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PLANT PROTECTION - Article

Oxidative stress and differential antioxidant enzyme activity in glyphosate-resistant and -sensitive hairy fleabane in response to glyphosate treatment

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ABSTRACT: Biochemical assays in a time-course were employed to evaluate stresses induced by glyphosate treatment in resistant and sensitive biotypes of *Conyza bonariensis*. Two experiments were conducted assessing glyphosate doses and time-course after treatment. The doses of glyphosate ranged from 0 to 11840 g ae·ha^{-1} and assessments performed until 552 h after glyphosate treatment (HAT). The objectives of this study were to evaluate the oxidative stress and differential antioxidant enzyme activity in glyphosate-resistant and -sensitive biotypes of hairy fleabane after glyphosate treatment. After treatment, both studied biotypes accumulated similar levels of shikimic-acid until 96 h. The sensitive biotype died at 192 HAT. Shikimic-acid and hydrogen peroxide (H_2O_2) accumulation in glyphosate-resistant biotype were transient and did not differ from untreated plants at 288 and 500 HAT, respectively. In both glyphosate-resistant and -sensitive biotypes, a correlation analysis established a cause-and-effect relationship after glyphosate treatment, which leads to shikimic-acid and hydrogen peroxide accumulation, lipid peroxidation (indicates tissue damage) and an enhancement in the activity of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) enzyme. However, in the glyphosate-resistant biotype, the oxidative stress and tissue damage were lower, and antioxidant enzyme activities SOD, CAT, and APX were higher than in the -sensitive biotype. It indicates that antioxidant enzyme in glyphosate-resistant biotype might be related to the glyphosate-resistance process in *Conyza bonariensis*. This study is the first report of differential antioxidant enzyme activity in hairy fleabane.

Key words: *Conyza bonariensis*, lipid peroxidation, shikimic-acid, herbicide resistance.

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INTRODUCTION

Hairy fleabane (*Conyza bonariensis* (L.) Cronq.), belonging to the botanical Asteraceae family, is native to the Americas and now has a cosmopolitan distribution (Shrestha et al. 2014; Bajwa et al. 2016). Hairy fleabane is highly competitive on crops and is among the most challenging weed species to management around the world (Shrestha et al. 2014; Bajwa et al. 2016; Concenço and Concenço 2016). According to Trezzi et al. (2015), the yield losses caused by one uncontrolled hairy fleabane plant·m^{-2} on soybean can arrive at 36%.

Glyphosate (N-(phosphonomethyl)glycine) is employed in many crop production systems to control a broad spectrum of annual and perennial grasses and broadleaf weeds (Duke and Powles 2008; Dill 2005). The widespread use of glyphosate is because of its high efficacy, environmental features and low cost when compared to other herbicides (Peng et al. 2010). However, the intensive use of glyphosate has been a factor in weeds evolving resistance, rendering glyphosate treatment ineffective (Baucom and Holt 2009) in at least 42 weed species (Heap 2018). Of these, glyphosate-resistant from *Conyza* genera *Conyza canadensis* L. (horseweed) was the first broadleaf weed to evolve glyphosate-resistance (GR), which was observed in the United States in 2000 (VanGessel 2001). The GR in *C. bonariensis* was first documented in 2005 in Brazil (Vargas et al. 2007).

Glyphosate directly inhibits the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) (Maroli et al. 2015) interrupting the shikimic acid pathway, which is a crucial pathway to carbon and nitrogen metabolism, causing the death in sensitive plants (Tzin and Galili 2010). In the shikimic acid pathway, EPSPS catalyzes the conversion of the shikimic acid to chorismate, which is the precursor for biosynthesis of aromatic amino acids tryptophan, phenylalanine, and tyrosine, and secondary metabolites in plants (Duke et al. 2003). Glyphosate acts as a competitive inhibitor concerning phosphoenolpyruvate (PEP), a substrate for EPSPS, to form a very stable complex between enzyme-herbicide and inhibits the product-formation reaction (Sammons et al. 1995).

The interruption of shikimic acid pathway disrupts the aromatic amino acid biosynthesis and causes alterations in the metabolic stoichiometric of carbon intermediates, which result in system wide-perturbations (Maroli et al. 2015). The EPSPS inhibition by glyphosate and consequently shikimic acid pathway blocking results in the shikimic-acid and reducing power (NADPH+H) accumulation (Schönbrunn et al. 2001) leading to oxidative stress in plants through reactive oxygen species (ROS) overproduction (Maroli et al. 2015; Cobb and Reade 2010). After glyphosate treatment and EPSPS inhibition, there are ROS generation because its action causes perturbations in the photosynthetic machinery through reducing ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activity and 3-phosphoglyceric acid (3-PGA) levels (Servaites et al. 1987; Sergiev et al. 2006). Also, the glyphosate' action causes the interruption on electron transport in the photosystem II (PSII). Tyrosine is required to plastoquinone regeneration, which is an electron acceptor in the PSII. The non-regeneration of plastoquinone in the PSII interrupts the electron transport, leading to energy accumulation (Cobb and Reade 2010). Therefore, the reduction of RuBisCo activity and 3-PGA levels, reducing power accumulation, and PSII blockage leads to ROS overproduction, cell damage, and glyphosate-sensitive plant die from 7 to 15 days after treatment (Cobb and Reade 2010).

The primary ROS are the superoxide radical (O_2•-), hydrogen peroxide (H_2O_2), the hydroxyl radical (OH•), and singlet oxygen ($^1O_2$) (Gill and Tuteja 2010). The ROS are highly reactive toxic molecules, causing lipid peroxidation (LPO), oxidation of DNA, RNA, and proteins, damage to cellular structures, and cell death (Foyer and Noctor 2005; Gill and Tuteja 2010). When the amount of ROS exceeds the plant's capacity to scavenging it through the action of the antioxidant system, occurs the LPO, the most destructive cellular process in living organisms, loss of cellular homeostasis, leading to loss of membrane integrity, and cell death (Foyer and Noctor 2005). The ROS may also serve as signaling molecules in response to stresses activating and controlling gene expression (Das and Roychoudhury 2014).

Plants use antioxidants systems (non-enzymatic and enzymatic) to cope with oxidative stress induced by ROS (Radwan and Fayez 2016). Among the main enzymatic antioxidants are the superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (Gill and Tuteja 2010; Caverzan et al. 2012; Mittler 2017). SOD provides the first line of defense against the toxic effects of elevated levels of ROS through removing $O_2^{\bullet-}$ by catalyzing its dismutation, one $O_2^{\bullet-}$ being reduced to H_2O_2 and another oxidized to O_2 (Gill and Tuteja 2010). CAT directly dismutate H_2O_2 into H_2O and O_2 and is indispensable for ROS detoxification during stressed conditions (Garg and Manchanda 2009). APX plays

the essential role in cope with ROS and protecting cells in higher plants, through scavenging H_2O_2 in water-water and ascorbate-reduced glutathione cycles and utilizing ascorbate as the electron donor (Gill and Tuteja 2010; Caverzan et al. 2012). APX has a higher affinity for H_2O_2 than CAT and peroxidases (POD), and it may have a more crucial role in the management of ROS during stress (Caverzan et al. 2012). The chloroplasts are the predominant source of ROS production as a consequence of the high O_2 concentration from photolysis and high energy produced by the photosynthetic electron transport chain. In this way, antioxidant enzyme concentration in the chloroplast is high, but it is also in the cytosol, mitochondria, and peroxisomes (Gill and Tuteja 2010).

Increase in antioxidant enzyme activity has been related to protection of glyphosate damages in glyphosate-resistant (GR) *Amaranthus palmeri* biotype indicating a potential role of antioxidant systems on resistance (Maroli et al. 2015). In hairy fleabane, studies have been reported greater constitutive activity of antioxidants enzyme in paraquat-resistant biotype than in a -sensitive and increased further following paraquat exposure (Shaaltiel and Gressel 1986; Ye and Gressel 2000). In these cases, injury symptoms were observed in both biotypes. However, the resistant plants survived paraquat treatment. To our knowledge, there are no reports of differential antioxidant enzyme activity protecting hairy fleabane of glyphosate perturbations. Investigating the oxidative stress and antioxidant enzyme activity in response to glyphosate treatment could provide useful information about the effects of ROS on DNA and RNA degradation. Nucleic-acid degradation evaluations are important especially previous to molecular studies that investigate herbicide resistance mechanisms in weeds like RNA-sequencing (RNA-Seq) and quantitative reverse transcription PCR (RT-qPCR), among others. Also, antioxidant enzyme activity could be related to many abiotic stress responses, in this case study, the herbicide resistance process in weeds (Maroli et al. 2015).

Thus, it is hypothesized that following glyphosate treatment, the glyphosate-resistant biotype of hairy fleabane suffers lower oxidative stress and tissue damage and has higher antioxidant enzyme activity than -sensitive biotype. Enhance in antioxidant enzyme activity in the resistant biotype could be related to protection against oxidative stress caused by glyphosate treatment. Therefore, the objectives of this research were to evaluate the oxidative stress and differential antioxidant enzyme activity assessing in glyphosate-resistant and -sensitive biotypes of hairy fleabane followed by glyphosate.

