Plant Physiol. (1991) 97, 280–287
0032-0889/91/97/0280/08/$01.00/0

Received for publication April 8, 1991
Accepted June 3, 1991

Physiological Basis for Differential Sensitivities of Plant Species to Protoporphyrinogen Oxidase-Inhibiting Herbicides[1]

Timothy D. Sherman, José M. Becerril[2], Hiroshi Matsumoto[3], Mary V. Duke, Judy M. Jacobs, Nicholas J. Jacobs, and Stephen O. Duke*

U.S. Department of Agriculture, Agricultural Research Service, Southern Weed Science Laboratory, P.O. Box 350, Stoneville, Mississippi 38776 (T.D.S., J.M.B., H.M., M.V.D., S.O.D.) and Department of Microbiology, Dartmouth Medical School, Hanover, New Hampshire 03756 (J.M.J., N.J.J.)

ABSTRACT

With a leaf disc assay, 11 species were tested for effects of the herbicide acifluorfen on porphyrin accumulation in darkness and subsequent electrolyte leakage and photobleaching of chlorophyll after exposure to light. Protoporphyrin IX (Proto IX) was the only porphyrin that was substantially increased by the herbicide in any of the species. However, there was a wide range in the amount of Proto IX accumulation caused by 0.1 millimolar acifluorfen between species. Within species, there was a reduced effect of the herbicide in older tissues. Therefore, direct quantitative comparisons between species are difficult. Nevertheless, when data from different species and from tissues of different age within a species were plotted, there was a curvilinear relationship between the amount of Proto IX caused to accumulate during 20 hours of darkness and the amount of electrolyte leakage or chlorophyll photobleaching caused after 6 and 24 hours of light, respectively, following the dark period. Herbicidal damage plateaued at about 10 nanomoles of Proto IX per gram of fresh weight. Little difference was found between *in vitro* acifluorfen inhibition of protoporphyrinogen oxidase (Protox) of plastid preparations of mustard, cucumber, and morning glory, three species with large differences in their susceptibility at the tissue level. Mustard, a highly tolerant species, produced little Proto IX in response to the herbicide, despite having a highly susceptible Protox. Acifluorfen blocked carbon flow from δ-aminolevulinic acid to protochlorophyllide in mustard, indicating that it inhibits Protox *in vivo*. Increasing δ-aminolevulinic acid concentrations (33–333 micromolar) supplied to mustard with 0.1 millimolar acifluorfen increased Proto IX accumulation and herbicidal activity, demonstrating that mustard sensitivity to Proto IX was similar to other species. Differential susceptibility to acifluorfen of the species examined in this study appears to be due in large part to differences in Proto IX accumulation in response to the herbicide. In some cases, differences in Proto IX accumulation appear to be due to differences in activity of the porphyrin pathway.

The *p*-substituted nitro-diphenyl ether herbicides cause photodynamic bleaching in sensitive plants by causing the accumulation of abnormally high levels of porphyrins, primarily Proto IX[4] (1, 2, 18, 19, 23–26, 31, 32, 35). Accumulation of Proto IX is due to strong inhibition of Protox (11, 12, 21, 22, 32, 34, 36), resulting in uncontrolled oxidation of the enzymic substrate, protoporphyrinogen IX, to Proto IX outside the porphyrin pathway (12, 18) and to deregulation of the porphyrin pathway (16, 20). Proto IX accumulation in plants treated with Protox-inhibiting herbicides is analogous to Proto IX accumulation in humans with a genetic defect in Protox, resulting in the disease variegate porphyria (4). In the presence of light and molecular oxygen, singlet oxygen is generated by Proto IX, resulting in membrane lipid peroxidation (5).

Considerable variation in susceptibility to these herbicides exists between plant species. Tolerance of soybeans to acifluorfen, a widely-used *p*-nitro-diphenyl ether herbicide, is due to metabolic detoxification of the herbicide (9). Acifluorfen was developed for use in soybeans. However, the mechanism of tolerance of other species to this herbicide is not understood. Tolerance could be related to the level of protection against photodynamic damage or to the effect of the herbicide on the tetrapyrrole pathway. Finckh and Kunert (8) found a wide range of tolerance among nine higher plant species to oxyfluorfen (a *p*-nitro-diphenyl ether closely related to acifluorfen) to be associated with the ratios of ascorbate to α-tocopherol within the affected tissue. They found ratios of approximately 10 to 15:1 (w/w) associated with species that were highly tolerant. Schmidt and Kunert (33) showed acifluorfen to cause increases in glutathione, ascorbate, and glutathione reductase levels in tolerant bean leaf tissues. Kenyon and Duke (14) found levels of these and other oxidative stress protectants to be reduced by acifluorfen in highly sensitive cucumber cotyledon tissues during oxidative stress.

Although these herbicides cause photodynamic stress as a result of abnormally high levels of porphyrin accumulation, no information exists concerning differential species sensitiv-

[1] This research was supported in part by an Overseas Research Fellowship from the Japanese Ministry of Education, Science, and Culture; the Fulbright/Ministry of Education and Science, Madrid, Spain; and United States Department of Agriculture Competitive Grant No. 9000705.

[2] Current address: Universidad del Pais Vasco/EHU, Facultad de Ciencias, Dep. Fisiología Vegetal y Ecología, Apartado 644, 48080 Bilbao, Spain.

[3] Current address: Institute of Applied Biochemistry, University of Tsukuba, Tsukuba, Ibaraki 305, Japan.

[4] Abbreviations: Proto IX, protoporphyrin IX; ALA, 5-aminolevulinic acid; Mg-Proto IX, Mg-protoporphyrin IX; Mg-Proto IX-ME, Mg-protoporphyrin IX monomethylester; Protox, protoporphyrinogen oxidase.

ity related to porphyrin synthesis. In this paper, we compare the herbicidal efficacy of acifluorfen with its ability to cause porphyrin synthesis in a collection of higher plant species with a wide range of susceptibility. Furthermore, we compare the *in vivo* effects on Proto IX accumulation with the *in vitro* effects of the herbicide on Protox in species with different susceptibilities. Our findings suggest that much of the variability in susceptibility to acifluorfen between the species studied is due to the capacity of the species to generate Proto IX in response to the herbicide.

