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In-field assessment of *EPSPS* amplification on fitness cost in mixed glyphosate-resistant and glyphosate-sensitive populations of Palmer amaranth (*Amaranthus palmeri*)

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Research Article

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Abstract

Comparing fitness of herbicide-resistant and herbicide-susceptible weed biotypes is important for managing herbicide resistance. Previous research suggests there is little to no fitness penalty from amplification of the 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene (a mechanism of glyphosate resistance) in Palmer amaranth (*Amaranthus palmeri* S. Watson) in controlled studies in the greenhouse or growth chamber. A field study was conducted in North Carolina at three locations naturally infested with *A. palmeri* to determine vegetative, reproductive, and germination fitness of plants with and without *EPSPS* amplification grown season-long with cotton (*Gossypium hirsutum* L.). Seed number was not correlated with *EPSPS* copy number. However, when plants were binned into two groups, those having an *EPSPS* copy number ≥2 (relative to reference genes) and those having an *EPSPS* copy number <2, plant fresh weight and seed number were 1.4 and 1.6 times greater, respectively, for plants with fewer than 2 *EPSPS* copies. *Amaranthus palmeri* height and seed germination, and yield of cotton, did not differ when comparing the two binned groups. These data suggest that *A. palmeri* plants with *EPSPS* amplification are relatively less fit in the absence of glyphosate, but this reduced fitness does not translate into differences in interference with cotton.

Introduction

Glyphosate resistance in Palmer amaranth (*Amaranthus palmeri* S. Watson) was first confirmed in Georgia in 2005 (Culpepper et al. 2006) and continues to threaten numerous crops in the United States (Heap 2022; Webster 2013). Characteristics of *A. palmeri* that contribute to its ability to dominate fields include high photosynthetic capacity via the C_4 photosynthetic pathway (Ehleringer 1983), rapid growth (Horak and Loughin 2000; Sellers et al. 2003), drought tolerance mechanisms (Place et al. 2008; Wright et al. 1999), shade-adaptive capabilities (Jha and Norsworthy 2009; Jha et al. 2008), prolonged germination (Steckel et al. 2004; Ward et al. 2013), immense fecundity (Schwartz et al. 2016; Webster and Grey 2015), and wide genetic variation (Chandi et al. 2013). These traits give *A. palmeri* a competitive advantage over most crops (Bensch et al. 2003; Burke et al. 2007; MacRae et al. 2013; Massinga et al. 2001; Monks and Oliver 1988). For example, 8 *A. palmeri* plants m^{-1} row are capable of reducing cotton (*Gossypium hirsutum* L.) yield by as much as 92% (MacRae et al. 2013; Morgan et al. 2001; Rowland et al. 1999). When coupled with widespread herbicide resistance, these characteristics cause significant yield loss and increased management costs (Culpepper et al. 2010; Klingaman and Oliver 1994; Morgan et al. 2001).

Amaranthus palmeri biotypes resistant to glyphosate (Group 9) and acetolactate synthase (ALS)-inhibiting (Group 2) herbicides are now commonplace (Heap 2022; Nandula et al. 2012; Poirier et al. 2014; Sosnoskie et al. 2011). The weed has also evolved resistance to triazine (Group 5) and 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibiting (Group 27) herbicides (Jhala et al. 2014; Kohrt et al. 2017). More alarming are the recent confirmations of *A. palmeri* biotypes resistant to protoporphyrinogen oxidase (PPO)-inhibiting (Group 14) herbicides, very-long-chain fatty-acid synthesis–inhibiting (Group 15) herbicides, synthetic auxin

(Group 4) herbicides, and glufosinate (Group 10) (Brabham et al. 2019; Heap 2022; Salas et al. 2016). Several mechanisms confer glyphosate resistance, including target-site mutation, gene amplification, vacuolar sequestration, reduced cellular absorption, and hypersensitive response (Sammons and Gaines 2014). Specific to gene amplification, *A. palmeri* evolved resistance to glyphosate via amplification of the 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene. Researchers who first discovered this mechanism of resistance reported glyphosate-resistant (GR) biotypes from Georgia to have 5- to greater than 160-fold more copies of the *EPSPS* gene than glyphosate-susceptible (GS) biotypes (Gaines et al. 2010).

