J. Pestic. Sci. **38**(3), 152–156 (2013)
DOI: 10.1584/jpestics.D13-014

Note

Role of metabolism in the selectivity of a herbicide, pyroxasulfone, between wheat and rigid ryegrass seedlings

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(Received February 28, 2013; Accepted May 20, 2013)

Metabolism of pyroxasulfone in a tolerant crop, wheat and a susceptible plant, rigid ryegrass, was studied using ^{14}C-pyroxasulfone. The main metabolites were a cysteine conjugate of the isoxazoline ring (M-26), deaminated M-26 (M-29) and a glucose conjugate of M-29, suggesting that the main metabolic route in both plants was the cleavage of the methylenesulfonyl linkage caused by glutathione conjugation. The difference in the metabolic activity was assumed to be one of the factors in determining the selectivity of pyroxasulfone between wheat and rigid ryegrass. © Pesticide Science Society of Japan

Keywords: pyroxasulfone, metabolism, selectivity, glutathione, glutathione *S*-transferase (GST).

Introduction

Pyroxasulfone(3-[5-(difluoromethoxy)-1-methyl-3-(trifluoromethyl)pyrazol-4-ylmethylsulfonyl]-4,5-dihydro-5,5-dimethyl-1,2-oxazole; code name KIH-485) is a novel pre-emergence herbicide for use in wheat,[1)] corn,[2–6)] soybean[7)] and other crops.[1,8–11)] This herbicide is effective on both grass and broadleaf weeds. In comparison with other currently available pre-emergence herbicides for wheat, application of pyroxasulfone at a rate of 100 g ai/ha provides an efficient control of both herbicide-resistant and susceptible annual ryegrass populations.[1)] This herbicide potently inhibits shoot elongation of weeds; however, selectivity was observed in growth inhibition between weeds and crops.[12,13)] We have already reported that pyroxasulfone is a potent inhibitor of the very long-chain fatty acid elongase (VLCFAE) of plants.[12)] The difference in the sensitivity of plant VLCFAEs to pyroxasulfone was partly involved in the selectivity of this herbicide.[13)]

In this study, we compared the pyroxasulfone metabolism in rigid ryegrass, a susceptible species, and in wheat, a tolerant species, to investigate the involvement of pyroxasulfone metabolism in the selectivity between crops and weeds.

Materials and Methods

1. Chemicals

1.1. Radio-labeled compound

^{14}C-labeled pyroxasulfone ([isoxazoline-3-^{14}C]pyroxasulfone) synthesized by Amersham Biosciences Co., Ltd. (United Kingdom) was used in these experiments. The specific radioactivity was 1.7 MBq/mg and the radiochemical purity was more than 99%.

1.2. Non-labeled compounds

Pyroxasulfone (white powder, mp 130.7°C, water solubility at 20°C 3.49 mg/L, vp 2.4×10^{-6} Pa) and the synthetic compounds, 2-amino-5-[1-(carboxylmethylamino)-3-(5,5-dimethyl-4,5-dihydroisoxazol-3-ylthio)-1-oxopropan-2-ylamino]-5-oxopentanoic acid (M-15), 2-amino-3-(5,5-dimethyl-4,5-dihydroisoxazol-3-ylthio)propanoic acid (M-26) and 3-(5,5-dimethyl-4,5-dihydroisoxazol-3-ylthio)-2-hydroxypropanoic acid (M-29) were used. These compounds were synthesized by KI Chemical Research Institute Co., Ltd. (Japan) and their purities were above 98%. The NMR data and MS data of these compounds are shown in Table 1.

2. Plant materials

Seeds of wheat (*Triticum aestivum* L. var. Bonnie Rock) and rigid ryegrass (*Lolium rigidum* Gaud.) were kindly provided by Prof. Stephen Powles of the University of Western Australia.

