MECHANISMS AND CONTROL OF WATERHEMP POPULATIONS RESISTANT TO GROUP 15 HERBICIDES

BY

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DISSERTATION

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ABSTRACT

Herbicides that inhibit very-long-chain fatty acids (VLCFAs) have been applied for preemergence (PRE) control of annual grasses and small-seed broadleaf weeds for over sixty years. Waterhemp is a small-seeded, summer annual weed species native to the Midwestern United States. The species can be challenging to control due to important biological characteristics, such as reproductive output, genetic variability, seed dormancy, and an ability to evolve resistance to herbicides from multiple site-of-action groups. These and other characteristics allow waterhemp to effectively interfere with row crops and reduce seed yield potential. Waterhemp is resistant to herbicides from seven site-of-action groups, with resistance to VLCFA-inhibiting herbicides the most recently evolved.

Control of two Illinois waterhemp populations (CHR and MCR) with the VLCFA inhibitor *S*-metolachlor was much less than expected in field research. An alternative VLCFA-inhibiting herbicide, acetochlor, remained relatively effective. Additional research was conducted to investigate VLCFA-inhibitor efficacy on each population in the field and greenhouse. Experiments identified few effective VLCFA-inhibiting herbicides for controlling CHR, and that both populations (CHR and MCR) displayed reduced sensitivity to acetochlor, dimethenamid-*P*, pyroxasulfone, and *S*-metolachlor under greenhouse conditions. The research presented herein was initiated in 2018 to identify the resistance mechanism(s) within CHR and MCR/SIR to the VLCFA-inhibitor, *S*-metolachlor.

Chapter 1 includes a literature review describing waterhemp biology, waterhemp resistance mechanisms, resistance to soil-applied herbicides, VLCFA-inhibiting herbicides, and herbicide detoxification in plants. Chapter 2 described my initial laboratory experiments that identified rapid *S*-metolachlor metabolism was the resistance mechanism within CHR and SIR.

CHR and SIR metabolized *S*-metolachlor twice as fast as sensitive waterhemp, but equally rapid as corn. Enzyme inhibitor studies suggested glutathione *S*-transferases (GSTs) and cytochrome P450s both might be involved in rapid *S*-metolachlor metabolism. Chapter 3 describes the experiments that directly investigated GSTs and microsomal P450s along with the *S*-metolachlor-treated metabolome of resistant waterhemp. Results identified that CHR and SIR possess approximately 2× more GST activity than sensitive waterhemp, but less than corn. Microsomal assays investigating P450 activity revealed CHR and SIR microsomes formed a single *O*-demethylated *S*-metolachlor metabolite at a significantly greater rate (>20-fold) than sensitive waterhemp or corn. Finally, mass spectrometry and β-glucosidase enzyme assays revealed GST-and P450-mediated detoxification activities of *S*-metolachlor with subsequent glucose-conjugates. Chapter 4 included results of three years of field research at multiple locations in Illinois. Two VLCFA-inhibitor resistant and two sensitive locations were included. While VLCFA-inhibiting herbicides provided poor control of resistant waterhemp, most remained efficacious against sensitive waterhemp, regardless of soil type.

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CHAPTER 1: LITERATURE REVIEW

Waterhemp: A Small Seeded Broadleaf with Significant Importance in Midwest Cropping Systems

Waterhemp [(*Amaranthus tuberculatus*(Moq) Sauer] is a small-seeded, summer-annual dicot weed species native to the Midwestern United States. The natural habitat of waterhemp includes disturbed environments, such as streambanks and lakeshores, which partially explains why it can thrive in the disturbed environments of agronomic fields (Sauer 1955; 1957). Widespread adoption of conservation tillage practices likely contributed to its increased presence in agronomic fields (Buhler 1995; Sauer 1957; Swanton et al. 1993). Additionally, widespread adoption of herbicide-tolerant crops beginning in the 1990s has favored its establishment in agronomic fields (Hilgenfield et al. 2004; Owen 2008). Today, waterhemp is widely distributed across North America, often in the same areas inhabited by other members of the *Amaranthaceae* family (Sauer 1957).

Waterhemp is phenotypically diverse, with stem color ranging from green to red to varigated, and statures from prostrate to erect. Plant height varies, but easily can exceed two meters tall (Horak and Loughin 2000; Sauer 1955). Waterhemp's morphology is relatively plastic, and believed to be adaptable by environment (Waselkov et al. 2020). Sex of the plant strongly influences various vegetative characteristics, with female plants usually larger in height and weight than male plants (Waselkov et al. 2020). Waterhemp utilizes the C4 carbon fixation pathway, which allows the species to excel in high light and temperature conditions (Steckel et al. 2007). Overall, waterhemp can be distinguished from other *Amaranthus* species by its

glabrous stems, lanceolate leaves that measure 2–10 cm long and 1–3 cm wide, and petioles that are shorter than the leaf itself (Sauer 1955).

Waterhemp is one of ten dioecious *Amaranthus*, and therefore obligate outcrossing, species native to North America (Murray 1940; Sauer 1955; 1957). Inflorescences arise from apical meristems and branch tips and can range from 3–35 cm (Horak et al. 1994; Sauer 1955; Steckel 2007). Male staminate and female pistillate flowers occur on separate plants (Murray 1940; Sauer 1955), and pollination occurs via wind (Mosyakin and Robertson 2003). The ratio of male to female plants within a population is generally weighted male, and genetic markers were identified to differentiate between the two sexes to aid in evolutionary research (Montgomery et al. 2019). Female plants are prolific seed producers, with up to a million seeds produced per female plant in optimum growing conditions (Hartzler et al. 2004; Sellers et al. 2003; Steckel et al. 2003). Additionally, waterhemp plants can complete their life cycle at any size given the proper photoperiod (Sauer 1957). Seeds are relatively small (1–1.5 cm) and can be viable as early as nine days after pollination (Bell and Tranel 2010; Sauer 1955). In addition, seeds possess high levels of dormancy that allows plants to emerge in multiple events throughout the growing season or retain viability in the soil for years (Buhler et al. 2001; Buhler and Hartzler 2001; Burnside et al. 1996; Hartzler et al. 1999).

Outcrossing of waterhemp results in high intraspecific genetic variability compared with self-pollinated species (Murray 1940). Genetic variability has likely contributed to waterhemp's evolution of resistance to herbicides representing multiple site-of-action (SOA) groups (Adhikary and Pratt 2015). To date, waterhemp has evolved resistance to herbicides from seven SOAs (Heap 2021). Moreover, multiple herbicide-resistance within a population is frequently reported. Resistance to herbicides from six SOAs has been documented within a single

population (Evans et al. 2019, Shergill et al. 2018; Strom et al. 2019), which limits chemical control methods. Resistance genes can be spread through pollen-mediated gene flow up to 800 m, thus allowing resistant populations to arise and spread across multiple geographies (Liu et al. 2012; Sarangi et al. 2017).

All afore mentioned characteristics makes waterhemp a challenge for growers. Interference with corn can decrease yield over 50%, especially when interference occurs early in the growing season (Steckel and Sprague 2004). In addition, soybean yield is reduced 40% when season-long interference occurs (Hager et al. 2002). Herbicide resistance and prolonged emergence necessitates multiple herbicide applications and effective use of multiple, effective herbicide SOAs including residual chemistries (Evans et al. 2016; Steckel et al. 2002). Waterhemp has established itself as a formidable opponent in agronomic cropping systems today, which likely will continue in the future. Long-term success in controlling this species requires an integrated approach utilizing chemical and non-chemical control strategies (Hager et al. 1997), with a sustainable system making no contributions to the soil-seed bank (Buhler et al. 1997; Schwartz-Lazaro and Copes 2019).

Herbicide Resistance and Mechanisms in Waterhemp

Waterhemp has demonstrated an ability to evolve resistance to herbicides that represent multiple SOAs. To date, waterhemp has developed resistance to inhibitors of acetolactate synthase (ALS), photosystem II (PSII), 5-enolpyruvylshikimate-3-phosphate (EPSPS), protoporphyrinogen oxidase (PPO), very-long-chain fatty acids (VLCFAs), 4-hydroxyphenylpyruvate dioxygenase (HPPD), and the synthetic auxin herbicides 2,4-D and

dicamba (Heap 2021). Populations with combinations of resistances to herbicides from up to six SOAs have been reported (Evans et al. 2019; Shergill et al. 2018; Strom et al. 2019).

ALS-inhibiting herbicides belong to the Weed Science Society of America (WSSA) Group 2 and kill sensitive plants by inhibiting the formation of the essential branched-amino acids leucine, isoleucine, and valine (Durner et al. 1991). ALS-inhibitor resistance is commonly reported in waterhemp and represents the herbicide group with the most reported resistant weed populations (Heap 2021). Waterhemp resistance to ALS inhibitors can be conferred by target-site and non-target site mechanisms. Target site resistance mechanisms include Trp574Leu, Ser653Asn, and Ser653Thr amino acid substitutions (Patzoldt and Tranel 2007). The ALS-inhibitor families that are affected vary by substitution (Patzoldt and Tranel 2007), but all decrease the sensitivity of the encoded enzyme to inhibition by the herbicide (Tranel and Wright 2002). Non-target site resistance to ALS inhibitors has also been reported, and is likely due to enhance herbicide metabolism (Guo et al. 2015).

PSII-inhibiting herbicides represent WSSA Groups 5, 6, and 7 and target the Q_B site of the D1 protein (Duke 1990). Photosynthetic electron transport is then blocked and rapid accumulation of reactive oxygen species results in plant death (Hess 2000). PSII resistance in waterhemp usually involves the Group 5 *s*-triazine herbicide, atrazine. A target site mutation of the *psbA* gene resulting in a Ser264 to Gly amino acid substitution commonly confers resistance to triazine herbicides (Hirschberg and McIntosh 1983), and has been reported in waterhemp (Foes et al. 1998). In addition, non-target site resistance to atrazine has been reported (Patzoldt et al. 2003), and involves rapid metabolism of the herbicide mediated by glutathione *S*-transferases (GSTs) (Evans et al. 2017; Ma et al. 2013; Vennapusa et al. 2018).

Glyphosate is an ESPS inhibitor belonging to the WSSA Group 9 herbicide class and kills plants by inhibiting the formation of the aromatic amino acids (tyrosine, tryptophan, and phenylalanine) via the shikimate pathway (Amrhein et al. 1980; Jaworski 1972). Glyphosate resistance in waterhemp was first reported in 1998 (Legleiter and Bradley 2008) and now is widespread across the Midwest (Heap 2021). The widespread distribution of glyphosate-resistant waterhemp populations is partially explained by pollen-mediated gene-flow (Liu et al. 2012; Sarangi et al. 2017). Multiple mechanisms of glyphosate resistance have been documented. One of the first identified and most abundant mechanisms in waterhemp was gene amplification of EPSPS (Bell et al. 2009; Chatham et al. 2013; 2015; Shaner et al. 2011; Tranel et al. 2011). In addition, reduced translocation contributes to glyphosate resistance (Nandula et al. 2013). A point mutation resulting in a Pro106 to Ser substitution has also been identified as a form of target site resistance in waterhemp (Bell et al. 2013; Chatham et al. 2015; Nandula et al. 2013).

PPO-inhibiting herbicides belong to the WSSA Group 14 class and act by inhibiting the PPO enzyme. Inhibition of PPO results in a depletion of chlorophyll in the chloroplast and subsequent leakage of protoporphyrinogen (Protogen) IX to the cytosol (Duke et al. 1991; Lee and Duke 1994). Protogen IX in the cytosol is then converted to protoporphyrin IX, and causes the formation of reactive oxygen species without antioxidant or enzymatic protection. Lipid peroxidation by the reactive oxygen species results in loss of membrane integrity, necrosis, and plant death (Grossman et al. 2011). Target-site resistance to PPO inhibitors in waterhemp is most commonly caused by a single codon deletion at position 210 (ΔG210) (Lee et al. 2008; Patzoldt et al. 2006; Thinglum et al. 2011), which results in a greatly reduced sensitivity to PPO-inhibitors (Dayan et al. 2010). Additional target site mutations of arginine (R128) and glycine

(G399A) have been reported in Palmer amaranth (*Amaranthus palmeri* S. Watson) (Giacomini et al. 2017; Rangani et al. 2019; Salas et al. 2016; Varanasi et al. 2018). Non-target site resistance to the PPO-inhibitor, carfentrazone, has been postulated through the application of metabolic inhibitors (Obenland et al. 2019).

HPPD inhibitors block the biosynthesis of plastoquinone and α-tocopherols, and indirectly deplete carotenoids needed for protection from reactive oxygen species (Mitchell et al. 2001; Norris et al. 1995; Van Almsick et al. 2009). HPPD inhibitors belong to the WSSA Group 27 class, and characteristic symptomology includes bleaching, chlorosis, and necrosis of plant meristems (Mitchell et al. 2001; Norris et al. 1995; van Almsick 2009). Resistance to HPPD inhibitors was first documented in 2011 at a location in McLean County, IL containing a population designated MCR (Hausman et al. 2011). Resistance to the HPPD inhibitors mesotrione and topramezone is due to enhanced metabolism relative to sensitive populations (Ma et al. 2013; Lygin et al. 2018). Resistance to HPPD inhibitors has been reported in other populations (Heap 2021), and increased HPPD gene expression has been postulated as a resistance mechanism in Palmer amaranth (Nakka et al. 2017).

Synthetic auxin herbicides, such as 2,4-D and dicamba, belong to the WSSA Group 4 class and act as growth regulators on susceptible plants (Sterling and Hall 1997). Herbicidal activity once in the plant involves the deregulation of auxin response pathways resulting in increased growth, formation of ethylene, abscisic acid, and eventually reactive oxygen species, ultimately resulting in plant death (Gaines 2020; Grossman 2010; Sterling and Hall 1997). Synthetic auxin herbicides, such as 2,4-D, are among the oldest herbicides utilized in the Midwest (Peterson et al. 2016), and 2-4, D-resistant waterhemp has been reported in multiple states (Bernards et al. 2012; Evans et al. 2019; Shergill et al. 2018). Much remains unkown

about the mechanisms of resistance, but resistance in a Nebraska waterhemp population is metabolism based, likely mediated by P450s (Figueiredo et al. 2018).

The most recent discovery of resistance in waterhemp is to Group 15 VLCFA inhibitors (Heap 2021; Strom et al. 2019). VLCFA inhibitors kill sensitive plants by inhibiting the VLCFA-elongase complex which subsequently results in the depletion of VLCFAs needed for cell membranes, cuticle waxes, and lipids (Bach and Faure 2010; Böger 2003). Sensitive plants generally fail to emerge or remain stunted after emergence when treated with a VLCFA-inhibiting herbicide (Deal and Hess 1980; Dhillon and Anderson 1972; Fuerst 1987; Pillai et al. 1979). Two Illinois waterhemp populations were reported resistant to the VLCFA-inhibitors acetochlor, dimethenamid-*P*, *S*-metolachlor, and pyroxasulfone through greenhouse assays (Strom et al. 2019). The mechanism of resistance to *S*-metolachlor is enhanced metabolism relative to sensitive populations, likely mediated by GSTs and P450s (Strom et al. 2020).

VLCFA-Inhibitor Mode of Action, Selectivity, and Resistance

Very-long-chain fatty acids (VLCFAs) consist of acyl carbon chains in excess of 18 and are essential for plant function. VLCFAs are present in lipid seed reserves, signaling molecules, and are main components of cellular membranes and cuticle waxes (Bach and Faure, 2010). VLCFA-inhibiting herbicides belong to the Weed Science Society of America Group 15 class of herbicides and inhibit emerging weed seedlings. Germination, however, is not inhibited. VLCFA-inhibitors are effective at controlling annual monocots, small-seeded dicots, and perennial nutsedge species (Fuerst 1987). Sensitive species generally fail to emerge or remain in an arrested state of growth after emerging (Deal and Hess 1980; Dhillon and Anderson 1972; Pillai et al. 1979). VLCFA-inhibiting herbicides are absorbed by both the roots and shoots of

emerging seedlings (Pillai et al. 1979). Shoot uptake is the primary mechanism of entry in monocot species (Knake et al. 1967; Pillai et al 1979). Inhibition of growth can occur to roots and shoots of affected plants (Pillai et al 1979).

VLCFA-inhibiting herbicides were originally discovered and developed in the 1950s (Hamm 1974), but their mode of action remained a mystery for decades until it was concluded the depletion of VLCFAs results in phytotoxicity (Böger 2003). Plants that are deficient in VLCFAs have unstable cells prone to lyse, which leads to plant death (Matthes et al. 1998). VLCFA-inhibitors target the VLCFA-synthase, encoded by the *FAE1* gene, in the four-subunit VLCFA-elongase complex within the endoplasmic reticulum (Böger 2003). VLCFA-synthase is a condensing enzyme and relies on a reactive cysteinyl sulfur (Ghanevati and Jaworski 2002) to perform a nucleophilic attack on either the natural substrate or herbicide (Böger et al. 2000; Götz and Böger 2004). Inhibition is irreversible, and once the herbicide binds it cannot be displaced (Götz and Böger 2004). VLCFA-inhibitors can inhibit several VLCFA-elongases in *Arabidopsis thaliana*, including *At5g43760, At104220, At1g25450, KCS1, KCS2, and FAE1* (Trenkamp et al. 2004). Regardless of the encoded enzyme inhibited, depletion of VLCFAs is the primary mechanism of action (Böger 2003), but many other secondary responses can result from high herbicide concentrations in the plant (Böger et al. 2000).

VLCFA inhibitors are an integral component of herbicide programs that provide residual weed control in important row crops including corn (*Zea mays* L.), soybean (*Glycine max* L. Merr), and cotton (*Gossypium hirsutum* L.) due to their application flexibility, crop safety, and residual control of annual monocot and small-seeded dicot weed species (Fuerst 1987). Selectivity is achieved in tolerant crops through enhanced herbicide metabolism relative to sensitive weed species (Jaworski 1969). Tolerant species are more efficient at metabolizing the

inhibitor through conjugation to glutathione (GSH) or homoglutathione (hGSH) (Breaux 1987; Joblankai and Dutka 1985) mediated by glutathione *S*-transferases (GSTs) (Cummins et al. 2011; Fuerst 1987). Once conjugated, the herbicide is no longer phytotoxic (Shimabukuru et al 1978). Tolerant crops generally possess either greater GST activity with herbicide substrates or GSH content compared with sensitive weed species, which allows tolerant crops to conjugate the herbicide more efficiently (Breaux 1987; Hatton et al. 1996). In cereal crops enhanced tolerance to VLCFA-inhibiting herbicides can be achieved using a herbicide safener (Gronwald 1989; Hatzios 1991; Riechers et al. 1996; 2010). In addition to conjugation to GSH, oxidative metabolism of VLCFA-inhibiting herbicidees by cytochrome P450s has been documented in several crops (Moreland and Corbin 1991; Moreland et al. 1993; 1995; Siminszky 2006).

Resistance to Group 15 herbicides is relatively rare. Worldwide, only five monocot and two dicot are resistant (Heap 2021). Most work in identifying resistance and mechanisms to VLCFA inhibitors has been with monocot species (Busi 2014). Resistance to the VLCFA inhibitors pyroxasulfone and flufenacet was determined enhanced metabolism in *Lolium spp.*, and in both cases, GSTs were the primary enzymes involved (Busi et al. 2018; Dücker et al. 2019). P450 involvement in resistance to *S*-metolachlor and pyroxasulfone in *Lolium* was also hypothesized using the P450 inhibitors malathion and phorate (Busi et al. 2017; Tardiff and Powles 1999). Fewer instances of VLCFA-inhibotor resitance in dicots have been reported. The first dicot species reported resistant was waterhemp (*Amaranthus tuberculatus* Moq Sauer) (Strom et al. 2019). In two resistant populations from Illinois, enhanced herbicide metabolism was identified as the resistance mechanism, likely due a combination of GST and P450 activities (Strom et al. 2020). VLCFA-inhibitor resistance was also documented in Arkansas Palmer amaranth (*Amaranthus palmeri* S. Watson) populations, and was hypothesized to be conferred by

GST-mediated metabolism via the GST inhibitor 4-chloro-7-nitrobenzofurazon (Brabham et al. 2019).

Implications of Resistance to Soil-Applied Herbicides in Waterhemp

Soil-applied, preemergence (PRE) herbicides are commonly used in integrated weed management systems to control troublesome weeds. PRE herbicides prevent or delay the emergence of weeds that would otherwise need to be controlled by postemergence (POST) herbicides, and can reduce early-season yield loss due to interference (Adcock and Banks 1991). In the Midwestern United States, waterhemp (*Amaranthus tuberculatus* Moq Sauer) is among the most important weeds targeted by chemical control practices (Sauer 1955; Steckel 2007). Waterhemp is a small-seeded, summer annual dicot that produces thousands of seedlings that emerge in multiple events throughout the growing season (Buhler and Hartzler 2001; Hartzler et al. 1999; Steckel 2007; Steckel et al. 2003). Additionally, waterhemp has evolved resistance to herbicides from multiple site-of-action groups (SOAs) (Heap 2021; Tranel 2020). To date, waterhemp has evolved resistance to herbicides that inhibit acetolactate synthase (ALS), photosystem II (PSII), 5-enolpyruvylshikimate-3-phosphate (EPSPS), protoporphyrinogen oxidase (PPO), very-long-chain fatty acids (VLCFAs), 4-hydroxyphenylpyruvate dioxygenase (HPPD), and the synthetic auxin herbicides 2,4-D and dicamba (Heap 2021). Most reports of resistance and mechanistic studies in waterhemp have focused on foliar-applied herbicides from the previously mentioned SOAs. ALS-, PSII-, PPO-, VLCFA-inhibitor and synthetic auxin herbicides possess soil-activity, but research on PRE resistance in waterhemp is limited.

Resistance in waterhemp to PPO inhibitors is most commonly conferred by a single codon deletion of glycine at position 210 (G210) (Lee et al. 2008; Patzoldt et al. 2006; Thinglum

et al. 2011), which greatly reduces the efficacy of PPO inhibitors (Dayan et al. 2010). Waterhemp resistant to foliar-applied PPO-inhibiting herbicides was first reported in Kansas in 2001 (Shoup et al. 2003) and now is prevalent across much of the Midwest (Lee et al. 2008; Schultz et al. 2015; Thinglum et al. 2011). PPO-inhibitor efficacy is dependent on the size of the target weed (Falk et al. 2006) and PPO-resistant waterhemp has demonstrated reduced sensitivity to soil-applied PPO inhibitors in growth chamber experiments (Wuerffel et al. 2015a). Germination of PPO-resistant populations possessing the ΔG210 codon deletion was greater than sensitive populations following PRE PPO-inhibitors. Additionally, early growth was greater in the resistant populations than sensitive populations (Wuerffel et al. 2015a). Soil-applied PPO-inhibitors also increased the frequency of resistant individuals within waterhemp populations (Wuerffel et al. 2015b). Under field conditions, soil-applied PPO inhibitors are efficacious on PPO-resistant waterhemp (Harder et al. 2012; Shoup et al. 2003), but control of a PPO-resistant population from Illinois ranged from 18–86% with PRE-applied saflufenacil, flumioxazin, and sulfentrazone 28 days after treatment (Evans et al. 2019).

Waterhemp resistant to HPPD inhibitors has been reported in several Midwestern states, including Illinois (Evans et al. 2019; Hausman et al. 2011; 2013), Iowa (McMullan and Green 2011), and Nebraska (Oliveira et al. 2017). Resistance to the foliar-applied HPPD inhibitors mesotrione and topramezone in an Illinois waterhemp population from McLean County is due to enhanced metabolism relative to sensitive populations, likely mediated by cytochrome P450s (Lygin et al. 2018; Ma et al. 2013). Several HPPD-inhibiting herbicides also possess soil activity, and are commonly applied in PRE programs (Luscombe and Pallett 1996; Wichert et al. 1999). Greenhouse dose-response experiments with PRE-applied mesotrione demonstrated resistant waterhemp had reduced sensitivity compared with sensitive populations (Hausman et al.

2013). Under field conditions, control of the HPPD-resistant population from McLean County, IL with PRE-applied isoxaflutole and mesotrione ranged 53–90% 30 days after treatment, but decreased to 25–87% 60 days after treatment (Hausman et al. 2013). Control of another HPPD-resistant population from Champaign County, IL with the same herbicides was 54–88% 28 days after treatment and 16–61% 42 days after treatment (Evans et al. 2019). Postemergence HPPD-inhibitors failed to control either population more than 76% (Evans et al. 2019; Hausman et al. 2011; 2016), and results demonstrated efficacy of PRE HPPD-inhibiting herbicides was also reduced (Evans et al. 2019; Hausman et al. 2013).

Atrazine is a PSII inhibitor that is used in the Midwestern United States both PRE and POST for waterhemp control (Anderson et al. 1996). Resistance in waterhemp can be conferred by a target site mutation of the *psbA* gene resulting in a Ser264 to Gly amino acid substitution (Foes et al. 1998; Hirschberg and McIntosh 1983) or by non-target site mechanisms involving rapid herbicide metabolism mediated by glutathione *S*-transferases (GSTs) (Evans et al. 2017; Ma et al. 2013; Patzoldt et al. 2003; Vennapusa et al. 2018). Most research has included atrazine applied POST, but metabolic atrazine-resistant populations from McLean County, IL and Adams County, IL also displayed reduced sensitivity to PRE-applied atrazine under greenhouse conditions (Ma et al. 2016). PRE control of the McLean County population with atrazine under field conditions did not exceed 22% 60 days after treatment (Hausman et al. 2013). Atrazine applied PRE also did not control the atrazine-resistant population from Champaign County, IL (Evans et al. 2019). Statewide surveys have demonstrated atrazine resistance in waterhemp is widespread, limiting atrazine as an effective option for waterhemp control (Anderson et al. 1996; Murphy et al. 2019; Shultz et al. 2015; Singh et al 2020; Vennapusa et al. 2018).