In some cases, herbicide resistance comes at a penalty, often referred to as a fitness cost. For example, Yanniccari et al. (2016) reported GR biotypes of perennial ryegrass (*Lolium perenne* L.) were shorter, had less leaf area and shoot biomass, and produced fewer seeds than GS biotypes. Triazine-resistant smooth pigweed (*Amaranthus hybridus* L.) is less efficient at photosynthesis and is shorter than susceptible wild-type species (Ahrens and Stoller 1983; Jordan 1996). Wu et al. (2018) reported a significant fitness cost in waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] associated with ALS resistance but not with resistance to atrazine, PPO-inhibiting herbicides, HPPD-inhibiting herbicides, or glyphosate. However, the researchers did note that *A. tuberculatus* resistant to glyphosate via amplification of the *EPSPS* gene decreased in frequency, while glyphosate resistance due to Pro-106-Ser *EPSPS* codon substitution increased over the course of the six generation (3-yr) experiment. Numerous greenhouse experiments have been conducted to determine whether glyphosate resistance imparts a fitness penalty in *A. palmeri*. Giacomini et al. (2014) reported no significant relationship between both final plant biomass and seed production with *EPSPS* copy number, although a negative relationship between *EPSPS* copy number and seed production was noted for one of the four pseudo-F_2 families tested. Similarly, researchers from Argentina and Australia found no correlation between *EPSPS* copy number and plant height or biomass for this weed (Vila-Aiub et al. 2014).

Determination of fitness costs associated with herbicide resistance is challenging due to the need to control for genetic background and because of the potential environmental dependence for manifestation of the fitness costs. (Keshtkar et al. 2019; Vila-Aiub et al. 2011). Furthermore, control of genetic background is necessary to directly relate pleiotropic effects on fitness cost with genes that confer resistance (Bergelson and Purrington 1996; Vila-Aiub et al. 2011). Otherwise, the differences in fitness between a resistant and susceptible biotype might simply be explained by differences in other traits. It is critical to keep in mind that the question about fitness cost due to resistance trait is not just theoretical, and ultimately, it is intended to determine the likelihood of the trait persisting or increasing in agroecosystems. The reality is that under field conditions, and especially in a dioecious species, genome architecture can change constantly, so the genetic context in which the resistance trait acts will likely differ from population to population and even from plant to plant (Leon et al. 2021). Therefore, studying multiple populations can help our understanding of the impact of the herbicide-resistance trait on the species and the range of fitness costs, if they exist, that can influence the spread of resistant biotypes (Bravo et al. 2017, 2018; Keshtkar et al. 2019). Although studies have indicated no fitness penalty associated with *EPSPS* amplification in *A. palmeri* under controlled conditions, fitness costs under field conditions have not been documented. The objective of this experiment was to study fitness costs of *EPSPS* amplification in *A. palmeri* under field conditions.

Materials and Methods

The experiment was conducted in North Carolina in 2014 near Clayton at the Central Crops Research Station (35.67°N, 78.51° W) and in a grower's field near Mount Olive (35.20°N, 77.96° W). The experiment was also conducted near Rocky Mount, NC, in 2015 at the Upper Coastal Plain Experiment Station (35.89°N, 77.67°W). Soil at Clayton was a Dothan loamy sand (fine-loamy, kaolinitic, thermic Plinthic Kandiudults) with 0.22% humic matter and pH 5.5. Soil at Mount Olive was a Lakeland sand (thermic, coated Typic Quartzipsamments) with 0.41% humic matter and pH 5.1. A Goldsboro fine sandy loam (fine-loamy, siliceous, subactive, thermic Aquic Paleudults) with 0.56% humic matter and pH 6.2 was present at Rocky Mount. Cotton cultivar 'Stoneville 4946GLB2' (Bayer CropScience, Research Triangle Park, NC, USA) was planted on May 3, 2014, and May 12, 2014, in Mount Olive and Clayton, respectively. At Rocky Mount, cotton cultivar 'Dyna-Gro 3385B2XF' (Loveland Products, Loveland, CO, USA) was planted on May 6, 2015. Cotton was seeded into conventionally tilled raised beds in Clayton and Rocky Mount and a strip-tillage system (Edmisten et al. 2022) in a desiccated wheat (*Triticum aestivum* L.) cover crop in Mount Olive. Cotton was planted at a seeding rate designed to achieve in-row populations of 15 plants m^{-1} with row spacing of 97 cm in Clayton and Mount Olive and 91 cm in Rocky Mount.

