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Resistance Mechanism to Bensulfuron-methyl in Biotypes of *Scirpus mucronatus* L. Collected in Chilean Rice Fields

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Two biotypes of *Scirpus mucronatus* not controlled with the herbicide bensulfuron-methyl in rice fields were characterized by using field, greenhouse, and laboratory techniques. Seeds were collected in two rice areas [Parral (R1) and Linares (R2)], where bensulfuron-methyl at 150 g ha^{-1} did not control *S. mucronatus*. A third seed sample of *S. mucronatus* susceptible (S) to bensulfuron-methyl was collected in an area from Chile. The dose−response studies confirmed resistance to bensulfuron-methyl in R1 and R2 *S. mucronatus* biotypes; ratios (R/S) of the ED_{50} values of resistant to susceptible plants were 1719 and 1627 for R1 and R2, respectively. The biotype R1 also showed strong cross-resistance (ratios ranging from 1719 to 43) to sulfonylureas (bensulfuron-methyl, cyclosulfamuron, ethoxysulfuron, imazosulfuron, and pyrazosulfuron-ethyl) and imidazolinone (imazamox) and a weak cross-resistance (ratio of 1.705) to pyrimidinyloxybenzoates (bispyribac-sodium), all ALS inhibiting herbicides used in rice. Absorption, translocation, and metabolism results did not explain the differences in susceptibility among biotypes. The in vitro assays confirmed cross-resistance to all ALS inhibitors tested and the level of cross resistance was bensulfuron-methyl > imazosulfuron ≫ cyclosulfamuron ≫ pyrazosulfuron-ethyl ≫ ethoxysulfuron > imazamox ≫ bispiribac-sodium. Molecular studies demonstrated that the Pro197His amino acid substitution on the ALS enzyme could explain the loss of affinity for the ALS-inhibiting herbicides.

KEYWORDS: *S. mucronatus*; bensulfuron-methyl; cross-resistance; ALS-inhibiting herbicides; amino acid substitution

INTRODUCTION

Rice is the largest crop in the world with 700 million t produced in 2005 and is the main source of carbohydrate consumed by humans in their diet (*1*). Rice yield can be reduced by 80% when weeds are left uncontrolled (*2*). However, even with weed control methods, escaped plants reduce rice yield by 35% (*3*).

Scirpus mucronatus (L.), also known as *Schoenoplectus mucronatus* (L.) Palla, belongs to the Cyperaceae plant family and is a major weed in rice crops in 43 countries of the world (*4*). This is a perennial species that reproduces by seeds, tubers, or stolons. Successful control of *S. mucronatus* in rice crop has been attained with acetolactate synthase (ALS; EC 4.1.3.18) inhibitor herbicides. ALS is the first enzyme that catalyzes the biosynthesis of the branched-chain amino acids, valine, leucine, and isoleucine. ALS inhibitors include herbicides from the chemical groups sulfonylurea (SU), imidazolinone, triazolopyrimidine sulfonanilide, sulfamoylurea, and pyrimidinylthiobenzoate (*5*−*7*).

Repeated use of ALS-inhibiting herbicides has selected weed-resistant biotypes. The first case of resistance was observed in *Lactuca serriola* only 5 years after the SU (chlorsulfuron) appeared on the market in 1982. Since then, there has been an increasing number of ALS-resistant species, being to date in 95 species (*8*). Three relevant mechanisms endowing herbicide resistance in weeds are lack to target enzyme sensitivity, increased herbicide metabolism, and impaired herbicide accumulation at the site of action (*6*, *9*−*11*). The main mechanism of resistance to ALS-inhibiting herbicides is an insensitive site of action caused by point mutation(s) in the ALS gene (*9*, *12*−*14*). This type of resistance, reported in the weed biotypes examined to date, has been found to be caused by a substitution in one of six conserved amino acids in the ALS enzyme: Ala122, Pro197, Asp376, Ala205, Trp574, or Ser653 (the amino acid number is standardized according to the *Arabidopsis thaliana* sequence) (*15*). Amino acid substitutions at Pro197 usually result in SU resistance but not IM resistance, whereas substitutions at Ala122 or Ser653 result in IM resistance but not SU resistance. Substitutions at Trp574 result in high levels of resistance (> 10-fold resistance) to both SUs and IMs. Substitutions at Ala205 also result in SU and IM resistance, but the levels of resistance to SUs are not high (< 10-fold resistance), and the levels of resistance to IMs are variable. Substitutions at Asp376 result in cross-resistance to five classes of ALS inhibitors (*15*). The high levels of resistance to SUs

