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Characterization of PPO-Inhibitor–Resistant Waterhemp (*Amaranthus tuberculatus*) Response to Soil-Applied PPO-Inhibiting Herbicides

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Waterhemp resistance to foliar applications of protoporphyrinogen oxidase (PPO)–inhibiting herbicides has become increasingly disconcerting given the widespread distribution of glyphosate resistance. Fortunately, soil-residual PPO-inhibiting herbicides remain efficacious in waterhemp populations resistant to PPO-inhibiting herbicides; however, these herbicides should theoretically select for the resistant biotype as herbicide concentrations diminish in the soil. Accordingly, the objectives of this research were twofold: (1) evaluate the efficacy of three PPO-inhibiting herbicides, foliar- and soil-applied, on PPO-resistant (PPO-R) and PPO-susceptible (PPO-S) waterhemp, and (2) investigate the differential effects of PPO-inhibiting herbicides on an R biotype and an S biotype during several discrete developmental events relevant to soil–residual herbicide activity (i.e., radicle protrusion, radicle elongation, and waterhemp emergence). Greenhouse and growth chamber experiments indicated that the R biotype was least sensitive to the diphenylether herbicide fomesafen, followed by sulfentrazone and flumioxazin; however, fomesafen plus *s*-metolachlor improved soil-residual efficacy over fomesafen alone. Growth stage considerably influenced the R : S ratio, decreasing from 38× to 3.4×, when comparing ratios generated from foliar applications and soil-residual applications measuring radicle protrusion, respectively. Overall, this research supports the use of full soil-residual herbicide rates, reinforcing the importance of best management practices to manage the spread of herbicide resistance.

Nomenclature: Flumioxazin; fomesafen; glyphosate; *s*-metolachlor; sulfentrazone; tall waterhemp, *Amaranthus tuberculatus* (Moq.) Sauer (syn. *rudis*) AMATA.

Key words: common waterhemp, tall waterhemp, herbicide resistance.

Overreliance on glyphosate and the subsequent selection for glyphosate-resistance has profoundly altered the success and tactics implemented for weed management in the past decade, forcing many growers to apply additional herbicide active ingredients to achieve desired levels of weed control (Hager et al. 2003; Riggins and Tranel 2012). These modified weed management strategies are often heavily reliant on soil-residual herbicides, especially in row crops, such as soybean [*Glycine max* (L.) Merr.] and cotton [*Gossypium hirsutum* (L.)], where herbicide-resistance has limited POST herbicide options (Norsworthy et al. 2012). Waterhemp is a pernicious broadleaf weed species in Midwestern soybean production because of its evolved resistance to herbicides from multiple sites of action, including herbicides that inhibit protoporphyrinogen oxidase (PPO) (Bernards et al. 2012; Dayan et al. 2014; Hausman et al. 2011; Tranel et al. 2011). Numerous reports of waterhemp with resistance to herbicides from several sites of action has further exacerbated the issue of herbicide resistance (Legleiter and Bradley 2008; Patzoldt et al. 2005). With continuous germination throughout the growing season, long-distance pollen dispersion, a high reproductive capacity, and a dioecious reproductive strategy, waterhemp has an ideal physiology for evolving and disseminating alleles conferring herbicide-resistance (Hartzler et al. 1999; Steckel 2007).

Waterhemp resistant to foliar-applications of PPO-inhibiting herbicides (PPO-R) was first discovered in 2001 in a Kansas soybean field (Shoup et al. 2003) and has subsequently been identified in three additional states: Illinois, Missouri, and Iowa (Heap 2014). The causal mechanism of PPO-R in waterhemp originates from a codon deletion on the nuclear-encoded gene (*PPX2L*) coding for the PPO enzyme, which is dual-targeted to the mitochondria and the chloroplasts (Patzoldt et al. 2006). This results in the loss of a glycine residue at the 210th amino acid position, deemed the ΔG210 mutation (Patzoldt et al. 2006). Loss of the glycine residue alters the architecture of the substrate binding domain, which does not result in a loss of binding

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affinity of the substrate protoporphyrinogen IX; rather, the glycine loss reduces the catalytic efficiency of PPO (Dayan et al. 2010). Consequently, competitive inhibition of the binding pocket via the herbicide is reduced, resulting in resistance to PPO-inhibiting herbicides (Dayan et al. 2010). In 2011, Thinglum et al. found that the ΔG210 mutation was responsible for PPO-R waterhemp biotypes in Illinois, Kansas, and Missouri, leading to the conclusion that the ΔG210 mutation was likely the only known mechanism of resistance to PPO inhibitors in waterhemp. In field conditions, soil-residual applications of PPO-inhibiting herbicides remain efficacious on waterhemp resistant to foliar-applied PPO-inhibiting herbicides, with greater than 90% residual control up to 14 d after treatment (DAT), depending on the active ingredient applied (Harder et al. 2012; Shoup et al. 2003). Therefore, PPO-inhibiting herbicides in soil-solution may have the potential to overcome evolved herbicide-resistance mechanisms very early in the plant life cycle, before emergence (i.e., radicle protrusion, radicle elongation). Some studies have indicated that differences in PPO inhibitor efficacy between herbicide-resistant and herbicide-susceptible biotypes are dependent on the size of the target plant at application (Falk et al. 2006), whereas others have reported growth stage as having a minimal effect (Koger et al. 2004). Differential response to herbicides among herbicide-resistant and herbicide-susceptible biotypes at various growth stages has led to speculation that the herbicide-resistant mechanism is linked to physiological or morphological changes during the developmental process. For example, germination differences of two soybean cultivars, with high and low sensitivity to sulfentrazone, were reportedly caused by differential absorption of the herbicide as early as imbibition and seedling emergence (Li et al. 2000).

