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A Trp574Leu Target-Site Mutation Confers Imazamox Resistance in Multiple Herbicide-Resistant Wild Poinsettia Populations from Brazil

Rafael R. Mendes [1], Hudson K. Takano [2], Rubem S. Oliveira, Jr. [1], Fernando S. Adegas [3], Todd A. Gaines [2] and Franck E. Dayan [2,*]

1 Agronomy Department, Maringá State University, 5790 Colombo Ave., Maringá, Paraná 87020-900, Brazil; rafaromero.mendes@gmail.com (R.R.M.); rsoliveirajr@gmail.com (R.S.O.J.)
2 Agricultural Biology, Colorado State University, 307 University Ave., Fort Collins, CO 80523, USA; hudsontakano@gmail.com (H.K.T.); todd.gaines@colostate.edu (T.A.G.)
3 Embrapa Soybean, Jõao Carlos Strass Rd., Londrina, Paraná 86001-970, Brazil; fernando.adegas@embrapa.br
* Correspondence: franck.dayan@colostate.edu; Tel.: +1-662-816-6214

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Abstract: Wild poinsettia (*Euphorbia heterophylla* L.) is an important weed species in southern Brazil, especially due to the evolution of multiple herbicide resistance (e.g., acetolactate synthase (ALS)-inhibitors, protoporphyrinogen oxidase inhibitors, and glyphosate). The mechanism of resistance to imazamox was investigated in two wild poinsettia populations (R1 and R2) from southern Brazil and compared to a known susceptible (S) population. Imazamox dose-response experiments revealed high levels of resistance: 45-fold and 224.5-fold based on dry biomass reduction, for R1 and R2, respectively. Extremely high concentrations of imazamox (20,000 µM) were not sufficient to provide 50% inhibition of ALS enzyme activity (I_{50}) for R1 or R2. Hence, resistance levels were estimated to be greater than 123-fold for both populations based on in vitro ALS assays. The *ALS* gene from all R1 and R2 plants had a Trp574Leu mutation. A genotyping assay was developed to discriminate resistant and susceptible alleles based on the Trp574Leu mutation.

Keywords: *Euphorbia heterophylla* L.; acetolactate synthase; target site mutation; herbicide resistance mechanism

1. Introduction

Wild poinsettia (*Euphorbia heterophylla* L.) is a broadleaf weed species spread across several agricultural regions of the globe such as South America, Mexico, and southern United States [1]. Wild poinsettia is a strong competitor in crop production environments, especially in no-till systems. Plants can reach up to 2 m in height and seeds can germinate over a large range of temperature and soil characteristics, pH, and moisture. Furthermore, seedlings can emerge from seeds buried up to 14 cm deep from the soil surface [2,3], and adult plants have a high leaf area index providing great ability for light competition against major broadleaf crops, such as soybeans, cotton, and peanuts [4–6]. Wild poinsettia interference can decrease soybean yields up to 5.1 kg ha^{-1} d^{-1} under simultaneous competition [7]. For dry beans, there was a 60% reduction in yield when high densities of wild poinsettia (more than 60 plants m^{-2}) were present [8].

Acetolactate synthase (ALS)-inhibiting herbicides block the production of the branched chain amino acids valine, leucine, and isoleucine in plants [9]. Herbicides targeting ALS are classified into five chemical classes: imidazolinones (IMI), sulfonylureas (SU), triazolopyrimidines (TRI), pyrimidiniothio (or oxy)-benzoates (PYR), and sulfonylamino-carbonyltriazolinones (SCT) [10]. There are more than

50 active ingredients which can be used at low rates, have low toxicity to animals, have selectivity in several crops, and provide relatively broad spectrum of weed control [11].

Prior to the introduction of the Roundup Ready® (RR) crops, ALS inhibitors were widely adopted for weed control in soybeans in the 1990s [12]. The selection pressure imposed by repeated post- and preemergence applications, combined with the relatively high initial frequency of resistant individuals in weed populations, resulted in multiple reports of ALS resistance around the world [13]. Currently, there are more cases of resistance to ALS inhibitors than to any other herbicide mode of action (MoA) [14]. Resistance to ALS inhibitors in wild poinsettia was first documented in 1994. Since then, several studies have shown that ALS-resistant populations are widespread in southern Brazil [15,16]. A few years later, multiple resistances to ALS and protoporphyrinogen oxidase (PPO) inhibitors were also confirmed in wild poinsettia [17]. More recently, multiple resistances to ALS-inhibitors and glyphosate were reported in a wild poinsettia population from Paraná State, Brazil [18]. Brazilian farmers started using alternative herbicides to control weeds after the evolution of ALS resistance. Protoporphyrinogen oxidase (PPO) inhibitors and glyphosate were efficient tools to manage ALS-resistant weeds before and during the RR era [19]. Twenty years after the first ALS resistance case, multiple resistances to ALS inhibitors have been documented in 48 weed species around the world including wild poinsettia. Many cases involve multiple resistances to ALS and PPO or ALS and glyphosate in the same population [14].

