

Dose-response and EPSPS gene sequencing reveal differential glyphosate susceptibility in *Eragrostis plana*

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Abstract: Background: *Eragrostis plana* Nees is the main invasive plant species in the grasslands of the Pampa Biome, southern Brazil. Crop-livestock integration has been used as a management strategy through the cultivation of soybean tolerant to glyphosate herbicide. However, in recent years, farmers have reported failures in the control of *E. plana*.

Objective: To evaluate the response of two *E. plana* biotypes to glyphosate (S - susceptible and R - resistant plants with suspected failure of control by the herbicide), as well as to investigate the mechanism involved in the differential response to the herbicide.

Methods: Dose-response assays were performed to estimate ED₅₀ and GR₅₀, based on seeds and clonal plants. The partial sequencing of the EPSPS gene was analysed. The gene expression of EPSPS, AKR, ABC-MRP8

Keywords: Herbicide; Invasive species; Gene expression; Mutation

and ABC-MRP10 were investigated, when the biotypes grown at 20/15°C or 35/30°C day/night temperature conditions.

Results: The ED₅₀ values of R biotype were 6634.18 and 4479.79 g ae. ha⁻¹ glyphosate in 2024 and 2025, respectively compared with S biotype (591.20 and 589.08 g ae. ha⁻¹). The GR₅₀ values were 103.71 and 1118.08 g ae. ha⁻¹ glyphosate for S and R, respectively. Dose-response assays using seed-derived plants (not clonal plants) showed considerable genetic variability, reflecting the natural condition in the field. Partial EPSPS gene sequencing revealed a heterozygous mutation at the Pro₁₀₆Leu position.

Conclusions: The results contribute to the understanding of glyphosate performance variability in *E. plana* populations, confirming the evolution for glyphosate resistance, and underscore the necessity of adopting integrated weed management strategies.

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1. Introduction

Eragrostis plana Nees is a perennial C4 grass species, predominantly allogamous and originally from South Africa (Medeiros, Focht, 2007). Introduced to southern Brazil through contaminated forage seed lots (Medeiros, Focht, 2007), the weed was initially considered a promising forage, and its seeds were commercially marketed. As many other species within the *Eragrostis* genus, *E. plana* maintains high productivity under arid conditions and intensive grazing, being associated with potential forage use. However, subsequent studies revealed its low nutritive value, prompting the prohibition of seed commercialization in the late 1970s (Medeiros, Focht, 2007).

It is estimated that *E. plana* occupy >1,000,000 hectares in the Pampa Biome, a region encompassing southern Brazil, as well as parts of Uruguay and northern Argentina (Boldrini, 2009). This region, characterized by its extensive livestock systems, harbors one of the world's most significant temperate and subtropical natural grasslands, noted for its biodiversity and high number of endemic species. The invasion of *E. plana* poses increasing threats to the biome biodiversity, in addition to causing significant economic losses for livestock (Boldrini, 2009). The performance of beef heifers in natural grasslands began to decline when *E. plana* tussock cover (upper stratum) reached between 34% and 44%, a level corresponding to weed dominance (Bremm et al., 2012).

The management of *E. plana* is based on two main strategies: (1) the restoration of invaded natural grasslands through an integrated method known as Integrated Method for Pasture Restoration - MIRAPASTO (Perez, 2015; Faleiro et al., 2021; Merotto et al., 2022), and (2) the adoption of crop-livestock integration, involving summer crops followed by winter pastures. The MIRAPASTO controls *E. plana* associating glyphosate use, soil fertility correction, the introduction of forage species through no-tillage seeding, and appropriate grazing management (Perez, 2015). Crop-livestock integration uses chemical desiccation of the grazed area (glyphosate) prior to the establishment of summer crops which provides effective control of *E. plana*. An analysis of the soil seed bank in area under crop-livestock integration over several years demonstrated the benefits of using soybean during summer combined with herbicide application (Lamego et al., 2020). It was effective in

preventing the replenishment of the soil seed bank by *E. plana* seeds.

Studies have evaluated chemical options for *E. plana* control (Goulart et al., 2009; Faleiro et al., 2021; Merotto et al., 2022). Glyphosate was applied at 1.080 g ae ha⁻¹, either alone or in combination with other herbicides (e.g., cyhalofop, imazethapyr), showing control >90% in plants of *E. plana* with four tillers (Faleiro et al., 2021). Applying MIRAPASTO method, glyphosate was used at 1,440 g ae ha⁻¹, especially during the summer, being effective for controlling *E. plana* tussocks, without causing injury to the native grasses (Merotto et al., 2022). Although different molecules have been evaluated, none has been more effective than glyphosate for controlling *E. plana* tussocks (Bastiani et al., 2021).

Recently, farmers have reported *E. plana* individuals escaping control in areas desiccated prior to soybean planting within crop-livestock integration system. When plant populations previously controlled by herbicides no longer respond to control, this may indicate a shift within the population driven by the selection pressure from repeated applications (Jasieniuk et al., 1996). The evolution of resistance to herbicides is an example of rapid, human-induced adaptation and is a major challenge for applied evolutionary biologists (Comont et al., 2019).

In this study, two *E. plana* biotypes were investigated: a resistant biotype following glyphosate applications, and a biotype from an area within the same field with no history of herbicide use, in southern Brazil. The hypothesis of this study is that *E. plana* biotypes exhibit differential responses to glyphosate, suggesting that the continuous application of this herbicide may be selecting individuals with reduced sensitivity in the population. Therefore, the objectives were to evaluate the response of two *E. plana* biotypes to glyphosate (S - susceptible and R - resistant plants), as well as to investigate the mechanism involved in the differential response to the herbicide.

2. Materials and Methods

2.1 Plant material

Seeds from *E. plana* plants that survived the herbicide treatment at the field were collected and mixed together, in Sao Gabriel, Rio Grande do Sul, Brazil (30°33'08.0"S 54°32'34.0"W). In a greenhouse, seeds were germinated in floating trays filled with soil. At the 2-3 tillers growth stage they were treated with glyphosate (Roundup Ready®, 720 g ae. ha⁻¹) using a CO₂ pressurized backpack, delivering 100 L ha⁻¹. At 28 days after treatment (DAT), surviving plants were transplanted into 4-L pots for crossing and generation advancement through seed production (designated as the R biotype). Seeds from an area within the same field with no history of herbicide use also were collected (S biotype).

