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Resistance to Fomesafen, Imazamox and Glyphosate in *Euphorbia heterophylla* from Brazil

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Abstract: *Euphorbia heterophylla* is a species of weed that was previously controlled by fomesafen, imazamox and glyphosate, but continued use of these herbicides has selected resistant populations from the Rio Grande do Sul (Brazil). One resistant (R) strain and one susceptible (S) strain to fomesafen, imazamox and glyphosate were compared, the latter by recurrent selection. Dose-response tests showed multiple resistance to these herbicides. The required imazamox concentration to inhibit ALS by 50% was approximately 16 times greater in the R population than in the S population. Based on the EPSPS activity results, the R population was 10 fold less sensitive to glyphosate than the S counterpart. In addition, basal EPSPS activity from R plants was 3.3 fold higher than the level detected on S plants. The Proto IX assays showed high resistance to fomesafen in the R population that accumulated less Proto IX than the S population. Malathion assays showed the participation of CytP450 in fomesafen resistance, but a molecular mechanism could also be involved. To our knowledge, this is the first characterisation of multiple resistance to these three groups of herbicides in *E. heterophylla* in the world.

Keywords: *Euphorbia heterophylla*; fomesafen; imazamox; glyphosate; multiple resistance; NTSR mechanisms; TSR mechanisms

1. Introduction

Euphorbia heterophylla (Wild poinsettia) is a summer annual plant with a short life cycle and with two or more generations per year. This species is a very common weed in South America that was later extended to Mexico and the Southern United States [1]. In soybean [Glycine max (L.) Merr.], *E. heterophylla* can cause a daily yield loss of up to 5.1 kg ha^{-1}, depending on the weed density [2]. Due to a high level of cross-pollination and elevated genetic recombination, there is high variability among populations of *E. heterophylla* which contributes to a rapid evolution of herbicide resistance [3,4].

Soybean is the most important crop in Brazil [5], and in 2018, occupied more than 30 million hectares corresponding to 45% of the cultivated area (~35 million ha) in the country [6]. In the 1980s, the control of *E. heterophylla* was achieved with protoporphyrinogen oxidase (PPO) inhibitor herbicides (fomesafen) and later in the mid-1980s, acetolactate synthase (ALS) inhibitor herbicides (mainly imidazolinones) began to be used [6]. However, in the early 2000s, biotypes of this species had already been selected for multiple resistance to fomesafen and imazamox [7]. On the other hand, the rapid adoption of glyphosate-resistant (GR) soybeans led to the exclusive use of glyphosate for the control *E. heterophylla* in the first years after the introduction of this technology. GR soybean varieties were officially introduced in Brazil in 2005; however, they began to be cultivated irregularly since 2000

in Rio Grande do Sul [6]. In 2007, biotypes of *E. heterophylla* with signs of glyphosate resistance the Rio Grande do Sul of Brazil, just a few years after the adoption of GR soybean [5]. This species has traditionally been considered tolerant to glyphosate; however, *E. heterophylla* was confirmed to be resistant to glyphosate in the Vale do Ivaí region, Paraná, in the 2018/2019 crop season [7,8].

Fomesafen inhibits the enzyme PPO (EC 1.3.3.4), which catalyses the last step in the pathway leading to haem and chlorophyll biosynthesis [9]. This enzyme inhibition eventually leads to the accumulation of protoporphyrin IX (Proto IX) in the cytosol and the generation of singlet oxygen that causes lipid peroxidation and cell death [10,11]. The PPO inhibitor herbicides were first commercialised in the 1960s and rapidly became important tools for weed management in soybean fields [12].

Imazamox belongs to the chemical family of imidazolinones within the ALS-inhibiting herbicides. It causes the inhibition of the ALS enzyme (EC 2.2.1.6), which is involved in the synthesis of the essential branched-chain amino acids isoleucine, leucine and valine [13,14]. Imazamox has been a very important tool used by farmers for the control of PPO herbicide-resistant *E. heterophylla* in soybean cultivation in Brazil [5,15,16].

Glyphosate is an inhibitor of the enzyme 5-enol-pyruvyl-shikimate-3-phosphate synthase (EC 2.5.1.19), its direct action is to block the biosynthesis of aromatic amino acids in plants [17,18]. It is the most important systemic herbicide in the world because it is relatively cheaper, controls several annual and perennial species, it does not have persistence in the soil and has low toxicity to mammals. Its use has increased strongly with the massive adoption of GR crops, no-till and conservation tillage practices [19,20].

The use of herbicides with different modes of action (MOA), alone or in a mixture, is one of the main tools to combat resistance to a specific group of herbicides [21–24]. However, a deficient weed control program that depends solely on chemical control has given rise to multiple resistances, which makes it difficult in the future to use alternative herbicides, especially when several resistance mechanisms (target sites and non-target sites) are found in the same population. Weeds achieve this condition because they contain an enormous amount of genetic variation that allows them to survive under different biotic and abiotic conditions [25].

As highlighted above, the occurrence of *E. heterophylla* resistant to ALS and PPO inhibitors in soybean fields in Brazil occurred more than 16 years ago [5]. In addition, due to the wide scale cultivation of GR varieties and the use of glyphosate for weed control, the resistance to this herbicide was also selected in *E. heterophylla*. Fortunately, no multiple resistance to these three groups of herbicides (ALS, EPSPS and PPO) has been documented, but that does not mean that there are no biotypes with this herbicide resistance profile in Brazilian soybean fields. The purpose of this work was to (1) study the levels of resistance to fomesafen, imazamox and glyphosate of the resistant (R) population obtained by recurrent selection and (2) determine the physiology and biochemical effects of these herbicides on R and susceptible (S) *E. heterophylla* populations.

