Protoporphyrin IX Content Correlates with Activity of Photobleaching Herbicides

Jose M. Becerril¹ and Stephen O. Duke*  
U.S. Department of Agriculture, Agricultural Research Service, Southern Weed Science Laboratory, P.O. Box 350, Stoneville, Mississippi 38776

ABSTRACT

Several laboratories have demonstrated recently that photobleaching herbicides such as acifluorfen and oxadiazon cause accumulation of protoporphyrin IX (PPIX), a photodynamic pigment capable of herbicidal activity. We investigated, in acifluorfen-treated tissues, the in vivo stability of PPIX, the kinetics of accumulation, and the correlation between concentration of PPIX and herbicidal damage. During a 20-hour dark period, PPIX levels rose from barely detectable concentrations to 1 to 2 nanomoles per 50 cucumber (Cucumis sativus L.) cotyledon discs treated with 10 micromolar acifluorfen. When placed in 500 micromoles per square meter per second PAR, PPIX levels decayed logarithmically, with an initial half-life of about 2.5 hours. PPIX levels at each time after exposure to light correlated positively with the cellular damage that occurred during the following 1 hour in both green and yellow (tentoxin-treated) cucumber cotyledon tissues. PPIX levels in discs incubated for 20 hours in darkness correlated positively with the acifluorfen concentration in which they were incubated. In cucumber, the level of herbicidal damage caused by several p-nitrodiphenyl ether herbicides, a p-chlorodiphenylether herbicide, and oxadiazon correlated positively with the amount of PPIX induced to accumulate by each of the herbicide treatments. Similar results were obtained with acifluorfen-treated pigweed and velvetleaf primary leaf tissues. In cucumber, PPIX levels increased within 15 and 30 minutes after exposure of discs to 10 micromolar acifluorfen in the dark and light, respectively. These data strengthen the view that PPIX is responsible for all or a major part of the photobleaching activity of acifluorfen and related herbicides.

The cause of the photobleaching activity of NDPE herbicides has been a continuing mystery (1, 20). Recent reports (8, 19, 21, 23–26, 30, 36) have linked accumulation of PPIX in tissues treated with NDPE, oxadiazole, cyclic imide, and other photobleaching herbicides to their ability to cause rapid photooxidative damage. These herbicides cause massive accumulation of PPIX in treated tissues and inhibitors of porphyrin synthesis strongly inhibit the activity of the herbicide. PPIX presumably acts as a photodynamic pigment, generating singlet oxygen in the presence of light and molecular oxygen. The absorption spectrum of PPIX, with peaks in the blue, green, and red spectral regions, fits the published action spectra for these herbicides (11, 12, 31, 32). Duke and Kenyon (7) argued that the toxic oxygen species in NDPE action is singlet oxygen and Haworth and Hess (15) recently demonstrated that singlet oxygen is the active toxic oxygen species in NDPE action. After dark incubation with the herbicide, the temperature independence of the light-induced photodestruction indicates that a photodynamic dye is responsible for the activity (17). If exposed to metabolic inhibitors, such as respiratory inhibitors, during the dark incubation period, the activity of NDPE herbicides is strongly reduced upon exposure to light (9, 22). Photobleaching NDPE, cyclic imide, and oxadiazole herbicides inhibit Chl synthesis (13, 34, 35). Furthermore, there is good evidence that photosynthetic light reactions are not required for activity of these herbicides (6, 9, 12, 15) or accumulation of PPIX in herbicide-treated tissues (25).

Photobleaching DPE herbicides were recently found to strongly inhibit protoporphyrinogen oxidase (26). As with the human genetic disease variegate porphyria (3), inactivated protoporphyrinogen oxidase results in uncontrolled autooxidation of protoporphyrinogen to PPIX, which apparently does not reenter the porphyrin pathway. These findings and most other available physiological data support the hypothesis that PPIX is the active photoreceptor generated by these herbicides. However, some further questions must be answered in order to unequivocally establish this as the only photodynamic compound involved.

