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Authors: Faleco, Felipe A., Oliveira, Maxwel C., Arneson, Nicholas J., Renz, Mark, Stoltenberg, David E., et al.

Source: Weed Technology, 36(5) : 597-608

Published By: Weed Science Society of America

URL: https://doi.org/10.1017/wet.2022.81

Weed Technology

www.cambridge.org/wet

Research Article

Cite this article: Faleco FA, Oliveira MC, Arneson NJ, Renz M, Stoltenberg DE, Werle R (2022) Multiple herbicide resistance in waterhemp (*Amaranthus tuberculatus*) accessions from Wisconsin. Weed Technol. **36**: 597–608. doi: 10.1017/wet.2022.81

Received: 25 July 2022
Revised: 7 October 2022
Accepted: 16 October 2022
First published online: 10 November 2022

Associate Editor:
William Johnson, Purdue University

Nomenclature:
atrazine; dicamba; fomesafen; glufosinate; glyphosate; imazethapyr; mesotrione; metribuzin; S-metolachlor; 2,4-D; waterhemp, *Amaranthus tuberculatus* (Moq.) Sauer; corn, *Zea mays* (L.); soybean, *Glycine max* (L.) Merr.

Keywords:
ALS inhibitor resistance; glyphosate resistance; PS II inhibitor resistance; auxin mimics resistance; herbicide efficacy

Author for correspondence:
Rodrigo Werle, Assistant Professor, Department of Agronomy, University of Wisconsin-Madison, 1575 Linden Dr., Madison, WI, 53706.
Email: rwerle@wisc.edu

Multiple herbicide resistance in waterhemp (*Amaranthus tuberculatus*) accessions from Wisconsin

Felipe A. Faleco[1], Maxwel C. Oliveira[2], Nicholas J. Arneson[3], Mark Renz[4], David E. Stoltenberg[4] and Rodrigo Werle[5]

[1]Graduate Student, Department of Agronomy, University of Wisconsin-Madison, Madison, WI, USA; [2]Postdoctoral Researcher, Department of Agronomy, University of Wisconsin-Madison, Madison, WI, USA; [3]Outreach Program Manager, Department of Agronomy, University of Wisconsin-Madison, Madison, WI, USA; [4]Professor, Department of Agronomy, University of Wisconsin-Madison, Madison, WI, USA and [5]Assistant Professor, Department of Agronomy, University of Wisconsin-Madison, Madison, WI, USA

Abstract

A comprehensive, Wisconsin state-wide assessment of waterhemp response to a diverse group of herbicide sites of action has not been conducted. Our objective was to characterize the response of a state-wide collection of waterhemp accessions to postemergence (POST) and preemergence (PRE) herbicides commonly used in corn and soybean in Wisconsin. Greenhouse experiments were conducted with more than 80 accessions from 27 counties. POST treatments included 2,4-D, atrazine, dicamba, fomesafen, glufosinate, glyphosate, imazethapyr, and mesotrione at 1× and 3× label rates. PRE treatments included atrazine, fomesafen, mesotrione, metribuzin, and S-metolachlor at 0.5×, 1×, and 3× label rates. Ninety-eight percent and 88% of the accessions exhibited ≥50% plant survival after exposure to imazethapyr and glyphosate POST 3× rate, respectively. Seventeen percent, 16%, and 3% of the accessions exhibited ≥50% plant survival after exposure to 2,4-D, atrazine, and dicamba, respectively, applied POST at the 1× rate. Survival of all accessions was ≤25% after exposure to 2,4-D or dicamba applied POST at the 3× rate, or glufosinate, fomesafen, and mesotrione applied POST at either rate evaluated. No plant of any accession survived exposure to glufosinate at either rate. Forty-five percent and 3% of the accessions exhibited <90% plant density reduction after exposure to atrazine applied PRE at the 3× rate and fomesafen PRE at the 1× rate, respectively. Plant density reduction of all accessions was ≥96% after exposure to fomesafen applied PRE at the 3× rate, or metribuzin, S-metolachlor, and mesotrione applied PRE at the 1× rate. Our results suggest that waterhemp resistance to imazethapyr and glyphosate applied POST is widespread in Wisconsin, whereas resistance to 2,4-D, atrazine, and dicamba applied POST is present to a lower extent. One accession (A75, Fond du Lac County) exhibited multiple resistance to imazethapyr, atrazine, glyphosate, and 2,4-D when applied POST. Overall, atrazine applied PRE was ineffective for waterhemp control in Wisconsin. Proactive resistance management and the use of effective PRE and POST herbicides are fundamental for waterhemp management in Wisconsin.

Introduction

Waterhemp is ranked as one of the most common and most troublesome weed species in the Midwestern United States, particularly in corn and soybean fields (Tranel 2021; Van Wychen 2019, 2020). With great adaptability and ability to rapidly evolve herbicide resistance, waterhemp was the first weed species to evolve resistance to herbicides that inhibit protoporphyrinogen oxidase (PPO) and hydroxyphenyl pyruvate dioxygenase (HPPD; Hausman et al. 2011; Shoup et al. 2003). Currently, in the United States, waterhemp has evolved resistance to seven herbicide sites of action (SOAs): acetolactate synthase (ALS), auxin mimics, photosynthesis at photosystem II – serine 264 binders (PS II), enolpyruvyl shikimate phosphate synthase (EPSPS), PPO, very long-chain fatty acid synthesis (VLCFA), and HPPD (Heap 2022; Tranel 2021). Moreover, a single waterhemp accession has been documented to be resistant to six SOAs (Shergill et al. 2018).

Waterhemp has become a steadily increasing concern in Wisconsin (Hammer et al. 2016; Stoltenberg 2018). In 2018, 85% of Wisconsin counties had reported its presence, a 25% increase compared with 2009 (Renz 2018; Zimbric et al. 2018). The first report of waterhemp herbicide resistance in Wisconsin was in 1999 when a population was confirmed to be resistant to ALS inhibitors (Zimbric et al. 2018). In 2013, two waterhemp accessions were confirmed to be resistant to glyphosate (Butts and Davis 2015). In 2018, the Wisconsin Cropping Systems Weed Science Survey (Werle and Oliveira 2018), with 286 respondents across 54 counties, reported waterhemp to be among the most troublesome weeds in Wisconsin cropping systems. Moreover, respondents perceived waterhemp as the weed species with the most frequent occurrence of glyphosate resistance. Currently, waterhemp in Wisconsin has been confirmed to be resistant to ALS-, EPSPS-, and PPO-inhibitor herbicides (Zimbric et al. 2018). Glyphosate-resistance has been confirmed in 28 counties, and multiple resistance to glyphosate and PPO inhibitors has been confirmed in 10 counties (Hammer et al. 2016; Zimbric et al. 2018).