2. Materials and Methods

2.1. Herbicides

The commercial herbicides used are shown in Table 1.

Table 1. Main characteristics of the herbicides used.

Herbicide	Company	Commercial Product	MOA (HRAC)	Field Dose (g ai ha^{-1})
Glyphosate [a]	Monsanto	36% *w/v*, Roundup® SL	EPSPS inhibiting-herbicide	720
Imazamox [b]	BASF	4% *w/w*, Pulsar ® 40	ALS inhibiting-herbicide	40
Fomesafen	Syngenta	25% *w/v*, Flex® 25 SL	PPO inhibiting herbicide	375

HRAC, Herbicide-Resistance Action Committee. [a] g ae ha^{-1}; [b] imazamox + 1L DASH (34.5% *w/v* methyl oleate/methyl palmitate, BASF).

2.2. Plant Material

Euphorbia heterophylla seeds resistant (R) and susceptible (S) to glyphosate were supplied by Prof. Ribas Vidal [5]. The R population was collected in Porto Alegre, the Rio Grande do Sul (Brazil). This population was confirmed to be resistant to fomesafen, imazamox, and moderately resistant to glyphosate [5,12]. The S population was confirmed as glyphosate-susceptible, and their seeds were collected in places never treated with herbicides over the last 15 years [5]. S seeds were stored at 4 °C, and each year their germination level was texted, maintaining a percentage greater than 80%.

2.3. Evolution of Glyphosate-Resistance under Recurrent Selection

During the period from 2011 to 2019, the R population was subjected to nine cycles of recurrent glyphosate selection under field conditions. Seeds of the R population were germinated in Petri dishes containing filter paper moistened with distilled water. The Petri dishes were placed in a growth chamber at 28/18 °C (day/night) with a photoperiod of 16 h, 850 μmol m^{-2} s^{-1} of photosynthetic photon flux and 60% relative humidity. Four days later, 100 seedlings were transplanted into small plots (experimental unit of 2 × 6 m^2 with plants spaced at 35 cm (approximately) in a square planting pattern) in the field of experimentation at the University of Córdoba (Spain); the plants were watered three days by week during the experiment. Plants were treated at the 4-leaf growth stage using a Pulverex backpack sprayer with a T coupling for the wand equipped with four flat fan nozzles at a spraying pressure of 200 kPa and calibrated to give a 200 L ha^{-1} volume. Glyphosate was applied at 540, 720, 900, 1080 and 1800 g ae ha^{-1}; after 5 months surviving plants finished their cycle and the seeds were harvested and used to repeat the selection process in successive years. The seeds collected from each dose of herbicide were cleaned and stored in a chamber at 4 °C until use. This selection process was repeated every year until 2019 in spring/summer, and during this last year a sequential application was made with fomesafen (plants with 3 leaves), imazamox (plants before tillering) and glyphosate (plants in tillering) at field dose (Table 1). The percentage of surviving plants was determined each year to evaluate the evolution of resistance to glyphosate and finally multiple resistance to fomesafen and imazamox. The confirmation of the multiple levels of resistance as well as the putative resistance mechanisms were carried out with the seeds obtained in 2019. The experiment of recurrent selection was carried out employing three replicates for each glyphosate-dose and year.

2.4. Dose-Response Assays

Plants from R and S populations were sprayed with the following increasing doses of glyphosate (0, X/16, X/8, X/4, X/2, X, 2X, 4X and 6X g ae ha^{-1}), imazamox (0, X/4, X/2, X, 4X, 8X, 10X and 20X g ai ha^{-1}) and fomesafen (0, X/4, X/2, X, 4X, 10X, 20X and 40X g ai ha^{-1}), with X being the field doses of every herbicide (Table 1). Five pots with one plant per pot were treated for each dose of herbicide. Spraying was carried out in a laboratory chamber equipped with an 8001 flat fan nozzle delivering 200 L ha^{-1} at a constant pressure of 200 kPa. Twenty-one days after treatment (DAT), plant mortality (LD_{50}) and dry weight (g) per plant (GR_{50}) were evaluated [26]. Data were expressed as percentages relative to the untreated control. The experiment was repeated twice at different times.

2.5. ALS Enzyme Activity

The ALS activity in response to imazamox was determined in vitro following the protocol described by Rojano-Delgado et al. [15]. Three grams of young leaf tissue of each biotype were ground separately using liquid N_2. Polyvinylpyrrolidone (0.5 g) was added to the fine powder as well as extraction buffer [1M K-phosphate buffer solution (pH 7.5), 10 mM sodium pyruvate, 5 mM $MgCl_2$, 50 mM thiamine pyrophosphate, 100 μflavin adenine dinucleotide, 12 mM dithiothreitol and glycerol–water (1:9, *v/v*)] in a proportion of 1:3 tissue:buffer. Samples were agitated for 10 min at 4 °C, filtered through cheesecloth and centrifuged (20,000× *g* for 20 min). The obtained supernatant was immediately used for the enzyme assays.

