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Confirmation and detection of novel acetolactate synthase- and protoporphyrinogen oxidase–inhibiting herbicide-resistant redroot pigweed (*Amaranthus retroflexus*) populations in North Carolina

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Research Article

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Abstract

Complaints of control failures with acetolactate synthase (ALS)- and protoporphyrinogen oxidase (PPO)-inhibiting herbicides on redroot pigweed (*Amaranthus retroflexus* L.) were reported in conventional soybean [*Glycine max* (L.) Merr.] fields in North Carolina. Greenhouse dose–response assays confirmed that the Camden County and Pasquotank County populations were less sensitive to ALS- and PPO-inhibiting herbicides compared with susceptible *A. retroflexus* populations, suggesting the evolution of resistance to these herbicides. Sanger sequencing of target genes determined the Camden County population carried a Trp-574-Leu mutation in the *ALS* gene and an Arg-98-Gly mutation in the *PPX2* gene, while the Pasquotank County population carried a His-197-Pro mutation in the *ALS* gene (first documentation of the mutation in the *Amaranthus* genus), but no mutation was detected in the *PPX2* gene. Single-nucleotide polymorphism (SNP) genotyping assays were developed to enable efficient screening of future control failures in order to limit the spread of these herbicide-resistant populations. In addition, preliminary testing of these assays revealed the three mutations were ubiquitous in the respective populations. These two populations represent the first confirmed cases of PPO-inhibiting herbicide-resistant *A. retroflexus* in the United States, as well as the first confirmed cases of this particular herbicide-resistance profile in *A. retroflexus* inhabiting North America. While no mutation was found in the *PPX2* gene of the Pasquotank County population, we suggest that this population has evolved resistance to PPO-inhibiting herbicides, but the mechanism of resistance is to be determined.

Introduction

Amaranthus spp. are pervasive and difficult to control in row-crop production in the United States (Sarangi et al. 2021). Moreover, control of *Amaranthus* spp. is further complicated, because these species have evolved resistance to most of the herbicides that can be applied (Shergill et al. 2018; Shyam et al. 2021; Tranel 2021). Historically, acetolactate synthase (ALS; EC 2.2.1.6; Group 2)-inhibiting herbicides were applied to control *Amaranthus* spp., but widespread resistance has limited their efficacy (Ferguson et al. 2001; Hinz and Owen 1997; Horak and Peterson 1995). Protoporphyrinogen oxidase (PPO; EC 1.3.3.4; Group 14)-inhibiting herbicides are applied extensively and intensively to control herbicide-resistant *Amaranthus* spp. in soybean [*Glycine max* (L.) Merr.] (Kniss 2018; Owen and Zelaya 2005). Recurrent use of these herbicides will result in the evolution of resistant weeds (Darwin 1859; Harper 1956).

Redroot pigweed (*Amaranthus retroflexus* L.) has historically been a problem weed in the Southeast but was displaced by Palmer amaranth (*Amaranthus palmeri* S. Watson) in the 2000s (Webster and Coble 1997; Webster and Nichols 2012). Historically, *A. retroflexus* was generally easy to control with herbicides (Ducar et al. 2004; Mayo et al. 1995). However, this is not the case in other parts of the world (Eleftherohorinos et al. 2000; Holm et al. 1997; Scarabel et al. 2007). This species has evolved resistance to three herbicide groups:

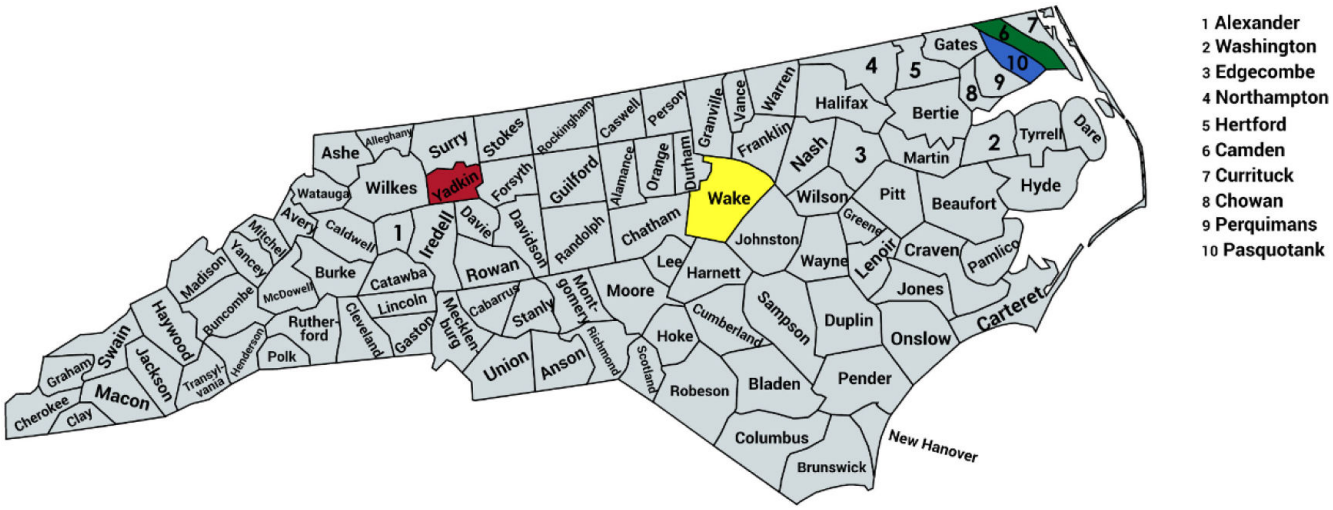

Figure 1. Map of North Carolina depicting the counties where the *Amaranthus retroflexus* populations were collected. The putative multiple herbicide–resistant *A. retroflexus* populations were collected in Camden County (green) and Pasquotank County (blue) in 2019 and 2020, respectively. The herbicide-susceptible *A. retroflexus* populations were collected in Wake County (yellow; S1) and Yadkin County (red; S2 and S3) in 2019. All *A. retroflexus* populations were collected from soybean fields.

ALS-, PPO-, and photosystem II (EC 1.10.3.9; Group 5)-inhibiting herbicides, and multiple herbicide–resistant populations have evolved (Heap 2022). *Amaranthus retroflexus* has not evolved resistance to as many herbicide groups to other *Amaranthus* spp., but this species was one of the first weeds to evolve herbicide resistance (Ferguson et al. 2001; Sibony et al. 2001; Warwick and Weaver 1980). While *A. retroflexus* has not evolved resistance to numerous herbicides compared with dioecious *Amaranthus* spp., *A. retroflexus* shares many of the same mechanism(s) of resistance, which reveals that the species has the capacity to evolve resistance to more herbicide(s) under recurrent selection pressure (Riggins and Tranel 2012; Tranel et al. 2017).

