Protoporphyrin Accumulation Induced by Peroxidizing Herbicides is Counteracted by Safeners

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A number of safeners like naphthalene-1,8-dicarboxylic acid anhydride (naphthalic anhydride) or dichloroacetyl-hexahydro-3,3,8-α-trimethylpyrrolo-[1,2 α]-pyrimidine-6-(2 H)-one (BAS 145138) drastically decreased the accumulation of protoporphyrin IX induced by a peroxidative cyclic imide (chlorophthalim, N-(4-chlorophenyl)-3,4,5,6-tetrahydrophthalimide), or *p*-nitrodiphenyl ether (acifluorfen methyl, 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic methylester). This effect was observed with etiolated maize and cress seedlings during a 16-h illumination period with these herbicides and 0.1 to 1 mM safener present. The safeners did not affect the inhibition of protoporphyrinogen oxidase, the target enzyme of peroxidative herbicides. Mixed function oxidase inhibitors did not influence this safening effect. A microsome preparation from safener-treated maize seedlings did neither degrade protoporphyrin IX nor protoporphyrinogen IX.

Introduction

Safeners are an established tool in chemical crop protection. They induce defense mechanisms in plants against herbicidal phytotoxicity. Often safeners are applied to seeds to protect the treated crop seedling only; there are, however, safeners available also for post-emergence leaf application (Stephenson and Yaacobi, 1991; for chemistry of safeners see Kömives and Hatzios, 1991). Safeners have no effect on uptake of herbicide by the plants nor do they compete for the target site. They act as inducers or triggers to express certain metabolic enzymes, which may catalyze reactions leading to breakdown of the herbicides applied. A prominent enzyme is the glutathione S-transferase which generally markedly increases by safener treatment initiating detoxication by glutathione conjugation (see *e.g.* Farago *et al.,* 1994). However, as was first discussed in more detail by Fedtke (see his scheme in Devine *et al.,* 1993) also microsomal mixed function monooxigenases may be induced by naphthalic anhydride (NA), dichlormid and others (for formulas see Table II). Today, the hypothesis has emerged that a row of enzymes can be induced simultaneously by increasing their build-up in the "safened" plant cell. Apparently, such triggering is not identical in all plants and may depend on the safener applied. Not many studies have been performed on additional metabolizing enzymes except for the two mentioned above. A safener-increased glutathione reductase was reported (Kömives *et al.,* 1985), and enzymes related to sulfur and glutathione metabolism (Farago *et al.,* 1994). Evidence was presented that glucoside hydroxylation is enhanced by the safener BAS 145138 (Lamoureux and Rusness, 1991) as well that esterases are increased in maize by NA treatment (Hatzios, 1993) or in barnyard grass (*Echinochloa crus-galli;* Romano *et al.,* 1994).

Safening has been studied in detail for some major crop herbicides (listed in Hatzios, 1991), and reports mostly refers to monocots like maize, sorghum, rice, wheat and other cereals. The major herbicide groups used are chloroacetamides, imidazolinones, sulfonylureas and arylphenoxypropionates. It was of interest that safening of maize was achieved with NA and chlorophthalim (Matsunaka and Wakabayashi, 1989) and recently with dichlormid or BAS 145138 and others using the peroxidizing herbicide V-53482, 7-fluoro-6-[(3,4,5,6-tetrahydro)phthalimido]-4-(2-propynyl)-1,4-benzoxazin-3-(2 H)-one. The stunting of corn shoots could be successfully relieved by soaking the seeds in 1 mM safener and applying V-53482

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pre-emergent to seeds germinating in vermiculite (Devlin and Zbiec, 1993).

We have studied various analogues of N-aryl-substituted cyclic imides previously with respect to formation light-induced peroxidative ethane formation, of protoporphyrin IX (Sandmann *et al.*, 1990; Watanabe *et al.*, 1992) and inhibition of protoporphyrinogen oxidase (Nicolaus *et al.*, 1993 a). In this report we present findings with safeners and peroxidizing herbicides relating to accumulation of protoporphyrin IX and inhibition of the target enzyme protoporphyrinogen oxidase.

Materials and Methods

Plant material

Disinfected maize seeds, *Zea mays,* strain Anjou, coated with TMTD (= Thiram, fungicide) were soaked for 7–8 h in tape water and then germinated in large closed petri dishes (5×18 cm ∅) on wet filter-paper in the dark for about 6 days. During soaking and germination the safener was present unless indicated otherwise in the tables. The upper part of the (etiolated) leaves were cut off, and 3–4 cm long leaf pieces (approx. 10–12 leaf pieces equivalent to about 0.5 g fresh weight per sample) immersed in a 1:5 diluted mineral medium of Böger (1969), pH 6.3, using smaller petri dishes (5×7 cm ∅). The dishes were illuminated for 16 h at 2000 lux, 22 °C, with peroxidizing herbicides and safeners added as indicated.

