Target-site resistance to ALS-inhibiting herbicides in *Amaranthus palmeri* from Argentina

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Abstract

BACKGROUND: Herbicide resistant weeds are a serious problem worldwide. Recently, two populations of *Amaranthus palmeri* with suspected cross-resistance to ALS-inhibiting herbicides (R1 and R2) were found by farmers in two locations from Argentina (Vicuña Mackenna and Totoras, respectively). We conducted studies to confirm and elucidate the mechanism of resistance.

RESULTS: We performed *in vivo* dose-response assays, and confirmed both populations had a strong resistance to chlorimuron-ethyl, diclosulam and imazethapyr when compared to a susceptible population (S). *In vitro* ALS activity inhibition tests only indicated a considerable resistance to imazethapyr and chlorimuron-ethyl, indicating that other non-target mechanisms could be involved in diclosulam resistance. Subsequently, molecular analysis of *als* nucleotide sequences revealed three single base-pair mutations conferring substitutions in amino acids previously associated with resistance to ALS inhibitors, A122, W574, and S653

CONCLUSION: This is the first report of *als* resistant alleles in *Amaranthus palmeri* from Argentina. The data support the involvement of a target-site mechanism of resistance to ALS inhibiting herbicides.

Keywords: weeds, *Amaranthus palmeri*, herbicide resistance, acetolactate synthase, ALS inhibitors, cross-resistance.

1. *Introduction*

For centuries, growing plants as food and fiber source has been one of the major human efforts. At present, with a world population over 7.5 billion people, any threat to production systems directly impacts global sustainability. One of these threats, if not the main one, remains the infestation of crops by wild plants with powerful adaptive and propagative abilities.[1] In the last four decades, herbicides were, by far, the main strategy used for weed control; largely replacing hand weeding and mechanical control tools.[2] Although herbicides contributed substantially to the high productivity of global agriculture, they did not result in the complete control of weeds. Instead, monoculture production systems and repeated use of herbicides with similar mechanism of action have led to herbicide resistance in weeds.[3-6] Worldwide, there are currently 252 weed species with biotypes resistant to one or more herbicides, and resistance has evolved for 23 of the 26 known herbicide sites of action.[7]

Palmer amaranth (*Amaranthus palmeri*) is one of the approximately 60 *Amaranthus* species native to the Americas,[8,9] and is the most troublesome for soybean (*Glycine max* L. Merr.), corn (*Zea mays* L.) and cotton (*Gossypium hirsutum* L.), all on the American continent.[10-16] *Amaranthus* species are characterized by having an extended period of germination, rapid growth, and prolific seed production.[17,18] However, Palmer amaranth is an annual dioecious broadleaf weed capable of growing 3 to 4 m tall,[19] producing unbranched terminal seedheads up to 0.5 m in length[20] and triggering seed production up to 600,000 seeds per female plant.[21] Moreover, compared with redroot pigweed (*Amaranthus retroflexus* L.), common waterhemp (*Amaranthus rudis* S.), and tumble pigweed (*Amaranthus albus* L.), Palmer amaranth has shown the greatest values for plant volume, dry weight, and leaf area.[22] Palmer amaranth has a long-term dormant

seed period, a large and aggressive growth, and a potential ability to acquire herbicide resistance by successfully outcrossing with other species from the same genus[12]. Consequently, this weed detrimentally affects crop growth and yield by effectively enhancing its competitiveness for resources like light, water, space, and nutrients.[23]

The first report of herbicide resistance in Palmer amaranth came in 1989 in South Carolina, United States.[24] Since then, the high selection pressure of modern farming systems has led to a rising emergence of resistance cases in this species, covering as much as six different mechanisms of action (5-enolpyruvylshikimate-3-phosphate synthase, EPSPS;acetolactate synthase, ALS; Photosystem II, 4-hydroxyphenylpyruvate dioxygenase, HPPD; microtubule inhibitors and protoporphyrinogen oxidase, PPO inhibitors) and biotypes developing multiple resistance to two or three of the mechanisms mentioned.[7] In Argentina, mucronate amaranth (*Amaranthus quitensis* L.) is largely considered the most problematic weed of recent times, with no official reports of the presence of *A. palmeri* until 2013.[13,25] Given the high tendency of *A. palmeri* to acquire resistance to several mechanisms of action, it is highly relevant to characterize the reported field resistances and intensify the studies regarding the molecular basis of them. This information will be useful in designing control strategies that allow farmers to prevent, understand and overcome this weed resistance problem without creating a similar one in the near future. Hence, the objectives of this work were to confirm the resistance of two suspected cross-resistant *A. palmeri* populations found in two Argentinian farms, and to characterize that resistance at the molecular level.