In this study, we investigated the mechanisms of resistance to ALS-inhibitors in two multiple-resistant wild poinsettia populations: R1 (multiple-resistant to ALS and PPO inhibitors) [20] and R2 (multiple-resistant to ALS-inhibitors and glyphosate) [18].

2. Materials and Methods

2.1. Plant Material

The two resistant populations were collected from soybean fields, R1 in Mamborê (24.19° S, 52.32° W, Paraná State, Brazil) and R2 in Kaloré (23°51′ S, 51°39′ W, Paraná State, Brazil). The susceptible (S) population was collected in a field that has never received any herbicide treatment (22.50° S, 47.12° W, São Paulo State, Brazil) and was confirmed to be sensitive to ALS inhibitors in preliminary greenhouse studies. Seeds were harvested from 20 plants, cleaned, and stored at 5 °C. The seeds were sown at a depth of 0.5 cm in 200-well flats filled with potting soil (Professional Growing Mix, Sun Gro®, Agawam, MA, USA). Seven days after emergence, seedlings were transplanted to 1 L-pots filled with the same potting soil and fertilized with 200 kg ha^{-1} of N–P–K 14–14–14 (Osmocote, Scotts Miracle-Gro, Marysville, WA, USA). Each pot had one plant and corresponded to one experimental unit for all subsequent greenhouse experiments described below. Plants were kept in the greenhouse (25/20 °C day/night, 16 h of daylength, manually watered daily) during all experimental period. A susceptible population (S) was collected in Engenheiro Coelho (22.09° S, 46.57° W, São Paulo State, Brazil) and grown as described above.

2.2. Whole-Plant Imazamox Dose-Response

When plants had three fully expanded leaves (10–12 cm-tall), they were treated with imazamox at 3.1, 12.5, 50, 200, 800, and 3200 g ha^{-1} for S and 12.5, 50, 200, 800, 3200, and 12,800 g ha^{-1} for R1 and R2. Untreated plants in each of the three populations were included as controls for comparison. Herbicides were applied using a chamber track sprayer (Generation 4, DeVries Manufacturing, Hollandale, MS, USA) calibrated to deliver 160 L ha^{-1} spray solution and equipped with an 8002EVS even flat-fan nozzle (TeeJet; Spraying Systems Co., Wheaton, IL, USA). Plant shoots were harvested and dried at 60 °C for two d before biomass measurements. Each combination of dose and population was tested on four replications and the experiment was conducted twice.

2.3. ALS Enzyme Assay

Fresh tissue (0.8 g) from meristematic leaves was collected from plants with 6–8 fully expanded leaves. Samples were ground in liquid nitrogen and homogenized with 4 mL of extraction buffer (4 mM thiamine pyrophosphate, 25 mM potassium phosphate, 5 mM magnesium chloride, 200 mM pyruvate, and 20 µM flavin adenine dinucleotide) at pH 7.5. The crude extract was filtered through cheesecloth and miracloth into 2 mL tubes. Then, tubes were centrifuged at 28,600× *g* for 5 min at 4 °C. Protein concentration was quantified using the Bradford method, and the crude extract was always maintained on ice.

Reactions were prepared in 1.5 mL tubes by adding 125 µL of extraction buffer and 125 µL of different herbicide concentrations. Preliminary tests were conducted for each population to determine the optimum range of herbicide concentrations to discriminate between S and R. For S, the herbicide concentrations were 1.9, 7.8, 31.2, 125, 500, and 2000 µM of imazamox. For the two resistant populations (R1 and R2), the reactions were performed at 19, 97, 312.5, 1250, 5000, and 20,000 µM of imazamox. The crude extract (125 µL) was added to the reaction tubes and samples were incubated at 37 °C for 1 h. The reaction was stopped by adding 62.5 µL of 2 N sulfuric acid and incubated for 15 min at 60 °C. After incubation, all samples received a fresh solution (435 µL) containing α-naphthol (2.5%), creatine (0.25%) and ammonium hydroxide (2 N). Tubes were incubated for 15 min at 60 °C and centrifuged at 28,600× *g* for 10 min at room temperature. Two blanks were included, one with no herbicide (dose zero) and other with no reaction (sulfuric acid added prior to the first incubation). An aliquot of 250 µL was transferred to microtiter plates and read at 535 nm absorbance. The blank samples were subtracted as background and values were expressed in percentage relative to dose zero. The enzyme assays were conducted twice with three replications for each population.

