Characterization of metabolic genes for multiple herbicide resistance in Palmer amaranth and optimization of metribuzin rates to control pigweed in soybean

by

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Abstract

Weed infestation is a significant threat to sustainable crop production, causing both direct and indirect crop losses. The most problematic weeds in the U.S. include Palmer amaranth (*Amaranthus palmeri* S. Watson) and waterhemp [*Amaranthus tuberculautus* (Moq). J. D. Sauer], known for their aggressive growth habits, competitiveness, and ability to evolve herbicide resistance. Currently, Palmer amaranth populations have evolved resistance to ten sites of action (SOA) of herbicides while waterhemp populations were documented to have resistance to at least seven SOAs. Herbicide resistance can evolve because of both target-site and non-target-site-based mechanisms. One of the most challenging non-target site resistance mechanisms is metabolism of herbicides due to increased activity of enzymes such as cytochrome P450s (P450) or glutathione-*S*-transferases (GSTs). Recently, a Palmer amaranth population (KCTR) from Kansas was found resistant to six SOAs of herbicides. Moreover, a predominance of metabolic resistance possibly mediated by P450 or GST enzyme activity was reported in this population. In this research, KCTR Palmer amaranth was used as a model to assess the molecular basis of metabolic resistance to multiple herbicides, which is the focus of Chapter 2 of this thesis. Rapidly evolving multiple herbicide-resistant (MHR) populations of both Palmer amaranth and waterhemp are already a challenge to growers, leaving limited chemical weed control options. However, metribuzin (photosystem II-inhibitor) can effectively control small-seeded broadleaf weeds including *Amaranthus* spp. when applied as a pre-emergence (PRE) herbicide. Despite the known activity of metribuzin growers are reluctant to use it as they are either unaware of metribuzin's efficacy or due to suspected crop injury. In Chapter 3, the use of metribuzin was evaluated as a potential PRE option to control MHR *Amaranthus* weeds, while documenting early-season soybean response. Overall the major

objectives of this thesis were to 1) identify the genes involved in metabolic resistance to multiple herbicides in Palmer amaranth (Chapter 2); 2) confirm and validate the activity of candidate genes in mediating herbicide metabolism (Chapter 2); 3) evaluate early season soybean growth and development following PRE applied metribuzin (Chapter 3); and 4) optimize metribuzin application rates for control of MHR Palmer amaranth and waterhemp populations (Chapter 3). Transcriptome analysis (Chapter 2) revealed 414, 129, 529, 688, and 152 genes differentially expressed in resistant plants following application of chlorsulfuron, 2,4-D, atrazine, lactofen, and mesotrione, respectively. Overexpression of *CYP72A219* gene isoforms was observed following mesotrione, chlorsulfuron, and lactofen treatment while *CYP704B1* overexpression was observed following 2,4-D and atrazine treatment. Additionally, isoforms of C-terminal domain GST were upregulated post-treatment with all herbicides except lactofen. Quantitative RT-PCR showed 3.4- to 6.6-fold and 5.9- to 12.4-fold overexpression of *CYP72A219* and *CYP704B1* transcripts, respectively, in KCTR Palmer amaranth. Field experiments evaluating metribuzin's efficacy (Chapter 3) revealed no significant impact on soybean height and yield even at application rates of 841 g ai ha^{-1} of metribuzin. Metribuzin at 630 and 315 g ai ha^{-1} delayed weed emergence more effectively than sulfentrazone and *S*-metolachlor, while rates of 525 and 315 g ai ha^{-1} were more effective in reducing weed density and biomass than sulfentrazone and *S*-metolachlor, respectively. Higher metribuzin rates (578 to 841 g ai ha^{-1}) achieved more than 95%, 90%, and 80% control of weeds at 14, 28, and 42 days after application, respectively. Overall, the outcome of this thesis uncovers key genes driving metabolic resistance to multiple herbicides in Palmer amaranth and demonstrates that higher rates of metribuzin can be safely used to control multiple herbicide-resistant Palmer amaranth and waterhemp in soybean, providing growers with a viable option for weed management

Table of Contents

List of Figures

List of Tables

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Dedication

I would like to dedicate this thesis to my incredible parents. My dad, Satyendra, who always made me taste the dirt and sweat even with the smallest day to day tasks, building a sense of hard work and moral character. My mom, Reeta, who nurtured me into a humble human cherishing the ups and downs of life. Although she could not continue her education after high school but knew the power of knowledge and always motivated me to pursue higher education. It is her dream that I fulfill through my work. The thread of their values and principles shapes who I am today, and for that, I am forever grateful.

Chapter 1 - Introduction and Literature Review

1.1 Weeds and Crop Production

Weeds have been a major concern for crop production since the dawn of agriculture. Over time, efforts to create arable land has opened new environments and allowed many species to become cosmopolitan weeds (Fried et al. 2009). Ever since, efforts have been made to either make the weed seed bank germinate when it does not compete with the crops or to eliminate the already germinated plants competing with the crops. Weeds are known to cause both direct and indirect crop losses. In a more direct way, they compete with crops for sunlight, water, nutrients and space decreasing both quality and quantity of yield. While indirectly they serve as a host for other insects and pathogen and hinder other crop management practices (Zimdahl 2018, Fried et al. 2017). Weeds are the most damaging pest and can cause a yield loss of 10- 40 % in various crops (Fried et al. 2017, Oerke 2006). Keeping in mind the growing population and limited arable land there is a need to develop management practices to mitigate this substantial crop yield loss caused by weeds. Introduction of synthetic chemicals as herbicides in the middle of 20th century helped in achieving effective weed control and reduced overall weed diversity. This made herbicides a primary choice for weed control by the growers but unfortunately overshadowed the traditional integrated weed management practices (Fried et al. 2009). This overreliance on herbicides over past few decades has accelerated the evolution of herbicide resistance in weeds.

With the accidental discovery of first chemical herbicide (2,4-D) in 1945, the market has been flooded with new chemicals over the decades. Each of these herbicides targets a specific biochemical or physiological process in plants, known as site of action (SOA). Differences in the

target site of herbicides results in herbicide selectivity. Table 1.1 highlights key herbicide sites of action, their mode of action, and commonly used active ingredients of some important groups.

Table 1.1 Herbicide classification based on sites of action.

Site of Action	Group	Herbicide examples	Mode of action
Acetyl CoA carboxylase (ACCase)-inhibitors	1	quizalofop, clethodim	Lipid synthesis inhibitors
Acetolactate synthase (ALS)- inhibitors	2	chlorsulfuron, imazamox	Amino acid synthesis inhibitors
Microtubule inhibitors	3	pendimethalin, trifluralin	Seedling root growth inhibitors
Various sites	4	2,4-D, dicamba	Growth regulators (Synthetic auxins)
Photosystem II (PS II)- inhibitors (serine 264 binders)	5	atrazine, metribuzin	Photosynthesis inhibitors
Photosystem II (PS II)- inhibitors (histidine 215 binders)	6	bentazone, pyridate	Photosynthesis inhibitors
Enolpyruvyl-shikimate-3-phosphate synthase (EPSPS)- inhibitors	9	glyphosate	Amino acid synthesis inhibitors
Glutamine synthetase (GS)- Inhibitor	10	glufosinate	Nitrogen metabolism inhibitors
Protoporphyrinogen oxidase (PPO)-inhibitors	14	lactofen, sulfenytrazone	Cell membrane disrupters
Very long-chain fatty acid (VLCFA)-inhibitors	15	*S*-metolachlor, pyroxasulfone	Seedling shoot growth inhibitors
Photosystem I electron diverters	22	paraquat, diquat	Cell membrane disrupters
Hydroxyphenylpyruvate dioxygenase (HPPD)- inhibitors	27	mesotrione, isoxaflutole	Pigment inhibitors

1.2 Evolution of Herbicide Resistance

Herbicide resistance evolves with existing genetic variability within a weed population that helps individual(s) in a population survive against the herbicide selection pressure (Jasieniuk et al. 1996). These survivors then reproduce and contribute to the next generation of resistance individuals. When herbicides with similar mode of action are used repeatedly, the number of survivors in the population increases and results in the spread of herbicide resistance (Jugulam and Shyam 2019). As of August 28th, 2024, 533 unique cases (species by site of action) of

herbicide resistant weeds have been reported around the world (Heap 2024). This includes 273 weed species enlisting 117 monocots such as Italian ryegrass (*Lolium perenne ssp. multiflorum* Lam. Husnot), barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.] and annual bluegrass (*Poa annua* L.) and 156 dicots such as Palmer amaranth (*Amaranthus palmeri* S. Watson), waterhemp [*Amaranthus tuberculatus* (Moq.) J. D. Sauer] and horseweed (*Conyza canadensis* L. Cronquist; Heap 2024). Weeds have evolved resistance to 21 of the 31 known herbicide SOAs and a total of 168 herbicide chemistries (Heap 2024). These cases of herbicide resistance have been reported in 101 crops across 72 countries. USA records the highest number of unique resistance cases (132) followed by Australia (91) and Canada (56; Heap 2024).

1.2.1 Types of Herbicide Resistance

Herbicide resistance can be classified as either target site (TSR) or non-target site (NTSR) based (Powles & Yu 2010; Yuan et al. 2007). TSR, usually a monogenic trait, evolves either due to any alteration in herbicide target site, reducing the efficacy of herbicide binding to the target site, or it could be due to herbicide target site abundance resulting from over-expression or amplification of target site (Gaines et al. 2020). On the other side, NTSR, generally a polygenic trait, is more complex and could be a result of multiple mechanisms working together from a whole plant level to molecular level. NTSR evolves due to reduced amount of total herbicide reaching the target site either due to reduced absorption, altered translocation, or as a result of enhanced metabolism and/or rapid sequestration of herbicide (Powles & Yu 2010; Yuan et al. 2007).

Reistance due to reduced absorption of herbicide is rare but have been reported for some foliar applied herbicides (Gaines et al. 2019), while NTSR based on altered translocation and sequestration tends to complement and co-exist with other resistance mechanism(s) including

herbicide metabolism (Rigon et al. 2020). Metabolism based resistance is a serious threat for weed management not ony because it has a potential for confiring resistance to multiple herbicdes at the same time but also it can bestow resistance to herbicides never used on the weed population or not yet disciovered (Yu & Powles 2014). Metabolism of herbicides is known to be mediated in three phases, namely Phase I, II and III biochemical reactions, by some specific family of gene. Phase I, mediated by cytochrome P450 monoxygenases (P450s) gene family, involves simple reactions such as oxidation, hydroxylation, dealkylation and deamination of herbicide molecule. Prodcuts of Phase I metabolism could possibly possess herbicidal activity. Phase II, reactions typically mediated by glutathione-*S*-transferases (GSTs) gene family, involves conjugation of Phase I metabolites with sugars or amino acids. Phase III, possibly mediated by ATP-binding cassette (ABC) transporters, include sequestration of herbicide parent compound and metabolites to vacuoles and cell walls (Gaines et al. 2020). Genes in P450 family have been reported to be involved in metabolism of herbicides belonging to multiple groups (e.g., Group 1, 2, 5, 6, 9, 14, 15, and 27) with genes from a single family *CYP81* dominating metabolism of multiple herbicides within and across weed species (Rigon et al 2020). Genes of GST family have been reported to metabolize herbicide belonging from Group 1, 2, 5, 6, 15 (Rigon et al. 2020) with Phi and Tau class being most important for herbicide metabolism (Edwards & Dixon 2005). Apart from P450s or GSTs, enymes such as acylamidases, esterases, aldo-keto reductases, glycosyl-, amino acid- and malonyl- transferases have also been reported to be involved at various phase I and II metabolism reactions either alone or in combination with P450s or GSTs (Rigon et al. 2020).

1.2.2 Herbicide Resistance in Palmer amaranth

Palmer amaranth, one of the most dynamic dicot weed with evolved resistance to

multiple herbicides. TSR in Palmer amaranth was reported against acetolactate synthase (ALS)-, enolpyruvylshikimate-3-phosphate synthase (EPSPS)-, protoporphyrinogen oxidase (PPO)-, and 4-hydroxyphenylpyruvate dioxygenase (HPPD)- inhibitors. Mutations in the target sites were found to be responsible for ALS- and PPO- inhibitor resistance (Chaudhari et al. 2020, Küpper et al. 2017, Nakka et al. 2017c, Copeland et al. 2018, Salas et al. 2016, Varanasi et al. 2018b). While EPSPS- and HPPD- inhibitor resistance was mediated by increased target site expression (Chahal et al. 2017, Chaudhari et al. 2020, Gaines et al. 2010, Koo et al. 2018, Nakka et al. 2017b). Palmer amaranth has also evolved NTSR involving reduced absorption and translocation imparting resistance to EPSPS- inhibitor (Dominguez-Valenzuela et al. 2017, Nandula et al. 2012), and enhanced metabolism as the primary mechanism for resistance to ALS-, photosystem II (PS II)-, PPO-, very long chain fatty acids inhibitors (VLCFA)-, HPPD-, glutamine synthetase (GS)- and microtubule- inhibitors as well as synthetic auxin herbicides (SAH) (Nakka et al. 2017a, Chahal et al. 2019a, Nakka et al. 2017c, Varanasi et al. 2018a, Küpper et al. 2018, Nakka et al. 2017b, Rangani et al. 2021, Rigon et al. 2023, Shyam et al. 2022a, González et al. 2021). Several genes of CYP and GST families have been suspected and/or confirmed for the rapid degradation of herbicide and are listed in Table 1.2.

Table 1.2 Genes from CYP and GST family reported for enhanced metabolism of different herbicide in Palmer amaranth

Site of action	Responsible genes	Validated gene expression	Validated in biological system	Reference
HPPD- inhibitors	*CYP72A219*	Yes	Yes	Rigon et al. 2023
HPPD- inhibitors	*CYP81E8*	Yes	No	Aarthy et al. 2022
VLCFA- inhibitors	*ApGSTU19, ApGSTF8, ApGSTF2,* and *ApGSTF2like*	Yes	No	Rangani et al 2021
GS- inhibitors	*CYP72A219*	Yes	No	Salas et al. 2018
SAH	*CYP81E8*	Yes	No	Giacomini et al. 2020

1.4 Palmer amaranth and waterhemp

Amaranthaceae is huge family encompassing about 65 genera and 900 species of plants (Rahman and Gulshana 2014). Specifically, the genus Amaranthus consists of 70 plant species collectively called pigweeds. Some Amaranthus species are edible and are used for grain production while others are used as ornamentals, animal feed or for medicinal purposes (Segura-Nieto et al. 1992). However, the Amaranthus genus also includes some agronomically important weeds species including smooth pigweed (*Amaranthus hybridus* L.), redroot pigweed (*Amaranthus retroflexus* L.), Powell's amaranth (*Amaranthus powellii* S. Watson), Palmer amaranth and waterhemp (Mosyakin and Robertson 2003). These weed species pose a serious threat to crop production around the globe. Among these weeds, the two dioecious species, Palmer amaranth and waterhemp have been reported to be the most troublesome weeds in the US cropping system (Van Wychen 2022). Palmer amaranth originated from southwest borders of US and Mexico while waterhemp is a native to the US Midwest (Sauer 1957). Both these weed species have evolved with an excellent set of biological characteristics which make them highly competitive. Rapid growth rate, prolific seed production (450,000 - 600,000 seeds per female plant; Keeley et al. 1987), ability to adapt to new environments and obligate outcrossing provide Palmer amaranth and waterhemp an upper hand in crop-weed competition (Sauer 1957). This genetic diversity is subjected to the enormous selection pressure imposed by herbicides specifically over the last two decades have helped accelerate the evolution of herbicide resistance in Palmer amaranth and waterhemp. Currently, Palmer amaranth populations have been confirmed with resistance to herbicides from 10 different sites of action (Heap 2024). A single Palmer amaranth population from Kansas has been reported with resistant to six different SOAs (Shyam et al. 2021). Waterhemp populations have also been reported resistant to

herbicides from seven different SOAs including: ALS-, PS II-, EPSPS-, PPO-, VLCFA- and HPPD- inhibitors and SAH (Heap 2024). A single waterhemp population from Illinois has been reported to have evolved resistance to five SOAs (Evans et al. 2019). All these factors contribute to wide distribution of Palmer amaranth and waterhemp and make them a menace for the growers by limiting their chemical weed control options. Infestation with Palmer amaranth has been reported to cause a yield loss of 91% in corn (*Zea mays* L.), 79% in soybean (*Glycine max* L. Merr), 22% in sorghum (*Sorghum bicolor* L. Moench) and 60% in cotton (*Gossypium hirsutum* L.) (Massinga et al. 2001, Bensch et al. 2003, Unruh 2013, MacRae et al. 2013). While waterhemp caused a yield loss of 74% in corn and 43% in soybean (Steckel et al. 2004, Hager et al. 2002).

