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Resistance of *Amaranthus hybridus* to glyphosate: detection, mechanisms involved and alternatives for integrated management

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Abstract: Background: Glyphosate-resistant *Amaranthus hybridus* biotypes have been observed in Rio Grande do Sul (RS) crops. Their impact on soybean yield is high due to rapid growth and competitiveness. Investigating the resistance mechanism guides management strategies. **Objective:** To confirm the evolution of glyphosate-resistant *A. hybridus* biotypes in southern RS, identifying the resistance mechanism and proposing alternative management strategies. **Methods:** Dose-response curve studies, investigation of EPSPS gene mutation, and analysis of integrated management practices were conducted to characterize glyphosate resistance in *A. hybridus* biotypes. **Results:** Three *Amaranthus hybridus* biotypes from Aceguá, Bagé, and Rosário do Sul (RS) exhibit triple mutations in the EPSPS gene, providing glyphosate resistance. Resistance levels are high (>10). Alternative mechanisms of action to glyphosate such as glufosinate-ammonium or 2,4-D choline salt (Enlist technology) in post-emergence and sulfentrazone + diuron (pre-emergence) control resistant plants without compromising soybean yield. **Conclusions:** Confirming *Amaranthus hybridus* resistance to glyphosate requires alternative control measures. Combining alternative mechanisms of action, such as those available in new technologies, is important and should be the main action in a control program. Important aspects of integrated management should be prioritized combined with herbicide use.

Keywords: Soybean; Pre-Emergent; Integrated Weed Management; Glyphosate

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1. Introduction

Soybean glyphosate resistant cultivars technology is widely used in Brazil and worldwide; its use without an established integrated management program selected resistant weeds (Heap, 2024). Among these species, *Amaranthus* spp. stands out in Rio Grande do Sul (RS); the genus comprises 60 species worldwide with annual cycle, sexual reproduction capable of producing up to 600,000 seeds per plant (Penckowski et al., 2020). As As C4 plant has competitive advantage over soybean (Brunetto, 2022).

Multiple resistance to glyphosate and acetolactate synthase (ALS) in smooth pigweed (*Amaranthus hybridus* L.) was first documented in RS (Mathioni et al., 2022). *A. hybridus* is a common weed in neighboring countries such as Argentina and Uruguay, infesting soybean and maize crops, causing profit losses to farmers (Larran et al., 2017; Larran et al., 2018). Since then, there have been increasing reports of control failures in crops due of this weed. One of the main consequences of weed resistance to herbicides is the increase in control costs, a fact that is often not emphasized in scientific journals, which can lead to increases of up to \$100 ha^{-1} depending on the species (Adegas et al., 2017). By the time a farmer perceives crop failure or lack of control, the infestation has already affected at least 30% of the area (Orson, 1999). Competition models predict soybean losses of around 60% with infestations of 10 plants m^{-2} of *A. hybridus* (Cousens, 1985). A more recent study indicates a 6.4% loss in soybean yield for each *A. hybridus* plant m^{-2} in the crop (Zandoná et al., 2022).

Chemical management has been widely used to control *A. hybridus* in soybean. However, repeated applications increase the selection pressure of herbicide-resistant biotypes. Herbicide molecules do not cause resistance but select individuals already resistant in a population (Markus et al., 2021).

A triple mutation in the enzyme enolpyruvylshikimate phosphate synthase (EPSPS) gene, providing a high level of glyphosate resistance has been reported in *A. hybridus* in Argentina (Perotti et al., 2019). The substitution of amino acids at positions 102 (ACA to ATA, Thr to Ile), 103 (GCC to GTG, Ala to Val), and at position 106, the most widely recognized (CCA to TCA, Pro to Ser), makes the plant resistant to glyphosate and has also been reported in Brazil (Mathioni et al., 2022; Sulzbach et al., 2023). For *A. hybridus*, previous studies indicated resistance to glyphosate and ALS enzyme inhibitors in biotypes in Argentina (Larran et al., 2017) and Brazil (Mathioni et al., 2022; Resende et al., 2022; Sulzbach et al., 2023).

To delay the evolution of resistance, integrated management is crucial. A study indicated that *A. hybridus* germinates at a temperature range between 30 and 40 ºC (Talaee et al., 2023), which is observed at RS state. Gazola (2021) obtained a 63.4% reduction in *A. hybridus* plants m^{-2} in a no tillage system after corn. In a two-year study conducted in Bagé (RS), Lamego et al. (2022) observed that the presence of Italian ryegrass residue, significantly contributed to reducing the emergence of *A. hybridus* seedlings in soybean succession.

