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Assessing Multiple-Herbicide Resistance in a 2,4-D Resistant Waterhemp (Amaranthus tuberculatus) Biotype from Nebraska – Student Research

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Abstract

A waterhemp biotype was recently reported resistant to 2,4-D in Nebraska. In addition to the repeated use of 2,4-D, atrazine and imazathapyr were reported by the farmer to be frequently used to control broadleaf weeds. Greenhouse experiments were conducted to confirm 2,4-D resistance and test for resistance to other herbicides including growth regulators (i.e. aminocyclopyrachlor, aminopyralid and picloram) and other herbicide modes-of-action (MoA) (i.e. atrazine, imazethapyr, lactofen, mesotrione, glufosinate, glyphosate, ametryn and chlorimuron-ethyl). A single dose response experiment showed reduced sensitivity in all three waterhemp biotypes to atrazine, imazethapyr and lactofen; therefore, they were generally considered resistant to those three herbicide MoA. None of the biotypes contained the Ser264 target-site mutation. Since the ametryn dose-response experiment resulted in all susceptible biotypes, atrazine resistance is suspected to be metabolism based. Trp574Leu mutation conferring broad cross-resistance to ALS-inhibiting herbicides including imidazolinones and sulfonylureas was identified in the two 2,4-D susceptible waterhemp biotypes. Trp574Leu mutation was not found in the 2,4-D resistant biotype, but Ser653 mutation conferring resistance to imidazolinones was identified. However, dose-response experiments showed reduced sensitivity of the 2,4-D resistant biotype to chlorimuron-ethyl. Despite the 50% survival rate or higher of plants treated with lactofen, results from sequencing the PPX2L gene conferring resistance to PPO-inhibitor herbicides were not quite clear. More research is needed to identify if these results indicate that little to no resistance to PPO-inhibitor herbicides, or a different resistance mechanism and/or genetic mutation, is conferring the lack of sensitivity to lactofen. The present manuscript confirms that the 2,4-D resistant biotype found in Nebraska is also resistant to herbicides belonging to PSII- and ALS-inhibitors. Additionally, the 2,4-D resistant biotype is resistant and has reduced susceptibility to some other growth regulator herbicides.

Key Words: Dose-response, injury, herbicide resistance, 2,4-D-resistant waterhemp, cross-resistance.

Introduction

The development of herbicide-resistant weeds represents a serious worldwide threat to agricultural production. Since the first case of Spreading dayflower (*Commelina diffusa* Burn.) resistant to 2,4-D reported in 1957 (Hilton, 1957), the number of resistant weed biotypes against various herbicides has been on the rise (Heap, 2015). Additionally, the land area infected with herbicide resistant weeds is increasing rapidly expected to reach an estimated area equivalent to 570,000 fields in the current year (Heap, 2015).

Although herbicide resistant weeds are considered an increasing problem, most of the cases of herbicide resistant weeds could be successfully managed. However, multiple-herbicide resistance reports are increasing in some of the more important weed species and they can constitute trouble in weed management (Tranel et al., 2011). According to Heap (2015), almost 100 weed species have evolved resistance to more than one herbicide mode-of-action (MoA). In crops, the most important grass weed species such as annual ryegrass (*Lolium rigidum* L.), barnyardgrass (*Echinochloa crus-galli* L.), wild oat (*Avena fatua* L.), annual bluegrass (*Poa annua* L.), blackgrass (*Alopecurus myusoroides* L.) and goosegrass (*Eleusine indica* L.) have evolved resistance to at least five MoA. Few dicot weed species have evolved resistance to several herbicide MoA compared to grass species. Although the *Amaranthus* family appears to be resistant to more than one herbicide MoA in most of the family members, waterhemp [*Amaranthus tuberculatus* (moq.) Sauer] has been reported resistant to six MoA including ALS inhibitors, PSII inhibitors (i.e. triazine), PPO inhibitors, ghyphosate, HPPD inhibitors and synthetic auxins (i.e. 2,4-D) (Heap, 2015).

The growth regulator herbicides, also called synthetic auxins, were the first selective organic herbicides to be developed. The selective control of broadleaf weeds in cereal grain crops by growth regulator herbicides has made this herbicide group one of the most widely used (Sterling and Hall, 1997). Although growth regulator herbicides are the oldest herbicides, their MoA is still unknown in detail. Growth regulator herbicides are in general structured similarly to indole-3-acetic acid, but over the years, various chemical classes of growth regulator herbicides, with different structures, weed spectra and types of selectivity have been synthesized and commercially introduced (Grossman, 2010).

Since the first two documented 2,4-D resistant weeds, wild carrot (*Daucus carota* L.) (Switzer, 1952) and spreading dayflower (*Commelina difussa* L.) biotypes in 1957 (Hilton,

1957), there has been a slow increase in the growth regulator resistant weeds principally concentrated in the 1990's (Heap, 2015). To date, 32 weed species have been reported to have evolved resistance to growth regulator herbicides (Heap, 2015) after more than 60 years of use. As a result of repeated application of the same herbicides, a high selection pressure was the common factor that seemed to be the key to the development of resistance to several herbicides classes, including growth regulator herbicides (Nandula, 2010). Sterling and Hall (1997) indicated the low incidence of growth regulator herbicide resistance was due to these herbicides having multiple modes- and sites-of-action and because they are not persistent in the soil.

Waterhemp is considered one of the most frequent and troublesome weeds in soybean [*Glycine max* (L.) Merr.] and corn (*Zea mays* L.) production systems in the Midwest (Webster, 2005; Hager and Sprague, 2002). Additionally, as mentioned above, waterhemp has been ranked as a top five weed species based on the poor response to six herbicide MoA groups to which waterhemp has had the ability to rapidly evolve resistance (Heap, 2015). This dynamic situation in which waterhemp is more prone to select resistance is in part favored by the fact that waterhemp is a dioecious species (Costea et al., 2005). Thus, outcrossing is assured and gene flow among and within populations occurs readily (Trucco et al., 2006). This attribute, along with a high-level seed production, provides large genetic variability and enough genetic material for selection pressure to take place. The potential for long-distance dispersal of resistance via wind-borne pollen is another important biological characteristic of waterhemp that helps herbicide resistance easily spread and stack with other herbicide resistant traits. This situation has lead to populations with multiple-herbicide resistance that limits chemical options for managing waterhemp (Tranel et al., 2011). In addition, where herbicide use is the primary weed control method, stacking results in additional selection pressure for the few herbicides that are still effective (Tranel et al., 2011).

