R128L in wild poinsettia

R128L Target Site Mutation in PPO2 Evolves in Wild Poinsettia (Euphorbia heterophylla) with Cross-Resistance to PPO-Inhibiting Herbicides

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Abstract

Wild poinsettia (*Euphorbia heterophylla* L.) is a troublesome broadleaf weed in grain production areas from 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 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 h after lactofen treatment (120 g ha⁻¹) in 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 R128L (homologous to R98L 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 monoxygenase 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 a R128L mutation in *PPO2* conferring cross resistance to PPO inhibitors in *E. heterophylla*.

Keywords: Protoporphyrin, lactofen, saflufenacil, pyraflufen-ethyl, flumioxazin, reactive oxygen species, *PPO1*, *PPO2*, R128L mutation, cytochrome P450 monoxygenase.
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 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 been again 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).

Protoporphyrinogen-oxidase-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 express the chloroplast and mitochondrial PPO enzyme isoforms, *PPO1* and *PPO2*, 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.) J.D.Sauer) (Patzoldt et al. 2006; Sarangi et al. 2019). Different mutations at arginine 128 (R128G or M) in PPO2 have also been reported in PPO-resistant Palmer amaranth (*Amaranthus palmeri* S.Watson) (Varanasi et al. 2017), as homologous to a R98L 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 (G399A) 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 a A212T amino acid change was
found conferring 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 (P450s) 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, *E. heterophylla* (three plants m\(^{-2}\)) caused up to 60% of yield losses due to weed interference (Machado et al. 2015). 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 (Meschede et al. 2002). Furthermore, this species also has high levels of genetic diversity as verified in 12 populations from Brazil, which contributes to a rapid evolution of herbicide resistance (Frigo et al. 2009).

Prior to 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 an *E. heterophylla* population from Brazil.
Material 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 seven years. 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). Three days 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, 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 (G1), and the same screening process was repeated twice to obtain a G3 generation with homogeneous resistance to lactofen. All subsequent experiments were conducted with the G3 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, USA). Then, five to seven days 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, USA) equipped with an
8002EVS even flat-fan nozzle (TeeJet; Spraying Systems Co., Wheaton, USA) calibrated to deliver 160 L ha\(^{-1}\) spray solution.

**Dose-response experiments**

A 2 × 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 3-4 fully expanded leaves (10-12 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\(^\circledast\), 297 g L\(^{-1}\), BASF, Ludwigshafen, Germany) at 0.98, 3.9, 15.7, 63, 252 and 1008 g ha\(^{-1}\) for S\(_{-}\)PPO and 1.9, 7.8, 31, 126, 504, 2016 g ha\(^{-1}\) for R\(_{-}\)PPO; pyraflufen-ethyl (Venue\(^\circledast\), 20 g L\(^{-1}\), Nichino America, Wilmington, USA) at 0.54, 2.2, 8.8, 34.4, 136 and 550 g ha\(^{-1}\) for both populations; and flumioxazin (Valor\(^\circledast\), 510 g L\(^{-1}\), Valent, Walnut Creek, USA) at 2.5, 10, 40, 160, 640 and 2520 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. Twenty-one days after application (DAA), plant shoots were collected and dried at 65 °C for five days to quantify their respective dry mass. Data were pooled from two experiments with four replications for each herbicide (n = 8).

**Protoporphyrin IX levels**

Twelve plants with 12 cm tall (4 leaf stage) were sprayed with 120 g ha\(^{-1}\) of lactofen. This rate provides a 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 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\(_4\)OH (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 21046) 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 SPD-M30A diode array detector coupled to an 8040 quadrupole mass spectrometer. The 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 maintained to 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, USA). Data were pooled from two experiments with three biological replications each (n = 6).

