SESSION 8B

THE BIOCHEMICAL MODE OF ACTION OF HERBICIDES

SESSION ORGANISER DR D. J. COLE

POSTERS 8B-1 to 8B-7

METABOLISM OF THE HERBICIDE SAFENER FENCHLORAZOLE-ETHYL IN WHEAT, BARLEY, AND *Digitaria ischaemum*

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ABSTRACT

Metabolism studies on foliarly applied [^{14}C]fenchlorazole-ethyl indicated that the chemical was rapidly metabolised in wheat, barley and *Digitaria ischaemum*. About 11%, 4% and 6% of total radioactivity extracted from wheat, barley and *D. ischaemum*, respectively, was identified as parent compound, 24 h after application. Based upon reverse-phase hplc, three different metabolites were found in all three plant species. Two of the major metabolites were identified as fenchlorazole (1-(2,4-dichlorophenyl)5-trichloromethyl-(1H)-1,2,4-triazole-3-carboxylicacid) and 1-(2,4-dichlorophenyl)-(1H)-1,2,4-triazole-3-carboxylic acid (Hoe 083348). Regardless of the plant species, there was no change in the relative amount of the unknown metabolite during the 24-h-time course study, whereas the quantities of fenchlorazole and Hoe 083348 decreased and increased, respectively. After 24 h, 15%, 12% and 34% of the total radioactivity was found as fenchlorazole and 57%, 67% and 45% as Hoe 083348 in wheat, barley and *D. ischaemum*, respectively. Furthermore, the quantity of Hoe 083348 detected in wheat (35%), barley (39%) and *D. ischaemum* (42%) equalled that of fenchlorazole after 6 h, 7 h and 22 h.

INTRODUCTION

The herbicide safener fenchlorazole-ethyl (ethyl-1-(2,4-dichlorphenyl)-5-trichloromethyl-1H-1,2,4-triazol-3-carboxylic acid), used in post-emergence application in combination with the herbicide fenoxaprop-ethyl (ethyl-(±)2-[4-[(6-chloro-2-benzoxazolyl)-oxy]phenoxy]propanoic acid), provides protection to wheat, rye and triticale without reduction of grass weed control (Bieringer *et al.*, 1989). Combination of fenchlorazole-ethyl with fenoxaprop-ethyl also improved the control of *Avena fatua* and *Setaria* spp. (Anderson *et al.*, 1989). Fenchlorazole-ethyl did not reduce foliar uptake or translocation of the herbicide, nor the degree of inhibition of acetyl-CoA carboxylase (Köcher *et al.*, 1989).

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Recently, Yaacoby et al. (1991) showed that fenchlorazole-ethyl eliminated, reduced and increased, respectively, the phytoxicity of fenoxaprop-ethyl to wheat, barley and Digitaria ischaemum. Fenchlorazole-ethyl increased both the rate of de-esterification of fenoxaprop-ethyl to fenoxaprop in all three species and the rate of metabolism of fenoxaprop to water-soluble metabolites in only wheat and barley. Little or no water-soluble metabolites of the herbicide were found in D. ischaemum.

Research is presently underway in our laboratory to further characterise the metabolites of fenoxaprop-ethyl in these tolerant and susceptible grass species. However, little is known about the metabolism and mode of action of fenchlorazole-ethyl and the importance of these mechanisms in the safening and sometimes synergising action of this compound on the herbicide fenoxaprop-ethyl. The purpose of the research reported herein was to determine if there were any qualitative and\or quantitative differences in the metabolism of fenchlorazole-ethyl in wheat, barley and D. ischaemum, and to isolate and identify possible metabolites of the safener.

MATERIALS AND METHODS

Seeds of wheat (Triticum aestivum L., cv. Fredrick), barley (Hordeum vulgare L., cv. Léger) and D. ischaemum (Schreb.) Muhl. were planted in 45 ml plastic pots containing a peat mixture (Promix BX, Premier Brands Inc., New Rochelle, NY). After emergence, the seedlings were reduced to 2 per pot. Plants were grown in a controlled environment growth-room maintained at 26/19±1°C day/night temperature with a 16-h photoperiod, light intensity of 450 $\mu E \cdot m^{-2} \cdot s^{-1}$ and relative humidity of 64/70% day/night.

Treatment solutions were prepared by dissolving Phenyl-U-radiolabelled [^{14}C]fenchlorazole-ethyl (sp act 2490.3 MBq/g) and fenchlorazole-ethyl (technical grade 97.9%) in an emulsifiable concentrate formulation blank (Hoechst AG, Frankfurt, Germany) to a concentration of 15 g/l. This was then diluted in water to give a final concentration of fenchlorazole-ethyl of 0.18 g/l and a specific activity of 10^7 dpm/ml.

Five X 1-μl droplets of the treatment solution were applied along the midvein, on the adaxial surface of 4-day old leaves of wheat, barley and D. ischaemum with a 1-5 μl micropipette. At intervals after application (4 h, 8 h, 16 h and 24 h) the treated shoots were rinsed with 10 ml of an aqueous solution containing 10% ethanol and 0.5% Tween 20. The washed shoots were then frozen until extraction. A 3-ml aliquot of the washing solution was mixed with 10 ml scintillation fluid (Ecosint A, National Diagnostic, Manoville, NJ, USA) and the radioactivity was determined by liquid scintillation spectrometry (LSS), using a Tri-Carb 460C Automatic Scintillation System (Packard Instrument Co.,Downers Grove, IL, USA). Leaf uptake of [^{14}C]fenchlorazole-ethyl was estimated by calculating the difference between the total radioactivity applied and that recovered in the washing solution. Total recoveries of radioactivity exceeded 90%.

Frozen tissues were homogenised in acetonitrile:water (7:3, v/v) with an Omni 1000 homogeniser (Omni International Inc., Waterbury, CT, USA) and the homogenate was centrifuged at 1800g for 15 min. Radiolabelled fenchlorazole-ethyl and its metabolites contained in the plant extract were concentrated by loading the supernatant (7-10 ml) on a reverse-phase preparative chromatography column (C_{18} Bakerbond SPE cartridge, J.T.Baker Inc., Phillipsburg, NJ, USA) activated by flushing with 6 ml acetonitrile followed by 6 ml distilled water. The radioactivity was eluted with 100% acetonitrile. Sample volumes were reduced by evaporation under a stream of nitrogen gas and filtered (0.22 μ; Micro Separation Inc., Westboro, MA, USA).

Radiolabelled fenchlorazole-ethyl and its metabolites were separated by high-performance liquid chromatography (hplc) on a Shimadzu Model LC-6A chromatograph, equipped with a 10 X 250 mm, C_{18} YMC-ODS column (YMC Inc. Morris Plains, NJ, USA). Chromatographic conditions consisted of a mobile phase of A = H_2O + 0.1% phosphoric acid (pH 2.5), B = acetonotrile and a 30 min convex #3 gradient from 0% B to 60% B followed by a 10 min linear gradient to 100% B; flow rate 2.0 ml/min; ambient temp. 22°C. The radiolabelled residues were detected and quantified by a Radiomatic FLO-ONE\Beta A-200 radioactivity flow detector (Radiomatic Instruments and Chemical Co., Inc., Tampa, FL, USA) and their retention times were compared with those of authentic standards of fenchlorazole-ethyl, fenchlorazole and 1-(2,4-dichlorophenyl)-(1H)-1,2,4-triazole-3-carboxylic acid (Hoe 083348). The unextracted radioactivity was quantified by combusting the extracted plant material in a Model OX-300 biological oxidizer (R.J. Harvey Instrument Corp., Hillsdale, NJ, USA). The resulting $^{14}CO_2$ was trapped in a carbon-14 scintillation fluid (R.J.Harvey Instrument Corp.) and quantified by LSS.

All experiments were repeated twice in a completely randomised design with three replicates per treatment. Each replicate consisted of 2 pots with 2 plants each.

RESULTS

The uptake of [^{14}C]fenchlorazole-ethyl was more rapid in barley than in wheat and D. ischaemum. When fenchlorazole-ethyl was applied alone, 63%, 98%, and 82% of the applied radioactivity was taken up by wheat, barley, and D. ischaemum, respectively, 4 h after treatment. After 24 h, the uptake in wheat and D. ischaemum was 85% of applied radioactivity. The amount of unextracted radioactivity, expressed as percentage of uptake, was about 44, 19, and 32 in wheat, barley and D. ischaemum, respectively, 24 h after the application of [^{14}C]fenchlorazole-ethyl, suggesting differences in metabolism and/or compartmentalisation of the radioactive metabolites of fenchlorazole-ethyl.

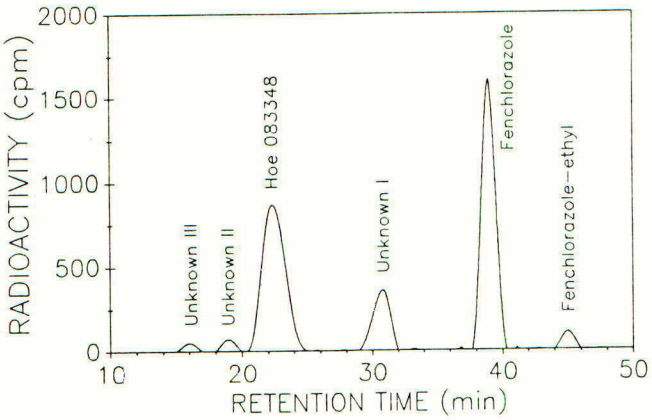

FIGURE 1. Typical chromatogram representing the hplc separation of [^{14}C]fenchlorazole-ethyl and its metabolites extracted from wheat 4 h after treatment. The retention times for authentic standards of fenchlorazole-ethyl, fenchlorazole and Hoe 083348 were 45 min, 39 min, and 22 min, respectively.

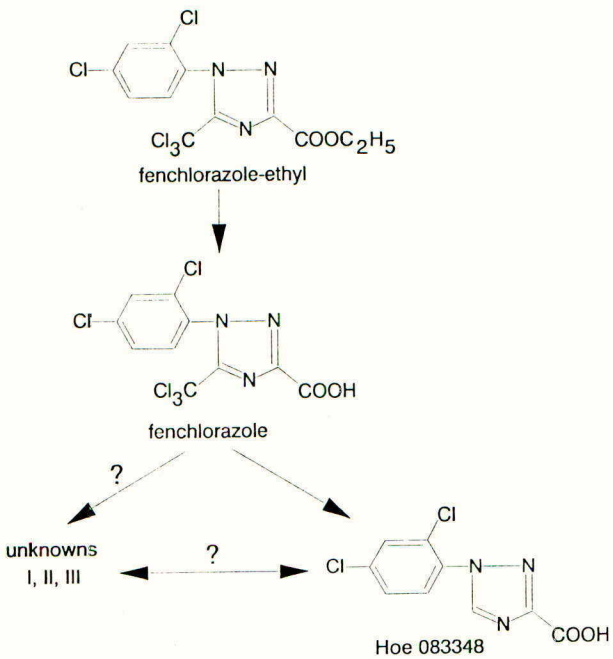

FIGURE 2. Hypothetical pathway for the metabolism of fenchlorazole-ethyl.

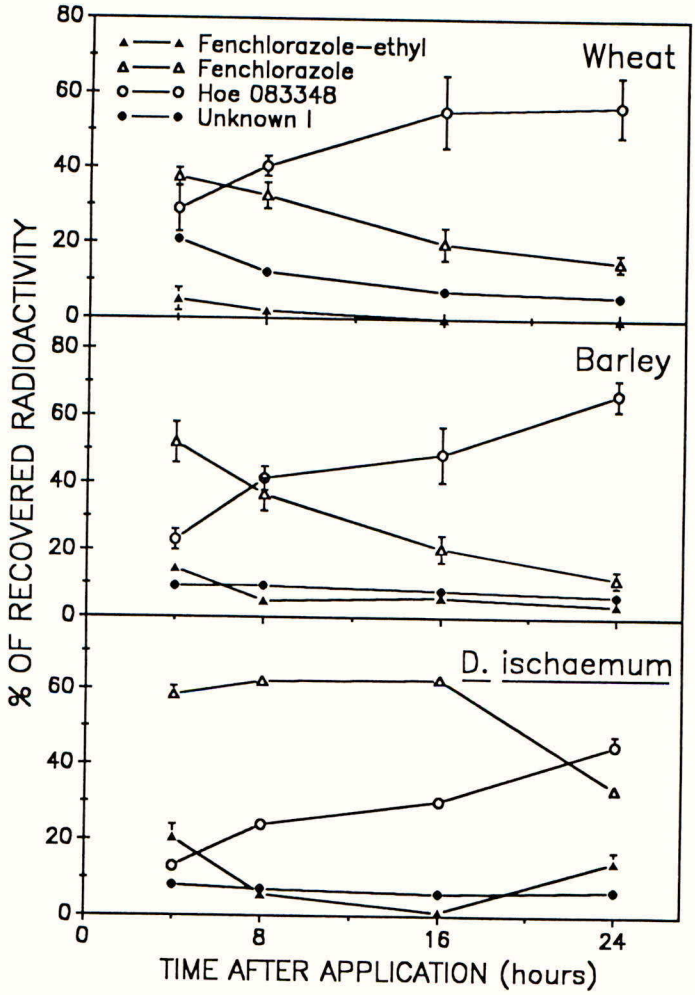

FIGURE 3. Metabolism of [^{14}C]fenchlorazole-ethyl after application to wheat, barley, and D. ischaemum. Vertical bars represent standard errors of the means (n=3).

Three major metabolites, with retention times of 22 min, 31 min and 39 min, respectively, were found in extracts of all the three plant species as represented by a typical chromatographic profile of wheat extract (Fig. 1). The metabolites having retention times of 39 min and 22 min were identical to the retention times of the respective authentic standards of fenchlorazole and Hoe 083348 (Fig. 2). The only major unidentified metabolite (Unknown I) had a retention time of 31 min in all three species (Fig. 1).

