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PPO2 Mutations in *Amaranthus palmeri*: Implications on Cross-Resistance

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Abstract: In Arkansas, resistance to protoporphyrinogen IX oxidase (PPO)-inhibiting herbicides in *Amaranthus palmeri* S. Wats. is mainly due to target site mutations. Although *A. palmeri* PPO-mutations are well investigated, the cross-resistance that each *ppo* mutant endows to weed populations is not yet well understood. We aimed to evaluate the response of PPO-resistant *A. palmeri* accessions, harboring the *ppo2* mutations Δ*G210* and *G399A*, to multiple PPO-inhibiting herbicides. Six resistant and one susceptible field accessions were subjected to a dose–response assay with fomesafen, and selected survivors from different fomesafen doses were genotyped to characterize the mutation profile. The level of resistance to fomesafen was determined and a cross-resistance assay was conducted with 1 and 2 times the labeled doses of selected PPO herbicides. The accession with higher predicted dose to control 50% of the population (ED50) had a higher frequency of Δ*G210*-homozygous survivors. Survivors harboring both mutations, and those that were Δ*G210*-homozygous, incurred less injury at the highest fomesafen rate tested (1120 g ai ha^{-1}). The populations with a high frequency of Δ*G210*-homozygous survivors, and those with individuals harboring Δ*G210* + *G399A* mutations, exhibited high potential for cross-resistance to other PPO herbicides. The new PPO–herbicide chemistries (saflufenacil, trifludimoxazin) generally controlled the PPO-resistant populations.

Keywords: *Amaranthus palmeri*; protoporphyrinogen IX oxidase (PPO); herbicide resistance; target-site resistance

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1. Introduction

The commercialization of genetically modified crops resistant to the highly effective, non-selective herbicide glyphosate has greatly impacted weed management. Although chemical control has been the main weed control method prior to the release of herbicide-resistant crops, the glyphosate-resistant technology allowed farmers to rely primarily on a single herbicide to control weeds, which reduced the diversity of weed management practices and chemistries used in a crop season [1–3]. Weed resistance to herbicides is the inevitable consequence of herbicide selection pressure. Relying on a single herbicide exerted tremendous selection pressure on weed populations, resulting in the evolution of many glyphosate-resistant weed species, including *Amaranthus* spp. [4,5]. The same is true with continuous use of herbicides with the same mode of action (MOA), as demonstrated by the global database on herbicide-resistant weeds [6].

Amaranthus palmeri S. Wats. (Palmer amaranth) is a highly competitive weed which is genetically compatible with other species in the Amaranthaceae family, including *A. spinosus* L. and *A. tuberculatus* (Moq.) Sauer [7–9]. *A. palmeri* is widely resistant to

acetolactate synthase (ALS)- and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)-inhibiting herbicides. In at least ten cases, a single *A. palmeri* population carries multiple resistance to ALS herbicides and glyphosate [6,10–12]. Herbicides inhibiting protoporphyrinogen oxidase (PPO, EC 1.3.3.4) have been used extensively to control ALS- and glyphosate-resistant *A. palmeri* populations. By inhibiting this enzyme, PPO-inhibitor herbicides stop the oxidation of protoporphyrinogen IX into protoporphyrin IX, which leads to accumulation of protoporphyrinogen IX. The excess protoporphyrinogen IX is exported into the cytoplasm, where it reacts with free oxygen, producing a photosensitive protoporphyrin IX. Upon exposure to light, protoporphyrin IX generates singlet oxygen, ultimately leading to cellular death in susceptible plants [13–17]. The PPO enzyme exists in two forms in plants; *PPO1* is located in the chloroplast, whereas *PPO2* is in the mitochondria and, in a few species, also in the chloroplast [18,19].

A. palmeri is resistant to PPO-inhibitor herbicides primarily due to target-site (TSR) mechanisms and, to a lesser extent, via non-target-site resistance (NTSR) mechanisms. Mutations in *PPO2* have been found in PPO-resistant Palmer amaranth. The first to be detected was the deletion of a glycine at position 210 (Δ*G210*), reported in *A. tuberculatus* [12,20–22]. Second was a substitution of arginine with glycine or methionine at the 128th position (*R128G* or *R128M*) [11,23,24]. This mutation was identified in *Ambrosia artemiisifolia* L. [25]. The substitution of glycine with alanine at position 399 (*G399A*) was the latest resistance-conferring *ppo2* mutation identified in *A. palmeri* [26].

