FROM CELL TO FIELD: UNDERSTANDING HERBICIDE RESISTANCE MECHANISMS IN KEY BROADLEAF WEEDS IN MICHIGAN TO PREDICT AND PROPOSE A SUSTAINABLE INTEGRATED WEED MANAGEMENT APPROACH

By

Juliano Ricardo Marchi Sulzback

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Crop and Soil Sciences – Master of Science

2024

ABSTRACT

Herbicide resistance is a constant threat to agriculture that heavily rely on chemical weed control. Traditionally, weed managers use a reactive approach to failures in weed control, where first a problem occurs and then the issue is remediated (Robert et al., 2016). However, it is possible to take a proactive approach to weed management, by predicting weed control issues using epidemiological approaches and/or rapidly identifying using modern diagnostics to reduce the impact of weeds on production. To this end, laboratory experiments and field validations were conducted to 1) optimize rapid molecular assays that identify resistant individuals of horseweed (*Erigeron canadensis* L.), Palmer amaranth (*Amaranthus palmeri* S. Watson), waterhemp (*Amaranthus tuberculatus* (Moq.) Sauer), and common ragweed (*Ambrosia artemisiifolia* L.), and 2) identify mechanisms conferring resistance to acetolactate synthase (ALS) and *5-enolpyrovylshikimate-3-phosphate synthase* (*EPSPS*) inhibiting herbicides. Nine single nucleotide polymorphisms (SNPs) were identified in five *ALS* positions known to confer herbicide resistance among all species surveyed. One SNP in *EPSPS* gene in horseweed, and increased *EPSPS* copy numbers in Palmer amaranth (20-160 copies) and waterhemp (2-7 copies) accessions were identified, which are known to confer resistance to glyphosate. The genotyping assays identified resistance mutations in >98% of cases and could be generated in two days. We confirm that rapid molecular resistance diagnostics is a valuable tool in herbicide resistance diagnostics, helping growers to confirm herbicide resistance in Michigan weeds. In another study, we set out to understand the relationship between management practices and herbicide resistance evolution in horseweed to glyphosate, dicamba and 2,4-D. Greenhouse experiments were conducted to 1) assess current resistance spectrum of horseweed in Michigan to those herbicides, and to 2) determine main factors that contribute to the presence of resistance in horseweed. Out of the 20 accessions screened, 60% were resistant to glyphosate, 35% to 2,4-D, and 20% to dicamba. Dose-response data were integrated with previous management history and environmental factors using odds-ratio analyses to rank which factors influenced the presence of resistance. Out of all significant pairwise comparisons, 44% were related to crop rotation frequency, 33% to previous herbicide-resistance status, and 22% to location. Results highlight that growers can proactively manage herbicide-resistance evolution of horseweed in Michigan by adopting integrated weed management techniques, especially crop rotations and herbicide rotations, to prevent the successive selection events that occur in low diversity management systems.

Dedicated to my Biggest Cheer Section: Julio Cezar Sulzback. Angelita Matilde Marchi Sulzback, Gabriela Camila Marchi Sulzback, Paula Fernanda Sulzbach, Adria Fontana Marchi, and Aline Rohden. Thank you for always believing in me.

ACKNOWLEDGEMENTS

First, I would like to acknowledge my professors and mentors Dr. Eric Patterson and Dr. Erin Burns for giving me the opportunity to pursue a Master's of Science degree at Michigan State. Being an international student in a country with such a different culture may be hard sometimes, but their knowledge regarding all things weed science and life, along with friendly interactions, patience in mentoring me, and constant encouragement have been a great help to me over the past years. I cannot put in words what this opportunity meant to me; all I can say is that their simple "YES" has dramatically changed my life, and I am eternally grateful for them. Thanks to them and Dr. Martin Chilvers for serving on my committee and reading this whole thesis.

None of my research would be possible without the assistance of the postdocs. I would like to acknowledge Ednaldo Borgato for serving me as a great friend, and consistently providing me insights on my research and how to better approach to resolve any issues. Thanks all my family who offered constant support and encouragement. Special thanks to my parents Julio and Angelita, and my sister Gabriela – they taught me that anything is possible if you believe. Special thanks to my partner Aline Rohden for her constant love and support, even in my darkest period as a MS student – she taught me that, regardless of what our goals are, we need to focus on the process. Thanks to my pets for the emotional support, especially Lili, who was a dog trapped in a cat's body and now is the most shining star in the sky – she taught me how to live life intensively, regardless on the time we have on Earth. Thanks to my Brazilian fellows for friendship and support. Thanks to my lab mates for sharing their knowledge with me and growing our learning curves together. I would also like to thanks to all the people who directly or indirectly helped me to be where I am, I would never be able to make it alone.

TABLE OF CONTENTS

CHAPTER I: *REACTIVE* VERSUS *PROACTIVE* APPROACHES TO HERBICIDE RESISTANCE MANAGEMENT

INTRODUCTION

Herbicide resistance is a constantly growing threat to agriculture in the parts of the world that heavily rely on them for weed control. In Michigan agroecosystems, horseweed (*Erigeron canadensis* L.), Palmer amaranth (*Amaranthus palmeri* S. Watson), waterhemp (*Amaranthus tuberculatus* (Moq.) Sauer), and common ragweed (*Ambrosia artemisiifolia* L.) are among the most troublesome herbicide resistant weed plants (MSU-PPD, 2024). Traditionally, weed managers use a reactive approach to failures in weed control, including herbicide resistance, where first a problem occurs and then the issue is remediated (Robert et al., 2016). However, it may be possible to take a proactive approach to weed management where weed control issues are predicted using a combination of epidemiological approaches and rapid diagnostics to predict problems and then adjust agricultural practices to reduce the likelihood of weed control failure. This thesis aims to develop molecular assays for rapid detection of common herbicide resistance mechanisms in Michigan dicot weeds so that farmers can make in-season decisions on weed control practices (Rapid, but still reactionary methods for herbicide resistance management) as well as understand and predict the main agronomic drivers of herbicide resistance so that farmers can take proactive measures to avoid the development of herbicide resistance in their properties and regions.

WHAT ARE WEEDS?

Weeds can be any plant that is unwanted by humans and this designation can be prescribed for any number of reasons. Weeds have been present in agriculture since it began 12000 years ago when humans began selecting some plants for food while others as undesirable (Zeder, 2011). Ultimately, their negative effects on crops include reducing crop emergence and establishment (Felton, 1976), reducing grain quality, and, most importantly reduced crop yield (Mousa et al., 2022), which directly results in economic losses for farmers (Gawęda et al., 2020; Latinia & Eisvand, 2021). Primarily, weeds are detrimental because they compete for water, soil nutrients, sunlight, and space, and are often more efficient than their crop counterparts (Gawęda et al., 2020; Latinia & Eisvand, 2021). In the United States, competitive weed species span a wide taxonomic range including both dicotyledonous and monocotyledonous plants. The most common troublesome weeds for row crops in North America include horseweed (*Erigeron candensis*),

common lambsquarters (*Chenopodium album*), giant, green, and yellow foxtail (*Setaria faberi*, *Setaria viridis*, and *Setaria pumila*, respectively), Palmer amaranth, redroot pigweed (*Amaranthus retroflexus*), waterhemp, common ragweed, and barnyardgrass (*Echinochloa* spp) to date (Heap, 2023).

High levels of crop production are not possible without weed control. If left unchecked, weeds can cause entire crop loss and therefore weed control is a multibillion-dollar industry (Abusteit, 1993; Soltani et al., 2017). For instance, weed species can cause yield loss up to 80% in soybean (*Glycine max* (L.) Merr.) (Keramati et al., 2008; Sebayang & Rifai, 2018), and 69% in corn (*Zea mayz* L.) (Ford et al., 2014; Soltani et al., 2021). There is a wide range of weed control practices, but broadly speaking they are broken into non-chemical control which includes mechanical, cultural, and biological control practices and chemical control (i.e. the use of high efficiency herbicides). Best practice for weed control integrates several practices from both chemical and nonchemical control as this delays weed evolution and maximized crop yield (Abbas et al., 2018; Hamill et al., 2004).

For each crop/weed combination there exists a critical period of weed control (CPWC) which provides a timeframe for farmers to remove weeds in their fields. Before this period, weeds have yet to emerge and control practices may be ineffective and afterward this period, the weeds have already begun to decrease crop yield (Mousa et al., 2022). The critical period for weed management is critical to understand as it minimizes establishment and resource usage by weeds (Dang et al., 2020) and thus preventing negative effects of weed species (Ferrell et al., 2021). This is especially important in situations where initial weed control practices are performed but fail. The time between identifying a weed control failure and being able to respond with an alternative strategy and stay within the CPWC is very short.

NON-CHEMICAL CONTROL

The first methods for non-chemical control are changes in agricultural practices broadly defined as "cultural practices". Cultural control practices are management strategies that support crop fitness so that they can outcompete weeds, or at least put up a fight. These strategies include: maintaining optimal pH for the crop plant, fertilization, crop rotation, and reduced fallowing (Rabin, 2012), all of which support crop fitness and help outcompete weeds for resources. For example, previous research has found that weed infestations are increased when crops are grown in monoculture in contrast when grown in crop rotation (Gawęda et al., 2020). Monoculture

systems have less crop diversity as well as a decreased diversity of weed control practices, these systems are less resilient and far less likely to outcompete weeds. These monoculture systems reduces herbicide rotation options, which also reduces site of action rotation, thus increasing selection pressure on a few sites-of-action and increasing issues with herbicide resistance. When crop rotation is adopted, the diversity of plants increases, as does the types of weed control practices available. This makes the cropping system more resilient making weed control more tractible both in the short and long term. Additionally, by adopting crop rotation, it is possible to rotate herbicides sites of action, and decreasing the likelihood of herbicide resistance issues. Ultimately, crop rotation interrupts the life cycle of weeds, thus depleting seeds from the seedbank, and suppressing weed competition. Therefore, the impacts of crop rotation on weed control can be massive and synergize with chemical weed control techniques for increasing weed control technique diversity (Gawęda et al., 2020).

Another non-chemical control method that is widely adopted are various forms of mechanical control. Mechanical control techniques physically destroy or inhibit weeds with mechanical implements such as plows, mowers, tillers, etc. Mechanical control methods can be a cheap, low tech, practice that both conventional and organic farmers can employ (Fang et al., 2023). Common implements that are used for mechanical weed control are harrows, weeders, and cultivators (Abbas et al., 2018). Studies from Gawęda et al. (2020) and Donald et al. (2001) evaluated the effect of mechanical control via tillage; results showed that there is a considerable increase in weed infestation under no-tillage compared to tillage conditions. Mechanical control techniques can both synergize and antagonize the efficacy cultural and chemical control practices as well as interact with other agronomic factors such as soil health and therefore should be carefully considered and implemented.

CHEMICAL CONTROL

Chemical weed control is the use of herbicides to control weedy plants. Each herbicide works by inhibiting an essential physiological process in a plant, their so-called mode-of-action. Within each mode-of action there are one or more specific protein targets, called the site-of-action (Gaines et al., 2020). Generally, the binding of the herbicide to the protein is irreversible, or at least much stronger than the native substrate of the protein (Gaines et al., 2020). The interference with the proteins normal function unbalances metabolic reactions and causes physiological anomalies such as dwarfing, wrinkling and twisted leaves, chlorosis and necrosis, and ultimately

death (Gunsolus & Curran, 1991). The first herbicide ever discovered was 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T) after World War II, another auxin-mimicking herbicide (Grossmann, 2010; Peterson et al., 2016). Therefore, the introduction of chemical control is relatively new (approximately 80 years) when compared to the entire history of weed control (Duke & Powles, 2008).

While Herbicides can be classified based on which types of plants they affect, how they injure the plant, when they are applied, their site of action, their mode of action, chemical family, etc., one of the most important features is selectivity (Gwatidzo et al., 2023). Herbicides are classified according to their mode of action in the plant, which is then separated into different sites of action. This classification and attributed a number to them, which indicates different sites of action (i.e. glyphosate group 9, ALS group 2 etc.). Herbicides can be broadly classified as selective or non-selective, where selective herbicides are herbicides that control specific weeds without injuring crops while non-selective herbicides control a broad-spectrum of plants in the fields, injuring both weedy plants and crops alike (Duke & Powles, 2008). There are many selective products as they are the safest to use in protecting specific crops while controlling specific weeds. For example, it is relatively common to control broadleaf weeds in grass crops or grass weeds in broadleaf crops. Examples of selective broadleaf herbicides are 2,4-D and dicamba as well as many other members of the Auxin-mimicking herbicides while an example of a grass herbicide is clethodim as well as many other members of the acetyl-CoA carboxylase (ACCase) inhibiting herbicides (Grossmann, 2010). An example of a non-selective herbicide is glyphosate (Duke & Powles, 2008; Gunsolus & Curran, 1991; Gwatidzo et al., 2023). This herbicide was relegated to bare-ground, burndown, and other total-vegetation control needs until the invention of genetically modified crops (GM crops) that resistant the herbicide which makes it highly selective for resistant crops (Duke & Powles, 2008; Gunsolus & Curran, 1991; Gwatidzo et al., 2023).

Farmers in North America have heavily adopted chemical control because it was efficient, inexpensive, and greatly simplifies weed control (Duke & Powles, 2008b; Norsworthy et al., 2012; Pingali, 2012) This adoption has made herbicides more-or-less essential to high-production row-crop agriculture (Heap, 2014). GM crops has further increased dependence on herbicides – simplifying weed control even further and making it even more cost-efficient (Sprent, 2011). These GM herbicide resistance traits have other benefits for growers such as the ability to do no-till agriculture which has been shown to improve soil health and fertility. The use of GM crops directly

affected herbicide usage worldwide because GM crops enable producers to change management strategies, such as using the nonselective herbicide glyphosate in established crops (Green, 2014). To date, 93% of soybean and 92% of corn acreage have the GM trait known as Roundup Ready®, making them resistant to glyphosate (USDA, 2023). The adoption of GM crops has also been international with countries such as Brazil and Argentina widely adopting GM soybeans for example (Duke & Powles, 2008). Unfortunately, GM crops have also decreased the diversity of herbicides used in many row-crop systems, leading to an over-reliance on a few chemicals, which, in turn, has led to a drastic increase in herbicide resistance evolution (Gunsolus & Curran, 1991; Heap, 2014).

RESISTANCE IN WEED SPECIES

According to the international herbicide resistance weed database, there are over 500 confirmed unique cases of herbicide resistance to 21 herbicide sites of action (Heap, 2023). Simply speaking, resistance is the evolved ability to survive field-level doses of an herbicide. Generally, resistant individuals have one or more alterations in their physiology that reduce the effectiveness of a given herbicide to control it. These alterations are due to mutation in their DNA which are therefore inheritable and selectable. Generally, it is assumed that these mutations occur naturally within weed population, but their frequency in the population is initially low (Gaines et al., 2020; Powles, 2008). When the same herbicide is used repeatedly, the initially rare mutant individuals (Gaines et al., 2020), increase in frequency and begin competing with crops (Duke & Powles, 2008b; Gaines et al., 2020; Gould et al., 2018; Hamill et al., 2004; Tranel et al., 2002). The speed of herbicide resistance evolution depends on several factors including mutation rate, population size, outcrossing frequency, allele dominance, and fitness (Gould et al., 2018; Yang & Kim, 2016). Perhaps the biggest determiner of resistance is the nature of the chemical-protein interaction. Some herbicides are prone to resistance from several common target site mutations (e.g. ALS-inhibitors) while other chemistries tend to require more complex or rare traits such as copy number variation of the target site (e.g. Glyphosate resistance). The factors that govern what specific mutations cause resistance to which herbicide are complex, leading to a case-by-case understanding of resistance mechanisms.

Resistance mechanisms are separated into two broad categories: target-site resistance (TSR) and non-target-site resistance (NTSR) (Gaines et al., 2020). Target-site resistance mechanisms include single nucleotide polymorphisms (SNPs) and insertions/deletions (In/Dels)

in the target protein or overexpression of herbicide-targeted enzyme, usually facilitated by gene amplification (Gaines et al., 2020). Target-site resistance mutations are generally rare as they can cause fitness penalties by changing the efficacy of the target enzyme, which are always critical enzymes for the plant to survive – that's what makes them good herbicide targets (Tranel et al., 2002). SNPs in the DNA of an organism change amino acids which in turn change the shape and efficiency of a protein. These usually decrease binding affinity of the enzyme for the herbicide while maintaining a certain level of affinity for its natural substrate (Gaines et al., 2020; Knezevic et al., 2016), reducing the herbicide's affinity for the herbicide relative to the normal substrate. In/Dels are insertions or deletions of nucleotide sequence in the DNA sequence that change amino acids (Gaines et al., 2020). Indels, however, are even less common as SNPs, as the changes they make in the amino acid sequence are larger and potentially carry large fitness penalties. Another TSR mechanism is gene amplification, which consists of multiple copies of the herbicide's target gene, and it is often referred as copy number variation (CNV). Copy number variation results in overexpression of the target-site enzyme, so the amount of herbicide applied is not sufficient to inhibit the overexpressed enzymes, thus the resistant plants are capable functioning normally in the presence of field rates of the herbicide (Gaines et al., 2020).

Mechanisms that confer NTSR are often related to physiological processes that reduce the amount of herbicide that reaches its target in the plant such as herbicide metabolism (the increase of herbicide's active ingredients degradation by metabolic pathways), overexpression of genes and proteins production that are not the herbicide target, reduced uptake and/or translocation (regulation of herbicide uptake by the plant, or the reduction of translocation of these molecules), and compartmentalization (the increase of sequestration and storage of these molecules in vacuoles) (Gaines et al., 2020; Jugulam & Shyam, 2019). Metabolism is the ability of the weed to break down the herbicide, generating metabolites that are less toxic or inactive (Dimaano & Iwakami, 2021). It is usually associated with the increased activity of enzymes (induced or not) that are part of detoxification pathways, including: cytochrome P450 monooxygenases, glutathione *S*-transferases (GSTs), and esterase (Gaines et al., 2020). Herbicide metabolism usually involves three phases: (I) initial reactions such as oxidation, reduction, or hydrolysis, (II) primary conjugation with internal substrates such as sugars, amino acids, or glutathione, and (III) secondary conjugation, the generation of insoluble residues, or storage in the vacuole. The first step involves processes to remove electrons (oxidation), add electrons (reduction), or breaking

down the molecule by adding water (hydrolysis). The second/third step, conjugation, is the process in which the primary metabolites are combined with sugars or amino acids resulting in reduced toxicity and greater solubility in water (Délye, 2013). This enables the herbicide to be transported out of the plant and eliminated or integrated in macro-structures like the cell wall.

Reduced absorption and translocation are generally associated with genetic or physiological alterations that change the ability of the active ingredients to enter and move within the plant, reducing the herbicide's effectiveness. Cuticle modification and changes in membrane transporters (e.g. ATP-binding cassette (ABC) transporters,) are examples of this type of herbicide resistance (Gaines et al., 2020; Menendez et al., 2023). The ABC transporters, specifically, perform active transport of a wide range of organic compounds through the membrane. Major facilitator superfamily transporters also perform transport through the membrane, whereas proton antiporters are involved in the exchange of protons of H^+ to other ions that can pass through the membrane (Menendez et al., 2023). One of the most notorious cases of herbicide resistance is with the ABC transporter activity inhibition and the herbicide 2,4-D. ABC transporters' activity is reduced, resulting in lower intracellular concentrations of 2,4-D, which reduces the efficacy of the herbicide (Goggin et al., 2016).

ACETOLACTATE SYNTHASE INHIBITOR (ALS; GROUP 2) RESISTANCE

Branched-chain amino acids are crucial for all organisms to survive and grow, as they are precursors of many metabolic reactions and essential for building proteins. A key protein in the formation of these amino acids is acetolactate synthase (ALS). ALS is directly responsible for the biosynthesis of acetolactate the precursor of branched-chain amino acids leucine, isoleucine, and valine (Umbarger, 1978). When branched-chain amino acid metabolic pathway is interrupted by the inhibition of ALS, the plant will die by amino acid starvation (Tranel et al., 2002). ALS-inhibitors (group 2 herbicides) (Gunsolus & Curran, 1991) are an important herbicide group that vary greatly in selectivity and may control annual and perennial broadleaf or grass weeds. ALS-inhibitors were first introduced in agriculture in 1982, with the usage of the herbicide chlorsulfuron, an herbicide that controls broadleaf weed species in cereal crops. Their diversity and high efficacy in controlling weeds at low rates (Ray, 1984), made these herbicides common for many crops. To date, there are five chemical families that have ALS as a site of action; the sulfonylureas (SUs), imidazolinones (IMIs), triazolopyrimidines (Types 1 and 2), pyrimidinyl benzoates, and triazolinones.

Even though ALS-inhibitor chemical families are diverse, there are 21 weed species described with resistance (Gaines et al., 2020), via both TSR and NTSR mechanisms. The mechanisms involved in TSR to group 2 herbicides are CNV (Iwakami et al., 2017) and SNPs in the ALS gene (Küpper et al., 2017). Iwakami et al. (2017) reported that ALS resistance in a population of shortawn foxtail (*Alopecurus aequalis*) was conferred by gene amplification. Furthermore, there are eight reported SNP mutations in ALS gene that confer resistance in positions Ala-122, Pro-197, Ala-205, Asp-376, Arg-377, Trp-574, Ser-653, and Gly-654 (Heap, 2023). For instance, the Pro197Ser mutation is often found in Group 2 resistant horseweed; this substitution has been shown to confers resistance to three chemical families of group 2 herbicides (imidazolinone, pyrimidinylthiobenzoic acid, and sulfonylurea) (Zheng et al., 2011). NTSR resistance to group 2 herbicides has be reported in only a few species, such as rigid ryegrass (*Lolium rigidum* Gaud.), and large crab-grass (*Digitaria sanguinalis* (L.) Scop.), which is conferred by an enhanced detoxification (Corbett & Tardif, 2006).

5-ENOLPYRUVYHLSHIKIMATE-3-PHOSPHATE SYNTHASE INHIBITOR (EPSPS; GROUP 9) RESISTANCE

Aromatic amino acids are also critical for plants, as plants use them as precursors to other molecules, including other amino acids, proteins, plant defense, lignin, and hormones as well as building blocks for proteins (Tzin & Galili, 2010). Aromatic amino acids are the product of the shikimate pathway, one of the largest secondary metabolite pathway in plants in terms of net carbon flow (Ghosh et al., 2012). Inhibiting any enzyme in this pathway results in a lack of aromatic amino acid production which causes a systemic dysregulation of the subsequent metabolic pathways and eventual death (Wang & Ching-Yuh, 2001). The only enzyme in this pathway that is an herbicide target is *5-enolpyruvylshikimate-3-phosphate synthase inhibitor (EPSPS)*, which converts phosphoenolpyruvate (PEP) and 3-phospho shikimate (S3P) into EPSP (Gunsolus & Curran, 1991). Glyphosate is the only registered disruptor of EPSPS and therefore the only member of a group 9 herbicides (Shaner et al., 2005).

To date, there is a total of 355 weed species with resistance to glyphosate, and the mechanisms of resistance are both TSR and NTSR (Heap, 2023). *EPSPS* TSR mechanisms commonly identified in weed species are SNPs (Takano et al., 2019), gene amplification (Gaines et al., 2011), and overexpression (Tani et al., 2015). The SNPs Pro106Ser and Thr102Ile as well as their double-mutation termed 'TIPS' (Thr102Ile & Pro106Ser) in the *EPSPS* gene can confer

resistance to glyphosate, as observed in goosegrass (*Eleusina indica* L. Gaertn.) (Takano et al., 2019). However, these SNPs often come with a mild to moderate fitness penalty, due to loss of efficiency of catalytic reactions, and accumulation of shikimate (Han et al., 2017; Sammons & Gaines, 2014). CNV of the *EPSPS* gene is the second most common TSR mechanism for glyphosate, occurring in both broadleaf and grass weeds (Gaines et al., 2010; Patterson et al., 2018). *EPSPS* is overexpressed due to these CNVs which in turn increases EPSPS concentration, and therefore it requires a higher amount of glyphosate to control these individuals compared to what is required to control a susceptible individual (Gaines et al., 2011, 2020).

EPSPS resistance can also be conferred via NTSR mechanisms, including reduced absorption and translocation (De Prado et al., 2005; Tani et al., 2015), rapid necrosis (Moretti et al., 2018; Van Horn et al., 2018), and metabolism (Pan et al., 2019). Tani et al. (2015) observed that the TSR and NTSR mechanism act together to confer glyphosate resistant horseweed, due to reduced translocation via ABC transporters and the overexpression of *EPSPS*. Also, rapid necrosis a novel mechanism of glyphosate NTSR has been reported in giant ragweed (*Ambrosia trifida* L.) (Moretti et al., 2018; Van Horn et al., 2018). These studies showed that rapid necrosis resulted in reduced translocation of glyphosate to its target site.

SYNTHETIC AUXIN (GROUP 4) RESISTANCE

Synthetic auxins are another important herbicide group in row crop production. These herbicides are part of group 4 and are frequently used in small grains, corn, and sorghum due to their natural selectivity in grass crops against broadleaves as well as in GE soybean (Enlist E3™soybean, Roundup Ready 2 XtendiMax®)(Todd et al., 2020). These herbicides mimic auxin molecules (Gunsolus & Curran, 1991), the main plant hormone responsible in regulating plant growth. When synthetic auxin herbicides are absorbed by the plant, the herbicide is translocated throughout the plant and they are thought to bind members of the Aux/IAA protein family. Aux/IAA is a protein that suppresses auxin response factors (ARF), and this suppression inhibits the transcription and expression of the plant hormone auxin (indole-3-acetic acid, or IAA) (Hagen & Guilfoyle, 2002). Herbicide binding releases Aux/IAA from ARFs and the auxin responsive gene starts to be expressed which leads to several abnormal plant growth phenotypes. Ultimately, synthetic auxin herbicides cause tissue necrosis, abnormal cell division and growth, and death (Gaines et al., 2020; Mockaitis & Estelle, 2008).

