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Confirmation of a five-way herbicide-resistant *Amaranthus tuberculatus* population in North Carolina

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Abstract

Amaranthus tuberculatus (waterhemp) is a pervasive weed of the Mid-west and -south United States and is not native to North Carolina but infestations in crop fields have been reported recently. *Amaranthus tuberculatus* has evolved resistance to seven herbicide groups and multiple herbicide-resistant populations are common where the species is native. The reported *A. tuberculatus* infestations in North Carolina have not been controlled with herbicides but no formal herbicide resistance characterisation has been conducted to date. Glasshouse dose–response experiments were conducted to determine the susceptibility of a population collected from Surry County, North Carolina to commonly applied postemergence herbicides compared to a herbicide-susceptible population collected from Story County, Iowa. The Surry County population survived labelled rates of imazethapyr, atrazine, glyphosate, fomesafen, and mesotrione; the Story County population was controlled with these herbicides. Further, 2,4-D, dicamba, and glufosinate effectively controlled the Surry and Story County populations. Molecular sequencing assays were subsequently conducted to determine if altered target sites facilitated resistance in the *acetolactate synthase* (*ALS*), *5-enolpyruvylshikimate-3-phosphate synthase* (*EPSPS*), *photosystem II* (*psbA*), and *protoporphyrinogen oxidase* (*PPX2*) genes. The Surry County population carried a $Trp_{574}Leu$ and ΔGly_{210} mutations in the *ALS* and *PPX2* gene, respectively. No mutations that would confer resistance were found in the *EPSPS* or *psbA* gene for either population. The results of both experiments provide evidence that a five-way herbicide-resistant *A. tuberculatus* population has encroached North Carolina. More research is needed to determine the mechanisms of resistance to atrazine, glyphosate, and mesotrione.

KEYWORDS
herbicide resistance, invasive species, weed management

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1 | INTRODUCTION

Amaranthus tuberculatus (Moq.) J.D. Sauer (waterhemp) is a small-seeded broadleaf that is native to the Midwest United States and historically inhabited riparian areas (Sauer, 1955). Changes in tillage and herbicide practices in the 1980s facilitated *A. tuberculatus* encroachment into crop fields (Buhler, 1995; Young, 2006). The species can reduce crop yield up to 75% if not controlled (Bensch et al., 2003; Steckel & Sprague, 2004). The competitiveness of the species is facilitated by prolonged emergence, rapid apical growth, and high fecundity (Hartzler et al., 2004; Wu & Owen, 2014). Since the encroachment to crop fields in the 1980s, *A. tuberculatus* has evolved resistance to seven herbicide groups and multiple herbicide-resistant populations are common (Heap, 2023; Shergill, Barlow, et al., 2018). The multiple herbicide-resistant populations do not incur a fitness penalty; thus, removing herbicide applications does not result in populations returning to being herbicide-susceptible (Jones et al., 2019; Wu et al., 2017).

Weed seeds can be moved long distances by animals, humans, and machinery (Benvenuti, 2007; Farmer et al., 2017). Non-native weed seeds, including *Amaranthus palmeri* S. Watson (Palmer amaranth), have been documented to be moved through various sources and become established far away from its original habitat (Hartzler & Anderson, 2016). Infestations of *A. tuberculatus* in North Carolina were first reported in 2015 (Everman, personal communication). Since *A. tuberculatus* is not native to North Carolina, the seeds were presumably introduced by agricultural machinery purchased in the Midwest United States (Everman, personal communication). The introduction of *A. tuberculatus* to North Carolina is a potential problem as the species can displace native species and generate new weed management problems (Trucco et al., 2009).