The parent compound was rapidly metabolised in all plant species with approximately 5%, 17% and 20% of extracted radioactivity remaining as fenchlorazole-ethyl in wheat, barley and D. ischaemum, respectively, 4 h after application of [^{14}C]fenchlorazole-ethyl (Fig. 3). Regardless of the plant species, there was no significant change in the quantity of Unknown I, during the 24-h time study. Unknown I accounted for less than 20% of total radioactivity in plant extracts of wheat and less than 10% in plant extracts of barley and D. ischaemum (Fig. 3). The amounts of fenchlorazole and Hoe 083348 decreased and increased with time, respectively. The relative amounts of fenchlorazole decreased from about 38%, 52%, and 58% of total radioactivity extracted 4 h after treatment to about 15%, 12%, and 34% of total radioactivity extracted 24 h after the application of [^{14}C]fenchlorazole-ethyl to wheat, barley, and D. ischaemum, respectively. During the same time course (4 h to 24 h after treatment), Hoe 083348 increased from about 29%, 23%, and 13% to 57%, 67% and 45% of radioactivity extracted from wheat, barley and D. ischaemum, respectively (Fig. 3). Furthermore, the quantity of Hoe 083348 detected in wheat (35%), barley (39%) and D. ischaemum (42%) equalled that of fenchlorazole after 6 h, 7 h, and 22 h, respectively.

Two minor metabolites were found only in wheat (Unknowns II, III; Fig. 1). The retention times of these two metabolites were 16 min and 19 min with their respective quantities not exceeding 7% and 16% of the extracted radioactivity during the 24-h-time course experiment. After base hydrolysis (1 M NaOH for 3 h) of the safener three compounds were produced that had the same respective retention times as those of the three major metabolites extracted from [^{14}C]fenchlorazole-ethyl treated wheat, barley and D. ischaemum .

DISCUSSION

Fenchlorazole-ethyl is a chemical that when added to the formulation of fenoxaprop-ethyl acts as a herbicide safener in wheat and barley and as a synergist in D. ischaemum. In earlier studies it was found that fenchlorazole-ethyl increased the conversion of fenoxaprop-ethyl to the more phytotoxic fenoxaprop in all three species, but it enhanced the detoxification of the free-acid to water soluble metabolites only in wheat and barley (Yaacoby et al., 1991).

In the 24-hour metabolism study reported here fenchlorazole-ethyl was rapidly metabolised in all three plant species tested with only 11%, 4% and 6% of total radioactivity in wheat, barley and D. ischaemum, respectively, remaining as the parent compound 24 h after treatment. These results suggest that the safening/synergising actions of fenchlorazole-ethyl are not actually due to the parent molecule, but, probably, due to one or a combination of metabolites of the parent molecule.

Three major metabolites were found in plant extracts of all the species. Two of the major metabolites, identified as fenchlorazole and Hoe 083348 decreased and increased in time, respectively, suggesting a rapid conversion of the parent compound to its free acid which was, in turn, dechlorinated to give a more water soluble product, Hoe 083348 (Fig. 2). The amount of the third metabolite (Unknown I) did not significantly change with time. It probably represents an intermediate metabolite or the final product of a secondary metabolic pathway. Unknown I, along with fenchlorazole and Hoe 083348, was produced upon basic hydrolysis of fenchlorazole-ethyl, indicating that it is a degradation product of the parent compound and not a conjugate.

Some differences in the degradation pattern of fenchlorazole-ethyl were found when the three species were compared. Firstly the conversion of fenchlorazole-acid to Hoe 083348 was faster in wheat and barley than D. ischaemum. Secondly two unidentified water soluble metabolites were found in wheat but not in barley or D. ischaemum. These metabolites were not obtained upon hydrolysis of the parent molecule and may represent further metabolism of the major metabolites or final products of secondary metabolic pathway. Thirdly the amount of bound radioactivity was significantly higher in wheat than in barley and D. ischaemum. These differences may partly account for the differences in the effects of fenchlorazole-ethyl on these three plant species.

The identity of Unknown I, II, III found in extracts of wheat, the significance of the bound material found in plant residues after extraction and the role of each degradation product of fenchlorazole-ethyl in safening or synergising the herbicidal effect of fenoxaprop-ethyl, at present are the objects of further investigation.

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CHARACTERISATION OF TWO GLUCOSYLTRANSFERASES ISOLATED FROM SOYBEAN ASSOCIATED WITH BENTAZON METABOLISM

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ABSTRACT

Glucosylation of the major bentazon metabolite, 6-hydroxybentazon by two glucosyltransferases isolated from tolerant soybean tissue has been demonstrated. A cytosolic kaempferol glucosyltransferase was isolated from 3 week-old light grown tissue extract. This enzyme had a relative molecular mass of 44,600, a pH optimum of 6.3 and Michaelis constants for kaempferol and 6-hydroxybentazon of 0.09 and 2.45 mM respectively. A membrane-bound glucosyltransferase whose primary substrate was the shikimic acid pathway intermediate, p-hydroxyphenylpyruvic acid, was isolated from 7 day-old etiolated tissue. This enzyme had a relative molecular mass of 53,000 (which could be dissociated to approximately 29,000), a pH optimum of 7.5 and Michaelis constants for p-hydroxyphenylpyruvic acid and 6-hydroxybentazon, of 0.11 and 1.96 mM respectively. These results suggest that 6-hydroxybentazon may be detoxified by several glucosyltransferases with overlapping specificity whose primary role involves the storage of endogenous metabolites.

INTRODUCTION

Bentazon is a selective herbicide used in major world crops, including soybean, to control broadleaf and sedge weeds. Differential metabolism has been established as the major basis of selective herbicide action. Bentazon is hydroxylated and then glucosylated (Figure 1) either at the 6- or 8- position of its aromatic ring in soybean (Connelly *et al.*, 1988) whereas only 6-hydroxybentazon has been reported in rice (Mine *et al.*, 1975). The hydroxylation/glucosylation route of bentazon has been confirmed more recently in soybean (Sterling and Balke, 1989; Leah *et al.*, 1991). Biochemical studies have to date largely concentrated on the first step of bentazon metabolism with increasing evidence of cytochrome p450 dependant aryl hydroxylation (Leah *et al.*, 1991; McFadden *et al.*, 1990) However, no biochemical data have been reported on the glucosylation of 6-hydroxybentazon. Herbicides which are hydroxylated and then metabolised to O-glucosides include bentazon, chlorotoluron and chlorsulfuron (Lamoureux *et al.*, 1989) although the distribution, location and substrate specificity of the enzymes responsible remains unknown.

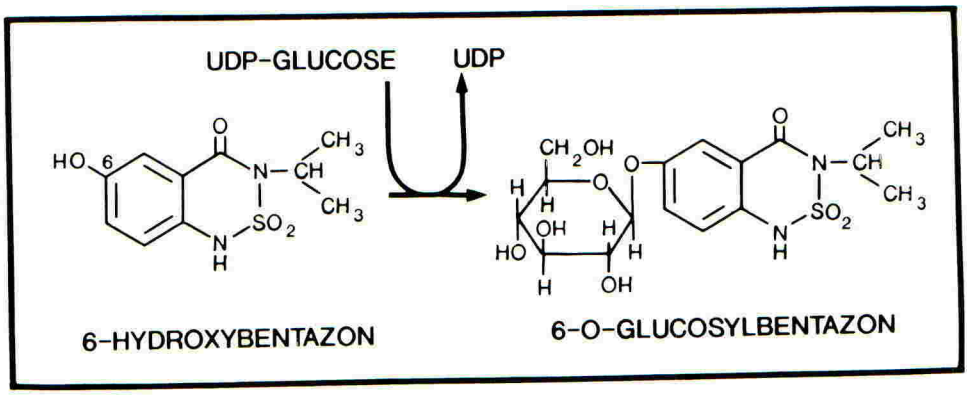

FIGURE 1. Glucosylation of 6-hydroxybentazon

An N-glucosyltransferase (GT) associated with metabolism of the herbicide metribuzin has been recently reported (Davis *et al.*, 1991). However, the physicochemical characteristics and substrate specificity have not been determined.

This report describes the isolation and characterisation of two UDP-glucose:phenol GTs with substrate specificity for quite different classes of endogenous secondary metabolites which are also associated with 6-hydroxybentazon glucosylation.

EXPERIMENTAL METHODS

Growth conditions

Soybean seedlings [*Glycine max* Fiskerby V] obtained from Suffolk Herbs (Sudbury, Suffolk) were grown in vermiculite at 18-25°C and watered daily from below. Plants were grown for 7 days in darkness to obtain etiolated tissue. Whole seedlings were washed free of vermiculite with distilled water and the roots and cotyledons removed and discarded immediately prior to use. Light grown plants were used after 3 weeks growth at 25°C with a 16 h photoperiod of 200-250 μmol m^{-2} s^{-1} photosynthetic photon flux density. First trifoliate leaves were washed with distilled water and used immediately.

Isolation of a p-hydroxyphenylpyruvate GT.

One week-old etiolated soybean seedlings (roots and cotyledons removed) were homogenised in an ice-cold mortar and pestle to produce a 100 mg ml^{-1} homogenate containing 50 mg ml^{-1} polyvinylpyrrolidone in isolation buffer containing 0.2 M Tris-HCl (pH 7.5), 200 mg ml^{-1} glycerol, 0.2 mM EDTA, 0.2 mM EGTA, 0.1 mM dithiothreitol and 0.1 M KCl. The homogenate was filtered through 100 μm nylon mesh and centrifuged at 16,000 x g for 10 min at 2°C. The supernatant was stored on ice while the pellet was re-suspended to 200 mg ml^{-1} in isolation buffer

and re-centrifuged as before. Pooled supernatants were solubilised by addition of Triton X-100 to 20 mg ml^{-1}. This extract was stirred on ice for 30 min and then centrifuged at 150,000 x g for 60 min at 2°C. The particle-free supernatant containing the solubilised enzyme was fractionated with solid ammonium sulphate and protein precipitating between 40-75% saturation was collected by centrifugation at 15,000 x g for 10 min at 2°C. The pellet was re-suspended in 25 mM Tris-HCl (pH 7.5) containing 50 mg ml^{-1} glycerol and 0.1 mM dithiothreitol and was desalted on Sephadex G-25 (1.6 x 14 cm) equilibrated with this buffer.

The eluate was applied to an ion-exchange column (DEAE-Sephacel, 3 x 18 cm) equilibrated with this buffer. Unbound protein was eluted at a flow rate of 30 ml hr^{-1}, followed by elution of the bound GT using a salt gradient of 0-0.8 M KCl. Fractions containing enzyme activity were eluted at 0.2-0.22 M KCl, pooled and concentrated by ammonium sulphate precipitation at 75% saturation. After centrifugation at 15,000 x g for 10 min at 2°C, the pellet was re-suspended in a minimal volume of buffer solution and desalted on a Sephadex G-25 column as previously described. The GT was isolated from this extract by gel filtration using a Sephacryl S-200 column (2.6 x 77 cm) equilibrated with 0.2 M Tris-HCl (pH 7.5) containing 50 mg ml^{-1} glycerol and 0.1 mM dithiothreitol. Enzyme was eluted at a flow rate of 10.2 ml h^{-1} and stored at -20°C or assayed immediately.

Isolation of a kaempferol GT

Fully-expanded first trifoliate leaves of 3 week-old soybean plants were washed in 25 mM Tris-HCl (pH 6.3) containing 50 mg ml^{-1} glycerol and 0.1 mM dithiothreitol. Leaves were homogenised in an ice cold mortar and pestle with this buffer solution containing 50 mg ml^{-1} polyvinylpyrrolidone to produce a 200 mg ml^{-1} homogenate prior to filtration through 100 μm nylon mesh. The filtrate was centrifuged at 16,000 x g for 10 min at 2°C. The supernatant was removed and stored on ice whilst the pellet was re-suspended to its original volume in isolation buffer and centrifuged as before. The supernatants were combined, adjusted to pH 6.3 and centrifuged at 100,000 x g for 60 min at 2°C.

The supernatant was filtered through 0.22 μm filters ('Acrodisc DLL', Gelman Sciences) before 0.5 ml aliquots were applied to a Mono-Q (HR 5/5, Pharmacia) ion-exchange FPLC column equilibrated with 25 mM Tris-HCl (pH 6.3) containing 50 mg ml^{-1} glycerol and 0.1 mM dithiothreitol. Bound enzyme was eluted at 0.33-0.36 M KCl from the column using a salt gradient of 0-0.5 M KCl. Active fractions were pooled, concentrated and applied to a Superose 12 gel filtration FPLC column (HR 10/30, Pharmacia) equilibrated with 0.2 M Tris-HCl (pH 6.3) containing 50 mg ml^{-1} glycerol and 0.1 mM dithiothreitol. Fractions were collected in 0.5 ml aliquots at a flow rate of 0.5 ml min^{-1} and a single active peak obtained at an elution volume of approximately 9.5 ml. The isolated enzyme was stored on ice at -20°C or assayed immediately.

Assays

Protein was assayed by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard (Sigma) and absorbance at 280 nm was used to monitor protein eluted by chromatography. Native M_r of the solubilised membrane-bound *p*-hydroxyphenylpyruvic acid GT and the soluble kaempferol GT were determined by Sephacryl S-200 and Superose 12 gel filtration respectively, as described for enzyme purifications. Dissociated M_r was estimated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) using a 10% gel.

GT activity was assayed using a modified method of Jain *et al.* (1989). The assay mixture contained 1.85 KBq of UDP-[U-^{14}C]glucose (8.44 GBq mmol^{-1}, ICN Biochemicals), 1 mM UDP-glucose, 4 mM phenolic substrate and enzyme extract in a total volume of 122 μl made up in 25 mM Tris-HCl (either pH 6.8 or 7.8 depending on the enzyme being assayed), containing 50 mg ml^{-1} glycerol and 0.1 mM dithiothreitol. The reaction was initiated by addition of enzyme extract followed by incubation in a 30°C water bath for 10-60 min. The reaction was stopped by addition of 1 ml of ice-cold distilled water. In each case, control assays containing no acceptor substrate for UDP-glucose were included. This mixture was made up to 5 ml in distilled water in a vacuum-filtration funnel (Whatman) fitted with two ion-exchange filters (25 mm diameter, DE-81, Whatman). This mixture was vacuum-filtered followed by two further washes with 5 ml of distilled water. Washed filters, to which unreacted UDP-glucose had bound, were removed and dissolved in scintillation fluid ('Hi-Safe', LKB) by vortex mixing followed by liquid scintillation spectrometry. A sample of the combined washings containing unbound glucosylated products was also assayed for ^{14}C activity. Boiled enzyme and substrate controls were included in each assay.