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due to changes in the ALS enzyme are thought to be caused by substitutions at Pro197, Asp376, or Trp574, according to the cross-resistance patterns described above.

Sulfonylurea-resistant *S. mucronatus* appeared in rice fields located in Parral (R1) and Linares (R2), in Chile. These paddy fields were cultivated with rice monoculture with the use of sulfonylurea herbicide-based mixtures for 10 consecutive years since 1994, and seeds were collected in 2004. This research was conducted to (1) quantify resistant levels to bensulfuron-methyl in two resistant (R1 and R2) biotypes of *S. mucronatus* and one susceptible (S) type; (2) examine cross-resistance of R1 an R2 biotypes to other ALS inhibiting herbicides; (3) examine absorption, translocation, and metabolism of ^{14}C-bensulfuron-methyl in both resistant and susceptible biotypes; and (4) conduct biochemical and molecular studies to understand the mechanism of action and interaction of ALS herbicides and its target site.

MATERIALS AND METHODS

Herbicides. ^{14}C-Bensulfuron-methyl (UL-ring-^{14}C-labeled, 53.1 μCi mL^{-1}) (^{14}C-BM) and technical-grade bensulfuron-methyl (98.5%) (BM) were supplied by E. I. DuPont de Nemours & Co (Nambsheim, France), whereas other herbicides for in vivo and in vitro studies were supplied as follows: bispyribac-sodium (98.0%) by Dr. A. Fischer, University of California, Davis; cyclosulfamuron (95.4%) by BASF; etoxysulfuron (98.5%) by Bayer; imazasulfuron (99.3%) by Takeda Chemical Industries; imazamox (98.5%) by BASF; and pyrazosulfuron-ethyl (98,3%) by Nissan Chemical Industries.

Plant Material and Growing Conditions. Seeds from two suspected sulfonylurea-resistant *S. mucronatus* (R1 and R2) biotypes were harvested in 2004 from locations within Parral and Linares provinces, Chile. These fields had been treated for 10 continuous years with sulfonylurea herbicide-based mixtures, mainly bensulfuron-methyl plus molinate. Seeds of *S. mucronatus* were also collected from paddy rice fields never treated with herbicides in Linares, hereafter referred to as S biotype. To overcome dormancy, seeds were stored at 4 °C for 3 months before use in the experiments.

For all experiments, seeds were sown in plastic pots (five seeds per pot) containing a clay loam soil, which were maintained in a growth chamber at 30/25 °C and a 16/8 h day/night period under 500 μmol/m^2/s photosynthetic photon flux density (PPDF) delivered by a mixture of incandescent and fluorescent lights, with an 80% relative humidity.

