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Multiple Resistance to Glyphosate and Acetolactate Synthase Inhibitors in Palmer Amaranth (*Amaranthus palmeri*) Identified in Brazil

Anita Küpper, Ednaldo A. Borgato, Eric L. Patterson, Acácio Gonçalves Netto, Marcelo Nicolai, Saul J. P. de Carvalho, Scott J. Nissen, Todd A. Gaines, and Pedro J. Christoffoleti*

Palmer amaranth is native to the United States, but was discovered in 2015 in Brazil. Palmer amaranth populations in Brazil were very difficult to control using glyphosate, which resulted in many changes to standard weed management practices. A genotyping assay was used to confirm that the population detected in Mato Grosso State, Brazil, was correctly identified as Palmer amaranth and that it was not tall waterhemp. Greenhouse dose–response curves and shikimate accumulation assays showed that the Brazilian population was highly resistant to glyphosate, with an LD50 value (3,982 g glyphosate ha^{-1}) more than twice the typical use rates and very little shikimate accumulation at 1 mM glyphosate concentrations in a leaf-disk assay. The Brazilian population was also resistant to sulfonylurea and imidazolinone acetolactate synthase (ALS) inhibitor herbicides. The resistance mechanisms in the Brazilian population were identified as increased *EPSPS* gene copy number for glyphosate resistance (between 50- and 179-fold relative *EPSPS* gene copy number increase) and two different alleles for target-site mutations in the *ALS* gene (W574L and S653N). These results confirm the introduction of Palmer amaranth to Brazil using a genetic marker for species identification, as well as resistance to glyphosate and ALS inhibitors.
Nomenclature: Glyphosate; Palmer amaranth, *Amaranthus palmeri* S. Wats; tall waterhemp, *Amaranthus tuberculatus* (Moq.) Sauer.
Key words: EPSPS copy number, multiple resistance, target-site mutation, acetolactate synthase, chlorsulfuron, *EPSPS*, imazethapyr, sulfometuron.

Palmer amaranth was reported in Brazil for the first time in 2015. It was found growing in cotton (*Gossypium hirsutum* L.) fields in Mato Grosso State (Andrade Júnior et al. 2015; Carvalho et al. 2015). Palmer amaranth is rarely found in South American countries; however, recent surveys conducted in Argentina have found that Palmer amaranth is established and problematic in southern Córdoba and San Luis states in soybean [*Glycine max* (L.) Merr.], peanuts (*Arachis hypogaea* L.), sorghum [*Sorghum bicolor* (L.) Moench], and corn (*Zea mays* L.) (Morichetti et al. 2013). Palmer amaranth is native to the United States and is originally from semi-arid regions (Sauer 1957).

Before Palmer amaranth's recent introduction, Brazilian weed scientists reported 10 *Amaranthus* species (Kissman and Groth 1999), including low amaranth (*Amaranthus deflexus* L.), smooth pigweed (*Amaranthus hybridus* L.), spiny amaranth (*Amaranthus spinosus* L.), redroot pigweed (*Amaranthus retroflexus* L.), and slender amaranth (*Amaranthus viridis* L.) (Carvalho et al. 2008). Palmer amaranth can be distinguished from other species by the absence of pubescence on the stem, a petiole longer than the leaf blade, and the presence of slightly spiny structures on the female flowers (Sauer 1957). Palmer amaranth is dioecious (Sauer 1957), and no other dioecious *Amaranthus* species are known to occur in Brazil. This flowering structure ensures cross-pollination (Franssen et al. 2001) and high genetic variability, factors that contribute to its adaptive and evolutionary success (Ward et al. 2013).

Palmer amaranth is an annual dicotyledonous species with C_4 photosynthesis and is able to survive extreme conditions of low humidity and high temperatures. It is a problematic species because it is highly competitive with agricultural crops (Ward et al. 2013). Palmer amaranth has become one of the most important weeds in cotton and soybean fields in the United States, especially since many populations have multiple herbicide resistance

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*First, third, seventh, and eighth authors: Graduate Student, Graduate Student, Professor, and Assistant Professor, Department of Bioagricultural Sciences and Pest Management, Colorado State University, Fort Collins, CO 80523; second, fourth, and ninth authors: Graduate Student, Graduate Student, and Associate Professor, Department of Crop Science, University of São Paulo, Piracicaba, SP 13418-000, Brazil; fifth author: Consultant, Agrocon Assessoria Agronômica, Santa Barbara do Oeste, SP 13450-971, Brazil; sixth author: Professor, Federal Institute of Education, Science and Technology of the South of Minas Gerais, Machado Campus, Machado, MG 37750-000, Brazil. [1]Corresponding author's E-mail: pjchrist@usp.br

(Nandula et al. 2012; Sosnoskie et al. 2011). A major problem in managing Palmer amaranth is the rapid selection and the potential for rapid dispersion of herbicide resistance.

