

First report of barnyardgrass resistant to glyphosate in Brazil

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Abstract: Background: Barnyardgrass (*Echinochloa crus-galli* (L.) P. Beauv.) is an annual weed affecting many field crops. A glyphosate-putative-resistant barnyardgrass population was found from a soybean field in southern Brazil.

Objective: This study aimed to confirm this resistance in Rio Grande do Sul (RS) and identify its mechanism. **Methods:** Dose-response curves were generated using a three-parameter nonlinear regression model to assess control efficacy and shoot dry weight reduction. Resistance factors (RF) were calculated across two generations from the resistant (BR20Esp016) and susceptible (BR19Esp001) populations. cDNA was synthesized from

total RNA extracted from the plants, and the EPSPS gene was sequenced from both populations. **Results:** Based on the glyphosate required to reduce dry weight by 50% (GR₅₀), the resistance biotype had RFs of 2.4 and 2.6 and values of 2.4 and 13.9 for the same variable for the self-fertilization generation (F2). A mutation was detected in the EPSPS gene, where proline (CCA) is replaced by alanine (GCA) at position 106, conferring resistance to EPSPS-inhibiting herbicides in barnyardgrass. **Conclusion:** Glyphosate resistance in the barnyardgrass biotype BR20Esp016 is due to target-site resistance associated with a mutation in the EPSPS gene.

Keywords: *Echinochloa Crus-galli*; EPSPs-Inhibiting Resistance; Mutation.

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1. Introduction

Soybean (*Glycine max*) is one of the most important crops due to its valuable, versatile, and nutritional content, with an estimated global production in the 2021/2022 growing season of 350.72 million metric tons, corresponding to 61% of the world oilseed production using 6% of the world's arable land use (Shea et al., 2020). Brazil, the United States, and Argentina produce approximately 81% of this total (Ates, Bukowski, 2022). The importance of this crop has increased over the years due to technological advances aimed at improving grain yield and genetically modified crops with herbicide resistance, such as Roundup Ready™ (RR) soybeans, which are resistant to glyphosate (Pagano, Miransari, 2016).

RR technology revolutionized weed management in many crops by enabling the use of glyphosate in post-emergence. However, one side effect of this technology was the increased selection pressure, which led to the evolution of weed resistance to glyphosate, which is today one of the most critical global threats to sustainable food production (Yu, Powles, 2014).

Weed resistance to herbicides is categorized into two mechanisms: (1) target-site resistance (TSR), caused by mutations that occur by simple or multiple amino acid substitutions disrupting the bind of target protein and herbicide, or by increased expression of target enzyme due to gene up-regulation, and (2) non-target-site resistance (NTSR) which involves mechanisms that reduce the amount of active herbicide reaching or the target site through physiological adaptations that the plant from herbicide's lethal effects (Gaines et al., 2020). Glyphosate [N-(phosphonomethyl)-glycine], the most widely used post-emergence, non-selective herbicide, has a unique mechanism for inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs) (Duke, Powles, 2008). To date, 58 glyphosate-resistant weed species have been reported worldwide, split between grass and broadleaf and are distributed across 31 countries (Heap, 2024).

Barnyardgrass (*Echinochloa crus-galli* (L.) P. Beauv.) has become one of the most difficult to control weeds globally. This cosmopolitan weed infests many crops and adapts to various environments, ranging from flooded areas on rice paddy fields to dryland conditions (Menegol et al., 2023). Barnyardgrass is widespread in tropical, northern, and southern temperate zones in field crops worldwide, ranking third in economic impact, characterized by its high ability to compete by high adaptability, rapid germination, and prolific seed production (Martinková, 2021). These characteristics of barnyardgrass make it difficult to control, and its management has relied heavily

on herbicides for over 70 years. However, this prolonged reliance has led to the evolution of herbicide-resistant biotypes due to direct selection pressure (Belz et al., 2022).