2.2 Screening for dose-response assays (clonal plants)

A high degree of genetic diversity is expected among plants within the same population because of the outcrossing requirement of *E. plana* (Fipke et al., 2022). Therefore, four S plants and four R were grown to dose-response assays. This approach made it possible to determine differences between the individuals from the same population without the confounding effects of genotypic or ecological differences (Salas et al., 2012). Composite seed samples from both biotypes were grown in trays in the greenhouse until the two-tiller stage. Tillers were separated and transplanted into separate pots to produce two clones of each seedling. One set of clones was sprayed with 2,160 g ae. ha⁻¹ of glyphosate to identify individuals with absence of control. Plants that survived at 28 DAT were considered with no control by glyphosate or resistant plants (R); otherwise, they were classified as susceptible (S). The non-treated clones corresponding to R1, R2, R3, and R4 and S1, S2, S3, and S4 plants were allowed to grow for subsequent experiments.

2.3 Dose-response assays

Two greenhouse experiments were performed, using clonal plants (Section 2.2) and seeds, in 2024 and 2025. Using clonal plants, the experimental design was completely randomized (CRD), with five replicates. The factorial arrangement comprised: A) S and R biotypes; B) glyphosate doses of 0, 45, 90, 180, 360, 720, 1,440 and 2,880 g ae ha⁻¹ to S; and 0, 360, 720, 1,440, 2,880, 5,760, 11,520 and 23,040 g ae ha⁻¹ to R. In 2025, two extra doses were added to S (5,760 and 11,520 g ae ha⁻¹) and R (46,080 and 92,160 g ae ha⁻¹) biotypes, respectively. All treatments were applied at seedlings with 4–6 tillers in a spray chamber at ETB/CPACT, delivering 200 L ha⁻¹, pressure of 1 bar, temperature of 24,8 °C and relative humidity (RH) was 86.8%.

Using seeds, the experimental design was CRD, with four replicates. The factorial arrangement comprised: A) S and R biotypes; B) Glyphosate doses of 0, 90, 180, 360, 720, 1,440, 2,880 and 5,760 g ae ha⁻¹ to S and 0, 360, 720, 1,440, 2,880, 5,760, 11,520 and 23,040 g ae ha⁻¹ to R, in 2024 and 2025. Seeds were sown in 300 mL pots filled with soil and seedlings with 4-6 tillers were sprayed according to the treatments.

In each experiment, a visual evaluation of control was performed 28 DAT, based on a percentage scale, where 0% indicated no symptom and 100% denoted total weed death (SBCPD, 1995). The remaining plants were cut close to the ground and placed in a forced air oven at 60 °C until they reached a constant weight to determine the shoot dry weight (SDW).

2.4 EPSPS gene sequencing

Young leaf tissues of R1, R2, R3 and R4, and S1, S2, S3 and S4 plants were collected for total RNA extraction using PureLink™ Plant RNA Reagent (ThermoFisher Scientific®). Oligo(dT) in the Super Script™ III Reverse Transcriptase

kit (ThermoFisher Scientific[®]) was used to synthesize cDNA. The primers used for the partial amplification of the EPSPS gene were designed using the Primer Designing Tool (NCBI, <https://www.ncbi.nlm.nih.gov/>) and Benchling. The coding sequence of the EPSPS gene from the *E. plana* transcriptome (data unpublished), was used as a reference. The primers designed were forward primer (F) 5'-GCCGACAAAGAGGCCAAAAG-3' and reverse primer (R) 5'-GCACCGAGCTGTTTCAATCC-3'. The polymerase chain reaction was performed in 50-μL reaction containing 2 μL of cDNA, 10 μL of 5X Taq Super Fi I buffer, 1 μL of 2 U μL⁻¹ Taq Super Fi I (Invitrogen[™]), 1 μL of 10mM dNTPs, 2 μL of each forward and reverse primer (10 μM), and nuclease-free water. Amplification was performed in a thermal cycler (Veriti Applied Biosystems) with an initial denaturing step of 95 °C for 30 s, followed of 40 cycles at 95 °C for 30 s, 60 °C for 20 s, and 72 °C for 45 s, with a final extension step at 72 °C for 5 min. Amplified fragment was sent for sequencing (GenOne Biotech). The resulting DNA sequences were cleaned, aligned using the EPSPS sequence of *Eleusine indica* (L.) Gaertn. Susceptible biotypes (KM387414.1; KM387415.1) and resistant biotypes (KM387413.1; KM387412.1) as reference and analysed for polymorphisms using Sequencer and MEGA X software.

2.5 Gene expression

R and S seeds were germinated in 300 mL pots filled with soil in a growth chamber (phytotron) under a light intensity of 400 μmol m⁻² s⁻¹, a photoperiod of 12 h light and day/night temperatures of 20 °C/15 °C, in March 2024. The experimental design was CRD arranged in a factorial scheme, where factor A corresponded to the S and R biotypes and factor B to the herbicide (with and without glyphosate spray, 720 g ae. ha⁻¹, RoundUp Transorb[®]), with three replicates. Seedlings were treated at 4–6 tillers growth stage. Two leaf tissue collections were performed at 48 and 168 hours after treatment (HAT), using bulked samples from two plants, with three biological replicates per sampling time point.

Total RNA was extracted, and cDNA was synthesized according to section 2.4. The reference genes used were *Elongation Factor 1-alpha* (*EF1α*), and *Tubulin* (*TUBF1R1*), previously evaluated for *E. plana*. RT-qPCR were conducted to assess the relative expression of the *Aldo-keto reductase* (*AKR*) gene (Pan et al., 2019), and *ABC-C MRP10* (*MRP10*) and *ABC-C MRP8* (*MRP8*), associated with herbicide sequestration mechanisms (Gaines et al., 2010; Fipke et al., 2022).

