Advances in Weed Science
formerly Planta Daninha

RESEARCH ARTICLE

Transgenerational memory of drought stress and low rates of glyphosate reduce the sensitivity of *Eragrostis plana* to the herbicide

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Abstract: Background: *Eragrostis plana* is the main invasive plant in the Pampa Biome of southern Brazil. This plant is highly competitive, tolerant to abiotic stresses, and very difficult to manage. Plants exposed to abiotic stresses and herbicides develop mechanisms that help alleviate or reduce damage caused by stressors and transmit this ability to the progenies.

Objective: The study's objectives were to ascertain whether acclimatization to stress due to drought and sub-lethal doses of glyphosate may decrease plant sensitivity to glyphosate and investigate the possible memory mechanisms involved in this process.

Methods: A population of *E. plana* was submitted to drought, glyphosate, or a combination of drought plus glyphosate for two generations. The progenies were analyzed for sensitivity to the herbicide glyphosate and for biochemical, metabolites, and molecular responses.

Results: When testing sensitivity to glyphosate in the $\mathbf{G_2}$ generations, the DRYxGLY (plants stressed with drought plus glyphosate) was the least sensitive (ED_{50} 443.0 g a.e. ha^{-1}), while CHK (without stresses) was the most sensitive (ED_{50} 278.0 g a.e. ha^{-1}). Evaluating the effects of glyphosate, the CHK population showed a greater accumulation of oxidative damage and shikimic acid. While DRYxGLY had greater antioxidant activity and higher expressions of the *EPSPS* and ABC-carrier *MRP10* genes.

Conclusions: Recurrent selection with drought stress and sub-lethal rate of glyphosate (DRYxGLY) showed reduced sensitivity to glyphosate in the second generation ($\mathbf{G_2}$). The conjunction of factors, including the upregulation of *EPSPS* and the ABC *MRP10* transporter, antioxidant enzymes seem to decrease the sensitivity of the DRYxGLY population to glyphosate.

Keywords: South African lovegrass; Acclimatization; Shikimic acid; Stress memory

Journal Information:
ISSN - 2675-9462
Website: http://awsjournal.org
Journal of the Brazilian Weed Science Society

How to cite: Fipke MV, Balbinot A, Viana VE, Gehrke VR, Kemmerich M, Dayan FE, Souza GM, Camargo ER, Avila LA. Transgenerational memory of drought stress and low rates of glyphosate reduce the sensitivity of Eragrostis plana to the herbicide. Adv Weed Sci. 2022;40:e020220039. https://doi.org/10.51694/AdvWeedSci/2022;40:00016

Approved by:
Editor in Chief: Carlos Eduardo Schaedler
Associate Editor: Lauren Lazaro

Conflict of Interest: The authors declare that there is no conflict of interest regarding the publication of this manuscript.

Received: June 1ST, 2022
Approved: September 15, 2022

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1. Introduction

Eragrostis plana (Ness), known as South African lovegrass or Tough lovegrass, is the primary invasive weed in over two million hectares of the Pampa Biome (Medeiros, Focht, 2007) in southern Brazil. South African lovegrass is a slow-growing C_4 grass with high seed production and dormancy, allelopathic activity, and tolerance to abiotic stresses (Bastiani et al., 2021).

The most used herbicide to control *E. plana* in the grassland is glyphosate (*N*-phosphonomethyl glycine). Glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), reducing the synthesis of aromatic amino acids, which are intermediate compounds in the shikimate pathway (glycolytic and phosphate pathways), proteins, and secondary metabolism (Maroli et al., 2015). The shikimic acid pathway route expends around 30% of the fixed carbon, which is used by plants to synthesize intermediate compounds, auxins, lignins, secondary metabolism, and aromatic amino acids (Maroli et al., 2015). Therefore, interruption of the shikimate pathway by glyphosate triggers several metabolic and physiological processes, leading to plant death (Gaines et al., 2020). In addition to the direct effects of EPSPS inhibition, glyphosate indirectly induces some ROS production, leading to lipid peroxidation, DNA, RNA, and protein oxidation, and slow cell death (Gomes et al., 2017; Maroli et al., 2015).

Despite the efficient action of glyphosate, plants have evolved multiple mechanisms to mitigate the herbicide effects (Gaines et al., 2020). The known resistance mechanisms are classified into target-site resistance (TSR) and non-target-site resistance (NTSR) mechanisms, being the latter the most complex, involving several genes and metabolic routes (Gaines et al., 2020). TSR mechanisms include mutation and upregulation of the target gene (Gaines et al., 2020). The main known NTSR mechanisms for glyphosate are reduction of absorption and translocation of the herbicide, metabolism of the molecule (forming mainly aminomethylphosphonic acid - AMPA), and exclusion of the herbicide (mainly in vacuole) (Gaines et al., 2020).

Abiotic stress and herbicide sub-lethal doses lead to several plant responses, such as NTSR regulation and other pleiotropic effects (Dyer, 2018). These defense responses are similar and interconnected (Burns et al., 2018). Therefore, it is expected

that plants adapted to abiotic stresses may have greater tolerance to herbicide stresses and vice-versa (Burns et al., 2018; Fipke et al., 2022). An increase in ROS-detoxifying enzymes promotes acclimation to drought and heat stress in *Conyza bonariensis* and *Abutilon theophrasti*, increasing the tolerance to paraquat and glyphosate (Dyer, 2018; Zhou et al., 2007).

As transcriptome, proteome, and plant metabolism changes caused by herbicide stress are similar to abiotic stresses, plants adapted to abiotic stresses are likely more tolerant to herbicides (Yuan et al., 2010). In this context, the study's objectives were to ascertain whether the transgenerational effect of acclimatization to stress due to water deficit and sub-lethal doses of glyphosate may decrease herbicide sensitivity and investigate whether antioxidant enzymes and other resistance mechanisms are involved in this process.

2. Material and Methods

2.1 Establishment of the populations

Seeds of *E. plana* were collected in an experimental field (bulk of seed) in the Embrapa located in Bagé (RS Brazil) and denominated $\mathbf{G_0}$. In the greenhouse facility at UFPel, the populations $\mathbf{G_1}$ and $\mathbf{G_2}$ were generated in 2017 and 2018 (Figure 1). In order to build the populations, the initial $\mathbf{G_0}$ population seeds were divided into four groups (each group with eight plants) and exposed to the treatments: untreated Check (CHK - without stress); drought treatment (DRY - water deficit until 35% of stomatal conductance in relation to unstressed plants); glyphosate treatment (GLY - 120 g a.e. ha^{-1} of glyphosate); DRY×GLY (water deficit until 35% of stomatal conductance and after recovery, sprayed with 120 g a.e. ha^{-1} of glyphosate). The dose of glyphosate (dose to caused 30% to injury, data are not shown) or level of drought were defined in the pre-experiment (data are not shown), aiming at plant stress. The drought stress was performed according to the methodology described by Bastiani et al. (2021). In each treatment group, eight plants were used. As *E. plana* is probably predominantly allogamous, the plants from each treatment were isolated (in different greenhouse, avoiding any crossing among treatments) and subsequently seeds were collected (in bulk), generating $\mathbf{G_1}$ population. $\mathbf{G_1}$ populations were planted and exposed to the same treatments to obtain $\mathbf{G_2}$ generation.

2.2 Dose-response curves with glyphosate

After obtaining all the population's offspring, a growth chamber experiment was conducted, with experimental units consisting of 1 L pots filled with sieved soil (Haplosol) organized in a randomized block design with six replications. Factor A included the different populations $\mathbf{G_0}$, $\mathbf{G_1}$ (CHK, DRY, GLY, and DRY×GLY) and $\mathbf{G_2}$ (CHK, DRY, GLY, and DRY×GLY), and factor B was of glyphosate doses (0, 90, 120, 180, 250, 360, 540 and 720 a.e. g ha^{-1}). All treatments were performed when plants reached an average of eight tillers, using glyphosate (Monsanto, Roundup Original™) applied with a CO_2 sprayer calibrated to deliver 150 L ha^{-1} of spray solution. At 35 days after application (DAA), the visual injury (%) and shoot dry weight (SDW) ratings were analyzed. The SDW was transformed as the percentage compared to the plants without herbicide (%) of each population and generation. The experiment was repeated twice.