3. Metabolism study of pyroxasulfone in wheat and rigid ryegrass

3.1. Treatment and cultivation

The wheat and rigid ryegrass were cultivated to the leaf stage 3 to 4, grown to a height of approximately 15 cm. The roots of 15 wheat seedlings were soaked in 50 mL of distilled water containing 50 μL of liquid fertilizer containing 10% phosphoric acid, 6% nitrogen and 5% potassium (HYPONex, HYPONex JAPAN CORP., LTD.) and 1.5 ppm of ^{14}C-pyroxasulfone, which corresponded to *ca.* 3.8 μM. Similarly, roots of 13 rigid ryegrass seedlings were soaked in 70 mL of distilled water containing 70 μL of the liquid fertilizer and 1.3 ppm of ^{14}C-pyroxasulfone, which corresponded to *ca.* 3.3 μM. No adverse effects were observed on the growth of seedlings in these concentrations.

Plant seedlings treated with ^{14}C-pyroxasulfone were cultivated in a greenhouse under natural conditions in June (15–25°C). After soaking in ^{14}C-pyroxasulfone solution for 1, 2, or 4 days,

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Published online August 14, 2013

Table 1. ^{1}H NMR data and MS data of authentic compound

Compounds	^{1}H NMR (δ_H, ppm)[a]	MS (m/z)[b]	MS/MS (m/z)[b]
Pyroxasulfone	1.52 (6H, s), 3.10 (2H, s), 3.87 (3H, s), 4.60 (2H, s), 6.87 (1H, t, $J_{H,F}$=71.9 Hz)	390 $[M-H]^-$, 450 $[M+CH_3CO_2]^-$	—
M-15	1.38 (6H, d, J=1.4 Hz), 2.07 (2H, d, J=1.4 Hz), 2.14–2.24 (2H, m), 2.56 (2H, t, J=7.1 Hz), 3.17–3.22 (1H, m), 3.54–3.59 (1H, m), 3.93 (2H, s), 4.02 (1H, t, J=6.5 Hz), 4.79 (1H, m), 8.33 (1H, br)	403 $[M-H]^-$	272, 254, 210, 179, 143, 128 (from 403)
M-26	1.31 (3H, s), 1.33 (3H, s), 2.89 (2H, s), 3.33–3.58 (2H, m), 4.13 (1H, m), 9.09 (2H, br)	260 $[M+CH_3CN+H]^+$, 219 $[M+H]^+$, 143 $[M-75]^+$	—
M-29	1.30 (6H, s), 2.87 (2H, s), 3.17 (1H, br), 3.30 (1H, br), 4.19 (1H, br)	220 $[M+H]^+$	202, 156, 121, 82 (from 220)

[a] ^{1}H NMR spectra of pyroxasulfone (in $CDCl_3$) and M-15 (in CD_3OD) were measured on a JEOL JMN-LA400 (400 MHz) spectrometer. ^{1}H NMR spectra of M-26 and M-29 (in DMSO-d_6) were measured on JEOL JMN-LA-300 (300 MHz) spectrometer.

[b] LC-ESI-MS spectra were measured on Thermo TSQ Quantum Discovery. Their analytical conditions were described in Materials and methods.

wheat seedlings and rigid ryegrass seedlings were used for extraction and fractionation.

3.2. Extraction and fractionation

Roots of plant seedlings were rinsed with 20 mL of acetonitrile, and all the seedlings were weighed. The seedlings were then homogenized using a Physcotron (NITI-ON Co., Ltd., Japan) in 150 mL of acetone/distilled water (3/1, v/v). After the removal of residue by filtration, the extracts were evaporated *in vacuo* and dissolved in 10 mL of acetonitrile/distilled water (1/1, v/v). The radioactivity of the extracts was measured by a liquid scintillation counter (LSC; TRI-CARB 2750TR/LL, PerkinElmer, United States) using AQUASOL-2 (PerkinElmer) as the scintillator. The radioactivity of the non-extractable residues of the seedlings was measured by LSC using a PERMAFLUOR E$^+$ scintillator (PerkinElmer) and CARBO-SORB E $^{14}CO_2$ trapping solution (PerkinElmer) following combustion by a sample oxidizer (Model 307, PerkinElmer).