The combination of effective postemergence (POST) and preemergence (PRE) herbicides, and multiple modes of action, as part of integrated weed management (IWM) program, is important to delay herbicide resistance evolution, preserve the usefulness of newly developed herbicide-resistant crops, and for the long-term economic success and sustainability of agricultural production (Norsworthy et al. 2012). In addition, the adoption of epidemiological approaches for herbicide monitoring and management, which systematically studies the extent, distribution, and determinants of a harmful organism, can greatly contribute to our efforts to understand the emergence, selection, and spread of herbicide resistance (Comont and Neve 2021). A comprehensive, Wisconsin state-wide assessment of waterhemp response to a diverse group of herbicide SOAs has not been conducted. Therefore, our objective was to characterize the response of a Wisconsin state-wide collection of waterhemp accessions to POST and PRE herbicides commonly used in corn and soybean crops. We hypothesized that ALS, EPSPS, and PPO inhibitors would be ineffective on most accessions, whereas auxin mimics, and inhibitors of PS II, glutamine synthetase, VLCFA, and HPPD would be effective.

Materials and Methods

Waterhemp Seed Collection

In the summer of 2018, the Wisconsin Cropping Systems Weed Science Program, in partnership with key collaborators (i.e., University of Wisconsin-Madison Nutrient and Pest Management Program, University of Wisconsin-Madison Division of Extension, Wisconsin Soybean Marketing Board, and Wisconsin Corn Promotion Board), released a protocol requesting stakeholders (i.e., farmers, agronomists, industry representatives, Extension educators, etc.) to collect seed samples from 20 waterhemp female plants from Wisconsin fields with unsatisfactory waterhemp management before crop harvest. Seed samples were pooled and composed the accession for that specific geographic location. Eighty-eight waterhemp accessions from 27 counties were collected and submitted by stakeholders to the Wisconsin Cropping Systems Weed Science Program (Figure 1) along with management information of the sampled fields from 2014 to 2018 (information presented in Supplementary Table S1). Seeds from each waterhemp accession were threshed, cleaned through a seed blower separator (Oregon Seed Blower; Hoffman Manufacturing, Inc., Corvallis, OR), and coldly stratified to improve seed germination. In the cold stratification procedure, all seeds of each accession were placed in a glass container with a thin layer of water, just enough to make seeds float, and stored in a dark environment at 5 C for 2 wk (adapted from Kohlhase et al. 2018). After this period, seeds were washed with water using a soil sieve mesh to retain the seeds, and dried on paper towels at room temperature for 24 h. Seeds were placed in plastic bags and stored at 5 C until the onset of experiments, which were conducted at the University of Wisconsin-Madison Walnut Street Greenhouses (43.076194°N, 89.423611°W), Madison, WI.

Waterhemp Response to POST Herbicides

The experiments were organized in a randomized complete block design with eight replications per treatment, and repeated over time (two experimental runs). Treatments were arranged as $A \times H \times D$ factorial with A representing the number of accessions, H the number of herbicides, and D the number of herbicide rates (1× and 3× the recommended label rates). Eight herbicides were evaluated (Table 1). The A and H factors evaluated at the same time varied across experiments due to seed availability and to allow for the research objectives to be accomplished promptly, particularly in 2020 during the COVID-19 global pandemic. Glyphosate, imazethapyr, and atrazine were evaluated in separate experiments on 88, 85, and 81 accessions, respectively. From the 81 accessions with enough seeds remaining, 29 were evaluated in the same experiment for dicamba and 2,4-D; and 26 were evaluated in the same experiment for glufosinate, fomesafen, and mesotrione. Each experiment included a nontreated control (NTC) of each accession.

Waterhemp seeds were planted at 1.5-cm depth in potting mix (Promix® HP Mycorrhizae; Premier Tech Horticulture, Rivière-du-Loup, QC, Canada) contained in 23-cm-diam disposable aluminum pans. Seedlings at the true 2-leaf stage were transplanted into 656-ml pots (D40H Deepots™; Stuewe & Sons Inc., Tangent, OR) filled with potting mix. The experimental unit was one seedling per pot. POST herbicide treatments were applied when plants reached 5 to 10 cm in height using a single-nozzle research track spray chamber (DeVries Manufacturing, Hollandale, MN) equipped with AI9502EVS or DG9502EVS nozzle (TeeJet Technologies®; Spraying Systems Co., Wheaton, IL) for systemic and contact herbicides, respectively. Due to vapor drift concerns within an enclosed environment (greenhouse), the dicamba and 2,4-D herbicide treatments were applied at the University of Wisconsin-Madison Arlington Agricultural Research Station (43.3026°N, 89.3454°W). Waterhemp plants were transported to this field location on the morning of the application and returned to the greenhouse at the end of the day to allow for herbicide absorption while minimizing unintended vapor drift issues. A CO_2-pressurized backpack spray boom with four TTI110015 nozzles (TeeJet Technologies®) was used for the application. A carrier volume of 140 L ha^{-1} was used in all applications (spray chamber and backpack). Plants were maintained in the greenhouse at 20 to 35 C with a natural ventilation system. Natural lighting was supplemented with 400-W high-pressure sodium light bulbs simulating a 16-h photoperiod. Plants were watered daily and fertigated weekly with 20-10-20 water-soluble fertilizer (Peters Professional®; ICL Fertilizers, Dublin, OH) delivering 500 ppm of nitrogen and potassium, respectively, and 250 ppm of phosphorus.

At 21 d after treatment (DAT), plant survival was assessed visually as dead (no green tissue; assessed value of 0) or alive (green tissue and evidence of regrowth; assessed value of 1; Figure 2). Accessions with ≥50% (± standard error) plant survival were classified as resistant to each herbicide × rate treatment (adapted from Schultz et al. 2015 and Vennapusa et al. 2018; adopted by Faleco et al. 2022). Aboveground biomass was harvested, and force air-dried at 52 C to constant mass. The biomass data were converted into percent biomass reduction compared to the NTC using Equation 1 (adapted from Wortman 2014). Seed production of survivor plants was not determined.

