INFLUENCE OF MESOTRIONE, ALS-INHIBITOR RESISTANCE, AND SELF-INCOMPATIBILITY ON GIANT RAGWEED MANAGEMENT IN SOYBEAN

by

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ABSTRACT

Giant ragweed (Ambrosia trifida L.) is an annual broadleaf plant capable of emergence throughout the cropping season, opportune colonization of disturbed soil, rapid biomass accumulation, and a propensity to evolve mutations that endow resistance to herbicides, all of which contribute to giant ragweed being one of the most challenging weeds to control in row-crop production. Many soybean growers rely on acetolactate synthase (ALS)-inhibiting herbicides such as cloransulam for control of giant ragweed prior to its emergence, though the spread of biotypes resistant to ALS inhibitors can render these herbicides largely ineffective. Mesotrione inhibits the 4-hydroxyphenylpyruvate dioxygenase (HPPD) enzyme, and applications of this herbicide have recently been approved for use in mesotrione-resistant soybean varieties. Field experiments demonstrated that preemergence applications of mesotrione resulted in greater control of giant ragweed populations segregating for ALS-inhibitor resistance than several other commonly used herbicide combinations. Where mesotrione was applied, giant ragweed biomass was reduced by an average of 84% relative to the nontreated, while treatments without mesotrione increased biomass by an average of 34% by suppressing competition from other weed species. Additionally, both soil- and agar-based bioassays demonstrated that combinations of mesotrione and metribuzin can be synergistic for control of giant ragweed.

Cloransulam was shown to result in strong selection for giant ragweed individuals with ALSinhibitor resistance, increasing the proportion of resistant plants that emerged at one field site from 15% to greater than 90% after a single preemergence application. This selection pressure was reduced when mesotrione was co-applied with cloransulam. However, no herbicide combination, including sequential applications of non-ALS-inhibiting herbicides, consistently resulted in a resistance frequency similar to the baseline if an ALS inhibitor was applied preemergence. Resistance to cloransulam and other ALS inhibitors is expressed in giant ragweed plants possessing at least one mutant (Trp574Leu) *ALS* allele. The distribution of this allele in one field violated the Hardy-Weinberg Equilibrium, despite the fact that *ALS* is a nuclear gene and the Trp574Leu mutation does not incur a fitness penalty. We suspected that the inheritance of this mutation may be linked with a gene or genes responsible for self-incompatibility (SI) in giant ragweed, and that linkage drag was disrupting pollination in resistant plants. This research provided evidence that giant ragweed does possess SI, as greater pollen retention, pollen tube growth, and seed set were observed in cross-pollinated plants compared with self-pollinated plants. Non-Mendelian inheritance of the Trp574Leu mutation was documented in crosses between plants from three different giant ragweed populations, indicating that the mutant *ALS* allele may be linked with an SI allele common to many plants because of a shared resistant ancestor. In crosses between plants from one population, production of resistant F1 seeds was 33% greater on average compared with the expectation under Mendelian inheritance.

Collectively, this research demonstrated that mesotrione may become a highly effective tool for control of giant ragweed in soybean. Applications of mesotrione can also reduce the selection for an increased frequency of ALS inhibitor-resistant biotypes induced by cloransulam, though a more robust weed management strategy may be necessary to maintain the long-term viability of ALS inhibitors. The need for sound weed management practices is underscored by the impact of the linkage of SI and *ALS* genes, which may be encouraging a more rapid spread of herbicide-resistance than was previously anticipated.

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

Production of soybean [*Glycine max* (L.) Merr.] has increased significantly over the last century and has become an integral component of modern agriculture. First introduced to North America from China in 1765, soybean now represents the largest agricultural export in the United States (Hymowitz 1984, USDA-Foreign Agricultural Sercives 2018). Soybean constitutes a considerable portion of global oilseed production, and it is a critical component of the global food supply (Ainsworth et al. 2012). One of the most detrimental factors in soybean production is competition from weeds, and herbicides are the primary tool employed by growers to control them (Oerke 2006).

In 2020, 94% of soybean hectares were planted with genetically engineered herbicideresistant varieties in an effort to combat weedy infestations (USDA-ERS 2020). However, overuse of and dependence on certain herbicides can, and has, resulted in the evolution and proliferation of herbicide-resistant weed biotypes (Heap 2014, Young 2006). The rate at which a weed population shifts from herbicide-sensitive to -resistant is affected by overall weed management tactics in addition to the reproductive strategies employed by that particular weed species and the genetic mechanism(s) responsible for resistance (Jasieniuk et al. 1996, Moss 2002). Ultimately, for effective and sustainable control of problematic weed species in the future, it is becoming increasingly important to integrate multiple effective herbicide modes of action in conjunction with non-chemical management tactics, and to fortify our understanding of weed biology and ecology (Beckie 2006, Norsworthy et al. 2012).

1.2 Acetolactate Synthase and Cloransulam

The acetolactate synthase (ALS) enzyme, also referred to as acetohydroxyacid synthase (AHAS), catalyzes the first committed step in the formation of the branched chain amino acids (BCAAs) L-valine, L-leucine, and L-isoleucine. Plants, fungi, archaea, and bacteria all possess active forms of ALS, while animals do not (Liu et al. 2016). Because it catalyzes an essential process with no structural counterpart in animals, ALS is a natural target for many synthetic herbicides and antimicrobial agents (Garcia et al. 2017, 2018). Though translated and expressed in the chloroplast, the *ALS* gene of higher plants is encoded in the nucleus (Smith et al. 1989, Tranel and Wright 2002). The coding region has been sequenced in several plant species, where a single-exon stretch of approximately 2000 nucleotides codes for *ALS* (Mazur et al. 1987). A chloroplast transit peptide located near the N-terminus directs the pro-ALS protein from the nucleus to the chloroplast, and subsequent cleavage yields the mature enzyme (Wright et al. 1998).

Thymine diphosphate, flavin adenine dinucleotide (FAD), and a divalent cation (usually Mg^{2+}) are required for efficient catalysis of the ALS enzyme (Duggleby 2006, Duggleby and Siew 2000, Liu et al. 2016). Isoleucine biosynthesis begins with the deamination of threonine and the dehydration of its β -carbon to form 2-ketobutyrate. Then, ALS facilitates the addition of a hydroxyethyl group from hydroxyethyl-TPP to 2-ketobutyrate to form 2-acetohydroxybutyrate. The formation of isoleucine is completed following additional isomerase, reductase, dehydratase, and aminotransferase activity. Biosynthesis of valine and leucine begins with pyruvate, which is condensed by ALS along with hydroxyethyl-TPP to form 2-acetolactate. Formation of valine and leucine is completed following subsequent enzymatic reactions (Bar-Ilan et al., 2001, Buchanan, Gruissem, & Jones, 2015, Liu et al., 2016, Park & Lee, 2010).

The precise structure of the heterodimeric ALS enzyme varies depending on the organism from which it is isolated. However, all ALS enzymes are built from a large catalytic subunit (LSU) and small regulatory subunit (SSU) (Eram et al. 2015, Garcia et al. 2017, Liu et al. 2016, 2017). Examination of this enzyme using X-ray crystallography was first accomplished in 2002 at a resolution of 2.6 Å (Pang et al. 2002). Additional structural properties of ALS were revealed upon subsequent crystallization following herbicide binding (McCourt et al. 2006). Two recently derived ALS enzyme complexes from *Saccharomyces cerevisiae* and *Arabidopsis thaliana* indicated four ALS enzymes can function cooperatively in the formation of a Maltese cross (Lonhienne et al. 2020).

Before ALS can initiate catalysis, it must first be activated by the small regulatory subunit. Coordination and synchronization of the catalytic active sites of adjacent LSUs is dependent upon induced dimerization. The SSU drives this conformational change by facilitating the formation of a salt bridge between LSU active sites though an interaction of its ACT domain(s) and the Q-loops of the LSUs, and ATP is critical for this regulatory mechanism to be implemented efficiently (Liu et al. 2016, Lonhienne et al. 2017, 2020). Of the three BCAAs, valine most strongly influences feedback inhibition. In *A. thaliana*, a cryo-EM map of an ALS enzyme complex has revealed that binding of valine to an ACT domain of the SSU results in the bending of the al helix. A subsequent shift in the α 2 helix as a result of this bending affects the interaction of the SSU and LSU, disrupting the salt bridge linking the Q-loops of the LSU. This leads to an expansion of the LSU that decreases its stability, and thus, its catalytic efficiency (Lonhienne et al., 2020). It has been proposed that the bending of the α 1 subunit is the result of a valine-induced coil-to-helix transition (Karanth & Sarma, 2013).

In addition to these regulatory mechanisms, synthetic chemicals can also be utilized to inhibit ALS. Herbicides that inhibit this enzyme have been a component of weed management programs since the early 1980's, and more than 50 unique ALS-inhibiting herbicides are currently used around the world to protect a plethora of essential agricultural crops from competition from weeds (Garcia et al. 2017, Yu and Powles 2014). Herbicidal inhibitors of ALS are derived from five chemical families: sulfonylureas, imidazolinones, triazolopyrimidines, pyrimidinyl-benzoates, and sulfonylamino-cabonyl-triazolinones. Several mechanisms of herbicidal activity have been suggested, namely, blockage of the substrate's route to the active site (McCourt et al. 2006) and degradation of ThDP (Garcia et al. 2017). Plant death following the use of an ALS-inhibiting herbicide was assumed to be resultant of a deficiency of BCAAs. In several experiments to confirm this hypothesis, plants with a deficit of BCAAs consequential of ALS-inhibition were supplied with BCAAs exogenously, resulting in reversed growth inhibition (Anderson and Hibberd 1985, Ray 1984, Rost and Reynolds 1985). However, more recent research instead implicates a buildup of the precursors 2-ketobutyrate and/or 2-aminobutyrate, depletion of other intermediates, and disruption of photosynthate translocation as possible explanations for the phytotoxic activity of ALS-inhibiting herbicides (Zhou et al. 2007). The precise physiological mechanisms responsible for the herbicidal effects resultant of ALS-inhibition are still being debated (Délye et al. 2018, Zhou et al. 2007).

Soon after their introduction, several ALS inhibitors were widely adopted in soybean production systems (Bellinder et al. 1994). Their use over vast acreage can be attributed to their broad spectrum control of many agronomically important weed species, soil-residual activity, low use rates, wide application windows, estimable crop safety, and low mammalian toxicity compared with many other herbicides available at that time (Mazur and Falco 1989). This widespread

adoption of ALS-inhibiting herbicides led to a subsequent decline in the total number of herbicide active ingredients applied to crops during the 1980's and 1990's (Bellinder et al. 1994, Hart et al. 1997, Mazur and Falco 1989). More recently, ALS inhibitors have also been evaluated for uses outside agriculture. One such study demonstrated the efficacy of the ALS-inhibiting herbicide chlorimuron-ethyl for treatment of fungal infections in mammals (Garcia et al. 2018).

Cloransulam-methyl is the common name for N-(2-carboxymethyl-6-chlorophenyl)-5ethoxy7-fluoro-(1,2,4)-triazolo[1,5c]-pyrimidine-2-sulfonamide. Cloransulam has been used as an ALS-inhibiting herbicide for control of broadleaf weeds in soybean since its commercial release in 1998 (Dow AgroSciences LLC 2011, Federal Register 1997). A member of the triazolopyrimidine family of ALS inhibitors, cloransulam is among the most efficacious herbicides for preemergence control of several large-seeded broadleaf weeds in soybean production (Franey and Hart 1999, Nelson and Renner 1998, Norsworthy et al. 2011a). Cloransulam induces phytotoxicity on susceptible plant species from soil-residual and foliar applications and can be applied both prior to and following soybean emergence at rates between 25-63 grams per hectare (Anonymous 2021). Cloransulam is often recommended for control of giant ragweed (*Ambrosia trifida* L.), a large-seeded broadleaf (Johnson et al. 2007). According to a multi-state university extension publication, cloransulam ranks as an 8 on a 10-point scale with 10 being the highest level of control of that weed species (Loux et al. 2017).

1.3 Mesotrione

The naturally-occurring triketone leptospermone (Figure 1.1) inhibits activity of the 4hydroxyphenylpyruvate dioxygenase (HPPD) enzyme, which was first observed by Reed Gray in 1997 during his study of allelopathic effects originating from the bottlebrush plant (*Callistemon* *citrinusL.*) (Knudsen et al. 2000). Leptospermone was later optimized as the syncarpic acid analog sulcotrione, which was not overly persistent in the soil and highly toxic to many broadleaf weed species (Knudsen et al. 2000). Application of an HPPD-inhibiting herbicide like sulcotrione results in a bleached appearance of susceptible plant tissue that ultimately stems from a reduction in carotenoid function (Carles et al. 2017, Hess 2000, Siefermann-Harms 1987). Biosynthesis of carotenoids is contingent upon activity of the enzyme phytoene desaturase (PD). Plastoquinone is biosynthesized by the HPPD enzyme and acts as an essential cofactor in the production of PD (Lee et al. 1997). When HPPD-inhibiting herbicides disrupt the production of PQ, affected plant tissues lack the appropriate carotenoids to quench triplet chlorophyll, which leads to the formation of damaging singlet oxygen (Carles et al. 2017, Hess 2000, Siefermann-Harms 1987). Additionally, insufficient production of carotenoids interrupts photosynthetic electron transfer between photosystem II and photosystem I, bringing about a deficiency of adenosine triphosphate (ATP) (Hess 2000).

Like sulcotrione, mesotrione (2-[4-(methylsulfonyl)-2-nitrobenzoyl]-cylohexane-1,3dione) is a member of the triketone family of herbicides that inhibit activity of the HPPD enzyme (Mitchell et al. 2001). The chemical structure of mesotrione is shown in Figure 1.1 alongside its predecessor, leptospermone. Along with good crop safety, initial product testing in field corn production revealed high phytotoxic activity on many broadleaf weed species and some grasses (Sutton et al. 2002). Mesotrione was first introduced in 2001 and has since been integrated into many different herbicide premix products (Givens et al. 2009, Sutton et al. 2002). Mesotrione is currently one of the most widely used herbicides employed by growers to manage weeds in corn production and was applied to nearly 30 percent of acres planted to corn in the United States in 2016 (Givens et al. 2009, USDA-NASS 2017).

Recently, genetic resistance to some HPPD inhibitors (mesotrione and isoxaflutole) has been engineered in soybean cultivars through the transformation event 'SYHT0H2' and the crossing of soybean lines 'FG7' and 'A5547-35'. SYHT0H2 represents the insertion of a mutant HPPD gene derived from oat (Avena sativa) with resistance to mesotrione conferred as a result of reduced enzymatic binding affinity for the herbicide molecule. This transformation also includes the insertion of a phosphinothricin acetyltransferase gene from S. viridochromogenes resulting in resistance to the glutamine synthetase-inhibitor glufosinate via rapid herbicide detoxification (Hawkes et al. 2011, Hipskind et al. 2012, USDA-APHIS 2013b). There is little published research regarding weed control programs for use in these cultivars, as they have not been released commercially. Soybean varieties generated from the hybrid cross of FG7 x A5547-35 are designated as LibertyLink[®]-GT27[®] and are resistant to mesotrione, isoxaflutole, glufosinate, and glyphosate (EPA reg. no. 70506-331, USDA-APHIS 2013a). Resistance to HPPD inhibitors is derived from the insertion of the hppdPfW336 gene from Pseudomonas fluorescens (USDA-APHIS 2013a, Boudec et al. 2001). These soybeans are commercially available and applications of mesotrione have been federally approved for the 2022 growing season (EPA reg. no. 70506-331).

1.4 Giant Ragweed

1.4.1 Biology and Distribution

Giant ragweed is a summer annual broadleaf plant capable of rapid growth and colonization of disturbed ground (Abul-Fatih and Bazzaz 1979, Basset and Crompton 1982). Though native to North America, this species can now be found throughout parts of Asia and Europe and is considered invasive in several regions (CABI 2022, Hovick et al. 2018, Montagnani et al. 2017). Giant ragweed is an extremely competitive habitat generalist and is often considered a pioneering species with the ability to disrupt both established and recently disturbed plant communities and agricultural fields (Chauvel et al. 2021, Montagnani et al. 2017, Shouhui et al. 2005). In general, giant ragweed is dicotyledonous, though tricotyledonous giant ragweed plants have been documented on rare occasions (Page et al. 2018, Wrensch and Paddock 1976, author's personal observation). Mature leaves generally present three primary lobes and are arranged oppositely along the plant's hairy stem.

Seeds produced by giant ragweed are inherently dormant, and if conditions are still unfavorable for germination when primary dormancy is broken, they may enter a period of secondary dormancy (Davis 1930). Germination of giant ragweed seeds is optimized at a burial depth of approximately 2 cm (Abul-Fatih and Bazzaz 1979). Seeds can remain viable in the soil for greater than nine years, but most seeds (~81 to 100%) will germinate after four growing seasons (Harrison et al. 2007). Dormancy of giant ragweed seeds collected for research purposes can be reduced through a period of after-ripening involving exposure to a period of cold sand stratification (Ballard et al. 1996, Davis 1930, Westhoven et al. 2008). Full or partial excision of the seed from the involucral hull can also serve to increase the germination rate of this species without the need for an after-ripening period (Harre et al. 2019, Page and Nurse 2015).

Abul-Fatih and Bazzaz (1979) attributed the success of this weed to its early and long germination period, high photosynthetic rate, rapid accumulation of biomass, and extensive genetic diversity. Cumulatively, these traits allow giant ragweed to dominate other annual weed species and compete effectively with corn and soybean crops (Abul-Fatih and Bazzaz 1979, Baysinger and Sims 1991). Giant ragweed densities of less than two plants per square meter have been shown to reduce soybean yields by 45 to 77% (Baysinger and Sims 1991, Webster et al.

1994). In addition to reducing crop yields, giant ragweed is capable of prolific pollen production. A single plant can produce over one billion grains of pollen during its lifecycle, and it is estimated that 10% of the U.S. population is allergic to pollen produced by giant ragweed plants (Gergen et al. 1987, Johnson et al. 2007).

A survey of U.S. Certified Crop Advisors indicated that giant ragweed was considered to be one of the most difficult weeds to manage in cropping systems in 45% of responding counties throughout the Midwest (Regnier et al. 2016). Results from this survey also indicated that giant ragweed is spreading from east to west across the Corn Belt. In Indiana, the expanding geography of giant ragweed was recently illustrated by an increase in the number of row-crop acres infested with giant ragweed from 2006 to 2014 (Harre et al. 2017).

High phenotypic plasticity is often associated with giant ragweed (Abul-Fatih and Bazzaz 1979). Hovick et al. (2018) documented a nearly 4-fold increase in reproductive allocation and a decrease in overall biomass accumulation in westernmost giant ragweed populations compared with its core native range in Ohio. This study also found that plants from agricultural populations emerged later than riparian populations, indicating adaptation of giant ragweed to agricultural practices such as burndown herbicide applications and spring tillage. This suggests that the recent spread of giant ragweed can be at least partially attributed to its adaptive potential, and data presented in Regnier et al. (2016) and Harre et al. (2017) demonstrates that giant ragweed infestations could occur over an even larger geography in the future.

1.4.2 Control in Agronomic Systems

Though giant ragweed is typically among the first summer annual weeds to germinate in the spring, tillage has not been an effective measure of control due to the wide germination period of this species (Barnes et al. 2004). Implementing no-till as a cultural practice for management of giant ragweed, which could theoretically decrease seed germination due to insufficient burial and increased predation, has been met with mixed success. Some studies report little impact of no-till management on giant ragweed (Moonen and Barberi 2004, Wilson and Foy 1990), while others have reported >88% seedbank depletion (Dong et al. 2020, Harrison et al. 2003). Another study showed that crop rotations that include perennial or fall-planted crops may be less conducive for giant ragweed germination and can facilitate seed predation and degradation, and that zero-weed thresholds preventing seed return eliminated 96% of giant ragweed seed from the seedbank over a period of two years (Goplen et al. 2017).

Effective foliar and soil-residual control of giant ragweed in soybean has been achieved through the use of ALS-inhibiting herbicides including sulfonylureas, imidazolinones, and triazolopyrimidines. Cloransulam has been shown to control greater than 90% of giant ragweed plants growing in competition with soybeans when appropriate rates were used and sufficient activating rainfall was received (Franey and Hart 1999). However, research has shown that in most cases, multiple herbicide applications are needed for season-long control of dense giant ragweed infestations in soybean fields (Baysinger and Sims 1992, Johnson et al. 2007, Vink et al. 2012). Several postemergence herbicides that target mechanisms other than ALS are recommended for control of giant ragweed in soybean, including glyphosate, glufosinate, fomesafen, dicamba, and 2,4-D (Loux et al. 2022). In corn production, mesotrione is often used for preemergence control of giant ragweed (Givens et al. 2009, Mitchell et al. 2001, Sutton et al. 2002). In general, the greatest control of giant ragweed has been observed with combinations of mesotrione and atrazine applied preemergence or mesotrione and dicamba applied postemergence (Belfry and Sikkema 2015, Carles et al. 2017, Loux et al. 2011).

The potential exists for synergistic herbicide interactions to occur between some herbicides available for use in HPPD inhibitor-resistant soybean for control of giant ragweed. Herbicide synergy has been defined as the cooperative action of two herbicides resulting in an observed response greater than the response predicted by a biologically appropriate reference model (Hatzio and Penner 1985). Synergistic effects have been observed between HPPD and photosystem II (PS II) inhibitors in numerous research studies (Abendroth et al. 2006, Armel et al. 2007, Hugie et al. 2008, Sutton et al. 2002). Mesotrione and metribuzin (a PS II inhibitor), are both labeled for preemergence use in HPPD inhibitor-resistant soybean varieties (EPA reg. no. 70506-331). Ditschun et al. (2013) documented synergy resultant of postemergence applications of isoxaflutole plus metribuzin in giant ragweed, and synergy on cultivated sunflower (Helianthus annuus L.) was observed by Abendroth et al. (2006) with postemergence combinations of mesotrione and metribuzin. Bollman et al. (2006) documented a synergistic response in giant ragweed control from preemergence applications of mesotrione and atrazine (a PS II inhibitor with the same binding site as metribuzin). Therefore, it is possible that a synergistic interaction may also be observed from preemergence applications of mesotrione and metribuzin on giant ragweed. However, this research has not yet been conducted as this combination of herbicides has not yet been widely adopted.

1.4.3 Herbicide Resistance

A plant is considered to be resistant to a herbicide when it possesses a heritable trait that allows it to survive a dose of a herbicide that would kill other members of the same species lacking that trait. The discovery of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) in 1941 marked the beginning of the "Chemical Era of Agriculture", and the first case of a herbicide resistant weed appeared within the next decade (Heap 2018, Timmons 2005). This case involved resistance to 2,4-D in wild carrot (*Daucus carota*) (Switzer 1957). By 2014, the number of herbicide-resistant weed species had grown to 210, and weeds had evolved resistance to 21 of the 25 known herbicide sites of action (Heap 2014).

Herbicide resistance mechanisms can be broadly classified as target-site (TSR) or nontarget-site (NTSR). Target-site resistance mechanisms can result from an alteration of the structure of the target protein, or from differential expression of the gene or genes encoding target proteins. Mechanisms of NTSR may reduce the amount of herbicide available to inhibit its target, or they may enhance a plant's ability to grow in the presence of the detrimental effects of a herbicide (Gaines et al. 2020). Mutations that confer resistance to herbicides typically occur naturally in a weed population at very low frequencies (Casale et al. 2019, Jasieniuk et al. 1996). Herbicides are the most important factor in predicting the change in the frequency of these mutations in weed populations, and higher selection pressure is associated with certain herbicidal characteristics including very high efficacy, a single target site, and frequent application (Jasieniuk et al. 1996). Reliance on singular herbicide modes of action to control weeds for multiple growing seasons applies perhaps the strongest selection pressure for resistance (Bellinder et al. 1994, Norsworthy et al. 2012, Young 2006). The rate of the spread of a resistance mutation in a population is also influenced by the reproductive strategy of that species and any substantial fitness penalty associated with the specific resistance mutation (Moss 2002, Vila-Aiub et al. 2015). Fitness penalties, also termed fitness costs or resistance costs, are derived from the evolutionary paradigm that plant strategies to adapt to new environments (i.e. herbicide selection) result in inherent tradeoffs in resource allocation, resulting in a less competitive biotype in the absence of those selection mechanisms (Vila-Aiub et al. 2011).

The first cases of resistance to ALS-inhibiting herbicides were identified in prickly lettuce (*Lactuca serriola* L.) and kochia (*Bassia scoparia* L.) in 1987, just five years after the introduction of the first ALS-inhibiting herbicide (Mallory-Smith et al. 1990, Primiani et al. 1990, Saari et al. 1994). Currently, more weed species have developed resistance to ALS-inhibiting herbicides than any other mode of action (Heap 2022). Though several different amino acid substitutions have been shown to convert the ALS enzyme from a susceptible to resistant form, most are resultant of a single point mutation with partial to complete dominance that reduces binding affinity for the herbicide molecule (Tranel and Wright 2002). In many cases, a herbicide-resistant ALS enzyme can retain full functionality, likely because these herbicides are not interacting with active or regulatory sites (Garcia et al. 2017).

The fact that a single nucleotide polymorphism (SNP) can result in high-level resistance, coupled with the fact that it is possible for an ALS enzyme to retain its functionality despite having several amino acid substitutions, may help to explain why baseline levels of resistance in many weed populations was exceptionally high, even prior to the commercialization of ALS-inhibiting herbicides (Tranel and Wright 2002, Yu and Powles 2014). As previously outlined, the risk of encountering resistance is intensified for herbicides with very high efficacy, a single target site, and frequent application, all of which are characteristics of ALS inhibitors and their use patterns (Jasieniuk et al. 1996, Norsworthy et al. 2012, Tranel and Wright 2002, Young 2006). Thus, historical use of these herbicides has applied strong selection pressure for the few resistant individuals naturally present in weed communities to become much more prevalent in the overall population (Shaner 2014).

In addition to SNP's conferring TSR to ALS inhibitors resultant of an altered binding site with reduced herbicidal affinity, metabolism-based NTSR mechanisms have been identified in some grass and broadleaf species (Délye et al. 2011, Délye et al. 2018, Devine and Shukla 2000, Scarabel et al. 2015, Yu and Powles 2014). Though the genes responsible for these cases of increased herbicide metabolism have not yet been identified, cytochrome P-450's likely play a significant role (Yu and Powles 2014).