2. *Materials and Methods*

2.1 Plant material/Seed collection

Seeds of Palmer amaranth were collected in three farms, sampling at least 100 plants combined into a single composite sample per field. In two of these farms, it was known that *A. palmeri* had survived herbicide applications of Derby® (a.i: imazethapyr 10% w/v) from Gleba , and in the third farm the population was susceptible to this herbicide mode of action.

The populations with suspected cross-resistance to ALS herbicides were named R1 (from Vicuña Mackenna, 33º55´20.68" S, 64º35´33.37" W) and R2 (from Totoras, 32° 34′ 59.88″ S, 61° 10′ 59.88″ W); and the susceptible population from Tucumán (27°17'45.36"S- 65°0'3.37"W) was referred as S.

2.2 Chemical compounds

For both *in vitro* and *in vivo* dose-response assays three herbicides were tested: Derby® (a.i: imazethapyr 10% w/v) from Gleba, VRILEC® (a.i: chlorimuron-ethyl 25% w/w) from Agrofina, and Spider® (a.i: diclosulam 84% w/w) from Dow AgroSciences. The active ingredients correspond to the ALS-inhibitor chemical families: imidazolinones (IMI), sulfonylureas (SU) and triazolopyrimidines (TP), respectively.

2.3 *In vivo* dose-response assays

To assess each herbicide effect, approximately 120 seedlings of the resistant and susceptible populations were grown in 9-cm plastic pots containing a mixture of soil, sand and perlite (70-20-10%, respectively), previously sterilized with methyl bromide. The experiment was conducted in a randomized complete block design with 5 replications per dose, using 3 plants per replication. When plants reached 4 to 6 true

leaves, different doses of herbicides were sprayed, depending on the population in order to obtain a good curve fit for each case. The following doses were used: 0, 0.0625X, 0.125X, 0.250X, 0.5X, 1X, 2X, 4X, 8X, 16X and 32X, with X=100 g ai/ha for imazethapyr, 50 g ai/ha for chlorimuron-ethyl, and 30 g ai/ha for diclosulam. These doses correspond to the recommended label rates. Herbicides were applied using a constant pressure backpack with flat fan nozzles at 206.84 kPa calibrated to deliver 75 L ha^{-1}. Three weeks after treatment, number of survivors was recorded. Dry weight was recorded harvesting the above-ground plant tissue, placing it in a paper bag and drying it at 60 ºC for 48 h. Data was expressed as percentage of plant survival and biomass reduction relative to the untreated control.

2.4 *In vitro* enzyme activity assays

Fresh leaves of 21-days-germinated plants from 6 plants of both resistant and susceptible populations were used to extract proteins according to Poston *et al*.[26] and enzyme activity was determined by measuring the amount of acetoin formed as a function of the doses of different herbicides tested, according to the modified method of Westerfeld.[27] This method allows the formation of a pink complex in a medium that also contains creatine and α-naphthol. The intensity of the color was quantified by measuring absorbance at 490 nm. Herbicide concentrations used for enzyme activity testing were: 0; 0.25; 1; 5; 250 and 1000 µM for imazethapyr; 0; 0.025; 0.1; 0.25; 1; 5; 25 and 250µM for chlorimuron-ethyl and diclosulam. Enzyme inhibition was computed as I_{50}, which represents the herbicide concentration required to reduce the enzyme activity by 50%, compared to the untreated control. There were three replications per treatment per population, and experiments were conducted three times.