Since *A. palmeri* is dioecious, it is an obligate outcrossing specie; thus, the accumulation of PPO mutations in the same plant via gene flow is expected. Indeed, some survivors of fomesafen treatment harbor Δ*G210* + *R128G* mutations in the same plant [27]. The authors did not report the level of resistance in these plants or if the mutations co-occurred in the same allele. The occurrence of multiple PPO mutations in the same allele would be rare if such a combination would compromise the enzyme function. The most likely scenario is for different mutations to occur in different plants within a field population. In a study of PPO-resistant *A. palmeri* accessions from four states in the USA, accessions with more than one *ppo* mutation were grouped in one cluster, and collectively exhibited stronger resistance [28]. Further evaluation revealed a few plants in these accessions accumulating the mutations Δ*G210* + *G399A* and *G399A* + *R128G* (in the same allele), and plants carrying Δ*G210* + *R128G* (may or may not occur in the same allele). How these double mutations might affect the degree of resistance at the plant level is yet to be understood. This study was conducted to evaluate the level of resistance to fomesafen conferred by the *ppo2* mutations Δ*G210* and *G399A* in PPO-resistant *A. palmeri* accessions, whether borne in separate plants or in the same plant or allele. The response of these accessions to other foliar-applied PPO herbicides was also evaluated.

2. Materials and Methods

2.1. Fomesafen Dose–Response Assay

A. palmeri accessions collected in 2017 and 2018 were tested for fomesafen resistance and genotyped for the presence of Δ*G210* and *G399A* mutations [28]. From this initial test, six populations (collected in 2017 from fields in Arkansas and Missouri) were selected based on their mutation profiles (Table 1). The six PPO-resistant accessions, and a susceptible standard (SS), were used in whole-plant bioassays to determine the resistance level to fomesafen. The accessions were expected to contain Δ*G210* (PHI-C and LAW-E), *G399A* (PHI-I and SC-C), and both mutations (NM-J and PEM-F). This experiment was conducted in a greenhouse located at the Altheimer Laboratory, University of Arkansas, Fayetteville, USA. Seeds were sown in 11 × 11 cm^2 pots filled with commercial potting soil (Sunshine® Premix No. 1; Sun Gro Horticulture, Bellevue, WA, USA) and thinned to 4 plants pot^{-1}. Plants were grown under a 14/10-h photoperiod and 32/25 ± 3 °C day/night temperature. The experiment was conducted twice and had four replications. Each replication was one pot. Seedlings, 8- to 10-cm tall (4- to 6-leaf stage), were sprayed with 6 doses of fomesafen (Flexstar®, Syngenta Crop Protection, Greensboro, NC, USA) from 0 to 1120 g ai ha^{-1}

for resistant populations, corresponding to 0 to 4× the recommended field dose. The dose range for SS was from 0 to 280 g ha^{-1}, corresponding to 0 to 1× the recommended dose. Fomesafen was applied with 0.5% *v/v* non-ionic surfactant (Induce, Helena Chemical, Collierville, TN, USA). Each replication was sprayed separately in a spray chamber with an air-propelled, motorized boom fitted with 1,100,067 nozzles (Teejet, Wheaton, IL, USA) calibrated to deliver 187 L ha^{-1}. At 3 weeks after treatment (WAT), plants were evaluated for injury. Visible injury (%) was rated on a scale of 0 to 100%, where 0 represented no effect, and 100% was dead [29,30]. The data were analyzed by regression using the "drc" package in the software R v. 4.0.3 [31]. A three-parameter log-logistic model was fitted to the data using Equation (1):

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

where Y is visible injury relative to the nontreated check (%), d is the upper horizontal asymptote; b is the slope around e, which is the herbicide dose causing 50% injury (ED50); and x is the herbicide dose [32]. The resistance index was the ratio of the ED50 values of the R accession and SS accession. There was no significant difference among runs; therefore, data from two runs were analyzed together. Injury of survivors (%) was also recorded to select plants for genotyping.

Table 1. Expected mutation profile of *A. palmeri* field accessions used in the experiment.

Accession	Origin State	Δ*G210*	*G399A*
LAW-E [a]	Arkansas	Present	Absent
NM-J	Missouri	Present	Present
PEM-F	Missouri	Present	Present
PHI-C	Arkansas	Present	Absent
PHI-I	Arkansas	Absent	Present
SC-C	Missouri	Absent	Present
SS [b]	Arkansas	Absent	Absent

[a] The resistant accessions were harvested in 2017. [b] The susceptible accession was harvested in 2018.

