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Coexistence of target-site and non-target-site mechanisms of glyphosate resistance in *Amaranthus palmeri* populations from Argentina

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ABSTRACT. *Amaranthus palmeri* S. Waston is currently one of the most problematic weeds worldwide. Biotypes with resistance to herbicides such as glyphosate and ALS inhibitors are now present in almost all Argentinian cultivable areas. In this work, we studied glyphosate resistance in three different populations, some of them previously characterized as resistant to ALS inhibitors. Dose-response curves were conducted in order to assess the effect of glyphosate on the survival and dry biomass of the populations. Subsequently, the presence of target-site resistance (TSR) was studied. Results confirmed the glyphosate resistance in the three populations, showing different levels of resistance, being R2 and R3 significantly more resistant than r1 population. A high prevalence of the P106S substitution was detected in the three resistant populations, while none increase in the relative EPSPS copy number was noticed. Some surviving plants without any of the TSR mechanisms for glyphosate were detected in R3 population, suggesting the presence of non-target-site resistance (NTSR).

Keywords: palmer amaranth; target-site resistance; EPSPS P106 substitution; *EPSPS* copy number; shikimate accumulation.

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Introduction

Herbicide resistant weeds are a major problem in agricultural systems worldwide. The extensive and repetitive use of single herbicides with poor diversification of management practices is the main reason regarding the increasing evolution of herbicide resistance (HR) weeds in the past decades (Shaner & Beckie, 2014). Currently, 262 different species have been reported to present HR to at least one active ingredient (Heap, 1999).

Glyphosate has been the most successful and used herbicide over the last 20 years, which simultaneously led to a rapid evolution of glyphosate resistance (GR) resulting in a significant limitation on chemical control options because of a low interest in new herbicide discovery (Heap & Duke, 2018). Although glyphosate was introduced in the market in 1974, no extensive reports of GR cases were recorded until the beginning of 2000, at the time that glyphosate-resistant crops were fully commercialized. Today, 50 species have evolved GR -15 of them currently present in Argentina- and several mechanisms were reported to cause this issue (Heap & Duke, 2018; Gaines, Patterson, & Neve, 2019).

P106 substitutions and *EPSPS* gene amplification are the most target-site reported mechanisms for GR (Sammons & Gaines, 2014), being recently discovered that the amplified gene can be carried in extrachromosomal circular DNA molecules in *Amaranthus palmeri* (Koo et al., 2018). Nonetheless, some other mechanisms involving different single (Li et al., 2018), double (Yu et al., 2015; Cruz et al., 2016; Takano et al., 2020) and even triple (García et al., 2019; Perotti et al., 2019) substitutions in the EPSPS were lately reported. Additionally, non-target site resistance (NTSR) mechanisms associated to reduced translocation, vacuole sequestration and rapid necrosis were also published (Peng et al., 2010; Yuan et al., 2010; Nol, Tsikou, Eid, Livieratos, & Giannopolitis, 2012; Domínguez-Valenzuela et al., 2017; Moretti et al., 2018; Van Horn et al., 2018).

Amaranthus palmeri S. Watson, commonly known as Palmer amaranth, is a broadleaf weed species native to North America. *A. palmeri* has unique germination, growing and reproductive traits that make it a highly-invasive and highly-adaptable species, becoming a perfect driver for HR (Ward, Webster, &

Steckel, 2013). Since its first detection in Argentina in 2013, populations with GR have already spread to almost the totality of the soybean cultivation area in this country (Morichetti et al., 2013; REM, 2018).

EPSPS gene amplification seems to be the primary genetic GR mechanism in this species worldwide (Ward et al., 2013; Sammons & Gaines, 2014) probably because of the high resistance levels and the lack of evidence for fitness costs associated with such mechanism (Vila-Aiub et al., 2014; Vila-Aiub, Yu, & Powles, 2019). However, two recent studies of *A. palmeri* populations from Argentina showed a reduced absorption and impaired herbicide translocation (Palma-Bautista et al., 2019) and a proline 106 to serine target-site substitution (Kaundun et al., 2019) as the major mechanisms. Deciphering the molecular basis of herbicide resistance is a key step in the understanding of the evolutionary processes that drive this phenomenon, and in the design of target-specific strategies to make a successful control through integrated weed management programs (Owen, 2016).