Growth regulator herbicides are recommended for use in fallow, turf, range, pasture, and cereal crop production for controlling a large spectrum of broadleaf weed species including waterhemp and other weed species belonging to *Amaranthus* family (Jhala et al., 2013; Hager et al., 2000). Transgenic soybeans, corn and cotton genetically modified to resist 2,4-D (Wright et al. 2010) and dicamba (Behrens et al. 2007) are close to be commercialized as tools that will help farmers to address weed changes in herbicide resistant weed management. Prolonged use of growth regulator herbicides on most of the weeds reported where resistance to this herbicide

group has been considered the main cause, resulted in an intensive selection pressure for the evolution of resistance. The incidence of auxinic herbicide resistance is low compared with other herbicide families such as the ALS- and PSII-inhibiting herbicides. However, the introduction of herbicide tolerant crops, such as 2,4-D resistant crops, could result in extensive changes in weed management and cropping systems by the frequent use of 2,4-D, thus raising the selection pressure on weed species.

In 2009, a grower from Nebraska contacted scientists from the University of Nebraska-Lincoln and reported that waterhemp plants survived and recovered after being treated with a recommended rate of 2,4-D. The grower also reported that a suspected 2,4-D resistant population was in a field were warm season grass had been growing since 1996 with annual applications of 2,4-D and PSII- and ALS-inhibiting herbicides to control broadleaf weeds. After greenhouse and field experiments, Bernards et al. (2012) confirmed that the waterhemp population was resistant to 2,4-D. Based on R:S ratio (Resistant : Susceptible), this study reported that the 2,4-D resistant biotype tolerated a 2,4-D dose approximately 10-fold higher than the susceptible biotype based on both I_{50} (50% visible injury) and GR_{50} (50% reduction in dry weight) values. The R:S ratio increased to 19 and 111 when results were extrapolated to I_{90} and GR_{90} estimates, respectively (Bernards et al., 2012). It is the sixth herbicide MoA group reported to which waterhemp has evolved resistance. Seed from the reported 2,4-D resistant waterhemp biotype was collected in 2010, and the current research experiment had the following objectives: 1) to confirm the resistance to 2,4-D in a Nebraska waterhemp biotype, 2) to determine if the 2,4-D resistant waterhemp biotype is also resistant to other herbicide MoA, 3) to determine the response of the 2,4-D resistant waterhemp biotype to other growth regulator herbicides, and 4) if multiple-herbicide resistance is detected, the molecular basis of herbicide resistance must be determined to reinforce previous findings and characterize the resistance mechanism.

Materials and Methods

Waterhemp biotypes

Seed from one 2,4-D resistant (FS) and two susceptible waterhemp (SE and SCAL) biotypes were used in the present study. Seed from the 2,4-D resistant waterhemp biotype reported by Bernards et al. (2012) was collected in a field planted with little bluestem grass [*Schizachyrium scoparium* (Michx.) Nash 'Camper'] located in Cass County, Nebraska. Seed

from two 2,4-D susceptible waterhemp biotypes was obtained from Nemaha and Clay Counties, Nebraska. Each population sample was a composite of at least 40 plants. Waterhemp seed was cleaned and stored at 4 C.

Plant growth

Experiments were conducted in greenhouses located on the East Campus of the University of Nebraska-Lincoln in Lincoln, Nebraska. Supplemental lighting (500 µmol $m^{-2}s^{-1}$) provided a 15 h photoperiod. The temperature during the day varied between 24 and 28 C while during the night varied between 18 and 22 C.

Waterhemp seed was germinated by placing it on moistened filter paper in petri dishes, sealing the petri dishes and placing them in the oven for 48-72 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 LTD, Saint-Modeste, Quebec, 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-Sierra Horticultural Co., Marysville, OH, USA). The seedlings were thinned to one plant per pot prior to herbicide treatments being applied.

Herbicide application

Herbicide treatments were applied to waterhemp plants when they were 8 to 12 cm tall and/or 5 to 8 fully expanded leaves. A chamber sprayer (DeVries Mfg. Corp., Hollandale, MN, USA) equipped with a TP8001E flat-fan nozzle tip (Spraying Systems Co., North Avenue, Wheaton, IL, USA) was used to make the herbicide application. The carrier volume used was 190 L ha^{-1} at a pressure of 207 kPa.

Herbicide single dose experiments

Fifty plants from each waterhemp population were treated in separated experiments with a single dose of six commercial herbicide formulations of atrazine, imazethapyr, lactofen, glyphosate, glufosinate and mesotrione as depicted in Table 1. Two single dose experiments were repeated. Visible injury estimates were made at 7, 14, 21 and 28 d after treatment (DAT) compared to untreated plants (controls) using a scale of 0 (no injury) to 100 (dead plants). At 28 DAT, plants were cut at the base and dried for 48 h in a forced air dryer at 65 C, and dry weight biomass was measured. Mean values and standard error bars were graphed using SigmaPlot 12.2 (Systat Software, Inc., San Jose, CA, USA).

Table 1. Herbicides and doses applied in the single dose screening on 2,4-D resistant and susceptible waterhemp biotypes.

Herbicide	Commercial brand	Dose g ae ha^{-1}	Site of action [a]	Additives [b]
Atrazine	AAtrex	2,240	PSII	COC
Imazethapyr	Pursuit	70	ALS	COC + AMS
Lactofen	Cobra	210	PPO	COC + AMS
Mesotrione	Callisto	105	HPPD	COC + AMS
Glufosinate	Ignite	322	GS	AMS
Glyphosate	Roundup PowerMax	867 [c]	EPSPS	AMS

[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).

