Mechanisms of Selective Action of a Protoporphyrinogen IX Oxidase-Inhibiting Herbicide Pyraflufen-ethyl between Wheat (*Triticum aestivum*) and Cleavers (*Galium aparine*)

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To elucidate the mechanisms of selective herbicidal action of pyraflufen-ethyl [ethyl 2-chloro-5-(4-chloro-5-difluoromethoxy-1-methylpyrazol-3-yl)-4-fluorophenoxyacetate], the intrinsic activity on the target enzyme, foliar deposition and absorption, and metabolism of the compound were compared between wheat (*Triticum aestivum* L.) and cleavers (*Galium aparine* L.). Although protoporphyrinogen IX oxidase activities from wheat and cleavers chloroplasts were almost equally inhibited by pyraflufen-ethyl, a foliar application of the compound caused much more accumulation of protoporphyrin IX in cleavers than in wheat. The foliar deposition and absorption of the compound were much greater in cleavers than in wheat. Metabolic detoxification in wheat was much greater than that in cleavers. These differences in foliar deposition and absorption, and in the rate of metabolic detoxification would play an important role in the accumulation of protoporphyrin IX and the resulting selectivity of pyraflufen-ethyl between wheat and cleavers.

Key words: pyraflufen-ethyl, herbicide, protoporphyrinogen IX oxidase (Protox), mechanism of selective action, wheat, cleavers.

INTRODUCTION

Pyraflufen-ethyl [ET-751, ethyl 2-chloro-5-(4-chloro-5-difluoromethoxy-1-methylpyrazol-3-yl)-4-fluorophenoxyacetate] is a new selective post-emergence herbicide for cereals and commercialized as ECOPART® flowable by Nihon Nohyaku Co., Ltd. This compound is highly effective against several important broad-leaved weeds, especially cleavers (*Galium aparine* L.). The herbicidal symptom of the compound was very similar to that of the herbicides inhibiting protoporphyrinogen IX oxidase (Protox, EC 1.3.3.4), that is, foliar application of the compound induces rapid necrosis or desiccation of the plant foliage in the presence of light. Horikoshi et al. revealed that pyraflufen-ethyl inhibited Protox activity extracted from tobacco (*Nicotiana tabacum* L.) cell lines and growth of *Escherichia coli* complemented with tobacco Protox cDNA. The selectivity of other Protox inhibiting herbicides has been primarily explained as a result of rapid metabolic degradation of the herbicide. Ishizuka et al. revealed that, in addition to the metabolism, the absorption of chlomethoxynil in plant played important roles in the selectivity between rice and barnyardgrass. Furthermore, several reports indicated that there was no physiological difference of Protox sensitivity to Protox inhibiting herbicides among plant species.

In this study, to clarify mechanisms of selective action of pyraflufen-ethyl between wheat (*Triticum aestivum* L.) and cleavers, differences in the intrinsic sensitivity of Protox, in the foliar deposition and absorption, and in the metabolism were investigated between the two species.

MATERIALS AND METHODS

1. Chemicals

Pyraflufen-ethyl, 2-chloro-5-(4-chloro-5-difluoromethoxy-1-methylpyrazol-3-yl)-4-fluorophenoxyacetic acid, 2-chloro-5-(4-chloro-5-difluoromethoxy-1-pyrazol-3-yl)-4-fluorophenoxyacetic acid and ethyl 2-chloro-5-(4-chloro-5-difluoromethoxy-1-methyl-[5,14C] pyrazol-3-yl)-4-fluorophenoxyacetate were synthesized in Nihon Nohyaku Co.,
L. Acifluorfen-methyl \{ methyl 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate \} was synthesized according to the method of Johnson.\(^{13}\) Protoporphyrin IX (Proto IX) and Proto IX standard were purchased from Sigma Chemical Co., Ltd. and Porphyrin Products (Logan, Utah, USA), respectively. Protoporphyrinogen IX (Protogen IX) was prepared by reducing Proto IX with sodium amalgam as described by Jacobs and Jacobs.\(^{14}\)

2. Plant Materials
Wheat (Triticum aestivum L. c.v. 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 (Galium aparine L.) to the 2-whorl stage with first axillary buds.

