

# Characterization of multiple herbicide-resistant waterhemp (*Amaranthus tuberculatus*) populations from Illinois to VLCFA-inhibiting herbicides

## Research Article

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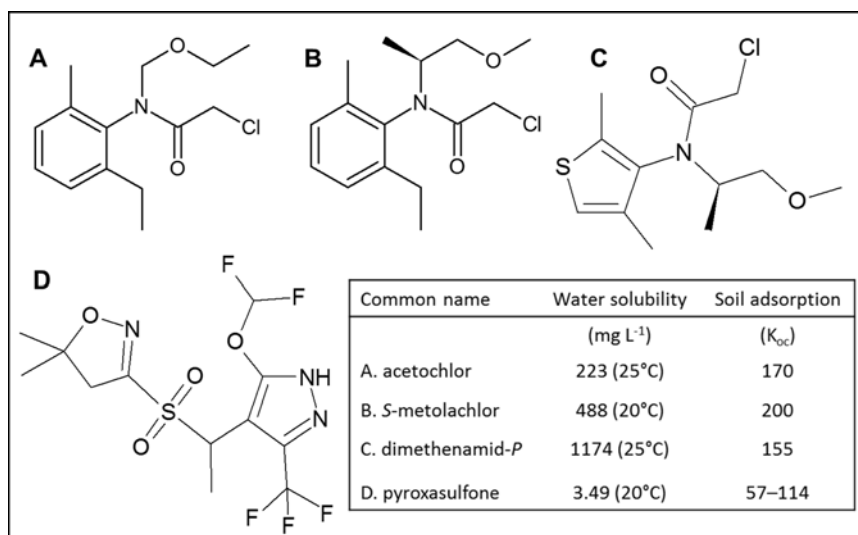
### Abstract

Field experiments were conducted in 2016 and 2017 in Champaign County, IL, to study a waterhemp [*Amaranthus tuberculatus* (Moq.) J. D. Sauer] population (CHR) resistant to 2,4-D and 4-hydroxyphenylpyruvate dioxygenase (HPPD)-, photosystem II-, acetolactate synthase (ALS)-, and protoporphyrinogen oxidase-inhibiting herbicides. Two field experiments were designed to investigate the efficacy of very-long-chain fatty-acid (VLCFA)-inhibiting herbicides, including a comparison of active ingredients at labeled use rates and a rate titration experiment. *Amaranthus tuberculatus* density and control were evaluated at 28 and 42 d after treatment (DAT). Nonencapsulated acetochlor, alachlor, and pyroxasulfone provided the greatest PRE control of CHR (56% to 75%) at 28 DAT, while metolachlor, S-metolachlor, dimethenamid-*P*, and encapsulated acetochlor provided less than 27% control. In the rate titration study, nonencapsulated acetochlor controlled CHR more than equivalent field use rates of S-metolachlor. Subsequent dose–response experiments with acetochlor, S-metolachlor, dimethenamid-*P*, and pyroxasulfone in the greenhouse included three multiple herbicide-resistant (MHR) *A. tuberculatus* populations: CHR-M6 (progeny generated from CHR), MCR-NH40 (progeny generated from Mclean County, IL), and ACR (Adams County, IL), in comparison with a sensitive population (WUS). Both CHR-M6 and MCR-NH40 are MHR to atrazine and HPPD, and ALS inhibitors and demonstrated higher survival rates (LD<sub>50</sub>) to S-metolachlor, acetochlor, dimethenamid-*P*, or pyroxasulfone than ACR (atrazine resistant but HPPD-inhibitor sensitive) and WUS. Based on biomass reduction (GR<sub>50</sub>), resistant to sensitive (R:S) ratios between CHR-M6 and WUS were 7.5, 6.1, 5.5, and 2.9 for S-metolachlor, acetochlor, dimethenamid-*P*, and pyroxasulfone, respectively. Values were greater for MCR-NH40 than CHR-M6, and ACR was the most sensitive to all VLCFA inhibitors tested. Complete control of all populations was achieved at or below a field use rate of acetochlor. In summary, field studies demonstrated CHR is not controlled by several VLCFA-inhibiting herbicides. Greenhouse dose–response experiments corroborated field results and generated R:S ratios (LD<sub>50</sub>) ranging from 4.5 to 64 for CHR-M6 and MCR-NH40 among the four VLCFA-inhibiting herbicides evaluated.

### Introduction

Waterhemp [*Amaranthus tuberculatus* (Moq.) J. D. Sauer] (Sauer 1955), a small-seeded, summer annual weed species, is controlled by most Group 15 very-long-chain fatty-acid (VLCFA)-inhibiting herbicides (Loux et al. 2018). *Amaranthus tuberculatus* is one of the most problematic weed species in corn (*Zea mays* L.) and soybean [*Glycine max* (L.) Merr.] production across the midwestern United States. This species is capable of producing thousands of seeds per female plant under suboptimal conditions (Steckel et al. 2003), and these seeds can be viable as early as 9 d after pollination (Bell and Tranel 2010). In addition, seed dormancy allows *A. tuberculatus* seedlings to emerge continually throughout the growing season (Buhler and Hartzler 2001; Hartzler et al. 1999). High reproductive output and prolonged emergence make residual chemical control essential for *A. tuberculatus* management (Steckel et al. 2002).

More troublesome than ecological factors is the inherent ability of *A. tuberculatus* to evolve resistance to herbicides from multiple sites of action (SOAs). *Amaranthus tuberculatus* is dioecious and an obligate-outcrossing species (Murray 1940; Sauer 1955), which increases intra-specific genetic variability compared with monoecious *Amaranthus* species. This high degree of



**Figure 1.** Chemical structures and physical properties of select very-long-chain fatty-acid-inhibiting active ingredients. Chemical structures generated via ACD/ChemSketch Freeware v. 17.01 (Advanced Chemistry Development, Toronto, ON, Canada; [www.acdlabs.com](http://www.acdlabs.com)). Chemical properties obtained from Shaner (2014) or Jhala (2017).

genetic variability allows weediness traits, including herbicide resistance, to spread among and within populations (Steckel 2007; Tranel et al. 2011). To date, *A. tuberculatus* is resistant to herbicides from six SOAs, including herbicides that inhibit 4-hydroxyphenylpyruvate dioxygenase (HPPD), photosystem II (PSII), acetolactate synthase (ALS), protoporphyrinogen oxidase (PPO), and 5-enolpyruvylshikimate-3-phosphate synthase and the synthetic auxin 2,4-D (Heap 2018). In addition, resistance to herbicides from various SOAs has stacked within *A. tuberculatus* populations, resulting in plants with resistance to herbicides from three, four, five, and six SOA groups (Bell et al. 2013; Evans 2016; Patzoldt et al. 2005; Shergill et al. 2018).

The biology of *A. tuberculatus* makes it well suited to compete with summer annual crops such as corn, soybean, and sorghum [*Sorghum bicolor* (L.) Moench] (Steckel 2007). In corn, the greatest yield losses result from interference up to the V6 growth stage, with the potential for more than 50% yield loss (Steckel and Sprague 2004). In total, 74% yield loss in corn is possible if season-long competition occurs (Steckel and Sprague 2004). *Amaranthus tuberculatus* interference can also cause more than 40% yield reduction in soybean (Hager et al. 2002b). Given the increasing frequency of populations with resistance to herbicides from multiple SOAs, integrated strategies are essential for *A. tuberculatus* management (Hager et al. 1997; Tranel et al. 2011). Incorporating multiple, effective SOAs each season is one strategy to reduce the frequency of resistant plants that survive and further contribute to the soil seedbank (Evans et al. 2016). VLCFA-inhibiting herbicides are labeled for use in many crops and remain an effective way to incorporate an additional SOA with residual activity into integrated management programs.