2.3 Physiological, biochemical, and profiling analysis of G_2 populations

The experiments were carried out in a growth chamber (temperature 32/28 °C and photoperiod of 12 h). The experiments had three replications and consisted of 8 L

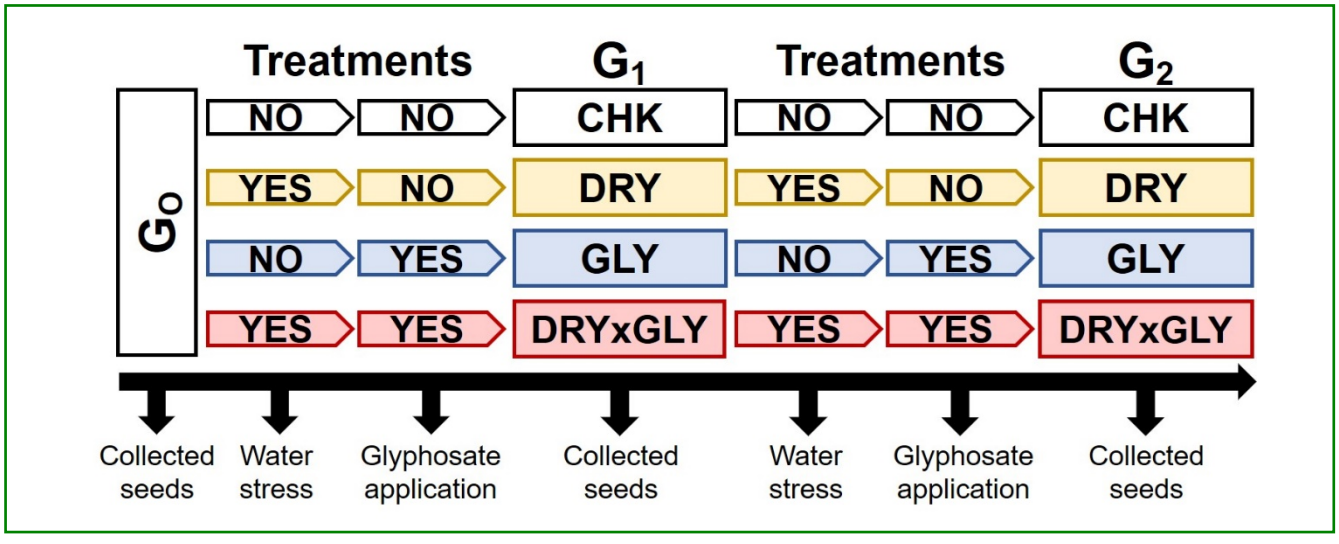

Figure 1 - Design of CHK (check), DRY (drought), GLY (glyphosate), and DRY×GLY (drought plus glyphosate) populations in two generations ($\mathbf{G_1}$ and $\mathbf{G_2}$) from this $\mathbf{G_0}$ *E. plana* plants

pots filled with the same soil described in the 2.2 section. Factor A consisted of populations of $\mathbf{G_2}$ generation: CHK and DRY×GLY (more and less sensitive glyphosate, respectively). Factor B: doses of 0 and 360 g a.e. ha^{-1} of glyphosate. The glyphosate dose used was the one that previously demonstrated plant injury without plant death, allowing the study of the physiological state of responses to the herbicide. The herbicide was applied when plants reached an average of 10 tillers. In experiment 1, plants were collected at 24, 48, 96, and 144 h after glyphosate application (HAA), and in experiment 2 were collected at 48, 96, and 192 HAA. Samples were kept in an ultra-freezer (-80 °C). Leaf samples (second and third fully expanded leaves) were collected and immediately frozen in liquid nitrogen and stored in an ultra-freezer (-80 °C).

2.3.1. *Measurements*

Stomatal conductance was measured with an LI-1600 steady-state porometer (Li-Cor Biosciences™ - Lincoln, NE USA). All plants were evaluated, taking measures in the adaxial leaf face.

H_2O_2 content and lipid peroxidation measurement were performed using leaf tissues (0.250 g) to determine H_2O_2 content according to Fipke et al. (2022); and malondialdehyde (MDA) determination was performed according to (Fipke et al., 2022; Velikova et al., 2000).

Antioxidant enzymes: were performed with leaf tissues (0.250 g) to extract and determine SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), APX (EC 1.11.1.11) activity adapted by Fipke et al. (2022).

2.3.2. *Quantification of glyphosate, AMPA, and aromatic amino acids*

The concentrations of glyphosate, AMPA, shikimic acid, phenylalanine, and tyrosine were determined in duplicate using High-Performance Liquid Chromatography (HPLC) coupled with Tandem Mass Spectrometry (MS/MS). First, the treated leaves were immersed in a 10-mL wash solution (water in pH 2.5 with phosphoric acid 6 M) and the unabsorbed glyphosate was removed from the leaf surface. Herbicide extraction was performed according to the methodology described by Gomes et al. (2015).

To quantify glyphosate and metabolites, HPLC-MS/MS system equipped with a mass spectrometer (Quadrupole-Orbitrap, Q-Exactive Focus, Thermo Scientific™). For separation, a Hypercarb (5 µm 250 Å, 50 mm x 3 mm) column was used, and as mobile phase: (A) 5 mM of ammonium acetate in water (pH 7.0) and (B) 5 mM of ammonium acetate in methanol (pH 7.0), in gradient condition. Calibration curves were prepared in water employing analytical standards with purity ≥99% (Sigma-Aldrich, St. Louis, MO, USA). Method linear range ($R^2 \geq 0.99$) was 1 to 500 µg L^{-1} for all compounds, and samples were diluted when necessary.

2.4 Gene expression analysis

In this study we evaluated the expression of the target genes *5-Enolpyruvylshikimate-3-phosphate synthase* gene (*EcEPSPS*) *Aldo-keto reductase* (*EcAKR*), *ABC-C Family MRP10* (*EcM10*), and *ABC-C Family MRP8* (*EcM11*). *EPSPS* was chosen because it is directly involved in synthesizing the enzyme inhibited by glyphosate (Gaines et al., 2010). The *AKR*, *M10*, *M11* genes are known to be involved in the process of glyphosate metabolism (Pan et al., 2019; Piasecki et al., 2019a).

Oligonucleotides for the target genes were obtained based on the *Eragrostis curvula's* genome available in the National Center for Biotechnology Information – NCBI (National Center for Biotechnology Information, 2020) and were designed using Primer3Plus software (Untergasser et al., 2007). In addition, rice (*Oryza sativa* L.) reference genes were used (Table 1). The GenBank IDs are *EcAKR* (TVU36522.1), *EcEPSPS* (AP014962.1), *EcMRP10* (TVU07241.,1), and *EcMRP8* (TVU00075.1).

The total RNA was extracted from leaves (three biological replicates) using PureLink™ (Plant RNA Reagent-Invitrogen™, Carlsbas, USA) following the manufacturer's instructions. RNA concentration, quality and integrity were assessed using NanoVue™ (GE Healthcare™, Buckinghamshire, UK) and agarose gel electrophoresis, respectively. RNA (1 µg) of each sample was treated with DNase I (Invitrogen) according to the manufacturer's recommendations and converted into

Table 1 - Oligonucleotides for the reference and target genes used to study the *Eragrostis plana* gene expression in RT-qPCR.

Gene	Forward (5'-3')	Reverse (5'-3')	Reference
OsACT1	CCTTCAACACCCCTGCTATG	CAATGCCAGGGAACATAGTG	Zhou et al., 2012
Os18S	CTACGTCCCTGCCCTTTGTACA	ACACTTCACCGGACCATTCAA	Jain et al., 2006
OsEF1α	TTTCACTCTTGGTGTGAAGCAGAT	GACTTCCTTCACGATTTCATCGTAA	Zhou et al., 2012
EcAKR	AGGCCGGTTACAGACACATC	CACTACGCCCTCCTGGAATA	
EcEPSPS	GACCGATTGGTGACTTGGTT	TCCTCCAATTCCCTTGACAC	
EcMRP10	CACAGCATTTGTTGCTCAGACT	GACTTCTGAATATCTCTCCGGTTG	
EcMRP8	AGTGGATTTCTTGCATAATGTTGA	GACTGTGCAATCATACCATCTCTC	

Os: *Oryza sativa* L.; Ec: *Eragrostis curvula*.

cDNA using oligo(dT) and the SuperScript™ III First-Strand Synthesis System kit (Invitrogen).

RT-qPCR experiments were performed in LightCycler™ 480 thermocycler with three biological and three technical replicates using oligonucleotides for the target and reference genes (Table 1), according to the MIQE Guidelines (Bustin et al., 2009). The amplification efficiency and specificity of each oligonucleotide were determined in validation experiments using four dilutions of cDNA. The reactions were performed following the protocol described by Fipke et al. (2022). Gene expression was calculated following the 2^(-delta-delta CT) method (Livak, Schmittgen, 2001), using CHK population without glyphosate (0 g a.e. ha^{-1}) at each time of collection as a baseline and normalized with *OsACT1*, *Os18S*, and *OsEF1α* reference genes.