MATERIALS AND METHODS

Biotype selection

As recommended by Burgos et al. (2013), we collected seeds from 54 biotypes of *Conyza sp.* (F_0 generation) in the Rio Grande do Sul state (RS), South of Brazil, in March 2016. Seeds from glyphosate-resistant (GR) biotypes were collected in agricultural areas with farmer complaints about hairy fleabane control in soybean cultivation that had been treated with glyphosate for a minimum of 5 years. The glyphosate-sensitive biotypes were collected in areas without glyphosate application. In both cases, seeds from each plant were placed in an individual paper bag and stored in a refrigerator (~4 °C) for two weeks. Subsequently, seeds of each biotype were germinated in trays containing sterilized soil and commercial substrate (Mac plant – Mec Prec, Brazil) 3:1, and watered daily in a greenhouse at 30 °C/20 °C day/night (± 4 °C) with 12-h photoperiod. Thirty days after emergence (30 DAE) seedlings of each biotype were transplanted to pots containing 2.0 L of soil and substrate mix (according described above). Thirty days after (60 DAE; rosette stage – plants 6 to 8 cm in diameter) plants were treated with glyphosate (1480 g ae·ha^{-1} – Roundup Original DI 370 g ae·L^{-1}; Monsanto) using CO_2 sprayer and 150 L·ha^{-1} of spray volume. Glyphosate-treated plants were considered resistant if they survived at 28 days after treatment (DAT).

Two biotypes of interest were selected from the municipality of Pelotas, 48 km apart from each other: B11R – glyphosate-resistant 32°04'05.91" S, 52°52'59.14" W; B17S – glyphosate-sensitive 31°49'15.15" S, 52°27'39.55" W. The *Conyza bonariensis* species of two selected biotypes was determined using molecular simple sequence repeats (SSR) markers through genotyping approach (Abercrombie et al. 2009; Marochio et al. 2017).

During two generations (F_0 and F_1) B11R and B17S non-treated plants were self-pollinated and GR segregation assessed by glyphosate treatment. In F_0 generation, segregation was assessed in 94 plants in each biotype (B11R and B17S) and F_1 generation, in 220 plants per biotype. Following glyphosate treatment (1480 g ae·ha^{-1}, 60 DAE; rosette stage), in both generations it was evaluated whether the plants were alive or died at 28 DAT. After that,

percentual segretating was calculated for B11R and B17S biotypes in both generations.

Glyphosate dose-response

Plants of the F_1 generation of B11R and B17S were cultivated until 60 DAE in a greenhouse. Plants were treated with glyphosate (Roundup Original DI 370 g ae·L^{-1}; Monsanto) at the following doses: 0; 92.2; 185; 370; 740; 1480; 2960; 5920; 11840; and 23680 g ae·ha^{-1}, with a CO_2 sprayer and 150 L·ha^{-1} of spray volume. The experiment was performed twice using a completely randomized design with four replicates (three plants per pot formed each replicate). Shoot material was harvested at 28 DAT and dried at 60 °C until constant weight to determine dry weight reduction compared with the non-treated control.

Dose-response curves for plant bioassays were obtained by a non-linear regression using the log-logistic Eq. 1:

$$y = C + (D - C)/ [1 + (x/GR_{50})^{b}] \quad (1)$$

where C is the lower limit; D is the upper limit; b is the slope at the GR_{50}; and GR_{50} indicates the glyphosate dose necessary to reduce dry weight by 50%. The resistance factor (RF) was calculated by the GR_{50} of the R biotype divided by that of the S biotype to estimate the resistance level. The regression parameters for each biotype were obtained using Sigma Plot® (version 12.5, SPSS Inc, Chicago, IL, USA).

Experimental design and evaluated variables

Two time-course experiments were performed in Pelotas, RS state, Brazil, in a greenhouse at 25 °C/15 °C day/night (± 3 °C) with 12-h photoperiod with five replicates (three plants per pot formed each replicate) in a completely randomized design. For both experiments, plants of F_1 generation of B11R and B17S were grown until 60 DAE as previously described. The experiment 1 (E1) was arranged in a three-factorial scheme 2 × 5 × 5. The first factor comprised two *C. bonariensis* biotypes (B11R and B17S); the second factor comprised five glyphosate doses: 0; 370; 1480; 5920 and 11840 g ae·ha^{-1}; and the third factor comprised evaluations of variables in five time-points after glyphosate treatment: 0; 12; 24; 48 and 96 hours after treatment (HAT).

The experiment 2 (E2) was arranged in a three-factorial scheme 2 × 2 × 8. The first factor comprised two *C. bonariensis* biotypes (B11R and B17S); the second factor comprised two glyphosate doses: 0 and 1480 g ae·ha^{-1}; and the third factor comprised evaluations of variables in eight times-points after glyphosate treatment: 0; 24; 48; 96; 192; 264; 384 and 552 HAT.

The evaluated variables in both E1 and E2 were: a) shikimic-acid content (SAC); oxidative stress and tissue damage according to b) ROS production – H_2O_2; c) lipid peroxidation measured using thiobarbituric acid-reactive substances (TBARS); activity of antioxidant enzymes – d) SOD; e) CAT; and f) APX.

For enzyme evaluations, the second and third fully expanded leaves (from apex) were harvested after glyphosate treatment according to each treatment. Enzyme evaluations were performed with five technical replicates, which were recorded on a Spectrophotometer Ultrospec 2000 UV-Visible (Pharmacia Biotech) in a 2-mL cuvette.

Whole-plant shikimic-acid bioassay

Shikimic-acid content (SAC) quantification was performed according to Singh and Shaner (1998) and Perez-Jones et al. (2007) with some modifications. Leaves of B11R and B17S biotypes were harvested after each treatment and stored at –80 °C. Leaf tissues were chopped, and 0.25 g of fresh weight samples were placed in 15-ml tubes containing 5 ml of 1.25 N HCl. The samples were mixed, placed at –80 °C until frozen, thawed at room temperature, and incubated at 37 °C for 45 min. Five technical samples of 125-µL were extracted from the tubes and mixed with reaction buffer [0.25% (w/v) periodic-acid and sodium(meta)periodate solution] and incubated at 37 °C for 30 min to allow shikimic acid oxidation. After incubation, samples were mixed with 1000-µL 0.6 N NaOH/0.22 M Na_2SO_3 and measured spectrophotometrically at 380 nm. The shikimic-acid content was determined based on a standard curve and expressed in µg·g^{-1} fresh weight (µg·g^{-1} FW).

Oxidative stress and tissue damage

Oxidative stress was determined according to levels of H_2O_2, as described by Sergiev et al. (1997) and tissue damage according to lipid peroxidation (Heath and Packer 1968). Lipid peroxidation was measured using species reactive to thiobarbituric acid (TBARS) by the accumulation of malondialdehyde (MDA), a product of lipid peroxidation. The solution of 10 mL of 0.1% trichloroacetic acid (TCA) →

was added to 1.0 g of processed leaf tissue and vortexed. Samples were centrifuged at 14000 rpm for 25 min at 4 °C. For H_2O_2 quantification, the supernatant was collected and five technical samples of 0.2 mL were added to 0.8 mL of phosphate buffer 10 mM (pH 7.0) and 1.0 mL of potassium iodide 1 M. The solution was kept for 10 min at room temperature. Absorbance was recorded at 390 nm. The concentration of H_2O_2 was determined using a standard curve with known concentrations of H_2O_2 and expressed in millimoles per gram of fresh weight ($mM \cdot g^{-1}$ FW). For the determination of TBARS, the supernatant was collected and five technical samples of 0.5 mL were added to 1.5 mL of TBARS 0.5% and TCA 10%. Samples were placed in a 90 °C water bath for 20 min, and immediately after cooled in an ice bath for 10 min. Absorbance was recorded at 532 and 600 nm. Non-specific absorption at 600 nm was subtracted from the reading at 532 nm. The MDA concentration was calculated using an extinction coefficient of 155 $mM^{-1} \cdot cm^{-1}$, and the results were expressed as nM MDA·g^{-1} of fresh weight (nM MDA·g^{-1} FW).

Enzyme assays

To determine the activity of antioxidant enzymes SOD, CAT, and APX, extraction was performed. From this extract the enzymes activities were calculated and expressed in active units (AU) per milligram of fresh weight (AU·mg^{-1} FW). To 1.0 g of processed leaf tissue with 0.1 g of polyvinylpyrrolidone (PVP), 4.5 mL of 200 mM phosphate buffer (pH 7.8), 90 µL of 10 mM EDTA, 900 µL of 200 mM ascorbic acid, and 3.51 mL of ultrapure water was added and mixed, and after centrifuged at 14000 rpm at 4 °C for 25 min. The supernatant was collected and used for all enzyme assays.

