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Assessment of Efficacy and Mechanism of Resistance to Soil-Applied PPO Inhibitors in *Amaranthus palmeri*

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Abstract: Resistance to protoporphyrinogen oxidase (PPO) inhibitors in Palmer amaranth is a major concern, given the high selection pressure and increasing number of populations with reduced sensitivity to PPO herbicides in the US. We evaluated the effect of five soil-applied herbicides on Palmer amaranth (*Amaranthus palmeri* S. Wats.) populations collected in 2014 and 2015 in Arkansas, USA. Soil-applied saflufenacil, sulfentrazone, and flumioxazin reduced the seedling emergence 91–100%; however, fomesafen and oxyfluorfen showed reduced (63–90%) efficacy on some populations. Target-site mutation (TSM) is the major mechanism of resistance to PPO herbicides; therefore, six populations showing resistance to soil-applied fomesafen were selected for molecular investigations. A total of 81 survivors were genotyped for all known resistance-conferring mutations. A total of 64% and 36% survivors had single and double TSMs, respectively, with 69% of plants carrying TSM in both alleles of *PPO2*. Three survivors from two populations showed an additional copy of *PPO2*, whereas all other survivors had one copy. Expression analysis showed 3- to 6-fold upregulation of *PPO2* in all plants from resistant populations tested. Transgenic overexpression of WT-*ApPPO2* and dG210-*Apppo2* in *A. thaliana* confirmed the reduced sensitivity to soil-applied fomesafen compared to the wild type. Collectively, PPO inhibitors applied pre-emergence are still effective in controlling populations resistant to foliar-applied PPO herbicides. Mechanically, elevated expression of resistant *PPO2*, alongside functional TSM, contribute to reduced sensitivity to soil-applied fomesafen.

Keywords: pre-emergence herbicide resistance; PPO inhibitors; *PPO2* gene

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1. Introduction

Palmer amaranth (*Amaranthus palmeri* S. Watson) is native to southwestern United States, and it is the most competitive and aggressive *Amaranthus* species that has become a devastating weed problem in several crops. Palmer amaranth has a high tendency to evolve resistance due to high genetic variability plus its dioecy nature requiring cross-pollination. To date, Palmer amaranth has evolved resistance to many herbicide modes of action including inhibitors of acetolactate synthase (ALS) (HRAC 2), photosystem II (PSII) (HRAC 6), protoporphyrinogen oxidase (PPO) (HRAC 14), enolpyruvyl shikimate-3-phosphate synthase (EPSPS) (HRAC 9), hydroxyphenyl pyruvate dioxygenase (HPPD) (HRAC 27), very long-chain fatty acid synthesis (HRAC 15), microtubule inhibitors (HRAC 3), synthetic hormones (HRAC 4), and most recently, glufosinate (HRAC 10) [1–3]. Recently, a Palmer amaranth population resistant to up to six herbicide modes of action (2,4-dichlorophenoxyacetic acid; ALS-, PSII-, EPSPS-, PPO-, and HPPD inhibitors) were documented in Kansas, USA [4].

Widespread occurrence of glyphosate-resistant populations led to heavy reliance on PPO-inhibitor herbicides, such as fomesafen and flumioxazin, for Palmer amaranth

control, specifically in soybean and cotton production. PPO inhibitors control a broad spectrum of weeds, generally have both soil and foliar activity, and cause rapid onset of phytotoxicity [5]. Several chemical families of PPO-inhibiting herbicides have been commercialized including diphenylethers (e.g., acifluorfen, fomesafen, lactofen, and oxyfluorfen), N-phenylphthalimide (e.g., flumioxazin and flumiclorac), phenylpyrazoles (e.g., fluazolate and pyraflufen-ethyl), oxadiazole (e.g., oxadiazon), oxazolidinones (e.g., pentoxazone), pyrimidinediones (e.g., saflufenacil), thiadiazole (e.g., fluthiacet-methyl), triazolinone (e.g., carfentrazone and sulfentrazone), and others (pyraclonil). These herbicides inhibit the PPO enzyme which catalyzes the conversion of protoporphyrinogen IX to protoporphyrin IX, the last common step for heme and chlorophyll biosynthesis [6]. There are two nuclear *PPO* genes in plants, *PPO1* and *PPO2*, which encode plastid- and mitochondria-targeted PPO isoforms, respectively [7]. In Palmer amaranth, *PPO2* isoforms are dual-targeted to both organelles [8,9]. Inhibition of PPO results in the accumulation of protophorphyrinogen IX, which leaks from the plastid to the cytoplasm where it is spontaneously oxidized into photosensitive protoporphyrin IX [10–12]. Upon exposure to light, protoporphyrin IX generates highly reactive oxygen species (ROS); singlet oxygen that cause lipid peroxidation, membrane destruction, and ultimately cell death [10,11].