A quantitative relationship has not been demonstrated between PPIX concentration in herbicide-treated tissues and herbicidal damage. Sandmann and Böger (30) have indicated the need for such studies. Kouji et al. (19) have reported primarily protochlorophyllide and not PPIX to accumulate in acifluorfen-treated cucumber cotyledon tissues. Thus, the question of whether other Chl intermediates are involved is not clear. The question of whether green tissues can accumulate enough PPIX in the light to explain the photodynamic damage has not been determined. Results of others (30) suggest that PPIX is too photolabile to accumulate in some plant tissues that are affected by these herbicides in the light. In the present report, we demonstrate that the photobleaching activity of a variety of NDPEs correlates with PPIX levels and that the photolabile pigment PPIX can remain in the tissue.
long enough in bright light to maintain a photodynamic effect. Furthermore, we demonstrate that accumulation of PPIX caused by photobleaching herbicides in light is a very rapid effect that is detectable within minutes of exposure to the herbicide. Our results strongly suggest that accumulation of PPIX alone can account for the photodynamic action of NDPE and oxadiazole herbicides.

**MATERIALS AND METHODS**

**Plant Material**

Cucumber seeds (*Cucumis sativus* L. [cv Straight Eight]) were planted in flats in a commercial greenhouse substrate (Jiffy-Mix; JPA, West Chicago, IL) and watered with distilled water. Plants were grown at 25°C for 7 to 8 d under 150 μmol/m²/s PAR white light and >90% relative humidity before cotyledons were harvested for use. Yellow cotyledons were obtained by imbibing the cucumber seeds in 80 μM tentoxin (Sigma Chemicals) for 24 h before planting as before (9, 10).

Locally collected redroot pigweed (*Amaranthus retroflexus* L.) and velvetleaf (*Abutilon theophrasti* Medic.) seeds were planted in 1.2-L pots. Pots were filled with Jiffy-Mix potting media and watered with tap water every 4 d until seedling establishment and were thereafter watered every other day with every other watering consisting of a dilute solution (0.25 g/L) of Peters 20-20-20 general purpose fertilizer. The plants were grown in the greenhouse and primary leaf tissue was harvested from 18- to 21-d-old plants.

**Measurements of Herbicidal Damage**

Electrolyte leakage was assayed as before (18) by cutting fifty 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 herbicides were used: acifluorfen [5-[2-chloro-4-(trifluoromethyl)phenyl]oxy]-2-nitrobenzoic acid], acifluorfen-methyl (the methyl ester of acifluorfen), fluorodifen [5-[2-nitro-(trifluoromethyl)oxy]-2-nitrobenzoic acid], oxadiazon [3-[2,4-dichloro-5-(1-methylethoxy)phenyl]-5-(1,1-dimethylethyl)-1,3,4-oxadiazol-2-(3H)-one], and oxyfluorfen [2-chloro-1-(3-ethoxy-4-nitrophenyl)-4(trifluoromethyl)benzene] were gifts of Rohm and Haas Co.; MC-15608 (an analog of acifluorfen-methyl with the nitro group substituted with a chloro group) was a gift of Dr. Gregory L. Orr of Colorado State University. The discs were then incubated at 25°C in darkness for 20 h before exposure to 500 μmol/m²/s PAR (bright light) or were exposed directly to bright light. All incubations were in growth chambers.

Electrolyte leakage was previously shown to be the most rapid effect of acifluorfen damage of several physiological parameters assayed (18). Cellular damage was measured by detection of electrolyte leakage into the bathing medium with a conductivity meter with the capability of assaying 1 mL of the bathing medium and returning it to the dish. 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. Electrolyte leakage experiments were repeated two to four times and within each experiment, treatments were triplicated. Results shown are means of triplicate samples from representative experiments.

**Chl Content**

Chl was extracted and assayed by the method of Hiscox and Israelstam (16). Fifty cotyledon or leaf discs were soaked for 24 h in darkness in 10 mL of dimethyl sulfoxide at room temperature. Extraction was complete at this time. Samples were then centrifuged at 500g for 10 min and the supernatants spectrophotometrically analyzed.