In 2019 (Camden County, NC) and 2020 (Pasquotank County, NC), complaints of control failures with ALS- and PPO-inhibiting herbicides on *A. retroflexus* were reported in conventional soybean fields. Specifically, the Camden County population was not controlled with imazethapyr (ALS) and lactofen (PPO), while the Pasquotank County population was not controlled with fomesafen (PPO) and thifensulfuron (ALS). Because multiple herbicide–resistant *A. retroflexus* is not common in North Carolina or the U.S. Southeast, the confirmation and rapid detection of plants possessing mutations that confer herbicide resistance would be crucial to minimize the spread of these biotypes (Evans et al. 2015; Laforest et al. 2022; Soteres and Peterson 2015; Wuerffel et al. 2015). Timely confirmation of herbicide-resistant plants is needed to implement effective control and cease the dispersal potential (Squires et al. 2021). Thus, the objectives of this research were (1) to determine if selected North Carolina *A. retroflexus* populations have evolved resistance to both ALS- and PPO-inhibiting herbicides, (2) to characterize the mechanism(s) of resistance in these populations, and (3) to develop an efficient detection assay to enable rapid herbicide-resistance detection.

Materials and Methods

Plant Material

Approximately 10 *A. retroflexus* plants that survived recurrent applications of ALS- and PPO-inhibiting herbicides during the 2019 and 2020 growing seasons were collected before soybean harvest in Camden County and Pasquotank County, NC, respectively (Figure 1). All collected plants exhibited herbicide injury (i.e., chemical excisions, chlorosis, leaf necrosis, and loss of apical dominance). The harvested plants were then stored at ambient air temperature (10 to 25 C) for approximately 1 mo to reduce plant moisture content while maintaining seed viability. After the storage period, the harvested plants were threshed by hand to remove seeds from the florets, and seeds were separated from plant residues using sieves and a forced-air column separator (South Dakota Seed Blower, Seedburo Equipment, Chicago, IL, USA). Seeds from individual plants were pooled for the locations where they were collected. The collected seeds were placed in a petri dish with a small amount of water and stored at 5 C for 2 wk to break dormancy. The petri dishes, without lids, were then placed into a dryer at 65 C for 48 h to reduce seed moisture content before storage (Leon et al. 2006). Approximately 10 plants from three herbicide-susceptible *A. retroflexus* populations (Wake County [S1]and Yadkin County [S2 and S3]) were collected in 2019 from soybean fields and handled as described earlier (Figure 1).

Whole-Plant Dose–Response Assay

Seeds from each *A. retroflexus* population were sown into separate 21 cm by 28 cm flats containing a 4:1 ratio of Sunshine® Mix #2 (Sun Gro Horticulture, Agawam, MA, USA) potting soil and sand with approximately 1 g of Osmocote® Flower Food Granules (14-14-14) (Scotts Company, Marysville, OH, USA). Plants were maintained in the greenhouse at 30/24 C diurnal fluctuation and topically watered to maintain field capacity water content. Sunlight was supplemented with 600 to 1,000 µmol m^{-2} s^{-1} PPFD of artificial light set to a 14-h photoperiod. Two plants were then transplanted at approximately 2 cm in height to 5-cm pots containing the same potting media with 1 g of pellet fertilizer. *Amaranthus retroflexus* plants were treated with herbicide when they reached 5 to 7.6 cm in height (4- to 6-leaf stage). Lactofen, fomesafen, imazethapyr, and thifensulfuron were applied at five rates that included the labeled adjuvants (Table 1). A nontreated control was included in each experiment. Herbicide treatments were applied with a CO_2-pressurized cabinet-mounted track

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Table 1. Herbicide and rates used in the dose–response experiments.

Target site[a]	Herbicide	Family	Rate[b] g ha^{-1}	Adjuvants[c]
ALS	Imazethapyr	Imidazolinone	0.7, 7, **70**, 700, 7000	Crop oil
	Thifensulfuron	Sulfonylurea	0.45, 4.5, **45**, 450, 4500	Ammonium sulfate, crop oil
PPO	Fomesafen	Diphenylether	14, 43, 140, **430**, 1400, 4300	Crop oil
	Lactofen	Diphenylether	22, 70, **220**, 700, 2200	Crop oil

[a]ALS, acetolactate synthase; PPO, protoporphyrinogen oxidase.
[b]Rates in bold represent a field-use rate.
[c]Crop oil and ammonium sulfate were at 1% v/v and 10 g L^{-1}, respectively.

sprayer calibrated to deliver 140 L ha^{-1} at 165 kPa with TeeJet® 8002EVS nozzles (TeeJet Technologies, Wheaton, IL, USA) 46 cm above the target weed height. Herbicide-treated *A. retroflexus* plants were not randomized until 24 h after treatment. The experimental design was completely randomized with each treatment (2 plants pot^{-1}) replicated four times. Each herbicide dose–response experiment was conducted twice, with each experimental run conducted in a different greenhouse. Only the Camden County, S1, and S2 populations were evaluated in the lactofen dose–response experiment. At 21 d after treatment, plant survival was recorded on a binomial scale where 0 equaled plant death (no green vegetative tissue) and 1 equaled plant survival (green meristem vegetative tissue).

Sanger Sequencing

Acetolactate Synthase

Seeds collected from each *A. retroflexus* population (Camden County, Pasquotank County, S1, S3) were sown and curated as described earlier. Two plants were sampled from each population. Young leaves were harvested from plants 10 to 20 cm in height (7- to 10-leaf stage), placed in microtubes, and ground into a fine powder with a micropestle. Because the *ALS* gene in *Amaranthus* spp. is ~2 kb long with a single exon, high-quality genomic DNA was extracted from the ground leaf tissue according to the protocol outlined in the Qiagen DNeasy Plant Mini Kit (Qiagen Sciences, Germantown, MD, USA). The Thermo Fisher Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to quantify DNA and normalized to 5 ng µl^{-1}. Polymerase chain reaction (PCR) conditions consisted of 23 µl of Promega GoTaq Green Master Mix (Promega Corporation, Madison, WI, USA), 25 µl of nuclease-free water, 5 µl of 10 µM forward primer, 5 µl of 10 µM reverse primer, and 10 µl of DNA for a total reaction volume of 68 µl. Thermal cycling conditions consisted of: (1) a 5-min initial denaturation at 95 C; (2) 11 cycles of a Touchdown PCR consisting of a 45-s denaturation at 95 C, 45 s of annealing at 65 to 54 C (decreasing 1 C every cycle), and a 1-min elongation at 72 C; (3) 30 cycles of standard PCR with a 45-s denaturation at 95 C, 45 s of annealing at 53 C, and a 1-min elongation at 72 C; (4) a 10-min final elongation at 72 C; and (5) a 4 C hold. Primers from McNaughton et al. (2005) were used for PCR and Sanger sequencing, with the exception of Primer 6R, which was replaced with 5′–GGAGAACAAAAYGTCRAGCA–3′, because the original Primer 6R did not amplify (Table 2). The new Primer 6R was based on the consensus ALS sequences in waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer], *A. palmeri*, Prince's feather (*Amaranthus hypochondriacus* L.), and smooth pigweed (*Amaranthus hybridus* L.) retrieved from CoGe (Lyons and Freeling 2008), designed using Primer3 default parameters to target the desired region (Untergasser et al. 2012), and ordered from Integrated DNA Technologies (Integrated DNA Technologies, Coralville, IA, USA). The new Primer 6R binds 242 bp further downstream than the original Primer 6R.

