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Confirmation of glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) populations in New York and responses to alternative chemistries

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Research Article

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Abstract

Palmer amaranth (*Amaranthus palmeri* S. Watson, AMAPA) is one of the most troublesome weeds in North America due to its rapid growth rate, substantial seed production, competitiveness and the evolution of herbicide-resistant populations. Though frequently encountered in the South, Midwest, and Mid-Atlantic regions of the United States, *A. palmeri* was recently identified in soybean [*Glycine max* (L.) Merr.] fields in Genesee, Orange, and Steuben counties, NY, where glyphosate was the primary herbicide for in-crop weed control. This research, conducted in 2023, aimed to (1) describe the dose response of three putative resistant NY *A. palmeri* populations to glyphosate, (2) determine their mechanisms of resistance, and (3) assess their sensitivity to other postemergence herbicides commonly used in NY crop production systems. Based on the effective dose necessary to reduce aboveground biomass by 50% (ED_{50}), the NY populations were 42 to 67 times more resistant to glyphosate compared with a glyphosate-susceptible population. Additionally, the NY populations had elevated *EPSPS* gene copy numbers ranging from 25 to 135 located within extrachromosomal circular DNA (eccDNA). Label rate applications of Weed Science Society of America (WSSA) Group 2 herbicides killed up to 42% of the NY populations of *A. palmeri*. Some variability was observed among populations in response to WSSA Group 5 and 27 herbicides. All populations were effectively controlled by labeled rates of herbicides belonging to WSSA Groups 4, 10, 14, and 22. Additional research is warranted to confirm whether NY populations have evolved multiple resistance to herbicides within other WSSA groups and to develop effective *A. palmeri* management strategies suitable for NY crop production.

Introduction

Across U.S. agricultural production, herbicides are indispensable tools for weed control due to their high efficacy and relatively low cost compared with other control options (Varanasi et al. 2016). However, herbicide resistance and the proliferation of herbicide-resistant (HR) weeds pose a major threat to various cropping systems across the world (Norsworthy et al. 2012; Westwood et al. 2018). The number of unique cases of HR weeds in the United States has more than quadrupled in the last 30 yr (Heap 2024). Furthermore, very few new herbicide sites of action (SOAs) have been released over the same period (Dayan and Duke 2020; Duke 2012; Shaner and Beckie 2014). While many weed species have evolved resistance to a single active ingredient or herbicide SOA, populations exhibiting resistance to multiple SOAs have been identified.

One such species is Palmer amaranth (*Amaranthus palmeri* S. Watson, AMAPA), a C_4 summer annual native to the southwestern United States and northern Mexico. It is consistently ranked as one of the most troublesome and economically significant weeds for U.S. corn (*Zea mays* L.) and soybean [*Glycine max* (L.) Merr.] production (Ehleringer 1983; Van Wychen 2022; Ward et al. 2013). This dioecious species poses a serious threat to cropping systems because of its rapid growth rate (Ward et al. 2013), prolific seed production (Keeley et al. 1987), potential for long-distance pollen dispersal (Sosnoskie et al. 2012), and morphological and phenological

plasticity in response to environmental conditions (Ehleringer 1983; Keeley et al. 1987; Spaunhorst et al. 2018). *Amaranthus palmeri* seed dispersal results from both natural and anthropogenic factors, including the movement of agricultural equipment required by mowing, tillage, and harvesting operations (Norsworthy et al. 2008); wildlife (Farmer et al. 2017); water movement (Norsworthy et al. 2014); and application of manure contaminated by ingested seeds (Yu et al. 2021). *A. palmeri*'s ability to dominate many agricultural landscapes following dispersal has been facilitated by the evolution of herbicide resistance, including glyphosate resistance.

Between 1987 and 2007, U.S. glyphosate usage increased from less than 5,000 Mg yr^{-1} to more than 80,000 Mg yr^{-1} due to the widespread adoption of glyphosate-resistant (GR) agronomic crops and the expansion of reduced- and no-tillage agriculture, which often relies heavily on herbicides for weed control (Battaglin et al. 2014; Benbrook 2016). GR populations of *A. palmeri* were first identified in Georgia (GA) in 2006 (Culpepper et al. 2006) and are now found in more than 28 U.S. states as well as in Argentina, Brazil, Mexico, South Africa, and Uruguay (Heap 2024; Küpper et al. 2018; Molin et al. 2020a). In the northeastern United States, GR populations have been reported in Connecticut (Heap 2024), Pennsylvania (DD Lingenfelter, personal communication), and New Jersey (TEB, personal observations). In the United States, *A. palmeri*'s mechanism of resistance to glyphosate relies on the amplification of the *EPSPS* coding gene contained within extrachromosomal circular DNA (eccDNA), thus increasing enzyme copy numbers (Gaines et al. 2010; Molin et al. 2018, 2020b). The conservation of the *EPSPS* replicon across GR *A. palmeri* populations suggests that widespread resistance originated from a single population (Molin et al. 2018, 2020a). *Amaranthus palmeri* is not solely resistant to glyphosate (Heap 2024). At this time, there have been 70 unique reports in the United States of *A. palmeri* resistance to many SOAs, alone or in combination, including acetolactate synthase (ALS)-, microtubule-, photosystem II (PSII)-, 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS)-, glutamine synthetase-, protoporphyrinogen oxidase- (PPO), very long-chain fatty acid synthesis (VLCFA)-, hydroxyphenylpyruvate dioxygenase (HPPD)-inhibiting and auxin-mimicking herbicides corresponding to WSSA Groups 2, 3, 5/6, 9, 10, 14, 15, 27, and 4, respectively (Heap 2024). For example, a population resistant to chlorsulfuron (WSSA Group 2), 2,4-D (WSSA Group 4), atrazine (WSSA Group 5), glyphosate (WSSA Group 9), and mesotrione (WSSA Group 27) was identified in Kansas (KS) (Kumar et al. 2019).

