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Weed Science 2017

Multiple-Herbicide Resistance in a 2,4-D–Resistant Waterhemp (*Amaranthus tuberculatus*) Population from Nebraska

Roberto J. Crespo, Ana B. Wingeyer, Greg R. Kruger, Chance W. Riggins, Patrick J. Tranel, and Mark L. Bernards*

A 2,4-D-resistant tall waterhemp population (FS) from Nebraska was evaluated for resistance to other T1R1 auxin receptor herbicides and to herbicides having alternative mechanisms of action using greenhouse bioassays and genetic markers. Atrazine, imazethapyr, lactofen, mesotrione, glufosinate, and glyphosate were applied in a single-dose bioassay, and tissue was collected from marked plants for genetic analysis. The FS population was not injured by atrazine or by imazethapyr. Approximately 50% of the plants survived lactofen and were actively growing 28 d after treatment. The population was susceptible to mesotrione, glufosinate, and glyphosate. Ametryn, chlorimuron-ethyl, 2,4-D, aminocyclopyraclor, aminopyralid, and picloram were applied in dose–response studies. The FS population was sensitive to ametryn, and the Ser-264-Gly substitution in the D1 protein was not detected, suggesting the lack of response to atrazine is not due to a target-site mutation. The FS population exhibited less than 50% injury to chlorimuron-ethyl at application rates 20 times the labeled use rate. The Ser-653-Asn acetolactate synthase (ALS) substitution, which confers resistance to imidazolinone herbicides, was present in the FS population. However, this does not explain the lack of response to the sulfonylurea herbicide, chlorimuron-ethyl. Sequencing of a portion of the *PPX2L* gene did not show the ΔG210 mutation that confers resistance to protoporphyrinogen oxidase–inhibiting herbicides, suggesting that other factors were responsible for waterhemp survival after lactofen application. The FS population was confirmed to be at least 30-fold resistant to 2,4-D relative to the susceptible populations. In addition, it was at least 3-fold less sensitive to aminopyralid and picloram, two other T1R1 auxin receptor herbicides, than the 2,4-D-susceptible populations were. These data indicated that the FS population contains both target and non–target site mechanisms conferring resistance to herbicides spanning at least three mechanisms of action: T1R1 auxin receptors, ALS inhibitors, and photosystem II inhibitors.

Nomenclature: 2,4-D; ametryn; aminocyclopyrachlor; aminopyralid; atrazine; chlorimuron-ethyl; glufosinate; glyphosate; imazethapyr; lactofen; mesotrione; picloram; tall waterhemp, *Amaranthus tuberculatus* (Moq.) Sauer. AMATU.

Key words: Cross-resistance, dose–response, herbicide resistance, injury.

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Although herbicide-resistant weeds represent a serious threat to agricultural production, when populations contain resistance to a single herbicide (or group of herbicides having the same mechanism of action), they can generally be managed successfully. However, populations that have evolved resistance to multiple herbicides spanning different mechanisms of action create significant management challenges (Tranel et al. 2011). Populations of more than 50 weed species have been reported resistant to herbicides with multiple mechanisms of action (Heap 2017). The most problematic weeds with multiple resistance in the midwestern and southern United States are waterhemp and Palmer amaranth (*Amaranthus palmeri* S. Wats.) (Hager and Sprague 2002; Webster 2005). Each species has evolved resistance to

herbicides spanning six mechanisms of action (acetolactate synthase [ALS] inhibitors, photosystem II [PSII] inhibitors, enolpyruvylshikimate-3-phosphate synthase [EPSPS] inhibitors, protoporphyrinogen oxidase [PPO] inhibitors, hydroxyphenylpyruvate dioxygenase [HPPD] inhibitors, and T1R1 auxin receptors [waterhemp] or microtubule inhibitors [Palmer amaranth]), and resistance to herbicides spanning five mechanisms of action has been identified in individual populations of waterhemp while resistance spanning three mechanisms of action has been reported in a single population of Palmer amaranth (Heap 2017; Schultz et al. 2015). Both species are dioecious (Costea et al. 2005), assuring outcrossing and gene flow among and within populations (Trucco et al. 2006). In addition, both species have high fecundity, and the combination of large genetic variability, high population density, and heavy reliance on herbicides for weed control have increased the frequency of resistant alleles and the stacking of herbicide-resistant traits in populations (Tranel et al. 2011).

A T1R1 auxin receptor herbicide (2,4-D) was the first synthetic-organic herbicide commercialized (Burnside 1996). Because T1R1 auxin receptors (synthetic auxins) selectively control broadleaf weeds in grass crops, this mechanism of action is one of the most widely used globally (Sterling and Hall 1997). The frequency of weed resistance to herbicides in this group is relatively low despite their widespread use since 1946 (Gustafson 2008), perhaps because they are often applied in mixtures with other herbicides or because of the complex ways they interfere with plant growth and their limited persistence in the soil (Sterling and Hall 1997). The first two documented 2,4-D–resistant weeds were wild carrot (*Daucus carota* L.) (Switzer 1957) and spreading dayflower (*Commelina diffusa* Burm. f.) (Hilton 1957). To date, 34 weed species have evolved resistance to synthetic auxin herbicides (Heap 2017). Transgenic soybean [*Glycine max* (L.) Merr.], corn (*Zea mays* L.), and cotton (*Gossypium hirsutum* L.) genetically modified with resistance to 2,4-D (Wright et al. 2010) and dicamba (Behrens et al. 2007) are tools that will help farmers to manage broadleaf weeds resistant to glyphosate. However, this will result in increased selection pressure for weeds, including waterhemp and Palmer amaranth, to evolve resistance to herbicides with this mechanism of action.