In the majority of cases, mutations that confer resistance to ALS-inhibiting herbicides do not result in a substantial fitness penalties (Yu and Powles 2014). Specifically, retained competitiveness of resistant biotypes has been demonstrated by two recent studies that evaluated a tryptophan-to-leucine substitution at the 574 amino acid position of the ALS gene (Trp574Leu), conferring TSR in several dicot weed species (Légère et al. 2013, Li et al. 2013). However, a significant reduction of growth and seed production in populations of Powell amaranth (*Amaranthus powellii* S. Wats) resistant to ALS inhibitors via the Trp574Leu mutation was documented by Tardif et al (2006), and Wu et al. (2018) observed a slight penalty associated with this same mutation in an artificially generated waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] population.

Consistent with what has been described regarding widespread native resistance to ALS inhibitors, a population of giant ragweed resistant to cloransulam was identified in Indiana in 1998, concurrent with the commercial launch of this herbicide (Dow AgroSciences LLC 2011, Federal Register 1997, Patzoldt and Tranel 2002). Resistance was conferred by the Trp574Leu mutation, resulting in a decrease in sensitivity to foliar applications of cloransulam of greater than 3000-fold (Patzoldt and Tranel 2002). This population was also found to be cross-resistant to imidazolines and sulfonylureas (Patzoldt and Tranel 2002). Since then, ALS inhibitor-resistant giant ragweed has been confirmed in seven U.S. states and Ontario, Canada (Heap 2022). These resistant

biotypes are not controlled with cloransulam applied either preemergence or postemergence (Taylor et al. 2002).

The Trp574Leu mutation is currently the only known mutation in giant ragweed that endows resistance to ALS inhibitors (Heap 2022, Marion et al. 2017, Tranel et al. 2020, Tranel and Wright 2002). As *ALS* is encoded in the nucleus, this mutation is transmitted through both pollen and seed (Tranel and Wright 2002). In a diploid species like giant ragweed (2n = 24), the inheritance of ALS should follow Mendelian principles (Bassett and Crompton 1982, Patzoldt and Tranel 2002, Payne et al. 1964). Since the resistant allele of the *ALS* gene in giant ragweed is dominant (or semi-dominant) over the susceptible allele without inducing a substantial fitness cost, it is not likely that a resistant population would become susceptible after a period of time in the absence of herbicide selection (Jasieniuk et al. 1996, Patzoldt and Tranel 2002).

Effective control of giant ragweed biotypes resistant to ALS inhibitors was achieved though foliar applications of glyphosate until the evolution and subsequent spread of biotypes possessing multiple-resistance to both ALS inhibitors and glyphosate (Gower et al. 2003, Heap 2018, Stachler 2008). In Indiana, populations resistant to glyphosate had spread from 15% of counties in 2006 to 39% in 2014, and 90% of these glyphosate-resistant populations were also resistant to ALS inhibitors (Harre et al. 2017). Currently, these are the only two modes of action to which giant ragweed has evolved resistance, but it certainly possible that this species will develop resistance to additional herbicides in the future. Resistance to HPPD-inhibiting herbicides has been documented in Palmer amaranth (*Amaranthus palmeri* S. Wats), waterhemp, and wild radish (*Raphanus raphanistrum* L.) (Heap 2022). Though giant ragweed populations with putative HPPD inhibitor-resistance have examined, control of these biotypes with HPPD inhibitors under

experimental conditions was similar to known susceptible populations (Hausman et al. 2011, Jhala et al. 2014, Jones 2018).

1.4.4 Pollination and Self-Incompatibility

Effective and sustainable weed management depends on a thorough understanding of chemical, biological, mechanical, and cultural control tactics, and each of these tactics is influenced by the reproductive biology of individual weed species. Weedy plant species employ a wide variety of reproductive strategies to spread across the landscape and pass along their genetic information. Many of these species spread primarily through seeds generated by the sexual recombination of male and female gametes during pollination, though some species may also proliferate asexually by vegetative propagation and other means. Giant ragweed is an annual plant that spreads exclusively through seed production (Abul-Fatih and Bazzaz 1979), though its pollination syndrome has not yet been investigated at the molecular level. Therefore, the pollination process will be reviewed in depth in the following paragraphs as it relates to giant ragweed, including those mechanisms that may impact the spread of herbicide resistance.

The female gametophyte serves two regulatory functions: to guide the pollen tube to into the embryo sac for appropriate discharge of its two sperm cells that fuse to the egg and central cell, and to distinguish between self- and non-self-pollen (Kessler and Grossniklaus 2011). Following germination of the pollen grain on the stigma surface, the pollen tube grows into the pistillate tissue and is directed down the transmitting tract to the ovary in a process known as preovular guidance (Mizuta and Higashiyama 2018). During this process, several signaling molecules including stigma-induced cysteine-rich adhesions (SCAs) in lilies and plantacyanin in *Arabidopsis* are involved in crosstalk between the stigma and the pollen grain. These adhesive molecules form

matrix of pectin at the tip of the pollen tube that facilitates guidance (Mizuta and Higashiyama 2018). Small cysteine-rich proteins (CRPs) have also been shown to participate in this process, and are implicated in several self-recognition mechanisms (Kessler and Grossniklaus 2011). Though many different biochemical mechanisms have been shown to participate in pollen tube guidance in various species, the modulation of Ca^{2+} channels [often by γ -aminobutyric acid (GABA)] and reactive oxygen species (ROS) are consistently involved in signaling pathways (Mizuta and Higashiyama 2018, Sankaranarayanan et al. 2020).

Once the pollen tube nears the ovary, it exits the transmitting tract between septum cells in a process known as emergence. Emergence may be regulated by expression of cation exchanger genes of the pollen tube through localized regulation of pH, though this assertion is still being debated (Higashiyama and Takeuchi 2015). The pollen tube then grows along the surface of the funiculus and, after reaching the micropyle, enters the ovule. Ovular guidance mechanisms known as LUREs (a type of CRP) facilitate this process (Palanivelu and Tsukamoto 2012). Synergid cells represent the "final gateway" into the female gametophyte. They flank the micropylar end of the embryo sac and function to attract pollen tubes and facilitate bursting. The fact that one of the synergid cells must undergo programmed cell death (PCD) following pollen tube entry for successful pollination was a key indicator of the strong female control of the pollination process (Kessler and Grossniklaus 2011). Less is known about the male determinants responsible for pollen rupture, though it may involve genes such as ANX1 and ANX2, Na⁺/K⁺ channels, and Ca²⁺ pumps to generate ion gradients between the synergid and the pollen tube. Many of these processes and interactions involve post-translational modifications and ROS, similar to those interactions observed during a plant's response to pathogens (Kessler and Grossniklaus 2011, Sankaranarayanan et al. 2020, Shi et al. 2017). Ultimately, pollination is successful when the

sperm cells carried by a pollen grain fertilize a female egg and central cell in a process known as double fertilization.

Many flowering plant species are monecious, with each individual plant possessing both pollen and ovules. This reproductive strategy leaves them vulnerable to inbreeding depression resulting from excessive self-pollination, though some species evolved strategies to ensure cross-pollination (De Nettancourt 2001). Development of carpel tissues like anthers and pistils physically separated female and male gametes and allowed greater opportunity for pollen discrimination (Heslop-Harrison 1975). Self-incompatibility (SI) is a process employed by nearly half of all flowering plant species to maintain genetic diversity by facilitating outcrossing (Hiscock and Kües 1999). When a self-pollination occurs in a self-incompatible plant, the pollination process may be halted at one or more of the following stages: pollen adhesion, pollen germination, pollen-tube growth, ovule fertilization, and embryo development (Figure 1.2) (Seavey and Bawa 1986). In many cases, a buildup of Ca^{2+} ions following the recognition of self-pollination causes a signaling cascade that ultimately results in the death of incompatible pollen (Franklin-Tong et al. 2002).

In many species, recognition of self-pollen involves a series of complex interactions between pollen and pistil that are governed by the *S*- (self-incompatibility) locus (Hiscock and Allen 2008, Takayama and Isogai 2005). When both male and female copies of the *S*-locus express the same alleles, an SI response is triggered. In the search for the molecular foundation of SI, many candidate genes have been identified, though they vary widely by species (Allen et al. 2011). For most SI species, the mechanisms responsible for an SI response fall into one of two distinct groups: gametophytic SI (GSI) and sporophytic SI (SSI). GSI is determined by the haploid genome of the pollen itself, while SSI is determined by the diploid genome of the plant that produced that pollen.

Complex dominance relationships can occur in SSI systems and, coupled with tissue-specific dominance and co-dominance interactions, can occasionally result in an incomplete SI response known as partial self-compatibility (PSC), also referred to as partial- or pseudo-SI (PSI). Plants possessing PSC are capable of some degree of self-pollination, and the degree of incompatibility can vary widely by biotype (Hiscock and Allen 2008). Additionally, SI interactions have been shown to be affected by modifier genes such as the *G*-locus. These modifiers can function to permit crossing between otherwise incompatible individuals with shared SI alleles and appear to be under gametophytic control (Brennan et al. 2002). Though there are notable exceptions, both GSI and SSI are often controlled by a single, multiallelic *S*-locus (Franklin-Tong and Franklin 2003).

Gametophytic SI is often found in the families Solanaceae, Rosaceae, and Plantaginaceae, where incompatible pollen tubes are arrested shortly after their penetration into the style (Oloumi and Rezanejhad 2009). The genus *Petunia* belongs to the Solanaceae family, and many species in this family are known to possess a GSI mechanism controlled by a single, multiallelic *S*-locus (Mcclure and Franklin-Tong 2006, Oloumi and Rezanejhad 2009). This SI response employs a S-ribonuclease (S-RNase)-based system as its sole pistil determinate used to identify and degrade pollen that shares in its *S*-haplotype (Mcclure and Franklin-Tong 2006). The catalytic mechanism employed by these S-RNases to degrade pollen tube RNA and stop growth is contingent on the cooperative activity of two active-site histidine residues, and its sequence specificity is based on its primary protein sequence, rather than post-translational glycosylation (Kao and Tsukamoto 2004). The pollen determinant of GSI in *Petunia* is the *S*-locus-encoded F-box protein SLF. As S-RNases can be found in both compatible and incompatible pollen tubes, SLF acts to inhibit S-RNases encoded by compatible *S*-loci in the cytoplasm of the pollen tube, ensuring its continued

development (Mcclure and Franklin-Tong 2006, Sims and Robbins 2009). This reaction is also contingent upon the action of other style-side factors and the ubiquitin pathway (Mcclure and Franklin-Tong 2006). Genes responsible for both male and female determinants are inherited together, with linkage enforced by locally suppressed recombination (Sims and Robbins 2009).

Sporophytic SI has been most thoroughly described in the Brassica family, and it is also controlled by a single, multi-allelic *S*-locus. Though pollen tube growth may be halted at any point during the pollen-stigma interaction, incompatible pollen tubes in SSI species rarely penetrate significantly into the stigma. When self-pollen is detected, allele-specific ligand-receptor binding occurs, signal transduction is activated in stigma papilla cells, and the pollen tube is rejected (Sehgal and Singh 2018). Many pollen grains cease growth prior to or immediately following germination (Hiscock and Allen 2008).

The female determinant in Brassica SSI is known as the stigma-specific *S*-receptor kinase (SRK), which binds the male-determined S-cysteine-rich (SCR/SP11) ligand (Hiscock and McInnis 2003, Kao and Tsukamoto 2004, Takayama et al. 2000). The SRK protein is characterized as a serine-threonine kinase, and its receptor domain is primarily responsible for the allelic specificity of the *S*-locus, though it can be enhanced by the *S*-locus glycoprotein SLG (Hiscock and McInnis 2003). Since SCR is expressed exclusively in the tapetum, the phenotypic expression of the SCR protein reflects the diploid genotype of the male sporophyte. A hypervariable loop protrudes from each SCR protein and is recognized by the SRK receptor region. Following recognition, incompatible SRK and SCR proteins, along with the M-locus protein kinase (MLPK), form a complex that results in the autophosphorylation of key serine and threonine residues of the SRK protein. The complex can then bind the Armandillo repeat-containing protein ARC1 in its phosphorylated SRK kinase domain. This activates a ubiquitin pathway involved in

pollen degradation (Hiscock and Allen 2008, Takayama and Isogai 2005). Signaling pathways involving Ca^{2+} have also been implicated in self-pollen rejection, but these mechanisms are yet to be elucidated (Sehgal and Singh 2018).

Dominance of *S*-alleles in the SSI system is thought to be differentially controlled in the *SRK* and *SCR* genes. It seems that heterozygous *SRK* alleles often exhibit codominance, while recessive *SCR* alleles can be completely masked by their dominant counterparts. Data suggest *SRK* dominance is determined post-transcriptionally, while *SCR* dominance is controlled during transcription and can be reversed on an epigenetic basis (Kakizaki et al. 2003). Interestingly, it appears that *SRK/SCR* gene pairs may have co-evolved in an ancestral *Brassica* species, as highlighted by sequence comparison (Hiscock and McInnis 2003). The *Brassica* system of SSI has been introduced successfully into the self-compatible species *Arabidopsis thaliana*, revealing new avenues of potential study (Hiscock and McInnis 2003, Yamamoto and Nishio 2014).

There is a lack of consensus in available literature concerning whether giant ragweed, an outcrossing member of the Asteraceae family, is self-incompatible. Contrary to most other wind-pollinated plants, pollen produced by giant ragweed falls passively downward towards female flowers which are positioned directly below the anthers, resulting in the deposition of large quantities of self-pollen on its own stigmas (Abul-Fatih and Bazzaz 1979, Bianchi et al. 1959). Therefore, SI could serve to promote outcrossing by reducing the impact of heavy depositions of self-pollen, thereby increasing genetic variability and minimizing the consequences of inbreeding.

Several studies have considered giant ragweed to be at least partially self-compatible, though reduced seed production and seedling vigor was noted in some cases (Bassett and Crompton 1982, Brabham et al. 2011, Ganie and Jhala 2017, Vincent and Cappadocia 1987). Another study reported a strong SI response when giant ragweed plants were only exposed to selfpollen, including very low to absent fruit set (Wrensch and Paddock 1976). These findings indicate that giant ragweed may possess PSC, though the extent of this response has not been investigated as none of the aforementioned research was designed to test for SI. "Leaky" SI responses, like what may be present in giant ragweed, have been reported in many other Asteraceae species considered to possess PSC (Ferrer and Good-Avila 2007). To date, there is a lack of literature investigating the prevalence and genetic mechanisms of SI in giant ragweed.

Similar discrepancies existed with research concerning common ragweed (Ambrosia artemisiifolia L.), a close relative of giant ragweed, until more recent research was conducted. One study confirmed that outcrossing rates in common ragweed were not affected by plant density, seed set of self-pollinated plants was dramatically reduced compared with cross-pollinated plants, and self-pollen germination and pollen tube growth was considerably less than cross-pollen (Friedman and Barrett 2008). Self-pollen tubes were almost always unable to penetrate the style due to formation of callose, a mechanism frequently seen in SI plants (Friedman and Barrett 2008, de Nettancourt 1977). This may not be the case for SI in giant ragweed, as one study reported 93% self-pollen germination and successful pollen tube growth (Vincent and Cappadocia 1987). However, as the objective of that research was to generate hybrids of common and giant ragweed, the authors did not report on successful fertilization, final seed set, or the resulting seedling vigor, and they did not compare self-pollinated and cross-pollinated plants. Another plant in the Asteraceae family, Oxford ragwort (Senecio squalidus L.), serves as a model species for the study of PSC. Oxford ragwort is a highly successful colonizing species of the British Isles (Hiscock 2000a, 2000b). This species possesses SSI controlled by a single, multiallelic S-locus, and the strength of SI is variable between individual plants. Some are strongly SI, while others clearly demonstrate PSC, and there is evidence of complex dominance relationships between *S*-alleles and influence of a *G*-locus (Brennan et al. 2002, Hiscock 2000b).

1.5 Justification of Research

Giant ragweed is one of the most competitive weeds currently infesting US soybean fields, and control of this species can be challenging. Presently available preemergence (PRE) and postemergence (POST) herbicide options for control of giant ragweed in soybean are becoming less effective due to the spread of biotypes resistant to ALS inhibitors and glyphosate. The introduction of soybean varieties with genetically-engineered resistance traits could allow for the use of HPPD-inhibiting herbicides like mesotrione for PRE control of giant ragweed. One goal of this research will be to evaluate several PRE herbicide combinations in a mesotrione-resistant soybean crop for soil-residual control of giant ragweed. Mesotrione-resistance traits could also allow for unique sequential herbicides applications (i.e. PRE followed by POST) compared with currently-available soybean varieties, so several sequential herbicide programs will be tested with the goal of effectively managing giant ragweed throughout the entire soybean growing season. A component of this research will include exploring the interaction of mesotrione and metribuzin when used in combination for soil-residual control of giant ragweed through both soil- and agarbased bioassays.

Cloransulam is currently among the highest rated herbicides for PRE control of susceptible giant ragweed in soybean and can result in significant selection pressure for resistance to ALS inhibitors. Simultaneous use of multiple effective herbicide modes of action may reduce this selection pressure for resistance. Another goal of this research will be to examine the frequency of resistance to ALS inhibitors in giant ragweed populations as influenced by combinations of cloransulam with other PRE herbicides such as mesotrione, and by sequential applications with non-ALS-inhibiting POST herbicides.

Understanding whether a species is self-compatible is critical when studying the inheritance and spread of herbicide resistance traits. Though giant ragweed is known to be an outcrossing species, there are very few published studies regarding the pollination syndrome of giant ragweed. Therefore, this research will pursue the identification and characterization of any self-incompatibility mechanisms that may be driving this outcrossing behavior. Preliminary research has indicated that the frequency of giant ragweed biotypes homozygous for the mutant allele that confers resistance to ALS-inhibiting herbicides to be far lower than expected levels in some populations. Thus, this research will seek to further explain this phenomenon in giant ragweed by examining the inheritance of this mutation through controlled greenhouse crosses, and whether its inheritance could be linked with a gene or genes involved with self-incompatibility.

Overall, completion of this research should provide growers with insights that will allow for improved control of giant ragweed and reduced selection pressure for resistance to currently effective herbicides. Additionally, it may provide a better understanding of the reproductive biology of giant ragweed and the inheritance and spread of resistance mechanisms.


Figure 1.1 Chemical structure of mesotrione (1) and leptospermone (2). From Mitchell et al (2001).



Figure 1.2 Incompatible and compatible pollinations in *Senecio squalidus*. Squash preparations of stigmas stained with aniline blue and viewed under UV light. (A, B) Incompatible pollination; pollen tube (arrow) blocked from entering papillae (P).
(C) Compatible pollination; pollen tubes penetrating stigma tissue. (D) Compatible pollen tube growing through transmitting tissue (arrow). Scale bars = 0.25 μm. From Allen et al. (2011).

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CHAPTER 2. CONTROL OF GIANT RAGWEED IN MESOTRIONE-RESISTANT SOYBEAN

2.1 Abstract

Preemergence (PRE) applications of mesotrione, a herbicide that inhibits the 4hydroxyphenolpyruvate dioxygenase (HPPD) enzyme, have recently gained regulatory approval in appropriately traited soybean varieties. Giant ragweed (Ambrosia trifida L.) is an extremely competitive broadleaf weed, and biotypes resistant to acetolactate synthase inhibitors (ALS-R) can be particularly difficult to manage with soil-residual herbicides in soybean production. This study investigated control of giant ragweed from PRE applications of cloransulam (32 g ai ha⁻¹), metribuzin (315 g ai ha⁻¹), and S-metolachlor (1600 g ai ha⁻¹) in a factorial design with and without mesotrione (177 g ai ha⁻¹) at two different sites over two years. Treatments with mesotrione were also compared with two commercial premix products: sulfentrazone (283 g ai ha⁻¹) and cloransulam (37 g ai ha⁻¹), and chlorimuron (19 g ai ha⁻¹), flumioxazin (69 g ai ha⁻¹), and pyroxasulfone (87 g ai ha⁻¹). At 42 days after planting, control, density reduction, and biomass reduction of giant ragweed were greater in treatments with mesotrione than any treatment without mesotrione over all site-years. Giant ragweed biomass was reduced by 84% in treatments with mesotrione, while treatments without mesotrione did not reduce biomass relative to the nontreated. Sequential herbicide treatments utilizing postemergence (POST) applications of glufosinate (655 g ai ha⁻¹) plus fomesafen (266 g ai ha⁻¹) and S-metolachlor (1217 g ai ha⁻¹) resulted in at least 97% control of giant ragweed at 42 days after planting, which was greater than POST applications of glufosinate alone in 3 of 4 site-years. These results demonstrate that a PRE application of mesotrione can be an impactful addition to soybean herbicide programs designed to manage giant

ragweed, with the potential to improve weed control and delay the onset of herbicide resistance by providing an additional effective herbicide site of action.

2.2 Introduction

Giant ragweed (Ambrosia trifida L.) is a dicotyledonous annual broadleaf in the Asteraceae family. Though it is native to North America with a core range in central Ohio, giant ragweed can now be found throughout parts of Asia and Europe (Hovick et al. 2018, Montagnani et al. 2017). This species has an early and wide germination period, high genetic diversity and germination polymorphism, and can be extremely competitive with crops like soybean (Abul-Fatih and Bazzaz 1979, Bassett and Crompton 1982, Baysinger and Sims 1991). As few as one giant ragweed plant per square meter has been shown to reduce soybean yields by up to 77%, due in part to the ability of giant ragweed to rapidly accumulate biomass and reach heights of up to 6 m (Bassett and Crompton 1982, Webster et al. 1994). Considered by many growers to be among the most troublesome weeds in soybean production, giant ragweed appears to be spreading westward across the Corn Belt, and the number of infested row-crop acres has been increasing (Barnes et al. 2004, Gibson et al. 2006, Harre et al. 2017, Regnier et al. 2016). Additionally, giant ragweed is adapting to agricultural environments and targeted control practices through higher reproductive allocation, altered emergence patterns, and the evolution of herbicide-resistance mechanisms, reflecting the high phenotypic plasticity that is often associated with this species (Abul-Fatih and Bazzaz 1979, Albert et al. 2011, Hovick et al. 2018, Patzoldt and Tranel 2002, Stachler 2008).

Several families of herbicides that inhibit the acetolactate synthase (ALS) enzyme are effective for foliar and soil-residual control of giant ragweed, though multiple applications of these herbicides coupled with other herbicide modes of action and non-chemical management tactics are

often necessary for season-long control of heavy infestations in soybean fields (Baysinger and Sims 1992, Franey and Hart 1999, Ganie et al. 2016, Johnson et al. 2007, Taylor et al. 2002). The substantial dependence on ALS inhibitors such as cloransulam for control of giant ragweed has contributed to the evolution and proliferation of biotypes resistant to ALS inhibitors (ALS-R) (Jasieniuk et al. 1996, Norsworthy et al. 2012, Tranel and Wright 2002). The presence of ALS-R giant ragweed was first documented in 1998 (Patzolt and Tranel 2002) and has since been confirmed in seven US states and Ontario, Canada (Heap 2022). These biotypes exhibit high-level cross-resistance to at least three classes of ALS-inhibiting herbicides (sulfonylureas, imidazolinones, and triazolopyrimidines) without incurring a fitness cost (Marion et al. 2017, Patzoldt and Tranel 2002).

Giant ragweed resistance to ALS inhibitors severely impacts effective management with preemergence (PRE), soil-residual herbicides (Loux et al. 2022, Taylor et al. 2002). In soybean production, these ALS-R biotypes were controlled with postemergence (POST) applications of glyphosate until the evolution and subsequent spread of glyphosate-resistant biotypes in 2004, and multiple-resistant biotypes in 2006 (Gower et al. 2003, Heap 2022, Stachler 2008). In 2016, a survey reported the suspicion and/or confirmation of giant ragweed with some form of herbicide resistance in 57% of responding midwestern counties, with multiple-resistant biotypes reported in 12 states (Regnier et al. 2016). A study conducted on giant ragweed populations collected from fields throughout the state of Indiana identified glyphosate-resistant giant ragweed plants in nearly all of the fields where ALS-R biotypes were found (Harre et al. 2017). Despite the widespread distribution of ALS-R biotypes, the use of ALS inhibitors for partial control of giant ragweed may still be warranted, as most populations appear to be segregating for resistance (Boe 2019, Harre et

al. 2017). In fact, biotypes susceptible to ALS inhibitors outnumbered resistant biotypes in more than 70% of the fields sampled in Indiana (Harre et al. 2017).

Though several POST herbicides are still effective for control of multiple-resistant giant ragweed (Barnett et al. 2013, Jhala et al. 2014a, Kaur et al. 2014, Loux et al. 2022, Norsworthy et al. 2010, Vink et al. 2012b), it can be difficult to provide the critical weed-free period of 8 to 10 wk after soybean emergence that is necessary to avoid substantial yield loss without the use of effective soil-residual herbicides (Baysinger and Sims 1991). In corn production, 4- hydroxyphenylpyruvate dioxygenase (HPPD)-inhibiting herbicides have been used for soil-residual control of several broadleaf weed species including giant ragweed (Givens et al. 2009, Mitchell et al. 2001, Sutton et al. 2002). In these systems, the HPPD inhibitor mesotrione is often applied PRE in combination with atrazine, a photosystem II (PS II) inhibitor. This strategy typically results in greater than 80% control of giant ragweed (Belfry and Sikkema 2015, Bollman et al. 2006, Loux et al. 2011, Soltani et al. 2011).

Preemergence applications of mesotrione have received federal approval for use in soybean varieties with genetically engineered resistance to HPPD inhibitors (EPA reg. no. 70506-331, USDA-APHIS 2013a, 2013b). Resistance is conferred by the insertion of a mutant *HPPD* gene derived either from oat (*Avena sativa* L.) in SYHT0H2 cultivars (Hawkes et al. 2011, Hipskind et al. 2012, USDA-APHIS 2013b), or from *Pseudomonas fluorescens* in LibertyLink[®]-GT27[®] varieties (Boudec et al. 2001, USDA-APHIS 2013a). Expression of HPPD enzymes derived from these mutant *HPPD* genes have a lower binding affinity for HPPD-inhibiting herbicides (Boudec et al. 2001, Hawkes et al. 2011), endowing soybean with resistance to rates of mesotrione similar to those used in corn production. Applying multiple, effective herbicide modes of action is one of the most effective tools for slowing the onset of herbicide resistance (Jasieniuk et al. 1996,

Norsworthy et al. 2012, Young 2006). As such, the joint application of HPPD inhibitors with other effective soybean PRE herbicides has the potential to both increase weed control and decrease selection for weed resistance in giant ragweed.