Given its competitiveness, confirmed resistance to numerous herbicide SOAs, and expected range expansion due to climate change (Eceiza et al. 2022), *A. palmeri* poses a major threat to cropping systems in the northeastern United States. *Amaranthus palmeri*'s presence in New York (NY) was first confirmed in 2019 in a soybean field in Steuben County. Additionally, sizable populations were reported in 2021 and 2022, also in soybeans, in Orange and Genesee counties, respectively (LMS, personal observations). In 2021, NY soybean production covered more than 131,000 ha and was valued at more than US$205 million. In the northeastern United States, diversified cropping systems are commonplace, often including both agronomic and specialty crops. Soybeans are commonly included in the crop rotation of vegetable production systems. Vegetables are important commodities in NY, which is a top 10 state for the production of specialty crops. NY has been ranked second in the United States for both cabbage (*Brassica oleracea* L.) and snap beans (*Phaseolus vulgaris* L.) and is one of the top 10 producing states for onions (*Allium cepa* L.), pumpkins (*Cucurbita pepo* L.), squash (*Cucurbita maxima* Duchesne), and sweet corn (*Zea mays* L.) (USDA-NASS 2024). *Amaranthus palmeri* is also becoming increasingly troublesome in several vegetable crops such as sweet potato [*Ipomea batatas* (L.) Lam.], pumpkin, and asparagus (*Asparagus officinalis* L.) (Boyd et al. 2022). Given that the same field preparation equipment can be used in both agronomic and specialty crop production, it is important to evaluate the efficacy of different herbicide options for managing *A. palmeri* once it has spread into vegetable systems. Controlling *A. palmeri* in vegetables may pose greater challenges due to the restricted availability of herbicides approved for use in these crops, hindering the necessary rotation of herbicides SOAs and increasing the risk of selecting for HR biotypes.

The purpose of this study was to (1) confirm the presence of GR *A. palmeri* populations in NY through dose–response studies, (2) determine the mechanism of resistance to glyphosate of these populations, and (3) assess their sensitivity to alternative herbicide SOAs commonly used in corn, soybean, and vegetable production. We hypothesize that the suspected GR accessions are resistant through the amplification of eccDNA containing the gene coding for EPSPS, thus significantly increasing enzyme copy numbers. Further, we hypothesize that the NY populations will exhibit reduced sensitivity to field use rates of at least one herbicide from WSSA Groups 2, 5, and 27.

Materials and Methods

Plant Material and Research Sites

Between 2019 and 2022, seeds were collected from three suspected GR *A. palmeri* populations present in soybean fields in Steuben, Orange, and Genesee counties, NY (Figure 1). Samples were harvested from 20 female plants that survived glyphosate applications made during the growing season. Field edges were avoided during sampling. Seed heads were threshed to separate the seeds, which were subsequently cleaned and stored at 4 C until the start of the experiments. The Steuben County population (NY-STE) was discovered and collected from a farm near Howard, NY (42.36°N, 77.51°W). Most fields within this operation were in a corn–soybean–alfalfa (*Medicago sativa* L.) rotation. The Orange County population (NY-ORA) was collected from a soybean and diversified vegetable farm near Florida, NY (41.33°N, 74.37°W). The Genesee County population (NY-GEN) was collected from a farm with an agronomic crop rotation located near Pavilion, NY (42.88°N, 78.02°W). In addition to these NY populations, a Nebraska (NE) glyphosate-susceptible (GS) *A. palmeri* population, collected from Keith County in 2017, was used in the experiments and is hereafter referred to as NE-S. Seeds from this population were provided Rodrigo Werle at University of Wisconsin–Madison, WI.

Glyphosate Dose Response

Greenhouse trials were conducted at Cornell AgriTech in Geneva, NY, between February and April 2023. The study was established as a completely randomized design (CRD) with 10 replications per treatment and was repeated once. Five to 10 *A. palmeri* seeds were planted in 7.6-cm-diameter pots filled with Lambert LM-111 growing media (Lambert, Rivière-Ouelle, Québec, Canada) and hand-thinned to one plant per pot after emergence. Growing media moisture was maintained for the duration of the experiment through daily irrigation. Greenhouses were set to a constant temperature of 25 C with a 16-h day length. Natural lighting was supplemented with 400-W high-pressure sodium lamps.

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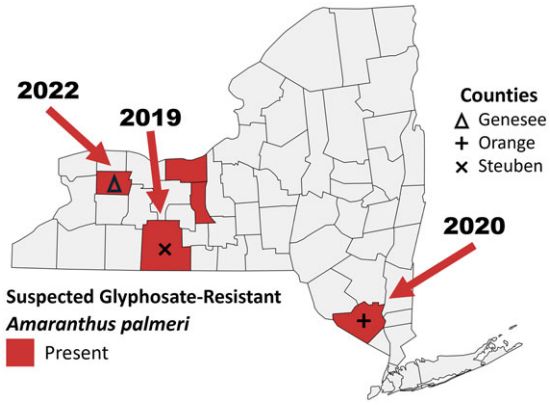

Figure 1. Map of New York showing Genesee (Δ), Orange (+), Seneca (no marker), Steuben (×), and Wayne (no marker) counties in which populations of *Amaranthus palmeri* have been found. Insufficient seed was obtained from populations in Wayne and Seneca counties to include them in this study.

All seedlings were grown to the 2- to 4-leaf stage, at which time glyphosate (Roundup PowerMax®, 540 g ae L^{-1}, Bayer Crop Science, St Louis, MO) was applied at 27, 54, 109, 218, 435, 870 (1X label application rate), 1,740, 3,480, and 6,960 g ae ha^{-1}. A nontreated control (NTC) was also included for comparison. Applications were made using a single-nozzle cabinet sprayer (DeVries Manufacturing, Hollandale, MN) equipped with a TeeJet® 8002VS nozzle (TeeJet Technologies, Glendale Heights, IL). The cabinet sprayer was calibrated to deliver a volume of 187 L ha^{-1} at 276 kPa. At 21 d after treatment (DAT), plant survival was evaluated visually as dead (no green tissue = 0) or alive (green tissue and evidence of regrowth = 1). Aboveground biomass was also collected and recorded at 21 DAT and dried at 60 C for 7 d, and then the dry weights were recorded. Biomass data were converted to percent biomass relative to the NTC using Equation 1:

$$\text{Percent biomass} = \frac{\text{DB}_{\text{EU}}}{\overline{\text{DB}}_{\text{NTC}}} \times 100 \quad [1]$$

with DB_{EU} representing the dry biomass of the experimental unit, and $\overline{\text{DB}}_{\text{NTC}}$ corresponding to the mean dry biomass of the NTC for each population.

