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Protoporphyrinogen oxidase (PPO) inhibitor–resistant waterhemp (*Amaranthus tuberculatus*) from Nebraska is multiple herbicide resistant: confirmation, mechanism of resistance, and management

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Abstract

A waterhemp [*Amaranthus tuberculatus* (Moq.) J. D. Sauer] biotype (designated as “NER”) collected from a soybean [*Glycine max* (L.) Merr.] production field in eastern Nebraska survived the POST application of fomesafen at the labeled rate. The objectives of this study were to (1) quantify the level of resistance to protoporphyrinogen oxidase (PPO) inhibitors (acifluorfen, fomesafen, and lactofen) applied POST, (2) determine the mechanism of PPO-inhibitor resistance in the NER biotype, (3) determine whether NER possessed multiple resistance to acetolactate synthase (ALS)-, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)-, and photosystem II (PSII)-inhibiting herbicides, and (4) control NER with POST soybean herbicides. A whole-plant dose–response bioassay revealed that the NER biotype was 4- to 6-fold resistant to PPO-inhibiting herbicides depending on the known susceptible biotype (S1 or S2) used for comparison. A Kompetitive Allele Specific PCR (KASP™) assay was developed and performed for rapid and robust detection of the ΔG210 mutation (deletion of a codon) in the *PPX2L* gene. All samples of the NER biotype tested positive for the ΔG210 mutation. Dose–response bioassays confirmed that the NER biotype was resistant to three additional herbicide sites of action. Chlorimuron and imazethapyr, both ALS inhibitors, applied at 32X the labeled rate resulted in <80% reduction in the aboveground biomass of the NER biotype. The same biotype was 3- and 7-fold resistant to glyphosate (EPSPS inhibitor) and atrazine (PSII inhibitor), respectively. Glufosinate, 2,4-D choline plus glyphosate, and dicamba were the only soybean POST herbicides that controlled NER effectively (≥92% aboveground biomass reduction). *Amaranthus tuberculatus* is the first confirmed weed species in Nebraska to evolve resistance to four distinct herbicide sites of action, leaving no POST herbicide choice for effective control in glyphosate-resistant and conventional (non-transgenic) soybean.

Introduction

Waterhemp [*Amaranthus tuberculatus* (Moq.) J. D. Sauer] is a small-seeded, summer annual weed indigenous to North America (Sauer 1957; Waselkov and Olsen 2014). Over the last 30 yr, *A. tuberculatus* has gone from virtual obscurity to being the most commonly encountered and troublesome weed in corn (*Zea mays* L.) and soybean [*Glycine max* (L.) Merr.] production fields in the midwestern United States, including Nebraska (Prince et al. 2012; Sarangi and Jhala 2018). *Amaranthus tuberculatus* is a highly competitive weed; for example, Steckel and Sprague (2004) reported that season-long interference of *A. tuberculatus* at 270 plants m^{-2} can reduce corn yield by 74%. A study in Illinois reported that *A. tuberculatus* allowed to compete with soybean up to 10 wk after soybean unifoliate expansion at a density up to 362 plants m^{-2} reduced soybean yield by 43% (Hager et al. 2002). Favorable biological attributes such as rapid growth rate, high net assimilation rate, prolific seed production, and smaller seed size have provided opportunities for *A. tuberculatus* to persist as the most successful and problematic weed in corn and soybean production systems in the midwestern United States (Costea et al. 2005; Hartzler et al. 2004; Horak

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and Loughin 2000; Sellers et al. 2003). Additionally, the ability of *A. tuberculatus* to survive and produce seeds under abiotic stresses such as water stress is another important biological attribute for its survival (Sarangi et al. 2016).

Amaranthus tuberculatus biotypes resistant to acetolactate synthase (ALS), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), 4-hydroxyphenylpyruvate dioxygenase, and photosystem II (PSII) inhibitors and synthetic auxins have been confirmed in Nebraska (Anderson et al. 1996; Bernards et al. 2012; Oliveira et al. 2017; Sarangi et al. 2015). Moreover, the dioecious nature of *A. tuberculatus* is believed to promote the rapid spread of herbicide-resistant traits through pollen-mediated gene flow and hasten the evolution of multiple herbicide–resistant biotypes (Sarangi et al. 2017b).

Protoporphyrinogen oxidase (PPO)-inhibiting herbicides were one of the key components of weed management in soybean before the commercialization of glyphosate-resistant crops; however, the widespread adoption of glyphosate-resistant crops in the United States substantially reduced the use of this chemistry (Dayan et al. 2017). The selection pressure imposed by the extensive use of glyphosate has led to the evolution of glyphosate-resistant weeds, with *A. tuberculatus* and Palmer amaranth (*Amaranthus palmeri* S. Watson) being the most problematic weeds in row-crop production fields in the United States. This has resulted in a resurgence of interest in using PPO-inhibiting herbicides in soybean for effective POST control of glyphosate-resistant weeds (Sarangi and Jhala 2019; Sarangi et al. 2017a; Whitaker et al. 2010).

PPO is the last enzyme in the common tetrapyrrole biosynthetic pathway that produces heme and chlorophyll (Matringe et al. 1992). Two nuclear genes, *PPX1* and *PPX2*, encode PPO in plants, where *PPX1* functions in the plastids and *PPX2* works in the mitochondria (Lermontova et al. 1997; Watanabe et al. 2001). The inhibition of PPO enzymes following a herbicide application results in the accumulation of protoporphyrinogen IX, which leaks from plastid to the cytoplasm, where it is oxidized into protoporphyrin IX (Matringe and Scalla 1988; Witkowski and Halling 1989). In the presence of sunlight, protoporphyrin IX in the cytoplasm generates highly reactive singlet oxygen, leading to the death of susceptible plants (Becerril and Duke 1989; Jacobs et al. 1991).

Amaranthus tuberculatus resistant to PPO-inhibiting herbicides was first reported in 2001 in Kansas in a continuous soybean production field with a history of repeated POST application of PPO-inhibiting herbicides (Shoup et al. 2003). By 2019, a total of 13 weed species had evolved resistance to PPO-inhibiting herbicides worldwide, with 4 weed species reported in the United States (Heap 2019c). The mechanism of PPO-inhibitor resistance in an *A. tuberculatus* biotype from Illinois was identified as the deletion of a codon in the coding sequence of *PPX2L* (a longer version of *PPX2*), resulting in the loss of glycine at position 210 (ΔG210). The mutation conferring PPO-inhibitor resistance in *A. tuberculatus* is unique in that it involves an amino acid deletion rather than a substitution (Patzoldt et al. 2006).

In 2016, the survival of *A. tuberculatus* after POST application of fomesafen and lactofen was reported by soybean growers in eastern Nebraska. This research was necessary to confirm whether putative resistant *A. tuberculatus* biotypes from Nebraska were resistant to PPO-inhibiting herbicides and cross-resistant or multiple resistant to other herbicide sites of action. The objectives of this research were to (1) confirm the existence of PPO inhibitor–resistant *A. tuberculatus* in Nebraska using a whole-plant dose–response bioassay in the greenhouse, (2) investigate the mechanism of PPO-inhibitor resistance in *A. tuberculatus*, (3) examine the potential multiple herbicide resistance in a putative PPO inhibitor–resistant biotype, and (4) evaluate the response of PPO inhibitor–resistant *A. tuberculatus* to soybean POST herbicides.

Materials and Methods

Plant Materials

Seven soybean fields in eastern Nebraska, where *A. tuberculatus* control failures were observed after application of PPO-inhibiting herbicides, were surveyed in the fall of 2016. The seedheads of at least 20 *A. tuberculatus* plants were collected from each field. Seeds were cleaned thoroughly using a seed blower (South Dakota Seed Blower, Seedburo Equipment, Des Plaines, IL) and stored separately in airtight polyethylene bags at 4 C for 6 mo. In 2017, composite seed samples from each site were planted separately in plastic trays (51 cm by 38 cm by 10 cm) containing potting mix (Berger BM1 All-Purpose Mix, Berger Peat Moss, Saint-Modeste, QC, Canada). Seedlings emerged 4 d after seeding, and the plants were kept in a greenhouse maintained at a 28/24 C day/night temperature with a 16-h photoperiod supplemented by metal-halide lamps. Sufficient water and nutrients (24-8-16, Miracle-Gro® Water Soluble All-Purpose Plant Food, Scotts Miracle-Gro Products, Marysville, OH) were supplied as needed.