No research evaluating mesotrione applied PRE in soybean for soil-residual control of giant ragweed is present in the literature. Because mesotrione has been an effective tool for management of this weed in corn production, the co-application of this herbicide with other soybean PRE herbicides may be a robust control tactic in future weed management programs. Therefore, the primary objectives of this study were to determine the extent of giant ragweed management and response of HPPD inhibitor-resistant soybean to 1) PRE applications of mesotrione alone and in combination with cloransulam, metribuzin, and/or *S*-metolachlor for control of predominantly susceptible or ALS-R giant ragweed populations, and 2) sequential herbicide combinations (i.e. PRE fb POST) utilizing either glufosinate or glufosinate plus fomesafen and *S*-metolachlor applied POST.

2.3 Materials and Methods

2.3.1 Field Experiments

Two field experiments were conducted in 2018 and 2019 at two sites near West Lafayette, Indiana: the Throckmorton Purdue Agriculture Center (TPAC; 40.17° N, 86.54° W), and a commercial field, TIP-1 (40.26° N, 87.04° W). The field at TPAC (pH 6.6, 2.9% OM) consisted primarily of a Toronto-Millbrook silt loam complex, whereas the TIP-1 field (pH 5.7, 1.8% OM) was a combination of Mahalasville, Treaty, and Rainsville silt loams. The frequency of ALS-R giant ragweed at TPAC and TIP-1 prior to initiating this research was 10 and 70%, respectively (Harre, unpublished data). Each experiment was initiated weed-free with an application of paraquat (Gramoxone 2.0 SL[®], Syngenta Crop Protection, Greensboro, NC) followed by a combination of disk and field cultivator prior to planting. Soybean was planted at 346,000 seeds ha⁻¹ in 76 cm rows at a depth of 2.5 to 5 cm. A SYHT0H2 soybean cultivar (maturity group 3; Syngenta) (Hipskind et al. 2012) was planted in May of 2018, and due to the deregulation of LibertyLink[®]-GT27[®] varieties, Stine 33GA13 soybean (USDA-APHIS 2013a) were planted in June of 2019. Plots were 3m by 9m with herbicides applied to the center 2 rows of each 4-row plot using a CO₂-pressurized backpack sprayer and a 2 m handheld spray boom equipped with extended-range, flat fan nozzles (XR 8002VS; TeeJet[®] Spraying Systems, Wheaton, IL 60187) delivering 140 L ha⁻¹ at 207 kPa. All PRE herbicides were applied immediately following planting, and only the area within the center two soybean rows was evaluated.

Two experiments were initiated at each site, with either PRE-only or sequential applications. The PRE-only experiment used a factorial design to evaluate the addition of mesotrione (177 g ai ha⁻¹) to other soybean PRE herbicide programs that included cloransulam (32 g ai ha⁻¹), metribuzin (315 g ai ha⁻¹) and/or *S*-metolachlor (1600 g ai ha⁻¹) for soil-residual control of giant ragweed and soybean response (Table 2.1). The other experiment was a factorial of PRE and POST herbicide treatments applied sequentially. Both experiments also included two commercial herbicide premixes commonly recommended for PRE control of giant ragweed in soybean: sulfentrazone (283 g ai ha⁻¹) and cloransulam (37 g ai ha⁻¹), and chlorimuron (19 g ai ha⁻¹), flumioxazin (69 g ai ha⁻¹), and pyroxasulfone (87 g ai ha⁻¹). The POST application included either glufosinate (655 g ai ha⁻¹) or glufosinate plus fomesafen (266 g ai ha⁻¹) and *S*-metolachlor (1217 g ai ha⁻¹). The POST applications were made at 21 days after planting (DAP) and included ammonium sulfate (N-PAK[®] AMS Liquid, Winfield Solutions, LLC, St. Paul, MN) at 10 g/l.

2.3.2 Frequency of Resistance to ALS Inhibitors

Both experiments were conducted at two different sites based on the expected prevalence of giant ragweed biotypes resistant to ALS inhibitors. The only mechanism known to confer ALS-R in giant ragweed is a SNP in the *ALS* gene (Trp754Leu) (Marion et al. 2017, Patzoldt and Tranel 2002). Thus, a high-throughput molecular assay designed by Harre et al. (2017) was appropriate for resistance screening (Délye et al. 2015, Yu and Powles 2014). In 2018, 16 and 71% of the plants sampled in the nontreated plots of each experiment were resistant to ALS inhibitors at TPAC and TIP-1, respectively. In 2019, the frequency of ALS-R at TPAC was considerably greater (57%) than the year prior, though it remained similar at TIP-1 (73%).

2.3.3 Data Collection

Visual soybean injury and giant ragweed control were rated at 14, 21, and 42 DAP on a scale of 0 to 100%, with 0 being no injury and 100 being plant death. These ratings were also taken at 28 and 35 DAP in the sequential experiment. Soybean stand counts were taken at 21 DAP by counting the number of plants in 1 m of each of the center two rows in each plot, and the average count per meter of row was analyzed. Giant ragweed density counts were taken at 21 and 42 DAP, and biomass was collected at 42 DAP and oven-dried at 50 C until the weight was constant. Density counts were taken by randomly placing a 0.5 m² quadrat at two different locations between the center two soybean rows in each plot, and the quadrates were placed in the same locations for the 21 and 42 DAP counts. Biomass was harvested from the same quadrat area used for density counts. Density and biomass data were combined over locations within each plot, yielding a total measured area of one square meter per plot for analysis. Both biomass and density measurements for each treated plot were analyzed as a percent reduction compared with the nontreated.

2.3.4 Experimental Design and Analysis

Treatments were arranged in a randomized complete block design with four replications. Each experiment was conducted twice at both sites over the 2018 and 2019 growing seasons. Data were checked for normality and constancy of variance using PROC UNIVARIATE in SAS[®] 9.4 (SAS Institute Inc., Cary, NC), or through visual inspection of histograms and quantile-quantile (Q-Q) plots of the residuals, and plots of residuals vs fitted values. All data were subjected to analysis of variance (ANOVA) using PROC GLIMMIX in SAS[®]. Treatment means were separated using Tukey-Kramer's Honest Significant Difference (HSD) at an alpha level of 0.05.

In the PRE-only experiment, fixed effects included a factorial of residual herbicide treatments, the addition of mesotrione, site, and year. Data were combined over sites, years, and residual herbicides other than mesotrione when these effects and their associated interactions were not significant (P > 0.05). Orthogonal contrasts were used to compare the combined efficacy of all treatments that included mesotrione with the commercial standards. In the Sequential experiment, fixed effects included a factorial of PRE and POST herbicide treatment, year, and site. Similarly, data were combined over years, sites, and treatments where appropriate.

2.4 **Results and Discussion**

2.4.1 Soybean Injury

Preemergence herbicide treatments did not cause soybean stand loss in either experiment (data not presented). In prior research evaluating soybean varieties that expressed the mutant *HPPD* gene from *Pseudomonas fluorescens* (*hppdPfW336*), soybean biomass was reduced by up to 25% after a PRE application of 210 g ai ha⁻¹ of mesotrione (Schultz et al. 2015). In the present study, both soybean varieties showed robust resistance to PRE applications of mesotrione at the

rate used in these experiments (177 g ai ha⁻¹). Though soybean biomass and yield were not evaluated, no bleaching symptomology consistent with injury from an HPPD inhibitor was observed in either experiment. Data for general soybean injury (stunting, chlorosis, leaf malformation) were combined over sites and separated by year based on ANOVA. In 2018, an average of 11% soybean injury was observed from applications of the premix of chlorimuron, flumioxazin, and pyroxasulfone at 14 DAP, though injury from this treatment was less than 6% at subsequent rating timings. This level of injury at 14 DAP was greater than all other treatments, where less than 5% injury was observed at each evaluation timing (data not presented). In 2019, soybean injury was less than 6% in all treatments between all rating timings (data not presented). As the difference in injury between years could be attributed primarily to the commercial premix that did not include mesotrione, soybean genetics related to mesotrione-resistance did not lead to the difference in injury between years.

Similar to the PRE-only experiment, 11% injury was observed in 2018 from PRE applications of the premix of chlorimuron, flumioxazin, and pyroxasulfone at 14 DAP in the sequential experiment, and less than 6% injury was observed in all treatments in 2019 (data not presented). For evaluations in the sequential experiment between 28 and 42 DAP, soybean injury data were combined over years, sites, and PRE treatments. Injury at 28 DAP (1 wk after the POST application) was greater in treatments that included glufosinate plus fomesafen and *S*-metolachlor (17%) than treatments that only included glufosinate (6%) ($t_1 = 16.82$, P < 0.0001). Soybean injury remained evident in treatments with glufosinate plus fomesafen and *S*-metolachlor at the 42 DAP (1%), which was greater than in treatments where only glufosinate was applied POST (1%) ($t_1 = 11.88 P < 0.0001$). Symptomology in the former treatment was consistent with applications

of fomesafen (bronzing, spray droplet-sized necrotic lesions), and similar levels of fomesafen injury have been observed in previous research (Hager et al. 2003, Legleiter and Bradley 2008).

2.4.2 Giant Ragweed Efficacy: PRE-Only Experiment

In the PRE-only experiment at 21 DAP, control of giant ragweed was 80% or greater across years and sites in treatments with mesotrione (data not presented). Efficacy in many treatments had declined by 42 DAP, resulting in the greatest differences between treatments at the 42 DAP timing. Thus, further discussion of the efficacy of these treatments on giant ragweed will consist of data collected at 42 DAP. The interaction between site and treatment was not significant for any response variable, so all data were combined over sites. Visual control data were also combined over years. Giant ragweed control was greater in treatments that included cloransulam (20 to 30%) than treatments that only included metribuzin and/or S-metolachlor (3 to 12%) (Table 2.2). Control was improved with the addition of mesotrione, including mesotrione alone, ranging from 82 to 90% (Table 2.2). Despite the factorial interaction of mesotrione with the other residual herbicides, the effect of mesotrione ($F_{1,206} = 2078$) was greater than the effect of the other herbicides $F_{7,206} = 11$), resulting in greater control in treatments with mesotrione (85%) compared with those same treatments without mesotrione (17%) ($F_{1,107} = 1621$, P < 0.0001). Including additional PRE herbicides with alternative modes of action did not increase giant ragweed control beyond mesotrione alone (Table 2.2). However, the addition of these herbicide mode of action groups should at least partially reduce the risk of giant ragweed evolving resistance to mesotrione.

Overall, control of giant ragweed with mesotrione in this experiment was similar to what other researchers have found in corn production. In a study summarizing data over two years across four midwestern states, control from the highest rate of mesotrione (210 g ai ha⁻¹) ranged

from approximately 75 to 90% (Bollman et al. 2006), similar to what has been reported by other researcher groups (Belfry and Sikkema 2015, Loux et al. 2011). In agreement with these prior studies, control of giant ragweed in treatments with mesotrione was 82 to 90% in this study (Table 2.2).

Giant ragweed density data were separated by year. In 2018, the density of giant ragweed was reduced more with cloransulam than treatments that included only metribuzin and/or Smetolachlor (Table 2.2). Density reduction was similar between treatments with cloransulam and treatments with mesotrione, despite mesotrione resulting in greater control (Table 2.2). While the number of giant ragweed plants may have been similar between these treatments in 2018, many plants in treatments where mesotrione was applied had emerged later than plants in treatments without mesotrione, which is demonstrated in the subsequent biomass data comparison. In 2019, density data were combined over herbicide treatments, as mesotrione was the only significant main effect and there was no interaction between the main effects. Giant ragweed density was reduced by 86% in treatments with mesotrione relative to the nontreated, while treatments without mesotrione reduced density by 32% (Table 2.3). Biomass data were combined over years, sites, and herbicides other than mesotrione. Giant ragweed biomass was reduced by 84% in treatments with mesotrione, while treatments without mesotrione increased biomass by 34% relative to the nontreated (Table 2.3). Although an increase in biomass may seem counterintuitive, these other herbicides reduced the competition from other weed species (data not presented), allowing the surviving giant ragweed plants to grow more vigorously in these plots compared with the nontreated plots.

The efficacy of mesotrione for control of giant ragweed was also compared with two soybean herbicide premixes that are considered to be commercial standards for residual control of giant ragweed: sulfentrazone and cloransulam, and chlorimuron, flumioxazin, and pyroxasulfone. Orthogonal contrasts determined that giant ragweed control, density reduction, and biomass reduction were greater for the pooled mesotrione treatments compared with each of the commercial premixes at 42 DAP (Table 2.4). Across all of the herbicide treatments in the PRE-only experiment, only those treatments that included mesotrione reduced giant ragweed biomass relative to the nontreated plots (Tables 2.3 and 2.4).

Control of giant ragweed with cloransulam was similar at both sites, despite a lower frequency of ALS-R at TPAC. Even at TPAC in 2018, when the frequency of ALS-R was 16% in the nontreated plots, control in treatments with cloransulam (but without mesotrione) was less than 40% at 42 DAP (data not presented). This was similar to what has been observed with PREapplied ALS inhibitors in previous research on giant ragweed populations that included ALS-R biotypes (Taylor et al. 2002). In 2018, the average density of giant ragweed in the nontreated plots at TPAC was greater than 70 plants per m². Even with an assumption of 100% control of susceptible plants with cloransulam, more than 10 resistant plants per m² would still remain, which could result in near-complete yield loss if not managed (Baysinger and Sims 1991, Webster et al. 1994). Other researchers have suggested that use of ALS inhibitors for control of ALS-R giant ragweed should be "de-emphasized" (Taylor et al. 2002). However, because this herbicide is highly effective on susceptible biotypes (Franey and Hart 1999, Loux et al. 2022), cloransulam still has value for partial control of segregating populations. When supplemented with other effective PRE herbicides like mesotrione, in conjunction with POST herbicides and non-chemical control tactics, the utility of ALS inhibitors like cloransulam may be sustainable (Boe 2019).

2.4.3 Giant Ragweed Efficacy: Sequential Experiment

Similar to the PRE-only experiment, treatments with mesotrione in the sequential experiment generally resulted in greater control of giant ragweed at 21 DAP than treatments without mesotrione, including the commercial standard premixes (data not presented). Glufosinate is often highly effective for foliar control of giant ragweed (Kaur et al. 2014, Wiesbrook et al. 2001). In this experiment, no emerged giant ragweed plants (10 to 15 cm) survived the POST application of either glufosinate or glufosinate plus fomesafen and *S*-metolachlor made at 21 DAP in any year or site. At 42 DAP, the main effect of PRE herbicide treatment and the interaction of the PRE treatment with POST applications were not significant. Therefore, data for each POST treatment were combined over respective PRE treatments.

Overall, similar trends were observed across response variables at 42 DAP. Control of giant ragweed at 42 DAP and biomass reduction were greater than 90%, regardless of which POST treatment was applied (Table 2.5). In 2018, giant ragweed control, density reduction, and biomass reduction were greater in treatments where glufosinate plus fomesafen and *S*-metolachlor were applied, compared with treatments that only included glufosinate (Table 2.5). The largest difference between treatments was observed in 2018, where giant ragweed density was reduced an additional 33% with the inclusion of fomesafen and *S*-metolachlor. In 2019, efficacy was similar between POST treatments at TPAC, while the inclusion of fomesafen and *S*-metolachlor increased the efficacy of the POST application at TIP-1 (Table 2.5). No significant precipitation was recorded at TPAC after the POST herbicides were applied in 2019, while TIP-1 received 3.7 cm of rainfall between 21 and 42 DAP (Table 2.6). The lack of activating rainfall at TPAC likely reduced the soil-residual activity of fomesafen at that site. Regardless, efficacy of both POST applications was 93% or greater across all response variables in 2019 (Table 2.5).

Glufosinate is rapidly degraded by soil microbes, and applications do not result in soilresidual weed control (Aulakh and Jhala 2015, Bartsch and Tebbe 1989, Takano and Dayan 2020). In the PRE-only experiment, applications of *S*-metolachlor alone did not control giant ragweed (Table 2.2). Several studies have shown that fomesafen can be highly effective for foliar control of giant ragweed (Barnett et al. 2013, Baysinger and Sims 1992, Norsworthy et al. 2011a, Taylor et al. 2002). This experiment demonstrates that soil-residual control of giant ragweed with fomesafen is also possible, given sufficient activating rainfall.

High-level resistance to foliar applications of fomesafen has been recently confirmed in a giant ragweed population from Wisconsin (Faleco et al. 2021), though characterization of this resistance mechanism is limited thus far. Fomesafen is a diphenylether herbicide that inhibits the protoporphyrinogen oxidase (PPO) enzyme. In common ragweed, an Arg98Leu mutation in the *PPX2* target-site gene has been shown to confer an 80-fold level of resistance to POST applications of fomesafen, and a 10-fold level resistance the PPO-inhibitor flumioxazin applied PRE (Rousonelos et al. 2012). Efficacy of fomesafen for soil-residual control of PPO inhibitor-resistant giant ragweed has not yet been evaluated, though resistance to both PRE and POST applications of fomesafen has been documented in several weed species (Heap 2022, Lillie et al. 2020). Whether PRE-applied PPO inhibitors are still effective for control of PPO inhibitor-resistant weeds is highly dependent on species and resistance mechanism, in addition to the specific rate and type of PPO inhibitor applied (Copeland et al. 2018, Lillie et al. 2020, Wuerffel et al. 2015).

Overall these results demonstrate that mesotrione can be more effective than many existing soybean herbicides for PRE control of multiple-resistant giant ragweed. A survey of giant ragweed infestations in Indiana indicated that biotypes susceptible to ALS inhibitors outnumber resistant biotypes in nearly half of ALS-R populations (Harre et al. 2017), and only one instance of

resistance to PPO inhibitors has been reported globally (Faleco et al. 2021), but not yet confirmed in a published research article. Mesotrione, cloransulam, and fomesafen can all contribute soilresidual control of giant ragweed populations that are segregating for resistance, which is important for managing weeds throughout the critical weed-free period of soybean. Sequential herbicide applications can be utilized in addition to non-chemical weed management tactics such as reduced tillage (Harrison et al. 2003), crop rotation (Goplen et al. 2017, Regnier et al. 2016), and cover crops (Regnier et al. 2016) to control giant ragweed throughout the soybean growing season and reduce selection for herbicide resistance. Integration of these management strategies is essential for sustainable management of this extremely competitive species with a propensity to develop resistance to herbicides.

Table 2.1. Sources of herbicides used in field experiments.

Common name	Trade name	Manufacturer	Manufacture location	Manufacture website
Mesotrione	Callisto®	Syngenta Crop Protection	Greensboro, NC	www.syngenta.com
Cloransulam	Firstrate [®]	Corteva Agriscience	Indianapolis, IN	www.corteva.com
Metribuzin	Tricor [®] DF	UPL NA Inc.	King of Prussia, PA	www.upl-ltd.com
S-metolachlor	Dual Magnum [®]	Syngenta Crop Protection	Greensboro, NC	www.syngenta.com
Sulfentrazone +	Authority [®] First DF	FMC Corp.	Philadelphia, PA	www.fmc.com
cloransulam				
Chlorimuron +	Fierce [®] XLT	Valent USA Corp.	Walnut Creek, CA	www.valent.com
flumioxazin +				
pyroxasulfone				
Glufosinate	Liberty [®] 280 SL	BASF Corp.	Research Triangle Park, NC	www.basf.com
S-metolachlor +	Prefix®	Syngenta Crop Protection	Greensboro, NC	www.syngenta.com
fomesafen		_		

	Con	trol ^b	Density 1 20	Density reduction 2018 ^c			
Herbicide	Without mesotrione	With mesotrione	Without mesotrione	With mesotrione			
			%				
None	-	82 a	-	86 a			
Cloransulam	20 bc	86 a	52 ab	77 a			
Metribuzin	4 d	82 a	9 bc	91 a			
S-metolachlor	3 d	83 a	7 c	76 a			
Cloransulam + metribuzin	29 b	87 a	69 a	76 a			
Cloransulam + S-	23 bc	88 a	59 a	82 a			
metolachlor							
Metribuzin + S-	12 d	82 a	13 bc	77 a			
metolachlor							
Cloransulam + metribuzin	30 b	90 a	53 ab	76 a			
+ S-metolachlor							

Table 2.2. Efficacy of preemergence herbicides with or without mesotrione on giant ragweed 42 days after planting at two sites in Indiana.^a

^a Means within a column under the same heading that are followed by the same letter are not different according to Tukey-Kramer's HSD ($\alpha = 0.05$). ^b Visual control ratings were combined over data collected in 2018 and 2019. ^c Density reduction in each treatment was determined relative to the nontreated.

Table 2.3. Combined efficacy of preemergence herbicides for control of giant ragweed 42 days after planting at two sites in Indiana.^a

Herbicide treatments ^b	Density reduction 2019 ^c	Biomass reduction ^d
		%
Without mesotrione	32 b	-34 b
With mesotrione	86 a	84 a

^aMeans within a column are not different according to Tukey-Kramer's HSD ($\alpha = 0.05$). ^b Data from preemergence herbicide treatments were combined into two groups based on the inclusion of mesotrione.

^c Density and biomass reduction were determined relative to the nontreated.

^d Biomass reduction was calculated from data collected in 2018 and 2019. Negative values indicate that giant ragweed biomass was greater in treated plots compared with the nontreated.

Table 2.4. Efficacy of mesotrione combinations on giant ragweed 42 days after planting compared with two commercial standard herbicide premixes at two sites in Indiana in 2018 and 2019.

		Density	Biomass
Herbicide	Control	reduction ^a	reduction
		%	
Mesotrione mixtures ^b	85	83	84
Sulfentrazone + cloransulam	22	45	-38
Chlorimuron + flumioxazin + pyroxasulfone	43	53	-10
Contrast 1 ^c	***	***	***
Contrast 2	***	***	***

^a Density and biomass reduction were determined relative to the nontreated. Negative values indicate that giant ragweed density and/or biomass were greater in treated plots compared with the nontreated.

^b Means pooled over all treatments that contained mesotrione.

^c Orthogonal contrast 1: mesotrione mixtures vs. sulfentrazone + cloransulam; Orthogonal contrast 2: mesotrione mixtures vs. chlorimuron + flumioxazin + pyroxasulfone. Significance designated as ***=P<0.001

Table 2.5. Efficacy^a of postemergence herbicides applied 21 days after planting on giant ragweed at two sites in Indiana.^b

	Control			Density reduction ^c			Biom	Biomass reduction		
	Combined	TPAC	TIP-1	Combined	TPAC	TIP-1	Combined	TPAC	TIP-1	
Herbicide ^d	2018	2019	2019	2018	2019	2019	2018	2019	2019	
					%					
Glufosinate	90 b	97 a	95 b	66 b	93 a	93 b	95 b	100 a	99 b	
Glufosinate +										
fomesafen +	98 a	97 a	97 a	93 a	95 a	97 a	100 a	100 a	100 a	
S-metolachlor										

^a Data were collected 42 days after planting. Means were calculated from data combined over preemergence herbicide treatments.

^b Means within a column with the same letter are not different according to Tukey-Kramer's HSD ($\alpha = 0.05$).

^c Density and biomass reduction were determined relative to the nontreated.

^d All postemergence applications included ammonium sulfate at 9.53 kg ha⁻¹.
Table 2.6. Weekly rainfall accumulation and average temperature at two Indiana field sites.^a

	2018				2019			
WAP	TPAC	TIP-1	TPAC	TIP-1	 TPAC	TIP-1	TPAC	TIP-1
	cı	m ——	(<u> </u>	ci	n ——	(<u> </u>
1	4.6	4.8	26	24	2.1	3.3	21	21
2	1.3	2	21	21	0.8	1.7	19	18
3	6.4	5.2	23	22	6.9	7.6	21	20
4	1.5	3.1	26	25	0	0	25	25
5	7.5	3	22	21	0	3.3	26	25
6	0.1	2.6	26	26	0	0.4	25	25

^a Data for TPAC and TIP-1 were generated by weather stations at the Throckmorton Purdue Agricultural Center and the Purdue University Airport (KLAF), respectively. Abbreviations: WAP, weeks after planting

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CHAPTER 3. INTERACTION BETWEEN MESOTRIONE AND METRIBUZIN FOR PREEMERGENCE CONTROL OF GIANT RAGWEED

3.1 Abstract

The 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitor mesotrione has recently gained regulatory approval for application in appropriately traited soybean varieties prior to emergence. To increase overall weed control and reduce selection pressure for herbicide resistance, growers planting these soybean varieties may apply mesotrione in combination with other herbicide sites of action. Synergistic interactions are often observed when HPPD inhibitors and photosystem II inhibitors are applied in combination. This study evaluated the interaction of mesotrione and metribuzin (a photosystem II inhibitor) when applied as a mixture for preemergence, soil-residual control of giant ragweed (Ambrosia trifida L.). A field experiment indicated that the interaction was additive, though only one rate of each herbicide was examined, and there was a large disparity between the efficacy of the active ingredients. Two bioassays were then used to characterize this interaction more thoroughly. In these bioassays, the relative potency of each herbicide was established, then mesotrione and metribuzin were combined in a multi-ray fixed-ratio design at 1:1, 1:2, and 2:1 ratios of their ED_{50} estimates when applied alone. The concave shape of the Isobolograms for all mixture ratios in both soil- and agar-based bioassays indicated the interaction between mesotrione and metribuzin was synergistic, which was quantified by calculating an interaction index (γ) for each mixture. The upper 95% confidence limit for each estimate of γ was less than 1 (synergistic) for all mixture ratios in both bioassays. Overall, these results indicate that the application of a mixture of mesotrione and metribuzin for preemergence, soil-residual control of giant ragweed is likely to be synergistic in commercial production systems.

However, the magnitude of this synergistic interaction may be reduced if one of the components lacks efficacy at the dose that is available to germinating seedlings.

3.2 Introduction

Giant ragweed (Ambrosia trifida L.) is a highly competitive broadleaf weed that can be difficult to manage in soybean production (Baysinger and Sims 1991, Regnier et al. 2016, Webster et al. 1994). Control of this species with soil-residual herbicides can be challenging, especially for growers faced with giant ragweed resistant to acetolactate synthase (ALS)-inhibiting herbicides (Johnson et al. 2007, Loux et al. 2022, Regnier et al. 2016). Preemergence (PRE) applications of 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibiting herbicides have been effective for soilresidual control of giant ragweed in corn production (Soltani et al. 2011, Taylor-Lovell and Wax 2001). Federal approval has been granted for applications of the HPPD-inhibitor mesotrione in appropriately traited soybean varieties (EPA reg. no. 70506-331). Soybean growers may choose to apply these herbicides in combination with metribuzin, a photosystem II (PS II) inhibitor, for PRE soil-residual control of giant ragweed. Multiple herbicide sites of action are often applied together for increased weed control and reduced selection pressure for resistance (Busi et al. 2020, Jasieniuk et al. 1996, Moss et al. 2019). Furthermore, there is potential for synergistic weed control when HPPD inhibitors and PS II inhibitors are applied as a mixture (Abendroth et al. 2006; Armel et al. 2007; Hugie et al. 2008; Sutton et al. 2002).