There is limited research regarding the mechanism of tolerance/resistance to soil-applied PPO-inhibitors in crops/weeds. Thus far, research in this area focused on foliar-applied PPO inhibitors since resistance to this herbicide group was first selected through foliar application. Recently, the genetic variation (total of 28 significant SNPs) in chromosome 3 was found to be associated with tolerance to soil-applied fomesafen in *Sorghum bicolor* [13]. The target gene, *PPO1* is not associated with tolerance to fomesafen in the same study. Similar results were reported regarding *Phaseolus Vulgaris* L. tolerance to pre-emergence application of sulfentrazone where multiple genomic regions were associated with the tolerant phenotype, but none of them were associated with the *PPO1* or *PPO2* gene [14]. Most of the understanding of the mechanism of resistance to PPO inhibitors come from studying weed populations. Resistance to foliar-applied PPO inhibitors is conferred by four PPO2 mutations dG210, R128G, R128M and G399A and one PPO1 mutation, A212T [8,15–17]. The A212T substitution in PPO1 is the first case involving this isoform that was suggested to confer resistance to oxadiazon in *Eleusine indica* [17]. Thus far, target site mutations in PPO2 are confirmed in most of the resistance cases. Out of all four PPO2 TSMs, dG210 is the primary mutation associated with resistance to PPO inhibitors. The substitution at R128 of PPO2 in *A. tuberculatus* and *A. palmeri* corresponds to the R98 mutation in *Ambrosia artemisiifolia*, where it was first found to be associated with resistance to PPO inhibitors. Recently, G399 to A399 substitution was found to confer broad resistance to PPO inhibitors. The presence of one of these functional mutations, either as single or in combination with another, is strongly associated with resistance to foliar-applied PPO herbicides. Computational modeling data revealed the possible effect of the mutation on the binding strength between the ppo2 mutant enzyme and the herbicide, or with its native substrate. The following conclusions can be drawn from all studies: 1. The binding pocket is enlarged by the deletion of G210 amino acid, which in turn allows easy movement of water molecule within the binding pocket, resulting in weak interaction with herbicide. 2. The substitution of G399 to A399 in PPO2 enzyme adds a methyl group, which orients itself into the binding pocket and makes the binding pocket smaller. 3. Mutation from R128 to G128 results in loss of hydrogen-bonding interactions with herbicide and absence of side chain from arginine, which allows water to enter the binding site and further weakens herbicide binding.

Resistance to PPO herbicides in Palmer amaranth was first detected in a population from Arkansas collected in 2011 in a retroactive large-scale screening for response to fomesafen [18]. Resistance to foliar-applied PPO inhibitors is now widespread among *A. palmeri* populations in the mid-southern USA [19]. PPO inhibitors applied to soil prior to crop or weed emergence remain important tools for controlling Palmer amaranth genotypes, which are resistant to foliar-applied PPO herbicides. However, some researchers reported

variable response to soil-applied PPO-inhibiting herbicides in *A. tuberculatus* and *A. palmeri* populations, showing reduced sensitivity to soil-applied diphenylether herbicide (i.e., fomesafen) relative to other PPO-inhibitor chemical families [20,21]. Effective management recommendations for multiple-resistant Palmer amaranth populations emphasized the use of residual herbicides to minimize weed population size, to boost the efficacy of foliar-applied herbicides. Our collective anecdotal experience informs us that the rate of resistance evolution to pre-emergence herbicides is slower compared to that of post-emergence herbicides. With the undeniable importance of soil-applied herbicides, it is necessary to understand their likelihood of selecting herbicide-resistant genotypes. Many studies showed that resistance evolution to foliar-applied PPO-inhibitor herbicides is mainly the consequence of selecting for certain target-site mutations in the *PPO2* gene. This study was conducted with the following aims: 1. Evaluate the response to soil-applied PPO herbicides. 2. Analyze the TSM profile and copy number in survivors from soil-applied fomesafen. 3. Analyze the expression and mutation profile of the target-site in survivors from soil-applied fomesafen. 4. Evaluate the level of resistance to fomesafen in transgenic *Arabidopsis* lines overexpressing *A. palmeri PPO2*.

2. Materials and Methods

2.1. Plant Materials

In late summer 2014 and 2015, Palmer amaranth samples were collected from fields with a history of glyphosate, glufosinate, or PPO-inhibiting herbicide use in Arkansas. Inflorescences from at least 10 female plants per field were collected, dried, and threshed. Equal amounts of seeds from each plant were mixed to make a composite seed sample to represent the field. A susceptible standard population (SS) was included for comparison. For resistance tests and other experiments, plants were grown in a greenhouse maintained at 32/25 °C ± 3 °C day/night temperature with a 16 h photoperiod. The resistance screening test (described in Section 2.2) was done in 2016, whereas the molecular investigation (described in Sections 2.3 and 2.4) was conducted in 2021–2022 using original seeds collected from fields. The seeds were stored in glass vials at 5 °C.