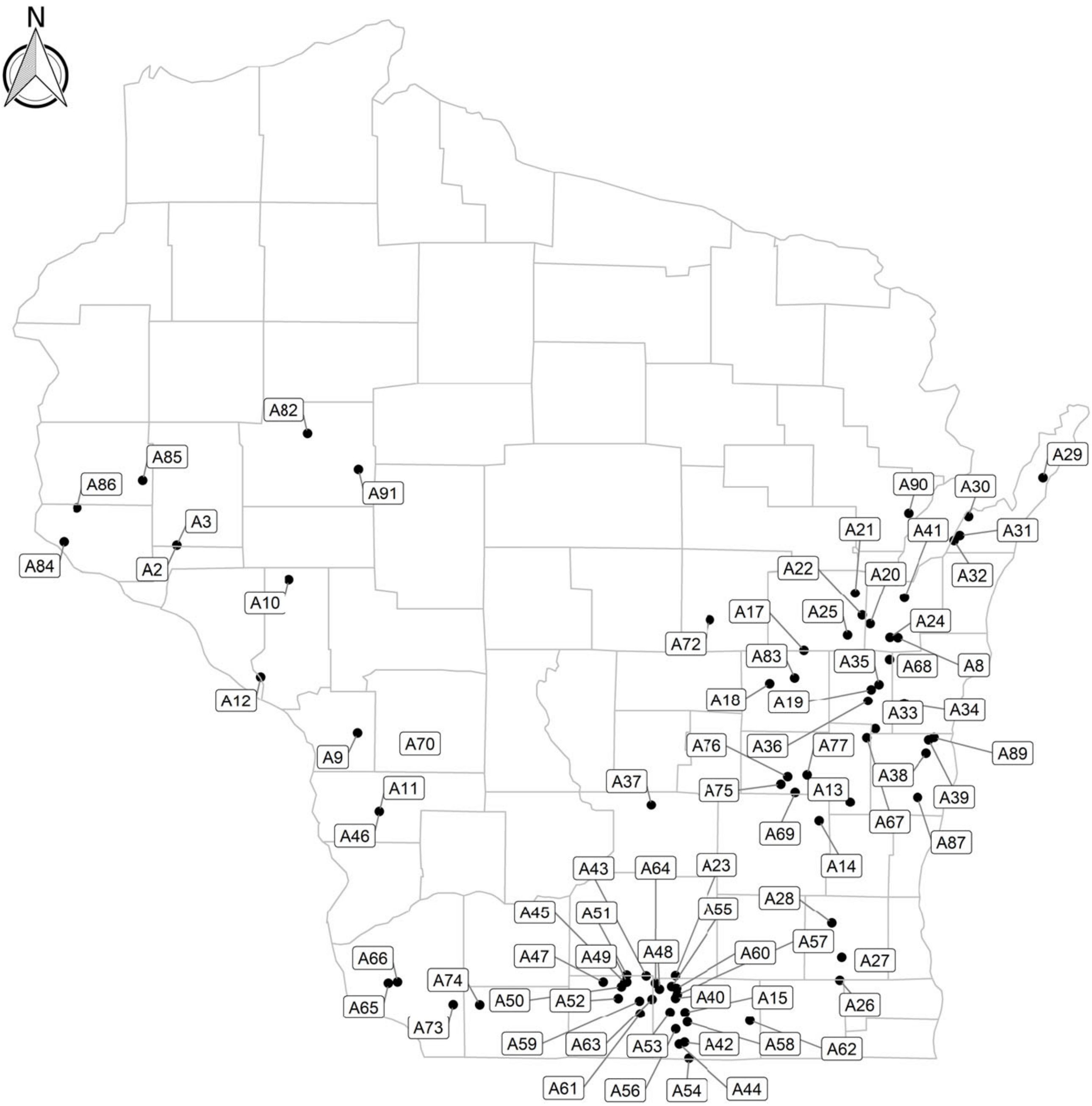

Figure 1. Geographic distribution of the 88 waterhemp accessions from 27 Wisconsin counties collected and submitted by stakeholders to the Wisconsin Cropping Systems Weed Science Program.

$$Biomass\ Reduction\ (\%) = \left(1 - \frac{BEU}{\overline{BNTC}}\right) \times 100 \qquad [1]$$

where BEU represents the biomass of the experimental unit and $\overline{BNTC}$ represents the biomass mean of the NTC for the respective accession.

Waterhemp Response to PRE Herbicides

The experiments were organized in a randomized complete block design, with four replications per treatment and repeated over time (two experimental runs). Treatments were arranged as $A \times H \times D$ factorial with A representing the number of accessions, H the number of herbicides, and D the number of herbicide rates (0.5×, 1×, and 3× the recommended label rate). Five herbicides were evaluated (Table 2). The A and H factors evaluated at the same time varied across experiments as described above. Fomesafen, S-metolachlor, and mesotrione were evaluated in the same experiment on 30 accessions. Atrazine and metribuzin were evaluated in the same experiment on 29 accessions. Each experiment included an NTC of each accession.

Experimental units consisted of approximately 190 seeds (measured by volume) planted 1.5 cm deep in 360-ml pot (8.9 cm Kord Traditional Square Pot; The HC Companies, Twinsburg, OH) filled with nonsterilized field soil (silty clay loam; 6.4 pH; 3.0% organic matter; 18% sand, 53% silt, and 30% clay by weight).

Table 1. Postemergence herbicide treatments used to evaluate the response of waterhemp accessions.[a]

Active ingredient	Trade name	Formulation	WSSA SOA[b]	Accessions evaluated	Rate[c] Herbicide 1×		Adjuvant HSOC[d]	AMS[d]	Herbicide manufacturer
					1×	3×	HSOC[d]	AMS[d]	
					—g ai or ae ha^{-1}—		v/v %	g ha^{-1}	
Imazethapyr	Pursuit®	2 L	ALS, Group 2	85	72	216	0.63	2,352	BASF Corporation, Research Triangle Park, NC
Dicamba	XtendiMax®	2.9 L	AM, Group 4	29	565	1,695	–	–	Monsanto Company, St. Louis, MO
2,4-D	Enlist One™	3.8 L	AM, Group 4	29	800	2,400	–	–	Dow AgroSciences, LLC, Indianapolis, IN
Atrazine	Aatrex®	4 L	PS II, Group 5	81	1,121	3,363	0.83	–	Syngenta Crop Protection, LLC Greensboro, NC
Glyphosate	Roundup PowerMax®	4.5 L	EPSPS, Group 9	88	864	2,592	–	2,184	Monsanto Company, St. Louis, MO
Glufosinate	Liberty®	280 SL	GS, Group 10	26	654	1,962	–	2,242	Bayer CropScience LP, Research Triangle Park, NC
Fomesafen	Flexstar®	1.88 SL	PPO, Group 14	26	263	789	0.5	1,428	Valent U.S.A Corporation, Walnut Creek, CA
Mesotrione	Callisto®	4 SC	HPPD, Group 27	26	106	318	0.5	1,428	Syngenta Crop Protection, LLC Greensboro, NC

[a]Abbreviations: ALS, acetolactate synthase; AM, auxin mimic; AMS ammonium sulfate; EPSPS, enolpyruvyl shikimate phosphate synthase; GS, glutamine synthetase; HPPD, hydroxyphenyl pyruvate dioxygenase; HSOC, high surfactant oil concentrate; L, liquid; SL, soluble liquid; SC, soluble concentrate; POST, postemergence; PPO, protoporphyrinogen oxidase; PS II, photosynthesis at photosystem II – serine 264 binders; SOA, site of action; WSSA, Weed Science Society of America.
[b]Group represents the herbicide SOA as classified by the WSSA.
[c]The 1× herbicide adjuvant rates were based on the respective herbicide label crop use directions for POST application in corn or soybean, and recommendations for controlling waterhemp when specified.
[d]A dash (-) indicates adjuvant was not included.

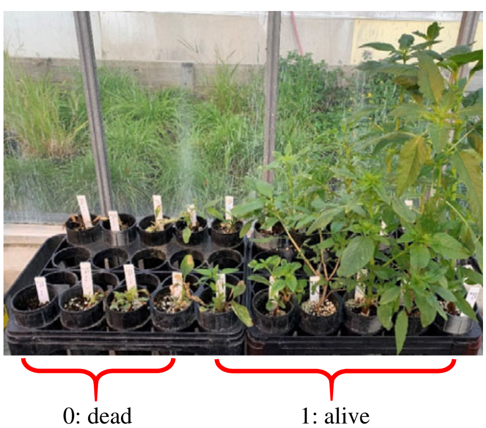

Figure 2. Plant survival rating used for herbicide resistance classification for waterhemp response to postemergence-applied herbicides.

