Biochemical Mechanism of Resistance to Imazapyr in Sugarcane Cell Selections

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Abstract

Selection of imazapyr-resistant sugarcane clones was evaluated at the whole-plant and cell levels. Field tolerance to imazapyr of 27 sugarcane clones was evaluated to categorize the degree of tolerance. The experiments were arranged in a split-plot in RCB with four dosages of imazapyr application (0, 0.156, 0.312 and 0.625 kg ai ha^{-1}) as main plots and 27 different sugarcane clones as sub plots. Sugarcane clones K95-282, K88-92, K97-32, K99-55, K95-87 and K93-219 showed relatively high tolerance to imazapyr at 0.625 kg ai ha^{-1} whereas sugarcane clones K99-5, K99-72, K99-85, K97-33 and K97-27 were susceptible to imazapyr at 0.156 kg ai ha^{-1}. Cell culture selection was attempted using callus and cell suspension culture induced from the tight young furled leaves of sugarcane clone K95-282. The callus and cell were cultured in liquid modified MS medium. A sugarcane cell line from K95-282 resistant to 1 μM imazapyr was obtained after 420 days of selection, using a stepwise selection with increasing concentrations of imazapyr from 0.1 to 1 μM. It was referred to as 1 μM imazapyr-resistant sugarcane cell line. This indicates that the resistant cells were 116.67 times more resistant to imazapyr than the normal cells. To establish the biochemical mechanism of resistance to imazapyr, acetolactate synthase (ALS) activity was determined in the normal and resistant cells. Based on the I_{50} values, ALS activity of the resistant cells was 6.50 times higher than that of the normal cells at various concentrations of imazapyr, from 0.01 to 100 μM. This performance suggests that the biochemical mechanism of imazapyr resistance in the sugarcane cell line appears to be an alteration at the target site, based on the ALS activity, leading to less sensitivity to imazapyr. The specific mutation(s) of the ALS gene that endows resistance in this cell line remains to be identified.

Keywords: acetolactate synthase (ALS), imazapyr, resistant-sugarcane cell line, sugarcane clone

Introduction

Sugarcane *(Saccharum* spp.) is a crop of major importance, providing about 65% of the sugar produced in the world. Relatively few pesticides are used for pest control in sugarcane crop production (Zambrano et al., 2003). In related prevention and management of weeds in sugarcane, however, herbicides are now used as the main method for weed control. Response of crop cultivars to herbicides depends on their inheritance within a plant species that enables the crop to escape the toxic effects of the herbicides. To develop a herbicide-tolerant crop, information is needed on the highest safe application rate of the herbicide, and how other weed control measures can be integrated for effective season-long weed control.

Imazapyr is a non-selective and a broad-spectrum imidazolinone herbicide. It controls many grass and broadleaf weeds in non-crop areas, and in plantation crops, such as rubber, oil palm and sugarcane (Cox, 1996; Osuna et al., 2003). This herbicide inhibits the activity of an enzyme, acetolactate synthase (ALS; EC 4.6.3.8), the first enzyme in the biosynthetic pathway to the branched-chain amino acids valine, leucine and isoleucine in plants, fungi and bacteria (Doggleby and Pang, 2000). ALS is nuclearly encoded, produced in the cytoplasm and transported via a transit peptide to the chloroplasts (Corbett and Tardif, 2006). Imidazolinone-tolerant plants with altered ALS genes and enzymes have been discovered in many crops (Rajasekaran et al., 1996; Wright and Penner, 1998a, 1998b). This makes it possible to develop imazapyr-tolerant sugarcane based on the resistance mechanism at the site of action.

ALS-inhibiting herbicides have become a major target of herbicide-tolerant crop plants development. A number of plants and cultured plant cells resistant to ALS-inhibiting herbicides have been generated, utilizing either conventional breeding, mutation breeding (or selection of somaclonal variants) or transgenic approaches (Sebastian et al., 1989; Swanson et al., 1989; Newhouse et al., 1991, 1992; Rajasekaran et al., 1996; Wright and Penner, 1998a, 1998b; Bae et al., 2002; Bailey and Wilcut, 2003). However, in recent years new herbicide-resistant crops have been developed using a variety of biotechnological approaches. Much current research focuses on strategies for developing and identifying the mechanisms of herbicide tolerance. Information is needed on the responses of more genotypes before a breeding program can be initiated for imazapyr tolerance in any particular region. Therefore, the objectives of the present study were to: (i) evaluate the differences in tolerance of 27 sugarcane clones to imazapyr at whole-plant level; (ii) select imazapyr-resistant sugarcane cell lines using *in vitro* mutant selection to generate herbicide-resistant crops; and (iii) determine the ALS activity in resistant and normal sugarcane cell lines to establish whether enhanced resistance was due to an alteration in the activity at the target site.