EPSPS Gene Copy Number Assay

Six replicate plants from the three NY and the NE-S population were grown to the 6- to 8-leaf stage under the conditions described earlier. One fully expanded leaf was harvested from the newest growth of each plant and stored at −80 C until further processing. To extract genomic DNA, the sample leaf was inserted into 1.5-ml microcentrifuge tubes containing metal beads and placed in a TissueLyser II (Qiagen Sciences, Germantown, MD) for one minute at 1,200 rpm. Genomic DNA was extracted using a modified cetyltrimethylammonium bromide protocol as described by Xin and Chen (2012). Following DNA extraction, the concentration of nucleic acid was quantified using a NanoDrop™ 2000 (Thermo Fisher Scientific, Waltham, MA). DNA concentrations were standardized to 1 ng μl^{-1} using sterile high-performance liquid chromatography (HPLC)-grade water and used for quantitative polymerase chain reaction (qPCR) as described below.

Quantitative real-time PCR was used to measure gene copy number of *EPSPS* relative to the *ALS* copy number of the NE-S population. Extracted DNA from the NY and NE-S populations were used for this assay. DNA from confirmed GR and GS populations originating from Pima County, AZ, were included as positive (AZ-R) and negative (AZ-S) controls (Molin et al. 2018); however, only three replicates were used due to the limited number of samples available. Primers EPSF1 (5′-ATGTTGGACGCTCTCAGAACTCTTGGT-3′) × EPSR8 (5′- TGAATTTCCTCCAGCAACGGCAA-3′) and ALSF2 (5′-GCTGCTGAAGGCTACGCT-3′) × ALSR2 (5′-GCG GGACT GAGTCAAGAAGTG-3′) were used as the EPSPS and ALS primers, respectively (Gaines et al. 2010; Tranel et al. 2004). ALS primers were included due to low variability in *ALS* gene sequences in *Amaranthus* spp., as demonstrated by Tranel et al. (2004).

Quantitative PCR was performed following the procedures described by Gaines et al. (2010). Each individual sample was run in duplicate. Each 20 µl reaction was composed of 2 µl of DNA template containing 10 ng of sample DNA, 1 µl of forward primers, 1 µl of reverse primers, 10 µl of SYBR® Green Master Mix (Bio-Rad Laboratories, Hercules, CA), and 6 µl of sterile HPLC-grade water. The 20-µl reactions were amplified by the following thermoprofile on a MyiQ real-time PCR detection system (Bio-Rad Laboratories): 95 C for 15 min, then 30 cycles of 95 C for 30 s and 60 C for 1 min. Real-time fluorescence data were captured during the amplification cycles. Negative controls containing primers without sample DNA or lacking both primers and sample DNA were included. No amplification products were observed in the negative controls.

Relative quantification of *EPSPS* copy number following the method developed by Gaines et al. (2010) was used to assess data from the qPCR experiment. Relative quantification of *EPSPS* copy number (ΔC_t) was calculated using Equation 2:

$$\Delta C_t = C_t^{ALS} - C_t^{EPSPS} \quad [2]$$

with C_t^{ALS} and C_t^{EPSPS} representing the number of cycles required for the fluorescence signal to exceed that of the background level (threshold cycle) for samples amplified using the ALS and EPSPS primers, respectively. Change in *EPSPS* copy number was reported as $2^{\Delta Ct}$ which is the multiplicative increase in *EPSPS* copy number relative to *ALS* copy number, the latter of which has proven monogenic inheritance in *Amaranthus* spp. (Trucco et al. 2005). Resistance to ALS-inhibiting herbicides does not result from *ALS* gene amplification; rather, it is caused by target-site mutations at amino acid sites, thus interfering with the binding of ALS inhibitors (Gaines et al. 2020).

EPSPS Cassette Marker Assay

Amaranthus palmeri DNA isolated for the *EPSPS* gene copy number assay was also used to confirm the presence of eccDNA in the suspected GR *A. palmeri* populations through PCR. Each reaction contained 10 ng of DNA template, EconoTaq DNA Polymerase (Lucigen Corporation, Middleton, WI), forward and reverse primers, and sterile HPLC-grade water, thus producing a 20-µl reaction. Two sets of primer pairs were used in this experiment. Primer pairs AW293 (5′-GTTATAGCAGCAATT CACCAG-3′) × AW275 (5′-CTAGTTGTTTCACTTGTTTGT GTG-3′) and AW216 (5′-GACCTGGGTTGTCTTCATTC-3′) × AW541 (5′-CGATGATCCAACCGTCCA-3′), henceforth referred to as eccDNA markers A (1,757 bp) and C (1,554 bp), respectively, were used to amplify regions of the eccDNA genome containing the amplified *EPSPS* gene (Molin et al. 2018). Reactions containing DNA from GR and GS populations as described for the

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EPSPS gene copy number assay were used as positive and negative controls, respectively. In addition, separate negative control reactions containing replicon-specific markers but no sample DNA were included in each amplification run. The PCR thermocycler settings were as follows: 4 min of denaturing at 94 C, followed by thirty 30-s cycles at 94 C, 30 s of annealing at 55 C, a 90-s extension period at 72 C, and a final 5-min extension period at 72 C. The presence or absence of the two eccDNA markers was detected using 1% agarose gel electrophoresis. No amplification products were observed in the negative controls.