Plant Response to ALS Inhibitor Herbicides. Dose−response experiments were conducted in the growth chamber to quantify resistance levels to herbicides in *S. mucronatus*. Treatments were applied to plants of all biotypes at the three- to four-leaf growth stage using a laboratory track sprayer equipped with a TeeJet 80.02E VS flat-fan nozzle delivering a spray volume of 200 L ha^{-1} at 200 kPa. For the resistant biotypes, each herbicide was tested using seven rates *D*/8, *D*/4, *D*/2, *D*, 2*D*, 4*D*, and 8*D*, *D* being the labeled field rate for each herbicide (BM, 51 g ha^{-1}; bispiribac-sodium, 25 g ha^{-1}; cyclosulfamuron, 60 g ha^{-1}; etoxysulfuron, 120 g ha^{-1}; imazasulfuron, 75 g ha^{-1}; imazamox, 70 g ha^{-1}; and pyrazosulfuron-ethyl, 21 g ha^{-1}). For the susceptible biotype, also seven rates were tested: *D*/1000, *D*/500, *D*/250, *D*/125, *D*/25, *D*/5, and *D*. Herbicide rates needed to inhibit fresh weight by 50% compared to the untreated control (ED_{50}) (*16*) were determined for each biotype. The resistance factor was computed as $ED_{50}(R)/ED_{50}(S)$. The experiments were repeated three times and were arranged in a completely randomized design with four replicates per dose. Shoot fresh weight data were expressed as percentage of untreated control. Data were pooled and fitted to a nonlinear, log−logistic regression model,

$$Y = c + \{(d - c)/[1 + (x/g)^b]\}$$

where Y is the shoot fresh weight expressed as percentage of the untreated control, c and d are the coefficients corresponding to the lower and upper asymptotes, b is the slope of the line, g is the herbicide rate at the point of inflection halfway between the upper and lower asymptotes and represents the ED_{50}, and x (independent variable) is the herbicide dose. Regression analysis was conducted using Graphpad Prism 3.03 statistical software (*11*).

Absorption and Translocation. ^{14}C-Bensulfuron-methyl (^{14}C-BM) was mixed with commercially formulated BM and codacide oil to prepare emulsions with a specific activity of 1 kBq μL^{-1} and a BM concentration of 1.5 g L^{-1} (corresponding to 51 g ha^{-1} BM at the spray volume of 200 L ha^{-1}). This formulation of labeled herbicide was applied to the adaxial surface of the second leaf of each plant in four 0.5 μL droplets using a microapplicator (Hamilton PB-600, Hamilton Co., Reno, NV) when plants were at the three- to four-leaf growth stage. A total of 2.083 kBq was applied on each plant for both absorption and translocation studies. Plants were harvested in batches of three plants at several time intervals after herbicide application (6, 12, 24, and 48 h) and separated into treated leaf, root, and remainder of the shoot. Unabsorbed ^{14}C-BM was removed from the leaf surface by rinsing the treated area with 3 mL of methanol 80% (v/v). Rinses from each batch were pooled and analyzed by liquid scintillation spectrometry (LSS) (Scintillation counter, Beckman LS 6500 TA, Beckman Instruments Inc., Fullerton, CA). Percent herbicide absorbed was expressed as [kBq in combusted tissue/(kBq in combusted tissue + kBq in leaf washes)] × 100. The experiment was replicated three times.

In the translocation studies, the treated plants of each biotype were sampled at the same periods described. Plant tissue was dried at 55 °C for 72 h and combusted in a sample oxidizer (Oxidizer, Tri Carb model 307, Packard Instrument Co., Downers Grove, IL) . The $^{14}CO_2$ evolved was trapped in a 10 mL mixture of Carbo-Sorb E and Permafluor E$^+$ (3:7 v/v) (Perkin-Elmer, Packard Bioscience BV, Groningen, The Netherlands). The radioactivity was quantified by LSS. The experiment was repeated three times. Means and standard errors (of the mean) were computed for all parts of plants, and means were tested for group differences and compared using an analysis of variance (ANOVA) and a Tukey HSD posthoc test.

