

Nontarget site resistance in Palmer amaranth [*Amaranthus palmeri* (S.) Wats.] confers cross-resistance to protoporphyrinogen oxidase-inhibiting herbicides

Research Article

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Abstract

Palmer amaranth is one of the most problematic weeds in cropping systems of North America, especially in midsouthern United States, because of its competitive ability and propensity to evolve resistance to several herbicide sites of action. Previously, we confirmed and characterized the first case of nontarget site resistance (NTSR) to fomesafen in a Palmer amaranth accession from Randolph County, AR (RCA). The primary basis of the present study was to evaluate the cross- and multiple-resistance profile of the RCA accession. The fomesafen dose-response assay in the presence of malathion revealed a lower level of RCA resistance when compared with fomesafen alone. The resistance index of the RCA accession, based on 50% biomass reduction, ranged from 63-fold (fomesafen alone) to 22-fold (malathion plus fomesafen), when compared with a 2007 susceptible, and 476-fold and 167-fold, respectively, relative to a 1986 susceptible check. The RCA accession was resistant to other protoporphyrinogen oxidase (PPO) inhibitors (i.e., flumioxazin, acifluorfen, saflufenacil) as well as the 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitor tembotrione and acetolactate synthase (ALS) inhibitor pyriithiobac sodium. Sequencing of the ALS gene revealed no point mutations, indicating that a target-site mechanism is not involved in conferring ALS-inhibitor resistance in the RCA accession. Of the three PPO-inhibiting herbicides tested in combination with the malathion, saflufenacil resulted in the greatest biomass reduction (80%; $P < 0.05$) and lowest survival rate (23%; $P < 0.05$) relative to nontreated plants. The application of cytochrome P450 or glutathione S-transferase inhibitors with fomesafen did not lead to any adverse effects on soybean, suggesting a possible role for these compounds for management of NTSR under field conditions. These results shed light on the relative unpredictability of NTSR in conferring herbicide cross- and multiple resistance in Palmer amaranth.

Weed resistance to herbicides may be conferred through two major mechanisms: target and nontarget site (Powles and Yu 2010). Target-site herbicide resistance is endowed by changes to the nucleotide sequence of the target-site gene causing missense mutation(s), leading to changes in the three-dimensional structure of the protein and hence herbicide affinity (Dayan et al. 2010; Tranel and Wright 2002). Gene amplification is another commonly reported target-site mechanism in herbicide-resistant plants (Gaines et al. 2010). In contrast, the more complex and less understood nontarget site resistance (NTSR) can be attributed to differences in herbicide uptake and translocation, metabolic detoxification, or sequestration mechanisms (Powles and Yu 2010; Yuan et al. 2007). It was only in the past decade that the significance and implications of NTSR have started to unfold.

Evolution of NTSR is facilitated by the gradual accumulation of resistant alleles within the individuals of a population, resulting in favorable allelic combinations over time (Délye et al. 2013). Outcrossing species are known to evolve NTSR mechanisms much faster than self-pollinating species (Délye et al. 2013). NTSR is considered a threat to sustainability of herbicide use for chemical weed control, mainly due to its unintended consequences such as resistance to herbicides from different site-of-action (SOA) groups (Délye et al. 2013; Petit et al. 2010). Previous reports have described NTSR mechanisms (mainly metabolic) in weeds for several SOAs, including acetyl coenzyme A carboxylase and inhibitors of photosystem II (PSII), 4-hydroxyphenylpyruvate dioxygenase (HPPD), 5-enolpyruvylshikimate-3-phosphate synthase, acetolactate synthase (ALS), and protoporphyrinogen oxidase (PPO) (Guo et al. 2015; Han et al. 2016; Kaundun et al. 2017; Ma et al. 2013; Nakka et al. 2017; Preston and Wakelin 2008; Varanasi et al. 2018b; Yu et al. 2009).