RESULTS

A time-course for the enzyme assay using kaempferol, *p*-hydroxyphenylpyruvic acid and 6-hydroxybentazon as respective substrates, was linear over a period of 60 min (Figure 2) producing standard errors no greater than 8% of the mean in all cases. Since the assay relies on UDP-glucose measurement and conveniently does not require chromatographic separation of glucosylated products, crude extracts which may contain competing endogenous substrates or competing enzymes can produce misleading results. Consequently, overall enzyme purification cannot be assessed since a semi-crude extract is required for accurate assay.

Characteristics of the two isolated GTs are summarised in Table 1. Similar specific activities were obtained for the two isolated enzymes using their respective primary substrates. The presence of glycerol and dithiothreitol were essential to maintain activity. The addition of divalent cations enhanced activity of the *p*-hydroxyphenylpyruvic acid GT and to a lesser

extent, the kaempferol GT at a concentration of 1 mM. Higher concentrations (10 mM) of cations produced over 50% inhibition Addition p-chloromercuribenzoate, an inhibitor of enzymes with accessible sulphydryl groups produced 85% inhibition of both enzymes at a concentration of 0.01 mM. 1 mM EGTA produced a decrease in activity (63% and 87% of control for p-hydroxyphenylpyruvic acid and Kaempferol GT respectively) which confirms a divalent cation requirement for the enzyme. The presence of EDTA and EGTA in crude extracts of the p-hydroxyphenylpyruvic acid GT prevented the loss of activity

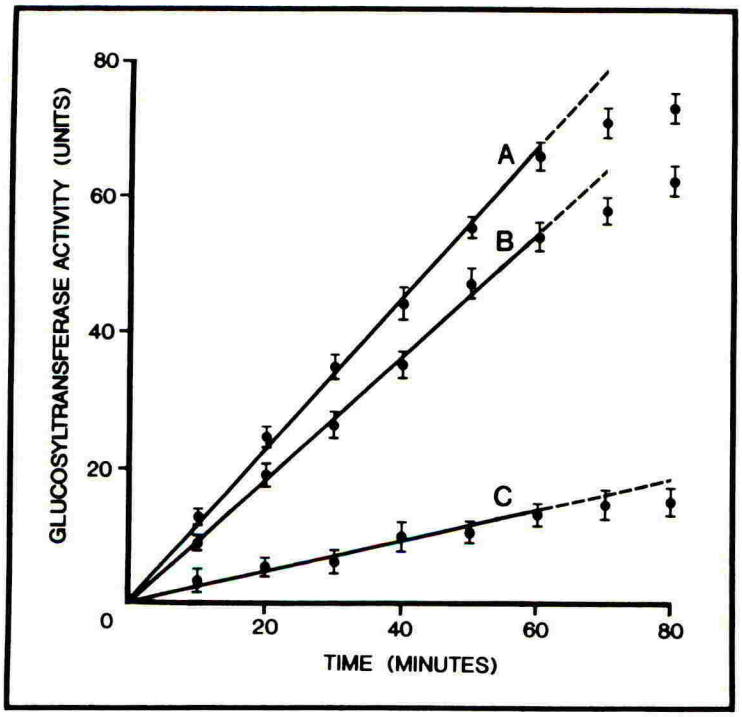

FIGURE 2. Typical time-courses for the glucosyltransferase assay using A. p-hydroxyphenylpyruvate, B. kaempferol and C. 6-hydroxybentazon. One unit of activity = 1 μmol of UDP-glucose utilised per hour at 30°C, pH 7.5 (A and C, using the membrane bound enzyme) or 6.3 (B, using the soluble enzyme).

possibly by inhibiting the activity of metalloproteases or high concentrations of divalent cations. The membrane-bound p-hydroxyphenylpyruvic acid GT could be readily solubilised from microsomal pellets obtained from a 16,000 x g for 10 min supernatant which was pelleted by centrifugation at 150,000 x g for 60 min, using 20 mg ml^{-1} Triton X-100.

The pH optima for the kaempferol and p-hydroxyphenylpyruvic acid GTs were 6.3 and 7.5 respectively providing

further evidence of the differing sub-cellular location and substrate specificity for these two enzymes. The p-hydroxyphenylpyruvic acid GT produced a slightly broader pH activity profile than the kaempferol enzyme. The native M_r of the p-hydroxyphenylpyruvic acid GT was estimated at 53,000 by Sephacryl S-200 gel filtration. The enzyme could be dissociated upon SDS-PAGE to 29,000 suggesting a dimeric protein. Native M_r for the kaempferol GT was estimated at 44,600 by Superose 12 gel filtration.

TABLE 1. Comparison of the properties of soluble and particulate glucosyltransferases in etiolated Soybean tissue.

	GLUCOSYLTRANSFERASE	
	SOLUBLE	PARTICULATE
PROPERTY	GT[a]	GT[b]
Primary substrate	Kaempferol	p-hydroxyphenyl pyruvate
Isolated specific activity	4.26 (U/mg)	3.85 (U/mg)
Km (Kaempferol)	0.09 mM	(not determined)
Km (p-hydroxyphenyl pyruvate)	0.29 mM	0.11 mM
Km (6-hydroxybentazon)	2.45 mM	1.96 mM
pH optimum	6.3	7.5
Relative molecular mass	44,600	53,000
Dissociated molecular mass	(not determined)	28,000
Effectors of activity:		
Divalent cations (1 mM)	stimulate	stimulate
(10 mM)	inhibit	inhibit
p-chloromercuri-benzoate (0.01 mM)	inhibit	inhibit

[a]Kaempferol glucosyltransferase. [b]p-hydroxyphenylpyruvic acid glucosyltransferase. For definition of units see Figure 2.

Over 30 additional phenols were assessed for substrate specificity and the majority of them could be glucosylated to some extent by both isolated enzymes. The kaempferol enzyme had a broader specificity which encompassed the major substrates of the p-hydroxyphenylpyruvic acid GT. Very similar Km values for the two enzymes with their respective primary substrates were obtained, both being about 0.1 mM. The p-hydroxyphenylpyruvic acid GT produced no activity with kaempferol and negligible activity with a range of flavonoids. However, the kaempferol enzyme had a Km value of 0.29 mM for p-hydroxyphenylpyruvic acid. Overlapping specificity was observed with low affinity substrates, including 6-hydroxybentazon. Km Values for 6-hydroxybentazon were over 20 times higher than those obtained for both enzymes using their respective primary substrates.

DISCUSSION

In this study it has been shown that 6-hydroxybentazon can be glucosylated by two enzymes with presumably different subcellular location and substrate specificities, and that the Km values for the metabolite were both at least 20-fold higher than those obtained for their respective optimal substrates. Consequently, for glucosylation to be significant, concentrations of 6-hydroxybentazon would have to be relatively high with respect to endogenous high affinity substrates. This is probably the case, since formulated bentazon is applied in the millimolar range, ensuring that metabolite accumulation is sufficiently high to permit effective glucosylation. It is also possible that the compartmentation of the enzyme permits access to a high concentration pool of xenobiotic to the exclusion of endogenous substrates.

The sequential shikimic acid metabolites, chorismic and prephenic and p-hydroxyphenylpyruvic acid produce increasing specific activities and corresponding decreasing Km values, for the membrane-bound enzyme. This trend is also reflected in activities obtained for the soluble kaempferol GT in spite of its structurally dissimilar primary substrate. While the biosynthetic pathways for flavonoids remain poorly defined, it is possible that several GTs are present in plant cells whose specificity is chiefly directed towards a few metabolites along a biosynthetic pathway, but which have a broad specificity for a diverse range of phenolics which include many xenobiotics. Overlapping substrate specificity would not only enable fine control of metabolite levels at key positions along a biosynthetic pathway but may also permit the most efficient detoxification route for a given pesticide so that the enzyme with the greatest affinity (lowest Km) for the xenobiotic can be exploited by the plant cell.

Since the metabolic status of the plant considerably affects secondary metabolism it is possible that the levels of endogenous substrate may dictate the rate of xenobiotic glucosylation. It has long been recognised that in most cases, the biosynthesis of flavonols such as kaempferol and quercetin is light-dependent (Harbourne, 1967). Levels of flavonoids are therefore generally much higher in light-grown tissue, causing much greater competitive inhibition of xenobiotic glucosylation . This confirms previous observations in our laboratory studying bentazon metabolism *in vivo* in soybean plants which showed that glucosyl hydroxybentazon accumulated more rapidly in leaves under dark conditions (Leah *et al.*, 1991) compared to light. This suggests that other GTs such as the membrane-bound p-hydroxyphenylpyruvate enzyme, may preferentially metabolise 6-hydroxybentazon in light conditions, even though both of the enzymes studied have similar Km values for this xenobiotic. It is interesting to note that a very similar p-hydroxyphenyl-pyruvate GT has been isolated from light-grown Soybean (Leah, J.M., 1991 unpublished) but which produced no activity with 6-hydroxybentazon, suggesting that GT isoenzymes may be present with the same primary substrate but differing broad

specificity.

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KINETICS OF CHLOROPHYLL FLUORESCENCE DECAY IN TRIAZINE-RESISTANT AND -SUSCEPTIBLE WEEDS

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ABSTRACT

The dark decay of the chlorophyll fluorescence rise, induced in leaves or chloroplasts by a short saturating flash, was used as a measure for electron transport from the quinone Q_A to the quinone Q_B. The decay was resolved into three distinct phases with first order rate constants of 2500 s^{-1}, 470 s^{-1} and 9 s^{-1}, which accounted for 50%, 25% and 25% of the signal amplitude respectively. DCMU inhibited the two faster phases but not the slow phase, indicating that the latter is not involved in open-chain electron transport. The rate constants for all three phases were lower in triazine resistant (R) compared to susceptible (S) biotypes, and the relative contribution of the slow phase in R lines was twice that obtained in S lines. These results indicate that the triazine resistance trait involves both inhibition of Q_A to Q_B electron transfer and increase in the steady state level of Q_A^-.

INTRODUCTION

Triazine resistant weeds have appeared over the last two decades in many parts of the world. In most cases, the resistance was found to be due to reduced affinity of the binding site for triazine herbicides. Several reports have linked the resistance trait to low ecological fitness while other studies did not confirm the inferiority of R biotypes (Schonfeld, *et al.*, 1987). The question of the relationship between triazine resistance and ecological fitness is thus still open (Rubin, 1991).

The resistance is caused by a mutation in D1, the Q_B-binding protein (Hirschberg & McIntosh, 1984), which is part of the PSII reaction centre complex and thus intimately involved in the primary electron transfer. The question can be reduced to whether changes in photosynthetic capacity in intact plants can be correlated with changes, induced by the mutation, in electron transport at the level of PSII.

The primary photochemical reaction resulting in the reduction of Q_A, is immediately followed by electron transport from Q_A to Q_B. As expected, the last reaction is modified in R biotypes due to the alteration in the Q_B binding site. There is however disagreement as to the specific effect of the mutation on the electron transfer reaction. Bowes *et al.*, (1980) reported that the rate of the Q_A to Q_B electron transfer in an R biotype of *Amaranthus retroflexus* was ten times slower than in the S biotype. Results of another study of several R and S lines (Jansen & Pfister, 1989), showed a threefold increase in $t_{½}$ for the Q_A to Q_B electron transfer, in R compared to S plants. On the other hand, a recent study showed no difference between R and S biotypes of *Chenopodium album* in the rate of the Q_A to Q_B electron transfer, but rather a displacement of the equilibrium state to a higher steady state concentration of Q_A^- (Etienne *et al.*, 1990).

In the current study we tried to resolve this disagreement, by a detailed kinetic analysis of the Q_A to Q_B electron transfer, in R and S lines of several plant species. This was achieved by measuring the chlorophyll fluorescence decay kinetics, following a short saturating flash. A similar analysis was also used in a comparative study of the heat sensitivity of the same R and S biotypes.

MATERIALS AND METHODS

Seeds of R and S lines of the following species were collected in fields and roadsides in Israel: *Amaranthus hybridus, Amaranthus blitoides, Alopecurus myosuroides, Lolium rigidum* and *Phalaris paradoxa*. Seeds of nearly isogenic R and S lines of *Brassica napus* were kindly provided by J. Gressel. The mutation was found to be a substitution of serine-264 by glycine, in the D1 protein, in all R biotypes except for *L. rigidum* and *A. myosuroides* in which the mutation has not been analysed yet. Plants were grown in a growth chamber under controlled environmental conditions. Experiments with intact leaves were carried out with 4-8 week old plants. Chloroplasts were isolated as previously described (Yaacoby *et al.*, 1986).

Fluorescence measurements were carried out with a chlorophyll fluorometer (PAM, H. Walz, Effeltrich, Germany). Intact leaves or chloroplast suspensions were dark adapted for 5 minutes before being exposed to an 8 μs saturating flash. The chlorophyll fluorescence time course was monitored with weak modulated light. Data points were registered at 25 μs intervals by a microcomputer equipped with a data acquisition card.

RESULTS

Kinetic analysis of the fluorescence decay

Illumination of dark-adapted leaves or chloroplasts by a single-turnover saturating flash, results in reduction of Q_A, the primary acceptor of PSII. This is evident as a sharp increase in chlorophyll fluorescence to a maximum amplitude. The fluorescence decay in the dark accordingly portrays the kinetics of Q_A^- re-oxidation. A kinetic analysis of the time course of fluorescence decay indicated the presence of three distinct phases, characterised by first order rate constants (Figure 1). The main difference between R and S lines was in the relative amplitude of the phases: the amplitude of the fast phase in R leaves was about half that measured in S leaves while the amplitude of the slow phase in R lines was about twice that of the S lines. In addition, the first order rate constants for the three phases were significantly lower in R plants of all species tested (Table 1).

The rate constants determined for the fast and medium phases are high enough to be associated with open-chain electron transfer, in both the R and S biotypes. The slow phase however, is not compatible with the rate limiting step of electron transfer through the plastoquinone pool (Sukenik, *et al.*, 1987) and is probably associated with non-productive reactions.

Effects of DCMU

The last conclusion is supported by experiments carried out with DCMU. Increasing concentrations of the herbicide inhibited the fluorescence decay rate. Figure 2A illustrates the effect of different DCMU concentrations on the flash-induced fluorescence signal in chloroplasts isolated from an S biotype of *A. blitoides*. A similar inhibition by DCMU was obtained with R plants, as well as with methabenzthiazuron, another urea herbicide. Atrazine and metribuzin inhibited the fluorescence decay in a similar manner in S lines but as expected, did not affect the decay kinetics of R chloroplasts.