For the ALS enzyme activity, 90 µL of enzyme extract was used with 110 µL of freshly prepared assay buffer [0.08 M K-phosphate buffer solution (pH 7.5), 0.5 M sodium pyruvate, 0.1 M $MgCl_2$, 0.5 mM thiamine pyrophosphate and 1000 µM flavin adenine dinucleotide]. The imazamox concentrations assayed were 0, 0.1, 1, 10, 100, 1000 and 10,000 µM. Aliquots of 250 µL of a solution 0.04 M K_2HPO_4 at pH 7.0 were added. This mixture was incubated for 1 h at 37 °C. Afterwards, the reaction was stopped by adding 50 µL of H_2SO_4/water 1:10 (*v*/*v*). To decarboxylate acetolactate to acetoin, the tubes with the mixture were heated for 15 min at 60 °C. A red complex (λ 520 nm) formed after the addition of 250 µL of creatine (5 g L^{-1} freshly prepared in water) and 250 µL of 1-naphthol (50 g L^{-1} freshly prepared in 5 N NaOH) before incubation at 60 °C for 15 min. Total protein content was determined by the Bradford method [27]. The experiment was carried out twice and with three replicates for each concentration of the herbicide.

2.6. EPSPS Enzyme Activity

The extraction and activity of the EPSPS enzyme were carried out following the methodology described by Palma-Bautista et al. [26]. Five grams of young leaf tissue from the R and S populations of *E. heterophylla* were ground with liquid N_2 to a fine powder. Samples were transferred to tubes containing 25 mL of extraction buffer (100 mM MOPS, 5 mM EDTA, 10% glycerol, 50 mM KCl, and 0.5 mM benzamidine) with 70 µL of ß-mercaptoethanol and 1% in polyvinylpolypyrrolidone (PVPP) to extract the EPSPS. Samples were vortexed for 5 min, avoiding foaming, and then centrifuged (18,000× *g*, 30 min, 4 °C). The supernatant was filtered using a cheesecloth in a cold beaker and the $(NH_4)_2$ SO_4 was slowly added while under continuous stirring (30 min) and at the end centrifuged (15,000× *g*, 30 min, 4 °C). This step was repeated once more to obtain a 70% $(NH_4)_2$ SO_4 (*w*/*v*) solution to precipitate the fraction that contained the EPSPS activity. The pellets obtained in the successive centrifugations were mixed and re-suspended in 1 mL assay buffer (100 mM MOPS, 1 mM $MgCl_2$, 10% glycerol (*v*/*v*), 2 mM sodium molybdate, 200 mM NaF). These samples were dialysed using Slide-A-Lyzer dialysis cassettes (1000-MWC, Thermo Scientific, Meridian, IL, USA) overnight in 2 L of dialysis buffer (100 mM MOPS and 5 mM EDTA) at 4 °C on a stir plate. The pH of the buffers was adjusted to 7.0.

The concentration of total protein soluble (TPS) was determined by Bradford assay [27]. The EPSPS enzyme activity was determined in a continuous assay quantifying the inorganic phosphate (Pi) released from shikimate-3-phosphate with the EnzCheck phosphate assay kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The glyphosate concentrations used were 0, 0.01, 0.1, 1, 10, 100 and 1000 µM. The amount Pi released was measured at 360 nm for 10 min in a spectrophotometer (DU-640, Beckman Coulter Inc., Fullerton, CA, USA). The EPSPS activity was calculated by determining the amount of Pi (µmol) released in µg of TSP^{-1} min^{-1}. EPSPS enzyme activity was expressed as a percentage of enzyme activity in the presence of glyphosate relative to the control (basal activity without glyphosate). Three replicates for each concentration in a completely randomised design were tested and the experiment was conducted twice.

2.7. Proto IX Assays

2.7.1. Without CytP450 Inhibitor

Determination of the Proto IX levels without a CytP450 inhibitor was carried out following the methodology described by Fernández-Moreno et al. [28].

Leaf disks (approx 0.1 g each) from each population were incubated individually in a 6 cm diameter Petri dish containing 6 mL of 2% (wt/v) sucrose, 1 mM of 2-(N-morpholine) ethanesulphonic acid, and different concentrations (100, 500, 1000, 2000 and 5000 µM) of fomesafen at technical grade during 20 h at 25 °C in darkness. The same experiment was conducted but without herbicide as control. All extractions of Proto IX from leaf discs were made under a dim, green light source. After incubation, the leaf disks were homogenised using 2 mL of a mix of methanol and NH_4OH 1N (9:1) at maximum

speed for 15 s and kept in the dark for 10 min. Homogenates were centrifuged at 3000× *g* for 3 min, and then supernatants were collected. Pellets were re-suspended in 1 mL of methanol in the dark for 10 min, collecting the supernatant. Supernatants from both centrifugations were combined and dried under an air stream. Concentrates were reconstituted in 1 mL of the methanol and filtered through a 0.2-µm nylon syringe filter to remove particles. The extracts were stored in opaque glass vials at −20 °C until their analysis by the HPLC method.

Fifty microliters of the reconstituted phase were injected into a Gold HPLC System Beckman Coulter 126 (Fullerton, CA, USA) equipped with a Jasco FP-1520 fluorescence detector (Easton, PA, USA), with excitation and emission wavelengths set at 400 and 630 nm, respectively, and a 25 cm × 4.6 mm, 5-micron particle size Phenomenex SphereClone® ODS column (Torrance, CA, USA). Deionised water was used as mobile phase A, and pure LC grade methanol as mobile phase B. The elution programme started by 60% mobile phase B and followed the linear gradient until 100% methanol in 10 min. After 30 min, the solvent system was returned to the initial conditions. The constant flow rate and column temperature were 1.0 mL/min and 25 °C, respectively. Proto levels in the extracts were quantified using a calibration curve obtained with a commercially available Proto IX standard from Sigma Aldrich (St. Louis, MO, USA). Data were expressed as nmol g^{-1} of fresh weight. All treatments were carried out three times using three replicates with and without herbicide in a completely randomised design.