Palmer amaranth is one of the most problematic weeds in North America, especially in midsouthern United States, due to its rapid growth rate, prolific seed production, and propensity to

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Table 1. Herbicide treatments (1×) applied for testing the cross- and multiple-resistance profile of Palmer amaranth accession from Randolph County, AR.

Herbicide	Rate (g ai or ae ha ⁻¹)	Trade name	Manufacturer	Address	Website
Flumioxazin	160	Valor	Valent USA Corp.	Walnut Creek, CA	https://www.valent.com
Saflufenacil	410	Sharpen	BASF Corp.	Research Triangle Park, NC	https://www.basf.com
Acifluorfen	420	Ultra Blazer	UPL Inc.	King of Prussia, PA	http://www.upi-usa.com
Tembotrione	92	Laudis	Bayer AG	Research Triangle Park, NC	https://www.bayer.com/
Atrazine	1120	AAtrex 4L	Syngenta Crop Protection	Greensboro, NC	http://www.syngentacropprotection.com
Dicamba	560	Clarity	BASF Corp.	Research Triangle Park, NC	https://www.basf.com
Pyriithiobac sodium	72	Staple LX	DuPont	Wilmington, DE	http://www.dupont.com

evolve resistance to several herbicide SOAs (Heap 2018; Korres and Norsworthy 2017; Van Wychen 2016). PPO inhibitors became a popular option for controlling ALS-inhibitor and glyphosate-resistant Palmer amaranth biotypes in soybean and cotton (*Gossypium hirsutum* L.) (Owen and Zelaya 2005; Rousonelos et al. 2012). However, repeated use of PPO-inhibiting herbicides for controlling *Amaranthus* spp. has resulted in the evolution of resistance to this SOA. To date, mostly target-site alterations have been reported to confer resistance to PPO inhibitors. The first target-site mechanism (namely, ΔG210) conferring PPO-inhibitor resistance was reported in waterhemp [*A. tuberculatus* (Moq.) Sauer] in Illinois (Lee et al. 2008; Patzoldt et al. 2006), followed by Palmer amaranth in Arkansas (Salas et al. 2016). Several other target-site mutations in the PPO enzyme of Palmer amaranth endowing PPO-inhibitor resistance have been confirmed: arginine to glycine/methionine (R128G/M), glycine to alanine (G399A), glycine to glutamic acid (G114E), and serine to isoleucine (S149I) (Giacomini et al. 2017; Rangani et al. 2018).

An alternative resistance mechanism to PPO inhibitors became more evident when Obenland et al. (2017) reported an NTSR mechanism to carfentrazone-ethyl in waterhemp. Varanasi et al. (2018b), however, confirmed and characterized the first documented case of NTSR to fomesafen in a Palmer amaranth accession from Randolph County, AR (RCA). Additional research on the cross-resistance patterns of the RCA accession was needed to understand the implications of NTSR. In light of the previous findings, the objectives of this study were to (1) determine the level of fomesafen resistance in the NTSR Palmer amaranth accession with/without malathion, (2) characterize cross-resistance patterns of the Palmer amaranth accession for different PPO class herbicides, and (3) determine the effects of malathion with other PPO-inhibiting herbicides (namely, flumioxazin, saflufenacil, and acifluorfen) in the Palmer amaranth accession.

Materials and Methods

Plant Materials

The Palmer amaranth accession under investigation was collected in 2016 from a cotton and soybean field in RCA (36° 29' 10"N, 90° 55' 37.92"W), as a part of a late-season survey to assess the severity of fomesafen resistance in the state (Varanasi et al. 2018a). The initial seed lot for the RCA accession was created by threshing 10 inflorescences. A homogeneous F1 population of the RCA accession was later created after one generation of selection and used for this study (Varanasi et al. 2018b). Two known susceptible biotypes from 2007 (S1) and 1986 (S2) were included for comparison in the dose-response and cross-resistance assays.

Greenhouse experiments were conducted in 2018 at the Alzheimer Laboratory, University of Arkansas, Fayetteville, AR.