MATERIALS AND METHODS

Plant Material

Seeds of velvetleaf (*Abutilon theophrasti* Medic.), cucumber (*Cucumis sativus* L. [cv Straight Eight]), mustard (*Brassica hirta* Moench), lambsquarters (*Chenopodium album* L.), redroot pigweed (*Amaranthus retroflexus* L.), alfalfa (*Medicago sativa* L.), tartary buckwheat (*Fagopyrum tataricum* [L.] Gaertn.), pitted morningglory (*Ipomoea lacunosa* L.), sicklepod (*Cassia obtusifolia* L.), spinach (*Spinacia oleracea* L.), and jimsonweed (*Datura stramonium* L.) were planted in 1.2-L pots containing two parts commercial potting mixture and one part perlite-vermiculite. The plants were grown in a greenhouse in which the temperature varied from 20 to 30°C or in growth chambers with 12-h photoperiods and temperature regimens of 23°C days and 19°C nights for morningglory, sicklepod, pigweed, velvetleaf, and lambsquarters and 20°C days and 15°C nights for mustard, tartary buckwheat, and spinach. Cucumbers were grown under continuous light for 7 d at 30°C. All chambers had 90 ± 5% RH and a PPFD of 450 μmol/m^2·s PAR. Unless otherwise noted, leaf tissues were harvested from 20-d-old sicklepod and morningglory plants; 30-d-old jimsonweed, velvetleaf, mustard, tartary buckwheat, spinach, and alfalfa plants; and 40- to 50-d-old pigweed and lambsquarters plants.

In one set of experiments, mustard and pitted morningglory were grown under continuous far-red light as before (6) at 25°C for 7 d before harvesting.

Herbicide Treatment

Treatment of plant tissues with herbicides was as before (15), by cutting 50 4-mm diameter cotyledon or leaf discs with a cork borer and washing them in 1% sucrose, 1 mM Mes (pH 6.5), and then placing them in a 6-cm diameter polystyrene Petri dish with 5 mL of the wash medium with or without test compounds. Technical-grade acifluorfen {5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid} was a gift of Rohm and Haas Co.[5] The discs were then incubated at 25°C in darkness for 20 h before exposure to 500 μE/m^2·s PAR. All incubations were in growth chambers.

[5] Mention of a trademark or product does not constitute endorsement of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

Herbicidal Damage

Cellular damage was measured by detection of electrolyte leakage into the bathing medium with a conductivity meter with the capacity to assay 1 mL of the bathing medium and return it to the dish (15). Because of differences in background conductivity of different treatment solutions, results are expressed as change in conductivity after exposure to light. Previous studies have shown that photobleaching herbicides have no significant effect on cellular leakage in darkness (7).

Photobleaching was determined by measuring Chl content after 24 h of exposure to 500 μE/m^2·s PAR at 25°C. Discs from each dish were soaked in 5 mL of DMSO in darkness at room temperature for 24 h, and total Chl in extracts was determined spectrophotometrically according to the method of Hiscox and Israelstam (10). All treatments for electrolyte leakage and photobleaching measurements were triplicated.

Porphyrin Determinations

All extractions for HPLC were made under a dim, green light source. Samples (50 discs) were homogenized in 6 mL of HPLC-grade methanol: 0.1 N NH_4OH (9:1, v/v) with a Brinkmann Polytron at 60% full power for 15 s. The homogenate was centrifuged at 30,000*g* for 10 min at 0°C and the supernatant was saved. The pellet was resuspended in 3 mL of basic methanol, sonicated for 5 min, and centrifuged at 30,000*g* for 10 min at 0°C. Supernatants were combined and evaporated to dryness at 40°C with a rotary evaporator. The residue was dissolved in 2 mL of HPLC-grade basic methanol and filtered through a 0.2 μm syringe filter. Samples were stored in light-tight (glass wrapped in aluminum foil) vials at −20°C until analysis by HPLC.

HPLC determinations were made as before (26) with a system composed of Waters Associates components that included two model 510 pumps, a model 712 autosampler, a Maxima 820 controller, and a model 990 photodiode spectrophotometric detector. A model 470 fluorescence detector preceded the model 990 detector. The column was a 250 × 4.6 mm (i.d.) Spherisorb 5 μm ODS-I reversed phase column preceded by a Bio-Rad ODS-5S guard column. The solvent gradient was composed of 0.1 M ammonium phosphate (pH 5.8) (solvent A) and HPLC-grade methanol (solvent B) at a flow rate of 1.4 mL/min. The solvent delivery program was as follows: 20% A in B from 0 to 10 min, a linear transition from 20 to 0% A in B from 10 to 18 min, and B only from 18 to 35 min. The injection volume was 50 μL. Commercial standards of Proto IX (Sigma Chemical Co.), Mg-Proto IX, and Mg-Proto IX-ME (Porphyrin Products, Inc.) were used. Pchlide was quantified by extracting Pchlide from etiolated tissues, quantifying it spectrophotometrically as before (2), and injecting spectrophotometrically-assayed Pchlide into the HPLC for calibration of the spectrofluorometric detector. Porphyrin detection was performed with fluorescence detector excitation and emission wavelength settings of 400 and 630 nm, respectively, for Proto IX; 415 and 595 nm, respectively, for Mg-Proto IX and Mg-Proto IX-ME; and 440 and 630 nm, respectively, for Pchlide. The photodiode array detector scanned from 300 to 700 nm to confirm all peaks. All porphyrin compound levels are expressed on a molar basis

per g of fresh weight. All treatments for porphyrin samples were triplicated.

