ORIGINAL ARTICLE / RESIDUE and SAFETY

Differential herbicide response of sulfonylurea-resistant *Monochoria vagnalis* accessions to sulfonylurea herbicides

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Abstract : Four sulfonylurea(SU)-resistant *Monochoria vaginalis*(*M. vaginalis*) accessions were tested for levels of resistance to four SU herbicides which have been widely using in paddy fields of Korea, based on whole plant response and sensitivity of the target enzyme, acetolactate synthase(ALS). The resistant Naju, Nonsan and Gimje accessions were not affected to the survival by treatment with recommended dose of all SU herbicides tested. The GR_{50} values for the Naju, Nonsan and Gimje accessions were 8- to 33-fold, 8- to 30-fold and 7- to 32-fold higher to recommended doses of all SU herbicides tested than the susceptible Cheongdo accession, respectively. However, the GR_{50} values for Kimhae accession displayed an intermediate response and was only 4-to 13-fold more resistant than the susceptible accession. The ALS I_{50} values for the Naju, Nonsan and Gimje accessions were 25- to 66-fold, 9- to 26-fold and 10- to 24-fold higher to recommended doses of all SU herbicides tested than the susceptible Cheongdo accession, respectively. However, the I_{50} value for Kimhae accession was 4- to 9-fold more resistant than the susceptible accession, as determined by I_{50} values of ALS.

Key words : ALS, *Monochoria vaginalis*, Resistance, sulfonylurea, herbicide

INTRODUCTION

According to Dyer *et al.*, (1993) and Malik *et al.*, (1996), the development of herbicide-resistant weeds was first observed in 1960. Since the first report of this resistance, various herbicide-resistant weeds have been reported including resistance to herbicides with different modes of action(Carol, 1994; Cavan & Mosss, 1997; Park *et al.*, 1999; Wang *et al.*, 2002). In recent decades, the most significant development was the discovery of SU-herbicides, which requires very low-rate with extremely low mammalian toxicity and kills plants by inhibiting the enzyme ALS that catalyses the first step in branched amino acid biosynthesis(Donald, 1992; Gressel *et al.*, 1990; Hensley, 1997; Van *et al.*, 1992).

Since the introduction of first SU herbicide in the early 1980s, chlorsulfuron, SU herbicides have been widely used in major cereal-growing areas to control or suppress broad leaf weeds and sedges. With the repeated use of the same SU-based herbicides, several weed species have developed resistance to SU-herbicides(Heap, 2007). The first reported case of a SU-resistance weed was that of prickly lettuce(*Lactuca serriola*) in 1987 followed by kochia (*Kochia scoparia*), common chickweed (*Stellaria media*), and Russian thistle (*Salsola iberica*). However, most of these species have been identified through continuous use of chlorsulfuron in wheat, soybean and corn fields of America, Europe and Australia.(Heap, 1997; Hensley, 1981))

More recently, the widespread and diverse SU-resistance problem has found in rice field of Korea and Japan, where SU herbicides such as bensulfuron-methyl

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and pyrazosulfuron-ethyl have been used continuously since 1990's(Itoh et al., 1999; Kohara et al., 1999; Park et al., 2007; Yoshida et al., 1999). SU-resistant weeds in seven annual and three perennial weeds as of 2007 have confirmed in rice field of Korea(Park, 2007)

Of these species, Monochoria vaginalis accounts for the cast majority of resistant sites. The SU-resistant M. vaginalis has been mainly identified in the western and southern areas practicing extensively in flooded directed-seeded rice, however, many farmer complaints to its control effect by SU herbicides have been provoking throughout Korea. Due to its ability to high germination rate, high seed

production and seed dispersal systems, M. vaginalis is considered a major weed pest in rice field of Korea. As of 2006, above 42,000 ha SU-resistant M. vaginalis had been documented.

The mechanism of SU-resistance in M. vaginalis is due to an altered form of ALS which is no longer inhibited by the herbicide(Park et al., 2007). The specific lesion has further been identified as an amino acid substitution at position 197, from proline to serine in Domain A resulting from a point mutation in the gene encoding ALS. While the mechanism of SU-resistance in one M. vaginalis accession was shown to be due to an insensitive ALS(Park, 2002), the physiological basis for differential response to ALS-inhibiting herbicides among resistant M. vaginalis populations is unknown.