2.2. Response to Soil-Applied PPO Herbicides

Approximately 120 Palmer amaranth seeds from 27 populations were sown in a 23 × 16.5 × 6.3 cm tray filled with 1.4 kg of 5:1 mixture of silt-loam field soil and commercial potting soil. The populations included 5 and 22 resistant populations from 2014 and 2015, respectively. Immediately after planting, trays were sprayed with recommended doses of fomesafen, flumioxazin, saflufenacil, sulfentrazone, and oxyfluorfen (Table S1). Treatments were applied in a spray chamber with a motorized spray boom fitted with 110067 nozzles calibrated to deliver 187 L ha^{-1} at 3.6 km/h. The trays were mist-irrigated following herbicide application to activate the herbicide. Later, the trays were sub-irrigated as needed to avoid physical damage to young seedlings. Seedlings were counted 21 days after treatment (DAT), and emergence was expressed as percent reduction relative to the corresponding non-treated trays of each population. Each herbicide was assessed in a separate experiment. The experiment was conducted in two runs in a randomized complete block design with two replications. Data were analyzed using JMP Pro v. 13 (SAS Institute, Inc., Cary, NC, USA).

2.3. Analyze the TSM Profile and Copy Number in Survivors from Soil-Applied Fomesafen

Survivors from six populations were analyzed for TSMs that endow resistance to soil-applied fomesafen. Treatment of soil with fomesafen (1x= 264 g ha^{-1}) was done as described in the above section. The experiment was conducted in two runs. Frequency of survivors was calculated by counting seedlings 21 DAT and expressed as percent emergence relative to the number of seedlings in the corresponding non-treated checks. A total of 81 (from Run1) survivors were tested for the presence of dGly210, R128G and G399A mutations which confer resistance to PPO-inhibiting herbicides in *Amaranthus* species. Two

leaf pieces (5 mm) from 10 to 17 plants were sampled from six populations that showed reduced sensitivity to the recommended dose of soil-applied fomesafen. Nontreated SS seedlings were used as controls. DNA was extracted from leaf tissues using a modified CTAB protocol [22]. Survivors were genotyped by pyrosequencing [23]. Using the same DNA from at least 50 survivors, copy number analysis was performed using two technical replicates. The primer pairs used for this experiment is listed in Table S2, and copy number was calculated using a single-copy reference gene (A36) [24].

2.4. Analyze the Expression and Mutation Profile the Target-Site in of Survivors from Soil-Applied Fomesafen

The *PPO1* and *PPO2* expression assay was performed using four resistant populations. Approximately 100 seeds were planted to flats filled with the same soil medium as described in Section 2.2 with two replications. Fomesafen was applied at 2x (528 g ha^{-1}) rate to ensure that survivors are truly resistant. Seedlings that survived were labelled at 21 DAT and allowed to grow for 15 more days to develop enough leaf material for RNA extraction. Tissues from nontreated plants were collected at 21 DAT so that both treated and nontreated plants were of the same growth stage. Approximately 50–70 mg leaf tissues were collected from three biological replicates, except the nontreated plants from 14-MIS-H (2 replicates). Nontreated tissues were collected from SS and 14-MIS-H. RNA extraction was done using Qiagen RNeasy mini kit (Qiagen, Hilden, Germany) using RLT buffer. A total of 1 µg of RNA was used to generate cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). Quantitative RT-PCR was carried out using SensiFast Kit (Bioline GmbH, Luckenwalde, Germany). Expression of *PPO1* and *PPO2* was measured against *Actin* gene. The primer pairs used are listed in Table S2. Small amount of tissue was also collected in separate tubes for genotyping PPO2 mutations in these plants. Genotyping was done by pyrosequencing as described previously.

2.5. Evaluate the Level of Resistance to Fomesafen in Transgenic Arabidopsis Lines Overexpressing A. palmeri PPO2

Transgenic *Arabidopsis* lines were generated as described in [25] using wild type (WT-*ApPPO2*) and the dGly210 mutant (dG210-*Apppo2*) PPO2 gene. Molecular analysis was carried out on transgenic lines (35S:WT-*ApPPO2* and 35S:dG210-*Apppo2*) to verify that transgenic lines were homozygous, and overexpression of the transgene was comparable between the two lines. Emergence counts were converted to a percentage of the nontreated control counts from each respective population. The data was analyzed using a log-logistic model with three parameters as described in the following equation:

$$y = f(x) = C + \frac{D - C}{1 + \exp(b(\log(x) - \log(e)))}$$

where C represents the lowest limit; D represents the upper limit; b is the slope of the curve around e; and the values of e correspond to the rate of fomesafen that reduces y (emergence) by 50%. This parameter is referred to as LD_{50}. Data were analyzed using the drc package in R® version 4.1.3 statistic ambient [26]. The three-parameter model was selected according to the best-fit criteria using the mselect function, which selects the best model based on the lesser log-likelihood values [27]. The corresponding regression parameters were determined for each population.

