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Original Article

Translocation and Metabolism of a Seed Fungicide, Pefurazoate, in Rice Seedlings

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Translocation and metabolism of a fungicide, pefurazoate (pent-4-enyl *N*-furfuryl-*N*-imidazol-1-ylcarbonyl-DL-homoalaninate, Healthied®)*, were investigated in rice seedlings after the seeds were treated with three different kinds of ^{14}C-labeled pefurazoate. Three weeks after seeding in a sand culture medium, the radioactivity in the seeds accounted for 81.3–86.3%, and decreased to 54.1–55.8% during the following two-week culture. The decrease was mostly due to the translocation to the shoots and the roots. Pefurazoate was so readily metabolized that the parent molecule was reduced to 49.1–58.2% of the total radioactivity in the seedlings three weeks after seeding. During the following two-week culture it was further reduced to 22.6–25.4%, remaining mostly in the seeds (90.2–93.9%). Major metabolites were 4-pentenyl (*RS*)-2-furfurylaminobutanoate, (*RS*)-2-furfurylaminobutanoic acid, 1-((*RS*)-1-carboxylatopropyl)-3-hydroxypyridinium, *N*-(furan-2-ylcarbonyl)-glycine, (*RS*)-2-aminobutanoic acid, imidazole, hydantoin and hydantoic acid. Conjugated metabolites were observed in small amounts. The metabolic pathway of pefurazoate in the rice plants was almost similar to that in soil.

INTRODUCTION

Pefurazoate is a seed fungicide for control of seed-borne pathogens of rice, such as *Gibberella fujikuroi*, *Cochliobolus miyabeanus* and *Pyricularia oryzae*.[1] Rice seeds disinfected with pefurazoate were cultured in a nursery soil and transferred to a paddy field after three weeks. Pefurazoate adhering to the seeds underwent degradation in the nursery and paddy soils. Its degradability in soils has been reported already.[2] The present paper describes the translocation and metabolism of pefurazoate absorbed in seeds.

MATERIALS AND METHODS

1. *Chemicals*

Three kinds of ^{14}C-pefurazoate (designated as F-, B- and I-labeled pefurazoate) (Fig. 1) with differently positioned ^{14}C were synthesized as follows.

F-labeled pefurazoate (furfuryl-labeled): Previously described.[2]

B-labeled pefurazoate (butanoate-labeled): By reaction of sodium [3,4-^{14}C]butanoate (specific activity: 0.94 GBq/mmol, radiochemical purity: 99.5%, product of CEA) with bromine, [3,4-^{14}C]2-bromobutanoic acid was obtained. [3,4-^{14}C]2-bromobutanoic acid was esterified with pentenyl alcohol. From this product butanoate-labeled pefurazoate was synthesized by the same method as that for F-labeled pefurazoate.[2]

I-labeled pefurazoate (imidazole-labeled): This was obtained by reaction of [2-^{14}C]imidazole (specific activity: 0.65 GBq/mmol, radiochemical purity: 98%, product of CEA) with carbamoyl chloride of 4-pentenyl (*RS*)-2-furfurylaminobutanoate.

* The chemical name of pefurazoate was given by British Standards Institution.

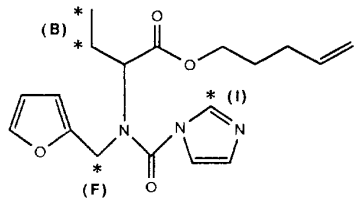

Fig. 1 Chemical structure and labeled-position of pefurazoate.

* Labeled position.
(F): furfuryl-labeled, (B): butanoate-labeled, (I): imidazole-labeled.

The radiochemical purity of the respective ^{14}C-pefurazoates was more than 97% by TLC (Silica gel plate, 60F$_{254}$, 20×20 cm, 0.25 mm thickness, Merck).

