Acifluorfen-methyl Effects on Porphyrin Synthesis in *Lemna pausicostata* Hegelm. 6746

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Acifluorfen-methyl [methyl 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate] (0.1–1 μM) caused electrolyte leakage from *Lemna pausicostata* plants after a 2-h lag when treated in white light (500 $\mu E\ m^{-2}\ s^{-1}$). When treated in darkness for 20 h and then exposed to light, herbicidal damage developed slowly compared with that in plants exposed to light and herbicide simultaneously. In light, acifluorfen-methyl caused an almost 100-fold accumulation of protoporphyrin IX during the first 2 h of treatment, followed by a decline in content. Protochlorophyllide levels in light were decreased by the herbicide. There was no effect on Mg–protoporphyrin IX, coproporphyrin, or uroporphyrin content. In darkness, protoporphyrin IX accumulation in herbicide treatments reached only 20–25% of that occurred in the light. Exogenous sucrose had no effect on dark accumulation of any porphyrin in any treatment. Exogenous hemin significantly reduced the efficacy to the herbicide. These data indicate that protoporphyrin IX is the only photodynamic compound causing herbicidal damage in acifluorfen-methyl-treated plants and that the herbicide is much more effective in light than in darkness because of a greater capacity to synthesize porphyrins in light.

INTRODUCTION

Several herbicides that cause rapid photobleaching of green plant tissues have recently been found to cause accumulation of unusually high concentrations of porphyrin precursors of chlorophyll in treated tissues (Matringe and Scalla, 1987, 1988a,b; Lydon and Duke, 1988; Duke et al., 1989; Sandman and Böger, 1988; Becerril and Duke, 1989a,b; Witkowski and Halling, 1988). The compounds include nitrodiphenyl ethers, cyclic imides, oxadiazoles, and a phenylpyrazole. In most cases, protoporphyrin IX was found to be the prevalent porphyrin pigment accumulating, and the level accumulated is generally strongly correlated with herbicidal damage [e.g., Becerril and Duke (1989a,b)]. Protoporphyrin IX is a strong photosensitizing pigment, and its absorption spectrum approximates the action spectra for these photobleaching herbicides (Ensminger and Hess, 1985; Gaba et al., 1988; Sato et al., 1987, 1988).

Protoporphyrin IX is caused to accumulate by inhibition of the enzyme that normally converts protoporphyrinogen to protoporphyrin IX, protoporphyrinogen oxidase (Matringe et al., 1989a,b; Witkowski and Halling, 1989). As with the genetic defect in this enzyme in humans which causes variegate porphyria (Deybach et al., 1981), blockage of the porphyrin pathway at this site results in uncontrolled autoxidation of the enzyme's substrate to protoporphyrin IX. Furthermore, flow of carbon into the porphyrin pathway is deregulated by inhibition of enzymatically produced protoporphyrin IX (Kouji et al., 1989). Loss of enzymatically produced protoporphyrin IX results in lower heme levels (Kouji et al., 1989; Masuda et al., 1990), and heme is a feedback inhibitor of 5-aminolevulinic acid synthesis (Castelfranco and Beale, 1983). Supplying herbicide-treated tissues with an exogenous source of heme resulted in an almost complete reduction in herbicidal damage (Masuda et al., 1990).

Although the molecular site of action of these herbicides appears to have been discovered, several details of the mode of action remain unclear. In some cases there have not been good correlations between the amount of porphyrin accumulation and the herbicidal damage (Sandmann and Böger, 1988). In other cases these herbicides have caused more profound effects on protochlorophyllide than on protoporphyrin IX accumulation (Kouji et al., 1988, 1989; Mayasich et al., 1989). Many of the above studies have been on excised tissues or detached cotyledons that may not accurately reflect porphyrin metabolism in intact plants. Matringe and Scalla (1988b) found quite different effects of acifuorfen-methyl and the pyridine derivative LS 82-556 on protoporphyrin IX accumulation in cotyledons of intact cucumber plants than in excised cotyledon tissues. Thus far, there has not been a comprehensive study of the effects of a protoporphyrinogen oxidase inhibiting herbicide on porphyrin synthesis in intact plants under various conditions. In this paper we make such a study, utilizing the aquatic higher plant *Lemna pausicostata*. This species offers the advantage of utilizing many individual plants of identical genetic background at each sampling, thus greatly reducing sampling error and variation due to genetic differences. Furthermore, this species has previously been shown to be quite sensitive to photobleaching diphenyl ether herbicides (Matsumoto et al., 1990), and we chose it to further extend these studies. Unlike any previous study, we have determined the effects of the herbicide on virtually all of the intermediates of chlorophyll synthesis, from uroporphyrin III to protochlorophyllide. Our results indicate that, unlike results with excised tissues, these herbicides are more effective on intact plants in light than in dark because of the capacity for greater porphyrin synthesis in the light. However, the pattern of porphyrin accumulation is very similar to that found in excised tissues of other species, with only protoporphyrin IX accumulating at sufficiently high levels to act as a photodynamic pigment.