2.4. ALS Sequencing

Young leaf tissue (100 mg) was collected, frozen in liquid nitrogen, and ground to a fine powder with TissueLyzer (Qiagen, Germantown, MD, USA). Total RNA was extracted with Direct-zol™ RNA MiniPrep Kit, (Zymo Research, Irvine, CA, USA), and RNA concentration was quantified by nanodrop (Termo Fisher Scientific, Waltham, MA, USA). The 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 conditions for the cDNA synthesis were 5 min at 25 °C, 20 min at 46 °C, and 1 min at 95 °C. Total cDNA was also quantified by nanodrop.

Two sets of primers were designed to sequence the amino acid positions where target-site mutations have been reported in ALS-resistant weeds. Primers were based on the wild poinsettia *ALS* sequences that are available in the herbicide target sequence database [21]. The primer set 1 (ALS1_F 5′-CACCGACGTGTTCGCCTAC and ALS1_R 5′ TGACTCCCTGCAAGGCTAATTTAAC) amplified an 889 bp-fragment including residues Ala122, Pro197, Ala205, Asp376, and Arg377. The primer set 2 (ALS2_F 5′ GGWACTGTTTATGCCAATTATGCTG and ALS2_R 5′ ATCTCCCATCACCCTCRGT) amplified a 920 bp-fragment covering positions Asp376, Arg377, Trp574, Ser653, and Gly654. Amino acid substitutions at these positions are well known for conferring ALS resistance in several species [22].

The polymerase chain reaction (PCR) was composed by 1.5 µL of each primer (forward and reverse), 12.5 µL of Econotaq Plus 2× Master Mix (Lucigen, Middleton, WI, USA), 1 µL of cDNA (20 ng), and nuclease free water was added to complete 25 µL final volume. PCR conditions were: 94 °C for 60 s, 35 cycles of 94 °C for 30 s, melting temperature (60 °C for both sets of primers) for 30 s, and 72 °C for 60 s, followed by a final extension at 72 °C for 10 min. The PCR products were verified by agarose gel (1%) and submitted to purification and DNA Sanger Sequencing (Genewiz, South Plainfield, NJ, USA). In total, three plants for each population were sequenced.

2.5. Homology Modeling of S- and R-ALS from Wild Poinsettia

The ALS amino acid sequences from wild poinsettia were aligned to the Arabidopsis sequence using EMBOSS Needle (https://www.ebi.ac.uk/Tools/psa/emboss_needle/). This information was used to build protein homology models based on the crystal structure (1Z8N) of Arabidopsis ALS with imazaquin in the binding domain [23] using Modeller [24]. The homology modeling pipeline was similar to that reported previously [25]. Briefly, the model was refined using GROMACS (version 2018.3) [26] on a 24-Core Intel® Xeon® 5600 series processors workstation. Steric clashes or inappropriate geometries were corrected through molecular dynamics simulation and evaluated using MolProbity (http://molprobity.biochem.duke.edu/) as described before. Residues involved in the dimerization of ALS were identified using PDBe PISA (http://www.ebi.ac.uk/pdbe/prot_int/pistart.html) and the active ALS enzyme was dimerized using HADDOCK 2.4 [27].

2.6. Docking of herbicides to S- and R-ALS

The structure of imazamox was downloaded as sdf files from PubChem (pubchem.ncbi.nlm.nih.gov), edited with Spartan18 (Wavefunction Inc., Irvine, CA, USA), and saved as saved as pdb files. The structure was converted to Autodock compatible pdbqt format using AutoDockTools (version 1.5.6). Imazamonx was docked to into the channel formed at the interface of the S- and R-ALS dimers using Autodock 4 and Autodock 4 [28]. The binding energy of the herbicides on the wild-type and resistant forms of ALS were obtained from the dock.dlg file. Proteins and ligand interactions were visualized using PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC, New York, NY 10036, USA) [29].