Total SOD activity was measured according to Peixoto et al. (1999) in a 2-mL reaction mixture containing 1.0 mL phosphate buffer 100 mM (pH 7.8), 400 µL of methionine 70 mM, 20 µL of ethylenediaminetetraacetic (EDTA) 10 µM, 390 µL of ultrapure water, 150 µL ρ-nitro blue tetrazolium chloride (NBT) 1 mM, 20 µL of enzyme extract. After the addition of 20 µL of riboflavin 0.2 mM last, the reaction was initiated. The samples were illuminated with fluorescent lamps at 4000 lx, 15 Watts for 10 min and after the absorbance at 560 nm was recorded. One activity unit (AU) of SOD was equivalent to the amount of enzyme necessary to inhibit 50% of NBT reduction at 560 nm. The results were expressed in AU·mg^{-1} of fresh weight·min^{-1} (AU·mg^{-1} FW·min^{-1}).

Catalase and ascorbate peroxidase activities were determined according to Azevedo et al. (1998). Catalase activity was evaluated according to the decline in absorbance for 1.5 min at 240 nm, which indicates the H_2O_2 consumption (extinction coefficient: 39.4 $mM \cdot cm^{-1}$). The 2-mL reaction mixture contained 1.0 mL of potassium phosphate buffer 200 mM (pH 7.0), 850 µL of ultrapure water, 100 µL of H_2O_2 250 mM, and 50 µL of enzyme extract last to initiate the reaction.

Following the decrease in absorbance at 290 nm for 1.5 min the ascorbate peroxidase activity was measured. The 2.0-mL reaction mixture contained 1.0 mL of potassium phosphate buffer 200 mM (pH 7.0), 750 µL of ultrapure water, 100 µL of ascorbic-acid (ASC) 10 mM, 50 µL of enzyme extract, and 100 µL of H_2O_2 2.0 mM last to initiate the reaction. The APX activity was calculated using an extinction coefficient of 2.9 $mM^{-1} \cdot cm^{-1}$.

For CAT and APX activities calculation purposes, the decrease of one absorbance unit was considered equivalent to one active unit (AU). From the total extract, the activities were calculated by the amount of extract that reduced the absorbance reading by one AU and expressed in AU·mg^{-1} of fresh weight·min^{-1} (AU·mg^{-1} FW·min^{-1}).

Statistical analysis

Statistical analysis was performed using the GLM package statement from SAS (version 9.0, SAS Institute Inc, Cary, NC, USA), and results fitted using Sigma Plot®. To test for normality (Shapiro-Wilk's test) and homogeneity of variance (Hartley's test), SAS Proc Univariate was used. Analysis of variance (ANOVA) was performed at F test ($p \leq 0.05$) using Proc Mixed. Test-t were applied in regressions at $p \leq 0.05$. Interactions among factors were verified at $p \leq 0.05$, and characters that presented significant interaction were split to simple effects referents to qualitative factor (biotypes). Effects of quantitative factors (glyphosate doses and times after treatment) were splitted using linear regressions at the highest significant polynomial degree, and equations from each level of factors in interaction were split ($p \leq 0.05$). Significant characters were submitted to linear correlations analysis and effects of biotypes and glyphosate doses isolated to verify the tendency of association between interest

characters at $p \leq 0.05$. To identify multivariate relation it was performed a Step-Wise multiple regression, where it was fixed how dependent variable H_2O_2, while SAC, TBARS, SOD, CAT, and APX were considered explanatory from the model.

For both E1 and E2, the ANOVA demonstrated interactions between factors at $p \leq 0.05$ for all evaluated enzymatic variables (Tables 1 and 2). Polynomial models of the second, third and fourth order were applied to describe enzyme results (Tables 3 and 4).

Table 1. Simple qualitative effect of separating the tissue damage and activity of antioxidant enzymes as a response of glyphosate treatment in resistant (B11R) and sensitive (B17S) biotypes of *Conyza bonariensis* as a function of time after treatment and doses. Experiment 1.

Time after treatment (h)	Biotype									
	B11R					B17S				
	Glyphosate doses (g ae·ha^{-1})									
	0	370	1480	5920	11840	0	370	1480	5920	11840
Shikimic acid (µg·g^{-1} FW)										
0	0.98 α	1.67 α	1.22 α	0.95 α	1.34 α	0.88 α	0.66 α	0.85 α	1.05 α	1.20 α
12	0.87 α	3.04 α	5.49 α	5.62 β	6.33 α	0.66 α	4.41 α	6.02 α	7.91 α	6.88 α
24	1.76 α	1.26 β	14.13 β	14.13 α	12.40 α	0.91 α	14.26 α	18.65 α	7.96 β	8.64 β
48	1.77 α	21.48 α	29.33 α	21.89 α	21.54 β	0.94 α	22.71 α	28.08 α	18.68 β	24.50 α
96	1.33 α	28.20 β	28.84 β	23.85 β	30.19 α	1.11 α	31.00 α	32.27 α	31.29 α	25.06 β
CV (%)	9.62									
H_2O_2 (mM·g^{-1} FW)										
0	13.02 α	17.00 α	15.34 α	19.66 β	16.68 β	15.00 α	19.68 α	14.36 α	28.00 α	21.66 α
12	14.02 α	24.66 α	38.32 α	46.68 α	33.00 β	15.68 α	26.68 α	30.34 β	40.68 β	36.68 α
24	13.68 α	20.66 β	28.98 α	46.32 α	59.68 α	14.02 α	27.00 α	27.98 α	45.34 α	59.34 α
48	14.34 α	44.32 α	59.68 α	95.00 α	100.10 β	14.34 α	40.66 β	61.02 α	78.00 β	276.32 α
96	14.68 β	63.02 β	75.22 β	94.00 β	74.66 β	18.32 α	451.00 α	383.00 α	416.02 α	371.66 α
CV (%)	3.74									
Thiobarbituric Acid-reactive Substances (nM MDA·g^{-1} FW)										
0	8.87 α	9.80 α	12.85 α	8.67 α	8.57 α	9.44 α	7.63 α	11.25 α	10.99 α	5.32 α
12	8.16 α	14.71 α	16.67 α	8.46 α	16.15 α	8.41 α	15.02 α	15.69 α	9.34 α	14.19 α
24	10.22 α	23.48 α	35.53 β	23.53 β	37.12 α	9.81 α	23.84 α	43.71 α	34.17 α	37.01 α
48	10.73 α	50.53 β	53.41 β	64.92 α	71.43 β	11.81 α	73.65 α	65.54 α	64.72 α	89.60 α
96	9.96 α	40.10 β	50.89 β	49.50 β	39.89 β	11.41 α	78.40 α	97.70 α	90.01 α	73.65 α
CV (%)	8.83									
Superoxide Dismutase (AU·mg^{-1} of FW·min^{-1})										
0	95.80 α	104.91 α	93.03 α	96.37 α	90.19 α	96.02 α	91.21 β	96.18 α	88.12 α	88.91 α
12	96.02 α	101.66 α	102.77 α	110.99 α	98.37 α	96.84 α	98.22 α	99.68 α	91.56 β	85.32 β
24	98.73 α	176.49 α	175.13 α	183.41 α	163.08 α	95.54 α	167.51 α	163.91 α	177.61 α	158.02 α
48	98.06 α	303.83 α	312.21 α	313.80 α	313.96 α	90.47 α	298.64 α	301.63 α	308.74 α	314.98 α
96	96.82 α	306.22 α	327.57 α	312.97 α	317.85 α	93.34 α	151.29 β	161.93 β	144.44 β	141.64 β
CV (%)	6.17									
Catalase (AU·mg^{-1} of FW·min^{-1})										
0	9.40 α	11.35 α	11.36 α	10.96 α	12.92 α	10.57 α	9.79 α	10.18 α	12.92 α	12.53 α
12	10.96 α	17.62 α	23.49 α	19.58 α	18.40 α	9.40 α	18.03 α	27.41 β	20.36 α	20.36 α
24	11.35 α	21.14 α	21.93 α	23.89 α	28.56 β	9.79 α	17.62 β	23.10 α	20.75 α	34.46 α
48	10.57 α	36.42 α	41.12 α	42.29 α	48.95 α	9.39 α	30.54 β	37.59 β	38.77 β	46.99 α
96	10.55 α	48.16 α	52.47 α	54.82 α	49.34 β	10.18 α	44.25 β	54.43 α	48.95 β	52.86 α
CV (%)	10.73									
Ascorbate peroxidase (AU·mg^{-1} of FW·min^{-1})										
0	21.28 α	20.22 α	19.68 α	21.81 α	21.81 α	23.41 α	18.62 α	22.88 α	22.88 α	24.47 α
12	19.68 α	23.94 α	37.77 α	26.60 α	25.00 α	21.81 α	24.47 α	38.83 α	27.66 α	26.07 α
24	20.75 α	28.73 α	52.14 α	62.77 α	68.63 α	21.81 α	30.32 α	45.75 α	48.95 β	45.75 β
48	20.74 α	197.38 α	269.73 α	269.20 α	261.75 α	22.88 α	126.09 β	121.30 β	122.36 β	130.88 β
96	19.15 α	226.11 α	293.14 α	310.70 α	314.95 α	20.22 α	204.29 β	258.03 β	295.80 β	281.97 β
CV (%)	6.85									

*Significant at $p \leq 0.05$; ^{NS} non-significant at $p \leq 0.05$; CV = coefficient of variation; Different symbols in each glyphosate dose and time indicate the difference between biotypes.