3.3. Determination of metabolites

Pyroxasulfone and its metabolites were identified by comparison with authentic compounds using TLC and LC-MS. For two-dimensional TLC analysis, an aliquot of each extract and authentic compounds were mixed and applied to silica gel 60 F_{254} chromatoplates (20×20 cm, 0.25 mm thick, Merck, Germany). The plates were initially developed with a mixture of ethyl acetate/chloroform/methanol/formic acid (6/6/1/1, v/v/v/v) and subsequently developed in a second dimension by a solvent mixture containing ethyl acetate/methanol/distilled water/formic acid (6/4/2/1, v/v/v/v). The radioactive spots were detected and measured by autoradiography with a Bio-Imaging Analyzer (Fuji BAS1000, Fuji Photo Film Co., Ltd., Japan), and verified by comparison with those of the authentic compounds visualized by UV irradiation.

For LC-MS analysis sample preparation, an aliquot of the extract was applied to the TLC plate. The plate was developed in one dimension with a mixture of ethyl acetate/methanol/distilled water/formic acid (6/4/2/1, v/v/v/v). The silica gel band corresponding to each metabolite was scratched out from the plate and the metabolites were extracted in a mixture of acetonitrile/distilled water (1/1, v/v). A portion of the extract was filtered using a 0.2 μm centrifuge filter before LC-MS injection. HPLC (Nanospace, UV6000, SHISEIDO, Japan) analyses were performed on a CAPCELL PAK C18 UG120 column (4.6 mm I.D.×250 mm, SHISEIDO) using mixtures of acetonitrile containing 0.5% acetic acid (solvent A) and distilled water containing 0.5% acetic acid (solvent B) as the mobile phase. The compounds were eluted in a stepwise manner (gradient elution) for which the run was initiated using 10% solvent A for 5 min, linearly changed to 50% solvent A over 35 min and held for 5 min, followed by increasing to 100% solvent A over 5 min and held for 5 min. At the end of the run, the column was conditioned with the starting solvent mixture. The flow rate was 1.0 mL/min and the column temperature was maintained at 35°C. The radioactive compounds were detected by a Solid Flow Cell Radiomatic 610TR (PerkinElmer) equipped with HPLC. Mass detection was performed on a TSQ Quantum Discovery (Thermo Electron Corporation, United States) according to the following conditions: electrospray ionization (ESI); 100–800 m/z Q1 Scan; 25 V collision energy; Split ratio 1/5. Similarly, LC-MS analysis of authentic compounds was conducted. Retention times of pyroxasulfone, M-15, M-26 and M-29 were 46.7 min, 7.8 min, 4.0 min and 14.0 min, respectively.

In glycoside hydrolysis reaction, β-glucosidase (Sigma-Aldrich, United States) and cellulase (Sigma-Aldrich) were used. The reaction mixture contained 3 mg of 2 units/mg β-glucosidase, 7 mg of 0.3 units/mg cellulase, 0.4 mL of acetate buffer (200 mM, pH 5.0) and 1.2 mL of the glucose conjugate dissolved in acetonitrile/distilled water (1/1, v/v). After the reaction mixture was incubated at 37°C for 12 hr, the aglycone was identified by co-TLC with authentic M-29.

Results

A day after treatment (day 1), 3.7 μg eq./g (amount of ^{14}C-compound equivalent to pyroxasulfone/plant fresh weight) of ^{14}C-compound was absorbed by wheat seedlings (Table 2). The absorbed ^{14}C-compound increased to 9.2 μg eq./g at day 4 and the ratio of extractable radioactivity to the total amount of absorbed radioactivity was more than 87.5% during 4 days (Table 3). The wheat seedlings extract showed 6 spots on TLC (Fig. 1A). Three spots were determined to be pyroxasulfone, M-26 and M-29 by co-TLC with authentic compounds and LC-MS

Table 2. Amount of radioactivity in plant seedlings treated wtih [isoxazoline-^{14}C]pyroxasulfone

Plant	Day after treatment	Plant fresh weight (g)	Amount of radioactivity in plants (μg eq.)[a]			Concentration[b] (μg eq./g)
			Extract	Unextractable residue	Total radioactivity	
Wheat	1	3.82	13.5	0.6	14.1	3.7
	2	4.30	23.5	2.4	25.9	6.0
	4	4.10	32.9	4.7	37.6	9.2
Rigid ryegrass	1	1.82	3.5	0.6	4.1	2.3
	2	1.89	5.3	0.6	5.9	3.1
	4	1.87	8.2	0.6	8.8	4.7

[a] Values are expressed as the amount equivalent to pyroxasulfone.
[b] Concentration is the amount of parent compound equivalent (μg eq.) to plant fresh weight (g).