**Quantification of reactive oxygen species**

Reactive oxygen species 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 (H₂O₂) and superoxide (O₂⁻) were measured by staining leaf discs in 3,3’-diaminobenzidine (DAB) or nitro blue tetrazolium chloride (NBT), respectively. The DAB solution contained 600 mL water and 0.3 g 3,3’-diaminobenzidine (pH 3.8). The NBT solution was composed of 600 mL water, 0.3 g nitro blue tetrazolium chloride, 40.8 g potassium phosphate monobasic (KH₂PO₄), and 3.9 g sodium azide. Ten leaf discs (5 mm) from treated plants were placed in 10 mL of each solution, then shaken for 1 h on Hg vacuum at 15 kPa. Leaf discs were washed with water prior to 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 discs were stored in 70% ethanol until the images were taken with a scanner (M479fdw, HP Color Laser Jet Pro MFP, Houston, TX USA). The leaf-disc images were converted to gray scale, and inverted using the CS3 Photoshop software (Adobe Systems, San Jose, CA, USA). Hydrogen peroxide
and superoxide measurements were taken from S\textsuperscript{-PPO} and R\textsuperscript{-PPO} samples for color intensity relative to untreated leaf discs used as background. Data were pooled from two experiments with ten replications each (n = 20).

**PPO1 and PPO2 amplification and sequencing**

**RNA extraction and cDNA synthesis**

One-hundred mg of fresh tissue from three R\textsuperscript{-PPO} and three S\textsuperscript{-PPO} plants were collected, frozen, and homogenized with a TissueLyzer (Qiagen, Germantown, MD USA). RNA extraction was conducted using a Direct-zol\textsuperscript{TM} RNA MiniPrep Kit, (Zymo Research, Irvine, CA USA). The total RNA was quantified using a spectrophotometer Nanodrop (Thermo Fisher Scientific, Waltham, MA USA), and cDNA was synthesized using 4 μL of iScript RT Supermix (Bio-Rad Laboratories, Hercules, 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 of amplification. The target sequence includes the region of three mutations (R128, G210, and G399) in the *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 Fischer Scientific\textsuperscript{®}, Whatham, USA). The best amplification products were obtained with PPO1\textsubscript{2F} (5’-TCCGKTGCTCAATCAGAG)
and PPO1_2R (5’- CCATCTATTAAGCATCTGTTCCG) for PPO1; and PPO2_2F (5’-GTGATRTAGGAGTGGGATTAGC) 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 reamplifying 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’-CTTCAACTGATTCCCTACGTCC and reverse 5’-TGAGGCGGTTCTTCTAAGC), PPO2 (forward 5’-AAAGTATGCGCATCAGAAAGAC and reverse 5’-TTACCGAGGAGTGTTGGTGAG) and ALS (forward 5’-GCCTTCCAGGAACTCGATTG and reverse 5’-AAACTGGACCAGGACGACC) were designed to amplify short fragments (up to 118 bp) of each gene. The reference gene (ALS) has been extensively used 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:

\[
PPO: \text{ALS relative expression} = 2^{(Ct_{ALS} - Ct_{PPO})}
\]  

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 monoxygenase inhibition**

Sixteen plants (4-leaf stage) of each population were sprayed with malathion (Malathion Spectracide, 500 g L⁻¹, Spectracide, St. Louis, 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 were included for comparison, as well as lactofen-only treated plants. 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.

**Data analysis**

For whole dose-response experiments, data were submitted to ANOVA, and a three-parameter log-logistic non-linear regression model was fitted (Streibig 1988):

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

where y is a dependent variable (dry mass response); a is the asymptote, x is the independent variable (dose), c is the rate producing 50% of a (GR₅₀), and b is the slope around c. The ratio GR₅₀ S⁻PPO / GR₅₀ 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, USA).

Results and Discussion

**Cross-resistance to PPO inhibitors in E. heterophylla**

The field rate of lactofen (180 g ha\(^{-1}\)) completely controlled S\(_{\text{PPO}}\) but did not reduce the dry mass by more than 80% in R\(_{\text{PPO}}\). The R\(_{\text{PPO}}\) population was 47.7-times more resistant to lactofen than S\(_{\text{PPO}}\), based on GR\(_{50}\) values (Figure 1 and Table 1). The R\(_{\text{PPO}}\) was also resistant to saflufenacil with RF of 8.6-fold (Figure 1 and Table 1). For pyraflufen-ethyl, the GR\(_{50}\) was 22.8 for R\(_{\text{PPO}}\) and 6.4 for S\(_{\text{PPO}}\) 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\(_{\text{PPO}}\) (Table 1). These results are consistent with other PPO-resistant *E. heterophylla* populations from Brazil (Trezzi et al. 2005; Trezzi et al. 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\(^{-1}\)) in 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, N-phenylphthalimid).

