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Asp376Glu Mutation and Enhanced Metabolism Controlling the Resistance to ALS-Inhibiting Herbicides in *Ixophorus unisetus* (J. Presl) Schltdl. from the Bajio, Mexico

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Abstract: A study was carried out to determine the ALS (acetolactate synthase)-inhibitor herbicide resistance in the Mexican grass *Ixophorus unisetus*, a troublesome weed in corn crops in Mexico. First, the resistance was confirmed in field screening assays. Eight populations that survived nicosulfuron treatment at a field rate of 40 g ai ha^{-1} were labeled as putative-resistant. Dose–response trials demonstrated a high resistance in the eight populations (GR_{50} from 140.33 to 245.46 g ai ha^{-1}). The synergism of malathion plus nicosulfuron demonstrated that the non-target-site resistance (NTSR) mechanism based on cytochrome P450 (Cyt-P450) was involved in five populations of *I. unisetus*. Molecular studies revealed that a single-nucleotide change occurs in the amino acid at position 376 (from GAT to GAG), which codifies from Asp-376 to Glu-376. This is the first time that Asp-376-Glu has been reported in this species. Assays in vitro and in vivo demonstrated *I. unisetus* cross-resistance to flucarbazone, penoxsulam, bispyribac-Na, and imazamox. No multiple resistance was found in two resistant populations exposed to different herbicides. Our results indicate that the lack of good control over Mexican grass in corn with ALS inhibitors is due to target-site mutation and NTSR mechanisms (Cyt-P450-mediated metabolism). A strategy should be established in Mexican fields to continue controlling this weed, including mechanical control practices and a good combination of the available pre- and post-emergence herbicides.

Keywords: cross-resistance; ALS activity; corn weeds; Asp376Glu; target-site mechanism; non-target-site mechanism

1. Introduction

Ixophorus unisetus (J. Presl Schltdl.), also known as Mexican grass [1], is a monophyletic plant and considered one of the most troublesome weeds in corn, soybean, sugarcane, citrus, banana, and agave crops from Mexico [2,3]. Moreover, in bean crops (*Phaseolus vulgaris*), the presence of this weed can result in a reduction in the yield and grain quality of at least 50% [4,5]. Additionally, it is considered to be a host plant for pest eggs, principally from the *Spodoptera* genus, in corn and soybean [6]. This weed is characterized by a high growth rate, high fertility, and reproduction by seed.

The application of selective herbicides on corn fields is required to reduce weed populations and thereby obtain high yields [7]. In Mexico, a list of selective post-emergence herbicides has been used in weed control, such as 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors (topramezone, tolpyralate, tembotrione, and mesotrione), acetolactate synthase (ALS) inhibitors (nicosulfuron, and foramsulfuron + iodosulfuron), and a photosystem II inhibitor (atrazine), among others. The application of these herbicides has been ongoing in Mexico for many years, and the emergence of resistance in *I. unisetus* was first observed in 2014 [8]. Herbicide resistance refers to the ability of a weed biotype to resist the effects of an herbicide, even when the herbicide is applied at the recommended rate under typical conditions that would otherwise eliminate the weed. [9]. Herbicide-resistance selection occurs over a few or several years, depending on the chemical group of the herbicide. Some of the key factors influencing herbicide resistance are the intensity of the selection pressure (applications per time/crop cycle) and the initial frequency of resistant biotypes [10].

Acetolactate synthase (EC 2.2.1.6) is the key enzyme in the biosynthesis of the branched-chain amino acid pathways valine, leucine, and isoleucine. As mentioned above, ALS is a target site of six chemical groups used for weed control (grass and broadleaf weeds): sulfonylureas (SUs); imidazolinone (IMI); triazolopyrimidine (TP); pyrimidinyl thiobenzoates (PTBs); sulfonanilides (SAs); and triazolinones (SCTs) [8,9,11].

Since the introduction of ALS-inhibiting herbicides in the 1980s, the appearance of weed-resistant biotypes has increased drastically worldwide [8]. In the first decade, 11 resistant biotypes were reported, including *I. unisetus* from Costa Rica. Nowadays, there are 170 biotypes annexed as resistant to ALS inhibitors globally [8]. Most of these biotypes have obtained resistance mechanisms based on target-site resistance (TSR) [12], which involves key amino acid substitution in *ALS* genes. Nine modifications in key amino acid positions (Ala122; Pro197; Ala205; Phe206; Asp376; Arg377; Trp574; Ser653; and Gly654) of the *ALS* gene that confers resistance to ALS inhibitors have been described [8,13,14]. In addition, several studies have demonstrated non-target-site resistance (NTSR) mechanisms principally based on enhanced metabolism; in these NTSR mechanisms, elevated levels of herbicide-detoxifying enzymes are involved, including cytochrome P450 mixed-function oxidases, UDP glucose-dependent glycosyltransferases (UGTs), glutathione-S transferases (GSTs), and membrane-associated ATP-binding cassette (ABC) drug transporter proteins [15].

Regarding the problem of *I. unisetus* in crop production, currently, Mexican farmers have adopted several chemical alternatives to control this weed in corn. However, the alternatives do not always have a different mode of action (MoA), and the intensive use of ALS inhibitors has recently increased. We hypothesized that this grass could have selected resistance and could spread this resistance to different corn-growing areas. In addition, we believe that different resistance mechanisms could be associated with this weed species due to the different responses in corn fields with *I. unisetus* infestations.

The aims of this work were as follows: (1) to detect the nicosulfuron resistance of *I. unisetus* in some corn-growing areas; (2) to determine the level of resistance to nicosulfuron; (3) to find the resistance mechanisms involved; and (4) to conduct the screening of the possible mechanisms of multiple resistance to herbicides.

2. Materials and Methods

2.1. Resistance Screening in Corn Fields

A field test was performed to confirm the resistance of *I. unisetus* in the Bajio, Mexico. Two areas, Jalisco and Guanajuato, considered as place 1 and place 2, respectively, were tested according to reports of farmers. A total of 15 farms were assayed: 9 farms were set up in place 1 and 6 farms in place 2. On each farm, three plots (three biological replications) were delimited; the plot dimensions were 2 × 10 m. The plot delimitation was performed based on the presence of *I. unisetus*. Moreover, the same number of plots was established as a control (without herbicide application) at each site. The herbicide was applied at a medium field dose (50 g ai ha^{-1}) using a pneumatic backpack sprayer with TeeJet 11002 flat-fan nozzle tips and calibrated to deliver 250 L ha^{-1} at 276 kPa when plants were at the

3- or 4-leaf stage. The plots were evaluated at 28 DAA. Four samples of fresh plant tissue from 0.25 m^2 were harvested from each plot and dried for 1 week at 60 °C. The results were presented as the percentage of dry weight with respect to the untreated plot.