Metabolism Studies. The metabolism of ^{14}C-BM was examined in the second leaf tissue of R1 and S plants at the three-to four-leaf stage of growth. The labeled herbicide was applied to the adaxial surface of the second leaf in four 0.5 μL droplets using a microapplicator. A total of 2.083 kBq was applied on each plant. Plants of R1 and S biotypes were sampled 6, 12, 24, and 48 h after treatment. Treated leaves were rinsed as above. An aliquot of the leaf rinse solution was assayed for radioactivity, and the remaining solution was stored at −20 °C until analysis. The treated plants were separated into treated leaf, roots, and remainder of shoots andt were ground in a mortar using 4 mL of 90% (v/v) methanol, and the homogenate was centrifuged at 20000*g* for 10 min at 4 °C. The pellet was rinsed with 90% methanol until ^{14}C was no longer extracted and then oven-dried at 60 °C for 48 h and combusted in a sample oxidizer. The supernatants were combined, evaporated to dryness at 40 °C under a stream of N_2 at 10 kPa, and redissolved in 300 μL of 80% methanol (v/v). ^{14}C-BM and its metabolites in the supernatant were identified by thin-layer chromatography (TLC) on 20 cm × 20 cm, 250 μm silica gel plates (silica gel 60, Merck, Darmstadt, Germany) and a dichloromethane/methanol/ammonium hydroxide (6:1:0.1, v/v/v) mobile phase. The radioactive zones were detected with a radiochromatogram scanner (Berthold LB 2821, Wildbald, Germany), and their chemical nature was identified by comparing their R_f value with that of the standard. The experiment was repeated three times.

ALS Activity Assay. The ALS response to ALS-inhibiting herbicides was determined in vitro using crude extracts isolated from R and S seedling biotypes as was described previously (*17*). The R/S ratio was calculated as $I_{50}(R)/I_{50}(S)$, I_{50} being the concentration of herbicide that caused a 50% reduction in ALS activity. Total protein content was measured using the Bradford

method (*18*). Maximum ALS specific activity (nmol of acetoin mg^{-1} of protein h^{-1}) was determined in the absence of herbicide. Data were pooled and fitted to the log−logistic model described before. Experiments were repeated three times.

DNA Extraction. Total genomic DNA was extracted from fresh plants at the four-leaf stage following a cetyl trimethylammonium bromide (CTAB)-based extraction method using a NucleoSpin Plant kit (Macherey-Nagel) and 250 mg of plant material. In all cases, DNA was spectrophotometrically quantified with a SmartSpec 3000 spectrophotometer (Bio-Rad) and then immediately used for PCR reactions or stored at −20 °C until its use.

Two sets of degenerated and universal primer pairs were used to amplify the regions containing the conserved domains C, A, and D (primers ALS-U-295 and ALS-L1170) and domains B and E (primers ALS-U-1580 and ALS-L-2160), under optimized conditions as previously described (*9*). Only genomic DNA was used because no introns were found in the amplified regions or in other sequences found in the databases. PCR products were separated in 1% agarose TAE (tris−acetate−EDTA) gels and extracted using a QiaQuick Gel Extract Band kit (Qiagen). Extracted bands were treated with 2 units of Klenow for 15 min at 37 °C and then were cloned into the *Eco*RV site of pBluescript KS II+. Recombinant plasmids were introduced into competent *Escherichia coli* DH according the Inoue method (*19*), positive colonies were PCR screened using the same primers that generated the PCR fragment and, then, plasmids with the desired inserts were isolated using a Nucleospin Plasmid Isolation Kit (Macherey Nagel) according the manufacturer's protocol. Inserts were sequenced by the central facilities of the University of Cordoba, using M13 forward and M13 reverse primers with an ABI PRISM 310 sequencer and ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit. At least three different clones of each PCR fragment were sequenced by their 5′ and 3′ ends, and the assembled sequences were analyzed for the presence of mutations.

Bioinformatics Analysis. Chromatograms resulting from sequentiation reactions were edited using the Chromas program (http://www.technelysium.com.au). Forward (5′) and reverse (3′) sequences of each PCR fragment were converted to FASTA format and assembled with the BioEdit program (*20*). Each assembled sequence was analyzed to avoid sequentiation errors, comparing forward and reverse chromatograms. Several assembled sequences of different clones corresponding to each PCR product were fully aligned and phylogenetically analyzed using the Clustal X program (*21*). General manipulation of nucleotides and amino acid sequences was done using the EMBOSS package (*22*).