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MATERIALS AND METHODS

Plant Material and Treatment with Herbicide. Cultures of *L. pausicostata* Hegelm. 6746 were initiated with inoculation of 30 fronds (10 colonies) to 50 mL of half-strength Hutner's medium (pH 6.4) in 1-L Erlenmeyer flasks. Plants were grown autotropically for a week in a growth chamber in continuous illumination (150 μE m^{-2} s^{-1}) at 25 °C. Then, plants in three flasks were combined and transferred to a 5-L plastic container with a 20-cm depth in which they were grown for another 1–2 weeks in 1.5 L of Hutner's medium. The medium was renewed every 3 days. Under these conditions, doubling time of the colonies was approximately 2 days.

Herbicide Treatment and Electrolyte Leakage. Fifty colonies of three fronds each were selected from culture and placed in 6-cm-diameter polystyrene Petri dishes with 5 mL of distilled water. Technical grade (>99%) acifluorfen-methyl was added in ethanol to a final ethanol concentration of 0.3% (v/v). Control plants were also exposed to 0.3% ethanol. Hemin (type I, bovine, from Sigma Chemical Co.) dissolved in 0.001 N NH_4OH was used in some experiments with 0.001 N NH_4OH in control dishes. The plants were then exposed to 500 μE m^{-2} s^{-1} of white light or kept in darkness at 25 °C. Electrolyte leakage from the plants was determined with a conductivity meter as described previously (Kenyon et al., 1985). The experiments were repeated, and results shown are means of triplicate treatments from representative experiments.

Chlorophyll and Porphyrin Assays. Chlorophyll was extracted and assayed by the methods of Hiscox and Israelstam (1979). Fifty colonies were soaked in 5 mL of dimethyl sulfoxide in darkness at room temperature for 24 h, and total chlorophyll in extracts was determined spectrophotometrically by the method of Arnon (1949).

All extractions for HPLC were made under a dim, green light source. Samples (50 colonies) for analysis of protoporphyrin IX, Mg-protoporphyrin IX, Mg-protoporphyrin IX monomethyl ester, and protochlorophyllide were homogenized in 6 mL of HPLC-grade methanol: 0.1 N NH_4OH (9:1 v/v) with a Brinkman Polytron at 60% full power for 15 s. The homogenate was centrifuged at 30000*g* for 10 min at 0 °C, and the supernatant was saved. The pellet was resuspended in 3 mL of methanol, sonicated for 5 min, and centrifuged at 30000*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.

Samples for analysis of coproporphyrin III and uroporphyrin III were homogenized in 3 mL of acetone/concentrated HCl (99:1 v/v) and centrifuged as above. The pellet was extracted again with 3 more mL of acetone/HCl by sonicating for 2 min and then centrifuged, combined with the first supernatant, and rotoevaporated as above. The residue was dissolved in 2 mL of acetone/0.1 N HCl (9:1 v/v) with sonication and then filtered before analysis by HPLC. This method also extracted protoporphyrin IX, so these data were included with the coproporphyrin III and uroporphyrin III data.

The HPLC system was composed of Waters Associates components which 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.

For analysis of protoporphyrin IX, Mg-protoporphyrin IX, Mg-protoporphyrin IX monomethyl ester, and protochlorophyllide, 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 0–10 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 protoporphyrin IX (Sigma Chemical Co.), Mg-protoporphyrin IX, and Mg-protoporphyrin IX monomethyl ester (Porphyrin Products, Inc.) were used. Protochlorophyllide was quantified by extracting protochlorophyllide from etiolated tissues, quantifying it spectrophotometrically according to the method of Anderson and Boardman (1964), and injecting spectrophotometrically assayed protochlorophyllide 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 protoporphyrin IX; 415 and 595 nm, respectively, for Mg-protoporphyrin IX and Mg-protoporphyrin IX monomethyl ester; and 440 and 630 nm, respectively, for protochlorophyllide. The photodiode array detector scanned from 300 to 700 nm to confirm all peaks. The retention times of Mg-protoporphyrin IX, protochlorophyllide, and protoporphyrin IX were 6.3, 11.3, and 16.5 min, respectively. High levels of Mg-protoporphyrin IX monomethyl ester are caused to accumulate by treatment of plant tissues with 2,2′-dipyridyl (Duggan and Gassman, 1974). The retention times of commercial Mg-protoporphyrin IX monomethyl ester and the principle component caused to accumulate by 2,2′-dipyridyl in a variety of plant tissues were 21.7 and 12.8 min, respectively. The spectrophotometric and spectrofluorometric properties of these two compounds were virtually identical, and the extinction coefficients of the commercial standard at several wavelengths were within 90% of the published values for Mg-protoporphyrin IX monomethyl ester (Granick, 1948). Amending tissue extracts with the commercial standard did not alter its retention time. We concluded that the commercial standard of Mg-protoporphyrin IX monomethyl ester contained an unknown ligand that altered its retention time, but not its spectral properties. Thus, we used it for quantification of Mg-protoporphyrin IX monomethyl ester.