A chemical interaction is considered synergistic when the combination of two or more different agents results in an effect greater than what would be expected if the two ingredients were acting independently (Greco 1995). Likewise, herbicide synergy has been defined as the cooperative action of two herbicides resulting in an observed response greater than that which is predicted by a biologically appropriate reference model (Hatzio and Penner 1985). Herbicide interactions are often classified by using factorial designs and Colby's method (also referred to as Bliss independence), and by using dose-response designs and Isobole analysis (Colby 1967, Loewe 1927, 1953, Ritz et al. 2021). As interactions are often highly dependent on rate (Hatzio and Penner 1985, Kelly and Chapman 1995), an Isobole analysis derived from complete dose-response experiments may be more appropriate for classifying these interactions than factorial designs (Armel et al. 2007, Ritz et al. 2021, Sørensen et al. 2007, Tallarida 2012). In traditional soil-based experiments, even if the applied rate of each herbicide is consistent, the amount of each of product that is available for uptake is affected by several factors associated with the variability of the soil matrix and precipitation (Curran 2016). An agar-herbicide solution can instead be used to maintain consistent availability of different herbicides throughout the duration of an experiment (Burgos et al. 2013). Thus, agar-based bioassays can be utilized to supplement soil-based assays in the characterization of herbicide interactions (Sukhoverkov and Mylne 2021).

Synergistic interactions have been observed between herbicides that inhibit HPPD and PS II in several weed species (Abendroth et al. 2006, Armel et al. 2007, Hugie et al. 2008, Sukhoverkov and Mylne 2021, Woodyard et al. 2009). For a more in-depth discussion of the biochemical nature of this interaction, see Armel et al. (2005). The interaction between atrazine (a PS II inhibitor) and mesotrione is among the most studied examples of herbicide synergy (Sukhoverkov and Mylne 2021). Applications of mesotrione plus atrazine for PRE or postemergence (POST) control of giant ragweed in corn production can be synergistic (Bollman et al. 2006, Woodyard et al. 2009). Additionally, applications of isoxaflutole (an HPPD inhibitor) plus metribuzin can be synergistic for POST control of giant ragweed (Ditschun et al. 2016), while POST synergy with mesotrione plus metribuzin was demonstrated on cultivated sunflower (Abendroth et al. 2006). Still, mixtures that are synergistic for control of one species may be additive or even antagonistic for others (Hatzio and Penner 1985). As the combination of mesotrione and metribuzin has not been widely adopted, this specific interaction has not been characterized.

Recent approval of mesotrione for PRE applications in soybean makes the joint application of mesotrione and metribuzin more likely than it has been in the past. Thus, this combination was evaluated for PRE control of giant ragweed under field conditions at a rate that may be used in soybean. Additionally, much of the previous work examining the interaction between HPPD inhibitors and PS II inhibitors was conducted using factorial designs and/or subjective datasets derived solely from visual observations (Abendroth et al. 2006, Armel et al. 2007, Bollman et al. 2006, Ditschun et al. 2016, Smith et al. 2019, Woodyard et al. 2009). Therefore, we conducted both agar- and soil-based bioassays and analyzed objective data with the Isobole method in a multiray fixed-ratio design (Sørensen et al. 2007, Tallarida 1992) to characterize the interaction of mesotrione and metribuzin for PRE control of giant ragweed.

3.3 Materials and Methods

3.3.1 Field Experiment

A field experiment was conducted in 2018 and 2019 at two sites in Indiana: the Throckmorton Purdue Agriculture Center (TPAC; 40.17° N, 86.54° W), and a commercial field, TIP-1 (40.26° N, 87.04° W). The field at TPAC (pH 6.6, 2.9% OM) consisted primarily of a Toronto-Millbrook silt loam complex, and the TIP-1 field (pH 5.7, 1.8% OM) was a combination of Mahalasville, Treaty, and Rainsville silt loams. Each experiment was initiated weed-free with an application of paraquat (Gramoxone 2.0 SL[®], Syngenta Crop Protection, Greensboro, NC) followed by a combination of disk and field cultivator prior to planting. Mesotrione-resistant

soybean was planted at 346,000 seeds ha⁻¹ in 76 cm rows at a depth of 2.5 to 5 cm. Plots were 3 m by 9 m, and herbicides were applied immediately after planting to the center 2 rows of each 4-row plot using a CO₂-pressurized backpack sprayer and a 2-m handheld spray boom equipped with extended-range, flat fan nozzles (XR 8002VS; TeeJet[®]t Spraying Systems, Wheaton, IL 60187) delivering 140 L ha⁻¹ at 207 kPa. Herbicide treatments included mesotrione (Callisto[®], Syngenta, Greensboro, NC, USA) and metribuzin (Tricor[®] DF, United Phosphorus, Inc., King of Prussia, PA) applied alone and combined at 177 and 315 g ai ha⁻¹ of each active ingredient, respectively. The experiment also included nontreated plots for comparison.

Herbicide efficacy on giant ragweed was evaluated using plant density and biomass accumulation at 42 days after planting (DAP). Giant ragweed plant density counts were taken by randomly placing a 0.5 m² quadrat at two different locations between the center two soybean rows in each plot. Biomass was harvested from the same quadrat area used for plant density and ovendried at 50 C until the weight was constant. Density and biomass data were combined over locations within each plot, yielding a total measured area of one square meter per plot. Both biomass and density measurements for each treated plot were analyzed as a percent reduction compared with the nontreated.

Treatments were arranged in a randomized complete block design with four replications, and this experiment was conducted twice at both sites over the 2018 and 2019 growing seasons. Data were tested for normality and constancy of variance through visual inspection of histograms and quantile-quantile (Q-Q) plots of the residuals and plots of residuals vs fitted values. The interaction of mesotrione and metribuzin was examined following Colby's method as outlined in Equation 1:

$$E = A + B - [(A * B)/100]$$
[1]

where *E* is the expected efficacy of the mixture of herbicides A and B assuming an additive interaction, and *A* and *B* represent the observed efficacy of each herbicide applied alone (Colby 1967). An interaction was considered to be synergistic when the observed efficacy of the herbicide mixture was greater than *E*, while efficacy less than *E* indicated antagonism. Significant differences between observed and expected values were determined by using PROC TTEST in SAS[®] 9.4 (SAS Institute Inc., Cary, NC) to conduct an independent, two-sample t-test.

3.3.2 Plant Materials for Bioassays

Though the seeds of giant ragweed are contained within a cypsela made up of a seed coat, pericarp, and involucre hull (Figure 3.1), the term "seed" is typically used in reference to the complete cypsela and will be used hereafter (Harre et al. 2019, Marzinek et al. 2008). In October of 2018, mature seeds were collected from several hundred giant ragweed plants growing in the TIP-1 field and stored at 4 C until utilized in these experiments. To partially alleviate dormancy, seeds were placed in mesh bags and buried in a 3:1 mixture of sand to soil and kept at 4 C for 2 to 3 months prior to planting (Westhoven et al. 2008). For the agar-based experiment, additional steps were taken to further improve germination using a technique modified from previous studies (Harre et al. 2019, Page and Nurse 2015, Westhoven et al. 2008). After soil stratification, seeds used in the agar-based bioassay were fully excised from the other cypsela components and soaked in water for 12 to 16 hours on a stir plate with gentle circulation.

3.3.3 Soil-Based Bioassay

The soil in this experiment was a sandy loam (pH 7.0, 3% organic matter) that had been passed through a 4-mm sieve. One layer of filter paper (Whatman #1) was placed in the bottom of round plastic pots (170 cm³) that were filled with 900 ml of soil. The pots were leveled to yield

an even planting surface, watered, and drained to field capacity one day prior to planting. Twentyfive giant ragweed seeds (with intact cypsela) were planted in each pot and tamped down to improve soil contact. Seeds were then covered with an additional 200 ml of soil, and the pots were leveled once more (seed depth was 1 cm). Pots were watered again to achieve field capacity (45 ml per pot).

Commercially formulated mesotrione (Callisto[®]) and metribuzin (Mauler[®], Valent, Walnut Creek, CA, USA) were applied immediately after planting using a track-mounted research sprayer (Generation III Research Sprayer, DeVries Manufacturing, Hollandale MN) delivering 140 L ha⁻¹ at 207 kPa with a single, even-fan XR8002 EVS nozzle (TeeJet[®] Technologies, Springfield, IL 62703). Mesotrione and metribuzin were applied at rates evenly spaced on a log₄ scale from 4 to 1,024 and 16 to 4096 g ai ha⁻¹, respectively. Mixtures of these herbicides were applied according to a multi-ray fixed-ratio design of 1:1, 1:2, and 2:1 ratios of their relative potency, which was determined in a pilot experiment (data not presented) (Ritz et al. 2021, Sørensen et al. 2007, Tallarida 2012). A treatment where no herbicide was applied was included for each dose-response curve. Each rate was replicated six times, and the experiment was conducted twice.

After these applications, pots were placed in a greenhouse maintained at 23 to 29 C with 16 h supplemental light (1,100 μ mol m⁻² s⁻¹ photon flux density) supplied by high-pressure sodium bulbs. Overhead irrigation designed to simulate rainfall was used to add another 45 ml water per pot immediately following herbicide application (Hausman et al. 2013, Umphres et al. 2018). This over-the-top watering method was used for the following 9 d to ensure distribution of the herbicide(s) throughout the soil profile (Table 3.1). Sub-irrigation was used for the remainder of the experiment to maintain adequate moisture for optimal growth without moving the herbicides beyond the range of uptake by plant roots, as determined in preliminary research. Aboveground

biomass was collected 14 days after planting (DAP). Fresh weights were recorded immediately after harvest, and dry weights were recorded after samples were kept at 38 C in an oven dryer until weight was constant (data not presented). The reduction in fresh biomass was determined for plants harvested from the treated pots relative to the nontreated pots, and the resulting data were transformed using the Yeo-Johnson method ($\lambda = 1.15$) prior to analysis. Nonlinear regression was conducted in accordance with a three-parameter log logistic model (Knezevic et al. 2007) (Equation 2) using the 'drc' package in R (Ritz and Streibig 2005) to obtain estimates of the effective herbicide dose that resulted in a 50% reduction in the response variable (ED₅₀).

$$f(x) = \frac{d}{1 + \exp(b(\log(x) - \log(e)))}$$
[2]

The coefficients b, d, and e denote the slope, upper limit, and inflection point, respectively. Confidence intervals were derived using the delta method (Ritz et al. 2021), and all estimates were back-transformed to their original scale for presentation. As the interaction of herbicide rate and experimental run was not significant according to analysis of variance (ANOVA) (P = 0.25), data were combined over runs.

3.3.4 Agar-Based Bioassay

A 1% w/v agar solution was autoclaved, and herbicides of the same commercial formulation used in the soil-based bioassay were added once the solution had cooled below 55 C. The dose ranges for mesotrione and metribuzin were 0.0005 to 5 and 0.005 to 50 μ M, respectively, on a log₁₀ scale, and a treatment without herbicide was also included for each dose-response curve. The relative potency of these herbicides was similarly determined through a separate pilot study (data not presented), and the concentration of each herbicide in mixture treatments was also based

on a multi-ray fixed-ratio design derived from 1:1, 1:2, and 2:1 ratios of their relative potency. Each concentration was replicated ten times, and the experiment was conducted twice.

After mixing thoroughly, 10-ml of the agar-herbicide solution was distributed into 15-ml glass culture tubes (Fisher Scientific, Waltham, MA, USA). Once the solution had solidified, one giant ragweed seed, separated from other cypsela components as described previously, was planted in each tube with the radical end of the seed oriented towards the bottom of the tube (Figure 3.1). The tubes were arranged in boxes designed to shield developing roots from direct light. After planting, the boxes were moved to a growth chamber with conditions set to simulate a field environment while also optimizing germination and growth. For the first 4 d, a 12 h, 23/18 C temperature regime and 2.5/21.5 h light/dark environment supplied by florescent bulbs (820 µmol m⁻² s⁻¹ photon flux density) was maintained, and the boxes were covered with 4 layers of black shade cloth. For the remainder of the experiment, the shade cloth was removed and the light/dark period was adjusted to 12 h and synchronized with the temperature regime. To account for water lost due to transpiration, and to provide nutrients for optimal growth, a 3% w/v solution of Miracle-Gro® fertilizer (24-8-16 Miracle-Gro Water-Soluble All-Purpose Plant Food, Scotts Miracle-Gro Products, Marysville, OH) was added at 7 and 11 DAP to each culture tube at a volume sufficient to completely cover the roots.

After 14 d, giant ragweed roots and shoots were extracted from the agar and imaged using a printer scanner. The total plant area represented in each image was calculated by adjusting RGB parameters in ImageJ software (Wayne Rasbaud, National Institutes of Health, Bethesda, MD; http://imagej.nih.govij) to quantify root and shoot tissue (Figure 3.1). Fresh weights were also recorded immediately after imaging (data not presented). Image data were transformed using the Yeo-Johnson method ($\lambda = -0.2$) prior to analysis. Each ED₅₀ estimate was determined by fitting the data to a four-parameter Weibull Type 1 model (Equation 3) using the 'drc' package in R, and associated confidence intervals were calculated using the delta method.

$$f(x) = c + (d - c)\exp(-\exp(b(\log(x) - \log(e))))$$
[3]

The coefficients b, c, d, and e denote the slope, lower limit, upper limit, and the inflection point between c and d, respectively. Estimates and confidence intervals were back-transformed to their original scale for presentation. Data were pooled over experimental runs due to a non-significant interaction between herbicide concentration and experimental run according to ANOVA (P = 0.68).

3.3.5 Isobole Analysis.

The Isobole method relies on the assumption that, in the absence of synergy or antagonism, the efficacy of the mixture of two or more components is equal to the combined efficacy of each component individually. This assumption of additivity is often tested by comparing the dose of each herbicide alone that results in 50% efficacy with the dose of the herbicide mixture that yields the same effect (Ritz et al. 2021, Sørensen et al. 2007, Tallarida 1992). Isobole analysis explores this relationship geometrically. For each bioassay, the ED₅₀ estimates of mesotrione and metribuzin alone were graphed on the axes of a Cartesian plane. A line, referred to as the line of independent action (or simply "Isobole"), was drawn between those two points. When a point representing the ED₅₀ of the mixture fell along that line, the interaction was considered additive, while a value that fell below or above the line indicated synergism or antagonism, respectively (Figure 3.2) (Armel et al. 2007). The interaction index (γ) was used to quantify the radial distance between the ED₅₀ of the mixture and the line of independent action, with respect to the origin (Huang et al. 2019, Ritz et al. 2021, Tallarida 2002). This term was derived by the isobolar relation described in Equation 4,

$$\gamma = \frac{a}{A} + \frac{b}{B}$$
[4]

where A and B were the ED_{50} estimates of herbicides A and B alone, and a and b were the doses of herbicides A and B as mixture components that resulted in the same effect level. Estimates of γ that were less than, equal to, or greater than one were considered synergistic, additive, or antagonistic, respectively (Tallarida 2002).

3.4 Results and Discussion

3.4.1 Field Experiment

Giant ragweed plant density data were combined over sites and years, as the interaction between these effects and the effect of herbicide treatment was not significant according to ANOVA (P > 0.05). Applications of mesotrione reduced giant ragweed density by 84% relative to the nontreated, while metribuzin reduced the density by 22% (Table 3.2). A t-test ($\alpha = 0.05$) indicated that the interaction between mesotrione and metribuzin was additive, as the observed density reduction from the mixture of these herbicides (88%) was not different than the expected mean assuming additivity (86%) (Table 3.2). Biomass reduction data were separated by year due to a significant interaction with herbicide treatment (P < 0.0001). In 2018, mesotrione reduced giant ragweed biomass by 83%, while metribuzin increased biomass by 77% (Table 3.2) by reducing competition from other weedy species (data not presented). In 2019, mesotrione and metribuzin alone reduced giant ragweed density by 82 and 19%, respectively. Biomass reduction data in both years resulted in an additive interaction, as observed and expected values in 2018 (91 and 70%, respectively) and 2019 (75 and 83%, respectively) were not different according to a ttest ($\alpha = 0.05$).

The interaction between HPPD and PS II inhibitors is one of the most well-characterized examples of herbicide synergy (Sukhoverkov and Mylne 2021). In corn production, mesotrione and atrazine are often applied together in anticipation of synergistic weed control (Bollman et al. 2006). In this experiment, mesotrione was applied with metribuzin, a PS II inhibitor that binds to the same active site as atrazine. Foliar applications of these two herbicides can be synergistic (Abendroth et al. 2006), though their interaction for soil-residual control has not been investigated. Results from this field experiment indicated that the interaction was additive. However, the implications of these data are limited, as only one dose of each herbicide was examined. Interactions are classified most effectively when the rate of each herbicide results in an intermediate level of control, and when multiple rates are examined (Kelly and Chapman 1995, Ritz et al. 2021). In this experiment, mesotrione resulted in very high efficacy on giant ragweed, reducing density and biomass by greater than 80% when applied alone, while metribuzin was largely ineffective (Table 3.2). Given the large differences in the efficacy of these herbicides at the rates utilized in this experiment, it may not be biologically reasonable to expect to fully characterize this interaction using this experimental design (Kelly and Chapman 1995, Ritz et al. 2021). Therefore, additional experiments were conducted to more thoroughly characterize this interaction by employing a wider range of herbicide doses under controlled conditions.

3.4.2 Germination and Symptomology in the Bioassays

Approximately 74% of giant ragweed seeds used in the soil-based bioassay had germinated by 14 DAP, with the majority emerging within 3 DAP. Germination of excised seeds used in the agar-based bioassay was nearly always successful within 24 h; only 1 of 600 failed to germinate. Interestingly, in both experiments, seeds exposed to even the highest herbicide doses grew normally for at least 7 DAP. Symptomology consistent with these herbicide modes of action was not readily apparent until 9 to 11 days after the initiation of each experiment. By 14 DAP, all plants in the agar-based bioassay that were exposed to a lethal herbicide dose were desiccated and completely necrotic (Figure 3.1 d). This was also true for the majority of plants growing in the highest doses of the soil-based bioassay (Figure 3.1 e). Perhaps, in the soil-based assay, the placement of the seeds relative to the herbicides did not allow for sufficient uptake prior to germination, which could have delayed herbicidal efficacy. Although, full herbicide doses were available to the seeds germinating in the agar-based assay, and the progression of symptomology was similar between these two experiments.

Both mesotrione and metribuzin are light-dependent herbicides (Hess 2000). Metribuzin displaces plastoquinone at the D1 binding site of PS II, which disrupts the flow of electrons and eventually results in the formation of singlet oxygen species and triplet chlorophyll that initiate lipid peroxidation (Funar-Timofei et al. 2017, Hess 2000). Uptake of mesotrione in susceptible plant species reduces the production of plastoquinone by inhibiting upstream processes, leading to similar light-induced phytotoxicity (Carles et al. 2017, Hess 2000, Siefermann-Harms 1987). Therefore, the seemingly delayed herbicidal effects of these two chemicals applied PRE can be partially attributed to their dependence on the progression of photosynthesis for activity. Based on personal observation in these experiments, this delay seemed to be exacerbated in giant ragweed compared with other smaller-seeded weed species (data not presented). This could be explained in the soil-based bioassay if the herbicide concentration was greater in the upper portion of the soil profile where the smaller-seeds weeds were germinating. However, at least 7 days passed between planting and symptomology on giant ragweed seeds in the agar-based bioassay as well, where the herbicide dose was constant throughout the agar profile. There is little published research examining the relationship between seed size and the progression of soil-residual herbicide efficacy. In theory, a larger seed could support seedlings with carbohydrate reserves for a longer period of time before these seedlings became fully dependent on the progression of photosynthesis to meet their energy requirements (McManamen et al. 2018).

3.4.3 Soil-Based Bioassay

A total of 3.3 cm of water was added to each pot through overhead irrigation over the first 10 d of this experiment to distribute the herbicides throughout the soil profile (Table 3.1). This amount of water was greater than what has been used in other experiments with *Amaranthus* spp. (Hausman et al. 2013, Umphres et al. 2018) to account for the deeper burial of giant ragweed seeds and the longer period of growth prior to herbicidal efficacy. At 14 DAP, the reduction in fresh giant ragweed biomass in each treated pot was compared with the average of the nontreated pots. Biomass reduction data were modeled using Equation 2 to estimate ED_{50} values for each herbicide or mixture, which were then plotted in Isobolograms (Figure 3.2).

Contrary to the proof proposed by Berenbaum (1989), Isoboles can be curvilinear if the relative potency of mixture components is different across the range of doses tested. Ignoring this principal and assuming the Isobole is linear can lead to mischaracterization of chemical interactions (Grabovsky and Tallarida 2004, Tallarida 2012), though these are often minimal when analyses are based on ED₅₀ estimates (Ritz et al. 2021). In the soil-based bioassay, the slope parameter (b) was not different between mesotrione and metribuzin applied alone (P = 0.87). This indicated that the relative potency of these herbicides was in fact constant across the range of doses tested. Therefore, when constructing the Isobologram, the line of independent action (Isobole) had a constant slope connecting the ED₅₀ estimates of mesotrione (24 g ai ha⁻¹) and metribuzin (46 g ai ha⁻¹) applied alone (Ritz et al. 2021, Tallarida 2012). After adding the ED₅₀ estimates of the herbicide mixtures, the concave shape each Isobologram indicated that the combination of

mesotrione and metribuzin was synergistic at all mixture ratios (Figure 3.2) (Armel et al. 2007). The resulting interaction indices were 0.76, 0.78, and 0.51 for the 1:1, 1:2, and 2:1 mixture ratios, respectively (Table 3.3). All values of γ and their associated 95% confidence intervals quantified the synergistic interaction observed in the Isobolograms, as all values were less than one (Table 3.3) (Ritz et al. 2021, Tallarida 2002). Dose-response curves used to calculate each ED₅₀ and γ value are shown in Figure 3.3, with model parameters listed in Table 3.4.

The lowest interaction index (indicative of the strongest synergy) was observed in the 2:1 mixture ratio of mesotrione to metribuzin ($\gamma = 0.51$). This mixture ratio was comprised of 13% mesotrione and 87% metribuzin, which was similar to another study reporting synergistic control of Palmer amaranth (*Amaranthus palmeri* S. Watson) and velvetleaf (*Abutilon theophrasti* Medik.) from POST applications of 14% mesotrione relative to 86% metribuzin (Abendroth et al. 2006). Isoxaflutole is an HPPD inhibitor that, like mesotrione, may be used in HPPD-resistant soybean varieties. One study reported both additive and synergistic interactions for PRE applications of isoxaflutole plus metribuzin for control of four broadleaf and three grass species (Smith et al. 2019). Though giant ragweed was not evaluated by Smith et al. (2019), both additivity and synergy on giant ragweed has been reported following foliar applications of isoxaflutole plus metribuzin (Ditschun et al. 2016).

3.4.4 Agar-Based Bioassay

To eliminate the variability associated with the soil matrix (e.g. soil adsorption, herbicide location in the soil profile, etc.) and from precipitation that may occur under field conditions, an agar-based bioassay was also utilized to simulate combinations of mesotrione and metribuzin for PRE control of giant ragweed. This method was implemented to ensure that both herbicides were available consistently at the proper ratio for seedling uptake. After 14 d, both the roots and shoots

of giant ragweed plants were easily extracted from the agar solution (Figure 3.1). The slopes of the dose-response curves derived from images and modeled using Equation 3 were not different for each herbicide applied alone (P = 0.85), so a linear Isobole analysis was conducted (Ritz et al. 2021, Tallarida 2012). Similar to the soil-based bioassay, Isobolograms indicated synergy at all mixture ratios (Figure 3.4, Table 3.5), with associated dose-response curves shown in Figure 3.5 and model parameters listed in Table 3.6. A mesotrione concentration of 0.17 uM resulted in a 50% reduction in giant ragweed seedling growth, while a 0.6 µM concentration of metribuzin was needed to reach the same effect level. A similar agar-based study demonstrated that the ED_{50} in Arabidopsis thaliana was reached at a mesotrione concentration of just 0.02 µM (Sukhoverkov and Mylne 2021), nearly 8-fold lower than the ED_{50} observed on giant ragweed in this study. The synergistic interactions observed in the Isobolograms were established by the interaction indices for the 1:1, 1:2, and 2:1 mixture ratios, which were 0.57. 0.69, and 0.71, respectively. Upper 95% confidence intervals were less than one for all values of γ (Table 3.5). In this bioassay, the 1:1 mixture ratio was comprised of 11% mesotrione and 89% metribuzin, respectively, and resulted in the lowest estimation of γ (0.57). These results indicate that, in the absence of soil, mesotrione and metribuzin can be synergistic for control of giant ragweed at the ratios tested.

This study represents the first evidence of synergy between PRE applications of an HPPD inhibitor (mesotrione) plus metribuzin for control of giant ragweed. In commercial production systems, the relative dose of each herbicide that is available for uptake and the position of those herbicides in the soil profile will vary throughout the growing season. As synergy was observed at all mixture ratios tested in these bioassays, synergy may occur when this combination of herbicides is used in commercial environments. However, the dynamics of this interaction may be altered, as differences in the soil type, rainfall patterns, and herbicide degradation rates that are

unique to specific environments all affect the available herbicide concentration of each herbicide. Results from the field experiment indicate that if uptake of one of the herbicides is insufficient for substantial activity, the potential for synergy may be reduced.

Table 3.1. Overhead irrigation applied to each pot in the soil-based bioassay following herbicide application.