1.5 Control of Palmer amaranth and Waterhemp

This scenario of rapid evolution of herbicide resistance in weed species, especially to triazines and ALS-inhibitors, is one of the major factors towards the introduction of glyphosate resistant (GR) crops in mid 1990s. GR crops were widely adopted in under five years with over 90% of planted corn, soybean and cotton in the US being GR by 2020 (USDA 2021). Prior to GR technology, weed management programs primarily included a combination of pre-emergence (PRE) and post-emergence (POST) herbicide from different SOAs (Kniss 2018) coupled with mechanical methods of weed control. Rapid adoption of GR crops by growers in the early 2000s drastically transformed weed management for row crops in the US. GR technology provided growers the flexibility to apply POST herbicide. This helped growers save time, labor and herbicide expenses and decreased their dependence on other chemical, mechanical and cultural weed control methods (Bradley et al. 2004; Johnson et al. 2000). PRE herbicides that have been long forgotten, are known to have an effective control of small seeded

broadleaf weeds including Palmer amaranth and waterhemp, the most economically damaging weeds in soybean (Oliveira et al. 2017, Whitaker et al. 2011). PRE herbicides provide weed control up to first 3 to 4 weeks after application which helps not only in reducing crop weed competition during the critical period of weed control but also helps in alleviating pressure on subsequent POST applications (Butts et al. 2017; Knezevic et al. 2019; Tursun et al. 2016). Moreover, effective PRE programs were found to be an important strategy to control herbicide-resistant weeds (Norsworthy et al. 2014). Keeping in mind the current scenario of herbicide resistance in weeds and fewer chemical weed control options, growers are getting back to their conventional methods, i.e., PRE herbicides with an 18% increase in the metribuzin (PS II-inhibitor) use in the soybean planted areas from 2006 to 2017 (USDA-NASS 2017).

1.6 Why Metribuzin?

PS II-inhibitor herbicides are widely known for their broad-spectrum weed control. These herbicides work by disrupting photosynthesis, specifically by binding to the D_1 protein in the electron transport chain within the chloroplast. These herbicides inhibit the flow of electrons through PS II, resulting in the formation of reactive oxygen species, which lead to lipid peroxidation and, ultimately, plant death. Two key PS II inhibitors include atrazine and metribuzin. Atrazine has a symmetric structure and belongs to the triazine chemical family, while metribuzin is asymmetric and belongs to the triazinone chemical family. In *Amaranthus* spp. resistance to group 5 herbicides is due enhanced herbicide metabolism via GSTs, which compromises atrazine's efficacy while metribuzin still remains effective. Recent research has suggested that metribuzin can provide good-to-excellent residual control of multiple herbicide resistant Palmer amaranth and waterhemp (Meyer et al. 2015). Metribuzin is labelled for PRE applications in soybean and is commonly applied as a pre-mix with herbicides in Group 2, 14

and 15. However, these premixes highlight the non-metribuzin part in the product. Whether used alone or as pre-mix metribuzin application rates are too low to achieve long-term residual control of Palmer amaranth and waterhemp. Despite the known efficacy of metribuzin, growers are reluctant to apply metribuzin due to the associated concerns of soybean injury. Metribuzin, like other soil applied herbicides, require adequate soil moisture for activation and being a cationic herbicide, its efficacy is also affected by other soil characteristics like texture, pH, and organic matter (Coble and Schrader 1973). However, a prolonged period of wet conditions after metribuzin application could lead to soybean injury symptoms such as interveinal chlorosis followed by necrosis which is more evident on unifoliate and first trifoliate leaves (Moomaw and Martin 1978; Osborne et al. 1995). Coarse textured soil with low organic matter content and high pH (>7) have been reported to pose a greater risk of soybean injury from metribuzin (Bollich et al 1985).

1.7 Background and Objective of this Thesis

With numerous cases of herbicide resistance across weed species there is a need to understand the basis of evolution of resistance mechanisms. TSR is relatively much better studied but there is still limited understanding of metabolism based NTSR. Palmer amaranth is an important dicot weed for such studies as it has evolved metabolic resistance to multiple herbicides across the US and can serve as a model to investigate NTSR. This knowledge will help us not only to understand the cause and spread of resistance at the molecular level but also to strategically develop future weed management practices. In 2020, a Palmer amaranth population (KCTR) from Kansas was reported resistance to six herbicide SOAs including ALS-, PS II-, EPSPS-, PPO, HPPD- inhibitors and SAH (Shyam et al. 2021). Except for EPSPS- inhibitors, resistance to other five SAO was found to be caused by enhanced metabolism of herbicide possibly mediated by

P450s for ALS- PPO, HPPD- inhibitors, SAH and GSTs for PS II- inhibitor (Shyam et al. 2021, 2022, Borgato et al. 2024, Aarthy et al 2022, Singh et al. 2023). Both CYP and GST are huge family of genes involved in biotic and abiotic stress management in plants, so it's important to pin down the specific genes/subfamily responsible for rapid metabolism of herbicides. Considering the extend of herbicide resistance and complexity of metabolism based NTSR, the Chapter 2 of this thesis is focused on identification and characterization of metabolic genes imparting multiple herbicide resistance in KCTR Palmer amaranth. POST herbicide resistance problems are likely to be managed by PRE herbicides, specifically using metribuzin due to its efficacy to control multiple herbicide resistant Palmer amaranth and waterhemp. Early-season crop injury and its translation to crop loss is always a concern for growers. Although some information about differential tolerance of soybean varieties to metribuzin is available but information on soybean response and yield impacts in response to various metribuzin rates under varied soil and environmental conditions is limited. Chapter 3 of the thesis evaluated the effectiveness of metribuzin as a PRE option to control multiple herbicide resistant Palmer amaranth and waterhemp population under field conditions while determining soybean response.

The dissertation was divided into the following three chapters (the hypotheses, specific objectives of each chapter are discussed separately):

Chapter 2: Deciphering Metabolic Resistance to Multiple Herbicides in Palmer amaranth (*Amaranthus palmeri* S. Watson) via Transcriptome Analysis

Chapter 3: Optimizing metribuzin rates for *Amaranthus* weed control in soybean

Chapter 4: Summary and conclusions

Chapter 2 - Deciphering Metabolic Resistance to Multiple Herbicides in Palmer amaranth (*Amaranthus palmeri* S. Watson) via Transcriptome Analysis

Abstract

Multiple herbicide resistance in Palmer amaranth (*Amaranthus palmeri* S. Watson) poses a serious threat to US crop production. A Palmer amaranth population (KCTR) from Kansas was found resistant to herbicides across six sites of action, including ALS-, PS II-, EPSPS-, PPO-, HPPD-inhibitors and synthetic auxins. Moreover, a predominance of metabolic resistance, possibly mediated by P450s or GSTs enzyme activity was reported in this population. This study aims to identify the specific genes involved in multiple herbicide metabolism in this Palmer amaranth population via transcriptome analysis. Vegetative clones were developed from three biological replicates of both resistant (KCTR/KCTR-G2) and susceptible (KSS) Palmer amaranth populations. Ten to 12 cm tall clones from each biological replicate were treated with labelled doses of chlorsulfuron, 2,4-D, atrazine, lactofen and mesotrione. Leaf samples were collected for RNA isolation at 6 hours after treatment with respective herbicides along with non-treated plants. Upon RNA sequencing, paired end reads generated were mapped to the Palmer amaranth transcriptome using HISAT2. Differential gene expression analysis revealed 414, 129, 529, 688 and 152 genes expressed differentially in resistant plants following chlorsulfuron, 2,4-D, atrazine, lactofen and mesotrione treatments, respectively. Isoforms of *CYP72A219* or *CYP704B1* and *GST C-terminal* were alternatively overexpressed across treatments. Transcripts for *CYP72A219* and *CYP704B1* show overexpression of 3.4- to 6.6-fold and 5.9- to 12.4-fold in resistant plants as validated by qRT-PCR. Functional validation of *CYP72A219* in homologous

system is ongoing. Identification of genes involved in multiple herbicide metabolism in Palmer amaranth is crucial to predict the evolutionary course of herbicide resistance in weed species.

2.1 Introduction

Palmer amaranth (*Amaranthus palmeri* S. Watson) is one of the most challenging competitors of crops in US agriculture. Palmer amaranth infestation can cause yield loss of up to 91% in major crops including corn (*Zea mays* L.), soybean (*Glycine max* L. Merr) and cotton (*Gossypium* spp. L.) (Klingaman and Oliver 1994, MacRae et al. 2013, Massinga et al. 2001, Bensch et al. 2003). At present, Palmer amaranth has been reported to evolve resistance to ten herbicide SOAs groups (Heap 2024) including: enolpyruvylshikimate-3-phosphate synthase (EPSPS)-, microtubule-, photosystem II (PS II)-, acetolactate synthase (ALS)-, protoporphyrinogen oxidase (PPO)-, 4-hydroxyphenylpyruvate dioxygenase (HPPD)-, very long chain fatty acids (VLCFA)- and glutamine synthetase (GS)- inhibiting as well as synthetic auxin herbicides (SAH; Heap 2024), leaving growers with fewer chemical weed control options.

Herbicide resistance is an evolutionary response to the selection pressure imposed by continuous use of herbicides with similar modes of action (Neve et al. 2009). Rapid evolution of multiple- and cross- resistance to herbicides in weeds over the past two decades is a serious threat to growers all around the globe (Heap 2024). Herbicide resistance can be broadly classified as target-site resistance (TSR) and non-target-site resistance (NTSR). TSR evolves due to any mutational changes in herbicide target or target site amplification or overexpression. These conformational changes in target site disturb the ratio of herbicide binding to the target site (Beckie and Tardif 2012, Jugulam and Shyam 2019). NTSR deals with mechanism(s) that reduce the amount of herbicides reaching the target site, either due to reduced absorption, altered translocation, enhanced metabolism or rapid sequestration (Yu and Powles 2014).

NTSR mechanism being complex, requires in depth understanding. Current efforts from researchers have established 4 gene families being actively involved in NTSR: cytochrome P450 monooxygenases (P450s), glutathione-*S*-transferases (GSTs), glycosyltransferases (GTs), and ATP-binding cassette (ABC) transporters (Yuan et al. 2007). P450s are known to be involved in Phase I xenobiotic metabolism, including metabolism of several herbicides (Siminszky 2006, Preston 2004, Yun et al. 2005). GSTs are another huge family of enzymes which are involved in Phase II metabolism by conjugation of herbicides and/or metabolites. Following GST action, metabolites are taken over by ATP-binding cassette (ABC) transporters, which are majorly involved in transporting conjugated metabolites to extra-cellular spaces (O'Keefe et al. 2002, Klein et al. 2006, Chen et al. 2019).

Palmer amaranth records cases of both TSR and NTSR mechanisms. TSR is relatively well studied and has been reported to confer resistance against ALS-, EPSPS-, PPO-, and HPPD-inhibitor herbicides (Chaudhari et al. 2020, Küpper et al. 2017, Copeland et al. 2018, Salas et al. 2016, Gaines et al. 2010, Nakka et al. 2017b , Nakka et al. 2017c, Varanasi et al. 2018b). NTSR mechanisms in Palmer amaranth include reduced absorption and translocation of EPSPS-inhibitors (Dominguez-Valenzuela et al. 2017, Nandula et al. 2012), and enhanced metabolism of ALS-, PS II-, PPO-, VLCFA-, HPPD-, GS-, microtubule- inhibitors and SAH. (Nakka et al. 2017a, Nakka et al. 2017b, Chahal et al. 2019, Varanasi et al. 2018a, Küpper et al. 2018, Nakka et al. 2017b, Rangani et al. 2021, Rigon et al. 2023, Shyam et al. 2021, Singh et al. 2023, González et al. 2021). Additionally, some of these studies suggested the indirect involvement of P450 and GST family genes, using enzyme inhibitors like malathion and NBD-Cl. Overexpression of specific CYP and GST have been previously associated with herbicide metabolism in Palmer amaranth including *CYP81E8* for HPPD- inhibitor and SAH resistance;

ApGSTU19, *ApGSTF8*, *ApGSTF2*, and *ApGSTF2like* for VLCFA- inhibitor resistance; and *CYP72A219* for GS- and HPPD- inhibitor resistance (Aarthy et al. 2022, Rangani et al. 2022, Salas et al. 2018, Giacomini et al. 2020). Only one study from Rigon et al. 2024, provides functional validation for *CYP72A219* gene for HPPD- inhibitor metabolism. Additionally, studies have reported co-existence of TSR and NTSR within the same population of Palmer amaranth (Chaudhari et al. 2020, Nakka et al. 2017b, Nakka et al. 2017c).

In 2021, a Palmer amaranth population (KCTR) from Kansas was reported resistant to six SOAs (Shyam et al. 2021) including: ALS-, EPSPS-, PSII-, PPO-, HPPD- inhibitors and SAH. KCTR Palmer amaranth rapidly metabolized 2,4-D, fomesafen, and tembotrione as compared to the susceptible plants (Shyam et al. 2022a, Borgato et al. 2024, Aarthy et al. 2022). CYP and GST enzyme inhibitor studies indicated a possible involvement of P450 or GST enzymes (Shyam et al. 2021) for all five herbicides tested except for glyphosate. Considering the limited understanding of the underlying molecular mechanism conferring metabolic resistance in Palmer amaranth, this study was conducted with the hypothesis that P450 and/or GST enzymes are involved in the metabolism of herbicides in Palmer amaranth. The aim of this study was to 1) conduct differential gene expression analysis and narrow down the candidate metabolic gene imparting resistance to multiple herbicides in Palmer amaranth and 2) confirm the expression level of candidate genes in non-treated samples.

2.2 Materials and Methods

2.2.1 Plant Material and Herbicide Treatment

Pseudo F_2 seeds were developed from the early screening survivors of the field collected, multiple herbicide-resistant population of Palmer amaranth (KCTR) from Kansas as described by Shyam et al. (2021). Additionally, KCTR-G2 seeds developed by Borgato et al. (2024), found

more homogeneous for lactofen resistance, were also used in the experiment as the survival percentage of KCTR to lactofen was low. The experiments were conducted in 3 batches. KSS was used as the susceptible population along with KCTR for batches 1 and 2, while KCTR-G2 for batch 3 as resistant population. Plants were raised from seeds in flat trays and seedlings at were transplanted cotyledon stage to individual pots (6 by 6 by 6.5 cm) containing commercial media (Pro-Mix® premium potting mix, Premier Tech Home and Garden, Mississauga, ON, Canada). Experiments were conducted under growth chamber conditions at 32/22 C day/night (d/n) temperatures, 60 ± 10% relative humidity, and 16/8 d/n photoperiod, provided by fluorescent bulbs delivering 500 μmol m^{-2} s^{-1} illumination at plant canopy level. Plants were watered as required. Ten healthy plants from each, resistant (KCTR, KCTR-G2) and susceptible (KSS) population were selected and numbered to produce vegetative clones from each biological replicate. At 12 to15 cm height, the top of the plant was clipped off to induce growth of side shoots. A week later the side shots were clipped off, dipped in rooting hormone (Fast Root Rooting Hormone, Miracle-Gro) and were transplanted separately for each plant to the flat trays containing commercial potting mix. Among the 10 plants selected, cuttings from KCTR 5, 7, 8 and KSS 5, 6, 7 produced healthy root system in two weeks, and were transplanted to individual pots as mentioned above and watered as needed. Two weeks later, single 10 to 12 cm tall clonal cuttings from each biological replicate of resistant and susceptible populations were treated with 2,4-D or mesotrione at field recommended rates (Table 2.1) along with a non-treated (NT) control and was labelled as batch 1. Due to the early prostate growth, getting multiple healthy clones at the same stage was difficult, so following the first batch of treatment, the NT clones of each biological replicate were further used to produce more clonal cuttings as mentioned above. Next set of clones from each biological replicate were treated with atrazine or

chlorsulfuron at field recommended rates (Table 1) along with a NT control and labelled as batch 2. Additionally, for the third batch, KCTR-G2 was used in place of KCTR, along with KSS. The seeds were germinated to prepare clonal cuttings as mentioned above. Plant number, KCTR-G2 1, 4, 8 and KSS 3, 5, 7 produced healthy root system and were transplanted to individual pots as mentioned earlier. Two weeks later, single 10 to 12 cm tall clonal cuttings from each, resistant and susceptible, biological replicate were treated with lactofen at field recommended rates (Table 2.1) along with a NT control. Detailed experimental design is discussed in Figure 2.1. Plant health and herbicide symptoms were evaluated 2 weeks after treatment (WAT).