In 2009 a farmer contacted scientists from the University of Nebraska–Lincoln and reported a waterhemp population that had survived the maximum labeled rates of 2,4-D. The field containing the putative resistant population had also received annual applications of atrazine and *S*-metolachlor in addition to 2,4-D. Greenhouse and field experiments confirmed that the waterhemp population was resistant to 2,4-D (Bernards et al. 2012). Seeds from the 2,4-D–resistant waterhemp population were collected in 2010 for use in this research. Our objectives were: (1) to evaluate the population for resistance to PSII inhibitors, ALS inhibitors, HPPD inhibitors, PPO inhibitors, EPSPS inhibitors, glutamine synthetase inhibitors, and additional herbicides from the T1R1 auxin inhibitors; and (2) to more accurately quantify the level of resistance to 2,4-D using higher 2,4-D doses in a greenhouse bioassay than were used in Bernards et al. (2012).

Materials and Methods

Waterhemp Populations. Seed from one 2,4-D–resistant (FS) and two 2,4-D–susceptible waterhemp (SE and SCAL) populations were used in this experiment. The FS population was collected in a field planted with little bluestem grass [*Schizachyrium scoparium* (Michx.) Nash 'Camper'] located in Cass County, NE (Bernards et al. 2012). The SE and SCAL populations were collected from soybean fields in Nemaha County and Clay County, NE, respectively. Each population sample was a composite of at least 40 plants. Waterhemp seed was cleaned and stored at 4 C.

Plant Growth. Herbicide bioassays were conducted in greenhouses located on the East Campus of the University of Nebraska–Lincoln in Lincoln, NE. Supplemental lighting (500 $\mu mol\, m^{-2} s^{-1}$) provided a 15-h photoperiod. Day temperatures varied between 24 and 28 C and night temperatures varied between 18 and 22 C.

Waterhemp seed was germinated by placing it on moistened filter paper in petri dishes, then sealing the petri dishes and placing them in an incubator for 48 to 72 h at 35 C (Ellis et al. 1985; Steckel et al. 2007). Two or three germinated waterhemp seedlings were transferred into growing mix (BM1® Growing Mix, Berger Peat Moss, Saint-Modeste, QC, Canada) in 10 by 10 by 12.5 cm black plastic pots. Plants were watered as needed and fertilized weekly with Miracle-Gro® fertilizer (Scotts Miracle-Gro, Marysville, OH). The seedlings were thinned to 1 plant pot^{-1} before herbicide treatments were applied.

Herbicide Application. Herbicide treatments were applied to waterhemp plants when they were 8- to 12-cm tall (5 to 8 fully expanded leaves).

Table 1. List of herbicides used.

Herbicide	Mechanism of action[a]	Trade name	Formulation	Rate range g ai ha^{-1}	Manufacturer	Additives[b]
Atrazine	PSII	Aatrex®	4L	2,240	Syngenta, Greensboro, NC	COC
Imazethapyr	ALS	Pursuit®	2L	70	BASF Research Triangle Park, NC	COC + AMS
Lactofen	PPO	Cobra®	2EC	210	Valent USA, Walnut Creek, CA	COC + AMS
Mesotrione	HPPD	Callisto®	4EC	105	Syngenta ,Greensboro, NC	COC + AMS
Glufosinate	GS	Ignite®	280SL	322	Bayer CropScience, Research Triangle Park, NC	AMS
Glyphosate	EPSPS	Roundup PowerMax®	SL	867[c]	Monsanto, St Louis, MO	AMS
Ametryn	PSII	Evik®	DF	123–2,240	Syngenta Crop Protection, Greensboro, NC	COC
Chlorimuron-ethyl	ALS	Classic®	DF	17–280	E.I. Du Pont de Nemours and Company, Wilmington, DE	COC
2,4-D	T1R1	Lo-Vol 4® Herbicide	EC	9–35,840[c]	Tenkōz, Alpharetta, GA	NIS
Aminocyclopyrachlor	T1R1	Imprelis™			E.I. du Pont de Nemours and Company, Wilmington, DE	NIS
Aminopyralid	T1R1	Milestone™			Dow AgroSciences, Indianapolis, IN	NIS
Picloram	T1R1	Tordon® 22K			Dow AgroSciences, Indianapolis, IN	

[a] Abbreviations for site of action: ALS, acetolactate synthase; EPSPS, enolpyruvylshikimate-3-phosphate synthase; GS, glutamine synthetase; HPPD, hydroxyphenylpyruvate dioxygenase; PPO, protoporphyrinogen oxidase; PSII, photosystem II.

[b] Abbreviations for additives: COC, crop oil concentrate at 1% (v/v); AMS, ammonium sulfate at 2.5% (v/v); NIS, nonionic surfactant at 0.25% (v/v).

[c] Acid equivalent (g ae ha^{-1}).

A chamber sprayer (DeVries Manufacturing, Hollandale, MN) equipped with a TP8001E flat-fan nozzle tip (TeeJet Spraying Systems, Wheaton, IL) was used to make the herbicide application. The carrier volume used was 190 L ha^{-1} at a pressure of 207 kPa with 1.6 km h^{-1} application speed.

Single-Dose Bioassays. The experiments were conducted in two experimental runs. Fifty plants from each waterhemp population were treated with a single dose of each of the first six herbicides listed in Table 1. Visible injury estimates were made at 7, 14, 21, and 28 d after treatment (DAT) and were compared with estimates for untreated plants (controls) using a scale of 0 (no injury) to 100 (dead plants). At 28 DAT, plants were severed at the base and dried for 48 h in a forced-air dryer at 65 C, after which dry weight biomass was measured. Mean values and standard error bars were graphed using SigmaPlot 12.2 (Systat Software, San Jose, CA).

Dose–Response Bioassays

Response to PSII- and ALS-inhibiting Herbicides. Dose–response experiments using ametryn or chlorimuron-ethyl (Table 1) were conducted on the FS and SE and SCAL waterhemp populations. The experimental design was a randomized complete block with 10 replications per treatment and experimental run. Five ametryn doses were applied: 0, 123, 560, 1,120, and 2,240 g ai ha^{-1}. In a separate experiment, six chlorimuron-ethyl doses were applied: 0, 17, 35, 70, 140, and 280 g ai ha^{-1}. Treatment solutions included a 1% (v/v) crop oil concentrate adjuvant. Each dose–response experiment was conducted in two experimental runs.

