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Emergence of multiple resistance to EPSPS and ALS herbicides in smooth pigweed (*Amaranthus hybridus*): a growing concern in Brazil

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Abstract

Recently, farmers in Brazil have observed a decline in efficacy of glyphosate, chlorimuron, and imazethapyr control of smooth pigweed (*Amaranthus hybridus* L.). The objectives of this study were to quantify the resistance of *Amaranthus* in Brazil to glyphosate and acetolactate synthase (ALS)-inhibiting herbicides, elucidate the mechanism of resistance, and assess the frequency of sensitivity shifts to glyphosate and chlorimuron in Brazil. Dose–response assays were conducted in a greenhouse with glyphosate, chlorimuron, and imazethapyr. This was followed by sequencing of the *EPSPS* and *ALS* genes. Additionally, 740 *Amaranthus* populations across several Brazilian states were monitored over 4 yr, subjected to a single discriminatory dose of glyphosate and chlorimuron. The populations BR18Asp051 and BR21Asp205 were resistant to glyphosate, chlorimuron, and imazethapyr. The elevated resistance level to glyphosate in these populations is attributed to multiple amino acid substitutions (TAP-IVS) in the *EPSPS* gene; and cross-resistance to sulfonylureas and imidazolinones is conferred by the Trp-574-Leu substitution in the *ALS* gene in both populations. Overall, resistance distribution indicated that 88% of the sampled populations were considered sensitive to glyphosate, while 66% were sensitive to chlorimuron. Furthermore, 10% of the samples demonstrated multiple resistance to both active ingredients. A shift in glyphosate sensitivity was observed in four states in Brazil; however, sensitivity shifts to chlorimuron were more widely dispersed in Brazilian agricultural regions.

Keywords: Chlorimuron; glyphosate; imazethapyr; resistance frequency; resistance mechanism

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Introduction

The evolution of herbicide-resistant weed populations represents a growing challenge for both contemporary and future agriculturalists. The repetitive applications of herbicides with the same active ingredient or mode of action have catalyzed this biological phenomenon (Evans et al. 2016; Neve et al. 2014). Such selection pressure has culminated in the selection of resistance to at least one herbicide in 272 weed species globally (Heap 2024). While herbicides are not the sole form of selection pressure in modern agriculture, their utilization precipitates more rapid changes, and the resultant weed resistance is more readily apparent (Coble and Schroeder 2016).

The genus *Amaranthus* encompasses multiple species, some of which are significant agricultural weeds (Gonçalves-Netto et al. 2016). In Brazil, approximately 10 *Amaranthus* species are recognized as agriculturally significant. To date, seven instances of herbicide resistance within *Amaranthus* species have been documented in Brazil, involving redroot pigweed (*Amaranthus retroflexus* L.), slender amaranth (*Amaranthus viridis* L.), Palmer amaranth (*Amaranthus palmeri* S. Watson), and smooth pigweed (*Amaranthus hybridus* L.) (Heap 2024). *Amaranthus hybridus* is a notably competitive weed for summer crops in South America and is characterized as an annual, erect, herbaceous species with glabrous stems, petiolate ovate leaves with sharp apices, terminal panicles forming inflorescences, and hermaphrodite flowers (Belgrano et al. 2008; Perotti et al. 2019). This species utilizes the C_4 photosynthetic pathway (Ferreira et al. 2003), exhibits high fecundity and seed longevity (Faccini and Vitta 2005), demonstrates emergence fluxes across varying periods of the crop cycles (Brighenti and Oliveira 2015), and, as a result, is becoming increasingly challenging to manage within cropping systems (Butts et al. 2016).

In Argentina, *A. hybridus* has been reported to exhibit resistance to acetolactate synthase (ALS) inhibitors (Tuesca and Nisensohn 2001), auxins (Dellaferrera et al. 2018), and glyphosate (García et al. 2019; Perotti et al. 2019), with some mechanisms of resistance to these herbicides having been elucidated. For instance, the mechanism conferring glyphosate resistance involves a triple substitution (TAP-IVS: Thr-102-Ile, Ala-103-Val, and Pro-106-Ser) in the EPSPS protein (García et al. 2019; Perotti et al. 2019; Sulzbach et al. 2024). In the case of ALS-inhibitor resistance, the predominant molecular mechanism resistance identified is target-site mutation. To date, 28 amino acid substitutions at 8 positions within the *ALS* gene have been documented to confer ALS-inhibitor resistance (Tranel et al. 2023). Biotypes of *A. hybridus* found in Argentina

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that are resistant to ALS-inhibiting herbicides exhibit a specific site mutation documented as substitutions Trp-574-Leu and Asp-376-Glu in the ALS amino acid sequence (Larran et al. 2018).

The weed management decisions made by growers significantly influence the selection of herbicide-resistant weeds within their fields. When poor weed control is observed, growers often respond by increasing herbicide doses, thereby intensifying the selection pressure for resistance (Bracamonte et al. 2016; González-Torralva et al. 2012). However, resistance can also be spread from one field to another through field machinery operations, high seed production by weeds, and seed crop contaminants, affecting regions previously unaffected by resistance (Penckowski et al. 2020). Resistance monitoring studies, aimed at understanding the frequency and distribution of resistant weeds in agricultural areas, serve as a crucial tool for increasing awareness about herbicide resistance and aiding in the adoption of integrated management strategies at the regional level (Schultz et al. 2015).

