PHENOTYPIC AND BIOCHEMICAL CHARACTERIZATION OF ATRAZINE AND HPPD-RESISTANT WATERHEMP (*AMARANTHUS TUBERCULATUS*) POPULATIONS

BY

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THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Crop Sciences
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2017

Urbana, Illinois

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ABSTRACT

Waterhemp (*Amaranthus tuberculatus*) is a dioecious, summer annual, broadleaf species that is native to the Midwest. Two decades ago it was not considered a major agricultural pest, but with the adoption of glyphosate-only postemergence (POST) herbicide programs without soil residual activity combined with no-till practices, it has become one of the most important weeds in Illinois and across the Midwest in regards to corn and soybean production. Research has indicated that multiple herbicide resistances can be found within one waterhemp plant, with one population in Champaign Co., IL exhibiting resistance to five different herbicide sites of action. Concerns exist about how to control waterhemp, especially as more populations are found to be resistant to most herbicides farmers use today.

Chapter 1 of this thesis includes a literature review of waterhemp biology, the history and use of atrazine, a photosystem II inhibitor, and carotenoid biosynthesis inhibitors, as well as the synergistic activity that has been observed when these two classes of herbicides are combined in a tank mixture. Chapter 2 covers previous research indicating that two atrazine-resistant populations of waterhemp (*Amaranthus tuberculatus*) from Illinois (designated ACR and MCR) displayed enhanced rates of atrazine metabolism via glutathione conjugation. Elevated constitutive expression levels of a single phi-class *GST*, named *AtuGSTF2*, correlated with atrazine resistance in ACR and MCR populations. Using this information, a discriminatory rate of 14.4 kg/ha was determined, and a POST study was conducted in the greenhouse to phenotype a segregating F_2 population (derived from an MCR x WCS cross). Genotypes falling into three distinct categories (RR, Rr, or rr) were tentatively assigned based on varying phenotypic responses. Basal *AtuGSTF2* expression levels were quantified and compared via RT-qPCR. Results indicated that each atrazine-resistant line (RR and Rr) tested displayed high *AtuGSTF2*

expression levels, ranging from 200- to 1140-fold greater than the low baseline levels detected in atrazine-sensitive lines (rr). Sequence analysis of RT-PCR products revealed several putative allelic variants of the *AtuGSTF2* gene among F_2 lines and their parent populations. These results demonstrate that constitutive *AtuGSTF2* expression correlates strongly with phenotype and may therefore represent the predominant GST that confers atrazine resistance in ACR and MCR.

Chapter 3 provides research that was conducted to explore the options for control of two HPPD- and atrazine-resistant waterhemp populations, SIR and NEB. The first objective was to determine the level of resistance to two HPPD-inhibiting herbicides; one that both populations had been exposed to previously (mesotrione) and another that had not been applied to either population (isoxaflutole). A dose-response experiment was conducted in the greenhouse at two different postemergence (POST) timings. Overall our findings did not indicate a consistent pattern in fold-resistance levels to isoxaflutole in taller plants. However, mesotrione applied POST to SIR showed a clear decrease in fold-resistance levels relative to EPOST. To further investigate potential management strategies of HPPD- and atrazine-resistant waterhemp populations in the field, our second objective was to conduct a POST herbicide interaction study and evaluate combinations of metribuzin and either isoxaflutole or mesotrione. This objective was designed to test two interconnected hypotheses: (1) HPPD-inhibitor activity contributes to synergism in a tank mix with metribuzin, and (2) metabolic atrazine resistance can be overcome by using a different PSII inhibitor (metribuzin). Results indicated that mesotrione at 52.5 g ai ha^{-1} combined with 191 g ai ha^{-1} of metribuzin displayed a synergistic effect on biomass reduction in SIR plants. However, all other combinations of either mesotrione or isoxaflutole and metribuzin resulted in an additive effect on biomass reduction in both the SIR and the NEB populations. These results give insight into how the joint activity between HPPD- and PSII-

inhibitors can be used to control metabolism-based, multiple herbicide-resistant waterhemp populations.

Chapter 4 summarizes the discussion and conclusions from Chapters 2 and 3 and identifies current limitations and future research goals for utilizing the herbicides we currently have available to control waterhemp, as herbicide resistance continues to evolve and no new modes of action are coming to market.

ACKNOWLEDGEMENTS

I would first like to thank my advisor, Dr. Dean Riechers, for offering me the opportunity to conduct research in his lab. Thank you for all of your advice, and for guiding me through my graduate school career. Also, thank you for giving me the opportunity to inspire young weed scientists, by allowing me to be your teaching assistant while I was here. I would also like to express my appreciation for the guidance of my committee members: Dr. Chance Riggins, Dr. Adam Davis, and Dr. Kris Lambert. I would also like to thank Syngenta Corporation for providing funding for this research, and for gifting us waterhemp seed to use in this research.

It was a pleasure and honor to work alongside the other members of the Riechers' Lab and Weeds Group including Loren Goodrich, Dr. Rong Ma, Seth Strom, Lanae Ringler, Dr. Yousoon Baek, Cody Evans, Olivia Obenland, and Dr. Anatoli Lygin. Thank you all for providing countless hours of help, love and support throughout my graduate school career. I would not have been able to make it through graduate school without all of our jokes, Triptych afternoons, laughing about Buzzfeed articles, Starbucks dates, Pandamonium mornings, and scientific rantings. I will never forget my family in N-335 and all of the time we spent together, complaining about R software and helping each other through the hard times. I will always be here for each and every one of you.

I would like to thank my family for never giving up on me. Every time I would call and say, "I can't do this anymore," you would always tell me, "you're smarter than you think; you've made it this far, and we know you can do anything you set your mind to." Your ability to turn tears into laughter will never be lost on me. I'm so thankful that I have a family as supportive as you, who is always willing to drop everything and come and help if need be. I know you'll

always be here in Illinois if I need you, and I promise to come back as often as I can. I love you all more than you know.

I would like to express my gratitude towards the whole Herbicide Evaluation team: Lisa Gonzini, Charlie Mitsdarfer, as well as Dr. Aaron Hager. Thank you all for sharing your weed and field expertise, and for adopting me the summer of 2017. I couldn't have finished graduate school without your willingness to pick me up and give me a place to work during a rough time.

Last, but definitely not least, I would like to acknowledge my boyfriend, Anthony. Thank you for walking into my life when I needed it most – only 1 month after I started this journey called graduate school. Thank you for listening to me go on about herbicides, my plants, my data, and everything in-between for hours on end. Thank you for all of the sushi dates, for adopting Chewie and Zelda with me, and for always being there for me. I don't know what I did to deserve such an amazing person in my life, but I'm sure glad you chose me to go through life with you, as well as your willingness to follow me as I continue my career as a Weed Scientist. "Until the sun rises in the west, and sets in the east. Until the rivers run dry and the mountains blow in the wind like leaves … my sun and stars."

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CHAPTER 1

LITERATURE REVIEW

1.1 Waterhemp (*Amaranthus tuberculatus*) Biology and Management

There are over seventy-five different species in the genus *Amaranthus* found worldwide, including both monoecious and dioecious species (Steckel, 2007). One of the ten dioecious species within this genus is called waterhemp (*Amaranthus tuberculatus* (Moq.) Sauer var. *rudis* (Sauer) Costea & Tardif, or syn. *A. rudis* Sauer) (Costea et al., 2005; Pratt and Clark, 2001). Waterhemp is identified by its glabrous stem, which can reach over 2 meters in height, with lanceolate leaves (Sauer, 1955; Steckel, 2007). Palmer amaranth (*Amaranthus palmeri*, S. Wats AMAPA) a close relative of waterhemp, is often confused with *A. tuberculatus*, but the most characteristic way to differentiate these two *Amaranthus* species is to compare the length of the petiole to the leaf. Waterhemp's petioles are typically shorter than the leaves, while Palmer amaranth's petioles are much longer (Steckel, 2007). Another differentiation is in their flowering structures, where waterhemp's structure ranges from 3–35 cm with branches, while Palmer amaranth's can be as long as 60 cm with no branches and sharp bracts (Steckel, 2007).

Waterhemp has become a major problem in the Midwest and South due to its summer annual life cycle and prolonged germination season that extends late into the summer, as well as its ability to compete with corn due to its C_4 photosynthetic physiology (Sauer, 1955). Due to its dioecious nature, waterhemp is an obligate outcrossing species, which has ensured that genes enabling herbicide resistance are rapidly spread throughout many different waterhemp populations (Trucco *et al.,* 2005; Steckel, 2007; Trucco *et al.,* 2007). Due to this outcrossing ability, many waterhemp populations are now resistant to a number of herbicides commonly used in maize (*Zea mays*) and soybean (*Glycine max*) production, which makes weed control very

difficult for farmers. Many of these resistance genes have been stacked within a single waterhemp population or an individual plant, resulting in multiple herbicide-resistant phenotypes (Heap, 2017). Waterhemp female plants possess the ability to produce up to 1 million seeds, and plants growing in a 68% shaded environment were still capable of producing up to 400,000 seeds (Steckel *et al.*, 2003). This presents an additional problem for farmers. If they are unable to control a waterhemp population because of a resistance issue and female plants produce seed, then the issue will be intensified because of the large amount of seeds that will be present in the soil seedbank in following years. Waterhemp seeds also have an innate ability to remain dormant and viable within that soil seed bank for several years (Buhler and Hartzler, 2001). The bottom line is that control of waterhemp is critical for farmers because waterhemp competition can reduce soybean and corn yields by as much as 56% and 74% respectively (Steckel *et al.*, 2007).

Unfortunately, waterhemp has become more prevalent in the Corn Belt region because of the increase in reduced- or no-till systems (Horowitz *et al.*, 2010) and the evolution of herbicide resistance. This creates an undisturbed environment within the field, thus allowing certain weeds to proliferate and go to seed. Waterhemp seeds are very small (1–1.5 mm) and tend to germinate when close to the soil surface (Steckel *et al.*, 2007), making this weed a huge problem for farmers over the past two decades (Hager *et al.*, 1997). Another problem that has contributed to resistance issues was the introduction of glyphosate-resistant crops in the mid-1990s (Bradshaw *et al.*, 1997). Glyphosate products control the market, with 90-95% of all soybean acres in Illinois being glyphosate resistant (Green, 2014). Glyphosate provides no residual activity, which provides no control for the prolonged germination period of waterhemp. Herbicide programs for farmers relied heavily on total postemergence (POST), one-pass programs because of the convenience that glyphosate provided, but this also selected for glyphosate-resistant weed

biotypes, such as waterhemp. Unfortunately, glyphosate resistance has been documented in numerous waterhemp populations (Heap, 2017), and even worse, this is not the only herbicide to which waterhemp has evolved resistance. There is also resistance to 4-hydroxyphenylpyruvate dioxygenase inhibitors, acetolactate synthase inhibitors, protoporphyrinogen oxidase inhibitors, 2,4-D, and photosystem II inhibitors in this species (Hausman *et al.*, 2011; Heap, 2017). Because of the increase of 3-, 4-, and 5-way stacks of herbicide resistance found within a single waterhemp plant, farmers are severely limited for options to control this troublesome weed.

1.2 History and Use of Atrazine

Atrazine [6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine], an *s*-triazine, is one of the most commonly used herbicides in maize in North America. In the United States alone, it is estimated that 32 million kg of active ingredient are applied annually (EPA, 2009). Atrazine is primarily used for dicot weed management in corn, sorghum, and sugarcane. Triazine herbicides contribute 85,000 American jobs and $4.8 billion to the U.S. economy due to increased crop yields and reduced input costs for farmers (Syngenta Corporation, 2015). Atrazine provides flexibility for farmers because it can be applied either preemergence (PRE) to the soil or POST, which provides residual control on sensitive dicot weeds up to 60 days depending on soil conditions (Krutz *et al.*, 2009). Not only is atrazine versatile in how it can be used, but it is also relatively inexpensive compared to other herbicides. In recent years, though, atrazine has been intensely scrutinized following the EPA's decision to evaluate the effect this herbicide has on human health, groundwater, and amphibians (EPA, 2009).

Atrazine is part of herbicide Group 5, which is one of the photosystem II (PSII) inhibiting herbicide families. Herbicides that inhibit the PSII mechanism competitively inhibit the binding

of plastoquinone (PQ) to the Q_b binding site of the D1 protein (Hess, 2000). In normal photosynthetic reactions, light energy is absorbed by the reaction center chlorophyll, P680. The excited electrons are then transferred to two PQ molecules at the Q_a and Q_b binding sites with the D2 and D1 proteins, respectively. When PSII herbicides block the Q_b binding site, chlorophyll is excited to a triplet state (Hess, 2000). Triplet chlorophyll, which is extremely unstable, then induces the rapid formation of singlet oxygen, which is also very unstable. Lipid peroxidation follows as a result of both triplet chlorophyll and singlet oxygen inducing lipid and hydroxyl radicals, and cell death quickly follows (Hess, 2000). This rapid cell death is recognized phenotypically as brown, necrotic tissue, beginning on the leaf margins in treated leaves, and eventually killing the entire plant. As mentioned before, atrazine is an *s*-triazine, but other chemicals that inhibit PSII belong to many other subclasses, including dinitrophenols, biscarbamates, phenyl ureas, nitriles, and triazinones or *as*-triazines (Duke, 1990).

Unfortunately, worldwide, there are 66 different species that are resistant to atrazine alone, including multiple *Amaranthus* species (Heap, 2017). This primarily resulted from several mutations in the *psbA* gene, which encodes the D1 protein and affects the Q_b binding site. Several amino acid substitutions result in a less-sensitive target site and confer weed resistance to atrazine. The most common substitution found in *Amaranthus* and other weeds is a serine to glycine mutation at amino acid position 264 (Hirschberg and McIntosh, 1983). If the most common mutation exists at the D1 protein, then there is usually resistance to other herbicides that also inhibit this site of action. This is referred to as cross-resistance. Plants with cross-resistance possess a mechanism that provides the plant with the ability to withstand herbicides from different chemical classes but within the same site of action. Additionally, metabolism-based resistance to PSII inhibitors can arise from enhanced detoxification (Ma *et al.*, 2013; Evans *et*

al., 2017), with the most notable case discovered in a population of velvetleaf (*Abutilon theophrasti*) (Gronwald *et al.*, 1989; Anderson and Gronwald, 1991). Currently, there have been experiments conducted in regards to cross-resistance between metribuzin and atrazine (Fuerst et al., 1986; Holt et al., 1993). In the case of metabolism-based atrazine resistance, experiments have not been reported that determine the effect of other PSII-inhibitors on these plants. Metabolism-based atrazine resistance due to enhanced detoxification by glutathione *S*-transferases (GSTs) was reported in waterhemp (Evans *et al.* 2017) and velvetleaf (Gronwald *et al.* 1989). By contrast, resistance to simazine, a different PSII-inhibitor but also an *s*-triazine, was due to elevated rates of cytochrome P450 monooxygenase (P450)-mediated oxidation in annual ryegrass (*Lolium rigidum*) in Australia (Burnet *et al.*, 1993). Tolerance to atrazine and other PSII herbicides in maize, grain sorghum, and wheat results from rapid detoxification mechanisms via GSTs (Dixon *et al.*, 2002). Resistance or natural tolerance occurs when the parent herbicide is metabolized by plant enzymes, rendering it more water soluble, immobile, and non-phytotoxic (Labrou *et al.*, 2015). Metabolism has to occur at a rate high enough to prevent significant herbicide binding to its particular site of action, which in the case of PSII inhibitors, detoxification has to occur faster than binding of the Q_b site of the D1 protein.