The encroachment of *A. tuberculatus* represents a new weed problem for North Carolina (Eastern, Mid-Atlantic, and Southeastern United States) farmers that must be controlled along other weeds of economic importance (Webster & Nichols, 2012). Many weeds evolved resistance to acetolactate synthase (ALS [EC 2.2.1.6]; Herbicide Group [HG] 2)- and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS [EC 2.5.1.19]; HG 9)-inhibiting herbicides in North Carolina (Heap, 2023; Mahoney et al., 2020). While resistance to photosystem II (PSII [EC 1.10.3.9]; HG 5)-, glutamine synthetase (EC 6.3.1.2; HG 10)-, protoporphyrinogen oxidase (PPO [EC 1.3.3.4]; HG 14)-, 4-hydroxyphenylpyruvate dioxygenase (HPPD [1.13.11.27]; HG 27)-inhibiting herbicides have been confirmed in North Carolina, the populations resistant to these herbicides are not widespread (Heap, 2023). Timely characterisation of the herbicide resistance profiles of introduced *A. tuberculatus* populations would inform and allow for sound recommendations for management with herbicides. The objective of the present research was to characterise the herbicide resistance profile of a selected *A. tuberculatus* population that has established recently in North Carolina.

2 | MATERIALS AND METHODS

Plant material. Seed heads from approximately 10 putative multiple herbicide-resistant *A. tuberculatus* plants were arbitrarily collected from a soyabean field in Surry County, North Carolina in the fall 2018; the first year the field had been recognised as infested with the species. A herbicide-susceptible *A. tuberculatus* population was collected from a wooded field edge at an Iowa State University research farm in Story County, Iowa in the fall 2006. This *A. tuberculatus* population has been used since the 1990s to represent a herbicide-susceptible population in previous research (Hinz & Owen, 1997; Jones et al., 2019). The seeds from each population were placed in a petri dish with a small amount of water and stored at 5°C for 2 weeks to break dormancy. The petri dishes without lids were then placed in a dryer at 65°C for 48 h to reduce their moisture content before storage (Leon et al., 2006).

Whole-plant dose–response assay. Seeds from the Surry and Story County populations were sown into separate 22 cm by 28 cm flats containing a 4:1 ratio of Sunshine Mix #2 potting soil and sand with approximately 1 g of Osmocote Flower Food Granules (14-14-14) (The Scotts Company LLC, Marysville, OH, USA). Plants were maintained in the greenhouse at 30/24°C diurnal temperature fluctuation and topically watered to maintain field capacity water content. Sunlight was supplemented with 600–1000 μmol m^{-2} s^{-1} PFFD of artificial light set to a 14-h photoperiod. Two plants were then transplanted when approximately 2 cm in height to 5-cm pots containing the same potting media with 1 g of the same pellet fertiliser.

The herbicides and labelled rates evaluated are listed in Table 1. Herbicide rates included the one (1×), two (2×) and four times (4×) the labelled rate for each respective herbicide. A nontreated control was included from each population for comparison. Treatments were applied when the plants reached 7.6–10 cm in height (4- to 6-leaf) with a CO_2-pressurised cabinet-mounted track sprayer calibrated to

TABLE 1 Herbicides labelled rates used in the dose–response assay to determine the resistance profile of the putative multiple herbicide-resistant (Surry County, NC) and the herbicide-susceptible (Story County, IA) *Amaranthus tuberculatus* populations.

Herbicide	Labelled rate (g ai or ae ha^{-1})	Herbicide group
2,4-D	550	4
Atrazine[a]	2225	5
Dicamba	560	4
Fomesafen[a]	280	14
Glufosinate[b]	450	10
Glyphosate[b]	1264	9
Imazethapyr[a]	70	2
Mesotrione[bc]	105	27

[a]Included concentrated crop oil at 1% v v^{-1}.
[b]Included ammonium sulphate at 10 g L^{-1}.
[c]Included methylated seed oil at 1% v v^{-1}.

deliver 140 L ha^{-1} at 165 kPa with Teejet 8002EVS nozzles (TeeJet Technologies, Wheaton, IL, USA) 46 cm above the target weed height. Herbicides were applied with labelled adjuvants based on manufacturer specifications (Table 1). The experiment was a completely randomised design and was conducted twice in separate glasshouses. Since resistance to ALS-inhibiting herbicides and glyphosate is reportedly common where *A. tuberculatus* is native (Heap, 2023; Schultz et al., 2015), imazethapyr and glyphosate treatments consisted of two replications (two plants replication^{-1}) to conserve space and resources (Mahoney et al., 2020). All other herbicide treatments consisted of four replications (two plants replication^{-1}). Twenty-one days after treatment, plant survival was recorded on a binomial scale where 0 (no green vegetative tissue) and 1 (visible green vegetative tissue) equaled plant death and survival, respectively.