Response to T1R1 Auxin Receptor Herbicides. The maximum rate of 2,4-D used in greenhouse bioassays by Bernards et al. (2012) was 2,240 g ae ha^{-1}, which was inadequate to control the resistant population. In the greenhouse bioassay reported in this paper, we used 2,4-D doses that matched the previous field bioassay (Bernards et al. 2012) to better characterize the level of resistance. The FS waterhemp was treated with 2,4-D at 0, 140, 280, 560, 1,120, 2,240, 4,480, 8,960, 17,920, and 35,840 g ha^{-1}. The SE and SCAL waterhemp populations were treated with 2,4-D at 0, 9, 18, 37, 70, 140, 560, 1,120, 2,240, and 4,480 g ha^{-1}. Dose–response experiments were also conducted using eight doses of each of the following herbicides: aminocyclopyrachlor, aminopyralid, and

Table 2. T1R1 auxin receptor herbicides and doses applied to 2,4-D–resistant and 2,4-D–susceptible waterhemp populations.

Herbicide	Treatment/doses								
	g ae ha^{-1}								
Aminocyclopyrachlor[a]	0	5	10	20	39	79	158	315	630
Aminopyralid[a]	0	11	22	44	88	175	350	700	1,400
Picloram									
2,4-D–susceptible	0	18	35	70	140	280	560	1,120	2,240
2,4-D–resistant	0	35	140	560	1,120	2,240	4,500	9,000	18,000

[a] Both susceptible and resistant populations received the same doses of aminocyclopyrachlor and aminopyralid.

picloram on the FS, SE, and SCAL populations (Table 1; see Table 2 for herbicide doses). In preliminary experiments the FS population was less injured by picloram than a 2,4-D-susceptible population (unpublished data), therefore, the FS population was treated with greater picloram doses compared with the susceptible populations. All dose–response experiments were arranged in a randomized complete block design with five replications each, and were conducted in two experimental runs. Treatments containing 2,4-D, aminocyclopyrachlor, and aminopyralid applications included nonionic surfactant (NIS) at 0.25% (v/v). Treatments containing picloram were applied without an adjuvant.

Data Collection and Statistical Analysis. Visible injury estimates were made at 7, 14, 21, and 28 DAT based on each particular herbicide injury symptom compared with untreated controls using a scale of 0 (no injury) to 100 (dead plants). At 28 DAT, all plants for each treatment at each dose–response experiment were harvested and dried for 48 h in a forced-air dryer at 65 C, after which dry weight biomass was recorded.

Visible injury estimates and dry weight at 28 DAT were analyzed using a nonlinear regression model with the 'drc' package (drc 1.2, Christian Ritz and Jens Strebig, R 2.5, Kurt Hornik, online) in R v. 2.3.0 (R statistical software, R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org) (Knezevic et al. 2007). Dose–response models were constructed using a four-parameter log-logistic equation (Equation 1) (Streibig et al. 1993; Seefeldt et al. 1995):

$$y = c + (d - c/1 + \exp(b(\log x - \log e))) \qquad [1]$$

where y is the response based on visible injury estimate or dry weight, c is the lower limit, d is the upper limit, x is the herbicide dose, e is the herbicide dose giving a 50% response (injury estimation [I_{50}] or dry weight reduction [GR_{50}]) between the upper and lower limit, and is also the inflection point, and b is the slope of the line at the inflection point. The ametryn or chlorimuron-ethyl doses needed to achieve 50%, 80%, and 90% visible injury estimates (I) and dry weight (GR) at 28 DAT were calculated. The relative level of resistance was expressed by calculating the R:S ratios between the I or GR values of the least susceptible biotype and the I or GR values of the most susceptible biotype (Beckie et al. 2000). Standard error bars shown in the figures were calculated for each treatment using mean and standard error functions in SigmaPlot 12.2 (Systat Software, San Jose, CA).

Waterhemp Molecular Analysis. The results of the first run of the single-dose herbicide bioassays led us to suspect that there might be resistance to ALS-, PSII- and PPO-inhibiting herbicides among the FS, SE, and SCAL populations. Prior to herbicide application in the second run of the single-dose herbicide experiment described above, a young fully expanded leaf was collected from each plant, placed in a labeled 1.5-ml Eppendorf tube, and then stored in a freezer at –20 C until sample analysis. After plants were valuated for herbicide response, tissue samples from five suspected ALS-, atrazine-, or lactofen-resistant plants and five susceptible plants for each population were selected for molecular evaluation. Genetic analyses were conducted in laboratories located at the University of Illinois at Urbana, IL. Samples were evaluated for the Trp-574-Leu mutation conferring resistance to sulfonylurea and imidazolinone herbicides and/or substitution at Ser-653, which confers resistance to imidazolinone herbicides (Patzoldt and Tranel 2007). Additionally, we tested for the presence of Ser-264-Gly, Ser-264-Thr, Val-219-Ile, Ala-251-Val, and Asn-266-Thr mutations in the *psbA* gene conferring resistance to PSII-inhibiting herbicides (Foes et al. 1998; Patzoldt et al. 2003). Samples with suspected resistance to PPO-inhibiting herbicides were evaluated for the 3-base pair deletion in the *PPX2L* gene (Lee et al. 2008).