The reactions were amplified using a LightCycler[®] 480 Real-Time PCR (Roche[™]) with: 1 μL of cDNA, 6 μL of 2X GoTaq[®] Green Master Mix (Promega), 0.18 μL each 10 μM forward and reverse primers, and 4.64 μL of DEPC-treated water, in a final reaction volume of 12 μL. The RT-qPCR conditions were: initial denaturation at 95 °C for 120 s; 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 20 s (with fluorescence detection during this step); followed by a

Table 1 - Primer sequences used to the relative expression of genes

Gene	Primer	Sequence
EPSPS AKR MRP10 MRP8	Os_qPCR_EPSPS_F	GACCGATTGGTGACTTGGTT
	Os_qPCR_EPSPS_R	TCCTCCAATCCCTTGACAC
	EcAKR_F	AGGCCGGTTACAGACACATC
	EcAKR_R	CACACGCCCTCCTGGAATA
	EcMRP10_F	CACAGCATTGTGTGCTCAGACT
	EcMRP10_R	GACTTCTGGAATATCTCTCCGGTTG
	EcMRP8_F	AGTGGATTCTGCATAATGTTGA
	EcMRP8_R	GACTGTGGCAATCATACCACTCTC

¹ Primers designed by Fipke et al. (2022).

melting curve analysis consisting of 95 °C for 5 s and 70 °C for 60 s. Three technical replicates were performed for each treatment. Relative quantification was calculated using the 2^{-ΔΔCt} method (Livak, Schmittgen, 2001).

A second experiment was conducted in June 2024, under the same conditions previously described. However, the temperatures in the phytotron were set at 35 °C/30 °C (day/night), with a 12 h light photoperiod and a light intensity of 350 μmol m⁻² s⁻¹. The treatments and analyses conducted were the same as those previously described.

2.6 Statistical Analysis

The herbicide concentration that caused 50 % dry weight reduction (GR₅₀) and plant mortality (ED₅₀) were calculated using nonlinear regression following equations:

Equation 1 (Ritz, Streibig, 2005):

$$Y = \frac{a}{1 + \left(\frac{x}{x_0}\right)^b} \quad (1),$$

where *a* is the upper asymptote (maximum response value); *b* is the slope of the curve; and *x*₀ is the x-value corresponding to 50 % of the maximum response (SDW – GR₅₀ or visual control – ED₅₀);

Equation 2 (Seber, Wild, 1989):

$$Y = \frac{a}{1 + e^{-\left(\frac{x-x_0}{b}\right)}} \quad (2),$$

where *a* is the upper asymptote; *x*₀ is the inflection point of the curve, i.e., the value at which 50 % of the maximum response is observed; and *b* is the slope parameter of the curve;

Equation 3 (Hill, 1910):

$$Y = \frac{ax^b}{c^b + x^b} \quad (3),$$

where *a* is the upper asymptote; *b* is the slope coefficient; and *c* is the x-value (dose/concentration) at which 50 % of the maximum response occurs, and,

Equation 4 (Ritz, Streibig, 2005):

$$Y = y_0 + \frac{ax^b}{c^b + x^b} \quad (4),$$

where y_0 is the lower asymptote; a is the difference between the upper and lower asymptotes (the sum of y_0 and a represents the maximum response); b is the slope coefficient; and c is the x-value that results in 50 % of the response between y_0 and $y_0 + a$.

A resistance index (RI) was calculated, $RI = ED_{50} \text{ resistant population} / ED_{50} \text{ susceptible population}$. Nonlinear regression analysis was performed in the R program and RStudio (R Core Team, 2019). Dose-response curves were fitted using SigmaPlot 15.0.

3. Results and Discussion

3.1 Dose-response assays

Clonal plants of R and S biotypes exhibited differences to glyphosate in dose-response. As shown in Table 2; Figure 1(a) and (b) plant injury and SDW are significantly different between the biotypes ($p \leq 0.05$). The ED_{50} values estimated for S and R clonal plants were 591 and 6,634 g ae ha⁻¹ of glyphosate, respectively at 28 DAT. The GR_{50} value estimated for S and R clonal plants were 104 and 1,118 g ae ha⁻¹ glyphosate, respectively.

The results found for dose-response assays with clonal *E. plana* plants in 2024 were repeated in 2025. As shown in Table 3 and Figure 2, both plant injury and SDW differed significantly between the biotypes. The estimated ED_{50} values for S and R clonal plants were 589.08 and 4,480 g ae ha⁻¹ of glyphosate at 28 DAT. The GR_{50} values for the S and R clonal plants were 211 and 1,648 g ae ha⁻¹ of glyphosate, respectively. In the first experiment, based on the ED_{50} and GR_{50} values respectively, the R biotype was at least 11.22 and 10.78-fold more resistant to glyphosate than the S biotype (Table 2). The second experiment in 2025, revealed