The same HPLC apparatus and columns were used for analysis of coproporphyrin III and uroporphyrin III. Standards of the dihydrochloride salts of these two protoporphyrin IX precursors were obtained from Porphyrin Products, Inc., Logan, UT. The solvent gradient was composed of 0.1 M ammonium phosphate (brought to pH 3.5 with phosphoric acid) (solvent A) and HPLC-grade methanol (solvent B) at a flow rate of 1.2 mL/min. The solvent delivery program was as follows: a linear transition from 60% B in A to 100% B in 15 min, followed by 20 min of 100% B and then 10 min of 60% B in A. The injection volume was 50 μL. Fluorescence detection was with an excitation wavelength of 398 nm and emission at 670 nm. Uroporphyrin III, coproporphyrin III, and protoporphyrin IX were detected at 7.9, 16.3, and 20.9 min of elution, respectively, with this method.

In previous studies with these methods (Becerril and Duke, 1989b), we found excellent recovery of commercial porphyrin standards from plant tissues augmented with various porphyrins. Furthermore, we found no conversion of these compounds to other porphyrin compounds during sample preparation and analysis with our methods. All porphyrin compound levels are expressed as moles per 50 colonies or per gram of fresh weight.

RESULTS AND DISCUSSION

Herbicidal Effects of Acifluorfen-methyl. Electrolyte leakage was a much more rapid and sensitive assay of herbicidal activity than was loss of chlorophyll. After 24 h of exposure to light and the herbicide, a chlorophyll reduction of approximately 50% was caused by 1 μM acifluorfen-methyl (Figure 1). However, in the same experiment there was a 400% increase in electrolyte leakage caused by the same herbicide concentration after only 6 h of exposure (Figure 1). Electrolyte leakage was previously shown to be one of the earliest detectable effects of herbicidal damage from acifluorfen in cucumber cotyledon disks (Kenyon et al., 1985).

Rapid electrolyte leakage was preceded by a 2-h lag period during which there was no detectable effect of the herbicide (Figure 2A). The lag period was unaffected by herbicide concentration in the range 0.1–1 μM. Electrolyte leakage reached a maximum after 8, 9, and 10 h in plants treated with 0.1, 0.33, and 1.0 μM, respectively.

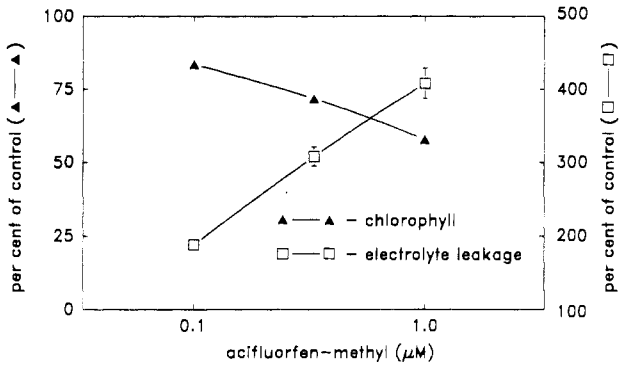

Figure 1. Effect of different concentrations of acifluorfen-methyl on chlorophyll content and electrolyte linkage of *L. pausicostata* colonies. Chlorophyll was assayed after 24 h and electrolyte leakage after 6 h of exposure to light during exposure to herbicide. The control level of chlorophyll was 131.3 ± 17.5 μg/50 colonies, and the control electrolyte leakage was 9 ± 3 μmho/cm. Error bars are ±1 SE of the mean. In some cases the symbol diameter is greater than the error.

In previous experiments with cucumber cotyledon disks, we found that incubation with the herbicide in darkness for 20 h reduced the lag period for herbicidal activity in the light (Duke et al., 1984). This was also the case with *L. pausicostata*; however, the activity of the herbicide was much lower than in plants exposed simultaneously to acifluorfen-methyl and light (Figure 2B). The effect was the same in (a) plants incubated in water during 20 h of darkness and then exposed to the herbicide and light simultaneously (data not shown), (b) plants exposed to the same herbicide solution in dark and light (Figure 2B), and (c) plants exposed to the herbicide in darkness and then transferred to the light and a fresh herbicide solution simultaneously (data not shown).