Screening for Fomesafen Resistance

Seedlings were thinned, and 80 to 150 plants were allowed per tray. Three trays for each of the seven biotypes were screened in the greenhouse to evaluate their response to fomesafen. Seedlings at 8- to 10-cm height were sprayed with fomesafen (Flexstar®, 225 g ai L^{-1}) at 263 g ha^{-1} plus crop oil concentrate (COC; Agri-Dex®) at 1% v/v and ammonium sulfate (N-Pak AMS Liquid) at 2.5% v/v using a single-tip spray chamber (DeVries Manufacturing, Hollandale, MN 56045) fitted with an 8001E nozzle (TeeJet® Technologies, Spraying Systems, Wheaton, IL 60187) calibrated to deliver 140 L ha^{-1} spray volume at 207 kPa pressure at a speed of 4 km h^{-1}. Surviving seedlings from each biotype were counted at 21 d after treatment (DAT), and the percentage of survival was calculated.

Whole-Plant Dose–Response Bioassay

A biotype (hereafter referred to as "NER") collected from a soybean field in Saunders County, NE (41.24°N, 96.50°W) survived the initial fomesafen screening in the greenhouse and was selected for the whole-plant dose–response bioassay to confirm resistance to PPO-inhibiting herbicides applied POST such as acifluorfen (Ultra Blazer®, 240 g ai L^{-1}, United Phosphorus, King of Prussia, PA), fomesafen, and lactofen (Cobra®, 240 g ai L^{-1}, Valent U.S.A., Walnut Creek, CA). The greenhouse dose–response bioassay was conducted in 2017 at the University of Nebraska–Lincoln. Experiments were repeated in time beginning 14 d after the first experiment. A greenhouse environment similar to that described earlier was maintained for the dose–response bioassay. An *A. tuberculatus* biotype from Illinois (hereafter referred as "ILR") with confirmed resistance to PPO-inhibiting herbicides was included in this study as a positive control. Two *A. tuberculatus* biotypes from Clay County (S1) and Saunders County, NE (S2), with a known history of effective control with PPO-inhibiting herbicides applied POST were included in this study for comparison.

Amaranthus tuberculatus seedlings were grown in 72-cell germination trays and transplanted at the first true-leaf stage into square plastic pots (10 cm by 10 cm by 12 cm) containing potting mix. A single *A. tuberculatus* plant was allowed to grow in each pot and was considered an experimental unit. Greenhouse experiments were laid out in a randomized complete block design with 10 replications. Seedlings of NER and ILR biotypes were sprayed at 8- to 10-cm height in the spray chamber using eight doses (0, 0.125X, 0.25X, 0.5X, 1X, 2X, 4X, and 16X) of PPO-inhibiting herbicides, where the labeled doses (1X) for acifluorfen, fomesafen, and lactofen were 420, 263, and 220 g ha^{-1}, respectively. The S1 and S2 biotypes were also sprayed with eight doses of these herbicides at rates of 0, 0.063X, 0.125X, 0.25X, 0.5X, 1X, 2X, and 4X. The COC at 1% v/v and ammonium sulfate at 2.5% v/v were mixed with all treatments.

Aboveground biomass of *A. tuberculatus* was harvested at 21 DAT and oven-dried at 65 C for 5 d. Biomass data were converted into percent biomass reduction compared with the nontreated control using the equation (Sarangi et al. 2017a):

$$\text{Aboveground biomass reduction (\%)} = \left[\frac{C-B}{C}\right] \times 100 \quad [1]$$

where C is the biomass of the nontreated control, and B is the biomass of a herbicide-treated plant.

Dose–Response Data Analysis

Aboveground biomass reduction data were regressed over the doses of acifluorfen, fomesafen, and lactofen using a four-parameter log-logistic function (Knezevic et al. 2007) in R (R Statistical Software, R Foundation for Statistical Computing, Vienna, Austria):

$$Y = c + \frac{d-c}{1+\exp[b(\log x - \log e)]} \quad [2]$$

where Y is the response variable (reduction in the aboveground biomass), x is the herbicide dose, c is the lower limit (i.e., zero), d is the estimated maximum value of Y, e represents the effective doses of herbicide needed to reduce the aboveground biomass by 50% (i.e., 50% of d or relative ED_{50}), and b is the slope around e. When d was not 100%, the ED_{50} (relative) values were adjusted using *type = "absolute"* function in the drc package in R to report the absolute ED_{50} values. The ED_{80} values were also estimated following a similar procedure. The resistance index (RI), the ratio between ED_{50} values of PPO inhibitor–resistant biotypes (NER or ILR) and susceptible biotypes (S1 or S2), was determined.

The goodness-of-fit parameters such as root mean-square error (RMSE) and model efficiency coefficient (E_f) were calculated using the equations (Sarangi et al. 2016):

$$\text{RMSE} = \left[\frac{1}{n}\sum_{i=1}^{n}(P_i - O_i)^2\right]^{1/2} \quad [3]$$

$$E_f = 1 - \left[\sum_{i-1}^{n}(O_i - P_i)^2 / \sum_{i=1}^{n}(O_i - \overline{O}_i)^2\right] \quad [4]$$

where P_i is the predicted value, O_i is the observed value, $\overline{O}_i$ is the mean observed value, and n is the total number of observations. A smaller RMSE value means a better fit, and an E_f value closer to 1 means more accurate predictions.

Screening for Target-Site Resistance in the PPX2L Gene

The Kompetitive Allele Specific PCR (KASP™) assay is a faster and less expensive method for genotyping SNPs (Broccanello et al. 2018; Patterson et al. 2017; Rosas et al. 2014) than other techniques, such as allele-specific PCR (Lee et al. 2008) or TaqMan® qPCR assays (Varanasi et al. 2018; Wuerffel et al. 2015a). Therefore, a novel assay using KASP™ was developed for the rapid and robust detection of the ΔG210 mutation in *PPX2L* alleles in the NER and ILR biotypes. A 50-mg sample of young meristematic leaf tissue was collected in a 1.5-ml microcentrifuge tube (Thermo Fisher Scientific, Waltham, MA) for each *A. tuberculatus* biotype—both the NER and ILR biotypes (which survived the application of acifluorfen, fomesafen, and lactofen at 4X and 16X doses in the dose–response bioassay) and the S1 and S2 biotypes (nontreated). A total of nine plants from NER and six plants from ILR biotypes were selected for leaf tissue collection. Three plants from the S1 and S2 biotypes were selected. The leaf samples were immediately frozen in liquid nitrogen and stored at −80 C until the commencement of the experiment.

Plant DNA Extraction

The DNA extraction and analysis were performed in the Molecular Weed Science Lab at Colorado State University using the Qiagen DNEasy Plant Mini Kit (Qiagen Science, Germantown, MD). DNA concentration and purity were measured in a spectrophotometer (NanoDrop 2000 Spectrophotometers, Thermo Fisher Scientific, Wilmington, DE), and samples were diluted to 5 ng µl^{-1}.

Primer Design

Three primers were designed for the KASP™ assay to distinguish between *A. tuberculatus* with the ΔG210 mutation (the resistant allele) and biotypes retaining the G210 codon (the susceptible allele). The forward primer for the susceptible allele was appended at the 5′ end with the sequence complementary to the HEX fluorophore-quencher (GAAGGTCGGAGTCAACGGATTagcgattgaggatctccaccac), while the forward primer for the resistant allele was appended at the 5′ end with the sequence complementary to the FAM fluorophore-quencher (GAAGGTGACCAAGTTCATGCTagcgattgaggatctccacatg). Additionally, one universal reverse primer (gttatgacccttttgttgcggg) was also designed for the KASP™ assay.

KASP™ Assay

A primer master mix including both forward primers and the reverse primer was made according to the KASP™ assay manufacturer's recommendations (LGC Genomics, Beverly, MA). The primers were resuspended in distilled water at 100 µM, and a primer master mix was assembled with 18 µl of resistant allele forward primer, 18 µl of the susceptible allele forward primer, 45 µl of the common reverse primer, and 69 µl of distilled water. The KASP™ master mix contained 432 µl of LGC Genomics Master Mix (which includes polymerase, dNTPs, buffer, and HEX- and FAM-tagged oligonucleotides) and 11.88 µl of the appropriate primer master mix.

The KASP™ reactions were assembled in a 96-well plate using 4 µl of the master mix with either 4 µl of water (no-template control) or 4 µl of genomic DNA (at a concentration of 5 ng µl^{-1}). The reactions were performed in a Bio-Rad CFX Connect (Bio-Rad Laboratories, Hercules, CA) following the standard KASP™ protocol. The PCR conditions for amplifying the *PPX2L* gene were activation at 94 C for 15 min, followed by 10 touchdown cycles of 20 s at 94 C (denaturing), 61 to 55 C for

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Table 1. The POST herbicides used for the whole-plant dose–response bioassays conducted at the University of Nebraska–Lincoln to confirm multiple herbicide–resistant *Amaranthus tuberculatus* in Nebraska[a].