The purposes of our research were to conduct a comprehensive analysis of the sensitivity of four resistant M. vaginalis accessions to several SU herbicides at the whole plant and target enzyme levels, and investigate to management of SU-resistant M. vaginalis to several alternative herbicides that do not inhibit ALS activity.

MATERIALS AND METHODS

Plant materials

In the fall of 2002, M. vaginalis seeds were collected from about 20 plants in rice fields of Naju, Nonsan, Gimje, Kimhae and Cheongdo. The fields of Naju, Nonsan, Gimje, Kimhae had been treated with SU-included herbicides for a least 8 consecutive years

before seed collection while the Cheongdo accession was from an untreated site. Seeds were stored at $2^{\circ}C$ until use. For greenhouse studies, the seeds of 5 accessions were planted 0.5mm in a growth chamber(E-15, Conviron's, Canada) maintained at $30\pm2^{\circ}C$ and 75% relative humidity, and 12 hrs photoperiod (dark/light). Twenty germinated seeds of each accession having plumule of 5mm in length were transplanted to a plastic pot(15 cm×15 cm×12 cm) containing clay loam paddy soil and maintained the water level to 5 cm depth.

Herbicide treatment

The herbicides were treated at 10 days after transplantation. The herbicidal response was determined from fresh weight against M. vaginalis treated with 1X and 10X the normal field use rates. Herbicides used (and 1X field rates in parentheses) to each accession of M. vaginalis were bensulfuron-methyl(51 g ha^{-1}), ethoxysulfuron(21 g ha^{-1}), imazosulfuron(75 g ha^{-1}), and pyrazosulfuron-ethyl(21 g ha^{-1}) formulated granular. At 20 days after herbicide treatment, the herbicidal responses by fresh weight were determined. All treatments for each measurement were triplicated. The experiment was conducted twice using a randomized complete block design with triplications. Data were subjected to analysis of variance and pooled over both experimental repetitions since there were no between-experiment interactions. Means of the five accessions within herbicide rate combinations were analyzed separately using the LSD test (P ≤ 0.05). Data are reported as percent reduction in biomass compared to the untreated controls of each accession.

ALS Extraction and Assay

Seeds of the susceptible and resistant biotype were planted in pots and placed in a growth chamber as described above. ALS was extracted from the shoots of 20-day-old seedlings(10 to 13 cm tall with 4 to 5 leaves) which were grown under the growth chamber (E-15, Conviron's, Canada) maintained at $25^{\circ}C$. The seedlings were homogenized in 3 volumes of buffer containing 0.1 M K_2HPO_4, pH 7.5, 1 mM sodium pyruvate, 0.5 mM $MgCl_2$, 0.5 mM thiamine-

pyrophosphate, 10 μM FAD and 10% v/v glycerol. The homogenate was filtered through 8 layers of cheesecloth and centrifuged at 27,000 xg for 20 min. ALS was precipitated from the supernatant fluid with $(NH4)_2SO_4$. The enzyme was collected at 25 to 50% saturation by centrifugation and the pellet dissolved in buffer containing 0.1 M K_2HPO_4, pH 7.5, 20 mM pyruvate, and 0.5 mM $MgCl_2$ and desalted on small column of Sephadex G-25 (Pharmacia PD-10) equilibrated with the same buffer. The desalted enzyme was used immediately for assays.

ALS assays were carried out in a final volume of 0.5 mL at 30°C. The final reaction mixture contained 20 mM K_2HPO_4, 20 mM sodium pyruvate, 0.5 mM thiamine-pyrophosphate, 0.5 mM $MgCl_2$, and 10 μM FAD and, its pH was adjusted to be 7.0. Then, by dissolving a range of amounts of pyrazosulfuron-ethyl in the mixtures, 0, 0.0001, 0.001, 0.01, 0.1, 1, 10 μM concentrations were prepared. Assays were prepared as described above to avoid the introduction of organic solvents into the reaction mix. Assays were initiated by adding enzyme (100 μL) and terminated by adding 50 μL of 6 N H_2OS_4. ALS was determined as described by Westerfield(1945) with the follow-ing modifications. The acidified reaction mixtures were heated for 15 min at 60°C after which 0.5 mL of 0.5°C w/v creatine was added. Next, 0.5 mL of 5% w/v α-naphthol, freshly prepared in 2.5 N NaOH, was added and the solutions were heated for an additional 15 min at 60°C . The absorbances of the solutions were then determined at 525 nm by using a spectrophotometer(UV4, Unicam, USA). Protein was determined by the method of Lowry *et al.* (1951). The I_{50} value for inhibition is defined as the concentration of herbicides which inhibits ALS activity by 50%.