Table 3. Ratio of pyroxasulfone and its metabolites in plant seedlings

Plant	Day after treatment	Percent in plant seedlings (%)										
		Pyroxasulfone	Identified metabolites				Unidentified metabolites					Total
			M-26	M-29	M-29-glc	Total	Uk-1	Uk-2	Uk-3	Uk-4	Others	
Wheat	1	20.0 (0.74)[a]	16.1	16.1	22.7	54.9 (2.03)	6.7	<0.5	3.9	<0.5	10.2	95.7
	2	10.0 (0.60)	14.7	10.6	33.8	59.1 (3.55)	8.3	<0.5	5.9	<0.5	7.4	90.7
	4	6.1 (0.56)	<0.5	9.6	45.2	54.8 (5.04)	3.6	<0.5	8.9	<0.5	14.1	87.5
Rigid ryegrass	1	46.4 (1.07)	13.7	3.6	4.6	21.9 (0.50)	4.6	1.8	2.7	3.6	4.4	85.4
	2	26.4 (0.82)	18.2	8.2	10.0	36.4 (1.13)	8.2	1.8	3.6	6.4	7.0	89.8
	4	9.1 (0.43)	24.6	10.0	13.7	48.3 (2.27)	8.2	1.8	8.2	7.3	10.3	93.2

[a] Values in parentheses indicate concentration (μg eq./g).

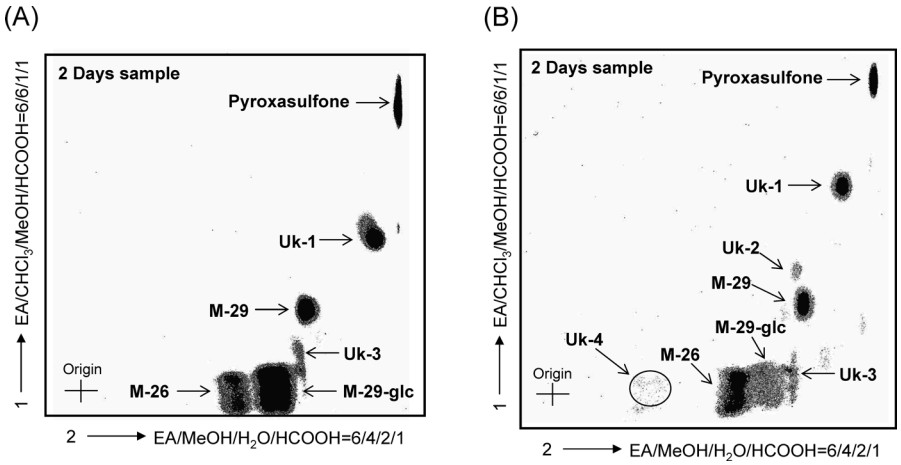

Fig. 1. TLC of metabolites present in the extract from wheat seedlings (A) and rigid ryegrass seedling (B) after [isoxazoline-3-^{14}C]pyroxasulfone treatment.

analysis. One of the spots was confirmed as a glucose conjugate of M-29 (M-29-glc), having molecular weight 381 (Fig. 2A), and from M-29 liberation on treatment with a mixture of glucose hydrolase, β-glucosidase and cellulase. The other spot (Uk-1), possessing a molecular weight of 189, was presumed to be the ring-opened form of M-26 (Fig. 2B). The mass spectrum of Uk-3 could not be measured. Among them, M-26, M-29, and M-29-glc were the main metabolites. In wheat, 20.0% of the total radioactivity remained as a parent compound and 54.9% was converted to its main metabolites a day after the treatment. The concentration of pyroxasulfone and main metabolites were 0.74 μg eq./g and 2.03 μg eq./g, respectively (Table 3). The ratio of M-26 and M-29 decreased with time, whereas the ratio of M-29-glc increased. The ratio of M-29-glc was the highest among the metabolites in 4 days.

