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Target-Site Resistance to Nicosulfuron in Johnsongrass (*Sorghum halepense*) from Chilean Corn Fields

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Johnsongrass is a common weed of corn in Chile, which is most often controlled by nicosulfuron, an acetohydroxyacid synthase (AHAS)-inhibiting herbicide. Recurrent nicosulfuron use has resulted in selection for resistant johnsongrass biotypes. We conducted studies to determine nicosulfuron resistance levels in two johnsongrass biotypes from Chile and to investigate if this resistance was target-site mediated. Whole-plant resistance to nicosulfuron was 33 and 46 times higher in resistant (R) than in susceptible (S) plants grown from seed and rhizomes, respectively. The nicosulfuron concentrations for 50% inhibition of AHAS enzyme activity in vitro were more than 11 times higher in R than in S plants. Sequencing analysis of the *AHAS* coding sequence revealed a Trp-574-Leu substitution in both R biotypes. This study shows that resistance to nicosulfuron in the two R biotypes is conferred by an altered target site. We also report the first consensus sequence of the johnsongrass *AHAS* gene corresponding to the known mutation sites conferring resistance to AHAS-inhibiting herbicides.

Nomenclature: Nicosulfuron; johnsongrass, *Sorghum halepense* (L.) Pers.; corn, *Zea mays* L.
Key words: Acetohydroxyacid synthase (AHAS), acetolactate synthase (ALS), AHAS-inhibiting herbicides, polyploidy.

Corn is the second most important annual crop in Chile, where it occupies approximately 141,000 ha per year with 23% of this area used for seed production (ODEPA 2013). Corn is grown in the central zone of the country, with 73% of the production harvested within the O'Higgins and Maule Regions. Genetically modified (GM) crops can only be grown for seed production in Chile, and transgenic varieties of corn resistant to glyphosate and glufosinate are the most commonly grown GM crops.

Johnsongrass is a perennial, highly competitive grass and an important weed in crops worldwide, such as corn (*Z. mays*), grain sorghum [*Sorghum bicolor* (L.) Moench], and soybean [*Glycine max* (L.) Merr] (Holm et al. 1977). This allotetraploid species (2n = 40) (Celarier 1958) is thought to be derived from naturally occurring crosses between *S. bicolor* and *Sorghum propinquum* (Kunth) Hichc. (both 2n = 20) followed by chromosome doubling (Bowers et al. 2003). Johnsongrass is difficult to control because it produces large quantities of seeds and rhizomes (McWhorter 1972). It is spread through agricultural areas mainly by dispersal of clonal fragments (rhizomes) or by seed dispersal after mostly self-pollination (Warwick and Black 1983), a strategy that would lead to increased homozygosity over time (Fernández et al. 2013). Full-season interference by heavy infestations of johnsongrass can reduce corn yields by 74% to 100% (Bendixen 1986).

Soil cultivation alone does not control johnsongrass effectively, because it can increase the density and spread short rhizomes. The sectioning of large rhizomes releases bud dormancy and results in greater number of emerging shoots (McWhorter 1972). Herbicides applied PRE can control johnsongrass plants from seed but are less effective against rhizome plants (McWhorter 1989). POST applications of AHAS-inhibiting herbicides in corn (Foy and Witt 1990) or acetyl coenzyme-A carboxylase (ACCase)-inhibiting herbicides in broadleaf crops (Kaloumenos and Eleftherohorinos 2009) provide very good control of both seedling and rhizome johnsongrass plants. Also, POST applications of glyphosate in conjunction with GM glyphosate-resistant crops can successfully control johnsongrass plants from seed and rhizomes as well.

The AHAS-inhibiting herbicide nicosulfuron labeled for POST use in corn is effective on

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seedling- and rhizome-emerged johnsongrass. This herbicide thus fills a critical void in POST weed management options for corn (Gubbiga et al. 1996), especially in Chile where it has been used since 1995. Recently, failures to control johnsongrass with commercial nicosulfuron applications have been reported for fields with a history of 3 to 10 yr of nicosulfuron use.

The target enzyme of nicosulfuron is AHAS (EC 2.2.1.6), also referred to as acetolactate synthase (ALS), which catalyzes the first committed step in the pathway for the biosynthesis of the branched chain amino acids valine, leucine, and isoleucine (Duggleby and Pang 2000). There are five chemical families of AHAS-inhibiting herbicides that have been commercialized: sulfonylureas (SU), imidazolinones (IMI), triazolopyrimidines (TP), pyrimidinyl(thio)benzoates (PTB), and sulfonylamino-carbonyl-triazolinones (SCT). Resistance to AHAS-inhibiting herbicides most often results from a single amino acid substitution in the AHAS enzyme, but can also arise from enhanced metabolism (Fischer et al. 2000; Yu and Powles 2014). Mutations in the herbicide-binding region prevent herbicide binding, rendering the altered enzyme insensitive to the herbicide (Corbett and Tardif 2006). Point mutations at eight positions in the *AHAS* gene causing amino acid substitutions at Ala-122, Pro-197, Ala-205, Asp-376, Arg-377, Trp-574, Ser-653, and Gly-654 have been reported to confer resistance to AHAS-inhibiting herbicides in weeds with substitutions at Pro-197 and Trp-574 being the most common resistance mutations (Beckie and Tardif 2012; Tranel et al. 2013). Various cross-resistance patterns result from the different mutations. For example, the Trp-574-Leu substitution confers resistance to all families of AHAS-inhibiting herbicides in many weed species, while the Ala-122-Thr substitution confers weed resistance to IMI but not to SU herbicides (Beckie and Tardif 2012; Powles and Yu 2010).

In 2000, the first case of johnsongrass resistance to nicosulfuron was reported in corn fields of Texas. Since that time, nicosulfuron-resistant johnsongrass has been identified in other US states and in México, Italy, and Venezuela, of which the mechanism of resistance was only studied for the Mexican biotypes and attributed to an insensitive altered target site (Heap 2014). However, the *AHAS* gene mutation(s) conferring resistance to nicosulfuron were not identified.

Given the relevance of johnsongrass as a troublesome weed and nicosulfuron for its control in Chilean corn production, the objectives of the present study were: to confirm and determine the level of resistance to nicosulfuron in johnsongrass biotypes from Chile; to elucidate if the mechanism of resistance relates to an altered AHAS enzyme, and, if so, to identify the mutation(s) possibly associated with such resistance.