Table 2.1 Herbicides used to treat KCTR, KCTR-G2 and KSS Palmer amaranth vegetative clones.

HARC group	Site of Action	Herbicide	Dose[a] (g ha^{-1})	Product	Adjuvant (v/v)[b]
2	ALS-inhibitor	Chlorsulfuron	18	Glean®	NIS 0.25%
4	SAH	2,4-D	560	2,4-D Amine®	-
5	PSII- inhibitors	Atrazine	2240	Aatrex 4L®	COC 1%
14	PPO-inhibitors	Lactofen	175	Cobra®	COC 1%
27	HPPD-inhibitors	Mesotrione	105	Callisto®	COC 1%

[a] Labelled dose to control Palmer amaranth.

[b] Abbreviations: NIS, non-ionic surfactant; COC, crop oil concentrate.

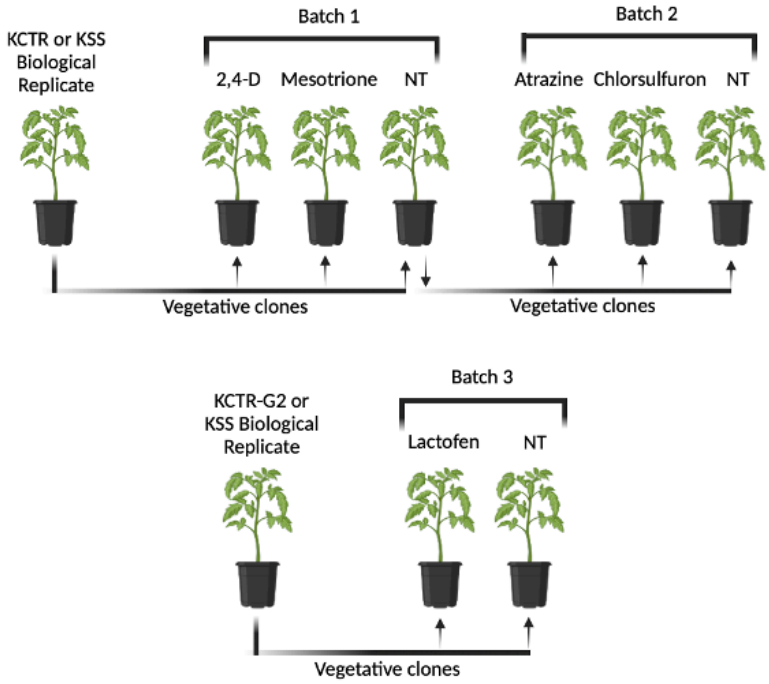

Figure 2.1 Experimental design for herbicide treatment of vegetative clones from KCTR, KCTR-G2 and KSS Palmer amaranth biological replicates.

2.2.2 RNA Isolation and Sequencing

The third or fourth fully expanded leaf was collected separately for each biological replicate 6 hours after treatment (HAT) with respective herbicides and from the NT plants. Leaf samples were flash frozen in liquid nitrogen and stored at -80°C until further processing. Total RNA was extracted using GeneJET Plant RNA Purification Mini Kit® (Thermo Fischer Scientific) following manufacturer's instructions. RNA quality and quantity was accessed using spectrophotometer (NanoDrop 1000, Thermo Scientific) and 1% agarose gel electrophoresis. High quality RNA samples were sent to Azenta life science® for paired end (150 bp) Illumina sequencing.

2.2.3 Bioinformatics and Statistical Analyses

Individual FASTQ files obtained from the RNA sequencing were uploaded to high-performance computing cluster at Kansas State University until further analyses. Quality of the raw RNA-Seq data was assessed and the adaptor sequences were trimmed using fastp with default parameters (Chen et al. 2018). Trimmed reads so obtained were aligned to the Palmer amaranth transcriptome (37Mb, 26,255 genes) using HISAT2 v 2.1.0 (Kim et al. 2019). SAM files so obtained were indexed and saved to BAM format using SAMtools v1.16.1 (Li et al. 2009). FastQC v0.11.7 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to get the quality scores of the filtered and aligned files, along with MultiQC (Ewels et al. 2016) to summarize all the quality parameters. The number of mapped reads per gene were calculated from the aligned BAM files using SAMtools. Counts matrix so generated was then subject to a pairwise differential gene expression (DGE) analysis using DEseq2 (Love et al. 2014). package in R-studio. DGE was conducted by contrasting KCTR / KCTR-G2 (R) against KSS (S) sample group (R vs S) following treatment with herbicides listed in Table 2.1 and without any treatment (NT), separately for each batch. Each comparison was labelled by the treatment name followed by the populations being compared. Custom scripts for all the bioinformatics analysis can be accessed on GitHub page (mohitmahey/Amaranthus_palmer_rna-seq). Candidate genes were selected based on the following criteria: (i) statistically significant differential expression between R and S genotypes, with an adjusted p-value < 0.05; (ii) log fold change ≥ 2; (iii) functional annotations associated to herbicide metabolism pathways; and (iv) upregulation in R genotypes compared to S genotypes.

2.2.4 cDNA Library and qRT-PCR Validation

Differential expression of candidate genes was confirmed via qRT-PCR using the same samples used for RNA sequencing. Total of 1 µg RNA was treated with DNase I (Thermo Scientific, Waltham, MA, USA) and the DNA free RNA was used for cDNA synthesis using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific).The cDNA libraries were constructed for all 48 samples and stored at -80°C. Two P450s annotated as *CYP72A219* (Gene ID: AmaPaChr16Ag245530) and *CYP704B1* (Gene ID: AmaPaChr10Ag173550) were selected as candidate genes for validation. The qRT-PCR primers were designed using PrimerQuest™ Tool and listed in Table 2.2. Quantitative RT-PCR was performed using StepOnePlus™ Real-Time PCR system (Applied BiosystemsTM, Waltham, MA, USA). The qRT-PCR reaction mixture consisted of 10 µL of PowerUp SYBR Green Master Mix (Applied Biosystems), 1 µL each of forward and reverse primers (10 mM) and 3 µl of 1:8 diluted cDNA. PCR was cycled as 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15s and 60°C for 1 min as per the manufacturer's instruction of the master mix (Applied Biosystems). The qRT-PCR reaction was conducted in 96-well plates, consisting of three technical replicates for each biological replicate of treated and control samples. Gene expression was calculated using the 2–ΔΔCT method for individual samples relative to the control samples from the same batch (Livak and Schmittgen 2001). The housekeeping gene, β- tubulin served as an internal control (Godar et al. 2015) with standard deviation of < 0.5, indicating stability across the samples. The experiments were repeated to ensure consistency and the resulting expression levels were subjected to a two-sided t-test in R-Studio.

Table 2.2 Primers used for qRT-PCR amplification of *CYP72A219* and *CYP704B1* gene in Palmer amaranth.

Gene ID	Annotation	Primer	Sequence (5`-3`)	Amplicon size (bp)
AmaPaChr16Ag245530	*CYP72A219*	Forward	GTTTATATCCCTGGTTGGAGGT	134
		Reverse	CCTTTGCCCTCTCTTCTCTATT	
AmaPaChr10Ag173550	*CYP704B1*	Forward	CAGGAGTTGTTGATGAGGATGA	95
		Reverse	GAGCAAAGGAGTTCTCAGGTAG	

2.2.5 Screening of F_1 Palmer amaranth Plants for Multiple Herbicides

Upon harvesting tissues for RNA seq experiment, the NT biological replicates of both resistant and susceptible populations were transferred to greenhouse and were allowed to cross pollinate under pollen exclusion tents. Seeds were produced for each batch separately. Later, seeds from batch 1 and 2 were pooled together and collectively labelled as batch 1+2 while those from batch 3 were kept separate. The seeds of susceptible parent (KSS), resistant parent (KCTR or KCTR-G2) and F_1 population from batch 1+2 and 3 were raised as described earlier. At 10 to 12 cm height, 20 plants from each resistant and susceptible parent population were treated separately with herbicides listed in Table 2.1, along with 40 plants from F_1 population. Batch 1 seedlings were used for treatment with 2,4-D, mesotrione, atrazine or chlorsulfuron while batch 3 seedlings were used for treatment with lactofen. The plants were evaluated as dead and alive at 1, 2 and 3 WAT. This data was later converted to survival percentage separately for each herbicide.

2.3 Results

2.3.1 Response of Palmer amaranth to Herbicide Treatment

Palmer amaranth vegetative clones displayed normal growth, and a well-developed root system similar to the plants grown from seeds. All herbicide treatments resulted in significant visual injury symptoms on both susceptible and resistant plants as evaluated at 2 WAT with respective herbicides (Figure 2.2). However, at 2 WAT, upon 2,4-D, atrazine and lactofen

treatment, the susceptible plants were completely dead, while resistant plants showed some initial injury but eventually recovered. For chlorsulfuron treatment, the susceptible plants were dead along with KCTR 5, while KCTR 7 and 8 showed stunted growth as compared to NT. For mesotrione treatment the susceptible plants showed significant injury of growing tissues at 2 WAT with no new growth later while the resistant plants survived.

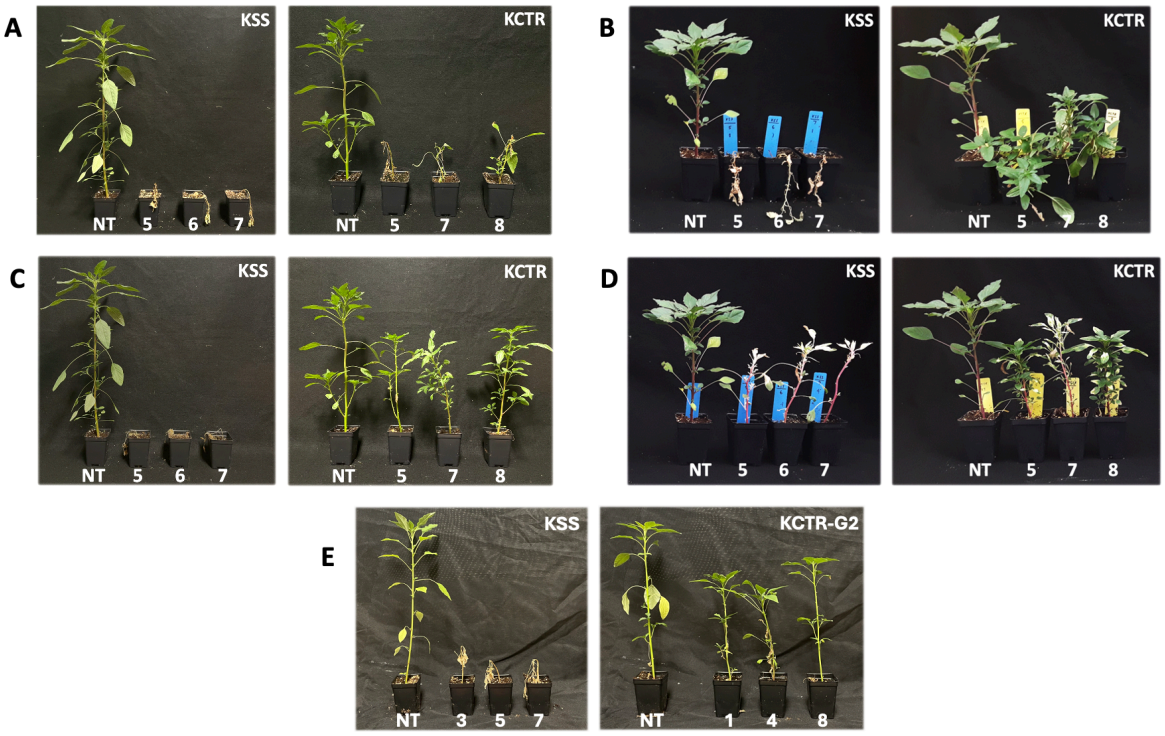

Figure 2.2 Response of KSS, KCTR and KCTR-G2 biological replicates to A) chlorsulfuron, B) 2,4-D, C) atrazine, D) mesotrione, and E) lactofen application 2 weeks after treatment. Numbers represent the biological replicate from which the vegetative clones were prepared. NT: Non-treated.

2.3.2 RNA Sequencing and Differentially Expressed Genes

An average of 77-93 million raw reads were generated for individual samples across batches and low-quality reads were trimmed off. Overall mapping rate to the transcriptome ranged from 66% to 81% across all samples. DGE analysis was conducted separately for all three batches to remove noise in biological signals arising from different timings the experiments were

conducted. No more than 3% of the transcriptome was differentially expressed for individual comparison indicating the efficiency of the experiments. According to the relative references to herbicide metabolism (Rigon et al. 2023, Jugulam and Shyam 2019), and the above listed criterion, the candidate genes were selected for each treatment comparison of resistant and susceptible plants.

In batch one comparing 2,4-D and mesotrione treatment along with NT, a total of 129,152 and 130 genes were differentially expressed in resistant plants as compared to susceptible respectively (Table 2.3). The NT comparison revealed overexpression of 2 P450s and 3 GSTs genes (Table 2.4). Overexpression of *CYP704B1* and C-terminal *GST* was constitutive following 2,4-D treatment with an additional overexpression of two ABC transporters from C and G family and a *UGT79B7* (Table 2.4). While 2 P450s (*CYP72A219 and CYP711A1*) and 2 GSTs (*GST parC and GST C-terminal*) were overexpressed following mesotrione treatment along with two ABC transporters from C and G family, similar to 2,4-D treatment (Table 2.4).

Table 2.3 Number of differentially expressed (up-regulated and down-regulated) genes following differential gene expression analysis in resistant and susceptible Palmer amaranth following herbicide application. NT: non-treated; R: resistant; S: susceptible.

Batch	Treatment groups	Differentially expressed genes	Upregulated	Downregulated
1	NT- RvsS	130	86	44
	2,4-D- RvsS	129	71	58
	Mesotrione- RvsS	152	76	76
2	NT- RvsS	269	169	100
	Atrazine- RvsS	529	229	300
	Chlorsulfuron- RvsS	414	184	230
3	NT- RvsS	109	50	59
	Lactofen- RvsS	688	253	435

Table 2.4 List of candidate genes overexpressed in resistant (R) plants as compared to susceptible (S) without any treatment and with treatment with 2,4-D and mesotrione. NT: non-treated ; R: resistant; S: susceptible.

Comparison	Gene ID	Annotation	Log2 FC
NT- RvsS	AmaPaChr10Ag173550	*CYP704B1like*	3.5
	AmaPaChr16Ag245530	*CYP72A219*	3.5
	AmaPaChr03Ag054610	*GST C-terminal*	2.6
	AmaPaChr09Ag156820	*GST parC*	3.0
	AmaPaChr15Ag232780	*GSTU7*	2.0
2,4-D- RvsS	AmaPaChr10Ag175070	*ABC C family*	4.9
	AmaPaChr05Ag086240	*ABC G28*	2.4
	AmaPaChr10Ag173550	*CYP704B1like*	4.5
	AmaPaChr03Ag054610	*GST C-terminal*	2.5
	AmaPaChr10Ag176690	*UGT79B7 like*	2.2
Mesotrione- RvsS	AmaPaChr10Ag175070	*ABC C15*	4.3
	AmaPaChr05Ag086240	*ABC G28*	2.5
	AmaPaChr11Ag184050	*CYP711A1*	2.2
	AmaPaChr16Ag245530	*CYP72A219*	3.4
	AmaPaChr03Ag054610	*GST C-terminal*	2.3
	AmaPaChr09Ag156830	*GST parC*	2.1

In batch two comparing NT along with atrazine and chlorsulfuron treatment, a total of 269, 529 and 414 genes were differentially expressed in resistant plants as compared to susceptible, respectively (Table 2.3). The NT comparison indicated overexpression 5 P450s, 6 GSTs, 2 UGTs and 1 ABC transporter (Table 2.5). Higher number of genes overexpressed from candidate gene family can be attributed to the involvement of these genes in other plant biotic and abiotic stresses which might have incurred due to vegetative cloning of plants. Notably, NT comparisons from both the batches had 2 P450s (*CYP72A219, CYP704B1*) and 3 GSTs (*GST parC, GST C-terminal and GSTU7*) in common. This outcome aligns with expectations, since these plant samples are vegetative clones from same mother plant. Overexpression of 2 P450s (*CYP704B1, CYP82D47*), *C-terminal GST* and ABC transporter from G family were constitutive following atrazine treatment with an additional overexpression of *CYP705*, ABC transporter

from C family and two UGTs (Table 2.5). While P450 *CYP72A219*, was overexpressed following chlorsulfuron treatment along with two ABC transporters from G family and 2 UGTs (Table 2.5).

