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Target Site-Based Resistance to ALS Inhibitors, Glyphosate, and PPO Inhibitors in an *Amaranthus palmeri* Accession from Mississippi

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Abstract

Extensive acceptance of glyphosate-resistant (GR) row crops coupled with the simultaneous increase in glyphosate usage has sped the evolution of glyphosate resistance in economically important weeds. GR *Amaranthus palmeri* populations are widespread across the state with some exhibiting multiple resistance to acetolactate synthase (ALS) inhibiting herbicides such as pyrithiobac. A GR and ALS inhibitor-resistant accession was also resistant to the protoporphyrinogen oxidase (PPO) inhibiting herbicide fomesafen. The PPO inhibitor resistance profile and multiple herbicide resistance mechanisms in this accession were investigated. In addition to fomesafen, resistance to postemergence applications of acifluorfen, lactofen, carfentrazone, and sulfentrazone was confirmed. There was no resistance to preemergence application of fomesafen, flumioxazin, or oxyfluorfen. Molecular analysis of the ALS gene indicated the presence of point mutations leading to single nucleotide substitutions at codons 197, 377, 574, and 653, resulting in proline-to-serine, arginine-to-glutamine, tryptophan-to-leucine, and serine-to-asparagine replacements, respectively. The resistant accession contained up to 87-fold more copies of the EPSPS gene compared to a susceptible accession. A mutation leading to a deletion of glycine at codon 210 (ΔG210) of PPO2 gene was also detected. These results indicate that the mechanism of resistance in the Palmer amaranth accession is target-site based, *i.e.*, altered target site for ALS and PPO inhibitor resistance and gene amplification for glyphosate resistance.

Keywords

Amaranthus palmeri, ALS Inhibitors, Glyphosate, Palmer Amaranth, PPO Inhibitors, Resistance

1. Introduction

The collective attributes of glyphosate herbicide, from its systemic action to its nonselective, wide range of postemergence activity, has contributed to its broad appeal throughout the world in both crop and noncrop lands since its commercialization in 1974. With the introduction of glyphosate-resistant (GR) crops in the mid-1990s, glyphosate was used selectively and predominantly for weed control in GR crops without concern for crop injury. The widespread adoption of GR crops around the world has led to overuse of the herbicide and reduced crop rotation, which resulted in the evolution of several GR weed biotypes. As of May 2020, GR populations have been reported for 48 weed species worldwide [1], including *Amaranthus palmeri* (S.) Wat. (Palmer amaranth).

Before the commercialization of GR crops, acetolactate synthase (ALS) inhibiting herbicides were used extensively for weed management in crop and noncrop areas. A major downside to the widespread use of ALS inhibitors has been the rapid and extensive evolution of resistance in several grasses and broadleaf weed populations across the world. For example, within 5 years of introduction of chlorsulfuron, the first ALS inhibitor to be commercialized, *Lactuca serriola* L. and *Kochia scoparia* (L.) Shrad populations became resistant [2] [3] [4]. As of May 2020, 165 weed species have been documented to be resistant to one or more ALS inhibitors [1]. Among these resistant weed species are several *Amaranthus* spp. including *A. palmeri*.

Resistance to multiple herbicides, such as glyphosate and ALS inhibitors, has been documented in *A. palmeri* [5] [6]. Almost all populations of *A. palmeri* in row-crop growing areas of Mississippi are considered to be resistant to glyphosate and ALS inhibitors. Loss of glyphosate and ALS inhibitors severely hampered control efforts against *A. palmeri*, leaving very few chemical options, such as glufosinate labeled for use in glufosinate-resistant crops and protoporphyrinogen oxidase (PPO) inhibitors for row crop growers in the United States.

PPO inhibitor resistant *A. palmeri* populations have been reported in Arkansas, Illinois, and Tennessee [1]. Lack of control of *A. palmeri* with PPO inhibitors has been sporadic in Mississippi, but more consistent in the past 24 - 36 months. *A. palmeri* plants in a GR soybean field in Stoneville, Washington County, MS were individually treated with fomesafen, a PPO inhibitor, two times (two weeks apart) at 0.42 kg·ai·ha^{-1}. Leaf tissue from 43 surviving plants was sampled and analyzed for the presence of a deletion mutation, ΔG210 [7]. Only one of the 43 plants, referred to as PA-R hereafter, revealed the presence of the above mutation. The other 42 plants were not analyzed further.