Chloroacetamide herbicides have been used for PRE control of grass and small-seeded dicot weeds since their discovery and commercialization in the 1950s (Hamm 1974). Active ingredients from this family belong to the Group 15 class of herbicides and control sensitive species by inhibiting the VLCFA elongase complex, resulting in depletion of VLCFAs in excess of 18 carbons (Bach and Faure 2010; Böger 2003). Sensitive dicot weed seedlings either fail to emerge or remain in an arrested state of growth soon after cotyledon expansion (Deal and Hess 1980; Dhillon and Anderson 1972; Fuerst 1987; Pillai et al. 1979).

Resistance to VLCFA inhibitors is a relatively rare occurrence. To date, only five species of grasses worldwide are resistant to herbicides from this class, with resistance in dicot weed species yet to be documented (Heap 2018). The low level of resistance to these PRE herbicides could be explained by naturally less intense selection pressure than POST herbicides, such as glyphosate, and the inability of surviving weeds to overcome subsequent POST herbicides (Somerville et al. 2017). In addition, mutations in VLCFA elongases are unlikely, as any change in the target site might alter the ability of normal substrates to bind (due to competitive inhibition kinetics) and, more likely, multiple enzymatic detoxification processes must be present in order to confer resistance (Böger 2003). In nearly all cases, resistance to VLCFA-inhibiting herbicides occurs in multiple herbicide-resistant (MHR) populations, potentially through cross-resistance mechanisms or indirect selection through the use of herbicides from other SOAs (Burnet et al. 1994; Busi and Powles 2016).

Research results from investigating a population of *A. tuberculatus* resistant to HPPD-inhibiting herbicides in Mclean County, IL (Hausman et al. 2011), indicated that S-metolachlor provided significantly less PRE control than anticipated (Hausman et al. 2013). This population (designated MCR) was subsequently characterized as resistant to HPPD-, ALS-, and PSII-inhibiting herbicides (Hausman et al. 2011, 2013, 2016). During research conducted on a separate population from Champaign County, IL, with resistance to HPPD, ALS, PSII, and PPO inhibitors and 2,4-D (designated CHR), similar observations in regard to less than expected control were made with S-metolachlor (Evans 2016). In both cases, another herbicide from the same class, acetochlor (Figure 1), remained relatively effective (Evans 2016; Hausman et al. 2013). Based on similarities between these MHR populations, experiments were designed to explore the consistent differences in PRE control among various VLCFA-inhibiting herbicides. The objectives of this research were to investigate the response of the CHR population to VLCFA-inhibiting herbicides and specifically compare acetochlor and S-metolachlor at different rates under field conditions. Experiments were subsequently initiated under greenhouse conditions to compare the response of CHR to VLCFA inhibitors with that of other populations, including the MCR population, for which the initial field

**Table 1.** Cumulative precipitation recorded at the Champaign County, IL, field location during 2016–2017 field experiments.

Days after herbicide application (DAA)	Precipitation	
	2016	2017
	cm	
0–3	0.66	3.1
0–7	0.66	3.9
0–42	23.3	11.4

observations of reduced efficacy had been made (Hausman et al. 2013).

## Materials and methods

### Characterization of a multiple herbicide-resistant *Amaranthus tuberculatus* population response to VLCFA-inhibiting herbicides under field conditions

#### General field methods

Field experiments were conducted in 2016 and 2017 at a site in Champaign County, IL, where the CHR population with multiple resistance to HPPD-, PPO-, PSII-, and ALS-inhibiting herbicides plus the synthetic auxin 2,4-D was documented (Evans 2016). Past row-crop history of the field included an annual rotation of non-GMO corn and glyphosate-resistant soybean.

The soil at the site is a Flanagan silt loam (fine, smectitic, mesic Aquic Argiudolls) with a pH of 5.5 and 4.8% organic matter. The soil was tilled before herbicide application to control existing vegetation, and experiments were established in nonplanted stale seedbeds. The natural *A. tuberculatus* population was evenly distributed across the location. Individual plots measured 3 by 7.6 m and were arranged in a randomized complete block design with four replications in 2016 and three replications in 2017. Cumulative precipitation during field experiments in 2016 and 2017 is presented in Table 1.

Two field experiments included a VLCFA-inhibitor comparison and a herbicide rate titration. For the herbicide-comparison experiment, eight commercially available VLCFA-inhibiting herbicides were selected and compared at 1X doses based on manufacturers' labeled recommendations for the soil type (Table 2). The rate titration experiment included three VLCFA inhibitors (non-encapsulated acetochlor, *S*-metolachlor, and pyroxasulfone), each applied at 0.5X, 1X, 2X, and 4X doses based on the 1X manufacturer recommended rate (Table 2). Pyroxasulfone is a pyrazole and is applied at a much lower use rate; it was included in experiments due to having the same SOAs and target weed spectrum as chloroacetamide herbicides (Nakatani et al. 2016; Tanetani et al. 2009, 2011). Treatments were applied on May 23, 2016, and June 14, 2017 with a pressurized CO<sub>2</sub> backpack sprayer calibrated to deliver 187 L ha<sup>-1</sup> at 276 kPa with a 3-m boom consisting of six AI110025VS nozzles (TeeJet® Technologies, P.O. Box 7900, Wheaton, IL 60187) spaced 51 cm apart.

Visual ratings of *A. tuberculatus* control were recorded at 28 and 42 d after treatment (DAT) using a scale of 0% (no control) to 100% (complete control). Ratings considered *A. tuberculatus* injury, biomass reduction, and emergence compared with a non-treated control. *Amaranthus tuberculatus* density was recorded within two 0.25-m<sup>2</sup> quadrats at 28 and 42 DAT. Quadrats were randomly placed in the center of each plot, and counts were taken within the same area both times. *Amaranthus tuberculatus* biomass was harvested from each quadrat at 42 DAT, samples were

combined and dried in a forced-air dryer at 65 C, and dry biomass was recorded.

#### Statistical analysis

Statistical analysis was conducted with SAS v. 9.4 (SAS Institute, 100 SAS Campus Drive, Cary, NC 27513). Biomass, density, and control ratings were analyzed by ANOVA in PROC MIXED. *Amaranthus tuberculatus* density measurements were analyzed as a repeated measure. For the VLCFA-inhibitor comparison study, treatment was considered a fixed effect, while in the rate titration study, treatment, rate, and their interactions were considered fixed effects. Year and block within year were considered random effects in both experiments. Initial analysis revealed a significant treatment by days after treatment interaction for *A. tuberculatus* density in the herbicide-comparison study. Mean estimates were separated by LSD using the SAS macro %pdmix800 (Saxton 1998) with  $\alpha = 0.05$ . The assumption of normally distributed residuals was reviewed with PROC UNIVARIATE, and homogeneous variance was verified with PROC GLM. Density values were subject to a square-root transformation to meet assumptions, and back-transformed data are presented as plants per square meter.

#### VLCFA-inhibitor greenhouse dose-response experiments

##### *Amaranthus tuberculatus* populations

Four *A. tuberculatus* populations were selected to characterize their response to *S*-metolachlor, acetochlor, dimethenamid-*P*, and pyroxasulfone. CHR-M6 is the progeny of greenhouse crosses and was confirmed resistant to atrazine and HPPD-, PPO-, and ALS-inhibiting herbicides plus 2,4-D (Evans 2016). Similarly, MCR-NH40, analogous to the HPPD inhibitor-resistant and atrazine-resistant NH41 population described in Hausman et al. (2013), was included for comparison with CHR-M6 (Evans 2016). Two HPPD inhibitor-sensitive populations (ACR, WUS) were included to calculate R:S ratios. ACR is resistant to atrazine (due to rapid metabolism; Ma et al. 2013) and ALS- and PPO-inhibiting herbicides (Patzoldt et al. 2005), while WUS is not resistant to any herbicide. All seeds were stratified according to methods previously described (Bell et al. 2013) and stored at 4 C for at least 20 d before experiment initiation to improve germination.