Table 2.5 List of candidate genes overexpressed in resistant (R) plants as compared to susceptible (S) without any treatment and with treatment with atrazine and chlorsulfuron. NT: non-treated ; R: resistant; S: susceptible.

Comparison	Gene ID	Annotation	Log2 FC
NT- RvsS	AmaPaChr05Ag086240	*ABC G28*	2.4
	AmaPaChr10Ag173550	*CYP704B1like*	3.5
	AmaPaChr16Ag245530	*CYP72A219*	3.1
	AmaPaChr09Ag155620	*CYP72A219 like*	2.7
	AmaPaChr14Ag221190	*CYP82D47 like*	2.8
	AmaPaChr17Ag255570	*CYP82G1 like*	3.5
	AmaPaChr09Ag156790	*GST*	3.1
	AmaPaChr03Ag054610	*GST* C-terminal	4.1
	AmaPaChr17Ag255900	*GST like*	5.1
	AmaPaChr09Ag156820	*GST parC*	3.3
	AmaPaChr09Ag156830	*GST parC*	2.9
	AmaPaChr15Ag232780	*GSTU7*	4.3
	AmaPaChr10Ag169600	*UGT*	2.5
	AmaPaChr15Ag236760	*UGT-C*	3.3
Atrazine- RvsS	AmaPaChr05Ag086240	*ABC G28*	2.5
	AmaPaChr10Ag173550	*CYP704B1like*	6.0
	AmaPaChr06Ag103700	*CYP705A22*	2.1
	AmaPaChr14Ag221190	*CYP82D47*	2.1
	AmaPaChr01Ag011390	*GST C-terminal*	2.5
	AmaPaChr03Ag054610	*GST C-terminal*	3.2
	AmaPaChr15Ag227100	*ABC C*	2.2
	AmaPaChr10Ag176690	*UGT79B7*	2.8
Chlorsulfuron- RvsS	AmaPaChr04Ag069790	*ABC G FAMILY*	7.1
	AmaPaChr05Ag086240	*ABC G28*	2.3
	AmaPaChr09Ag155620	*CYP72A219 like*	2.0
	AmaPaChr10Ag176690	*UGT79B7*	2.4

In batch three comparing NT along with lactofen treatment a total 109 and 688 genes were differentially expressed in resistant plants as compared to susceptible, respectively (Table 2.2). The NT comparison indicated overexpression of an ABC transporter, while no P450s or GSTs were overexpressed (Table 2.6). Interestingly, isoform of *CYP72A219* gene was found overexpressed following lactofen treatment with an additional overexpression 4 P450s, 2 UGTs and 3 ABC transporters from B, C and G family (Table 2.6).

Table 2.6 List of candidate genes overexpressed in resistant (R) plants as compared to susceptible (S) without any treatment and with treatment with lactofen. NT- non-treated; R: resistant; S: susceptible.

Comparison	Gene ID	Gene annotation	Log2 FC
NT- RvsS	AmaPaChr15Ag227100	uncharacterized *ABC*	2.8
Lactofen- RvsS	AmaPaChr15Ag227100	*ABC C*	2.3
	AmaPaChr05Ag086170	*ABC B9*	9.0
	AmaPaChr09Ag157410	*ABC B25*	2.2
	AmaPaChr09Ag155940	*ABC C3*	2.7
	AmaPaChr13Ag206220	*ABC G14*	4.2
	AmaPaChr08Ag141950	*CYP710A11like*	2.4
	AmaPaChr06Ag113330	*CYP78A9 like*	2.3
	AmaPaChr03Ag055770	*CYP81D1 like*	5.3
	AmaPaChr01Ag006470	*CYP83B1 like*	2.7
	AmaPaChr16Ag245440	*CYP72A219 like*	2.6
	AmaPaChr12Ag196590	*UGT76F1*	2.6
	AmaPaChr06Ag108730	*UGT79B30 like*	3.6

Overall, we see over expression of multiple genes from candidate families overexpressed in resistant plants as compared to susceptible following herbicide treatment. Importantly gene isoforms of *CYP72A219*, *CYP704B1* and *GST C-terminal* depicts a significant overexpression alternatively in batch 1, 2 and 3 comparisons as discussed above.

2.3.3 P450 Gene Expression

Since the P450 gene family is known to be involved in Phase I herbicide metabolism of multiple herbicides (Rigon et al. 2020), so we investigated mRNA transcript abundance of

CYP72A219, *CYP704B1* gene(s) in resistant and susceptible plants without any herbicide treatment. The qRT-PCR results show greater ($\alpha \leq 0.05$) average transcript levels of both *CYP72A219* and *CYP704B1* genes in the resistant plants as compared to the susceptible for both batch 1 and batch 2 validating the results from RNA-seq experiment. Transcripts for gene *CYP72A219* show 3.4- and 6.6-fold overexpression in resistant plants for batch 1 and 2 respectively (Figure 2.3 A). While *CYP704B1* transcripts show 12.4- and 5.9-fold overexpression in resistant plants for batch 1 and 2 respectively (Figure 2.3 B). Increased transcript level is likely to play an important role in increased expression.

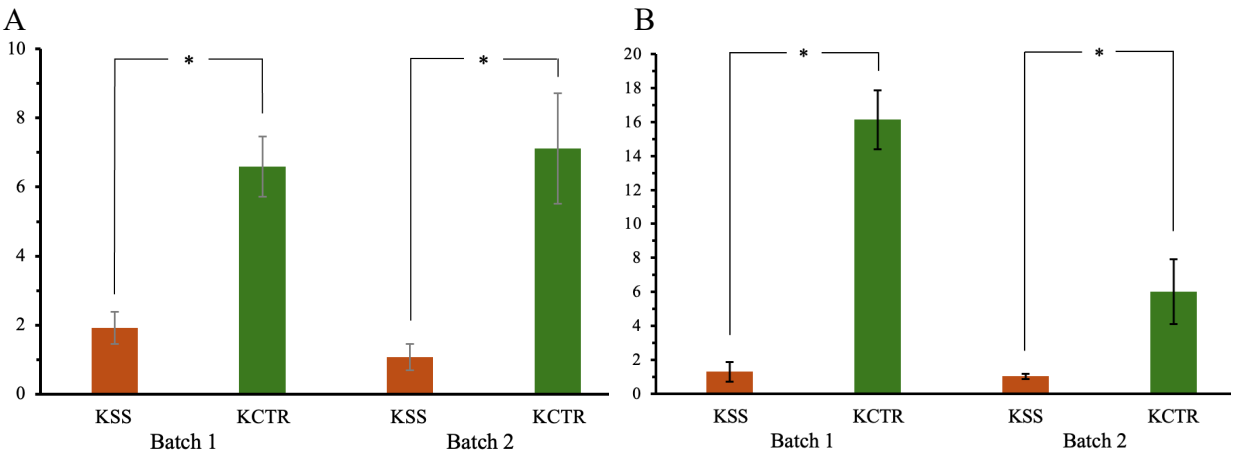

Figure 2.3 Bar graphs depicting transcript levels of A) *CYP72A219* and B) *CYP704B1* validated by qRT-PCR in non-treated KSS and KCTR biological replicates from batch 1 and batch 2 respectively without herbicide treatment. Asterisks represent significant differences among KSS and KCTR plants as identified by two-sided t-test ($\alpha = 0.05$).

2.3.4 Response of F_1 Palmer amaranth Plants to Multiple Herbicides

Survival percentage show a clear pattern of inherited herbicide resistance in the F_1 generation for multiple herbicides as evaluated 3 WAT. For 2,4-D, mesotrione, atrazine and lactofen, the F_1 plants exhibit survival rates of 75-100 % , which is much closer to the resistant parent than the susceptible parent (Table 2.7). However, the F_1 plants treated with chlorsulfuron

exhibited ~35% survival rate which is intermediate between resistant and susceptible parent (Table 2.7). Further inheritance patterns will be studied with F_2 population.

Table 2.7 Survival percentage of resistant and susceptible Palmer amaranth parent along with F_1 when treated with different herbicide at 3 weeks after treatment.

Herbicide	Population	Survival %
Chlorsulfuron	Resistant parent	80
	Susceptible parent	0
	F_1	35
2,4-D	Resistant parent	80
	Susceptible parent	0
	F_1	75
Atrazine	Resistant parent	100
	Susceptible parent	10
	F_1	90
Lactofen	Resistant parent	100
	Susceptible parent	0
	F_1	100
Mesotrione	Resistant parent	100
	Susceptible parent	0
	F_1	92.5

2.4 Discussion

2.4.1 Chlorsulfuron Resistance

Resistance to ALS- inhibitor is predominantly bestowed by target site mutation of single amino acid across weed species (Jugulam and Shyam 2019) although NTSR haven been reported in certain grass species like barnyard grass (Riar et al. 2012) and dicots like waterhemp (Shergill et al. 2018) and Palmer amaranth (Nakka et al. 2017c). However, co-existence of TSR and NTSR have been reported in some weed species (Bai et al. 2019, Chen et al. 2019) including Palmer amaranth (Nakka et al. 2017c). Studies using inhibitors of P450 enzyme activity highlighted the possible involvement of these enzymes in metabolizing chlorsulfuron in rigid ryegrass and the KCTR Palmer amaranth population (Busi et al. 2017, Shyam et al. 2021).

However, research towards understanding of molecular basis of ALS-inhibitor resistance is very limited in dicot weeds. Our current research reported over-expression of *CYP72A219 Like, UGT79B7* and two genes from ABC transporter G family, in response to chlorsulfuron application in KCTR Palmer amaranth plants. The limited number of overexpressed genes related to herbicide metabolism may be attributed to the poor response of resistant biological replicates to chlorsulfuron. These genes are in concordance to a recent study where Duhoux et al. (2015) reported possible involvement of P450s from families *CYP72A* and *CYP81B1*, GTs and one GST for pyroxsulum resistance in ryegrass (*Lolium* spp.). NTSR to ALS inhibitors was also credited to multiple P450s from *CYP71* and *CYP81* family in grass species including American sloughrass (*Beckmannia syzigachne* Steud.) and blackgrass (*Alopecurus myosuroides* Huds.) (Wang et al. 2021, Gardin et al. 2015). Overexpression of GSTs from theta and tau class have also been reported in resistant American sloughgrass following mesosulfuron-methyl application (Wang et al. 2021). However, we didn't find any GSTs overexpression in our experiment. Additionally, ABC transporters have previously been reported for compartmentalization of GST conjugates from sulfonylurea family of ALS-inhibitor herbicides (Goldberg-Cavalleri et al. 2023)

2.4.2 2,4-D Resistance

NTSR led by enhanced metabolism of 2,4-D have been reported in nine weed species so far including wild radish (*Raphanus raphanistrum*), waterhemp (*Amaranthus tuberculatus*) and Palmer amaranth (Torra et al. 2024). The metabolism of 2,4-D in weed species involves a complex network of genes and enzymes carrying out hydroxylation of 2,4-D followed by possible conjugation by GSTs or GTs (Torra et al. 2017, Torra et al. 2024). Previous studies revealed indirect involvement of P450s in Phase I metabolism in all weed species including

waterhemp and Palmer amaranth (Figueiredo et al. 2018, Shyam et al. 2022a). Our current research reported over-expression of *CYP704B1,* a *C-terminal domain GST* (possibly from phi class), *UGT79B7* and two genes from *C*- and *G*- family of ABC transporters*,* in response to 2,4-D application in resistant Palmer amaranth plants. Previously *CYP81E8* have been suspected to be involved in 2,4-D metabolism in waterhemp along with ABC transporters, including *ABCC10* (Giacomini et al. 2020). GSTs, particularly from the phi and tau class, play crucial roles in SAH detoxification and overexpressed in dicamba resistant waterhemp along UGT85A and UGT2 (Bobadilla et al. 2024). Members from ABC G family were previously reported to be involved in fluroxypyr resistance in kochia along with multiple P450s and GTs (Todd et al. 2024). As suggested by McCauley et al. (2020), the SAH resistance mechanism typically involves a rapid downregulation of photosynthetic machinery, coupled with increased expression of genes related to oxidative stress response and hormone metabolism. The coordinated upregulation of these metabolic genes, often observed in clusters suggests shared regulatory mechanisms, enabling resistant weeds to rapidly detoxify and compartmentalize 2,4-D (Giacomini et al. 2020). Moreover, Shyam et al. (2022) discussed that 2,4-D resistance trait in KCTR Palmer amaranth is controlled by multiple genes.

2.4.3 Atrazine Resistance

NTSR based resistance to atrazine in Palmer amaranth and other weed species is predominantly attributed to metabolism by GSTs (Nakka et al. 2017a, Chahal et al. 2019, Shyam et al. 2021). Information on genes involved in atrazine metabolism is very limited. Our current research reported over-expression three P450s (*CYP704B1, CYP705A2* and *CYP82D47*)*,* two *C-terminal domain GST* (possibly from phi class), *UGT79B7* and two genes from *C*- and *G*- family of ABC transporters*,* in response to chlorsulfuron application in resistant Palmer amaranth

plants. Primary function of CYP704B1 is associated with pollen development but *CYP705A* and *CYP82D*, belonging to the CYP71 clan, are more likely to be involved in herbicide metabolism (Bai et al 2022). The involvement of phi-class GSTs in atrazine resistance has been further corroborated by the identification of *AtuGSTF2* in atrazine resistant waterhemp (Evans et al. 2017).

2.4.4 Lactofen Resistance

Lactofen belongs to the PPO-inhibitor class of herbicides. PPO-inhibitor resistance in weed species is predominated by amino acid substitution or deletion in *PPX2* gene (Rangani et al. 2019, Patzoldt et al. 2006). However, NTSR due to enhanced metabolism of PPO herbicides also exists and have been reported in Palmer amaranth (Varanasi et al. 2018a, Borgato et al. 2024) and waterhemp (Obenland et al. 2019). Pre-treatment with enzyme inhibitors, malathion and NCBD-Cl, indicated possible involvement of both P450s and GSTs in fomesafen metabolism in Palmer amaranth (Varanasi et al. 2018a, Borgato et al. 2024). While target-site resistance is well-documented, metabolic resistance mechanisms have not been fully elucidated for PPO inhibitors. Our current research reported over-expression of multiple P450s including, *two UGTs (UGT79B30* and *UGT76F1*) and multiple genes from *B*-, *C*- and *G*- family of ABC transporters, in response to lactofen application in resistant Palmer amaranth plants. Genes from *CYP710A and CYP78A* family are responsible for sterol biosynthesis and plant growth and senescence respectively (Zhou et al. 2023, Ghosh 2017). While *CYP83B1* acts as a key regulator of auxin biosynthesis in Arabidopsis (Bak et al. 2001). Our candidate Phase I catalyzing enzymes focuses on *CYP81*D1 and isoform of *CYP72A219,* as genes from these subfamilies have been reported for catabolism reaction, scavenging reactive oxygen species (ROS) and metabolism of herbicides

across multiple SOAs (Ghosh 2017, Wang et al. 2020, Rigon et al. 2020, Rigon et al. 2024). No specific genes have been identified or validated for PPO metabolism in weeds species.

2.4.5 Mesotrione Resistance

Mesotrione belongs to the HPPD- inhibitors. Resistance to HPPD- inhibitors is dominated by metabolism based NTSR (Jugulam and Shyam 2019), however TSR also exist in Palmer amaranth (Nakka et al. 2017b). Mesotrione metabolism in Palmer amaranth and waterhemp was reported due to rapid 4-hydroxylation followed by glycosylation of herbicide molecule (Küpper et al. 2018, Kaundun et al. 2017). Our current research reported over-expression of *CYP711A1* and *CYP72A219*, two GSTs with C-terminal domain and genes from *C*- and *G*- family of ABC transporters, in response to mesotrione application in resistant Palmer amaranth plants. In 2022, Aarthy et al. reported overexpression of *CYP81E8* in the KCTR population, however our RNA seq experiment did not reveal a significant overexpression post mesotrione application. Overexpression of *CYP72A219* has been reported in mesotrione and tembotrione resistant Palmer amaranth plants (Küpper et al. 2018, Rigon et al. 2024). Moreover, this gene was functionally validated to metabolize mesotrione in yeast cells (Rigon et al. 2024). We hypothesize that Phase II metabolism of mesotrione could possibly be mediated by associated GSTs from Phi class followed by compartmentalization by ABC transporters.