Acifluorfen-methyl Effects on Porphyrins. Protoporphyrin IX content rose rapidly after simultaneous exposure to light and acifluorfen-methyl, peaking at 2 h and then rapidly decreasing (Figure 3A). At 2 h there was almost 100-fold more protoporphyrin IX in acifluorfen-methyl-treated plants than in control plants. In very similar experiments with cucumber cotyledon disks, acifluorfen caused rapid increases in protoporphyrin IX also, with maximal accumulation of protoporphyrin IX by 12 h, followed by a rapid decrease in protoporphyrin IX content (Becerril and Duke, 1989). In *L. pausicostata*, the protoporphyrin IX accumulation phase and the lag phase in electrolyte leakage coincided (Figures 2A and 3A). The start of herbicide-induced electrolyte leakage and of loss of protoporphyrin IX occurred together. In cucumber, the beginning of electrolyte leakage preceded the beginning of the loss of protoporphyrin IX somewhat; however, loss of proporphyrins was correlated with electrolyte leakage (Becerril and Duke, 1989a). This is to be expected because electrolyte leakage correlates with broken plasmalemmas and loss of cellular contents (Kenyon et al., 1985).

The protoporphyrin IX lost from the tissues could not be accounted for in the bathing solution. After 6 h of light exposure, only 0.006 ± 0.002 and 0.002 ± 0.001 nmol of protoporphyrin IX/50 colonies were found in the bathing solution of treated and control plants, respectively. Similar results were found with cucumber cotyledon disks (H. Matsumoto, unpublished data). Protoporphyrin IX is more stable in vitro than in vivo (Becerril and Duke, 1989a). Furthermore, it is much more soluble in lipid than in aqueous media and is thus unlikely to be substantially released from membranes into the media.

Protochlorophyllide content decreased in light in both treated and untreated plants; however, the decrease was much faster in acifluorfen-methyl-treated plants than in control plants (Figure 3B). Very little Mg-protoporphyrin IX was detected, and the levels decreased at a similar rate in both control and acifluorfen-methyl-treated plants (Figure 3C). There was even less Mg-protoporphyrin IX monomethyl ester in these tissues, and these levels also decreased similarly in both control and herbicide-treated plants (data not shown). These data demonstrate unequivocally that protochlorophyllide, Mg-protoporphyrin IX, or Mg-protoporphyrin IX monomethyl ester cannot be responsible for the photodynamic damage caused by acifluorfen-methyl in these plants. Thus, of the porphyrins assayed, protoporphyrin IX is the porphyrin likely to be responsible for the photodynamic action of the herbicide.

The dose/response kinetics of the effects of acifluorfen-methyl on protoporphyrin IX content (Figure 4) paralleled those of the herbicidal activity of this diphenyl ether (Figure 1). No similar relationship existed for the other chlorophyll precursors (Figure 4; data not shown for Mg-protoporphyrin IX monomethyl ester).

The pattern of porphyrin accumulation in acifluorfen-methyl-treated plants in darkness (Figure 5) was quite different from that observed in the light. Protoporphyrin IX accumulation was rapid for 2 h and then stopped, reaching a stationary level for the ensuing 18 h. The stationary state that was reached was a third to a fourth of that of the maximum levels that accumulated in treated tissues in the light. There was also a much reduced herbicidal action of acifluorfen-methyl after a 20-h dark incubation compared to simultaneous exposure to light and the herbicide (Figure 2). Protochlorophyllide accumulated at approximately the same rate over the 20-h incubation period in both control and herbicide-treated plants after the first 2 h. Since this level of protochlorophyllide caused no herbicidal activity in untreated plants (Figure 2B), it is doubtful that protochlorophyllide could be involved in the herbicidal action of acifluorfen-methyl. No accumulation of Mg-protoporphyrin IX (Figure 5) or its monomethyl ester (data not shown) occurred in either treatment.

Addition of 1% sucrose ot the media had no effect on accumulation of any of the porphyrins in untreated acifluorfen-methyl-treated plants in darkness (data not shown). Thus, a lack of photosynthate is probably not the cause of the cessation of protoporphyrin IX accumulation in acifluorfen-methyl-treated plants after 2 h of darkness.

Upon exposure of dark-incubated tissues to light, protoporphyrin IX accumulation reached a maximum of only 2 nmol/50 colonies, regardless of whether herbicide exposure was continuous, replenished after the dark incubation, or provided only after the dark incubation (data not shown). Thus, the reduced herbicidal activity after dark incubation is apparently due to a reduced capacity for porphyrin synthesis after 20 h of darkness, rather than to herbicide degradation in darkness.

Protoporphyrinogen oxidase inhibiting herbicides have strong effects on protoporphyrinogen oxidase in mammalian liver (Matringe et al., 1989a,b). Some biphenyl compounds cause uroporphyrin III and coproporphyrin III accumulation in animal liver tissues (Lamprecht et al., 1988). Accumulation of protoporphyrin IX as well as uroporphyrin III and coproporphyrin III is symptomatic of porphyria. There have been no published reports of the effects of diphenyl ether herbicides on uroporphyrin or coproporphyrin in animal or plant tissues. No strong effects of acifluorfen-methyl were found on content of co-

