# ESTABLISHING THE VALUE OF ALS-INHIBITING HERBICIDES IN FIELDS WITH CONFIRMED WEED RESISTANCE TO ALS-INHIBITING HERBICIDES

by

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*To my parents Ken and Sharon Boe*

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# ABSTRACT

Author: Boe, Jodi, E. MS
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Title: Establishing the Value of ALS-Inhibiting Herbicides in Fields with Confirmed Weed Resistance to ALS-Inhibiting Herbicides
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Acetolactate synthase (ALS) inhibitors are a widely used class of selective herbicides used to control grass and broadleaf weeds. The repeated use of ALS-inhibiting herbicides has selected for biotypes of weeds resistant to ALS inhibitors, especially in the weeds most problematic to growers in the Midwest. While ALS inhibitor use seems futile, new mechanisms of herbicide action are not predicted to be commercialized in the near future to solve this problem. This leads to the main objective of this research, determining what value ALS inhibitors provide in controlling populations of weeds with resistance to ALS inhibitors.

Field experiments with soil-applied (PRE) applications of ALS inhibitors on horseweed (*Erigeron canadensis*) and tall waterhemp (*Amaranthus tuberculatus* var. *rudis*) exhibited higher efficacy than would be expected given the frequency of the ALS resistance trait in the population. Whereas control of these species with POST-applied applications was similar or less than the proportion of the population characterized as susceptible using molecular techniques. Soil-applied applications, therefore, resulted in relatively greater control than POST applications in populations with known ALS-inhibitor-resistance mechanisms.

Greenhouse experiments showed that overall resistance ratios were higher for PRE applications of ALS inhibitors in horseweed, tall waterhemp, and Palmer amaranth

(*Amaranthus palmeri*). However, $GR_{50}$ values decreased for both susceptible and resistant biotypes for the PRE applications compared to POST, suggesting the biologically effective dose of these herbicides is lower in soil residual applications. This research found that PRE applications of ALS inhibitors resulted in some level of control on horseweed and tall waterhemp classified as resistant to ALS inhibitors due to the higher efficacy of PRE herbicide applications.

Genetic analysis assessing the amino acid substitutions that confer resistance to ALS inhibitors in tall waterhemp confirmed a difference in selection pressure between PRE and POST applications and between ALS active ingredients in tall waterhemp. Applications of chlorimuron PRE at 11 g ai $ha^{-1}$ selected for 35% homozygous W574L genotypes and at 44 g $ha^{-1}$ selected for 70% homozygous W574L genotypes. An increase of homozygous W574L individuals along with a decrease in heterozygous individuals from 65 (11 g $ha^{-1}$) to 29% (44 g $ha^{-1}$) suggests that W574L is semi-dominant in tall waterhemp and that high labeled rates of chlorimuron applied PRE can partially overcome the heterozygous W574L-resistance mechanism. In horseweed, no difference in selection pressure was observed between application timing or between chlorimuron or cloransulam. A new mutation conferring ALS-inhibitor resistance in horseweed was discovered, a Pro197Leu amino acid substitution, with resistance ratios of 21X to chlorimuron and 8.6X to cloransulam. These resistance ratios are slightly less than those reported for the Pro197Ala and Pro197Ser amino acid substitutions in conferring ALS-inhibitor resistance in horseweed.

Finally, a survey of 42 populations of tall waterhemp in Indiana counties with confirmed ALS-inhibitor resistant populations of tall waterhemp found that all

populations contained at least 16% individuals with the W574L amino acid substitution, 35 populations contained at least 1% individuals with the S653N substitution, and 9 populations contained at least 1% individuals with the S653T substitution. Taking into consideration the three mutations tested, 8 of the 42 populations tested contained <50% ALS-inhibitor resistant individuals within the population. Using the same tall waterhemp populations as collected in the survey, Next-Generation Sequencing was used to determine if other amino acid substitutions conferring resistance to ALS inhibitors existed. Results from WideSeq revealed that 10 other amino acid substitutions in the ALS protein may be conferring resistance in tall waterhemp in Indiana: A122T, A122N, A122S, P197T, P197L, P197H, D376E, and G654F. Further research from this survey also suggests that metabolic resistance to ALS inhibitors is likely a contributor to resistance in tall waterhemp in Indiana.

This research suggests that ALS-inhibiting herbicides, more specifically chlorimuron, would provide the greatest contribution to management of tall waterhemp. Chlorimuron would perform best when used in soil residual applications and in populations of tall waterhemp containing either individuals susceptible to chlorimuron or individuals heterozygous for ALS inhibitor resistance conferred by the W574L mutation. This research also demonstrates the specificity of the amino acid substitutions in the ALS protein and by weed species to realize the benefit of these herbicides for management of weeds resistant to ALS inhibitors. Molecular characterization of target site resistance to ALS inhibitors has traditionally been considered relatively simple. However, we found 11 new amino acid substitutions that confer resistance to ALS inhibitors in horseweed and tall waterhemp. The complexity of ALS inhibitor resistance calls for the use of

methods such as NGS to detect all potential resistance mutations in a timely manner and for the use of tests detecting metabolic resistance. Overall, this research demonstrates that ALS inhibitors still provide some utility for management of weed populations classified as resistant to ALS inhibitors and that the resistance mechanisms in horseweed and tall waterhemp are more numerous than previously reported.

# CHAPTER 1. LITERATURE REVIEW

## 1.1 Introduction

Corn and soybeans represent the two largest crop commodities in terms of sales value in the United States at $67.3 and $38.7 billion annually, respectively (USDA-NASS 2014).Weeds threaten the profitability of these crops by directly competing with them and causing crop loss (Dille et al. 2016, Oerke 2006, Soltani et al. 2016). Herbicides are the primary weed management tool implemented by farmers in agronomic crops and can help prevent major losses from weeds (Oerke 2006).

The advent of genetically modified crops containing herbicide tolerance traits has allowed growers to control problematic weeds while reducing tillage and the number of active ingredients used per year (Fawcett and Towery 2002, Young 2006). An example of a widely adopted herbicide-resistance trait in corn and soybean production systems is the glyphosate-resistance trait (Fernandez-Cornejo et al. 2014). The overall efficacy of glyphosate in corn and soybean production has decreased over time in areas with weeds that have developed resistance to glyphosate. Glyphosate resistance, and herbicide resistance in general, is a growing concern for weed scientists and growers alike, as novel cases of herbicide resistance continue to increase each year (Heap 2019).

Development of new herbicide modes of action does not seem to be a viable means of controlling herbicide-resistant weeds (Duke 2011, Peters and Strek 2017). Therefore, growers must implement practices that either manage or reduce the risk of herbicide resistance (Norsworthy et al. 2012) while making the most out of the weed control tools that are currently available.

## 1.2 ALS-Inhibiting Herbicides

### 1.2.1 Overview/Popularity

Acetolactate synthase (ALS), or acetohydroxy acid synthase (AHAS) inhibitors are an important class of selective herbicides used to control grass and broadleaf weds in many different cropping systems. Acetolactate synthase inhibitors include five chemical families: imidazolinone (IMI), pyrimidinylthiobenzoate (PB), sulfonylaminocarbonyltriazolinone (SCT), sulfonylurea (SU), and triazolopyrimidine (TP) (WSSA 2014). Sulfonylureas and IMIs were the first families developed for commercial use (Devine et al. 1993). In US corn production, TPs and SCTs are the most commonly used ALS-inhibiting herbicide families by kilograms active ingredient applied and by percent hectares applied at 142,882 and 75,750 kg and 13 and 9%, respectively (USDA 2015). In US soybean production, IMIs and SUs are the most commonly used ALS-inhibiting herbicide classes by kilograms active ingredient applied and by percent hectares applied at 237,682 and 119,295 kg and 13 and 17%, respectively (USDA 2015).

Acetolactate synthase inhibitor use has remained popular since the introduction of ALS inhibitors in the early 1980s, due to their low mammalian toxicity, wide window of application, broad-spectrum weed control, length of soil activity, superb crop safety, and low use rate (Mazur and Falco 1989). Acetolactate synthase inhibitors are highly toxic to target plants; hence, low required use rates (grams per hectare versus kilograms per hectare). Low ALS inhibitor use rates have been credited with the decline of total herbicide active ingredient applied to crops during the 1980s (Bellinder et al. 1994). Currently, the Weed Science Society of America recognizes 49 active ingredients within the ALS-inhibitor site of action (WSSA 2017). This is more than any other herbicide site of action.

Use of ALS-inhibiting herbicides in U.S. soybean production peaked in 1994 with approximately 91% of soybean hectares applied with an ALS inhibitor (USDA 2015) (Figure 1.1). Acetolactate synthase inhibitor use in corn peaked later, in 1999, with 53% of corn hectares applied with an ALS inhibitor (USDA 2015). After the peak of ALS inhibitor use in soybean, total ALS inhibitor use declined to a low in 2005 with only 10% of soybean hectares receiving an application of an ALS inhibitor (USDA 2015), an 81% reduction from 1994 levels. A similar trend occurred in corn. Peak use of ALS inhibitors occurred in corn in 1999 and dropped to a low of 15% of corn hectares with ALS-inhibitor applications in 2010 (USDA 2015), a 66% reduction. After reaching lows in both corn and soybean, the use of ALS inhibitors has more recently been increasing (Figure 1.1).

The trend of U.S. ALS-inhibitor use follows inversely with the trend of glyphosate use by farmers in the United States. Recent upturn of ALS-inhibitor use could be related to the increase in glyphosate-resistant weeds that are now found throughout the soybean growing regions of the United States (Heap 2019).

### 1.2.2 Physiology

Acetolactate synthase inhibitors function by blocking the acetolactate synthase (ALS) enzyme, thereby inhibiting branched-chain amino acid (BCAA) synthesis. Branched-chain amino acids are required for protein synthesis and normal plant growth, as well as providing precursors for a number of secondary metabolites like cyanogenic glycosides, glucosinolates, and acyl-sugars (Buchanan et al. 2015). The ALS enzyme is the first of four shared steps of synthesis of all three of the BCAAs (Figure 1.2) with two parallel BCAA synthesis pathways. The ALS enzyme catalyzes the decarboxylation of

pyruvate and the condensation of the decarboxylated pyruvate to either pyruvate or 2-ketobutyrate, depending on which parallel pathway is catalyzed: either the leucine and valine pathway or the isoleucine pathway (Buchanan et al. 2015). Plant ALS activity is feedback inhibited by the end products of the pathway, valine, leucine, and isoleucine.

Acetolactate synthase inhibitors are not thought to bind to the ALS enzyme active site. In both plant and yeast ALS enzymes, SU and IMI molecules bind "within the substrate access channel" (Duggleby et al. 2008) of the enzyme and consequently block 2-ketobutyrate and pyruvate from binding to the active site. The sulfonylurea and imidazolinone molecules bind to overlapping sites, which may explain patterns of cross-resistance found in ALS-inhibitor resistant plant biotypes (Duggleby et al. 2008). Blocking the substrate access channel shuts down BCAA production.

Plant growth stops rapidly after BCAA synthesis is inhibited, but the exact cause of plant death from the application of ALS inhibitors has not been elucidated (WSSA 2014). Plant death from ALS-inhibitor application was originally thought to be solely due to the starvation of BCAAs, as exogenous additions of BCAAs caused plant recovery. This theory fit neatly into the idea that BCAA starvation slowed protein synthesis and then cell division, leading to slow plant death (Shaner and Singh 1993). In conflict with this theory, further research showed that plants treated with ALS-inhibitors still had reduced or normal levels of BCAAs due to protein turnover (Rhodes et al. 1987, Scheel and Casida 1985). Therefore, starvation of BCAAs does not seem to the sole reason for plant death due to ALS inhibition. Further downstream effects are implicated, as discussed in Zhou et al. (2007) and Zabalza et al. (2013).

Despite rapid plant growth inhibition, other phenotypic responses to ALS-inhibitors in sensitive species develop slowly. Visual symptoms of ALS-inhibition include chlorosis, shortened internodes, diminished root and shoot growth, increased anthocyanin production, and various leaf deformities (Blair and Martin 1988). Symptoms vary depending on species, with the most consistent symptom being chlorosis of shoot meristems. Preemergence applications of sulfonylurea herbicides do not affect seed germination and usually allow for normal cotyledon development. After cotyledon expansion, however, subsequent seedling growth is affected and true leaves often are prevented from emerging (Blair and Martin 1988).

Extremely low amounts of ALS-inhibiting herbicides are required for reductions in normal ALS-enzyme activity. Inhibitor constants (Ki) describe inhibitor potency and are calculated as the concentration of inhibitor required to produce half of maximum enzyme inhibition. Low Ki values for herbicides translate into low use rates for biological activity. Different ALS-inhibitor families and active ingredients interact with the ALS-enzyme substrate access channel differently, however, contributing to differences in potency. The SUs are generally accepted to be 100 times more potent than IMIs (Duggleby et al. 2008), with PCs being as potent as SUs (Böger et al. 2002). The structure of SUs allow active ingredients within the family to bind further into the substrate access channel, thus allowing more contacts to be made with the channel. Sulfonylureas make >50 hydrophobic contacts and five hydrogen bonds to residues of the ALS enzyme. Increased contacts and anchoring by hydrogen bonds decreases overall Ki. In contrast, IMIs make 12 hydrophobic contacts and 1 hydrogen bond to ALS residues (Duggleby et al. 2008). It is presumed that the other families of ALS inhibitors, based on

cross-resistance patterns observed in ALS-inhibitor resistant weeds, also bind to overlapping sites in the ALS-active site channel.

Differing binding behavior of SUs and IMIs has implications for resistance. Residues A122, P197, A205, and D376 in the ALS enzyme either anchor the aromatic ring of SUs or are located near the aromatic ring *ortho* substituent. Mutations at these residues would therefore affecting binding of SUs with the ALS enzyme (Duggleby et al. 2008). Many interactions occur between the W574 residue and the heterocyclic ring of SUs. The W574 residue also contributes to shape definition of the ALS substrate access channel. Mutations of W574 would therefore reduce the number of contacts SUs make with the ALS enzyme and weaken binding of other ALS inhibitors to the ALS enzyme (Duggleby et al. 2008). Residues A122, W574, and S653 are involved in the anchoring the disubstituted dihydroimidazolonone ring of IMIs and residue D376 is involved in anchoring the carboxylated aromatic ring of IMIs. Mutations in these residues would therefore affect IMI binding (Duggleby et al. 2008). Single nucleotide polymorphisms that confer resistance to ALS inhibitors have been reported in weeds for all residues mentioned in this paragraph (Foes et al. 1998, Patzoldt and Tranel 2007, Zheng et al. 2011, Matzrafi et al. 2015, Larran et al. 2017, Nakka et al. 2017).

Acetolactate-synthase inhibitor uptake is rapid (Blair and Martin 1988), and occurs through roots and foliage. Translocation of ALS inhibitors occurs in the xylem and phloem and is translocated in smaller amounts when applied to foliage versus the roots (Blair and Martin 1988). Postemergence applications of ALS-inhibitors require the addition of an adjuvant for foliar absorption (Kirkwood 1993). Younger leaves tend to be more sensitive to ALS inhibitors than older leaves (Shim et al. 2003), and it has been

observed that older plants decrease in susceptibility to sulfonylureas (Blair and Martin 1988). Crop tolerance and selectivity of ALS inhibitors is due to differential metabolism. Metabolic mechanism of detoxification depends on the crop and specific ALS-inhibitor active ingredient involved. For example, the SU chlorimuron-ethyl is metabolized in corn through cytochrome $P_{450}$ mediated ring-hydroxylation, whereas the same herbicide is metabolized in soybeans through glutathione S-transferase mediated glutathione conjugation (Monaco et al. 2002).

## 1.3 Herbicide Resistance

Herbicides have been a laborsaving and cost-effective way to combat weeds and prevent extensive yield loss from weed competition since their introduction for use in agriculture. Unfortunately, herbicide use under non-ideal conditions (e.g. below-label herbicide rates, excessive weed height, non-conducive weather conditions, poor water quality, etc.) and overreliance on herbicides for weed control has caused herbicide resistance to become a major concern for farm managers and weed scientists alike (Norsworthy et al. 2012). Herbicide resistance is the ability of a weed biotype to survive an herbicide application at doses previously effective for control of the species (WSSA 2017b). General herbicide resistance is reviewed in Powles and Holtum (1994) and Powles and Yu (2010).

Herbicide resistance is not a new phenomenon and originates with origin of synthetic herbicide use. The first reported incidence of herbicide resistance occurred in 1957 when a wild carrot (*Daucus carrota*) biotype was confirmed resistant to 2,4-D, a growth regulator herbicide (Switzer 1957). Resistance has only grown in magnitude since this first report and occurrence is not limited to any one country or continent. Since then,

494 unique cases of confirmed weed resistance have been reported worldwide (Heap 2019). Over 160 of those unique cases have been reported in the United States (Heap 2019). Worldwide, almost 90 weed species exhibit multiple resistance (Heap 2019). Multiple resistance occurs when a weed biotype is resistant to two or more herbicides with differing sites of action (Powles and Preston 1995). Cross resistance occurs when a weed biotype is resistant to two or more herbicide families that share the same site of action (Powles and Preston 1995)

Twelve biotypes of herbicide-resistant weed species are documented in Indiana (Table 1.1). Nine of these weed biotypes are resistant to ALS inhibitors. Five of the twelve biotypes exhibit multiple resistance. All instances of multiple resistance in Indiana include resistance to ALS inhibitors. Further, four species especially troublesome to Indiana growers (giant ragweed [Regnier et al. 2016, Van Wychen 2016], tall waterhemp, horseweed, and Palmer amaranth [Van Wychen 2016]) exhibit resistance to both ALS inhibitors and glyphosate.

When weeds developed resistance to ALS inhibitors, soybean growers that utilized glyphosate-resistant soybean varieties were still able to use glyphosate to manage such weeds. However, the development of multiple resistant weed biotypes with resistance to both glyphosate and ALS inhibitors leaves soybean growers with few herbicide options for weed control. Herbicide options available for POST control of Palmer amaranth and tall waterhemp without glyphosate and ALS-inhibitors include the PPO inhibitors acifluorfen, fomesafen, and lactofen; the photosystem-II inhibitor bentazon; and dicamba and glufosinate in dicamba- and glufosinate-tolerant soybean

system. POST options for control of horseweed resistant to glyphosate and ALS inhibitors are more limited and include dicamba and glufosinate (Loux et al. 2018).

## 1.4 Resistance to ALS Inhibitors

### 1.4.1 Overview

Resistance to ALS-inhibiting herbicides can occur through two mechanisms: target-site resistance (TSR) as the predominant form and non-target site resistance (NTSR), which is less reported and likely more prevalent than currently documented (Tranel and Wright 2002, Yu and Powles 2013). Target-site resistance is caused by a mutation in a gene that encodes for the target site of an herbicide, whereas NTSR includes a number of mechanisms that confer resistance via mechanisms outside of the target site.

The first case of ALS-inhibitor resistance was reported 1986 in rigid ryegrass (*Lolium rigidum* Gaud.) as a perceived byproduct of weeds evolving resistance to photosystem II inhibitors and conferred by enhanced metabolism, a NTSR mechanism (Heap and Knight 1986). Direct use of ALS inhibitors leading to weed resistance was first documented in 1987, five years after the introduction of the first ALS-inhibiting herbicide chlorsulfuron, in the weeds kochia (Primiani et al. 1990) and prickly lettuce (*Lactuca serriola*) (Mallory-Smith et al. 1990). The use of ALS-inhibiting herbicides was common in the 1990s and, ultimately, in 1998 the herbicide site of action group with the most unique cases of evolved resistance in weed species became the ALS inhibitors (Tranel and Wright 2002), surpassing the triazine SOA (WSSA group #5). By 2018, 160 weed species were reported as resistant to ALS inhibitors (Heap 2019). Triazines remain

second in terms of herbicide classes with the most weed species with evolved resistance (Heap 2019).

### 1.4.2 Biochemistry

The most reported type of ALS-inhibitor resistance is due to TSR, or more specifically, single nucleotide polymorphisms (SNPs) in the *ALS* gene, resulting in amino acid substitution (Yu and Powles 2013). Depending on the specific position within the gene, amino acid changes in the ALS enzyme can reduce the binding efficiency of ALS inhibitors to the enzyme and render plant ALS enzymes less sensitive or insensitive to the herbicides (Yu and Powles 2013). Twenty-eight unique amino acid substitutions exist in eight highly conserved regions of the *ALS* gene (Tranel et al. 2018). The sites most commonly found to have mutations conferring ALS-inhibitor resistance are the Pro-197 and Trp-574 sites.

Non-target-site resistance to ALS-inhibiting herbicides occurs through increased herbicide metabolism, which rapidly detoxifies the herbicide to sub-lethal concentrations within the plant (Tranel and Wright 2002, Yu and Powles 2013). Other NTSR mechanisms have been found to play minor roles in ALS-inhibitor resistance (Riar et al. 2013, White et al. 2002), but only on rare occasion. Non-target site resistance to ALS inhibitors can occur based on selection from other herbicides, such as the case of the first documented case of ALS-inhibitor resistance (Heap and Knight 1986), and the first report of tall waterhemp resistant to ALS-inhibitors through NTSR (Guo et al. 2015). Weeds reported to be resistant to ALS-inhibitors through NTSR include rigid ryegrass (*Lolium rigidum* Gaud.), blackgrass (*Alopecurus myosuroides* Huds.), rigid brome (*Bromus rigidum* Roth), wild oat (*Avena fatua* L.), late watergrass [*Echinochloa phyllopogon*

(Stapf.) Koss.], wild mustard (*Sinapis arvensis* L.), corn poppy (*Papaver rhoeas* L.), silky windgrass (*Apera spica-venti*), water starwort (*Myosoton aquaticum*), turnip weed (*Rapistrum rugosum*), flixweed (*Descurainia sophia* L.), tall waterhemp, and Palmer amaranth (*Amaranthus palmeri* S. Watson) (Babineau et al. 2017, Guo et al. 2015, Hatami et al. 2016, Liu et al. 2015, Nakka et al. 2017, Rey-Caballero et al. 2017, Shergill et al. 2018b, Yang et al. 2016, Yu and Powles 2013). Accumulation of both types of resistance mechanisms within a single species biotype has been reported and adds to the challenge of managing herbicide resistance (Babineau et al. 2017, Christopher et al. 1992, Liu et al. 2015, Rey-Caballero et al. 2017, Shergill et al. 2018b, Yang et al. 2016).

The determination of the crystalline structure of the herbicide-bound *Arabidopsis thaliana* ALS catalytic subunit has helped scientists determine the sites where imidazolinones and sulfonylureas bind to the ALS enzyme (Duggleby et al. 2008). As previously mentioned, this work contributed to the understanding that ALS-inhibiting herbicides block the channel that leads to the ALS active site. Differences in herbicide compound structure translate into differences in binding efficacy. These differences have implications for resistance endowing amino acid substitutions. When combined, sulfonylureas and imidazolinones bind to eighteen amino acid residues in the ALS enzyme. Of these eighteen amino acids, ten are shared between sulfonylureas and imidazolinones, two are unique to imidazolinones and six are unique to sulfonylureas (Duggleby et al. 2008, McCourt and Duggleby 2006). When these residues are subject to an amino acid substitution, the change in composition alters the binding site of sulfonylurea and imidazolinone herbicides to the ALS enzyme and may or may not confer resistance. Similar work has not been done on the binding patterns of the

pyrimidinylthiobenzoate, sulfonylaminocarbonyltriazolinone, and triazolopyrimidine family ALS inhibitors. Despite a greater understanding of how ALS-inhibitors bind to the ALS enzyme, the authors concluded that resistance response to amino acid substitutions in the ALS enzyme are complex and exact phenotypic response to certain mutations could not be predicted.

The response of weeds with these assorted mutations in the ALS enzyme can vary widely relative to the specific chemical family or herbicide active ingredient. For example, the Pro-197-Ser substitution in common groundsel (*Senecio vulgaris* L.) confers high-level resistance to sulfonylureas when the residue proline is replaced with serine (Délye et al. 2016). However, when leucine replaced proline at residue 197, the common groundsel biotype is susceptible to sulfonylureas (Délye et al. 2016). Further instances of response variance are evident in the ALS Mutation Database (Tranel et al. 2018). In conclusion, magnitudes of resistance (MOR; syn. resistance ratio) and cross-resistance profiles of respective amino acid substitutions are dependent not only on the specific amino acid substitution, but on the individual weed species the mutation exists and the specific ALS-inhibiting active ingredient, not just the chemical family (Yu and Powles 2013).

### 1.4.3 Inheritance

The ALS enzyme is encoded in the nucleus of the plant, whereas the BCAA pathway is located in the chloroplast (Schulze-Siebert et al. 1984). A transit peptide directs the nuclear encoded enzyme to the chloroplast. Nuclear encoding of the enzyme has implications for the inheritance of ALS-inhibitor resistance conferred by TSR. Mutated *ALS* genes conferring ALS-inhibitor resistance are spread by both pollen and

seed. It has also been shown that the TSRs to ALS- and PPO-inhibitors are linked in tall waterhemp (Tranel et al. 2017).

The level of dominance exhibited by TSR ALS-inhibitor-resistant genes varies among plant species (Tranel and Wright 2002). In some weeds, such as prickly lettuce, annual sowthistle, wild sunflower, oriental mustard (*Sisymbrium orientale*), and eastern black nightshade, resistance is incompletely dominant (Ashigh et al. 2008, Boutsalis et al. 1999, Kolkman et al. 2004). In others, like kochia, corn poppy, common cocklebur, resistance is completely dominant (Ashigh et al. 2008, Scarabel et al. 2004).

Level of dominance of ALS-inhibitor resistance genes has been an important subject in crop breeding. Conventional breeding techniques have been used to breed ALS-inhibitor resistance into crops such as corn, soybeans, sugarbeets, sunflowers, and rice (although the current state of these varieties for commercial use is greatly diminished due to widespread weed resistance to ALS-inhibitors). Dominance influences the associated magnitude of resistance of the ALS-inhibitor resistance. The semidominant nature of the ALS-inhibitor resistance trait in soybean has lead to the thought that chemical rogueing could be employed to purify commercial lines of soybeans with a selective herbicide treatment that kills soybeans heterozygous for the ALS-inhibitor-resistant trait, but not those homozygous for the trait (Sebastian et al. 1989). Dominance level dictates which traits and at what level should be included in male and female lines to confer the highest level of resistance for ALS-inhibitors in corn and sunflowers (Kolkman et al. 2004, Newhouse et al. 1991). The ALS-inhibitor resistance trait in sugarbeet is also semidominant (Hart et al. 1993).

The inheritance patterns for NTSR to ALS inhibitors is less clear, due to the inherent complexity of the biochemical processes associated with NTSR and the limited amount of genomic information available related to weed species (Yuan et al. 2007). In a study of cytochrome P450 metabolic resistance to diclofop-methyl and chlorsulfuron in rigid ryegrass, it was found that P450 herbicide resistance was nuclear inherited and exhibited a high level of dominance over susceptibility (Busi et al. 2011). In a biotype of blackgrass, it was found that at least one to three dominant loci or one recessive locus are involved in NTSR-based ACCase- and ALS-inhibitor resistance. Further, it was predicted that the accumulation of up to at least three of these NTSR loci is required to confer resistance (Petit et al. 2010). In a dicot species, corn poppy, NTSR was polygenic and arose through accumulation of NTSR loci (Scarabel et al. 2015). In short, NTSR inheritance is complex, as NTSR is assumed to be polygenic, and not yet fully understood, whereas TSR is usually conferred by a single gene.

### 1.4.4 Fitness

The Pro-197-His, Trp-574-Leu, and Gly-654-Glu mutations in the ALS enzyme in prickly lettuce, Powell amaranth (*Amaranthus palmeri* S. Wats.) and tall waterhemp, and imidazolinone-resistant rice (*Oryza sativa*), respectively, have been shown to confer a fitness penalty (Alcocer-Ruthling et al. 1992b, 1992a, Sha et al. 2007, Wu et al. 2017, Tardif et al. 2006). The associated fitness cost for the Pro-197-His and Trp-574-Leu mutations are thought to be due to either decreased ALS activity or reduced feedback inhibition (Vila-Aiub et al. 2009).

Other studies conducted on ALS-inhibitor resistance mutations have found little in the way of fitness costs related to these mutations (Vila-Aiub et al. 2009, Yu and

Powles 2013). This is consistent with the findings at the enzyme level, that ALS-inhibitor resistance mutations do not generally confer costs to enzyme efficiency (Yu and Powles 2013).

Generalizations about fitness costs for mutations conferring resistance to ALS inhibitors have been cautioned against, as fitness costs for amino acid substitutions across species are not consistent (Vila-Aiub et al. 2009). Nevertheless, many mutations that confer ALS-inhibitor resistance do not confer a fitness penalty. Without a fitness penalty, mutations conferring ALS inhibitor resistance are likely to remain within field populations once they are present and the use of those herbicides have been discontinued (Yu and Powles 2013).

## 1.5 Herbicide Resistance Management

The presence of herbicide resistance signals to growers that they need to be implementing best management practices to either prevent or manage herbicide resistance. Norsworthy et al. (2012) outlined 13 best management practices (BMPs) that growers can utilize to reduce the risk of herbicide resistance. Two key recommendations for growers stated the need to diversify weed management practices (e.g. chemical and non-chemical methods) and use multiple herbicide sites of action (SOA).

Using multiple herbicide SOAs includes applying different SOAs throughout the growing season in sequential applications, using SOA rotations, and utilizing combinations of multiple herbicide SOA groups. This strategy works to delay weed resistance evolution by decreasing selection pressure from one SOA and reduces the probability that resistant individuals within weed populations survive and reproduce (Norsworthy et al. 2012). In general, using herbicide tank mixtures is more effective at

delaying herbicide resistance than annual herbicide rotations (Diggle et al. 2003, Evans et al. 2015, Reboud and Beckie 2009).

Despite fervent recommendation by weed scientists and crop protection specialists, using multiple herbicides with multiple, effective SOAs was one of the least-adopted practices for resistance management implemented by growers (Frisvold et al. 2009). One reason that growers are not using multiple modes of action for weed management is the associated cost of doing so (Frisvold et al. 2009, Hurley and Frisvold 2016). Further, growers have been instructed to stop using an herbicide once a weed has been declared resistant to it (Patzoldt et al. 2002). This instruction has led to a perception among growers that once a weed has been declared resistant to an herbicide, they should stop using that herbicide altogether. However, when ALS inhibitors are excluded from the weed management strategy the potential for these herbicides to control susceptible individuals and other susceptible weed species that infest the field goes unrealized. This leaves out another SOA that could be reducing the risk of herbicide resistance to the other herbicides used in that year for weed management. Using ALS inhibitors in confirmed ALS-resistant weed populations could potentially reduce the number of weeds exposed to selection from other herbicide SOA groups. This herbicide SOA diversity is essential for long-term herbicide-resistance management (Norsworthy et al. 2012).

## 1.6 Resistant Weed Population Surveys

Geographical surveys for confirming weed resistance to herbicides are important in understanding the development, distribution and growth of herbicide-resistant weeds (Burgos et al. 2013). These surveys typically collect samples within a defined geographical area and then screen the collected samples to detect herbicide resistance

using herbicide treatments or molecular analysis. Often, surveys will conclude that an entire population is resistant when one individual plant is found to be resistant (Anderson et al. 1998, Beckie et al. 2000, 2011, Bourgeois and Morrison 1997, Patzoldt et al. 2002). This was cautioned against in a review on confirming resistance to herbicides (Beckie et al. 2000) and recommended that individual plant resistance frequency without a characterization (a population being called resistant or susceptible) be presented as a result. However, that recommendation has not consistently been followed. Characterizing a population as resistant or susceptible without giving data on resistance frequency does not take into account the number of susceptible individuals remaining in the population that could still be controlled by the herbicide studied.

Some surveys on herbicide resistance are more conservative and descriptive with labeling a field as "resistant". For example, wild radish (*Raphanus raphanistrum*) populations were characterized as being resistant when 20% of the individuals being screened survive (Walsh et al. 2007) Further classifications were used with the determinants "developing resistance" and "susceptible" when 1 to 19% of wild radish plants survive the screen and when 0% wild radish plants survive the screen, respectively.

Susceptible individuals (when reported) are almost always present among herbicide-resistant individuals, especially in surveys during the early epidemiology of herbicide resistance. Very few populations in these surveys have been found to contain 100% resistant individuals (Wise et al. 2009). Populations that have both susceptible and resistant individuals are segregating populations, or populations that exhibit phenotypic variance for a certain trait such as herbicide response. This heterogeneity is likely due to

the existence of resistant and susceptible individuals existing in the soil seedbanks (Wise et al. 2009), but may vary due to the mating system of the weed species studied.