Table 1. Nicosulfuron control of johnsongrass biotypes collected in Chilean corn farms assessed 15 and 30 d after treatment (DAT) with 105 g nicosulfuron ha^{-1} (double the recommended field rate). Data are visual ratings on a 0 to 100 scale (0 = no control, 100 = complete control); each value is the mean ± standard error (n = 8).

	Control (%)	
Biotype	15 DAT	30 DAT
Codegua	75.0 ± 3.7	96.2 ± 2.6
Coinco	73.7 ± 4.6	80.0 ± 5.0
Curepto	62.5 ± 3.1	72.5 ± 1.6
Doñihue 1	65.0 ± 5.0	71.2 ± 3.5
Doñihue 2	25.0 ± 1.8	35.0 ± 5.0
Melipilla 1	80.0 ± 5.6	92.5 ± 3.6
Melipilla 2	68.7 ± 3.9	88.8 ± 4.8
Malloa	55.0 ± 2.6	66.2 ± 7.8
Nancagua	7.5 ± 3.1	11.3 ± 2.9
Peñaflor	80.0 ± 4.6	91.2 ± 2.3
Rancagua	58.7 ± 2.2	76.2 ± 2.6
San Joaquín[a]	82.5 ± 3.6	—
Talagante	75.0 ± 3.7	97.5 ± 1.6

[a] Susceptible biotype.

Materials and Methods

Plant Material Collection and Preliminary Screening. During the spring of 2010, johnsongrass seeds and rhizomes were collected from 12 corn fields in the Chilean central valley (Table 1), between the Metropolitan Region and the O'Higgins Region (from 33°26′S, 70°39′W to 34°10′S, 70°43′W). These fields had been treated annually with nicosulfuron (52.5 g ha^{-1}) for more than 3 yr, and johnsongrass control had become erratic with this herbicide in recent years. Herbicides with other mechanisms of action had also been used in the different locations sampled (Table 2). At each location, seeds and rhizomes were collected randomly from approximately 20 surviving plants after nicosulfuron application. Seeds and rhizomes were also collected from San Joaquin, a site in the Metropolitan Region that had not been exposed to any herbicide application. This biotype (hereafter referred to as SJ) was considered nicosulfuron-susceptible (S). Plants obtained from the rhizomes collected were screened with

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Table 2. Cropping years, crop rotation, and seasons of herbicide use (2006 and 2011) at the johnsongrass biotype collection sites.

	Cropping history		WSSA herbicide group[a]					
			2[b]	5[c]	27[d]	15[e]	10[f]	9[g]
Biotype	Years	Crop rotation	No. of seasons					
Coinco	5	No	4[1], 3[2]	3	1[1]	3[1], 1[2]	3	4
Codegua	7	No	5[1]	5	4[1]	2[4], 3[2]	5	5
Curepto	14	Yes	2[1],1[2]	6	1[1]	3[4]	1	6
Doñihue 1	6	No	6[1]	4		2[2], 1[3]	3	6
Doñihue 2	8	No	6[1]	5		6[2]		6
Melipilla 1	4	Yes	3[1]	3	3[2]			3
Melipilla 2	7	Yes	3[1]	5		5[2]	1	1
Malloa	6	No	4[1]	4	1[1]	4[1], 1[2]	3	4
Nancagua	12	No	6[1]	6	3[1]	4[2]		
Peñaflor	7	No	1[1], 2[1]				2	5
Rancagua	12	No	6[1]	6		1[1], 5[2]		1
Talagante	5	No	3[1], 2[2]	4			1	4

[a] Shaner DL, ed (2014) Herbicide Handbook. 10th edn. Lawrence, KS: Weed Science Society of America. Pp 11–15.
[b] [1] Nicosulfuron; [2] dicamba + nicosulfuron.
[c] Atrazine.
[d] [1] Topramezone; [2] Tembotrione.
[e] [1] Metolachlor; [2] atrazine + metolachlor; [3] acetochlor; [4] alachlor.
[f] Ammonium glufosinate.
[g] Glyphosate.

nicosulfuron from January to March 2011 at the School of Agronomy and Forestry, Pontificia Universidad Católica de Chile in Santiago. Screening was conducted in the greenhouse (20 to 33 C and 16 h photoperiod under supplemental 400 µmol m^{-2} s^{-1} photosynthetic photon-flux density from high-pressure sodium lamps). Rhizomes were cut into 3-cm segments with only one bud each. Six rhizomes per pot were planted (3 cm depth) in 1.1 L pots containing a mixture of peat : perlite (3 : 1) and fertilized with 27.0 g of a commercial N–P–K (16–8–12) plus micronutrients mixture (Basacote® Plus 6M, COMPO Expert Spain S.L., Barcelona, Spain) and were thinned down to two uniform plants per pot. Plants were irrigated regularly. At the four- to five-leaf stage (30-cm height), plants from all sites were screened with 2X nicosulfuron (for X = 52.5 g ha^{-1}) using a commercial formulation (FUROR®, Agrícola Nacional S.A.C, Santiago, Chile) plus adjuvant (0.1% v/v nonionic surfactant, ZOOM® 50, Agrícola Nacional S.A.C., Santiago, Chile). The herbicide was applied with a backpack sprayer using a flat fan-nozzle to deliver a volume of 240 L ha^{-1} under 150 kPa. Fifteen and 30 d after treatment (DAT), control was visually rated on a 0 to 100 scale (0 = no control, 100 = complete control of all plants). Three replicates were used for the nontreated (control) and eight for the treated pots. Treatments (combinations of biotypes and nicosulfuron rates) were completely randomized. The experimental unit was one pot with two plants.

The two most resistant populations, biotypes Doñihue 2 (D) and Nancagua (N) and the susceptible SJ biotype were used in the dose-response experiments with rhizomes, the AHAS activity in vitro assays, and the *AHAS* gene sequencing subsequently described. Six individual rhizomes from each of the three biotypes were propagated in the greenhouse in 10-L pots containing the same substrate and fertilizer described earlier to produce needed rhizomes.

Whole-Plant Dose Response. Two experiments were conducted in the greenhouse using plants grown from seeds or from rhizomes of the resistant (D and N) and the susceptible (SJ) biotypes.