Table 2. Primer sequences used in polymerase chain reaction (PCR) amplification and Sanger sequencing for the *Amaranthus retroflexus ALS* and *PPX2* genes.

Gene	Primer	Sequence (5′→3′)
ALS	ALS 1F	CTTCAAGCTTCAACAATG
	ALS 1R	CCAGTAGCACGAGCGTAG
	ALS 2F	GATGTYCTCGTYGARGCTCT
	ALS 2R	AAYCAAAACAGGYCCAGGTC
	ALS 3F	GTGTTAGATGTTGAGGATATTCC
	ALS 3R	GCATTCCCAACATATGAA
	ALS 4F	GTGGCTAGTACTTTAATGGGG
	ALS 4R	GAGGTCAGCCATTGGCGA
	ALS 5F	ATTCCTCCGCARTATGCSATT
	ALS 5R	CAGRTAWGGTCCTGGRGTATCC
	ALS 6F	TTCTTCCGAAATCTTCCC
	ALS 6R	GGAGAACAAAAYGTCRAGCA
PPX2	PPX2 1F	GCAACATTTCTGAGCGGGAA
	PPX2 1R	GCAGCGGGATTTGAAGGTAG
	PPX2 2F	CGATGATCTTGGGCTTCGTG
	PPX2 2R	GCAAACACAGAGCCAAACCT
	PPX2 3F	TGTTCAGGAAAGTGTTGGCG
	PPX2 3R	GCGAATTGGAGCAGTGACAA
	PPX2 4F	AGAAGGAAAAGGGTGGAGGAG
	PPX2 4R	GGATAAGAACTCCGAAGCCCT
	PPX2 5F	TTGTCACTGCTCCAATTCGC
	PPX2 5R	CGGGAATGCATTGCTCCAAA
	PPX2 6F	CTACATTTGTCGGAGGAAGC
	PPX2 6R	TTATGCGGTCTTCTCATCCATCTTCAC

Following PCR, 8 µl of PCR product was run at 110 V for 1 h on a 1.25% agarose gel with 3X Biotium GelRed (Biotium, Fremont, CA, USA) and visualized by UV transillumination to ensure amplification. The remaining 60 µl of PCR product was purified using the Zymo Research DNA Clean & Concentrator Kit (Zymo Research, Irvine, CA, USA) per the manufacturer's instructions with a final elution volume of 12 µl. Purified PCR products were quantified using PicoGreen and normalized to 10 ng µl^{-1}. Sanger sequencing reactions consisted of 2 µl of purified PCR product, 1 µl of 10 µM primer, and 9 µl of nuclease-free water. Sanger sequencing was performed by the North Carolina State University Genomic Sciences Laboratory. Raw sequence data were trimmed for quality and aligned to a previously published *A. retroflexus ALS* gene sequence (GenBank accession no.: AF363369.1) (McNaughton et al. 2005), using Sequencher 5.4.6 (Gene Codes, Ann Arbor, MI, USA).

Protoporphyrinogen Oxidase

Seeds from each *A. retroflexus* population were sown and curated as described earlier. Two plants were sampled from each population. Young leaves were harvested from plants 10 to 20 cm in height, placed in microtubes, and ground into a fine powder with a micropestle. Because the *PPX2* gene in *Amaranthus* spp. is ~10 kb with 18 introns spanning ~84% of the gene length but only ~1.6 kb of coding sequence, sequencing genomic DNA was deemed inefficient. Therefore, RNA was isolated using the Sigma-Aldrich

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Table 3. Primers used in the single-nucleotide polymorphism polymerase genotyping (PCR-allele competitive extension [PACE]) assays to detect putative causal resistance mutations identified by Sanger sequencing in the *Amaranthus retroflexus ALS* and *PPX2* genes.

Genes	Primer	Sequence (5′→3′)
ALS	His-197-Pro common	TGCTGATGCACTTCTTGACT
	His-197-Pro wild type	GAAGGTGACCAAGTTCATGCTAGTACCAATCATACGCCGGG
	His-197-Pro resistant mutant	GAAGGTCGGAGTCAACGGATTTACCAATCATACGCCGGT
	Trp-574-Leu common	GGTATGTATGTGCCCGGTTAGCTTT
	Trp-574-Leu wild type	GAAGGTGACCAAGTTCATGCTAATCAACATTTAGGTATGGTTGTTCAATG
	Trp-574-Leu resistant mutant	GAAGGTCGGAGTCAACGGATTCAATCAACATTTAGGTATGGTTGTTCAATT
PPX2	Arg-98-Gly common	TAGTAGCACCGGAAGACCAT
	Arg-98-Gly wild type	GAAGGTGACCAAGTTCATGCTAGTTGCCAATTTCACAAAATAAAA
	Arg-98-Gly resistant mutant	GAAGGTCGGAGTCAACGGATTAGTTGCCAATTTCACAAAATAAAG

Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St Louis, MO, USA) and converted to cDNA using the Promega ImProm-II™ Reverse Transcription System (Promega Corporation, Madison, WI, USA), both per the manufacturer's instructions. PCR and Sanger sequencing were as described earlier for the *ALS* gene. Primers were designed for PPX2 based on two partial *A. retroflexus* coding sequences (GenBank accession nos.: MK716317 and MK71618), a partial *A. palmeri* coding sequence (GenBank accession no.: KY882137) (Giacomini et al. 2017), and the *A. palmeri* genomic sequence (Montgomery et al. 2020) using Primer3 (Table 2). MK716317 was used as the reference sequence alignment in Sequencher.

Single-Nucleotide Polymorphism Genotyping

Single-nucleotide polymorphism (SNP) genotyping assays (PCR-allele competitive extension [PACE]) (3cr Bioscience, Essex, UK) were designed for the SNPs identified via Sanger sequencing using the "Allele-Specific Primers and Allele-Flanking Primers" option of BatchPrimer3 (Magoč and Salzberg 2011). The following changes were made to the default parameters: Minimum Primer Tm = 55, Optimal Primer Tm = 57, Maximum Primer Tm = 60, Max Tm Difference = 2, Minimum Product Size = 50 bp, Optimum Product Size = 50 bp, and Maximum Product Size = 100 bp (Hulse-Kemp et al. 2015). Primers used are listed in Table 3. The three primers comprising each PACE assay were combined per the manufacturer's instructions (46 µl of nuclease-free water, 30 µl of 100 µM common primer, and 12 µl of 100 µM of each allele-specific primer). All three assays were designed so that the putative resistance mutation allele always attracted the HEX fluorophore, while the susceptible allele always attracted the FAM fluorophore.

