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Distribution of Herbicide Resistances and Molecular Mechanisms Conferring Resistance in Missouri Waterhemp (*Amaranthus rudis* Sauer) Populations

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A survey of soybean fields containing waterhemp was conducted just prior to harvest in 2012 to determine the scope and extent of herbicide resistance and multiple herbicide resistances among a sample of Missouri waterhemp populations. Resistance was confirmed to glyphosate and to acetolactate synthase (ALS), protoporphyrinogen oxidase (PPO), photosystem II (PSII), and 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors, but not to 2,4-D. Of the 187 populations tested, 186 exhibited resistance to chlorimuron. The proportions of populations with atrazine or glyphosate resistance were similar, with 30 and 29% of the populations surviving the 3× rates. Lactofen resistance was observed in 5% of the populations, whereas mesotrione resistance was only found in 1.6% of the populations. All populations tested were susceptible to 2,4-D at the 3× rate. At least 52% of the waterhemp populations tested exhibited resistance to herbicides from two mechanism of action. Resistance to atrazine plus chlorimuron as well as glyphosate plus chlorimuron was present in 29% of the populations. Three-way resistance, primarily comprised of resistance to atrazine plus chlorimuron plus glyphosate, was present in 11% of the populations. Resistance to herbicides from four mechanisms of action was found in 2% of the populations, and one population exhibited resistance to herbicides from five mechanisms of action. DNA analysis of a subsample of plants revealed that previously documented mechanisms of resistance in waterhemp, including the ΔG210 deletion conferring PPO-inhibitor resistance, the $Trp^{574}Leu$ amino acid substitution conferring ALS-inhibitor resistance, and elevated 5-enolypyruvyl-shikimate-3-phosphate synthase copy number and the Pro106Ser amino acid substitution resulting in glyphosate resistance, explained survival in many, but not all, instances. Atrazine resistance was not explained by the $Ser^{264}Gly$ D1 protein substitution. Overall, results from these experiments indicate that Missouri soybean fields contain waterhemp populations with resistance to glyphosate, ALS-, PPO-, PSII-, and HPPD-inhibiting herbicides, which are some of the most common mechanisms of action currently utilized for the control of this species in corn and soybean production systems. Additionally, these results indicate that slightly more than half of the populations tested exhibit resistance to more than one herbicide mechanisms of action. Managing the current resistance levels in existing populations is of utmost importance. The use of multiple, effective herbicide modes of action, both preemergence and postemergence, and the integration of optimum cultural and mechanical control practices will be vital in the management of Missouri waterhemp populations in the future.

Nomenclature: 2,4-D; atrazine; chlorimuron; glyphosate; lactofen; mesotrione; waterhemp, *Amaranthus tuberculatus* (Moq.) Sauer var. *rudis* (Sauer); corn, *Zea mays* L.; soybean, *Glycine max* (L.) Merr.

Key words: Herbicide resistance, survey.

Waterhemp is the most prominent and troublesome weed in agronomic crops in Missouri, Iowa, and Illinois (Bradley 2013; Bradley et al. 2007; Hager et al. 2000; Legleiter and Bradley 2008; Rosenbaum and Bradley 2013; Waggoner and Bradley 2011). Its extended period of germination, rapid growth rate, and prolific seed production are all characteristics that have enabled this weed to thrive in current corn and soybean production systems (Hartzler et al. 1999, 2004; Sauer 1957). Waggoner and Bradley (2011) reported that waterhemp was found in 87% of the Missouri soybean fields that were surveyed at an average population density of 22 plants m^{-2}, and resulted in yield losses up to 545 kg ha^{-1}. Corn and soybean can suffer substantial yield losses due to waterhemp. Hager et al. (2002) found that 10 wk of waterhemp interference at population densities from 89 to 362 plants m^{-2} resulted in a 43% soybean yield loss. Cordes et al. (2004) reported waterhemp population densities from 82 to 445 plants m^{-2} resulted in a 10 to 36% corn yield reduction. Many producers rely primarily and often solely on herbicides for weed control; therefore, increasing selection pressure is placed on weed populations to evolve

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resistance (Powles 2008; Young 2006). Because waterhemp is dioecious, gene mutation is more likely and this species can transfer its genes easily from male to female plants via pollen (Costea et al. 2005; Hausman et al. 2011; Steckel 2007; Tranel et al. 2011).