**Protoporphyrin IX accumulates in sensitive E. 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 indicates that lactofen is not inhibiting PPO activity by either no longer binding to the catalytic domain or by some defense mechanism reducing the amount of herbicide reaching the target site (e.g., altered uptake and translocation or increased metabolism).

**Injury is dependent on accumulation of reactive oxygen species**

Both R-PPO and S-PPO generated ROS at 24 HAT compared to untreated plants. However, S-PPO showed 5 and 4.5 higher accumulation of H$_2$O$_2$ 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 they accumulate less proto at 24 HAT compared to S-PPO (Figure 2).

**R128L mutation identified in PPO2**

The PCR products were 987 bp for PPO1 and 1015 bp for PPO2. *E. 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 *C. sinensis* (XM_028195431.1). By aligning S-PPO and R-PPO
sequences, no mutations were found in \textit{PPO1} as the two populations shared 100\% identity in all sequenced plants. For \textit{PPO2}, in contrast, we found 35 single nucleotide polymorphisms (SNP) between \textit{S-PPO} and \textit{R-PPO}. Nineteen SNPs were synonymous, whereas 16 provided codon substitution (R128L, N150S, V165F, S170N, C176Y, D179G, D185G, Q194K, K242N, P245S, E288G, N316D, D321N, L349P, T367A, and K374R). Among these 16 amino acid changes, the only codon substitution unique to \textit{R-PPO} was R128L, found in a conserved region of \textit{PPO2} when compared to all aligned species (Figure 4). All of the \textit{R-PPO} plants had a leucine (L) instead of an arginine (R) in \textit{S-PPO} plants (nucleotide substitution from CGG to CTG).

The R128L mutation in \textit{PPO2} was previously reported in \textit{A. artemisiifolia} as homologous to R98L. A \textit{PPO2} mutant (L128) sequence from \textit{A. tuberculatus} was cloned into \textit{E. coli} to prove that R128L confers resistance to PPO inhibitors \textit{in vitro} (Rousonelos et al. 2012). \textit{Echerichia coli} colonies containing L128 were 31-fold more resistant to lactofen than the R128 wild-type. For both \textit{E. heterophylla} and \textit{A. artemisiifolia} R128L normally confers high levels of resistance to diphenyl-ethers and pyrimidinedione, low or intermediate levels to phenylpyrazole, and sensibility to \textit{N-phenylphthalimid} (Table 1). In \textit{A. palmeri}, other amino acid polymorphisms in R128 are possible, such as R128M and R128G conferring resistance to fomesafen (Giacomini et al. 2017).

In spinach and several other species, \textit{PPO2} encodes a dual-targeting peptide that directs \textit{PPO2} to both chloroplast and mitochondria (Dayan et al. 2018; Patzoldt et al. 2006; Watanabe et al. 2001). This means that a target site mutation in \textit{PPO2} leads to resistance in both organelles. In contrast, if \textit{PPO2} was not dual-targeted, a target site mutation may 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 (chloroplast or mitochondria). For example, the A212T mutation found in \textit{E. indica} \textit{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, R128 in *E. heterophylla* is equivalent to R98 in tobacco. It has been demonstrated that in tobacco, the positively charged residue R98 (along with other conserved residues F392, L356, and L372) 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 R98 with alanine (a non-polar 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 R128 (equivalent to R98 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 R128L substitution introducing a non-polar 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 R128 is localized in a conserved domain of PPO2 (Figure 4) and nearby 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 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, 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 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 a *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 P450s inhibitor) was sprayed prior to fomesafen and lactofen in a *E. heterophylla* population from Southwest of 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 ∆G210 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. 1998; Dayan et al. 1997a; Dayan et al.
In conclusion, *E. heterophylla* is the first reported species harboring a R128L mutation in *PPO2* in South America. This is supported by lower levels of protoporphyrin and ROS in R-*PPO* compared to S-*PPO*. At the plant level, the R128L 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*.