DNA Isolation and SNP Genotyping

Seeds were sowed and curated as described earlier. The DNA was extracted from tissue collected from 32 plants (10 to 20 cm in height; 7- to 10-leaf stage) from each *A. retroflexus* population as described earlier. Eight pseudo-F_1 plants were created by mixing a 1:1 ratio of the DNA from the S1 population with the each of other *A. retroflexus* populations to determine whether the PACE assay could successfully detect plants that were heterozygous for each mutation. The PACE thermal cycling conditions followed the manufacturer's instructions, except that 33 cycles were used in the third step instead of 30. Following PCR, plates were read with a BMG Labtech GmbH PHERAstar (BMG Labtech, Incorporate, Cary, NC, USA), and data were analyzed in the North Carolina State University Peanut Breeding and Genetics SNP caller (Andres and Dunne 2022; Dunne 2022). Data were reported as the normalized fluorescence (Rn) of HEX and FAM to the internal ROX standard. Each PACE assay was tested with 32 plants (10 to 20 cm in height) from all tested *A. retroflexus* populations along with two negative controls for a total of 202 samples.

Crude DNA

Seeds were sown and curated as described earlier. Fresh leaf tissue was collected from 36 plants (10 to 20 cm in height; 7- to 10-leaf stage) from each *A. retroflexus* population to more broadly and rapidly determine the presence of the mutations in each population. Twelve negative controls were included to bring the total sample number to 192. Tissue was placed in 96–round well microplates (Spex Sample Prep, Metuchen, NJ, USA) containing a 4-mm stainless steel bead (Spex Sample Prep), capped (Spex Sample Prep), and placed in liquid N_2. Tissue was ground at 1,350 rpm for 15 s in a Spex Sample Prep 1600 MiniG centrifuge (Spex Sample Prep). After grinding, 50 µl of 100 mM NaOH, 2% Tween 20 (Sigma-Aldrich, St Louis, MO, USA) was added to each well, and plates were vortexed vigorously for 15 s. Microplates were then placed in an oven at 65 C for 10 min. Two hundred microliters of 100 mM Tris-HCl, 2 mM EDTA was then added to each well followed by 200 µl of nuclease-free water. Each microplate was shaken vigorously for 15 s and then centrifuged until reaching 3,000 rpm. Thirty microliters of supernatant was removed and added to 120 µl of deionized water. One microliter of extracted DNA with normalization and quantification was used for PCR as described earlier, except that 27 cycles were used in the third PCR step. The entire process took less than 4 h and cost ~\$0.25 sample^{-1} (\$50 total).

Statistical Analysis

Whole-Plant Dose–Response Assay

Plant survival data were subjected to ANOVA using the PROC GLIMMIX in SAS v. 9.4 (Statistical Analysis Software, Cary, NC, USA). *Amaranthus retroflexus* population and rates were considered fixed effects, while the experimental run and repetition were considered random effects.

Dose–response curves for plant survival were fit with a three-parameter log-logistic equation:

$$y = a/[1 + (x / x0)b] \quad [1]$$

where a is the upper asymptote, x is the herbicide rate, $x0$ equals the LD_{50} (lethal dose to control 50% of the population [survival]) rate, and b is the slope at $x0$. The LD_{50} of each *A. retroflexus* population was derived using the regression equations. The resistance ratio (R/S) was calculated by dividing the LD_{50} of the putative herbicide-resistant populations by the LD_{50} of the herbicide-susceptible populations.

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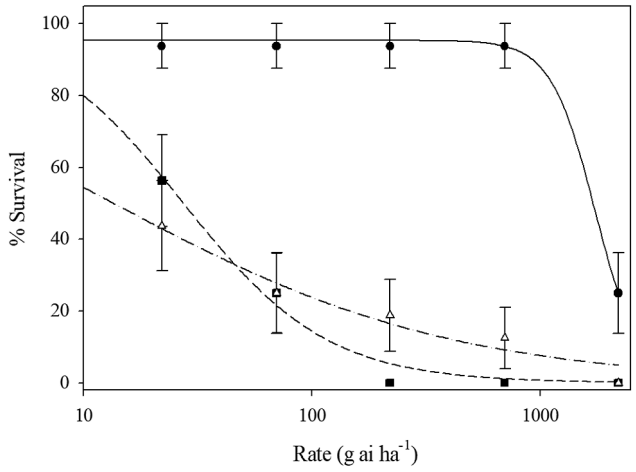

Figure 2. Dose–response curve fit to a three-parameter log-logistic equation for plant survival of the *Amaranthus retroflexus* populations (putative resistant: Camden County; susceptible: Wake County [S1] and Yadkin [S2] County) treated with lactofen. Error bars represent the standard error of the mean. Camden County: filled circles; Wake County: open triangles; Yadkin County: filled squares.

Results and Discussion

Whole-Plant Dose–Response

Lactofen

Plant survival was affected by *A. retroflexus* population and lactofen rate ($P < 0.0001$) with a significant interaction ($P < 0.0001$); thus, plant survival data were analyzed separately by *A. retroflexus* population and rate. The lactofen LD_{50} values were 1,745, 14, and 28 g ai ha^{-1} for the Camden County, S1, and S2 populations, respectively (Figure 2; Table 4). The LD_{50} for the Camden County population is significantly higher than the maximum labeled lactofen rate (220 g ha^{-1}) applied to row crops in North Carolina. The calculated R/S values were 124 and 63 for the Camden County population when compared with the S1 and S2 populations, respectively. The high level of differential susceptibility observed in this experiment was similar to that of other experiments confirming PPO-inhibiting herbicide-resistant *A. retroflexus* populations (Du et al. 2021; Wang et al. 2019).

The Pasquotank County population was collected after the initial lactofen dose–response assay, but a replicated experiment was conducted to determine the susceptibility compared with the S1 population (the most susceptible population in the initial assay). The lactofen LD_{50} for the Pasquotank County population was 50 g ha^{-1}, and the S1 population was controlled with all tested rates (data not shown). While the results of experiment cannot be directly compared with the initial dose–response assay, the Pasquotank County population survived lactofen rates that were lethal to the S1 population.

Fomesafen

Plant survival was affected by *A. retroflexus* populations and fomesafen rate ($P < 0.0001$) with a significant interaction ($P < 0.0001$); thus, plant survival data were analyzed across *A. retroflexus* population and rates. All Camden County plants survived the tested fomesafen rates; thus, neither LD_{50} values nor R/S could be calculated (Figure 3; Table 4). This result suggests that the Camden County population is significantly less susceptible to fomesafen compared with other confirmed PPO-inhibiting herbicide-resistant *A. retroflexus* populations (Du et al. 2021; Wang et al. 2019).