Recently, field crop extension scientists and farmers have observed diminished control of *Amaranthus* spp. with glyphosate, chlorimuron, and imazethapyr herbicides in Brazil. Consequently, the primary objective of this study was to quantify the resistance level to glyphosate, chlorimuron, and imazethapyr in four *Amaranthus* populations from southern Brazil and to investigate the mechanism underlying resistance to glyphosate and ALS inhibitors. Additionally, this study aimed to determine the frequency of shifts in sensitivity to glyphosate and chlorimuron in Brazil.

Materials and Methods

Plant Material and Growth

Seeds from two plants from two *Amaranthus* populations with putative resistance to glyphosate, chlorimuron, and imazethapyr were collected from distinct soybean [*Glycine max* (L.) Merr.] fields in Brazil. Additionally, seeds from two reference populations known to be sensitive were obtained; these populations had no history of exposure to glyphosate or ALS-inhibiting herbicides (Table 1).

Plants from the putatively resistant population were treated with glyphosate (1,000 g ae ha^{-1}, Roundup WG®, 720 g ae kg^{-1}, Monsanto from Brazil LTDA, 1100 Domingo Jorge Road, São Paulo, SP 047779-900, Brazil), chlorimuron (20 g ai ha^{-1}, Classic WG®, 250 g ai kg^{-1}, Du

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Pont from Brazil S.A, 506 Alameda Itapecuru Road, Barueri, SP 06454-080, Brazil), and imazethapyr (100 g ai ha^{-1}, Pivot SL®, 100 g ai L^{-1}, 5756 Industrial Domingos Giomi District, Indaiatuba, SP 13347-390, Brazil). Surviving plants were maintained in the greenhouse for seed production. Seeds from *Amaranthus* populations were germinated in 0.3-L pots filled with a commercial substrate (BASE substratos, Basaplant tubetes). Plants were potted individually and grown in a greenhouse at 32 C/ 25 C (day/night) under a 16-h photoperiod until they reached the 4-leaf stage.

Whole-Plant Dose–Response Curve

The dose–response curve was plotted for two generations of *Amaranthus* (G1 and G2). The experimental design was completely randomized in a 6 by 12 factorial scheme for glyphosate and a 5 by 9 factorial design for chlorimuron and imazethapyr. The first factor was the *Amaranthus* population (Table 1) and the second factor was herbicide. For Herbicide dosages were: glyphosate (Roundup WG®, 720 g kg^{-1}) at 0, 45, 90, 180, 360, 720, 1,440, 2,880, 5,760, 11,520, 23,040, and 46,080 g ha^{-1}; chlorimuron (Classic WG®, 250 g kg^{-1}) at 0, 2.5, 5.0, 10, 20, 40, 80, 160, and 320 g ha^{-1}; and imazethapyr (Pivot SL® 100 g L^{-1}) at 0, 12.5, 25, 50, 100, 200, 400, 800, and 1,600 g ha^{-1}. Each combination of population and dose was replicated four times. The application was performed in a spray chamber equipped with flat-fan nozzles (XR110.02, TeeJet®, Wheaton, USA) calibrated to deliver 200 L ha^{-1} at 200 kPa pressure.

Aboveground biomass of each plant was harvested at 21 d after treatment (DAT) and oven-dried for 3 d at 60 C for biomass (dry weight) determination. The biomass reduction (BR) data were converted into percent biomass reduction relative to the nontreated control plants (Wortman 2014) using Equation 1:

$$\text{Biomass reduction (\%)} = \frac{(C - B)}{C} \times 100 \qquad [1]$$

where C represents the mean biomass of the four nontreated control replicates, and B represents the biomass of an individually treated experimental plant.

Data were analyzed using ANOVA, and a nonlinear logistic regression model was fit using Equation 2:

$$y = \frac{a}{[1+\left(\frac{x}{b}\right)c]} \qquad [2]$$

In this equation, y represents the control, x represents the herbicide dose (g ha^{-1}), a represents the maximum value, b represents the dose providing 50% response (GR_{50}), and represents the

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curve slope around *b*. The R_F was calculated as the ratio of the GD_{50} values among the putative resistant and sensitive populations.

DNA Extraction

Leaf tissue from herbicide-treated greenhouse-grown plant (4 plants per tested population) was utilized for DNA extraction. Genomic DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega) adhering to the manufacturer's protocol. DNA quantification was conducted using the Denovix instrument, and DNA integrity was assessed on a 1% agarose gel. All DNA samples were diluted to a concentration of 50 ng μl^{-1}, and a 2.0 µl aliquot was used for PCR amplification.

Amaranthus Species Identification

The species of the four populations (BR18Asp050, BR21Asp206, BR18Asp051, and BR21Asp205) were identified using three molecular approaches. The first approach was based on the methodology developed by Wright et al. (2016). Briefly, the methodology is based on a PCR assay using the amplification of a region on intron 1 of the *EPSPS* gene, which considers seven *Amaranthus* species: *A. palmeri*, spiny amaranth (*Amaranthus spinosus* L.), waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer], *A. retroflexus*, *A. hybridus*, prostrate pigweed (*Amaranthus blitoides* S. Watson), and *A. viridis*. For this approach, positive controls were used for *A. palmeri*, *A. spinosus*, *A. hybridus*, and *A. viridis*. The second approach was based on Sanger sequencing of PCR fragments amplified from the internal transcribed spacer (ITS) region as reported previously (Murphy and Tranel 2018; Sulzbach et al. 2023). And the third approach was based on Sanger sequencing of the *EPSPS* intron 1 region (Wright et al. 2016). All primers and PCR conditions were performed as reported in the cited publications.