For the experiment using seedling plants, seeds were immersed in sodium hypochlorite bleach following Vila-Aiub et al. (2007) to overcome seed dormancy. The treated seeds were placed in 1.1 L pots containing the same substrate and fertilized as described earlier, and were covered with 1 cm of the substrate mix. After emergence, seedlings were thinned down to two seedlings per pot. For the experiment with shoots emerging from rhizomes, plants were grown from the new rhizomes obtained as described in the preceding section. Plants of both experiments were treated at the four- to five-leaf

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Table 3. Nucleotide sequences of oligonucleotide primer sets used for amplification of different fragments of the johnsongrass *AHAS* gene. The degeneration used is the standard code: b (not A), n (any), m (A or C), r (A or G), and y (C or T).

Set	Primer name	Sequence (5′–3′)	Targeted mutation[a]
1	ALS-U-295[b]	AGGGGCGCCGATAtyctygtbgargc	Ala-122, Pro-197, Ala-205
	ALS-L-1170[b]	ACAGTCCCATGCatnccmarcat	
2	AHAS-Sb F	CTGTTCTTTATGTTGGTGGT	Asp-376, Arg-377, Trp-574, Ser-653, Gly-654
	AHAS-Sb R	TATCTGTAGCAAAAGGCACT	

[a] Numbering of amino acids using as reference *Arabidopsis thaliana AHAS* gene (X51514).
[b] Primers designed by Prado et al. 2004.

stage (25 to 30-cm height) with nicosulfuron using eight rates ranging from 0 to 1,680 g ha^{-1} (biotypes D and N) or from 0 to 420 g ha^{-1} (susceptible SJ biotype). The herbicide formulation and spraying conditions were the same as described earlier. Twenty-one DAT, shoot fresh weight per pot was measured (Yasuor et al. 2009). The experiments followed a completely randomized design with three replicates per treatment (factorial combination of nicosulfuron rates and biotypes). The experimental unit was one pot with two plants. Both experiments were carried out twice.

AHAS Inhibition In Vitro Assay. The AHAS activity was measured ''in vitro'' using a modification of Ray's method by detection of acetoin obtained after the decarboxylation of the reaction product, acetolactate, in the presence of acid (Ray 1984). Following the procedure of Fischer et al. (2000) and Osuna et al. (2002), leaf tissue (3.5 g) from shoots at the three- to four-leaf stage was used. The nicosulfuron used at active ingredient (technical grade, 96.67 % purity; Agrícola Nacional S.A.C, Santiago, Chile) concentrations of 0, 10^{-2}, 10^{-1}, 1, 10, 10^2, and 10^3 μM. The assay was conducted three times, each time with a new biological sample, and the enzyme activity for each herbicide concentration was determined in triplicate.

***AHAS* Gene Sequencing.** DNA from three plants from each biotype was extracted using 100 mg of young leaf tissue using a DNeasy Plant Mini Kit (QIAGEN Inc., Valencia, CA). Genomic DNA was quantified by NanoDrop 2000c (Thermo Scientific, CA). Primers were designed for amplification of conserved domains where mutation sites endowing resistance to AHAS-inhibiting herbicides have been previously identified (Table 3) (Powles and Yu 2010; Tranel et al. 2013). Because no information regarding the *AHAS* gene sequence of johnsongrass was available, the *AHAS* gene sequences of the following Poaceae species from the National Center for Biotechnology Information (NCBI; Bethesda, MD: http://www.ncbi.nlm.nih.gov) database were used for primer design: grain sorghum (*S. bicolor*) (XM002452104), rice (*Oryza sativa* L.) (AY885675), Italian ryegrass (*Lolium multiflorum* Lam.) (AF310684), and downy brome (*Bromus tectorum* L.) (AF488771). ClustalX2 was used for sequence alignment and primers were designed using the Primer3 software. Also, a set of universal and degenerate primers designed by Prado et al. (2004) was tested. The primer pairs finally employed with their complete sequences and the targeted point mutations (reportedly conferring resistance to AHAS-inhibiting herbicides) corresponding to the amplified region of the gene are presented in Table 3. For the primer pair ALS-U-295/ALS-L-1170, polymerase chain reaction (PCR) was conducted using 30 μL reaction mixture of 1X Phusion HF Buffer, 1.5 mM $MgCl_2$, 0.5 μM of each forward and reverse primer, 0.3 mM dNTP mix (Invitrogen, CA), one unit Phusion® High-Fidelity DNA Polymerase (Thermo Scientific), and 30 ng of genomic DNA. PCR was conducted in a Veriti™ thermocycler (Applied Biosystems, CA) with an initial 2-min DNA denaturation cycle at 98 C, followed by 35 cycles at 98 C for 10 s, 71 C for 15 s, and 72 C for 1 min; and a final extension cycle at 72 C for 10 min. PCR with primer pair AHAS-Sb F/AHAS-Sb R was conducted in 30 μL reaction mixture 1X High Fidelity PCR Buffer, containing 2.0 mM $MgSO_4$, 0.2 μM of each forward and reverse primer, 0.2 mM dNTP mix, one unit Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen) and 30 ng genomic DNA. PCR cycles included one cycle of 94 C for 2 min, and 35 cycles of 94 C for 30 s, 57 C for 30 s, and 68 C for 1.2 min of elongation. Amplified fragments were purified in 1% agarose gel with Wizard® SV Gel and PCR Clean-up System (Promega, Madison, WI), and cloned in *Escherichia coli* DH5α using a pGem®-T Easy Vector (Promega). Plasmid DNA was extracted using an AxyPrep Plasmid Miniprep (Axygen, CA). At least six selected

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clones from three plants per biotype were sent for sequencing to Macrogen Korea (Seoul, Korea) plasmid sequencing service (www.macrogen.com). DNA sequences from the R and S biotypes were analyzed using ClustalX2.

Statistical Analysis. The dose-response data from repeated experiments were pooled for analysis, because there were no differences between experiments and no treatment by treatment interactions. Pooled data were subjected to nonlinear regression analysis using a three-parameter log-logistic equation (Ritz 2010):

$$y = d \Big/ \left[1 + (x/ED_{50})^{b}\right] \qquad [1]$$

Where y is the fresh weight, d is the mean response when the herbicide rate approaches zero (upper limit), ED_{50} is the herbicide dose that results in 50% growth reduction with respect to the nontreated control and the lower limit (assumed to be zero by this model), b is the slope of the line at ED_{50}, and x is the nicosulfuron rate. ED_{50} was calculated with fresh weight values (g pot^{-1}) and for presentation purposes data in Figure 1 are expressed as percentage of the nontreated control (Ritz 2010).