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

Dose-response experiments

i. *Response to non-growth regulator herbicides*

Separated dose-response experiments using ametryn [N-ethyl-N'-(1-methylethyl)-6-(methylthio)-1,3,5-triazine-2,4-diamine] (Evik, DF 76%, Syngenta Crop Protection, NC, USA) and chlorimuron ethyl [2-[[[[(4-chloro-6-methoxy-2-pyrimidinyl)amino]carbonyl] amino]sulfonyl]benzoic acid] (Classic, DF 25%, DuPont Crop Protection, DE, USA) herbicides were performed for 2,4-D resistant and susceptible waterhemp biotypes. The experiments were arranged using a randomized complete block design with 10 repetitions per treatment. The dose-response experiments were repeated twice. Five ametryn doses were applied: 0, 123, 560, 1,120, 2,240 g ai ha^{-1}. In a separate experiment, six chlorimuron-ethyl doses were applied: 0, 17, 35, 70, 140 and 280 g ha^{-1}. Both herbicides had 1% of crop oil concentrate added as an adjuvant.

ii. *Response to growth regulator herbicides*

To confirm the resistance in the previously reported 2,4-D resistant waterhemp biotype by Bernards et al. (2012), herbicide treatments included higher doses above the labeled 2,4-D rate for corn than those doses used by previous researchers. 2,4-D resistant waterhemp biotype was treated with 2,4-D (2-ethylhexyl ester of 2,4-dichlorophenoxyacetic acid) (Lo-Vol 4 Herbicide, Tenkoz Inc., Alpharetta, GA, USA) at 0, 140, 280, 560, 1,120, 2,240, 4,480, 8,960,

17,920, 35,840 g ae ha^{-1}. In separate dose-response experiments, 2,4-D susceptible waterhemp biotypes were treated with 2,4-D at 0, 9, 18, 37, 70, 140, 560, 1,120, 2,240, 4,480 g ae ha^{-1}. Similarly, dose-response experiments were conducted using eight doses of each of the following herbicides (Table 2): aminocyclopyrachlor (6-amino-5-chloro-2-cyclopropyl-4-pyrimidinecarboxylic acid) (Imprelis Herbicide, DuPont, Wilmington, DE, USA), aminopyralid (4-amino-3,6-dichloropyridine-2-carboxylic acid) (Milestone Herbicide, Dow AgroSciences LLC, Indianapolis, IN, USA) and picloram (4-amino-3,5,6-trichloropicolinic acid) (Tordon 22k Herbicide, Dow AgroSciences LLC, Indianapolis, IN, USA) (Table 2) on the three previously mentioned waterhemp biotypes. Preliminary results from dose response experiments with picloram showed a lower visible injury estimate on 2,4-D resistant waterhemp biotype than 2,4-D susceptible biotype (data not shown). Therefore, higher picloram doses were applied on 2,4-D resistant waterhemp biotype than those susceptible biotypes. All dose-response experiments were arranged in a randomized complete block design with five repetitions each, and were conducted twice. Treatments containing 2,4-D, aminocyclopyrachlor and aminopyralid applications included nonionic surfactant (NIS) at 0.25% (v/v) while the experiments using picloram were applied without adjuvant.

Table 2. Growth regulator herbicides other than 2,4-D and doses applied in the dose-response experiments on 2,4-D resistant and susceptible waterhemp biotypes.

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

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 to 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, dried for 48 h in a forced air dryer at 65 C, and 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) package in R 2.3.0 (R statistical software, R Foundation for Statistical Computing, Vienna, Austria. URL: 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):

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

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 dose needed to achieve the 50, 80 and 90% based on the visible injury estimates (I) and dry weight (GR) at 28 DAT was calculated. The relative level of resistance was expressed by calculating the R:S ratios between the I or GR values of the less 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, Inc., San Jose, CA).

Molecular analysis of resistance

Prior to herbicide application in the second run of the single herbicide dose experiment depicted above, a smaller fully expanded leaf per plant was obtained, placed in an identified 1.5 ml eppendorf tube and then stored in a freezer at -20 C until the samples were needed. As a result of the single dose experiments, it was suspected that the tested populations were resistant to ALS-, PSII- and PPO-inhibiting herbicides. Then, ten tissue samples per population from five suspected ALS, atrazine and lactofen resistant plants and five susceptible plants were selected, and molecular experiments were conducted in laboratories located at the University of Illinois at Urbana, IL. These samples were used to determine if the Trp574Leu mutation conferring resistance to sulfonylurea and imidazolinone herbicides, and/or Ser653 mutation conferring resistance to imidazolinone herbicides were present (Patzoldt and Tranel 2007). Additionally, the presence of Ser264Gly, Ser264Thr, Val219Ile, Ala251Val and Asn266Thr mutations in the PsbA

gene conferring resistance to PSII inhibiting herbicides were tested (Foes et al. 1998; Patzoldt et al. 2003). For suspected resistant to PPO-inhibiting herbicides, the three-base-pair deletion in the PPX2L gene were tested (Lee et al. 2008).

DNA-based testing was used to detect a mutation in the ALS gene which consisted of DNA isolation from the leaf tissue samples and amplification of the region B of ALS gene by using PCR technique. The following primers AmALS-F2: 5´-TCCCGGTTAAAATCATGCTC and AmALS-R2: 5´-CTAAACGAGAGAACGGCCAG were used (Foes et al 1998). A PCR-RFLP assay was used to digest the PCR product with a specific restriction enzyme (MfeI) and incubated for 6 h at 37 C. After digestion, DNA fragments were separated on a 2% agarose gel and visualized with a Kodak Gel Logic 1500 Imaging System. Individual plants were classified as homozygous for the L570 ALS allele, heterozygous, or homozygous for the W570 allele based on the presence of DNA fragments with approximate base pair sizes of 950, both 950 and 1,300, or 1,300, respectively (Foes et al. 1999).

