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Original Article

Mode of Growth Inhibitory Activity and Metabolism of Imazosulfuron in Excised Roots of Plants

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Growth inhibition activity of imazosulfuron, 1-(2-chloroimidazo[1, 2-*a*]pyridin-3-ylsulfonyl)-3-(4, 6-dimethoxypyrimidin-2-yl)urea, against excised pea and soybean roots was investigated. Fifty percents growth inhibition concentrations of imazosulfuron (I_{50}) against pea and soybean roots were 17.6 and 54.2 ppb, respectively. When the excised pea roots were incubated in the culture medium containing 25 ppb of imazosulfuron and 1 mM of either branched-chain amino acids or intermediates of their biosynthetic pathways, the growth inhibition activity of excised roots was alleviated by the combination of isoleucine and valine or α-ketoisovalerate and α-keto-β-methyl-*n*-valerate. These findings suggest that imazosulfuron exhibits inhibition of acetolactate synthase which catalyses the biosynthetic pathways of branched-chain amino acids. Metabolic fate of imazosulfuron in excised pea roots was studied using the ^{14}C-labeled compounds. Imazosulfuron was predominantly demethylated to afford a monodemethyl derivative (HMS) in the roots, and hydrolytically cleaved at the sulfonylurea bond to give a sulfonamide (IPSN) and an aminopyrimidine (ADPM) in the culture medium. Since HMS did not inhibit the growth of excised roots, metabolic fate of imazosulfuron is involved in a selectivity between plants.

INTRODUCTION

Imazosulfuron, 1-(2-chloroimidazo[1,2-*a*]pyridin-3-ylsulfonyl)-3-(4,6-dimethoxypyrimidin-2-yl)urea, is a novel sulfonylurea herbicide developed by Takeda Chemical Industries, Ltd. Imazosulfuron exhibits a potent herbicidal activity against annual and perennial weeds especially *Cyperus serotinus, Cyperus difformis, Sagittaria pygmaea* and *Scirpus juncoides* at low application rate of 9 g/10 a in paddy field.

It has been reported that growth of the excised root of plants in sterilized culture medium is significantly inhibited by addition of sulfonylurea herbicides,[1–4] and measurement of decrease in the root growth length provides a quantitative bioassay for evaluating effects of the herbicides on plants.

This paper deals with the effect of imazosulfuron on growth of excised pea and soybean roots, and metabolism study of imazosulfuron in excised pea root using ^{14}C-labeled compounds.

MATERIALS AND METHODS

1. Chemicals

[Imidazopyridine-3-^{14}C]imazosulfuron ([imi-^{14}C]imazo) and [pyrimidine-5-^{14}C]imazosulfuron ([pyr-^{14}C]-imazo) were synthesized from [2-^{14}C]monochloroacetate and [2-^{14}C]diethylmalonate, respectively, by Nemoto & Co., Ltd., Japan (Fig. 1). The specific radioactivities of [imi-^{14}C]- and [pyr-^{14}C]imazo were 2.061 GBq/mmol and 2.057 GBq/mmol, respectively, and radiochemical purities were more than 99% by HPLC analysis.

The following authentic compounds were prepared in our laboratories: imazosulfuron, 1-(2-chloroimidazo[1, 2-*a*]pyridin-3-ylsulfonyl)-3-(4-hydroxy-6-methoxypyrimidin-2-yl)urea (HMS) and 2-chloroimidazo[1,2-*a*]pyridine-3-sulfonamide (IPSN). 2-Amino-4, 6-dimethoxypyrimidine (ADPM) was prepared by Tateyama Kasei Co., Ltd., Japan. α-Ketoisovalerate (KIV) and α-keto-β-methyl-*n*-valerate (KMV) were purchased from Tokyo Kasei Kogyo Co., Ltd., Japan. Isoleucine (Ile), leucine (Leu), valine (Val), α-ketobutyrate (KBA), pyruvate (PRV) and all other chemicals were purchased from

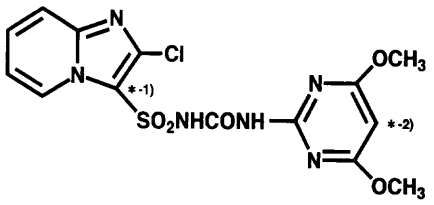

Fig. 1 Chemical structure of imazosulfuron.
*: ^{14}C-labeled position. 1) [imi-^{14}C], 2) [pyr-^{14}C].