Response to Alternative Chemistries

In addition to the glyphosate dose−response study, the sensitivity of the NE-S and NY populations to alternative chemistries was also evaluated. Treatments consisted of registered postemergence herbicides commonly used in field corn, soybean, and vegetable production (Table 1) applied within the rate range recommended by the label. Plants were grown under the same greenhouse conditions as previously described for the glyphosate dose −response study. The experiment was conducted as a CRD. Based on emergence success, 6 to 10 replicate plants for each population by herbicide combination were used to evaluate responses to alternate chemistries. The study was conducted twice. Herbicides were applied when plants reached the 2- to 4-leaf stage. The same cabinet sprayer and application settings as those described for the dose−response study were used. At 21 DAT, plant survival was rated as dead (no green tissue = 0) or alive (green tissue and evidence of regrowth = 1). This was used to calculate percent mortality for each combination of herbicide and population. Aboveground biomass was subsequently collected, dried at 60 C for 7 d, and weighed. Relative biomass was calculated using Equation 1.

Statistical Analysis

All statistical analyses were performed using RStudio v. 2023.06.1 (R Core Team 2023). ANOVA confirmed that there were no significant differences between the experimental runs, and data were pooled across runs. The assumption of normality was not violated; thus, no transformation of the data was required. Three- and four-parameter log-logistic functions were created using the DRC package v. 3.0-1 (Ritz et al. 2015). Lack-of-fit tests were run on each model using the *modelFit* function within the DRC package (Ritz et al. 2015). We failed to reject the null hypothesis and concluded that the models fit the data well. ANOVA was performed to assess whether the three- and four-parameter log-logistic models differed significantly. ANOVA confirmed no significant differences in fit between the models; thus the simpler model was chosen. The three-parameter model was used to ascertain the dose of glyphosate required to reduce the biomass of each *A. palmeri* population relative to the NTC by 50% (ED_{50}) using Equation 3 (Knezevic et al. 2007):

$$Y = \frac{d}{1 + \exp[b(\log x - \log e)]} \quad [3]$$

where Y is the aboveground dry biomass expressed as a percentage of the mean biomass of the NTC and x is the herbicide application rate. Parameter d is the upper limit of the log-logistic curve. The ED_{50} is represented by e, while b is the relative slope around parameter e. Using the *ED* function within the DRC package, the dose of glyphosate required to reduce the biomass of each population relative to the NTC by 90% (ED_{90}) was also calculated. The resistance level, expressed as the R/S ratio, was calculated by dividing the ED_{50} of each suspected GR population by the ED_{50} of the susceptible population. Statistical analysis was not conducted on the mortality data, as the data were reported as percent dead for each herbicide treatment.

For the *EPSPS* gene copy number assay and the alternative chemistries experiment, a linear mixed models were fit to determine the effect of population on relative *EPSPS* gene copy number and the effects of herbicide and population on biomass accumulation using the LME4 package v. 1.1-32 (Bates et al. 2015). To account for the imbalance in the number of replicates, the Satterthwaite approximation for degrees of freedom was used (Kuznetsova et al. 2017). Herbicides, populations, and their interaction were treated as fixed effects, while experimental runs were considered a random effect. ANOVA was performed followed by Tukey's honestly significant difference pairwise comparisons ($\alpha = 0.05$) using the EMMEANS package v. 1.8.8 (Lenth 2023). The normality and homoskedasticity assumptions were not violated, thus no transformations of the data were required.

Results and Discussion

Dose–Response Experiment

NY populations were not effectively controlled by glyphosate compared with the NE-S population, which had 100% mortality at the 870 g ae ha^{-1} rate (data not shown). Conversely, at the same glyphosate dose, percent mortality observed for the NY-GEN, NY-ORA, and NY-STE populations was 15%, 25%, and 10%, respectively. None of the NY populations were completely controlled by the highest dose (6,960 g ae ha^{-1}) of glyphosate, although 85% to 95% mortality was observed.

The dose of glyphosate required to reduce the aboveground dry biomass of the NY populations by 50% ranged from 565 to 902 g ae ha^{-1} (Figure 2; Table 2). The ED_{50} value for NY-ORA (565 g ae ha^{-1}) differed significantly from that of NY-STE (902 g ae ha^{-1}), but not NY-GEN (849 g ae ha^{-1}). The ED_{50} values of the NY populations were within the lower part of the range of reported ED_{50} values for GR *A. palmeri* in the United States. Based on our estimated ED_{50} values, the computed R/S ratios of the NY-GEN, NY-ORA, and NY-STE populations were 68, 42, and 64, respectively (Table 2). These values were within the range of the reported ED_{50}-based R/S ratios for GR populations in the United States. Similar ED_{50} values were reported from GA and New Mexico (NM) at glyphosate rates of 560 and 458 g ae ha^{-1}, respectively (Culpepper et al. 2006; Mohseni-Moghadam et al. 2013). Conversely, glyphosate applied at 1,320 g ae ha^{-1} caused a 50% biomass reduction in an NE GR population (Chahal et al. 2017). With respect to R/S values, Chahal et al. (2017) observed that GR populations from NE were 37 to 40 times more resistant to glyphosate than the susceptible population. On the upper end of the range of R/S values, Norsworthy et al. (2008) reported that an Arkansas (AR) GR population's resistance levels were 79 to 115 times greater than that of the susceptible population. On the lower end of the range, Culpepper et al. (2006) found that a GR population from GA was six times more resistant than the GS population. Similarly, Kumar et al. (2018) reported that a multiple HR population from KS had R/S ratios ranging from 7 to 14. Glyphosate rates required to reduce the biomass of NY populations by 90% ranged from 2,196 to 5,311 g ae ha^{-1}

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Table 1. Postemergence herbicides used to assess the response of *Amaranthus palmeri* populations to herbicide sites of action commonly used in New York agriculture