Days after planting	Run 1	Run 2
	cn	n ———
0	0.25	0.25
1	0	0
2	0.27	0.14
3	0.55	0.33
4	0.38	0.36
5	0.38	0.36
6	0.41	0.38
7	0.25	0.47
8	0.22	0.16
9	0.22	0.41
10	0.36	0.44
Cumulative	3.29	3.29

Table 3.2. Efficacy of mesotrione and metribuzin applied alone and as a mixture at two fields for control of giant ragweed, with expected values derived using Colby's method and assuming additivity. ^a

		Biomass reduction	Biomass reduction
Treatment	Density reduction ^b	2018	2019
		%	
Mesotrione	84	83	82
Metribuzin	22	-77	19
Mixture (expected)	88 (86)	91 (70)	75 (83)
Observed vs. expected ^c	P = 0.64	P = 0.26	P = 0.36

^a Efficacy was evaluated through density and biomass reduction relative to the nontreated.

^b Density reduction data for 2018 and 2019 were combined due to a non-significant interaction according to ANOVA.

^c A t-test was used to compare observed and expected treatment means, with expected means calculated using Equation 1 (Colby 1967).

Table 3.3. Interaction indices (γ) calculated using Equation 4 and estimates derived using non-linear regression^a to model biomass data that quantified the response of giant ragweed to mesotrione and metribuzin in the soil-based bioassay after 14 d.

Mixture ratio ^b	γ^{c}	95% CI	Interaction ^d
1:1	0.76	[0.57-0.95]	Synergy
1:2	0.78	[0.56-0.99]	Synergy
2:1	0.51	[0.35-0.66]	Synergy

^a Dose-response curves were generated using a three-parameter log logistic model (Equation 2).

^b The mixture ratio describes the relative concentration of mesotrione and metribuzin in each mixture as defined by their potency ratio.

^c Estimates used to calculate γ were back-transformed after the Yeo-Johnson method ($\lambda = 1.15$) was used prior to analysis.

^d Estimates of γ that are less than 1 indicate synergy (Tallarida 2002).

Table 3.4. Regression parameters utilized in a three-parameter log logistic model (Equation 2) to describe the effect of mesotrione and metribuzin on giant ragweed in the soil-based bioassay after 14 d. ^a

		_		Parameter ^b	
Herbicide (mixture ratio) ^c	ED50	95% CI	b	с	d
	——— g a	i ha ⁻¹ ———			
Mesotrione (1:0)	24	[12-35]	-0.9	34	170
Metribuzin (0:1)	46	[27-65]	-1	73	159
Mesotrione + metribuzin (1:1)	33	[25-41]	-1.4	50	169
Mesotrione + metribuzin (1:2)	35	[25-44]	-1.1	29	169
Mesotrione + metribuzin (2:1)	21	[14-27]	-1.3	53	164

^a Abbreviations: ED₅₀, calculated dose resulting in 50% reduction in fresh weight; b, slope of the line; c, lower limit; d, upper limit.

^b Estimates of each parameter and ED₅₀ value were calculated from data that were transformed using the Yeo-Johnson method ($\lambda = 1.15$) prior to analysis, with back-transformed ED₅₀ values and their respective confidence intervals presented here.

^c The mixture ratio describes the relative concentration of mesotrione and metribuzin in each dose-response curve as defined by their potency ratio.

Table 3.5. Interaction indices (γ) calculated using Equation 4 and estimates derived using non-linear regression^a to model image data that quantified the response of giant ragweed to mesotrione and metribuzin in the agar-based bioassay after 14 d.

Mixture ratio ^b	γ^{c}	95% CI	Interaction ^d
1:1	0.57	[0.35-0.83]	Synergy
1:2	0.69	[0.44-0.98]	Synergy
2:1	0.71	[0.45-0.99]	Synergy
0 D	1		

^a Dose-response curves were generated using a four-parameter Weibull Type 1 model (Equation 3).

^b The mixture ratio describes the relative concentration of mesotrione and metribuzin in each mixture as defined by their potency ratio.

^c Estimates used to calculate γ were back-transformed after the Yeo-Johnson method ($\lambda = -0.2$) was used prior to analysis.

^d Estimates of γ that are less than 1 indicate synergy (Tallarida 2002).

Table 3.6. Regression parameters utilized in a four-parameter Weibull Type 1 model (Equation 3) to describe the effect of mesotrione and metribuzin on giant ragweed in the agarbased bioassay after 14 d. ^a

		_		Parameter ^b	
Herbicide (mixture ratio) ^c	ED50	95% CI	b	с	d
	· · · · · · · · · · · · · · · · · · ·	μΜ			
Mesotrione (1:0)	0.17	[0.09-0.25]	0.59	0.25	1
Metribuzin (0:1)	0.60	[0.32-0.96]	0.7	0.33	1.06
Mesotrione + metribuzin (1:1)	0.27	[0.16-0.39]	0.65	0.29	1.08
Mesotrione + metribuzin (1:2)	0.37	[0.23-0.52]	0.96	0.28	1.06
Mesotrione + metribuzin (2:1)	0.29	[0.18-0.40]	1.26	0.29	1.05

^a Abbreviations: ED₅₀, calculated dose resulting in 50% reduction of total plant area; b, slope of the line; c, lower limit; d, upper limit.

^b Estimates of each parameter and ED₅₀ value were calculated from data that were transformed using the Yeo-Johnson method ($\lambda = -0.2$) prior to analysis, with back-transformed ED₅₀ values and their respective confidence intervals presented here.

^c The mixture ratio describes the relative concentration of mesotrione and metribuzin in each dose-response curve as defined by their potency ratio.



Figure 3.1 (a) Giant ragweed seed and cypsela components including, from left to right, the involucre hull, pericarp, seed coat, and seed. (b) Excised giant ragweed seeds planted in the agar-based bioassay. (c) Giant ragweed roots and shoots extracted from agar before and after processing in ImageJ. (d, e) Photos of giant ragweed after growing for 14 d in the agar-based bioassay (d) and the soil-based bioassay (e) arranged from left to right by ascending herbicide concentration.



Figure 3.2. Isobolograms illustrating the interaction of mesotrione and metribuzin in a soil-based bioassay for preemergence control of giant ragweed. Ratios reflect the concentration of mesotrione relative to metribuzin in each mixture as defined by their relative potency. The dose of each herbicide or mixture that resulted in 50% efficacy after 14 d is represented by solid points. Points representing a mixture (red) that fall below the Isobole (green line) indicate synergy.



Figure 3.3. Dose-response curves modeling the reduction of giant ragweed biomass in the soil-based bioassay after 14 d as a function of preemergence herbicide rate. Data were transformed with the Yeo-Johnson method ($\lambda = 1.15$) and fit to a three-parameter log-logistic function (Equation 2), with associated parameters listed in Table 3.4. Mixtures reflect the relative concentration of mesotrione to metribuzin at fixed ratios based on their relative potency.



Figure 3.4. Isobolograms illustrating the interaction of mesotrione and metribuzin in an agar-based bioassay simulating preemergence control of giant ragweed. Ratios reflect the concentration of mesotrione relative to metribuzin in each mixture as defined by their relative potency. The dose of each herbicide or mixture that resulted in 50% efficacy after 14 d is represented by solid points. Points representing a mixture (red) that fall below the Isobole (green line) indicate synergy.



Figure 3.5. Dose-response curves modeling giant ragweed seedling growth in the agar-based bioassay after 14 d as a function of preemergence herbicide rate. Total plant area was quantified using image analysis in ImageJ. Data were transformed with the Yeo-Johnson method ($\lambda = -0.2$) and fit to a four-parameter Weibull Type 1 function (Equation 3), with associated parameters listed in Table 3.6. Mixtures reflect the relative concentration of mesotrione to metribuzin at fixed ratios based on their relative potency.
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CHAPTER 4. INFLUENCE OF HERBICIDE MIXTURES AND SEQUENTIAL APPLICATIONS ON THE PREVALENCE OF GIANT RAGWEED BIOTYPES RESISTANT TO ALS INHIBITORS

4.1 Abstract

Cloransulam-methyl is an acetolactate synthase (ALS)-inhibiting herbicide often used to control giant ragweed (Ambrosia trifida L.) in soybean production. Heavy reliance on herbicides that target ALS has resulted in the spread of giant ragweed biotypes with resistance to these herbicides (ALS-R) endowed by a point mutation in the ALS gene. Mesotrione, which inhibits the 4-hydroxyphenolpyruvate dioxygenase (HPPD) enzyme, is now approved for preemergence (PRE) application in appropriately traited soybean varieties. Applications of herbicide mixtures that include mesotrione and sequential applications of herbicides with alternative modes of action may lessen the selection for an increased frequency of ALS-R giant ragweed (FOR) induced by cloransulam in populations with established resistance. This study examined the FOR as influenced by PRE-only and sequential herbicide regimes at two field sites with indigenous ALS-R. Across all site-years in the PRE-only experiment, all treatments increased the FOR in surviving giant ragweed plants beyond initial resistance frequency levels. Treatments with cloransulam, but without mesotrione, increased the FOR to greater than 90% across site-years, while the addition of mesotrione resulted in a lower FOR compared to cloransulam without mesotrione. Treatments with mesotrione also resulted in a lower FOR than the commercial premix of sulfentrazone and cloransulam, and a FOR similar to a premix of chlorimuron, flumioxazin and pyroxasulfone. In some instances, sequential applications of glufosinate plus fomesafen and S-metolachlor applied postemergence eliminated the selection for an increased FOR as a result of PRE-applied ALS inhibitors. However, the magnitude of this effect was dependent on environmental conditions, and

no treatment eliminated this selection pressure in every site-year. Overall, PRE herbicide mixtures that include mesotrione and sequential applications with overlapping, effective soil-residual herbicides can reduce the increase in the FOR induced by PRE applications of an ALS inhibitor. Nevertheless, a more robust approach with additional herbicide or non-chemical weed management strategies is necessary to consistently prevent an increase in the FOR in populations where ALS-R is established.

4.2 Introduction

Herbicides that inhibit acetolactate synthase (ALS) have been an integral component of numerous weed management systems since their introduction in the early 1980s, and more than 50 unique ALS-inhibiting herbicides are used globally to protect a plethora of essential agricultural crops (Bellinder et al. 1994, Garcia et al. 2017, Tranel and Wright 2002). The popularity of these herbicides among growers is often attributed to broad-spectrum control of many agronomically important weed species, soil-residual activity, low use rates, good crop safety, and low mammalian toxicity (Mazur and Falco 1989). The widespread adoption of ALS inhibitors led to a decline in the total number of herbicide active ingredients applied to crops during the two decades following their release (Bellinder et al. 1994, Hart et al. 1997, Mazur and Falco 1989). Though several factors influence the evolution and spread of herbicide resistance traits, exclusive dependence on a single mode of action results in strong selection pressure for resistance (Evans et al. 2016, Jasieniuk et al. 1996, Norsworthy et al. 2012). Weed resistance to ALS inhibitors (ALS-R) was confirmed shortly after the commercialization of this chemistry (Mallory-Smith et al. 1990, Primiani et al. 1990), and today, more weed species have evolved resistance to these herbicides than to any other herbicide mode of action (Heap 2022).

Herbicidal inhibitors of ALS do not resemble native substrates, products, transition-state intermediates, or regulatory compounds that interact with this enzyme during normal function (Garcia et al. 2017), and herbicide molecules do not bind directly to the enzyme's active site (Gaines et al. 2020, McCourt et al. 2006). Instead, these herbicides block the route that substrates must take to reach the active site of ALS (McCourt et al. 2006), and they also appear to degrade an essential enzymatic cofactor, thiamine diphosphate (ThDP) (Garcia et al. 2017). Resistance to ALS inhibitors often results from a structural change at the site where the herbicide molecule binds to ALS. In many cases, mutations responsible for ALS-R do not affect the structure of the catalytic center of ALS. As a result, these mutations typically do not influence overall plant fitness in the absence of herbicide selection (Gaines et al. 2020, Tranel and Wright 2002). Considerable overlap exists in the binding sites of ALS inhibitors belonging to several different chemical families, which contributes to the cross-resistance observed in many plant species as a result of a single mutation (Gaines et al. 2020, Garcia et al. 2017, Tranel and Wright 2002). Together, these characteristics may partially explain the rapid evolution and widespread prevalence of ALS-R compared with resistance to other herbicide modes of action (Heap 2014).

Cloransulam-methyl is an ALS inhibitor that was among the most effective herbicides for preemergence (PRE), soil-residual control of giant ragweed (*Ambrosia trifida* L.) in soybean production until the evolution of ALS-R (Franey and Hart 1999, Paztolt and Tranel 2002, Taylor et al. 2002). Giant ragweed is an extremely competitive summer annual dicot that poses a significant threat to soybean production when left uncontrolled (Baysinger and Sims 1991, Webster et al. 1994). A substitution of leucine for tryptophan at position 574 of *ALS* is the only known mechanism to endow ALS-R in giant ragweed (Marion et al. 2017, Patzoldt and Tranel 2002). One or two mutant alleles in this diploid species (2n = 24) confers resistance to rates of

cloransulam that are at least 100-fold greater than those used in soybean, without a cost to overall plant fitness (Marion et al. 2017, Patzoldt and Tranel 2002). This mutation also confers cross-resistance to the ALS-inhibiting herbicides imazethapyr and chlorimuron (Patzoldt and Tranel 2002). Resistant giant ragweed biotypes have been confirmed in seven US states and Ontario, Canada (Heap 2022), though many of these populations are segregating for resistance (Harre et al. 2017). Glyphosate was highly effective for control of ALS-R giant ragweed, until this species evolved glyphosate-resistance as well (Heap 2022, Stachler 2008). Resistance to a third class of herbicides, protoporphyrinogen oxidase (PPO) inhibitors, has recently been reported (Faleco et al. 2021). Though several postemergence (POST) herbicides (i.e. glufosinate) are still effective on giant ragweed, use of soil-residual herbicides is important to maintain weed control during the critical weed-free period of soybean. As ALS inhibitors such as cloransulam are often ranked as the most effective herbicides for soil-residual control of giant ragweed in soybean (Johnson et al. 2007, Loux et al. 2022), the spread of ALS-R biotypes represents a considerable threat to season-long weed management.

Both foliar and soil-residual applications of ALS inhibitors have the potential to select for ALS-R biotypes (Norsworthy et al. 2012, Tranel and Wright 2002). Herbicide mixtures that utilize multiple modes of action with activity on overlapping weed spectrums are often recommended to reduce the selection pressure for resistance (Bellinder et al. 1994, Diggle et al. 2003, Norsworthy et al. 2012). The efficacy of this strategy was proven experimentally in a study of foliar herbicide applications on a population of field pennycress (*Thlaspi arvense* L.) that was segregating for ALS-R. Over four years, foliar applications combining the ALS inhibitor ethametsulfuran and two herbicides with different modes of action did not increase the frequency of resistance to ALS inhibitors (FOR) beyond initial levels. However, applications of ethametsulfuran alone increased

the FOR by 24 to 81% (Beckie and Reboud 2009). Herbicide mixtures in this study were effective at preventing an increase in the FOR in part because each mixture component had a different target site and effectively controlled field pennycress (Beckie and Reboud 2009, Wrubel and Gressel 1994). Mixtures of soil-residual herbicides also have the potential to reduce selection pressure for resistance if, in addition to these same criteria, each mixture component has similar persistence in the soil (Wrubel and Gressel 1994). This concept has not been demonstrated experimentally using primary research. Nevertheless, herbicide mixtures have been shown to increased control of weed biotypes resistant to one of two mixture components, which would lead to a decrease in overall seed production and delay the selection for resistance (Wuerffel et al. 2015).

Soybean varieties resistant to mesotrione, an inhibitor of the 4-hydroxyphenolpyruvate dioxygenase (HPPD) enzyme, have been developed using genetic engineering (Boudec et al. 2001, Hawkes et al. 2011). Some varieties have been granted regulatory approval (USDA-APHIS 2013a, 2013b), and applications of mesotrione are now permitted prior to soybean emergence (EPA reg. no. 70506-331). A mutant *HPPD* gene from oat (*Avena sativa* L.) endows resistance to HPPD inhibitors in SYHT0H2 cultivars (Hawkes et al. 2011, Hipskind et al. 2012, USDA-APHIS 2013b), while resistance to these herbicides in LibertyLink[®] GT27[®] varieties results from the insertion of a mutant *HPPD* gene from *Pseudomonas fluorescens* (Boudec et al. 2001, USDA-APHIS 2013a). Both of these mutant *HPPD* genes facilitate the expression of an HPPD enzyme with a reduced binding affinity for HPPD inhibitors, compared to the HPPD enzyme native to soybean (Boudec et al. 2001, Hipskind et al. 2012). Co-application of mesotrione and other soybean PRE herbicides with cloransulam has the potential to reduce selection pressure for ALS-R compared with cloransulam alone. Therefore, the objectives of this study were to compare the FOR in two giant ragweed populations that were segregating for ALS-R as a result of 1) factorial combinations of

cloransulam plus mesotrione, metribuzin, and *S*-metolachlor applied PRE in addition to two commercial premix standards, and 2) sequential applications of these herbicides applied PRE followed by POST herbicides with alternative modes of action.

4.3 Materials and Methods

4.3.1 Site Selection and Preparation

Two field experiments were conducted in 2018 and 2019 at two sites in Indiana. The site at the Throckmorton Purdue Agricultural Center (TPAC; 40.17° N, 86.54° W) consisted primarily of a Toronto-Millbrook silt loam complex. The other site, a commercial field known as TIP-1 (pH 5.7, 1.8% OM), was a combination of Mahalasville, Treaty, and Rainsville silt loams. A giant ragweed survey indicated that ALS-R individuals comprised 10% of the population at TPAC and 70% at TIP-1 (Harre, unpublished data). An application of paraquat (Gramoxone 2.0 SL[®], Syngenta Crop Protection, Greensboro, NC) followed by a combination of disk and field cultivator tillage events were conducted prior to planting to ensure the experiments were established under weed-free conditions. Soybean was planted in 76 cm rows at a depth of 2.5 to 5 cm and a seeding rate of 346,000 seeds ha⁻¹. In 2018, a SYHT0H2 soybean cultivar was planted (maturity group 3, Syngenta), while a LibertyLink[®] GT27[®] variety (Stine[®] 33GA13) was planted in 2019.

4.3.2 Herbicide Treatments

Experiments were established in 3 m by 9 m plots containing four soybean rows, with herbicides applied to the center 2 m of each plot using a CO₂-pressurized handheld spray boom calibrated to deliver 140 L ha⁻¹ at 207 kPa with extended-range, flat fan nozzles (XR 8002VS; TeeJet Spraying Systems, Wheaton, IL 60187). In the PRE-only experiment, cloransulam (32 g ai ha⁻¹) was applied alone and in combination with metribuzin (315 g ai ha⁻¹), *S*-metolachlor (1600

g ai ha⁻¹), and both of these herbicides (Table 4.1). These four treatments were evaluated with and without the addition of mesotrione (177 g ai ha⁻¹). In 2019, an additional treatment was incorporated which examined mesotrione applied alone. The sequential experiment examined several PRE herbicide combinations (Table 4.2) at the same rates followed by a postemergence (POST) application of glufosinate plus fomesafen (266 g ai ha⁻¹) and *S*-metolachlor (1217 g ai ha⁻¹). The POST herbicides were applied 21 days after planting (DAP) and included ammonium sulfate (N-PAK[®] AMS Liquid, Winfield Solutions, LLC, St. Paul, MN) at 10 g/l. Both the PRE-only and sequential experiments included two commercial-standard premixes applied PRE: sulfentrazone (283 g ai ha⁻¹) and cloransulam (37 g ai ha⁻¹), and chlorimuron (19 g ai ha⁻¹), flumioxazin (69 g ai ha⁻¹) and pyroxasulfone (87 g ai ha⁻¹). These treatments were also followed by the stated POST herbicide application in the sequential experiment. These standard herbicide premixes, both of which included an ALS inhibitor, are often recommended for control of giant ragweed in soybean (Loux et al. 2022). Additionally, each experiment included nontreated plots that represented the baseline frequency of resistance to ALS inhibitors.

4.3.3 Detection of the Trp574Leu Mutation

Giant ragweed tissue (15 mg) was collected from the youngest fully expanded true leaf of up to 25 plants that emerged in each plot at 21 DAP, and again at 42 DAP in the sequential experiment only. Each sample was stored at -20 C until the DNA was extracted using a modified CTAB protocol originally designed by Saghai-Maroof et al. (1984). A TaqMan[®] SNP genotyping assay developed by Harre et al. (2017) and synthesized by ABI (Applied BioSystems Inc., Grand Island, NY 14072) was utilized to classify each sample as homozygous-susceptible, heterozygous, or homozygous-resistant based on the relative florescence of VIC and FAM fluorophores. For each sample, a 10-µl reaction was prepared with 4.8 µl of PCR-grade water, 2 µl of GoTaq Flexi buffer, 1.2 μ l of 25 mM MgCl₂, 0.4 μ l of 10 mM dNTP, 0.5 μ l of 20X primers and TaqMan[®] probes, 0.1 μ l of GoTaq Flexi polymerase (5 U μ l⁻¹), and 1 μ l of genomic DNA. Reactions were amplified using a CFX384 RT-PCR detection system (Bio-Rad Laboratories, Hercules, CA 94547) with the following cycle conditions: 2 min at 95 C; 39 cycles of 95 C for 15 s and 60 C for 1 min; followed by a plate read after every cycle (Harre et al. 2017).

4.3.4 Experimental Design and Analysis

Treatments in each experiment were arranged in a randomized complete block design with four replications. Giant ragweed plants that were classified as homozygous-resistant and heterozygous were considered to possess the ALS-R phenotype (Marion et al. 2017, Patzoldt and Tranel 2002). A FOR value was calculated for each plot by dividing the number of resistant plants by the number of successfully classified plants in each plot and multiplying by 100%. Data were tested for normality and constancy of variance, and analysis of variance (ANOVA) was conducted using PROC GLIMMIX in SAS[®] 9.4 (SAS Institute Inc., Cary, NC).

In the PRE-only experiment, herbicide treatment, the addition of mesotrione, and site-year were considered main effects, while replication was considered a random effect. Data were pooled when any main effects or interactions were not significant (P > 0.05), and treatment means were separated using Tukey-Kramer's HSD ($\alpha = 0.05$). Additionally, orthogonal contrasts were conducted using PROC GLM in SAS[®] to compare the FOR as a result of treatments that included both cloransulam and mesotrione, each of the commercial-standard premix herbicides, and the nontreated. In the experiment with sequential applications, treatments were grouped as outlined in Table 4.2, as FOR in treatments within a group were not different according to ANOVA (P > 0.05) (data not presented). Orthogonal contrasts were used to determine whether the FOR was different in treatments where an ALS inhibitor was applied than in treatments where no ALS

inhibitors were applied, both before and after the POST herbicide application. Additionally, paired t-tests were used to determine whether the FOR in each treatment or treatment group differed between the 21 and 42 DAP collection timings.

4.4 **Results and Discussion**

4.4.1 Initial Resistance Frequency

The initial resistance frequency based on the phenotype of individual plants was determined in each site-year by sampling plants from the nontreated plots in both experiments. The initial FOR at TPAC was 16% in 2018 and 57% in 2019. At that site, experiments were conducted in adjacent fields between 2018 and 2019 to align with the corn and soybean rotation being employed, which may explain the variability in the resistance frequency between years. At TIP-1, the same field was used in both years, and the initial FOR was 71 and 73% in 2018 and 2019, respectively. An increase in the FOR as a result of a herbicide application becomes more difficult to detect as the initial FOR approaches 100%, due to a decrease in the theoretical maximum treatment effect (Wuerffel et al. 2015). Data from these experiments support this claim, as the largest treatment differences were observed at TPAC in 2018 (Tables 4.3 and 4.5).

4.4.2 **PRE-only Experiment**

In the factorial portion of the PRE-only experiment, mesotrione and site-year were the only significant main effects. Thus, the inclusion of S-metolachlor and/or metribuzin did not affect the selection for ALS-R from PRE applications of cloransulam. Data from TPAC in 2018 were analyzed separately from the other three site-years due to a significant interaction according to ANOVA. Selection for ALS-R was most evident at TPAC in 2018, where the FOR was increased from 15% in the nontreated to between 87 and 97% in treatments containing cloransulam without

mesotrione (Table 4.3). When mesotrione was added to those treatments, the average FOR was 48% (Table 4.3). In the other site years (TIP-1 in 2018, TPAC and TIP-1 in 2019), treatments containing cloransulam and mesotrione resulted in a lower FOR (85%) than treatments with cloransulam and without mesotrione (94%) at 21 DAP (Table 4.3). The FOR as a result of mesotrione applied alone (without cloransulam) in 2019 was not different than the nontreated at either site (data not presented).

These results suggest that mesotrione was persisting in the soil at a biologically effective dose longer than cloransulam and reducing the selection for ALS-R individuals, despite having a shorter soil half-life than cloransulam (Table 4.4) (Shaner 2014). As the concentration of mesotrione in the weed germination zone of the soil profile declined, the herbicide concentration available to suppress giant ragweed was also reduced. When more giant ragweed plants emerged as a result, the concentration of cloransulam would have been lower relative to the concentration on the day of application, allowing for increased emergence of the susceptible biotype (Wrubel and Gressel 1994). Applying mixtures of multiple, effective herbicide modes of action is often cited as one of the most effective methods for preventing the evolution and spread of herbicide resistance (Beckie and Reboud 2009, Diggle et al. 2003, Evans et al. 2016, Moss et al. 2019, Norsworthy et al. 2012, Powles et al. 1997). These results support this recommendation by providing the first experimental evidence that mixtures of PRE-applied, soil-residual herbicides can reduce selection for an increased FOR in weed populations with established resistance.

This research is evidence that PRE mixtures of mesotrione with ALS inhibitors and other herbicide groups for control of giant ragweed in soybean have the potential to prolong the utility of ALS inhibitors, even in fields with a history of ALS-R biotypes. However, further analysis was needed to determine whether the FOR in mixture treatments was similar to the initial FOR. Orthogonal contrasts were used to compare the FOR between the nontreated, combinations of mesotrione and cloransulam, and the commercial-standard premixes. The FOR from the premix of sulfentrazone and cloransulam was greater than treatments with cloransulam and mesotrione (Table 4.3), though the half-life of sulfentrazone is considerably greater than both mesotrione and cloransulam (Table 4.4). This could be explained by the comparatively low efficacy of sulfentrazone in the soil for control of giant ragweed in the absence of an ALS inhibitor (Krausz and Young 2003), compared with the high efficacy of mesotrione (Belfry and Sikkema 2015, Bollman et al. 2006, Loux et al. 2011). The combined efficacy of flumioxazin and pyroxasulfone limited the selection of ALS-R induced by chlorimuron to the same extent that mesotrione limited the selection induced by cloransulam (Table 4.3). Even though including mesotrione in treatments with cloransulam resulted in a lower FOR than cloransulam without mesotrione, these treatments still selected for an increased FOR relative to the nontreated (Table 4.3).