##### Plant culture

Plastic cell-pack inserts (384 cm<sup>3</sup> per cell) were filled halfway with growth medium (1:1:1 mixture of soil, peat, and sand, with a pH of 6.4 and 3.5% organic matter) and slow-release fertilizer (Scotts Osmocote® Classic 13-13-13, Scotts Company, 14111 Scottslawn Road, Marysville, OH 43041) was added. Each cell was then filled to the top, lightly tamped, and then soaked in water for 12 h to provide a uniformly moist seedbed. Fifteen seeds from each population were sown on the soil surface in a 3 by 5 grid with 1 cm between each of the outside rows and the side of the insert. After sowing, 10 ml of the same growth medium was passed through a 3.5-mm sieve and spread evenly across the surface. Plants were supplied weekly with water-soluble fertilizer (Peters® 20-20-20, Everris NA, P.O. Box 3310, Dublin, OH 43016) and greenhouse conditions were maintained at 28/22 C day/night with a 16-h photoperiod. Natural sunlight was supplemented with mercury-halide lamps to provide 800  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photon flux at the soil surface.

**Table 2.** Very-long-chain fatty-acid-inhibiting herbicides, rates, and source information for field studies at the Champaign County, IL, site (2016–2017).<sup>a</sup>

Common name	Trade name	Application rate	Manufacturer
		kg ai ha <sup>-1</sup>	
Acetochlor <sup>b</sup>	Warrant <sup>®</sup>	2.5	Bayer CropScience, St Louis, MO 63137; <a href="http://www.cropscience.bayer.com">www.cropscience.bayer.com</a>
Acetochlor	Harness <sup>®</sup>	2.7	Bayer CropScience, St Louis, MO 63137; <a href="http://www.cropscience.bayer.com">www.cropscience.bayer.com</a>
S-metolachlor	Dual Magnum <sup>®</sup>	2.1	Syngenta Crop Protection, Greensboro, NC 27419; <a href="http://www.syngentacropprotection.com">www.syngentacropprotection.com</a>
S-metolachlor <sup>c</sup>	Dual II Magnum <sup>®</sup>	2.1	Syngenta Crop Protection, Greensboro, NC 27419; <a href="http://www.syngentacropprotection.com">www.syngentacropprotection.com</a>
Metolachlor	Stalwart <sup>®</sup>	2.2	SipcamAgro, Durham, NC 27713; <a href="http://www.sipcamadvan.com">www.sipcamadvan.com</a>
Dimethenamid- <i>P</i>	Outlook <sup>®</sup>	1.1	BASF Corporation Agricultural Products, Research Triangle Park, NC 27709; <a href="http://www.agro.basf.com">www.agro.basf.com</a>
Alachlor	Intrro <sup>®</sup>	3.6	Bayer CropScience, St Louis, MO 63137; <a href="http://www.cropscience.bayer.com">www.cropscience.bayer.com</a>
Pyroxasulfone	Zidua <sup>®</sup>	0.2	BASF Corporation Agricultural Products, Research Triangle Park, NC 27709; <a href="http://www.agro.basf.com">www.agro.basf.com</a>

<sup>a</sup> Herbicides were applied to bare ground before weed emergence.

<sup>b</sup> Encapsulated formulation.

<sup>c</sup> Contains the herbicide safener benoxacor.

**Table 3.** Mean estimates<sup>a</sup> of *Amaranthus tuberculatus* control at 28 and 42 d after treatment (DAT), density at 28 and 42 DAT, and recovered biomass at 42 DAT of very-long-chain fatty-acid-inhibiting herbicides (2016–2017).

Herbicide	Rate	Control		Density		Biomass
		28 DAT	42 DAT	28 DAT	42 DAT	42 DAT
	kg ai ha <sup>-1</sup>	%		plants m <sup>-2</sup>		g m <sup>-2</sup>
Acetochlor <sup>b</sup>	2.5	23 b	3 d	79 bcd	79 bcd	284 abc
Acetochlor	2.7	75 a	42 a	13 a	14 a	135 a
S-metolachlor	2.1	16 b	6 d	126 d	104 cd	427 c
S-metolachlor <sup>c</sup>	2.1	27 b	10 cd	95 cd	97 cd	381 bc
Metolachlor	2.2	14 b	3 d	136 d	134 d	425 c
Dimethenamid- <i>P</i>	1.1	13 b	3 d	96 cd	94 cd	407 c
Alachlor	3.6	67 a	31 ab	21 ab	26 ab	241 ab
Pyroxasulfone	0.24	56 a	21 bc	34 abc	36 abc	276 abc
Nontreated	—	—	—	152 d	137 d	402 bc

<sup>a</sup> Mean estimates followed by the same letter within a column are not significantly different at  $\alpha = 0.05$  (separated by LSD using the SAS macro %pdmix800).

<sup>b</sup> Encapsulated formulation.

<sup>c</sup> Contains the herbicide safener benoxacor.

### Herbicide application

All herbicides were applied PRE at increasing rates on a base 3.16 logarithmic scale. Each population was treated with *S*-metolachlor at rates ranging 8.4 to 8,390 g ai ha<sup>-1</sup>, acetochlor at rates ranging from 2.5 to 2,530 g ai ha<sup>-1</sup>, dimethenamid-*P* at rates ranging from 3.4 to 3,470 g ai ha<sup>-1</sup>, or pyroxasulfone at rates ranging from 0.8 to 780 g ai ha<sup>-1</sup>. Herbicides were applied with a compressed-air research sprayer (Generation III Research Sprayer, DeVries Manufacturing, 86956 State Highway 251, Hollandale, MN 56045) fitted with a single TeeJet<sup>®</sup> 80015EVS nozzle set 46 cm above the medium surface and calibrated to deliver 187 L ha<sup>-1</sup> at 275 kPa. After herbicide application, a method modified from Busi and Powles (2016) was used to add an additional 25 ml of sieved growth medium on top of the treated medium to simulate mechanical incorporation of the herbicide. Pots were then returned to the greenhouse and maintained beneath an overhead misting system. The mister was equipped with 0.4 L min<sup>-1</sup> nozzles (Netafim<sup>™</sup> USA, 5470 East Home Avenue, Fresno, CA 93727) and programmed to cycle three times daily in order to maintain adequate soil moisture.

### Data collection and statistical analysis

In all experiments, each treatment by population combination consisted of three replications. Dose–response experiments with acetochlor and *S*-metolachlor were performed simultaneously and conducted twice. Dose–response experiments with dimethenamid-*P* and pyroxasulfone were performed simultaneously and conducted three times. At 21 DAT, surviving seedlings were counted, and aboveground biomass was harvested. Harvested plants were placed in a forced-air dryer set at 65 C for 7 d,

and dry biomass was recorded. Statistical analysis was performed using R software v. 3.4.2. Pooled biomass and survival data were then analyzed using nonlinear regression in the DRC package in R (Knezevic et al. 2007). Data derived from plant population effects within acetochlor, *S*-metolachlor, dimethenamid-*P*, and pyroxasulfone herbicide treatments were analyzed separately. The four-parameter dose–response logistic function was fit using the following equation:

$$y = c + \frac{d-c}{1 + \exp\{b[\log(x) - \log(LD_{50}/GR_{50})]\}} \quad [1]$$

where *b* is the slope of the curve, *c* is the lower asymptote, *d* is the upper asymptote, and LD<sub>50</sub> and GR<sub>50</sub> are the 50% reduction in seedling survival and plant biomass, respectively. Initially LD<sub>50</sub> and GR<sub>50</sub> values were calculated for each individual experimental run. Effective dose values were then analyzed by ANOVA via a general linear mixed-effects model in the NLME package in R to test the main effects of treatment, population, and their interactions. Experimental run was considered a random effect in order to test the significance of main effects across both experimental runs.

## Results and Discussion

### Characterization of a multiple herbicide-resistant *Amaranthus tuberculatus* population response to VLCFA-inhibiting herbicides under field conditions

#### VLCFA-inhibitor comparison

*Amaranthus tuberculatus* control was 75% or less at 28 DAT for all herbicides tested (Table 3). Nonencapsulated acetochlor, alachlor,



**Table 4.** Mean estimates<sup>a</sup> of *Amaranthus tuberculatus* control at 28 and 42 d after treatment (DAT) from the very-long-chain fatty-acid-inhibitor rate titration study (2016–2017).