The LD_{50} value for the Pasquotank County population was 595 g ha^{-1}, significantly higher than the rate of fomesafen (290 g ai ha^{-1}) commonly applied to row crops in North Carolina (Figure 3; Table 4). The LD_{50} values were 36, 94, and 174 g ha^{-1} for the S1, S2, and S3 populations, respectively (Figure 3; Table 4). The R/S values ranged from 3.4 to 17 when herbicide-susceptible populations were compared with the Pasquotank County population (Table 4). The R/S values from these experiments were lower than those of confirmed target-site PPO-inhibiting herbicide-resistant *A. retroflexus* populations from China (Du et al. 2021; Wang et al. 2019). The R/S values from these experiments were also lower than those of confirmed PPO-inhibiting herbicide-resistant *A. palmeri* and *A. tuberculatus* populations facilitated by metabolism (Obenland et al. 2019; Varanasi et al. 2018). Despite the variable R/S across herbicide-susceptible *A. retroflexus* populations, the R/S values were always greater than 1 for the Pasquotank County population, suggesting the evolution of resistance to fomesafen (Burgos 2015).

Imazethapyr

Plant survival was affected by *A. retroflexus* populations and imazethapyr rates ($P < 0.0001$) with a significant interaction ($P < 0.0001$); thus, plant survival data were analyzed across *A. retroflexus* population and rate. All Camden County plants survived the tested imazethapyr rates; thus, neither LD_{50} values nor R/S could be calculated (Figure 4; Table 4). High survival of other ALS-inhibiting herbicide-resistant *A. retroflexus* populations when treated with imazethapyr has been documented (Scarabel et al. 2007). The LD_{50} value for the Pasquotank County population was 95 g ai ha^{-1}, higher than the rate of imazethapyr (70 g ai ha^{-1}) commonly applied to row crops in North Carolina (Figure 4; Table 4). The LD_{50} values were 0.02, 3, and 2.7 g ha^{-1} for the S1, S2, and S3 populations, respectively (Figure 4; Table 4). The R/S values ranged from 32 to 4,750 when herbicide-susceptible populations were compared with the Pasquotank County population (Table 4). Similarly high levels of differential susceptibility have been documented for other ALS-inhibiting herbicide-resistant *A. retroflexus* populations (Chen et al. 2015; Sibony et al. 2001).

Thifensulfuron

Plant survival was affected by *A. retroflexus* populations and imazethapyr rates ($P < 0.0001$) with a significant interaction ($P < 0.0001$); thus, plant survival data were analyzed across *A. retroflexus* population and rate. The LD_{50} values for the Camden County and Pasquotank County populations were >4,500 and 2,771 g ai ha^{-1}, respectively; these rates are significantly higher than the maximum labeled rate of thifensulfuron (45 g ai ha^{-1}) (Figure 5; Table 4). The LD_{50} values were 0.3, 2, 0.3 g ai ha^{-1} for the S1, S2, and S3 populations, respectively (Figure 4; Table 4). The R/S values were greater than 1,000 when herbicide-susceptible populations were compared with the Camden County and Pasquotank County populations (Table 4). These results were similar to those that have been reported for ALS-inhibiting herbicide-resistant *A. retroflexus* populations, as described earlier.

Sanger Sequencing

Acetolactate Synthase

The full-length 2,010-bp coding sequence of the *ALS* gene was amplified and sequenced from all eight individual plants. Neither the S1 nor S3 population had nonsynonymous mutations relative to the susceptible reference sequence (GenBank accession

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Table 4. Parameter estimates from the three-parameter log-logistic equation for plant survival of the *Amaranthus retroflexus* populations treated with lactofen, fomesafen, imazethapyr, and thifensulfuron.[a]

Herbicide	Population[b]	Regression parameters[c]							
		a	*x*0	*b*	r^2	LD_{50} (SE)	R/S_{Wake}	$R/S_{Yadkin\ (A)}$	$R/S_{Yadkin\ (B)}$
Lactofen	Camden County	95.3	1,745.1	4.5	0.99	1,745 (188)	124	63	—
	Wake County	100	13.5	0.6	0.99	14 (5)			
	Yadkin (A) County	99.7	27.5	1.4	0.99	28 (3)			
Fomesafen	Camden County	—[d]	—	—	—	—	NA	NA	NA
	Pasquotank County	101	595.2	1.3	0.93	595 (160)	17	6	3.4
	Wake County	98.5	36.1	0.6	0.89	36 (24)			
	Yadkin (A) County	99.1	94.1	0.9	0.94	94 (38)			
	Yadkin (B) County	93.3	173.6	1.8	0.98	174 (20)			
Imazethapyr	Camden County	—	—	—	—	NA	NA	NA	NA
	Pasquotank County	102.3	94.8	1	0.99	95 (19)	4750	32	35
	Wake County	99.6	0.02	0.1	0.81	0.02 (0.1)			
	Yadkin (A) County	99.7	3	0.4	0.99	3 (0.9)			
	Yadkin (B) County	99.3	2.7	0.7	0.99	2.7 (0.5)			
Thifensulfuron	Camden County	100.2	7,642.2	1	0.99	>4,500	>15,000	>2,250	>15,000
	Pasquotank County	100.3	2,771.4	1.1	0.99	2,771 (66)	9,236	1,386	9,236
	Wake County	99.9	0.3	0.4	0.99	0.3 (0.01)			
	Yadkin (A) County	99.9	1.7	2	0.99	2 (0.2)			
	Yadkin (B) County	99.7	0.3	0.6	0.99	0.3 (0.1)			

[a]Abbreviations: LD_{50}, lethal dose (g ha^{-1}) to control 50% of the population; R/S, resistance ratio (LD_{50} resistant population:LD_{50} susceptible population); NA, not achieved.
[b]Putative resistant: Camden County and Pasquotank County; susceptible: Wake County (S1) and Yadkin County (A [S2] and B [S3]).
[c]*a* is the upper asymptote, *x*0 equals the LD_{50}, and *b* is the slope at *x*0.
[d]Dashes indicate that all plants survived the tested rates of the respective herbicide.

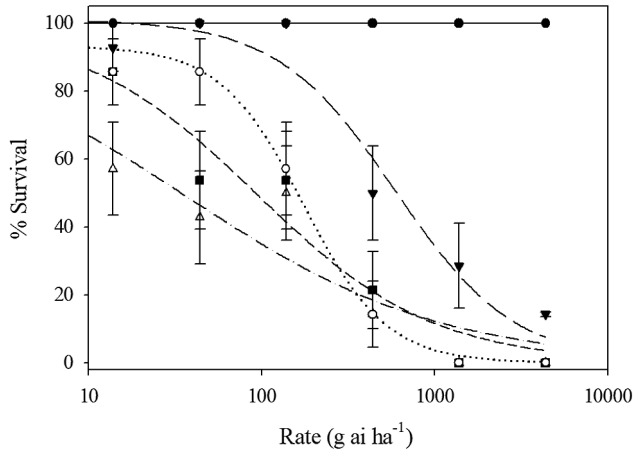

Figure 3. Dose–response curve fit to a three-parameter log-logistic equation for plant survival of the *Amaranthus retroflexus* populations (putative resistant: Camden County and Pasquotank County; susceptible: Wake County [S1] and Yadkin County [A (S2)] and [B (S3)]) treated with fomesafen. Error bars represent the standard error of the mean. Camden County: filled circles; Pasquotank County: upside-down filled triangles; Wake County: open triangles; Yadkin County (A): filled squares; Yadkin County (B): open circles.