The synthetic procedure of unlabeled authentic pefurazoate and four reference compounds, PFAB,* FAB,* HPB* and FG,* were prepared as reported previously.[2] Other reference compounds (AB,* IM,* HY* and HYA*) were purchased from Sigma Chemical Company (U.S.A.).

2. *Culture*

Seeds of rice (*Oryza sativa* L. cv. Nihonbare) were dipped at 20°C for three days in the 1000 ppm ^{14}C-pefurazoate solutions (seeds/solution =1: 1, volume/volume) prepared from 20% wettable-powder formulation.

Ten seeds were dipped for three days, put in a Wagner pot (1/5000 a) packed with sand (river sand/vermiculite=3: 1, volume/volume) and cultured in a growth cabinet at 20°C-daytime and 16°C-night for 3–5 weeks. They were fed with a culture solution (pH 5.5) containing mineral nutrients, *i.e.* N, P, K, Mg and Fe components.

3. *Absorption Test with Seeds*

Seeds dipped for one or three days in the ^{14}C-pefurazoate solution by the method mentioned above were successively soaked with water for two or four days and washed in 3 ml acetone for 15 sec. Radioactivity in the washings was determined by liquid scintillation spectrometry (LSC, Tricarb 2000CA, Packard) with a Clear-Sol scintillator (Nakarai Chemical). Radioactivity in the seeds washed with acetone was determined similarly after treatment with an autocombustion system (ASC-113, Aloka).

4. *Analytical Methods*

4.1 *Extraction*

The seedlings were divided into shoots, roots and seeds. Each part was macerated overnight with aqueous acetone (acetone/water= 10: 1) (plant/solvent=1: 20, weight/volume), homogenized and shaken at room temperature for 30 min. After filtration of the resulting suspension with a membrane filter (PTFE, 0.2 μm, Toyo Roshi), the residue was refluxed with 80% aqueous ethanol (residue/solvent=1: 40, weight/volume). Both aqueous acetone and aqueous ethanol extracts were combined to make an extracted fraction. The unextracted fraction was classified as bound residue.

4.2 *Fractionation and determination of the extracted fraction*

The extracted fraction was concentrated *in vacuo* below 35°C and separated to two fractions: one, benzene-extracted fraction obtained by extracting with benzene (concentrated aqueous extracted fraction/benzene= 4: 10) and the other, water-soluble fraction by lyophilizing and resolubilizing with methanol. Each fraction was analyzed by TLC using solvent systems shown in Table 1.

Individual solutions (10–50 μl) of each fraction were spotted on silica gel plates and developed with solvent system 1. When a solution containing both pefurazoate and PFAB was analyzed, solvent system 2 was also used. ^{14}C-compounds separated on the silica gel plate were detected by autoradiography (^{3}H ARG film, Konica) and their radioactivity was determined by LSC. Pefurazoate and its metabolites were identified by co-chromatography with reference compounds.

4.3 *Conjugates in water-soluble fraction*

The water-soluble fraction from which methanol had been removed by evaporation *in vacuo* was treated with either β-D-glucosidase (Type 2, Sigma) or cellulase (Type 2,

* PFAB: 4-pentenyl (*RS*)-2-furfurylaminobutanoate, FAB: (*RS*)-2-furfurylaminobutanoic acid, HPB: 1-((*RS*)-1-carboxylatopropyl)-3-hydroxypyridinium, FG: *N*-(furan-2-ylcarbonyl)glycine, AB: (*RS*)-2-aminobutanoic acid, IM: imidazole, HY: hydantoin, HYA: hydantoinic acid.

Table 1 *Rf* values of pefurazoate and its metabolites by TLC.