Plant Material

Each field site was dedicated to weed science research and naturally infested with a segregating field population of *A. palmeri* at densities greater than 100 plant m^{-2} and a long history (>15 yr) of recurrent glyphosate use. Expression of glyphosate resistance varied across populations and was approximately 40% to 60% at Clayton and Rocky Mount and 67% to 80% at Mount Olive. Level of glyphosate resistance was estimated from the population's response to glyphosate in previous experiments at each field site. As mentioned previously, control of genetic background is necessary to associate fitness cost with genes that confer resistance (Bergelson and Purrington 1996; Vila-Aiub et al. 2011); genetic background in this experiment was controlled by utilizing these three field sites naturally infested with *A. palmeri* segregating for *EPSPS* amplification.

One hundred to 175 *A. palmeri* plants per population were randomly selected at 3 wk after cotton planting and were covered with plastic cups to allow a broadcast application of glufosinate (Liberty® 280 herbicide, Bayer CropScience) at 656 g ai ha^{-1} plus acetochlor (Warrant® herbicide, Monsanto, St Louis, MO, USA) at 1,260 g ai ha^{-1} to control all other weeds. Cups were removed within 30 min of application. Other than the selected *A. palmeri* plants, weeds were removed by hand weeding throughout the season. Experimental fields were approximately 0.2 to 0.4 ha in size. The final plant density of *A. palmeri* was 1 plant 19 m^{-2}, which constituted a single plot; plots were spaced approximately 2.6 m apart. At 6 wk after planting, a single recently emerged leaf, approximately 1 cm^{-2} in size, was removed from each *A. palmeri* plant and stored on dry ice before being shipped to the University of Illinois to determine *EPSPS* copy number relative to a 1-copy reference gene (*CPS*, which encodes the large subunit of

carbamoylphosphate synthetase) number using quantitative PCR as described by Chatham et al. (2015). *Amaranthus palmeri* plants with an *EPSPS* copy number <2 were considered GS, whereas plants with *EPSPS* copy number ≥2 were considered GR.

Amaranthus palmeri height was recorded at 15 wk after planting, with aboveground fresh weight determined at physiological maturity in mid-September of each year before seed rain. Cotton yield within 1 m of the *A. palmeri* plant and between 1 and 2 m from the *A. palmeri* plant was determined by hand picking cotton when bolls were fully open in October or November. *Amaranthus palmeri* reproductive inflorescences from female plants were removed, dried, and threshed to determine seed production.

Germination Assay

Seed production per plant was determined by counting seeds in 0.06- to 0.081-g subsamples to calculate 100-seed weight of the cleaned seed. One hundred seeds from each female plant were seeded in separate 25 by 53 cm greenhouse trays containing potting soil mix (Sun Gro® Fafard 4P Mix, Agawam, MA, USA). The greenhouse was maintained at 35 ± 5 C with metal-halide lighting (400 μmol m^{-2} s^{-1}; Hubbell Lighting, Greenville, SC, USA) supplementing natural light for 14 h daily; greenhouse trays were lightly irrigated twice daily. To determine germination percentage, the number of emerged *A. palmeri* seedlings was recorded at 14 d after seeding.