In this work, we aimed at deciphering the molecular mechanisms causing GR in three *A. palmeri* populations from Argentina, two of them showing also resistance to AcetoLactate Synthase (ALS) inhibitors, as previously described (Larran et al., 2017).

Material and methods

Plant material

Seeds of Palmer amaranth (*A. palmeri*) were collected in four soybean fields, harvesting at least 100 mature seedheads that were combined into a single composite sample per field. In three of these, it was known that *A. palmeri* had survived at least five consecutive glyphosate applications (Roundup Full II®), and in the fourth field the population was known to be susceptible to this herbicide mode of action. Populations with suspected GR from three provinces were named r1 (Tucumán, 27°17′45.36″ S, 65°0′3.37″ W), R2 (Córdoba, 33º55′20.68″ S, 64º35′33.37″ W), and R3 (Santa Fe, 32°34′59.88″ S, 61°10′59.88″ W). The susceptible population (Córdoba, 33º55′20.68″ S, 64º35′33.39″ W), was referred as S. S and R2 populations were very close to each other, thus having the same geographic coordinates. Populations r1, R2 and R3 were previously assessed for ALS inhibiting-herbicide resistance, where they were named S, R1, and R2, respectively (Larran et al., 2017).

Dose-response curves

To assess glyphosate 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). Pots were placed in a growing chamber at 25ºC and 16/8 photoperiod. The experiment was arranged 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 glyphosate 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 = 1,040 g a.i. ha^{-1} (Roundup Full II®), corresponding to the recommended label glyphosate rate. This experiment was repeated twice. Spraying conditions and results analysis were conducted exactly as described in Larran et al. (2017) study.

Pro106 mutation screening

Genomic DNA from at least 15 1x-surviving plants of each population was obtained using Wizard Genomic Extraction Kit® (Promega Corp., Madison, WI). An aliquot of these DNAs was subjected to a derived Cleaved Amplified Polymorphic Sequence (dCAPS) assay, in order to detect substitutions at P106 in each single *A. palmeri* plant. PCR and digestion were performed as described previously (Chatham et al., 2015), with modifications in the reverse primer to make it specific for local *Amaranthus* spp. populations (EPSdcapsR1: TCCAGCAACGGCAACCGCAGCTGTCCATG). Bands were visualized on 6% polyacrylamide gel stained with SYBR Safe (Invitrogen®). Control DNAs from previously-sequenced samples were included to validate the fidelity of the test. Plants presenting the undigested band were classified as having substitutions at P106. No digestion or partial digestion indicate homo and heterozygosis for the mutation, respectively. These results were validated by sequencing a subset of the samples.

EPSPS sequencing

DNA from a subset of surviving plants of each population was used as template to amplify an EPSPS conserved region containing the P106 (Gaines et al., 2010). Moreover, some samples were used to obtain the complete *EPSPS* sequence. cDNA sequence from *EPSPS* of Palmer Amaranth (FJ861243.1) was used as reference for alignments. Alignments were performed using Muscle algorithm from Unipro Ugene v1.11.2 software.31. All sequences were translated and aligned in order to search for amino acid substitutions.

Relative *EPSPS* copy number

Quantitative real-time PCR (qPCR) was performed to measure relative *EPSPS* copy number. Primers Egf (5′-ATGTTGGACGCTCTCAGAACTCTTGGT-3′) and Egr (5′-TGAATTTCCTCCAGCAACGGCAA-3′) previously designed (Gaines et al., 2010) were used to amplify a 195 bp gene fragment. Reactions were conducted in a final volume of 13 µL, containing 1x Real mix qPCR (Biodynamics, Buenos Aires, Argentina), 200 nM of gene-specific primers, and 50 ng of genomic DNA. Each biological sample was processed in triplicate. Amplifications were performed in a Rotor-Gene Q thermocycler (Qiagen), set as follows: 2 min. at 95°C, 40 cycles of 15 s at 95°C, 30 s at 58°C, 30 s at 72°C. Specificity of amplification products was assessed through the building of melting curves at the end of cycling (86 cycles of 10 s from 72 to 95°C, with a 0.5ºC increase per cycle after cycle 2). Amplification efficiency for each reaction was independently calculated and considered in data processing. Comparative Ct quantifications were calculated with REST software (Relative Expression Software Tool V 2.0.7 for Rotor Gene, Corbett Life Sciences – Pfaffl, Horgan, & Dempfle, 2002), using the actin gene as reference. Results were expressed as fold increase in *EPSPS* copy number in resistant plants relative to S plants.