2.5 Conclusion

Understanding the underlying molecular basis of resistance, specifically metabolism based NTSR is a key aspect in predicting the course of evolution of herbicide resistance in weeds. In a remarkable display of adaptive evolution, the KCTR Palmer amaranth population has developed resistance to multiple herbicides across six SOAs, including those it has never encountered. In this study we identified specific genes of P450, GST, GT and ABC transporter

family which may be directly or indirectly linked to metabolism of herbicides across 5 SOAs in KCTR Palmer amaranth plants.

Unlike TSR, NTSR mechanisms are largely driven by gene regulation. Delye (2013) described NTSR as a dynamic process which involve majorly two types of genes: ‘protectors’, which interferes directly with herbicide action and includes P450s, GSTs, GTs, esterases, transporters, and oxidases and the ‘regulators’, which controls the expression of the ‘protectors’ and includes transcription factors, micro-RNAs, and kinases (Delye 2013). While research has focused on understanding the role of protectors, little is known about the specific regulators involved in overexpression of the gene involved in herbicide metabolism. Regulation of gene expression itself is a very complex process and involves post -transcription, -translational controls and protein expression of the gene of interest. Elucidating regulation of gene expression is crucial to understanding metabolism based NTSR to herbicides and warrants further investigation.

Chapter 3 - Optimizing Metribuzin Rates for *Amaranthus* Weed Control in Soybean

Abstract

Palmer amaranth (*Amaranthus palmeri* S. Wats) and waterhemp [*Amaranthus tuberculautus* (Moq). J. D. Sauer] pose a serious threat to major crop production systems in the US including corn, soybean, and cotton. Rapid evolution of herbicide resistance in both species warrant alternate options for weed control. Metribuzin provides good control of herbicide-resistant *Amaranthus* spp. when applied as a pre-emergence (PRE) herbicide. However, despite its proven efficacy, many growers remain either unaware of metribuzin's potential or hesitant to use it due to concerns over crop injury. Field experiments were conducted in 2022 and 2023 at 15 sites across the US to investigate residual control of waterhemp and Palmer amaranth by PRE application of metribuzin in soybean. Experimental sites either had Palmer amaranth or waterhemp as the dominant weed species with multiple herbicide-resistant populations. A total of 17 PRE treatments were evaluated, including 13 doses of metribuzin ranging from 210 to 841 g ai ha^{-1}, sulfentrazone (420 g ai ha^{-1}), and *S*-metolachlor (1790 g ai ha^{-1}), along with a non-treated control and a weed-free check. Visual weed control and soybean injury were recorded 14, 28 and 42 days after application (DAA) of PRE herbicides. Additionally, weed density, weed biomass, and soybean height were recorded 28 DAA followed by soybean yield. Data were pooled together for both weed species. Weed control was analyzed as a function of metribuzin dose, time and other soil and environmental differences among locations using a generalized additive model. Crop injury of no more than 5% was predicted even with 841 g ai ha^{-1} of metribuzin. Metribuzin at 630 and 525 g ai ha^{-1} was more effective than sulfentrazone in delaying weed emergence and reducing weed density, while 315 g ai ha^{-1} of metribuzin was

found more effective than *S*-metolachlor in both aspects. Metribuzin dose of 578 to 871 g ai ha $^{-1}$ provided more than 95, 90 and 80% weed control at 14, 28 and 42 DAA. Higher metribuzin doses of 578 to 841 g ai ha^{-1} could be safely used by the growers to effectively control herbicide-resistant Palmer amaranth and waterhemp.

3.1 Introduction

The Amaranthus genus consists of approximately 50 species with their origin in the Americas (Kigel 1994). Among these, the two dioecious species, Palmer amaranth (*Amaranthus palmeri* S. Wats) and waterhemp [*Amaranthus tuberculautus* (Moq). J. D. Sauer] are consistently ranked among the most problematic weeds in the US cropping system, especially in soybean (Van Wychen 2022). Palmer amaranth densities of 0.33 to10 plants m^{-1} of row, at 8 to12 weeks after emergence, have been reported to reduce soybean yield by 17 to 68%, respectively (Klingaman and Oliver 1994). Soybean yield losses of 43% were reported after ten weeks of waterhemp interference at densities of 89 to 362 plants m^{-2} (Hager et al. 2002). Selection of herbicide resistant biotypes of Palmer amaranth and waterhemp over the years has contributed to the problem not only by increasing the production costs in soybean but also complicating weed management decisions. Currently, Palmer amaranth populations in the US have been confirmed with resistance to herbicides from ten SOAs including: ALS-, microtubule-, PS II-, EPSPS-, PPO-, VLCFA-, HPPD- and GS- inhibitors as well as SAH (Heap 2024). Waterhemp resistance in the US is also reported to herbicides from seven different SOAs including: EPSPS-, ALS-, PS II-, PPO-, and HPPD- , VLCFA- inhibiting and SAH (Heap 2024).

Introduction of glyphosate resistant (GR) crops in late 1990s propelled weed management programs to be more focused on post-emergence herbicides (POST) in place of the combinations of pre-emergence (PRE) + POST options, thus accelerating the evolution of

herbicide resistance (Duke 2015, Givens et al. 2009, Powles 2008). Oliveira et al. (2017) reported benefits of using PRE herbicides to control annual broadleaf and herbicide-resistant weeds, including Palmer amaranth and waterhemp. PRE herbicides not only provide weed control during the first 3 to 4 weeks after crop planting, but also reduce the selection pressure on POST herbicide applications (Butts et al. 2017; Knezevic et al. 2019; Tursun et al. 2016). Additionally, effective PRE programs were found to be an important strategy to control herbicide-resistant weeds (Norsworthy et al. 2014). The current scenario of herbicide resistance in weeds has led to fewer chemical weed control options prompting growers to increase the use PRE herbicides. Between 2006 and 2017, the area of soybean fields treated with metribuzin, sulfentrazone and *S*-metolachlor has increased by 16%, 21%, and 15%, respectively (USDA-NASS 2017).

Metribuzin is an asymmetric triazinone herbicide, that inhibits the flow of electrons through photosystem II, generating reactive oxygen species which ultimately leads to plant death. Recent research demonstrates that metribuzin can provide good to excellent residual control of herbicide-resistant waterhemp and Palmer amaranth (Meyer et al. 2015; Vyn et al. 2007). Metribuzin is an important part of commercially available premixes for soybean, as it can be naturally metabolized by soybean plants. However, when metribuzin is used, the dose frequently is too low to achieve the needed duration of residual control of *Amaranthus* species. Also, for the soybean producers who adopt metribuzin as a PRE option there is always an associated concern for early-season soybean injury possibly translating to lower yield. Typical metribuzin injury involves interveinal chlorosis leading to necrosis, evident on unifoliate and first trifoliate leaves, with greater risk of injury in high pH (>7) and/or low organic matter soils (Hartzler 2017).

However, like most soil applied herbicides the effective dose of metribuzin is influenced by the interaction between the herbicide and other soil and environmental parameters that affects herbicide activation, retention, adsorption and transportation. Soybean tolerance to metribuzin was greatly influenced by metribuzin dose, soil organic matter, and amount of rainfall following herbicide treatment as suggested by Coble and Schrader (1973). However, many soybean producers might be either unaware of metribuzin's efficacy or hesitant to apply it because of soybean injury concerns. We hypothesize that metribuzin can provide excellent control of Palmer amaranth and waterhemp with no adverse effects on soybean growth and development. The objectives of this study were to 1) determine the optimal metribuzin dose for residual control of herbicide-resistant Palmer amaranth and waterhemp under varied soil and environmental conditions; 2) evaluate early-season growth and development of soybean following PRE application of metribuzin at multiple doses and 3) determine whether potential early-season herbicide-induced injury could affect soybean yield.

3.2 Materials and Methods

Field experiments were conducted during 2021 and 2022 at 15 sites in the US: Arkansas, Illinois, Indiana, Kansas, Kentucky, Louisiana, Michigan, Missouri, Nebraska, North Dakota, Ohio, Tennessee and Wisconsin. Additional information on management practices for each site is summarized in Table 3.1. Soil characteristics and rainfall data for the duration of the experiment are summarized in Table 3.2. Site-years offer variability in soil texture, organic matter (1.5 to 6.6 %), pH (5.5 to 8.0), interval between application and first precipitation (0 to 18 days DAA) and cumulative precipitation 42 DAA (46 to 343 mm). Naturally occurring infestations of herbicide resistant Palmer amaranth and waterhemp were utilized at each site (Table 3.1)

Table 3.1 Management practices for 32 site-years included in this study.

Site year	Location	Soybean variety	Seeding rate (seeds/ha)	Row spacing (m)	Planting date	Herbicide application	Metribuzin product	Plot size (m)	Weed[a]	Resistance profile (HRAC group)
Arkansas 2023	Fayetteville, AR	P48A14E	375000	0.91	12-May	13-May	Tricor 4F	3.7x7.6	PA	2,9
Arkansas 2022	Fayetteville, AR	AG48XF0	375000	0.91	12-May	13-May	Tricor 4F	3.7x7.6	PA	2,9
Illinois 2022	Sidney, IL	AG33XF2	350000	0.76	4-Jun	4-Jun	Metricor DF	3x7.6	WH	2,4,5,9,14,15,27
Illinois 2023	Sidney, IL	AG33XF2	350000	0.76	18-May	18-May	Metricor DF	3x7.6	WH	2,4,5,9,14,15,27
Indiana 2022	Francesville, IN	AG29XF1	367500	0.76	11-May	11-May	Metricor DF	2x9.1	WH	2,4,5,9,14,27
Indiana 2023	Francesville, IN	AG30XF2	350000	0.76	10-May	11-May	Metricor DF	2x9.1	WH	2,4,5,9,14,27
Iowa 2023	-	-	-	-	-	-	-	-	-	-
Kansas 2022	Manhattan, KS	GH3982X	300000	0.76	23-May	23-May	Metricor DF	3x9.1	PA	2,9,14
Kansas 2023	Manhattan, KS	GH3982X	300000	0.76	31-May	31-May	Metricor DF	3x9.1	PA	2,9,14
Kentucky 2022	Princeton, KY	Xitavo	350000	0.76	16-May	16-May	Metricor DF	3x9.1	PA	2,9,(14)
Kentucky 2023	Princeton, KY	XO4522E	350000	0.76	25-Apr	25-Apr	Metricor DF	3x9.1	PA	2,9,(14)
Louisiana 2022	Alexandria, LA	P49T62E	312500	0.97	9-May	10-May	Glory 4L	3.9x9.1	PA	9
Louisiana 2023	-	-	-	-	-	-	-	-	-	-
Michigan 2022	Lansing, MI	P24T35E	375000	0.76	24-May	24-May	Metribuzin 75WG	3x7.6	WH	2,9
Michigan 2023	Lansing, MI	AG26XF3	375000	0.76	23-May	23-May	Metribuzin 75WG	3x7.6	WH	2,9
Mississippi 2022	-	AG48XF2	325000	1	28-Apr	-	Tricor 4F	4x7.6	PA	-
Mississippi 2023	-	AG48XF2	325000	1	17-Apr	-	Tricor 4F	4x7.6	PA	-
Missouri 2022	Columbia, MO	MorSoy3861XE	350000	0.76	6-Jul	6-Jul	Metricor DF	2x15.2	WH	9,14

Missouri 2023	Columbia, MO	XO3752E	375000	0.76	3-May	3-May	Metricor DF	2x15.2	WH	9,14
Nebraska 2022	Clay Centre, NE	AG 27XF1	335000	0.76	18-May	18-May	Metricor DF	3x9.1	PA	2,9
Nebraska 2023	Clay Centre, NE	NK31-J9XF	337500	0.76	25-Apr	25-Apr	Metricor DF	3x9.1	PA	2,9
North Dakota 2022	Palmerville, ND	AG 09XF0	390000	0.76	6-Jun	7-Jun	Tricor 4F	2x9.1	PA	2,4,5,9,27
North Dakota 2022	Fargo, ND	AG 09XF0	390000	0.76	24-May	24-May	Tricor 4F	2x9.1	WH	2,9
North Dakota 2023	Fargo, ND	AG 07XF2	390000	0.76	21-May	22-May	Tricor 4F	2x9.1	WH	2,9
Ohio 2022	Charleston, OH	P35T15E	375000	0.76	24-May	24-May	Metricor DF	3x9.1	WH	-
Ohio 2023	Charleston, OH	P35T15E	387500	0.76	11-May	11-May	Metricor DF	3x9.1	WH	-
S Illinois 2022	Carbondale, IL	AG38XF1	350000	0.76	31-May	1-Jun	Sencor 75DF	2x10.7	WH	-
S Illinois 2023	Carbondale, IL	AG38XF1	350000	0.76	19-May	20-May	Sencor 75DF	2x10.7	WH	-
Tennessee 2022	Holston, TN	AG45XF0	350000	0.76	1-Jun	2-Jun	Metricor DF	3x9.1	PA	2,9
Tennessee 2023	Holston, TN	-	350000	0.76	26-May	26-May	Sencor 75DF	2x9.1	PA	2,9
Wisconsin 2022	Brooklyn, WI	NK22-C43E3	350000	0.76	23-May	23-May	Metribuzin 75DF	3x9.1	WH	2,4,5,9,14
Wisconsin 2023	Brooklyn, WI	NK22-C43E3	350000	0.76	17-May	17-May	Metribuzin 75DF	3x7.6	WH	2,4,5,9,14

[a] Abbreviations: PA, Palmer amaranth; WH, waterhemp.

Table 3.2 Soil characteristics and precipitation records.

	Soil Characteristics								Precipitation records				
Site year	Organic matter (%)	pH	Texture	Sand (%)	Silt (%)	Clay (%)	Moisture at application	Temperature at application (C)	Amount of first PPT (mm)[a]	Cumulative PPT 42 DAA (mm)[a]	Interval between application and first PPT (DAA)[ab]	Days between application and cumulative 12.7mm PPT (DAA)[ab]	Irrigation
Arkansas2023	1.55	6.3	silt loam	19	73	7.5	adequate	20	2	146	0	1	Rainfed
Arkansas 2022	2	6.2	silt loam	18	71	10	adequate	24	1	133	2	4	Rainfed
Illinois 2023	5	5.5	silt loam	28	51	21	dry	24	2	48	18	23	Rainfed
Indiana 2022	2.4	7.5	sandy loam	75	14	11	normal	22	3	149	4	10	Rainfed
Indiana 2023	3.2	6.7	sandy loam	74	16	10	normal	21	17	170	2	2	Rainfed
Kansas 2022	3	6.1	silt loam	10	76	14	good	21	18	343	1	1	Rainfed
Kansas 2023	2.6	6.2	silt loam	10	76	14	good	22	26	144	1	1	Rainfed
Kentucky 2022	2.5	6.1	silt loam	10	74	14.9	dry	18	18	102	5	5	Rainfed
Kentucky 2023	2.5	6.1	silt loam	10	74	14.9	damp	17	11	128	2	4	Rainfed
Louisiana 2022	2.3	8	silt loam	12	70	18	fair	26	15	158	5	5	Rainfed
Michigan 2022	2.6	6.1	sandy clay loam	52	23	25	fair	21	7	88	1	3	Rainfed

Michigan 2023	1.3	6.1	loam	36	45	18	fair	21	5	48	19	32	Rainfed
Missouri 2022	1.9	6.8	silt loam	12	67	20	very dry	33	2	108	0	5	Rainfed
Missouri 2023	1.8	5.9	silt loam	12.5	67.5	20	dry	30	2	101	3	11	Rainfed
Nebraska 2022	2.5	6.5	silty clay loam	17	58	25	good	19	1	100	5	6	Rainfed
Nebraska 2023	2.5	6.5	silty clay loam	17	58	25	good	19	4	99	2	13	Rainfed
North Dakota 2022	6.6	7.4	loam	33	46	20	good	20	2	112	1	5	Rainfed
North Dakota 2022	5.3	8	silt clay	2	41	56	good	20	3	122	1	6	Rainfed
North Dakota 2023	5.3	8	silt clay	2	41	56	dry	21	2	121	1	8	Rainfed
Ohio 2022	2.3	6.6	loam	35	40	25	dry	23	18	150	1	1	Rainfed
Ohio 2023	3.2	7	loam	40	35	25	dry	22	22	94	1	1	Rainfed
S Illinois 2022	2	6.9	silt loam	8	72	20	fair	24	10	91	6	6	Rainfed
S Illinois 2023	1.8	6.4	silt loam	3	78	19	fair	21	12	70	1	1	Rainfed
Tennessee 2022	2.5	6.3	silt loam	24	54	22	fair	28	13	46	2	2	Rainfed
Tennessee 2023	2.5	5.8	loam	36	40	24	dry	24	15	162	1	1	Irrigated
Wisconsin 2022	2	7.9	loam	40	41	19	wet	21	39	165	2	2	Irrigated

Wisconsin 2023	1.9	6.9	loam	39	43	18	damp	19	3	89	2	7	Irrigated

[a,b] Abbreviations: PPT, precipitation; DAA, days after application of herbicide.