RESULTS AND DISCUSSION

Whole-Plant Bioassay. The *S. mucronatus* accessions R1 and R2 originated from paddy fields used as monoculture rice production, which had been continuously treated with BM herbicide since 1994. However, the efficacy of the herbicides on these biotypes had declined strongly since 2003, and *S. mucronatus* R1 and R2 became dominant weeds in these fields (*23*).

Both accessions exhibited similar levels of resistance to BM in whole-plant response bioassay. The BM rates needed for ED_{50} for biotypes R1 and R2 were very different from that for the S biotype. The resistance factors were 1718.9 and 1627.4 for R1 and R2 accessions, respectively. This suggests that the rate necessary to inhibit growth of these biotypes by 50% is > 3 orders of magnitude greater than the rate needed to control the S population (**Table 1**).

Table 1. Parameters of the Equation[a] Used To Calculate the Bensulfuron-Mmethyl Dose Required for 50% Plant Injury (ED_{50}) of Resistant (R1, R2) and Susceptible (S) Biotypes of *S. mucronatus*

biotype	*c*	*d*	*b*	ED_{50} (g ha^{-1})	pseudo r^{2b}	*P*[c]	RF[d]
R1	1.472	99.784	3.1439	319.546	0.99	<0.001	1718.9
R2	1.235	99.57	2.8367	302.53	0.99	<0.001	1627.4
S	2.788	100	1.3746	0.1859	0.98	<0.001	

[a] Equation $Y = c + \{(d - c)/[1 + (x/g)^b]\}$, where *Y* is the percentage of plant injury, *x* (independent variable) is the herbicide rate, *c* and *d* are the lower and the upper asymptotes, *b* is the slope of the line, and ED_{50} is the effective dose required for 50% plant injury. Data were pooled and fitted to nonlinear regression model. Data are means of four replicates. [b] Approximate coefficient of determination of nonlinear models with a defined intercept calculated as pseudo $r^2 = 1 -$ (sums of squares of the regression/corrected total sums of squares). [c] Probability level of significance of the nonlinear model. [d] RF = resistant factor = ED_{50} of resistant/ED_{50} of susceptible biotype.

On the basis of the above-ground fresh weight, the resistance factor for biotype R1 ranged from 1.597 to 1.7 for the herbicides bensulfuron-methyl and bispiribac-sodium, respectively. The S biotype was completely controlled with less than half of the respective recommended field rates of bensulfuron-methyl, bispiribac-sodium, cyclosulfamuron, ethoxysulfuron, imazamox, imazosulfuron, and pyrazosulfuron-ethyl (**Table 2**). The cross-resistance ratio values to these ALS-inhibiting herbicides were bensulfuron-methyl > imazosulfuron ≫ cyclosulfamuron ≫ pyrazosulfuron-ethyl ≫ ethoxysulfuron > imazamox ≫ bispiribac-sodium (**Table 2**). Other ALS-resistant weed species (*Cyperus difformis*, *Schoenoplectus mucronatus*, *Alisma plantago-aquatica*, *Echinochloa phyllopogon*, and *Monochoria vaginalis*) found in rice fields have shown different patterns of cross-resistance (*13*, *14*, *24*−*26*).

Absorption and Translocation. There were no significant differences in absorption and translocation between resistant (R1) and susceptible (S) *S. mucronatus* biotypes (**Table 3**). At 48 HAT, about 70% of recovered radioactivity had penetrated into the plants of both R1 and S biotypes. Maximum absorption depends on herbicide and species assayed. Maximum absorption in *E. cruss-galli* resistant and susceptible biotypes of propanil was 90% at 72 HAT (*27*). The foliar penetration of other herbicides such as cyhalofop-butyl (CB) was faster than that of propanil in *E. oryzoides*. In this case, CB absorption reached 77% only 24 HAT (*11*). The maximum uptake of BM evaluated on *A. plantago aquatica* was 41% after 96 h of root incubation (*14*). For all of these species the absorption/uptake rate could not explain differences in herbicide resistance found in field rice. Up to today, resistance due to lack of herbicide absorption/translocation/sequestration has been found in only *L. rigidum* and *L. multiflorum* to diclofop-methyl and glyphosate (*10*, *28*), *L. rigidum* to paraquat (*29*), and *Conyza* spp. to glyphosate (*30*−*32*).