Wako Pure Chemical Industries, Ltd., Japan.

2. Culture Solution

A Murashige-Skoog (MS) culture medium[5] supplemented with 20 g/*l* of sucrose[6] was used for root growth inhibition study. The pH of the medium was adjusted to 5.5 prior to autoclaving. Test solutions were prepared with the MS medium at the concentration of 5, 10, 25, 50 and 100 ppb for imazosulfuron, and 50, 200 and 1000 ppb for HMS. Mixture solutions of either 1 mM of Ile, Leu and/or Val or 1 mM of KBA, KIV, KMV, and/or PRV which are intermediates of the Ile-Val pathways in the presence of 25 ppb imazosulfuron were prepared with the MS medium to examine the alleviation effect of root growth inhibition. Each of mixture solutions prepared was filtrated using a MILLEX®-GS (Nihon Millipore Ltd., Japan). The sterilized solution obtained by filtration was incubated after addition of excised roots of the plants. All procedures were conducted under sterilized conditions.

3. Growth Inhibition Study

The seeds of pea (*Pisum sativum* L. cv. Usui) and soybean (*Glycine max* Merr. cv. Okuhara Wase) were sterilized by soaking in 5% NaOCl solution twice for 5 min followed by rinsing with sterilized water twice for 3 min. The sterile seeds were germinated on an absorbent cotton wetted with 10 ml of MS medium in a Petri dish (ϕ 9.0 cm), and incubated at 25°C for 48 to 72 hr in the dark. The seed roots were cut 10 mm long including the root tip with a razor in sterilized water. Ten excised roots were incubated in the MS medium (50 ml) in a 200-ml flask containing imazosulfuron, Ile, Leu, Val, KBA, PRV, KIV and/or KMV at 25°C for 72 hr in the dark with gentle gyratory shaking (60 rpm). Then the roots were sampled to determine the average of net growth length and standard deviations of the ten excised roots. The I_{50} values for inhibition which show 50% growth inhibition activity of the excised roots are defined for imazosulfuron concentration. The following Dixon's equation was employed to calculate I_{50};

$$I_{50} = [\text{imazo}]/(100/\text{Act} - 1),$$

where [imazo] and Act mean imazosulfuron concentration and percent activity, respectively.

4. Metabolism Study

Metabolism study of imazosulfuron in excised pea roots was conducted using ^{14}C-labeled compounds. Ten pea roots were incubated with 50 ml of the MS medium in a 200-ml flask containing either 25 ppb [imi-^{14}C]- or [pyr-^{14}C]imazo at 25°C for 72 hr in the dark. After incubation, the roots and culture medium were collected separately and the roots were homogenized in 30 ml of 50% acetonitrile aqueous solution using a Bio-Mixer (BM-1, Nihonseiki Co., Ltd., Japan). After centrifuging at 2000×*g* for 4 min, the resultant residue was resuspended in 30 ml of 50% acetonitrile aqueous solution. The suspension was homogenized and centrifuged as described above. The resultant residue was resuspended in 30 ml of acetonitrile and the suspension was homogenized and centrifuged in the same manner as above. The combined supernatant, residue and the culture medium were radioassayed. The supernatant and the culture medium were used for identification of the metabolites of imazosulfuron by thin-layer chromatograthy (TLC).

5. Radioassay

Radioactivities in the culture solution, supernatant extracts and silica gel regions on TLC plate were quantified by a liquid scintillation counter (LSC, LS6000TA, Beckman Instruments, Inc., U.S.A.) with scintillation cocktail A [12 *l* toluene, 1, 4-bis(2-methylstyryl)benzene (bis-MSB) 12 g, 2, 5-diphenyloxazole (DPO) 15 g, 5.16 *l* nonion-NS 210 (Nihon Yushi Co., Ltd., Japan)]. The counting efficiency was corrected by the external standard method and expressed as dpm. The residue of the extracts was combusted to analyse ^{14}C-carbon dioxide with an automatic sample combustion system (ASC-113, Aloka Co., Ltd., Japan).

6. Thin-layer Chromatography (TLC)

Precoated silica gel 60 F_{254} chromatoplate (20 cm×20 cm, 0.25 mm thickness, E. Merck, F. R. G) was used for analysis of the metabolites. The solvent systems used in TLC were as follows; A, ethyl acetate-methanol (4 : 1, v/v); B, chloroform-methanol (9 : 1, v/v); C, ethyl acetate-methanol-acetic acid (8 : 1 : 1, v/v/v). The *Rf*

Table 1 Authentic compounds and their *Rf* values on thin layer chromatography.