3. Foliar Application of Pyraflufen-ethyl and Visual Assessment of Herbicidal Efficacy and Crop Safety
Pyraflufen-ethyl \[2.5\% emulsion concentrate (EC)\] at 0.6 or 6 g a.i./ha was foliar-applied to wheat and cleavers in a spray box (0.5 × 0.5 × 0.5 m). A spray volume was 600 liter water/ha. After the application, the plants were raised under continuous light (light intensity: 100 \(\mu\)E/m²/sec) at 25°C in a growth chamber (Takayama Seisakusho, Kyoto, Japan). After 42 hr of the application, phytotoxicity based on a degree of necrosis was evaluated and rated by 0 (no effect) to 10 (completely killed).

4. Extraction and Determination of Proto IX in Whole Plants
After 18 hr of the foliar application of pyraflufen-ethyl, the shoots of the plants (ca. 0.2 g) were cut into 2 mm sections and homogenated in 4 ml of HPLC-grade methanol/1 M perchloric acid (1/1, v/v) with a Brinkman Polytron at 60% full power for 20 sec. The homogenate was centrifuged at 30,000 × g for 10 min at 4°C, and the supernatant was stored in vials at −20°C until HPLC analysis.

The HPLC separation and fluorometric determination of Proto IX were performed by a modification of the method of Bonkovsky et al.\(^{15}\) 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, a 150 × 6.0 mm (i.d.), 5 \(\mu\)m. Recovery of Proto IX from plant tissues was 94.4±6.4%. Proto IX concentration was expressed on a molar basis per g fresh weight. Results were presented as the means and standard deviations of 3 replications.

5. Assay of Chloroplastic Protox Activity of the Plants
Chloroplasts were prepared from wheat and cleavers \via their protoplasts by a modified method of Murata et al.\(^{16}\) Modifications for this study were as follows. Concentrations of cellulase Onozuka R-10 (Yakult Biochemicals, Nishinomiya, Japan) and pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Tokyo, Japan) in digestion medium were 2 and 0.35% for wheat, 3 and 0.35% for cleavers, respectively. The leaves were cut into small segments (1 to 2 mm in width) with a razor blade, vacuum-infiltrated in 10 volumes (v/w) of the digestion medium and digested for 3.5 hr at 30°C under illumination at a light intensity of 100 \(\mu\)E/m²/sec. Chloroplasts isolation from protoplasts was carried out without bovine serum albumin. Chlorophyll and protein contents in the chloroplast preparations were determined by the method of Arnon\(^{17}\) and Bradford,\(^{18}\) respectively. The reaction mixture for Protox activity determination contained 100 mM HEPES-KOH (pH 7.8), 1 mM EDTA, 5 mM DTT and 20 \(\mu\)M Protogen IX. The test compound was added to the reaction mixture as acetone solution. The final concentration of acetone in reaction mixture was 0.1% (v/v). The reaction was initiated by adding 0.1 ml of chloroplast preparations equivalent to 14 \(\mu\)g chlorophyll to 0.1 ml of the reaction mixture, incubated at 30°C for 60 min, and stopped by adding 0.2 ml of HPLC-grade methanol/1 M perchloric acid (1/1, v/v). The reaction mixture was sonicated for 5 min and centrifuged at 3000 × g for 5 min. Supernatants were stored in vials at −20°C until HPLC analysis as described above. The rate of auto-oxidation of Protogen IX in the presence of heat-denatured chloroplast preparations was subtracted from the enzyme activity. Protox activities calculated on a molar basis per mg protein per hour were expressed as the means and standard deviations of 3 replications.

