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Inheritance of resistance to *S*-metolachlor in a waterhemp (*Amaranthus tuberculatus*) population from central Illinois

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Research Article

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Abstract

Waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] is a dioecious weed that has evolved resistance to very-long-chain fatty-acid elongase (VLCFAE)–inhibiting herbicides via rapid metabolism. Although detoxification enzyme activities are associated with *S*-metolachlor resistance in two multiple herbicide–resistant (MHR) *A. tuberculatus* populations from Illinois, the genetic basis of resistance is unknown. Therefore, our goal was to investigate inheritance of *S*-metolachlor resistance in the Stanford, Illinois–resistant (SIR) population. Specifically, our research objectives were to: (1) generate a uniformly resistant, full-sib near-inbred line ($DK_{3\text{-}2}$) via three generations of recurrent selection for resistance using preemergence *S*-metolachlor; (2) develop *A. tuberculatus* populations segregating for *S*-metolachlor resistance via reciprocal single-plant (one male × one female) full-sib mating of $DK_{3\text{-}2}$ and a VLCFAE-inhibiting herbicide-sensitive population, SEN; (3) quantify *S*-metolachlor resistance levels in parental lines and their F_1 progenies via greenhouse dose–response analysis; and (4) evaluate inheritance of *S*-metolachlor resistance in F_2 progenies. Dose–response analysis using six to eight *S*-metolachlor concentrations (0.015 to 15.0 µM, varying per population) generated lethal dose (LD) estimates of 50% (LD_{50}) and 90% (LD_{90}) for SIR, SEN, $DK_{3\text{-}2}$, and F_1 progenies. LD estimates indicated $DK_{3\text{-}2}$ has a higher magnitude of *S*-metolachlor resistance than the SIR population, demonstrating single crosses significantly increased *S*-metolachlor resistance in $DK_{3\text{-}2}$. Levels of *S*-metolachlor resistance in F_1 populations were intermediate compared with $DK_{3\text{-}2}$ and SEN. Segregation of *S*-metolachlor resistance in F_2 families from the paternal-derived lines fit a single-gene model (R:S = 3:1), indicating a single, dominant gene confers *S*-metolachlor resistance in SIR. However, F_2 segregation results from the maternal-derived lines fit a duplicate recessive epistasis model (R:S = 9:7), indicating a second recessive gene may also modify *S*-metolachlor resistance in SIR. Results and germplasm derived from this research can assist in identifying the gene(s) conferring resistance to *S*-metolachlor in *A. tuberculatus*.

Introduction

Waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] is a pernicious weed species limiting corn (*Zea mays* L.) (Steckel and Sprague 2004), soybean [*Glycine max* (L.) Merr.] (Hager et al. 2002b), and grain sorghum [*Sorghum bicolor* (L.) Moench.] (Feltner et al. 1969) production in the United States. *Amaranthus tuberculatus* is a summer annual dicot with C_4 physiology and is a dioecious, diploid ($2n = 32$) outcrossing weed species (Steckel 2007). It is characterized by high fecundity, with female plants producing up to a million seeds that persist in the soil seedbank for years combined with multiple emergence events per year (Hager et al. 2002a; Steckel et al. 2007). The dioecious biology of *A. tuberculatus* contributes to its rich genetic diversity, adaptability, and evolutionary success (Kreiner et al. 2022) despite application of various weed control strategies (Tranel 2021). To date, *A. tuberculatus* is resistant to herbicides from seven site-of-action groups (Heap 2023). Most recently, it was postulated that dicamba resistance in a multiple herbicide–resistant (MHR) *A. tuberculatus* population from central Illinois displayed moderate heritability and is potentially controlled by multiple genes (Bobadilla et al. 2022). The growing number of resistance cases to preemergence herbicides among *A. tuberculatus* populations (Evans et al. 2019; Strom et al. 2019) warrants innovative strategies that quickly detect resistance as well as improve herbicide efficacy (Tataridas et al. 2022).

Previous studies have characterized the genetics and inheritance of herbicide resistance in *A. tuberculatus* (Bobadilla et al. 2022; Huffman et al. 2015; Kohlhase et al. 2018; Oliveira et al. 2018). Importantly, however, these previous studies only investigated foliar-applied herbicides. Field populations of resistant *A. tuberculatus* are rarely purified via recurrent selection beyond a single generation before crossing with a sensitive population. Genetically purified populations should theoretically have an increased frequency of resistance alleles if subjected to herbicide

selection during inbreeding (Falconer 1989; Kohlhase et al. 2018). Three generations of recurrent selection designed to increase homozygosity of an Iowa *A. tuberculatus* population were utilized for creation of an F_1 in previous genetic research, which led to reproducible segregation of mesotrione resistance in F_2 lines (Kohlhase et al. 2018). However, starting without a homogenous, homozygous parent population of mesotrione-resistant *A. tuberculatus* may have contributed to unclear segregation results in another genetic study (Huffman et al. 2015).

Resistance to preemergence herbicides is a trait of interest warranting further research to investigate the mechanisms involved. Resistance and inheritance studies of the very-long-chain fatty-acid elongase (VLCFAE)-inhibiting preemergence herbicide pyroxasulfone (Busi et al. 2014, 2018; Busi and Powles 2016) and preplant-incorporated herbicide triallate (Brunton et al. 2021) have been conducted in rigid ryegrass (*Lolium rigidum* Gaudin.). Resistance to pyroxasulfone in *L. rigidum* is metabolism based and is characterized by upregulation of glutathione *S*-transferase (*GST*) and cytochrome P450 (*P450*) genes (Busi et al. 2014, 2018; Busi and Powles 2016). Resistance to *S*-metolachlor in an *A. tuberculatus* population from Stanford, Illinois (SIR) occurs via P450- and GST-catalyzed detoxification mechanisms acting in concert (Strom et al. 2020, 2021). However, research has not been reported that clearly establishes the genetic basis and inheritance of *S*-metolachlor resistance in *A. tuberculatus*.