Palmer amaranth seed (RCA accession, S1, and S2) were germinated in plastic trays (25 cm × 55 cm), and 1 week after germination, seedlings at the one- to two-leaf stage were transplanted into 50-well plastic trays (25 cm × 55 cm) filled with potting mix (Sunshine premix No. 1[®]; Sun Gro Horticulture, Bellevue, WA). Soybean (var. CZ 4748LL, Bayer Crop Science) seeds were also planted in plastic pots (8-cm height × 10-cm diameter) filled with potting mix. Initially, four soybean seeds were planted in each pot, which were later thinned to one plant at the seedling stage. All plants were grown under a 16-h photoperiod, 35/25 C day/night temperature, and watered daily throughout the study.

Fomesafen (with or without Malathion) Dose-Response Experiment

To determine the level of resistance in the RCA accession, seedlings at the four- to six-leaf stage were treated with increasing rates of fomesafen with or without malathion. The following fomesafen rate structures were used for the RCA accession and known susceptible biotypes (S1 and S2) based on the 1× rate of fomesafen at 395 g ai ha⁻¹: 0.06×, 0.12×, 0.25×, 0.5×, 1×, 2×, 4×, and 8× for the RCA accession and 0.003×, 0.007×, 0.015×, 0.03×, 0.06×, 0.12×, 0.25×, and 0.5×, for S1 and S2. A nonionic surfactant was included at 0.25% v/v in all treatments. Plants were sprayed with malathion (Hi-Yield[®] Malathion; Hi-Yield Chemical Company, Bonham, TX) at 1,500 g ai ha⁻¹ 2 h before fomesafen treatments (Oliveira et al. 2018). Treatments were applied using a research track sprayer equipped with two flat-fan spray nozzles (TeeJet spray nozzles; Spraying Systems Co., Wheaton, IL) calibrated to deliver 187 L ha⁻¹ of herbicide solution at 269 kPa, moving 1.6 km h⁻¹.

Herbicide Cross- and Multiple Resistance Studies

To determine the cross- and multiple resistance profile of the RCA accession, seedlings at four- to six-leaf stage were treated with field recommended rates of the herbicides listed in Table 1. A known S2 biotype was included as a check and treated with the aforementioned herbicides for comparison. All herbicide treatments included label-recommended adjuvants: crop oil concentrate (Superb[®] HC; Winfield Solutions, St. Paul, MN) at 1% v/v for flumioxazin, saflufenacil, and atrazine; nonionic surfactant (Induce[®]; Helena Agri-Enterprises, Collierville, TN) at 0.25% v/v for acifluorfen, pyriithiobac sodium, and dicamba; and methylated seed oil (Loveland Products Inc., Greeley, CO) at 1% v/v for tembotrione.

Treatment of RCA Accession with Malathion and PPO Inhibitors

To investigate the effects of malathion with PPO-inhibiting herbicides, seedlings (four- to six-leaf stage) of the RCA accession were treated separately with flumioxazin, saflufenacil, and acifluorfen

(Table 1) with or without malathion (1,500 g ai ha⁻¹). Malathion was applied 2 h before the herbicide treatments. All PPO inhibitor treatments included label-recommended adjuvants as previously discussed.

Treatment of Soybean with Fomesafen, P450, and Glutathione S-Transferase Inhibitors

Soybean seedlings (V2) were treated separately with fomesafen (395 g ai ha⁻¹), P450 inhibitor malathion (1,500 g ai ha⁻¹), glutathione S-transferase (GST) inhibitor 4-chloro-7-nitrobenzofurazan (NBD-Cl; Sigma-Aldrich Ltd., St. Louis, MO) (270 g ai ha⁻¹), malathion followed by (fb) fomesafen, or NBD-Cl fb fomesafen under greenhouse conditions (16-h photoperiod, 35/25 C day/night). The NBD-Cl rate of 270 g ai ha⁻¹ was chosen on the basis of a study conducted in atrazine-resistant waterhemp (Ma et al. 2016). Malathion was applied 2 h and NBD-Cl 2 d before fomesafen treatment.