2.5 Statistics analysis

Data were tested for homogeneity of variance and normality. Visual injury and SDW data were fitted with a nonlinear sigmoid ($y = a/(1+\exp(-(x-ED_{50})/b))$) and log-logistic ($y = a/[1 + (x/GR_{50})^b]$) regressions model. The sensitivity reduction rate was calculated with ED_{50} or GR_{50} (DRY, GLY or DRY×GLY) / ED_{50} or GR_{50} (CHK) in the same generation.

The stomatal conductance, H_2O_2 content, lipid peroxidation, antioxidant enzyme activity, glyphosate, AMPA, shikimate acid, L-tyrosine, and L-phenylalanine concentration were subjected to analysis of variance, calculated confidence intervals (CI; 95%). For plants of the same population with and without herbicide, a t-test was performed. The statistical analysis was conducted using SAS University Edition™ (SAS Institute, Inc.™, Cary, NC, USA) statistical program.

3. Results and Discussion

3.1 Dose-response curves for populations to check for sensitivity shift

In **G_0** plants (Figure 2A), the dose of glyphosate required to cause 50% plant injury (ED_{50}) was 173 g a.e. ha^{-1} (Table 2). Regarding **G_1** and **G_2** generation, CHK populations showed

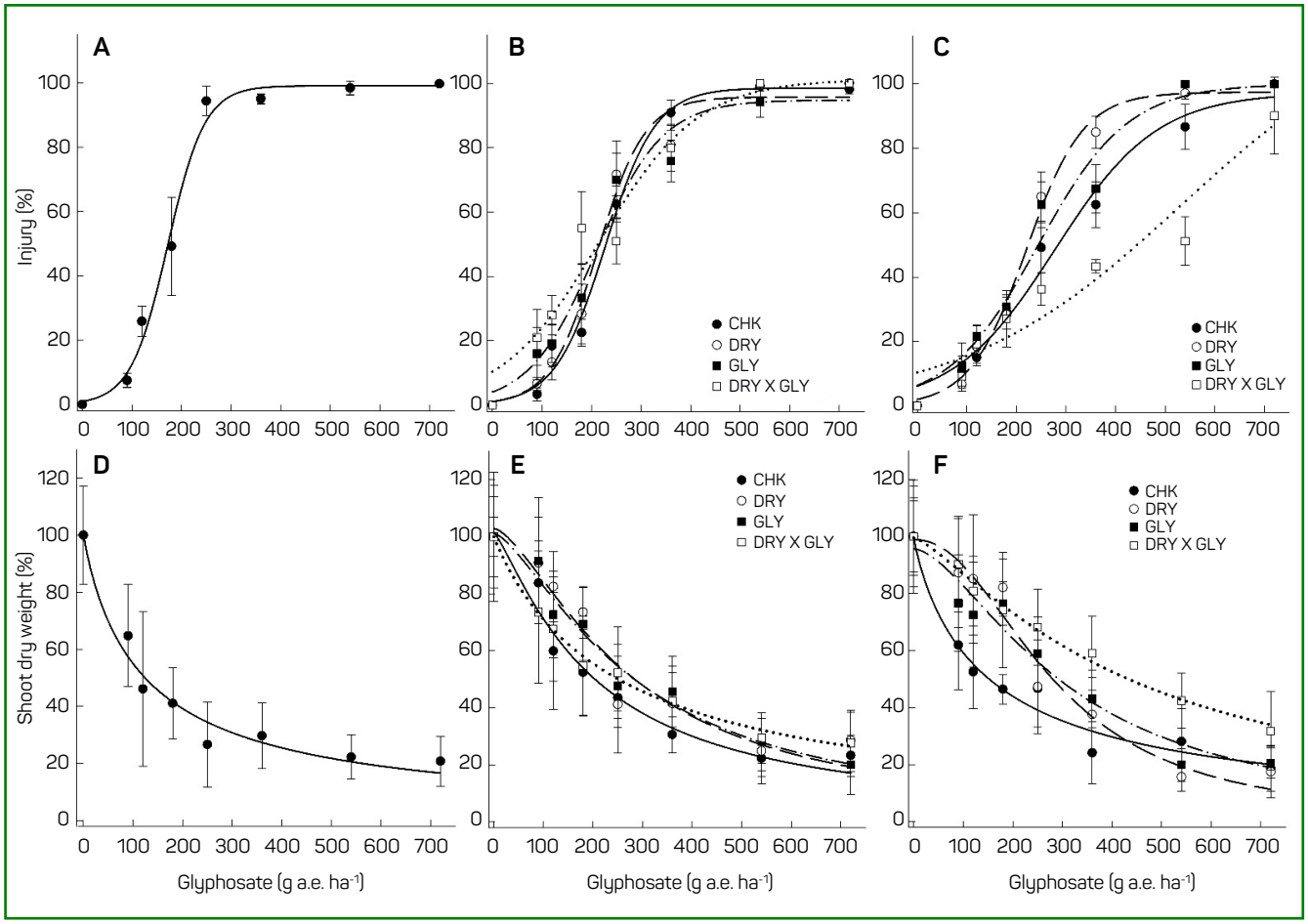

Figure 2 - Visual injury (**A**, **B** and **C**) and shoot dry weight (**D**, **E** and **F**) of *Eragrostis plana* populations from generations **G_0** (**A** and **D**), **G_1** (**B** and **E**), and **G_2** (**C** and **F**) at 35 DAA of glyphosate doses. Parameters estimated for the curves are presented in Table 1. Vertical error bars represent a 95% confidence interval

an increased dose of glyphosate compared to $\mathbf{G_0}$, with ED_{50} 229 and 278 g a.e. ha^{-1}, respectively. In $\mathbf{G_1}$ there were no differences in the ED_{50} of the DRY, GLY, and DRY×GLY populations compared to the CHK population. When analyzing the $\mathbf{G_2}$ generation, the DRY×GLY population presented the highest ED_{50}, 443 g a.e. ha^{-1}, among populations. In $\mathbf{G_2}$ generation, the sensitivity reduction rate (Table 2) showed 1.59 in the DRY×GLY population.

In the dose of glyphosate required to cause 50% of shoot dry weight reduction (GR_{50}), there were no differences between the CHK populations of the $\mathbf{G_0}$, $\mathbf{G_1}$, and $\mathbf{G_2}$ generations, with 122, 199, and 152 g a.e. ha^{-1}, respectively. In $\mathbf{G_1}$ generations, there were no differences in GR_{50} between populations. However, in the $\mathbf{G_2}$ generation, the DRY, GLY, and DRY×GLY populations presented higher GR_{50} (284, 308, and 436 g a.e. ha^{-1}, respectively) than the CHK population. In $\mathbf{G_2}$ generation, the sensitivity reduction rate demonstrated 1.85, 2.02, and 2.85 for DRY, GLY, and DRY×GLY populations, respectively.

These results indicated that the offspring of plants exposed to water stress decreased sensitivity to glyphosate, mainly in $\mathbf{G_2}$. Plants from the DRY×GLY population, which received both stresses (drought followed by a sub-lethal dose of glyphosate), were less sensitive to glyphosate. It is well known that plants exposed to previous stresses may have improved tolerance to other stresses, a phenomenon related to memory mechanisms in plants (Galviz et al., 2020). For instance, in another study, *E. plana* submitted to drought, quizalofop, and drought plus quizalofop had a lesser sensitivity to quizalofop than non-stressed plants (Fipke et al., 2022). Consequently, the current study also sought to determine the possible biochemical mechanisms involved in priming for glyphosate reduced sensitivity in *E. plana*.

3.2 Physiological, biochemical, and metabolites analysis of G_2 populations

3.2.1. *Stomatal conductance*

At 24 and 48 HAA (Figures 3A and 3B), all plants treated with glyphosate had lower stomatal conductance

Table 2 - Parameters estimates for the curves for visual injury and shoot dry weight of *Eragrostis plana* populations of glyphosate doses.