The current research was designed to investigate inheritance of *S*-metolachlor resistance in an MHR *A. tuberculatus* population (SIR). Our working hypothesis is that the *S*-metolachlor resistance trait in SIR is controlled by a single, dominant, nuclear-encoded gene. Specific aims of our study were to: (1) genetically advance (i.e., increase homozygosity of) the SIR field population via a breeding scheme designed for a dioecious species combined with recurrent selection; (2) optimize a phenotyping assay for VLCFAE-inhibiting, preemergence herbicides; (3) develop F_2 populations segregating for resistance to the VLCFAE-inhibiting herbicide, *S*-metolachlor; and (4) evaluate the inheritance patterns of *S*-metolachlor resistance in these segregating populations.

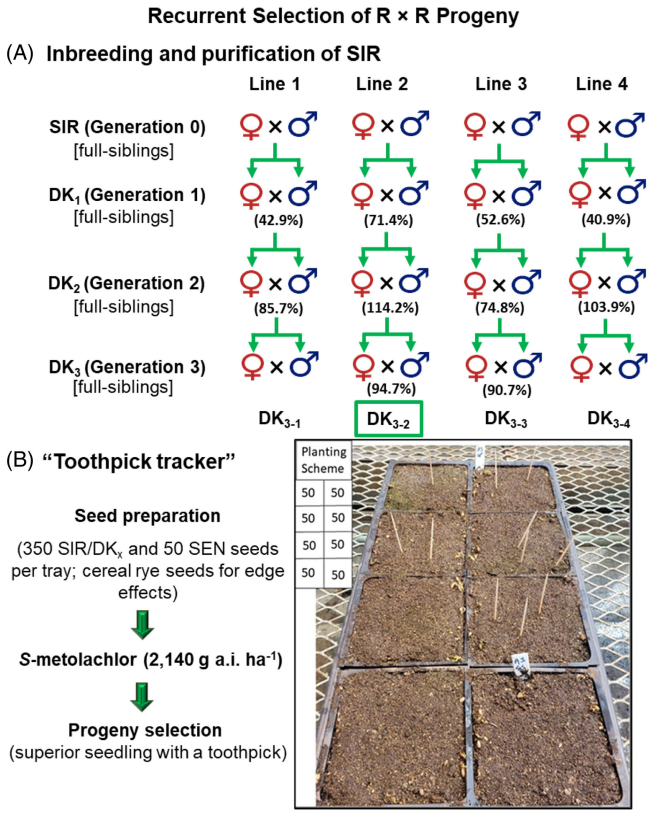

Figure 1. Recurrent selection of SIR *Amaranthus tuberculatus*. (A) Breeding scheme for selection of uniform, highly *S*-metolachlor–resistant progeny derived from the original SIR field population. Four crosses per generation utilized single male and female resistant plants to select uniformly resistant lines. Numbers in parentheses below DK lines in Generations 1, 2, and 3 represent percent survivorship following recurrent selection with 2,140 g ai ha^{-1} *S*-metolachlor (four lines per generation). Survivorship of lines 3-1 and 3-4 was not determined, because lines 3-2 and 3-3 were the most uniform, displayed the highest percent survivorship in Generation 1, and yielded ample viable seeds for analysis. Line DK_{3-2} (green rectangle) was ultimately chosen for subsequent crosses with sensitive plants. (B) The "toothpick tracker" method of screening for survival at each step following 2,140 g ha^{-1} *S*-metolachlor preemergence treatment at 14 d after treatment. Cereal rye (*Secale cereale* L.) seeds were planted along the edges of all trays to reduce possible border effects.

Materials and Methods

Generation of Full-Sib Near-Inbred Resistant Lines and Parent Selection

Two *A. tuberculatus* populations (SIR and SEN) previously investigated for responses to the VLCFAE-inhibiting herbicide *S*-metolachlor (Kerr 2021) were used as parental populations in this study. SIR is a subpopulation derived from the original MCR (McLean County, Illinois–resistant) *A. tuberculatus* population (Hausman et al. 2011; Jacobs et al. 2020; O'Brien et al. 2018). The SIR/MCR population is resistant to herbicides from five site-of-action groups (Concepcion et al. 2021; Heap 2023). The second *A. tuberculatus* population (SEN) is a standard sensitive population used in previous research (Kaundun et al. 2017; O'Brien et al. 2018) and was previously confirmed sensitive to *S*-metolachlor (Kerr 2021).

To create a uniform, highly resistant population, male and female SIR plants were intermated (Figure 1A) before creation of F_1 and segregating F_2 *A. tuberculatus* populations (Figure 2). Three generations of single-plant, full-sib mating and recurrent selection were conducted with male and female SIR plants. Four full-sib near-inbred lines were ultimately obtained, and each line was screened for survival to 2,140 g ai ha^{-1} *S*-metolachlor at each cycle via a "toothpick tracker" method in the greenhouse (Figure 1B). Before planting, seeds were surface sterilized and cold stratified in 0.15% agarose (w/w) for at least 30 d, as previously described (Bell et al. 2013). Large cell pack inserts (801 Daisy Trays, Greenhouse Megastore, 70 Eastgate Drive, Danville, IL 61834) were filled halfway with soil mix (1:1:1 soil:peat:torpedo sand) with a pH of 6.4 and 3.5% organic matter. A 15-g portion of slow-release fertilizer (Osmocote 13-13-13, Scotts Miracle-Gro Company, 14111 Scottslawn Road, Marysville, OH 43041) was added, then the remainder of the insert was filled with soil mix, patted firmly, and allowed to saturate with water overnight, as previously described (Strom et al. 2019). Stratified *A. tuberculatus* seeds from SIR were planted onto the moistened soil surface into seven of the eight inserts, while SEN was planted in the eighth insert to ensure herbicide efficacy before selection and transplanting (Figure 1B). *S*-metolachlor was applied at a rate of 2,140 g ha^{-1} using a research-grade cabinet sprayer (Generation III Research Sprayer, DeVries Manufacturing, 86956 State Highway 251, Hollandale, MN 56045). Immediately following herbicide treatment, each insert was lightly covered with nontreated soil mix and gently pressed. *S*-metolachlor–treated flats were placed under