As of 2014, numerous waterhemp populations within the United States have been reported to be resistant to herbicides from one or more of the following sites of action: inhibitors of EPSPS, ALS, PPO, PSII, and HPPD, and growth regulators (Heap 2014). One population of waterhemp from Illinois and one from Iowa evolved multiple resistances to herbicides from four sites of action (Bell et al. 2013; Heap 2014). Populations of waterhemp throughout Missouri, Iowa, and Kansas have evolved multiple resistances to herbicides from two or more modes of action (Heap 2014; Legleiter and Bradley 2008). In Missouri, waterhemp with resistance to glyphosate and ALS-, PPO-, or PSII-inhibiting herbicides has been documented (Heap 2014; Legleiter and Bradley 2008). In 2005, a waterhemp biotype that exhibited three-way resistance to glyphosate, ALS inhibitors, and PPO inhibitors was confirmed in Platte County, Missouri (Heap 2014; Legleiter and Bradley 2008).

Several mechanisms have been discovered to explain these herbicide resistances in waterhemp. ALS-inhibitor resistance has been conferred mostly through a target-site point mutation, with $Trp^{574}Leu$ amino acid substitution being most common (Patzoldt and Tranel 2007), but also through a non–target-site mechanism that appears to be metabolism-based (Guo et al. 2013; Warwick et al. 2010). Glyphosate resistance has been conferred through EPSPS gene amplification, a $Pro^{106}Ser$ amino acid substitution in the EPSPS enzyme, and a non–target-site mechanism (Bell et al. 2013; Chatham et al. 2013; Nandula et al. 2013). Resistance to PPO-inhibiting herbicides is highly conserved and is conferred by a codon deletion at amino acid 210 in the *PPX2* gene (Patzoldt et al. 2006). PSII-inhibitor herbicide resistance may be confirmed by a $Ser^{264}Gly$ amino acid mutation in the psbA enzyme (Foes et al. 1998; Mechant et al. 2008), but non–target-site resistance to atrazine is common in waterhemp as well (Patzoldt et al. 2003). Ma et al. (2013) reported that, contrary to the aforementioned resistance mechanisms, there were no alterations in the *HPPD* sequence or *HPPD* expression in HPPD-inhibitor–resistant waterhemp. Rather, the mechanism of resistance was determined to be enhanced oxidative metabolism (Ma et al. 2013).

Surveys are practical tools to gather important information to assist in educating producers on the current weed resistance status and distribution within a given geographic area (Beckie et al. 2000; Givens et al. 2009; Johnson and Gibson 2006; Rosenbaum and Bradley 2013). Rosenbaum and Bradley (2013) conducted a survey of weedy soybean fields at harvest in 2008 and 2009 to determine the extent and distribution of glyphosate resistance in Missouri waterhemp populations. They found that 69% of the Missouri waterhemp populations sampled were resistant to glyphosate across 54 counties in the state (Rosenbaum and Bradley 2013). However, currently there is no information as to the extent of multiple herbicide resistances in Missouri waterhemp populations.

The objectives of this research were to determine the level of herbicide resistance and multiple herbicide resistances among a sample of Missouri waterhemp populations to herbicides spanning six mechanisms of action. In addition, some plants were evaluated for the presence of known resistance mechanisms to support whole-plant greenhouse data and to provide an indication of the frequencies at which these mechanisms exist.

Materials and Methods

Whole-Plant Resistance Evaluation. *Plant Materials and Growth Conditions*. In 2012, waterhemp seed samples were collected from 187 soybean fields across 57 counties in the primary corn and soybean production areas in Missouri. Sites for seed collection were chosen arbitrarily, but based on the presence of waterhemp in soybean fields just prior to soybean harvest. At each survey location, approximately 5 to 10 female waterhemp seed heads were harvested and the global positioning system coordinate was recorded (Figure 1). Mature seeds were gleaned from waterhemp seed heads and combined into a sample representative of that waterhemp population. Seeds were treated with a 50 : 50 water and commercial bleach solution for 10 min, washed with water, suspended in a 0.15% agarose solution, and stored at 4 C for 6 wk prior to the start of the experiments to improve germination. Seeds from each population were broadcast into 25- by 50-cm greenhouse flats containing commercial potting medium (Premier Tech Horticulture, Quakertown, PA), which was used to cover the seedbed to a thickness of approximately 6 mm following planting. After emergence, one- to two-leaf seedlings were transplanted into cones 4 cm in diam and 4 cm deep (Cone-tainers, Stewe and Sons Inc., Tangent, OR) containing a 4 : 1 mixture of commercial potting