Metabolism or detoxification has been divided into three different phases, and there are several classes of plant enzymes that mediate these reactions. Phase I is characterized by oxidative enzymes such as P450s. P450s are found in all domains of life and are involved in dealkylation, deamination, decarboxylation, and other oxidative reactions that result in more water soluble or reactive products that are ready for Phase II detoxification (Riechers *et al.*, 2010). Phase II detoxification involves glycosyltransferases and GSTs, which are characterized by their ability to conjugate xenobiotics (i.e., foreign compounds) with a sugar molecule or

reduced glutathione (GSH), respectively. These conjugated molecules are then targeted for Phase III detoxification. ATP-binding cassette (ABC) transporters are the major proteins involved in the process of Phase III detoxification (Riechers *et al.*, 2010). ABC transporters use energy from the hydrolysis of ATP to actively transport a variety of different conjugated substrates to the vacuole or to be deposited in the cell wall (Riechers *et al.*, 2010).

GST enzymes are found in all eukaryotic cells and are well-studied due to their ability to detoxify a wide variety of endogenous and xenobiotic substrates (Riechers *et al.*, 2010; Labrou *et al.*, 2015). GSTs are characterized by their ability to catalyze the rapid conjugation of GSH with a xenobiotic substrate, such as herbicides and other pesticides. The GSH-conjugated compound is rendered more water soluble, immobile, and non-phytotoxic, and targeted for Phase III detoxification to the vacuole for long-term storage, catabolism, or degradation (Edwards and Dixon, 2005; Riechers *et al.*, 2010; Labrou *et al.*, 2015). There are many different classes of GSTs, with phi and tau classes being the most abundant in plants (Labrou *et al.*, 2015). These two classes are well known for their ability to conjugate a wide variety of herbicides with GSH, leading to herbicide selectivity in crops or weed resistance (Edwards and Dixon, 2005). Both of these classes are functionally active as both homodimers and heterodimers, and contain an essential catalytic serine residue near the N-terminus of the protein (Labrou *et al.*, 2015). Tau and phi class GSTs are involved in herbicide metabolism and resistance in weeds such as barnyard grass (*Echinochola crus-galli*) (Carey III *et al.*, 1997), *Arabidopsis* (Smith *et al.*, 2004) and black grass (*Alopecurus myosuroides*) (Cummins *et al.*, 2013).

Atrazine tolerance in cereal crops such as maize and grain sorghum is a direct result of rapid metabolism due to GST activities (Timmerman, 1989). Utilizing rapid metabolism as a biochemical resistance mechanism to alleviate the herbicidal stress of atrazine allows these

plants to avoid decreased productivity (such as a fitness cost) that typically results from an altered D1 target site (Holt *et al.*, 1993). This tolerance mechanism is similar to what was observed in two atrazine-resistant waterhemp populations from Illinois (Ma *et al.*, 2013; Evans *et al.*, 2017).

1.3 History and Use of HPPD Inhibitors

HPPD-inhibiting herbicides, or benzoylcyclohexane-1,3-dione herbicides, represent the newest commercially available class for use in corn and other cereal crops. These herbicides include three different chemical classes: the triketones, isoxazoles, and pyrazolones. In 1977, Zeneca scientists (now Syngenta) in California noticed how weeds didn't grow around the California bottlebrush plant, *Callistemon citrinus* (Mitchell *et al.*, 2001). Upon further investigation, scientists found that the bottlebrush plant was excreting a natural allelochemical from its roots called leptospermone. Scientists began testing this compound and found it to be a unique, moderately active herbicide that produced bleaching symptoms in susceptible broadleaf and grass weeds (Mitchell *et al.*, 2001). Furthermore, in 1982, scientists uncovered a benzoylcyclohexanedione compound when attempting to generate a sethoxydim analog (Lee *et al.*, 1998; Mitchell *et al.*, 2001). Upon further investigation and the removal of a methyl group on the cyclohexanedione moiety, herbicidal activity was significantly increased, especially on a wide range of susceptible broadleaf species, and maize was found to be tolerant to this synthesized compound (Lee *et al.*, 1998; Mitchell *et al.*, 2001). Thus, HPPD triketone herbicides were discovered. Since the discovery of the triketones, other chemical and agricultural companies have created their own HPPD herbicides, including isoxazoles and pyrazolones, the latter of which was actually used in rice (*Oryza sativa*) in Japan during the 1970s and 1980s by

the name of pyrazolate (Kawakubo *et al.*, 1979; Konotsune and Kawakubo, 1977; Yamaoka *et al.*, 1988). Each of the chemical classes are now commercially used in corn, including mesotrione, sulcotrione and tembotrione (triketones), isoxaflutole (an isoxazole) and topramezone (a pyrazolone).

HPPD-inhibiting herbicides are unique in that they can be applied PRE to the soil or POST applied to the plant. These herbicides inhibit 4-hydroxyphenylpyruvate dioxygenase, the key enzyme in the biosynthesis of plastoquinone (PQ) and tocopherols, which leads to the common bleaching symptoms of the meristem associated with these herbicides. The white tissue color stems from the fact that when PQ is inhibited, carotenoid synthesis is indirectly inhibited as well. PQ is responsible for accepting electrons during carotenoid synthesis. When the HPPD enzyme is inhibited, homogentisic acid (HGA) is depleted and PQ, tocopherols, and carotenoids are slowly depleted as well (Pallett *et al.*, 1998). Without PQ, phytoene, a carotenoid precursor, cannot be converted to phytofluene by phytoene desaturase (PDS). Carotenoids are important for protecting the plant from photooxidation by quenching triplet chlorophyll and preventing singlet oxygen from forming (Triantaphylidès and Havaux, 2009). Without carotenoids, the plant is susceptible to these destructive radicals that result in membrane and pigment destruction, leading to the characteristic bleaching symptoms.

The only two weed species that have evolved resistance to HPPD-inhibiting herbicides are waterhemp and Palmer amaranth (Heap, 2017). The first case of HPPD-resistant waterhemp occurred in a seed corn production field in McLean County, IL, USA, where the progeny grown from seed collected in this field survived foliar applications of mesotrione, tembotrione and topramezone (Hausman *et al.*, 2011). Dose-response experiments indicated at least a 10-fold level of resistance to mesotrione relative to sensitive biotypes (Hausman *et al.*, 2011). The

mechanism of resistance to mesotrione was not due to an altered target site, but was attributed to rapid herbicide metabolism via P450s (Ma *et al.*, 2013). A waterhemp population from Nebraska (designated as NEB) displayed at least a 31-fold level of resistance to mesotrione relative to a sensitive population (Kaundun *et al.*, 2017). Resistance to mesotrione in NEB was due to enhanced detoxification of the parent compound by P450s, thus mirroring the selectivity basis of mesotrione in tolerant corn (Hawkes *et al.*, 2001) and similar to the earlier report by Ma *et al.* (2013) in HPPD-resistant waterhemp (designated as MCR).

As stated before, Phase I detoxification is characterized by oxidative enzymes such as P450s. P450s are found in all living organisms and utilize NADPH, molecular oxygen, and electrons from P450 reductase to convert xenobiotics, such as herbicides, into non-phytotoxic products (Riechers *et al.*, 2010). The metabolism of mesotrione in corn is due to P450-catalyzed ring hydroxylation (Hawkes *et al.*, 2001). It is still unknown how many P450s are responsible for this rapid metabolism. Ma *et al.* (2013) confirmed that the MCR population utilized the same P450-catalyzed pathway as in corn to metabolize mesotrione. To date, the overall number of specific P450(s) involved in mesotrione metabolism in HPPD-resistant *Amaranthus* is not yet known. Many researchers have tried to answer this question of how many P450s could be involved in herbicide tolerance in corn (Rowe et al., 1989; Porpiglia et al., 1990), soybeans (Ahrens, 1990) and other small grains (Miller, 1998; Miller and Dalrymple, 1990) by using the synergistic interaction observed between insecticides and some herbicides, since some of these insecticides have been found to inhibit P450 activities (Kreuz and Fonné-Pfister, 1992).

1.4 Synergistic Activity Between PSII- and HPPD-Inhibitors

Much research has gone into understanding the interaction of PSII-inhibiting herbicides in combination with HPPD-inhibiting herbicides. This tank mix is effective because of the synergistic activity observed when these two classes of herbicides are combined (Sutton *et al.*, 2002; Abendroth *et al.*, 2006; Hugie *et al.*, 2008; Woodyard *et al.*, 2009). As previously mentioned, HPPD inhibitors such as mesotrione and isoxaflutole (more specifically its diketonitrile metabolite) inhibit the conversion of 4-hydroxyphenylpyruvic acid (HPPA) to homogentisic acid (HGA) (Lee *et al.*, 1998; Pallett *et al.*, 1998). The inhibition of the HPPD enzyme indirectly leads to a reduction in plastoquinone, which is an essential electron acceptor for phytoene desaturase, thus reducing the amount of carotenoids produced (Hess, 2000; Pallett *et al.*, 1998). A reduction in plastoquinone means there is less substrate for atrazine to compete with, enabling the PSII inhibitors to inhibit the target site by reducing competition for binding. A reduction of HGA inevitably blocks the production of α-tocopherol, which is an important antioxidant within the chloroplast membranes involved in quenching free radicals (Pallett *et al.*, 1998). When atrazine binds to the D1 protein, it interferes with the normal electron transport in photosystem II. Because these excited electrons are free within the cell, excited chlorophyll is arrested in a triplet state (Hess, 2000). Triplet chlorophyll then induces the rapid formation of singlet oxygen. Lipid peroxidation follows as a result of both triplet chlorophyll and singlet oxygen inducing lipid and hydroxyl radicals (Hess, 2000). This injury induced by the radicals could be exacerbated when a PSII inhibitor is combined in tank mix with an HPPD inhibitor, since radical-scavenging α-tocopherol and carotenoids are present at decreased amounts within thylakoid membranes following HPPD inhibition (Pallett *et al.*, 1998).

Tank mixing herbicides with different sites of action, rather than applying either herbicide alone or in rotation, is a proposed method for delaying the development of resistance (Evans *et al*., 2016). Unfortunately, waterhemp and Palmer amaranth have developed resistance to both PSII and HPPD inhibitors (Heap, 2017). Typically, the Colby equation (Colby, 1967) is used to determine a synergistic or antagonistic effect using the combination of HPPD and PSII inhibitors. Previous research has shown synergistic activity on broadleaf weeds, where the combined herbicidal activity in a tank mix is greater than the expected sum of activity when the two herbicides are applied alone (Abendroth *et al.*, 2006; Hugie *et al.*, 2008; Sutton *et al.*, 2002). This synergistic effect was observed on both triazine-sensitive (TS) and triazine-resistant (TR) biotypes of redroot pigweed (Hugie *et al.*, 2008), as well as other TR weed species (Sutton *et al.*, 2002). A synergistic reaction was shown when atrazine was applied PRE and an HPPD inhibitor applied POST in a metabolism-based TR velvetleaf population from Wisconsin (Woodyard *et al.*, 2009). The synergistic interaction between PSII and HPPD inhibitors continues to be an important option for weed control in corn, even for resistant species.

1.5 Research Objectives

Waterhemp is one of the most important agronomic weeds to date. Since its spread into farmer's fields within the past 30 years, waterhemp has voraciously competed with crops for limited resources while evolving resistance to a variety of different herbicides in a numerous amount of ways. Multiple resistance cases are becoming a normal occurrence, and farmers are even more desperate for control options to combat this weed species. Since commercialization of the HPPD-inhibiting herbicides in the late 1990s, there has been no new chemistry brought to the market that could be a viable option. Because of this lack of new technology, it is crucial to

understand the mechanisms of resistance in waterhemp and to critically consider new ways to combat this and other *Amaranthus* species, using the tools we currently have at hand.

Atrazine is one of the oldest, but still most widely used, herbicides for weed management in corn and grain sorghum. Unfortunately, there are 66 different species worldwide that are resistant to this herbicide alone (Heap, 2017). Atrazine is still used today, though, because it is inexpensive and is flexible in its application. Not only is there a lot of resistance associated with atrazine, but there have been reports of this herbicide leaching into groundwater and affecting amphibians (EPA, 2009). An interesting phenomenon is observed when PSII inhibitors are combined with HPPD inhibitors. Specifically, a synergistic effect between these two chemical families occurs when tank mixed, where the herbicides combined are more effective (*i.e.* have more activity or cause more injury) than either herbicide alone. This is an important tool for farmers to utilize. Some herbicides, such as Lumax™ and Lexar™, are currently labeled for use in corn that combine these two herbicide sites of action (SoA) in one convenient mix. A problem arises when this tank mix is applied to a population that is resistant to one or the other SoA, or even both. Essentially, only one effective herbicide is being applied to that plant, while still selecting for resistance to the other herbicide. This is why there is a call for action to understand how these plants, in particular waterhemp, are able to evolve resistance to these herbicides and survive each year and discover different ways of combatting these resistant populations.

Based on these circumstances that farmers are currently facing on a day-to-day basis, the overall objective of the research herein is to provide a better understanding of these resistances, and to help identify a way to control these resistant waterhemp populations in an economical, sustainable, and environmentally-friendly way so that everyone could benefit. Previous research demonstrated that atrazine resistance in two populations of waterhemp from Illinois (designated

ACR and MCR) resulted from a non-target site resistance (NTSR) mechanism, as indicated by the lack of mutation in the *psbA* gene and rapid accumulation of polar metabolites with the same retention time as a synthetic GSH-atrazine standard (Ma *et al.*, 2013). Using this information, it was determined that one transcript, identified as a phi-class GST (*AtuGSTF2*), displayed higher constitutive expression in both atrazine-resistant waterhemp populations (Evans *et al*., 2017); therefore, I hypothesized that expression of this *GST* is likely associated with atrazine resistance in MCR and ACR. Chapter 2 aims to further characterize elevated *AtuGSTF2* expression levels and identify sequence variants by using an F_2 population segregating for atrazine resistance.

Chapter 3 investigates the response of two different HPPD- and atrazine-resistant waterhemp populations to two different HPPD inhibitors. Both populations had previous exposure to mesotrione and tembotrione (both triketones), while neither had been exposed to isoxaflutole. Isoxaflutole has previously only been applied PRE in corn. However, with the inclusion of the corn safener cyprosulfamide and introduction of HPPD-resistant soybean technology, there is a possibility that isoxaflutole could be used early POST in soybean and corn in the near future. In this study, the two multiple resistant populations (designated SIR and NEB) were treated with mesotrione or isoxaflutole at two different POST timings. Results were then analyzed and compared to two HPPD-sensitive populations (designated ACR and SS) to evaluate the fold-level of resistance to these two herbicides. Based on the results, a herbicide interaction study was conducted using metribuzin, an *as*-triazine, in combination with either mesotrione or isoxaflutole. The main objective was to test the hypothesis that by using a different PSII inhibitor, combined with either of these HPPD inhibitors, the synergistic activity between HPPD inhibitors and triazines typically observed in a sensitive population will be regained.

Chapter 4 summarizes the discussion and conclusions from Chapters 2 and 3 and identifies current limitations and future research goals for utilizing the herbicides we currently have available to control waterhemp, as herbicide resistance continues to evolve and no new modes of action are coming to market. Overall, the objective of the research presented herein was to identify options to control herbicide-resistant waterhemp populations using the tools we currently have, and to do so in an economical, sustainable, and environmentally-safe way so that farmers can benefit.