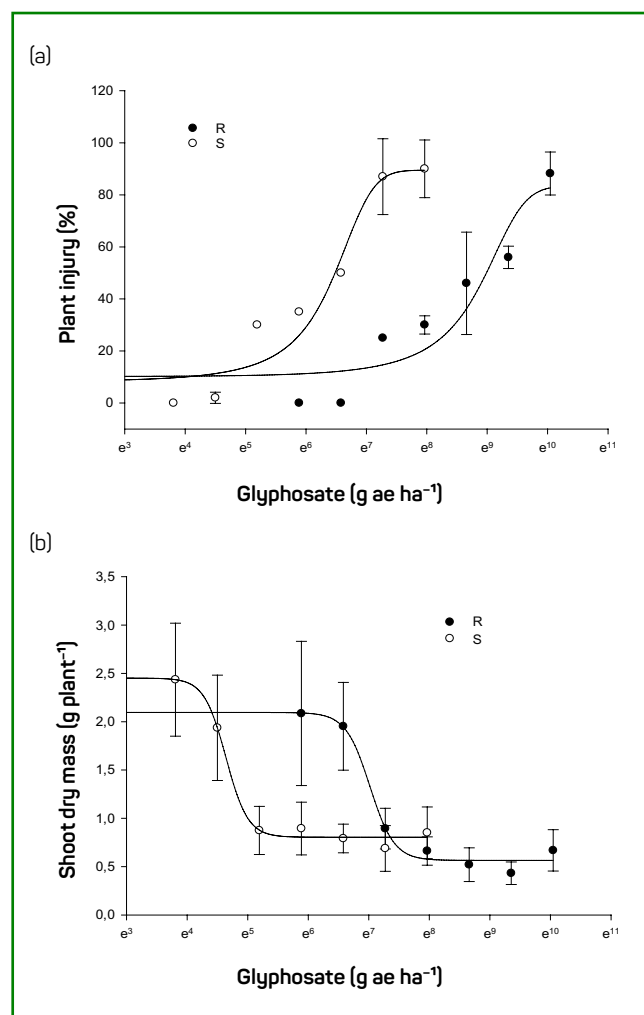


Figure 1 - Dose-response curves for plant injury (%) (a) and shoot dry mass (g plant⁻¹) (b) of *E. plana* resistant (R) and susceptible (S) clonal plants treated with glyphosate. Plants were evaluated 28 days after treatment (DAT), 2024. The vertical bar represents the 95% confidence ($p \leq 0.05$)

Table 2 - Parameters of the logistic-sigmoid equation^a and Hill sigmoid equation^b used to calculate the glyphosate dose required to reduce survival for 50% (ED_{50}) and 50% dry weight reduction (GR_{50}) in *Eragrostis plana* biotypes (S – susceptible; R – resistant plants), 28 days after treatment (DAT), 2024

Biotype	a	B	ED_{50} and GR_{50} (g ae ha ⁻¹)	R ²	RI ^b
28 DAT (Plant injury (%))					
S	89.44	258.97	591.20	0.91	--
R	83.30	3362.78	6634.18	0.83	11.22
28 DAT (SDW)					
S	1.64	-5.54	103.71	0.98	--
R	1.53	-5.01	1118.08	0.97	10.78

^a Equations 2 and 4, described in section 2.6

^b Resistant index = $ED_{50}R / ED_{50}S$

Table 3 - Parameters of the logistic-sigmoid equation ^a and Hill sigmoid equation ^b used to calculate the glyphosate dose required to reduce survival for 50% (ED ₅₀) and 50% dry weight reduction (GR ₅₀) in <i>Eragrostis plana</i> biotypes (S – susceptible; R – resistant plants), 28 days after treatment (DAT), 2025					
Biotype	a	b	ED ₅₀ and GR ₅₀ (g ae ha ⁻¹)	R ²	RI ^b
28 DAT (Plant injury [%])					
S	101.11	-1.77	589.08	0.98	--
R	94.93	-2.39	4479.79	0.98	7.60
28 DAT (SDW)					
S	0.80	-1.79	211.28	0.95	--
R	0.66	-1.64	1647.92	0.95	7.79

^a Equations 1 and 4, described in section 2.6.

^b Resistant index = ED₅₀R / ED₅₀S

that R biotype was 7.60 and 7.79-fold more resistant to glyphosate than the S biotype (Table 3).

In the first dose-response assay conducted with seeds, the S biotype showed over 80 % control at 14 DAT (data not shown) and was completely controlled with 180 g ae ha⁻¹ glyphosate at 21 DAT (Table 4; Figure 3). The R biotype showed over 80% control only at glyphosate rates starting from 1,440 g ae ha⁻¹, with near-complete control (close to 100%) achieved between 2,880 and 5,760 g ae ha⁻¹. When dose-response with seeds were repeated in 2025, both biotypes required higher glyphosate rates to reach over 80 % control. At 21 DAT, the glyphosate rates needed to achieve >80 % control were 1,440 and 5,760 g ae. ha⁻¹ for the S and R biotypes, respectively (Table 4, Figure 3). Based on the ED₅₀ values (Tables 4 and 5), the R biotype was 2.00- and 5.22-foldmore resistant to glyphosate than the S biotype.

The non-linear models provided a good description of the relationship between lethal dose (ED₅₀) and glyphosate rate, with R² values of 0.87 or higher (Tables 4 and 5). However, for SDW, non-linear models could not be fitted to the data (Figures 3 and 4). To reduce the broad genetic variability expected in a cross-pollinated species such as *E. plana*, both seed-derived and clonal plants were used in the dose-response experiments, following the approach proposed by Salas et al. (2012). Nonetheless, the variation in response to herbicide within a population reflects the different degrees of *E. plana* injury from glyphosate observed among plants of the same age in growers' fields. The SDW results from seed-derived plants highlights this variability, as they more closely represent the genetic heterogeneity found under field conditions.

The dose-response assays confirmed differences between the *E. plana* biotypes. When using plants up to the 6-tiller growth stage, higher glyphosate rates were required to control the R biotype compared to the S biotype. The GR₅₀ values estimated from clonal plants for S biotype (103.7 and 211.3 g ae ha⁻¹ of glyphosate) were consistent with those previously reported by Bastiani et al. (2021) for plants at the full tillering stage (20 to 30 tillers per plant), 312 g ae ha⁻¹