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.

Shikimic acid accumulation after *in vitro* glyphosate treatment

Shikimic acid accumulation was measured according to the protocol described by Shaner, Nadler-Hassar, Henry, and Koger (2005). Ten 4 mm diameter leaf discs from eight plants of each population were collected and placed into 20 mL vials containing the assay solution. Each experimental unit contained 10 mM ammonium phosphate (pH 4.4), 0.1% (v v^{-1}) Tween 80, plus glyphosate at 0 (controls) and 100 µM. Vials were incubated for 16 hours in growth chamber under 150 µM m^{-2} s^{-1} of light intensity. Then, the reaction was stopped with the adding of 250 µL of 1.25 N HCl and shikimic acid levels were determined as described previously (Cromartie & Polge, 2000). Finally, absorbance was read at 380 nm in a spectrophotometer (PerkinElmer Lambda Bio+) and values were expressed as µg of shikimic acid per g of fresh weight using a shikimic acid standard (Sigma-Aldrich) curve. Each treatment was performed in triplicate.

Shikimic acid accumulation after *in vivo* glyphosate application

Plants in a 4-6 leaf stage from each population were treated with 1080 g a.i. ha^{-1} of glyphosate (RoundUp Full II®) as described in dose-response curves section and leaves were used to quantify the accumulation of shikimic acid at 0, 24, 48, 72 and 96 hours after application, following the methodology detailed in the *in vitro* glyphosate treatment section.

Results and Discussion

Glyphosate resistance in *A. palmeri* populations

Dose-response experiments indicated that both dry biomass and survival rate of r1, R2, and R3 plants were significantly higher than S plants (See Figure 1A and B, respectively). The range of glyphosate doses used allowed a good curve fit for all biotypes (see R^2 values in Table 1). Resistant Factors (Rf) ranged between 24.4 and 44.5 for dry biomass and between 28.0 and 209.8 for survival rate (Table 1). R2 and R3 populations were significantly more resistant than r1 population (p-values < 0.01), which was named as lowercase ‘r’ because the survival rate was barely higher than 10% at the recommended glyphosate dose. It is important to highlight that S population was markedly susceptible to glyphosate (LD_{50} = 10.6 and GR_{50} = 28.3, Table 1), thus triggering high Rf values. Nevertheless, GR_{50} and LD_{50} parameters for these resistant populations are in the order of magnitude as those previously reported for this species (Gaines et al., 2010; Domínguez-Valenzuela et al., 2017; Palma-Bautista et al., 2019; Kaundun et al., 2019).