Materials and Methods

Field Selection of Imazapyr-Tolerant Sugarcane Clones

Field selection experiments were conducted at the Field Station of Central Research on Sugarcane, Phanom Thuan, Kanchanaburi, Thailand, in 2005. The experiments were arranged in a split-plot in RCB with four dosages of imazapyr application as main plots and 27 different sugarcane clones listed in Table 2 as sub plots, with three replications. Forty-five days after planting (at the 4-6 leaf stage), imazapyr was applied at 0, 0.156, 0.312 and 0.625 kg ai ha^{-1}. The herbicide was applied at a pressure of 2.1 kg cm^{-2} and spray volume of 500 L ha^{-1} with a laboratory sprayer having a 8004 flat fan T-jet. BASF (Thai) Limited generously provided a commercial formulation of imazapyr, which contains 12.3% w/v 2-(4-isopropyl-4-methy-5-oxo-2-imida-zolin-2-yl) nicotinic acid as a soluble liquid. The degree of tolerance was evaluated at 30, 45 and 60 days after application (DAA) by visually evaluating plants for chlorotic and necrotic symptoms, counting the number of stalks ha^{-1} and also by measuring plant height (from soil surface to the visible dewlap of the plant). Crop injury was visually scored on a scale of 0 to 100, with 0 indicating no effect and 100 indicating plant death. Herbicide concentrations causing 50% inhibition (I_{50}) and reduction of growth (GR_{50}) were also determined. Data were analyzed by analysis of variance (ANOVA), and means were separated using Duncan's multiple range test with $P = 0.01$.

Selection of Imazapyr-Resistant Sugarcane Cell Line

Selection of sugarcane cell line resistant to imazapyr was conducted through tissue culture by the stepwise selection method using callus and cell suspension culture derived from the tight young furled leaves of sugarcane clone K95-282. Calli were induced on modified MS medium (Murashige and Skoog, 1962) supplemented with 3 mL L^{-1} 2,4-D, 100 mg L^{-1} myo-inositol, 500 mg L^{-1} hydrolyzed casein, 2% sucrose, 7 g agar powder and 10% coconut wter (pH 5.7) at 28°C. After 4 weeks, calli

were transferred to fresh medium. To initiate cell suspensions, the 0.5 g calli were transferred to 45 mL of liquid MS medium in 125 mL Erlenmeyer flasks on a gyrating shaker (120 rpm) at 28°C. The liquid MS medium composition was the same as the callus inducing medium, except that agar powder was omitted. The technical-grade imazapyr (2-propanamine, 2-(4,5-dihydro-4-methyl-4-(1-methyl-ethyl)-5-oxo-1*H*-imidazol-2-yl)-3-pyridine-carboxylate; purity 98.8%), the isopropylamine salt, was provided by BASF (Thai) Limited. Imazapyr was dissolved in distilled water and filter-sterilized with a membrane filter and added to the medium to give final concentrations of 0, 0.0001, 0.001, 0.01, 0.1, 1.0, 10, 100 and 1,000 μM before the cells were sub-cultured. Cell growth response was determined by measuring packed cell volume (PCV) at 0, 5, 7, 10 and 14 days after treatment. At 2-week intervals, the cell suspensions were sub-cultured by transferring 5 mL of PCV cells to 45 mL of fresh medium. The suspension cells were treated with the lowest concentration of imazapyr. Sub-culturing was done at 2-week intervals by transferring the cell suspensions to fresh medium which maintained the same concentration of the herbicide. When the imazapyr-treated cells, growth rate was recovered to the same level as that of the normal cells (without imazapyr), they were transferred into the medium containing a higher concentration of imazapyr. Sub-culturing was repeated for several passages until highly resistant cells were obtained. Data were analyzed by analysis of variance (ANOVA), and means were separated using Duncan's multiple range test with P = 0.05. Parts of the data were presented as means and standard errors of the sample means.