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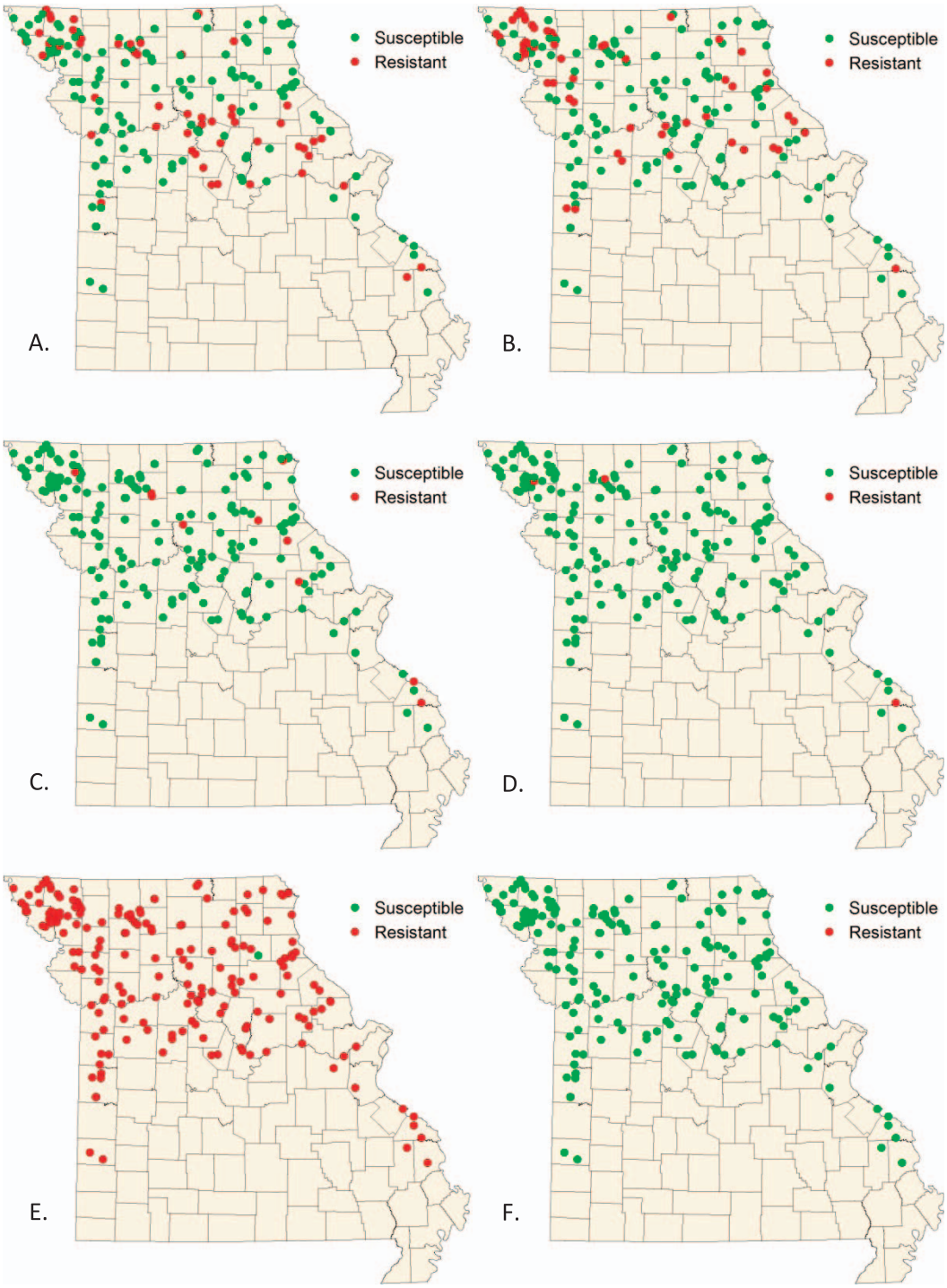

Figure 1. Distribution of waterhemp samples collected in the 2012 survey with resistances to the 3× rate of (A) glyphosate, (B) atrazine, (C) lactofen, (D) mesotrione, (E) chlorimuron, and (F) 2,4-D.

medium to sand. Plants were maintained in a greenhouse at 25 to 30 C, watered and fertilized as needed, and provided with artificial lighting from metal halide lamps (600 µmol photon m^{-2} s^{-1}) simulating a 16-h-photoperiod day.

Experimental Design. The trial design was a factorial arrangement of treatments in a randomized complete block with six replications (individual plants), where the factors were population, herbicide, and rate. The experiment was repeated once.

Treatment, Evaluation, and Data Collection. The herbicides and rates evaluated are listed in Table 1. Herbicide rates included the standard labeled rate (1×) and three times the standard labeled rate (3×) for each respective herbicide. A nontreated control was included from each population for comparison.

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Table 1. Sources of materials and rates used in the experiment.