2.2. Response to Other Foliar-Applied PPO Herbicides

The seven accessions (including SS) used in the dose–response assay were also tested with selected PPO herbicides (Table 2). This experiment was conducted in similar conditions as the dose–response assay. A total of 36 seedlings per accession (16 seedlings in the first run and 20 seedlings in the second run), mostly 7- to 10-cm and 4- to 6-leaf stage, were treated with 1× and 2× the recommended doses of PPO herbicides, carfentrazone, flumioxazin, saflufenacil, and trifludimoxazin. Carfentrazone and flumioxazin were sprayed with 0.25% NIS (*v/v*). Saflufenacil was applied with 1% methylated seed oil (*v/v*) and 1% ammonium sulfate (*w/v*). Trifludimoxazin was applied with 1% methylated seed oil (*v/v*). Nontreated check was used as reference. Herbicide application followed the methodology explained above. After herbicide application, plants were grouped in the greenhouse by herbicide and dose. The accessions were completely randomized within each dose and herbicide group. At 3 WAT, injury per survivor and mortality (%) were assessed. Survivors showing injury higher than 90% were classified as susceptible because such severely injured plants would not survive in the field, in competition with the crop and other weeds. The mortality data were subjected to ANOVA using Proc GLIMMIX function in SAS v. 9.4 (SAS Institute, Cary, NC, USA). Mortality data did not follow a normal distribution based on the Shapiro–Wilk test [33]; therefore, a beta distribution was assumed for this response analysis [34]. Student's t test ($p < 0.05$) was used to compare treatment means.

Table 2. Common name, trade name, and chemical family of PPO-inhibiting herbicides in the study.

Common Name [a]	Trade Name	Chemical Family	Field Rate
			g ai ha^{-1}
Carfentrazone	Aim® 2EC	Aryl triazinone	280
Flumioxazin	Valor® SX 51WDG	N-phenylphthalimide	71.5
Fomesafen	Flexstar® 1.88SL	Diphenyl ether	280
Saflufenacil	Sharpen® 4F	Pyrimidinedione	25
Trifludimoxazin	Tirexor™ [b]	Triazinone	30

[a] Protoporphyrinogen IX oxidase inhibitors. [b] Commercial name in Australia; not registered in the USA.

2.3. Detection of Mutations by TaqMan Genotyping Assay

DNA was extracted from leaf tissues of selected survivors showing less than 85% injury from both runs of the "2.1. Fomesafen Dose–Response Assay". Leaf tissues were collected from survivors at 1×, 2×, and 4× the recommended dose of fomesafen ha^{-1} for the accessions LAW-E, NM-J, PEM-F, and PHI-I; at 1× and 2× for the accession SC-C; and at 1× for the accession PHI-C. Following the protocol previously used [28], the tissues were placed separately in 1.5- mL Eppendorf tubes (VWR International LLC, Radnor, PA, USA) with two 2.4-mm metal beads (VWR International LLC). The tubes were stored in −80 °C until processed. The tubes with leaf tissues and beads were ground in a laboratory mixer mill (MM400, Retsch GmbH, Haan, Germany) for 15 s at 30 Hz. Genomic DNA was extracted using a modified CTAB protocol [35] and quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The extracted DNA was diluted to about 150 ng uL^{-1}. Plant samples of each accession were genotyped individually for the presence of the target-site mutations Δ*G210* and/or *G399A*.

DNA samples were diluted to a concentration of 150 ng μL^{-1}. The diluted samples were used in the TaqMan® SNP Genotyping Assay. Fluorescent probes were used to discriminate between the resistant and susceptible alleles of the *ppo2* (Δ*G210* and *G399A*) gene. For Δ*G210* detection, the forward (5′-TGATTATGTTATTGACCCTTTTGTTGCG-3′) and reverse (5′-GAGGGAGTATAATTTATTTACAACCTCCAGAA-3′) primer pair was used [23]. Probes overlapping the Δ*G210* mutation, targeting wild type (5′-TTGAGGATCTCCACCACATG-3′) and positive Δ*G210* (5′-CGATTGAGGATCTCCACATG-3′), were used [36]. For *G399A* detection, the forward (5′-TGTTTATTTGATAAACATATCATAGAATCTAATGCTAGTTTCTT-3′) and reverse (5′-AGCACGATCAGGAAACATCATAGAC-3′) primers were used. Probes overlapping the *G399A* mutation, targeting wild type (5′-ACGTCGCAGGTACTTT-3′) and positive Δ*G210* (5′-CGTCGCAGCTACTTT-3′), were used [28].

The qPCR reaction mixture (5.5 μL) consisted of 1 μL GoTaq® Flexi buffer (Promega, Madison, WI, USA), 0.6 μL 25 mM $MgCl_2$ (Promega), 0.25 μL 10 mM dNTP mix (Promega), 0.25 μL primer-probe mix (Thermo Fisher Scientific, Waltham, MA, USA), 0.05 μL GoTaq® Flexi DNA polymerase (Promega), and 1 μL of genomic DNA. The qPCR was conducted using a CFX96 Real-Time PCR machine (Bio-Rad, Hercules, CA, USA) using the following conditions: 3 min at 95 °C, followed by 40 cycles of 15 s denaturation at 95 °C, 1 min at 60 °C, followed by a plate read at the end of every cycle. The plates included a known homozygous and heterozygous resistant allele for each mutation and a homozygous susceptible. Allelic discrimination was performed using the Bio-Rad CFX Manager™ software (BioRad, Hercules, CA, USA) based on the relative fluorescence units. This data was used to describe the profile of individual survivors per accession and dose.