A greenhouse experiment conducted by Falk et al. (2006) reported R : S ratios of 6.3×, 2.5×, and 2.6× following soil-residual applications of the diphenylether herbicides acifluorfen, fomesafen, and lactofen, respectively. Significant emergence of the resistant (R) biotype, ranging from 15 to 62% relative emergence depending on the herbicide, was recorded at 0.25× the field use rates, whereas < 2% of the susceptible (S) biotypes emerged at the same rate. Meanwhile, the labeled 1× rates provided considerable control of both the R biotype and the S biotype. Although Falk et al. (2006) provided compelling evidence that soil-residual PPO-inhibiting herbicides may select for PPO-R biotypes, it must be noted (as it is by Falk et al. 2006) that diphenylether herbicides typically are not applied for soil-residual control (Dayan and Duke 2010). PPO-inhibiting herbicides from the triazolinones (sulfentrazone) and *N*-phenyl-phthalimides (flumioxazin) are more commonly used in soil-residual applications and represent an essential component of herbicide-resistant weed management in soybean and cotton (Dayan and Duke 2010). For example, Harder et al. (2012) observed > 90% soil-residual control of an R biotype from Missouri using sulfentrazone and flumioxazin 28 d after application.

Given that PPO-inhibiting herbicides still provide adequate soil-residual control of R biotypes, PPO-inhibiting herbicides remain one of the most viable options for control of many herbicide-resistant *Amaranthus* spp. in soybean and cotton. With the increasing importance of soil-residual herbicides, it is critical to further elucidate the complexities surrounding the selection for herbicide-resistant biotypes following soil-residual herbicide applications. To address these complexities, research was conducted in four growth chamber and greenhouse experiments in 2011, 2012, and 2013 to investigate the efficacy of three PPO-inhibiting herbicides: fomesafen, sulfentrazone, and flumioxazin each from distinct chemical families (diphenylethers, triazolinones, and *N*-phenyl-phthalimides, respectively) for control of PPO-R and PPO-S waterhemp biotypes. The primary objectives of this research were to (1) assess the efficacy of three foliar-applied and soil-applied PPO-inhibiting herbicides on PPO-R and PPO-S waterhemp, and (2) investigate the differential effects of PPO-inhibiting herbicides on an R biotype and an S biotype soon after seed germination, in the absence of soil.

Materials and Methods

To investigate the two objectives, four experiments were conducted at the Horticultural Research Center greenhouses at Southern Illinois University. The four experiments consisted of two greenhouse experiments and two petri dish assays carried out in a growth chamber. Seeds from the R biotype were collected in 2011 from plants that survived 220 g ai ha^{-1} of commercially formulated, foliar-applied lactofen in a field near Carlyle, IL. The presence of the ΔG210 mutation of *PPX2L* was confirmed in the progeny of female waterhemp plants by Wuerffel et al. 2014 (unpublished data) using the detection method described by Lee et al.

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(2008). The target-site mutation is currently the only known mechanism of resistance to PPO inhibitors in waterhemp, leading to the conclusion that this is likely the case for most PPO-R waterhemp from the Carlyle population. Seeds from the S biotype were collected in 2002 from multiple female waterhemp plants with typical sensitivity to PPO-inhibiting herbicides. Both seed lots were kept at 4 C for long-term storage. Before initiating the experiment, all seeds were treated with a bleach solution (2% sodium hypochlorite) for 10 min for scarification and sterilization and were then rinsed for 3 min with double distilled (dd) H_2O to remove residual sodium hypochlorite.

Statistical Analysis of Rate-Response Experiments. The statistical program, SAS 9.2 (SAS Institute Inc., 100 SAS Campus Drive, Cary, NC 27513) was employed for all statistical analyses. The NLIN procedure was used to generate dose–response curves using a four- or five-parameter, nonlinear regression model (Schabenberger et al. 1999). From the nonlinear regression, GR_{50} (the dose at which 50% inhibition of the response variable is achieved) values were derived. To compare across the four experiments, the GR_{50} from the R biotype was divided by the GR_{50} from S biotype to produce an R : S ratio.

For all experiments, the range, the scale, and the center of the herbicide rate distributions were based on preliminary data for fomesafen only or from previously published research. The distributions of sulfentrazone and flumioxazin rates were based on the relative (fraction of the recommended labeled rate) fomesafen rate distribution; hence, nonlinear regression for sulfentrazone and flumioxazin is only applied where an adequate range of responses was generated. For cases in which nonlinear regression was not appropriate, mixed-model ANOVA analyses were performed using PROC MIXED, where replications and experimental runs were considered random effects and all other factors were considered fixed effects. Means were separated with Tukey-Kramer's honestly significant difference (HSD) in conjunction with the PDMIXED macro, which slices designated factors into comparable groups and provides means separation (Davis et al. 2010).