Statistical Analysis

Data for *EPSPS* copy number and *A. palmeri* height, plant biomass, seed number, and germination percentage were subjected to ANOVA using PROC GLIMMIX in SAS (SAS Software v. 9.4, SAS Institute, Cary, NC, USA) to test for main effect of population, glyphosate-resistance binary designation (yes or no), and the interaction of population and resistance designation. Seed cotton yield within 1 m of the weed and from 1 to 2 m away from the weed was also subjected to ANOVA using PROC GLIMMIX in SAS along with cotton yield from a weed-free section. Significance between data for GR and GS biotypes was determined using a standard *t*-test at $P \leq 0.05$. Differences in cotton yield in presence of GR and GS biotypes and the weed-free control were separated using Fisher's protected LSD test at $P \leq 0.05$. Regression coefficients were constructed using the PROC REG in SAS for the following relationships: *EPSPS* copy number versus plant height, *EPSPS* copy number versus biomass, *EPSPS* copy number versus seed number, and *EPSPS* copy number versus seed germination at $P \leq 0.05$. Additional regression coefficients included seed production versus plant height, seed number versus biomass, and plant height versus biomass.

Results and Discussion

Average *EPSPS* copy number for *A. palmeri* plants with ≥2 *EPSPS* copies was 41.3, 35.9, and 36.2 at Mount Olive, Clayton, and Rocky Mount, respectively (Table 1). *EPSPS* copy number for *A. palmeri* plants with <2 *EPSPS* copies from these respective populations was 0.8, 0.5, and 0.7, respectively. Fewer female *A. palmeri* plants having <2 *EPSPS* copies relative to females having ≥2 *EPSPS* copies were present at the end of the season at Mount Olive, while roughly the same number were present at Clayton and Rocky Mount (Table 1). During the period of time when these experiments were conducted, fields often had varying frequencies of GR *A. palmeri*.

Table 1. Number of female *Amaranthus palmeri* plants and average *EPSPS* copy number and range at Mount Olive, Clayton, and Rocky Mount, NC, USA.

Population	Binary designation	Average *EPSPS* copy number	Range of *EPSPS* copy number	Number of plants
Mount Olive	≥2 *EPSPS copies*	41.3	6.9–128.0	13
Mount Olive	<2 *EPSPS* copies	0.8	0.3–1.3	3
Clayton	≥2 *EPSPS copies*	35.9	3.9–56.1	9
Clayton	<2 *EPSPS* copies	0.5	0.3–0.7	7
Rocky Mount	≥2 *EPSPS copies*	36.2	21.6–47.4	7
Rocky Mount	<2 *EPSPS* copies	0.7	0.2–1.2	7

Table 2. Regression coefficients for *Amaranthus palmeri EPSPS* copy number, height at 15 wk after planting, plant fresh weight at physiological maturity, seed production, and germination.[a]

Correlation variables	P > *F*	Regression coefficient
EPSPS copy number vs. *A. palmeri* height	0.2721	0.04
EPSPS copy number vs. *A. palmeri* biomass	0.7590	0.01
EPSPS copy number vs. *A. palmeri* seed number	0.8184	−0.01
EPSPS copy number vs. *A. palmeri* seed germination	0.3374	0.04
A. palmeri height vs. *A. palmeri* biomass	0.9199	0.01
A. palmeri height vs. *A. palmeri* seed number	0.9643	0.01
A. palmeri biomass vs. *A. palmeri* seed number	<0.0001	0.53

[a]Data are pooled over three populations.

EPSPS copy number was not correlated with *A. palmeri* height, plant biomass, seed number, or seed germination (Table 2). These results are consistent with previous research (Giacomini et al. 2014; Vila-Aiub et al. 2014) showing no relationship between *EPSPS* copy number and biology of this weed, including seed production. While *A. palmeri* height was not correlated with plant biomass or seed number, plant biomass and seed number were correlated (Table 2).