Palmer amaranth introduced in Brazil is resistant to glyphosate, requiring doses higher than 4,500 g ae ha^{-1} to reduce plant growth by 80%, a threshold at which control with glyphosate is no longer considered economically viable (Carvalho et al. 2015). Dose–response studies also confirmed that these populations were cross-resistant to the acetolactate synthase (ALS) inhibitors chlorimuron, imazethapyr, and cloransulan, confirming a case of multiple resistance (Gonçalves Netto et al. 2016).

Given the importance of correct species identification and determining herbicide response to inform appropriate management decisions, the objectives of this research were to (1) confirm the identification of the new *Amaranthus* species discovered in Brazil; (2) confirm and characterize the multiple resistance to glyphosate and ALS inhibitor herbicides; and (3) identify the mechanisms conferring ALS and glyphosate resistance in the Brazilian population.

Materials and Methods

Plant Material. The glyphosate-resistant (BR-R) Palmer amaranth population was collected from a field site in Ipiranga do Norte, Mato Grosso, Brazil. The glyphosate-susceptible (GA-S) Palmer amaranth population was originally collected in 2004 from the University of Georgia Ponder Farm Research Station (Culpepper et al. 2006). A known tall waterhemp population from Nebraska was used for species identification (Bernards et al. 2012).

Species-Diagnostic Marker. Tall waterhemp is another dioecious species in the *Amaranthus* genus but is not known to be present in Brazil. Additionally, a single-nucleotide polymorphism in the *ALS* gene has been used to genetically identify tall waterhemp and Palmer amaranth (Tranel et al. 2002). To determine whether the dioecious *Amaranthus* individuals collected in Brazil were Palmer amaranth or tall waterhemp, a genotyping protocol was developed using the *ALS* polymorphism. Approximately 50 mg of young leaf tissue from three untreated GA-S individuals, six untreated BR-R individuals, and three known tall waterhemp individuals were used for DNA extraction using a modified cetyltrimethylammonium bromide (CTAB) extraction protocol (Doyle 1991). Samples were placed in tubes, a metal bead was added, and then the tubes were frozen in liquid nitrogen. The samples were ground using a Qiagen TissueLyser II (Qiagen, Valencia, CA) for 1 min at 30 oscillations s^{-1}. The ground tissue was incubated with 500 µl of 2 × CTAB buffer with 4 µl of 2-mercaptoethanol at 50 C for 15 min. The suspension was then incubated for 15 min with 500 µl of 24:1 chloroform:isoamyl alcohol with gentle agitation and then centrifuged for 15 min at 15,000 × *g*. The aqueous phase was removed and reseparated using another 500 µl of 24:1 chloroform:isoamyl alcohol and was centrifuged for 5 min at 15,000 × *g*. The aqueous phase was once again removed and then precipitated with 50 µl sodium acetate (3M, pH 5.2) and 1,650 µl of 100% ethanol. After 15 min at room temperature, samples were centrifuged for 15,000 × *g* for 15 min. All liquid was removed, and the pellets were rinsed with 70% ethanol and allowed to dry. Dry pellets were resuspended in water. DNA concentration and quality were determined using a micro-spectrophotometer (NanoDrop 2000 Spectrophotometer, Thermo Fisher Scientific, Wilmington, DE).

Amaranthus KASP Genotyping. To determine whether individuals in the BR-R population were Palmer amaranth or tall waterhemp, a Kompetitive Allele Specific PCR (KASP™) assay was developed to genotype a species-diagnostic single-nucleotide polymorphism (SNP) located at base pair 678 in the *ALS* coding sequence (Tranel et al. 2002). The assay was performed using six control individuals, (three GA-S Palmer amaranth individuals, three Nebraska tall waterhemp individuals) and six untreated individuals from the BR-R population. SNP678 is a cytosine (C) in tall waterhemp individuals and a thymine (T) in Palmer amaranth individuals. Two species-diagnostic forward primers for SNP678 were developed that were identical, except the final 3′ nucleotide, which pairs with SNP678. Additionally, each forward primer contained nucleotides at its 5′ end specific for either a HEX- or FAM-labeled oligo contained in the KASP Master Mix (LGC Genomics, Beverly, MA) (tall waterhemp forward primer: 5′-GAAGGTGACCAAGTTCATGCTAAA AAGAAAGCTTCCTTAACAATTCTAGGG-3′; Palmer amaranth forward primer: 5′-GAAGGTCG GAGTCAACGGATTAAAAAGAAAGCTTCCTTAAC AATTCTAGGA-3′). For PCR, a universal reverse primer (5′-GTTGAGGTAACTCGATCCATTACTA AGC-3′) was developed that was complementary in both *Amaranthus* species.