Kinetic analyses of the fluorescence decay in the presence of DCMU showed that the rate constants for the three phases were practically unaffected while their relative amplitudes changed significantly (Figure 2B). About 0.1 μM DCMU was required for 50% inhibition of the fast phase amplitude, and 0.6 μM DCMU was required for a similar inhibition of the medium phase. The slow phase was not inhibited and its amplitude actually increased as a consequence of the inhibition of the faster components.

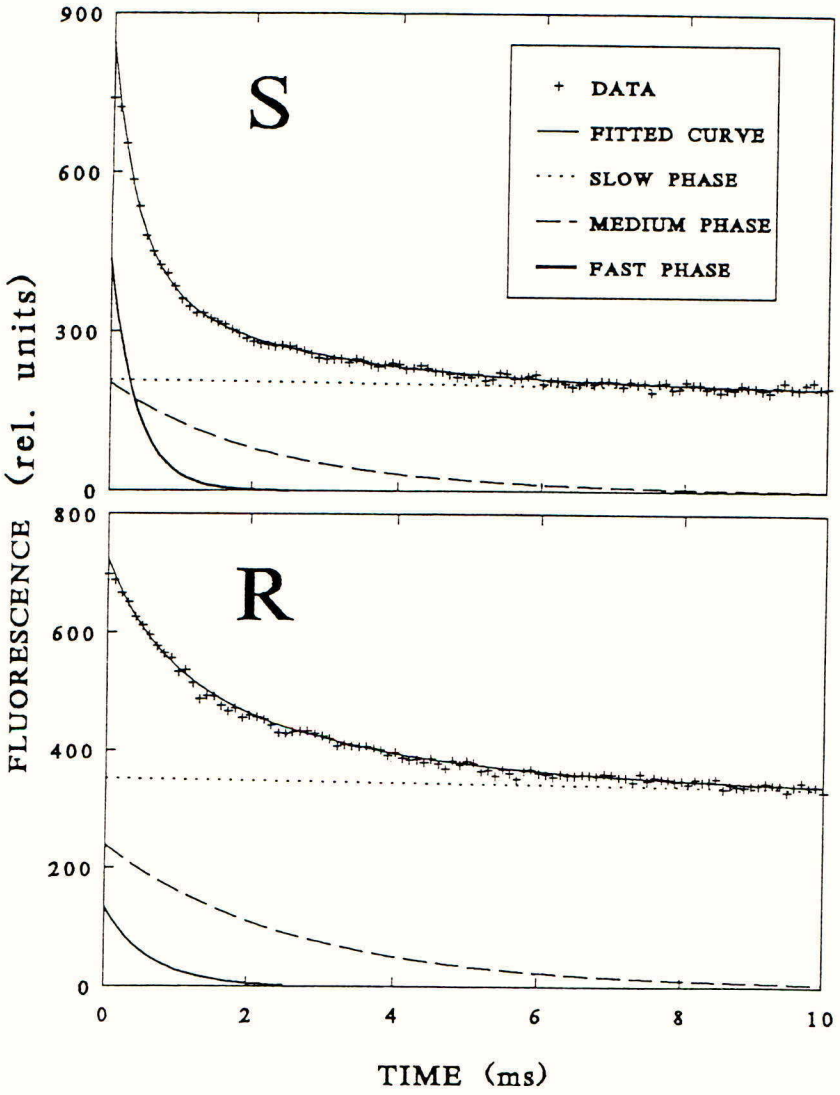

FIGURE 1. Kinetic components of the chlorophyll fluorescence decay following a saturating single-turnover flash, in leaves of susceptible (S) and resistant (R) biotypes of *Amaranthus hybridus*. The data represent an average of 4 separate measurements.

TABLE 1. Relative amplitudes and first order rate constants of the three phases of chlorophyll fluorescence decay, following a light flash. The data represent an average of 10 measurements carried out with R and S leaves of each of the following species: *A. hybridus, A. blitoides, A. myosuroides, L. rigidum, P. paradoxa* and *B. napus*.

Phase	Relative Amplitude (%) S	R	Rate Constant (s^{-1}) S	R
Fast	56 ± 9	24 ± 7	2500 ± 300	1500 ± 400
Medium	21 ± 7	35 ± 5	470 ± 60	330 ± 50
Slow	23 ± 4	41 ± 7	9 ± 3	6 ± 3

Effects of a short heat shock

A short exposure of detached leaves to temperatures in the range of 40°C to 45°C reduced the overall extent of the flash-induced fluorescence rise, and also modified the decay process. The triazine resistance trait was found to involve increased sensitivity to heat. For example, a one minute exposure of *A. myosuroides* leaves to 43°C resulted in 47% inhibition of the fluorescence rise in the S biotype and 74% in the R biotype. The main effect was usually obtained within 1-3 minutes of exposure to elevated temperatures.

In R leaves, the heat treatment abolished the fast phase of the fluorescence decay. The medium phase was also partially inhibited, and the relative contribution of the slow phase increased as a result of these effects. Only minor effects of the heat treatment on the fluorescence decay were observed with S leaves (Figure 3).

DISCUSSION

The polyphasic nature of the fluorescence decay, following exposure of dark adapted leaves to a single turnover flash, has been previously reported (Bowes *et al.*, 1980; Etienne *et al.*, 1990). The first order rate constants for the different phases calculated from the results of Etienne *et al.*, (1990) are roughly similar to those reported here. The two faster phases seem to be associated with the Q_A to Q_B electron transfer, and thereby with a linear electron transport. This is based upon both their rate constants which are compatible with the overall potential rate and on their sensitivity to DCMU. The slow phase is insensitive to DCMU, and its rate constant is too low to be associated with the linear electron transport. It probably represents the combination of electron leaks (or back-reactions) from both Q_A^- and Q_B^-.

The comparative kinetic analysis of fluorescence decay in R and S plants, indicated effects of the mutation on both the rate constants and the relative amplitudes of the different phases. The rate of Q_A to Q_B was significantly lower in R biotypes of all species tested. In addition, the relative amplitude of the slow phase in the R lines was about twice that measured in S lines, while the fast phase amplitude in R lines was half that of S lines.

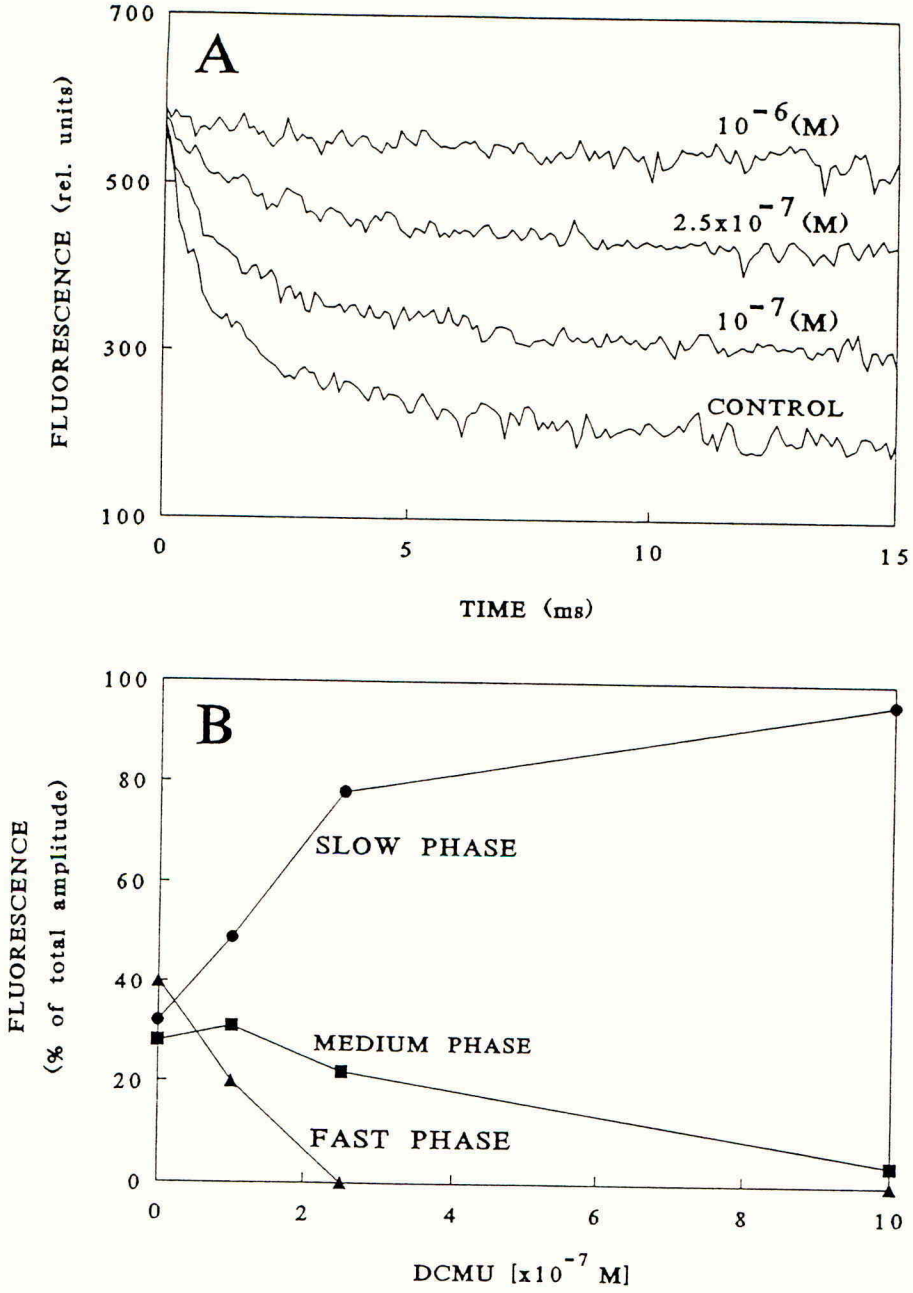

FIGURE 2. **A.** Effect of DCMU concentration on the chlorophyll fluorescence decay in chloroplasts from *Amaranthus blitoides*. **B.** Effects of DCMU concentration on the amplitudes of the three kinetic phases, obtained by analysis of the data in A. The analysis was carried out as illustrated in Figure 1. The data represent an average of 4 separate measurements.

The fluorescence level established at the end of the second phase (i.e., about 10 ms after the flash), can be used as a relative measure for the steady state concentration of Q_A^-, on that time scale. As could have been expected, but at variance with the results of Etienne *et al.*, (1990), the increase in the steady-state Q_A^- concentration, observed in R lines, was largely correlated with a decrease in the rate of Q_A to Q_B electron transfer (Table 1). This rate decrease is probably the cause of the increase in Q_A-concentration.

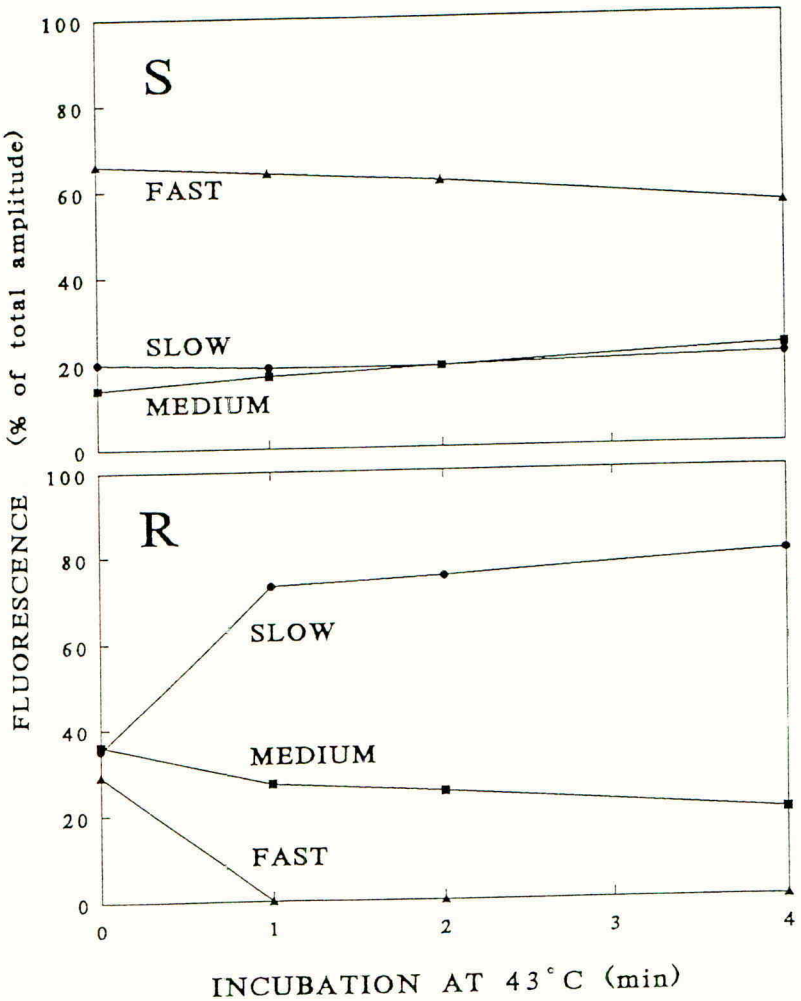

FIGURE 3. Effect of incubation time at 43°C on the amplitudes of the three phases of the chlorophyll fluorescence decay in R and S leaves of *Alopecurus myosuroides*. The data represent an average of 4 measurements carried out with different leaves.

The rate of Q_A to Q_B electron transfer in R plants was, on the average, about 40% slower than in S plants (Table 1). This partial reaction is faster than steady state electron transport by at least one order of magnitude in both S and R biotypes. Although the deleterious effects of the mutation are usually evident at low light intensities, they might fade at higher intensities, where R plants were indeed shown to be as fit as the S biotypes (Schonfeld *et al.,* 1987; Benyamini *et al.,* 1991).

As previously reported (Ducruet & Ort, 1988), photosynthetic reactions of R plants were found to be more sensitive to high temperatures than those of S plants. Elevated leaf temperatures were found to reduce the initial fluorescence rise in both R and S biotypes but more so in triazine-resistant plants. This reduction might be due to either an obstruction of electron transfer at the oxidizing side of PSII, or enhancement of non-radiative de-excitation at the expense of fluorescence. Based on the available evidence, we can not discriminate between these two explanations.

The fast phase of the fluorescence decay was inhibited by 1 min exposure to 43°C in R but not in S leaves (Figure 3). Evidently the mutation renders the Q_A to Q_B electron transfer step is more vulnerable to damage by heat shock, as previously concluded from different experiments (Ducruet & Ort, 1988).