Resistance has been shown to be conferred by both TSR and NTSR mechanisms to group 4 herbicides. A few polymorphisms have been described that cause TSR, primarily in members of the Aux/IAA protein family (Walsh et al., 2006). For example, LeClere et al. (2018) found a mutation in the coreceptor IAA16 (Aux/IAA16) that confers resistance to dicamba in kochia while Figueiredo et al. (2022) identified a deletion of 27 nucleotides (9 amino acids) in the degron tail of IAA2 (Aux/IAA2), that caused resistance to 2,4-D in waterhemp. In both cases. AuX/IAA proteins became less sensitive to their Group 4 partners.

To date, both metabolism and reduced translocation mechanisms have been found that cause NTSR to auxin mimic herbicides. These processes can be complex and variable depending on the herbicide and plant species. One example is reduced activity of ABC transporters, which results in decreased mobility of 2,4-D, thus diminishing the amount of active ingredient reaching the target tissue (Goggin et al., 2016). The resistance to group 4 herbicides is obviously advantageous in agronomic systems where they are used frequently; however, resistance mechanisms for synthetic auxins often carry severe fitness penalties as they change the regulation of critical plant hormones, mainly indole acetic acid. LeClere et al. (2018) determined that mutation in BsIAA16 that confers cross resistance to dicamba and 2,4-D in kochia reduces seed mass production to less than 50% when compared to a non-mutant individual.

BROADLEAF WEED SPECIES IN MICHIGAN

Globally speaking, grass weeds are generally the more damaging weed species; however, in North America, and especially in Michigan it is often broadleaves that are the most difficult to control, especially in terms of herbicide resistance. In Michigan, our primary herbicide resistant weeds include the broadleaf weed species horseweed, Palmer amaranth, waterhemp, and common ragweed (MSU-PPD, 2024). These weeds are problems in many of the major row, vegetable, and tree crops in Michigan (Felton, 1976), leading to losses in grain quality, biomass production, and yield (Mousa et al., 2022). Ultimately, these negative effects lead to economic losses for farmers (Gawęda et al., 2020; Latinia & Eisvand, 2021). Therefore, it is necessary to comprehend their biology in order to determine the best approach to effectively manage these species.

Horseweed (family Asteraceae) is a C_3 monoecious summer *or* winter annual weed species that can self-pollinating and reproduce sexually (Mulligan & Findlay, 1970). Horseweed is a cosmopolitan weed species and does not require specific climatic conditions to grow (Weaver, 2001). These plants have two forms during their life cycle, the vegetative stage (or rosette) and the

mature stage (or bolt) (Buhler & Owen, 1997). When in the rosette stage, their root system is aggressive, which promotes establishment and nutrient and water use (Weaver, 2001). During the bolting stage, each plant can produce 200,000 seeds (Buhler & Owen, 1997). Due to seed's morphology, these seeds can be dispersed by wind to long distances (Bhowmik & Bekech, 1993). Herbicide resistance has been described for groups 2, 5, 9 and 22 in this horseweed (Heap, 2023).

Palmer amaranth (family Amaranthaceae) is a C_4 dioecious summer annual weed species, cross-pollinating and reproduces sexually (Jha et al., 2008; Sauer, 1955; Ward et al., 2013). Palmer amaranth is a cosmopolitan weed species and requires 24/10°C day/night (d/n) and 15 hour photoperiod to grow (Assad et al., 2017; Roberts & Florentine, 2022). Their deep root system is aggressive and quickly exhausts water and nutrients from the soil, causing severe damage to agricultural lands (Bensch et al., 2003; Menges, 1988). When photoperiod decreases, these plants start to emit their inflorescence, and during the reproduction stage, each plant can produce 200,000 to 1,000,000 seeds. Seeds are small and easily dispersed in shorts distances through gravity; but they can be dispersed to medium and long distances when field operation are done, such as harvesting (Menges, 1988). This weedy species is arguably the most troublesome weed plant to control worldwide due resistance to most major groups of herbicides including groups 2, 3, 4, 5, 9, 10, 14, 15, and 27 (Heap, 2023).

Waterhemp (family Amaranthaceae) is a C_4 dioecious summer annual weed species, cross-pollinating and reproduces sexually (Assad et al., 2017; Sauer, 1955). Waterhemp is a cosmopolitan weed species and requires similar conditions than Palmer amaranth to grow though better adapted to the cooler and wetter climates found I Michigan where it is endemic (Roberts & Florentine, 2022). Their root system are also similar to Palmer amaranth (deep and aggressive) and they quickly compete for water and nutrients from the soil, causing economic losses on agricultural lands (Weaver & McWilliams, 1980). When photoperiod become shorter, these plants start to emit their inflorescence, and during reproduction stage, each plant can produce 200,000 to 600,000 seeds (Assad et al., 2017). Similarly to Palmer amaranth, waterhemp's seeds are small which is dispersed through gravity in short distances; but it can be dispersed in medium or long distances with the use of implements, such as a harvester (Menges, 1988). This weedy species has evolved resistance to various herbicides, including groups 2, 4, 5, 9, 14, 15, 27 (Heap, 2023).

Common ragweed (family Asteraceae) is a C_3 monoecious summer annual weed species, cross-pollinating and reproduces sexually (Essl et al., 2015). Common ragweed is a cosmopolitan

weed species and grows in a wide thermal range (8-30°C) and longer photoperiod of 14 hours to vegetatively grow (Deen et al., 1998). When photoperiod is less than 14 hours, these plants start their reproductive stage (Deen et al., 1998). During the reproductive stage, each plant can produce a wide range of seeds, varying from 3,000 to 62,000 (Dickerson & Sweet, 1971). Common ragweed seeds can be dispersed through several pathways, but dispersal is most significant via agricultural machinery and mowing machines (short to long distances) (Karrer & Vitalos, 2009). Ragweed is probably best known for being a major seasonal allergen as well as for its incredible seed longevity and dormancy (Fleming et al., 2023). This weedy species has adapted to herbicides of groups 2, 4, 5, 9, and 14 (Heap, 2023).

METHOD FOR A MORE SUSTAINABLE WEED MANAGEMENT PROGRAM

Weed control still relies heavily on chemical control, and the recurrent usage of herbicides will increase selection pressures and therefore herbicide-resistance evolution. The inevitability of this phenomena is now clear after the characterization hundreds of documented cases of resistance evolution, showing how fragile and non-sustainable chemical-only control really is. However, recent advance of agricultural technologies and our increased understanding of how plants evolve, has led to an understanding of the importance of diversified management strategies - an integrated weed management program seems to be able mitigate or at least delay future resistance issues. Perhaps most important for delaying resistance is crop rotation and diversity; this practice enables farmers to alternate herbicides' SOA according to the crop, selecting weed populations in multiple different directions. In addition to crop rotation, supporting crop competitiveness with optimized fertilizing programs helps crops to outcompete weeds by quickly, most importantly reaching maximum crop canopy quickly and shading emerging weed seedlings. Fertilizing and fallowing also serve to restore nutrients and soil health, which are essential for a competitive advantage for crops. Chemistry will still continue to be essential in conventional agriculture. The development and adoption of new bioengineered crops (such as Roundup Ready 2 XtendiMax® - glyphosate and dicamba, Enlist E3™ - 2,4-D choline, glyphosate, and glufosinate tolerant) will broaden the usage of existing SOA, providing alternatives for farmers for control; furthermore, the discovery and development of new herbicides molecules will hopefully provide alternatives for farmers now that it is clear that glyphosate will no longer be a solo product. New technologies are also being developed that will heavily alter integrated weed management. Most importantly drone technologies that rapidly identify and map weeds may greatly impact future weed control. These

drones lead to technologies such as electric-weeding , see-and-spray (allows direct applications of herbicides not able to be broadcast), direct mechanical control, etc. that would be otherwise infeasible without robotic autonomy. Applying integrated weed management principles with upcoming technologies adoption will provide effective and sustainable weed management strategies for farmers, avoiding or slowing herbicide resistance evolution.

BIBLIOGRAPHY

Abbas, T., Zahir, Z. A., Naveed, M., & Kremer, R. J. (2018). Limitations of Existing Weed Control Practices Necessitate Development of Alternative Techniques Based on Biological Approaches. In *Advances in Agronomy* (Vol. 147, pp. 239–280). Academic Press Inc. https://doi.org/10.1016/bs.agron.2017.10.005

Abusteit, E. E.-D. O. (1993). Weed Competition in Soybeans *Glycine max* L. *Journal of Agronomy & Crop Science*.

Agostinetto, D., Silva, D. R. O. da, & Vargas, L. (2018). Soybean yield loss and economic thresholds due to glyphosate resistant hairy fleabane interference. *Arquivos Do Instituto Biológico*, *84*(0). https://doi.org/10.1590/1808-1657000022017

Assad, R., Reshi, Z. A., Jan, S., & Rashid, I. (2017). Biology of Amaranths. *Botanical Review*, *83*(4), 382–436. https://doi.org/10.1007/sl2229-017-9194-l

Bensch, C. N., Horak, M. J., & Peterson, D. (2003). Interference of Redroot Pigweed (*Amaranthus retroflexus*), Palmer Amaranth (*A. palmeri*), and Common Waterhemp (*A. rudis*) in Soybean. *Weed Science*, *51*(1), 37–43. https://www.jstor.org/stable/4046623?seq=1&cid=pdf-

Bhowmik, P. C., & Bekech, M. M. (1993). Horseweed (*Conyza canadensis*) seed production, emergence, and distribution in no-tillage and conventional-tillage corn (*Zea mays*). *Agronomy (Trends in Agril. Sci.)*, 67–71. https://doi.org/https://www.researchgate.net/publication/256505935

Buhler, D. D., & Owen, M. D. K. (1997). Emergence and Survival of Horseweed (*Conyza canadensis*). *Weed Science*, *45*(1), 98–101. https://www.jstor.org/stable/4045719?seq=1&cid=pdf-

Corbett, C. A. L., & Tardif, F. J. (2006). Detection of resistance to acetolactate synthase inhibitors in weeds with emphasis on DNA-based techniques: A review. *Pest Management Science*, *62*(7), 584–597. https://doi.org/10.1002/ps.1219

Dang, Y. P., Dalal, R. C., & Menzies, N. W. (2020). *No-till Farming Systems for Sustainable Agriculture* (Y. P. Dang, R. C. Dalal, & N. W. Menzies, Eds.). Springer International Publishing. https://doi.org/10.1007/978-3-030-46409-7

De Prado, J. L., Osuna, M. D., Heredia, A., & De Prado, R. (2005). *Lolium rigidum*, a pool of resistance mechanisms to ACCase inhibitor herbicides. *Journal of Agricultural and Food Chemistry*, *53*(6), 2185–2191. https://doi.org/10.1021/jf049481m

Deen, W., Hunt, T., & Swanton, C. (1998). Influence of temperature, photoperiod, and irradiance on the phenological development of common ragweed (*Ambrosia artemisiifolia*). *Weed Science*, *46*(5), 555–560.

Délye, C. (2013). Unravelling the genetic bases of non-target-site-based resistance (NTSR) to herbicides: A major challenge for weed science in the forthcoming decade. *Pest Management Science*, *69*(2), 176–187. https://doi.org/10.1002/ps.3318

Dickerson, C. T., & Sweet, R. D. (1971). Common Ragweed Ecotypes. *Weed Science*, *19*(1), 64–66. https://www.jstor.org/stable/4041819

Dimaano, N. G., & Iwakami, S. (2021). Cytochrome P450-mediated herbicide metabolism in plants: current understanding and prospects. In *Pest Management Science* (Vol. 77, Issue 1, pp. 22–32). John Wiley and Sons Ltd. https://doi.org/10.1002/ps.6040

Donald, W. W., Kitchen, N. R., Sudduth, & Kenneth A. (2001). Between-Row Mowing Banded Herbicide to Control Annual Weeds and Reduce Herbicide Use in No-till Soybean (*Glycine max*) and Corn (*Zea mays*). *Weed Technology*, *15*, 576–584. https://doi.org/10.1614/0890

Duke, S. O., & Powles, S. B. (2008). Glyphosate: A once-in-a-century herbicide. *Pest Management Science*, *64*(4), 319–325. https://doi.org/10.1002/ps.1518

Essl, F., Biró, K., Brandes, D., Broennimann, O., Bullock, J. M., Chapman, D. S., Chauvel, B., Dullinger, S., Fumanal, B., Guisan, A., Karrer, G., Kazinczi, G., Kueffer, C., Laitung, B., Lavoie, C., Leitner, M., Mang, T., Moser, D., Müller-Schärer, H., … Follak, S. (2015). Biological Flora of the British Isles: *Ambrosia artemisiifolia*. *Journal of Ecology*, *103*(4), 1069–1098. https://doi.org/10.1111/1365-2745.12424

Fang, H., Xu, G., Xue, X., Niu, M., & Qiao, L. (2023). Study of Mechanical-Chemical Synergistic Weeding on Characterization of Weed–Soil Complex and Weed Control Efficacy. *Sustainability (Switzerland)*, *15*(1). https://doi.org/10.3390/su15010665

Felton, W. L. (1976). *The influence of row spacing and plant population on the effect of weed competition in soybeans (*Glycine max*)*. 926–931.

Ferrell, J. A., Macdonald, G. E., & Devkota, P. (2021). *Weed Management in Soybeans 1*. https://edis.ifas.ufl.edu

Fleming, M. B., Stanley, L., Zallen, R., Chansler, M. T., Brudvig, L. A., Lowry, D. B., Weber, M., & Telewski, F. W. (2023). The 141-year period for Dr. Beal's seed viability experiment: A hybrid surprise. *American Journal of Botany*, *110*(11). https://doi.org/10.1002/ajb2.16250

Ford, L., Soltani, N., Robinson, D. E., Nurse, R. E., McFadden, A., & Sikkema, P. H. (2014). Canada fleabane (*Conyza canadensis*) control with preplant applied residual herbicides followed by 2,4-D choline/glyphosate DMA applied postemergence in corn. *Canadian Journal of Plant Science*, *94*(7), 1231–1237. https://doi.org/10.4141/CJPS2013-403

Gaines, T. A., Duke, S. O., Morran, S., Rigon, C. A. G., Tranel, P. J., Anita Küpper, & Dayan, F. E. (2020). Mechanisms of evolved herbicide resistance. In *Journal of Biological Chemistry* (Vol. 295, Issue 30, pp. 10307–10330). American Society for Biochemistry and Molecular Biology Inc. https://doi.org/10.1074/jbc.REV120.013572

Gaines, T. A., Shaner, D. L., Ward, S. M., Leach, J. E., Preston, C., & Westra, P. (2011). Mechanism of resistance of evolved glyphosate-resistant palmer amaranth (*Amaranthus palmeri*). *Journal of Agricultural and Food Chemistry*, *59*(11), 5886–5889. https://doi.org/10.1021/jf104719k

Gaines, T. A., Zhang, W., Wang, D., Bukun, B., Chisholm, S. T., Shaner, D. L., Nissen, S. J., Patzoldt, W. L., Tranel, P. J., Culpepper, A. S., Grey, T. L., Webster, T. M., Vencill, W. K., Sammons, R. D., Jiang, J., Preston, C., Leach, J. E., & Westra, P. (2010). Gene amplification confers glyphosate resistance in *Amaranthus palmeri*. *Proceedings of the*

National Academy of Sciences of the United States of America, *107*(3), 1029–1034. https://doi.org/10.1073/pnas.0906649107

Gawęda, D., Haliniarz, M., Bronowicka-Mielniczuk, U., & Łukasz, J. (2020). Weed infestation and health of the soybean crop depending on cropping system and tillage system. *Agriculture (Switzerland)*, *10*(6), 1–20. https://doi.org/10.3390/agriculture10060208

Ghosh, S., Chisti, Y., & Banerjee, U. C. (2012). Production of shikimic acid. In *Biotechnology Advances* (Vol. 30, Issue 6, pp. 1425–1431). https://doi.org/10.1016/j.biotechadv.2012.03.001

Goggin, D. E., Cawthray, G. R., & Powles, S. B. (2016). 2,4-D resistance in wild radish: Reduced herbicide translocation via inhibition of cellular transport. *Journal of Experimental Botany*, *67*(11), 3223–3235. https://doi.org/10.1093/jxb/erw120

Gould, F., Brown, Z. S., & Kuzma, J. (2018). Wicked evolution: Can we address the sociobiological dilemma of pesticide resistance? *Science*, *360*(6390), 728–732. https://www.science.org

Green, J. M. (2014). Current state of herbicides in herbicide-resistant crops. In *Pest Management Science* (Vol. 70, Issue 9, pp. 1351–1357). John Wiley and Sons Ltd. https://doi.org/10.1002/ps.3727

Grossmann, K. (2010). Auxin herbicides: Current status of mechanism and mode of action. *Pest Management Science*, *66*(2), 113–120. https://doi.org/10.1002/ps.1860

Gunsolus, J. L., & Curran, W. S. (1991). Herbicide Mode of Action and Injury Symptoms. In *order* (Vol. 612).

Gwatidzo, V., Chipomho, J., & Parwada, C. (2023). Understanding Mechanisms of Herbicide Selectivity in Agro-Ecosystems: A Review. *Advanced Chemicobiology Research*, 77–86. https://doi.org/10.37256/acbr.2120232351

Hagen, G., & Guilfoyle, T. (2002a). Auxin-responsive gene expression: genes, promoters and regulatory factors. *Plant Molecular Biology*, *49*, 373–385.

Hagen, G., & Guilfoyle, T. (2002b). Auxin-responsive gene expression: genes, promoters and regulatory factors. *Plant Molecular Biology*, *49*, 373–385.

Hamill, A. S., Jodie S, Mallory-Smith, & Carol A. (2004). Contributions of Weed Science to Weed Control and Management. *Weed Technology*, *18*, 1563–1565. https://doi.org/10.1614/0890

Han, H., Vila-Aiub, M. M., Jalaludin, A., Yu, Q., & Powles, S. B. (2017). A double EPSPS gene mutation endowing glyphosate resistance shows a remarkably high resistance cost. *Plant Cell and Environment*, *40*(12), 3031–3042. https://doi.org/10.1111/pce.13067

Heap. (2014). Global perspective of herbicide-resistant weeds. *Pest Management Science*, *70*(9), 1306–1315. https://doi.org/10.1002/ps.3696

Heap, I. (2023, September 26). *The International Herbicide-Resistant Weed Database*. Www.Weedscience.Org.

Hill, E. (2024). *History & Map of Herbicide-Resistant Weeds In Michigan*. History & Map of Herbicide-Resistant Weeds In Michigan. https://www.canr.msu.edu/pestid/resources/plant-and-weed-identification/map

Holm, L., Doll, J., Holm, E., Pancho, J., & Herberger, J. (1997). *World weeds: Natural histories and distribution*. John Wiley & Sons, Inc.

Iwakami, S., Shimono, Y., Manabe, Y., Endo, M., Shibaike, H., Uchino, A., & Tominaga, T. (2017). Copy number variation in acetolactate synthase genes of thifensulfuron-methyl resistant *Alopecurus aequalis* (shortawn foxtail) accessions in Japan. *Frontiers in Plant Science*, *8*. https://doi.org/10.3389/fpls.2017.00254

Jha, P., Norsworthy, J. K., Riley, M. B., Bielenberg, D. G., & Bridges, W. (2008). Acclimation of Palmer Amaranth (*Amaranthus palmeri*) to Shading. *Weed Science*, *56*(5), 729–734. https://doi.org/10.1614/ws-07-203.1

Jugulam, M., & Shyam, C. (2019). Non-target-site resistance to herbicides: Recent developments. *Plants*, *8*(10). https://doi.org/10.3390/plants8100417

Karrer, G., & Vitalos, M. (2009). Dispersal of *Ambrosia artemisiifolia* seeds along roads: the contribution of traffic and mowing machines. *Neobiota*, *8*, 53–60. https://www.researchgate.net/publication/290827967

Keramati, S., Pirdashti, H., Esmaili, M. A., Abbasian, A., & Habibi, M. (2008). The critical period of weed control in soybean (*Glycine max* (L.) Merr.) in north of iran conditions. *Pakistan Journal of Biological Sciences*, *11*(3), 463–467. https://doi.org/10.3923/pjbs.2008.463.467

Knezevic, S. Z., Jhala, A., & Gaines, T. (2016). Herbicide Resistance and Molecular Aspects. In *Encyclopedia of Applied Plant Sciences* (Vol. 3, pp. 455–458). Elsevier Inc. https://doi.org/10.1016/B978-0-12-394807-6.00025-3

Küpper, A., Borgato, E. A., Patterson, E. L., Netto, A. G., Nicolai, M., Carvalho, S. J. P., Nissen, S. J., Gaines, T. A., & Christoffoleti, P. J. (2017). Multiple resistance to glyphosate and acetolactate synthase inhibitors in Palmer amaranth (*Amaranthus palmeri*) identified in Brazil. *Weed Science*, *65*(3), 317–326. https://doi.org/10.1017/wsc.2017.1

Latinia, E., & Eisvand, H. R. (2021). *Soybean responses to nutrients deciency, and the possibility of detecting this deciency using chlorophyll fluorescence technique*. https://doi.org/10.21203/rs.3.rs-489649/v1

LeClere, S., Wu, C., Westra, P., & Sammons, R. D. (2018). Cross-resistance to dicamba, 2,4-D, and fluroxypyr in *Kochia scoparia* is endowed by a mutation in an AUX/IAA gene. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(13), E2911–E2920. https://doi.org/10.1073/pnas.1712372115

Menendez, J., Rojano-Delgado, M. A., & De Prado, R. (2023). *Differences in Herbicide Uptake, Translocation, and Distribution as Sources of Herbicide Resistance in Weeds*. *13*, 33. https://pubs.acs.org/sharingguidelines

Menges, R. M. (1988). Allelopathic Effects of Palmer Amaranth (*Amaranthus palmeri*) on Seedling Growth. *Weed Science*, *36*(3), 325–328. https://about.jstor.org/terms

Mockaitis, K., & Estelle, M. (2008). Auxin receptors and plant development: A new signaling paradigm. In *Annual Review of Cell and Developmental Biology* (Vol. 24, pp. 55–80). https://doi.org/10.1146/annurev.cellbio.23.090506.123214

Moretti, M. L., Bobadilla, L. K., & Hanson, B. D. (2021). Cross-resistance to diquat in glyphosate/paraquat-resistant hairy fleabane (*Conyza bonariensis*) and horseweed (*Conyza canadensis*) and confirmation of 2,4-D resistance in *Conyza bonariensis*. *Weed Technology*, *35*(4), 554–559. https://doi.org/10.1017/wet.2021.11

Moretti, M. L., Sosnoskie, L. M., Shrestha, A., Wright, S. D., Hembree, K. J., Jasieniuk, M., & Hanson, B. D. (2016). Distribution of *Conyza* sp. in Orchards of California and Response to Glyphosate and Paraquat. *Weed Science*, *64*(2), 339–347. https://doi.org/10.1614/ws-d-15-00174.1

Moretti, M. L., Van Horn, C. R., Robertson, R., Segobye, K., Weller, S. C., Young, B. G., Johnson, W. G., Douglas Sammons, R., Wang, D., Ge, X., d'Avignon, A., Gaines, T. A., Westra, P., Green, A. C., Jeffery, T., Lespérance, M. A., Tardif, F. J., Sikkema, P. H., Christopher Hall, J., … Schulz, B. (2018). Glyphosate resistance in *Ambrosia trifida*: Part 2. Rapid response physiology and non-target-site resistance. *Pest Management Science*, *74*(5), 1079–1088. https://doi.org/10.1002/ps.4569

Mousa, R., Abouzied, K., & El Attar, M. (2022). Estimation of Critical Period for Weed Control in Field Pea (*Pisum sativum* L.) Under Different Seeding Rates. *Alexandria Science Exchange Journal*, *43*(4), 625–635. https://doi.org/10.21608/asejaiqjsae.2022.274019

MSU-PPD. (2024). *Plant & pest diagnostics*. Plant & Pest Diagnostics. Https://Www.Canr.Msu.Edu/Pestid/Index.

Mulligan, G. A., & Findlay, J. N. (1970). Reproductive systems and colonization in Canadian weeds. *Can. J. Bot.*, *48*, 859–860.

Norsworthy, J. K., Ward, S. M., Shaw, D. R., Llewellyn, R. S., Nichols, R. L., Webster, T. M., Bradley, K. W., Frisvold, G., Powles, S. B., Burgos, N. R., Witt, W. W., & Barrett, M. (2012). Reducing the Risks of Herbicide Resistance: Best Management Practices and Recommendations. *Weed Science*, *60*(SP1), 31–62. https://doi.org/10.1614/ws-d-11-00155.1

Pan, L., Yu, Q., Han, H., Mao, L., Nyporko, A., Fan, L. J., Bai, L., & Powles, S. (2019). Aldo-keto reductase metabolizes glyphosate and confers glyphosate resistance in Echinochloa colona. Plant Physiology, 181(4), 1519–1534. https://doi.org/10.1104/pp.19.00979

Patterson, E. L., Pettinga, D. J., Ravet, K., Neve, P., & Gaines, T. A. (2018). Glyphosate resistance and EPSPS gene duplication: Convergent evolution in multiple plant species. Journal of Heredity, 109(2), 117–125. https://doi.org/10.1093/jhered/esx087

Peterson, M. A., McMaster, S. A., Riechers, D. E., Skelton, J., & Stahlman, P. W. (2016). 2,4-D Past, Present, and Future: A Review. *Weed Technology*, *30*(2), 303–345. https://doi.org/10.1614/wt-d-15-00131.1

Pingali, P. L. (2012). Green revolution: Impacts, limits, and the path ahead. In *Proceedings of the National Academy of Sciences of the United States of America* (Vol. 109, Issue 31, pp. 12302–12308). https://doi.org/10.1073/pnas.0912953109

Powles, S. B. (2008). Evolved glyphosate-resistant weeds around the world: Lessons to be learnt. *Pest Management Science*, *64*(4), 360–365. https://doi.org/10.1002/ps.1525

Rabin, J. (2012). *A Review of Cultural Weed Control Practices sustainable-farming.rutgers.edu/a-review-of-cultural-weed-control-practices*.