Cress seedlings (*Lepidium sativum* from K. Sperling, Lüneburg, Zulassungs-Nr. 60) germinated in large petri dishes as above for 3–4 days on wet filter-paper without pre-soaking under conditions as noted above. Thereafter, 30 to 40 seedlings (1 g) together with the additions indicated were immersed for incubation and illuminated as was done with maize leaf pieces.

Isolation of protoporphyrinogen oxidase (protox; protoporphyrin IX: oxygen oxidoreductase, EC 1.3.3.4)

We followed the procedure as published (Nicolaus *et al.*, 1993 b) with minor modifications relating to the centrifugation steps. The first step was for 2 min at 700–800×*g*, the second one for 5 min at 4000×*g*. Protoporphyrinogen (protogen) was prepared from protoporphyrin IX (free acid).

Determination of protoporphyrin IX (proto IX)

After light incubation the plant material was washed once with 1:5 diluted mineral medium, and each sample extracted with 19 ml of 80% (v/v) alkaline acetone including 10 mM Hepes/NaOH buffer, [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid] taken from a 100 mM stock, pH 8, and 0.01 N ammonia. In case of maize 0.3% (w/v) polyvinylpyrrolidone (Fluka) was included. Extraction was done for 1.5 h at 60 °C. Subsequently, the extract was partitioned twice with 20 and 5 ml *n*-hexane (Merck, purified, for chromatography) and the fluorescence at 630–632 nm determined as described (Nicolaus *et al.*, 1993 b). To standardize the determination 5×10^{-8} to 10^{-7} M authentic proto IX (sodium salt) was added to each sample after each measurement and the fluorescence measured again to take into account a possible quenching.

Microsomes: Preparation and assay

For microsome preparation hypocotyls of etiolated maize seedlings were used which have been incubated with 10^{-4} M NA for 16 h. Homogenization was done after Lord (1988) with the gradient procedure omitted, while the differential centrifugation steps were performed after Fonné-Pfister *et al.* (1990). The detailed procedure will be published elsewhere. The basic assay medium included: 150 mM Tricine/NaOH, pH 7.5, N-[tris-(hydroxymethyl)methyl]glycine, 10 mM KCl, 1 mM EDTA, 1 mM $MgCl_2$ and 12% sucrose. Additions were as follows: 0.75 mM NADP and NADPH, respectively, 2.5 mM glucose-6-phosphate or 2.5 mM isocitrate, 3 units/ml of the corresponding dehydrogenases, and 1 μM proto IX or, alternatively, protogen. The basic assay medium was also used to homogenize the hypocotyls. Using protogen as substrate an aliquot of the assay was stopped with the same volume of methanolic perchlorate (1 M Na-perchlorate in 50% methanol) to oxidize the protogen. Then proto IX was extracted with alkaline acetone as described and measured by fluorescence.

Fine chemicals

The safeners were prepared as 0.1 M acetone stock solutions, the NA stock was 2×10^{-2} M. The

final acetone concentration in the assays was 1% v/v or lower and was used as control. Piperonylbutoxide was bought from Riedel de Haën (Hannover). Naphthalic anhydride, 1-aminobenzotriazole and protoporphyrin IX (free acid and sodium salt), were purchased from Sigma (Munich). For further chemicals see Acknowledgements.

Acifluorfen methyl was prepared as a 10^{-2} M methanolic stock solution. A 10^{-2} M stock of chlorophthalim was made with acetone.

Reliability

The data on formation of proto IX induced by herbicides and safeners exhibited a strong deviation between treatments of separate seedlings material (± 30%). Within a given plant sample, however, only ± 10% deviation of four determinations of proto IX levels and protox activities was found. The tables represent data of a typical experiment.

Results and Discussion

Table I demonstrates a substantial accumulation of protoporphyrin IX in maize seedlings treated with either the imide-type peroxidizing herbicide chlorophthalim or the *p*-nitrodiphenyl ether acifluorfen methyl. With naphthalic anhydride (NA) present during germination and herbicide treatment formation of proto IX is drastically depressed, the best results obtained with 10^{-4} M safener. The safening effect is about the same with the two herbicides of different chemical structure. Phytotoxicity of these herbicides is decreased by the safeners as can be judged by the greenish appearance of the leaves after the light incubation. Those treated with the herbicides alone looked yellow and brownish. Proto IX formation in maize seedlings during a 16-h dark period was only 10% of the light experiment. The safening effect, however, was present nevertheless.