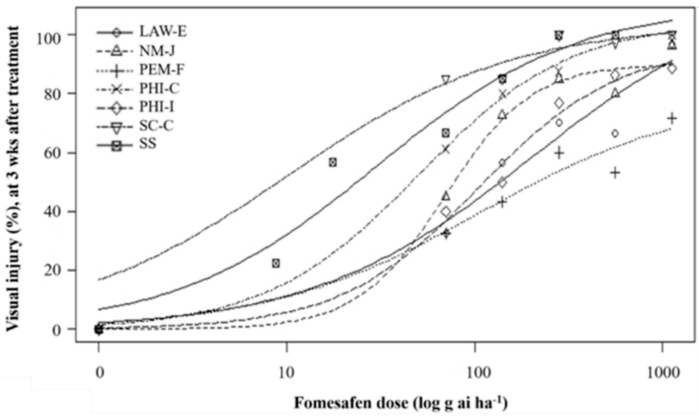
3. Results

3.1. Fomesafen Dose–Response Assay

To determine the resistance level to fomesafen, a dose–response bioassay was conducted with LAW-E, NM-J, PEM-F, PHI-C, PHI-I, SC-C, and SS accessions. Except for SC-C, none of the resistant accessions were completely controlled at 1× dose (280 g ha^{-1}) of fomesafen (Figure 1). Regardless of accession, survivors at 280 g ha^{-1} fomesafen showed

a wide range of injury (from no symptoms to severe plant necrosis and stunting). The approximate fomesafen dose that would cause 50% injury (ED50) varied widely among the resistant accessions. The ED50 ranged from 55 to 171 g ha^{-1} with the order of resistance level as follows: LAW-E > PEM-F > PHI-I > NM-J > PHI-C. The resistance levels (R/S) ranged from 2- to 7-fold (Figure 2). It turned out that SC-C was more sensitive to fomesafen (ED50 = 13 g ha^{-1}) than the SS population (ED50 = 24 g ha^{-1}). SC-C was included in this test because rare individuals harboring the G399A mutation were detected in this population in the general resistance screening. This case highlights the fact that rare resistant individuals would already have been selected in the field several years prior to detection of field-level resistance [22].

Figure 1. *Amaranthus palmeri* accessions susceptible and resistant to fomesafen in greenhouse dose–response experiment. Pictures were taken 3 weeks after treatment with 6 doses of fomesafen, Altheimer Laboratory, University of Arkansas, Fayetteville, USA 2020. Each letter represents one specific accession: (**A**) susceptible; (**B**) LAW-E; (**C**) NM-J; (**D**) PEM-F; (**E**) PHI-C; (**F**) PHI-I; (**G**) SC-C. The first pot to the left of each photo was nontreated. Fomesafen doses were in g ai ha^{-1}.



Accession	ED_{50} (g ai ha^{-1})	Resistance Factor (RF)
Susceptible	24 (± 9)	1
LAW-E	171 (± 76)	7
NM-J	67 (± 9)	3
PEM-F	149 (± 49)	6
PHI-C	55 (± 18)	2
PHI-I	128 (± 69)	5
SC-C	13 (± 10)	0.5

Figure 2. Resistance levels of *Amaranthus palmeri* accessions to fomesafen in greenhouse bioassays, Altheimer Laboratory, University of Arkansas, Fayetteville, USA 2020. The resistance factor was calculated as the ratio of *ED50 R/ED50SS*. Symbols and lines represent actual and predicted herbicide injury responses, respectively. Data were fitted to a non-linear, three-parameter log-logistic regression function $Y = d/1 + \exp[\log(x) - \log(e)]$. Values in parenthesis are standard errors of the mean ($p < 0.05$).

3.2. Response to Other Foliar-Applied Herbicides

The response of the same accessions to other PPO-inhibiting herbicides tested was as follows: carfentrazone < flumioxazin < saflufenacil < trifludimoxazin. Except for carfentrazone, the interaction between accession and herbicide dose was not significant (Table 3). The accessions differed significantly across all herbicides tested, averaged across doses. The Student's *t* test ($p < 0.05$) was used to separate accession x dose interaction means for carfentrazone and the mean injury of accessions in response to all other herbicides (Table 4). At 1× of carfentrazone, all fomesafen-resistant accessions were more tolerant to the herbicide than SS. PHI-C and SC-C were as susceptible to the 2× dose of carfentrazone as the SS. Overall, LAW-E and PEM-F were less sensitive than SS to all PPO-inhibiting herbicides tested. However, most survivors of trifludimoxazin treatment, regardless of accession, had ≥90 injury at 21 d after treatment (data not shown), indicating that such individuals were tougher to kill, but not necessarily resistant. Plants that regrow from this level of injury would be resistant. The resistance frequency and resistance level to trifludimoxazin was low. At 2× trifludimoxazin, the mortality rates of fomesafen-resistant accessions ranged from 92 to 100%.