The use of pre-emergent herbicides has proven to be an interesting alternative for resistant *A. hybridus* control in soybean crops (Pedroso et al., 2020), allowing for their establishment in a weed-free area, thus minimizing competition. For *A. hybridus*, some herbicides have provided good control, such as combinations of [imazethapyr + flumioxazin], [sulfentrazone + diuron], or individual molecules such as flumioxazin and sulfentrazone (Brunetto, 2022). Post-emergence few options are efficient and limited its early applications which target small plants (usually up to 10 cm).

New technologies involve the use of soybean cultivars resistant to choline salt 2,4-D (Enlist), as an alternative for *A. hybridus* control in heavily infested areas or especially when complementing pre-emergent herbicides. However, there have been reports of *A. hybridus* resistant to 2,4-D, dicamba, and glyphosate in Argentina (Dellaferrera et al., 2018), reinforcing the need for integrated management to delay premature loss of the tool.

The aim of this study was to investigate glyphosate resistance in *A. hybridus* biotypes from the southern half of RS, determining the resistance mechanism and proposing effective alternatives that promote integrated weed management. The hypothesis of the work is that biotypes of *Amarathus hybridus* in RS state are evolving as resistant to glyphosate herbicide due to mutation in the enzyme's target gene and integrated management based on alternative mechanisms of action can control resistant biotypes.

2. Materials and Methods

Seeds of suspected glyphosate-resistant *A. hybridus* were collected from the municipalities of: Aceguá (R1 - 31°45'43"S 54°16'42"W), Bagé (R2 - 31°18'40"S 54°01'15"W), and Rosário do Sul (R3 - 30°17'25"S 55°03'20"W) in RS, in 2019/20. Susceptible seeds from Bagé (S1 - 31°32'27"S 54°07'16"W) and Pedras Altas (S2 – 31°55'01"S 53°52'33"W), RS, were used as controls. The R1, R2 and R3 seeds were harvested in bulk from 5 to 10 plants that survived application of glyphosate in the field. S1 and S2 seeds were harvested the same way in areas without previous herbicide use.

2.1 Resistance confirmation: dose-response curves

Seeds were germinated in pots (300 ml) filled with soil and commercial soil mixture, in the greenhouse of Embrapa Pecuária Sul, Bagé (RS). When the plants reached the 2-4 leaf stage, they were sprayed. The study was in a completely randomized design (CRD), with four replications.

The treatments were arranged in a factorial scheme, where factor A consisted of the *A. hybridus* biotypes (R1, R2, R3, S1, and S2), and factor B the herbicide doses (glyphosate): 0; 0.125; 0.25; 0.5; 1; 1.5; 2; 2.5; and 3x the recommended dose of 720 g a.e ha^{-1} each. The treatments were applied with a CO_2-pressurized backpack sprayer, with 110.015 fan type nozzles, spaced 50 cm apart, at a constant pressure of 210 KPa, adjusted to a spray volume of 140 L ha^{-1}. Shoot dry matter (SDM) was determined 28 days after application (DAA), by drying the plant material in an oven at 60 °C until reaching constant mass.

2.2 Investigation of EPSPS target site mutations

Two leaves from individual three plants of *A. hybridus* biotypes confirmed as resistant (R1, R2, and R3) and susceptible (S1) to glyphosate were collected for DNA extraction at the Embrapa Clima Temperado Molecular Biology Laboratory, Pelotas (RS). The CTAB protocol (Doyle & Doyle, 1990) was used, starting from 100 mg of leaves ground in liquid nitrogen. DNA samples were stored at -20 °C until further use.

Two primers were used to amplify partial EPSPS gene of *A. hybridus*, according to Perotti et al. (2019): Primer F: (5'- ATGTTGGACGCTCTCAGAACTCTTGGT-3') and Primer R: (5'- TGAATTTCCTCCAGCAACGGCAA-3'). PCRs were performed with High-Fidelity DNA polymerase (Thermo Fisher, USA), and reactions were prepared as follows: 1 µL of 50 ng DNA, 0.4 µL of 10mM dNTPs, 1.2 µL of 2.5 nM MgCl2, 1 µL of 100 nM primers (each), 0.4 µL of 5U Taq polymerase (Platinum High Fidelity), 4 µL of 1X buffer, and milliQ H_2O to 20 µL. The amplification program consisted of activation at 98 °C for 30 s, 35 cycles at 98 °C for 10 s, 57 °C for 10 s (anneling temperature), 72 °C for 1 min 10 s, 72 °C for 5 min, and a 4 °C hold. PCR products were subjected to 2% agarose gel electrophoresis, with 3 µL of PCR product and 4 µL containing gelred + bromophenol blue. The expected fragment was 195bp for EPSPs in *A. hybridus* as described by Perotti et al. (2019). Amplification products were sequenced using the Sanger method. Sequencing reads were aligned with a reference EPSPS from *A. hybridus* (GenBank, MH482844.1 and MH482843.1), as described by Perotti et al. (2019). Alignment was performed using BioEdit v1.11.2 software. Nucleotide sequences were translated and subsequently aligned to search for amino acid substitutions.