EPSPS Sequencing

The presence of the TAP-IVS triple substitution in the samples was verified using a PCR-based assay (Mathioni et al. 2022), and a 195-bp fragment of the *EPSPS* gene was screened for novel mutations through amplification and sequencing. The primer set used was EPSF1 (5′-ATGTTGGACGCTCTCAGAACTCTTGGT-3′) and EPSR8 (5′-TGAATTTCCTCCAGCAACGGCAA-3′), reported by Gaines et al. (2010). PCR reactions were carried out in a 25-µl volume containing 100 ng of DNA and the following reagents: 200 µM dNTPs, 1.5 mM $MgCl_2$, 200 nM primers, and 1 unit of GoTaq G2 Hot Start DNA Polymerase. PCR cycling conditions were as follows: 95 C for 2 min, 40 cycles of 95 C for 30 s, 58 C for 30

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s, and 72 C for 30 s; final extension was performed at 72 C for 5 min. The amplification and expected size of amplicons were verified on 1% agarose gels. PCR products were cleaned before sequencing using the ExoSAP-IT Express (Thermo Fisher) following the manufacturer's instructions. Sequencing reactions were prepared using the BigDye Terminator v. 3.1 chemistry (Thermo Fisher) and sequenced on a genetic analyzer sequencing instrument (AB 3500, Applied BioSystems). The *EPSPS* sequences from *A. hybridus* (MH482844.1, MH482843.1) were used for analysis, and alignment was performed with the UGene (v. 1.45.0, Unipro) software (Okonechnikov et al. 2012).

ALS Sequencing

The *ALS* gene was amplified with primers designed for this study based on the GenBank *ALS* sequence MH036304.1 for *A. hybridus*: Ahy-ALS-F1 (5′-ATGGCGTCCACTTCTTCAAACC-3′), Ahy-ALS-F2 (5′-GACCTGTGCTGTATACTGGAG-3′), Ahy-ALS-R1 (5′-CTCCAGTATACAGCACAGGTC-3′), Ahy-ALS-R2 (5′-CTTACAACCGCATCGCCCTTCG-3′), and Ahy-ALS-R3 (5′- CTAATAAGCCCTTCTTCCATCACC-3′). These primers amplify fragments containing all the known plant *ALS* mutations associated with resistance. The PCR reactions were performed in 25 µl with 100 ng of DNA and the following reagents: 200 µM dNTPs, 1.5 mM $MgCl_2$, 200 nM primers, and 1 unit of GoTaq G2 Hot Start DNA Polymerase. PCR cycling conditions were as follows: 95 C for 3 min, 40 cycles of 95 C for 30 s, 58 C for 30 s, and 72 C for 90 s; final extension was performed at 72 C for 5 min. The amplification and expected size of amplicons were verified on 1% agarose gels. PCR products were cleaned before sequencing using the ExoSAP-IT Express (Thermo Fisher) following the manufacturer's instructions. Sequencing reactions were prepared using the BigDye Terminator v. 3.1 chemistry (Thermo Fisher) and sequenced on a genetic analyzer sequencing instrument (AB 3500, Applied BioSystems). The *ALS* sequences from *A. hybridus* (MH036304.1 and MH036306.1) were used for analysis, and alignment was performed with the UGene (v. 1.45.0, Unipro) software (Okonechnikov et al. 2012).

Frequency of Shifts in Sensitivity to Glyphosate and Chlorimuron in Amaranthus Populations across Brazil

Seed collection was carried out in areas where *Amaranthus* plants exhibited survival after herbicide application. A total of 740 samples were collected over a period of 4 yr (2018 to 2021). Seeds were obtained from agricultural fields located in 266 municipalities across eight Brazilian

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states. The seed collection methodology adhered to the protocol suggested by Burgos et al. (2013). Upon identification of a crop site with herbicide application failures, seeds were randomly collected from a minimum of 10 mature plants and subsequently combined to form a single composite sample. Each seed sample was catalogued with site-specific information, including geographic coordinates, state, and municipality.

In greenhouse experiments, the seeds from all *Amaranthus* populations were germinated and planted in 1-L pots filled with a commercial substrate; here, one pot contained eight plants. Each combination of population and herbicide was replicated three times (24 plants tested per population) in a completely randomized design. A single discriminatory dose was utilized to determine the shift in sensitivity to glyphosate and chlorimuron, using the recommended field doses of 720 and 20 g ha^{-1} for glyphosate and chlorimuron, respectively. Control plants, including both glyphosate and chlorimuron-sensitive (BR18Asp050) and resistant (BR18Asp051), were included as reference standards in all assays.

Glyphosate and chlorimuron treatments were administered when the plants reached the 4- to 6-leaf stage. The application was performed in a spray chamber equipped with flat-fan nozzles calibrated to deliver 200 L ha^{-1} at 200 kPa pressure. Plant mortality was evaluated at 21 DAT using the scale described in Table 2. After each population was classified based on the shift sensitivity criteria, the data were grouped by class (Table 2). Maps of the frequency and dispersion of *Amaranthus* populations were created for each herbicide using TIBCO Spotfire 10.3.1 Analyst®. In the maps, color-code classification was used to identify each sample (Table 2).