Although the purpose of herbicide resistance surveys is to determine the distribution of herbicide resistance in a certain geography, data on the frequency of individuals within collected populations where susceptible remain a product of the research. Data from surveys of horseweed in Indiana (Kruger et al. 2009a) and tall waterhemp in Kansas (Falk et al. 2005) show that 49 to 83% of populations confirmed as ALS-inhibitor resistance, contained susceptible plants composing greater than 50% of the entire population.

## 1.7 Relationship of Magnitude of Resistance on the Opportunity to Overcome Resistance

### 1.7.1 Magnitude of Resistance

The magnitude of resistance (MOR) of a resistant weed biotype is expressed as the ratio of an herbicide dose to elicit a specific plant response (some measure of plant growth or enzyme activity) on a resistant (R) plants divided by the herbicide dose to elicit the same response on a susceptible (S) plant (Beckie et al. 2000). A herbicide dose that results in a growth reduction of 50% of the observed response may be noted as the $GR_{50}$ value; likewise, a lethal dose to result in a 50% mortality rate would be the $LD_{50}$ value. The MOR may also be referred to as the resistance ratio or resistance factor.

### 1.7.2 PRE vs. POST

Herbicide application timings relative to weed growth are performed prior to weed seedling emergence from the soil (preemergence, PRE) or after the weed has emerged from the soil (postemergence, POST). Preemergence applications rely on the

soil persistence of the herbicide and relatively high herbicide concentrations to inhibit the growth of a germinating weed seedling as water from the soil solution is imbibed. Thus, the herbicide is acting on the smallest size weed as possible during germination and prior to seedling emergence when the plant requires the least amount of herbicide for lethality. Postemergence herbicides are applied to the weed following emergence from the soil on a seedling that is relatively larger than a seedling still under the soil. Thus, the biologically effective dose of an herbicide required by an emerged weed is greater for a postemergence herbicide application than a soil residual herbicide application.

These differences between PRE and POST applied herbicides (sizes of targeted plants, number of active sites in targeted plants, and herbicide concentration) have implications for weed resistance management. Preemergence applications have been shown to control herbicide-resistant weeds by overcoming the resistance mechanisms found in tall waterhemp for HPPD inhibitors (Hausman et al. 2013), PPO inhibitors (Wuerffel et al. 2015), and atrazine (Ma et al. 2016). These specific resistance mechanisms were all considered to be relatively low-level, suggesting that the resistance mechanism wasn't extremely robust. Therefore, the high concentrations of the herbicides in the soil acting on a germinating seedling were able to overcome the low-level resistance mechanism. Likewise, populations of kochia with resistance to dicamba found that soil residual applications of dicamba were more efficacious than foliar applications of dicamba (Ou et al. 2018). The tall waterhemp and kochia populations tested expressed relatively low magnitudes of resistance enabled by the specific resistance mechanism.

### 1.7.3 Opportunities to Overcome Resistance for Improved Field Management

#### 1.7.3.1 Giant Ragweed

Giant ragweed is a problematic summer annual weed common in Indiana. Pollen grains can travel up to 1 km away from the distributing plant (Raynor et al. 1970). Giant ragweed exhibits a high degree of out-crossing, due to wind pollination, although the species is able to self-pollinate as well (Bassett and Crompton 1982). Giant ragweed is a diploid species with 24 chromosomes (Mulligan 1957). Giant ragweed's ability to germinate early and handle variable growing conditions, its high net assimilation rate, plastic growth habit, and capability to compete with and drastically reduce crop yield make it a formidable weed in cropping systems (Abul-Fatih and Bazzaz 1979, Baysinger and Sims 1991).

Giant ragweed control with PRE herbicides in soybean is fair to poor and depends on soil moisture and giant ragweed population (Baysinger and Sims 1992). On susceptible populations, cloransulam-methyl at 36 or 72 g $ha^{-1}$ was the most effective PRE ALS-inhibitor treatment investigated, obtaining 60 and 70% control, respectively (Taylor et al. 2002). The other PRE ALS-inhibiting treatments included chlorimuron, imazaquin, imazethapyr, and flumetsulam. Control from these treatments ranged from 12 to 50% (Taylor et al. 2002).

In field populations of giant ragweed with confirmed resistance to ALS inhibitors, the POST application of ALS inhibitors were generally more effective than PRE applications (Taylor et al. 2002). When a dose-response experiment was conducted in the greenhouse on this population a MOR of > 1,000X was reported for chlorimuron (SU) and cloransulam-methyl (TP) and 26.3X for imazamox (IMI) (Taylor et al. 2002). The presumed mechanism of resistance for this population of ALS-inhibitor resistant giant

ragweed was the Trp-574-Leu amino acid substitution, as this is the only reported resistance mechanism in giant ragweed.

Perhaps the only opportunity to "overcome" the ALS-inhibitor mechanism of resistance in giant ragweed [Trp-574-Leu amino acid substitution (Patzoldt and Tranel 2002)] by applying an ALS-inhibiting herbicide PRE would be with an imidazolinone herbicide, like imazaquin, the IMI herbicide imazamox conferred the lowest MOR in the dose-response experiments conducted by Taylor et al. (2002).

#### 1.7.3.2 Tall Waterhemp

Tall waterhemp is a dioecious summer annual weed that is highly competitive and is well known for its propensity to accumulate herbicide resistance traits (Tranel et al. 2011). Competing in soybeans and corn, tall waterhemp can cause up to 43 and 74% yield reduction, respectively (Hager et al. 2002, Steckel and Sprague 2004). Germination of tall waterhemp occurs throughout the growing season (Hartzler et al. 1999, 2004), making effective weed control challenging. Seed production can top one million seeds under full light conditions (Steckel et al. 2003) and viable seed is produced in as little as nine days after pollination (Bell and Tranel 2010). Viability in the soil seedbank is relatively low, with maximum persistence of only four years (Steckel et al. 2007). Presently, tall waterhemp has developed resistance to six unique herbicide sites of action (Heap 2019) with one population containing resistance to all six SOAs (Shergill et al. 2018a).

The first instance of tall waterhemp resistant to ALS inhibitors was identified in 1991 and populations of tall waterhemp have been accumulating resistance to ALS inhibitors ever since (Tranel et al. 2011). Factors that contribute to the spread of ALS-

inhibitor resistance in tall waterhemp include the dioecious nature of tall waterhemp, a high initial frequency of resistance-conferring mutations, low associated fitness costs (Wu et al. 2017), and extensive use of ALS inhibitors for control of tall waterhemp (Tranel and Wright 2002). Resistance to ALS inhibitors in tall waterhemp is not limited to TSR, as it has been discovered that a biotype is resistant to ALS inhibitors through NTSR (Guo et al. 2015, Shergill et al. 2018a). All reported mechanisms that confer resistance to ALS inhibitors in tall waterhemp are listed in Table 1.2, along with associated MORs.

Prior to resistance development, susceptible biotypes of tall waterhemp were controlled by thifensulfuron and imazethapyr applied POST at levels higher than 97% (Mayo et al. 1994). Another field study found that thifensulfuron and imazethapyr applied POST resulted in slightly less control than reported by Mayo et al. (1994) at 77 to 84% and 77 to 89%, respectively (Sweat et al. 1998). Sweat et al. (1998) included an additional ALS inhibitor, imazamox applied POST, and found POST control to be 93 to 95%. In contrast, PRE applications of ALS inhibitors on tall waterhemp provided more consistent, and generally higher, control. PRE-applied imazaquin, cloransulam, and imazethapyr provided 97 to 100% control in field conditions (Sweat et al. 1998). Interestingly, cloransulam applied POST resulted in only 38% control, whereas PRE application resulted in 99% control, suggesting a differential response in application timing for the TP active ingredient. On a tall waterhemp biotype resistant to ALS inhibitors through an unknown mechanism, PRE applications of imazaquin and cloransulam provided greater control than POST application. Levels of control for imazethapyr PRE and POST on the resistant tall waterhemp biotype were similar (Sweat

et al. 1998). A later study showed slightly different results, that PRE applications of imazethapyr and cloransulam resulted in less control than post applications at 2 and 14% control PRE and 14 and 58% control reported for the two active ingredients (Vyn et al. 2007).

In the absence of ALS-inhibitor resistance, imazethapyr applied PRE has been shown to have greater control of tall waterhemp than POST applications (Sprague et al. 1997). However, imazethapyr applied PRE or POST on a resistant (unknown mechanism) tall waterhemp biotype with a magnitude of resistance of >1270X, unsurprisingly, achieved little or no control (Sprague et al. 1997).

Although variable results have been observed, it seems the most likely opportunity of seeing a difference in PRE and POST applications in ALS-inhibitor-resistant tall waterhemp is PRE applications of TP active ingredients such as cloransulam. Cloransulam showed greater control when applied PRE versus POST in susceptible populations of tall waterhemp. Further, with the W574L resistance mechanism, the associated magnitude of resistance to TPs has been characterized as 32X (Foes et al. 1998), compared to MORs of >1000X for both IMI and SU families (Patzoldt and Tranel 2007).

A second avenue of value for ALS inhibitors in tall waterhemp include control of susceptible individuals. Surveys of herbicide resistance in tall waterhemp have been conducted on both the molecular and phenotypic response level and across the Corn Belt, in Kansas, Missouri, and Illinois. A molecular-based survey of 93 tall waterhemp individuals from 17 fields in Illinois and one in Missouri showed that none of the fields characterized were absent of the Trp574Leu resistance mechanism and that only nine of

the total individuals were susceptible to glyphosate and inhibitors of PPO and ALS (Tranel et al. 2011). This survey may have underestimated actual ALS-inhibitor resistance by not considering other resistance mechanisms, and it may have overestimated ALS-inhibitor resistance by selecting fields for the survey that contained tall waterhemp suspected to be glyphosate resistant. In a greenhouse- and molecular-based survey of tall waterhemp in Missouri, 186 of 187 populations of tall waterhemp were resistant to chlorimuron (Schultz et al. 2015). Populations were considered resistant if >50% of individuals tested survived a 3X rate of the active ingredient. Further molecular studies showed that 75 of 92 chlorimuron-resistant plants tested contained the Trp574L mutation, 20 and 55 at the homozygous and heterozygous level, respectively. The remaining 17 plants were suspected to contain a NTSR mechanism (Schultz et al. 2015).

Earlier surveys have shown less frequency of ALS-inhibitor resistance in tall waterhemp populations. Falk et al. (2005) reported that, out of 30 populations tested, all contained at least one plant resistant to imazethapyr. However, 22 of the 30 populations contained a majority of plants still susceptible to imazethapyr. In a survey of 59 tall waterhemp populations in Illinois, 90% of populations contained at least one individual resistant to either thifensulfuron or imazethapyr (Patzoldt et al. 2002). It was unclear from the 2002 survey what percentage of populations contained a majority ALS-inhibitor-susceptible individuals. From tall waterhemp surveys, it appears that there may be a small percentage of individuals still susceptible to ALS inhibitors. No such survey has been performed on tall waterhemp found in Indiana and no survey has determined the

frequency of the different mutations conferring resistance to ALS-inhibitors and at what level of zygosity.

#### 1.7.3.3 Horseweed

Horseweed is a winter or summer annual weed species found throughout the United States (Davis and Johnson 2008, Regeher and Bazzaz 1979). Horseweed is a diploid plant, contains 18 chromosomes, and is primarily self-pollinated (Mulligan and Findlay 1970, Thébaud and Abbott 1995) with an outcrossing rate estimated to be 4% (Smisek 1995). Due to increasing no-till production, especially in soybean, horseweed has become a problematic weed in Indiana (Kruger et al. 2009b, Weaver 2001). The small seed and adaptations for wind dispersal are key factors in seed rain spreading over long distances; while the highly autogamous reproduction means resistance development in this species can spready rapidly and can cover a large geographic area in a relatively short time (Owen and Zelaya 2005).

The first recorded instance of ALS-inhibitor resistance in horseweed was in 1993 in Israel (Heap 2019). Since then, biotypes of horseweed have been found that have multiple resistance to ALS inhibitors plus either glyphosate or photosystem II inhibitors (Heap 2019). Target site mutations that confer resistance to ALS inhibitors include Pro197Ala, Pro197Ser, Ala205Val, Asp376Glu, and Trp574Leu (Table 1.2). Non-target site resistance to ALS inhibitors has not been reported in horseweed. In a survey conducted in Indiana, 100% of 266 horseweed biotypes contained horseweed individuals that were susceptible to cloransulam (Kruger et al. 2009a). Further, 96% of the 266 biotypes contained individuals susceptible to chlorimuron. Looking at populations with a majority of individuals susceptible to either cloransulam or chlorimuron, 83% and 49%

of the populations, respectively, contained a majority of individuals susceptible to either herbicide (Kruger et al. 2009a). Interestingly, only five of the 266 populations tested exhibited multiple resistance to glyphosate and ALS inhibitors (Kruger et al. 2009a). A lack of populations with multiple resistance to ALS inhibitors and glyphosate is similar to what Trainer et al. (2005) observed in Ohio, although with a non-random sampling technique.

In the absence of ALS-inhibitor resistance, chlorimuron plus tribenuron applied early fall or early spring provided > 90% control of horseweed (Davis et al. 2010). Imazaquin applied POST provided 41% control (Moseley and Hagood 1990). Limited POST application options exist in soybean production with cloransulam and chlorimuron, along with the non-ALS-inhibitors dicamba, glufosinate, and glyphosate, being the only herbicides that control horseweed (Loux et al. 2018).

Opportunities may still exist to use ALS-inhibiting herbicides to control horseweed populations classified as resistant to this SOA group. Although a long period of time has elapsed since a survey has been conducted on horseweed with ALS inhibitor resistance in Indiana, populations may still exist that maintain susceptibility to ALS inhibitors. Further, with relatively low magnitudes of resistance to ALS inhibitors with TSR mechanisms found in the United States, there may be opportunity for control of ALS-R horseweed populations with PRE applications.

#### 1.7.3.4 Palmer amaranth

Palmer amaranth is a dioecious summer annual weed (Sauer 1957), present in the Midwestern and Southern United States (Ward et al. 2013). Factors that make Palmer amaranth difficult to manage in agronomic systems include an extended germination

period (Guo and Al-khatib 2003, Jha and Norsworthy 2009), $C_4$ metabolism (Wang et al. 1992), water stress tolerance (Ehleringer 1983), and extremely high growth rate (Horak and Loughin 2000). An in-depth review of the biology, management, and herbicide resistant history was provided by Ward et al. (2013).

For practical purposes, Palmer amaranth is a diploid with a chromosome number 2n=34 (Gaines et al. 2012, Ward et al. 2013). Since Palmer amaranth is dioecious, it is an obligate outcrosser (Franssen et al. 2001). Despite obligate outcrosser status, Palmer amaranth females have been found to produce viable seed without a pollen source (Ribeiro et al. 2014). Male pollen can spread 300 m from its source, contributing to the spread of herbicide resistance traits from an isolated male to susceptible females (Sosnoskie et al. 2012). Seed production is prolific under ideal and non-ideal growing conditions (600,000 seeds plant$^{-1}$ and $\leq$ 80,000 seeds plant$^{-1}$ respectively) (Keeley et al. 1987). Seed viability has been observed as soon as 14 days after flowering (Keeley et al. 1987). These traits have helped Palmer amaranth develop resistance to 6 total mechanisms of action (Heap 2019).

Resistance of Palmer amaranth to ALS inhibitors was first discovered in Kansas in 1991 (Horak and Peterson 1995). Since, Palmer amaranth has developed resistance to ALS-inhibitors through four unique mutations of the *ALS* gene and through one NTSR mechanism (Table 1.2). In a survey of Palmer amaranth in Georgia, none of the 66 accessions were susceptible to imazapic; although a high level of variability was evident throughout the accessions, thought to be due to the mix of susceptible and resistant individuals within the accessions studied (Wise et al. 2009). Accessions collected from across the southern U.S. also showed variation in their response to pyrithiobac, an ALS

inhibitor in the PTB family. Of the 47 accessions studied, none showed complete susceptibility to pyrithiobac, but average response ranged from 20 to 94% control, suggesting a mix of susceptible and resistant individuals within accessions (Bond et al. 2006). In a recent survey of 41 populations of Palmer amaranth in Indiana, all populations had 60 and 90% of plants survive a 3X rate of chlorimuron and cloransulam. The Trp574Leu mutation accounted for resistance in all but four of these populations (Spaunhorst 2016).

Before resistance to ALS inhibitors developed, susceptible Palmer amaranth biotypes were controlled by imazamox, imazethapyr, and thifensulfuron applied POST with 88, 81, and 83% control, respectively (Sweat et al. 1998). In the same research, control of Palmer amaranth with PRE applications of imazaquin, imazethapyr, and cloransulam was 100% for all herbicides. Similar to the response of tall waterhemp to cloransulam, PRE control of Palmer amaranth was much greater than POST control (100 vs. 26%, respectively) (Sweat et al. 1998).

## 1.8 Review of Literature Summary

The rapid spread and high frequency of ALS-inhibitor resistance has led to a cautious attitude towards the use of ALS-inhibiting herbicides as the sole source of control of problematic weed species, and rightly so. However, with more weeds becoming resistant to other herbicide sites of action and with no new herbicide modes of action coming onto the market, growers need to maximize current weed management practices and herbicides. This research aims to further characterize the value of using ALS inhibitors in situations where they have the potential to be used in weed populations with confirmed ALS-inhibitor resistance.

## 1.9 Justification

Globally, 156 weed biotypes have been confirmed resistant to ALS inhibitors. Further, Indiana has 12 species of herbicide resistant weeds, nine of which are resistant to ALS inhibitors, and five of them exhibit multiple resistance. Of the five species in Indiana exhibiting multiple resistance, four are resistant to both ALS inhibitors and glyphosate. The lack of novel herbicide sites of action being discovered and/or commercialized justifies a greater emphasis on the optimization of the current herbicide groups available. Improvements in weed management, even in the face of increasing herbicide-resistant weed infestations, must come from a greater understanding of how the herbicides contribute to unique weed management situations. Common recommendations and grower practices have diminished the role of ALS-inhibiting herbicides in weed management strategies due to the perceived low value in the presence of weeds with resistance to ALS inhibitors. However, this narrow or simplistic approach leaves out the possibility of controlling susceptible individuals from a segregating population with individuals resistant to ALS inhibitors. Furthermore, the possibility exists that the resistance mechanisms found in weeds may be minimized by altering the application method of the herbicide, such as using a PRE application instead of POST. The opportunity to include these considerations in best management practices for herbicide-resistant biotypes warrants further investigation into directed use of ALS-inhibiting herbicides.

The general hypothesis of this research is that ALS inhibitors will serve some role in terms of managing important weed species in Indiana in field populations that have been confirmed as resistant to ALS-inhibitors. Research objectives include the following:

1) Determine if PRE applications of ALS inhibitors increase the control of weed populations (tall waterhemp, Palmer amaranth, and horseweed) with confirmed ALS-inhibitor resistance relative to the same herbicides applied POST.
2) Quantify differences in selection pressure for ALS-inhibitor resistance alleles in weed populations between PRE and POST applied ALS inhibitors
3) Characterize the frequency of susceptible and resistant individuals in putative ALS-inhibitor resistant tall waterhemp populations in Indiana while assessing a high-throughput method of resistance confirmation.

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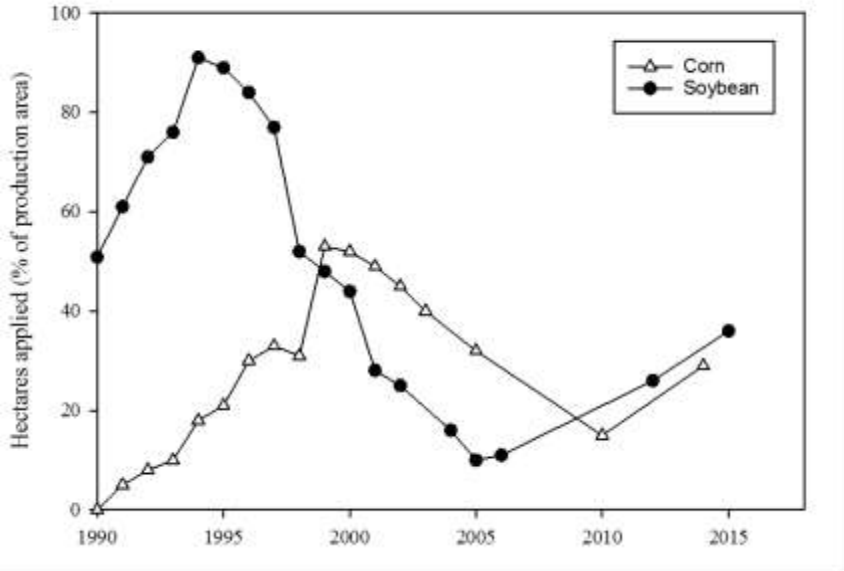


Figure 1.1. U.S. ALS-inhibitor use in corn and soybean production, expressed as a percent of production area. Figure 1.1shows use from 1990 to 2015. Data adapted from the U.S. Department of Agriculture, National Agricultural Statistics Service, Agricultural Chemical Use Database (USDA 2015).

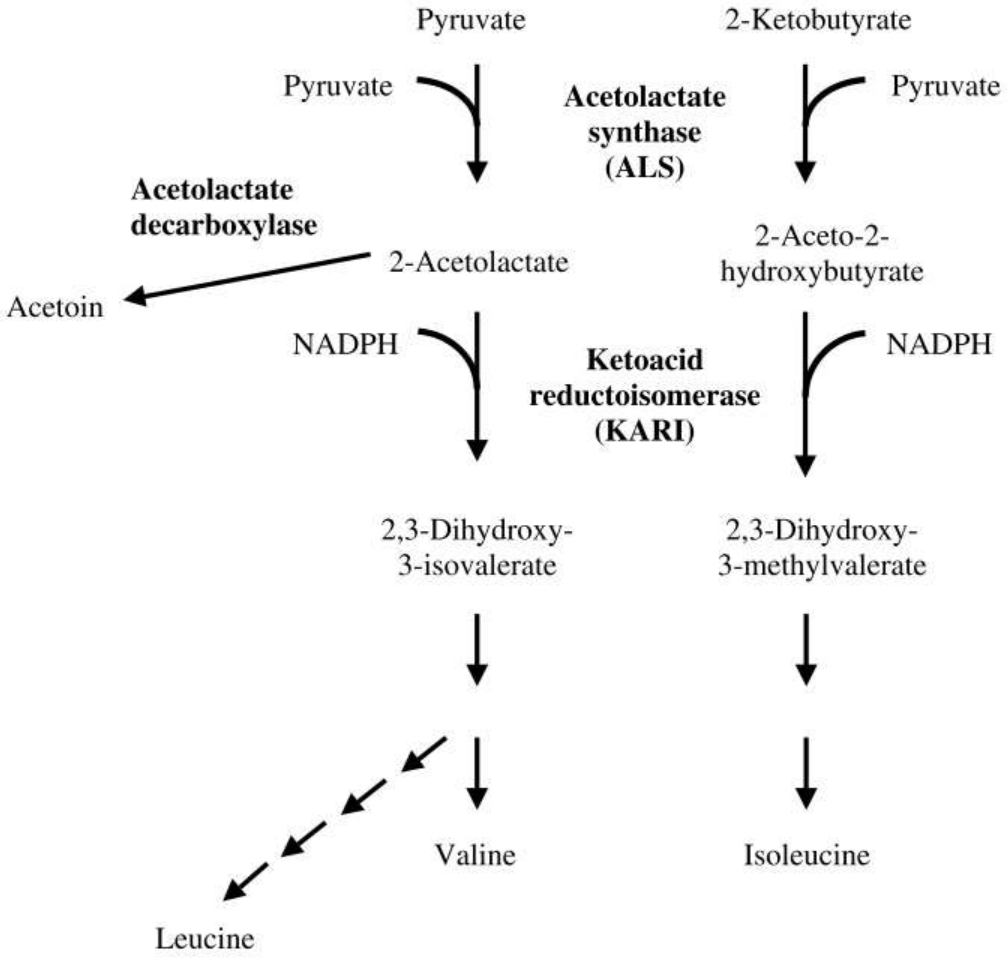


Figure 1.2. Branched-chain amino acid synthesis pathway showing parallel pathways that produce isoleucine and valine and leucine (Corbett and Tardif 2006).

Table 1.1. Biotypes of herbicide resistant weed species in Indiana.

| Common name | Species | Herbicide resistance | Citation |
|---|---|---|---|
| Redroot pigweed | *Amaranthus retroflexus* L. | Photosystem II inhibitors | Heap 2019 |
| Common lambsquarters | *Chenopodium album* L. | Photosystem II inhibitors | Heap 2019 |
| Jimsonweed | *Datura stramonium* L. | Photosystem II inhibitors | Heap 2019 |
| Kochia | *Kochia scoparia* (L.) Schrad. | ALS and Photosystem II inhibitors | Heap 2019 |
| Common ragweed | *Ambrosia artemisiifolia* L. | ALS inhibitors | Heap 2019 |
| Giant ragweed | *Ambrosia trifida* L. | ALS inhibitors<br>ALS and EPSP synthase inhibitors | Harre et al. 2017 |
| Giant foxtail | *Setaria faberi* Herm. | ALS inhibitors | Heap 2019 |
| Tall waterhemp | *Amaranthus tuberculatus* (Moq.) Sauer | ALS inhibitors<br>ALS and EPSPS synthase inhibitors | Heap 2019<br>Bradley 2015 |
| Horseweed | *Erigeron canadensis* (L.) Cronq. | ALS inhibitors<br>ALS and EPSP synthase inhibitors | Heap 2019<br>Kruger et al. 2009 |
| Johnsongrass | *Sorghum halepense* (L.) Pers. | ALS inhibitors | Heap 2019 |
| Shattercane | *Sorghum bicolor* (L.) Moench spp. *arundinaceum* (Desv.) de Wet & Harlan | ALS inhibitors | Heap 2019 |
| Palmer amaranth | *Amaranthus palmeri* S. Wats | ALS inhibitors<br>ALS and EPSP synthase inhibitors | Heap 2019<br>Legleiter and Johnson 2013 |

Table 1.2. Described resistance mechanisms associated with ALS inhibitor resistance in tall waterhemp, horseweed, and Palmer amaranth.

| | | Magnitude of Resistance by Group #2 Family | | | | |
|---|---|---|---|---|---|---|
| Weed species | *ALS* amino acid substitution | IMI | SU | TP | PTB | Source |
| Tall waterhemp | Trp-574-Leu | >2000 | >34000 | >32 | N/A | Foes et al. 1998<br>Patzoldt and Tranel 2007 |
| | Ser-653-Asn | 860 | 1 | N/A | N/A | Patzoldt and Tranel 2007 |
| | Ser-653-Thr | 74 | 1 | N/A | N/A | |
| | Enhanced CP450 activity | 8.9-19 | 5.8-11 | 90 | 2.8 | Guo et al. 2015 |
| Horseweed | Pro-197-Ala | 0.9 | 40 | 50 | 72 | Zheng et al. 2011 |
| | Pro-197-Ser | 0.1 | 25 | 70 | 55 | |
| | Asp-376-Glu | 9.1 | 34 | 33 | 580 | |
| | Ala-205-Val | >29 | 24 | N/A | >44 | Matzrafi et al. 2015 |
| | Trp-574-Leu | >29 | >42 | N/A | >44 | |
| Palmer amaranth | Ala-122-Ser | N/A | N/A | N/A | N/A | Larran et al. 2017 |
| | Pro-197-Ser | N/A | 275 | N/A | N/A | Nakka et al. 2017 |
| | Trp-574-Leu | >112000 | >150-700 | N/A | 112 | Molin et al. 2016 |
| | Ser-653-Asn | N/A | N/A | N/A | N/A | Berger et al. 2016, Larran et al. 2017, Molin et al. 2016 |
| | Enhanced CP450 activity | N/A | N/A | N/A | N/A | Nakka et al. 2017 |

# CHAPTER 2. DIFFERENTIAL RESPONSES OF HERBICIDE RESISTANT HORSEWEED, TALL WATERHEMP, AND PALMER AMARANTH, TO PRE- VS. POST-APPLIED ALS INHIBITORS

## 2.1 Abstract

Multiple resistance to ALS inhibitors and glyphosate in problematic weed species has made weed management especially difficult in soybean production. Since limited herbicide options exist, herbicide use should be optimized to improve the efficiency of chemical weed management. Field and greenhouse experiments were conducted to determine if a change in application timing from postemergence (POST) to preemergence (PRE) can increase the efficacy of select ALS inhibitors in populations of horseweed, tall waterhemp, and Palmer amaranth, containing resistance to ALS inhibitors. In field experiments, PRE applications of ALS inhibitors resulted in greater control of horseweed and tall waterhemp than POST applications in populations with known target site resistance mechanisms to ALS-inhibitors. Herbicide efficacy within field populations of ALS-inhibitor-resistant horseweed and tall waterhemp with PRE applications of ALS inhibitors was greater than expected given the recorded frequency of susceptible individuals. The 44 g ai $ha^{-1}$ rate of chlorimuron applied PRE (92 and 86%) resulted in similar levels of control as PRE-applied fomesafen (99 and 95%) and atrazine (95 to 98%) 21 DAT at the Lafayette site in 2016 and 2017 respectively. Greenhouse experiments showed that overall resistance ratios were higher for PRE applications of ALS inhibitors in horseweed, tall waterhemp and Palmer amaranth. However, the biologically effective herbicide dose was lower for both susceptible and resistant biotypes using PRE applications compared to POST applications, which supports the theory that

field applications for soil residual activity may overcome the target site resistance. In horseweed, a new target site mutation conferring ALS-inhibitor resistance, a Pro197Leu amino acid substitution, was discovered. Greenhouse experiments demonstrated this genotype confers resistance ratios of 21X to chlorimuron and 8.6X to cloransulam. In conclusion, PRE applications of ALS-inhibiting herbicides can control susceptible individuals and individuals with target site resistance mechanisms within horseweed and tall waterhemp populations. Thus, if alternative herbicide options are limited, some ALS inhibitors can still contribute as a functional herbicide site of action group with other effective herbicide groups or non-chemical practices for weed management.

## 2.2 Introduction

Acetolactate-synthase (ALS) inhibitors are an important class of herbicides used to control weeds in a variety of cropping systems. Use of ALS inhibitors has been popular since the introduction of the herbicides in the early 1980s. Sustained popularity of ALS inhibitors is due to low mammalian toxicity, a wide window of application, broad-spectrum weed control, soil-residual length, high crop safety, and low use rates (Mazur and Falco 1989). The use of ALS-inhibiting herbicides in U.S. corn and soybean production, peaked in 1999 and 1994, respectively, with approximately 91% of soybean hectares and 53% of corn hectares receiving an ALS-inhibitor application (USDA 2015). Use of these herbicides declined progressively after the development of ALS-inhibitor resistant weed populations (Tranel and Wright 2002) and the introduction of glyphosate-tolerant crops (Trainer et al. 2005). However, in recent years, ALS-inhibitor use has increased, most likely due to the rise and spread of glyphosate-resistant weeds.

Weed scientists caution growers from using ALS-inhibiting herbicides alone due to the high propensity of ALS inhibitors to select for resistant biotypes (Guttieri et al. 1995, Patzoldt et al. 2002, Tranel and Wright 2002). Currently, 160 species have developed resistance to ALS inhibitors (Heap 2018). Further, numerous problematic weeds in the Midwestern U.S. have developed multiple resistance to ALS inhibitors and glyphosate, increasing the difficulty of weed management, especially in soybean production (Harre et al. 2017, Kruger et al. 2009, Schultz et al. 2015, Spaunhorst 2016). The growing prevalence of PPO-inhibitor resistance in tall waterhemp (*Amaranth tuberculatus* (Moq.) J.D. Sauer) and Palmer amaranth (*Amaranthus palmeri)* (Mansfield et al. 2017, Varanasi et al. 2018) increases the challenge of controlling herbicide resistant tall waterhemp, especially for postemergence (POST) applications to soybeans.

The development of new herbicide mechanisms of action does not seem to be a sustainable means of controlling herbicide-resistant weeds (Duke 2011, Peters and Strek 2017) as the discovery and subsequent commercialization of novel herbicide modes of action has stagnated. Growers must therefore implement practices that either manage or reduce the risk of herbicide resistance (Norsworthy et al. 2012) while making the most of the weed control tools that are currently available.