Barnyardgrass belongs to the genus *Echinochloa*, which includes approximately 50 species with diverse forms and taxonomies, due to hybridization and environment adaptation (Rampoldi et al., 2017). The genus *Echinochloa* currently has 99 confirmed cases of herbicide resistance across 36 countries (Heap, 2024). Of these, 57 cases are in barnyardgrass, with 54 cases in *E. crus-galli* var. *crus-galli*, two in *E. crus-galli* var. *formosensis*, and one in *E. crus-galli* var. *zelayensis*, including reports of single, cross, and multiple resistance. So far, it has been reported for the following herbicide classes: Auxin mimics, Acetolactate Synthase (ALS) inhibitors, Acetyl CoA Carboxylase (ACCase) inhibitors, Microtubule assembly inhibitors, PSII inhibitors, Very Long-Chain Fatty Acid Synthesis inhibitors, and EPSPs inhibitors (Heap, 2024). Of these cases, only 11 have documented resistance mechanisms, which correspond to TSR by mutation for glyphosate, fenoxaprop, atrazine, foramsulfuron, penoxulam, nicosulfuron, aminosulfuron, bispiribac, and imazamox (Heap, 2024).

Historically, barnyardgrass infestations in Brazil were problematic in rice crops due to the flooding conditions, but the increase in soybeans crop rotation, this issue has expanded (Andres et al., 2012). Recently, field crop extensionists and farmers observed decreased glyphosate control of barnyardgrass in southern Brazilian soybean crops. In this context, this study aimed to determine the existence of a barnyardgrass-resistant population to glyphosate in the RS state and its resistance mechanism.

2. Materials and Methods

The putative-resistant barnyardgrass population was collected in a field where glyphosate failed to control it. Plants from this population were collected in March 2020 in a soybean field located in Santa Rosa (27,9660 ° S; 54,5649 ° W), RS State, Brazil, and designated as BR20Esp016. Seeds from a reference-susceptible biotype were collected from a field with no history of glyphosate application in Cosmopolis (22.6069° S; 47.2205° W), São Paulo State, Brazil, and labeled as BR19Esp001.

The collected seeds were used as a first-generation (F1) assay. F1 plants were grown in isolation until maturity, self-pollinated, and their seeds were collected as the second generation (F2). F2 plants were then used for subsequent assays to confirm resistance heritability. All experiments were carried out with corresponding replicates over time.

2.1 *Echinochloa* species determination

The plants were grown to the reproductive stage in a greenhouse and exsiccates were prepared and sent to the Herbarium of the Santa Maria-Department of Biology

(SMDB) at the Federal University of Santa Maria, located in Santa Maria, RS - Brazil, for identification based on morphological characteristics.

2.2 Dose-response curve

Seeds from both barnyardgrass populations were germinated at 28 °C/ 24 °C (day/night) in trays filled with the commercial substrate. Four plants were transplanted into 1 L pots at the two-leaf stage. The pots were pre-filled with sieved loam soil. Plants were grown under controlled conditions in a growth chamber in Pelotas Federal University, RS State, at 28 °C/ 24 °C in a 12 h/12 h photoperiod light/dark (700 µmol m⁻² s⁻¹) and constant relative humid of 70 ± 5%, until they reached the four-leaf stage, at which point when the experiments were conducted.

The dose-response curve experiments were performed at Pelotas Federal University in 2022 for two generations of barnyardgrass (F1 and F2). The experimental design was completely randomized in a factorial arrangement with four replications. Factor A was barnyardgrass populations (resistant and susceptible), and factor B was the glyphosate (Roundup Original®, 480 g a.e L⁻¹) doses: at 0; 57.6; 115.2; 165; 216; 360; 740; 1,090; 1,440; 2,880; 5,760; and 11,520 g ae ha⁻¹. The recommended field dose of glyphosate for burndown in Brazil is 1,440 g ae ha⁻¹. Herbicide application was performed with a backpack sprayer, equipped with a 2 m boom and four flat-fan nozzles (TeeJet® AIXR110015 nozzles - Spraying Systems, North Avenue and Schmale Road, Wheaton, IL 60187) spaced 0.5 m apart, calibrated to deliver 150 L ha⁻¹ of spray solution.

Barnyardgrass control was assessed visually at 21 days after treatment (DAT), using a scale of 0% to 100%, in which 0% indicates no herbicide symptoms and 100% represents plant death. The aboveground dry weight of each barnyardgrass plant was harvested at 21 DAT and oven-dried for 72 h at 65°C and weighed. The shoot dry weight data were converted to percent shoot dry weight reduction compared to the non-treated control plants (Wortman, 2014) using the following formula:

$$\text{Shoot dry weight reduction (\%)} = \frac{(C-B)}{C} \times 100 \quad (1)$$

where C is the mean shoot dry weight of the four non-treated control replicates, and B is the shoot dry weight of an individual treated experimental unit.