Herbicide[b]	Trade name	Site of action[c]	Manufacturer	Biotype and herbicide doses	Labeled dose (1X)
					g ae or ai ha^{-1}
Chlorimuron	Classic®	ALS	E.I. du Pont de Nemours and Company, Wilmington, DE	NER: 0, 0.25X, 0.5X, 1X, 2X, 4X, 16X, and 32X PAW: 0, 0.125X, 0.25X, 0.5X, 1X, 2X, 4X, and 16X	13.1
Imazethapyr	Pursuit®	ALS	BASF Corporation, Research Triangle Park, NC	NER: 0, 0.25X, 0.5X, 1X, 2X, 4X, 16X, and 32X PAW: 0, 0.125X, 0.25X, 0.5X, 1X, 2X, 4X, and 16X	70
Glyphosate	Roundup PowerMax®	EPSPS	Monsanto Company, St Louis, MO	NER: 0, 0.25X, 0.5X, 1X, 2X, 4X, 16X, and 32X DOD: 0, 0.25X, 0.5X, 1X, 2X, 4X, 16X, and 32X S1: 0, 0.063X, 0.125X, 0.25X, 0.5X, 1X, 2X, and 4X	870
Atrazine	AAtrex® 4L	PSII	Syngenta Crop Protection, LLC, Greensboro, NC	NER: 0, 0.25X, 0.5X, 1X, 2X, 4X, 16X, and 24X S1: 0, 0.063X, 0.125X, 0.25X, 0.5X, 1X, 2X, and 4X	2,240

[a] *Amaranthus tuberculatus* biotypes collected from Saunders, Pawnee, and Dodge counties of Nebraska were designated as NER, PAW, and DOD, respectively. A known glyphosate- and atrazine-susceptible biotype (S1) was collected from Clay County, NE, and included in this study for comparison.
[b] Crop oil concentrate (COC; Agri-Dex®, Helena Chemical, Collierville, TN 38017) at 1% v/v was included in the chlorimuron, imazethapyr, and atrazine treatments; nonionic surfactant (NIS; Induce®, Helena Chemical), at 0.25% v/v was included in the glyphosate treatment; and ammonium sulfate (N-Pak® AMS Liquid, Winfield Solutions, St Paul, MN 55164) at 2.86 kg ai ha^{-1} was mixed with the treatments of chlorimuron, imazethapyr, and glyphosate.
[c] Abbreviations: ALS, acetolactate synthase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; PSII, photosystem II.

60 s (dropping 0.6 C per cycle for annealing and elongation), 23 C for 30 s (for accurate plate reading), followed by 26 cycles at 94 C for 20 s, 55 C for 60 s, and 23 C for 30 s. Real-time data for fluorescence were recorded with the plate reads at the end of every amplification cycle. Fluorescence data from the cycle showing the greatest distinction between clusters without any background amplification (29 to 31 of the amplification phase) were used for making genotype calls.

Data Analysis

The HEX and FAM fluorescence data for the individual samples were transformed into the percentage of relative fluorescence units (RFUs) for each fluorophore using the equation (Oliveira et al. 2018):

$$\mathrm{RFU}(\%) = \frac{X - \mathrm{RFU}_{\min}}{\mathrm{RFU}_{\max} - \mathrm{RFU}_{\min}} \times 100 \qquad [5]$$

where X is the HEX or FAM fluorescence for an individual data point, and $\mathrm{RFU}_{\min}$ and $\mathrm{RFU}_{\max}$ are the lowest and highest fluorescence signal, respectively, from a reaction. A stepwise linear discriminant analysis was performed using R statistical software for discriminating the three clusters (homozygous for the resistant or susceptible *PPX2L* alleles, or heterozygous) based on their similarity in HEX and FAM fluorescence values.

Screening for Multiple Herbicide Resistance

The NER biotype was tested for resistance to ALS (chlorimuron and imazethapyr), EPSPS (glyphosate), and PSII (atrazine) inhibitors in the greenhouse at the University of Nebraska–Lincoln in 2017. Whole-plant dose–response bioassays were conducted separately for each herbicide following the procedure described earlier for the PPO-inhibiting herbicides, and the experiments were repeated in time. The susceptibility of the S1 biotype to atrazine and glyphosate was known; therefore, the S1 biotype was included in the dose–response study for comparison. However, the S1 biotype had previously been identified as resistant to ALS-inhibiting herbicides (Sarangi et al. 2015); therefore, an *A. tuberculatus* biotype collected from Pawnee County, NE ("PAW"), with a relatively higher sensitivity to ALS-inhibiting herbicides was included in the dose–response bioassay. A confirmed glyphosate-resistant *A. tuberculatus* biotype from Dodge County, NE ("DOD"), was also included in the glyphosate dose–response bioassay as a positive control (Table 1). Aboveground biomass reduction data were recorded at 21 DAT and regressed over the herbicide doses using Equation 2.

Response to POST Soybean Herbicides

The efficacy of POST soybean herbicides was evaluated for aboveground biomass reduction of NER. Treatments included registered POST soybean herbicides and their tank mixes (Table 2). The study was conducted in the greenhouse under the same growing conditions described for the dose–response study. Herbicide doses were selected based on the labeled doses for soybean (Table 2) and applied when plants were 10- to 15-cm tall. The response of the PPO inhibitor–susceptible biotype (S1) was also evaluated in this study. Experiments were repeated in time, beginning 14 d after the first experiment. Treatments were arranged in a randomized complete block design with five replications. Aboveground biomass data were recorded at 21 DAT and converted into percent biomass reduction using Equation 1.

Statistical Analysis

Data were subjected to ANOVA using PROC GLIMMIX in SAS v. 9.4 (SAS Institute, Cary, NC). Experimental run, replication, and all interactions containing either of these effects were considered random effects. Herbicide treatment was considered a fixed effect in the model. Aboveground biomass reduction data for the nontreated control were excluded from the analysis, as all the replicated values were zero. To satisfy the assumptions of ANOVA, normality and homogeneity of variance were tested using PROC UNIVARIATE in SAS. Data were arc-sine square-root transformed before analysis; however, back-transformed original mean values are presented based on the interpretation of the transformed

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Table 2. The POST soybean herbicides and doses used in a greenhouse study at the University of Nebraska–Lincoln to determine the response of *Amaranthus tuberculatus* biotypes.

Herbicide	Trade name	Site of action[a]	Dose	Manufacturer	Adjuvants[a,b]
			g ae or ai ha^{-1}		
Chlorimuron + thifensulfuron-methyl	Synchrony® XP	ALS	7.46	E.I. du Pont de Nemours and Company, Wilmington, DE	COC + AMS
Glufosinate	Liberty® 280 SL	GLS	740	Bayer CropScience LP, Research Triangle Park, NC	AMS
Fluthiacet-methyl	Cadet™	PPO	7.2	FMC Corporation, Philadelphia, PA	NIS + AMS
Saflufenacil[c]	Sharpen®	PPO	50	BASF Corporation, Research Triangle Park, NC	MSO + AMS
Fluthiacet-methyl + fomesafen	Marvel™	PPO	190	FMC Corporation	NIS + AMS
Fomesafen + glyphosate	Flexstar® GT 3.5	PPO + EPSPS	1,110	Syngenta Crop Protection, LLC, Greensboro, NC	NIS + AMS
Imazethapyr + glyphosate	Extreme®	ALS + EPSPS	910	BASF Corporation	NIS + AMS
Bentazon	Basagran® 5L	PSII	1,120	Winfield Solutions, LLC, St Paul, MN	COC + AMS
2,4-D choline + glyphosate	Enlist Duo™	SA	2,200	Corteva Agriscience, Indianapolis, IN	——
Dicamba DGA	XtendiMax™	SA	1,120	Monsanto Company, St Louis, MO	NIS

[a] Abbreviations: ALS, acetolactate synthase; AMS, ammonium sulfate; COC, crop oil concentrate; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; GLS, glutamine synthetase; MSO, methylated seed oil; NIS, nonionic surfactant; PPO, protoporphyrinogen oxidase; PSII, photosystem II; SA, synthetic auxins.
[b] AMS at 2.86 kg ai ha^{-1}; COC at 1.0% v/v; MSO at 1.0% v/v; and NIS at 0.25% v/v rate were mixed.
[c] Saflufenacil is labeled for preplant or PRE applications in soybean.

Table 3. Estimates of the regression parameters, model goodness of fit, and acifluorfen doses required to reduce the aboveground biomass of *Amaranthus tuberculatus* biotypes by 50% (ED_{50}) and 80% (ED_{80}) at 21 d after acifluorfen treatments in a greenhouse whole-plant dose–response bioassay conducted at the University of Nebraska–Lincoln[a].