A total of 17 treatments were evaluated to determine the effective dose of metribuzin to control Palmer amaranth and waterhemp. Treatment information is summarized in 3.3, which includes 13 treatments of metribuzin ranging from 210-841 g ai ha^{-1} (each subsequent treatment with 52 g ai ha^{-1} higher dose), along with two comparison herbicides, sulfentrazone and *S*-metolachlor at field recommended dose, a non-treated control (NT) and weed-free check (WF). Herbicides were applied either at the time of planting or 1 day after planting using carbon dioxide pressurized backpack sprayers calibrated to deliver 140 L ha^{-1} at most locations. The application rate and nozzle used at each site is listed in Table 3.4. Plot size was approximately 3 m x 9.1 m and differed slightly between locations primarily due to row spacing and management practice unique to each location. Soybean were planted at 300,000-390,000 seeds ha^{-1} following preplant tillage to prepare a weed-free site at trial establishment. Weed free plots were maintained either by hand weeding, hoeing or herbicides unique to each location to reduce the effects of weed on soybean growth and development.

Table 3.3 List of herbicide treatments applied pre-emergence in soybean.

Treatment number	Treatment	Dose (g ai or g ae ha^{-1})
1	Non-treated	-
2	Weed-free	-
3	Metribuzin	210
4	Metribuzin	263
5	Metribuzin	315
6	Metribuzin	368
7	Metribuzin	420
8	Metribuzin	473
9	Metribuzin	525
10	Metribuzin	578
11	Metribuzin	631

12	Metribuzin	683
13	Metribuzin	736
14	Metribuzin	788
15	Metribuzin	841
16	Sulfentrazone	420
17	*S*-metolachlor	1790

Table 3.4 Application information of pre-emergence herbicide across site years

Site years	Pressure (psi)	Nozzle type	Nozzle spacing (in)	Ground speed (km h^{-1})	Application rate (L ha^{-1})
Arkansas 2023	40	AIXR 110015	20	4.8	140
Arkansas 2022	40	AIXR 110015	20	4.8	140
Illinois 2022	30	XR 110025	20	4.8	187
Illinois 2023	30	XR 110025	16	4.8	187
Indiana 2022	25	XR 8002	20	4.8	140
Indiana 2023	25	XR 8002	20	4.8	140
Kansas 2022	28	TT 11002	20	4.8	140
Kansas 2023	28	TT 11002	20	4.8	140
Kentucky 2022	35	XR 11002	20	4.8	140
Kentucky 2023	32	XR 11002	20	4.8	140
Louisiana 2022	35	AIXR 11002	19	5.6	140
Michigan 2022	30	AIXR 11003	20	6.1	178
Michigan 2023	30	AIXR 11003	20	6.1	178
Missouri 2022	24	XR 8002	20	4.8	140
Missouri 2023	24	XR 8002	20	4.8	140
Nebraska 2022	30	AIXR 110015	20	4.8	140
Nebraska 2023	30	AIXR 110015	20	4.8	140
North Dakota2022	28	AIXR 11002	20	4.8	140
North Dakota 2022	28	AIXR 11002	20	4.8	140
North Dakota 2023	28	AIXR 11002	20	4.8	140
Ohio 2022	44	AIXR 110015	20	4.8	140
Ohio 2023	44	AIXR 110015	20	4.8	140
S Illinois 2022	30	TTI 8002	-	-	140
S Illinois 2023	30	AIXR 11002	-	-	140
Tennessee 2022	38	AIXR 11002	-	4.8	-
Tennessee 2023	-	-	-	-	-
Wisconsin 2022	34	TTI 110015	20	4.8	140

Wisconsin 2023	39	TTI 110015	20	4.8	140

Weed parameters were recorded only for Palmer amaranth and waterhemp at respective locations and will collectively be referred to as *Amaranthus* weeds. Weed control was accessed at 14, 28 and 42 DAA of herbicide. Each experimental unit was rated visually from 0 to 100 % control relative to non-treated control, with 0% being no weed control and 100% being complete weed control. Additionally, weed density and above ground weed biomass per square meter were collected at 28 DAA. Soybean injury was visually accessed at 14, 28 and 42 DAA on a scale of 0 to 100% injury relative to NT, with 0% being no injury and 100% being completely dead soybean plants. Additionally, the height of 6 soybean plants per plot was measured at 28 DAA followed by soybean yield at maturity at some locations (Table S3.1).

3.3 Statistical Analysis

Data from Illinois 2022, Iowa 2023, Louisiana 2023, and Mississippi 2022 and 2023 were excluded from further analysis due to technical errors or experimental failure, making it insufficient for drawing any conclusions. All data was processed and analyzed using RStudio with respective packages. Soil moisture data was a categorical variable and so was reduced to three categories: Dry for dry and very dry site years, fair for fair, adequate and good site years and moist for damp and moist site years. Sowing date was transformed to Julian days to maintain consistency. Principal component analysis (PCA) was conduct using *prcomp* function in R to evaluate the differences across site years (Venables and Ripley 2002). The following variables were included for calculating eigenvectors: soil texture (sand, silt and clay %), soil organic matter (OM), soil temperature at time of herbicide application, soil pH, interval between application and first precipitation, amount of first precipitation, days between application and cumulative 12.7 mm precipitation and cumulative precipitation 42 DAA. Following PCA, a

random forest approach was used to estimate the importance score of covariates determining weed control using *randomForest* (Liaw and Wiener 2002) package in R. Weed control was selected as the predicted variable and was evaluated as a liner function of metribuzin dose, weed control days after application, soil OM, soil pH, soil texture, interval between application and first precipitation, amount of first precipitation, days between application and cumulative 12.7 mm precipitation, cumulative precipitation 42 DAA, seeding rate, row spacing, soil temperature at application, soil moisture at application and planting date.

Weed control was analyzed using generalized additive model (GAM) from *mgcv* package in R (Wood 2011). Weed control was evaluated as a function of metribuzin dose, soil OM, soil pH, soil texture, interval between application and first precipitation, amount of first precipitation, days between application and cumulative 12.7 mm precipitation, cumulative precipitation 42 DAA, soil temperature, and soil moisture at application. Weed control, weed density and weed biomass data were analyzed separately using GAM as a function of metribuzin dose. Crop injury data were analyzed separately for 14, 28 and 42 DAA using a GAM, with injury as a function of metribuzin dose. Predicted crop response was then plotted against the metribuzin dose. The *emmeans* package was used to estimate marginal means for soybean height and yield data across treatments (Lenth 2023). The Estimated means were then subjected to pairwise comparison using Tukey's HSD ($\alpha = 0.05$). Sulfentrazone and *S*-metolachlor treatments were evaluated independently from metribuzin doses, where crop injury and all weed parameters were analyzed similar to crop injury and yield as discussed above.

3.4 Results

A PCA biplot was developed to summarize the geographical differences in terms of soil and precipitation parameters. Dimensions 1 and 2 combined explain 48.4% variability among

site-years. We observed that Illinois 2023 and Michigan 2023 site years were plotted away from the other locations in terms of interval between application and first precipitation and days between application and cumulative 12.7 mm precipitation (Figure 3.1). Illinois 2023 received first precipitation 18 days after herbicide application and 23 days to accumulate 12.7 mm precipitation (Table 3.2). While Michigan 2023 received first precipitation 19 days after herbicide application and 32 days to accumulate 12.7 mm precipitation (Table 3.2). Data from Illinois and Michigan 2023 was not analyzed with other site-years as precipitation in first 2 weeks of herbicide application is an important factor for herbicide activation and subsequent weed control. All site-years except Illinois 2023 and Michigan 2023, were clustered together for further analysis with some variation in soil and precipitation parameters (Figure 3.1).

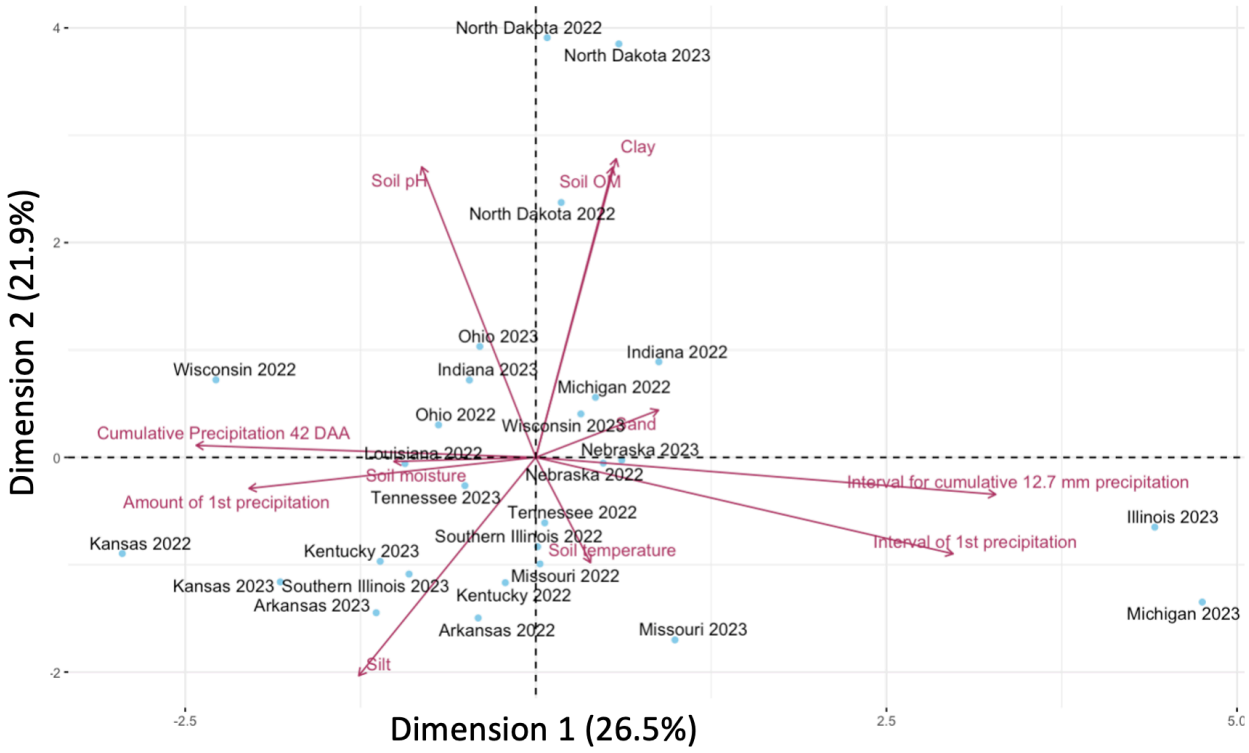

Figure 3.1 Principal component biplot for soil texture (sand, silt and clay), soil organic matter (OM) , soil temperature at herbicide application, soil moisture, soil pH, precipitation interval post herbicide application, amount of 1st precipitation, interval for cumulative 12.7 mm

precipitation and cumulative precipitation 42 days after application (DAA) parameters across site years. Site years are represented by state location followed by experimental year.

In the context of random forests, a variable importance plot quantifies the contribution of each variable to the overall predictive accuracy of the model. As expected, metribuzin dose was the biggest factor in predicting weed control followed by weed control days after application. Weed control days after application represents, how deep into the growing season, we are accessing weed control (14, 28 or 42 DAA). Cumulative precipitation and amount of first precipitation were the two biggest co-variates among the precipitation parameters (Figure 3.2). Soil OM, soil texture and soil pH were the major co-variates among the soil parameters. Seeding rate and row spacing had a minimal effect on predicting weed control which has also been reported previously by Lammers (2022). Therefore, these two variables were excluded from further analysis (Figure 3.2).

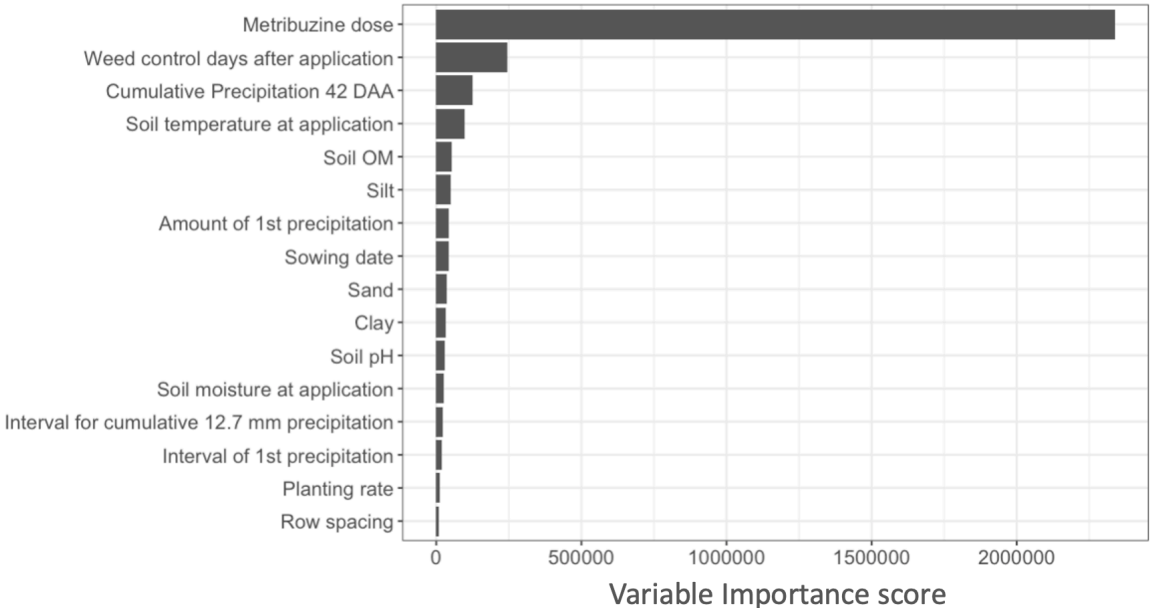

Figure 3.2 Variable importance plot for covariates determining the weed control based on random forest model.

3.4.1 Weed Control

Amaranthus weed control was accessed using a GAM and all the co-variates had a significant effect in predicting weed control (Table 3.5). Percent weed control increased with an increasing dose of metribuzin. Effectiveness of metribuzin to control weeds decreases as we go deeper into the growing season with overall highest control at 14 DAA and lowest at 42 DAA. At 14 DAA, 473 g ai ha^{-1} of metribuzin provided 95% weed control with the maximum weed control of 98% achieved with 841 g ai ha^{-1} of metribuzin. Metribuzin dose of 420 g ai ha^{-1} provided better weed control (94%) than either sulfentrazone (91.8%) or *S*-metolachlor (87.6%; Table 3.6). While at 28 DAA, we could not achieve 95% weed control even at the highest dose of metribuzin used. However, 90% weed control was achieved using 630 g ai ha^{-1} metribuzin with a marginal increase after that, which was still better than estimated mean weed control by sulfentrazone (85%) and S metolachlor (73.2%; Table 3.6). For the lower doses of metribuzin (210 to 420 g ai ha^{-1}) weed control decreased at 42 DAA as compared to 14 and 28 DAA. Eighty percent and 85% weed control was achieved with 578 and 683 g ai ha^{-1} respectively at 42 DAA. Weed control gradually increases with an increasing dose of metribuzin at 42 DAA with a maximum control 89% achieved at 841 g ai ha^{-1} (Figure 3.3). Metribuzin at 578 and 683 g ai ha^{-1} provided better weed control than sulfentrazone (81.3%), while metribuzin dose of 368 g ai ha^{-1} provided better weed than *S*-metolachlor at 42 DAA (Table 3.6).