2.7. Genotyping Assay for Trp574Leu

A Kompetitive Allele Specific Assay (KASP) was developed to discriminate S and resistant (R) plants based on a single nucleotide polymorphism (SNP) in the codon codifying the Trp574Leu mutation (TGG in S; TTG in R). Two forward primers were designed differing only at the SNP: 5′-GCATTTGGGTATGGTTGTACAATG for S (FAM) and 5′-GCATTTGGGTATGGTTGTACAATT for R1 and R2 (HEX). The universal reverse primer was 5′-CCCCAAATAAGTATGAGCTCG. The primer mix consisted of 18 µL of each forward primer, 45 µL of the universal reverse primer, and 69 µL of nuclease free water. The KASP master mix was composed by 6 µL of primer mix and 216 µL of 2× KASP (LGC Biosearch Technology, Novato, CA, USA). The KASP reactions were performed with 4 µL of KASP master mix and 4 µL of cDNA (20 ng). Ten cDNA samples were used, five from R1 plants and five from R2 plants. Resistant plants confirmed to contain the Trp547Leu mutation (based on ALS sequencing data) and previously known susceptible individuals were included as controls. In addition, the controls were mixed in three different proportions to represent heterozygous controls (S:R 3:1, 1:1, 1:3). Nuclease free water was added in two samples with no cDNA which were considered as non-treated controls (NTC). Real time PCR was conducted at 94 °C for 15 min, ten cycles of 94 °C for 20 s, 61 °C decreasing to 55 °C for 60 s (0.6 °C touchdown), followed by 35 cycles of 94 °C for 20 s, and 55 °C for 60 s. In the end, FAM and HEX fluorescence channels were read by cooling samples to 30 °C for 10 s.

2.8. Data Analysis

Dose-responses and enzyme assays were subjected to ANOVA and a four-parameter non-linear regression model was fitted [30]. Model parameters were estimated using the *drc* package in R software [31]:

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

where y is the dependent variable (dry biomass or ALS activity), a is the asymptote, x is the independent variable (imazamox dose), c is the rate providing 50% of a (GR_{50}, or I_{50}), and b is the slope around c.

The ratio *c* (R1 or R2)/*c* (S) was calculated to estimate the resistance factor (RF). For the genotyping assay, data was presented as percentage relative to the highest FAM and HEX fluorescence values.

3. Results and Discussion

3.1. Imazamox Dose-Response

Imazamox resistance was confirmed for both R1 and R2 populations. Lethal doses for 50% of biomass reduction (GR_{50}) were 1084.3 and 5434 g ha^{-1} for R1 and R2, respectively (Table 1). Considering that field rates can vary from 40 to 50 g ha^{-1} [32] and the S population had a GR_{50} of 24.2 g ha^{-1}, R1 and R2 can be considered highly resistant to imazamox (Figure 1). While the GR_{50} values was 5-times higher for R2 than R1 (RF = 224.5 vs. 45, see Table 1), a t-test between these two GR_{50} values revealed a non-significant difference (p-value = 0.086), suggesting that the resistance level cannot be distinguished between R1 and R2 populations.

Table 1. Dose-response parameters and resistant factors (RF) for two imazamox-resistant wild poinsettia (*Euphorbia heterophylla*) populations based on dry biomass and enzyme activity.

Population	*a*	*b*	*c* (GR_{50}, I_{50})	R^2	RF [1]
Dry biomass (*c* in g ha^{-1})					
S	102	0.8	24.2	0.91	-
R1	96.5	0.4	1084.3	0.75	45
R2	101.3	0.5	5434.0	0.65	224.5
Enzyme activity (*c* in µM)					
S	104	0.6	163	0.92	-
R1	NA	NA	>20,000	NA	>123
R2	NA	NA	>20,000	NA	>123

[1] Calculated by ratio of *c* R1 or R2/*c* S. Model fit: $y = a/[1 + (x/c)^b]$. R1 is also resistant to PPO-inhibiting herbicides and R2 is also resistant to glyphosate. NA: model not adjusted.