Ray, T. B. (1984). Site of Action of Chlorsulfuron: Inhibition of Valine and Isoleucine Biosynthesis in Plants. *Plant Physiol*, *75*, 827–831. https://academic.oup.com/plphys/article/75/3/827/6079908

Robert, M., Thomas, A., & Bergez, J. E. (2016). Processes of adaptation in farm decision-making models. A review. *Agronomy for Sustainable Development*, *36*(4). https://doi.org/10.1007/s13593-016-0402-x

Roberts, J., & Florentine, S. (2022). A review of the biology, distribution patterns and management of the invasive species *Amaranthus palmeri* S. Watson (Palmer amaranth): Current and future management challenges. In *Weed Research* (Vol. 62, Issue 2, pp. 113–122). John Wiley and Sons Inc. https://doi.org/10.1111/wre.12520

Sammons, R. D., & Gaines, T. A. (2014). Glyphosate resistance: State of knowledge. In *Pest Management Science* (Vol. 70, Issue 9, pp. 1367–1377). John Wiley and Sons Ltd. https://doi.org/10.1002/ps.3743

Sarangi, D., Tyre, A. J., Patterson, E. L., Gaines, T. A., Irmak, S., Knezevic, S. Z., Lindquist, J. L., & Jhala, A. J. (2017). Pollen-mediated gene flow from glyphosate-resistant common waterhemp (*Amaranthus rudis* Sauer): Consequences for the dispersal of resistance genes. *Scientific Reports*, *7*. https://doi.org/10.1038/srep44913

Sauer, J. (1955). Revision of the Dioecious Amaranths. *Madroño*, *13*(1), 5–46.

Sebayang, H. T., & Rifai, A. P. (2018). The effect of soil tillage system and weeding time on the growth of weed and yield of soybean (*Glycine max* (L.) Merril). *Journal of Degraded and Mining Lands Management*, *5*(3), 1237–1243. https://doi.org/10.15243/jdmlm.2018.053.1237

Shaner, D. L., Nadler-Hassar, T., Brien Henry, W., & Koger, C. H. (2005). A rapid in vivo shikimate accumulation assay with excised leaf discs. In *Weed Science* (Vol. 53).

Soltani, N., DIlle, J. A., Burke, I. C., Everman, W. J., Vangessel, M. J., Davis, V. M., & Sikkema, P. H. (2017). Perspectives on Potential Soybean Yield Losses from Weeds in North America. In *Weed Technology* (Vol. 31, Issue 1, pp. 148–154). Weed Science Society of America. https://doi.org/10.1017/wet.2016.2

Soltani, N., Shropshire, C., & Sikkema, P. H. (2021). Control of glyphosate-resistant horseweed (*Conyza canadensis*) with tiafenacil mixes in corn. *Weed Technology*, *35*(6), 908–911. https://doi.org/10.1017/wet.2021.44

Sprent, J. (2011). Microbes for Legume Improvement. By M. S. Khan, A. Zaidi and J. Musarrat. New York: Springer (2010), pp. 534, £153.00. ISBN 978-3-211-99752-9. *Experimental Agriculture*, *47*(2), 415–415. https://doi.org/10.1017/s0014479710001250

v

Steckel, L. E., & Gwathmey, C. O. (2009). Glyphosate-Resistant Horseweed (*Conyza canadensis*) Growth, Seed Production, and Interference in Cotton. *Weed Science*, *57*(3), 346–350. https://doi.org/10.1614/ws-08-127.1

Takano, H. K., Mendes, R. R., Scoz, L. B., Lopez Ovejero, R. F., Constantin, J., Gaines, T. A., Westra, P., Dayan, F. E., & Oliveira, R. S. (2019). Proline-106 EPSPS mutation imparting glyphosate resistance in goosegrass (*Eleusine indica*) Emerges in South America. *Weed Science*, *67*(1), 48–56. https://doi.org/10.1017/wsc.2018.71

Tani, E., Chachalis, D., & Travlos, I. S. (2015). A Glyphosate Resistance Mechanism in *Conyza canadensis* Involves Synchronization of EPSPS and ABC-transporter Genes. *Plant Molecular Biology Reporter*, *33*(6), 1721–1730. https://doi.org/10.1007/s11105-015-0868-8

Todd, O. E., Figueiredo, M. R. A., Morran, S., Soni, N., Preston, C., Kubeš, M. F., Napier, R., & Gaines, T. A. (2020). Synthetic auxin herbicides: finding the lock and key to weed resistance. In *Plant Science* (Vol. 300). Elsevier Ireland Ltd. https://doi.org/10.1016/j.plantsci.2020.110631

Tranel, P. J., Wright, & Terry R. (2002). Resistance of weeds to ALS-inhibiting herbicides: what have we learned? *Weed Science*, *50*(6), 700–712. https://doi.org/10.1614/0043

Trezzi, M. M., Balbinot Jr., A. A., Benin, G., Debastiani, F., Patel, F., & Miotto Jr., E. (2013). Competitive ability of soybean cultivars with horseweed (*Conyza boranensis*). *Planta Daninha*, *31*(3), 543–550.

Tzin, V., & Galili, G. (2010). New Insights into the shikimate and aromatic amino acids biosynthesis pathways in plants. In *Molecular Plant* (Vol. 3, Issue 6, pp. 956–972). Oxford University Press. https://doi.org/10.1093/mp/ssq048

Umbarger, H. E. (1978). AMINO ACID BIOSYNTHESIS AND ITS REGULATION. In *Annual review of biochemistry* (Vol. 47, Issue 1, pp. 533–606). www.annualreviews.org

USDA. (2023, July). *Agriculture Across Michigan*. https://www.nass.usda.gov/Quick_Stats/Ag_Overview/stateOverview.php?state=MICHIGAN

Van Horn, C. R., Moretti, M. L., Robertson, R. R., Segobye, K., Weller, S. C., Young, B. G., Johnson, W. G., Schulz, B., Green, A. C., Jeffery, T., Lespérance, M. A., Tardif, F. J., Sikkema, P. H., Hall, J. C., McLean, M. D., Lawton, M. B., Sammons, R. D., Wang, D., Westra, P., & Gaines, T. A. (2018). Glyphosate resistance in *Ambrosia trifida*: Part 1. Novel rapid cell death response to glyphosate. *Pest Management Science*, *74*(5), 1071–1078. https://doi.org/10.1002/ps.4567

Walsh, T. A., Neal, R., Merlo, A. O., Honma, M., Hicks, G. R., Wolff, K., Matsumura, W., & Davies, J. P. (2006). Mutations in an auxin receptor homolog AFB5 and in SGT1b confer resistance to synthetic picolinate auxins and not to 2,4-dichlorophenoxyacetic acid or indole-3-acetic acid in Arabidopsis. *Plant Physiology*, *142*(2), 542–552. https://doi.org/10.1104/pp.106.085969

Wang, & Ching-Yuh. (2001). Effect of Glyphosate on Aromatic Amino Acid Metabolism in Purple Nutsedge (*Cyperus rotundus*). *Weed Technology*, *15*(4), 628–635. https://doi.org/10.1614/0890

Ward, S. M., Webster, T. M., & Steckel, L. E. (2013). Palmer Amaranth (*Amaranthus palmeri*): A Review. *Weed Technology*, *27*(1), 12–27. https://doi.org/10.1614/wt-d-12-00113.1

Weaver, S. E. (2001). The biology of Canadian weeds. 115. *Conyza canadensis*. *Canadian Journal Plant Science*, *81*, 867–875. https://doi.org/https://doi.org/10.4141/P00-196

Weaver, S. E., & McWilliams, E. L. (1980). The Biology of Canadian Weeds. 44. *Amaranthus retroflexus* L., *A. powellii* S. Wats. and *A. hybridus* L. *Can. J.* , *60*, 1215–1234.

Yang, Y. Y., & Kim, J. G. (2016). The optimal balance between sexual and asexual reproduction in variable environments: A systematic review. In *Journal of Ecology and Environment* (Vol. 40, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s41610-016-0013-0

Zeder, M. A. (2011). The origins of agriculture in the Near East. *Current Anthropology*, *52*(SUPPL. 4). https://doi.org/10.1086/659307

Zheng, D., Kruger, G. R., Singh, S., Davis, V. M., Tranel, P. J., Weller, S. C., & Johnson, W. G. (2011). Cross-resistance of horseweed (*Conyza canadensis*) populations with three different ALS mutations. *Pest Management Science*, *67*(12), 1486–1492. https://doi.org/10.1002/ps.2190

CHAPTER II: OPTIMIZING MOLECULAR ASSAYS FOR GLYPHOSATE AND ALS-INHIBITOR RESISTANCE DIAGNOSTICS IN FOUR WEED SPECIES

ABSTRACT

Herbicide-resistant weeds pose a threat to food production in modern agriculture, causing US\$32 billion dollars in crop production losses worldwide. In Michigan, highly troublesome and widespread weeds include waterhemp, Palmer amaranth, common ragweed, and horseweed, with accessions that are resistant to glyphosate (Group 9) and ALS-inhibitors (Group 2), major herbicide sites of action utilized in soybean and corn cropping systems. Molecular assays for rapid resistance diagnostics to confirm the in-field status of herbicide resistance can assist with more effective, timely, and proactive management. In this research, we developed and tested PCR-based assays to identify target-site resistance mechanisms to both herbicide groups through Sanger sequencing and *EPSPS* copy number variation. Nine different SNPs were identified in five *ALS* positions known to confer herbicide resistance among all species surveyed. Pro197Ser was the most frequent in horseweed and common ragweed accessions, whereas Trp574Leu was the predominant mutation in Palmer amaranth and waterhemp. Four horseweed accessions contained the Pro106Ser mutation in the *EPSPS* gene, which confers resistance to glyphosate. Additionally, waterhemp and Palmer amaranth had 2-7 and 20-160 copies of *EPSPS*, respectively. The assays were validated by comparing genotyping of several field-collected accessions of unknown resistance status with known resistant and susceptible accessions. The efficacy of genotyping assays was > 98%, and required only two days, confirming that molecular assays are a robust tool for rapid resistance diagnostics. These assays can help growers evaluate herbicide resistance status in weed populations within the same growing season, allowing them to adopt effective management practices.

KEYWORDS

Amaranthus, Diagnostics, Herbicide Resistance, Horseweed, Molecular Biology, Ragweed

INTRODUCTION

Herbicide-resistant weeds are one of the major threats to sustainable, profitable agriculture, causing approximately 32 billion dollars in crop production losses annually in the United States (Pimentel et al., 2000; USDA, 2015). Currently, there are 272 species of weeds with resistance to at least one site of action, with many populations displaying multiple and/or cross-resistance (Heap, 2023). Furthermore, resistance has evolved at least once to 21 of the 31 known herbicide sites of action (Heap, 2023). The causes of herbicide resistance are broadly classified into two major categories: target- (TSR) and non-target-site resistance (NTSR) mechanisms. TSR mechanisms include mutations in the gene that codifies the herbicide target enzyme which in turn leads to reduced herbicide binding, or increased production of the herbicide target enzyme, often caused by gene copy number variation (CNV) (Gaines et al., 2020). NTSR mechanisms include reduced or enhanced herbicide detoxification, vacuolar sequestration, and reduced absorption/translocation (Jugulam & Shyam, 2019). Herbicide resistance evolves when relatively rare alleles in a population are selected for by the repeated use of the same chemistry or chemistries with the same target enzyme. Due to their high efficiency, relatively low cost, enhanced crop safety, and ease of use, herbicides are ubiquitous in row crop farming in the United States making herbicide resistance all but inevitable in weeds that compete with those crops. In Michigan row crops some of the most troublesome weeds are horseweed (*Conyza canadensis*), Palmer amaranth (*Amaranthus palmeri*), waterhemp (*Amaranthus tuberculatus*), and common ragweed (*Ambrosia artemisiifolia*). In the past, these weeds were effectively controlled in other United States regions and countries with herbicides that inhibit the acetolactate synthase (ALS, Group 2) and *5-enolpyrovylshikimate synthase* (EPSPS, Group 9). However, the repeated use of these herbicides has led to resistance to these site-of-action, with first report in Michigan in 2004 by the Michigan State University's Plan and Pest Diagnostics (MSU-PPD, 2024).

TSR to glyphosate and ALS-inhibitors is often found in Palmer amaranth, waterhemp, common ragweed, and horseweed. To date, nine amino acids positions in the ALS enzyme were discovered in weed species conferring resistance to ALS-inhibiting herbicides (Table 2.1). The most common ALS mutations are a Tryptophan to Leucine replacement at amino acid position 574 (Trp574Leu) and several changes to the Proline at position 197 (Pro197---). TSR to ALS-inhibitors have previously been identified in horseweed, common ragweed, Palmer amaranth, and waterhemp, and several mutations have been previously reported for each species (summarized in

Table 2.1). Resistance to glyphosate (the only commercially used Group 9 herbicide) can also be caused by mutations in its target protein, EPSPS (Table 2.1). A mutation in Proline to Serine at position 106 (Pro106Ser) is the most common and has been identified in horseweed (Beres et al., 2020), Palmer amaranth (Kaundun et al., 2019), and waterhemp (Nandula et al., 2013). However, the most common glyphosate resistance mechanism for Palmer amaranth and waterhemp is extra *EPSPS* gene copies, which results in an overproduction of EPSPS protein and therefore the recommended dose is no longer sufficient to control these plants (Dillon et al., 2017; Koo et al., 2018). It should be noted that the presence of TSR does not eliminate the co-occurrence of NTSR mechanism in the same plant, and that different mechanisms can be additive (Laforest et al., 2021; Page et al., 2018).

One of the most important tasks in the battle against herbicide-resistant weeds is to quickly and accurately diagnose resistant populations, so that proper control measures can be implemented – the faster resistance can be determined and management practices altered, the greater the chances of delaying its spread. This is similar to epidemiological principles of disease control (Comont & Neve, 2021). We distinguish diagnostics methodologies into two large groups, so-called 'traditional diagnostics' and 'molecular diagnostics'. The traditional dose-response curve (DRC) method to diagnose herbicide resistance (Burgos et al., 2013) has been used for almost 40 years (Carpenter, 1986), but it can have severe time and space limitations, besides being labor-intensive. One DRC experiment takes at minimum 30 days from the time of seed germination until the final evaluation, thus it is not a suitably quick diagnostic method to inform critical periods for weed control. Generally, results of DRC experiments cannot be utilized in the same growing season for weed control, resulting in economic consequences, such as the need of additional herbicide applications, and contribution of herbicide-resistant seeds into the soil seedbank. Molecular diagnostic tools can be a remedy for this limitation. These assays are becoming more cost-effective, can be run on any tissue with DNA (even seeds from the seedbank), and are effective for several common mechanisms, especially TSR. The speed of molecular diagnostics allows growers to take action immediately, reducing the negative economic and environmental costs associated with herbicide-resistant weeds. Molecular diagnostics provide the additional benefit of describing the mechanism of resistance (TSR and NTSR) which may be useful from a basic biology or evolutionary standpoint (Sarangi et al., 2017). TSR mechanisms are common and relatively easy to diagnose. For TSR mechanisms, DNA is extracted followed by either PCR diagnostics like

KASP or TaqMan or simply by Sanger sequencing of the target gene (Cutti et al., 2021; Kutasy et al., 2021). NTSR diagnostics on the other hand may require careful plant tissue collection, gene expression detection through qPCR, or biochemical work such as high-pressure liquid chromatography (Han et al., 2021; Pan et al., 2019). It should be noted that molecular diagnostic methods can only detect if the herbicide target is altered or overexpressed; if individuals tested have a resistance mechanism different than the one tested, for instance as NTSR, it is possible to call a false negative. Molecular diagnostics should only be used for confirming resistance, not confirming susceptibility.

Scientists have developed many non-molecular, quick herbicide resistance diagnostic tests for different herbicides and weed species, such as leaf-disc assays in Palmer amaranth (Wu et al., 2021), or the quick agar assay (RISQ test) in *Lolium* spp. (Kaundun et al., 2011). These tests, however, do not provide any herbicide resistance mechanism information (TSR or NTSR) and need to be thoroughly validated with reference lines. Molecular diagnostic techniques can provide faster results, in addition to providing information on DNA mutations or genomic rearrangements. Considering the importance of early herbicide resistance detection and the reproducibility of molecular markers, the objective of this study was to design and optimize a few molecular diagnostics assays to identify glyphosate and ALS resistance in horseweed, Palmer amaranth, waterhemp, and common ragweed, the most prevalent resistant species in Michigan. Additionally, this study presents improved primers and protocols for these target sites to robustly work on all field-collected samples in the state and all greenhouse-grown plants, valuable information for the adoption of these assays as reliable tools for resistance identification.

MATERIALS AND METHODS

Plant material and DNA extraction

Five accessions of Palmer amaranth, waterhemp, horseweed, and common ragweed were collected from soybean and corn producing areas across multiple counties in Michigan (hereafter referred as "greenhouse accessions"). Accessions are defined as seeds from one individual on a farmer's property. These accessions were stored at 4°C and further used to develop and test molecular markers to confirm the occurrence of common TSR mechanisms for glyphosate and ALS-inhibitors reported in each species (Table 2.1). Previously, these accessions were phenotypically characterized as either resistant or susceptible to the labeled rate of glyphosate (1090 g a.e. ha^{-1}, Roundup PowerMax®), thifensulfuron (4.5 g a.e. ha^{-1}, Harmony SG®), and

cloransulam (17.64 g a.e. ha^{-1}, FirstRate$^®$) by Michigan State University (MSU) Plant & Pest Diagnostics (MSU-PPD, 2024).

For the genotyping experiments, seeds were broadcast in plastic trays filled with potting soil, and upon emergence, seedlings were transplanted into individual pots. Ten individuals from five collections of each species were sampled for DNA extraction. Approximately 100 mg of young leaf tissue was collected from each individual plant, and the DNA was extracted using the DNeasy$^®$ Plant Pro kit (Qiagen, Strasse 1, 40724 Hilden, Germany) with one modification to the manufacturer protocol. The lysis buffer was added to the tubes containing the leaf sample and two metal beads, followed by a grinding step using a Tissue Lyzer II (Qiagen) with frequency of 30 oscillations per second (30/s) for 2 mins. The subsequent steps were performed following the protocol without modifications. DNA was quantified in a Nanodrop One (Thermo Scientific, 5225 Verona Rd., Madison, WI 53711) and diluted to 50 ng µL^{-1}.

To verify the robustness of the assays and their applicability to field collections, 14, 3, 21 and 19 additional field samples of horseweed, Palmer amaranth, waterhemp, and common ragweed, respectively, were tested with the molecular assays developed. These samples were collected and sent by growers and MSU Extension Educators from different counties in Michigan, and hereafter are referred to as "field collections".

ALS and EPSPS gene sequencing

Several primers pairs were designed to amplify large fragments of the *ALS* gene from each species so that it could be sequenced, covering the most frequent known herbicide-resistance mutations in each species. Primers were designed for waterhemp, horseweed, and common ragweed based on the sequences available in GenBank (EF157821.1, HM067014.1, MT415954.1, and KX870184.1, respectively), and for Palmer amaranth we used the same primers described in Whaley et al. (2006) (Figure 2.1, Table 2.2). After trying several combinations, primers that worked most robustly (i.e. on all samples, greenhouse and field accessions) are reported here (Table 2.2).

To investigate the occurrence of SNPs in horseweed, , we identified three *EPSPS* genes in the published genome by protein function annotation, EPSPS1, EPSPS2, and EPSPS3 (Genbank: AY545666.1, AY545667.1, and AY545668.1) (Laforest et al, 2020). One of the copies (*EPSPS3*) was incomplete, missing four of the expected 8 exons, and therefore not a functional copy of *EPSPS*. To the best of our knowledge, it was unclear whether mutations in the *EPSPS1*, *EPSPS2*,

or both, cause resistance in horseweed. We tested the primers from Beres et al. (2020), and observed sequence similarity with EPSPS2, and then designed new primer set to amplify EPSPS1 (Table 2.2).The *EPSPS1* and *EPSPS2* specificity was checked based on fragment size in agarose gel (Figure 2.8). Glyphosate resistance in Palmer amaranth and waterhemp were tested for CNV (next section). Additionally, we sequenced *EPSPS* gene of three individuals from five accessions of waterhemp in order to find Pro106 mutation (as previously found and indicated in Table 2.1), which was not found. Common ragweed was not tested for TSR because the mechanism in this species had not been clarified until recent description, where Laforest et al. (2024) found that the TSR in EPSPS is through CNV.

All PCR were made with 12.5 µL of GoTaq G2 Green Master Mix (Promega, 2800 Woods Hollow Road, Madison, WI 53711), 1 µL of forward and 1 µL reverse primers (10 mM), 8.5 µL of molecular grade water, and 2 µL of DNA (50 ng µL^{-1}). PCR cycles were as follows: 95°C for 5 min, and 39 cycles of 95°C for 30 s, annealing temperature (Table 2.2) for 30 s, 72°C during the extension time (Table 2.2), and 72°C for 8 min. The amplification was verified using agarose gel electrophoresis at 1.5%. Further, PCR products were purified using Wizard SV Gel and PCR Clean-up System (Promega), and samples were sent to the MSU Genomics Core for Sanger sequencing. *ALS* and *EPSPS* sequences were translated using Expasy and aligned using ApE (Davis & Jorgensen, 2022). The same sequence used to design primers from the GenBank were included as reference when aligning for comparisons.

EPSPS copy number variation

Palmer amaranth and waterhemp samples were tested for *EPSPS* CNV. The reactions were prepared with 10 µL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, 1000 Alfred Nobel Drive Hercules, California 94547), 1 µL of forward and 1 µL of reverse primers (10 mM), 2 µL of DNA (10 ng µL^{-1}), and 6 µL of molecular grade water, totaling 20 µL reactions. Forward and reverse primers of *EPSPS*, and housekeeping control (*ALS* and *β-tubulin*) were previously published (Gaines et al., 2010, Godar et al., 2015) and are listed in Table 2.3. The real-time qPCR cycles started with 3 min at 95°C, followed with 30 cycles of 95°C and 1 min at 60°C, with a standard melting curves included in a CFX 96 Real-time system thermocycler (Bio-Rad). The assay was performed with five biological replicates per accession, and two technical replicates. A known susceptible sample was included in each run for comparison. The *EPSPS* copy number was estimated as relative to *ALS* and/or *β-tubulin* in a known susceptible sample.

RESULTS

ALS sequencing

Nine different SNPs were identified in five *ALS* positions known to confer herbicide resistance among all greenhouse accessions and species sequenced (Figure 2.2). The Pro197 substitution showed more diversity of amino acid replacement, Pro197Ile, Pro197Thr, Pro197Leu, and Pro197Ser than others identified. Overall, 36% of Palmer amaranth contained the *ALS* wild-type (WT) susceptible allele. One greenhouse accession of Palmer amaranth did not have any *ALS* mutations, and the remaining four accessions showed a mixture of resistant plants carrying different mutations and susceptible plants. The *ALS* mutations identified in Palmer amaranth were Pro197Thr, Pro197Ile, Pro197Ile/Thr, Asp376Glu, Trp574Leu, and Ser653Asn (Figure 2.2; Figure 2.3; Table 2.4). Heterozygosity for *ALS* mutation was present in 42% of the Palmer amaranth plants screened (Figure 2.3). Waterhemp greenhouse accessions did not show any completely susceptible accession. Four out of five accessions had a mixture of resistant and susceptible plants, and one accession had only resistant plants (Figure 2.2). The presence of resistant alleles was observed in 74% out of 50 plants sequenced. The *ALS* mutations identified in waterhemp were Pro197Thr, Pro197Ser, and Trp574Leu. *ALS* heterozygosity was present in 53% of resistant waterhemp plants screened (Figure 2.3). Two common ragweed greenhouse accession out of five showed all plants with *ALS* WT allele, while the other three showed a mixture of *ALS* mutations and WT allele. The mutations identified in common ragweed were Pro197Leu, Pro197Ser, Ala205Val, and Trp574Leu (Figure 2.2; Table 2.4). *ALS* heterozygosity was present in 28% of the plants screened (Figure 2.3). Horseweed, the only self-pollinated species studied here, showed two accessions with all plants carrying *ALS* WT allele and three accessions with all plants carrying *ALS* mutations (Figure 2.2). The ALS mutations identified were Pro197Ser and Pro197Thr (Figure 2.2; Table 2.4). No genotype mixture and heterozygosity were identified in horseweed accessions.

Horseweed EPSPS sequencing

Three copies of *EPSPS* gene were identified in the horseweed genome (Laforest et al., 2020) (GenBank: AY545666.1, AY545667.1, and AY545668.1). Two of these (*EPSPS1* and *EPSPS2*) were deemed potentially functional as they were complete with the requisite eight exons and had the proper start and stop codons (Figure 2.7), while one (*EPSPS3*) was considered a pseudogene as it did not encode a full-length EPSPS protein. *EPSPS1* sequencing did not show any mutation in all greenhouse accessions sequenced; however, four out of five accessions showed

the Pro106Ser mutation in *EPSPS2*, no heterozygosity was identified (Table 2.4; Figure 2.4). One accession had *EPSPS1* and *EPSPS2* WT allele for all 10 plants sequenced and was susceptible.