Results and Discussion

Amaranthus Species Identification

The four populations (BR18Asp050, BR21Asp206, BR18Asp051, and BR21Asp205) used in the plant dose–response curve were analyzed using the three approaches described in " Materials and Methods." When the PCR-based approach described by Wright et al. (2016) was used, the four populations presented positive bands (positive amplification) for the primers AW473+AW483 and AW471+AW482 (Wright et al. 2016), which amplified fragments of *A. hybridus* and *A. retroflexus*, respectively. These *EPSPS* intron 1 fragments were Sanger sequenced, resulting in a 1,053-bp sequence that was analyzed and compared with the *A. hybridus* (KT833344) and *A. retroflexus* (KT833348) sequences. A total of 18 single-nucleotide polymorphisms (SNPs) were

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observed among these two species, and the analyzed populations showed 13 SNPs identical to *A. hybridus*. When the ITS sequences for the four biotypes were analyzed, the alignment rendered identical (100% similarity) sequences among the four biotypes with 100% similarity to *A. hybridus* AMACHY-S (Sulzbach et al. 2024) and 99.5% similarity to a sample deposited in NCBI as being *A. retroflexus* (KF493795). Taking all the results together, we conclude that the four populations in this study belong to the *A. hybridus* species.

Whole-Plant Dose–Response Curve

Dose–response analysis indicated a reduction in biomass correlating with increased herbicide dose. However, significant differences were observed among populations. Notably, some populations exhibited high-level resistance to glyphosate (Figure 1A). Resistance factors, based on the GD_{50}, were 34 and 129 for BR18Asp051 and BR21Asp205, respectively (Table 3). The sensitive populations displayed GD_{50} values of 4.20 and 29.75 g ha^{-1} for BR18Asp050 and BR21Asp206, respectively. Even the highest dose tested in this study (32×) failed to effectively control the BR18Asp051 and BR21Asp205 populations.

The initial report of glyphosate-resistant *A. hybridus* in Argentina emerged in 2013, with subsequent studies from the same region indicating high levels of resistance (R_F = 314) (Heap 2024; Perotti et al. 2019). Another study in Argentina reported a resistance factor of 125 for *A. hybridus* (García et al. 2020), while Resende et al. (2022) identified lower resistance factors (13, 14, and 15) in the same *A. hybridus* populations from Paraná State in Brazil. The resistance factor in this study surpassed those previously cited; however, BR19Asp050 was the most sensitive population with a GD_{50} of 4.20, whereas other studies reported higher GD_{50}, values: 66.6 g e.a ha^{-1} (Perotti et al. 2019), 17.7 g e.a ha^{-1} (García et al. 2020), and 227.63 g e.a ha^{-1} (Resende et al. 2022).

Glyphosate was first introduced commercially in the early 1970s; despite its widespread use, resistant weeds were not reported until 1996 (Powles et al. 1998). Currently, glyphosate-resistant weeds impact hundreds of millions of acres in the United States and other countries, such as Argentina and Brazil, that have adopted genetically engineered crops (Bain et al. 2017). In Brazil, 11 glyphosate-resistant species have been reported, leading to crop yield losses, complicating management, and increasing weed control costs.

For the ALS-inhibiting herbicides, resistance was also observed in the evaluated populations. The resistance factors based on GD_{50} were 15 and 35 for the BR18Asp051 and

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BR21Asp205 populations, respectively (Table 3). In the case of the BR18Asp051 population, the highest chlorimuron dose (320 g ai ha^{-1}) almost achieved complete control of the population, but the BR21Asp205 population did not achieve 80% control with this dose (Figure 1B). The resistance factors for imazethapyr were 6 and 47 for BR18Asp051 and BR21Asp205 populations, respectively (Table 3). Even at the maximum imazethapyr dose, neither population showed effective control (Figure 1C).

Amaranthus hybridus populations from Illinois, USA, exhibited a resistance factor exceeding 152 for an ALS-inhibiting herbicide (Maertens et al. 2004). In Argentina, a resistance factor of 38 was observed for *A. hybridus* (García et al. 2020). A Brazilian study detected cross-resistance to ALS-inhibiting herbicides in an *A. hybridus* population, with resistance factors of 6.9 and 6.5 for chlorimuron and metsulfuron-ethyl, respectively, although, resistance to imazethapyr was not confirmed (Mendes et al. 2022). Moreover, the recommended rates for each herbicide were insufficient to achieve 90% control of the resistant population based on GD_{90} parameter estimation (Mendes et al. 2022).

Since the 1960s, ALS-inhibiting herbicides have been marketed globally (Garcia et al. 2017). They remain crucial in burndown programs and weed management systems including glyphosate-resistant species for major crops such as soybeans (Santos et al. 2016; Zobiole et al. 2018). In South America, farmers have relied for decades on the residual herbicide imazethapyr and subsequently glyphosate postemergence for managing *Amaranthus* species (García et al. 2020). However, their continuous applications may have been selected for *A. hybridus* populations with multiple resistance. In this study, the BR18Asp051 and BR21Asp205 populations were cross-resistant to chlorimuron and imazethapyr (ALS inhibitors), and also exhibited multiple resistance to glyphosate (EPSPS inhibitor) and chlorimuron and imazethapyr (ALS inhibitors).

