Weed Science

www.cambridge.org/wsc

Smooth pigweed (*Amaranthus hybridus*) and unresolved *Amaranthus* spp. from Brazil resistant to glyphosate exhibit the EPSPS TAP-IVS substitution

Estéfani Sulzbach[1], Guilherme Menegol Turra[1], Luan Cutti[1], Leonardo Vicente Ellert Kroth[2], Patrick J. Tranel[3], Aldo Merotto Jr[4] and Catarine Markus[4]

[1]PhD Student, Department of Crop Science, Federal University of Rio Grande do Sul, Porto Alegre, Brazil; [2]Undergraduate Student, Department of Crop Science, Federal University of Rio Grande do Sul, Porto Alegre, Brazil; [3]Professor, Department of Crop Sciences, University of Illinois at Urbana–Champaign, IL, USA and [4]Professor, Department of Crop Science, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

Research Article

Cite this article: Sulzbach E, Turra GM, Cutti L, Kroth LVE, Tranel PJ, Merotto A Jr, Markus C (2024) Smooth pigweed (*Amaranthus hybridus*) and unresolved *Amaranthus* spp. from Brazil resistant to glyphosate exhibit the EPSPS TAP-IVS substitution. Weed Sci. **72**: 48–58. doi: 10.1017/wsc.2023.70

Received: 29 July 2023
Revised: 6 November 2023
Accepted: 14 November 2023
First published online: 24 November 2023

Associate Editor:
Mithila Jugulam, Kansas State University

Keywords:
ALS; gene flow frequency; glyphosate herbicide resistance; Triple amino acid substitution; copy number

Corresponding author:
Catarine Markus;
Email: catarine.markus@ufrgs.br

Abstract

The presence of glyphosate-resistant smooth pigweed (*Amaranthus hybridus* L.) biotypes has increased in southern Brazil in recent years, presenting the triple amino acid substitution TAP-IVS in 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) previously found in Argentina. Some of these biotypes have morphological characteristics of *A. hybridus* and redroot amaranth (*Amaranthus retroflexus* L.). The present study aimed to identify, through molecular markers, the herbicide-resistant species of *Amaranthus* from Brazil that have the TAP-IVS substitution and to analyze the occurrence of pollen-mediated gene flow (PMGF) as the source of the TAP-IVS substitution in these biotypes. Six biotypes were evaluated using internal transcribed spacer (*ITS*) sequences, of which two (AMACHY-S and CAMAQ-R) were molecularly classified as *A. hybridus* and four (AMACRET-S, AMACVI-S, ARRGR-R, and SAOJER-R) were unclassified. Interestingly, all the glyphosate-resistant biotypes (ARRGR-R, SAOJER-R, and CAMAQ-R) had the TAP-IVS substitution and an increase in *EPSPS* relative copy number; however, only CAMAQ-R was confirmed as *A. hybridus*. Although the biotypes ARRGR-R and SAOJER-R are closely related to *A. hybridus* and green pigweed (*Amaranthus powellii* S. Watson), their species identity could not be resolved. The biotype SAOJER-R also was resistant to acetolactate synthase (ALS)-inhibiting herbicides due to a tryptophan to leucine substitution at position 574 in ALS. The evaluation of 119,746 seedlings in an intraspecific hybridization study of *A. hybridus* indicated an outcrossing frequency of 0.09%. In contrast, an absence of interspecific hybridization (*A. hybridus* × unclassified biotype, AMACVI-S) was found after screening 111,429 offspring. Unclassified biotypes might be derived from one or more ancient hybridization events and subsequently evolved the triple mutation independently. Alternatively, such biotypes could have evolved from recent hybridization events, which occur at a frequency below the level of detection in our study.

Introduction

The genus *Amaranthus* (Amaranthaceae) encompasses 75 species distributed in tropical and temperate regions (Costea et al. 2001a). Around 65 species are monoecious (Bayón 2015), and 10 are dioecious (Sauer 1955). All species are diploid with 32 or 34 chromosomes, except spleen amaranth (*Amaranthus dubius* Mart. ex Thell.), which is tetraploid (Grant 1959). Within *Amaranthus*, 20 species are weeds widely spread worldwide (Costea et al. 2001b). Weedy species of this genus are often dominant weeds and challenging to control due to their high growth rate, fecundity, stress tolerance, genetic variability, and ability to evolve herbicide resistance (Faccini and Vitta 2005).

Smooth pigweed (*Amaranthus hybridus* L.) is native to South America and is currently recognized as a major weed in agricultural production systems (Larran et al. 2018). It can reduce soybean grain yield by 82%, and the presence of one *A. hybridus* plant per square meter can result in a 6.4% yield reduction (Soltani et al. 2017; Zandoná et al. 2022). The importance of this species is increasing, including in Brazil, as resistance cases are reported (García et al. 2020; Perotti et al. 2019; Resende et al. 2020; Whaley et al. 2006).

Currently, *A. hybridus* populations have been identified with resistance to inhibitors of photosystem II, acetolactate synthase (ALS), protoporphyrinogen oxidase, 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS), and to the synthetic auxin herbicides (Heap 2023).

Amaranthus hybridus biotypes from Brazil were identified with resistance to the EPSPS inhibitor, glyphosate, and to ALS-inhibiting herbicides (Mendes et al. 2022; Resende et al. 2020). However, the mechanism of resistance to ALS-inhibiting herbicides in biotypes from Brazil has not been characterized. Interestingly, glyphosate-resistant biotypes from southern Brazil have the same triple amino acid substitution Thr-102-IIe, Val-103-Ala, and Pro-106-Ser (TAP-IVS) (Mathioni et al. 2022) that was reported for the first time in Argentina (Perotti et al. 2019). However, limited information is available about the dispersion of the *EPSPS* triple mutation in Brazil populations.

The rapid distribution of *A. hybridus* resistant biotypes with the EPSPS TAP-IVS substitution in southern Brazil has been associated with high seed production, distribution through agricultural machinery, and seed crop contaminants (Penckowski et al. 2020). It is worth noting that farmers and field technicians have observed significant morphological variation in glyphosate-resistant *Amaranthus* biotypes, including those suspected to be redroot pigweed (*Amaranthus retroflexus* L.), making it difficult to accurately identify and classify certain species.

Glyphosate resistance caused by the triple TAP-IVS substitution is thought to be a rare event, which may indicate that its large spread is also caused by pollen-mediated gene flow (PMGF). Morphological admixtures that have been observed among *Amaranthus* species in South America support interspecies PMGF. Previous studies have confirmed interspecies gene flow among *Amaranthus* species, including between monoecious and dioecious species (e.g., Gaines et al. 2012; Tranel et al. 2002; Trucco et al. 2005).

Consequently, the goal of this research was to address the role of PMGF in the dissemination of the EPSPS TAP-IVS triple substitution in *Amaranthus* populations. Our specific objectives were (1) to classify the species of *Amaranthus* from Brazil with resistance to herbicides; (2) identify the occurrence of resistance to ALS-inhibiting herbicides and glyphosate and identify the target site–resistance mechanisms; and (3) determine *A. hybridus* outcrossing rates in both intra- and interspecific crosses.

Material and Methods

Plant Materials

Mature seeds of resistant *Amaranthus* spp. populations (ARRGR-R, SAOJER-R, and CAMAQ-R) were harvested from three regions in the state of Rio Grande do Sul, Brazil, near Arroio Grande (29.32°S, 52.5°W), São Jerônimo (29.57° S, 51.43° W), and Camaquã (30.51°S, 51.48°W). The populations were collected in glyphosate-resistant (GR) soybean fields during the 2019 to 2020 crop season. These areas had been treated with glyphosate more than 5 yr, approximately. In addition, seeds of four putative susceptible biotypes (AMACHY-S, AMACVI-S, EEA-S, and AMARET-S) were collected nearby. For all populations, seeds were obtained from 20 plants and pooled.

The progeny from each population was screened in a greenhouse at a temperature of 25 ± 3 C and a photoperiod of 14/10 h (day/night). The seeds were germinated in plastic trays with substrate based on expanded vermiculite, peat, perlite, and rice husk. Seedlings were transplanted individually into 200-ml pots and grown until the 4-leaf stage, when the herbicides were sprayed. Up to 10 plants of each biotype were used for the screening. The glyphosate doses were 0 (control) and 1,080 g ha^{-1} (Roundup Original® DI, Monsanto of Brazil Lt da, São José dos Campos, SP, Brazil). Herbicides were applied using a greenhouse spray chamber (Generation III, DeVries Manufacturing, Hollandale, MN, USA). The spray volume used was 200 L ha^{-1}, applied with a TJ8002E nozzle at a pressure of 280 kPa and a speed of 1.2 m s^{-1} (TeeJet Technologies, Wheaton, IL, USA). Plants that survived 1,080 g ha^{-1} of glyphosate were selected 21 d after herbicide application and self-pollinated. Progeny were subjected to a second generation of glyphosate selection and self-pollination to obtain more homogenous populations.