The data of control percentage and shoot dry weight reduction were subjected to a non-linear regression using three parameters log-logistic model using the following formula:

$$f(x) = \frac{d}{[1 + \exp(b(\log(x) - \log(e)))]} \quad (2)$$

where $f(x)$ represents the control or shoot dry weight reduction, b is the slope around the dose providing 50% response (I_{50}), c is the higher limit, x is glyphosate dose (g ae ha^{-1}), and e is the I_{50} (Ritz et al., 2015). From the adjusted models across different generations and time points, the effective doses for 50% and 90% control of the population were calculated for both biotypes, along with their resistance factors (RF) and repeated over time.

The statistical analysis was conducted using the *drc* package in R® version 3.5.2 GUI 1.70. The lack-of-fit test was used to select the model with best adjustment for the different parameters at the other evaluated variables. The effective doses were estimated at 50% and 90% of control (ED_{50} – ED_{90}) and shoot dry weight reduction (GR_{50} and GR_{90}). The resistance factor (RF) was calculated as the ratio of ED_{50}/ED_{50} and GR_{50}/GR_{50} values between resistant and susceptible biotypes; resistance confirmation is when $RF > 1.0$. Simultaneous 95% confidence intervals were calculated and multiple comparisons of means by the Tukey test with $p < 0.05$, employing the *multcomp* package, for F1 and F2 and their repetition over time.

2.3 Determination of resistance mechanism

Leaf tissue was collected from plants of BR20Esp016 and BR19Esp001. RNA extraction was performed using PureLink™ Plant RNA Reagent (Invitrogen) following the manufacturer's recommendations, and the pellet was dried in the flow chamber, and it was diluted in 20 μL of UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen) and stored in ultra-freezer at -80°C . The concentration and quality of the total RNA were evaluated in spectrophotometry using NanoVue™ (GE Healthcare). The integrity of the RNA samples was analyzed in electrophoresis in 2% agarose gel. Each sample was converted into cDNA using oligo(dT) and the SuperScript™ III First-Strand Synthesis System kit (Invitrogen) using the T100 Thermal Cycler Bio-Rad®, according to the manufacturer's recommendations.

The 687 bp EPSPS gene fragment was amplified using the primer set AW-F (5'-TATCCGAGGGGACTACAGTG-3') and AW-R (5'-CTGCACCAGCCAAGAAATAG-3') (Figure 1), designed based on the genome of barnyardgrass available at GenBank: GCA_900205405.1 (YE et al., 2020). PCR reactions were performed in 25 μL with the following reagents: 6.5 μL of Ultrapure Water, 2 μL of cDNA, 2 μL of each primer, and 12.5 μL GoTaq® Green Master Mix (Promega). PCR cycle conditions were as follows: 95 $^{\circ}\text{C}$ for 4 min, 40 cycles of 95 $^{\circ}\text{C}$ for 60 secs, 55,7 $^{\circ}\text{C}$ for 45 secs, 72 $^{\circ}\text{C}$ for 90 secs, the final extension of 72 $^{\circ}\text{C}$ for 10 min, and 4 $^{\circ}\text{C}$ hold. Amplicons were verified on 1.5% agarose gels for amplification and expected size with a Ladder 1 Kb molecular marker (Ludwig).

PCR products were purified with Wizard® SV Gel and PCR Clean-Up System, according to the protocol of the fabricants. The samples were sent to ACTGene Molecular Analyses company for sequencing using Sanger method on an in-house Genetic Analyzer sequencing instrument (AB 3500, Applied BioSystems) with their Forward and Reverse primers for the five biotypes. The EPSPS gene sequences obtained for each biotype were compared with the genome of barnyardgrass using MUSCLE alignment software (Edgar, 2004).

3. Results and Discussion

3.1 Echinochloa species determination

Morphological analyses of the *Echinochloa* exsiccates confirmed that the samples were *Echinochloa crus-galli* (L.) P. Beauv., as determined by the SMDB Herbarium.

3.2 Dose-response curve

The dose-response analysis showed significant results for both biotypes, BR20Esp016 (R) and BR19Esp001 (S), indicating an increase of control and in the reduction on the shoot dry weight with the increase of herbicide doses.