A variety of safeners as documented in Table II are instrumental with cress seedlings as well as with maize (not documented). Apparently this "safening" effect is not restricted to monocots. We have not really checked for different activities of safeners but routinely NA and BAS 145138 proved to be best at 10^{-4} to 10^{-3} M. The safeners themselves do not produce any proto IX increase above the control.

When cress seedlings were germinated in the presence of BAS 145138, then thoroughly washed with tape water and treated with AFM minus safener, a high although somewhat lowered proto IX level *vs.* control was observed, which got no safener treatment during germination (data not shown). Apparently, a safener has to be present during the herbicide attack to give an optimum effect. When adding the safener together with AFM at the beginning of the 16 h light incubation of the cress seedlings, a strongly decreased proto IX level was observed of 50 to 70% below the unsafened control. The optimum decrease of proto IX accumulation, however, is seen with seedlings being in contact with the safener from start of germination. The data are listed in lines 3 and 4 of Table IV, column (c).

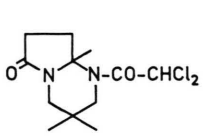
To find the reason for the drastic decrease of herbicide-induced proto IX levels by safeners isolated protox from untreated maize seedlings was assayed for inhibition under the influence of safeners. As shown in Table III acifluorfen methyl strongly inhibits protox (comp. Nicolaus *et al.*, 1994). Adding the herbicide together with BAS 145138 the same inhibition was observed excluding the possibility that the safener will alle-

Table I. Protoporphyrin IX formation (nmol/g fresh weight) and its counteraction by naphthalic anhydride (NA): concentration dependency assayed with maize seedlings.

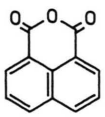
Assay	NA present					
	0	10^{-5} M	2×10^{-5} M	7×10^{-5} M	10^{-4} M	10^{-3} M
Control, 1% acetone, v/v	1.5	–	–	–	1.6	1.3
(+) Chlorophthalim, 5×10^{-7} M	16	15	8	5	3.5	3.0
(+) Acifluorfen methyl, 5×10^{-8} M	24	–	–	10	6.2	6.0

Table II. Test with different safeners (10^{-3} M): protoporphyrin IX formation (nmol/g fresh weight) with cress seedlings.

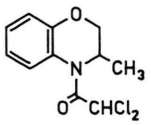
Assay	(−) Safener	BAS 145138	NA	Benoxacor	Oxabetrinil	Fenchlorim	Dichlormid
Control, 1% acetone (v/v)	1.4	0.8	0.6	1.3	1.8	1.7	1.6
(+) Acifluorfen methyl, 5×10^{-8} M	28	8	4	10	8	14	9



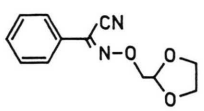
BAS 145 138



Naphthalic anhydride (NA)



Benoxacor



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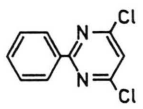
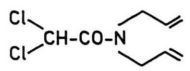


Fenclorim



Dichlormid (R-25788)

Table III. Check for influence of safener BAS 145138 on inhibition of isolated protoporphyrinogen oxidase from maize seedlings.

Additions	nmol proto IX formed / mg protein × h (−) Safener	(+) Safener, 10^{-3} M
Control	8.2	7.9
(+) Acifluorfen methyl, 2×10^{-9} M	3.7	3.7
5×10^{-9} M	–	3.0
10^{-8} M	1.5	1.6

viate the inhibition of protox by AFM in the cell which subsequently may lead to decreased tetrapyrrole accumulation (and to less herbicidal injury of the intact plant).

In a next experimental series cress seedlings were incubated with different additions, namely 1% acetone (= control), safener, herbicide, and herbicide plus safener, respectively, as shown in lines (1) to (4) of Table IV. The seedlings so pretreated were extracted for their protox and the isolated enzyme was assayed for inhibition with acifluorfen methyl ± safener as indicated in columns (a), (b). Section (1) repeats the findings of Table III with maize protox, namely that presence of BAS 145138 in the assay does not affect the inhibitory potency of AFM. The same holds for 10^{-4} M NA (data not shown). In section (2) BAS 145138 was present during the 16 h light incubation period. Activity and inhibition of protox by AFM obtained from these safener-treated seedlings was not altered either, the degree of inhibition was found about the same as from the control incubation of section (1). Light incubation of cress seedlings with 4×10^{-9} M AFM reduced the protox activity in the plant (section (3) of Table IV). Conceivably, protein synthesis is impaired by AFM during treatment, although a substantial amount of proto IX was formed in the seedlings (column (c)). Again strong inhibition of the isolated protox by added AFM ± BAS 145138 was observed. In section (4) the same light incubation of seedlings with AFM + safener resulted in higher protox activity, since the safener relieved the phytotoxic effect of AFM. The degree of AFM inhibition of isolated cress protox of this sample was almost identical to that of sections (1) or (2). This experiment gives proof that also the *incubation* of the seedlings with a safener over a 16-h period does not alter the protox toward an impaired inhibitor affinity.