2.3 Management Alternatives

A field experiment was conducted in 2022/23 at Embrapa Pecuária Sul, in a randomized complete block design (RCBD), with four replications. During the winter season, the area was seeded with Italian ryegrass. Nineteen days before soybean sowing 1,080 g ae ha^{-1} of glyphosate + [168 + 345.60 g ai ha^{-1}] of clethodim + fluroxypir-methyl + 0.5% v/v mineral oil were applied, in 200 L ha^{-1} of water solution. Soybeans were sown in 2x6m experimental units, on November 23, 2022. The following cultivars were used: BMX Torque I2X (5.7), BMX Vênus CE3 (5.7), and CZ15B70 IPRO/RR (5.7), tolerant, respectively, to glyphosate and dicamba; glyphosate, 2,4-D choline, and glufosinate-ammonium; and only to glyphosate. The treatments were applied using a CO_2-pressurized backpack sprayer, at 2.3 bar pressure and a solution volume of 100 L ha^{-1} (Table 1).

A. hybridus counts were conducted at 30 DAA A and B across the entire plot (12 m^2). To minimize the drought in 2022/23 season, the experimental area was irrigated four times with 25 mm.

Table 1. Treatments applied for *Amaranthus hybridus* control. Embrapa Pecuária Sul, Bagé (RS), 2022/23.

Cultivar	Application A – sowing day (g ae/ai ha^{-1})	Application B (V4-V5**)
I2X[1]	glyphosate (960) + dicamba (480)* + glufosinate-ammonium (400)	glyphosate (720)
I2X	glyphosate (960) + dicamba (480)* + (sulfentrazone + diuron) (175+350) + glufosinate- ammonium (400)	glyphosate (720)
CE	glufosinate-ammonium (400)	(2,4-D*** + glyphosate) (585+615)
CE	glufosinate-ammonium (400)	glyphosate + glufosinate-ammonium (720+400)
CE	glufosinate-ammonium (400)	(2,4-D*** + glyphosate) (585 + 615) + glufos-inate-ammonium (400)
CE	(sulfentrazone + diuron) (175+350) + glufosinate-ammonium (400)	(2,4-D** + glyphosate) (585+615)
CE	(sulfentrazone + diuron) (175+350) + glufosinate-ammonium (400)	glyphosate + glufosinate-ammonium (720+400)
CE	(sulfentrazone + diuron) (175+350) + glufosinate-ammonium (400)	(2,4-D*** + glyphosate) (585+615) + glufos-inate-ammonium (400)
IPRO	glufosinate-ammonium (400)	glyphosate + fomesafen (720+250)
IPRO	(sulfentrazone + diuron) (175+350) + glufosinate-ammonium (400)	glyphosate + fomesafen (720+250)

[1] I2X = BMX Torque I2X (5.7); CE = BMX Vênus CE3 (5.7); IPRO = CZ15B70 IPRO/RR (5.7).
* Addition of Xtend Protect (1L).
** Soybean growth stages: V4 pl ants are 20 to 27 cm tall with four fully developed trifoliate leaf nodes whereas at V5, plants are 25 to 30 cm tall with five fully developed trifoliate leaf nodes.
***Choline salt.

A second run of the field experiment was conducted in 2023/24 at Embrapa Pecuária Sul, in a RCBD, with four replications. The conditions previously to soybean seeding were the same on 2022/23. Soybean cultivars were sown in 2x6m experimental units, on December 12, 2023. At this time, only cultivars BMX Torque I2X (5.7) and BMX Vênus CE3 (5.7) were used. Glufosinate-ammonium spray was adding as “Application C” (Table 2). The treatments were applied using a CO_2-pressurized backpack sprayer, at 2.3 bar pressure and a solution volume of 100 L ha^{-1} (Table 2).