2.2. Dose–Response Curves

An herbicide test to inhibit growth by 50% was performed (GR_{50}). Seeds from eight resistant populations (five from Jalisco and three from Guanajuato) and one susceptible population were harvested (from Jalisco) and sent to the University of Cordoba to conduct the dose–response assay. The seeds were germinated in pots with peat moss only. Seedlings were placed in individual pots (one plant per pot) with 50 g of substrate (sand/peat moss (1:1)). When plants were in the BBCH 13 foliar stage [16], 20, 40, 80, 160, 320, and 640 g ha^{-1} of nicosulfuron was applied. The herbicide application was performed with a bench-type track sprayer (Devries Manufacturing, Hollandale, MN, USA), equipped with a TeeJet 8002 EVS flat-fan nozzle, adjusted to 200 kPa and a field application volume of 200 L ha^{-1}. The experiment was repeated twice in a completely randomized design. Ten plants per dose of herbicide were used.

2.3. Synergism of Herbicide plus Malathion

An experiment was conducted to determine the possible interaction of herbicide nicosulfuron (40 g ai ha^{-1}) application with malathion (1000 g ai ha^{-1}). Ten plants (one plant per pot) from nine populations (from Jalisco and Guanajuato) were applied with the same calibration and conditions as described in the previous section. Malathion was applied first and an hour later nicosulfuron was applied. At twenty-one DAA, the plants were cut at the ground level and the fresh-tissue weight was registered (Precisa XB 120 A, Dietikon, Switzerland). The experiment was repeated two times in a completely randomized design.

2.4. Molecular Analyses

Ten plants from three populations, one susceptible and two previously identified as resistant, were used. In this resistance selection procedure, one population (IxR4) represented the resistant group from Jalisco and the other one (IxR7) represented the group from Guanajuato. Both populations were selected due to their responses in field and laboratory assays. Six conserved domains (CAD, F, and BE) described in previous studies [17] were analyzed to identify putative changes in single nucleotides and, thus, changes in amino acids. To amplify the conserved domains, two pairs of primers were selected from previous studies and another one was designed in this work. The primers were constructed with the help of Geneious Prime software version 2023.0.1 and using a sequence from an accession of *Setaria viridis* (GenBank ID: KF020514). The primers (pair 1) designed here to amplify the domain "CAD" were Ix-1F 5′ACATCCTCGAGTCCC TC 3′ and Ix-1R 5′ CGTCGA CCAGGTAGTTG-3′; the primers (pair 2) designed by Laplante et al. [17] to amplify the domain "F" were 2-F 5′GAGTTGTGCCGCTTTGTGGAG3′ and 2-R 5′GCCTTGGCCGCTTGTAAGTG 3′; and the primers (pair 3) to amplify the domain "BE" were BE1 5′ GTC TTG GGG CTA TGG GAT TT 3′ and ECH3-R 5′ TCC TGC CAT CAC CTT CCA GGA 3′ (reported by Amaro-Blanco et al. [18] and Panozzo et al. [19], respectively). The schema of the amplified regions is shown in Figure 1.

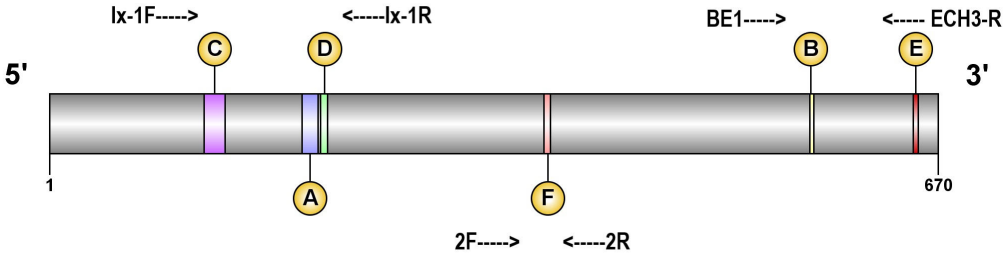

Figure 1. Primers used to amplify all conserved domains of the *ALS* gene of three *Ixophorus unisetus* populations. C, A, D, F, B, and E are the six known conserved domains.

DNA Isolation and PCR Amplification

Young foliar tissue was obtained from thirty plants (ten per population). The samples were immediately placed in liquid nitrogen (N_2) and then freeze-dried (LyoQuest, H140 BeiJer, Telstar, Terrassa, Barcelona) at −50 °C between 10 and 0.03 mBar for approximately 48 h. DNA isolation was performed from ~15 mg lyophilized tissue. PCR amplifications were performed using GoTaq DNA Polymerase (Promega, Madison, WI, USA) in a 50 µL mixture, including 10 µL of 5X Colorless GoTaq Flexi Buffer; 5 µL of MgCl2 (25 mM); 1.875 µL of each primer (10 µM); 1 µL of dNTPs (10 mM); 0.5 µL GoTaq DNA Polymerase; and 20 ng of DNA. The thermocycler program was as follows: 5 min at 92 °C; 38 cycles of 30 s at 95 °C and 30 s at 62 °C (pairs 1 and 3) and 65 °C (pair 2); 45 s at 72 °C; and 5 min at 72 °C. PCR products were purified using Speed Tools PCR Clean-Up Kit (Biotools, B&M Labs, Madrid, Spain), following the manufacturer's instructions. The samples were sequenced using an external service (STABVIDA, Caparica, Portugal). The sequencing results were analyzed using Geneious Prime software. The ten sequences from the *I. unisetus* populations were aligned, and a consensus sequence was made using Geneious alignment (global alignment with free-end gaps) [20]. Then, an alignment was performed using the consensus sequence of *I. unisetus* and accession KF020514 from *Setaria viridis*.

