Protoporphyrin IX Accumulation in *Lemna paucicostata* Hegelm. Caused by Diphenyl Ether Herbicides and Their Herbicidal Activity

Shuuichi Kojima*,** Hiroshi Matsumoto*,*** and Kozo Ishizuka*

Abstract: The relationship between protoporphyrin IX (Proto IX) levels and herbicidal activity of diphenyl ether herbicides in intact higher plant *Lemna paucicostata* Hegelm. strain 441 was determined. Rapid accumulation of Proto IX in the plant was observed after simultaneous exposure to light and oxyfluorfen [2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene]. At 4 hr, the accumulation reached the maximum and this was followed by a rapid decrease. The rate of decrease was slowed when the plants were transferred to darkness after 4 hr light irradiation, although some Proto IX degradation continued in the dark. This suggests that not only photo-decomposition but degradation and/or metabolism which occurred in darkness are involved in the decrease. Proto IX levels accumulated in the plants and electrolyte leakage from the plants were increased with increasing concentration of oxyfluorfen. A positive correlation was found between Proto IX content and electrolyte leakage. Diphenyl ether herbicides used in the study had differential activity in the accumulation of Proto IX. A positive correlation was also found between Proto IX levels and their herbicidal activity to the plant.

These data suggest that Proto IX is the principal compound responsible for the rapid electrolyte leakage from plants, and that variation in the herbicidal activity of the diphenyl ethers observed in the study is caused by differential activity in accumulation of Proto IX.

Key words: diphenyl ethers, oxyfluorfen, herbicidal activity, protoporphyrin IX, *Lemna paucicostata*

Introduction

Several chemical classes of herbicides including diphenyl ethers, oxadiazoles and cyclic imides cause peroxidation of membrane lipids followed by bleaching of plant tissues. These chemicals act on the chlorophyll synthesizing pathway and increase the levels of tetrapyrroles which are intermediates of the pathway in plant cells. In most cases, protoporphyrin IX (Proto IX) was found to be the prevalent tetrapyrrole accumulating in diphenyl ether herbicide-treated tissues^{2,10)}. Because Proto IX is a strong photosensitizing pigment which generates singlet oxygen and its absorption spectrum coincides with the action spectra of the herbicides⁹⁾, Proto IX is considered the principal compound responsible for the activity of the photobleaching herbicides. Inhibitors of porphyrin synthesis have been reported to counteract the herbicidal activity^{6,15)}. Matringe *et al.*⁷⁾ followed by Witkowski and Halling¹⁶⁾ reported

* Institute of Applied Biochemistry, University of Tsukuba, Tsukuba, Ibaraki 305, Japan

** Current address: Odawara Research Center, Nippon Soda Co. Ltd. 345 Takada, Odawara, Kanagawa 250-02, Japan

***To whom correspondence should be addressed.

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that the herbicides inhibited protoporphyrinogen oxidase which converted protoporphyrinogen IX (Proto IX) to Proto IX at very low concentration. Blockage at this site apparently leads to autooxidation of Proto IX to form Proto IX, as has been observed when this enzyme is inactive due to genetic lesions in humans⁹⁾ and yeast¹⁴⁾.

A quantitative relationship between Proto IX levels in cucumber cotyledon disks treated with diphenyl ether herbicide acifluorfen and its herbicidal activity was demonstrated by Becerril and Duke¹⁾. But in some cases there has not been good correlation between Proto IX levels in peroxidizing herbicide-treated tissues and herbicidal damage¹²⁾. In other cases, it was reported that the chlorophyll intermediate related to the photobleaching activity was not Proto IX but protochlorophyllide (Pchlde)⁹⁾.

Because many of the above studies have been on excised tissues, detached cotyledons or unicellular algae, they may not accurately indicate porphyrin metabolism in intact plants. Matsumoto and Duke¹⁰⁾ have made a study of acifluorfen-methyl effects on porphyrin synthesis utilizing an aquatic higher plant, *Lemna paucicostata*. They determined the effects of the herbicide on virtually all of the chlorophyll intermediates from uroporphyrinogen III to Pchlde, and indicated that Proto IX was the only porphyrin accumulated by the herbicide.

In this paper, we determined the relationship of Proto IX levels and herbicidal activity of diphenyl ethers in *L. paucicostata*.

Materials and Methods

Plant material

The aquatic floating plant, *Lemna paucicostata* Hegelm. strain 441 was grown axenically with half strength Hutner's medium containing 1% sucrose (w/v) as previously described⁹⁾.