Forward and reverse primers were mixed according to the manufacturer's recommendations and

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included 18 µl tall waterhemp forward primer (FAM label) at 12 µM, 18 µl Palmer amaranth forward primer (HEX label) at 12 µM, and 45 µl of universal reverse primer at 30 µM. Primer mix was brought up to 150 µl with 10 mM Tris-HCl (pH 8.3). A master mix was then generated from 11.8 µl of primer mix and 432 µl of LGC Genomics Master Mix. Final reactions were mixed in a 96-well, optically clear plate by combining 4 µl of *Amaranthus* DNA at 5 ng/µl with 4 µl of LGC Genomics Master Mix plus primers. PCR was performed on a CFX Connect (Bio-Rad Laboratories, Hercules, CA) with the following cycling protocol: 94 C for 15 min; followed by 10 cycles of 94 C for 20 s, 61 decreasing to 55 C for 60 s (0.6 C touchdown per cycle); followed by 26 cycles of 94 C for 20 s, and 55 C for 60 s. An endpoint fluorescence reading was taken by cooling the plate to 30 C for 30 s and reading the plate in both the HEX and FAM fluorescent channels. HEX and FAM fluorescence was corrected by removing the background fluorescence observed in a no-template control. Fluorescence was plotted in a two-dimensional scatter plot so that the test BR-R population individuals could be compared with known tall waterhemp and Palmer amaranth samples. Tall waterhemp samples (primers labeled with FAM) were expected to have high fluorescence intensity for FAM but not HEX, while Palmer amaranth samples (primers labeled with HEX) were expected to have high fluorescence intensity for HEX but not FAM. Clustering of the test BR-R individuals with either high FAM or high HEX fluorescence intensity would indicate their identity as tall waterhemp or Palmer amaranth, respectively. Each KASP-genotyped sample was independently confirmed using the *Eco*RV PCR-RFLP developed by Tranel et al. (2002) (unpublished data).

Greenhouse Glyphosate Dose Response. A dose–response experiment was carried out in the greenhouse of the Weed Research Laboratory at Colorado State University in Fort Collins, CO, to quantify the level of glyphosate resistance. Seeds from the BR-R and GA-S Palmer amaranth populations were planted on 1% agar medium and placed in a refrigerator at 4 C for 7 d. They were then transferred to a germination bench at room temperature with 12/12 h of day/night to stimulate rapid and simultaneous germination. Germinated seedlings were then transplanted into commercial potting soil (Professional Growing Mix, Sun Gro® Horticulture, Vancouver, Canada) in 5 by 5 cm inserts. They were treated with glyphosate at 8- to 10-cm height and kept in a greenhouse where they were maintained at 24 ± 2 C temperatures and 15/9 h day/night photoperiods supplemented with metal-halide lamps (400 µmol m^{-2} s^{-1}) and watered twice daily. The experiment was arranged in a randomized complete block design with three replicates. Each replicate contained six individuals for each population and dose, and four individuals for each population and dose when the experiment was repeated. A glyphosate dose response was conducted using 0, 0.05, 0.08, 0.2, 0.4, 0.8, 1.6, and 4.8 kg ae ha^{-1} with commercially formulated glyphosate (potassium salt, Roundup WeatherMax®, Monsanto, St. Louis, MO). When the experiment was repeated, an additional dose of 8 kg glyphosate ha^{-1} was included. Applications were made using an overhead track sprayer (DeVries Manufacturing, Hollandale, MN) equipped with a flat-fan nozzle tip (TeeJet® 8002EVS, Spraying System, Wheaton, IL) calibrated to deliver 187 L ha^{-1} of spray solution at 172 kPa. Survival was recorded after 21 d, defined as any plant showing new growth. Dose–response analysis was conducted using the 'drc' package in R (Knezevic et al. 2007; R Core Team 2015). Survival data (proportion) were analyzed using the three-parameter log-logistic model in the 'drc' package:

$$y = \frac{D}{\left[1 + \left(\frac{x}{\mathrm{LD50}}\right)^{b}\right]} \quad [1]$$

where y = survival; x = glyphosate dose (g ae ha^{-1}); D = upper limit; b = slope; and LD50 = dose causing 50% reduction in survival.

Shikimate Assay. Twenty individuals from each of the BR-R and the GA-S populations were grown in the greenhouse and tested for glyphosate resistance using an in vivo leaf-disk assay. Three technical replicates (5-mm leaf disks) from each individual per population were sampled, following the procedure described by Shaner et al. (2005). The excised leaf disks were placed into 96-well microtiter plates containing 10 mM ammonium phosphate buffer and molecular grade glyphosate at the doses of 100, 500, and 1,000 µM. Shikimate levels were read at 380 nm on a fluorescence plate reader (BioTek™ Synergy™ 2 multi-mode microplate reader, Winooski, VT). A shikimate standard curve was used to quantify shikimate accumulation (ng shikimate $µl^{-1}$) in the samples. Data were analyzed using a *t*-test to compare shikimate accumulation between BR-R and GA-S.