The results presented here confirm earlier reports that the change in D1 induced by the mutation, in addition to causing resistance to triazine herbicides also involves modifications in the Q_A to Q_B electron transfer, as well as in the sensitivity of this step to high temperatures. The changes in this reaction seem however to be relatively limited, so that under appropriate conditions R plants might appear to be as fit as the wild type.

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NITRODIPHENYL ETHER AND PHENYLIMIDE RESISTANCE OF A TOBACCO BIOTYPE IS DUE TO ENHANCED INDUCIBILITY OF ITS ANTIOXIDANT SYSTEMS

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ABSTRACT

Activities of the enzymes ascorbate peroxidase (AP), glutathione reductase (GR) and glutathione S-transferase (GST), levels of non-protein thiols (mostly glutathione, GSH) and ascorbic acid (AA) were determined in leaves of tobacco plants of differential sensitivity to the nitrodiphenyl ether herbicides acifluorfen and bifenox and to the experimental N-phenylimide derivative S-23142. In leaves of untreated plants the AA content was 40% higher in the resistant biotype as compared to the sensitive ones. However, the levels of GSH, AP, GR, and GST did not differ significantly in the two biotypes. In the resistant leaves stressed by these herbicides the activity of AP readily increased while in the sensitive leaves it did not change. The level of GSH and the activities of GR and GST markedly increased in both biotypes after herbicide stress, but the induction in the resistant leaves was consistently stronger in each case. Enhanced inducibility of antioxidant systems seems to be involved in resistance of tobacco to nitrodiphenyl ether and N-phenylimide stress.

INTRODUCTION

The herbicidal mode of action of acifluorfen and related nitrodiphenyl ethers has been recently discovered (Matringe & Scalla, 1988). In addition to their effects on the secondary metabolism of plants (Komives & Casida, 1983), they inhibit the biosynthesis of chlorophyll thereby leading to an accumulation of photooxidative tetrapyrroles (Lydon & Duke, 1988; Yanase & Ando, 1989; Sato et al., 1991). Phytotoxicity in the light is a consequence of oxidative stress by increased production of reduced, active oxygen derivatives (Matringe & Scalla, 1988). Herbicidal effects of the experimental compound S-23142 (N-(4-chloro-2-fluoro-5-propargyloxyphenyl)-3,4,5,6-tetrahydrophthalimide) are due to the same mechanism of action (Sato et al., 1991).

Leaf cells contain superoxide dismutase (SOD, E.C. 1.15.1.1.) to decompose superoxide radical anions and an efficient ascorbate (AA) - glutathione (GSH) system in the chloroplast to scavenge hydrogen peroxide (Halliwell & Foyer, 1978; Nakano & Asada, 1981). Ascorbate peroxidase (AP, E.C. 1.11.1.11.) and

glutathione reductase (GR, E.C. 1.6.4.2.) participate in this system. Higher levels of these two enzymes and that of SOD were found in the chloroplast stromal extracts (but not in total cell extracts) of a paraquat resistant biotype of Conyza bonariensis (Shaaltiel et al., 1988).

Cucumber leaf discs treated with acifluorfen contained strongly decreased levels of GSH and GR activity (Kenyon & Duke, 1985). In contrast, bean leaves responded to exposure to this herbicide by increased concentrations of GSH and elevated activity of GR (Schmidt & Kunert, 1986). The increased level of antioxidant systems was suggested to be a general strategy to limit toxic peroxidation (Schmidt & Kunert, 1986).

GSH S-transferase enzymes (GST, E.C. 2.5.1.18.) catalyse the reactions of GSH with many xenobiotics and electrophilic metabolites. These conjugation reactions, with few exceptions, are considered as detoxication processes. In addition to this role, GST enzymes may also function as GSH peroxidase by catalysing the reaction between GSH and lipid hydroperoxides (Komives & Dutka, 1989). The inducibility of GST isoenzymes by herbicide safeners has been described in higher plants (Komives, Dutka, 1989; Dean et al., 1990).

Increased activity of SOD was measured in a superoxide-resistant tobacco biotype which had been regenerated from callus after in vitro selection by paraquat (Furusawa & Mizuguchi, 1988). In the present study the hydrogen peroxide-scavenging systems have been examined in this superoxide-resistant biotype of tobacco. In order to gain a deeper insight in the mechanism of herbicide resistance the effects of the nitrodiphenyl ethers acifluorfen and bifenox and the N-phenylimide S-23142 on these redox systems have been investigated.

MATERIALS AND METHODS

Plant material

Seeds of in vitro selected superoxide-resistant and sensitive tobacco plants Nicotiana tabacum L., cv. Samsun, were obtained from Dr.I. Furusawa, Kyoto University. They were planted in individual pots containing sand and soil (1:1). Plants were grown in a greenhouse at 18-23 ^{o}C with supplemental light at 160 μE m^{-2} s^{-1} for 8 hours per day. Leaves of 65-70 d plants were excised from identical leaf positions and were treated by placing their petioles in solutions of herbicides (1 μM - 1 mM or up to solubility limits for diphenyl ethers; 10 nM - 10 μM for S-23241),or in tap water. Leaves were exposed to continuous light (120 μE m^{-2} s^{-1} at 22 ^{o}C).

Enzyme assays

Leaf material (1 g) was homogenised in 3 ml cold 0.2 M TRIS/HCl buffer pH 7.8 containing 3% soluble polyvinylpyrro-

lidone and 0.1 mM EDTA-Na. The homogenate was strained through muslin and centrifuged at 8,000 g for 20 min. The supernatants were used as enzyme source.

Activity of AP was determined according to Nakano & Asada (1981), except that in our experiments 0.5 mM hydrogen peroxide and 0.25 mM AA were used. GR (Halliwell & Foyer, 1978) and GST (Habig et al., 1974) activities were assayed spectrophotometrically.

Other assays

The spectrophotometric methods of Mukherjee & Choudhuri (1983), De Kok & Graham (1989), and Smith (1985) were used to determine the levels of AA, non-protein thiols, and GSH, respectively.

RESULTS

Hydrogen peroxide-scavenging systems in untreated plants

In the leaves of superoxide-resistant tobacco plants the AA content was approximately 40% higher than in the sensitive ones. However, the level of non-protein thiols and the activities of AP, GR and GST did not differ significantly between the two biotypes. Glutathione accounted for 76 % of the non-protein thiol content both in the resistant and sensitive plants.

Herbicide resistance of leaves

Herbicide phytotoxicity was tested at various concentrations. The severity of phytotoxic symptoms increased at higher doses and longer treatment times, with tissue necrosis requiring at least 2-5 days to appear. The sequential symptoms of the leaves were appearance of water soaked spots, yellowing, wilting and desiccation first evident at 24 h and loss of chlorophyll usually after the appearance of first necrotic lesions. Leaves of the superoxide resistant plants proved to be tolerant to both nitrodiphenyl ethers and S-23142. This latter compound caused phytotoxic symptoms at much lower concentrations than the nitrodiphenyl ethers investigated. The resistant leaves were able to tolerate 10 μM S-23142, 250 μM acifluorfen and 750 μM bifenox for 96 h.

Nitrodiphenyl ether stress

Nitrodiphenyl ethers at 50 μM considerably altered the level of hydrogen peroxide-scavenging systems in tobacco leaves under continuous illumination. In leaves stressed by these herbicides the AA content increased equally in both biotypes after 24 h, then declined to the control level (Tables 1-4).

TABLE 1. Effect of 50 μM acifluorfen and bifenox and 2 μM S-23142 on hydrogen peroxide-scavenging systems in sensitive tobacco leaves after 48 h exposure under continuous illumination

	Control	Acifluorfen	Bifenox	S-23142
Ascorbic acid (μmol/g FW)	1.92 ±0.20	2.73 ±0.15	2.06 ±0.18	2.34 ±0.24
Non-protein thiols (μmol/g FW)	0.32 ±0.03	0.74 ±0.0	0.49 ±0.01	0.80 ±0.10
Ascorbate peroxidase (μmol AA/g FW/min)	20.3 ±2.0	19.5 ±1.7	18.1 ±1.9	20.9 ±1.9
GSH reductase (μmol NADPH/g FW/min)	0.31 ±0.03	0.72 ±0.02	0.46 ±0.06	0.58 ±0.07
GSH S-transferase (μmol/g FW/min)	0.16 ±0.02	2.42 ±0.30	0.61 ±0.08	1.55 ±0.10

TABLE 2. Effect of 50 μM acifluorfen and bifenox and 2 μM S-23142 on hydrogen peroxide-scavenging systems in resistant tobacco leaves after 48 h exposure under continuous illumination

	Control	Acifluorfen	Bifenox	S-23142
Ascorbic acid (μmol/g FW)	2.81 ±0.24	3.59 ±0.25	3.15 ±0.32	4.16 ±0.14
Non-protein thiols (μmol/g FW)	0.27 ±0.03	1.04 ±0.02	0.5 ±0.07	0.87 ±0.10
Ascorbate peroxidase (μmol AA/g FW/min)	20.3 ±2.2	31.7 ±1.9	26.8 ±3.0	25.8 ±2.7
GSH reductase (μmol NADPH/g FW/min)	0.30 ±0.03	1.13 ±0.02	0.59 ±0.03	0.70 ±0.05
GSH S-transferase (μmol/g FW/min)	0.14 ±0.03	4.72 ±0.29	1.04 ±0.10	2.21 ±0.30

Nitrodiphenyl ether stress resulted in a significant increase of non-protein thiol content both in the resistant and sensitive leaves. In sensitive leaves the thiol content reached 232 % of the untreated controls after 48 h exposure to acifluorfen, then declined to the control level after 96 h. In resistant leaves the thiol content increased more rapidly. Thiols reached significantly higher levels than in the sensitive leaves and their level remained above that of the untreated control throughout the experimental period (Tables 1-4).

Significantly increased ascorbate peroxidase activity was found in the resistant, but not in the sensitive biotype when leaves were exposed to nitrodiphenyl ether herbicides. The AP activity in the resistant leaves reached five times that of the control after 96 h of treatment with acifluorfen.

TABLE 3. Effect of 50 μM acifluorfen on the hydrogen-peroxide scavenging systems at various exposure times in sensitive tobacco leaves under continuous illumination

	Content/activity*, % control			
	24 h	48 h	72 h	96 h
Ascorbic acid	173	165	96	83
Non-protein thiols	164	232	184	118
Ascorbate peroxidase	112	96	96	83
GSH reductase	141	232	346	392
GSH S-transferase	882	1512	1720	1488

*See Table 1 for units.

TABLE 4. Effect of 50 μM acifluorfen on the hydrogen peroxide-scavenging systems at various exposure times in resistant tobacco leaves under continuous illumination

	Content/activity*, % control			
	24 h	48 h	72 h	96 h
Ascorbic acid	140	128	104	87
Non-protein thiols	362	360	297	225
Ascorbate peroxidase	128	153	244	516
GSH reductase	283	377	565	890
GSH S-transferase	1315	3371	2807	2135

*See Table 1 for units.

Nitrodiphenyl ethers increased the activity of GR in both biotypes, however the induction was stronger in the resistant leaves during the entire course of treatment than in the leaves of the sensitive biotype. After 96 h of exposure to acifluorfen the GR activity was nine times that of the control in the resistant leaves.

Nitrodiphenyl ether treatment led to a massive and rapid induction of GST in both biotypes. Similar to the results obtained with GR, this induction was more consistent in the resistant leaves.

S-23142 stress

S-23142 affected the above processes very similarly to those of the nitrodiphenyl ethers. However, it proved to be a much more effective inducer: micromolar concentrations of this compound resulted in great changes in all the parameters investigated in this study (Tables 1 and 2).

DISCUSSION

Differential sensitivity of plants to the herbicides paraquat and acifluorfen has been attributed to alterations in the capability of the plants to detoxify active oxygen species generated by the herbicide (Shaaltiel et al., 1988; Schmidt & Kunert, 1986). Thus, higher levels of SOD, AP and GR were detected in Conyza bonariensis plants that were resistant to paraquat (Shaaltiel et al., 1988). Increased synthesis of GSH (Farago & Brunold, 1990) and induction of GR (Smith et al., 1989) have been observed in plants after various stress effects, including acifluorfen stress (Schmidt, Kunert, 1986).

The in vitro-selected paraquat- (superoxide-) resistant tobacco plants used in our experiments proved to be cotolerant to nitrodiphenyl ethers and the N-phenylimide S-23142. The leaves of resistant tobacco plants contained more AA (by 40%) than the sensitive ones. This phenomenon can contribute to the resistance against oxidative damage not only because AA participates in the scavenging of hydrogen peroxide but also because of its role in the direct regeneration of vitamin E under peroxidative conditions (Finckh & Kunert, 1985). However, the differences in the contents of AA, non protein thiols, as well as in the activities of AP, GR, and GST in the paraquat resistant and susceptible tobacco biotypes were rather small or insignificant to account for the degree of the observed herbicide resistance.

Exposure of the plants to herbicides that generate active oxygen derivatives, however, led to changes of sufficient magnitude to explain resistance. The nonprotein thiol content significantly increased in tobacco leaves exposed to the above herbicides and to a lesser extent in the sensitive leaves. Glutathione was found to be the major thiol in the tobacco biotypes according to earlier reports (Rennenberg, 1982).

Nitrodiphenyl ether and N-phenylimide stress markedly increased the activities of AP, GR and especially that of GST. The rate of induction was higher in each case in the resistant leaves. Induction of GST by chloroacetamide and thiolcarbamate herbicides in maize, sorghum and rice (Komives & Dutka, 1989) and by herbicide safeners in sorghum (Dean et al., 1990) has been described. The mechanism of induction is unknown. Our findings show that oxidative stress may also stimulate GST activity and this response may take place also in a dicotyledonous plant. The results of this study indicate the possibility of decreasing plant susceptibility to nitrodiphenyl ethers, N-phenylimides or to other types of oxidative environmental stress by preliminary application of low doses of these herbicides for stimulating plant defense mechanisms. Experiments are in progress to investigate this hypothesis.

The observation that superoxide-resistant tobacco responds to oxidative stress more rapidly and with higher activity than the sensitive one suggests that herbicide resistance is due to the enhanced inducibility of the detoxification systems in the resistant plants.

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A COMPARATIVE STUDY OF THE EFFECTS OF THREE BLEACHING HERBICIDES ON CAROTENOID BIOSYNTHESIS IN GALIUM APARINE.