The most recently evolved resistance in waterhemp is to herbicides that inhibit the formation of VLCFAs (Heap 2021; Strom et al. 2019; 2020). VLCFA inhibitors are soil-applied to control annual monocot and small-seeded dicot species, such as waterhemp (Fuerst 1987). Currently, two populations in Illinois (collected from Champaign County and McLean County) have been confirmed resistant to VLCFA-inhibiting herbicides (Strom et al. 2019). The mechanism of resistance to the VLCFA inhibitor, *S*-metolachlor, is enhanced metabolism relative to sensitive populations, likely mediated by GSTs and cytochrome P450s (Strom et al. 2020). Both populations demonstrated resistance to acetochlor, dimethenamid-*P*, pyroxasulfone, and *S*-metolachlor under greenhouse conditions (Strom et al. 2019), and overall PRE control in the field was poor (Evans et al. 2019; Hausman et al. 2013; Strom et al. 2019). VLCFA-inhibitors have been a staple in herbicide programs for over sixty years (Hamm 1974), and resistance in waterhemp could become more widespread if integrated control strategies are not employed.

Resistance to ALS-inhibiting herbicides in waterhemp is assumed to be present in nearly every field the species inhabits (Tranel 2020; Tranel et al. 2011). ALS resistance in waterhemp is widely characterized and is endowed due to various target-site or possible non-target site mechanisms (Guo et al. 2015; Patzoldt and Tranel 2007; Tranel and Wright 2002). Most research has focused on POST-applied herbicides, but ALS inhibitors applied PRE do not control ALS-resistant waterhemp (Evans et al. 2019; Hausman et al. 2013; Benoit et al. 2019). Waterhemp has also developed resistance to synthetic auxin herbicides, such as 2,4-D (Bernards et al. 2012; Evans et al. 2019; Shergill et al. 2018). Synthetic auxins are predominately POST-applied, but since certain syentetic auxins possess soil-residual activity, PRE resistance to these herbicides could evolve next in waterhemp.

The previously mentioned cases of PRE herbicide resistances in waterhemp demonstrate that resistance does not only affect POST-applied herbicides. The general assumption is that resistance has been selected for in waterhemp exclusively from foliar-applied herbicides; however, there is no empirical evidence that soil-applied herbicides did not select for resistance. Resistance to soil-applied herbicides is a relatively recent discovery in waterhemp, but has been long studied in monocot species, such as *Lolium* (Busi 2014). Agronomic cropping practices using chemical weed control methods impose selection pressures on weed populations, resulting in weed species shifts and the evolution of resistant populations (Baker 1991; Owen and Zelaya 2005). Plants that emerge from soils treated with PRE herbicides are subject to POST herbicides, and if controlled, do not pass resistance alleles to the next generation (Somerville et al. 2017). PRE herbicides are persistent (Curran 2016; Horowitz 1969; Miller et al. 1978), however, and emerging seedlings are exposed to the herbicide for a long period of time relative to many POST herbicides. The concentration of PRE herbicides decreases over time due various factors in the environment, eventually less than what is needed to control emerging seedlings. The emerged seedlings are thus likely exposed to sub-lethal doses of the herbicide. Previous studies have reported low-dose selection of PRE herbicides such as pyroxasulfone can hasten the evolution of resistance in *Lolium rigidum* (Busi and Powles 2016). It is possible that low-dose selection occurs when emerged seedlings are exposed to sub-lethal rates of PRE herbicides.

Failures of PRE herbicides to control waterhemp are not always due to resistance. Many diverse environmental factors can contribute to their efficacy (Splittstoesser and Derscheid 1962). Most PRE herbicides require adequate rainfall for incorporation and subsequent activity. The timing and amount of precipitation is essential for PRE activity, and if too much time passes after application and sufficient rainfall does not occur, overall activity will be reduced (Armel et

al. 2003; Jhala 2017; Stewart et al. 2010; 2012). In addition, too much rainfall can be detrimental to herbicidal activity due to leaching and rapid dissipation of the herbicide (Chesters et al. 1989; Jursík et al. 2013; Lewan et al. 2009). Ambient air and soil temperature can also impact PRE herbicide activity, and increasing temperatures can increase or decrease weed control (Mulder and Nalewaja 1978). Warm, moist soils can also result in the loss of certain PRE herbicides from the soil via volatilization (Prueger et al. 2005, 2017).

Edaphic factors are especially important with PRE herbicides. Organic matter content can drastically influence the availability of PRE herbicides to emerging seedlings, and in general, bioavailibility decreases with increasing organic matter content (Harrison et al. 1976). The tillage practices employed in each field influence the amount of crop residue and organic matter in soils (Unger and McCalla 1980), and PRE herbicide availability and subsequent activity is generally less in fields under conservation tillage programs with abundant crop residues (Johnson et al. 1989; Kapusta 1979; Kapusta and Strieker 1976). Other edaphic factors, such as cation exchange capacity, pH, and clay content, can also correlate to overall PRE herbicide availibility (Sebastian et al. 2017; Shaner et al. 2006; Stougaard et al. 1990). Organic matter content can also influence the amount of microbial degradation of certain PRE herbicides (Rice et al. 2002; Shaner et al. 2006). Soils with high organic matter generally have greater microbial activity than those with low organic matter, and thus result in increased microbial degradation of PRE herbicides (Rice et al. 2002; Wu et al. 2011). In addition, repeated use of similar PRE herbicides can shift the microbial community to metabolize certain herbicides, and result in rapid microbial degradation (Baily and Coffey 1986; Mueller et al. 2017; Shaner and Henry 2007; Sanyal and Kulshrestha 1999).

The information presented herein demonstrates that resistance to PRE herbicides has evolved in waterhemp, and highlights the need for integrated management strategies as well as new chemical control methods. The continued use of herbicide mixtures with POST and residual activity are ever important, and have demonstrated an ability to delay the evolution of herbicide resistance (Busi et al. 2020). Each season, multiple, effective herbicide SOAs should be employed (Evans et al. 2016), and overlapping PRE residual herbicides can aid the efficacy of an effective POST program by reducing the number of emerged seedlings to control (Chahal et al. 2018; Steckel et al. 2002). Non-chemical control strategies are also an important component of any integrated weed management program. Cover crops are an emerging resource for control of annual weeds in Midwestern cropping systems that are also compatible with many chemical weed management practices (Cornelius and Bradley 2017; Jha et al 2020; Loux et al 2017). Post-harvest weed seed destruction and manual removal of surviving weeds are also effective methods for limiting the number of seeds returned to the soil seedbank each year (Buhler et al. 1997; Schwartz-Lazaro and Copes 2019; Shergill et al. 2020; Walsh et al. 2012).

Every grower, retailer, and crop protection professional needs to understand all viable tools at their disposal for waterhemp control. Regular field scouting is essential in identifying possible PRE resistance in waterhemp and identifying effective management strategies. PRE herbicide resistance often manifests as a shorter duration of control; this can be more difficult to detect than immediate failures to control emerged weeds observed when resistance occurs to POST herbicides (Hager 2019). The Champaign County and McLean County waterhemp populations discussed herein possess resistance to ALS-, HPPD-, VLCFA-inhibitors, and atrazine (Evans et al. 2019; Hausman et al. 2011; 2013; Strom et al. 2019). Most PRE herbicides are applied in combinations with other previously mentioned SOAs, and if a population

possesses resistance to all of them, control might not be sufficient even with mixtures. Control of multiple-herbicide resistant waterhemp populations is still possible, however, if every effective resource is utilized, and will be sustainable if the net yield of seed back into the seed-bank each year is zero (Buhler et al. 1997; Schwartz-Lazaro and Copes 2019).

Plant Herbicide Metabolism Occurs in Phases

Plants are constantly exposed to foreign chemicals or xenobiotics from their surrounding environment. Plants perform multiple phases of metabolism to detoxify xenobiotics, including herbicides (Riechers et al. 2010; Sandermann 1992). Differential metabolism of herbicides also results in selectivity of herbicides between crop and weed species (Cole 1994; Lamoureux et al. 1991). Four phases of plant metabolism (I-IV) occur and have unique properties and enzymes involved (Kreuz et al. 1996). Phase I reactions commonly involve cytochrome P450 enzymes and are oxidative reactions that can either detoxify or activate herbicides. The reactions are diverse and include hydroxylation, demethylation, *N*-dealkylation, and hydrolysis (Hamberger and Bak 2013; Mansuy 1998). An example reaction is aryl hydroxylation of sulfonylurea herbicides in corn (*Zea mays*) (Moreland et al. 1993). Phase I reactions often predispose the herbicide to subsequent Phase II reactions (Siminszky 2006; Werk-Reichhart and Feyereisen 2000). Phase II reactions include conjugation reactions that incorporate sugars or amino acids onto the herbicide (Kreuz et al. 1996; Siminszky 2006; Werk-Reichhart and Feyereisen 2000), resulting in non-phytotoxic metabolites (Shimabukuru et al. 1978). Glucosyltransferases mediate conjugation reactions incorporating sugars (Kreuz et al. 1996; Lamoureux et al. 1991), while glutathione *S* transferases (GSTs) catalyze the conjugation of the tripetide glutathione (GSH) to herbicides with electrophilic sites (Cummins et al. 2011; Kreuz et al. 1996; Lamoureux et al.

1991; Riechers et al. 2010). Example reactions include the conjugation of GSH to atrazine or inhibitors of very-long-chain fatty acids (Breaux 1987; Frear and Swanson 1970; Hatton et al. 1996). Phase III of herbicide metabolism involves transport and compartmentalization of herbicide metabolites to the vacuole, cell wall, or extracellular space (Sandermann 1992) mediated by ATP-binding cassette tonoplast transporters (Schulz and Kolukisaoglu 2006). Phase IV occurs following compartmentalization and GSH-herbicide conjugates are catabolized to recycle component amino acids, which explains detection of various cysteine and dipeptide herbicide conjugates when investigating GST-mediated herbicide metabolism (Lamoureux and Rusness 1981; 1993; Martin et al. 2007; Riechers et al. 1996; Wolf et al. 1996).

Cytochrome P450s and Glutathione *S*-Transferases: Two Important Herbicide Detoxification Enzyme Families

Cytochrome P450s (P450s) are one of the largest super-families of plant enzymes and are very diverse (Mizutani and Ohta 2010; Werk-Reichhart and Feyereisen 2000), often with less than 20% amino-acid identity among genes (Graham and Peterson 1999). P450s receive their name from their heme group active site, which reaches a maximum light absorbance at 450 nm (Nielsen and Moller, 2005). P450 nomenclature is designated based on amino acid sequence identity, with families including enzymes with over 40% sequence identity and subfamilies including enzymes with over 55% sequence identity. All P450 enzymes have the root symbol *CYP* (Nelson 2006), and over 200 P450 encoding genes have been identified in *Arabidopsis thaliana* (Werk-Reichhart and Feyereisen 2000). P450s are important in Phase I plant metabolic reactions including hydroxylation, demethylation, *N*-dealkylation, and hydrolysis (Hamberger and Bak 2013; Mansuy 1998). P450 substrate specificity is low, and reactions and substrates

involving P450s can be surprising (Siminszky 2006; Werk-Reichhart and Feyereisen 2000). Most herbicide metabolizing P450s are membrane-bound (to the endoplasmic reticulum) external oxygenases that require an electron donor such as NADPH or NADH (Gordeziani et al. 2016; Werk-Reichhart et al. 2000). The general reaction involves the enzyme binding to the substrate, reduction of the heme group to a ferrous state, and insertion of one oxygen atom into the substrate. Electrons are transferred from NADPH by NAD(P)H-reductase enzymes to the heme center of the enzyme and oxygen. Without reductase enzymes or electron donors, the enzyme will fail to oxidize the substrate (Gordeziani et al. 2016; Werk-Reichhart et al. 2000, Siminszky 2006). In herbicide metabolism studies, P450 activity is often determined using microsomal protein and various herbicide substrates (Moreland et al. 1993; 1995; Persons and Schuler 1995; Polge and Barrett 1995) or indirectly tested using P450 inhibitors such as organophosphate insecticides or tetcyclasis (Baerg et al. 1996; Ma et al. 2013; Kreuz and Fonné-Pfister 1992). Oxidative reactions mediated by P450s usually partially or fully detoxify the herbicide and predispose it to further Phase II conjugation reactions (Siminszky 2006; Werk-Reichhart and Feyereisen 2000), but can also activate pro herbicides such as thiocarbamates or clomazone (Ferhatoglu and Barrett 2006; Fuerst 1987; Nandula et al. 2019).

Glutathione *S*-transferases (GSTs) are a diverse family of soluble enzymes, and are classified based on their amino acid sequence identity and gene structure. GSTs are dimers composed of two polypeptide subunits and typically have a native molecular mass of around 50 kDa. Five main classes of GSTs have been identified in plants, with *phi* and *tau* classes the most abundant (Dixon et al. 1998; 2002; Mohsenzadeh et al. 2011). Numerous GST-encoding genes have been identified in *Arabidopsis thaliana,* and GSTs can make up 2% of total protein from cereal grass crops (Dixon et al. 2002). GSTs are important in phase II plant metabolic reactions

that conjugate glutathione or homoglutathione (legume crops) to xenobiotics, such as herbicides, with electrophilic sites (Cummins et al. 2011; Kreuz et al. 1996; Lamoureux et al. 1991; Riechers et al. 2010). Each dimer consists of two catalytic sites, one of which is specific to GSH and the other (hydrophobic H-site) binds to the substrate (Dixon et al. 2002; Mannervik and Danielson 1988; Marrs 1996). Once GSH is incorporated into the substrate, it is considered non-toxic and is then transferred to the vacuole (Cummins et al. 2011; Shimabukuru et al. 1978). Once in the vacuole, glutathione can then be recycled to reuse its component amino acids (Lamoureux and Rusness 1981; 1993; Martin et al. 2007; Riechers et al. 1996; Wolf et al. 1996). GSTs in plants have been extensively studied largely due to their involvement in herbicide metabolism, but are also very important enzymes for other plant functions such as biotic and abiotic stress response and cell signaling pathways (Edwards et al. 2000; Marrs 1996).

As mentioned previously, GSTs and P450s are both important in plant herbicide metabolism. Safeners can increase the concentration of antioxidants such as GSH in cereal crops. Safeners also induce activity of herbicide detoxification enzymes such as GSTs and P450s (Gronwald 1989; Hatzios 1991; Riechers et al. 1996, Riechers et al. 2010) and vacuolar transport of herbicide conjugates (Pang et al. 2012), thus enhancing the overall stepwise metabolic process of herbicide detoxification.

Attributions

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experimental design were provided by Dean E. Riechers and Shiv S. Kaundun. Statistical analysis advice was provided by Adam S. Davis, and manuscript preparation assistance was provided by Aaron G. Hager, Nicholas J. Seiter, Adam S. Davis, Shiv S. Kaundun, and Dean E. Riechers. Support and equipment for conducting thin-layer chromatography was provided by Peter Yau at the Proteomics Facility at the University of Illinois, and financial support was provided by Syngenta Crop Protection. Experiments and final statistical analysis were developed and conducted by Seth A. Strom.

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CHAPTER 2: METABOLIC RESISTANCE TO S-METOLACHLOR IN TWO WATERHEMP (*AMARANTHUS TUBERCULATUS*) POPULATIONS FROM ILLINOIS, UNITED STATES

Abstract

Two waterhemp (*Amaranthus tuberculatus*) populations from Illinois demonstrating multiple-resistance to ALS-, HPPD-, and PSII-inhibiting herbicides (designated CHR and SIR) also displayed reduced sensitivity to very-long-chain fatty acid (VLCFA)-inhibiting herbicides, including *S*-metolachlor. We hypothesized that a physiological mechanism, such as enhanced metabolism, could be responsible for the reduced efficacy of *S*-metolachlor. Metabolism experiments indicated that resistant populations degraded *S*-metolachlor more rapidly than sensitive populations and equally as rapid as corn 2–24 hours after treatment (HAT). Resistant waterhemp and corn metabolized 90% (DT_{90}) of absorbed *S*-metolachlor in less than 3.2 h whereas DT_{90} values for sensitive waterhemp exceeded 6 h. The glutathione *S*-transferase inhibitor 4-chloro-7-nitrobenzofurazon and cytochrome P450-inhibitor malathion decreased the amount of *S*-metolachlor metabolized in resistant waterhemp at 4 HAT but not in sensitive waterhemp or corn, and altered the abundance of certain metabolites in resistant waterhemp. Results from this research demonstrate that resistance to *S*-metolachlor in these waterhemp populations is due to enhanced herbicide metabolism relative to sensitive populations. In addition, our results indicate resistant waterhemp might utilize metabolic pathway(s) more intricate than either sensitive waterhemp or corn based on their metabolite profiles.

Introduction

Waterhemp [*Amaranthus tuberculatus* (Moq.) J.D. Sauer] (Sauer 1955) is one of the most troublesome weed species in Midwestern U.S. agronomic systems. Waterhemp is a small-seeded, summer-annual dicot that utilizes the C4 carbon fixation pathway and can produce thousands of seeds per female plant, even in suboptimal growth conditions (Steckel 2007; Steckel and Sprague 2003). Prolonged seedling emergence (Buhler and Hartzler 2001; Hartzler et al. 1999) is one characteristic that enables waterhemp to effectively compete with crops such as corn (*Zea mays* L.) and soybean (*Glycine max* (L.) Merr.) throughout the growing season (Steckel et al. 2002). Corn yield loss can exceed 50% from waterhemp interference before the V6 growth stage (Steckel and Sprague 2004). In addition, interference with soybean can reduce yield over 40% (Hager et al. 2002).

Waterhemp is dioecious, which results in obligate outcrossing and high intraspecific diversity when compared to self-pollinated weed species (Murray 1940; Sauer 1955). High intraspecific diversity has facilitated the evolution of resistance in waterhemp to herbicides representing several site-of-action groups (Steckel et al. 2007; Tranel et al. 2011), including inhibitors of 4-hydroxyphenylpyruvate dioxygenase (HPPD), photosystem II, acetolactate synthase, 5-enolpyruvylshikimate-3-phosphate synthase, protoporphyrinogen oxidase, very-long-chain fatty acid (VLCFA) elongases, and the synthetic auxin herbicide 2,4-D (Heap 2020). Even more troublesome are populations or individual plants that are multiple herbicide-resistant (MHR). Currently, two six-way resistant waterhemp populations have been reported (Evans et al. 2019; Shergill et al. 2018; Strom et al. 2019), and among these MHR populations, resistance to herbicides that inhibit VLCFA elongases has most recently been added (Heap 2020; Strom et al. 2019).

VLCFA-inhibiting herbicides belong to the Weed Science Society of America Group 15 class and control sensitive species by inhibiting the synthesis of VLCFAs in excess of eighteen carbons (Bach and Faure 2010; Böger 2003). VLCFA-inhibiting herbicides are an integral component of soil-applied preemergence (PRE) and postemergence (POST) programs that provide residual weed control in important row-crops including corn, soybean, and cotton (*Gossypium hirsutum* L.) due to their application flexibility, crop safety, and control of annual grasses and small-seeded dicot weed species (Fuerst 1987). Cases of weed resistance to VLCFA inhibitors have been infrequent compared with herbicides that inhibit other sites of action (Heap 2020). Prior to confirmation of resistance in waterhemp and Palmer amaranth (*Amaranthus palmeri* S. Watson) (Brabham et al. 2019; Strom et al. 2019), resistance to VLCFA inhibitors only occurred in five grass species worldwide (Heap 2020), despite being utilized for over sixty years (Hamm 1974). Efficacy of VLCFA inhibitors is greatly influenced by climatic characteristics and edaphic factors (Jhala 2019; Shaner et al. 2006; Wu et al. 2011), which could partially explain the relatively few reports of resistance. Other hypotheses for infrequent resistance to VLCFA inhibitors include lower selection intensity compared with POST herbicides and target-site alterations that could confer fitness penalties to the plant (Böger 2003; Somerville et al. 2017).

Previous field research demonstrated that several VLCFA-inhibiting herbicides did not effectively control two MHR waterhemp populations from Illinois (Evans et al. 2019; Hausman et al. 2013; Strom et al. 2019). Progeny generated from each population also were less sensitive to acetochlor, dimethenamid-*P*, pyroxasulfone, and *S*-metolachlor in greenhouse dose-response experiments (Strom et al. 2019). Crop tolerance to VLCFA inhibitors is due to rapid metabolism through glutathione *S*-transferase (GST) activities relative to sensitive weed species (Breaux

1987; Cummins et al. 2011; Fuerst 1987; Hatton et al 1996) and GST-mediated metabolism plays a major role in conferring resistance to the VLCFA-inhibiting herbicides flufenacet and pyroxasulfone in *Lolium spp* (Busi et al. 2018; Dücker et al. 2019). Mechanistic research describing resistance to VLCFA inhibitors has not been reported in dicot weed species, however. We hypothesized that enhanced metabolic detoxification in these two waterhemp populations could confer resistance to the VLCFA inhibitor *S*-metolachlor. The objectives of this research are to (1) quantify and compare *S*-metolachlor metabolism in MHR and sensitive waterhemp populations, and (2) utilize a combination of synthetic metabolite standards and metabolic inhibitors to investigate enzymatic detoxification pathway(s) involved in MHR waterhemp.

Materials and Methods

Chemicals

Radiolabeled [URL-^{14}C] *S*-metolachlor (555 MBq mmol^{-1}) was supplied by Syngenta Crop Protection (Greensboro, North Carolina 27409). Non-labeled analytical grade *S*-metolachlor (98% pure) and malathion (99.8% pure) were purchased from Chem Service, Inc. (West Chester, Pennsylvania 19381). All other analytical grade chemicals and reagents were purchased through Fisher Scientific (Thermo-Fisher, Hanover Park, Illinois 60133) or Sigma Chemical (Millipore Sigma, St. Louis, Missouri 63102).

Populations and plant materials

Two previously characterized MHR waterhemp populations (CHR and SIR) were selected. CHR originated from Champaign County, IL (Evans et al. 2019) and SIR originated from Stanford, IL and is derived from the MCR population described in previous research

(Hausman et al. 2013; Hausman et al. 2011). Both populations are resistant to VLCFA-inhibiting herbicides (Strom et al. 2019). ACR and WUS populations are sensitive to VLCFA inhibitors and were selected for comparison (Strom et al. 2019). A corn hybrid (B73xMo17) also was included for comparison to waterhemp since corn is naturally tolerant to *S*-metolachlor via rapid GST-mediated metabolism (Fuerst 1987). All waterhemp seeds were stratified to improve germination according to methods previously described (Bell et al. 2013) and stored at 4°C for at least 20 days prior to initiating experiments.

All plants were grown under greenhouse conditions (28/22 °C day/night with a 16 h photoperiod) in 509 cm^3 cell pack inserts containing vermiculite soaked in a commercial hydroponic fertilizer solution (Peters Hydroponic Special 5-11-26; ICL Specialty Fertilizers, Summerville, South Carolina 29483) at one-third strength supplemented with 0.15 g L^{-1} $Ca(NO_3)_2$. Natural sunlight was supplemented with mercury halide lamps to provide 800 µmol m^{-2} s^{-1} photon flux at the soil surface. New hydroponic solution was added every two days until plants attained the desired growth stage. For all metabolism experiments described below, waterhemp seedlings were harvested when the second true leaf had fully expanded (approximately 2–3 cm), while corn shoots were harvested prior to the first leaf unfurling (approximately 4–5 cm). For all experiments, a single biological replicate (experimental unit) consisted of a sample containing ten waterhemp seedlings or five corn shoots.

Thin-layer chromatography of S-metolachlor metabolism in waterhemp and corn

Greenhouse-grown plants were transferred to a growth chamber set at 28/22°C day/night with a 16 h photoperiod 24 h prior to experiments. The following day, ten waterhemp seedlings or five corn shoots were washed with deionized water and incubated for 2 h in a single 100 mL

sample cup per population containing 40 mL of the hydroponic solution described previously plus 5 µM radiolabeled *S*-metolachlor (3.0 kBq mL^{-1}; 555 MBq $mmol^{-1}$ specific activity). At 2 h, plant samples were triple rinsed with deionized water, fresh weights were recorded, frozen in liquid nitrogen, and stored at -20°C until processing. Plant samples for additional time points (> 2 h) were triple rinsed with deionized water, then transferred to a new 100 mL sample cup per population containing 40 mL of fresh hydroponic solution without herbicide. Plants were then removed, frozen, and stored at 4, 12, or 24 h after treatment (HAT). Time-course experiments were independently conducted twice (24 HAT results are not shown).

Following incubation, frozen tissue samples (approx. 0.25 g waterhemp or 0.75 g corn) were ground in liquid nitrogen and total radioactivity was extracted in 1 mL of 90% (v/v) methanol. After the first extraction and centrifugation at 12,000 *x* g, the supernatant was removed and remaining plant material was re-extracted in an additional 1 mL of 90% methanol. Following centrifugation, the pellet was discarded and supernatant removed and combined with the first supernatant, yielding a total final volume of 2 mL. The methanolic extracts were concentrated to incipient dryness with nitrogen gas, reconstituted in 400 µL of 90% methanol, vortexed, centrifuged, and radioactivity in each sample was quantified by liquid scintillation spectrometry. Samples were stored at -20°C, then removed and stored at 4°C for one day prior to TLC.

Aliquots containing 50 Bq (3000 dpm) were spotted on the preadsorbent zone of a channeled (20 x 20 cm) 250 µm silica TLC plate (Whatman Silica Gel 150A; GE Healthcare Life Sciences, Marlborough, Massachusetts 01752) along with 16.7 Bq (1000 dpm) of radiolabeled *S*-metolachlor and synthetic metabolite standards (synthesis protocol described below). Plates were developed once with a solvent system containing

chloroform/methanol/formic acid/water (75:25:4:2 by volume) as described previously (Miller et al. 1996). Plates were removed from the chamber after the solvent front had migrated to the top of the plate (20 cm). Plates were then air dried, wrapped in plastic film, and stored in a cassette facing a 20 x 40 cm phosphorimage plate (BAS-IIIs; Fuji Film Holdings America Corporation, Valhalla, NY 10595) for 24 h. Storage phosphor plates were imaged using a GE Typhoon phosphor image scanner (Typhoon 9400; GE Healthcare Life Sciences) and ImageQuant software (ImageQuant TL version 8.1; GE Healthcare Life Sciences) at the University of Illinois Proteomics Facility.