	Regression parameters (±SEM)		Model goodness of fit		Effective doses (±SEM)			
Biotype	*b*	*d*	RMSE	E_f	ED_{50}	ED_{80}	Predicted value at 1X dose[b]	RI[c]
					——g ai ha^{-1}——		%	
ILR	−0.8 (±0.1)	87.8 (±3.0)	7.4	0.9	83 (±11)	1,121 (±521)	73	8
NER	−1.0 (±0.2)	88.4 (±2.7)	12.5	0.8	49 (±6)	392 (±170)	80	4
S1	−1.2 (±0.3)	94.4 (±1.3)	5.7	0.9	11 (±3)	39 (±5)	93	——
S2	−1.6 (±0.4)	95.1 (±1.0)	5.2	0.9	11 (±3)	30 (±3)	95	——

[a] Abbreviations: E_f, model efficiency coefficient; ILR, confirmed PPO inhibitor–resistant *A. tuberculatus* biotype from Illinois; NER, putative PPO inhibitor–resistant biotype from Nebraska; RI, resistance index; RMSE, root mean-square error; S1, PPO inhibitor–susceptible biotype from Clay County; S2, PPO inhibitor–susceptible biotype from Saunders County; SEM, standard error of the mean.
[b] Acifluorfen labeled dose (1X) = 420 g ai ha^{-1}.
[c] RI was determined using the ratio between ED_{50} values of the NER or ILR and the susceptible biotypes (S1 or S2).

data. Treatment means were separated using Fisher's protected LSD test at a significance level of 0.05.

Results and Discussion

PPO-Inhibitor Dose–Response Bioassay

Amaranthus tuberculatus biotypes collected from eastern Nebraska were screened for PPO-inhibitor resistance in the greenhouse using a single dose of fomesafen (263 g ha^{-1}), and six of seven biotypes showed less than 5% survival; however, 63% of plants from the NER biotype survived the initial screening (unpublished data). It is evident that PPO inhibitor–resistant *A. tuberculatus* is not widespread in Nebraska, but that some plants less sensitive (or resistant) to fomesafen are present. Similarly, Crespo et al. (2017) also found an *A. tuberculatus* biotype in Nebraska that had reduced sensitivity to lactofen.

Acifluorfen applied POST at the labeled dose (420 g ha^{-1}) reduced the aboveground biomass of the S1 and S2 biotypes by 93% and 95%, respectively (Table 3). However, the same dose caused ≤80% biomass reduction of the ILR and NER biotypes (Figure 1A; Table 3). Acifluorfen doses required for 50% (ED_{50}) and 80% (ED_{80}) biomass reduction of the NER biotype were 49 and 392 g ha^{-1}, respectively, compared with 83 and 1,121 g ha^{-1}, respectively, for the ILR biotype. The ED_{50} values for the susceptible biotypes were 11 g ha^{-1}. The comparisons of relative potency between dose–response curves showed that the ED_{50} values of ILR and NER were higher ($P < 0.05$) than the ED_{50} values of the susceptible biotypes (data not shown). The dose response of the NER biotype to acifluorfen is depicted in Figure 2A.

Shortly after the PPO-inhibiting herbicide application, resistant plants from the NER and ILR biotypes exhibited injury symptoms typically found with this chemistry, including chlorosis, necrosis, and crinkling of leaves; however, within 10 d of herbicide application, new growth arose from the apical meristem and/or the axillary buds (Figure 2B). A similar phenomenon was also observed in the PPO inhibitor–resistant *A. tuberculatus* biotypes from Illinois (Patzoldt et al. 2005) and Kansas (Shoup et al. 2003). Results of acifluorfen dose–response bioassay confirmed that the putative PPO inhibitor–resistant biotype (NER) was 4-fold resistant to acifluorfen compared with the susceptible biotypes (Table 3).

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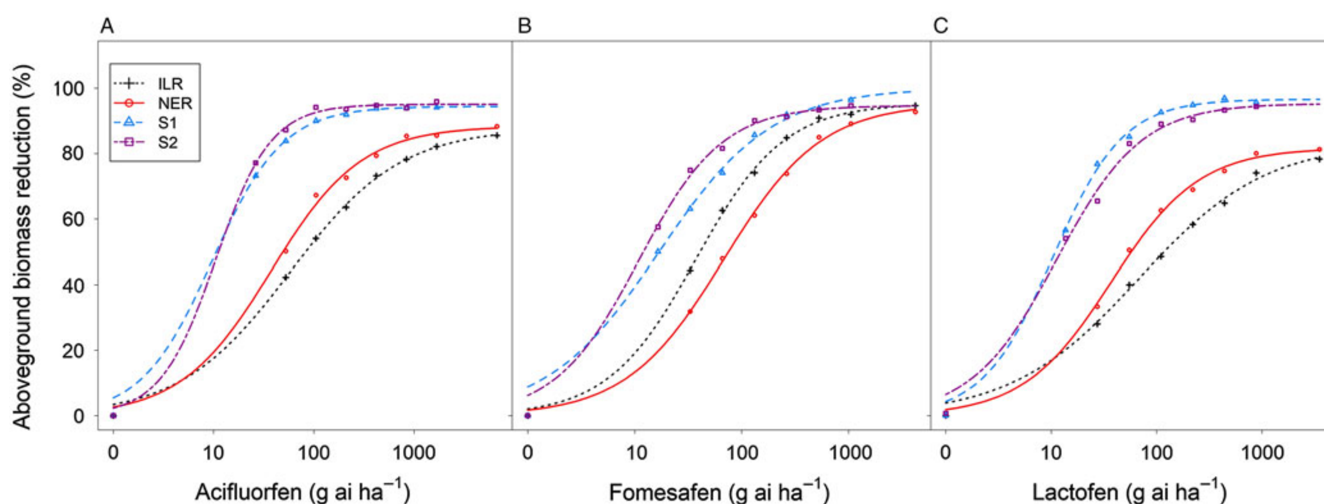

Figure 1. Dose–response curves of a putative protoporphyrinogen oxidase (PPO) inhibitor–resistant *Amaranthus tuberculatus* biotype from Nebraska (NER), a known PPO inhibitor–resistant biotype from Illinois (ILR), and two known PPO inhibitor–susceptible biotypes from Nebraska (S1 and S2). Graphs presenting the effect of (A) acifluorfen, (B) fomesafen, and (C) lactofen for the aboveground biomass reduction of *A. tuberculatus* biotypes at 21 d after herbicide treatment in the whole-plant dose–response bioassays conducted in the greenhouse at the University of Nebraska–Lincoln.

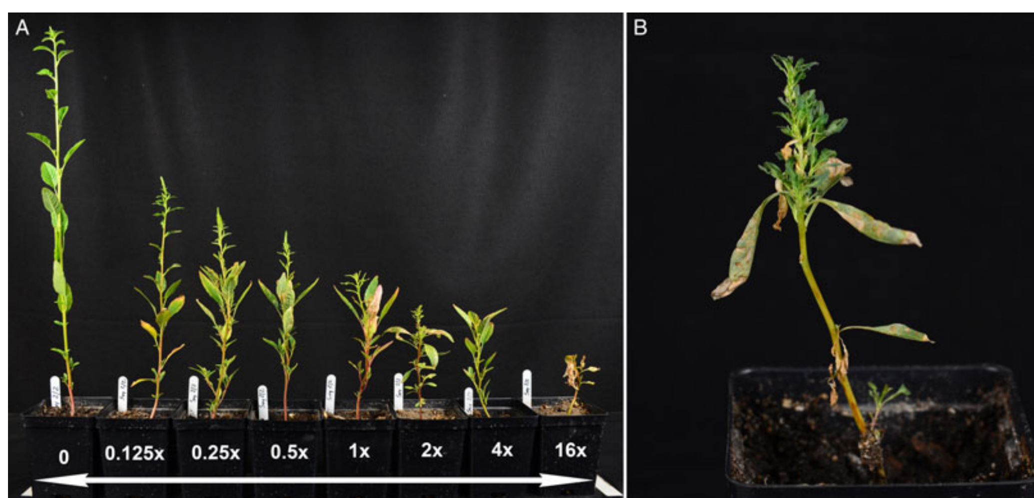

Figure 2. Response of a putative protoporphyrinogen oxidase (PPO) inhibitor–resistant *Amaranthus tuberculatus* biotype from Nebraska (NER) to POST application of acifluorfen at 21 d after treatment. (A) Dose–response of *A. tuberculatus*, where 1X = 420 g ai ha^{-1}; and (B) phenotypic response of PPO inhibitor–resistant *A. tuberculatus* plants: old tissue exhibits bronzing and necrosis from the damage caused by acifluorfen applied at the 2X dose, whereas new tissue shows minimal damage.