Morphological and Molecular Identification

The morphological traits of the biotypes were analyzed following two keys for the Amaranthaceae. The key described by Vasconcellos (1986) evaluated the male flower (three to five stamens), fruit dehiscence (dehiscence or indehiscent), presence of intra-axillary spines, and size of bracts relative to ovary. The synopsis of the subgenus *Amaranthus* in Venezuela described by Pinto and Velásquez (2010) was used to evaluate stems (glabrous or pubescent) and bract form (spinescent or lanceolate) and size (bracts larger or smaller than sepals).

Two different approaches were performed for the molecular identification of *Amaranthus* species. The first is based on the fragment sizes of intron 1 of *EPSPS* (Wright et al. 2016). For amplification of intron 1, three primer pairs—AW473_F × AW483_R, AW471_F × AW482_R, and AW477_F × AW493_R—were selected for *A. hybridus*, *A. retroflexus*, and slender amaranth (*Amaranthus viridis* L.), respectively (Supplementary Table 1). Supplementary Table 1 describes the PCR conditions and respective fragment sizes (bp). PCR products were analyzed for the presence or absence of a fragment and their size in a 1.5% agarose gel stained with ethidium bromide.

The second molecular approach was the amplification of a 648-bp fragment of an internal transcribed spacer (*ITS*), which constitutes a conserved region located between two subunits of ribosomal RNA (rRNA) that differentiate nine species of *Amaranthus* (Murphy and Tranel 2018). The amplification of the ITS5-ITS4 region was obtained with specific primers from the 18S, 5.8S, and 28S conserved regions of rRNA using primers ITS5_F and ITS4_R (Wetzel et al. 1999). The primers, PCR conditions, and size of PCR products (bp) are listed in Supplementary Table 1. The region amplified was purified (ExoSAP-IT™, Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA), and the purified product was used for Sanger sequencing. A multiple alignment was performed with *ITS* sequences from biotypes described in the "Plant Materials" section plus all 72 sequences of the *ITS* region described in Murphy and Tranel's (2018) study that were obtained from the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov). Subsequently, a neighbor-joining tree was constructed from the alignment. The multiple alignment was generated using MAFFT, and a phylogenetic tree was created using Interactive Tree of Life (iTOLv6), following the protocol described by Murphy and Tranel (2018).

Dose–Response Assay

Herbicide susceptibility and resistance were quantified for each population using the progeny from the second generation of glyphosate selection described earlier. The resistance levels to glyphosate, chlorimuron, and imazethapyr were evaluated through a dose–response curve experiment with a completely randomized design. For glyphosate, factor A consisted of the susceptible

https://doi.org/10.1017/wsc.2023.70 Published online by Cambridge University Press

AMACHY-S and resistant ARRGR-R, SAOJER-R, and CAMAQ-R biotypes. Factor B was the doses of glyphosate (Roundup Original® MAIS, 480 g ae L^{-1}, Monsanto of Brazil) with 0, 8.4, 16.8, 33.7, 67.5, 135, 270, and 540 g ha^{-1} for the susceptible biotype and 0, 540, 1,080, 2,160, 4,320, 8,640, 17,280, and 34,560 g ha^{-1} for the resistant biotypes. For imazethapyr, factor A consisted of the susceptible AMACHY-S and SAOJER-R resistant biotype, and factor B was imazethapyr (Imazetapir Nortox, 212 g ae L^{-1}, Nortox S.A., Arapongas, PR, Brazil) with doses of 0, 3.3, 6.6, 13.2, 26.5, 53, 106, and 212 g ha^{-1} for the susceptible biotype and 0, 106, 848, 1,696, 3,392, 6,784, 13,568, and 27,136 g ha^{-1} for the resistant biotype. In the chlorimuron dose–response assay, factor A was the susceptible AMACHY-S and SAOJER-R resistant biotype, and factor B was the doses of chlorimuron (Clorim, 250 g ai kg^{-1}, UPL of Brazil, Ituverava, SP, Brazil) with 0, 0.3, 0.6, 1.2, 2.5, 5, 10, and 20 g ha^{-1} for the susceptible biotype and 0, 20, 80, 160, 320, 640, 1,280, and 2,560 g ha^{-1} for the resistant biotype. Surfactant at 0.5% v/v (Dash®, BASF S.A. Guaratinguetá, SP, Brazil) and oil mineral at 1.0% v/v (Assist®EC, BASF S.A.) were applied with imazethapyr and chlorimuron, respectively. Resistant and susceptible populations were treated as previously described. Each treatment had four replicates with one plant per pot, and the experiment was carried out twice.

The evaluations of herbicide control and fresh and dry shoot weights were carried out 21 d after the application of treatments (DAT). Herbicide control was visually evaluated using a percentage scale, in which 0% corresponds to the absence of plant injury and 100% to complete necrosis and plant death.

Mutations in the EPSPS and ALS Coding Sequences

Young leaf tissues from resistant and susceptible biotypes were sampled and frozen in liquid nitrogen. DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle 1987). The DNA was diluted to a final concentration of 50 ng μL^{-1}. Forward gly.a.3F and reverse gly.a.4R primers were used to amplify a fragment of approximately 195 bp of *EPSPS* (Lorentz et al. 2014), encompassing the EPSPS triple amino acid substitution. The PCR conditions are described in Supplementary Table 1.

Four primer pairs were designed based on the *A. hybridus* *ALS* sequence (GenBank MH036304.1) using Primer3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Supplementary Table 1). These primers amplify fragments containing all the known plant *ALS* mutations associated with herbicide resistance. The PCR conditions are described in Supplementary Table 1. PCR products were analyzed by agarose gel electrophoresis, and purified with ExoSAP-IT™. Sanger sequencing was used to detect target-site mutations for *EPSPS* and *ALS*. The obtained sequences were analyzed using *A. hybridus* (GenBank MG595171.1) as a reference for *EPSPS* and *Arabidopsis thaliana* (L.) Heynh. (GenBank X51514.1) as a reference for *ALS*.

Relative Genomic EPSPS Gene Copy Number

Quantitative real-time PCR (qPCR) was used to estimate the relative genomic *EPSPS* copy number. The *ACTIN* gene was used as a reference (Lorentz et al. 2014), and the primer sets of forward gly.a.3F and reverse gly.a.4R were used to amplify a fragment of approximately 195 bp of the *EPSPS* gene (Lorentz et al. 2014). The primers and the qPCR conditions are described in Supplementary Table 1. Amplifications were carried in a QuantStudio 3 Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific). Melting curves were performed at the end of the cycling to control primer amplification specificity. Amplification efficiency was independently calculated in each reaction and was considered in data processing, according to Perotti et al. (2019). Twelve plants from each resistant (ARRGR-R, SAOJER-R, and CAMAQ-R) and susceptible biotype (AMACHY-S and AMACVI-S) were processed in triplicate. Data were analyzed by the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen 2001), with the susceptible biotype (AMACHY-S) as the standard control, to obtain relative copy number.

Pollen-mediated Gene Flow (PMGF) Study: Wind-mediated Gene Flow

The experiments were conducted during the 2020 to 2021 and 2021 to 2022 cropping seasons at the Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil. The experimental design followed the PMGF wind-mediated gene flow described by Murray et al. (2002) and Walsh et al. (2012), which consisted of 12 receptor plants (susceptible plants, -S) surrounded by one donor plant (resistant plant, -R), without the presence of a crop species and physical barrier. Two experiments were performed using the CAMAQ-R population as a donor plant. The receptor plant was from the AMACHY-S population for Experiment 1 and from the AMACVI-S population for Experiment 2. The distance between the resistant plant (donor) and the susceptible plants was 0.3 to 0.6 m to avoid physical contact between plants. Each set of 13 plants formed a repetition, and in total, eight repetitions with 4.5-m distance between them were assembled. Experiments 1 and 2 were separated by 145 m (Supplementary Figure 1), isolated by native vegetation of 3 m height without the presence of *Amaranthus* species. Each experiment was repeated (2020 to 2021 and 2021 to 2022).

To ensure overlapping flowering periods, seeds of the donor-resistant biotype (CAMAQ-R) and the receptor-susceptible biotypes (AMACHY-S and AMACVI-S) were germinated at six periods with intervals of 7 d. Resistant biotypes were screened for glyphosate at the 3- to 4-leaf stage at a dose of 1,080 g ha^{-1} (Roundup Original® MAIS). In both years, plants were kept in a greenhouse until the beginning of the development of the floral structure. After that, plants were transplanted into 11-L pots and allocated as described above. Plants were irrigated daily. Seed collection (progeny) for the first year began in December 2020 and ended in early February 2021. For the second year, the collections were initiated at the end of March 2022 and finalized in May 2022. Seed harvesting was performed weekly, in bulk, within each repetition.