Quantification of S-metolachlor metabolism in waterhemp using HPLC

Plants were grown and prepared as previously described for TLC experiments, but the herbicide incubation and wash steps were modified as shown in Fig. 2.1 to conserve radiolabeled *S*-metolachlor. Samples of ten waterhemp seedlings or five corn shoots were placed in a 5 mL Eppendorf tube containing 1 mL of hydroponic solution and 100 µM radiolabeled *S*-metolachlor (5.6 kBq mL^{-1}; 55 MBq $mmol^{-1}$ specific activity) for 2 h. At 2 h, plant samples were triple rinsed with deionized water, fresh weights were recorded, frozen in liquid nitrogen, and stored at -20°C until processing. Plant samples for additional time points (> 2 h) were triple rinsed with deionized water, then transferred to a 60 mL sample cup per population containing 2 mL of fresh hydroponic solution without herbicide. Plants were then removed, frozen, and stored at 3, 4, 12, or 24 HAT. Time-course experiments were conducted twice with three replications per population per time-point per experiment. Recovery of absorbed radiolabeled *S*-metolachlor averaged 80% for all HPLC experiments (Section 2.4 and 2.5).

Frozen tissue samples were processed using the methods described previously for TLC experiments with the following modifications: concentrated methanolic extracts were reconstituted in 400 µL of 50% (v/v) acetonitrile and plant samples containing 133 Bq (8000 dpm) were analyzed by HPLC. Reverse-phase HPLC was performed with a PerkinElmer Flexar LC system (Model N2910401, Perkin Elmer, Akron, Ohio 44311) at a flow rate of 1 mL min^{-1} using a C18 column (Nucleodur C18 Pyramid, 5 µm x 250 mm; Macherey-Nagel, Düren, Germany). Eluent A was 0.1% (v/v) formic acid in water and eluent B was 0.1% (v/v) formic acid in acetonitrile. Sample volumes of 77 µL were injected and extractable radioactivity was resolved with a 19 min stepwise gradient starting at 90:10 A:B. Steps included a linear gradient to 75:25 A:B in 5 min, 60:40 A:B in 3 min, 40:60 A:B in 2 min, 15:85 A:B in 2 min, and finally 5:95 A:B in 3 min followed by a 4 min isocratic hold. Following each 19 min run, the column was returned to 90:10 A:B in 5 min and equilibrated for 3 min before subsequent injections. Radiolabeled compounds were detected with a β-RAM Radio HPLC-detector (Model 4; LabLogic Systems, Tampa, FL 33619) and Ultima-Flow M cocktail (PerkinElmer). The amount of *S*-metolachlor at each time point was determined by peak integration in Laura Software (Version 4.2.3.37; LabLogic Systems) as a percentage of total radioactivity detected. Peak retention times (R_T) for *S*-metolachlor, the cysteine (CYS) conjugate of *S*-metolachor, and the glutathione (GSH) conjugate of *S*-metolachlor (described below) were 17.4, 12.5, and 12.4 min, respectively.

S-metolachlor metabolism in the presence of metabolic inhibitors

Metabolism experiments with radiolabeled *S*-metolachlor and inhibitors were conducted to identify possible metabolic pathways and detoxification enzymes that might be responsible for

enhanced metabolism in CHR and SIR. Plants were grown and prepared as previously described for TLC and HPLC studies, but the ACR population was excluded. The GST inhibitor 4-chloro-7-nitrobenzofurazon (NBD-Cl) (Cummins et al. 2013; Ma et al. 2016; Ricci et al. 2005) and malathion, an organophosphate insecticide that inhibits certain cytochrome P450 activities (Abass et al 2009; Kreuz and Fonné-Pfister 1992), were tested for their ability to alter *S*-metolachlor metabolism rates and profiles in waterhemp and corn. Previous whole-plant studies have utilized organophosphate insecticides or NBD-Cl to investigate weed resistances to various herbicides (Brabham et al. 2019; Busi et al. 2017; Figueiredo et al. 2018; Ma et al. 2016; Ma et al. 2013; Obenland et al. 2019; Tardiff and Powles 1999; Varanasi et al. 2018).

Ten waterhemp seedlings or five corn shoots were placed in a 5 mL Eppendorf tube containing 1 mL of nontreated hydroponic solution or solution containing 100 µM malathion, 100 µM NBD-Cl, or both for a 1 h pre-incubation period. After 1 h, samples were then transferred to a separate 5 mL Eppendorf tube containing the same solutions (± inhibitors), but with the addition of 100 µM radiolabeled *S*-metolachlor (5.6 kBq mL^{-1}; 55 MBq $mmol^{-1}$ specific activity) for 2 h. At 2 h, samples were triple rinsed with deionized water and transferred to a new 60 mL cup per sample containing 2 mL of the same solutions (± inhibitors) without *S*-metolachlor. At 4 HAT, plant samples were triple rinsed with deionized water, fresh weights were recorded, frozen in liquid nitrogen, and stored at -20°C until processing. Frozen tissue samples were processed and analyzed using the same methods previously described for HPLC experiments (Section 2.4). Metabolic inhibitor experiments were independently conducted twice with three replications per population per treatment per experiment.

In vitro formation of synthetic standards

Synthetic GSH and CYS conjugates of *S*-metolachlor were prepared *in vitro* as previously described (Riechers et al. 1996). Reactions were carried out in 2 mL Eppendorf tubes containing 60 mM TAPS buffer (unadjusted pH 8.5), 10 mM GSH or L-CYS, and 1 µM radiolabeled *S*-metolachlor (555 MBq $mmol^{-1}$) in 1 mL of deionized water. Reaction mixtures were incubated for 24 h at 35°C and then terminated by adjusting the final volume to 1.5% formic acid. Following termination, reaction mixtures were partitioned once against 1 volume of methylene chloride to remove non-conjugated *S*-metolachlor. The conjugates of *S*-metolachlor in the aqueous phase were purified and concentrated by solid-phase extraction (SPE) using a preparative (3 mL) C18 column (200 mg; Chromabond C18ec; Machery-Nagel, Düren, Germany). Columns were pre-conditioned with 2 volumes of methanol followed by 2 volumes of acidified water (0.1% formic acid). The aqueous phase was then loaded onto the column under vacuum at 1 mL min^{-1}, washed with 1 mL of acidified water, dried under vacuum, and synthetic conjugates were eluted in 2 x 0.5 mL methanol and used as analytical standards for TLC and HPLC analysis.

Statistical analysis

Degradation rates of *S*-metolachlor from pooled HPLC experiments were calculated via a non-linear regression model in the *drc* package of R software (version 3.4.3, R Foundation for Statistical Computing) (Knezevic et al. 2007). The three-parameter logistic function was fitted utilizing the following equation:

$$Y = \frac{d}{1+\exp[b(\log x - \log DT50/DT90)]} \quad [1]$$

The logistic function consists of three-parameters: *b* is the slope of the curve, *d* is the upper asymptote, and the DT_{50} or DT_{90} are time needed to degrade 50% (DT_{50}) and 90% (DT_{90}) of the parent radiolabeled compound, respectively.

For metabolism experiments with metabolic inhibitors, data were analyzed by ANOVA in PROC GLIMMIX of SAS 9.4 (SAS Institute, Inc., Cary, NC 27513) fitted with a Beta distribution and a Logit link function. The response variable was the percentage of radiolabeled *S*-metolachlor detected with population, treatment, and their interaction as fixed effects. Random effects included experiment run and sample replication nested within run. Mean separations were conducted by Fishers Least Significant Difference (LSD) (α=0.05). Data are presented by population due to significant treatment (P<0.0001), population (P=0.0062), and treatment x population (P<0.0001) interactions.

Results

Thin layer chromatography of S-metolachlor metabolism in waterhemp and corn

TLC was conducted to compare the relative abundances of *S*-metolachlor among populations and species between 2 and 24 HAT. Visual inspection of the compound in each lane that co-chromatographed with parent *S*-metolachlor demonstrated that resistant waterhemp (CHR and SIR) metabolized more *S*-metolachlor than either sensitive population (ACR and WUS) at 4 HAT (Fig. 2.2A) and 12 HAT (Fig. 2.2B). In addition, the abundance of *S*-metolachlor in each resistant waterhemp population was similar to naturally tolerant corn (Fig. 2.2A,B).

Metabolites with similar migration distances as the synthetic GSH and CYS conjugates of *S*-metolachlor were observed in all waterhemp populations and corn. Interestingly, the relative amounts of the metabolite that co-chromatographed with the *S*-metolachlor-GSH conjugate in

each waterhemp population were equal to or less than corn at each respective time point (Fig. 2.2A,B). Both resistant waterhemp populations also formed metabolites that were not present in sensitive waterhemp or corn (Fig. 2.2A,B). This result indicates that resistant waterhemp might utilize different metabolic pathways or enzymes than either sensitive waterhemp or corn, in addition to GST-mediated conjugation with GSH (Fuerst 1987). TLC experiments demonstrated that CHR and SIR are resistant to *S*-metolachlor through enhanced metabolism, and served as a guide for further experimentation to quantify differences in *S*-metolachlor degradation.

Quantification of S-metolachlor metabolism in waterhemp using HPLC

Time-course experiments (2 to 24 HAT) were conducted to quantify rates of *S*-metolachlor metabolism in resistant (CHR and SIR) and sensitive (WUS and ACR) waterhemp in comparison to corn. CHR and SIR degraded *S*-metolachlor more rapidly than either sensitive population ($P<0.001$) and at a similar rate as corn ($P>0.1$) (Table 2.1) based on the curves generated (Fig. 2.3) and comparison of times to degrade 50% (DT_{50}) and 90% (DT_{90}) of absorbed *S*-metolachlor (Table 2.1). In addition to the calculated DT values, HPLC analysis qualitatively supported TLC results. Metabolites in each sample co-chromatographed with the synthetic GSH and CYS conjugates of *S*-metolachor (R_T of 12.4 and 12.5 min, respectively) (Fig. 2.4). The presence of several metabolites in each sample with R_T values between 11 and 14 min may represent degradation products of the initial GSH conjugate, including dipeptide conjugates (Lamoureaux and Rusness 1981; Lamorueux and Rusness 1993; Martin et al. 2007; Wolf et al. 1996), as well as various oxidation products. Interestingly, one polar metabolite (R_T=3.3 min) was detected only in CHR and SIR (Fig. 2.4).

S-metolachlor metabolism in the presence of metabolic inhibitors

Each waterhemp population and corn responded differently to each inhibitor in combination with *S*-metolachlor at 4 HAT (Fig. 2.5). The only treatment that significantly inhibited metabolism of *S*-metolachlor in WUS was the combination of malathion and NBD-Cl (Fig. 2.5C), which increased the amount of parent herbicide remaining 1.2-fold (45% to 54%). In contrast, each inhibitor alone and in combination significantly increased the amount of *S*-metolachlor in CHR and SIR (Fig. 2.5A,B). Only 2.5% of absorbed *S*-metolachlor was detected in CHR without an inhibitor. However, addition of malathion, NBD-Cl, or both inhibitors increased the amount of *S*-metolachlor remaining 5.6-, 3.8-, and 7-fold, respectively (Fig. 2.5A). Similar to CHR, SIR without an inhibitor contained 1.1% of the parent herbicide (Fig. 2.5B). The addition of malathion increased *S*-metolachlor remaining 5.4-fold while NBD-Cl treatment resulted in a 5.8-fold increase, and the combination of malathion and NBD-Cl yielded a 13.7-fold increase of parent herbicide remaining (Fig. 2.5B). *S*-metolachlor metabolism in corn was not affected by the addition of either or both inhibitors and the amount of *S*-metolachlor remaining was 2% or less for all treatments (Fig. 2.5D).

Representative chromatograms of CHR and SIR extracts from metabolic inhibitor studies at 4 HAT were virtually identical qualitatively, so only results for SIR are shown (Fig. 2.6). In addition to increasing the amount of parent herbicide (Fig. 2.5B), malathion and/or NBD-Cl altered the metabolite profile in SIR extracts (Fig. 2.6). Malathion reduced the abundance of several metabolites, including the unknown polar metabolite (R_T=3.3 min; Fig. 2.6B) only detected in CHR or SIR extracts (Fig. 2.4), indicating this compound results from oxidative metabolism. NBD-Cl reduced the abundance of GSH and CYS conjugates (and possibly dipeptide catabolites; Fig. 2.6C), indicating that inhibition of GSTs in SIR capable of

metabolizing *S*-metolachlor had occurred. The combination of malathion and NBD-Cl clearly increased the abundance of *S*-metolachlor (Fig. 2.6D) to a level greater than with either inhibitor applied alone (Fig. 2.6B, C). Collectively, results from these experiments indicate both GSTs and P450s might play major roles in rapidly metabolizing *S*-metolachlor in MHR waterhemp.

Discussion

CHR and SIR are the first reported waterhemp populations that display resistance to VLCFA-inhibiting herbicides (Heap 2020). Results from our current mechanistic research support previous greenhouse findings that both populations are resistant to *S*-metolachlor (Strom et al. 2019). Time-course experiments using TLC and HPLC demonstrated that resistant populations metabolize *S*-metolachlor faster than sensitive populations and equally as fast as corn. Our findings are similar to investigations of VLCFA-inhibitor resistance in *Lolium spp*., where DT_{50} values for flufenacet in resistant *Lolium spp*. populations were less than one hour compared with more than seven hours in sensitive populations (Dücker et al. 2019). In addition, higher levels of pyroxasulfone were quantified in VLCFA-inhibitor sensitive *Lolium rigidum* than in resistant populations during time-course experiments (Busi et al. 2018).

Extensive research has been conducted to investigate natural crop tolerance to various VLCFA-inhibiting herbicides, but mechanistic research investigating resistance in a weed species, especially dicot weeds, is limited. In general, certain crops and weeds are tolerant to VLCFA-inhibiting herbicides due to rapid conjugation to GSH or homoglutathione resulting from higher levels of GST activity (measured using herbicide substrates) and/or GSH content than sensitive species (Breaux 1987; Hatton et al. 1996). GSH-herbicide conjugates formed in the cytosol are considered non-phytotoxic (Cummins et al. 2011; Shimabukuro et al. 1978) but

following vacuolar transport the GSH tripeptide moiety is typically catabolized by sequential hydrolysis of its component amino acids and recycled in the vacuole, resulting in detection of herbicide-CYS conjugates (Lamoureaux and Rusness 1981; Martin et al. 2007). This detoxification process in cereal crops can be further increased through the addition of a herbicide safener (Gronwald 1989; Hatzios 1991; Riechers et al. 1996; Riechers et al. 2010).

In addition to GSH conjugation reactions catalyzed by GSTs, oxidative metabolism of several VLCFA-inhibitor substrates by P450s occurs in some plant species (Moreland and Corbin 1991; Moreland et al. 1993; Moreland et al. 1995; Siminszky 2006). Plant P450 enzymes are encoded by large multigene families (Mizutani and Ohta 2010) and are capable of catalyzing many different reactions in plant primary and secondary metabolism (Hamberger and Bak 2013), including oxidation of herbicides to less phytotoxic intermediates that are predisposed to further conjugation to amino acids, sugars, or possibly GSH (Siminszky 2006; Werck-Reichhart et al. 2000). Microsomal fractions containing P450s metabolize VLCFA inhibitors (such as metolachlor) in corn and mung bean (Moreland and Corbin 1993; Moreland and Corbin 1995) and oxidative metabolites of metolachlor have been identified in corn (Xie et al. 2010), but these metabolites have not been detected in a resistant dicot weed to date.

Metabolism of most selective herbicides occurs more slowly in sensitive weed species than tolerant crops, such as with pyroxasulfone selectivity between *Lolium* and wheat (Tanetani et al. 2013). It also is possible that herbicide-resistant weeds could possess different metabolic pathways (Pan et al. 2019) or generate different metabolites within the same pathway than in tolerant crops. For example, SIR primarily generated ring-hydroxylated metabolites of the HPPD-inhibitor topramezone whereas corn generated an *N*-demethylated product (Lygin et al. 2018). Both sensitive and resistant waterhemp populations examined in our study metabolized

S-metolachlor through conjugation to GSH, which is supported by co-chromatography of plant-extracted metabolites with corresponding synthetic GSH and CYS conjugate standards. However, the presence of unknown metabolites detected in chromatograms from resistant waterhemp but not in corn (Fig. 2.2, 2.4) also demonstrates the possibility of resistant waterhemp utilizing a different or additional detoxification pathway.

Metabolism of parent *S*-metolachlor was partially reduced in CHR and SIR after treatment with a P450 and/or GST inhibitor (Fig. 2.5). SIR rapidly detoxifies atrazine through GSH conjugation (Evans et al. 2017; Ma et al. 2013) and the HPPD-inhibiting herbicides mesotrione (Ma et al. 2013) and topramezone (Lygin et al. 2018) via oxidative metabolism. Plant detoxification processes are generally complex and substrate specific (Edwards and Owen 1989; Riechers et al. 2010; Werck-Reichhart et al. 2000). We hypothesize that the previously characterized metabolic mechanisms and enzymes in SIR (Lygin et al. 2018; Ma et al. 2013) could confer cross-resistance to *S*-metolachlor, or alternatively multiple resistance to *S*-metolachlor may be conferred by distinct genes and metabolic enzymes. Further research using LC-MS methods is underway to identify and quantify the initial metabolites in resistant waterhemp to comprehensively understand potential resistance mechanism(s) to *S*-metolachlor and the implications for managing MHR waterhemp populations in the field.

Conclusions

CHR and SIR possess metabolic resistance to *S*-metolachlor, possibly due to more complex metabolic pathway(s) than in sensitive waterhemp or tolerant corn. The dioecious biology of waterhemp could lead to resistance arising or spreading to other populations (Liu et al. 2012; Sarangi et al. 2017). Rapid metabolism of *S*-metolachlor in resistant waterhemp has

serious implications for growers by reducing the efficacy and duration of residual control (Hager 2019) with products containing *S*-metolachlor or other VLCFA-inhibiting herbicides (Evans et al. 2019; Strom et al. 2019; Hausman et al. 2013). Understanding if MHR waterhemp detoxification pathways are distinctive from tolerant crops could provide insight for the crop protection industry by informing herbicide discovery efforts aimed at avoiding or inhibiting current resistance mechanisms, leading to new selective products and management recommendations in the future. An integrated management system incorporating diverse chemical, cultural, and mechanical practices is an essential component of a sustainable program that limits additions to the soil-seed bank (Buhler et al. 1997; Schwartz-Lazaro and Copes 2019).

Table

Table 2.1 Times required to degrade 50% (DT_{50}) or 90% (DT_{90}) of absorbed radiolabeled *S*-metolachlor in waterhemp (*Amaranthus tuberculatus*) seedlings and corn (*Zea mays*) shoots, with corresponding 95% confidence intervals in parentheses. Degradation of *S*-metolachlor was measured during a time course at 2, 3, 4, 12, and 24 hours after treatment as described in Methods.

Population[a]	DT_{50}	DT_{90}
	Time (Hours)	
ACR (S)	2.9 (2.7–3.0)	6.3 (5.7–6.9)
WUS (S)	2.9 (2.7–3.0)	7.4 (6.4–8.3)
CHR (R)	1.7 (1.6–1.8)	3.2 (3.0–3.5)
SIR (R)	1.6 (1.5–1.8)	2.7 (2.5–3.0)
Corn (NT)	1.7 (1.6–1.8)	2.7 (2.5–3.0)

[a]Letters in parentheses describe the phenotype of each population in response to *S*-metolachlor. S, *S*-metolachlor-sensitive waterhemp; R, *S*-metolachlor-resistant waterhemp; NT, naturally tolerant to *S*-metolachlor (hybrid B73xMo17).

Figures

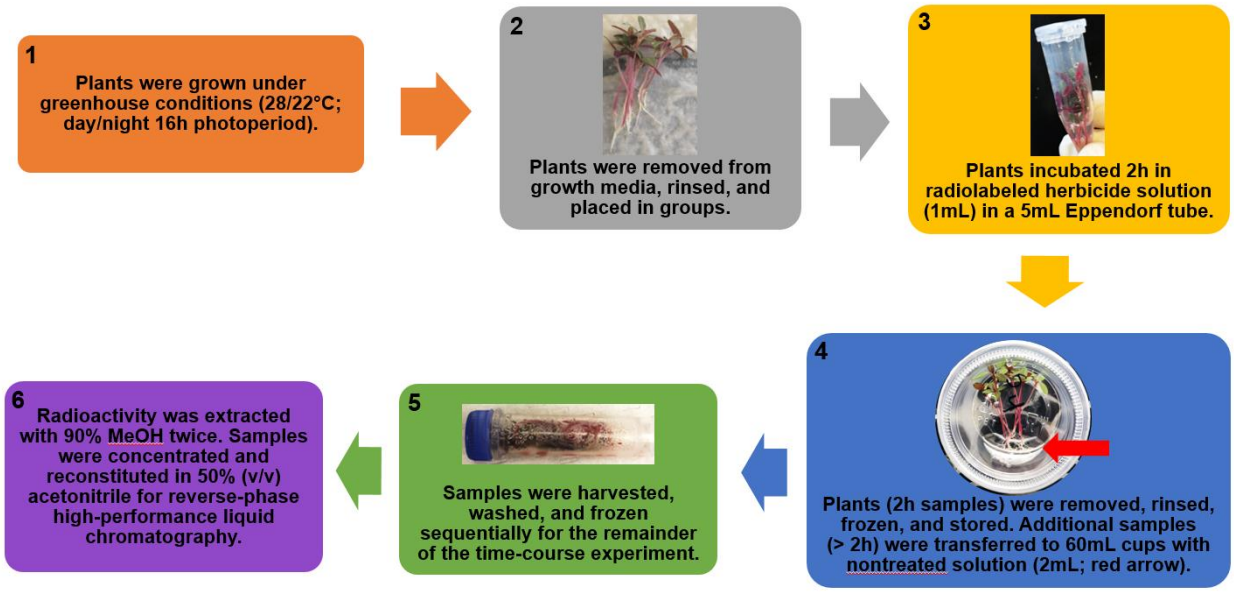

Figure 2.1 Flow diagram of methods used to prepare waterhemp seedlings for *S*-metolachlor metabolism studies and HPLC analysis.

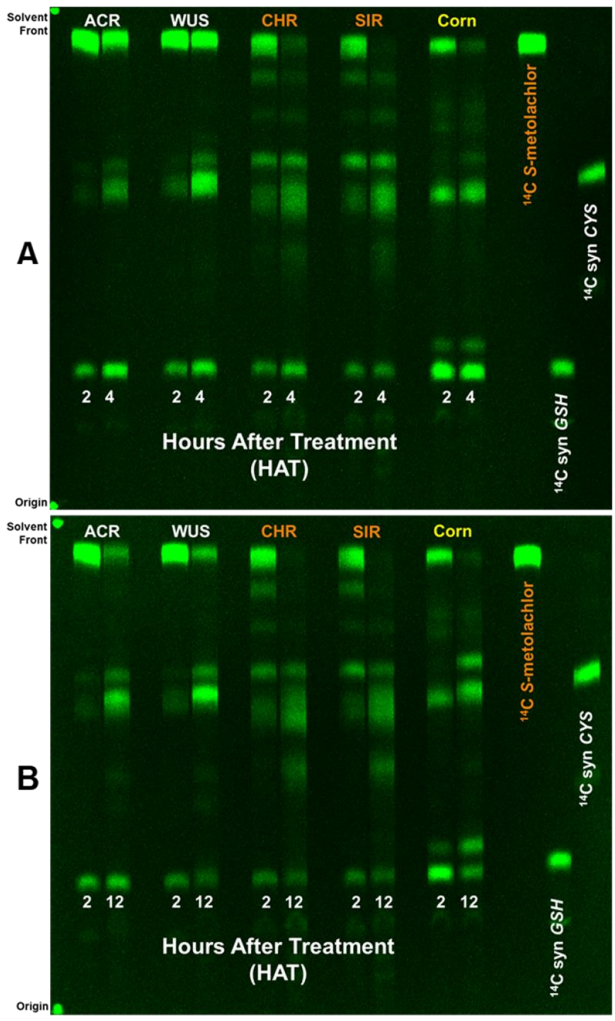

Figure 2.2 Thin-layer chromatography (TLC) analysis of radiolabeled *S*-metolachlor and its metabolites extracted from corn (*Zea mays*) shoots and *S*-metolachlor-sensitive (ACR, WUS) or resistant (CHR, SIR) waterhemp (*Amaranthus tuberculatus*) seedlings 2 and 4 HAT (**A**) or 2 and 12 HAT (**B**) with 5 µM radiolabeled *S*-metolachlor (3.0 kBq Ml^{-1}; 555 MBq $mmol^{-1}$ specific activity). Methanolic extracts from each plant sample or standard containing 50 Bq (3000 dpm) or 16.7 Bq (1000 dpm), respectively, were spotted per lane on a silica gel TLC plate and developed in chloroform/methanol/formic acid/water (75:25:4:2 by volume). ^{14}C syn GSH, synthetic *S*-metolachlor-glutathione standard; ^{14}C syn CYS, synthetic *S*-metolachlor-cysteine standard.