A. hybridus counts were conducted at 30 DAA of A, B and C applications across the entire plot (12 m^2).

2.4 Statistical Analysis

Data from the dose response study were analyzed for homoscedasticity and submitted to analysis of variance (ANOVA). When statistically significant ($p<0.05$), data were adjusted to the log-logistic nonlinear regression model using SigmaPlot 12.0 software (Sigmaplot, 2012), and GR_{50} values were calculated from the parameters of the equation (Seefeldt et al., 1995), which relates plant response (shoot dry mass) to the herbicide dose. Values were adjusted to the logistic-type sigmoid regression equation: $y = a / [1 + (x / x0)^b]$, where: y = control percentage; x = herbicide dose; and a, x0 and b equation parameters, where a is the difference between the maximum and minimum points on the curve, x0, the dose providing 50% of the variable response, and b the curve gradient. The resistance factor (RF) was calculated by the ratio between GR_{50} of the resistant and susceptible accessions.

Data from the field studies were analyzed for homoscedasticity and submitted to ANOVA. When needed, data transformation was done. If significance was found ($p<0.05$), Duncan’s mean test was conducted using RStudio software (R Core Team, 2023).

3. Results and Discussion

3.1 Dose-Response Curve

The resistance of the three *A. hybridus* biotypes to the glyphosate herbicide (R1, R2, and R3) was confirmed (Figure 1). In a comparison with two susceptible biotypes (S1 and S2), biotype R1 demonstrated highest levels of resistance (Figure 1). For R1, RF was equivalent to 12.69x when compared to S1 and 30.29x when compared to S2 biotypes. For biotype R2, RF were lower, although still confirming glyphosate resistance (Figure 1, Table 3). However, biotype R3 behaved differently from the others; the values obtained were not adjustable to a dose-response curve but showed a hormetic behavior (data not shown).

3.2 Mechanism of Resistance

The partial sequences of the EPSPS gene from R1 and R2 biotypes confirmed as glyphosate resistance (dose-response studies) were aligned. Even not statistically

Table 2. Treatments applied for *Amaranthus hybridus* control. Embrapa Pecuária Sul, Bagé (RS), 2023/24.

Cultivar	Application A – sowing day (g ae/ai ha⁻¹)	Application B (V4-V5**) (g ae/ai ha⁻¹)	Application C (8 DAA B) (g ea/ia ha⁻¹)
I2X	glyphosate (960) + dicamba (480)* + glufosinate-ammonium (400)	glyphosate (720)	-
I2X	glyphosate (960) + dicamba (480)* + (sulfentrazone + diuron) (175+350) + glufosinate-ammonium (400)	glyphosate (720)	-
CE	glufosinate-ammonium (400)	(2,4-D*** + glyphosate) (585+615)	-
CE	glufosinate-ammonium (400)	glyphosate + glufosinate-ammonium (720+400)	-
CE	glufosinate-ammonium (400)	(2,4-D*** + glyphosate) (585 + 615)	glufosinate-ammonium (400)
CE	(sulfentrazone + diuron) (175+350) + glufosinate-ammonium (400)	(2,4-D*** + glyphosate) (585+615)	-
CE	(sulfentrazone + diuron) (175+350) + glufosinate-ammonium (400)	glyphosate + glufosinate-ammonium (720+400)	-
CE	(sulfentrazone + diuron) (175+350) + glufosinate-ammonium (400)	(2,4-D*** + glyphosate) (585+615)	glufosinate-ammonium (400)
CE	glufosinate-ammonium (400)	glyphosate + fomesafen (720+250)	-
CE	(sulfentrazone + diuron) (175+350) + glufosinate-ammonium (400)	glyphosate + fomesafen (720+250)	-

[1]I2X = BMX Torque I2X (5.7); CE = BMX Vênus CE3 (5.7);
* Addition of Xtend Protect (1L).
** Soybean growth stages: V4 plants are 20 to 27 cm tall with four fully developed trifoliate leaf nodes whereas at V5, plants are 25 to 30 cm tall with five fully developed trifoliate leaf nodes.
***Choline salt.

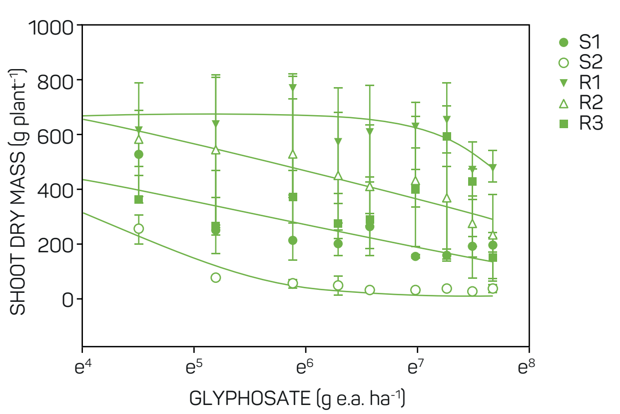

Figure 1 - Dose-response curve analysis in biotypes of *A. hybridus* from the state of Rio Grande do Sul, Brazil: S1-Bagé, S2-Pedras Altas, R1-Aceguá, R2-Bagé, R3-Rosário do Sul. Embrapa Pecuária Sul, Bagé (RS), 2019/20