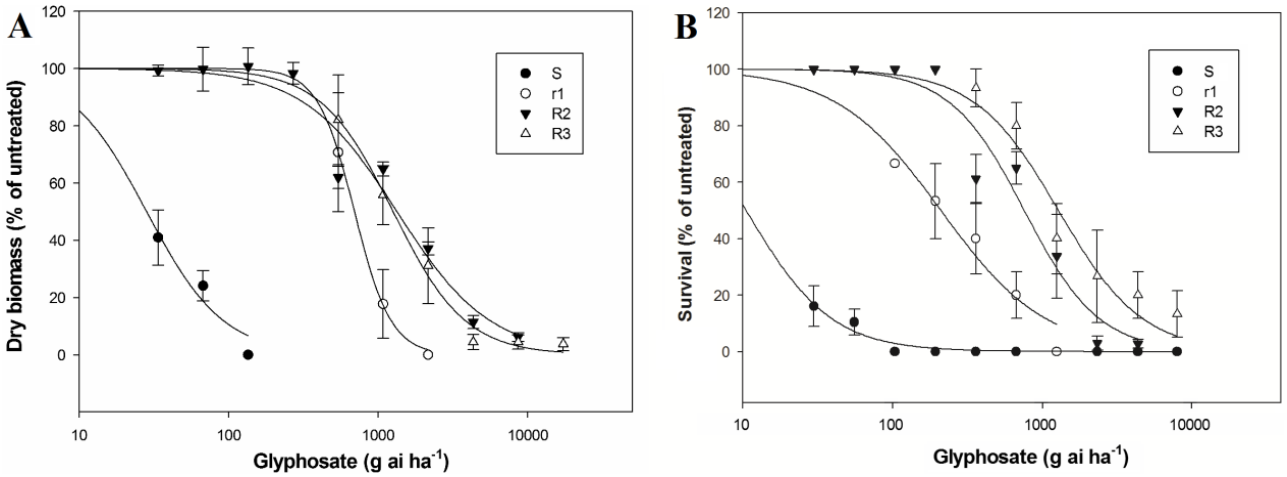

Figure 1. *In vivo* dose-response curves with glyphosate in *A. palmeri* populations. Plants of r1, R2, R3 and S biotypes were subjected to the application of different doses of glyphosate. Results are expressed as a percent of dry biomass (A) and survival rate (B) in comparison to untreated control groups.

Table 1. Dose-response parameters. LD_{50} and GR_{50} units are g ai ha^{-1}. Resistance factors (Rf) were calculated using concentrations of inhibitors required to reduce survival and dry weight by 50%. Different letters indicate statistically significant differences.

Population	Dry weight				Survival			
	GR_{50}		Rf	R^2	LD_{50}		Rf	R^2
S	28.3 ±	5.1[a]		0.99	10.6 ±	2.7[a]		0.99
r1	694.1 ±	10.0[b]	24.5	0.99	297.0 ±	38.9[b]	28.0	0.98
R2	1336.8 ±	171.1[c]	47.2	0.97	1239.3 ±	172.4[c]	116.9	0.96
R3	1260.6 ±	63.3[c]	44.5	0.99	2223.6 ±	303.9[d]	209.8	0.96

P106S substitution as target-site mechanism for GR.

P106 substitutions and gene amplification were studied in each 1x-surviving plant from each population through dCAPS and qPCR, respectively. The P106S substitution was present in all r1 and R2 plants, and in many R3 plants (Figure 2). While all r1 plants were homozygous for P106S substitution, all R2 plants also presented the P106 replacement but half of them as heterozygous. On the other side, some of the R3 plants presented the P106 substitution (all in homozygosis) and some of them not. Partial EPSPS sequences were deposited in GenBank database under the accession numbers MK069608 and MK069609. No additional substitutions were detected within the EPSPS coding sequence (accession number: MT724694). Finally, no plants with an amplified EPSPS copy number were detected in the three populations (Table 2).

Interestingly, some plants from R3 population did not present any of the target-site resistance (TSR) mechanisms explored (Table 2), indicating that NTSR mechanisms may be present, as recently reported for *A. palmeri* in Mexico and other populations from Argentina (Domínguez-Valenzuela et al., 2017; Palma-Bautista et al., 2019). As this population presented the highest GR levels (Table 1), it may be inferred that an extra mechanism in addition to the proline substitution is contributing with the high levels of GR.

Shikimic acid accumulation after *in vivo* versus *in vitro* glyphosate treatments

Shikimic acid levels detected after *in vivo* glyphosate spraying (*in plant* treatment) were higher in S population in comparison to the resistant populations. This difference was notoriously increased at 96 hours after application (Figure 3A).

On the other hand, diverse levels of shikimic acid accumulation after *in vitro* glyphosate treatment were found between populations as well as between plants from the same population (R3) with different genotypes (Figure 3B). Resistant plants with the P106S substitution (genotypes r1, R2 and $R3_1$) accumulated a smaller amount of shikimic acid than the susceptible plants (S), consistently with the different EPSPS isoforms found in each population. Resistant plants containing a wild type EPSPS isoform ($R3_2$) accumulated a similar amount of shikimic acid than susceptible plants. These results indicate that the NTSR mechanism carried by $R3_2$ plants was not operating in those experimental conditions.