Single nucleotide polymorphism genotyping. Additional seeds from the Story and Surry County populations were sown and curated as described above. Four plants were sampled in triplicate from each population. Young leaves were harvested from non-treated plants when 10–20 cm in height (6- to 10-leaf), placed in microtubes, and ground into a fine powder with a micropestle. High-quality genomic DNA was extracted from the ground leaf tissue with the Qiagen DNeasy Plant Mini Kit (Qiagen Sciences, Germantown, MD, USA). Both *A. tuberculatus* populations were genotyped with PCR-Allele Competitive Extension assays ([PACE] 3cr Bioscience, Essex, UK) previously designed to target single nucleotide polymorphisms (SNPs) previously associated with ALS-inhibiting herbicide resistance (Jones et al., 2022).

The PACE assay followed the manufacturer's recommendations for thermal cycling, read with a BMG Labtech GmbH PHERAstar (BMG Labtech, Incorporate, Cary, NC, USA) and analysed with a custom single nucleotide polymorphism (SNP)-calling web application (https://snp-caller.herokuapp.com, Andres & Dunne, 2022). Data were reported as the normalised fluorescence (Rn) of HEX and FAM to the internal ROX standard, where the herbicide-resistant allele was tagged with the HEX fluorescence and the wild type (herbicide-susceptible) allele was tagged with the FAM fluorescence.

Sanger Sequencing of 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*), *photosystem II* (*psbA*), and *protoporphyrinogen oxidase* (*PPX2*) *genes*. Additional seeds from each *A. tuberculatus* population were sown and curated as described above. Four plants were sampled from each population. Young leaves were harvested from non-treated plants 10–20 cm in height, and RNA was isolated using the Sigma-Aldrich 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. Polymerase chain reaction (PCR) conditions consisted of 23 µL Promega GoTaq Green Master Mix (Promega Corporation, Madison, WI, USA), 25 µL nuclease-free water, 5 µL 10 µM forward primer, 5 µL 10 µM reverse primer, and 10 µL DNA. Thermal cycling was a standard Touchdown PCR with 40 total cycles. After amplification, target DNA was Sanger-sequenced at the North Carolina State University Genomic Sciences Laboratory.

TABLE 2 Primer sequences used in PCR amplification for single nucleotide polymorphism genotyping and Sanger sequencing for the *Amaranthus tuberculatus acetolactate synthase* (*ALS*), *5-enolpyruvylshikimate-3-phosphate synthase* (*EPSPS*), *photosystem II* (*psbA*) *protoporphyrinogen oxidase* (*PPX2*) genes.