Table 3 - Statistical parameters of glyphosate dose-response curve analysis in *Amaranthus hybridus* biotypes. Embrapa Pecuária Sul, Bagé (RS), 2019/20

Parameters[1]		R^2	GR50	FR	
A	b		(g ae ha⁻¹)	R/S1	R/S2
S1 Biotype					
636.72	0.62	0.69	221.63	-	-
S2 Biotype					
448.15	1.57	0.94	92.81	-	-
R1 Biotype					
769.38	1.55	0.75	2811.94	12.69	30.29
R2 Biotype					
685.62	1.21	0.70	1548.29	6.98	16.68

Log-logistic equation: $y=a/[1+(x/x0)^b]$

confirmed in the dose-response trials, we opted to keep analyzing the R3 biotype on further analyses. The biotype S1 was aligned and compared to the sequences deposited in GenBank (MH82843.1 and MH 482844.1, respectively for resistant and susceptible *A. hybridus*, described by Perotti et al. (2019).

The three populations R1, R2, and R3 (Figure 2) evaluated in this study exhibit triple mutation (TAP-IVS) in the EPSPS gene, first reported in Argentina (Perotti et al., 2019), but already confirmed in biotypes from RS and Paraná (PR) states (Mathioni et al., 2022; Sulzbach et al., 2023). The high resistance levels found in three populations from Ponta Grossa (PR) (RF between 13 and 15) also suggest that the same triple mutation may be occurring (Resende et al., 2022).

The most found mutation in EPSPS (P106S) was first observed in *Eleusine indica* and later in other weed species; it provides a low resistance level (Baerson et al., 2002). Subsequently, double mutations known as TIPS (T102I + P106S) were reported to have a high resistance level (Han et al., 2017). Finally, the TVA-IVS triple mutation

(T102I, A103V, and P106S) found in this work also exhibits high resistance (Perotti et al., 2019), and although rare, has been observed in Brazilian populations of *A. hybridus* (Mathioni et al., 2022; Sulzbach et al., 2023). Thus, according to our results, we believe the TVA-IVS triple mutation is the principal mechanism of glyphosate resistance in the *A. hybridus* biotypes evaluated. However, we cannot highlight the influence of other mechanisms since we did not investigate them.

Of the three resistant populations assessed, R3 did not show multiple resistance to ALS inhibitors, being controlled by chlorimuron (20 g ha^{-1}) and imazethapyr (106 g ha^{-1}) in post-emergence (Lamego et al., 2021). However, the same test showed that biotypes R1 and R2, respectively, are not controlled by the same herbicides. In this study, primers from the literature were used (Larran et al., 2017), partial sequences of the ALS gene were amplified in the three resistant (R1, R2, and R3) and susceptible (S1) biotypes. The results were inconclusive, and further analysis is ongoing.

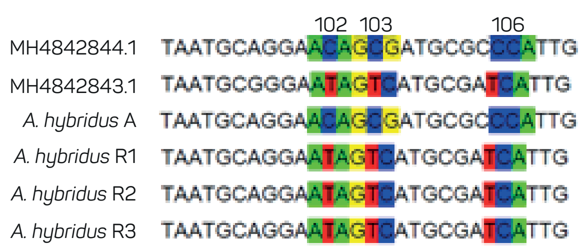

Figure 2 - Alignment of partial EPSPS sequences from *Amaranthus hybridus* biotypes. R1 = Aceguá, R2 = Bagé, R3 = Rosário do Sul, and S = Bagé. Reference sequences: MH48244.1 as susceptible and MH482843.1 as resistant (Genbank, Perotti et al., 2019)

3.3 Management Alternatives

Field experiments will be presented separated once there is an additional treatment in 2023/24. It is important to highlight that drought was reported in 2022/23, which was not in 2023/24 (Figure 3). To minimize the drought stress, the experimental area was irrigated four times (in a volume of 25 mm each time) in 2022/23.

The most effective *A. hybridus* control in 2022/23 experiment, was observed in treatments with post-emergence application of choline salt of 2,4-D, preceded by sulfentrazone + diuron in pre-emergence (Table 4). *A. hybridus* control was ineffective in treatments with dicamba, without 2,4-D application, and/or alternative options to glyphosate in post-emergence. *A. hybridus* stands out for its emergence throughout the soybean cycle, making the use of pre-emergence applied herbicides (PRE) and their residual effect essential, complemented by post-emergent control. Brunetto (2022) achieved good *A. hybridus* control indices with PRE sulfentrazone + diuron application. Additionally, 2,4-D showed control above 80%.