Acetolactate Synthase Activity

Inhibition of acetolactate synthase activity by imazapyr was compared between the resistant and normal cells according to the modified procedures of Osuna et al. (2003). Both types of suspension-cultured cells were used seven days after subculture, during the linear phase of the culture growth cycle. Each 2 g sample of sugarcane cells was ground with an extraction buffer (3 mL g^{-1} cell) and polyvinyl-pyrrolidone (PVPP; 0.5 g). The extraction buffer contained 0.1 M potassium phosphate (KH_2PO_4 / K_2HPO_4), pH 7.5, 1 mM sodium pyruvate, 0.5 mM $MgCl_2$, 0.5 mM thiamine pyrophosphate, 10 μM flavin adenine dinucleotide (FAD), 12 mM dithio-threitol (DTT) and glycerol (1:9, v/v). The homogenate was filtered through four layers of cheesecloth and centrifuged (at 27,000 g for 15 min). The protein fraction including the ALS enzyme was precipitated from the crude extract at 50% saturation of $(NH_4)_2SO_4$. The pellet was resuspended in the extraction buffer (0.3 mL), and the resuspension was applied to a Sephadex G-25 PD-10 column, previously equilibrated with the elution buffer, pH 7.5 (0.1 M potassium phosphate (KH_2PO_4 / K_2HPO_4), pH 7.5, 20 mM sodium pyruvate, 0.5 mM $MgCl_2$). The desalted protein extract was immediately used for ALS enzyme activity assays.

ALS activity was assayed by adding 0.05 mL of enzyme extract to 0.1 mL of freshly prepared assay buffer [0.08 M potassium phosphate (KH_2PO_4 / K_2HPO_4), pH 7.5, 0.15 M sodium pyruvate, 1.5 mM $MgCl_2$, 160 μM FAD], and to various concentrations of technical-grade imazapyr at 0.01-100 μM. Dibasic potassium phosphate (0.04 M, pH 7.0) was added to achieve a final mixture volume of 0.25 mL. After mixture incubation (37°C for 1 h), the reaction was stopped by adding 0.05 mL of H_2SO_4 (3 M). The reaction tubes were then heated (15 min at 60°C) to facilitate decarboxylation of acetolactate to acetoin. Acetoin was detected as a coloured complex (A_{525} nm) formed after the addition of 0.25 mL of creatine (5 g L^{-1}), freshly prepared in water) and 0.25 mL of α-naphthol (50 g L^{-1}), freshly prepared in 2.5 M NaOH) and incubation (60°C for 15 min) (Westerfeld, 1945). Background was determined using control vials, in which the reaction was stopped before the incubation, and subtracted. Total protein content in crude ALS extracts was determined by the method of Bradford (1976) using bovine serum albumin for the standard curve. The level of resistance was defined by inhibitor concentration required for 50% inhibition of enzyme activity (I_{50}) and expressed as the resistance index. Concentrations of herbicides required for I_{50} of ALS activity were calculated from linear plots of inhibition percentages against the logarithm of concentrations. Three experiments were conducted per cell line, and each sample at each herbicide concentration was assayed in triplicate. Data were analyzed by analysis of variance (ANOVA), and means were separated

using Duncan's multiple range test with P = 0.05. Parts of the data were presented as means and standard errors of the sample means.