POST Greenhouse Experiment. Methods and rate structure were based on previous research investigating POST applications of PPO-inhibiting herbicides on PPO-R and PPO-S waterhemp biotypes under greenhouse conditions (Patzoldt et al. 2005). Waterhemp from the R and S biotypes were planted at 0.2 and 0.1 g pot^{-1}, respectively, in a growing media (Fafard Growing Mix 2, Conrad Fafard Inc. P.O. Box 790, Agawam, MA 01001) and thinned to 1 plant per 10-cm by 10-cm pot. Waterhemp was fertilized with a 20–20–20 (N–P–K) product (JR Peters, Inc., 6656 Grant Way, Allentown, PA 18106) solution once per week after reaching 7 to 8 cm tall. Plants were maintained in a 16 h photoperiod with 430-W sodium lighting providing 250 μmol m^{-2} s^{-1} of supplemental photosynthetically active radiation. Air temperature was maintained near 32 (± 5) C during the course of the experiment. Once plants reached 10 to 12 cm in height, herbicide applications were made using a single-nozzle, CO_2 spray chamber with an EVS8002 nozzle (TeeJet Technologies, P.O. Box 7900, Wheaton, IL 60187) set to deliver 187 L ha^{-1} of carrier at 207 kPA pressure. Before herbicide application, waterhemp plants were sorted into blocks based on minor height differences, then randomly assigned a treatment. Fomesafen (Reflex, Syngenta Crop Protection, LLC, P.O. Box 18300, Greensboro, NC 27419), sulfentrazone (Spartan, FMC Corporation, 1735 Market St., Philadelphia, PA 19103), and flumioxazin (Valor SX, Valent U.S.A. Corporation, 1600 Riviera Ave., Suite 200, Walnut Creek, CA 94596), were applied at rates ranging from 0.329 to 3,290 g ai ha^{-1} (labeled 1× rate = 329 g ai ha^{-1}), 0.21 to 2,100 g ai ha^{-1}, and 0.142 to 1,420 g ai ha^{-1}, respectively, with five rates evenly spaced along a log_{10} scale. Each treatment included 1% (v/v) crop oil concentrate (Prime Oil, Winfield Solutions, LLC, St. Paul, MN 55164), and nontreated plants were included for comparison. Herbicide efficacy was visually assessed at 3, 7, and 14 DAT. Aboveground biomass was harvested after the 14 DAT visual rating, dried in an oven for 72 h at 65 C, then weighed. The experimental design was a completely crossed three-way factorial (two populations by three herbicides by five rates) structured in a randomized complete block with eight replications per experiment. The experiment was conducted twice. Blocks were rearranged in the greenhouse at 7 DAT in an attempt to reduce variability from greenhouse conditions. Dry weight data were subjected to log-transformation before analysis; however, the untransformed data were used for means representation with the analysis from the transformed data.

Soil-Residual Greenhouse Experiment. Experimental methods and herbicide rate structure were

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based on previous research (Falk et al. 2006) involving soil-residual applications of PPO-inhibiting herbicides to greenhouse pots containing PPO-R waterhemp. Two-hundred waterhemp seeds from the R and S biotypes were sown in separate 10-cm by 10-cm pots filled with a top layer of 2.5 cm containing sandy-loam field soil (pH 6.3; 2.5% organic material) and a bottom layer containing 7.5 cm of growing media. Seeds were placed randomly in the pot; however, special consideration was taken to avoid uneven distribution of seed and seed placement within 2 cm of the pot edge. Seeds were covered with 1 to 2 mm of dry field soil and then lightly misted to avoid disturbing seed placement. Damping-off of waterhemp seedlings, caused by *Rhizoctonia*, *Pythium*, *Phytophthora*, and *Fusarium* spp., can cause significant waterhemp seedling mortality, even in steam-sterilized soil. To avoid significant seedling mortality from pathogens, pots were treated using a 50-ml drench with two fungicides that provide control of the aforementioned fungi and oomycetes: 5.63 mg of etridiazole and 9.38 mg of thiophanate-methyl (Banrot, Scotts, 14111 Scottslawn Rd., Marysville, OH 43041). The following day, pots were sprayed using the previously described herbicide-application methods. Each population was sprayed with a staggered-rate structure encompassing seven rates, evenly spaced on a $\log_{3.16}$ scale between 1.32 to 13,200 g ha^{-1}, 0.89 to 8,900 g ha^{-1}, 0.34 to 3,400 g ha^{-1}, 1.32 to 13,200 g ha^{-1} + 6.05 to 60,500 g ha^{-1} for fomesafen, sulfentrazone, flumioxazin, and fomesafen + *s*-metolachlor (Dual Magnum, Syngenta Crop Protection), respectively. Approximately 0.6 to 0.8 cm of overhead water was gently applied following herbicide application to leach the herbicide into the soil profile, then moisture was maintained with subirrigation for the remainder of the experiment (Harder et al. 2012). Greenhouse conditions were maintained under the same conditions listed previously for the POST experiment. Seedling emergence was enumerated every other day for 10 d and is expressed as percentage of emergence of the nontreated control, from which the GR_{50} and R : S ratios were calculated. Waterhemp seedlings were considered fully emerged when the cotyledons emerged from the soil and the adaxial leaf surfaces of the cotyledons were no longer touching each other. The experiment was designed as a completely crossed three-way factorial (two populations by four herbicides by seven rates) with treatments organized in a randomized complete block, with two runs separated temporally and six replications per run, spatially blocked. Emergence data were subjected to log-transformation before analysis; however, the untransformed data were used for means representation with the analysis from the transformed data.

Radicle Elongation Growth Chamber Experiment. Waterhemp seeds from the R and S biotypes were sown in 100-mm petri dishes (Fisher Scientific, 300 Industry Drive, Pittsburg, PA 15275), lined with one sheet of filter paper (Whatman No. 2, GE Healthcare Life Sciences, 800 Centennial Ave., Piscataway, NJ 08854), and presoaked with 5 ml of ddH_2O. Approximately 7 to 8 g total of seed for each population was planted in dishes at 0.5 g $dish^{-1}$ (approximately 2,500 seeds), sealed with Parafilm (Fisher Scientific), and placed in the growth chamber before herbicide treatment. Growth chamber conditions were set to optimize waterhemp germination, with 14/10 h, 30/20 C day/night temperature conditions (Zelaya and Owen 2005), supplemented by 130 to 150 µmol m^{-2} s^{-1} of inflorescent light (Leon et al. 2004). Once most waterhemp from both populations germinated, 20 waterhemp seedlings from each population, with radicles no longer than 1 mm, were removed from the 100-mm dish and transplanted to 50-mm petri dishes (separate petri dish for each population) lined with blue blotter paper (Anchor Paper Co., 480 Broadway St., St. Paul, MN 55101), then treated with 2.5 ml of herbicide solution. Pregerminated seedlings were chosen to evaluate radicle elongation for several reasons: (1) waterhemp germination is highly dependent on the growing conditions of the female parent and environmental conditions before germination; therefore, germination and emergence patterns can be highly variable among populations; (2) the R and S biotypes had low initial germination, but both would germinate for extended periods; (3) preliminary research suggested that selection for resistance did not take place before germination, which would be biologically compatible with the mode of action for PPO-inhibiting herbicides, considering light is required for activity. Given the aforementioned difficulties, pregerminated seedlings were used to measure radicle elongation, and a separate experiment was conducted to measure radicle protrusion (i.e., visible germination). Several researchers have also used pregerminated seedlings when encountering similar struggles when testing the efficacy of various herbicides (Beckie et al. 1990; Zelaya and Owen 2005).

