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Arg-128-Leu target-site mutation in *PPO2* evolves in wild poinsettia (*Euphorbia heterophylla*) with cross-resistance to PPO-inhibiting herbicides

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Research Article

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Abstract

Wild poinsettia (*Euphorbia heterophylla* L.) is a troublesome broadleaf weed in grain production areas in South America. Herbicide resistance to multiple sites of action has been documented in this species, including protoporphyrinogen oxidase (PPO) inhibitors. We investigated the physiological and molecular bases for PPO-inhibitor resistance in a *E. heterophylla* population (R_{PPO}) from Southern Brazil. Whole-plant dose–response experiments revealed a cross-resistance profile to three different chemical groups of PPO inhibitors. Based on dose–response parameters, R_{PPO} was resistant to lactofen (47.7-fold), saflufenacil (8.6-fold), and pyraflufen-ethyl (3.5-fold). Twenty-four hours after lactofen treatment (120 g ha^{-1}) POST, R_{PPO} accumulated 27 times less protoporphyrin than the susceptible population (S_{PPO}). In addition, R_{PPO} generated 5 and 4.5 times less hydrogen peroxide and superoxide than S_{PPO}, respectively. The chloroplast PPO (*PPO1*) sequences were identical between the two populations, whereas 35 single-nucleotide polymorphisms were found for the mitochondrial PPO (*PPO2*). Based on protein homology modeling, the Arg-128-Leu (homologous to Arg-98-Leu in common ragweed [*Ambrosia artemisiifolia* L.] was the only one located near the catalytic site, also in a conserved region of *PPO2*. The cytochrome P450 monooxygenase inhibitor malathion did not reverse resistance to lactofen in R_{PPO}, and both populations showed similar levels of *PPO1* and *PPO2* expression, suggesting that metabolic resistance and *PPO* overexpression are unlikely. This is the first report of an Arg-128-Leu mutation in *PPO2* conferring cross-resistance to PPO inhibitors in *E. heterophylla*.

Introduction

Herbicides targeting protoporphyrinogen oxidase (PPO) were first commercialized in the 1960s (Dayan et al. 2018) and rapidly became important tools for weed management in soybean [*Glycine max* (L.) Merr.] fields. However, the use of PPO-inhibiting herbicides declined significantly after the introduction of transgenic glyphosate-resistant (GR) soybeans in 1996. More recently, PPO inhibitors have again been widely used in response to the evolution of GR weeds (Duke 2018). This increase in use has intensified the selection pressure exerted by these herbicides, and a number of species have evolved resistance to PPO inhibitors (Heap 2020).

PPO inhibitors are contact herbicides, because they cause rapid accumulation of protoporphyrin IX (proto), the product of the reaction catalyzed by the enzyme (Dayan et al. 2020; Duke and Dayan 2018). Proto is light-reactive and able to transfer electrons to other molecules, generating reactive oxygen species (ROS) (Becerril and Duke 1989), which leads to membrane peroxidation and plant cell death (Watanabe et al. 1992). Two nuclear-encoded genes, *PPO1* and *PPO2*, express the chloroplast and mitochondrial PPO enzyme isoforms, respectively (Dayan et al. 2018).

Target-site mutations in the amino acid sequence of *PPO2* are the most common mechanism of resistance to PPO inhibitors in plants. A glycine deletion at position 210 (ΔG210) in PPO2 confers resistance to PPO inhibitors in waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] (Patzoldt et al. 2006; Sarangi et al. 2019). Different mutations at Arg-128 (Arg-128-Gly or Met) in PPO2 have also been reported in PPO-resistant Palmer amaranth (*Amaranthus palmeri* S. Watson) (Varanasi et al. 2017), as homologous to a Arg-98-Leu mutation first found in common ragweed (*Ambrosia artemisiifolia* L.) (Giacomini et al. 2017; Rousonelos et al. 2012). A glycine to alanine substitution at position 399 (Gly-399-Ala) in PPO2 was also confirmed to

cause resistance to fomesafen in *A. palmeri* (Rangani et al. 2019). More recently, a single point mutation in *PPO1* leading to an Ala-212-Thr amino acid change was found to confer resistance to oxadiazon in goosegrass [*Eleusine indica* (L.) Gaertn.] for the first time (Bi et al. 2020). In addition to target-site mutations, resistance to PPO inhibitors was partially reverted by malathion, a cytochrome P450 monooxygenase (P450) inhibitor, suggesting that metabolic resistance to PPO herbicides could also evolve in *A. palmeri* (Varanasi et al. 2018) and *A. tuberculatus* (Obenland et al. 2019).

Wild poinsettia (*Euphorbia heterophylla* L.) is one of the most troublesome annual weeds in South America. In dry beans (*Phaseolus vulgaris* L.), *E. heterophylla* (3 plants m^{-2}) caused up to 60% of yield losses due to weed interference (Machado et al. 2015). In soybean, *E. heterophylla* can cause a daily yield loss of up to 5.1 kg ha^{-1}, depending on the weed density (Meschede et al. 2002). Furthermore, this species also has high levels of genetic diversity, as verified in 12 populations from Brazil, a factor that contributes to a rapid evolution of herbicide resistance (Frigo et al. 2009).