In the translocation study, the radioactivity recovered on both biotypes revealed a similar distribution of ^{14}C-BM. The BM was widely distributed among leaves and roots, with appreciable acropetal or basipetal herbicide translocation (or both) in both R1 and S biotypes from 3 to 48 HAT. Although the translocation increased with time, it does not constitute a differential mechanism of resistance between R1 and S biotypes.

Metabolism Studies. The ^{14}C-BM metabolic pattern was not different in both *S. mucronatus* R1 and S biotypes at 48 HAT (**Table 4**). The amount of polar metabolites of BM was higher in the roots than in the shoots. TLC analysis revealed the presence of the herbicide BM (R_f = 0.70) and of two polar metabolites (pm) (pm1 with R_f = 0.30 and pm2 with R_f = 0.10) in the different parts of R and S biotypes of *S. mucronatus* (**Table 4**). Apolar metabolites (R_f > 0.70) were not found in any biotype. Results

Table 2. Parameters of the Equation[a] Used To Calculate the Herbicide Dose Required for 50% Plant Injury (ED_{50}) of Resistant (R1) and Susceptible (S) Biotypes of *S. mucronatus* Using Different Herbicides

herbicide	accession	*c*	*d*	*b*	ED_{50} (g ha^{-1})	pseudo r^b	P^c	RF[d]
bensulfuron-methyl	R	1.472	99.784	3.143	319.54	0.99	<0.001	1718.9
	S	2.788	100	1.376	0.1859	0.98	<0.001	
bispiribac-sodium	R	1.076	99.99	1.569	23.2	0.98	<0.0001	1.705
	S	2.471	100	1.5	13.6	0.99	<0.0001	
cyclosulfamuron	R	1.343	99.72	1.296	155.0	0.99	<0.0001	775
	S	3.293	100	2.005	0.2	0.99	<0.0001	
ethoxysulfuron	R	2.191	100	3.734	1576.8	0.99	<0.0001	59.501
	S	2.275	99.76	5.637	26.5	0.99	<0.0001	
imazamox	R	2.714	100	1.956	8.6	0.99	<0.0001	43
	S	0.976	100	4.239	0.2	0.98	<0.0001	
imazosulfuron	R	2.612	100	5.799	562.6	0.98	<0.0001	1406.5
	S	0.718	100	3.550	0.4	0.99	<0.0001	
pyrazosulfuron-ethyl	R	3.374	100	2.856	49.0	0.99	<0.0001	122.5
	S	0.020	100	3.211	0.4	0.98	<0.0001	

[a] Equation $Y = c + \{(d - c)/[1 + (x/g)^b]\}$, where *Y* is the percentage of plant injury, *x* (independent variable) is the herbicide rate, *c* and *d* are the lower and upper asymptotes, *b* is the slope of the line, and ED_{50} is the effective dose required for 50% plant injury. Data were pooled and fitted to nonlinear regression model. Data are means of four replicates. [b] Approximate coefficient of determination of nonlinear models with a defined intercept calculated as pseudo r^2 = 1 - (sums of squares of the regression/corrected total sums of squares). [c] Probability level of significance of the nonlinear model. [d] RF = resistant factor = ED_{50} of resistant/ED_{50} of susceptible biotype.