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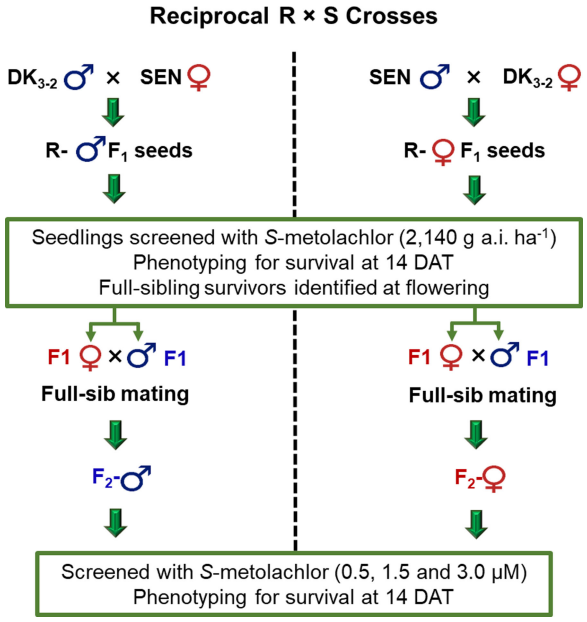

Figure 2. Generation of F_1 and F_2 families of SIR *Amaranthus tuberculatus* derived from reciprocal crosses between full-sib near-inbred line, $DK_{3\text{-}2}$, and a very-long-chain fatty-acid elongase (VLCFAE) inhibitor–sensitive population, SEN. Each cross per generation used single male and female plants. F_1 seedlings were screened for survival in soil mix from 2,140 g ai ha^{-1} *S*-metolachlor at 14 d after treatment, while F_2 seedlings were screened and phenotyped with three concentrations of *S*-metolachlor (0.5, 1.5, and 3.0 µM) using the preemergence resistance identification method (PRIM) soilless assay (Kerr 2021).

an overhead misting system fitted with 0.4 L min^{-1} nozzles and set on a timer to deliver water three times per day to ensure herbicide incorporation, as previously described (Strom et al. 2019).

Amaranthus tuberculatus seedlings emerging within the first 7 to 10 d were identified and tracked using toothpicks placed at their bases (Figure 1B). After 14 d, living seedlings marked with a toothpick were recorded (compared with the untreated flat) and were subsequently transplanted for crossing experiments. Seedlings identified as resistant (no visual injury symptoms or growth reduction relative to control plants; Krähmer et al. 2019; Pillai et al. 1979) were transplanted into 720-cm^3 pots using BM6 potting soil (Berger BM6 General Purpose Mix, Value-Added Mixing Plant, Watsonville, CA 95076) with slow-release fertilizer (Osmocote®) added, then allowed to grow under mercury-halide lamps delivering 800 µmol m^{-2} s^{-1} at 28/22 C day/night with a 16/8-h photoperiod. As *A. tuberculatus* plants developed into the desired size for producing large amounts of seed, they were placed in framed pollination chambers using 2.5-cm polyvinyl chloride and pollination bags capable of housing one male and one female (full sibs). Each pollination chamber was then placed in a separate greenhouse room using the same growing conditions previously mentioned, but with a 12-h photoperiod to stimulate flowering. Seeds from the full-sib crosses were stratified, planted, treated, and selected with *S*-metolachlor using the strategy described above for an additional two generations.

After three generations of recurrent selection for each resistant line (Figure 1A), survivorship was used as the primary method for identifying the most suitable parental population, henceforth called $DK_{3\text{-}2}$ (Supplementary Table S1). Mean survivorship (per generation) calculated for the original SIR field population, DK_1, DK_2, and DK_3 following treatment with 2,140 g ha^{-1} *S*-metolachlor in soil was 39%, 52%, 95%, and 93%, respectively. Full-sib near-inbred lines were not advanced to a fourth generation of recurrent selection due to the degree of genetic gain in percent survivorship in lines $DK_{3\text{-}2}$ and $DK_{3\text{-}3}$ (Figure 1A). Survival rates of line $DK_{3\text{-}2}$ were 39% (original SIR field population), 71%, 114% and 95%, respectively, from Generation 0 to Generation 3. In Generation 2, the survival of line $DK_{3\text{-}2}$ exceeded 100% due to the nontreated flat having lower than normal germination rates. Among the DK_3 lines generated (Supplementary Table S1), $DK_{3\text{-}2}$ consistently demonstrated the highest survival rates following three cycles of recurrent selection with *S*-metolachlor (Figures 1 and 3). Additionally, potential inbreeding depression was observed beyond two generations of full-sib crossing (data not shown). Inbreeding may have affected seed germination rates and aboveground physiology of mature *A. tuberculatus* plants, which ultimately reduced overall seed output.

