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Response and Acetolactate Synthase Activity in Different Rice Cultivars(*Oryza sativa* L.) to Cinosulfuron

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Cinosulfuron에 내한 벼 품종간의 생육반응과 Acetolactate Synthase 활성에 미친 영향

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ABSTRACT

Acetolactate synthase(ALS) activity was determined in germinating seedlings of two rice cultivars treated with cinosulfuron [3-(4,6-dimethoxy-1,3,5-triazin-2-yl)-1-[2-methoxyethoxy)-phenylsulfonyl]-urea]. IR 74(Indica type) was more tolerant than Hwajinbyeo(Japonica type) under various rates of cinosulfuron applied at the pregermination stage. *In vitro* response of ALS activity in the two rice cultivars was similar to I_{50} values(cinosulfuron concentration required for 50% inhibition of ALS activity) of about 23ppb. *In vivo,* ALS activity of IR 74 increased as the seedlings grew, but that of Hwajinbyeo dropped at 5 days after 10ppm cinosulfuron treatment and shoot growth of Hwajinbyeo lagged at 4 to 5 days after herbicide treatment. ALS activity and shoot growth of Hwajinbyeo was resumed from cinosulfuron-induced inhibition at 6 days after cinosulfuron treatment. The differential response of ALS activity in two different rice cultivars against cinosulfuron may not be due to difference of ALS sensitivity, but rather due to different metabolic inactivation rates of cinosulfuron.

Key words : Cinosulfuron, Acetolactate synthase, Rice cultivar

INTRODUCTION

The sulfonylurea herbicides are known to inhibit the activity of acetolactate synthase(ALS), a key enzyme in the biosynthesis of branched chain amino acids (BAAs), valine, isoleucine and leucine[11,19]. Chlorsulfuron, one of the sulfonylurea herbicides, selectively inhibits the cell cycle in root tips without apparently affecting any other metabolic process. It was suggested that chlorsulfuron inhibits cell cycle progression by blocking the G_2 into mitosis and G_1 into S phase through inhibition of cell cycle specific RNA synthesis[14]. However, the inhibitory effect of cell cycle can be blocked or reversed by adding BAAs to the

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culture medium. Further, Rost et al.[13] proposed that the plant cell cycle progression was not blocked by the reduction of BAAs pool in itself, but a toxic intermediate, such as α-amino-n-butyrate[12], α-ketobutyrate[6], or some other intermediates probably might inhibit the cell cycle specific protein, and thereby plant cell division and growth would be inhibited. Accumulation of α-ketobutyrate caused by inhibition of the progression of the BAAs biosynthesis partially mediated the herbicidal activity of ALS inhibitors[6].

The selective action of sulfonylurea herbicides between crops and weed plants can be attributed to the rapid metabolism of the herbicides to inactive products in crop species[16]. On the other hand, their resistance was assumed to involve the reduction of ALS sensitivity[15]. Exceptionally annual ryegrass(*Lolium rigidum*) have a wheat-like detoxification system[3]. The resistance mechanism of mutants induced is based on one or two base pair substitution of ALS gene resulting in various forms of less sensitive ALS enzyme[7].

Cinosulfuron [3-(4,6-dimethoxy-1,3,5-triazin-2-yl)-1-[2-methoxyethoxy)-phenylsulfonyl]-urea] is a sulfonylurea herbicide used for the control of broadleaf weeds and annual and perennial sedges in rice[10], a slight phytotoxicity in rice caused by cinosulfuron can be safed by dymron [1-(α,α-dimethylbenzyl)-3-p-tolylurea] application, showing a safening effect[2].

This study was conducted to determine effect of cinosulfuron on the growth response and ALS activity of two rice cultivars, and effect of BAAs on cinosulfuron inhibition.