Genomic DNA Isolation

Young leaf tissue was collected from the Palmer amaranth survivors 2 wk after treatment (WAT) with pyriithiobac sodium. Tissue from seven RCA and two S plants were collected, placed in liquid nitrogen, and stored at -80 C until further use. Genomic DNA (gDNA) was isolated from the leaves using a modified cetyltrimethylammonium bromide protocol (Doyle and Doyle 1987). The quantity and quality of isolated gDNA were determined using a spectrophotometer (NanoDrop 1000; Thermo Fisher Scientific, Waltham, MA) and agarose gel electrophoresis.

ALS Gene-Specific Primer Designing and Sequencing

To amplify and sequence the ALS gene (approximately 2,000 bp) from the RCA and S accessions, the following primers were used: forward 5'-CTGCAATCATCCATTTACGCTATC-3' and reverse 5'-TCCAACCAACTAATAAGCCCTTC-3'. Primers were designed on the basis of the sequence information from *Amaranthus* spp. available at GenBank (accession U55852.1), using OligoAnalyzer v. 3.1 (Integrated DNA Technologies, Coralville, IA) (Owczarzy et al. 2008) and checked for specificity using the BLAST tool at the National Center for Biotechnology Information (Bethesda, MD).

Polymerase chain reactions (PCRs) were performed in a T100 thermal cycler (Bio-Rad, Hercules, CA) for amplification of the ALS gene. A 50- μ l reaction volume consisted of 10 μ l of 5 \times GoTaq Flexi buffer (Promega, Madison, WI), 4 μ l of 25 mM MgCl₂ (Promega), 1 μ l of 10 mM dNTP mix (Promega), 0.25 μ l of GoTaq Flexi DNA Polymerase (Promega), 1 μ l each of forward and reverse gene-specific primers, 2 μ l of gDNA, and 30.75 μ l of nuclease-free water (Thermo Fisher Scientific). PCR conditions for amplifying the ALS gene were 95.0 C for 3 min, 35 cycles of 95.0 C for 30 s, 53.0 C for 30 s, and 72.0 C for 2 min, fb 72.0 C for 7 min.

The PCR products were run on 0.8% agarose gel with a 1-kb marker to confirm amplicon size, purified using the GeneJet PCR purification kit (Thermo Fisher Scientific), and quantified with a NanoDrop spectrophotometer. The purified PCR products (25 to 50 ng μ l⁻¹) were sent for Sanger DNA sequencing (Genewiz, South Plainfield, NJ), and aligned using MultAlin to identify point mutations (Corpet 1988).

Table 2. Summary parameters describing the response of aboveground dry biomass from resistant (Randolph County, Arkansas,) and susceptible (S1 and S2) Palmer amaranth accessions to increasing rates of fomesafen 2 WAT.

Biotype ^b	Herbicide Dose for GR ₅₀ ^c	SE
RCA _F	238.24***	42.21
RCA _{F+M}	83.81***	21.46
S1	3.78	0.69
S2	0.50	0.24

^aThe greenhouse experiment was conducted at the Altheimer Laboratory, University of Arkansas, Fayetteville, AR.

^bAbbreviations: RCA, Randolph County, AR; RCA_F, Randolph County, AR, accession treated with fomesafen alone; RCA_{F+M}, Randolph County, AR, accession treated with fomesafen plus malathion; S1, known susceptible from 2007; S2, known susceptible from 1986; GR₅₀, 50% biomass reduction; WAT, weeks after treatment.

^cGR₅₀ values of RCA_F and RCA_{F+M} were highly significant compared with the S1 and S2 biotypes ($P < 0.001$).