Before GR soybean, acetolactate synthase (ALS)-inhibiting herbicides were widely adopted by Brazilian farmers in the mid-1980s and 1990s. Consequently, *E. heterophylla* populations evolved resistance to ALS herbicides across most soybean production areas of Brazil (Gelmini et al. 2001). Some PPO inhibitors (e.g., lactofen and fomesafen) then became an important tool to manage *E. heterophylla* in conventional (non-GR) soybean fields (de Oliveira et al. 2006). Trezzi et al. (2005) reported *E. heterophylla* populations with multiple resistance to ALS and PPO inhibitors (Heap 2020), but the mechanism(s) of resistance are unknown. Therefore, our objective was to confirm resistance to PPO-inhibiting herbicides and characterize the resistance mechanisms, such as target-site mutations in *PPO1* and *PPO2*, gene amplification, and herbicide metabolism in a *E. heterophylla* population from Brazil.

Materials and Methods

Plant Growth and Spraying Conditions

Seeds of *E. heterophylla* were collected in a soybean field (Mamborê, Paraná State, Brazil; 24.19°S, 52.32°W) where lactofen had been sprayed at least once a year for the preceding 7 yr. Seeds were collected from 20 plants, cleaned, labeled as resistant (R_{PPO}), and stored at 4 C until the experiments were conducted. Seeds for the susceptible (S_{PPO}) population were collected in another field with no record of lactofen application (São Paulo State; 22.09°S, 46.57°W), as described for the R_{PPO} population. Seeds of R_{PPO} were sown in 3-L pots filled with potting soil (MecPlant®, Telêmaco Borba, BR, Brazil). At 3 d after emergence, the seedlings were thinned to keep two plants of equivalent size per pot. Once they reached the 3-leaf stage, plants were sprayed with lactofen (Cobra®, 240 g L^{-1}, Valent, Walnut Creek, CA, USA) at 180 g ha^{-1} to eliminate contamination with susceptible plants. This screening was conducted with 20 pots (40 plants in total). The seeds from surviving plants were collected (G_1), and the same screening process was repeated twice to obtain a G_3 generation with homogeneous resistance to lactofen. All subsequent experiments were conducted with the G_3 generation of R_{PPO}.

Seeds from R_{PPO} and S_{PPO} were sown in 200-insert flats filled with potting soil (Professional Growing Mix, Sun Gro®, Agawam, MA, USA). Then, at 5 to 7 d after germination, seedlings were transplanted to 1-L pots (one plant per pot represented one experimental unit for all experiments) filled with the same potting soil. For all experiments, seeds and plants were maintained under greenhouse conditions, with an air temperature of 25 C (day)/20 C (night) and under 16 h daylength, and plants were manually watered daily as needed. Herbicide treatments for all experiments were applied using a chamber track sprayer (Generation 4, DeVries Manufacturing, Hollandale, MN, USA) equipped with an 8002EVS even flat-fan nozzle (TeeJet®, Spraying Systems, Wheaton, IL, USA) calibrated to deliver 160 L ha^{-1} of spray solution.

Dose–Response Experiments

A 2 by 6 factorial design was used in which R_{PPO} and S_{PPO} populations were the first factor, and herbicide doses formed the second factor. When plants had 3 to 4 fully expanded leaves (10- to 12-cm tall), lactofen was applied at 0.35, 1.4, 5.6, 22.5, 90, and 360 g ha^{-1} for S_{PPO} and at 0.69, 2.7, 11.2, 45, 180 and 720 g ha^{-1} for R_{PPO}; saflufenacil (Sharpen®, 297 g L^{-1}, BASF, Ludwigshafen, Germany) at 0.98, 3.9, 15.7, 63, 252, and 1,008 g ha^{-1} for S_{PPO} and 1.9, 7.8, 31, 126, 504, 2016 g ha^{-1} for R_{PPO}; pyraflufen-ethyl (Venue®, 20 g L^{-1}, Nichino America, Wilmington, DE, USA) at 0.54, 2.2, 8.8, 34.4, 136, and 550 g ha^{-1} for both populations; and flumioxazin (Valor®, 510 g L^{-1}, Valent) at 2.5, 10, 40, 160, 640, and 2,520 g ha^{-1} for both populations. Preliminary experiments were conducted to establish the best range of doses for each population and each herbicide. Untreated plants from both, R_{PPO} and S_{PPO}, were included as control treatments for all herbicides. At 21 d after application (DAA), plant shoots were collected and dried at 65 C for 5 d to quantify their respective dry masses. Data were pooled from two experiments with four replications for each herbicide ($n = 8$).

Protoporphyrin IX Levels

Twelve 12-cm-tall (4-leaf-stage) plants were sprayed with 120 g ha^{-1} of lactofen. This rate provides clear visual discrimination between the populations, leading to plant death in S_{PPO} but plant survival in R_{PPO}. Leaf tissue (~300 mg) was collected at 24 h after treatment (HAT) and ground with liquid nitrogen. Proto was extracted by homogenizing the powder in 2 ml of methanol:0.1 M NH_4OH (9:1), and centrifuged at 5,000 × *g* for 15 min (Dayan et al. 2015). The supernatants were filtered through a 0.25-µm nylon membrane filter, and proto levels were determined by liquid chromatography–mass spectrometry (LC-MS/MS) analysis. The LC-MS/MS system (Shimadzu Scientific Instruments, Columbia, MD, USA) consisted of a Nexera X2 UPLC (2 LC-30 AD pumps), a SIL-30 AC MP autosampler, a DGU-20A5 Prominence degasser, a Kinetex 2.6 µm F5 100 A LC column, and a SPD-M30A diode array detector coupled to an 8040 quadrupole mass spectrometer. Solvent A was 10 mM ammonium acetate (pH 5.6), and solvent B was methanol. The gradient started at 50% B and increased linearly to 70% B until 8 min, followed by a linear gradient to 90% B until 11 min. The mobile phase remained at 90% B until 13 min, then returned to 50% B at 13.5 min, and was maintained at 50% until the end of the run (15 min). The flow rate was 0.4 ml min^{-1}, and 5 µl of the samples were analyzed. Proto levels were quantified based on the external calibration curve (P8293, Sigma-Aldrich, St Louis, MO, USA). Data were pooled from two experiments with three biological replications each ($n = 6$).