2.7.2. With CytP450 Inhibitor

For this assay, we used whole plants at the four to the six-leaf stage, that were sprayed without and with malathion (2000 g ai ha^{-1}). After approximately 120 min, the fomesafen was applied at 375 g ai ha^{-1} to the populations. The doses were based on previous studies [29,30]. Non-fomesafen incubation was used because the metabolism is a slower mechanism.

Plants not treated with fomesafen were used as controls. Ten plants from each population and fomesafen and malathion dose were harvested at 96 HAT using leaf discs (approximately 0.1 g each).

All Proto IX extractions from the leaf discs were carried out following the methodology described by Fernández-Moreno et al. [28]. Proto IX levels in the extracts were also quantified using a calibration curve obtained with a commercially available Proto IX standard from Sigma Aldrich (St. Louis, MO, USA). All treatments were carried out three times using three replicates with and without herbicide in a completely randomised design.

2.8. Statistical Analysis

The herbicide concentration causing a 50% reduction in plant growth (GR_{50}), the dose required to kill 50% of the population (LD_{50}) and the herbicide concentration causing a 50% inhibition of enzyme activity (I_{50}) were calculated by a non-linear regression analysis adjusted to a three-parameter model using the R package drc (R Core Team) (Equation (1)):

$$Y = (d)/[1 + \exp(b(\log(x) - \log(e)))] \quad (1)$$

where d is the coefficient corresponding to the upper asymptote, the coefficient b is the slope at the inflection point, and the herbicide concentration required to inhibit shoot growth or enzyme activity by 50% (i.e., GR_{50}, LD_{50} or I_{50}, respectively), and x is the herbicide dose. The resistance index (RI = R/S) was computed as R-to-S GR_{50}, GR_{50} or I_{50} ratios.

Data on the field evolution of glyphosate, basal EPSPS activity and the proto IX were subjected to ANOVA using Statistix 10.0 (Analytical Software, Tallahassee, FA, USA). Percentage data were transformed (arcsine of the square root) to meet model assumptions of normality of the error distribution and variance homogeneity. Model assumptions were graphically inspected. When needed, differences between means were separated using Tukey's honestly significant difference test. The replicates of the experiments were pooled due to the lack of statistical differences between them.

3. Results

3.1. Evolution of Glyphosate Resistance under Recurrent Selection

The selection pressure exerted by glyphosate on a population of *E. heterophylla* was rapid, and after 9 years of recurrent selection, the maximum dose in which around 90% of plants survived to the herbicide increased from 540 to 1800 g ae ha^{-1} (Table 2). The original R2011 population survived 540 g ae ha^{-1}, while only 20.67% of plants survived from that population treated with 720 glyphosate (field doses in Brazil), but in 2019 a survival rate of 98.00% was obtained as a product of the recurrent selection (Table 2). Similarly, plants treated independently with 900, 1080 and 1800 g ae ha^{-1} of glyphosate had survival rates of 94, 96 and 89% after 7, 5 and 3 years of application, respectively (Table 2).

Table 2. Evolution of glyphosate-resistant *E. heterophylla* treated with different glyphosate doses during the 2011 to 2019 period.

GLYPHOSATE % Survival Plants					
Application Year	**540 ***	**720 ***	**900 *[a]**	**1080 *[b]**	**1800 *[c]**
2011	90.33 ± 3.51	20.67 ± 3.06	-	-	-
2012	97.33 ± 3.06	52.00 ± 7.21	-	-	-
2013	100	72.00 ± 7.21	21.67 ± 7.64	-	-
2014	100	84.67 ± 5.03	40.00 ± 2.00	-	-
2015	100	92.00 ± 2.00	65.67 ± 3.51	13.33 ± 3.06	-
2016	100	93.33 ± 3.05	83.33 ± 3.06	24.67 ± 3.06	-
2017	100	98.00 ± 2.00	90.00 ± 2.00	63.33 ± 6.11	24.67 ± 5.03
2018	100	97.33 ± 3.06	94.33 ± 5.51	94.00 ± 2.00	60.00 ± 2.00
2019	100	98.00 ± 2.00	94.33 ± 4.93	96.00 ± 5.29	89.33 ± 3.06

* doses expressed as g ae ha^{-1}; [a] 2013 seed plants treated at 720 g ae ha^{-1}; [b] 2014 seed plants treated at 900 g ae ha^{-1}; [c] 2016 seed plants treated at 1080 g ae ha^{-1}.

3.2. Dose-Response Assays

The LD_{50} values were far less for the S population, than the ones that survived to higher doses used in soybean cultivation in southern Brazil (Table 3 and Figure 1). Based on the GR_{50} values of the R population, we calculated resistance factors (RF: GR_{50} of R/GR_{50} of S) from 13.6, 19.1 and 68.1 for glyphosate, imazamox and fomesafen (Table 3 and Figure 1).

Table 3. Parameters of the log-logistic equation used (Equation (1)) to calculate the fomesafen, imazamox and glyphosate rates required for 50% reduction dry weight (GR_{50}), and % survival (LD_{50}) expressed as a percentage of the mean untreated control of the R and S *E. heterophylla* populations.