Screening for Resistant Hybrids

The glyphosate-resistance trait was used as a phenotypic and genotypic marker to screen plants from the receptor plants. The screening was carried out with a seed-soaking bioassay adapted from Zelaya and Owen (2005). Seeds were incubated in 24-well cell culture cluster plates (CoStar 3524, Corning Incorporated, Porto Alegre, RS, Brazil) with a germination paper of 16 mm in diameter. Twenty seeds were soaked with 500 µl of distilled deionized water (untreated control) or with the herbicide treatments. Each plate was wrapped with plastic film to avoid evaporation. Plates were placed in a germinator chamber at 30 C and 14-h diurnal conditions and 20 C and 10-h nocturnal conditions.

The experiment was arranged in a factorial completely randomized design. The progenies from the crosses for the intraspecific hybridization of *A. hybridus* (CAMAQ-R × AMACHY-S) and for interspecific hybridization of *A. hybridus* × unclassified biotype

https://doi.org/10.1017/wsc.2023.70 Published online by Cambridge University Press

(CAMAQ-R × AMACVI-S) comprised factor A, along with the negative (seeds of susceptible biotypes) and positive controls (seeds of resistant biotype) in herbicide solution. Factor B comprised the control solution (dose 0) and glyphosate solution (dose 3.2 mM). The dose of 3.2 mM was selected by a previous study performed with a dose–response assay according to Zelaya and Owen (2005). Plant survival was evaluated at 14 d of incubation based on the positive and negative control observations. Seed germination, seedling hypocotyl, and radicle length were recorded at 2 wk after treatment. Germination was defined by radicle protrusion from the seed. Gene flow frequency calculations were performed using the number of glyphosate-surviving seedlings in relation to the total number of viable seeds, based on the no-herbicide control. The seedlings surviving the herbicide solution were selected and stored in 1.5-ml Eppendorf microtubes. These materials were stored in a freezer (−20 C) until genomic DNA extraction. DNA extraction and amplification of *EPSPS* were performed according to previous studies, and the triple substitution was used to confirm hybridization.

Statistical Analysis

The ANOVAs of all dose–response curves were analyzed for significance ($P < 0.05$). The dose–response curves were fit by a four-parameter log-logistic nonlinear regression model:

$$y = c + \{d - c/1 + \exp[b(\log(x) - \log(e))]\} \quad [1]$$

where y is the variable response; x is the herbicide dose; parameter b is the slope; parameter d is the upper limit; parameter c is the lower limit; and parameter e is the effective dose for 50% control (ED_{50}). The data were analyzed using the DRC package from the statistical software R v. 4.2.1 (Knezevic et al. 2007). This package provides the curve fit, equation parameters, and resistance factor (RF) significances. Log-logistic significant parameters (b, d, c, and e) (P-value < 0.05) mean good model fit. ANOVA ($P < 0.05$) was also carried out for relative genomic *EPSPS* gene copy number; if statistical significance was found, means were compared using Tukey's test ($P < 0.05$). The frequency of PMGF ranged from 0 to 1 and followed a binomial distribution (dead or alive). For PMGF, a power analysis for binomial probabilities was performed to determine the statistical precision of hybridization with the sample size used in this experiment. The theoretical frequency used was 0.001 at $\alpha = 0.05$ for hybridization and was compared with the observed hybridization frequencies as described in Jhala et al. (2011).

Results and Discussion

Morphological and Molecular Identification

The available morphological keys allowed for the identification of the EEA-S biotype as *A. viridis* (Supplementary Table 3). The presence of a glabrous stem, flowers with four tepals, male flower with three stamens, and non-spinescent bracts (Pinto and Velásquez 2010; Vasconcellos 1986) was decisive in the differentiation and morphological identification of this biotype as *A. viridis*. The other biotypes have pubescent stems, flowers with five tepals (except AMACVI-S and AMARET-S, which have four tepals), and bracts of varying shapes and sizes. For instance, the bracts of AMACHY-S, ARRGR-R, SAOJER-R, and CAMAQ-R range from spiky to a well-defined apex, while the bracts of susceptible populations AMACVI-S and AMARET-S have less-defined features (Supplementary Table 3).

The size of bracts is the key characteristic to differentiate between *A. hybridus* and *A. retroflexus* (Vasconcellos 1986). Differentiating between *A. hybridus* and *A. retroflexus* based on morphological characteristics, such as bract size, is challenging. The morphological evaluation through the keys described by Vasconcellos (1986) and Pinto and Velásquez (2010) did not allow the classification of six out of seven biotypes evaluated. The morphological similarities between the two species make distinguishing them difficult, especially in the vegetative phase (Horak et al. 1994). Hybridization between *Amaranthus* species can also lead to the formation of hybrids with intermediate characteristics that cannot be reliably distinguished using traditional morphological strategies (Murray 1940). As a result, *A. hybridus* and *A. retroflexus* may have been, in some cases, misidentified for a long time in Brazil.

In the genotypic study, amplification of intron 1 of the *EPSPS* allowed for differentiation of only the EEA-S biotype as *A. viridis*. The ARRGR-R, SAOJER-R, CAMAQ-R, AMACHY-S, AMACVI-S, and AMARET-S biotypes each yielded PCR products for both *A. hybridus*– and *A. retroflexus*–specific primer sets. However, AMARET-S had weak bands for both sets of primers (Supplementary Figure 2). This suggests that methods described by Wright et al. (2016) were validated against a limited range of plants/samples and geographic origins and may not represent the *Amaranthus* species found in Brazil.

The amplification of the *ITS* region allowed the classification of three out of seven biotypes. Phylogenetic analysis classified AMACHY-S and CAMAQ-R biotypes as *A. hybridus* and EEA-S as *A. viridis* (Figure 1). *ITS* sequences have been widely used to resolve phylogenetic issues and to select candidate plant barcodes (Baldwin et al. 1995). The biotypes ARRGR-R, SAOJER-R, and AMACVI-S appeared to be most closely related to *A. hybridus* and *A. powellii*. Although the *ITS* sequences of these three biotypes were clearly within the *hybridus*/*powellii*/*retroflexus* clade, their species identity was not resolved. The biotype AMARET-S is closely related to prostrate pigweed (*Amaranthus albus* L.) and mat amaranth (*Amaranthus blitoides* S. Watson) (Figure 1). The biotype AMACHY-S was used as susceptible biotype in further studies because its species identity was better resolved. Through morphological analysis and two different molecular study strategies, it was possible to verify that only EEA-S showed identical classification results between evaluations (Table 1). These results show that identifying *Amaranthus* species using phenotypic traits alone is challenging, and molecular strategies may be necessary. Based on our results, the *ITS* proved to be the most promising strategy for identifying *Amaranthus* species. Despite its limitations, many studies have shown that *ITS* is advantageous for identifying species of this genus (Murphy et al. 2017; Xu et al. 2017). Phylogenetic trees based on *ITS* suggested that *Amaranthus* species from China could be divided into five sections and two subsections. *Amaranthus retroflexus* and *A. powellii* formed a clade together, and foxtail amaranth (*Amaranthus caudatus* L.) and *A. hybridus* were closely related (Xu et al. 2017). Palmer amaranth (*Amaranthus palmeri* S. Watson) was successfully detected in mixed samples using *ITS* with a quantitative PCR method (Murphy et al. 2017). The increasing cases of *Amaranthus* species with resistance to glyphosate and ALS-inhibiting herbicides in Brazil have aroused interest in identifying them. Molecular techniques are reliable; however, it is necessary to obtain positive controls related to known samples of each species to verify distinguishable single-nucleotide polymorphisms to validate the identification of biotypes from southern Brazil.

https://doi.org/10.1017/wsc.2023.70 Published online by Cambridge University Press

Table 1. Morphological identification and two molecular approaches to identify the *Amaranthus* biotypes from the present study.

Biotype	Morphological identification[a]	Intron 1 of *EPSPS*[b]	*ITS* region[c]
AMACHY-S	*A. retroflexus* and *A. hybridus*	*A. retroflexus* and *A. hybridus*	*A. hybridus*
AMACVI-S	*A. retroflexus* and *A. hybridus*	*A. retroflexus* and *A. hybridus*	Unclassified genotype
AMACRET-S	*A. retroflexus* and *A. hybridus*	*A. retroflexus* and *A. hybridus*	Unclassified genotype
EEA-S	*A. viridis*	*A. viridis*	*A. viridis*
ARRGR-R	*A. retroflexus* and *A. hybridus*	*A. retroflexus* and *A. hybridus*	Unclassified genotype
SAOJER-R	*A. retroflexus* and *A. hybridus*	*A. retroflexus* and *A. hybridus*	Unclassified genotype
CAMAQ-R	*A. retroflexus* and *A. hybridus*	*A. retroflexus* and *A. hybridus*	*A. hybridus*

[a]Morphological identification keys used: Amaranthaceae of Rio Grande do Sul (Vasconcellos 1986) and a synopsis of the *Amaranthus* subgenus in Venezuela (Pinto and Velásquez 2010).
[b]First molecular approach: amplification of intron 1 of *EPSPS* (Wright et al. 2016).
[c]*ITS*, internal transcribed spacer. Second molecular approach: amplification of the ITS5-ITS4 region (Murphy and Tranel 2018).