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Quantification of ROS

ROS were measured in R_{PPO} and S_{PPO} plants at 24 HAT with 120 g ha^{-1} of lactofen as described elsewhere (Takano et al. 2019). The levels of hydrogen peroxide (H_2O_2) and superoxide (O_2^-) were measured by staining leaf disks in 3,3′-diaminobendizine (DAB) or nitro blue tetrazolium chloride (NBT), respectively. The DAB solution contained 600 ml of water and 0.3 g of DAB (pH 3.8). The NBT solution was composed of 600 ml of water, 0.3 g of NBT, 40.8 g of potassium phosphate monobasic (KH_2PO_4), and 3.9 g of sodium azide. Ten leaf disks (5 mm) from treated plants were placed in 10 ml of each solution, then shaken for 1 h at Hg vacuum at 15 kPa. Leaf disks were washed with water before bringing to boil at 95 C in 70% ethanol solution. The solution was replaced three times (10 min each) to remove leaf pigments, and the leaf disks were stored in 70% ethanol until the images were taken with a scanner (M479fdw, HP Color LaserJet Pro MFP, Houston, TX, USA). The leaf-disk images were converted to gray scale and inverted using CS3 Photoshop software (Adobe Systems, San Jose, CA, USA). Hydrogen peroxide and superoxide measurements were taken from S_{PPO} and R_{PPO} samples for color intensity relative to untreated leaf disks used as background. Data were pooled from two experiments with 10 replications each ($n = 20$).

PPO1 and PPO2 Amplification and Sequencing

RNA Extraction and cDNA Synthesis

One-hundred milligrams of fresh tissue per plant were collected from three R_{PPO} and three S_{PPO} plants, frozen, and homogenized with a TissueLyzer (Qiagen, Germantown, MD, USA). RNA extraction was conducted using a Direct-zol™ RNA MiniPrep Kit, (Zymo Research, Irvine, CA, USA). The total RNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and cDNA was synthesized using 4 µl of iScript RT Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 1 µl of RNA (100 ng), and 15 µl of nuclease-free water. The synthesis of cDNA was performed following the cycle: 5 min at 25 C, 20 min at 46 C, and 1 min at 95 C.

Primer Design and PCR Conditions

Because *E. heterophylla PPO* sequences are unavailable, primers were designed by aligning *PPO1* and *PPO2* sequences from four species belonging to the Euphorbiaceae family: *Manihot esculenta* (OAY48824.1 and R_OAY29614.1), *Ricinus communis* (XP_002515173.1 and XM_002509502.3), *Hevea brasiliensis* (XM_021820427.1 and XM_021790241.1), and *Jatropha curcas* (XP_012083019.1 and XP_012070726.1). Three sets of primers were designed for both *PPO1* and *PPO2* to evaluate which one provided the best-quality amplification. The target sequence includes the region of three mutations (Arg-128, Gly-210, and Gly-399) in *PPO2* that are known to impart resistance to PPO inhibitors, as well as the corresponding sequence in *PPO1*. Primer quality was assessed using the Multiple Primer Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). The best amplification products were obtained with PPO1_2F (5′-TCCGKTGCTCAATCACAGAG) and PPO1_2R (5′- CCATCTATTAAGCATTCTGTCCG) for *PPO1*; and PPO2_2F (5′-GTGATTRTAGGAGGTGGAATTAGC) and PPO2_3R (5′- TGCACGATCTGGAAACATC) for *PPO2*.

The PCR was conducted with 2 µl (20 ng) of cDNA, 2 µM of forward and reverse primers, and 12.5 µl of Econotaq Plus 2X Master Mix (Lucigen, Middleton, WI, USA). Nuclease-free water was used to obtain a final volume of 25 µl. The PCR settings were: 97 C for 60 s, 34 cycles of 97 C for 30 s, melting temperature (52 C for *PPO1* and 49.8 C for *PPO2*) for 30 s, and 72 C for 90 s. The final extension was at 72 C for 10 min. PCR products were separated in agarose gel (1%) and purified using a Zymo Gel Recovery Kit (Zymo Research, Irvine, CA, USA) before reamplification under the same PCR conditions. The final PCR products were purified with the same gel extraction kit and sequenced by Sanger DNA sequencing (Genewiz, South Plainfield, NJ, USA). Sequences from R_{PPO} and S_{PPO} were aligned to different species, including *A. palmeri*, *A. artemisiifolia*, tobacco (*Nicotiana tabacum* L.), and *Arabidopsis*.