Chemicals

Oxyfluorfen [2-chloro-1-(3-ethoxy-4-nitrophenyl)-4-trifluoromethylbenzene] and chlo-methoxynil [2,4-dichloro-1-(methoxy-4-nitrophenoxy)benzene] were the gift of Rohm and Haas and Ishihara Sangyo Kaisha, respectively. Bifenox [methyl 5-(2,4-dichlorophenoxy)-2-nitrobenzoate, chlornitrofen [2,4,6-trichloro-1-(4-nitrophenoxy)benzene] and nitrofen [2,4-dichloro-1-(4-nitrophenoxy)benzene] were purchased from Wako Pure Chemical Industries, Ltd. Osaka Japan.

Herbicide treatment and determination of electrolyte leakage

One-tenth gram of the plant (about 45–50 colonies) was transferred to a 100 ml beaker containing 50 ml of double distilled water, and herbicides were added to it as ethanol solution. The final ethanol concentration was 0.1%. Then the plant was exposed to light ($200 \mu\text{E}/\text{m}^2 \cdot \text{sec}$) in a growth chamber at 25°C. Phytotoxic effects of the herbicides were evaluated by detection of electrolyte leakage from the plant into the bathing solution with a conductivity meter as previously described⁹⁾.

Determination of Proto IX

All extractions of Proto IX were made under a dim, green light source. Samples (0.1 g *L. paucicostata*) were homogenized in 6 ml of HPLC-grade methanol/0.1 N NH_4OH (9/1, v/v) with a Teflon homogenizer. The homogenate was centrifuged at $30,000 \times g$ for 10 min and the supernatant was saved. The pellet was resuspended in 3 ml of HPLC-grade methanol, sonicated for 5 min and centrifuged at $30,000 \times g$ for 10 min. Supernatants were combined and evaporated to dryness at 40°C with a rotary evaporator. The residue was dissolved in 1 ml of the basic methanol and filtered through a $0.22 \mu\text{m}$ syringe filter. Samples were stored in brown vials at -20°C until analysis by HPLC. The HPLC system was composed of a HPLC-pump (Shimadzu LC-6A) and a fluorescence

spectrophotometer (Japan Spectroscopic FP-777) as a detector. The column was a Shim-pack CLC-ODS (150×60 mm) preceded by a Shimadzu ODS guard column. Elution solvent was HPLC-grade methanol/20 mM ammonium phosphate buffer (pH 5.8) (9/1, v/v) and the flow rate was 1.4 ml/min. Excitation and emission wavelength of the fluorescence detector were 400 and 630 nm, respectively.

Results and Discussion

Rapid accumulation of Proto IX in *L. paucicostata* was observed after simultaneous exposure to light ($200 \mu\text{E}/\text{m}^2\cdot\text{sec}$) and $1 \mu\text{M}$ oxyfluorfen (Fig. 1). The accumulation reached half of the maximum level after 1 hr and maximum at 4 hr. This suggests that oxyfluorfen penetrates to chloroplasts and disturbs porphyrin synthesis in the plastid very rapidly. A similar Proto IX accumulation pattern in acifluorfen treated cucumber cotyledon disks¹⁾ and in acifluorfen-methyl treated *L. paucicostata*¹⁰⁾ has been reported.

After 4 hr of light exposure, there was a rapid decrease of Proto IX in the plant (Fig. 1). The rate of decrease was slowed when the plant was transferred to darkness after 4 hr, however, Proto IX degradation also occurred in the dark. Proto IX is known as a photolabile compound and Becceril and Duke¹⁾ indicated that its half-life in cucumber leaf disks was about 2.5 hr. In our study, accumulated Proto IX decreased to less than half of the maximal level within 2 hr. The results suggest that not only photo-decomposition but degradation and/or metabolism of Proto IX in darkness are involved in its reduction mechanism. Leaking of Proto IX from the plant may not be concerned with the decrease because little of the compound was detected in the bathing solution¹⁰⁾. Accumulation of Proto IX by the herbicide also occurred in darkness and peaked at 2 hr (Fig. 1); however, the maximal level was

Fig. 1. Effect of light condition on Proto IX accumulation in *L. paucicostata* treated with $1 \mu\text{M}$ oxyfluorfen. Error bars are SE of the mean.

about a quarter of that in light indicating that light enhances Proto IX accumulation. Mayer and Beale¹¹⁾ recently reported that the activity of δ -aminolevulinic acid (ALA) synthesizing enzyme was light dependent; therefore, stimulation of ALA synthesis may be involved in the light enhanced accumulation of the compound. Amount of Proto IX in non-treated plants was below the limit of detection ($10 \text{ pmol}/0.1 \text{ g f.w.}$) throughout the 8 hr light period (Fig. 1).