Treatment	Rate	Control	
		28 DAT	42 DAT
	X <sup>b</sup>		
	kg ha <sup>-1</sup>		%
Acetochlor	0.5	1.4	44 cd
	1	2.7	67 bc
	2	5.4	85 ab
	4	10.8	94 a
S-metolachlor	0.5	1.1	10 e
	1	2.1	10 e
	2	4.2	20 e
	4	8.4	45 bcd
Pyroxasulfone	0.5	0.12	27 de
	1	0.24	40 de
	2	0.48	63 abc
	4	0.96	74 abc

<sup>a</sup> Mean estimates followed by the same letter within a column are not significantly different at  $\alpha = 0.05$  (separated by the SAS macro %pdmix800).

<sup>b</sup> Relative rate based on manufacturers' labeled recommendations.

and pyroxasulfone provided the greatest control of the CHR population, providing 75%, 67%, and 56% control, respectively. Control did not exceed 27% for all other treatments. In addition, nonencapsulated acetochlor, alachlor, and pyroxasulfone provided the greatest density reduction at 28 DAT (12, 21, and 34 plants m<sup>-2</sup>, respectively), which corresponds with visual estimations of control. *Amaranthus tuberculatus* densities among other treatments were not different from those of nontreated plots.

Results at 28 DAT are similar to previous studies conducted from 2014 to 2015 at the CHR location. In previous studies, a labeled rate of nonencapsulated acetochlor provided 88% control of CHR, while S-metolachlor did not exceed 46% control at 28 DAT (Evans 2016). At the MCR location, where a large difference in efficacy between S-metolachlor and nonencapsulated acetochlor was initially documented (Hausman et al. 2013), greater than 83% control was measured with a labeled rate of nonencapsulated acetochlor, while S-metolachlor provided less than 18% control at 30 DAT. In contrast, *A. tuberculatus* control greater than 98% and 95% with nonencapsulated acetochlor and S-metolachlor, respectively, has been reported at 28 d after planting (DAP) (Steckel et al. 2002), which is much greater than control documented in the present research.

In addition to the differences noted between nonencapsulated acetochlor and S-metolachlor, pyroxasulfone provided less control of CHR than demonstrated with other HPPD inhibitor-resistant and atrazine-resistant populations. Greater than 87% control was recorded for the MCR *A. tuberculatus* population (Hausman et al. 2013) and 83% to 95% control was reported for an HPPD inhibitor-resistant population from Nebraska (Oliveira et al. 2017). Control of MCR with alachlor was 62% to 78% at 30 DAT (Hausman et al. 2013), which is consistent with the results from the present research.

No PRE treatment exceeded 42% control at 42 DAT (Table 3). Control with nonencapsulated acetochlor was 42%, while alachlor and pyroxasulfone provided 31% and 21% control, respectively. All other treatments provided less than 10% control. Plots treated with nonencapsulated acetochlor, alachlor, and pyroxasulfone provided the greatest density reduction at 42 DAT (14, 26, and 36 plants m<sup>-2</sup>, respectively), similar to results at 28 DAT. *Amaranthus tuberculatus* densities among other treatments were not different

from those of nontreated plots. Only two treatments (nonencapsulated acetochlor and alachlor) reduced biomass more than the nontreated control at 42 DAT. Biomass from plots treated with nonencapsulated acetochlor and alachlor was 135 and 241 g m<sup>-2</sup>, respectively.

In previous studies conducted at the CHR location from 2014 to 2015, control with labeled rates of nonencapsulated acetochlor and S-metolachlor was 70% and 5%, respectively, at 42 DAT (Evans 2016). Control with nonencapsulated acetochlor was greater than that recorded in the present research at 42 DAT, but the difference in control with nonencapsulated acetochlor and S-metolachlor remained similar. In contrast, 57% control of *A. tuberculatus* with S-metolachlor and 61% control with nonencapsulated acetochlor at 56 DAP has been reported (Steckel et al. 2002). Moreover, greater than 80% control of *A. tuberculatus* has been achieved with S-metolachlor at 8 wk after planting (Johnson et al. 2012), and 80% control with racemic metolachlor has been documented at 4 wk after planting (Hager et al. 2002a).

Results at 28 and 42 DAT also revealed a large difference in PRE control with encapsulated and nonencapsulated acetochlor. The dissimilar control of CHR could be due to the extended-release characteristics of the encapsulated formulation. Initially, emerging seedlings in plots treated with encapsulated acetochlor were likely exposed to less active herbicide than those in plots treated with nonencapsulated acetochlor, resulting in less control and higher plant densities at 28 and 42 DAT.

#### VLCFA-inhibitor rate titration

Nonencapsulated acetochlor provided greater control of CHR at 28 DAT when compared with 0.5X or 1X rates of pyroxasulfone or any rate of S-metolachlor (Table 4). A 1X rate of nonencapsulated acetochlor controlled CHR 67%, which is slightly less than control reported for the comparison study (Table 3). Treatments providing the greatest control were nonencapsulated acetochlor at 2X (85%) and 4X (94%) rates and pyroxasulfone at 2X (63%) and 4X (74%) rates. S-metolachlor, regardless of rate, did not provide greater than 45% control of CHR. Previous studies at the CHR location at 28 DAT reported 79% to 95% control with nonencapsulated acetochlor, 48% to 84% control with pyroxasulfone, and 20% to 62% control with S-metolachlor applied at 0.5 to 2X rates (Evans 2016).

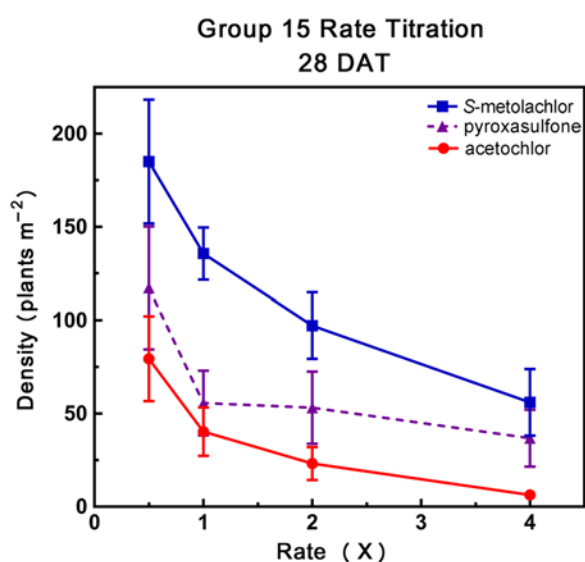
In general, *A. tuberculatus* density (plants m<sup>-2</sup>) at 28 DAT decreased as herbicide rate increased (Figure 2). Densities in plots treated with nonencapsulated acetochlor were 4 to 73 plants m<sup>-2</sup>. Pyroxasulfone-treated plots had densities ranging from 32 to 117 plants m<sup>-2</sup>, and plots treated with S-metolachlor had plant densities ranging from 52 to 204 plants m<sup>-2</sup>. Selected 1 df contrasts were performed between increasing rates of nonencapsulated acetochlor and a 4X rate of S-metolachlor (Table 5). *Amaranthus tuberculatus* densities at 28 DAT in plots treated with 0.5X, 1X, and 2X rates of nonencapsulated acetochlor were not significantly different from those of plots treated with a 4X rate of S-metolachlor.

All treatments, regardless of rate, provided less control of the CHR population by 42 DAT than at 28 DAT (Table 4). Overall, the highest levels of control were achieved with nonencapsulated acetochlor or pyroxasulfone at 2X and 4X rates. S-metolachlor at a 4X rate provided 23% control at 42 DAT, and all other rates provided less than 10% control. Previous studies at the CHR location at 42 DAT reported 54% to 85% control with nonencapsulated acetochlor, 17% to 75% control with pyroxasulfone, and 5% to 39% control with S-metolachlor applied at 0.5 to 2X rates (Evans 2016).