Three ALS-inhibitor active ingredients, imazethapyr, chlorimuron-ethyl, and cloransulam-methyl, are labelled for preemergence (PRE) and postemergence (POST) applications in soybean. Research in giant ragweed resistant to ALS inhibitors showed no distinguishable difference in the efficacy of those herbicides between application timings (Taylor et al. 2002). Previous research with other sites of action, however, has shown that PRE applications can maintain greater efficacy than POST applications despite resistance

at the POST application timing. This has been documented with HPPD inhibitors (Hausman et al. 2013), PPO-inhibitors (Falk et al. 2006, Wuerffel et al. 2015), and atrazine (Ma et al. 2016). One reason that PRE applications provided greater control than POST applications is the phenomenon that plants generally tend to be more susceptible to herbicides at early growth stages (Shim et al. 2003, Zawierucha and Penner 2001). Reduced sensitivity of POST-applied cloransulam, imazethapyr, and chlorsulfuron has been observed when applied to later growth stages versus earlier growth stages in some weeds (Franey and Hart 1999, Klingaman et al. 1992, O'Sullivan 1982). As viable herbicide options continue to decline, the use of any herbicides should be optimized to provide the greatest contribution to the overall weed management strategy. Therefore, the primary objective of this research was to determine if ALS-inhibitor application timing (PRE vs. POST) influences the efficacy of ALS inhibitors in populations of horseweed (*Erigeron canadensis* L.), tall waterhemp, and Palmer amaranth with varying frequencies of resistance to ALS inhibitors. The secondary objective included characterizing a newly discovered mutation conferring resistance to ALS inhibitors in horseweed.

## 2.3 Materials and Methods

### 2.3.1 Responses of ALS-Inhibitor-Resistant Tall Waterhemp and Horseweed Populations to PRE- and POST-Applied Herbicides under Field Conditions

Field experiments were conducted to investigate the efficacy of ALS inhibitors in tall waterhemp and horseweed populations with histories of resistance to ALS-inhibiting herbicides. The tall waterhemp field experiment was conducted three times over two years at two locations in Indiana: Lafayette in 2016 and 2017 and Farmland in 2017.

Molecular assays were conducted to determine the frequency of resistance to ALS inhibitor resistance within the weed population at each site (Table 2.1). Herbicide treatments included ALS inhibitors with PRE and POST activity with non-ALS inhibitors used for comparison (Table 2.2). Chlorimuron was of specific interest since this herbicide has demonstrated soil residual activity on tall waterhemp populations classified as resistant to ALS inhibitors (Hustedde 2011). Tall waterhemp experiments included chlorimuron at three rates (11, 22, and 44 g ai $ha^{-1}$), imazethapyr (70 g ae $ha^{-1}$), atrazine (1121 and 2242 g ai $ha^{-1}$), and fomesafen (330 g ai $ha^{-1}$). Multiple rates of chlorimuron were included to represent the range in use of the herbicide across PRE and POST applications and over broad geographies with varying carryover and soil pH restrictions. Tall waterhemp experiments were initiated in stale seedbed free of vegetation using an application of paraquat and glyphosate prior to trial establishment. Postemergence applications were made when tall waterhemp was 5- to 10-cm in height. One day prior to POST applications, 25 tall waterhemp plants were randomly selected and marked in each plot receiving a POST application. Marked plants were monitored for the remainder of the growing season

The horseweed field experiment was conducted at Brookston and West Lafayette, Indiana in 2017. Greenhouse bioassays and molecular assays were conducted to determine the frequency of ALS-inhibitor resistance at each site. Horseweed experiments included three rates of chlorimuron (11, 22, and 44 g $ha^{-1}$), cloransulam (35 g ai $ha^{-1}$), metribuzin (280 g ai $ha^{-1}$), and saflufenacil (25 g ai $ha^{-1}$). Plots assigned for a PRE application were treated with paraquat and glufosinate to remove existing vegetation and to allow for accurate PRE control ratings while maintaining an adequate density of

horseweed for POST treatment analysis. Postemergence herbicide treatments were applied when horseweed was 4- to 11-cm in diameter during the rosette growth stage. These research methods allowed the PRE and POST applications to be performed on the same day. One day prior to POST applications, 20 horseweed plants were randomly selected and marked in each plot receiving a POST application. Marked plants were monitored for the remainder of the growing season.

Herbicide treatments for both tall waterhemp and horseweed experiments were applied with a $CO_2$-pressurized backpack sprayer with a 4-nozzle boom with 50 cm nozzle spacing using XR8002 VS nozzles, at 140 L $ha^{-1}$ and 276 kPa pressure. All field experiments were conducted in non-crop areas to reduce competition and potential spray interference for the target weed species. Plots were 3 m by 7.6 m long with nontreated borders along each plot.

Visual estimates of control were made using a 0 to 100 rating scale (0 = no injury; 100 = complete plant death). Ratings for PRE applications commenced once tall waterhemp started to emerge. At the Lafayette site, emergence occurred 1 d after PRE application. At the Farmland site, tall waterhemp emergence did not occur until 3 d after PRE application. Evaluations for tall waterhemp control included 7 d after POST treatments were made (DAPT) and 14, 21, 28, and 35 d after emergence and treatment for PRE and POST applications, respectively. Marked plants were rated at 7 and 14 DAPT and given a rating of either dead or alive 35 DAPT. Tall waterhemp density was determined 1d prior to POST treatment and biomass per plant determined at 14 DAPT.

In horseweed experiments, visual control estimates were taken at 7, 14, 21, 28 and 35 DAT. Aboveground biomass of marked plants were harvested 35 DAT at the soil

surface, dried for 72 h at 41 C for weight determination, and converted to biomass reduction, compared to the nontreated control. Horseweed density and inflorescent plant density was determined at 35 and 120 DAT, respectively.

The experimental design was a three-way factorial of site year, treatment and application timing, with factors completely crossed. The factorial was structured within a randomized complete block design using four replications. A nontreated control was included in all field experiments for comparison. Tall waterhemp and horseweed control data were analyzed using the MIXED procedure in SAS (Version 9.4, SAS Institute Inc., Cary, NC) using replication and location as random effects. Visual control data for tall waterhemp were analyzed as the arcsine transformation and density data analyzed as the square root transformation, with backtransformed means presented. Dry weight data was not transformed for analysis. Means were separated using Tukey's HSD ($\alpha$ = 0.05).

### 2.3.2 Responses of ALS-Inhibitor Resistant Tall Waterhemp, Horseweed, and Palmer Amaranth Biotypes to PRE- and POST-Applied Herbicides under Greenhouse Conditions

General greenhouse conditions included supplemental lighting with high pressure sodium bulbs set to a 16-h photoperiod and temperature maintained at 26 to 29 C. Plants were watered as needed and fertilized weekly using Jack's Professional 20-20-20 (N-P-K) general-purpose fertilizer (JR Peters Inc., Allentown, PA, USA). Herbicide treatments were applied using a single-nozzle, $CO_2$ spray chamber with a TP8002E nozzle (TeeJet Technologies, Wheaton, IL) set to deliver 140 L $ha^{-1}$ of carrier at 276 kPa pressure. All treatments included crop oil concentrate adjuvant (Prime Oil, Winfield Solutions, LLC, St. Paul, MN) at 1% v/v. Before herbicide application, plants were sorted into blocks based on minor height differences, then randomly assigned a treatment. After treatment,

plants were returned to the greenhouse and maintained in the environmental conditions described above. All POST experiments were arranged in a randomized complete block design, included six replicates, and conducted twice.

#### 2.3.2.1 Postemergence Horseweed Methods

Horseweed seed collected from a single horseweed plant in Parke County, Indiana was confirmed susceptible to ALS-inhibitors through preliminary greenhouse assays and was designated S HW. Horseweed seed collected from a single horseweed plant from the West Lafayette field site was confirmed resistant to ALS-inhibitors through preliminary greenhouse assays and was designated R HW. Resistant and susceptible seed was sown into separate plastic 25- by 50-cm flats with potting media (Fafard Growing Mix 2, Conrad Fafard Inc., Agawam, MA) for germination. Once the horseweed seedlings reached the two-leaf growth stage (approximately 14 d after seeding), seedlings were transplanted into 10- by 10-cm plastic pots filled with potting media. Herbicide treatments were applied when horseweed rosettes reached a diameter of 3.75- to 6.5-cm.

Dose-response experiments included two ALS-inhibiting herbicides, chlorimuron and cloransulam since these herbicides are used commercially in soybean for control of horseweed. Chlorimuron was applied to S HW at rates of 0, 0.05, 0.15, 0.44, 0.67, 0.89, 1.33, and 4 g $ha^{-1}$ and to R HW at rates of 0, 1.33, 4, 12, 36, 108, and 2916 g $ha^{-1}$. Cloransulam was applied to S HW at rates of 0, 0.025, 0.074, 0.22, 0.67, and 2 g $ha^{-1}$ and to R HW at rates of 0, 0.67, 2, 6, 18, 54, and 162 g $ha^{-1}$. Each herbicide dose structure was based on preliminary research with the goal of creating a dose-response curve comprising rates that caused a range of plant response from no visual injury to complete-plant death. Visual estimates of control were taken at 7, 14, 21, and 28 DAT.

Aboveground biomass was harvested after the 28 DAT visual rating, dried for 72 h at 41 C for weight determination, and converted to biomass reduction values relative to the average of the non-treated control plants.

2.3.2.2 Postemergence Tall Waterhemp and Palmer Amaranth Methods

Tall waterhemp and Palmer amaranth seed susceptible and resistant to ALS inhibitors was used for PRE and POST *Amaranthus* dose-response experiments. Tall waterhemp seed confirmed as susceptible and resistant (S WH, R WH) to ALS inhibitors was produced by crossing plants in the greenhouse originally obtained from the Lafayette field site. Greenhouse crosses resulted in the R WH line being 100% homozygous for the W574L mutation and the S WH line being 100% susceptible to chlorimuron. Susceptible and resistant Palmer amaranth (S PA, R PA) seed was produced by crossing plants in the greenhouse, originally from a Tippecanoe County, Indiana population. After crossing, S and R *Amaranthus* seed was sown into separate plastic 25- by 50-cm flats with potting media for germination in the greenhouse at 23 to 29 C. Once the *Amaranthus* species seedlings reached the one-leaf growth stage (approximately 7 d after seeding), seedlings were transplanted into 10- by 10-cm plastic pots filled with the aforementioned potting media. Herbicide treatments were applied when Palmer amaranth reached a height of 1.5 to 6 cm and ranged from the fourth to seventh leaf stage. Tall waterhemp was treated when plants reached a height of 1.5 to 5 cm and the fourth to fifth leaf growth stage.

Dose-response experiments included one ALS-inhibiting herbicide, chlorimuron. Herbicide rate structure was based off of thifensulfuron-methyl rates reported in Patzoldt and Tranel (2007). Chlorimuron was applied to S *Amaranthus* species at rates of 0, 0.0044, 0.044, 0.44, 4.4, and 44 g $ha^{-1}$ and to R *Amaranthus* at rates of 0, 0.44, 4.4, 44,

440, and 4400 g $ha^{-1}$. Before herbicide application, *Amaranthus* were sorted into blocks based on minor height differences then randomly assigned a treatment. The experimental unit was one plant. The experimental design was a completely-crossed two-way factorial (2 biotypes x 6 rates) with six replications. Blocks were re-arranged in the greenhouse every 7 d in an attempt to reduce variability from greenhouse conditions. Visual estimates of control were taken at 7, 14, and 21 DAT. Aboveground biomass was harvested after the 21 DAT visual rating, dried for 72 h at 41 C for weight determination, and converted to biomass reduction values relative to the average of the non-treated control plants.

2.3.2.3 Preemergence Greenhouse Experiments

Experimental methods were designed to be similar to previous research involving PRE applications of PPO-inhibiting herbicides (Falk et al. 2006, Wuerffel et al. 2015). The same weed seed sources that were used for POST experiments were used for PRE experiments. Horseweed seed was prepared for planting by placing 0.1 ml sand in a 1.5 ml vial, with either 0.033g S HW seed or 0.022g R HW seed. Sand was used as a medium to help disperse the fine horseweed seed across the soil surface. Tall waterhemp seed amounts were 55 and 20 seeds $pot^{-1}$ for the S WH and R WH accessions, respectively. Palmer amaranth amounts were 30 and 50 seeds $pot^{-1}$ for the S PA and R PA accessions, respectively. Seed amounts were determined based off germination percentages obtained in preliminary research experiments (data not shown). Seeds were sown in separate 10 $cm^2$ flats filled with a bottom layer of 100 ml potting media and a 350 ml top layer of a 1:1:1 mixture of potting media, sand, and field soil (pH 7.1, 6.1% OM) with overall pH of 7.3 and 3.6% OM. Seeds were covered with 20 ml of the 1:1:1 soil mixture and then lightly watered to avoid disturbing seed placement. The following day, pots were sprayed

using the previously described herbicide application methods. For the PRE experiments, each population was sprayed with the same herbicide dose structure as was used in the POST experiment, with or without the addition of one additional lower rate to account for the potentially increased susceptibility to PRE-applied herbicides. Pots were then watered with 30 ml, equivalent to 3 cm of rain, to simulate an activating rain. Pot moisture was regulated with sub-irrigation for the remainder of the experiment (Harder et al. 2012, Wuerffel et al. 2015). Greenhouse conditions were maintained as described previously for the POST experiment. Seedling emergence was enumerated every other day from the outset of the experiment. Visual control data was collected at 7, 14, and 21 DAT for the horseweed and tall waterhemp experiments, and at 7 and 14 DAT for the Palmer amaranth experiment. At 21 DAT for horseweed and tall waterhemp and 14 DAT for Palmer amaranth, above ground biomass was harvested and weighed for fresh weight determination, dried for 72 h at 41 C for weight determination, and converted to biomass reduction values relative to the average of the non-treated control plants. The horseweed and *Amaranthus* experiments were run separately. The experimental design for the horseweed experiment was a completely-crossed three-way factorial (2 biotypes x 2 herbicides x 7 to 9 doses) and the design for the *Amaranthus* experiments was a completely-crossed two-way factorial (2 biotypes x 7 rates) with treatments organized in a randomized complete block design. Horseweed and *Amaranthus* experiments included six replications were conducted twice.

### 2.3.3 Statistical Analysis of Greenhouse Experiments

Dry weight data for horseweed, tall waterhemp, and Palmer amaranth were analyzed using nonlinear regression to determine the herbicide rate required to reduce dry

weight by 50% ($GR_{50}$) using the 'drc' package in R (Knezevic et al. 2007, Ritz and Strebig 2016). Regression parameters for all accessions were estimated using a three-parameter log-logistic equation:

$$Y = d/1 + \exp[b(\log x - \log e)] \quad [1]$$

Where Y is the response (dry weight as a percent of the nontreated control); *d* is the upper limit; *e* is the inflection point, or the $GR_{50}$; and *b* is the relative slope around *e*.

## 2.4 Results and Discussion

### 2.4.1 Field Studies

#### 2.4.1.1 Tall Waterhemp

Significant location by treatment by time-of-application (TOA) interactions were present for the tall waterhemp data, so data are presented separately by site year (Table 2.3). Differential response to time of application was observed at the Lafayette site during 2016 and 2017 (Table 2.3). Preemergenece applications of chlorimuron at all three rates resulted in greater control than POST applications in 2016 and 2017. In 2016, a differential response to timing was also observed with imazethapyr, with the PRE application resulting in greater control (34%) compared to POST applications, which resulted in little to no control across all site years (Table 2.3). Differences between ALS-inhibiting herbicides were also observed at both years of the Lafayette site. Chlorimuron-treated plots resulted in greater control (15 to 92%) than imazethapyr (5 to 34%) in this population of ALS-inhibitor-resistant tall waterhemp. As the rate of chlorimuron increased, control also increased at the Lafayette tall waterhemp site for PRE- (65 to 92%) and POST-applied (35 to 66%) treatments in 2016 and in PRE-applied treatments in 2017 (66 to 86%) (Table 2.3). Contrary to the two site years at Lafayette, no

differences were observed between application timing or between ALS inhibitors at the Farmland site by 21 DAT. Overall weed control for the Farmland site was also lower than at the Lafayette site.

In general, the comparison standard treatments of atrazine and fomesafen resulted in greater control of these tall waterhemp populations classified as ALS-resistant than all of the ALS-inhibiting herbicides (Tables 2.3, 2.4). However, the high rate of chlorimuron applied PRE (92 and 86%) did reach similar levels of control as PRE-applied fomesafen (99 and 95%) and atrazine (95 to 98%) 21 DAT at the Lafayette site in 2016 and 2017, respectively.

Previous research on ALS-S tall waterhemp demonstrated POST control with imazethapyr was similar to chlorimuron, in the 85 to 99% range (Mayo et al. 1994, Sweat et al. 1998). Our results at the Lafayette site highlights that POST-applied imazethapyr results in little to no control while POST-applied chlorimuron resulted in significantly greater activity (Table 2.3). This difference in activity between chlorimuron and imazethapyr suggests a resistance mechanism is present in the population that causes a differential response between active ingredients, ultimately affecting imazethapyr more than chlorimuron. Molecular assays confirmed this, as the population contained a high frequency of both the Trp574Leu and Ser653Asn amino acid substitutions. The Trp574Leu mutation confers a high level of resistance to imidazolinone, sulfonylurea, and triazolopyrimidine family ALS inhibitors (Foes et al. 1998, Patzoldt and Tranel 2007). The Ser653Asn mutation confers high-level resistance to only imidazolinone family ALS inhibitors (Patzoldt and Tranel 2007). Control of plants with the Ser653Asn mutation by chlorimuron must have occurred at the Lafayette site, hence greater control

with POST-applied chlorimuron versus POST-applied imazethapyr. This confirms on the field level that tall waterhemp with the Ser653Asn amino acid can still be controlled by alternative ALS inhibitors. Further, the frequency of the Trp574Leu resistance mechanism may explain differences in POST-applied chlorimuron activity between the 2016 and 2017 Lafayette site years. When the frequency of Trp574Leu was lower (33% in 2016), POST control was higher (38 to 66%) and when the frequency of Trp574Leu was higher (70% in 2017), POST control was lower (15 to 19%). Response to PRE-applied ALS inhibitors remained relatively the consistent between the two site years (Table 2.3). Resistance frequency and the type of resistance mutations present clearly influences ALS-inhibitor efficacy.

At the Farmland site, no differences existed in tall waterhemp control among any of the ALS inhibitors. This is interesting because the resistance frequency of any ALS-inhibiting mechanism (Trp574Leu) we screened at this site was 52%, yet less than 50% control was achieved by any ALS inhibitor (Table 2.3). This is in contrast with results from Lafayette, where control with ALS inhibitors was occasionally greater than the susceptible frequency. Further, because the Ser653Asn mechanism was not present at Farmland, the efficacy of imazethapyr was similar to chlorimuron, unlike Lafayette, which consisted of a population with a high frequency of the Ser653Asn and Trp574Leu mutations. The lack of response to increasing chlorimuron doses would suggest that an unknown resistance mechanism may exist at the Farmland site, as the mechanism has a high magnitude of resistance, which would rule out the alternative mechanism as being solely NTSR. This new mechanism of resistance likely exhibits cross-resistance between

imazethapyr and chlorimuron, as both active ingredients caused similar levels of control PRE and POST at Farmland.

The POST-applied ALS inhibitors resulted in control of susceptible individuals within the plants marked prior to application. Average control of the marked plants at the Lafayette site in 2016 and 2017 showed that there was no difference in response to increasing rates of chlorimuron applied POST (Table 2.5). Chlorimuron applications resulted in greater control (44 to 49%) than imazethapyr (8%). Atrazine at the highest rate and fomesafen resulted in 97 and 99% control, respectively, at Lafayette 2016 and 2017 (Table 2.5). The response of marked plants at the Farmland site showed similar results as overall plot control. Little to no control of marked tall waterhemp plants was evident for both ALS inhibitors applied POST at Farmland (Table 2.5). Mortality of marked plants followed closely with average visual control ratings and with reported genotypes associated with each site. For Lafayette in both site years, mortality in response to the application of an ALS inhibitor was similar to the percent of individuals without a known target site mutation (Table 2.1). At Farmland, mortality in response to ALS inhibitors was not similar to the percent of susceptible individuals in the population, further supporting the hypothesis that an unknown resistance mechanism may exist in the population.

Sweat et al. (1998) reported POST-control of an ALS-inhibitor resistant population of tall waterhemp (unknown resistance mechanism) to be 25 to 27% with imazethapyr and 49 to 59% with thifensulfuron, an ALS inhibitor in the same chemical family (sulfonylurea) as chlorimuron. Patzoldt and Tranel (2002) conducted a survey of 59 tall waterhemp populations across Illinois and found control responses with

imazethapyr and thifensulfuron to be variable across the six tall waterhemp plants tested from each population. Variability in the Patzoldt et al. (2002) survey was attributed to the dioecious nature of tall waterhemp and the presence of the Trp574Leu mutation and an unknown resistance mechanism, later found out to be Ser653Asn (Patzoldt and Tranel 2007). This is the first report where genotyping is employed to estimate what percentage of a tall waterhemp population contains TSR ALS-inhibitor-resistance mechanisms. Variability in response to ALS inhibitors in a population of tall waterhemp can be attributed to the frequency of resistance mechanisms present in the population. Importantly, our research also shows that PRE applications of ALS inhibitors achieved greater control than POST applications in the presence of both the Trp574Leu and Ser653Asn resistance mechanisms.

#### 2.4.1.2 Horseweed

Significant location-by-treatment-by-timing interactions were present; therefore, data are presented separately by site year (Table 2.6). At the Brookston field site, a differential PRE vs. POST response with PRE applications providing greater control than POST applications was observed with chlorimuron at the middle (83 vs. 58%) and high rates (89 vs. 65%), cloransulam (94 vs. 61%), and metribuzin (99 vs. 44%). All PRE treatments, except for chlorimuron at the lowest rate, and POST-applied saflufenacil, resulted in similar control at 21DAT, ranging from 83 to 98%. Differences in plant density between PRE and POST applications were observed solely with the low rate of chlorimuron and metribuzin. Cloransulam and metribuzin applied PRE and saflufenacil applied PRE and POST resulted in the greatest density reduction (Table 2.6). By 35 DAT, only cloransulam and metribuzin PRE treatments resulted in greater control than

POST treatments. The greatest control values PRE at 35 DAT were obtained with cloransulam, metribuzin and saflufenacil at 89, 98, and 98% control, respectively (data not shown). Chlorimuron (high rate) and cloransulam applied PRE reduced inflorescent plant density by 65 and 87%, respectively (data not shown).

At the West Lafayette site, greater control was observed for PRE-applied vs. POST-applied herbicides for every herbicide treatment at both rating times, except for the saflufenacil treatment, which resulted in 100% control of horseweed at both application timings at 21 DAT (Table 2.6). This consistent differential PRE vs. POST response was likely an artifact of the lack of horseweed germination after PRE plots were burned down for PRE application. Horseweed emergence can occur primarily in the fall, spring, or a mix of fall and spring (Bhowmik and Bekech 1993, Davis and Johnson 2008). Fall-applied herbicides can influence spring emergence of horseweed (Davis et al. 2010), though it was not a possible factor influencing the West Lafayette site. The Brookston field site contained a mixture of fall-emerged and spring-emerged horseweed; whereas, the West Lafayette site was primarily a fall-emerging population. Although horseweed has been observed to emerge into July (Bhowmik and Bekech 1993; Davis et al. 2010), no emergence occurred after initial burndown of PRE plots at the West Lafayette site. Therefore, all PRE-applied herbicide treatments at the West Lafayette site provided near 100% control at all rating times (Table 2.6). At the Brookston site, germination of horseweed occurred throughout the experiment. This difference in germination period explains the significant interaction between location, herbicide, and rate and the greater control associated with PRE-applied herbicides versus POST-applied herbicides in horseweed at the West Lafayette location.

The ALS inhibitors applied POST reduced the biomass of marked horseweed plants by over 50% at 14 DAT (Table 2.7). Chlorimuron at all rates, cloransulam, and metribuzin reduced horseweed biomass by similar amounts and resulted in similar control and mortality rate (Table 2.7). Although biomass was reduced by all ALS inhibitors, overall percent mortality of marked plants at 14DAT was low (Table 2.7), at 3 to 5%. Similar responses, reduction in overall biomass, and visual injury symptoms due to ALS inhibitor application, but then regrowth, were observed when chlorsulfuron was applied to 6- to 8-leaf common lambsquarters and Tartary buckwheat (O'Sullivan 1982). Both species also flowered and produced seed after initial injury symptoms. The more likely fate of marked horseweed plants treated with POST applications of ALS inhibitors would be a biomass reduction and subsequent flower and seed production.

Overall, PRE applications of chlorimuron at the high rate and cloransulam suppressed the horseweed population at Brookston, despite a high frequency of ALS-inhibitor resistance among individual plants (Table 2.1). Cloransulam application resulted in slightly greater control than chlorimuron. This is in contrast with greenhouse dose-response experiments performed by Trainer et al. (2005), where the susceptible horseweed biotype was more sensitive to chlorimuron in cloransulam. However, horseweed control recommendations for soybeans before widespread ALS inhibitor resistance reported glyphosate and cloransulam as being the two best options for POST herbicide efficacy (Kruger et al. 2009).

The field results for horseweed and tall waterhemp were similar in that PRE applications of ALS inhibitors generally resulted in greater control than POST applications. Important differences exist, however, in that the rate of chlorimuron was

more influential for horseweed control than tall waterhemp. Further, the alternative ALS inhibitor used in horseweed, cloransulam, provided greater control than chlorimuron, whereas the alternative ALS inhibitor in tall waterhemp, imazethapyr, provided less control of tall waterhemp in some cases. These differences in herbicide efficacy across herbicide active ingredients, application rates, and application timings emphasizes the importance of understanding the specific herbicide resistance mechanisms for the opportunity to optimize the efficacy of ALS inhibitor herbicides in weed populations with segregating resistance traits.

### 2.4.2 Greenhouse Dose-Response Experiments

#### 2.4.2.1 POST Greenhouse Experiments

Postemergence applications of chlorimuron on tall waterhemp and Palmer amaranth resulted in resistance ratios of 281X and 790X, respectively (Table 2.8). The resistance ratio for sulfonylurea herbicides in tall waterhemp with the Trp574Leu has been reported to be >34,000X for thifensulfuron (Patzoldt and Tranel 2007). Although the current experiment uses chlorimuron instead of thifensulfuron, the resulting resistance ratio of 281X is much less than expected. This is likely due to the difference in sensitivity of the biotypes used in this dose-response experiment, possible differences in greenhouse growing conditions, and the growth stage of the plants when they were treated. The resistance ratio for Palmer amaranth of 790X aligns closely with results reported by Molin et al. (2016), in that applications of the sulfonylurea trifloxysulfuron resulted in a resistance ratio of >700X. Molin et al. (2016) also found that with a different sulfonylurea herbicide, nicosulfuron applications resulted in a resistance ratio of >150X,

showing that variation in resistance ratio can occur based on the active ingredient within the ALS inhibitor chemical family used.

The population of horseweed used for greenhouse studies was found to have a previously unreported amino acid substitution conferring resistance to ALS inhibitors at the Proline-197 position with a substitution to leucine. Previous research has reported two other substitutions in horseweed at the Pro197 position with substitutions to alanine and serine (Zheng et al. 2011). However, both the Brookston and West Lafayette field populations contained only the Pro197Leu mutation. Associated resistance ratios with the two previously reported Pro197 mutations vary among active ingredient used and between which amino acid substitutes for proline.

In our horseweed experiment, POST applications of cloransulam and chlorimuron resulted in resistance ratios of 8.6 and 21X, respectively (Table 2.8). The resistance ratio for cloransulam is less than what was reported for both the Pro197Ala and Pro197Ser mutations, but the resistance ratio for chlorimuron is close to the resistance ratio reported for Pro197Ser (Zheng et al. 2011). A different susceptible biotype was used in this experiment than what was used in previous experiments which rules out any meaningful examination of resistance ratios across research groups, in addition to the specific plant growth conditions and application parameters involved.

#### 2.4.2.2 PRE Greenhouse Experiments

The PRE greenhouse experiments were performed to investigate soil residual activity of ALS inhibitors on ALS-S and –R biotypes of tall waterhemp, Palmer amaranth, and horseweed. Biomass was collected at 21 DAT for tall waterhemp and horseweed and at 14 DAT for Palmer amaranth. Chlorimuron did not prevent

germination of any weed species and expanded cotyledons were observed before injury symptoms were apparent. Initial injury symptoms were not observed in Palmer amaranth and horseweed until 7 and 9 DAT in tall waterhemp, and mostly in pots treated with the highest rate of herbicide applied. Symptoms included general stunting and chlorosis of leaf tissue. Increases in chlorimuron rate on the *Amaranthus* species did not increase mortality by harvest time, but did, along with cloransulam, increase mortality in horseweed (data not shown).

Biomass reduction data from all PRE experiments for the three weed species were subjected to non-linear regression. In all three species, $GR_{50}$ values for all biotypes and herbicide combinations decreased from POST to PRE applications. The magnitude of decrease in $GR_{50}$ value was much larger in the susceptible biotypes than the resistant biotypes, resulting in increased resistance ratios for PRE applications versus POST. The PRE $GR_{50}$ value for R PA was half of the POST R PA $GR_{50}$ value (Table 2.8). Likewise, the $GR_{50}$ value for S PA decreased by a factor of 12.5X when the herbicide was applied PRE versus POST (Table 2.8). The $GR_{50}$ values for PRE-applied treatments were 33 to 99% less than when applied POST for all species, resistant or susceptible. Application from POST to PRE in R WH reduced the $GR_{50}$ by 82% and in S WH reduced the $GR_{50}$ by 99% (Table 2.8). Changing application from POST to PRE with chlorimuron in R HW reduced the $GR_{50}$ by the least of any species x herbicide combination at 33% (Table 2.8). The $GR_{50}$ value for S HW applied with chlorimuron was reduced by 83% when application timing was changed from POST to PRE (Table 2.8). In both R and S HW with cloransulam as the treatment, $GR_{50}$ value was reduced by 60% (Table 2.8).

Resulting resistance ratios for PRE applications of chlorimuron in tall waterhemp and Palmer amaranth were 5346 and 4418X, respectively (Table 2.8). In horseweed, chlorimuron and cloransulam applied as a PRE resulted in resistance ratios of 85 and 8.6X, respectively (Table 2.8). Differences in resistance ratios between application timings are attributed to the decreased $GR_{50}$ values of PRE applications versus larger POST $GR_{50}$ values. The decreased $GR_{50}$ values for PRE applications caused PRE resistance ratios to be larger than all POST applications for all combinations of species and herbicides excluding horseweed applied with cloransulam, where resistance ratios for both PRE and POST applications were the same.

Overall, this research demonstrates that PRE applications of ALS inhibitors provide greater efficacy than POST applications in field populations of tall waterhemp and horseweed and greenhouse lines of Palmer amaranth with varying levels of ALS-inhibitor resistance. Our research also demonstrates that chlorimuron has greater potential than imazethapyr to control ALS-R tall waterhemp in field conditions, that cloransulam and chlorimuron at high field use rates have similar potential to control ALS-R horseweed PRE and POST, and that increasing chlorimuron rate in tall waterhemp and horseweed increases herbicide activity. Greenhouse experiments emphasize the biologically effective dose in PRE applications are dramatically reduced compared with POST applications of ALS inhibitors which can explain how the difference in application timing can partially overcome the resistance mechanisms we tested in our research across weed populations. These results are similar to what has been observed in HPPD-inhibitor-, metabolism-based-PSII-inhibitor-, and PPO-inhibitor resistance, in that PRE applications of the active ingredient provides greater control despite the existence of

resistance at the POST application timing. Our results are exceptional due to the perceived high-level resistance associated with ALS target-site resistance compared to the resistance mechanisms associated with HPPD-, PSII-, and PPO-inhibitors.

This research also discovered and characterized a new mutation conferring ALS-inhibitor resistance in horseweed, the Pro197Leu mutation. The Pro197Leu mutation has a MOR of 21 and 8.6X for POST applications of chlorimuron and cloransulam, respectively. Future research should investigate the suspected unknown resistance mechanism present at the Farmland tall waterhemp site. We are not encouraging the use of ALS-inhibiting herbicides for specific management of weed populations with resistance to these herbicides. Rather, these herbicides can still contribute as a part of a best management practice by controlling weed species and biotypes that remain susceptible to group ALS-inhibiting herbicides while providing a different herbicide mode of action to mitigate the evolution of herbicide resistance to other effective herbicides used in the overall weed management strategy. As viable herbicide options dwindle due to the evolution of multiple herbicide resistance, the optimization of herbicide use becomes even more imperative.