ALS activity data are presented as percent of untreated control activities for each accession. Regression equations of these data were used to calculate I_{50} values as described by Ray(1984). The I_{50} is defined as the herbicide concentration needed to inhibit ALS specific activity by 50%. Approximate standard errors(SE) associated with I_{50} values were calculated as described using

$$SE(I_{50}) = SE(y/I_{50})[b_1 + 2(b_2)I_{50}]^{-1}$$

where y=herbicide concentration, b_1=linear coefficient, and b_2=quadratic coefficient. Data are reported as averages of two experiments conducted with different plant material, with two determinations from enzyme extracts in each experiment.

RESULTS AND DISCUSSION

The response of five accessions to treatment with four herbicides is shown in Table 1. Fresh weights(GR_{50}) of the Naju, Nonsan and Gimje accessions were not affected by at the normal field use rate of SU herbicides applied, and reduced by about from 30% to 40% to untreated plant fresh weight even at 10X the normal field use rate of all herbicides. In contrast, Cheongdo accession was much more sensitive to the other resistant accessions: fresh weight was reduced by more than 90% by these treatments. The Kimhae accession was less resistant than the Naju, Nonsan and Gimje accessions to all sulfonylurea herbicides tested, fresh weight ranged from 39% to 58% to untreated plant fresh weight at 1X herbicide doses, indicating an intermediate level of resistance. Thus, the Kimhae accession displayed a low and reasonably uniform level of resistance to the SU-herbicides tested. And the resistant degree among Naju, Nonsan and Gimje accessions was not distinct difference to the herbicides tested. At the 1X herbicide doses, fresh weights of the susceptible Cheongdo accession ranged from 3% (pyrazosulfuron-ethyl) to 12% (ethoxysulfuron) of untreated plant fresh weight.

To compare whole plant response of *M. vaginalis* accessions with in vitro ALS sensitivity, plant extracts were assayed for ALS activity in the presence of the four SU herbicides tested(Fig. 1). ALS activity form the resistant Naju, Nonsan, and Gimje accessions was highly resistant to SU herbicides and was not completely inhibited even at the 10uM concentration. As seen from the whole plant experiments, ALS from the Kimhae accession displayed an intermediate level of sensitivity to SU herbicides tested and was only slightly less sensitive than the susceptible accession, while ALS from the Cheongdo accession was very sensitive to SU herbicides inhibition. Regression equations of data in

Table 1. Fresh weights(GR50) expressed as a percent of control of Naju, Nonsan, Gimje, Kimhae and Cheongdo *Monochoria vaginalis* accessions 20 days after treatment with four sulfonylurea herbicides, bensufuron-methyl(BSM), ethoxysulfuron, imazosulfuron and pyrazosulfuron-ethyl(PSE) at 1X and 10X field application rates

Accession	Fresh weight as affected by herbicide							
	BSM		Ethoxysulfuron		Imazosulfuron		PSE	
	1X[a]	10X	1X	10X	1X	10X	1X	10X
	percent of control							
Naju	105	75	98	71	107	68	100	75
Nonsan	98	62	95	61	98	57	91	65
Gimje	97	68	91	65	100	61	98	69
Kimhae	48	20	42	17	45	18	39	18
Cheongdo	9	0	12	0	5	0	3	0
LSD(0.05)	4.8	5.9	4.7	6.2	4.7	4.4	4.5	6.8