Genes	Primer	Sequence (5′-3′)
ALS	Trp_{574}Leu common	GGTATGTATGTGCCCGGTTAGCTTT
	Trp_{574}Leu Wild Type	GAAGGTGACCAAGTTCATGCTAATC AACATTTAGGTATGGTTGTTCAATG
	Trp_{574}Leu Resistant Mutant	GAAGGTCGGAGTCAACGGATTCAAT CAACATTTAGGTATGGTTGTTCAATT
EPSPS	EPSPS_F1	TCAGCCAAGAAACAAAGCGA
	EPSPS_R1	GCAAGTTGTCGACCACTGTT
	EPSPS_F2	ACCCAAAACCCACTTACCCA
	EPSPS_R2	GAAACAGACCACCACAACCC
	EPSPS_F3	AACAGTGGTCGACAACTTGC
	EPSPS_R3	CGATCCAGAGAGCTTGACCT
	EPSPS_F4	GGTTGTGGTGGTCTGTTTCC
	EPSPS_R4	TCGGATGAAGAACCTGTCCC
	EPSPS_F5	GTCAAGCTCTCTGGATCGGT
	EPSPS_R5	TTCACTCTCCAGCTAGCCAC
	EPSPS_F6	TGACTGTCAAGGGTTGTGGA
	EPSPS_R6	GCTACTCAATGCTTGGCGAA
psbA	psba_F1	ACCAAGATTTTACCATGACTGCA
	psba_R1	GTTCCCACTCACGACCCATA
	psba_F2	CCTTCATAGCTGCTCCTCCA
	psba_R2	TGCTCAGCCTGGAATACGAT
	psba_F3	CAATCTGGGAAGCGGCATC
	psba_R3	ACCATGAGCAGCTACGATGT
	psba_F4	TATGGGTCGTGAGTGGGAAC
	psba_R4	GGCCAAGCAGCTAAGAAGAA
	psba_F5	ATCGTATTCCAGGCTGAGCA
	psba_R5	CCATACCAAGGTTAGCACGG
	psba_F6	GCTATGCATGGTTCCTTGGT
	psba_R6	AGAAAACGGCATTGCTCCTT
PPX2	PPX2_F1	GCAACATTTCTGAGCGGGAA
	PPX2_R1	GCAGCGGGATTTGAAGGTAG
	PPX2_F2	CGATGATCTTGGGCTTCGTG
	PPX2_R2	GCAAACACAGAGCCAAACCT
	PPX2_F3	TGTTCAGGAAAGTGTTGGCG
	PPX2_R3	GCGAATTGGAGCAGTGACAA
	PPX2_F4	AGAAGGAAAAGGGTGGAGGAG
	PPX2_R4	GGATAAGAACTCCGAAGCCCT
	PPX2_F5	TTGTCACTGCTCCAATTCGC
	PPX2_R5	CGGGAATGCATTGCTCCAAA
	PPX2_F6	CTACATTTGTCGGAGGAAGC
	PPX2_R6	TTATGCGGTCTTCTCATCCATCTTCAC