Generation of F_1 and F_2 Families

The full-sib near-inbred *A. tuberculatus* line, $DK_{3\text{-}2}$, was used to choose R parents in subsequent reciprocal crosses with SEN. Stratified seeds of $DK_{3\text{-}2}$ and the SEN population were planted, screened, and tracked for survival to 2,140 g ha^{-1} *S*-metolachlor using the method outlined in Figure 1B. Corresponding nontreated seedlings of each population were also prepared. Selected seedlings from $DK_{3\text{-}2}$ and SEN at a similar growth stage were then transplanted after 14 d into larger pots, as previously described. At flowering, pollination chambers were constructed with each chamber containing one $DK_{3\text{-}2}$ male parent and one SEN female parent. These crosses and the respective reciprocal cross (one $DK_{3\text{-}2}$ female parent and one SEN male parent) were placed in separate greenhouse rooms and grown under the same conditions previously described. Mature female plants were harvested, and seed was threshed and stratified (F_1 progeny) for full-sib crosses to produce F_2 progeny (Figure 2). The two F_1 *A. tuberculatus* lines derived from $DK_{3\text{-}2}$ × SEN and SEN × $DK_{3\text{-}2}$ cross are designated F_1-♂ and F_1-♀, respectively (Figure 1B; Supplementary Table S1). Maternal and paternal F_1 progenies were grown and selected using the previously described method (Figure 1B). Before flowering, two pairs of F_1-♂ siblings and two pairs of F_1-♀ siblings were placed in pollination chambers, producing four F_2 families designated as F_2-♂-1, F_2-♂-2, and F_2-♀-1, F_2-♀-2 (Supplementary Table S1).

Soilless Hydroponic Plant Culture

Different concentrations of formulated *S*-metolachlor (Dual Magnum®, Syngenta Crop Protection, Greensboro, NC 27419) were used for dose–response experiments. These herbicide concentrations were previously identified as effective for differentiating dose responses to *S*-metolachlor among *A. tuberculatus* populations using a soilless greenhouse assay described previously (Kerr 2021). Dry, medium-textured, exfoliated vermiculite (Vermiculite, Thermo-O-Rock East, New Eagle, PA 15067) was used to fill plastic cell pack inserts (31801 Deep Insert, BFG Supply, 3708 Enterprise Drive, Suite 180, Janesville, WI 53546), which were arranged in the greenhouse using a randomized complete block design with "replicate" as a blocking factor. Greenhouse conditions were set at 28/22°C day/night under a 16-h photoperiod and maintained throughout the two independent experimental runs (separated in time). Supplemental light was provided using mercury-halide lamps providing 800 µmol m^{-2} s^{-1} photon flux at the vermiculite surface. Pots were subirrigated every second day with 150 ml water-soluble fertilizer (Peters Hydroponic

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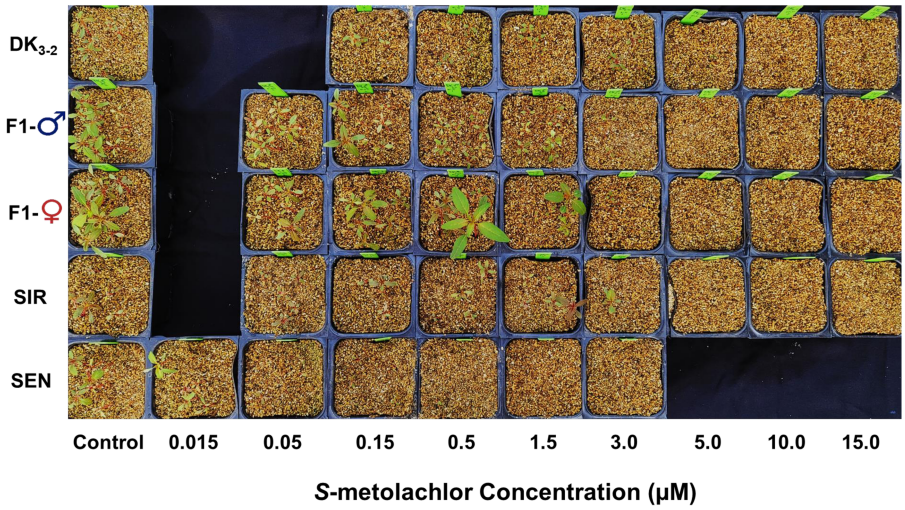

Figure 3. Representative plants from five *Amaranthus tuberculatus* populations at 14 d after treatment with *S*-metolachlor: DK_{3-2}, full-sib near-inbred line and R parent; $F_1♂$- F_1 paternal-derived R line; $F_1♀$, F_1 maternal-derived R line; SIR, original field population; SEN, sensitive parent. Treated pots are arranged from left to right with increasing *S*-metolachlor concentrations ranging from 0.015 to 15.0 µM. Nontreated controls are included for comparison on the left for each population. Herbicide treatments were applied using the preemergence resistance identification method (PRIM) soilless assay (Kerr 2021).

Special 5-11-26, ICL Specialty Fertilizers, Summerville, SC, USA) not containing *S*-metolachlor.