EPSPS Gene Resistance Mechanism

Sequencing of the 195-bp fragment of the *EPSPS* gene revealed a triple amino acid substitution in all plants from glyphosate-resistant populations BR18Asp051 and BR21Asp205 (Figure 2). Four nucleotide replacements resulted in codon changes: ACA for ATA (Thr-102-Ile), GCG for GTC (Ala-103-Val), and CCA for TCA (Pro-106-Ser).

Simultaneously, substitutions at positions 102 and 106 (TIPS) were previously identified in species such as goosegrass [*Eleusine indica* (L.) Gaertn.] (Yu et al. 2015), hairy beggarticks

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(*Bidens pilosa* L.) (Alcántara De La Cruz et al. 2016), and greater beggarticks (*Bidens sulbalternans* DC.) (Takano et al. 2020). Recent studies by Perotti et al. (2019) and García et al. (2020) also reported this triple substitution (TAP-IVS) in *A. hybridus* from Argentina correlating with a high resistance factor, akin to the finding of this study.

The resistance to glyphosate in the analyzed populations is mainly caused by the triple mutations in the *EPSPS* gene; however, other resistance mechanisms cannot be ruled out, such an increase in the number of *EPSPS* gene copies, an increase in gene expression, change in herbicide absorption and translocation, and metabolism. *Amaranthus hybridus* biotypes from Argentina showed reduced glyphosate translocation and signs of *EPSPS* gene amplification; however, these mechanisms seem to have made a small contribution to herbicide resistance (García et al. 2019). On the other hand, a higher copy number of the *EPSPS* gene was identified in some biotypes of this species in Argentina and Brazil (García et al. 2020; Sulzbach et al. 2024); however, the resistant plants showed expression levels similar to those of the susceptible individuals (Perotti et al. 2018).

ALS Gene Resistance Mechanism

The sequencing analysis of the *ALS* gene identified only a point mutation at position 574 in the *ALS* gene when comparing resistant populations BR18Asp051 and BR21Asp205 with the ALS–inhibitor susceptible populations BR18Asp050 and BR21Asp206. No other mutation was observed in these two populations. This mutation, a T to G nucleotide change, resulted in a codon shift from TTG (leucine) to TGG (tryptophan) (Figure 3). The same substitution was reported in an *A. hybridus* population from Argentina, conferring cross-resistance to ALS-inhibiting herbicides (Larran et al. 2018). Other studies have noted similar cross-resistance patterns for ALS inhibitors to imidazolinones (IMIs) and sulfonylureas (SUs) (Tranel et al. 2023).

Mutations conferring resistance to ALS inhibitors in various weeds species can occur at eight positions within the *ALS* genes (Tranel et al. 2023). Cross-resistance patterns among ALS-inhibitor classes vary depending on the specific amino acid position and substitution (Shaner 1999). For instance, resistance to IMI herbicide is often due to an Ala-122 or Ser-653 substitution, which also confers a lower level of resistance to SU herbicides (Bernasconi et al. 2016; Tranel et al. 2023). Conversely, a Pro-197 substitution typically results in SU herbicide resistance with low or no cross-resistance to IMI herbicides (Guttieri et al. 1992). Cross-

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resistance usually involves an Ala-205 or Trp-574 substitution, with the latter conferring higher resistance levels (García et al. 2020).

The Trp-574-Leu mutation identified in the BR18Asp051 and BR21Asp205 populations is associated with high level of resistance to SU and IMI herbicides. In Illinois, USA, the same mutation in *A. hybridus* was linked to substantial resistance to imazamox and thifensulfuron (Maertens et al. 2004).

Frequency of Shifts in Sensitivity to Glyphosate and Chlorimuron in Amaranthus spp. Populations across Brazil

Overall, 88% of the Brazilian populations were deemed sensitive to glyphosate, with mortality rates between 90% and 100%. Additionally, 2% of populations exhibited mortality rates between 20% and 89% (yellow classification), distributed across nine municipalities. A mortality rate between 0% and 19% (red classification) was observed in 10% of the total population, spread over 30 different municipalities. Sensitivity shifts to glyphosate were noted in four states: Rio Grande do Sul, Paraná, São Paulo, and Santa Catarina. (Figures 4A and 5A).

Most of the populations with altered glyphosate sensitivity were in Rio Grande do Sul, where 273 populations were tested. Of these, 77% were sensitive (green classification), 4% were yellow, and 19% were red (Figure 5A). Yellow and red classifications were found in 7 and 16 municipalities, respectively. In Paraná, of the 200 tested populations, 2.5% were yellow and 6% were red, with these classifications found in 13 and 4 municipalities, respectively. In Santa Catarina and São Paulo, glyphosate sensitivity shifts were detected in two and one locations, respectively (Figure 5A).

Chlorimuron sensitivity shifts in *Amaranthus* spp. are widespread across various Brazilian regions (Figure 4B). Of all samples analyzed, 66% were sensitive to chlorimuron (green classification), 11% yellow, and 23% were red (Figure 5B). The most severe situation was in the midwest, particularly in Mato Grosso state, where only 15% of the population was sensitive to chlorimuron. The first ALS–inhibitor resistant *A. hybridus* population in Brazil was reported in 2011 in Mato Grosso's cotton (*Gossypium hirsutum* L.) fields (Heap 2024).