3. Results

3.1. Response to Soil-Applied PPO-Inhibiting Herbicides

The pre-emergence test was conducted to examine the response of selected Palmer amaranth populations to five soil-applied PPO herbicides, namely: flumioxazin, fomesafen, oxyfluorfen, saflufenacil, and sulfentrazone. The efficacy of flumioxazin and saflufenacil were similar on all the 27 populations tested, causing 91 to 100% reduction in emergence (Table 1). Sulfentrazone reduced seedling emergence >90% in all populations except for

15-CRI-D (86%). On the other hand, the populations differed in response to diphenylether herbicides fomesafen and oxyfluorfen. Soil-applied fomesafen, reduced seedling emergence ≥89% except for 15-GRE-A, 14-MIS-H, and 15-CLA-A, which were controlled 83%, 74%, and 63%, respectively. Oxyfluorfen reduced seedling emergence ≥87% for the majority of populations; however, four populations were only moderately controlled (68–81%). Interestingly, three of these four oxyfluorfen-recalcitrant populations (14-MIS-H, 15-CLA-A, and 15-GRE-A; highlighted in bold, Table 1) were the same populations that were least sensitive to soil-applied fomesafen. This indicates cross-resistance of these populations to soil-applied fomesafen and oxyfluorfen.

Table 1. Seedling emergence reduction in Palmer amaranth populations treated with various soil-applied herbicides at the recommended dose.

Population [a]	Seedling Emergence without Herbicide Treatment	Seedling Emergence Reduction [b]				
		Flum	Fom	Saf	Sulf	Oxy
	plants 380 cm^{-2}	----------%----------				
14-CLA-D	53	100	100	100	100	96
14-CRI-C	44	100	100	100	100	99
14-CRI-G	40	100	99	98	98	96
14-MIS-E	50	100	99	98	99	99
14-MIS-H	39	91	**74**	96	96	**77**
15-CLA-A	64	98	**63**	97	91	**77**
15-CLA-B	60	100	100	100	100	100
15-CON-A	50	100	99	100	100	100
15-CRI-A	62	100	89	98	98	87
15-CRI-B	50	100	99	100	100	99
15-CRI-C	58	100	93	100	98	94
15-CRI-D	57	97	92	100	86	96
15-GRE-A	68	97	**83**	100	95	**68**
15-IND-A	38	100	98	100	100	98
15-LAW-A	68	100	100	100	100	100
15-LAW-B	46	100	100	100	100	100
15-LAW-C	52	99	98	100	100	100
15-LEE-A	35	100	100	100	99	97
15-LEE-B	75	100	100	100	100	100
15-MIS-A	55	100	100	100	100	100
15-MIS-B	48	99	99	100	100	100
15-MIS-C	60	100	94	100	100	92
15-MIS-D	65	97	89	100	100	92
15-MIS-E	47	97	90	100	99	88
15-MIS-F	49	98	95	99	100	94
15-PHI-A	58	99	95	99	100	81
15-PRA-A	43	100	100	100	100	96
SS [c]	120	100	100	100	100	100
$LSD_{0.05}$ [d]		NS	9	NS	5	7

[a] Palmer amaranth population collected in 2014 and 2015. [b] Flu = flumioxazin (70.6 g ha^{-1}), Fom = fomesafen (280 g ha^{-1}), Saf = saflufenacil (49.3 g ha^{-1}), Sul = sulfentrazone (280 g ha^{-1}), and Oxy = oxyfluorfen (280 g ha^{-1}) are PPO-inhibiting herbicides. [c] SS = sensitive standard population. [d] Fisher's protected LSD to compare populations within herbicide treatment.

3.2. Analysis of the TSM Profile and Copy Number in Survivors from Soil-Applied Fomesafen

Six populations, *viz*., 15-GRE-A, 14-MIS-H, 15-CLA-A, 15-CRI-A, 15-MIS-D, and 15-MIS-E, that showed reduced sensitivity to soil-applied fomesafen were selected along with SS population for molecular investigations. Analysis of TSM profile was done on 10 to 17 resistant plants from each population. Target-site mutation(s) was found in all 81 (100%) survivors analyzed with 52 plants (64%) carrying a single TSM, and 29 plants (36%) carrying a double *ppo2* mutation. The deletion of G210 codon (dGly210) was the primary TSM we detected, which occurred alone or with one other TSM (dGly210 + Ala399 or dGly210 +

Gly128), as shown in Table 2. Survivors from 15-GRE-A harbored single (dGly210+/+) and double (dGly210+/−, Ala399−/+) mutations equally, but when single mutation was present, it was homozygous. Similarly, most survivors from 15-CLA-A and 14-MIS-H carried two resistant alleles of *ppo2* gene. The majority of survivors from 15-CLA-A and 14-MIS-H harbored single (dGly210+/+) and double (dGly210+/−, Gly128−/+) mutations, respectively. On the other hand, equal or a greater number of survivors from 15-CRI-A, 15-MIS-D, and 15-MIS-E contained only one resistant allele of *ppo2* gene. Sixty-nine percent of survivors carried two resistant alleles of *ppo2* (Table 2). The three populations, 15-GRE-A, 14-MIS-H, and 15-CLA-A with the majority of survivors harboring two resistant alleles also showed higher frequency of survivors with soil-applied fomesafen compared to other populations that consist of more plants carrying a single mutant allele of *ppo2* (Table S3). The frequency of survivors from foliar-applied fomesafen was retrieved from a previous study [28] and compared to the frequency of survivors from pre-emergence application in the current experiment. All six populations showed less survivors from pre-emergence application compared to post-emergence application (Table S4). The total of 50 survivors from six populations were also examined to determine the copy number of *PPO2* gene and only three plants from two populations (14-MIS-H and 15-CRI-A) showed an additional copy (Figure S1). The rest contained only a single copy of *PPO2*.