These results do not align with findings from Beckie and Rebound (2009), where combinations of an ALS inhibitor and other herbicide modes of action completely eliminated selection for ALS-R. In that study, foliar applications of the mixture of ethametsulfuron plus bromoxynil/MCPA may have completely controlled all emerged field pennycress, and the concentration of ethametsulfuron in the soil may have been too low to exert selection for ALS-R in later-emerging plants. In the present study, the effect of including mesotrione, metribuzin and *S*-metolachlor was insufficient to completely eliminate the selection pressure from PRE applications of cloransulam (Table 4.3). Thus, other weed management tactics such as effective POST herbicides and non-chemical methods may be required to prevent the surviving herbicide-resistant plants from producing seed and increasing the frequency of ALS-R alleles in the weed soil seedbank (Jasieniuk et al. 1996, Moss et al. 2019, Norsworthy et al. 2012).

4.4.3 Sequential Experiment

In the sequential experiment, the prevalence of ALS-R giant ragweed as a result of PREapplied ALS inhibitors was evaluated both prior to and following a POST application of glufosinate plus fomesafen and S-metolachlor. Treatments without an ALS inhibitor resulted in an FOR similar to the nontreated according to ANOVA (data not presented), therefore, those treatments were grouped (Table 4.2). Additionally, the treatments of cloransulam plus mesotrione plus S-metolachlor with and without metribuzin were grouped (Table 4.2), as metribuzin did not affect the FOR according to ANOVA (data not presented). Class comparisons via orthogonal contrasts were used to determine whether the FOR was different at 21 or 42 DAP between the no-ALS inhibitor treatments and the treatments or treatment groups where an ALS inhibitor was applied PRE.

Data collected at TIP-1 in 2018 were excluded, as a combination of poor germination and high herbicide efficacy resulted in an insufficient sample size of emerged giant ragweed plants in many treatments. Data collected at TPAC in 2018 were analyzed separately, while data collected in 2019 were combined over sites. Similar to the PRE-only experiment, all PRE applications of an ALS inhibitor at TPAC in 2018 increased the FOR at 21 DAP relative to treatments where no ALS inhibitor was applied (Table 4.5). Similarly, an increase in the FOR was also observed in 2019 at 21 DAP in treatments with cloransulam, but not in the premix treatment of chlorimuron, flumioxazin, and pyroxasulfone (Table 4.5).

The POST application of glufosinate plus fomesafen and *S*-metolachlor was made immediately after tissue was collected at 21 DAP and completely controlled all giant ragweed plants that had emerged from the PRE herbicide applications in all site-years. As a result, plants that were sampled in treated plots at the 42 DAP collection timing emerged after the POST herbicides were applied. By the time these plants emerged, they may have been exposed to less selection for ALS-R, as the concentration of the PRE-applied ALS inhibitors (cloransulam and chlorimuron) should have declined over time. Additionally, the POST application that included fomesafen had the potential to further reduce the selection for ALS-R individuals as a result of PRE-applied ALS inhibitors by adding an overlapping component of soil-residual control (Barnett et al. 2013, Baysinger and Sims 1992, Norsworthy et al. 2011b, 2012, Sarangi and Jhala 2019, Taylor et al. 2002).

After the POST herbicides were applied at TPAC in 2018, the FOR at 42 DAP in treatments of cloransulam with and without mesotrione were not different than the treatments where no ALS inhibitors were applied (P > 0.05) (Table 4.5). This indicated that the combination of multiple, effective PRE herbicides and sequential applications of products with soil-residual activity have the potential to allow for the sustainable use of ALS inhibitors in fields where ALS-R biotypes are present. However, the efficacy of this strategy may vary based on environmental conditions and the active ingredients used. In 2019, all treatments except the premix of chlorimuron, flumioxazin, and pyroxasulfone selected for an increased FOR, even after the POST herbicides were applied (Table 4.5). A lack of rainfall following the POST herbicide application at TPAC in 2019 did not allow for soil uptake of fomesafen in this site-year (Table 4.6). This may partially explain why a significant increase in the FOR was observed in treatments in 2019 where it was not evident in the year prior.

At TPAC in 2018, the FOR declined between the 21 and 42 DAP collection timings in all treatments with cloransulam, but remained similar where chlorimuron was applied (Table 4.5). This may be indicative of the greater soil half-life of chlorimuron compared with cloransulam (Table 4.4). To a lesser degree, the FOR was also lower in the no-ALS-inhibitor treatments at 42 DAP compared with 21 DAP in this site-year (Table 4.5), indicating that some of the decline in

the FOR from 21 to 42 DAP may have been a result of differential emergence of the resistant and susceptible biotypes. Changes in germination patterns due to genetic differences between populations has been documented in giant ragweed (Hovick et al. 2018). However, in the present study, this difference was not consistent between site-years as the FOR in the no-ALS-inhibitor group of treatments was similar across collection timings in 2019 (Table 4.5). The only treatment where the FOR declined over time in 2019 was cloransulam plus *S*-metolachlor plus metribuzin (Table 4.5). Regardless of site-year, the FOR was more consistent between treatments after the POST herbicides were applied, compared with the large treatment differences observed prior to the POST application (Table 4.5). This indicates that the influence of PRE herbicides on the FOR may be underestimated in some instances where sequential POST herbicides are used.

This study confirmed selection pressure from PRE-applied ALS inhibitors when as many as five additional herbicide modes of action groups were included between two separate applications (Table 4.5). No combination of herbicides that included an ALS inhibitor consistently resulted in a FOR similar to initial levels across all site-years (Tables 4.3 and 4.5). Though a slight fitness penalty has been associated with the Trp574Leu mutation of *ALS* in waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] (Wu et al. 2018), the Trp574Leu mutation in giant ragweed has not demonstrated any negative growth parameters as a result of this herbicide resistance mechanism (Marion et al. 2017). As such, the FOR is not likely to decline substantially over time, even in the absence of selection pressure (Yu and Powles 2014). The results in the present study highlight the tendency of applications of ALS inhibitors to steadily increase the FOR after the mutation has been established within a population, fortifying the importance of the integration of non-chemical management tactics such as cover crops, tillage, and crop rotation to maintain the long-term viability of this class of herbicides.

Resistance to ALS inhibitors in giant ragweed is referred to as "target-site resistance" (TSR), as the mutation that endows resistance occurs in the gene that codes for the herbicide target. Applying mixtures of multiple herbicide active ingredients is often cited as being more effective for delaying the evolution of TSR than rotating between different active ingredients over time, especially when considering ALS inhibitors (Beckie and Reboud 2009, Diggle et al. 2003, Gaines et al. 2020, Powles et al. 1997). However, overreliance on herbicide mixtures may instead select for generalist, "non-target-site resistance" (NTSR) mechanisms such as enhanced metabolic detoxification. This has been demonstrated by both mathematical models and experimental research studies across multiple agricultural and medical disciplines (Comont et al. 2020, Fardisi et al. 2019, Lagator et al. 2013, Tamma et al. 2012, Vestergaard et al. 2016). Metabolism-based NTSR mechanisms present an additional challenge to weed management strategies that rely heavily on herbicides, as they are often unpredictable and may act on herbicides from several unrelated chemical classes (Gaines et al. 2020). Theoretically, plants endowed with generalist resistance mechanisms could exhibit resistance to a new herbicide active ingredient, herbicide chemical family, or herbicide mode of action group even before selection pressure was exerted by the novel chemistry.

At present, there is no published evidence of NTSR to ALS inhibitors in giant ragweed, though the mechanisms of resistance to both glyphosate and PPO inhibitors have yet to be elucidated at the molecular level. Our study highlights that herbicide mixtures and sequential applications can be effective for reducing the influence of an ALS inhibitor on the FOR in fields infested with ALS-R giant ragweed biotypes. However, no combination of herbicides eliminated the selection for an increased FOR due to a PRE-applied ALS inhibitor in every site-year, and relying solely on these chemical-based weed management strategies also has the potential to select for more generalist resistance mechanisms in giant ragweed, and in other weed species. Ultimately, growers should strive to completely eliminate weed seed production using both chemical and nonchemical tactics to prevent the initial establishment of resistant weed biotypes, and to preserve the efficacy of herbicides in the future.

Table 4.1. Sources of herbicides used in field experiments.

Common name Trade name		Manufacturer	Manufacturer location	Manufacturer website	
Mesotrione	Callisto®	Syngenta Crop Protection	Greensboro, NC	www.syngenta.com	
Cloransulam Firstrate [®]		Corteva Agriscience	Indianapolis, IN	www.corteva.com	
Metribuzin Tricor [®] DF		UPL NA Inc.	King of Prussia, PA	www.upl-ltd.com	
S-metolachlor	Dual Magnum [®]	Syngenta Crop Protection	Greensboro, NC	www.syngenta.com	
Sulfentrazone +	Authority [®] First DF	FMC Corp.	Philadelphia, PA	www.fmc.com	
cloransulam					
Chlorimuron +	Fierce [®] XLT	Valent USA Corp.	Walnut Creek, CA	www.valent.com	
flumioxazin +					
pyroxasulfone					
Glufosinate	Liberty [®] 280 SL	BASF Corp.	Research Triangle Park, NC	www.basf.com	
S-metolachlor +	Prefix®	Syngenta Crop Protection	Greensboro, NC	www.syngenta.com	
fomesafen					

Table 4.2. Preemergence herbicide treatments utilized in the sequential experiment and their grouping for orthogonal contrasts. a

Treatment	Contrast grouping			
Nontreated	No ALS inhibitor			
Mesotrione + S-metolachlor				
Mesotrione + metribuzin + S-metolachlor				
Cloransulam + metribuzin + S-metolachlor	Cloransulam without mesotrione			
Cloransulam + mesotrione + S-metolachlor	Cloransulam with mesotrione			
Cloransulam + mesotrione + metribuzin + S-metolachlor				
Sulfentrazone + cloransulam	Commercial standard 1			
Chlorimuron + flumioxazin + pyroxasulfone	Commercial standard 2			
^a Treatment means within a group did not differ according to ANOVA (P>0.05).				

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		Combined site-
Herbicide treatment(s)	TPAC 2018	years ^b
	——FOF	R (%) ——
Nontreated (baseline)	15	70
Cloransulam	97	95
Cloransulam + metribuzin	87	94
Cloransulam + S-metolachlor	98	93
Cloransulam + metribuzin + S-metolachlor	93	92
Mesotrione + cloransulam	54	88
Mesotrione + cloransulam + metribuzin	47	76
Mesotrione + cloransulam + S-metolachlor	48	88
Mesotrione + cloransulam + metribuzin + S-metolachlor	41	86
	ns ^c	ns
Cloransulam without mesotrione	94	94
Cloransulam with mesotrione	48	85
	***	****
Orthogonal contrasts: ^d		
C1- cloransulam with mesotrione vs nontreated	48 vs 15 ***	82 vs 70 **
C2- cloransulam with mesotrione vs cloransulam and sulfentrazone	48 vs 94 ****	82 vs 97 **
C3- cloransulam with mesotrione vs chlorimuron.	48 vs 59	82 vs 83
flumioxazin and pyroxasulfone	ns	ns

Table 4.3. Frequency of giant ragweed biotypes resistant to ALS inhibitors in the PRE-only experiment that had emerged by 21 DAP.^a

^a Abbreviations: ALS, acetolactate synthase; PRE, preemergence; DAP, days after planting; FOR, frequency of resistance.

^b Combined site-years included TIP-1 2018, TIP-1 2019, and TPAC 2019.

^c The interaction between mesotrione and the other herbicides was not significant (P>0.05), so means are also presented for pooled treatments with and without mesotrione.

^d Orthogonal contrasts: C1, cloransulam with mesotrione vs nontreated; C2, cloransulam with mesotrione vs cloransulam and sulfentrazone premix; C3, cloransulam with mesotrione vs chlorimuron, flumioxazin and pyroxasulfone premix. Significance is designated as **=P<0.01, ***=P<0.001, ***=P<0.001, ns = not significant (P>0.05).

Table 4.4. Soil half-lives of residual herbicides applied in field experiments.

Half-life ^a	
16	
40	
9	
11.9 to 17.5	
121 to 302	
	Half-life ^a 16 40 9 11.9 to 17.5 121 to 302

^a Average half-life in days as described by Shaner (2014).

			Change from
Year/ PRE herbicide treatment groups ^b	21 DAP ^c	42 DAP	21 to 42 DAP^{d}
2018	— FOR	. (%) ——	ΔFOR
No ALS inhibitor	24	10	-14*
Cloransulam without mesotrione	97*	31	-66*
Cloransulam with mesotrione	55*	25	-30*
Cloransulam + sulfentrazone	94*	40*	-54*
Chlorimuron + flumioxazin + pyroxasulfone	52*	48*	-4
2019			
No ALS inhibitor	68	67	-1
Cloransulam without mesotrione	95*	81*	-14*
Cloransulam with mesotrione	81*	77*	-4
Cloransulam + sulfentrazone	91*	81*	-10
Chlorimuron + flumioxazin + pyroxasulfone	75	71	-4

Table 4.5. Frequency of resistance to ALS inhibitors in giant ragweed at 21 and 42 DAP, and the change in the FOR from a postemergence herbicide application in 2018 and 2019. ^a

^a The postemergence application consisted of glufosinate plus fomesafen and *S*-metolachlor applied immediately after sampling at 21 DAP. Abbreviations: ALS, acetolactate synthase; FOR, frequency of resistance; Δ FOR, change in the frequency of resistance; DAP, days after planting ^b Treatments were grouped as outlined in Table 4.2. Data in 2018 represent only the TPAC site, while data in 2019 represent both the TPAC and TIP-1 sites.

^c Asterisks (*) in the 21 and 42 DAP columns signify treatments with a FOR different than the group of no-ALS inhibitor treatments based on orthogonal contrasts (P<0.05).

^d Asterisks represent a significant difference in the FOR between 21 and 42 DAP based on a paired t-test (P<0.05).

Table 4.6. Weekly rainfall accumulation and average temperature at two Indiana field sites. ^a

	2018				2019			
WAP	TPAC	TIP-1	TPAC	TIP-1	 TPAC	TIP-1	TPAC	TIP-1
	cm C		C —	cm		——C——		
1	4.6	4.8	26	24	2.1	3.3	21	21
2	1.3	2	21	21	0.8	1.7	19	18
3	6.4	5.2	23	22	6.9	7.6	21	20
4	1.5	3.1	26	25	0	0	25	25
5	7.5	3	22	21	0	3.3	26	25
6	0.1	2.6	26	26	0	0.4	25	25

^a Data for TPAC and TIP-1 were generated by weather stations at the Throckmorton Purdue Agricultural Center and the Purdue University Airport (KLAF), respectively. Abbreviations: WAP, weeks after planting

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CHAPTER 5. LINKAGE OF GENES ENCODING SELF-INCOMPATIBILITY AND ACETOLACTATE SYNTHASE IMPACTS THE INHERITANCE OF HERBICIDE RESISTANCE IN GIANT RAGWEED

5.1 Abstract

A Trp574Leu mutation in the nuclear gene acetolactate synthase (ALS) endows giant ragweed (Ambrosia trifida L.) with resistance to herbicides that inhibit the resulting acetolactate synthase enzyme. Mendelian inheritance may not be universally applicable to this resistance mechanism, as far fewer homozygous-mutant plants were found growing in a commercial soybean field (TIP-1) than would be expected under the Hardy-Weinberg Equilibrium. The present study investigated whether giant ragweed possesses self-incompatibility (SI), and whether linkage drag between ALS and SI genes could be responsible for the unexpected distribution of ALS alleles. Up to a 98% outcrossing rate of the Trp574Leu mutation and greater pollen retention, pollen tube growth, and seed set in cross-pollinated plants compared with self-pollinated plants were determined in greenhouse experiments. These results demonstrated that giant ragweed possesses SI, though the precise mechanism may integrate elements of both sporophytic and late-acting SI. Non-Mendelian inheritance of the Trp574Leu mutation was observed in F1 progeny groups generated by crossing giant ragweed plants from three different parental populations. These results suggest that, in many cases, the mutant ALS allele may be linked with an SI allele common to many of the resistant plants because of shared ancestry, prompting an SI response and preventing the formation of homozygous-mutant progeny. In some cases, production of wild-type, susceptible progeny was similarly reduced. Furthermore, homozygous-mutant F1 seeds were produced from some crosses at frequencies that aligned with Mendelian inheritance, indicating a recombination event between ALS and SI genes in the parental linage, or perhaps a lineage that included multiple,

unique resistant ancestors. Across all experiments, the proportion of resistant progeny was 33% greater in crosses between giant ragweed plants originating from the TIP-1 field, compared with expectations under Mendelian predictions. A heightened proportion of resistant progeny could facilitate a much faster distribution of herbicide resistance throughout a field, underscoring the importance of sound herbicide resistance management strategies.

5.2 Introduction

Management of herbicide-resistant weeds is critical to the sustainability of many agricultural production systems (Oerke 2006, Owen 2016). Herbicides that inhibit acetolactate synthase (ALS) are widely used throughout the world to protect the yields of many different crops from weedy competition, though more weed species have developed resistance to ALS inhibitors than any other herbicide class (Bellinder et al. 1994, Garcia et al. 2017, Heap 2022). An application of the ALS-inhibiting herbicide cloransulam-methyl is among the most effective measures for preemergence control of giant ragweed (Ambrosia trifida L.) in soybean production (Franey and Hart 1999, Loux et al. 2022, Vink et al. 2012c). However, giant ragweed biotypes resistant to several ALS inhibitors including cloransulam can now be found throughout at least seven U.S. states and Ontario, Canada (Heap 2022, Patzoldt and Tranel 2002). Resistance to ALS inhibitors in giant ragweed is conferred by a missense mutation (Trp574Leu) in the ALS gene that greatly reduces the binding affinity of the resulting ALS enzyme for the herbicide molecule (Marion et al. 2017, Patzoldt and Tranel 2002). Giant ragweed is diploid (2n = 24) (Bassett and Crompton 1982, Payne et al. 1964), and a single mutant copy of ALS endows resistance to cloransulam doses at least 100-fold greater than those used in soybean production (Marion et al. 2017). As ALS is a nuclear gene, and because the Trp574Leu mutation does not incur a fitness penalty, resistance to ALS inhibitors in giant ragweed was assumed to follow Mendelian

inheritance (Ghanizadeh et al. 2019, Marion et al. 2017, Patzoldt and Tranel 2002, Tranel et al. 2020, Tranel and Wright 2002). Therefore, once established, this resistance-conferring mutant allele should remain in the gene pool at a frequency in concordance with the Hardy-Weinberg equilibrium (HWE) for many subsequent generations, even in the absence of herbicide selection.

The frequency of Trp574Leu alleles was examined in giant ragweed plants growing in two Indiana soybean fields in 2018 and 2019, with a summary presented in Table 5.1. The number of homozygous-mutant individuals at the TIP-1 field was far lower than expected and was in violation of the HWE, given the persistence of a high frequency of heterozygous plants identified in multiple surveys dating back to 2014 (Harre unpublished data, Table 5.1). One of the fundamental assumptions of the HWE is random mating in a population (Hardy 1908, Weinberg 1908). In nearly half of all flowering plant species, the intermating compatibility of different individuals is driven by a self-incompatibility (SI) system (de Nettancourt 1977). Self-incompatibility often serves as a post-pollination, prezygotic mechanism to reduce or eliminate self-pollination through a complex system of molecular crosstalk between the paternal gametophyte (pollen) and the maternal sporophytic tissue in the pistil (Hiscock and Allen 2008, Igic et al. 2008, de Nettancourt 1977, De Nettancourt 2001). The cross-compatibility of related plants can also be reduced when the same SI phenotype is expressed by both mating individuals (Wright 1939). SI interactions are determined by the compatibility of unique SI alleles derived from several genes that are expressed in the pollen and pistil, and these genes are contained within a single locus (S) in most plant families (Franklin-Tong and Franklin 2003, Hiscock and Allen 2008, De Nettancourt 2001, Takayama and Isogai 2005). A herbicide selection event in the past may have removed a significant portion of the giant ragweed plants at the TIP-1 field, thereby restricting mate availability due to a lack of SI allele diversity and disrupting the expected prevalence of the
Trp574Leu mutation (Figure 5.1). Interestingly, similar observation of giant ragweed plants in a different field revealed homozygous mutants at approximately expected frequencies (Table 5.1).

Giant ragweed is generally regarded as a wind-pollinated outcrossing species (Abul-Fatih and Bazzaz 1979, Bassett and Crompton 1982), though no mechanism of SI has been proposed in peer-reviewed literature. Giant ragweed is monecious with imperfect flowers, and there is considerable overlap in the period of anthesis between male and female reproductive structures (Bassett and Crompton 1982). Densely clustered distal racemiform branches comprise each male inflorescence, while pistillate flowers are contained within gynoecium at leaf axils and bracts (Curtis and Lersten 1995) (Figure 5.2). Anthers are positioned just above the pistils, resulting in heavy deposition of self-pollen on female flowers (Figure 5.2 b). Several studies have considered giant ragweed to be at least partially self-compatible, though reduced seed production and seedling vigor was noted in some instances of self-pollination (Bassett and Crompton 1982, Brabham et al. 2011, Ganie and Jhala 2017, Vincent and Cappadocia 1987, Wrensch and Paddock 1976). However, none of these studies were designed to examine SI.

Common ragweed (*Ambrosia artemisiifolia* L.), of the same genus as giant ragweed, has been shown to possess SI (Friedman and Barrett 2008). Though self-pollinated common ragweed plants are still able to set seed, total seed production is reduced compared with cross-pollinated plants (Friedman and Barrett 2008). This phenomenon of "leaky" SI is often referred to as partial or pseudo self-compatibility (PSC) (Hiscock 2000a) or pseudo self-incompatibility (PSI) (Ferrer and Good-Avila 2007). A system of PSC presents distinct advantages to colonizing species such as common and giant ragweed (Chauvel et al. 2021, Montagnani et al. 2017), as species with PSC may obtain the heterotic benefits associated with outcrossing while retaining the ability to set a limited amount of seed if a source of cross-pollen is not available (Hiscock 2000b). The evolution of herbicide resistance traits would be favored in species with PSC compared with strict SI. Mutations that confer resistance to herbicides are thought to occur naturally at very low frequencies prior to selection (Casale et al. 2019, Jasieniuk et al. 1996). In species incapable of self-pollination, mutant plants that survive the initial selecting herbicide application would not be able to pass on their genetic information to subsequent generations if all other suitable mates were controlled by the herbicide. By retaining the capacity for limited self-pollination, species with PSC will pass on resistance-conferring mutations in the event that only one plant survives. Continued selection for the phenotypically dominant Trp574Leu mutation in giant ragweed with repeated applications of ALS inhibitors has the potential to rapidly shift the population structure to the resistant biotype (Beckie and Reboud 2009, Norsworthy et al. 2012). This selection process would undoubtably lead to numerous resistant plants throughout the field, though many or all of these individuals may ultimately share a common resistant ancestor (Figure 5.1).

If giant ragweed does possess SI, it is possible that linkage drag between the *ALS* gene and a gene or genes responsible for SI could lead to the distorted segregation of *ALS* alleles observed at the TIP-1 field site by restricting the compatibility between resistant individuals due to their genetic similarity. There exists substantial evidence of suppressed recombination in and around the region of the *S*-locus in several plant species (Charlesworth 2002, Kamau et al. 2007, Schierup and Vekemans 2008, Sims and Robbins 2009, Takuno et al. 2007). Close chromosomal proximity of *ALS* and SI genes, along with locally repressed recombination, may have resulted in the mutant, resistance-conferring *ALS* alleles to be linked with a common SI allele in giant ragweed individuals sharing a common ancestor. This proposed linkage of *ALS* and SI genes could serve to repress the formation of giant ragweed seeds that were homozygous for the Trp574Leu mutation, as pollen and pistils carrying the mutation would trigger an SI response ending in pollination failure (Figure

5.1). To investigate this hypothesis, this study first sought to establish whether giant ragweed possesses a functional SI system. Lacking the necessary genomic data in this species to directly investigate linkage between *ALS* and SI genes, a series of crosses was conducted to test for indirect evidence of such a linkage, and to explore the implications of this phenomenon on the management and spread of herbicide resistance.

5.3 Materials and Methods

5.3.1 Plant Materials

Three distinct parental populations were derived from seeds harvested from giant ragweed plants growing naturally in agricultural fields in Indiana. A composite sampling method was used at each of the following locations: Ti18 (40.26° N, 87.04° W), Tp18 (40.17° N, 86.54° W), and Ba14 (39.10° N, 85.51° W). A fourth population (Rg21) was generated in an isolated greenhouse cross between four giant ragweed plants from the Tp18 that were homozygous for the Trp574Leu mutation of *ALS* using methods described herein. All seeds were collected from mature, desiccated plants when their involucres had turned from green to brown (Figure 5.2), and both hollow and malformed involucres were discarded.

Giant ragweed seeds are inherently dormant due to physical barriers and chemical inhibition (Ballard et al. 1996, Davis 1930, Schutte et al. 2012). Dormancy was alleviated by stratifying seeds in moist, sandy soil at 4 C for at least 2 mo, or by fully excising each seed from the pericarp and involucre hull and soaking for 48 hr in water with aeration (Harre et al. 2019, Page and Nurse 2015, Westhoven et al. 2008). Giant ragweed seeds were then planted 1.5 cm deep in pots containing a 2:1 mixture of commercial potting media (Sun Gro[®] seedling mix, Sun Gro Horticulture, Bellevue, WA) and sand. All plants were grown in a greenhouse, fertilized

biweekly (24-8-16 Miracle-Gro Water-Soluble All-Purpose Plant Food, Scotts Miracle-Gro Products, Marysville, OH), and watered as needed. Greenhouses were maintained at 23 to 29 C with natural lighting supplemented by a 16-hr photoperiod, which was supplied by high-pressure sodium bulbs that delivered 1,100 μ mol m⁻² s⁻¹ photon flux density. Pots were drenched with a 0.025% v/v solution of spirotetramat (Kontos[®], Bayer CropScience, St. Louis, MO) when insect feeding was observed, and all plant foliage was sprayed with a solution of pyraclostrobin (Headline[®], BASF Corporation, Research Triangle Park, NC) at 3% v/v plus a non-ionic surfactant (Activator 90, Loveland Products, Inc., Greeley, CO) at 0.25% v/v to control *Pseudoperonospora* spp. when present.