Solvent system[a] No.	Compound											
	P-1 P-5	Pefurazoate	PFAB	FAB	P-2	HPB	P-3 P-4 P-6	FG	AB	IM	HY	HYA
1	0.85	0.82	0.82	0.46	0.41	0.27	0.20	0.36	0.17	0.67	0.34	0.30
2	—	0.75	0.66	0.30	—	0.17	—	0.42	0.17	0.29	0.38	0.16
3	—	0.91	—	0.38	—	—	—	—	—	—	—	—
4	—	0.30	0.54	0.00	—	0.00	—	0.01	—	0.08	—	—
5	—	—	—	—	—	—	—	—	—	0.37	—	—
6	—	0.89	—	0.45	—	0.09	—	0.59	—	—	—	—
7	—	—	—	—	—	—	—	—	—	0.67	—	—
8	—	0.96	—	—	—	—	—	—	—	—	0.49	0.43
9	—	0.03	0.49	—	—	—	—	—	—	—	—	—

[a] Solvent system (volume ratio): 1, $CHCl_3$/EtOH/28%NH_4OH (2/4/1); 2, *n*-BuOH/AcOH/H_2O (5/1/1); 3, AcOEt/MeOH/HCOOH (8/8/1); 4, $C_6H_5CH_3$/AcOEt (1/5); 5, $(CH_3)CO/CHCl_3$/*n*-BuOH (3/3/4/1); 6, AcOEt/MeOH/AcOH (10/5/1); 7, *i*-PrOH/28%NH_4OH (40/1); 8, *n*-PrOH/28% NH_4OH (3/1); 9, *n*-C_6H_{12}/AcOEt (4/1).

Sigma) in phosphate buffer (pH 5). The reaction product was lyophilized and resolubilized with methanol. The methanolic solution was analyzed by TLC with solvent system 2.

4.4 Bound residue[3,4]

The bound residue (1–5 g) was treated with 0.03 M sodium hydroxide for 15 min with a sonicator (VS-100, Verboclear). The filtrate was classified as protein fraction. The precipitate was shaken with 100 ml acidic Van Soest solution (0.5 M sulfuric acid containing 2% cetyltrimethylammonium bromide) for 1 hr. The resulting solubilized fraction was classified as hemicellulose fraction. The precipitate was shaken with dimethyl sulfoxide containing 0.2% sulfuric acid at 140°C for 5 hr. The filtrate was classified as lignin fraction and its precipitate as cellulose fraction.

Radioactivity in the soluble fractions, *i.e.* protein, hemicellulose and lignin fraction, were determined by LSC. Radioactivity in the cellulose fraction was calculated by subtracting the radioactivity of the soluble fractions from that of the total bound residue. Radioactive compounds in the protein and hemicellulose fraction were subjected to TLC with solvent system 2 by almost the same method as that for the conjugate hydrolysate after neutralizing their fractions.

The net amount of each fraction was determined gravimetrically from the oven-dried sample.

RESULTS AND DISCUSSION

1. Absorption of Pefurazoate by Seeds[5]

Rice seeds were soaked conventionally for one day with a seed fungicide and for the following four days with water. In the present metabolism experiment the seeds were soaked with pefurazoate solution for three days and with water another two days in order to improve the absorption of pefurazoate.

The carrying ratio indicating the radioactivity carried by the seeds (on and in) was 23.2% of the applied amount, and the absorption ratio indicating the true intake by the seeds was 20.9% of the applied amount. The carrying ratio increased with the time of soaking with pefurazoate and the absorption ratio was not much affected by soaking with water (data not shown). This indicated that most of pefurazoate was in the seeds not on the hulls.

2. Distribution of Radioactivity in Seedlings (Table 2)

The total radioactivity in the seedlings cultured for three to five weeks after seeding scarcely fluctuated (100–113.2%). This showed that the exudation of metabolites from the roots, the evolution of volatile metabolites such as CO_2 and the uptake from the culture me-

dium, into which pefurazoate was carried by seeds, were negligible.

Radioactivity was distributed in the seedlings cultured for three weeks as follows: With F-labeled pefurazoate, 7.1% in the shoots, 86.3% in the seeds and 6.6% in the roots, and these ratios were almost same with B- and I-labeled pefurazoate. In every case, 81.3–86.3% of the total radioactivity remained in the seeds.