The *AHAS* inhibition in vitro assay was analyzed using a four-parameter log-logistic equation (Price et al. 2012):

$$y = c + \left\{(d - c) \Big/ \left[1 + (x/I_{50})^{b}\right]\right\} \qquad [2]$$

Where y is the AHAS activity, c and d are the lower and upper asymptotes at high and zero herbicide concentrations, respectively; b is the slope and I_{50} is the effective dose causing 50% reduction of the AHAS activity with respect to the control and the lower limit. Regression curves were fitted using SAS NLIN and plotted using Sigmaplot® software (version 10.0). Values of ED_{50} and I_{50} were considered to be statistically different when their respective 95% confidence intervals did not overlap (Alarcón-Reverte et al. 2013). Resistance indices (RI) from the plant-dose response and the AHAS assays were calculated as R to S ratios of the ED_{50} or the I_{50}.

Results and Discussion

Corn is generally grown as a monoculture in Chile and only 25% of the corn farms in this study exhibited some degree of crop rotation. Five different herbicide groups have been used in the region sampled (Table 2) to control johnsongrass in

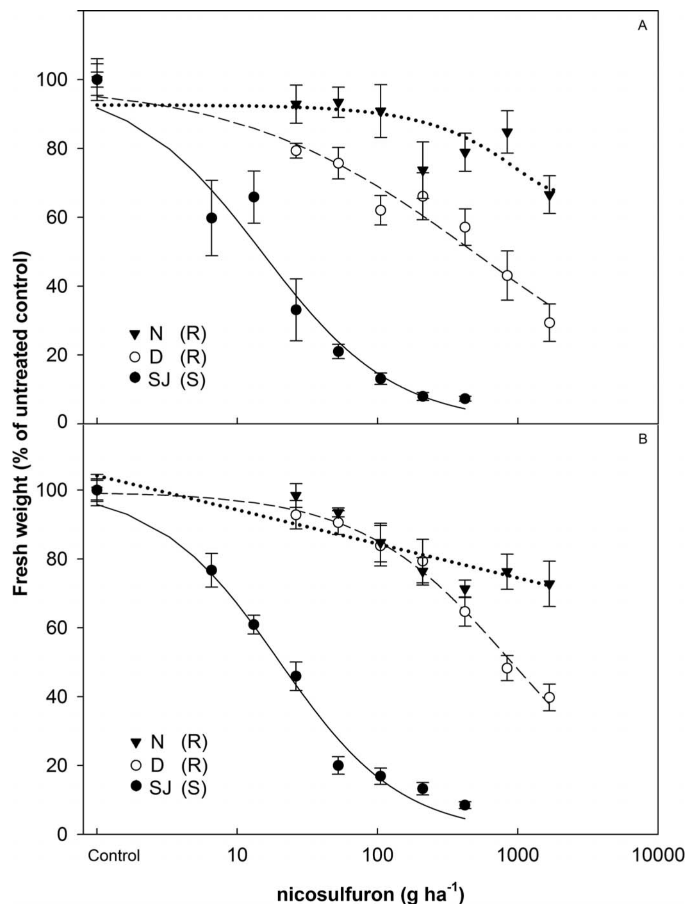

Figure 1. Shoot fresh weight responses of resistant (N, ▼ and D, ○) and susceptible (SJ, ●) johnsongrass biotypes, from seeds (a) and rhizomes (b), to increasing rates of nicosulfuron. Plants were sprayed at the four- to five-leaf stage (30 cm height) and harvested 21 d after treatment. Data are fresh weights expressed as percent of the mean nontreated control; each data point is the mean ± standard error (n = 6). Regression parameters are presented in Table 4.

corn with various levels of effectiveness (e.g., tembotrione controls seedlings but not established plants with rhizomes). However, in all sampled fields the only AHAS-inhibiting herbicide used was nicosulfuron.

Preliminary Resistance Screening. By 30 DAT, visual ratings of johnsongrass control across the 12 sampled sites ranged from 100% (for the susceptible SJ plants) to 11% (Table 1). As mentioned earlier, out of the 12 sites, johnsongrass control was least at Doñihue 2 and Nancagua where selection for nicosulfuron resistance was presumed to be at an advanced stage. Locations with control levels between 60% and 70% may represent fields at intermediate stages of selection for nicosulfuron resistance. Resistance to herbicides can evolve in johnsongrass and spread throughout large areas following recurrent use by the same herbicide (Heap

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Table 4. Parameter estimates for the log-logistic equation (Equation 1) used to calculate the herbicide concentration required for 50% reduction of aboveground fresh weight (ED_{50}) of three johnsongrass biotypes grown from seeds and from rhizomes in dose-response experiments and ratios of the ED_{50} values of resistant to susceptible biotypes (Resistant Index [RI]).

	Plant source											
	Seedlings						Rhizome plants					
Biotype	*d*	*b*	ED_{50}		*P* value	RI[b]	*D*	*b*	ED_{50}		*P* value	RI[b]
			g ha^{-1}	95% CI[a]					g ha^{-1}	95% CI[a]		
N	Model did not fit[c]		> 1,680	—	—	> 109.0	Model did not fit[d]		> 1,680	—	—	> 84.4
D	39.9	0.5	502.3	218.8–785.9	< 0.0009	33.2	49.6	0.8	916.6	627.5–1205.7	< 0.0001	46.0
SJ	33.1	0.9	15.1	8.5–21.7	< 0.0001	—	40.42	0.9	19.9	15.8 – 24.3	< 0.0001	—

[a] Abbreviation: CI, confidence interval.
[b] RI is the ratio of the ED_{50} values of resistant to susceptible biotypes.
[c] Fresh weight of nontreated control equals 35.04 ± 1.6 g plant^{-1}.
[d] Fresh weight of nontreated control equals 33.7 ± 1.1 g plant^{-1}.

2014). This may explain the finding of widespread infestations of glyphosate R johnsongrass throughout glyphosate-R soybean fields in the provinces of Salta and Tucumán in Argentina, that were regularly treated with this herbicide (Vila-Aiub et al. 2007). Similarly, weeds R to AHAS-inhibiting herbicides readily evolve where AHAS-inhibiting herbicides are repeatedly used over an extended period of time on a certain area (Busi et al. 2012; Merotto et al. 2010; Shaner et al. 1997). Previous research (Shaner et al. 1997) has determined that resistance to AHAS-inhibiting herbicides evolves much slower when they are part of a more diverse weed management program. This may be the case in Chilean corn fields where diversification of herbicide use could have limited the spread of nicosulfuron resistance in the fields we surveyed.