2.5 Cloning and sequencing of *als* gene

Genomic DNA was extracted from fresh leaf tissue of ten plants of each population (S, R1 and R2) using Wizard Genomic Extraction Kit® (Promega Corp., Madison, WI), and genomic bulks were used as template for *als* gene amplification with primers designed from *als* sequences of related species published in available database.[28,29] Complete *als* gene was amplified with primers X1 and X2 (see Table 1) using Q5® High-Fidelity DNA polymerase (New England Biolabs, inc.). Reactions consisted of ~100 ng DNA, 500 nM primers, 1.5 mM $MgCl_2$, 200 µM dNTPs, 1X buffer, 1 polymerase unit, and H_2O to 50 µL. Cycle conditions were as follows: 98 ºC for 1 min, 35 cycles of 98 ºC for 10 s, 57 ºC for 30 s, and 72 ºC for 1 min, 72 ºC for 7 min, and a 12 ºC hold. Amplification products were ligated to pGEM®-T easy vector (Life Technologies, Grand Island, NY, USA) as described in the technical manual. Chemically competent DH5α *Escherichia coli* cells were prepared and transformed according to Sambrook and Russell[30] and transformants were selected for isolation on LB plus 100 µg mL^{-1}ampicilin plates. Insert integration was checked carrying out a specific colony PCR, using primers X3 and X4 (see Table 1). Overnight cultures of positive colonies were used to make plasmid minipreparation, according to Wizard® Plus SV Minipreps DNA Purification System protocol (Promega Corp., Madison, WI). Eight positive-recombinant DNA molecules from each population were sequenced (Marcogen Inc. Korea), employing T7 and SP6 primers for the pGEM-T vector backbone and X5, X6, and X7 primers designed to cover the entire *als* gene sequence (see Table 1). This cloning strategy allows the unambiguous determination of the complete sequence of individual *als* alleles.

2.6 *als* sequences analysis

To manually generate complete R1, R2 and S *als* sequences, sequencing reads were aligned with reference *als* sequences from *Amaranthus* genus obtained from NCBI. Alignment was performed using MUSCLE algorithm from Unipro Ugene v1.11.2 software.[31] All sequences were translated and aligned, to search for aminoacid substitutions.

2.7 Statistical analysis

Dose-response curves were obtained by a non-linear log-logistic regression model using SigmaPlot (version 11.0, Systat Software, Inc., San Jose, CA, USA) according to the following equation:

$$y = min + \frac{(max - min)}{1 + (\frac{x}{b})^a}$$

where *y* is the percent of dry weight or plant survival, *x* is the herbicide concentration (rate), *min* and *max* are the lower and the upper limits of the curve, respectively, *a* is the slope at the inflexion point, and *b* is the herbicide dose rate required to reduce dry weight by 50% (GR_{50}) or to reduce the plant survival by 50% (LD_{50}).

A different nonlinear regression analysis was applied to fit ALS activity data to a sigmoidal three parametric logistic curve using SigmaPlot (version 11.0, Systat Software, Inc., San Jose, CA, USA) according to the following equation:

$$y = \frac{a}{1 + (\frac{x}{I50})^b}$$

where *y* is the ALS activity, *x* is the herbicide concentration (rate), *a* is the upper response limit, *b* is the slope of the curve to relate effect of herbicide dose and

concentration and I_{50} is the herbicide concentration required to cause a 50% reduction in ALS enzyme activity.

Data from the experiments were tested using one-way analysis of variance (ANOVA). Minimum significant differences were calculated by the Holm-Sidak Test ($\alpha = 0.05$) using the Sigma Stat Package. The resistance factor (Rf) was calculated by dividing the determined GR_{50}, LD_{50} or I_{50} value of the resistant populations by that of the susceptible population to quantify the level of resistance.

3. *Results and Discussion*

3.1 *In vivo* dose-response assays

In vivo dose-response assays showed that both resistant populations (R1 and R2) proved to be highly cross resistant to all ALS inhibitors tested (imazethapyr, chlorimuron-ethyl and diclosulam), as shown in Figs. 1 and 2. The resistance factors (Rf) were > 9.3 and > 11 for GR_{50} and LD_{50}, respectively (Table 2).

Plant survival percentage at the recommended label rate (1X) was around 100% of the untreated control for all the herbicides (Fig. 2). Simultaneously, the remaining biomass of R plants at dose 1X ranged from 80 to 100% of the untreated control, except for imazethapyr curve in R2, which showed a remaining biomass value of ~45% at the recommended rate (Fig. 1).