Table 3. Absorption (Percent Recovered Radioactivity) and Translocation (Percent of Absorbed Radioactivity) of ^{14}C-Bensulfuron-methyl in Resistant (R1) and Susceptible (S) Biotypes of *S. mucronatus*

			translocation[a]		
biotype	HAT	absorption[a]	treated leaf	rest of shoots	roots
R1	6	5.5 ± 0.8 C	87.4 ± 4.1 A	12.6 ± 1.3 G	0 H
	12	11.7 ± 2.3 C	74.3 ± 4.9 B	16.9 ± 3.2 FG	8.8 ± 0.7 GH
	24	34.6 ± 7.4 B	60.4 ± 5.8 C	27.3 ± 4.7 EF	12.3 ± 2.5 G
	48	68.4 ± 5.2 A	42.3 ± 3.6 D	40.8 ± 4.4 D	16.9 ± 2.6 FG
S	6	8.8 ± 3.2 C	90.3 ± 9.3 A	9.7 ± 1.9 GH	0 H
	12	20.1 ± 4.7 BC	84.4 ± 7.1 AB	7.7 ± 3.1 GH	7.9 ± 1.7 GH
	24	32.4 ± 3.8 B	58.4 ± 3.2 C	26.1 ± 5.3 EF	15.5 ± 2.7 G
	48	71.7 ± 10.1 A	41.8 ± 9.1 D	37.0 ± 4.3 DE	21.2 ± 3.7 FG

[a] Means within a column followed by the same letter are not significantly different at the 5% level as determined by the Tukey test. Values ± standard error of the mean, 0 = nondetected

Table 4. Percentage of the Residual Absorbed Radioactivity Represented by Bensulfuron-methyl (BM) and Its Metabolites Detected by TLC in Extracts of Resistant (R1) and Susceptible (S) Plants of *S. mucronatus*, Evaluated 48 h after Foliar Application

	metabolites[a]					
	R1			S		
plant part	polar	BM	apolar	polar	BM	apolar
treated leaf	76.2 ± 3.8	23.8 ± 2.6	nd	75.6 ± 2.3	24.4 ± 1.3	nd
rest of shoots	73.6 ± 4.2	26.4 ± 1.7	nd	77.0 ± 6.1	22.9 ± 0.9	nd
roots	89.4 ± 5.1	10.6 ± 0.3	nd	85.7 ± 4.9	14.3 ± 1.3	nd

[a] Values ± standard error of the mean. nd, nondetected.

from the literature (*33*, *34*), which analyzed root extracts from rice seedling, identified these polar metabolite as the methyl-α-(4-hydroxy-6-methoxypyrimidin-2-yl)carbamoylsulfamoyl-*o*-toluate (pm1), which could be conjugated with glucose, and the methyl-α-aminosulfonyl-*o*-toluate (pm2).

As there were no significant differences among R and S biotypes of *S. mucronatus* for BM absorption, translocation, and metabolism, it was assumed that these processes could not explain herbicide resistance.

ALS Activity Assay. The specific activities in vitro of ALS extracted from shoot tissue of *S. mucronatus* R and S biotypes were similar, with acetoin production (nmol of acetoin mg^{-1} of protein h^{-1}) equivalent to 697 (±39) and 763 (±54) for R and S biotypes, respectively. These results suggest that herbicide resistance was not due to an overexpression of the target enzyme in the R biotype.

In vitro, the resistance factors to ALS-inhibiting herbicides were, from highest to lowest, bensulfuron-methyl > imazosulfuron ≫ cyclosulfamuron ≫ ethoxysulfuron > imazamox > pyrazosulfuron-ethyl > bispiribac-sodium (**Table 5**). Then, resistance factors determined in ALS in vitro assays indicate stronger cross-resistance to sulfonylurea (bensulfuron-methyl, cyclosulfamuron, ethoxysulfuron, imazosulfuron, and pyrazosulfuron-ethyl) and imidazolinone herbicides (imazamox) than to pyriminyl(thio)benzoates (bispiribac-sodium), the same as found at whole plant level (**Table 2**). These results suggest the mechanism of resistance for this biotype is an altered ALS enzyme. The basis of resistance in *S. mucronatus* to these herbicides appears to be similar to that of *S. juncoides* (*35*), *Lindernia* spp. (*36*), or *Monochoria vaginalis* (*25*), in which resistance to ALS inhibitors has been linked to a reduced sensitivity of the target site to the herbicides.