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***EPSPS* Gene Copy Number.** Genomic DNA was used to determine 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) copy number using real-time quantitative PCR (qPCR). Twenty individuals from each of the GA-S and BR-R populations were grown in small pots, and young leaf tissue was collected from each individual for genomic DNA. The samples were immediately frozen in liquid nitrogen and stored at −80 C. Genomic DNA was extracted using the DNEasy Plant Mini Kit (Qiagen, Valencia, CA) and quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). DNA concentrations were adjusted to 5 ng μl^{-1}, and primer sets and qPCR conditions were used as previously described (primers ALSF2 and ALSR2, EPSF1 and EPSR8) (Gaines et al. 2010). Threshold cycles (C_t) for *EPSPS* and *ALS* were recorded by a CFX Connect™ Real-Time PCR Detection System thermal cycler (Bio-Rad Laboratories, Hercules, CA). Relative *EPSPS* gene copy number was calculated as $2^{-\Delta Ct}$, with $\Delta Ct = [(Ct, ALS) - (Ct, EPSPS)]$ (Gaines et al. 2010). Triplicate technical replications were used to calculate the mean and standard error of the increase in *EPSPS* gene copy number relative to *ALS*.

Single-Dose ALS Testing. Single-dose experiments were conducted to assess resistance to the ALS inhibitors chlorsulfuron, sulfometuron-methyl (both sulfonylureas), and imazethapyr (imidazolinone). These experiments were conducted in the greenhouse at Colorado State University in Fort Collins, CO, under the same conditions as described in the glyphosate dose-response experiment. Individuals from BR-R and GA-S were treated at 8- to 10-cm height. The experiments with sulfonylureas used 28 individuals per population and treatment combination, and the experiments with imazethapyr used 36 individuals for each population. Chlorsulfuron (Glean, DuPont, Wilmington, DE) and sulfometuron-methyl (Oust, Bayer CropScience, Research Triangle Park, NC) were applied at 88 g ai ha^{-1} and 315 g ai ha^{-1}, respectively. Imazethapyr (Pursuit, BASF, Research Triangle Park, NC) was applied at 61 g ai ha^{-1} with 1.25% v/v crop oil concentrate. Height (cm), dry weight (g), and survival data were collected 21 d after treatment. The data were analyzed using ANOVA, and LSD with $P = 0.05$ was used for multiple comparison adjustment.

***ALS* Gene Sequence.** Approximately 50 mg of young leaf tissue was sampled from each of three untreated GA-S individuals, six untreated BR-R individuals, nine BR-R individuals that survived 315 g ai ha^{-1} sulfometuron-methyl, and nine BR-R individuals that survived 88 g ai ha^{-1} chlorsulfuron. DNA was extracted as described for the species-diagnostic marker. The full-length *ALS* gene was amplified by PCR using the forward primer: 5′-ATGGCGTCCACTTCAACAAACC-3′ and reverse primer 5′-CTAATAAGCCCTTCTTCCATCACCC -3′. Thirty microliter reactions were mixed following the standard protocol provided with Thermo Scientific Phusion polymerase and 20 ng of template genomic DNA. Reactions were cycled 32 times with the following three-step protocol: 98 C for 10 s, 62 C for 20 s, and 72 C for 90 s. PCR products were run on a 1% agarose gel to verify single band amplification. The expected bands at 2,010 bp were excised from the gel and purified following the standard protocol provided by the QIAquick Gel Extraction Kit from Qiagen. Purified PCR products were sequenced using both the amplification primers listed above and the following four sequencing primers: Seq_FP1 5′-AGTTTGTATTGCCACTTCTGGTCC-3′, Seq_FP2 5′-GAAATCCTCGCCAATGGCTGAC-3′, Seq_RP1 5′- GTCAGCCATTGGCGAGGATTTC-3′, Seq_RP2 5′-TGGACCAGAAGTGGCAATACAAAC-3′. Sanger sequencing reads were analyzed using A Plasmid Editor (aPe, version 2.0.49) and heterozygous base pairs were identified in the sequence trace files by manual inspection. Translated amino acid sequences obtained from BR-R and GA-S were compared with a known susceptible Palmer amaranth ALS amino acid sequence (Molin et al. 2016), GenBank protein accession AMS38337.1.