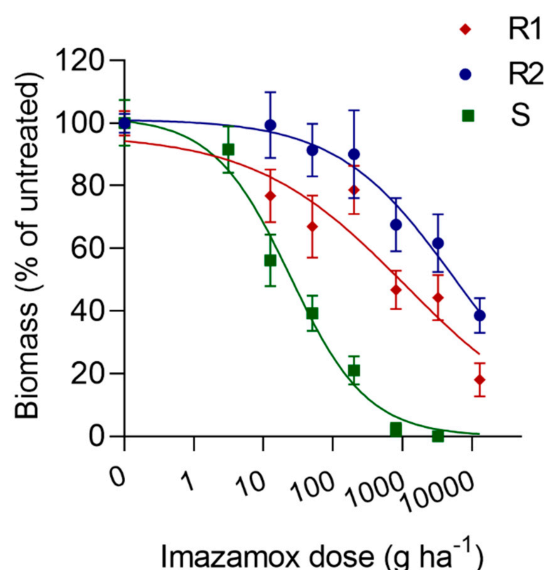

Figure 1. Dose-response curves for two imazamox-resistant wild poinsettia (*Euphorbia heterophylla*) populations. R1 is also resistant to PPO-inhibiting herbicides and R2 is also resistant to glyphosate. Model fit: $y = a/[1 + (x/c)^b]$. Error bars represent the standard error of the means (n = 8).

Imazamox resistance has been reported in several species worldwide, such as common ragweed (*Ambrosia artemisiifolia*) [33], downy brome (*Bromus tectorum*) [34], and wild mustard (*Sinapis arvensis*) [35]. ALS-resistant wild poinsettia was first reported in 1998 [36] and has spread across the southern

states in Brazil [37]. Similar to what was found for R1 and R2, high levels of resistance have been documented for IMI herbicides. In two populations from Paraná State, the RF were higher than 24-fold for imazethapyr [18]. Another population from São Paulo State had a RF higher than 7.8-fold for imazaquin [37]. More recently, extremely high levels of resistance to imazamox (RF = 168-fold) was reported in a population from Rio Grande do Sul State [38].

3.2. ALS Enzyme Assay

The ALS response to imazamox at the enzyme level correlated with the whole-plant response in the greenhouse (Figure 2). The I_{50} for the S population was 65.6 μM whereas the highest concentration of imazamox (20,000 μM) was not sufficient to inhibit 50% of the enzyme activity in R1 and R2 (Table 1). Consequently, the I_{50} (*c* parameter for the log-logistic equation) values were not estimated for R1 and R2 (Table 1). However, the RF value is >123-fold for the two populations.

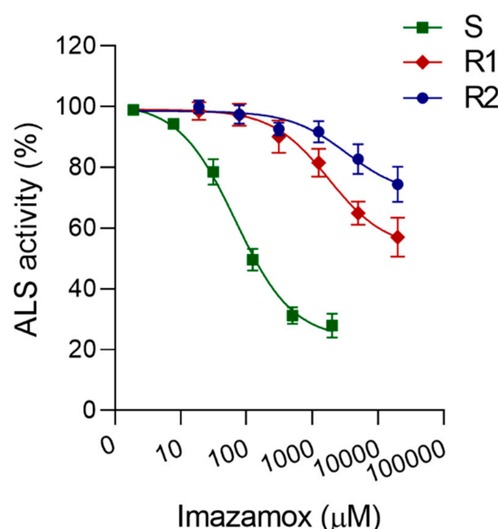

Figure 2. Acetolactate synthase (ALS) enzyme activity in two imazamox-resistant (**R**) and one susceptible (**S**) wild poinsettia (*Euphorbia heterophylla*) populations in response to increasing imazamox concentrations. R1 is also resistant to PPO-inhibiting herbicides and R2 is also resistant to glyphosate. Model fit: $y = a/[1 + (x/c)^b]$. Error bars represent the standard error of the means ($n = 6$).

The ALS enzyme assay has been used to complement whole-plant dose-response experiments in the investigation of ALS resistance in weeds [39]. Since this assay isolates the enzyme response in the presence of the herbicides, it can be used to predict potential target-site mutations [40,41]. For example, in tribenuron and imazamox resistant *Rapistrum rugosum* population, the RF at the enzyme level was greater than 270-fold, while the plant response in the greenhouse resulted in RF ranging from 40 to 370-fold [42]. In the same study, target site mutation at Trp574 and Pro197 imparting resistance were reported. Another target-site mutation (Ser653Leu) was reported in a different wild poinsettia population conferring imazamox resistance at the enzyme level (RF = 16-fold) [38].

3.3. ALS Sequencing

The *ALS* sequences were identical between the three populations except for a single nucleotide polymorphism (SNP) at position 574, based on Arabidopsis (*Arabidopsis thaliana*) *ALS* gene. A nucleotide change from T for G resulted in a codon change from tryptophan (TTG) to leucine (TGG) (Figure 3). The Trp574Leu mutation was identified in all R1 and R2 plants that were sequenced. This SNP is the second most common target-site mutation conferring ALS resistance to weeds. Other amino acid substations at this position are also possible, such as Trp574Gly [43], Trp574Met [44], or Trp574Arg [45]. However, Trp574Leu is found in 96% of all species containing a SNP at the Trp574 codon [22]. In contrast, a wild poinsettia population from Rio Grande do Sul harbors a Ser653Leu mutation

conferring ALS resistance [38], suggesting that more than one target-site mutation can occur across different populations. The *ALS* sequences of wild poinsettia populations were deposited at GenBank (S-accession number MT479177, R1- and R2-accession number MT479178).