**Measurements of PPIX**

All extractions were made under a dim, green light source. Samples (50 discs) were homogenized in 6 mL of HPLC-grade methanol with a glass homogenizer. The homogenate was centrifuged at 30,000g 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 30,000g 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 methanol and filtered through a 0.2 μm syringe filter. Samples were stored in light-tight (amber glass wrapped in aluminum foil) vials at −20°C until analysis by HPLC.

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 Shimadzu model RF-535 fluorescence detector preceded the model 990 detector. The column was a 250 × 4.6 mm (i.d.) Spherisorb 5 μm ODS-1 reversed phase column preceded by a Bio-Rad ODS-5S guard column. The solvent gradient was composed of 0.1 M ammonium phosphate monobasic (pH 5.6) (solvent A) and HPLC-grade methanol (solvent B) at a flow rate of 1.6 mL/min. The gradient was as follows: a linear transition from 15 to 0% A in B from 0 to 20 min. The injection volume was 50 μL.

Commercial standards of PPIX (Sigma Chemical Co.), Mg-PPIX, and Mg-PPIXDE (Porphyrin Products, Inc.) were used. Porphyrin detection was performed with fluorescence detector excitation and emission wavelength settings of 400 and 630 nm, respectively, for PPIX and 415 and 595 nm, respectively, for Mg-PPIX, Mg-PPIXE, and Mg-PPIXDE. The photodiode array detector scanned from 300 to 650 nm to confirm all peaks. The retention times of Mg-PPIX, Mg-PPIXE, PPIX, and Mg-PPIXDE were 6.1, 7.8, 10.2, and 15.5 min, respectively. Recovery of commercial standards from plant tissues augmented with PPIX, MgPPIX, and MgPPIXDE was 81, 84, and 88%, respectively. Augmenting homogenates with...
MgPPIX or MgPPIXDE standards, we found no conversion of these compounds to PPIX with our methods.

PPIX levels are expressed on a molar basis per 50 cotyledon discs (one experimental unit). When expressed on a protein basis, the relative effects of the treatments were the same, since none of the treatments were found to have a significant effect on protein content.

**Protein Determinations**

Fifty cotyledon discs were homogenized in 10 mL of 0.1 M sodium phosphate buffer (pH 7.0) containing 1% NaCl. The homogenate was centrifuged at 20,000g for 20 min and the supernatant used for determination of protein according to the method of Bradford (2).

**RESULTS**

**Correlations of Herbicidal Damage with PPIX Levels**

Tissues were incubated with different concentrations of acifluorfen for 20 h in darkness. After the dark incubation, PPIX levels in all treatments were determined. Parallel treatments were exposed to continuous bright light and herbicidal damage was assessed. An almost linear log-linear relationship was found between acifluorfen concentration and PPIX accumulation in the discs (Fig. 1A). The maximum level of PPIX induced by the herbicide on a protein basis was about 40 nmol/100 mg of protein. Mg-PPIX was detected at low levels in both acifluorfen-treated and control tissues, whereas ALA greatly increased the levels of Mg-PPIX in treated tissues (data not shown). An almost identical relationship was found between acifluorfen treatment and cellular leakage as measured by electrolyte leakage 1 h after exposure to light (Fig. 1B). Cellular leakage of electrolytes is one of the first detectable effects of acifluorfen that can be measured (18). It precedes malondialdehyde evolution and photobleaching of photosynthetic pigments. A plot of PPIX levels after the 20 h dark period versus cellular leakage at 1 h after exposure to light (Fig. 2A), indicates that a threshold level of PPIX of about 0.2 nmol/50 discs is necessary for herbicidal damage to occur within 1 h. Tentoxin-treated tissues produced much more PPIX at lower doses of acifluorfen than did green tissues (Fig. 2B). However, the amount to PPIX that correlated with a particular level of cellular leakage was lower for tentoxin-treated than for green tissues (Fig. 2).