Results and Discussion

Species-Diagnostic Marker. The genotyping assay used to amplify an SNP within the *ALS* gene that distinguishes Palmer amaranth from tall waterhemp clearly grouped the BR-R individuals with Palmer amaranth GA-S individuals (Figure 1) due to the high HEX fluorescence intensity produced from the Palmer amaranth HEX-labeled forward primer. Known tall waterhemp individuals were clearly distinguished by the tall waterhemp allele at this SNP position, as shown by high FAM fluorescence intensity produced from the FAM-labeled forward primer specific for tall waterhemp. Therefore, the dioecious *Amaranthus* population collected from Mato Grosso State, Brazil is Palmer amaranth and not tall waterhemp. The *Eco*RV PCR-RFLP developed by Tranel et al. (2002) also produced the same

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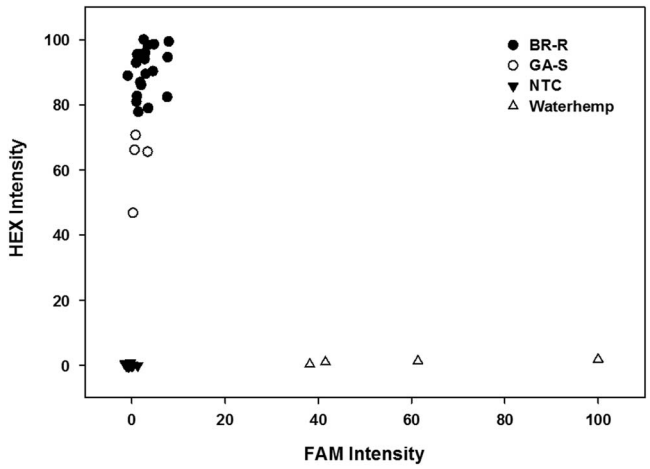

Figure 1. Genotyping assay using KASP in which Palmer amaranth forward primers in the KASP assay were labeled with HEX, and tall waterhemp forward primers were labeled with FAM. Clustering of the Brazilian population (BR-R) together with known Palmer amaranth (GA-S) for high HEX fluorescence intensity confirms that BR-R is Palmer amaranth. Known tall waterhemp samples showed expected high FAM fluorescence intensity, and no-template control (NTC) had no fluorescence for HEX or FAM.

species identification as our KASP assay (unpublished data).

Greenhouse Dose–Response Curves. The data from two repeated greenhouse dose–response experiments were combined for analysis. The BR-R population was glyphosate resistant (Table 1; Figure 2), with an LD50 of 3,982 g ha^{-1}. The LD50 for GA-S was 169 g ha^{-1}, resulting in a resistance factor (R/S) of 24 (Table 1; Figure 2). This resistance factor confirms the resistance of the Brazilian Palmer amaranth population to glyphosate and demonstrates that the BR-R population has an LD50 higher than two times the typical commercial glyphosate rate (ranging from 800 to 1,000 g ae ha^{-1}).

Shikimate Assay. Shikimate is an important intermediate in the biosynthesis of the aromatic amino acids phenylalanine, tyrosine, and tryptophan. EPSPS inhibition by glyphosate results in shikimate accumulation (Herrmann and Weaver 1999; Shaner et al. 2005; Steinrücken and Amrhein 1980). There was a clear difference in shikimate accumulation between the two populations tested at all three glyphosate doses (Figure 3), with GA-S accumulating significantly more shikimate than BR-R. Increasing glyphosate doses, for doses of up to 1,000 µM, did not cause shikimate accumulation in BR-R, a clear metabolic marker for glyphosate resistance. The lack of shikimate accumulation in the BR-R plants corroborates results obtained from the dose–response curves (Table 1; Figure 2), with both confirming glyphosate resistance in the BR-R population. These results also confirm the findings of Carvalho et al. (2015) reporting glyphosate resistance of this Palmer amaranth population from the state of Mato Grosso, Brazil.

***EPSPS* Gene Copy Number.** The qPCR technique was used to quantify *EPSPS* gene copy number relative to *ALS*. Between 1.1 and 1.5 relative *EPSPS* gene copies were measured in the GA-S population, and between 50 and 179 relative *EPSPS* gene copies were measured in the BR-R population. Increased *EPSPS* gene copy number was highly correlated with reduced shikimate accumulation in BR-R individuals, while wild-type, single-copy *EPSPS* was highly correlated with high shikimate accumulation in GA-S individuals (Figure 4). Individuals in the BR-R population have *EPSPS* gene duplication that results in EPSPS overexpression as a mechanism of glyphosate resistance, first reported in Palmer amaranth (Gaines et al. 2010).

A previously reported glyphosate-resistant Palmer amaranth population from Georgia had a reported LD50 of 1,600 g ha^{-1} and between 40 and 100 relative *EPSPS* gene copies (Gaines et al. 2011). The BR-R population had an LD50 of 3,982 g ha^{-1} (Table 1) and *EPSPS* gene duplication between 50

Table 1. Confirmation of glyphosate resistance in Palmer amaranth from Brazil (BR-R) compared with a known glyphosate-susceptible population from Georgia (GA-S) in two repeated greenhouse dose–response experiments.[a]

Population	D[b]	LD50[c]	b[d]	R/S[e]	P value
BR-R	1.0	3,982 (310)	3.1	23.5 (3.3)	<0.0001
GA-S	0.96	169 (19)	1.8		

[a] Plant survival expressed as a proportion was used in a three-parameter log-logistic equation (Equation 1).
[b] Upper limit.
[c] Herbicide dose in g ae ha^{-1} that causes 50% reduction in survival; standard error in parentheses.
[d] Slope.
[e] Ratio of LD50 for BR-R to LD50 for GA-S expressed as R/S resistance factor; standard error in parentheses.