FOMESAFEN										
	Aboveground dry weight (%)					Survival (%)				
Population	***d***	***b***	**GR_{50} (g ai ha^{-1})**	**RI [a]**	***p***	***d***	***b***	**LD_{50} (g ai ha^{-1})**	**RI**	***p***
R	100.3	2.0	3290.1 (190.4)	19.1	<0.0001	101.2	4.8	10,420.8 (735.4)	32.1	<0.0001
S	102.3	2.2	172.4 (20.6)			100.4	3.8	324.1 (28.6)		
IMAZAMOX										
	Aboveground dry weight (%)					Survival (%)				
Population	***d***	***b***	**GR_{50} (g ai ha^{-1})**	**RI**	***p***	***d***	***b***	**LD_{50} (g ai ha^{-1})**	**RI**	***p***
R	100.9	1.0	490.8 (57.6)	68.1	<0.0001	100.1	3.1	799.1 (77.1)	47.8	<0.0001
S	101.1	1.2	7.2 (0.5)			101.3	2.7	16.7 (0.6)		
GLYPHOSATE										
	Aboveground dry weight (%)					Survival (%)				
Population	***d***	***b***	**GR_{50} (g ae ha^{-1})**	**RI**	***p***	***d***	***b***	**LD_{50} (g ae ha^{-1})**	**RI**	***p***
R	101.8	1.4	976.1 (88.2)	13.6	0.0003	100.3	4.3	2621.8 (182.3)	19.8	<0.0001
S	102.1	1.7	71.5 (8.3)			102.6	2.0	131.9 (10.3)		

[a] Resistance index (RI = GR_{50} or LD_{50} R/ GR_{50} or LD_{50} S).

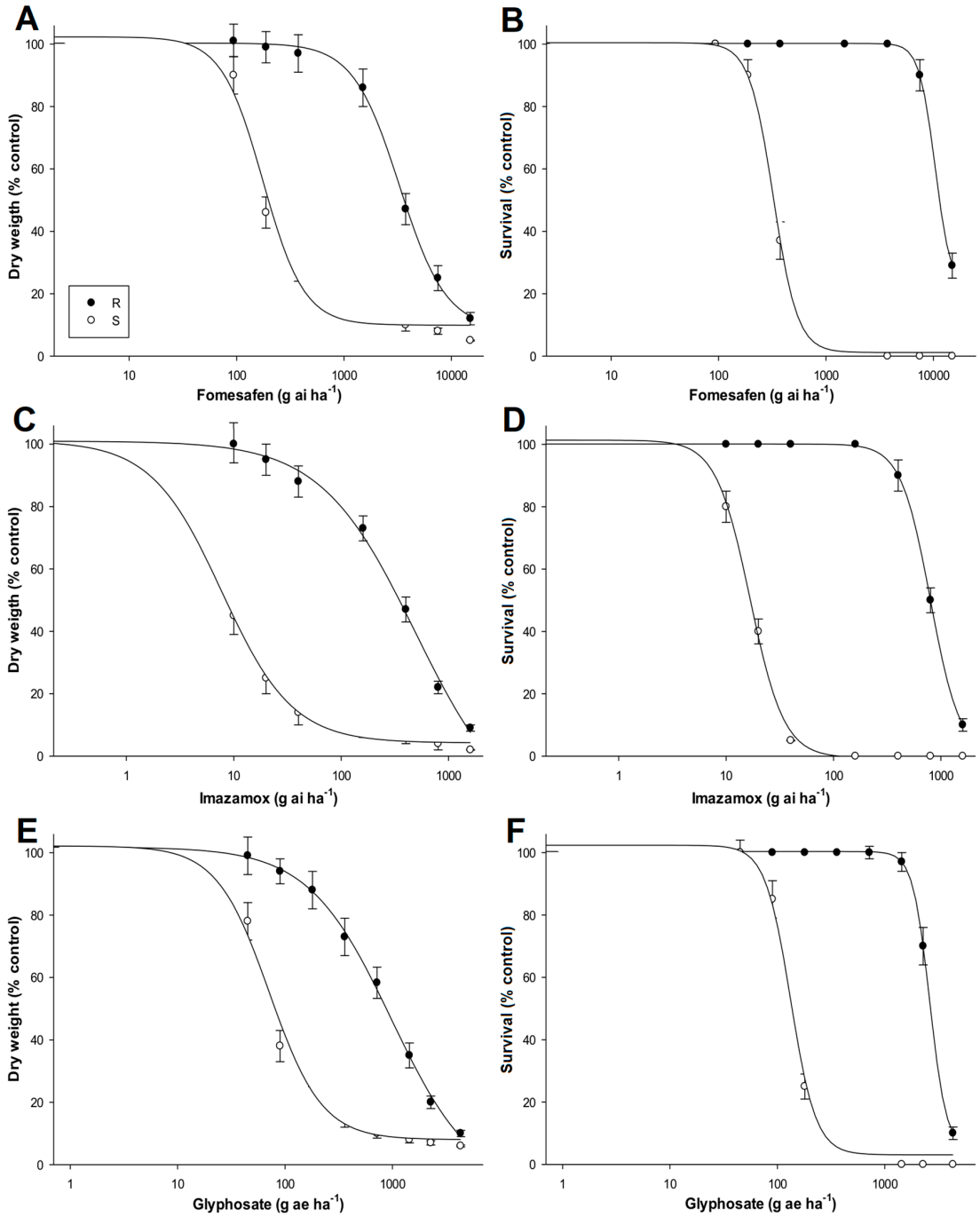

Figure 1. Dose-response curves (dry weight reduction and survival rates) of fomesafen (**A**,**B**), imazamox (**C**,**D**) and glyphosate (**E**,**F**) in resistant (R) and susceptible (S) populations of *E. heterophylla* populations from Brazil. Vertical bars ± standard error (n = 10).