PPO1 and PPO2 Expression

The same cDNAs synthesized from three S_{PPO} and three R_{PPO} plants were used for quantitative real-time PCR (qPCR) to quantify the expression of *PPO1* and *PPO2* relative to *ALS* (reference gene). Primers for *PPO1* (forward: 5′-CTTCAACTGATTCCTCACGTCC; and reverse: 5′-TGAGGCCAGTTCCTTCTAAGC), *PPO2* (forward: 5′-AAAGTATGCCGCACAGAAAGAGAC; and reverse: 5′-TTACCGAGGAGAGTGTTGGTGAG), and *ALS* (forward: 5′-GCCTTCCAGGAAACTCCGATTG; and reverse: 5′-AAAC TGGACCAGGACGACC) were designed to amplify short fragments (up to 118 bp) of each gene. The reference gene (*ALS*) has been used extensively for gene amplification in other studies (Brunharo et al. 2019; Chen et al. 2017; Gaines et al. 2010; Takano et al. 2020; Wiersma et al. 2015) The qPCR was run with the following conditions: 95 C for 10 min, 40 cycles of 95 C for 20 s, 55 C (*PPO1*) and 62 C (*PPO2*) for 1 min, and 59 C for 1 min. The subsequent melting curve was performed, increasing the temperature from 59 to 95 C with steps of 0.5 C for 5 s. Gene expression was calculated using the following equation:

$$\text{PPO: ALS relative expression} = 2^{(\text{Ct ALS} - \text{Ct PPO})} \quad [1]$$

where Ct is the cycle threshold. Data were pooled from two experiments with three biological replications and two technical replications ($n = 12$).

Effect of Cytochrome P450 Monooxygenase Inhibition

Sixteen plants (4-leaf stage) of each population were sprayed with malathion (Malathion Spectracide®, 500 g L^{-1}, Spectracide, St Louis, MO, USA) at 1,000 g ha^{-1}, and half of them were sprayed with lactofen (120 g ha^{-1}) 24 h later. Untreated control plants and lactofen-only treated plants were included for comparison. Plants were kept under greenhouse conditions until 21 DAA, when they were visually evaluated (0% means no control and 100% means plant death). Shoot tissue was also collected and dried at 65 C for dry-mass quantification. The experiment was conducted twice in a completed randomized design with eight replications per treatment ($n = 8$).

Data Analysis

For whole dose–response experiments, data were subjected to ANOVA, and a three-parameter log-logistic nonlinear regression model was fit (Streibig 1988):

$$y = \frac{a}{[1 + (\frac{x}{c})^b]} \quad [2]$$

where y is a dependent variable (dry-mass response), a is the asymptote, x is the independent variable (dose), c is the rate

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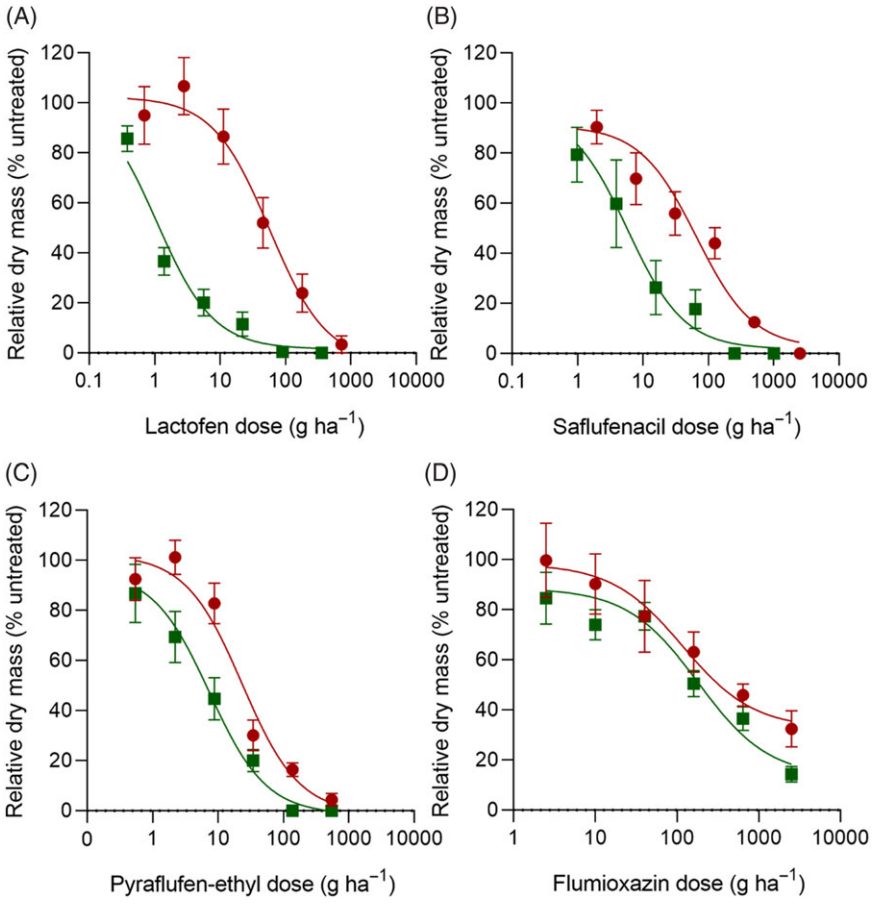

Figure 1. Dose–response curves for *Euphorbia heterophylla* susceptible (S_{PPO}) and resistant (R_{PPO}) to protoporphyrinogen oxidase (PPO)-inhibiting herbicides treated with (A) lactofen, (B) saflufenacil, (C) pyraflufen-ethyl, and (D) flumioxazin. The three-parameter log-logistic nonlinear regression model was fit: $y = a/[1 + (x/c)^b]$. Bars represent the standard errors of the means.

producing 50% of *a* (GR_{50}), and *b* is the slope around *c*. The ratio GR_{50} S_{PPO}/GR_{50} R_{PPO} was calculated to estimate the resistance factor (RF). Model selection (log-logistic three or four parameters) was based on previous literature, following guidelines described elsewhere (Ritz et al. 2015).