MATERIALS AND METHODS

Rice cultivars, Hwajinbyeo was provided by the Kyungpook Provincial Rural Development Administration and IR 74 from IRRI. These cultivars were chosen as the plant materials because they have shown differential response to cinosulfuron in our preliminary test. The technical grade of cinosulfuron was provided by Kyungnong Corperation Ltd.. Cinosulfuron was dissolved in dimethyl sulfoxide and diluted with distilled water to the desired concentrations. The concentration of dimethyl sulfoxide was maintained within 0.2% in solutions.

Effect of Cinosulfuron on the Growth of Rice Seedlings.

Seeds of two rice cultivars were imbibed at 30℃ for 20h in the dark and pregerminated in the incubator maintained at 30℃ in the dark. Pregerminated seeds were transferred into plastic Petri dishes containing 10ml of various concentrations of cinosulfuron and maintained at 30℃ in the dark. Growth of shoot and root was determined at 7 days after herbicide application. The experiment was conducted three times with two replications. The data presented are the means of all experiments. The herbicide concentrations(GR_{50} values) at which 50% of plant growth was inhibited as compared to the untreated control were calculated from the data by plotting them on the log normal paper, determining where the graph intersected the 50% line.

Effect of Valine, Leucine and Isoleucine on Cinosulfuron Inhibition.

Pregerminated seeds were placed in Petri dishes containing 10ppm cinosulfuron without or with 1mM each of valine, leucine and isoleucine and Petri dishes were maintained at 30℃ in the dark. The seedlings were harvested and separated into shoot and root after 7-day incubation and dry weights of each part were measured after drying at 70℃ for 3 days. This test was conducted three times with two replications.

Acetolactate Synthase Activities of Two Rice Cultivars Affected by Cinosulfuron.

Two cultivars were grown on the condition as discribed in experiment I. Shoot tissues were harvested at the 3, 4, 5, 7 days after herbicide application for *in vivo* ALS activity. For *in vitro* ALS activity, the shoot of 4-day old seedlings grown in the herbicide-free solution was harvested. ALS was extracted as described by Ray[11] with some modifications. Tissue samples were harvested and washed with distilled water, blotted dry with tissue paper, and frozen in liquid nitrogen and ground with a pestle and mortar. Two volumes of extraction buffer containing 100mM potassium phosphate, pH7.5, 1mM sodium pyruvate, 0.5mM $MgCl_2$, 0.5mM thiamine pyrophosphate, 10μM FAD and 10%(v/v) glycerol were added and homogenized the extract. The homogenate was filtered through a fine nylon mesh and centrifuged at 15,000rpm for 15min at 4℃. The supernatant was brought to 50% saturation with $(NH_4)_2SO_4$ and allowed to stand for 1h on ice. The enzyme collected by centrifugation was dissolved in 100mM potassium phosphate buffer (pH7.5) containing 20mM sodium pyruvate and 0.5mM $MgCl_2$, and desalted on Sephadex G-25 column(Pharmacia PD-10) equilibrated with the same buffer. The desalted enzyme was used immediately for assays.

ALS activity was assayed as previously outlined by Singh et al.[18]. The reaction solutions(2mL) contained the enzyme with 50mM potassium phosphate buffer(pH7.5) containing 100mM sodium pyruvate, 10mM $MgCl_2$, 1mM thiamine pyrophosphate, 10μM FAD, and the indicated concentrations of cinosulfuron for *in vitro* ALS activity. This reaction solution was incubated at 37℃ for 1h. The reaction was stopped by the addition of 100μℓ 6N H_2SO_4 and allowed to decarboxylate at 60℃ for 15min. The decarboxylated acetoin was quantified by the method of Westerfeld[21] Each of 1%(w/v) creatine and 10%(w/v) α-naphthol(freshly prepared in 6 N NaOH) by 0.51ml was added to the acidified reaction solution and the solution was incubated at 60℃ for 15min. The absorbance of the solution was measured at 525nm. Protein was determined according to the method of Bradford[1] using bovine serum albumin as the standard. Each assay was run in duplicate and the experiments were repeated three times.

RESULTS AND DISCUSSION

Growth of Rice Seedlings Affected by Cinosulfuron.