Table 3. *p* values from ANOVA for mortality 3 weeks after treatment with different PPO herbicides.

Factors Evaluated	ANOVA *p* Values			
	Carfentrazone	Flumioxazin	Saflufenacil	Trifludimoxazin
Accession	<0.0001	<0.0001	<0.0001	0.0277
Dose	0.0002	0.1332	0.0201	0.0556
Acc × Dose	0.0389	0.5124	0.4518	0.8619

3.3. Detection of Mutations by TaqMan Genotyping Assay

The presence of Δ*G210* and *G399A ppo2* mutations was observed in survivors of accessions NM-J, PEM-F, and SC-C (Table 5). PEM-F and SC-C had survivors with both mutations found in the same plant. Only one individual from SC-C survived the 2× dose of fomesafen, and this plant carried both mutations. This individual was excluded from the other comparisons. Based on the dose–response assay SC-C was sensitive to fomesafen, but it contained rare individuals that are resistant to PPO inhibitors. Accession PEM-F, which also contained plants harboring both *ppo2* mutations, was the second most resistant to fomesafen. A total of 32% of survivors of this accession had both mutations. In all

survivors genotyped, both mutations were heterozygous. Therefore, Δ*G210* and *G399A* may or may not co-exist in the same allele of the survivors tested.

Table 4. Response of fomesafen-resistant *Amaranthus palmeri* accessions to the 1× and 2× rates of PPO-inhibiting herbicides, Altheimer Laboratory, University of Arkansas, Fayetteville, USA 2020.

Accession	Dose [a]	Mortality							
		Carfentrazone		Flumioxazin		Saflufenacil		Trifludimoxazin	
		%							
LAW-E	1×	27	*	16	*	54	*	87	*
	2×	31	*	10		78		92	
NM-J	1×	41	*	18	*	86	ns	90	ns
	2×	93	ns	42		93		94	
PEM-F	1×	30	*	10	*	75	*	87	*
	2×	42	*	11		83		94	
PHI-C	1×	66	*	95	ns	93	ns	100	ns
	2×	83	*	99		100		100	
PHI-I	1×	37	*	20	*	93	ns	94	ns
	2×	58	*	47		92		100	
SC-C	1×	55	*	87	ns	89	ns	100	ns
	2×	93	ns	96		93		100	
SS [b]	1×	100		100		100		100	
	2×	100		100		100		100	

[a] Recommended field rate (1×) per herbicide in g ai ha^{-1}: carfentrazone, 280, and flumioxazin, 71.5, with 0.25% NIS (*v*/*v*); saflufenacil, 25, with 1% *v*/*v* methylated seed oil and 1% *w*/*v* ammonium sulfate; trifludimoxazin, 30, with 1% *v*/*v* methylated seed oil. [b] Susceptible population (SS). * Significant difference ($p < 0.05$) compared to susceptible standard. [ns] No significant difference from the susceptible standard.

Table 5. Genotype and zygosity of *Amaranthus palmeri* survivors from treatments with 280, 560, and 1120 g ha^{-1} fomesafen (Flexstar® 1.88 EC) + 0.5% *v*/*v* NIS surfactant.

Accession	No. of Plants Genotyped	Δ*G210* Only		*G399A* Only		Δ*G210* + *G399A*	WT [c]	WT Injury (%)		
		RR [a]	Rr [b]	RR	Rr			Min	Max	Average
LAW-E [d]	16	11	4	-	-	-	1	50	50	50
NM-J [d]	13	-	2	2	4	-	5	40	70	45
PEM-F [d]	22	2	6	-	6	7	1	50	50	50
PHI-C [e]	4	-	2	-	-	-	2	30	30	30
PHI-I [d]	16	-	-	-	1	-	15	30	60	46
SC-C [f]	4	-	1	1	1	-	1	50	50	50

[a] Homozygous; [b] Heterozygous; [c] Mechanism of resistance was not investigated.; [d] Leaf tissues from survivors of 280, 560 and 1120 g fomesafen ha^{-1}. [e] Leaf tissues from survivors of 280 g fomesafen ha^{-1}. [f] Leaf tissues from survivors of 280 and 560 g fomesafen ha^{-1}.