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#codon         569 570 571 572 573 574 575 576 577 578 579

Protein        Gly Met Val Val Gln Trp Glu Asp Arg Fen Ser
                                   Leu

S.             GGT ATG GTT GTA CAA TGG GAA GAC CGA TTT TAC
R1 sample 1.   GGT ATG GTT GTA CAA TTG GAA GAC CGA TTT TAC
R1 sample 2.   GGT ATG GTT GTA CAA TTG GAA GAC CGA TTT TAC
R1 sample 3.   GGT ATG GTT GTA CAA TTG GAA GAC CGA TTT TAC
R2 sample 1.   GGT ATG GTT GTA CAA TTG GAA GAC CGA TTT TAC
R2 sample 2.   GGT ATG GTT GTA CAA TTG GAA GAC CGA TTT TAC
R2 sample 3.   GGT ATG GTT GTA CAA TTG GAA GAC CGA TTT TAC
               *** *** *** *** *** *  * *** *** *** *** ***
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Figure 3. Acetolactate synthase (*ALS*) sequences of imazamox susceptible (**S**) and resistant (**R1** and **R2**) wild poinsettia (*Euphorbia heterophylla*). A single nucleotide change from T for G at the 574 codon (underlined letter) results in a Trp574Leu mutation in all resistant plants. R1 is also resistant to PPO-inhibiting herbicides and R2 is also resistant to glyphosate.

3.4. Biding of Imazamox to ALS of R and S Wild Poinsettia

A comparison between S- and R-ALS from wild poinsettia and Arabidopsis revealed a high similarity in the amino acids involved in the dimerization of the active enzyme. A total of 85 residues interact at the interface of the two subunits (Figure 4A), and 82 of these were identical between wild poinsettia and Arabidopsis. Most of the residues (75%) have lipophilic properties and the remaining 25% residues are charged. Consequently, the interface between to two ALS monomers involves interactions between hydrophobic domains stabilized by a few electrostatic anchors (Figure 4B).

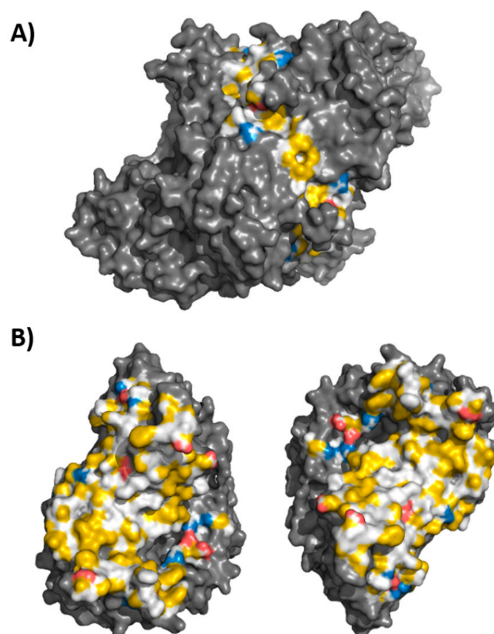

Figure 4. Active ALS from wild poinsettia showing (**A**) the ensemble dimer and (**B**) the interfaces of each monomer. Inspection of the interface revealed large hydrophobic domains stabilized by distributed electrostatic anchors. Residues involved in the interaction were colored according to each residue properties and the others were colored 50% grey.

The Trp574Leu mutation cause an increase in the volume of the channel because of the side chain of leucine occupies a much smaller space than that of tryptophan (as shown by the asterisk in Figure 5A,B). These two models were used to determine the binding of imazamox to wild poinsettia ALS based on the residues known to interact with imazaquin [23]. A total of 25 poses were generated for each domain (i.e., S-ALS and R-ALS). While the calculated binding energies of imazamox to the wildtype and resistant forms of ALS were similar, at −6.78/2 and −6.28 kcal/mol respectively. However, a closer analysis of the preferred docking poses obtained revealed a striking difference in the distribution of the various orientations relative to the known orientation of imazaquin (Figure 5C,D). The opening of the cavity resulting from the leucine mutation changes the potential positions of imazamox binding, resulting in reduced herbicidal activity.