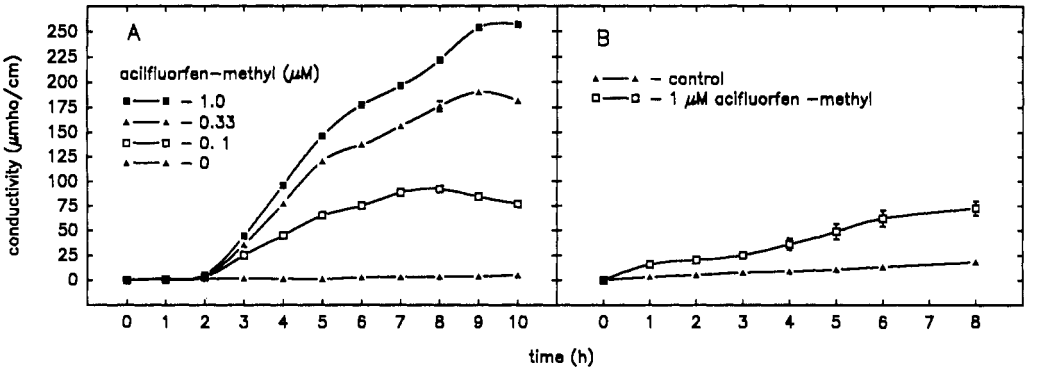

Figure 2. Effect of acifluorfen-methyl on electrolyte leakage of 50 *L. pausicostata* colonies upon immediate exposure to the herbicide and light (A) or upon exposure to light following 20 h of incubation in the herbicide solution in darkness (B). Error bars are ±1 SE of the mean. In some cases the symbol diameter is greater than the error.

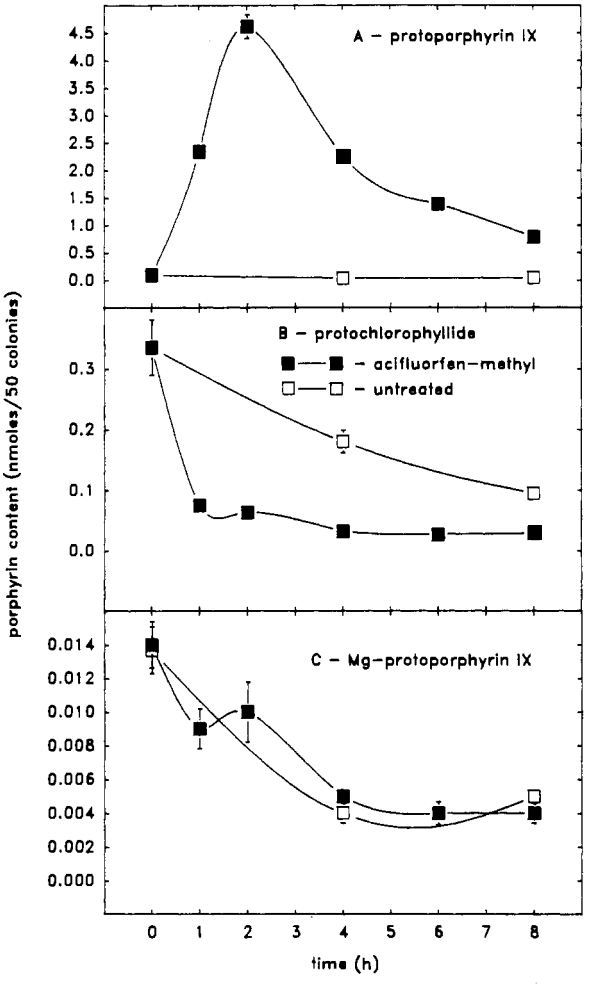

Figure 3. Effect of 1 μM acifluorfen-methyl on chlorophyll precursors in *L. pausicostata* colonies upon immediate exposure to light and the herbicide. Error bars are ±1 SE of the mean. In some cases the symbol diameter is greater than the error.

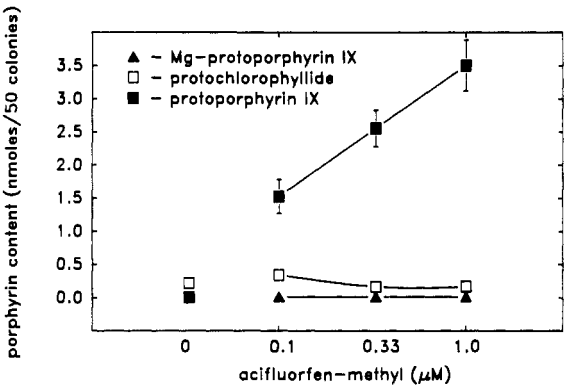

Figure 4. Effect of different concentrations of acifluorfen-methyl on chlorophyll precursors in *L. pausicostata* colonies after 2 h exposure to light and the herbicide. Error bars are ±1 SE of the mean. In some cases the symbol diameter is greater than the error.