2.5. Cross-Resistance In Vitro Assay (ALS Activity)

Ixophorus unisetus plants, labeled as IxR4, IxR7, and IxS, were used to determine the ALS activity when samples were exposed to different herbicide concentrations. For these studies, 5 g of fresh tissue was collected from plants at the BBCH 13–14 foliar stage. The foliar tissue was introduced into liquid N_2 and pulverized using a porcelain mortar. Immediately, a mix-labeled "A" was combined with shredded tissue and 0.5 g of polyvinylpyrrolidone (PVP). An extraction buffer composed of 1 M K of phosphate (pH 7.5), 10 mM of sodium pyruvate, 5 mM of $MgCl_2$, 50 mM of thiamine pyrophosphate, 100 µM of flavin adenine dinucleotide, 12 mM of dithiothreitol (DTT), and glycerol–water (1:9 v/v) was added in a 1:3 (w/v) ratio of mix A: buffer. The mixture was magnetically stirred for 10 min in a cold chamber at 4 °C. Then, the solution was filtered with cheesecloth and immediately centrifuged (44,800× g for 20 min). The methodology followed was the same as the one described by Hatami et al. [21]. Herbicide concentrations of technical-grade nicosulfuron and penoxsulam (0, 0.001, 0.01, 0.1, 1, 10, 100, and 1000 µM) and imazamox, bispyribac-Na, and flucarbazone (0, 0.01, 0.1, 1, 10, 100, 1000, and 10,000 µM) were added to the reaction mix and incubated for 1 h at 37 °C. After this time, 50 µL of H_2SO_4 (3 M) was added, and the reaction was stopped by incubating it at 60 °C for 15 min. The acetoin formed after the decarboxylation of acetate by the addition of 0.25 mL of creatine (5 g L^{-1}) and 0.25 mL of α-naphthol (50 g L^{-1}, freshly prepared in 5 M NaOH) and was detected following subsequent incubation at 65 °C for 15 min. The amount of acetoin formed was measured at 520 nm using a spectrophotometer (Beckman Coulter, DU 640, Brea, CA, USA). Three replicates per herbicide concentration were used, and the assay was repeated two times.

2.6. Cross-Resistance In Vivo Assay

To understand the spectra of ALS resistance, in addition to the in vitro assay, different herbicides were applied with different doses over two resistant populations (IxR4 and IxR7) and one susceptible population. The applications were performed with the equipment and calibration described in the Dose–Response Curves section. Commercial products are described in Section 2.9 (Table 1). Imazamox, bispyribac-Na, and penoxsulam were applied at doses of 0, 10, 20, and 40 g ai ha^{-1}. Flucarbazone was applied at 0, 15, 30, and 60 g ia ha^{-1}. The treated plants were placed under greenhouse conditions and maintained until evaluation. Twenty-eight days after the treatments, the plants were cut to ground level and weighed. A percentage was calculated considering the average of the untreated plants (absence of herbicide). The experiment was repeated, with ten replicates per population in a completely randomized design.

Table 1. Herbicide-resistance assays used in the evaluation of Mexican grass (*Ixophorus unisetus*) throughout this work.

Tradename	Active Ingredient	MoA WSSA/HRAC [a]	Timing [b]	Field Dose (g ai ha^{-1})
Herbicide-resistance field screening and dose–response curves				
SANSON® 4 SC	Nicosulfuron	2	Post	40
Herbicide and Cyt-P450-inhibitor synergism				
SANSON® 4 SC	Nicosulfuron	2	Post	40
INMAR 50	Malathion	-	-	1000
Cross-resistance in vivo assay				
Everest 70	Flucarbazone	2	Post	25
Pulsar®	Imazamox	2	Post	40
Nomine®	Bispyribac-Na	2	Post	40
Viper	Penoxsulam	2	Post	20
Multiple-resistance screening				
Asgard®	Petoxamida	15	Pre	1800
Anthem-Maxx 518	Piroxasulfone + Fluthiacet	15 + 14	Pre	376.7 + 9.8
Roundup	Glyphosate	9	Post	720
Leopard 5%	Quizalofop	1	Post	100
Laudis 20%	Tembotrione	27	Post	120
Callisto 100	Mesotrione	27	Post	150
Convey®	Topramezone	27	Post	26.9
Raker PRO®	Tolpyralate	27	Post	40
Elumis	Nicosulfuron + Mesotrione	2 + 27	Post	60 + 150

[a] Mode of action from the Herbicide Resistance Action Committee (HRAC) and Weed Science Society of America (WSSA). [b] Time of herbicide application: pre-emergency (Pre) or post-emergency (Post). g ai ha^{-1} = grams of active ingredient per hectare.

2.7. Multiple-Resistance Screening

The main objective of this assay was to detect possible multiple resistance due to enhanced metabolism. Herbicides and doses are shown in Table 1. The application of the herbicides was performed with a bench-type track sprayer (Devries Manufacturing, Hollandale, MN, USA) equipped with a TeeJet 8002 EVS flat-fan nozzle, adjusted to 200 kPa and an application volume of 200 L ha^{-1}. For pre-emergence assays, 100 manually scarified seeds were placed into pots (8 × 8 × 12 cm) pre-filled with sand–peat moss (1:1) and fertilizer. Two pots were used for the treatments and two were untreated as the control. Emerged plants were evaluated, and the percentage of the control was calculated. Post-emergence applications were performed at the BBCH 13–14 foliar stage, and plants were

grown as described in the Dose–Response Curves section. At twenty-eight DAA, plant mortality was evaluated. Plants were considered dead when they did not show growth and/or had meristem necrosis. The experiment was performed twice with the same application and climate conditions.

2.8. Data Analysis

The nicosulfuron rates necessary to reduce plant growth (GR_{50}), plant mortality (LD_{50}), and enzyme activity (I_{50}) were estimated using the transformation of the plant weight to a percentage with respect to the untreated control. The rates were used to create dose–response curves with non-linear regression analyses with a three-parameter log-logistic model using the equation $y = d/[1 + (\log(x) - \log I)^b]$, where d is the coefficient of the upper limit; b is the slope of the curve in the point of inflection halfway between the upper and lower curves (fitted to zero); e is the herbicide dose in GR_{50}, LD_{50}, and I_{50}; and x is the herbicide dose (depending on the herbicide) [22]. Resistance indices (RIs) were calculated dividing the GR_{50}, LD_{50}, or I_{50} of the putative-resistant population by the same parameter as the sensitive population.

Analysis of variance (ANOVA) was performed on the nicosulfuron + Cyt-P450 inhibitor, cross-resistant in vivo assay, and multiple-resistance screening assays. For repeated trials, no significant interactions were found for any trial and the data were pooled for analysis. When statistically necessary ($p < 0.01$) in the experiments, means comparison was performed using Tukey's test ($\alpha = 0.05$).

2.9. Chemicals

The herbicides presented in Table 1 were used in different trials. Imazamox, penoxsulam, flucarbazone, and bispyribac-Na of analytical grade (Sigma-Aldrich, Madrid, Spain) were used (all with purities of 99%) in the cross-resistance in vitro assay.