The soil was watered immediately after planting and before herbicide application to facilitate seed germination. Preemergence herbicide treatments were applied using the spray chamber and carrier volume described above, equipped with a AI9502EVS nozzle (TeeJet Technologies®). Plants were watered daily and fertigated weekly with 20-10-20 water-soluble fertilizer (Peters Professional®) delivering 500 ppm of nitrogen and potassium, respectively, and 250 ppm of phosphorus. The daily watering promoted PRE herbicide activation in soil following application. Environmental conditions in the greenhouse were the same as described above for the POST experiments.

At 28 DAT, emerged plants per experimental unit were counted. The count data were converted into percent plant density reduction compared with the NTC using Equation 2 (adapted from Wortman 2014).

$$Plant\ Density\ Reduction\ (\%) = \left(1 - \frac{PCEU}{\overline{PCNTC}}\right) \times 100 \quad [2]$$

where $PCEU$ represents the plant counts of the experimental unit and $\overline{PCNTC}$ represents the plant counts mean of the NTC for the respective accession.

Herbicide × rate treatments that provided <90% (± standard error) plant density reduction were classified as ineffective for each accession (adapted from Vennapusa et al. 2018; adopted by Faleco et al. 2022).

Assessment of Targe-Site Resistance for EPSPS- and PPO-inhibitor Herbicides

Target-site resistance for EPSPS- and PPO-inhibitor herbicides was assessed for the 26 accessions evaluated in the fomesafen POST experiment, using leaf tissue from five plants per accession. These accessions were also evaluated in the glyphosate POST experiment. The assessments were conducted by the University of Illinois Plant Clinic (Urbana, IL) using the methodology described by Chatham et al. (2015), which identifies *EPSPS* gene amplification for glyphosate resistance, and the methodology described by Wuerffel et al. (2015), which identifies ΔG210 protoporphyrinogen oxidase mutation for PPO resistance.

Statistical Analyses

A generalized linear mixed model with Gaussian distribution was fitted to the biomass reduction data (POST experiment) and plant density reduction data (PRE experiment) using the GLMMTMB package version 1.0.2.1 (Brooks et al. 2017). Analysis of variance

Table 2. Preemergence herbicide treatments used to evaluate the response of waterhemp accessions.[a]

Active ingredient	Trade name	Formulation	WSSA SOA[b]	Accessions evaluated	Herbicide rate[c] 0.5×	1×	3×	Herbicide manufacturer
					—— g ai ha^{-1}——			
Atrazine	Aatrex®	4 L	PS II, Group 5	29	560.5	1,121	3,363	Syngenta Crop Protection, LLC Greensboro, NC
Metribuzin	Tricor®	75 DF	PS II, Group 5	29	262.5	525	1,575	United Phosphorus, Inc., King of Prussia, PA
Fomesafen	Flexstar®	1.88 SL	PPO, Group 14	30	131.5	263	789	FMC Corporation, Philadelphia, PA
S-metolachlor	Dual II Magnum®	7.64 EC	VLCFA, Group 15	30	892.5	1,785	5,355	Syngenta Crop Protection, LLC Greensboro, NC
Mesotrione	Callisto®	4 SC	HPPD, Group 27	30	135	270	810	Syngenta Crop Protection, LLC Greensboro, NC

[a]Abbreviations: DF, dry flowable; EC, emulsifiable concentrate; HPPD, hydroxyphenyl pyruvate dioxygenase; L, liquid; PPO, protoporphyrinogen oxidase; PS II, photosynthesis at photosystem II – serine 264 binders; SL, soluble liquid; SC, soluble concentrate; SOA site of action; VLCFA, very long-chain fatty acid synthesis; WSSA, Weed Science Society of America.
[b]Group represents the herbicide SOA as classified by the WSSA.
[c]The 1× herbicide rate was based on the respective herbicide label crop use directions for preemergence application in corn or soybean on medium not highly erodible soils with 3.0% organic matter, and recommendations for controlling waterhemp when specified.

type II Wald Chi-square was performed followed by Tukey's honestly significant difference test ($\alpha = 0.05$) pairwise comparisons using the EMMEANS package version 1.5.4 (Lenth 2020). To have a general assessment of the response of waterhemp accessions from Wisconsin to the POST and PRE herbicide treatments, herbicide and rate were considered as fixed effects, whereas accession and experimental run as random effects. Both response variables were logit-transformed to improve normality assumptions (Barnes et al. 2020; Davies et al. 2019, 2020; Striegel et al. 2020; Warton and Hui 2011). Back transformed means are presented for ease of result interpretation. Statistical analyses were performed using R software version 4.0.3 (R Core Team 2020) and RStudio software version 1.4.1103 (RStudio Team 2021).

Results and Discussion

Waterhemp Response to POST Herbicides

Ninety-eight percent and 88% of the accessions exhibited ≥50% plant survival after exposure to imazethapyr and glyphosate POST at the 3× rate, respectively (Figure 3). Seventeen percent, 16%, and 3% of the accessions exhibited ≥50% plant survival after exposure to 2,4-D, atrazine, and dicamba POST at the 1× rate, respectively. Survival of all accessions was ≤25% after exposure to 2,4-D or dicamba POST at the 3× rate, or glufosinate, fomesafen, and mesotrione POST at either rate evaluated in this study. No plant of any accession survived exposure to glufosinate at either rate.

Among the 26 accessions evaluated for all herbicides at the 1× rate applied POST, 58% exhibited ≥50% survival after exposure to imazethapyr and glyphosate (Figure 4; herbicide treatments applied separately, not tank mixed); 12% after exposure to imazethapyr, glyphosate, and atrazine; and other 12% after exposure to imazethapyr, glyphosate, and 2,4-D. One accession (A75, Fond du Lac County) exhibited ≥50% survival after exposure to imazethapyr, atrazine, glyphosate, and 2,4-D POST at the 1× rate.

ANOVA exhibited a significant two-way interaction between herbicide and rate for biomass reduction ($P < 0.0001$). For the POST 1× rate, biomass reduction did not differ among glufosinate, mesotrione, and fomesafen (≥97%; Figure 5), which was greater than for atrazine, 2,4-D, and dicamba (95%, 95%, 94%, respectively), followed by glyphosate (35%) and imazethapyr (27%). For the POST 3× rate, biomass reduction did not differ among glufosinate, mesotrione, fomesafen, 2,4-D, dicamba, and atrazine (≥97%), which was greater than for glyphosate (69%) and imazethapyr (33%).