Results and Discussion

Field Selection of Imazapyr-Tolerant Sugarcane Clones

In this study, the reactions of locally-adapted sugarcane clones to imazapyr were evaluated at whole-plant level. Within a few hours after treatment with imazapyr, synthesis of DNA and cell division stops. Next, plant growth stops, complete death of the plant occurs slowly, taking as long as a month after treatment (Cox, 1996). The effects of imazapyr on the physiological response to crop visual injury, plant height, and number of stalks ha^{-1} were measured to categorize the degree of tolerance. At 45 DAA, analysis of variance showed significant differences between sugarcane clone and dosage at the P = 0.01 level (Table 1). Although the interaction between dosage and clone is not significant, there are significant of the yield component in each sugarcane clone presented in Table 2. For crop visual injury, K84-200, K88-92, K93-347, K95-156, K95-234, K95-282, K97-32, K99-55 and K99-61 had significantly higher tolerance to imazapyr than most of the other clones with K88-92 having the lowest mean visual injury score. Among the susceptible clones, K97-27, K97-33 and K99-5 had the highest mean visual injury score but were not significantly different from the rest of the susceptible clones. Relative plant height was significantly different among the 27 clones tested. While all of the clones showed definite stunting of growth compared to the control, K88-92, K92-80, K95-87, K95-156, K95-282, K95-283 and K97-29 had significantly higher relative plant height than the rest of the clones while K97-27, K97-33, K99-5, K99-49, K99-72 and K99-85 were significantly shorter. Imazapyr treatment significantly affected the relative number of stalks ha^{-1} which ranged from a low of 48.15% to as high as 80.83%. The clones with the lowest number of stalks ha^{-1} were K97-33, K99-72, K99-49, K97-29 and K97-27, respectively, while K95-282, K99-56, K93-347, K99-55, K88-92 and K97-32 were the highest. Table 3 shows the mean visual crop injury, relative plant height and relative number of stalks ha^{-1} averaged across 27 sugarcane clones at 45 DAA. Increasing concentration of imazapyr significantly resulted to an increase in visual injury and a decrease in relative plant height and relative number of stalks ha^{-1}. In addition, a similar response was also observed at 30 and 60 DAA. The main effects of dosage and genotype were significant in all traits at the P = 0.01 level, although the interaction between them was not significant (data not shown).

Table 1 Mean squares from the analysis of variance of 3 traits related to imazapyr resistance in 27 sugarcane clones determined at 45 days after application[1/].

Source of variation	df	Visual injury	Plant height (cm)	Number of stalk (stalk ha^{-1})
Block	2	246.15	702.61	2573.67
Herbicide Dosage	3	94894.96**	31673.56**	54256.85**
Error (a)	6	83.54	1290.34	1640.96
Sugarcane Clone	26	84.19**	208.57**	608.17**
Dosage x Clone	78	31.07 ns	99.12 ns	396.02 ns
Error (b)	208	27.90	42.13	370.81

[1/] ** Significantly different at the 0.01 probability level.
ns Non-significant (P > 0.01).

Table 2 Means of crop visual injury, plant height and number of stalks per hectare in 27 sugarcane clones averaged across 4 dosages of imazapyr determined at 45 days after application[1/].

Clone	Visual injury[2/]	Plant height[3/] (% of control)	Number of stalks[4/] (stalk ha^{-1})
K84-200	47.50 cde	72.14 bc	66.59 cd
K88-92	43.33 e	76.13 ab	75.49 ab
K92-80	49.17 bcd	74.06 abc	71.42 abc
K92-213	50.00 a-d	66.29 cde	65.72 cd
K93-219	50.83 a-d	72.95 bc	62.06 de
K93-347	48.33 bcd	70.77 bcd	76.64 ab
K95-84	50.00 a-d	68.28 cd	68.45 cd
K95-87	51.67 abc	74.49 abc	70.95 abc
K95-156	45.84 de	74.23 abc	70.41 abc
K95-161	50.83 a-d	68.76 cd	70.29 abc
K95-234	47.50 cde	66.52 cde	62.18 de
K95-282	49.17 de	81.51 a	80.83 a
K95-283	51.67 abc	78.41 ab	66.38 cd
K97-27	54.17 a	47.14 e	48.15 f
K97-29	49.17 bcd	75.74 ab	53.26 ef
K97-32	48.33 bcd	72.10 bc	73.16 abc
K97-33	54.17 a	46.30 e	56.49 ef
K99-5	54.17 a	39.29 f	60.12 de
K99-45	49.17 bcd	62.73 de	72.94 abc
K99-49	52.50 ab	44.08 ef	54.82 ef
K99-55	48.33 bcd	64.67 de	75.60 ab
K99-56	50.00 a-d	69.67 bcd	78.09 ab
K99-59	50.00 a-d	69.47 bcd	65.02 cd
K99-61	47.50 cde	60.85 de	71.39 abc
K99-72	51.67 abc	41.73 ef	56.33 ef
K99-82	52.50 ab	60.47 de	66.92 cd
K99-85	52.50 ab	41.42 ef	61.83 de
CV (%)	10.58	27.76	27.97

[1/] a-f means followed by the same letters in each trait are not significantly different at the 0.01 probability level by DMRT.
[2/] Crop injury rating on a scale of 0 = no injury to 100 = plant death.
[3/] Plant height = (plant height × 100)/plant height of the untreated control.
[4/] Number of stalks per hectare = (number of stalks × 100)/ number of stalks of the untreated control.