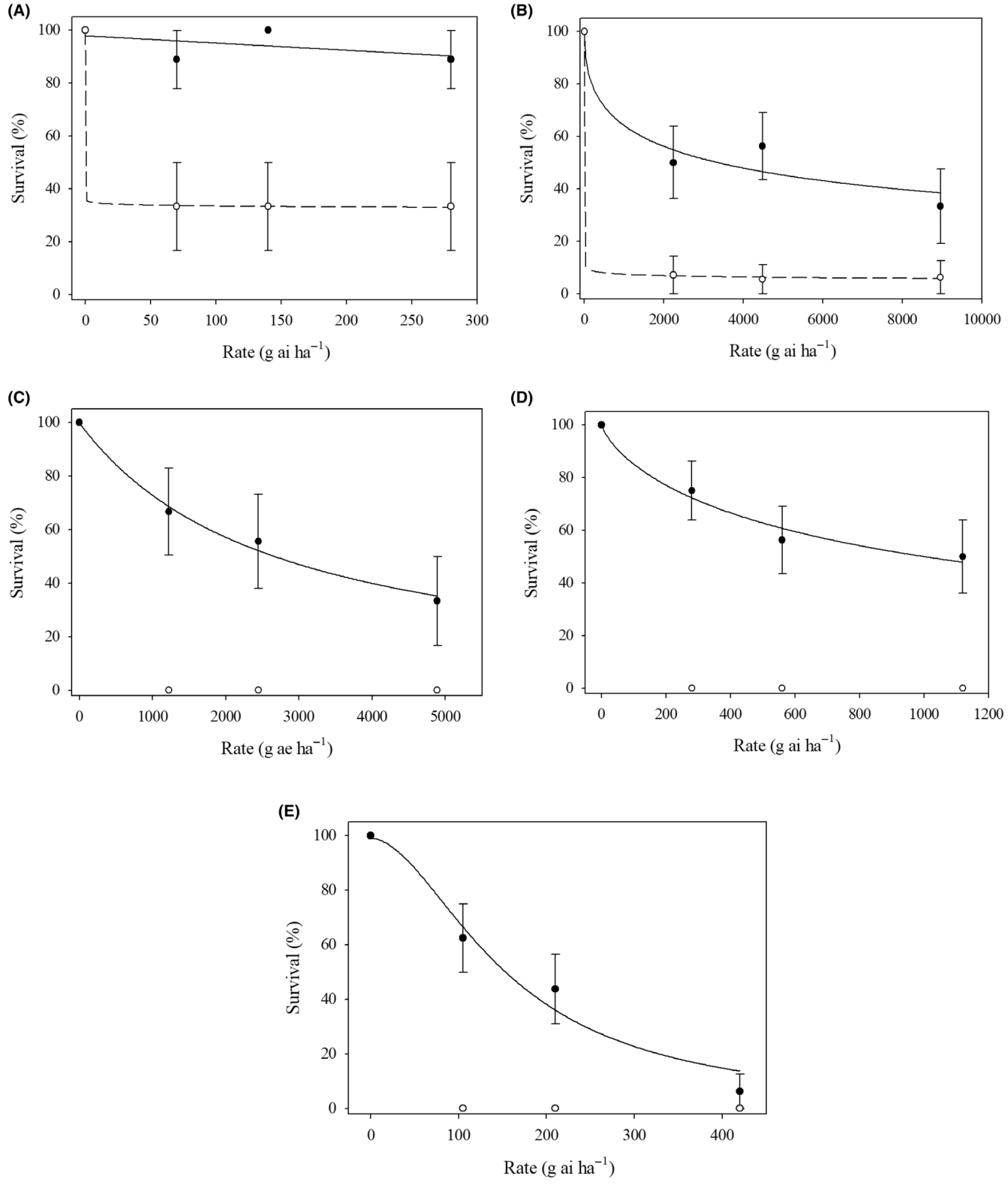


FIGURE 1 Survival of the herbicide-susceptible (Story County, IA; white circle) and the putative multiple herbicide-resistant (Surry County, NC; black circle) *Amaranthus tuberculatus* populations to postemergence applications of imazethapyr (A), atrazine (B), glyphosate (C), fomesafen (D), and mesotrione (E) 21 days after treatment. Error bars represent the standard error of the mean.

The *EPSPS* and *psbA* primers were designed using the *A. tuberculatus* (Genbank accession number: FJ869881.1) and the *Amaranthus hybridus* coding sequence (Genbank accession number: K01200.1) using Primer3 respectively (Table 2). Primers were designed for *PPX2* based on two partial *A. retroflexus* coding sequences (GenBank accession numbers: MK716317 and MK71618), a partial *A. palmeri* coding sequence

(GenBank accession number: KY882137) (Giacomini et al., 2017), and the *A. palmeri* genomic sequence (Montgomery et al., 2020) using Primer3 (Table 2). Raw sequence data were trimmed for quality and aligned to a previously published *A. tuberculatus EPSPS* (GenBank accession number: FJ869881.1), *A. hybridus psbA* (Genbank accession number: K01200.1), and *A. tuberculatus PPX2* gene sequence using Sequencher 5.4.6 (Gene Codes Corporation, Ann Arbor, MI, USA).

2.1 | Statistical analysis

Whole-plant dose–response assay. Plant survival data were subjected to analysis variance using the GLIMMIX procedure in SAS 9.4 (Statistical Analysis Software, Cary, NC, USA). *Amaranthus tuberculatus* population and herbicide rate 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 (1)

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

where a is the upper asymptote, x is the herbicide rate, $x0$ equals the LD_{50} (lethal dose to control 50% of the population) rate, and b is the slope at $x0$. The LD_{50} of each *A. tuberculatus* population was derived from the regression equations. Deviations of survival across tested rates and LD_{50} values will provide evidence if the Surry County population is resistant.

3 | RESULTS

3.1 | Whole-plant dose–response

Each herbicide had a significant population by rate interaction for survival ($p < 0.05$); thus, survival was analysed separately for population and rate for each herbicide.