The fomesafen dose–response bioassay revealed that the level of resistance in the NER biotype was higher than that of the confirmed PPO inhibitor–resistant biotype from Illinois (ILR) (Figure 1B). Biologically effective doses of fomesafen required for 50% reduction in the aboveground biomass (ED_{50}) of ILR, NER, S1, and S2 were 41, 75, 17, and 12 g ha^{-1}, respectively (Table 4). The labeled dose of fomesafen (263 g ha^{-1}) caused 85% and 75% biomass reduction of the ILR and NER biotypes, respectively; however, that dose resulted in 91% and 92% reduction in the biomass of the S1 and S2 biotypes, respectively. Thus, the NER biotype showed a 4- to 6-fold resistance to fomesafen compared with the known susceptible biotypes.

The labeled dose of lactofen (220 g ai ha^{-1}) resulted in 58% and 71% biomass reduction of the ILR and NER biotypes, respectively (Table 5). Lactofen applied at the labeled dose showed the least injury to the ILR and NER biotypes compared with acifluorfen and fomesafen. Shoup et al. (2003) also reported that the PPO inhibitor–resistant *A. tuberculatus* biotype from Kansas had a higher level of resistance to lactofen compared with acifluorfen and fomesafen.

Effective doses of lactofen required to reduce aboveground biomass of the NER biotype by 50% (ED_{50}) and 80% (ED_{80}) were 58 and 1,526 g ha^{-1}. The ED_{50} values for the susceptible biotypes (S1 and S2) were 11 and 12 g ha^{-1} (Figure 1C; Table 5). Comparison of the effective doses showed that the ED_{50} values were similar for the ILR and NER biotypes (P = 0.26); however, the values were higher for the resistant biotypes than the susceptible biotypes (data not shown). The RI for the NER biotype was 5; however, the values ranged from 10 to 11 for the ILR biotype depending on the susceptible biotypes used for comparison (Table 5).

The RMSE values for the dose–response bioassays of the PPO-inhibiting herbicides ranged between 5.2 and 19.5, with the E_f values ranging from 0.6 to 0.9, showing a good fit of the four-parameter log-logistic model (Tables 3–5).

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Table 4. Estimates of the regression parameters, model goodness of fit, and fomesafen doses required to reduce the aboveground biomass of *Amaranthus tuberculatus* biotypes by 50% (ED_{50}) and 80% (ED_{80}) at 21 d after fomesafen treatments in a greenhouse whole-plant dose–response bioassay conducted at the University of Nebraska–Lincoln[a].

Biotype	Regression parameters (±SEM)		Model goodness of fit		Effective doses (±SEM)		Predicted value at 1X dose[b]	RI[c]
	b	*d*	RMSE	E_f	ED_{50}	ED_{80}		
					g ai ha^{-1}		%	
ILR	−1.1 (±0.2)	95.3 (±2.5)	8.7	0.9	41 (±4)	179 (±41)	85	2 to 3
NER	−0.9 (±0.1)	95.4 (±3.7)	15.1	0.8	75 (±10)	387 (±121)	75	4 to 6
S1	−0.8 (±0.2)	100.0 (±6.1)	14.3	0.8	17 (±3)	90 (±46)	91	—
S2	−1.1 (±0.2)	94.7 (±2.5)	9.2	0.9	12 (±2)	50 (±13)	92	—

[a] Abbreviations: E_f, model efficiency coefficient; ILR, confirmed PPO inhibitor–resistant *A. tuberculatus* biotype from Illinois; NER, putative PPO inhibitor–resistant biotype from Nebraska; RI, resistance index; RMSE, root mean-square error; S1, PPO inhibitor–susceptible biotype from Clay County; S2, PPO inhibitor–susceptible biotype from Saunders County; SEM, standard error of the mean.
[b] Fomesafen labeled dose (1X) = 263 g ai ha^{-1}.
[c] RI was determined using the ratio between ED_{50} values of the NER or ILR and the susceptible biotypes (S1 or S2).

Table 5. Estimates of the regression parameters, model goodness of fit, and lactofen doses required to reduce the aboveground biomass of *Amaranthus tuberculatus* biotypes by 50% (ED_{50}) and 80% (ED_{80}) at 21 d after lactofen treatments in a greenhouse whole-plant dose–response bioassay conducted at the University of Nebraska–Lincoln[a].

Biotype	Regression parameters (±SEM)		Model goodness of fit		Effective doses (±SEM)		Predicted value at 1X dose[b]	RI[c]
	b	*d*	RMSE	E_f	ED_{50}	ED_{80}		
					g ai ha^{-1}		%	
ILR	−0.7 (±0.1)	83.5 (±5.7)	10.3	0.8	118 (±37)	5,278 (±2216)	58	10 to 11
NER	−1.0 (±0.3)	81.7 (±4.2)	19.5	0.6	58 (±12)	1,526 (±471)	71	5
S1	−1.3 (±0.3)	96.6 (±2.2)	9.4	0.9	11 (±1)	35 (±7)	95	—
S2	−1.1 (±0.2)	95.3 (±3.2)	11.6	0.9	12 (±2)	52 (±16)	92	—

[a] Abbreviations: E_f, model efficiency coefficient; ILR, confirmed PPO inhibitor–resistant *A. tuberculatus* biotype from Illinois; NER, putative PPO inhibitor–resistant biotype from Nebraska; RI, resistance index; RMSE, root mean-square error; S1, PPO inhibitor–susceptible biotype from Clay County; S2, PPO inhibitor–susceptible biotype from Saunders County; SEM, standard error of the mean.
[b] Lactofen labeled dose (1X) = 220 g ai ha^{-1}.
[c] RI was determined using the ratio between ED_{50} values of the NER or ILR and the susceptible biotypes (S1 or S2).

Mechanism of PPO-Inhibitor Resistance

A KASP™ assay designed to evaluate the PPO-inhibitor resistance mechanism (a target-site resistance) in *A. tuberculatus* was able to distinguish the resistant biotypes from the susceptible biotypes. Four clusters were identified in the linear discriminant analysis, including three genotype clusters and one no-template control, and the RFU values for the FAM (resistant alleles) ranged from 80.3% to 100% in the homozygous condition and from 32.8% to 41.2% in the heterozygous condition (Figure 3). Results revealed that all samples of the NER biotype tested positive for the ΔG210 mutation, but that the individuals selected for the KASP™ assay were heterozygous. The same mutation was also present in the plants of a known PPO inhibitor–resistant biotype, ILR, but in a homozygous condition (Figure 3). As expected, the KASP™ assay detected the susceptible allele containing the G210 codon in both *A. tuberculatus* biotypes that were phenotypically susceptible to PPO-inhibiting herbicides. No ambiguous sample was detected in the 18 samples tested using the KASP™ assay.

A target-site resistance mechanism involving a codon deletion in the *PPX2L* gene, resulting in the loss of a glycine residue at the position 210 (ΔG210) of the PPO enzyme, confers PPO resistance in the NER biotype. Surveys have indicated that the ΔG210 mutation in PPO inhibitor–resistant *A. tuberculatus* was widespread in Illinois, Kansas, and Missouri (Shergill et al. 2018b; Thinglum et al. 2011; Wuerffel et al. 2015a). The presence of ΔG210 was also confirmed in PPO inhibitor–resistant *A. palmeri* in Arkansas (Salas et al. 2016). Two additional mutations of the *PPX2* gene (R128G and R128I mutations encoding for a glycine [G] or an isoleucine [I] substitution for an arginine at the 128th [R128] site) likely to confer resistance to PPO inhibitors were recently detected in *A. tuberculatus* biotypes collected from the Midwest; however, it was suspected that the interspecific gene flow among *Amaranthus* spp. aided in accumulation of these mutations along with the G210 codon deletion in *A. tuberculatus* (Nie et al. 2019). In our study, these mutations were not tested in the NER biotype.

Multiple Herbicide Resistance

The NER biotype showed a high level of resistance to ALS-inhibiting herbicides. Chlorimuron and imazethapyr at labeled doses (13.1 and 70 g ai ha^{-1}, respectively) caused 41% and 27% aboveground biomass reduction of the NER biotype, respectively. The highest doses of these herbicides (32X the labeled doses) were not able to provide 80% reduction of the aboveground biomass. The biologically effective doses of chlorimuron and imazethapyr for 50% reduction in the aboveground biomass of the NER biotype were 35 and 235 g ha^{-1}, respectively (Table 6). RIs of the NER biotype for ALS-inhibiting herbicides tested in this study were >7.0. It was evident that the NER biotype was cross-resistant to chlorimuron and imazethapyr and that the level of resistance was high. The high level of resistance to ALS-inhibiting herbicides in *A. tuberculatus* biotypes has also previously been documented in several states in the Midwest (Foes et al. 1998; Shergill et al. 2018a), including Nebraska (Sarangi et al. 2015).