Resistance to ALS, PS II, and EPSPS inhibitors in waterhemp has been widely reported in the United States (Evans et al. 2019; Heap 2022; Sarangi et al. 2019; Singh et al. 2020; Vieira et al. 2018). Murphy et al. (2019) reported that atrazine and glyphosate resistance was very frequent among waterhemp accessions evaluated from Ohio, whereas lactofen resistance was less frequent. In their study, a target-site resistance mechanism was observed for lactofen and glyphosate, but not for atrazine. Vennapusa et al. (2018) reported that atrazine applied POST was ineffective in the majority of waterhemp accessions evaluated from Nebraska, with the non-target site resistance (NTSR) mechanism via glutathione S-transferase present. Schryver et al. (2017) confirmed imazethapyr, glyphosate, and atrazine resistance in 100%, 82%, and 76% of the accessions from Ontario, Canada. In their experiment, 61% of the accessions were resistant to all three herbicides. Moreover, several waterhemp accessions have been confirmed to be resistant to multiple SOAs, including auxin mimics (Bernards et al. 2012; Crespo et al. 2017; Schultz et al. 2015), with a single waterhemp accession being resistant to six herbicide SOAs (Shergill et al. 2018).

Between 2014 and 2018, ALS inhibitors were applied at least once in 67% of the fields where the accessions with ≥50% survival after exposure to imazethapyr at the 3× rate applied POST were sampled, with predominance of flumetsulam (commercial tank mix with acetochlor and clopyralid) applied PRE in corn, and imazethapyr (commercial tank mix with glyphosate) applied POST in soybean (Supplementary Table S1). Widespread occurrence of ALS inhibitor resistance in waterhemp is a good example of how important it is to preserve herbicide SOAs. This resistance began appearing in several Midwest U.S. states in the early 1990s and became widespread within about 5 yr after rapid adoption of this SOA for waterhemp management (Heap 2022; Tranel 2021). In recent years, this resistance has been the norm rather than the exception, being present in essentially every field accession of waterhemp and in naturalized riparian populations from Ohio (Tranel 2021; Waselkov 2013). Moreover, research has demonstrated that the ALS inhibitor resistance fitness cost may vary depending on the weed species. For instance, Werle et al. (2016, 2017) reported a lack

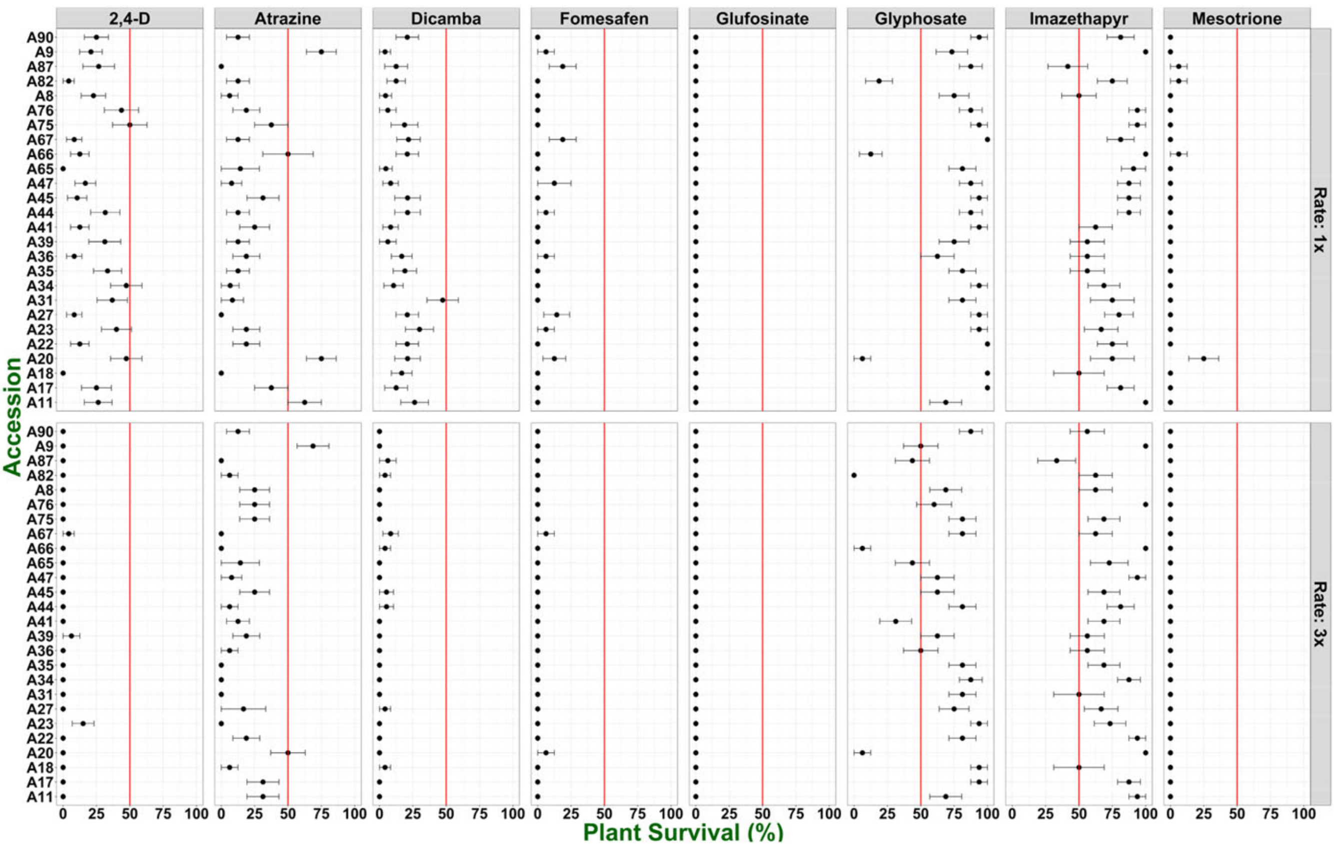

Figure 3. Waterhemp plant survival (± standard error) in response to postemergence-applied herbicides. Accessions with survival ≥50% (represented by the red line) were classified as resistant to each herbicide × rate treatment. Data from the 26 accessions evaluated for all herbicides applied postemergence are presented.

of strong ALS inhibitor resistance fitness cost in shattercane [*Sorghum bicolor* (L.) Moench ssp. *drummondii* (Nees ex Steud.) de Wet ex Davidse] and johnsongrass [*Sorghum halepense* (L.) Pers.]. On the other hand, Wu et al. (2018) reported ALS inhibitor resistance fitness cost in waterhemp, but not for resistance to PS II, EPSPS, PPO, and HPPD inhibitors.

In our study, elevated survival after exposure to atrazine applied POST was not very frequent (16% of the accessions exhibited ≥50% survival after exposure to atrazine at the 1× rate applied POST; Figure 3), nor was a lack of biomass reduction observed (95% biomass reduction at the 1× rate applied POST; Figure 5). Although atrazine is one of the most widely used corn herbicides in Wisconsin (USDA-NASS 2015, 2017, 2018, 2019), between 2014 and 2018, atrazine was applied at least once in only 35% of the fields from which the 81 accessions evaluated for atrazine were collected (Supplementary Table S1). In contrast, atrazine was applied at least once in 69% of the fields from which the accessions with ≥50% survival after exposure to atrazine at the 1× rate applied POST were sampled. We believe that the reduced use of atrazine in most of the sampled fields during this 5-yr period that preceded seed collection, and perhaps for a longer period, minimized selection pressure for atrazine resistance. Additionally, the Wisconsin rules and regulations for atrazine use are more restrictive than the Federal standards, such as establishing maximum application rates given soil texture and use pattern, and established atrazine prohibition areas (WI-DATCP 2021, ATCP 30.31). All the accessions with ≥50% survival after exposure to atrazine at the 1× rate applied POST were sampled from fields outside the established atrazine prohibition areas (Figure 4).