Green primary leaf tissues of redroot pigweed and velvetleaf responded to acifluorfen similarly to cucumber in terms of the relationship between cellular leakage and PPIX caused to accumulate by the herbicide (Fig. 3). Chl content of the tissues

![Figure 1](image1.png)

**Figure 1.** Effects of different concentrations of acifluorfen on PPIX (A) and herbicidal activity as measured by electrolyte leakage from damaged cells (B). Cucumber cotyledon discs were incubated in herbicide solutions for 20 h in darkness and then exposed to light. PPIX was measured at the end of the dark period and conductivity was measured after 1 h of exposure to light.

![Figure 2](image2.png)

**Figure 2.** Relationship between cellular damage (conductivity of electrolytes leaked) and PPIX of green tissue from Figure 1 (A) and from yellow cucumber cotyledon tissues produced by treatment with tentoxin (B). In B, the numbers associated with the data points represent the micromolar concentrations of acifluorfen used to obtain that datum.

![Figure 3](image3.png)

**Figure 3.** Relationship between PPIX and cellular damage caused by various concentrations (shown in µM units beside each datum) of acifluorfen. Primary leaf discs of redroot pigweed (A) and velvetleaf (B) seedlings were incubated in herbicide solutions for 20 h in darkness and then exposed to light. PPIX was measured at the end of the dark period and conductivity was measured after 1 and 4 h of exposure to light for velvetleaf and pigweed, respectively. Error bars are 1 se of the mean.
after 24 h of white light and PPIX content at the beginning of the light period were negatively correlated (Fig. 4).

**Different Herbicides**

There was generally a positive correlation between PPIX content after a 20 h dark incubation in different photobleaching herbicides and the ensuing herbicidal activity in the light (Fig. 5). In an experiment with 10 μM of either flurodifen, nitrofen, oxyfluorfen, or oxadiazon, there was an almost fourfold range in PPIX content among these different treatments after the dark incubation and a twofold range in activity of the herbicides after 2 h of exposure to light (Fig. 5A). In a similar experiment, with 10 μM acifluorfen, acifluorfen-methyl, oxyfluorfen, oxadiazon, and MC-15608, an active analog of acifluorfen-methyl in which the nitro group is substituted with a chlorine (27), protoporphyrin IX content after the 20-h dark incubation and activity of the herbicides after 5 h of light exposure were also positively correlated (Fig. 5B). Thus, with a structurally diverse group of photobleaching herbicides, the light-induced herbicidal activity correlates positively with the amount of PPIX caused to accumulate before exposure to light.

**Photostability of PPIX**

Cotyledon discs were incubated in 10 μM acifluorfen for 20 h in darkness and then exposed to continuous bright light for 9 h. PPIX content was measured after the dark period and at various times after exposure to light (Fig. 6). It took about 2 h for PPIX levels to decay to half the original levels. PPIX decayed at an initial half-life of about 4 h in vitro when placed in the bathing buffer under the same light to which the plant tissues were exposed (data not shown). An almost linear relationship exists between log of the PPIX levels remaining at each sampling time and the electrolyte leakage occurring during the 1 h following the PPIX sampling time (Fig. 7).

**Time Courses of PPIX Accumulation in Darkness and in Light**

When simultaneously placed in darkness and in 10 μM acifluorfen, a large linear increase in PPIX could be detected.

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**Figure 4.** Relationship between PPIX and Chi loss caused by various concentrations (shown in μM units beside each datum) of acifluorfen. Primary leaf discs of redroot pigweed (A) and velvetleaf (B) seedlings were incubated in herbicide solutions for 20 h in darkness and then exposed to light. PPIX was measured at the end of the dark period and Chi content was measured after 24 h of exposure to light. The Chi level in the untreated tissues of both species was approximately 80 μM per 50 discs. Error bars are 1 se of the mean.

**Figure 5.** Relationship between PPIX and cellular damage caused by 10 μM of each of four (A) or five (B) different photobleaching herbicides. Cucumber cotyledon discs were incubated in herbicide solutions for 20 h in darkness and then exposed to light. PPIX was measured at the end of the dark period in both experiments. In A, the conductivity was measured after 2 h of exposure to light. In B, conductivity was measured after 5 h of exposure to light. Error bars are 1 se of the mean.

**Figure 6.** PPIX content of cucumber cotyledons during exposure to light after 20 h of dark exposure to 10 μM acifluorfen. Zero time is the beginning of light exposure. Error bars are 1 se of the mean.