Additionally, DNA sequencing was performed on the amplified gene in the PCR product to confirm the results from PCR-RFLP assay in the suspected ALS-resistant plants and to identify the Ser264 mutation in the PsbA gene for atrazine resistance. Standard techniques as described previously by Foes et al. (1998) were used. The primers for ALS and PsbA genes used in the sequencing were also the same as used previously (Foes et al. 1998) and described above.

Results and Discussion

Herbicide single dose experiments

The 50 plant average visible injury estimates at 28 DAT of both 2,4-D resistant (FS) and susceptible (SCAL and SE) biotypes treated in two separate experiments with one single dose of six different herbicide MoA are presented in Figure 1. At 28 DAT all untreated plants for all herbicides did not show visible injury (data not shown). Atrazine injured 2,4-D resistant and susceptible biotypes scored less than 10% on the average visible injury scale at 28 DAT for both experimental runs (Figure 1). Plants belonging to FS biotype showed 1% or less visible injury at 28 DAT when treated with imazethapyr in both runs. Plants belonging to both, SE and SCAL biotypes showed no higher visual injury than 26 and 42%, respectively when treated with imazethapyr (Figure 1).

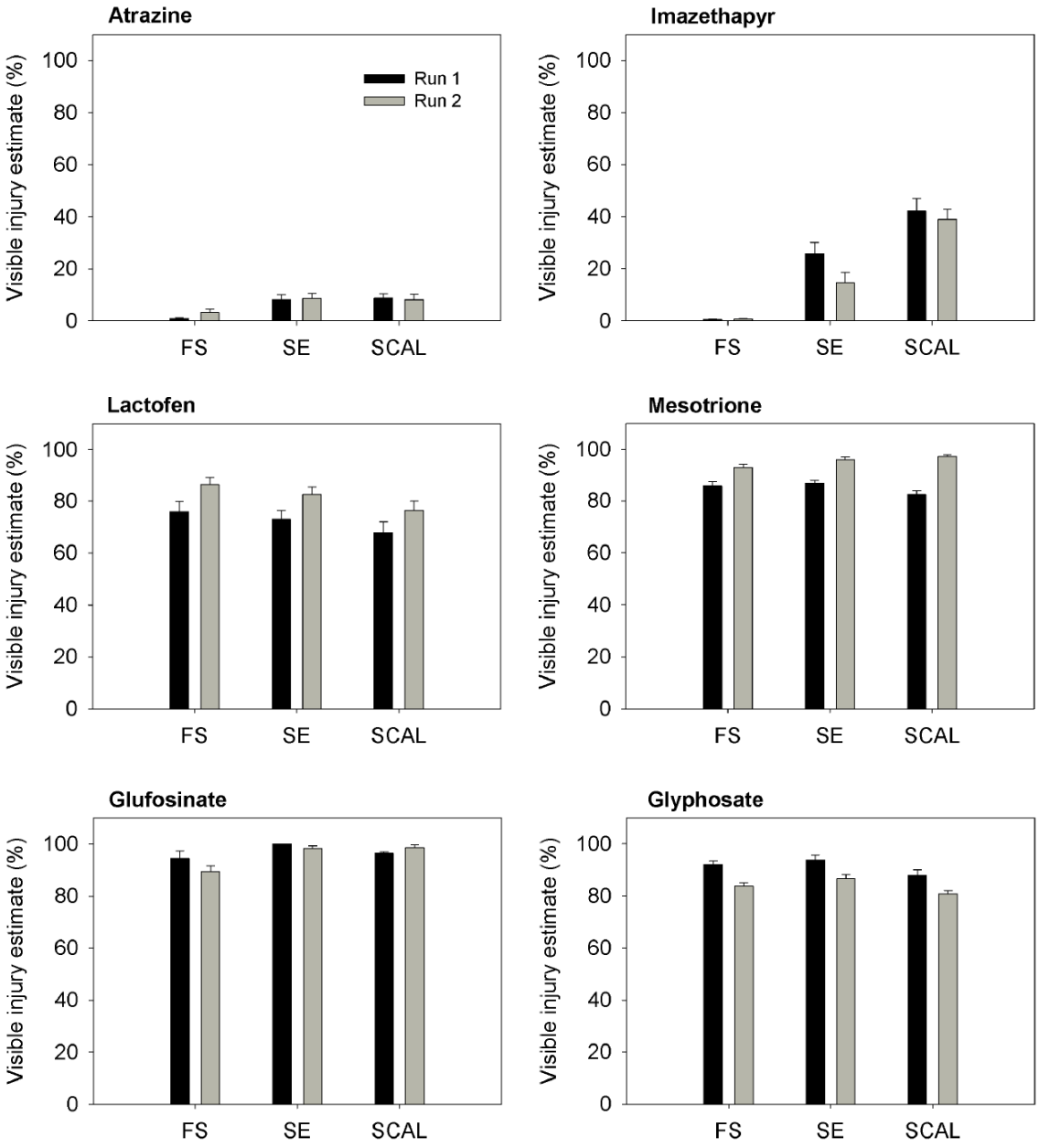

Figure 1. Visible injury estimates from two experimental runs of the 2,4-D resistant (FS) and susceptible (SE and SCAL) waterhemp biotypes to a single labeled dose of atrazine, imazethapyr, lactofen, mesotrione, glufosinate and glyphosate. Vertical bars represent ± the standard error of the mean. Data represent the average of 50 plants per experimental run.

Lactofen injury ranged between 62 and 69% in the first experimental run, and between 70 and 78% in the second run for all three waterhemp biotypes. Lactofen strongly injured plants belonging to all waterhemp biotypes in the first two days after treatment, and typical symptoms

such as chlorosis, necrosis and crinkling were observed. However, 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 (data not shown). Although lower visible injury estimates occurred, the same symptoms were observed by Shoup and Al-Khatib (2005) who reported the first case of PPO-resistant waterhemp in Kansas in 2001. Average visible injury estimates were 80% or higher for three waterhemp biotypes in both experimental runs for mesotrione, glufosinate and glyphosate (Figure 1). All plants belonging to biotype SE showed 100% control when treated with glufosinate (Figure 1).