The shoot and seminal root growth of Hwajinbyeo was more inhibited by cinosulfuron treatment in pregerminated seeds for 7 days at the all rates than those of IR 74(Fig. 1). The concentrations required for 50% inhibition of the shoot growth, as compared to the untreated control, were about 6ppm for Hwajinbyeo and above 100ppm for IR 74, respectively. Fifty % inhibition of the seminal root elongation(GR_{50} values) was made at 0.5ppm for Hwajinbyeo and 10ppm for IR 74. However, in terms of dry weight of two rice cultivars, degree of growth inhibition of Hwajinbyeo was much greater than that of IR 74. Three ppm cinosulfuron inhibited 50% of shoot growth of Hwajinbyeo, but IR 74 was inhibited only 40% at 100ppm cinosulfuron. The similar trend of rice cultivar response to sulfonylurea herbicides was confirmed by other researchers[9,23]. Yuyama et al[23] suggested that Japonica type rice cultivars were generally more sensitive to bensulfuron methyl than rice cultivars of Indica type in field test. Ohno et al.[9] also reported the similar observation, and the differential response among the different ecogeographic races resulted from the difference of translocation, degradation in roots and metabolic inactivation of bensulfuron methyl.

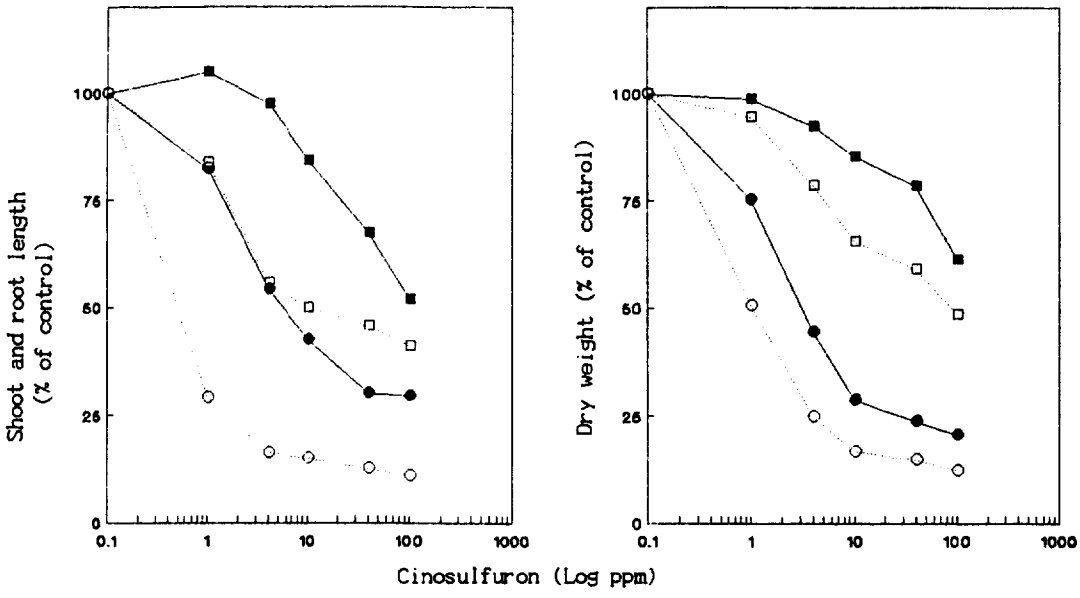

Fig. 1. The effect of cinosulfuron on the growth of the rice cultivars. The cinosulfuron was treated with various concentrations at germinated seeds. Shoot and root elongation (A)and dry weight (B)were measured after 7d growth.
■-■ ; shoot of IR 74, □···□ ; root of IR 74, ●-● ; shoot of Hwajinbyeo,
○···○ ; root of Hwajinbyeo.

Effect of Valine, Leucine and Isoleucine on Cinosulfuron Inhibition.