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CHAPTER 2

BIOCHEMICAL CHARACTERIZATION OF METABOLISM-BASED ATRAZINE RESISTANCE IN *AMARANTHUS TUBERCULATUS* AND IDENTIFICATION OF AN EXPRESSED *GST* ASSOCIATED WITH RESISTANCE[1]

2.1 Abstract

Rapid detoxification of atrazine in naturally tolerant crops such as maize (*Zea mays*) and grain sorghum (*Sorghum bicolor*) results from glutathione *S*-transferase (GST) activity. In previous research, two atrazine-resistant waterhemp (*Amaranthus tuberculatus*) populations from Illinois, U.S.A. (designated ACR and MCR), displayed rapid formation of atrazine-glutathione (GSH) conjugates, implicating elevated rates of metabolism as the resistance mechanism. Our main objective was to utilize protein purification combined with qualitative proteomics to investigate the hypothesis that enhanced atrazine detoxification, catalysed by distinct GSTs, confers resistance in ACR and MCR. Additionally, candidate *AtuGST* expression was analysed in an F_2 population segregating for atrazine resistance. ACR and MCR showed higher specific activities towards atrazine in partially purified ammonium sulfate and GSH affinity-purified fractions compared to an atrazine-sensitive population (WCS). One-dimensional electrophoresis of these fractions displayed an approximate 26-kDa band, typical of GST subunits. Several phi- and tau-class GSTs were identified by LC-MS/MS from each population, based on peptide

[1] Previously published as:
Evans, A.F. Jr., O'Brien, S.R., Ma, R., Hager, A.G., Riggins, C.W., Lambert, K.N. and Riechers, D.E. (2017) Biochemical characterization of metabolism-based atrazine resistance in *Amaranthus tuberculatus* and identification of an expressed *GST* associated with resistance. Plant Biotechnol. J., 15, 1238-1249, doi: 10.1111/pbi.12711
Author contributed all information involving F_2 lines and sequencing.

similarity with GSTs from *Arabidopsis*. Elevated constitutive expression of one phi-class GST, named *AtuGSTF2*, correlated strongly with atrazine resistance in ACR and MCR and segregating F_2 population. These results indicate that *AtuGSTF2* may be linked to a metabolic mechanism that confers atrazine resistance in ACR and MCR.

2.2 Introduction

There are over 75 species in the genus *Amaranthus* found worldwide, including both monoecious and dioecious species (Mosyakin and Robertson, 2003). A dioecious species called waterhemp (*Amaranthus tuberculatus* (Moq.) Sauer var. *rudis* (Sauer) Costea & Tardif or syn. *A. rudis* Sauer) (Costea *et al*., 2005; Pratt and Clark, 2001) has become a major problem in the United States due to several ecological, biological, and genetic factors (Steckel, 2007). For example, waterhemp is difficult to selectively manage in maize and soybean (*Glycine max*) production systems because it is a summer annual with a prolonged germination period (Costea *et al*., 2005; Hartzler *et al*., 1999). In addition, the obligate outcrossing nature and dioecious biology of waterhemp facilitates the spread of genes conferring herbicide resistance via pollen flow throughout natural populations (Costea *et al*., 2005; Tranel *et al*., 2011). Multiple genes or alleles conferring resistance can occur within single waterhemp populations or individual plants due to strong herbicide selection pressures, resulting in multiple-resistant phenotypes (Heap, 2016). For example, resistance to herbicides that inhibit 4-hydroxyphenyl-pyruvate dioxygenase (HPPD), protoporphyrinogen oxidase, acetolactate synthase (ALS), EPSP synthase, photosystem II (PS II) and the auxin herbicide 2,4-D has been reported in waterhemp populations (Hausman *et al*., 2011; Heap, 2016; Patzoldt *et al*., 2005).

Atrazine is a commonly used herbicide for weed management in maize (LeBaron *et al*., 2011). PS II-inhibiting herbicides such as atrazine inhibit the light reactions of photosynthesis by competing with plastoquinone for the Q_b binding site of the D1 protein (Fuerst and Norman, 1991; Hess, 2000), thus blocking the flow of electrons to cytochrome b_6f and subsequently triggering the rapid formation of triplet chlorophyll followed by singlet oxygen in the presence of light (Krieger-Liszkay, 2005; Triantaphylidès and Havaux, 2009) in sensitive dicots. In contrast, natural tolerance in maize and grain sorghum is due to the high constitutive activity of glutathione *S*-transferase (GSTs) that can use atrazine as a substrate, leading to rapid metabolic detoxification in these crops (Timmerman, 1989). The most common mechanism conferring atrazine resistance in dicot weeds is an insensitive target-site protein. A point mutation in the *psbA* gene (which encodes the D1 protein) frequently identified in atrazine-resistant weeds results in a SER to GLY mutation at amino acid 264, which confers an approximate 1000-fold level of resistance compared with sensitive biotypes (Devine and Preston, 2000; Hirschberg and McIntosh, 1983). By contrast, evolved resistance to atrazine in velvetleaf (*Abutilon theophrasti*) has been linked to elevated GST activity (Anderson and Gronwald, 1991; Gray *et al*., 1996). Similarly, GST-based detoxification mechanisms have also been documented in several resistant grass weeds (Cummins *et al*., 2013; Reade *et al*., 2004; Yu and Powles, 2014). Rapid metabolism of atrazine in multiple-herbicide-resistant waterhemp resulted in a several hundred-fold resistance level compared to atrazine-sensitive plants (Evans, 2016).

GSTs are found in both plants and animals and are a widely studied class of primarily cytosolic (Mashiyama *et al*., 2014), dimeric enzymes mainly due to their detoxification abilities (Dixon *et al*., 2010; McGonigle *et al*., 2000; Wagner *et al*., 2002). Plant GST subunits belong to

several different classes, including theta, zeta, lambda, phi, tau and glutathione-dependent dehydroascorbate reductases (DHARs), based on sequence similarity, essential catalytic residues and immunological cross-reactivity (Edwards and Dixon, 2005; Frova, 2006; Mashiyama *et al*., 2014). The most common subclasses of plant GSTs are the phi and tau classes (Labrou *et al*., 2015), although the relative proportions differ depending on species (Chi *et al*., 2011). Phi-class GSTs were among the first GSTs shown to catalyse herbicide detoxification reactions in maize (Fuerst *et al*., 1993; Holt *et al*., 1995; Irzyk and Fuerst, 1993; Jepson *et al*., 1994).

Previous research demonstrated that atrazine resistance in two populations of waterhemp from Illinois (designated ACR and MCR; Hausman *et al*., 2011) results from non-target-site resistance (NTSR) mechanism(s), as indicated by the lack of a mutation in the *psbA* gene and rapid accumulation of a polar metabolite with the same retention time (via reverse-phase HPLC) as a synthetic GSH-atrazine standard in resistant populations (Ma *et al*., 2013). Therefore, we hypothesize that rapid formation of this metabolite results from increased GST activity in ACR and MCR compared to an atrazine-sensitive population (WCS; Hausman *et al*., 2011) and that this increased activity results from either higher constitutive expression of GST(s) or the presence of novel GST isoforms with greater affinity towards atrazine. As a result, the objectives of this study were to (i) determine whether differences in GST activity exist between atrazine-resistant and atrazine-sensitive waterhemp populations, (ii) utilize ammonium sulfate (AMS) fractionation combined with GSH affinity chromatography to partially purify GSTs from each population and obtain peptide sequences, (iii) search a waterhemp transcriptome database to identify partial cDNA sequences encoding *GSTs* and (iv) determine whether expression of candidate *GST(s)* correlates with whole-plant phenotypic responses to atrazine in the glasshouse,

using an F_2 population segregating for atrazine resistance (Huffman *et al*., 2015). Our results demonstrate that basal expression levels of a single candidate gene, named *AtuGSTF2,* correlate strongly with the atrazine-resistant phenotype in ACR and MCR and segregating F_2 population.

2.3 Materials and Methods

2.3.1 Phenotyping of whole-plant responses to atrazine in an F_2 population

A total of 32 F_2 plants were randomly selected from the original MCR × WCS cross (Huffman *et al*., 2015). Vegetative clones derived from these original F_2 'parent' plants were used to study segregation of whole-plant responses and gene expression due to the large amount of genetic variability in waterhemp (Ma *et al*., 2013; Steckel, 2007). Clones were grown and handled as described previously (Ma *et al*., 2015). When sufficient clones had been generated to represent each F_2 line (Ma *et al*., 2015), a dose–response study was conducted to compare the response of the 32 F_2 lines to foliar-applied atrazine. When plants reached 10–12 cm in height, they were treated with atrazine at rates evenly spaced along a 3.16 log scale (Hausman *et al*., 2011), ranging from 3.2 g/ha to 10,000 g/ha, and included 1% crop oil concentrate (COC) as a spray adjuvant. Control plants were treated with water plus COC only.

This initial study broadly determined which F_2 lines were sensitive or resistant to atrazine. GR_{50} values for the sensitive lines ranged from 25 to 69 g/ha, significantly lower than the maximum field-use rate of 2.2 kg/ha (Ma *et al*., 2016). However, complete plant death was never achieved in atrazine-resistant lines, and estimated GR_{20} values for all resistant lines were greater than the field-use rate. A discriminatory rate was then determined to distinguish between RR and Rr plants. Atrazine rates ranged from 1.2 kg/ha to 28.8 kg/ha and included 1% COC and

2.5% liquid AMS as spray adjuvants. Due to the large degree of variability at the highest rate tested, the discriminatory rate of 14.4 kg/ha was determined for distinguishing between resistant genotypes. By comparison, a much lower rate of atrazine (985 g/ha) had been used previously to distinguish between resistant (RR and Rr) and sensitive genotypes in this F_2 population (Huffman *et al*., 2015), but a different growth medium and nutrient system was utilized compared with the methods described herein. Experiments were independently conducted at least twice with five replications per treatment. Aboveground biomass was harvested at 12 DAT, dried in an oven at 65° C, and dry weight data were combined and analyzed by LSD ($P = 0.1$) using PROC GLM in SAS (Release 9.2) to determine significant differences among F_2 lines.

2.3.2 Constitutive *AtuGSTF2* expression and genotyping in a segregating F_2 population

Total RNA was extracted from nontreated waterhemp tissues and prepared using previously described methods (Riechers et al., 2003). Total RNA concentrations were determined using a NanoDrop spectrometer and rRNA quality was confirmed by visual analysis in agarose-formaldehyde gels. First-strand cDNA synthesis was performed using the Maxima H-Minus cDNA synthesis kit (Thermo-Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol using 500 ng total RNA. The following parameters were then used for RT-PCR, with 1 uL of first-strand cDNA reaction: initial denaturing at 95^o C for 4.5 min, then 30 amplification cycles consisting of 95^o C for 50 s, 56^o C for 55 s and 72^o C for 1 min, followed by final extension at 72^o C for 8.5 min. RT-PCR products were visualized with 1% agarose gels stained with ethidium bromide.

RT-qPCR was performed with total RNA isolated from each waterhemp population using the same tissues and growth stage as described previously. Original, gene-specific primers were designed to specifically amplify *AtuGSTF2*. Stable, constitutive expression of *AtuBTUB1* was demonstrated under the experimental conditions and waterhemp growth stage used in these studies as determined by <1-fold magnitude of differences in CT values. Primer efficiencies for RT-qPCR ranged from 95% to 99% for *AtuGSTF2* and *AtuBTUB1* amplifications from cDNA. RT-qPCR was conducted using the 7900 HT Sequence Detection System (PerkinElmer, Applied Biosystems, Waltham, MA, USA) and reactions performed in 20 µL volumes following the manufacturer's protocol (Syber® Green RNA-to C_T™ 1-Step Kit; Applied Biosystems, Waltham, MA, USA). The protocol was as follows: 48° C for 30 min, 95° C for 10 min, 40 cycles at 95° C for 15 s, 60° C for 1 min and a melting curve at 95° C for 15 s, 60° C for 15 s and 95° C for 15 s. Dissociation curves for each reaction were analyzed to ensure only one replicon was amplified. Gene expression in each sample was calculated relative to transcript levels in WCS and the *AtuBTUB1* reference gene using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). For analysis of *AtuGSTF2* expression in segregating F_2 lines, experiments were independently conducted twice with three technical replications per RNA sample. Data from each experiment were combined and *AtuGSTF2* relative expression values were analyzed by LSD ($P = 0.1$) using PROC GLM in SAS (Release 9.2) to determine significant differences among F_2 lines.

2.3.3 Sequence analysis of individual *AtuGSTF2* and *AtuBTUB* amplicons from cDNA

Total RNA was extracted from nontreated waterhemp tissues (using methods described earlier) from different F_2 lines, with at least three representative lines from each putative genotype. Gene-specific primers (Table 2.2) were used to amplify *AtuGSTF2* and *AtuBTUB*

alleles using methods described earlier for RT-qPCR. RT-PCR products were purified directly from each reaction using the QIAquick™ PCR Purification Kit (Qiagen Inc., Valencia, CA, USA). Purified amplicons were then ligated into a pCR™4-TOPO cloning vector and transformed into competent *E. coli* cells (TOPO TA™ Cloning Kit, Invitrogen, Waltham, MA, USA). Plasmids were purified using the I-Blue Mini Plasmid Kit (IBI Scientific, Peosta, IA, USA) and submitted for sequencing. Amplicons were sequenced from a total of seven different F_2 lines (two RR, three Rr and two rr), plus the original MCR and WCS populations, originating from at least two different colonies per transformation reaction.

2.4 Results and Discussion

2.4.1 Phenotyping atrazine responses, constitutive *AtuGSTF2* expression and genotyping in a segregating F_2 population

Due to the large degree of variability at the highest atrazine rate tested (28.8 kg/ha), a discriminatory rate (14.4 kg/ha) was determined as optimal for distinguishing between resistant genotypes. In order to determine whether constitutive expression of *AtuGSTF2* also correlated with phenotypic responses in the F_2 population (Huffman *et al*., 2015), 10- to 12-cm plants were treated with foliar-applied atrazine at this rate. Treated plants revealed significant phenotypic differences among segregating F_2 lines, as shown in Figure 2.1a and further described below. Plants from several F_2 lines that rapidly developed healthy, new green tissue following atrazine treatment and were as tall as nontreated controls were tentatively assigned a homozygous (RR) atrazine-resistant genotype (Figure 2.1b). By comparison, plants from numerous F_2 lines that developed less green meristematic tissue than RR lines following atrazine treatment, were

stunted, and did not grow significantly taller after application were tentatively assigned a heterozygous (Rr) atrazine-resistant genotype (Figure 2.1c). Plants from several F_2 lines died at this discriminatory rate within 7 days after treatment and were assigned an atrazine-sensitive (rr) genotype (Figure 2.1d).

Dry weight reductions and constitutive *AtuGSTF2* expression (relative to WCS) in 10 representative lines from the F_2 population are summarized in Table 2.1. Phenotypic responses resulting from atrazine treatment at 14.4 kg/ha correlated strongly with basal expression of *AtuGSTF2* (Table 2.1). In general, dry weight (biomass accumulation) and expression data followed the same trend, where dry weights were much higher in putative RR plants (lines 11 and 22), mainly because these plants developed a significant amount of green tissue following atrazine treatment (Figure 2.1b). Furthermore, lines 10, 21, 23, 31 and 32 displayed intermediate dry weights and *AtuGSTF2* expression values (Table 2.1), consistent with a putative Rr genotype. By comparison, rr lines did not accumulate biomass following atrazine treatment and eventually died at this rate (Figure 2.1d; Table 2.1).

These whole-plant results are consistent with the corresponding *AtuGSTF2* expression levels in each line, which were extremely high in RR and Rr lines by comparison with rr lines (Table 2.1), ranging from approximately 200-fold (line 10) to 1140-fold (line 22) higher. When considering all RR and Rr lines together, the mean *AtuGSTF2* expression value of 3304 units is 661-fold greater than the mean expression of 5 units for all rr lines. The large difference in *AtuGSTF2* expression between resistant and sensitive genotypes indicates the robustness of utilizing this constitutively expressed gene as a marker for identifying metabolic-based atrazine-resistant genotypes in this F_2 population, as well as in MCR, ACR and possibly other NTSR waterhemp populations yet to be analysed.

Exceptions to the overall strong association were noted, as evidenced by a weak fit within a discrete statistical category when considering both dry weight reductions and their *AtuGSTF2* expression (*e.g*. lines 23 and 32; Table 2.1). Based on the statistical groupings and categorization of phenotypic responses displayed in the glasshouse (Table 2.1), however, genotypes assigned for the atrazine-resistance trait in these lines are consistent with the F_2 population segregating for resistance in a 3:1 ratio (Huffman *et al*., 2015); visually more Rr lines were identified than either homozygous genotype (RR or rr) from the original 32 F_2 lines investigated.