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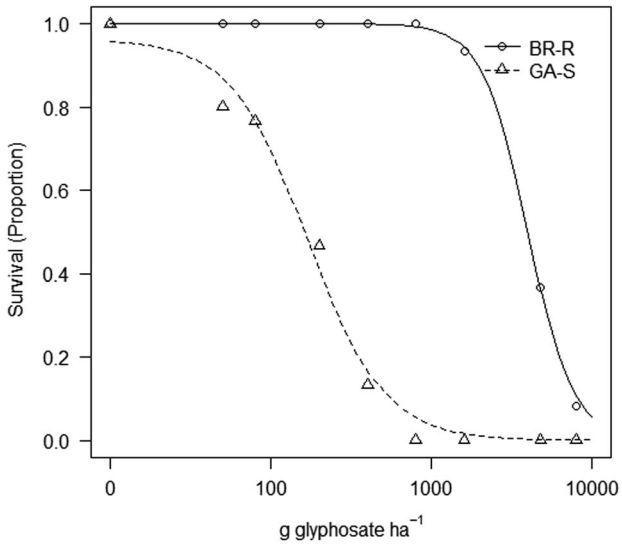

Figure 2. Greenhouse glyphosate dose–response curves for plant survival of a glyphosate-resistant Palmer amaranth population from Brazil (BR-R) and glyphosate-susceptible Palmer amaranth from Georgia (GA-S) expressed as the proportion of survivors (Equation 1).

and 179 copies, suggesting that glyphosate resistance in BR-R is due to increased *EPSPS* gene copies. Increased *EPSPS* gene copy number as a glyphosate resistance mechanism has also been reported in Italian ryegrass [*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot] (Salas et al. 2012), tall waterhemp (Chatham et al. 2015a, 2015b; Lorentz et al. 2014), spiny amaranth (Nandula et al. 2014), ripgut brome (*Bromus diandrus* Roth) (Malone et al. 2016), kochia [*Kochia scoparia* (L.) Schrad.] (Wiersma et al. 2015), and goosegrass (*Eleusine indica* (L.) Gaertn.] (Chen et al. 2015).

Single-Dose ALS Testing. A high percentage of BR-R individuals survived treatment with chlorsulfuron, sulfometuron, and imazethapyr (Table 2). The GA-S population was completely controlled by all three ALS herbicides. Plant height and dry weight also indicated that BR-R individuals were resistant to all three ALS herbicides, while GA-S individuals were susceptible (Table 2). The high survival rate of BR-R after ALS herbicide treatment is consistent with previous observations of high-level ALS herbicide resistance in Palmer amaranth (Burgos et al. 2001; Guo et al. 2015; Whaley et al. 2007; Wise et al. 2009).

***ALS* Gene Sequencing.** Sequencing the *ALS* gene from both populations resulted in the identification of two independent mutations in the BR-R *ALS* gene sequence, resulting in a change from tryptophan to leucine at position 574 (W574L) and serine to asparagine at position 653 (S653N) (Table 3; Figure 5). The W574L mutation is known to confer resistance to both imidazolinones and sulfonylureas, while the S653N mutation is known to confer resistance only to imidazolinones (Burgos et al. 2001; Franssen et al. 2001; McCourt et al. 2006; Patzoldt and Tranel 2007; Powles and Yu 2010; Sprague et al. 1997; Tranel and Wright 2002). Nearly all BR-R individuals were either heterozygous or homozygous for W574L, while 10 of 24 sequenced individuals were heterozygous for S653N; none were homozygous for S653N. No individuals carried both W574L and S653N mutations within the same allele. ALS resistance is inherited as a dominant trait (Powles and Yu 2010; Tranel and Wright 2002), which explains the high survival rate of heterozygous

Table 2. A glyphosate-resistant Palmer amaranth population from Brazil (BR-R) is resistant to sulfonylurea and imidazolinone ALS herbicides.[a]

Line	*n*	Herbicide	Alive (%)		Height (cm)		Dry weight (g)	
BR-R	28	Untreated	100 (0)	A	22.8 (8.2)	AB	2.3 (0.5)	A
BR-R	28	Chlorsulfuron	96 (20)	A	16.4 (6.0)	C	1.8 (0.8)	BC
BR-R	28	Sulfometuron	88 (33)	A	18.7 (8.4)	BC	1.5 (0.7)	C
GA-S	28	Untreated	100 (0)	A	27.5 (6.4)	A	2.1 (0.7)	AB
GA-S	28	Chlorsulfuron	0 (0)	B	8.1 (5.2)	D	0.9 (0.5)	D
GA-S	28	Sulfometuron	0 (0)	B	8.0 (4.3)	D	0.8 (0.5)	D
BR-R	36	Untreated	100 (0)	A	17.8 (3.3)	B	1.3 (0.5)	B
BR-R	36	Imazethapyr	100 (0)	A	19.3 (3.0)	AB	1.8 (0.3)	A
GA-S	36	Untreated	100 (0)	A	20.5 (4.8)	A	1.5 (0.5)	B
GA-S	36	Imazethapyr	0 (0)	B	7.4 (3.3)	C	0.8 (0.4)	C