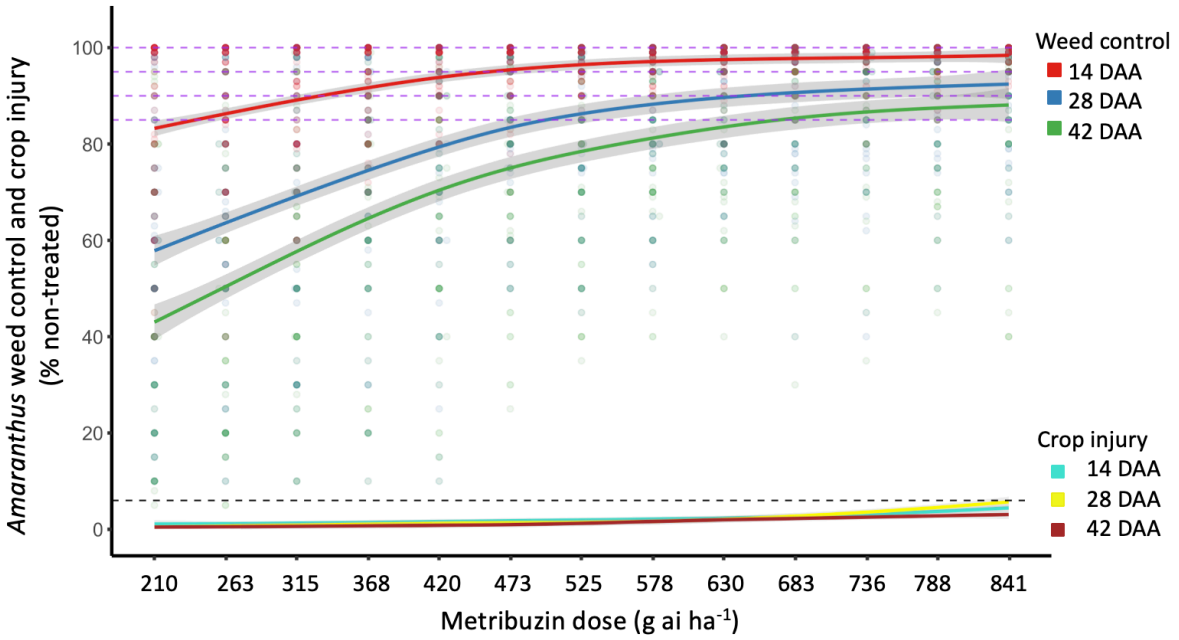

Figure 3.3 Soybean injury and Amaranthus weed control across metribuzin doses. Dotted black line represents 5% crop injury levels and dotted purple lines represent 100%, 95%, 90%, and 85% weed control respectively. Each dot represents an individual weed control data point across treatments.

Table 3.5 Estimated parameter values of the generalized additive model for weed control as a function of predicting variables across site years.

Intercept	Predicting variable		Slope	p-value
41.1	Metribuzin dose (g ai ha^{-1})		3.9	<0.01
	Weed control days after application (days)		-0.42	<0.01
	Soil OM (%)		0.032	0.013
	Soil pH		0.095	<0.01
	Clay (%)		-0.39	<0.01
	Sand (%)		-0.39	<0.01
	Silt (%)		-0.4	<0.01
	Soil Moisture	Fair	-0.30	<0.01
		Moist	-0.56	<0.01
	Soil temperature at application (°C)		0.11	<0.01
	Interval between application and first precipitation (days)		0.003	<0.01
	Amount of first precipitation (mm)		0.012	<0.01
	Days between application and cumulative 12.7 mm precipitation (days)		-0.075	<0.01
	Cumulative precipitation 42 days after application (mm)		0.0010	<0.01

Table 3.6 Percent weed control estimated as a function of herbicide treatment.

Interval (DAA)[a]	Treatment	Mean percent control[b]		P-value
14	Sulfentrazone	91.8	a	<.01
	S-metolachlor	87.6	a	0.07
28	Sulfentrazone	85	a	<.01
	S-metolachlor	73.2	b	<.01
42	Sulfentrazone	81.3	a	<.01
	S-metolachlor	59	b	<.01

[a] Abbreviation: DAA, days after application of herbicide

[b] Means within a column followed by lowercase letters represent significant differences identified by separation of means for each interval using Tukey's honest significant difference (HSD) test ($\alpha = 0.05$)

3.4.1.1 Weed Emergence

Weed emergence was recorded for nine site-years (Table S3.1). Weeds emerged as early as 6 days after planting in some of the NT plots. We observed an overall delayed weed emergence for both site years at Missouri and Ohio compared to other locations. Sulfentrazone and *S*-metolachlor delayed weed emergence by 27 and 21 days after planting, respectively (Figure 3.4). Metribuzin doses of 630 to 841 g ai ha^{-1} delayed weed emergence longer than sulfentrazone and *S*-metolachlor. While 841 g ai ha^{-1} of metribuzin delayed emergence to an average of 33 DAA.

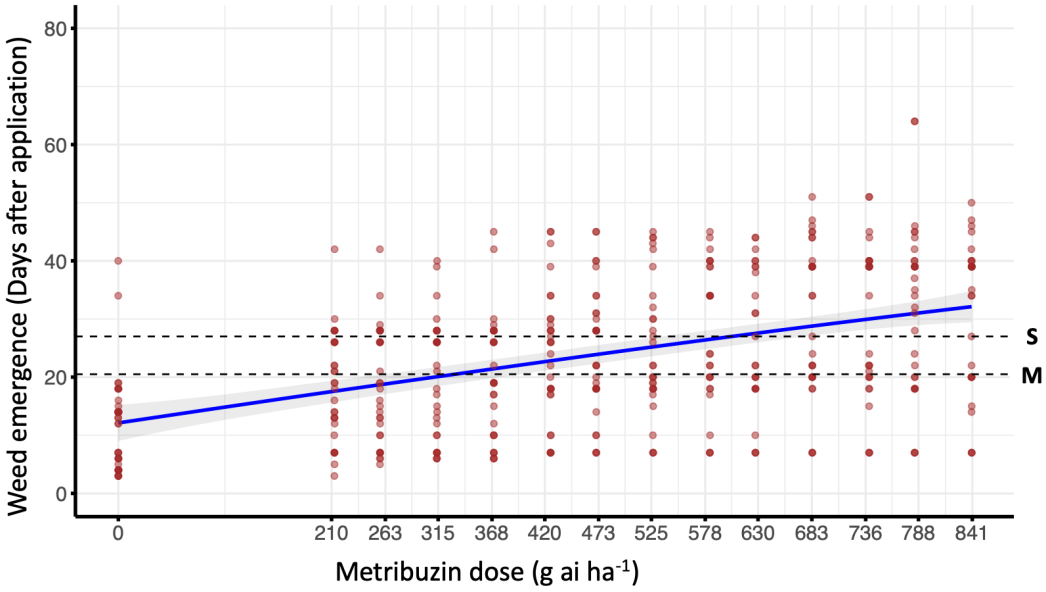

Figure 3.4 Amaranthus weed emergence from nine site-years across metribuzin doses. Regression line represents weed emergence as a function of metribuzin dose. Dashed lines represent significantly different mean weed emergence for sulfentrazone (S; 27 days after planting) and *S*-metolachlor (M; 20 days after planting) as identified with Tukey's honest significant difference (HSD) test ($\alpha = 0.05$). Each dot represents an individual weed emergence data point across treatments.

3.4.1.2 Weed Density and Weed Biomass

Weed density was recorded for 20 site years (Table S3.1). The lowest weed density was observed for North Dakota 2023 with average of 9 waterhemp plants m^{-2} and the greatest density at Missouri 2023 with average of 1300 waterhemp plants m^{-2}. Herbicide application reduced weed density. We observed the lowest weed densities for 525 to 841 g ai ha^{-1} of metribuzin which was similar to sulfentrazone treatment (Figure 3.5). *S*-metolachlor treatment reduced weed density similar to lower doses of metribuzin (210 to 315 g ai ha^{-1}; Figure 3.5) . Weed biomass was recorded for 16 site years (Table S3.1). Weed biomass followed a similar trend as weed density where Kansas and Missouri sites for 2023 accumulated the highest weed biomass in NT plots. Herbicide application reduced weed biomass. We observed less weed biomass at 28 DAA

for 525-841 g ai ha^{-1} of metribuzin (Figure 3.6). While sulfentrazone and *S*-metolachlor had a similar mean weed biomass of 6 and 9.4 g per m^{-2} at 28 DAA respectively .

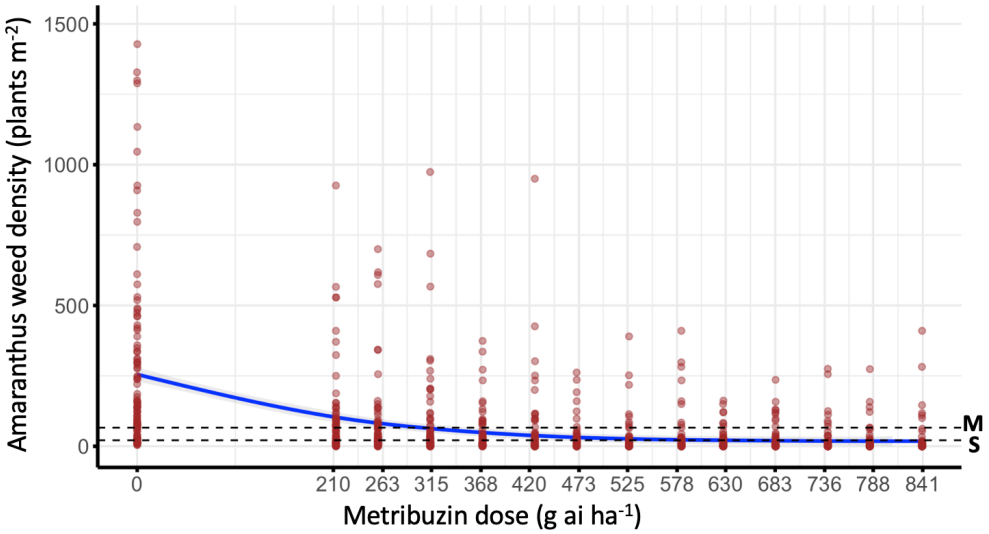

Figure 3.5 Amaranthus weed density from 20 site-years across metribuzin doses. Regression line represents weed density as a function of metribuzin dose. Dashed lines represent significantly different mean weed density for sulfentrazone (S; 21 plants) and *S*-metolachlor (M; 66 plants) as identified with Tukey's honest significant difference (HSD) test ($\alpha = 0.05$). Each dot represents an individual weed density data point across treatments.

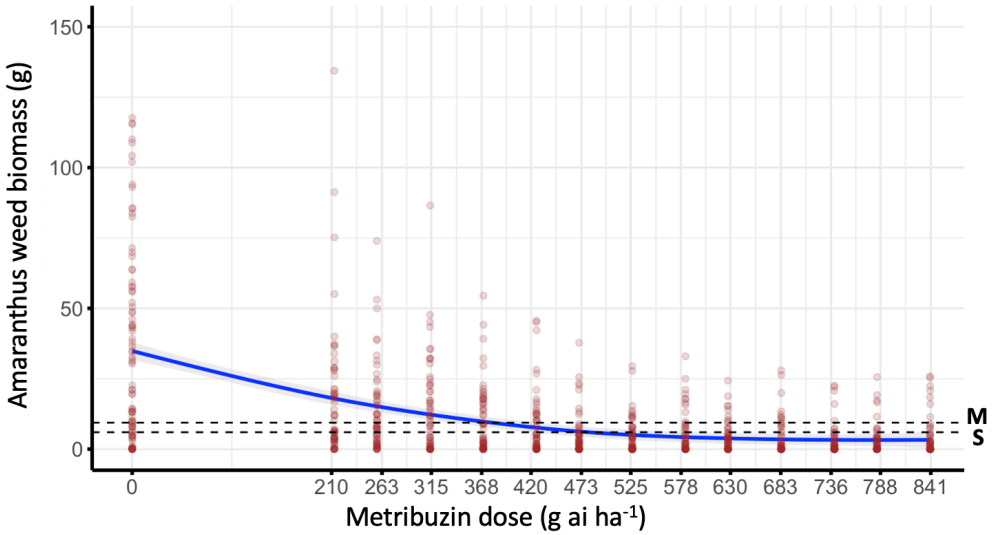

Figure 3.6 Amaranthus weed biomass from 16 site-years across metribuzin doses. Regression line represents weed biomass as a function of metribuzin dose. Dashed lines represent similar estimated mean weed biomass for sulfentrazone (S; 6g) and *S*-metolachlor (M; 9g) as identified

with Tukey's honest significant difference (HSD) test (α = 0.05). Each dot represents an individual weed biomass data point across treatments.

3.4.2 Crop Response

Crop Injury increased with an increasing dose of metribuzin with predicted injury of no more than 5% even at the highest dose of metribuzin (841 g ai ha^{-}1; Figure 3.3). Locations including Ohio 2022, and Tennessee 2023 reported up to 10% crop injury at 14 DAA which recovered at 28 and 42 DAA (data not shown). Arkansas 2022 and Louisiana 2022 reported higher injury ranging up to 20% even at the end of the season (data not shown). There was no concerning injury with either sulfentrazone or *S*-metolachlor (Table 3.7).

Table 3.7 Percent crop injury estimated as a function of herbicide treatment.

Interval (DAA)[a]	Treatment	Mean percent control[b]	P-value
14	Sulfentrazone	3.7 a	<.01
	S-metolachlor	2.8 a	0.32
28	Sulfentrazone	5.9 a	<.01
	S-metolachlor	1.4 b	<.01
42	Sulfentrazone	4.9 a	<.01
	S-metolachlor	1 b	<.01

[a] Abbreviation: DAA, days after application of herbicide

[b] Means within a column followed by lowercase letters represent significant differences identified by separation of means for each interval using Tukey's honest significant difference (HSD) test (α = 0.05)

3.4.2.1 Soybean Height

Soybean height was recorded for 20 site-years at 28 DAA (Table S3.1). Soybean growth varied across site-years depending on the relative weather conditions and soybean variety. Overall, there was no significant difference in soybean height among all treatments, especially comparing the non-treated and weed-free plots to those with herbicide treatment (Figure 3.7).

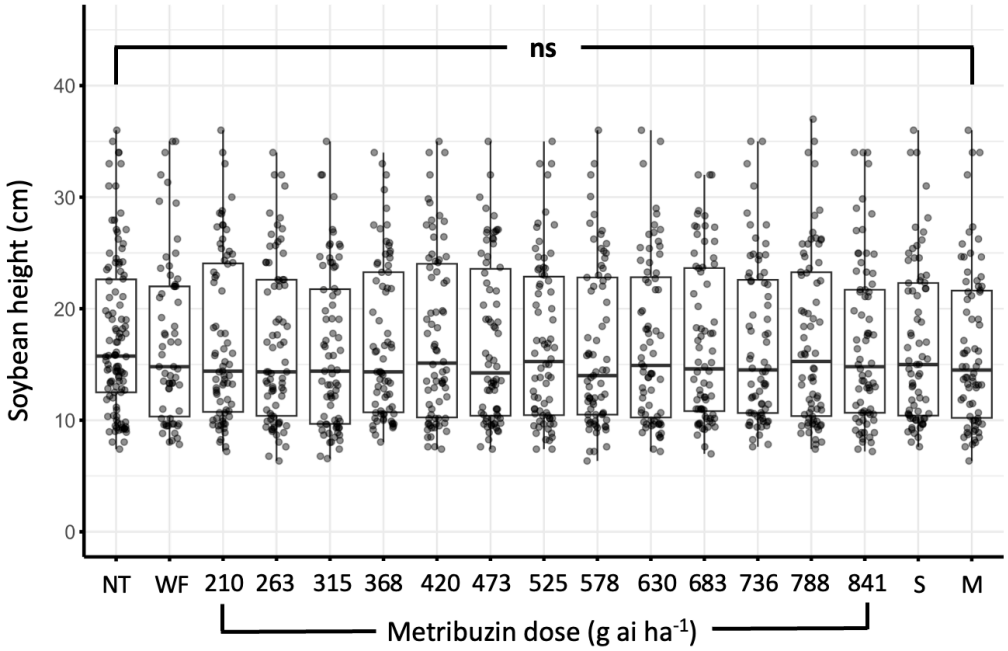

Figure 3.7 Soybean height from 20 site-years across herbicide treatment. Ns represent no significant differences identified by separation of means using Tukey's honest significant difference (HSD) test (α = 0.05). NT: non-treated; WF: weed-free; S: sulfentazone; M: *S*-metolachlor. Each dot represents an individual soybean height data point across treatments.