**Figure 7.** Relationship between the PPIX levels in Figure 6 on a log scale and herbicidal damage occurring during the 1 h following each PPIX measurement. Error bars are 1 se of the mean.
in tissues exposed to the herbicide after as little as 15 min (Fig. 8). No lag phase was detected. After 1 h, the levels of PPIX were as much as one-third as high as levels found after the 20 h dark incubation time. The level of PPIX produced during the 20 h incubation varied almost twofold, probably due to condition and age of the tissue.

When placed in continuous bright light and 10 \( \mu \text{M} \) acifluorfen simultaneously, there was an 8 h lag period in cellular damage as determined by cellular leakage (Fig. 9). However, a dramatic increase in PPIX content could be measured within 30 min (Fig. 9). The PPIX levels continued to increase up to 12 h, a time when cellular damage was increasing rapidly. After this time, PPIX decreased to about 150 pmol/50 discs and remained stable.

**DISCUSSION**

Although correlations do not establish causal relationships, in general, all results presented here indicate that PPIX is the photoreceptor responsible for the activity of NDPE and NDPE-like herbicides. Cellular leakage caused by the herbicide and the amount of PPIX caused to accumulate by the herbicide correlated positively (Figs. 2-5, 7). This correlation was relatively independent of the photobleaching herbicide used (Fig. 5) or the species tested (Figs. 2-4). The levels of PPIX that accumulated in response to the various herbicides ranged from about 20 to 60 nmol/100 mg of protein in green cucumber tissues. These levels compare favorably with the levels reported by Rebeiz et al. (29) to accumulate in green cucumber tissues treated with ALA and dipyridyl. However, the capacity of cut cotyledon discs and intact cotyledons to produce porphyrins may differ. Indeed, we found approximately 25 nmol of PPIX per 100 mg of protein to accumulate in cotyledon discs exposed to 0.1 mM ALA plus 5 mM dipyridyl after only 2 h in the dark. This value is similar to what Rebeiz et al. (29) found in cotyledons of sprayed plants after 17 h of darkness.

We previously speculated that yellow tissues are more sensitive to photobleaching herbicides than are green tissues because they lack the photosynthetic reducing capacity to generate protectants (6, 7). To support this hypothesis, we have shown that a smaller concentration of PPIX in the tissue is correlated with a particular herbicidal effect in yellow (tentoxin-treated) than in green tissues (Fig. 2). However, we were surprised to find that acifluorfen was much more effective in causing accumulation of PPIX in these virtually achlorophyllous tissues than in green tissues. Thus, the hypersensitivity of tentoxin-treated tissues is probably due to both reduced protection and increased effect of the herbicide on PPIX production. Protochlorophyll(ide) synthesis in tentoxin-treated tissues is slower than in untreated tissues (10). Since, in the present study, we found increased PPIX synthesis in tentoxin-treated tissues, the tentoxin-impaired step in protochlorophyll(ide) synthesis is probably at a point in the Chl pathway after protoporphyrinogen oxidase.

PPIX was not photostable *in vivo* (Fig. 6); however, its concentration was above that which correlated with herbicidal activity during a period of several hours of exposure to light, during which massive cellular damage occurred. We were somewhat surprised to find that PPIX was more photostable in the aqueous buffer in which the tissue was floated than *in vivo*. PPIX is photoxidized by singlet oxygen which it generates in the presence of molecular oxygen and light (4, 33). This process may be more rapid *in vivo* because molecular oxygen is much more soluble in the lipid membrane environment than in more aqueous environments (14) and PPIX is a lipophilic molecule. Also, in a membrane, singlet oxygen rapidly begins the production of lipid peroxides, resulting in autocatalytic propagation of peroxidative reactions in the presence of molecular oxygen (14). These peroxidative reactions may play a role in the oxidative destruction of PPIX *in vivo*.