Compound	*Rf* value		
	A	B	C
Imazosulfuron	0.60	0.67	0.89
ADPM	0.82	0.76	0.90
IPSN	0.82	0.53	0.85
HMS	0.44	0.04	0.54

Solvent system: A, ethyl acetate-methanol (4 : 1, v/v); B, chloroform-methanol (9 : 1, v/v); C, ethyl acetate-methanol-acetic acid (8 : 1 : 1, v/v/v).

values for the authentic standards are listed in Table 1. The metabolites in the extract of plants and the culture solution were identified by two-dimensional TLC co-chromatography with authentic standards followed by TLC-autoradiography detected by a Bioimaging analyzer (FUJIX BAS2000, Fuji Photo Film Co., Ltd., Japan).

RESULTS AND DISCUSSION

As shown in Table 2, imazosulfuron strongly inhibited the growth of excised pea and soybean roots. As imazosulfuron concentrations in the medium increased from 5 or 10 to 100 ppb, the growth rates of excised pea and soybean roots decreased from 82 to 28% and from 70 to 37%, respectively, and the I_{50} values for pea and soybean roots were 17.6 and 54.2 ppb, respectively.

Ray showed that chlorsulfuron inhibited the excised pea root growth at the concentration as low as 10 ppb.[1] Takeda *et al.* reported that the growth of excised pea roots in the presence of 30 ppb of bensulfuron methyl was inhibited 80% in comparison with the control.[4] Our results presented here suggested that the root growth inhibition of imazosulfuron was almost in the same level as those of the other sulfonylurea herbicides.

Many studies on the mode of action of sulfonylurea herbicides have been made to define the site of action of the herbicides in plants.[1-4,7,8] The biochemical mechanism of plant growth inhibition by the herbicides was explained by LaRossa & Schloss[8] who demonstrated that sulfometuron methyl inhibited acetolactate synthase (ALS) from *Salmonella typhimurium*. These findings had been extended to plant tissue using excised pea root culture.[1] Ray[1] and Takeda *et al.*[4] showed that ALS prepared from a variety of plants were inhibited by sulfonylurea herbicides. Imazosulfuron, according to Tanaka *et al.*,[9] also inhibited ALS from rice plant which was resistant to imazosulfuron and such weeds as *Cyperus serotinus, Echinochloa oryzicola* and *Sagittaria pygmaea*, which were susceptible to imazosulfuron, with the I_{50} values of 5.8, 8.3, 10.7 and 23.9 ppb, respectively.

In order to supplement the data prepared by Tanaka *et al.*,[9] the effect of the branched-chain amino acids or the intermediates involving their biosynthetic pathways on growth of excised pea roots were investigated. As shown in Table 3, an addition of a combination of 1 mM of Ile and Val or a combination of 1 mM of Ile, Leu and Val into the medium containing 25 ppb of imazosulfuron significantly protected (108 and 96%) from root growth inhibition by imazosulfuron compared with the root growth in the medium containing imazosulfuron alone (37%). However, the growth inhibition of the roots by imazosulfuron was not alleviated (38 to 44%) by the addition of either Ile, Leu or Val alone into the medium. A mixture of 1 mM of KBA and PRV slightly alleviated the root growth inhibition (73%) in the presence of 25 ppb of imazosulfuron. On the other hand, mixture of the penultimate intermediates such as KIV and KMV, provided a significant protection (112%) on root growth inhibition as shown in Table 4.

Accordingly, it is obvious that imazosulfuron exerts its

Table 2 The effect of imazosulfuron or HMS on the growth of excised roots.

Plant	Compound	(ppb)	Average net[a] root length (mm)	% of root[b] growth
Pea	Control	0	9.0±1.3	100
	Imazosulfuron	5	7.4±1.0	82
		10	5.0±1.9	56
		25	3.8±1.6	42
		50	2.8±0.7	31
		100	2.5±1.5	28
	HMS[c]	50	9.8±1.4	102
		200	9.2±1.3	109
		1000	9.6±1.4	106
Soybean	Control	0	20.4±3.4	100
	Imazosulfuron	10	14.2±1.2	70
		50	8.6±0.8	42
		100	7.6±0.8	37
	HMS	50	20.6±1.0	101
		200	21.2±1.2	104
		1000	21.8±1.5	107

[a] Values are the mean±S.D. of ten roots tested.
[b] Percent indicates the relative root growth when compared with the control.
[c] HMS : 1-(2-chloroimidazo[1, 2-*a*]pyridin-3-ylsulfonyl)-3-(4-hydroxy-6-methoxypyrimidin-2-yl)urea.