Although there is not a substantial difference between R1 and R2 plant survival curves, the biomass reduction effects were stronger for R2 than R1 for the three herbicides, as observed in Figs. 1 and 2. Thus, both variables provided complementary information to compare the resistance levels of R1 and R2.

Interestingly, in the absence of herbicides, both populations displayed significantly higher biomass values compared to the susceptible population (200% and 155% for R1 and R2, respectively).

The results presented herein confirm that R1 and R2 populations of *A. palmeri* exhibit cross-resistance to three active ingredients belonging to different chemical families: imidazolinones (IMI), sulphonylureas (SU) and triazolopyrimidines (TP).

3.2 *In vitro* enzyme activity assays

There were no significant differences in the levels of *in vitro* ALS activity between susceptible and resistant populations in the absence of herbicides. Specific activity of untreated controls ranged between 0.02–0.04 U mg^{-1}, similarly to values obtained for other plant crude extracts.[32,33]

Experiments were set up to include three replicates at different concentrations (up to seven data points) in order to have a good distribution within the curve fitting. Based on I_{50} values (Table 3), both R populations have ALS isoforms with increased resistance to the three herbicides tested, compared to the S population. According to Rf values these effects are considerably high for imazethapyr (Rf ranging from 30 to 40-fold), moderate for chlorimuron-ethyl (Rf ranging from 5 to 10-fold) and low, but still significant for diclosulam (around 2.5-fold).

These results strongly support the hypothesis of a target-site resistance (TSR) mechanism in these *A. palmeri* resistant populations.

3.3 Molecular insight into ALS resistance

The study of the sequences of individual clones allowed a comparative analysis of the variability between R and S populations and within each population. All S population ALS sequences had a 100% amino acids identity between them, whereas for the R1 and R2 populations, several clones presented mutations causing amino acids substitutions. All substitutions found in the complete open reading frame are listed in Table 4 as well as the accession numbers of the complete sequences added to the NCBI database.

We identified four and five allelic versions in R1 and R2 populations, respectively (see Table 4). Even though we have worked with genomic bulks, this represents a high number, considering that *Amaranthus* sp. are diploid plants that contain one copy of the *als* gene.[34] However, strong selection pressure for resistance to ALS inhibitors, a variety

of amino acid changes that can confer resistance, and the outcrossing nature of *A. palmeri* all could contribute to the high number of polymorphisms we observed.

In R1 population, the replacement of a tryptophan by a leucine at position 574 of the polypeptide chain was found in two clones. Due to the later adoption of TP herbicides, this substitution has been more frequently associated with resistance to SU and IMI in a great variety of weed species;[36] but there are some reports that also associate such substitution with resistance to TP.[37,38] Thus, the W574L substitution itself could explain the cross-resistance observed in R1 population.

Tardif *et al.*[39] found that W574L mutation appears to have several pleiotropic effects on the growth and development of *Amaranthus powelli* plants. However, the absence of a biomass reduction and/or differential ALS activity under non-selection pressure between R and S populations does not provide any clue of fitness cost in our study. Nevertheless, a much more complex methodological framework is necessary to unequivocally associate a fitness cost to the resistant trait, as it has been exhaustively reviewed by Vila-Aiub *et al.*[40]

In R2 population, the previously reported substitution S653N was found in five clones. This mutation has been almost exclusively linked IMI resistance, conferring low resistance to SU in some cases.[36,41] Interestingly, a new substitution that replaces an alanine by a serine at position 122 appeared in two clones. Although several replacements at this position (122) have been reported to confer resistance in plants, a serine has been found only in yeasts until now.[42] Since none of the known substitutions present in R2 population justify the high level of resistance to diclosulam, a contribution of the new substitutions found and/or a non-target-site resistance (NTSR) mechanism, mainly to TP resistance, cannot be discarded in this population.

Although W574L triggers broad cross-resistance to all ALS-inhibiting herbicides families with higher magnitudes than S653N[43], the higher Rf from *in vivo* assays in R1 over R2 populations can not be wholly explained by W574L itself, since R2 population seem to have a higher frequency of plants with a target-site mutation (see Table 4). Moreover, *in vitro* assays showed no significant differences between the two resistant populations, suggesting a likely contribution of a NTSR mechanism in R1 population.