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ABSTRACT

Seedlings of *Galium aparine* were treated, post-emergence, at the 1-2 leaf stage with several bleaching herbicides (diflufenican, amitrole and dichlormate). The chlorophyll, carotenoid and carotenoid-precursor content of the leaf tissue was monitored as a function of leaf age and development using a rapid screening technique involving high-performance liquid chromatography. Following treatment, newly developed tissues emerged bleached and showed the accumulation of a number of carotenoid precursors. Diflufenican treatment resulted in the accumulation of phytoene and phytofluene whereas dichlormate caused accumulation of all the desaturation products leading to ζ-carotene. Amitrole treatment caused desaturation products and lycopene to accumulate. Significantly, the cotyledon tissue which was present at the time of spraying and which remained green throughout the course of the experiment, also showed the accumulation of high levels of precursors in the presence of the normal chloroplast pigments. Differences in the geometric and hydroxylated nature of the accumulated precursors were found to differ as a function of leaf age and development.

INTRODUCTION

The carotenoids of higher plant photosynthetic tissues are synthesised and located within the chloroplast where they serve two important functions; light-harvesting and photoprotection. The second of these processes is generally thought to be essential for the survival of the plant through the quenching of triplet-excited chlorophyll molecules and the scavenging of singlet oxygen. Inhibition of carotenogenesis by herbicide action (or stress conditions) may impair the normal photoprotective mechanism resulting in damage to the plant. Carotenoids also play an important role in the construction of the photosynthetic apparatus, thus their absence will affect the structure of the pigment-protein complexes and the incorporation of the chlorophyll molecules.

The interference in the biosynthetic pathway of these carotenoids is therefore an attractive target site for a number of herbicide molecules. Several of these "bleaching" herbicides are known to cause chlorosis of treated photosynthetic tissues as a consequence of a reduction in, or the complete absence of the normal chloroplast carotenoids (Ridley, 1977). These herbicides may directly inhibit the desaturation and / or cyclisation reactions leading to the formation of α- and ß-carotene and the xanthophylls.

The action of a number of herbicides that cause inhibition of carotenoid biosynthesis have been investigated in this study. Diflufenican is a potent inhibitor of phytoene desaturase in a range of plant species (Britton et al, 1987; Barry and Pallett, 1988). Dichlormate acts through the inhibition of desaturation reactions up to ζ-carotene desaturase (Burns et al, 1971; Barry and Pallett, 1990), whereas amitrole will block desaturation reactions and lycopene cyclisation (Britton et al, 1989; Barry and Pallett, 1990; Young et al, 1990).

The application of these inhibitors to plant tissues will result in the accumulation of a range of biosynthetic intermediates caused by the inhibition of the following biosynthetic step. Previous studies have used a rapid screening technique to detect and characterise these intermediates in small amounts of treated plant tissue using diode-array high-performance liquid chromatography (Britton et al, 1987; Young et al, 1990; Barry and Pallett, 1988, 1990). This technique has been employed in this study in order to examine the effect of the post-emergence application of these herbicides on *Galium aparine*. Leaf tissues, representing the developmental stages of the plant (cotyledons, 1st. leaf and emerging leaves), were removed for pigment analysis at regular intervals. The nature and the levels of the precursors produced in the different developmental stages of the plant are discussed.

MATERIALS AND METHODS

Plant material.

The plant species used in this study was *Galium aparine* which was maintained under glasshouse conditions with supplemental lighting. For the post-emergence application of the herbicides plants at the 1-2 leaf stage were sprayed with 250 g/ha technical grade diflufenican (N-(2,4-difluorophenyl)-2-(3-trifluoromethylphenoxy)-3-(pyridine carboxamide), 1.0 Kg/ha dichlormate (3,4-dichlorobenzyl methyl carbamate) or 1.0 Kg/ha amitrole (1 H-1,2,4-triazol-3-ylamine). These compounds were dissolved in acetone and sprayed using a laboratory pot sprayer. Control plants were treated with acetone. Following treatment, leaf material, reflecting the different developmental stages of the plant, i.e. cotyledon, 1st. leaf and emerging leaves, was removed and weighed. These were immediately extracted for pigment analysis.

Extraction of pigments

The leaf material (approx. 100mg) was homogenised in ethanol (dried over molecular sieve) and the homogenate filtered through a cotton wool plug. The solvent was evaporated under a steady stream of nitrogen and the samples stored dry, under a nitrogen atmosphere at -20^{o}C prior to analysis by high-performance liquid chromatography.

High-performance liquid chromatography

Carotenoids, chlorophylls and carotenoid precursors were separated and quantified on a Spherisorb ODS2 reversed-phase column (25.0 x 0.46 cm) using the chromatographic conditions described in Young et al (1990).

RESULTS AND DISCUSSION

Plants were treated post-emergence with the bleaching herbicides at the 1-2 leaf stage and monitored for up to 7 days following spraying. A visual examination of the plants showed that the existing tissues (cotyledons) remained green following treatment. In contrast, the newly developing tissues, 1st. leaf and emerging leaves, were either partially or heavily bleached respectively. Similar observations were made by Barry and Pallett (1990) in developing *Ipomea* seedlings but no examination of the precursor content of these tissues was made.

An initial analysis of control and treated tissues by HPLC was performed. In the control, untreated, tissues the normal higher plant pigment composition was found. Importantly, no precursors in the carotenoid biosynthetic pathway were detected in these tissues. In contrast, the separation of pigments from leaves from the same growth stage treated post-emergence with diflufenican showed that the normal, cyclic, carotenoids and chlorophylls are much reduced or absent. A number of precursors were detected in these extracts; phytoene, phytofluene, mono-hydroxyphytoene, lycopene and ζ-carotene. A much more rigorous analysis of the different developmental stages was then performed.

Cotyledons

The total chlorophyll and total coloured carotenoid content of the cotyledons of *G. aparine* following treatment are shown in Fig. 1. Although visually these tissues remained green and healthy throughout the course of the experiment, there is clearly a marked decrease in their pigment content with all herbicides. Dichlormate-treated cotyledons, however, showed necrotic lesions after 7 days. In all cases the ratio of chlorophyll a : b was constant (data not shown); i.e. there was no preferential loss of chlorophyll a which would indicate photodestruction of the reaction centres.

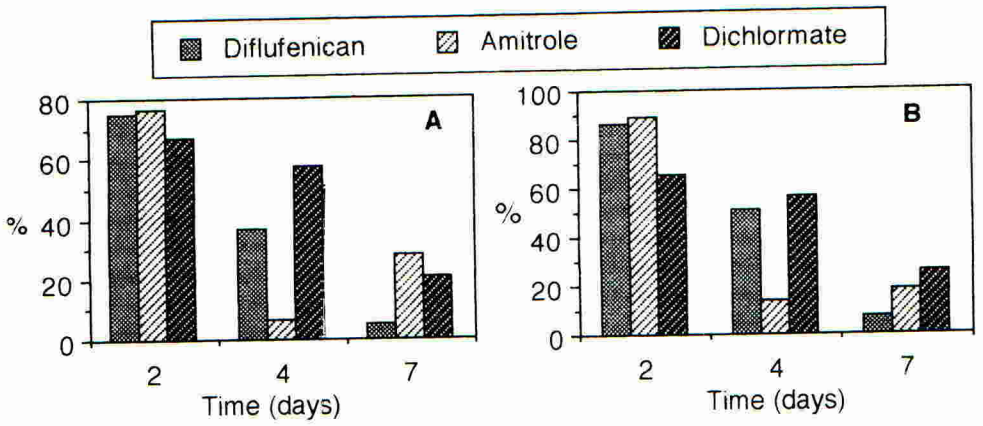

FIGURE 1. Total chlorophyll (A) and total coloured carotenoid (B) content of cotyledons of *G. aparine* following treatment with bleaching herbicides. The data shown are expressed as a percentage of the values for the control tissues and are the mean of 2 determinations.

The accumulation of precursors in the cotyledons as a result of herbicide treatment is shown in Table 1. Treatment with diflufenican resulted in an accumulation of mainly Cis-phytoene, together with a small amount of all-Trans-phytoene, and phytofluene. Towards the end of the experiment, when degradation of cellular components was likely, an increasing proportion of the accumulated precursors was made up of mono-hydroxyphytoene. No attempt was made to fully characterize what is a complex mixture of different geometrical isomers, some of which may result from the photo-sensitized oxidation of phytoene (Musker, D., Young, A. and Britton, G. unpublished data). The significance, if any, of mono-hydroxyphytoene in the biosynthesis of the chloroplast carotenoids is not known. Clearly, diflufenican inhibited the turnover of chloroplast carotenoids in these green tissues by inhibiting desaturation

Treatment with amitrole initially resulted in the accumulation of ζ-carotene. Other intermediates such as phytoene (Cis- and all-Trans-), mono-hydroxyphytoene and lycopene were also detected after 4 and 7 days. This shows that amitrole inhibits both desaturation and cyclisation reactions in *G. aparine*. Dichlormate also resulted in a range of accumulated intermediates in the carotenoid biosynthetic pathway; phytoene (Cis- and all-Trans-) phytofluene, mono-hydroxyphytoene and, especially, ζ-carotene. No evidence for the inhibition of cyclisation reactions was seen.

	Diflufenican			Amitrole			Dichlormate		
DAY	2	4	7	2	4	7	2	4	7
PRECURSOR									
Cis-phytoene	0.232	0.319	0.199	n.d.	0.006	0.011	0.171	0.085	0.023
Trans-phytoene	0.043	n.d.	n.d.	n.d.	0.013	0.011	0.069	0.109	n.d.
Cis-phytofluene	0.023	0.052	0.010	n.d.	n.d.	n.d.	0.060	0.101	0.010
Trans-phytofluene	n.d.	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	n.d.	n.d.
ζ-carotene	n.d.	n.d.	n.d.	0.027	0.166	0.016	1.093	2.060	0.027
lycopene	n.d.	n.d.	n.d.	n.d.	0.013	0.008	n.d.	n.d	n.d.
OH-phytoene	n.d.	0.053	0.064	n.d.	0.009	0.009	0.017	0.044	0.019
Total precursors	0.298	0.424	0.273	0.027	0.228	0.115	1.631	2.737	0.253
Ratio Chl:Carot	4.100	3.100	2.270	4.080	2.100	5.470	4.880	4.430	2.840
Ratio Col: Non-col Carot	5.940	2.010	1.170	0.228	1.590	2.160	2.870	0.440	2.350

TABLE 1. Precursor content of cotyledons of *Galium aparine* (μg / g FW) following treatment with bleaching herbicides. The data shown are the means of at least 2 separate determinations by high-performance liquid chromatography.

1st. leaf and emerging leaves

These tissues were either partially developed (1st. leaf) or absent when spraying took place. Subsequent development during the course of the experiment produced partial chlorosis of the 1st. leaf and complete bleaching of the emerging leaves. The effect of the 3 herbicides on the pigment composition of these leaves is shown in Figure 2. The full time course is not represented due to lack of development or tissue degradation. Whereas diflufenican reduced the chlorophyll and carotenoid content of the 1st. leaf and emerging leaves, the application of the other compounds, amitrole and dichlormate, initially resulted in an increase in the levels of both classes of pigment on a μg/g F.W. basis. These herbicides are known to have additional effects on growth and development of the plant and may have suppressed overall cell growth and expansion without specifically suppressing chloroplast development. Levels of chlorophylls and coloured carotenoids are particularly low in the emerging leaves, where levels are as low as 10% of the control tissues at the same stage of development. The ratio of chlorophyll a : b in these tissues remained constant during the course of the experiment.

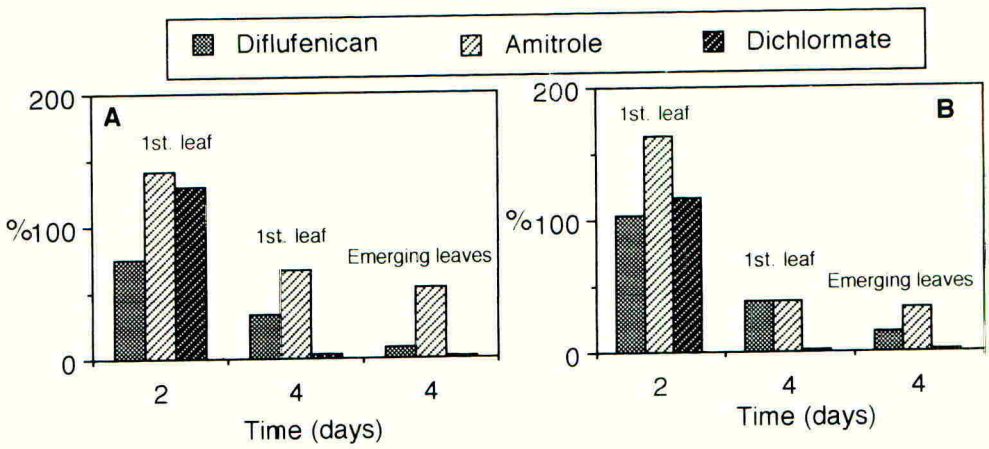

FIGURE 2. Total chlorophyll (A) and total coloured carotenoid (B) content of the 1st. leaf and emerging leaves of *G. aparine* following treatment with bleaching herbicides. The data shown are expressed as a percentage of the values for the control tissues and are the mean of 2 determinations.

	Diflufenican		Amitrole		Dichlormate	
Day	2	4	2	4	2	4
PRECURSOR						
Cis-phytoene	0.248	0.491	n.d.	0.026	0.084	0.021
Trans -phytoene	0.057	0.173	n.d.	n.d.	0.032	n.d.
Cis-phytofluene	0.010	0.367	n.d.	0.016	n.d	0.013
Trans-phytofluene	0.006	0.040	n.d.	n.d.	n.d.	0.003
ζ-carotene	n.d	n.d.	0.009	0.452	0.009	0.124
lycopene	n.d.	n.d.	n.d.	0.035	n.d.	n.d.
OH-phytoene	0.026	0.158	n.d.	n.d.	n.d.	n.d.
Total precursors	0.347	1.229	0.009	0.530	0.124	0.161
Ratio Chl:Carot	3.040	3.320	3.680	6.270	14.81	0.290
Ratio Col: Non-col Carot	4.660	0.800	0.009	2.010	0.880	0.380

TABLE 2. Precursor content of the first leaves of *Galium aparine* (μg / g FW) following treatment with bleaching herbicides.