In general, *A. tuberculatus* density (plants m<sup>-2</sup>) at 42 DAT decreased as herbicide rate increased (Figure 3). Densities in plots

**Table 5.** Select contrasts of *Amaranthus tuberculatus* density at 28 and 42 d after treatment (DAT) and recovered biomass at 42 DAT between a rate of *S*-metolachlor compared with increasing rates of acetochlor from the very-long-chain fatty-acid-inhibitor rate titration study (2016–2017).

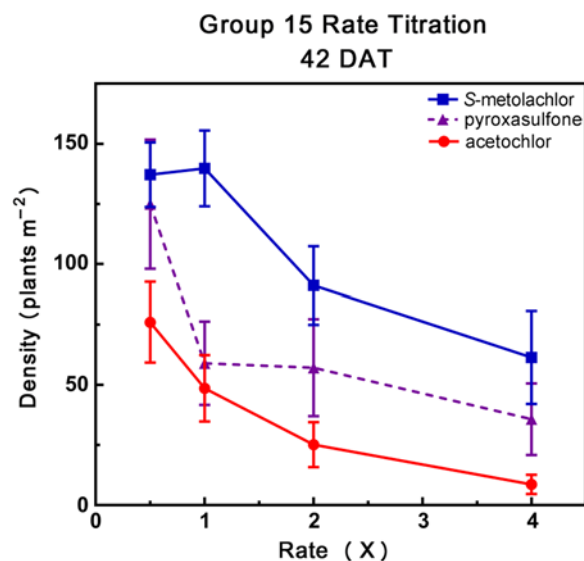
Contrast	Density		Biomass
	28 DAT	42 DAT	42 DAT
	—P-value—		
<i>S</i> -metolachlor (4) < acetochlor (0.5)	0.38	0.46	0.47
<i>S</i> -metolachlor (4) < acetochlor (1)	0.57	0.80	0.38
<i>S</i> -metolachlor (4) < acetochlor (2)	0.13	0.15	0.12
<i>S</i> -metolachlor (4) < acetochlor (4)	0.03	0.04	0.01



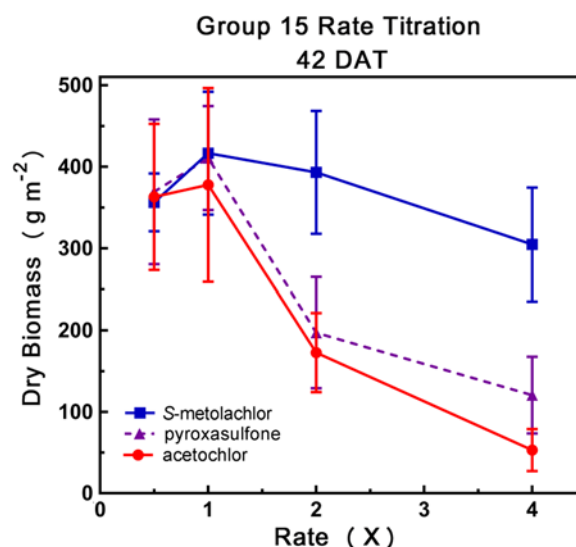
**Figure 2.** Relationship of *Amaranthus tuberculatus* density at 28 DAT at the Champaign County, IL, location in response to increasing rates of three very-long-chain fatty-acid inhibitors (2016–2017). A continuous line with solid squares signifies treatments of *S*-metolachlor; a discontinuous line with solid triangles signifies treatments of pyroxasulfone; and a continuous line with solid circles represents treatments of acetochlor. Symbols on each line signify the mean  $\pm$  SE.

treated with nonencapsulated acetochlor were 7 to 71 plants  $m^{-2}$ . Pyroxasulfone-treated plots had densities ranging from 31 to 118 plants  $m^{-2}$ , and plots treated with *S*-metolachlor had plant densities ranging from 56 to 137 plants  $m^{-2}$ . Selected 1 df contrasts between increasing rates of nonencapsulated acetochlor and a 4X rate of *S*-metolachlor are presented (Table 5). *Amaranthus tuberculatus* densities at 42 DAT in plots treated with 0.5X, 1X, and 2X rates of nonencapsulated acetochlor were not significantly different from plots treated with a 4X rate of *S*-metolachlor.

In all treatments at 42 DAT, biomass decreased in response to 1X to 4X rates of each herbicide (Figure 4). Biomass from plots treated with nonencapsulated acetochlor ranged from 356 to 38  $g m^{-2}$  and pyroxasulfone ranged from 393 to 104  $g m^{-2}$  as rates increased. Overall, reduction in plant biomass was variable, and *S*-metolachlor-treated plots had biomass ranging from 417 to 285  $g m^{-2}$  as rates increased. Surprisingly, 1X rates of all treatments had slightly greater biomass than plots treated with 0.5X rates. Plots treated with 1X rates had lower *A. tuberculatus* densities than plots treated with 0.5X rates (Figure 3). This unusual observation may be explained by lower intraspecific competition or, potentially, hormesis (Belz and Duke 2014). Selected 1 df contrasts between increasing rates of acetochlor and a 4X rate of *S*-metolachlor at



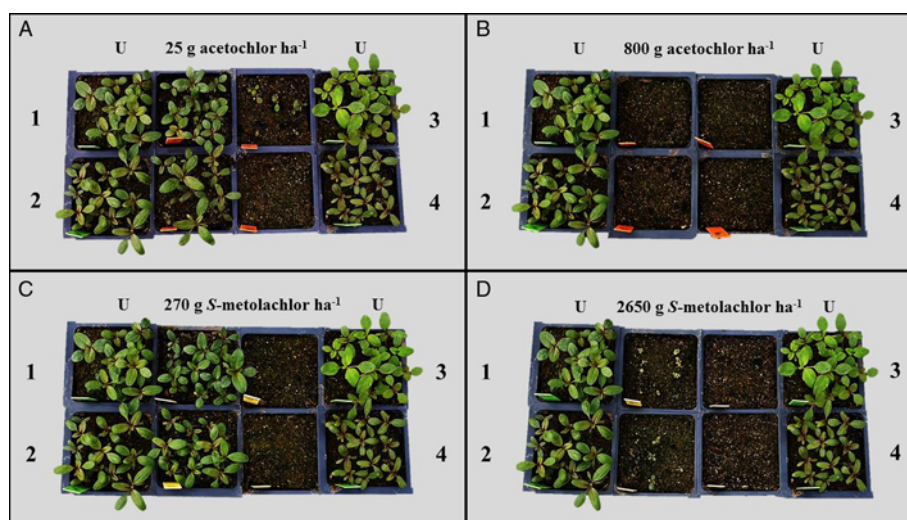
**Figure 3.** Relationship of *Amaranthus tuberculatus* density at 42 DAT at the Champaign County, IL, location in response to increasing rates of three very-long-chain fatty-acid inhibitors (2016–2017). A continuous line with solid squares signifies treatments of *S*-metolachlor; a discontinuous line with solid triangles signifies treatments of pyroxasulfone; and a continuous line with solid circles represents treatments of acetochlor. Symbols on each line signify the mean  $\pm$  SE.



**Figure 4.** Relationship of accumulated biomass per square meter ( $g m^{-2}$ ) at 42 DAT at the Champaign County, IL, location in response to increasing rates of three very-long-chain fatty-acid inhibitors (2016–2017). A continuous line with solid squares signifies treatments of *S*-metolachlor; a discontinuous line with solid triangles signifies treatments of pyroxasulfone; and a continuous line with solid circles represents treatments of acetochlor. Symbols on each line signify the observed mean  $\pm$  SE.

28 and 42 DAT (Table 5) showed that biomass recovered from plots treated with 0.5X, 1X, and 2X rates of nonencapsulated acetochlor were not significantly different from that recovered from plots treated with a 4X rate of *S*-metolachlor.