To determine the protective effect of BAAs on growth inhibition induced by cinosulfuron, 1mM (final concentration) of Val, Ile, and Leu, respectively, was added to the 10ppm cinosulfuron solution. The shoot and root growth of Hwajinbyeo treated with 10ppm cinosulfuron in the absence of amino acids supplementation became 28.7% and 16.8% of the untreated control, whereas those of IR 74 applied with 10ppm cinosulfuron without amino acids supplementation were 85.2% and 65.3% of herbicide-free cultured plant(Table 1). Ten ppm cinosulfuron containing the BAAs increased the shoot and root growth of Hwajinbyeo from 28.7% to 96.9% and 16.8% to 60.9% of the untreated one, while there was very small recovery in the shoot and root growth of IR 74, from 85.2% to 95% and 65.3% to 73.3%, as compared to herbicide-free treatment. Addition of 1mM Val, Ile and Leu to 10ppm cinosulfuron

Table 1. Protective effect of Val, Leu and Ile on cinosulfuron inhibition of the growth of rice seedlings.

Cultivars		Cinosulfuron concentration(ppm)	Amino acids[a)] 0mM	Amino acids[a)] 1mM
			····· *mg/plant* ·····	
Hwajinbyeo	Shoot	0	2.59(100)[b)]	2.42(93.4)
		10	0.74(28.7)	2.51(96.9)
	Root	0	1.45(100)	1.04(71.7)
		10	0.24(16.8)	0.88(60.9)
IR 74	Shoot	0	3.77(100)	3.76(99.7)
		10	3.21(85.2)	3.46(95.0)
	Root	0	1.76(100)	1.54(87.5)
		10	1.15(65.3)	1.29(73.3)

a) Amino acids such as Val, Leu and Ile were spontaneously treated with 10ppm cinosulfuron to germinated seed.

b) The values are given as the average dry weight of the two parts, the % of control are presented in parenthesis.

solution showed the marked recovery of the shoot growth inhibition by cinosulfuron in two rice cultivars, showing about 3.38-fold in Hwajinbyeo. However, root growth was partially recovered by the addition of BAAs.

Ray[11] indirectly accounted for the site of action of chlorsulfuron in pea by the study of supplementation of amino acids where supplementation of Val and Ile was able to reverse the growth Inhibition of pea root and seedlings caused by chlorsulfuron. The protective effect of BAAs supplementation on rice suspension-cultured cells treated with bensulfuron methyl was also observed by Sengnil et al.[17]. The fact that supplementation of Val, Ile and Leu ruled out cinosulfuron-induced growth inhibition indicates that the primary target site of cinosulfuron might be ALS.

Acetolactate Synthase Activity of Two Rice Cultivars Affected by Cinosulfuron.

The extractable levels of ALS from 4-day old seedlings of Hwajinbyeo and IR 74 were 5.7μM and 6.4μM acetoin/mg protein/h, respectively. The sensitivities of ALS to cinosulfuron under this assay condition were similar to I_{50} values(herbicide concentration required for 50% inhibition of ALS activity) of about 23ppb(Fig. 2). The shoot growth of IR 74 in the presence of 10ppm cinosulfuron showed a linear growth type, and after 7-day exposure the average shoot length of IR 74 was 84% of the untreated control. The extractable level of ALS from IR 74 grown in the presence of 10ppm cinosulfuron increased as the shoot grew (Fig. 3). The shoot growth of Hwajinbyeo lagged at 3 to 5 days after treatment with 10ppm cinosulfuron. After 7-day exposure to cinosulfuron, the average shoot length of Hwajinbyeo was 42.6% of the untreated control, showing 57.4% inhibition. The extractable level of ALS from Hwajinbyeo dropped at 5 days after cinosulfuron application (Fig. 4). The reduction of ALS activity at this