The nature of the precursors accumulated in these developing tissues as a result of herbicide action is shown in Tables 2. and 3. In the 1st. leaves the relative amount of mono-hydroxyphytoene increases greatly when compared to the levels of phytoene following diflufenican treatment, perhaps indicative of photooxidative production from phytoene. Levels of phytofluene also accounted for a much higher proportion of the total precursors 4 days after treatment. This was also observed in the cotyledons with diflufenican treatment. Again, the significance of this is not fully understood.

	Diflufenican	Amitrole	Dichlormate
Day	4	4	4
PRECURSOR			
Cis-phytoene	0.059	0.029	0.003
Trans-phytoene	0.005	n.d.	n.d.
Cis-phytofluene	0.054	0.029	n.d.
Trans-phytofluene	0.008	n.d	n.d.
ß-carotene	n.d	0.201	0.011
lycopene	n.d	0.017	n.d
OH-phytoene	0.033	0.020	n.d.
Total precursors	0.158	0.296	0.015
Ratio Chl. Carot	2.010	5.570	4.860
Ratio Col. Non-col Carot	0.730	1.310	0.880

TABLE 3. Precursor content of the emerging leaves of *Galium aparine* (µg / g FW) following treatment with bleaching herbicides. The data shown are the means of at least 2 separate determinations by high-performance liquid chromatography.

In plants treated with diflufenican, the 1st. and emerging leves contained high levels of both cis- and trans-isomers of phytoene and phytofluene, and of mono-hydroxyphytoene, levels of which increased over time. In contrast, this pattern was not seen in the cotyledon tissues.

CONCLUSIONS

Treatment of *G. aparine* at the 1-2 leaf stage with diflufenican, amitrole or dichlormate resulted in the formation of newly developed tissue which was highly bleached. Examination of these tissues showed an expected carotenoid

precursor composition, indicating inhibition of desaturation and cyclisation reactions. Importantly, the cotyledons which were present at the time of spraying also showed the presence of high levels of precursors, even though the tissue remained green. Levels of precursors in the cotyledons were often much higher than those recorded for the bleached tissues. Subtle differences in the nature of these precursors were observed for the different developmental stages of the plant. The bleaching of newly developed tissues clearly reflects that inhibition of desaturation and cyclisation reactions prevents coloured carotenoid biosynthesis. This appears essential for normal chloroplast development as this bleached tissues contain plastids at the proplastid stage (Barry and Pallett, 1988).

The precursor accumulation data for dichlormate and amitrole indicate a multisite action on carotenoid biosynthesis. However, the enzymes involved in these reactions are membrane bound and it is likely that dichlormate and amitrole inhibit only the desaturation of ζ-carotene and lycopene cyclisation respectively and the accumulation of earlier precursors seen in this study may be due to a feedback effect on the earlier reactions.

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INTERACTION OF MAIZE CYTOCHROME P_{450} WITH SAFENERS AND 1-AMINOBENZOTRIAZOLE

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ABSTRACT

The inhibitory action of EPTC, EPTC-sulfoxide (EPTC-SO), 1-aminobenzotriazole (ABT) and safeners on cytochrome P_{450} *in vitro* was investigated spectrophotometrically on microsomes of etiolated maize seedlings. While EPTC, EPTC-SO, ABT, N,N-diallyl-2,2-dichloroacetamide (dichlormid) and 2-dichloromethyl-2-methyl-1,3-dioxolane (MG-191) were not inhibitors of oxidised enzyme at mM levels, naphthalic anhydride blocked the enzyme at 100 μM. Metabolic intermediate complexes were not detected after a short incubation of chemicals and microsomes in the presence of NADPH. Under metabolic reducing conditions both ABT and safeners markedly decreased the cytochrome P_{450} level in the reaction medium indicating that the safeners acted in a similar manner to ABT.

INTRODUCTION

Based on mammalian analogies and insecticide synergist experiments it has been speculated that cytochrome P_{450}-monooxygenase enzymes may play a role in the plant metabolism and phytotoxicity of the thiocarbamate herbicide EPTC (Casida *et al.*, 1975). Moreover, according to recent results and hypotheses these enzymes are involved in safener action as well (Fedtke, 1987).

Because of the difficult detection of plant cytochrome P_{450} limited direct data on the influence of thiocarbamates or safeners on the level and activity of the maize enzyme were available (Kőmíves & Dutka, 1989).

Using the advantages of a diode-array spectrophotometer a program was started to clarify the interaction of the chemicals and enzymes mentioned. We have reported that the known plant cytochrome P_{450} inhibitor ABT safened maize against EPTC phytotoxicity (Barta & Dutka, 1991) and the cytochrome P_{450} level of this plant was elevated by pretreatments of EPTC and safener combinations (Barta & Dutka, 1990).

These results suggest that cytochrome P_{450} enzymes can oxidise EPTC to EPTC-SO thereby increasing detoxication of parent molecule and that the safening effect is at least partially due to safener-cytochrome P_{450} interactions. The objective of the present study was to determine if EPTC, EPTC-oxides, ABT, and various safeners are *in vitro* inhibitors of microsomal cytochrome P_{450} of etiolated maize seedlings.

MATERIALS AND METHODS

Chemicals

EPTC (boiling point 112 °C/2.1 kPa) and NA (melting point 268-269 °C) were purified commercial samples. EPTC-SO, ABT, dichlormid, and MG-191 were prepared in this laboratory by literature methods.

Plant material

Forty grams of maize (Pioneer 3737) grains were washed five times with 200 ml water, planted in a plastic pot filled with a mixture of 1500 g river sand and 250 ml of water and kept for 5 days in the dark at 25 °C and 100 % relative humidity.

For NA pretreatment 15 g of washed maize grains were placed at the top of the three-quarters of the moistened sand. The grain layer was then sprayed with a mixture of 0, 2.5, 5, 10 or 75 mg NA and 5 ml 0.1 % Tween 20 in water and covered by the remaining quarter of the sand.

Isolation of microsomal fraction

Maize microsomal fractions were isolated by the method of Vanden Bossche *et al.* (1987) from 40 g etiolated shoot portions (2 pots) while microsomes of NA pretreated seedlings were prepared from one pot.

Spectrophotometric analyses

Instruments

Spectra were recorded on HP 8452A Diode-array UV/Visible Spectrophotometer (Hewlett-Packard, Palo Alto, U.S.A.) coupled with HP 89500 UV/VIS ChemStation PC controller (Hewlett-Packard) in subtract spectra task mode using air as blank. The signal averaging time was 5s throughout these experiments.

Determination of cytochrome P_{450} content

The cytochrome P_{450} content of the microsomal fractions was determined by measuring the carbon monoxide difference spectrum of the reduced enzyme (Vanden Bossche *et al.* 1987).

The subcellular fraction was diluted in 0.1 M potassium phosphate buffer (pH=7.4) to give a cytochrome P_{450} content of approximately 0.1 µM and poured into a cuvette. The cytochrome P_{450} isozymes were reduced for 1 minute with a few grains of sodium dithionite and a spectrum was recorded. The cuvette was saturated with carbon monoxide by bubbling the gas for 45 seconds and closing tightly. One minute later a second spectrum was recorded and the difference spectrum was computed. The enzyme concentration was calculated from changes in the absorbance increment between 450 and 490 nm using 91 $cm^{-1}mM^{-1}$ extinction coefficient.

Since one spectrum was computed from 50 scans concentrated samples having up to 2 absorbance units were measured with small standard error.

Effect of chemicals on unreduced enzymes

Five µl dimethyl sulfoxide (DMSO) or solutions of chemicals dissolved in DMSO were added to 1245 µl diluted microsomes before reduction by dithionite. After standing for one minute cytochrome P_{450} concentration was

determined as described above.

In other experiments a standard spectrum of the microsomal solution containing blank DMSO was retained by the computer. After recording the same spectrum in the presence of the chemicals difference spectra were computed from these spectra for all of the chemicals. Because of relatively high absorption of NA at investigated concentrations a further subtraction of the spectrum of NA dissolved in water was made.

Investigations under metabolic conditions

Incubation mixtures in phosphate buffer (pH=7.4, 1.25 ml) contained: microsomes equivalent to 0.1 μM cytochrome P_{450}; 50 μM NADPH; 5 μl DMSO and the chemical investigated.

Formation of metabolic intermediate complexes was investigated by subtracting of spectra of the same mixture after and before incubation for 5 minutes at 28 °C.

The mixtures were also incubated for 30 minutes at 28 °C. Decrease of the cytochrome P_{450} concentration was measured and compared to sample containing only DMSO.

RESULTS AND DISCUSSION

According to our earlier findings mixtures of EPTC with dichlormid or MG-191 safeners elevated the cytochrome P_{450} level of etiolated maize seedlings (Barta & Dutka, 1990). Ten μM dichlormid pretreatment or phytotoxic 5 g/kg NA seed dressing increased the level of this enzyme about by 20 percent as well. In TABLE 1. we show that a non-phytotoxic rate of NA seed dressing (0.66 g/kg) significantly elevates the enzyme level investigated providing proof both to the direct influence of NA on cytochrome P_{450} and explanations for effectiveness of this safener against diverse types of herbicides (Hatzios, 1989).

TABLE 1. Effect of NA seed dressing on cytochrome P_{450} level of etiolated corn seedlings*.

NA dose (g/kg)	0	0.16	0.33	0.66	5.0
Cytochrome P_{450} (%)**	100[a]	93[a]	105[a]	140[b]	113[ab]
Standard deviation***	29	25	26	20	90

* Values followed by the same letter are not significantly different at the 5 % level.
** Percentage of untreated control.
Cytochrome P_{450} level = 97 pmole/mg protein.
***Calculated from six replicates.

The cytochrome P_{450} enhancing effect of NA corresponds to the mechanism of EPTC safeners previously suggested (Casida *et al.*, 1975). Namely, an increase in the level of a specific cytochrome P_{450} may cause faster sulfoxidation and subsequent glutathione conjugation of EPTC thereby causing more rapid detoxication. However, it is known that cytochrome P_{450} inducers

in vivo may be inhibitors *in vitro* (Ortiz de Montellano & Reich, 1986). Because of the protecting effect of the plant cytochrome P_{450} inhibitor ABT and its derivatives the question arises whether a similar situation exists in the case of safeners as well. Moreover, an enzyme induction and inhibitory effect was reported in cultured wheat cells for oxime ether safeners (Mougin *et al.*, 1991). Consequently, our chemicals were tested for possible enzyme inhibition.

In the first experiments microsomes containing cytochrome P_{450} were treated with chemicals before reduction according to Ortiz de Montellano *et al.* (1984) who showed that mammalian cytochrome P_{450} was inhibited by ABT. Under these conditions neither DMSO, nor EPTC, EPTC-SO, ABT, dichlormid and MG-191 at 100 μM, nor ABT and MG-191 at 1 mM were inhibitory. It is interesting to note that these chemicals had no effect on the enzyme activity *in vivo* as well. At the same time NA markedly decreased the remaining enzyme activity (TABLE 2.). The IC_{50} concentration was similar to that of other plant cytochrome P_{450} inhibitors i.e. 50 μM.

TABLE 2. Effect of NA treatment on cytochrome P_{450} levels in microsomes prior to dithionite reduction.

NA (μM)	0	10	20	50	100	1000
Cytochrome P-450 (%)*	100	89	82	53	5	0

*Percentage of control treated by DMSO.
Cytochrome P_{450} level = 0.15 μM.

This cytochrome P_{450} inhibitory effect is generally accompanied by the formation of an enzyme-inhibitor complex which has a typical spectrum with a maximum in the region of 380-430 nm. This spectrum was found for NA only by very precise comparison when the difference spectrum was corrected for absorption of the inhibitor NA. The maximum appeared at 405 nm and had an extinction coefficient similar to that of the carbon monoxide complex (the spectrum not shown here). Binding spectra were not seen with the other non-inhibitor chemicals.

Appearance of cytochrome P_{450} inhibition may require the presence of NADPH, the natural reductant of this P_{450} (Ortiz de Montellano & Reich, 1986). Sometimes inhibitors form metabolic intermediate complexes after short incubation periods which can be demonstrated by spectroscopy. Neither ABT nor MG-191, even at 1 mM, gave this spectrum after 5 minutes incubation.

Metabolism of inhibitors may take place after prolonged incubation with cytochrome P_{450}. Inhibitors acting by suicide mechanisms, for instance ABT on mammalian cytochrome P-450, are simultaneously metabolised to end-products and in a minor part to reactive intermediates which deactivate the enzyme (Ortiz de Montellano & Reich, 1986). Other inhibitors form only reversible enzyme-substrate complexes. When chemicals were incubated for 30 minutes under these conditions an ABT-like inhibition was observed (TABLE 3.). Namely, after incubation a decreased or zero cytochrome P-450 level was found in the reaction medium indicating that carbon monoxide could not displace the inhibitor from the complex formed.

Data of TABLE 3. make it probable that the inhibitory action of ABT in maize and mammalian cytochrome P_{450} is similar. However, clarification of the exact mechanism would require isolation of ABT metabolites and the ABT-enzyme complex. It is remarkable that dichlormid and MG-191 safeners are not only more effective protecting agents *in vivo* but stronger inhibitors *in vitro*.

TABLE 3. Cytochrome P_{450} concentration of reaction medium after 30 minutes incubation in the presence of 0.4 % DMSO, 50 μM NADPH and 100 μM inhibitor.

Inhibitor	-	EPTC	ABT	MG-191	Dichlormid
Cytochrome P-450 (%)*	100	108	50	6	0
Standard deviation**	12	25	9	3	0

* Percentage of DMSO treated control.
Cytochrome P_{450} level = 0.15 μM.
**Calculated from 5 replicates.

Comparing the effect of NA and the other two safeners there is a clear difference in inhibitory action. NA elevates the cytochrome P_{450} level of maize in preplant application and can couple directly to the enzyme. The influence of dichlormid and MG-191 on cytochrome P_{450} is not so strong: they have no inducing effect and act as inhibitors under metabolic conditions. This difference exists in the safener action as well. NA is a more general but less effective safener than dichlormid or MG-191 (Hatzios, 1989).

The suicide mechanism of safeners proposed does not conflict with the known metabolic pathway of EPTC. The metabolism of a suicide inhibitor would lead only partially to formation of irreversible enzyme-substrate complex. Thus it is possible that safeners do not prevent but only slow EPTC oxidation so as to allow the GSH-GST system to detoxify EPTC-SO, the product of oxidation. Secondly, it is also possible that dichlormid and MG-191 accelerate EPTC detoxication by induction of the enzyme as was proposed above for the mechanism of NA.