The only *ppo2* mutation present in LAW-E and PHI-C survivors was Δ*G210*. Based on accession-level responses to fomesafen and to other PPO herbicides, LAW-E had the highest resistance to PPO-inhibitor herbicides. The majority of genotyped survivors from LAW-E (11 out of 16) had homozygous Δ*G210* mutation (Table 5). The high frequency of Δ*G210*-homozygous individuals, and the high resistance to PPO inhibitors in this accession, indicate that the occurrence of Δ*G210* mutation on both alleles confers high resistance level to PPO herbicides resulting in <50% injury (Figure 3). Overall, across accessions, the Δ*G210* mutation was present in most survivors up to the highest dose of fomesafen (1120 g ai ha^{-1}).

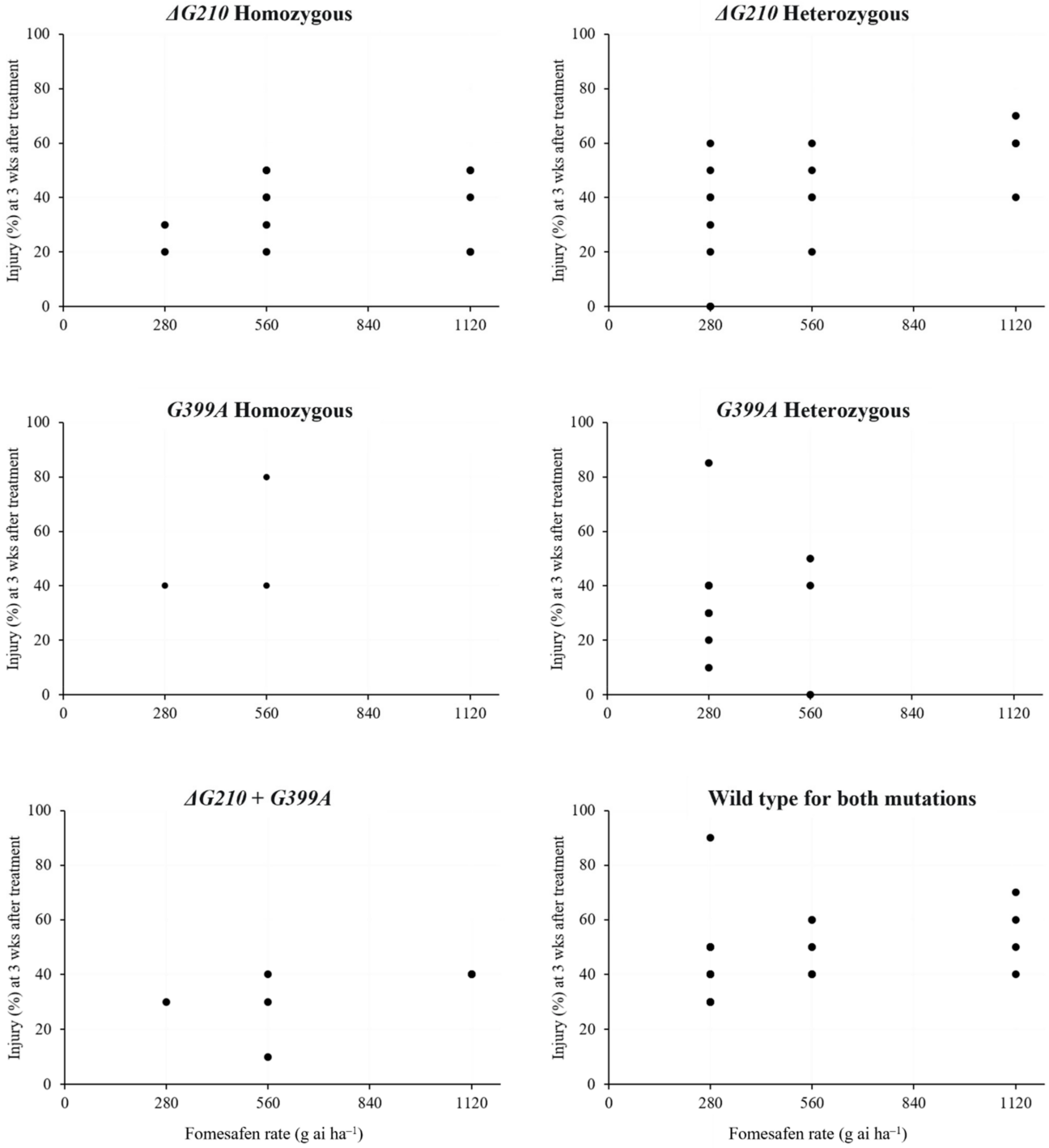


Figure 3. Injury (%) of Amaranthus *palmeri* survivors from treatments with 280, 560 and 1120 g ha^{-1} fomesafen (Flexstar® 1.88 EC) + 0.5% *v*/*v* nonionic surfactant separated by genotype. Each dot in the graphs may represent multiple survivors.