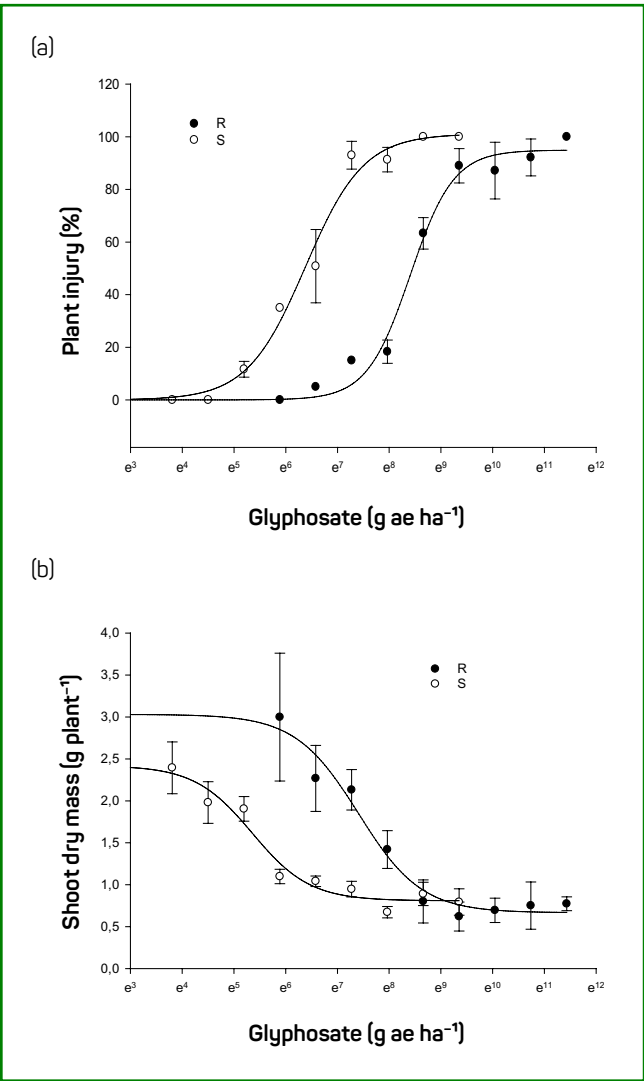


Figure 2 - Dose-response curves for plant injury (%) (a) and shoot dry mass (g plant⁻¹) (b) of *E. plana* resistant (R) and susceptible (S) clonal plants treated with glyphosate. Plants were evaluated 28 days after treatment (DAT), 2025. The vertical bar represents the 95% confidence ($p \leq 0.05$)

for GR_{50} and $461.4 \text{ g ae ha}^{-1}$ for LD_{90} , which corresponds to the dose required to achieve 90% plant control. These findings indicate the R biotype represents a population shift driven by the selection pressure from repeated glyphosate applications. Therefore, the dose-response experiments confirmed that the putative herbicide-resistant *E. plana* biotype (R) is resistant to glyphosate herbicide.

Repeated herbicide use imposes strong selection pressure on weed populations, favouring the survival and reproduction of individuals carrying alleles that confer a fitness advantage under these conditions (Jasieniuk et al., 1996). Over time, this process leads to shifts in allele frequencies, gradually altering the genetic composition of the population. Cross-pollinated species such as *E. plana* (Fipke et al., 2022) exhibit high levels of genetic diversity within populations, promoting allele recombination and increasing phenotypic variability, which in turn enhances the potential for differential responses to herbicide selection pressure (Jasieniuk, Brûlé-Babel & Morrison, 1996). Gene flow through pollen further facilitates the spread of alleles associated with reduced herbicide sensitivity among individuals and populations. Under intense selection pressure, the frequency of such alleles tends to increase (Délye et al., 2013). Therefore, cross-pollinated species are more prone to evolve mechanisms that alter herbicide response compared to predominantly self-pollinated species (Délye et al., 2013).

3.2 Mechanisms involved in failure of control by glyphosate

3.2.1 Partial sequencing of the EPSPS enzyme gene

A 236 bp region of the EPSPS gene was sequenced from the same four R and S clonal plants used in dose-response assays. The partial EPSPS sequence of the R plants reveal mutation at Pro₁₀₆ (Figure 5). Nucleotide polymorphisms CCG to CTG were detected in all R plants, resulting in

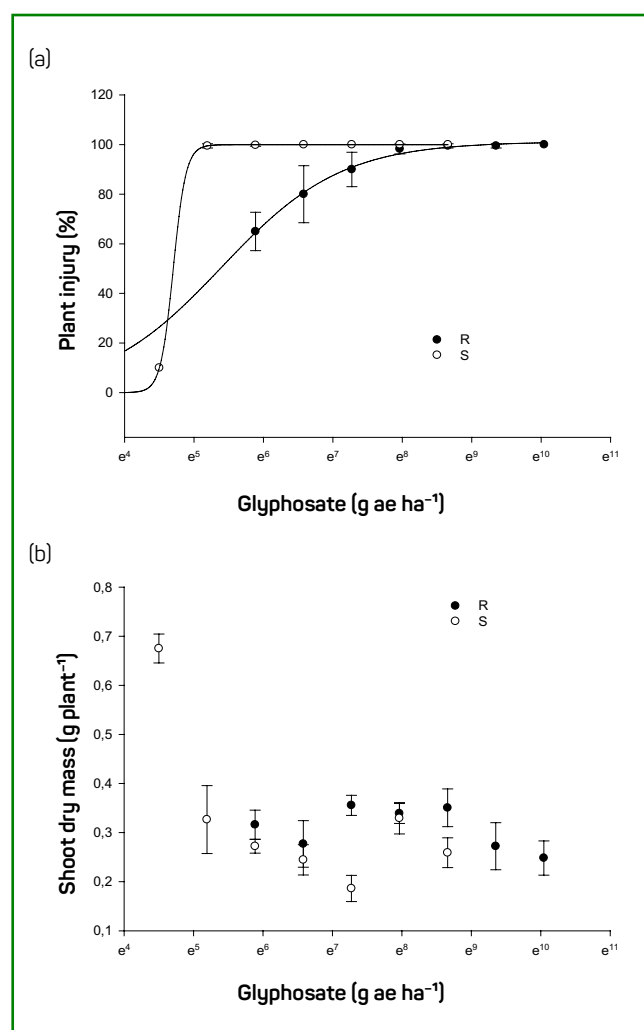


Figure 3 - Dose-response curves for plant injury (%) (a) and shoot dry mass (g plant^{-1}) (b) of *E. plana* resistant (R) and susceptible (S) plants treated with glyphosate. Plants were evaluated 21 days after treatment (DAT), 2024. The vertical bar represents the 95% confidence ($p \leq 0.05$)

Table 4 - Parameters of the Hill-sigmoid equation^a used to calculate the glyphosate dose required to reduce survival for 50% (ED_{50}) in *Eragrostis plana* biotypes (S – susceptible; R – resistant plants), 21 days after treatment (DAT), 2024

Biotype	a	B	ED_{50} (g ae ha^{-1})	R^2	RI^b
S	99.95	10.95	109.97	1.0	--
R	101.18	1.15	220.58	0.99	2.00

^aEquation 3, described in section 2.6.