In summary, NA, dichlormid and MG-191 safeners are inhibitors of cytochrome P_{450} enzyme of maize. The mechanism of inhibitory action of NA differs from that of the other compounds. The role of the inhibition of this enzyme by safeners and ABT in EPTC metabolism and phytotoxicity is under continued study in this laboratory.

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STUDIES ON THE ACTION OF THE NEW GROWTH RETARDANT CGA 163'935

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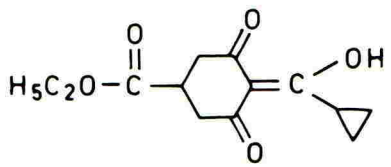
ABSTRACT

The new growth retardant CGA 163'935 acts on a wide variety of monocotyledonous and dicotyledonous plants and is a highly efficient antilodging agent in small grain cereals and oilseed rape as well as a growth inhibitor of turf grasses. In contrast to herbicidal cyclohexanediones, this compound does not interfere with fatty acid biosynthesis. Kaurene oxidase, a typical target of growth retardants, is unaffected by CGA 163'935. This paper shows, for barley as a model plant, that CGA 163'935 treatment strongly reduces the level of the active gibberellin, GA_1, while the level of its immediate biosynthetic precursor, GA_{20}, is elevated. Levels of other phytohormones analysed do not vary or are only slightly affected. *In vitro*, CGA 163'935 strongly inhibits GA_{20}-3ß-hydroxylase of barley seedlings. Growth effects of treated plants can be alleviated by the application of a physiologically active gibberellin (GA_3). Thus, CGA 163'935 appears to act selectively through a reduction of the level of active gibberellin in treated plants, by inhibiting the 3ß-hydroxylase required for the conversion of inactive gibberellins into the active, 3ß-hydroxylated hormones.

INTRODUCTION

The acylcyclohexanedione CGA 163'935 (ethyl 4-cyclopropyl(hydroxy) methylene-3,5-dioxocyclohexanecarboxylate), the active principle of Moddus®, is a new plant growth retardant developed by Ciba-Geigy Ltd., Basle, mainly for turfgrass inhibition and as an antilodging agent for cereals and oilseed rape (Amrein et al., 1989):



CGA 163'935

CGA163'935 is active on a wide variety of monocotyledonous and dicotyledonous species (Kerber et al., 1989) and rates of application vary from 100-600 g a.i./ha, depending on the crop. In contrast to herbicidal cyclohexanediones, this compound does not interfere with fatty acid

biosynthesis. More specifically, the enzyme acetyl-CoA-carboxylase (ACC) is not affected by CGA 163'935 up to 10^{-4} M. Efficient ACC inhibiting herbicides typically show IC_{50}-concentrations in the range of 10^{-7} M. The classical target of many growth retardants (e.g. triazoles, norbornenodiazetines) is the enzyme kaurene oxidase, located at an early stage of gibberellin biosynthesis. This enzyme is unaffected by CGA 163'935 up to 10^{-4} M. For comparison, triazole-type growth inhibitors are active in the 10^{-8} M range. Several acylcyclohexanediones have been shown to inhibit late steps in gibberellin biosynthesis which are catalysed by Fe^{2+}-dependent, ascorbate-stimulated, soluble dioxygenases that use 2-oxoglutarate as co-substrate (Hedden, 1991; see Rademacher, 1991, for a review). The resulting drop in the levels of active gibberellins is thought to be responsible for growth inhibition. The aim of the present investigation was to determine the mode of action of CGA 163'935 *in vivo* by comparing the levels of several phytohormones in treated as well as untreated barley and *in vitro* by determining the activity of the GA_{20}-3ß-hydroxylase in cell-free extracts from barley seedlings.

METHODS

Barley plants (*Hordeum vulgare* L. cv. 'Iban' or 'Golf') were grown in standard soil or vermiculite in phytotron chambers (day: 20 °C, 0.250 $mE/m^2/s^1$ PAR, night: 18 °C; 70 % rel. humidity, 11 h photoperiod). An emulsifiable concentrate (EC 250) of CGA 163'935 containing 250 g/l a.i. was suitably diluted with water and sprayed onto plants until run-off, or added to the vermiculite at the time of sowing. Plant tissues were harvested, frozen in liquid N_2 and extracted with 100 % and then 80 % methanol. The acidic hormone fraction was obtained by reversed-phase cartridge (C_{18}) chromatography using standard techniques (Weiler et al., 1986) [1-^{14}C]-IAA and GA_1-/GA_{20}-[7-O-C^3H_3]methylester were used as internal standards. ABA was determined by ELISA and IAA by HPLC-ELISA according to Weiler et al. (1986). Phaseic acid was determined by ELISA (Weiler, in prep.) and GA_5, GA_{20} and GA_1 were determined by HPLC-RIA using selective antisera whose cross-reactions against GA_1 to GA_{53} were known (Kurogochi and Weiler, unpublished). The basic technique was as in Yamaguchi et al. (1987). For the cell-free analysis of 3ß-hydroxylase activity, 20,000 g supernatants from 2d old etiolated barley seedlings were fractionated by ammonium sulfate precipitation similarly as in Albone et al. (1989). The proteins precipitating by 35-55% satd. ammonium sulfate were used as the source of enzyme. Incubation conditions are detailed in the results section. GA_{20} was used as the substrate.

RESULTS

In vivo studies

The application of low amounts of CGA 163'935 to barley seedlings resulted in a clear reduction of growth (Tables 1 and 2). The levels of indolylacetic acid (IAA) and phaseic acid (PA) in treated plants remained unaltered and abscisic acid (ABA) levels were somewhat increased. By contrast, CGA 163'935 drastically lowered the level of extractable GA_1,

TABLE 1. Growth and levels of several phytohormones in 12 day old barley seedlings, 5 days after treatment with CGA 163'935 (250 mg/l a.i., sprayed until run-off, control: EC blank).

Parameter	control	treated	% of control
Plant height	24.80± 1.80	18.30± 1.50	74
IAA	1540.00±110.0	1470.00±60.00	96
ABA	12.40± 0.64	18.10± 0.81	146
PA	94.00± 7.90	86.00±15.10	91
GA_1	1.33± 0.15	0.36± 0.12	27
GA_{20}	1.08± 0.03	3.74± 0.07	346

PA, phaseic acid. Height in cm (means ± s.d. from 18 replicates), hormone levels in pmol/g fresh weight, means ± s.d. of 6 replicates .

TABLE 2 A. Growth and levels of GA_1 and GA_{20} in barley seedlings after pre-emergence application of CGA 163'935.

Days after treatment	Plant height % of control	GA_{20} % of control	GA_1 % of control
4	57	400	26
5	50	1114	39
6	46	324	28
7	24	335	41

Substrate: vermiculite; treatment: substrate soaked in CGA 163'935, 250 mg/l a.i., at time of sowing; controls: EC blank; each value average from three replications.

TABLE 2 B. Growth inhibition and levels of GA_1 and GA_{20} in barley seedlings, 4 days after treatment with CGA 163'935 (indicated mg/l a.i., sprayed until run-off, control: EC blank).

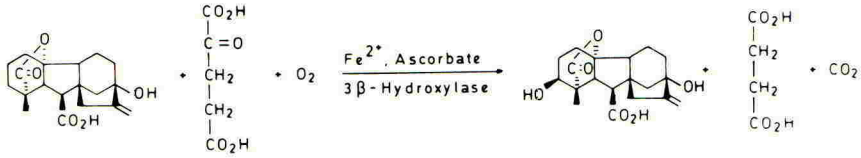
CGA 163'935	Height [cm]	% of control	GA_{20} level	% of control	GA_1 level	% of control
Control	19.1±1.2	100	1.96±0.2	100	1.70±0.2	100
60	14.9±0.9	78	4.41±0.2	225	0.91±0.1	54
125	14.7±1.1	77	4.98±0.3	254	0.61±0.1	36
250	14.5±1.3	76	5.12±0.0	261	0.47±0.0	28

Gibberellins in pmol/g fresh weight, means ± s.d. of 6 replicates; height, means ± s.d. for 18 replicates.

while the level of GA_{20} was clearly elevated (Table 1). The level of GA_1 was significantly lower compared to that in control plants even 12 h after application (not shown). Pre-emergence application of CGA 163'935 (Table 2A) produced the same effect. Plant height was strongly reduced, GA_1 levels severely reduced and levels of GA_{20} elevated suggesting an inhibition of conversion of GA_{20} to GA_1. The magnitude of decrease in GA_1 content and the concomitant rise in GA_{20}-levels in treated plants were dependent on the applied dose of CGA 163'935 (Table 2B). Approximately half maximum reduction in the level of GA_1 was achieved when spraying the seedlings with 60 mg/l a.i., corresponding to a dose of only 30-50 µg of a.i. per plantlet. This dose was sufficient to produce almost the full growth retardation, since a further increase in CGA 163'935 dose, although reducing GA_1 levels even further, did not reduce growth any further.

In vitro studies

From the *in vivo* experiments, it appeared that CGA 163'935, like other cyclohexanediones, interfered in the formation of GA_1 from its direct precursor, GA_{20}. We therefore tested if the enzyme GA_{20}-3ß-hydroxylase was a target of CGA 163'935 action in barley. The enzyme catalyses the reaction:



GA_{20} **GA_1**

To assay the enzyme, we developed an antibody-based technique for the cell-free quantification of enzymatic activity using unlabeled GA_{20} as substrate. We found that a partially purified extract from 2d-old barley seedlings converted GA_{20} into GA_1 in the presence of O_2, Fe^{2+}, ascorbate and 2-oxoglutarate. The general properties of the enzyme are given in Table 3.

TABLE 3. Properties of GA_{20} 3ß-hydroxylase from 2 d old barley seedlings.

Parameter	Value
Precipitation by ammonium sulfate	35 to 55 % saturation
Specific activity	0.5 fkat/mg protein
Km, 2-oxoglutarate (with 0.25µM GA_{20})	0.75 mM
Km, GA_{20} (with 5.0 mM 2-oxoglutarate)	0.94 µM
vmax (standard conditions)	0.9 fkat/mg protein
pH optimum	7.0
Ki, CGA 163'935 (with 0.2 - 1 mM 2-oxoglutarate and 0.25 µM GA_{20})	4.7 µM

Standard assay conditions, if not mentioned otherwise: 0.5 mM Fe^{2+}, 1.5 mM ascorbate, 5.0 mM 2-oxoglutarate, 0.25 µM GA_{20}, O_2, 30 °C, 5 mg protein, 2 ml assay volume.

The conversion of GA_{20} to GA_1 was strongly inhibited by micromolar levels of CGA 163'935 (Fig. 1). From double-reciprocal and Dixon plots, at low levels of both CGA 163'935 and 2-oxoglutarate, competition between the cosubstrate and the retardant was deduced with K_i,app. for CGA 163'935 being 4.7 µM. However, at higher levels of CGA 163'935 and 2-oxoglutarate, the inhibitor did not behave in a purely competitive manner.

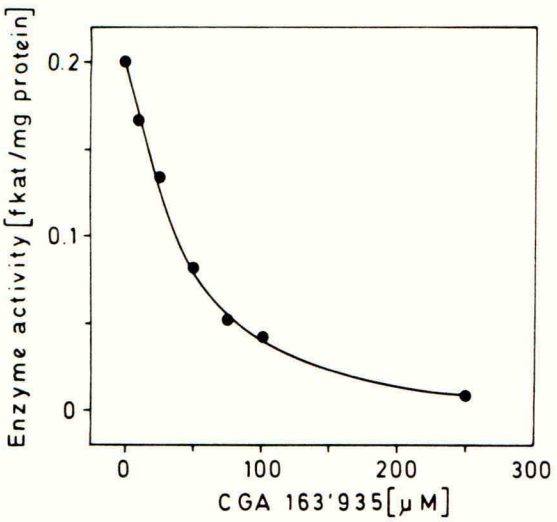


FIGURE 1. Inhibition of the conversion of GA_{20} to GA_1 in a cell-free system from 2 d old barley seedlings by CGA 163'935. Pure a.i. was used; standard assay conditions as specified in TABLE 3; 2-oxoglutarate, 0.75 mM.

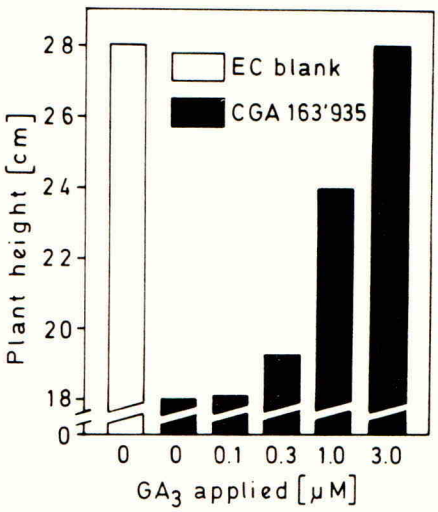


FIGURE 2. Reversal of growth inhibition of barley seedlings treated with CGA 163'935 by application of exogenous GA_3. Five d old plants were treated with 250 g a.i./l CGA 163'935 until run-off with or without GA_3. Plant height was determined 18 d after treatment.

Effects of exogenous gibberellin

Application of low levels of GA_3, a gibberellin known to promote elongation growth in barley similar to GA_1 (Fig. 2), along with CGA 163'935, fully restored growth. At 3 µM exogenous GA_3, the growth of treated plants reached that of untreated control plants.

DISCUSSION

Although other GAs have not been analyzed in the present study, the fact that growth of barley plants treated with CGA 163'935 could be completely restored to normal with micromolar levels of GA_3 (Fig. 2) virtually rules out other major physiologically relevant sites of action of the growth retardant. The data presented above strongly support the conclusion that CGA 163'935 inhibits growth in barley through a reduction in the level of active gibberellins, notably GA_1. Furthermore, this reduction appears to be caused by an inhibition of GA_{20}-3ß-hydroxylase activity. All growth retarding cyclohexanediones studied so far thus appear to exert their biological action through an inhibition of the terminal (activation) step in the biosynthesis of gibberellins, notably of GA_1 (Hedden, 1991; for review, see: Rademacher, 1991). This inhibition probably results from competition of the co-substrate of Fe^{2+}/ascorbate-dependent dioxygenases, 2-oxoglutarate, with the growth retardant, when both are present at low levels.

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