At 4× fomesafen, survivors that carried (Δ*G210* + *G399A*) or homozygous Δ*G210* mutation incurred similar levels of injury (20 to 50%), while the survivors that did not carry Δ*G210* or *G399A* mutation, or those that showed the presence of heterozygous Δ*G210* mutation, incurred a higher injury from 40 up to 70% (Figure 3). Regardless of accession and fomesafen rate, this pattern was the same across all the genotyped survivors (78 total). This indicates that survivors carrying the Δ*G210* in both alleles and other mutations co-existing with *G399A* will recover better than those with *G399A* alone or those harboring mutations other than Δ*G210* or *G399A*.

Unlike Δ*G210*, homozygous *G399A* individuals were rare among the populations tested. Only three out of 78 survivors genotyped harbored this mutation in the homozygous state (Table 4). When *G399A* occurred by itself, the heterozygous survivors incurred 20–80% injury while the homozygous ones had 40–80% injury (Figure 3). Thus, the relationship between the occurrence of *ppo2* mutation(s) in field-selected plants and resistance level is not straightforward. Of the 16 survivors of 4× fomesafen, none harbored the *G399A* mutation by itself, either heterozygous or homozygous.

The accession PHI-I which harbors plants carrying the *ppo2 G399A* mutation was classified as resistant in the dose–response assay. However, only one out of sixteen survivors exhibited the *G399A* mutation. The other survivors were wild type for both mutations. Since the *PPO2* gene of these plants was not sequenced nor tested for other PPO mutations, the mechanism of resistance in these plants is not known.

4. Discussion

4.1. Resistance Level to Fomesafen and Overall Response to Other Foliar-Applied Herbicides

The ED50 values estimated in this study were similar to those reported previously for *A. palmeri* field populations resistant to PPO-inhibitor herbicides. The susceptible population used in this study seemed to have a higher tolerance to fomesafen than the susceptible standards used in previous studies, although direct comparison cannot be made across studies. The resistance index values obtained here were slightly lower compared to those reported in other studies [11,22,26,37]. Wide-ranging ED50 values were also reported for PPO-resistant *A. palmeri* populations from other states. Higher ED50 values (up to 614 g fomesafen ha^{-1}) were estimated for PPO-resistant populations from Kentucky carrying Δ*G210* mutation [38]. On the other hand, low ED50 values (from 12.4 to 28.5 g fomesafen ha^{-1}) were reported for populations from Tennessee [27]. Even with low ED50 values, these Tennessee populations had survivors when treated with up to 3360 g ha^{-1} fomesafen.

The accessions exhibiting high resistance levels to fomesafen also showed cross-resistance to other PPO-inhibitor herbicides. Similar results were previously obtained in studies of different PPO-resistant Amaranthaceae species [11,39,40]. Regarding the overall response to other PPO herbicides, the accessions tested here responded similarly to other *A. palmeri* accessions tested previously [11]. It was expected that saflufenacil and the newest PPO-inhibitor chemistry, trifludimoxazin, would have the highest levels of control. Saflufenacil is applied pre- and postemergence to field corn, cotton, and soybean to control susceptible broadleaf weeds, including *A. palmeri*. Previous results showed saflufenacil as a potent herbicide option for *A. palmeri* [41–43]. However, whenever saflufenacil was applied to fomesafen-resistant populations, its efficacy declined significantly [11,44]. Various cross-resistance levels to PPO-chemistries were reported previously among highly fomesafen-resistant *A. palmeri* and *A. tuberculatus* populations [11,44,45]. The common pattern is that the great majority of fomesafen-resistant populations are susceptible to saflufenacil, and populations that are cross-resistant to trifludimoxazin have not yet evolved at the field level. However, individuals with cross-resistance to trifludimoxazin have already been selected in a few field populations. A premix formulation of saflufenacil and trifludimoxazin was launched this year in Australia. This formulation is more effective in burndown application than other burndown herbicides and is a promising tool for the control of PPO–herbicide-tolerant or -resistant weeds [46–49]. Nevertheless, to curtail further resistance evolution, it is crucial to use this new product with other herbicide modes of action.