PRE sulfentrazone + diuron combined with glufosinate-ammonium, and post-emergent application of 2,4-D choline salt resulted in the highest grain yield (3,457 kg ha^{-1}) (data not shown). Nevertheless, this result reflects the best treatments observed for *A. hybridus* control (Table 4). Glufosinate-ammonium sprayed with soybean sowing and glyphosate + fomesafen in post-emergence, did not control *A. hybridus* efficiently, resulting in the lowest soybean yield (1,989 kg ha^{-1}) (data not shown).

In 2023/24, glufosinate-ammonium spray was added (application C - 8 DAA application B), after some treatments (Table 5). The most effective *A. hybridus* control were observed with post-emergence application of choline salt of 2,4-D or preceded by sulfentrazone + diuron in pre-emergence. The addition of glufosinate-ammonium

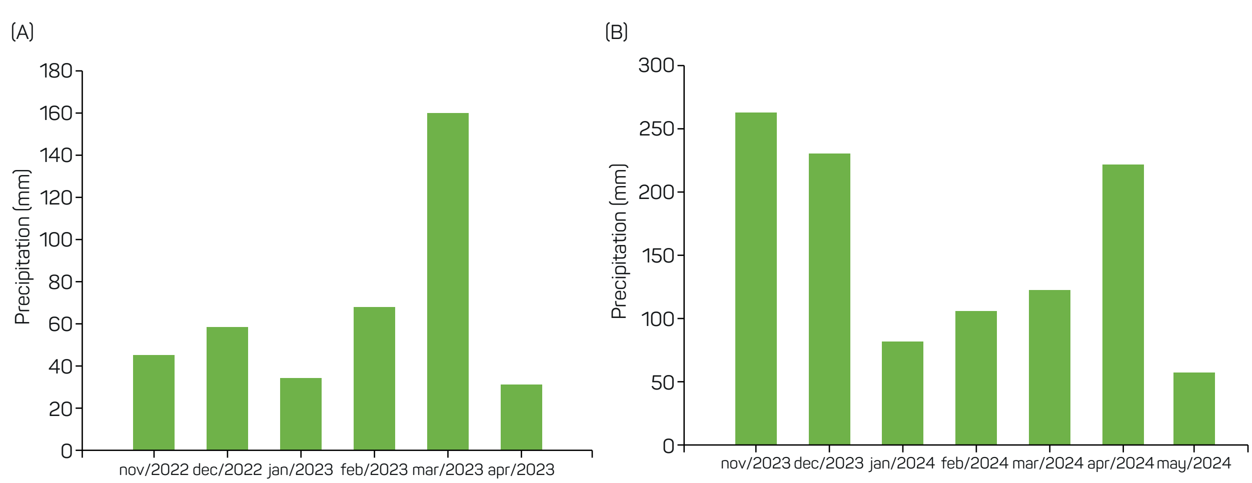

Figure 3 - Precipitation (mm) during the soybean cycle, recorded: A) from soybean seeding (November, 2022) until harvest (April, 2023); B) from soybean seeding (December, 2023) until harvest (May, 2024), according to the Brazilian National Meteorology Institute (INMET)

Table 4 - *Amaranthus hybridus* per square meter at 30 days after application (DAA) of treatments A and B, and at soybean pre-harvest. CPPSul, Bagé/RS, 2022/23.

Cultivar	Application A – sowing day (g ae/ai ha^{-1})	Application B (V4-V5**)	A. hybridus m^{-2} 30 DAA - Application A	A. hybridus m^{-2} 30 DAA - Application B	A. hybridus m^{-2} pre-harvest
I2X[1]	glyphosate + dicamba + glufosinate-ammonium	glyphosate	26.44 ab	23.38 ab	19.00 ab
I2X	glyphosate + dicamba + (sulfentrazone + diuron) + glufosinate- ammonium	glyphosate	14.29 b	20.10 ab	21.16 ab
CE	glufosinate-ammonium	(2,4-D*** + glyphosate)	55.29 a	4.81 b	4.00 cd
CE	glufosinate-ammonium	glyphosate + glufos-inate-ammonium	59.60 a	41.71 a	23.00 ab
CE	glufosinate-ammonium	(2,4-D*** + glyphosate) + glufosinate-ammonium	72.10 a	5.12 b	6.02 cd
CE	(sulfentrazone + diuron) + glufosinate-ammonium	(2,4-D*** + glyphosate)	11.83 b	0.40 b	1.29 d
CE	(sulfentrazone + diuron) + glufosinate-ammonium	glyphosate + glufos-inate-ammonium	17.60 b	11.87 b	5.38 cd
CE	(sulfentrazone + diuron) + glufosinate-ammonium	(2,4-D*** + glyphosate) + glufosinate-ammonium	9.25 b	0.35 b	2.02 cd
IPRO	glufosinate-ammonium	glyphosate + fomesafen	29.23 ab	29.63 ab	40.00 a
IPRO	(sulfentrazone + diuron) + glufosinate-ammonium	glyphosate + fomesafen	6.75 b	4.63 b	12.00 bc
CV. (%)	-	-	35.25[3]	40.70[3]	38.90[3]