*** P -value of < 0.001 .

Statistical Analysis

Experiments were conducted in two separate runs and three replications (10 plants per replication). Data on percent survivors and dry biomass (oven-dried at 65 C for 3 d) were collected 2 WAT. Dry biomass data were expressed as a percentage of the nontreated plants. The soybean experiment was carried out in three separate runs, each run consisting of six replications (one plant per replication). Aboveground fresh soybean biomass was collected at 2 WAT and converted into percent biomass reduction relative to the nontreated plants. ANOVA was performed, and data were analyzed using the JMP Pro 13.1 (SAS Institute Inc., Cary, NC).

Dose-response data were analyzed using the *drc* package in R 3.1.2 (R Development Core Team 2018). The relationship between herbicide rate and aboveground dry biomass was established using a four-parameter log-logistic model (Seefeldt et al. 1995), described as follows:

$$Y = C + \frac{D - C}{1 + \exp\{b[\log(x)] - \log(\text{GR}_{50})\}} \quad (1)$$

where Y is the response (aboveground dry biomass) expressed as a percentage of the nontreated control, C is the lower limit of Y , D is the upper limit of Y , b is the slope of the curve around the effective herbicide dose for 50% biomass reduction (GR_{50}), and x is the herbicide dose. The resistance index (resistance divided by susceptibility [R/S]) was calculated using GR_{50} values. There was no significant interaction between runs; therefore, data were pooled ($P > 0.05$).

Results and Discussion

Level of PPO-Inhibitor Resistance (with or without Malathion)

The fomesafen doses that caused 50% reduction in the aboveground dry biomass of the S1 (2007) and S2 (1986) were 3.78 and 0.50 g ai ha⁻¹, respectively, whereas for the RCA accession, those doses were 238 g ai ha⁻¹ (fomesafen only [RCA_F]) and 83.8 g ai ha⁻¹ (fomesafen plus malathion [RCA_{F+M}]) (Table 2). Based on GR_{50} , the presence of malathion increased the efficacy of fomesafen by approximately 64%. There was a downward shift in the fomesafen dose-response curve for RCA accession in the presence of malathion (Figure 1). Varanasi et al. (2018b) had earlier reported partial reversal of fomesafen resistance in the RCA accession after application of malathion preceding fomesafen treatment at 263 g ai ha⁻¹. Based on the GR_{50} values, the resistant indices (RCA_F/S1 and RCA_{F+M}/S1) for fomesafen were estimated to be

Table 3. Cross-resistance profile of Palmer amaranth RCA accession to different herbicide modes of action.^a

Treatments	RCA Accession ^b		S2	
	Survival ^c	Biomass ^d	Survival	Biomass
	%			
PPO inhibitor				
Flumioxazin ^e	60.0 c	55.7 b	0	12.7 bc
Saflufenacil ^f	34.7 d	19.3 c	0	14.4 bc
Acifluorfen ^g	78.7 b	69.3 b	0	19.0 b
HPPD inhibitor				
Tembotrione ^h	69.3 bc	26.9 c	0	17.0 bc
PS II inhibitor				
Atrazine ⁱ	4.0 e	19.3 c	0	16.2 bc
Auxinic inhibitor				
Dicamba ^j	0 e	27.6 c	0	9.3 c
ALS inhibitor				
Pyrithiobac sodium ^k	94.7 a	87.5 a	0	42.7 a

^aHerbicides were applied to RCA seedlings at the four- to six-leaf stage and evaluated 2 wk after treatment. The greenhouse experiment was conducted at the Altheimer Laboratory, University of Arkansas, Fayetteville.

^bAbbreviations: ALS, acetolactate synthase; HPPD, 4-hydroxyphenylpyruvate dioxygenase; PPO, protoporphyrinogen oxidase; PSII, photosystem II; RCA, Randolph County, AR; S2, 1986 susceptible check.

^cMeans with no common letter(s) within a column are significantly different according to Fisher's protected LSD test ($P < 0.05$).

^dPercent biomass relative to nontreated control.

^eApplication rate: 160 g ai ha⁻¹.