Whole-Plant Dose Response. The dose-response assay on whole plants from both seeds and rhizomes confirmed that biotypes D and N are both resistant to nicosulfuron (Figure 1). Plants from these biotypes were less affected by nicosulfuron than plants from the susceptible SJ biotype. Treatment of seedlings with the recommended field rate of nicosulfuron for johnsongrass control in Chile (52.5 g ha^{-1}) reduced shoot growth of S plants by more than 80%, while shoot growth in plants from R biotypes N and D was reduced by less than 10% and 25%, respectively (Figure 1). Similarly, the same treatment reduced shoot growth of S plants emerging from rhizomes more than 80%, while shoot growth was reduced by less than 10% for plants from R biotypes N and D (Figure 1). The ED_{50}-based resistance level (RI) of the D biotype was 33.2 and 46 for seedling and rhizomatous plants, respectively (Table 4). At 32-fold the recommended field rate (1,680 g nicosulfuron ha^{-1}) plants from the R biotype N had a fresh weight reduction of 30%, for both seedling and plants grown from rhizomes (Figure 1). Due to the weak dose response, the regression model could not be fitted to calculate an ED_{50} for the N biotype; this parameter would have a value outside the dose range used. Thus, the ED_{50} for the N biotype is expressed as > 1,680 g ha^{-1} and the RI would be > 109.0 and > 84.4 for seedlings and plants grown from rhizomes, respectively (Table 4). The high resistance level of D and N plants would be compatible with reduced sensitivity of the target AHAS enzyme to inhibition by the herbicide (Merotto et al. 2009).

In Vitro AHAS Assay. Results from the assay in our study suggested the presence of an altered, nicosulfuron-insensitive, target site in both R biotypes (D and N). In the absence of nicosulfuron, the AHAS specific activity was not different among biotypes; acetoin levels were 9.5 (± 0.51), 10.8 (± 0.3), and 8.9 (± 0.2) nmol mg^{-1} protein min^{-1} for SJ, N and D plants, respectively. However, enzyme incubation under increasing nicosulfuron concentrations resulted in differential AHAS activity responses between enzyme extracts from R (N and D) and S (SJ) plants (Figure 2). The AHAS enzyme extracted from plants of johnsongrass biotypes N and D was 11 and 16 times less

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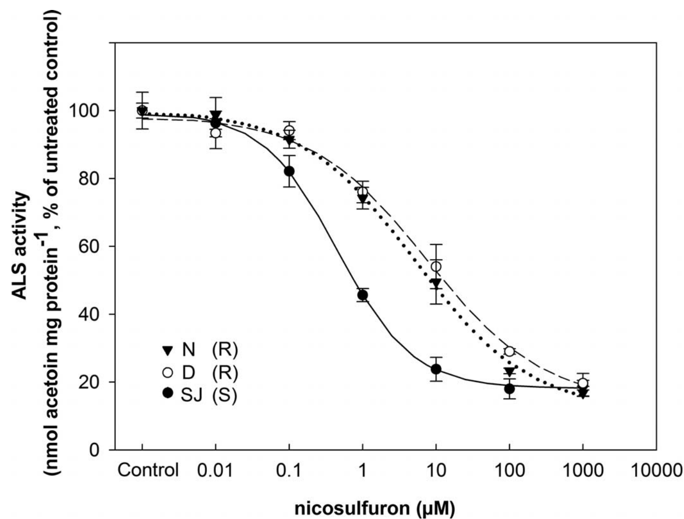

Figure 2. In vitro inhibition of acetohydroxyacid synthase (AHAS) specific activity by nicosulfuron in tissue extracts from three- to four-leaf stage shoots of resistant (N, ▼ and D, ○) and susceptible (SJ, ●) johnsongrass biotypes. Data are nmol of acetoin mg^{-1} of protein min^{-1} expressed as percent of no herbicide control; each data point is the mean ± standard error ($n = 3$). Regression parameters are presented in Table 5.

sensitive to nicosulfuron than that of S plants according to I_{50}-based RI values, respectively (Table 5). However, there was inconsistency between resistance at the enzyme and whole-plant levels. Although the I_{50}-values were similar for D and N plants, nicosulfuron insensitivity at the whole plant level was greater for the N biotype. Lower sensitivity to in vitro assays compared to whole-plant responses has also been documented for other polyploid species, *Bidens subalternans* DC. (Lamego et al 2009), barnyardgrass [*Echinochloa crus-galli* (L.) Beauv.] (Panozzo et al. 2013), and monochoria [*Monochoria vaginalis* (Burm.f.) C.Presl ex Kunth] (Kuk et al. 2003). Differential expression of mutated and "wild type" alleles or homoeologous *AHAS* gene copies in polyploid species can cause variable contributions of the insensitive altered AHAS to the total AHAS enzyme pool ("dilution effect"), which could cause discrepancies between resistance at enzyme vs. whole-plant level (Panozzo et al. 2013; Riar et al. 2013; Yu et al. 2013). Although in the present study the specific AHAS activity was similar between R and S biotypes in the absence of herbicide, the relative expression of mutated and "wild type" variants of the *AHAS* gene is unknown for plants in our study. Contribution by unknown nontarget site mechanisms to the overall plant resistance could be another reason for differences between enzyme assays and whole-plant responses. Although there are fewer cases of non-target site resistance to AHAS-inhibiting herbicides, enhanced herbicide metabolism has been well documented as a mechanism of resistance in other species (Fischer et al. 2000; Veldhuis et al. 2000; Yasuor et al. 2009). Both an insensitive target-site and enhanced herbicide metabolism can contribute to the resistance observed in certain biotypes to AHAS-inhibiting herbicides (Figueroa et al. 2007; Kuk et al. 2003; Riar et al. 2013).

***AHAS* Gene Sequencing.** Mutations in the *AHAS* gene coding for a conserved herbicide binding region prevent herbicide binding, making the altered enzyme insensitive to the herbicide (Corbett and Tardif 2006). Structurally different AHAS-inhibiting herbicides orientate differently in the herbicide binding region, with partial overlap. Thus, a particular amino acid substitution within the herbicide binding region can confer resistance to some but not to other AHAS-inhibiting herbicide (Powles and Yu 2010).