For the remaining experiments, means were subjected to ANOVA and compared by *t*-test (proto and ROS) or Tukey's test (*PPO* expression and P450s) ($P < 0.05$). All data were analyzed in R software (R Foundation for Statistical Computing, Vienna, Austria), and graphs were plotted using GraphPad 8 Prism (La Jolla, CA, USA).

Results and Discussion

Cross-resistance to PPO Inhibitors in Euphorbia heterophylla

The field rate of lactofen (180 g ha^{-1}) completely controlled S_{PPO} but did not reduce the dry mass by more than 80% in R_{PPO}. The R_{PPO} population was 47.7 times more resistant to lactofen than S_{PPO}, based on GR_{50} values (Figure 1; Table 1). The R_{PPO} was also resistant to saflufenacil with an RF of 8.6-fold (Figure 1; Table 1). For pyraflufen-ethyl, the GR_{50} was 22.8 for R_{PPO} and 6.4 for S_{PPO}, resulting in an intermediate level of resistance (3.5-fold). For flumioxazin, differences in GR_{50} parameters between the two populations were not significant, and thus resistance to flumioxazin was not confirmed in R_{PPO} (Table 1). These results are consistent with other PPO-resistant *E. heterophylla* populations from Brazil (Trezzi et al. 2005, 2006; Xavier et al. 2018). Trezzi et al. (2005) studied two populations with high RF to fomesafen (39- and 62-fold) that were controlled with sulfentrazone (600 g ha^{-1}) and flumioxazin (50 g ha^{-1}) PRE. In a *E. heterophylla* population from Virotino city, the RF was 15.1-fold for saflufenacil and 4.9-fold for flumiclorac (same chemical group as flumioxazin) (Xavier et al. 2018). Regardless of the conditions adopted by different laboratories, the resistance levels and cross-resistance patterns primarily depend on the population and its resistance mechanism. *Euphorbia heterophylla* resistant to PPO inhibitors is widespread across Brazilian agricultural areas, especially in the south (Prigol et al. 2014; Trezzi et al. 2006). Here, we demonstrate that R_{PPO} is resistant to at least three chemical groups of PPO inhibitors (diphenyl ether, pyrimidinedione, and *N*-phenylphthalimid).

Protoporphyrin IX Accumulates in Sensitive Euphorbia heterophylla

Lactofen treatment caused 27 times higher proto accumulation in S_{PPO} than R_{PPO} (Figure 2). This is consistent with the differences in lactofen sensitivity between R_{PPO} and S_{PPO}, as observed in the whole dose–response experiments. Similar to our results, a susceptible population accumulated 19 times more proto than a PPO-resistant population after fomesafen treatment (Rojano-Delgado et al. 2019). Low levels of proto in R_{PPO} indicate that lactofen is not inhibiting PPO activity, either because it is no longer binding

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Table 1. Regression parameters and resistance factors (RFs) for *Euphorbia heterophylla* susceptible (S_{PPO}) and resistant (R_{PPO}) to protoporphyrinogen oxidase (PPO)-inhibiting herbicides.

Herbicide	Population	*a*	*b*	*c*[a] GR_{50}	P-value	RF[b]
				g ha^{-1}		
Lactofen	R_{PPO}	101.6	1.13	52 (±12)	<0.0001	47.7
	S_{PPO}	102.5	1.09	1.1 (±0.3)	0.0001	
Saflufenacil	R_{PPO}	98.8	0.66	48 (±22)	0.03	8.6
	S_{PPO}	99.7	0.82	5.6 (±2)	0.0071	
Pyraflufen-ethyl	R_{PPO}	99.4	1.4	22.8 (±4.1)	<0.0001	3.5
	S_{PPO}	98.4	0.8	6.4 (±1.9)	0.0017	
Flumioxazin	R_{PPO}	102.7	0.5	448 (±232)	0.0569	2
	S_{PPO}	95.6	0.5	217 (±122)	0.0802	

[a]Means of two experiments with four replicates each ($n = 8$) followed by SE.
[b]Calculated by GR_{50} in R_{PPO} /GR_{50} in S_{PPO} according to three-parameter nonlinear log-logistic regression model: $y = a/[1 + (x/c)^b]$.

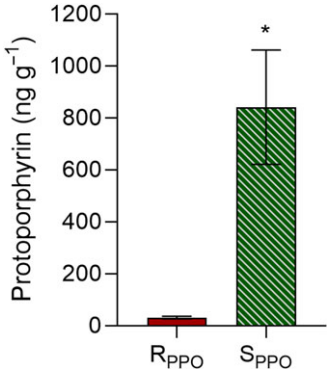

Figure 2. Protoporphyrin IX (proto) accumulation in *Euphorbia heterophylla* susceptible (S_{PPO}) and resistant (R_{PPO}) to protoporphyrinogen oxidase (PPO)-inhibiting herbicides 24 h after treatment with 120 g ha^{-1} of lactofen. S_{PPO} accumulated 20-fold more proto than R_{PPO}. *Means are significantly different by *t*-test ($P \leq 0.05$).