Table 2. The *PPO2* mutation profile of survivors from 1x soil-applied fomesafen treatment.

Population	Number of Plants Genotyped	Genotype of Each Mutation								
		dGly210		Gly128		Ala399		dGly210 Gly128	dGly210 Ala399	Gly128 Ala399
		+/+	+/−	+/+	+/−	+/+	+/−	+ −/− +	+ −/− +	+ −/− +
15-GRE-A	17	9	0	0	0	0	0	0	8	0
14-MIS-H	13	0	2	0	0	1	0	8	1	1
15-CLA-A	12	9	2	0	0	0	0	1	0	0
15-CRI-A	14	0	0	0	2	3	5	2	0	2
15-MIS-D	11	0	1	0	3	1	3	0	2	1
15-MIS-E	14	2	3	1	2	1	2	2	0	1
SS	4	0	0	0	0	0	0	0	0	0
SUM	81	20	8	1	7	6	10	13	11	5
		52							29	

+/+ Homozygous; mutation is present in both alleles of *ppo2*. +/− Heterozygous; mutation is present only in one allele of *ppo2*. + −/− + Presence of two heterozygous mutations, each in a different allele of *ppo2*.

Cells highlighted in blue represents the number of plants containing two resistant alleles; the rest are the number of plants containing one resistant allele of *ppo2* gene.

3.3. Analysis of Expression and Mutation Profile of the Target-Site in Survivors from Soil-Applied Fomesafen

A smaller subset of survivors representing four populations were selected to analyze the expression of *PPO1* and *PPO2*. All the treated plants from selected resistant populations showed 3- to 6-fold upregulation of *PPO2* gene compared to nontreated SS population (Figure 1). The expression of *PPO1* was not upregulated above 2-fold in the tested plants, whereas that of *PPO2* was upregulated in all plants when compared to nontreated SS. The expression of *PPO2* was also elevated 3-fold in two of the nontreated plants tested from 14-MIS-H. Plants that were treated (survived 2x rate of soil-applied fomesafen) from 15-GRE-A and 15-CRI-A showed 4 to 6-fold increase, whereas 14-MIS-H and 15-CLA-A showed approximately 3 to 4.5-fold upregulation in *PPO2* expression. The TSM profile of plants used in expression analysis showed that all survivors harbor TSM (Figure 1). Only two nontreated plants were included from 14-MIS-H with the intention to select the susceptible plant from resistant population, but 14-MIS-H showed the higher frequency (73%) of survivors with foliar-applied fomesafen contributed by TSM [28]. Thus, nontreated plants

chosen from 14-MIS-H lower the chance of selecting true sensitive from this population; however, the upregulation of *PPO2* expression in all nontreated plants analyzed indicates constitutive upregulation of *PPO2* in the resistant field population.