^b Resistant index = $ED_{50}R / ED_{50}S$

Table 5 - Parameters of the sigmoid equation^a used to calculate the glyphosate dose required to reduce survival for 50% (ED_{50}) in *Eragrostis plana* biotypes (S – susceptible; R – resistant plants), 21 days after treatment (DAT), 2025

Biotype	a	B	ED_{50} (g ae ha^{-1})	R^2	RI^b
S	87.96	140.55	370.31	0.92	--
R	87.72	914.40	1935.12	0.87	5.22

^a Equation 2, described in section 2.6

^b Resistant index = $ED_{50}R / ED_{50}S$

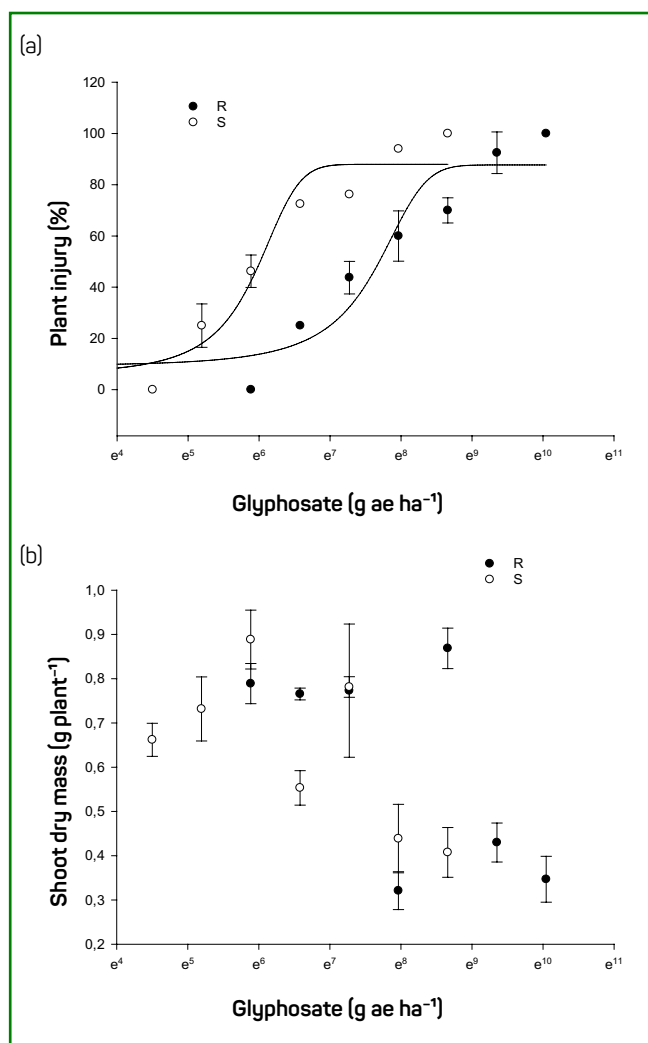


Figure 4 - Dose-response curves for plant injury (%) (a) and shoot dry mass (g plant^{-1}) (b) of *E. plana* resistant (R) and susceptible (S) plants treated with glyphosate. Plants were evaluated 21 days after treatment (DAT), 2025. The vertical bar represents the 95% confidence ($p \leq 0.05$)

a substitution of Pro₁₀₆Leu. A double peak observed at the second nucleotide base of the codon corresponding to amino acid position 106 in R plants, resulting in an ambiguity represented by the base “Y,” which can correspond to either thymine (T) or cytosine (C), suggests the presence of heterozygous alleles, with part of the copies encoding Proline (CCG) and others encoding Leucine (CTG) at position 106. Being *E. plana* diploid, according to a study analyzing two populations from southern Brazil using flow cytometry (unpublished data), the double peak found only in plants sampled from the R biotype confirms heterozygosity for the mutation.

The mutation at position Proline 106 is among the most frequently associated with glyphosate resistance, particularly in species such as *E. indica*, *Lolium* spp., *Amaranthus* spp., *Conyza* spp., among others (Ngo et al.,

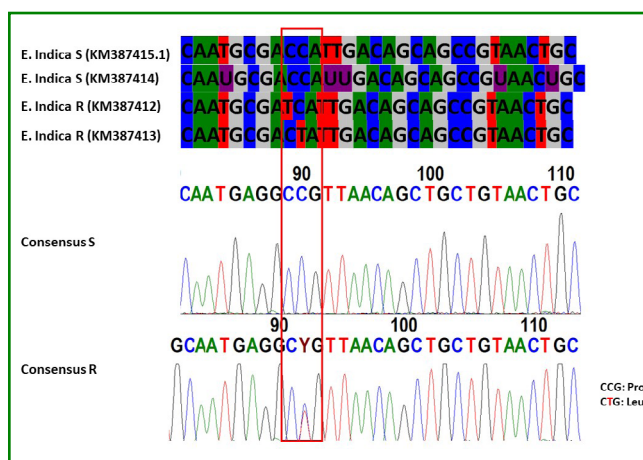


Figure 5 - Resistant and sensitive *E. plana* DNA electropherogram for the *EPSPS* gene. The overlapping of peaks in R at position Pro₁₀₆ shows changes in the second nucleotide base (cytosine to thymine), that is changing proline (CCG) to leucine (CTG). The alignments were compared with *EPSPS* gene of *Eleusine indica* (NCBI: KM387414 and KM387415.1 as susceptible and KM387412.1 and KM387413.1 as resistant to glyphosate)

2016; Chen et al., 2023). This mutation leads to a reduction in herbicide affinity for the active site of the *EPSPS* enzyme, thereby decreasing glyphosate efficacy without compromising enzyme functionality (Chen et al., 2020). These findings corroborate the results observed in dose-response assays for *E. plana* and suggest that strong selection pressure is acting on the *EPSPS* target site. This is consistent with field observations where exclusive reliance on glyphosate for *E. plana* management, either through the integrated MIRAPASTO method or via crop-livestock integrated systems, is favouring the selection of the Pro106Leu mutation.