3. Results

3.1. Herbicide-Resistance Field Screening

Nicosulfuron resistance (NR) was observed in field assays. From the Jalisco sites tested, farms 1, 3, 4, 5, and 6 revealed high herbicide resistances in comparison with plots 2, 7, 8 and 9. In at least one repetition, no visual toxicity symptoms were observed in the plants, and the dry weight was higher in the treated plants compared with the control ones (Figure 2). From the Guanajuato test, three farms revealed up to 80% dry-weight reductions. In contrast, plots 12, 13, and 15 revealed resistances due to poor dry-weight decreases.

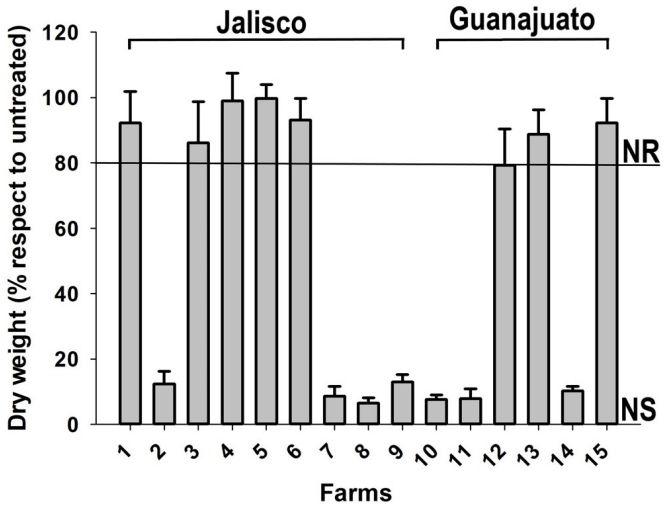

Figure 2. Percentages of dry weight (m^2) of different farms treated with nicosulfuron (50 g ai ha^{-1}) in corn fields infested with Mexican grass (*Ixophorus unisetus*). NR: nicosulfuron-resistant; NS: nicosulfuron-susceptible.

3.2. Dose–Response Curves

Several responses were observed according to the GR_{50} values (Figure 3). At 5 days after treatment (DAT) at 80 g ai ha^{-1}, IxS showed chlorosis symptoms at the apical meristem and young leaves, followed by the necrosis of all leaves 12–15 days later, whereas IxR1–IxR8 did not present any visual injuries. Based on the GR_{50} values, there were differences between the populations collected in Jalisco vs. Guanajuato. The ones from Jalisco were the most resistant, with GR_{50} values from 186.6 to 245.46 g ai ha^{-1} (Table 2), while those from Guanajuato had from 140.3 to 155 g ai. These differences can also be observed in the RIs, which ranged from 7.6 to 10.1 in the Jalisco populations, while the Guanajuato populations ranged from 5.7 to 6.3 (Table 2).

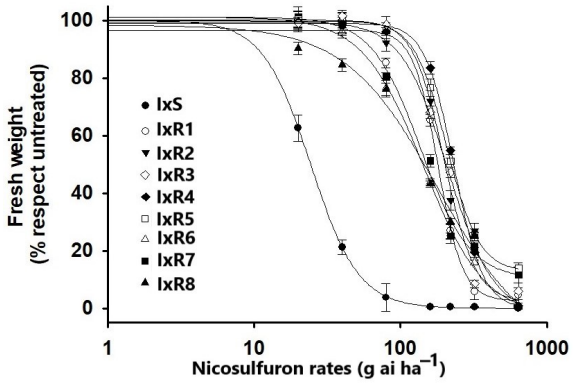

Figure 3. Dose–response curves of nine Mexican grass (*Ixophorus unisetus*) populations exposed to different rates of nicosulfuron. Error bars represent the standard error of the means (n = 20).

Table 2. Data obtained from non-linear regression of nine populations of Mexican grass (*Ixophorus unisetus*) from Mexico on resistance to nicosulfuron.

Population Code	Place	b	d	GR_{50}	95% CI		RI [a]
					Lower	Upper	
IxS	Jalisco	2.6	99.9	24.3	22.2	26.4	--
IxR1	Jalisco	3.98	97.2	186.6	176.5	196.7	7.6
IxR2	Jalisco	2.9	98.9	214.4	201.9	226.8	8.8
IxR3	Jalisco	2.7	100.7	237.2	223.6	250.8	9.7
IxR4	Jalisco	4.6	98.3	245.5	235.0	255.8	10.1
IxR5	Jalisco	3.61	100.1	209.5	198.6	220.3	8.6
IxR6	Guanajuato	2.4	99.9	140.3	128.4	152.1	5.7
IxR7	Guanajuato	2.0	102.5	155.0	141.8	168.1	6.3
IxR8	Guanajuato	1.7	96.1	151.9	135.1	168.5	6.2

[a] Resistance indices calculated dividing the GR_{50} of the resistant population (IxR1–IxR8) by the GR_{50} of IxS. b is the slope of the curve at the point of inflection halfway between the upper and lower curves (fitted at zero), and d is the coefficient of the upper limit.

3.3. Nicosulfuron and Malathion Synergism

The application of nicosulfuron alone or in a mixture with malathion showed different responses in the *I. unisetus* samples from Jalisco and Guanajuato. First, the putative-susceptible (IxS) *I. unisetus* had serious visual injury caused by the herbicide, but the plants did not die. Conversely, all plants died when exposed to malathion. In IxR1–IxR5, differences were observed when the plants were sprayed with nicosulfuron and malathion (Figure 4). Quantitative differences were also visualized (ranging from 1.3 to 1.94 g). In contrast, populations IxR6, IxR7, and IxR8 did not have visual injuries with either the nicosulfuron alone or nicosulfuron-plus-malathion treatment.