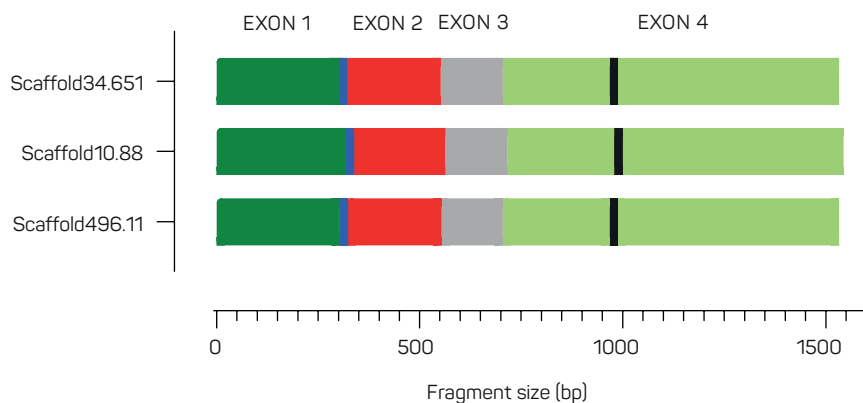


Figure 1 - Location model used to amplify the EPSPS gene in barnyardgrass, with forward (blue) and reverse (black) oligonucleotides design based on GenBank: GCA_900205405.1 for Scaffold34.651 (1536bp) Scaffold10.88 (1551 bp), Scaffold34.651 (1553bp)

These variables were fitted to a three-parameter log-logistic model, which provided the best fit for the dose-response data, as confirmed by formal significance testing. The p-values for all variables across different assays ranged from 0.06 to 0.99, indicating a good fit of the model to the data (Table 1).

For control at 21 DAT of the biotype BR20Esp016 in both generations (F1 and F2), RF values were greater than 1.00. However, the effective dose to control 50% of the population was lower than the recommended dose of 1,440 g ae ha⁻¹, with an ED₅₀ of 306 and 863 g ae ha⁻¹, respectively. These values generated RFs of 2.2 and 7.3. However, the effective doses to control 90% of the population were 2,100 and 4,439 g ae ha⁻¹, doses higher than the recommended commercial dose, values that generated RFs of 2.5 and 15.3, respectively, compared to the susceptible biotype (Figure 2).

For shoot dry weight reduction, the results mirrored the control outcomes, with RF > 1.00 for the resistant (R) biotype in both F1 and F2 generations. For R biotype, the GR₅₀ values were 335 and 323 g ae ha⁻¹ for F1 and F2, respectively, resulting in RFs of 2.4 and 2.6 compared to the S biotype, which had GR₅₀ values of 140 and 111 g a.e. ha⁻¹. The GR₉₀ value exceeded the recommended dose only for R in the F2 generation, with 4,685 g a.e. ha⁻¹ and an RF of 13.9, while for F1, it was 995 g a.e. ha⁻¹, with an RF of 2.9, compared to the S values of 412 and 336 g a.e. ha⁻¹ for each generation (Figure 2).

Previous studies have evaluated cases of glyphosate resistance in *Echinochloa colona*, such as the case of Gaines et al. (2012), who evaluated a glyphosate-resistant population from Austria and obtained RF values of 8 and

6 for control and shoot dry weight reduction, respectively. Similarly, Nandula et al. (2018) examined populations from Mississippi and Tennessee in the United States, reporting RF values of 4 and 7. Alarcón-Reverte et al. (2014) studied biotypes from California, finding an RF value of 6.6 for ED₅₀. Studies in barnyardgrass evaluated 13 different populations of the Iberian Peninsula, finding RF values between 1.3 and 21.7 (Vázquez-García et al., 2021).

The RF values obtained in our study for ED₅₀ and GR₅₀ lower than those previous studies, except for the RF value for ED₅₀ of F2 generation, which is similar. Nevertheless, all calculated variables in the dose-response curves indicate that the BR20Esp016 biotype is resistant to glyphosate compared to BR19Esp001.

Evaluating the heritability of resistance observed in the biotypes across two generations, it was found that the RFs of the second generation were consistently higher than those of the first, with no significant differences between them. This confirms that the observed resistance is heritable and tends to increase over generations. The increase was particularly noticeable in the ED₅₀ values, which was also evident in the ED₉₀ and GR₉₀ values. This is attributed to the high selection pressure applied to the population, as resistant plants were self-pollinated while growing in isolation. This self-pollination accelerates the spread of resistance and may lead to greater stabilization within the population by effectively reducing the flow of susceptible alleles, thus excluding genetic variability from cross-pollination (Kuester et al., 2016).