[a]POP	[b]Gen	[c]Equation parameters (SE)				[d]D_{50}	CI 95%	Sensitivity reduction rate
		a	b	R^2	P			
[e]Visual injury								
CHK	G_0	99.0 (1.92)**	37.29 (3.68)**	0.96	<0.01	172.9	(166.2-178.7)	1.00
CHK	G_1	98.5 (1.57)**	50.59 (3.59)**	0.98	<0.01	228.8	(222.6-234.2)	1.00
DRY	G_1	95.7 (1.99)**	47.42 (4.47)**	0.96	<0.01	216.2	(207.9-223.3)	0.94
GLY	G_1	94.8 (2.92)**	67.19 (8.16)**	0.92	<0.01	215.9	(201.8-226.9)	0.94
DRY×GLY	G_1	101.3 (3.76)**	99.15 (13.07)**	0.91	<0.01	215.3	(185.8-244.7)	0.94
CHK	G_2	97.2 (3.00)**	102.8 (9.19)**	0.95	<0.01	278.0	(253.1-302.8)	1.00
DRY	G_2	97.4 (1.50)**	57.24 (3.65)**	0.98	<0.01	223.4	(217.3-228.9)	0.80
GLY	G_2	100.0 (2.86)**	90.73 (8.42)**	0.94	<0.01	244.1	(231.8-253.7)	0.88
DRY×GLY	G_2	124.9 (32.50)**	222.06 (47.45)**	0.86	<0.01	443.0	(425.6-462.7)	1.59
Shoot dry weight								
CHK	G_0	100.6 (8.64)**	0.91 (0.22)**	0.60	<0.01	122.4	(55.8-189.0)	1.00
CHK	G_1	101.4 (6.05)**	1.24 (0.18)**	0.77	<0.01	199.4	(144.0-254.7)	1.00
DRY	G_1	102.8 (7.10)**	1.51 (0.27)**	0.68	<0.01	273.7	(194.6-352.9)	1.37
GLY	G_1	101.1 (6.74)**	1.46 (0.26)**	0.70	<0.01	278.9	(198.8-358.9)	1.39
DRY×GLY	G_1	100.0 (8.06)**	0.97 (0.25)**	0.56	<0.01	251.2	(130.1-372.2)	1.25
CHK	G_2	99.9 (5.89)**	0.89 (0.55)**	0.73	<0.01	152.5	(94.5-210.5)	1.00
DRY	G_2	98.8 (6.47)**	2.19 (0.43)**	0.74	<0.01	283.6	(218.6-348.6)	1.85
GLY	G_2	95.6 (6.29)**	1.64 (0.30)**	0.72	<0.01	308.5	(226.9-390.2)	2.02
DRY×GLY	G_2	99.5 (6.41)**	1.31 (0.70)**	0.63	<0.01	435.9	(294.0-577.7)	2.85

[a]POP: Populations of *Eragrostis plana*, CHK (no stress), DRY (drought), GLY (glyphosate), and DRY×GLY (drought followed by glyphosate).
[b]Gen: generations of *Eragrostis plana* populations submitted to different stresses.
[c]Equation parameters used, SE: standard error of estimate, *p<0.05, **p<0.01, R^2: Adjusted R-squared, P: model probability.
[d]Herbicide dose required to injury (ED_{50}) or shoot dry weight (GR_{50}) by 50%, confidence interval (CI 95%).
[e]Visual injury equation was nonlinear sigmoid (y=a/(1+exp(-(x-ED_{50})/b))); Shoot dry weight equation was log-logistic y = a/[1 + (x/GR_{50})^b].

than untreated plants (without glyphosate). DRY×GLY population glyphosate-treated at 24, 48, and 144 HAA (Figures 3A and 3B) also presented reduced stomatal conductance compared with untreated plants.

In our study, the reduction in stomatal conductance in all populations was a typical response to glyphosate (Figure 3). However, this response is not necessarily involved in harmful effects caused by the herbicide in plants. *Lolium perenne* resistant to glyphosate had a stomatal closure regulation; however, it was linked to carbon fixation optimization according to assimilated demands (Yanniccari et al., 2012). Stomatal conductance can be used as a biomarker in plants sensitive to glyphosate (Yanniccari et al., 2012).

3.2.2. *H_2O_2 content and lipid peroxidation*

Glyphosate-treated CHK population produced 82 and 80% higher hydrogen peroxide than untreated plants at 96 and 144 HAA (Figure 4a). At 144 HAA, the glyphosate-treated DRY×GLY population (Figure 4b) also showed higher hydrogen peroxide than untreated plants. Depending on the efficiency of redox homeostasis, it can be related to apparent antagonistic functions such as, on the one hand, signaling to prevent stressful situations and, on the other hand, causing oxidative stresses (Moretti et al., 2017).

Inhibition of EPSPS by glyphosate indirectly induces ROS production (Maroli et al., 2015). This unregulated accumulation of H_2O_2 and other ROS causes lipid peroxidation and loss of membrane integrity, and, ultimately, cell death (Foyer, Noctor, 2005). The glyphosate-treated CHK population showed the highest lipid peroxidation levels (Figure 4c), with an increase of 49, 75, and 70% (48, 96, and 144 HAA, respectively). On the other hand, primed DRY×GLY plants (Figure 4d) had no ROS increases in the evaluated periods.

Accumulation of H_2O_2 and the concomitant increase of lipid peroxidation (Figure 4) could explain the susceptibility of the CHK population to glyphosate. At 96 and 144 HAA, the high production of H_2O_2 likely triggered an increase in lipid peroxidation. At 48 HAA, although H_2O_2 apparently did not induce lipid peroxidation, it could be reached by other types of ROS responsible for this process. In *C. bonariensis*, susceptible plants showed 5.2 and 3.2-fold higher in H_2O_2 and lipid peroxidation than untreated plants (Piasecki et al., 2019a). *Salix miyabeana* plants subjected to glyphosate had an increase in the production of H_2O_2 after 6 HAA, reaching a peak at 72 HAA. Likely, from 6 HAA, a gradual increase in lipid peroxidation has occurred (Gomes et al., 2017).

3.2.3. *Antioxidant enzymes*

SOD activity differed only at 24 and 96 HAA in the glyphosate-treated DRY×GLY population compared to the untreated plants (Figure 5b). In offspring of plants DRY×GLY population, it was observed to have a higher baseline of SOD activity than untreated CHK population. All plants presented increased SOD activity following glyphosate application, with 16 and 56% for CHK and DRY×GLY, respectively, demonstrating that plants submitted to stresses in previous generations

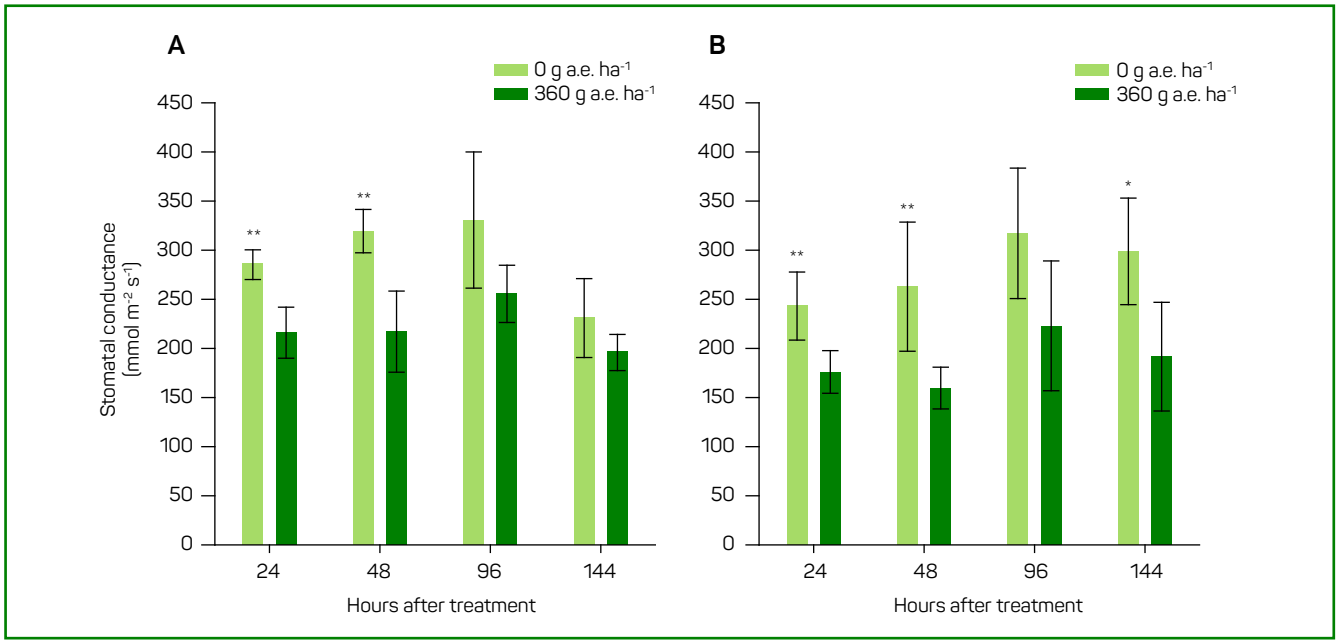

Figure 3 - Stomatal conductance (mmol m^{-2} s^{-1}) of *Eragrostis plana* populations CHK (**A**, no stress) and DRY×GLY (**B**, drought plus glyphosate) with and without herbicide at 24, 48, 96, and 144 h after application of glyphosate. Error bars represent 95% confidence intervals. *t-test (α=0.05); **t-test (α=0.01)