ALS Gene Sequences. The nucleotide sequences of the domain A region for R *S. mucronatus* biotype differed from that of the S biotype by a single nucleotide substitution (CCT to CAT) at variable Pro codon of domain A, predicting a Pro in the S but a His in the R biotype. The same amino acid change was seen in *L. serriola* (*37*, *38*), *Raphanus raphanistrum* (*39*), *Papaver rhoeas* (*40*), and *S. juncoides* var. Ohwianus (*41*). No nucleotide differences between S and R *S. mucronatus* were observed in other ALS domains studied (B−E) (data not shown).

Comparison of this mutation with the resistance pattern of the same population, previously determined by enzyme activity

Table 5. Parameters of the Equation[a] Used To Calculate the Herbicide Concentration Required for 50% Reduction of the ALS activiy (I_{50}) of Resistant (R) and Susceptible (S) Biotypes of *S. mucronatus*

herbicide	accession	*c*	*d*	*b*	I_{50}	pseudo r^2[b]	*P*[c]	RF[d]
bensulfuron-methyl	R	3.711	98.463	2.044	1163.0	0.975	<0.0001	161.527
	S	1.043	100.00	1.2036	7.2	0.984	<0.0001	
bispiribac-sodium	R	8.073	99.94	1.525	157.0	0.988	<0.0001	5.322
	S	1.253	98.743	1.819	29.5	0.985	<0.0001	
cyclosulfamuron	R	7.719	99.718	1.982	3.2	0.982	<0.0001	106.666
	S	0.423	100.00	1.194	0.03	0.99	<0.0001	
ethoxysulfuron	R	1.879	97.812	2.328	7.3	0.96	<0.0001	24.333
	S	0.368	97.563	2.149	0.3	0.967	<0.0001	
imazamox	R	1.169	97.911	1.875	1105.7	0.96	<0.0001	10.925
	S	1.562	98.492	1.171	101.2	0.981	<0.0001	
imazosulfuron	R	1.655	99.729	1.725	162.2	0.982	<0.0001	147.454
	S	1.21	99.267	1.923	1.1	0.978	<0.0001	
pyrazosulfuron-ethyl	R	5.768	97.242	1.645	149.5	0.964	<0.0001	6.826
	S	1.482	99.59	1.49	21.9	0.989	<0.0001	

[a] Equation $Y = c + \{(d - c)/[1 + (x/g)^b]\}$, where *Y* is the percentage of plant injury, *x* (independent variable) is the herbicide concentration, *c* and *d* are the lower and upper asymptotes, *b* is the slope of the line, and I_{50} is the effective dose required for 50% reduction of ALS activity. Data were pooled and fitted to nonlinear regression model. Data are means of four replicates. [b] Approximate coefficient of determination of nonlinear models with a defined intercept calculated as pseudo $r^2 = 1 -$ (sums of squares of the regression/corrected total sums of squares). [c] Probability level of significance of the nonlinear model. [d] RF = resistant factor = I_{50} of resistant /I_{50} of susceptible biotype.

experiments, conferred a common resistance pattern found in another species to SU (high resistance) and little or none to PTB (*15*). In our study we found high resistance to IMI as well, with resistance ratios of 43 and 1406.5 for imazamox and imazosulfuron, respectively. Pro197His mutations have so far conferred no or weak resistance to IMI. In some of the species the corresponding cross-resistance pattern was not fully determined. Studies of the sequence containing the Asp376 must be carried out to complete this experiment.

Resistance in many cases has been attributed to single point mutations, which can occur at multiple sites in the ALS gene, resulting in a variable pattern of cross-resistance between the classes of ALS inhibitors (*15*). Because no other differences were found between R and S biotypes, it can be concluded that this point mutation could explain the resistance of the R biotype. The main advantage of the identification of the point mutations leading to resistance is that the resistance can be classified as target-site resistance without any additional tests (*42*). Furthermore, knowing the mutation and its site seems to facilitate a forecast of the expected levels of resistance and cross-resistance to other ALS inhibitors.

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