Ten percent of the 740 samples tested nationwide showed control rates below 90% for both glyphosate and chlorimuron (Figure 6). These were in the states of Rio Grande do Sul, Santa Catarina, Pananá, and São Paulo. The populations showing sensitivity shift to the

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herbicides may include different *Amaranthus* species. Therefore, a crucial advancement in understanding *Amaranthus* resistance in Brazil involves accurate identification of these species.

Resistant *A. hybridus* plants or seeds may have been introduced to Brazil from other countries, as many producers import forage seeds from neighboring countries, and significant movement of machinery and people occurs between these regions. Another point is that the resistance mechanism observed in this study, for glyphosate (TAP-IVS mutation), has already been found in populations from Argentina (García et al. 2020; Larran et al. 2018; Perotti et al. 2019). The Occurrence of a triple mutation in the same gene is rare, suggesting that the resistant populations are likely due to the dissemination of seeds from resistant populations from Argentina (Sulzbach et al. 2024). Although the long-term use of ALS herbicide in soybeans and cotton could explain the spread of chlorimuron resistance across southern and midwestern Brazil.

In Brazil, the first area with *Amaranthus* populations exhibiting glyphosate resistance was identified in 2016 (Heap 2024); thus, the number and distribution of areas where glyphosate was ineffective in this study are concerning. This suggests that in a short period, *Amaranthus* species could become a significant challenge for Brazilian agriculture.

In conclusion, the collective findings of this study underscore the need to reevaluate current weed management programs in Brazil. The adoption of preemergence herbicides is still low compared with other countries facing herbicide-resistant *A. hybridus* (e.g., Argentina and the United States). Diversifying herbicide mode of action, mandating the use of preemergence herbicides, and increasing the use of combined mechanisms may become more crucial strategies. Our data also emphasize the importance of an effective herbicide-resistant monitoring program, which could help prevent weed resistance by focusing efforts on regional issues and increasing stakeholders' awareness in each area.

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Table 1. Identification and location of the *Amaranthus hybridus* populations studied

ID	City	State	Latitude	Longitude
BR18Asp050 (sensitive)	Cosmópolis	São Paulo	-31,59-4556	-52,331472
BR21Asp206 (sensitive)	Florínea	São Paulo	-22,844444	-50,566389
BR18Asp051	Pelotas	Rio Grande do Sul	-22,606920	-47,220580
BR21Asp205	Boa Vista da Aparecida	Paraná	-25,370567	-53,395478

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Table 2. Color code for glyphosate and chlorimuron sensitivity shifts in *Amaranthus* populations at 21 d after treatment (DAT)

Mortality category — % —	No. of surviving plants	Color code
90–100	0–4	Green
20–89	4–27	Yellow
0–19	26–32	Red

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Table 3. Glyphosate, chlorimuron, and imazethapyr doses required for biomass reduction (GR) of 50% in the BR18Asp050, BR21Asp206, BR18Asp051, and BR21Asp205 populations at 21 d after treatment, and resistance factors (RF) for *Amaranthus hybridus* populations

	Regression parameters[b]							
Populations[a]	*a*	*b*	*c*	r^2	GD_{50}[b]	Lower CI	Upper CI	RF
	Glyphosate							
BR18Asp050	101.8	5.3	−0.15*	0.88	4.2	1.2	7.2	—
BR21Asp206	101.6*	30.4*	−1.38*	0.98	29.7	4.2	54.6	—
BR18Asp051	96.4*	479.0*	−0.43*	0.99	572.0	564.0	579.0	34*
BR21Asp205	472.0*	3,508.0	−0.42*	0.96	2,200.0	1,913.0	2,487.0	129*
	Chlorimuron							
BR18Asp050	100.3*	0.6	−1.46*	0.99	0.7	0.4	0.9	—
BR21Asp206	99.1*	2.8*	−0.85*	0.99	2.9	2.6	3.4	—
BR18Asp051	192.6*	425.0*	−0.26*	0.98	26.8	13.4	40.1	15*
BR21Asp205	572.3*	62,445.0	−0.34*	0.94	63.0	32.0	94.0	35*
	Imazethapyr							
BR18Asp050	100.3*	4.9*	−1.69*	0.99	4.7	4.0	5.3	—
BR21Asp206	101.6*	26.2*	−1.12*	0.98	25.6	22.6	28.6	—
BR18Asp051	81.76*	60.0*	−1.01*	0.99	94.1	84.3	103.0	6*
BR21Asp205	270.4*	21,433.0	−0.26*	0.99	714.0	654.0	774.0	47*

[a] *Amaranthus* putative resistant: BR18Asp051 and BR21Asp205; susceptible: BR18Asp050 and BR21Asp205.

[b] Herbicide dose (g ha^{-1}) to control 50% of the variable (GD_{50}); lower/upper confidence interval (CI) of the variable *e* (ED_{50}); RF, resistance factor (resistant/susceptible).

*Statistically significant at $P < 0.05$.