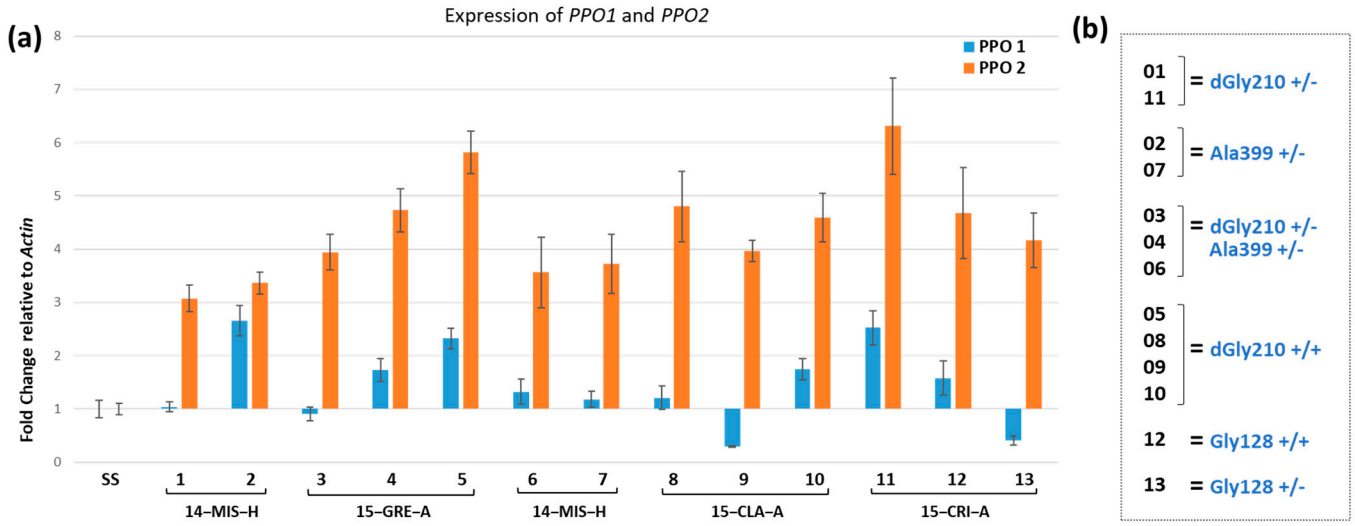

Figure 1. Expression analysis of *PPO1* and *PPO2* gene in *A. palmeri* seedlings (**a**) nontreated (SS, 1 and 2) and treated (3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13) with soil-application with 2x fomesafen. Each bar represents the relative expression (fold change) of each gene in nontreated and treated seedlings compared to nontreated SS plants. Data are means ± SE of three biological replicates (except nontreated from 14-MIS-H). Expression of *PPO1* and *PPO2* were normalized against *Actin*. (**b**) Genotyping of each sample used in expression analysis.

3.4. Evaluation of the Level of Resistance to Fomesafen in Transgenic Arabidopsis Lines Overexpressing A. palmeri PPO2

To understand the role of increased expression of *PPO2* toward resistance to pre-emergence application of fomesafen, we analyzed two transgenic *Arabidopsis* lines that overexpressed wild type (35S:WT-*ApPPO2*) and mutant (35S:dG210-*Apppo2*) genes from *A. palmeri* driven by 35S promotor. The ED50 of non-transgenic *Arabidopsis* line was 0.4 g ai/ha (Figure 2, Table 3). The ED50 value of WT-*ApPPO2* and dG210-*Apppo2* overexpressing lines were higher than that of the non-transgenic line (Figure 2, Table 3), indicating that overproduction of either form of Palmer amaranth PPO2 (wild type or mutant) in *Arabidopsis* reduced its sensitivity to fomesafen applied preemergence.

Table 3. Parameters estimate of the dose–response curve used to calculate the herbicide dose required for decrease the 50% emergence (LD_{50}) of each *Arabidopsis* line evaluated three weeks after the application.

Population	B [1] (±SE)	D [2] (±SE)	LD_{50} (g ai/ha)	95% CI [3] (g ai/ha)		RI [4]	*p*-Value [5] Compared to WT	*p*-Value [6] Compare between PPO2
				Upper	Lower			
WT	2.3 (0.7)	97.0 (6.1)	0.4	0.3	0.5	-	-	-
35S:WT-*ApPPO2*	2.6 (1.3)	84.5 (84.5)	20.0	13.6	26.3	51.8	3.0×10^{-4}	-
35S:dG210-*Apppo2*	1.5 (0.4)	92.3 (3.6)	32.4	19.4	45.4	84.0	8.9×10^{-4}	0.134

SE, standard error; LD, lethal doses; [1] Slope around GR_{50} and ED_{50}. [2] Upper limit for all plants (% emergence). [3] Confidence interval for LD_{50}. [4] Resistance ratio = 35S:WT-*ApPPO2*/ WT and 35S:dG210-*Apppo2*/ WT. [5,6] *p*-value for *t*-test comparing LD_{50} parameter.