Three unreported substitutions, M601I (R1), P84H (R2) and A282D (R1 and R2) were found outside the five ALS conserved domains, which were previously associated to TSR[44] (see Fig. 3). Although it is more likely that they represent a natural genetic variation, it remains to be elucidated if they contribute to the resistant phenotypes.

These molecular results show, for the first time, the presence of TSR mechanism in *A. palmeri* Argentinian populations to ALS inhibitors.

4. *Conclusions*

We have confirmed the suspected cross-resistance to ALS-inhibiting herbicides in two populations of *A. palmeri* found in Argentina. This is the first report of TSR to ALS-inhibiting herbicides for this species in this country. We have found two amino acids substitutions in acetolactate synthase from the resistant populations that were previously reported as responsible of TSR in plants: W574L (R1) and S653N (R2). Interestingly, the substitution A122S, only reported in yeasts up to now, has been found for the first time in plants (R2 population). The *in vivo* characterization of each purified homozygote progeny together with the heterologous expression of each *als* allele will be useful to evaluate the individual contribution of each substitution to the whole resistance phenotype.

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Tables

Table 1. Primers used in polymerase chain reactions.

Name	Sequence (5'-3')
X1	CTTCAAGCTTCAACAATGG
X2	CCTACAAAAAGCTTCTCCTCT
X3	CCAGAAAAGGTTGCGATGTT
X4	ATGCTTGGTAATGGATCGAG
X5	GGCAACCTAGAAAGATACCC
X6	CCTGAATGGCGTATTGCGGAGG
X7	GGAGGGAGGAGTTGAATGAGCAG

Table 2. Dose-response parameters. GR_{50}/LD_{50} units are g ai/ha. Resistance factors (Rf) were calculated using concentrations of inhibitors required to reduce dry weight or plant survival by 50%. For each herbicide, parameters with different letters indicate statistically significant differences.

		Dry weight		Plant survival	
Herbicide	**Population**	**GR_{50}**	**Rf**	**LD_{50}**	**Rf**
imazethapyr	R1	2996 ± 206 [a]	788	2288 ± 135 [a]	211
	R2	78.8 ± 25.9 [b]	21	1327 ± 50 [b]	123
	S	3.8 ± 0.6 [c]		10.82 ± 2.82 [c]	
chlorimuron-ethyl	R1	>800	>9.3	>800	>11
	R2	>800	>9.3	>800	>11
	S	86.2 ± 12.5		73 ± 17	
diclosulam	R1	4397 ± 3214 [a]	600	>960	>78
	R2	128 ± 39 [b]	17	>960	>78
	S	7.32 ± 0.82 [c]		12.2 ± 1.5	

Table 3. Parameters of ALS activity fit curves. I_{50} unit is µM. Resistance factors (Rf) were calculated using concentrations of inhibitors required to reduce ALS activity by 50% (I_{50}). Resistance factors can only be compared within a chemical family. For each herbicide, I_{50} values with different letters indicate statistically significant differences.

		Parameters	
Herbicide	**Population**	**I_{50}**	**Rf**
imazethapyr	R1	17.50 ± 9.09 [a]	33.68
	R2	21.98 ± 6.23 [a]	42.30
	S	0.5196 ± 0.3418 [b]	
chlorimuron-ethyl	R1	0.0098 ± 0.0020 [a]	6.53
	R2	0.0145 ± 0.0054 [a]	9.67
	S	0.0015 ± 0.0004 [b]	
diclosulam	R1	0.0136 ± 0.0032 [a]	2.27
	R2	0.0171 ± 0.0029 [a]	2.85
	S	0.0060 ± 0.0006 [b]	

Table 4. Amino acid differences found in ALS open reading frame of resistant *Amaranthus palmeri* populations compared to the susceptible population. Previously reported substitutions are in bold. Amino acids are numbered according to *A. thaliana* ALS sequence, with the exception of A282D, which is numbered according to *Amaranthus retroflexus* ALS sequence, since it falls into an indel.