Palmer amaranth and waterhemp EPSPS copy number variation

EPSPS copy number was analyzed using both *ALS* and *β-tubulin* as housekeeping genes for Palmer amaranth (Figure 2.5A) and waterhemp (Figure 2.5B). Consistently, when the *ALS* gene was used as a control, it predicted twice as many *EPSPS* copies when compared to *β-tubulin* when running on the same DNA sample (Figures 2.5A and 2.5B). The *β-tubulin* gene was therefore rejected as a control gene, due to presence of multiple copies in the genome and the potential for multi-locus priming, *ALS* was used in further *EPSPS* CNV prediction. In Palmer amaranth individuals from the greenhouse accessions, an *EPSPS* copy number increase was found in all plants in four out of the five accessions, with 20 individuals out of 25 tested in total, with CNV ranging between 20-160 additional copies (Figure 2.5C), and one population being fully susceptible. *EPSPS* copy numbers varied across accessions and within individuals from the same accession. In waterhemp greenhouse accessions, an *EPSPS* copy number increase was found in seven individuals (across three accessions) out of the total 25 individuals (five accessions), ranging from 2-7 copies. The *EPSPS* CNV varied within and across waterhemp accessions (Figures 2.5D).

Validation of assays in field collections

Palmer amaranth, waterhemp, common ragweed, and horseweed were collected from fields spanning eight counties around Michigan to validate the molecular markers. First, the *ALS* gene from all individuals from all four species was sequenced to detect mutations (Figure 2.6A), while Palmer amaranth and waterhemp individuals were used to validate the *EPSPS*-CNV (Figure 2.6B). Individuals with and without *ALS* mutations were detected in the field for all four studied species. Palmer amaranth was collected in Allegan county and two collections carried the *ALS* gene with the Trp574Leu mutation and one with the WT allele (Table 2.5). Out of 19 waterhemp collections across seven counties, 14 did not show any *ALS* mutation, while five had Trp574Leu or Ser653Asn, and no Pro197 substitutions (Table 2.5). The Ser653Asn had not been detected in any waterhemp greenhouse accessions. Out of 19 common ragweed collections, two contained the Trp574Leu mutation, one the Ala205Val mutation, and none had the Pro197 substitution (Table 2.5). Out of 14 horseweed collections, eight had mutations at the Pro197 position, either Pro197Ser or Pro197Leu (Table 2.5). The Pro197Leu substitution was not detected in the greenhouse accessions.

The *EPSPS*-CNV assay showed that two out of three field collections of Palmer amaranth had low CNV, varying between 1 and 7 copies of *EPSPS* (Table 2.5, Figure 2.6B), and one collection was resistant with 21 copies of *EPSPS* (Table 2.5, Figure 2.6B). All populations tested were from Allegan county, as Palmer amaranth incidence is predominantly in the southwest region of Michigan. Five of the 21 field collections of waterhemp had more than five *EPSPS* copies which we consider resistant (Table 2.5, Figure 2.6B), varying between one to eight *EPSPS* copies. Glyphosate-resistant waterhemp individuals were primarily found in Allegan county as well.

DISCUSSION

ALS sequencing

SNPs that confer resistance to *ALS*-inhibitors were found in most greenhouse grown accessions and many of the field collections for Palmer amaranth, waterhemp, common ragweed, and horseweed. The frequency and nature of these SNPs vary from species to species and accession to accession. Overall, mutations at the Pro197 and Trp574 positions predominated all others, however rare mutations at Ala205 and Asp376 were also detected in some individuals. Populations that had these rare mutations generally had another SNP as well, with Ala205 and Asp376 being less frequent. There are biological variations regarding the phenotype that each allele results in different species; however, having several SNPs in a population may result in a greater evolutionary advantage if several ALS chemistries are being used as some may be favored by certain chemistries over others. Mutations at Pro197 are known to confer high-level resistance (greater than 10-fold) to sulfonylurea (Guttieri et al., 1992), pyrithiobac-sodium (pyrimidinyl benzoates), and triazolopyrimidine chemical families (Matzrafi et al., 2015; Zheng et al., 2011). Pro197Thr confers high resistance to sulfonylureas and triazolopyrimidines (greater than 10-fold) in kochia (*Bassia scoparia*) (Guttieri et al., 1995), but remains susceptible to imidazolinones. However, the same mutation in prickly lettuce (*Lactuca serriola*), confers moderate resistance (lower than 10-fold) to imidazolinones and triazolopyrimidine, and high resistance (greater than 10-fold) to sulfonylureas (Preston et al., 2006). The Ala205Val mutation is relatively unique among the plants we sequenced; however, it has been previously observed in common ragweed (Loubet et al., 2021). It has been also reported for other species, including common cocklebur (*Xanthium strumarium*) (Bernasconi et al., 1995), common sunflower (*Helianthus annuus*) (Kolkman et al., 2004), redroot pigweed (*Amaranthus retroflexus*) (McNaughton et al., 2005), eastern black nightshade (*Solanum ptycanthum*) (Ashigh & Tardif, 2009), horseweed (Matzrafi et al., 2015), and

annual bluegrass (*Poa annua*) (Brosnan et al., 2016), causing at least moderate resistance to all chemistry families. Palmer amaranth populations had a relatively unique mutation at Asp376, however, this SNP was previously reported in Kohrt et al. (2017) and Palmieri et al. (2022). Asp376Glu confers high level resistance to all ALS chemistries (imidazolinones, pyrimidinyl benzoates, sulfonylureas, triazolopyrimidines, and triazolinones) in Powell amaranth (*Amaranthus powellii*) according to Ashigh et al. (2009), and high levels of resistance to imidazolinones in redroot pigweed (Huang et al., 2016). The mutation Trp574Leu confers high level resistance to imidazolinone, sulfonylurea, and triazolopyrimidine chemical groups in waterhemp (Patzoldt et al., 2001). This mutation is relatively common in weeds in general, as it is reported in 41 species to date (Heap, 2023), including waterhemp (Patzoldt & Tranel, 2007), kochia (Foes et al., 1999), Palmer amaranth (Molin et al., 2016), and annual bluegrass (*Poa annua*) (McElroy et al., 2013). One Palmer amaranth greenhouse accession contained the mutation Ser653Asn (Figure 2.2, Tables 2.4 and 2.5). This SNP confers moderate to high level resistance to all chemical families in the ALS group (such as imidazolinones, sulfonylureas, triazolinones, and others) across narrow and broadleaf species including Palmer amaranth (Molin et al., 2016), waterhemp (Patzoldt & Tranel, 2007), and wild oat (*Avena fatua*) (Beckie et al., 2012); this mutation is currently reported in six species to date (Heap, 2023).

In horseweed, all individuals with an ALS mutation were homozygous and furthermore, all populations were homogenously resistant or susceptible. This is in stark contrast to Palmer amaranth, waterhemp, and common ragweed, where heterozygosity was reported at 42%, 64%, and 28% respectively, with no collections being homogeneous. In fact, several of these populations still had high proportions of susceptible individuals. This is most likely due to horseweed being primarily self-pollinating while the others can or must obligately outcross. Specifically, we would like to point out the importance of manually looking at Sanger sequencing chromatograms when calling resistance SNPs, as heterozygous locations are often miscalled WT by sequence calling software, although they would still be resistant as these mutations are known to be dominant (Figure 2.3B). Misinterpretation of heterozygous loci as WT would lead to false negatives and overpredict the number of susceptible individuals from field collections. Overall, the most frequent SNPs were in the Pro197 and Trp574 positions which suggests that selection with ALS chemistry favors these mutations over others, as they provide broad spectrum ALS resistance.

Glyphosate resistance mechanisms in horseweed

Despite horseweed being among the first weeds to evolved resistance to glyphosate (VanGessel, 2001), the molecular basis of resistance in this species is still somewhat ambiguous. Glyphosate resistance in horseweed is thought to be primary due to decreased translocation to the growing points through sequestration in the vacuole (Xe et al. 2010), however, the genetic mechanisms of this phenotype have not yet been identified (Cardinali et al., 2015; Feng et al., 2004; Koger & Reddy, 2005). Furthermore, a mutation in *EPSPS* at the Pro106Ser locus has also been reported in horseweed to cause resistance to glyphosate, as well as in eleven other species including moderate resistance in goosegrass (*Eleusine indica*) (Kaundun et al., 2008) waterhemp (*Amaranthus tuberculatus (=A. rudis)*) (Nandula et al., 2013), and high levels in Palmer amaranth (Dominguez-Valenzuela et al., 2017). Recently the genome of horseweed has been published, which revealed that horseweed has three copies of *EPSPS*, two of which seem functional, while a third seems to be incomplete and, therefore, pseudogenized (Laforest et al., 2020). Many Asteraceae have three copies due to two polyploid events in the family's evolutionary history (Huang et al., 2016). In this project we developed *EPSPS*-copy-specific primers that amplify either *EPSPS1* or *EPSPS2* but not both simultaneously, so that resistance SNPs can be properly attributed to each of these homologs. The Pro106Ser in the *EPSPS2* gene was found in four out of five greenhouse populations with only one being glyphosate-resistant without an *EPSPS* SNP in either *EPSPS1* or *EPSPS2* (Table 2.4). The Pro106 SNP in *EPSPS2* has been shown to cause intermediate resistance to glyphosate in horseweed (Page et al., 2018). In contrast to the CNV resistance mechanisms in the *Amaranthus* species, a mutation in the Pro106 locus changes EPSPS's interaction with glyphosate, decreasing binding. It also changes EPSPS's affinity for its native substrate, phosphoenolpyruvate (PEP) carboxylase, sometimes causing a fitness penalty (Vila-Aiub, 2019); however, with a still unmutated copy of *EPSPS (i.e. EPSPS1)*, it is unclear whether that would be the case in horseweed. Regardless, the *EPSPS2* mutation seems to be more frequent in glyphosate-resistant horseweed populations in Michigan than previously thought and is either complimenting NTSR mechanisms previously described or possibly the sole source of resistance in some cases (Fisher et al., 2023). More work into glyphosate resistance mechanisms in horseweed are needed for robust molecular diagnostics to be viable in this species.

The methods developed and optimized here for early TSR detection of resistance to *ALS* and *EPSPS*-inhibitor herbicides were proven to be robust and reliable. Our assays worked nearly

100% of the time, with the major limitation being the reliability of accurate Sanger sequencing reactions, which works between 93% and 99% of the time. Also, genotyping multiple individuals from a single accession can provide insights regarding the frequency of resistant alleles at the population level. Modern use of Oxford Nanopore Technology (ONT) for amplicon sequencing may reduce this unreliability severely as ONT amplicon sequencing provides hundreds or thousands of reads of single molecules that are very long. Even though ONT is error prone, they are random and through consensus it is easy to access the true sequence at any locus. The primary drawback with target site mutation sequencing *ALS* and *EPSPS2* is that the results can only prove that individuals are resistant in the case where a SNP is found; however, the lack of a target site SNP does not guarantee that the plant is susceptible, as NTSR mechanisms or unidentified mechanisms could contribute to herbicide resistance. Therefore, target site molecular diagnostics should still be complimented with traditional diagnostics, or non-molecular, quick tests, such as leaf-disc-assays (Wu et al. 2021), mentioned earlier to access whether a population is herbicide susceptible or resistant.

EPSPS CNV in Palmer and waterhemp

Glyphosate resistance in waterhemp and Palmer amaranth are commonly attributed to increased *EPSPS* copy number, which causes an overexpression of the EPSPS protein so that the protein pool becomes greater than reasonably inhibited by glyphosate (Gaines et al., 2010; Lorentz et al., 2014). The *EPSPS* CNV is easily diagnosable using standard genomic qPCR methodologies that has been previously described for both species. The estimation of *EPSPS* CNV was determined through the $2^{-\Delta Ct}$ method (Livak & Schmittgen, 2001). Our results showed that the amplification of the *EPSPS* gene in our populations varied between 1 to 160 in Palmer amaranth and 1 to 8 in waterhemp populations (Table 2.3, Figure 2.5C, Figure 2.9A-D); similar to findings by Gaines et al. (2010) for Palmer amaranth, and Chatham et al. (2015) for waterhemp. In their work, Gaines et al. (2010) and Chatham et al. (2015) suggest a baseline of 30 *EPSPS* copies to prescribe resistance in Palmer amaranth and four copies for resistance in waterhemp respectively; however, more work should be done to identify exactly how many copies is needed for each species to survive the recommended field dose of glyphosate. In kochia, another EPSPS-CNV glyphosate-resistant species, more than four copies can confer field-level resistance (Gaines et al., 2016). Therefore, in this work any individual with copy number equal or greater than four was considered resistant or at least moderately resistant. With this as our baseline, four out of five greenhouse

Palmer amaranth populations and three out of five waterhemp populations were classified as resistant. In the field, two of three Palmer amaranth individuals and six of twenty-one waterhemp individuals showed resistance through EPSPS CNV.

Several housekeeping genes have been proposed for the genomic qPCRs for *EPSPS*-CNV assays. Originally, others have used *ALS* but this practice has been questioned as ALS is an herbicide target and theoretically may become a CNV as well (Gaines et al., 2010). To this end, others have used housekeeping genes like *β-tubulin* as they may be more stable in copy number (Godar et al., 2015). In our work, we compared *ALS* and *β-tubulin* as housekeeping genes and we rejected *β-tubulin* as a control gene for qPCR assays, as it underestimates the actual CNV of the tested species. *β-tubulin* is part of a multi-gene family and the primers previously reported bind to at least two of these genes and therefore underestimates the number of amplified copies of *EPSPS* by half. *ALS* performed as expected and was a stable single-copy gene in our plants, therefore, provide an accurate estimation on the EPSPS CNV.

CONCLUSIONS

Molecular diagnostics can be an effective tool for the development of integrated pest management plans, especially when controlling herbicide resistance is critical. They allow for rapid and empirical detection of resistance mechanisms and can support real-time management changes within the growing season. One of the benefits of the SNPs sequencing using molecular diagnostics for the farmers is the timeframe reduction for *ALS* and *EPSPS*-resistance detection. By performing molecular diagnostics, the drastic reduction of time allows farmers to act in the same cropping season, potentially avoiding further herbicide resistance selection in their areas. Additionally, the low costs attributed to detecting herbicide resistance early in the cropping season is another advantage of molecular diagnostics as compared with traditional phenotyping assays. Farmers may be able to avoid spraying herbicides that are fated to fail in controlling target weeds. However, molecular diagnostics require large amounts of knowledge about dominant resistance mechanisms for a given weed and herbicide combination as well as the expertise to develop, optimize, run, and interpret the results. In this work, we present a tested and robust set of PCR-based assays for detecting TSR mechanisms for ALS and EPSPS inhibiting chemistries in the major broadleaf weeds of the upper Midwest, as well as detailed methodology for others to adopt these assays in their resistance surveys. In some cases, these assays were previously developed but were redesigned here to be robust, working on nearly all individuals in the greenhouse or field, and in other cases we designed the assays *de novo*. These early-season molecular diagnostics for herbicide resistance detection can be implemented in tandem with traditional whole plant assays to more quickly diagnose TSR resistance and help with the discovery of NTSR resistance mechanisms by quickly ruling out TSR.

BIBLIOGRAPHY

Ashigh, J., Corbett, C. A. L., Smith, P. J., Laplante, J., & Tardif, F. J. (2009). Characterization and diagnostic tests of resistance to acetohydroxyacid synthase inhibitors due to an Asp376Glu substitution in *Amaranthus powellii*. *Pesticide Biochemistry and Physiology*, *95*(1), 38–46. https://doi.org/10.1016/j.pestbp.2009.06.002

Ashigh, J., & Tardif, F. J. (2009). An amino acid substitution at position 205 of acetohydroxyacid synthase reduces fitness under optimal light in resistant populations of *Solanum ptychanthum*. *Weed Research*, *49*(5), 479–489. https://doi.org/10.1111/j.1365-3180.2009.00717.x

Beckie, H. J., Warwick, S. I., & Sauder, C. A. (2012). Basis for Herbicide Resistance in Canadian Populations of Wild Oat (*Avena fatua*). *Weed Science*, *60*(1), 10–18. https://doi.org/10.1614/ws-d-11-00110.1

Beres, Z. T., Giese, L. A., Mackey, D. M., Owen, M. D. K., Page, E. R., & Snow, A. A. (2020). Target-site EPSPS Pro-106-Ser mutation in Conyza canadensis biotypes with extreme resistance to glyphosate in Ohio and Iowa, USA. *Scientific Reports*, *10*(1). https://doi.org/10.1038/s41598-020-64458-7

Bernasconi, P., Woodworth, A. R., Rosen, B. A., Subramanian, M. V, & Siehl, D. L. (1995). A Naturally Occurring Point Mutation Confers Broad Range Tolerance to Herbicides That Target Acetolactate Synthase. *The Journal of Biological Chemistry*, *270*(29), 17381–17385. https://doi.org/https://doi.org/10.1074/jbc.270.29.17381

Brosnan, J. T., Vargas, J. J., Breeden, G. K., Grier, L., Aponte, R. A., Tresch, S., & Laforest, M. (2016). A new amino acid substitution (Ala-205-Phe) in acetolactate synthase (ALS) confers broad spectrum resistance to ALS-inhibiting herbicides. *Planta*, *243*(1), 149–159. https://doi.org/10.1007/s00425-015-2399-9

Burgos, N. R., Tranel, P. J., Streibig, J. C., Davis, V. M., Shaner, D., Norsworthy, J. K., & Ritz, C. (2013). Review: Confirmation of Resistance to Herbicides and Evaluation of Resistance Levels. *Weed Science*, *61*(1), 4–20. https://doi.org/10.1614/ws-d-12-00032.1

Cardinali, V. C. B., Dias, A. C. R., Mueller, T. C., Abercrombie, L., Stewart Jr., C. N., Tornisielo, V. L., & Christoffoleti, P. J. (2015). Shikimate Accumulation, Glyphosate Absorption and Translocation in Horseweed Biotypes. *Planta Daninha*, *33*(1), 109–118. https://doi.org/https://doi.org/10.1590/S0100-83582015000100013

Carpenter, J. R. (1986). A Method for Presenting and Comparing Dose-Response Curves. In *Journal of Pharmacological Methods* (Vol. 15). Elsevier Science Publishing Co., Inc. https://doi.org/https://doi.org/10.1016/0160-5402(86)90009-4

Chatham, L. A., Wu, C., Riggins, C. W., Hager, A. G., Young, B. G., Roskamp, G. K., & Tranel, P. J. (2015). EPSPS Gene Amplification is Present in the Majority of Glyphosate-Resistant Illinois Waterhemp (*Amaranthus tuberculatus*) Populations. *Weed Technology*, *29*(1), 48–55. https://doi.org/10.1614/wt-d-14-00064.1

Comont, D., & Neve, P. (2021). Adopting epidemiological approaches for herbicide resistance monitoring and management. *Weed Research*, *61*(2), 81–87. https://doi.org/10.1111/wre.12420

Cutti, L., Rigon, C. A. G., Kaspary, T. E., Turra, G. M., Markus, C., & Merotto, A. (2021). Negative cross-resistance to clomazone in imazethapyr-resistant *Echinochloa crus-galli* caused by increased metabolization. *Pesticide Biochemistry and Physiology*, *178*. https://doi.org/10.1016/j.pestbp.2021.104918

Davis, M. W., & Jorgensen, E. M. (2022). ApE, A Plasmid Editor: A Freely Available DNA Manipulation and Visualization Program. *Frontiers in Bioinformatics*, *2*. https://doi.org/10.3389/fbinf.2022.818619

Dillon, A., Varanasi, V. K., Danilova, T. V., Koo, D. H., Nakka, S., Peterson, D. E., Tranel, P. J., Friebe, B., Gill, B. S., & Jugulam, M. (2017). Physical mapping of amplified copies of the 5-Enolpyruvylshikimate-3-Phosphate synthase gene in Glyphosate-Resistant *Amaranthus tuberculatus*. *Plant Physiology*, *173*(2), 1226–1234. https://doi.org/10.1104/pp.16.01427

Dominguez-Valenzuela, J. A., Gherekhloo, J., Fernández-Moreno, P. T., Cruz-Hipolito, H. E., Alcántara-de la Cruz, R., Sánchez-González, E., & De Prado, R. (2017). First confirmation and characterization of target and non-target site resistance to glyphosate in Palmer amaranth (*Amaranthus palmeri*) from Mexico. *Plant Physiology and Biochemistry*, *115*, 212–218. https://doi.org/10.1016/j.plaphy.2017.03.022

Feng, P. C. C., Tran, M., Chiu, T., Sammons, R. D., Heck, G. R., & CaJacob, C. A. (2004). Investigations into glyphosate-resistant horseweed (*Conyza canadensis*): retention, uptake, translocation, and metabolism. *Weed Science*, *52*(4), 498–505. https://doi.org/10.1614/ws-03-137r

Fisher, J. L., Sprague, C. L., Patterson, E. L., & Schramski, J. A. (2023). Investigations into differential glyphosate sensitivity between two horseweed (*Conyza canadensis*) growth types. *Weed Science*, *71*(1), 22–28. https://doi.org/10.1017/wsc.2022.69

Foes, M. J., Liu, L., Vigue, G., Stoller, E. W., Wax, L. M., & Tranel, P. J. (1999). A Kochia (*Kochia scoparia*) Biotype Resistant to Triazine and ALS-Inhibiting Herbicides. *Weed Science*, *47*(1), 20–27. https://doi.org/doi:10.1017/S0043174500090603

Gaines, T. A., Barker, A. L., Patterson, E. L., Westra, E. P., & Kniss, A. R. (2016). EPSPS Gene copy number and whole-plant glyphosate resistance level in Kochia scoparia. *PLoS ONE*, *11*(12). https://doi.org/10.1371/journal.pone.0168295

Gaines, T. A., Duke, S. O., Morran, S., Rigon, C. A. G., Tranel, P. J., Anita Küpper, & Dayan, F. E. (2020). Mechanisms of evolved herbicide resistance. In *Journal of Biological Chemistry* (Vol. 295, Issue 30, pp. 10307–10330). American Society for Biochemistry and Molecular Biology Inc. https://doi.org/10.1074/jbc.REV120.013572

Gaines, T. A., Zhang, W., Wang, D., Bukun, B., Chisholm, S. T., Shaner, D. L., Nissen, S. J., Patzoldt, W. L., Tranel, P. J., Culpepper, A. S., Grey, T. L., Webster, T. M., Vencill, W. K., Sammons, R. D., Jiang, J., Preston, C., Leach, J. E., & Westra, P. (2010). Gene amplification confers glyphosate resistance in *Amaranthus palmeri*. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(3), 1029–1034. https://doi.org/10.1073/pnas.0906649107

Godar, A. S., Varanasi, V. K., Nakka, S., Prasad, P. V. V., Thompson, C. R., & Mithila, J. (2015). Physiological and molecular mechanisms of differential sensitivity of Palmer amaranth (*Amaranthus palmeri*) to mesotrione at varying growth temperatures. *PLoS ONE*, *10*(5). https://doi.org/10.1371/journal.pone.0126731

Guttieri, M. J., Eberlein, C. V, Mallory-Smith, C. A., Thill, D. C., & Hoffman, D. L. (1992). DNA Sequence Variation in Domain A of the Acetolactate Synthase Genes of Herbicide-Resistant and -Susceptible Weed Biotypes. *Weed Science*, *40*(4), 670–677. https://doi.org/doi:10.1017/S0043174500058288

Guttieri, M. J., Eberlein, C. V, & Thill, D. C. (1995). Diverse Mutations in the Acetolactate Synthase Gene Confer Chlorsulfuron Resistance in Kochia (*Kochia scoparia*) Biotypes. *Weed Science*, *43*(2), 175–178. https://doi.org/doi:10.1017/S0043174500081029

Han, H., Yu, Q., Beffa, R., González, S., Maiwald, F., Wang, J., & Powles, S. B. (2021). Cytochrome P450 CYP81A10v7 in *Lolium rigidum* confers metabolic resistance to herbicides across at least five modes of action. *Plant Journal*, *105*(1), 79–92. https://doi.org/10.1111/tpj.15040

Heap, I. (2023, September 26). *The International Herbicide-Resistant Weed Database*. Www.Weedscience.Org.