**Acknowledgment**

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Table 1. Regression parameters and resistance factor (RF) for *Euphorbia heterophylla* susceptible (S-\textsuperscript{PPO}) and resistant (R-\textsuperscript{PPO}) to PPO-inhibiting herbicides.

<table>
<thead>
<tr>
<th>Herbicide</th>
<th>Population</th>
<th>(a)</th>
<th>(b)</th>
<th>(c^{a})</th>
<th>(P)-value</th>
<th>RF (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactofen</td>
<td>R-\textsuperscript{PPO}</td>
<td>101.6</td>
<td>1.13</td>
<td>52 (± 12)</td>
<td>&lt;0.0001</td>
<td>47.7</td>
</tr>
<tr>
<td></td>
<td>S-\textsuperscript{PPO}</td>
<td>102.5</td>
<td>1.09</td>
<td>1.1 (± 0.3)</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Saflufenacil</td>
<td>R-\textsuperscript{PPO}</td>
<td>98.8</td>
<td>0.66</td>
<td>48 (± 22)</td>
<td>0.03</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>S-\textsuperscript{PPO}</td>
<td>99.7</td>
<td>0.82</td>
<td>5.6 (± 2)</td>
<td>0.0071</td>
<td></td>
</tr>
<tr>
<td>Pyraflufen-ethyl</td>
<td>R-\textsuperscript{PPO}</td>
<td>99.4</td>
<td>1.4</td>
<td>22.8 (± 4.1)</td>
<td>&lt;0.0001</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>S-\textsuperscript{PPO}</td>
<td>98.4</td>
<td>0.8</td>
<td>6.4 (± 1.9)</td>
<td>0.0017</td>
<td></td>
</tr>
<tr>
<td>Flumioxazin</td>
<td>R-\textsuperscript{PPO}</td>
<td>102.7</td>
<td>0.5</td>
<td>448 (± 232)</td>
<td>0.0569</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>S-\textsuperscript{PPO}</td>
<td>95.6</td>
<td>0.5</td>
<td>217 (± 122)</td>
<td>0.0802</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Means of two experiments with four replicates each (\(n=8\)) followed by standard error.

\(^b\) Calculated by \(GR_{50}^{R-PPO} / GR_{50}^{S-PPO}\) according to three-parameter non-linear log-logistic regression model: \(y = a/[1+(x/c)^b]\).
Table 2. Effect of the P450 monooxygenase inhibitor malathion on lactofen response by R-<sub>PPO</sub> (PPO resistant) and S-<sub>PPO</sub> (PPO susceptible) *Euphorbia heterophylla*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Visual injury (%)</th>
<th>Relative dry mass (% of untreated)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R&lt;sub&gt;PPO&lt;/sub&gt;</td>
<td>S&lt;sub&gt;PPO&lt;/sub&gt;</td>
</tr>
<tr>
<td>Untreated</td>
<td>0 b</td>
<td>0 b</td>
</tr>
<tr>
<td>Malathion</td>
<td>3 b</td>
<td>3 b</td>
</tr>
<tr>
<td>Lactofen</td>
<td>47 a</td>
<td>98 a</td>
</tr>
<tr>
<td>Malathion / lactofen</td>
<td>51 a</td>
<td>99 a</td>
</tr>
</tbody>
</table>

Means followed by the same letters in columns are not significantly different (Tukey’s test, p<0.05).

Data were pooled from two experiments with eight replication each (n=16).

“/”: 24 h between malathion and lactofen applications.