3.3. EPSPS Enzyme Activity

Based on the EPSPS activity data, a log-logistic model was fitted for each population (Figure 2A). From there, the required glyphosate concentration to inhibit EPSPS by 50% (I_{50}) was estimated, which was approximately 10 times greater in the R population than in the S population (Table 4).

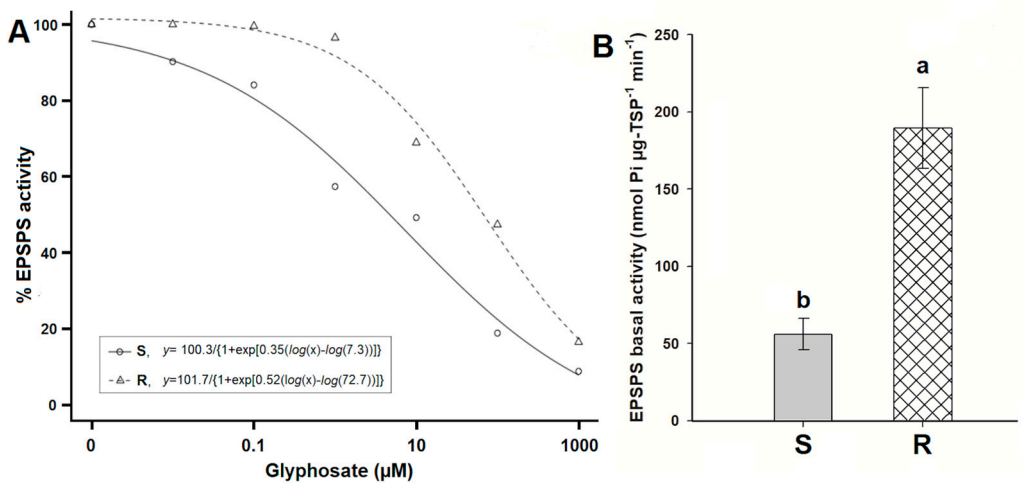

Figure 2. EPSPS enzyme activity (expressed in %) in presence of different glyphosate concentrations (**A**) and EPSPS basal activity (**B**) of R and S *E. heterophylla* populations; where different letters in each column-population mean differences statistically by the Tukey test ($p < 0.05$). Vertical bars ± standard error (n = 9).

Table 4. Parameters of the log-logistic model (Equation (1)) of the inhibition of EPSPS enzymatic activity in response to glyphosate concentration for R and S *E. heterophylla* populations.

Populations	*d*	*b*	R^2	I_{50}	RF [a]	*p*-Value
R	101.77	0.52	0.99	72.77	9.98	<0.0001
S	100.33	0.35	0.99	7.29		

[a] Resistance factor (RF = I_{50} R/I_{50} S).

Apparent differences were found between S and R population in the enzyme basal activities that were 56.20 ± 2.47 and 189.51 ± 4.48 nmol Pi μg TSP^{-1} min^{-1}, respectively (Figure 2B).

3.4. ALS Enzyme Activity

The quantity of imazamox needed to inhibit the ALS activity by 50% (I_{50}) in R plants was 651.13 μM, while that in the S population was 40.64 μM (Table 5 and Figure 3A). In other words, the R population was 1602 fold more resistant to imazamox than the S counterpart (Table 5). No differences were found in the basal enzyme activities (Figure 3B).

Table 5. Parameters of the log-logistic model (Equation (1)) of the inhibition of ALS enzymatic activity in response to imazamox concentration for R and S *E. heterophylla* populations.

Populations	*d*	*b*	R^2	I_{50}	RF [a]	*p*-Value
R	101.29	0.54	0.99	651.13	16.02	<0.0001
S	92.51	0.55	0.99	40.64		

[a] Resistance factor (RF= I_{50} R/I_{50} S).

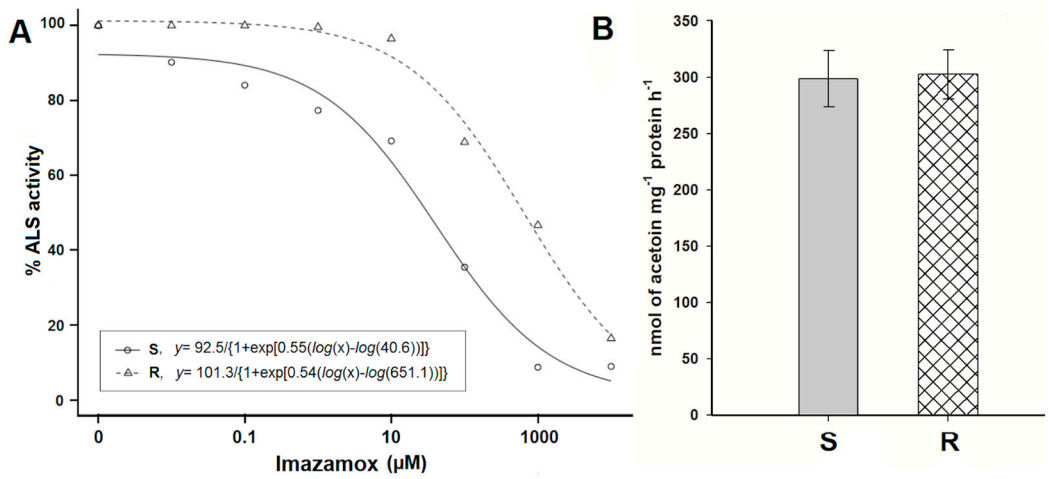

Figure 3. ALS enzyme activity (expressed in %) in presence of different imazamox concentrations (**A**) and ALS basal activity (**B**). Vertical bars ± standard error (n = 9).