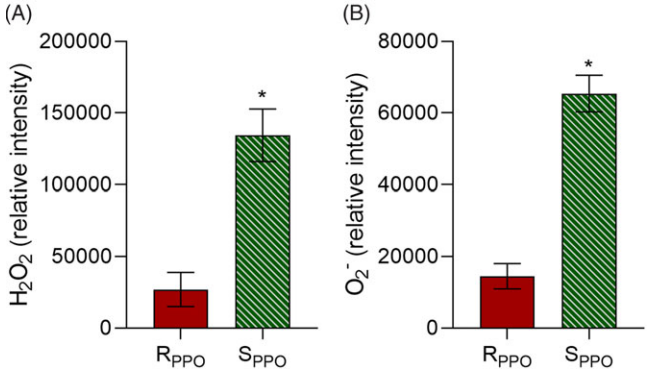

Figure 3. Reactive oxygen species (ROS) produced by *Euphorbia heterophylla* susceptible (S_{PPO}) and resistant (R_{PPO}) to protoporphyrinogen oxidase (PPO)-inhibiting herbicides at 24 h after treatment with lactofen (120 g ha^{-1}). Means represent (A) hydrogen peroxide (H_2O_2) and (B) superoxide (O_2^-) levels. *Means are significantly different by *t*-test ($P \leq 0.05$).

to the catalytic domain or because some defense mechanism is reducing the amount of herbicide reaching the target site (e.g., altered uptake and translocation or increased metabolism).

Injury Is Dependent on Accumulation of ROS

Both R_{PPO} and S_{PPO} generated ROS at 24 HAT compared with untreated plants. However, S_{PPO} showed 5 and 4.5 higher accumulation of H_2O_2 and O_2^- than R_{PPO}, respectively (Figure 3). In susceptible plants, proto accumulates following PPO inhibition and is then exported from the chloroplast to the cytoplasm, where it is oxidized into proto that accumulates in high concentrations (Lee et al. 1993). Because proto has photodynamic properties, fast lipid peroxidation in the presence of light occurs in response to ROS formation (Matringe et al. 1989). Consequently, R_{PPO} is less likely to produce ROS, because plants accumulate less proto at 24 HAT compared with S_{PPO} (Figure 2).

Arg-128-Leu Mutation Identified in PPO2

The PCR products were 987 bp for *PPO1* and 1,015 bp for *PPO2*. *Euphorbia heterophylla PPO1* had 86% similarity with *H. brasiliensis* (XM_021820427.1) and 85% with *M. esculenta* (XM_021757904.1), whereas *PPO2* was 82% similar to *H. brasiliensis* (XM_021790241.1) and 76% to *Clonorchis sinensis* (XM_028195431.1). When S_{PPO} and R_{PPO} sequences were aligned, no mutations were found in *PPO1*, as the two populations shared 100% identity in all sequenced plants.

For *PPO2*, in contrast, we found 35 single-nucleotide polymorphisms (SNPs) between S_{PPO} and R_{PPO}. Nineteen SNPs were synonymous, whereas 16 provided codon substitution (Arg-128-Leu, Asn-150-Ser, Val-165-Phe, Ser-170-Asn, Cys-176-Tyr, Asp-179-Gly, Asp-185-Gly, Gln-194-Lys, Lys-242-Asn, Pro-245-Ser, Glu-288-Gly, Asn-316-Asp, Asp-321-Asn, Leu-349-Pro, Thr-367-Ala, and Lys-374-Arg). Among these 16 amino acid changes, the only codon substitution unique to R_{PPO} was Arg-128-Leu, found in a conserved region of *PPO2* when compared with all aligned species (Figure 4). All of the R_{PPO} plants had a leucine instead of an arginine in S_{PPO} plants (nucleotide substitution from CGG to CTG).

The Arg-128-Leu mutation in *PPO2* was previously reported in *A. artemisiifolia* as homologous to Arg-98-Leu. A *PPO2* mutant (Leu-128) sequence from *A. tuberculatus* was cloned into *Escherichia coli* to prove that Arg-128-Leu confers resistance to PPO inhibitors in vitro (Rousonelos et al. 2012). *Echerichia coli* colonies containing Leu-128 were 31-fold more resistant to lactofen than the Arg-128 wild type. For both *E. heterophylla* and *A. artemisiifolia* Arg-128-Leu normally confers high levels of resistance to diphenyl-ethers and pyrimidinedione, low or intermediate levels to phenylpyrazole, and sensibility to *N*-phenylphthalimid (Table 1). In *A. palmeri*, other amino acid polymorphisms in Arg-128 are possible, such as Arg-128-Met and Arg-128-Gly conferring resistance to fomesafen (Giacomini et al. 2017).

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                                  ...128...150...165...170...176...179...185...194...
RPPO E. heterophylla              ...KLY...SSI...PVF...RNE...VYN...SGD...VGE...GKE...
SPPO E. heterophylla              ...KRY...SNI...PFF...RSE...VCN...SDT...VDE...GQE...
SPPO Amaranthus palmeri           ...KRY...SNF...PFL...KRN...LSD...EHV...VGE...GKE...
SPPO Ambrosia artemisiifolia      ...KRY...SSF...PFL...KTS...-SD...EP-...VGG...GKE...
Nicotiana tabacum                 ...KRY...SNF...PIL...KKL...VSD...SH-...VSG...GKE...
Arabidopsis thaliana              ...KRY...SSV...PFL...KKS...VSD...ASA...VSE...GQE...