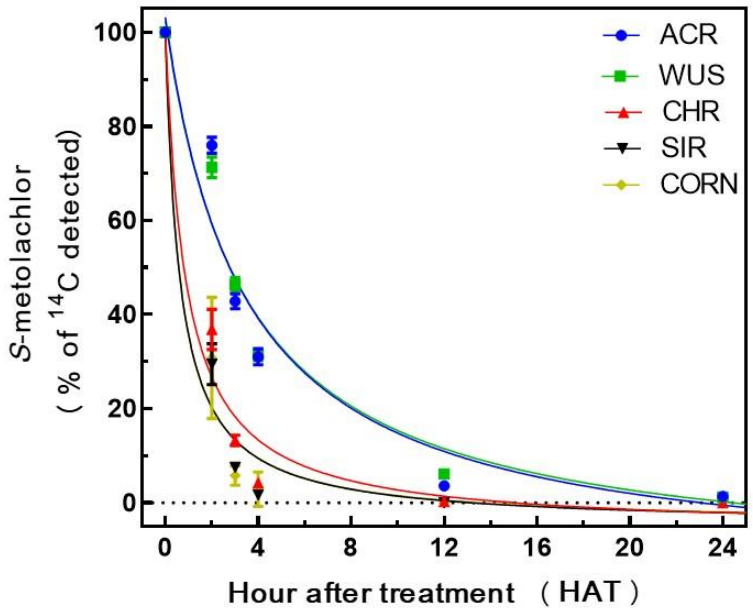

Figure 2.3 Degradation curves of *S*-metolachlor extracted from corn (*Zea mays*) shoots and *S*-metolachlor-sensitive (ACR, WUS) or resistant (CHR, SIR) waterhemp (*Amaranthus tuberculatus*) seedlings during a 24 h time course. Lines in each graph were fitted using the equation: ($Y = \frac{d}{1+\exp[b(\log x - \log DT50/DT90)]}$), and each symbol represents the observed mean (±SE) amount of *S*-metolachlor as a percentage of total radioactivity detected by HPLC analysis.

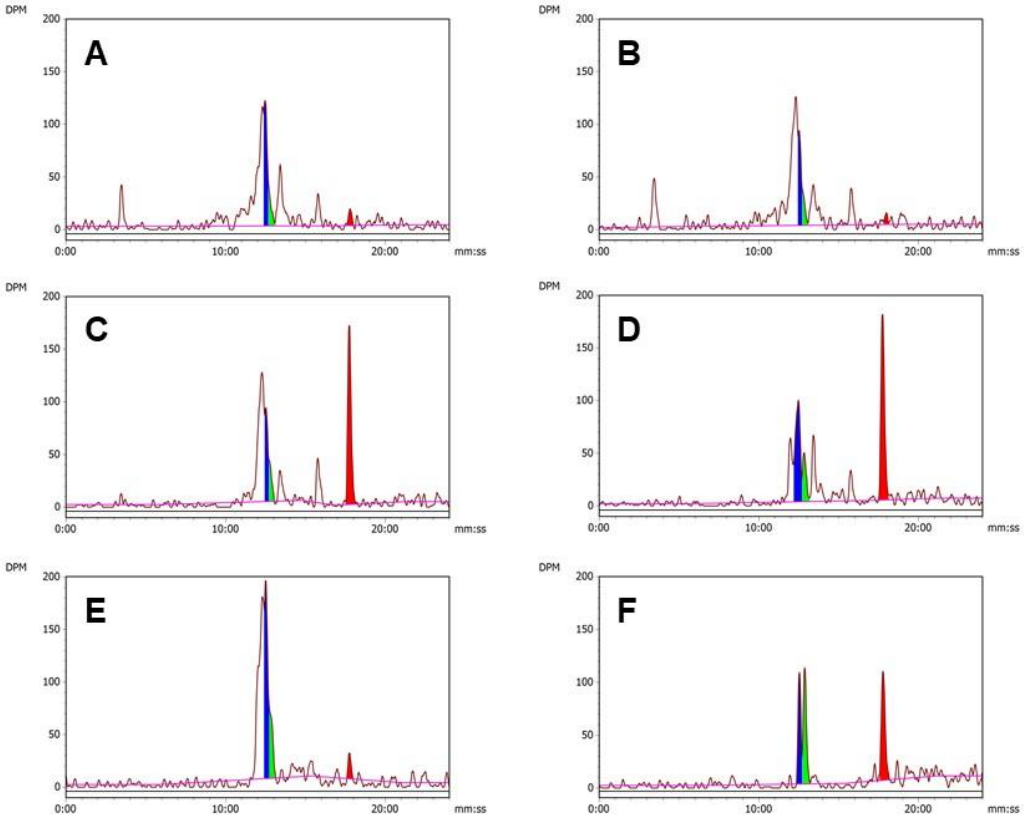

Figure 2.4 Representative HPLC chromatograms from extracts of *S*-metolachlor-resistant (**A**, CHR; **B**, SIR) and sensitive (**C**, ACR; **D**, WUS) waterhemp (*Amaranthus tuberculatus*) seedlings or corn (*Zea mays*) shoots € at four hours after treatment (HAT) with 100 µM radiolabeled *S*-metolachlor (5.6 kBq mL^{-1}; 55 MBq $mmol^{-1}$ specific activity). Four HAT chromatograms are shown since they best represent the array of metabolites formed during the 24 h degradation study (Fig. 2.3). Regions shaded in red in each panel correspond to parent *S*-metolachlor, peak retention time (R_T) of 17.4 min. Green and blue shaded regions in each panel correspond to CYS and GSH conjugates of *S*-metolachlor (R_T=12.5 and 12.4 min, respectively). Panel **F**, co-injection of all three standards.

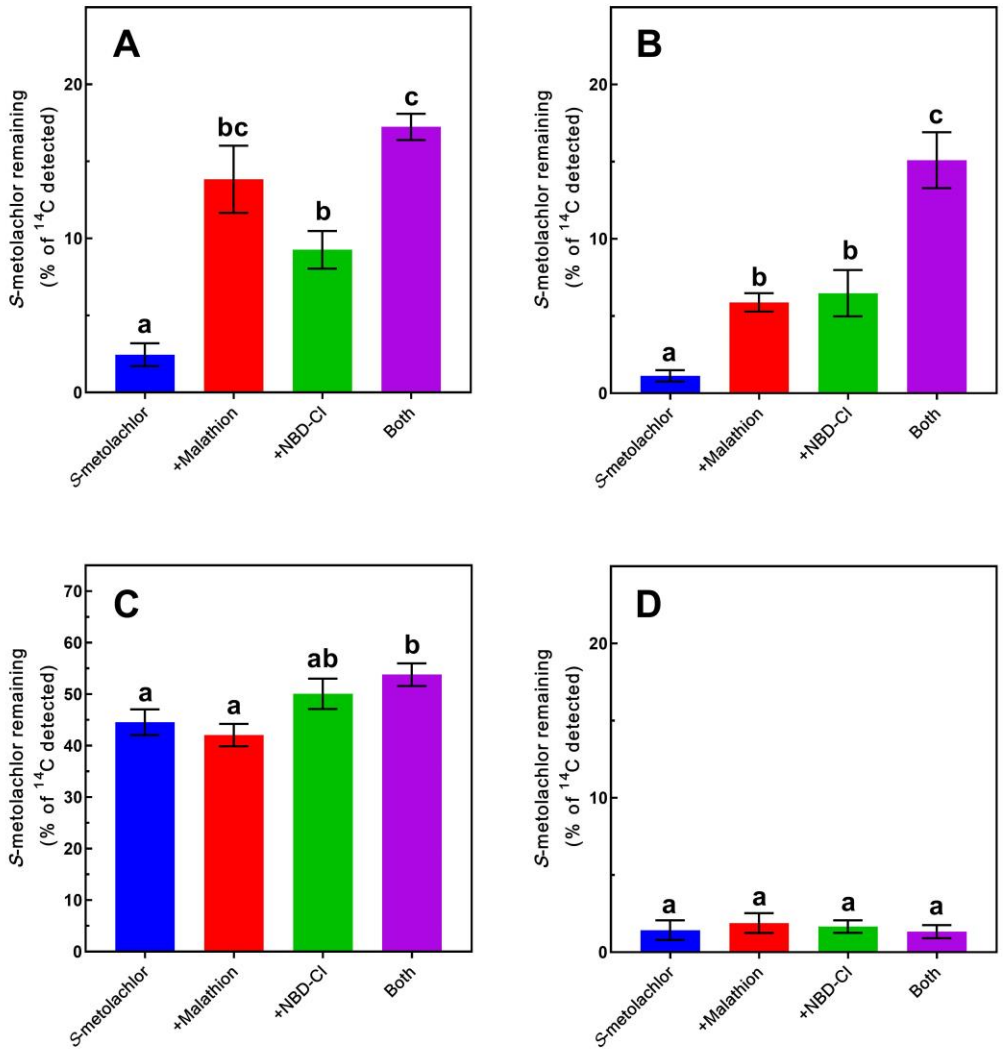

Figure 2.5 Effects of malathion (P450 inhibitor) and 4-chloro-7-nitrobenzofurazon (GST inhibitor) on *S*-metolachlor metabolism in resistant (**A**, CHR; **B**, SIR) and sensitive (**C**, WUS) waterhemp (*Amaranthus tuberculatus*) seedlings or corn (*Zea mays*) shoots (**D**) at four hours after treatment. Plants were pre-incubated for one hour before treatment with 100 µM radiolabeled *S*-metolachlor (5.6 kBq mL^{-1}; 55 MBq $mmol^{-1}$ specific activity) ± inhibitors. Each bar represents the mean (±SE) percentage of *S*-metolachlor quantified by HPLC. Mean separations were conducted by Fishers protected LSD (α=0.05) and values sharing the same letter within each panel are not significantly different.

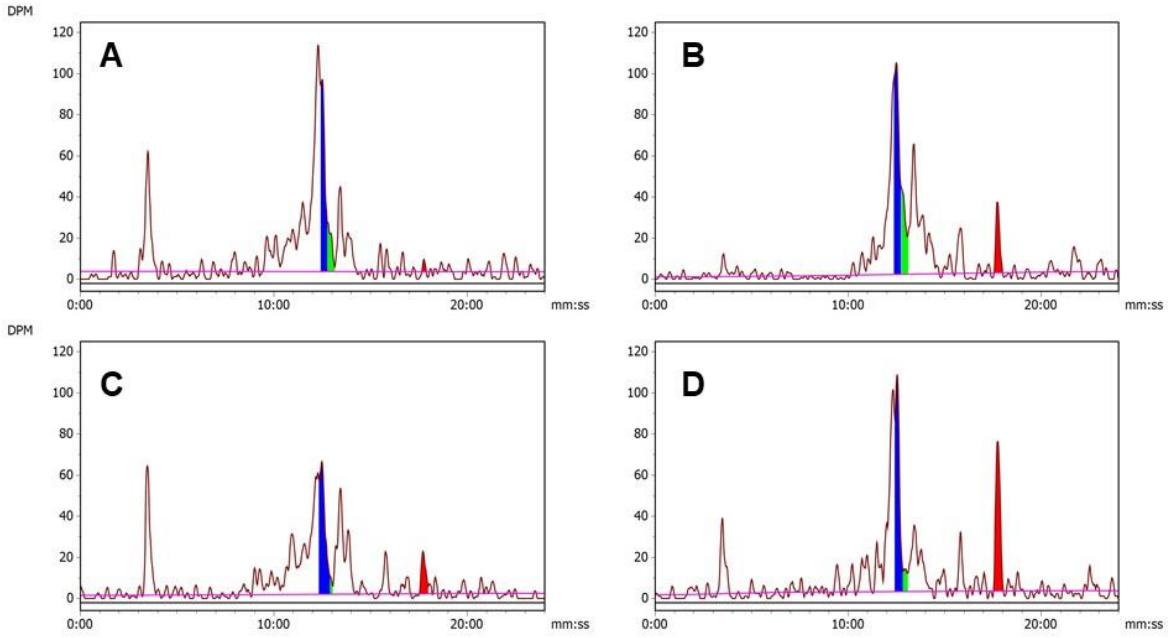

Figure 2.6 Representative HPLC chromatographs from *S*-metolachlor-resistant waterhemp (*Amaranthus tuberculatus*) SIR extracts from the metabolic inhibitor experiment (Fig. 2.5B) at four hours after treatment. Plants were pre-incubated for one hour before treatment with 100 µM radiolabeled *S*-metolachlor (5.6 kBq mL^{-1}; 55 MBq $mmol^{-1}$ specific activity) ± inhibitors. **A**, SIR seedlings treated with *S*-metolachlor only; **B**, SIR seedlings treated with *S*-metolachlor plus 100 µM malathion (P450 inhibitor); **C**, SIR seedlings treated with *S*-metolachlor plus 100 µM 4-chloro-7-nitrobenzofurazon (GST inhibitor); **D**, SIR seedlings treated with *S*-metolachlor plus both inhibitors at 100 µM each. Regions shaded in red in each panel correspond to parent *S*-metolachlor, peak retention time (R_T) of 17.4 min. Green and blue shaded regions in each panel correspond to CYS and GSH conjugates of *S*-metolachlor (R_T=12.5 and 12.4 min, respectively).

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CHAPTER 3: METABOLIC PATHWAYS FOR *S*-METOLACHLOR DETOXIFICATION DIFFER BETWEEN TOLERANT CORN AND MULTIPLE-RESISTANT WATERHEMP

Abstract

Resistance to the very-long-chain fatty acid (VLCFA)-inhibiting herbicide, *S*-metolachlor, in multiple-herbicide resistant populations (CHR and SIR) of waterhemp (*Amaranthus tuberculatus*) is conferred by rapid metabolism compared with sensitive populations. However, enzymatic pathways for *S*-metolachlor metabolism in waterhemp are unknown. Enzyme assays using *S*-metolachlor were developed to determine specific activities of glutathione *S*-transferases (GSTs) and cytochrome P450 monooxygenases (P450s) from CHR and SIR seedlings to compare with tolerant corn and sensitive waterhemp (WUS). GST activities were greater (~2-fold) in CHR and SIR compared to WUS, but much less than corn. In contrast, P450s in microsomal extracts from CHR and SIR formed *O*-demethylated *S*-metolachlor, and their NADPH-dependent specific activities were greater (>20-fold) than corn or WUS. Metabolite profiles of *S*-metolachlor generated via untargeted and targeted liquid chromatography-mass spectrometry from CHR and SIR differed from WUS, with greater relative abundance of *O*-demethylated *S*-metolachlor and *O*-demethylated *S*-metolachlor-glutathione conjugates formed by CHR and SIR. Treatment of CHR and SIR extracts with β-glucosidase indicated glucose conjugates, likely generated by glucosyl transferases following *S*-metolachlor *O*-demethylation by P450s. In summary, results demonstrate *S*-metolachlor metabolism in resistant waterhemp involves the metabolic activities of P450s, GSTs, and glucosyl transferases acting in concert, but the initial *O*-demethylation reaction confers resistance.

Introduction

Waterhemp (*Amaranthus tuberculatus* var *rudis* Moq. Sauer) is a common, small-seeded dicot weed species in the Midwestern United States (Sauer 1955; Steckel 2007). Waterhemp is a prolific seed producer capable of generating thousands of seeds per female plant, and seedlings emerge throughout the entire growing season (Buhler and Hartzler 2001; Hartzler et al. 1999; Steckel 2007; Steckel et al. 2003). Waterhemp is extremely competitive and can reduce corn (*Zea mays* L.) and soybean (*Glycine max* (L.) Merr) grain yield (Hager et al. 2002; Steckel and Sprague 2004). Waterhemp is also dioecious, and outcrossing results in high intraspecific diversity compared with self-pollinated weed species (Murray 1940; Sauer 1955). High intraspecific diversity favors resistance evolution (Adhikary and Pratt 2015), and waterhemp has evolved resistance to herbicides representing seven sites-of-action (SoAs) groups as a species (Heap 2021; Tranel 2020; Tranel et al. 2011). Most recently, waterhemp has evolved resistance to inhibitors of very-long-chain fatty acids (VLCFAs) (Heap 2021), and multiple resistance to herbicides from up to six SoAs has been reported within populations (Evans et al. 2019; Shergill et al. 2018; Strom et al. 2019).

VLCFAs with acyl chains in excess of 18 carbons are essential for the formation of cell membranes, cuticle waxes, and lipids (Bach and Faure 2010) and are formed by the membrane-bound VLCFA-elongase complex (Fehling and Mukherjee 1991; Haslam and Kunst 2013). The VLCFA-elongase complex consists of four enzymes (Haslam and Kunst 2013) and *S*-metolachlor is a competitive inhibitor of the first subunit, VLCFA synthase, resulting in depletion of VLCFAs (Böger 2003; Trenkamp et al. 2004). *S*-metolachlor is a resolved formulation of racemic metolachlor, which normally exists in four stereoisomers (Muller et al.

2001), but the *S*-isomers provide the majority of herbicidal activity (Moser et al. 1983). *S*-metolachlor is effective for preemergence control of annual monocot and small-seeded dicot weeds (Fuerst 1987), including waterhemp and Palmer amaranth (*Amaranthus palmeri* S. Watson), in corn, grain sorghum (*Sorghum bicolor* L. Moench ssp. *bicolor*), and soybeans.

Metabolic herbicide resistance has recently become more common in waterhemp and typically involves either glutathione *S*-transferases (GSTs) or cytochrome P450 monooxygenases (P450s) (Nandula et al. 2019, Tranel 2020; Yu and Powles 2015). P450 enzymes are encoded by large multigene families, with over 240 genes encoding P450s in *Arabidopsis thaliana* (Dimaano and Iwakami 2020; Mizutani and Ohta 2010; Paquette et al. 2009; Schuler 2015). The overall catalytic reaction of P450s typically results in the incorporation of one oxygen atom into the substrate (Siminszky 2006; Werk-Reichhart and Feyereisen 2000). Substrates and reactions are diverse, including ring and aryl hydroxylation, *N*- and *O*-dealkylation, and hydrolysis (Hamberger and Bak 2013; Li et al. 2020; Mansuy 1998). Previous reports demonstrated microsomal fractions containing P450s from crop species oxidize metolachlor as a substrate (Moreland et al. 1990; 1993; 1995). Additionally, several oxidative metabolites have been identified in corn (Xie et al. 2010). P450-mediated herbicide metabolism has been postulated as a resistance mechanism to the VLCFA inhibitors pyroxasulfone and *S*-metolachlor in *Lolium* (Busi 2014; Busi et al. 2017; Tardiff and Powles 1999) and *S*-metolachlor in waterhemp (Strom et al. 2020).

Natural tolerance to VLCFA-inhibiting herbicides is due to Phase II reactions mediated by GSTs (Fuerst 1987). GSTs are encoded by a diverse family of genes, with over 50 genes in *Arabidopsis thaliana* (Estévez and Hernández 2020), and GST subunits can also form homo- and heterodimers (Dixon et al. 2002). GSTs catalyze conjugation of reduced glutathione (GSH) or

homoglutathione to electrophilic herbicides in the cytosol, forming a non-phytotoxic compound (Cummins et al. 2011; Mashiyama et al. 2014; Shimabukuru et al. 1978) that is typically pumped into the vacuole by Phase III transporters in the tonoplast (Riechers et al. 2010). In general, tolerant crops or weed species have higher GST activity, GSH content, or both compared with sensitive species (Breaux 1987; Hatton et al. 1996). Mechanistic research of VLCFA-inhibitor-resistant dicot weed species is limited, but greenhouse and laboratory experiments with the GST inhibitor 4-chloro-nitrobenzofurazon suggested GSTs might be involved in conferring *S*-metolachlor resistance in waterhemp (Strom et al. 2020) and Palmer amaranth (Brabham et al. 2019).

Previously, we reported two multiple herbicide-resistant waterhemp populations from Illinois (CHR from Champaign County; SIR from McLean County) were resistant to VLCFA inhibitors (Strom et al. 2019). We then determined that the mechanism of *S*-metolachlor resistance was enhanced herbicide metabolism compared with sensitive waterhemp (Strom et al. 2020). However, our previous research did not unequivocally determine the enzyme families responsible for enhanced *S*-metolachlor metabolism. Based on our previous results, we hypothesized the pathways for *S*-metolachlor metabolism in CHR and SIR are different than the tolerance mechanism in corn, and that *S*-metolachlor detoxification involves both GSTs and P450s (Strom et al. 2020). To directly test this hypothesis, we first developed methods for waterhemp seedlings to study the activity of each enzyme with *S*-metolachlor as substrate. The objectives of our present study are to: (1) determine the relative contribution of GSTs and P450s in enhanced *S*-metolachlor metabolism, and (2) investigate early metabolites formed during detoxification of *S*-metolachlor and deduce metabolic pathways in CHR and SIR.

Materials and Methods

Chemicals

Radiolabeled [URL-^{14}C] *S*-metolachlor (555 MBq mmol^{-1}) was supplied by Syngenta Crop Protection (Greensboro, North Carolina 27409). Non-labeled analytical grade *S*-metolachlor (98% pure) was purchased from Chem Service, Inc. (West Chester, Pennsylvania 19381). All other analytical grade chemicals and reagents were purchased through Fisher Scientific (Thermo-Fisher, Hanover Park, Illinois 60133) or Sigma Chemical (Millipore Sigma, St. Louis, Missouri 63102).

Waterhemp populations and plant materials

Two previously-characterized multiple herbicide-resistant waterhemp populations (CHR and SIR) were selected. CHR originated from Champaign County, IL (Evans et al. 2019) and SIR originated from McLean County, IL and is analogous to the MCR population described in previous research (Hausman et al. 2011; 2013). Both populations are resistant to VLCFA-inhibiting herbicides due to enhanced herbicide metabolism (Strom et al. 2020). The WUS population is sensitive to VLCFA inhibitors (Strom et al. 2019) and was selected for comparison. Corn (hybrid B73 x Mo17) was included for comparison due to natural corn tolerance to *S*-metolachlor via rapid GST-mediated metabolism (Fuerst 1987). All waterhemp seeds were stratified to improve germination according to methods previously described (Bell et al. 2013) and stored at 4°C for at least 20 d prior to initiating experiments.

All plants were grown under greenhouse conditions (28/22 °C day/night with a 16 h photoperiod) in 509 cm^3 cell pack inserts containing vermiculite soaked in a commercial hydroponic fertilizer solution (Peters Hydroponic Special 5-11-26; ICL Specialty Fertilizers,

Summerville, South Carolina 29483) at one-third strength supplemented with 0.15 g L^{-1} $Ca(NO_3)_2$. Natural sunlight was supplemented with mercury halide lamps to provide 800 µmol m^{-2} s^{-1} photon flux at the soil surface. New hydroponic solution was added every two d until plants attained the desired growth stage. For all experiments, waterhemp seedlings were harvested when the second true leaf had fully expanded (approximately 2–3 cm), while corn shoots were harvested prior to the first leaf unfurling (approximately 4–5 cm). For enzymatic assays, whole waterhemp seedlings and corn shoots were then rinsed in deionized H_20 to remove vermiculite, patted dry, sorted into 1 g fresh weight samples, placed in containers and frozen in liquid N_2. Samples were stored at -80°C until experiments were initiated. Samples for all liquid chromatography-mass spectrometry (LC-MS) and β-glucosidase experiments consisted of ten waterhemp seedlings (approximately 0.25 g).

Tissue homogenization and protein preparation for GST-activity assays

Frozen 1 g samples were pulverized in a mortar pre-chilled with liquid N_2 and protein was extracted in 3 volumes of extraction buffer (200 mM Tris-Cl (pH 7.8), 1 mM Na_2EDTA, 5 mM 2-mercaptoethanol, plant protease inhibitor cocktail (Protease inhibitor Cocktail VI; bioWORLD, Dublin, OH), and polyvinylpolypyrrolidone (PVPP; 75 mg mL^{-1})). Samples were then centrifuged for 20 min at 15,000 *x g* and the supernatant (crude protein extract) was removed and transferred to a clean 15 mL centrifuge tube. Crude protein extracts were then precipitated by adjusting the final volume to 80% (v/v) acetone (4°C) (Lester et al. 2004). Acetone precipitation was conducted to concentrate protein from each tissue sample. Precipitated protein was then re-centrifuged at 15,000 *x g* for an additional 10 min and the supernatant was decanted. Acetone-precipitated protein was gently dried on ice with N_2 gas and stored at -80°C.

On the same day protein samples were used in enzymatic assays, precipitated protein was resuspended in the same extraction buffer described previously (0.5 mL waterhemp; 0.8 mL corn) without PVPP. Protein was resuspended by gently stirring with a paintbrush dipped in buffer and remaining plant pigments were removed with a desalting column (Zeba Spin Desalting Column, MWCO 7000 Da, ThermoFisher Scientific, Waltham, MA 02451) preconditioned in three column volumes of protein extraction buffer without PVPP. Protein concentrations within samples was determined by a Bradford assay (Bradford 1976) utilizing a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific).

Waterhemp and corn GST-activity assays with S-metolachlor

GST activity assays with *S*-metolachlor were modified from previous studies with the herbicides atrazine (Evans et al. 2017) or dimethenamid (Riechers et al. 1996b). Reaction mixtures consisted of 100 mM Tris-Cl (pH 7.8), 10 mM GSH, partially purified protein extracts, and 0.2 mg mL^{-1} BSA. Negative controls were included to determine the non-enzymatic conjugation rate of GSH with *S*-metolachlor and protein was replaced by extraction buffer without PVPP. Assay mixtures were incubated for five min at 30°C for equilibration then reactions were initiated by adding radiolabeled *S*-metolachlor (100 µM final concentration; 29.6 MBq $mmol^{-1}$ specific radioactivity) to adjust the final volume to 500 µL. Reactions were incubated for 30 min at 30°C and were terminated by the addition of 50 µL glacial acetic acid.

Reaction mixtures were partitioned once against 900 µL methylene chloride to separate unconjugated *S*-metolachlor from its polar GSH-*S*-metolachlor metabolite. Samples were vortexed and briefly centrifuged at 10,000 *x g*. The following day, radioactivity was determined in 200 µL of the aqueous phase by liquid scintillation spectrometry (LSS). Enzymatic

conjugation rates were determined by subtracting the average radioactivity from the aqueous phase of negative control reactions (no protein added) from the total activity measured in experimental reactions. Specific activity was determined based on protein concentration and the corrected total activity determined by LSS. Units of GST activity are reported as pmol GSH-*S*-metolachlor conjugated mg protein^{-1} min^{-1}. GST activity results represent the combined data from three independent experiments with two protein extracts per population per experiment and three technical replications per protein extract.