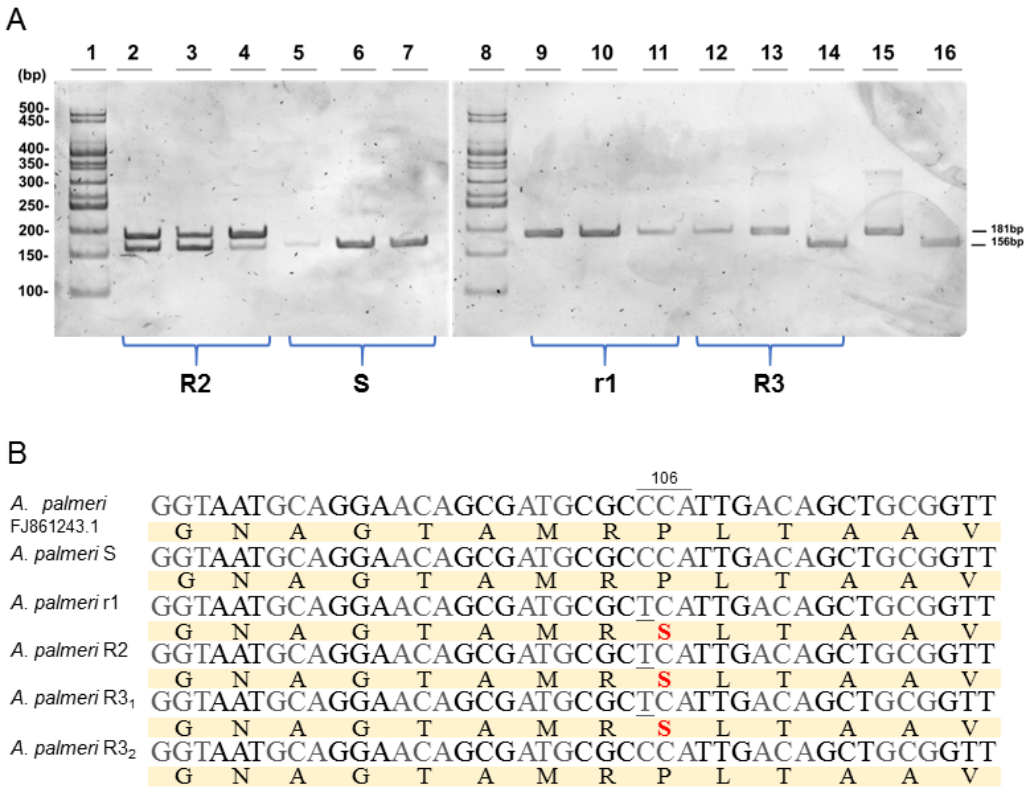

Figure 2. A: dCAPS for Pro106 mutation screening. Three samples of each population are shown in this representative gel. The wild-type sequence produces a single digested band of 156 bp (samples load in lanes 5-7 and 14, and the control load in lane 16) while the mutant sequence produces an undigested band of 181 bp (samples load in lanes 9-13, and the control load in lane 15). The presence of two bands indicates heterozygosis (lanes 2-4). B: Sequence analysis allowed the confirmation of polymorphisms occurring in the EPSPS region surrounding EPSPS 106 amino acid (numbered according to *Zea mays*) in individual plants from each population.

Table 2. TSR characterization for GR. P106S substitution was present in r1, R2 and R3 populations and there was not any significant increase of EPSPS copy number. P and S indicate a Proline or a Serine residue at position 106 of EPSPS, respectively.

Population	Genotypes of survivors plants	Relative EPSPS copy nº
r1	SS	1.3 ± 0.3[a]
R2	PS	2 ± 1[a]
	SS	2.1 ± 0.4[a]
R3	PP	2 ± 1[a]
	SS	1.9 ± 0.9[a]