Active ingredient[a]	WSSA Group[b]	Herbicide family	Product formulation	Herbicide manufacturer	Application rate[c]
					g ai or ae ha^{-1}
Chlorimuron-ethyl	2	Sulfonylureas	Classic®	AMVAC, Newport Beach, CA	13.1
Cloransulam-methyl	2	Triazolopyrimidine	FirstRate®	AMVAC, Newport Beach, CA	35.3
Halosulfuron-methyl*	2	Sulfonylureas	Sandea®	Gowan, Yuma, AZ	58.5
Rimsulfuron*	2	Sulfonylureas	Matrix® SG	Corteva, Indianapolis, IN	70.1
2,4-D	4	Phenoxy-carboxylates	Embed® Extra	Corteva, Indianapolis, IN	733*
Dicamba	4	Benzoic acid	XtendiMax®	Bayer Crop Science, St Louis, MO	559*
Atrazine	5	Triazine	Aatrex® 4L	Syngenta Crop Protection, Greensboro, NC	1,244
Prometryn*	5	Triazine	Caparol® 4L	Syngenta Crop Protection, Greensboro, NC	1,122
Linuron	5	Substituted ureas	Lorox® DF	Tessenderlo Kerley, Phoenix, AZ	841
Glufosinate-ammonium	10	Phosphinic acids	Rely® 280	Bayer Crop Science, St Louis, MO	595
Flumioxazin	14	*N*-phenyl-imides	Chateau® SW	Valent, Walnut Creek CA	70.1
Fomesafen*	14	Diphenyl ethers	Reflex®	Syngenta Crop Protection, Greensboro, NC	421
Oxyfluorfen	14	Diphenyl ethers	Goaltender®	NuFarm, Alsip, IL	210
Paraquat*	22	Bipyridilium	Gramoxone® SL 3.0	Syngenta Crop Protection, Greensboro, NC	754
Mesotrione*	27	Triketone	Callisto®	Syngenta Crop Protection, Greensboro, NC	105
Topramezone†	27	Pyrazoles	Impact®	AMVAC, Newport Beach, CA	24.5

[a]Active ingredients displayed with an asterisk (*) and a dagger (†) included nonionic surfactant at 0.25% (v/v) and methylated seed oil at 1% (v/v) in the spray mix, respectively.
[b]WSSA, Weed Science Society of America.
[c]Rates displayed with an asterisk (*) are acid equivalent (ae) while those without an asterisk are active ingredient (ai).

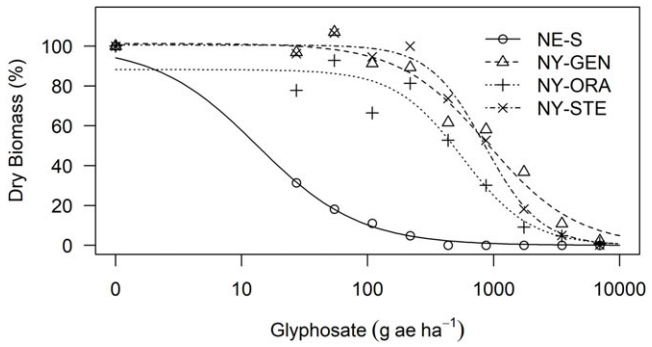

Figure 2. Dose–response curves of glyphosate-resistant (GR) populations of *Amaranthus palmeri* from Genesee (NY-GEN), Orange (NY-ORA), and Steuben (NY-STE) counties in New York and a glyphosate-susceptible (GS) population from Nebraska (NE-S) showing relative biomass at 21 d after treatment. Relative biomass was calculated with the following equation: Relative biomass $= \frac{DB_{EU}}{\overline{DB}_{NTC}} \times 100$ with DB_{EU} representing the dry biomass of the experimental unit and $\overline{DB}_{NTC}$ representing the mean biomass of 10 nontreated control replicates for the appropriate population.

(Table 2). Given *A. palmeri*'s abundant seed production, high levels of control are required to reduce its in-field persistence and subsequent spread to new areas. Results show that effective control of NY populations with glyphosate will not be achievable at labeled use rates.

EPSPS Gene Copy Number and Confirmation of *EPSPS* Cassette Presence

The NY populations had higher *EPSPS* copy numbers than the known susceptible populations from NE and AZ. The relative *EPSPS* gene copy numbers of NY-GEN, NY-ORA, and NY-STE averaged 55, 47, and 91, respectively (Figure 3). These results strongly suggest that amplification of the *EPSPS* gene confers glyphosate resistance in the NY populations as described by Gaines et al. (2010) for GR *A. palmeri* plants collected from GA. Chahal et al. (2017) found that *A. palmeri* plants with at least 30 copies of the *EPSPS* gene survived exposure to glyphosate applied at 870 g ae ha^{-1}. Molin et al. (2018) reported that the *EPSPS* gene copy numbers of GR *A. palmeri* populations from AZ, Delaware (DE), GA, KS, Maryland (MD), and Mississippi (MS), respectively, ranged from 6 to 61 copies. Other members of Amaranthaceae have evolved resistance to glyphosate through the same mechanism. For example, Gaines et al. (2016) confirmed that increased *EPSPS* gene copy number was the resistance mechanism of several GR kochia [*Bassia scoparia* (L.) A.J. Scott] populations collected from sugar beet (*Beta vulgaris* L.) and wheat (*Triticum aestivum* L.)–chemical fallow fields across the Great Plains region of the United States.

Significantly higher gene-copy number was observed in the NY-STE plants than in the NY-ORA plants. Although the relationship between gene copy number and level of resistance was not explicitly tested in this experiment, our results appear to indicate that higher gene copy numbers of NY-STE may be responsible for the higher level of resistance (R/S = 64) observed in this population as compared with NY-ORA (R/S = 42). Vila-Aiub et al. (2014) found that plants with higher amplification of the *EPSPS* gene displayed higher levels of resistance compared with those with lower amplification of the *EPSPS* gene.