Table 2. Parameters of the logistic equation and resistance factors (RFs) of weed control, fresh shoot weight, and dry shoot weight at 21 d after the application of treatments for glyphosate, imazethapyr, and chlorimuron.

		Regression parameters[b]						
Herbicide	Biotypes[a]	*b*	*c*	*d*	*e* (ED_{50})	Lower CI	Upper CI	RF
Glyphosate	Control							
	AMACHY-S	−0.62*	0.24	117.83*	19.22	5.87	32.56	—
	ARRGR-R	−0.97*	−1.45	122.41*	9,156.97	2,957.14	15,356.81	476.50*
	SAOJER-R	−1.55*	−2.13	113.43*	9,857.27	6,140.85	13,573.69	513.00*
	CAMAQ-R	−1.85*	−1.77	91.12*	5,879.68	4,448.70	7,310.67	306.00*
	Fresh shoot weight							
	AMACHY-S	1.47*	−0.11	9.41*	11.51	8.98	14.03	—
	ARRGR-R	0.84*	−3.25	9.19*	9,806.37	1,141.22	18,471.52	852.00*
	SAOJER-R	1.51*	−0.34	6.89*	8,973.96	4,109.33	13,838.59	779.60*
	CAMAQ-R	3.60*	0.36	6.16*	5,583.36	4,557.99	6,608.72	485.00*
	Dry shoot weight							
	AMACHY-S	1.60*	0.02	1.89*	10.99	8.45	13.54	—
	ARRGR-R	0.90*	−0.27	1.77*	3,968.96	1,541.41	6,396.51	361.10*
	SAOJER-R	1.24*	−0.03	1.45*	6,015.33	1,998.66	10,032.00	547.30*
	CAMAQ-R	2.47*	0.06	1.25*	4,452.92	3,322.69	5,573.16	405.20*
Imazethapyr	Control							
	AMACHY-S	−0.50*	−0.03	106.67*	0.81	0.38	1.237	—
	SAOJER-R	−1.62*	4.56*	106.13*	3,759.41	3,368.09	4,150.73	4,658.5*
	Fresh shoot weight							
	AMACHY-S	0.81*	0.06	7.36*	0.55	−0.46	1.53	—
	SAOJER-R	4.11*	0.29*	1.03*	2,289.30	2,138.81	2,439.80	4,147.3*
	Dry shoot weight							
	AMACHY-S	0.94	0.06	1.22*	0.56	−0.86	1.98	—
	SAOJER-R	2.21*	0.046	1.59*	1,485.10	1,327.52	1,642.69	2,652.0*
Chlorimuron	Control							
	AMACHY-S	−1.56*	0.46	99.23*	0.45	0.38	0.52	—
	SAOJER-R	−0.97*	−2.18	104.43*	337.35	199.15	475.55	418.0*
	Fresh shoot weight							
	AMACHY-S	2.27*	−0.01	9.68*	0.54	0.45	0.63	—
	SAOJER-R	1.82*	0.67	7.48*	317.26	228.18	406.34	574.7*
	Dry shoot weight							
	AMACHY-S	1.84*	0.06	1.41*	0.46	0.34	0.566	—
	SAOJER-R	1.24*	0.10	0.95*	209.10	88.84	329.36	373.4*

[a]*Amaranthus* putative resistant: ARRGR-R, SAOJER-R, and CAMAQ-R; susceptible: AMACHY-S.
[b]*b*, slope; *c*, lower limit; *d*, upper limit; *e*, herbicide dose (g ha^{-1}) to control 50% of the variable (ED_{50}); lower/upper confidence interval (CI) of the variable *e* (ED_{50}); RF, resistance factor (resistant/susceptible).
*Statistically significant ($P < 0.05$).

Confirmation of Resistance and Resistance Level

Herbicide ED_{50} values for all biotypes are presented in Table 2. The glyphosate ED_{50} values of control at 21 DAT for AMACHY-S, ARRGR-R, SAOJER-R, and CAMAQ-R biotypes were 19, 9,157, 9,857, and 5,880 g ha^{-1}, respectively. These results indicate that the ARRGR-R, SAOJER-R, and CAMAQ-R biotypes are 477-, 513-, and 306-fold less sensitive to glyphosate, respectively, than the AMACHY-S biotype, presenting a high RF level (RF > 300) (Table 2). The RF of biotypes with resistance to glyphosate identified in Argentina in 2018 was 314 (Perotti et al. 2019). Furthermore, RF of the fresh and dry shoot weight measurements also showed a high RF level for ARRGR-R, SAOJER-R, and CAMAQ-R biotypes, where the RF was 852, 780, and 485 g ha^{-1} for fresh shoot weight and 361, 547, and 405 g ha^{-1} dry shoot weight, respectively (Table 2). Biotypes of *A. hybridus* previously identified with resistance to glyphosate in Brazil showed a dose of glyphosate to reduce the shoot weight ranging from 3,019 to 3,316 g ha^{-1} (Resende et al. 2020). Thus, the RFs from biotypes studied in the

https://doi.org/10.1017/wsc.2023.70 Published online by Cambridge University Press

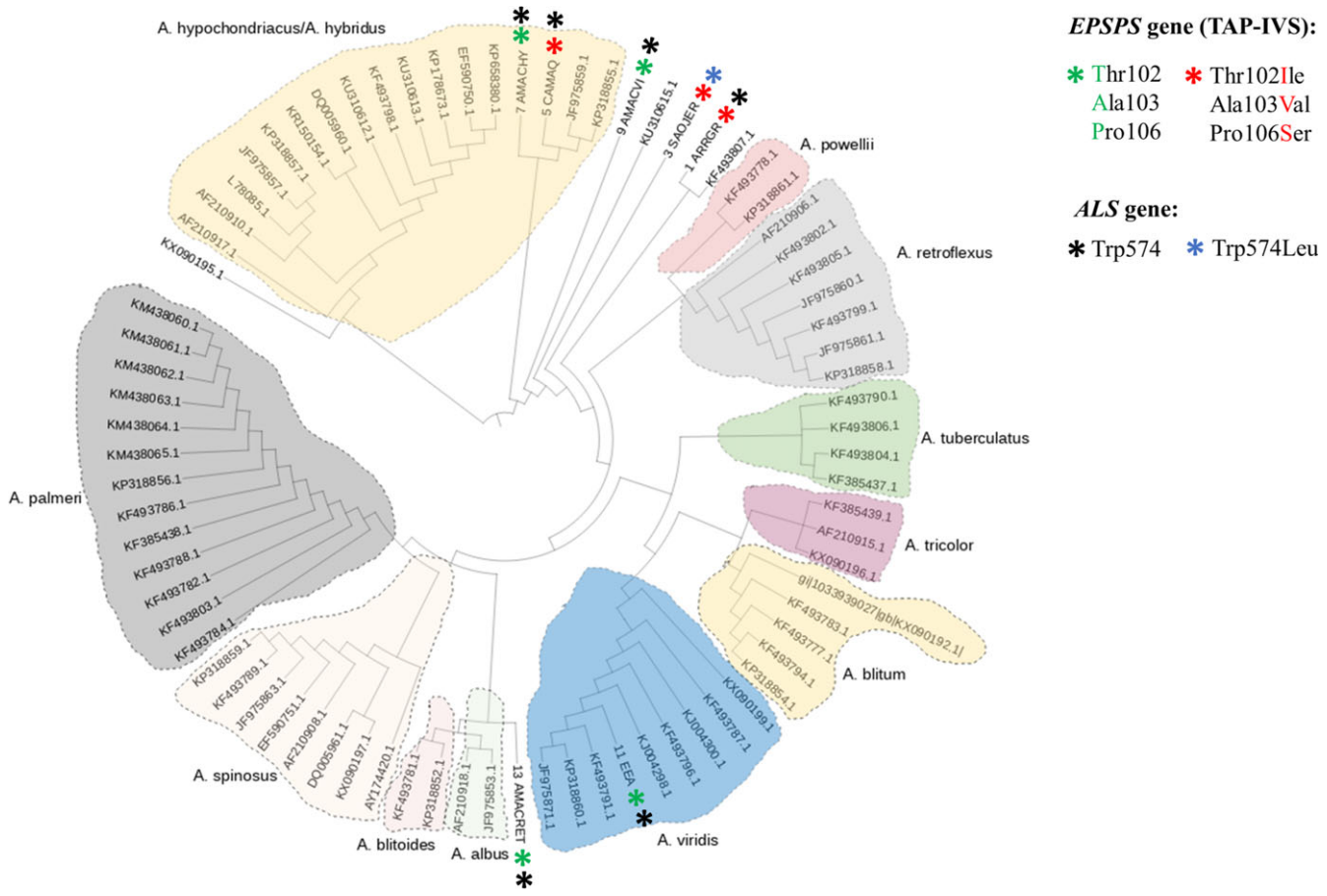

Figure 1. Radial phylogenetic tree of reference *Amaranthus* with internal transcribed spacer (*ITS*) sequences extracted from Murphy and Tranel (2018) and *ITS* sequences of AMACHY-S, AMACVI-S, AMARET-S, EEA-S, ARRGR-R, SAOJER-R, and CAMAQ-R biotypes from this study. Asterisks (*): green, biotypes susceptible to glyphosate; red, resistant biotypes with the TAP-IVS mutation in *EPSPS* gene; from *ALS* gene: biotypes susceptible to (black) and biotypes resistant to (blue) acetolactate synthase (ALS)-inhibiting herbicides.

present study were similar to those found in Argentina and other reports of glyphosate-resistant populations in Brazil. This experiment confirmed the resistance of ARRGR-R, SAOJER-R, and CAMAQ-R to glyphosate.