Malathion dose: 1000 g ha<sup>-1</sup>; Lactofen dose: 120 g ha<sup>-1</sup>.
Figure 1. Dose-response curves for R-PPO and S-PPO E. heterophylla treated with A) lactofen, B) saflufenacil, C) pyraflufen-ethyl, and D) flumioxazin. The three parameter log-logistic non-linear regression model was fit: $y = a/[1+(x/c)^b]$. Bars represent the standard error of the means.
Figure 2. Protoporphyrin IX (proto) accumulation in PPO resistant (R<PPO>) and susceptible (S<PPO>) Euphorbia heterophylla at 24 h after treatment with 120 g ha<sup>-1</sup> of lactofen. S<PPO> showed 20-fold proto than R<PPO>. *means are significantly different by t-test (p≤0.05).
Figure 3. Reactive oxygen species (ROS) produced by S-POO and R-POO Euphorbia heterophylla at 24 h after treatment with lactofen (120 g ha\(^{-1}\)). Means represent A) hydrogen peroxide (H\(_2\)O\(_2\)) and B) superoxide (O\(_2^-\)) levels. *means are significantly different by t-test (p≤0.05).
<table>
<thead>
<tr>
<th>Species</th>
<th>Protein Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wild poinsettia R-PPO</strong></td>
<td>...KLY...SSI...PVF...RNE...VYN...SGD...VGE...GKE...</td>
</tr>
<tr>
<td><strong>Wild poinsettia S-PPO</strong></td>
<td>...KRY...SNI...PFF...RSE...VCN...SDT...VDE...GQE...</td>
</tr>
<tr>
<td><strong>Palmer amaranth S-PPO</strong></td>
<td>...KRY...SNF...PFL...KRN...LSD...EHV...VGE...GKE...</td>
</tr>
<tr>
<td><strong>Common ragweed S-PPO</strong></td>
<td>...KRY...SSF...PFL...KTS...-SD...EP...-VGG...GKE...</td>
</tr>
<tr>
<td><strong>Tobacco</strong></td>
<td>...KRY...SNF...PFL...KKL...VSD...SH...VSG...GKE...</td>
</tr>
<tr>
<td><strong>Arabidopsis</strong></td>
<td>...KRY...SSV...PFL...KKS...VSD...ASA...VSE...GQE...</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wild poinsettia R-PPO</strong></td>
<td>...242...245...291...319...324...352...370...377...</td>
</tr>
<tr>
<td><strong>Wild poinsettia S-PPO</strong></td>
<td>...SNF...SSK...LQS...GDK...QNS...FPL...TAF...VBO...</td>
</tr>
<tr>
<td><strong>Palmer amaranth S-PPO</strong></td>
<td>...SKF...SPK...LES...GNK...QDS...FLL...TTF...VQK...</td>
</tr>
<tr>
<td><strong>Common ragweed S-PPO</strong></td>
<td>...SMV...SSR...LQS...DQN...PQO...FLL...STF...VQK...</td>
</tr>
<tr>
<td><strong>Tobacco</strong></td>
<td>...SKL...SPK...INS...HKR...REE...FLL...TTF...VRY...</td>
</tr>
<tr>
<td><strong>Arabidopsis</strong></td>
<td>...TKF...AAK...LDS...NET...QNF...PQL...TAF...VQR...</td>
</tr>
</tbody>
</table>

**Figure 4.** PPO2 protein sequence showing polymorphisms between S-PPO and R-PPO

Figure 5. Overlay of the catalytic domain of the crystal structure of PPO2 from tobacco (slate) and the homology model of the R-\textit{PPO} from \textit{Euphorbia heterophylla} (cyan). The R98 amino acid (sensitive tobacco) is shown in darker slate color, and the corresponding L128 mutation in \textit{E. heterophylla} R-\textit{PPO} is shown in red. The mutation R128L [homologous to R98L in tobacco and \textit{Ambrosia artemisiifolia}] alters lactofen binding.
Figure 6. *PPO1* (A) and *PPO2* (B) expression relative to *ALS* in S*-PPO* and R*-PPO* Euphorbia heterophylla from Brazil. Data were pooled from two experiments (*n* = 12). Differences are not significant between S*-PPO* and R*-PPO* (*p* < 0.05).