Primer pair ALS-U-295/ALS-L-1170 amplified a fragment of 772 bp, and primer pair AHAS-Sb F/AHAS-Sb R amplified a fragment of 1,364 bp. Assembly of the sequences from these fragments

Table 5. Parameter estimates for the log-logistic equation (Equation 2) used to calculate the herbicide concentration required for inhibition of the acetohydroxyacid synthase (AHAS)-enzyme specific activity (nmol mg^{-1} protein min^{-1}, and I_{50} values obtained of three johnsongrass biotypes in AHAS in vitro experiments and ratios of the I_{50} values of resistant to susceptible biotypes (Resistant Index [RI]).

Biotype	*b*	*c*	*d*	I_{50}		P value	RI[b]
				µM	95% CI[a]		
N	0.5	1.2	10.8	5.16	2.34–7.98	> 0.0001	11.5
D	0.5	1.2	8.7	7.46	1.77–13.14	> 0.0001	16.6
SJ	0.8	1.7	9.5	0.45	0.19–0.71	> 0.0001	—

[a] Abbreviation: CI, confidence interval.
[b] RI is the ratio of the I_{50} values of resistant to susceptible biotypes.

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AHAS gene N  CACCTGGGGATGGTGGTGCAGTKGGAGGACAGGTTCTAT
               H  L  G  M  V  V  Q W/L E  D  R  F  Y
AHAS gene D  CACCTGGGGATGGTGGTGCAGTKGGAGGACAGGTTCTAT
               H  L  G  M  V  V  Q W/L E  D  R  F  Y
AHAS gene SJ CACCTGGGGATGGTGGTGCAGTGGGAGGACAGGTTCTAT
               H  L  G  M  V  V  Q  W  E  D  R  F  Y
             ------------------------------------------574--------------------------------
```

Figure 3. Partial DNA sequencing of the *AHAS* gene of plants from nicosulfuron-resistant (N and D) and –susceptible (SJ) johnsongrass biotypes. Observed polymorphisms are marked in bold and correspond to the Trp-574 position of the *A. thaliana AHAS* gene (X51514).

produced a 1,977 bp DNA sequence of the *AHAS* gene, which included conserved domains where mutation sites endowing resistance to AHAS-inhibiting herbicides have been previously identified (Table 3) (Powles and Yu 2010; Tranel et al. 2013). These sequences were submitted to GenBank (NCBI) as accession numbers KJ538785, KJ538786, and KJ638787 for the SJ, D, and N biotypes, respectively. The deduced amino acid sequence of this fragment contained 659 residues (including stop codon) without introns showing a high level of identity (90% to 99%) with other *AHAS* gene sequences from the following Poaceae species (% sequence identity and GenBank number is indicated): grain sorghum (99%, XM002452104.1), corn (96%, X63553.1), and rice barnyardgrass [*Echinochloa phyllopogon* (Stapf) Koso-Pol.] (90%, AB636380.1 and AB63381.1). In the R biotypes, a point mutation (TGG to TTG) resulting in an amino acid substitution from tryptophan to leucine at position 574 (Trp-574-Leu) of the AHAS enzyme was identified (Figure 3). No other polymorphisms were observed in the different biotypes. Analysis of cloned fragments from both R biotypes revealed sequences with and without the Trp-574-Leu substitution. Flow cytometry analysis (Plant Cytometry Services, Postbus 299, 5480 AG Schijndel, The Netherlands) confirmed the tetraploidy of the johnsongrass plants in this study (data not shown), establishing at least the existence of two *AHAS* gene copies; the presence of mutated and ''wild-type'' alleles was suggested in other tetraploid species with multiple copies of the *AHAS* gene (Panozzo et al. 2013; Riar et al. 2013). Studies of plants with herbicide-resistant mutated AHAS enzymes are most common in diploid species. Recently, studies in polyploid species have also been conducted and show that higher plants can have different numbers of *AHAS* genes depending on the level of ploidy (Grula et al. 1995; Iwakami et al. 2012; Lamego et al. 2009; Rutledge et al. 1991; Scarabel et al. 2010).

The mutation identified in both R johnsongrass biotypes has been reported to confer broad cross-resistance across different groups of AHAS-inhibiting herbicides, including nicosulfuron. Therefore, gene expression causing the substitution at Trp-574-Leu in the AHAS enzyme of both R biotypes is the most likely cause of the nicosulfuron resistance observed in this study (Beckie and Tardif 2012; Kaloumenos et al. 2013; Tranel et al. 2013).

Resistance to nicosulfuron in other weeds has also been attributed to target site mutations. Amino acid substitutions at Trp-574-Leu in the *AHAS* gene of early watergrass [*Echinochloa oryzicola* (Ard.) Fritsch] conferred cross-resistance to penoxsulam, bispyribac-sodium, imazamox, nicosulfuron, and several sulfonylurea herbicides (Kaloumenos et al. 2013). Mutations causing substitutions for serine at position 653 and for glycine at position 654 were associated with cross-resistance to imazapyr, nicosulfuron, flucarbazone, and pyrithiobac in green foxtail [*Setaria viridis* (L.) Beauv.] (Laplante et al. 2009).

We found the Trp-574-Leu mutation in sequences from all plants sampled from both R biotypes; Trp-574-Leu is reportedly the most common amino acid substitution conferring herbicide resistance to AHAS-inhibiting herbicides across 27 species (Heap 2014). Johnsongrass plants carrying the Trp-574-Leu substitution may have been abundant among individuals in the R biotypes from our study, given the negligible impact this mutation has been shown to have on AHAS functionality and plant fitness in other species (e.g., rigid ryegrass (*Lolium rigidum* Gaudin) (Yu et al. 2010). However, the presence of other undetected mutations among our R plants cannot be ruled out.