The radioactivity in the seeds decreased with the elapse of culture time, accounting for 54.1–54.9% in the seeds cultured with any ^{14}C-pefurazoate for five weeks. As the radioactivity in the seeds decreased, that in the shoots and the roots increased. The radioactivity in the seeds was readily translocated to the shoots and the roots.

3. *Fate of Pefurazoate and Its Metabolites* (Table 3)

A number of metabolites were produced in each seedling treated with F-, B- and I-labeled pefurazoate during the three-week culture.

With F-labeled pefurazoate, PFAB, FAB, HPB, FG, P-1, P-2 and P-3 were major metabolites, among which the chemical structures of P-1, P-2 and P-3 were not identified. These metabolites were less than several percents of the total radioactivity in the seedlings and fluctuated only slightly during the culture, whereas the residue and the other hydrophilic minor metabolites increased. Pefurazoate,

Table 2 Distribution of radioactivity in seedlings.

(Distribution ratio, %)[a]

Labeled compound	Part of seedlings	Time after seeding (week)		
		3	4	5
F-label	Shoots	7.1	20.6	30.3
	Seeds	86.3	64.6	54.9
	Roots	6.6	15.9	19.1
	Total	100	101.1	104.3
B-label	Shoots	10.7	17.7	27.4
	Seeds	81.5	62.4	55.8
	Roots	7.8	22.1	23.1
	Total	100	102.2	106.4
I-label	Shoots	10.2	16.5	33.8
	Seeds	81.3	73.8	54.1
	Roots	8.5	11.1	25.3
	Total	100	101.4	113.2

[a] % of total radioactivity in three-week-cultured seedlings.

Table 3 Fate of pefurazoate and its metabolites in seedlings.

(Distribution ratio, %)[a]

Labeled compound	Detected compound	Time after seeding (week)		
		3	4	5
F-label	P-1[b]	1.3	3.0	4.1
	Pefurazoate	56.6	38.5	24.4
	PFAB	3.4	4.1	4.4
	FAB	3.6	3.5	3.9
	P-2[b]	1.2	1.7	2.3
	FG	0.9	0.9	1.6
	HPB	3.2	3.7	5.1
	P-3[b]	0.5	0.9	1.5
	Others[c]	2.9	3.9	8.5
	Residue	26.4	40.9	48.5
	Total	100	101.1	104.3
B-label	P-1	2.8	2.5	1.7
	Pefurazoate	49.1	31.3	27.0
	PFAB	3.1	3.5	2.4
	FAB	2.3	2.4	1.9
	P-2	1.4	1.8	1.1
	HPB	1.3	1.6	1.3
	AB	2.6	2.9	3.8
	P-4[b]	0.0	0.1	0.1
	Others	5.6	6.3	6.2
	Residue	31.8	49.8	60.9
	Total	100	102.2	106.4
I-label	P-5[b]	1.9	2.4	2.1
	Pefurazoate	58.2	42.2	25.6
	IM	15.3	18.3	20.0
	HY	0.1	0.4	1.0
	HYA	0.0	0.1	0.3
	P-6[b]	0.1	0.1	0.1
	Others	2.2	2.7	2.8
	Residue	22.2	35.2	61.3
	Total	100	101.4	113.2

[a] % of radioactivity in three-week-cultured seedlings.

[b] P-1, -2, -3, -4, -5, -6 are the unidentified metabolites.

[c] Others contain minor metabolites except for identified ones.

Table 4 Dissipation and translocation of pefurazoate in seedlings.