2.4.2 Sequence analysis of individual *AtuGSTF2* amplicons from parent waterhemp populations and individual F_2 lines

Sequencing results from a total of 11 individual RT-PCR products (185-bp) indicated the presence of identical transcripts (*AtuGSTF2.2*) in each Rr or RR F_2 line tested (5 total; lines 10, 11, 22, 31 and 32). In addition, four individual amplicons derived from the MCR population (four different plants) possessed this same sequence, which differed from the original waterhemp transcriptome sequence (*AtuGSTF2.1*) by one conservative amino acid change within this region (Figure 2.2a). In contrast, analysis of two different amplicons from two rr genotypes (lines 7 and 26) and four individual amplicons from the WCS population (two different plants) revealed three sequences; the *AtuGSTF2.2* allele (from all resistant plants tested) and the *AtuGSTF2.1* allele, plus an additional allele (*AtuGSTF2.3*) found only in line 26 (Figure 2.2a).

Sequence variants of *AtuGSTF2* identified from the limited amount of amplicons are consistent with the dioecious, outcrossing nature of waterhemp. Sequence analysis of additional F_2 lines and individual clones may yield more allelic variants of the genes listed in Figure 2.2a-b.

However, the lack of polymorphisms among all *AtuGSTF2* amplicons sequenced from atrazine-resistant plants thus far (15 total) suggests a single haplotype containing the R allele (*AtuGSTF2.2*) in MCR and the F_2 population. This haplotype might occur if higher constitutive expression in resistant genotypes, as compared to sensitive genotypes, results from genetic variability that exists within the promoter (or untranslated regions) several kb upstream of the *AtuGSTF2* gene (Mahmood *et al*., 2016). Further analysis is required, however, because the 185-bp *AtuGSTF2* sequence represents *c.a*. 30% of the coding region (Figure 2.2c).

Sequence alignment of AtGSTF2 with the partial amino acid sequence of AtuGSTF2.1 (Figure 2.2c) revealed 67% identity, although this is a preliminary comparison as the AtuGSTF2.1 sequence only represents a portion of the full-length protein. The sequence of maize ZmGSTF2, a phi-class GST (previously called maize GST II, GST IV, or GST-27), was included for comparison because its involvement in herbicide detoxification and stress responses has been well documented (Edwards *et al*., 2000; Holt *et al*., 1995; Irzyk and Fuerst, 1993; Jepson *et al*., 1994). Comparison of the full-length sequences of ZmGSTF2 and AtGSTF2 showed 39% identity and comparison of ZmGSTF2 with the partial AtuGSTF2.1 sequence revealed 44% identity, which is within the expected range for interspecific comparisons of GSTs within a subclass (Labrou *et al*., 2015; Yang *et al*., 2009). It is important to note, however, that the *c.a*. 30% of AtuGSTF2.1 aligns closely with the N-terminus of AtGSTF2 (Figure 2.2c). Plant GST sequences from the same subclass are strongly conserved in the N-terminal domain of the protein, which typically corresponds with Exon 1 of the genomic sequence (Frova, 2006; Labrou *et al*., 2015), relative to the C-terminus. Interestingly, the diagnostic phi-class waterhemp GST peptide KVLDVYEARL is present in AtGSTF2 and ZmGSTF2, although the ZmGSTF2 sequence contains one conservative amino acid change (Figure 2.2c).

2.4.3 Discussion

NTSR mechanisms to herbicides in weeds (such as enhanced herbicide detoxification) have drawn great interest in recent years, particularly in grass weed species (Cummins *et al*., 2013; Gaines et al., 2014; Reade *et al*., 2004; Yu and Powles, 2014). However, metabolic resistance in dicots is not well characterized and remains markedly under-explored, particularly regarding the underlying biochemical mechanisms, enzymes and specific genes in these species (Anderson and Gronwald, 1991; Gray *et al*., 1996; Ma *et al*., 2013). Atrazine resistance in dicots is typically conferred by a point mutation in the plastidic target-site gene *psbA* (encoding the D1 protein in PS II), leading to decreased atrazine binding (reviewed by Devine and Preston, 2000). In contrast to previous research aimed at sequencing *psbA*, our primary goal was to characterize total and specific GST activities from atrazine-resistant MCR and ACR populations and compare with activities in the atrazine-sensitive population WCS, thereby following up on previous atrazine metabolism findings (Ma *et al*., 2013).

In spite of a significant enrichment in specific activity in MCR and ACR (and WCS to a lesser extent) protein extracts throughout the purification scheme, fold-purification levels were much lower than previously reported in cereal crops (Gronwald and Plaisance, 1998; Irzyk and Fuerst, 1993; Riechers *et al*., 1997; Timmerman, 1989) for affinity-purified GSTs. These lower fold-purification levels in our research may have resulted from use of photosynthetic tissues instead of etiolated seedling, shoot or coleoptile tissues from cereals (Irzyk and Fuerst, 1993; Riechers *et al*., 1997) or loss of activity during sample processing following initial extract preparation through GSH affinity purification as protein fractions become more dilute. However, these results establish a framework for continued mechanistic investigations of evolved resistance to atrazine, other pesticides or metabolism of environmental/endogenous toxins by

GSTs in weedy *Amaranthus*. In addition, these findings pave the way for new biotechnology applications aimed towards overcoming metabolic resistance in weedy plants.

2.4.4 Possible underlying mechanisms for elevated basal *AtuGSTF2* expression in waterhemp

AtuGSTF2 displayed higher constitutive expression in both atrazine-resistant waterhemp populations as well as in resistant F_2 lines segregating as a single-gene trait. Greater transcript abundance of the *AtuGSTF2* gene may contribute to elevated GST activity (data not shown) and higher levels of GSH-atrazine metabolites formed in ACR and MCR compared with WCS (Ma *et al*., 2013). Thus far, higher GST-specific activities with atrazine quantified in partially purified ACR and MCR protein extracts can only be associated with higher constitutive expression of *AtuGSTF2*. Further experiments are required, however, to obtain the entire open reading frame for expression and biochemical analyses of the recombinant AtuGSTF2 enzyme (with atrazine as substrate) because plant genomes contain dozens of *GST* genes and isozymes (Chi *et al*., 2011; McGonigle *et al*., 2000; Riechers *et al*., 2010) that contribute to total activity. From the standpoint of gene regulation, the potential for induction of *AtuGSTF2* expression by atrazine pretreatment in waterhemp should be examined in future research.

Higher basal expression levels of *AtuGSTF2* could be due to a mutation, insertion or varying degrees of methylation in the *AtuGSTF2* promoter or untranslated regions (Mahmood *et al*., 2016), an alteration in a DNA-binding protein, or a protein regulating mRNA stability. *GSTs* are unevenly dispersed throughout plant genomes (Dong *et al*., 2016; Lan *et al*., 2009) or found in clusters of duplicated genes (Soranzo *et al*., 2004; Xu *et al*., 2002),

thus promoting gene evolution and functional diversification (Kaltenegger and Ober, 2015; Liu *et al*., 2013). Alternatively, a mutation in a single transcription factor (TF) protein that binds to *GST*promoters could coordinately activate the expression of multiple *AtuGST*s. An analogous situation occurs with the maize Opaque-2 TF protein and DNA-binding One Zinc Finger (Dof) protein OBP1 (Noguero *et al*., 2013), which together regulate the transcription of numerous zein genes (Li *et al*., 2015; Vicente-Carbajosa *et al*., 1997). Interestingly, an OBP1 protein identified in *Arabidopsis* regulates expression of the *GST6* gene (renamed *AtGSTF8*; Wagner *et al*., 2002) in a similar manner (Chen *et al*., 1996). However, additional genomic sequence analyses of *AtuGSTF2* from resistant and sensitive plants are required to fully understand the resistance mechanism, because the alignments in Figure 2.2 only represent an estimated 30% of the *AtuGSTF2.1* coding sequence.

2.4.5 Transcript profiling techniques may reveal the entire watehemp GSTome

In weedy grass species without entire genomic sequences available, RNAseq has recently been utilized for the characterization of evolved resistance mechanisms (Chen *et al*., 2017; Duhoux *et al*., 2015; Gaines *et al*., 2014). Global transcriptional analyses of distinct genotypes identified from the segregating F_2 population (Table 2.1), using the available waterhemp genome and transcriptome (Lee *et al*., 2009; Riggins *et al*., 2010) along with the sequenced grain amaranth (*Amaranthus hypochondriacus*) genome and transcriptome (Sunil *et al*., 2014) as references, could more accurately determine the number of expressed *GST* genes (Chi *et al*., 2011; Labrou *et al*., 2015) in waterhemp and may enable further confirmation of the specific *AtuGST* gene(s) correlating with resistance. If RNAseq analyses indicated

that *AtuGSTF2* (or other metabolic enzymes) are the major genetic factor(s) correlating with resistance then further biochemical and molecular characterizations could be performed with the full-length AtuGSTF2 coding region, such as transgenic plant analysis with an atrazine-sensitive dicot such as *Arabidopsis* or tobacco. In addition, genomic cloning of *AtuGSTs* and subsequent bioinformatic comparisons with other herbicide-detoxifying plant *GSTs* (Cummins *et al*., 2011; Edwards *et al*., 2000; Frova, 2006) may reveal regulatory motifs or sequence variations between resistant and sensitive waterhemp plants (Mahmood *et al*., 2016).

2.4.6 New biotechnology applications towards improving resistant-weed management

Basal expression levels of *AtuGSTF2* could be used as a molecular marker for screening putative resistant waterhemp populations to exclude or confirm metabolic resistance to atrazine. A similar approach was utilized following the discovery of a key mutation associated with metabolic resistance to dichlorodiphenyl-trichloroethane (DDT) in the mosquito *Anopheles funestus* (Riveron *et al*., 2014). A genomewide transcriptional analysis was conducted in which the most highly upregulated gene was identified as a *GST* (termed *GSTe2*), which was confirmed to confer resistance to DDT and cross-resistance to pyrethroid insecticides through transgenic expression in sensitive *Drosophila*. The molecular basis for resistance resulted from both quantitative and qualitative mechanisms; increased expression in resistant mosquitos combined with a point mutation in the wild-type *GSTe2* gene in which LEU was substituted for PHE (Riveron *et al*., 2014).

In addition to improved resistance screening methods, the coding sequence of *AtuGSTF2* could be utilized to engineer targeted gene knockout strategies such as RNAi directed to a

specific *Amaranthus GST* (Yu and Powles, 2014) or to synthesize new chemical inhibitors of herbicide-detoxifying GSTs (Cummins *et al*., 2013; Lamoureux and Rusness, 1986; Ma *et al*., 2016). RNAi-based knockdown techniques have been used successfully in insect systems where insecticide-detoxifying P450s have been targeted (Bautista *et al*., 2009; Zhu *et al*., 2016), thus regaining activity of the insecticide in resistant populations. Polynucleotide-based gene knockdown systems are also being generated to overcome herbicide resistance in weeds (Sammons *et al*., 2015), which to date have been targeted primarily towards herbicide target-site proteins. However, additional knowledge of specific herbicide-detoxifying isozymes in weeds, such as those belonging to large, multigene GST and P450 families, provides a new opportunity to regain herbicide activity in multiple-resistant weeds. The findings presented herein support the conclusion that increased basal expression of a specific herbicide-detoxifying GST is associated with atrazine resistance in MCR and ACR, which may ultimately confer atrazine resistance, but might also lead to innovative and integrated weed management strategies.

2.5 Tables and Figures

Figure 2.1 Whole-plant responses of representative F_2 lines, 12 days after treatment (DAT) with foliar-applied atrazine. Waterhemp seedlings (10-12 cm tall) were treated with atrazine at the discriminatory rate of 14.4 kg/ha, including crop oil concentrate (1% v/v) and liquid ammonium sulfate (2.5% v/v) as adjuvants. (a) Top-down view of treated plants, from left to right: homozygous (RR) resistant, heterozygous (Rr) resistant and sensitive (rr) phenotypic responses (12 DAT). (b) Side view of typical RR response at 12 DAT. (c) Side view of typical Rr response at 12 DAT. (d) Side view of rr response at 12 DAT (i.e. complete death). For the bottom panels (b-d), the plant on the far left is an untreated control.

Table 2.1 Phenotypes of selected segregating F_2 lines, grouped according to dry weight (as a percent of the untreated control) or *AtuGSTF2* expression (relative to β-tubulin; *AtuBTUB1*).

F_2 Line	Dry Weight	Grouping	*AtuGSTF2* relative expression	Grouping	Atrazine genotype
22	54.1 (±7)	A	5742 (±2219)	A	RR
11	59.6 (±14)	A	5515 (±2244)	A	RR
21	33.9 (±5)	B	2546 (±1313)	BC	Rr
32	29.1 (±7)	BC	4233 (±1831)	AB	Rr
10	25.7 (±2)	CD	1008 (±489)	CD	Rr
31	20.0 (±4)	D	2998 (±1951)	BC	Rr
23	13.7 (±3)	E	1089 (±516)	CD	Rr
26	10.9 (±3)	E	4 (±1)	D	rr
27	5.1 (±1)	E	9 (±5)	D	rr
7	2.7 (±1)	E	2 (±1)	D	rr

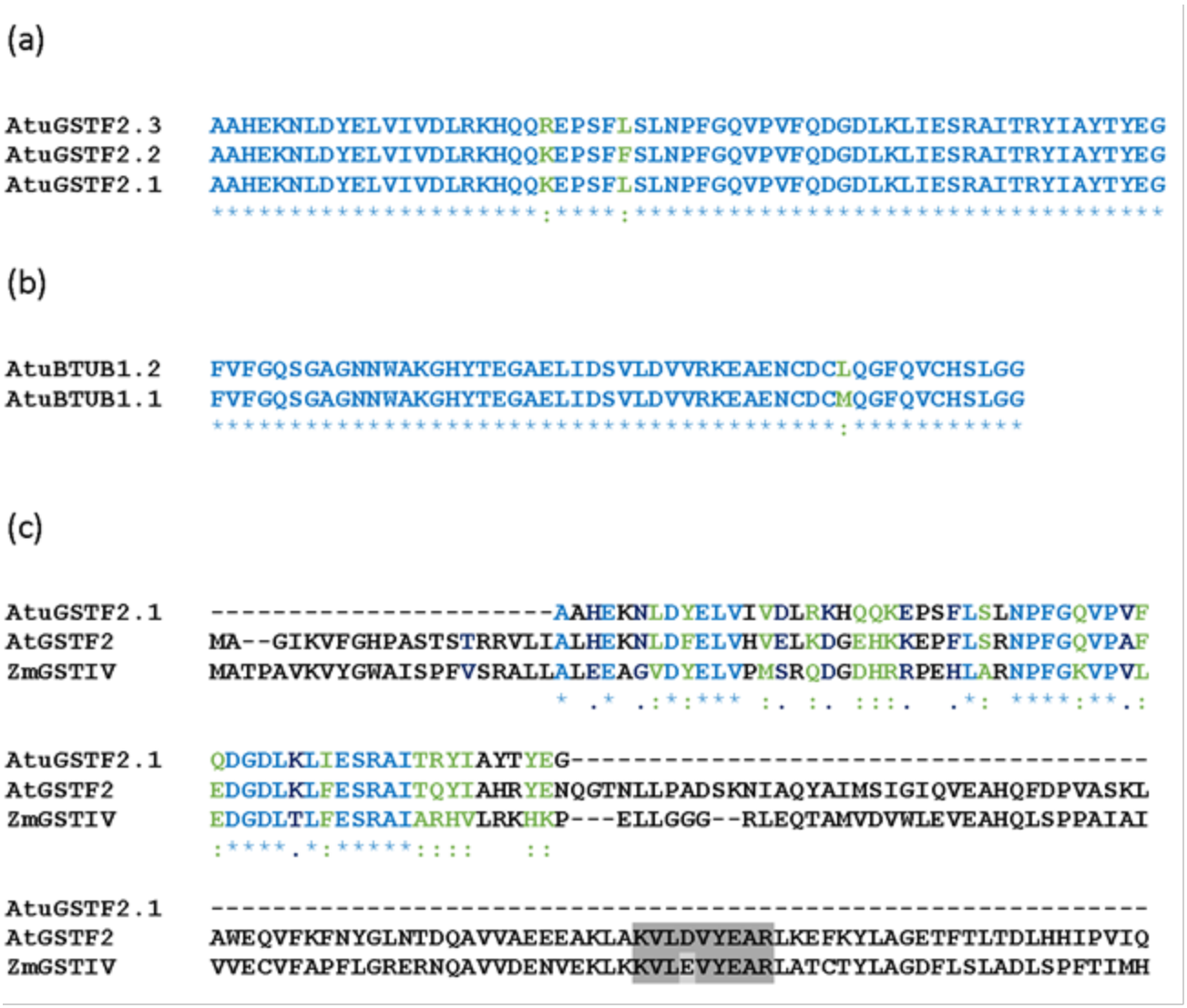

Figure 2.2 Partial cDNA sequences of *AtuGSTF2* and *AtuBTUB1* alleles expressed in several F_2 lines from the segregating population described by Huffman et al., (2015). Deduced amino acid alignments of different (a) AtuGSTF2 or (b) β-tubulin (AtuBTUB1) proteins, encoded by partial cDNAs. (c) Partial AtuGSTF2.1 sequence aligned with the corresponding region of the best-matched *Arabidopsis* protein (AtGSTF2) and maize ZmGSTF2 (all phi-class GSTs). Amino acids highlighted in gray in AtGSTF2 and ZmGSTF2 represent the corresponding region of AtuGSTF2 where the diagnostic peptide KVLDVYEAR was identified by LC-MS (Figure 3).