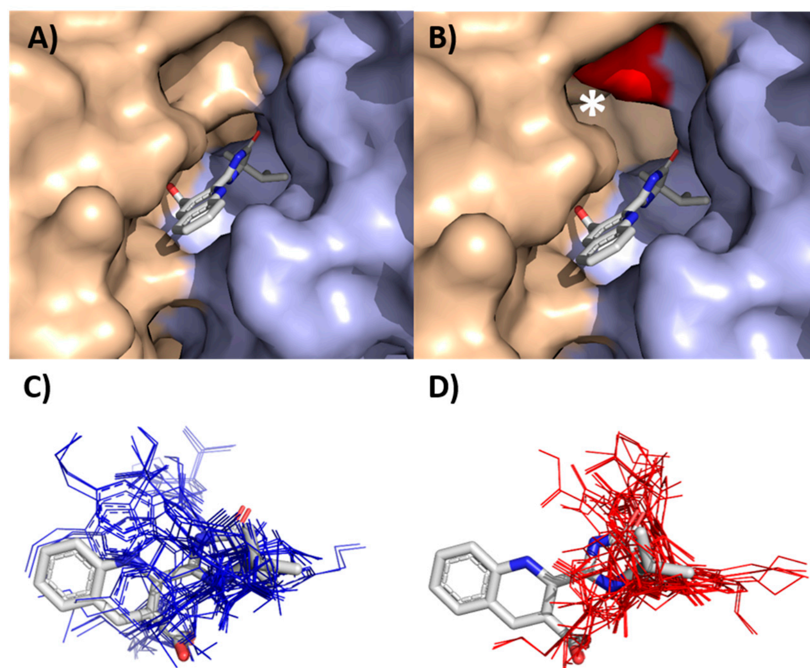

Figure 5. Docking of imazamox to (**A**) S-ALS and (**B**) R-ALS from wild poinsettia. The herbicide binding site is at the interface between two ALS subunits shown in pale yellow and light blue. Imazaquin is positioned in the channel according to coordinate from 1Z8N crystal structure. The leucine mutation is shown in red in panel B. The white asterisk points to the larger opening in the substrate channel resulting from the Trp to Leu mutation. Distribution of imazamox docking poses in (**C**) S-ALS in blue and (**D**) R-ALS in red, relative to the position of imazaquin from the 1Z8N crystal structure.

3.5. Genotyping Assay for Trp574Leu

Susceptible control plants (S) amplified the FAM fluorescence indicating that they have the nucleotide G at position 574. By contrast, resistant control plants confirmed to have the Trp574Leu mutation by sequencing (R1 and R2–R) amplified the HEX fluorescence, confirming that they have the nucleotide T at position 574. Different mixture ratios between S and R cDNAs amplified both alleles (FAM and HEX), therefore they fell into the middle of the scatter plot (Figure 6). Ten R samples amplified only the HEX fluorescence, indicating that they contain a leucine (nucleotide T) instead of a tryptophan (nucleotide G) at position 574 of the *ALS* gene. Two R2 plants amplified both FAM and HEX alleles, suggesting that they could be heterozygous for Trp574Leu at the 3:1 (R:S) proportion. Considering that wild poinsettia is tetraploid, heterozygous plants are heterozygous for one of the homeologs and homozygous resistant for the other. These results demonstrate that the genotyping assay can be used to screen ALS resistant wild poinsettia plants harboring a Trp574Leu mutation even if they are heterozygous.