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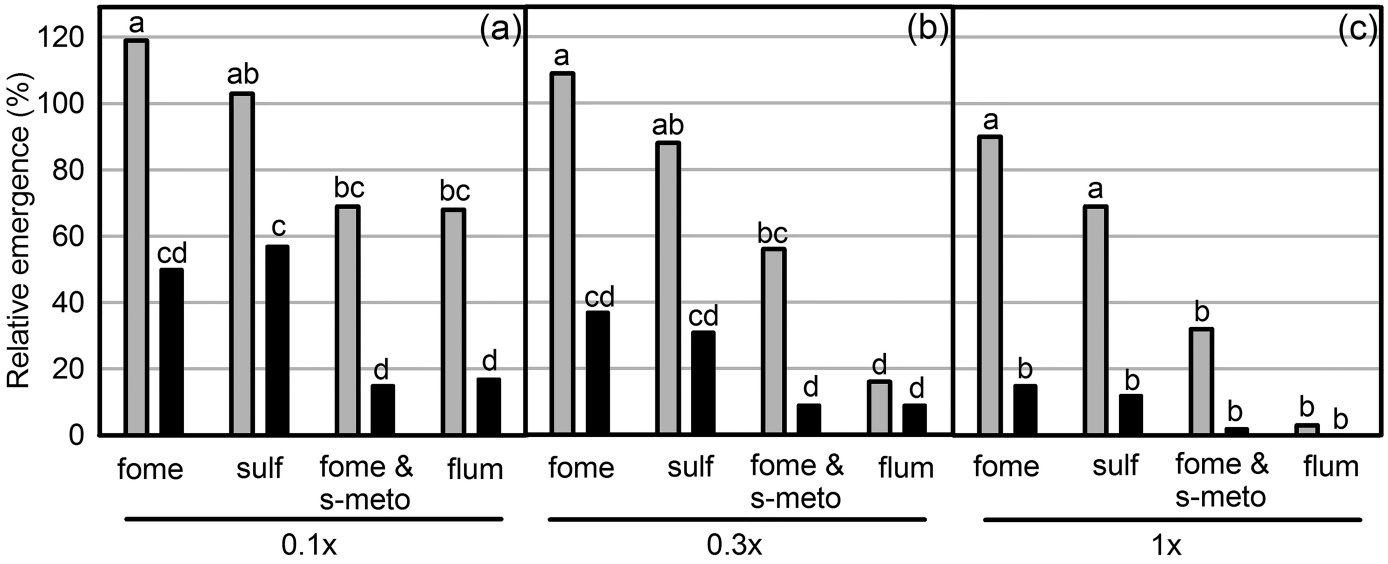

Figure 1. Relative cumulative waterhemp emergence of protoporphyrinogen oxidase inhibitor-resistant (PPO-R) (grey) and susceptible (PPO-S) (black) biotypes 10 d after treatment from four soil-residual herbicides applied at (a) 0.1×, (b) 0.3×, and (c) 1× labeled rates. The 1× labeled rates were as follows: fomesafen at 420 g ai ha^{-1}, sulfentrazone at 280 g ai ha^{-1}, flumioxazin at 107 g ai ha^{-1}, and fomesafen at 420 g ai ha^{-1} + *s*-metolachlor at 1,910 g ai ha^{-1}. Means were separated using Tukey's honestly significant difference ($\alpha = 0.05$). Abbreviations: fome, fomesafen; sulf, sulfentrazone; s-meto, *s*-metolachlor; and flum, flumioxazin.

Herbicide treatments consisted of five rates per herbicide, ranging from 0.005 mM through 50 mM on a $\log_{10}$ scale with a nontreated control for comparison. The experiment was designed as completely crossed three-way factorial (two populations by three herbicides by six rates) organized in a randomized complete block with three spatial replications, and the experiment was conducted twice. Pictures of each dish were taken at application to establish a baseline radicle length and, subsequently, every 24 h following application for 72 h. ImageJ software (ImageJ, U.S. National Institutes of Health, Bethesda, MD 20892) was used to measure individual radicle lengths in each picture at each of the four evaluation timings. All data were transformed by subtracting the average baseline radicle length of the control dish from the individual radical length with the treatment, then dividing the quotient by the average growth in the nontreated control petri dish.

Germination Growth Chamber Experiment. Experimental methods for the germination experiment directly paralleled the latter growth chamber experiment with minor variations; therefore, methods are identical unless explicitly stated. Before germination, 100 seeds from the R and S biotypes were sown directly into separate 50-mm petri dishes containing blue blotter paper and 2.5 ml of the herbicide solution. Germinated seeds were enumerated every other day for 10 d. A *germinated seed* was defined as a seed whose radicle had protruded from the seed coat and endosperm, with the radicle being at least 1 mm in length, which was deemed *visible germination* (Rashid et al. 2010; Wolf et al. 1984). Each treatment had three spatial replications within the growth chamber, and the experiment was conducted twice. Data for raw cumulative germination 10 DAT were subjected to log-transformation before normalizing the two populations relative to their nontreated controls. Data were also expressed with the following germination indices, relative to nontreated controls, with respect to waterhemp biotype: speed of germination, accumulated speed of germination, and the coefficient rate of germination, as described by Anjum and Bajwa (2005).