Table 2.2 Primers used for RT-qPCR expression analysis.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
AtuGSTF2	GCACCCAACGTGTATTAG	AGTAAGGGGTGTTCCTTG
AtuBTUB1	AGATTTTTCGCCCGGATAAC	TCCCATTCCAGATCCTGTTC

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CHAPTER 3

QUANTIFYING RESISTANCE TO ISOXAFLUTOLE AND MESOTRIONE AND INVESTIGATING THEIR INTERACTION WITH METRIBUZIN APPLIED POST-EMERGENCE IN *AMARANTHUS TUBERCULATUS*

3.1 Abstract

Previous research reported resistance to mesotrione and other 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibiting herbicides in waterhemp (*Amaranthus tuberculatus*). Herein, experiments were conducted to quantify the level of resistance to mesotrione and isoxaflutole in the NEB (for Nebraska HPPD-resistant) and SIR (for Stanford, Illinois HPPD-resistant) waterhemp populations, which differ in their field-use histories and levels of resistance to mesotrione. Foliar responses of these two populations were compared to ACR (HPPD-sensitive but atrazine-resistant) and SEN (for herbicide sensitive). A greenhouse dose-response study was conducted with each herbicide at two different postemergence (POST) timings: an EPOST (5 cm tall or 4-5 true leaves) and POST (10 cm tall or 8-9 true leaves). SIR was 10-fold resistant to isoxaflutole and 32-fold resistant to mesotrione EPOST compared to ACR, and NEB was 4-fold resistant to isoxaflutole and 7-fold resistant to mesotrione EPOST compared to ACR. Furthermore, SIR was 17-fold resistant to isoxaflutole and 21-fold resistant to mesotrione POST compared to ACR, while NEB was 3-fold resistant to isoxaflutole and 7-fold resistant to mesotrione POST compared to ACR. Overall these findings indicated greater fold-resistance to mesotrione relative to isoxaflutole within each timing. However, POST treatments to SIR showed contrasting effects on fold-resistance levels relative to EPOST. To further investigate

potential management strategies of HPPD-resistant waterhemp populations in the field, a POST herbicide interaction study was conducted using combinations of metribuzin and either isoxaflutole or mesotrione. Following dose-response analysis of several sublethal metribuzin rates, 191 g ai ha^{-1} was chosen for interaction studies since this rate caused an approximate 20% biomass reduction to SIR and NEB plants. This metribuzin rate was combined with either a 0x, 0.5x, 1x, or 2x field-use rate of either isoxaflutole or mesotrione. Results indicated that mesotrione at 52.5 g ai ha^{-1} combined with 191 g ai ha^{-1} of metribuzin displayed a synergistic effect on biomass reduction in SIR plants. However, all other combinations of either mesotrione or isoxaflutole and metribuzin resulted in an additive effect on biomass reduction in both the SIR and the NEB populations. These results give insight into how the joint activity between HPPD- and PSII-inhibitors can be used to control metabolism-based, multiple herbicide-resistant waterhemp populations.

3.2 Introduction

Waterhemp (*Amaranthus tuberculatus* (Moq.) Sauer var. *rudis* (Sauer) Costea & Tardif, or syn. *A. rudis* Sauer) (Costea et al., 2005; Pratt and Clark, 2001) is a small-seeded dicot species that has become a major problem in corn (*Zea mays*) and soybean (*Glycine max*) production in North America (Steckel, 2007). Waterhemp can reduce corn yields by up to 74% (Steckel and Sprague, 2004) and soybean yields more than 40% (Hager *et al.*, 2002b). Waterhemp is one of the dioecious species within the genus *Amaranthus,* leading to obligate outcrossing within the species (Murray, 1940) and a large degree of genetic diversity (Trucco *et al.*, 2009). Waterhemp control is crucial because outcrossing leads to the spread of different herbicide resistances via

pollen between populations. Additionally, waterhemp produces up to one million seeds per female plant (Hartzler *et al.*, 2004; Steckel *et al.*, 2003) that can remain dormant and viable in the soil for up to four years (Buhler and Hartzler, 2001; Burnside *et al.*, 2003) as well as germinate and emerge well into the summer growing season (Hartzler *et al.*, 2004). The increase in reduced- or no-till systems, combined with the variability in herbicide responses (Patzoldt *et al.*, 2002) and the evolution of herbicide resistance (Heap 2017), have led to a dramatic increase in waterhemp infestations in Illinois cropping systems within the last twenty years (Hager *et al.*, 1997; Steckel, 2007). Together these biological factors and management practices have made this species a huge problem for farmers across the Midwest (Steckel, 2007).

Herbicides that inhibit 4-hydroxyphenylpyruvate dioxygenase (HPPD) are commonly used in corn and other cereal crops for dicot weed management. HPPD herbicides directly inhibit the HPPD enzyme, which is a key enzyme in the biosynthesis of plastoquinone (PQ) and tocopherols. These herbicides also indirectly inhibit carotenoid biosynthesis since PQ is an electron acceptor required for phytoene desaturase activity in the carotenoid biosynthetic pathway (Lee *et al.,* 1998; Pallett *et al.,* 2001). Sensitive plants die due to loss of carotenoids, which leads to the distinctive white or 'bleaching' color of treated plants due to the oxidation of chlorophyll in the presence of light (Hess, 2000; Pallett *et al.,* 2001). Triplet chlorophyll and singlet oxygen are also produced, which lead to further chlorophyll destruction and subsequent membrane damage in the chloroplast and thylakoids (Pallett *et al.,* 2001). New leaves and meristems are primarily affected due to the systemic nature of these herbicides.

PQ also serves an important role as a membrane-soluble electron carrier in PSII (Hess, 2000). PSII-inhibiting herbicides such as atrazine or metribuzin inhibit the light reactions of photosynthesis by reversibly competing with PQ for the Q_b binding site of the D1 protein (Hess,

2000). This inhibition blocks the flow of electrons to cytochrome b_6f, subsequently triggering the rapid formation of triplet chlorophyll, followed by singlet oxygen, in the presence of light (Hess, 2000) in sensitive plants. Previous research demonstrated the synergistic activity of a postemergence (POST) tank mixture of mesotrione and PSII inhibitors, where the combination of these two herbicides produced greater activity than the sum of either herbicide applied alone (Abendroth *et al.*, 2006; Sutton *et al.*, 2002). Synergistic herbicidal activity has the potential to reduce costs for farmers and lower the amount of herbicides entering the environment (Kudsk and Mathiassen, 2004; Streibig and Jensen, 2000).

Tank mixing herbicides with different sites of action, rather than applying either herbicide alone or in rotation, is a method proposed for delaying the development of resistance (Diggle *et al.*, 2003; Evans *et al.*, 2016). Unfortunately, both waterhemp and Palmer amaranth (*Amaranthus palmeri*) have developed resistance to both PSII and HPPD inhibitors (Heap, 2017). Typically, the Colby equation is used to determine a synergistic, additive, or antagonistic effect using the combination of HPPD and PSII inhibitors relative to each herbicide applied alone (Colby, 1967). Previous research has shown synergistic activity on broadleaf weeds (Abendroth *et al.*, 2006; Hugie *et al.*, 2008; Sutton *et al.*, 2002), which was also observed on both triazine-sensitive and site-of-action based triazine-resistant (TR) redroot pigweed (*Amaranthus retroflexus*) biotypes (Hugie *et al.*, 2008) and other weed species (Sutton *et al.*, 2002). Additionally, a synergistic interaction was documented when atrazine was applied preemergence (PRE) and mesotrione was applied POST sequentially in a metabolism-based AR velvetleaf population (Woodyard *et al.*, 2009b). As a result, the synergism between PSII and HPPD inhibitors continues to be an important option for weed control in corn, even for TR populations. In contrast, HPPD inhibitors applied to a known HPPD- and symmetrical (*s*)-

triazine-resistant waterhemp population (termed MCR) provided only partial control (Hausman *et al.* 2011; Hausman *et al.*, 2013), and *s*-triazine-treated plots (PRE) were similar to the nontreated plots. However, the potential for POST synergism between HPPD inhibitors and metribuzin, an asymmetrical (*as*)-triazine, in metabolism-based HPPD- and atrazine-resistant populations has not been examined. This topic warrants further investigation because there is a possibility of combining HPPD inhibitors plus metribuzin early POST in soybean and corn in the near future with inclusion of the corn safener cyprosulfamide and following introduction of HPPD-resistant soybean technology.

The research described herein explores potential control options for two HPPD- and *s*-triazine-resistant waterhemp populations, SIR (from Stanford, Illinois; same field site as the MCR population described in Hausman *et al.* 2011) and NEB (from Nebraska). Both populations demonstrated enhanced mesotrione metabolism via cytochrome P450 monooxygenases (P450s) (Ma *et al.*, 2013; Kaundun *et al.*, 2017). The first objective was to determine the level of resistance to two HPPD-inhibiting herbicides; one that both populations had been exposed to previously (mesotrione) and another that had not been applied to either population (isoxaflutole). Herbicides were applied at two different heights (5- or 10-cm) to simulate different POST spray timings. For the second objective, joint activity of either mesotrione or isoxaflutole at varying rates combined with a single rate of metribuzin POST was evaluated in SIR and NEB. This objective was designed to test two interconnected hypotheses: (1) HPPD-inhibitor activity contributes to synergism in a tank mix with metribuzin, and (2) metabolic atrazine resistance can be overcome by using a different PSII inhibitor (metribuzin). This is based on our assumption that the glutathione *S*-transferase (GST) most likely responsible for detoxifying atrazine in waterhemp (Evans *et al.*, 2017) may only recognize specific herbicide substrates within the

triazine family (i.e., *s*-triazines), and thus typical herbicide activity might be observed in multiple-resistant plants.

3.3 Materials and Methods

3.3.1 Waterhemp populations

Seeds from all populations were stratified in 0.1% agarose solution at 4ºC for 30 days. A listing of all populations and their corresponding documented resistances are shown in Table 3.1.

3.3.2 Greenhouse plant culture

All plants used in these experiments were germinated from seeds sown in 12 x 12 cm flats containing a commercial potting medium[1]. Emerged seedlings (2 cm) were transplanted into 950 cm^3 pots (one seedling per pot) containing a 3:1:1:1 mixture of potting mix:soil:peat:sand that included a slow-release fertilizer[2]. Greenhouse conditions were maintained at 28/22º C day/night with a 16:8 h photoperiod. Natural sunlight was supplemented with mercury halide lamps to provide 800 μmol m^{-2} s^{-1} photon flux at the plant canopy.

3.3.3.1 Quantifying resistance to foliar-applied isoxaflutole or mesotrione at two different timings

Uniformly sized waterhemp plants were treated with isoxaflutole or mesotrione. The early postemergence (EPOST) timing was applied when plants reached 5 cm in height with 4-5

true leaves. The POST timing was applied when plants reached 10 cm in height with 8-9 true leaves. The HPPD inhibitors and their respective application rates included isoxaflutole ranging from 0.8 to 420 g ha^{-1} and mesotrione from 1.6 to 840 g ha^{-1}, with a log(2) spacing factor. The formulation of isoxaflutole did not include the maize safener cyprosulfamide. Herbicides were applied using a compressed air research sprayer[3] fitted with a TeeJet 80015 EVS nozzle[4] calibrated to deliver 185 L ha^{-1} at 275 kPa. All treatments included methylated seed oil (MSO, 1% v/v) and liquid ammonium sulfate (AMS, 2.5% v/v). Following application, plants were placed on greenhouse benches in a randomized complete block design. Each treatment was replicated 3 times, and the experiment was conducted three times. Visual assessment of plant responses was recorded at 7, 14 and 21 days after treatment (DAT) using a scale ranging from 0 (no plant injury) to 100 (plant mortality). At 21 DAT, all aboveground plant tissue was harvested and dried at 65ºC for 7 days. Dry weights were recorded and converted to a percentage of the untreated control for each population. Dry weight data were analyzed using a non-linear regression model with the dose-response curve package (Knezevic *et al.* 2007) in the R statistical computing environment.[5] The dose-response model was constructed using the equation:

$$y = c + \frac{d - c}{1 + \exp\{b[\log(x) - \log(GR50)]\}}$$

The four-parameter non-linear logistic model is described as follows: *b* is the slope of the curve, *c* is the lower limit, *d* is the upper limit and GR_{50} is 50% reduction in dry weight. The GR_{50} values were compared using the linear mixed-effects model procedure in R. After comparisons were made, population, treatment, and the interaction of population and treatment were determined significant ($F_{3,144} = 21.5$, $p < 0.001$).

3.3.3.2 Response to foliar-applied HPPD inhibitors combined with metribuzin

Waterhemp plants from the SIR and NEB populations were grown under the greenhouse conditions described in Section 3.3.2. Waterhemp plants 10 cm in height (8-9 true leaves) were treated with isoxaflutole, mesotrione or metribuzin alone or in a tank-mix combination of an HPPD inhibitor and metribuzin. The HPPD inhibitors and their respective application rates were isoxaflutole at 26.3, 52.5 and 105 g ha^{-1} and mesotrione at 52.5, 105 and 210 g ha^{-1}. Though isoxaflutole is typically applied preplant incorporated or PRE, the label states that it can be applied early POST in corn. Mesotrione can also be applied PRE or POST. Metribuzin was applied alone at 191 g ha^{-1}. This metribuzin rate was determined based on an initial dose-response study to determine a rate that elicited an approximate 20% biomass reduction. Herbicide treatment applications were made using the compressed air research spray system described above. All treatments included methylated seed oil (MSO, 1% v/v) and liquid ammonium sulfate (AMS, 2.5% v/v). Following application, plants were placed on greenhouse benches in a randomized complete block design (by rep and position on the bench) with seven replicate blocks. The experiment was conducted three times, using a different randomization scheme each time. Visual assessment of plant responses was recorded at 7 and 14 DAT using a scale ranging from 0 to 100 as described previously. At 14 DAT, all aboveground plant tissue was harvested and dried at 65^{o} C for 7 days.

The assessment of joint activity of herbicides with independent modes of action is most commonly calculated using variants of the Multiplicative Survival Model (MSM) (Flint *et al.*, 1988; Green *et al.*, 1997; Kelly and Chapman, 1995; Streibig *et al.*, 1998), where the MSM utilizes Colby's equation for herbicide joint action through analysis of various quantitative

observations, including plant biomass (fresh or dry weight), growth suppression, or percent survival (Colby, 1967; Gowing, 1960). Colby's equation is expressed as:

$$E = \frac{(XY)}{100}$$

Values are expressed as growth (or dry weights) as a percent of control; E is the expected growth with application of herbicides A + B in combination; X and Y are the growth observed with herbicide A or B, respectively, applied at specific rates.