Table 2. Simple qualitative effect of separating the cellular damage and activity of antioxidant enzymes as a response of glyphosate treatment in resistant (B11R) and sensitive (B17S) biotypes of *Conyza bonariensis* as a function of time after treatment and doses. Experiment 2.

Time after treatment (hours)	Biotype			
	B11R		B17S	
	Glyphosate doses (g ae·ha^{-1})			
	0	1480	0	1480
Shikimic acid (µg·g^{-1} FW)				
0	2.28 α	1.33 α	1.27 α	1.95 α
24	1.47 α	22.55 α	1.37 α	20.12 β
48	1.33 α	28.51 α	1.59 α	29.92 α
96	2.49 α	31.22 α	0.84 α	30.37 α
192	3.27 α	17.31 β	3.49 α	31.37 α
264	2.12 α	10.95 α	2.11 α	0.00 β
384	3.08 α	7.73 α	0.00 β	0.00 β
552	3.61 α	6.91 α	0.00 β	0.00 β
CV (%)	16.23			
H_2O_2 (mM g^{-1} FW)				
0	15.33 α	14.33 α	14.66 α	13.66 α
24	15.00 α	34.66 α	12.00 α	29.66 α
48	30.66 α	67.66 α	29.99 α	59.00 α
96	33.33 α	100.66 β	26.66 α	328.00 α
192	24.33 α	108.33 β	25.99 α	256.66 α
264	27.33 α	84.33 α	20.91 α	0.00 β
384	26.66 α	77.00 α	0.00 β	0.00 β
552	27.33 α	58.66 α	0.00 β	0.00 β
CV (%)	15.55			
Thiobarbituric Acid-reactive Substances (nM MDA·g^{-1} FW)				
0	13.05 α	17.65 α	15.74 α	13.57 α
24	11.71 α	27.45 α	13.88 α	30.55 α
48	11.09 α	42.32 β	12.18 α	49.34 α
96	10.99 α	35.66 β	11.71 α	90.06 α
192	11.09 α	28.02 β	10.01 α	57.75 α
264	9.54 α	27.71 α	11.37 α	0.00 β
384	13.52 α	37.26 α	0.00 β	0.00 β
552	13.67 α	19.61 α	0.00 β	0.00 β
CV (%)	16.57			
Superoxide Dismutase (AU·mg^{-1} of FW·min^{-1})				
0	95.79 α	101.52 α	83.27 α	112.17 α
24	112.90 α	190.13 β	90.86 α	260.09 α
48	98.73 α	298.25 α	92.38 α	265.12 β
96	114.05 α	307.88 α	101.94 α	153.64 β
192	96.81 α	327.47 α	105.48 α	196.63 β
264	114.56 α	314.82 α	103.58 α	0.00 β
384	104.11 α	311.03 α	0.00 β	0.00 β
552	108.44 α	274.26 α	0.00 β	0.00 β
CV (%)	18.53			
Catalase (AU·mg^{-1} of FW·min^{-1})				
0	8.61 α	8.61 α	9.79 α	9.39 α
24	7.83 α	28.58 α	9.79 α	22.32 β
48	9.39 α	46.99 α	9.39 α	45.81 α
96	9.00 α	55.99 β	8.61 α	65.00 α
192	9.39 α	56.78 α	10.18 α	24.28 β
264	9.39 α	55.99 α	8.68 α	0.00 β
384	9.00 α	54.82 α	0.00 β	0.00 β
552	9.39 α	37.69 α	0.00 β	0.00 β
CV (%)	16.41			
Ascorbate peroxidase (AU·mg^{-1} of FW·min^{-1})				
0	28.19 α	25.00 α	22.87 α	16.49 α
24	17.55 α	31.38 α	23.94 α	34.04 α
48	21.28 α	253.24 α	22.34 α	155.35 β
96	19.15 α	293.14 α	19.68 α	269.73 α
192	20.74 α	290.48 α	19.68 α	115.97 β
264	30.32 α	362.83 α	21.94 α	0.00 β
384	18.08 α	367.09 α	0.00 β	0.00 β
552	23.40 α	301.25 α	0.00 β	0.00 β
CV (%)	25.91			

*Significant at $p \leq 0.05$; ^{NS} non-significant at $p \leq 0.05$; CV = coefficient of variation; Different symbols in each glyphosate dose and time indicate the difference between biotypes.

Table 3. Regression[1], mean square error (MSE) and determination coefficient (R^2) for the polynomial model of evaluated variables extracted from glyphosate-resistant (B11R) and sensitive (B17S) *Conyza bonariensis* biotypes as a response to glyphosate dose (g ae·ha^{-1}) and time after glyphosate treatment. Experiment 1.