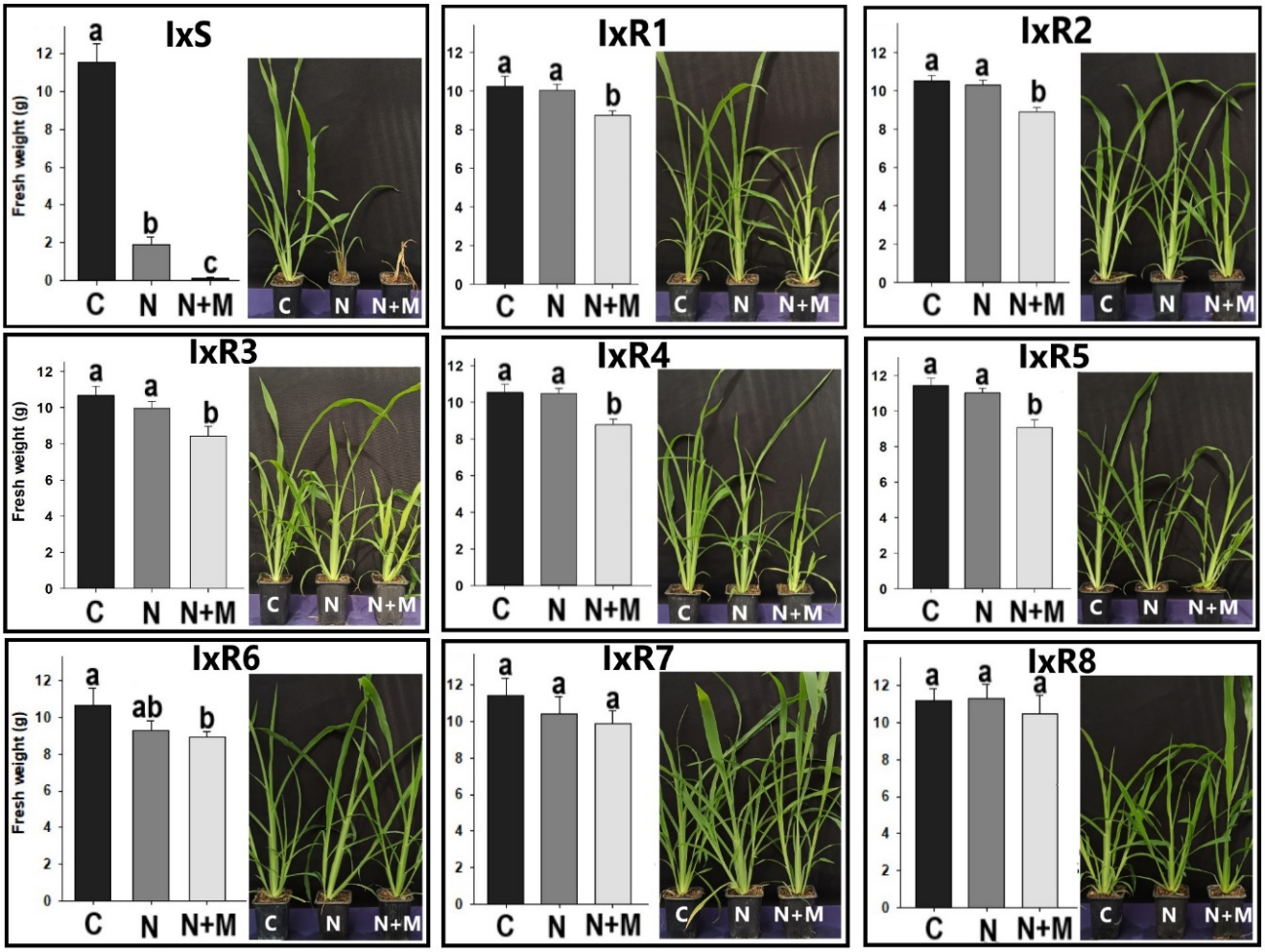

Figure 4. Fresh weights of *Ixophorus unisetus* populations treated with nicosulfuron (N), nicosulfuron + malathion (N + M), or untreated control (C). Bars represent the mean of plants (n = 10). Letters are the aggrupation after Tukey's test. The same letter indicates no significant differences between treatments.

3.4. Partial ALS Gene Sequencing

Partial *ALS* gene sequencing was performed in three *I. unisetus* populations (one susceptible and two resistant) covering nine known resistance-conferring point mutation sites. The sequence alignment of resistant plants (IxR4 and IxR7) showed a single-nucleotide change, from GAT to GAG, in amino acid position 376, which results in a change from aspartic acid to glutamic acid (Asp376Glu). No other nucleotide changes were found in the three *I. unisetus* populations (Figure 5).