Waterhemp and corn microsome preparation

Frozen tissue samples (3 g) were pulverized in a mortar pre-chilled with liquid N_2 and protein was extracted in 5 volumes of extraction buffer (200 mM Tris-Cl (pH 7.8), 1 mM Na_2EDTA, 250 mM sucrose, 40 mM ascorbic acid, 5 mM dithiothreitol (DTT), plant protease inhibitor cocktail, and PVPP (75 mg mL^{-1})). Samples were then centrifuged for 20 min at 15,000 *x g* and the supernatant (crude protein extract) was removed, filtered through cheese cloth, and transferred to a clean 10.4 mL ultracentrifuge tube (16 x 76 mm polycarbonate centrifuge bottle, Beckman Coulter Life Sciences, Brea, CA 92821). Samples were then centrifuged at 100,000 *x g* for 60 min (Optima LE-80K Ultracentrifuge, Beckman Coulter Life Sciences). The soluble portion (supernatant) was then discarded and the high-speed pellet containing the microsomal protein was rinsed three times with 1 mL of 200 mM Tris-Cl (pH 7.8) and resuspended in 330 µL storage buffer (200 mM Tris-Cl (pH 7.8), 3 mM DTT, and 30% v/v glycerol) by gently stirring the pellet with a clean paintbrush previously dipped in storage buffer. Microsomal protein was then stored on ice until needed for microsomal assays the same day. Protein

concentrations within samples were determined (Bradford 1976) utilizing a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific).

Oxidation of S-metolachlor by waterhemp and corn shoot microsomes

Microsomal activity assays with *S*-metolachlor were conducted similarly to previous studies with several different herbicide substrates and crops (Moreland et al. 1990; 1993; 1995; Polge and Barrett 1995). Reaction mixtures consisted of 100 mM Tris-Cl (pH 7.4), 5 µM radiolabeled *S*-metolachlor (555 MBq $mmol^{-1}$), and microsomal protein. Negative controls where protein was replaced by clean storage buffer were included to determine if non-enzymatic oxidation of *S*-metolachlor had occurred. Assay mixtures were allowed to incubate for five min at 30°C for equilibration and reactions were initiated by the addition of NADPH (1 mM final concentration; Cayman Chemicals, Ann Arbor, MI) adjusting the final assay volume to 500 µL. Several concentrations of NADPH, NADH (Research Products Intl. Mount Prospect, IL 60056), or both were originally tested as electron donors, but a 1 mM final concentration of NADPH was optimum (data not shown). Reactions were incubated for 25 min at 30°C and were terminated by the addition of 50 µL glacial acetic acid. Reaction mixtures were then frozen in liquid N_2 and stored at -20°C.

Reaction mixtures were partitioned three times with 1 mL ethyl acetate. Samples were vortexed and briefly centrifuged at 10,000 *x g* to denature protein at the interface of the aqueous and organic phases. The organic phase was then transferred to a clean 5 mL Eppendorf tube, and following the third partitioning, the ethyl acetate was evaporated under a stream of N_2 gas. Each sample was then reconstituted in 100 µL acetonitrile (50% v/v) for conducting high performance

liquid chromatography (HPLC). Percent recovery of radiolabeled compounds exceeded 95% (data not shown) and radioactivity was not detected in the aqueous phase following partitioning.

Quantification of microsome-generated S-metolachlor metabolites

Reverse-phase HPLC was performed with a PerkinElmer Flexar LC system (Model N2910401, Perkin Elmer, Akron, Ohio 44311) at a flow rate of 1 mL min^{-1} using a C18 column (Nucleodur C18 Pyramid, 5 µm x 250 mm; Macherey-Nagel, Düren, Germany). Eluent A was 0.1% (v/v) formic acid in water and eluent B was 0.1% (v/v) formic acid in acetonitrile. Sample volumes of 77 µL were injected and extractable radioactivity was resolved with a 19 min stepwise gradient starting at 90:10 A:B. Steps included a linear gradient to 75:25 A:B in 5 min, 60:40 A:B in 3 min, 40:60 A:B in 2 min, 15:85 A:B in 2 min, and finally 5:95 A:B in 3 min followed by a 4 min isocratic hold. Following each 19 min run, the column was returned to 90:10 A:B in 5 min and equilibrated for 3 min before subsequent injections.

Radiolabeled compounds were detected with a β-RAM Radio HPLC-detector (Model 4; LabLogic Systems, Tampa, FL 33619) and Ultima-Flow M cocktail (PerkinElmer). The amount of oxidative *S*-metolachlor metabolites for each assay was determined by peak integration in Laura Software (Version 4.2.3.37; LabLogic Systems). Peaks were then compared by co-chromatography to a non-labeled *O*-demethylated *S*-metolachlor metabolite (supplied by Syngenta Ltd., UK) (Fig. 3.1). Specific activity was determined based on protein concentration and the amount of radiolabeled oxidative metabolites detected. Units of microsomal activity are reported as pmol *S*-metolachlor oxidized mg $protein^{-1}$ min^{-1}. Microsomal activity results represent the combined data from three independent experiments with two protein extracts per population per experiment and two assays per protein extract. A third assay for each protein

extract was included with water substituted for NADPH to determine if product formation was NADPH dependent. In addition, non-enzymatic reactions were included in each experiment by replacing microsomal protein with storage buffer to determine if non-enzymatic formation of metabolites occurred. Peak retention times (R_T) for *S*-metolachlor and *O*-demethylated *S*-metolachlor were 17.4 and 16.0 min, respectively.

Untargeted liquid chromatography-mass spectrometry

Greenhouse-grown plants were transferred to a growth chamber set at 28/22 °C day/night with a 16 h photoperiod 24 h prior to experiments. Samples of ten waterhemp seedlings or five corn shoots were washed with deionized water and placed in a 5 mL Eppendorf tube containing 1 mL of hydroponic solution plus 100 µM non-labeled *S*-metolachlor for 2 h. At 2 h, plant samples were triple rinsed with deionized water, fresh weights were recorded, frozen in liquid nitrogen, and stored at -80°C until processing. Plant samples for additional time points (> 2 h) were triple rinsed with deionized water, then transferred to a 60 mL sample cup per population containing 2 mL of fresh hydroponic solution without herbicide. Plants were then removed, frozen, and stored at 4 and 12 h after treatment. Additional samples were treated using the same methods, but *S*-metolachlor was excluded during the 2 h treatment process to enable comparisons of the metabolome from treated and non-herbicide treated samples.

Following incubation, frozen tissue samples (approx. 0.25 g) were ground in liquid N_2 and compounds were extracted in 1 mL of 90% (v/v) methanol. After the first extraction and centrifugation at 12,000 *x g*, the supernatant was removed and remaining plant material was re-extracted in an additional 1 mL of 90 % (v/v) methanol. Following centrifugation, the pellet was discarded, supernatant removed and combined with the first supernatant, yielding a total final

volume of 2 mL. Quality control samples were then prepared by combining aliquots of each sample (treated and nontreated) (Dunn et al. 2011). Synthetic standards of *S*-metolachlor and its respective GSH, CYS-GLY, and CYS conjugates along with *O*-demethylated S-metolachlor and its respective GSH, CYS-GLY, and CYS conjugates were included. Standards were synthesized *in vitro* (described below) or supplied by Syngenta. All samples were stored at -80°C until further analysis.

Samples were submitted to the Roy J. Carver Metabolomics Facility at the University of Illinois for analysis using methods previously described (Elolimy et al. 2019). Prior to analysis, all samples were spiked with 4-chloro-DL-PHE as an internal standard. Samples were then analyzed with a Q-Exactive MS system (Thermo. Bremen, Germany). LC separation was conducted with a Dionex Ultimate 3000 series HPLC equipped with a Phenomenex C18 column (4.6 x 100mm, 2.6 µm). Mobile phases consisted of A (H_2O, 0.1% formic acid v/v) and B (Acetonitrile, 0.1% formic acid v/v). The flow rate was set at 0.25 mL min^{-1} with linear gradient starting at 100 % A for 3 min. The gradient then transitioned to 100% B (20–30 min) and returned to 100% A (31–36 min). Twenty µL of each sample was injected and the autosampler temperature was set at 15°C. Mass spectra were then acquired under both positive (sheath gas flow rate: 45; aux gas flow rate: 11; sweep gas flow rate: 2; spray voltage: 3.5 kV; capillary temp: 250°C; Aux gas heater temp: 415°C) and negative electrospray ionization (sheath gas flow rate: 45; aux gas flow rate: 11; sweep gas flow rate: 2; spray voltage: −2.5 kV; capillary temp: 250°C; Aux gas heater temp: 415°C). The full scan mass spectrum resolution was set to 70,000 with scan range of *m/z* 67 ~ *m/z* 1,000, and AGC target was 1E6 with a maximum injection time of 200 ms.

LC-MS data were further analyzed with Thermo Compound Discoverer software (v. 2.1 SP1) for chromatographic alignment and compound feature identification and quantitation. Multivariate analysis of peak areas was then conducted using unsupervised principal component analysis (PCA) in SIMCA software v.15 (Umetrics, Sweden) to visualize trends in the metabolomics data between resistant and sensitive waterhemp populations. Prior to analysis, values were log transformed and Pareto scaled to minimize the disequilibrium between metabolites produced at high and low concentrations within the waterhemp seedlings (van den Berg et al. 2006).

Targeted, high-resolution liquid chromatography-mass spectrometry

Plants were treated and processed as described for untargeted LC-MS experiments. During the first extraction of compounds in 90% (v/v) methanol, 100 µL of 100 µM acetochlor (a similar chloroacetamide) was added to each sample as an internal standard. Samples were then analyzed at the Roy J. Carver Metabolomics Facility at the University of Illinois. Standards of ten *S*-metolachlor metabolites were either supplied by Syngenta or synthesized *in vitro* (described below). Metabolites were the same as investigated in untargeted experiments, with the addition of the γ-GLU-CYS conjugates of *S*-metolachlor and *O*-demethylated *S*-metolachlor. LC-MS separation was then conducted using the same method used in untargeted experiments (Elolimy et al. 2019), except that the 4-chloro-DL-PHE was not included. Peak areas were then determined for each of the included standards in Xcalibur Software 4.1.31.9 (Thermo, Bremen, Germany). Relative values for each standard from treated plant samples were calculated by dividing the peak area of each metabolite by the peak area of acetochlor in each sample and the quotient was corrected for the fresh weight of each sample, yielding a relative abundance of each

metabolite among populations, similar to previous experiments with topramezone (Lygin et al. 2018). Targeted LC-MS experiments were conducted twice with three replications per population per time-point.

Analysis of putative glucose conjugates

Plants were grown and prepared as previously described (Strom et al. 2020), but the incubation step was modified to include 100 µM radiolabeled *S*-metolachlor (11.2 kBq mL^{-1}; 111 MBq $mmol^{-1}$ specific activity). Plants were incubated for 2 h in *S*-metolachlor-treated hydroponic solution. At 2 h, samples were triple rinsed in deionized water and transferred to a new 60 mL cup containing 2 mL of nontreated hydroponic solution. At 12 h after treatment, plant samples were triple rinsed in deionized water, fresh weights recorded, frozen in liquid N_2, and stored at -20°C until further analysis.

Extractable radioactivity was removed twice in 90% (v/v) methanol as described previously (Strom et al. 2020). The methanolic extract was then evaporated to incipient dryness under a stream of N_2 gas and reconstituted in 500 µL of 50% acetonitrile (v/v). Radioactivity was then quantified by LSS. Assays were then conducted to determine if glucose conjugates of *S*-metolachlor metabolites were present in samples from the CHR, SIR, and WUS waterhemp populations studied in enzymatic and LC-MS experiments. Assays included 6 mg mL^{-1} β-glucosidase (from sweet almond, >4 U mg^{-1}; Sigma), 0.2 µg mL^{-1} BSA, and *S*-metolachlor-treated plant extract (15,000 dpm; 250 Bq) in 50 mM sodium phosphate buffer (pH 6) in a final volume of 2 mL. Assay tubes were incubated at 37°C for 24 h, then terminated by freezing in liquid N_2. Reactions were also performed without the addition of β-glucosidase to compare

metabolite changes between enzyme treated and nontreated samples. Experiments were conducted twice, with three replications per population per treatment.

Prior to reverse-phase HPLC analysis, samples were purified and concentrated by solid-phase extraction (SPE) using a preparative (3 mL) C18 column (200 mg; Chromabond C18ec; Machery-Nagel, Düren, Germany). Columns were pre-conditioned with 2 volumes of methanol followed by 2 volumes of water acidified with 0.1% formic acid. The sample was then loaded onto the column under vacuum at 1 mL min^{-1}, washed with 1 mL of acidified water, dried under vacuum, and metabolites were eluted in 2 x 0.5 mL methanol. The methanol was then evaporated under a stream of N_2 gas and samples were reconstituted in 100 µL of 50% (v/v) acetonitrile.

Reverse-phase HPLC was performed similar to microsomal assays, but radiolabeled compounds were resolved with a longer stepwise gradient (26 min) starting at 90:10 A:B for improved resolution of metabolites. Steps included a linear gradient to 75:25 A:B in 5 min, 60:40 A:B in 5 min, 40:60 A:B in 5 min, 15:85 A:B in 5 min, and finally 5:95 A:B in 3 min followed by a 3 min isocratic hold. Following each 26 min run, the column was returned to 90:10 A:B in 4 min and equilibrated for 3 min before subsequent injections. Radiolabeled compounds were detected with a β-RAM Radio HPLC-detector (Model 4; LabLogic Systems, Tampa, FL 33619) and Ultima-Flow M cocktail (PerkinElmer). Chromatograms of each sample were then analyzed and *S*-metolachlor metabolites were compared between enzymatic and non-enzymatic reactions in Laura Software (Version 4.2.3.37; LabLogic Systems).

In vitro formation of synthetic standards

Synthetic GSH, CYS-GLY, γ-GLU-CYS and CYS conjugates of *S*-metolachlor and *O*-demethylated *S*-metolachlor were prepared *in vitro* as previously described (Riechers et al. 1996a). Reactions were carried out in 2 mL Eppendorf tubes containing 60 mM TAPS ([Tris(hydroxymethyl)methylamino]propanesulfonic acid) buffer (unadjusted pH 8.5), 10 mM GSH, dipeptide or L-CYS, and 20 µM *S*-metolachlor or *O*-demethylated *S*-metolachlor in 1 mL of deionized water. Reaction mixtures were incubated for 24 h at 35°C and then terminated by adjusting the final volume to 1.5% formic acid. Following termination, reaction mixtures were partitioned once against 1 volume of methylene chloride to remove non-conjugated *S*-metolachlor or *O*-demethylated *S*-metolachlor. The conjugates of *S*-metolachlor in the aqueous phase were purified and concentrated by SPE using a preparative (3 mL) C18 column (200 mg; Chromabond C18ec; Machery-Nagel, Düren, Germany). Columns were pre-conditioned with 2 volumes of methanol followed by 2 volumes of acidified water (0.1% formic acid). The aqueous phase was then loaded onto the column under vacuum at 1 mL min^{-1}, washed with 1 mL of acidified water, dried under vacuum, and synthetic conjugates were eluted in 2 x 0.5 mL methanol. The methanol was then evaporated under a stream of nitrogen gas and standards were reconstituted in 200 µL of 90% methanol for use as analytical standards in LC-MS.

Statistical analysis

Specific activities from GST- and microsomal assays with *S*-metolachlor were analyzed in SAS 9.4 (SAS Institute, Inc., Cary, NC, USA). The assumptions of homogenous variance and normality of residuals were reviewed with PROC UNIVARIATE and PROC GLM, respectively. Data from GST and microsomal assays were then analyzed by a generalized linear mixed model

in PROC GLIMMIX fitted with a normal distribution and an identity link function. The response variable was the pmol *S*-metolachlor (GSH conjugated or oxidized) mg protein^{-1} min^{-1} with population as a fixed effect. Random effects included experiment run, protein sample nested within run, and assay nested within protein sample and experimental run. Mean separations were then conducted by least significant difference (LSD) (α = 0.05).

Results

Resistant waterhemp and corn possess greater GST activity with S-metolachlor than sensitive waterhemp

GST activity of three waterhemp populations (CHR, SIR, WUS) and corn was measured using radiolabeled *S*-metolachlor as substrate. CHR and SIR are resistant to *S*-metolachlor via enhanced metabolism, WUS is sensitive to *S*-metolachlor (Strom et al. 2019), and corn is naturally tolerant via rapid GST-mediated metabolism (Fuerst 1987). Specific GST activities from each population varied and the population effect was significant (P <0.0001; Table 3.1). Corn had 5-fold more activity than WUS and 2.6–3.0 fold more activity than SIR and CHR, respectively (Table 3.1). Specific GST activities from CHR and SIR were similar, but were 1.7–2.0 fold greater than WUS, respectively. These results demonstrate that CHR and SIR have elevated GST activity with *S*-metolachlor compared with WUS. Although our previous research demonstrated CHR and SIR metabolize *S*-metolachlor as rapidly as corn (Strom et al. 2020), these results indicate CHR and SIR do not possess equivalent GST activity as tolerant corn (Table 3.1), thus indicating additional enzyme(s) are involved.

Microsomes prepared from resistant waterhemp oxidize S-metolachlor

Oxidative activities of microsomes prepared from the waterhemp populations and corn were analyzed using radiolabeled *S*-metolachlor as substrate. Preliminary experiments demonstrated product formation was linear during the first 25 min of incubation at 30°C, and reactions in excess of 25 min did not reveal additional metabolite formation (data not shown). Extracts from each waterhemp population formed a single major metabolite, which was tentatively identified as *O*-demethylated *S*-metolachlor by co-chromatography (R_T 16.0 min) with an authentic standard (Fig. 3.1; 3.2A-C). The identity of this metabolite was further verified by liquid chromatography-mass spectrometry (LC-MS). The metabolite formed by CHR and SIR microsomes had the same mass-to-charge ratio (*m/z*) as the authentic standard (*m/z* 270) under positive ionization mode. Additionally, the fragmentation pattern of the metabolite formed by CHR and SIR microsomes was the same as the standard, including major fragments with *m/z* of 176, 184 and 252.

O-demethylated *S*-metolachlor was more abundant in assays using CHR and SIR microsomes than from corn or WUS (Fig. 3.2A-D). Specific activities of CHR and SIR microsomes were much greater than corn or WUS (P <0.0001; Table 3.2). CHR had 21–30-fold higher specific P450 activity than WUS or corn, respectively. SIR had 28–39-fold higher specific activity than WUS or corn, respectively, and 1.3-fold greater specific activity than CHR. Product formation was also active enzyme and NADPH dependent (Fig. 3.2 D, F), corresponding to the requirement of P450 enzymes to exist in a reduced state for catalytic activity (Werk-Reichhart and Feyereisen 2000; Siminszky 2006). These results demonstrate CHR and SIR microsomes have a greater ability to oxidize *S*-metolachlor than the WUS population and corn, thus contributing to rapid *S*-metolachlor metabolism previously reported (Strom et al. 2020).

Untargeted LC-MS reveals differences between resistant and sensitive waterhemp

Untargeted liquid chromatography-mass spectrometry (LC-MS) was conducted to compare the metabolite profiles of CHR, SIR, and WUS following treatment with unlabeled *S*-metolachlor. Principal component analysis (PCA) of metabolite features demonstrated the metabolite profiles of CHR and SIR were different compared to WUS (Fig. 3.3A), with population (PC1) and herbicide treatment (PC2) explaining 35.1% and 10.4% of the variability in the dataset, respectively. Metabolite profiles of *S*-metolachlor-treated seedlings differed among populations and time points (Fig. 3.3B), with h after treatment accounting for an additional 10% of the variability. Cross referencing with the accurate mass and peak retention times of the analytical standards was conducted with parent *S*-metolachlor, *O*-demethylated *S*-metolachlor, and *in vitro* synthesized GSH, CYS-GLY, and CYS conjugates of *S*-metolachlor and *O*-demethylated *S*-metolachlor. Identities of these putative *S*-metolachlor metabolites were confirmed on the loadings plot (Fig. 3.4).

LC-MS revealed *S*-metolachlor and GSH-*S*-metolachlor conjugates (and its catabolites) were more characterizing for WUS, whereas *O*-demethylated *S*-metolachlor and its respective GSH-derived conjugates and catabolites were characterizing for CHR and SIR (Fig. 3.4). These results support the *in vitro* GST and P450 assays and indicate oxidative metabolism and GST-mediated conjugation reactions involving *S*-metolachlor occur in resistant populations. These untargeted experiments guided subsequent targeted LC-MS experiments to quantify the relative abundance of each metabolite in *S*-metolachlor-treated seedlings of each waterhemp population.

Abundances of S-metolachlor-derived metabolites differ in resistant and sensitive waterhemp seedlings

Targeted high-resolution LC-MS was conducted to investigate *S*-metolachlor metabolites formed in CHR, SIR, and WUS. *S*-metolachlor, *O*-demethylated *S*-metolachlor and their respective GSH, CYS-GLY and γ-GLU-CYS dipeptide, and CYS conjugates were included and relative abundances determined 2, 4, and 12 h after treatment. At each time point, the relative abundance of *S*-metolachlor was less in CHR and SIR than in WUS (Fig. 3.5). In contrast, the GSH, CYS-GLY, γ-GLU-CYS, and CYS conjugates of *S*-metolachlor were similar or in greater relative abundance in WUS than in CHR or SIR during the time course (Fig. 3.5). However, CHR and SIR formed more *O*-demethylated *S*-metolachlor initially than WUS and the GSH, CYS-GLY, and CYS conjugates of *O*-demethylated *S*-metolachlor were more abundant in CHR and SIR than WUS (Fig. 3.6). The γ-GLU-CYS dipeptide conjugate of *O*-demethylated *S*-metolachlor was not detected. These results imply that GST- and P450 activities are involved in *S*-metolachlor detoxification in resistant waterhemp.

Putative glucose conjugates are more abundant in resistant waterhemp than sensitive waterhemp

Following Phase I metabolic reactions (such as *O*-demethylation and hydroxylation), oxidized herbicides are predisposed to Phase II sugar conjugation, such as glucose, catalyzed by UDP-dependent glucosyl transferases (Osmani et al. 2009; Riechers et al. 2010; Siminszky 2006; Werk-Reichhart and Feyereisen 2000). Extracts from CHR, SIR, and WUS previously treated with radiolabeled *S*-metolachlor were subsequently treated with β-glucosidase to determine if metabolite profiles from β-glucosidase-treated and nontreated samples contained glucose conjugates. One polar metabolite (R_T 3.3 min) was detected in all populations, but was more

abundant in CHR and SIR than WUS (Fig. 3.7). Without β-glucosidase treatment, this polar metabolite accounted for 20 and 24% of total radioactivity detected in CHR and SIR extracts, respectively, but only 6% in WUS. The abundance of this putative glucose conjugate decreased 5, 7, and 1% for CHR, SIR, and WUS, respectively, following treatment with β-glucosidase. β-glucosidase treatment concurrently increased abundance of *O*-demethylated *S*-metolachlor (R_T 19.4 min) in each extract (Fig. 3.7).

Discussion

Results from our current research expand upon previous findings that resistance to *S*-metolachlor in waterhemp is due to enhanced metabolism (Strom et al. 2020). Enzyme assays with *S*-metolachlor as substrate indicated CHR and SIR have increased GST activity compared to WUS but less than corn, which is naturally tolerant via this mechanism (Fuerst 1987). Further investigation of microsomal P450 activity revealed that CHR and SIR possess a greater ability to oxidize *S*-metolachlor through *O*-demethylation than WUS or corn, and this oxidation reaction appears to be the predominant resistance mechanism. P450 and GST activities likely contribute to resistance in CHR and SIR and both enzymes metabolize *S*-metolachlor. Furthermore, we hypothesize GSTs also metabolize *O*-demethylated *S*-metolachlor.

Investigations into VLCFA-inhibitor resistance overall are limited and have mostly focused on monocot species. Our results represent the benchmark report of enzymatic processes underlying *S*-metolachlor resistance in a dicot weed species. Previously, the involvement of GSTs in *S*-metolachlor resistance was postulated in Palmer amaranth (Brabham et al. 2019) and waterhemp (Strom et al. 2020) based on results with a chemical GST inhibitor. GSH conjugation was also proposed as a mechanism for VLCFA-inhibitor resistance in *Lolium spp.*

(Busi et al. 2018; Dücker et al. 2019). The vast majority of research on GST activities and metabolism of VLCFA-inhibiting herbicides, however, has focused on crop selectivity mechanisms (Cummins et al. 2011; Riechers et al. 2010). In general, certain crop and weed species are endowed with natural tolerance to VLCFA-inhibiting herbicides through rapid conjugation to GSH or homoglutathione (Cummins et al. 2011; Fuerst 1987) due to greater GSH content, GST activity with herbicide substrates, or both than sensitive species (Breaux 1987; Hatton et al. 1996). Furthermore, herbicide detoxification via GST activities in cereal crops is induced by herbicide safeners (Gronwald 1989; Hatzios 1991; Riechers et al. 2010). Our results with *S*-metolachlor are in accord with natural corn tolerance via GST activity (Fuerst 1987), but the elevated specific GST activities of CHR and SIR relative to WUS do not appear to be the resistance mechanism.