Variable	Biotype		Regression[1]	MSE	R^2
Shikimic acid	B11R	0	$y=0.81858+0.03063x-0.00026173x^2$	0.388	0.38*
Shikimic acid	B11R	370	$y=1.6722+0.6551x-0.0659x^2+0.0018x^3-0.000012x^4$	1.085	0.99*
Shikimic acid	B11R	1480	$y=0.9968+0.3035x+0.0122x^2-0.0001287x^3$	1.302	0.99*
Shikimic acid	B11R	5920	$y=0.9536+0.0013x+0.0435x^2-0.0010x^3+0.0000060x^4$	1.268	0.98*
Shikimic acid	B11R	11840	$y=0.87758+0.540913x-0.0024459x^2$	1.160	0.99*
Shikimic acid	B17S	0	$y=0.8868-0.0580x+0.0042x^2-0.000089x^3-0.000000532x^4$	0.137	0.58*
Shikimic acid	B17S	370	$y=0.6646-0.2687x+0.0644x^2-0.0014x^3+0.0000087x^4$	1.373	0.98*
Shikimic acid	B17S	1480	$y=0.8522-0.3006x+0.0818x^2-0.0018x^3-0.000011x^4$	0.867	0.99*
Shikimic acid	B17S	5920	$y=1.0552+1.2031x-0.0699x^2+0.0015x^3-0.0000096x^4$	1.392	0.99*
Shikimic acid	B17S	11840	$y=1.1974+0.9277x-0.0525x^2+0.0013x^3-0.0000089x^4$	1.383	0.98*
H_2O_2	B11R	0	$y=13.4260$	2.799	0.032^{NS}
H_2O_2	B11R	370	$y=17+1.8953x-0.1433x^2+0.0034x^3-0.00002231x^4$	2.980	0.98*
H_2O_2	B11R	1480	$y=15.34+4.9067x-0.3309x^2+0.0073x^3-0.00004549x^4$	3.048	0.98*
H_2O_2	B11R	5920	$y=19.66+4.9095x-0.2972x^2+0.0068x^3-0.00004366x^4$	2.137	0.99*
H_2O_2	B11R	11840	$y=16.68+0.4954x+0.0927x^2-0.0018x^3+0.00000915x^4$	2.804	0.99*
H_2O_2	B17S	0	$y=15.4661-0.070588x+0.00104046x^2$	2.144	0.34*
H_2O_2	B17S	370	$y=19.4144+1.37006x-0.07104x^2+0.00107921x^3$	2.574	0.99*
H_2O_2	B17S	1480	$y=14.36+3.0102x-0.1836x^2+0.0038x^3-0.0000195x^4$	2.556	0.99*
H_2O_2	B17S	5920	$y=28.4068+1.2928x-0.0396x^2+0.00071086x^3$	2.672	0.99*
H_2O_2	B17S	11840	$y=21.660+2.9912x-0.2520x^2+0.0098x^3-0.0000741x^4$	2.703	0.99*
Thiobarbituric acid-reactive substances	B11R	0	$y=9.037925$	2.560	0.043^{NS}
Thiobarbituric acid-reactive substances	B11R	370	$y=10.0404-0.01305x+0.03215x^2-0.0002995x^3$	2.277	0.98*
Thiobarbituric acid-reactive substances	B11R	1480	$y=12.8516-1.0087x+0.1448x^2-0.00306x^3+0.00001785x^4$	2.789	0.98*
Thiobarbituric acid-reactive substances	B11R	5920	$y=8.4072-0.6170x+0.06404x^2-0.0005537x^3$	2.281	0.99*
Thiobarbituric acid-reactive substances	B11R	11840	$y=8.5680-0.3868x+0.1069x^2-0.0019x^3+0.000009473x^4$	3.174	0.98*
Thiobarbituric acid-reactive substances	B17S	0	$y=9.13664$	2.597	0.134^{NS}
Thiobarbituric acid-reactive substances	B17S	370	$y=7.6388+0.9554x-0.0493x^2+0.0019x^3-0.000015006x^4$	2.944	0.99*
Thiobarbituric acid-reactive substances	B17S	1480	$y=11.2516-1.7686x+0.2352x^2-0.0051x^3+0.00003087x^4$	3.013	0.99*
Thiobarbituric acid-reactive substances	B17S	5920	$y=10.9938-2.2873x+0.2302x^2-0.0045x^3+0.00002620x^4$	2.875	0.99*
Thiobarbituric acid-reactive substances	B17S	11840	$y=5.0474+0.1905x+0.0601x^2-0.0005697x^3$	3.374	0.99*
Superoxide dismutase	B11R	0	$y=96.78659$	9.834	0.0008^{NS}
Superoxide dismutase	B11R	370	$y=104.9096-6.1163x+0.6120x^2-0.0111x^3+0.0000586x^4$	10.285	0.99*
Superoxide dismutase	B11R	1480	$y=93.0266-3.7183x+0.4694x^2-0.0081x^3+0.00004089x^4$	10.316	0.99*
Superoxide dismutase	B11R	5920	$y=96.3716-3.0452x+0.4441x^2-0.0078x^3+0.00003987x^4$	10.881	0.99*
Superoxide dismutase	B11R	11840	$y=88.4491-0.87204x+0.1991x^2-0.001720x^3$	10.986	0.99*
Superoxide dismutase	B17S	0	$y=95.95514$	7.883	0.034^{NS}
Superoxide dismutase	B17S	370	$y=91.2108-3.8467x+0.4558x^2-0.00764x^3+0.00003518x^4$	10.690	0.98*
Superoxide dismutase	B17S	1480	$y=94.1578-1.3150x+0.21502x^2-0.0020206x^3$	11.024	0.98*
Superoxide dismutase	B17S	5920	$y=88.1202-6.0333x+0.6647x^2-0.0122x^3+0.00006311x^4$	7.444	0.99*
Superoxide dismutase	B17S	11840	$y=88.9168-5.4785x+0.5250x^2-0.00821x^3+0.00003545x^4$	11.152	0.99*
Catalase	B11R	0	$y=10.426350$	2.155	0.0044^{NS}
Catalase	B11R	370	$y=11.8015+0.2831x+0.0083x^2-0.00007621x^3$	2.789	0.96*
Catalase	B11R	1480	$y=11.3564+2.3010x-0.1431x^2+0.0032x^3-0.00002011x^4$	3.169	0.96*
Catalase	B11R	5920	$y=11.5583+0.4319x+0.0081x^2-0.00008273x^3$	2.941	0.97*
Catalase	B11R	11840	$y=12.7670+0.3265x+0.0173x^2-0.000174x^3$	2.299	0.98*
Catalase	B17S	0	$y=9.868320$	1.777	0.00^{NS}
Catalase	B17S	370	$y=9.79+1.4893x-0.0891x^2+0.0019x^3-0.000012336x^4$	2.670	0.96*
Catalase	B17S	1480	$y=10.1816+3.2987x-0.2029x^2+0.0042x^3-0.00002578x^4$	3.007	0.97*
Catalase	B17S	5920	$y=13.8922+0.16205x+0.01215x^2-0.00010456x^3$	3.478	0.94*
Catalase	B17S	11840	$y=11.3559+1.0519x-0.006447x^2$	3.687	0.95*
Ascorbate peroxidase	B11R	0	$y=20.8820$	2.528	0.045^{NS}
Ascorbate peroxidase	B11R	370	$y=20.2170+2.3584x-0.2789x^2+0.0098x^3-0.00007306x^4$	7.255	0.99*
Ascorbate peroxidase	B11R	1480	$y=19.6850+4.3251x-0.3722x^2+0.0125x^3-0.00009213x^4$	7.457	0.99*
Ascorbate peroxidase	B11R	5920	$y=21.8130-0.0430x-0.0115x^2+0.0045x^3-0.00004259x^4$	6.823	0.99*
Ascorbate peroxidase	B11R	11840	$y=21.8130-1.2041x+0.1068x^2+0.0015x^3-0.00002327x^4$	6.864	0.99*
Ascorbate peroxidase	B17S	0	$y=22.90360$	3.946	0.045^{NS}
Ascorbate peroxidase	B17S	370	$y=18.6210+1.5862x-0.1480x^2+0.0051x^3-0.00003724x^4$	7.381	0.99*
Ascorbate peroxidase	B17S	1480	$y=22.8770+2.7618x-0.1724x^2+0.0048x^3-0.00003169x^4$	5.464	0.99*
Ascorbate peroxidase	B17S	5920	$y=22.7875-0.3235x+0.0669x^2-0.000354x^3$	5.522	0.99*
Ascorbate peroxidase	B17S	11840	$y=24.8534-1.0792x+0.0976x^2-0.000609x^3$	8.189	0.99*

*Significant at $p \leq 0.05$; ^{NS} non-significant at $p \leq 0.05$.

Table 4. Regression[1], mean square error (MSE) and determination coefficient (R^2) for the polynomial model of evaluated variables extracted from glyphosate-resistant (B11R) and sensitive (B17S) *Conyza bonariensis* biotypes as a response to glyphosate dose (g ae·ha^{-1}) and time after glyphosate treatment. Experiment 2.

Variable	Biotype	Rate (g ae·ha^{-1})	Regression[1]	MSE	R^2
Shikimic acid	B11R	0	$y=2.2964-0.0615x+0.001208x^2-0.00000724x^3+0.000000017x^4$	0.627	0.64*
Shikimic acid	B11R	1480	$y=2.1458+1.0016x-0.0109x^2+0.00004614x^3-0.000000086x^4$	2.430	0.95*
Shikimic acid	B17S	0	$y=1.1998+0.0547x-0.0019x^2+0.0000227x^3-0.000000106x^4$	0.409	0.89*
Shikimic acid	B17S	1480	$y=1.5440+1.2984x-0.0238x^2+0.000218x^3-0.000000980x^4$	1.647	0.99*
H_2O_2	B11R	0	$y=14.3015+0.0263x+0.0095x^2-0.000125x^3+0.00000060x^4$	6.159	0.52*
H_2O_2	B11R	1480	$y=12.6273+1.1204x+0.000767x^2-0.0000416x^3+0.00000013x^4$	9.045	0.93*
H_2O_2	B17S	0	$y=12.3267+0.30203x-0.001486x^2+0.00000163x^3$	5.892	0.77*
H_2O_2	B17S	1480	$y=21.5298-4.7439x+0.1986x^2-0.001716x^3+0.00000599x^4$	20.172	0.98*
Thiobarbituric acid-reactive substances	B11R	0	$y=12.1651-0.01238x+0.00002926x^2$	2.343	0.16*
Thiobarbituric acid-reactive substances	B11R	1480	$y=16.8011+0.7957x-0.00923x^2+0.00004050x^3-0.00000007x^4$	3.931	0.81*
Thiobarbituric acid-reactive substances	B17S	0	$y=15.8364-0.1427x+0.00245x^2-0.00002345x^3+0.00000011x^4$	1.241	0.96*
Thiobarbituric acid-reactive substances	B17S	1480	$y=14.1699+0.19604x+0.2146x^2-0.000229x^3+0.00000084x^4$	5.347	0.97*
Superoxide dismutase	B11R	0	$y=104.05809$	15.103	0.01^{NS}
Superoxide dismutase	B11R	1480	$y=98.7349+5.6050x-0.0501x^2+0.000203x^3-0.00000038x^4$	23.960	0.92*
Superoxide dismutase	B17S	0	$y=82.4703+0.5084x-0.007305x^2+0.00005262x^3-0.000000016x^4$	12.632	0.93*
Superoxide dismutase	B17S	1480	$y=110.2537+12.4663x-0.3103x^2+0.00294x^3-0.0000127x^4$	43.604	0.86*
Catalase	B11R	0	$y=8.723882$	1.520	0.03^{NS}
Catalase	B11R	1480	$y=8.0414+1.1566x-0.009980x^2+0.0000377x^3-0.000000067x^4$	2.523	0.98*
Catalase	B17S	0	$y=10.2686-0.0573x+0.000734x^2-0.00000284x^3$	1.581	0.88*
Catalase	B17S	1480	$y=8.9403+03284x+0.0178x^2-0.000246x^3+0.000001123x^4$	5.673	0.95*
Ascorbate peroxidase	B11R	0	$y=27.5582-0.5382x+0.0115x^2-0.000107x^3+0.000000048x^4$	4.162	0.55*
Ascorbate peroxidase	B11R	1480	$y=9.6795+1.8663x+0.0848x^2-0.00119x^3-0.000005834x^4$	36.661	0.93*
Ascorbate peroxidase	B17S	0	$y=22.9555+0.0808x-0.00259x^2+0.00001849x^3-0.000000004x^4$	4.136	0.86*
Ascorbate peroxidase	B17S	1480	$y=12.7873-1.0183x+0.1368x^2-0.00154x^3+0.00000664x^4$	44.045	0.83*

*Significant at $p \leq 0.05$; *Significant at $p \leq 0.05$; ^{NS} non-significant at $p \leq 0.05$..