Analysis of the *ALS* gene was done by isolating DNA from leaf tissue samples and using PCR to amplify region B of the *ALS* gene, which encompasses the Trp-574-Leu mutation. The following primers were used: AmALS-F2: 5′-TCCCGGTTAAAAT CATGCTC; and AmALS-R2: 5′-CTAAACGAGA GAACGGCCAG (Foes et al. 1998). The Trp-574-Leu mutation in the *ALS* gene creates a recognition site for the *Mfe*I restriction enzyme, thus a PCR-RFLP assay was conducted as previously described by Foes et al. (1999) and Schultz et al. (2015). After digestion, DNA fragments were separated on a 1% agarose gel and visualized with a Kodak Gel Logic 1500 Imaging System. Individual plants were classified as homozygous for the L574 ALS allele, heterozygous or homozygous for the W574 allele based on the presence of DNA fragments with approximate base pair sizes of 389 bp (homozygous for L574) or 440 bp (uncut, homozygous for W574). Fragments smaller than 51 bp usually are not visible on the gel.

Additionally, we looked for mutations at the Ser-653 site of the *ALS* gene that are known to confer resistance to imidazolinone herbicides in waterhemp (Patzoldt and Tranel 2007). Five FS plants that tested negative for the Trp-574-Leu mutation and two 2,4-D sensitive plants that tested positive for Trp-574-Leu were examined. Mutations at position 653 were confirmed by sequencing and by allele-specific PCR using codon-specific primers (Patzoldt and Tranel 2007). PCR products were separated in a 1% agarose gel containing ethidium bromide and visualized with UV light.

DNA sequencing was also performed to identify the Ser-264 mutation in the *psbA* gene for atrazine resistance. Total DNA was extracted from leaf tissue, and a region of the chloroplast *psbA* gene encoding the Dl protein was selectively amplified with primers AmpsbAsF1: 5′-ATGAGGGTTACAGATTTGGTC and AmpsbAsR1: 5′-AGATTAGCACGGTTGAT GATA. Digestion products were separated by electrophoresis through a 1% agarose gel and visualized under UV light with ethidium bromide staining (Schultz et al. 2015).

Samples with suspected resistant to PPO-inhibiting herbicides were evaluated for the 3-base pair deletion in the *PPX2* gene (Lee et al. 2008). DNA was extracted from leaf tissue samples, and allele-specific primers described previously by Lee et al. (2008) were used to screen samples for the codon deletion in the gene that results in the deletion of Gly-210. Products from PCR amplification and digestion were fractionated in 2% agarose gel containing ethidium bromide and visualized with UV light (Lee et al. 2008; Schultz et al. 2015).

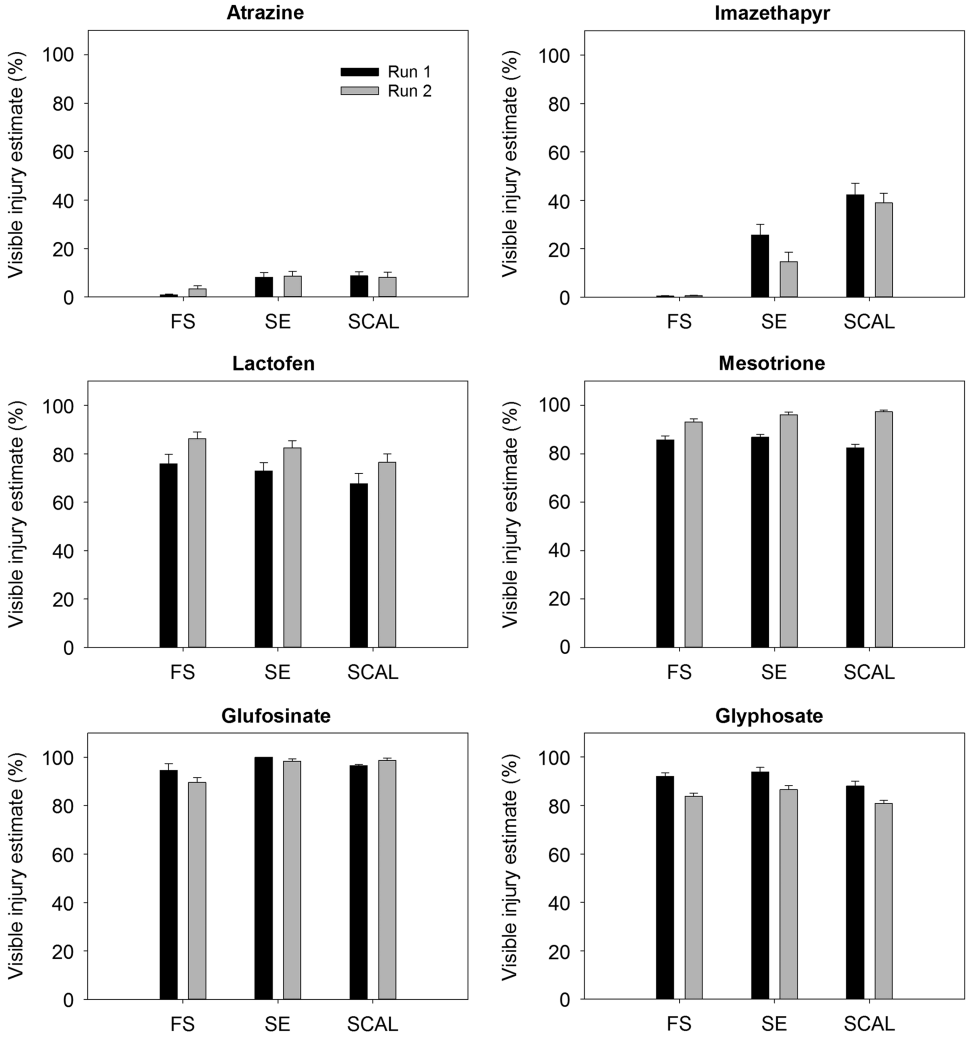

Figure 1. Visible injury estimates from two experimental runs of the 2,4-D–resistant (FS) and 2,4-D–susceptible (SE and SCAL) waterhemp populations to a single labeled dose of atrazine (2,240 g ai ha^{-1}), imazethapyr (70 g ai ha^{-1}), lactofen (210 g ai ha^{-1}), mesotrione (105 g ai ha^{-1}), glufosinate (322 g ai ha^{-1}), and glyphosate (867 g ai ha^{-1}). Vertical bars represent the standard error of the mean. Data represent the average of 50 plants $population^{-1}$ $herbicide^{-1}$ for each experimental run.