Results and Discussion

POST Greenhouse Experiment. Before conducting soil-residual experiments, fomesafen, sulfentrazone, and flumioxazin were foliar-applied to the R and S biotypes to establish R : S ratios for each herbicide. Following herbicide applications, rapid necrosis of leaf tissue developed with 24 to 48 h after application in both the R and S biotypes; however, necrosis was slower to develop and less severe in the R biotype. Plants that were not fully controlled recovered quickly, accumulating significant regrowth within 7 DAT. Foliar applications of fomesafen, sulfentrazone, and flumioxazin resulted

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Table 1. List of resistant to susceptible (R : S) ratios generated from multiple studies investigating the efficacy of foliar-applied PPO-inhibiting herbicides on protoporphyrinogen oxidase inhibitor-resistant (PPO-R) waterhemp biotypes.

Publication	Chemical family	Herbicide ai	GR_{50}[a]		R : S ratio	Evaluation type[b]
			R biotype	S biotype		
			g ai ha^{-1}			DAT
Present study[c,d]	Diphenylether	Fomesafen	621.0	16.4	38×	Relative dry wt; 14
	Triazolinone	Sulfentrazone	148.2	46.1	3.2×	
	N-phenyl-phthalimide	Flumioxazin	176.2	6.1	29×	
Shoup et al. (2003)[e]	Diphenylether	Acifluorfen	302.4	8.8	35×	Visual herbicide
		Fomesafen	165.2	19.8	8.3×	efficacy rating; 14
		Lactofen	106.9	1.3	82×	
	Triazolinone	Sulfentrazone	106.1	26.6	4×	
Patzoldt et al. (2005)	Diphenylether	Acifluorfen	6.6	3.0	2.2×	Relative dry wt; 15
		Fomesafen	8.0	1.3	6.2×	
		Lactofen	21.0	0.9	23×	
	N-phenyl-phthalimide	Flumiclorac	9.0	3.1	2.9×	
		Flumioxazin	1.9	0.4	4.8×	
	Triazolinone	Sulfentrazone	13.0	2.5	5.2×	
Patzoldt et al. (2006)[a]	Diphenylether	Lactofen	12.5–21	0.4	31–53	Relative dry wt; 14

[a] Abbreviations: GR_{50}, the dose at which 50% inhibition of the response variable is achieved; R : S ratio, ratio of GR_{50}-R to GR_{50}-S biotypes; R biotype, resistant biotype; S biotype, susceptible-biotype.

[b] Relative dry weights from all studies consisted of aboveground biomass only.

[c] Herbicide rate structure, crop oil concentrate rate, and application height paralleled methodology used by Patzoldt et al. (2005).

[d] All herbicides within the present study fit the four-parameter log-logistic model described by Schabenberger et al. (1999).

[e] Shoup et al. (2003) used 0.5% (v/v) crop oil concentrate, applied herbicides at 1/8× to 8× the labeled rates on a $\log_2$ scale, and made the herbicide application when waterhemp was 13 to 18 cm in height.

in R : S ratios of 38×, 3.2×, and 29×, respectively, which were greater than many previously reported R : S ratios for these herbicides, with the exception of sulfentrazone (Table 1). For example, Patzoldt et al. (2005) reported R : S ratios of 6.2×, 5.2×, and 4.8× for the latter three herbicides, respectively. Research by Shoup et al. (2003) found R : S ratios of 8.3× and 4× for fomesafen and sulfentrazone. Although R : S ratios are greater in the present experiment, R : S ratios of a similar magnitude as those derived from the data presented have been obtained from other PPO-inhibiting herbicides. Shoup et al. (2003) also reported R : S ratios of 34× and 82× following foliar applications of the diphenylether herbicides acifluorfen and lactofen, respectively (Table 1). As mentioned by Shoup et al. (2003), differences in R-biotype sensitivity may vary within a chemical family based on the herbicide that is applied most frequently. These differences could be due to one of several possibilities: (1) the R biotype has reduced sensitivity to higher doses when compared with other R biotypes, (2) the S biotype has greater sensitivity to lower doses when compared with other S biotypes, or (3) the latter two possibilities are not mutually exclusive and both are true to some extent. Average GR_{50} values derived from applications of fomesafen, sulfentrazone, and flumioxazin in the Patzoldt et al. (2005) experiment were 1.4 and 7.6 g ha^{-1} for S and R biotypes, respectively. In the present experiment, the average GR_{50} values were much higher: 22.8 g ha^{-1} for the S biotype and 315 g ha^{-1} for R biotype. Therefore, for the R and S biotypes, there was an average of a 16× and 41× increase, respectively, when comparing the two studies. The reduced sensitivity observed in both R and S biotypes from this experiment indicates that these populations could be inherently less sensitive to PPO-inhibiting herbicides, indicating the possible presence of an additional low-level resistance mechanism, especially because the sensitivity of the R biotype was reduced to a greater extent. Another possibility is that these differences could be an effect of unknown experimental dissimilarities among the studies (i.e., light intensity before or after application).

Soil-Residual Greenhouse Experiment. The soil-residual greenhouse experiment was performed to investigate the residual activity of three soil-residual PPO-inhibiting herbicides (fomesafen, sulfentrazone, and flumioxazin) by measuring their soil activity on the R and S biotype at 10 DAT. Fomesafen + *s*-metolachlor was also included because fomesafen is rarely applied alone for effective soil-residual control of *Amaranthus* spp. in soybean. Injury symptoms of emerged water-