Johnsongrass reproduces sexually and vegetatively. Mutations in the *AHAS* gene conferring herbicide resistance are generally inherited as partially dominant nuclear genes; thus resistance can be spread by both seed and pollen (Tsuji et al. 2003). Further studies to investigate the genetic diversity of the R and S johnsongrass biotypes across Chilean corn fields to elucidate the relative contribution of independent evolutionary events and of seed and rhizome dispersal to the spread of AHAS-inhibiting herbicide resistance will be critical to devise mitigation strategies. For example, gene flow of nontarget site resistance to glyphosate has been limited among johnsongrass biotypes in soybean fields of northern Argentina, where independent evolutionary events appear to have determined the current spread of resistance (Fernández et al. 2013). In such cases, in-field

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strategies to decrease the intensity of herbicide selection would be prioritized over attempts to reduce seed movement across distant locations (Tsuji et al. 2003). Future studies on AHAS-resistance evolution in this species will need to clarify the number of genes involved, their expression and inheritance (Riar et al. 2013).

This is the first report of a johnsongrass *AHAS* gene sequence corresponding to the conserved regions where mutations are known to endow herbicide resistance and a point mutation in that region causing a Trp-574-Leu substitution in the johnsongrass AHAS enzyme. Previous studies with other species have demonstrated the association between this mutation and broad-spectrum resistance across five chemical families of AHAS-inhibiting herbicides (Beckie and Tardif 2012; Tranel et al. 2013). Field expression of this AHAS-enzyme alteration would preclude the use of AHAS-inhibiting herbicides in the corn farms where our R biotypes originated and in those other farms where suboptimal control suggests intermediate levels of selection for nicosulfuron resistance. Similarly, the introduction of imidazolinone-resistant (Clearfield®) corn would also be precluded in those farms. The diversification of weed control (crop rotation, rotation of herbicides with different modes of action) observed in many of the corn farms surveyed (Table 2) may have reduced selection pressure and limited the spread of nicosulfuron resistance across corn farms (Gressel and Segel 1990). This observation, together with the detection of a target-site resistance mechanism, suggests johnsongrass control in these Chilean corn fields should contemplate the use of herbicides with a different mode of action. However, the contribution of nontarget site endowed resistance, such as enhanced metabolism cannot yet be discarded and requires further study, because it endows resistance to different and unrelated herbicides, thus compromising the effectiveness of herbicide rotation to mitigate resistance evolution (Yuan et al. 2010).

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Literature Cited

Alarcón-Reverte R, García A, Urzúa J, Fischer AJ (2013) Resistance to glyphosate in junglerice (*Echinochloa colona*) from California. Weed Sci 61:48–54

Beckie HJ, Tardif FJ (2012) Herbicide cross resistance in weeds. Crop Prot 35:15–28

Bendixen LE (1986) Corn (*Zea mays*) yield in relationship to johnsongrass (*Sorghum halepense*) population. Weed Sci 34:449–451

Bowers JE, Abbey C, Anderson S, Chang Ch, Draye X, Hoppe AH, Jessup R, Lemke C, Lennigton J, Li Z, Liu S, Luo L, Marler BS, Ming R, Mitchell SE, Qiang D, Reischmann K, Schulze SR, Skinner DN, Wang Y, Kresovich S, Schetrz KF, Paterso AH (2003) A high-density genetic recombination map of sequence-tagged sites for sorghum, as a framework for comparative structural and evolutionary genomics of tropical grains and grasses. Genetics 165:367–386

Busi R, Gaines TA, Walsh MJ, Powles SB (2012) Understanding the potential for resistance evolution to the new herbicide pyroxasulfone: field selection at high doses versus recurrent selection at low doses. Weed Res 52:489–499

Celarier RP (1958) Cytotaxonomic notes on the subsection Halepensia of the genus Sorghum. Bull Torr Bot Club 85:49–62

Corbett CL, Tardif FJ (2006) Detection of resistance to acetolactate synthase inhibitors in weeds with emphasis on DNA-based techniques: a review. Pest Manag Sci 62:584–597

Duggleby RG, Pang SS (2000) Acetohydroxyacid synthase. J Biochem Mol Biol 33:1–36

Fernández L, de Haro LA, Distefano AJ, Martínez MC, Lía V, Papa JC, Olea I, Tosto D, Hopp HE (2013) Population genetics structure of glyphosate-resistant johnsongrass (*Sorghum halepense* L. Pers) does not support a single origin of the resistance. Ecol Evol 3:3388–3400

Figueroa R, Gebauer M, Fischer AJ, Kogan M (2007) Resistance to bensulfuron-methyl in water plantain (*Alisma plantago-aquatica*) populations from Chilean paddy fields to bensulfuron-methyl in water populations from Chilean paddy fields. Weed Technol 22:602–608

Fischer AJ, Bayer DE, Carriere MD, Ateh CM, Yim K-O (2000) Mechanisms of resistance to bispyribac-sodium in an *Echinochloa phyllopogon* accession. Pestic Biochem Physiol 68:156–165

Foy CL, Witt HL (1990) Johnsongrass control with DPX-V9360 and CGA-136872 in corn (*Zea mays*) in Virginia. Weed Technol 4:615–619

Gressel J, Segel LA (1990) Modelling the effectiveness of herbicide rotations and mixtures as strategies to delay or preclude resistance. Weed Technol 4:186–198

Grula JW, Hudspeth RL, Hobbs SL, Anderson DM (1995) Organization, inheritance and expression of acetohydroxyacid synthase genes in the cotton allotetraploid *Gossypium hirsutum.* Plant Mol Biol 28:837–846

Gubbiga NG, Worsham AD, Corbin FT (1996) Root/rhizome exudation of nicosulfuron from treated johnsongrass (*Sorghum halepense*) and possible implications for corn (*Zea mays*). Weed Sci 44:455–460

Heap I (2014) The International Survey of Herbicide Resistant Weeds. http://www.weedsience.org. Accessed February 16, 2014.

Holm LG, Plucknett DL, Pancho JV, Herberger JP (1977) The World's Worst Weeds: Distribution and Biology. Honolulu, Hawaii: University Press of Hawaii. 609 p