The objectives of this research were to 1) characterize the magnitude of resistance to fomesafen and putative resistance to glyphosate and selected ALS inhibitors; 2) determine cross-resistance to selected PPO inhibitors, applied preemergence (PRE) and/or POST; and 3) elucidate the physiological and molecular mechanism(s) of resistance to ALS inhibitors, glyphosate, and PPO inhibitors in the PA-R accession.

2. Materials and Methods

2.1. Plant Growth and Herbicide Treatment Evaluations

Experiments involving herbicide responses on *A. palmeri* seedlings were performed in a greenhouse at the Jamie Whitten Delta States Research Center of USDA-ARS in Stoneville, Mississippi. The greenhouse was set to 25/20˚C ± 3˚C day/night temperature under ambient conditions. All molecular studies were conducted at University of Illinois, Urbana, Illinois. Seed from a wild type/susceptible *A. palmeri* accession, (susceptible to all major herbicide families, data not shown) designated as PA-S, was included in all experiments. PA-S seed was sown at a depth of 0.5 cm in plastic trays (50 cm × 20 cm× 6 cm) containing a commercial potting mix [formulated Canadian sphagnum peat moss, coarse perlite, bark ash, starter nutrient charge (with gypsum) and slow release nitrogen and dolomitic limestone (Metro-Mix 360, Sun Gro Horticulture, Bellevue, WA) and then watered. Two weeks after germination, 2.5-cm tall seedlings were trans-planted into 8 cm × 8 cm × 7 cm pots containing the same potting mix.

PA-R plants were generated from a parent a male plant (*A. palmeri* is dioecious with male and female reproductive organs developing on different plants) using a cloning procedure described before [8]. Briefly, an axillary branch, approximately 3 cm long, was cut from the stem and lateral leaves removed leaving 4 leaves per stalk. The cut end was lightly coated with Rootone rooting hormone (TechPac, Lexington, KY) and placed in moist growth media as above. The plantlets were kept in indirect sunlight for 3 wk, then transplanted into larger pots, and watered and fertilized as described before.

For PRE studies, the soil used in studies on herbicide effects on *A. palmeri* was a Bosket sandy loam (Bosket sandy loam, fine-loamy, mixed, thermic Mollic Hapludalfs Twenty-five seeds of PA-S were planted at a depth of 0.5 cm and covered with additional soil. Ten PA-R cloned plantlets were transplanted into each pot immediately after herbicide application. Pots were watered instantly after herbicide application to activate the herbicide and as needed thereafter. Emerged PA-S and transplanted PA-R seedlings that remained herbicide injury-free were counted 4 wk after treatment (WAT).

All herbicide treatments were applied using an air-pressurized indoor spray chamber (DeVries Manufacturing Co., Hollandale, MN) equipped with a nozzle mounted with 8002E flat-fan tip (Spraying Systems Co., Wheaton, IL) delivering 190 L·ha^{-1} at 220 kPa to *A. pameri* plants that were 5- to 10-cm tall and had four

to six fully expanded leaves. All herbicide treatments were evaluated for efficacy based on the following: percent control ratings (0 = no injury, 100 = dead) were recorded 3 WAT in studies with PPO inhibitors applied POST. PRE PPO inhibitor efficacy was measured as percent decrease in cumulative emergence of seedlings with an active growing point compared with a nontreated control and glyphosate and ALS inhibitors were evaluated by measuring above ground shoot dry weight 3 WAT. An individual plant represented one replication in POST treatments, whereas an individual pot with 25 seeds or 10 plantlets represented a single replication in PRE studies. There were 4 replications per treatment in all herbicide response studies and studies were repeated.

2.2. Herbicide Dose Response

PA-R and susceptible PA-S plants were treated with fomesafen (Reflex®, Syngenta Crop Protection, Greensboro, NC) at 1/8X, 1/4X, 1/2X, 1X (0.42 kg·ai·ha^{-1}), 2X, 4X, and 8X rates; glyphosate at 1/8X, 1/4X, 1/2X, 1X (0.84 kg·ae·ha^{-1}), and 2X for PA-S and 1/2X, 1X, 2X, 4X, and 8X for PA-R; ALS inhibitors: imazaquin (Scepter®, AMVAC Chemical Corporation, Los Angeles, CA), pyrithiobac (no 8X) (Staple®, Corteva Agriscience, Indianapolis, IN), and trifloxysulfuron (Envoke®, Syngenta Crop Protection) were applied in same dose range as glyphosate with the 1X rate being 0.14, 0.11, and 0.015 kg·ai·ha^{-1}, respectively. A non-treated control was included with each set of dose responses. A nonionic surfactant (Induce®, Helena Chemical Company, Collierville, TN) at 0.25% v/v and a crop oil concentrate (Agri-Dex®, Helena Chemical Company) at 1% v/v were included with all ALS inhibitors and PPO inhibitors, respectively.