The ED_{50} control values at 21 DAT for AMACHY-S and SAOJER-R biotypes for imazethapyr were 0.8, and 3,759 g ha^{-1}, respectively (Table 2). For chlorimuron, the values were 0.4 and 337 g ha^{-1}, respectively (Table 2). These results indicate that the SAOJER-R biotype is 4,659- and 418-fold less sensitive to imazethapyr and chlorimuron than the AMACHY-S biotype, respectively, with high RF levels (Table 2). Another study showed that *A. hybridus* with resistance to imazethapyr was 200- to 600-fold (Whaley et al. 2006). In the present study, fresh and dry shoot weight measurements also showed a high RF level for the SAOJER-R biotype; RF was 4,147 g ha^{-1} for fresh shoot weight and 2,652 g ha^{-1} dry shoot weight for imazethapyr and 317 g ha^{-1} for fresh shoot weight and 209 g ha^{-1} dry shoot weight for chlorimuron (Table 2). This experiment confirmed the cross-resistance of SAOJER-R to ALS-inhibiting herbicides.

Multiple resistance to ALS- and EPSPS-inhibiting herbicides in *A. hybridus* was first verified in 2014 in Argentina (Heap 2023). In 2018, a population of *A. hybridus* was found in Brazil with multiple resistance to glyphosate and chlorimuron (Heap 2023). However, in those cases, the mechanism(s) of resistance has not been characterized (Mathioni et al. 2022; Resende et al. 2020). Recently, a study identified and characterized multiple resistance to ALS and EPSPS inhibitors for the first time in populations of *A. hybridus* in Argentina (García et al. 2020). García et al.'s (2020) study showed resistance to imazamox with $ED_{50} > 700$ g ha^{-1} and RF of 39. The present study also showed high-level resistance rate to the imazethapyr herbicide with RF of 4,658.

Target-Site Mutation and Increase in Copy Number

The triple amino acid substitution TAP-IVS in the *EPSPS* gene was confirmed in the biotypes with glyphosate resistance (Figure 2A). Sequences of susceptible and triple-mutated biotypes were aligned with the resistant *A. hybridus* from Argentina (García et al. 2020; Perotti et al. 2019) (Supplementary Figure 3). Our results also showed ATA^{102}, GTC^{103}, and TCA^{106} codons for the resistant biotypes, the same as those identified in biotypes from Argentina; on the other hand, ACA^{102}, GCG^{103}, and CCA^{106} were verified for the susceptible biotype (Figure 2A). The chromatogram of the CAMAQ-R biotype presents double peaks in the first nucleotide of the TCA^{106} codon (Figure 2A) that were also verified in the different self-fertilized generations G1 to G2, showing that the locus was not heterozygotes (Supplementary Figure 4). Similarly, the double peaks were found in a previous study with *A. hybridus* in Brazil (Mathioni et al. 2022). The analysis of relative genomic *EPSPS* gene copy number indicates that there is more than one

https://doi.org/10.1017/wsc.2023.70 Published online by Cambridge University Press

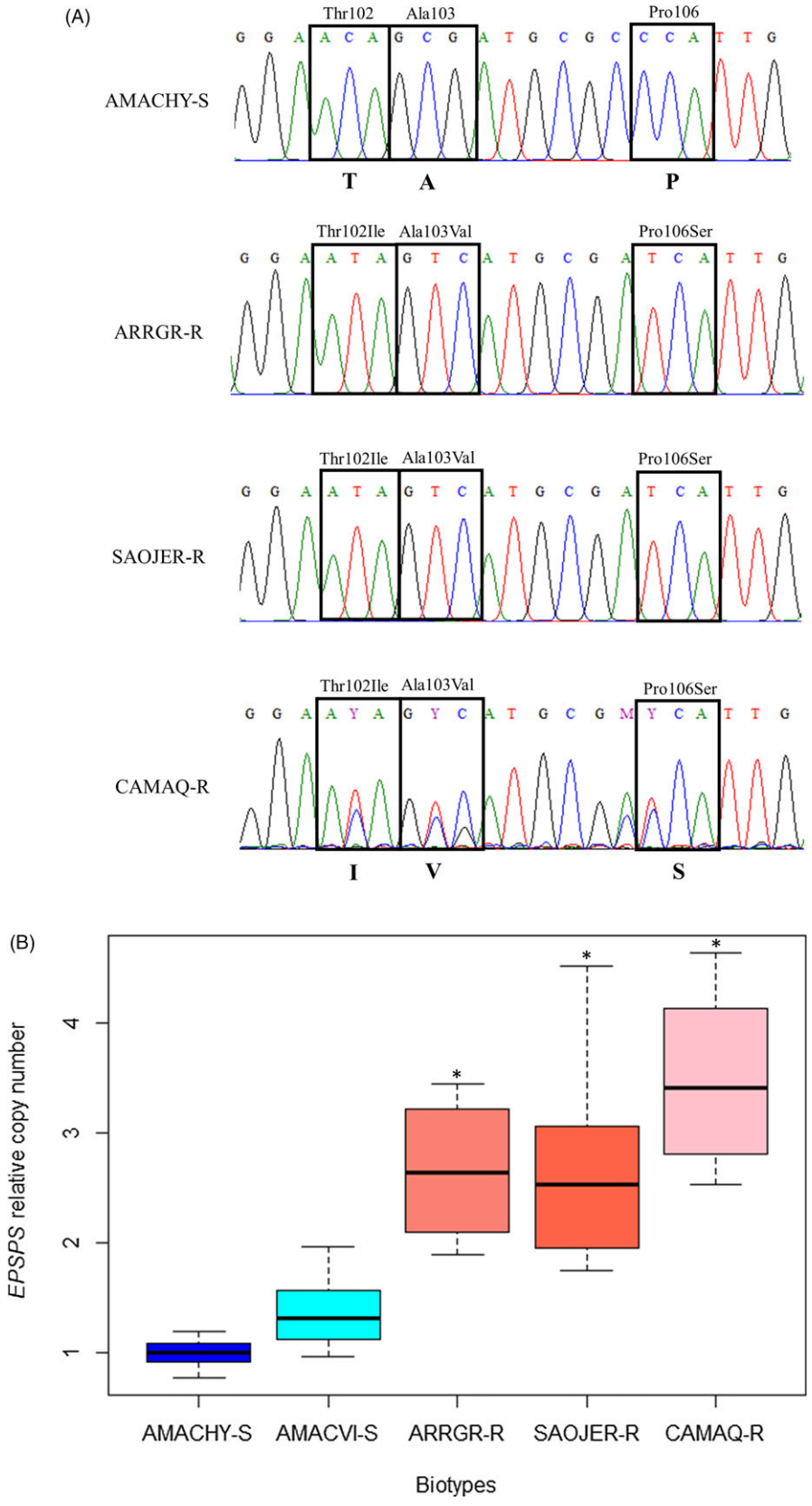

Figure 2. (A) Chromatogram with susceptible biotype without mutation (AMACHY-S), and biotypes with the triple amino acid substitution Thr-102-Ile, Val-103-Ala, and Pro-106-Ser (TAP-IVS) mutation (ARRGR-R, SAOJER-R, and CAMAQ-R) in the *EPSPS* gene. (B) *EPSPS* relative copy number in susceptible (AMACHY-S and AMACVI-S) and resistant biotypes (ARRGR-R, SAOJER-R, and CAMAQ-R). The results were normalized using the AMACHY-S sample as the reference. *EPSPS* relative copy number was relative to the *ACTN* gene. An asterisk (*) indicates statistical difference according to Tukey's test ($P < 0.05$) compared with AMACHY-S. Bars indicate standard error of the mean.

https://doi.org/10.1017/wsc.2023.70 Published online by Cambridge University Press

EPSPS locus in ARRGR-R, SAOJER-R, and CAMAQ-R biotypes (Figure 2B). These results are similar to those found in a study conducted by Perotti et al. (2019). Thus, CAMAQ-R probably has at least one locus from a susceptible population, which explains the double peaks in the chromatogram (Figure 2A and 2B). The presence of multiple *EPSPS* copies could also explain the ambiguity in species identification using the *EPSPS* marker.