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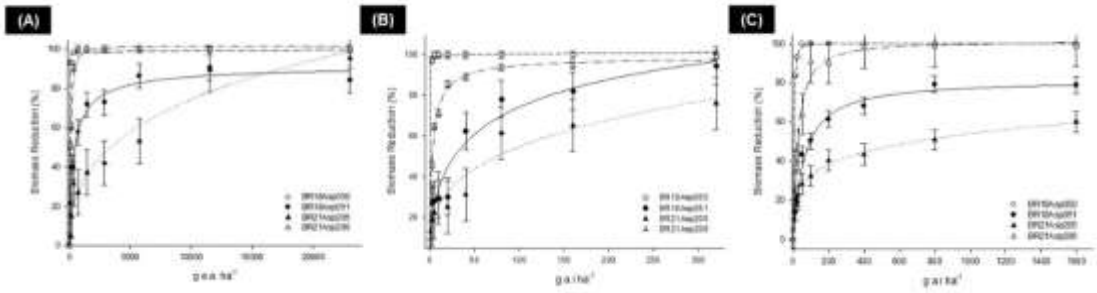

Figure 1. Glyphosate biomass reduction (A), chlorimuron biomass reduction (B), and imazethapyr biomass reduction (C) responses in BR18Asp050, BR21Asp206, BR18Asp051, and BR21Asp205 populations of *Amaranthus hybridus* at 21 d after treatment (DAT).

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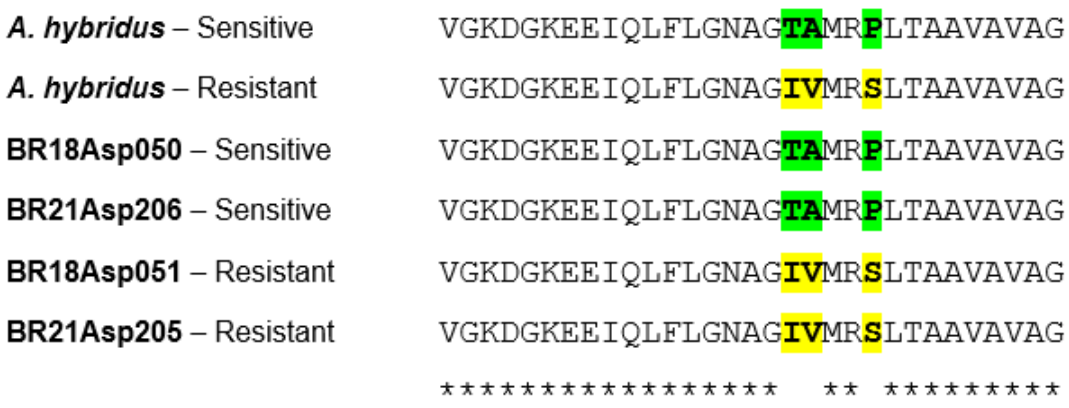

Figure 2. Alignment of EPSPS protein fragments showing the TAP-IVS triple substitution (Thr-102-Ile, Ala-103-Val, and Pro-106-Ser) (highlighted). Sequences S (sensitive, highlighted in green) and R (resistant, highlighted in yellow) are the reference sequences (GenBank accession nos. AXN57302.1 and AXN57301.1).

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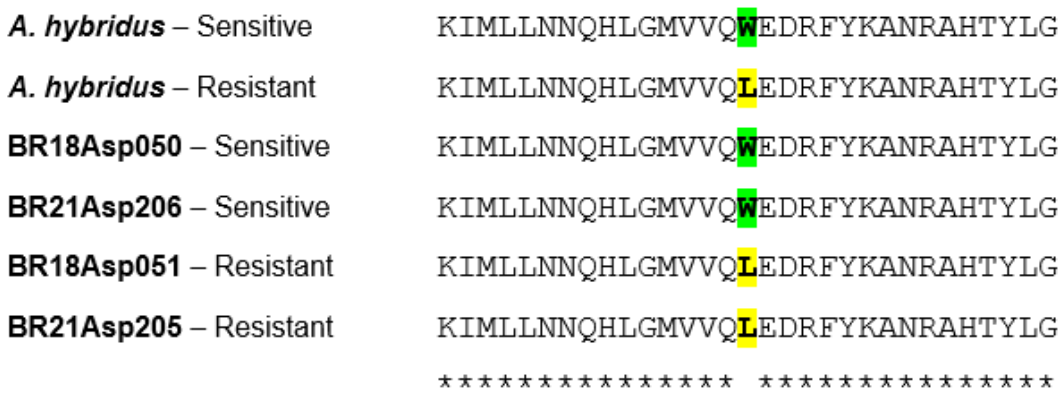

Figure 3. Alignment of *Amaranthus hybridus* ALS protein fragments showing the Trp-574-Leu (W574L) substitution (highlighted). Sensitive sequence with the tryptophan amino acid highlighted in green, and resistant sequence with the leucine amino acid highlighted in yellow for the reference sequences (GenBank accession nos. MH036304.1 and MH036306.1).