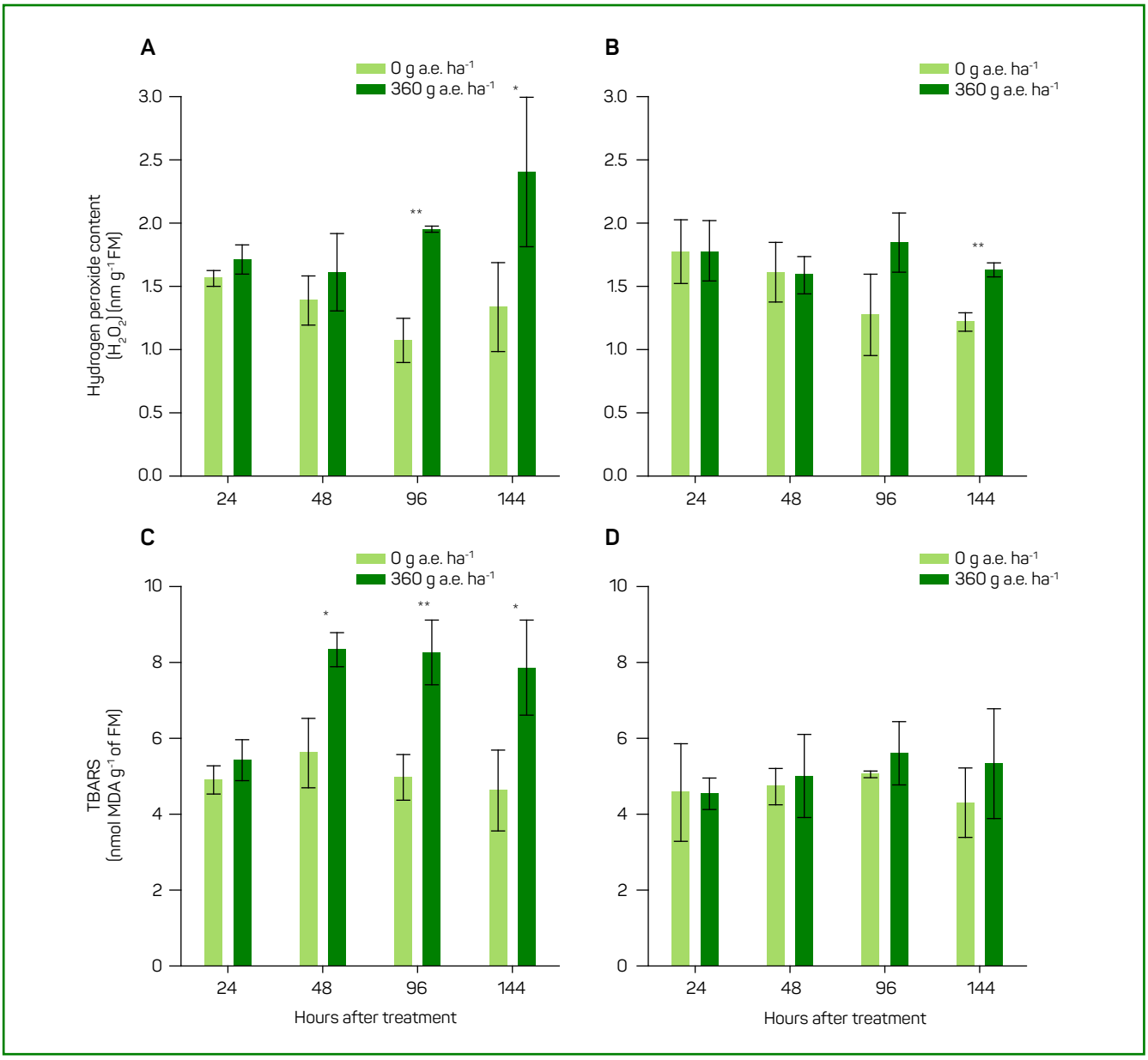

Figure 4 - Hydrogen peroxide content (**A** and **B**) and thiobarbituric acid-reactive substances (**C** and **D**) at 24, 48, 96, and 144 h of *Eragrostis plana* populations CHK (**A** and **C**, no stress) and DRY×GLY (**B** and **D**, drought plus glyphosate) with and without herbicide hours after application of glyphosate. Error bars represent a 95% confidence interval. *t-test (α=0.05); **t-test (α=0.01)

abet stimulated SOD activity. SOD is a crucial enzyme in regulating oxidative stress. For instance, *C. bonariensis* GLY resistant had 1.6-fold SOD activity than the susceptible biotype (Piasecki et al., 2019a).

CHK population exhibited differences in CAT activity at 24, 48, and 144 HAA (Figure 5c), showing increases of 81, 60, and 49% in glyphosate-treated plants than the untreated. However, this increase in CAT activity in the CHK population was like or less than the glyphosate-treated DRY×GLY population. In 144 HAA (Figure 5d), the glyphosate-treated DRY×GLY population presented the highest CAT activity. APX activity (Figure 5f) exhibited differences for the DRY×GLY population (24 and 96 HAA). CHK population did not differ from APX activity with or without quizalofop-treated (Figure 5e).

SOD, CAT, and APX enzymes play an essential role in ROS detoxification. In *C. bonariensis*, a glyphosate-resistant biotype demonstrated higher CAT and APX activity than a susceptible biotype when the herbicide was applied (Piasecki et al., 2019a). Application of glyphosate in *S. miyabeana* stimulated ROS production and, consequently, increased enzymatic activities of CAT and APX (Gomes et al., 2017). Antioxidant enzymes SOD, CAT, and APX are responsible for reducing oxidative stress and complement important resistance mechanisms enabling plants to tolerate herbicide glyphosate (Maroli et al., 2015).