Table 1 - Estimated glyphosate doses required to control and shoot dry weight biomass reduction of 50% and 90% in two generations (F1 and F2), 21 days after treatment (DAT) and their resistance factors (RF) for the resistant biotype. Regression estimated parameters for the dose-response curve of susceptible (BR20Ecr001) and resistant (BR20Esp016) biotypes of barnyardgrass for control (ED₅₀ and ED₉₀) and shoot dry weight reduction (GR₅₀ and GR₉₀) evaluated at 21 days after treatment with glyphosate

Generations	Biotypes	Regression parameters		Control (ED ₅₀ ¹)			Control (ED ₉₀ ²)		
		b	p-value ³	g ae ha ⁻¹	95% IC	RF ⁴	g ae ha ⁻¹	95% IC ⁴	RF
F1	BR19Esp001	-2.81	0.06	139	89-242	-	471	331-613	-
	BR20Esp016	-1.63		306	244-643	2.2	2100	1172-3030	4.4
F2	BR19Esp001	-2.44	0.65	118	99-137	-	291	179-404	-
	BR20Esp016	-0.78		863	415-1312	7.3	4439	1942-6936	15.3
		Regression parameters		Shoot dry weight reduction (GR ₅₀ ¹)			Shoot dry weight reduction (GR ₉₀ ²)		
		b	p-value ³	g ae ha ⁻¹	95% IC	RF ⁴	g ae ha ⁻¹	95% IC ⁴	RF
F1	BR19Esp001	-1.94	0.99	140	77-202	-	412	148-675	-
	BR20Esp016	-1.72		335	149-520	2.4	995	516-2051	2.4
F2	BR19Esp001	-1.97	0.99	111	73-162	-	336	146-528	-
	BR20Esp016	-0.79		323	257-489	2.6	4685	2065-7740	13.9

¹ ED₅₀ or GR₅₀ glyphosate dose resulting in a 50% control or shoot dry weight reduction ²ED₉₀ or GR₉₀ glyphosate dose resulting in a 90% control or shoot dry weight reduction. ³ p-value for the lack-of-fit test for three parameters model, at the different evaluated variables ⁴ RF Resistance factor = LD₅₀/LD₅₀ or GR₉₀/GR₉₀ of BR19Esp001(susceptible)/ BR20Esp016. ⁴IC= confidence interval with 95% significance.