Imazethapyr. The LD_{50} values were 1750 and approximately 1 g ai ha^{-1} for the Surry and Story County population respectively (Figure 1; Table 3). While the LD_{50} value for the Surry County population was extrapolated outside of the data range, this result suggested that the population would survive high imazethapyr rates. Average survival was greater than 90% and 30% for the Surry and Story County population across all tested rates respectively (Figure 1). The apparent separation of survival between the populations suggest that the Surry County population is imazethapyr-resistant. The Story County population survived lethal rates of imazethapyr, which could suggest that the population was shifting from susceptible to resistant at the time of collection (Hinz & Owen, 1997). While the Story County population may not be a true representation of a herbicide-susceptible population, due to decades of selection ALS-inhibiting herbicides, susceptible *A. tuberculatus* in the native regions is very rare.

Atrazine. The LD_{50} value for the Surry County populations was 3360 g ai ha^{-1}, which is approximately 3× the maximum labelled rate (Figure 1; Table 3). The LD_{50} value for the Story County population was 13 g ai ha^{-1} (Figure 1; Table 3). Average survival was never less than 30% or greater than 10% for the Surry and Story County populations across all tested rates respectively (Figure 1). The differential survival between the two populations across the tested rates suggests that the Surry County population is atrazine-resistant.

Glyphosate. The LD_{50} value for the Surry County population was 2654 g ae ha^{-1}, which is approximately 2× the maximum labelled rate. No LD_{50} value could be calculated for the Story County population as no plants survived any glyphosate treatment (Figure 1; Table 3). Average survival was never less than 30% for the Surry County population across all tested glyphosate rates (Figure 1). The fact that Surry County plants survived lethal rates indicates the population is glyphosate-resistant.

Fomesafen. The LD_{50} value for the Surry County population was 998 g ai ha^{-1}, which is approximately 3.5× the commonly applied rate (Figure 1; Table 3). No LD_{50} value could be calculated for the Story County population as no plants survived any fomesafen treatment (Figure 1; Table 3). Average survival was never less than 50% for the Surry

TABLE 3 Parameter estimates from the three-parameter logistic regression for plant survival of the *Amaranthus tuberculatus* populations (putative resistant: Surry County, North Carolina; susceptible: Story County, Iowa) treated with imazethapyr, atrazine, glyphosate, fomesafen and mesotrione applied postemergence.

		Regression parameters[a]					
Herbicide	Population	*a*	*x*0	*b*	r^2	LD_{50} (g ai/ae ha^{-1})	R/S
Imazethapyr	Surry county	95.3	1745.1	4.5	0.99	1745	122
	Story county	100.0	13.5	0.6	0.99	14	
Atrazine	Surry county	99.9	3380.0	0.5	0.94	3380	277
	Story county	100.0	3E-07	0.1	0.99	3E-07	
Glyphosate	Surry county	99.7	2676.8	1	0.99	2677	NA
	Story county	-[b]	-	-	-	-	
Fomesafen	Surry county	100.2	993.0	0.8	0.98	993	NA
	Story county	-	-	-	-	-	
Mesotrione	Surry county	99.1	155.0	1.8	0.97	155	NA
	Story county	-	-	-	-	-	

Abbreviations: LD_{50}, lethal dose to control 50% of the population; NA, not achieved; R/S, resistance ratio [LD_{50} resistant population LD_{50} susceptible population^{-1}].

[a]*a* is the upper asymptote, *x*0 equals the LD_{50}, and *b* is the slope at *x*0.

[b]No plants survived any herbicide treatment.

County population across all tested rates (Figure 1). Since the Surry County plants survived lethal rates, this population is fomesafen-resistant.

Mesotrione. The LD_{50} value for the Surry County population was 152 g ai ha^{-1}, which is approximately 1.5× the maximum labelled post-emergence rate (Figure 1; Table 3). No LD_{50} value could be calculated for the Story County population as no plants survived any mesotrione treatment (Figure 1; Table 3). Average survival was never less than 40% for the Surry County population until the 4× rate (420 g ai ha^{-1}) where plant survival was less than 10% (Figure 1). The Surry County population is mesotrione-resistant as demonstrated by surviving lethal rates.

The survival of the Surry County population to treatments of imazethapyr, atrazine, glyphosate, fomesafen, and mesotrione were similar when compared to research conducted on other multiple herbicide-resistant *A. tuberculatus* populations (Evans et al, 2019; Shergill, Barlow, et al., 2018). These results further suggest the Surry County population is resistant to five different herbicides. No plants from either population survived the 2,4-D, dicamba and glufosinate treatments (data not shown). These results suggest the Surry County *A. tuberculatus* population is likely still susceptible to these herbicides.