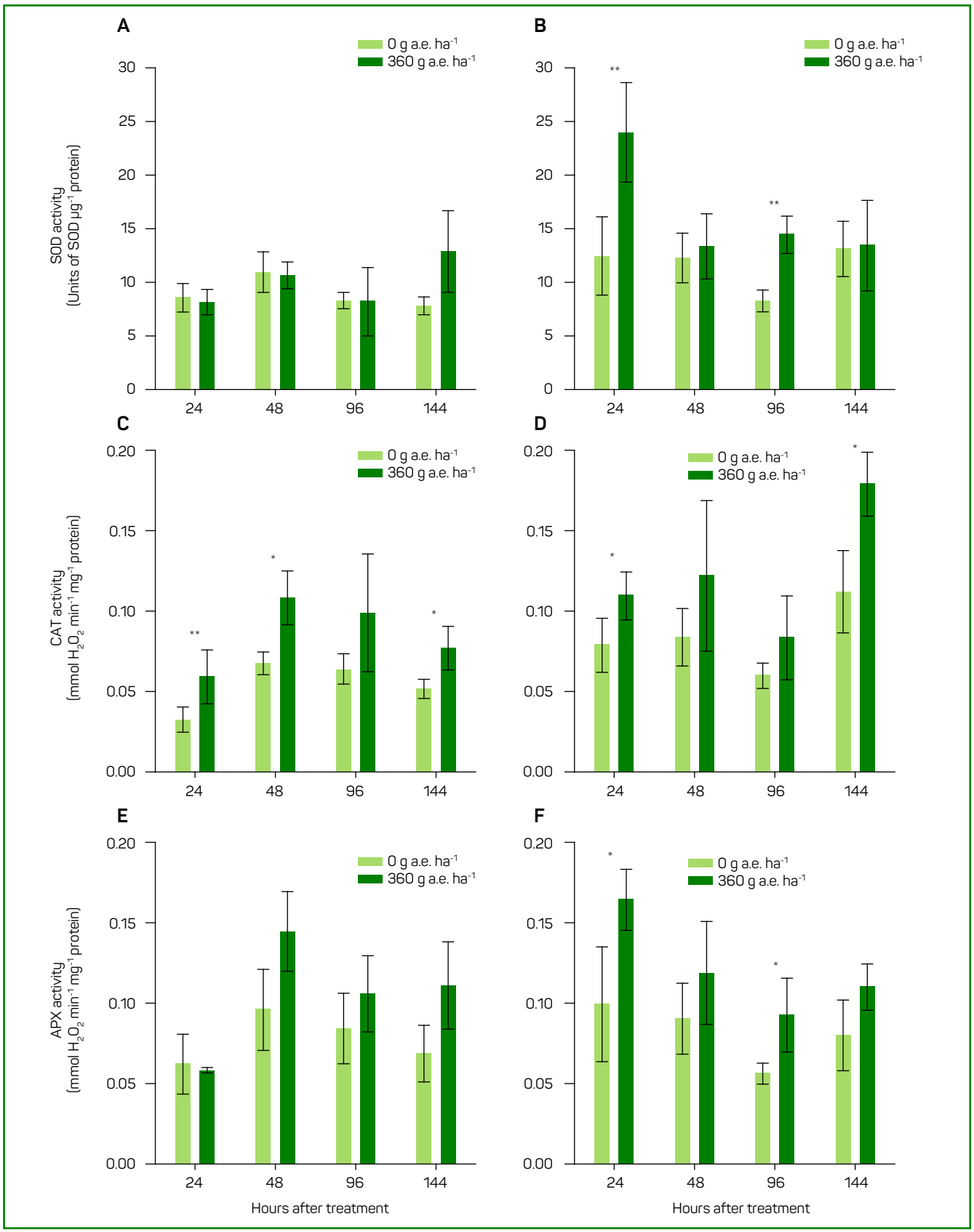

Figure 5 - Superoxide dismutase activity (**A** and **B**), catalase activity (**C** and **D**), and ascorbate peroxidase activity (**E** and **F**) at 24, 48, 96 and 144 h of *Eragrostis plana* populations CHK (**A**, **C** and **E**, no stress) and DRY×GLY (**B**, **D** and **F** drought plus glyphosate) with and without herbicide hours after application of quizalofop. Error bars represent a 95% confidence interval. *t-test (α=0.05); **t-test (α=0.01)

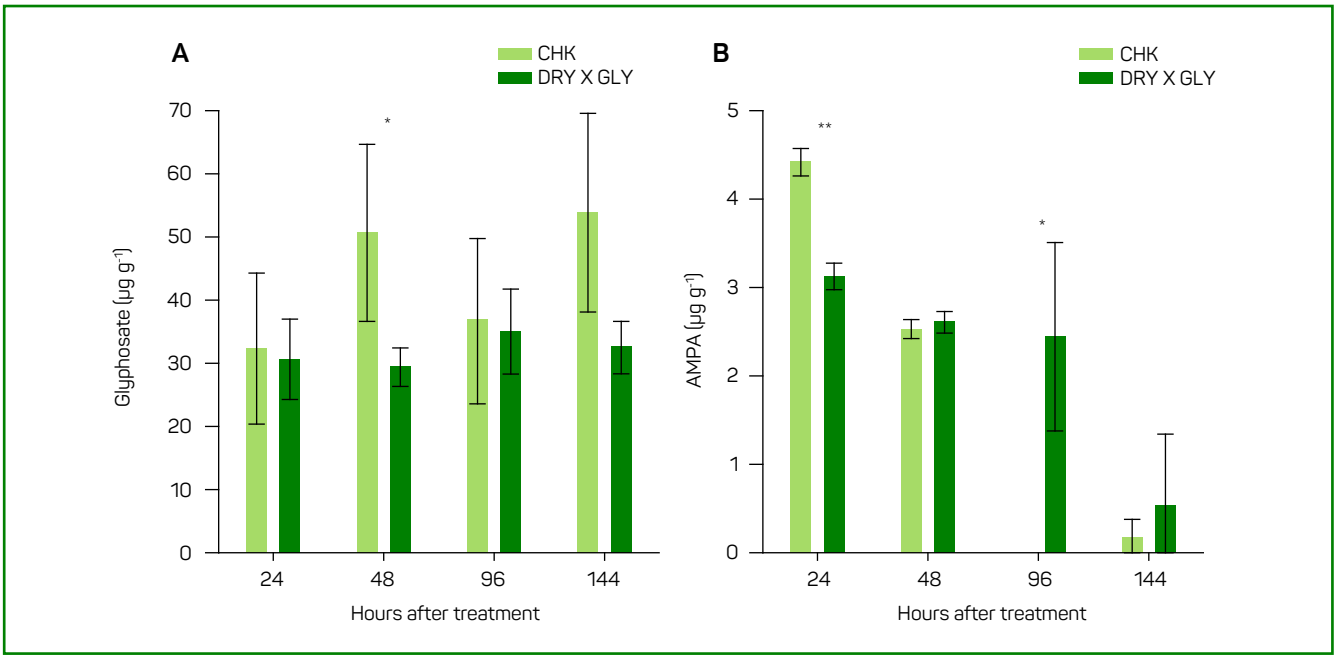

Figure 6 - Glyphosate (**A**) and AMPA (**B**) concentration (µg g^{-1}) of *Eragrostis plana* populations (CHK – no stress and DRYxGLY – drought plus glyphosate) at 24, 48, 96, and 144 h after applying glyphosate. Error bars represent a 95% confidence interval. *t-test (α=0.05); **t-test (α=0.01)

3.2.4. *Glyphosate, AMPA, shikimic acid, and amino acids concentration in plants*

Glyphosate concentration in CHK and DRYxGLY populations (Figure 6a) reveals that in the CHK population, there was 72% higher glyphosate concentration at 48 HAA compared to the DRYxGLY population. There were no differences in glyphosate concentration regarding sampling times in CHK and DRYxGLY populations. In CHK and DRYxGLY populations, glyphosate concentration remained similar in the leaf tissues until 144 HAA, indicating low translocation and/or metabolization in the leaf tissues.

Glyphosate degradation in plants generates metabolites AMPA and glyoxylate (Rojano-Delgado et al., 2012). Herein, AMPA metabolite was found in both populations CHK and DRYxGLY (Figure 6b). In the CHK population, AMPA was detected only at 24 and 48 HAA, with a concentration of 4.4 and 2.5 µg g^{-1}. In the DRYxGLY population, AMPA was found in all evaluation periods, with the highest concentration at 24 HAA and the lowest at 144 HAA (3.1 and 0.8 µg g^{-1}, respectively).

These results indicated that there was detoxification of glyphosate forming AMPA and that it was present in both more sensitive and less sensitive populations to glyphosate (CHK and DRYxGLY, respectively). However, AMPA levels represent 13.6 and 4.9% of glyphosate concentration at 24 and 48 HAA in the CHK population, while in the DRYxGLY population, AMPA levels were equivalent to 10.2, 8.8, 6.9, and 1.6% of the glyphosate concentration at 24, 48, 96 and 144 HAA, respectively.

EPSPS inhibition by glyphosate leads to rapid accumulation of shikimic acid and reduced synthesis of aromatic amino acids (Rojano-Delgado et al., 2012). All glyphosate-treated plants had a greater accumulation of shikimic acid than plants without glyphosate (Figure 7a). At all evaluated periods, the CHK population showed a higher concentration of shikimic acid than DRYxGLY populations, exhibiting an increase of 291, 97, 35, and 195% at 24, 48, 96, and 144 HAA, respectively. These results explain the lower sensitivity of DRYxGLY compared to CHK population. Comparing both populations, when the plants were not treated with glyphosate, there were no differences in shikimic acid concentration.

In the glyphosate-treated CHK population, an increase in shikimic acid was observed after 24 HAA and later remained constant at 48 and 144 HAA. At 96 HAA, a reduction in shikimic acid was detected, consistent with the lower glyphosate concentration (Figure 6a) in the CHK population. In the glyphosate-treated DRYxGLY population, shikimic acid accumulation initiates at 24 HAA, reaching the highest concentration at 48 and 96 HAA and reducing to 144 HAA. Plants with faster and higher shikimic acid accumulation over time demonstrate substantial inhibition of EPSPS and, therefore, greater sensitivity to glyphosate (Tani et al., 2015). *Conyza canadensis* plants susceptible to glyphosate showed higher shikimic acid accumulation than resistant plants (Tani et al., 2015).