2.3. PPO Inhibitor Cross Resistance

PRE herbicide treatments included flumioxazin at 0.11 kg·ai·ha^{-1}, fomesafen at 0.43 kg·ai·ha^{-1}, oxyfluorfen at 0.56 kg·ai·ha^{-1}, and nontreated. POST herbicide treatments were acifluorfen, lactofen, saflufenacil, carfentrazone, and sulfentrazone at 0.56, 0.22, 0.09, 0.04, and 0.42 kg·ai·ha^{-1}, respectively. A crop oil concentrate was included at 1% v/v with each treatment.

2.4. Molecular Analysis

To check for known target-site resistance mutations in the PPX2 gene, genomic DNA was extracted from eight PPO-inhibitor-resistant and two PPO-inhibitor-sensitive *A. palmeri* samples using a modified CTAB protocol [9]. A TaqMan-based quantitative PCR approach was used to detect the presence of any ΔG210, R128G, and R128M mutations in PPO2, following previously described protocols [10] [11]. These same ten samples were also checked for known mutations in the EPSPS and ALS genes. EPSPS gene amplification and EPSPS P106S mutation were detected via quantitative PCR and a derived cleaved amplified polymorphic sequences (dCAPS) assay, respectively, following previously described protocols [12]. The ALS gene was amplified using primers specific to the

5' and 3' untranslated regions of *A. palmeri* (ALS-5UTR-F: 5'-CTTCAAGCTTCAACAATG and ALS-3UTR-R: 5'-CCTACAAAAAGCTTCTCCTCTATAAG). PCR reactions included approximately 100 ng DNA, 5 µL Taq polymerase (New England Biolabs, Ipswich, MA, USA), 1.0 mM $MgCl_2$, 0.2 mM each deoxyribonucleotide triphosphate (dNTP), and 0.1 µM of the forward and reverse primers. The thermocycler protocol was as follows: denaturation for 5 min at 95˚C; 34 cycles of 95˚C denaturation for 30 s, 50 ˚C primer annealing for 30 s, and 72˚C extension for 2 min; final extension step of 5 min at 72˚C. Each PCR product was run out on 1% agarose gel and the ~2 kb band was excised and purified from the gel using a QIAquick Gel Extraction Kit (QIAGEN Inc., Germantown, MD, USA). The purified product was sequenced using an ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., Beverly, MD, USA) using the forward and reverse primers (ALS-5UTR-F; ALS-3UTR-R) as well as a third primer to capture the middle of the *ALS* gene (ALS-F2: 5'-GTATCTTTCTAGGTTGCCTAAACC). The sequenced products were then purified and electrophoresed on an ABI 3730xl Capillary DNA Analyzer by the W.M. Keck Center at the University of Illinois. After trimming low-quality bases using Sequencher 5.4 software (Gene Codes Corp., Ann Arbor, MI, USA), the sequences were aligned and analyzed using CLC Sequence Viewer (QIAGEN Inc., Redwood City, CA, USA).

2.5. Statistical Analysis

Data from dose-response and cross-resistance studies were subjected to analysis of variance using the general linear model procedure in SAS 9.4 (SAS Institute, Cary, NC). Data from the two experiments were combined because there were no significant interactions between experiments. Nonlinear regression was used to define a three-parametric power equation $y = y_0 + ax^b$ to relating the herbicide dose effects (x) on shoot dry weight (y), where y_0 is an asymptote, a is a constant, and b is the slope of the curve. Equation parameters were calculated with SigmaPlot 12.5 (Systat Software Inc., San Jose, CA). ED_{50} (effective dose to achieve 50% control) and GR_{50} (dose required to reduce shoot dry weight by 50%) estimates were derived from curves fit in SigmaPlot at 50% control or reduction in shoot dry weight. In the cross-resistance experiment, means were separated using Fisher's protected LSD at P = 0.05.