Table 3 Means of crop visual injury, plant height and number of stalks per hectare in 4 dosages of imazapyr averaged across 27 sugarcane clones determined at 45 days after application[1/].

Dosage (kg ai ha^{-1})	Visual injury[2/]	Plant height[3/] (% of control)	Number of stalks[4/] (stalk ha^{-1})
0	0 d	100 a	100 a
0.156	56.42 c	83.21 b	82.79 b
0.312	66.67 b	59.61 c	63.44 c
0.625	76.67 a	37.15 d	22.29 d
CV (%)	18.31	28.45	26.22

[1/] a-d means followed by the same letters in each trait are not significantly different at the 0.01 probability level by DMRT.
[2/] Crop injury rating on a scale of 0 = no injury to 100 = plant death.
[3/] Plant height = (plant height × 100)/plant height of the untreated control.
[4/] Number of stalks per hectare = (number of stalks × 100)/ number of stalks of the untreated control.

The field tolerance to imazapyr of 27 sugarcane clones was evaluated to categorize the degree of tolerance. Based on growth response of sugarcane clones to imazapyr traits, the I_{50} value of imazapyr on visual injury was over 0.312 kg ai ha^{-1} for the sugarcane tolerant clones, while the corresponding value was a little over 0.156 kg ai ha^{-1} for the susceptible clones. The susceptible clones were severely injured (injury rating greater than 65%) at 0.312 kg ai ha^{-1}, while the tolerant clones were severely injured at 0.625 kg ai ha^{-1}. Plant height and number of stalks ha^{-1} of the tolerant and susceptible clones were affected by the treatments to a similar extent, as was the degree of visual injury. Sugarcane clones K95-282, K88-92, K97-32, K99-55, K95-87 and K93-219 showed relatively high tolerance to imazapyr at 0.625 kg ai ha^{-1} whereas sugarcane clones K99-5, K99-72, K99-85, K97-33 and K97-27 were susceptible to imazapyr at 0.156 kg ai ha^{-1}. It is interesting that the response of crop cultivars to herbicides depends on their inheritance within a plant species, that enables the crop to escape the toxic effects of the herbicides. From this, the level of resistance can range from low susceptibility at recommended field rates of the herbicide to complete resistance. It is concluded from these results that sugarcane clone K95-282 is tolerant to herbicide representatives of imazapyr.

Selection of Imazapyr-Resistant Sugarcane Cell Line

The results showed that there were significant differences in the effect of imazapyr concentrations on the growth of normal and resistant cells. The inhibition of growth escalated as imazapyr concentration was increased. Growth was strongly inhibited at 1 μM imazapyr (Figure 1a). At first, the normal cells were cultured in liquid MS medium supplemented with 0.1 μM imazapyr. Their growth rate was found to be low at the early stage of the selection, and gradually reached the same rate as the normal cells, after 252 days (data not shown). In the next step, the cell suspensions were transferred to 1 μM imazapyr. The growth rate had recovered to the same level as that of the normal cells after 168 days. The selection of the imazapyr-resistant sugarcane cell line was finished at 1 μM imazapyr, because cells died at higher concentrations (Figure 1b). The sugarcane cell line resistant to 1 μM imazapyr was obtained after 420 days of selection. The normal cells attained the I_{50} value at 0.06 μM imazapyr and were dead at 7.00 μM. At this concentration, resistant cells were still able to grow normally. Based on the I_{50} values, the resistance index of resistant cells was 116.67-fold higher than that of the normal cells (Figure 2 and Table 4).