Safeners can induce *de novo* synthesis of cytochrome P450-dependent mixed function oxidases which are involved in many detoxification reactions with imidazolinones, sulfonylureas and other herbicides (Frear *et al.*, 1991; see Hatzios, 1991 for review). If the decrease of proto IX levels as observed here is due to an oxidative degradation, inhibitors of (plant) mixed function oxidases like piperonylbutoxide (PBO) or 1-aminobenzotriazole (ABT) should alleviate the safener effect leading to an increased level of proto IX. As shown in Table V, these inhibitors had little influence on the safener effect, using either maize or

Table IV. Inhibition of protoporphyrinogen oxidase (protox) activity from cress seedlings, which were incubated with different additions. Germination was done on wet filter-paper. Then 4-day-old seedlings were incubated as indicated during a 16-h light period (2000 lux, 22 °C) with acetone (1), safener (2), AFM (3), and AFM + safener (4), respectively, using 4×10^{-8} M acifluorfen methyl (AFM) and/or 10^{-3} M BAS 145138. The control included 1% acetone, v/v. Then inhibition of enzyme activity was determined with the additions as indicated (+); for the additions AFM was 4×10^{-9} M, and BAS 145138, 10^{-4} M.

No.	(a) Incubation condition, (+) additions to isolated enzyme	(b) Protox activity $\left[\frac{\text{nmol proto IX}}{\text{mg protein} \times \text{h}}\right]$	(c) Proto IX formed [nmol/g fr. wt.]
(1)	Seedlings incubated in 1% acetone (control incubation)		0.36
	Control, (−) herbicide	9.6	
	(+) Acifluorfen methyl	2.7	
	(+) Acifluorfen methyl, BAS 145138	2.7	
(2)	Seedlings incubated with BAS 145138		0.40
	Control, (−) herbicide	9.1	
	(+) Acifluorfen methyl	3.7	
	(+) Acifluorfen methyl, BAS 145138	2.9	
(3)	Seedlings incubated with acifluorfen methyl		5.1
	Control, (−) herbicide	2.4	
	(+) Acifluorfen methyl	1.2	
	(+) Acifluorfen methyl, BAS 145138	1.2	
(4)	Seedlings incubated with acifluorfen methyl plus BAS 145138		0.8
	Control, (−) herbicide	4.0	
	(+) Acifluorfen methyl	1.7	
	(+) Acifluorfen methyl, BAS 145138	1.7	

Table V. Herbicide-induced accumulation of protoporphyrin IX (nmol/g fresh weight) in seedlings under the influence of naphthalic anhydride and mixed function oxidase inhibitors.

Species; incubation conditions	No additions	+NA	+PBO	+NA, +PBO	+NA, +ABT
Maize					
Control	0.8	0.6	0.5	0.6	0.6
(+) Chlorophthalim, 5×10^{-7} M	27	1.7	19	1.7	2.7
Cress					
Control	0.4	0.5	0.4	1.0	0.4
(+) Chlorophthalim, 4×10^{-7} M	25	5	18	5.5	6

NA, naphthalic anhydride, 10^{-4} M; PBO, piperonylbutoxide, 5×10^{-5} M; ABT, 1-aminobenzotriazole, 10^{-4} M.

cress seedlings. Also the proto IX level of the controls treated with herbicide only did not change.

Additionally we have assayed whether an active microsome preparation from etiolated maize seedlings would react with proto IX or protoporphyrinogen as substrates. The reactions (see Materials and Methods) were followed during 4 h but no spectral change of proto IX and no decrease of protoporphyrinogen was observed.

Summarizing then: A strong decrease of proto IX accumulation by safeners was observed with two herbicides of different chemical structure making unlikely their possible safener-induced degradation during the 16-h incubation period. The findings indicate that the drastic alleviation of proto IX accumulation as induced by safeners is neither due to an impaired inhibition of protox nor to an oxidative degradation of the accumulated tetrapyrrole. Further studies are needed to elucidate alternative degradative mechanisms of either proto IX or its precursor.

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