[1] I2X = cv. BMX Torque I2X; CE = cv. Vênus CE3; IPRO = cv. CZ15B70 IPRO/RR.
[2] The same letters do not differ according to Duncan's test, at 5% probability.
[3] The data was transformed using $\sqrt{X+1}$.
[Ns] Not significant.
* Addition of Xtend Protect (1L).
** Soybean growth stages: V4 plants are 20 to 27 cm tall with four fully developed trifoliate leaf nodes whereas at V5, plants are 25 to 30 cm tall with five fully developed trifoliate leaf nodes.
***Choline salt.

sprayed 8 DAA of application B may help with control but it is not mandatory if spraying 2,4-D or PRE sulfentranzone + diuron previoulsy. Glufosinate-ammonium is efficient in *A. hybridus* control and applied on soybean seeding and later (V4-V5 soybean growth stages mixed with glyphosate) was also very efficient, not being required another spray (application C).

The technology involving the post-emergent soybean herbicide Enlist® containing 2,4-D (choline salt) is an important tool in mitigating high *A. hybridus* infestation, ensuring a harvest without adverse effects on yield and reinfestation. Pre-emergent herbicides have been an important management tool for resistant *A. hybridus*, since they reduce the number of plants that will need post-emergent management. However, using glufosinate-ammonim at seeding time and mixed with glyphosate at V4-V5 soybean growth stage is also important to *A. hybridus* resistance control.

3.4 Perspectives

The resistance of *A. hybridus* to glyphosate is widespread in the South of RS and rapidly advancing throughout the state. In addition to weed characteristics such as high prolificacy, which favors seed dissemination, and gene flow (Tranel et al., 2002; Sulzbach et al., 2023), the failure to clean agricultural machinery, especially harvesters, may be one of the important factors contributing to exacerbating the problem. The situation observed in Argentina, where the sharing of machinery possibly spread R biotypes to other regions, strengthens this hypothesis (Perotti et al., 2019). The recent case of *Amaranthus palmeri*, a quarantine pest, in cotton machines in Brazil imported from the USA (MAPA, 2021), also reinforces this alarming scenario. Producers should consider this factor a priority, since the cost of prevention is always lower than the solution to resistance (Adegas et al., 2017).

The triple mutation found in glyphosate-resistant biotypes reinforces the fact that the change of herbicide mechanisms of action is essential; RR soybean technology has resulted in significant improvements but has also caused a certain "complacency" in weed management. Crop rotation in areas heavily infested with *A. hybridus* is a feasible option; the cultivation of maize, sorghum, or summer forages such as Sudan grass, common in the southern half of RS, allows the use of alternative molecules such as atrazine. However, it is important to emphasize the need for planning because there are already reports of *A. hybridus* evolution resistant to atrazine (Maertens et al., 2004).

Table 5 - *A. hybridus* per square meter at 30 days after application (DAA) of treatments A and B. CPPSul, Bagé/RS, 2023/24.

Cultivar	Application A – sowing day	Application B (V4-V5**)	Application C (8 DAA B)	A. hybridus 30 DAA - Application A	A. hybridus 30 DAA - Application B	A. hybridus m^{-2} 30 DAA - Application C
I2X[1]	glyphosate (960) + dicamba (480)* + glufosinate-ammonium (400)	glyphosate (720)	-	14.55 a	10.78 a	6.67 a
I2X	glyphosate (960) + dicamba (480)* + (sulfentrazone + diuron) (175+350) + glufosinate-ammonium (400)	glyphosate (720)	-	0.63 c	2.08 c	1.96 bc
CE	glufosinate-ammonium (400)	(2,4-D*** + glyphosate) (585+615)	-	6.51 b	0.88 cd	0.48 d
CE	glufosinate-ammonium (400)	glyphosate + glufosinate-ammonium (720+400)	-	7.52 b	1.23 cd	0.42 d
CE	glufosinate-ammonium (400)	(2,4-D*** + glyphosate) (585 + 615)	glufosinate-ammonium (400)	11.40 ab	0.83 cd	0.90 cd
CE	(sulfentrazone + diuron) (175+350) + glufosinate-ammonium (400)	(2,4-D*** + glyphosate) (585+615)	-	1.29 c	0.25 d	0.13 d
CE	(sulfentrazone + diuron) (175+350) + glufosinate-ammonium (400)	glyphosate + glufosinate-ammonium (720+400)	-	1.92 c	0.40 d	0.27 d
CE	(sulfentrazone + diuron) (175+350) + glufosinate-ammonium (400)	(2,4-D*** + glyphosate) (585+615)	glufosinate-ammonium (400)	0.77 c	0.00 d	0.13 d
CE	glufosinate-ammonium (400)	glyphosate + fomesafen (720+250)	-	10.40 ab	5.02 b	2.63 b
CE	(sulfentrazone + diuron) (175+350) + glufosinate-ammonium (400)	glyphosate + fomesafen (720+250)	-	0.69 c	0.27 d	0.23 d
CV. (%)				26.87[3]	19.33[3]	54.38[4]