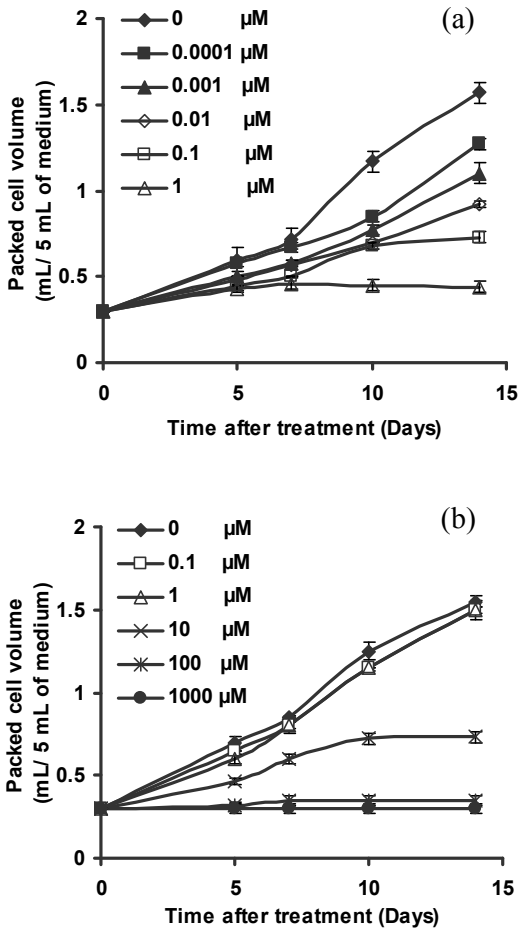

Figure 1 The effect of imazapyr on the growth response of normal (a) and resistant (b) sugarcane cell lines determined at 5, 7, 10 and 14 days after treatment at various concentration of herbicide. Points are the average of three replicates ± standard errors.

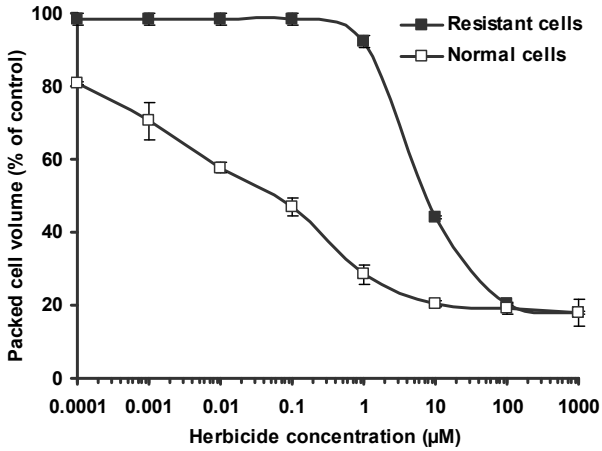

Figure 2 Growth response of normal (□) and resistant (■) sugarcane cells to imazapyr was determined 14 days after treatment. Points are the average of three replicates ± standard errors.

There have been many reports on herbicide resistant crops derived from tissue culture selection. Imidazolinone-resistant sugarbeet cells were obtained by somatic cell selections (Wright and Penner 1998a, 1998b). Soybean cells were screened for Protox-inhibiting herbicides resistance (Pornprom et al., 1994; Warabi et al., 2001). The selection of glufosinate resistant cells by the stepwise selection of herbicides was successful in soybean (Pornprom et al., 2000). Bae et al. (2002) selected rice (*Oryza sativa* L. cv. Ilpumbyeo) cells resistant to cyhalofop-butyl. Zambrano et al. (2003) reported *in vitro* selection of a glyphosate-tolerant sugarcane cellular line. It was suggested that cell suspension cultures should be frequently used to investigate herbicide target sites and their involvement in herbicide actions relating to resistance mechanisms. In addition, the target genes conferring resistance have been isolated and correlated with the biochemical mechanisms and/or molecular bases of resistance. In this study, the biochemical mechanism at the target site of imazapyr action was studied to elucidate the resistance mechanism of sugarcane cell selection. The results from this study could subsequently be attributed to the index of herbicide resistance.

Acetolactate Synthase Activity

ALS activity was measured to provide evidence about the biochemical mechanism of imazapyr-resistance in sugarcane cell selections. The ALS activity was assayed in both normal and resistant cells. Cells were assigned as herbicide-treated normal cells (NT), herbicide-free normal cells (NF), herbicide-treated resistant cells (RT), and herbicide-free resistant cells (RF). The results showed that the specific ALS activity in terms of acetoin production from both NF and RF were not different (data not shown). Since there was no significant difference in specific activity between the RF and NF, resistance was not due to an overproduction of ALS at the target site in the resistant cells.