Protox Assays

Cotyledons of 7- to 9-d-old, far-red-grown mustard, cucumber, or morningglory seedlings or leaves of dark-grown barley seedlings were homogenized with a Sorvall Omnimixer for 30 s at full speed using a fresh weight to volume ratio of 1:5. Homogenization buffer consisted of 10 mM Hepes (pH 7.7 at 2°C), 330 mM sorbitol, 1 mM EDTA, 1 mM $MgCl_2$, and 5 mM cysteine. Homogenate was filtered through two layers of Miracloth and crude cell debris was removed by centrifugation at 150*g* for 1 min at 4°C. Plastids were collected from this supernatant by centrifugation at 6000*g* for 15 min at 4°C. Plastids were disrupted in a small volume of extract buffer (homogenization buffer in which 1 mM DTT is substituted for 5 mM cysteine) by sonication twice for 10 s on ice. The extracts were resuspended in assay buffer and stored at −80°C until use. No loss of activity was noted in samples stored under these conditions. Prior to assay, protein concentration was determined by the method of Bradford (3) with BSA as a standard, and extracts were adjusted to 4 mg protein/mL in extract buffer.

Protoporphyrinogen IX was prepared according to Jacobs and Jacobs (13) with the following changes. Proto IX stock solution (0.5 mM) was reduced to protoporphyrinogen IX with approximately one-eighth volume of freshly ground sodium amalgam. The resulting colorless solution was adjusted to pH 8 by addition of an equal volume of (5× strength) assay buffer, consisting of 500 mM Hepes, pH 7.5, with 25 mM EDTA. Residual amalgam and porphyrin aggregates were removed by passing the solution through a 0.22-μm nylon syringe filter. DTT was added to the protoporphyrinogen solution to a final concentration of 2 mM. The resulting preparation was stable in dim light at room temperature for at least 2 h.

Prior to assay, plastid extracts were thawed and sonicated for two 5-s periods at 0 to 4°C. Acifluorfen was added in a volume of 2 μL of ethanol to 200 μL of extract. Ethanol was added to control treatments. The extract was allowed to incubate on ice for 30 min with or without the herbicide. The assay mixture consisted of 100 mM Hepes (pH 7.5, 30°C), 5 mM EDTA, 2 mM DTT, and about 2 μM protoporphyrinogen IX. The reaction was initiated by addition of 0.1 mL of extract (± herbicide) to 0.9 mL of assay mixture and monitored for 2 min at 30°C. Fluorescence was monitored directly from the assay mixture using a Shimadzu RF-5000U, temperature-controlled, recording spectrofluorometer with excitation at 395 nm and emission monitored at 622 nm. The reaction rate was essentially constant over this 2-min period. Autooxidation in the presence of heat-inactivated extract was negligible.

RESULTS AND DISCUSSION

Tetrapyrrole Levels

Under the conditions of our experiments, Proto IX is the only porphyrin compound in all species to consistently accumulate to much higher levels in acifluorfen-treated than in untreated tissues. However, the effect of acifluorfen on Proto IX content varied greatly between species. Table I provides examples of porphyrin profiles of several species tested. In some cases, Pchlide levels were reduced up to 50% or increased by as much as twofold by the herbicide. This range of effects of acifluorfen on Pchlide levels has been found in cucumber cotyledon tissues of varying types of treatment and physiological conditions (2, 5, 18). Mg-Proto IX levels were very low in control and acifluorfen-treated tissues of all species. Mg-Proto IX-ME was detected in only some of the species; however, there were no effects of acifluorfen on its accumulation (data not shown).

In a broader survey of the effects of acifluorfen on Proto IX accumulation, an increase of about 20- to 600-fold over the control levels was found in more than 10 different species (Table II). The absolute amount of Proto IX varied in acifluorfen-treated tissues from a low of 0.33 nmol/g fresh weight in mustard to about 12 nmol/g fresh weight in lambsquarters. The percentage increase figures are a less reliable indicator of the herbicidal effect because of the error in measuring very small levels of Proto IX in control tissues. Discrepancies between some of the values in Tables I and II for the same species are due to tissue age differences. The effect of the herbicide was generally reduced in older tissues. For example, the velvetleaf tissue sampled in Table I was about 7 weeks old, but that of Table II was 3 weeks old.

Closer examination of the effects of acifluorfen on Proto IX accumulation in tissues of two species that produced little Proto IX (mustard and spinach) and two that produced high levels (morningglory and lambsquarters) in response to acifluorfen revealed different dose-response curves (Fig. 1). At all herbicide concentrations, there was significantly more Proto IX than in the control treatment. However, in mustard and spinach there was no increase in the effect between 33 μM and 1 mM. Acifluorfen caused a significant increase of Proto IX in both morningglory and lambsquarters with concentrations between 33 μM and 1 mM.

Relationship between Proto IX Accumulation and Herbicidal Effects

The herbicidal activity of 0.1 mM acifluorfen as measured by cellular leakage after 6 h of light exposure or Chl loss after

Table I. *Effects of 0.1 mM Acifluorfen on Accumulation of Tetrapyrroles in Leaf Discs of Several Species after 20 h of Exposure in Darkness*

Con = control; AF = acifluorfen.

Species	Proto IX		Mg Proto IX		Pchlide	
	Con	AF	Con	AF	Con	AF
	nmol/g fresh wt					
Lambsquarters	0.02	12.34	0.02	0.01	8.12	9.11
Morningglory	0.09	4.10	0.08	0.06	0.24	0.28
Mustard	0.04	0.75	0.05	0.06	0.64	0.66
Pigweed	0.03	6.92	0.04	0.02	1.71	1.76
Sicklepod	0.02	1.91	0.01	0.02	3.55	6.11
Spinach	0.01	0.74	0.00	0.00	3.62	2.47
Velvetleaf	0.01	1.77	0.04	0.06	13.81	5.29
Cucumber	0.15	5.75	0.02	0.04	6.76	7.06

Table II. *Effects of 0.1 mM Acifluorfen on Proto IX Accumulation in Leaf Discs during a 20-h Dark Incubation*

Some data from Table I are repeated.