3. Results and Discussion

3.1. Fomesafen Dose Response

Response of PA-R and PA-S plants to fomesafen dose is represented in **Figure 1**. The ED_{50} values of PA-R and PA-S for fomesafen were 3.30 and 0.06 kg·ha^{-1} indicating that the PA-R accession is 55-fold more resistant to the herbicide compared to PA-S. This level is higher than the 6- to 21-fold resistance reported in certain *A. palmeri* populations from Arkansas populations [9].

3.2. Glyphosate Dose Response

Response of PA-R and PA-S plants to glyphosate dose is represented in Figure 2. The GR_{50} values of PA-R and PA-S for glyphosate were 7.55 and 0.07 kg·ha^{-1} indicating the PA-R accession is 108-fold more resistant to the herbicide compared to PA-S. Earlier reports of *A. palmeri* biotypes resistant to glyphosate from Mississippi were only 15- to 18-fold [6].

3.3. ALS Inhibitors Dose Response

Figures 3-5 represent response of PA-R and PA-S plants to increasing doses of pyrithiobac, imazaquin, and trifloxysulfuron, respectively. The GR_{50} values of PA-S were 0.006, 0.0012, and 0.0008 kg·ha^{-1} for pyrithiobac, imazaquin, and trifloxysulfuron, respectively. Similar GR50 values could not be assessed for the PA-R accession because the chosen nonlinear regression model did not fit the dose response. This was most likely due to the reduction in shoot dry weight being 0%, 0%, and 33% of the nontreated control with pyrithiobac, imazaquin, and trifloxysulfuron, respectively, even at the highest rates applied. Since the GR_{50} value estimates for the PA-R accession would seemingly lie outside the dose range, it would be more accurate to report R/S ratios as greater than a certain value based on the highest tested dose. This approach is frequently followed when resistance to a herbicide has already been widely reported and resistance is confirmed in a study followed by additional research and analysis. Therefore, the reported R/S ratios were based on extrapolated data and constrained to fit within the accuracy of their estimation. A similar procedure was used in an earlier report [13], where R/S values for ALS-inhibitor resistanr *A. palmeri* and *A. spinosus* L. (spiny amaranth) were calculated based on inferred data. Thus, the R/S values of the PA-R accession were >72, >933, and >78 for pyrithiobac, imazaquin, and trifloxysulfuron, respectively.

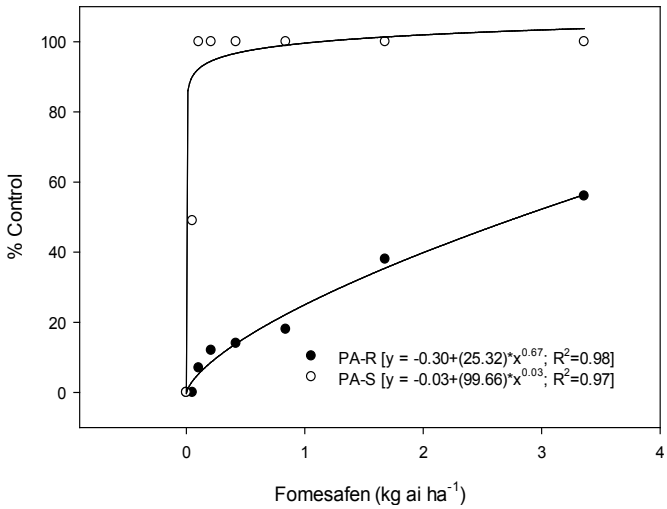

Figure 1. Fomesafen dose response as % control of resistant PA-R and susceptible PA-S *A. palmeri* plants 3 WAT.

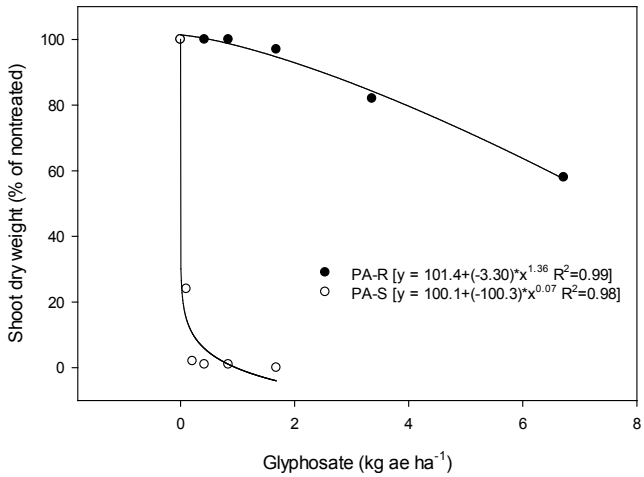

Figure 2. Glyphosate dose response as % shoot dry weight reduction of resistant PA-R and susceptible PA-S *A. palmeri* plants 3 WAT.