RESULTS AND DISCUSSION

Biotype selection and glyphosate dose-response

From the 54 collected hairy fleabane biotypes, two of them from the same geographical region were selected according to their high and low sensitivity to glyphosate, respectively for glyphosate-resistant (B11R) and glyphosate-sensitive (B17S) biotypes based on presenting symptoms after glyphosate treatment. Segregation experiments performed in the first (F_0) and second (F_1) self-pollinating generations evaluated at 28 DAT presented results of 100% of alive in B11R and 100% of death in B17S. This result indicates that both biotypes are non-segregating.

To determine the level of glyphosate resistance in the selected biotypes of hairy fleabane, we conducted dose-response experiments. The data were fitted to the non-linear log-logistic model, and after that used to estimate resistance factor (RF) (Fig. 1; Table 5). Shoot dry weight (SDW) in both biotypes decreased when the glyphosate dose increased. However, there was a different dose-response between the glyphosate-resistant and -sensitive biotypes, which could be observed in curve slope and confidence intervals (Fig. 1).

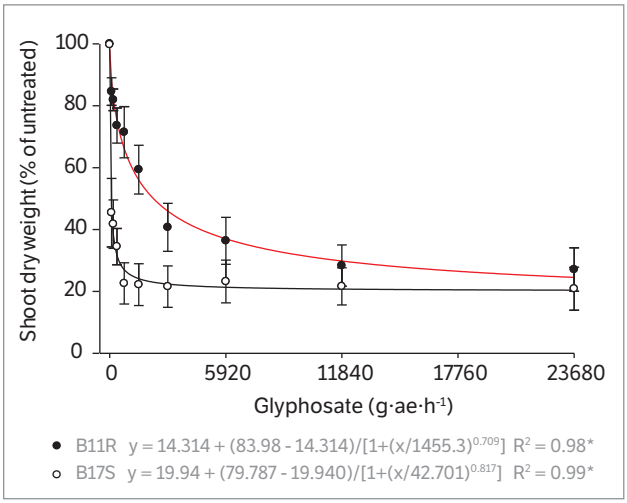

Figure 1. Glyphosate dose-response of glyphosate-resistant (B11R) and sensitive (B17S) *Conyza bonariensis* biotypes at 28 DAT. Lines are the response curves predicted from non-linear regression. Symbols represent mean dry weight of two experiments (% reduction of untreated) of four replicates, and bars the confidence intervals (CI) with 95 %.

On average of two experiments, the RF (GR_{50}) of glyphosate-resistant biotype was 18.4 (Table 5). According to HRAC (Herbicide Resistance Action Committee 2012; http://hracglobal.com/herbicide-resistance/confirming-resistance), an RF > 10 is considered a high-level of resistance.

Table 5. Parameter estimates of glyphosate dose resulting in 50% reduction of shoot dry weight (GR_{50}) in glyphosate-resistant (B11R) and sensitive (B17S) *C. bonariensis* biotypes determined at 28 DAT and based on a four-parameter log-logistic model (Eq. 1).

Herbicide	B11R	B17S	RF[b]
Herbicide	GR_{50}[a]	GR_{50}[a]	RF[b]
Glyphosate	2502.2 (954.5-4050)	135.8 (65.5-206.2)	18.4

Resistance levels were indicated by the resistance factor (RF). GR_{50}, herbicide dose causing 50 % growth reduction of plants; [b] RF (resistance factor) = GR_{50} (R)/GR_{50} (S).

Whole-plant shikimic-acid bioassay

Plants of both resistant (B11R) and -sensitive (B17S) biotypes treated with glyphosate accumulated higher levels of shikimic-acid content (SAC) than their respective non-treated control (Figs. 2a, 3a; Tables 1-4). In E1 both biotypes (B11R and B17S) had similar patterns of shikimic-acid accumulation after glyphosate treatment. Results presented in Table 1 suggest

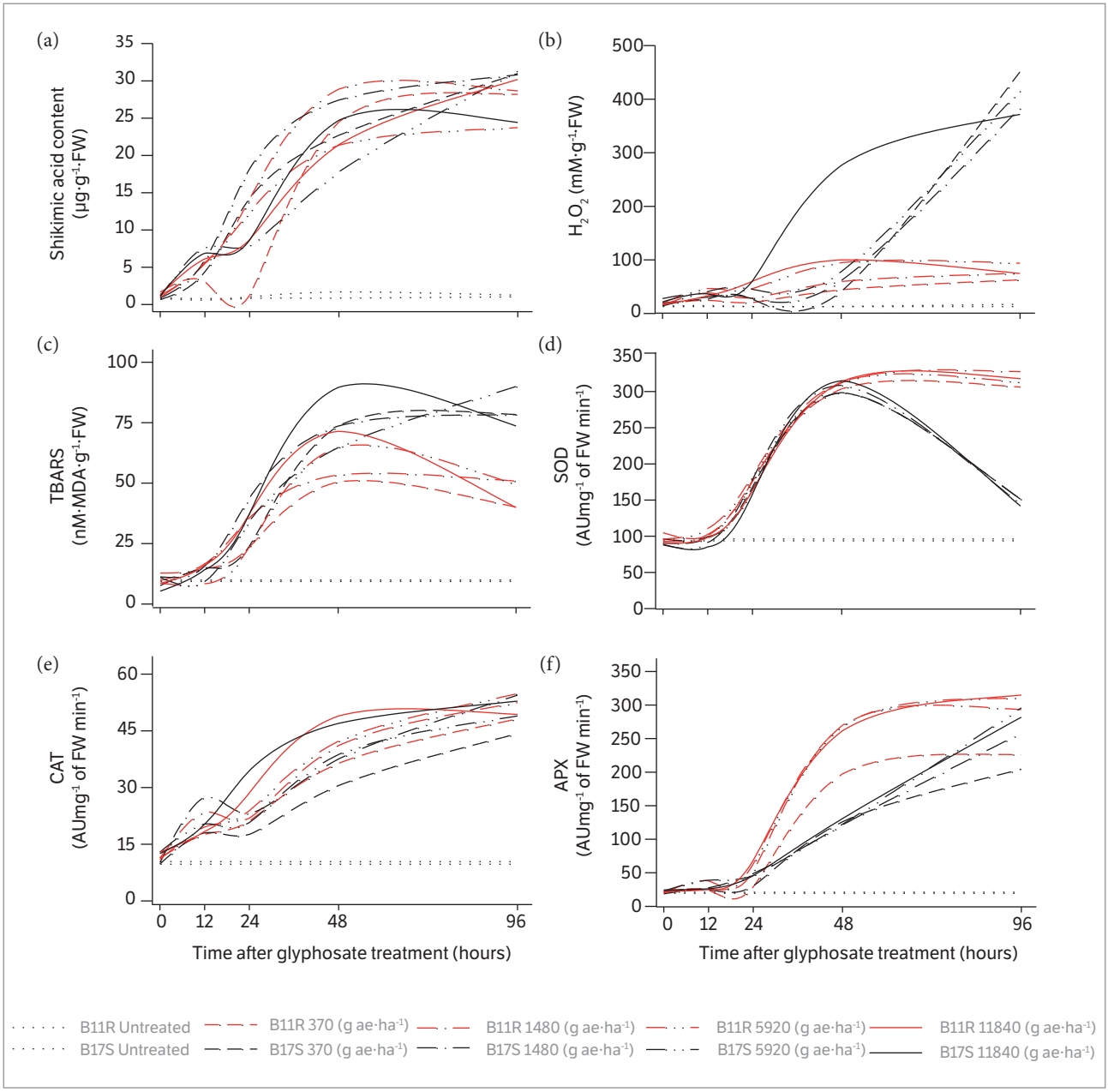

Figure 2. Tissue damage and antioxidant enzyme activities of glyphosate-resistant (B11R) and sensitive (B17S) *Conyza bonariensis* biotypes in a range of time points after glyphosate treatment and doses. Regression equations and statistics are presented in Table 3. Experiment 1. H_2O_2 = hydrogen peroxide; TBARS = thiobarbituric acid-reactive substances; SOD = superoxide dismutase; CAT = catalase; APX = ascorbate peroxidase.