Herbicide Screening of Parent, F_1, and F_2 Populations

Amaranthus tuberculatus populations F_1-♂, F_1-♀, F_2-♂-1, F_2-♂-2, F_2-♀-1, and F_2-♀-2 were treated with three concentrations of *S*-metolachlor (0.5, 1.5, and 3.0 µM) under greenhouse conditions using a soilless assay (Kerr 2021). The two experimental runs evaluated 91 F_1-♂, 94 F_1-♀, 168 F_2-♂-1, 161 F_2-♂-2, 172 F_2-♀-1, and 145 F_2-♀-2 seedlings at 14 d after treatment. Segregation data for [F_2-♂-1 and F_2-♂-2] or [F_2-♀-1 and F_2-♀-2] were pooled, resulting in a total of 329 F_2-♂ and 317 F_2-♀ seedlings evaluated using two phenotypic classes: resistant (R) and sensitive (S). Due to the lack of genetic markers, R and S phenotypes were based solely on plant survival, which precluded the ability to identify possible heterozygotes. The full-sib near-inbred *A. tuberculatus* line (DK_{3-2}), original SIR field population, SEN, F_1-♀, and F_1-♂ were chosen for dose–response experiments to compare resistance levels after treatment with various *S*-metolachlor concentrations (Kerr 2021). The DK_{3-2} line was treated with seven *S*-metolachlor concentrations ranging from 0.15 to 15.0 µM, and SEN was treated with six *S*-metolachlor concentrations ranging from 0.015 to 3.0 µM. The F_1-♀, F_1-♂, and SIR populations were treated with eight *S*-metolachlor concentrations ranging from 0.05 to 15.0 µM. Dose–response experiments using these concentrations were conducted with two experimental runs (separated in time) using the preemergence resistance identification method (PRIM) soilless assay described previously (Kerr 2021).

Statistical Analysis

Survival data were collected 14 d after treatment from two independent experimental runs consisting of surviving plant counts and respective aboveground biomass. Dose–response analysis was conducted with the DRC package in R (v. 4.2.1) and RStudio (v. 2022.07.1) using a three-parameter logistic regression model (Knezevic et al. 2007) with Equation 1:

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

where d is the upper limit, b is the slope of the curve, and e is the 50% reduction in seedling survival (LD_{50}). A three-parameter model was used instead of other log-logistic models based on lack-of-fit tests conducted at $\alpha = 0.05$, indicating all *A. tuberculatus* populations could be combined in the same model and accurately compared.

Chi-square (χ^2) goodness-of-fit tests (Cochran 1952) were used to test whether the F_2 segregation patterns of R and S *A. tuberculatus* seedlings after treatment with *S*-metolachlor (0.5, 1.5, and 3.0 µM) followed a single dominant gene or multiple gene model. The χ^2 was calculated using Equation 2:

$$\chi^2 = \sum \{[(O_i - E_i)^2]/E_i\} \quad [2]$$

where O_i is the observed frequency count for the ith level of the categorical variable, and E_i is the expected frequency count for the ith level of the categorical variable. The null hypothesis (H_0) in this study was: "survival ratio to 1.5-µM *S*-metolachlor in the F_2 population is not significantly different from a 3:1 (R:S) single-gene model." If the χ^2 goodness-of-fit tests for each χ^2 estimate resulted in $P \geq 0.05$, H_0 is accepted; otherwise, H_0 is rejected, and the alternative hypothesis (H_a), which was "survival ratio to 1.5-µM *S*-metolachlor in the F_2 population is significantly different from a 3:1 (R:S) single-gene model," is accepted. The H_a would also lead to further hypotheses and experimental procedures to investigate the potential multigenic nature of *S*-metolachlor resistance in MHR *A. tuberculatus*, which was beyond the scope of the current study. The ratio of alive (R) to dead (S) plants relative to nontreated controls for each F_2 family were fit to one-gene and two-gene phenotypic models at $\alpha = 0.05$.

The probability of resistance was calculated using an odds ratio described previously (Kohlhase et al. 2018) by analyzing an equal number of progenies from each F_1 and F_2 line. The odds ratio is the ratio between the odds of resistance in F_1 versus F_2 families. If the ratio is >1, the probability is higher in the F_1; if the ratio is <1,

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Table 1. Lethal dose (LD) estimates of 50% (LD_{50}) and 90% (LD_{90}) *Amaranthus tuberculatus* control with *S*-metolachlor at 14 d after treatment in five populations using the preemergence resistance identification method (PRIM) soilless assay (Kerr 2021).

Population[a]	LD_{50}[b]	LD_{90}[b]	R/S[c]
	μM		
SIR	0.9 (±0.1)	11.7 (±2.3)	4.6 (±0.1)
DK_{3-2}	3.3 (±0.3)[d]	17.3 (±2.7)[d]	16.5 (±0.3)
F_1-♂	1.9 (±0.1)[d]	4.1 (±0.5)[d]	9.6 (±0.1)
F_1-♀	0.9 (±0.1)	7.4 (±1.3)	4.7 (±0.1)
SEN	0.2 (±0.02)	0.7 (±0.1)	—

[a]SIR, Stanford, Illinois–resistant population from McLean County, IL. SEN, standard sensitive population used in previous research (Kaundun et al. 2017; Kerr 2021; O'Brien et al. 2018).
[b]Estimated LD values are expressed as *S*-metolachlor concentrations (μM) followed by standard errors of the mean in parentheses.
[c]Resistant-to-sensitive (R/S) ratios derived from LD_{50} estimates of each *A. tuberculatus* population relative to SEN.
[d]LD estimate is significantly higher than the LD_{50} of the field population, SIR ($P < 0.05$).

the probability is higher in the F_2; and if the ratio = 1, the probability is equal among F_1 and F_2 lines. The normality of data was analyzed using a Kolmogorov-Smirnov test at $\alpha = 0.05$.

Results and Discussion

Dose–Response Analysis of SIR, SEN, and F_1 Lines

In the current study, we aimed to identify the genetic basis of *S*-metolachlor resistance in the *A. tuberculatus* population, SIR. Three cycles of recurrent selection were first carried out to increase homozygosity of the SIR population and produce the DK_{3-2} line (Figure 1A); for example, survival rates of DK_{3-2} were 39% (SIR population), 71%, 114%, and 95%, respectively, proceeding from Generation 0 to Generation 3. Recurrent selection also increased the level of *S*-metolachlor resistance compared with SIR, which is exemplified by a significantly higher LD_{50} value (3.7-fold) in DK_{3-2} compared with SIR (Table 1; Figure 4). If the flow of pollen from one male to one female *A. tuberculatus* plant is restricted, all loci conferring resistance can in theory be stacked into one population and compared with the original field population. Generation of reciprocal of DK_{3-2} and SEN (R × S) subsequently yielded F_1 progenies (Figure 2).