On the other hand, the selection pressure associated with the over-use of glyphosate may help to explain our findings that 88% of the accessions exhibited ≥50% survival after exposure to glyphosate at the 3× rate applied POST. Between 2014 and 2018, glyphosate was applied at least once in 90% of the fields from which these accessions were sampled (Supplementary Table S1). Glyphosate resistance is a good example of the critical need to reduce over-reliance on single approaches to weed management. The first case of glyphosate resistance in weeds was reported in 1996 as a rigid ryegrass (*Lolium rigidum*) accession evolved resistance after 15 yr of multiple glyphosate treatments (Pratley et al. 1996, 1999). Around the same time, Powles et al. (1998) reported glyphosate resistance in a different rigid ryegrass accession collected from an orchard where glyphosate had been used two or three times a year for 15 yr to control weeds within rows of trees. Both authors strongly emphasized the importance of integrated weed management and careful use of selective herbicides to preserve the efficacy of glyphosate. Rosenbaum and Bradley (2013) reported that glyphosate-resistant waterhemp were more likely to occur in fields with no other weed species present at the end of the season, continuous cropping of soybean, exclusive use of glyphosate for several consecutive seasons, and waterhemp plants showing obvious signs of surviving herbicide treatment compared to fields characterized with glyphosate-susceptible waterhemp. They suggested that these four site parameters, and certain combinations of them, serve as predictors of glyphosate resistance in future waterhemp populations.

The A20 and A75 accessions (≥50% survival after exposure 1× 2,4-D applied POST), and the A31 accession (≥50% survival after

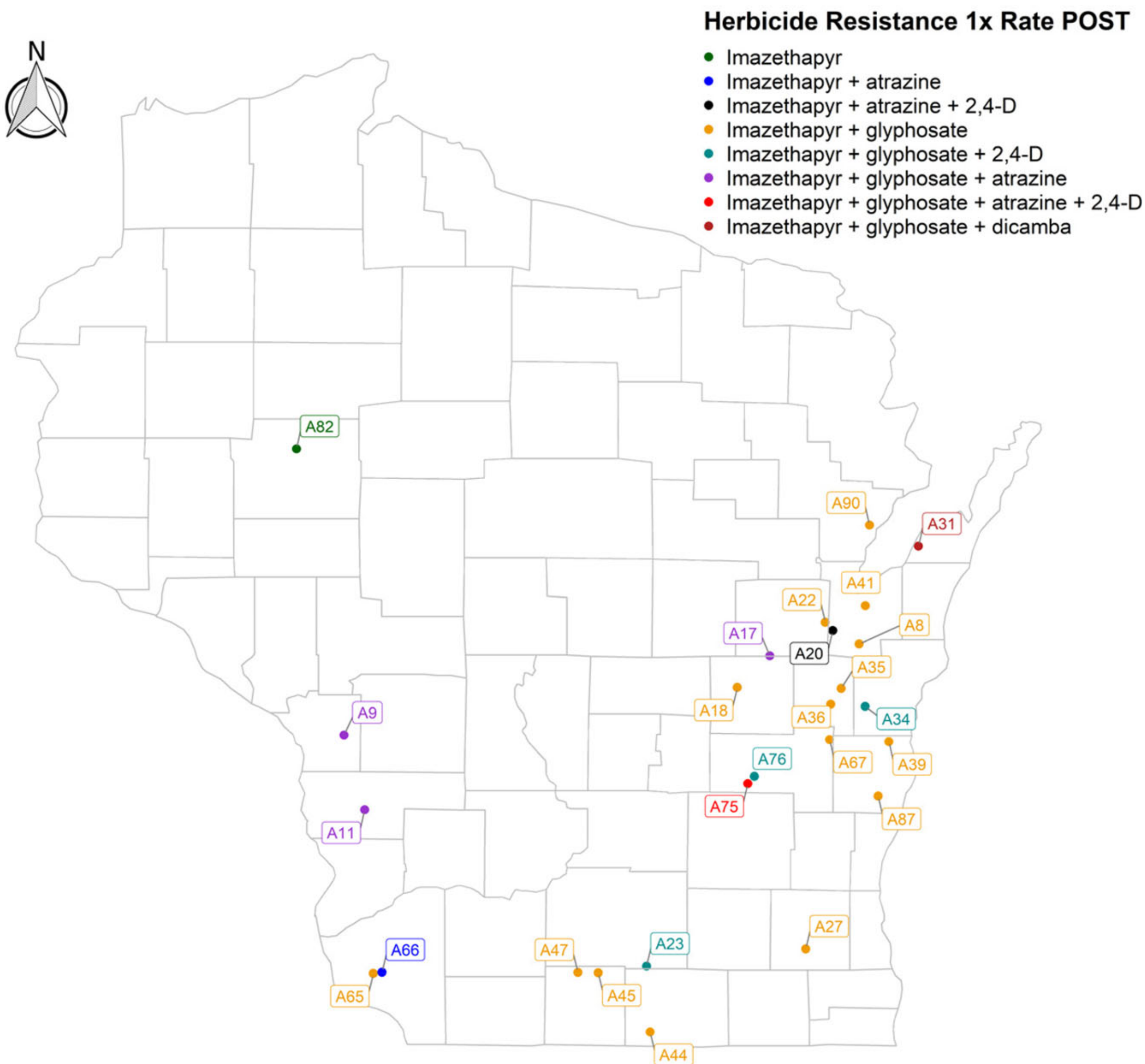

Figure 4. Geographic distribution of Wisconsin waterhemp accessions exhibiting herbicide resistance 1× rate applied postemergence. Herbicide treatments were applied separately (not tank mixed). Data from the 26 accessions evaluated for all herbicides applied postemergence are presented.

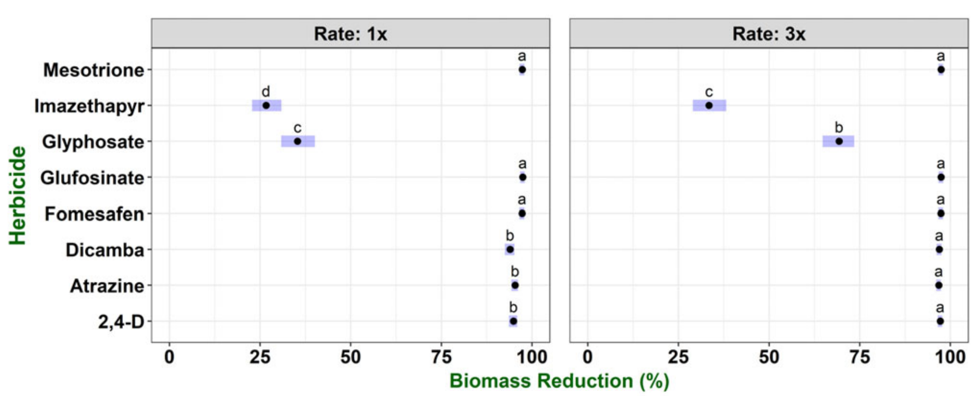

Figure 5. Waterhemp biomass reduction represented by the two-way interaction between postemergence-applied herbicide and rate. Accessions were considered as a random effect. The blue boxes represent the 95% confidence intervals. Treatments with the same letters did not differ according to Tukey’s honestly significant difference test at $\alpha = 0.05$.

exposure 1× dicamba applied POST) were not exposed to any auxin mimic between 2014 to 2018 (Supplementary Table S1). These results may be a possible indicative for NTSR metabolic resistance, which means plants can evolve resistance to herbicides that had never been sprayed in the field (Rigon et al. 2020; Shyam et al. 2021, Yu and Powles 2014).