3.5. Proto IX Assays

3.5.1. Without CytP450 Inhibitor

The Proto IX accumulation was significantly different at 500 μM of fomesafen, in that R population accumulated significantly less Proto IX than the S population (Table 6 and Figure 4A). In R plants, the Proto IX accumulation seems to be constant from 1000 to 5000 μM.

Table 6. Proto IX accumulation (expressed as nmol per gram of fresh weight ± standard error of the mean n = 9) in both R and S *E. heterophylla* populations treated with different fomesafen concentrations.

Populations	Fomesafen Concentration (μM)				
	100	500	1000	2000	5000
R	0.39 ± 0.08	0.44 ± 0.15	0.5 ± 0.17	0.56 ± 0.21	0.61 ± 0.19
S	0.41 ± 0.09	1.84 ± 0.17	2.69 ± 0.33	4.02 ± 0.72	5.73 ± 0.65

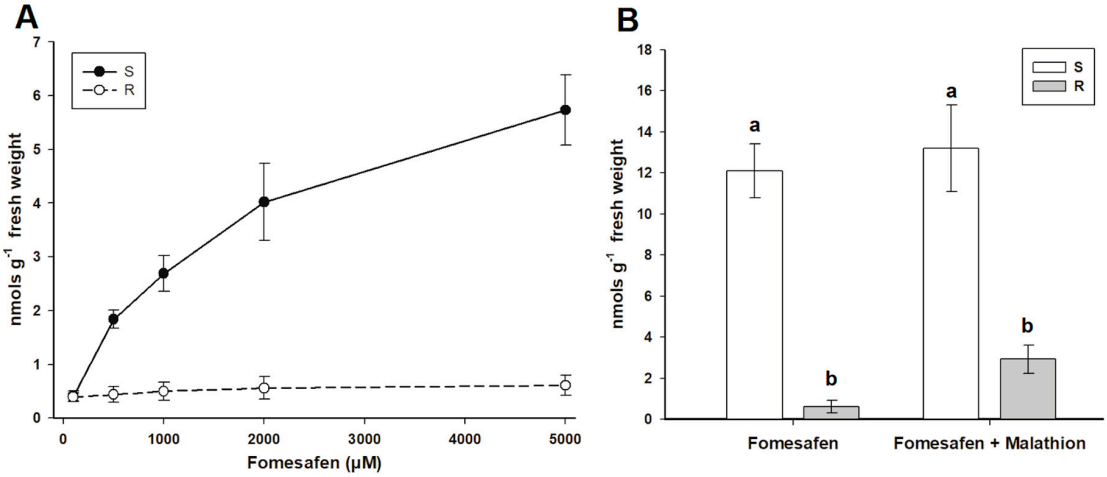

Figure 4. (**A**) Proto IX accumulation in R and S *E. heterophylla* populations with different fomesafen concentrations. (**B**) Proto IX accumulation in plants treated with a field dose (375 g ai ha^{-1}) of fomesafen without and with malathion (Cyt-P450 inhibitor); where different letters within each population differ statistically according to Tukey's HSD ($p < 0.05$) ± standard error of the mean (n = 9).

3.5.2. With CytP450 Inhibitor

The R *E. heterophylla* population accumulated significantly less Proto IX than the S population with and without malathion (Table 7 and Figure 4B). However, while in the S population the accumulation was approximately the same with and without the CytP450 inhibitor, the application of malathion on R plants produced an increase in the Proto IX levels, approximately five times higher compared to the treatment of fomesafen without the inhibitor (Table 7 and Figure 4B).

Table 7. Proto IX accumulation (expressed in nmol per gram of fresh weight ± standard error of the mean n = 9) in R and S *E. heterophylla* populations treated with a field dose of fomesafen without and with malathion (CytP450 inhibitor).

Populations	Fomesafen	With Malathion
R	0.63 ± 0.31a	2.93 ± 0.72b
S	12.08 ± 1.28a	13.23 ± 2.08a

Different letters within each population differ statistically by the Tukey test ($p < 0.05$).

4. Discussion

4.1. Physiological Assays

The weed survival after repeated applications of glyphosate (as the unique control tool) for more than five consecutive years triggers evolutionary adaptations that result in resistance to glyphosate [25,31,32]. In our case, the evolution of the R population (2011) was very fast, and with five years of repeated annual glyphosate applications of 720 g ae ha^{-1} (field dose in glyphosate-resistant soybean), the survival percentage reached 92% (Table 2). At the same time, the resistance level continued increasing exponentially, and 89% of surviving plants were recorded at the maximum evaluated rate (1800 g ae ha^{-1}) after 3 years of recurrent selection with that dose (Table 2). In this recurrent selection, high levels of resistance were achieved after nine years (one application per year), however, under field conditions, the time to select for resistance could be less, because the areas used for GR soybeans production in the summer cycle (safra) are generally occupied with GR maize in the winter cycle (safrinha) [6]. In addition, often more than one application of glyphosate is made per crop season, summer or winter, if GR varieties are grown [6].