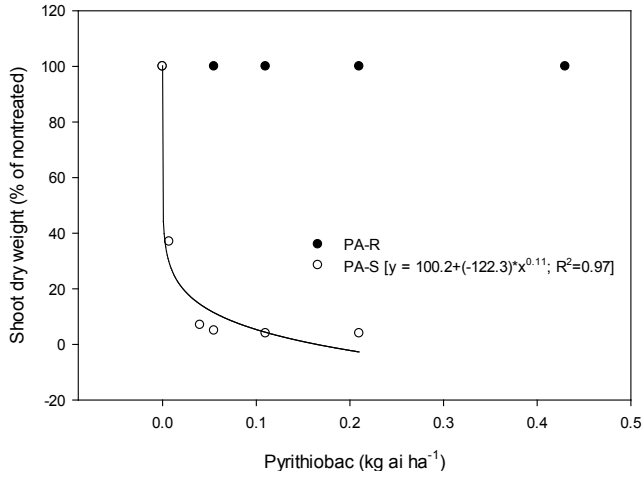

Figure 3. Pyrithiobac dose response as % shoot dry weight reduction of resistant PA-R and susceptible PA-S *A. palmeri* plants 3 WAT.

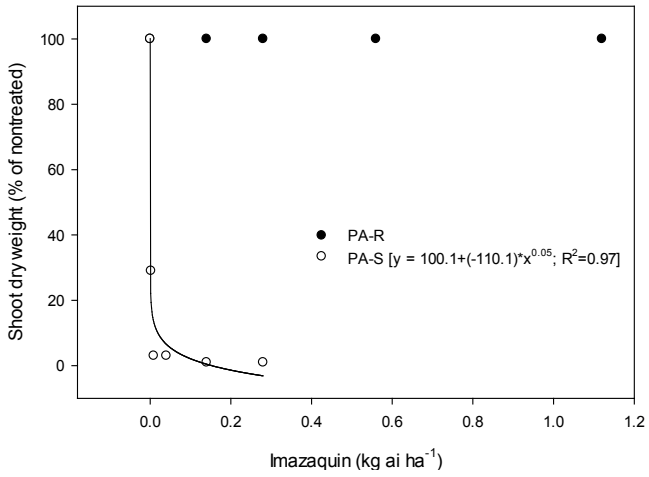

Figure 4. Imazaquin dose response as % shoot dry weight reduction of resistant PA-R and susceptible PA-S *A. palmeri* plants 3 WAT.

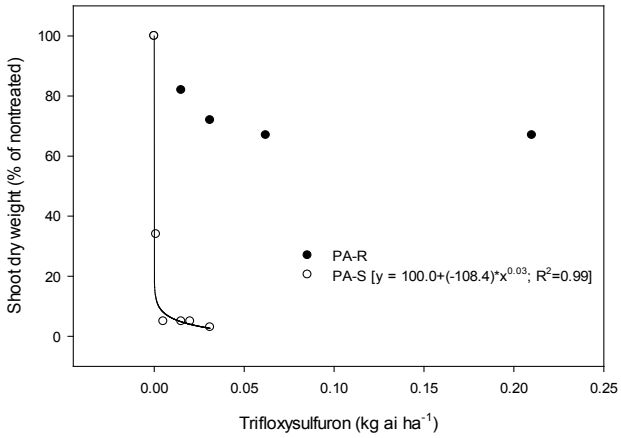

Figure 5. Trifloxysulfuron dose response as % shoot dry weight reduction of resistant PA-R and susceptible PA-S *A. palmeri* plants 3 WAT.

3.4. Cross Resistance to PPO Inhibitors

Both PA-R and PA-S accessions were controlled 100% by flumioxazin, fomesafen and oxyfluorfen, all applied PRE, thereby indicating lack of any cross-resistance in the PA-R accession (Table 1). A PRE dose-response study that includes doses less and more than the respective herbicide doses used for flumioxazin, fomesafen, and oxyfluorfen would provide a better understanding of resistance to herbicides applied PRE. Several PPO inhibiting herbicides applied POST were ineffective in controlling the PA-R accession, except saflufenacil (Table 1). Acifluorfen, lactofen, carfentrazone, and sulfentrazone provided 63%, 6%, 52%, and 18% control, respectively, of PA-R plants, while saflufenacil provided 95% control. PA-S plants were completely controlled by all herbicides evaluated. The above results indicate the PA-R accession is cross-resistant to selected herbicides applied POST, but not to some when treated PRE.