Results and Discussion

Single-Dose Bioassays. All three populations (FS, SCAL, and SE) showed less than 10% injury from atrazine (Figure 1). Two of the populations were collected from fields with long histories of atrazine use (FS and SCAL). The FS population was exposed to annual applications of atrazine beginning in 1996 (Bernards et al. 2012), and the SCAL population was from a University of Nebraska–Lincoln research farm where atrazine was frequently used to manage weeds in corn and sorghum (unpublished data). The third population (SE) was from a soybean–corn field that likely had a history of atrazine use. Anderson et al. (1996) reported that 92% of suspected atrazine-resistant waterhemp populations from southeast Nebraska were indeed resistant. Consequently, it was not surprising that all three populations showed little injury after being treated with labeled field rates of atrazine. However, the absence of a susceptible control prevents us from definitively concluding that they are resistant to atrazine.

None of the three populations were completely controlled by imazethapyr (Figure 1). Plants from the FS population were not sensitive to imazethapyr at 28 DAT. Injury to plants from the SE and SCAL populations was more variable, but averaged less than 30% and 45%, respectively. Resistance to ALS-inhibiting herbicides is presumed to be widespread among waterhemp populations in Nebraska (Bernards et al. 2011), and the response observed in these bioassays supports that assumption. The lack of response in the FS population was somewhat surprising, because the field where the seed was collected had not been in corn or soybean production since 1995, and the owner did not report the use of ALS-inhibiting herbicides in the management of the little bluestem growing there. However, the first reports of ALS-resistant waterhemp in the midwestern United States were made in 1993 (Heap 2017). The ALS resistance may have been in the population prior to the field being converted to little bluestem, or it may have been introduced through pollen-mediated gene flow from waterhemp in nearby corn and soybean fields, or introduced as a seed contaminant (Horak and Peterson 1996).

Waterhemp injury caused by lactofen was similar among the three populations, and ranged between 62% and 69% in the first bioassay run and 70% and 78% in the second run (Figure 1). Lactofen injury symptoms in the first 2 DAT included chlorosis, necrosis, and crinkling. Plants produced new growth within 14 DAT, and more than half of the plants in each biotype and run recovered and were actively growing at 28 DAT (unpublished data). Shoup and Al-Khatib (2005) noted similar symptoms in the first case of PPO inhibitor–resistant waterhemp reported in Kansas, but less severe final injury estimates. All three waterhemp populations were sensitive to glufosinate, glyphosate, and mesotrione, and injury estimates were 80% or higher for each (Figure 1).

Dose–Response Bioassays

Response to PSII- and ALS-inhibiting Herbicides. The labeled rate of 2,240 g ha^{-1} of ametryn resulted in visual injury ratings of 77% for the FS population and 93% for the SE and SCAL populations (Figure 2). Plants from FS population were less sensitive to ametryn than the SE or SCAL populations, based on 28 DAT visual injury estimates (Table 3; Figure 2) but not dry weight reduction (Table 4; Figure 3). The R:S ratio between the FS and most susceptible population never exceeded 2, suggesting there is no resistance to ametryn among these populations.

The FS population was less sensitive to chlorimuron-ethyl than the SE or SCAL populations based on visual injury estimates (Figure 4; Table 3) and dry weight reduction (Figure 5; Table 4). The R:S ratios were 7.1 for 50% visual injury (I_{50}) and 3.7 for 50% dry weight reduction (GR_{50}). None of the populations were controlled at the 80% visual injury level at the maximum rate tested of 280 g ha^{-1}, which is 21 times greater than the labeled use rate of 13 g ha^{-1}. The dose required to

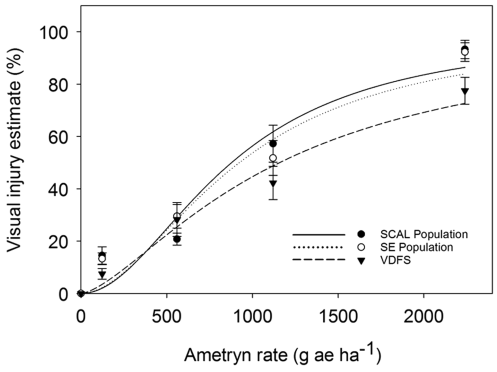

Figure 2. Visual injury estimate as affected by ametryn dose for 2,4-D–resistant and 2,4-D–susceptible waterhemp biotype at 28 DAT in greenhouse bioassays. Regression parameters are provided in Table 2. Data represent the mean of two experimental runs and 10 replications per experiment. The error bars represent the standard error for each data point.

Table 3. Visible injury estimate (I) regression parameters, ametryn (Evik® DF, Syngenta) and chlorimuron-ethyl (Classic® DF, DuPont™) doses necessary to achieve I_{50}, I_{80}, and I_{90} values, and standard errors (se) at 28 DAT for 2,4-D–resistant (FS) and 2,4-D–susceptible (SE and SCAL) waterhemp populations from Nebraska.

	Regression parameters[a]			
Biotype	*b*	I_{50} (± se)	I_{80} (± se)	I_{90} (± se)
		g ai ha^{-1}		
Ametryn				
FS	−1.48	1,158 (135)	2,953 (707)	5,107 (1,808)
SE	−1.86	923 (150)	1,945 (509)	3,007 (1,194)
SCAL	−1.97	878 (108)	1,773 (347)	2,673 (796)
R:S[b]		1.3	1.7	1.9
Chlorimuron-ethyl				
FS	−0.79	243 (66)	1,405 (889)	3,922 (3,406)
SE	−0.75	89 (14)	569 (209)	1,684 (904)
SCAL	−0.51	34 (6)	516 (205)	2,544 (1,655)
R:S[b]		7.1	2.7	2.3

[a] Regression parameters were estimated using a four-parameter log-logistic equation, $y = c + (d - c/1 + \exp(b(\log x - \log e)))$, where c represents the lower limit (0 = no injury), d represents the upper limit (100 = plant death), b represents the slope of the line at the inflection point, and e represents the herbicide dose necessary to provide 50% injury (I_{50}).