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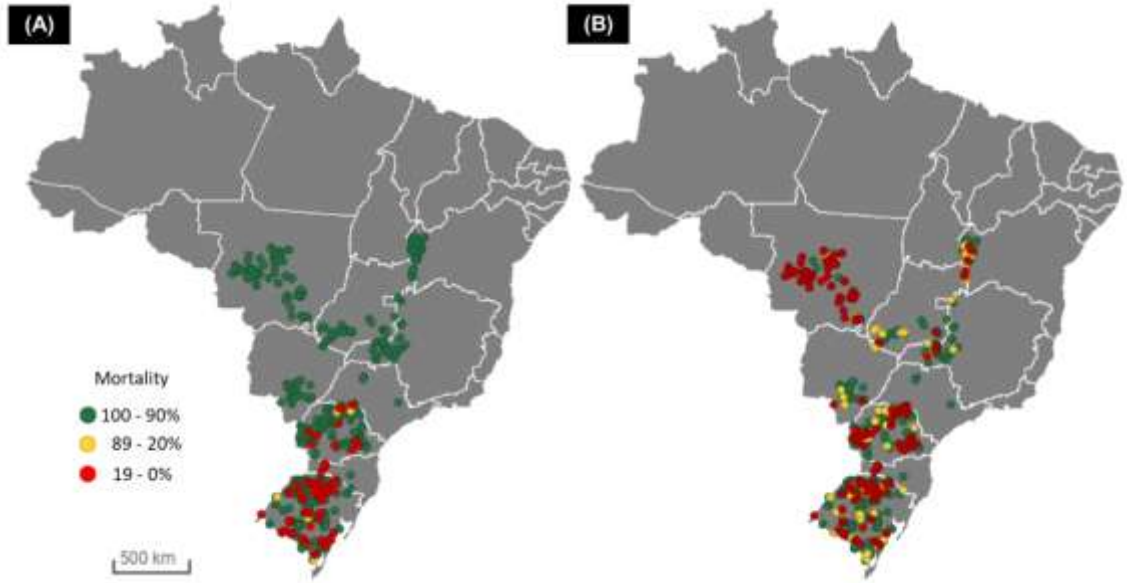

Figure 4. Distribution of *Amaranthus* populations based on glyphosate (A) and chlorimuron (B) sensitivity in Brazil.

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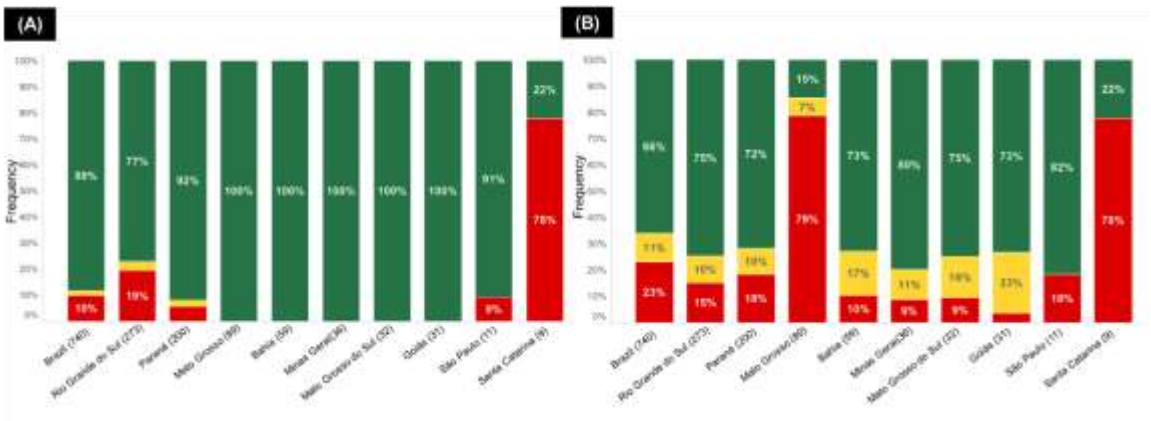

Figure 5. Frequency (%) of glyphosate (A) and chlorimuron (B) tested populations and total population (in parenthesis) of *Amaranthus* for selected states in Brazil.

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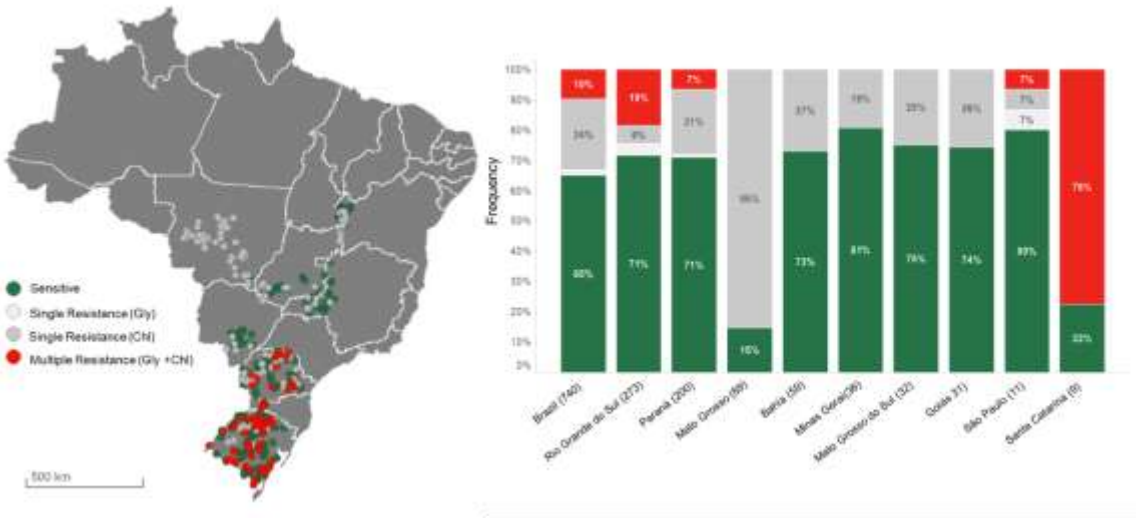

Figure 6. Frequency distribution (%) and number of populations (in parenthesis) of *Amaranthus* with single and multiple resistance to glyphosate and chlorimuron for selected states in Brazil.

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