[a] Plants were treated at 8- to 10-cm height. Survival, height, and dry weight data collected 21 d after treatment for single-dose treatments of chlorsulfuron (88 g ha^{-1}), sulfometuron (315 g ha^{-1}), and imazethapyr (61 g ha^{-1}) on BR-R and GA-S (glyphosate- and ALS-susceptible Palmer amaranth from Georgia). Standard deviation shown in parentheses; *n*, number of individuals tested per dose; letters within a column indicate significant difference at P = 0.05.

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Table 3. Two target-site *ALS* mutations are present in ALS-resistant Palmer amaranth from Brazil (BR-R).[a]

			Mutation and genotype[b]	
			W574L	S653N
Population	Treatment	Individual	TGG → TTG	AGC → AAC
BR-R	Chlorsulfuron	1	HET	HOMO S
		2	HET	HOMO S
		3	HOMO S	HET
		4	HOMO R	HOMO S
		5	HET	HOMO S
		6	HOMO S	HET
		7	HET	HET
		8	HOMO S	HET
		9	HET	HOMO S
	Sulfometuron	1	HOMO S	HET
		2	HET	HOMO S
		3	HET	HOMO S
		4	HOMO R	HOMO S
		5	HET	HOMO S
		6	HET	HET
		7	HET	HET
		8	HOMO R	HOMO S
		9	HET	HET
	Untreated	1	HET	HOMO S
		2	HET	HET
		3	HOMO R	HOMO S
		4	HET	HOMO S
		5	HET	HET
		6	HOMO R	HOMO S
GA-S	Untreated	1	HOMO S	HOMO S
		2	HOMO S	HOMO S
		3	HOMO S	HOMO S

[a] All BR-R individuals that survived a single-dose treatment with chlorsulfuron or sulfometuron had at least one ALS resistance–conferring allele at position W574 (resistant allele L) or S653 (resistant allele N) of ALS. All individuals from GA-S were homozygous for the susceptible allele at W574 and S653.

[b] HOMO R, homozygous resistant allele (dark cells); HET, heterozygous (gray cells); HOMO, homozygous susceptible allele (light cells).

ALS mutants in BR-R. No other *ALS* mutations were detected, but four BR-R individuals that survived either chlorsulfuron or sulfometuron were homozygous for the susceptible allele at W574, and all four were heterozygous for S653N (Table 3). Since the S653N is not known to confer resistance to sulfonylurea herbicides, these four individuals may have a different, non–target site resistance mechanism. A different and undetected *ALS* target-site mutation is considered unlikely, as the entire *ALS* gene was sequenced. All sequenced GA-S individuals were homozygous susceptible for both W574 and S653 (Table 3; Figure 5).

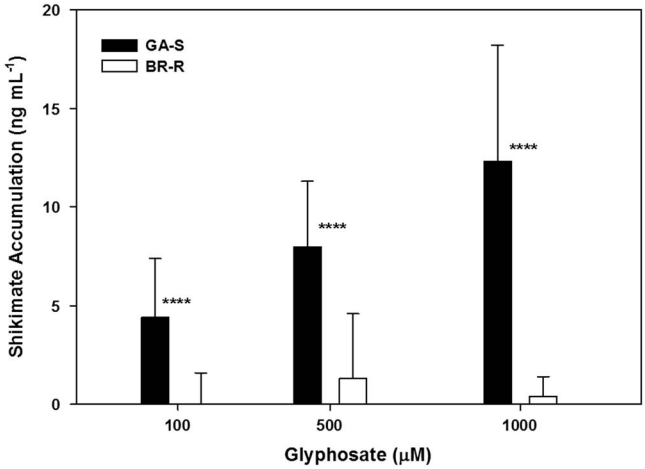

Figure 3. Shikimate accumulation in glyphosate-resistant Palmer amaranth from Brazil (BR-R) and glyphosate-susceptible Palmer amaranth from Georgia (GA-S) at three glyphosate doses. Mean from 20 biological replications with standard deviation; **** P-value < 0.0001 between GA-S and BR-R at each dose.

In wild radish (*Raphanus raphanistrum* L.), the S653N mutation confers resistance to the imidazolinones, while homozygous W574L confers resistance to sulfonylureas, imidazolinones, and triazolpirimidines, three chemical families of ALS inhibitors (Yu et al. 2012). The presence of W574L gives high levels of resistance to these three chemical groups in tall waterhemp, while S653N is usually linked to imidazolinone resistance (Patzoldt and Tranel 2007), as observed here.