[a]1X application rates in rice field of are 51, 21, 75, and 21 g ha^{-1} for bensufuron-methyl, ethoxysulfuron, imazosulfuron and pyrazosulfuron-ethyl, respectively.
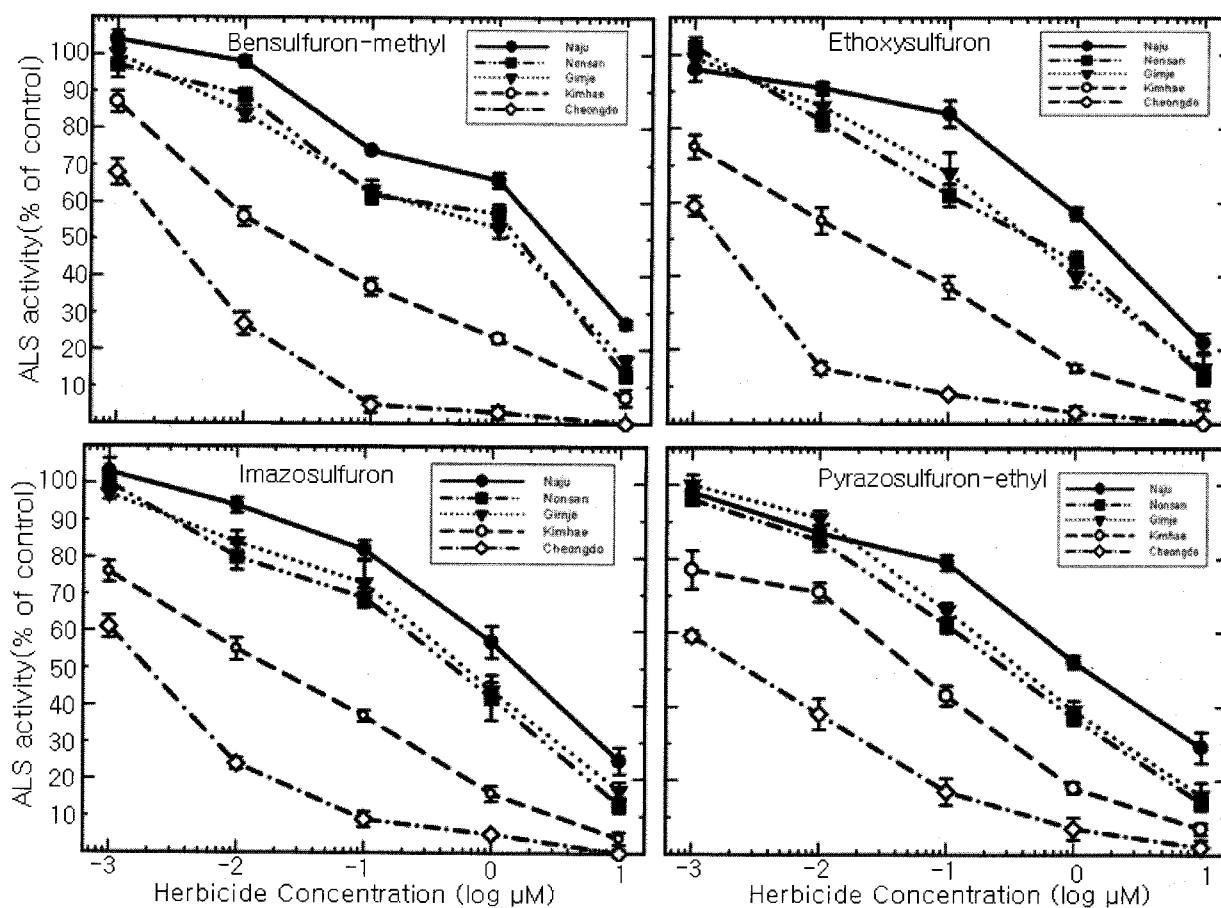

Fig. 1. Specific activity of ALS extracted from Naju, Nonsan, Gimje, Kimhae and Cheongdo *Monochoria vaginalis* accessions assayed in the presence of sulfonylurea herbicides. Vertical bars are SE of means.

Figure 1 were used to calculate I50 values for the four herbicides (Table 2). In all cases, the Naju accession displayed the highest level of resistance of the five accession. The I50 values for the Naju accession ranged from 150-fold (imazosulfuron) to 471-fold (bensulfuron-methyl) higher than the susceptible Cheongdo accession.

Table 2. The I_{50} values for four sulfonylurea herbicides of ALS extracted from Naju, Nonsan, Gimje, Kimhae and Cheongdo *Monochoria vaginalis* accessions

Accession	I_{50} values(nM)			
	BSM[a]	Ethoxysulfuron	Imazosulfuron	PSE
Naju	2,825(118)[b]	2,420(98)	1,348(74)	1,795(91)
Nonsan	1,097(200)	987(17)	724(14)	692(21)
Gimje	1,021(78)	972(28)	802(82)	726(60)
Kimhae	43(3.7)	57(5.1)	39(2.8)	73(3.4)
Cheongdo	6(0.4)	8(0.7)	9(0.6)	7(0.3)

[a]BSM : bensufuron-methyl, PSE : pyrazosulfuron-ethy.
[b]SE of I_{50} values in parentheses.