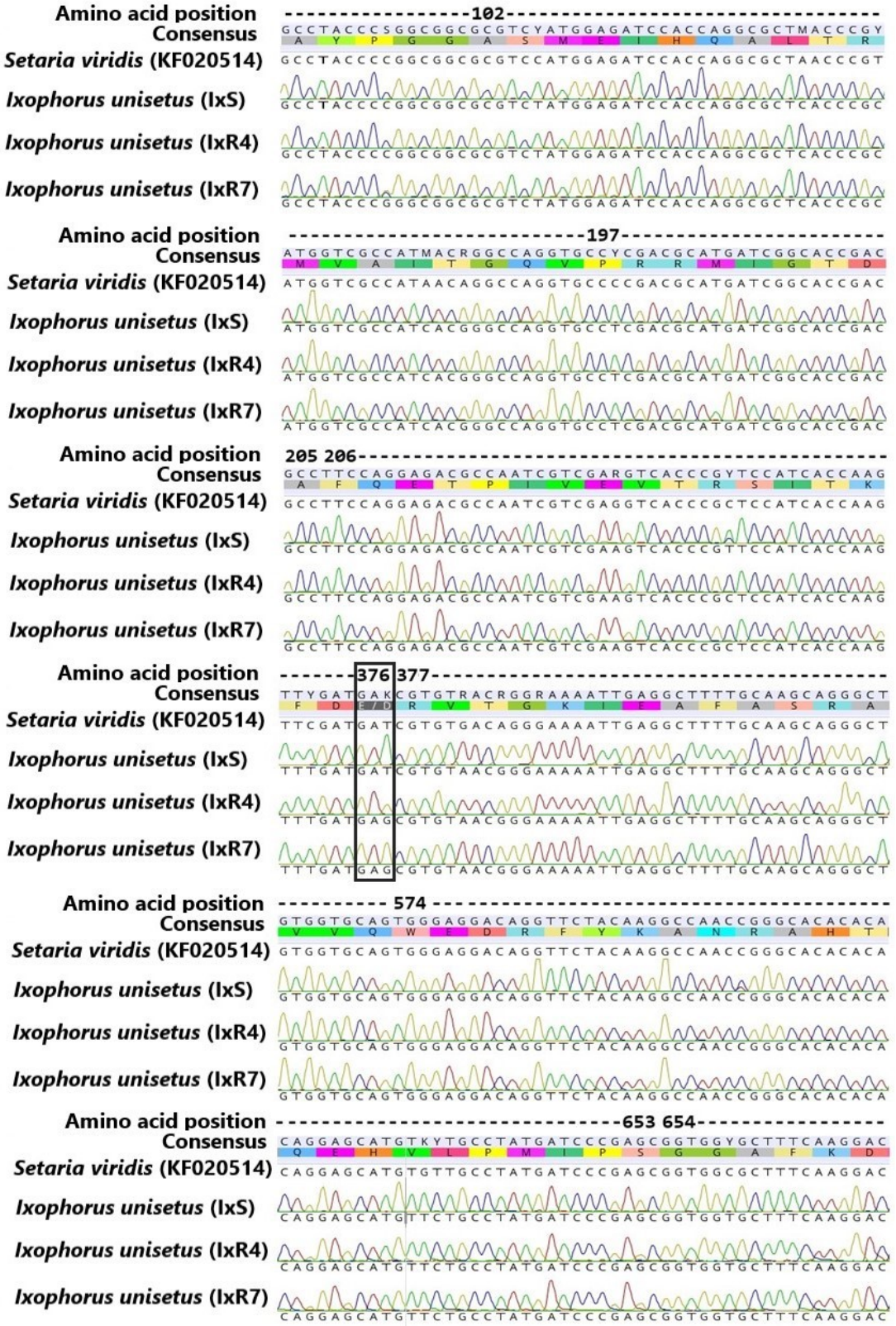

Figure 5. Alignment of partial *ALS* gene of three Mexican grass (*Ixophorus unisetus*) populations compared with *Setaria viridis* (NCBI accession KF020514).

3.5. Cross-Resistance In Vivo Assay

A clear cross-resistance to ALS-inhibiting herbicides was observed because the two resistant *I. unisetus* populations (IxR4 and IxR7) survived the field doses of all the herbicides

tested (Figure 6). IxR4 and IxR7 showed serious injuries with imazamox and bispyribac-Na, causing the fresh weight to decrease by around 65% (Figure 6A, C). High cross-resistance was observed with the flucarbazone and penoxsulam herbicides because the reduction in fresh weight was less than 20%. In addition, Tukey's test demonstrated that there were no differences between the two R populations for any of the herbicide rates used and grouped them into group "a" (Figure 6). Susceptible (IxS) plants died with the field doses of the herbicides.

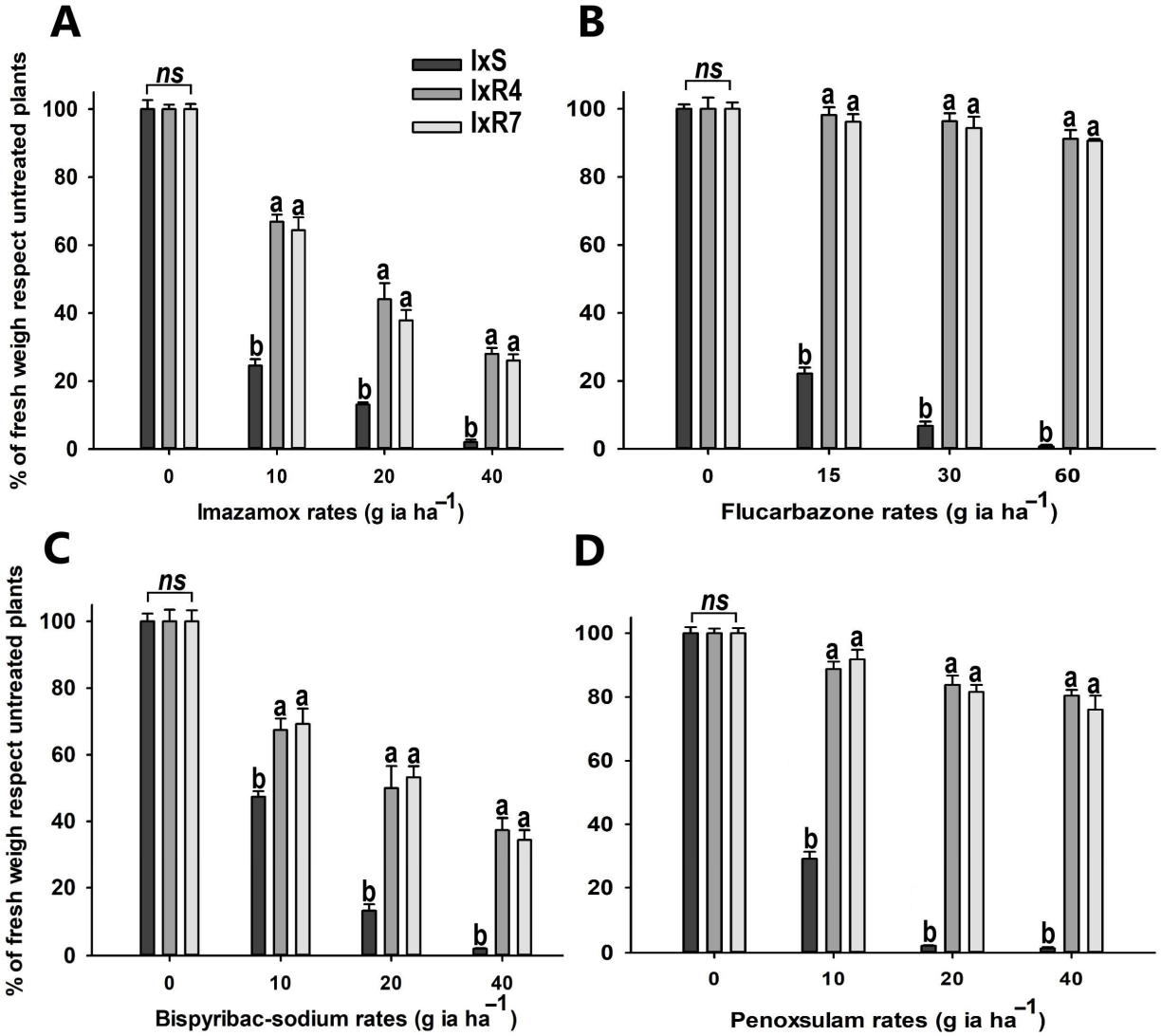

Figure 6. Fresh weights of *Ixophorus unisetus* populations tested (IxS, IxR4, and IxR7) after four ALS-inhibiting herbicides (**A**) imazamox, (**B**) flucarbazone, (**C**) bispyribac-Na, and (**D**) penoxsulam. Bars represent the error mean of the plant treated (n = 20). Different letters represent the grouping after Tukey's test. ns= no significant differences.

3.6. Cross-Resistance In Vitro Assay

The *I. unisetus* (IxS) population was highly susceptible to ALS activity in all the herbicide comparison tests. However, the IxR4 and IxR7 populations showed different levels of enzyme activity. High resistance was observed to some herbicides, such as nicosulfuron, flucarbazone, and penoxsulam, and medium resistance was observed to bispyribac-Na and imazamox (Figure 7). The enzyme activity decreased at 50% in the IxS population with 0.03 µM of nicosulfuron, whereas IxR4 and IxR7 needed 2.3 and 2.8 µM, respectively. Regarding flucarbazone, penoxsulam, the I_{50} activity of IxR4, and IxR7 was

199.5, 168.4 µM of flucarbazone and5.1, and 6.1 µM for penoxsulam, respectively. The response of resistant populations with imazamox and bispyribac-Na was low in comparison with the other herbicides; however, the IxS response was even lower (Figure 7).