However, when both types of cells were treated with 0.01-100 µM imazapyr, the total ALS activity in the herbicide-treated normal cells and the herbicide-treated resistant cells decreased, was significantly different. Based on the I_{50} values, the ALS activity in the resistant cells (RT; I_{50}=28.60 µM)

Table 4 I_{50} for imazapyr determined from the growth response and ALS activity of normal and resistant sugarcane cells.

Type of cells	Growth response	ALS activity
	I_{50}[1/] (---------- µM----------)	
Resistant cells	7.00 ± 0.03	28.60 ± 1.10
Normal cells	0.06 ± 0.04	4.40 ± 0.20
Resistance index[2/]	116.67	6.50

[1/] I_{50} ± standard error represents the average of three replications.
[2/] Resistance index = I_{50} value of resistant cells/ I_{50} value of normal cells.

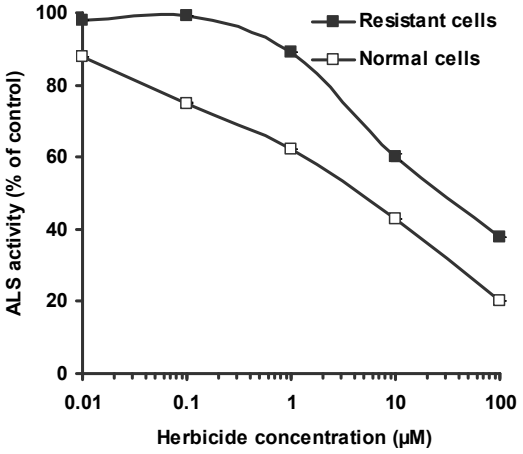

Figure 3 Inhibition of ALS activity in normal (□) and resistant (■) sugarcane cells by imazapyr. Points are the average of three replicates ± standard errors.

was higher than that of the normal cells (NT; I_{50} = 4.40 µM). The ALS activity of resistant cells was approximately 6.50-fold greater than that of normal cells at the enzyme level (Figure 3 and Table 4). This study revealed that ALS from the cells with target site-based resistance was less affected by imazapyr than that from normal cells. These data suggest that the higher activity of ALS in resistant cells was due to less sensitivity to imazapyr conferring the mechanism of resistance. These results were consistent with earlier reports on the mechanisms of ALS-inhibitors resistance in canola (Swanson et al., 1989), soybean (Sebastian et al., 1989), maize (Newhouse et al., 1991; Bailey and Wilcut, 2003), wheat (Newhouse et al., 1992), cotton (Rajasekaran et al., 1996), sugarbeet (Wright and Penner, 1998a, b), and rice (Bae et al., 2002). In most cases, ALS-inhibitor resistance has resulted from an altered ALS enzyme with reduced sensitivity to the herbicides. These results strongly support the hypothesis that herbicide resistance can be due to target site-based mechanisms and impart a reduced affinity for imazapyr.

Crop resistance to herbicides is typically conferred by one of three mechanisms: resistance at the site of action, metabolic detoxification and prevention of the herbicide from reaching the site of action (Tan et al., 2005). Developing one or more of these three mechanisms through chemical mutagens may provide herbicide resistance in plants. This indicates that plant cells (target sites of herbicide) may have inherently different mechanism (s) for herbicide resistance. From these results, the mechanism of resistance was apparently due to an alteration of the ALS at the target site showing the reduced sensitivity of ALS in imazapyr-resistant plant cells. Furthermore, the specific resistance endowing mutation of the ALS gene remains to be identified.

The development of ALS-tolerant sugarcane clones provides additional economical weed control options for sugarcane growers. The use of imazapyr in these tolerant varieties has been effective in controlling a wide spectrum of grass and broadleaf weeds with good crop safety. Currently, weed control in sugarcane often requires multiple herbicide applications or several cultivations or hand hoeings due, in part, to the wide spectrum of weeds and marginal crop tolerance to herbicides. Sugarcane clones tolerant to newer herbicides are potentially useful for future genotype development programs. Alternatively, it is highly possible that the resistance to imazapyr in the resistant cells selected from this study is stable and can be manifested in the calli-regenerated plants. These genetic resources should be conserved and studied further. Their role in the breeding of imazapyr-resistant sugarcane should be determined.