6. Determination of the Foliar Deposition and Absorption of Foliar-applied Pyraflufen-ethyl and Metabolism of the Absorbed Compound
[Pyrazole-5\(^{14}\)C]pyraflufen-ethyl (5.6 MBq/mg, 2.5% EC) at 0.6 or 6 g a.i./ha was foliar-applied to wheat and cleavers and raised as described above. After 1, 18, and 42 hr of the application, shoots were weighed and non-absorbed radioactivity was rinsed with 30 ml of acetone for 15 sec. Absorbed radioactivity in the shoots was extracted with 40 ml of acetone/methanol (1/1, v/v) for 30 min with sonication. After the extraction, the shoots were combusted with a sample oxidizer (Model 307, Packard Instrument Co., USA). Radioactivity in aliquots of the rinsed acetone and the extract and in the combusted samples was determined by a liquid scintillation counter (Wallac-Model 1409, Pharmacia-Wallac oy., Finland). The other aliquot of the extract was
evaporated to dryness at 40°C under N₂ gas stream. The residue was dissolved in methanol and analyzed by TLC on silica gel 60F₂₅₄ plates (Merck; #5715) with reference standards for metabolites identification. Developing solvents of TLC analysis were toluene/ethyl acetate (9/1, v/v), chloroform/methanol/acetic acid (20/1/1, v/v) or chloroform/methanol/ammonium hydroxide (6/3/1, v/v). Radioactive bands on the TLC plates and their relative radioactivity were detected by radioluminography using Bio-Imaging Analyzer BAS-2000 (Fuji Photo Film Co., Ltd., Japan). The reference standards were visualized by UV light. Amount of the absorption was the sum of the radioactivity in the extract and that in the combusted samples. Foliar deposition was total amount of the radioactivity in each sample. Foliar deposition and foliar absorption were expressed on a molar basis/g fresh weight. The amount of absorbed radioactivity divided by that of total foliar radioactivity gave the uptake ratio. Results were presented as the means of duplicate.

RESULTS AND DISCUSSION

1. Mode of Action and Selectivity at the Target Enzyme Level

After 42 hr of the foliar application, pyraflufen-ethyl (2.5% EC) at 0.6 or 6 g a.i./ha showed strong phytotoxicity against cleavers, but gave a little effect against wheat (Table 1). Several reports indicated that pyraflufen-ethyl inhibited Protox⁴, ¹¹) and that Proto IX content in leaves correlated with herbicidal activity of Protox inhibiting herbicides.⁹,¹⁹) Therefore, we identified an amount of Proto IX in each plant after a foliar application of pyraflufen-ethyl. After 18 hr of the treatment, high level of Proto IX accumulated in cleavers, while little accumulation took place in wheat (Table 2). Although the determination of Proto IX in plants was carried out only at 18 hr, the amount of the accumulated Proto IX in cleavers was about a hundred times higher than that in wheat. Since Proto IX content in leaves correlated with herbicidal activity of Protox inhibiting herbicides,⁹,¹⁹) it was considered that the selective action of pyraflufen-ethyl between wheat and cleavers resulted from the difference in the accumulated Proto IX in each plant.

To examine the inhibitory activity of pyraflufen-ethyl on Protox activities at enzyme level, chloroplasts were isolated from wheat and cleavers via their protoplasts. Because we could not directly isolate chloroplasts from cleavers due to its hard cell wall, we employed a digestion technique via their protoplasts. Protox activity was determined by measuring Proto IX formed from Protogen IX with a HPLC equipped with a fluorescence detector. In this method, Protox activities of untreated chloroplasts of wheat and cleavers were 20.2 and 16.4 nmol Proto IX/mg protein/hr, respectively. These values were almost equal to those of other species measured by the other methods.⁹-¹¹, ²⁰) Furthermore, the I₅₀ value of acifluorfen-methyl on wheat chloroplastic Protox (3.40±0.37 nM, data not shown) was the same order as that on maize etioplastic Protox (4 nM) reported by Matringe et al.²⁰) These results suggested that the effect of pyraflufen-ethyl on Protox activity was determined properly in our experiments. The I₅₀ values of pyraflufen-ethyl on Protox of wheat and cleavers chloroplasts were 1.67±0.20 and 1.24±0.17 nM, respectively (Fig. 1). This suggested that the intrinsic sensitivity of target enzyme, Protox was not important for the selective

![Fig 1](image-url)
action of pyraflufen-ethyl in the whole plants. Therefore, other factor(s) such as foliar absorption and/or detoxification are probably involved in the selectivity. Several other reports also indicated that there was no physiological difference of the Protox sensitivity to pyraflufen-ethyl\(^1\) and other Protox inhibiting herbicides\(^9\)–\(^11\) among plant species.