The mutation at the Pro-106 position has already been identified to confer resistance in many weeds (De Carvalho et al. 2012; Gaines et al. 2012). It should be noted that mutations in this locus result in low levels of resistance, generally ranging from 2- to 10-fold, when present in populations without other mechanisms of resistance (Baerson et al. 2002; Takano et al. 2019; Wakelin and Preston 2006). Furthermore, these substitutions do not confer an adaptive cost in plants (Sammons and Gaines 2014). Single substitutions found at positions Gly-101 and Thr-102 confer a high level of resistance to glyphosate due to the reduced size of the glyphosate binding site. However, this reduction also affects the binding of the enzyme with its substrate, phosphoenolpyruvate (Funke et al. 2009). The double mutation Thr-102-Ile and Pro-106-Ser, named TIPS, identified in goosegrass [*Eleusine indica* (L.) Gaertn.] (Yu et al. 2015) and hairy beggarticks (*Bidens pilosa* L.) (Alcántara-de la Cruz et al. 2016), also provides a high level of resistance to glyphosate. In Paraguay, the replacement of Thr-102-Ile and Pro-106-Thr (TIPT) was identified in greater beggarticks (*Bidens subalternans* DC) and confers high-level resistance to glyphosate (Takano et al. 2020). The triple mutation (TAP-IVS) was identified in biotypes of *A. hybridus* in Argentina and Brazil (García et al. 2020; Mathioni et al. 2022; Perotti et al. 2019). Due to the difficulty of occurrence of triple mutation in the same gene, because it is a rare event, it is suggested that the resistant biotypes existing in southern Brazil are due to the dissemination of seeds of resistant biotypes from Argentina. However, the origin of the EPSPS TAP-IVS substitution is unknown, and it is still possible to consider that its origin is in Brazil, even though it was reported earlier in Argentina.

Based on alignment with the *A. thaliana* reference sequence (GenBank X51514.1), the positions Ala-122, Pro-197, Ala-205, Ala-376, Arg-377, Trp-574, Ser-653, and Gly-654 associated with ALS mutations were analyzed (Figure 3). The sequencing of *ALS* revealed the presence of a predicted tryptophan to leucine substitution at position 574 (Trp-574-Leu) (Figure 3) in the individuals resistant to ALS herbicides (SAOJER-R biotype). This point mutation is well known to confer resistance to ALS-inhibiting herbicides in roughfruit amaranth (*Amaranthus rudis* Sauer), great ragweed (*Ambrosia trifida* L.), kochia [*Bassia scoparia* (L.) A.J. Scott], and rough cocklebur (*Xanthium strumarium* L.) (Tranel and Wright 2002). In addition, this mutation has already been described in *A. hybridus* biotypes from the United States (Whaley et al. 2006), and it is the first time described in Brazil. Thus, SAOJER-R has the EPSPS TAP-IVS substitution and ALS Trp-574-Leu target-site mechanisms conferring resistance to glyphosate and ALS inhibitors, respectively.

Intra- and Interspecific Hybridization

Initially, we established experimental conditions to evaluate for response to glyphosate utilizing a seedling assay that permitted large-scale screening of individuals. The seedling assay revealed the dose of 3.2 mM glyphosate to distinguish susceptible from resistant biotypes (Supplementary Table 2; Supplementary Figure 5), similar to a study with waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] (Zelaya and Owen 2005). Moreover, the results were obtained after 14 days of incubation, which is faster than conventional whole-plant assays.

In wind-mediated gene flow trials, hybridization was detected under field research conditions between resistant and susceptible *Amaranthus* biotypes (Table 3). The intraspecific hybridization was evaluated in 82,206 and 37,540 individuals for the first and second seasons (2020 to 2021 and 2021 to 2022), respectively. The number of survivors were 73 and 34 for the experiments carried out in 2020 to 2021 and 2021 to 2022, respectively (Table 3). Thus, the observed rates for intraspecific hybridization between CAMAQ-R (*A. hybridus*) × AMACHY-S (*A. hybridus*) in 2020 to 2021 and 2021 to 2022 were 0.089% and 0.091%, respectively. Hybrids from the cross of *A. hybridus* biotypes were confirmed with *EPSPS* sequencing (Supplementary Figure 6), and the triple mutation was found in all 12 samples sequenced from each season (data not shown).

Even though *Amaranthus hybridus* is considered a predominantly self-pollinating species due to the proximity of the male and female flowers, it can be pollinated by wind, and the male and female flowers are separate (Murray 1940). Our study showed that gene flow might be a relevant contribution to the dispersion of TAP-IVS substitution from *A. hybridus* in South America. Pollen spread contributes to a higher frequency of herbicide-resistant plants compared with the expected initial frequency of herbicide-resistant individuals (10^{-8} to 10^{-9}) in herbicide-unselected populations, and it plays an important role in resistance evolution by increasing the incidence of herbicide-resistant weeds (Beckie et al. 2019). This is the first documentation of dispersal by PMGF of alleles from the triple mutation in *A. hybridus*. However, the source of the EPSPS TAP-IVS substitution remains unknown.

Interspecific hybridization was not observed in the study with CAMAQ-R (*A. hybridus*) × AMACVI-S (unclassified genotype) in a total of 84,769 and 26,660 individuals analyzed in seasons 2020 to 2021 and 2021 to 2022, respectively (Table 3). However, the possibility of such hybridization cannot be entirely excluded. It should be considered that it might occur at a lower frequency, which needs to be evaluated in a larger sample size (Table 3). For example, the interspecific hybridization among species within the *Amaranthus* and *Ambrosia* genera enables the transference of herbicide-resistance alleles, even at relatively low levels, which requires evaluating a large number of individuals (Jhala et al. 2021).

Interspecific hybridization was reported in *A. palmeri*, transferring glyphosate resistance to *A. hybridus* at a rate of 0.01% (Gaines et al. 2012). Introgression between *A. tuberculatus* and *A. hybridus* was verified only in one direction, from *A. hybridus* to *A. tuberculatus* (Trucco et al. 2009). High rates of hybridization of 5.9% were found between *A. tuberculatus* and *A. hybridus* (Trucco et al. 2005). Hybridization between other species of *Amaranthus* was also reported (Gaines et al. 2012; Oliveira et al. 2018). Therefore, the observed hybridization in *Amaranthus* species appears to play a significant role in explaining instances of resistance spread, as observed in the findings in this study.

Gene flow of traits is a process that depends on many factors, such as particular genes and alleles, genetic inheritance of the trait, species ploidy, fitness costs, and species ecological traits (Jasieniuk et al. 1996). The interspecific transference of herbicide resistance within *Amaranthus* is mostly seen in dioecious species, which are different from *A. hybridus*, *A. powellii* and *A. retroflexus*, that are monoecious species. The transference of mesotrione resistance due to cytochrome P450 metabolism in *A. tuberculatus* to *A. palmeri*,

https://doi.org/10.1017/wsc.2023.70 Published online by Cambridge University Press