Huang, C. H., Zhang, C., Liu, M., Hu, Y., Gao, T., Qi, J., & Ma, H. (2016). Multiple Polyploidization Events across Asteraceae with Two Nested Events in the Early History Revealed by Nuclear Phylogenomics. *Molecular Biology and Evolution*, *33*(11), 2820–2835. https://doi.org/10.1093/molbev/msw157

Huang, Z., Chen, J., Zhang, C., Huang, H., Wei, S., Zhou, X., Chen, J., & Wang, X. (2016). Target-site basis for resistance to imazethapyr in redroot amaranth (*Amaranthus retroflexus* L.). *Pesticide Biochemistry and Physiology*, *128*, 10–15. https://doi.org/10.1016/j.pestbp.2015.10.011

Jugulam, M., & Shyam, C. (2019). Non-target-site resistance to herbicides: Recent developments. *Plants*, *8*(10). https://doi.org/10.3390/plants8100417

Kaundun, S. S., Hutchings, S. J., Dale, R. P., Bailly, G. C., & Glanfield, P. (2011). Syngenta "RISQ" test: A novel in-season method for detecting resistance to post-emergence ACCase and ALS inhibitor herbicides in grass weeds. *Weed Research*, *51*(3), 284–293. https://doi.org/10.1111/j.1365-3180.2011.00841.x

Kaundun, S. S., Jackson, L. V., Hutchings, S. J., Galloway, J., Marchegiani, E., Howell, A., Carlin, R., McIndoe, E., Tuesca, D., & Moreno, R. (2019). Evolution of target-site resistance to glyphosate in an *Amaranthus palmeri* population from Argentina and its expression at different plant growth temperatures. *Plants*, *8*(11). https://doi.org/10.3390/plants8110512

Kaundun, S. S., Zelaya, I. A., Dale, R. P., Lycett, A. J., Carter, P., Sharples, K. R., & McIndoe, E. (2008). Importance of the P106S Target-Site Mutation in Conferring Resistance to Glyphosate in a Goosegrass (*Eleusine indica*) Population from the Philippines. *Weed Science*, *56*(5), 637–646. https://doi.org/10.1614/ws-07-148.1

Koger, C. H., & Reddy, K. N. (2005). Role of absorption and translocation in the mechanism of glyphosate resistance in horseweed (*Conyza canadensis*). *Weed Science*, *53*(1), 84–89. https://doi.org/10.1614/ws-04-102r

Kohrt, J. R., Sprague, C. L., Nadakuduti, S. S., & Douches, D. (2017). Confirmation of a three-way (glyphosate, ALS, and atrazine) herbicide-resistant population of palmer amaranth (*Amaranthus palmeri*) in Michigan. *Weed Science*, *65*(3), 327–338. https://doi.org/10.1017/wsc.2017.2

Kolkman, J. M., Slabaugh, M. B., Bruniard, J. M., Berry, S., Bushman, B. S., Olungu, C., Maes, N., Abratti, G., Zambelli, A., Miller, J. F., Leon, A., & Knapp, S. J. (2004). Acetohydroxyacid synthase mutations conferring resistance to imidazolinone or sulfonylurea herbicides in sunflower. *Theoretical and Applied Genetics*, *109*(6), 1147–1159. https://doi.org/10.1007/s00122-004-1716-7

Koo, D. H., Molin, W. T., Saski, C. A., Jiang, J., Putta, K., Jugulam, M., Friebe, B., & Gill, B. S. (2018). Extrachromosomal circular DNA-based amplification and transmission of herbicide resistance in crop weed *Amaranthus palmeri*. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(13), 3332–3337. https://doi.org/10.1073/pnas.1719354115

Kutasy, B., Farkas, Z., Kolics, B., Decsi, K., Hegedűs, G., Kovács, J., Taller, J., Tóth, Z., Kálmán, N., Kazinczi, G., & Virág, E. (2021). Detection of target-site herbicide resistance in the common Ragweed: Nucleotide polymorphism genotyping by targeted amplicon sequencing. *Diversity*, *13*(3). https://doi.org/10.3390/d13030118

Laforest, M., Martin, S. L., Bisaillon, K., Soufiane, B., Meloche, S., & Page, E. (2020). A chromosome-scale draft sequence of the Canada fleabane genome. *Pest Management Science*, *76*(6), 2158–2169. https://doi.org/10.1002/ps.5753

Laforest, M., Martin, S. L., Bisaillon, K., Soufiane, B., Meloche, S., Tardif, F. J., & Page, E. (2024). The ancestral karyotype of the Heliantheae Alliance, herbicide resistance, and human allergens: Insights from the genomes of common and giant ragweed. *Plant Genome*. https://doi.org/10.1002/tpg2.20442

Laforest, M., Soufiane, B., Patterson, E. L., Vargas, J. J., Boggess, S. L., Houston, L. C., Trigiano, R. N., & Brosnan, J. T. (2021). Differential expression of genes associated with non-target site resistance in *Poa annua* with target site resistance to acetolactate synthase inhibitors. *Pest Management Science*, *77*(11), 4993–5000. https://doi.org/10.1002/ps.6541

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, *2*(5), 402–408. https://doi.org/10.1006/m

Lorentz, L., Gaines, T. A., Nissen, S. J., Westra, P., Strek, H. J., Dehne, H. W., Ruiz-Santaella, J. P., & Beffa, R. (2014). Characterization of glyphosate resistance in Amaranthus tuberculatus Populations. *Journal of Agricultural and Food Chemistry*, *62*(32), 8134–8142. https://doi.org/10.1021/jf501040x

Loubet, I., Caddoux, L., Fontaine, S., Michel, S., Pernin, F., Barrès, B., Le Corre, V., & Délye, C. (2021). A high diversity of mechanisms endows ALS-inhibiting herbicide resistance in the invasive common ragweed (*Ambrosia artemisiifolia* L.). *Scientific Reports*, *11*(1). https://doi.org/10.1038/s41598-021-99306-9

Matzrafi, M., Lazar, T. W., Sibony, M., & Rubin, B. (2015). Conyza species: distribution and evolution of multiple target-site herbicide resistances. *Planta*, *242*(1), 259–267. https://doi.org/10.1007/s00425-015-2306-4

McElroy, J. S., Flessner, M. L., Wang, Z., Dane, F., Walker, R. H., & Wehtje, G. R. (2013). A Trp 574 to Leu Amino Acid Substitution in the ALS Gene of Annual Bluegrass (*Poa annua*) Is Associated with Resistance to ALS-Inhibiting Herbicides. *Weed Science*, *61*(1), 21–25. https://doi.org/10.1614/ws-d-12-00068.1

McNaughton, K. E., Letarte, J., Lee, E. A., & Tardif, F. J. (2005). Mutations in ALS confer herbicide resistance in redroot pigweed (*Amaranthus retroflexus*) and Powell amaranth (*Amaranthus powellii*). *Weed Science*, *53*(1), 17–22. https://doi.org/10.1614/ws-04-109

Molin, W. T., Nandula, V. K., Wright, A. A., & Bond, J. A. (2016). Transfer and Expression of ALS Inhibitor Resistance from Palmer Amaranth (*Amaranthus palmeri*) to an *A. spinosus × A. palmeri* Hybrid. *Weed Science*, *64*(2), 240–247. https://doi.org/10.1614/ws-d-15-00172.1

MSU-PPD. (2024). *Plant & pest diagnostics*. Plant & Pest Diagnostics. Https://Www.Canr.Msu.Edu/Pestid/Index.

Nandula, V. K., Ray, J. D., Ribeiro, D. N., Pan, Z., & Reddy, K. N. (2013). Glyphosate Resistance in Tall Waterhemp (*Amaranthus tuberculatus*) from Mississippi is due to both Altered Target-Site and Nontarget-Site Mechanisms. *Weed Science*, *61*(3), 374–383. https://doi.org/10.1614/ws-d-12-00155.1

Page, E. R., Grainger, C. M., Laforest, M., Nurse, R. E., Rajcan, I., Bae, J., & Tardif, F. J. (2018). Target and Non-target site Mechanisms Confer Resistance to Glyphosate in Canadian Accessions of *Conyza canadensis*. *Weed Science*, *66*(2), 234–245. https://doi.org/10.1017/wsc.2017.69

Palmieri, V. E., Alvarez, C. E., Permingeat, H. R., & Perotti, V. E. (2022). A122S, A205V, D376E, W574L and S653N substitutions in acetolactate synthase (ALS) from *Amaranthus palmeri* show different functional impacts on herbicide resistance. *Pest Management Science*, *78*(2), 749–757. https://doi.org/10.1002/ps.6688

Pan, L., Yu, Q., Han, H., Mao, L., Nyporko, A., Fan, L. J., Bai, L., & Powles, S. (2019). Aldo-keto reductase metabolizes glyphosate and confers glyphosate resistance in *Echinochloa colona*. *Plant Physiology*, *181*(4), 1519–1534. https://doi.org/10.1104/pp.19.00979

Patzoldt, W. L., Patrick J, Alexander, Anita L, Schmitzer, & Paul R. (2001). A common ragweed population resistant to cloransulam-methyl. *Weed Science*, *49*, 485–490. https://doi.org/10.1614/0043

Patzoldt, W. L., & Tranel, P. J. (2007). Multiple ALS Mutations Confer Herbicide Resistance in Waterhemp (*Amaranthus tuberculatus*). *Weed Science*, *55*(5), 421–428. https://doi.org/10.1614/ws-06-213.1

Pimentel, D., Lach, L., Zuniga, R., & Morrison, D. (2000). Environmental and Economic Costs of Nonindigenous Species in the United States. *Bioscience*, *50*, 53–65. https://doi.org/https://doi.org/10.1641/0006-3568(2000)050[0053:EAECON]2.3.CO;2

Preston, C., Stone, L. M., Rieger, M. A., & Baker, J. (2006). Multiple effects of a naturally occurring proline to threonine substitution within acetolactate synthase in two herbicide-resistant populations of *Lactuca serriola*. *Pesticide Biochemistry and Physiology*, *84*(3), 227–235. https://doi.org/10.1016/j.pestbp.2005.07.007

Sarangi, D., Tyre, A. J., Patterson, E. L., Gaines, T. A., Irmak, S., Knezevic, S. Z., Lindquist, J. L., & Jhala, A. J. (2017). Pollen-mediated gene flow from glyphosate-resistant common waterhemp (*Amaranthus rudis* Sauer): Consequences for the dispersal of resistance genes. *Scientific Reports*, *7*. https://doi.org/10.1038/srep44913

USDA, N. A. S. S. (2015). *Chapter Three. Prices Paid Program*. Https://Www.Nass.Usda.Gov/Surveys/Guide_to_NASS_Surveys/Prices/Chapter%20Three%20Prices%20Paid%2003092015.Pdf.

Vila-Aiub, M. M. (2019). Fitness of herbicide-resistant weeds: Current knowledge and implications for management. In *Plants* (Vol. 8, Issue 11). MDPI AG. https://doi.org/10.3390/plants8110469

Whaley, C. M., Wilson, H. P., & Westwood, J. H. (2006). ALS resistance in several smooth pigweed (*Amaranthus hybridus*) biotypes. *Weed Science*, *54*(5), 828–832. https://doi.org/10.1614/ws-05-040r.1

Wu, C., Varanasi, V., & Perez-Jones, A. (2021). A nondestructive leaf-disk assay for rapid diagnosis of weed resistance to multiple herbicides. *Weed Science*, *69*(3), 274–283. https://doi.org/10.1017/wsc.2021.15

Zheng, D., Kruger, G. R., Singh, S., Davis, V. M., Tranel, P. J., Weller, S. C., & Johnson, W. G. (2011). Cross-resistance of horseweed (*Conyza canadensis*) populations with three different ALS mutations. *Pest Management Science*, *67*(12), 1486–1492. https://doi.org/10.1002/ps.2190

Figure 2.1. Primers (forward, middle, and reverse) covering *ALS* positions known to confer herbicide resistance. A middle primer was used to sequence the Palmer amaranth fragment. Horseweed primers skip the Ala122, Ser653, and Gly654 positions, while waterhemp primers skip the Ala122.
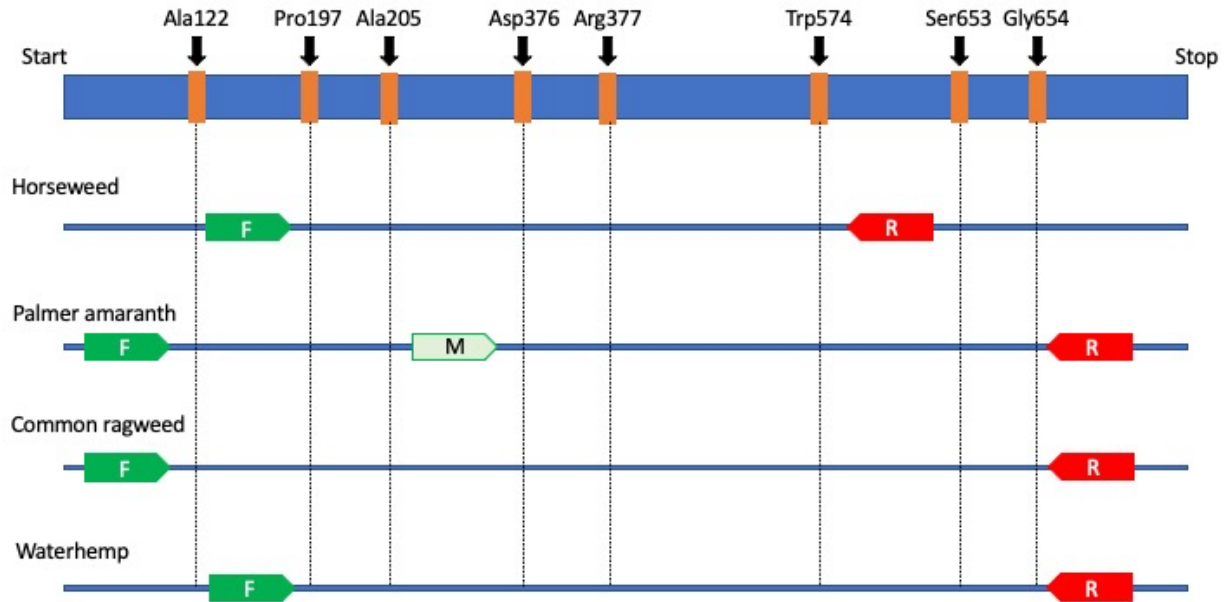
Figure 2.2. Frequency of *ALS* mutations in horseweed (ERICA), common ragweed (AMBEL), waterhemp (AMATU), and Palmer amaranth (AMAPA). Five greenhouse accessions of each species had 10 plants sequenced. Field plants were collected in soybean and corn fields in Michigan to address the efficiency of the molecular markers tested in the greenhouse accessions.
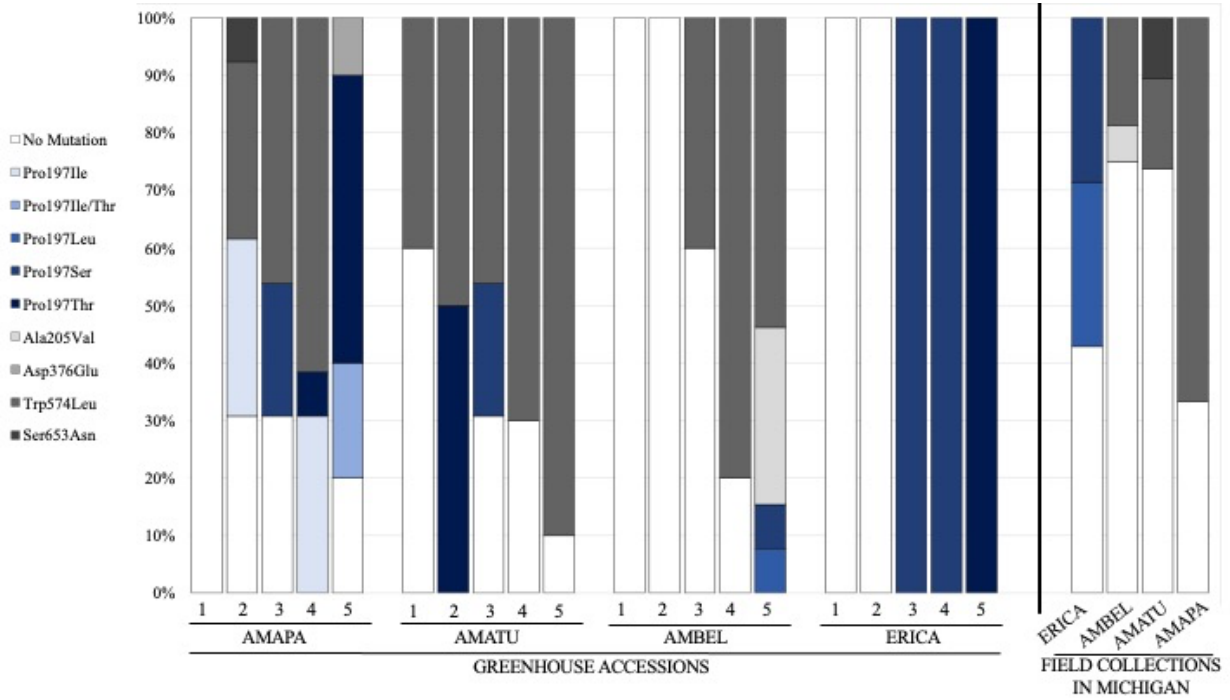
Figure 2.3. Chroma of all *ALS* mutations found in accessions of four weed species, Palmer amaranth (A), waterhemp (B), common ragweed (C), and horseweed (D) resistant and susceptible.
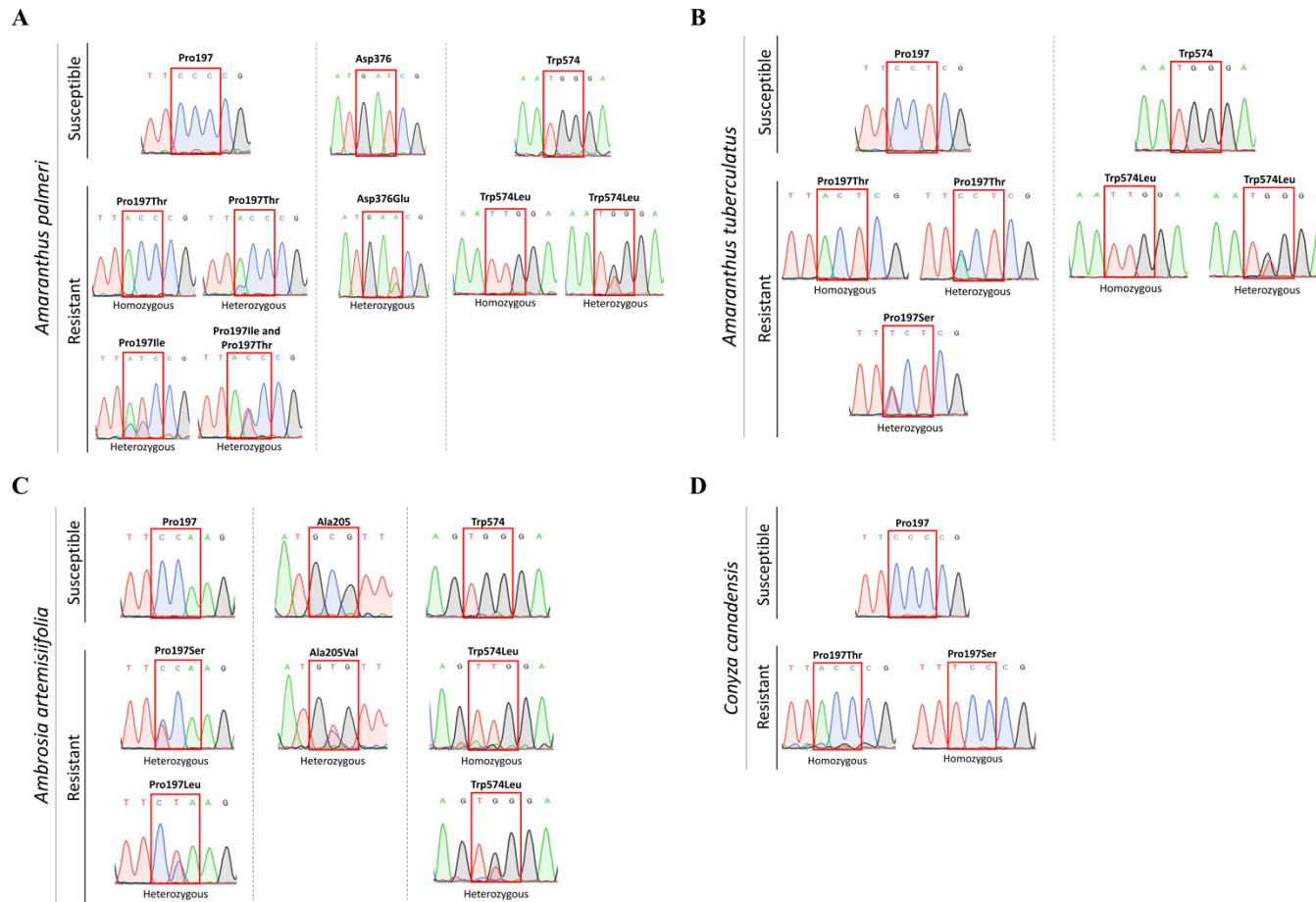
Figure 2.4. Chroma of *EPSPS* positions known to confer herbicide resistance in *EPSPS1* and *EPSPS2* from resistant and susceptible accessions of horseweed (*Conyza canadensis*). There is the mutation Pro106Ser in *EPSPS2*.
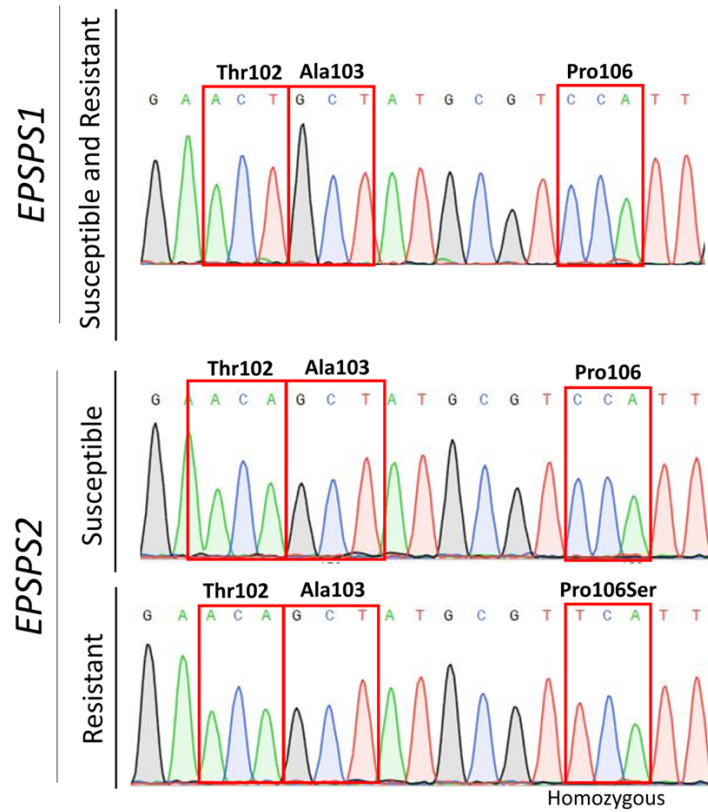
Figure 2.5. *EPSPS* copy number variation analysis for three Palmer amaranth (A) and waterhemp (B) accessions using two different housekeeping genes, *ALS* and *β-TUB*. *EPSPS* copy number variation analysis in five plants of five Palmer amaranth (C) and waterhemp (D) greenhouse accessions using housekeeping *ALS* gene.

Figure 2.6. Michigan State map showing the location of field collections of four weed species used to validate the molecular markers. (A) Collections showing ALS-inhibitors resistance or susceptibility based on mutations detected with molecular markers. (B) Collections of Palmer amaranth and waterhemp showing glyphosate resistance or susceptibility based on EPSPS copy number variation.