3.4.2.2 Soybean Yield

Soybean yield was recorded for seven site-years at maturity (Table S3.1) with overall lower yields recorded at Kansas 2023 and Kentucky 2023 averaging under 17 ton ha^{-1}. Maximum yield was recorded for weed free plots averaging at 34 ton ha^{-1} at Nebraska 2023. Surprisingly, herbicide application significantly increased soybean yield, comparable to the weed free plot, considering we had no POST application in our study (Figure 3.8). Soybean yield following metribuzin application at 315 to 841 g ai ha^{-1} and sulfentrazone were similar to the weed free plots. Lower yields were observed for *S*-metolachlor treatment and 210 g ai ha^{-1} of metribuzin (Figure 3.8).

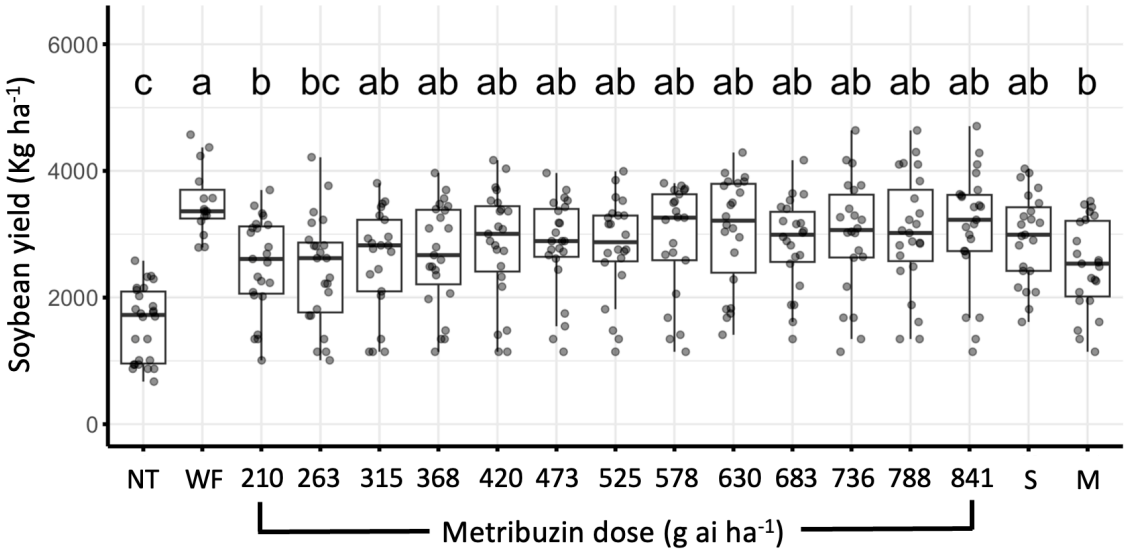

Figure 3.8 Soybean yield from seven site-years across herbicide treatment. Lowercase letters represent significant differences identified by separation of means using Tukey's honest significant difference (HSD) test (α = 0.05). NT: non-treated; WF: weed-free; S: sulfentazone; M: *S*-metolachlor. Each dot represents an individual soybean yield data point across treatments.

3.5 Discussion

In this study effective control of *Amaranthus* weeds was found, particularly at higher doses of metribuzin (578 to 841 g ai ha^{-1}). The amount of metribuzin to be applied PRE was significantly influenced by the timing of weed control assessment. Maximum control of 98% was achieved at 841 g metribuzin ha^{-1} at 14 DAA, outperforming sulfentrazone (91.7%) and *S*-metolachlor (87.6%). The efficacy of metribuzin declined later into the growing season. At 28 DAA, 90% control was achieved with 630 g ai ha^{-1} of metribuzin. Contrastingly, Meyer et al. (2016) reported 69% control of Palmer amaranth at 4 weeks after treatment of metribuzin (420 g ai ha^{-1}) which was less than *S*-metolachlor (89%; 1068 g ai ha^{-1}) under coarse textured low OM soil. However, both *S*-metolachlor and metribuzin treatments recorded similar Palmer amaranth and waterhemp densities (Meyer et al. 2016). In our study, 42 DAA, though the metribuzin efficacy declined, especially at lower doses (210–420 g ai ha^{-1}), the highest dose (841 g ai ha^{-1})

still provided 89% control. In contrast, *S*-metolachlor exhibited a sharp decline in efficacy, dropping to 59% control at 42 DAA. These results suggest that metribuzin, at higher doses (578 to 841 g ai ha^{-1}), offers superior long-term weed control compared to sulfentrazone and *S*-metolachlor. However, previous research suggests that sulfentrazone (280 g ai ha^{-1}) and *S*-metolachlor (1787 g ai ha^{-1}) had better residual control of Palmer amaranth than metribuzin (563 g ai ha^{-1}) (Ribeiro et al 2021b). This conclusion is based on the greenhouse bioassays conducted on silty loam soils at a depth of 0 to 10 cm with OM ranging from 2.6% to 3.1% and pH of 6.5 and 7 (Ribeiro et al 2021b). Growers can select the herbicides dose based on the desired weed control and soil and environmental factors using the parameters listed in Table 3.5. Selection of PRE herbicide and POST application plans have been evaluated in depth by Meyer et al (2015).

Furthermore, this study highlights the efficacy of metribuzin on herbicide-resistant Amaranthus weed populations. Palmer amaranth and waterhemp populations at experimental sites were resistant to multiple herbicides (mostly to Group 2 and 9 herbicides) as listed in Table 3.1. Specifically, populations at Illinois, Indiana, and North Dakota were resistant to POST application of group 5 herbicides (potentially atrazine) and still be controlled effectively by metribuzin. This suggests that metribuzin remains an effective tool for managing multiple herbicide-resistant populations of Palmer amaranth and waterhemp.

The findings of this research show that there were no concerning levels of soybean injury resulting from the application of metribuzin, even at the highest dose of 841 g ai ha^{-1}. The variability in injury across Ohio 2022, Tennessee 2023, Arkansas 2022 and Louisiana 2022 could be attributed to low OM and heavy rainfall post application of herbicide as herbicide adsorption is known to be strongly correlated with soil OM and texture (Blumhorst et al. 1990, Gundy and Dille 2022). Additionally, extended periods of cool, wet soil conditions during crop

emergence are known to reduce soybean's ability to metabolize PRE herbicides, potentially leading to soybean injury (Moomaw and Martin 1978, Osborne et al. 1995). Soybean plants, due to their indeterminate growth habit, are known to compensate for herbicide injury occurring during early developmental stages, by producing additional branches (Cox and Cherney 2011, Weidenhamer et al. 1989, Ribeiro et al 2021a). This could possibly be the reason we observed no negative effects on soybean height and yield in our study following metribuzin application. In previous report metribuzin (560 g ai ha^{-1}) and sulfentrazone (280 g ai ha^{-1}) applied PRE caused no yield reduction in soybean grown in loam soil texture with soil organic matter ranging from 1.7 to 2.2% and pH from 6.7 to 7.5 (Arsenijevic et al 2021). However, Arsenijevic et al (2021) reported 22% reduced green canopy at V2 growth stage and 10% lower plant stand at maturity with sulfentrazone compared with the control. But the overall yield increased by 3% following metribuzin and sulfentrazone treatment as compared to the control due to production of more seeds per plant. Interestingly, in our study sulfentrazone and *S*-metolachlor did not cause concerning injury levels. This contradicts with earlier findings by Taylor–Lowell et al. (2001) who reported injury to 15 soybean varieties ranging from 4% to 61% when sulfentrazone was applied at three different doses (112, 224, and 446 g ai ha^{-1}), with higher injury observed under prolonged wet and cool conditions after soybean planting. Differential tolerance of soybean verities to PRE herbicides have been reported previously by researchers (Taylor–Lowell et al. 2001), however our study lacks enough power to evaluate the varietal tolerance.

3.5 Conclusion

Amid the accelerating evolution of herbicide-resistant weeds in the US, PRE herbicides have regained importance for building effective weed management strategies. The critical period

of weed control (CPWC) in soybean is between 10 and 36 days after emergence, depending on soybean cultivar and weed species as suggested by Silva et al. (2011). Knezevic et al. (2019) found that PRE herbicides can delay the CPWC by 2-6 weeks, reducing the need for multiple POST applications during the season.

The experimental sites in this study were selected with either Palmer amaranth or waterhemp as a dominant weed. These weed species are highly competitive and can inhibit the growth of other weed species. Early season control of *Amaranthus* can create opportunities for grasses and large-seeded broadleaf weeds to thrive, posing new challenges as metribuzin has limited efficacy on large seeded-broadleaf weeds and grasses. To address this, growers should adopt a combination of PRE and POST herbicides tailored to local weed populations which will also help in delaying the evolution of herbicide resistance to PRE herbicides. Popular PRE herbicide options like sulfentrazone, pyroxasulfone, flumioxazin, and *S*-metolachlor are frequently used by soybean growers. The weed control efficacy of PRE herbicides likely outweighs the concerns associated to early-season injury. Based on the results of this study, higher doses of metribuzin ranging from 578 to 841 g ai ha^{-1}, can be judiciously incorporated into herbicide rotation strategies with the above mentioned PREs, to provide a broad spectrum of residual weed control.

Chapter 4 - Summary and Conclusion

Weeds are one of the most economically damaging pests in agriculture. Within the US crop production system, Palmer amaranth and waterhemp have been identified as the most troublesome weed species. These two Amaranthus species possess an excellent set of biological characteristics including rapid growth rate, prolific seed production and exceptional adaptability to diverse environmental conditions. Furthermore, the rapidly evolving problem of herbicide resistance in both Palmer amaranth and waterhemp populations has significantly added to growers' weed management challenges.

Chapter 2 of this thesis elucidates a remarkable display of adaptive evolution in KCTR Palmer amaranth, demonstrating resistance to multiple herbicides across 6 SOAs, including those it has never encountered. Notably, 4 of the 5 Groups of herbicides tested in our study, except for chlorsulfuron, share a common mechanistic feature: their ability to induce oxidative stress via ROS production and ultimately leading to plant death. To withstand the herbicide-induced stress, the weed populations likely evolved mechanisms to rapidly metabolize herbicides and decrease ROS production. In this study we identified specific genes of P450, GST, GT and ABC transporter family which may be directly or indirectly linked to metabolism of herbicides across 5 SOAs in KCTR Palmer amaranth plants. Specifically, isoforms of *CYP72A219* for mesotrione, chlorsulfuron and lactofen metabolism and *CYP704B1* for 2,4-D and atrazine metabolism. Isoforms of *C-terminal domain GST*, possibly from phi class, were upregulated post treatment with all herbicides except lactofen. Moreover, expression of some P450s was induced only after herbicide treatment such as *CYP711A* for mesotrione*, CYP705A22, CYP82D47* for atrazine and *CYP710A11, CYP78A9, CYP81D1* and *CYP83B1* for lactofen. Not all these enzymes are linked specifically with herbicide metabolism, but studies show that it's not unusual for P450s to be

multifunctional, and their functions can often be attributed to the selectivity of some herbicides (Dimaano et al. 2021, Brazier-Hicks et al. 2022). Apart from P450s and GSTs, we observed overexpression of GTs (*UGT79B7*, *UGT76F1* and *UGT79B30 like*) and ABC transporter from B, C and G family, which were induced upon herbicide application. UGTs are substrate specific enzymes and members from class D and E have been previously reported to be involved in xenobiotic conjugation (Brazier-Hicks et al. 2018, Caygill et al. 2023, Wang et al. 2024). ABC transporters are also known to be important in drug detoxification in plants and members of class C and G are known to catalyze compartmentalization of herbicide conjugates produced by GSTs (Goldberg-Cavalleri et al. 2023, Caygill et al. 2023, Pan et al. 2021). Previously, 2,4-D resistance in the KCTR Palmer amaranth population was found to be a quantitative trait regulated by multiple genes (Shyam et al. 2022b). The F_2 progeny developed from the survivors of F_1 plants will be helpful to understand the inheritance chlorsulfuron, atrazine, lactofen and mesotrione resistance and to identify the QTLs associated with the resistant traits in KCTR Palmer amaranth.

Understanding the underlying molecular basis of resistance, particularly metabolism based NTSR is crucial for predicting the course of evolution of herbicide resistance in weed populations. Research is in progress to validate candidate genes in homologous system. Unlike TSR, NTSR mechanisms are complex and largely driven by alterations in gene regulation. While substantial research has been conducted on understanding the role of key metabolic enzymes, little do we known about the specific regulators involved in overexpression of these genes. Elucidating regulation of gene expression is crucial to understanding metabolism-based NTSR to herbicides and warrants further investigation.

In response to the rapidly evolving herbicide-resistant weeds population across the US, growers are in urgent need of alternative weed control strategies. PRE herbicides have traditionally been a foundation for effective weed management and have regained importance over the past decade. Metribuzin, a PS II- inhibitor, despite being labelled for PRE use in soybean production, remains underutilized due to growers' concerns of potential crop injury. Additionally, metribuzin is known to effectively control of herbicide resistant Palmer amaranth and waterhemp, two of the most challenging weeds for growers. In Chapter 3 of this thesis, we found that 578 to 841 g ai ha^{-1}can delay weed emergence and reduce weed density and biomass more effectively than sulfentrazone and *S*-metolachlor. Optimal weed control of 98% was achieved at 841 g metribuzin ha^{-1} at 14 DAA, surpassing the efficacy of sulfentrazone (91.7%) and *S*-metolachlor (87.6%). Although, the efficacy of metribuzin declined later into the growing season, 90% control was achieved with 630 g ai ha^{-1} of metribuzin 28 DAA. At 42 DAA, though the metribuzin efficacy declined, particularly at lower doses (210–420 g ai ha^{-1}), the highest dose of 841 g ai ha^{-1} still provided 89% control. In contrast, *S*-metolachlor exhibited a sharp decline in efficacy, dropping to 59% control at 42 DAA. These finding suggests that when applied at higher doses of 578 to 841 g ai ha^{-1} , metribuzin offers superior long-term weed control compared to sulfentrazone and *S*-metolachlor. However, it is important to note that previous research by Ribeiro et al. (2021b) suggests that sulfentrazone (280 g ai ha^{-1}) and *S*-metolachlor (1787 g ai ha^{-1}) provided better residual control of Palmer amaranth than metribuzin (563 g ai ha^{-1}). These differences could be attributed to the MHR populations of Palmer amaranth and waterhemp in this study, which are better controlled with metribuzin rather than sulfentrazone and *S*-metolachlor.

Despite concerns over early-season crop injury, the efficacy of PRE herbicides generally outweighs these risks. It is noteworthy that even at the highest dose of metribuzin used in our study (841 g ai ha^{-1}) we did not observe any significant crop injury. This low phytotoxicity was validated by no significant impacts on soybean height and yield following metribuzin application. This information will be helpful in alleviating growers concerns regarding crop safety. The judicious use of PRE herbicide combinations along with POST applications could offer a viable strategy for managing MHR weed populations. The selection of PRE herbicide and development of effective POST application plans have been comprehensively evaluated by Meyer et al (2015).

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Appendix A - Supplementary Material

Table S3.1 Status of various soybean and weed parameters recorded across site-years.

Location	Soybean parameters					Weed parameters					
	Soybean response			Height	Yield	Control			Density	Biomass	Emergence
	14 DAA[a]	28 DAA[a]	42 DAA[a]			14 DAA[a]	28 DAA[a]	42 DAA[a]			
Arkansas 2022											
Arkansas 2023											
Illinois 2023											
Indiana 2022											
Indiana 2023											
Kansas 2022											
Kansas 2023											
Kentucky 2022											
Kentucky 2023											
Louisiana 2022											
Michigan 2022											
Michigan 2023											
Missouri 2022											
Missouri 2023											
Nebraska 2022											
Nebraska 2023											
North Dakota 2022											
North Dakota 2022											
North Dakota 2023											
Ohio 2022											
Ohio 2023											
S Illinois 2022											
S Illinois 2023											
Tennessee 2022											
Tennessee 2023											

Wisconsin 2022											
Wisconsin 2023											

[a] Abbreviation: DAA, days after application of herbicide.

* Green color represents correct data; grey color represents missing data; and red color represents data recorded on a different timing than the protocol.