Tyrosine and phenylalanine are aromatic amino acids from the shikimic acid pathway (Fernández-Escalada et al., 2017). Between CHK and DRYxGLY, there was a difference in L-tyrosine concentration only for untreated plants (Figure 7b). In glyphosate-treated CHK population had increased L-tyrosine concentration compared to untreated plants at 48 and 96 HAA. However, at 144 HAA, there was

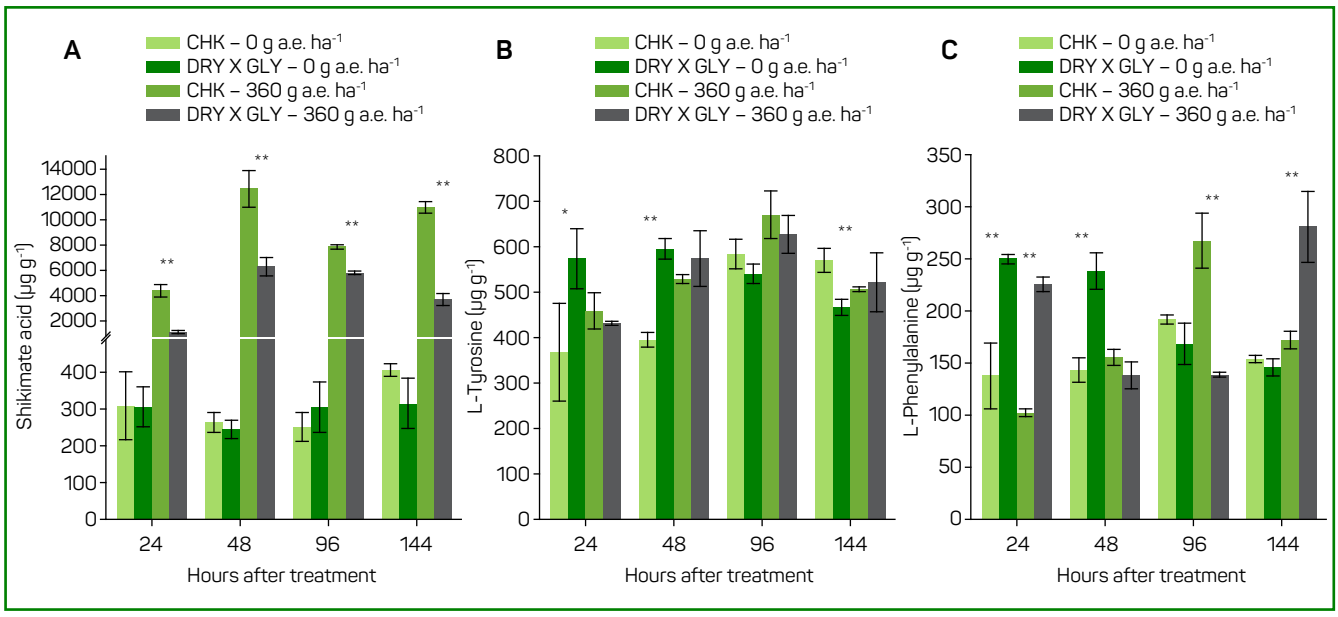

Figure 7 - Shikimate acid (**A**), L-tyrosine (**B**), and L-phenylalanine (**C**) concentration (µg g^{-1}) of *Eragrostis plana* populations (CHK – no stress and DRYxGLY – drought plus glyphosate) at 24, 48, 96 and 144 h after applying glyphosate. Error bars represent a 95% confidence interval. t-test was performed between populations in same glyphosate dose; *t-test (α=0.05); **t-test (α=0.01)

an inversion, and glyphosate-treated plants presented a reduction of 11%. Regarding the DRYxGLY population, contrasts were found at 24 and 96 HAA, with a difference of -25% and 16% in glyphosate-treated plants compared to untreated plants.

The L-phenylalanine concentration (Figure 7c) varied between populations at different times and glyphosate application. In the glyphosate-treated CHK population, an increased L-phenylalanine concentration was observed from the initial evaluation at 24 HAA until 96 HAA and a reduction at 144 HAA (Figure 7C). From the glyphosate-treated DRYxGLY population, differences of -9, -42, -18, and 92% occurred compared to untreated plants (24, 48, 96, and 144 HAA, respectively).

Amino acid concentrations usually vary among plants; however, the increase in phenylalanine and tyrosine concentrations may be due to increased protein turnover following glyphosate treatment (Fernández-Escalada et al., 2017). In *Amaranthus palmeri,* susceptible to glyphosate, an increase in phenylalanine and tyrosine concentration at 72 HAA was reported. In contrast, in an *A. palmeri* population resistant to glyphosate, differences in concentration of these amino acids were not detected (Fernández-Escalada et al., 2017). Also, *Lolium multiflorum* plants susceptible to glyphosate showed a higher concentration of tyrosine than plants resistant to 72 HAA, while no changes in phenylalanine concentrations were observed (Barroso et al., 2018).

3.3 Gene expression

In this study, *EPSPS* expression was relative to the untreated CHK population (Figure 8A). At 48 HAA, *EPSPS* was downregulated in glyphosate-treated CHK and DRYxGLY populations. However, *EPSPS* was upregulated relative to the untreated CHK population in all other treatments and evaluation periods. At 96 HAA, the glyphosate-treated DRYxGLY population demonstrated the highest *EPSPS* expression, with 4-fold more transcripts than the untreated CHK population and 1.92-fold more than the untreated DRYxGLY population. At 192 HAA, glyphosate-treated CHK and untreated DRYxGLY populations showed the higher *EPSPS* expressions, with 2.3-fold and 2.6-fold, respectively, compared to the untreated CHK population.

A mechanism of resistance to glyphosate that has been widely studied is the upregulation of the *EPSPS* gene. Increased copies of the *EPSPS* gene allows for a higher synthesis of the EPSPS enzyme, as there is more enzyme than glyphosate molecules for inhibition, making the plant resistant (Gaines et al., 2010). Plants *C. bonariensis* and *C. canadensis,* both glyphosate-resistant, *EPSPS* expression was observed two times compared to susceptible plants. However, the authors report other NTSR mechanisms involved (Dinelli et al., 2008). In *A. palmeri*, it was found that increased expression of *EPSPS* was responsible for the resistance (Gaines et al., 2010). The increase in *EPSPS* expression was explained by the amplification of genes, where several copies of the gene are present in the genome (Gaines et al., 2010).

Studies have recently shown that plants that have submitted stress, such as herbicides, transmit pre-regulated expression patterns to the progeny (transgenerational) through epigenetic processes (Délye et al., 2013; Margaritopoulou et al., 2018). In *C. canadensis* resistant to glyphosate, there was an upregulation mechanism of

EPSPS, and it was reported that this upregulation was due to the DNA methylation process affecting regions of *EPSP-synthase 1* (Margaritopoulou et al., 2018).

The *AKR* gene at 48 HAA was downregulated in all populations compared to the CHK population without herbicide (Figure 8B). The CHK population with herbicide did not differ in expression levels at any time. In contrast, the DRYxGLY population showed a relative increase of 1.1 and 0.6-fold at 96 HAA and 2.6 and 1.6-fold at 192 HAA compared to the CHK population without herbicide. DRYxGLY population had no difference in glyphosate application at any evaluation time. 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). However, this superfamily is also involved in the metabolism of xenobiotics, secondary metabolism, and protection of osmolytes. It was recently reported that *EcAKR4-1* is involved in the metabolization of glyphosate (Pan et al., 2019). However, it is reported that *Echinochloa colona* had resistance to glyphosate by Pro-106-Thr EPSPS target-site mutation (McElroy, Hall, 2020).

These data suggest that the DRYxGLY population that has undergone two generations of drought stress and a sub-lethal dose of glyphosate was able to positively regulate *AKR* gene expression compared to the CHK population. A previous report demonstrates that *E. colona* could detoxify glyphosate, forming AMPA, by the *AKR* enzyme. The *EcAKR4-1* gene was upregulated in this species (Pan et al., 2019).

ABC-transporter MRP8 (*M11*) was differentially expressed among treatments (Fig 8C). The glyphosate-treated CHK and DRYxGLY populations were not very responsive to *M11*, except for CHK at 96 HAA, where an *M11* was upregulated. At 192 HAA, there was 1.7-fold *M11* expression in the DRYxGLY population without herbicide. These results demonstrate that *M11* was not responsive to glyphosate in *E. plana* and suggest that other ABC-transporter codifying gene may be involved in glyphosate responses in this species.