Giant ragweed is a short-day plant (Bassett and Crompton 1982, Mann 1942), and reproductive structures are often visible by late-August when growing in its native range of North America (Goplen et al. 2016). In these greenhouse experiments, after a period of vegetative growth lasting 6 to 8 wk under a 16-hr photoperiod, uniform floral development was encouraged by reducing the photoperiod to 12 hr. This was accomplished by transferring the plants to a growth chamber that supplied 700 to 1000 μ mol m⁻² s⁻¹ photon flux density with either florescent or LED light bulbs. Reproductive growth in some plants was initiated in the greenhouse, though this was only possible when the ambient photoperiod was 12 hr or less and the duration of supplemental light was adjusted accordingly. After 18 d under the 12-hr photoperiod, a 16-hr photoperiod was reinstated without affecting the progression of reproduction.

5.3.2 Classification of ALS Alleles

In giant ragweed, resistance to ALS-inhibiting herbicides is conferred by a Trp574Leu mutation in the *ALS* gene (Harre et al. 2017, Marion et al. 2017, Patzoldt and Tranel 2002). In these experiments, the Trp574Leu mutation was identified in both plants and seeds of giant

ragweed. Genomic DNA was extracted from leaf tissue (15 mg) using a modified cetyltrimethylammonium bromide (CTAB) protocol (Saghai-Maroof et al. 1984). Prior to extracting DNA from the seeds, each seed was cut in half lengthwise as to retain portions of the embryo and endosperm in both halves. These halved seeds were placed in 2-ml centrifuge tubes and soaked in 1 ml of deionized water for 12 hr. A vortex was used to separate the seed from the seed coat, pericarp, and involucre hull, then each half-seed was transferred to a 1.5-ml centrifuge tube or a 96-well polypropylene plate (DOT Scientific Inc., Burton, MI) for extraction. A purification step with phenol, chloroform, and isoamyl alcohol (25:24:1) was added to the CTAB protocol when extracting DNA from seeds to increase quality (Kamiya and Kiguchi 2003).

Primers and TaqMan[®] probes were synthesized by ABI (Applied Biosystems, Waltham, MA) and designed to flank position 574 of *ALS* to distinguish between mutant and wild-type alleles. Forward and reverse primers were 5'-ACTTAACAATCAGCATTTGGGTATGGT-3' and 5'-CTAAGTAGGTATGAGCCCGATTCG -3', respectively. Probes used to overlap the mutation site were 5'-CCGATCCTC<u>CCA</u>CTGAA-3' [FAM fluorophore (wild-type allele)] and 5'-CCGATCCTC<u>CAA</u>CTGAA-3' [VIC fluorophore (mutant allele)]. For each sample, a 10-µl reaction was prepared with 4.8 µl of PCR-grade water, 2 µl of GoTaq Flexi buffer, 1.2 µl of 25 mM MgCl2, 0.4 µl of 10 mM dNTP, 0.5 µl of 20X primers and TaqMan[®] probes, 0.1 µl of GoTaq Flexi polymerase (5 U µl-1), and 1 µl of genomic DNA. A CFX384 RT-PCR system (Bio-Rad Laboratories, Hercules, CA) and a 2-step PCR protocol were used to amplify each reaction under the following cycle conditions: 2 min at 95 C, then 39 cycles of 95 C for 15 s and 60 C for 1 min followed by a plate read after each cycle. Samples were classified as either wild-type, heterozygous, or homozygous-mutant using Bio-Rad CFX manager software based on the relative florescence of each fluorophore.

The accuracy of allelic discrimination using the qPCR assay and DNA extracted from giant ragweed seeds was confirmed by planting ten seeds from each of six F1 populations in the greenhouse. The genotype of each emerged plant was ascertained using a non-destructive DNA extraction assay from leaf tissue, and segregation ratios were compared between the two extraction methods within each F1 population. Additionally, all emerged F1 plants were sprayed with cloransulam (Firstrate[®], Corteva Agriscience, Indianapolis, IN) at a rate of 42 g ai ha⁻¹ using a track-mounted research spray cabinet (Generation III Research Sprayer, DeVries Manufacturing, Hollandale MN) at the 4-leaf growth stage. Ammonium sulfate (N-PAK[®] AMS Liquid, Winfield Solutions, LLC, St. Paul, MN) and a non-ionic surfactant (Activator 90) were included in the spray mix at 10 g/l and 0.25% v/v, respectively. Plants were classified as either resistant or susceptible 21 d after application using visual observation and a dry-weight biomass measurement, and the continuity between genotypic and phenotypic characterization was examined. Additionally, in a number of samples, the allelic classification via qPCR was confirmed through sequencing with the Sanger method (Sanger et al. 1977) without discrepancy (Nie, unpublished data).

5.3.3 Self-Incompatibility

Three experiments were conducted to compare self- and cross-pollination in giant ragweed. As giant ragweed is monecious, each "female" plant utilized in a cross was created through emasculation (Brabham 2011). Anthers were visible before releasing mature pollen, so they could be removed prior to the deposition of self-pollen onto the pistils. A passive-pollination technique was used in the first experiment, in which plants were gently shaken each day during the 2 wk following the initiation of anthesis to encourage the natural release of pollen from the anthers. In this experiment, individual self- or cross-pollinations were isolated in separate greenhouses, and all plants originated from the Ti18 population. Seed production per plant was compared using analysis of variance (ANOVA) and an independent Student's t-test ($\alpha = 0.05$).

Three giant ragweed populations (Ti18, Tp18, and Ba14) were used in an experiment evaluating pollen retention, pollen tube growth, and seed set in a more diverse group of plants. Emasculated branches on each female plant were bagged to prevent passive pollination, and both self- and cross-pollinations were conducted by brushing anthers cut from paternal plants against the styles of emasculated branches. This manual-pollination technique allowed for many unique crosses to be conducted in the same greenhouse, with minimal cross-contamination. Female plants from each of the three populations were pollinated by paternal plants from each population on separate branches, with one branch left unpollinated. Three branches on each paternal plant were also emasculated and covered, with self-pollinations conducted using the same technique. This pollination scheme was replicated six times, resulting in 18 self-pollinations and 54 crosspollinations representing three distinct populations.

After 48 hr, between six and nine styles from each unique pollination were dissected and fixed in 3:1 solution of ethanol and acetic acid for at least 24 hr, washed twice using deionized water, and softened in a 1M sodium hydroxide solution for 3 hr. Following two additional washes with deionized water, pistils were stained for 24 hr with 0.1% w/v methyl blue dissolved in 0.1M potassium phosphate (Liu et al. 2012). Styles were then transferred to a microscope slide and observed using a Nikon Eclipse Ti2-E microscope (Nikon[®] Corp., Melville, NY). The number of pollen grains adhered to each style and the number of pollen tubes that had reached the style surface were quantified, in addition to the number of seeds produced per node. Analysis of variance was conducted with the pollination method (self, cross, or unpollinated) as a main effect, while maternal population and replicate were considered random effects. Data for each pollination

method were combined over populations when a significant interaction was absent. Orthogonal contrasts were used to compare the mean of each response variable between pollination methods. All data were square-root-transformed prior to analysis to improve the normality of the residuals, with back-transformed means presented.

In the third experiment, a mixture of both self- and cross-pollen was used to simultaneously pollinate the same giant ragweed flowers. The Trp574Leu mutation at position 574 of ALS was used as a genetic marker to determine the paternal origin of each seed produced as a result of these pollinations (Goodwillie et al. 2004, Vogler and Stephenson 2001). For brevity, individual giant ragweed plants and seeds are referred to hereafter as wild-type, heterozygous, or homozygousmutant as determined by their genotype with respect to the Trp754Leu mutation of ALS. Parental plants in this experiment included four homozygous wild-type individuals from the Tp18 population and four homozygous-mutant individuals from the Rg21 population. Seven branches on each plant were emasculated and bagged. Pollen was collected from the non-emasculated branches of reciprocal pairs of homozygous wild-type and mutant plants and mixed together in a 1:1 ratio $(\pm 4\%)$ by weight. This pollen mixture was then applied to the styles of three emasculated branches belonging to each of the parental plants using a nylon paintbrush. Thus, each parent plant was both a pollen recipient and a pollen donor. Three other emasculated branches on each plant were pollinated with self-pollen only. A final emasculated branch on each plant was left unpollinated.

After mature seeds were harvested, the genotype of at least 15 seeds from each F1 population was established using DNA extraction and qPCR as described previously (n = 138 total seeds genotyped). Heterozygous seeds indicated that the ovule had been fertilized by cross-pollen, while homozygous seeds (either wild-type or mutant) were the result of successful self-pollination.

A Chi-square test was used to evaluate whether progeny groups from each reciprocal pair deviated from the null hypothesis that the ratio of homozygous (selfed) to heterozygous (outcrossed) progeny would be 1:1. Total seed production per node was also compared between pollination methods, with means separated using the GLIMMIX procedure in SAS[®] 9.4 (SAS Institute Inc., Cary, NC) and Tukey-Kramer's adjustment for multiple comparisons.

5.3.4 Inheritance of Resistance to ALS Inhibitors

Three additional experiments were conducted to examine the inheritance of a Trp574Leu mutation at position 574 of *ALS* in the F1 and S1 progeny of giant ragweed. The genotype of each parental plant used in these experiments was confirmed at least twice using a qPCR assay designed by Harre et al. (2017), and this same assay was also used to classify the genotype of the seeds produced as a result of each self- or cross-pollination. Chi-square tests were performed to assess the segregation of alleles in each F1 or S1 population, with the null hypothesis that the Trp574Leu mutation would follow Mendelian inheritance.

The first experiment was conducted using open, passive pollination that occurred as pollen was released over time, with four crosses and three self-pollinations isolated in separate greenhouses. To confirm that the mutant allele was carried in both parental gametes, a set of reciprocal crosses was conducted between wild-type and heterozygous plants from the Ti18 population. To investigate whether homozygous-mutant F1 seeds would be produced under controlled greenhouse conditions, heterozygous female and male plants from the Ti18 population were crossed. Additionally, heterozygous, non-emasculated plants from the Ti18 and Tp18 populations were crossed with the same objective. Finally, one wild-type and two heterozygous plants from the Ti18 population from the Ti18 population from the Ti18 population for 100 seeds in each F1 or S1 population if at least 200 seeds were

produced by the maternal parent. If fewer than 200 seeds were produced, then half of the total number of seeds were genotyped.

In the remaining experiments, individual branches were emasculated, and bags were used to cover female flowers before and after pollination. This technique prevented passive pollination until it could be conducted by hand with pollen from the desired male plant or plants. In several species with SI, self-compatibility has been restored using techniques such as bud pollination (Hiratsuka et al. 1985, Hiscock and Dickinson 1993) and a saline treatment (Hiscock 2000a). If a gene or genetic involved with SI in giant ragweed was linked with the *ALS* gene, overcoming SI and restoring self-compatibility could also serve to restore the normal inheritance of the Trp574Leu mutation.

In an attempt to use the bud pollination technique, immature pistils on four heterozygous female plants were cross-pollinated with mature pollen from heterozygous donors. Pistils were classified as "immature" when the visible portion of the style was less than 1 mm in length. Cross-pollinations were also conducted between the same pairs of parent plants after anthesis was reached in the female plants and their pistils were mature. Pistils from four additional heterozygous giant ragweed plants were treated with a solution of sodium chloride (1 or 2.5% w/v) and Tween-20 (0.1% v/v) (MilliporeSigma, Burlington, MA) 30 to 60 min prior to pollination. These pistils were then pollinated with pollen from a heterozygous donor, and both self- and cross-pollinations were conducted. These pollinations were also conducted between the same pairs of plants utilizing pistils that were not treated with the saline solution. All parental plants utilized in this experiment were from the Ti18 population, and all pollinations were made using a nylon paintbrush. The segregation of the Trp574Leu mutation of *ALS* in each of the resulting F1 populations was compared between traditional pollinations and those pollinations designed to overcome SI.

The third experiment investigated the inheritance of the Trp574Leu mutation of *ALS* in the Ti18, Tp18, and Ba14 populations. The diversity of SI alleles between parents from different populations would likely be greater compared with crosses between plants from the same population, and a greater diversity in SI alleles would decrease the chance that both parents would express incompatible SI phenotypes (Busch and Schoen 2008). Therefore, it was presumed that due to genetic linkage with SI, Mendelian inheritance of the Trp574Leu mutation would occur more frequently in crosses where each parent was from a different population.

Heterozygous males from each population were used to pollinate wild-type and heterozygous females in an adaptation of the North Carolina II (NC II) factorial mating design (Comstock and Robinson 1952). Every male was crossed with a female from each of the three populations, and similarly, each female was pollinated by three males from different populations. This was performed by cutting multiple mature anthers from each male plant and brushing them against the pistils on isolated, emasculated branches belong to the female plants. Thus, three halfsibling progeny groups originated from each parental plant, representing both internal (males and females from the same population) and external (males and females from different populations) crosses. This technique was replicated three times, yielding 54 unique parental combinations. Additionally, three homozygous-mutant males from the Tp18 population were used to pollinate heterozygous females from each population in nine added crosses. Twenty seeds from each F1 population were genotyped using the qPCR assay as described previously. The binary outcome of either Mendelian or non-Mendelian inheritance according to Chi-square analysis χ^2 (P < 0.05) was compared between populations generated as a result of internal and external crosses using PROC LOGISTIC in SAS®. Additionally, for each set of parental genotypes, a one-sample t-test was

used to compare the observed number of mutant alleles and resistant seeds with the expected number under Mendelian inheritance.

5.4 Results and Discussion

5.4.1 Growth and Reproduction

Under a 16-hr photoperiod, giant ragweed plants grown in the greenhouse reached heights of up to 2 m by 8 wk after planting. Vertical growth ceased around 10 d after the photoperiod was reduced to 12 hr, though branching continued for an additional 2 wk. Anthers were the first reproductive structures to become visible, the first of which emerged from the apical meristem of the primary shoot approximately 10 to 12 d after the photoperiod was decreased. Mature pollen was released from the anthers 5 to 10 d after they emerged (Figure 5.2 b). Pistils were visible 3 d after the anthers, and their styles and were receptive to pollen prior to anthesis in the male inflorescences (Bassett and Crompton 1982). After the first flowers were observed at the primary shoot apex, floral development continued over the next 10 d, steadily progressing downward toward the branches originating closest to the base of the plant. Anthers were formed at the apex of each shoot and at most nodes, while pistils developed at the axils of each node and occasionally at the involucres of the male inflorescences. Each pistil contained a single branched style leading to a common embryo (Figure 5.2 a). On average, there were 12 pistils at each node, and the highest density of pistils was found within gynoecium located closest to the apical meristems (data not presented). The development of reproductive tissues in the giant ragweed plants utilized in these greenhouse experiments was similar to what has been described for common ragweed (Essl et al. 2015).

Seeds were formed 7 to 10 d after pollination and turned from green to brown as they matured over the next 3 to 4 wk (Figure 5.2). Collectively, the time between generations was approximately 15 wk. Averaged across all experiments, individual giant ragweed plants produced 600 seeds when they were allowed to passively cross-pollinate over time (n = 22 plants). Seed production was somewhat lower than what has been reported for giant ragweed plants growing in agricultural fields, which typically produce 1,000 to 2000 seeds (Brabham et al. 2011, Goplen et al. 2016). On average, six seeds were formed per node when cross-pollinations were conducted by hand, which was half the approximate number of pistils at each node (data not presented). Seed size appeared to be controlled almost exclusively by the maternal parent (data not presented).

5.4.2 Self-Incompatibility

Though a wide array of SI mechanisms can be found throughout the angiosperm clades, many of them lead to a similar downstream effect: seed production is reduced or eliminated in self-pollinated plants (De Nettancourt 2001). One metric used in these experiments to investigate whether giant ragweed possesses SI was seed production, which was compared between self- and cross-pollinated plants grown in a greenhouse. Using the passive-pollination technique in the first experiment, cross-pollinated plants (n = 3 plants) produced 431 ± 62 (mean \pm SE) seeds per plant. This was greater than self-pollinated plants (n = 4 plants), which produced 109 ± 38 seeds (t_5 = 4.72, P = 0.005). However, evidence of reduced seed set in self-pollinated plants was insufficient to conclude giant ragweed possesses SI, as other phenomena such as inbreeding depression can similarly affect the reproductive success of selfed individuals (Charlesworth 1989).

The SI response to incompatible pollen typically occurs at the style surface in species from the Asteraceae family such as giant ragweed (Ferrer and Good-Avila 2007, De Nettancourt 2001). Incompatible pollen is often rejected as it germinates on the style, or shortly after pollen tube penetration. Therefore, in addition to seed production, pollen retention and pollen tube growth was compared between pollination methods (cross, self, or unpollinated) utilizing giant ragweed plants from three different populations. This experiment was conducted by hand-pollinating emasculated branches, allowing for many more unique pollinations to be conducted compared with the passive-pollination strategy used in the previous experiment.

Data were combined across populations, as none of the response variables were affected by the population of the maternal plant, and the interaction between the three giant ragweed populations and pollination method was not significant (P > 0.05). The number of pollen grains adhered to each style, the number of pollen tubes that had reached each style, and the number of seeds formed per node were 72 to 83% lower in self-pollinated flowers than in cross-pollinated flowers (Table 5.2). Some plants had a much stronger SI response than others, demonstrated by the observation of as few as one and as many as 157 pollen grains on self-pollinated styles. This may be attributed to the genetic variability inherent to these mixed populations, with different plants at various points on a spectrum between SI and PSC (Ferrer and Good-Avila 2007, Igic et al. 2008). The number of pollen tubes per style and seeds produced per node was similar between self-pollinated flowers and unpollinated flowers (Table 5.2). This was likely caused by the occasional emergence of additional anthers on the "unpollinated" branches after the initial emasculation.

These results confirm SI for giant ragweed, though the magnitude of the SI response was somewhat weaker than what has been observed in other plants from this family (Friedman and Barrett 2008, Hiscock 2000a). In addition to a mechanism that results in initial pollen rejection at the style surface, giant ragweed may also possess a late-acting form of SI, wherein some degree of selfing is permitted later in anthesis (Goodwillie and Weber 2018). Furthermore, the growth of some self-pollen tubes may be slowed by the SI system without being stopped entirely, which would reduce their "competitiveness" compared with cross-pollen tubes (Mcclure et al. 2000, Rangappa Thimmaiah et al. 2018). This PSC-type response would provide a distinct advantage to a colonizing species such as giant ragweed, as isolated plants would still be able to set a reduced number of seeds (Goodwillie and Weber 2018).

In addition to seed production, the next experiment investigated the rate of outcrossing when both self- and cross-pollen were equally available and delivered simultaneously to each pistil by utilizing a genetic paternity marker (Trp574Leu of *ALS*). Comparable techniques have been used to investigate SI in *Campanula rapunculoides* (Vogler and Stephenson 2001) and *Leptosiphon jepsonii* (Goodwillie et al. 2004). Similar to the other experiments, seed production per node was greater in flowers that were pollinated with the mixture of self- and cross-pollen (4.9 \pm 0.6) than those flowers that only received self-pollen (2.1 \pm 0.4) ($t_{14} = 5.41$, P = 0.0003). Genotypic results were analogous between reciprocal crosses, all of which deviated from the null hypothesis of a 1:1 ratio of homozygous to heterozygous progeny (Table 5.3). Across all reciprocal pairs, only 2% of the F1 seeds were homozygous. These data indicated that the outcrossing rate of giant ragweed can be as high as 98% when self- and cross-pollen are equally available. This rate of outcrossing was similar to what has been in observed in other members of the Asteraceae family (Ellstrand et al. 1978, Friedman and Barrett 2008, Galloway et al. 2003, Godt and Hamrick 1995).

5.4.3 Inheritance of Resistance to ALS Inhibitors

Acetolactate synthase is encoded by the nuclear gene *ALS*. As such, inheritance of the Trp574Leu mutation of *ALS* in giant ragweed that confers resistance to ALS inhibiting-herbicides should conform to Mendelian principals, so long as necessary assumptions like random mating are

met. The presence of homozygous-mutants would be naturally limited under field conditions, as giant ragweed is an outcrossing species, and heterozygous plants with only one mutant allele are endowed with high-level resistance (Bassett and Crompton 1982, Marion et al. 2017, Patzoldt and Tranel 2002). Thus, it is unlikely that homozygous-mutants would possess a significant fitness advantage under herbicide selection compared with heterozygous plants. Regardless, this mutant allele should follow Mendelian inheritance.

The segregation ratio of this mutation departed from the predicted inheritance pattern in all F1 populations in initial controlled greenhouse crosses (Table 5.4). The cross between two heterozygous plants from the Ti18 population should have produced F1 progeny at a segregation ratio of 1:2:1, yet homozygous seeds (either wild-type or mutant) were rare to nonexistent. These data corroborated the field survey results presented in Table 5.1, wherein homozygous mutant plants were underrepresented in the field where the Ti18 population was collected (TIP-1). Wild-type seeds were produced at approximately expected frequencies when one of the parent plants was from the Tp18 population, yet homozygous-mutant seeds were absent from those crosses as well (Table 5.4).

The resistance-conferring mutation was transmitted by both the male and female gametes based on reciprocal crosses between heterozygous and wild-type plants, though the expected 1:1 ratio of wild-type to heterozygous seeds was not found. Instead, nearly all of the progeny were heterozygous (Table 5.4). As such, these seeds would express the resistant phenotype upon germination (Marion et al. 2017, Patzoldt and Tranel 2002). This was confirmed by growing a total of 46 plants from six different F1 populations in the greenhouse. All plants classified as heterozygous using the qPCR assay survived an application of the ALS-inhibiting herbicide cloransulam at the maximum labeled use rate (Anonymous 2017), while all wild-type plants were

susceptible (data not presented). The relative proportion of resistant and susceptible plants in each F1 population was similar to what was reflected in the genotypic ratios determined using DNA extracted from seeds (data not presented).

Homozygous-mutant seeds were not formed in the cross between plants collected from the TIP-1 field and the TPAC field (Tp18), despite identifying a considerable number of homozygousmutant plants in the field survey conducted at the latter site (Tables 5.1 and 5.4). If a linkage between *ALS* and SI genes was responsible for the segregation distortion observed in these F1 populations, it is possible that the mutant *ALS* allele was linked with an SI allele that resulted in the expression of the same (and therefore, incompatible) SI phenotype in all of the parental plants used in these crosses. This may have prevented the union between gametes that possessed the mutant *ALS* allele due to an SI response, while the union between mutant and wild-type gametes did not trigger this response.

If the inheritance of *ALS* in giant ragweed was linked with one or more genes that regulated SI, disruption of the SI process could serve to restore Mendelian inheritance of the Trp574Leu mutation. Bud pollination was used in an attempt to overcome SI, and the resulting F1 seeds were genotyped. Segregation of *ALS* alleles in seeds formed after bud pollination did not differ from traditionally pollinated pistils (data not presented). All seeds formed in these crosses were heterozygous, indicating that the SI system may have been fully functional when the immature pistils were pollinated (Hiscock 2000a). Careful dissection of the pistils prior to the emergence of the style may have improved the efficacy of this technique by allowing for pollination to be conducted earlier in the development of the styles. Yet, all attempts to use this method in this experiment damaged the pistils and resulted in abortion. In another attempt to overcome SI, a saline solution was used to treat the pistils prior to pollination. However, both concentrations of

NaCl (1 and 2.5% w/v) were toxic to the pistils. The styles became discolored and wilted within 30 min of applying the saline solution, and no seeds were produced on these treated flowers.

Salt solutions at concentrations between 1 to 5% w/v have been used without significant damage to pistils of several other plant species (Carafa and Carratù 1997, Hiscock 2000b). Giant ragweed pistils appear to be more sensitive to NaCl than some other species, so any additional attempts to use saline treatments to disrupt SI in giant ragweed should utilize an NaCl concentration below 1% w/v. Techniques such as bud pollination and saline treatments are not effective for overcoming SI in all species (Bianchi and Gibbs 2021). Mentor or companion pollination (pollination with a mixture of self-and cross-pollen) has also been shown to overcome SI (De Nettancourt 2001, Vilanova et al. 2006), though this technique would likely be ineffective in giant ragweed based on the results of the outcrossing experiment as described previously. Additional chemical treatments, high temperatures, increased carbon dioxide concentration, and a myriad of other tactics have been used to overcome SI (Muñoz-Sanz et al. 2020, De Nettancourt 2001), many of which could be adapted in additional attempts to restore full self-compatibility in giant ragweed in future experiments. Instead, the final experiment in this study was designed to examine the segregation of ALS alleles between plants from a wider geography, and the implications of these unique inheritance patterns on the management and spread of herbicideresistance.

An adaptation of the NC II mating design (Comstock and Robinson 1952) was utilized to examine the inheritance of resistance to ALS inhibitors endowed by the Trp574Leu mutation in crosses within and between the Ti18, Tp18, and Ba14 populations. This breeding technique is typically used to evaluate the combining ability of inbred crop lines, wherein males and females from different lines are intermated in a factorial design. In this experiment, crosses within and between populations were conducted using multiple anthers cut from the same male plant and separate, isolated branches on female plants. This allowed for abnormal inheritance patterns to be evaluated as a function of the presumed relatedness of the parental plants. Mating should have been less restricted in external crosses than in internal crosses due to a greater diversity of SI alleles between populations (Busch and Schoen 2008). However, neither the parental population nor the type of cross (internal or external) were effective in predicting whether the segregation ratio in each F1 population would follow Mendelian genetics (Tables 5.5, A.1, and A.2). Non-Mendelian segregation ratios were observed in 59% of the crosses between heterozygous plants, and in both internal and external crosses between all three populations (Table 5.5). Both normal and distorted segregation ratios were observed in half-sibling progeny groups. Overall, there was a slight decrease in the number of mutant ALS alleles identified within the F1 populations generated as a result of the crosses between heterozygous plants (Table 5.6). Conversely, there was a slight increase in the number of seeds produced that would express the resistant phenotype in these F1 populations (Table 5.7). Both of these deviations were resultant of a greater number of heterozygous F1 progeny than would be expected under Mendelian inheritance.