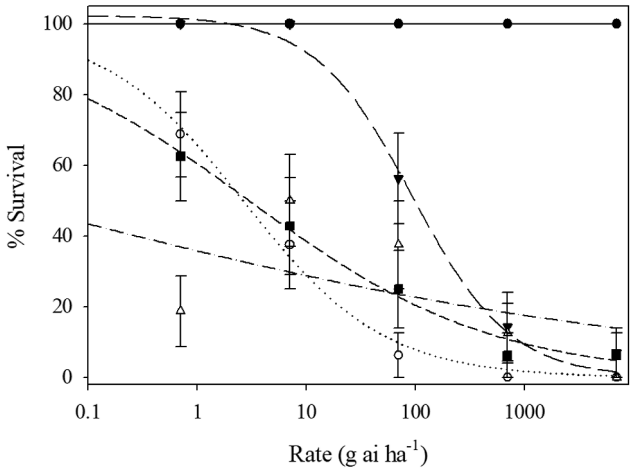

Figure 4. Dose–response curve fit to a three-parameter log-logistic equation for plant survival of the *Amaranthus retroflexus* populations (putative resistant: Camden County and Pasquotank County; susceptible: Wake County [S1] and Yadkin County [A (S2)] and [B (S3)]) treated with imazethapyr. Error bars represent the standard error of the mean. Camden County: filled circles; Pasquotank County: upside-down filled triangles; Wake County: open triangles; Yadkin County (A): filled squares; Yadkin County (B): open circles.

no.: AF363369) (McNaughton et al. 2005). Relative to the reference sequence, the Camden County population had a single nonsynonymous mutation, a GàT transversion at position 1718 of the coding sequence resulting in an amino acid change from tryptophan to leucine at amino acid position 574 (Trp-574-Leu) of the resulting gene sequence (Supplemental Figure 1). Meanwhile, relative to the reference sequence, the Pasquotank County population also had a single nonsynonymous mutation, a CàA transversion at position 575 of the coding sequence resulting in an amino acid change from proline to histidine at amino acid position 197 (Pro-197-His) of the resulting gene sequence (Supplemental Figure 1). Both these mutations confer high levels of resistance to all families of ALS-inhibiting herbicides by decreased binding affinity (Tranel and Wright 2002; Yang et al. 2018). While this mutation has not been reported to date within *Amaranthus* spp., it is known to confer resistance to all families of ALS-inhibiting herbicides in other weed species (Tranel et al. 2022). Both the Camden County and Pasquotank County population exhibited cross-resistance to imazethapyr and thifensulfuron (Figures 4 and 5; Table 4). However, previous research has shown that the imidazolinone herbicides can be more

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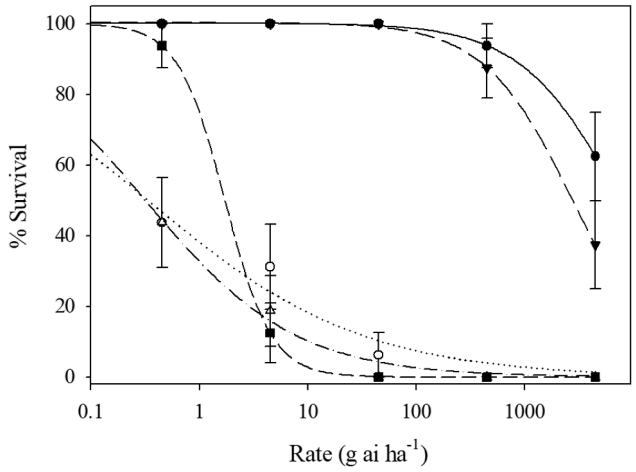

Figure 5. Dose–response curve fit to a three-parameter log-logistic equation for plant survival of the *Amaranthus retroflexus* populations (putative resistant: Camden County and Pasquotank County; susceptible: Wake County [S1] and Yadkin County [A (S2)] and [B (S3)]) treated with thifensulfuron. Error bars represent the standard error of the mean. Camden County: filled circles; Pasquotank County: upside-down filled triangles; Wake County: open triangles; Yadkin County (A): filled squares; Yadkin County (B): open circles.

efficacious on broadleaf weeds with a mutation at this position due to binding affinity (Li et al. 2017; Yang et al. 2018; Yu et al. 2003). This result was similar for the Pasquotank County population being more highly controlled by imazethapyr compared with thifensulfuron (Table 4).

PPO

Based on the four available reference sequences, the *A. retroflexus PPX2* gene was predicted to stretch 1,518 bp and produce a protein 505 amino acids long. The primers used here reliably amplified and sequenced 1,490 bp stretching from position 18 to 1508 in the coding sequence or 7 to 503 in the protein sequence of the predicted *A. retroflexus PPX2* gene. Both the MK716317 reference and the Camden County population exhibited an AàG transition at position 292 of the coding sequence in the *PPX2* gene. This resulted in an amino acid substitution from arginine to glycine at amino acid position 98 (Arg-98-Gly) of the resulting gene sequence (Supplemental Figure 1). This mutation has been previously documented to confer resistance to PPO herbicides in *A. palmeri*, *A. retroflexus*, and common ragweed (*Ambrosia artemisiifolia* L.) (Dayan et al. 2018; Du et al. 2021; Giacomini et al. 2017; Rousonelos et al. 2012). The Arg-98-Gly is considered homologous to the Arg-128-Gly mutation, with the difference attributed to whether or not a 30–amino acid targeting/signal peptide is ascribed to the N-terminal end of the protein (Du et al. 2021; Huang et al. 2020; Nie et al. 2019; Patzoldt et al. 2006; Varanasi et al. 2018). Five other nonsynonymous mutations were detected by Sanger sequencing, although none of these five were unique to *A. retroflexus* populations that displayed resistance to PPO-inhibiting herbicides in the whole-plant dose–response assay. One of these mutations was a Asp-384-Asn found in the Camden County and S2 populations. The other four mutations (Trp-214-Lys, Val-411-Ile, Ala-423-Glu, and Asn-446-Asp) were found in the S1 population and the MK716317 reference. Because these mutations are present in herbicide-resistant and herbicide-susceptible populations, it is unlikely they are involved in the resistance mechanism.