Species	Control	Treated	Treated ÷ Control
	nmol/g fresh wt		*-fold increase*
Alfalfa	0.20 ± 0.01	3.82 ± 0.05	19
Jimsonweed	0.28 ± 0.03	6.48 ± 1.03	23
Lambsquarters	0.02 ± 0.00	12.34 ± 0.80	617
Morningglory	0.09 ± 0.01	4.10 ± 0.45	46
Mustard	0.02 ± 0.02	0.33 ± 0.06	17
Pigweed	0.03 ± 0.00	6.92 ± 0.50	231
Sicklepod	0.02 ± 0.00	1.91 ± 0.44	96
Spinach	0.01 ± 0.00	0.74 ± 0.11	87
Tartary buckwheat	0.21 ± 0.01	7.64 ± 0.64	36
Velvetleaf	0.03 ± 0.01	9.68 ± 1.69	285
Cucumber	0.15 ± 0.02	5.75 ± 0.32	38

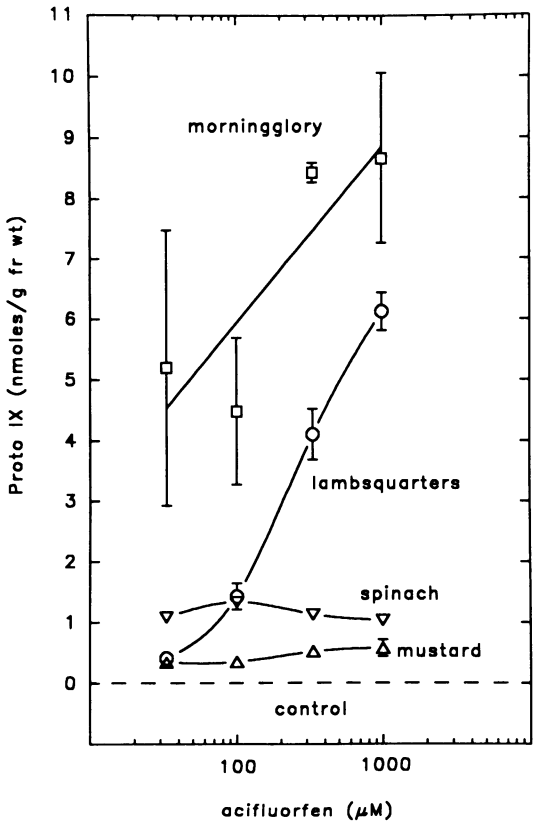

Figure 1. Proto IX accumulation in tissues of four species in response to increasing acifluorfen dosage. Leaf discs were incubated in darkness in herbicide solutions for 20 h before Proto IX was assayed. Control values (indicated by dashed line) of the four species were not significantly different between species. Error bars = ± 1 SE.

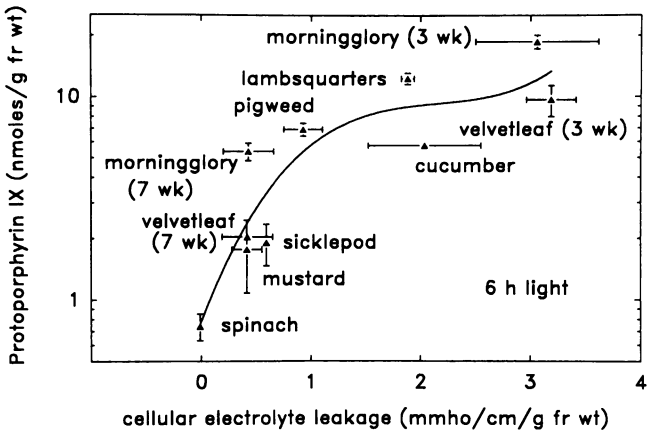

Figure 2. Accumulation of Proto IX after exposure to 0.1 mM acifluorfen for 20 h in darkness *versus* the cellular leakage of tissue from different species treated with 0.1 mM acifluorfen during a subsequent 6 h exposure to light. Error bars = ± 1 SE.

24 h of light exposure (Figs. 2 and 3) varied considerably between species. A broad range of sensitivity to this herbicide was found between the species assayed and between tissues of the same species, but of different age. We previously found that *Lemna pausicostata* was tolerant to acifluorfen as a sodium salt, but sensitive to acifluorfen as the methyl ester (26), apparently because the plants did not absorb the ionized form of the herbicide. This was not the case with either mustard or spinach, the two most acifluorfen-tolerant species of our study. Treatment with 33 μM acifluorfen-methyl had no bleaching effect on either species, whereas it was very effective on velvetleaf (data not shown).

A roughly curvilinear relationship was found between the log of Proto IX content of 0.1 mM acifluorfen-treated tissues of different species after the 20-h dark period and electrolyte leakage from these tissue during 6 h of subsequent exposure to light (Fig. 2). The shape of the curve and the relative

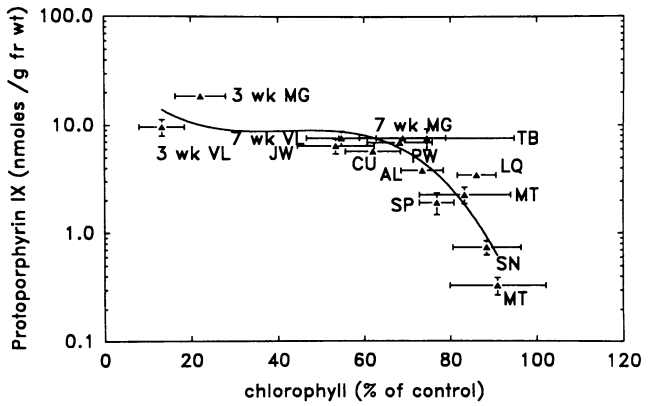

Figure 3. Accumulation of Proto IX after exposure to 0.1 mM acifluorfen for 20 h in darkness *versus* the loss of Chl in a subsequent 24 h exposure to light in 10 different species. AL = alfalfa; CU = cucumber; JW = jimsonweed; LQ = lambsquarters; MG = morningglory; MT = mustard; PW = pigweed; SN = spinach; SP = sicklepod; TB = tartary buckwheat; VL = velvetleaf. Error bars = ± 1 SE.

position of different species on the curve changed slightly with sampling time, because the kinetics of leakage for each species varied (data not shown). However, the general curvilinear relationship remained the same.