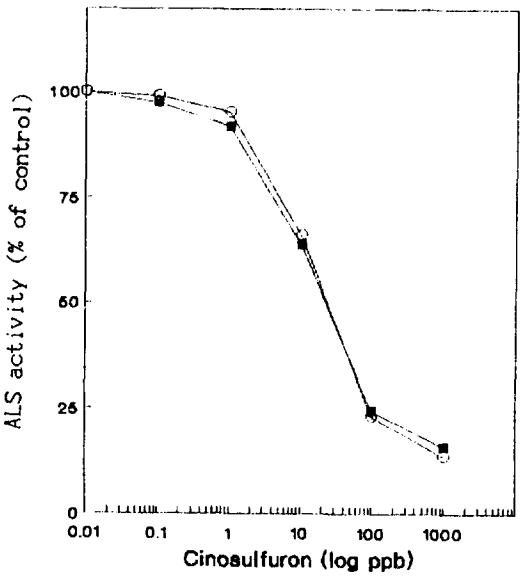

Fig. 2. In vitro effects of cinosulfuron on ALS activity from 4 days-old seedlings of IR 74 (○) and Hwajinbyeo (■).

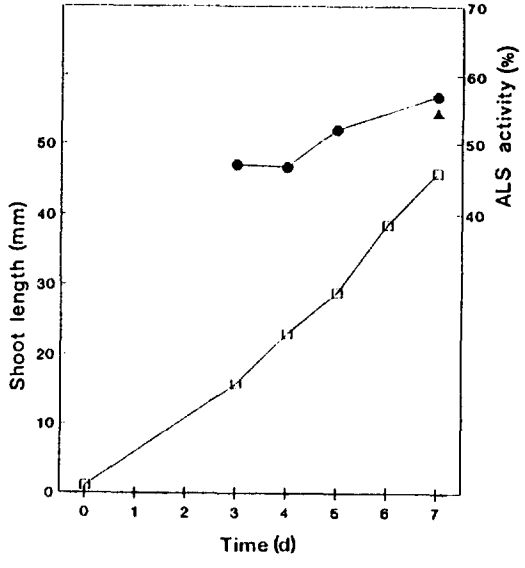

Fig. 3. Shoot growth (□-□) and ALS activity (●-●) of IR 74 as affected by 10ppm cinosulfuron. ▲ : Shoot length of untreated control.

stage may be related to retardation of the shoot growth of Hwajinbyeo. At 5 days after cinosulfuron exposure, ALS activity started to recover,

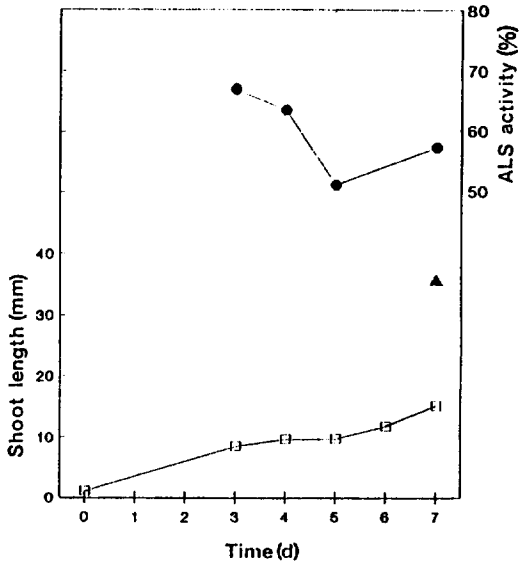

Fig. 4. Shoot growth (□ - □) and ALS activity (●-●) of Hwajinbyeo as affected by 10ppm cinosulfuron ▲ ; Shoot length of untreated control.

and the shoot growth of Hwajinbyeo increased simultaneously.

Very similar trend of ALS activities in the two rice cultivars against various concentrations of cinosulfuron was observed from the test of *in vitro* enzyme activity. From these results, it is unlikely that higher tolerance of IR 74 to cinosulfuron may be due to the cinosulfuron-insensitive ALS. Further it was suggested that sulfonylurea herbicide selectivity between crops and weeds may be caused by rapid metabolic inactivation in crop plant[16] and varied tolerance among crop cultivars might be resulted from difference in translocation and metabolism of herbicide[8]. In this study, the difference of *in vivo* ALS activity between Hwajinbyeo and IR 74 may be induced by differential metabolic rate, which resulted in difference of herbicide rates reached to the site of action and thus it may cause differential response between two cultivars.