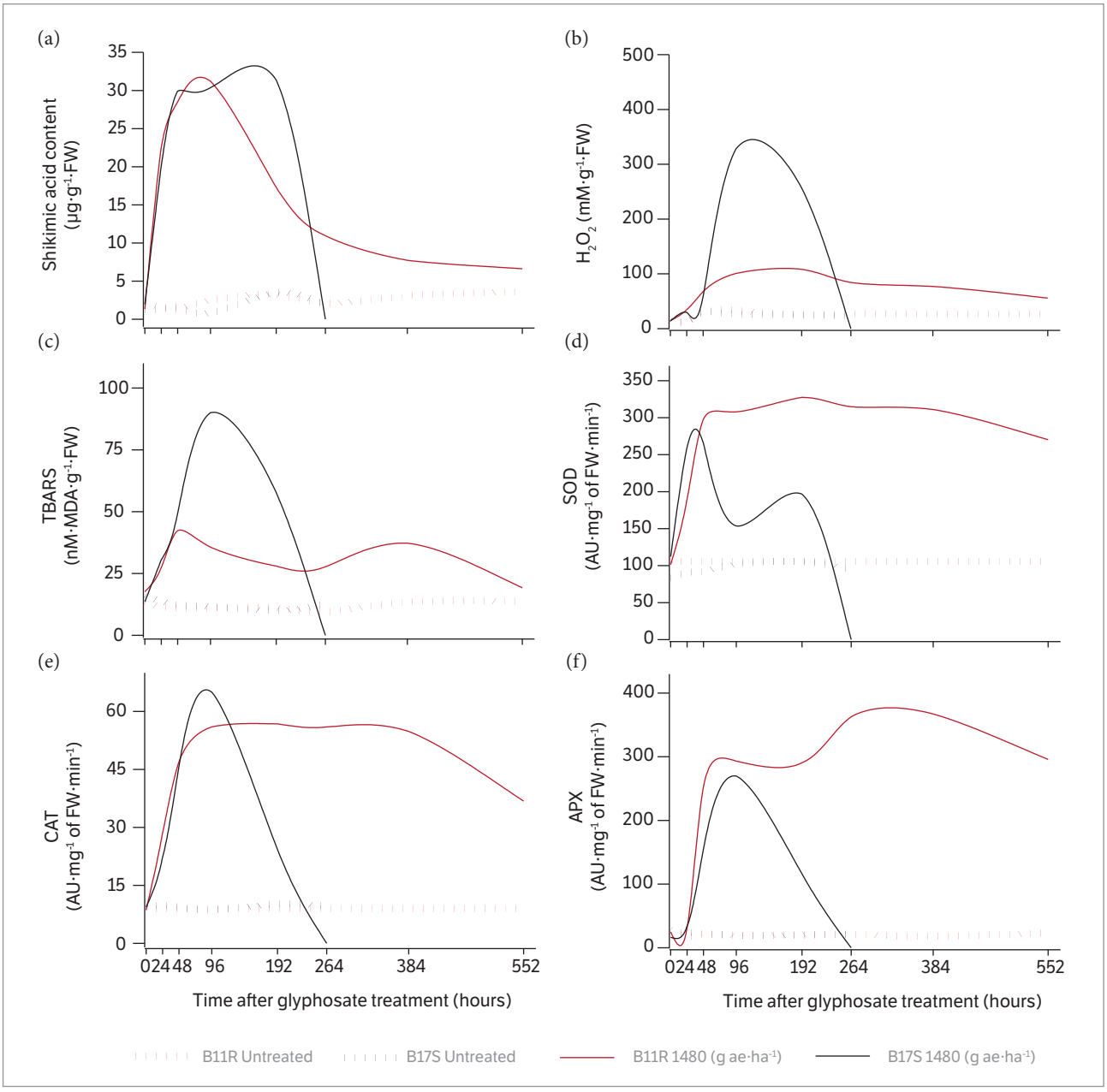

Figure 3. Tissue damage and antioxidant enzyme activities of glyphosate-resistant (B11R) and -sensitive (B17S) *Conyza bonariensis* biotypes in a range of time points after glyphosate treatment and doses. Regression equations and statistics are presented in Table 4. Experiment 2. H_2O_2 = hydrogen peroxide; TBARS = thiobarbituric acid-reactive substances; SOD = superoxide dismutase; CAT = catalase; APX = ascorbate peroxidase.

that there are differences between glyphosate doses, but it is not a clear pattern. At low glyphosate concentrations (370 g ae·ha^{-1}), the SAC in B11R biotype response was slower than B17S at 24 HAT. It also appears that the B11R never reaches the highest level of shikimic acid concentrations seen in B17S biotype at 96 HAT, except for the highest studied dose. These results could indicate reduction of glyphosate absorption, transport or sequestering, as reported in other *Conyza* species studies (Feng et al. 2004; Ferreira et al. 2008; Ge et al. 2014; Cardinali et al. 2015; Tani et al. 2015; Moretti et al. 2017; Kleinman and Rubin 2017). However, when glyphosate dose increased, the levels of SAC in both B11R and B17S biotypes were similar (Table 1). It indicates that reduction in glyphosate absorption, transport or sequestering are not preventing glyphosate to reach its target enzyme EPSPS.

On average (E2 - 0 to 192 HAT), SAC levels were 9.3 and 13.3-fold higher than controls for B11R and B17S biotypes, respectively. After of 96 HAT, the SAC levels decreased gradually in B11R until 288 HAT when did not differ from non-treated control (confident interval not shown). In B17S the SAC abruptly reduced after 192 HAT because the plants died (Fig. 3a). The primary consequence of blocking the shikimate pathway, shikimic-acid accumulation, in B11R was transient and at 288 HAT (~12 days) did not differ from non-treated control on E2 (Fig. 3a). The EPSPS inhibition could be reduced with time, allowing the enzyme to process the available shikimate-3-phosphate (S_3P), thereby reducing its concentration (Mueller et al. 2003).

Shikimic-acid accumulation in plants tissue was linearly correlated with glyphosate concentration and is a strategy to evaluate the glyphosate-resistance mechanism (Nol et al. 2012). In this way, our results of SAC levels indicate that the glyphosate-resistance mechanism of B11R biotype has non-target site resistance (NTSR). However, further studies are necessary to probe the glyphosate-resistance mechanism such as determining EPSPS gene sequence and expression patterns as well as glyphosate absorption and transport patterns. The glyphosate target-site resistance alleles have low to no known natural variation, which has led to a few cases of target-site resistance (TS). Thus, NTSR might represent the main mechanism of resistance to glyphosate (Yuan et al. 2007). Also, the glyphosate resistance in *C. canadensis* in the United States (Peng et al. 2010; Moretti et al. 2017), Greece (Nol et al. 2012), and *C. bonariensis* in the United States (Moretti et al. 2017), Australia (Hereward et al. 2018), and Israel (Kleinman and Rubin 2016) is not the result of EPSPS mutations or overexpression, but due to a NTSR.

Oxidative stress and tissue damage

In E1 higher levels of H_2O_2 (indicate oxidative stress) and TBARS (indicate lipid peroxidation) were observed in B17S than B11R at 96 HAT for all glyphosate-doses (Figs. 2b and 2c; Figs. 3b and 3c; Tables 1-4). On E2, the accumulation of H_2O_2 and TBARS in B17S were on average (from 0 to 552 HAT) 1.7 and 1.4-fold higher than in B11R, while at 96 HAT (peak) it was 3.3 and 2.5-fold, respectively (Figs. 3b and 3c; Table 2). The H_2O_2 and TBARS levels at 96 HAT in B11R were 3 and 3.2-fold higher than non-treated plants, and on average (from 0 to 552 HAT) the levels were 2.7 and 2.5-fold higher than non-treated, respectively. In B17S, the H_2O_2 and TBARS levels at 96 HAT were 12.3 and 7.7 higher than non-treated plants, and on average (from 0 to 552 HAT) 5.2 and 3.2-fold higher than non-treated, respectively (Figs. 3b and 3c).

In plants, glyphosate action also leads to ROS production and oxidative stress, which may be a secondary effect of blocking the shikimate pathway (Ahsan et al. 2008). Therefore, SAC accumulation (primary effect) led to higher H_2O_2 levels (secondary effect), and consequent lipid peroxidation (LPO), the most damaging ROS process known (Gill and Tuteja 2010), indicated by TBARS levels (Figs, 2b and 2c; Figs. 3b and 3c). The lower levels of ROS production (H_2O_2) and lipid peroxidation (TBARS) in glyphosate-treated B11R than B17S indicates that the resistant biotype had minor tissue damages after glyphosate treatment. Although B11R had lower levels of H_2O_2 and TBARS regarding B17S, it presented higher levels than non-treated control plants indicating that oxidative stress also occurred, however, in a lesser scale than B17S, which died (Fig. 1 and Table 5).

In the B17S, H_2O_2 and TBARS levels peaked at 96 HAT and decreased abruptly after this time (E2) until reaching zero at 264 HAT, which matches with the death of plants. Irreversible damage to cellular tissues may have occurred beginning at this time, such as a loss of cellular homeostasis leading to cell death in sensitive plants (Figs. 3b and 3c). In plants, low concentrations of H_2O_2 acts as a signal molecule, and at high concentrations leads to plant cell death (Gill and Tuteja 2010). In E2 at 500 HAT (~21 days), the levels of H_2O_2 and TBARS in B11R non-differed from the non-treated control. Thus, this result suggests that the detoxification process of the H_2O_2 in glyphosate-resistant biotype took around 500 h after glyphosate treatment. However, it appears that even after 500 h, H_2O_2 level is still 2-fold untreated. It looks like ROS is still being produced, just at a lower rate.