Herbicide	Formulation	Rate (kg ai ha^{-1})	Manufacturer	Address
Mesotrione	4 SC	0.11 (1×) 0.31 (3×)	Syngenta	Greensboro, NC
Glyphosate	3 L	0.84 (1×) 2.53 (3×)	Tenkoz	Alpharetta, GA
2,4-D amine	3.8 L	0.53 (1×) 1.59 (3×)	Tenkoz	Alpharetta, GA
Lactofen	2 L	0.18 (1×) 0.53 (3×)	Valent U.S.A.	Walnut Creek, CA
Chlorimuron	25 DG	0.012 (1×) 0.035 (3×)	Dupont	Wilmington, DE
Atrazine	4 L	1.12 (1×) 3.37 (3×)	Syngenta	Greensboro, NC

Treatments were applied when plants reached 8 to 12 cm in height, using a compressed-air laboratory spray chamber equipped with an 8001EVS nozzle (Teejet Spraying Systems, Wheaton, IL) delivering 220 L ha^{-1} at 234 kPa. Resistance was characterized based on the number of plants that survived the 3× rate and were capable of continued growth 21 d after application (DAA) as suggested by Beckie et al. (2000) and Rosenbaum and Bradley (2013). Rosenbaum and Bradley (2013) classified waterhemp populations as resistant to glyphosate if survival was 60% or more at the 2× rate. In this experiment, waterhemp populations were considered resistant if 50% or more of the plants averaged across both runs survived the 3× herbicide application and were capable of growth and reproduction. Survivorship in response to each treatment was determined and recorded for each plant 21 DAA.

Molecular Examination of Resistance Mechanisms. *Sample Preparation*. Plant sampling to examine resistance mechanisms was conducted during the second run of the whole-plant screening, and selection of populations for sampling was guided in part based on results from the first screening. Prior to herbicide application, tissue samples were taken from the new growth of six plants from each of 26 potential PPO-resistant populations to determine if the ΔG210 codon deletion in the *PPX2* gene was present. These same plants were tracked through the remainder of the experiment to determine survival or death following the application of the 3× rate of lactofen. Three weeks after the herbicide applications, additional tissue samples were arbitrarily collected from the new growth of 92 plants from 41 populations that survived the 3× rate of chlorimuron, from 93 plants from 36 populations that survived the 3× rate of glyphosate, and from 19 plants from populations in 19 different counties (one plant per county) that survived the 3× rate of atrazine to test for the mechanisms of resistance for these respective herbicides. Tissue was stored at −80 C until DNA extraction. All DNA was extracted from frozen leaf tissue using the hexadecyltrimethylammonium bromide method previously described by Doyle and Doyle (1990). Quality and quantity of DNA were examined using a spectrophotometer (NanoDrop 1000 spectrophotometer, Thermo Fisher Scientific, 81 Wyman St., Waltham, MA 02451), and samples were diluted to either 10 ng μl^{-1} for quantitative polymerase chain reaction (qPCR) or to 10% of the original concentration for all other downstream applications.

EPSPS Gene Amplification. Relative *EPSPS* copy number was determined using real-time qPCR as described previously (Délye et al. 2014; Ma et al. 2013).

EPSPS Point Mutation. Detection of the point mutation responsible for the Pro^{106}Ser substitution was carried out using a derived cleaved amplified polymorphic sequences (dCAPS) assay designed in the manner described by Délye et al. (2014). A portion of the *EPSPS* gene containing the codon at position 106 was amplified using the forward primer EPSf1 (5′-ATG TTG GAC GCT CTC AGA ACT CTT GGT-3′) and reverse primer eps106wt-R3 (5′-CTC CAG CAA CGG CAA CCG CAA CTG TCC ATG-3′), which includes a single mismatch to introduce a *Nco*I restriction site in wild-type alleles. After PCR, resulting amplicons were digested with the enzyme *Nco*I (New England BioLabs Inc., 240 County Road, Ipswich, MA 01938-2723), and products were fractionated on a 2% agarose gel containing 0.5 μg ml^{-1} ethidium bromide and visualized with ultraviolet light.

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ALS Trp^{574}*Leu Mutation*. The Trp^{574}Leu substitution in ALS results in the addition of an *Mfe*I recognition site. Primers AmALS-F2 (5′-TCC CGG TTA AAA TCA TGC TC-3′) and AmALS-R2 (5′-CTA AAC GAG AGA ACG GCC AG-3′) were used to amplify the region of the ALS gene encompassing the Trp^{574}Leu substitution, and the amplified product was digested with *Mfe*I, as described for kochia [*Kochia scoparia* (L.) Schrad.] (Foes et al. 1999).

ALS Sequencing. Regions A and B of *ALS* previously described by Foes et al. (1998) were amplified with PCR and visualized using gel electrophoresis to confirm the presence and size of the correct amplicon. The remaining PCR products were then purified (E.Z.N.A. Cycle Pure Kit, Omega Bio-Tek, Inc., 400 Pinnacle Way, Suite 450, Norcross, GA 30071) and sequenced (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems Inc., 850 Lincoln Centre Dr., Foster City, CA 94404) using both the forward and reverse primers for regions A and B. Products of the sequencing reaction were analyzed by the W.M. Keck Center for Comparative and Functional Genomics using an AB 3730xl DNA analyzer (Applied Biosystems Inc.). Sequences were then aligned to a waterhemp sequence from an ALS inhibitor–sensitive line (EF157818) in GenBank with MEGA6 (Tamura et al. 2013).