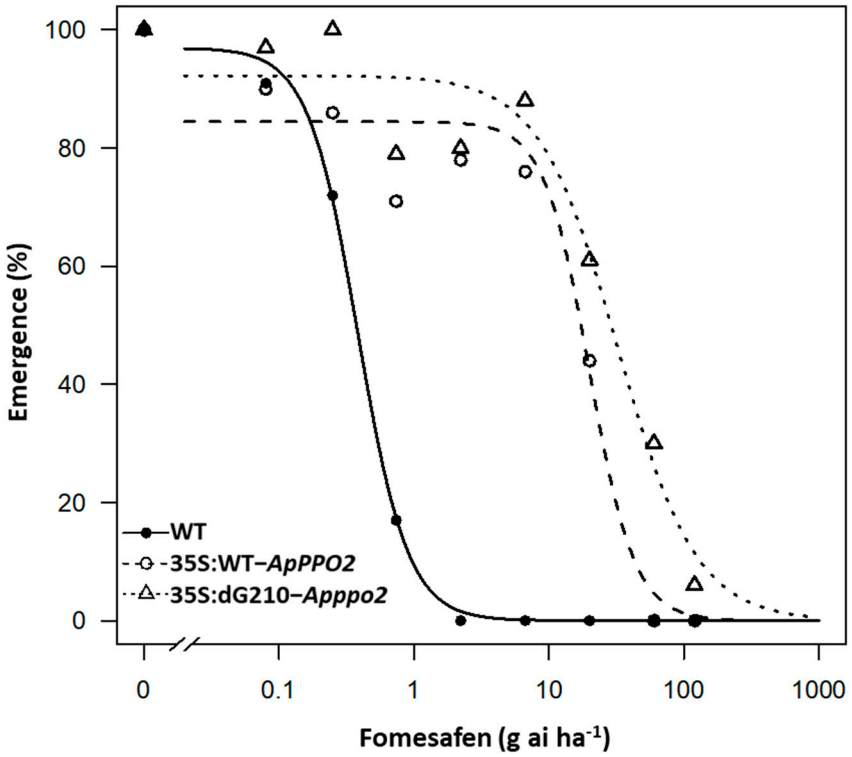

Figure 2. The dose–response curves for percentages of germinated transgenic *Arabidopsis* lines; WT (solid line, black circles), 35S:WT−*ApPPO2* (broken line, white circles), and 35S:dG210−*Apppo2* (dotted line, white triangles) to increasing doses of soil-applied fomesafen.

4. Discussion

Residual herbicides are an integral component of herbicide-resistant weed management. It is important to evaluate their impact on the evolution of weed resistance. In this study, the efficacy of soil-applied PPO herbicides on Palmer amaranth was highest for saflufenacil, sulfentrazone, and flumioxazin, followed by fomesafen and oxyfluorfen, respectively. Similar information was reported by other research groups [20,21,29], where PPO-resistant tall waterhemp was the least sensitive to the diphenylether herbicide fomesafen followed by sulfentrazone and flumioxazin. A previous study we conducted to test the response of these populations to foliar-applied PPO herbicides showed that the three populations, 15-GRE-A, 14-MIS-H, and 15-CLA-A were also among the top 10 most resistant populations to foliar-applied fomesafen [28]. Among the 2014 populations, 14-MIS-H was the least sensitive to foliar-applied fomesafen. Similarly, 15-GRE-A and 15-CLA-A were highly recalcitrant to foliar-applied fomesafen with most of its survivors incurring minimal injury of <10% [28]. The reduced sensitivity to diphenylether herbicides could be attributed to higher usage frequency of herbicides belonging to this chemical family relative to other PPO chemical families [30]. This is supported by the fact that resistance to PPO inhibitors is due primarily to dG210 mutation of PPO2, which confers broad cross-resistance to Group 14 herbicides. Diphenylether herbicides were the first widely used family of PPO-inhibiting herbicides, and have been used tremendously in soybean, and later on in cotton, for broadleaf weed control [20]. Reduced sensitivity to soil-applied diphenylether herbicides among PPO-resistant populations means that fomesafen and oxyfluorfen are no longer reliable in managing Palmer amaranth. While resistance evolution to PPO-inhibiting herbicides is relatively recent, and resistance to soil-applied PPO herbicides is at an early phase, broadscale management strategy has already been sought by researchers and implemented by progressive crop growers. Integrated weed management is now the mainstream message disseminated by Extension Weed Specialists in the US.

Understanding the genetic evolution of a population that results in resistance to a given herbicide is complex. Resistance to foliar-applied PPO-inhibiting herbicides in Palmer amaranth is becoming increasingly common with TSM as the major mechanism identified so far. In this study, all survivors from soil-applied fomesafen harbored known resistance-conferring mutations (Table 2) indicating TSM is essential to overcome the lethality of soil-applied fomesafen to germinating seeds and young seedlings. The presence of mutation in both alleles, either as a single homozygous mutation or two different heterozygous mutations (one in each allele), should increase the chance of survival. At the same time, greater reduction in frequency of survivors with soil-applied compared to foliar-applied fomesafen (Table S4) indicates that TSMs alone do not allow survival from pre-emergence application of fomesafen. The chloroplastic PPO1 and mitochondrial PPO2 isoforms are both targets of PPO inhibitors. Yet, the resistance mechanisms that evolved to PPO inhibitors are associated with PPO2 isoform only [8,15,16,31]. Finding the genes that are differentially expressed between conditions is an integral part of understanding the molecular basis of phenotypic variation most likely mediated by change in protein level. In the present study, we found that survivors of double the field rate of soil-applied fomesafen showed selective upregulation of *PPO2* gene, but not *PPO1*, which is in sync with the occurrence of resistance-conferring TSMs exclusively in *PPO2* of Palmer amaranth. The basis for this selection is the dual targeting of PPO2 enzyme to two organelles: chloroplast and mitochondria. Thus, the constitutive or induced elevation in expression of *PPO2* gene suggest that it may directly contribute to resistance against soil-applied herbicide at the germination stage. This is considering that three-to-six-fold increase in *ppo2* expression most likely translate into an increased level of herbicide-resistant PPO2 enzyme as TSM was found in all survivors from resistant populations, the majority of which, harbor two resistant alleles of *ppo2* (Table 2). These mutations have a significant magnitude of effect to individually confer resistance. In another words, these are functional mutations and overexpression of a resistant mutant *ppo2* increases the ability of germinating seedlings to survive soil-applied PPO-inhibiting herbicides.