(Distribution ratio of pefurazoate, %)[a)]

Labeled compound	Part of seedlings	Time after seeding (week)		
		3	4	5
F-label	Shoots	0.0	0.2	0.4
	Seeds	98.7 (98.7)[b)]	66.0 (97.1)	40.4 (94.0)
	Roots	1.3	1.8	2.2
	Total	100	68.0	43.0
B-label	Shoots	0.4	0.6	1.5
	Seeds	98.6 (98.6)	60.2 (94.5)	49.6 (90.2)
	Roots	1.0	2.9	3.9
	Total	100	63.7	55.0
I-label	Shoots	1.1	0.7	0.7
	Seeds	97.6 (97.6)	70.6 (97.2)	41.4 (94.3)
	Roots	1.3	1.3	1.8
	Total	100	72.6	43.9

a) % of pefurazoate in three-week-cultured seedlings.

b) The value in parenthesis shows % of total pefurazoate in each seedlings.

which accounted for 56.6% of the total radioactivity in the seedlings cultured for three weeks, was readily metabolized in the next two weeks, decreasing to 24.4%. The rates of metabolism were similarly obtained with B- and I-labeled pefurazoate.

When B-labeled pefurazoate was used, major metabolites were PFAB, FAB, HPB, AB, P-1, P-2 and P-4. Major metabolites with F-labeled pefurazoate, were the same excluding AB and P-4. P-4 was not identified.

When I-labeled pefurazoate was used, major metabolites were IM and P-5, the former accounting for 15.3–20.0% of the total radioactivity in the seedlings. HY and HYA were minor metabolites, and P-6 was not identified.

Since most of pefurazoate (90.2–98.7% in the seedlings) remained immobilized in the seeds with every ^{14}C-pefurazoate as shown in Table 4, it was clear that pefurazoate was metabolized in the seeds, without being translocated elsewhere.

4. *Metabolic Pathway of Pefurazoate*

From the identification of metabolites produced from three different kinds of ^{14}C-pefurazoate, a metabolic pathway was proposed as shown in Fig. 2; The metabolism of pefurazoate was mainly initiated from elimination of the imidazolylcarbonyl group to produce PFAB. From the eliminated group imidazole was released *via* decarboxylation and transformed to hydantoic acid *via* hydantoin. PFAB was de-esterified to FAB, which yielded

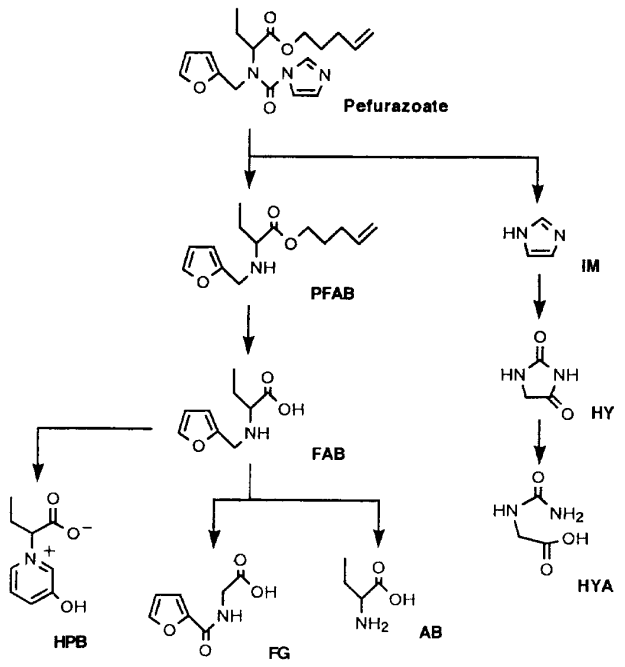

Fig. 2 Proposed pathway of pefurazoate metabolism in rice plant.

Table 5 Fractionation of residue.

(%)[a]

Fraction	Labeled compound		
	F-label	B-label	I-label
Protein	9.2 (53.4)[b]	5.7 (48.7)	4.0 (66.5)
Hemicellulose	38.1 (35.3)	41.1 (40.1)	42.1 (24.8)
Lignin	32.7 (2.6)	26.3 (4.1)	30.9 (3.3)
Cellulose	20.0 (8.7)	26.8 (7.1)	23.0 (5.4)
Total residue	100 (100)	100 (100)	100 (100)

[a] % of total radioactivity of residue.
[b] The value in parenthesis shows the substantial content determined by gravimetric analysis.