PPO ΔG^{210} *Mutation*. Allele-specific primers described previously by Lee et al. (2008) were used to screen samples for the codon deletion in the *PPX2* gene that results in the deletion of Gly^{210}.

D1 Ser^{264}*Gly Mutation*. A fragment of the chloroplast *psbA* gene, which encodes the D1 protein, was amplified with primers described by Foes et al. (1998). Following PCR, 5 µl of each reaction was added to 20 µl of a digestion mixture and incubated at 37 C overnight. The digestion mixture contained the restriction enzyme BfaI (2.5 units µl^{-1}), 1× concentration of the supplied buffer (New England Biolabs Inc.) and ultrapure water. Digested products were separated on a 1% agarose gel and visualized under ultraviolet light with ethidium bromide staining. Digestion with BfaI produces cleaved products only for the non-mutated form of the gene.

Results and Discussion

Whole-Plant Resistance Evaluation. Resistance was confirmed to herbicides from five of the six modes of action tested (Table 2; Figure 1). Of the 187 populations tested, 186 exhibited resistance to chlorimuron. Atrazine and glyphosate resistances were similar with 30 and 29% of the populations surviving the 3× rates, respectively. Lactofen resistance was observed in 5% of the populations whereas mesotrione resistance was only found in 1.6% of the populations. All populations tested were susceptible to 2,4-D at the 3× rate.

Although the resistance threshold used in this experiment was 50% or greater survival to the 3× rate, data from the 1× rate were collected and included to show the potential future resistances in Missouri waterhemp populations (Table 2). There were no differences in the percentage of waterhemp populations that survived 1× and 3× rates of chlorimuron. High levels of resistance to chlorimuron and other ALS inhibitors have been well documented within the literature (Patzoldt et al. 2005; Patzoldt and Tranel 2007; Shoup et al. 2003). However, the number of populations resistant to the 1× rate of atrazine, glyphosate, lactofen, and mesotrione exceeded the level of resistance to the 3× rate of these same herbicides in every instance. Patzoldt et al. (2003) also observed differences in waterhemp survival between high and low application rates of atrazine. They attributed this response to a non–target-site mechanism of resistance. Unlike resistance to ALS inhibitors, current evidence suggests that glyphosate target-site mechanisms of resistance do not confer an absolute resistance level. In most cases of glyphosate resistance in *Amaranthus* species to date, overproduction of EPSPS reduces the ability of glyphosate to successfully bind to all EPSPS copies within the plant due to a higher ratio of EPSPS proteins to glyphosate (Powles 2010). Chatham et al. (2013) observed increasingly higher levels of glyphosate-resistant waterhemp control with each incremental increase in glyphosate rate across five separate waterhemp populations in the Midwest. Four of these populations were confirmed to have an elevated EPSPS copy number whereas one population had the Pro^{10}6Ser amino acid substitution. An approximate 20% increase in control was also seen in a Mississippi glyphosate-resistant waterhemp population in response to increasing the glyphosate rate from 1× to 2× (Nandula et al. 2013). With regard to lactofen, the greater survival of waterhemp to the 1× compared to the 3× rate may have occurred because the resistance mechanism was overwhelmed and, since lactofen is a cell membrane disruptor, plant tissue may have been damaged to the extent that effective control was achieved. Thinglum et al. (2011) also reported increased control of PPO

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Table 2. Comparison of herbicide resistance in Missouri waterhemp populations.