A similar relationship was also found between the log of Proto IX content and Chl bleaching after 24 h light (Fig. 3). Again, the curve appears to plateau near 10 nmol of Proto IX/g fresh weight. The relative positions of the different species on this curve are similar to those in Figure 2. Younger tissues generally accumulated more Proto IX and bleached more than older tissues of the same species. In a separate experiment in which tissues of lambsquarters were exposed to different concentrations of acifluorfen, an excellent correlation was found between herbicidal damage and Proto IX accumulation (Fig. 4). These data conformed closely to the curve of Figure 3. The uniformity of the data between species was surprising. This suggests that Proto IX is about equally active as an herbicidal agent in many of the species examined. Furthermore, these data suggest that the differential sensitivity to acifluorfen between species is due in large part to differences in acifluorfen-induced Proto IX accumulation. One possible cause of this could be differences between species in the sensitivity of Protox to the herbicide.

Others (27, 28) have argued that dark accumulation of Proto IX in cut leaf discs treated with Protox inhibitors has little relationship to what happens in intact plants in the field. They base their argument on their findings that relatively little Proto IX accumulates in the dark compared with the light in intact plants treated with these herbicides, a result reported by others (25). We have found very high levels of Proto IX accumulation in the dark in acifluorfen-treated cucumber seedlings (5), but less accumulation in dark than in light in acifluorfen-methyl-treated *L. pausicostata* plants (26). The reasons and conditions for differences between Proto IX accumulation in light and dark in whole plants requires further study. Nevertheless, we recently found excellent correlations between the effects of several pyrazole phenyl ether Protox inhibitors on whole plants and their capacity to cause

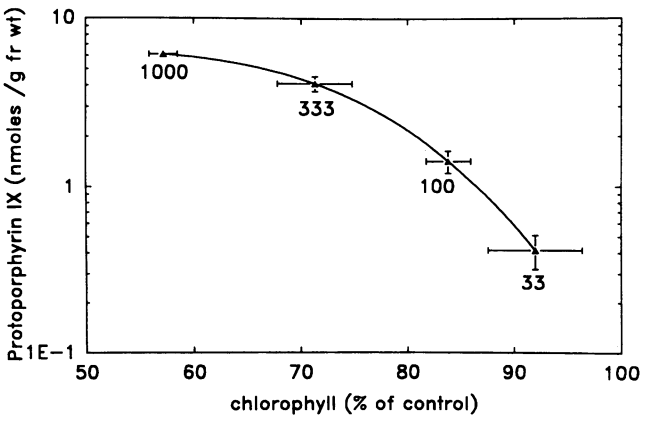

Figure 4. Accumulation of Proto IX in 70-d-old lambsquarters after exposure to different concentrations (numbers next to data points are μM concentrations) of acifluorfen for 20 h in darkness *versus* the loss of Chl in a subsequent 24-h exposure to light. Error bars = ± 1 SE.

Proto IX accumulation in leaf discs in the dark (34). The results of the present study may not extrapolate to the field in every case; however, the relative herbicidal efficacy of acifluorfen that we found between species in the bioassays used in these studies are similar to those of whole plants. In intact plants, cuticular penetration of the herbicide can play an important role in herbicidal efficacy. Our bioassay largely removes this confounding factor.

More Proto IX may have been synthesized after exposure to light, further contributing to herbicidal damage. However, in previous studies, we found that much more Proto IX accumulates in the dark than in light in cut leaf discs (1). Proto IX levels decrease rapidly upon exposure to light in cucumber, lambsquarters, and morningglory (ref. 1 and data not shown from the present study) with an initial half-life of 1 to 2 h. Furthermore, the lag period before herbicidal damage could be detected by electrolyte leakage was only 1 to 2 h for most species. With such rapid cellular damage, we doubt that much more Proto IX would have been synthesized. We chose a 6-h time point to obtain a better range of values than at 1 or 2 h.

Protox Effects

We chose morningglory, cucumber, and mustard (three species with highly divergent sensitivities to acifluorfen as an herbicide; see Figs. 2 and 3) for examination of *in vitro* Protox sensitivity. Protox preparations from green tissues cannot be assayed for activity because of background fluorescence from Chl. Therefore, we grew plants under far-red light in order to cause photomorphogenic cotyledon expansion without significant Chl accumulation. Another reason that these species were chosen is that they have sufficiently large cotyledons for Protox preparations and for assay of herbicidal activity of far-red-grown tissues. Protox preparations from dark-grown barley was used as a comparison, because this is one of the few species for which data on diphenyl ether herbicide effects on Protox are available (11). The I_{50} values for the inhibition of Protox from these tissues by acifluorfen varied little (Fig. 5); the values for pitted morningglory, mustard, barley, and cucumber were 1, 1.5, 2, and 4 μM, respectively. Far-red-grown mustard is as tolerant to acifluorfen as green, white-light-grown mustard (data not shown), indicating that the Protox of far-red-grown mustard is not different than that of the enzyme in green mustard tissues.