We considered the lack of correlation between *EPSPS* copy number and fitness-associated traits may have occurred because *EPSPS* amplification affects those traits more in a qualitative rather than a quantitative fashion. Stated differently, we hypothesized that plants with, for example, 10 or 100 EPSPS copies would incur fitness costs relative to wild-type plants, but the relative fitness among plants expressing increased copy number would be similar. Therefore, we treated *EPSPS* copy number as a binary variable, with those having an *EPSPS* copy number ≥2 (relative to reference genes) and those having an *EPSPS* copy number <2 making up the two groups. Analyzed this way, the main effect of *EPSPS* copy number was significant for *A. palmeri* plant biomass and seed number (Table 3). The interaction of *EPSPS* copy number and population was not significant for these measurements. *Amaranthus palmeri* height and seed germination were not affected by *EPSPS* copy number or the interaction of *EPSPS* copy number and population.

When pooled over the three populations, *EPSPS* copy number was 37.8 for plants having ≥2 *EPSPS* copies and 0.6 for plants having <2 *EPSPS* copies (Table 4). *Amaranthus palmeri* height for plants having ≥2 *EPSPS* copies (141 cm) and plants having <2 *EPSPS* copies (144 cm) were not different. *Amaranthus palmeri* plant biomass and seed number were 1.4 and 1.6 times greater for plants having <2 *EPSPS* copies ($n = 17$) compared with plants

Table 3. Analysis of variance for *EPSPS* copy number, height at 15 wk after planting, biomass, number of seed, and germination of seed for *Amaranthus palmeri* plants based on binary designation of glyphosate resistance.

Source of variation	Degrees of freedom	*EPSPS* copy number		Height		Biomass		Seed number		Germination	
		F-value	P > *F*	*F*-value	P > *F*	*F*-value	P > *F*	*F*-value	P > *F*	*F*-value	P > *F*
Population	2	0.1	0.9221	100.9	<0.0001	2.1	0.1380	1.8	0.1844	0.1	0.7114
Binary designation[a]	1	38.7	<0.0001	0.1	0.7305	4.2	0.0478	6.4	0.0157	1.4	0.2502
Population × binary designation	2	0.1	0.9333	0.5	0.6277	0.3	0.7238	0.5	0.6268	1.0	0.3282
Error	40	—	—	—	—	—	—	—	—	—	—
Coefficient of variation (%)		72.4	—	16.8	—	56.7	—	61.5	—	33.5	—

[a]*EPSPS* copy number ≥2 or *EPSPS* copy number <2.

Table 4. *EPSPS* copy number, height at 15 wk after planting, plant biomass, seed production, and seed germination for *Amaranthus palmeri* plants based on binary designation of glyphosate resistance.[a]

Binary designation[b]	*EPSPS* copy number[b]	Height	Biomass	Seed number	Seed germination
	no. plant^{-1}	cm	g plant^{-1}	no. plant^{-1}	%
EPSPS copy number ≥2	37.8 (4.1)	141 (10)	4,503 (350)	342,175 (38,610)	37 (3)
EPSPS copy number <2	0.6 (0.1)*	144 (16)	6,420 (1,020)*	557,543 (131,140)*	31 (2)

[a]SEs for means in parentheses.
[b]Data are pooled over three populations.
*Significance at P < 0.05 using a paired *t*-test.

Table 5. Cotton yield with season-long interference from *Amaranthus palmeri* plants based on binary designation of glyphosate resistance in sections 0 to 1 m and 1 to 2 m from the weed.

Binary designation[a]	Cotton yield[b]	
	0 to 1 m from the weed[c]	1 to 2 m from the weed
	g m^{-1}	
EPSPS copy number ≥2	121 (13) b	168 (27) b
EPSPS copy number <2	115 (23) b	162 (23) b
Weed free	191 (21) a	191 (21) a

[a]Data are pooled over three populations.
[b]SEs for means in parentheses.
[c]Means within a column followed by the same letter are not significantly different based on Fisher's Protected LSD test at P ≤ 0.05.

having ≥2 copies ($n = 29$). Seed size was similar for the two *A. palmeri* biotypes, with 100-seed weights of 32.9 and 36.4 mg for plants having <2 *EPSPS* copies and plants having ≥2 copies, respectively (data not shown). Seed germination did not differ for *A. palmeri* plants having <2 *EPSPS* copies or those having ≥2 copies, and no difference in cotton yield was observed after season-long interference with the two biotypes regardless of distance from the *A. palmeri* plant (Table 5). Cotton yield in the presence of a single *A. palmeri* plant was 62% of weed-free cotton within 1 m of the weed and 87% at a distance of 1 to 2 m from the weed.