                                  ...242...245...291...319...324...352...370...377...
RPPO E. heterophylla              ...SNF...SSK...LGS...GDK...QNS...FPL...TAF...VRQ...
SPPO E. heterophylla              ...SKF...SPK...LES...GNK...QDS...FLL...TTF...VKQ...
SPPO Amaranthus palmeri           ...STL...LSK...LQC...-NN...EDQ...FSL...TAF...VKR...
SPPO Ambrosia artemisiifolia      ...SMV...SSR...LQS...DQN...FQQ...FLL...STF...VKQ...
Nicotiana tabacum                 ...SKL...SPK...LNS...HKR...EEE...FLL...TTF...VKY...
Arabidopsis thaliana              ...TKF...AAK...LDS...NET...QNP...FQL...TAF...VKR...
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Figure 4. PPO2 protein sequence showing polymorphisms between *Euphorbia heterophylla* susceptible (S_{PPO}) and resistant (R_{PPO}) to protoporphyrinogen oxidase (PPO)-inhibiting herbicides and alignment with sequences from other species. Green and red represent codon substitutions between S_{PPO} and R_{PPO} *E. heterophylla*. Only the Arg-128-Leu mutation is located in a conserved domain of *PPO2* (gray highlighted bar). The Arg-128 position is homologous to Arg-98 in *Ambrosia artemiisifolia* (GenBank AFJ04119.1) and tobacco (GenBank XP_016496870.1). *Amaranthus palmeri*, GenBank MF583744.1, and *Arabidopsis thaliana*, GenBank NP_001190307.1.

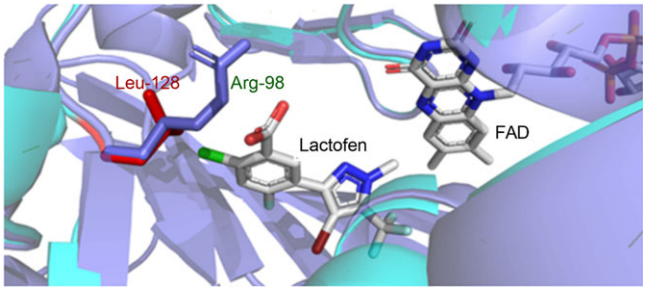

Figure 5. Overlay of the catalytic domain of the crystal structure of PPO2 from tobacco (slate blue) and the homology model of the *Euphorbia heterophylla* resistant (R_{PPO}) to protoporphyrinogen oxidase (PPO)-inhibiting herbicides (cyan). The Arg-98 amino acid (sensitive tobacco) is shown in darker slate blue color, and the corresponding Leu-128 mutation in *E. heterophylla* R_{PPO} is shown in red. The mutation Arg-128-Leu (homologous to Arg-98-Leu in tobacco and *Ambrosia artemisiifolia*) alters lactofen binding.

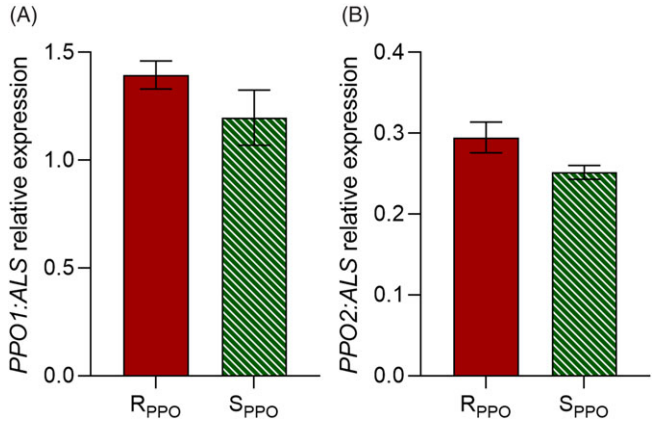

Figure 6. *PPO1* (A) and *PPO2* (B) expression relative to *ALS* in *Euphorbia heterophylla* susceptible (S_{PPO}) and resistant (R_{PPO}) to protoporphyrinogen oxidase (PPO)-inhibiting herbicides from Brazil. Data were pooled from two experiments ($n = 12$). Differences are not significant between S_{PPO} and R_{PPO} ($P < 0.05$).

In spinach (*Spinacia oleracea* L.) and several other species, *PPO2* encodes a dual-targeting peptide that directs PPO2 to both chloroplasts and mitochondria (Dayan et al. 2018; Patzoldt et al. 2006; Watanabe et al. 2001). This means that a target-site mutation in *PPO2* leads to resistance in both organelles. In contrast, if *PPO2* was not dual-targeted, a target-site mutation might not provide the same degree of herbicide resistance at the plant level (Rangani et al. 2019). It is possible that some herbicides may prefer one of the targets (chloroplasts or mitochondria). For example, the Ala-212-Thr mutation found in *E. indica PPO1* confers resistance to oxadiazon but not to other PPO inhibitors, indicating that oxadiazon may target only PPO1 (Bi et al. 2020). The presence of a dual-target sequence should be investigated in *E. heterophylla*.

The plant crystal structure of PPO2 was obtained from tobacco (1SEZ) (Koch et al. 2004) and used as a template for protein alignment with the sequences from S_{PPO} and R_{PPO}. According to this alignment, Arg-128 in *E. heterophylla* is equivalent to Arg-98 in tobacco. It has been demonstrated in tobacco that the positively charged residue Arg-98 (along with other conserved residues Phe-392, Leu-356, and Leu-372) is involved in coordinating the substrate within the active site by forming a salt bridge with one of the propionic acid side chains of protogen. Substituting Arg-98 with alanine (a nonpolar residue) decreased the affinity of PPO for its substrate 8-fold by disrupting this interaction (Heinemann et al. 2007). The complex lactofen-R_{PPO} illustrates the importance of Arg-128 (equivalent to Arg-98 in tobacco) to herbicide binding in *E. heterophylla* by interacting with the carboxy group on one of the rings of the herbicide (Figure 5). Likely, the Arg-128-Leu substitution introducing a nonpolar residue similar to that of alanine reported in tobacco may also negatively alter the binding of both the substrate protogen and lactofen.