SNP Genotyping Assays

All three PACE assays performed as expected, confirming the presence or absence of the three mutations (*ALS*: Trp-574-Leu, Pro-197-His; *PPX2*: Arg-98-Gly) in all sampled plants from each population as determined by Sanger sequencing, respectively (Figure 6). This suggests each mutation is widespread within the population and is likely to be driven to fixation under continuous selection. All pseudo-F_1 plants from the Camden–S1 and Pasquotank–S1 combinations successfully grouped as heterozygous for the respective mutations (Figure 6). All plants from the populations that did not exhibit a specific mutation from Sanger sequencing grouped as homozygous for the wild-type allele (Figure 6).

SNP Genotyping Assays—Crude DNA

The Trp-574-Leu marker identified all 36 Camden County plants as possessing this mutation (Figure 7). Two plants from the S3 population clustered intermediately, and one negative control appears to have been contaminated with DNA from the Camden County population. However, the mutation was not found definitively in any plant from any of the other *A. retroflexus* populations (Figure 7). It is possible that due to a greater starting concentration of genomic DNA in the non-normalized crude DNA, some of the negative controls began to produce Rn_FAM signal and migrate along the *x* axis. However, the negative controls remained below the group formed by the four populations lacking the mutation.

The Pro-197-His marker identified all 36 Pasquotank County plants as possessing this mutation (Figure 7). One plant from the S3 population appears to carry this mutation; however, it had the lowest Rn_HEX/Rn_FAM ratio of any individual in the resistant cluster, indicating this may be the result of contamination. The mutation was not found in any other plant from any of the other four *A. retroflexus* populations, including the previously non-tested S2 population. (Figure 7). As with the Trp-574-Leu assay, the negative controls began to produce Rn_FAM signal and migrate along the *x* axis but continued to group below the four populations lacking the mutation.

The Arg-98-Gly marker identified 31 out of the 36 sampled Camden County plants as possessing this mutation (Figure 7). The remaining five plants failed to amplify, implying that the assay can detect *A. retroflexus* plants carrying this mutation with at least 85% accuracy. No other plants were identified as possessing this mutation (Figure 7). While 11 of the 12 negative controls failed, as expected, the clustering between resistant and susceptible plants was not as pronounced as in the previous two *ALS* assays. Furthermore, more plants from all populations failed to amplify, which likely indicates the wild-type primers may need to be redesigned to increase affinity.

The PACE assays enable rapid detection of specific DNA mutations that confer herbicide resistance without the need to conduct Sanger sequencing. This is especially important for genes with large base pair sizes (i.e., *PPX2*), because RNA isolation and conversion to cDNA can be circumvented, saving time and money. Dose–response assays are a proven way to confirm herbicide-resistant weeds, but the assays can take several weeks to months to complete and may not provide a timely answer to farmers if the weed is herbicide resistant (Burgos 2015; Burgos et al. 2013). Dose–response assays also require a significant amount of labor and space to conduct. The PACE assays significantly reduce the time needed to confirm whether a weed has evolved resistance conferred by a specific mutation.

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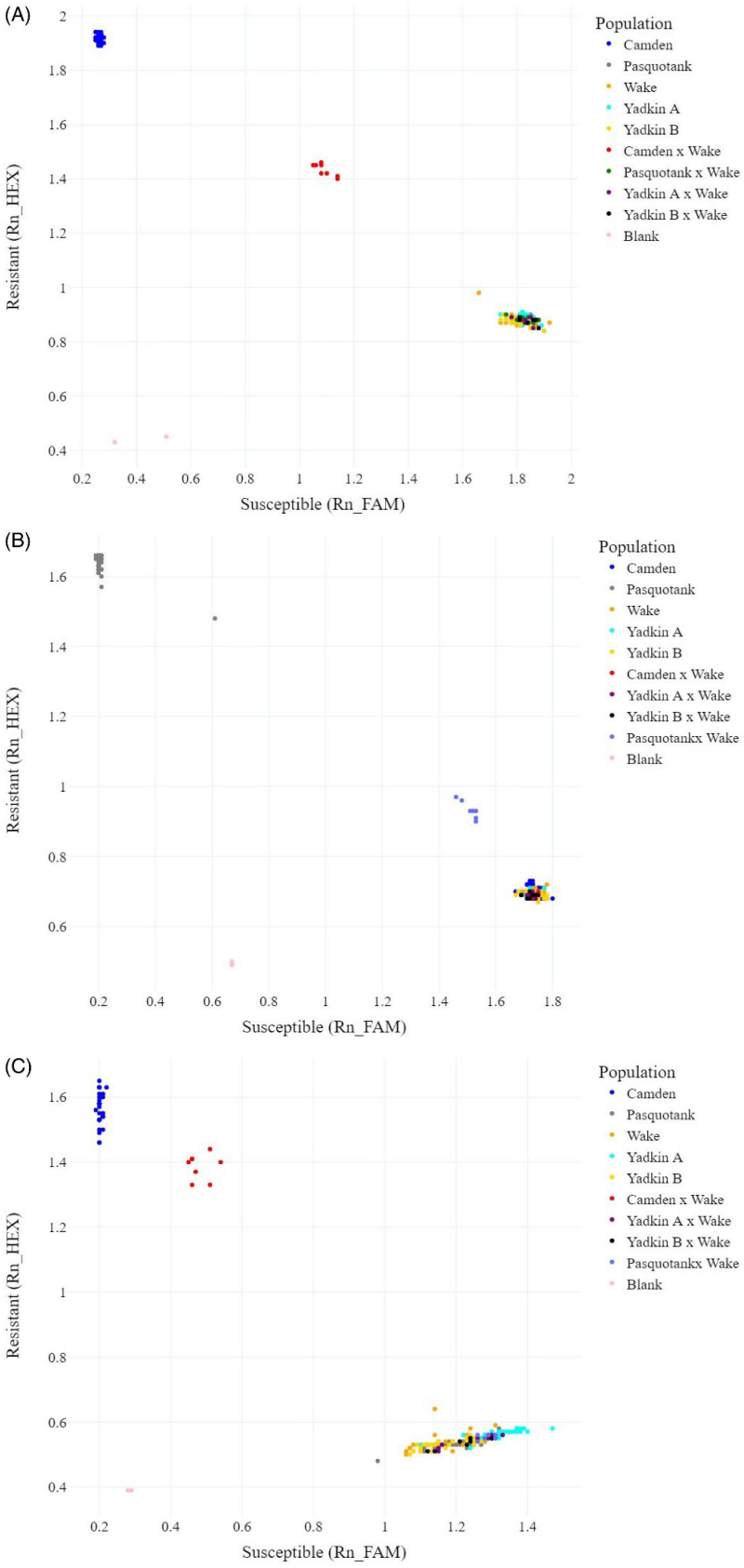

Figure 6. Graphical representation of the PCR-based genotyping (PCR-allele competitive extension [PACE]) assays completed with high-quality DNA for the Trp-574-Leu (A), Pro-197-His (B) mutation in the *ALS* gene, and the Arg-98-Gly (C) mutation in the *PPX2* gene of *Amaranthus retroflexus*. Thirty-two plants per population were sampled.