CHR and SIR microsomal activities with *S*-metolachlor revealed large differences compared to WUS, however. Xenobiotic-metabolizing P450s in plants are integral membrane proteins in the ER that catalyze the insertion of one oxygen atom into lipophilic substrates (Dimaano and Iwakami 2020; Siminszky 2006; Werk-Reichhart and Feyereisen 2000). Microsomes isolated from sorghum, corn and mung bean (*Vigna radiata* L. cv 'Berken') metabolize metolachlor via *O*-demethylation (Moreland et al. 1990; 1993; 1995). Our results show that microsomes from CHR and SIR also *O*-demethylate *S*-metolachlor. P450s require an electron donor (typically NADPH or NADH) and ER-bound cytochrome P450 reductase activity (Siminszky 2006; Werk-Reichhart and Feyereisen 2000). The requirement of NAPDH for formation of *O*-demethylated *S*-metolachlor by CHR and SIR microsomes strongly supports P450-mediated *O*-demethylation activity. Previous metabolic assays with *S*-metolachlor using the P450 inhibitor malathion indirectly suggested P450 involvement in VLCFA inhibitor-

resistant waterhemp (Strom et al. 2020). Our current results directly determined that CHR and SIR microsomes oxidize *S*-metolachlor.

LC-MS experiments identified both *O*-demethylated *S*-metolachlor and GSH-*S*-metolachlor conjugates in waterhemp extracts, which support our results with *in vitro* enzyme assays. After conjugation, GSH-herbicide conjugates are transported into the vacuole by ABC transporters (Bartholomew et al. 2002; Yazaki 2006). GSH conjugates are catabolized inside the vacuole, yielding various dipeptide and CYS conjugates (Lamoureux and Rusness 1981; 1993; Martin et al. 2007; Wolf et al. 1996). The relative abundance of GSH, CYS-GLY, and CYS *S*-metolachlor conjugates were either equal, or more abundant, in WUS compared with CHR or SIR. By contrast, *O*-demethylated *S*-metolachlor and its respective GSH, CYS-GLY, and CYS conjugates were more abundant in CHR and SIR than WUS.

The difference in abundance of *S*-metolachlor, *O*-demethylated *S*-metolachlor, and their metabolites among CHR, SIR, and WUS demonstrates the metabolic pathway of *S*-metolachlor in resistant waterhemp differs from sensitive waterhemp. Sensitive waterhemp primarily utilizes GSH conjugation for *S*-metolachlor detoxification, whereas multiple Phase I and II detoxification enzymes are involved in resistant waterhemp. Identification of *O*-demethylated *S*-metolachlor-GSH conjugates indicates that P450s and GSTs metabolize *S*-metolachlor independently or sequentially. *O*-demethylation was previously considered a secondary metabolic reaction for *S*-metolachlor in plants (Coleman et al. 2002), but our findings indicate greater importance in resistant waterhemp. Additionally, rapid GSH conjugation of *O*-demethylated *S*-metolachlor in resistant waterhemp may completely detoxify this Phase I metabolite, in addition to other Phase II conjugation reactions. Greater abundance of presumed glucose conjugates in resistant waterhemp supports previous findings of Phase II conjugation following Phase I reactions

(Moreland et al. 1990; Siminszky 2006; Werk-Reichhart and Feyereisen 2000), and implies that *S*-metolachlor detoxification in resistant waterhemp involves the concerted effort of multiple enzymes.

In many cases of weed resistance conferred by rapid herbicide metabolism, the same detoxification enzymes and pathways are utilized by tolerant crops in which the herbicide is applied (Dücker et al. 2019; Ma et al. 2013; Tanetani et al. 2013; Yu and Powles 2014). However, this is not always the case. For example, SIR formed ring-hydroxylated metabolites of the HPPD-inhibitor topramezone while corn generated a *N*-demethylated product (Lygin et al. 2018), although both reactions are likely catalyzed by P450(s). Our research demonstrates that the metabolic pathway for *S*-metolachlor in CHR and SIR is more intricate than corn or WUS, with multiple enzymes acting in concert to facilitate detoxification. Our results also indicate enhanced herbicide metabolism and non-target-site resistance is not always conferred by a single metabolic enzyme or enzyme family.

Conclusions

S-metolachlor has remained one of the most widely used herbicide active ingredients for weed management in corn and soybean since commercialization in the late 1990s (Blaser et al. 1999; USDA 2020). Metabolic resistance to *S*-metolachlor, however, reduces the duration and effectiveness of *S*-metolachlor for control of VLCFA inhibitor-resistant waterhemp populations (Hager 2019; Strom et al. 2019). Presently, the extent of VLCFA inhibitor resistance in waterhemp is unknown, but the dioecious biology and reproductive efficiency of waterhemp favor continued resistance evolution (Liu et al. 2012; Sarangi et al. 2017). The present research, however, presents novel opportunities for the development of new chemistries and

recommendations for growers. For example, understanding the intricacies of *S*-metolachlor metabolism in waterhemp provides insight into novel approaches for weed management, such as silencing resistance genes, inhibiting metabolic enzymes, or developing resistance-breaking chemistries (Kaundun 2020). Additionally, the enzyme assays developed for investigating *S*-metolachlor resistance in waterhemp will be useful for studying GSTs and P450s associated with herbicide resistance, adaptive stress responses, and primary and secondary metabolism in *Amaranthus* species (Bhuiyan et al. 2007; Tanaka et al. 2008). Integrating all viable chemical and non-chemical management practices is essential to control resistant waterhemp populations, and will be sustainable if new contributions of seed to the soil-seedbank are limited each season (Buhler et al. 1997; Schwartz-Lazaro and Copes 2019).

Tables

Table 3.1 Glutathione *S*-transferase activities from partially purified extracts of three waterhemp (*Amaranthus tuberculatus*) populations and corn (*Zea maize*), measured using radiolabeled *S*-metolachlor as substrate.

Population	Protein Per Assay	Total Activity[a]	Specific Activity[b]
	mg	pmol	pmol mg^{-1} min^{-1}
CHR	0.1	434	138 bc
SIR	0.1	462	156 b
WUS	0.1	265	79 c
Corn[c]	0.1	1320	403 a

[a]Total activity represents pmol *S*-metolachlor conjugated in a 30 min assay.

[b]Mean specific activities were separated by Fisher's LSD and values followed by the same letter are not significantly different (α=0.05).

[c]Corn (hybrid B73 x Mo17) was included to represent a tolerant crop.

Table 3.2 Activity of microsomal extracts from three waterhemp (*Amaranthus tuberculatus*) populations and corn (*Zea maize*), measured using radiolabeled *S*-metolachlor as substrate.

Population	Protein Per Assay	Total Activity[a]	Specific Activity[b]
	mg	pmol	pmol mg^{-1} min^{-1}
CHR	0.1	105	51 b
SIR	0.1	137	66 a
WUS	0.1	5.5	2.4 c
Corn[c]	0.2	6.7	1.7 c

[a]Total activity represents pmol *S*-metolachlor oxidized in a 25 min assay.

[b]Mean specific activities were separated by Fisher's LSD and values followed by the same letter are not significantly different (α=0.05).

[c]Corn (hybrid B73 x Mo17) was included to represent a tolerant crop.

Figures

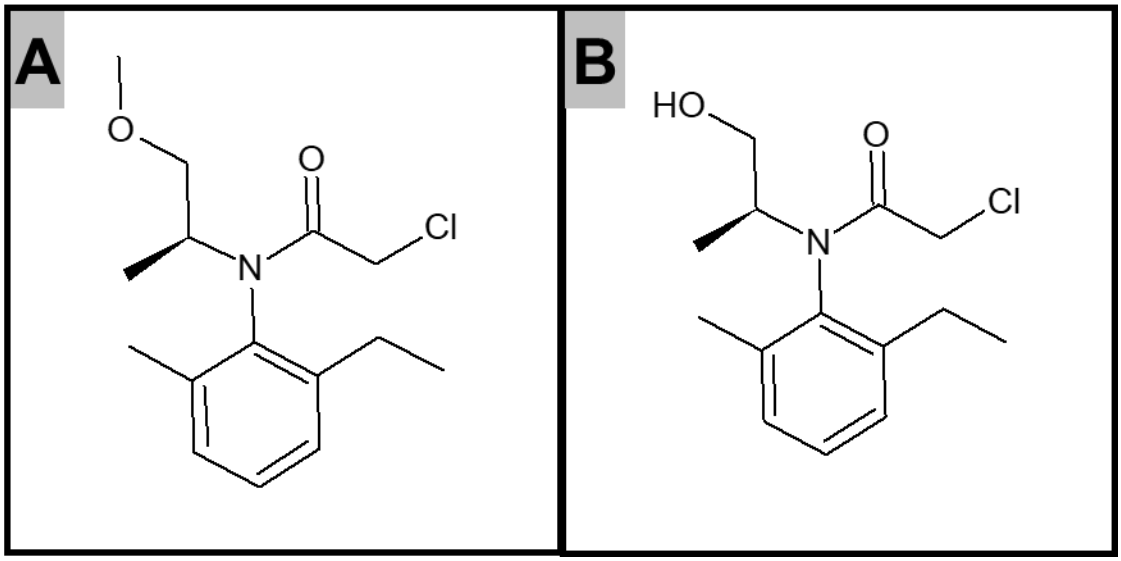

Figure 3.1 Structures of parent *S*-metolachlor (**A**) and *O*-demethylated *S*-metolachlor (**B**).

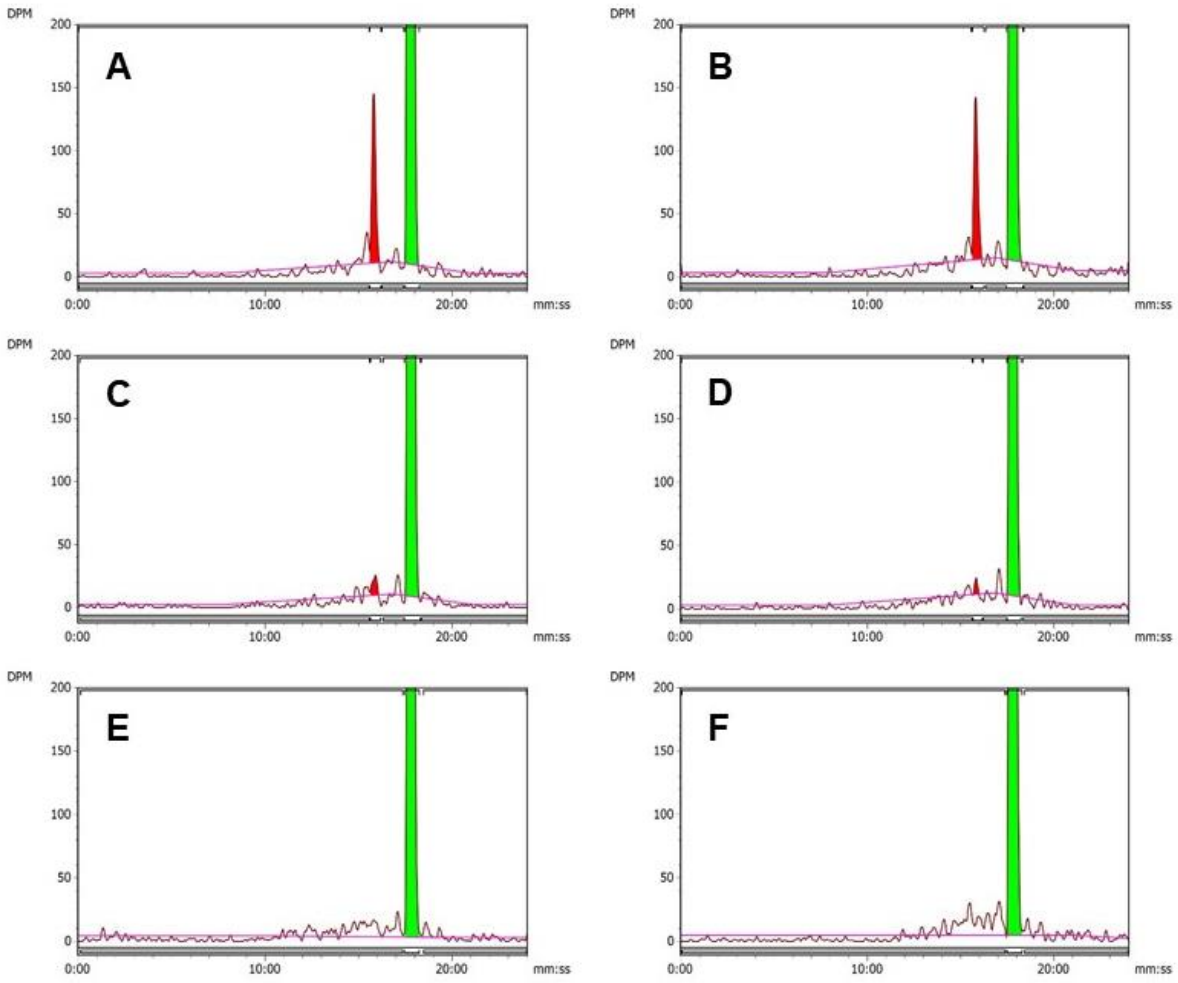

Figure 3.2 Representative reverse-phase HPLC chromatograms from microsomal assays of VLCFA-inhibitor-resistant (**A**, CHR; **B**, SIR) and sensitive (**C**, WUS) waterhemp (*Amaranthus tuberculatus*) seedlings or corn (*Zea mays*) shoots (**D**) after 25 min incubation. Regions shaded in red in each panel correspond to *O*-demethylated *S*-metolachlor, peak retention time (R_T) of 16 min. Green-shaded regions correspond to *S*-metolachlor (R_T = 17.4 min). Panel **E**, non-enzymatic reaction. Panel **F**, VLCFA-inhibitor-resistant (SIR) waterhemp without NADPH.

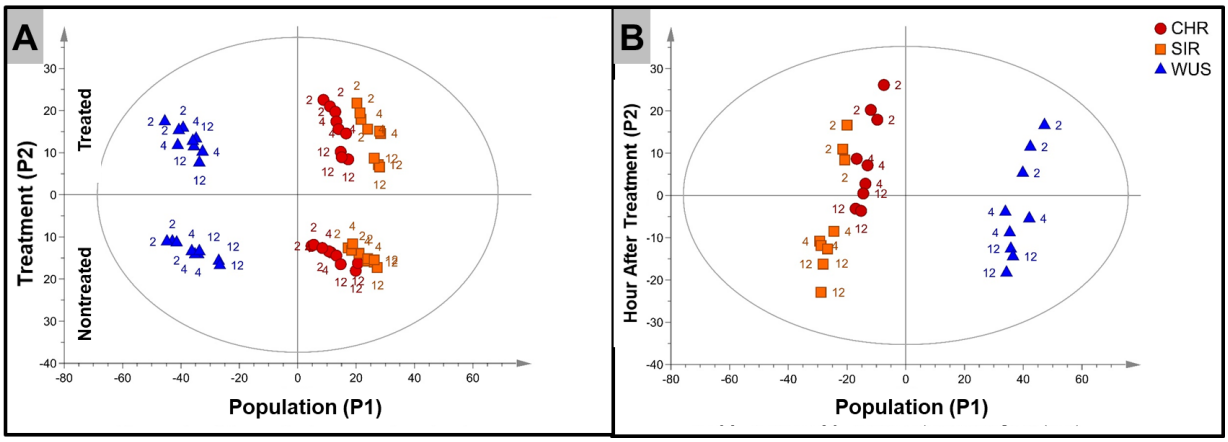

Figure 3.3 Unsupervised principal component analysis of the metabolite profiles of VLCFA-inhibitor-resistant (CHR, SIR) and sensitive (WUS) waterhemp (*Amaranthus tuberculatus*) seedlings. Data point labels correspond to h after treatment that the sample was harvested. Panel **A**, discrimination of the metabolite profiles with population (P1) and treatment (P2). Panel **B**, separation of metabolite profiles of samples with population (P1) and h after treatment (P2).

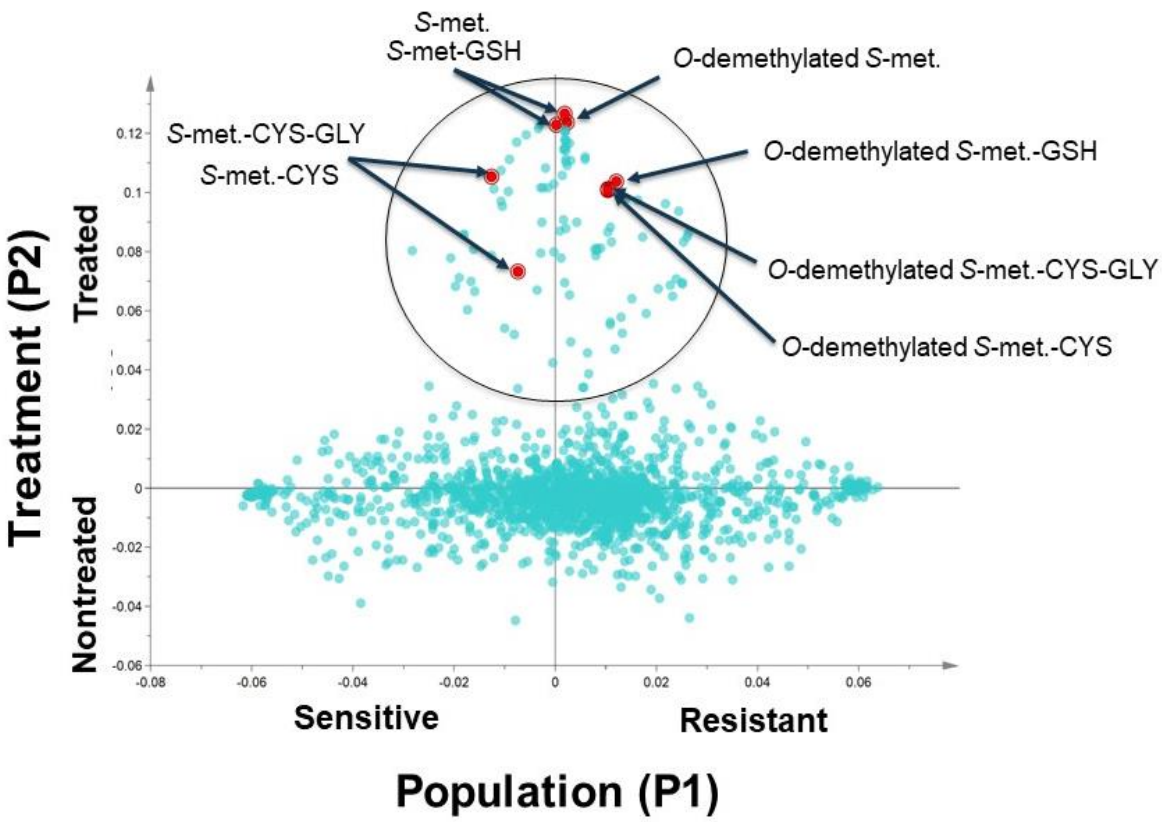

Figure 3.4 Loadings plot of all metabolites from VLCFA-inhibitor-resistant (CHR, SIR) and sensitive (WUS) waterhemp (*Amaranthus tuberculatus*) seedlings. Metabolites highlighted in red correspond to *S*-metolachlor metabolite standards.

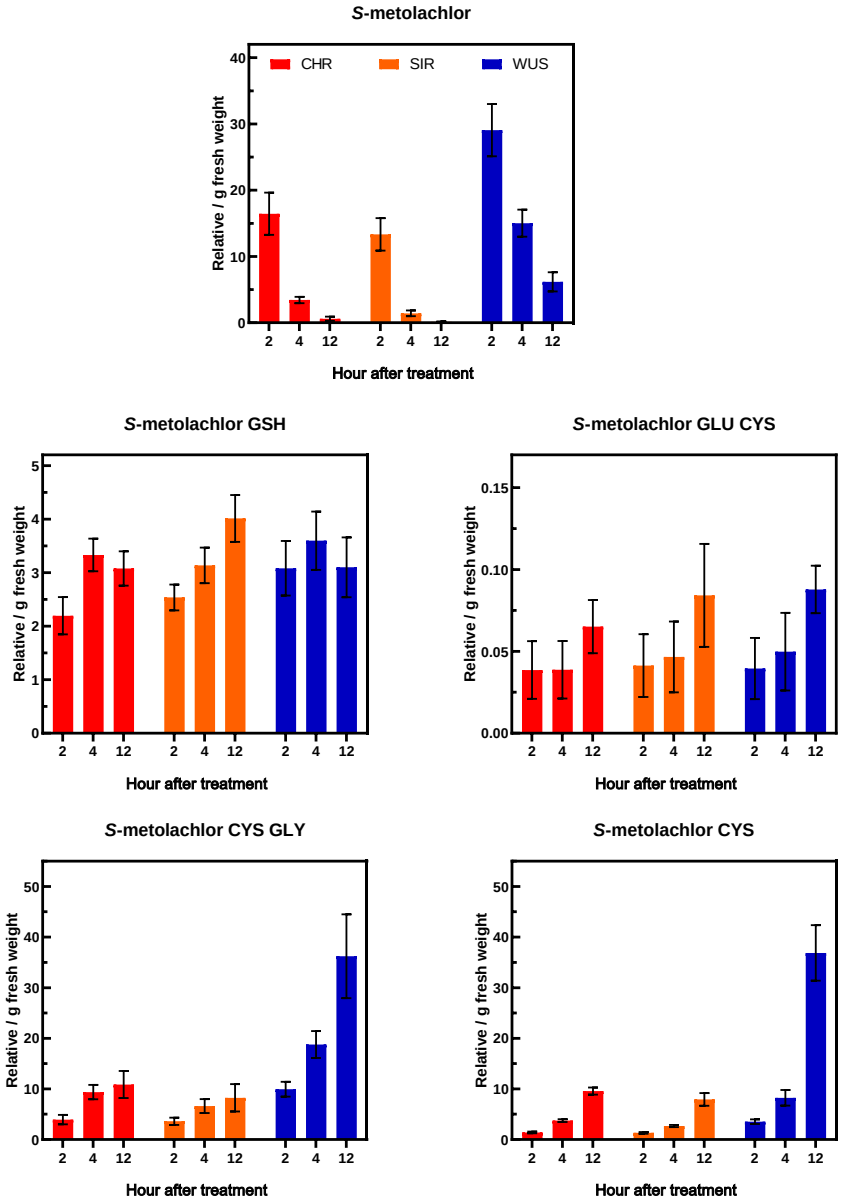

Figure 3.5 Relative abundances of *S*-metolachlor and its respective GSH, γ-GLU-CYS, CYS-GLY, and CYS conjugates from extracts of VLCFA-inhibitor-resistant (CHR and SIR) and sensitive (WUS) waterhemp (*Amaranthus tuberculatus*) populations. Relative values were determined by dividing the peak area of each metabolite by the peak area of the internal standard (acetochlor) and the fresh weight of each sample.

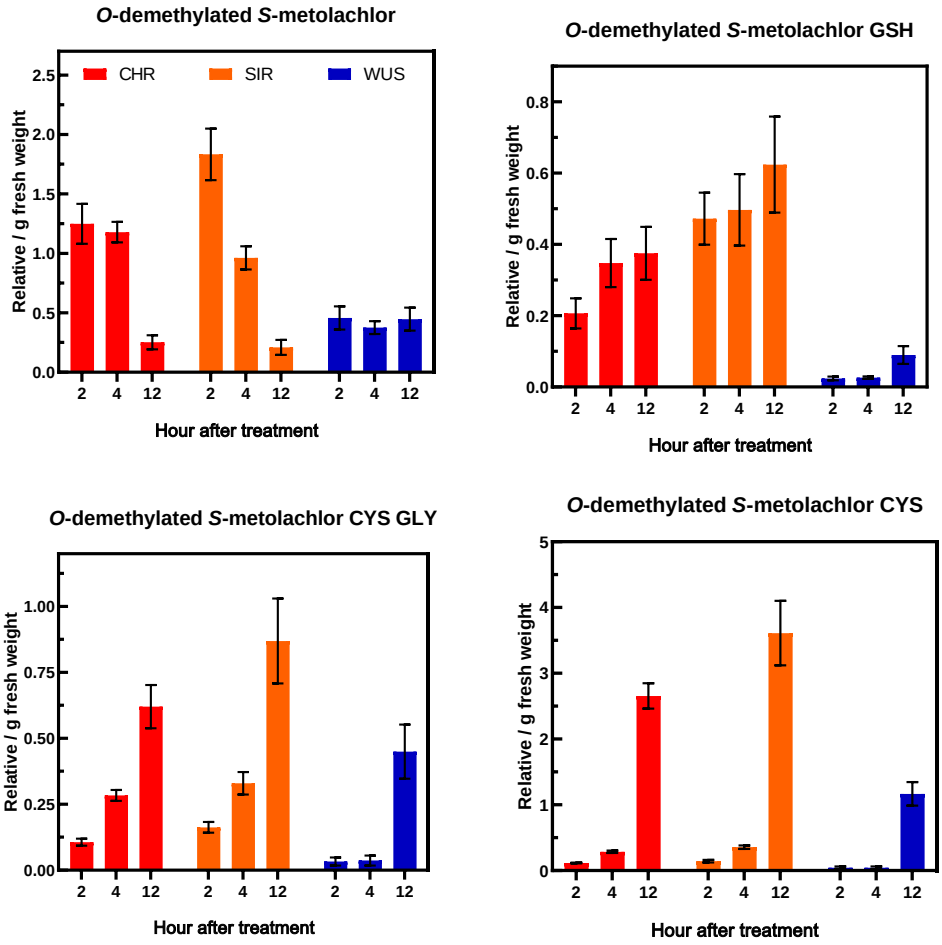

Figure 3.6 Relative abundances of *O*-demethylated *S*-metolachlor and its respective GSH, CYS-GLY, and CYS conjugates from extracts of VLCFA-inhibitor-resistant (CHR and SIR) and sensitive (WUS) waterhemp (*Amaranthus tuberculatus*) populations. Relative values were determined by dividing the peak area of each metabolite by the peak area of the internal standard (acetochlor) and the fresh weight of each sample.