Dose–response analysis of SIR, SEN, line DK_{3-2}, paternal F_1 (F_1-♂), and maternal F_1 (F_1-♀) families resulted in different responses to *S*-metolachlor (Table 1; Figures 3 and 4). As expected, the LD_{50} value of SIR was significantly higher than that of SEN. However, DK_{3-2} exhibited a significantly higher LD_{50} than SIR, indicating a successful purification of the original SIR population after three cycles of full-sib mating (Figure 1). The F_1-♂ and F_1-♀ progeny displayed intermediate responses (measured by LD_{50} and LD_{90} values) to *S*-metolachlor relative to parental lines DK_{3-2} and SEN (Table 1; Figure 4). However, the LD_{50} value of F_1-♂ was significantly higher than that of SIR, whereas F_1-♀ had an LD_{50} value similar to SIR (Table 1), which may reflect heterosis in both F_1 progeny (Figure 3). In addition, calculated R/S ratios based on LD_{50} values in DK_{3-2} and F_1-♂ were 16.5- and 9.6-fold higher than for SEN, whereas SIR and F_1-♀ were only 4.6- and 4.7-fold higher (Table 1). Interestingly, the maternal F_1-♀ line demonstrated higher survival levels at *S*-metolachlor concentrations exceeding 1.5 μM compared with the paternal F_1-♂ line (Figures 3 and 4). As a result, it is possible that: (1) the F_1-♀ possesses additional resistance or sensitivity genes (see discussion below 'Probabilities of *S*-Metolachlor Resistance and Segregation Analysis in F_2 Families' regarding F_2 populations), (2) these resistance genes

Table 2. Probabilities of resistance from pooled reciprocal crosses in F_1 and F_2 *Amaranthus tuberculatus* generations at three concentrations of *S*-metolachlor 14 d after treatment using the preemergence resistance identification method (PRIM) soilless assay (Kerr 2021).

S-metolachlor[a]	SE	*z*-value	Pr > |*z*|	Odds ratio[b]
μM				
0.5	0.03	4.7×10^{-16}	1.0	1.02
1.5	0.04	-2.3×10^{-15}	1.0	0.85
3.0	0.07	5.6×10^{-17}	1.0	0.84

[a]Estimated values are expressed as *S*-metolachlor concentrations (μM) followed by their respective standard errors of the mean.
[b]The odds ratio measures the resistance probability of the F_1 generation compared with the resistance probability of the F_2 generation (odds of resistance in F_1/odds of resistance in F_2). If ratio is >1, the probability of resistance is higher in the F_1; if ratio is <1, the probability of resistance is higher in the F_2; if ratio = 1, the probability of resistance is the same.

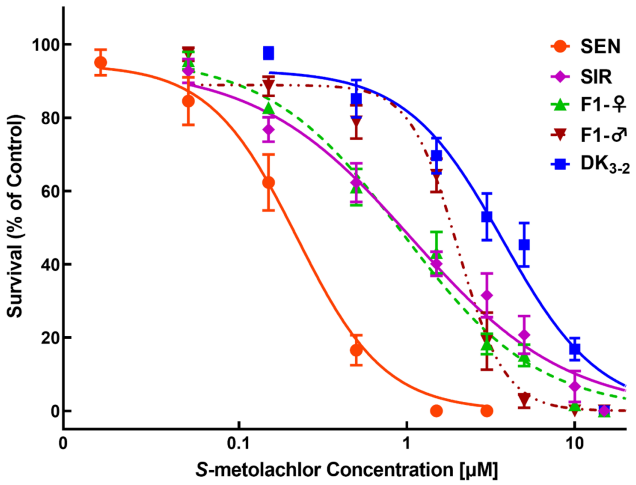

Figure 4. *Amaranthus tuberculatus* survival in response to increasing concentrations of *S*-metolachlor in a dose–response experiment of four *A. tuberculatus* populations using the preemergence resistance identification method (PRIM) soilless assay (Kerr 2021). Survival data were collected 14 d after treatment by recording the number of living plants as a percent of the untreated control. Lines in each graph were fit using Equation 1:

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

where *d* is the upper limit, *b* is the slope of the curve, and *e* is the 50% reduction in seedling survival (LD_{50}). Each error bar represents ± SE. SEN, S parent; SIR, original field population; F_1-♀, F_1 maternal-derived R line; F_1-♂, F_1 paternal-derived R line; DK_{3-2}, purified R × R population and R parent.

are inducible by *S*-metolachlor (compared with F_1-♂), (3) unknown maternal effects are present, or (4) unequal inheritance was rendered by cellular concentration-dependent resistance mechanisms (Nobusawa et al. 2013; Nobusawa and Umeda 2012). Alternatively, variations in parentally biased gene expression between maternal and paternal lines could also be explained by an epigenetic phenomenon called genomic imprinting (Batista and Köhler 2020; Pignatta et al. 2014). However, further research is needed to test these theories.

Probabilities of *S*-Metolachlor Resistance and Segregation Analysis in F_2 Families

Pooled probabilities of resistance based on survival data from F_1 and F_2 families treated with three concentrations of *S*-metolachlor indicated equal probabilities at 0.5 μM but higher probabilities in

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Table 3. Pooled probabilities of resistance of the F_1 and F_2 *Amaranthus tuberculatus* SIR generations within reciprocal crosses at three concentrations of *S*-metolachlor at 14 d after treatment using the preemergence resistance identification method (PRIM) soilless assay (Kerr 2021).

