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Original Article

Accumulation of Protoporphyrinogen IX prior to Protoporphyrin IX in Intact Plants Treated with Protoporphyrinogen IX Oxidase-Inhibiting Herbicides*

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A combination of two conventional methods of porphyrins analysis revealed a significant accumulation of protoporphyrinogen IX (Protogen) prior to protoporphyrin IX (Proto IX) in cucumber (*Cucumis sativus* L.) cotyledons immediately after the foliar application of a protoporphyrinogen IX oxidase (Protox)-inhibiting herbicide, pyraflufen-ethyl. The accumulation of Protogen peaked at 4 to 7 hr and then decreased with the increase of Proto IX. Although a similar time-course of Protogen accumulation was observed in cucumber cotyledons treated with another Protox-inhibiting herbicide, acifluorfen, the amount of Proto IX accumulated was 2 to 3 times lower than that after the pyraflufen-ethyl treatment. Furthermore, a foliar application of pyraflufen-ethyl caused a significant accumulation of Protogen rather than Proto IX in cleavers (*Galium aparine* L.) after 7 hr, while little accumulation of Protogen and Proto IX took place in wheat (*Triticum aestivum* L.).

Keywords: pyraflufen-ethyl, acifluorfen, herbicide, protoporphyrinogen IX oxidase, protoporphyrin IX.

INTRODUCTION

Pyraflufen-ethyl, ethyl 2-chloro-5-(4-chloro-5-difluoromethoxy-1-methylpyrazol-3-yl)-4-fluorophenoxyacetate, developed by Nihon Nohyaku Co., Ltd. is a selective post-emergence herbicide for cereals and highly effective against several important broad-leaved weeds, especially cleavers (*Galium aparine* L.).[1,2] We reported that pyraflufen-ethyl was a potent protoporphyrinogen IX oxidase (Protox, EC 1.3.3.4) inhibitor and its selective effect on wheat (*Triticum aestivum* L.) and cleavers was due to differences of foliar deposition and absorption, and the rate of metabolic detoxification.[2]

Treatment with Protox-inhibiting herbicides is known to cause an accumulation of protoporphyrin IX (Proto IX; product of the enzyme) in plants. The mechanism is explained as follows. Protox-inhibiting herbicides inhibit chloroplastic Protox, and cause an accumulation of protoporphyrinogen IX (Protogen) in chloroplasts. Then the accumulated Protogen leaks out of chloroplasts and is rapidly oxidized to Proto IX by herbicide-resistant peroxidases and/or auto-oxidation in the cytoplasm.[3–5] Although Jacobs and Jacobs[6] showed that Protogen was solely accumulated in isolated barley (*Hordeum vulgare* L.) plastids treated with a Protox-inhibiting herbicide, acifluorfen (5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid), there has been no report on the accumulation of Protogen in intact plants which should be the first biological response after a foliar application of the Protox-inhibiting herbicide.

In this report, we describe the accumulation of Protogen prior to Proto IX in intact plants after a foliar application of the Protox-inhibiting herbicide, pyraflufen-ethyl or acifluorfen, revealed by a combination of two conventional methods of porphyrins analysis.

MATERIALS AND METHODS

1. Chemicals

Pyraflufen-ethyl was synthesized in Nihon Nohyaku Co., Ltd.[7] Acifluorfen was synthesized according to the method of Johnson.[8] Proto IX and Mg-Proto IX standards were purchased from Porphyrin Products, Inc. (Logan, Utah, USA). Protogen was prepared by reducing Proto IX, purchased from Sigma Chemical Co., Ltd. with sodium amalgam as described by Jacobs and Jacobs.[9] To prevent auto-oxidation of Protogen, dithiothreitol was added to the stock solution (400 μM Protogen) at a final concentration of 200 mM.

* Pyraflufen-ethyl: A Potent Protox Inhibiting Herbicide (Part 3): See Ref. 2 for Part 2.

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2. Plant Materials

Cucumber (*Cucumis sativus* L., cv. Junkei Shiyoh) was raised in a plastic pot (9 cm in diameter) containing clay loam soil in a greenhouse (25±3°C/18±3°C, day/night) to the 1-leaf stage for 8 days. Wheat (cv. Mercia) was raised in a plastic pot (9 cm in diameter) containing clay loam soil in a greenhouse (22±3°C/15±3°C, day/night) to the 3-leaf stage and cleavers to the 2-whorl stage with first axillary buds as described previously.[2)]

3. Foliar Applications of Compounds, Sampling of Leaf Discs or Shoots and Assessment of Herbicidal Efficacy

Before the foliar application of herbicides, the plants were kept in the dark for 12 hr at 25°C in a growth chamber (Takayama Seisakusho, Kyoto, Japan). Pyraflufen-ethyl [2.5% emulsifiable concentrate (EC)] at 6 g a.i./ha or acifluorfen (2.5% EC) at 600 g a.i./ha was foliar applied to the plants in a spray box (0.6×0.6×0.6 m). The splay volume was 1000 liter water/ha. After the application, the plants were kept under continuous light (light intensity: 100 μE/m^2/sec) at 25°C in the growth chamber.