Molin et al. (2016) reported both the W574L and the S653N mutation in Palmer amaranth, and showed the transfer of the W574L mutation from Palmer amaranth to hybrids between Palmer amaranth and spiny amaranth. Resistance to ALS inhibitors was reported in mucronate amaranth (*Amaranthus quitensis* H.B.K.) and Palmer amaranth from Argentina, with the S653N mutation observed only in mucronate amaranth but not in Palmer amaranth (Berger et al. 2016). The study concluded that the Palmer amaranth population in Argentina likely has a different ALS resistance mechanism than target-site mutation. While not definitive, the absence of S653N in Palmer amaranth from Argentina and the presence of both W574L and S653N in Palmer amaranth from Brazil suggest that independent introductions of Palmer amaranth may have occurred in the two countries.

This is the first study confirming through molecular methods the introduction of Palmer amaranth in Brazil and the molecular mechanisms of multiple resistance within this population to

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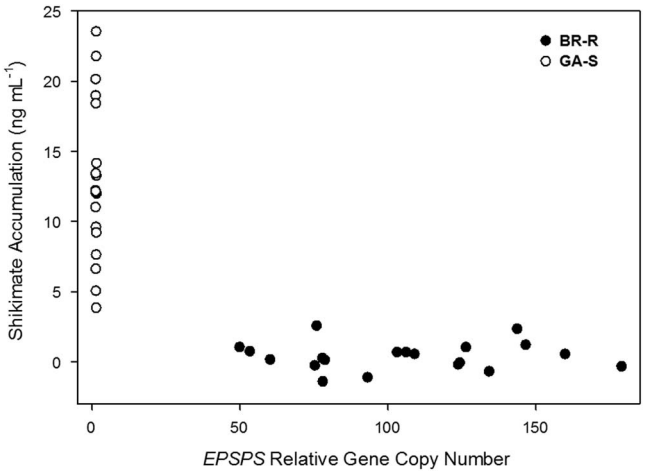

Figure 4. *EPSPS* relative genomic copy number and shikimate accumulation after treatment of leaf disks with 1,000 µM glyphosate in glyphosate-resistant Palmer amaranth from Brazil (BR-R) and glyphosate-susceptible Palmer amaranth from Georgia (GA-S).

```
>AMS38337.1 NNQHLGMVVQWEDRFYKANRA HQEHVLPMIPSGAAFKDTITE
>GA-S1      NNQHLGMVVQWEDRFYKANRA HQEHVLPMIPSGAAFKDTITE
>GA-S2      NNQHLGMVVQWEDRFYKANRA HQEHVLPMIPSGAAFKDTITE
>GA-S3      NNQHLGMVVQWEDRFYKANRA HQEHVLPMIPSGAAFKDTITE
>BR-R1      NNQHLGMVVQWEDRFYKANRA HQEHVLPMIPSGAAFKDTITE
>BR-R2      NNQHLGMVVQLEDRFYKANRA HQEHVLPMIPNGAAFKDTITE
>BR-R3      NNQHLGMVVQLEDRFYKANRA HQEHVLPMIPSGAAFKDTITE
>BR-R4      NNQHLGMVVQWEDRFYKANRA HQEHVLPMIPNGAAFKDTITE
>BR-R5      NNQHLGMVVQLEDRFYKANRA HQEHVLPMIPSGAAFKDTITE
```

Figure 5. Alignment of ALS amino acid sequences from known ALS-susceptible Palmer amaranth (GenBank AMS38337.1), glyphosate- and ALS-susceptible Palmer amaranth from Georgia (GA-S), and glyphosate- and ALS-resistant Palmer amaranth from Brazil (BR-R) individuals, showing W574L and S653N mutations in BR-R individuals.

glyphosate and ALS inhibitors. The resistance mechanisms are, respectively, increased *EPSPS* gene copy number and target-site mutations in *ALS* (W574L and S653N). Both mechanisms confer high resistance levels to these herbicides. Other known glyphosate resistance mechanisms such as vacuole sequestration (Ge et al. 2010, 2012) and reduced translocation (e.g., Vila-Aiub et al. 2012; Wakelin et al. 2004) were not investigated. Increased *ALS* gene expression was also not investigated as a potential resistance mechanism. While these mechanisms have not yet been reported in Palmer amaranth, they cannot be ruled out based on the results of this study. Integrated management practices should be adopted in places where Palmer amaranth is found in Brazil, such as tank-mixing herbicides with different mechanisms of action, using PRE herbicides, crop rotation, and integrating cover crops (e.g., DeVore et al. 2013; Price et al. 2012) for more effective Palmer amaranth control. Future research should focus on population genetics to determine the geographic route by which Palmer amaranth was introduced in Brazil and how to prevent possible new introductions of this and other species.

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