Waterhemp Response to PRE Herbicides

Forty-five percent and 3% of the accessions exhibited <90% plant density reduction after exposure to atrazine applied PRE at the 3× rate and fomesafen applied PRE at the 1× rate, respectively (Figure 6). Three percent of the accessions exhibited <90% plant density reduction after exposure to S-metolachlor or mesotrione applied PRE at the 0.5× rate. Plant density reduction of all accessions was ≥96% after exposure to fomesafen applied PRE at the 3× rate, or to metribuzin, *S*-metolachlor mesotrione applied PRE at the 1× rate.

ANOVA exhibited a significant two-way interaction between herbicide and rate for plant density reduction ($P < 0.0001$). At the 0.5× rate, plant density reduction did not differ for *S*-metolachlor, metribuzin, and mesotrione (≥97%; Figure 7), which was greater than that for fomesafen (96%), and atrazine (77%). At the 1× and 3× rates, plant density reduction for *S*-metolachlor, metribuzin, mesotrione, and fomesafen (≥97%) was greater than that for atrazine (≤93%).

Preemergence herbicides have a very important role to play in integrated weed management. However, biotic and abiotic factors such as interactions among weather, soil, microorganisms, and herbicide, might affect the performance of PRE herbicides (Dao and Lavy 1978; Fang et al. 2015; Houot et al. 2000; Jing et al. 2020; Takeshita et al. 2019). For example, Vennapusa et al. (2018) reported more effective waterhemp control with atrazine applied PRE rather than POST, although it was still unsatisfactory in both cases. In contrast, our study found greater atrazine performance when it was applied POST rather than PRE. Comparing the soil characteristics from the study by Vennapusa et al. 2018 (loam, 6.4 pH, 1.7% organic matter) vs. the characteristics of soil in our study (silty clay loam, 6.4 pH, 3.0% organic matter; 18% sand, 53% silt, and 30% clay by weight), our soil contained a greater amount of organic matter and clay. Higher amounts of organic matter and/or clay is generally associated with increased adsorption of S-triazines (Talbert and Fletchall 1965), and therefore, we believe this condition might help to elucidate our results.

The use of reduced PRE herbicide rates as an attempt to reduce costs, herbicide carryover, and/or environmental impacts may increase the selection pressure and lead to rapid herbicide resistance evolution (Belz 2020; Manalil et al. 2011; Maxwell and Mortimer 1994; Norsworthy 2012; Tehranchian et al. 2017; Vieira et al. 2020). Our results suggest that herbicides applied PRE at the 0.5× label rate might provide reduced waterhemp control. Consequently, the reliance on herbicides applied POST may increase and, in the end, the short-term economic benefits associated with using reduced herbicide rates are quickly outweighed by the future costs related to herbicide resistance evolution and spread (Gressel 1997).

Assessment of Targe-Site Resistance for EPSPS- and PPO-inhibitor Herbicides

Fifty percent of the 26 accessions evaluated exhibited both *EPSPS* gene amplification and ΔG210 protoporphyrinogen oxidase mutation, 35% exhibited only the *EPSPS* gene amplification, 4% exhibited the ΔG210 protoporphyrinogen oxidase mutation only, and 11% did not exhibit these target-site alterations (data not shown).

Comparing the target-site assessment results to the glyphosate POST experiment, all accessions containing the *EPSPS* gene amplification also exhibited ≥50% plant survival after exposure to glyphosate applied POST at the 1× rate, supporting our resistance classification methodology. Three accessions (A15, A57, and A76) exhibited ≥50% plant survival after exposure to glyphosate applied POST at the 1× rate but did not exhibit the *EPSPS* gene amplification. This evidence warrants further investigation of other glyphosate resistance mechanisms, such as amino acid substitution (*P106S*) and reduced glyphosate translocation (Bell et al. 2013; Nandula et al. 2013).

Comparing the target-site assessment results to the fomesafen POST experiment, 54% of the 26 accessions evaluated exhibited the ΔG210 protoporphyrinogen oxidase mutation, whereas no accession exhibited ≥50% plant survival after exposure to fomesafen applied POST at the 1× rate. We believe that these accessions might have a low-level resistance to PPO inhibitors that we were not able to detect in the POST experiment. Oliveira et al. (2021) also observed high mortality of Palmer amaranth (*Amaranthus palmeri* S. Watson) in greenhouse conditions, even with most of the accessions containing the ΔG210 protoporphyrinogen oxidase mutation. They suggested several factors that may help to understand this phenomenon, such as having 1× as the lowest herbicide rate, ideal greenhouse conditions for herbicide application and performance compared to the field, or the opposite, with limited root growth due to pot size affecting plant ability to overcome herbicide effects. This warrants further investigation.

Best management practices, as proposed by Norsworthy et al. (2012), are of paramount importance for long-term sustainability of weed management, particularly in cases of NTSR. Avoiding new introductions of waterhemp, preventing established infestations from reproducing, and preventing seed movement are important; equipment cleaning and weed-free crop seeds may help in this context. Enhancing crop competitiveness, routinely scouting fields, diversifying and mixing herbicide SOAs as often as possible, and respecting the labeled herbicide rates and recommended weed sizes are necessary. Continued community efforts, education, training, economic incentives, and policies are of critical importance to move farmers to more sustainable weed management systems (Liu et al. 2020; Moss 2019; Peterson et al. 2018). Research, development, and successful implementation of innovative weed management tools such as biopesticides, computer vision, decision tools, robotics, and machine learning may also play important roles in near future and mitigate the reliance on herbicides (Arakeri et al. 2017; Coleman et al. 2019; Fennimore and Cutulle 2019; McCool et al. 2018; Panpatte and Ganeshkumar 2021; Westwood et al. 2018).