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Table 2.1. Test site characteristics for field studies conducted in Indiana in 2016 and 2017.

| Field site | Weed species | Mechanism of resistance to ALS inhibitors[a] | Herbicide application dates |
|---|---|---|---|
| Lafayette 2016 | Tall waterhemp | W574L: 33%<br>S653N: Unknown | PRE: 6-3-16<br>POST: 6-27-16 |
| Lafayette 2017 | Tall waterhemp | W574L: 61%<br>S653N: 70% | PRE: 5-25-17<br>POST: 6-17-17 |
| Farmland | Tall waterhemp | W574L: 52%<br>S653N: 0% | PRE: 6-3-17<br>POST: 7-14-17 |
| West Lafayette | Horseweed | P197L: 91% | PRE and POST: 4-12-17 |
| Brookston | Horseweed | P197L: 76% | PRE and POST: 4-17-17 |

[a]Abbreviations: W574L, Trp574Leu; S653N, Ser653Asn; P197L, Pro197Leu.

Table 2.2. Sources of material and rates used in field experiments.

| Herbicde | Trade name | Formulation[a] | Manufacturer | Location | Rate(s)[b] |
|---|---|---|---|---|---|
| Atrazine | Aatrex® | 4 L | Syngenta | Greensboro, NC | 1121, 2242 g ai $ha^{-1}$ |
| Chlorimuron | Classic® | 25 DG | DuPont | Wilmington, DE | 11, 22, 44 g ai $ha^{-1}$ |
| Cloransulam | FirstRate® | 84 DG | DuPont | Wilmington, DE | 35 g ai $ha^{-1}$ |
| Fomesafen | Flexstar® | 1.88 SL | Syngenta | Greensboro, NC | 330 g ai $ha^{-1}$ |
| Imazethapyr | Pursuit® | 2 L | BASF | Research Triangle Park, NC | 71 g ae $ha^{-1}$ |
| Metribuzin | Sencor® | 75 DF | Bayer CropScience | Research Triangle Park, NC | 280 g ai $ha^{-1}$ |
| Saflufenacil | Sharpen® | 2.85 SC | BASF | Research Triangle Park, NC | 25 g ai $ha^{-1}$ |

[a]Abbreviations: DG, dispersible granule; L, liquid; SC, suspension concentrate; SL, soluble liquid.
[b]All rates were applied PRE and POST. POST applications included crop oil concentrate (COC) (Prime Oil, Winfield Solutions, LLC, St. Paul, MN) at 1%v/v.

Table 2.3. Control of tall waterhemp in populations with ALS-inhibitor resistance at 21 d after treatment, as affected by application timing in field experiments conducted in Lafayette and Farmland, IN in 2016 and 2017.

| | | Control[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | Lafayette 2016 | | Lafayette 2017 | | Farmland | |
| Herbicide | Rate | PRE | POST | PRE | POST | PRE | POST |
| | g ha $^{-1}$ | ----------%---------- | | | | | |
| Chlorimuron | 11 | 65 c | 43 d | 66 de | 15 f | 43 cde | 14 ef |
| | 22 | 69 c | 38 d | 69 de | 19 f | 20 ef | 4 f |
| | 44 | 92 ab | 66 c | 86 bc | 16 f | 44 cde | 16 ef |
| Imazethapyr | 71 | 34 d | 5 e | 10 fg | 5 g | 33 def | 10 f |
| Atrazine | 1121 | 97 ab | 90 b | 97 a | 55 e | 56 bcd | 73 abc |
| | 2242 | 95 ab | 95 ab | 98 a | 78 cd | 60 abc | 66 abc |
| Fomesafen | 330 | 99 a | 96 ab | 95 ab | 88 b | 74 ab | 91 a |

[a]Means followed by the same letter within a site and year are not significantly different according to Tukey's LSD ($\alpha$ = 0.05).

Table 2.4. Density evaluation of a population of tall waterhemp with ALS-inhibitor resistance at 14 d after POST treatments as affected by application timing of ALS inhibitor in field experiments conducted in Lafayette and Farmland, IN in 2017. Results are pooled across site years.

| | | Density[ab] | | | |
|---|---|---|---|---|---|
| Herbicide | Rate | PRE | | POST | |
| | g ha $^{-1}$ | ---------- % of nontreated | | ---------- | |
| Nontreated | | 100 | d | 100 | d |
| Chlorimuron | 11 | 56 | cd | 85 | cd |
| | 22 | 62 | cd | 94 | d |
| | 44 | 64 | cd | 64 | cd |
| Imazethapyr | 71 | 87 | cd | 92 | cd |
| Atrazine | 1121 | 49 | bc | 26 | ab |
| | 2242 | 62 | cd | 32 | ab |
| Fomesafen | 330 | 16 | a | 8 | a |

[a] Densities are presented as percentage of respective nontreated control.
[b]Means followed by the same letter are not significantly different according to Tukey's LSD ($\alpha$=0.05).

Table 2.5. Average rating and mortality for marked tall waterhemp plants at 14 d after POST treatment, as affected by herbicide active ingredient in field experiments conducted in Lafayette and Farmland, IN in 2016 and 2017. Control values for the Lafayette field site were pooled across years.

| | | Control[a] | | | | Mortality | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Herbicide | Rate | Lafayette '16 & '17 | | Farmland | | Lafayette '16 | | Lafayette '17 | | Farmland | |
| | g ha $^{-1}$ | ----------%---------- | | | | ----------%---------- | | | | | |
| Chlorimuron | 11 | 44 | c | 4 | D | 41 | b | 17 | cd | 2 | C |
| | 22 | 44 | c | 3 | D | 36 | b | 26 | c | 0 | C |
| | 44 | 49 | c | 5 | D | 49 | b | 15 | cd | 0 | C |
| Imazethapyr | 71 | 8 | d | 3 | D | 6 | c | 3 | d | 0 | C |
| Atrazine | 1121 | 87 | b | 71 | C | 92 | a | 71 | b | 57 | B |
| | 2242 | 97 | a | 78 | B | 97 | a | 96 | a | 72 | A |
| Fomesafen | 330 | 99 | a | 92 | A | 97 | a | 98 | a | 77 | A |

[a]Means followed by the same letter within a site and year are not significantly different according to Tukey's LSD ($\alpha$=0.05).

Table 2.6. Horseweed control ratings at 21 d after treatment and density counts as affected by application timing and herbicide active ingredient in a field study conducted in Brookston and West Lafayette, IN in 2017.

| | | Control[a] | | | | | | | | Density[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Brookston | | | | West Lafayette | | | | Brookston | | | | West Lafayette | | | |
| Herbicide | Rate | PRE | | POST | | PRE | | POST | | PRE | | POST | | PRE | | POST | |
| | g ha $^{-1}$ | ---------- | | | | %---------- | | | | ---------- | | | | % of nontreated[c] ---------- | | | |
| Chlorimuron | 11 | 52 | cd | 53 | cd | 100 | a | 35 | c | 47 | c | 113 | e | 20 | a | 60 | bc |
| | 22 | 83 | ab | 58 | cd | 100 | a | 28 | c | 43 | cd | 83 | de | 0 | a | 71 | bc |
| | 44 | 89 | a | 65 | bc | 100 | a | 31 | c | 36 | bc | 21 | bc | 0 | a | 119 | c |
| Cloransulam | 35 | 94 | a | 61 | cd | 100 | a | 35 | c | 11 | ab | 40 | bc | 0 | a | 111 | c |
| Metribuzin | 280 | 99 | a | 44 | d | 100 | a | 14 | d | 1 | a | 27 | bc | 0 | a | 102 | c |
| Saflufenacil | 25 | 98 | a | 99 | a | 100 | a | 90 | b | 3 | a | 1 | a | 0 | a | 40 | b |

[a]Means followed by the same letter within a field site are not significantly different according to Tukey's LSD ($\alpha$ = 0.05).

Table 2.7. Average biomass, rating, and weight of marked horseweed at 14 d after POST treatments in field experiments conducted in Brookston and West Lafayette, IN in 2017. Values are pooled across locations.

| Herbicide | Rate | Biomass[a] | Control | Mortality |
|---|---|---|---|---|
| | g ha $^{-1}$ | g plant$^{-1}$ | % | % of NTC[b] |
| NTC[b] | | 1.5 c | | |
| Chlorimuron | 11 | 0.7 b | 28 b | 3 b |
| | 22 | 0.6 b | 30 b | 3 b |
| | 44 | 0.5 b | 37 b | 3 b |
| Cloransulam | 35 | 0.5 b | 40 b | 5 b |
| Metribuzin | 280 | 0.8 b | 41 b | 20 b |
| Saflufenacil | 25 | 0.1 a | 96 a | 71 a |

[a]Means in followed by the same letter within a column are not significantly different (LSD at $\alpha$=0.05).

[b]Abbreviations: NTC, nontreated control.

Table 2.8. Model parameters from log-logistic analysis predicting responses for R and S horseweed, tall waterhemp, and Palmer amaranth accessions. Three-parameter log-logistic model based on biomass reduction as determined by percent of biomass reduction 21 d after treatment in tall waterhemp and Palmer amaranth and 28 d after treatment in horseweed. Data from repeated experiments were pooled for analysis.[a]

| | | | | Dose-response model parameters[b] | | | |
|---|---|---|---|---|---|---|---|
| | | | | Dry weight | | | |
| Weed species | ALS-inhibitor | Application timing | Accession | *b* | *d* | $ED_{50}$ | $ED_{50}$ R:S |
| | | | | | % of control | g ai $ha^{-1}$ | |
| Tall waterhemp | Chlorimuron | PRE | R | 0.63 | 132.70 | 82.21 | 4110.50 |
| | | | S | 0.33 | 102.97 | 0.02 | |
| | | POST | R | 0.41 | 94.20 | 444.16 | 281.11 |
| | | | S | 0.38 | 99.72 | 1.58 | |
| Palmer amaranth | Chlorimuron | PRE | R | 0.45 | 116.85 | 265.10 | 4418.33 |
| | | | S | 0.64 | 94.48 | 0.06 | |
| | | POST | R | 0.48 | 102.13 | 592.56 | 790.08 |
| | | | S | 1.07 | 98.93 | 0.75 | |
| Horseweed | Chlorimuron | PRE | R | 1.03 | 108.27 | 5.93 | 84.71 |
| | | | S | 0.53 | 100.00 | 0.07 | |
| | | POST | R | 1.58 | 103.02 | 8.88 | 21.14 |
| | | | S | 1.77 | 107.99 | 0.42 | |
| | Cloransulam | PRE | R | 1.57 | 107.22 | 1.81 | 8.62 |
| | | | S | 1.17 | 104.05 | 0.21 | |
| | | POST | R | 1.18 | 103.15 | 4.57 | 8.62 |
| | | | S | 1.71 | 102.93 | 0.53 | |

[a]All POST treatments included 1% v/v crop oil concentrate.

[b]*b*, relative slope around *e* ($ED_{50}$); *d*, upper asymptote; $ED_{50}$, effective dose of herbicide that decreased shoot dry mass by 50% relative to nontreated control plants.

# CHAPTER 3. ASSESSING GENETIC CHANGES FROM ALS-INHIBITOR APPLICATIONS ON ALS-INHIBITOR RESISTANT WEED POPULATIONS

## 3.1 Abstract

The value of acetolactate synthase (ALS)-inhibiting herbicides (group #2) has been reduced in the face of widespread herbicide resistance in weeds. However, the impact of varying the use of ALS inhibitors on the genetic dynamics within a segregating population have largely been oversimplified and disregarded because more effective management options have been available. With the number of effective herbicides continuing to decline we need to understand the implications of using group #2 herbicides on resistant biotype selection and if certain ALS-inhibiting herbicides select for different resistance mutations than others. Field and lab research was conducted to determine the interaction of application timing (PRE vs. POST) and select ALS-inhibitor active ingredients on the frequency of ALS-inhibitor-resistant individuals in surviving populations of tall waterhemp and horseweed. Experiments were conducted at four locations, two in tall waterhemp and two in horseweed, with populations segregating for ALS-inhibitor resistance. Chlorimuron applied PRE at 11 and 44 g ai $ha^{-1}$ in tall waterhemp selected for 35 and 71% homozygous W574L genotypes, respectively. An increase of homozygous W574L individuals, along with a decrease in heterozygous individuals from 65% at the 11 g $ha^{-1}$ rate to 29% at the 44 g $ha^{-1}$ rate suggests that W574L is semi-dominant in tall waterhemp and that high labeled rates of chlorimuron applied PRE can overcome the heterozygous W574L-resistance mechanism. In horseweed, chlorimuron and cloransulam applied PRE and POST selected for a greater

number of resistant alleles than the non-treated checks, but no difference was detected in P197L allele frequency among group #2 herbicide treatments or application timings. Overall, altering the use of chlorimuron in terms of application timing and dose for control of tall waterhemp can influence the frequency of surviving resistant individuals and the zygosity of those individuals, as well as overcome the heterozygous W574L resistance mechanism.

## 3.2 Introduction

Herbicides that inhibit the acetolactate synthase (ALS) enzyme have foliar- and/or soil-residual activity and have been used in a wide variety of crops for the control of an even wider variety of weed species. The prevalent use of ALS-inhibiting herbicides eventually led to widespread weed resistance that includes 160 different species across the globe (Heap 2018). Use of ALS inhibitors has continued despite concerns of widespread resistance (USDA 2015, Trainer et al. 2005). Two weed species that have become especially problematic in the Midwest, tall waterhemp [*Amaranth tuberculatus* (Moq.) J.D. Sauer] and horseweed (*Erigeron canadensis*), have evolved multiple resistance to ALS-inhibiting herbicides and glyphosate (Kruger et al. 2009, Schultz et al. 2015). Tall waterhemp resistant to ALS-inhibitors (ALS-R) contain either a single-nucleotide polymorphism (SNP) on the gene coding for the ALS enzyme (*ALS*) or have enhanced cytochrome P450 (CP450) activity (Guo et al. 2015, Powles and Yu 2010), with target-site resistance being the most commonly reported (Patzoldt and Tranel 2007). In horseweed, ALS-inhibitor resistance is conferred by SNPs in the ALS gene (Zheng et al. 2011).

Different target site mutations within the ALS enzyme confer differing levels of resistance to each chemical family of ALS inhibitors and different weed species (Table 3.1). Target-site resistance (TSR) mutations usually confer a high level of resistance in field and greenhouse settings to foliar-applications. However, sparse research has been reported on the response of these ALS-R weed species to soil residual applications of ALS inhibitors. Previous research has shown that PRE applications of ALS-inhibitors in populations of tall waterhemp and horseweed with confirmed resistance to ALS-inhibitors result in more control than POST applications (Hustedde 2011). Research to quantify selection of resistant genotypes by specific herbicide treatments under field conditions is limited to tall waterhemp in response to soil-residual PPO-inhibitor applications (Wuerffel et al. 2015b). This research confirmed that soil-residual applications of PPO-inhibitors do select for the **Δ**G210 mutation, which confers resistance to PPO inihibitors. Our goal was to take this question further with ALS inhibitors, asking whether or not PRE applications of ALS inhibitors select for different frequencies of resistance alleles in the surviving populations than POST applications. Hence, the first objective of this research was to determine if the application timing, PRE vs. POST, of ALS-inhibiting herbicides influences selection for resistant biotypes and genotypes. A secondary objective was to determine the influence of different ALS inhibitor active ingredients on selection of resistance traits. The determination of genotypic response to application timing and specific ALS inhibitor active ingredient can help quantify the risk for resistance development when using certain ALS inhibitor active ingredients or when using different herbicide application timings.

## 3.3 Materials and Methods

### 3.3.1 Selection for R-Biotypes in Tall Waterhemp and Horseweed Populations Segregating for ALS-Inhibitor Resistance

Field experiments were performed to quantify changes in allele frequency for resistance traits in tall waterhemp and horseweed for ALS-inhibiting herbicides in response to soil-residual and foliar-applied herbicides. The research was conducted at agronomic field sites with segregating weed populations for ALS-inhibitor resistance (Table 3.2). The tall waterhemp experiment was conducted in 2017 at two field sites in Indiana: Farmland and Lafayette. Herbicide treatments included ALS inhibitors with PRE and POST activity with non-ALS inhibitor herbicides for comparison (Table 3.3). Chlorimuron was of specific interest since this herbicide demonstrated soil residual activity on tall waterhemp populations classified as resistant to ALS inhibitors (Hustedde 2011). Tall waterhemp experiments included chlorimuron at three rates (11, 22, and 44 g ai $ha^{-1}$), imazethapyr (70 g ae $ha^{-1}$), atrazine (1121 and 2242 g ai $ha^{-1}$), and fomesafen (330 g ai $ha^{-1}$). Tall waterhemp experiments were initiated in stale seedbed free of vegetation using an application of paraquat and glyphosate prior to trial establishment. Postemergence herbicide treatments were applied when tall waterhemp was 5- to 10- cm in height.

The horseweed field experiment was conducted in 2017 at two field sites in Indiana: Brookston and West Lafayette. Horseweed experiments included three rates of chlorimuron (11, 22, and 44 g $ha^{-1}$), cloransulam (35 g ai $ha^{-1}$), metribuzin (280 g ai $ha^{-1}$), and saflufenacil (25 g ai $ha^{-1}$). Plots assigned for a PRE application were treated with paraquat and glufosinate to remove existing vegetation and to allow for easier collection of emerged horseweed tissue after herbicide treatment. Postemergence herbicide

treatments were applied when horseweed was 4- to 11-cm in diameter during the rosette growth stage. These research methods allowed the PRE and POST applications to be performed on the same day.

Herbicide treatments for both tall waterhemp and horseweed experiments were applied with a $CO_2$-pressurized backpack sprayer with a 4-nozzle boom with 50 cm nozzle spacing using XR8002 VS nozzles, at 140 L $ha^{-1}$ and 276 kPa pressure. All field experiments were conducted in non-crop areas to reduce plant competition and potential spray interference for the target weed species. Plots were 3 m by 7.6 m long with nontreated borders along each plot.

Genomic DNA was obtained from leaf tissue collected at each site to analyze how each herbicide application affected frequency of resistance. Leaf tissue samples were collected from young, fully expanded leaves in the uppermost node of plants for genetic analysis. Prior to POST herbicide application, tissue was collected from 25 random tall waterhemp and horseweed plants from within each plot that were within the targeted spray height (tall waterhemp 5- to 10- cm tall; horseweed 4- to 11-cm diameter). Each plant sampled prior to herbicide application was marked with a wire flag and rated for visual control at 7 and 14 days after treatment (DAT), and rated as either dead or alive 35 DAT. This first collection would serve as a baseline of genotypic frequency for each POST treatment and would allow for the correlation of genotype to final efficacy of the herbicide treatment applied. Following both herbicide applications (PRE and POST), 25 additional individuals were sampled for genetic analysis. The first 25 plants to emerge from the PRE-treated plots were collected and 25 random plants that survived POST herbicide applications were sampled in POST-treated plots. Once collected, samples

were stored at -20 C until processed for the detection of the respective target site mutations in the ALS enzyme, as described below.

The experimental design for was a three-way factorial of site, treatment, and application timing, with factors completely crossed. The factorial was structured within a randomized complete block design using four replications. A nontreated control was included in all field experiments for comparison.

### 3.3.2 Molecular Techniques for Detection of ALS-Inhibitor Resistance

#### 3.3.2.1 Development of SNP Genotyping Method to Detect ALS-R Tall Waterhemp and Horseweed

Preliminary genetic analysis of the weeds at the field sites was conducted to document the presence of the different target site mutations in horseweed and tall waterhemp that confer resistance to ALS-inhibitors (Table 3.1). In order to quickly genotype ALS-inhibitor resistance in each collected tissue sample and determine genotypic selection pressure from each treatment, assays were developed following the TaqMan® technique. This methodology has been used by other researchers to detect SNPs conferring herbicide resistance in a number of weeds (Délye et al. 2010, Harre et al. 2017, Spaunhorst 2016, Warwick et al. 2008, 2010, Wuerffel et al. 2015a), but has yet to be adopted for all ALS-R-conferring mutations in tall waterhemp and horseweed. This method allows for the differentiation of resistant and susceptible alleles present in a DNA sample in the same reaction, thus allowing determination of individual plants that are heterozygous or homozygous for the resistance allele.

Tall waterhemp tissue was collected from both experimental sites. Genomic DNA (gDNA) was extracted from collected tissue using a modified cetyl trimethylammonium bromide (CTAB) method originally developed by Saghai-Maroof et al. (1984). Sites in

the ALS gene known to contain known mutations conferring resistance to ALS-inhibitors were sequenced (Table 3.1). The Trp574Leu and Ser653Asn mutations were found in the Lafayette population and only the Trp574Leu mutation was found in the Farmland population. For horseweed, plants from both locations were screened in the greenhouse for ALS-inhibitor resistance (data not shown). Plants found to survive a 48 g $ha^{-1}$ dose of cloransulam were considered resistant (Kruger et al. 2009) and subsequently tissue sampled and sequenced at locations in the ALS gene known to contain mutations conferring ALS-inhibitor resistance (Table 3.1). At both the Brookston and Lafayette locations, a new SNP was discovered, the Pro197Leu mutation, which is different from the two previously reported Pro197 amino acid substitutions conferring ALS-inhibitor resistance in horseweed (Zheng et al. 2011). Horseweed tissue collected from the Brookston field site also contained the Asp376Glu mutation.

Primers and TaqMan® probes were synthesized by ABI (Applied BioSystems, Grand Island, NY) to flank amino acid positions (Table 3.4) and discriminate between resistant and susceptible alleles using cDNA accessions from Genbank (Table 3.4). Extracted gDNA from field experiment samples were then genotyped using the TaqMan® assay. A 10-µl reaction was prepared for each sample using 5 µl $ddH_2O$, 2 µl 5X GoTaq Flexi buffer, 1.25 µl 25 mM $MgCl_2$, 0.1 µl 10 mM dNTP, 0.5 µl 20X primers and TaqMan® probes, 0.1 µl GoTaq Flexi polymerase (5 U $\mu l^{-1}$), and 1 µl gDNA. Reactions were amplified by a CFX384 RT-PCR detection system (Bio-Rad Laboratories, Hercules, CA). Cycling conditions were as follows: 3 min at 95 C; 39 cycles of 95 C for 10 s and 60 C for 1 min; followed by a plate read after every cycle. Positive controls from known ALS-R and ALS-S individuals were included in each reaction. Relative fluorescence of

each probe was used to distinguish between homozygous-resistant, homozygous-susceptible, and heterozygous *ALS* alleles using Bio-Rad CFX Manager software.

### 3.3.3 Statistical Analysis

Plants that are heterozygous for ALS target site mutations are often phenotypically resistant to POST-applied ALS inhibitors (Tranel and Wright 2002). Therefore, when calculating frequency of resistance (FOR), individuals that are both heterozygous (SR) and homozygous (RR) are considered resistant. The FOR was then calculated by taking the number of resistant individuals within a treatment divided by the total number of plants tested within the treatment. All FOR data was subjected to ANOVA using PROC MIXED (SAS 9.4, SAS Institute Inc., Cary, NC). Fixed effects for FOR analysis were herbicide treatment and time of application with random effects of location and replication. The genotype of each mutation [homozygous wildtype (SS), heterozygous (SR), and homozygous resistant (RR)] was analyzed using multinomial logistic regression (MLR) with the PROC GLIMMIX procedure in SAS. The main effect was treatment, which included application timing of either PRE or POST, herbicide active ingredient, and rate, with replication and location as random effects. Multinomial logistic regression is ideal to use in this situation, as the response variable analyzed is categorical (genotype). This type of regression works by breaking regression out into logits, or individual logistic functions. Each logit uses a fitted model to describe the log of probability of a response over the probability of the reference response. Due to different treatment trends, tall waterhemp data from the Lafayette and Farmland site were analyzed separately. The following is a general baseline category model with three response categories:

$$\log\left(\frac{\Pr(y=1)}{\Pr(y=2)}\right) = \alpha_1 + X\beta_{1i} + U\gamma_{ij} \begin{cases} i = 1,2,\ldots,15 \\ j = 1,2,3,4 \end{cases}$$

$$\log\left(\frac{\Pr(y=3)}{\Pr(y=2)}\right) = \alpha_2 + X\beta_{2i} + U\gamma_{ij} \begin{cases} i = 1,2,\ldots,15 \\ j = 1,2,3,4 \end{cases}$$

where response 2 serves as the reference category for both the logits, $\alpha$ is the intercept (in this case, the intercept is the coefficient for the nontreated control), β is the coefficient for fixed variable *X* (treatment; β for the nontreated control = 0), and $\gamma$ is the coefficient for random variable *U* (replication). Random variable $U\gamma_{ij}$ is Normally distributed. The nontreated control for PRE and POST applications were combined prior to MLR analysis as the two timings were not significantly different from each other.

## 3.4 Results and Discussion

### 3.4.1 Selection for R-Biotypes in Tall Waterhemp and Horseweed Populations Segregating for ALS-Inhibitor Resistance

#### 3.4.1.1 Tall Waterhemp Frequency of Resistance

A significant location-by-treatment-by-time of application interaction occurred while analyzing the FOR data so locations are presented separately. At the Farmland site, only the W574L mutation was found so plants are considered resistant if they contained the W574L mutation. At the Lafayette field site, both W574L and S653N were present. Since the W574L mutation confers broad resistance to sulfonylurea and imidazolinone ALS inhibitors, and the S653N mutation confers resistance to only imidazolinone herbicides, three FOR analyses are presented. One analysis considers the FOR of the W574L mutation at the Lafayette field site (Table 3.5), the W574L mutation at the Farmland field site (Table 3.5), the S653N mutation at the Lafayette field site (Table 3.6),

and another considering the FOR when accounting for the presence of either mutation at the Lafayette field site (Table 3.7).

The time of application-by-herbicide treatment interaction was non-significant so data are presented by herbicide treatment only, pooled over application timing. For the two analyses on the FOR of the W574L mutation only at the Lafayette and Farmland sites (Table 3.5), the time of application-by-treatment interaction was non-significant so data are also presented by herbicide treatment, pooled over application timing. At the Lafayette site, chlorimuron at all rates increased the frequency of resistance compared to the nontreated control, resulting in FOR values near 100%. As expected, fomesafen and atrazine applied at both rates did not influence the FOR (Table 3.5). Imazethapyr reduced the FOR of W574L compared to the nontreated control. This is probably due in part to the increased frequency of individuals with the S653N mutation after imazethapyr treatment (Table 3.6) and the fact that the two resistance mutations are mutually exclusive; that is, the two mutations occur on the same gene so the chances of having RR W574L and RR S653N in the same individual tall waterhemp plant would be extremely rare. The result is still puzzling, however, as it is unlikely that imazethapyr killed any of the ALS-inhibitor resistant individuals as the magnitudes of resistance for both mutations (W574L and S653N) were very high (Table 3.1). Other proposed explanations include the death of ALS S individuals within imazethapyr-treated plots. The nontreated control plots had on average of 5% individuals without either resistance mutation and imazethapyr-treated plots had on average 3% individuals without either resistance mutation. However, this difference only accounts for a 2% change in resistance frequency and is likely within the margin of error and the difference in FOR for W574L between the

nontreated control and imazethapyr treated plots was 19% (Table 3.5). This suggests something else may be influencing the results.

From further genotypic analysis that considers the heterozygosity of both the W574L and S653N mutations together, we understand that the W574L and S653N resistance mutations are mutually exclusive, or that a tall waterhemp plant homozygous for W574L is unlikely to have the S653N resistance mutation in any form. In fact, from our analysis, we found none of the 1,600 individuals genotyped from the Lafayette field study contained both W574L RR and S653N RR (Table 3.10).

When looking at the Lafayette tall waterhemp site, we know that resistance mutations can be present in different combinations, such as a combination of an individual that is RS for W574L and RS for S653N (Table 3.10). When we only look at FOR at an individual plant level based on presence at any level of either resistance mutation, we leave out the potential effect combinations of these two mutations may be having on selection. Again, the instance of imazethapyr selecting for fewer tall waterhemp individuals resistant by the W574L mutation stands unexplained. This unknown garners further attention and highlights the importance of studying combinations of resistance genotypes to understand how they may influence resistance level and selection for herbicide-resistance traits.

At the Farmland site, the nontreated check has an initial frequency of 48% for the W574L mutation. This would have been a small enough percentage to allow for a noticeable increase in W574L after herbicide application and selection. However, no change in the FOR was observed following herbicide application (Table 3.5). The lack of change in the FOR at Farmland may be due to an alternative resistance mutation or

mechanism, or some other factor besides herbicides applying selection pressure. This would support visual control estimates reported in Chapter 2. In Chapter 2, the authors found that overall control was less than the proportion of ALS-S tall waterhemp in the population. The observations that show no difference in the frequency of resistance among any of the treatments or between any of the treatments and the nontreated control, suggest that the W574L resistance mutation is not being selected for or against and that an alternative mechanism is somehow masking selection differences. It also suggests that the unknown resistance mutation confers cross-resistance to both the sulfonylurea and imidazolinone families of ALS inhibitors, as there was no difference in the FOR between the nontreated control and chlorimuron or imazethapyr. Future research should be conducted to determine if another resistance mechanism is present in this population.

#### 3.4.1.2 Horseweed Frequency of Resistance

Data are presented by the main effects or their interactions, depending on the results from the ANOVA. At the West Lafayette site, only the Pro197Leu mutation was found so horseweed are considered resistant if they contained the P197L mutation (Table 3.8). At the Brookston field site, the P197L, Asp-376-Glu, and D376E mutations were found (Table 3.8). The D376E mutation was found at a very small percentage, not in any of the nontreated check plots (data not shown), and only in the first block of the study, so the mutation was considered for analyzing overall frequency of resistance, but was not analyzed specifically as a separate SNP.

At the West Lafayette field site all of the PRE-applied ALS-inhibitor treatments increased overall resistance frequency compared to the nontreated control, increasing resistance frequency from 91% to 100% (Table 3.8). No differences in resistance

frequency were observed between PRE and POST applications of ALS inhibitors. There was a difference in frequency of resistance between PRE- and POST- applied metribuzin and saflufenacil, but neither treatment differed from the nontreated controls (Table 3.8). Despite near 100% control at the West Lafayette field site at 14 days after treatment (Chapter 2), ALS-inhibitor applications were still increasing the frequency of resistance compared to nontreated checks.

At the Brookston field site, all POST ALS-inhibitor treatments increased the FOR compared to the nontreated control. It is unclear if there would be a difference in selection between the two active ingredients as all resulting FOR values were near 100%. The FOR values were not different across herbicide application timings. The only exception was for saflufenacil applied POST resulting in 30% of the individuals with the P197L mutation and the PRE treatment resulting in 75% of the individuals with the P197L mutation (Table 3.8). From what was observed in Chapter 2, both the PRE- and POST-applied saflufenacil treatments resulted in control near 99% at the Brookston horseweed site. The change in the FOR for the P197L mutation in response to saflufenacil application timing may be indirectly influenced by the small sample size of individual surviving plants to the POST application (17 plants) due to the high level of herbicide efficacy. High levels of saflufenacil activity has been reported for foliar applications (Owen et al. 2011, Waggoner et al. 2011) and through soil residual activity of saflufenacil (Grossmann et al. 2010) that may have extended beyond the germination period of most horseweed at the Brookston field site.

The breakdown of individuals and their respective genotype sheds insight into the most likely form the P197L resistance mutation will take in horseweed at the Brookston

site (Table 3.9). Relatively few individuals were shown to be heterozygous for the P197L mutation in the nontreated control, unlike W574L in tall waterhemp, where the most common individual was heterozygous for W574L and heterozygous for S653N (Table 3.10). The prevalence of horseweed individuals homozygous for P197L within this population is likely due to the propensity of horseweed to self-pollinate (Smisek 1995). Future research could investigate whether or not horseweed heterozygous for P197L has a higher or lower level of resistance compared to individuals homozygous for P197L. An increase in resistance level for individuals homozygous for P197L vs. individuals heterozygous for P197L might explain why differences between PRE and POST applications were observed in tall waterhemp, but not necessarily in horseweed (Chapter 2).

Our investigation into the genotypes present in the Brookston horseweed field population confirms Gould's (1995) conclusion that in selfing species, resistance will persist in homozygous form versus heterozygous form. In horseweed, with little outcrossing, a homozygous resistant individual will produce almost all RR progeny while individuals heterozygous for the resistance trait will produce approximately 25% homozygous resistant progeny. Over time, in populations of a selfing weed species, the frequency of heterozygous resistant individuals will decline (Charlesworth 1992). Although horseweed populations with resistance to ALS inhibitors may shift to predominantly homozygous individuals under selection from solely-applied ALS inhibitors, susceptible individuals within resistant populations may be controlled with ALS inhibitors, as well as other species of weeds that exist in agronomic fields and remain susceptible to ALS inhibitors.