3.5. Molecular Sequence Analysis and Resistance Mechanisms

Only 3 (all heterozygous) of 10 PA-R plants tested positive for a mutation leading to the deletion of glycine at codon 210 (ΔG210) of PPO2 gene (data not shown) (10). No point mutations were detected at the 106 locus of *EPSPS* in the PA-R plants; they had 22 - 87 more copies of EPSPS compared to the PA-S plants (data not shown). Molecular analysis of the ALS gene from 8 PA-R plants indicated the presence of point mutations leading to single nucleotide substitutions at codons 197, 377, 574, and 653, resulting in proline-to-serine, arginine-to-glutamine, tryptophan-to-leucine, and serine-to-asparagine replacements, respectively (Table 2).

The above results from molecular sequence analysis indicate that the PA-R accession has target-site based resistance mechanisms to multiple herbicide modes of action, *i.e.*, altered target site for ALS and PPO inhibitor resistance and gene amplification for glyphosate resistance. Similar results of target-site based resistance to PPO inhibitors and multiple resistance to ALS and PPO inhibitors and glyphosate were reported in *A. palmeri* populations from Arkansas and Indiana, respectively [14] [15].

Table 1. Control of PA-R *A. palmeri* accession with selected PPO inhibiting herbicides applied PRE and POST.

Herbicide	Timing[a]	Control[b]
		%
Flumioxazin	PRE	100a
Fomesafen	PRE	100a
Oxyfluorfen	PRE	100a
Acifluorfen	POST	63b
Lactofen	POST	6
Carfentrazone	POST	52b
Sulfentrazone	POST	18
Saflufenacil	POST	95a

[a]PRE, preemergence; POST, postemergence. [b]Means followed by different letters are significantly different from each other within timing according to Fisher's protected LSD at P = 0.05.

Table 2. Codons and amino acids at eight loci (with known point mutations) of the ALS gene in 8 PA-R and 2 PA-S plants.

Codins:								
	Ala122	**Pro197**	**Ala205**	**Asp376**	**Arg377**	**Trp574**	**Ser653**	**Gly654**
R1	GCA	CCC	GCT	GAT	CGA/CCA	TGG/TTG	AGC	GGC
R2	GCA	CCC	GCT	GAT	CGA	TGG	AGC	GGC
R3	GCA	CCC	GCT	GAT	CGA/CGT	TGG	AGC/AAC	GGC
R6	GCA	CCC	GCT	GAT	CGA	TGG	AGC	GGC
R7	GCA	CCC	GCT	GAT	CGA	TGG/TTG	AGC	GGC
R8	GCA	CCC	GCT	GAT	CGA	TGG	AGC	GGC
R9	GCA	CCC	GCT	GAT	CGA	TGG	AGC/AAC	GGC
R10	GCA	CCC/TCC	GCT	GAT	CGA/CAA	TGG	AGC	GGC
S1	GCA	CCC	GCT	GAT	CGA	TGG	AGC	GGC
S2	GCA	CCC	GCT	GAT	CGA	TGG	AGC	GGC/GGT
Amino acids:								
R1	A	P	A	D	R/P	W/L	S	G
R2	A	P	A	D	R	W	S	G
R3	A	P	A	D	R	W	S/N	G
R6	A	P	A	D	R	W	S	G
R7	A	P	A	D	R	W/L	S	G
R8	A	P	A	D	R	W	S	G
R9	A	P	A	D	R	W	S/N	G
R10	A	P/S	A	D	R/Q	W	S	G
S1	A	P	A	D	R	W	S	G
S2	A	P	A	D	R	W	S	G

4. Conclusion

Growers in Mississippi and across the United States must implement short- and long-term integrated herbicide resistance management practices comprising chemical, mechanical, and cultural strategies to combat multiple herbicide resistant *A. palmeri* populations such as the one reported here. Short-term control methods may include targeted spraying of resistant plants with drones equipped with precision sprayers. Long-term practices could include implementation of cover crops, crop rotation, modified row spacing, and remote sensing-hyperspectral imaging technologies.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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