[b] R:S is the resistant:susceptible ratio between the least susceptible biotype and the most susceptible biotype.

reduce dry weight 80% (GR_{80}) ranged from 41 to 131 g ha^{-1}. Lovell et al. (1996) reported a 330-fold resistance based on visible injury compared with the susceptible waterhemp biotype with chlorimurom-ethyl. Other studies have used thifensulfuron in bioassays and reported 28-, 490-, 18,000- and 34,000-fold differences between resistant and susceptible waterhemp populations (Lovell et al. 1996; McMullan and Green 2011; Patzoldt et al. 2005; Patzoldt and Tranel 2007). This bioassay, however, did not use a known susceptible biotype, so we cannot conclusively confirm herbicide resistance (Beckie et al. 2000), even though the rates required to control these populations greatly exceeded commercial use rates.

Response to T1R1 Auxin Receptor Herbicides. The FS population was approximately 50-fold resistant to 2,4-D relative to the SCAL population based on visual injury (I_{80}) and dry weight reduction (GR_{80})

Table 4. Dry weight reduction (GR) regression parameters, ametryn (Evik® DF, Syngenta) and chlorimuron-ethyl (Classic® DF, DuPont™) doses necessary to achieve GR_{50}, GR_{80}, and GR_{90}, and standard errors (se) at 28 DAT for 2,4-D–resistant (FS) and 2,4-D–susceptible (SE and SCAL) waterhemp populations from Nebraska.

	Regression parameters[a]					
Biotype	*c*	*d*	*b*	GR_{50} (± se)	GR_{80} (± se)	GR_{90} (± se)
				g ai ha^{-1}		
Ametryn						
FS	0.6	17.3	0.64	180 (86)	1,541 (829)	5,419 (4,798)
SE	0.4	17.6	0.76	156 (44)	970 (280)	2,828 (1,286)
SCAL	0.4	17	0.93	232 (46)	1,032 (230)	2,470 (815)
R:S[b]				1.5	1.6	2.2
Chlorimuron-ethyl						
FS	4.6	12.7	0.85	26 (7)	131 (49)	339 (206)
SE	3.2	15.6	1.00	10 (5)	41 (12)	93 (48)
SCAL	1.6	14.5	0.66	7 (3)	56 (14)	199 (95)
R:S[b]				3.7	3.2	3.6

[a] Regression parameters were estimated using a four-parameter log-logistic equation, $y = c + (d - c/1 + \exp(b(\log x - \log e)))$, where, where c represents the lower limit (minimum dry weight for each biotype), d represents the upper limit (maximum dry weight for each biotype), b represents the slope of the line at the inflection point, and e represents the herbicide dose necessary to provide 50% reduction in dry matter (GR_{50}).

[b] R:S is the resistant:susceptible ratio between the least susceptible biotype and the most susceptible biotype.

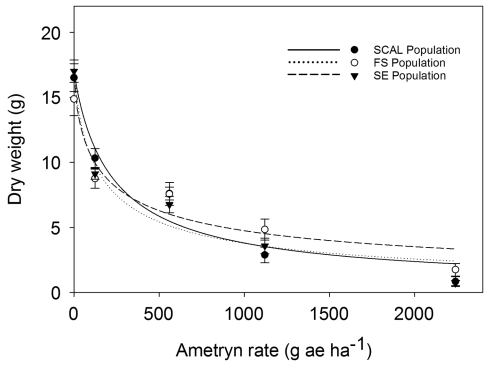

Figure 3. Percent dry weight reduction relative to untreated control as affected by ametryn dose at 28 DAT of 2,4-D–resistant and 2,4-D–susceptible waterhemp populations in greenhouse bioassays. Regression parameters are given in Table 3. Data represent the mean of two experimental runs and 10 replications per experiment. The error bars represent the standard error for each data point.

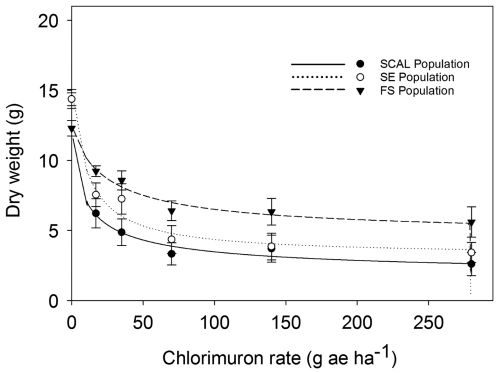

Figure 5. Percent dry weight reduction relative to untreated control as affected by chlorimuron-ethyl dose at 28 DAT of 2,4-D–resistant and 2,4-D–susceptible waterhemp populations in greenhouse bioassays. Regression parameters are given in Table 3. Data represent the mean of two experimental runs and 10 replications per experiment. The error bars represent the standard error for each data point.

(Tables 5 and 6). In the current study, the maximum 2,4-D dose of 35,840 g ha^{-1} was adequate to kill (100% visible injury at 28 DAT) waterhemp plants of the FS population. Thus, the log-logistic model estimate of the I_{80}, I_{90}, GR_{80}, and GR_{90} for the FS population are more reliable estimates than those reported by Bernards et al. (2012), in which the maximum 2,4-D dose was 2,240 g ha^{-1}. Doses of 2,4-D greater than 24,000 g ha^{-1} were required to achieve 90% injury and 90% dry weight reduction in the FS population.