### 3.4.2 Multinomial Logistic Regression

Although there was no statistical difference between application timings in the FOR analysis for the two tall waterhemp sites, observations within the data suggested that herbicide application timing in the Lafayette population of tall waterhemp was important. The results presented above focused only at the frequency of resistance based on the presence of resistance mutations at any level, homozygous or heterozygous, as previous researchers have done (Wuerffel et al. 2015b). Wuerffel et al. (2015b) found that there was no difference in survival percentage between individuals homozygous or heterozygous for the ΔG210 mutation after applications of fomesafen. Analysis of control data in this same research configuration (Chapter 2) showed that PRE applications of chlorimuron resulted in greater control than POST applications, suggesting that there may be a difference in response based on genotype of the resistant ALS allele. Further, we know there are two mutations present in the Lafayette population so we wanted to understand how surviving populations are selected for based on the interaction of these two ALS target site mutations, W574L and S653N. To accomplish this, we used multinomial logistic regression to analyze genotypic data.

For this analysis, 1,586 tall waterhemp individuals were successfully genotyped with TaqMan® assays to determine if an individual plant had either W574L or S653N and at what heterozygosity level (Table 3.10). The most common genotype in the nontreated control was heterozygous for each mutation (SR W574L, SR S653N). This is in contrast with the most common genotype observed in the nontreated control for horseweed (Table 3.9), but makes sense for an obligate outcrosser such as tall waterhemp (Costea et al. 2005, Gould 1995). No tall waterhemp individuals were found that contained both W574L RR and S653N RR (Table 3.10).

No herbicide treatment increased or decreased the frequency of the W574L SS and S653N SS genotype (Figure 3.1c). This is likely because there were only 5 individuals in the nontreated control that were this genotype. All PRE applications of chlorimuron decreased this genotype from 5 to 0 individuals. The frequency of individuals with the W574L SS and S653N RS genotype under selection from all three rates of chlorimuron, PRE and POST, decreased compared to the nontreated control (Figure 3.1a). The S653N resistance mutation does not confer resistance to sulfonylurea herbicides, so our observation is logical (Table 3.1) that chlorimuron controlled most individuals with the W574L SS S653N RS genotype. Applications of PRE-applied chlorimuron at 11g and 44 g $ha^{-1}$ and imazethapyr decreased the frequency of the W574L SS S653N RS genotype compared to respective POST treatments (Figure 3.1a). Imazethapyr increased the frequency of the W574L SS S653N RR genotype compared to the nontreated control (Figure 3.1b). Atrazine applied POST at 1121 g $ha^{-1}$ increased the frequency of the W574L SS S653N RS and W574L SS S653N RR genotype individuals (Figure 3.1a, Figure 3.1b). No clear reasoning can explain why a significant increase in frequency occurred for this treatment. However, the S653N mutation and atrazine resistance gene could potentially be linked. Previous research has shown that the *PPX2L* and *ALS* genes are linked, two genes that when mutated can confer resistance to two seemingly unrelated sites of action (PPO- and ALS-inhibitor resistance) (Tranel et al. 2016). Our research did not test any tall waterhemp populations for triazine resistance.

One of our most important findings is that as the rate of chlorimuron increases, the more frequent the W574L RR S653N SS genotype becomes (Figure 3.1f). The increase of genotypes RR for W574L and associated decrease of genotypes RS for

W574L (Figures 3.1d, e) leads us to the conclusion that PRE applications of chlorimuron and higher rates of chlorimuron are able to control tall waterhemp heterozygous for the W574L mutation and subsequently select for individuals homozygous for W574L.

Two surprising results from this analysis is that PRE applications of imazethapyr decreased the overall frequency of W574L RS S653N SS individuals compared to the nontreated control (Figure 3.1d). At the same time, fomesafen applied POST increased the frequency of W574L RS S653N SS and W574L RR S653N SS compared to the nontreated control (Figures 3.1d, f). This may be the result of the *PPX2L* and *ALS* genes being linked, although Tranel et al. (2016) indicated this linkage may not have any affect in field conditions. The ΔG210 mutation on *PPX2L* was found at a frequency of 4.5% at the Lafayette field site (data not shown).

Our research shows a strong selection pressure towards more homozygous resistant individuals in response to PRE applications of ALS inhibitors, which suggests that at the field level, ALS-R individuals (due to the W574L mutation) can still be controlled with high rates of chlorimuron applied PRE, as opposed to the same treatment applied POST.

## 3.5 Implications

Viable herbicide options in soybean production have become increasingly limited for control of weeds with multiple herbicide resistance traits. To manage weeds with established herbicide resistance mechanisms and mitigate the potential to evolve weed resistance to the few remaining, effective herbicides we must deploy an integrated approach that optimizes herbicide use. Our research highlights the importance of the soil residual activity (i.e. PRE) and high application rates of ALS-inhibiting herbicides for

control of tall waterhemp heterozygous resistant with the W574L mutation. Furthermore, our research demonstrates that specific target site mutations can respond differently at the population level to selection from herbicides. Thus, field-level management for tall waterhemp populations with the S653N mutation may respond differently to the inclusion of ALS-inhibitors than tall waterhemp populations with the W574L mutation, especially if chlorimuron is used in PRE applications at high rates. All these observations are a product of the biologically effective dose of the herbicide overcoming these target site resistance mechanisms. Currently, the biologically effective dose for the TSR mechanisms studied in this research falls within current herbicide label application rates and use patterns.

This research documents the effectiveness of ALS-inhibiting herbicides for control of weed individuals that are heterozygous for some ALS target site mutations. These observations may be short-lived as weed populations quickly responded towards genotypes that were less sensitive to these herbicides. Weed management should never focus on a single herbicide as the sole means of controlling a weed species. Thus, a herbicide program that integrates multiple effective herbicide modes of action, as well as non-chemical control practices, can optimize the use ALS-inhibiting herbicides to potentially remove individuals with “low-level” herbicide resistance mechanisms and other susceptible weed species to mitigate resistance evolution to other effective herbicides.

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Table 3.1. Currently reported ALS-inhibitor resistance-conferring mutations in tall waterhemp and horseweed, with associated magnitudes of resistance.

| Species | *ALS* amino acid position | Magnitude of Resistance by ALS-Inhibitor Family | | | | Citation |
|---|---|---|---|---|---|---|
| | | Imidazolinone | Sulfonylurea | Triazolopyrimidine | Pyrimidinylthiobenzoate | |
| | | ----------R/S---------- | | | | |
| Tall waterhemp | Trp-574-Leu | >1000 | >614 | >32 | N/A | (Foes et al. 1998) |
| | Ser-653-Asn | 860 | N/A | N/A | N/A | |
| | Ser-653-Thr | 74 | N/A | N/A | N/A | (Patzoldt and Tranel 2007) |
| Horseweed | Pro-197-Ala | N/A | 40 | 50 | 72 | (Zheng et al. 2011) |
| | Pro-197-Ser | N/A | 25 | 70 | 55 | (Matzrafi et al. 2015) |
| | Asp-376-Glu | 9.1 | 34 | 33 | 580 | (Zheng et al. 2011) |
| | Ala-205-Val | >29 | 24 | N/A | >44 | |
| | Trp-574-Leu | >29 | 42 | N/A | >44 | (Matzrafi et al. 2015) |

Table 3.2. Test site characteristics for field studies conducted in Indiana in 2017.

| Test site | Weed species | Mechanisms of resistance to ALS inhibitors[ab] |
|---|---|---|
| Lafayette | Tall waterhemp | W574L: 61%<br>S653N: 70% |
| Farmland | Tall waterhemp | W574L: 52%<br>S653N: 0% |
| West Lafayette | Horseweed | P197L: 91% |
| Brookston | Horseweed | P197L: 76%<br>D376E: 0% |

[a]Abbreviations: W574L, Trp574Leu; S653N, Ser653Asn; P197L, Pro197Leu.
[b]Frequencies of resistance mutations calculated as the percentage of individuals within the nontreated controls with each mutation.

Table 3.3. Sources of material and rates used in field experiments.

| Herbicde | Trade name | Formulation[a] | Manufacturer | Location | Rate(s)[b] |
|---|---|---|---|---|---|
| Atrazine | Aatrex® | 4 L | Syngenta | Greensboro, NC | 1121, 2242 g ai $ha^{-1}$ |
| Chlorimuron | Classic® | 25 DG | DuPont | Wilmington, DE | 11, 22, 44 g ai $ha^{-1}$ |
| Cloransulam | FirstRate® | 84 DG | DuPont | Wilmington, DE | 35 g ai $ha^{-1}$ |
| Fomesafen | Flexstar® | 1.88 SL | Syngenta | Greensboro, NC | 330 g ai $ha^{-1}$ |
| Imazethapyr | Pursuit® | 2 L | BASF | Research Triangle Park, NC | 71 g ae $ha^{-1}$ |
| Metribuzin | Sencor® | 75 DF | Bayer CropScience | Research Triangle Park, NC | 280 g ai $ha^{-1}$ |
| Saflufenacil | Sharpen® | 2.85 SC | BASF | Research Triangle Park, NC | 25 g ai $ha^{-1}$ |

[a]Abbreviations: DG, dispersible granule; L, liquid; SC, suspension concentrate; SL, soluble liquid.
[b]All rates were applied PRE and POST. POST applications included crop oil concentrate (COC) (Prime Oil, Winfield Solutions, LLC, St. Paul, MN) at 1%v/v.

Table 3.4. Descriptions of TaqMan probes.

| Species | *ALS* amino acid substitution | Primer sequence[abc] | Probe sequence (5' to 3') |
|---|---|---|---|
| Tall waterhemp | Trp574Leu | 5'-CCGGTTAAAATCAGCTCTTGAACAAT-3' | ATCGATCTTCCAATTGAA (VIC) |
| | | 5'-TGTGCCCGGTTAGCTTTGTAAA-3' | TCGATCTTCCCATTGAA (FAM) |
| | Ser653Asn | 5'-GTAATCGTACCACATCAGGAGCAT-3' | ATGATCCCTAGCGGTGCC (VIC) |
| | | 3'-AGCCCTTCTTCCATCACCCT-5' | ATGATCCCTAACGGTGCC (FAM) |
| Horseweed | Pro197Leu | 5'-CCCGTCGTTGCCATCAC-3' | CCAAGTTCCCCGGCGAA (VIC) |
| | | 3'-TCTTGAAAAGCATCAGTTCCGATCA-'5 | CCAAGTTCTCCGGCGAA (FAM) |
| | Asp376Glu | 5'-GGATTTGTTGCTTGCGTTTGG-'3 | CAGTCACACGGTCATCA (VIC) |
| | | 3'-TGAACAATCTTAGCTCTACTAGCAAAAGC-5' | CAGTCACACGTTCATCA (FAM) |

[a]Forward primer sequence followed by reverse primer sequence.
[b]Tall waterhemp primer and probe sequences based off of complete cds from *A. tuberculatus* (Genbank EF157819.1; 2010 bp)
[c]Horseweed primer and probe sequences based off of partial cds from *E. canadensis* (Genbank HM067014.1; 1818 bp)

Table 3.5 The influence of herbicide rate and active ingredient on tall waterhemp frequency of resistance considering only the W574L mutation at the Lafayette and Farmland, IN sites in 2017. Time of application was not significant so both applications are combined for an average.

| | | W574L FOR[ab] | | | |
|---|---|---|---|---|---|
| Herbicide | Rate | Lafayette | | Farmland | |
| | g ha $^{-1}$ | ----------% | | ---------- | |
| NTC[b] | | 64 | bc | 48 | a |
| Chlorimuron | 11 | 97 | d | 57 | a |
| | 22 | 95 | d | 59 | a |
| | 44 | 98 | d | 61 | a |
| Imazethapyr | 71 | 45 | a | 61 | a |
| Atrazine | 1121 | 56 | ab | 59 | a |
| | 2242 | 52 | ab | 51 | a |
| Fomesafen | 330 | 71 | c | 63 | a |

[a]Means followed by the same letter within a site and year are not significantly different according to Tukey's LSD (α=0.05).
[b]Abbreviations: FOR, frequency of resistance; NTC, nontreated control.

Table 3.6. The influence of herbicide rate and active ingredient on tall waterhemp frequency of resistance considering only the S653N mutation at the Lafayette, IN field site in 2017.

| | | S653N FOR[ab] | | | |
|---|---|---|---|---|---|
| Herbicide | Rate | PRE | | POST | |
| | g ha $^{-1}$ | ----------%---------- | | | |
| NTC[b] | | 67 | ef | 77 | ef |
| Chlorimuron | 11 | 35 | bc | 65 | e |
| | 22 | 24 | ab | 64 | de |
| | 44 | 17 | a | 49 | cd |
| Imazethapyr | 71 | 93 | g | 81 | fg |
| Atrazine | 1121 | 65 | e | 72 | ef |
| | 2242 | 78 | ef | 72 | ef |
| Fomesafen | 330 | 63 | de | 44 | c |

[a]Means followed by the same letter within a field site are not significantly different according to Tukey's LSD ($\alpha = 0.05$).
[b]Abbreviations: FOR, frequency of resistance; NTC, nontreated control.

Table 3.7. The influence of herbicide rate and active ingredient on tall waterhemp frequency of resistance considering both the W574L and S653N mutations at the Lafayette, IN field site in 2017.

| | | Overall FOR[ab] | | | |
|---|---|---|---|---|---|
| Herbicide | Rate | PRE | | POST | |
| | g ha $^{-1}$ | ----------%---------- | | | |
| NTC[b] | | 94 | bc | 97 | bc |
| Chlorimuron | 11 | 100 | c | 100 | c |
| | 22 | 100 | c | 99 | bc |
| | 44 | 100 | c | 99 | bc |
| Imazethapyr | 71 | 98 | bc | 99 | bc |
| Atrazine | 1121 | 94 | bc | 95 | bc |
| | 2242 | 96 | bc | 84 | a |
| Fomesafen | 330 | 93 | b | 97 | bc |

[a]Means followed by the same letter within a field site are not significantly different according to Tukey's LSD ($\alpha = 0.05$).
[b]Abbreviations: FOR, frequency of resistance; NTC, nontreated control.

Table 3.8. The influence of herbicide rate and active ingredient on horseweed frequency of resistance considering both the P197L mutation at the West Lafayette, IN site and the P197L and D376E resistance mutations at the Brookston, IN site in 2017.

| | | Overall FOR[ab] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Brookston | | | | West Lafayette | | | |
| Herbicide | Rate | PRE | | POST | | PRE | | POST | |
| | g ha $^{-1}$ | ----------%---------- | | | | | | | |
| NTC[b] | | 81 | bc | 72 | b | 88 | a | 96 | b |
| Chlorimuron | 11 | 98 | cd | 98 | cd | 100 | b | 99 | b |
| | 22 | 94 | cd | 100 | d | 100 | b | 100 | b |
| | 44 | 96 | cd | 98 | cd | 100 | b | 100 | b |
| Cloransulam | 35 | 94 | cd | 100 | d | 100 | b | 100 | b |
| Metribuzin | 280 | 67 | b | 74 | b | 82 | a | 97 | b |
| Saflufenacil | 25 | 75 | b | 30 | a | 88 | a | 98 | b |

[a]Means followed by the same letter within a field site are not significantly different according to Tukey's LSD ($\alpha = 0.05$).
[b]Abbreviations: FOR, frequency of resistance; NTC, nontreated control.

Table 3.9. Horseweed genotypes identified by herbicide and application timing at the Brookston field site in 2017. N size was 97 to 100 for all treatments, with the exception of the saflufenacil POST treatment due to mortality and limited survivors.

| | | | Genotypic Composition[a] | |
|---|---|---|---|---|
| Herbicide | Rate | Genotype | PRE | POST |
| | g ha $^{-1}$ | P197L | ----------%---------- | |
| Nontreated | | SS | 19 | 27 |
| | | SR | 15 | 16 |
| | | RR | 65 | 54 |
| Chlorimuron | 11 | SS | 2 | 2 |
| | | SR | 20 | 8 |
| | | RR | 78 | 89 |
| | 22 | SS | 6 | 0 |
| | | SR | 3 | 4 |
| | | RR | 90 | 96 |
| | 44 | SS | 4 | 2 |
| | | SR | 5 | 8 |
| | | RR | 90 | 90 |
| Cloransulam | 35 | SS | 6 | 0 |
| | | SR | 6 | 8 |
| | | RR | 86 | 91 |
| Metribuzin | 280 | SS | 33 | 26 |
| | | SR | 7 | 11 |
| | | RR | 60 | 61 |
| Saflufenacil | 25 | SS | 25 | 7 |
| | | SR | 5 | 2 |
| | | RR | 69 | 8 |

[a]Genotype frequencies that do not add up to 100% are due to rounding.

Table 3.10. Genotypes that make up each treatment at the Lafayette tall waterhemp site in 2017. PRE and POST nontreated control totals were combined prior to analysis. N size was 97 to 100 for all treatments.

| | | | | Genotypic Composition[a] | |
|---|---|---|---|---|---|
| Herbicide | Rate | Genotype[b] | | PRE | POST |
| | g ha $^{-1}$ | W574L | S653N | ----------%---------- | |
| Nontreated | | SS | SS | | 5 |
| | | SR | SS | | 21 |
| | | RR | SS | | 2 |
| | | SS | SR | | 17 |
| | | SR | SR | | 41 |
| | | RR | SR | | 0 |
| | | SS | RR | | 14 |
| | | SR | RR | | 0 |
| | | RR | RR | | 0 |
| Chlorimuron | 11 | SS | SS | 0 | 0 |
| | | SR | SS | 30 | 29 |
| | | RR | SS | 35 | 6 |
| | | SS | SR | 0 | 5 |
| | | SR | SR | 34 | 59 |
| | | RR | SR | 0 | 0 |
| | | SS | RR | 0 | 1 |
| | | SR | RR | 1 | 0 |
| | | RR | RR | 0 | 0 |
| | 22 | SS | SS | 0 | 1 |
| | | SR | SS | 16 | 23 |
| | | RR | SS | 59 | 12 |
| | | SS | SR | 1 | 3 |
| | | SR | SR | 23 | 56 |
| | | RR | SR | 0 | 0 |
| | | SS | RR | 0 | 5 |
| | | SR | RR | 0 | 0 |
| | | RR | RR | 0 | 0 |
| | 44 | SS | SS | 0 | 1 |
| | | SR | SS | 12 | 34 |
| | | RR | SS | 71 | 15 |
| | | SS | SR | 0 | 2 |
| | | SR | SR | 17 | 44 |
| | | RR | SR | 0 | 0 |
| | | SS | RR | 0 | 1 |

Table 3.10 continued

| | | | | | |
|---|---|---|---|---|---|
| | | SR | RR | 0 | 1 |
| | | RR | RR | 0 | 0 |
| Imazethapyr | 71 | SS | SS | 2 | 1 |
| | | SR | SS | 3 | 15 |
| | | RR | SS | 2 | 3 |
| | | SS | SR | 14 | 34 |
| | | SR | SR | 43 | 23 |
| | | RR | SR | 0 | 0 |
| | | SS | RR | 36 | 23 |
| | | SR | RR | 0 | 0 |
| | | RR | RR | 0 | 0 |
| Atrazine | 1121 | SS | SS | 6 | 5 |
| | | SR | SS | 23 | 20 |
| | | RR | SS | 6 | 3 |
| | | SS | SR | 15 | 31 |
| | | SR | SR | 36 | 21 |
| | | RR | SR | 0 | 0 |
| | | SS | RR | 12 | 19 |
| | | SR | RR | 1 | 0 |
| | | RR | RR | 0 | 0 |
| | 2242 | SS | SS | 4 | 12 |
| | | SR | SS | 13 | 13 |
| | | RR | SS | 5 | 2 |
| | | SS | SR | 24 | 24 |
| | | SR | SR | 35 | 34 |
| | | RR | SR | 0 | 0 |
| | | SS | RR | 18 | 14 |
| | | SR | RR | 0 | 0 |
| | | RR | RR | 0 | 0 |
| Fomesafen | 330 | SS | SS | 7 | 3 |
| | | SR | SS | 24 | 32 |
| | | RR | SS | 6 | 21 |
| | | SS | SR | 23 | 16 |
| | | SR | SR | 32 | 24 |
| | | RR | SR | 0 | 2 |
| | | SS | RR | 8 | 2 |
| | | SR | RR | 0 | 0 |
| | | RR | RR | 0 | 0 |

[a]Genotype frequencies that do not add up to 100% are due to rounding.

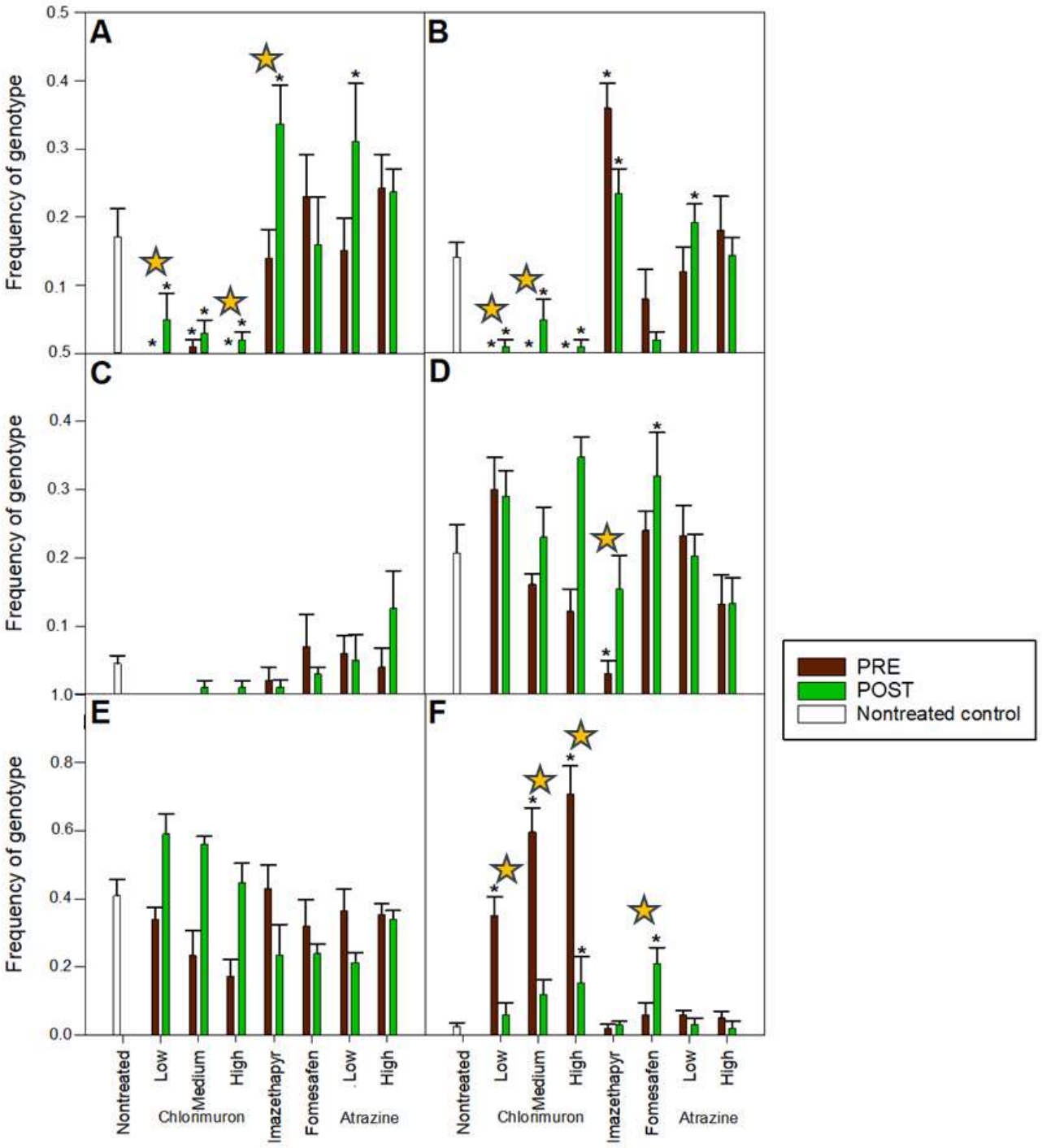


Figure 3.1. Effect of herbicides on specific tall waterhemp ALS-inhibitor resistant genotypes at the Lafayette field site. Charts are, in alphabetical order (A) W574L SS S653N RS (B) W574L SS S653N RR (C) W574L SS S653N SS (D) W574L RS S653N SS (E) W574L RS S653N RS (F) W574L RR S653N SS. *Indicates significant difference in frequency between a treatment and the nontreated control. Stars indicate significant difference in frequency between a POST and PRE application. The reference genotype for Multinomial Logistic Regression was W574L (SR) and S653N (SR).

# CHAPTER 4. DISTRIBUTION OF MUTATIONS CONFERRING ALS-INHIBITOR RESISTANCE IN TALL WATEREHMP IN INDIANA

## 4.1 Abstract

Management of tall waterhemp with multiple resistance to herbicides is one of the greatest challenges in soybean production in the U.S., with resistance to ALS-inhibiting herbicides being arguably the most common. Since resistance to ALS inhibitors is almost always confirmed in some plants found within field populations the perception is that these herbicide offer little value to waterhemp management. However, the practice of not using ALS inhibitors in weed management plans discounts the potential for control of susceptible individuals. A survey was conducted in the Fall of 2017 to determine the frequency of tall waterhemp plants that remain susceptible to ALS inhibitors within and across field populations in Indiana. Individuals within populations were genotyped for known SNP target site mutations (W574L, S653N, or S653T amino acid substitutions). In all populations (42) at least 16% of the individuals had the W54L amino acid substitution, 35 populations contained at least 1% of the individuals with the S653N mutation, and 9 populations contained at least 1% of the individuals with the S653T mutation. Taking into consideration the three mutations tested, 8 of the 42 populations contained <50% resistant individuals within the population. Results from Next-Generation Sequencing showed that 10 other amino acid substitutions in the ALS enzyme may be conferring resistance in tall waterhemp in Indiana: A122T, A122N, A122S, P197T, P197L, P197S, P197H, D376E, and G654F. Using pooled samples with NGS showed allelic frequency results similar to those achieved with SNP genotyping. Whole

plant greenhouse experiments revealed that metabolic resistance to ALS inhibitors is likely present in populations of tall waterhemp in Indiana. The combined greenhouse and NGS results suggest that ALS-inhibitor resistance in Indiana tall waterhemp was not well characterized previously and if future resistance screening is to occur on the state level, molecular methods such as NGS and greenhouse assays testing for metabolic resistance may need to be employed to detect all potential resistance mechanisms in a timely matter. Based on genotypic frequency results, there does exist significant value for certain ALS inhibitors to control susceptible tall waterhemp populations in Indiana.

## 4.2 Introduction

Tall waterhemp (*Amaranthus tuberculatus* var. *rudis*) is one of the most troublesome weeds throughout the Midwestern US due to widespread infestations and propensity for evolving resistance to herbicides (Tranel et al. 2011). Tall waterhemp has evolved resistance to seven different herbicide sites of action (Heap 2019) and biotypes have been reported to have multiple resistance to six sites of action (Shergill et al. 2018). The increased prevalence across the Midwest of tall waterhemp with multiple resistance to glyphosate, ALS-, and/or PPO-inhibitors has raised concern over the utility of POST-applied herbicides for soybean production (Patzoldt et al. 2005, Mansfield et al. 2017, Shergill et al. 2018). This is especially true for ALS inhibitors as resistance to this site of action has contributed to the mindset that ALS-inhibiting herbicides have little value in tall waterhemp populations today (Patzoldt et al. 2002). When ALS-inhibiting herbicides are avoided in weed management programs, the potential for these herbicides to control susceptible individuals goes unrealized. Field surveys conducted in the past have shown that, despite the prevalence of ALS-inhibitor-resistant tall waterhemp populations,

susceptible individuals remain within these populations (Falk et al. 2005, Kruger et al. 2009). Characterization of SNPs conferring ALS-inhibitor resistance has shown that some SNPs do not provide cross resistance to all ALS-inhibitor families (Table 4.1). Results from Chapter 2 and 3 also show that the zygosity of the Trp574Leu amino acid substitution in tall waterhemp can affect the efficacy of the ALS inhibitor chlorimuron. Thus, the potential for ALS inhibitors to contribute to management of tall waterhemp will not be realized until we know the specific target-site resistance mutations, the frequency of susceptible and resistant individuals in field populations, and the zygosity of the resistance mutations

The first instance of tall waterhemp resistant to ALS inhibitors was identified in 1991. Since then, many more tall waterhemp populations have evolved resistance to ALS inhibitors and several other mutations conferring ALS-inhibitor resistance have been discovered (Tranel et al. 2011). Resistance to ALS inhibitors in tall waterhemp is not limited to target-site resistance (TSR), as biotypes resistant to ALS inhibitors through non-target site resistance (NTSR) have been reported (Guo et al. 2015, Shergill et al. 2018). Inheritance of ALS-R by TSR is via a single gene with amino acid substitutions conferring resistance on two conserved regions of the ALS gene (Table 4.1). The ALS gene codes for the ALS enzyme, which is encoded in the nucleus. The ALS enzyme is the first of four shared steps of branched-chain amino acid synthesis (Buchanan et al. 2015). Results from Chapter 3 indicated that the W574L allele conferring resistance to ALS-inhibitors in tall waterhemp displayed incomplete dominance, with higher rates of chlorimuron selecting for more homozygous-resistant individuals than heterozygous individuals. The difference in sensitivity to ALS inhibitors between homozygous-

resistant (RR) tall waterhemp and heterozygous (RS) individuals remains undetermined, as well as how frequent the RR and RS genotypes occur in field populations.

The overall objective of this research was to document the frequency of susceptible individuals present in field populations of tall waterhemp with confirmed resistance to ALS inhibitors. The secondary objectives included determining if populations studied conform to Hardy-Weinburg and elucidating any population trends for TSR alleles if the soil seedbank and seed collected from mother plants deviated in the frequency of resistance alleles. Although only three TSR mutations have been described to confer resistance to ALS inhibitors in tall waterhemp, it is likely that other mutations, and potentially metabolic resistance, exist as other mutations in the ALS gene have been reported in additional *Amaranthus* species (Table 4.1). Assessing individual weed samples for resistance mutations can be time consuming and labor intensive. Therefore, an additional objective of this research was to assess the utility of a WideSeq bulking method to quickly analyze samples for resistance screening and to determine if other mutations in the ALS gene may also be contributing to tall waterhemp resistance to ALS-inhibiting herbicides.

## 4.3 Materials and Methods

### 4.3.1 Seed Collection and Sources

Tall waterhemp seed accessions were collected during September and October of 2017 from putative herbicide-resistant tall waterhemp in agronomic fields across Indiana. Collections were made in 14 counties with three populations collected from each county (42 populations total; Figure 4.1). Individual counties were selected based on past confirmation of ALS-inhibitor resistance in tall waterhemp and, therefore, were theorized

to present the regions with the greatest history or selection pressure for resistance to ALS inhibitors. Fields were selected based on the observation of poor control of tall waterhemp (i.e. plants with seed production near soybean harvest) at the time of collection. Each field population consisted of a bulk seed sample, assembled from seed from five individuals per field [as outlined by Burgos et al. (2013) for outcrossing species] and a bulk soil sample, consisting of six separate 292 $cm^3$ soil samples, taken across the field in a W-pattern with a bulb planter to a depth of 7.62 cm. At each field, GPS coordinates were recorded. Seed samples were dried at 40 C in a dryer for 1 wk and hand threshed thereafter. Clean seed was stored in plastic bags at 4 C until used in the greenhouse. Soil samples were also combined and stored in plastic bags, remaining in 4 C storage for at least 3 mos.

### 4.3.2 Plant Propagation

Before planting, bulk seed sample seeds were treated with a 9:1 water and commercial bleach solution for 10 min, washed with water, dried, and stored at 4 C 1 to 3 d prior to the start of experiments to improve germination. Each population was grown in separate 25- by 25-cm flats containing commercial potting media (Fafard Growing Mix 2, Conrad Fafard Inc., P.O. Box 790, Agawam, MA) and placed in the greenhouse with day and night temperatures of 30 and 25 C, respectively. Natural lighting was supplemented by high-pressure sodium bulbs delivering 1,100 μmol $m^{-2}s^{-1}$ photon flux density set to a 16-h photoperiod. Plants were grown to a size where they could be tissue sampled, around the 1- to 2-leaf stage.

Bulk soil samples were sown in the greenhouse to germinate tall waterhemp in the seedbank. Vermiculite was placed in the bottom of a hole-less 25- by 50- cm flat, then

covered with landscape fabric. Soil samples were then placed on top of the landscape fabric and spread across the flat. The samples were watered in and covered with a clear plastic dome to promote germination and removed at seedling emergence. Flats were kept moist for the duration of the grow-out. Flats were grown out until enough tall waterhemp were large enough to be tissue sampled at the 1- to 2-leaf stage. Soil samples layered in the flats were remixed as needed to promote further seed germination. Horseweed (*Erigeron canadensis* L.) that emerged in any flat was also sampled for this research.