All three NY populations contain the *EPSPS* replicon; eccDNA markers A and C were amplified in the AZ-R and all NY populations (Figure 4). The bands were similar in size, pattern, and position relative to the DNA ladder. The AZ-S and NE-S populations failed to amplify and produce PCR products, implying the absence of eccDNA. Results from this study support previous findings by Molin et al. (2020b), who reported that the mechanism of resistance is the amplification of the *EPSPS* replicon, or the approximately 400-kb eccDNA containing the *EPSPS* gene and 58 other genes that encode other competitive functions. Further, this mechanism is unique to GR *A. palmeri* plants (Molin et al. 2020a, 2020b). Koo et al. (2018) demonstrated that eccDNA is transmitted both mitotically and meiotically during cell division, leading to the rapid evolution of glyphosate resistance. The *EPSPS* replicon is reported to be highly conserved across GR *A. palmeri* populations from AZ, DE, GA, KS, MD, and MS, strongly suggesting that resistance to glyphosate in the United States originated from a single population despite its widespread prevalence (Molin et al. 2018, 2020a).

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Table 2. Estimation of regression parameters and glyphosate dose required for 50% (ED_{50}) and 90% (ED_{90}) reduction in biomass of *Amaranthus palmeri* populations at 21 d after treatment

A. palmeri population[a]	Regression parameters (±SE)[b]		Glyphosate[c]		
			ED_{50} (±SE)	ED_{90} (±SE)	R/S[d]
	b	*d*	g ae ha^{-1}		
NE-S	1.07 (±0.33)	99.98 (±5.74)	13.33 (±5.36)	103.24 (±35.62)	—
NY-GEN	1.24 (±0.16)	101.31 (±3.60)	901.99 (±124.13)	5,311.44 (±1,165.05)	67.67
NY-ORA	1.62 (±0.34)	88.20 (±3.78)	565.34 (±76.01)	2,195.91 (±577.81)	42.42
NY-STE	1.97 (±0.30)	100.53 (±2.94)	848.84 (±81.61)	2,579.42 (±447.60)	63.68

[a]NE-S, glyphosate-susceptible (GS) population from Keith County, NE; NY-GEN, NY-ORA, and NY-STE, glyphosate-resistant (GR) populations from Genesee County, Orange County, and Steuben County, NY respectively.
[b]Regression parameters were estimated using a three-parameter log-logistic model, $Y = \frac{d}{1 + \exp[b(\log x - \log e)]}$, where *b* represents the slope of the curve at the inflection point, *d* represents the upper limit, and *e* represents the dose of glyphosate needed to cause 50% biomass reduction (ED_{50}) compared with the nontreated control (NTC).
[c]ED_{50}, the effective dose of glyphosate required to reduce the biomass of each population relative to the NTC by 50%; ED_{90}, the effective dose of glyphosate required to reduce the biomass of each population relative to the NTC by 90%.
[d]R/S represents the resistant:susceptible ratio between the known susceptible (NE-S) and the suspected resistant NY populations (NY-GEN, NY-ORA, NY-STE). The R/S ratio was calculated by dividing the ED_{50} of each suspected GR population by the ED_{50} of the GS population.

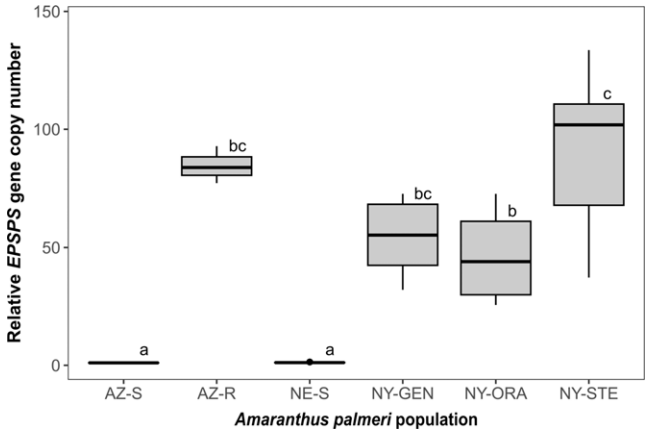

Figure 3. The relative 5-enol-pyruvylshikimate-3-phosphate synthase (*EPSPS*) gene copy numbers of glyphosate-resistant (GR) and glyphosate-susceptible (GS) *Amaranthus palmeri* populations ($n = 6$ biological replicates). The GR populations included a characterized check from Arizona (AZ-R) and the accessions from Genesee (NY-GEN), Orange (NY-ORA), and Steuben (NY-STE) counties in New York. The GS populations included characterized accessions from Nebraska (NE-S) and Arizona (AZ-S).

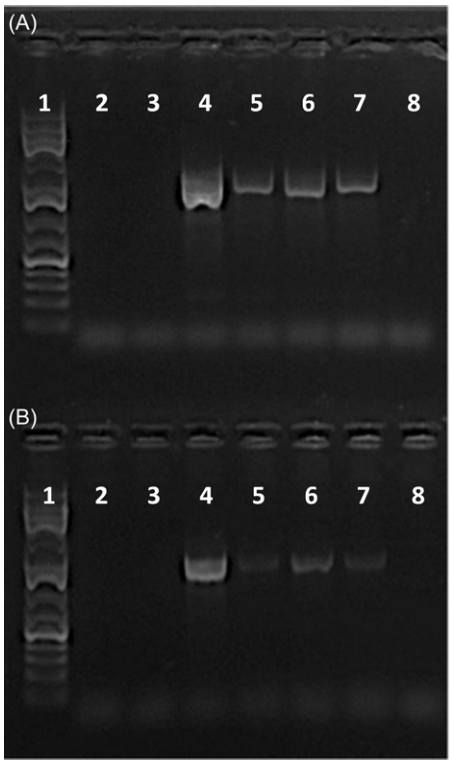

Figure 4. Gel image illustrating polymerase chain reaction (PCR) analysis of the 5-enol-pyruvylshikimate-3-phosphate synthase (*EPSPS*) extrachromosomal circular DNA (eccDNA) cassette markers (A) A (1,757 bp) and (B) C (1,554 bp) in glyphosate-resistant (GR) and glyphosate-susceptible (GS) *Amaranthus palmeri* populations from Arizona, Nebraska, and Genesee, Orange, and Steuben counties in New York. Lanes include (1) 1-kb ladder, (2) no template negative control, (3) Arizona susceptible (AZ-S), (4) Arizona resistant (AZ-R), (5) Genesee (NY-GEN), (6) Orange (NY-ORA), and (7) Steuben (NY-STE) counties, and (8) Nebraska susceptible (NE-S). Individuals from all New York populations amplified both *EPSPS* cassette primers similar to the Arizona resistant positive control. Each sample tested displayed results similar to those shown in the figure.