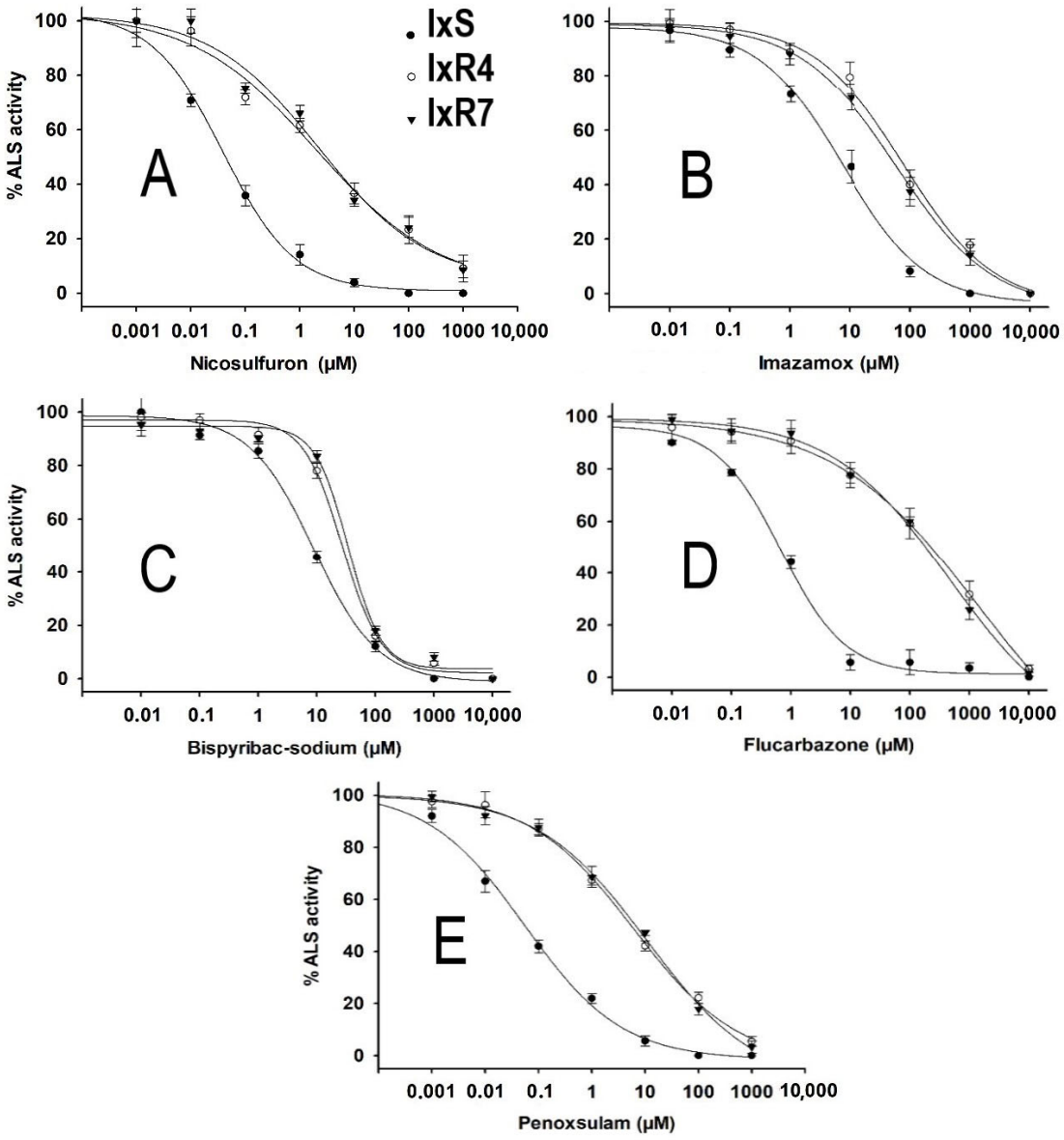

Figure 7. Dose–response curves of acetolactate synthase activity (I_{50}) expressed as percentage when *Ixophorus unisetus* (S and R) was exposed to (**A**) nicosulfuron, (**B**) imazamox, (**C**) bispyribac-Na, (**D**) flucarbazone, and (**E**) penoxsulam. RIs: resistance indices.

3.7. Multiple-Resistance Screening Test

Overall, no multiple resistance was observed in the resistant *I. unisetus* populations. The herbicides petoxamide, pyroxasulfone + fluthiacet, glyphosate, quizalofop, topramezome, tolpyralate, and mesotrione + nicosulfuron showed up to a 90% level of control over the *I. unisetus* populations. The herbicides tembotrione and mesotrione showed lower control levels in the three populations (Table 3). In these herbicides, two or three plants per treatment remained alive.

Table 3. Plant mortality with different herbicide treatments on *Ixophorus unisetus* populations (IxS, IxR4, and IxR7).

Treatment	Plant Mortality (%) [a]					
	IxS		IxR4		IxR7	
Petoxamide	100 ± 0	a	100 ± 0	b	100 ± 0	a
Pyroxasulfone + Fluthiacet	100 ± 0	a	90 ± 0	b	95 ± 7	ab
Glyphosate	100 ± 0	a	100 ± 0	a	100	a
Quizalofop	100 ± 0	a	100 ± 0	a	100 ± 0	a
Tembotrione	85 ± 7	bc	85 ± 0	b	75 ± 7	c
Mesotrione	85 ± 7	bc	90 ± 0	b	85 ± 7	bc
Topramezone	100 ± 0	a	90 ± 14	ab	100 ± 0	a
Tolpyralate	100 ± 0	a	95 ± 7	ab	100 ± 0	a
Nicosulfuron + Mesotrione	100 ± 0	a	100 ± 0	a	100 ± 0	a

[a] Plant mortality represents the mean of plants from replicates of the experiment (n = 20). Different letters correspond to the grouping of Tukey's test.

4. Discussion

The first case of resistance in *I. unisetus* was reported in Costa Rica in 1988 [8]. That report exposed the high level of survival of plants with ALS inhibitors (mostly imidazolinones). Later, the first case in Mexico with high resistance to nicosulfuron (SU) was reported in 2014. However, there are no reports of the resistance mechanisms involved in the resistance of *I. unisetus*. This situation is now a problem because the knowledge of its resistance mechanisms is the basis of good integrated weed management (IWM), in which the use of other chemical alternatives could be used [23].