### 4.3.3 Molecular Techniques for Detection of ALS Resistance

#### 4.3.3.1 Development of SNP Genotyping Method to Detect ALS-R Tall Waterhemp and Horseweed

Tissue was collected from young fully expanded leaves in the uppermost node of plants for genetic analysis. To compare two different methods for resistance-conferring allele detection, two leaf disc samples were collected from each plant: one small (2 cm in diameter) and one large (4 cm in diameter). For tall waterhemp, over 50 plants were tissue sampled from each flat. For horseweed, all plants that emerged from any soil flat were tissue sampled. For the single-nucleotide polymorphism detection experiment, as described in this paragraph, the large tissue sample was used. Genomic DNA (gDNA) was extracted from the collected tissue individually using a modified cetyl trimethylammonium bromide (CTAB) method originally developed by Saghai-Maroof et al. (1984).

Before conducting the research, several mutations that confer resistance to ALS-inhibitors in tall waterhemp were known (Table 4.1). In order to quickly genotype ALS-inhibitor resistance in each collected tissue sample and confirm NGS results, assays were developed following the TaqMan® technique. TaqMan® assays are allele specific and

provide the level of zygosity of the mutation assayed. The same probes utilized in Chapter 3 were used to detect the W574L and S653N mutations in tall waterhemp and the P197L and D376E mutations in horseweed. An additional probe was developed to detect the presence of the third mutation, S653T, that confers tall waterhemp resistance to ALS inhibitors. Primers and TaqMan® probes were synthesized by ABI (Applied BioSystems, Grand Island, NY) to flank amino acid position 653 of *ALS* and to discriminate between resistant (Threonine) and susceptible alleles (Serine) using the EF157819.1 reference sequence (Patzoldt and Tranel 2007). Forward and reverse primers were 5'-GTAATCGTACCACATCAGGAGCAT-3' and 3'-AGCCCTTCTTCCATCACCCT-'5, respectively. Probes used to overlap the mutation site were 5'-ATGATCCCTAGCGGTGCC-3' (VIC) and 5'-ATGATCCCTAACGGTGCC-3' (FAM). A 10-µl reaction was prepared for each sample using 5 µl $ddH_2O$, 2 µl 5X GoTaq Flexi buffer, 1.25 µl 25 mM $MgCl_2$, 0.1 µl 10 mM dNTP, 0.5 µl 20X primers and TaqMan® probes, 0.1 µl GoTaq Flexi polymerase (5 U $\mu l^{-1}$), and 1 µl gDNA. Reactions were amplified by a CFX384 RT-PCR detection system (Bio-Rad Laboratories, Hercules, CA). Cycling conditions were as follows: 3 min at 95 C; 39 cycles of 95 C for 10 s and 60 C for 1 min; followed by a plate read after every cycle. One positive control from known ALS-R and ALS-S individuals were included in each reaction. Relative fluorescence of each probe was used to distinguish between homozygous-resistant, homozygous-susceptible, and heterozygous *ALS* alleles using Bio-Rad CFX Manager software.

#### 4.3.3.2 WideSeq Analysis

To investigate an alternative method of testing for resistance-conferring mutations within populations of weeds, WideSeq, a next-generation sequencing (NGS) method, was

conducted. This method allows for the amplification of whole genes and, therefore, the detection of mutations anywhere within the gene. It also allows for the detection of alleles from multiple plants at once and therefore pooled samples can be used to decrease overall workload. The small tissue sample collected from 50 plants of each tall waterhemp population with known genotype from TaqMan® assays were pooled together in one sample prior to gDNA extraction, using the same methods as described above. The *ALS* region of pooled samples was amplified via PCR. The amplification product was purified and submitted for WideSeq analysis through the Purdue Genomics Core. WideSeq was performed using the Illumina MiSeq (Illumina, San Diego, CA) platform. Primer pairs for WideSeq were designed using tall waterhemp and horseweed ALS coding sequence (GenBank accession EF157819.1 and HM067014.1, respectively) to generate amplicons encompassing codons crucial for sensitivity to ALS inhibitors (Délye et al. 2015). An amplicon for the full *ALS* gene length could not reliably be obtained The full-length forward primer for tall waterhemp *ALS* was 5'-GTTGCGATGTTCTCGTTGAAGCTCTTGAACGT-3' and the full-length reverse was 5'-CTAATAAGCCCTTCTTCCATCACCCTCTGTGATGGT-3'. For horseweed, the full length forward primer was 5'-TATACAGTCCTCTGGACACAAACCCATCACTACCAC -3' and reverse 5'-TCGTTCTGCCATCACCCTCGGTGATCACATCCAT-3'. BBMap (Bushnell 2016) was used to map generated reads to the reference gene (GenBank EF157819.1 for waterhemp). The reference sequence for tall waterhemp *ALS*, EF15789.1, contained the W574L amino acid substitution so special care was taken to ensure the mapped sequence was read correctly at that amino acid position. Integrative

Genomics Viewer (IGV; Robinson et al. 2011, Thorvaldsdóttir et al. 2013) was used to view and summarize output generated by NGS. Mapping quality threshold was set to Phred-20.

### 4.3.4 Malathion Experiment

Malathion experiments were conducted to determine if cytochrome P450 mediated non-target site resistance was present in populations of tall waterhemp collected in Tippecanoe and Randolph Counties. Soil samples were collected from one field in each county in the Fall of 2017, refrigerated at 4C for three months and placed in 25- by 50- cm plastic flats, lined with potting media. Tall waterhemp that emerged from the soil seedbank were transplanted to 10 $cm^2$ pots once they reached the 1- to 2- leaf stage. Chlorimuron (Classic® 25 DG, DuPont, Wilmington, DE) was applied to seedlings (1.5- to 6.5-cm tall, 3.5- to 7- leaf stage) at two rates (4.4 g ai $ha^{-1}$ and 44 g $ha^{-1}$) with and without malathion insecticide (Spectracide Malathion insect spray concentrate, Spectrum Group, Division of United Industries, P.O. Box 142642, St. Louis, MO), a known cytochrome P450 inhibitor. Chlorimuron treatments included crop oil concentrate adjuvant (Prime Oil, Winfield Solutions, LLC, St. Paul, MN) at 1% v/v. Malathion was applied as previously described (Ma et al. 2013), with malathion applied at a rate of 2,000 g ai $ha^{-1}$ 1 h before a foliar application of chlorimuron, including 0.25% nonionic surfactant (Activator 90, Loveland Products, Inc., Greeley, CO). A soil drench of 5mM malathion solution (50 mL $pot^{-1}$) was applied 2 d after herbicide treatment. Plants were evaluated for visual control at 3, 7, and 14 DAT. Above ground biomass was harvested after the 14 DAT rating and dried for 72 hours at 41 C for dry weight determination. Populations were blocked based on minor height differences and arranged within a

randomized complete block design. Each experiment included eight replicates and was repeated in time.

### 4.3.5 Statistical Analysis

Observed genotypic frequencies were gathered from 50 tall waterhemp plants grown from the bulk seed collection from each of the 42 field populations surveyed. Individual field populations were considered the experimental unit in this analysis. A chi-square analysis was conducted using R software (R Foundation for Statistical Computing, Vienna, Austria) to test the observed genotypic frequencies of each population against the expected frequencies based on Hardy-Weinberg Equilibrium (HWE). Allelic frequencies of each population were calculated using equations [1] and [2], where *A* = frequency of the resistant allele, *a* = frequency of the susceptible allele, *n* = number of individuals within the population, and SS, RS, and RR represent the observed ($^{obs}$) number of homozygous-susceptible, heterozygous, and homozygous-resistant individuals, respectively.

$$A = \frac{2(count\ of\ RR^{obs}) + (count\ of\ RS^{obs})}{2n} \quad [1]$$

$$a = \frac{2(count\ of\ SS^{obs}) + (count\ of\ RS^{obs})}{2n} \quad [2]$$

Hardy-Weinberg expected genotypic frequencies were calculated with equation [3] using the allelic frequencies as calculated above. The symbols $A^2$, $2Aa$, and $a^2$ equal expected genotypic frequencies for RR, RS, and SS individuals, respectively.

$$A^2 + 2Aa + a^2 = A + a = 1 \quad [3]$$

The expected number of individuals within each genotype was calculated by multiplying the expected genotypic frequency by the sample size. Resulting quantities

represent expected ($^{exp}$) values of RR, RS, and SS genotypes. Genotypes provided from the TaqMan® assays were used in this analysis. Since the W574L mutation was the most common SNP found across populations and because it would be the most likely mutation selected for, it was the only mutation considered for analysis. The W574L mutation would be the most likely mutation selected for considering the W574L mutation confers cross-resistance to ALS inhibitors in the imidazolinone, sulfonylurea, and triazolopyrimidine families, whereas the S653N and S653T mutations confer resistance to ALS inhibitors in the imidazolinone family (Table 4.1).

Chi-square analyses were completed to compare the ratio of RR:SS:RS W574L individuals from seed-collection populations to the respective soil-collected population ratios to determine whether or not resistance frequency is different between the two samples. Only field populations that had 40 or greater individuals emerge from the soil-collected sample were considered for analysis. This resulted in 21 of the 42 populations to have a seed-collected and soil-collected genotype comparison.

The inbreeding coefficient ($F_{is}$) was calculated next to determine whether or not populations had more or less heterozygous individuals than expected. Tall waterhemp is an obligate outcrossing species so $F_{is}$ is a measure of inbreeding among siblings, or biparental inbreeding (Heywood 1993, Nason and Ellstrand 1995). Therefore, a positive $F_{is}$ indicates a lack of heterozygous (RS) individuals while a negative value indicates an excess of RS individuals (Guttieri et al. 1998). The equation to calculate $F_{is}$ is the following (Guttieri et al. 1998):

$$F_{is} = \frac{RR^{obs} - RS^{exp}}{RS^{exp}} \quad [4]$$

Biomass data from the malathion experiment for Randolph and Tippecanoe counties were subjected to ANOVA using PROC MIXED in SAS (Version 9.4, SAS Institute Inc., Cary, NC). No significant interaction existed between treatment and run, so runs were combined. Means were separated using Fisher's protected LSD ($\alpha = 0.05$).

## 4.4 Results and Discussion

### 4.4.1 Survey

Results from SNP genotyping of seed-collected population samples showed that at least 10% of the individuals tested from each of the 42 tall waterhemp populations contained the W574L mutation (Table 4.2). The W574L allele was the most common of the three mutations detected via TaqMan® assay and is the only mutation currently described in tall waterhemp that confers broad cross-resistance to each of the ALS-inhibiting herbicide families used to control tall waterhemp (Table 4.1). The percentage of individuals containing W574L within each field varied within and across counties. Six of the 42 fields contained 90% or more individuals with the W574L mutation. Sixteen of the 42 fields contained 50% or less individuals with the W574L mutation. Conversely, 38% of the fields surveyed contained less than a 50% infestation of tall waterhemp plants with the W574L mutation. Thus, herbicides from the sulfonylurea family such as chlorimuron may still provide meaningful efficacy that could contribute to weed management if no other resistance mechanisms are present in these fields.

When considering the two other described mutations that confer resistance to ALS-inhibitors in tall waterhemp, S653N and S653T, overall frequency of resistance for each population tends to increase compared to only considering the presence of the W574L mutation (Table 4.2). The S653N and S653T confer resistance to only the

imidazolinone family of ALS-inhibiting herbicides (Table 4.1). Thirty-five of the 42 populations tested contained at least one individual resistant to ALS-inhibitors by the S653N mutation, and 8 of the 42 populations tested contained at least one individual resistant by the S653T mutation. Since amino acid substitutions at the 653 position confer resistance to only imidazolinones, the presence of either S653N or S653T may be remnants of selection performed when imidazolinone herbicides such as imazethapyr were the predominant ALS-inhibitor family chemistry used (USDA 2015).

Overall, 1,966 (46.8%) mutant *ALS* alleles were detected. The prevalent mutation, W574L, was identified in 1,625 (39%) alleles. The less prevalent resistant alleles, S653N and S653T, were found in 300 (7.1%) and 43 (1.0%) alleles, respectively. All populations contained wild-type *ALS* alleles, due to the prevalence of heterozygous individuals, a product of the obligate outcrossing nature of tall waterhemp, the semi-dominant nature of ALS-inhibitor resistance conferred by mutations in the *ALS* gene, and the potential presence of other resistance-conferring mutations that have not yet been described.

Among the 2,100 plants tested from 42 populations, 2 (0.047%) *ALS* alleles contained two distinct mutations (BE1 contained one plant with a W574L SR and S653T RR genotype and DU1 contained one plant with a W574L SR and S653N RR genotype). This may be an error of SNP genotyping or a rare event, as all other *ALS* alleles (2,098) contained either one or zero unique resistance mutations. If a rare event, this would suggest recombination on the chromosome to allow for two ALS-R mutations on an allele.

### 4.4.2 Hardy-Weinberg and Inbreeding Coefficient Calculations

Adherence or deviation from Hardy-Weinberg equilibrium (HWE) can be an important tool in understanding the population genetics of a studied species. Assumptions that underline the theory include: 1) population size is infinite, 2) discrete generations, 3) random mating with respect to the allele, 4) no mutation, 5) no migration, and 6) no association between the genotypes and sex (Reilly 2009). Assumptions 2, 4, and 6 are satisfied considering ALS inhibitor resistance in tall waterhemp, as tall waterhemp is a summer annual (Sauer 1957), the *de novo* mutation rate in the ALS gene is not unusually high in tall waterhemp (Tranel and Wright 2002, Casale and Tranel 2018), and the ALS gene is encoded in the nucleus so it is not sex-linked (Tranel and Wright 2002). Considering the assumptions met, analyzing Hardy-Weinberg disequilibrium may be helpful to measure assumptions 1, 3, and 5.

Hardy-Weinberg equilibrium within each of the 42 populations from the tall waterhemp survey using the W574L mutation as the W574L mutation confers broad-cross resistance to ALS inhibitors and is most likely the mutation driving selection by ALS inhibitors. Only one of the populations tested was found to be in significant disequilibrium, Sullivan 2, which had a sample consisting of 20 RR, 29 RS and 1 SS individuals. Recent selection by an ALS inhibitor, survival of mother plants, and subsequent seed collection may explain the lack of SS individuals in the population and the subsequent disconformity to HWE. The overall lack of deviations from HWE may indicate that, over time in the absence of selection pressure, tall waterhemp populations slowly regress back to a state of expected heterozygosity. The ALS inhibitors are seldom, if ever, used as the primary means of controlling tall waterhemp in modern soybean or

corn production. This has implications for management, as previous research has shown that tall waterhemp heterozygous for ALS resistance may be less resistant to ALS inhibitors than tall waterhemp homozygous for ALS resistance (Chapter 2). This may be an area of value that weed managers can take advantage of with preemergence applications of sulfonylurea herbicides such as chlorimuron, in combination with other herbicides with different mechanisms of action.

In obligate outcrossing species such as tall waterhemp, the inbreeding coefficient ($F_{is}$) is an estimate of the degree to which sibling mating, or biparental inbreeding is occurring (Heywood 1993, Nason and Ellstrand 1995). The coefficient $F_{is}$ estimates the fractional reduction in heterozygosity compared to a random-mating population with the same allele frequencies (Guttieri et al. 1998). Therefore, a random-mating population at equilibrium will have an inbreeding coefficient of 0 while a value closer to 1 indicates less heterozygous individuals than expected and therefore biparental inbreeding (Guttieri et al. 1998) and values closer to -1 indicate an excess of heterozygous individuals than expected. Inbreeding coefficients derived from studied populations ranged from -0.36 to 0.28. Nineteen populations had inbreeding coefficients that approximated zero, 10 had inbreeding coefficients of 0.10 and above, and 14 had inbreeding coefficients that were -0.10 or below. This relatively tight range of inbreeding coefficient values may explain why no population was found to deviate from HWE. Again, this would support that there is a lack of selection pressure by ALS-inhibiting herbicides in these populations of tall waterhemp. In a similar study conducted by Wuerffel (2014) tall waterhemp populations with confirmed resistance to PPO inhibitors and confirmed applications of PPO inhibitors in the year before the survey was conducted showed a range of inbreeding coefficient

values of 0.37 to 0.85. No inbreeding coefficient value in the present study, negative or positive is greater than 0.37.

A difference in phenotypic response to xenobiotics between heterozygous and homozygous resistant individuals has been a keystone of resistance management in insects (Gould 1995). Several insecticide resistance traits have been found to be partially recessive; insecticide resistance management, therefore, focused on controlling RS and SS individuals while not controlling RR individuals (Gould 1995). Applying this concept to weed control is theoretically more difficult, as Gould (1995) presumed that the frequency of RR individuals in a population would predominate in a short period of time under intense selection pressure due to the sessile nature of plants and the limited distance of pollen dispersal. Predomination of RR individuals would even occur in obligate outcrossing species such as tall waterhemp (Gould 1995).

The only studies that have quantified resistance traits and their manifested genotype in field populations are those conducted by Guttieri et al. (1998) and Wuerffel (2014). Guttieri et al. (1998) used chlorsulfuron resistance in kochia (*Kochia scoparia*) as a phenotypic marker to discriminate among RR, RS, and SS genotypes. Wuerffel (2014), like the present study, used SNP genotyping in tall waterhemp to discriminate RR, RS, and SS genotypes for the ΔG210 deletion conferring resistance to PPO inhibitors. Out of 9 populations studied, Guttieri et al. (1998) reported that one had a high $F_{is}$, 0.32, 6 had negligible $F_{is}$'s, and the remaining 4 had large negative $F_{is}$'s, ranging from -0.35 to -0.52, similar to the present study. The population with the high $F_{is}$ was said to be due to assertive mating, thanks to a recent immigration event. The four populations with negative inbreeding coefficients were said to be due to selection by ALS-inhibiting

herbicides during the crop year, causing an underrepresentation of SS genotypes or because of some sort of heterozygous advantage (Guttieri et al. 1998). Although the current study does not include herbicide application data, recent selection by an ALS inhibitor could explain the large negative $F_{is}$ values associated with 4 of the 14 populations with relatively large negative $F_{is}$ values, Dubois 1, Hendricks 1, Posey 4 and Sullivan 3. The remaining ten populations with large negative $F_{is}$ values and a relatively high percentage of SS individuals may indicate that other resistance mutations exist in the population or that there is some sort of advantage of either not having the W574L or retaining the RS form. A slight (1%) fitness penalty has been reported in tall waterhemp containing the W574L mutation (Wu et al. 2017).

Overall, our findings suggest that over time, even in conditions that favor biparental inbreeding, populations can still have a low inbreeding coefficient. This may indicate that resistance traits, such as the W574L resistance mutation may persist over time in the heterozygous state.

### 4.4.3 Soil- vs. Seed-Collected Samples

The comparison of new seed rain versus the soil seedbank can indicate the trend in herbicide resistance for the overall field population. Not every soil collection produced 50 individual tall waterhemp plants to tissue sample and genotype. Therefore, only populations that had 40 or greater individuals tested are presented (Table 4.4). For the comparison between soil- and seed-collected samples, only the W574L mutation was used as this made the analysis robust. This analysis is justified because the W574L mutation is the most frequent ALS-inhibitor resistance mutation found in tall waterhemp in Indiana, and this mutation has broad cross-resistance to all ALS-inhibitor family

chemistries used in tall waterhemp, making it the driving mutation in populations of ALS-inhibitor resistant tall waterhemp. Six of the 21 populations with adequate soil-collected individuals contained a different make-up of W574L genotypes in the soil collection versus the seed-collected individuals. Half of the populations that exhibited a different soil- vs. seed-collected response had a higher percentage of resistant individuals by the W574L mutation in the seed-collected sample and half of the populations had a higher percentage of resistant individuals by the W574L mutation in soil-collected sample. No general trend emerged from this analysis, furthering the idea that resistance profiles change on a field by field basis depending on weed management practices.

### 4.4.4 WideSeq

A primer for the 5' end of the *ALS* gene was difficult to create (Délye et al. 2015, Panozzo et al. 2013) so the primer for tall waterhemp started at bp 271. This still allowed for an amplicon that included amino acid 122, an important amino acid whose substitution can confer resistance to ALS inhibitors.

Tall waterhemp and horseweed are diploid species (Thébaud and Abbott 1995, Waselkov and Olsen 2014), each with one copy of the ALS gene; therefore, two ALS alleles are expected for each individual plant. In pools of 50 tall waterhemp plants, 100 copies of the ALS gene are expected to be present. Not every read generated by NGS is quality so reads are filtered and then mapped to the reference gene. The number of reads mapped in each pooled sample ranged from 4,806 to 98,950 (data not shown). A more descriptive statistic on quality of reads provided from NGS is depth of read. Depth of read refers to the number of reads created for each bp. Average depth of read is, therefore, the total number of mapped reads divided by the number of bp in the amplicon

created. Average depth reads are listed in Table 4.5. Since 100 alleles are expected in each pool of plants, the average number of times each individual allele is expected to be sequenced is the depth of read value divided by 100. This value is also included in Table 4.5. Depth of read varied for each individual bp within each WideSeq sample and the actual number of reads $bp^{-1}$ increased for bps located in the middle of the gene and decreased for those located at the 5' and 3' end of the gene. Our threshold for considering nucleotide substitutions as being present in the pooled sampled was 1.0%, as 1 allele in a pooled sample of 100 is 0.01. This would be sensitive enough to pick up a nucleotide substitution present in the heterozygous state in one of 50 diploid tall waterhemp individuals.

#### 4.4.4.1 Identifying Resistance-Conferring Mutations

Integrative Genomics Viewer was utilized to identify sequences within the mapped reads that contained non-synonymous mutations in 8 ALS codons known to confer resistance to ALS inhibitors. Results from the analysis are listed in Table 4.5. All populations contained at least one non-synonymous mutation and all populations contained at least 5% alleles with the W574L substitution. Before this research, mutations conferring resistance to ALS inhibitors in tall waterhemp occurred at 2 positions: codon 574 and 653. Within the genus *Amaranthus*, 6 of the 8 possible conserved regions have been found mutated that confer resistance to ALS inhibitors (Table 4.1). Therefore, it seemed likely that other mutations in the ALS gene must occur in tall waterhemp that confer ALS-inhibitor resistance outside of the two previously described locations.

Our NGS results show that 6 out of 8 possible conserved locations in the ALS gene may contain mutations that confer resistance to ALS inhibitors (Table 4.6). Non-

synonymous mutations were found at codons 197, 376, 574, 653, and 654 (Tables 4.5, 4.6). No non-synonymous mutations were detected at codons 205 or 377. Amino acid transitions that have not been previously reported in tall waterhemp included substitutions from Ala to Thr, Asn, and Ser at codon 122; substitutions from Pro to Thr, Leu, Ser, and His at codon 197; substitutions from Asp to Glu at codon 376; and substitutions from Gly to Phe at codon 654 (Tables 4.5, 4.6). The most prevalent resistance allele was W574L, found in 37% of all alleles tested. This is the default amino acid substitution that many researchers use to confirm ALS-inhibitor resistance in tall waterhemp (Schultz et al. 2015, Tranel et al. 2011). The second-most prevalent resistance allele was Asn653 followed by Glu376 (Table 4.6). The Glu376 mutation likely confers broad-cross-resistance to the ALS-inhibitor families used in tall waterhemp, as such a resistance pattern has been reported previously in *Amaranthus* genus species (Table 4.1). Future research will include creating primers to sequence each of the amplicons generated and confirm what was reported through NGS with Sanger sequencing. Future research should also include growing out populations with certain new mutations and confirming that the substitutions reported do confer resistance to ALS inhibitors. Further, that research should also characterize resistance ratios for herbicides within the ALS inhibitor chemical families.

Another objective of this research was to compare resistant allele frequencies between TaqMan assay results and results reported by NGS. In general, results from SNP genotyping and NGS were similar, reporting similar allelic frequencies for the W574L, S653N, and S653T resistance mutations. The average difference between reported number of alleles from SNP genotyping and NGS was 2.8, 1.8, and 0.8, for the W574L,

S653N, and S653T resistance mutations, respectively (Table 4.5). The larger number for the W574L mutation is most likely due to the higher frequency of W574L in the populations compared to S653N and S653T. Differences in allele frequency between SNP genotyping and NGS for the W574L mutation may be due to error in preparing the 50 equally-sized tissue samples or error in tissue crushing for DNA extraction.

The wide array of possible mutations conferring resistance to ALS inhibitors in tall waterhemp cause us to recommend that research or service agencies that provide screening for target site resistance SNPs for ALS-inhibiting herbicides in tall waterhemp conduct a similar survey of the weed across their respective state and submit bulked tall waterhemp samples for NGS. Upon completion of NGS and an analysis of the results, new SNP genotyping probes could be created that reflect the ALS-inhibitor-resistance-conferring mutations that are most commonly found in the state, offering a more accurate result for submitters. Alternately, NGS may be the most simple and time-effective way of assessing tall waterhemp samples for ALS-inhibitor resistance in the future, considering the sheer number of codons involved with ALS-inhibitor resistance and the sometimes large number of possible amino acid substitutions associated with some codons (i.e. 122, 197, 376, and 653).

### 4.4.5 Malathion Experiment

In addition to TSR, NTSR has been reported in populations of tall waterhemp (Guo et al. 2015, Shergill et al. 2018). To determine whether or not NTSR resistance due to cytochrome P450 monooxygenase activity is present in tall waterhemp in Indiana, two populations from counties included in the tall waterhemp survey were used in this experiment. Individual tall waterhemp from both populations were treated with an ALS

inhibitor, with or without malathion, a CP450 inhibitor. Responses were then compared (Figures 4.2, 4.3). Herbicide doses were selected based on the response of a susceptible population of tall waterhemp where 4.4 and 44 g $ha^{-1}$ of chlorimuron resulted in approximately 80 and 100% control, respectively (Chapter 2). Malathion alone did not result in substantial injury to the treated plants compared to the nontreated control (Figures 4.2, 4.3). Both Tippecanoe and Randolph County populations are known to be populations segregating for ALS-inhibitor resistance. In general, the addition of malathion to chlorimuron at both chlorimuron rates resulted in less biomass (more sensitive) than the respective chlorimuron treatment alone, and the malathion alone treatment (Figures 4.2, 4.3). These results are consistent with other studies that have used malathion in combination with other herbicides to confirm the presence of enhanced CP450 activity (Guo et al. 2015, Nakka et al. 2017). The populations that were used in this experiment were both populations segregating for ALS-inhibitor resistance due to different resistance mutations, i.e. the Tippecanoe County population with both W574L and S653N and the Randolph County population with W574L and an unknown, suspected resistance mutation. This experiment confirmed that enhanced CP450 activity also plays an important role in resistance to chlorimuron in both of these populations. Approximately 75% of tall waterhemp tested exhibited resistance to chlorimuron via enhanced CP450 activity.

Despite years of research on the subject of ALS inhibitor-resistance, there are still new insights to gain on the most common type of herbicide resistance worldwide. Data presented confirm that some populations tested have a majority of individuals that do not have the W574L mutation. Further, a majority of the populations tested had the W574L

resistance mutation present in the heterozygous form, suggesting that this form of W574L may predominate in populations over time. Fortunately, for weed managers, sulfonylurea herbicide applications can still provide meaningful control of tall waterhemp heterozygous for W574L (Chapter 2). Unfortunately, for weed managers, tall waterhemp populations may contain more resistance-conferring mutations than previously thought. WideSeq analysis uncovered eight new amino acid substitutions in the studied tall waterhemp populations that previous researchers have found confer resistance to ALS inhibitors in other *Amaranthus* species. Metabolic resistance mediated by enhanced CP450 activity was also confirmed in two tall waterhemp populations. The value of ALS-inhibiting herbicides will vary in different tall waterhemp populations, but will most likely come from PRE applications, in combination with other effective modes of action. Future utility of ALS inhibitors in tall waterhemp may come down to knowing what resistance mutations exist in each field, as pest management is accomplished most successfully on the field scale.

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Table 4.1. Known amino acid substitutions that confer ALS-inhibitor resistance and their associated magnitudes of resistance in *Amaranthus* species.

| *ALS* amino acid position | *Amaranthus* species | Magnitude of resistance by ALS-inhibitor family | | | | Citation |
|---|---|---|---|---|---|---|
| | | Imidazolinone | Sulfonylurea | Triazolopyrimidine | Primidinylthiobenzoate | |
| | | ----------R/S---------- | | | | |
| Ala-122-Thr | *retroflexus* | 33 | S | N/A | N/A | (Ferguson et al. 2001, McNaughton et al. 2005) |
| | *powellii* | 769 to 3,438 | S | N/A | N/A | |
| | *hybridus (syn: quitensis)* | 730 to 1,350 | 0 to 7.0 | S | 0.01 to 0.3 | (Poston et al. 2000, Whaley et al. 2006) |
| Ala-122-Ser | *palmeri* | N/A | N/A | N/A | N/A | (Larran et al. 2017) |
| Pro-197-Leu | *retroflexus* | 4 to 63 | 6 to 127 | 20 to 35 | 11 | (Sibony et al. 2001) |
| Pro-197-Ser | *blitoides* | S | R | R | R | (Sibony and Rubin 2003) |
| | *palmeri* | N/A | 275 | N/A | N/A | (Nakka et al. 2017) |
| Ala-205-Val | *retroflexus* | 95 | S | N/A | N/A | (Ferguson et al. 2001, McNaughton et al. 2005) |
| Asp-376-Glu | *hybridus (syn: quitensis)* | 60 | 1,300 to 3,261 | 174 | 213 | (Whaley et al. 2011) |
| | *powellii* | 28 | 23 | 10 | 47 | (Ashigh et al. 2009) |
| | *retroflexus* | 19 | N/A | N/A | N/A | (Huang et al. 2016) |
| Trp-574-Leu | *tuberculatus* var. *rudis* | >1000 | >614 | >32 | N/A | (Foes et al. 1998) |
| | *blitoides* | R | R | R | R | (Sibony and Rubin 2003) |
| | *retroflexus* | 13 to 168 | 270 to 1,104 | N/A | N/A | (Ferguson et al. 2001, McNaughton et al. 2005) |
| | *powellii* | 916 to 1,284 | 1,257 to 2,416 | N/A | N/A | |

Table 4.1 continued

| | | | | | | |
|---|---|---|---|---|---|---|
| | *palmeri* | >11,200 | >150 to >700 | N/A | 112 | (Molin et al. 2016) |
| Ser-653-Thr | *powellii* | 3.8 to 174 | S | N/A | N/A | (Ferguson et al. 2001, McNaughton et al. 2005) |
| | *tuberculatus* var. *rudis* | 74 | 1 | N/A | N/A | (Patzoldt and Tranel 2007) |
| | *retroflexus* | >90 | N/A | N/A | N/A | (Chen et al. 2015) |
| Ser-653-Asn | *tuberculatus* var. *rudis* | 860 | 1 | N/A | N/A | (Patzoldt and Tranel 2007) |
| | *hybridus (syn: quitensis)* | 261 to 508 | 2 to 25 | 0.08 to 10 | 29 to 88 | (Whaley et al. 2006) |
| | *palmeri* | N/A | N/A | N/A | N/A | (Berger et al. 2016, Larran et al. 2017, Molin et al. 2016) |

Table 4.2. Multiple tall waterhemp populations resistant to ALS-inhibitors in Indiana: site location and frequency of resistance alleles from seed-collected samples. Each population consists of 50 individuals, genotyped by TaqMan® assay.

| | | | Mutation FOR[a] | | | |
|---|---|---|---|---|---|---|
| County | Population | GPS coordinates | W574L | S653N | S653T | Overall FOR |
| | | (N,W) | ----------%---------- | | | -------%------- |
| Benton | BE1 | 40.531, 87.223 | 62 (40) | 2 (2) | 6 (5) | 64 |
| | BE2 | 40.537, 87.328 | 56 (36) | 2 (1) | 0 (0) | 56 |
| | BE3 | 40.582, 87.393 | 30 (16) | 12 (6) | 2 (1) | 38 |
| Dubois | DU1 | 38.364, 86.897 | 100 (83) | 2 (2) | 0 (0) | 100 |
| | DU2 | 38.489, 87.042 | 62 (36) | 2 (1) | 0 (0) | 62 |
| | DU3 | 38.216, 86.870 | 34 (17) | 38 (26) | 0 (0) | 68 |
| Gibson | GI1 | 38.282, 87.825 | 68 (45) | 4 (2) | 0 (0) | 70 |
| | GI2 | 38.322, 87.677 | 68 (45) | 10 (5) | 0 (0) | 70 |
| | GI3 | 38.232, 87.463 | 72 (49) | 2 (1) | 0 (0) | 74 |
| Hendricks | HE1 | 39.906, 86.655 | 92 (64) | 0 (0) | 0 (0) | 92 |
| | HE2 | 39.777, 86.649 | 70 (42) | 38 (22) | 2 (2) | 86 |
| | HE3 | 39.815, 86.535 | 78 (48) | 2 (1) | 0 (0) | 78 |
| Knox | KN1 | 38.556, 87.523 | 70 (44) | 42 (25) | 0 (0) | 90 |
| | KN2 | 38.645, 87.437 | 64 (39) | 2 (1) | 2 (2) | 68 |
| | KN3 | 38.620, 87.363 | 40 (23) | 16 (8) | 0 (0) | 52 |
| Newton | NE1 | 40.748, 87.498 | 70 (45) | 4 (2) | 2 (1) | 74 |
| | NE2 | 40.885, 87.489 | 64 (37) | 42 (27) | 4 (2) | 92 |
| | NE3 | 40.936, 87.331 | 34 (19) | 4 (2) | 46 (28) | 70 |
| Posey | PO1 | 37.995, 87.991 | 16 (8) | 46 (26) | 0 (0) | 56 |
| | PO3 | 37.941, 87.979 | 74 (52) | 4 (2) | 0 (0) | 76 |
| | PO4 | 37.905, 87.776 | 98 (71) | 4 (2) | 0 (0) | 98 |
| Randolph | RA1 | 40.287, 85.038 | 18 (9) | 2 (1) | 2 (1) | 22 |
| | RA2 | 40.249, 85.070 | 38 (23) | 0 (0) | 0 (0) | 38 |
| | RA3 | 40.284, 85.091 | 10 (5) | 0 (0) | 0 (0) | 10 |
| Shelby | SH1 | 39.498, 85.854 | 70 (44) | 10 (5) | 0 (0) | 78 |
| | SH2 | 39.526, 85.897 | 40 (22) | 4 (2) | 2 (1) | 44 |
| | SH3 | 39.570, 85.806 | 66 (44) | 12 (6) | 0 (0) | 72 |
| Spencer | SP1 | 38.027, 87.127 | 72 (53) | 2 (1) | 0 (0) | 74 |
| | SP2 | 37.914, 87.177 | 60 (41) | 16 (9) | 0 (0) | 70 |
| | SP3 | 37.833, 87.069 | 38 (21) | 0 (0) | 0 (0) | 38 |
| Sullivan | SU1 | 39.247, 87.448 | 50 (29) | 2 (1) | 0 (0) | 52 |
| | SU2 | 39.125, 87.541 | 38 (19) | 4 (2) | 0 (0) | 42 |
| | SU3 | 39.204, 87.447 | 98 (69) | 0 (0) | 0 (0) | 98 |
| Tippecanoe | TI1 | 40.384, 86.852 | 36 (23) | 34 (19) | 0 (0) | 68 |
| | TI2 | 40.384, 86.909 | 38 (20) | 16 (8) | 0 (0) | 54 |
| | TI3 | 40.301, 86.845 | 50 (31) | 22 (11) | 0 (0) | 70 |
| Vanderburgh | VA1 | 37.987, 87.463 | 92 (67) | 22 (12) | 0 (0) | 100 |
| | VA2 | 38.100, 87.652 | 64 (41) | 6 (3) | 0 (0) | 64 |
| | VA3 | 38.149, 87.474 | 80 (50) | 0 (0) | 0 (0) | 80 |
| Warrick | WA1 | 37.991, 87.286 | 94 (72) | 0 (0) | 0 (0) | 94 |
| | WA2 | 38.095, 87.382 | 82 (54) | 14 (7) | 0 (0) | 88 |
| | WA3 | 38.050, 87.286 | 48 (29) | 68 (49) | 0 (0) | 96 |

[a]Number in perentheses is the frequency of the respective resistance alleles in the 50 waterhemp plants per field population.