Population	**Allelic version (and frequency)**	**ALS substitution**	**Accession number**
S	A (8/8)	–	KY781916
R1	A (1/8)	–	
	B (5/8)	A282D	KY781917
	C (1/8)	**W574L**	KY781918
	D (1/8)	**W574L** – M601I	KY781919
R2	B (2/8)	A282D	
	E (2/8)	**A122S** – A282D	KY781920
	F (1/8)	A282D – **S653N**	KY781921
	G (1/8)	P84H – A282D – **S653N**	KY781922
	H (2/8)	**S653N**	KY781923

Figure captions

Figure 1. *In vivo* dose-response (Dry biomass) curves with ALS-inhibiting herbicides in *A. palmeri*. Plants of R1, R2 and S populations were subjected to the application of different doses of imazethapyr (a), chlorimuron-ethyl (b) and diclosulam (c). Results are expressed as a percent of remaining biomass in comparison to untreated control groups. Arrows indicate doses applied to field.

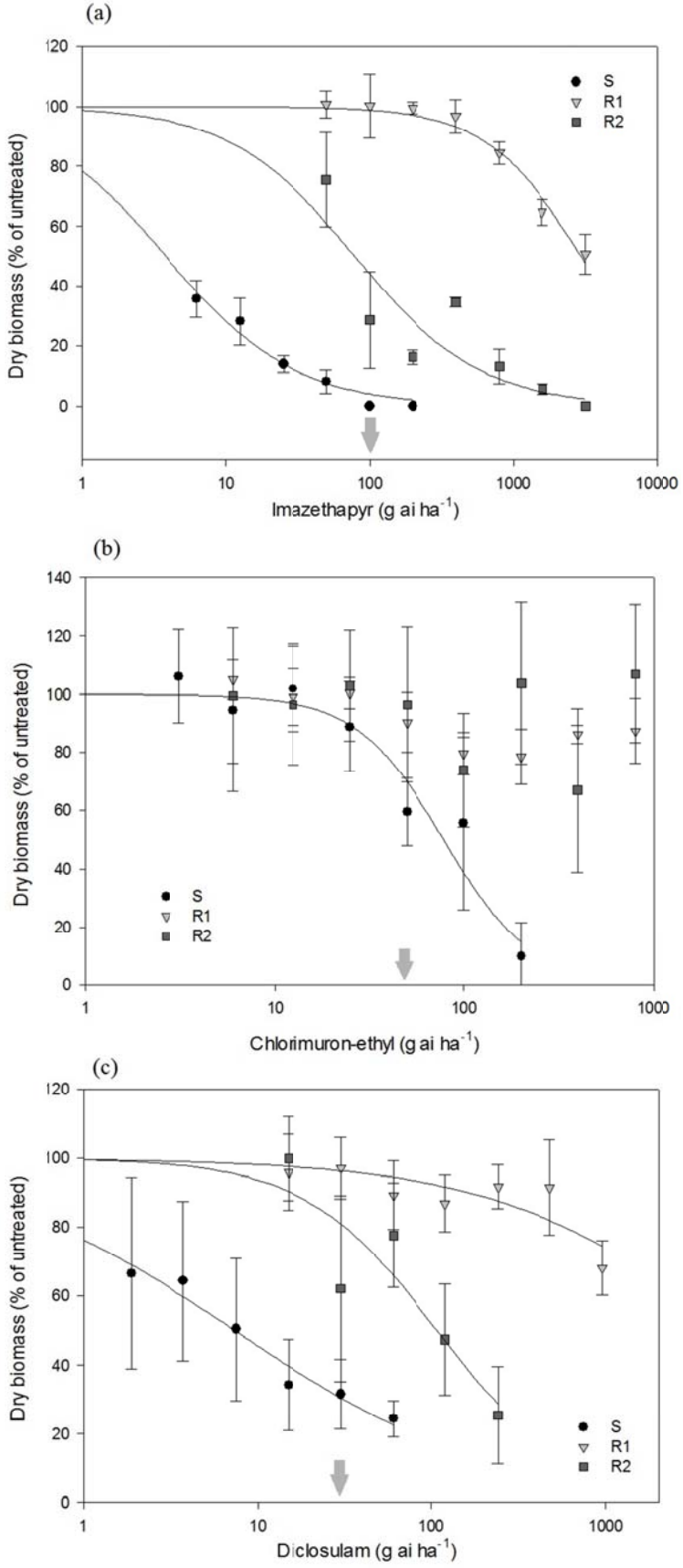

Figure 2. ***In vivo*** **dose-response (Plant survival) curves with ALS-inhibiting herbicides in** ***A. palmeri*****.** Plants of R1, R2 and S populations were subjected to the application of different doses of imazethapyr (a), chlorimuron-ethyl (b) and diclosulam (c). Results are expressed as a percent of plant survival in comparison to untreated control groups. Arrows indicate doses applied to field.