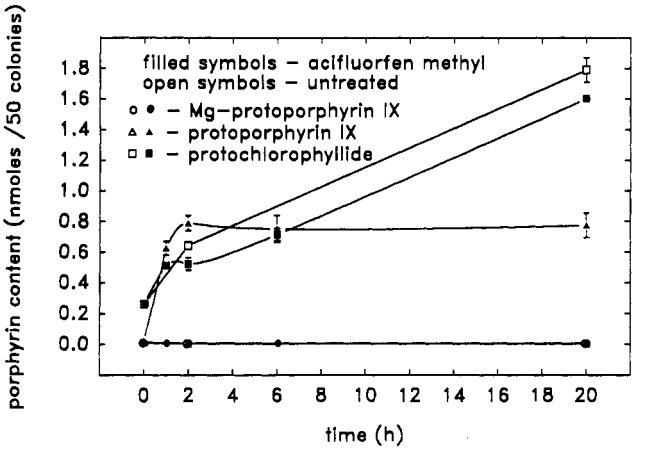

Figure 5. Effect of 1 μM acifluorfen-methyl on chlorophyll precursors in *L. pausicostata* colonies during incubation in darkness. Error bars are ±1 SE of the mean. In some cases the symbol diameter is greater than the error. Data points for both control and acifluorfen-methyl treatment Mg-protoporphyrin IX and control protoporphyrin IX are superimposed.

proporphyrin III or uroporphyrin III, the immediate precursors of protoporphyrin IX (Table I).

Addition of hemin causes a reduction in the activity of acifluorfen-methyl (Figure 6). Hemin had no effect when supplied with the herbicide simultaneously with exposure to light (data not shown); however, exposure to both the herbicide and hemin during 20 h of darkness before exposure to light allowed the hemin to exert an effect. Uptake of hemin or heme may be much slower than that of the herbicide. A similar treatment of acifluorfen-methyl-treated cultured tobacco cells by Masuda et al. (1990) resulted in reduced oxygen consumption of a cell homogenate (a measure of protoporphyrin IX stimulated lipid peroxidation) and reduced 5-aminolevulinic acid synthesis that was stimulated by the herbicide. Thus, the increase in protoporphyrin IX caused by acifluorfen-methyl in *L. pausicostata* is also probably due to decreased feedback inhibition by heme.

The results presented in this paper with intact *L. pausicostata* plants support our previous findings with excised cucumber tissues (Becerril and Duke, 1989a,b) that protoporphyrin IX is the only porphyrin compound between protoporphyrinogen and chlorophyll that is involved in the photodynamic action of diphenyl ether herbicides. We

Table I. Accumulation of Porphyrins in *L. pausicostata* after 20 h of Exposure to 1 μM Acifluorfen-methyl in Darkness

porphyrin	pmol/50 colonies control	pmol/50 colonies treated
uroporphyrin III	23 ± 1	27 ± 1
coproporphyrin III	4 ± 1	9 ± 1
protoporphyrin IX	6 ± 2	369 ± 38

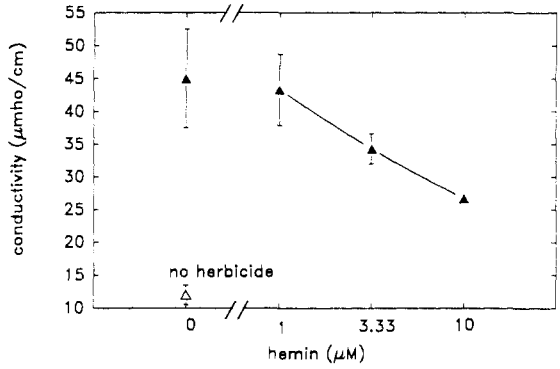

Figure 6. Effect of different hemin concentrations on electrolyte leakage from 50 *L. pausicostata* colonies treated with 1 μM acifluorfen-methyl after exposure to 8 h of light. The colonies were incubated in darkness for 20 h in both heme and the herbicide before exposure to light. Error bars are ±1 SE of the mean. In some cases the symbol diameter is greater than the error.

found no evidence, as others have suggested (Kouji et al., 1988, 1989; Mayasich, 1989), that protochlorophyllide is involved in any way with this activity. In intact cucumber plants, we have found acifluorfen-induced increases in protochlorophyllide (Duke et al., 1990). However, the increases were only 2-fold normal levels, whereas protoporphyrin IX concentrations were increased several hundredfold over the control levels.