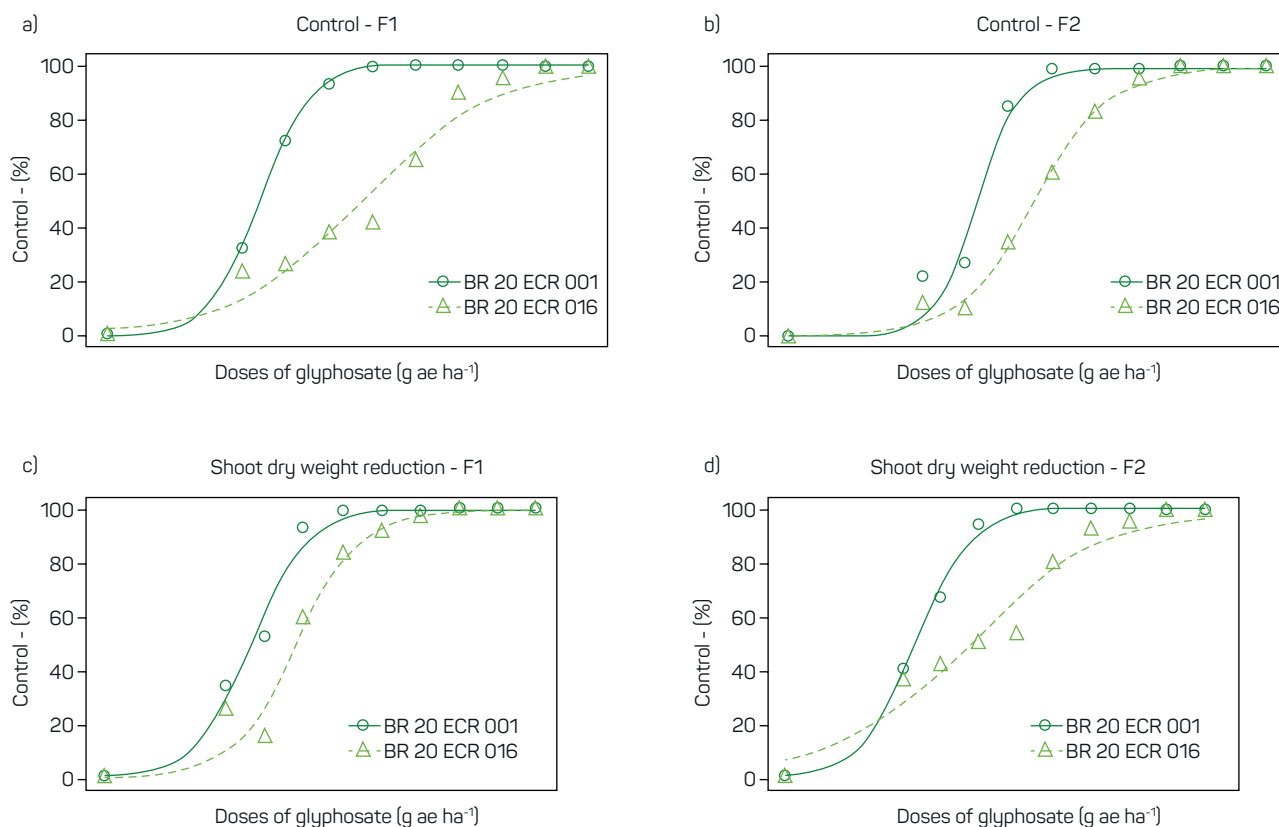


Figure 2 - Dose-response curve for weed control for F1 generation (A) and F2 generation (B) and for shoot dry weight reduction on F1 (C) and F2 (D) adjusted values of glyphosate susceptible (BR20ECR001 -o-) and resistant barnyardgrass biotype (BR20ESP016 -Δ-), at 21 days after treatment with glyphosate

3.3 Determination of resistance mechanism

This study focused on the glyphosate-susceptible (S) and resistant (R) barnyardgrass biotypes, specifically targeting the analysis of the EPSPS gene to identify TSR mutations, influenced by the dose-response results, which showed lower RFs values compared to those seen in NTSR cases. In previous studies, higher RF values (greater than 9.4) are often associated with metabolism-based resistance, where glyphosate is degraded into non-toxic metabolites (Vázquez-García et al., 2021).

Studies identifying mutations in various locations of the EPSPS gene sequence have reported a range of substitutions, including, Met104Asn, Ala103Val, Pro106 with a Ser, Thr, or Leu, Leu107Met and Ser110Ala, (Alarcón-Reverte et al. 2014, Bracamonte et al., 2019; García et al., 2019; Han et al., 2016; McElroy, Hall, 2020; Perotti et al., 2018), leading to resistance levels similar to those found in this study.

TSR analysis from our study identified an amino acid substitution in the sequenced fragments of the glyphosate-resistant biotype, BR20Esp016 (Figure 3). The nucleotide changes from CCA to GCA resulted in a codon substitution from proline to alanine at position 106 of the protein (Pro-106-Ala). In contrast, the

codon of the susceptible biotype continued to encode proline as expected.

No other substitutions were observed in the sequenced EPSPS fragment. However, the possibility of mutations in positions not covered by the sequence cannot be ruled out. Nonetheless, it is hypothesized that this is unlikely due to the low resistance factor values, as studies have shown that double mutations in the EPSPS gene result in biotypes with high resistance to glyphosate (Sammons, Gaines, 2014).

These findings are consistent with studies on *E. colona*, which reported single target-site mutations in the EPSPS gene at position 106, substituted with tryptophan (Pro-106-Thr), serine (Pro-106-Ser), or leucine (Pro-106-Leu) (Alarcón-Reverte et al., 2014; Han et al., 2016; McElroy, Hall, 2020). Substitutions at Pro106 cause a slight narrowing of the glyphosate/PEP binding site cavity, conferring low-level glyphosate resistance while preserving EPSPS functionality (Chen et al., 2015).