It was expected that the FS biotype would also show a lack of response to atrazine since the grower who reported the lack of control with 2,4-D (Bernards et al. 2012) also reported atrazine was annually applied since 1996 to control broadleaf weeds in the same field. Early in the 1990's, Anderson et al. (1996) confirmed that 92% of the suspected atrazine resistant waterhemp populations from southeast Nebraska were indeed resistant to that herbicide. Then because of this, it was not surprising that SE and SCAL biotypes also show low visible injury after being treated with labeled field rates of atrazine.

Waterhemp has also been reported resistant to imazethapyr, an imidazolinone group belonging to ALS-inhibiting herbicide, early in 1990's in Iowa, Missouri and Kansas (Heap, 2015), which are the neighboring U.S. states in southeastern Nebraska. Low response to imazethapyr in all three waterhemp biotypes was expected. While the 2,4-D resistant biotype, FS, was completely insensitive to imazethapyr at 28 DAT, SE and SCAL biotypes showed higher and more variable response than FS biotype at 28 DAT. However, SE and SCAL responses were substantially lower that the optimum (> 80% visible injury) control for the labeled field rate.

Dose response experiments

i. *Response to non-growth regulator herbicides*

In addition to the single dose experiment findings previously presented, results from dose-response experiments using ametryn and chlorimuron-ethyl are shown in Table 3 and 4, and Figures 2 to 5. Waterhemp plants from FS biotype always needed higher ametryn dose than SE and SCAL biotypes to reach 50, 80 and 90% of both visible injury (Table 3; Figure 2) and 80 and 90% dry weight reduction (Table 4; Figure 3). Recommended labeled rate of 2,240 g ai ha^{-1} of ametryn injured 77% of FS biotype at 28 DAT, which was lower than 93% injury on SE and

SCAL biotypes (Table 3). At the highest ametryn dose used in the present study, two of the 10 repetitions belonging to FS biotype were less affected at the beginning of the treatment and partially recovered at 28 DAT. However, the 2,4-D resistant biotype, FS, was less than 2-fold more tolerant to ametryn than both 2,4-D susceptible waterhemp biotypes, SE and SCAL based on visual injury (Table 3) and dry weight reduction (Figure 3) values. This low R:S ratio suggests there is not resistance to ametryn in any of the waterhemp biotypes evaluated in the present experiments.

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 susceptible (SE and SCAL) waterhemp biotypes from Nebraska.

Biotype	Regression parameters			I_{80} (±se)		I_{90} (±se)	
	b	I_{50} (±se)					
		g ae 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[a]		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[a]		7.1		2.7		2.3	

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}).

[a] R:S = Resistant:Susceptible ratio between the least susceptible biotype and the most susceptible biotype.

Table 4. Dry weight reduction (GR) regression parameters, ametryn (Evik DF, Syngenta) and chlorimuron (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 susceptible (SE and SCAL) waterhemp biotypes from Nebraska.

Biotype	Regression parameters			GR_{50} (±se)		GR_{80} (±se)		GR_{90} (±se)	
	c	d	b						
				g ae 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[a]				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[a]				3.7		3.2		3.6	

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}).

[a] R:S = Resistant:Susceptible ratio between the least susceptible biotype and the most susceptible biotype.

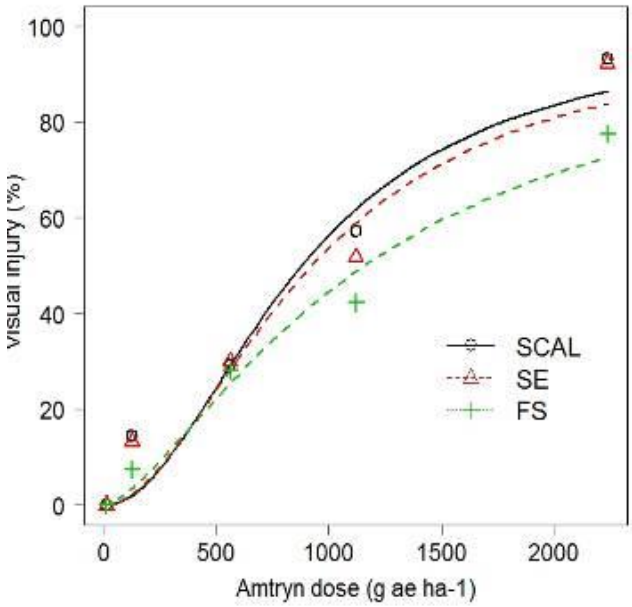

Figure 2. Visual injury estimate as affected by ametryn dose for 2,4-D resistant and susceptible waterhemp biotype at 28 DAT in greenhouse bioassays. Regression parameters are provided in Table 2. Data represent the mean of two experiments and four replications per experiment. The error bars represent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

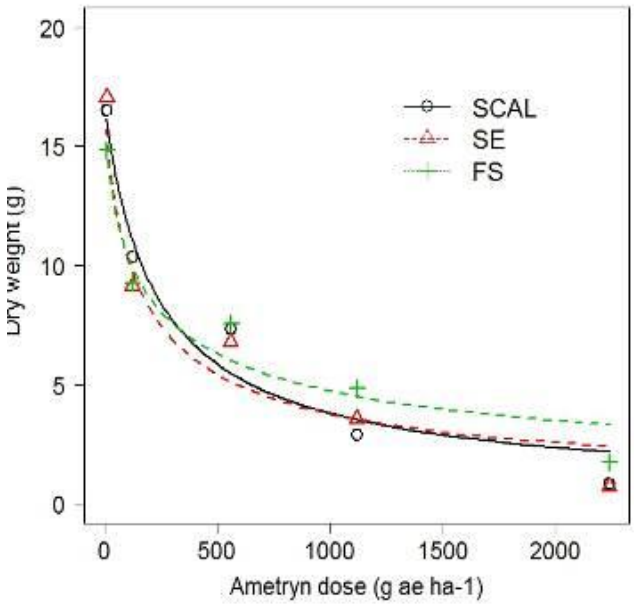

Figure 3. Percent dry weight reduction relative to untreated control as affected by ametryn dose at 28 DAT of 2,4-D resistant and susceptible waterhemp biotypes in greenhouse bioassays. Regression parameters are given in Table 3. Data represent the mean of two experiments and four replications per experiment.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