At 1, 4, 7 and 10 hr after the foliar application of the compounds, 16 discs (4 mm in diameter) were excised from the cucumber cotyledons and weighed. Herbicidal efficacy was assessed as the reduction in fresh weight per 16 discs. Results were presented as means with standard deviations of 6 replications.

In the case of wheat and cleavers, their shoots (60 to 200 mg) were weighed and cut into 2 mm sections at 7 hr after the foliar application of pyraflufen-ethyl.

4. Extraction and Determination of Proto IX

Leaf discs excised from cucumber cotyledons or slices of wheat and cleavers shoots were homogenized with 3 to 4 ml of HPLC-grade MeCN/1 M perchloric acid (9/1, v/v; acidic solvent) with a handy micro homogenizer, Physcotron NS-310E (Microtec Co., Ltd., Chiba, Japan) at full power for 15 sec. Although Jacobs and Jacobs[6)] used MeOH/1 M perchloric acid (1/1, v/v), we employed HPLC-grade MeCN/1 M perchloric acid (9/1, v/v) to prevent the formation of Proto IX methyl. Preliminary experiments showed that 4 μM Protogen (corresponding to ca. 170 nmol/g fresh weight of leaf discs) was not auto-oxidized to Proto IX in the acidic solvent as well as in MeOH/1 M perchloric acid (1/1, v/v) as described by Jacobs and Jacobs.[6)] The homogenate was centrifuged at 3,000×*g* for 5 min at room temperature, and the supernatant was immediately analyzed by HPLC with a fluorescence detector. The separation and fluorometric determination of Proto IX were performed by a modification of the method of Bonkovsky *et al.*[10)] as described previously.[2)] The HPLC system was composed of Waters Associates components which included a Model 600S pump system, a Model 717 autosampler, a millenium controller, a Model 486 UV detector, and a Model 474 fluorescence detector. The column was a Wakosil 5C18 ODS reversed-phase column, 150×6.0 mm (i.d.), 5 μm. The recovery of the commercial Proto IX standard (ca. 85 nmol/g fresh weight of leaf discs) was 94.4±3.7%. The Proto IX concentration was compensated with the recovery rate and expressed on a molar basis per g fresh weight. Results were presented as means with standard deviations of 3 replications.

5. Extraction and Determination of Protogen

Protogen was extracted and determined as Proto IX after the conversion of Protogen to Proto IX through auto-oxidation in basic solvent. Leaf discs excised from cucumber cotyledons or slices of wheat and cleavers shoots were homogenized with 3 to 4 ml of HPLC-grade MeOH/0.1 M NH_4OH (9/1, v/v; basic solvent)[11–20)] with the handy micro homogenizer at full power for 15 sec. The homogenate was centrifuged at 3,000×*g* for 5 min at room temperature. An aliquot (0.5 ml) of the supernatant was evaporated to dryness at 40°C under N_2 gas stream. The residue was dissolved in 0.5 ml of the basic solvent and immediately analyzed by HPLC as described above. The recovery of the commercial Proto IX standard (ca. 85 nmol/g fresh weight) from leaf discs of cucumber cotyledons was 69.1±0.5%. The Proto IX (including auto-oxidized Protogen) concentration was compensated with the recovery rate and expressed on a molar basis per g fresh weight. Preliminary experiments showed that the evaporation of the extracted basic solution was necessary for the complete auto-oxidation of 4 μM Protogen (corresponding to ca. 170 nmol/g fresh weight of leaf discs) to Proto IX.

6. Calculation of Protogen Concentration in Plants

Jacobs and Jacobs[6)] reported that Mg-Proto IX was demetalated and measured as Proto IX in acidic solvents. Therefore, we preliminary determined the concentration of Mg-Proto IX extracted with MeOH/0.1 M NH_4OH (9/1, v/v).[11–16)] The Mg-Proto IX concentration was less than 0.1 nmol/g fresh weight (data not shown) and thus negligible in the calculation of Proto IX concentration in plants. Therefore, the Protogen concentration was calculated by subtracting the Proto IX concentration in the acidic solvent from that in the basic solvent (including auto-oxidized Protogen). Results were presented as means with standard deviations of 3 replications.