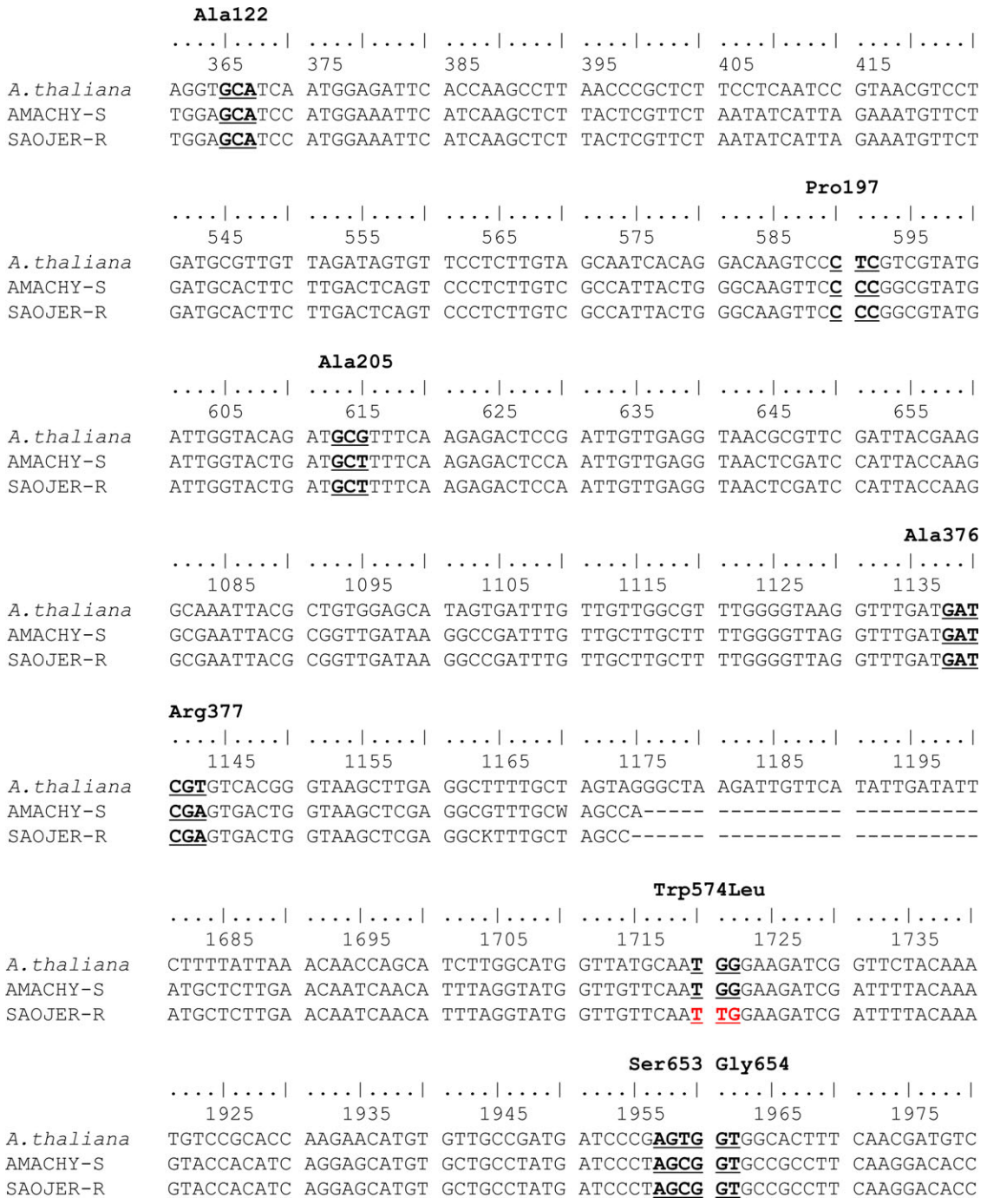

Figure 3. Sequence alignment of the *ALS* gene using *Arabidopsis thaliana* (GenBank X51514.1) as the reference and acetolactate synthase (ALS)-susceptible AMACHY-S and ALS-resistant SAOJER-R *Amaranthus* biotypes. The sequences presented here show the eight positions with known *ALS* mutations associated with resistance to ALS-inhibiting herbicides. Red nucleotide bases indicate the codon with mutation site position Trp-574-Leu.

both obligate outcrossing species, occurred under field conditions at a rate of 0.1%. When intraspecific transfer was evaluated, gene flow varied in distance and direction. The 90% reduction in PMGF was 13.1 and 26.1 m in each assessment year. PMGF under field conditions might be accelerating the increase of herbicide resistance in important weeds for the United States, such as *A. palmeri* and *A. tuberculatus* (Oliveira et al. 2018). However, it should be noted that similar studies are important to understand the dynamics of dispersion of resistance to glyphosate and ALS inhibitors in *A. hybridus*.

We showed that some biotypes that have the EPSPS TAP-IVS substitution and have morphological characteristics of *A. hybridus* potentially are hybrids of *A. hybridus* and *A. powellii* (Figure 1), explaining the morphological variation observed in the field by farmers. This can mean that these biotypes may be derived from one or more past hybridization events. In this case, the triple mutation could have been in Brazil for a long time, or the triple mutation may not be such a rare event for *EPSPS*. Additionally, a hybrid biotype used in the study showed resistance to ALS inhibitors caused by a target-site mechanism, with the presence of

https://doi.org/10.1017/wsc.2023.70 Published online by Cambridge University Press

Table 3. Frequency of pollen-mediated gene flow (PMGF) between resistant and susceptible *Amaranthus hybridus* biotypes, and between a resistant *A. hybridus* biotype and an unclassified species of *Amaranthus* under field research conditions at the Federal University of Rio Grande do Sul in seasons 2020–2021 and 2021–2022.

Donor parent × receptor parent	Season	Germinated seeds[a]	Surviving seedlings[b]	Hybridization	
				Frequency[c]	Power[d]
CAMAQ-R (*A. hybridus*) × AMACHY-S (*A. hybridus*)	2020–2021	82,206	73	0.00089	0.95
CAMAQ-R (*A. hybridus*) × AMACHY-S (*A. hybridus*)	2021–2022	37,540	34	0.00091	0.95
TOTAL		119,746		0.00090	
CAMAQ-R (*A. hybridus*) × AMACVI-S (unclassified genotype)	2020–2021	84,769	0	0	0.95
CAMAQ-R (*A. hybridus*) × AMACVI-S (unclassified genotype)	2021–2022	26,660	0	0	0.95
Total		111,429		<0.000027[e]	

[a]Minimum sample size for theoretical frequency (0.001) of gene flow considering analysis power of 95% is 55,870, according to Jhala et al. (2011).
[b]Seedlings that survived 3.2 mM of glyphosate.
[c]PMGF frequency was evaluated as number of surviving seedlings/number of emerged seedlings.
[d]Sample power for 95% confidence interval ($1 - \beta$), $\alpha = 0.05$.
[e]Estimated with 95% confidence, based on simple binomial distribution.

the Trp-574-Leu mutation described for the first time in Brazil for *Amaranthus*. It is possible to infer that the predominantly autogamous *A. hybridus* has a cross-fertilization rate that contributes to the spread of resistance to glyphosate and other herbicides. PMGF is a pathway for the spread of the EPSPS TAP-IVS substitution and dispersal of resistance to glyphosate. Furthermore, PMGF may favor the accumulation of resistance mechanisms through the pyramiding of genes and the acquisition of novel adaptive traits.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/wsc.2023.70

Acknowledgments. We thank Milene Ferro for analyzing some data and the Foundation for Research Support of the State of Rio Grande do Sul–FAPERGS for funding this research (grant no. 21/2551-0000607-2). No conflicts of interest have been declared.

References

Alcántara-de la Cruz R, Fernández-Moreno PT, Ozuna CV, Rojano-Delgado AM, Cruz-Hipolito HE, Domínguez-Valenzuela JA, Barro F, de Prado R (2016) Target and non-target site mechanisms developed by glyphosate-resistant hairy beggarticks (*Bidens pilosa* L.) populations from Mexico. Front Plant Sci 7:1–12

Baerson SR, Rodriguez DJ, Tran M, Feng Y, Biest NA, Dill GM (2002) Glyphosate-resistant goosegrass. Identification of a mutation in the target enzyme 5-enolpyruvylshikimate-3-phosphate synthase. Plant Physiol 129:1265–1275

Baldwin BG, Sanderson M, Porter JM, Wojciechowski MF, Campbell CS, Donoghue MJ (1995) The *ITS* region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann Mo Bot Gard 82:247–277

Bayón ND (2015) Revisión taxonómica de las especies monoicas de *Amaranthus* (Amaranthaceae): *Amaranthus* subg. *Amaranthus* y *Amaranthus* subg. Albersia. Ann Mo Bot Gard 101:261–383

Beckie HJ, Busi R, Bagavathiannan MV, Martin SL (2019) Herbicide resistance gene flow in weeds: under-estimated and under-appreciated. Agric Ecosyst Environ 283:1–11

Costea M, Sanders A, Waines G (2001a) Preliminary results toward a revision of the *Amaranthus hybridus* species complex (Amaranthaceae). SIDA Contrib Bot 19:931–974

Costea M, Sanders A, Waines G (2001b) Notes on some little known *Amaranthus* taxa (Amaranthaceae) in the United States. SIDA Contrib Bot 19:975–992

De Carvalho LB, Alves PLDCA, González-Torralva F, Cruz-Hipolito HE, Rojano-Delgado AM, De Prado R, Gil-Humanes J, Barro F, Luque De Castro MD (2012) Pool of resistance mechanisms to glyphosate in *Digitaria insularis*. J Agric Food Chem 60:615–622

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Faccini D, Vitta JI (2005) Germination characteristics of *Amaranthus quitensis* as affected by seed production date and duration of burial. Weed Res 45: 371–378

Funke T, Yang Y, Han H, Healy-Fried M, Olesen S, Becker A, Schönbrunn E (2009) Structural basis of glyphosate resistance resulting from the double mutation Thr97 → Ile and Pro101 → Ser in 5-enolpyruvylshikimate-3-phosphate synthase from *Escherichia coli*. J Biol Chem 284:9854–9860

Gaines TA, Cripps A, Powles SB (2012) Evolved resistance to glyphosate in junglerice (*Echinochloa colona*) from the tropical or driver region in Australia. Weed Technol 26:480–484