Mechanism of Mustard Tolerance

Mustard is a species that accumulates very little Proto IX and morningglory and cucumber represent species that accumulate relatively more Proto IX in response to acifluorfen. In mustard, the lack of Proto IX accumulation could be due to either lack of *in vivo* inhibition of Protox (even though the *in vitro* inhibition was strong; see Fig. 5) or to a limited capacity to synthesize substrate for porphyrins. The most likely explanation is the latter, because the large increase in Proto IX accumulation caused by ALA plus acifluorfen, compared with acifluorfen alone (Fig. 6), indicates that acifluorfen is an effective *in vivo* Protox inhibitor in these tissues, and that, with sufficient substrate, larger amounts of Proto

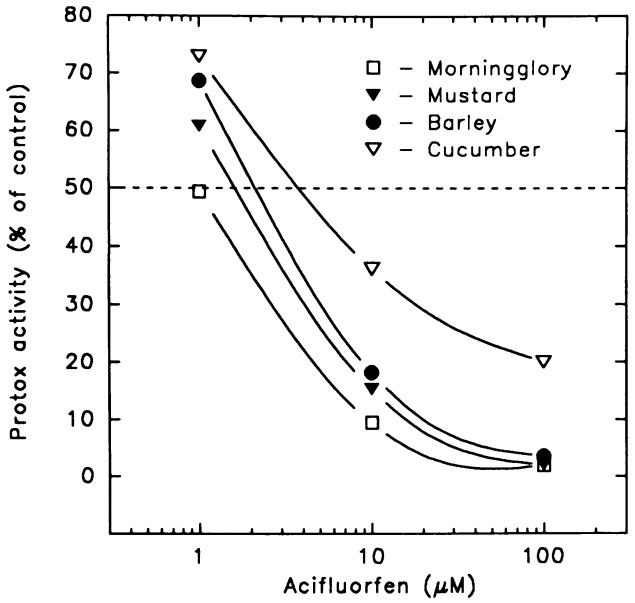

Figure 5. Inhibition of Protox from far-red light-grown morningglory, barley, cucumber, and mustard by different concentrations of acifluorfen. Enzyme activity rates of the control preparations were 20.5, 10.8, 28.5, and 55.2 nmol Proto IX/min·mg protein for barley, cucumber, morningglory, and mustard, respectively.

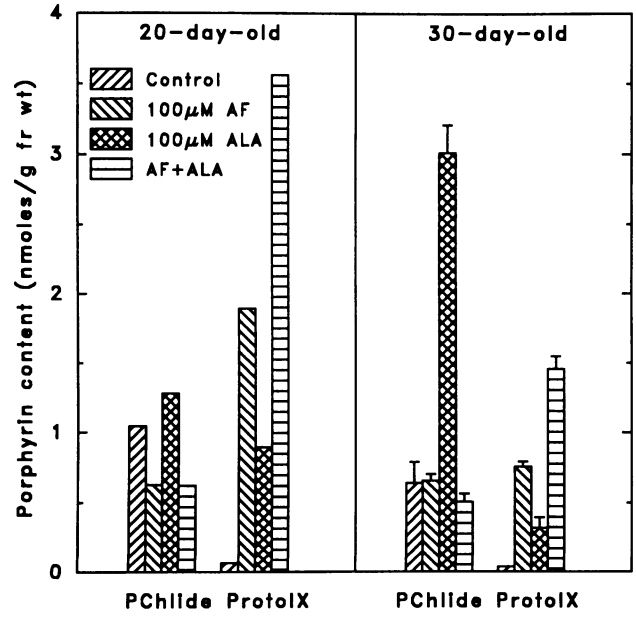

Figure 6. Effects of 0.1 mM acifluorfen (AF) and ALA, individually or together, on Pchlide and Proto IX accumulation of leaf discs from 20- and 30-d-old mustard plants after 20 h incubation in darkness. Error bars = ± 1 SE.

IX may accumulate. Furthermore, acifluorfen significantly reduces the ALA-induced accumulation of Pchlide. These conclusions are consistent with the dose-response results with mustard (Fig. 1). Compared with other species, mustard and spinach appeared to lose their capacity to further respond to higher doses of acifluorfen. This is perhaps due to saturation of the effect at low herbicide doses, due to lack of porphyrin synthesis capacity.

If so, addition of sufficient substrate should result in accumulation of sufficient Proto IX for herbicidal activity. Increasing concentrations of ALA with a constant dose of acifluorfen resulted in increasing Proto IX levels (Fig. 7). Acifluorfen prevented the added ALA from being converted to Pchlide. The relationship between Proto IX in each of the treatments of Figure 7 and the resulting herbicidal damage after exposure to light was positive (Fig. 8), supporting the view that mustard is tolerant to acifluorfen due to porphyrin substrate limitations. In fact, if the data from Figure 8 were plotted on Figure 2, mustard would appear somewhat more sensitive to Proto IX than most other species.

Another explanation that has been used to explain tolerance to photobleaching diphenyl ether herbicides is an enhanced capacity to detoxify singlet oxygen and lipid peroxides (8, 17). We examined the relative sensitivity of mustard, morningglory, and lambsquarters to rose bengal, a photodynamic dye that acts much like Proto IX to generate singlet oxygen. In these studies, the concentration of rose bengal required to cause a 50% reduction in Chl after 24 h exposure to light was about 30 μM for mustard and lambsquarters and about 300 μM for morningglory. Thus, there was no evidence that mustard was less sensitive to singlet oxygen than other species.

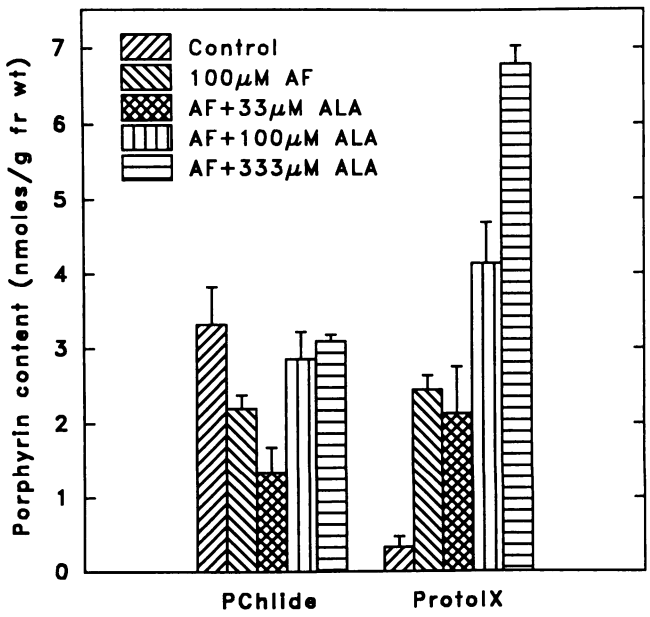

Figure 7. Effects of 0.1 mM acifluorfen (AF) with or without logarithmically increasing ALA concentrations on Pchlide and Proto IX content in leaf discs from 32-d-old mustard plants after 20 h in darkness. Error bars = ± 1 SE.