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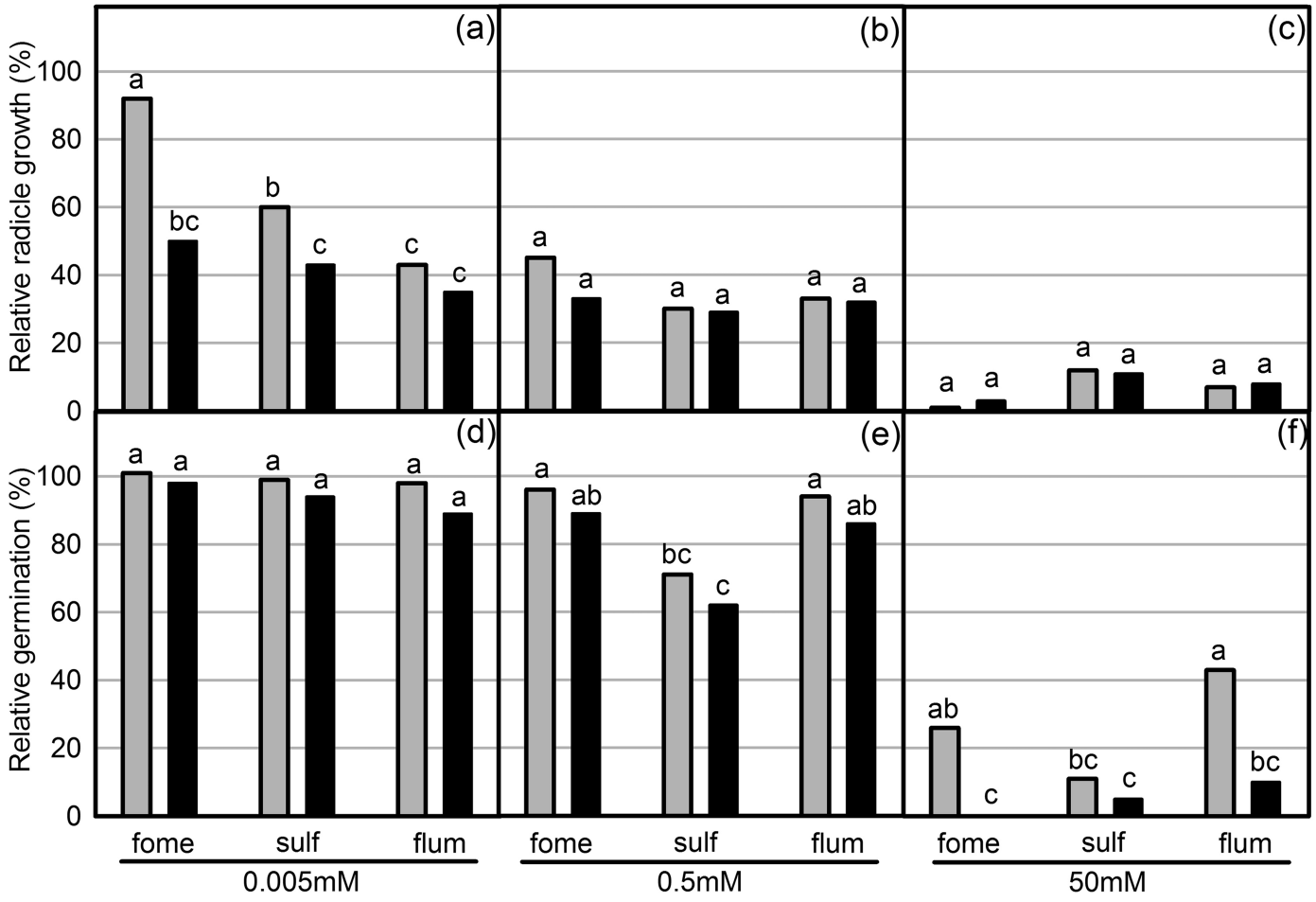

Figure 2. (a–c) Relative radicle elongation and (d–f) relative cumulative waterhemp germination of protoporphyrinogen oxidase inhibitor-resistant (PPO-R) (grey) and susceptible (PPO-S) (black) biotypes 48 h after treatment (HAT) or 10 d after treatment (DAT), respectively, with three soil-residual herbicides applied from 0.005 to 50 mM on a $\log_{10}$ scale (data for 0.05 and 5 mM not shown). Means are separated using Tukey's honestly significant difference ($\alpha = 0.05$). Abbreviations: fome, fomesafen; sulf, sulfentrazone; flum, flumioxazin.

hemp seedlings were similar to those characterized by Falk et al. (2006). Significant differences in efficacy among the four herbicides for the S biotype, were only identifiable at 0.1× rates, with > 50% relative waterhemp emergence for fomesafen and sulfentrazone, and < 20% for fomesafen + *s*-metolachlor and flumioxazin (Figure 1). On the other hand, evaluation of the R biotype resulted in significant differences among herbicides at all three rates, including the labeled 1× rate (Figure 1). At the labeled 1× rate, relative emergence of the R biotype was > 60% for fomesafen and sulfentrazone, whereas emergence was < 40% for fomesafen + *s*-metolachlor and flumioxazin. All herbicides conferred < 20% relative emergence of the S biotype at the labeled 1× rate (Figure 1).

Significant differences between the R and S biotypes were only detectable at the 0.1× and 0.3× rates for fomesafen + *s*-metolachlor, and only at the 0.1× rate for flumioxazin (Figure 1). Significant differences between the R and S biotypes were detectable at the 0.1×, 0.3×, and 1× rates for fomesafen and sulfentrazone (Figure 1). Overall, herbicide efficacy on the emergence of PPO-R waterhemp was ranked flumioxazin > fomesafen + *s*-metolachlor > sulfentrazone = fomesafen. Surprisingly, Falk et al. (2006) came to the opposite conclusion, where fomesafen and lactofen vastly outperformed flumioxazin. However, the 1× labeled rate used by Falk et al. (2006) (1,015 g ha^{-1}) was nearly 2.5× greater than the 1× rate labeled for soil-residual applications in southern Illinois (420 g ha^{-1}), which undoubtedly contributed to the surprisingly long-lasting soil-residual activity of fomesafen. Indeed, the 1× labeled herbicide rate could vary depending on regional labeling restrictions; conversely, regional variations in 1× labeled rates are unlikely to change by an order of magnitude and are minor when compared with the range of rates examined in this experiment.