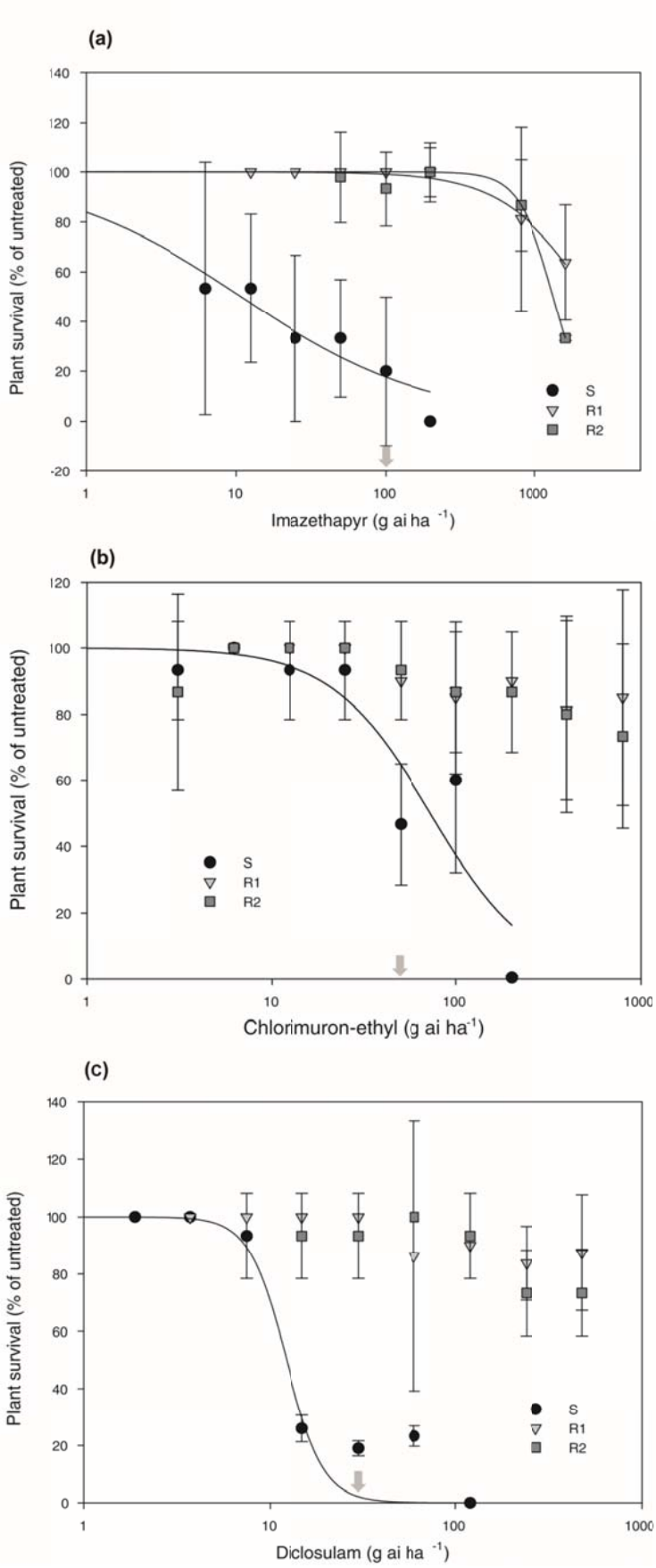

Figure 3. Scheme of the amino acid substitutions found in *als* gene from R populations of *A. palmeri*. Black, grey and empty arrows indicate substitutions found in R1, R2 and in both of them, respectively. Only previously reported substitutions (at positions 122, 574 and 653) fall into the conserved domains of the ALS protein (named A to E). Amino acids are numbered according to *A. thaliana* ALS sequence.