FG, AB and HPB. FG was transformed *via* dealkylation of ω and ω-1 carbons of the carboxylate moiety and oxidation of furfuryl carbon. AB was produced *via* oxidation of furfuryl carbon followed by hydrolysis of its product. The transformation to HPB was driven by the condensation reaction between the amine-nitrogen of FAB and the reactive position-5 carbon in the furan ring of FAB caused by cleavage of its bond between the position-5 carbon and oxygen.

5. *Analysis of Conjugate and Bound Residue*

The water-soluble fractions in the seedlings cultured for five weeks after treatment with each labeled pefurazoate were analyzed for conjugates. Each fraction showed 1.3–3.7% of the radioactivity at the original position on the TLC plate, which seemed to correspond to the conjugated metabolites. When the fractions were treated in advance with β-D-glucosidase or cellulase, the original band on the TLC plate almost disappeared, and FAB, HPB and IM increased evidently. It suggested that these metabolites formed a fair amount of glucose conjugates. Although the –OH, –NH– and –COOH groups of these metabolites appeared to be responsible for the formation of the conjugates, further study is needed to elucidate the type of conjugates.

The bound residue in the seedlings cultured for five weeks was analyzed and the result is shown in Table 5. In every seedling, the hemicellulose fraction surpassed the other fractions and the protein fraction was the least. These distribution ratios were quite different from those obtained by gravimetric analysis.[6] Furthermore, PFAB, FAB, HPB, FG, IM and HY were found in the protein and hemicellulose fraction, as shown in Table 6. This showed that the radioactivity in the bound residue was not from the assimilation but mostly from the noncovalent binding of metabolites (PFAB, FAB, *etc.*) to plant constituents.

Table 6 Analysis of hemicellulose fraction and protein fraction.

(%)[a]

Labeled compound	Detected compound	In hemicellulose fraction	In protein fraction
F-label	PFAB	11.2	—[b]
	FAB	—	44.7
	HPB	—	11.2
	FG	10.2	—
B-label	PFAB	14.7	—
	FAB	—	46.9
	HPB	—	14.2
I-label	IM	19.6	38.4
	HY	14.1	—

[a] % of radioactivity in each fraction.
[b] Not detected.

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要　　約

水稲幼苗中における種子殺菌剤ペフラゾエートの移行および代謝

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水稲種籾に浸漬処理したペフラゾエート（4-ペンテニル=(*RS*)-2-[フルフリル(イミダゾール-1-イルカルボニル)アミノ]ブタノアート）の幼苗期の水稲における移行および代謝を，三種の ^{14}C 標識ペフラゾエートを用いて調べた．砂培土に播種後3週目における水稲体内の ^{14}C は，三種の標識体において 81.3～86.3% が種籾中に分布していたが，5週目には 54.1～55.8% に減少した．その減少は，ほぼ茎葉および根への移行によるものであった．一方，ペフラゾエートの代謝は活発で，3週目に幼苗中全 ^{14}C の 49.1～58.2% 存在していたペフラゾエートは，5週目には 22.6～25.4% に減少し，その大部分 (90.2～93.9%) は種籾に存在していた．主要代謝物として，4-ペンテニル=(*RS*)-2-フルフリルアミノブタノアート，(*RS*)-2-フルフリルアミノブタノアート，1-[(*RS*)-1-カルボキシラートプロピル]-3-ヒドロキシピリジニウム，*N*-(フラン-2-イルカルボニル)グリシン，(*RS*)-2-アミノブタン酸，イミダゾール，ヒダントイン，ヒダントイン酸が同定された．抱合体の生成はわずかであった．これらの代謝物から，水稲中における代謝経路は土壌中とほぼ同じであると考えられた．