The ALS-inhibiting herbicides have been used for more than 30 yr, targeting most of the weed species in major agronomic crops

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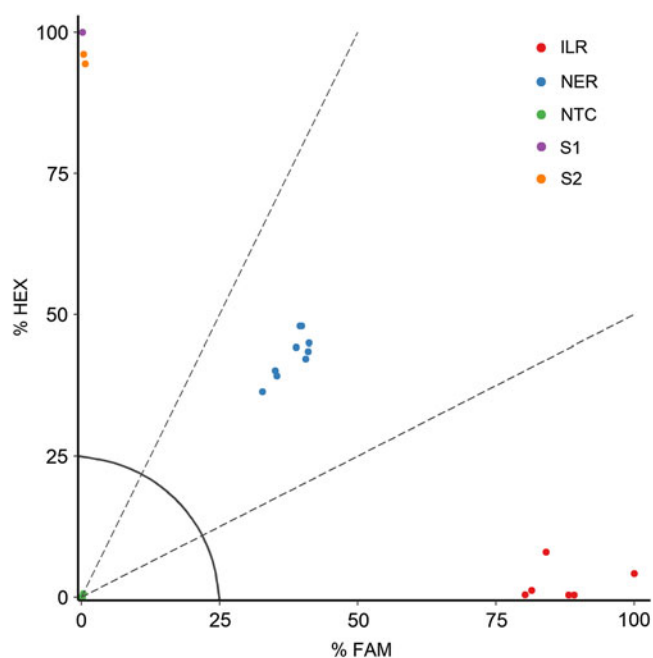

Figure 3. Results of the Kompetitive Allele Specific PCR (KASP™) assay showing the presence of target-site mutation (ΔG210) in a putative protoporphyrinogen oxidase (PPO) inhibitor–resistant *Amaranthus tuberculatus* biotype from Nebraska (NER) and a known PPO inhibitor–resistant biotype from Illinois (ILR). No-template control (NTC) and known PPO inhibitor–susceptible biotypes (S1 and S2) were included for comparison, and the HEX and FAM fluorescence data were transformed into the percentage of relative fluorescence units. Dashed lines represent the cutoffs for making genotyping calls, and the solid quarter circle represents the cutoff for no amplification.

in the United States, which resulted in high selection pressure on weed species (Tranel and Wright 2002). Moreover, the ALS enzyme is vulnerable to gene point mutations that confer resistance. ALS inhibitor–resistant weed species, including *Amaranthus* spp., are widely distributed in Nebraska (Sarangi and Jhala 2018; Sarangi et al. 2015). Tranel et al. (2017) revealed that the corresponding genes for resistance to ALS- and PPO-inhibiting herbicides (*ALS* and *PPX2*) were genetically linked in a multiple herbicide–resistant *A. tuberculatus* biotype found in Illinois. In our study, the mechanism for ALS-inhibiting herbicide resistance was not tested.

Glyphosate applied at the labeled dose (870 g ae ha^{-1}) resulted in 70% aboveground biomass reduction of the NER biotype (Table 6). Glyphosate doses needed for 50% reduction in the biomass reduction (ED_{50}) of the NER and S1 biotypes were 371 and 111 g ha^{-1}, respectively. A known glyphosate-resistant *A. tuberculatus* biotype (designated "DOD") from Nebraska showed a relatively high level of resistance to glyphosate compared with the NER biotype, and the ED_{50} value for the DOD biotype was 1,046 g ha^{-1} for biomass reduction (Table 6). Comparison of biologically effective doses of glyphosate showed that the ED_{50} value for the S1 biotype was lower ($P < 0.05$) than values for the NER and DOD biotypes (data not shown). The RIs were 3 and 9 for the NER and DOD biotypes, respectively.

Glyphosate-resistant *A. tuberculatus* is widespread in the eastern part of Nebraska (Vieira et al. 2018). Sarangi et al. (2015) revealed that *A. tuberculatus* biotypes collected from seven counties in eastern Nebraska were 3- to 39-fold resistant to glyphosate compared with known susceptible biotypes. Moreover, a total of 18 states in the midwestern and southern United States have confirmed the presence of glyphosate-resistant *A. tuberculatus* (Heap 2019a).

The atrazine dose required for 50% aboveground biomass reduction (ED_{50}) of the NER biotype was 1,323 g ai ha^{-1} (Table 6). The labeled dose of atrazine (2,240 g ha^{-1}) provided 62% biomass reduction of the NER biotype. The ED_{50} value for the NER biotype was higher ($P < 0.05$) than that of the susceptible biotype (S1) (data not shown), and based on the ED_{50} values, the NER biotype was 7-fold resistant to atrazine compared with the S1 biotype. Atrazine-resistant *A. tuberculatus* in Nebraska was first reported in 1990 (Anderson et al. 1996), and *A. tuberculatus* resistant to atrazine applied POST is widespread in Nebraska: a 2014 survey of 85 fields in eastern Nebraska revealed that atrazine-resistant *A. tuberculatus* was present in 73% of all fields surveyed (Vennapusa et al. 2018). The occurrence of atrazine-resistant *A. tuberculatus* with a high level of resistance is also widespread in other states in the Midwest (Heap 2019b): for example, a recent report suggested that a multiple herbicide–resistant biotype from Missouri showed 7- to 19-fold resistance to atrazine (Shergill et al. 2018a).

Response of the NER Biotype to POST Soybean Herbicides

With the confirmed resistance to four herbicide sites of action, there are limited POST herbicide choices for soybean growers to control the NER biotype. The ALS-inhibiting herbicides applied alone or in mixture with glyphosate reduced the aboveground biomass of the NER biotype up to 43% (Table 7). Glufosinate, 2,4-D choline plus glyphosate, and dicamba caused ≥92% aboveground biomass reduction of the NER biotype. Therefore, it is evident that the multiple herbicide–resistant NER biotype can only be controlled effectively using POST herbicides in glufosinate-, 2,4-D-, and dicamba-resistant soybean. Similarly, in a previous study, Sarangi et al. (2015) reported that glufosinate applied POST at 594 g ai ha^{-1} reduced aboveground biomass up to 93% in glyphosate-resistant *A. tuberculatus* biotypes collected from soybean fields in eastern Nebraska. Additionally, Chahal et al. (2015) reported that glyphosate-resistant *A. tuberculatus* at 10-cm height was controlled 90% with 2,4-D choline plus glyphosate applied at 1,640 g ha^{-1}. An *A. tuberculatus* biotype from Nebraska was previously confirmed with 10-fold resistance to 2,4-D and reduced sensitivity to dicamba in a continuous grass seed–production pasture (Bernards et al. 2012); therefore, proper stewardship is needed to preserve herbicide-resistant soybean technologies against the spread of multiple herbicide–resistant weeds.

Saflufenacil, a PPO-inhibiting herbicide, is labeled for preplant or PRE applications in soybean, and a stakeholders' survey in 2015 revealed that saflufenacil was one of the most commonly used preplant herbicides in Nebraska (Sarangi and Jhala 2018). Saflufenacil applied at the labeled dose reduced aboveground biomass 77% in the NER biotype compared with 94% biomass reduction of PPO inhibitor–susceptible biotype (S1) (Table 7). Salas-Perez et al. (2017) reported that fomesafen-resistant *A. palmeri* biotypes from Arkansas showed higher sensitivity to saflufenacil than other foliar-applied PPO-inhibiting herbicides.

Practical Implications

Results of this study confirmed that the NER biotype was multiple resistant to herbicides from four sites of action (ALS, EPSPS, PPO, and PSII inhibitors) and that *A. tuberculatus* is the first weed species in Nebraska showing resistance to PPO-inhibiting herbicides. The ΔG210 mutation in the *PPX2L* gene conferred the PPO-inhibitor resistance in the NER biotype. Additionally,

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Table 6. Estimates of herbicide doses resulting in 50% (ED_{50}) and 80% (ED_{80}) reduction in the aboveground biomass of *Amaranthus tuberculatus* biotypes at 21 d after treatment in the greenhouse whole-plant dose–response bioassay conducted at the University of Nebraska–Lincoln.[a]

Herbicide	Biotype	Effective doses (±SEM)		Predicted value at 1X dose[c]	RI[d]
		ED_{50}	ED_{80}		
		g ae or ai ha^{-1}		%	
Chlorimuron	NER	35 (±8)	>419[b]	41	>12[e]
	PAW	3 (±1)	>210[b]	69	—
Imazethapyr	NER	235 (±119)	>2,240[b]	27	>7[e]
	PAW	35 (±16)	>1,120[b]	60	—
Glyphosate	DOD	1,046 (±142)	3,837 (±1,235)	44	9
	NER	371 (±56)	1,576 (±559)	70	3
	S1	111 (±15)	364 (±114)	90	—
Atrazine	NER	1,323 (±215)	11,411 (±6,426)	62	7
	S1	198 (±34)	846 (±354)	98	—

[a] Abbreviations: DOD, confirmed glyphosate-resistant *A. tuberculatus* biotype from Dodge County; NER, PPO inhibitor–resistant biotype from Nebraska; PAW, biotype collected from Pawnee County; RI, resistance index; S1, glyphosate- and atrazine-susceptible biotype from Clay County; SEM, standard error of the mean.
[b] Exact values could not be estimated, as the selected dose range did not cover sufficiently large doses for this biotype.
[c] The labeled doses (1X) of chlorimuron, imazethapyr, glyphosate, and atrazine are 13.1, 70, 870, and 2,240 g ai ha^{-1}, respectively.
[d] RI was determined using the ratio between ED_{50} values of the resistant biotypes and susceptible biotypes.
[e] RI for the NER biotype was calculated based on the ED_{50} values of the PAW biotype; however, no known susceptible biotype was available for comparison.