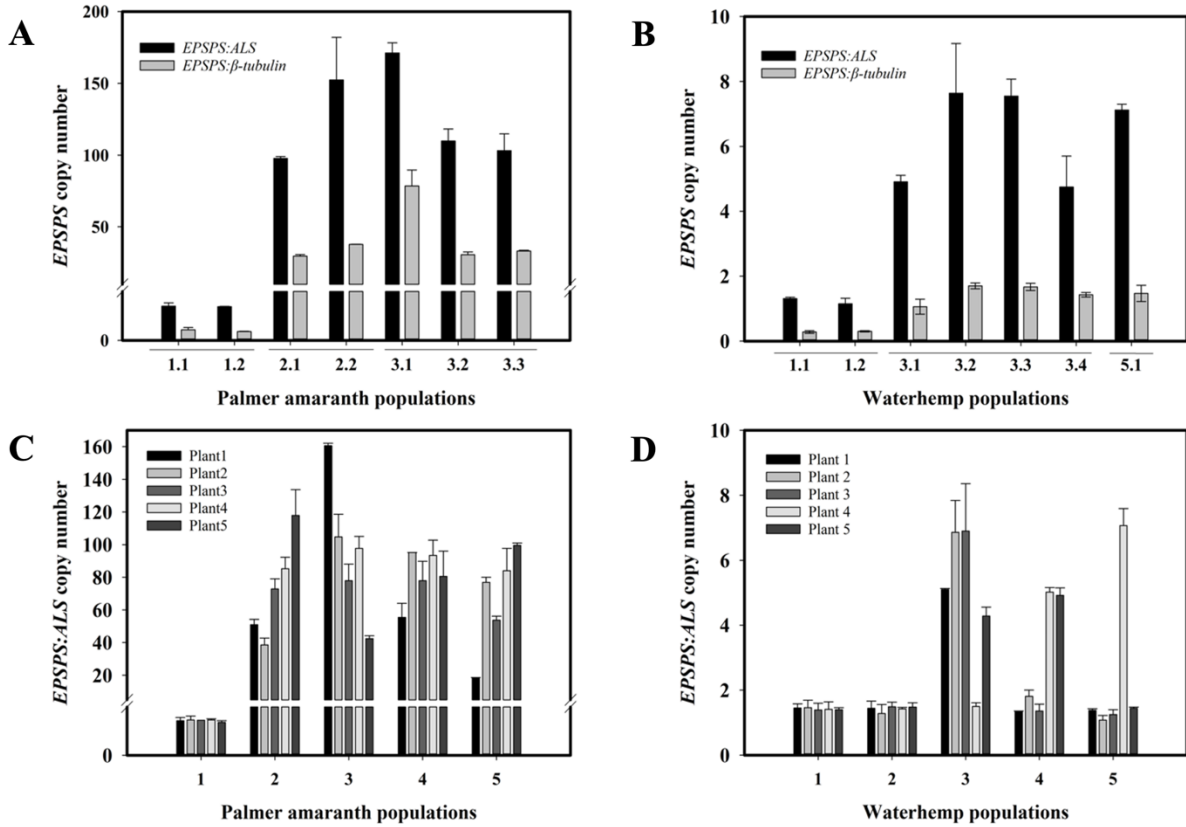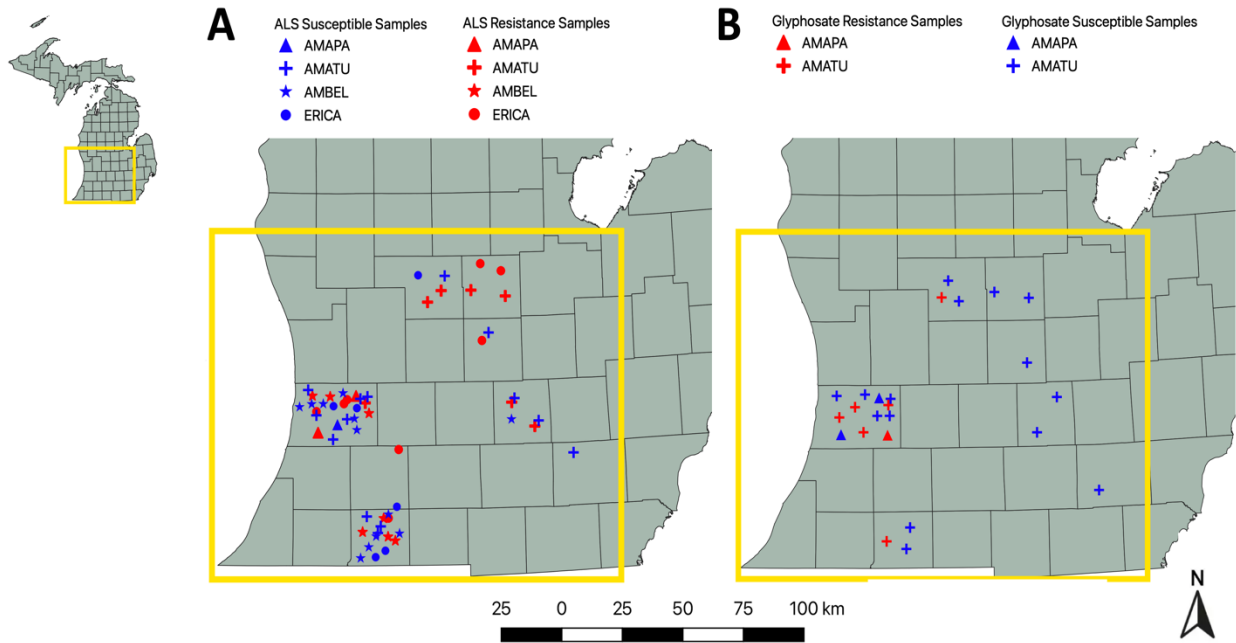Figure 2.7. Alignment of translated *EPSPS1* and *EPSPS2* genes from horseweed showing the identity between both. Amino acids in red refer to known positions where mutations confer glyphosate resistance.

```
EPSPS1    MAATHINTT-NIAHNLQATTSLSKTQTPSIKSQPFLSFGPKHKNPIAHFSVSSNNNRNLG      59
EPSPS2    MAVHINNSNIPIFNTSNLTTQNPSSKSSSFLSFGSNFKNPLKNNNNNYTSVSCNVKNNK      60
          **      *      *      **         *  *       *   *      *  *  *

EPSPS1    KKCLIVSAVATTEK---PSTVPEIVLQPIKEISGTVNLPGSKSLSNRILLLAALAEGTTI     116
EPSPS2    NPFKVSAFSATSTKEKPSKAPEEIVLKPIQEISGTVHLPGSKSLSNRILLLAALSEGTTV     120
               **  *            **** **  *****.* ****************** ****

EPSPS1    VDNLLNSDDVHYMLGALRTLGLNVEEDVAIKRAIVEGCGGVFPVGKEAKDDIQLFLGNAG     176
EPSPS2    VDNLLNSDDVHYMLGALRALGLNVEENSAIKRAIVEGCGGVFPVGKEAKDEIQLFLGNAG     180
          ****************** *******   ***************.***** *********

EPSPS1    TAMRPLTAAVTAAGGNSSYILDGVPRMRERPIGDLVTGLKQLGADVDCSLGTNCPPVRVV     236
EPSPS2    TAMRPLTAAVTAAGGNSSYILDGVPRMRERPIGDLVTGLKQLGANVDCSLGTNCPPVRVV     240
          ********************************************* ***************

EPSPS1    GGGGLPGGKVKLSGSISSQYLTALLMASPLALGDVEIEIIDKLISIPYVEMTLKLMERFG     296
EPSPS2    GSGGLPGGKVKLSGSISSQYLTSLLMAAPLALGDVEIEIIVDKLISVPYVEMTLKLMERFG     300
          * ********************** **** *********** ***** ***************

EPSPS1    VSVEHSDSWDQFFIRGGQKYKSPGNAYVEGDASSASYFLAGAAITGGTITVEGCGTSSLQ     356
EPSPS2    VSVEHSDTWDRFHVRGGQKYKSPGNAYVEGDASSASYFLAGAAITGGTVTVEGCGTSSLQ     360
          ******* ** *  ********************************** ************

EPSPS1    GDVKFAEVLGQMGAEVTWTENSVTVKGPPRDSSGRKHLRAVDVNMNKMPDVAMTLAVVAL     416
EPSPS2    GDVKFAEVLGQMGAEVTWTENSVTVKGPPRNSSGRGHLRPVDVNMNKMPDVAMTLAVVAL     420
          ****************************** **** *** ********************

EPSPS1    YADGPTAIRDVASWRVKETERMIAICTELRKLGATVEEGPDYCVITPPEKLNVTAIDTYD     476
EPSPS2    YADGPTAIRDVASWRVKETERMIAICTELRKLGATVEEGPDYCVITPPEKLNVTAIDTYD     480
          ************************************************************

EPSPS1    DHRMAMAFSLAACADVPVTIKDPSCTRKTFPDYFEVLQRFAKH     519
EPSPS2    DHRMAMAFSLAACAEVPVTIKDPGCTRKTFPDYFEVLERYTKH     523
          ************** ******* ***********.* *  **
```

47

Figure 2.8. Agarose gel showing specific fragment size produced by *EPSPS1* and *EPSPS2* primer pairs used to sequence and address the mutations.
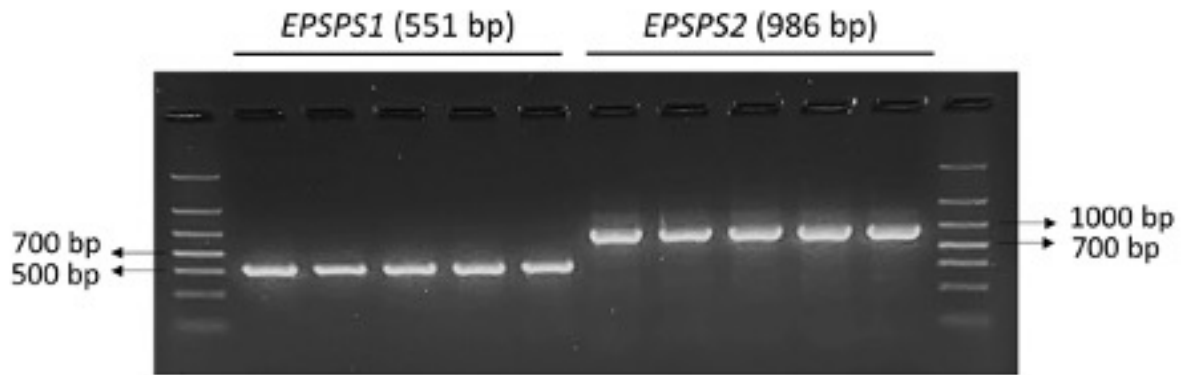
Figure 2.9. *EPSPS* and *ALS* qPCR amplification curves. (A) *Amaranthus palmeri* S. Watson individual without *EPSPS* CNV; (B) *Amaranthus palmeri* S. Watson individual with *EPSPS* CNV; (C) *Amaranthus tuberculatus* (Moq.) Sauer individual without *EPSPS* CNV; (D) *Amaranthus tuberculatus* (Moq.) Sauer individual with *EPSPS* CNV.
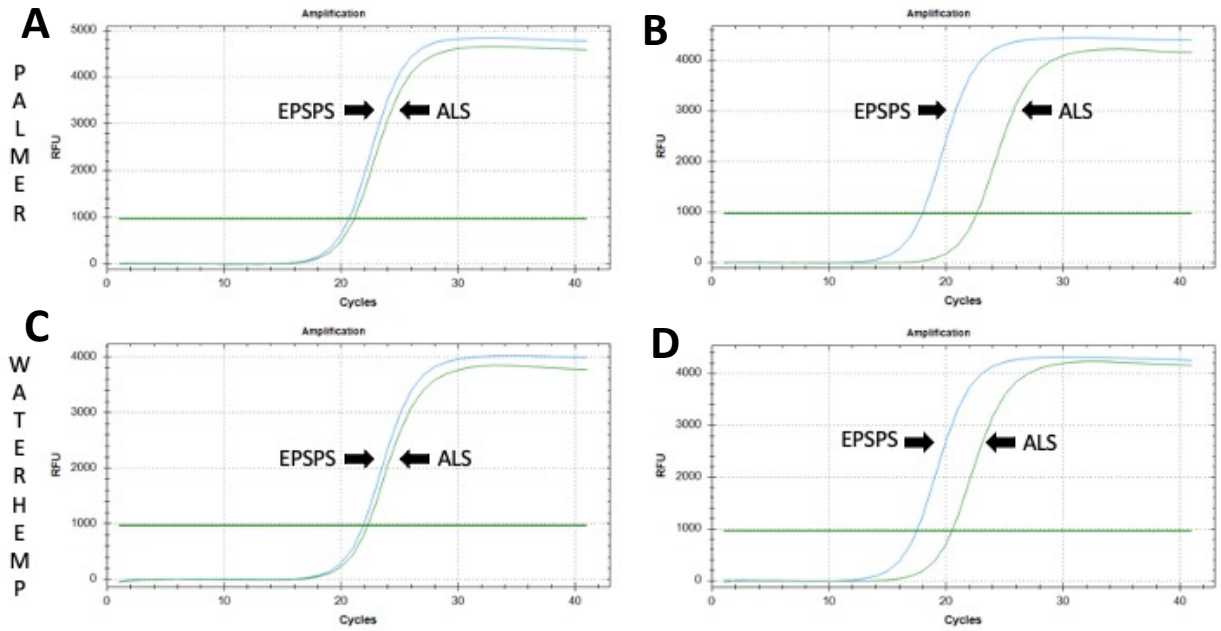
Table 2.1. Known mutations in the *ALS* and *EPSPS* genes conferring ALS- and *EPSPS*-inhibitors herbicide resistance in horseweed, common ragweed, Palmer amaranth, and waterhemp.

Mutation	Species			
	Common ragweed	Palmer amaranth	Waterhemp	Horseweed
ALS				
Ala122	No	Yes	No	No
Pro197	Yes	Yes	No	Yes
Ala205	Yes	Yes	No	Yes
Phe206	No	No	No	No
Asp376	No	Yes	No	Yes
Arg377	No	No	No	No
Trp574	Yes	Yes	Yes	Yes
Ser653	No	Yes	Yes	No
Gly654	No	No	No	No
References	(Loubet et al., 2021; Rousonelos et al., 2012)	(Küpper et al., 2017; Palmieri et al., 2022; Singh et al., 2019)	(Patzoldt & Tranel, 2007)	(Matzrafi et al., 2015; Zheng et al., 2011)
EPSPS				
Thr102	No	No	No	No
Ala103	No	No	No	No
Pro106	No	Yes	Yes	Yes
References		(Kaundun et al., 2019)	(Nandula et al., 2013)	(Beres et al., 2020)

Table 2.2. Molecular markers and PCR conditions utilized to sequence *ALS* gene from four weed species, horseweed, common ragweed, Palmer amaranth, and waterhemp, and *EPSPS* gene from horseweed.

Species	Primer	Sequence (5' - 3')	Fragment size (bp)	Annealing temperature (°C)	Extension time (min)	References
ALS						
Common ragweed	Forward	AGCTTTGGAACGTGAAGGC	1677	56	1:30	
	Reverse	ATTTCGTTCTGCCATCGCC		56		
Palmer amaranth	Forward	TCCTCGCCGCCCTCTTCAAATC	1990	60	2:00	(Whaley et al., 2006)
	Middle	AGGTTGCCTAAACCCAC		60		
	Reverse	CAGCTAAACGAGAGAACGGCCAG		60		
Waterhemp	Forward	GGTTTTCGCTGCTGAAGG	1584	54	1:45	
	Reverse	AGCCCTTCTTCCATCACC		54		
Horseweed	Forward	AGATCCACCAAGCTCTCACG	1400	57	1:45	
	Reverse	CTTCGGCAAACTTCAACATGTTTGG		57		
EPSPS1						
Horseweed	Forward	TCAGAGCAACATTCGAGGAGTC	551	62	1:00	
	Reverse	CAATTGGTTAAAGGTAGAAGGAGG		62		
EPSPS2						
Horseweed	Forward	GGACTACTGTTGTAGACAACTTG	986	62	1:00	
	Reverse	GTGGGCAGTTTGTACCGAGA		62		

Table 2.3. *EPSPS*, *ALS*, and *β-TUB* molecular markers utilized to address *EPSPS* copy number variation genes in Palmer amaranth, and waterhemp.

Primer	Primer sequence (5' to 3')	Fragment size (bp)	Annealing temperature (°C)	References
		EPSPS		
Forward	ATGTTGGACGCTCTCAGAACTCTTGGT	195	60	(Gaines et al.,
Reverse	TGAATTTCCTCCAGCAACGGCAA		60	2010)
		ALS		
Forward	GCTGCTGAAGGCTACGCT	118	60	(Gaines et al.,
Reverse	GCGGGACTGAGTCAAGAAGTG		60	2010)
		β-TUB		
Forward	ATGTGGGATGCCAAGAACATGATGTG	157	60	(Godar et al.,
Reverse	TCCACTCCACAAAGTAGGAAGAGTTCT		60	2015)

Table 2.4. Summary of molecular markers diagnostic for five greenhouse accession of four weed species targeting *ALS* and *EPSPS* genes.

Species	Population No.	Sulfonylurea resistant?	Glyphosate Resistant?	ALS Mutation	EPSPS Mutation/CNV	Michigan County
Horseweed	1	No	Yes	No mutation[+]	Pro106Ser[+]	Shiawassee
Horseweed	2	No	Yes	No mutation[+]	Pro106Ser[+]	Kalamazoo
Horseweed	3	Yes	Yes	Pro197Ser[+]	Pro106Ser[+]	Branch
Horseweed	4	Yes	Yes	Pro197Ser[+]	Pro106Ser[+]	Montcalm
Horseweed	5	Yes	Yes	Pro197Thr[+]	No mutation[+]	Cass
Palmer amaranth	1	No	No	No mutation[+]	1-2	Jackson, TN
Palmer amaranth	2	Yes	Yes	Pro197Ile[*] Trp574Leu[*] Ser653Asn[*]	40-120	Hillsdale
Palmer amaranth	3	No	Yes	Pro197Leu[*] Trp574Leu[*]	40-160	Mason
Palmer amaranth	4	Yes	Yes	Pro197Ile[*] Pro197Thr[*] Trp574Leu[*+]	55-95	Ottawa
Palmer amaranth	5	Yes	Yes	Pro197Ile/Thr[*] Pro197Thr[*+] Asp376Glu[*]	20-100	Barry
Waterhemp	1	Yes	No	Trp574Leu[+*]	1	St. Clair
Waterhemp	2	Yes	No	Pro197Thr[+*] Trp574Leu[+*]	1	Sanilac
Waterhemp	3	Yes	Yes	Pro197Ser[*] Trp574Leu[*]	1-7	Sanilac
Waterhemp	4	Yes	Yes	Trp574Leu[+*]	1-5	Lenawee
Waterhemp	5	Yes	Yes	Trp574Leu[+*]	1-7	-
Common ragweed	1	Yes	No	No mutation[+]	-	Montcalm
Common ragweed	2	Yes	No	No mutation[+]	-	Ingham
Common ragweed	3	Yes	No	Trp574Leu[+*]	-	Shiawassee
Common ragweed	4	Yes	No	Trp574Leu[+*]	-	Clinton

Table 2.4. (cont'd)

Common ragweed	5	Yes	No	Pro197Leu*	-	Shiawassee
				Pro197Ser*		
				Ala205Val*		
				Trp574Leu+*		

Table 2.5. Summary of molecular markers diagnostic for field collections of four weed species targeting *ALS* and *EPSPS* genes.

Species	Field accession	County	ALS Mutation	EPSPS
Horseweed	1	Allegan	No mutation[+]	-
Horseweed	2	Allegan	Pro197Ser[+]	-
Horseweed	3	Allegan	Pro197Leu[+]	-
Horseweed	4	Allegan	No mutation[+]	-
Horseweed	5	Allegan	Pro197Leu[+]	-
Horseweed	6	Clinton	Pro197Ser[+]	-
Horseweed	7	Montcalm	No mutation[+]	-
Horseweed	8	Gratiot	Pro197Ser[+]	-
Horseweed	9	Gratiot	Pro197Ser[+]	-
Horseweed	10	Kalamazoo	No mutation[+]	-
Horseweed	11	Kalamazoo	Pro197Leu[+]	-
Horseweed	12	St. Joseph	Pro197Leu[+]	-
Horseweed	13	St. Joseph	No mutation[+]	-
Horseweed	14	St. Joseph	No mutation[+]	-
Palmer amaranth	1	Allegan	Trp574Leu[+]	1.53 copies
Palmer amaranth	2	Allegan	No mutation[+]	7.10 copies
Palmer amaranth	3	Allegan	Trp574Leu[*]	21.62 copies
Waterhemp	1	Washtenaw	No mutation[+]	2.6 copies
Waterhemp	2	Clinton	No mutation[+]	2.7 copies
Waterhemp	3	Gratiot	-[*]	#
Waterhemp	4	Gratiot	-[*]	#
Waterhemp	5	Allegan	No mutation[+]	4.2 copies
Waterhemp	6	Allegan	No mutation[+]	5.6 copies
Waterhemp	7	Allegan	Ser653Asn[*]	7.3 copies
Waterhemp	8	Allegan	Trp574Leu[*]	3.8 copies
Waterhemp	9	Allegan	Trp574Leu[*]	1.5 copies
Waterhemp	10	Allegan	No mutation[+]	1.4 copies

Table 2.5. (cont'd)

Waterhemp	11	Allegan	No mutation[+]	8.4 copies
Waterhemp	12	Allegan	No mutation[+]	1.6 copies
Waterhemp	13	Allegan	No mutation[+]	1.6 copies
Waterhemp	14	Montcalm	Trp574Leu[+]	2.5 copies
Waterhemp	15	Montcalm	Ser653Asn[+]	6.7 copies
Waterhemp	16	Montcalm	No mutation[+]	1.4 copies
Waterhemp	17	Ingham	No mutation[+]	1.6 copies
Waterhemp	18	Ingham	No mutation[+]	2.5 copies
Waterhemp	19	St. Joseph	No mutation[+]	6.7 copies
Waterhemp	20	St. Joseph	No mutation[+]	1.4 copies
Waterhemp	21	St. Joseph	No mutation[+]	0.9 copies
Common Ragweed	1	Allegan	No mutation[+]	
Common Ragweed	2	Allegan	Trp574Leu[+]	
Common Ragweed	3	Allegan	No mutation[+]	
Common Ragweed	4	Allegan	Trp574Leu[+]	
Common Ragweed	5	Allegan	Trp574Leu[+]	
Common Ragweed	6	Allegan	No mutation[+]	
Common Ragweed	7	Allegan	No mutation[+]	
Common Ragweed	8	Allegan	No mutation[+]	
Common Ragweed	9	Allegan	No mutation[+]	
Common Ragweed	10	Ingham	No mutation[+]	
Common Ragweed	11	St. Joseph	No mutation[+]	
Common Ragweed	12	St. Joseph	No mutation[+]	
Common Ragweed	13	St. Joseph	No mutation[+]	
Common Ragweed	14	St. Joseph	No mutation[+]	
Common Ragweed	15	St. Joseph	Ala205Val[*]	
Common Ragweed	16	St. Joseph	No mutation[+]	
Common Ragweed	17	St. Joseph	No mutation[+]	
Common Ragweed	18	St. Joseph	No mutation[+]	
Common Ragweed	19	St. Joseph	No mutation[+]	

CHAPTER III: AGRONOMIC PREDICTORS OF HERBICIDE-RESISTANCE FOR PROACTIVE MANAGEMENT IN MICHIGAN

ABSTRACT

Herbicide-resistant weeds threaten modern agriculture production. In Michigan, horseweed (*Erigeron canadensis* L.) is among the most troublesome weeds, and glyphosate was widely used to control *E. canadensis*. Due to extreme selection pressure imposed by heavy glyphosate usage, glyphosate-resistant *E. canadensis* is widespread. New technologies to control resistant *E. canadensis* are being introduced in the form of multiple herbicide-resistance traits into glyphosate-resistant soybean (e.g. dicamba or 2,4-D choline). These new soybean varieties will likely increase the use of 2,4-D and dicamba thus increasing the resistance selection pressure in *E. canadensis*. Predicting agronomic factors that drive herbicide-resistance evolution can serve as an effective proactive tool to advise practitioners to modify management strategies. Therefore, the objectives of this study are: 1) conduct dose-response assays to assess current resistance spectrum of *E. canadensis* collected in Michigan and 2) predict and determine the main factors in row crop production that contribute to resistance evolution in these accessions. Dose-response assays were conducted to evaluate the herbicide sensitivity spectrum to glyphosate, dicamba, and 2,4-D in 20 *E. canadensis* accessions collected from eight Michigan counties. Out of the 20 accessions, 60% were resistant to glyphosate, 35% to 2,4-D, and 20% to dicamba. Pearson's correlation coefficient of dose-response values were positive in all comparisons (2,4-D-dicamba, $r = 0.35$; dicamba-glyphosate, $r = 0.15$; 2,4-D-glyphosate, $r = 0.21$). Dose-response data were integrated in odds ratio analyses to access the influence that previous management history had on the occurrence of resistance. Out of the significant pairwise comparisons, 44% were related to crop rotation frequency, 33% to previous herbicide-resistance status, and 22% to location collected. Results highlight that growers have the ability to proactively manage herbicide-resistance evolution progression of *E. canadensis* in Michigan by adopting integrated weed management techniques to slow successive selection events that occur in low diversity management systems.

KEYWORDS: Herbicide-resistance evolution, multiple resistance, *Erigeron canadensis*, auxinic herbicides, glyphosate, resistance spectrum

INTRODUCTION

Horseweed (*Erigeron canadensis* L.) is an agriculturally important weed worldwide (Holm et al., 1997). In the United States, it is found in annual and perennial crops, such as soybean (*Glycine max* (L.) Merr.), corn (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), orchards, and vineyards (Moretti et al., 2016; Steckel & Gwathmey, 2009). *Erigeron canadensis* competition can reduce yield by 32-69% in corn (Ford et al., 2014; Soltani et al., 2021) and 40-90% in soybean (Agostinetto et al., 2018; Trezzi et al., 2013; Weaver, 2001). *Erigeron canadensis* is difficult to control due to numerous factors. First, *E. canadensis* is the most widespread glyphosate-resistant weed in the world and it is also resistant to other common sites of action (SOA) such as acetolactate synthase (ALS)-inhibitors (Heap, 2014). Second, *E. canadensis* has an extremely aggressive root system and plants can grow up to 2.30 m tall (Bhowmik & Bekech, 1993; Weaver, 2001). Third, *E. canadensis* is a highly prolific seed producer; a single plant can produce up to 200,000 windblown seeds per year (Bhowmik & Bekech, 1993; Weaver, 2001). Wind-mediated seeds can travel long-distances and infest a wide variety of different crops, even if they are relatively distant (Shields et al., 2006), which helps to explain the cosmopolitan nature of this species (Heap, 2014; Holm et al., 1997). Furthermore, the large amount of seed produced may also account for high amounts of genetic diversity both regionally and internationally despite *E. canadensis* being a primarily self-pollinating species (Gaines et al., 2020; Powles, 2008). Fourth, *E. canadensis* can emerge in either the fall or spring, making it both a summer and winter annual, depending on environmental conditions (Schramski et al., 2021). Taken together, these traits make *E. canadensis* one of the most troublesome weed species.

Glyphosate has been widely used to control *E. canadensis* in both the fall and spring as part of burndown herbicide programs as well as in postemergence in-season applications to glyphosate-resistant crops (i.e. Roundup Ready™). Glyphosate-resistant *E. canadensis* was reported in the early 2000s, shortly after the widespread adoption of glyphosate-resistant crops, and is among the first glyphosate-resistant species identified (VanGessel, 2001). Due to the extreme selection pressure imposed by heavy glyphosate usage, glyphosate-resistant *E. canadensis* became frequent and has rapidly spread across the US, limiting the effective use of this chemistry and therefore glyphosate-resistant crop technology (Davis et al., 2008; Flessner et al., 2015; Heap, 2023; Koger et al., 2004). *Erigeron canadensis* accessions resistant to glyphosate, ALS, photosystem I (PSI), and photosystem II (PSII) inhibitors have all been identified in the United

States (Heap, 2023; Moretti et al., 2021). Several of these accessions are actually resistant to two SOA, termed multiple resistant. To date, multiple resistant *E. canadensis* accessions include resistance to: glyphosate and ALS, glyphosate and PSI, and triazine and ALS inhibitors (Byker et al., 2013; Davis et al., 2009; Matzrafi et al., 2015; Moretti et al., 2013). Specifically in Michigan, glyphosate-resistant *E. canadensis* accessions were first reported in 2007 in Mason county and have since become widespread, especially in combination with ALS inhibitors (MSU-PPD, 2024).