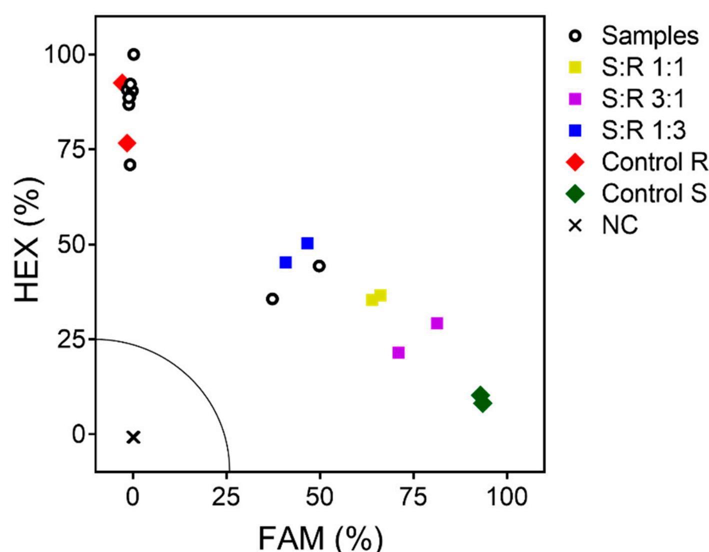

Figure 6. Kompetitive Allele Specific (KASP) assay to genotype the Trp574Leu target site mutation in tetraploid wild poinsettia (*Euphorbia heterophylla*). The FAM fluorophore represents tryptophan (TGG) while HEX is associated to leucine (TTG) at position 574. Green and red diamonds represent different proportions of R and S genotypes, respectively. Open circles are cDNA samples of R1 and R2 populations analyzed in this study. Most plants were homozygous for Trp574Leu, except two R2 plants that were heterozygous at the 3:1 (R:S) proportion.

Genotyping assays have been widely used to discriminate weed species that are morphologically difficult to identify [46,47]. They are also important assays to identify herbicide-resistant weeds if the resistance mechanism involves a target-site mutation. Based on a Ser653Asn mutation, a genotyping assay was efficient to detect homozygous and heterozygous plants within ALS-resistant borage (*Borago officinalis*) populations [48]. Similarly, a genotyping assay was developed to identify resistant alleles containing double amino acid substitutions (Trp102Ile and Pro106Thr) within the tetraploid genome of glyphosate resistant *Bidens subalternans* populations [49]. While herbicide-based screenings are the most used method to identify resistant weeds [50], these experiments require collecting seeds in the field, growing plants in the greenhouse, and spraying herbicides on many individual plants. This process can be very time-consuming and costly. A species-specific genotyping assay provides a quick and easy method to discriminate plants based on slight differences in their DNA such as target site mutations [47]. However, this type of assay only identifies known mutations; therefore a negative result does not preclude the existence of other mutations.

Non-target site resistance mechanisms remain to be explored given that they can co-exist with target-site resistance mechanisms conferring ALS resistance [51]. However, the in planta resistance magnitude was consistent with the enzyme response in vitro, suggesting that the Trp574Leu mutation could be the only mechanism of resistance in R1 and R2. This mutation has already been broadly studied in weeds and often results in a broad cross-resistance profile to all chemical families of ALS inhibiting herbicides [22]. Defining the cross-resistance pattern should be the next step in this research, to understand which ALS herbicides R1 and R2 are resistant to, including those that are used in soybean and corn, such as chlorimuron-ethyl, nicosulfuron (SU), cloransulam, diclosulam (TRI), imazethapyr, and imazaquin (IMI).

Wild poinsettia is a tetraploid species with extremely high genetic variability within and among different populations [52,53]. Furthermore, the presence of hermaphrodite flowers, non-cleistogamous reproductive organs, and pollinating insects suggest that wild poinsettia may have at least some level of cross-fertilization [54]. If that is true, cross-pollination can contribute to the spread target-site resistance alleles more rapidly across different plants, populations, and fields [55].

The findings of this research complement previous studies on R1 and R2 populations. While R1 plants were selected for PPO resistance due to a target site mutation in PPO2 (Arg128Leu) [20], R2 evolved a double amino acid substitution (Thr102Ile and Pro106Thr, TIPT) in EPSPS [21]. More importantly, the same R1 and R2 cDNA samples were evaluated for glyphosate, PPO, and ALS resistance, indicating that one mutation (Trp574Leu) was likely selected first by the intense use of ALS-inhibitors in the 1990s. Later on, Arg128Leu evolved in R1 conferring PPO-resistance and TIPT mutant plants were selected in R2 by glyphosate applications. ALS resistant wild poinsettia populations seem to have fixed the Trp574Leu allele even though ALS inhibitors have not been used for a long time, suggesting that the mutation is unlikely to provide any fitness cost to those plants [56].

4. Conclusions

Both R1 (PPO resistant) and R2 (glyphosate resistant) wild poinsettia populations were also confirmed as resistant to imazamox. High levels of resistance were measured for the two populations 224-fold for dry biomass; and >301-fold for in vitro enzyme activity). A target-site mutation Trp574Leu previously identified in several weed species was identified for the first time in wild poinsettia, which accounts for the mechanism of resistance to imazamox in R1 and R2. A molecular genotyping assay based on the Trp574Leu amino acid substitution was proven to rapidly detect ALS-resistant wild poinsettia plant samples.

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