Table 7. Control and biomass reduction of PPO inhibitor–resistant (NER) and PPO inhibitor–susceptible (S1) *Amaranthus tuberculatus* biotypes in response to soybean POST herbicides at 21 d after treatment in the greenhouse study conducted at the University of Nebraska–Lincoln.[a,b]

Herbicide	Dose	NER	S1
	g ae or ai ha^{-1}	%	
Nontreated control	—	0	0
Chlorimuron + thifensulfuron-methyl	7.46	4 e	41 d
Glufosinate	740	92 a	91 ab
Fluthiacet-methyl	7.2	60 bc	68 c
Saflufenacil	50	77 b	94 a
Fluthiacet-methyl + fomesafen	190	60 bc	88 ab
Fomesafen + glyphosate	1,110	72 b	93 a
Imazethapyr + glyphosate	910	43 cd	84 b
Bentazon	1,120	26 d	24 e
2,4-D choline + glyphosate	2,200	93 a	93 a
Dicamba DGA	1,120	93 a	95 a

[a] Data were arc-sine square-root transformed before analysis; however, back-transformed original mean values are presented based on the interpretation of the transformed data.
[b] Means presented within each column with no common letter(s) are significantly different according to Fisher's protected LSD at a significance level of 0.05.

the results of the PPO-inhibitor dose–response bioassays most likely underestimated the level of resistance in the NER biotype because of the presence of heterozygous resistant plants, which was also confirmed in the KASP™ assay. Moreover, Patzoldt et al. (2006) confirmed that PPO-inhibitor resistance in *A. tuberculatus* is an incomplete dominant trait.

A recent stakeholders' survey in Nebraska indicated that PPO-inhibiting herbicides are used frequently in soybean for preplant (e.g., saflufenacil), PRE (flumioxazin and sulfentrazone), and POST (fluthiacet-methyl and lactofen) weed control (Sarangi and Jhala 2018). Non-transgenic soybean growers are also primarily relying on PPO inhibitors for POST control of *Amaranthus* spp. (Sarangi and Jhala 2019). Though it is reported that the evolution rate of PPO inhibitor–resistant weed biotypes is relatively slow compared with ALS-inhibitor resistance (Dayan et al. 2017; Riggins and Tranel 2012), pollen- and seed-mediated gene flow in *A. tuberculatus* may play an important role in spreading this biotype (Sarangi et al. 2017b).

The efficacy of soil-applied PPO-inhibiting herbicides was not tested in this study for the NER biotype; however, a variable sensitivity of the PPO inhibitor–resistant *A. tuberculatus* to soil-applied PPO inhibitors was reported previously (Patzoldt et al. 2005; Wuerffel et al. 2015b). Shoup et al. (2003) reasoned that a PPO inhibitor–resistant biotype might have reduced sensitivity to a particular PPO-inhibiting herbicide chemical family that has been used most frequently in a system. Moreover, Wuerffel et al. (2015b) reported that plant growth stages considerably impacted the sensitivity of *A. tuberculatus* to PPO-inhibiting herbicides. Umphres et al. (2018) reported that *A. palmeri* resistant to POST-applied fomesafen had greater sensitivity to soil-applied PPO-inhibiting herbicides such as flumioxazin and saflufenacil.

While dicamba, 2,4-D choline, or glufosinate were effective for controlling the multiple herbicide–resistant *A. tuberculatus* biotype (NER), relying on a single herbicide or the herbicides with a same site of action will enhance selection pressure. Therefore, diversified weed management approaches, including cultural, mechanical, and chemical weed management and implementation of herbicide programs with multiple sites of action, are needed for sustainable management of weeds.

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References

Anderson DD, Roeth FW, Martin AR (1996) Occurrence and control of triazine-resistant common waterhemp (*Amaranthus rudis*) in field corn (*Zea mays*). Weed Technol 10:570–575

Becerril JM, Duke SO (1989) Protoporphyrin IX content correlates with activity of photobleaching herbicides. Plant Physiol 90:1175–1181

Bernards ML, Crespo RJ, Kruger GR, Gaussoin R, Tranel PJ (2012) A waterhemp (*Amaranthus tuberculatus*) population resistant to 2, 4-D. Weed Sci 60:379–384

Broccanello C, Chiodi C, Funk A, McGrath JM, Panella L, Stevanato P (2018) Comparison of three PCR-based assays for SNP genotyping in plants. Plant Methods 14:28

Chahal PS, Aulakh JS, Rosenbaum K, Jhala AJ (2015) Growth stage affects dose response of selected glyphosate-resistant weeds to premix of 2, 4-D choline and glyphosate (Enlist DuoTM Herbicide). J Agric Sci 7:1–10

Costea M, Weaver SE, Tardif FJ (2005) The biology of invasive alien plants in Canada. 3. *Amaranthus tuberculatus* (Moq.) Sauer var. *rudis* (Sauer) Costea & Tardif. Can J Plant Sci 85:507–522

Crespo RJ, Wingeyer AB, Kruger GR, Riggins CW, Tranel PJ, Bernards ML (2017) Multiple-herbicide resistance in a 2, 4-D-resistant waterhemp (*Amaranthus tuberculatus*) population from Nebraska. Weed Sci 65:743–754

Dayan FE, Barker A, Tranel PJ (2017) Origins and structure of chloroplastic and mitochondrial plant protoporphyrinogen oxidases: implications for the evolution of herbicide resistance. Pest Manag Sci 74:2226–2234

Foes MJ, Liu L, Tranel PJ, Wax LM, Stoller EW (1998) A biotype of common waterhemp (*Amaranthus rudis*) resistant to triazine and ALS herbicides. Weed Sci 46:514–520

Hager AG, Wax LM, Stoller EW, Bollero GA (2002) Common waterhemp (*Amaranthus rudis*) interference in soybean. Weed Sci 50:607–610

Hartzler RG, Battles BA, Nordby D (2004) Effect of common waterhemp (*Amaranthus rudis*) emergence date on growth and fecundity in soybean. Weed Sci 52:242–245

Heap I (2019a) Herbicide resistant tall waterhemp globally. The International Survey of Herbicide Resistant Weeds. http://weedscience.org/Summary/Species.aspx?WeedID=219. Accessed: February 8, 2019

Heap I (2019b) Weeds resistant to photosystem II inhibitors. The International Survey of Herbicide Resistant Weeds. http://weedscience.org/Summary/MOA.aspx?MOAID=4. Accessed: February 8, 2019

Heap I (2019c) Weeds resistant to PPO inhibitors. The International Survey of Herbicide Resistant Weeds. http://weedscience.org/Summary/MOA.aspx?MOAID=8. Accessed: February 8, 2019

Horak MJ, Loughin TM (2000) Growth analysis of four *Amaranthus* species. Weed Sci 48:347–355

Jacobs JM, Jacobs NJ, Sherman TD, Duke SO (1991) Effect of diphenyl ether herbicides on oxidation of protoporphyrinogen to protoporphyrin in organellar and plasma membrane enriched fractions of barley. Plant Physiol 97:197–203

Knezevic SZ, Streibig JC, Ritz C (2007) Utilizing R software package for dose-response studies: the concept and data analysis. Weed Technol 21:840–848

Lee RM, Hager AG, Tranel PJ (2008) Prevalence of a novel resistance mechanism to PPO-inhibiting herbicides in waterhemp (*Amaranthus tuberculatus*). Weed Sci 56:371–375

Lermontova I, Kruse E, Mock HP, Grimm B (1997) Cloning and characterization of a plastidal and a mitochondrial isoform of tobacco protoporphyrinogen IX oxidase. Proc Natl Acad Sci USA 94:8895–8900

Matringe M, Camadro JM, Block MA, Joyard J, Scalla R, Labbe P, Douce R (1992) Localization within chloroplasts of protoporphyrinogen oxidase, the target enzyme for diphenylether-like herbicides. J Biol Chem 267:4646–4651

Matringe M, Scalla R (1988) Studies on the mode of action of acifluorfen-methyl in nonchlorophyllous soybean cells. Plant Physiol 86:619–622