Table 4.3. Hardy-Weinberg Equilibrium (HWE) esults derived from 42 tall waterhemp populations collected in Indiana. Genotype of the W574L resistance mutation was used for HWE calculations.

| Population | Tall waterhemp plants | | | | | | $F_{is}$ | $\chi^{2}$[b,c] |
|---|---|---|---|---|---|---|---|---|
| | RR | | RS | | SS | | | |
| | ----------no.[a]---------- | | | | | | | |
| Benton 1 | 9 | (18) | 22 | (44) | 19 | (38) | 0.08 | 0.35 |
| Benton 2 | 8 | (16) | 20 | (40) | 22 | (44) | 0.13 | 0.87 |
| Benton 3 | 1 | (2) | 14 | (28) | 35 | (70) | -0.04 | 0.09* |
| Dubois 1 | 33 | (66) | 17 | (34) | 0 | (0) | -0.20 | 2.10* |
| Dubois 2 | 5 | (10) | 26 | (52) | 19 | (38) | -0.13 | 0.83 |
| Dubois 3 | 0 | (0) | 17 | (34) | 33 | (66) | -0.20 | 2.10* |
| Gibson 1 | 11 | (22) | 23 | (46) | 16 | (32) | 0.07 | 0.25 |
| Gibson 2 | 11 | (22) | 23 | (46) | 16 | (32) | 0.07 | 0.25 |
| Gibson 3 | 13 | (26) | 23 | (46) | 14 | (28) | 0.08 | 0.32 |
| Hendricks 1 | 18 | (36) | 28 | (56) | 4 | (8) | -0.22 | 2.32 |
| Hendricks 2 | 7 | (14) | 28 | (56) | 15 | (30) | -0.15 | 1.12 |
| Hendricks 3 | 9 | (18) | 30 | (60) | 11 | (22) | -0.20 | 2.04 |
| Knox 1 | 9 | (18) | 26 | (52) | 15 | (30) | -0.06 | 0.15 |
| Knox 2 | 7 | (14) | 25 | (50) | 18 | (36) | -0.05 | 0.13 |
| Knox 3 | 3 | (6) | 17 | (34) | 30 | (60) | 0.04 | 0.08* |
| Newton 1 | 10 | (20) | 25 | (50) | 15 | (30) | -0.01 | 0.01 |
| Newton 2 | 5 | (10) | 27 | (54) | 18 | (36) | -0.16 | 1.25 |
| Newton 3 | 2 | (4) | 15 | (30) | 33 | (66) | 0.03 | 0.03* |
| Posey 1 | 0 | (0) | 8 | (16) | 42 | (84) | -0.09 | 0.38* |
| Posey 2 | 10 | (20) | 21 | (42) | 19 | (38) | 0.13 | 0.87 |
| Posey 3 | 15 | (30) | 22 | (44) | 13 | (26) | 0.12 | 0.70 |
| Posey 4 | 22 | (44) | 27 | (54) | 1 | (2) | -0.31 | 4.85* |
| Randolph 1 | 0 | (0) | 9 | (18) | 41 | (82) | -0.10 | 0.49* |
| Randolph 2 | 4 | (8) | 15 | (30) | 31 | (62) | 0.15 | 1.17* |
| Randolph 3 | 0 | (0) | 5 | (10) | 45 | (90) | -0.05 | 0.14* |
| Shelby 1 | 9 | (18) | 26 | (52) | 15 | (30) | -0.06 | 0.15 |
| Shelby 2 | 2 | (4) | 18 | (36) | 30 | (60) | -0.05 | 0.12* |
| Shelby 3 | 11 | (22) | 22 | (44) | 17 | (34) | 0.11 | 0.57 |
| Spencer 1 | 17 | (34) | 19 | (38) | 14 | (28) | 0.24 | 2.19 |
| Spencer 2 | 11 | (22) | 19 | (38) | 20 | (40) | 0.21 | 2.30 |
| Spencer 3 | 2 | (4) | 17 | (34) | 31 | (62) | -0.02 | 0.03* |
| Sullivan 1 | 4 | (8) | 21 | (42) | 25 | (50) | -0.02 | 0.02* |
| Sullivan 2 | 0 | (0) | 19 | (38) | 31 | (62) | -0.23 | 2.75* |
| Sullivan 3 | 20 | (40) | 29 | (58) | 1 | (2) | -0.36 | 6.33*† |
| Tippecanoe 1 | 5 | (10) | 13 | (26) | 32 | (64) | 0.27 | 3.54* |
| Tippecanoe 2 | 1 | (2) | 18 | (36) | 31 | (62) | -0.13 | 0.78* |
| Tippecanoe 3 | 6 | (12) | 19 | (38) | 25 | (50) | 0.11 | 0.62* |
| Vanderburgh 1 | 21 | (42) | 25 | (50) | 4 | (8) | -0.13 | 0.85 |
| Vanderburgh 2 | 9 | (18) | 23 | (46) | 18 | (36) | 0.05 | 0.12 |
| Vanderburgh 3 | 10 | (20) | 30 | (60) | 10 | (20) | -0.20 | 2 |
| Warrick 1 | 25 | (50) | 22 | (44) | 3 | (6) | -0.09 | 0.42* |
| Warrick 2 | 13 | (26) | 28 | (56) | 9 | (18) | -0.13 | 0.81 |
| Warrick 3 | 5 | (10) | 19 | (38) | 26 | (52) | 0.08 | 0.30* |

Table 4.3 continued

[a]Number in perenthesis is the percentage of total individuals within the population sample that were the respective W574L genotype.
[b]Only one Chi-Square analysis resulted in a significant p-value ($\alpha = 0.05$), indicated by [†].
[c*]Indicates that assumptions for chi-square analysis were not met because of the small number of expected individuals (<5). A synthetic set of numbers was used to generate the resulting chi-square value.

Table 4.4. Summary of genotypic results of soil seedbank-collected individuals, genotyped by TaqMan® assay. Number of individuals per population varied based on how many plants emerged out of the soil samples collected. Only those populations with 40 or more individuals tested are included in this table.

| | | | Mutation FOR[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | W574L | | | | | | | |
| County | Population | Individuals | WT | Het | Hom | Overall R | S653N | S653T | Overall FOR | $\chi^{2b}$ |
| | | ---no.--- | ------no.------ | | | -----%---- | --------%------- | | --%-- | |
| Dubois | DU1 Seed | 50 | 0 | 17 | 33 | 100 (83) | 2 (2) | 0 (0) | 100 | 10.042* |
| | DU1 | 50 | 4 | 27 | 19 | 92 (65) | 0 (0) | 0 (0) | 92 | |
| | DU2 Seed | 50 | 19 | 26 | 5 | 62 (36) | 2 (1) | 0 (0) | 62 | 6.62* |
| | DU2 | 50 | 9 | 29 | 12 | 82 (53) | 22 (11) | 0 (0) | 96 | |
| | DU3 Seed | 50 | 33 | 17 | 0 | 34 (17) | 38 (26) | 0 (0) | 68 | 8.13* |
| | DU3 | 50 | 25 | 18 | 7 | 50 (32) | 16 (12) | 0 (0) | 66 | |
| Gibson | GI1 Seed | 50 | 16 | 23 | 11 | 68 (45) | 4 (2) | 0 (0) | 70 | 1.38 |
| | GI1 | 46 | 20 | 17 | 9 | 57 (38) | 15 (8) | 0 (0) | 70 | |
| | GI3 Seed | 50 | 14 | 23 | 13 | 72 (49) | 2 (1) | 0 (0) | 74 | 3.05 |
| | GI3 | 50 | 7 | 26 | 17 | 86 (60) | 4 (2) | 0 (0) | 86 | |
| Hendricks | HE3 Seed | 50 | 11 | 30 | 9 | 78 (48) | 2 (1) | 0 (0) | 78 | 1.59 |
| | HE3 | 50 | 16 | 28 | 6 | 68 (40) | 6 (3) | 0 (0) | 70 | |
| Knox | KN1 Seed | 50 | 15 | 26 | 9 | 70 (44) | 42 (25) | 0 (0) | 90 | 0.46 |
| | KN1 | 50 | 12 | 28 | 10 | 76 (48) | 10 (5) | 2 (1) | 84 | |
| | KN2 Seed | 50 | 18 | 25 | 7 | 64 (39) | 2 (1) | 2 (2) | 68 | 7.39* |
| | KN2 | 50 | 8 | 26 | 16 | 84 (58) | 0 (0) | 4 (2) | 88 | |
| | KN3 Seed | 50 | 30 | 17 | 3 | 40 (23) | 16 (8) | 0 (0) | 52 | 4.35 |
| | KN3 | 50 | 26 | 14 | 10 | 48 (34) | 12 (6) | 0 (0) | 60 | |
| Newton | NE2 Seed | 50 | 18 | 27 | 5 | 64 (37) | 42 (27) | 4 (2) | 92 | 1.45 |
| | NE2 | 50 | 23 | 21 | 6 | 54 (33) | 34 (19) | 18 (11) | 80 | |
| Posey | PO3 Seed | 50 | 13 | 22 | 15 | 74 (52) | 4 (2) | 0 (0) | 76 | 1.33 |
| | PO3 | 50 | 15 | 25 | 10 | 70 (45) | 10 (6) | 0 (0) | 74 | |

Table 4.4 continued

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Randolph | RA3 Seed | 50 | 45 | 5 | 0 | 10 (5) | 0 (0) | 0 (0) | 10 | 0.61 |
| | RA3 | 50 | 48 | 2 | 0 | 4 (2) | 4 (2) | 0 (0) | 8 | |
| Shelby | SH1 Seed | 50 | 15 | 26 | 9 | 70 (44) | 10 (5) | 0 (0) | 78 | 8.34* |
| | SH1 | 50 | 23 | 12 | 15 | 54 (42) | 6 (3) | 0 (0) | 56 | |
| | SH2 Seed | 50 | 30 | 18 | 2 | 40 (22) | 4 (2) | 2 (1) | 44 | 0.46 |
| | SH2 | 50 | 29 | 20 | 1 | 42 (22) | 4 (2) | 0 (0) | 46 | |
| | SH3 Seed | 50 | 17 | 22 | 11 | 40 (22) | 4 (2) | 2 (1) | 44 | 1.48 |
| | SH3 | 50 | 14 | 28 | 8 | 72 (44) | 6 (3) | 0 (0) | 76 | |
| Sullivan | SU2 Seed | 50 | 31 | 19 | 0 | 38 (19) | 4 (2) | 0 (0) | 42 | 2.38 |
| | SU2 | 50 | 27 | 21 | 2 | 46 (25) | 12 (6) | 2 (1) | 54 | |
| | SU3 Seed | 50 | 1 | 29 | 20 | 98 (69) | 0 (0) | 0 (0) | 98 | 10.96* |
| | SU3 | 50 | 11 | 28 | 11 | 78 (50) | 4 (2) | 0 (0) | 78 | |
| Vanderburgh | VA1 Seed | 50 | 4 | 25 | 21 | 92 (67) | 22 (12) | 0 (0) | 100 | 2.60 |
| | VA1 | 50 | 9 | 25 | 16 | 82 (57) | 16 (8) | 0 (0) | 84 | |
| | VA2 Seed | 50 | 18 | 23 | 9 | 64 (41) | 6 (3) | 0 (0) | 64 | 4.41 |
| | VA2 | 50 | 9 | 27 | 14 | 82 (55) | 14 (7) | 0 (0) | 82 | |
| | VA3 Seed | 50 | 10 | 30 | 10 | 80 (50) | 0 (0) | 0 (0) | 80 | 0.07 |
| | VA3 | 50 | 9 | 31 | 10 | 82 (51) | 14 (7) | 0 (0) | 86 | |
| Warrick | WA1 Seed | 50 | 3 | 22 | 25 | 94 (72) | 0 (0) | 0 (0) | 94 | 1.95 |
| | WA1 | 46 | 5 | 24 | 17 | 89 (63) | 0 (0) | 0 (0) | 46 | |

[a]Number in perentheses is the frequency of the respective resistance allele.
[b]* indicates p-value less than 0.05.

Table 4.5. NGS Sequencing results performed on a single pool of 50 uniform tall waterhemp tissue samples. An additional population of tall waterhemp and two horseweed populations are included at the bottom of the table.

| Population | Number of reads[a] | Number of reads containing a mutant codon (%)[b] A122 | P197[c] | D376E | W574L[c] | S653N[c] | S653T[c] | G654F |
|---|---|---|---|---|---|---|---|---|
| | | ----------no.---------- | | | | | | |
| BE1 | 2640 (26.4) | 0 (0.0) | Thr 133 (1.2%) | 699 (3.7%) | 5630 (33.8%) *40* | 221 (5.0%) *2* | 142 (3.2%) *5* | 0 (0.0) |
| BE2 | 3261 (32.6) | 0 (0.0) | Thr 3166 (34.5%) Leu 526 (5.6%) | 0 (0.0) | 4217 (33.1%) *36* | 65 (1.8%) *1* | 0 (0.0) *0* | 0 (0.0) |
| BE3 | 3131 (31.3) | 0 (0.0) | Thr 667 (10.0%) | 0 (0.0) | 1592 (15.2%) *16* | 147 (5.4%) *6* | 38 (1.9%) *1* | 0 (0.0) |
| DU1 | 3146 (31.5) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 5450 (81.5%) *83* | 0 (0.0) *2* | 0 (0.0) *0* | 0 (0.0) |
| DU2 | 3091 (30.9) | Thr 561 (33.4%) Asn 63 (3.7%) | 0 (0.0) | 0 (0.0) | 1875 (31.5%) *36* | 0 (0.0) *1* | 0 (0.0) *0* | 0 (0.0) |
| DU3 | 2126 (21.3) | Thr 11 (1.3%) | 0 (0.0) | 0 (0.0) | 310 (14.8%) *17* | 130 (27.7%) *26 both mutations no discrepancy* | 32 (6.8%) *0 both mutations (ACT/ACC) - discrepancy* | 0 (0.0) |
| GI1 | 2643 (26.4) | Asn 307 (14.6%) | 0 (0.0) | 0 (0.0) | 3618 (41.9%) *45* | 50 (2.1%) *2 both mutations, no discrepancy* | 0 (0.0) *0* | 0 (0.0) |

Table 4.5 continued

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| GI2 | 2917 (29.2) | Asn 51 (6.3%) Thr 12 (1.5%) | 0 (0.0) | 66 (1.3%) | 1048 (40.2%) *45* | 50 (8.1%) *5 both mutations, no discrepancy* | 0 (0.0) *0* | 0 (0.0) |
| GI3 | 2821 (28.2) | Asn 287 (13.9%) Thr 22 (1.1%) | 0 (0.0) | 0 (0.0) | 3917 (49.0%) *49* | 46 (2.1%) *1 both mutations, no discrepancy* | 30 (1.4%) *0 (Both – ACT/ACC)* | 0 (0.0) |
| HE1 | 2940 (29.4) | 0 (0.0) | Thr 69 (1.7%) | 512 (7.1%) | 3809 (65.9%) *64* | 0 (0.0) *0* | 0 (0.0) *0* | 0 (0.0) |
| HE2 | 3014 (30.1) | 0 (0.0) | 0 (0.0) | 1274 (16.5%) | 2666 (39.9%) *42* | 463 (6.3%) *22 both mutations, discrepancy (AAT/AAC)* | 39 (1.9%) *2* | 0 (0.0) |
| HE3 | 3181 (31.8) | Thr 24 (1.3%) | Thr 846 (16.8%) | 0 (0.0) | 3343 (47.0%) *48* | 25 (1.4%) *1* | 0 (0.0) *0* | 0 (0.0) |
| KN1 | 3168 (31.7) | Thr 32 (2.0%) | Thr 48 (1.0%) | 0 (0.0) | 2818 (44.2%) *44* | 358 (22.8%) *25* | 0 (0.0) *0* | 0 (0.0) |
| KN2 | 2400 (24.0) | Asn 75 (8.9%) | Leu 214 (7.4%) | 0 (0.0) | 988 (38.0%) *39* | 0 (0.0) *1* | 14 (2.0%) *2* | 0 (0.0) |
| KN3 | 2790 (27.9) | Asn 45 (3.1%) | 0 (0.0) | 0 (0.0) | 1134 (16.9%) *23* | 145 (8.4%) *8* | 0 (0.0) *0* | 0 (0.0) |
| NE1 | 662 (6.62) | 0 (0.0) | Thr 152 (31.3%) | 0 (0.0) | 227 (47.7%) *45* | 3 (3.5%) *2* | 1 (1.2%) *1* | 2 (2.4%) |
| NE2 | 2994 (29.9) | 0 (0.0) | Thr 155 (2.9%) Leu 126 (2.4%) | 0 (0.0) | 3371 (40.8%) *37* | 523 (23.4%) *27* | 24 (1.1%) *2* | 147 (6.7%) |

Table 4.5 continued

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| NE3 | 3022 (30.2) | 0 (0.0) | Thr 56 (1.1%) | 0 (0.0) | 1335 (17.3%) *19* | 145 (6.6%) *2* | 556 (25.2%) *28* | 0 (0.0) |
| PO1 | 2427 (24.3) | Ser 27 (1.9%) | 0 (0.0) | 0 (0.0) | 402 (6.0%) *8* | 465 (27.1%) *26* | 0 (0.0) *0* | 0 (0.0) |
| PO3 | 1801 (18.0) | Asn 162 (27.9%) | 0 (0.0) | 0 (0.0) | 819 (48.7%) *52* | 5 (1.5%) *2* | 0 (0.0) *0* | 0 (0.0) |
| PO4 | 3148 (31.5) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2329 (69.3%) *71* | 17 (2.3%) *2* | 0 (0.0) *0* | 0 (0.0) |
| RA1 | 3008 (30.1) | 0 (0.0) | 0 (0.0) | 3084 (41.1%) | 734 (10.4%) *9* | 0 (0.0) *1* | 38 (2.2%) *2* | 0 (0.0) |
| RA2 | 2354 (23.5) | Thr 11 (2.1%) | 0 (0.0) | 1728 (51.4%) | 299 (17.3%) *23* | 0 (0.0) *0* | 0 (0.0) *0* | 0 (0.0) |
| RA3 | 3446 (34.5) | 0 (0.0) | 0 (0.0) | 4295 (70.5%) | 306 (5.8%) *5* | 0 (0.0) *0* | 0 (0.0) *0* | 0 (0.0) |
| SH1 | 2943 (29.4) | 0 (0.0) | 0 (0.0) | 942 (11.5%) | 2585 (35.5%) *44* | 0 (0.0) *5* | 131 (6.5%) *0 ACC – only one, discrepancy* | 0 (0.0) |
| SH2 | 3392 (33.9) | 0 (0.0) | 0 (0.0) | 1081 (18.5%) | 901 (18.3%) *22* | 35 (2.5%) *2* | 20 (1.4%) *1* | 0 (0.0) |
| SH3 | 2578 (25.8) | 0 (0.0) | Ser 61 (1.5%) His 68 (1.7%) | 2235 (34.4%) | 2592 (44.0%) *44* | 136 (9.2%) *6* | 0 (0.0) *0* | 0 (0.0) |
| SP1 | 3271 (32.7) | Asn 16 (1.2%) | Thr 426 (11.7%) | 0 (0.0) | 2388 (45.3%) *53* | 17 (1.3%) *1* | 0 (0.0) *0* | 0 (0.0) |
| SP2 | 1749 (17.5) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 569 (41.3%) *41* | 0 (0.0) *9* | 31 (8.1%) *0 ACC* | 0 (0.0) |
| SP3 | 2907 (29.1) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1090 (19.3%) *21* | 0 (0.0) *0* | 0 (0.0) *0* | 0 (0.0) |

Table 4.5 continued

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| SU1 | 3090 (30.9) | Thr 12 (1.0%) | Thr 156 (4.4%) | 0 (0.0) | 1303 (26.1%) *29* | 0 (0.0) *1* | 0 (0.0) *0* | 0 (0.0) | |
| SU2 | 2902 (29.0) | Asn 32 (1.8%) | Thr 266 (5.5%) | 0 (0.0) | 1371 (19.2%) *19* | 58 (3.4%) *2* | 0 (0.0) *0* | 0 (0.0) | |
| SU3 | 3067 (30.7) | Asn 28 (1.6%) | | Leu 86 (1.9%) | 0 (0.0) | 4075 (65.7%) *69* | 0 (0.0) *0* | 0 (0.0) *0* | 0 (0.0) |
| TI1 | 2905 (29.1) | 0 (0.0) | | Thr 223 (9.2%) | 0 (0.0) | 816 (20.4%) *23* | 240 (16.7%) *19* | 0 (0.0) *0* | 0 (0.0) |
| TI2 | 2700 (27.0) | 0 (0.0) | | Thr 64 (1.6%) Ser 513 (12.4%) Leu 669 (16.2%) | 110 (1.7%) | 1189 (19.2%) *20* | 137 (8.7%) *8* | 0 (0.0) *0* | 0 (0.0) |
| TI3 | 3204 (32.0) | 0 (0.0) | | Thr 609 (11.2%) | 0 (0.0) | 1772 (27.9%) *31* | 183 (12.1%) *11* | 0 (0.0) *0* | 0 (0.0) |
| VA1 | 3140 (31.4) | 0 (0.0) | | 0 (0.0) | 0 (0.0) | 2150 (65.6%) *67* | 76 (9.2%) *12* | 0 (0.0) *0* | 0 (0.0) |
| VA2 | 2947 (29.5) | Asn 257 (12.8%) Thr 19 (1.0%) | | 0 (0.0) | 145 (1.7%) | 2760 (36.7%) *41* | 59 (3.0%) *3* | 0 (0.0) *0* | 0 (0.0) |
| VA3 | 2144 (21.4) | Asn 12 (1.9%) Thr 20 (1.3%) | | 0 (0.0) | 0 (0.0) | 876 (50.3%) *50* | 0 (0.0) *0* | 0 (0.0) *0* | 0 (0.0) |
| WA1 | 3032 (30.3) | Asn 25 (1.2%) | | 0 (0.0) | 0 (0.0) | 5395 (67.7%) *72* | 0 (0.0) *0* | 0 (0.0) *0* | 0 (0.0) |
| WA2 | 2913 (29.1) | Asn 39 (1.2%) | | Leu 90 (1.7%) | 101 (1.3%) | 3578 (48.0%) *54* | 140 (7.0%) *7* | 36 (1.8%) *0 ACT* | 0 (0.0) |
| WA3 | 2843 (28.4) | Asn 53 (4.4%) | | 0 (0.0) | 0 (0.0) | 1352 (25.0%) *29* | 843 (53.1%) *49* | 0 (0.0) *0* | 0 (0.0) |
| PO2 | 3170 (31.7) | 0 (0.0) | | 0 (0.0) | 0 (0.0) | 2402 (49.8%) *41* | 331 (26.3%) *28* | 0 (0.0) *0* | 0 (0.0 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| HW1 | 2294 (22.9) | Leu 1033 (3.7%) *6* | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | | 0 (0.0) | 0 (0.0) |

Table 4.5 continued

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| HW3 | 4118 (41.2) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |

[a]Numbers in parentheses represent the sequencing coverage depth for the amplicon in the population considered (i.e. the average number of times the region corresponding to the amplicon is expected to have been sequenced in each of the 100 ALS gene copies present in each population)

[b]Percentage of the NGS sequence reads containing the mutant codon relative to the total number of sequence reads corresponding to the ALS amplicon.

[c]Number in italics (for the W574L and S653N columns; P197L for horseweed) represents the number of alleles carrying the mutation in the population out of 100, as identified by SNP genotyping.

[d]No population contained an amino acid substitution at codon Ala205.

Table 4.6. Summary of polymorphisms in the ALS gene that have previously been shown to confer resistance to ALS inhibitors. Summary is based on results of next-generation sequencing on pooled collections of tall waterhemp collected in 42 fields in Indiana.

| Codon at amino acid position | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 122 | | | 197 | | | 376 | | | 574 | | | 653 | | | 654 | | |
| GCT | Pops[b] | Alleles[c] | CCT | Pops | Alleles | GAT | Pops | Alleles | TGG | Pops | Alleles | AGC | Pops | Alleles | GGT | Pops | Alleles |
| | no. | no. | | no. | no. | | no. | no. | | no. | no. | | no. | no. | | no. | no. |
| Thr ACT | 10 | 46 | Thr ACT | 15 | 144 | Glu GAA | 1 | 4 | Leu TTG | 42 | 1532 | Asn AAC, AAT | 28 | 282 | Phe TTT | 2 | 9 |
| Asn AAT[a] | 15 | 105 | Leu CTT | 6 | 36 | Glu GAG | 12 | 257 | | | | Thr ACC, ACT | 14 | 68 | | | |
| Ser TCT | 1 | 2 | Ser TCT | 2 | 14 | | | | | | | | | | | | |
| | | | His CAT | 1 | 2 | | | | | | | | | | | | |

[a]One population had a small percentage of individuals with amino acid substitution to Asn with the AAC-coded polymorphism.
[b]Out of 42 populations.
[c]Out of 4200 alleles.

Table 4.7. Malathion response of tall waterhemp populations collected from Tippecanoe and Randolph Counties in Indiana. Means separated by Fisher's protected LSD (α = 0.05). Same letters within a site are not significantly different.

| | | Biomass Reduction[ab] | | | |
|---|---|---|---|---|---|
| Treatment | Rate | Tippecanoe | | Randolph | |
| | g ha $^{-1}$ | ----------%---------- | | | |
| Nontreated control | | 100 | a | 100 | a |
| Malathion | 2000 | 91 | ab | 105 | a |
| Chlorimuron | 4.4 | 68 | bc | 89 | ab |
| | 44 | 54 | cd | 58 | bc |
| Chlorimuron + Malathion | 4.4 + 2000 | 34 | de | 40 | cd |
| | 44 + 2000 | 22 | e | 21 | d |

[a]Percent of nontreated control.
[b]Means followed by the same letter within a column are not significantly different according to Tukey's LSD (α = 0.05).

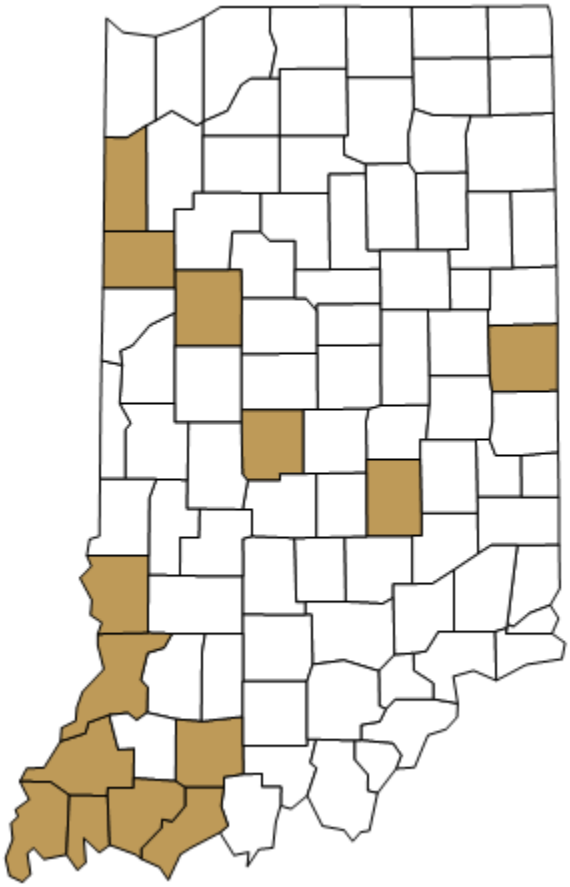

Figure 4.1. Indiana counties surveyed for ALS inhibitor resistant tall waterhemp are highlighted. Three fields within each highlighted county were sampled. Seed and soil samples were collected from 42 fields in the fall of 2017.