The VLCFA inhibitors included at the CHR site possess different physicochemical properties, such as water solubility and soil-binding characteristics (Figure 1), and their efficacy can be affected by environmental factors and the timing of *A. tuberculatus* emergence. Greenhouse dose–response experiments were designed to compare VLCFA-inhibiting herbicides with a uniform soil type



**Figure 5.** Response of four *Amaranthus tuberculatus* populations at 14 DAT to (A) 25 g acetochlor ha<sup>-1</sup>, (B) 800 g acetochlor ha<sup>-1</sup>, (C) 270 g *S*-metolachlor ha<sup>-1</sup>, and (D) 2,650 g *S*-metolachlor ha<sup>-1</sup>. In each frame, outside columns are nontreated (signified by “U”) and treated containers are located in the center below the labeled rate. Populations (1, CHR-M6; 2, MCR-NH40; 3, WUS; 4, ACR) are displayed with nontreated nearest number and corresponding treatment directly adjacent. Herbicide treatments were applied directly to the soil PRE.

and watering regimen. Limiting environmental variability allowed comparison of the responses of CHR and other *A. tuberculatus* populations to various VLCFA-inhibiting herbicides.

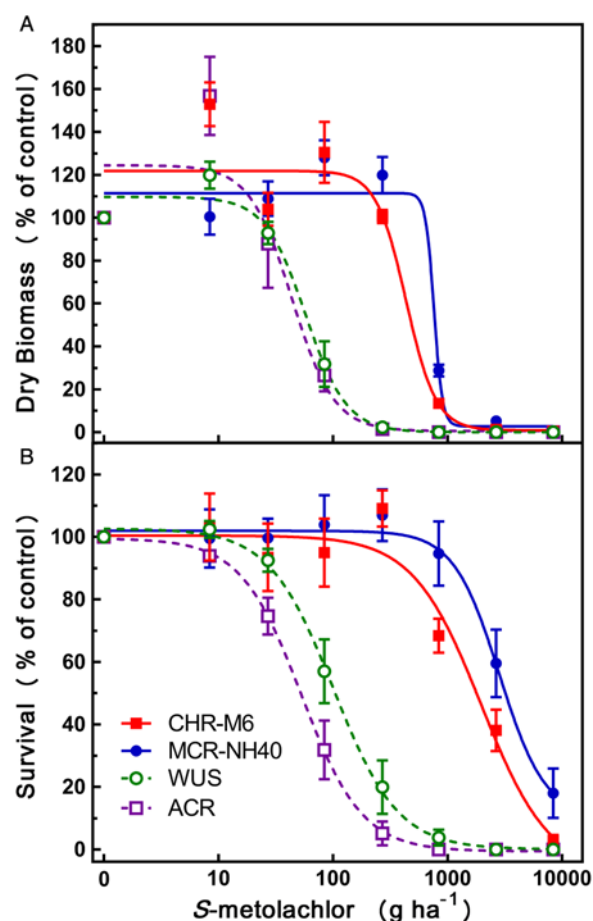
#### VLCFA-inhibitor greenhouse dose-response experiments

Following herbicide application, *A. tuberculatus* seedlings had emerged consistently in each insert by 5 DAT. Plants from each population initially appeared healthy, but by 7 DAT, treated plants began exhibiting less vigor than nontreated plants. This pattern continued throughout the entire experiment, and affected plants remained in an arrested state of growth or died during the 21-d period (unpublished data). Similar symptomology has been previously reported for various dicot species following treatment with VLCFA-inhibiting herbicides (Deal and Hess 1980; Dhillon and Anderson 1972; Fuerst 1987). Initial ANOVA analysis of LD<sub>50</sub> and GR<sub>50</sub> values revealed significant population, treatment, and population by treatment interactions across experimental replications.

#### Dose response to *S*-metolachlor

Both HPPD inhibitor-sensitive populations (ACR and WUS) were controlled at 270 g ha<sup>-1</sup> or less by 21 DAT (Figures 5 and 6). In contrast, both CHR-M6 and MCR-NH40 survived 2.7 kg ha<sup>-1</sup> (Figure 5), and on occasion, replications (individual containers) of either population survived 8.4 kg ha<sup>-1</sup>. Survivors from replicates treated with 2.7 or 8.4 kg ha<sup>-1</sup> were severely stunted, but plants continued growing with at least one true leaf. Extra inserts treated with 2.7 kg ha<sup>-1</sup> were allowed to persist past 21 DAT and eventually progressed through full reproductive development (unpublished data).

Dose-response curves to *S*-metolachlor (Figure 6) and LD<sub>50</sub> values (Table 6) indicate a positive shift in rate needed to control either HPPD inhibitor-resistant and atrazine-resistant population (CHR-M6 or MCR-NH40). Based on LD<sub>50</sub> values, calculated resistant to sensitive (R:S) values for CHR-M6 and MCR-NH40 are 17.9 and 33.3, respectively, when compared with WUS, and 34.3 and 63.8 in comparison with ACR, respectively. Similar to LD<sub>50</sub> values, GR<sub>50</sub> values also demonstrated a positive shift in



**Figure 6.** Herbicide dose-response experiments of four *Amaranthus tuberculatus* populations treated with soil-applied *S*-metolachlor. Data were collected at 21 DAT. (A) Dry biomass as a percentage of nontreated control, and (B) mean survival of *A. tuberculatus* seedlings as a percentage of nontreated control. Lines in each graph were fit using Equation 1:  $(y = c + \frac{d-c}{1 + \exp[b(\log(x) - \log(LD_{50}/GR_{50})] )})$ , and each symbol represents the observed mean  $\pm$  SE. CHR-M6, continuous line and full squares; MCR-NH40, continuous line and full circles; WUS, discontinuous line and open circles; ACR, discontinuous line and open squares.



**Table 6.** Pooled greenhouse dose–response data to assess differences among *Amaranthus tuberculatus* populations.<sup>a</sup>

Population	LD <sub>50</sub> GR <sub>50</sub>	
	g ha <sup>-1</sup>	
	Response to <i>S</i> -metolachlor	
CHR-M6	1808 (±329)	431 (±61)
MCR-NH40	3360 (±624)	742 (±237)
ACR	53 (±9)	44 (±7)
WUS	101 (±16)	57 (±9)
	Response to acetochlor	
CHR-M6	178 (±24)	72 (±33)
MCR-NH40	226 (±34)	80 (±2)
ACR	13 (±2)	5 (±1)
WUS	40 (±4)	12 (±2)
	Response to dimethenamid- <i>P</i>	
CHR-M6	729 (±221)	65 (±15)
MCR-NH40	1462 (±314)	102 (±24)
ACR	26 (±8)	6 (±2)
WUS	35 (±15)	12 (±4)
	Response to pyroxasulfone	
CHR-M6	65 (±23)	5 (±1)
MCR-NH40	153 (±37)	8 (±2)
ACR	9 (±3)	1 (±0.2)
WUS	10 (±4)	2 (±0.3)

<sup>a</sup> Estimated LD<sub>50</sub> and GR<sub>50</sub> values followed by their standard errors of the mean in parentheses.