3.2 | Single nucleotide polymorphism genotyping

The Trp_{574}Leu marker identified all Surry County plants as possessing this mutation while all Story County plants were identified as possessing the wild type allele (Figure 2). While only one family of ALS-inhibiting herbicides was used in the whole-plant dose–response, this mutation confers resistance to many families within this herbicide group that are applied to control *A. tuberculatus* (Tranel et al., 2022). The utility of the commonly applied ALS-inhibiting herbicides applied pre- or postemergence is lost since the Surry County plants possess the Trp_{574}Leu mutation in the *ALS* gene (Tranel & Wright, 2002).

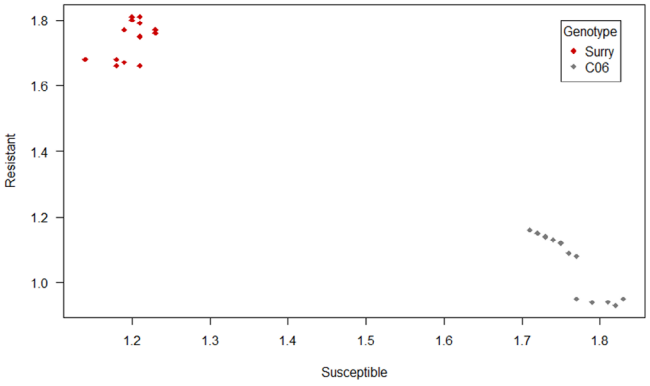


FIGURE 2 Graphical representation of the PCR-based genotyping (PACE) assays completed for the Trp_{574}Leu (mutation in the *acetolactate synthase* (*ALS*) gene of *Amaranthus tuberculatus*. The red dots represent the Surry County, North Carolina population and the grey dots (CO6) represent the Story County, Iowa population.

3.3 | Sanger sequencing

EPSPS. No mutations were found at the positions that have been documented for glyphosate-resistant weeds (Data not shown) (Murphy & Tranel, 2019; Nandula et al., 2013).

psbA. No mutations were found at positions that have been documented for atrazine-resistant weeds (Lu et al., 2019; Thiel & Varrelmann, 2014; Vennapusa et al., 2018). No other consistent polymorphisms were found in the gene of the Surry County population that could confer resistance (Data not shown).

PPX2. Compared to the reference and the Story County sequence, the Surry County population exhibited a codon deletion (GGT) at base pair position 630–632 of the coding sequence in the *PPX2* gene. This resulted in a glycine codon deletion at amino acid position 210 (ΔGly_{210}) of the resulting gene sequence (Figure 3). This mutation has been previously documented and most ubiquitous in *A. tuberculatus* to confer resistance to PPO-inhibiting herbicides (Patzoldt et al., 2006; Schultz et al., 2015).

4 | DISCUSSION

The herbicide resistance profile (HG 2, 5, 9, 14, and 27) and mechanisms of the Surry County population were similar to that of multiple herbicide-resistant populations that exist in the Midwest United States (Bell et al., 2013; Sarangi et al., 2019; Shergill, Barlow, et al., 2018). Three out of the four Surry County plants carried a Trp_{116}Cys mutation in the *EPSPS* gene, but this mutation has not been documented to confer glyphosate resistance (Data not shown) (Murphy & Tranel, 2019). Many other glyphosate-resistant *A. tuberculatus* populations do not exhibit target site mutations due to the amplification of the gene itself (Chatham et al., 2015). In addition to gene amplification, albeit rare, differential absorption and translocation cannot be ruled out as this mechanism has been exhibited by resistant *A. tuberculatus* and related species