In rigid ryegrass seedlings, the absorbed ^{14}C-compound was 2.3 μg eq./g at day 1 and increased to 4.7 μg eq./g on day 4 (Table 2). The concentration of absorbed radioactivity in rigid ryegrass

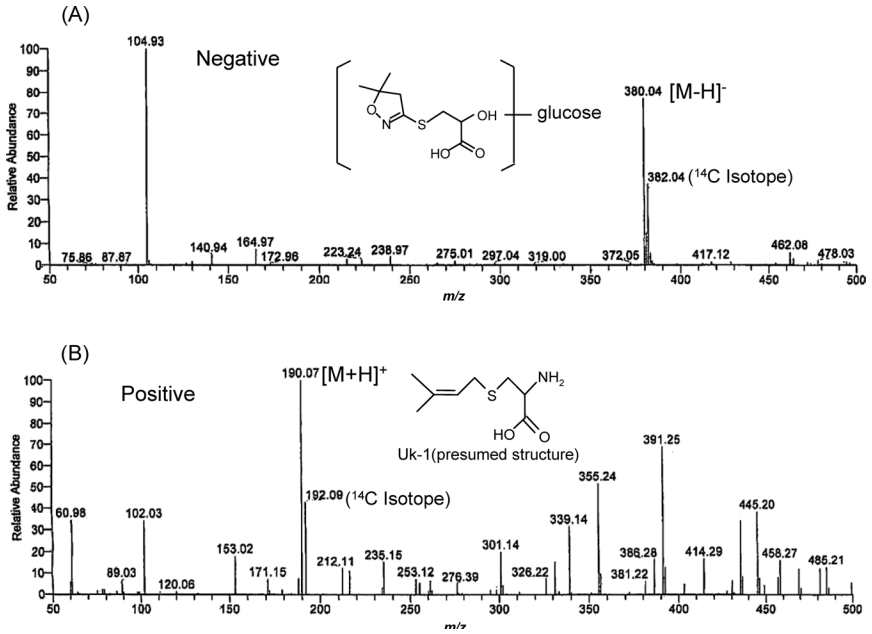

Fig. 2. LC-MS spectrum of M-29-glc (A) and Uk-1 (B) isolated from the seedlings by TLC.

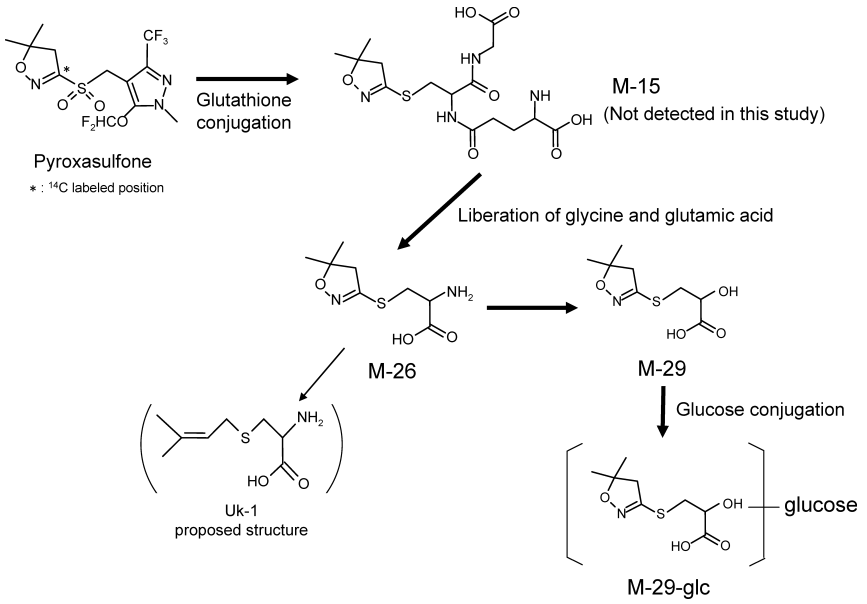

Fig. 3. Schematic representation of proposed route of pyroxasulfone metabolism in wheat and rigid ryegrass. Bold arrow shows the major route of pyroxasulfone metabolism.