While Colby's equation is a commonly used method for calculating herbicide joint activity within the MSM, a statistical test for analyzing herbicide joint activity is still necessary. A model proposed by Flint *et al.* (1988) was used to apply a statistical test to Colby's equation by utilizing slope comparisons. With regards to determining increased herbicidal activity when combining mesotrione or isoxaflutole with metribuzin POST in this research, the experiments were designed for application to a wide range of growth data, and to fit the statistical method described by Flint *et al.* (1988), which is similar to previous work on mesotrione-atrazine interactions (Hugie *et al.*, 2008; Woodyard *et al.*, 2009a; 2009b). A log-transformation was performed on the plant dry weight biomass data to account for heterogeneity of variances and allow slope comparisons (Flint *et al.* 1988), and to describe nonlinear effects of herbicide joint action (Hugie *et al.*, 2008; Woodyard *et al.*, 2009a; 2009b), where mesotrione, isoxaflutole and metribuzin alone and in combination were compared with control plants to determine joint herbicide action in the resistant populations. The herbicide joint action analysis was performed using the mixed procedure in SAS[6] software for slope comparisons (SAS, 2004).

3.4 Results and Discussion

3.4.1 Quantifying resistance to foliar-applied isoxaflutole or mesotrione at two different plant heights

Treatment of SIR, NEB, ACR, and SEN plants with a range of isoxaflutole rates resulted in typical herbicide-response curves, with decreasing dry weights observed with increasing rates EPOST (Figure 3.1) and POST (Figure 3.2). GR_{50} values determined with curve-fitting software were 21.5, 7.8, 2.1, and 0.9 g isoxaflutole ha^{-1} for SIR, NEB, ACR, and SEN, respectively, at the EPOST treatment. For the POST treatment, the GR_{50} values were 31.9, 6.3, 1.8, and 1.2 g isoxaflutole ha^{-1}, respectively. ACR and SEN were not significantly different from each other, so only ACR was used as the sensitive population to determine fold-resistance levels. Based on these GR_{50} values, the relative level of resistance to isoxaflutole in SIR was 10-fold EPOST and 17-fold POST, compared with 4-fold EPOST and 3-fold POST in NEB.

Treatment to all populations with a range of mesotrione rates resulted in typical herbicide response curves as well, with decreasing dry weights observed with increasing rates EPOST (Figure 3.3) and POST (Figure 3.4). GR_{50} values were 149, 34.8, 4.7, and 4.2 g mesotrione ha^{-1} for SIR, NEB, ACR, and SEN, respectively, at the EPOST treatment. GR_{50} values were 104, 35.2, 4.9, and 4.9 g mesotrione ha^{-1} for the POST treatment, respectively. As with isoxaflutole, ACR and SEN were not significantly different from each other, so only ACR was used as the sensitive population. The relative level of resistance to mesotrione in SIR was 32-fold EPOST and 21-fold POST, compared with 7-fold for both EPOST and POST for NEB. Visual assessments of plant injury 21 DAT were consistent with dry weight data in revealing different rate responses among the populations for both herbicides (data not shown).

Overall these findings indicated greater fold-resistance to mesotrione relative to isoxaflutole within each timing. Both the SIR and NEB populations were previously exposed to mesotrione, which may have selected for greater resistance to mesotrione relative to isoxaflutole (Hausman *et al.*, 2011; Kaundun *et al.*, 2017). However, POST treatments to SIR showed contrasting effects on fold-resistance levels relative to EPOST. For example, the fold-resistance in SIR to isoxaflutole increased while the resistance levels in SIR to mesotrione decreased. These opposite effects result from an increase in the GR_{50} at the POST timing relative to EPOST in SIR following isoxaflutole treatment, combined with a decrease in the GR_{50} at the POST timing relative to EPOST in ACR. In contrast, the inverse of this scenario occurred following mesotrione treatment in each population. The underlying basis for the different trends in R/S ratios between herbicide treatments as plant height increased may be due to several reasons: biokinetic factors such as herbicide uptake and translocation, herbicide metabolism or P450 expression differences between plant heights, or other physiological factors.

SIR is more resistant than NEB as compared to ACR at all treatments (Figures 3.1-3.4). The mechanism of resistance in the MCR waterhemp population from McLean County, IL, USA was attributed to rapid herbicide metabolism via P450s (Ma *et al.*, 2013), which is similar to previous results in the NEB population (Kaundun *et al.*, 2017). The metabolism of mesotrione in corn is also due to P450-catalyzed ring hydroxylation (Hawkes *et al.*, 2001). It is still unknown how many P450s are responsible for rapid mesotrione metabolism in HPPD-resistant waterhemp. Based on our results, it is possible that the SIR population more rapidly metabolizes isoxaflutole and mesotrione, which could be attributed to differences in P450 expression in the leaves or higher substrate specificity for herbicide-detoxifying P450s in SIR relative to NEB.

Surprisingly, the MCR population (same field site as SIR) had a GR_{50} value of 48.5 g ha^{-1} (Hausman *et al.*, 2011), which is significantly lower than the GR_{50} value of 162 g ha^{-1} for mesotrione in the NEB population (Kaundun *et al.*, 2017). However, differences exist between these studies. MCR plants were treated with mesotrione at 10- to 12-cm whereas NEB plants were treated at 7 cm. NEB plants were grown under different growing conditions, with a light intensity of 180 µmol $m^{-2}s^{-1}$ and temperatures of 24/18ºC day/night (Kaundun *et al.*, 2017). In this study, all populations were grown under 800 µmol m^{-2} s^{-1} photon flux at the plant canopy with 28/22º C day/night cycles (Hausman *et al.*, 2011). These differences in growth parameters might account for the different results obtained between our studies and previous research. HPPD-inhibiting herbicides indirectly inhibit carotenoid biosynthesis (Hess, 2000). Carotenoids play a number of important roles in plants, but the most critical one is to protect chlorophyll from photodegradation under high light intensity by quenching the excess energy released (Ramel *et al.*, 2012). Because NEB and MCR were exposed to different light intensities and temperatures during the experiments, this could have a direct impact on how active the herbicide was within the treated plants and thus affected fold-resistance levels.

3.4.2 Response to foliar-applied HPPD inhibitors combined with metribuzin

Synergism between mesotrione and metribuzin was detected in SIR plants when 52.5 g ai ha^{-1} of mesotrione was paired with metribuzin at a constant rate of 191 g ai ha^{-1} in a tank mix (Table 3.2). However, the estimate values indicated an additive effect as the rate of mesotrione increased in combination with metribuzin. As the rate of mesotrione increased, more biomass reduction occurred from mesotrione alone. Therefore, a significant interaction between the two herbicides was not determined. The absence of a synergistic interaction is similar to a

metabolism-based atrazine resistant velvetleaf biotype, when mesotrione and atrazine were applied in temporally-spaced herbicide applications (Woodyard *et al.,* 2009b). The underlying mechanism was attributed to the ability of the velvetleaf biotype to rapidly detoxify the atrazine, which could prevent the interaction from occurring (Woodyard *et al.*, 2009b).

Antagonism was not detected at any mesotrione or isoxaflutole rates (Table 3.2, Table 3.3, Table 3.4, Table 3.5) (*i.e.* no mixture provided significantly less control than expected, or gave a positive estimate that was closer to 1). However, due to the fact that rates were chosen based on our previous dose-response data, we did not anticipate that the stand-alone rates would result in complete death in these populations. While the combinations of mesotrione or isoxaflutole with metribuzin did not result in synergistic activity in the NEB population, the results are additive (Table 3.4, Table 3.5). Additive activity was also observed in the SIR population in regards to the isoxaflutole and metribuzin combinations (Table 3.3) and the 105 and 210 g ai ha^{-1} rates of mesotrione combined with metribuzin (Table 3.2). Again, our statistical analysis shows estimates close to 0, and associated P values that are not significant (> 0.05), which results in an additive response for these tank-mix combinations.

The lack of detecting synergism with isoxaflutole plus metribuzin may be due to the fact that isoxaflutole is a proherbicide (Pallett *et al.,* 1998). Proherbicides are inactive, non-phytotoxic chemicals that are metabolized by enzymes within the plant to yield an active, herbicidal metabolite (Jeschke, 2016). Isoxaflutole is applied in an inactive form, but following plant uptake it is converted to the diketonitrile (DKN) active metabolite (Pallett *et al*., 1998; 2001). DKN acts upon the HPPD enzyme to prevent the conversion of HPPA to HGA (Pallett *et al.*, 1998). We hypothesize that the conversion of isoxaflutole to DKN could be a rate-limiting step in determining the timing of foliar activity of HPPD-inhibiting herbicides within these

populations in relation to metribuzin. In contrast, mesotrione is applied in its active form and therefore does not require a bioactivation step, which may allow it to directly interact with the phytotoxicity caused by metribuzin.

Metribuzin is an *as*-triazine herbicide while atrazine is an *s*-triazine. Within the PSII inhibitor class of herbicides, all triazine herbicides interact with the D1 protein in a similar manner (Fuerst *et al.*, 1986; Hess, 2000). However, differences in metabolism for these two triazines exist among crops and weeds. Atrazine is typically applied in corn and grain sorghum because they possess high levels of GSTs that detoxify atrazine via conjugation with reduced glutathione (Dixon *et al.*, 2002). Metribuzin is typically applied in soybeans, tomatoes, and potatoes, but is also applied PRE in corn. Metribuzin is detoxified via rapid GST-catalyzed conjugation with homoglutathione within soybeans, which is a reaction unique to legumes (Frear *et al.*, 1985). Though there is a difference in metabolism due to the form of glutathione that is conjugated with the substrate, there is a possibility that the GST(s) responsible for this metabolism in waterhemp is only able to recognize one form of the herbicide. Thus, it is possible that the GST(s) responsible for metabolism-based atrazine resistance in these waterhemp populations (Evans *et al.*, 2017) recognize atrazine for detoxification but do not recognize metribuzin as a substrate, despite the fact that it is still a triazine. This potential scenario differs from that in site-of-action based TR dicots, where mutations in the *psbA* gene (encoding the D1 protein) typically confer cross-resistance to atrazine and metribuzin (Fuerst *et al.*, 1986).

This unique combination of isoxaflutole or mesotrione combined with metribuzin POST has not been previously investigated. Our results show synergism between mesotrione and metribuzin in SIR plants when 52.5 g ai ha^{-1} of mesotrione was paired with metribuzin at a constant rate of 191 g ai ha^{-1} in a tank mix (Table 3.2). These findings provide fundamental

insight into how a metabolism-based, HPPD- and atrazine-resistant waterhemp population can be controlled using tools already available. The rates used in our research that elicited this synergistic response are both below typical field-use rates, which is important for the environment and the sustainability of these herbicides in the future.

3.4.3 Implications and future research

The evolution of herbicide resistance within waterhemp continues to increase (Heap, 2017), without the introduction of any new novel modes of action being commercialized (Cole *et al.*, 2000; Duke and Dayan, 2015). Effective utilization of the herbicides still currently available is thus imperative until a new herbicide is brought to market. Previous research has demonstrated the value of the synergistic interactions between HPPD and PSII inhibitors, in particular in herbicide-resistant weeds (Hugie *et al.*, 2008; Woodyard *et al.*, 2009b; Hausman *et al.*, 2013). Our research also shows that even in an atrazine- and HPPD-resistant population, the combination of metribuzin and mesotrione or isoxaflutole can be an option for control in corn production.

Tank mixing herbicides with different sites of action, rather than applying either herbicide alone or in rotation, is a proposed method for delaying the development of resistance (Evans *et al.*, 2016). While our interaction study determined that only a single rate combination (among those tested) was synergistic on one HPPD- and atrazine-resistant population, it provides insight into how the combination of two herbicides can be used as a control option for metabolism-based, multiple-resistant waterhemp. It is important to note, though, that a biotype of rigid ryegrass (*Lolium rigidum*) in Australia was found to be resistant to atrazine due to a target

site mutation, and upon further testing, was also found to be cross resistant to metribuzin, even though there were no reports of previous exposure to metribuzin (Burnet *et al.,* 1991). It is therefore critical to know exactly what type of resistance (TS vs. NTSR) you are dealing with before following a recommendation and making management decisions.

Future research should be conducted to further understand the observed effects as indicated by our data at the other rate combinations. Previous studies have included many more combinations of an HPPD inhibitor and a PSII inhibitor, such as where the rates of each herbicide were varied, where the other was held constant. This would help us determine the directionality of this synergistic interaction, whether the HPPD inhibitor or metribuzin is more responsible for the biomass reductions we observed in our experiments. Before a recommendation could be made to farmers as to how specific herbicide combinations could be beneficial, a study that included more combinations of rates, such as those that utilize a GR_{50} rate of an HPPD inhibitor with various metribuzin rates, would need to be done. In our synergism study, we used 0x, 0.5x, 1x and 2x field use rates for isoxaflutole and mesotrione. If you compare this to the actual GR_{50} values identified in our dose-response study, for example, the GR_{50} rate for isoxaflutole was 21.5 at the EPOST treatment and 31.9 for the POST treatment for the SIR population. Our research could provide more insight and give us a better understanding of beneficial tank mixes with controlling multiple resistant waterhemp populations.

3.5 Source of Materials

[1]LC1. Sun Gro Horticulture, 15831 N.E. 8th Street, Bellevue, WA 98008.

[2]Osmocote 13-13-13 slow release fertilizer. The Scotts Company, 14111 Scottslawn Rd.,

Marysville, OH 43041.

[3]Generation III Research Sprayer. DeVries Manufacturing, 28081 870th Ave., Hollandale, MN 56045.

[4]TeeJet 80015EVS. TeeJet Technologies, P.O. Box 7900, Wheaton, IL 60187.

[5]R. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org

[6]SAS. SAS Institute Inc., SAS Circle, Box 8000, Cary, NC 27512-8000.

3.6 Tables and Figures

Table 3.1 Waterhemp populations used in this study and their known herbicide resistances.

Population	Resistances[a]
SIR – Stanford, IL[b]	HPPD, PSII[c], ALS
NEB – Platte Co., NE	HPPD, PSII[c]
ACR – Adams Co., IL	PPO, ALS, PSII
SEN – Azlin Seed; Leland, MS	None

[a] Resistances listed in Table 3.1 are further described at weedscience.com. [b] Same field site as the MCR population described by Hausman *et al.*, 2011. [c] PSII resistance is to *s*-triazines (*i.e.* atrazine) specifically.