Matringe and Scalla (1988b) found herbicide-induced protoporphyrin accumulation in intact cucumber plants to be light stimulated. In cotyledons of intact younger cucumber seedlings (Duke et al., 1990) or in excised cucumber cotyledons floated on sucrose (Lydon and Duke, 1988; Becerril and Duke, 1989a), this was not the case. Our present data show that acifluorfen-methyl-induced protoporphyrin IX accumulation is more pronounced in light than in darkness in intact *L. pausicostata* plants; however, synthesis could proceed for 2 h in darkness. The finding that supplemental sucrose had no effect on the patterns of porphyrin accumulation in darkness supports the view that diphenyl ether induced protoporphyrin IX synthesis is limited by a regulatory factor other than photosynthate in darkness. The fact that protochlorophyllide synthesis continued after the cessation of herbicide-induced protoporphyrin IX synthesis supports this hypothesis. Similarly, Nicolaus et al. (1989) found that heterotrophically growth algae treated with a photobleaching diphenyl ether herbicide accumulated protoporphyrin IX in both dark and light, but accumulated more in light than in darkness. Autotrophically growth algae treated with the herbicide accumulated no protoporphyrin IX in darkness, but accumulated high levels in the light. Apparently, the capacity to synthesize porphyrins is more substrate dependent in this system.

CONCLUSIONS

Our data are consistent with all of our previous data on the effects of photobleaching diphenyl ether and oxadiazole herbicides on excised and intact plant tissues (Lydon and Duke, 1988; Becerril and Duke, 1989a,b; Duke et al., 1989, 1990). These data indicate that protoporphyrin IX is the only chlorophyll precursor between 5-aminolevulinic acid and chlorophyll that is significantly involved in the photodynamic action of these herbicides. Blockage of heme synthesis may deregulate the porphyrin synthesis pathway, leading to even greater carbon flow into the pathway than might normally be expected. Furthermore, we conclude that differences in effects in light and dark and in intact versus excised tissues are the result of differential regulation of porphyrin synthesis in light and darkness, as well as a reduction in capacity to synthesize porphyrins in light after a prolonged dark incubation.

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LITERATURE CITED

Anderson, J. M.; Boardman, N. K. Studies of the greening of dark-grown bean plants. II. Development of photochemical activity. *Aust. J. Biol. Sci.* **1964**, *17*, 93–101.

Arnon, D. I. Copper enzymes in isolated chloroplasts. Polyphenol oxidases in *Beta vulgaris*. *Plant Physiol.* **1949**, *24*, 1–15.

Becerril, J. M.; Duke, S. O. Protoporphyrin IX content correlates with activity of photobleaching herbicides. *Plant Physiol.* **1989a**, *90*, 1175–1181.

Becerril, J. M.; Duke, S. O. Acifluorfen effects on intermediates of chlorophyll synthesis in green cucumber tissues. *Pestic. Biochem. Physiol.* **1989b**, *35*, 119–126.

Castelfranco, P. A.; Beale, S. I. Chlorophyll biosynthesis: Recent advances and areas of interest. *Annu. Rev. Plant Physiol.* **1983**, *34*, 241-278.

Deybach, J. C.; de Verneuil, H.; Nordmann, Y. The inherited enzymatic defect in porphyria variegata. *Hum. Genet.* **1981**, *58*, 425–428.

Duggan, J.; Gassman, M. Induction of porphyrin synthesis in etiolated bean leaves by chelators of iron. *Plant Physiol.* **1974**, *53*, 206–215.

Duke, S. O.; Vaughn, K. C.; Meeusen, R. L. Mitochondrial involvement in the mode of action of acifluoren. *Pestic. Biochem. Physiol.* **1984**, *21*, 368–376.

Duke, S. O.; Lydon, J.; Paul, R. N. Oxadiazon activity is similar to that of *p*-nitro-diphenyl ether herbicides. *Weed Sci.* **1989**, *37*, 152–160.

Duke, S. O.; Becerril, J. M.; Sherman, T. D.; Matsumoto, H. Photosensitizing porphyrins as herbicides. *ACS Symp. Ser.* **1990**, in press.

Ensminger, M. P.; Hess, F. D. Action spectrum of the activity of acifluorfen-methyl, a diphenylether herbicide, in *Chlamydomonas eugametos*. *Plant Physiol.* **1985**, *77*, 503–505.

Gaba, V.; Cohen, N.; Shaaltiel, Y.; Ben-Amotz, A.; Gressel, J. Light-requiring acifluorfen action in the absence of bulk photosynthetic pigments. *Pestic. Biochem. Physiol.* **1988**, *31*, 1–12.

Granick, S. Magnesium protoporphyrin as a precursor of chlorophyll in *Chlorella*. *J. Biol. Chem.* **1948**, *175*, 333–342.

Hiscox, J. D.; Israelstam, G. F. A method for the extraction of chlorophyll from leaf tissues without maceration. *Can. J. Bot.* **1979**, *57*, 1332–1334.

Kenyon, W. H.; Duke, S. O.; Vaughn, K. C. Sequence of effects of acifluorfen on physiological and ultrastructural parameters in cucumber cotyledon discs. *Pestic. Biochem. Physiol.* **1985**, *24*, 240–250.

Kouji, H.; Masuda, T.; Matsunaka, S. Action mechanism of diphenyl ether herbicides; Light-dependent O_2-consumption in diphenyl ether treated tobacco cell homogenate. *J. Pestic. Sci.* **1988**, *13*, 495–499.