RESULTS AND DISCUSSION

1. Changes of Protogen and Proto IX Concentration in Cucumber Cotyledons after a Foliar Application of Pyraflufen-ethyl or Acifluorfen

Figure 1A shows the changes of Protogen and Proto IX concentrations in cucumber cotyledons after a foliar application of pyraflufen-ethyl. Protogen increased for 7 hr after the treatment. The accumulated Protogen then rapidly decreased and had almost disappeared at 10 hr after the treatment. On the other hand, the increase in Proto IX was slow until 4 hr, but gradually accelerated from 7 to 10 hr after the treatment. The

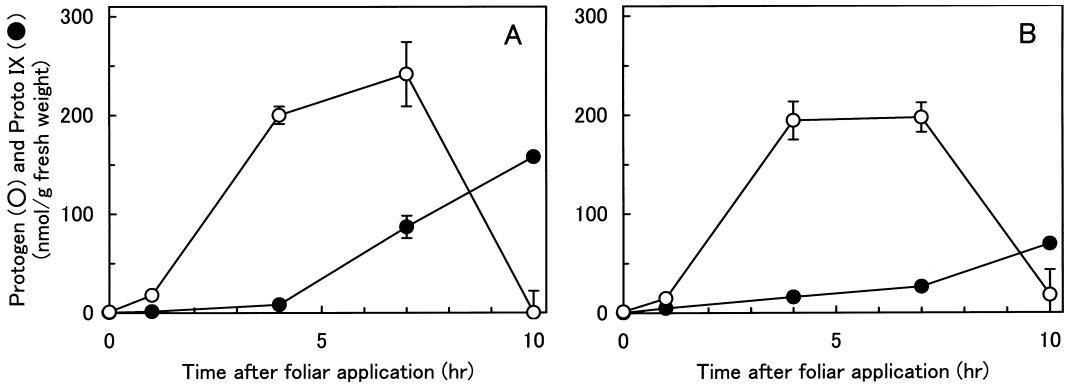

Fig. 1. Time course of Protogen (○) and Proto IX (●) accumulation in cucumber cotyledons after foliar applications of pyraflufen-ethyl (A) and acifluorfen (B). Results are presented as means with standard deviations of 3 replications.

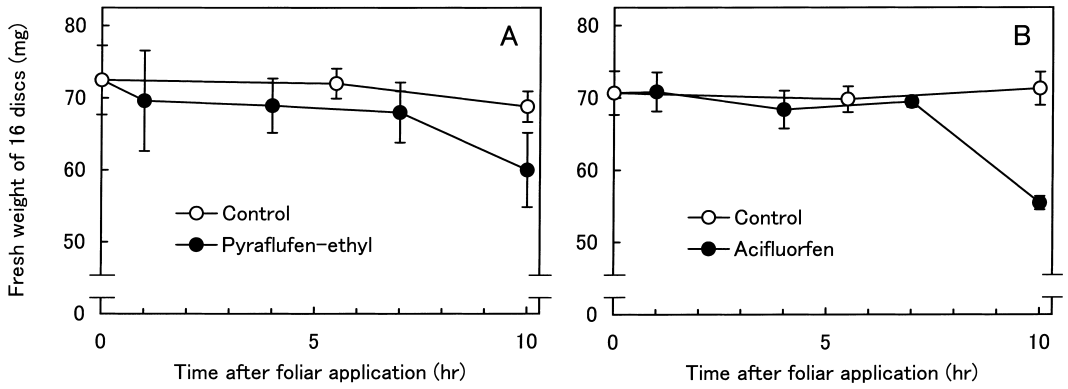

Fig. 2. Time course of the change in fresh weight of 16 discs of cucumber cotyledons after foliar applications of pyraflufen-ethyl (A) and acifluorfen (B). Results are presented as means with standard deviations of 6 replications.

accumulated Protogen would be oxidized to Proto IX by herbicide-resistant peroxidases and/or auto-oxidation in the cytoplasm[3–5] and endoplasmic reticulum.[21] Although a similar time-course of Protogen accumulation was observed in cucumber cotyledons treated with another Protox-inhibiting herbicide, acifluorfen (Fig. 1B), the amount of Proto IX that accumulated after the acifluorfen treatment was 2 to 3 times lower than that after the pyraflufen-ethyl treatment. Retzlaff and Böger[21] reported that endoplasmic reticulum from corn (*Zea mays* L.) hypocotyls had Protox activity, and that the enzyme was less sensitive to several Protox-inhibiting herbicides than that from thylakoids but the I_{50} values of Protox from endoplasmic reticulum differed with the herbicide tested. Future studies should focus on the sensitivity of Protox activity in the endoplasmic reticulum from cucumber cotyledons to various herbicides to elucidate the mechanism behind the difference in Proto IX accumulation.