In terms of rice seedling growth, based on GR_{50} values of shoot dry weight, IR 74 showed about 33-fold more tolerant than that of Hwajinbyeo(Fig. 1). The fact that cinosulfuron toxicity was reversed by supplementation of BAAs indicates that ALS may be the site of cinosulfuron action(Table 1). However, it is assumed that differential response of two rice seedlings to cinosulfuron may not be due to sensitivity of target site(Fig. 2). When two rice cultivars were grown in cinosulfuron solution, ALS activity of IR 74 increased as the shoot grew, showing a linear type growth(Fig. 3). However, ALS activity of Hwajinbyeo decreased at 5 days after cinosulfuron application and again increased at 6 days after treatment, and at the same time very slow shoot growth was observed during 3 to 5 days after herbicide application(Fig. 4). Takeda et al.[20] suggested that Indica type rice was more tolerant than Japonica type to bensulfuron methyl, one of the sulfonylurea herbicides because of the more rapid herbicidal inactivation in tolerant plant. If a herbicide is rapidly detoxified, the amount of active herbicide translocated to the target site will be reduced. Forlani et al.[4] proposed that naturally occurring differences in ALS levels in the roots of corn inbred lines contributed largely to the differential *in vivo* response affected by chlorsulfuron treatment. Although IR 74 was less inhibited by cinosulfuron and it had higher ALS content, there is no correlation between seedling growth and ALS content which can support tolerance of rice cultivars used in this study.

Inherent rice cultivar tolerance to bensulfuron methyl[8], one of sulfonylurea herbicides, resulted from the rapid metabolic inactivation, O-demethylation of the pyrimidine ring of bensulfuron methyl into herbicidally inactive 4-hydroxy-6-methoxy-pyrimidinyl derivative. Yuyama et al.[22] indicated that bensulfuron methyl was rapidly metabolised by shoot but not by root. However, it is known that rice ALS is very sensitive to bensulfuron methyl as ALS of bensulfuron methyl

sensitive weeds[23], and the similar results were also reported by other sulfonylurea herbicide like pyrazosulfuron ethyl[5].

In this experiment, cinosulfuron was treated at pregerminated seed stage in which nutrients including amino acids can be translocated from seed to the developing shoot. Thus the inhibition of the biosynthesis of BAAs by cinosulfuron may not probably be the major cause of the retardation of rice seedling growth. It is assumed that different response of rice cultivars to cinosulfuron observed in this study will partially depend on other factors rather than ALS properties, such as the rate of herbicide metabolism in the plant cell, and the levels of enzymes capable of inactivating herbicide although they were not studied here.

摘 要

제초제 cinosulfuron에 대해서 감수성이 각각 다른 두 품종을 선발해서 반응차이의 원인을 비교 검정하였다. 벼 어린식물체의 반응시험에서는 Indica type인 IR 74가 Japonica type인 화진벼보다 cinosulfuron에 대해서 높은 저항성을 보였다. 두 품종에서 추출한 ALS 효소의 활성은 cinosulfuron에 대해 비슷한 억제작용을 보였다. 10ppm cinosulfuron 처리에 대한 두 벼 품종간의 생육반응과 효소활성의 비교에서는 ALS효소 활성과 생육이 일치하는 경향을 보였다. IR 74의 효소활성과 생육은 시간이 지남에 따라 증가하는 경향이 있었으나, 화진벼에 있어서는 제초제 처리 후 5일째에 효소활성이 떨어졌으며 이시기에 유묘의 생육도 상당히 억제 되었다.

두 벼 품종의 ALS효소는 cinosulfuron에 대해서 비슷한 감수성을 지닌 것으로 나타났으며 cinosulfuron을 처리한 유식물로부터 추출 가능한 효소의 양에서 차이가 나는 것으로 보아 다른 요인 즉 대사속도의 차이에 의해서 두 벼 품종의 생육반응이 다르게 나타난 것이 아닌가 사료된다.

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