2. Difference in Foliar Deposition and Absorption
Foliar deposition and absorption of \([\text{pyrazole-5-}^{14}\text{C}]\) pyraflufen-ethyl (6 g a.i./ha) were compared between wheat and cleavers. At each sampling time, the foliar radioactivity of cleavers was about 3 to 5 times higher than that of wheat (Fig. 2A). Time dependent increase of recovered radioactivity on the basis of fresh weight of cleavers would be due to the decrease of fresh weight by wilting. On the other hand, slight decrease of the radioactivity of wheat would result from the increase of fresh weight by its growth. After 1 hr of the application, the amount of absorbed radioactivity in cleavers was about 7 times higher than that in wheat. The amount of the compound absorbed in cleavers increased in a time dependent manner, while that in wheat remained almost constant. After 42 hr of the foliar application, the amount of absorbed compound in cleavers reached to almost 13 times higher than that in wheat. Figure 2B shows that the uptake ratio of the compound in cleavers was also 2 to 3 times higher than that in wheat. These results revealed that a large amount of foliar uptake of the compound by cleavers resulted from not only high spray deposition but also high absorption rate. The differences in the foliar deposition and absorption of the compound might play an important role in the selectivity of pyraflufen-ethyl between wheat and cleavers. The foliar deposition and uptake of pyraflufen-ethyl would vary according to shape, surface structure and composition of epicuticular wax of plant leaves. Willingham et al.\(^2\) reported that although the amount of absorbed acifluorfen-sodium correlated with its herbicidal activity, the relationship between composition of epicuticular wax, wetting and absorption of the compound was complex. Further study of these aspects would be needed to understand the differences in the foliar deposition and absorption of pyraflufen-ethyl between wheat and cleavers in more detail.

3. Difference in Metabolic Detoxification
Figure 3 shows the metabolic pathway of pyraflufen-ethyl in plants. Table 3 shows the \(Rf\) values of pyraflufen-ethyl and its metabolites. Major metabolites identified in leaves were metabolites I and II. Although metabolite II appeared to be further metabolized to a polar metabolite (Metabolite III), it was not characterized well due to the low content. To obtain more information on metabolite III, further metabolism of methyl ester of metabolite II in suspension-cultured tobacco (Nicotiana tabacum L.) BY-2 cells was investigated under dark. A polar metabolite was extracted from the
culture and purified by several kinds of chromatography.
Considering the results of NMR and MS analysis of
acetylated and methylated polar metabolite, metabolite
III seemed to be a glucose conjugate of metabolite II
data not shown).
To elucidate the mechanisms of the selectivity of
pyraflufen-ethyl, the rate of the metabolism of the com-
pound in the two species and the inhibitory effect of the
metabolites on Protox activity were examined. Because
the absorption in cleavers was about 10 times higher than
that in wheat (Fig. 2A), the time course of metabolism of
pyraflufen-ethyl was compared between the plants not
only at the same application rate but also at the rate in
which similar amount of the compound was absorbed
(Fig. 4). In cleavers at 6 g a.i./ha, the amount of the
parent compound on the basis of fresh weight depended
on the amount of the absorbed radioactivity, which
increased in a time dependent manner. However, in
wheat at 6 g a.i./ha and cleavers at 0.6 g a.i./ha, the
amount of the parent compound remained almost con-
stant for 42 hr. In wheat, metabolite I was rapidly
produced and then it decreased with the increase of
metabolites II and III. Metabolite III appeared to be
produced from metabolite II. In cleavers, metabolite I
increased with time and the metabolites II and III were
minor products. These results indicated that ethyl ester
hydrolysis of pyraflufen-ethyl (formation of metabolite I)
was very rapid in both plants. On the other hand,
N-demethylation and subsequent metabolism in wheat
were much faster than those in cleavers. Considering
that the Protox was localized in chloroplasts and mito-
chondria, it seems that not the parent compound but its
metabolites play an important role in the herbicidal
activity in the whole plants.