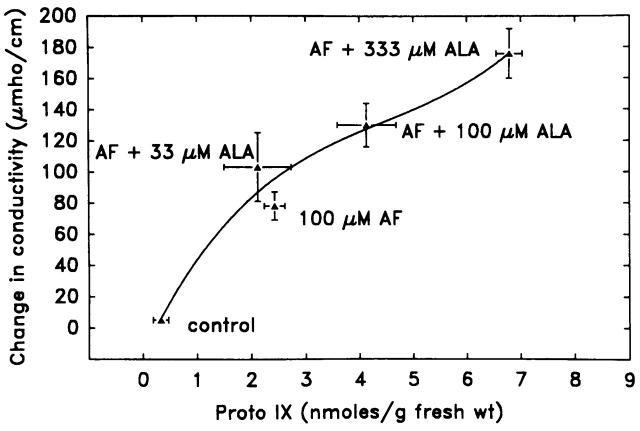

Figure 8. Relationship between the Proto IX levels of Figure 7 and the electrolyte leakage from these tissues 6 h after exposure to 0.5 $mE/m^2 \cdot s$ white light. AF = acifluorfen; error bars = ± 1 SE.

CONCLUSIONS

Acifluorfen substantially increased Proto IX content of all species. However, there was a wide range in the magnitude of effect between species. There was little or no effect on content of other Chl intermediates in any species. Generally, there was less Proto IX accumulation in response to the herbicide in older tissues.

The similarity in the relationship between Proto IX accumulation and herbicidal damage between species is remarkable (Figs. 2 and 3). As in Figure 5, we had previously demonstrated very good correlations between herbicidal damage and Proto IX accumulation within a single species by several methods (1). With another class of Protox inhibitors, we have found good correlations between herbicidal damage to intact plants, *in vitro* Protox inhibition, and capacity to induce Proto IX accumulation in tissue sections (34). Although Matringe *et al.* (23) have also found positive correlations, others (27–29, 31, 32) have found poor correlations between herbicidal effects of Protox inhibitors and Proto IX accumulation caused by them. To find a positive correlation between a cause and an effect, the cause and the effect must be measured at appropriate times. We suggest that, in cases in which no relationship has been found, the timing of these measurements has been inappropriate.

In general, our results indicate that there could be several different mechanisms of tolerance to acifluorfen and, perhaps, to other Protox-inhibiting herbicides. Others have shown that soybeans are tolerant due to rapid metabolic degradation of the herbicide (9). Some species apparently generate much less Proto IX in response to these herbicides than do others. Within a species, this may explain the relative tolerance of older tissues. Although we did not make an exhaustive survey of Protox susceptibility to acifluorfen, because of the technical problems in obtaining sufficient etiolated or far-red-grown seedling tissues of some species, the data that we have obtained indicate that an insensitive Protox is unlikely. Certainly, high accumulation of Proto IX in response to the herbicide indicates strong *in vivo* inhibition of Protox, although other factors are also involved in Proto IX accumulation (see below). In mustard, a species that produces small amounts of Proto IX in response to even very high levels of the herbicide, Protox was as sensitive to acifluorfen as Protox of morningglory and cucumber, two highly sensitive species. Because the Proto IX accumulation response to acifluorfen treatment was greatly enhanced by ALA, the reason for low accumulation with acifluorfen treatment alone appears to be low rates of carbon flow through the porphyrin pathway compared with other species.

Why certain species differ in the amount of Proto IX that they generate when Protox is blocked may be related to differences between species in regulation of the porphyrin pathway. Hemin, the oxidized form of heme, is a feedback inhibitor of the porphyrin pathway (20, 30). Inhibition of its synthesis by inhibition of Protox should eventually deregulate the porphyrin pathway when free heme pools are reduced. Several aspects of this regulatory system could differ between species, resulting in the differential effects of acifluorfen that we have observed.

LITERATURE CITED

1. **Becerril JM, Duke SO** (1989) Protoporphyrin IX content correlates with activity of photobleaching herbicides. Plant Physiol **90:** 1175–1181
2. **Becerril JM, Duke SO** (1989) Acifluorfen effects on intermediates of chlorophyll synthesis in green cucumber tissues. Pestic Biochem Physiol **35:** 119–126
3. **Bradford MM** (1976) A rapid and sensitive method for the quantification of microgram quantities of protein-dye binding. Anal Biochem **72:** 248–254
4. **Deybach JC, de Verneuil H, Nordmann Y** (1981) The inherited enzymatic defect in porphyria variegata. Hum Genet **58:** 425–428
5. **Duke SO, Becerril JM, Sherman TD, Matsumoto H** (1991) Photosensitizing porphyrins as herbicides. Am Chem Soc Symp Ser **449:** 371–386
6. **Duke SO, Kenyon WH** (1986) Photosynthesis is not involved in the mechanism of action of acifluorfen in cucumber (*Cucumis sativus*). Plant Physiol **81:** 882–888
7. **Duke SO, Vaughn KC, Meeusen RL** (1984) Mitochondrial involvement in the mode of action of acifluorfen. Pestic Biochem Physiol **21:** 368–376
8. **Finckh BF, Kunert KJ** (1985) Vitamins C and E: an antioxidative system against herbicide-induced lipid peroxidation in higher plants. J Agric Food Chem **33:** 574–577
9. **Frear DS, Swanson HR, Mansager ER** (1983) Acifluorfen metabolism in soybean: diphenylether bond cleavage and the formation of homoglutathione, cysteine, and glucose conjugates. Pestic Biochem Physiol **20:** 299–310
10. **Hiscox JD, Israelstam GF** (1979) A method for the extraction of chlorophyll from leaf tissues without maceration. Can J Bot **57:** 1332–1334
11. **Jacobs JM, Jacobs NJ, Borotz SE, Guerinot ML** (1990) Effects of the photobleaching herbicide, acifluorfen-methyl, on protoporphyrinogen oxidation in barley organelles, soybean root mitochondria, soybean root nodules, and bacteria. Arch Biochem Biophys **280:** 369–375
12. **Jacobs JM, Jacobs NJ, Sherman TD, Duke SO** (1991) Effect of diphenyl ether herbicides on oxidation of protoporphyrinogen to protoporphyrin in organellar and plasma membrane enriched fractions of barley. Plant Physiol **97:** 197–203
13. **Jacobs NJ, Jacobs JM** (1982) Assay for enzymatic protoporphyrinogen oxidase, a late step in heme synthesis. Enzyme **28:** 206–219
14. **Kenyon WH, Duke SO** (1985) Acifluorfen effects on endogenous antioxidants and protective enzymes. Plant Physiol **79:** 862–866