4.2. ppo2 Mutations Patterns among Survivors: Implications on Cross-Resistance to PPO Herbicides

The predominance of Δ*G210* mutation among the survivors was expected, since this mutation is predominant among PPO–herbicide-resistant *A. palmeri* and *A. tuberculatus* across the US [11,20,24,28,35]. The mutation *G399A* occurred in a smaller number of plants. Interestingly, among the survivors from the highest fomesafen dose, none carried the mutation *G399A* by itself (heterozygous or homozygous). In a previous study, a field population of *A. palmeri* exclusively harboring the *G399A* mutation also did not survive the 4× dose (1053 g ha^{-1}) of fomesafen [26]. Thus far, the G399A mutation has been reported in a few *A. palmeri* populations in Arkansas, Kansas, Missouri, and Tennessee [26,28,50]. Further research is necessary to fully characterize the physiological effect of G399A substitution and to see if it contributes to increased resistance levels with complementary resistance mechanisms.

Although several researchers studied the resistance level of plants harboring *ppo2* mutations, these studies were conducted with survivors from 1× doses. Up to this point, data on the *ppo2* mutation profile of individuals surviving higher doses are not yet available. In the highest fomesafen dose used in this study (1120 g ai ha^{-1}), the accumulation of two mutations (Δ*G210* + *G399A*) in heterozygous state and single mutation (Δ*G210*) in homozygous state conferred numerically lower injury levels compared to the other mutation profiles at this dose. Although there is no previous information regarding the level of resistance provided by the accumulation of two resistance-conferring *ppo2* mutations in the same plant, there are some studies showing the effects of multiple mutations. The accumulation of different target site mutations in the same plant has been reported in *Eleusine indica* (L.) Gaertn also [51]. The EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) mutations *P106S* and *T102I* were found to occur in the same allele of glyphosate-resistant *E. indica*, conferring high resistance (180×) to glyphosate. When compared to the resistant population with plants harboring only the P106S mutation, the double mutant *E. indica* was 32× more resistant than the single mutant. On the downside, a severe fitness cost was observed in the double *epsps* mutant goosegrass plants [52]. Similarly, the accumulation of two *als* mutations in the same plant was detected in *A. palmeri* from Arkansas [53]. The authors concluded that the accumulation of ALS mutations did not affect the resistance level since all resistant plants across populations harbor one common mutation (*W574L*), independent of the combination.

The Δ*G210* mutation, even in the heterozygous state, endows resistance to PPO herbicides [21]. The presence of double mutation (Δ*G210* + *G399A*) in heterozygous state indicates that both mutations most likely occurred in different alleles, given that Palmer amaranth is diploid. Additionally, the survivors that showed homozygous single Δ*G210* mutation had the highest levels of resistance to fomesafen and the populations comprised of this genotype had low mortality across the PPO herbicides tested. Although the number of populations studied here was low, these observations suggest that if both alleles of *ppo2* carry a mutation (Δ*G210* homozygous or Δ*G210* + *G399A*), the plant may acquire a higher level of resistance to fomesafen. Additionally, our data indicates that Δ*G210* is more likely to occur alone or in combination with *G399A* on both alleles. The strong resistance level by individuals carrying homozygous Δ*G210* mutation has been reported previously. Homozygous Δ*G210* F1 crosses of *A. palmeri* had the highest ED50 for fomesafen compared to heterozygous ones and those harboring both Δ*G210* and *R128G* mutations [54]. The high resistance level of the accession accumulating mutations cannot be solely attributed to this accumulation, since only 32% of the survivors were carrying both mutations.

One of the accessions that exhibited resistance to fomesafen and to two other PPO herbicides was primarily comporised of wild type individuals for both mutations investigated here. Neither the presence of R128 substitutions nor NTSR mechanisms were investigated in this study. Even though TSR is the prevalent mechanism of resistance among PPO-inhibitor-resistant *A. palmeri* populations, other researchers have reported or suggested the possible existence of NTSR mechanisms based on the absence of target-site mutations in some PPO–herbicide-resistant plants [11,20,22,26,37]. PPO-resistant *A. palmeri*

and *A. tuberculatus* populations harboring NTSR mechanisms of resistance were identified in Arkansas and Illinois, respectively, based on plant response to P450 inhibitor application ahead of the PPO herbicides [55,56].

5. Conclusions

The *ppo2* Δ*G210* mutation is the primary mechanism of resistance to PPO-inhibitor herbicides among *A. palmeri* accessions. The high frequency of homozygous Δ*G210* carriers confers high population-level resistance to fomesafen, reflected in higher predicted ED50 values for fomesafen. Plants that survive the highest fomesafen rate (1120 g ha^{-1}), which showed homozygous Δ*G210* or heterozygous Δ*G210* + *G399A*, are more resistant than heterozygous Δ*G210* alone. The *G399A* mutation by itself, either heterozygous or homozygous, was not detected among survivors treated with 1120 g ha^{-1} fomesafen; therefore, this mutation may not confer an equal resistance level as homozygous Δ*G210*. High frequency of homozygous Δ*G210* plants and individuals accumulating Δ*G210* + *G399A* confers high resistance to PPO herbicides.

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