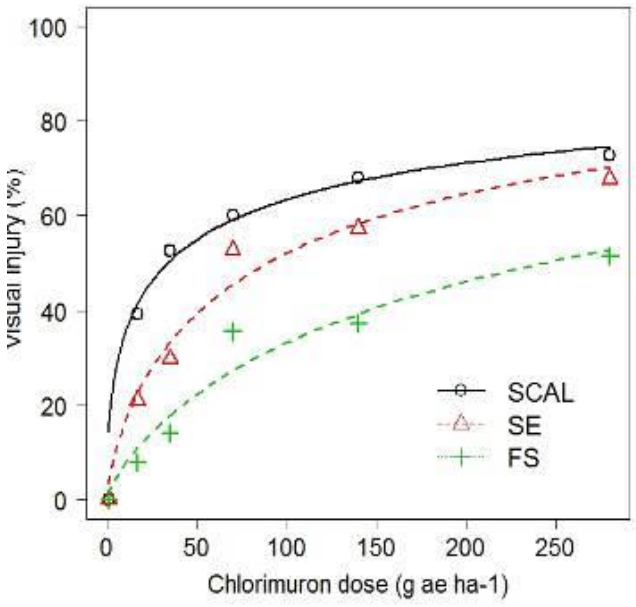

Figure 4. Visual injury estimate as affected by chlorimuron-ethyl dose for 2,4-D resistant and susceptible waterhemp biotype at 28 DAT in greenhouse bioassays. Regression parameters are provided in Table 2. Data represent the mean of two experiments and four replications per experiment. The error bars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

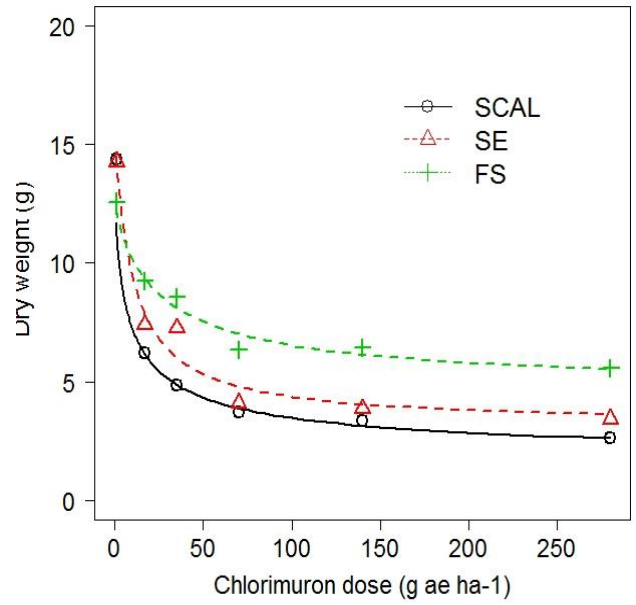

Figure 5. Percent dry weight reduction relative to untreated control as affected by chlorimuron-ethyl dose at 28 DAT of 2,4-D resistant and susceptible waterhemp biotypes in greenhouse bioassays. Regression parameters are given in Table 3. Data represent the mean of two experiments and four replications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

The dose-response experiment conducted with chlorimuron-ethyl also showed that FS biotypes needed the highest doses to reach I and GR values (Table 3; Figure 4). The R:S ratios calculated for 80 and 90% visible injury (Table 3) and dry growth reduction (Figure 4) varied between 2.3 and 3.6, while the R:S ratio for the 50% injury was 7.1 (Table 3). For the FS biotype, an estimated dose of 243 g ha^{-1} was predicted to result in 50% visible injury estimate, while for the most susceptible biotype, SCAL, 34 g ha^{-1} was the predicted dose to cause 50% visible injury (Table 3). Based on the predicted values, and on the fact that 1,405 g ha^{-1} of chlorimuron-ethyl (40 times higher than the labeled rate for soybean) was needed to reach an optimum control ($\geq$ 80% injury) of the FS biotype, it would suggest the FS biotype was resistant to chlorimuron-ethyl. However, it is important to point out that the reported experiment was not using a known susceptible biotype to contrast with a putative resistant biotype to confirm herbicide resistance (Beckie et al., 2000). Preliminary dose-response experiments (data not shown) using ACR and WCS waterhemp biotypes reported sulfonylurea (i.e. thifensulfuron) resistant and susceptible respectively by Patzoldt et al. (2005), which suggested resistance in the FS biotype. Those preliminary results showed 5% injury of ACR biotype when treated with 405 g ha^{-1} of chlorimuron-ethyl while biotype WCS was 75% injured with the same dose. Those results confirm previous reports by Patzoldt et al. (2005) and suggest that FS biotype is resistant to sulfonylurea herbicides.

ii. *Response to growth regulator herbicides*

A previous dose-response experiment confirmed the 2,4-D resistant waterhemp biotype as reported by Bernards et al. (2012). In the current study, the maximum level of resistance expressed as R:S ratio for GR_{90} was lower than the ratio reported by Bernards et al. (2012). The rank between I or GR ratio to reach 50% control, and those values to reach 90% control, showed less variation than values reported by Bernards et al. (2012). Resistant biotype, FS was 53 and 35 times more resistant than 2,4-D susceptible biotypes based on I_{50} and GR_{50}, respectively (Tables 5 and 6). FS biotype needed a 2,4-D dose 44 times higher than susceptible ones to be 90% injured (Table 5). 2,4-D susceptible biotypes, SE and SCAL, were 50% injured by minimal doses of 91 and 86 g ha^{-1} of 2,4-D, and strongly injured (> 90%) by 830 g ha^{-1} of 2,4-D or less (Table 5). In contrast to the previous report by Bernards et al. (2012), 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 confirmed 2,4-D resistant biotype. Thus, the log-logistic model prediction could be

more accurate to predict the 2,4-D doses needed to reach 90% visible injury or reduction in dry weight. For the resistant biotype, FS, I_{90} and GR_{90} values of 28,545 and 24,722 g ha^{-1} were predicted which are more closely related than those values reported by Bernards et al. (2012).