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                                                  210
Story_County_Susceptible          QESVGEFFERHFGKEFVDYVIDPFVAGTCGGDPQSLSMHHTFPEVWNIEKRFGSVFAGLI   240
Surry_County_Resistant            QESVGEFFERHFGKEFVDYVIDPFVAGTCG-DPQSLSMHHTFPEVWNIEKRFGSVFAGLI   239
A.tuberculatus_chromosome_25984   QESVGEFFERHFGKEFVDYVIDPFVAGTCGGDPQSLSMHHTFPEVWNIEKRFGSVFAGLI   240
                                  ****************************.*****************************
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FIGURE 3 Visual representations of the the *PPX2* gene sequence of *Amaranthus tuberculatus* populations (putative multiple herbicide-resistant: Surry County, NC [Surry]; herbicide-susceptible: Story County, IA [CO6]). The reference *A. tuberculatus* sequence of the gene is reported at the bottom of the figure. The Surry County population exhibits a codon deletion (glycine), denoted in the figure by '-', at the 210–212 codon position (enclosed by the rectangle).

(Nandula et al., 2013). Other *A. tuberculatus* populations exhibit atrazine resistance without a mutation in the *psbA* gene (Patzoldt et al., 2006). Additionally, it is common for non-target site resistance (e.g., metabolism via glutathione *S*-transferase) to atrazine to be present along with mesotrione resistance (e.g., metabolism via cytochrome P450) as demonstrated by the Surry County population (Jacobs Jr et al., 2020).

This result provides evidence that this population likely was introduced from the Midwest United States as originally presumed. Ceasing the spread of *A. tuberculatus* in North Carolina will be dependent on rapid detection and rapid response. Since the Surry County population exhibited previously characterised resistance mechanisms, SNP-genotyping (as used for detecting the $Trp_{574}Leu$ in this research) can be conducted rapidly to inform the farmer what herbicides will or will not be effective on inhabiting populations (Jones et al., 2022; Tian & Darmency, 2006). While there are other resistance mechanism(s) that were not observed in this research, additional sequencing can be conducted on newly found populations and/or primers can be developed based on findings or previous literature.

The number of effective herbicide options to control the Surry County population is limited. While the auxin herbicides (i.e., 2,4-D and dicamba) effectively controlled the Surry County population, *A. tuberculatus* populations resistant to dicamba and 2,4-D have been confirmed in the Midwest United States (Bobadilla et al., 2022; Shergill, Barlow, et al., 2018). Evolved resistance to glufosinate has been confirmed in *A. palmeri* in the midsouth United States and North Carolina (Heap, 2023; Noguera et al., 2022). While these herbicides will likely be effective for controlling the herbicide-resistant *A. tuberculatus* populations found in North Carolina, farmers will need to steward these herbicides to reduce selection pressure (Matzrafi et al., 2020; Tehranchian et al., 2017). In addition, the utility of all PPO-inhibiting herbicides applied postemergence is lost due to the ΔGly_{210} mutation in the *PPX2* gene (Lillie et al., 2020; Sarangi et al., 2019). While PPO-inhibiting herbicides applied pre-emergence may likely provide some control on this *A. tuberculatus* population, we do not recommend that these herbicides be relied on solely (Lillie et al., 2020; Wuerffel et al., 2015).

The Surry County *A. tuberculatus* population represents the first five-way herbicide-resistant weed species in the Northeast, Mid-Atlantic, and Southeast United States row crops (Heap, 2023). The confirmation of this five-way herbicide-resistant *A. tuberculatus* population potentially will increase weed control costs and complexity in North Carolina. While this *A. tuberculatus* population is currently isolated within North Carolina, management must limit the further establishment within the state and regions (Ervin & Frisvold, 2016; Yu et al., 2021). The fact that *A. tuberculatus* is not native to North Carolina demonstrates the ability for plant material to be transported long distances and successfully colonise new areas with environmental conditions different than its natural habitat. Future research should investigate the control provided by commonly applied preemergence herbicides on the Surry County *A. tuberculatus* population. *Amaranthus tuberculatus* populations with similar herbicide resistance profiles exhibited resistance to very-long-chain fatty-acid-inhibiting herbicides (Strom et al., 2019). Additionally, *A. tuberculatus* populations should be characterised as reported to extension specialists to determine if other herbicide resistance profiles exist in the state.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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