^fApplication rate: 410 g ai ha⁻¹.

^gApplication rate: 420 g ai ha⁻¹.

^hApplication rate: 92 g ai ha⁻¹.

ⁱApplication rate: 1,120 g ai ha⁻¹.

^jApplication rate: 560 g ae ha⁻¹.

^kApplication rate: 72 g ai ha⁻¹.

63- and 22-fold, respectively. In contrast, the fomesafen resistance indices relative to the S2 accession ($RCA_F/S2$ and $RCA_{F+M}/S2$) were 476- and 167-fold, respectively. Previously, the resistant index for the RCA accession based on the S2 accession was reported to be 18-fold (Varanasi et al. 2018b). The higher level of the fomesafen resistance observed in the present study is due to the use of a more homogeneous F1 population instead of a field-derived population for the initial dose-response experiment. Studies conducted with other SOA herbicides have shown an increase in resistance levels with repeated selection in Palmer amaranth (Norsworthy 2014; Tehranchian et al. 2017; Varanasi et al. 2018c).

Resistance Profile of the RCA Accession

Nontarget site resistance confers cross-resistance to different herbicides within the same SOA group (Délye et al. 2013; Vencill et al. 2012). Therefore, the cross- and multiple-resistance profiles of the RCA accession were tested against different herbicides applied at field rates and evaluated 2 WAT (Table 1). Treatment with pyrithiobac sodium showed the highest percent survival (94.7%) fb acifluorfen (78.7%), tembotrione (69.3%), flumioxazin (60%), and saflufenacil (34.7%) (Table 3). In comparison, Palmer amaranth survival was 0% and 4% for dicamba and atrazine applications, respectively, indicating the RCA accession is still sensitive to these herbicides (Table 3). The susceptible check (S2) used in this experiment was completely controlled by the aforementioned herbicides with 0% survival, when compared with a nontreated control (Table 3). In general, percent biomass (relative to nontreated plants) exhibited similar trends with percent survival. These findings indicate the fomesafen-resistant RCA accession is cross-resistant to other PPO-inhibiting herbicides (i.e., flumioxazin, acifluorfen, saflufenacil) and has multiple

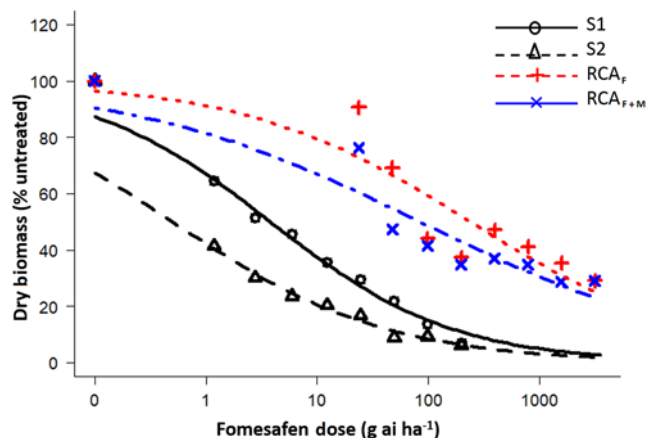


Figure 1. Dose-response assay using a four-parameter log logistic model for fomesafen-resistant Palmer amaranth Randolph County, AR (RCA), and susceptible accessions. S1, susceptible accession from 2007; S2, susceptible accession from 1986; RCA_F , Randolph County, AR, treated only with fomesafen; RCA_{F+M} , Randolph County, AR, accession treated fomesafen plus malathion.