The frequent distortion in the segregation ratios of F1 populations generated from crosses between all three parental populations was surprising, and some F1 populations lacked homozygous seeds entirely (Table 5.5). Thus, the mutant *ALS* allele may have been linked with a common SI allele in the majority of crosses, signifying that there may have been a common resistant ancestor between many of the individuals, irrespective of parental population. Recombination is often suppressed within and around the chromosomal regions where SI genes are located (Charlesworth 2002, Kamau et al. 2007, Schierup and Vekemans 2008, Sims and Robbins 2009, Takuno et al. 2007). This may have served to maintain the linkage between the mutant *ALS* allele and an allele or alleles involved with SI in a majority of the plants utilized in this study, despite a considerable number of isolated breeding cycles. Nevertheless, homozygousmutant seeds were produced in 41% of these crosses (Table 5.5). This may have occurred as a result of rare recombination events leading to the linkage of mutant allele to compatible SI alleles in some parent plants, or perhaps additional, unrelated resistant ancestors were present in the Tp18 and Ba14 populations (Figure 5.1).

In crosses between heterozygous females and homozygous-mutant males, normal segregation ratios were observed in most cases (Table A.1). However, three crosses produced F1 progeny that were exclusively heterozygous. When wild-type females were crossed with heterozygous males, only 15% of the F1 populations deviated from Mendelian inheritance (Table A.2). This indicated that there may have been a greater diversity of SI alleles linked with wild-type *ALS* alleles compared to mutant *ALS* alleles, which aligns the present theory (Figure 5.1). The number of unique SI alleles linked with wild-type *ALS* alleles would not be restricted to the same extent as mutant alleles, as these wild-type allele would not provide an advantage under herbicide selection.

Most self-incompatibility mechanisms identified in Asteraceae have been characterized as sporophytic SI (SSI), where the phenotypic expression of SI alleles is determined by the diploid genomes of the parent plants (sporophytes) (Allen et al. 2011, Ferrer and Good-Avila 2007). The other predominant form of SI is referred to as gametophytic SI (GSI), where the SI phenotype of the male gametophyte is determined by the haploid genome of the pollen grain itself (Mcclure and Franklin-Tong 2006, Oloumi and Rezanejhad 2009). Complex dominance relationships can occur in SSI systems and, coupled with tissue-specific dominance and co-dominance interactions, can occasionally result in a partial compatibility between related individuals (Brennan et al. 2011,

Hatakeyama et al. 1998). Giant ragweed likely possesses a form of SSI, as GSI is not prevalent in the Asteraceae family, and because the SI response appears to take place primarily at the style surface or shortly after pollen tube germination (Hiscock and Allen 2008). However, linkage of *ALS* and a singular *S*-locus does not explain the inheritance patterns of Trp574Leu mutation observed in this study. For instance, under SSI, all pollen grains produced by a plant would express the same SI phenotype because the genes involved with SSI are expressed in the tapetum, which is under diploid control and involves both of the SI alleles derived from the sporophyte (Hiscock and McInnis 2003, Takayama et al. 2000, Takayama and Isogai 2005). For a linkage between *ALS* and SI genes to explain the inheritance of resistance to ALS-inhibitors in giant ragweed, some level of gametophytic control in both maternal and paternal tissues is necessary to explain why, in some instances, pollen grains carrying the mutant *ALS* allele were only compatible with ovules that possess the wild-type allele, and vice versa (Tables 5.4, 5.5, and A.2).

Sporophytic SI interactions have been shown to be affected by modifier genes like the *G*-locus, which may be under gametophytic control and are capable of permitting crossing between otherwise incompatible individuals (Brennan et al. 2002). These SI systems have been referred to cryptic SI (CSI). Rather than being linked with the *S*-locus, *ALS* may instead be linked with one of these modifier loci, which could explain some of the complex inheritance patterns observed in this study. Finally, in addition to a primary SSI mechanism resulting in initial pollen rejection at the style surface, giant ragweed may also possess a gametically controlled late-acting form of SI to which *ALS* is linked, resulting in post-zygotic incompatibility or even ovular inhibition (Seavey and Bawa 1986, Zhang et al. 2016, Zhao et al. 2022).

Full genomic sequencing of this species and identification of genes involved with SI will allow for further exploration of this theory. Regardless, the results from this study clearly demonstrate that giant ragweed possess SI, resulting in an outcrossing rate of as high as 98%. Resistance to ALS-inhibiting herbicides endowed by the Trp574Leu mutation has been shown to deviate from Mendelian inheritance patterns in giant ragweed plants from three different fields across the state of Indiana, despite the causal mutation occurring in a nuclear gene and lacking a fitness penalty. Linkage between ALS and a gene involved with SI could explain this segregation distortion. In crosses between plants from the Ti18 population across all three inheritance experiments, the proportion of resistant progeny increased by an average of 33%, compared with expectations under Mendelian predictions (data not presented). This indicates that resistance to ALS inhibitors can be disseminated throughout a field much faster than previously anticipated. These results emphasize the importance of the integration of weed management tactics that mitigate the evolution and spread of herbicide resistance, such as rotating active ingredients and applying multiple, effective modes of action simultaneously, while also employing non-chemical strategies such as cover crops, tillage, and rotation between crops with differing life cycles (Evans et al. 2016, Jasieniuk et al. 1996, Moss et al. 2019, Norsworthy et al. 2012). Additionally, the unusual inheritance patterns described in this study could present a unique opportunity to study the effects of population fragmentation on the evolution and expression of self-incompatibility through the lens of herbicide resistance and intensive selection pressure on these linked loci.

Table 5.1. Distribution of alleles with respect to position 574 of *ALS* in giant ragweed plants growing in two Indiana soybean fields in 2018 and 2019. ^a

	Genotype		
Field	Wild-type	Heterozygous	Homozygous-mutant
		—— no. plants ——	
TIP-1	112 (165)	291 (186)	0 (53)
TPAC	345 (345)	175 (175)	21 (21)

^a The predicted allelic distribution assuming Mendelian inheritance and the Hardy-Weinberg Equilibrium is presented in parentheses.

Table 5.2. Comparison of pollen retention, pollen tube growth, and seed production in selfand cross-pollinated giant ragweed plants from three populations using orthogonal contrasts. ^a

Pollination method	Pollen grains ^b	Pollen tubes	Seeds
	——— no. per style ———		no. per node
Cross	85	42	6
Self	24	8	1
Control	4	1	1
Cross vs self	***	***	****
Cross vs control	****	****	****
Self vs control	*		ns

^a Significance designated as .=P<0.1, *=P<0.05, ***=P<0.001, ****=P<0.0001, ns = not significant (P>0.05)

^b All data were square-root-transformed prior to analysis, with back-transformed means presented.

Table 5.3. Genotypic classification of giant ragweed seeds produced as a result of pollination with a mixture of self- and cross-pollen from homozygous parents. ^a

	Allelic segregation ^b		Chi-square ^c	
Reciprocal cross	Heterozygous	Homozygous	χ^2	P-value
	—— no. s	eeds ——		
1	32	1	29.12	< 0.0001
2	33	1	30.12	< 0.0001
3	36	0	36	< 0.0001
4	34	1	31.11	< 0.0001

^a Heterozygous seeds were formed following cross-pollination, while homozygous seeds were formed as a result of self-pollination.

^b The Trp574Leu mutation of the nuclear gene acetolactate synthase was used as a paternity marker.

^c Chi-square tests were conducted assuming a 1:1 ratio of heterozygous to homozygous seeds would be produced in the absence of self-incompatibility.

Table 5.4. Segregation of a Trp574Leu mutation of ALS in F1 populations generated by passively pollinating giant ragweed plants in isolated greenhouses.^a

	Allelic segregation			Chi-sc	luare ^b
Pedigree ^c	aa	Aa	AA	χ^2	P-value
		— no. seeds —			
Aa x aa	1	99	0	96.01	< 0.0001
aa x Aa	11	89	0	60.84	< 0.0001
Aa x Aa	2	98	0	92.24	< 0.0001
Aa x Aa ^d	30	70	0	34	< 0.0001
Aa ^d x Aa	40	60	0	36	< 0.0001
Aa	27	72	1	32.88	< 0.0001
Aa	0	32	1	29.18	< 0.0001
aa	38	0	0	-	-

^a Abbreviations: *ALS*, acetolactate synthase; a, wild-type allele; A, mutant allele.

^b Chi-square tests were conducted assuming Mendelian inheritance of the Trp574Leu mutation. ^c The genotype of the maternal parent is listed first. Only one parental genotype is listed for selfpollinations. ^d Plant was from the Tp18 population. All other plants were from the Ti18 population.

		Allelic segregation		Chi-square ^c		
Replicate ^b	Pedigree	aa	Aa	AA	χ^2	P-value ^d
			– no. seeds –			
1	Ba14 x Ba14	6	12	2	2.4	ns
2	Bal4 x Bal4	2	12	6	2.4	ns
3	Ba14 x Ba14	6	9	5	0.3	ns
1	Ba14 x Ti18	0	20	0	20	****
2	Ba14 x Ti18	5	10	5	0	ns
3	Ba14 x Ti18	0	20	0	20	****
1	Bal4 x Tp18	2	13	5	2.7	ns
2	Bal4 x Tp18	2	14	4	3.6	ns
3	Bal4 x Tp18	4	13	3	1.9	ns
1	Ti18 x Ba14	6	14	0	6.8	*
2	Ti18 x Ba14	6	14	0	6.8	*
3	Ti18 x Ba14	4	6	10	6.8	*
1	Ti18 x Ti18	0	20	0	20	****
2	Ti18 x Ti18	3	17	0	10.7	**
3	Ti18 x Ti18	5	15	0	7.5	*
1	Ti18 x Tp18	5	15	0	7.5	*
2	Ti18 x Tp18	5	15	0	7.5	*
3	Ti18 x Tp18	3	15	2	5.1	ns
1	Tp18 x Ba14	5	15	0	7.5	*
2	Tp18 x Ba14	7	13	0	6.7	*
3	Tp18 x Ba14	4	14	2	3.6	ns
1	Tp18 x Ti18	3	17	0	10.7	**
2	Tp18 x Ti18	5	15	0	7.5	*
3	Tp18 x Ti18	7	12	1	4.4	ns
1	Tp18 x Tp18	5	15	0	7.5	*
2	Tp18 x Tp18	6	14	0	6.8	*
3	Tp18 x Tp18	5	10	5	0	ns

Table 5.5. Segregation analysis^a of giant ragweed seeds produced by crossing heterozygous plants from three populations.

^a Allelic segregation was based on a Trp574Leu mutation of *ALS* that endows resistance to acetolactate synthase-inhibiting herbicides. Abbreviations: *ALS*, acetolactate synthase; a, wild-type allele; A, mutant allele.

^b Each cross was replicated three times using separate maternal and paternal plants from each parental population.

^c Expected values were based on Mendelian inheritance in 20 giant ragweed seeds from each F1 population.

^d Significance designated as *=P<0.05, **=P<0.01, ****=P<0.001, ns = not significant (P>0.05)

Table 5.6. Segregation of the Trp574Leu mutation of *ALS* in 20 F1 seeds generated in crosses between giant ragweed plants from three populations.

Pedigree ^a	Expected ^b	Observed	t-value ^c	P-value
	—— no. mutai	nt alleles ——		
Aa x Aa	20	18	3.39	0.0022
aa x Aa	10	10	0.29	0.7727
Aa x AA	30	25	2.48	0.0476

^a The genotype of the maternal parent is listed first. Data were combined over parental populations and replicates. Abbreviations: *ALS*, acetolactate synthase; a, wild-type allele; A, mutant allele.

^b Expected values represent the total number of mutant alleles that would be expected in 20 seeds from each F1 population based on Mendelian inheritance of *ALS* alleles.

^c Differences between observed and expected values were determined using a one-sided t-test.

Table 5.7. Production of giant ragweed seeds with resistance to acetolactate synthaseinhibiting herbicides combined over crosses between giant ragweed plants from three distinct populations.

Pedigree ^a	Expected ^b	Observed	t-value ^c	P-value
	— no. resist	ant seeds —		
Aa x Aa	15	16	2.26	0.0325
aa x Aa	10	10	0.29	0.7727

^a The genotype of the maternal parent is listed first. Heterozygous seeds will express the resistant phenotype upon germination. Abbreviations: a, wild-type allele; A, mutant allele.

^b Expected values represent the number of resistant seeds that would be expected out of 20 seeds from each F1 population based on Mendelian inheritance of *ALS* alleles.

^c Differences between observed and expected values were determined using a one-sided t-test.



Figure 5.1. Illustration of the theory of linked acetolactate synthase (*ALS*) and self-incompatibility (SI) genes in giant ragweed (*Ambrosia trifida* L.) over several generations (G). G₀: A population with one resistant plant (orange) that is heterozygous (Aa) for the Trp574Leu mutation, five susceptible (aa), wild-type plants (green), and an assortment of six unique SI alleles (S₁₋₆). G₁: Selfed progeny of the resistant plant in G₀ after a herbicide application eliminated all susceptible plants prior to pollination. All resistant plants share the same SI alleles, and additional wild-type plants, though homozygous mutant plants are absent because the mutant *ALS* allele is still linked with S₁. This stage represents the Ti18 population. G_n: In successive generations, a recombination event has occurred, linking A with S₃ and integrating homozygous-mutant (AA) plants (blue) into the population. This stage represents the Tp18 and Ba14 populations.



Figure 5.2. Pictures of the reproductive structures of giant ragweed (*Ambrosia trifida* L.). (a) Emasculated node with pistils and branched styles contained within gynoecium, (b) deposition of self-pollen from anthers positioned just above the pistils, (c) immature seeds, and (d) mature seeds.

5.5 Literature Cited

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APPENDIX A. SUPPLEMENTARY DATA

		Segregation			Chi-square ^c	
Replicate ^b	Pedigree	aa	Aa	AA	χ^2	P-value ^d
			– no. seeds –			
1	Ba14 x Tp18	0	20	0	20	***
2	Ba14 x Tp18	0	12	8	0.8	ns
3	Ba14 x Tp18	0	11	9	0.2	ns
1	Ti18 x Tp18	0	20	0	20	***
2	Ti18 x Tp18	1	3	0	-	-
3	Ti18 x Tp18	0	20	0	20	***
1	Tp18 x Tp18	0	9	11	0.2	ns
2	Tp18 x Tp18	3	10	7	0.5	ns
3	Tp18 x Tp18	1	9	10	0.1	ns

Table A.1. Segregation analysis^a of giant ragweed seeds produced by crossing heterozygous females with homozygous-mutant males from three populations.

^a Allelic segregation was based on a Trp574Leu mutation of *ALS* that endows resistance to acetolactate synthase-inhibiting herbicides. Abbreviations: *ALS*, acetolactate synthase; a, wild-type allele; A, mutant allele.

^b Each cross was replicated three times using separate maternal and paternal plants from each parental population.

^c Expected values were based on Mendelian inheritance in 20 giant ragweed seeds from each F1 population. Homozygous wild-type seeds were a result of unintended self-pollination and were not included when calculating the Chi-square statistic.

^d Significance designated as *=P<0.05, **=P<0.01, ****=P<0.0001, ns = not significant (P>0.05)

		Segregation			Chi-square ^c	
Replicate ^b	Pedigree	aa	Aa	AA	χ^2	P-value ^d
			– no. seeds –			
1	Ba14 x Ba14	14	6	0	3.2	ns
2	Ba14 x Ba14	8	12	0	0.8	ns
3	Ba14 x Ba14	6	3	0	-	-
1	Ba14 x Ti18	14	6	0	3.2	ns
2	Ba14 x Ti18	11	9	0	0.2	ns
3	Ba14 x Ti18	10	10	0	1	ns
1	Ba14 x Tp18	12	8	0	0.8	ns
2	Ba14 x Tp18	8	12	0	0.8	ns
3	Ba14 x Tp18	8	12	0	0.8	ns
1	Ti18 x Ba14	1	4	0	-	-
2	Ti18 x Ba14	8	12	0	0.8	ns
3	Ti18 x Ba14	9	11	0	0.2	ns
1	Ti18 x Ti18	10	10	0	1	ns
2	Ti18 x Ti18	14	6	0	3.2	ns
3	Ti18 x Ti18	2	18	0	12.8	***
1	Ti18 x Tp18	8	12	0	0.8	ns
2	Ti18 x Tp18	10	10	0	1	ns
3	Ti18 x Tp18	8	12	0	0.8	ns
1	Tp18 x Ba14	15	5	0	5	*
2	Tp18 x Ba14	5	15	0	5	*
3	Tp18 x Ba14	8	12	0	0.8	ns
1	Tp18 x Ti18	8	12	0	0.8	ns
2	Tp18 x Ti18	12	8	0	0.8	ns
3	Tp18 x Ti18	5	15	0	5	*
1	Tp18 x Tp18	10	10	0	1	ns
2	Tp18 x Tp18	12	8	0	0.8	ns
3	$T_n 18 \times T_n 18$	0	11	0	0.2	ne

Table A.2. Segregation analysis^a of giant ragweed seeds produced by crossing wild-type females with heterozygous males from three populations.

3Tp18 x Tp1891100.2nsa Allelic segregation was based on a Trp574Leu mutation of ALS that endows resistance to
acetolactate synthase-inhibiting herbicides.Abbreviations: ALS, acetolactate synthase; a,
wild-type allele; A, mutant allele.

^b Each cross was replicated three times using separate maternal and paternal plants from each parental population.

^c Expected values were based on Mendelian inheritance in 20 giant ragweed seeds from each F1 population.

^d Significance designated as *=P<0.05, **=P<0.01, ****=P<0.001, ns = not significant (P>0.05)

		Total seed	Deformed		Mendelian
Replicate ^b	Pedigree	production	seeds ^c	Seed weight ^d	inheritance ^e
		no. seeds	%	g/100 seeds	
1	Ba14 x Ba14	22	14	6.9	yes
2	Ba14 x Ba14	79	11	2.7	yes
3	Ba14 x Ba14	15	40	3	yes
1	Ba14 x Ti18	53	23	-	no
2	Ba14 x Ti18	86	8	5	yes
3	Ba14 x Ti18	33	15	7.2	no
1	Ba14 x Tp18	65	23	10.5	yes
2	Ba14 x Tp18	89	10	3.6	yes
3	Ba14 x Tp18	62	23	5.5	yes
1	Ti18 x Ba14	17	35	4.3	no
2	Ti18 x Ba14	116	6	2.8	no
3	Ti18 x Ba14	47	32	1.6	no
1	Ti18 x Ti18	51	6	3.8	no
2	Ti18 x Ti18	32	28	3	no
3	Ti18 x Ti18	15	0	3.9	no
1	Ti18 x Tp18	64	5	4.1	no
2	Ti18 x Tp18	39	13	2.5	no
3	Ti18 x Tp18	28	7	2.5	yes
1	Tp18 x Ba14	24	38	2.2	no
2	Tp18 x Ba14	27	4	4.8	no
3	Tp18 x Ba14	23	35	3.7	yes
1	Tp18 x Ti18	137	7	2	no
2	Tp18 x Ti18	193	7	4.7	no
3	Tp18 x Ti18	35	0	2.9	yes
1	Tp18 x Tp18	47	4	2.1	no
2	Tp18 x Tp18	10	20	4.7	no
3	Tp18 x Tp18	27	19	2.8	yes

Table A.3. Characteristics of F1 seeds produced by crossing heterozygous giant ragweed plants^a from three populations.

^a Maternal and paternal plants in each cross were heterozygous for the Trp574Leu mutation at position 574 of *ALS*.

^b Each cross was replicated three times using separate maternal and paternal plants from each parental population.

^c Seeds were considered deformed when they were significantly malformed and/or aborted with hollow involucral hulls. The number of deformed seeds was divided by the total number of seeds produced in each cross and multiplied by 100%.

^d Seed weights were normalized based on the predicted weight of 100 seeds, with estimates derived using the average weight of 10 seeds from each cross.

^e Seed production in each cross was considered to follow Mendelian Inheritance with respect to the Trp574Leu mutation at position 574 of *ALS* by genotyping 20 seeds from each F1 population and conducting Chi-square analysis of the resulting segregation ratio (P>0.05), with allelic segregation presented in Table 5.5.

VITA

Benjamin C. Westrich

EDUCATION

Doctor of Philosophy in Weed Science – Purdue University______Anticipated May 2022 Dissertation Title: "Influence of Mesotrione, ALS-Inhibitor Resistance, and Self-Incompatibility on Giant Ragweed Management in Soybean" Major Professor: Dr. Bryan G. Young GPA: 3.91

Bachelor of Science – Southern Illinois University Carbondale______May 2018 Crop, Soil, and Environmental Management & Agribusiness Economics GPA: 3.94 – *Summa Cum Laude*

SKILLS

Technical

- Design, execution, and evaluation of field and greenhouse-based biological research trials
 - Pesticide application utilizing equipment including ATV and tractor-mounted sprayers, hand-booms, and research-grade track sprayers
 - Field equipment operation including planting and planter calibration, fertilizer application, tillage, and harvest
 - Proficiency in hauling equipment (Class D)
- Development of novel methods for the germination and cross-pollination of giant ragweed
- Design, optimization, and execution of laboratory procedures including DNA extraction, PCR, microscopy, and agar media preparation for fungal culture and herbicide bioassays
- Data organization and analysis using ARM, SAS 9.4, R Studio, and Microsoft Office programs

Communication

- Presentation of research findings at scientific meetings
- Sale and distribution of pesticide and seed care products alongside retail agronomists
- Use of emerging digital microscope technology to educate growers and agronomists on plant-pathogen interactions
- Undergraduate education as a teaching assistant for Introductory Weed Science courses
- Collaboration with agronomists, graduate students, student workers, and interns for the design and implementation of research trials and demonstrations

RESEARCH AND PROFESSIONAL EXPERIENCE

Purdue University

Graduate Research Assistant_____May 2018-present

• Designed, implemented, and evaluated weed science research projects using field, greenhouse, and molecular techniques

Syngenta Crop Protection

Syngenta Crop Protection	
Research Assistant May-	Sep 2016, Feb-May 2018
• Designed, conducted, evaluated, and presented field-based researce	ch trials and
demonstrations	····· ··· ··· ··· ··· ··· ··· · · · ·
• Maintained research sites and equipment, managed product invent	tory, and organized data
Sales Assistant	May-Aug 2017
• Assisted retail customers in selling, distributing, and evaluating he	erbicides, fungicides,
insecticides, and seed care products	
Southern Illinois University Carbondale	
Undergraduate Researcher	Jan 2015-Apr 2016
 Designed and implemented two soil science research projects eval 	luating methods to
improve the efficiency of nitrogen fertilizer usage	
Undergraduate Research AssistantOct 2014-May 2	017 (excluding summers)
• Assisted in research operations in soil and plant pathology laborat	ories
Southern FS — Eldorado Location	
Retail Location Intern	May-Aug 2015
• Assisted in retail operations including crop scouting, sales, and on	eration of a bulk seed
treater	
TEACHING AND LEADERSHIP APPOINTMENTS	
Teaching Assistant Introductory Wood Science	Aug Dec 2010 & 2021
 Teaching Assistant, Introductory weed Science Montor, Changellor's Scholar and University Honors Programs 	Aug-Dec 2019 & 2021
• Mentor, Chancehor's Scholar and University Honors Programs	Aug 2015-Dec 2018
• Vice President, Kappa Alpha Order Fraternity	100V 2015 - 100V 2016
• Senator, Undergraduate Student Government	Aug 2015-Jan 2016
• Agricultural Student Advisory Council Executive Committee	Aug 2015-Jan 2016
PROFESSIONAL INVOLVEMENT	
Weed Science Society of America	Jan 2021-present
 North Central Weed Science Society 	Sep 2018-present
 SIUC Agronomy Society 	Jan 2016-May 2018
• SIUC Agbassadors	May 2015-May 2016
• SIUC Collegiate FFA	Oct 2014-Aug 2016
• Saluki Ambassadors	Sep 2014-May 2015
HONORS AND AWARDS	
• 2 nd place, WSSA PhD Poster Contest	2022
• Bilsland Dissertation Fellowship	2021
• 1 st place graduate individual, NCWSS Student Weed Contest	2021
• 1 st place team spraver calibration. NCWSS Student Weed Contest	2021
• 1 st place overall team. NCWSS Student Weed Contest	2021
• 1 st place, NCWSS Paper Contest	2020
• 1 st place, NCWSS Poster Contest	2019
• 2 nd place, NCWSS Paper Contest	2019
• 1 st place team spraver calibration. Weed Olympics	2019
• 2 nd place overall team. Weed Olympics – North Central Region	2019
• Chancellor's Scholarshin (full coverage of tuition fees room and	board) 2014-2018
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• Dean's List, SIUC	2014-2018
• Farm Credit Scholarship	2014
Stone Seed Scholarship	2014
 IAA Foundation Scholarship 	2014
Illinois State Scholar	2014

ORAL PAPER AND POSTER PRESENTATIONS

- Westrich BC, Young BG (2022) Interaction of Mesotrione and Metribuzin for Soil-Residual Control of Giant Ragweed. Weed Science Society of America Annual Meeting.
- Westrich BC, Young BG (2021) Inheritance of Resistance to ALS-Inhibitors in Giant Ragweed is Controlled by Multiple Genetic Factors. Page 48 *in* Unofficial Proceedings of the North Central Weed Science Society
- Westrich BC, Young, BG (2021) Using an Agar-Based Bioassay to Characterize the Interaction Between Mesotrione and Metribuzin on Giant Ragweed. Page 19 *in* Unofficial Proceedings of the North Central Weed Science Society
- Westrich BC, Sankaranarayanan S, Kessler SA, Young BG (2020) Patterns in the Inheritance of Resistance to ALS-Inhibiting Herbicides in Giant Ragweed Infers Link with Self-Incompatibility. Page 95 *in* Proceedings of the North Central Weed Science Society
- Westrich BC, Young, BG (2019) Preemergence Applications of Mesotrione for Control of ALS-Resistant Giant Ragweed in Liberty-Link GT27 Soybean. Page 51 *in* Proceedings of the North Central Weed Science Society. Columbus, OH: North Central Weed Science Society
- Westrich BC, Young BG (2019) Utilizing Multiple, Effective Herbicide Modes of Action Can Strongly Influence Selection Pressure for ALS-Resistance in Giant Ragweed. Page 140 *in* Proceedings of the North Central Weed Science Society. Columbus, OH: North Central Weed Science Society
- Westrich BC, Mansfield BC, Young BG (2018) Benefits of Mesotrione in the Residual Control of ALS-Resistant Giant Ragweed in MGI Soybean. Pages 78-79 *in* Proceedings of the North Central Weed Science Society. Milwaukee, WI: North Central Weed Science Society
- Westrich BC, Cook R (2016) Nitrogen Loss and Residue Breakdown Potential for Fall-Applied Diammonium Phosphate. ASA, CSSA, and SSSA Annual Meeting. Phoenix, AZ.