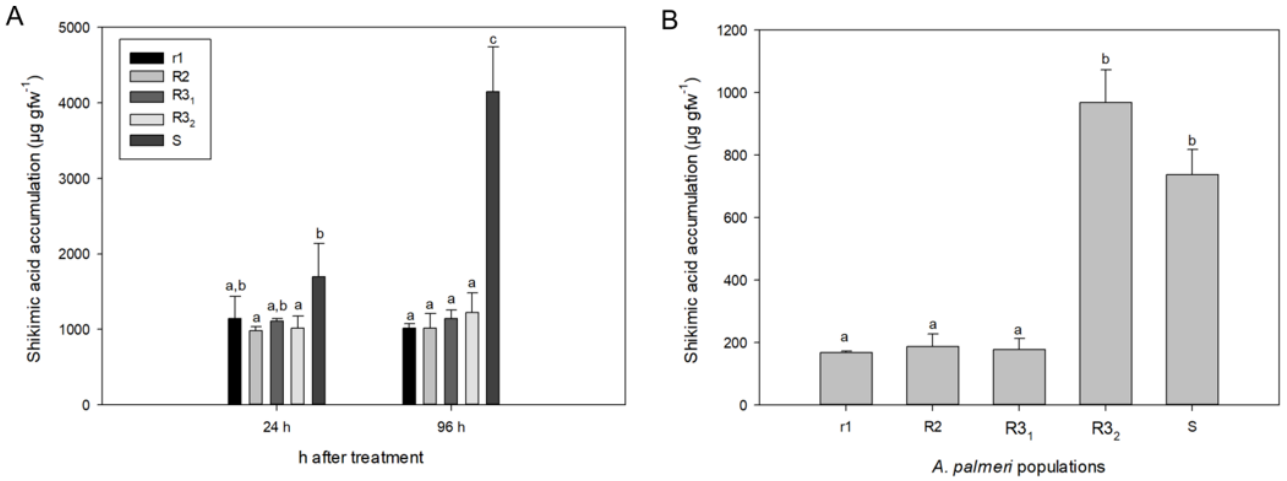

Figure 3. A: Shikimic acid accumulation after *in vivo* glyphosate application. Each determination was performed 24 or 96 hours after glyphosate application at the recommended rate. B: Shikimic acid accumulation after *in vitro* glyphosate treatment. Both experiments were carried out on the same individual plants, previously genotyped (S, r1, R2, R3$_1$ and R3$_2$). Vertical bars represent the standard error of the mean. Different letters indicate statistically significant differences.

When glyphosate is incubated *in vitro* with leaf discs, it bypasses the ordinary absorption and penetration processes occurring in plants. In contrast, when glyphosate is sprayed *in vivo*, the herbicide uptake relies on an effective absorption, penetration, and translocation. Thus, the comparative analysis of the shikimic acid accumulation profiles in each experimental condition would presumably allow the detection of a reduced foliar glyphosate absorption, since this mechanism would be skipped in the *in vitro* treatment, eliminating its contribution. Likewise, resistance due to a reduced translocation of the herbicide to meristematic tissues would not be significantly contributing to *in vitro* tests. In contrast, a NTSR given by an increased metabolism would be assessed in both types of trials.

Our results suggest that the NTSR mechanism present in R3 population may be a reduced absorption or translocation, but an accurate experimental validation of the proposed methodology is necessary in order to make such a statement. An experimental approach with radiolabeled herbicides, for instance, would allow to unequivocally identify a reduced movement of the herbicide (Goggin, Cawthray, & Powles, 2016). In concordance with our observations, a genotype-dependent shikimic acid accumulation after *in vitro* glyphosate treatment was observed in *A. palmeri* populations with the P106S substitution in EPSPS and a reduced absorption and translocation of glyphosate. That is, these populations accumulated lower shikimic acid levels than those populations with only the NTSR mechanism (Domínguez-Valenzuela et al., 2017).

Interestingly, a reduced absorption and an impaired translocation were recently reported as NTSR mechanisms conferring GR in a population of *A. palmeri* from Córdoba province, Argentina (Palma-Bautista et al., 2019). Nevertheless, such resistance involved exclusively NTSR mechanisms. At the same time, other study reported a proline 106 to serine target-site mutation as the major GR mechanism operating in another population from the same province (Kaundun et al., 2019). Therefore, the co-existence of both mechanisms in R3 population shows, one more time, the consequences of a continuous selection pressure and explains why the GR levels from R3 population were higher than those from the populations carrying single mechanisms.

In this context, it is essential to mention that multiple resistance to glyphosate and ALS-inhibiting herbicides has become extremely widespread in Argentinian Pampean region, limiting the chemical control strategies to the rotation and mixture of herbicides with a few additional sites of action, such as protoporphirinogen oxidase inhibitors, photosystem II inhibitors and synthetic auxins (Chahal, Aulakh, Jugulam, & Jhala, 2015; REM, 2018). The integration of non-chemical control practices into weed management programs is an emergent need in our agricultural systems, especially if we aim to slow down the evolution of herbicide resistance weed populations, and avoid the arising of new resistance mechanisms, as it was recently reviewed by Perotti, Larran, Palmieri, Martinatto, and Permingeat (2020).

Conclusion

This work represents the first report of TSR and NTSR co-existence in a GR *A. palmeri* population from Argentina and complements the characterization previously conducted over these populations, where ALS-inhibitors resistance was confirmed and associated to several target substitutions.

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