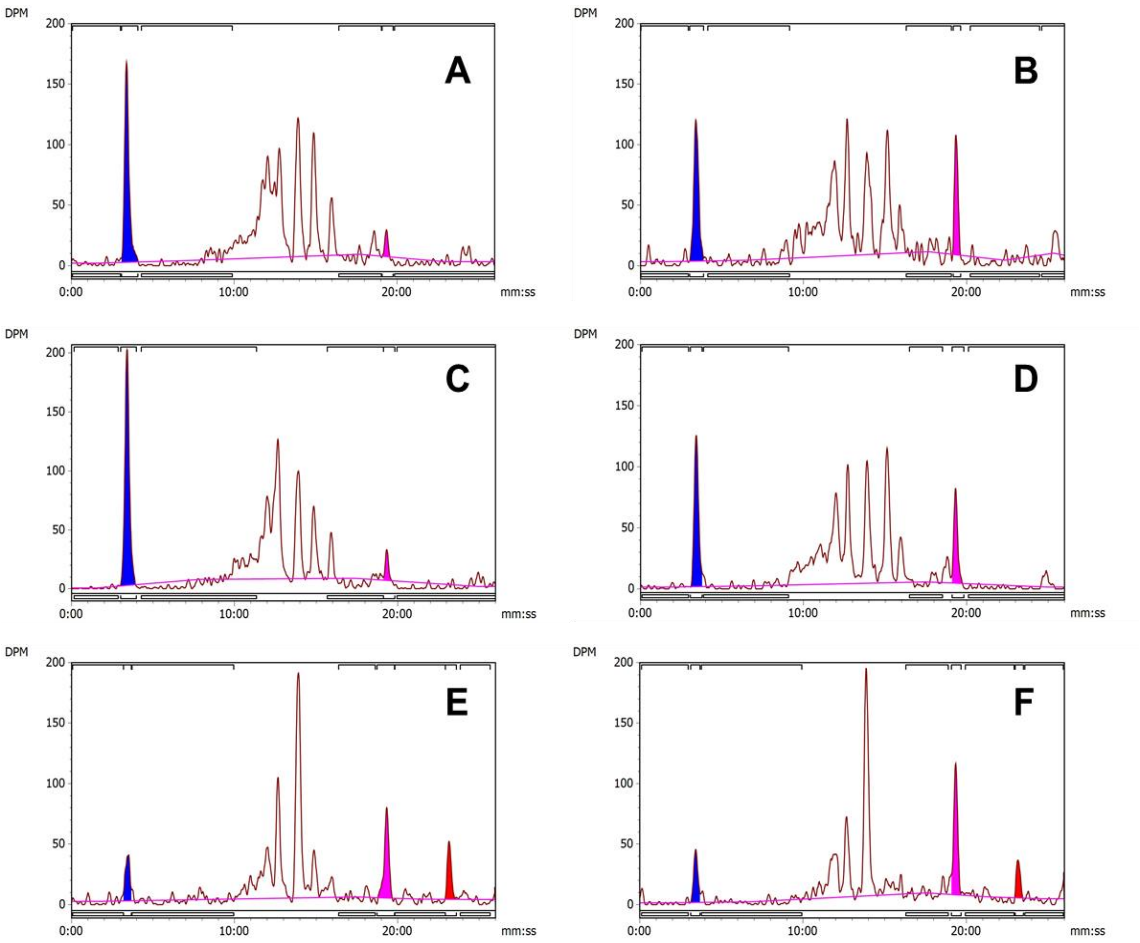

Figure 3.7 Representative reverse-phase HPLC chromatograms of waterhemp (*Amaranthus tuberculatus*) samples harvested 12 h after treatment with radiolabeled *S*-metolachlor. Panel A, VLCFA-inhibitor-resistant (CHR) without β-glucosidase treatment and Panel B, with treatment. Panel C, VLCFA-inhibitor-resistant (SIR) without β-glucosidase treatment and Panel D, with treatment. Panel E, VLCFA-inhibitor-sensitive (WUS) without β-glucosidase treatment and Panel F, with treatment. β-glucosidase-treated samples contained the enzyme at 6 mg mL-1 (>4 U mg-1). Blue-shaded regions correspond to a putative glucose conjugate (RT 3.3 min). Pink-shaded regions correspond to *O*-demethylated S-metolachlor (RT 19.4 min) and red-shaded regions correspond to *S*-metolachlor (RT 23.2 min).

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CHAPTER 4: CONTROL OF WATERHEMP (*AMARANTHUS TUBERCULATUS*) AT MULTIPLE LOCATIONS IN ILLINOIS WITH SINGLE PREEMERGENCE APPLICATIONS OF GROUP 15 HERBICIDES

Abstract

Group 15 herbicides have been widely used for preemergence control of annual monocot and small-seeded dicot weed species, such as waterhemp, since their discovery in the 1950s. Group 15 herbicides are often applied in combination with active ingredients that also possess residual activity, which can make their contribution to preemergence control difficult to quantify. Field experiments were designed to investigate the efficacy of eight Group 15 herbicides applied at their minimum and maximum labeled rates for control of Illinois waterhemp populations. Four different locations were selected, two of which contained previously characterized Group 15-resistant waterhemp populations in Champaign County (CHR) and McLean County (MCR). Two locations with Group 15-sensitive waterhemp populations included the University of Illinois South Farm in Urbana, IL and the Orr Research Center in Perry, IL. Soils at the CHR, MCR, and Urbana locations contained greater than 3% organic matter, but less than 3% organic matter at Perry. Non-encapsulated acetochlor and alachlor provided greater than 91% control of CHR and MCR 28 DAT, whereas other Group 15 herbicides provided less than 61 and 76% control of CHR, and MCR, respectively. In contrast, all Group 15 herbicides provided greater than 81 and 88% control of the Perry and Urbana populations, respectively, 28 DAT. Waterhemp control decreased by 42 DAT, especially for the Group 15-resistant CHR and MCR populations. Overall, Group 15 herbicides remain effective for controlling sensitive waterhemp, but most are not effective for controlling Group 15-resistant waterhemp populations. Proper

herbicide stewardship and integrated weed management practices should be implemented to maintain Group 15 herbicide efficacy for waterhemp management in the future.

Introduction

Waterhemp (*Amaranthus tuberculatus* Moq. Sauer) is a small-seeded, summer annual, dicot weed species that is among the most challenging to control in crops such as corn (*Zea mays* L.) or soybean (*Glycine max* L. Merr) in the Midwestern U.S. (Sauer 1955; Steckel 2007). Waterhemp can reduce corn yield over 50% when interference occurs early in the growing season (Steckel and Sprague 2004). Additionally, soybean yield can be reduced 40% through season long interference (Hager et al. 2002b). Waterhemp utilizes the C4 carbon fixation pathway and can produce in excess of one million seeds per female plant (Steckel 2007; Steckel et al. 2003). Seeds are viable within two weeks of pollination and frequently dormant (Bell and Tranel 2010). Seed dormancy enables waterhemp to emerge in multiple cohorts throughout the growing season (Buhler and Hartzler 2001; Hartzler et al. 1999). The prolonged emergence challenges growers by necessitating use of herbicides with soil-residual activity often followed by postemergence herbicides and nonchemical control measures (Steckel et al. 2002).

Waterhemp is dioecious and requires outcrossing for successful pollination (Murray 1940; Sauer 1955). Outcrossing results in high intraspecific genetic variability compared with self-pollinated weed species (Murray 1940). High genetic variability and outcrossing likely has contributed to the frequent evolution and widespread distribution of herbicide resistance (Adhikary and Pratt 2015; Steckel 2007; Tranel et al. 2011; Tranel 2020). To date, waterhemp has evolved resistance to inhibitors of acetolactate synthase (ALS), photosystem II (PSII), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), protoporphyrinogen oxygenase (PPO), 4-

hydroxyphenylpyruvate dioxygenase (HPPD), synthetic auxins, and most recently, very-long-chain fatty acids (VLCFAs) (Heap 2020). Multiple resistance to herbicides representing up to six different site of action (SOA) groups has also been reported within populations (Evans et al. 2019; Shergill et al. 2018; Strom et al. 2019).

VLCFA-inhibiting herbicides belong to the Weed Science Society of America Group 15 herbicide class and provide preemergence (PRE) control of annual monocot and small-seeded dicot weeds (Fuerst 1987), including waterhemp and Palmer amaranth (*Amaranthus palmeri* S. Watson). VLCFAs consist of acyl chains in excess of 18 carbons and are essential for the formation of cell membranes, cuticle waxes, and lipids (Bach and Faure 2010). Group 15 VLCFA inhibitors control sensitive weed species by inhibiting the VLCFA-elongase complex in plant cells and subsequent formation of VLCFAs (Böger 2003). Sensitive weed species either fail to emerge or remain in an arrested state of growth after cotyledon expansion (Deal and Hess 1980; Dhillon and Anderson 1972; Fuerst 1987; Pillai et al. 1979). Group 15 herbicides are among the oldest classes of herbicides utilized in cropping systems and were originally discovered and developed in the 1950s (Hamm 1974).

Resistance to Group 15 VLCFA-inhibitors has been relatively infrequent in comparison to herbicides from other SOA groups, and has been reported in only five monocot and two dicot weed species (Heap 2020). We recently reported Illinois waterhemp populations from McLean county (MCR) and Champaign county (CHR) are resistant to Group 15 herbicides (Strom et al. 2019; 2020). Control of each population in the field was poor with most Group 15 herbicides (Evans et al. 2019; Hausman et al. 2013; Strom et al. 2019). Group 15 herbicides are subject to environmental factors that influence their activity. Precipitation is especially important for Group 15 herbicide incorporation and subsequent weed control (Jhala 2017). Edaphic factors

also influence PRE activity of Group 15 herbicides. In general, higher herbicide rates are needed for fine-textured soils with high organic matter. Soils with high organic matter also tend to have high microbial activity which could reduce residual weed control with Group 15 herbicides (Shaner et al. 2006; Wu et al. 2011).

Group 15 herbicides are commonly applied in combinations or tank mixtures with herbicides from other SOA groups, and their contribution to weed control can often be difficult to quantify. The objective of our experiments was to investigate Group 15 herbicides applied alone to control waterhemp at multiple locations in Illinois with differing soil types. The four field locations included two Group 15-resistant and two Group 15-sensitive waterhemp populations.

Materials and Methods

Site selection

Field experiments were conducted from 2018–2020 at four locations in Illinois to investigate the efficacy of Group 15 herbicides for residual waterhemp control. Two locations were chosen with Group 15-resistant waterhemp populations: one in Champaign County (Evans et al. 2019; Strom et al. 2019), and one from McLean County (Hausman et al. 2011; 2013; Strom et al. 2019). These locations are designated CHR and MCR, respectively, to coincide with the characterized weed populations. The soil at CHR is a Flanigan silt loam (Fine, smectitic, mesic Aquic Argiudolls) with a pH of 5.5 and 4.8% organic matter. The soil at MCR is a Sable silty clay loam (fine-silty, mixed, superactive, mesic Typic Endoaquolls) with a pH of 6.4 and 3.2% organic matter. Two additional locations with Group 15 sensitive waterhemp populations were selected. One was at the University of Illinois Agronomy Research and Education Center in

Urbana, IL (designated Urbana). The soil at Urbana is a Drummer silty clay loam (fine-silty, mixed, superactive, mesic Typic Endoaquolls) with a pH of 6.7 and 5.5 % organic matter. The other field site was at the University of Illinois Orr Research Center in Perry, IL (designated Perry). The soil at the site is a Downsouth silt loam (Fine-silty, mixed, superactive, mesic Mollic Oxyaquic Hapludalfs) with a pH of 6.2 and 2.5 % organic matter. The Urbana and Perry waterhemp populations were confirmed resistant to PPO inhibitors and glyphosate using whole plant greenhouse tests (Perry) or molecular marker assays (Urbana) by the University of Illinois Plant Clinic (data not shown).

General field methods

Eight Group 15 herbicides were applied at their respective minimum and maximum labeled rates based on the manufacturers recommendation according to soil type (Table 4.1). Field experiments were initiated at all locations within seven days of a forecasted rainfall event. Cumulative precipitation for each location is presented in Table 4.2. Experiments were initiated at CHR May 17, June 11, and June 2 in 2018, 2019, and 2020, respectively. MCR experiments were initiated May 2, May 22, and May 13, for 2018, 2019, and 2020, respectively. Experiments were established May 17 and June 19 at Urbana for 2019 and 2020, respectively, and April 30, June 14, and May 12 at Perry for 2018, 2019, and 2020, respectively. Data from 2018 MCR was excluded due to lack of timely initial rainfall and no observable herbicide activity (Table 4.2).

The soil at each location was tilled prior to herbicide application to control existing vegetation. Individual plots measured 3 × 7.6 m with treatments arranged in a randomized complete block design with four replications at each location. Treatments were applied with a pressurized CO_2 backpack sprayer calibrated to deliver 187 L ha^{-1} at 276 kPa with a 3-m boom

consisting of six AI110025VS nozzles (Teejet Technologies, P.O. Box 7900, Wheaton, IL 60187) spaced 51 cm apart. Herbicide efficacy was assessed 28 and 42 days after treatment (DAT) using visual ratings of percent waterhemp control recorded on a scale of 0 (no control) to 100% (complete control). Ratings considered waterhemp density, injury, and biomass reduction compared with a nontreated control. Waterhemp density per square meter was recorded from a consistent quadrat location in the middle of each plot at each evaluation timing, and above-ground biomass was subsequently harvested 42 DAT. Above-ground biomass was then dried in a forced air dryer at 65°C.

Statistical Analysis

Statistical analysis was conducted with SAS 9.4 (SAS Institute, Inc., 100 SAS Campus Drive, Cary, NC 27513). Waterhemp control and density were analyzed by ANOVA using a generalized linear mixed model (PROC GLIMMIX). The assumption of normally distributed residuals was reviewed with PROC UNIVARIATE and homogeneous variance was checked with PROC GLM. Waterhemp control was fitted to a Beta distribution with a logit link function (Davis 2018). Waterhemp density and above ground biomass were fitted to a negative binomial distribution with a log link function (O'Hara and Kotze 2010). We first determined if there were interactions of herbicide treatment and location. Fixed effects were location, Group 15 herbicide, rate, and their interactions. Random effects included year and block nested within year. Initial analysis revealed location and location by Group 15 herbicide interaction effects were significant. Each location was then analyzed separately to explore control at each location. Analysis did not indicate a significant interaction of Group 15 herbicide and rate. Mean estimates were then separated by Fishers Least Significant Difference (LSD) (α=0.05) and

treatment means pooled between rates. Listed means represent the data scale and were back transformed following analysis.

Results and Discussion

Champaign County resistant location

Group 15 herbicide was significant (P<0.001) and by 28 DAT, only non-encapsulated acetochlor and alachlor provided 91% or greater control of CHR, whereas control with all other Group 15 herbicides was 61% or less (Table 4.3). Control with metolachlor and *S*-metolachlor did not exceed 37%. Non-encapsulated acetochlor and alachlor reduced waterhemp densities 92–95% 28 DAT (Table 4.3). Dimethenamid-*P*, encapsulated acetochlor, and pyroxasulfone reduced waterhemp density 72–77% 28 DAT while metolachlor or *S*-metolachlor reduced waterhemp density 42–62%.

Control with all Group 15 herbicides decreased by 42 DAT (Table 4.3). Non-encapsulated acetochlor and alachlor provided 85–88% control of CHR, respectively, whereas control was 40% or less for the other Group 15 herbicides. These results with non-encapsulted acetochlor are consistent with previous reports in which non-encapsulated acetochlor controlled CHR over 70%, while control with other Group 15 products was significantly less (Evans et al. 2019; Strom et al. 2019). Waterhemp densities in nontreated plots decreased by 42 DAT, potentially due to intraspecific competition. Non-encapsulated acetochlor and alachlor reduced waterhemp density 88 and 84%, respectively, 42 DAT. Other Group 15 herbicides reduced waterhemp density up to 65% 42 DAT, while metolachlor and *S*-metolachlor reduced density only 26–45%. Average biomass was 336 g m^{-2} for nontreated plots 42 DAT and non-encapsulated acetochlor reduced biomass 81%. Alachlor reduced biomass 74%, but biomass

reduction did not exceed 41% for all other Group 15 active ingredients. Biomass m^{-2} from plots treated with either *S*-metolachlor formulation, metolachlor, or pyroxasulfone were not significantly different from the nontreated control.

McLean County resistant location

Control of MCR with Group 15 herbicides 28 DAT was greater than previously reported by Hausman et al. (2013). Group 15 herbicide was significant (P=0.007) and non-encapsulated acetochlor, alachlor, and pyroxasulfone controlled MCR 94–98% (Table 4.4). Control with other treatments exceeded 62% (Table 4.4). Metolachlor and *S*-metolachlor controlled MCR 62–73%, whereas control was 17–53% in previous research 30 DAT (Hausman et al. 2013). All Group 15 herbicides reduced waterhemp density 28 DAT (Table 4.4). Non-encapsulated acetochlor and alachlor reduced waterhemp density 94 and 91%, respectively, while metolachlor and *S*-metolachlor reduced waterhemp density 64–72%. Encapsulated acetochlor reduced waterhemp density only 48%.

By 42 DAT non-encapsulated acetochlor, alachlor, and pyroxasulfone provided the greatest control (85–94%), while control among the other Group 15 herbicides was not different and ranged from 39–54%. Hausman et al. (2013) reported 7–55% control of MCR 60 DAT. Waterhemp density in nontreated plots was 69 plants m^{-2} and all treatments significantly reduced waterhemp density. Encapsulated acetochlor reduced waterhemp density only 32%. Waterhemp densities in metolachlor, *S*-metolachlor, and dimethenamid-*P* treated plots were not different and were 45–55% less than the nontreated. Non-encapsulated acetochlor reduced waterhemp density the most (90%) 42 DAT. All Group 15 herbicides reduced waterhemp biomass 42 DAT. Biomass from nontreated plots was 83 g m^{-2} and non-encapsulated acetochlor provided the

greatest reduction in biomass (96%). Biomass from plots treated with encapsulated acetochlor, *S*-metolachlor, metolachlor, and dimethenamid-*P* were not different and were 48–65% less than the nontreated.

Urbana, IL location

Group 15 herbicides were effective for control of the Urbana waterhemp populations 28 DAT (Fig 4.1). Group 15 herbicide was significant (P=0.0013) and control with each Group 15 herbicide was 88% or greater (Table 4.5). Control with non-encapsulated acetochlor, alachlor, and pyroxasulfone was 98%. *S*-metolachlor, encapsulated acetochlor, and dimethenamid-*P* provide up to 96% control which is greater than reported at the Group 15 herbicide-resistant locations (Tables 4.3,4.4). Results from Urbana are consistent with previous reports where non-encapsulated acetochlor, *S*-metolachlor, dimethenamid-*P* and pyroxasulfone provided greater than 85% control of waterhemp 28 DAT (Hedges et al. 2019; Schryver et al. 2017; Steckel et al. 2002). Waterhemp density in nontreated plots averaged 122 plants m^{-2} and was reduced at least 95% with non-encapsulated acetochlor, alachlor, and pyroxasulfone. Encapsulated acetochlor reduced waterhemp density 89%, while *S*-metolachlor and dimethenamid-*P* reduced density 80–84%. Metolachlor reduced waterhemp density only 67%.

By 42 DAT waterhemp control with all Group 15 herbicides decreased (Table 4.5), but control with all treatments, with the exception of metolachlor, remained 80% or greater. Control with non-encapsulated acetochlor, alachlor, and pyroxasulfone was at least 95% 42 DAT. In general, waterhemp densities in the treated plots increased by 42 DAT, whereas densities in the nontreated plot decreased slightly (Table 4.5). Non-encapsulated acetochlor, alachlor, and pyroxasulfone decreased waterhemp density 93, 94, and 93%, respectively, whereas density

reduction with other treatments was 83% or less. All treatments also reduced waterhemp biomass m^{-2} 42 DAT when compared with the nontreated, with pyroxasulfone reducing biomass 99%.

Perry, IL location

The Perry, IL location was the only location with soils containing less than 3% organic matter. Results from Perry were similar to the Urbana location and Group 15 herbicide was significant (P=0.0016). At 28 DAT all treatments controlled waterhemp 81% or greater (Table 4.6). Control among treatments was not different, with the exceptions of encapsulated acetochlor and metolachlor which provided 88 and 81% control, respectively. All treatments reduced waterhemp density, with acetochlor, alachlor, and pyroxasulfone reducing density 93–96%. Other treatments reduced waterhemp density up to 87%, but encapsulated acetochlor and metolachlor reduced densities the least.

Control at Perry decreased by 42 DAT, but the Group 15 herbicides provided greater than 80% control with the exception of encapsulated acetochlor and metolachlor. Control at Perry 42 DAT with racemic metolachlor was greater than observed at the CHR and MCR locations (Table 4.3, 4.4), but less than previous reports where racemic metolachlor provided over 80% control up to 60 DAT (Hager et al. 2002a; Oliveira et al. 2017). Control was greatest with non-encapsulated acetochlor, alachlor, and pyroxasulfone (92–95%). Waterhemp densities in treated plots numerically increased 42 DAT (Table 4.6), but remained significantly less than the nontreated. Encapsulated acetochlor, *S*-metolachlor, and metolachlor reduced waterhemp density 59–73%. All herbicides reduced above-ground biomass 42 DAT compared with the nontreated. Nontreated biomass averaged 102 g m^{-2} and biomass reduction ranged from 79–99% across

treatments. Non-encapsulated acetochlor and pyroxasulfone reduced waterhemp biomass 98–99%.

Implications

Only non-encapsulated acetochlor and alachlor controlled CHR 91% or greater 28 DAT; these two Group 15 herbicides along with pyroxasulfone controlled MCR 94% or greater 28 DAT. Control of these Group 15-resistant populations with other Group 15 herbicides ranged 20–76% 28 DAT. All treatments reduced CHR and MCR density 28 DAT compared with a nontreated, but non-encapsulated acetochlor and alachlor consistently reduced waterhemp density the most among all Group 15 Herbicides. Previous greenhouse research has demonstrated resistance ratios calculated from dose-response experiments for CHR and MCR are variable and are greatest with *S*-metolachlor and least with acetochlor (Strom et al. 2019). In contrast, control of Group 15-sensitive waterhemp at Urbana was 93% or greater with all treatments except metolachlor, while the Group 15-sensitive waterhemp at Perry was controlled at least 93% with all treatments except metolachlor and encapsulated acetochlor 28 DAT. Even though most Group 15 herbicides controlled sensitive waterhemp, resistance in the CHR and MCR populations should serve as a reminder that resistance to Group 15 herbicides has evolved and, if present, will reduce efficacy under field conditions.

Strom et al. (2020) demonstrated CHR and MCR seedlings rapidly metabolize *S*-metolachlor. CHR and MCR also demonstrated reduced sensitivity to pyroxasulfone, dimethenamid-*P*, and acetochlor in greenhouse dose-response experiments (Strom et al. 2019). Mechanisms for reduced sensitivity to these other Group 15 herbicides have not been identified, but might be similar to those conferring resistance to *S*-metolachlor. Resistance to soil-applied

herbicides under field conditions can be difficult to accurately identify. In general, resistance to soil-applied herbicides is manifest as a reduced duration of residual weed control (Hager 2019).

Poor weed control with soil-residual herbicides, including Group 15 herbicides, is not always due to resistance. Many climatic and edaphic factors influence herbicide activity and duration of residual control (Stewart et al. 2010). The timing and amount of precipitation in relation to herbicide application are essential for incorporation of surface-applied Group 15 herbicides (Jhala 2017). In addition, photodegradation can occur if excess time elapses between herbicide application and movement into the soil (Shaner 2014). In general, fine soil textures with high organic matter, require increased herbicide rates. Soils with higher organic matter also tend to have elevated microbial communities which hasten herbicide degradation, and thus, decrease residual control (Beestman and Deming 1974; Long et al. 2014; Shaner et al. 2006; Wu et al. 2011).

The magnitude and distribution of resistance to Group 15 herbicides is currently not well understood. A recent five-year study of preemergence herbicides in Iowa discovered most Group 15 herbicides provided greater than 80% control of waterhemp throughout the season, but there were instances where control was much less (Jha 2020). We have identified only two populations in Illinois resistant to Group 15 herbicides at this time (Strom et al. 2019; 2020). The dioecious biology of waterhemp contributes to the rapid spread of resistance traits if proper management and chemical stewardship practices are not implemented (Liu et al. 2012; Sarangi et al. 2017). Growers, applicators, and crop protection professionals should understand the effective weed control methods available for each field. The continued use of best management practices, such as applications of herbicides from multiple, effective sites of action should be utilized (Evans et al. 2016), including those with soil-residual activity. In addition, overlapping

residual herbicides can enhance the probability of an effective post-emergence program (Chahal et al. 2018; Steckel et al. 2002).

Non-chemical control methods should also be considered in integrated management strategies. Cover crops, such as cereal rye, have demonstrated promise in reducing weed densities and are compatible with many herbicide programs (Cornelius and Bradley 2017; Jha et al. 2020; Loux et al. 2017). Additionally, post-harvest seed destruction and manual removal of weeds can be incorporated into sustainable weed management programs with the goal of limiting the number of seeds reintroduced into the soil seed bank each year (Bhuler et al. 1997; Schwartz-Lazaro and Copes 2019; Shergill et al. 2020; Walsh et al. 2012).

Tables

Table 4.1 Group 15 herbicides, rates, and source information for field studies at multiple locations in Illinois with differing soil types (2018 to 2020). Herbicides were applied to bare ground prior to weed emergence.