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References

Bae, C., L. Young-Ill, L. Yong-Pyo, S. Yong-Won, L. Do-Jin, Y. Deuk-Chum and L. Hyo-Yeon. 2002. Selection of herbicide tolerant cell lines from γ-ray-irradiated cell cultures in rice (*Oryza sativa* L. cv. Ilpumbyeo). J. Plant Biotechnol. 4:123-127.

Bailey, A.W. and W.J. Wilcut. 2003. Tolerance of imidazolinone-resistant corn (*Zea mays*) to diclosulam. Weed Technol. 17:60-64.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Corbett, C.L. and F.J. Tardif. 2006. Detection of resistance to acetolactate synthase inhibitors in weeds with emphasis on DNA-based techniques: a review. Pest Manag. Sci. 62:584-597.

Cox, C. 1996. Herbicide fact sheet imazapyr. J. Pesticide Reform. 16:16-20.

Duggleby, R.G. and S.S. Pang. 2000. Acetohydroxyacid synthase. J. Biochem. Mol. Biol. 33:1-36.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 18:100-127.

Newhouse, K.E., B.K. Singh, D.L. Shaner and M. Stidham. 1991. Mutations in corn (*Zea mays* L.) conferring resistance to imidazolinones. Theor. Appl. Genet. 83:65-70

Newhouse, K.E., W. Smith, M. Starrett, T. Schaefer and B.K. Singh. 1992. Tolerance to imidazolinone herbicides in wheat. Plant Physiol. 100:882-886.

Osuna, M.D., A.J. Fischer and R. De Prado. 2003. Herbcides resistance in *Aster squamatus* conferred by a less sensitive form of acetolactate synthase. Pest Manag. Sci. 59:1210-1216.

Pornprom, T., H. Matsumoto, K. Usui and K. Ishizuka. 1994. Characterization of oxyfluorfen tolerance in selected soybean cell line. Pestic. Biochem. Physiol. 50:107-114.

Pornprom, T., S. Surawattananon and P. Srinives. 2000. Ammonia accumulation as an index of glufosinate-tolerant soybean cell lines. Pestic. Biochem. Physiol. 68:102-106.

Rajasekaran, K., J.W. Grula and D.M. Anderson. 1996. Selection and characterization of mutant cotton (*Gossypium hirsutum* L.) cell lines resistant to sulfonylurea and imidazolinone herbicides. Plant Sci. 199:115-124.

Sebastian, S.A., M.G. Fader, F.J. Ulrich, R.D. Forney and S.R. Chaleff. 1989. Semidominant soybean mutation for resistance to sulfonylurea herbicides. Crop Sci. 29:1403-1408.

Swanson, E.B., M.J. Hergesell, M. Arnoldo, D.W. Sippell and R.S.C. Wong. 1989. Microspore mutagenesis and selection: Canola plants with field tolerance to imidazolinones. Theor. Appl. Genet. 78:525-530.

Tan, S., R.R. Evans, L.M. Dahmer, K.B. Singh and L.D. Shaner. 2005. Imidazolinone-tolerant crops: history, current status and future. Pest Manag. Sci. 61:246-257.

Warabi, E., K. Usui, Y. Tanaka and H. Matsumoto. 2001. Resistance of a soybean cell line to oxyfluorfen by overproduction of mitochondrial protoporphyrinogen oxidase. Pest Manag. Sci. 57:743-748.

Westerfeld, W.W. 1945. A colorimetric determination of blood acetoin. J. Biol. Chem. 161:495-502.

Wright, T. and D. Penner. 1998a. Cell selection and inheritance of imidazolinone resistance in sugar beet (*Beta vulgaris*). Theor. Appl. Genet. 96:612-620.

Wright, T. and D. Penner. 1998b. *In vitro* and whole-plant magnitude and cross-resistance characterization of two imidazolinone-resistant sugarbeet (*Beta vulgaris*) somatic cell selections. Weed Sci. 46:24-29.

Zambrano, A.Y., J.R. Demey and V. Gonzalez. 2003. *In vitro* selection of a glyphosate-tolerant sugarcane cellular line. Plant Mol. Biol. Rep. 21:365-373

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