[1] I2X = cv. BMX Torque I2X; CE = cv. Vênus CE3.
[2] The same letters do not differ according to Duncan's test, at 5% probability.
[3] The data was transformed using √X+1.
[4] The data was transformed using Log (X+1).
* Addition of Xtend Protect (1L).
** Soybean growth stages: V4 plants are 20 to 27 cm tall with four fully developed trifoliate leaf nodes whereas at V5, plants are 25 to 30 cm tall with five fully developed trifoliate leaf nodes.
***Choline salt.

Integrated systems with crop cultivation in summer and livestock farming in winter are part of the agricultural scenario of the southern half of RS. Livestock grazing residue can be combined with *A. hybridus* management and the use of PRE, provided there is remaining plant matter (residue). Field technician reports demonstrate that when the livestock area is handed over to the "plower" in the leasing system, there is scarcely any physical barrier to contribute to integrated management (lack of residue). Previous studies confirm how crop residue can contribute within the logic of integrated management (Lamego et al., 2022).

Another point to consider is that cattle do not prefer *A. hybridus* seeds. However, the provision of hay contaminated with resistant *A. hybridus* seeds is indeed a problem and an opportunity to favor their dispersal via zoochory. Viero et al. (2018) demonstrated the potential for the dispersal of herbicide-resistant barnyardgrass (*Echinochloa crus-galli)* and red rice seeds via endozoochory. Additionally, Schaedler et al. (2021) found the potential for endozoochoric dispersal of ryegrass resistant to herbicide. A study coordinated by the same research group noted that *A. hybridus* seeds remain viable after passing through the digestive tract of cattle and birds (unpublished data).

Superior *A. hybridus* control was ensured when herbicide use was combined with straw residue from cover crops with higher shoot dry mass production (Italian ryegrass and rye) (Unpublished data). This emphasizes that cover crops, commonly cultivated at RS among soybean seasons, can contribute to *A. hybridus* suppression. This was confirmed by a study conducted with different cover crops (rye, oats, turnip, and Italian ryegrass) aimed at controlling species of the genus *Amaranthus* spp. in the USA, combined with herbicide use, showed potential for rye in an integrated herbicide management program (Loux et al., 2017).

The southern half of RS has expanded its soybean cultivation area driven by the commodity price. However, it is also a traditional area for irrigated rice. Albeit slowly, there is an entry or adaptation of *A. hybridus* plants to the wetter environments of lowland rice-growing areas. It is important to be aware of this adaptation, which can be rapid, since hybridization may occur between species of the genus *Amaranthus*, increasing genetic variability (Tranel et al., 2002) and favoring their adaptation to new environments.

4. Conclusions

A. hybridus biotypes from southern RS exhibit triple mutations in the EPSPS gene as a mechanism of resistance to glyphosate. Alternative mechanisms of action such as 2,4-D choline salt in post-emergence in technology that allows its use, combined with pre-emergent application and cover crop residue in winter improve the management and reduce the exclusive dependence on chemical control that could lead to the evolution of new resistance cases. Other resistance mechanisms were not investigated in the present study and need further investigation.

Author's contributions

All authors read and agreed to the published version of the manuscript. FPL and MOB: conceptualization of the manuscript and development of the methodology. YMSM, JRN, COL and RCP: data collection and curation. YMSM, COL and FPL: data analysis. FPL, YMSM and COL: data interpretation. FPL and MOB: funding acquisition and resources. FPL: project administration. FPL: supervision. FPL, YMSM and COL: writing the original draft of the manuscript. FPL, JRN, YMSM and COL: writing, review and editing.

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