Kouji, H.; Masuda, T.; Matsunaka, S. Action mechanism of diphenyl ether herbicides; Stimulation of 5-aminolevulinic acid synthesizing system activity. *Pestic. Biochem. Physiol.* **1989**, *33*, 230–238.

Lambrecht, R. W.; Sinclair, P. R.; Bement, W. J.; Sinclair, J. F.; Carptenter, H. M.; Buhler, D. R.; Urquhart, A. J.; Elder, G. H. Hepatic uroporphyrin accumulation and uroporphyrinogen decarboxylase activity in cultured chick-embryo hepatocytes and in Japanese quail (*Coturnix coturnix japonica*) and mice treated with polyhalogenated aromatic compounds. *Biochem. J.* **1988**, *253*, 131–138.

Lydon, J.; Duke, S. O. Porphyrin synthesis is required for photobleaching activity of the *p*-nitrosubstituted diphenyl ether herbicides. *Pestic. Biochem. Physiol.* **1988**, *31*, 74–83.

Masuda, T.; Kouji, H.; Matsunaka, S. Action mechanism of diphenyl ether herbicides—decrease in heme content stimulates 5-aminolevulinic acid synthesis. *Pestic. Biochem. Physiol.* **1990**, *36*, 106–114.

Matringe, M.; Scalla, R. Induction of tetrapyrroles by diphenylether-type herbicides. *Proc. Br. Crop Protect. Conf.* **1987**, *9B*, 981–988.

Matringe, M.; Scalla, R. Studies on the mode of action of acifluorfen-methyl in non-chlorophyllous soybean cells: accumulation of tetrapyrroles. *Plant Physiol.* **1988a**, *86*, 619–622.

Matringe, M.; Scalla, R. Effects of acifluorfen-methyl on cucumber cotyledons: protoporphyrin accumulation. *Pestic. Biochem. Physiol.* **1988b**, *32*, 164–172.

Matringe, M.; Camadro, J.-M.; Labbe, P.; Scalla, R. Protoporphyrinogen oxidase as a molecular target for diphenyl ether herbicides. *Biochem. J.* **1989a**, *260*, 231–235.

Matringe, M.; Camadro, J.-M.; Labbe, P.; Scalla, R. Protoporphyrinogen oxidase inhibition by three peroxidizing herbicides: oxadiazon, LS 82-556 and M&B 39279. *FEBS Lett.* **1989b**, *245*, 35–38.

Matsumoto, H.; Kojima, S.; Ishizuka, K. Characteristics of herbicidal injury by diphenyl ether herbicides oxyfluorfen and bifenox in *Lemna pausicostata* Hegelm. *Weed Res.* **1990**, *35*, 36–43.

Mayasich, J. M.; Nandihalli, U. B.; Liebl, R. A.; Rebeiz, C. A. Accumulation of photodynamic tetrapyrroles in intact plants induced by acifluorfen. *Proc. Weed Sci. Soc. Am.* **1989**, *29*, 90.

Nicolaus, B.; Sandmann, G.; Watanabe, H.; Wakabayashi, K.; Böger, P. Herbicide-induced peroxidation: Influence of light and diuron on protoporphyrin IX formation. *Pestic. Biochem. Physiol.* **1989**, *35*, 192–201.

Sandmann, G.; Böger, P. Accumulation of protoporphyrin IX in the presence of peroxidizing herbicides. *Z. Naturforsch.* **1988**, *43C*, 699–704.

Sato, R.; Nagano, E.; Oshio, H.; Kamoshita, K.; Furuya, M. Wavelength effect of the action of a *N*-phenylimide S-23142 and a diphenylether acifluorfen-ethyl in cotyledons of cucumber (*Cucumis sativus* L.) seedlings. *Plant Physiol.* **1987**, *85*, 1146–1150.

Sato, R.; Nagano, E.; Oshio, H.; Kamoshita, K. Activities of the *N*-phenyl imide S-23142 in carotenoid-deficient seedlings of rice and cucumber. *Pestic. Biochem. Physiol.* **1988**, *31*, 213–220.

Witkowski, D. A.; Halling, B. P. Accumulation of photodynamic tetrapyrroles induced by acifluorfen-methyl. *Plant Physiol.* **1988**, *87*, 632–637.

Witkowski, D. A.; Halling, B. P. Inhibition of plant protoporphyrinogen oxidase by the herbicide acifluorfen-methyl. *Plant Physiol.* **1989**, *90*, 1239–1242.

Yanase, D.; Andoh, A. Porphyrin synthesis involvement in diphenyl ether-like mode of action of TNPP-ethyl, a novel phenylpyrazole herbicide. *Pestic. Biochem. Physiol.* **1989**, *35*, 70–80.

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Registry No. Acifluorfen-methyl, 50594-67-7; uroporphyrin III, 18273-06-8; coproporphyrin III, 14643-66-4; protoporphyrin IX, 553-12-8.