The studies of the dose-response carried out in S and R *E. heterophylla* populations show high resistance to fomesafen, imazamox and glyphosate (Table 2 and Figure 1). In the case of dicotyledonous weeds, only four cases with multiple resistances (three sites of action) to these herbicides have been confirmed worldwide. The first was a population of *Amaranthus palmeri* detected in 2016 in Arkansas (USA) [33], the second was a population of *A. tuberculatus* detected in 2017 in Ontario (Canada) [34], and both species were found in glyphosate-resistant cotton and soybeans fields [33]. The third population was *Epilobium ciliatum* confirmed in 2018 in Lolol (Chile) in an olive orchard [35] and the fourth a *Parthenium hystherophorus* population confirmed in 2020 in Monte Cristi (Dominican Republic) in a field of banana orchards [26].

4.2. Biochemical Assays

The activity of the ALS enzyme showed that there were no differences in the basal activity, but differences were found between the populations in the amount of imazamox necessary to inhibit the ALS activity by 50%. These results could imply the presence of a TSR mechanism for imazamox (IMI) and cross-resistance to other ALS chemical families cannot be discarded, as has been demonstrated for another population collected in Nova Boa Vista Li Perau, Brazil [15]. This R population has also shown that resistance to imazamox is due to two resistance mechanisms, the main one was the *Ser653Asn* mutation and a secondary mechanism due to the exudation of the ^{14}C-imazamox by roots [15].

However, Mendes et al. [16] have studied resistance to imazamox in two populations collected from soybean fields in Mamborê and Kaloré (State of Paraná, Brazil) that showed a *Trp574Leu* mutation.

Significant differences in the EPSPS basal activity and response to glyphosate concentration were detected between S and R populations, concluding that resistance levels depend on the TSR mechanism [10,32]. Our results showed a resistance index of 9.98, which indicates that one or more mutations could be involved in resistance to glyphosate. In addition, the R populations presented a basal enzyme activity 3.37 times greater than that detected in the S population, which implies that other molecular mechanisms such as copy number and/or overexpression of EPSPS could also be involved in the resistance of *E heterophylla* to glyphosate (Table 5 and Figure 3B). Glyphosate resistance in dicot weeds is due to different mechanisms grouped and commonly known as non-target site resistance (NTSR) and target site resistance (TSR) mechanisms [32,36]. The TSR mechanisms are those related to the EPSPS, either by a loss of affinity between the linking enzyme and glyphosate caused by mutations and/or by the EPSPS overexpression as have been concluded for species such as *Amaranthus* spp, *Bidens pilosa* and *B. subalternans*, among others [37–41].

Differences in poor control and resistance factors between S and R populations confirm the high resistance of *E. heterophylla* to fomesafen (Table 3 and Figure 1). The accumulation of Protoporphyrin IX (Proto IX) due to the application of PPO inhibitor herbicides has been a widely used tool for the determination of resistance in weeds, where susceptible weeds accumulate much more of this metabolite [10,26,42]. Our results show that the accumulation of Proto IX in the S population was 7.18 times greater than in the R population treated with 2000 µM of fomesafen (Table 6 and Figure 4A), which is in agreement with the results obtained by other authors on dicot weeds such as *E. ciliatum* [42], *P. hysterophorus* [26] and recently on a population of *E. heterophylla* [11]. The use of malathion as an inhibitor of CytP450 would interfere with metabolisation of fomesafen in non-toxic products and therefore increased the accumulation of Proto IX in R plants, helping us to be able to interpret the possible mechanisms involved in resistance to PPO (Table 7 and Figure 4B). The application of fomesafen + malathion did not affect the accumulation of Proto IX in the S population; however, this treatment increased by 4.66 times the accumulation of Proto IX on the R population compared to the treatment done under the same conditions but only with fomesafen (Table 7 and Figure 4B). Nevertheless, the accumulation of Proto IX detected on R plants treated with fomesafen and malathion represented 22% of the Proto IX measured on S plants (Table 7). This result could be due to two mechanisms; due to the enhanced metabolism of fomesafen (NTSR), which is in the fixation stage since it is not yet able to fully confer resistance to fomesafen, or due to a low fomesafen sensitivity of PPO putatively associated with a TSR. In contrast, a trial conducted in a different population of *E. heterophylla* treated with lactofen (PPO inhibitor) and lactofen + malathion concluded that herbicide metabolism is not involved in resistance and only a mutation in PPO can explain the high level of resistance to lactofen [8]. In addition, molecular studies have shown that a mutation in the PPO gene at position 98 is the most likely resistance mechanism involved in *Ambrosia artemisiifolia* [43] and *A. palmeri* [44].

5. Conclusions

The first case of multiple resistances to ALS, EPSPS and PPO in *Euphorbia heterophylla* collected from a soybean field in Southern Brazil has been confirmed, which was obtained by recurrent selection from 2011 to 2019. Biochemical analyses suggest that resistance to imazamox and glyphosate is based on TSR, while fomesafen resistance would be based on TSR and NTSR mechanisms. The condition of multiple resistance to three MOAs (ALS, EPSPS and PPO) could occur soon in Brazilian soybean fields, as demonstrated by the recurrent selection, if alternatives to weed management, other than herbicides, are not included.

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