3.2.2 *EPSPS*, *AKR*, *MRP8* and *MRP10* gene expression

In this study, *EPSPS*, *AKR*, *MRP8* and *MRP10* expression were relative to S biotype untreated (or not applied with herbicide). Under 20/15 °C temperature regime at 48 HAT, *EPSPS* was downregulated in S and R glyphosate-treated (Figure 6). However, *EPSPS* was upregulated in all other treatments at 168 HAT.

The *AKR* gene at 48 HAT was downregulated only in R biotype (not applied with herbicide – NAP) (Figure 6). In this case, *AKR* was upregulated in all evaluated time in S biotype treated with glyphosate. *Aldo-keto reductase* is a superfamily of enzymes responsible for catalyzing the reduction of NAD(P) H-dependent aldehydes and ketones (Pan et al., 2019). This superfamily is also involved in the metabolism of xenobiotics, secondary metabolism, and protection of osmolytes being *EcAKR4-1* involved in the metabolization of glyphosate (Pan et al., 2019). Therefore, the *AKR* gene investigated here is not involved in glyphosate metabolization.

ABC-transporter MRP8 was differentially expressed among biotypes (Figure 6). *MRP8* at 48 HAT was upregulated in R biotype untreated with the herbicide. At 168 HAT, there was 9.1-fold *MRP8* expression in the R biotype without herbicide (NAP). These results demonstrate that *MRP8* was not responsive to glyphosate in *E. plana*. *MRP10* was upregulated 48 HAT in all treatments (Figure 6). *MRP8* and *MRP10* genes are ABC-transporters related to weed resistance to glyphosate, acting on the active transport of this molecule to the vacuole. In resistant *C. canadensis*, an upregulation of *MRP8* and *MRP10* was reported upon glyphosate treatment (Tani et al., 2015).

35/30 °C (day/night) temperature regime (Figure 7), at 48 HAT, *EPSPS* was downregulated in all conditions of herbicide treatment when compared with S biotype without herbicide. At 168 HAT, R biotype glyphosate-treated was significantly upregulated (~2.4-fold). *MRP10* was downregulated in R biotype independently of herbicide use

at 48 HAT. At 168 HAT (Figure 7), *MRP10* was significantly upregulated in R biotype glyphosate treated or AP (~3.0-fold). *AKR* and *MRP8* expression could not be analysed under these temperature conditions, as the primers failed the validation tests (data not shown).

Our results suggest increased expression of *EPSPS* is not involved in the differential response to glyphosate as well as the other genes investigated. In most cases an increase in the expression levels of a gene is usually correlated with a higher copy number (Salas et al., 2012; Gaines et al., 2016), which was also not investigated in this study. In glyphosate-resistant *E. indica* a (CT6) insertion in the 5'-UTR of the *EPSPS* gene modifies transcriptional regulation and leads to gene overexpression, conferring resistance (Zhang et al., 2021). The Pro₁₀₆Leu mutation combined with *EPSPS* gene amplification (2.6-fold increase relative to susceptible populations) confers resistance in *E. indica* (Chen et al., 2023).