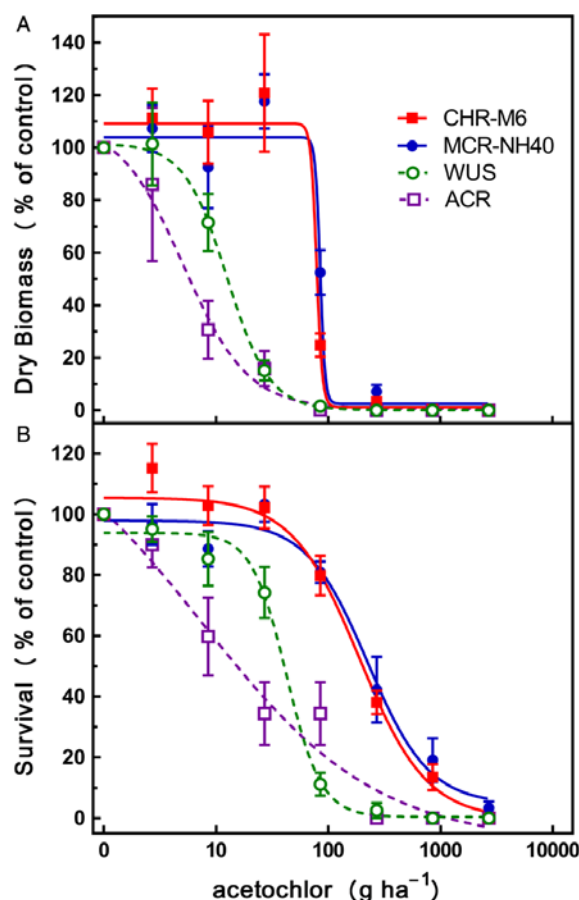
the rate needed to reduce aboveground biomass. R:S values for CHR-M6 and MCR-NH40 were 7.5 and 12.9, respectively, in comparison with WUS, and 9.9 and 17.1 when compared with ACR, respectively. The large difference in GR<sub>50</sub> R:S values and those calculated from LD<sub>50</sub> estimates can be attributed to the low overall biomass recovered from containers treated with higher herbicide rates.

#### Dose response to acetochlor

Complete mortality of both HPPD inhibitor-sensitive populations (ACR and WUS) was achieved at lower rates of acetochlor ( $\leq 80$  g ha<sup>-1</sup>) compared with either CHR-M6 or MCR-NH40. Both CHR-M6 and MCR-NH40 were completely controlled at 800 g ha<sup>-1</sup> (Figure 5). Similar to *S*-metolachlor, dose–response curves for acetochlor indicate a positive shift in rate needed to reduce the aboveground biomass and surviving seedlings from CHR-M6 and MCR-NH40 (Figure 7), but complete control was achieved at rates lower than applied in the field. R:S ratios were 4.5 and 5.7 for CHR-M6 and MCR-NH40, respectively, when compared with WUS, and 14.1 and 17.9 when compared with ACR, respectively, based on calculated LD<sub>50</sub> values (Table 6). R:S ratios calculated from GR<sub>50</sub> values were 6.1 and 6.7 for CHR-M6 and MCR-NH40, respectively, when compared with WUS, and 13.2 and 14.6 when compared with ACR, respectively.

#### Dose response to dimethenamid-*P*

CHR-M6 and MCR-NH40 survived higher rates of dimethenamid-*P* (1.1 kg ha<sup>-1</sup>) compared with either ACR or WUS, for which complete mortality was achieved at or below 350 g ha<sup>-1</sup> (Figures 8 and 9). Surviving MCR-NH40 or CHR-M6 plants in replicates treated with 1.1 or 3.5 kg ha<sup>-1</sup> dimethenamid-*P* were severely stunted but still actively growing at 21 DAT, similar to *S*-metolachlor treatments. Dose–response curves (Figure 9) also indicate a positive shift in rate for CHR-M6 and MCR-NH40 needed to reduce aboveground biomass and surviving seedlings. R:S ratios were 21.1 and



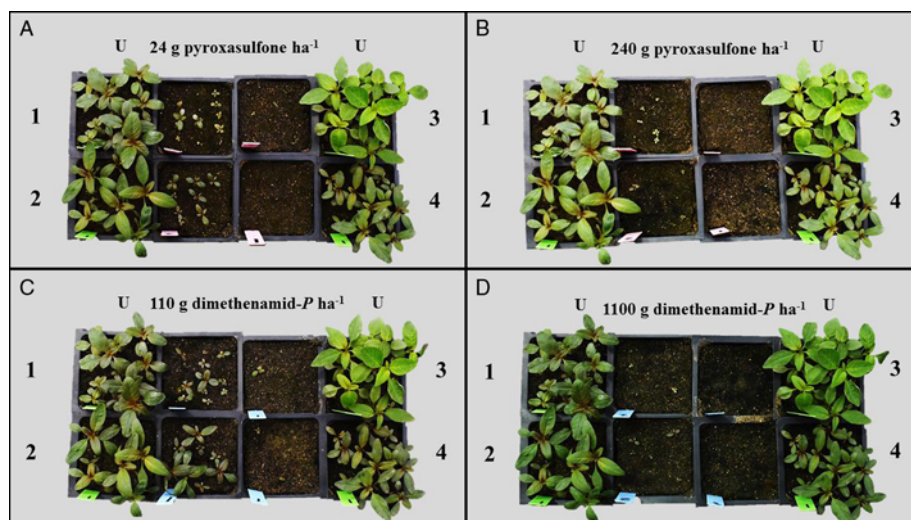
**Figure 7.** Herbicide dose–response experiments of four *Amaranthus tuberculatus* populations treated with soil-applied acetochlor. Data were collected at 21 DAT. (A) Dry biomass as a percentage of nontreated control, and (B) mean survival of *A. tuberculatus* seedlings as a percentage of nontreated control. Lines in each graph were fit using Equation 1:  $(y = c + \frac{d-c}{1 + \exp\{b[\log(x) - \log(LD_{50}/GR_{50})]\}})$ , and each symbol represents the observed mean  $\pm$  SE. CHR-M6, continuous line and full squares; MCR-NH40, continuous line and full circles; WUS, discontinuous line and open circles; ACR, discontinuous line and open squares.

42.2 for CHR-M6 and MCR-NH40, respectively, when compared with WUS, and 28 and 56.1 when compared with ACR, respectively, based on calculated LD<sub>50</sub> values (Table 6). R:S ratios calculated from GR<sub>50</sub> values were 5.5 and 8.7 for CHR-M6 and MCR-NH40, respectively, when compared with WUS, and 11.9 and 18.8 when compared with ACR, respectively.

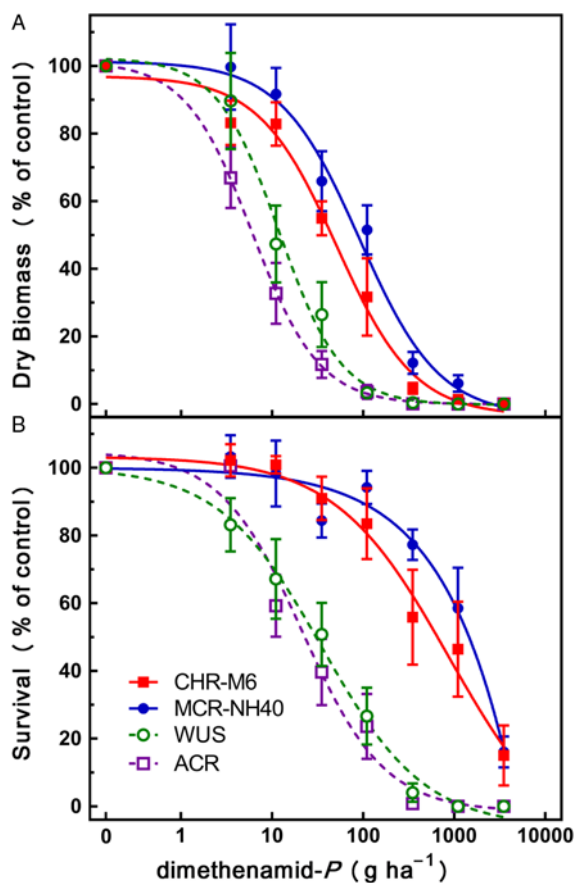
#### Dose response to pyroxasulfone

Complete control of either HPPD inhibitor-sensitive population (ACR or WUS) was achieved at or below 78 g ha<sup>-1</sup> pyroxasulfone, whereas CHR-M6 and MCR-NH40 survived 240 and occasionally 780 g ha<sup>-1</sup> (Figures 8 and 10). Survivors from replicates treated with high rates of pyroxasulfone ( $\geq 240$  g ha<sup>-1</sup>) were severely stunted, similar to plants treated with high rates of *S*-metolachlor or dimethenamid-*P*. Dose–response curves (Figure 10) demonstrate that CHR-M6 and MCR-NH40 require higher rates to reduce aboveground biomass and seedling survival, similar to all previously mentioned active ingredients. R:S ratios were 6.5 and 15.4 for CHR-M6 and MCR-NH40, respectively, when compared with WUS, and 7.2 and 17 when compared with ACR, respectively, based on calculated LD<sub>50</sub> values (Table 6). R:S ratios calculated from GR<sub>50</sub> values were 2.9 and 4.9 for CHR-M6 and MCR-NH40,