In addition, ALS from the Naju accession was generally 25- to 66-fold more resistant than enzyme form the Kimhae accession. The I_{50} values for the Kimhae accession were again intermediate, averaging about 7-fold greater than the Cheongdo accession.

These results demonstrate differential sensitivity of *M. vaginalis* accessions to SU herbicides at the whole plant and target enzyme levels, and resistant accessions of Naju, Nonsan and Gimje was high resistance to susceptible accession of Cheongdo. The resistant Naju *M. vaginalis* accession showed a higher degree of resistance than the other accessions to SU herbicides tested, a difference that may be due to the type of mutation in their respective ALS genes(Park et al., 2007). In another SU-resistant *M. vaginalis* accession, sequence analysis of a 401bp region within the ALS gene demonstrated the presence of a point mutation leading to an amino acid substitution at proline for serine 198 in Domain A of ALS(Park et al., 2007). Although we do not know which specific mutations occur in the resistant *M. vaginalis* accessions, apparently they confer significantly different levels of resistance to the SU herbicides. The intermediate response of the Kimhae accession is also of interest, since it may reflect a only low levels of SU resistance. Resistance level differences among these accessions were not due to altered amounts of the ALS enzyme, since ALS specific activity was similar in all cases to the response of whole plant by four SU herbicides tested. We do not believe that altered uptake, translocation, or metabolim contributed to the differences in whole plant resistance measured here, since these processes were not affected

in SU-resistant *M. korsakowii* which is the same family to *M. vaginalis*(Park et al., 2002). In any case, ALS sensitivity differences among the five accessions are sufficient to explain whole plant resistance differences. If herbicide resistance initially arises from scattered individual plants, then the gene flow (pollen and seed dispersal) characteristics of each species will influence the makeup and variability of resistant populations. Since resistance to ALS inhibitor appears to be inherited as a simple, partially dominant trait(Itoh et al., 1994; Gerwick, 1993), weed species may develop homogeneous resistant populations by outcrossing and high seed dispersal or variable lines within resistant populations by selfing and low seed dispersal. In the case of *M. vaginalis*, the accessions tested here indicate that resistance levels may be highly variable among populations. However, prediction of within-population variability is difficult, since *M. vaginalis* appears to be primarily self-pollinated unlikely *M. korsakowii*, and yet has an extremely efficient means of seed dispersal. A better understanding of this variability and the underlying genetic mechanism is clearly needed in order to develop more effective strategies to manage resistant weeds and prevent further out breaks.

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서로 다른 지역에서 채집된 Sulfonylurea계 제초제 저항성 물달개비의 제초제 반응 차이

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요약 : 4개의 서로 지역 계통별 sulfonylurea(SU)계 제초제 저항성 물달개비의 저항성 정도 차이를 구명하기 위하여 SU계 제초제들에 대한 식물체 및 acetolactate synthase(ALS) 반응 차이를 감수성 계통과 비교 분석하였다. 나주, 논산, 김제 지역에서 채집된 저항성 물달개비들은 SU계 제초제들의 기준량에서 거의 영향을 받지 않았다. 사용된 SU계 제초제들의 기준량에 대한 나주, 논산, 김제 지역계통의 건물중 50% 억제 제초제 농도인 GR_{50}은 감수성계통(청도) 보다 각각 8~33배, 8~30배, 7~32배 높게 나타났다. 그러나 김해 채집계통의 GR_{50}은 감수성 채집계통에 비해 4~13배 높게 나타나 중간정도의 저항성을 보였다. 나주, 논산, 김제에 대한 ALS 50% 억제 제초제 농도인 I_{50}은 감수성 채집계통에 비해 각각 25~66배, 9~26배, 10~24배 높게 나타났다. 그러나 김해 채집계통의 GR_{50}은 감수성 채집계통에 비해 4~9배 높게 나타났다.

색인어 : 물달개비(*Monochoria vaginalis*), 저항성, 제초제, acetolactate synthase(ALS), sulfonylurea