Factor	Resistance			
	1× Rate		3× Rate	
	No. of resistant populations	% of populations	No. of resistant populations	% of populations
Herbicide[a]				
Chlorimuron	186	99.5	186	99.5
Atrazine	96	51	56	30
Glyphosate	108	58	55	29
Lactofen	20	11	10	5
Mesotrione	27	14	3	1.6
2,4-D Amine	1	0.5	0	0
Two-way resistances[b]				
2,4-D + chlorimuron	1	0.5	0	0
2,4-D + mesotrione	1	0.5	0	0
Atrazine + chlorimuron	96	51	54	29
Atrazine + lactofen	11	6	4	2
Atrazine + glyphosate	53	28	19	10
Atrazine + mesotrione	18	10	3	1.6
Chlorimuron + glyphosate	107	57	55	29
Chlorimuron + lactofen	19	10	9	5
Chlorimuron + mesotrione	27	14	3	1.6
Glyphosate + lactofen	15	8	3	1.6
Glyphosate + mesotrione	17	9	3	1.6
Lactofen + mesotrione	2	1	1	0.5
Total populations with two-way resistance	157	84	98	52
Three-way resistances[b]				
2,4-D + chlorimuron + mesotrione	1	0.5	0	0
Atrazine + chlorimuron + glyphosate	53	28	18	10
Atrazine + chlorimuron + lactofen	11	6	3	1.6
Atrazine + chlorimuron + mesotrione	18	10	3	1.6
Atrazine + glyphosate + mesotrione	13	7	3	1.6
Atrazine + glyphosate + lactofen	7	4	2	2
Atrazine + lactofen+ mesotrione	1	0.5	1	0.5
Chlorimuron + glyphosate + lactofen	14	7	3	1.6
Chlorimuron+ glyphosate+ mesotrione	17	9	3	1.6
Chlorimuron + lactofen + mesotrione	2	1	1	0.5
Glyphosate + lactofen + mesotrione	2	1	1	0.5
Total populations with three-way resistance	73	39	20	11
Four-way resistances[b]				
Atrazine + chlorimuron + glypnosate + mesotrione	13	7	3	1.6
Atrazine + chlorimuron + glypnosate + lactofen	7	4	2	1
Atrazine + glypnosate + lactofen + mesotrione	1	0.5	1	0.5
atrazine + chlorimuron + lactofen + mesotrione	1	0.5	1	0.5
Chlorimuron + glyphosate + lactofen + mesotrione	2	1	1	0.5
Total populations with four-way Resistance	20	11	4	2
Five-way resistances[b]				
Atrazine + chlorimuron + glyphosate + lactofen + mesotrione	1	0.5	1	0.5
Total populations with five-way resistance	1	0.5	1	0.5

[a] Appropriate adjuvants were added based on label recommendations for each respective herbicide.
[b] Herbicide combination data is compiled from the single herbicide application data. Herbicides were not tank-mixed.

inhibitor–resistant waterhemp as the herbicide rate increased. Enhanced oxidative metabolism, as noted by Ma et al. (2013), is the only known mechanism of HPPD resistance in waterhemp to date. HPPD resistance was found in only 1.6% of the Missouri waterhemp populations, most likely due to the lack of frequency of HPPD-inhibiting herbicide use in Missouri corn and soybean production systems as compared to glyphosate, ALS-inhibiting herbicides, or atrazine.

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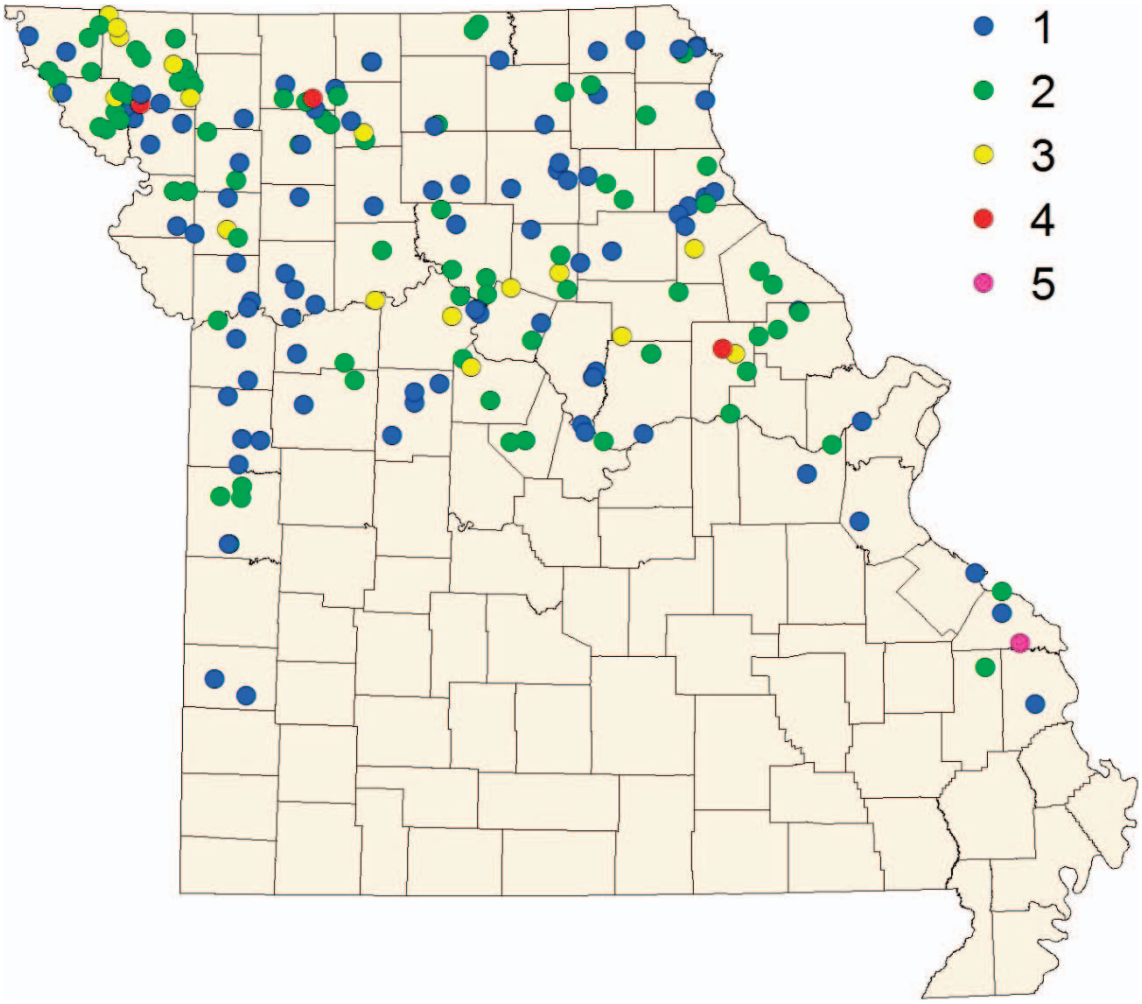