In this work, we confirm a high resistance to the SU family of herbicides, with GR_{50} values and RIs from 5.7 to 10.1. *I. unistus* from two Mexican corn-growing zones (Jalisco and Guanajuato) were tested for nicosulfuron resistance, and the responses were zone-dependent. The Jalisco populations ranged from 180 to 250 g ai ha^{-1}, whereas the Guanajuato populations were below 160 g ai ha^{-1}. The responses are probably due to different practices of weed management coupled with resistance-mechanism interaction. It is widely recognized that employing a wide range of tools and techniques over time can effectively mitigate the resistance selection pressure in weed management. This approach groups a range of strategies that go beyond the mere use of herbicides [24,25].

With the use of malathion, an inhibitor of the Cyt-P450 enzyme complex, it was found that enhanced metabolic activity contributes to the resistances of five *I. unisetus* populations (IxR1–IxR5). This result is remarkable because the five populations come from the same Mexican zone (Jalisco). This is the first evidence of resistance mechanisms in *I. unisetus* worldwide. Conversely, an interesting point to highlight is the response of IxS with nicosulfuron alone and nicosulfuron plus malathion. With the lowest field rate of nicosulfuron (40 g ai ha^{-1}), plants did not die; however, when malathion was added, all the plants died. In other susceptible grassweed species, malathion does not always reverse GR_{50}. For instance, Christopher et al. [26] found that the malathion reversed GR_{50} in *Lolium rigidum*, while Mei et al. [27] reported that it did not reverse the GR_{50} in *Digitaria sanguinalis*. Our study could be a case of "from tolerance to resistance", where metabolism as a resistance mechanism was selected in the populations from Jalisco.

To understand the resistance mechanisms based on the target site, we partially sequenced the *ALS* gene in three *I. unisetus* populations. In alignment with the other grassweed (*S. viridis*), a change from Asp376 to Glu376 was found. In this work, the change was present in all the plants tested from the two resistant populations, but in none of the susceptible ones (IxS). It is known that Asp376Glu can contribute to a reduced sensibility to ALS-inhibiting herbicides in weeds [28]. Although the level of the resistance is variable, plants exhibiting this mutation had a high resistance to sulfonylureas [8]. Our study confirms these findings regarding the low sensibility to nicosulfuron. However, there is still the

question of herbicide resistance in other ALS families. The target-site mutation Asp376Glu has been found in several broadleaf and grassweed species, such as *Alopecurus japonicus* [29], *Lolium perenne* [30] *Sorghum halepense* [31], and recently *S. viridis* [28]. As shown in Table 4, the resistance spectrum is variable, but in all cases, resistance to sulfonylureas is high.

Table 4. Revision of cross-resistance in grassweeds with Asp376Glu mutation in the *acetolactate synthase* gene.

Species [a]	Herbicide Family Abbreviations [b,c]				
	SU	**IMI**	**SCT**	**PTB**	**TP**
Ixophorus unisetus (studied here) (SW)	R	r	R	r	R
Sorghum halepense (SW)	R	S	R	R	ND
Alopecurus japonicus (WW)	R	R	R	r	R
Setaria viridis (SW)	R	R	ND	ND	ND
Lolium perenne (WW)	R	r	R	ND	R

[a] Summer weed (SW); winter weed (WW). [b] Sulfonylureas (SUs); imidazolinones (IMIs); triazolinones (SCTs); pyrimidinyl thiobenzoates (PTBs); triazolopyrimidine—type 1 (TP). [c] R: high resistance; r: low resistance; S: susceptible; ND: not demonstrated.

Mutations could affect the binding of the herbicide, altering the stability of the herbicide–enzyme complex by changing the three-dimensional structure of the target site [32,33]. Here, we propose three possible reasons: (i) the plants have different biological cycles (summer or winter weeds), which affect the conditions under which the herbicides act (Table 4); (ii) the morphology and physiology of the plants can affect the pathway of the herbicide from the leaf to the target-site enzyme; (iii) the structure of the enzyme.

Regarding the cross-resistance in vitro assay, our results confirm that *I. unisetus* from the two zones from Mexico have resistance to five ALS-inhibiting herbicide families. The high resistance to sulfonylureas, triazolinones, and triazolopyrimidine—type 1 has been found in grasses such as *A. japonicus* and *L. perenne*. The low resistance to imidazolinones corresponds to *L. perenne*, but not to *S. halepense*. Additionally, low resistance to PTB applies only to *A. japonicus*. Therefore, differences in the *ALS* gene sequence are thought to be the most likely reason for the differences in herbicide susceptibility [30].

The tests for the cross-resistance in vivo agree with the above results. The resistant populations tested (IxR4 and IxR7) survived all the herbicides. The responses of both R populations were very similar, which can be attributed to the mutation Glu376. Enhanced metabolism could participate only in sulfonylurea resistance; however, research with Cyt-P450 inhibitors is necessary to prove this hypothesis.

Within NTSR mechanisms, enhanced metabolism could be a major factor in weeds with low susceptibility to herbicides. Nowadays, enhanced metabolism is considered a very important cause that can confer resistance to herbicides with different modes of action (including those that have not yet been marketed) [27,34]. Here, we studied different herbicides with the aim of discriminating possible multiple resistance. Only two herbicides (mesotrione and tembotrione) showed minor impacts on resistant populations (including the sensitive population IxS). Although the average percentage of control is acceptable, further assays are required to discriminate for low tolerance, as well as testing with other metabolism inhibitors.

5. Conclusions

In brief, the resistance of *I. unisetus* from two corn-growing zones of México (the Bajio, México) was demonstrated using dose–response and molecular trials. One population showed high susceptibility to nicosulfuron; nevertheless, its survival at the lowest field dose demonstrated that this species has a naturally low tolerance. Conversely, eight populations showed high nicosulfuron resistance. Overall, the TSR-mutation-based cross-resistance mechanism (Asp376Glu) was involved in the nicosulfuron resistance in the Jalisco and Guanajuato populations. In contrast, the NTS Cyt-P450-based resistance mechanism was

only involved in the populations from Jalisco. Cross-resistance in vitro and in vivo assays indirectly demonstrated that NTSR was involved only in the response to nicosulfuron. The screening for multiple resistance allowed us to identify possible alternative chemicals for *I. unisetus* control; however, other nonchemical measures need to be integrated with alternative MoAs in order to manage this weed in a sustainable manner.

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