In conclusion, our results suggest that ≥88% of the accessions evaluated are resistant (≥50% survival) to both imazethapyr and glyphosate applied POST. Seventeen percent, 16%, and 3% of the accessions are resistant to 2,4-D, atrazine, and dicamba applied POST, respectively. All accessions were susceptible (<50% survival) to glufosinate, fomesafen, and mesotrione applied POST. The A75 accession (Fond du Lac County, WI) exhibited multiple resistance to imazethapyr, glyphosate, atrazine, and 2,4-D applied POST. Moreover, atrazine and fomesafen applied PRE were ineffective (<90% plant density reduction) for 45% and 3%, respectively, of the accessions evaluated. Metribuzin, S-metolachlor, and mesotrione applied PRE effectively controlled (≥90% plant density

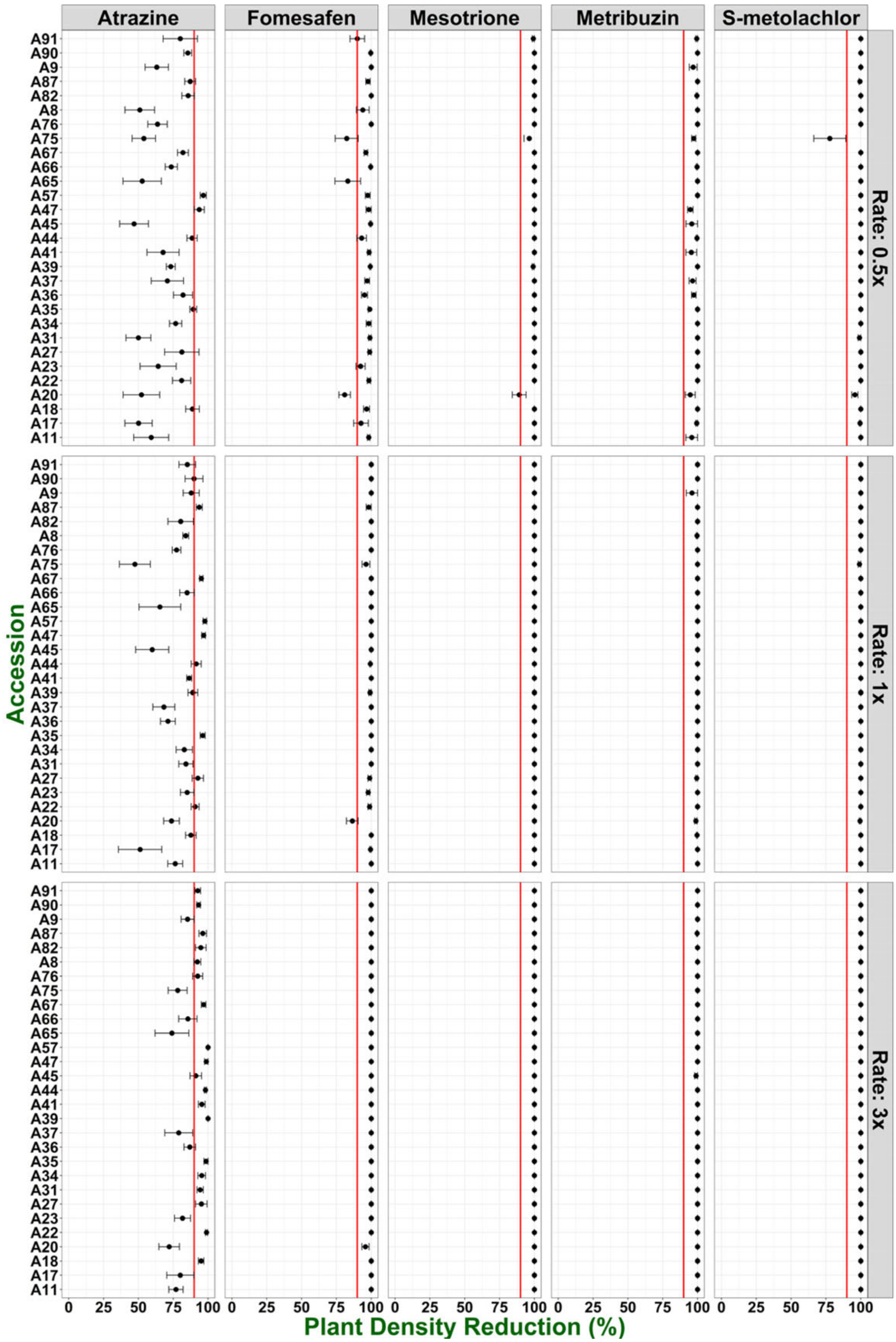

Figure 6. Waterhemp plant density reduction (± standard error) in response to preemergence-applied herbicides. Treatments with plant density reduction <90% (represented by the red line) were classified as ineffective. Data from the 29 accessions evaluated for all herbicides applied preemergence are presented.

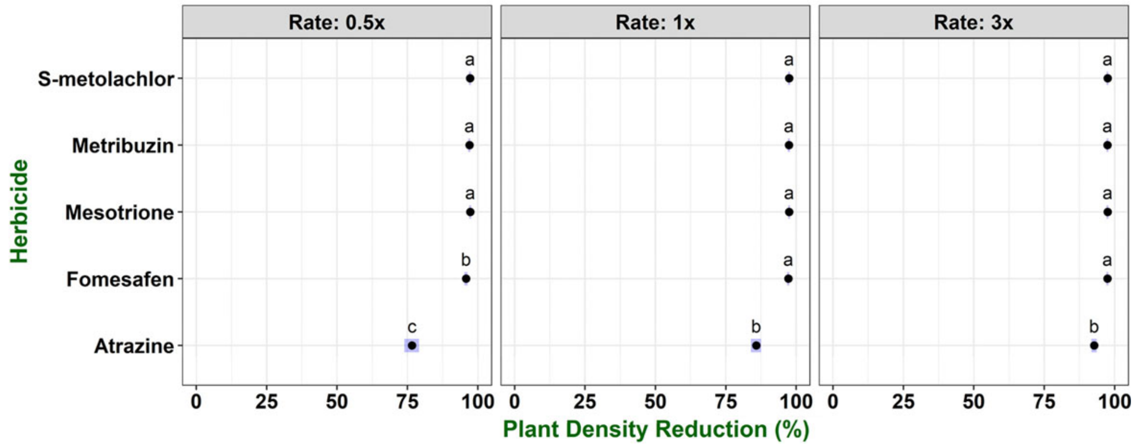

Figure 7. Waterhemp plant density reduction represented by the two-way interaction between preemergence-applied herbicide and rate. Accessions were considered as a random effect. The blue boxes represent the 95% confidence intervals. Treatments with the same letters did not differ according to Tukey's honestly significant difference test at α = 0.05.

reduction) each accession at 1× and 3× rates. Herbicides applied PRE at the 0.5× rate provided reduced waterhemp control and might increase the reliance on herbicides applied POST. Proactive resistance management and the use of effective PRE and POST herbicides as part of an integrated weed management program, are fundamental for waterhemp management in Wisconsin.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/wet.2022.81

Acknowledgments. We thank the Wisconsin Soybean Marketing Board for funding Felipe Faleco's graduate research assistantship, the stakeholders for collecting and sending seed samples, students and staff in the University of Wisconsin-Madison Cropping Systems Weed Science Program for their technical assistance with the greenhouse experiments, and Diane Elizabeth Plewa and the University of Illinois Plant Clinic for conducting the molecular analyses. No conflicts of interest have been declared.

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