New technologies to control resistant accessions of *E. canadensis* are currently being introduced in the form of multiple herbicide-resistance traits into glyphosate-resistant crops. Specifically, in soybean, new varieties include: Roundup Ready 2 Xtend® (glyphosate and dicamba-resistant), Roundup Ready 2 XtendFlex® (glyphosate, dicamba, and glufosinate resistant), Enlist E3™ (2,4-D choline, glyphosate, and glufosinate resistant), and LibertyLink GT27® (glyphosate, glufosinate, and isoxaflutole resistant). Roundup Ready 2 Xtend® was first commercially available in 2017 while Enlist E3™ was first available in 2020 (Dodson, 2022). The introduction of these multiple herbicide-resistant soybean varieties primarily enables farmers to apply auxin mimicking herbicides (group 4) after soybean emergence. These new soybean varieties will likely increase the use of 2,4-D or dicamba for in-season weed control in soybean in addition to applications already happening in corn which is naturally tolerant to these chemistries. This recurrent use of auxinic herbicides will increase the selection pressure for auxin resistance in *E. canadensis*.

Due to the insurgence of herbicide-resistant *E. canadensis* in the United States, especially in a relatively small timeframe (Holm et al., 1997; VanGessel, 2001), and considering new crop-resistant technologies, we are in critical need of information pertaining to the main contributing factors that select for resistance and how growers can alter use practices to delay resistance evolution. One approach is to use epidemiology theory to proactively - not reactively – predict and manage herbicide-resistance evolution (Comont et al., 2019; Comont & Neve, 2021). By epidemiologically understanding the main drivers of herbicide-resistance evolution, we could educate growers and the agricultural industry as early as possible on how to avoid herbicide-resistance evolution and to preserve the use of group 4 resistant crops (Evans et al., 2016). Therefore, the objectives of this study are: 1) conduct dose-response assays to assess current resistance spectrum of *E. canadensis* accessions collected in Michigan and 2) predict and

determine the main factors in row crop production that contribute to herbicide-resistance evolution in these accessions.

MATERIALS AND METHODS

Dose-Response Assay

Greenhouse dose-response experiments were conducted from August 2021 to September 2023 at Michigan State University in East Lansing, MI, USA. The experimental design consisted of a randomized complete block design with four replications repeated once. We utilized 20 *E. canadensis* accessions in this study collected from eight counties in Michigan (Table 3.1). Seeds were hand threshed from the plant material and cleaned. Once the seeds were cleaned, the accessions were planted in flats containing potting media and placed in the greenhouse (16h light at 26 C; 8h dark at 18 C). At the presence of two true leaves, each plant was transplanted into a 12 by 12 cm pot (Shuttle Pot®, East Jordan Plastics, Inc.) containing the same media. When transplants reached approximately 12 cm in diameter the below herbicide treatments were made. Plants were watered daily and fertilizer (NPK 15-07-25, ICL Specialty Fertilizers) was applied weekly.

The dose-response experiment consisted of nine rates of the following herbicides: glyphosate (Roundup PowerMAX® 3, 575 g ae L^{-1}, Bayer), dicamba (XtendiMax®, 350 g ae L^{-1}, Bayer), and 2,4-D choline (Enlist One™, 455 g ae L^{-1}, Corteva Agriscience) (Table 3.1). For glyphosate the dose treatments were: 0, 0.25, 0.5, 1, 2, 4, 8, 16, and 32 times the recommended labeled rate of 1.26 kg ae ha^{-1}. For dicamba and 2,4-D the dose treatments were: 0, 0.016, 0.031, 0.062, 0.125, 0.25, 0.33, 0.5, and 1 times the recommended labeled rate of 0.56 kg ae ha^{-1} and 1.07 kg ae ha^{-1}, respectively. Additionally, 2% v/v dry ammonium sulfate was included with glyphosate and 2,4-D treatments. Herbicide treatments were applied using a single-track sprayer (Generation 4, DeVries Manufacturing, Inc., Hollandale, MN) equipped with an 8001E TeeJet flat-fan nozzle (TeeJet Technologies, Wheaton, IL) calibrated to deliver 187 L ha^{-1} at 193 kPa of pressure. Visual injury ratings were performed in 7, 14, and 21 days after treatment (DAT). Aboveground dry biomass was obtained 21 DAT. Plants were cut at the soil surface and dried at 66 C for 7 days before dry biomass was recorded.

Statistical Analysis

Three and four-parameter log-logistic models as well as a three-parameter Weibull model were fit to the data to determine the pattern of biomass reduction per herbicide and accession

(Equations 1, 2 and 3), where the independent variable was herbicide rate, and the dependent variable was dry biomass using the DRC package in R (Ritz et al., 2015). Model fit was assessed using the DRC modelFit function in R, following the methods outlined in Knezevic et al. (2007). Models that have p-values >0.05 were chosen for the analysis with a few exceptions in which models that yielded the smallest standard error values were chosen for analysis (Table 3.2).

$$f(x) = \frac{d}{1+[\frac{x}{(ED50)b}]} \qquad [1]$$

$$f(x) = d + \frac{(a-d)}{1+[\frac{x}{(ED50)b}]} \qquad [2]$$

$$f(x \mid c, \lambda, k) = 1 - e^{\left[\frac{(x-c)^k}{\lambda}\right]} \qquad [3]$$

For all the equations, $f(x)$ represents the effect of the herbicide at a given dose x, a is the response level when the dose x is the highest, d is the response level when the dose x is the lowest, ED_{50} is the dose causing 50% biomass reduction, and b is Hill's slope, which is how steep the dose-response curve is (Muse et al., 2021). For the Weibull function, $f(x/c,\lambda,k)$ is the probability that the ED_{50} is either less than or equal to a given dose x, c is the location parameter, λ is the scale parameter, and k is the shape parameter (Hallinan, 1993). Accessions with ED_{50} values greater than the glyphosate recommended field use rate were considered resistant and lower than the field use rate were considered susceptible for subsequent analysis. Accessions with ED_{50} values greater than dicamba and 2,4-D recommended field use rates were considered reduced sensitivity and lower than field use rate were considered susceptible for subsequent analysis.

To investigate the association amongst ED_{50} values from different herbicides, correlation analysis was performed using Pearson's correlation coefficient (Equation 4) using the Hmisc package in R (Harrell Jr, 2023).

$$r = \frac{(n \sum x y) - (\sum x)*(\sum y)}{\sqrt{[n \sum x^2 - (\sum x)^2] \cdot [(n \sum y^2 - (\sum y)^2]}} \qquad [4]$$

For this equation, r represents the correlation coefficient, x and y represent the individual data points for ED_{50} values to be correlated, and x^2 and y^2 represent the average of the respective sets of ED_{50} values.

Dose-response data were further analyzed using logistic regression to access the influence previous management history had on the occurrence of resistant accessions. Odds ratio (OR) analysis was performed to verify the strength and direction of association between two variables. For this study, odds ratio analyses were performed using the herbicide ED_{50} values (glyphosate, dicamba, and 2,4-D) generated in the dose-response assays in objective 1, location the accession was collected, previous herbicide-resistance screening, and eight-year crop rotation history in the location the accessions were collected (Table 3.3). Michigan State University Plant and Pest Diagnostics previously evaluated these accessions for herbicide response (screened with one and four times the recommend field use rate) to glyphosate, 2,4-D amine, and cloransulam (MSU-PPD, 2024). Crop rotation data from 2015-2022 were collected from the USDA-NASS Cropland Data Layer using the CroplandCROS web application (USDA, 2022). Once crop rotation information was extracted the frequency of a particular crop grown in that rotation was calculated for each rotation and categorized as: high (greater than 50% of the years contained that crop), medium (20-50% of the years contained that crop), and low (less than 20% of the years contained that crop).

RESULTS AND DISCUSSION

Dose-Response Assay - Glyphosate

Out of the 20 *E. canadensis* accessions evaluated in this study, 12 (60%) were resistant to glyphosate (i.e. survived the recommended field use rate of 1.26 kg ae ha^{-1}) (Table 3.1). Susceptible accession ED_{50} values varied substantially, ranging between <0.32 kg ae ha^{-1} (the lowest dose applied) to 1.15 kg ae ha^{-1} (~0.9 times the field use rate). Half of the susceptible accessions were collected from separate specific locations but within Ingham County, while the remaining accessions were distributed across Montcalm, Isabella, and Delta counties. Resistant accession ED_{50} values also varied widely; ranging between 1.85 kg ae ha^{-1} (1.5 times the field use rate) to >40.32 kg ae ha^{-1} (the highest rate applied). The accessions with ED_{50} values >40.32 kg ae ha^{-1} were collected from Macomb and Montcalm counties. Interestingly, out of the seven accessions collected in Montcalm country, six were resistant to glyphosate, with ED_{50} values ranging from 2.79 to >40.32 kg ae ha^{-1}.

Adopting Roundup Ready® (i.e. glyphosate-resistant) soybean simplified weed management, was economical, and saved growers multiple passes through the field for weed management. The adoption rate of Roundup Ready® soybean increased dramatically since introduction in 1996, reaching nearly 90% of acreage in the United States by 2008 (Dodson, 2022). Michigan has followed suit, with 93% of soybean and 92% of corn hectarage being Roundup Ready® (USDA, 2023). Annual glyphosate usage in Michigan is approximately 8.06 and 6.43 million liters in soybean and corn, respectively. In the short term, this single SOA weed control tactic allowed for higher yields with greatly reduced cost (Duke & Powles, 2009); however, it also led to successive selection events which in turn selected for widespread resistance in many troublesome weed species, including *E. canadensis* (Green, 2007). Perhaps unsurprisingly then, from our initial dose-response results we observed that Michigan counties that are the largest producers of corn and soybean (Table 3.3) also have the highest level of glyphosate resistance (Table 3.1).

Dose-Response- 2,4-D and Dicamba

Out of the 20 accessions screened in our study, 7 (35%) had reduced sensitivity to 2,4-D (i.e. survived the recommended field use rate of 1.07 kg ae ha^{-1}) and 4 (20%) had reduced sensitivity to dicamba (i.e. survived the recommended field use rate of 0.56 kg ae ha^{-1}) (Table 3.1). These populations were considered as having 'reduced sensitivity' to 2,4-D and dicamba as they survived the highest dose applied; however, it is unclear since we did not apply a dose greater than the field use rate if they would be resistant in an agronomic setting. Overall, ED$_{50}$ values for 2,4-D and dicamba varied widely across all 20 accessions, ranging between less than the lowest dose applied (<0.02 kg ae ha^{-1} and <0.01 kg ae ha^{-1}for 2,4-D and dicamba, respectively) to the recommended field use rate (1.07 kg ae ha^{-1} and 0.56 kg ae ha^{-1} for 2,4-D and dicamba, respectively). Susceptible accessions for 2,4-D were found in all counties screened, except for Macomb and Cass, and in all counties for dicamba. Accessions with reduced sensitivity to 2,4-D were collected from Cass (1 accession), Ingham (1 accession), Isabella (1 accession), Macomb (1 accession), and Montcalm (3 accessions) counties, while accessions with reduced sensitivity to dicamba were collected from Ingham (1 accession), Isabella (1 accession), and Montcalm (2 accessions) counties.

To combat the rise in glyphosate resistance, agricultural companies have recently developed and released soybean varieties, Xtend/XtendFlex® and Enlist E3™, that are resistant to

the auxinic herbicides dicamba or 2,4-D, respectively (Skelton et al., 2017). We screened our accessions of *E. canadensis* with 2,4-D and dicamba in an attempt to understand baseline sensitivity in this species in Michigan before these technologies are widely adopted. Our results show that in counties with high frequency of glyphosate-resistant *E. canadensis* we also see a corresponding decrease in auxinic herbicide sensitivity. Perhaps unsurprisingly, we see this pattern the most in Michigan counties that are the largest producers of corn and soybean (USDA, 2024). For instance, 45% of all accessions with reduced sensitivity to auxin herbicides were collected from Montcalm County (Table 3.1), which is the 22nd producer of corn and 26th in soybean out of 83 counties in Michigan (USDA, 2024). Auxin mimicking herbicides have been extensively used in corn for a long time due to its natural tolerance, therefore it is possible usage in corn started the selection process for auxinic resistance in *E. canadensis* that has the potential to be exacerbated by the future increased usage of auxinic herbicides in soybean.

Dose-Response- Correlation

Overall, 30% of collected accessions are resistant or have reduced sensitivity to two or more of the herbicides tested. Amongst these multiple resistant populations, five are resistant to glyphosate and 2,4-D (Cass, Macomb, and Montcalm counties), two are resistant to glyphosate and dicamba (Montcalm county), and three have reduced sensitivity to both 2,4-D and dicamba (Ingham and Montcalm counties) (Table 3.1). Furthermore, two of collected accessions are resistant to all three herbicides assayed (Montcalm county) while five are susceptible to all three herbicides tested (Delta, Ingham, and Montcalm counties).

To investigate the relationship between herbicide response to each SOA tested, Pearson's correlation analysis amongst ED_{50} values was performed. Pearson's correlation coefficient (r) ranges from -1 to 1 (negative and positive correlation, respectively), where a value of: 0 indicates no, $0 < r < \pm0.20$ weak, $\pm0.20 < r < \pm0.40$ moderate, $\pm0.40 < r < \pm0.80$ strong, and $\pm0.80 < r < \pm1.00$ very strong correlation (Pearson, 1895).

When comparing ED_{50} values amongst accessions treated with the auxinic herbicides 2,4-D and dicamba, Pearson's correlation coefficient was moderately positive, $r = 0.35$, indicating that increases in the ED_{50} for 2,4-D corresponds to increases in the ED_{50} value for dicamba (Figure 1). Furthermore, when comparing ED_{50} values amongst accessions treated with 2,4-D and glyphosate, Pearson's correlation coefficient was also moderately positive, $r = 0.21$, indicating glyphosate and 2,4-D resistance are positively correlated as well (Figure 1). Finally, when comparing ED_{50} values

amongst accessions treated with dicamba and glyphosate, Pearson's correlation coefficient was weakly positive, r = 0.15 (Figure 1).

The moderately positive Pearson's correlation coefficient between dicamba and 2,4-D is interesting, as these herbicides are both group 4 herbicides, but are from different chemical families and have been shown to have different protein binding partners, IAA16 and IAA2, respectively (LeClere et al., 2018; Todd et al., 2020). Even though these herbicides are from different chemical families, non-target-site resistance (NTSR) mechanism(s) could be responsible for the moderate, but positive, correlation between these herbicides. Although not reported yet in *E. canadensis*; these NTSR mechanisms have been implied in cross-resistance for group 4 herbicides in wild radish (*Raphanus raphanistrum* L.) (Goggin et al., 2016).

The moderate and weak correlation between glyphosate and 2,4-D and glyphosate and dicamba and glyphosate is notable. Regardless of strength, it is interesting to find a correlation between glyphosate and auxin mimicking herbicides, as these herbicides are from different SOA and chemical families. Furthermore *E. canadensis* is a strongly self-pollinated species and thus lacks the ability to rapidly stack multiple resistance traits in a single individual or population which is predicted in outcrossing species such as Palmer amaranth (*Amaranthus palmeri* S. Watson) and waterhemp (*Amaranthus tuberculatus* (Moq.) Sauer) (Gaines et al., 2020; Powles, 2008). These positive correlations are particularly concerning from a management perspective where growers will likely turn to Xtend/XtendFlex® and Enlist E3® technologies in an attempt to control glyphosate-resistant weeds, yet these populations seem primed to develop resistance to group 4 herbicides already via previous exposure to group 4 herbicides used in grass crops.

Odds Ratio-Previous Herbicide-Resistance

Dose-response data were further analyzed using logistic regression to access the influence previous management history had on the occurrence of resistant accessions. Odds ratio analyses were performed using the ED_{50}'s generated from the dose-response assays performed in objective 1 (Table 3.1), previous herbicide-resistance screening performed at Plant and Pest Diagnostics at MSU (MSU-PPD, 2024), county the accession was collected, and crop rotation information from the location the accession was collected using the USDA-NASS Cropland Data Layer CroplandCROS web application (USDA, 2022) (Table 3.3). In total, 174 pairwise comparisons were performed for the analysis (Supplementary Table S1).

Amongst all combinations, nine were statistically significant (p-value <0.20, Table 3.4) (Andrade 2019). Out of the significant pairwise comparisons, 33% involved comparisons of herbicide-resistance phenotypes identified in objective 1 to different SOA previously screened (MSU-PPD, 2024). First, if an *E. canadensis* accession has reduced sensitivity to 2,4-D the odds the accession is glyphosate-resistant increases by 20% (OR = 0.20, p = 0.19, Table 3.4). Second, if an *E. canadensis* accession is resistant to cloransulam the odds the accession is glyphosate-resistant increases by 15% (OR = 0.15, p = 0.08, Table 3.4). Finally, if an *E. canadensis* accession has reduced sensitivity to 2,4-D the odds the accession has reduced sensitivity to dicamba increases by 8% (OR = 0.08, p = 0.03, Table 3.4).

Overall, our results suggest that resistance to one herbicide is associated to resistance to another in *E. canadensis*, a finding that is supported by previous literature. In general, when one herbicide fails to control a weed, the primary alternative is to use a different herbicide chemical family or SOA to control those individuals, which can often lead to multiple herbicide-resistance in which accessions are resistant to herbicides with different herbicide chemical families or SOA (Beckie & Tardif, 2012). Specifically, the co-occurrence of ALS and glyphosate resistance in *E. canadensis* has been reported by Byker et al. (2013). Furthermore, cross-resistance has occurred in wild mustard (*Sinapis arvensis* L.) to three auxin mimicking herbicides from different chemical families (2,4-D, dicamba, and/or quinclorac) (Heap & Morrison, 2002). Another case reported multiple resistance in *E. sumatrensis* Retz. to paraquat, glyphosate, and chlorimuron (Albrecht et al. 2020). The results found in this study highlight the propensity of *E. canadensis* to evolve multiple resistance, especially in accessions that were already resistant to one SOA.

Odds Ratio-Location

The second most influential variable was location, where combinations between the counties the accessions were collected were analyzed to resistance. Specifically, glyphosate-resistant accessions were 2,400% (OR = 24.00, p = 0.04, Table 3.4) more likely to occur in Ingham County, or 1,200% (OR = 12.00, p = 0.13, Table 3.4) more likely to occur in Isabella County when glyphosate resistance was already present in the neighboring county, Montcalm (Table 3.4, Figure 2). This is not surprising as these counties have similar crop rotations: corn, soybean, and winter wheat and thus share herbicide SOAs used in those rotations (USDA, 2022). Interestingly, Isabella and Montcalm counties share a boarder, and because *E. canadensis* seed dispersal is wind-

mediated, the spread of resistance is potentially favored by the short distance between these counties.

Odds Ratio-Crop Rotation Frequency

The third most impactful variable was crop rotation frequency. When corn is planted between 20 to 50% of the years evaluated (medium frequency), the odds of *E. canadensis* having reduced dicamba sensitivity increases by 600% (OR = 6.00, p = 0.15, Table 3.4). When soybean is planted in <20% of the years evaluated (low frequency), the odds of *E. canadensis* having reduced 2,4-D sensitivity increases by 560% (OR = 5.60, p = 0.17, Table 3.4). When soybean is planted in >50% of the years (high frequency), the odds of *E. canadensis* having reduced 2,4-D sensitivity increases by 18% (OR = 0.18, p = 0.17, Table 3.4). Finally, when winter wheat is planted in <20% of years, the odds of *E. canadensis* having reduced dicamba sensitivity increases by 15% (OR = 0.15 p = 0.17, Table 3.4).

Crop rotation dictates what herbicide SOAs are used within a particular crop and across the rotation. With the introduction of auxinic resistant soybean varieties, there is potential that this SOA will be used more frequently or even continuously in the crop rotation, especially for soybean-corn rotations (Figure 2A and B). Furthermore, using the same herbicide SOA increases the selection pressure and speed at which *E. canadensis* evolves resistant to those SOA (Evans et al., 2016). Specifically, the propensity of *E. canadensis* to evolve dicamba resistance increased in our study when corn was present at medium frequency in the rotation. Furthermore, our finding that having soybean represented at low frequency in the crop rotation leads to an increase in 2,4-D resistance is supported by the high frequency of grass crops (i.e. corn and pasture) as the main components of these low soybean rotations (Table 3.3, Figure 2). Overall, these results suggest that having a medium to high frequency of grass crops, which are naturally tolerant to auxinic herbicides, in the rotation will predispose *E. canadensis* to have reduced sensitivity to auxinic herbicides when they are used more frequently in the future as adoption of auxinic resistant soybean increases. This is potentially already occurring as our data found that there was a marginal increase in 2,4-D resistance in high frequency soybean rotations (Table 3.4).

The epidemiological approach utilized in this study begins to elucidate the main agronomic predictors of herbicide-resistance in *E. canadensis* in Michigan. These predictors reiterate the importance of diverse management strategies and crop rotations to prevent future resistance evolution. Alarmingly, we are already able to find *E. canadensis* accessions that have reduced

sensitivity to 2,4-D and dicamba, therefore it is possible that the introduction of Enlist™ and Xtend® technologies may only offer short-term relief and control of glyphosate-resistant weeds (Evans et al., 2016; LeClere et al., 2018; Todd et al., 2020) if used without other non-chemical management strategies. Not coincidentally, counties in the same agroclimatic zone are prone to have similar food production systems and therefore comparable overall management strategies and resistance issues (Evans et al., 2016).

Perhaps expectedly, similarity in food production and management strategies favors the evolution and spread of herbicide-resistance in *E. canadensis*, highlighting lack of diversity is a major factor in resistance evolution. Furthermore, in a wind dispersed species such as *E. canadensis*, once herbicide-resistance is established in a certain county, the proximity between counties potentially leads to the spread of herbicide-resistant accessions in neighboring counties. The epidemiological approach performed in this study focused on evaluating a medium sized data set of categorical variables that are controllable by farmers. However, other drivers may be influencing the shift towards herbicide resistance of *E. canadensis* in Michigan, which will require more research to explore and integrate to form robust predictions. Overall, growers have the ability to *proactively* manage herbicide-resistance evolution progression of *E. canadensis* in Michigan by adopting integrated weed management (IWM) techniques to slow successive selection events that occur in low diversity management systems. Specifically, IWM principles of crop rotation diversity and thus herbicide SOA diversity will enable farmers to diminish the threat of herbicide-resistance evolution in *E. canadensis* on their properties and regions.

BIBLIOGRAPHY

Agostinetto, D., Silva, D. R. O. da, & Vargas, L. (2018). Soybean yield loss and economic thresholds due to glyphosate resistant hairy fleabane interference. *Arquivos Do Instituto Biológico*, *84*(0). https://doi.org/10.1590/1808-1657000022017

Albrecht, A. J. P., Pereira, V. G. C., de Souza, C. N. Z., Zobiole, L. H. S., Albrecht, L. P., & Adegas, F. S. (2020). Multiple resistance of *Conyza sumatrensis* to three mechanisms of action of herbicides. *Acta Scientiarum - Agronomy*, *42*, 1–9. https://doi.org/10.4025/actasciagron.v42i1.42485

Beckie, H. J., & Tardif, F. J. (2012). Herbicide cross resistance in weeds. *Crop Protection*, *35*, 15–28. https://doi.org/10.1016/j.cropro.2011.12.018

Bhowmik, P. C., & Bekech, M. M. (1993). Horseweed (*Conyza canadensis*) seed production, emergence, and distribution in no-tillage and conventional-tillage corn (*Zea mays*). *Agronomy (Trends in Agril. Sci.)*, 67–71. https://doi.org/https://www.researchgate.net/publication/256505935

Byker, H. P., Soltani, N., Robinson, D. E., Tardif, F. J., Lawton, M. B., & Sikkema, P. H. (2013). Occurrence of glyphosate and cloransulam resistant Canada fleabane (*Conyza canadensis* L. Cronq.) in Ontario. *Canadian Journal of Plant Science*, *93*(5), 851–855. https://doi.org/10.4141/CJPS2013-039

Comont, D., Hicks, H., Crook, L., Hull, R., Cocciantelli, E., Hadfield, J., Childs, D., Freckleton, R., & Neve, P. (2019). Evolutionary epidemiology predicts the emergence of glyphosate resistance in a major agricultural weed. *New Phytologist*, *223*(3), 1584–1594. https://doi.org/10.1111/nph.15800

Comont, D., & Neve, P. (2021). Adopting epidemiological approaches for herbicide resistance monitoring and management. *Weed Research*, *61*(2), 81–87. https://doi.org/10.1111/wre.12420

Davis, V. M., Gibson, K. D., & Johnson, W. G. (2008). A Field Survey to Determine Distribution and Frequency of Glyphosate-Resistant Horseweed (*Conyza Canadensis*) in Indiana. *Weed Technology*, *22*(2), 331–338. https://doi.org/10.1614/wt-07-147.1

Davis, V. M., Kruger, G. R., Stachler, J. M., Loux, M. M., & Johnson, W. G. (2009). Growth and Seed Production of Horseweed (*Conyza canadensis*) Populations Resistant to Glyphosate, ALS-Inhibiting, and Multiple (Glyphosate + ALS-Inhibiting) Herbicides. *Weed Science*, *57*(5), 494–504. https://doi.org/10.1614/WS-09-024

Dodson, L. (2022, September). *USDA ERS - Recent Trends in GE Adoption*. Www.Ers.Usda.Gov. Https://Www.Ers.Usda.Gov/Data-Products/Adoption-of-Genetically-Engineered-Crops-in-the-u-s/Recent-Trends-in-Ge-Adoption/.

Duke, S. O., & Powles, S. B. (2009). Glyphosate-Resistant Crops and Weeds: Now and in the Future. *AgBioForum*, *12*(3 & 4), 346–357.