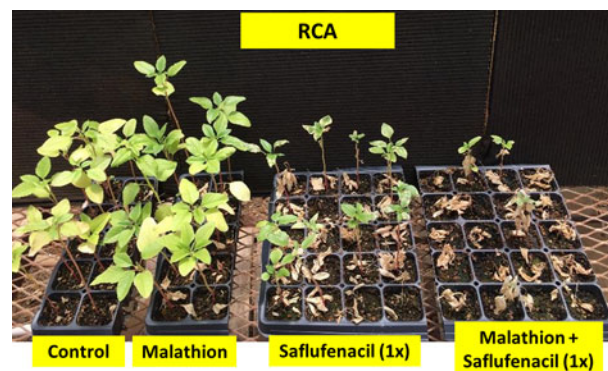


Figure 2. Palmer amaranth Randolph County, AR (RCA), accession treated with P450 inhibitor malathion (1,500 g ai ha⁻¹), saflufenacil (410 g ai ha⁻¹), or malathion followed by saflufenacil. Photograph taken 2 wk after treatment.

resistance to HPPD (i.e., tembotrione) and ALS (i.e., pyrithiobac sodium) inhibitors, and glyphosate. Because of the wide variability in Palmer amaranth accessions, resistance to the HPPD inhibitors mesotrione and tembotrione was found to be widespread in Arkansas and is most likely due to a nontarget site-based mechanism (Singh et al. 2018).

ALS Gene Sequencing

The ALS gene was sequenced from RCA plants that survived the 1X (72 g ai ha⁻¹) application rate of pyrithiobac sodium. No point mutations at positions 122, 197, 205, 376, 377, 574, 653, and 654 in the ALS enzyme were identified in the RCA accession. The mutations at eight locations spread over five different domains (A to E) in the ALS enzyme are known to confer varying levels of ALS-inhibitor resistance in weed species (Tranel and Wright 2002; Yu and Powles 2014). It is concluded that a target-site mechanism based on a point mutation is not involved in conferring pyrithiobac resistance in the RCA accession, and resistance may be a consequence of increased cytochrome P450 activity selected by PPO inhibitors or by prior exposure to ALS inhibitors. Guo et al. (2015) reported similar results with an *A. tuberculatus* population resistant to HPPD and PSII inhibitors.

Table 4. Treatment of Palmer amaranth RCA accession with malathion and PPO inhibitors.^{a,b}

Treatments	RCA ^c	
	Survival ^d	Biomass ^e
	%	
Malathion ^f	100 a	95.9 a
Flumioxazin ^g	91.7 ab	41.6 bc
Malathion fb flumioxazin	83.3 b	27.2 bcd
Acifluorfen ^h	88.3 ab	44.9 b
Malathion fb acifluorfen	89.0 ab	39.5 bc
Saflufenacil ⁱ	63.3 c	24.8 cd
Malathion fb saflufenacil	23.3 d	20.1 d

^aFlumioxazin, acifluorfen, and saflufenacil.

^bHerbicides were applied to RCA seedlings at the four- to six-leaf stage and evaluated 2 wk after treatment. The greenhouse experiment was conducted at the Altheimer Laboratory, University of Arkansas, Fayetteville, AR.

^cAbbreviations: fb, followed by; PPO, protoporphyrinogen oxidase; RCA, Randolph County, AR.

^dMeans with no common letter(s) within a column are significantly different according to Fisher's protected LSD test ($P < 0.05$).

^ePercent biomass relative to nontreated control.

^fApplication rate: 1,500 g ai ha⁻¹.

^gApplication rate: 160 g ai ha⁻¹.

^hApplication rate: 420 g ai ha⁻¹.

ⁱApplication rate: 410 g ai ha⁻¹.

Table 5. Effect of fomesafen, cytochrome P450 (malathion), and glutathione S-transferase (NBD-Cl) inhibitors on soybean.^a

Treatment ^b	Soybean Fresh Biomass ^{c,d}
	%
Malathion ^e	98.6 a
NBD-Cl ^f	96.1 ab
Fomesafen ^g	81.3 c
Malathion + fomesafen	89.2 bc
NBD-Cl + fomesafen	85.9 c

^aTreatments were applied at V2 stage of soybean and evaluated 2 wk after treatment. The greenhouse experiment was conducted at the Altheimer Laboratory, University of Arkansas, Fayetteville, AR.