The FS population was less susceptible to aminocyclopyrachlor, aminopyralid, and picloram herbicides than the SE or SCAL populations based on visual injury estimates (Table 5). The R:S ratios for I_{50} were 2.4 for aminocyclopyrachlor, 4.7 for aminopyralid, and 4.7 for picloram. When the analyses were based on dry weight reduction, the FS population was less susceptible to aminopyralid and picloram than the SE or SCAL populations, but more susceptible to aminocyclopyrachlor than the SCAL population (Table 6). None of the T1R1 auxin inhibitor herbicides evaluated were exceptionally effective in controlling these waterhemp populations. In general, the labeled use rates of aminocyclopyrachlor (80 g ae ha^{-1}), aminopyralid (88 g ae ha^{-1}), and picloram (280 g ae ha^{-1}) were inadequate to achieve 90% visual injury or dry weight reduction for any of the populations (Tables 5 and 6). In particular, the FS population required 7-, 11-, and 16-fold higher doses than recommended field rates for aminocyclopyrachlor, aminopyralid, and picloram, respectively, based on visible injury estimates (Table 5). The synthetic auxin herbicides we evaluated are labeled for pasture and range applications where waterhemp is less likely to be a troublesome weed and are not used in corn or soybean. Bernards et al. (2012) found the FS population to have 3-fold resistance to dicamba based on visual injury estimates but less than 2-fold resistance for dry weight reduction.

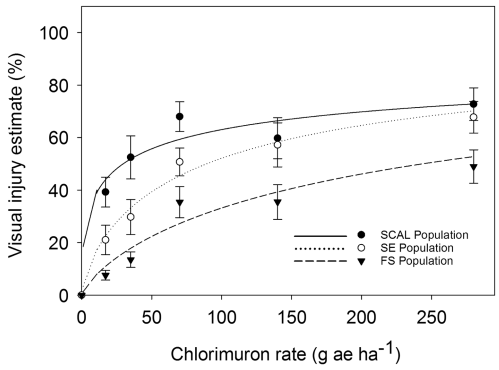

Figure 4. Visual injury estimate as affected by chlorimuron-ethyl dose for 2,4-D–resistant and 2,4-D–susceptible waterhemp biotype at 28 DAT in greenhouse bioassays. Regression parameters are provided in Table 2. Data represent the mean of two experimental runs and 10 replications per experiment. The error bars represent the standard error for each data point.

Waterhemp Molecular Analysis. Based on the responses of the FS, SE, and SCAL populations to atrazine, ALS-inhibiting herbicides, and lactofen, we evaluated each population for the presence of alleles that confer resistance to those herbicides. A serine to glycine substitution at amino acid number 264 of the D1 protein (encoded by the chloroplastic *psbA* gene) has been associated with atrazine resistance in

Table 5. Visible injury estimate (I) regression parameters, 2,4-D (Lo-Vol 4®, Tenkōz), aminocyclopyrachlor (Imprelis™, DuPont™), aminopyralid (Milestone™, Dow AgroSciences™) and picloram (Tordon® 22k, Dow AgroSciences) doses necessary to achieve I_{50}, I_{80}, and I_{90} values, and standard errors (se) at 28 DAT for 2,4-D–resistant (FS) and 2,4-D–susceptible (SE and SCAL) waterhemp populations from Nebraska.

	Regression parameters[a]			
Biotype	*b*	I_{50} (± se)	I_{80} (± se)	I_{90} (± se)
		g ae ha^{-1}		
2,4-D				
FS	−1.20	4,560 (464)	14,476 (2,390)	28,454 (6,519)
SE	−0.99	91 (14)	368 (82)	832 (262)
SCAL	−1.09	86 (12)	309 (68)	650 (206)
R:S[a]		53	47	44
Aminocyclopyrachlor				
FS	−0.82	38 (5)	206 (43)	553 (167)
SE	−1.00	17 (2)	67 (12)	152 (38)
SCAL	−0.87	16 (2)	78 (15)	200 (55)
R:S[a]		2.4	3.1	3.6
Aminopyralid				
FS	−0.88	80 (8)	385 (59)	967 (212)
SE	−1.09	17 (1)	61 (5)	129 (17)
SCAL	−0.87	18 (2)	87 (12)	222 (48)
R:S[b]		4.7	6.3	7.5
Picloram				
FS	−0.66	166 (25)	1,357 (229)	4,631 (1,136)
SE	−0.73	35 (6)	230 (46)	693 (211)
SCAL	−0.65	43 (7)	365 (82)	1,276 (443)
R:S[b]		4.7	5.9	6.7

[a] Regression parameters were estimated using a four-parameter log-logistic equation, $y = c + (d - c/1 + \exp(b(\log x - \log e)))$, where c represents the lower limit (0 = no injury), d represents the upper limit (100 = plant death), b represents the slope of the line at the inflection point, and e represents the herbicide dose necessary to provide 50% injury (I_{50}).

[b] R:S is the resistant:susceptible ratio between the least susceptible biotype and the most susceptible biotype.

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other species (Devine and Preston 2000; Hirschberg and McIntosh 1983). Sequencing results of the *psbA* gene of two atrazine-resistant plants of each of the waterhemp populations (FS, SE, and SCAL) did not identify the Ser-264 mutation. Patzoldt et al. (2003) reported triazine resistance in some Illinois waterhemp populations conferred by a nuclear-inherited, non–target site mechanism. All three populations were sensitive to ametryn (Tables 3 and 4), another PSII-inhibiting herbicide. Ametryn binding is not affected by the Ser-Gly substitution. Susceptibility to ametryn is consistent with other waterhemp populations resistant to atrazine but lacking a target-site mutation (Patzoldt et al. 2003). Because the non–target site mechanism of triazine resistance can be transmitted by seed and/or pollen, it is expected to be distributed more rapidly than the target-site mechanism due to the long-distance dispersal of wind-borne pollen and obligate outcrossing in dioecious *Amaranthus* species (Costea et al. 2005; Tranel et al. 2011; Trucco et al. 2006). Based on the complete lack of response to atrazine in the single-dose bioassay combined with the absence of the Ser-264 mutation that confers target-site resistance in all three waterhemp populations, we speculate that these populations likely have a non–target site resistance mechanism to atrazine.