https://doi.org/10.1614/WS-D-14-00167.1 Published online by Cambridge University Press

Iwakami S, Uchino A, Watanabe H, Yamasue Y, Inamura T (2012) Isolation and expression of genes for acetolactate synthase and acetyl-CoA carboxylase in *Echinochloa phyllopogon*, a polyploid weed species. Pest Manag Sci 68:1098–1106

Kaloumenos NS, Chatzilazaridou SL, Mylona PV, Polidoros AN, Eleftherohorinos IG (2013) Target-site mutation associated with cross-resistance to ALS-inhibiting herbicides in late watergrass (*Echinochloa oryzicola* Vasing.). Pest Manag Sci 69:865–873

Kaloumenos NS, Eleftherohorinos IG (2009) Identification of a johnsongrass (*Sorghum halepense*) biotype resistant to ACCase-inhibiting herbicide in northern Greece. Weed Technol 23:470–476

Kuk YI, Jung HII, Kwon OD, Lee DJ, Burgos NR, Guh JO (2003) Sulfonylurea herbicide-resistant *Monochoria vaginalis* in Korean rice culture. Pest Manag Sci 59:949–961

Lamego FP, Charlson D, Delatorre CA, Burgos NR, Vidal RA (2009) Molecular basis of resistance to ALS-inhibitor herbicides in greater Beggarticks. Weed Sci 57:464–481

Laplante J, Rajcan I, Tardif FJ (2009) Multiple allelic forms of acetohydroxyacid synthase are responsible for herbicide resistance in *Setaria viridis.* Theor Appl Genet 119:577–585

McWhorter CG (1972) Factors affecting johnsongrass rhizome production and germination. Weed Sci 20:41–45

McWhorter CG (1989) History, biology and control of johnsongrass. Rev Weed Sci 4:85–121

Merotto A Jr, Jasieniuk M, Fischer AJ (2010) Distribution and cross-resistance patterns of ALS-inhibiting herbicide resistance in smallflower umbrella sedge (*Cyperus difformis*). Weed Sci 58:22–29

Merotto A Jr, Jasieniuk M, Osuna MD, Vidotto F, Ferrero A, Fischer AJ (2009) Cross-resistance to herbicides of five ALS-inhibiting groups and sequencing of the ALS Ggne in *Cyperus difformis* L. J Agric Food Chem 57:1389–1398

ODEPA. 2013. Cultivos Anuales: Superficie, Producción Y Rendimientos. http://www.odepa.cl. Accessed October 6, 2013

Osuna MD, Vidotto F, Fischer AJ, Bayer DE, De Prado R, Ferrero A (2002) Cross-resistance to bispyribac-sodium and bensulfuron-methyl in *Echinochloa phyllopogon* and *Cyperus difformis.* Pestic Biochem Physiol 73:9–17

Panozzo SL, Scarabel L, Tranel PJ, Sattin M (2013) Target-site resistance to ALS inhibitors in the polyploid species *Echinochloa crus-galli.* Pestic Biochem Physiol 105:93–101

Powles SB, Yu Q (2010) Evolution in action: plants resistant to herbicides. Annu Rev Plant Biol 61:317–347

Prado M, De Prado R, Franco AR (2004) Design and optimization of degenerated universal primers for the cloning of the plant acetolactate synthase conserved domains. Weed Sci 52:487–491

Price WJ, Shafii B, Seefeldt SS (2012) Estimation of dose–response models for discrete and continuous data in weed science. Weed Technol 26:587–601

Ray TB (1984) Site of action of chlorsulfuron. Plant Physiol 75:827–831

Riar DS, Norsworthy JK, Srivastava V, Nandula V, Bond JA, Scott RC (2013) Physiological and molecular basis of acetolactate synthase-inhibiting herbicide resistance in barnyardgrass (*Echinochloa crus-galli*). J Agric Food Chem 61:278–289

Ritz C (2010) Toward a unified approach to dose-response modeling in ecotoxicology. Environ Toxicol Chem 29:220–229

Rutledge RG, Hattori J, Miki BL (1991) Molecular characterization and genetic origin of the brassica napus acetohydroxyacid synthase multigene family. Mol Gen Genet 229:31–40

Scarabel L, Locascio A, Furini A, Sattin M, Varotto S (2010) Characterisation of ALS genes in the polyploid species *Schoenoplectus mucronatus* and implications for resistance management. Pest Manag Sci 66:337–344

Shaner DL, Feist DA, Retzinger EJ (1997) SAMOA TM: One company's approach to herbicide-resistant weed management. Pest Manag Sci 51:367–370

Tranel PJ, Wright TR, Heap IM (2013) ALS Mutations from Herbicide-resistant Weeds. http://www.weedscience.com. Accessed October 22, 2013

Tsuji R, Fischer AJ, Yoshimo M, Roel A, Hill JE (2003) Herbicide-resistant late watergrass (*Echinochloa phyllopogon*): similarity in morphological and amplified fragment length polymorphism traits. Weed Sci 51:740–747

Veldhuis LJ, Hall LM, O'Donovan JT, Dyer W, Hall JC (2000) Metabolism-based resistance of a wild mustard (*Sinapis arvensis* L.) biotype to ethametsulfuron-methyl. J Agricul Food Chem 48:2986–2990

Vila-Aiub MM, Balbi MC, Gundel PE, Ghersa CM, Powles SB (2007) Evolution of glyphosate-resistant johnsongrass (*Sorghum halepense*) in glyphosate-resistant soybean. Weed Sci 55:566–571

Warwick SI, Black LD (1983) The biology of Canadian weeds. 61 *Sorghum halepense* (L.) Pers. Can J Plant Sci 63:997–1014

Yasuor H, Osuna MD, Ortiz A, Saldaín NE, Eckert JW, Fischer AJ (2009) Mechanism of resistance to penoxsulam in late watergrass [*Echinochloa phyllopogon* (Stapf) Koss.]. J Agricul Food Chem 57:3653–3660

Yu Q, Ahmad-Hamdani MS, Han H, Christoffers MJ, Powles SB (2013) Herbicide resistance-endowing ACCase gene mutations in hexaploid wild oat (*Avena fatua*): insights into resistance evolution in a hexaploid species. Heredity 110:220–231

Yu Q, Han H, Vila-Aiub MM, Powles SB (2010) AHAS herbicide resistance endowing mutations: effect on AHAS functionality and plant growth. J Exp Bot 61:3925–3934

Yu Q, Powles SB (2014) Resistance to AHAS inhibitor herbicides: current understanding. Pest Mang Sci 70: 1340–1350

Yuan JS, Abercrombie LLG, Cao Y, Halfhill MD, Peng Y, Hu J, Rao MR, Heck GR, Larosa TJ, Sammons RD, Wang X, Ranjan P, Johnson DH, Wadl PA, Scheffler BE, Rinehart TA, Trigiano RN, Stewart N, Jr (2010) Functional genomics analysis of horseweed (*Conyza canadensis*) with special reference to the evolution of non-target-site glyphosate resistance. Weed Sci 58:109–117

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