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Table 2. Germination indices of protoporphyrinogen oxidase inhibitor-resistant (PPO-R) and susceptible (PPO-S) biotypes from the soil-less growth-chamber experiments.[a,b]

Herbicide concentration[c]	Relative germination indices, 10 DAT[d]							
	Total germination		Speed of germination		Speed of accumulated germination		Coefficient of rate of germination	
	R	S	R	S	R	S	R	S
mM	%							
Fomesafen								
0	100 a	100 a	100 a	100 a	100 ab	100 ab	100 a	100 a
5	92 a	68 b	81 a	38 bc	82 ab	35 cd	103 a	103 a
50	26 c	0 d	3 c	0 c	5 d	0 d	81 a	0 b
Sulfentrazone								
0	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100 a
5	6 d	0 d	1 c	0 c	1 d	0 d	20 b	0 b
50	11 d	5 d	2 c	0 c	2 d	0 d	31 b	23 b
Flumioxazin								
0	100 ab	100 ab	100 ab	100 ab	100 ab	100 ab	100 a	100 a
5	104 a	91 ab	131 a	73 bc	135 a	73 bc	103 a	101 a
50	43 c	10 d	10 d	1 d	10 d	1 d	79 ab	40 b

[a] Abbreviations: DAT, days after treatment; R, resistant biotype; S, susceptible biotype.
[b] Means are separated using Tukey's honestly significant difference ($\alpha = 0.05$) and are compared within herbicide and germination index, across herbicide rate and waterhemp biotype.
[c] Relative germination indices were expressed relative to nontreated controls for waterhemp biotype. Indices were calculated from equations listed by Anjum and Bajwa (2005).
[d] Data for concentration < 5 mM are not shown because of a lack of statistical significance among treatments.

Germination and Radicle Elongation Growth Chamber Experiments. PPO-inhibiting herbicides are photodynamic herbicides that only produce phytotoxic effects in the light (Duke et al. 1991); therefore, we hypothesized that the selection for PPO-R biotypes likely occurs after radicle protrusion, given that light is highly attenuated but not completely blocked by the soil and the seed coat (Fenner 2000; Tester and Morris 1987). A PPO-inhibiting herbicide at a high enough concentration could seemingly cause other secondary effects on the seed that may disrupt visible germination (i.e., disruption of hormone balance or sensitivity, osmotic regulation, or protease and amylase activity) (Goggin and Powles 2014; Guillaumot et al. 2009; Kucera et al. 2005). Moreover, if these secondary effects were independent of PPO-enzyme inhibition and chlorophyll/heme biosynthesis, the presence/absence of the ΔG210 mutation in the germinating seedling would have a minimal effect on the protrusion of the radicle.

Interestingly, there was a differential response in relative cumulative germination 10 DAT between R and S biotypes, indicating the negative response to the herbicide may be due, in part, to inhibition of the PPO-enzyme within the growing embryo (Figure 2). A number of studies have found correlations between porphyrin accumulation and germination in several species, lending credibility to the idea that PPO-inhibiting herbicides may affect the growing embryo (Guillaumot et al. 2009; Korkmaz and Korkmaz 2009; Wang et al. 2005). However, this differential response was not observed until the concentration reached 5 mM; at which point, only fomesafen resulted in a 25% difference (data not shown) between R and S biotypes. A 33% difference among biotypes was observed for flumioxazin at 50 mM (Figure 2f). Differences among herbicides were observed at the higher concentrations as well (Figures 2e and 2f). Several germination indices were calculated to determine whether these herbicides could adversely or differentially affect the speed and rate of germination of R and S biotypes, but results were very similar to the total germination (Table 2). The observation that the presence/absence of the ΔG210 mutation may have effects on radicle protrusion reinforces the idea that PPO-inhibiting herbicides and the subsequent accumulation of porphyrins may affect very early stages of seed germination. It is conceivable that rupture of the seed coat (testa) may not have been affected; however, radicle protrusion was differentially affected. Given that testa rupture was not recorded, further experiments recording testa rup-

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Table 3. List of resistant to susceptible (R : S) ratios generated following the application of fomesafen to protoporphyrinogen oxidase inhibitor-resistant (PPO-R) and susceptible (PPO-S) waterhemp biotypes at several application timings.[a]

Application timing	Evaluation timing	Evaluation type	GR_{50}[b]		R : S ratio
			R biotype	S biotype	
			g ai ha^{-1}		
POST[c]	14 DAT	Dry weight	621 (± 160)	16.4 (± 4.1)	38
Seedling emergence[d]	10 DAT	Cumulative emergence[e]	1,385 (± 181)	42.4 (± 6.12)	33
Radicle elongation[c]	72 HAT	Radicle growth	0.19 (± 0.08)	0.01 (± 0.007)	17
	48 HAT		0.33 (± 0.133)	0.02 (± 0.013)	15
	24 HAT		1.15 (± 0.37)	0.15 (± 0.06)	7.7
Germination[c]	10 DAT	Cumulative germination[f]	26.2 (± 3.2)	7.62 (± 0.73)	3.4

[a] Abbreviations: GR_{50}, the dose at which 50% inhibition of the response variable is achieved; R : S ratio, ratio of GR_{50}-R to GR_{50}-S biotypes; R biotype, resistant biotype; S biotype, susceptible-biotype; DAT, days after treatment; HAT, hours after treatment.
[b] The (±) represents the standard error in g ai ha^{-1}.
[c] Modeled using a four-parameter model described by Schabenberger et al. (1999).
[d] Modeled using a five-parameter model described by Schabenberger et al. (1999).
[e] Waterhemp was considered emerged upon full cotyledonary expansion.
[f] Waterhemp was considered germinated when the radicle exceeded 1 mm in length.

ture and radicle protrusion may better elucidate the observed effect.