Sandmann and Böger (30) found a cyclic imide herbicide to cause PPIX to accumulate in the light in the relatively insensitive green alga *Bumilleriopsis*, but not in the highly sensitive *Scenedesmus*. PPIX accumulation was greatly increased by this herbicide in *Scenedesmus* in the dark, however. Their explanation was that the same protective systems that provide resistance to the photobleaching herbicide in *Bumilleriopsis* also provides protection to PPIX. This does not explain how PPIX could be the photodynamic agent of cyclic imide damage to *Scenedesmus*. This study is hard to interpret because of the extended exposure (3 d) to light and herbicide.
PPIX could have risen and then fallen to undetectable levels as the cells were damaged or killed, similar to our findings with cucumber (Fig. 9). Our results clearly show that PPIX can accumulate in the light in a highly sensitive species.

The most dramatic finding was the speed with which acifluorfen caused accumulation of PPIX (Figs. 8 and 9). We found large increases in PPIX levels within 15 and 30 min in the dark and light, respectively. The kinetics of accumulation of PPIX are not similar to the kinetics of absorption of the herbicide. Earlier research from our laboratory showed that absorption of 14C-acifluorfen by cucumber cotyledons is linear over a period of 20 h (4) (our unpublished data). Autoradiographs also indicated very slow absorption. Using these previous data, estimates of 0.7 and 1.4 μM in the total leaf disc at 15 and 30 min, respectively, can be made. The concentration around the periphery of the disc after 15 and 30 min of exposure is probably considerably higher. Still, considering the speed of PPIX accumulation, the actual concentration of herbicide required for the effect must be very low.

Indeed, Matringe et al. (26) have shown that the acifluorfen-methyl I₅₀ for protoporphyrinogen oxidase of maize etioplasts is only 4 nM and that of potato mitochondria was only 0.4 nM. These concentrations should be rapidly reached in much of the tissue.

Kouji et al. (19) indicated that the ferrochelatase of higher plants is not inhibited by DPE herbicides. Matringe et al. (26) found that these herbicides inhibited both the magnesium- and ferro-chelatase activity of etioplasts with I₅₀ of 80 to 100 μM, respectively. However, they found DPE herbicides to be up to five orders of magnitude more inhibitory to protoporphyrinogen oxidase. The in vivo inhibition of this enzyme apparently leads to unregulated autooxidation of protoporphyrin to PPIX and the PPIX produced in this way apparently is no longer available to the porphyrin pathway. They found the protoporphyrinogen oxidase of the mitochondria and etioplast to be similarly affected. Thus, as with the iron-chelating compound 2,2’-dipyrildyl that has been shown to stimulate massive accumulation of porphyrins by blocking heme synthesis and thereby deregulating porphyrin synthesis by reducing heme levels (5), blockage of protoporphyrinogen oxidase is also likely to cause deregulation of the porphyrin pathway.

The suggestion by Kouji et al. (19), that these herbicides stimulate synthesis of ALA without blocking porphyrin synthesis, is apparently incorrect. The stimulation of ALA synthesis that they observed as probably due to deregulation of the porphyrin pathway. In green cucumber cotyledon tissue, they found protochlorophyll(id)e to accumulate after treatment in the dark with acifluorfen-methyl. However, in this paper, and in previous papers from our laboratory and others (8, 19, 21, 23–26, 30, 36), only PPIX has been found to accumulate in green tissues treated in the dark with DPE and other photobleaching herbicides. There is no evidence yet that the PPIX formed in the presence of these photobleaching herbicides is available to the porphyrin pathway. Further, protochlorophyll(id)e and Chl synthesis was demonstrated to be inhibited by the herbicides in two of these studies (21, 36).

Just as the photodynamic herbicide combination of ALA and 2,2’-dipyrildyl causes accumulation of photodynamic porphyrins (28, 29), the photobleaching oxadiazole, cyclic imide, and DPE herbicides apparently kill plants by the same general mechanism. However, unlike the photodynamic herbicide of Rebeiz et al. (28, 29), only PPIX appears to accumulate as a photosensitizing agent with these herbicides. The data of this paper demonstrate strong positive correlations between herbicide-induced PPIX concentrations and herbicidal action in a variety of tissues and under a variety of conditions.

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**LITERATURE CITED**