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 susceptible (SE and SCAL) waterhemp biotypes from Nebraska.

Biotype	Regression parameters			I_{80} (±se)		I_{90} (±se)	
	b	I_{50} (±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[a]		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[a]		4.7		5.9		6.7	

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}).

[a] R:S = Resistant:Susceptible ratio between the least susceptible biotype and the most susceptible biotype.

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 susceptible (SE and SCAL) waterhemp biotypes from Nebraska.

Biotype	Regression parameters					GR_{80} (±se)		GR_{90} (±se)	
	c	d	b	GR_{50} (±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[a]				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[a]				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	

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}).

[a] R:S = Resistant:Susceptible ratio between the least susceptible biotype and the most susceptible biotype.

Results of multiple dose-response experiments with aminocyclopyrachlor, aminopyralid and picloram herbicides, different than 2,4-D, are shown in Tables 5 and 6. 2,4-D resistant waterhemp biotype, FS, was always shown to be the most tolerant to all three herbicides than 2,4-D susceptible biotypes, SE and SCAL when results are based on visible injury (Table 5). The R:S ratios varied between 2.4 and 7.5 based on visible injury estimates (Table 5) and between 1.9 and 6.1 based on dry weight reduction (Table 6). SE and SCAL biotypes showed similar response to aminocyclopyrachlor, aminopyralid and picloram based on visible injury estimates (Table 5); however, when based on dry weight reduction, biotype SE was shown to be more susceptible than biotype SCAL for all three growth regulators other than 2,4-D (Table 6). Biotype FS showed 2.4 and 3.6-fold greater tolerance to aminocyclopyrachlor based in I_{50} and I_{90} values. For aminopyralid and picloram, FS biotype was 4.7-fold more tolerant than the other two biotypes based on I_{50} value. Based on I_{90} values, biotype FS expressed a 7.5- and 6.7-fold difference for aminopyralid and picloram, respectively compared to SE and SCAL biotypes. Interestingly, biotype SCAL showed a higher level of tolerance to aminocyclopyrachlor than FS and SE biotypes based on GR values (Tables 6).

These results suggest that growth regulator herbicides such as aminocyclopyrachlor, aminopyralid and picloram should be effective to reach 50% injury when used at recommended field rates of 80, 88 and 280 g ha^{-1}, respectively in all three waterhemp biotypes. However, those recommended rates did not reach 80% injury when applied on 2,4-D resistant waterhemp biotype, FS. Surprisingly, any waterhemp biotype was 90% injured or higher with recommended field rates (Table 5). Results indicate that not only was the FS biotype resistant to the 2,4-D, it was also resistant to other growth regulator herbicides. FS biotype needed 7, 11 and 16-fold higher dose than recommended field rates for aminocyclopyrachlor, aminopyralid and picloram respectively, based on visible injury estimates (Table 6). The maximum labeled rates of the three tested growth regulator herbicides, other than 2,4-D, provided at least 80% control on SE and SCAL biotypes. An exception was SCAL biotype when it was treated with picloram in which, the recommended field rate (280 g ha^{-1}) was not enough to reach 80% injury. These results also suggested that aminocyclopyrachlor, aminopyralid and picloram may play a key role in postemergence application on 2,4-D susceptible biotypes, but not on the 2,4-D resistant biotype, FS. The resistance of this biotype of waterhemp to 2,4-D and the reduced sensitivity to other growth regulator herbicides presents practical problems as this herbicide MoA is frequently used

on corn and small grain crops. Additionally, this could be problematic in the near future when 2,4-D and also dicamba resistant corn, soybean and cotton will be available in the market. Appropriate stewardship practices that minimize the selection pressure on 2,4-D resistant waterhemp biotypes, but also on other waterhemp populations should be imposed to reduce the herbicide resistance evolution.

Molecular analysis of resistance

From previous single dose experiments, labeled field rates of atrazine and imazethapyr achieved poor control of all three waterhemp biotypes (Figure 1). Plants belonging to all three biotypes were strongly injured by lactofen two days after treatment. However, more than 50% of the treated plants started to actively recover and new leaves and stems were observed at 28 DAT (Figure 1). Therefore, previous results suggested that both, 2,4-D resistant and susceptible waterhemp biotypes could be resistant to those three herbicide MoA (Figure 1). In addition to the differential response to imidazolinone, dose-response experiment results suggested that 2,4-D resistant waterhemp biotype, FS, would also have a differential response to sulfonylurea herbicides (Table 2). Further studies focused on confirming the presence of a genetic mutation in the PsbA gene conferring resistance to atrazine, and Trp574Leu (i.e. conferring resistance to imidazolinones and sulfonylureas) and Ser653Asn (i.e. conferring resistance to imidazolinones) mutations in the ALS gene. Also, a three-base-pair deletion in the PPX2L gene conferring resistance to PPO- inhibiting herbicides was tested.