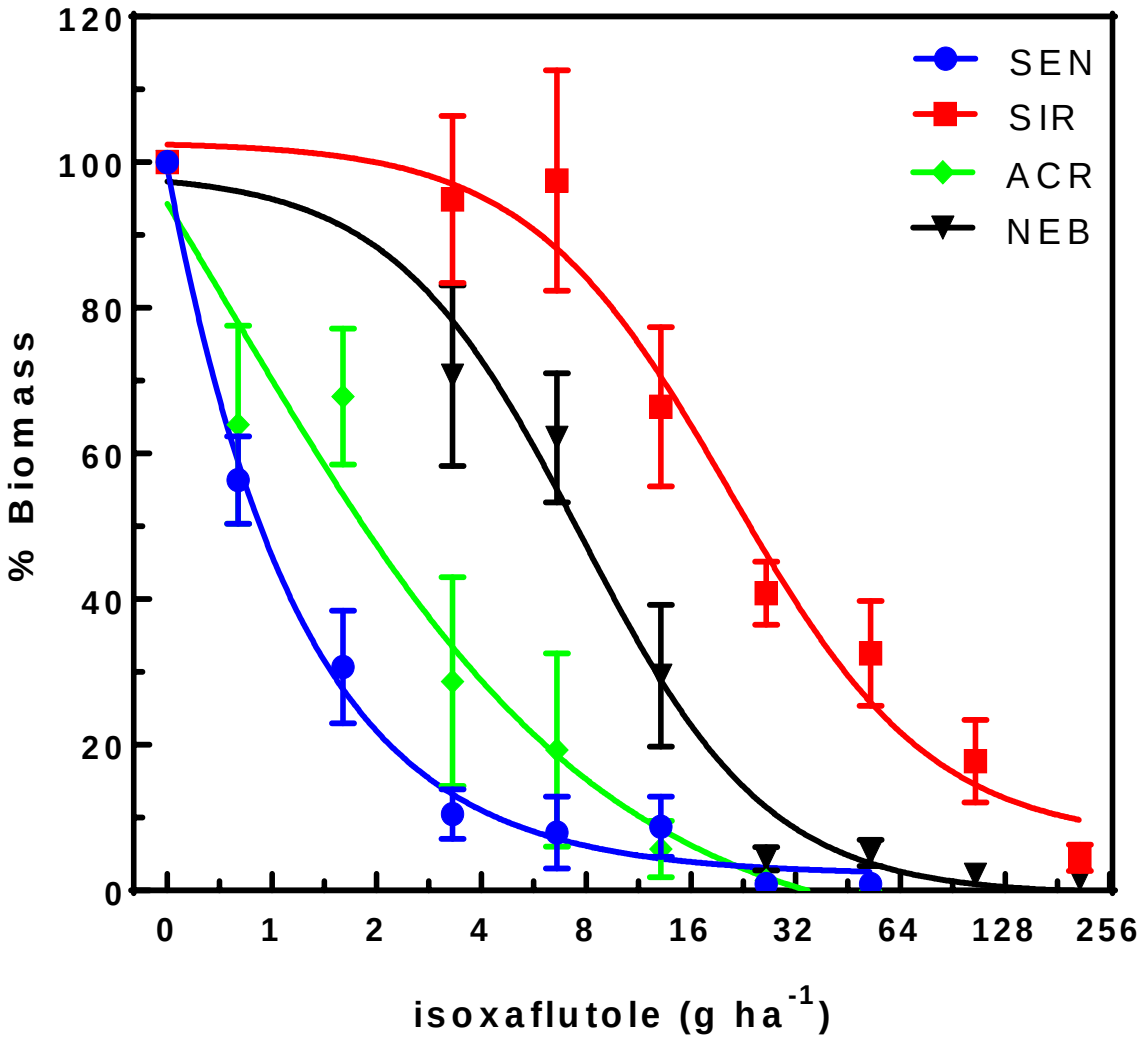

Figure 3.1 Isoxaflutole dose-response curves (EPOST timing) for HPPD-inhibitor-sensitive populations SEN and ACR and the HPPD-inhibitor-resistant populations SIR and NEB. Plant dry weights were obtained 21 DAT. Plants were treated EPOST at 5 cm in height (4-5 true leaves).

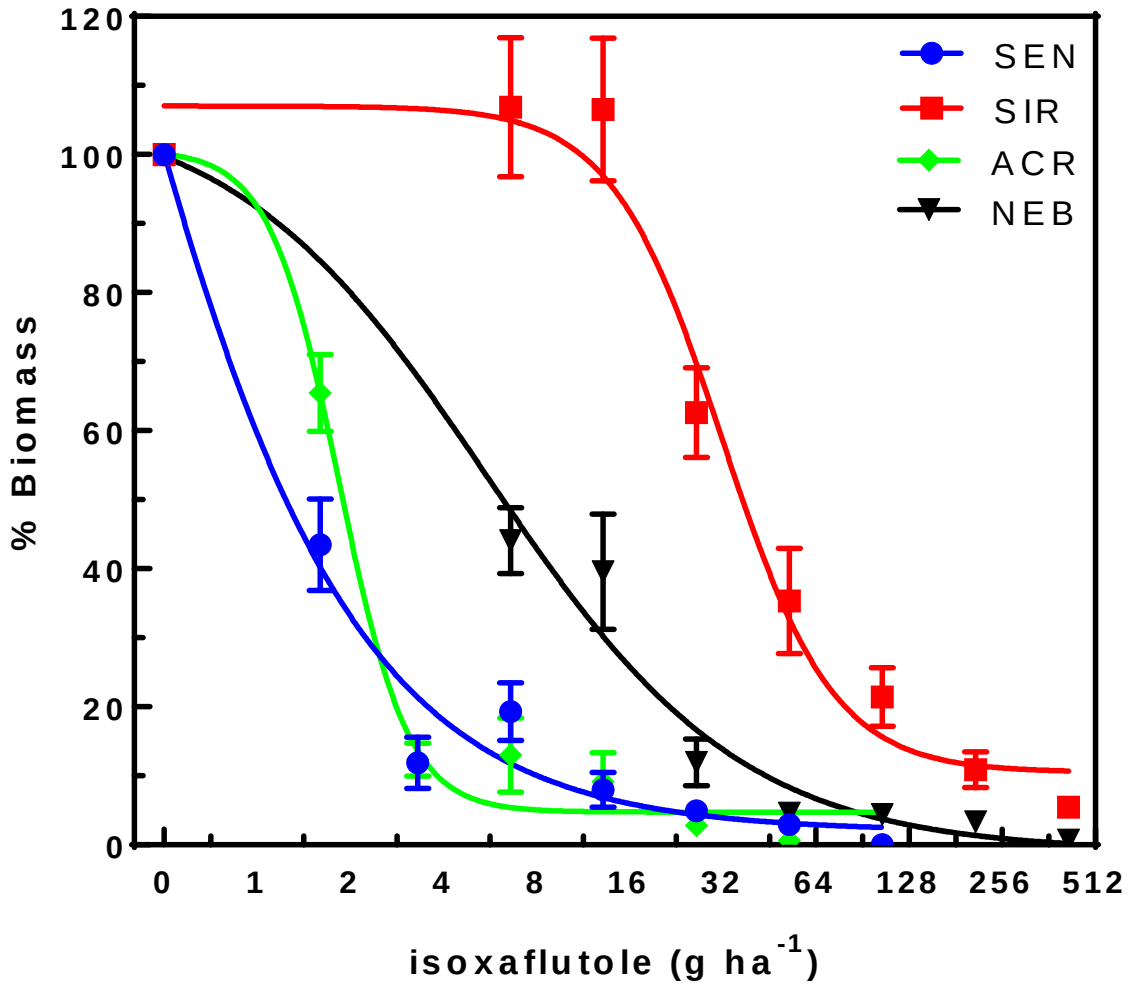

Figure 3.2 Isoxaflutole dose-response curves (POST timing) for HPPD-inhibitor-sensitive populations SEN and ACR and the HPPD-inhibitor-resistant populations SIR and NEB. Plant dry weights were obtained 21 DAT. Plants were treated POST at 10 cm in height (8-9 true leaves).

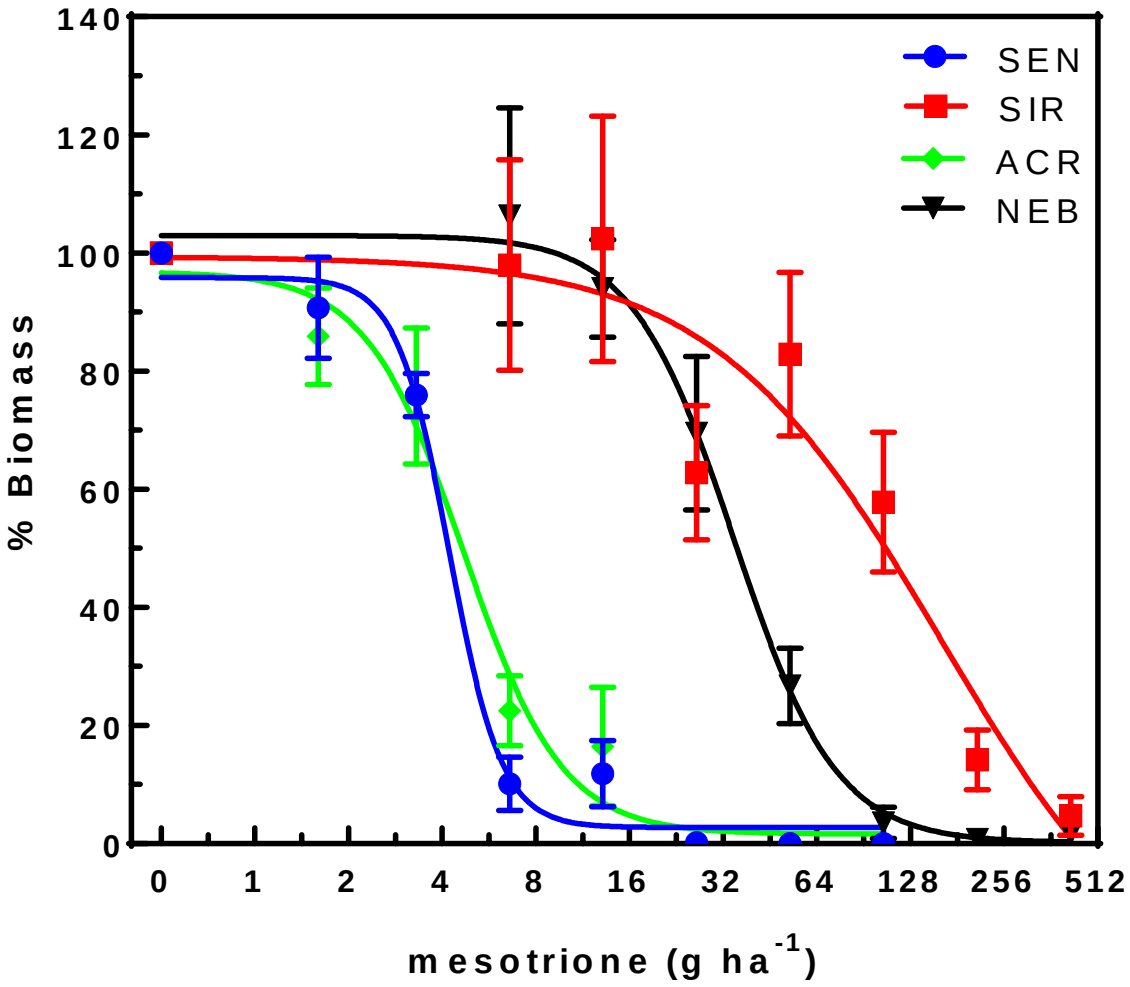

Figure 3.3 Mesotrione dose-response curves (EPOST timing) for HPPD-inhibitor-sensitive populations SEN and ACR and the HPPD-inhibitor-resistant populations SIR and NEB. Plant dry weights were obtained 21 DAT. Plants were treated EPOST at 5 cm in height (4-5 true leaves).

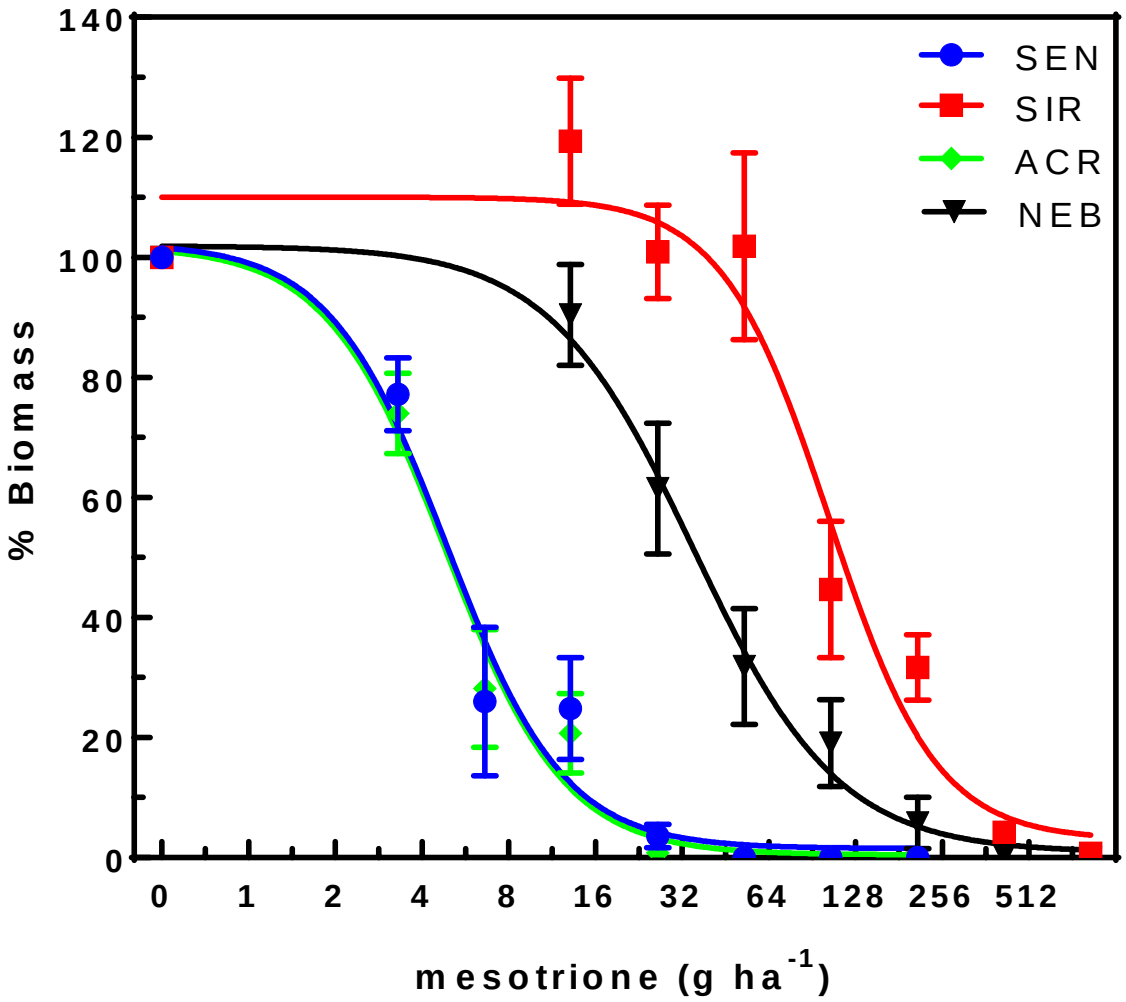

Figure 3.4 Mesotrione dose-response curves (POST timing) for HPPD-inhibitor-sensitive populations SEN and ACR and the HPPD-inhibitor-resistant populations SIR and NEB. Plant dry weights were obtained 21 DAT. Plants were treated POST at 10 cm in height (8-9 true leaves).

Table 3.2 Herbicide joint activity of mesotrione and metribuzin applied in a tank-mix in Stanford, IL-resistant (SIR) waterhemp population. Joint activity was determined as described in Materials and Methods.

Herbicide rate (g ai ha^{-1})				
Mesotrione	Metribuzin	P value	Estimate[a]	Joint activity
52.5	191	0.0423	-0.00092 (±3.6E-4)	Synergistic
105	191	0.1175	-0.00066 (±3.6E-4)	Additive
210	191	0.5750	-0.00021 (±3.6E-4)	Additive

[a] Estimate, deviation of slope magnitude from parallel or assumed "additivity" determined from reduction in aboveground plant biomass.

Table 3.3 Herbicide joint activity of isoxaflutole and metribuzin applied in a tank-mix in Stanford, IL-resistant (SIR) waterhemp population. Joint activity was determined as described in Materials and Methods.

Herbicide rate (g ai ha^{-1})				
Isoxaflutole	Metribuzin	P value	Estimate[a]	Joint activity
26.3	191	0.3958	0.000296 (±3.2E-4)	Additive
52.5	191	0.5949	0.000182 (±3.2E-4)	Additive
105	191	0.5384	0.000211 (±3.2E-4)	Additive

[a] Estimate, deviation of slope magnitude from parallel or assumed "additivity" determined from reduction in aboveground plant biomass.

Table 3.4 Herbicide joint activity of mesotrione and metribuzin applied in a tank-mix in Nebraska-resistant (NEB) waterhemp population. Joint activity was determined as described in Materials and Methods.