Table 3 \( R_f \) values of pyraflufen-ethyl and
metabolites in plants.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( R_f ) value in solvent system</th>
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<tbody>
<tr>
<td></td>
<td>A*</td>
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<tr>
<td>Pyraflufen-ethyl</td>
<td>0.28</td>
</tr>
<tr>
<td>Metabolite I</td>
<td>0.00</td>
</tr>
<tr>
<td>Metabolite II</td>
<td>0.00</td>
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<tr>
<td>Metabolite III</td>
<td>0.00</td>
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* Solvent systems; A: toluene/ethyl acetate (9/1, v/v),
B: chloroform/methanol/acetic acid (20/1/1, v/v),
C: chloroform/methanol/ammonium hydroxide (6/3/
1, v/v).

Fig. 4 Metabolism of the absorbed pyraflufen-ethyl in shoots of wheat and cleavers.
Results were presented as the means of duplicate.

Fig. 5 Effect of pyraflufen-ethyl and its metabolites on
Protox activity in wheat chloroplasts.
Protox activity in untreated chloroplasts of wheat was 20.2
nmol Proto IX/mg protein/hr. O: pyraflufen-ethyl, ■:
metabolite I, ●: metabolite II, □: Metabolite III. Results
were presented as the means and standard deviations of 3
replications.
significant role in selectivity between crops and weeds. To confirm whether the metabolism of pyraflufen-ethyl is detoxification or not, inhibitory effect of the metabolites on Protox activity in wheat chloroplasts was examined (Fig. 5). Metabolite III was isolated with preparative TLC plates [chloroform/methanol/acetic acid (15/4/1, v/v)]. The \( I_{50} \) value of metabolite I for wheat chloroplastic Protox was 0.45±0.05 nM, which was surprisingly about one fourth of the parent compound, pyraflufen-ethyl (1.67±0.20 nM). The \( I_{50} \) value of metabolite II was 5.13±0.38 nM, which was about 11 times higher than that of metabolite I. Metabolite III was inactive even at 1000 nM. These results suggested that pyraflufen-ethyl absorbed by both plants underwent a bioactivation by ester hydrolysis at first, and then this more active metabolite I was rapidly converted to the inactive metabolite III via metabolite II in wheat. On the other hand, the detoxification that followed ester hydrolysis was slow in cleavers. These differences in metabolism seemed to be important factors for the selective action as well as those of the foliar deposition and absorption.

In conclusion, the inhibitory activity of pyraflufen-ethyl against target enzyme, Protox was not important in the selectivity between wheat and cleavers. Distinct differences were observed in foliar deposition and absorption, and in the rate of metabolic detoxification of pyraflufen-ethyl between both plants. A combination of these factors in complicated manner will be certainly involved in the selectivity of pyraflufen-ethyl as post-emergence herbicide for wheat.

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REFERENCES


要 約

プロトボルフィリノゲン IX オキシンダーゼ阻害型除草剤ピラフシレンエチルのコムギとヤエムグラに対する選択性発現機構

村田伸治，山下亜紀，木村幸夫，元場一彦
馬潤 勉，三浦友三

広葉雑草，特にヤエムグラに対して卓効を示す新規ムギ用除草剤であるピラフシレンエチル [ethyl 2-chloro-5-(4-chloro-5-difluoromethoxyl-1-methylpyrazol-3-yl) 4-fluorophenoxyacetate] のコムギとヤエムグラに対する選択性発現機構を解明するために，本化合物の Protox 阻害活性ならびに本化合物を茎葉処理した時の Proto IX の蓄積量，本化合物の葉面付着，吸収および代謝を両植物間で比較した。また，植物代謝物の Protox 阻害活性を測定した。ピラフシレンエチルの標的酵素である葉緑体由来 Protox の感受性には両植物間に差が認められなかったが，本化合物の茎葉処理によりヤエムグラにのみ Proto IX が顕著に蓄積した。茎葉処理した本化合物の葉面付着と葉面中への吸収はコムギと比較してヤエムグラで顕著に大きく，吸収された本化合物に対する解毒代謝能はコムギに比較してヤエムグラでは極めて小さかった。これらの違いにより，選択性除草活性が発現していると考えられた。