For a long time, the role of ROS was associated with the loss of seed viability through desiccation or aging of seeds [32,33], but recently ROS homeostasis was demonstrated as a crucial factor in switching from seed dormancy to germination phase [34]. The concept of "oxidative window for germination" was determined, which defines an upper limit of ROS level that can prevent germination, and a lower threshold level below which germination cannot occur [33]. ROS activity is generally lower (below oxidative window) in dormant stage compared to non-dormant stage. During seed imbibition, ROS begins to accumulate through resumption of metabolic activities (within the oxidative window) [33,35]. As the entire process of germination is modulated largely by external conditions, the ROS that are generated at that stage perceive the environmental cue that in turn triggers hormone signaling associated with germination [35]. At the same time, antioxidants are activated to prevent excessive ROS accumulation. Thus, ROS homeostasis is maintained in a range that allows ROS signaling, but not ROS-induced damage. In other words, under appropriate external conditions, ROS must be present within the oxidative limit for allowing completion of germination process. It is not yet clearly understood how environmental factors fine tune ROS production, but non-optimal environmental conditions, such as heat, cold or drought at germination stage, are associated with oxidative stress and lipid peroxidation that in turn prevent radicle emergence [36–39]. Inhibition of PPO2 enzyme by foliar-applied PPO inhibitors result immediately in accumulation of ROS that led to increased internal ROS contents in cells. The same response is expected from soil-applied PPO herbicides, only that it would take minimal amounts of ROS to kill germinating seedlings. Considering the physiology of seed germination and mode-of-action of PPO inhibitors, avoidance of excessive ROS production or maintenance of the oxidative window would be necessary for seedling germination and growth. At the germination stage, an increased pool of PPO2 enzyme in survivors may be enough to oxidize all available protoporphyrinogen to protoporphyrin after competitive inhibition by fomesafen. Consequently, there would be

none or less free protoporphyrinogen that could leak to cytoplasm in survivors compared to WT plants where protoporphyrinogen is oxidized by peroxidases, leading to ROS accumulation.

Since the selected Palmer amaranth populations are cross-resistant to other foliar-applied herbicides [28], the role of common non-target site resistance factors that help alleviate herbicide stress cannot be ignored. Thus, increased expression of *PPO2* gene may remain necessary along with other factors that are required to counteract the oxidative stress arising from exposure to soil-applied herbicide. Collectively, these small-effect mechanisms can lend enough protection, allowing survival of some seedlings. Increased expression of target gene has been associated with increased copy number in glyphosate- and glufosinate-resistant *A. palmeri* populations [2,3,40]. In the present study, we did not find copy number variation of *PPO2* in most of the survivors (Figure S1); therefore, the elevated expression of *PPO2* may be driven by enhanced activity of promotor. Perturbance in the upstream regulatory region of *PPO2* may be associated with fitness consequences as the frequency of survivors with pre-emergence herbicides was always lower compared to that of foliar application. Further investigations are needed to understand if resistance to pre-emergence herbicides is linked to a single locus, that is only *PPO2*, or if it is associated with multiple genes.

Although *PPO2* expression driven by 35S promotor would be strong in transgenic *Arabidopsis* lines and cannot be compared to the level of upregulation found in resistant Palmer amaranth populations, the transgenic experiments in this current research and that of a previous one [25] confirmed that increased pool of WT and mutant forms of PPO2 enzyme can reduce the sensitivity to herbicide at the germinating stage in a susceptible model plant. Moreover, compared to overexpression of WT-*ApPPO2*, overexpression of dG210-*Apppo2* gene showed greater reduction in sensitivity to soil-applied fomesafen (Table 3) indicating greater efficiency of resistant form of PPO2 enzyme.

5. Conclusions

Field use rates of pre-emergence PPO herbicides are generally effective in controlling most of the glyphosate-resistant and foliar PPO-herbicide-resistant Palmer amaranth populations. Target-site mutations, primarily dG210 alone or in combination with either G128 or A399, contribute to resistance to soil-applied fomesafen. Furthermore, upregulation of *PPO2* increases tolerance to soil-applied fomesafen. We hypothesize that higher expression of *PPO2* gene in addition to the presence of functional mutations increases the protection against soil-applied fomesafen. Identification of additional factors involved in herbicide detoxification or mitigation of oxidative stress contributing to the evolution of resistance to pre-emergence PPO inhibitors is necessary. Selection of resistant plants with TSM and low-level NTSR mechanisms by preemergence herbicides could lead to increased resistance to soil-applied PPO herbicides.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13020592/s1, Figure S1: Copy number of PPO2 gene in survivors from 1x soil-applied fomesafen treatment; Table S1: Common, trade names, and manufacturers of herbicides used in the study; Table S2: List of primers used in the copy number and expression assay; Table S3: Comparison of frequency of two resistant allele with the frequency of survivors to soil-applied fomesafen (1x) among six resistant *A. palmeri* population; Table S4: Comparison between frequency of survivors with foliar- and soil-applied fomesafen (1x).

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