Most cases of ALS resistance in *Amaranthus* weed species are conferred by mutations in the *ALS* gene. Using a PCR-RFLP technique, we analyzed the ALS locus for five plants of each of the three waterhemp populations. Broad cross-resistance to imidazolinone and sulfonylurea herbicides is conferred by the Trp-574-Leu mutation, but it was not present in the FS population. The Trp-574-Leu mutation was identified in one plant from the SCAL population and in three plants of the SE population. Using gene sequencing, we identified a Ser-653-Asn mutation that confers resistance to imidazolinone herbicides in all five FS plants that were sequenced, which provided genetic confirmation for the lack of response to imazethapyr observed in the single-dose bioassay (Figure 1). However, the FS population was less susceptible to chlorimuron-ethyl, a

Table 6. Dry weight reduction (GR) regression parameters, 2,4-D (Lo-Vol 4®, Tenkōz), aminocyclopyrachlor (Imprelis™, DuPont™), aminopyralid (Milestone™, Dow AgroSciences™), and picloram (Tordon® 22k, Dow AgroSciences) doses necessary to achieve GR_{50}, GR_{80}, and GR_{90}, and standard errors (se) at 28 DAT for 2,4-D–resistant (FS) and 2,4-D–susceptible (SE and SCAL) waterhemp populations from Nebraska.

	Regression parameters[a]					
Biotype	*c*	*d*	*b*	GR_{50} (± se)	GR_{80} (± se)	GR_{90} (± se)
				g ae ha^{-1}		
2,4-D						
FS	0.4	20.5	0.8	1,451 (277)	8,683 (2,484)	24,722 (10,236)
SE	0.4	17.1	0.7	42 (9)	319 (102)	1,049 (491)
SCAL	1.6	14.5	1.3	58 (14)	168 (55)	312 (145)
R:S[b]				35	52	79
Aminocyclopyrachlor						
FS	0.5	17.9	0.9	8 (1)	38 (6)	93 (23)
SE	0.5	16.7	1.0	7 (1)	25 (4)	54 (13)
SCAL	0.8	15.8	0.8	13 (3)	65 (17)	169 (65)
R:S[b]				1.9	2.6	3.1
Aminopyralid						
FS	0.5	20.6	0.7	74 (11)	486 (86)	1,462 (385)
SE	0.5	17.1	0.7	20 (6)	146 (44)	472 (238)
SCAL	1.6	14.5	1.3	42 (13)	126 (70)	241 (192)
R:S[a]				3.7	3.9	6.1
Picloram						
FS	1.1	24.4	0.7	42 (6)	272 (40)	813 (178)
SE	0.8	22.0	0.7	10 (3)	76 (13)	254 (75)
SCAL	1.0	22.9	0.8	17 (2)	87 (11)	230 (48)
R:S[a]				4.2	3.6	3.5

[a] Regression parameters were estimated using a four-parameter log-logistic equation, $y = c + (d - c/1 + \exp(b(\log x - \log e)))$, where *c* represents the lower limit (0 = no injury), *d* represents the upper limit (100 = plant death), *b* represents the slope of the line at the inflection point, and *e* represents the herbicide dose necessary to provide 50% dry weight reduction (GR_{50}).

[b] R:S is the resistant:susceptible ratio between the least susceptible biotype and the most susceptible biotype.

sulfonylurea herbicide, than the SE or SCAL populations, where the Trp-574-Leu mutation was present (Figures 4 and 5; Tables 3 and 4). We did not sequence the entire *ALS* gene, so it is possible that other mutations may exist or that the FS population may also metabolize sulfonylurea herbicides, as has been reported previously in waterhemp (Guo et al. 2015).

The only mechanism reported to confer resistance to PPO-inhibiting herbicides in waterhemp is a 3-base pair deletion in the *PPX2L* gene, referred to as the ΔG210 mutation (Lee et al. 2008; Patzoldt et al. 2006; Shoup et al. 2003; Tranel et al. 2011). Despite more than 50% of the plants from all populations surviving lactofen in the single-dose bioassay, none of the plants contained the deletion. PPO resistance has not been reported in any waterhemp populations in Nebraska. Because all of the plants were severely injured immediately following the application of lactofen (unpublished data), and all three populations responded similarly to the treatment in both runs, it is unlikely that the FS population is resistant to PPO-inhibiting herbicides.

The FS waterhemp population first reported by Bernards et al. (2012) is also resistant to ALS-inhibiting herbicides and to the PSII-inhibiting herbicide atrazine. Resistance to ALS-inhibiting herbicides was confirmed by the presence of at least one mutation known to confer resistance. Resistance to atrazine is likely due to a non–target site mechanism because mutations conferring target-site resistance to atrazine were not present and the population was susceptible to ametryn but showed no response to atrazine. Two other Nebraska waterhemp populations, SE and SCAL, also contained mutations conferring resistance to ALS-inhibiting herbicides and responded to atrazine and ametryn similarly to the FS population. The FS population was less susceptible to the T1R1 auxin receptor herbicides aminopyralid and picloram than the two other waterhemp populations. All three populations were susceptible to lactofen, mesotrione, glufosinate, and glyphosate. The field where

the FS population evolved was planted to a perennial crop in 1996 that was mowed each fall and burned each spring through 2011. In addition, it received an annual spring application of a triple mechanism of action herbicide tank mix (*S*-metolachlor, atrazine, and 2,4-D) followed by an annual application of 2,4-D. In short, resistance evolved even where there was diversity in cultural tactics and herbicide mechanisms of action. Resistance to ALS-inhibiting herbicides and atrazine may have been present in the population prior to the little bluestem being established, based on when resistance to those herbicides was first documented in the midwestern United States. This example emphasizes the need for weed managers to prevent seeds returning to the soil, in addition to using diverse cultural tactics and mixtures of effective herbicides.

Acknowledgments

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