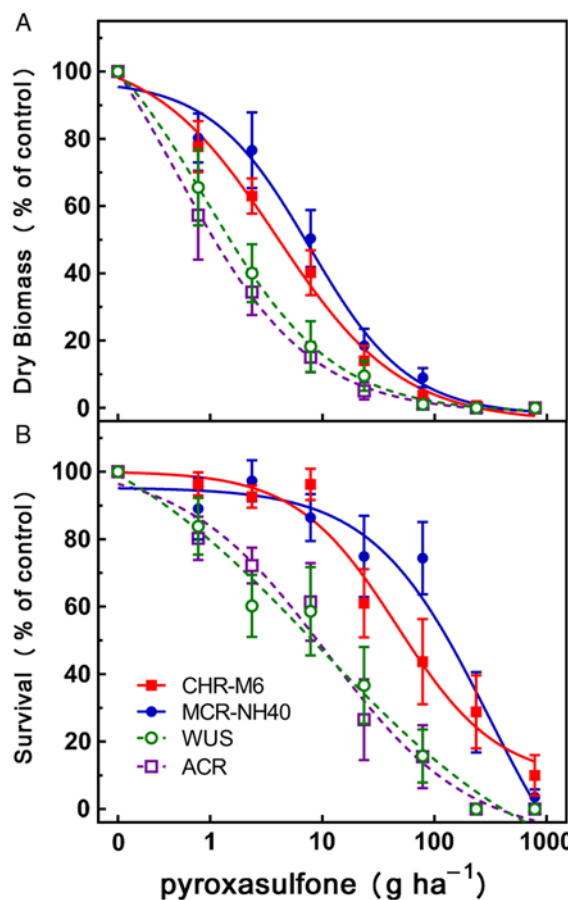




**Figure 8.** Response of four *Amaranthus tuberculatus* populations at 14 DAT to (A) 24 g pyrooxasulfone ha<sup>-1</sup>, (B) 240 g pyrooxasulfone ha<sup>-1</sup>, (C) 110 g dimethenamid-P ha<sup>-1</sup>, and (D) 1,100 g dimethenamid-P ha<sup>-1</sup>. In each frame, outside columns are nontreated (signified by “U”), and treated containers are located in the center below the labeled rate. Populations (1, CHR-M6; 2, MCR-NH40; 3, WUS; 4, ACR) are displayed with nontreated nearest number and corresponding treatment directly adjacent. Herbicide treatments were applied directly to the soil PRE.



**Figure 9.** Herbicide dose-response experiments of four *Amaranthus tuberculatus* populations treated with soil-applied dimethenamid-P. Data were collected at 21 DAT. (A) Dry biomass as a percentage of nontreated control, and (B) mean survival of *A. tuberculatus* seedlings as a percentage of nontreated control. Lines in each graph were fit using Equation 1:  $(y = c + \frac{d-c}{1 + \exp\{b[\log(x) - \log(LD_{50}/GR_{50})]\}})$ , and each symbol represents the observed mean  $\pm$ SE. CHR-M6, continuous line and full squares; MCR-NH40, continuous line and full circles; WUS, discontinuous line and open circles; ACR, discontinuous line and open squares.



**Figure 10.** Herbicide dose-response experiments of four *Amaranthus tuberculatus* populations treated with soil-applied pyrooxasulfone. Data were collected at 21 DAT. (A) Dry biomass as a percentage of nontreated control, and (B) mean survival of *A. tuberculatus* seedlings as a percentage of nontreated control. Lines in each graph were fit using Equation 1:  $(y = c + \frac{d-c}{1 + \exp\{b[\log(x) - \log(LD_{50}/GR_{50})]\}})$ , and each symbol represents the observed mean  $\pm$ SE. CHR-M6, continuous line and full squares; MCR-NH40, continuous line and full circles; WUS, discontinuous line and open circles; ACR, discontinuous line and open squares.

respectively, when compared with WUS, and 4.0 and 6.8 when compared with ACR, respectively. The R:S ratios from the present research are similar to previous reports on several MHR rigid ryegrass (*Lolium rigidum* Gaudin) populations, for which 8.1-fold (Brunton et al. 2018) and 3.6-fold (Busi et al. 2017) resistances to pyroxasulfone were documented based on LD<sub>50</sub> values.

### Summary and management implications

Overall, field results indicate a wide range of efficacy among the VLCFA inhibitors compared at the CHR site. Greenhouse experiments corroborated field results, demonstrating an increase in VLCFA-inhibitor rates required to reduce biomass and survival of either MHR population with resistances to atrazine and HPPD inhibitors (CHR-M6 and MCR-NH40). Conversely, ACR, which shares only atrazine resistance with CHR-M6 and MCR-NH40, was the most sensitive to all VLCFA-inhibiting herbicides tested.

Natural crop tolerance to chloroacetamide herbicides is achieved through rapid conjugation to glutathione in corn or homoglutathione in soybean (Breux 1987; Fuerst 1987). Higher levels of glutathione S-transferase (GST) activity typically occur in tolerant crop seedlings versus sensitive weed species when assaying with chloroacetamide herbicide substrates (Hatton et al. 1996). Plants from the MCR population contain the metabolic capacity necessary for oxidative metabolism of mesotrione as well as GST-mediated detoxification of atrazine (Ma et al. 2013). It is reasonable to hypothesize that either or both of these metabolic mechanisms could confer cross-resistance to certain VLCFA inhibitors or, alternatively, that multiple metabolic resistance could occur via distinct enzymes for each herbicide chemistry. As mentioned previously, it is interesting that the ACR population (metabolic atrazine-resistant only) was the most sensitive to all VLCFA-inhibiting herbicides tested. However, additional mechanistic research is required to directly test these hypotheses.

A similar detoxification process for VLCFA-inhibiting herbicides may occur in CHR and MCR, but it is unclear why acetochlor is less affected than other VLCFA inhibitors. One possibility is that acetochlor might be less affected because GST- and cytochrome P450-mediated detoxification mechanisms in plants are complex and substrate specific (Edwards and Owen 1989; Riechers et al. 2010; Werck-Reichhart et al. 2000). Also, VLCFA-inhibiting herbicides target the VLCFA elongase encoded by the *FAE1* gene (Böger 2003), and previous research demonstrated that VLCFA-inhibiting herbicides can inhibit more than one VLCFA-elongase enzyme in *Arabidopsis thaliana*, including *At5g43760*, *At104220*, *At1g25450*, *KCS1*, *KCS2*, and *FAE1* (Trenkamp et al. 2004). Acetochlor may inhibit more of these condensing enzymes (or different enzymes) than other VLCFA-inhibiting active ingredients tested on CHR and thus remains more effective.

Overall, this initial report of a dicot species with resistance to VLCFA-inhibiting herbicides will undoubtedly be followed by other species and poses serious management implications. Early POST applications or tillage may be needed to compensate for the reduced duration of PRE activity, along with the best management practices of using multiple effective SOAs per season (Evans et al. 2016) and sequential applications of residual herbicides (Steckel et al. 2002). Further research including herbicide degradation studies in plants, target-site sequence analysis, or genetics and inheritance studies are needed to determine the underlying physiological mechanism(s) for the resistance of CHR and MCR to VLCFA-inhibiting herbicides. Understanding how these “old chemistries” perform in today’s agronomic systems will be crucial

for maintaining them as effective SOAs for residual control of small-seeded broadleaves in the future.

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