Response to Alternative Herbicide Chemistries

All populations were controlled 90% to 100% at 21 DAT following applications of prometryn and linuron (WSSA Group 5), glufosinate-ammonium (WSSA Group 10), and paraquat (WSSA Group 22) at labeled rates (Table 3). Mortality of 85% to 100% and 95% to 100% was observed for plants treated with fomesafen and flumioxazin, respectively, while oxyfluorfen caused 60% to 75% mortality of all populations. Mortality following application of ALS-inhibiting herbicides ranged from 0% to 42% across the NY populations, indicating that none of the suspected GR accessions were effectively controlled by the WSSA Group 2 herbicides tested in this study. The mortality of the NE-S population to WSSA Group 2 herbicides ranged from 20% (halosulfuron-methyl) to 70% (rimsulfuron); 33% and 42% mortality was observed for cloransulam-methyl and chlorimuron-methyl, respectively. NY-GEN plants were not effectively controlled by atrazine, with 90% survival observed as compared with only 15%, 40%, and 35% for NY-ORA, NY-STE, and NE-S, respectively. Mesotrione caused variable levels of mortality for the different populations; 90%, 75%, 35%, and 30% of the NE-S, NY-ORA, NY-GEN, and NY-STE plants were controlled, respectively. Across all populations, mortality ranged from 75% to 100% for 2,4-D and dicamba.

Relative *A. palmeri* biomass did not differ significantly between the experimental runs ($P = 0.126$); therefore, data were pooled across runs (Figure 5). Due to substantial mortality, there were no significant differences among populations with respect to relative biomass following treatment with 2,4-D, dicamba, prometryn,

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Table 3. Percent mortality of Nebraska and New York *Amaranthus palmeri* populations following applications of several postemergence herbicides[a]

Active ingredient[b]	WSSA Group[c]	Herbicide family	*A. palmeri* mortality at 21 DAT[d]			
			NE-S	NY-GEN	NY-ORA	NY-STE
			%			
Chlorimuron-ethyl	2	Sulfonylureas	42	25	25	42
Cloransulam-methyl	2	Triazolopyrimidine	33	8	0	8
Halosulfuron-methyl*	2	Sulfonylureas	20	15	10	15
Rimsulfuron*	2	Sulfonylureas	70	30	10	10
2,4-D	4	Phenoxy-carboxylates	100	80	100	75
Dicamba	4	Benzoic acid	85	95	100	90
Atrazine	5	Triazine	65	10	85	60
Prometryn*	5	Triazine	100	90	100	100
Linuron	5	Substituted ureas	100	100	100	100
Glufosinate-ammonium	10	Phosphinic acids	100	100	100	100
Flumioxazin	14	*N*-phenyl-imides	100	95	100	95
Fomesafen*	14	Diphenyl ethers	100	85	100	100
Oxyflurofen	14	Diphenyl ethers	75	60	65	70
Paraquat*	22	Bipyridilium	100	100	100	100
Mesotrione*	27	Triketone	90	35	75	30
Topramezone†	27	Pyrazoles	100	65	100	85

[a]Populations with mortality ≤50% were considered ineffectively controlled by an herbicide.
[b]Active ingredients displayed with an asterisk (*) and a dagger (†) included nonionic surfactant at 0.25% (v/v) and methylated seed oil at 1% (v/v) in the spray mix, respectively.
[c]WSSA, Weed Science Society of America.
[d]DAT, days after treatment; NE-S, glyphosate-susceptible population from Keith County, NE; NY-GEN, NY-ORA, NY-STE, glyphosate-resistant populations from Genesee County, Orange County, and Steuben County, NY, respectively.

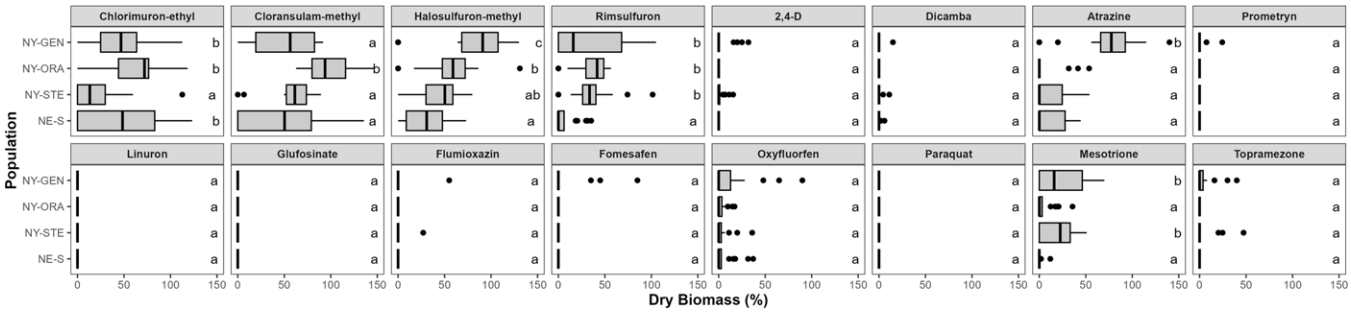

Figure 5. Relative biomass at 21 d after treatment of *Amaranthus palmeri* populations from Nebraska (NE-S) and Genesee (NY-GEN), Orange (NY-ORA), and Steuben (NY-STE) counties in New York in response to herbicide. Relative biomass is expressed as a percent of the mean nontreated control (NTC) and calculated using the following equation: Relative biomass $= \frac{DB_{EU}}{\overline{DB}_{NTC}} \times 100$ with DB_{EU} representing the dry biomass of the experimental unit and $\overline{DB}_{NTC}$ representing the mean biomass of 6 nontreated control replicates for the appropriate population. Treatments with the same letters did not significantly differ according to Tukey's honestly significant difference ($\alpha = 0.05$).