Figure 2. Distribution of waterhemp with multiple herbicide resistances collected in the 2012 survey.

At least 52% of the waterhemp populations tested exhibited resistance to herbicides from two mechanisms of action (Table 2; Figure 2). Resistance to atrazine plus chlorimuron as well as glyphosate plus chlorimuron was present in 29% of the populations. Three-way resistance was present in 11% of the populations with resistance to atrazine plus chlorimuron plus glyphosate present in 10% of the populations. Resistance to herbicides from four mechanisms of action was shown in 2% of the populations whereas only one population exhibited resistance to herbicides from five mechanisms of action.

Molecular Examination of Resistance Mechanisms. DNA analysis indicated that all sampled plants resistant to lactofen in the greenhouse experiment contained the ΔG210 deletion mutation in the *PPX2* gene (Table 3). To date, this mutation remains the only known mechanism of PPO resistance in waterhemp. Based on anecdotal observations, it was expected that a higher percentage of the populations screened would have been resistant to lactofen. Therefore, plants were sampled prior to lactofen application so that plants that did not survive lactofen could be evaluated for the presence of the PPO ΔG210 deletion. Of 135 plants sampled that did not survive lactofen, 37 contained the ΔG210 deletion (data not shown). The use rates in this study were 0.18 (1×) and 0.53 (3×) kg lactofen ha^{-1}. Thinglum et al. (2011) reported increased control of PPO-resistant waterhemp as the lactofen rate increased. To determine the frequency of PPO-resistant waterhemp in populations from three states, Thinglum et al. (2011) used 0.11 kg of lactofen ha^{-1}. The presence of the ΔG210 deletion in several plants that did not survive the 3× rate of lactofen indicates that the greenhouse data underestimated the frequency of resistance to PPO inhibitors.

Of the 92 plants sampled that exhibited resistance to chlorimuron, 75 contained the $Trp^{574}Leu$ amino acid substitution (Table 3), with 20 and 55 being homozygous and heterozygous for the resistance allele, respectively (data not shown). All but one of

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Table 3. Analysis of known gene alterations in resistant and susceptible waterhemp plants following 3× rates of selected herbicide treatments.

Herbicide treatment	Total no. of plants screened	No. of resistant plants screened	No. of resistant plants with mutation	No. of resistant plants heterozygous for mutation	No. of sensitive plants screened	No. of sensitive plants without mutation
Lactofen	155[a]	20[a]	20[a]	n/a[b]	135[a]	98[a]
Chlorimuron	92[c]	92[c]	75[c]	55[c]	n/a[b]	n/a[b]
Glyphosate	93[d]	93[d]	19[d]	16[d]	n/a[b]	n/a[b]
Glyphosate	93[e]	93[e]	82[e]	n/a[b]	n/a[b]	n/a[b]
Atrazine	19[f]	19[f]	0[f]	n/a[b]	n/a[b]	n/a[b]

[a] Plants genotyped for a ΔG210 deletion in the PP2XL enzyme.
[b] Data not collected for this treatment.
[c] Plants genotyped for the mutation causing a $Trp^{574}Leu$ amino acid substitution in the acetolactate synthase enzyme.
[d] Plants genotyped for the mutation causing a $Pro^{106}Ser$ amino acid substitution in the 5-enolypyruvyl-shikimate-3-phosphate synthase (EPSPS) enzyme.
[e] Plants analyzed for increased copies (≥ twofold) of the *EPSPS* gene.
[f] Plants genotyped for the mutation causing a $Ser^{264}Gly$ amino acid substitution in the D1 protein.