^bAbbreviation: NBD-Cl, 4-chloro-7-nitrobenzofurazan.

^cPercent biomass relative to nontreated control.

^dMeans with no common letter(s) within a column are significantly different according to Fisher's protected LSD test ($P < 0.05$).

^eApplication rate: 1,500 g ai ha⁻¹.

^fApplication rate: 270 g ai ha⁻¹.

^gApplication rate: 395 g ai ha⁻¹.

Effect of Malathion on Control with PPO Herbicides

Varanasi et al. (2018b) had reported that malathion partially reversed fomesafen resistance in the RCA accession. The present study investigated the response of the RCA accession to malathion with and without other PPO-inhibiting herbicides. Treatment with malathion fb saflufenacil resulted in a decrease in the survival rate (23%; $P < 0.05$) compared with saflufenacil applied alone (63%; $P < 0.05$) (Table 4; Figure 2). On the contrary, no reduction in percent survival and biomass was observed for RCA accession after malathion fb flumioxazin or malathion fb acifluorfen treatments, compared with herbicides applied alone (Table 4). Similar to our earlier studies with fomesafen (Varanasi et al. 2018b), the results show that malathion improved control with saflufenacil by partially reversing the PPO-resistant phenotype (Figure 2). Organophosphate insecticides such as malathion and synergists target different classes of cytochrome P450s; therefore, it is likely that a different set of cytochrome P450s are involved in conferring

metabolic resistance to flumioxazin and acifluorfen compared with saflufenacil and fomesafen. Additional investigations using radio-labeled herbicides are necessary to confirm these results.

Soybean Response to Fomesafen, P450, and GST Inhibitors

Previous research has suggested the use of cytochrome P450 and GST inhibitors as alternative approaches to manage nontarget-site herbicide resistance in weed species (Cummins et al. 2013; Pan et al. 2012). Therefore, the effects of fomesafen with P450 and GST inhibitors on soybean injury were evaluated to verify whether these compounds could be used under field conditions to manage NTSR. No reduction relative to the nontreated plants was observed in soybean fresh biomass 2 WAT with malathion (98.6%) or NBD-Cl (96.1%) (Table 5). Similarly, no biomass reduction occurred when plants were treated with malathion plus fomesafen (89.2%), NBD-Cl plus fomesafen (85.9%), and fomesafen (81.3%) alone, showing that application of P450 or GST inhibitors in combination with fomesafen does not lead to significantly adverse effects on soybean (Table 5). The P450/GST inhibitor treatments in combination with fomesafen caused initial injury (10%) to the plants; however, the injury was transient, and plants recovered within a week.

In conclusion, the nontarget-site fomesafen-resistant Palmer amaranth accession exhibited cross-resistance to selected PPO-, HPPD-, and ALS-inhibiting herbicides (i.e., flumioxazin, acifluorfen, saflufenacil; tembotrione; and pyriithiobac sodium, respectively) in addition to glyphosate. These data lead to further validation of earlier studies showing unpredictable resistance to different herbicide chemistries due to NTSR (Délye et al. 2011). Similar to our previous research with fomesafen (Varanasi et al. 2018b), the cytochrome P450 inhibitor malathion enhanced the effect of saflufenacil in the RCA accession. However, future metabolic research using radioisotopes (¹⁴C), analyses by gas chromatography–mass spectrometry, and liquid chromatography–mass spectrometry are needed to confirm these findings and identify parent herbicide and metabolites. Identification of the cytochrome P450 gene(s) conferring metabolic resistance to fomesafen and saflufenacil are key components toward understanding the mechanism and evolution of NTSR. Prevalence of cross- and multiple resistance in Palmer amaranth accessions emphasizes the need for adoption of integrated weed management strategies in combating the evolution of herbicide-resistant weeds. This work highlights the need for new research approaches toward the important, but still relatively unknown, nontarget-site herbicide resistance.

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