Gaines TA, Ward SM, Bukun B, Preston C, Leach JE, Westra P (2012) Interspecific hybridization transfers a previously unknown glyphosate resistance mechanism in *Amaranthus* species. Evol Appl 5:29–38

García MJ, Palma-Bautista C, Vazquez-Garcia JG, Rojano-Delgado AM, Osuna MD, Torra J, De Prado R (2020) Multiple mutations in the EPSPS and ALS genes of *Amaranthus hybridus* underlie resistance to glyphosate and ALS inhibitors. Sci Rep 10:1–11

Grant WF (1959) Cytogenetic studies in *Amaranthus*: III. Chromosome numbers and phylogenetic aspects. Can J Genet Cytol 1:313–328

Heap I (2023) The International Herbicide-Resistant Weed Database. www.weedscience.org. Accessed: May 1, 2023

Horak MJ, Peterson DE, Chessman DJ, Wax LM (1994) Pigweed Identification: A Pictorial Guide to the Common Pigweeds of the Great Plains. Volume S80. Manhattan: Kansas State University Cooperative Extension Service. 13 p

Jasieniuk M, Brûlé-Babel AL, Morrison IN (1996) The evolution and genetics of herbicide resistance in weeds. Weed Sci 44:176–193

Jhala AJ, Bhatt H, Topinka K, Hall LM (2011) Pollen-mediated gene flow in flax (*Linum usitatissimum* L.): can genetically engineered and organic flax coexist? Heredity 106:557–566

Jhala AJ, Norsworthy JK, Ganie ZA, Sosnoskie LM, Beckie HJ, Mallory-Smith CA, Liu J, Wei W, Wang J, Stoltenberg DE (2021) Pollen-mediated gene flow and transfer of resistance alleles from herbicide-resistant broadleaf weeds. Weed Technol 35:173–187

Knezevic SZ, Streibig JC, Ritz C (2007) Utilizing R software package for dose-response studies: the concept and data analysis. Weed Technol 21:840–848

Larran AS, Lorenzetti F, Tuesca D, Perotti VE, Permingeat HR (2018) Molecular mechanisms endowing cross-resistance to ALS-inhibiting herbicides in *Amaranthus hybridus* from Argentina. Plant Mol Biol Report 36:907–912

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25: 402–408

Lorentz L, Gaines TA, Nissen SJ, Westra P, Strek HJ, Dehne HW, Ruiz-Santaella JP, Beffa R (2014) Characterization of glyphosate resistance in *Amaranthus tuberculatus* populations. J Agric Food Chem 62:8134–8142

Mathioni SM, de Oliveira C, Lemes LN, Ozório EG, Rosa DD (2022) PCR-based assay to detect the EPSPS TAP-IVS substitution in *Amaranthus hybridus*. Adv Weed Sci 40:1–7

Mendes RR, Silva VFV, Ferreira LAI, Oliveira RS Jr (2022) Sulfonylurea resistance in *Amaranthus hybridus* from Southern Brazil. Ver Ceres 69:374–378

Murphy BP, Plewa DE, Phillippi E, Bissonnette SM, Tranel PJ (2017) A quantitative assay for *Amaranthus palmeri* identification. Pest Manag Sci 73:2221–2224

Murphy BP, Tranel PJ (2018) Identification and validation of *Amaranthus* species-specific SNPs within the ITS region: applications in quantitative species identification. Crop Sci 58:304–311

Murray BC, Morrison IN, Friesen LF (2002) Pollen-mediated gene flow in wild oat. Weed Sci 50:321–325

Murray MJ (1940) The genetics of sex determination in the family Amaranthaceae. Genetics 25:409–431

Oliveira MC, Gaines TA, Patterson EL, Jhala AJ, Irmak S, Amundsen K, Knezevic SZ (2018) Interspecific and intraspecific transference of metabolism-based mesotrione resistance in dioecious weedy *Amaranthus*. Plant J 96:1051–1063

Penckowski LH, Maschietto EHG, Borsato EF, Adegas FS, Moreira LSO, Bianchi MA, Rizzardi MA, Oliveira RS Jr, Dornelles SHB (2020) *Amaranthus* resistant to glyphosate. Revista Fund ABC 39:9–12

Perotti VE, Larran AS, Palmieri VE, Martinatto AK, Alvarez CE, Tuesca D, Permingeat HR (2019) A novel triple amino acid substitution in the EPSPS found in a high-level glyphosate-resistant *Amaranthus hybridus* population from Argentina. Pest Manag Sci 75:1242–1251

Pinto WC, Velásquez GO (2010) Sinopsis del subgénero *Amaranthus* (Amaranthus, Amaranthaceae) en venezuela. Acta Bot Venez 33:329–356

Resende LS, Christoffoleti PJ, Gonçalves A, Presoto JC, Nicolai M (2020) Glyphosate-resistant smooth-pigweed (*Amaranthus hybridus*) in Brazil. Adv Weed Sci 40:1–6

Sammons RD, Gaines TA (2014) Glyphosate resistance: state of knowledge. Pest Manag Sci 70:1367–1377

Sauer J (1955) Revision of the dioecious amaranths. Madrono 13:5–46

Soltani N, Dille JA, Burke IC, Everman WJ, Vangessel MJ, Davis VM, Sikkema PH (2017) Perspectives on potential soybean yield losses from weeds in North America. Weed Technol 31:148–154

Takano HK, Fernandes VNA, Adegas FS, Oliveira RS, Westra P, Gaines TA, Dayan FE (2020) A novel TIPT double mutation in EPSPS conferring glyphosate resistance in tetraploid *Bidens subalternans*. Pest Manag Sci 76:95–102

Takano HK, Mendes RR, Scoz LB, Ovejero RFL, Constantin J, Gaines TA, Westra P, Dayan FE, Oliveira RS Jr (2019) Proline-106 EPSPS mutation imparting glyphosate resistance in goosegrass (*Eleusine indica*) emerges in South America. Weed Sci 67:48–56

Tranel PJ, Wassom JJ, Jeschke MR, Rayburn AL (2002) Transmission of herbicide resistance from a monoecious to a dioecious weedy *Amaranthus* species. Theor Appl Genet 105:674–679

Tranel PJ, Wright TR (2002) Resistance of weeds to ALS-inhibiting herbicides: what have we learned? Weed Sci 50:700–712

Trucco F, Jeschke MR, Rayburn AL, Tranel PJ (2005) *Amaranthus hybridus* can be pollinated frequently by *A. tuberculatus* under field conditions. Heredity 94:64–70

Trucco F, Tatum T, Rayburn AL, Tranel PJ (2009) Out of the swamp: unidirectional hybridization with weedy species may explain the prevalence of *Amaranthus tuberculatus* as a weed. New Phytol 184:819–827

Vasconcellos JMO (1986) Amaranthaceae of Rio Grande do Sul, Brazil. Roessléria 8:107–137

Walsh KD, Puttick DM, Hills MJ, Yang RC, Topinka KC, Hall LM (2012) Short communication: first report of outcrossing rates in camelina [*Camelina sativa* (L.) Crantz], a potential platform for bioindustrial oils. Can J Plant Sci 92:681–685

Wakelin AM, Preston C (2006) A target-site mutation is present in a glyphosate-resistant *Lolium rigidum* population. Weed Res 46:432–440

Wetzel DK, Horak MJ, Skinner DZ (1999) Use of PCR-based molecular markers to identify weedy *Amaranthus* species. Weed Sci 47:518–523

Whaley CM, Wilson HP, Westwood JH (2006) ALS resistance in several smooth pigweed (*Amaranthus hybridus*) biotypes. Weed Sci 54:828–832

Wright AA, Molin WT, Nandula VK (2016) Distinguishing between weedy *Amaranthus* species based on intron 1 sequences from the 5-enolpyruvyl-shikimate-3-phosphate synthase gene. Pest Manag Sci 72:2347–2354

Xu H, Li ZY, Li JS (2017) Phylogenetic analysis of alien species of *Amaranthus* in China based on ITS sequences. Guihaia 37:139–144

Yu Q, Jalaludin A, Han H, Chen M, Douglas Sammons R, Powles SB (2015) Evolution of a double amino acid substitution in the 5-enolpyruvylshikimate-3-phosphate synthase in *Eleusine indica* conferring high-level glyphosate resistance. Plant Physiol 167:1440–1447

Zandoná RR, Barbieri GF, Schmitz MF, Do Amarante AA, Göebel JGS, Agostinetto D (2022) Economic threshold of smooth pigweed escaped from a herbicide program in Roundup Ready® soybean. Adv Weed Sci 40:1–7

Zelaya IA, Owen MDK (2005) Differential response of *Amaranthus tuberculatus* (Moq ex DC) JD Sauer to glyphosate. Pest Manag Sci 61:936–950

https://doi.org/10.1017/wsc.2023.70 Published online by Cambridge University Press