Antioxidant enzyme activities

In E1 both glyphosate-treated biotypes when compared with non-treated controls had increased SOD, CAT, and APX activities, independently of dose and time after glyphosate treatment (Figs. 2d, 2f, 3d and 3f; Tables 1-4). In E2 at 96 HAT the activities of SOD, CAT, and APX in B11R were 2.7, 6.2, 15.3-fold higher than non-treated control, and in B17S 1.5, 7.5, 13.7-fold higher than non-treated control, respectively (Figs. 3d and 3f; Table 2). On E2, on average (from 0 to 552 HAT) SOD, CAT and APX activities in B11R were 2.5, 4.8, and 11.3-fold higher than non-treated control, →

and in B17S 1.7, 2.9, and 4.5-fold higher than non-treated control. Comparisons between treated biotypes activities of SOD, CAT, and APX after glyphosate treatment show higher activities in B11R than in B17S, mainly after 96 HAT (Figs. 3d and 3f; Table 2). On average (from 0 to 552 HAT) in E2, the activities of SOD, CAT, and APX in the B11R were 1.6, 1.5, and 2.4-fold higher than in B17S, respectively. In general, the SOD, CAT, and APX activities decline after 384 HAT, indicating a transient response to oxidative stress. In fact, all oxidative stress markers are still high after 500 h.

Other studies have reported the transient antioxidant enzyme activities in *C. bonariensis* and *Ambrosia trifida* in response to paraquat and glyphosate treatment, respectively (Ye and Gressel 2000; Harre et al. 2018). Glyphosate-mediated changes in antioxidant status have been reported to other species. In maize leaves, glyphosate treatment resulted in increased of H_2O_2 levels, and lipid peroxidation (Sergiev et al. 2006). Similar results were reported in rice (Ahsan et al. 2008) and peanut (Radwan and Fayez 2016). Also, a potential role of antioxidant systems in glyphosate resistance was reported in *Amaranthus palmeri* (Maroli et al. 2015).

In the present study, plants of both glyphosate-resistant and -sensitive biotypes treated with glyphosate presented significant positive correlation between SAC, H_2O_2 and TBARS contents, and the activities of SOD, CAT, and APX (Table 6). It establishes a relation of cause-effect between glyphosate treatment and SAC, H_2O_2 and TBARS production, as well as between treatment and SOD, CAT and APX antioxidant activities in both glyphosate-resistant and -sensitive biotypes. Therefore, according to correlation results, the glyphosate treatment causes an accumulation of shikimic-acid in leaf tissue, leading to increase in oxidative stress and consequent lipid peroxidation, followed by enhancing in antioxidant enzyme activities (SOD, CAT, and APX) in both studied →

Table 6. Pearson correlation coefficients estimates of variables shikimic-acid content (SAC), hydrogen peroxide (H_2O_2), thiobarbituric acid-reactive substances (TBARS), superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) from glyphosate-resistant (B11R) and sensitive (B17S) *Conyza bonariensis* biotypes as a response to glyphosate (g ae·ha^{-1}) treatment.

			Shikimic acid content				H_2O_2				Thiobarbituric acid-reactive substances			
			B11R		B17S		B11R		B17S		B11R		B17S	
			0	1480	0	1480	0	1480	0	1480	0	1480	0	1480
Shikimic acid	B11R	0	-				0.034^{NS}				0.217^{NS}			
		1480		-				0.401*				0.627**		
	B17S	0			-				0.630**				0.539*	
		1480				-				0.769**				0.913**
H_2O_2	B11R	0	0.034^{NS}				-				-0.076^{NS}			
		1480		0.401*				-				0.437*		
	B17S	0			0.630**				-				0.637**	
		1480				0.769**				-				0.902**
Thiobarbituric acid-reactive substances	B11R	0	0.217^{NS}				-0.076^{NS}				-			
		1480		0.627**				0.437*				-		
	B17S	0			0.539*				0.637**				-	
		1480				0.913**				0.902**				-
Superoxide dismutase	B11R	0	–0.132^{NS}				0.039^{NS}				-0.140^{NS}			
		1480		0.396*				0.849**				0.557*		
	B17S	0			0.687**				0.797**				0.864**	
		1480				0.797**				0.374*				0.640**
Catalase	B11R	0	0.152^{NS}				0.092^{NS}				0.003^{NS}			
		1480		0.415*				0.899**				0.598**		
	B17S	0			0.678**				0.731**				0.886**	
		1480				0.849**				0.750**				0.931**
Ascorbate peroxidase	B11R	0	0.017^{NS}				–0.143^{NS}				-0.151^{NS}			
		1480		0.071^{NS}				0.791**				0.436*		
	B17S	0			0.632**				0.697**				0.886**	
		1480				0.762**				0.798**				0.883**

....continue

Table 6. Continuation...

			Superoxide dismutase				Catalase				Ascorbate peroxidase			
			B11R		B17S		B11R		B17S		B11R		B17S	
			0	1480	0	1480	0	1480	0	1480	0	1480	0	1480
Shikimic acid	B11R	0	–0.132^{NS}				0.152^{NS}				0.017^{NS}			
		1480		0.396*				0.415*				0.071^{NS}		
	B17S	0			0.687**				0.678**				0.632**	
		1480				0.797**				0.849**				0.762**
H_2O_2	B11R	0	0.039^{NS}				0.092^{NS}				–0.143^{NS}			
		1480		0.849**				0.899**				0.791**		
	B17S	0			0.797**				0.731**				0.697**	
		1480				0.374*				0.750**				0.798**
Thiobarbituric acid-reactive substances	B11R	0	–0.140^{NS}				0.003^{NS}				–0.151^{NS}			
		1480		0.557*				0.598**				0.436*		
	B17S	0			0.864**				0.886**				0.886**	
		1480				0.640**				0.931**				0.883**
Superoxide dismutase	B11R	0	-				–0.181^{NS}				–0.118^{NS}			
		1480		-				0.958**				0.891**		
	B17S	0			-				0.896**				0.831**	
		1480				-				0.676**				0.563**
Catalase	B11R	0	–0.181^{NS}				-				0.021^{NS}			
		1480		0.958**				-				0.882**		
	B17S	0			0.896**				-				0.851**	
		1480				0.676**				-				0.938**
Ascorbate peroxidase	B11R	0	–0.118^{NS}				0.021^{NS}				-			
		1480		0.891**				0.882**				-		
	B17S	0			0.831**				0.851**				-	
		1480				0.563**				0.938**				-

*correlation coefficients of linear correlation (n = 40) significant at 5.00% of probability to t-test; ** correlation coefficients of linear correlation (n = 40) significant at 1.00% of probability to t-test; ^{NS} linear coefficients of correlation (n = 40) non-significant to the t-test.

glyphosate-resistant and -sensitive hairy fleabane biotypes (Table 6).

The lower oxidative stress, tissue damage, and higher antioxidant enzyme activities in B11R than in B17S indicates that antioxidant systems in glyphosate-resistant biotype could be related to resistance and is playing an important role in glyphosate resistance process. A recent study related the role of the antioxidant enzyme in reducing the herbicide damage in glyphosate resistance process (Délye 2013; Maroli et al. 2015). To our knowledge, there are no reports of resistance to glyphosate in *Conyza* spp. related to antioxidant mechanisms. This is the first report of differential antioxidant enzyme activity that could be related to glyphosate resistance in hairy fleabane. This type of resistance might pose a more significant threat to agriculture because the multi-herbicide resistance and multi-gene involvement in the mechanisms are considered the worst types of resistance (Yuan et al. 2007; Duke 2011; Délye 2013; Délye et al. 2013).

CONCLUSION

Glyphosate treatment on glyphosate-resistant and -sensitive biotypes resulted in a similar pattern of shikimic-acid accumulation until 96 hours after treatment for all glyphosate studied doses, but it is not a clear pattern. Shikimic-acid content in resistant biotype was transient and did not differ from non-treated control at 288 hours after treatment (~12 days). The shikimic-acid accumulation leads to oxidative stress and tissue damage occurrence in both biotypes. However, the oxidative stress and tissue damage occurred in glyphosate-sensitive biotype were higher than in -resistant. In response to glyphosate-induced stresses, the activities of antioxidant →

enzyme superoxide dismutase, catalase, and ascorbate peroxidase increase in glyphosate-resistant and -sensitive biotypes. However, the enzyme activities in glyphosate-resistant biotype were higher than in -sensitive. Thus, the results of the present research indicate that antioxidant enzyme might be related to glyphosate resistance in hairy fleabane.

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AUTHORS' CONTRIBUTION

Conceptualization, C.P., D.A., L.M., and L.V.; Data curation and statistical analysis, C.P. and I.R.C.; Formal analysis, C.P., J.C., and F.A.P.G.; Funding acquisition, C.P., D.A., and L.V.; Investigation, C.P.; Methodology, C.P.; Project administration, D.A. and L.V.; Software, C.P. and I.R.C.; Supervision, L.V. and C.N.S.J.; Writing—original draft, C.P.; Writing—review and editing, C.P., A.C., and C.N.S.J.

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