Population[a]	*S*-metolachlor[b]	SE	*z*-value	Pr > |*z*|	Odds ratio[c]
	μM				
F_2-♂	0.5	0.07	1.8×10^{-16}	1.0	0.94
	1.5	0.06	-3.2×10^{-16}	1.0	0.71
	3.0	0.11	−0.16	0.9	0.68
F_2-♀	0.5	0.09	-1.3×10^{-15}	1.0	1.12
	1.5	0.09	-1.6×10^{-16}	1.0	1.00
	3.0	0.08	0	1.0	1.01

[a]Reciprocal crosses were generated from crossing a very-long-chain fatty-acid elongase (VLCFAE) inhibitor–sensitive population (SEN) with a purified parent derived from multiple herbicide–resistant *A. tuberculatus*, SIR ($DK_{3\text{-}2}$). F_2-♂ were derived from the parent cross (SEN-♀ × $DK_{3\text{-}2}$-♂), whereas F_2-♀ were derived from parent cross ($DK_{3\text{-}2}$-♀ × SEN-♂).
[b]Estimated values are expressed as *S*-metolachlor concentrations (μM).
[c]The odds ratio measures the resistance probability of the F_1 generation compared with the resistance probability of the F_2 generation within each reciprocal cross (odds of resistance in F_1/odds of resistance in F_2). If ratio is >1, the probability of resistance is higher in the F_1; if ratio is <1, the probability of resistance is higher in the F_2; if ratio = 1, the probability of resistance is the same.

Table 4. Chi-square (χ^2) goodness-of-fit analysis of pooled paternal-derived (F_2-♂) and maternal-derived (F_2-♀) F_2 *Amaranthus tuberculatus*, SIR populations.[a]

	χ^2 test						
	No. of plants			One locus[b]		Two loci[c]	
Population	Total	Observed (alive, dead)	Survival ratio	χ^2	P-value	χ^2	P-value
F_2-♂	329	246, 83	0.75	0.01	0.92[d]	45.9	<0.001
F_2-♀	317	193, 124	0.61	33.69	<0.001	2.8	0.09[e]

[a]Survival values were derived from the ratio of number of living plants to total seedlings at 14 d after treatment with 1.5-μM *S*-metolachlor using the preemergence resistance identification method (PRIM) (Kerr 2021). χ^2 values were calculated using Equation 2: $\chi^2 = \Sigma[(O_i - E_i)^2]/E_i\}$ (Cochran 1952).
[b]Expected values are calculated from a 0.75:0.25 resistant:sensitive (3:1) ratio.
[c]Expected values are calculated from a 0.5625:0.4375 resistant:sensitive (9:7) ratio.
[d]Calculated P-value is greater than 0.05; population fits one-gene model.
[e]Calculated P-value is greater than 0.05; population fits two-gene model.

the F_2 at 1.5 and 3.0 μM, with odds ratios of 0.85 and 0.84, respectively (Table 2). A higher probability of resistance in the F_2 generation at the higher herbicide concentrations could indicate a greater number of dominant alleles present in the F_2 at each contributing locus (Kohlhase et al. 2018). When considering reciprocal F_2 families separately, however, a difference was noted in the calculated probabilities of resistance when comparing maternal- and paternal-derived F_1 and F_2 lines at 0.5-, 1.5-, and 3.0-μM *S*-metolachlor (Table 3). F_2-♂ lines exhibited higher probabilities of resistance than their F_1 parents at each concentration, whereas the F_2-♀ lines exhibited similar (at 1.5 and 3.0 μM) or slightly lower (at 0.5 μM) probabilities of resistance as their F_1 parents (Table 3). A potential explanation for these different trends in probabilities is that maternal-derived F_1 and F_2 populations may contain a putative recessive allele at a second, unlinked locus that lowers the magnitude of *S*-metolachlor resistance (or increases sensitivity) in the F_2 population compared with the F_1, which is not present in the paternal-derived F_1 and F_2 populations created in our study (further described below).

The χ^2 tests for goodness of fit were performed with F_2 segregation data generated from only 1.5-μM *S*-metolachlor (Table 4). A 3:1 (R:S) phenotypic ratio indicates a single dominant gene model. A phenotypic ratio of 9:7 (R:S) is consistent with a two-gene, complementary gene action model, indicative of an epistatic interaction between two unlinked loci (Falconer 1989). Chi-square tests for goodness of fit at 0.5- and 3.0-μM *S*-metolachlor concentrations were not pursued in this study. Segregation analysis at 0.5-μM *S*-metolachlor was deemed limited, because this concentration was likely not effective in killing 100% of sensitive plants, based on LD_{90} values in Table 1. Additionally, 3.0-μM *S*-metolachlor likely killed a significant portion of resistant plants by overwhelming metabolic resistance, leading to possibly inaccurate survival data compared with segregation results obtained with 1.5 μM (Table 4).

Results of the χ^2 goodness of fit test among segregating (F_2-♂) F_2 families based on survivorship of seedlings at 1.5-μM *S*-metolachlor indicate that inheritance of *S*-metolachlor resistance fits a one-locus model ($\alpha = 0.05$; Table 4). Therefore, a single, dominant gene governs the 3:1 (R:S) phenotypic ratio and confers resistance to *S*-metolachlor at the 1.5-μM concentration in F_2-♂ lines (Table 4). By contrast, segregation of F_2-♀ lines best fits a two-locus model of 9:7 (R:S), consistent with two complementary genes wherein one homozygous recessive locus (rr) interacts with dominant allele(s) at a different locus (R-) controlling resistance (Bernardo 2014; Falconer 1989). The most plausible biochemical explanation for the single, dominant gene model is that *S*-metolachlor resistance in SIR is conferred by a single *P450* gene encoding a P450 enzyme that rapidly detoxifies the herbicide (Strom et al. 2020, 2021). However, the two-gene model described above is consistent with a single *P450* gene that primarily confers resistance, but a second recessive gene (if homozygous) decreases the magnitude of *S*-metolachlor resistance by an unknown mechanism.