Atrazine resistance

Sequencing results of the region A in the Psb gene of two plants per waterhemp biotype (i.e. 2,4-D resistant and susceptible biotypes) showed identical sequences. In these waterhemp biotypes the Ser264 mutation in the PsbA gene for atrazine resistance was not identified. The other three target-site mutations (i.e. Val219Ile, Ala251Val and Asn266Thr) that were reported to confer resistance to atrazine were not identified in the sequenced region. These results are consistent with those results reported by Patzoldt et al. (2003) and confirm that triazine resistance in all three waterhemp biotypes can be conferred by a non-target-site mechanism. The high visual injury estimates previously reported in ametryn dose-response experiment on all three waterhemp biotypes (Table 2) are coincident with lacking a target-site mutation and also confirm the molecular/genetic results. Since the non-target-site mechanism of triazine resistance can be transmitted by seed and/or pollen, the spreading of this resistance mechanism was

expected to be faster than the target-site mechanism. As reported by Costea et al. (2005), the high capacity of *Amaranthus* sp.to evolve to herbicide resistance is given because waterhemp is a dioecious species (Costea et al. 2005). Thus, outcrossing is assured and gene flow among and within populations occurs readily (Trucco et al. 2006). This attribute, together with a high seed production, provides large genetic variability and enough genetic material for selection pressure to take place. The potential for long-distance dispersal of resistance via wind-borne pollen and seed is another important biological characteristic of waterhemp that helps herbicide resistance easily spread and stack with other herbicide resistant traits (Tranel et al. 2011).

ALS resistance

Using PCR-RFLP technique, the ALS locus was analyzed using tissue samples obtained from five plants of each of the three waterhemp biotypes. Difference in alleles at the ALS gene locus, and whether the plants were "heterozygous" (i.e. one copy of each resistance and susceptible alleles, on different DNA strands) or "homozygous" (i.e. two copies of the same allele, resistance or susceptible allele, on different DNA strands) was determined for the named locus. Four plants from SCAL biotype were heterozygous for the ALS locus while the other one was categorized as homozygous susceptible for the same locus. All five plants from FS biotype were homozygous susceptible for the ALS locus. Plants from SE biotype were mixed results as follows: two homozygous susceptible, one homozygous resistant and two heterozygous for the ALS locus.

Most of the ALS resistance cases in *Amaranthus* sp. are conferred by mutations in the ALS gene. Trp574Leu mutation conferring broad cross-resistance to ALS-inhibiting herbicides including imidazolinones and sulfonylureas was identified in one plant of SCAL biotype and in other plants of SE biotype, which agreed with RFLP results. Trp574Leu mutation lacked in all five plants belonging to FS biotype, but Ser653 mutation conferring resistance to imidazolinones was found in all FS plants. These findings confirm that FS biotype is resistant to imazethapyr. However, those results contradict the suspected resistance to chlorimurom-ethyl, a sulfonylurea herbicide (Table 2). In the present experiment, dose-response experiments showed a R:S ratio of 7.1 based in the I_{50} value which would suggest resistance to chlorimuron-ethyl. This ratio value result is slightly higher than the value reported by Sobony and Rubin (2003) using chorimurum-ethyl on *Amaranthus blitoides* (S.) Watson, a relative species of waterhemp, *Amaranthus tuberculatus*. Lovell et al. (1996) reported at least 330-fold resistance based on visible injury

compared to the susceptible waterhemp biotype with chlorimurom-ethyl. Several studies have used thifensulfuron, other sulfonylurea herbicide, and reported 28-, 490-, 18,000- and 34,000-fold differences between waterhemp resistant and susceptible biotypes (Lovell et al. 1996; Patzoldt et al. 2005; Patzoldt and Tranel 2007; McMullan and Green 2011). In contrast to the previously hypothesized sulfonylurea resistance in FS biotype, older studies would suggest that 7.1 R:S ratio is enough magnitude to confirm resistance. Lovell et al. (1996) reported high degree of cross-resistance between imidazolinone and sulfonylurea herbicides in a waterhemp biotype even when field history did not show a continuous use of imidazolinone herbicides. This suggests that seed and/pollen from highly tolerant natural waterhemp populations could play an alternative key role as another mechanism of imidazolinone resistance evolution. Additionally, manure application and harvest equipment can contribute as sources of resistance transference from field to field (Horak and Peterson 1996).

Lactofen resistance

In order to test the three-pair deletion, also called ΔG210 mutation, conferring resistance to PPO herbicides, the sequencing of PPX2L gene was carried out. Despite greenhouse results suggesting resistance to lactofen (plant survival higher than 50% and no more than 78% of visual injury average from 50 plants), the sequencing results were not quite clear. Previous researchers reported the presence of the three-pair deletion in the PPX2L gene in waterhemp populations from Illinois, Kansas and Missouri (Shoup et al. 2003; Patzoldt et al. 2006; Lee et al. 2008). As reported by Tranel et al. (2011), the three pair deletion has been the only mechanism reported to date conferring resistance to PPO-inhibiting herbicides. Results reported in the current study could indicate that little to no resistance to PPO-inhibitor herbicides, or a different resistance mechanism and/or genetic mutation is conferring the lack of sensitivity to lactofen.

These experiments have demonstrated that the waterhemp population previously reported resistant to 2,4-D by Bernards et al. (2012) has also evolved resistance to herbicides from ALS- and PSII-inhibiting MoA. Additionally, this population showed resistance to other growth regulator herbicides such as aminopyralid and picloram. This finding severely limits the herbicide option to control the 2,4-D resistant waterhemp biotype. Other herbicides with different MoA such as mesotrione, glufosinate, and glyphosate have been shown to still be effective to control the 2,4-D resistant waterhemp biotype. However, the reliance on only one of those herbicides to control the biotype could probably lead to the evolution of multiple herbicide

MoA resistance. It is more than clear that the repeated annual application of 2,4-D and herbicides belonging to PSII- and ALS-inhibitors has resulted in multiple herbicide resistance. The present report is particularly important because waterhemp is a frequent and problematic weed in soybean and corn in the Midwest and this weed has been reported resistant to six herbicide MoA (Heap 2013). This scenario has been, in part, favored by the fact that waterhemp is a dioecious species and therefore, outcrossing and gene flow occur among populations. Also, waterhemp produces a high amount of seed providing large genetic variability. This could allow the enrichment and accumulation of resistance genes through the long-distance dispersal of resistance via wind-borne pollen and seed. Further research should be conducted to elucidate the resistance mechanism to 2,4-D in the 2,4-D resistant waterhemp biotype. Also, resistance to lactofen should be investigated and if it is found, the mechanism responsible for resistance to lactofen should be elucidated.

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