Table 3 The effect of the branched-chain amino acids on the growth of excised pea roots in the presence of imazosulfuron.

Imazosulfuron (ppb)	Amino acid[a]	Average net[b] root length (mm)	% of root[c] growth
0 (Control)	None	16.2±1.3	100
25	None	6.0±1.1	37
25	Ile	6.1±0.7	38
25	Leu	7.0±0.4	43
25	Val	7.1±0.3	44
25	Ile, Leu	7.1±0.3	44
25	Ile, Val	17.5±0.5	108
25	Leu, Val	6.9±0.3	43
25	Ile, Leu, Val	15.6±3.0	96

[a] The concentration of amino acid was 1 mM each.
[b] Values are the mean±S.D. of ten roots tested.
[c] Percent indicates the relative root growth when compared with the control.

Table 4 The effect of intermediates of the Ile-Val pathways on the growth of excised pea roots in the presence of imazosulfuron.

Imazosulfuron (ppb)	Intermediate	Average net[a] root length (mm)	% of root[b] growth
0 (Control)	0	16.8±0.7	100
25	0	7.2±0.4	43
25	KBA+PRV[c]	12.3±1.2	73
25	KIV+KMV	18.8±1.1	112

[a] Values are the mean±S.D. of ten roots tested.
[b] Percent indicates the relative root growth when compared with the control.
[c] KBA: α-ketobutyrate, KIV: α-ketoisovalerate, KMV: α-keto-β-methyl-*n*-valerate, PRV: pyruvate.
The concentration of intermediate was 1 mM each.

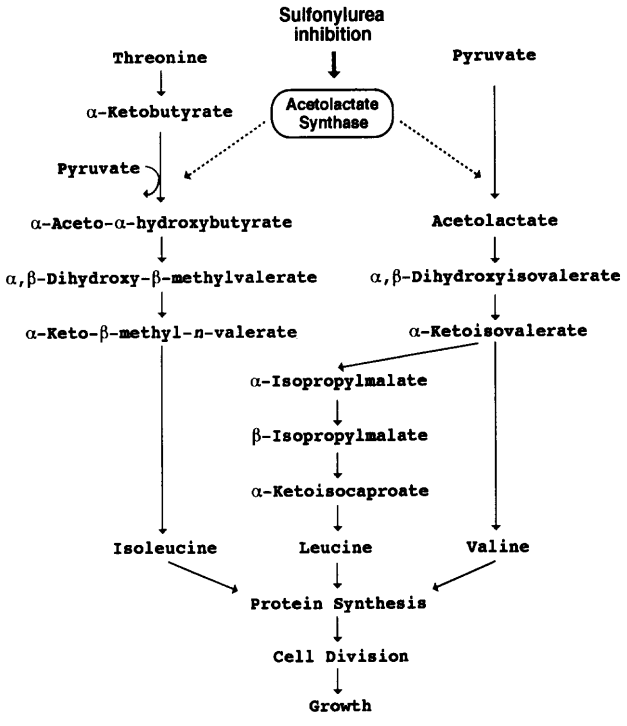

Fig. 2 Biosynthetic pathways of isoleucine, leucine and valine.

herbicidal activity by inhibiting the biosynthesis of the branched-chain amino acids such as Ile and Val in plant (Fig. 2). This finding supports the above-mentioned results which were reported by Tanaka *et al.*[9]

Study on metabolism in the excised pea roots was conducted using [imi-^{14}C]- and [pyr-^{14}C]imazo. The results of identification of the metabolite are summarized in Table 5. In the roots, HMS, a monodemethylated derivative of imazosulfuron, was identified as the major metabolite by TLC cochromatography with the authentic standard. In the culture medium, IPSN and ADPM given by the hydrolytic cleavage of sulfonylurea bond of imazosulfuron were detected as the major metabolites. Proposed metabolic pathways of imazosulfuron in pea roots are shown in Fig. 3.

The growth inhibition activity of HMS, a demethylated major metabolite of imazosulfuron, on excised pea and soybean roots was investigated. HMS showed no growth inhibition activity against these roots, even at the concentration of 1000 ppb (Table 2). Thus it was considered unlikely that HMS exhibited inhibition activity on root growth.