These data are the only published results in the peer-reviewed literature with a mixed population of *A. palmeri* segregating for *EPSPS* amplification under field conditions in cotton demonstrating a fitness penalty for *EPSPS* copy number associated with seed production. Evaluation of fitness, especially in dioecious species, is difficult due to the changing genome architecture, which can differ from population to population (Leon et al. 2021). Furthermore, we considered the possibilities of a quantitative or qualitative relationship between fitness cost and *EPSPS* amplification. Because the frequencies of glyphosate-resistance mechanisms changed over time, Wu et al. (2018) hypothesized *A. tuberculatus* plants resistant to glyphosate via Pro-106-Ser *EPSPS* codon substitution were more fit than biotypes resistant to the herbicide by amplification of the *EPSPS* gene, which suggests a fitness cost may exist. While differences in plant biomass and seed production were noted between plants having *EPSPS* copy number <2 and those having ≥2 copies, interference with cotton and possibly other crops will not be affected by resistance. While there is a significant difference in seed production between plants having *EPSPS* copy number <2 and those having ≥2 copies, the amount of seed produced by plants having ≥2 copies remains substantial.

Another important consideration is that in a species such as *A. palmeri* with high genetic variability and obligate outcrossing, the genetic architectures in which the resistance allele(s) operate are also variable within populations. Furthermore, other traits that affect fitness are also under selection, and they could additively (or synergistically/antagonistically) increase or mitigate the fitness change caused by the resistance allele(s) (Leon et al. 2021; Leon and van der Laat 2021). In fact, Bravo et al. (2017, 2018) documented in commercial farms the existence of *A. palmeri* populations in which GR biotypes were taller and produced more biomass than GS biotypes. They also found that the differences in growth were at least partially due to increased nutrient-use efficiency in the former compared with the latter. Later, Leon and van der Laat (2021) provided evidence that those differences were the result of selection pressure in the different production systems from which the populations originated. Therefore, interpreting the presence or absence of fitness costs in *A. palmeri* and other dioecious or outcrossing species with high genetic diversity must be done cautiously, because the final fitness that weeds exhibit in the field is highly determined by genes other than those controlling herbicide resistance.

It is not clear why we did not observe a quantitative relationship between fitness cost and *EPSPS* amplification but did observe a significant fitness cost when *EPSPS* amplification was analyzed as a qualitative trait. Intuitively, one would expect more copies would result in more fitness cost due to greater resource drain on the plant or greater perturbation of the shikimate pathway and, therefore, a quantitative relationship. However, one possibility is that increased copies of *EPSPS* do not confer a fitness cost. Instead, perhaps one

or more genes that are present within the *EPSPS* amplicon and that might play a role in fostering DNA amplification (Molin et al. 2020) act in a qualitative fashion to impart a fitness cost.

A practical goal for determining reproductive fitness costs of herbicide resistance is predicting the utility of herbicide rotation as a herbicide-resistance mitigation strategy. In this study, we did not compare fitness between *A. palmeri* males having <2 *EPSPS* copies and males having ≥2 copies. Also, because increased *EPSPS* copy number is not inherited as a single-gene trait (Gaines et al. 2010), it is difficult to extrapolate how our observed fitness cost in *A. palmeri* seed production would decrease the frequency of GR individuals over time if the population was not exposed to glyphosate. However, if one simply assumes that all GR and GS females produce only GR and GS progeny, respectively, and one further assumes no impact by relative male fitness and no seed dormancy, then even after 5 yr, a population starting with 75% GR individuals would still have about 20% GR individuals. With seed dormancy, the decrease in GR frequency would occur even more slowly. However, a return to glyphosate use would dramatically increase glyphosate resistance, but perhaps it could be an effective option for managing a population of GR *A. palmeri* if used in rotation only once every 5 to 10 yr.

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