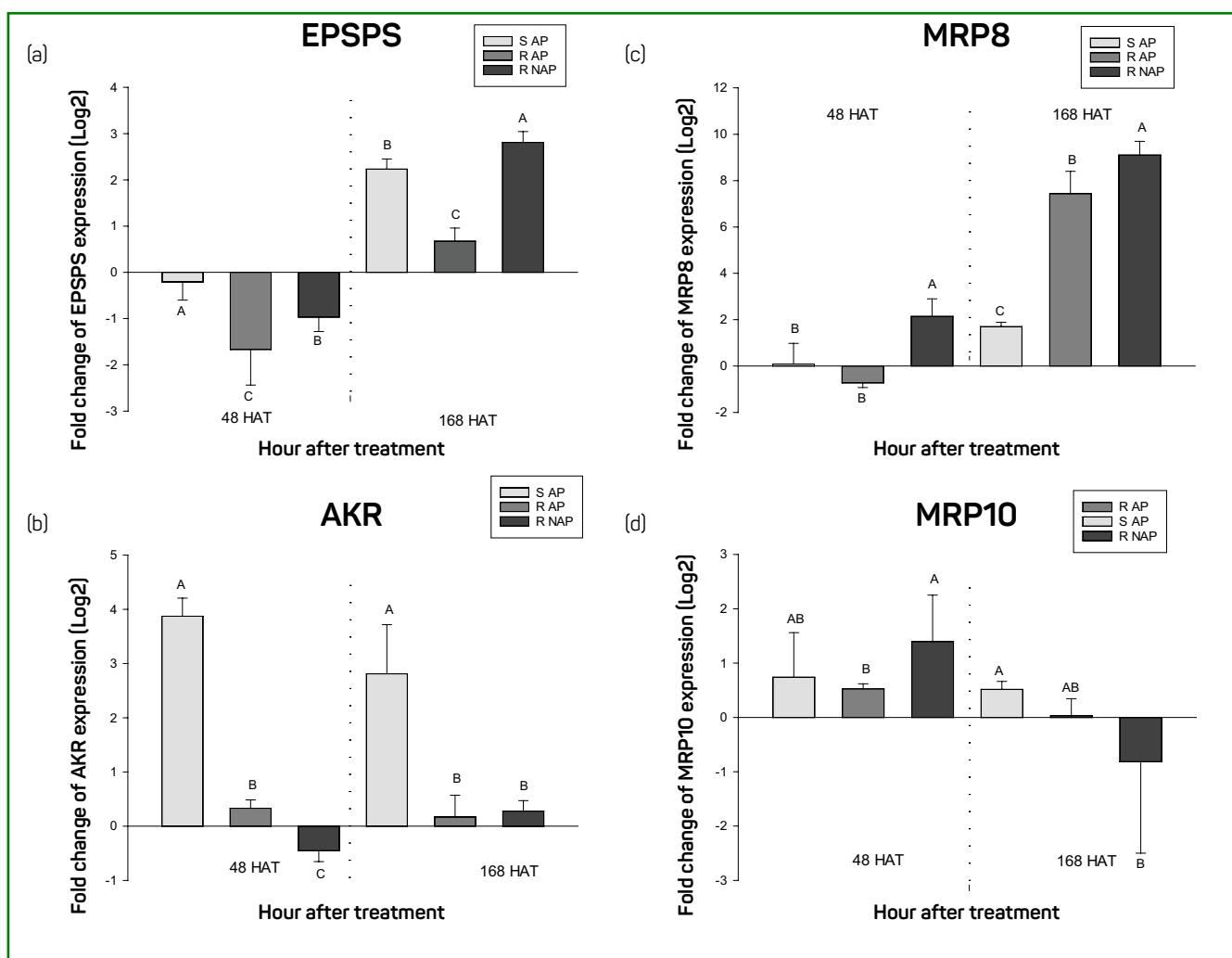


Figure 6 - (a) *EPSPS*, (b) *AKR*, (c) *MRP8* and (d) *MRP10* relative gene expression in *E. plana* susceptible (S) and resistant plants (R) to glyphosate application (720 g ae ha⁻¹), 48 HAT and 168 HAT. S and R AP – applied with herbicide and R NAP – not applied with herbicide, under 20/15 °C (day/night) temperature. Uppercase letters indicate comparisons between biotypes at each sampling point. *Tubulin* and *EF1α* were used as reference genes

This is the first report of point mutation in the EPSPS gene in *E. plana*, suggesting that the observed differences in dose-response assays may be explained by the evolution of the Pro₁₀₆Leu mutation. The differential response between biotypes indicates strong selective pressure on field population, which is consistent with the management strategies implemented for *E. plana* (Perez, 2015; Lamego et al., 2020). From a practical weed management perspective, seed production may favour the spread of biotypes with reduced control, even under low herbicide selection pressure, maintaining or increasing the frequency of altered alleles (Neve et al., 2003; Délye et al., 2013). This represents a determining factor for the dissemination and expansion of populations with differential responses across various areas (Neve et al., 2003; Délye et al., 2013), particularly because seeds constitute the primary means of weed spread

via endozoochorous dispersal (Medeiros, Focht, 2007; Perez, 2015; Faleiro et al., 2022); cattle avoid the tussocks during grazing but readily consume the inflorescences, and, since animal quarantine practices are often not observed, the spread of resistance may be facilitated. A novel and pro-active assessment of adaptive potential for herbicide resistance is possible as showed for *Alopecurus myosuroides*, based in a direct epidemiological link between historical glyphosate selection and current population-level sensitivity (Comont et al., 2019). This approach provides compelling evidence of directional selection for glyphosate insensitivity in advance of reports of field resistance. Initiatives like this should be considered reducing the dependency of glyphosate aiming to contain the spread of *E. plana* and to protect the biodiversity of Pampa biome.

4. Conclusions

Resistant plants of the *E. plana* population after glyphosate use are being selected in the field due to the high selection pressure imposed by the continuous use of this herbicide. This ongoing selection is evidenced by the identification of the Pro₁₀₆Leu point mutation in the EPSPS gene among surviving individuals. To prevent further spread and impact of *E. plana* in the Pampa Biome, it is essential to adopt innovative management strategies with less reliance on glyphosate.

Author's contributions

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

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Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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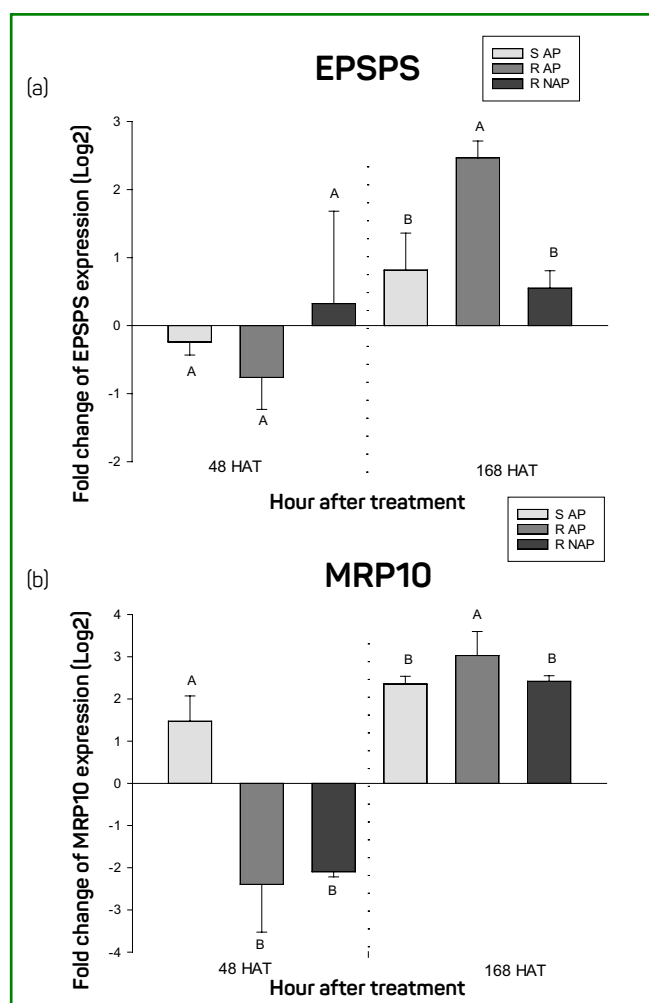


Figure 7 - (a) *EPSPS* and (b) *MRP10* relative gene expression in *E. plana* susceptible (S) and resistant plants (R) to glyphosate application (720 g ae ha⁻¹, 48 HAT and 168 HAT. S and R AP – applied with herbicide and R NAP – not applied with herbicide, under 35/30 °C (day/night) temperature. Uppercase letters indicate comparisons between biotypes at each sampling point. *Tubulin* and *EF1α* were used as reference genes

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