There are numerous reports on the accumulation of Proto IX after the application of Protox-inhibiting herbicides.[11–20] However, the auto-oxidation of Protogen to Proto IX is not well understood, because Protogen is completely auto-oxidized to Proto IX in basic solutions *via* evaporation. Therefore, Fig. 1 represents the first experimental results in intact plants, showing Protogen accumulates to high levels prior to Proto IX after the foliar application of Protox-inhibiting herbicides. Jacobs *et al.*[22] reported that young cucumber leaves could decompose Protogen to nonporphyrin products. Therefore, a decrease in Protogen might be stoichiometrically incompatible with an increase in Proto IX.

Figures 2A and 2B show the reduction in the fresh weight of leaf discs of cucumber cotyledons after the application of each compound. The fresh weight did not decrease until 7 hr after the application and decreased significantly at 10 hr. Although the amount of Proto IX accumulated after the acifluorfen treatment was 2–3 times lower than that after the pyraflufen-ethyl treatment (Fig. 1), the degree of desiccation was approximately equal in both cases (Fig. 2). These results indicate that the amount of Proto IX accumulated in cucumber cotyledons after the acifluorfen treatment was enough to desiccate cucumber cotyledons. From a comparison of Figs. 1 and 2, it is clear that Proto IX began to accumulate in the cucumber cotyledons prior to the desiccation. These results are consistent with the well-accepted phytotoxic mechanism whereby accumulated Proto IX acts as a strong photosensi-

Table 1. Accumulation of Protogen and Proto IX in wheat and cleavers at 7 hr after the foliar application of pyraflufen-ethyl

Species	Control		Treated*	
	Protogen	Proto IX	Protogen	Proto IX
	(nmol/g fresh weight)			
Wheat	<0.10	<0.10	0.99± 0.57	0.17±0.05
Cleavers	<0.10	0.28±0.14	66.4 ±13.6	13.5 ±1.19

* Application rate of pyraflufen-ethyl was 6 g a.i./ha. Results are presented as means with standard deviations of 3 replications.

tizer generating singlet oxygen and leads to the peroxidative destruction of cellular membranes and bleaching of tissues in plants in light.[3–5)]

2. Protogen and Proto IX Accumulation in Wheat and Cleavers after a Foliar Application of Pyraflufen-ethyl

We previously reported the mechanisms of selective action of pyraflufen-ethyl in wheat and cleavers.[2)] Although the chloroplastic Protox isolated from these two plants had a similar sensitivity to the compound, a foliar application of the compound caused a much greater accumulation of Proto IX in cleavers than in wheat. Distinct differences were observed in the foliar deposition and absorption, and in the rate of metabolic detoxification of the compound between these plants. We considered that a complicated combination of factors would cause the difference in accumulation of Proto IX between the species and the resulting selectivity of pyraflufen-ethyl as a post-emergence herbicide for wheat.

Here, the accumulation of Protogen and Proto IX in each of these plants after a foliar application of pyraflufen-ethyl was re-examined using a combination of two conventional methods of porphyrins analysis (Table 1). Although the measurement of Proto IX and Protogen concentrations in both plants was carried out only 7 hr after the foliar application, high levels of Proto IX and Protogen were detected in cleavers, while little accumulation took place in wheat. The concentrations of Protogen and Proto IX in cleavers were 66.4±13.6 and 13.5±1.2 nmol/g fresh weight, respectively, a difference of the amount of Protogen and Proto IX was approximately 5-fold. The concentrations of Protogen and Proto IX accumulated in cleavers were less than those in cucumber cotyledons, and the ratio of Protogen/Proto IX was higher in cleavers than cucumber cotyledons at 7 hr after the application (Table 1 and Fig. 1A). In wheat, the concentrations of Protogen and Proto IX were much lower than those in cleavers and cucumber cotyledons. The finding that neither Proto IX nor Protogen accumulated in wheat after a foliar application of pyraflufen-ethyl suggests that the chloroplastic Protox in wheat is little inhibited *in vivo*. This supports our previous study on the mechanisms of selective action of pyraflufen-ethyl in wheat and cleavers.[2)]

In conclusion, a combination of two conventional methods of porphyrins analysis revealed that Protogen began to accumulate in cucumber cotyledons immediately after a foliar application of the Protox-inhibiting herbicide pyraflufen-ethyl or acifluorfen, and then decreased with the increase of Proto IX. Namely, the treatment of the Protox-inhibiting herbicide caused an accumulation of the substrate (Protogen) of Protox in the leaves, just as treatment with imazaquin[23)] or glufosinate[24)] resulted in an accumulation of the substrate (2-ketobutyrate or ammonium) of each target enzyme. Furthermore, the foliar application of pyraflufen-ethyl caused a significant accumulation of Protogen rather than Proto IX in cleavers, but little accumulation took place in wheat. This is the first report demonstrating a significant accumulation of Protogen prior to Proto IX in intact plants treated with Protox-inhibiting herbicides.

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