Evans, J. A., Tranel, P. J., Hager, A. G., Schutte, B., Wu, C., Chatham, L. A., & Davis, A. S. (2016). Managing the evolution of herbicide resistance. *Pest Management Science*, *72*(1), 74–80. https://doi.org/10.1002/ps.4009

Flessner, M. L., McElroy, J. S., McCurdy, J. D., Toombs, J. M., Wehtje, G. R., Burmester, C. H., Price, A. J., & Ducar, J. T. (2015). Glyphosate-Resistant Horseweed (*Conyza canadensis*) Control with Dicamba in Alabama. *Weed Technology*, *29*(4), 633–640. https://doi.org/10.1614/wt-d-14-00089.1

Ford, L., Soltani, N., Robinson, D. E., Nurse, R. E., McFadden, A., & Sikkema, P. H. (2014). Canada fleabane (*Conyza canadensis*) control with preplant applied residual herbicides followed by 2,4-D choline/glyphosate DMA applied postemergence in corn. *Canadian Journal of Plant Science*, *94*(7), 1231–1237. https://doi.org/10.4141/CJPS2013-403

Gaines, T. A., Duke, S. O., Morran, S., Rigon, C. A. G., Tranel, P. J., Anita Küpper, & Dayan, F. E. (2020). Mechanisms of evolved herbicide resistance. In *Journal of Biological Chemistry* (Vol. 295, Issue 30, pp. 10307–10330). American Society for Biochemistry and Molecular Biology Inc. https://doi.org/10.1074/jbc.REV120.013572

Goggin, D. E., Cawthray, G. R., & Powles, S. B. (2016). 2,4-D resistance in wild radish: Reduced herbicide translocation via inhibition of cellular transport. *Journal of Experimental Botany*, *67*(11), 3223–3235. https://doi.org/10.1093/jxb/erw120

Green, J. M. (2007). Review of Glyphosate and Als-inhibiting Herbicide Crop Resistance and Resistant Weed Management. *Weed Technology*, *21*(2), 547–558. https://doi.org/10.1614/wt-06-004.1

Hallinan, A. J. (1993). A Review of the Weibull Distribution. *Journal of Quality Technology*, *25*(2), 85–93. https://doi.org/10.1080/00224065.1993.11979431

Harrell Jr. (2023). *Hmisc: Harrell Miscellaneous. R package version 5.1-2*.

Heap. (2014). Global perspective of herbicide-resistant weeds. *Pest Management Science*, *70*(9), 1306–1315. https://doi.org/10.1002/ps.3696

Heap, I. (2023, September 26). *The International Herbicide-Resistant Weed Database*. Www.Weedscience.Org.

Heap, & Morrison, I. N. (2002). Resistance to auxin-type herbicides in wild mustard (*Sinapis arvensis* L.) populations in western Canada. *Weed Sci. Soc. Am. Abstr.*, *55*, 32.

Hill, E. (2024). *History & Map of Herbicide-Resistant Weeds In Michigan*. History & Map of Herbicide-Resistant Weeds In Michigan. https://www.canr.msu.edu/pestid/resources/plant-and-weed-identification/map

Holm, L., Doll, J., Holm, E., Pancho, J., & Herberger, J. (1997). *World weeds: Natural histories and distribution*. John Wiley & Sons, Inc.

Knezevic, S. Z., Streibig, J. C., & Ritz, C. (2007). Utilizing R Software Package for Dose-Response Studies: The Concept and Data Analysis. *Weed Technology*, *21*(3), 840–848. https://doi.org/10.1614/wt-06-161.1

Koger, C. H., Poston, D. H., Hayes, R. M., & Montgomery, R. F. (2004). Glyphosate-Resistant Horseweed (*Conyza canadensis*) in Mississippi. *Weed Technology*, *18*(3), 820–825. https://doi.org/10.1614/wt-03-218r

LeClere, S., Wu, C., Westra, P., & Sammons, R. D. (2018). Cross-resistance to dicamba, 2,4-D, and fluroxypyr in *Kochia scoparia* is endowed by a mutation in an AUX/IAA gene. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(13), E2911–E2920. https://doi.org/10.1073/pnas.1712372115

Matzrafi, M., Lazar, T. W., Sibony, M., & Rubin, B. (2015). Conyza species: distribution and evolution of multiple target-site herbicide resistances. *Planta*, *242*(1), 259–267. https://doi.org/10.1007/s00425-015-2306-4

Moretti, M. L., Bobadilla, L. K., & Hanson, B. D. (2021). Cross-resistance to diquat in glyphosate/paraquat-resistant hairy fleabane (*Conyza bonariensis*) and horseweed (*Conyza canadensis*) and confirmation of 2,4-D resistance in *Conyza bonariensis*. *Weed Technology*, *35*(4), 554–559. https://doi.org/10.1017/wet.2021.11

Moretti, M. L., Hanson, B. D., Hembree, K. J., & Shrestha, A. (2013). Glyphosate Resistance Is More Variable Than Paraquat Resistance in a Multiple-Resistant Hairy Fleabane (*Conyza bonariensis*) Population. *Source: Weed Science*, *61*(3), 396–402. https://www.jstor.org/stable/43700219?seq=1&cid=pdf-

Moretti, M. L., Sosnoskie, L. M., Shrestha, A., Wright, S. D., Hembree, K. J., Jasieniuk, M., & Hanson, B. D. (2016). Distribution of *Conyza* sp. in Orchards of California and Response to Glyphosate and Paraquat. *Weed Science*, *64*(2), 339–347. https://doi.org/10.1614/ws-d-15-00174.1

MSU-PPD. (2024). *Plant & pest diagnostics*. Plant & Pest Diagnostics. Https://Www.Canr.Msu.Edu/Pestid/Index.

Muse, A. H., Mwalili, S. M., & Ngesa, O. (2021). On the Log-Logistic Distribution and Its Generalizations: A Survey. *International Journal of Statistics and Probability*, *10*(3), 93. https://doi.org/10.5539/ijsp.v10n3p93

Pearson, K. (1895). . *In Proceedings of Royal Society, 241-58.*

Powles, S. B. (2008). Evolved glyphosate-resistant weeds around the world: Lessons to be learnt. *Pest Management Science*, *64*(4), 360–365. https://doi.org/10.1002/ps.1525

Ritz, C., Baty, F., Streibig, J. C., & Gerhard, D. (2015). Dose-response analysis using R. *PLoS ONE*, *10*(12). https://doi.org/10.1371/journal.pone.0146021

Schramski, J. A., Sprague, C. L., & Patterson, E. L. (2021). Environmental cues affecting horseweed (*Conyza canadensis*) growth types and their sensitivity to glyphosate. *Weed Science*, *69*(4), 412–421. https://doi.org/10.1017/wsc.2021.27

Shields, E. J., Dauer, J. T., VanGessel, M. J., & Neumann, G. (2006). Horseweed (*Conyza canadensis*) seed collected in the planetary boundary layer. *Weed Science*, *54*(6), 1063–1067. https://doi.org/10.1614/ws-06-097r1.1

Skelton, J. J., Simpson, D. M., Peterson, M. A., & Riechers, D. E. (2017). Biokinetic Analysis and Metabolic Fate of 2,4-D in 2,4-D-Resistant Soybean (*Glycine max*). *Journal of Agricultural and Food Chemistry*, *65*(29), 5847–5859. https://doi.org/10.1021/acs.jafc.7b00796

Soltani, N., Shropshire, C., & Sikkema, P. H. (2021). Control of glyphosate-resistant horseweed (*Conyza canadensis*) with tiafenacil mixes in corn. *Weed Technology*, *35*(6), 908–911. https://doi.org/10.1017/wet.2021.44

Steckel, L. E., & Gwathmey, C. O. (2009). Glyphosate-Resistant Horseweed (*Conyza canadensis*) Growth, Seed Production, and Interference in Cotton. *Weed Science*, *57*(3), 346–350. https://doi.org/10.1614/ws-08-127.1

Todd, O. E., Figueiredo, M. R. A., Morran, S., Soni, N., Preston, C., Kubeš, M. F., Napier, R., & Gaines, T. A. (2020). Synthetic auxin herbicides: finding the lock and key to weed resistance. In *Plant Science* (Vol. 300). Elsevier Ireland Ltd. https://doi.org/10.1016/j.plantsci.2020.110631

Trezzi, M. M., Balbinot Jr., A. A., Benin, G., Debastiani, F., Patel, F., & Miotto Jr., E. (2013). Competitive ability of soybean cultivars with horseweed (*Conyza boranensis*). *Planta Daninha*, *31*(3), 543–550.

USDA. (2022). *CroplandCROS*. Https://Croplandcros.Scinet.Usda.Gov/.

USDA. (2023, July). *Agriculture Across Michigan*. https://www.nass.usda.gov/Quick_Stats/Ag_Overview/stateOverview.php?state=MICHIGAN

USDA. (2024, April 15). *"USDA - National Agricultural Statistics Service - Michigan - County Estimates."* Www.Nass.Usda.Gov, Www.Nass.Usda.Gov/Statistics_by_State/Michigan/Publications/County_Estimates/Index.Php.

VanGessel, M. J. (2001). Glyphosate-resistant horseweed from Delaware. *Weed Science*, *49*(6), 703–705. https://doi.org/10.1614/0043

Weaver, S. E. (2001). The biology of Canadian weeds. 115. *Conyza canadensis*. *Canadian Journal Plant Science*, *81*, 867–875. https://doi.org/https://doi.org/10.4141/P00-196

Figure 3.1. Pearson's correlation analysis between the mean herbicide dose (ED_{50} in kg ae ha^{-1}) required for 50% biomass reduction in 20 *Erigeron canadensis* L. accessions collected from eight counties in Michigan. ED_{50} values were calculated using dry plant biomass harvested 21 days after treatment using the DRC package in R. Field recommended labeled rates are 1.26, 0.56, and 1.07 kg ae ha^{-1} for glyphosate, dicamba, and 2,4-D, respectively.
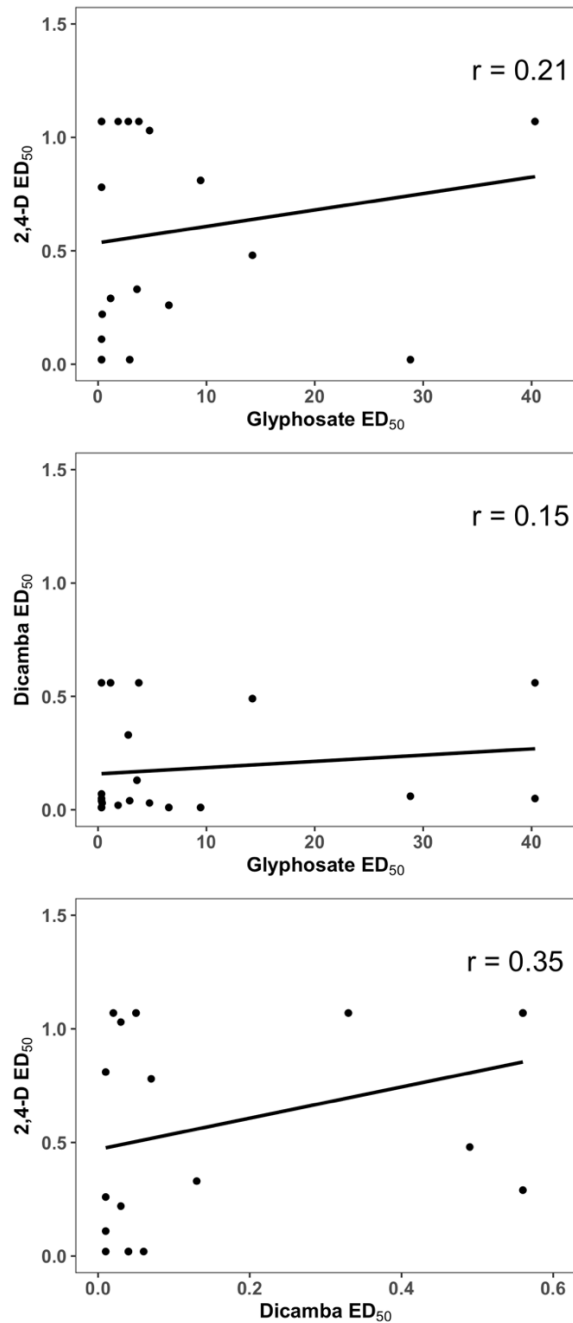
Figure 3.2. Location and resistance phenotype of *E. canadensis* accessions that were collected throughout Michigan. Crop rotation data was collected from USDA-NASS Cropland Data Layer using the CroplandCROS web application from an eight year period (2015-2022, USDA, 2022) soybean (A), corn (B), winter wheat (C), potatoes (D), and pasture (E).
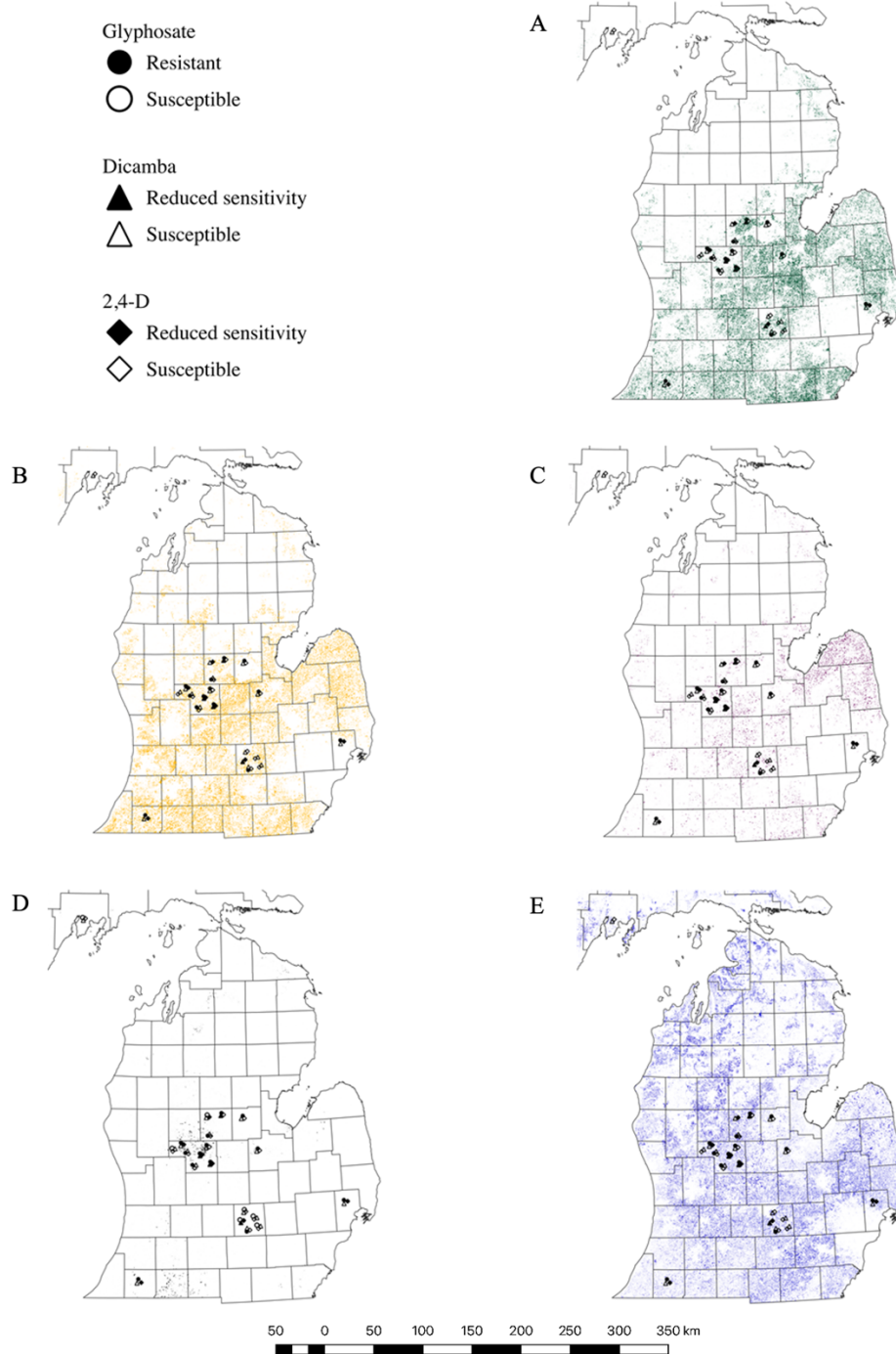
Table 3.1. Mean herbicide dose (ED_{50} in kg ae ha^{-1} ± [SE])[a] required for 50% biomass reduction in 20 *Erigeron canadensis* L. accessions collected from eight counties in Michigan.

Accession location	Glyphosate	Dicamba	2,4-D
	---------------------------- ED_{50} kg ae ha^{-1}-----------------------		
Cass	1.85 (1.34)	0.02 (0.00)	>1.07
Delta	<0.32	0.04 (0.04)	0.02 (0.62)
Ingham	<0.32	0.01 (0.49)	0.11 (0.55)
Ingham	<0.32	>0.56	>1.07
Ingham	<0.32	0.07 (0.13)	0.78 (0.13)
Ingham	4.75 (3.27)	0.03 (0.01)	1.03 (0.48)
Ingham	<0.32	<0.01	<0.02
Isabella	<0.32	0.05 (0.03)	>1.07
Isabella	1.15 (0.75)	>0.56	0.29 (0.24)
Isabella	6.53 (2.98)	<0.01	0.26 (0.04)
Macomb	>40.32	0.05 (0.06)	>1.07
Midland	3.59 (2.69)	0.13 (0.08)	0.33 (0.17)
Montcalm	0.38 (0.33)	0.03 (0.01)	0.22 (0.20)
Montcalm	2.79 (1.47)	0.33 (0.13)	>1.07
Montcalm	3.76 (2.89)	>0.56	>1.07
Montcalm	2.92 (0.53)	0.04 (0.04)	0.02 (0.01)
Montcalm	9.46 (20.28)	<0.01	0.81 (0.52)
Montcalm	14.25 (20.75)	0.49 (1.01)	0.48 (0.11)
Montcalm	>40.32	>0.56	>1.07
Saginaw	28.82 (19.19)	0.06 (0.03)	<0.02

[a]ED_{50} calculated using dry plant biomass harvested 21 days after treatment using the DRC package in R. Field recommended labeled rates are 1.26, 0.56, and 1.07 kg ae ha^{-1} for glyphosate, dicamba, and 2,4-D, respectively.

Table 3.2. List of statistical models used to generate mean herbicide dose (ED$_{50}$ in kg ae ha^{-1} ± [SE]) required for 50% biomass reduction in 20 *Erigeron canadensis* L. accession collected from eight counties in Michigan.[a]

Accession location	Model			Model Fit		
	Glyphosate	Dicamba	2,4-D	Glyphosate	Dicamba	2,4-D
	---------------Function--------------			---------------P-value--------------		
Cass	LL.3	LL.4	LL.3	0.99	0.42	0.007
Delta	LL2.3	LL.4	LL.4	0.15	0.31	0.48
Ingham	LL.4	LL.3	LL2.3	0.98	0.29	0.96
Ingham	W1.3	LL.3	LL2.3	0.03	0.08	0.38
Ingham	LL.3	LL.3	LL.3	0.06	0.89	0.61
Ingham	LL.4	LL.4	LL.3	0.93	0.17	0.09
Ingham	W1.4	W2.3u	LL2.3	0.73	0.26	0.71
Isabella	LL.4	LL.4	LL.3	0.26	0.32	0.99
Isabella	LL.3	LL2.3	LL.3	0.79	0.06	0.84
Isabella	LL.4	LL2.4	LL.4	0.64	0.65	0.83
Macomb	LL.3	LL.4	LL2.3u	0.99	0.98	0.11
Midland	LL2.3	LL.3	LL.3	0.11	0.14	0.03
Montcalm	LL.4	LL.4	LL.3	0.60	0.23	0.95
Montcalm	LL.4	LL.3	LL.3	0.04	0.91	0.14
Montcalm	LL.3	LL2.3	LL2.3	0.14	0.73	0.54
Montcalm	LL2.3	LL.4	LL.4	0.79	0.99	0.99
Montcalm	LL.4	LL2.4	LL.3	0.88	0.75	0.84
Montcalm	LL.4	LL2.3	LL.4	0.45	0.64	0.55
Montcalm	LL2.4	LL.3	LL.3u	0.84	0.79	0.33
Saginaw	LL.3	LL.4	LL2.4	0.94	0.42	0.29

[a]Models were chosen using the modelFit function in R (Knezevic et al., 2007). LL3, log logistic three-parameter model; LL3u, log logistic three-parameter model with upper limit of 1; LL2.3, log logistic type 2 three-parameter model; LL2.3u, log logistic type 2 three-parameter model with upper limit of 1; LL.4, log logistic four-parameter model; LL2.4, log logistic type 2 four-

Table 3.2. (cont'd)

parameter model; W1.3, Weibull three-parameter model; W1.4, Weibull four-parameter model; W2.3u, Weibull type 2 three-parameter model with upper limit of 1; W2.4, Weibull type 2 four-parameter model.

Table 3.3. Categorical variables associated with *Erigeron canadensis* L. accessions used in odds ratio analysis.

Accession location[a]	Phenotype[b]	Previous herbicide-resistance[c]	Corn	Soybean	Winter wheat	Potato	Pasture
			----Frequency of crop in rotation (2015-2022)[d]----				
Cass	G^R, D^{RS}, E^S	None	High	Low	Low	High	Low
Delta	G^S, D^S, E^S	Cloransulam	High	High	Low	Low	Low
Ingham	G^S, D^S, E^S	None	Medium	High	Low	Low	Low
Ingham	G^S, D^{RS}, E^{RS}	None	High	Medium	Low	Low	Low
Ingham	G^S, D^{RS}, E^S	None	High	High	Low	Low	Low
Ingham	G^R, D^{RS}, E^S	None	Low	Low	Low	Low	High
Ingham	G^S, D^S, E^S	None	Medium	Medium	Low	Low	Medium
Isabella	G^S, D^{RS}, E^S	None	Medium	High	Low	Low	Low
Isabella	G^S, D^S, E^{RS}	Cloransulam	Medium	High	Medium	Low	Low
Isabella	G^R, D^S, E^S	Cloransulam	Medium	High	Low	Low	Low
Macomb	G^R, D^{RS}, E^S	Cloransulam	Low	Low	Low	Low	High
Midland	G^R, D^S, E^S	Cloransulam	High	Low	Medium	Low	Low
Montcalm	G^S, D^S, E^S	Cloransulam	High	High	Low	Low	Low
Montcalm	G^R, D^{RS}, E^{RS}	Cloransulam	Medium	High	Low	Low	Low
Montcalm	G^R, D^{RS}, E^{RS}	Cloransulam	Medium	High	Medium	Low	Low
Montcalm	G^R, D^S, E^S	Cloransulam	Medium	High	Low	Low	Low
Montcalm	G^R, D^{RS}, E^S	Cloransulam	Low	High	Low	Low	Low
Montcalm	G^R, D^S, E^{RS}	Cloransulam	Medium	High	Low	Low	Low
Montcalm	G^R, D^{RS}, E^{RS}	Cloransulam	Medium	Low	Low	Low	High
Saginaw	G^R, D^S, E^S	Cloransulam	High	Medium	Low	Low	Low

[a]County accession collection from in Michigan

[b]Phenotyped through does-response assays in objective 1 of this study; G, glyphosate; D, dicamba; E, 2,4-D; R, resistant; RS, reduced sensitivity; S, susceptible.

Table 3.3. (cont'd)

[c]The Plant and Pest Diagnostics at Michigan State University previously evaluated these accessions for herbicide response (screened with one and four times the recommend field use rate) to glyphosate, 2,4-D, dicamba, and cloransulam (MSU-PPD, 2024).

[d]Crop rotation data was collected from USDA-NASS Cropland Data Layer using the CroplandCROS web application (USDA, 2022). Once crop rotation information was extracted the frequency of a particular crop grown in that rotation was calculated for each rotation and categorized as: high (greater than 50% of the years contained that crop), medium (20-50% of the years contained that crop), and low (less than 20% of the years contained that crop).

Table 3.4. Results of odds ratio analysis of significant pairwise comparisons amongst categorical variables (p ≤ 0.2).

General categorical variable	Comparison			Odds ratio	P-value
Previous herbicide-resistance[a]	2,4-D RS[b]	VS	Glyphosate R	0.20	0.19
	Cloransulam R	VS	Glyphosate R	0.15	0.08
	2,4-D RS	VS	Dicamba RS	0.08	0.03
Location (counties in MI)[c]	Glyphosate R Montcalm	VS	Glyphosate R Ingham	24.00	0.04
	Glyphosate R Montcalm	VS	Glyphosate R Isabella	12.00	0.13
Crop rotation frequency[d]	Corn M	VS	Dicamba RS	6.00	0.15
	Soybean L	VS	2,4-D RS	5.6	0.17
	Soybean H	VS	2,4-D RS	0.18	0.17
	Winter wheat L	VS	Dicamba RS	0.15	0.17

[a]Plant and Pest Diagnostics at Michigan State University previously evaluated these accessions for herbicide response (screened with one and four times the recommend field use rate) to glyphosate, 2,4-D, dicamba, and cloransulam (MSU-PPD, 2024).

[b]Phenotyped through does-response assays in objective 1 of this study; R, resistant; RS, reduced sensitivity.

[c]County accession collection from in Michigan

[d]Crop rotation data was collected from USDA-NASS Cropland Data Layer using the CroplandCROS web application (USDA, 2022). Once crop rotation information was extracted the frequency of a particular crop grown in that rotation was calculated for each rotation and categorized as: high (H = greater than 50% of the years contained that crop), medium (M = 20-50% of the years contained that crop), and low (L = less than 20% of the years contained that crop)