Nie H, Mansfield BC, Harre NT, Young JM, Steppig NR, Young BG (2019) Investigating target-site resistance mechanism to the PPO-inhibiting herbicide fomesafen in waterhemp and interspecific hybridization of *Amaranthus* species using next generation sequencing. Pest Manag Sci, 10.1002/ps.5445

Oliveira MC, Gaines TA, Patterson EL, Jhala AJ, Irmak S, Amundsen K, Knezevic SZ (2018) Interspecific and intraspecific transference of metabolism-based mesotrione resistance in dioecious weedy *Amaranthus*. Plant J 96:1051–1063

Oliveira MC, Jhala AJ, Gaines T, Irmak S, Amundsen K, Scott JE, Knezevic SZ (2017) Confirmation and control of HPPD-inhibiting herbicide–resistant waterhemp (*Amaranthus tuberculatus*) in Nebraska. Weed Technol 31:67–79

Patterson EL, Fleming MB, Kessler KC, Nissen SJ, Gaines TA (2017) A KASP genotyping method to identify northern watermilfoil, Eurasian watermilfoil, and their interspecific hybrids. Front Plant Sci 8:752

Patzoldt WL, Hager AG, McCormick JS, Tranel PJ (2006) A codon deletion confers resistance to herbicides inhibiting protoporphyrinogen oxidase. Proc Natl Acad Sci USA 103:12329–12334

Patzoldt WL, Tranel PJ, Hager AG (2005) A waterhemp (*Amaranthus tuberculatus*) biotype with multiple resistance across three herbicide sites of action. Weed Sci 53:30–36

Prince JM, Shaw DR, Givens WA, Owen MDK, Weller SC, Young BG, Wilson RG, Jordan DL (2012) Benchmark study: I. Introduction, weed population, and management trends from the benchmark survey 2010. Weed Technol 26:525–530

Riggins CW, Tranel PJ (2012) Will the *Amaranthus tuberculatus* resistance mechanism to PPO-inhibiting herbicides evolve in other *Amaranthus* species? Int J Agron 2012:305764

Rosas JE, Bonnecarrère V, Pérez de Vida F (2014) One-step, codominant detection of imidazolinone resistance mutations in weedy rice (*Oryza sativa* L.). Electron J Biotechnol 17:95–101

Salas RA, Burgos NR, Tranel PJ, Singh S, Glasgow L, Scott RC, Nichols RL (2016) Resistance to PPO-inhibiting herbicide in Palmer amaranth from Arkansas. Pest Manag Sci 72:864–869

Salas-Perez RA, Burgos NR, Rangani G, Singh S, Refatti JP, Piveta L, Tranel PJ, Mauromoustakos A, Scott RC (2017) Frequency of Gly-210 deletion mutation among protoporphyrinogen oxidase inhibitor-resistant Palmer amaranth (*Amaranthus palmeri*) populations. Weed Sci 65:718–731

Sarangi D, Irmak S, Lindquist J, Knezevic S, Jhala AJ (2016) Effect of water stress on the growth and fecundity of common waterhemp (*Amaranthus rudis*). Weed Sci 64:42–52

Sarangi D, Jhala AJ (2018) A statewide survey of stakeholders to assess the problem weeds and weed management practices in Nebraska. Weed Technol 32:642–655

Sarangi D, Jhala AJ (2019) Palmer amaranth (*Amaranthus palmeri*) and velvetleaf (*Abutilon theophrasti*) control in no-tillage conventional (non–genetically engineered) soybean using overlapping residual herbicide programs. Weed Technol 33:95–105

Sarangi D, Sandell LD, Knezevic SZ, Aulakh JS, Lindquist JL, Irmak S, Jhala AJ (2015) Confirmation and control of glyphosate-resistant common waterhemp (*Amaranthus rudis*) in Nebraska. Weed Technol 29:82–92

Sarangi D, Sandell LD, Kruger GR, Knezevic SZ, Irmak S, Jhala AJ (2017a) Comparison of herbicide programs for season-long control of glyphosate-resistant common waterhemp (*Amaranthus rudis*) in soybean. Weed Technol 31:53–66

Sarangi D, Tyre AJ, Patterson EL, Gaines TA, Irmak S, Knezevic SZ, Lindquist JL, Jhala AJ (2017b) Pollen-mediated gene flow from glyphosate-resistant common waterhemp (*Amaranthus rudis* Sauer): consequences for the dispersal of resistance genes. Sci Rep 7:44913

Sauer J (1957) Recent migration and evolution of the dioecious amaranths. Evolution 11:11–31

Sellers BA, Smeda RJ, Johnson WG, Kendig JA, Ellersieck MR (2003) Comparative growth of six *Amaranthus* species in Missouri. Weed Sci 51:329–333

Shergill LS, Barlow BR, Bish MD, Bradley KW (2018a) Investigations of 2, 4-D and multiple herbicide resistance in a Missouri waterhemp (*Amaranthus tuberculatus*) population. Weed Sci 66:386–394

Shergill LS, Bish MD, Jugulam M, Bradley KW (2018b) Molecular and physiological characterization of six-way resistance in an *Amaranthus tuberculatus* var. *rudis* biotype from Missouri. Pest Manag Sci 74:2688–2698

Shoup DE, Al-Khatib K, Peterson DE (2003) Common waterhemp (*Amaranthus rudis*) resistance to protoporphyrinogen oxidase-inhibiting herbicides. Weed Sci 51:145–150

Steckel LE, Sprague CL (2004) Common waterhemp (*Amaranthus rudis*) interference in corn. Weed Sci 52:359–364

Thinglum KA, Riggins CW, Davis AS, Bradley KW, Al-Khatib K, Tranel PJ (2011) Wide distribution of the waterhemp (*Amaranthus tuberculatus*) ΔG210 *PPX2* mutation, which confers resistance to PPO-inhibiting herbicides. Weed Sci 59:22–27

https://doi.org/10.1017/wsc.2019.29 Published online by Cambridge University Press

Tranel PJ, Wright TR (2002) Resistance of weeds to ALS-inhibiting herbicides: what have we learned? Weed Sci 50:700–712

Tranel PJ, Wu C, Sadeque A (2017) Target-site resistances to ALS and PPO inhibitors are linked in waterhemp (*Amaranthus tuberculatus*). Weed Sci 65:4–8

Umphres AM, Steckel LE, Mueller TC (2018) Control of protoporphyrinogen oxidase inhibiting herbicide resistant and susceptible Palmer amaranth (*Amaranthus palmeri*) with soil-applied protoporphyrinogen oxidase–inhibiting herbicides. Weed Technol 32:95–100

Varanasi VK, Brabham C, Norsworthy JK, Nie H, Young BG, Houston M, Barber T, Scott RC (2018) A statewide survey of PPO-inhibitor resistance and the prevalent target-site mechanisms in Palmer amaranth (*Amaranthus palmeri*) accessions from Arkansas. Weed Sci 66:149–158

Vennapusa AR, Faleco F, Vieira B, Samuelson S, Kruger GR, Werle R, Jugulam M (2018) Prevalence and mechanism of atrazine resistance in waterhemp (*Amaranthus tuberculatus*) from Nebraska. Weed Sci 66:595–602

Vieira BC, Samuelson SL, Alves GS, Gaines TA, Werle R, Kruger GR (2018) Distribution of glyphosate-resistant *Amaranthus* spp. in Nebraska. Pest Manag Sci 74:2316–2324

Waselkov KE, Olsen KM (2014) Population genetics and origin of the native North American agricultural weed waterhemp (*Amaranthus tuberculatus*; Amaranthaceae). Am J Bot 101:1726–1736

Watanabe N, Che FS, Iwano M, Takayama S, Yoshida S, Isogai A (2001) Dual targeting of spinach protoporphyrinogen oxidase II to mitochondria and chloroplasts by alternative use of two in-frame initiation codons. J Biol Chem 276:20474–20481

Whitaker JR, York AC, Jordan DL, Culpepper AS (2010) Palmer amaranth (*Amaranthus palmeri*) control in soybean with glyphosate and conventional herbicide systems. Weed Technol 24:403–410

Witkowski DA, Halling BP (1989) Inhibition of plant protoporphyrinogen oxidase by the herbicide acifluorfen-methyl. Plant Physiol 90:1239–1242

Wuerffel RJ, Young JM, Lee RM, Tranel PJ, Lightfoot DA, Young BG (2015a) Distribution of the ΔG210 protoporphyrinogen oxidase mutation in Illinois waterhemp (*Amaranthus tuberculatus*) and an improved molecular method for detection. Weed Sci 63:839–846

Wuerffel RJ, Young JM, Matthews JL, Young BG (2015b) Characterization of PPO-inhibitor-resistant waterhemp (*Amaranthus tuberculatus*) response to soil-applied PPO-inhibiting herbicides. Weed Sci 63:511–521

https://doi.org/10.1017/wsc.2019.29 Published online by Cambridge University Press