the populations evaluated for the $Trp^{574}Leu$ mutation had at least one plant with this mutation. Four plants from the population lacking this mutation were further evaluated by partial sequencing of the ALS gene. Mutations conferring $Ser^{653}Asn$ or $Ser^{653}Thr$ ALS substitutions were found in three of the four plants (data not shown). However, Patzoldt and Tranel (2007) reported that waterhemp biotypes with these ALS mutations were resistant to imidazolinone but not sulfonylurea herbicides (including chlorimuron, based on a leaf-disc assay). Observed resistance to chlorimuron in plants lacking the $Trp^{574}Leu$ substitution, possibly including even those plants with substitutions at Ser^{653}, may have been due to a non–target-site mechanism. Guo et al. (2013) found that a non–target-site, metabolism-based mechanism was responsible for ALS resistance in a waterhemp population from Illinois.

Ninety-three glyphosate-resistant plants from the greenhouse experiment were sampled to determine potential mechanisms of resistance. An elevated EPSPS copy number (≥ twofold) was found in 82 plants (Table 3). The $Pro^{106}Ser$ substitution was found in 19 plants (Table 3), with 3 and 16 appearing to be homozygous and heterozygous for the mutation, respectively (data not shown). Some plants possessed both mechanisms: of those with an elevated EPSPS copy number, one appeared to be homozygous and 11 heterozygous for the $Pro^{106}Ser$ amino acid substitution. Of the resistant plants, four had neither an elevated EPSPS copy number nor the $Pro^{106}Ser$ substitution. These plants may have a novel non–target-site mechanism of resistance or one similar to the GR waterhemp population from Mississippi documented by Nandula et al. (2013). Teaster and Hoagland (2014) also suggest that there are other, unknown, mechanisms of glyphosate resistance in *Amaranthus* species.

Of 19 plants that survived 3× atrazine, none contained the $Ser^{264}Gly$ D1 substitution, which is the common basis of atrazine resistance in many weeds, including waterhemp (Foes et al. 1998). As previously reported, non–target-site atrazine resistance exists in waterhemp and confers a lower magnitude of resistance than does target-site resistance (Patzoldt et al. 2003). That there was a large difference in apparent atrazine resistance between the 1× and 3× atrazine rates, as discussed above, is consistent with the presence of a low-magnitude, non–target-site resistance mechanism in many if not all of the Missouri populations. It is also possible, however, that a target-site mutation other than $Ser^{264}Gly$ is present in some of the Missouri populations (Powles and Yu 2010). Patzoldt et al. (2002) indicated that non–target-site atrazine resistance was much more common than target-site resistance in waterhemp populations from Illinois as well.

In conclusion, results from these experiments indicate that Missouri soybean fields contain waterhemp populations with resistances to glyphosate and to ALS-, PPO-, PSII-, and HPPD-inhibiting herbicides, which comprise some of the most common mechanisms of action currently utilized for the control of this species in corn and soybean production systems. Additionally, these results indicate that slightly more than half of the populations tested exhibit resistance to herbicides from more than one mechanism of action. ALS-inhibiting herbicides are not effective in controlling many waterhemp populations and will likely continue to be unsuccessful in the future. ALS-inhibitor resistance in waterhemp was first documented in the United States in 1993 (Heap

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2014). Twenty years later ALS-inhibitor resistance is present in most Missouri waterhemp, and in most other areas in the United States where waterhemp occurs (Heap 2014). Glyphosate and atrazine are still effective on some waterhemp populations, but resistances to these herbicides now occurs across such a wide geographical region that, based on previous experiences with ALS-inhibiting herbicide resistance in waterhemp, resistance in future waterhemp populations may be inevitable. Resistance to lactofen and mesotrione is present in only a small proportion of waterhemp populations in Missouri at this time, although resistance to lactofen may have been underestimated. As of yet, no resistance to 2,4-D has been discovered in Missouri waterhemp. The results from the DNA analysis of the subsample of plants that survived applications of glyphosate, PPO inhibitors, and ALS inhibitors revealed that many of the same mechanisms of resistance documented in previous research were present in Missouri waterhemp and, with the exception of PPO inhibitors, there appear to be multiple mechanisms that confer resistance. Looking to the future, it is unlikely that reversal of herbicide resistance is possible. Managing the current resistance levels in existing populations is likely the most plausible action. The use of multiple effective herbicides with different mechanisms of action, both PRE and POST, along with the integration of optimum cultural and mechanical control practices such as crop rotation, narrow row spacings, cover crops, and between-row cultivation will be vital to managing Missouri waterhemp populations in the future.

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