15. **Kenyon WH, Duke SO, Vaughn KC** (1985) Sequence of effects of acifluorfen on physiological and ultrastructural parameters in cucumber cotyledon discs. Pestic Biochem Physiol **24:** 240–250
16. **Kouji H, Masuda T, Matsunaka S** (1989) Action mechanism of diphenyl ether herbicides: stimulation of 5-aminolevulinic acid synthesizing system activity. Pestic Biochem Physiol **33:** 230–238
17. **Kunert K, Dodge AD** (1989) Herbicide-induced radical damage and antioxidative systems. *In* P Böger, G Sandmann, eds, Target Sites of Herbicide Action. CRC Press, Boca Raton, FL, pp 45–63
18. **Lehnen LP, Sherman TD, Becerril JM, Duke SO** (1990) Tissue and cell localization of acifluorfen-induced porphyrins in cucumber cotyledons. Pestic Biochem Physiol **37:** 239–248
19. **Lydon J, Duke SO** (1988) Porphyrin synthesis is required for photobleaching activity of the *p*-nitrosubstituted diphenyl ether herbicides. Pestic Biochem Physiol **31:** 74–83
20. **Masuda T, Kouji, Matsunaka S** (1990) Action mechanism of diphenyl ether herbicides—decrease in heme content stimulates 5-aminolevulinic acid synthesis. Pestic Biochem Physiol **36:** 106–114
21. **Matringe M, Camadro J-M, Labbe P, Scalla R** (1989) Protoporphyrinogen oxidase as a molecular target for diphenyl ether herbicides. Biochem J **260:** 231–235
22. **Matringe M, Camadro J-M, Labbe P, Scalla R** (1989) Protoporphyrinogen oxidase inhibition by three peroxidizing herbicides: oxadiazon, LS 82–556 and M&B 39279. FEBS Lett **245:** 35–38
23. **Matringe M, Clair D, Scalla R** (1990) Effects of peroxidizing herbicides on protoporphyrin IX levels in non-chlorophyllous soybean cell culture. Pestic Biochem Physiol **36:** 300–307
24. **Matringe M, Scalla R** (1988) Studies on the mode of action of acifluorfen-methyl in non-chlorophyllous soybean cells: accumulation of tetrapyroles. Plant Physiol **86:** 619–622
25. **Matringe M, Scalla R** (1988) Effects of acifluorfen-methyl on cucumber cotyledons: protoporphyrin accumulation. Pestic Biochem Physiol **32:** 164–172
26. **Matsumoto H, Duke SO** (1990) Acifluorfen-methyl effects on porphyrin synthesis in *Lemna paucicostata* Hegelm. 6746. J Agric Food Chem **38:** 2066–2071
27. **Mayasich JM, Nandihalli UB, Leibl RA, Rebeiz CA** (1990) The primary mode of action of acifluorfen-Na in intact seedlings is not via tetrapyrrole accumulation during the first dark period following treatment. Pestic Biochem Physiol **36:** 259–268
28. **Nandihalli UB, Leibl RA, Rebeiz CA** (1991) Photodynamic herbicides VIII. Mandatory requirement of light for the induction of protoporphyrin IX accumulation in acifluorfen-treated cucumber. Pestic Sci **31:** 9–23
29. **Nicolaus B, Sandmann G, Watanabe H, Wakabayashi K, Böger P** (1989) Herbicide-induced peroxidation: influence of light and diuron on protoporphyrin IX formation. Pestic Biochem Physiol **35:** 192–201
30. **Padmanaban G, Ventkateswar V, Rangarajan PN** (1990) Haem as a multifunctional regulator. Trends Biochem **14:** 492–496
31. **Sandmann G, Böger P** (1988) Accumulation of protoporphyrin IX in the presence of peroxidizing herbicides. Z Naturforsch **43c:** 699–704
32. **Sandmann G, Nicolaus B, Böger P** (1990) Typical peroxidative parameters verified with mung-bean seedlings, soybean cells and duckweed. Z Naturforsch **45c:** 512–517
33. **Schmidt A, Kunert KJ** (1986) Lipid peroxidation in higher plants. The role of glutathione reductase. Plant Physiol **82:** 700–702
34. **Sherman TD, Duke MV, Clark RD, Sanders EF, Matsumoto H, Duke SO** (1991) Pyrazole phenyl ether herbicides inhibit protoporphyrinogen oxidase. Pestic Biochem Physiol (in press)
35. **Witkowski DA, Halling BP** (1988) Accumulation of photodynamic tetrapyrroles induced by acifluorfen-methyl. Plant Physiol **87:** 632–637
36. **Witkowski DA, Halling BP** (1989) Inhibition of plant protoporphyrinogen oxidase by the herbicide acifluorfen-methyl. Plant Physiol **90:** 1239–1242