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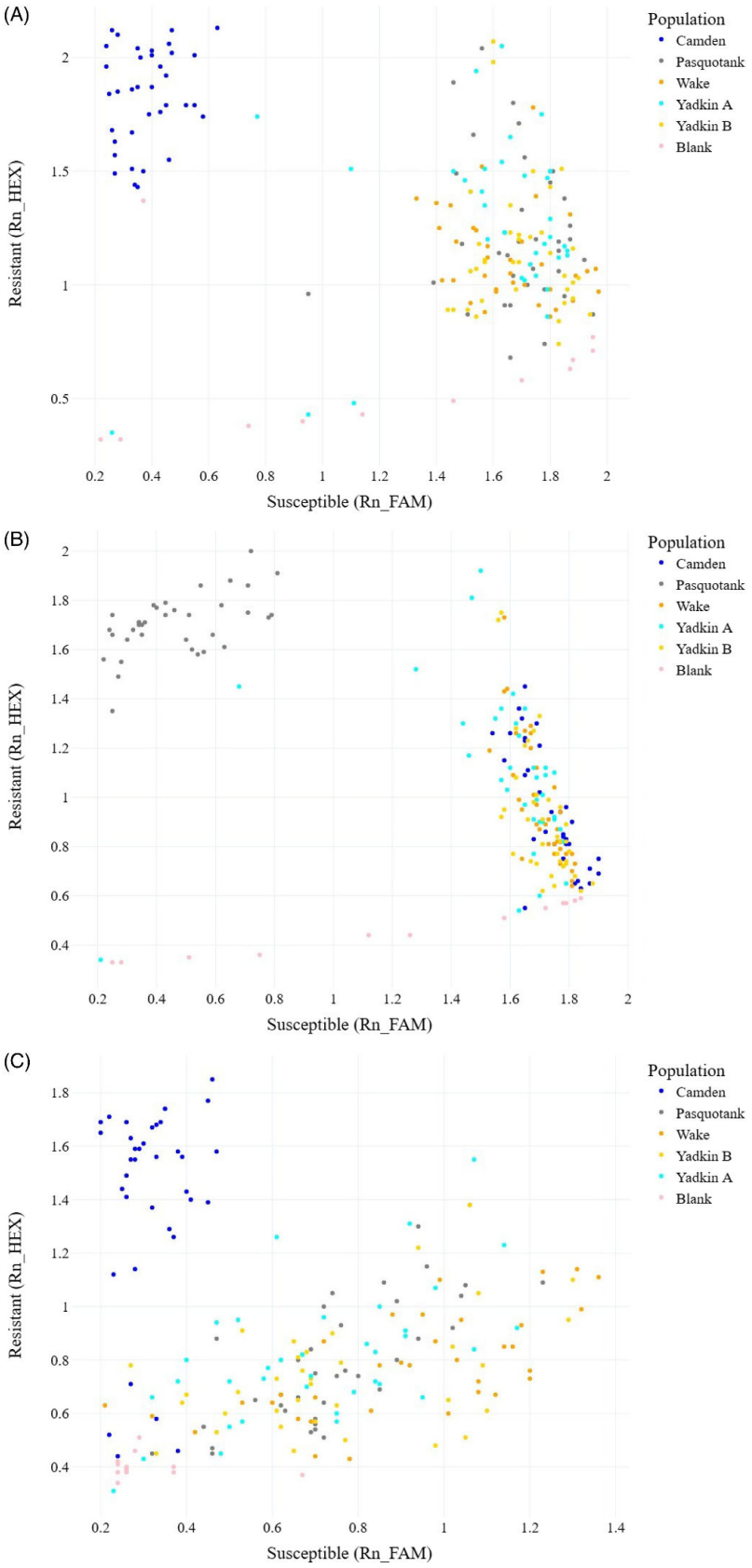

Figure 7. Graphical representation of the polymerase chain reaction (PCR)-based genotyping (PCR-allele competitive extension [PACE]) assays completed with crudely extracted DNA for the Trp-574-Leu (A), Pro-197-His (B) mutation in the *ALS* gene, and the Arg-98-Gly (C) mutation in the *PPX2* gene of *Amaranthus retroflexus*. Thirty-six plants were sampled per population.

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Additionally, while not as accurate as the high-quality DNA assay, the crude DNA PACE assay can serve as a rapid and low-cost assay to confirm whether an *A. retroflexus* population has evolved herbicide resistance facilitated by a specific target-site mutation in the tested genes. Crudely extracted DNA SNP genotyping assays have been utilized to confirm herbicide resistance in other species with success (Délye et al. 2002: Tian and Darmency 2006; Wuerffel et al. 2015). By extension, many *A. retroflexus* plants putatively possessing this mutation could be genotyped rapidly at low cost, thus facilitating timely implementation of effective control to slow the spread of these isolated biotypes into other regions. This assay could be beneficial for high-throughput diagnostics for samples collected and submitted by County Extension agents during the growing season for other weed species and genes conferring herbicide resistance (Laforest et al. 2022; Squires et al. 2021; Tataridas et al. 2022). More optimization of the crude DNA assay wild-type primers may be needed to ensure no false positives are being reported (Délye et al. 2002; Tian and Darmency 2006). More primers will have to be designed as more mutation(s) are documented and for the assay to be adapted to other species.

The evolution of two distinct ALS- and PPO-inhibiting herbicide-resistant *A. retroflexus* populations represents the first case of PPO-inhibiting herbicide-resistant *A. retroflexus* and of this particular herbicide-resistance profile in the North America. While resistance to the ALS-inhibiting herbicides is facilitated by mutations in the target site, the Camden County and Pasquotank County *A. retroflexus* exhibited two distinct mutations. Additionally, the Pro-197-His mutation in the *ALS* gene of the Pasquotank County *A. retroflexus* population has not been documented in any *Amaranthus* spp. to date, but is not unexpected due to the plethora of species confirmed to carry this mutation (Beckie and Tardif 2012; Tranel et al. 2022). The Pasquotank County population represents the third species to evolve non–target site resistance to PPO-inhibiting herbicides (Obenland et al. 2019; Varanasi et al. 2018). This finding is concerning, as non–target site resistance mechanisms can confer resistance to non-related herbicides (Yu and Powles 2014). Further research is needed to determine the mechanism of resistance to the PPO-inhibiting herbicides in the Pasquotank County *A. retroflexus* population and whether the plants exhibit resistance to non-related herbicides. As previously stated, *A. retroflexus* has historically been easy to control with herbicides; however, the complexity of controlling weeds in crops that rely heavily on the ALS- and PPO-inhibiting herbicides will increase.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/wsc.2023.4

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