A genetic mechanism that could explain why paternally versus maternally derived F_2 lines differed in their segregation ratios, and ultimately fit either one-gene or two-gene models (Table 4), is that the different SEN parent plants (SEN-♂ and SEN-♀) utilized in the reciprocal crosses (Figure 2) may not have been equally *S*-metolachlor sensitive. This scenario might have occurred, as the SEN population was not screened for sensitivity to *S*-metolachlor, but instead was presumed to be uniformly sensitive. For example, the SEN parent plant used to create the maternally derived lines (SEN-♂) may have been heterozygous or homozygous recessive for increased sensitivity to *S*-metolachlor at a second, different

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locus. A similar scenario was postulated to occur wherein two imidazolinone-resistant eastern black nightshade (*Solanum ptycanthum* Dunal) populations with identical target-site mutations and rates of imazamox metabolism exhibited different levels of whole-plant resistance (Volenberg et al. 2007).

S-Metolachlor Resistance: Mechanisms and Implications for Amaranthus tuberculatus Management

Our research posits a single, major gene confers *S*-metolachlor resistance in the SIR population and the full-sib near-inbred line, $DK_{3\text{-}2}$. Similarly, resistance to another VLCFAE-inhibiting herbicide, pyroxasulfone, in *L. rigidum* is conferred by a single, semi-dominant allele (Busi et al. 2014). The *S*-metolachlor resistance trait likely resulted from multiple years of preemergence and postemergence herbicide usage with the same sites-of-action (and/or similar biokinetic properties), which selected for gene(s) and enzyme(s) that metabolize multiple herbicides in SIR (Concepcion et al. 2021; Hausman et al. 2011, 2013; Jacobs et al. 2020; Obenland et al. 2019; Strom et al. 2019). However, it has yet to be determined genetically if these resistance cases are examples of cross-resistance (i.e., pleiotropic) or multiple resistance.

The crossing strategy used in our research (one R male × one R female) combined with recurrent selection produced a superior resistant line, $DK_{3\text{-}2}$, from a heterogenous SIR field population (Figure 1). This strategy was necessary, because the original SIR population had displayed variable responses in dose–response experiments with *S*-metolachlor compared with a different MHR (including VLCFAE-inhibitor resistance; Strom et al. 2019) *A. tuberculatus* population from central Illinois, CHR (Kerr 2021), indicating the possibility of segregation for resistance gene(s) in SIR.

It is possible a VLCFAE inhibitor–resistant population existed at the McLean County, Stanford, Illinois, site before field scouts noticed the lack of chemical control, particularly because *S*-metolachlor is typically not used alone for managing *A. tuberculatus* (Strom et al. 2022). Furthermore, resistant waterhemp may have occurred before Group 5 (PSII inhibitors; e.g., atrazine and simazine) and Group 27 herbicides (HPPD inhibitors; e.g., mesotrione, topramezone, tembotrione) no longer controlled weed escapes (Hausman et al. 2011; Strom et al. 2019). Thus, resistance to VLCFAE-inhibiting herbicides in *A. tuberculatus* represents a relatively new trait of interest that warrants further research to investigate the underlying gene involved and to better understand how selection pressures shaped the evolutionary history and successful adaptation of *A. tuberculatus* (Kreiner et al. 2022).

The $DK_{3\text{-}2}$ line as well as maternal- and paternal-derived F_1 and F_2 lines (Supplementary Table S1) provide unique genetic stocks for identifying the precise molecular mechanism conferring VLCFAE-inhibiting herbicide resistance. Although *S*-metolachlor resistance in *A. tuberculatus* is primarily conferred by enhanced metabolism—where P450 (primary) and GST (secondary) enzymes play important detoxification roles (Strom et al. 2020, 2021)—it is also possible transcription factors may affect expression of these metabolic genes or that altered target-site genes encoding the large family of plant VLCFAE elongases (Böger 2003; Haslam and Kunst 2013; Krähmer et al. 2019; Trenkamp et al. 2004) may play a secondary role. Ongoing work in our lab utilizing proteomic, transcriptomic, and tissue-specific expression analysis in roots, etiolated shoots, and leaves is aimed at identifying which specific metabolic enzyme confers VLCFAE-inhibitor resistance in *A. tuberculatus*. Future gene mapping research, using the pseudo-F_2 progenies generated by this work (Figure 2) combined with publicly available genomic resources (Montgomery et al. 2020), will aim to identify resistance genes and alleles. Metabolism-based phenotyping could also be conducted on our pseudo-F_2 lines to assist in comprehensively understanding the molecular-genetic basis of *S*-metolachlor resistance in SIR, which may ultimately lay the foundation for understanding VLCFAE-inhibiting herbicide resistance in additional MHR *A. tuberculatus* populations (Strom et al. 2019) as well as in other dicots (Brabham et al. 2019). A further understanding of the genetics and inheritance of VLCFAE inhibitor resistance in dioecious amaranths is crucial to assist with the identification of genes conferring resistance to preemergence herbicides, as well as to develop molecular markers for identification of resistant populations and streamline research efforts for improved management of *A. tuberculatus*.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/wsc.2023.63

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