In summary, this finding is supported by the fact that Arg-128 is localized in a conserved domain of *PPO2* (Figure 4) and near the binding site of lactofen (Figure 5). Mutations in the same amino acid position were previously found in *A. artemisiifolia* and *A. palmeri* resistant to PPO inhibitors (Giacomini et al. 2017; Sarangi et al. 2019). Inheritance studies conducted with other *E. heterophylla* populations showed a single nuclear and dominant gene involved with PPO-inhibitor resistance (Brusamarello et al. 2016). A single and dominant resistance allele supports our results for a target-site mutation as the only mechanism of resistance.

PPO1 *and* PPO2 *Have Similar Expression Levels in* S_{PPO} *and* R_{PPO}

The relative expression of *PPO1* ranged from 1.3 to 1.8 in S_{PPO} plants and from 1.2 to 1.6 in R_{PPO} plants (Figure 6). For *PPO2*,

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Table 2. Effect of the P450 monooxygenase inhibitor malathion on lactofen response by *Euphorbia heterophylla* susceptible (S_{PPO}) and resistant (R_{PPO}) to protoporphyrinogen oxidase (PPO)-inhibiting herbicides.[a]

Treatment	Visual injury		Relative dry mass	
	R_{PPO}	S_{PPO}	R_{PPO}	S_{PPO}
	%		% of untreated	
Untreated	0 b	0 b	100 A	100 a
Malathion	3 b	3 b	109 A	86 a
Lactofen	47 a	98 a	49 B	2 b
Malathion/lactofen[b]	51 a	99 a	37 B	1 b

[a]Means followed by the same letters in columns are not significantly different (Tukey's test, $P \leq 0.05$). Data were pooled from two experiments with eight replications each ($n = 16$).
[b]There were 24 h between malathion and lactofen applications. Malathion dose: 1,000 g ha^{-1}; lactofen dose: 120 g ha^{-1}.

expression levels were 0.24 to 0.25 in S_{PPO} and 0.27 to 0.28 in R_{PPO}. Differences between populations were not significant for *PPO1* ($P = 0.13$) nor *PPO2* ($P = 0.053$). While transgenic studies demonstrated that overexpression of PPO1 or PPO2 in plants can impart resistance to PPO inhibitors (Ha et al. 2003; Jung et al. 2004; Lermontova and Grimm 2000; Warabi et al. 2001), this mechanism of resistance has not been identified in any PPO-resistant weed population to date and is not involved in PPO-inhibitor resistance in *E. heterophylla* analyzed in this study.

Inhibitor of P450 Monooxygenases Does Not Restore Herbicidal Activity

Metabolism of PPO inhibitors by these enzymes in weeds was first found in an *A. palmeri* population from Arkansas, in which malathion partially reversed fomesafen resistance (Varanasi et al. 2018). In *E. heterophylla*, malathion application at 24 h before lactofen treatment did not decrease R_{PPO} capacity to survive against the herbicide (Table 2). Lactofen, with or without malathion, caused similar visual injury of 50% and reduced dry mass by approximately 43% in relation to untreated plants (Table 2). These results suggest that P450s inhibited by malathion are not associated with lactofen metabolism in *E. heterophylla*. Similar results were found when the organophosphate methamidophos (a potent P450 inhibitor) was sprayed before application of fomesafen and lactofen in a *E. heterophylla* population from southwestern Parana State (Trezzi et al. 2009). Organophosphate insecticides such as malathion are cytochrome P450 inhibitors in plants, and they can reverse resistance when herbicide metabolism involves these enzymes (Busi et al. 2017; Keith et al. 2015). In a population of *A. tuberculatus* resistant to PPO inhibitors, carfentrazone-ethyl provided higher levels of control in the presence of malathion than in the absence of the insecticide. This population did not have the ΔG210 in *PPO2*; thus, increased enhanced metabolism could be involved (Obenland et al. 2019). While cytochrome P450 enzymes can metabolize some PPO inhibitors to impart crop selectivity (Dayan et al. 1997a, 1997b, 1998; Strang and Rogers 1974), they are normally not involved in the mechanism of resistance to PPO inhibitors in weeds (Dayan et al. 1996, 2014).

In conclusion, *E. heterophylla* is the first reported species harboring an Arg-128-Leu mutation in PPO2 in South America. This is supported by lower levels of protoporphyrin and ROS in R_{PPO} compared with S_{PPO}. At the plant level, the Arg-128-Leu mutation confers resistance to the PPO inhibitors lactofen (47.7-fold), saflufenacil (8.6-fold), and pyraflufen-ethyl (3.5-fold). Malathion treatment did not affect the response to lactofen in R_{PPO}, suggesting that herbicide metabolism is unlikely. Both PPO1 and PPO2 have similar expression levels in R_{PPO} and S_{PPO}. Future research will focus on determining PPO activity in vitro as well as kinetics data from S_{PPO} and R_{PPO}. Molecular markers could also be developed to survey a large number of samples based on the codon polymorphism in residue 128 of PPO2.

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