It should be noted that when reviewing the chromatogram at position 106 (Figure 3b), two peaks are evident at the beginning of this codon, indicating the possibility that the nucleotide in this position is a C or G, in the forward

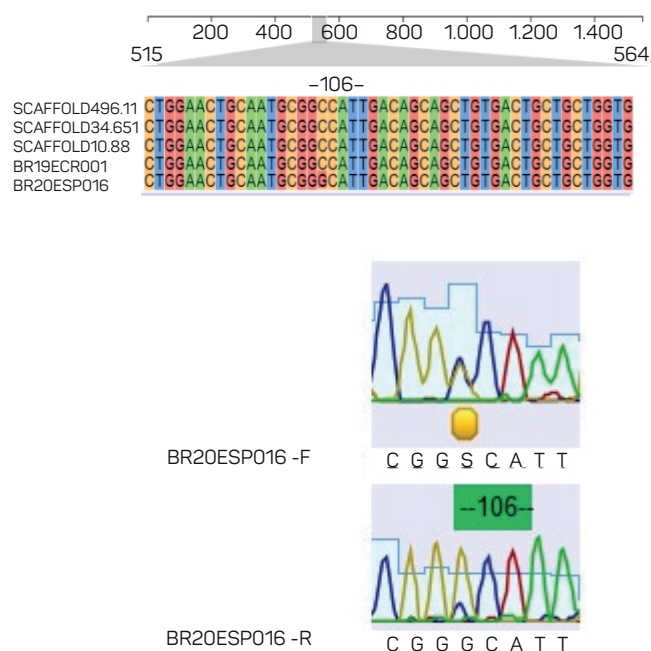


Figure 3 - Substitution analysis of the EPSPS genes at codon 106 in susceptible and resistant barnyardgrass biotypes, compared to the three available scaffold sequences (YE et al., 2020). a) Alignment of EPSPS gene fragment showing the substitution from CCA to GCA in the consensus sequence and b) Sanger sequencing chromatograms of the resistant biotype, illustrating the substitution in the forward and reverse sequences

sequence. Glyphosate-resistant polyploid *E. colona* plants showed similar results, with double nucleotide peaks in the EPSPS gene, suggesting the presence of mutations (Han et al., 2016). Barnyardgrass is an allohexaploid species that has six subgenomes with multiple homeoalleles, this genetic diversity allows for different copies of the EPSPs gene to be expressed at various levels with some copies having mutations while others do not (Carranza et al., 2023; Powles, Yu, 2014; Wu et al., 2022).

It is important to emphasize that the presence of this mutation does not rule out the possibility of other resistance mechanisms, whether TSR resistance through an increase in EPSPS gene copy number or NTSR involving changes in absorption, translocation, or metabolism. Therefore, further studies are necessary to evaluate these additional mechanisms.

Understanding the mechanisms of resistance of weeds to herbicides is essential for predicting their evolutionary trajectory and implementing integrated management practices to reduce selection pressure and prevent the emergence of new resistances. This study represents the first documentation of barnyardgrass resistance in soybean cultivation in Rio Grande do Sul and Brazil. Based on these findings, strategies for the integrated management for this problematic weed in this system can be developed.

These strategies include the use of herbicides with different mechanisms of action from glyphosate, as well as implementing other integrated management practices, such as crop rotation, mechanical control, and sustainable management techniques. Such approaches aim to reduce the population of resistant biotypes and ensure the continued effectiveness of herbicides, ultimately promoting more sustainable agricultural production.

4. Conclusions

The BR20Esp016 barnyardgrass biotype collected from soybean field in the State of Rio Grande do Sul, Brazil, is resistant to glyphosate compared with a susceptible biotype. Resistance is heritable from F1 to F2, with RF between 2.2. and 7.3 for ED₅₀, respectively, and 2.4 and 2.9 for GR₅₀. Additionally, we confirmed that the resistance mechanism in barnyardgrass is attributed to target site mutation where proline (CCA) is replaced by alanine (GCA) at position 106 in the EPSPs gene. Determining the resistance mechanism represents a significant advancement, enabling the proposal of future control strategies for crops infested with the resistant biotype.

Author's contributions

All authors read and agreed to the published version of the manuscript. CO, SM, LA, EC, and AR; conceptualization of the manuscript and development of the methodology. CO, SM, LA, and AR; data collection and curation. CO, SM, and LA; data analysis. CO, SM, SD, LA, and AR; data interpretation. EO, LL, and LA; funding acquisition and resources. CO, LL, and LA; project administration. LA: supervision. CO, SM, LA, and AR; writing the original draft of the manuscript. CO, SM, LA, EO, SD, LL, EC, and AR; writing, review, and editing.

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