Table 5 Percents of radioactive compounds extracted from excised pea roots treated with [imi-^{14}C] or [pyr-^{14}C]imazosulfuron (imazo).

Metabolite	% of ^{14}C treated			
	[imi-^{14}C]imazo		[pyr-^{14}C]imazo	
	In root	In culture	In root	In culture
1. ADPM	—	—	ND	33.5 (36.3)
2. Imazosulfuron	0.8 (45.3)	66.6 (68.0)	1.5 (43.8)	50.1 (54.2)
3. IPSN	ND	31.3 (32.0)	—	—
4. HMS	0.7 (36.4)	ND	1.5 (44.6)	8.8 (9.5)
5. Unknown	0.1 (7.4)	ND	ND	ND
Unextractable ^{14}C	0.2 (10.9)	—	0.4 (11.6)	—
Subtotal	1.8 (100)	97.9 (100)	3.4 (100)	92.4 (100)
Total	99.7		95.8	

The data in the parenthesis are expressed as percents of ^{14}C in the root or the culture medium. —: not analyzed, ND: not detected.

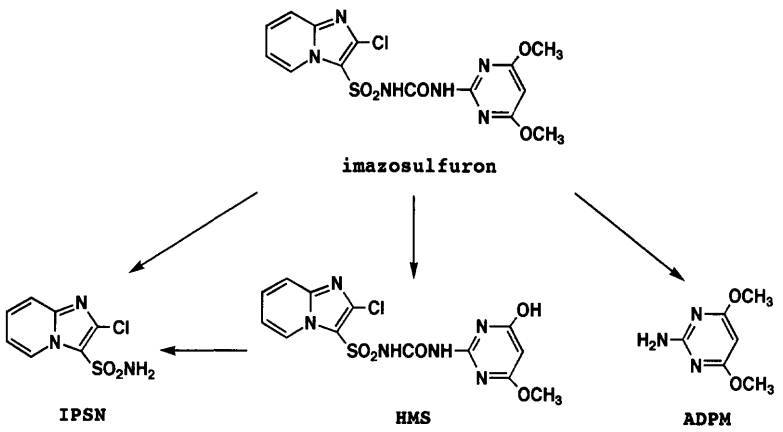

Fig. 3 Proposed metabolic pathways of imazosulfuron in pea roots.

ALS sensitivity to imazosulfuron of susceptible and resistant plants, which was reported by Tanaka *et al.*,[9] does not appear to account for the selectivity between these plants. Kamizono *et al.*[10] has revealed that HMS is the major metabolite in excised leaves of rice plant and *C. serotinus*, and the difference in metabolic rates of imazosulfuron to HMS between these plants is concerned in the selectivity. From the fact in the present paper, that HMS was the major metabolite of imazosulfuron in the roots, and that HMS showed no growth inhibition activity, the selectivity between susceptible and resistant plants was based on metabolic inactivation as has been reported for other sulfonylurea herbicides.[4]

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要　　約

イマゾスルフロンの植物切断根における生長抑制作用機構および代謝

清水直史，坂本順子，神園博史
太田一成，田代茂喜

イマゾスルフロンのエンドウおよびダイズ切断根に対する伸長阻害活性を調べた．本化合物の50%伸長阻害濃度はそれぞれ17.6 ppbおよび54.2 ppbと求められた．濃度25 ppbのイマゾスルフロンを含む培養液中に分岐鎖アミノ酸，あるいはそれらの生合成経路における中間体（各濃度1 mM）を加えて，エンドウの切断根を培養した．イソロイシンおよびバリンあるいはα-ケトイソ吉草酸およびα-ケト-β-メチル-n-吉草酸の組合せにより，切断根の伸長抑制作用が軽減されたことから，イマゾスルフロンは分岐鎖ア

ミノ酸の生合成を触媒するアセト乳酸合成酵素を阻害することが示唆された．イマゾスルフロンの^{14}C-標識化合物を用いて，エンドウ切断根中における代謝分解性を検討した．イマゾスルフロンは根部中においては優先的に脱メチル化反応を受けてモノ脱メチル体（HMS）を，培養液中ではスルホニル尿素結合が加水分解を受けてスルホンアミド体（IPSN）およびアミノピリミジン体（ADPM）を与えた．HMS は切断根伸長を阻害しなかったことから，イマゾスルフロンの植物における選択性は，その代謝分解性に起因すると推察された．