linuron, glufosinate-ammonium, flumioxazin, fomesafen, oxyfluorfen, paraquat, and topramezone. For the chlorimuron-ethyl treatment, the NY-STE population had significantly lower relative biomass compared with the other populations; there were no significant differences with respect to biomass among the NE-S, NY-GEN, and NY-ORA populations. Chlorimuron-ethyl reduced the biomass of the NY-STE, NE-S, NY-GEN, and NY-ORA by 75%, 52%, 56%, and 42%, respectively. The NY-ORA population had significantly higher relative biomass compared with the other populations following treatment with cloransulam-methyl. Halosulfuron-methyl reduced the biomass of NY-GEN, NY-ORA, NY-STE, and NE-S by 11%, 43%, 57%, and 71%, respectively; the NY-GEN and NY-ORA populations differed significantly from NE-S. Overall, the NY-ORA and NY-GEN populations appeared less sensitive to various ALS-inhibiting chemical families than the reference sensitive population, warranting further investigation to assess potential cross-resistance to ALS-inhibiting herbicides. With respect to atrazine, relative biomass was significantly higher for NY-GEN (74%) compared with all other populations (6% to 14%). Mesotrione reduced the mean percent biomass of NY-GEN and NY-STE by 75% and 80% respectively, while the NE-S and NY ORA populations were almost completely controlled. Data from this study suggest that the NY-GEN population, in addition to being resistant to glyphosate, may be less sensitive to WSSA Group 2 herbicides, atrazine, and possibly mesotrione. The NY-STE and NY-ORA populations may also be less sensitive to some ALS-inhibiting chemistries. The responses of the NY populations to atrazine and mesotrione also merit further evaluation. The poor control exhibited by some of the alternative herbicide chemistries tested in this experiment may be due to dose; only one rate per active ingredient was selected for evaluation in this study. More robust screening is required to fully describe *A. palmeri* control potential. Ongoing dose–response studies are being conducted to quantify the levels and assess the mechanisms of potential resistance from NY populations to chlorimuron-ethyl, cloransulam-methyl, atrazine, and mesotrione.

This work confirms the presence *A. palmeri* resistant to glyphosate in NY. The suspected GR populations have resistance levels ranging from 42 to 67 times that of the susceptible NE

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population. The reduced efficacy of ALS, PSII, and HPPD inhibitors in controlling populations assessed in this study is consistent with previous reports. Several *A. palmeri* populations have developed resistance to single herbicides belonging to WSSA Groups 2, 3, 5, 6, 9, 10, 14, 15, and 27. Additionally, populations resistant to more than one herbicide SOA have been identified (Heap 2024). For example, Faleco et al. (2022) found a newly introduced *A. palmeri* population in Wisconsin that showed resistance to atrazine, glyphosate, and imazethapyr. While Chahal et al. (2017) found that glufosinate-ammonium effectively controlled the multiple-resistance populations from NE, Priess et al. (2022) confirmed the existence of a population resistant to glufosinate-ammonium and suspected resistance to imazethapyr, pendimethalin, 2,4-D, glyphosate, fomesafen, *S*-metolachlor, mesotrione, and tembotrione in AR. Finally, a population resistant to WSSA Groups 2, 4, 5, 6, 9, 14, and 27 was reported in KS (Shyam et al. 2021).

The adoption of herbicide-tolerant crops over the last few decades has significantly reduced the diversity of SOAs in several cropping systems (Kniss 2018). The overreliance on single SOAs, such as glyphosate, has hastened the evolution and proliferation of herbicide resistance across several weed species and production systems (Boyd et al. 2022; Culpepper et al. 2006; Norsworthy et al. 2012). While the newly introduced NY populations are still controlled by several of the herbicide SOAs tested, specifically auxin mimics, PPO inhibitors, and photosystem I electron diverters, this sensitivity might be temporary. To maintain the efficacy of these herbicides, efforts must be made to reduce selection pressure through the deployment of diversified weed management techniques including prevention, cultural practices, and mechanical options for control (Norsworthy et al. 2012). While practicing integrated weed management offers several benefits, the short-term costs may not favor implementation of best management practices that provide delayed economic benefit. Efforts to facilitate the adoption of multifaceted approaches to weed management must be ongoing.

The presence of GR *A. palmeri* in NY and its reduced sensitivity to alternative chemistries only increases the challenges facing NY growers, as other HR species are present in the state, including common lambsquarters (*Chenopodium album* L.), smooth pigweed (*Amaranthus hybridus* L.), waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer], common ragweed (*Ambrosia artemisiifolia* L.), common groundsel (*Senecio vulgaris* L.), and horseweed [*Conyza canadensis* (L.) Cronquist]. This is particularly concerning, especially considering that very few new herbicide SOAs have been introduced over the last three decades. The number of herbicides available to NY corn, soybean, and vegetable growers is diminishing and unlikely to rebound because of the rapidly changing regulatory environment due to future compliance with the Endangered Species Act. It is also possible that resistance could develop relatively quickly to new herbicides released to market because of increased selection pressure in response to the lack of other chemical control options. Considering all these factors, growers should look to integrated weed management for guidance and incorporate non–chemical based control options into their production systems. These include mechanical methods such as tillage and cultivation, preventative measures like harvest weed seed control, and novel technologies, including electrical weed control. Furthermore, growers and land managers must be cautious of practices that can introduce *A. palmeri* and further its spread. This weed can easily be introduced to new areas on agricultural equipment, through irrigation, and by birds (Boyd et al. 2022; Norsworthy et al. 2014; Proctor 1968). Equipment, especially items purchased from areas where *A. palmeri* is common, should be thoroughly inspected and cleaned. With climate change, *A. palmeri* is expected to spread farther north (Eceiza et al. 2022). Successful eradication is possible but unlikely. Regardless, management efforts will require rapid establishment of a regulatory framework, access to funds, collaboration among various partners, concerted efforts toward educating the public, and actively addressing new infestations. Agricultural professionals and the public must remain vigilant and take steps to prevent future introductions, eradicate infestations early, and limit their spread.

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