seedlings was approximately one-half compared to wheat seedlings. The extract from rigid ryegrass seedlings showed 8 spots on TLC. Of these, 6 spots were determined to be the same compounds (pyroxasulfone, M-26, M-29, M-29-glc, Uk-1 and Uk-3) obtained from wheat (Fig. 1). The other 2 metabolites, Uk-2 and Uk-4, were not identified. The ratios of the parent compound and the main metabolites in rigid ryegrass were 46.4% and 21.9%, respectively, 1 day after treatment. The concentrations of pyroxasulfone and the main metabolites were 1.07 μg eq./g and 0.50 μg eq./g, respectively (Table 3). The ratios of M-26, M-29, and M-29-glc increased with time, and the ratio of M-26 was the highest in 4 days. In rigid ryegrass, the ratio of pyroxasulfone was twice as high as that in wheat, and the total ratio as well as the concentration of the main metabolites was about one-third of that in wheat 1 day after treatment.

Discussion

^{14}C-Pyroxasulfone was metabolized in both wheat and rigid ryegrass seedlings, and M-26, M-29 and M-29-glc were identified as its main metabolites. Although M-15 (a glutathione conjugate of the isoxazoline ring) was not detected in this study, these metabolites were assumed to be formed *via* the glutathione conju-

gation. Glutathione conjugation of pesticides by various types of glutathione *S*-transferases (GSTs) has been previously reported.[14–18] Earlier studies showed that the glutathione conjugate of pesticides in plants is generally catabolized to the cysteine conjugate by liberating glycine and glutamic acid from glutathione moiety.[19,20] Additionally, the selectivity of chloroacetamides, which are VLCFAE-inhibiting herbicides like pyroxasulfone, was attributed to the high activity of GSTs in crops.[21–23] In our preliminary metabolism study of pyroxasulfone, a glutamyl-cysteine conjugate of the isoxazoline ring, the metabolite in which glycine was liberated from glutathione of M-15, was detected as a molecular ion (positive, *m*/*z* 348) by LC-MS in wheat (data not shown) as shown in several compounds.[19,24] Accordingly, in the case of pyroxasulfone, M-15 was presumed to be formed initially, subsequently liberating glycine and glutamic acid to form M-26. Furthermore, oxidative deamination[19,20] of M-26 led to M-29, and M-29-glc was consequently formed by the glucose conjugation of M-29. These metabolic processes indicated that there was no difference in the main route of pyroxasulfone metabolism between wheat and rigid ryegrass (Fig. 3). Therefore, the cleavage of the methylenesulfonyl linkage by glutathione conjugation of the isoxazoline ring plays a significant role in the detoxification of pyroxasulfone in plants.

The ratio of pyroxasulfone and its metabolites in wheat and rigid ryegrass 1 and 2 days after the treatment, showed a difference in its decomposition rate. In wheat, the residual ratio of the parent compound was lower and the ratio of its main metabolites was higher than in rigid ryegrass, and rapid conversion to M-29-glc was observed. It is suggested that there are differences in the physiological activities toward the metabolism of pyroxasulfone.

In this study, we compared the pyroxasulfone metabolism in a tolerant crop, wheat and a susceptible plant, rigid ryegrass at a seedling leaf stage of 3 to 4. Consequently, the difference in the metabolism activity was assumed to be one of the factors in determining the selectivity of pyroxasulfone, in addition to the difference in the inhibition of target enzymes, VLCFAEs.[13] Further clarification of the mechanism of the selectivity of pyroxasulfone can be obtained by examination of its *in vitro* conjugation activities in plants and identifying the molecular species of plant GSTs possessing similar conjugation activities.

Acknowledgements

We are grateful to Prof. Stephen B. Powles of the University of Western Australia for a kindly gifting wheat and rigid ryegrass seeds and to KI Chemical Research Institute Co., Ltd. for supplying authentic compounds.

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