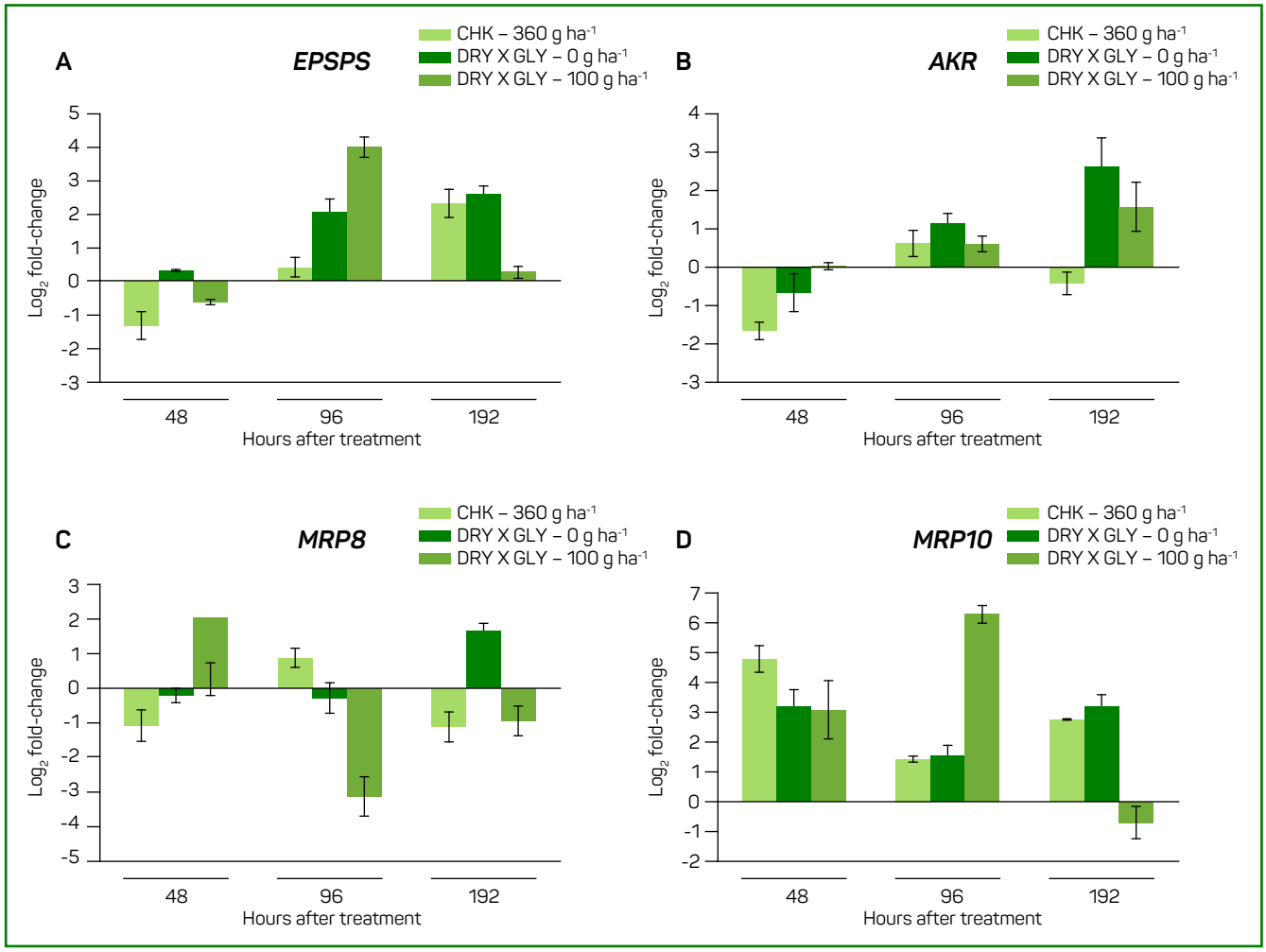

Figure 8 - Relative expression levels in log_2 of *EPSPS* (A), *AKR* (B), *MRP8* (C), and *MRP10* (D) gene in leaves of *Eragrostis plana* populations CHK – with 360 g a.e. ha-1 of glyphosate (CHK- 360 g ha^{-1}– dark bars), DRYxGLY – without herbicide (DRYxGLY-0 g ha^{-1}–dark gray bars) and DRYxGLY – 360 g a.e. ha^{-1} of glyphosate (DRYxGLY-360 g ha^{-1}–gray bars) at 48, 96 and 192 h after application of glyphosate. Vertical error bars represent 95% confidence intervals

ABC-transporter MRP10 (*M10*) was upregulated in almost all populations and times analyzed (Figure 8D). In the glyphosate-treated CHK population, *M10* was upregulated in all periods analyzed, with 4.8-fold, 1.4-fold, and 2.8-fold at 48, 96, and 192 HAA, respectively. *M10* was upregulated in the glyphosate-treated DRYxGLY population at 48 and 96 HAA and downregulation at 192 HAA. The higher difference between populations was detected at 96 HAA, where glyphosate-treated DRYxGLY showed the highest expression of *M10*, 6.3-fold, thus being upregulated in this period. The untreated DRYxGLY population also showed upregulation of the *M10* gene, demonstrating a greater baseline expression than the untreated CHK population.

M10 and *M11* genes are ABC-transporters located in the tonoplast (Moretti et al., 2017). These transporters are related to weed resistance to glyphosate, acting on the active transport of this molecule to the vacuole (Moretti et al., 2017; Piasecki et al., 2019b). In *C. canadensis* and *C. bonariensis,* the *ABC- transporters M10* and *M11* are involved in resistance to glyphosate (Piasecki et al., 2019b; Tani et al., 2015). In resistant *C. canadensis*, an upregulation of *M10* and *M11* was reported upon glyphosate treatment (Nol et al., 2012). The *ABC-transporter M10* was 2.3-fold overexpressed in the resistant *C. canadensis* than the sensitive biotype (Tani et al., 2015). The authors suggest that the *ABC-transporters* resistance in glyphosate-treated plants is mainly due to the exclusion of glyphosate in the vacuole and reduced herbicide translocation to the other plant tissues (Nol et al., 2012; Tani et al., 2015).

3.4 Enhanced mechanisms of response to glyphosate in *E. plana*

The ability of plants to adapt to environmental stresses allows regulating their metabolism to increase their tolerance (Délye et al., 2013). Mechanisms of resistance to herbicides that do not involve a site of action are the same as those used by plants in responding to abiotic stresses (Délye et al., 2013; Fipke et al., 2022). According to our results, *E. plana* $\mathbf{G_1}$ and $\mathbf{G_2}$ generations, when exposed to combined stress (drought plus a sub-lethal dose of glyphosate), allowed a decrease in sensitivity compared with plants that did not undergo any stress. This transgenerational effect can be caused by epigenetic processes, allowing differentiated regulation of the expression of stress tolerance mechanisms, such as antioxidant machinery (Galviz et al., 2020). However, there is a need for further studies to explain the epigenetic role in this observed effect.

In addition to the antioxidant processes presented, it is suggested that the reduced sensitivity of the DRYxGLY population to glyphosate is due to multiple mechanisms. It is important to note that a high accumulation of shikimic acid was detected in both populations. However, the DRYxGLY population had a lower shikimate acid concentration than the CHK population in all evaluation periods (Figure 7a). The increased expression of *EPSPS* in DRYxGLY at 96 HAA (Figure 8a) may be one factor in reducing shikimic acid concentration at 144 HAA (Figure 7a). The tyrosine and phenylalanine amino acids pool showed contrasting responses (Figures 7b and 7c).

Regarding the involved NTSR mechanisms, it was found that the concentration of glyphosate in the leaf tissue remained constant over time, which may demonstrate a reduction in translocation to other plant tissues (Figure 6A). Also, AMPA was detected due to glyphosate detoxification (Figure 6B), together with the increase in *AKR* expression (Figure 6B). However, AMPA concentration was relatively low to explain the difference in sensitivity to glyphosate. Also, an increased expression of *M10* in DRYxGLY at 96 HAA (Figure 8D) may help exclude glyphosate in the vacuole and, consequently, reduce its translocation to other plant tissues.

4. Conclusions

Eragrostis plana exposed to combined stress (drought followed by a sub-lethal dose of glyphosate) showed induced transgenerational memory allowing acclimation to stressful conditions and decreasing sensitivity to glyphosate. The increased activity of the enzimes SOD, CAT, and APX following homeostatic adjustment in the DRYxGLY population, relieves glyphosate's oxidative stress. Additionally, the set of mechanisms such as the possible metabolization of glyphosate in AMPA and the upregulation of *AKR,* and the upregulation of *MRP10* (exclusion of glyphosate in the vacuole) seem to be involved in decreasing glyphosate sensitivity in *Eragrostis plana* DRYxGLY population.

Author's contributions

All authors read and agreed to the published version of the manuscript. MVF, VEV, MK, GMS, ERC and LAA: conceptualization of the manuscript and development of the methodology. MVF, AB, VEV, VRG and MK: data collection and curation. MVF, AB, VRG and VEV: data analysis. MVF, VEV, MK, FED, GMS, LAA: data interpretation. ERC and LAA: funding acquisition and resources. LAA: supervision. MVF and VEV: writing the original draft of the manuscript. MVF, AB, VEV, VRG, MK, FED, GMS, ERC and LAA: writing, review and editing:

Funding

This research was funded by CAPES - financing code 001 for Marcus Vinícius Fipke and Vívian Ebeling Viana by CAPES/PNPD; by CNPq for the Research Fellowship of Luis Antonio de Avila/N.Proc. 310830/2019-2 and for the Universal grant Universal 2018 - CNPq N.Proc. 426714/2018-0; by FAPERGS - Program for the internationalization of Graduate Programs - FAPERGS/CAPES 06/2018: 19/2551-0000715-9.

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