Radicle elongation was more sensitive to PPO-inhibiting herbicides when compared with germination, as expected. Radicle growth of the S biotype was reduced to < 50% for all herbicides, even at the lowest concentration (0.005 mM), whereas overall germination and the speed of germination were unaffected at this concentration (Figures 2a and 2d). Radicle growth of the R biotype responded differently to fomesafen, sulfentrazone, and flumioxazin at the 0.005 mM concentration, resulting in radicle growth that was 92, 60, and 43% of the nontreated control, respectively (Figure 2a). Furthermore, radicle growth of the R biotype was 88 and 40% greater than the S biotype at 0.005 mM concentration for fomesafen and sulfentrazone, respectively (Figure 2a). Overall, the high sensitivity of radicle elongation overshadows the low sensitivity of the germination considering that concentrations that select for germination of the R biotype simultaneously provide considerable reductions in radicle elongation. Additionally, herbicide concentrations likely do not reach the 5 to 50 mM level in the soil considering that those rates are near the concentrations of the herbicide often mixed in the spray tank before spraying; therefore, these data suggest that the effect of PPO-inhibiting herbicides on radicle protrusion is likely negligible, especially in selecting for resistance. Therefore, selection for resistance from soil-residual herbicides seemingly occurs from damage incurred sometime after radicle protrusion and before, or shortly after, emergence.

Summary of Fomesafen Dose–Response Curves. Fomesafen data from all four experiments were subjected to nonlinear regression. Some studies have found that differences among R and S biotypes were positively correlated with plant size, i.e., earlier herbicide applications result in a lower R : S ratio because of reduced sensitivity of the R biotype at earlier growth stages. The R : S ratios ranged from 38× to 3.4× when comparing the effect of fomesafen aboveground biomass reduction of a 10- to 12-cm plant and radicle protrusion of the seed coat, respectively (Table 3). Even within the radicle elongation experiment, the response of R and S biotypes differentiated further with time, with observed R : S ratios of 7.7×, 15×, and 17× at 24, 48, and 72 h after treatment, respectively (Table 3). Analogous soil-residual greenhouse experiments conducted by Falk et al. (2006) resulted in an R : S ratio of 2.5× following the soil-residual application of fomesafen, indicating once again that the results obtained from the R biotype used in this experiment are further separated in their response to PPO-inhibiting herbicides when compared with the S biotype.

Overall, this research supports the idea that the response of R biotypes to PPO-inhibiting herbicides is highly dependent on the growth stage of the target plant, the herbicide active ingredient applied, and the individual response of each R biotype. The R biotype used in this study was least sensitive to fomesafen, followed by sulfentrazone, followed by flumioxazin. Other research groups have used R biotypes with greater sensitivity to fomesafen. However, they also reported reduced sensitivity of R biotypes to other

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diphenylethers, such as lactofen and acifluorfen, while simultaneously reporting greater sensitivity to sulfentrazone and flumioxazin (Falk et al. 2006; Patzoldt et al. 2005). Therefore, when considering the sensitivity of all reported PPO-R waterhemp biotypes, the diphenylether herbicides may have a propensity for reduced sensitivity, resulting in higher R : S ratios, whereas sulfentrazone and flumioxazin have consistently resulted in lower R : S ratios, especially when soil-applied. Shoup et al. (2003) reasoned that a PPO-R biotype may have reduced sensitivity to the chemical family most frequently applied before selection for the R biotype. The finding that diphenylether herbicides have reduced sensitivity in multiple PPO-R waterhemp biotypes suggests that herbicides within this family may have been involved in the selection for resistance to PPO inhibitors. This idea is further substantiated when considering that diphenylether herbicides are primarily used for POST control of *Amaranthus* species and have historically been applied POST to a greater number of soybean and cotton hectares than other chemical families classified as PPO-inhibiting herbicides. We found that when fomesafen was combined with a long-chain fatty acid inhibitor, *s*-metolachlor, soil-residual herbicide efficacy on the R biotype was equal to that of flumioxazin and greater than that of sulfentrazone alone under the greenhouse conditions in this experiment. Additional experimentation should be conducted to better understand whether the observed selection for resistance has implications in field conditions, and if so, to what extent.

Based on the current state of knowledge regarding the sensitivity of PPO-R waterhemp, the best recommendation for mitigating the selection for PPO-R biotypes would be to apply flumioxazin, sulfentrazone, or a diphenylether in combination with another effective herbicide site of action for soil-residual control of *Amaranthus* species. Furthermore, given that these data suggest selection for PPO-R waterhemp primarily takes place during radicle elongation, it may be beneficial to include soil-residual herbicides with significant activity before the latter developmental events in an effort to reduce the selection for PPO-R during radicle elongation. Unfortunately, there is limited information regarding the activity of herbicides before radicle protrusion; therefore, additional research must follow before this recommendation is legitimized.

Because this research indicates that resistant individuals have the potential to emerge before susceptible individuals as herbicide concentrations dissipate in the soil, POST weed management decisions must be based on the first emerging weeds, not the field average following a soil-residual herbicide application. Soil-applied PPO-inhibiting herbicides are essential tools for management of herbicide-resistant *Amaranthus* spp. in soybean and cotton production; therefore, soybean and cotton growers are likely to continue to rely heavily on herbicides within this site of action, especially for soil-residual applications. The common practice of applying reduced rates of soil-residual herbicides (Blackshaw et al. 2006, Muyonga et al. 1996), especially PPO-inhibiting herbicides, needs to be discouraged because this would potentially increase the selection pressure for PPO-R waterhemp. Under reduced residual herbicide rates more individual seedlings would be exposed to sublethal concentrations of the herbicides sooner, possibly during the primary germination period of waterhemp. Full residual herbicide rates would theoretically extend the length of which lethal concentrations are maintained high enough during the primary emergence period of waterhemp for control of even PPO-R individuals. In conclusion, this research supports the best management practices of using effective herbicides from multiple sites of action and full herbicide use rates to prevent and/or delay the selection for novel instances of herbicide resistance, while slowing the spread of existing herbicide-resistant biotypes.

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