Proto IX content and electrolyte leakage in *L. paucicostata* were determined under different concentrations of oxyfluorfen (Fig. 2). Proto IX levels and conductivity in the bathing solution were measured at 4 hr and 12 hr after simultaneous exposure to light and the herbicide, respectively. Matsumoto and Duke¹⁰⁾ indicated that electrolyte leakage determination was rapid and sensitive assay

Fig. 2. Relationship between protoporphyrin IX levels and electrolyte leakage caused by different concentrations of oxyfluorfen in *L. paucicostata*. Proto IX and conductivity were measured after 4 hr and 12 hr exposure to light and the herbicide, respectively. Error bars are SE of the mean.

for herbicidal activity of diphenyl ethers to the plant. In this paper, we also found a positive correlation between Proto IX levels and herbicidal damage determined by electrolyte leakage. Since Proto IX is known to a photosensitizing pigment which causes peroxidation of membrane lipids, the results suggest that electrolyte leakage from the plant is caused by accumulated Proto IX.

Proto IX accumulation by diphenyl ether herbicides which have different herbicidal activities on *L. paucicostata* was determined (Fig. 3). Proto IX level and electrolyte leakage were also determined at 4 hr and 12 hr, respectively, after exposure to light and 1 μM of each herbicide. The herbicides caused accumulation of different amounts of Proto IX in the plant. Again, a positive correlation was found between Proto IX levels and herbicidal damage. Similar correlation was seen from the results of conductivity determina-

Fig. 3. Relationship between protoporphyrin IX levels and electrolyte leakage caused by 1 μM of *p*-nitrodiphenyl ether herbicides in *L. paucicostata*. Proto IX and conductivity were measured after 4 hr and 12 hr exposure to light and the herbicide, respectively. Error bars are SE of the mean.

tion at 8 and 10 hr (data not shown).

Our data indicated that diphenyl ether herbicides caused Proto IX accumulation in intact *L. paucicostata* plants and that the accumulation preceded electrolyte leakage from the plants. Amounts of Proto IX in the plants were significantly correlated with herbicidal activity of the diphenyl ethers. This suggests that Proto IX is the principal compound responsible for the membrane peroxidation which leads to the electrolyte leakage from the plant, and that variation in the herbicidal activity of the diphenyl ethers observed in the study is caused by their differential ability to accumulate Proto IX.

Involvement of singlet oxygen generated

by photodynamic reaction of Proto IX has been suggested in the initiation of peroxidation of lipid membrane. Haworth and Hess³⁾ indirectly detected the production of singlet oxygen in oxyfluorfen treated pea thylakoids. However, others proposed the possible involvement of superoxide anion radical in the reaction¹³⁾. The nature of initiation of the peroxidation reaction must be clarified.

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アオウキクサにおけるジフェニルエーテル系除草剤によるプロトポルフィリン IX の蓄積とその殺草活性

小島修一*・松本 宏*・石塚皓造*

摘 要

ジフェニルエーテル系除草剤は生体膜を構成する脂質の過酸化を引き起こし、膜を破壊する作用を持つ。この過酸化をもたらし要因として、光の存在下で活性酸素を生成する働きをもつクロロフィル合成中間体の異常蓄積が指摘されている。その中でもプロトポルフィリン IX (Proto IX) は、剤の処理後植物体内に急速に蓄積することから、ジフェニルエーテル系除草剤の作用の原因物質であると推定されている。しかし、これらの研究は培養細胞や切断組織などを用いて行われてきており、無傷の植物体を用いた研究例はほとんどない。そこで本研究ではアオウキクサ (*Lemna paucicostata* Hegelm.) を無傷の植物材料とし、Proto IX の蓄積と殺草活性との関係を調べた。

アオウキクサに光照射下で oxyfluorfen を処理したところ、体内に急速に Proto IX が蓄積し、4 時間後

に最大となり、その後は急速に減少した (Fig. 1)。また 4 時間後に植物を暗所に移すと減少速度はわずかに抑制されたが、蓄積した Proto IX は暗所でも分解することが示された (Fig. 1)。このことは Proto IX の減少に光分解が関与していること、ならびに暗所でも起こる分解、もしくは代謝が関与していることを示唆した。oxyfluorfen の処理後も継続して暗所においた場合でも Proto IX の蓄積がみられたが、ピーク時でも蓄積量は明所でのその 1/4 程度であった (Fig. 1)。oxyfluorfen の処理濃度が増加するにつれて Proto IX 蓄積量と電解質の漏出量が増加し、これらの間には正の相関がみられた (Fig. 2)。さらに数種のジフェニルエーテル系除草剤をそれぞれ 1 μ M で処理したところ、これらによる Proto IX 蓄積量と電解質の漏出量との間にも正の相関があった (Fig. 3)。これらのことから、アオウキクサに対するジフェニルエーテル系除草剤の殺草活性は Proto IX 蓄積量に依存しており、Proto IX が電解質の漏出を引き起こす原因物質であることが強く示唆された。

キーワード：ジフェニルエーテル, オキシフルオルフェン, 殺草活性, プロトポルフィリン IX, アオウキクサ

* 筑波大学応用生物化学系