Common name	Trade name	Application rate[a] Organic Matter <3%	Organic Matter >3%	Manufacturer
		kg ha^{-1}		
acetochlor[b]	Warrant	1.3	2.3	Bayer CropScience, St. Louis, MO
		2.3	2.5	
acetochlor	Harness	1.7	2.2	Bayer CropScience, St. Louis, MO
		2.2	2.7	
S-metolachlor	Dual Magnum	1.4	1.8	Syngenta Crop Protection, Greensboro, NC
		1.8	2.1	
S-metolachlor[c]	Dual II Magnum	1.4	1.8	Syngenta Crop Protection, Greensboro, NC
		1.8	2.1	
metolachlor	Stalwart	1.5	1.9	SipcamAgro, Durham, NC
		1.9	2.2	
dimethenamid-*P*	Outlook	0.7	1.0	BASF, Research Triangle Park, NC
		1.0	1.1	
alachlor	IntRRo	2.2	2.8	Bayer CropScience, St. Louis, MO
		3.1	3.4	
pyroxasulfone	Zidua	0.1	0.1	BASF, Research Triangle Park, NC
		0.2	0.2	

[a]Application rates were chosen based on manufacturer's labeled recommendations for each soil type and two rate structures were chosen by organic matter content.

[b]Encapsulated formulation

[c]Contains the safener benoxacor

Table 4.2 Cumulative precipitation at field locations in Illinois during 2018–2020 field experiments.				
		Precipitation (cm)		
Location	Days after treatment	2018	2019	2020
Champaign Co. (CHR)[a]	7	0.2	3.4	10.6
	14	4.2	7.2	12.0
	42	33.9	11.2	22.7
McLean Co. (MCR)[a]	7	0.5	5.8	9.2
	14	0.7	7.6	9.4
	42	5.4	19.6	15.8
Urbana[b]	7	–	4.4	7.1
	14	–	8.1	9.0
	42	–	16.5	21.3
Perry[c]	7	1.8	7.5	1.5
	14	2.2	11.6	7.2
	42	8.5	18.0	25.2

[a]Data acquired using a Watchdog 2000 Series Weather Station, Spectrum Technologies Inc., 3600 Thayer Ct., Aurora, IL 60504

[b]Data acquired from the Illinois State Water Survey, Prairie Research Institute, 2204 Griffith Dr., Champaign, IL 61820

[c]Data acquired from the U.S. Department of Commerce National Oceanic & Atmospheric Administration, Savoy 0.9 N, IL US US1ILCP0082, National Centers for Environmental Information 151 Patton Ave, Asheville, NC 2880

Table 4.3 Mean estimates[a] of waterhemp control 28 and 42 days after treatment (DAT), density 28 and 42 DAT, and recovered biomass 42 DAT of Group 15 herbicides at the Champaign County, IL (CHR) location (2018–2020).

	Control		Density		Biomass
Herbicide	28 DAT	42 DAT	28 DAT	42 DAT	42 DAT
	— % —		— plants m^{-2} —		g m^{-2}
acetochlor[b]	56 b	39 b	49 d	46 d	219 b
acetochlor	93 a	88 a	10 f	15 e	64 e
S-metolachlor	37 c	20 bc	72 c	67 c	291 ab
S-metolachlor[c]	30 cd	20 bc	79 c	66 c	262 abc
metolachlor	20 d	13 c	111 b	89 b	300 a
dimethenamid-*P*	61 b	28 bc	44 d	42 d	198 c
alachlor	91 a	85 a	15 e	19 e	89 d
pyroxasulfone	57 b	40 b	52 d	43 d	253 abc
Nontreated	—	—	190 a	121 a	336 a

[a]Mean estimates with the same letter within a column are not significantly different at α=0.05 separated by LSD.

[b]Encapsulated formulation

[c]Contains the herbicide safener benoxacor

Table 4.4 Mean estimates[a] of waterhemp control 28 and 42 days after treatment (DAT), density 28 and 42 DAT, and recovered biomass 42 DAT of Group 15 herbicides at the McLean County, IL (MCR) location (2018–2020)[b].

Herbicide	Control		Density		Biomass
	28 DAT	42 DAT	28 DAT	42 DAT	42 DAT
	— % —		— plants m^{-2} —		g m^{-2}
acetochlor[c]	65 c	39 b	45 b	47 b	43 c
acetochlor	98 a	94 a	5 f	7 e	3 e
S-metolachlor	73 c	52 b	24 cd	31 c	38 bc
S-metolachlor[d]	71 c	48 b	25 cd	33 c	34 bc
metolachlor	62 c	40 b	31 c	38 bc	35 bc
dimethenamid-*P*	76 bc	54 b	21 d	32 c	29 c
alachlor	97 a	90 a	8 ef	13 d	6 d
pyroxasulfone	94 ab	85 a	10 e	13 d	7 d
Nontreated	—	—	86 a	69 a	83 a

[a]Mean estimates with the same letter within a column are not significantly different at α=0.05 separated by LSD.

[b]Data from 2018 were excluded due to lack of initial rainfall.

[c]Encapsulated formulation

[d]Contains the herbicide safener benoxacor

Table 4.5 Mean estimates[a] of waterhemp control 28 and 42 days after treatment (DAT), density 28 and 42 DAT, and recovered biomass 42 DAT of Group 15 herbicides at the Urbana, IL location (2019–2020).

Herbicide	Control		Density		Biomass
	28 DAT	42 DAT	28 DAT	42 DAT	42 DAT
	— % —		— plants m^{-2} —		g m^{-2}
acetochlor[b]	96 b	88 abc	14 d	18 d	58 c
acetochlor	98 a	95 ab	5 e	8 e	24 d
S-metolachlor	95 b	84 abc	19 cd	24 cd	74 bc
S-metolachlor[c]	93 b	80 bc	24 c	26 bc	117 b
metolachlor	88 c	68 c	40 b	35 b	126 b
dimethenamid-*P*	94 b	87 abc	19 cd	20 b	86 bc
alachlor	98 a	96 a	5 e	7 e	6 e
pyroxasulfone	98 a	95 ab	6 e	8 e	2 f
Nontreated	—	—	122 a	108 a	345 a

[a]Mean estimates with the same letter within a column are not significantly different at α=0.05 separated by LSD.

[b]Encapsulated formulation

[c]Contains the herbicide safener benoxacor

Table 4.6 Mean estimates[a] of waterhemp control 28 and 42 days after treatment (DAT), density 28 and 42 DAT, and recovered biomass 42 DAT of Group 15 herbicides at the Perry, IL location (2018–2020).

Herbicide	Control		Density		Biomass
	28 DAT	42 DAT	28 DAT	42 DAT	42 DAT
	— % —		— plants m^{-2} —		g m^{-2}
acetochlor[b]	88 bc	79 c	21 bc	22 bcd	9 cd
acetochlor	98 a	95 a	5 e	6 fg	2 ef
S-metolachlor	96 a	86 bc	12 d	15 de	7 d
S-metolachlor[c]	93 ab	80 c	19 cd	26 bc	18 bc
metolachlor	81 c	60 d	32 b	33 b	21 b
dimethenamid-*P*	94 ab	85 bc	14 cd	18 cd	7 d
alachlor	98 a	92 ab	6 e	9 ef	3 e
pyroxasulfone	98 a	94 a	4 e	5 g	1 f
Nontreated	—	—	91 a	81 a	102 a

[a]Mean estimates with the same letter within a column are not significantly different at α=0.05 separated by LSD.

[b]Encapsulated formulation

[c]Contains the herbicide safener benoxacor

Figure

Figure 4.1 Representative images of Group 15 herbicide efficacy for control of Group 15-resistant waterhemp (*Amaranthus tuberculatus*) population (CHR) and a sensitive (Urbana) population, 28 days after treatment.

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CHAPTER 5: SUMMMARY AND IMPLICATIONS OF RESEARCH FINDINGS

Waterhemp (*Amaranthus tuberculatus* Moq. Sauer) is among the most challenging weed species to control in the Midwestern United States (Steckel 2007). Previous field research had demonstrated that two multiple-herbicide resistant populations from McLean County, IL and Champaign County, IL (designated MCR and CHR) were not controlled with herbicides that inhibit the formation of very-long-chain fatty acids (Evans et al. 2019; Hausman et al. 2013; Strom et al. 2019). The populations also displayed resistance to the VLCFA-inhibiting herbicides acetochlor, dimethenamid-*P*, pyroxasulfone, and *S*-metolachlor under greenhouse conditions in dose-response experiments (Strom et al. 2019). My findings were the inaugural reports of resistance to VLCFA-inhibitors in waterhemp, and in any dicot weed species globally.

My research focused on the VLCFA-inhibitor *S*-metolachlor, and I quickly discovered CHR and MCR are resistant due to enhanced herbicide metabolism relative to sensitive waterhemp. Additionally, CHR and SIR metabolize *S*-metolachlor as rapidly as corn, which utilizes glutathione *S*-transferase (GST) mediated metabolism for natural tolerance (Fuerst 1987). Using enzymatic inhibitors, however, I found that the metabolic mechanisms in CHR and SIR might differ from corn, with multiple detoxification enzymes involved (Strom et al. 2020). GST-activity assays using waterhemp and corn identified CHR and SIR possess increased GST-activity with *S*-metolachlor as substrate compared to sensitive population, but much less than corn, despite being able to metabolize *S*-metolachlor just as quickly. Subsequent *in vitro* enzymatic assays investigating microsomal cytochrome P450 activity from waterhemp and corn microsomes revealed a different finding. Microsomes isolated from CHR and SIR formed a single oxidative metabolite via *O*-demethylation at a significantly greater rate than sensitive

waterhemp or corn. The product was NADPH-dependent, strongly indicating P450-mediated detoxification activities (Gordeziani et al. 2016; Werk-Reichhart et al. 2000). Formation of the same metabolite from microsomal fractions has been previously reported in several crops (Moreland and Corin 1991; Moreland et al. 1993; 1995), but my results are the first reports from waterhemp. Subsequent investigations of the metabolome from CHR and SIR revealed a more diverse metabolite profile than sensitive waterhemp, and supported enzymatic assays by identifying multiple metabolites derived from activities by GSTs, P450s, or both in combination. Assays utilizing β-glucosidase enzymes also revealed that conjugation to sugars likely follows initial oxidative metabolism, which is commonly reported for hydroxylated or de-alkylated herbicides (Siminszky 2006; Werk-Reichhart and Feyereisen 2000). Overall, these findings are the first mechanistic explanation of VLCFA-inhibitor resistance in a dicot weed, and change the current paradigm of single enzyme families mediating herbicide resistance.

Non-target site resistance, such as metabolism, is becoming more prevalent in waterhemp (Tranel 2020). Non-target site resistance mechanisms, including enhanced metabolism, pose a significant challenge to the agrochemical industry because potential cross-resistance patterns and underlying genetic control of resistance remain unclear (Beckie and Tardif 2010; Yu and Powles 2014). SIR discussed herein possesses multiple distinct detoxification mechanisms to mesotrione, topramezone, and atrazine (Lygin et al. 2018; Ma et al. 2013), and the relation of any of the previously reported mechanisms to *S*-metolachlor resistance remains unknown. In the future, transcriptome analysis and inheritance studies need to be conducted to elucidate candidate gene(s) related to *S*-metolachlor resistance and how they are inherited (Ward et al. 2012). Further investigation into other VLCFA-inhibitors should be conducted to determine if similar

mechanisms that confer resistance to *S*-metolachlor effect herbicides such as acetochlor, dimethenamid-*P*, or pyroxasulfone.

Resistance to VLCFA-inhibitors is interesting to study in the lab, but also poses real world challenges for growers. Many factors influence the activity of VLCFA-inhibitors each season, and resistance within the weed population could be one (Splittstoesser and Derscheid 1962). Under field conditions, growers might notice less or a shorter duration of residual control than normally expected (Hager 2019). During a three-year period, my studies found that VLCFA-inhibitors do not provide utility for controlling resistant populations such as CHR and MCR. Control of sensitive waterhemp populations in Illinois was still achieved. Although VLCFA-inhibitors were originally developed for controlling monocot species over sixty years ago (Hamm 1974), they remain an effective option for waterhemp today, and in the future, as long as proper chemical stewardship practices are maintained.

Metabolic resistance causes uncertainty in regards to controlling resistant weed populations in the future, but also poses new opportunities for weed management. With gene-editing and silencing technologies becoming more mainstream, identifying potential resistance genes will be more important than ever. No herbicide is completely immune to resistance development, and many forms of non-chemical practices, such as tillage, cover crops, flaming, electricity, and post-harvest seed destruction, can be effective (Cornelius and Bradley 2017; Jha et al 2020; Loux et al 2017; Schwartz-Lazaro and Copes 2019; Shergill et al. 2020; Walsh et al. 2012). The goal of each weed control practitioner should be a weed control program that yields no contributions to the soil seedbank each season (Buhler et al. 1997; Schwartz-Lazaro and Copes 2019). If this goal is achieved, even multiple-herbicide resistant waterhemp can be controlled.

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APPENDIX A: INITIAL MICROSOMAL ASSAY FIGURES

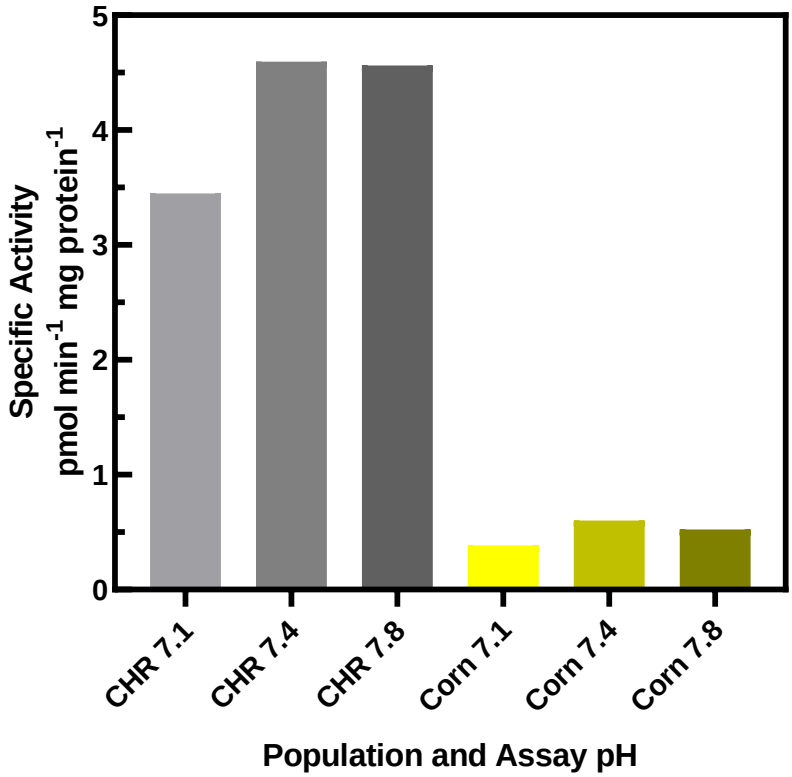

Figure A.1 Initial microsomal assay with very-long-chain fatty acid (VLCFA)-inhibitor resistant (CHR) waterhemp (*Amaranthus tuberculatus*) and naturally tolerant corn (*Zea mays*; B73 x Mo17) to determine the optimum assay pH. Assay duration was 2 h at 30°C with pH conditions of 7.4 and 7.8 yielding the greatest specific activity (pmol mg protein^{-1} min^{-1}).

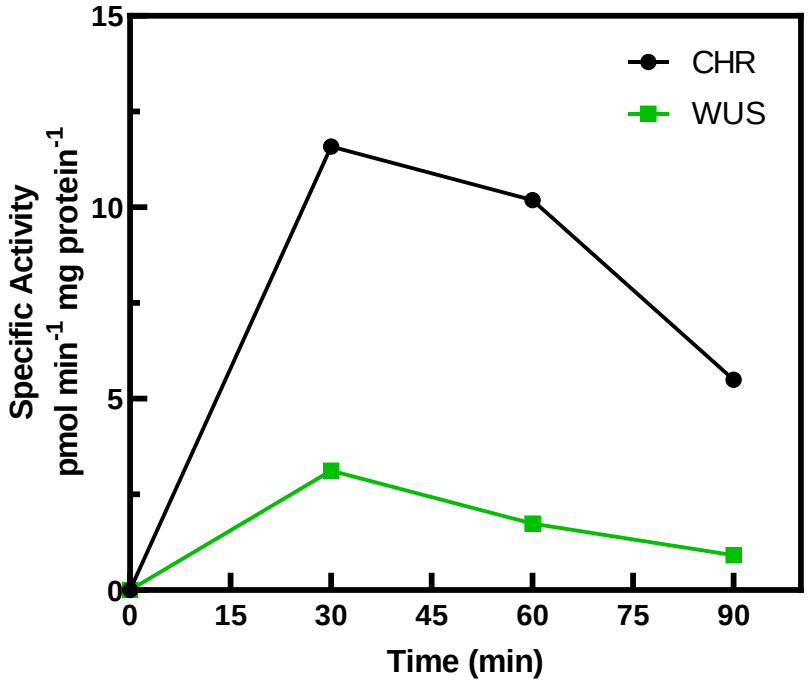

Figure A.2 Initial microsomal assay with very-long-chain fatty acid (VLCFA)-inhibitor resistant (CHR) and sensitive (WUS) waterhemp (*Amaranthus tuberculatus*) to determine the optimum assay duration. Assays times were 30, 60, and 90 min at 30°C and assay pH was 7.8. Assays longer than 30 min resulted in a decrease in specific activity (pmol mg protein^{-1} min^{-1}).

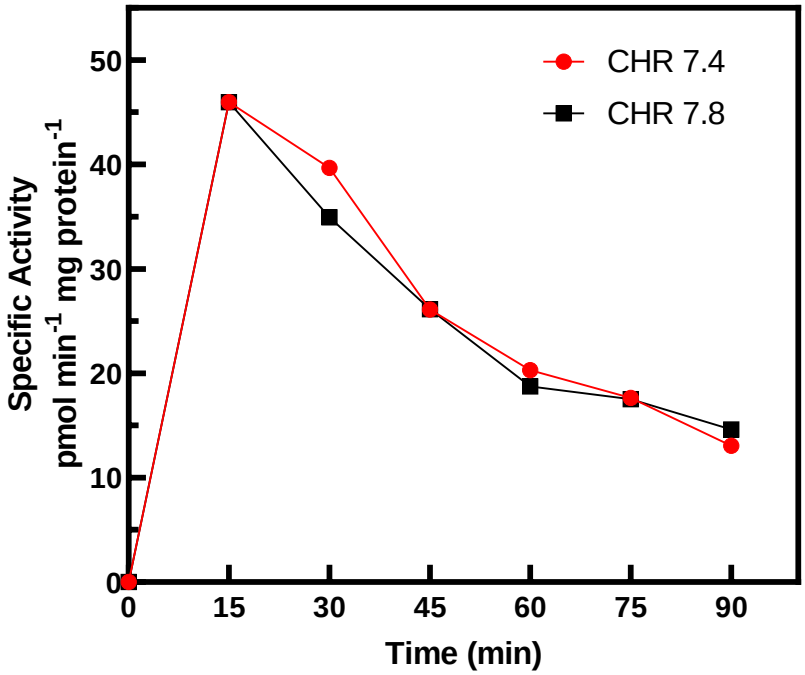

Figure A.3 Initial microsomal assay with very-long-chain fatty acid (VLCFA)-inhibitor resistant (CHR) waterhemp (*Amaranthus tuberculatus*) to further investigate the optimum assay duration and pH. Assays were completed every 15 min for 90 min at 30°C with assay pH values of 7.4 and 7.8. Assays longer than 30 min resulted in a decrease in specific activity (pmol mg protein^{-1} min^{-1}) and pH 7.4 was optimum.

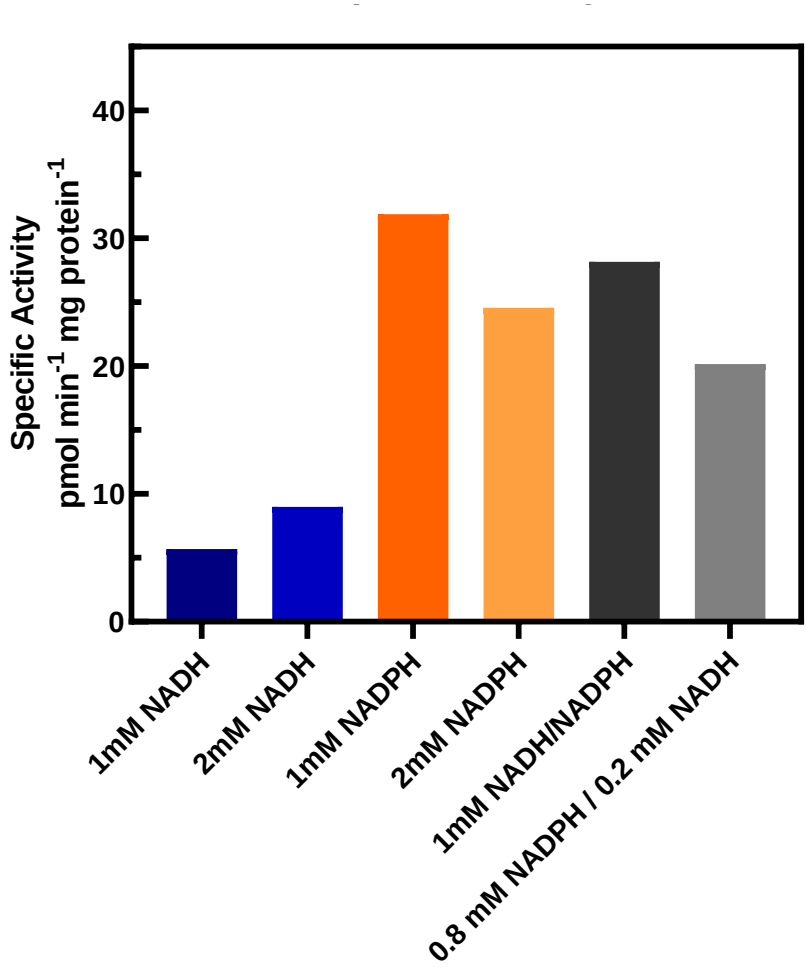

Figure A.4 Initial microsomal assay with very-long-chain fatty acid (VLCFA)-inhibitor resistant (CHR) waterhemp (*Amaranthus tuberculatus*) to determine the optimum electron donor and concentration. Assays time was 30 min at 30°C and assay pH was 7.4. A final concentration of 1mM NADPH as the electron donor yielded the greatest specific activity (pmol mg protein^{-1} min^{-1}).

APPENDIX B: STANDARDS USED IN METABOLOMICS EXPERIMENTS

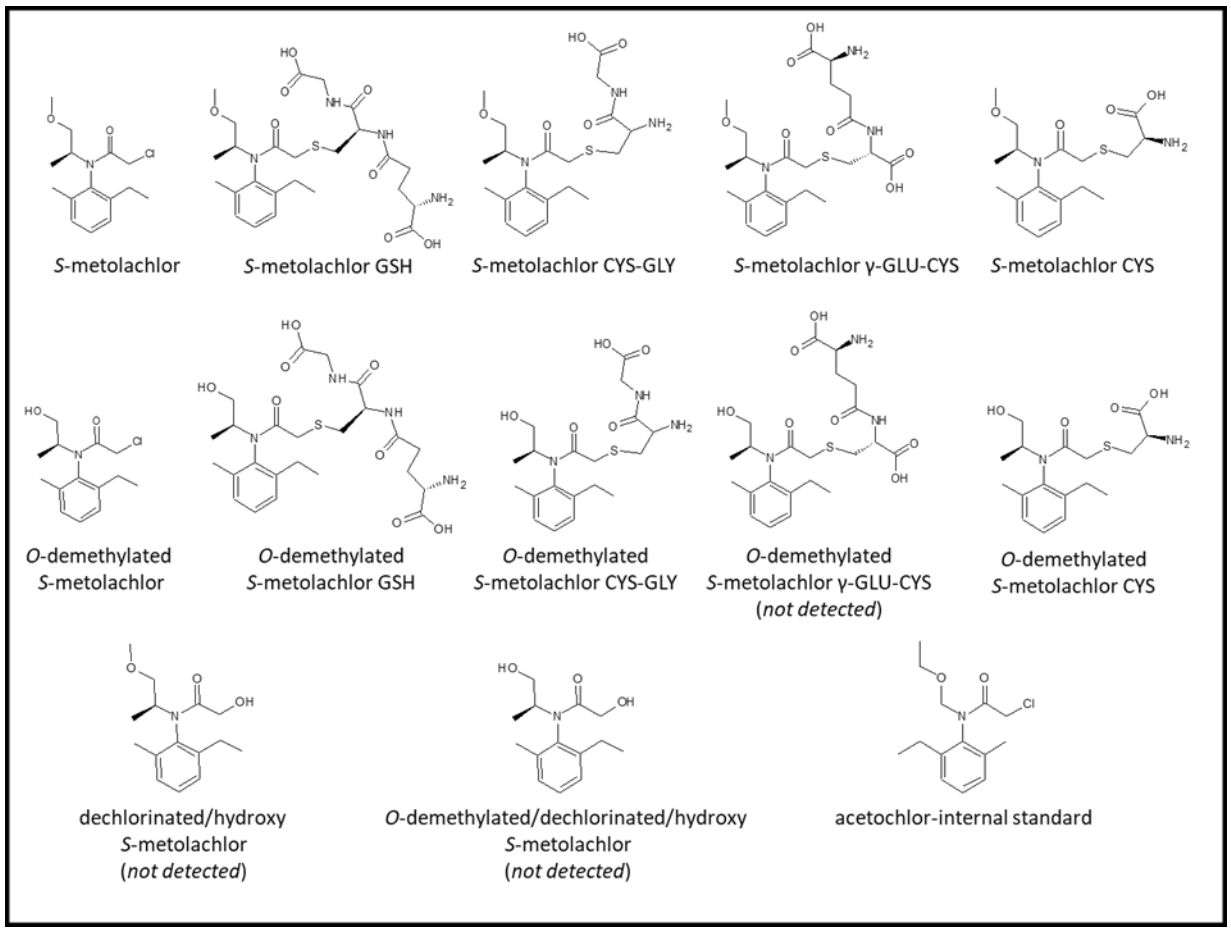

Figure B.1 Metabolite standards included in targeted and untargeted metabolomics experiments.