Herbicide rate (g ai ha^{-1})				
Mesotrione	Metribuzin	P value	Estimate[a]	Joint activity
52.5	191	0.4558	-0.00038 (±4.8E-4)	Additive
105	191	0.4986	0.000342 (±4.8E-4)	Additive
210	191	0.3001	0.000539 (±4.8E-4)	Additive

[a] Estimate, deviation of slope magnitude from parallel or assumed "additivity" determined from reduction in aboveground plant biomass.

Table 3.5 Herbicide joint activity of isoxaflutole and metribuzin applied in a tank-mix in Nebraska-resistant (NEB) waterhemp population. Joint activity was determined as described in Materials and Methods.

Herbicide rate (g ai ha^{-1})				
Isoxaflutole	Metribuzin	P value	Estimate[a]	Joint activity
26.3	191	0.1074	0.000902 (±4.8E-4)	Additive
52.5	191	0.1703	0.000743 (±4.8E-4)	Additive
105	191	0.1539	0.000778 (±4.8E-4)	Additive

[a] Estimate, deviation of slope magnitude from parallel or assumed "additivity" determined from reduction in aboveground plant biomass.

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CHAPTER 4

SYNOPSIS OF RESEARCH AND FUTURE IMPACTS

4.1 Synopsis and Impacts

Waterhemp (*Amaranthus tuberculatus*) is one of the worst agronomic weeds in Illinois and across the Midwest in regards to corn and soybean production. It is a summer annual, with prolonged germination throughout the entire growing season, and possesses C_4 photosynthetic physiology, which gives this weed the inherent ability to grow rapidly and voraciously compete with crops for limited resources. Waterhemp is dioecious, which ensures that the genes enabling herbicide resistance are rapidly spread through many different waterhemp populations (Steckel, 2007; Hausman *et al.*, 2011; Ma *et al.*, 2013). The ability for this one species to evolve resistance to herbicides at an astronomical rate has caused headaches for farmers and chemical companies alike. Not only is herbicide resistance a problem for farmers, but one female waterhemp plant is capable of producing almost 1 million seeds (Steckel *et al.*, 2003), which can exacerbate an already enormous problem if not controlled early in the growing season.

The research presented herein demonstrates how difficult it can be to control herbicide-resistant waterhemp populations. Non-target site atrazine resistance in waterhemp could be more common than growers realize, and unfortunately, metabolic resistance in dicots is not well characterized and remains markedly under-explored, particularly regarding the underlying biochemical mechanisms, enzymes and specific genes in these species (Anderson and Gronwald, 1991; Gray *et al.*, 1996; Ma *et al.*, 2013). As my research with the segregating F_2 population demonstrated, these populations could survive applications to more than 28 times the current field use rate of atrazine. Homozygous (RR) resistant plants withstood applications up to

28x the typical field use rate (1000 g ha^{-1}) of atrazine. The GST we identified that is associated with atrazine resistance is expressed 200- to 1140-fold higher in atrazine-resistant plants as compared to sensitive plants. While this may not be true for all metabolic-resistant waterhemp populations, our research demonstrates how important metabolic-based atrazine resistance is and why farmers need to be aware of their control options. Our results from these studies can lead to beneficial research in the future, including new insights for biotechnology applications aimed toward overcoming metabolic resistance in weedy plants and as a new marker for identification of other metabolism-based atrazine-resistant waterhemp biotypes.

One important area of research that should be conducted in regards to our results from the atrazine study is identifying whether this *GST* is the single gene responsible for this resistance, or if another mechanism, such as gene copy number, could be influencing the high levels of expression we observed. *AtuGSTF2* displayed higher constitutive expression in both atrazine-resistant waterhemp populations as well as in resistant F_2 lines segregating as a single-gene trait. Greater transcript abundance of the *AtuGSTF2* gene may contribute to elevated GST activity (data not shown) and higher levels of GSH-atrazine metabolites formed in ACR and MCR compared with WCS (Ma *et al.*, 2013). Thus far, higher GST-specific activities with atrazine quantified in partially purified ACR and MCR protein extracts can only be associated with higher constitutive expression of *AtuGSTF2*. Further experiments are required, however, to obtain the entire open reading frame for expression and biochemical analyses of the recombinant AtuGSTF2 enzyme (with atrazine as substrate) because plant genomes contain dozens of *GST* genes and isozymes (Chi *et al.*, 2011; McGonigle *et al.*, 2000; Riechers *et al.*, 2010) that contribute to total activity. From the standpoint of gene regulation, the potential for induction

of *AtuGSTF2* expression by atrazine pretreatment in waterhemp should be examined in future research.

Higher basal expression levels of *AtuGSTF2* could be due to a mutation, insertion or varying degrees of methylation in the *AtuGSTF2* promoter or untranslated regions (Mahmood *et al.*, 2016), an alteration in a DNA-binding protein, or a protein regulating mRNA stability. *GSTs* are unevenly dispersed throughout plant genomes (Dong *et al.*, 2016; Lan *et al.*, 2009) or found in clusters of duplicated genes (Soranzo *et al.*, 2004; Xu *et al.*, 2002), thus promoting gene evolution and functional diversification (Kaltenegger and Ober, 2015; Liu *et al.*, 2013). Alternatively, a mutation in a single transcription factor (TF) protein that binds to *GST* promoters could coordinately activate the expression of multiple *AtuGST*s. An analogous situation occurs with the maize Opaque-2 TF protein and DNA-binding One Zinc Finger (Dof) protein OBP1 (Noguero *et al.*, 2013), which together regulate the transcription of numerous zein genes (Li *et al.*, 2015; Vicente-Carbajosa *et al.*, 1997). Interestingly, an OBP1 protein identified in *Arabidopsis* regulates expression of the *GST6* gene (renamed *AtGSTF8*; Wagner *et al.*, 2002) in a similar manner (Chen *et al.*, 1996). However, additional genomic sequence analyses of *AtuGSTF2* from resistant and sensitive plants are required to fully understand the resistance mechanism, because our alignments only represent an estimated 30% of the *AtuGSTF2.1* coding sequence.

Basal expression levels of *AtuGSTF2* could be used as a molecular marker for screening putative resistant waterhemp populations to exclude or confirm metabolic resistance to atrazine. A similar approach was utilized following the discovery of a key mutation associated with metabolic resistance to dichlorodiphenyl-trichloroethane (DDT) in the mosquito *Anopheles funestus* (Riveron *et al.*, 2014). A genome-wide transcriptional analysis was conducted in which

the most highly upregulated gene was identified as a *GST* (termed *GSTe2*), which was confirmed to confer resistance to DDT and cross-resistance to pyrethroid insecticides through transgenic expression in sensitive *Drosophila*. The molecular basis for resistance resulted from both quantitative and qualitative mechanisms; increased expression in resistant mosquitos combined with a point mutation in the wild-type *GSTe2* gene in which LEU was substituted for PHE (Riveron *et al.*, 2014).

In addition to improved resistance screening methods, the coding sequence of *AtuGSTF2* could be utilized to engineer targeted gene knockout strategies such as RNAi directed to a specific *Amaranthus GST* (Yu and Powles, 2014) or to synthesize new chemical inhibitors of herbicide-detoxifying GSTs (Cummins *et al.*, 2013; Lamoureux and Rusness, 1986; Ma *et al.*, 2016). RNAi-based knockdown techniques have been used successfully in insect systems where insecticide-detoxifying P450s have been targeted (Bautista *et al.*, 2009; Zhu *et al.*, 2016), thus regaining activity of the insecticide in resistant populations. Polynucleotide-based gene knockdown systems are also being generated to overcome herbicide resistance in weeds (Sammons *et al.*, 2015), which to date have been targeted primarily towards herbicide target-site proteins. However, additional knowledge of specific herbicide-detoxifying isozymes in weeds, such as those belonging to large, multigene GST and P450 families, provides a new opportunity to regain herbicide activity in multiple-resistant weeds. The findings presented herein support the conclusion that increased basal expression of a specific herbicide-detoxifying GST is associated with atrazine resistance in MCR and ACR, which may ultimately confer atrazine resistance, but might also lead to innovative and integrated weed management strategies.

The results of my HPPD dose-response and synergy study bring about a more practical way farmers can start to deal with some of these resistant waterhemp populations in their fields,

using tools that are currently available. My HPPD dose-response study was conducted using four different populations (SIR, NEB, ACR and SEN), treated with isoxaflutole or mesotrione, at two different postemergence heights: early postemergence (EPOST) at 5 cm, and postemergence (POST) at 10 cm. Three takeaways from this study included: There was a greater fold-resistance to mesotrione relative to isoxaflutole within each timing. Both the SIR and NEB populations were previously exposed to mesotrione, which may have selected for greater resistance to mesotrione relative to isoxaflutole (Hausman *et al.*, 2011; Kaundun *et al.*, 2017). The fold-resistance in SIR to isoxaflutole increased while the resistance levels in SIR to mesotrione decreased. Overall, though, SIR is more resistant than NEB as compared to ACR at all treatments. The underlying basis for the different trends in R/S ratios between herbicide treatments as plant height increased may be due to several reasons: biokinetic factors such as herbicide uptake and translocation, herbicide metabolism or P450 expression differences between plant heights, or other physiological factors.

The mechanism of resistance in the MCR waterhemp population from McLean County, IL, USA was attributed to rapid herbicide metabolism via P450s (Ma *et al.*, 2013), which is similar to previous results in the NEB population (Kaundun *et al.*, 2017). It is still unknown how many P450s are responsible for rapid mesotrione metabolism in HPPD-resistant waterhemp. Based on our results, it is possible that the SIR population more rapidly metabolizes isoxaflutole and mesotrione, which could be attributed to differences in P450 expression in the leaves or higher substrate specificity for herbicide-detoxifying P450s in SIR relative to NEB.

Surprisingly, the MCR population (same field site as SIR) had a GR_{50} value of 48.5 g ha^{-1} (Hausman *et al.*, 2011), which is significantly lower than the GR_{50} value of 162 g ha^{-1} for mesotrione in the NEB population (Kaundun *et al.*, 2017). However, differences exist between

these studies. MCR plants were treated with mesotrione at 10- to 12-cm whereas NEB plants were treated at 7 cm. NEB plants were grown under different growing conditions, with a light intensity of 180 µmol $m^{-2}s^{-1}$ and temperatures of 24/18ºC day/night (Kaundun *et al.*, 2017). In this study, all populations were grown under 800 µmol m^{-2} s^{-1} photon flux at the plant canopy with 28/22º C day/night cycles (Hausman *et al.*, 2011). These differences in growth parameters might account for the different results obtained between our studies and previous research. HPPD-inhibiting herbicides indirectly inhibit carotenoid biosynthesis (Hess, 2000). Carotenoids play a number of important roles in plants, but the most critical one is to protect chlorophyll from photodegradation under high light intensity by quenching the excess energy released (Ramel *et al.*, 2012). Because NEB and MCR were exposed to different light intensities and temperatures during the experiments, this could have a direct impact on how active the herbicide was within the treated plants and thus affected fold-resistance levels.

The ability of one weed population to detoxify herbicides, similarly to corn, calls for researchers to start exploring options for control that have never been done previously. My synergy study provides new insight to how farmers could potentially combat resistant plant species, using metribuzin in combination with an HPPD inhibitor. The evolution of herbicide resistance within waterhemp continues to increase (Heap, 2017), without the introduction of any new novel modes of action being commercialized (Cole *et al.*, 2000). Until a new herbicide is brought to market, we need to effectively utilize the herbicides that are still currently available. Previous research has demonstrated the value of the synergistic interactions between HPPD and PSII inhibitors, in particular in herbicide-resistant weeds (Hugie *et al.*, 2008; Woodyard *et al.*, 2009a; 2009b; Hausman *et al.*, 2013). My research also shows that even in an atrazine- and

HPPD-resistant population, the combination of metribuzin and mesotrione or isoxaflutole can be an option for control in corn production.

Unfortunately, many weeds will have resistance to more than one herbicide, and those herbicides may or may not be in related chemical families. Plants with cross-resistance possess a mechanism that provides the plant with the ability to withstand herbicides from different chemical class but within the same site of action. For example, a single point mutation in the enzyme acetolactate synthase may provide resistance to the sulfonylurea and imidazolinone herbicide families. Plants with multiple-resistance possess more than one mechanism that provides plants with the ability to withstand herbicides from different chemical families. In this case, herbicide options are extremely limited, because there could be many different resistance mechanisms within that plant. In relation to our study, experiments have been conducted in regards to investigating cross-resistance between metribuzin and atrazine (Fuerst *et al.*, 1986; Holt *et al*., 1993). If the most common mutation exists at the D1 protein (the serine to glycine conversion at amino acid 264), then there is usually resistance to both metribuzin and atrazine (Fuerst *et al.*, 1986). In the case of metabolism-based atrazine resistance, experiments have not been reported that determine the effect of other PSII-inhibitors on these plants. In our case, when we screened confirmed metabolic-based atrazine-resistant populations with metribuzin, they were almost completely killed at the typical field use rate. My synergy study provides evidence that by simply changing the PSII-inhibitor involved in the tank mix, you can regain activity and even kill these metabolic-resistant plants.

Waterhemp and Palmer amaranth are currently the only weeds that have been confirmed resistant to HPPD inhibitors specifically (Heap, 2017). As more multiple-resistant cases of waterhemp biotypes occur we need to stay vigilant. It is our responsibility as weed scientists to

help farmers combat these resistant populations, while also trying to prolong the occurrence of more resistance, using the tools we currently have. In the scenario presented here, an atrazine- and HPPD-resistant population was controlled using a combination of metribuzin and an HPPD inhibitor. This is important, because of three things: First, HPPD inhibitors are relatively expensive. If they are not effective on a specific population, you are essentially just throwing away money. Our results herein show that the combination of metribuzin with mesotrione at a 0.5x rate in the SIR population has a synergistic interaction. This brings about the second point, which is that PSII inhibitors, such as atrazine, have a negative perception when it comes to the environment. There have been reports of triazines being found in ground water, or causing crop injury due to carryover situations. Being able to use a significantly decreased amount of a PSII inhibitor in combination with an HPPD inhibitor, but still gain adequate control of a resistant population, is crucial for sustainability of the ecosystem and the environment.

The third important point to take from this research is the idea of tank mixing herbicides with different sites of action, rather than applying either herbicide alone or in rotation, as a proposed method for delaying the development of resistance. While the results of our synergy study suggest only a single rate that was tested to be synergistic on one of our resistant populations, it provides insight to how the combination of two herbicides can be used as a control option. Future research will need to be performed to further understand the physiological basis for the additive effects our data indicate at the other combinations. We could be observing these results because of the relatively small size of the experiment that was conducted. Previous studies have included many more combinations of an HPPD inhibitor and a PSII inhibitor. Before a recommendation could be made to farmers as to how this particular combination could be beneficial to them, a study that included more combinations of rates, even those that make use

of a GR_{50} rate of an HPPD inhibitor with various metribuzin rates, would need to be done. A past study investigated the synergistic interaction of atrazine and mesotrione by utilizing chlorophyll fluorescence imaging (Woodyard *et al.*, 2009b). This experiment showed that it might be possible that the GST-mediated detoxification system is inducible by either atrazine itself or by reactive oxygen species that are produced in response to the herbicide treatment (Hess, 2000; Woodyard *et al.*, 2009b).

A large field study could give a better idea as to how a combination of metribuzin and isoxaflutole or mesotrione could be used in a real-world situation. Many more combinations and rates of both herbicides could then be applied across a large study. Previous research has explored the directionality of the synergistic response between mesotrione and atrazine, and whether one herbicide or the other was responsible for the reduction in biomass observed in a triazine-resistant redroot pigweed population (Hugie *et al.*, 2008). A field study might provide insight as to how much injury a corn crop would experience from these combinations, if any. Environmental impacts such as drought and sunlight needed for herbicide activation could also be assessed. Ultimately, this could provide more insight and give us a better understanding of just how beneficial this tank mix could be for managing multiple herbicide-resistant waterhemp.

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