# APPENDIX

Table A.1. Model summary for multinomial logistic regression (n = 1564) for the Lafayette tall waterhemp location.

| Treatments | Unstandardized | 95% Confidence Interval | | |
|---|---|---|---|---|
| | Coefficient B (SE) | Lower | Odds Ratio | Upper |
| W574L RS + S653N RS vs. W574L SS + S653N SS | | | | |
| Intercept | -2.1519 (0.44)*** | | 0.116 | |
| Chlorimuron 11.2 g PRE | -11.1183 (122.28) | <0.001 | <0.001 | >999.99 |
| Chlorimuron 22.4g PRE | -10.1750 (93.03) | <0.001 | <0.001 | >999.99 |
| Chlorimuron 44.8g PRE | -9.4660 (75.45) | <0.001 | <0.001 | >999.99 |
| Chlorimuron 11.2g POST | -11.9294 (139.57) | <0.001 | <0.001 | >999.99 |
| Chlorimuron 22.4g POST | -2.0079 (1.07) | 0.016 | 0.134 | 1.094 |
| Chlorimuron 44.8g POST | -1.7599 (1.07) | 0.021 | 0.172 | 1.408 |
| Imazethapyr 70.6 g PRE | -1.0229 (0.80) | 0.075 | 0.360 | 1.725 |
| Imazethapyr 70.6 g POST | -1.0789 (1.08) | 0.041 | 0.340 | 2.809 |
| Fomesafen 330 g PRE | 0.5322 (0.54) | 0.591 | 1.703 | 4.903 |
| Fomesafen 330 g POST | -0.004323 (0.70) | 0.242 | 0.958 | 3.788 |
| Atrazine 1121.2g PRE | 0.2564 (0.56) | 0.433 | 1.292 | 3.853 |
| Atrazine 2242.4g PRE | -0.1232 (0.63) | 0.258 | 0.884 | 3.034 |
| Atrazine 1121.2g POST | 0.6214 (0.60) | 0.570 | 1.861 | 6.078 |
| Atrazine 2242.4g POST | 1.0378 (0.54) | 0.974 | 2.823 | 8.184 |
| W574L RS + S653N RS vs. W574L RS + S653N SS | | | | |
| Intercept | -0.6878 (0.20)** | | 0.503 | |
| Chlorimuron 11.2 g PRE | 0.5618 (0.32) | 0.940 | 1.754 | 3.273 |
| Chlorimuron 22.4g PRE | 0.3246 (0.38) | 0.656 | 1.384 | 2.916 |
| Chlorimuron 44.8g PRE | 0.3361 (0.43) | 0.608 | 1.399 | 3.221 |
| Chlorimuron 11.2g POST | -0.02316 (0.30) | 0.543 | 0.977 | 1.759 |
| Chlorimuron 22.4g POST | -0.2025 (0.32) | 0.440 | 0.817 | 1.518 |
| Chlorimuron 44.8g POST | 0.4291 (0.30) | 0.851 | 1.536 | 2.772 |
| Imazethapyr 70.6 g PRE | -1.9751 (0.63)** | 0.040 | 0.139 | 0.476 |
| Imazethapyr 70.6 g POST | 0.2586 (0.39) | 0.608 | 1.295 | 2.759 |
| Fomesafen 330 g PRE | 0.3983 (0.33) | 0.774 | 1.489 | 2.865 |
| Fomesafen 330 g POST | 0.9742 (0.33)** | 1.377 | 2.649 | 5.097 |
| Atrazine 1121.2g PRE | 0.2403 (0.33) | 0.664 | 1.272 | 2.435 |
| Atrazine 2242.4g PRE | -0.3048 (0.38) | 0.350 | 0.737 | 1.552 |
| Atrazine 1121.2g POST | 0.6407 (0.37) | 0.921 | 1.898 | 3.912 |
| Atrazine 2242.4g POST | -0.1738 (0.41) | 0.376 | 0.840 | 1.878 |

Table A.1. continued

| | | | | |
|---|---|---|---|---|
| W574L RS + S653N RS vs. W574L RR + S653N SS | | | | |
| Intercept | -2.2676 (0.37)*** | | 0.104 | |
| Chlorimuron 11.2 g PRE | 2.2957 (0.44)*** | 4.176 | 9.932 | 23.620 |
| Chlorimuron 22.4g PRE | 3.2093 (0.44)*** | 10.354 | 24.762 | 59.218 |
| Chlorimuron 44.8g PRE | 3.6805 (0.46)*** | 16.138 | 39.666 | 97.494 |
| Chlorimuron 11.2g POST | -0.01794 (0.57) | 0.323 | 0.982 | 2.983 |
| Chlorimuron 22.4g POST | 0.7252 (0.49) | 0.793 | 2.065 | 5.381 |
| Chlorimuron 44.8g POST | 1.1898 (0.48)* | 1.292 | 3.287 | 8.359 |
| Imazethapyr 70.6 g PRE | -0.8044 (0.82) | 0.091 | 0.447 | 2.207 |
| Imazethapyr 70.6 g POST | 0.2273 (0.72) | 0.307 | 1.255 | 5.128 |
| Fomesafen 330 g PRE | 0.5862 (0.58) | 0.577 | 1.797 | 5.600 |
| Fomesafen 330 g POST | 2.1341 (0.48)*** | 3.324 | 8.449 | 21.480 |
| Atrazine 1121.2g PRE | 0.4779 (0.58) | 0.521 | 1.613 | 4.988 |
| Atrazine 2242.4g PRE | 0.3200 (0.60) | 0.420 | 1.377 | 4.511 |
| Atrazine 1121.2g POST | 0.3261 (0.72) | 0.338 | 1.386 | 5.675 |
| Atrazine 2242.4g POST | -0.2993 (0.82) | 0.148 | 0.741 | 3.721 |
| W574L RS + S653N RS vs. W574L SS + S653N RS | | | | |
| Intercept | -0.8393 (0.24)** | | 0.432 | |
| Chlorimuron 11.2 g PRE | -25.4038 (0)*** | . | <0.001 | . |
| Chlorimuron 22.4g PRE | -2.3080 (1.04)* | 0.013 | 0.099 | 0.760 |
| Chlorimuron 44.8g PRE | -22.0395 (0)*** | . | <0.001 | . |
| Chlorimuron 11.2g POST | -1.6506 (0.51)** | 0.071 | 0.192 | 0.522 |
| Chlorimuron 22.4g POST | -2.1110 (0.63)*** | 0.035 | 0.121 | 0.415 |
| Chlorimuron 44.8g POST | -2.2733 (0.75)** | 0.024 | 0.103 | 0.451 |
| Imazethapyr 70.6 g PRE | -0.2961 (0.37) | 0.359 | 0.744 | 1.540 |
| Imazethapyr 70.6 g POST | 1.1964 (0.34)*** | 1.692 | 3.308 | 6.469 |
| Fomesafen 330 g PRE | 0.4987 (0.34) | 0.840 | 1.647 | 3.228 |
| Fomesafen 330 g POST | 0.4125 (0.38) | 0.711 | 1.511 | 3.208 |
| Atrazine 1121.2g PRE | -0.04973 (0.37) | 0.460 | 0.951 | 1.969 |
| Atrazine 2242.4g PRE | 0.4498 (0.34) | 0.810 | 1.568 | 3.035 |
| Atrazine 1121.2g POST | 0.3261 (0.72) | 1.708 | 3.397 | 6.759 |
| Atrazine 2242.4g POST | 0.3104 (0.39) | 0.638 | 1.364 | 2.918 |
| W574L RS + S653N RS vs. W574L RR + S653N RS | | | | |
| Intercept | -13.8356 (72.77) | | 0.000 | |
| Chlorimuron 11.2 g PRE | -0.5133 (160.80) | <0.001 | 0.599 | >999.99 |
| Chlorimuron 22.4g PRE | -0.1855 (164.55) | <0.001 | 0.831 | >999.99 |
| Chlorimuron 44.8g PRE | 0.0749 (167.26) | <0.001 | 1.078 | >999.99 |

Table A.1. continued

| | | | | |
|---|---|---|---|---|
| Chlorimuron 11.2g POST | -1.0557 (160.04) | <0.001 | 0.348 | >999.99 |
| Chlorimuron 22.4g POST | -0.9825 (158.60) | <0.001 | 0.374 | >999.99 |
| Chlorimuron 44.8g POST | -0.7668 (159.65) | <0.001 | 0.465 | >999.99 |
| Imazethapyr 70.6 g PRE | -0.7809 (161.55) | <0.001 | 0.458 | >999.99 |
| Imazethapyr 70.6 g POST | -0.1395 (160.72) | <0.001 | 0.870 | >999.99 |
| Fomesafen 330 g PRE | -0.4267 (157.32) | <0.001 | 0.653 | >999.99 |
| Fomesafen 330 g POST | 10.4748 (72.73) | <0.001 | >999.99 | >999.99 |
| Atrazine 1121.2g PRE | -0.5409 (157.25) | <0.001 | 0.582 | >999.99 |
| Atrazine 2242.4g PRE | -0.5299 (157.97) | <0.001 | 0.589 | >999.99 |
| Atrazine 1121.2g POST | -0.04456 (159.23) | <0.001 | 0.956 | >999.99 |
| Atrazine 2242.4g POST | -0.4861 (162.84) | <0.001 | 0.615 | >999.99 |
| | | | | |
| W574L RS + S653N RS vs. W574L SS + S653N RR | | | | |
| Intercept | -0.9874 (0.22)*** | | 0.373 | |
| Chlorimuron 11.2 g PRE | -21.7322 (0)*** | . | <0.001 | . |
| Chlorimuron 22.4g PRE | -20.1130 (0)*** | . | <0.001 | . |
| Chlorimuron 44.8g PRE | -18.7245 (0)*** | . | <0.001 | . |
| Chlorimuron 11.2g POST | -3.0765 (1.02)** | 0.006 | 0.046 | 0.343 |
| Chlorimuron 22.4g POST | -1.4351 (0.52)* | 0.087 | 0.238 | 0.655 |
| Chlorimuron 44.8g POST | -2.7885 (1.03)* | 0.008 | 0.062 | 0.463 |
| Imazethapyr 70.6 g PRE | 0.8088 (0.31)* | 1.213 | 2.245 | 4.157 |
| Imazethapyr 70.6 g POST | 0.9882 (0.37)* | 1.308 | 2.686 | 5.515 |
| Fomesafen 330 g PRE | -0.4038 (0.45) | 0.275 | 0.668 | 1.620 |
| Fomesafen 330 g POST | -1.5065 (0.77) | 0.049 | 0.222 | 1.002 |
| Atrazine 1121.2g PRE | -0.1110 (0.40) | 0.410 | 0.895 | 1.954 |
| Atrazine 2242.4g PRE | 0.3243 (0.36) | 0.679 | 1.383 | 2.817 |
| Atrazine 1121.2g POST | 0.8875 (0.38)* | 1.142 | 2.429 | 5.164 |
| Atrazine 2242.4g POST | 0.04645 (0.43) | 0.448 | 1.048 | 2.452 |
| | | | | |
| W574L RS + S653N RS vs. W574L RS + S653N RR | | | | |
| Intercept | -10.6084 (22.84) | | 0.000 | |
| Chlorimuron 11.2 g PRE | 7.0844 (22.84) | <0.001 | >999.99 | >999.99 |
| Chlorimuron 22.4g PRE | -0.2167 (52.02) | <0.001 | 0.805 | >999.99 |
| Chlorimuron 44.8g PRE | 0.0444 (52.85) | <0.001 | 1.045 | >999.99 |
| Chlorimuron 11.2g POST | -1.0935 (50.68) | <0.001 | 0.335 | >999.99 |
| Chlorimuron 22.4g POST | -1.0216 (50.27) | <0.001 | 0.360 | >999.99 |
| Chlorimuron 44.8g POST | 6.8172 (22.84) | <0.001 | 913.453 | >999.99 |
| Imazethapyr 70.6 g PRE | -0.8074 (51.28) | <0.001 | 0.446 | >999.99 |
| Imazethapyr 70.6 g POST | -0.1650 (50.94) | <0.001 | 0.848 | >999.99 |
| Fomesafen 330 g PRE | -0.4527 (50.05) | <0.001 | 0.636 | >999.99 |

Table A.1. continued

| | | | | |
|---|---|---|---|---|
| Fomesafen 330 g POST | -0.2461 (51.71) | <0.001 | 0.782 | >999.99 |
| Atrazine 1121.2g PRE | 7.0205 (22.84) | <0.001 | >999.99 | >999.99 |
| Atrazine 2242.4g PRE | -0.5538 (50.32) | <0.001 | 0.575 | >999.99 |
| Atrazine 1121.2g POST | -0.06381 (50.75) | <0.001 | 0.938 | >999.99 |
| Atrazine 2242.4g POST | -0.4528 (54.48) | <0.001 | 0.636 | >999.99 |

[a]Significance designated as *=P<0.05, **=P<0.005, ***=P<0.001

Table A.2 Summary of NGS reads for each sample completed and respective breakdown of reads for each possible *ALS* resistance mutation.

| Population | Number of reads[a] | Number of reads containing a mutant codon (%)[b] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | A122 | P197[c] | A205 | D376 | 377 | W574L[c] | S653N[c] | S653T[c] | G654F |
| | | ----------no.---------- | | | | | | | | |
| BE1 | 2640 (26.4) | 0 (0.0) | Thr (ACT) 133 (1.2%) | 0 (0.0) | Glu (GAA) 699 (3.7%) | 0 (0.0) | 5630 (33.8%) | (AAC) 221 (5.0%) | (ACC) 142 (3.2%) | 0 (0.0) |
| BE2 | 3261 (32.6) | 0 (0.0) | Thr (ACT) 3166 (34.5%) Leu (CTT) 526 (5.6%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 4217 (33.1%) | (AAC) 65 (1.8%) | 0 (0.0) | 0 (0.0) |
| BE3 | 3131 (31.3) | 0 (0.0) | Thr (ACT) 667 (10.0%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1592 (15.2%) | (AAC) 147 (5.4%) | (ACC) 38 (1.9%) | 0 (0.0) |
| DU1 | 3146 (31.5) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 5450 (81.5%) | 0 (0.0 | 0 (0.0) | 0 (0.0) |
| DU2 | 3091 (30.9) | Thr (ACT) 561 (33.4%) Asn (AAT) 63 (3.7%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1875 (31.5%) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| DU3 | 2126 (21.3) | Thr (ACT) 11 (1.3%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 310 (14.8%) | (AAT/AAC) 130 (27.7%) | (ACT/ACC) 32 (6.8%) | 0 (0.0) |
| GI1 | 2643 (26.4) | Asn (AAT) 307 (14.6%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 3618 (41.9%) | (AAT/AAC) 50 (2.1%) | 0 (0.0) | 0 (0.0) |

Table A.2 continued

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| GI2 | 2917 (29.2) | Asn (AAT) 51 (6.3%) Thr (ACT) 12 (1.5%) | 0 (0.0) | 0 (0.0) | Glu (GAG) 66 (1.3%) | 0 (0.0) | 1048 (40.2%) | (AAT/AAC) 50 (8.1%) | 0 (0.0) | 0 (0.0) |
| GI3 | 2821 (28.2) | Asn (AAT) 287 (13.9%) Thr (ACT) 22 (1.1%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 3917 (49.0%) | (AAT/AAC) 46 (2.1%) | (ACT/ACC) 30 (1.4%) | 0 (0.0) |
| HE1 | 2940 (29.4) | 0 (0.0) | Thr (ACT) 69 (1.7%) | 0 (0.0) | Glu (GAG) 512 (7.1%) | 0 (0.0) | 3809 (65.9%) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| HE2 | 3014 (30.1) | 0 (0.0) | 0 (0.0) | 0 (0.0) | Glu (GAG) 1274 (16.5%) | 0 (0.0) | 2666 (39.9%) | (AAT/AAC) 463 (6.3%) | (ACC) 39 (1.9%) | 0 (0.0) |
| HE3 | 3181 (31.8) | Thr (ACT) 24 (1.3%) | Thr (ACT) 846 (16.8%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 3343 (47.0%) | (AAC) 25 (1.4%) | 0 (0.0) | 0 (0.0) |
| KN1 | 3168 (31.7) | Thr (ACT) 32 (2.0%) | Thr (ACT) 48 (1.0%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2818 (44.2%) | (AAT) 358 (22.8%) | 0 (0.0) | 0 (0.0) |
| KN2 | 2400 (24.0) | Asn (AAT) 75 (8.9%) | Leu (CTT) 214 (7.4%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 988 (38.0%) | 0 (0.0) | (ACC) 14 (2.0%) | 0 (0.0) |
| KN3 | 2790 (27.9) | Asn (AAT) 45 (3.1%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1134 (16.9%) | (AAT/AAC) 145 (8.4%) | 0 (0.0) | 0 (0.0) |
| NE1 | 662 (6.62) | 0 (0.0) | Thr (ACT) 152 (31.3%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 227 (47.7%) | (AAC) 3 (3.5%) | (ACC) 1 (1.2%) | Phe (TTT) 2 (2.4%) |

Table A.2 continued

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| NE2 | 2994 (29.9) | 0 (0.0) | Thr (ACT) 155 (2.9%) Ile (CTT) 126 (2.4%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 3371 (40.8%) | (AAC) 523 (23.4%) | (ACC) 24 (1.1%) | Phe (TTT) 147 (6.7%) |
| NE3 | 3022 (30.2) | 0 (0.0) | Thr (ACT) 56 (1.1%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1335 (17.3%) | (AAC) 145 (6.6%) | (ACC) 556 (25.2%) | 0 (0.0) |
| PO1 | 2427 (24.3) | Ser (TCT) 27 (1.9%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 402 (6.0%) | (AAT/AAC) 465 (27.1%) | 0 (0.0) | 0 (0.0) |
| PO3 | 1801 (18.0) | Asn (AAT) 162 (27.9%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 819 (48.7%) | (AAT) 5 (1.5%) | 0 (0.0) | 0 (0.0) |
| PO4 | 3148 (31.5) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2329 (69.3%) | (AAT) 17 (2.3%) | 0 (0.0) | 0 (0.0) |
| RA1 | 3008 (30.1) | 0 (0.0) | 0 (0.0) | 0 (0.0) | Glu (GAG) 3084 (41.1%) | 0 (0.0) | 734 (10.4%) | 0 (0.0) | (ACC) 38 (2.2%) | 0 (0.0) |
| RA2 | 2354 (23.5) | Thr (ACT) 11 (2.1%) | 0 (0.0) | 0 (0.0) | Glu (GAG) 1728 (51.4%) | 0 (0.0) | 299 (17.3%) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| RA3 | 3446 (34.5) | 0 (0.0) | 0 (0.0) | 0 (0.0) | Glu (GAG) 4295 (70.5%) | 0 (0.0) | 306 (5.8%) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| SH1 | 2943 (29.4) | 0 (0.0) | 0 (0.0) | 0 (0.0) | Glu (GAG) 942 (11.5%) | 0 (0.0) | 2585 (35.5%) | 0 (0.0) | (ACC) 131 (6.5%) | 0 (0.0) |

Table A.2 continued

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| SH2 | 3392 (33.9) | 0 (0.0) | 0 (0.0) | 0 (0.0) | Glu (GAG) 1081 (18.5%) | 0 (0.0) | 901 (18.3%) | (AAT) 35 (2.5%) | (ACC) 20 (1.4%) | 0 (0.0) |
| SH3 | 2578 (25.8) | 0 (0.0) | Ser (TCT) 61 (1.5%) His (CAT) 68 (1.7%) | 0 (0.0) | Glu (GAG) 2235 (34.4%) | 0 (0.0) | 2592 (44.0%) | (AAT) 136 (9.2%) | 0 (0.0) | 0 (0.0) |
| SP1 | 3271 (32.7) | Asn (AAT) 16 (1.2%) | Thr (ACT) 426 (11.7%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2388 (45.3%) | (AAT) 17 (1.3%) | 0 (0.0) | 0 (0.0) |
| SP2 | 1749 (17.5) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 569 (41.3%) | 0 (0.0) | (ACC) 31 (8.1%) | 0 (0.0) |
| SP3 | 2907 (29.1) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1090 (19.3%) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| SU1 | 3090 (30.9) | Thr (ACT) 12 (1.0%) | Thr (ACT) 156 (4.4%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1303 (26.1%) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| SU2 | 2902 (29.0) | Asn (AAT) 32 (1.8%) | Thr (ACT) 266 (5.5%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1371 (19.2%) | (AAT/AAC) 58 (3.4%) | 0 (0.0) | 0 (0.0) |
| SU3 | 3067 (30.7) | Asn (AAT) 28 (1.6%) | Leu (CTT) 86 (1.9%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 4075 (65.7%) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| TI1 | 2905 (29.1) | 0 (0.0) | Thr (ACT) 223 (9.2%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 816 (20.4%) | (AAC) 240 (16.7%) | 0 (0.0) | 0 (0.0) |
| TI2 | 2700 (27.0) | 0 (0.0) | Thr (ACT) 64 (1.6%) Ser (TCT) 513 (12.4%) Leu (CTT) 669 (16.2%) | 0 (0.0) | Glu (GAG) 110 (1.7%) | 0 (0.0) | 1189 (19.2%) | (AAC) 137 (8.7%) | 0 (0.0) | 0 (0.0) |

Table A.2 continued

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| TI3 | 3204 (32.0) | 0 (0.0) | Thr (ACT) 609 (11.2%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1772 (27.9%) | (AAT/AAC) 183 (12.1%) | 0 (0.0) | 0 (0.0) |
| VA1 | 3140 (31.4) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2150 (65.6%) | (AAT/AAC) 76 (9.2%) | 0 (0.0) | 0 (0.0) |
| VA2 | 2947 (29.5) | Asn (AAT/AAC) 257 (12.8%) Thr (ACT) 19 (1.0%) | 0 (0.0) | 0 (0.0) | Glu (GAG) 145 (1.7%) | 0 (0.0) | 2760 (36.7%) | (AAT/AAC) 59 (3.0%) | 0 (0.0) | 0 (0.0) |
| VA3 | 2144 (21.4) | Asn (AAT) 12 (1.9%) Thr (ACT) (1.3%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 876 (50.3%) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| WA1 | 3032 (30.3) | Asn (AAT) 25 (1.2%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 5395 (67.7%) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| WA2 | 2913 (29.1) | Asn (AAT) 39 (1.2%) | Leu (CTT) 90 (1.7%) | 0 (0.0) | Glu (GAG) 101 (1.3%) | 0 (0.0) | 3578 (48.0%) | (AAT/AAC) 140 (7.0%) | (ACT) 36 (1.8%) | 0 (0.0) |
| WA3 | 2843 (28.4) | Asn (AAT) 53 (4.4%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1352 (25.0%) | (AAT/AAC) 843 (53.1%) | 0 (0.0) | 0 (0.0) |
| PO2 | 3170 (31.7) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2402 (49.8%) | (AAT/AAC) 331 (26.3%) | 0 (0.0) *0* | 0 (0.0 |
| HW1 | 2294 (22.9) | 0 (0.0) | Leu (CTC)1033 (3.7%) *6* | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| HW3 | 4118 (41.2) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |

Table A.2 continued

[a]Numbers in parentheses represent the sequencing coverage depth for the amplicon in the population considered (i.e. the average number of times the region corresponding to the amplicon is expected to have been sequenced in each of the 100 ALS gene copies present in each population)
[b]Percentage of the NGS sequence reads containing the mutant codon relative to the total number of sequence reads corresponding to the ALS amplicon.
[c]Number in italics (for the W574L and S653N columns; P197L for horseweed) represents the number of alleles carrying the mutation in the population out of 100, as identified by SNP genotyping.
[d]No population contained an amino acid substitution at codon Ala 205.

Table A.3. Summary of genotypic results of soil seedbank-collected individuals, genotyped by TaqMan® assay. Number of individuals per population varied based on how many plants emerged out of the soil samples collected. All populations are shown despite lack of emergence.

| | | | Mutation FOR[a] | | | |
|---|---|---|---|---|---|---|
| County | Population | Individuals | W574L | S653N | S653T | Overall FOR |
| | | ----no.---- | ----------no.---------- | | | -------%------- |
| Benton | BE1 | 5 | 40 (20) | 0 (0) | 40 (20) | 80 |
| | BE2 | 17 | 47 (29) | 6 (3) | 12 (6) | 53 |
| | BE3 | 2 | 0 (0) | 0 (0) | 0 (0) | 0 |
| Dubois | DU1 | 50 | 92 (65) | 0 (0) | 0 (0) | 65 |
| | DU2 | 50 | 82 (53) | 22 (11) | 0 (0) | 96 |
| | DU3 | 50 | 50 (32) | 16 (12) | 0 (0) | 66 |
| Gibson | GI1 | 46 | 57 (38) | 15 (8) | 0 (0) | 70 |
| | GI2 | 9 | 67 (33) | 11 (6) | 0 (0) | 78 |
| | GI3 | 50 | 86 (60) | 4 (2) | 0 (0) | 86 |
| Hendricks | HE1 | 37 | 86 (64) | 3 (1) | 0 (0) | 89 |
| | HE2 | 0 | n/a | n/a | n/a | n/a |
| | HE3 | 50 | 68 (40) | 6 (3) | 0 (0) | 70 |
| Knox | KN1 | 50 | 76 (48) | 10 (5) | 2 (1) | 84 |
| | KN2 | 50 | 84 (58) | 0 (0) | 4 (2) | 88 |
| | KN3 | 50 | 48 (34) | 12 (6) | 0 (0) | 60 |
| Newton | NE1 | 11 | 64 (50) | 0 (0) | 0 (0) | 64 |
| | NE2 | 50 | 54 (33) | 34 (19) | 18 (11) | 80 |
| | NE3 | 4 | 100 (50) | 25 (13) | 0 (0) | 100 |
| Posey | PO1 | 29 | 45 (24) | 10 (5) | 0 (0) | 52 |
| | PO3 | 50 | 70 (45) | 10 (6) | 0 (0) | 74 |
| | PO4 | 11 | 82 (59) | 9 (5) | 0 (0) | 91 |
| Randolph | RA1 | 18 | 22 (14) | 6 (3) | 6 (3) | 33 |
| | RA2 | 3 | 0 (0) | 0 (0) | 0 (0) | 0 |
| | RA3 | 50 | 4 (2) | 4 (2) | 0 (0) | 8 |
| Shelby | SH1 | 50 | 54 (42) | 6 (3) | 0 (0) | 56 |
| | SH2 | 50 | 42 (22) | 4 (2) | 0 (0) | 46 |
| | SH3 | 50 | 72 (44) | 6 (3) | 0 (0) | 76 |
| Spencer | SP1 | 17 | 94 (68) | 0 (0) | 0 (0) | 94 |
| | SP2 | 3 | 67 (50) | 33 (17) | 0 (0) | 67 |
| | SP3 | 1 | 1 (50) | 0 (0) | 0 (0) | 100 |
| Sullivan | SU1 | 38 | 58 (32) | 13 (7) | 0 (0) | 66 |
| | SU2 | 50 | 46 (25) | 12 (6) | 2 (1) | 54 |
| | SU3 | 50 | 78 (50) | 4 (2) | 0 (0) | 78 |
| Tippecanoe | TI1 | 11 | 55 (32) | 0 (0) | 0 (0) | 55 |
| | TI2 | 5 | 40 (30) | 20 (10) | 0 (0) | 50 |
| | TI3 | 1 | 0 (0) | 100 (50) | 0 (0) | 100 |

Table A.3 continued

| | | | | | | |
|---|---|---|---|---|---|---|
| Vanderburgh | VA1 | 50 | 82 (57) | 16 (8) | 0 (0) | 84 |
| | VA2 | 50 | 82 (55) | 14 (7) | 0 (0) | 82 |
| | VA3 | 50 | 82 (51) | 14 (7) | 0 (0) | 86 |
| Warrick | WA1 | 46 | 89 (63) | 0 (0) | 0 (0) | 46 |
| | WA2 | 19 | 68 (58) | 0 (0) | 0 (0) | 68 |
| | WA3 | 25 | 48 (26) | 56 (32) | 0 (0) | 84 |

[a]Number in perentheses is the frequency of the respective resistance allele.

# VITA

# Jodi Boe

## EDUCATION

**Purdue University** West Lafayette, Indiana May 2016 – May 2019 (Expected)
**Master of Science** Weed Science
**GPA** 3.88/4.0
**Thesis Research** Investigating the Value of ALS-Inhibiting Herbicides in ALS-Inhibitor Resistant Populations of Weeds
**Advisor** Bryan G Young, Professor of Weed Science

**North Dakota State University** Fargo, North Dakota September 2011 – May 2016
**Bachelor of Science** Crop and Weed Sciences and Agricultural Economics
**Minor** Soil Science
**GPA** 3.904/4.00 *Summa cum laude*
**Dean's List** 10 consecutive semesters

## EXPERIENCE

**BASF** Madison, Wisconsin February 2018 – Present
Technical Service Representative – Wisconsin and Northern Illinois

- Serve as technical lead for sales team
- Coordinate with external cooperators to demonstrate product performance and agronomic concepts
- Educate customers on proper use of products and technical aspects of agronomy

**Purdue University** West Lafayette, Indiana May 2016 – July 2018
Graduate Research/Teaching Assistant

- Conducted field and greenhouse research investigating populations weeds with ALS-inhibitor resistance
- Set up field trials for weed science research, prepared treatments and applied them using standard small plot research procedures
- Performed DNA extractions, SNP genotyping, and Next-Generation Sequencing
- Trained undergraduate and graduate students in lab and greenhouse techniques
- Teaching assistant for "Introductory Weed Science" (BTNY 304), Fall 2017

**BASF** Raleigh, North Carolina July 2018 – February 2018
Professional Development Program – Technical Services

- Collaborated with Technical Market Managers, Tech Service Representatives, and external design group to update Product Reference Guides for distribution for eight unique business districts
- Managed and conducted training events for launch of an internal agronomy education tool

- Coordinated data collection for 2018 plant health performance trials

**North Dakota State University** Fargo, North Dakota August 2015 – November 2015
World Food Crops Undergraduate Teaching Assistant, Messersmith Teaching Fellowship

- Observed class lab instruction twice every week and assist in lab instruct once every week with Dr. Ed Deckard
- Held study sessions to help students understand course material
- Assessed student learning through lab quiz grading

**North Dakota State University** Fargo, North Dakota November 2013 – May 2015
Department of Plant Sciences, Potato Agronomy Undergraduate Research Assistant

- Drafted and advised in the writing of an extension bulletin related to potatoes and potato cultivation
- Assisted potato agronomy graduate students in extracting nightshade seed for weed control experiments
- Entered data into Microsoft Excel from studies conducted over the summer

**BASF** Seymour, Illinois May 2015 – August 2015
Technical Crop Production Agronomist/Ag Professional Development Program Intern

- Worked with a variety of agronomic tools to discover their potential use with BASF products. Agronomic tools included a soil nitrate tester, liquid fertilizer applicator, under canopy plant protection applicator, and mobile crop scouting application
- Collaborated with a soil scientist from the University of Illinois to design and carry out a study on nitrate nitrogen and its presence in soil over time
- Collected data on corn disease levels versus time at the Midwest Research Farm (MWRF) in Seymour and at a farm in Paris, IL
- Lead work on an internal mobile photo app with the goals of discovering potential uses and providing feedback and recommendations to the app developer
- Presented findings to supervisors at the MWRF, growers on MWRF grower tours, and company leaders at the MWRF and at the BASF Regional Meeting in Indianapolis, IN

**Bayer CropScience** Sabin, Minnesota May 2014 – August 2014
Research Associate/Northern Field Technology Station Intern

- Prepared chemicals for application in research trials and assisted in trial layout
- Worked collaboratively with a team of interns to accomplish station-wide tasks
- Operated equipment of various sizes for trial crop and weed planting and for station and plot maintenance
- Recorded efficacy of compounds on cereals, along with stand and pest counts

**Northern Plains Cooperative – CHS** Selby, South Dakota June 2013 – August 2013
Sales Agronomist Intern

- Met and exceeded customer expectations by assisting them with weed identification, crop scouting and pesticide selection

- Forged relationships with growers through effective communication that involved active listening and empathy
- Built sales agronomy skills through chemical, seed, and fertilizer plant management

**Central Ag Consulting** Edgeley, North Dakota
Crop Scout Intern May 2012 - August 2012

- Connected theories about crop production and soil management learned from class to hands on, real world experiences
- Learned critical agronomy competencies by scouting fields, identifying pests, and taking stand counts
- Effectively communicated findings from the field to growers
- Demonstrated ability to work independently through accurate observations of field problems and competent navigation

**Family Farm** Golden Valley, North Dakota September 2007 – September 2011
General Laborer

- Assisted in farm management operations that included caring for laying hens; maintaining lawns, gardens, pastures and fences; and growing small grains
- Developed basic knowledge of agricultural terminology and nomenclature related to basic farm operations

## PROCEEDINGS AND PUBLICATIONS

- Boe JE, Nie H, Young JM, Young BG (2018) Effect of soil- vs. foliar-applied ALS-inhibiting herbicides on control of ALS-resistant horseweed (*Conyza canadensis*). Paper presented at Weed Science Society of America (January) *3rd Place M.S. Student Oral Contest*
- Boe JE, Nie H, Young BG (2017) Frequency of target-site resistance and susceptibility to ALS-inhibiting herbicides in Indiana waterhemp populations. Poster presented at North Central Weed Science Society meeting (December)
- Boe JE, Nie H, Young BG (2017) Interaction of application timing, herbicide active ingredient, and specific target-site mutation on the selection of ALS-inhibitor resistant horseweed and tall waterhemp. Paper presented at North Central Weed Science Society meeting (December)
- Boe JE, Nie H, Young BG (2016) Do ALS-inhibiting herbicides have any value when targeting fields with weeds resistant to those herbicides? Paper presented at North Central Weed Science Society meeting (December) *1st Place Student Contest*
- Robinson, A., J. Garden-Robinson, J. Boe, A. Dhuyvetter. 2015. From Garden to Table: My Potatoes Turned Green Now What? *North Dakota State University Extension Publication* A1768. North Dakota State University. https://www.ag.ndsu.edu/pubs/plantsci/rowcrops/a1768.pdf

## PROFESSIONAL DEVELOPMENT

- Proficient in SAS 9.4, JMP Edition 12, R statistical software, SigmaPlot, ARM software, Integrative Genomics Viewer, and Microsoft Office Suite of products

## INTERNATIONAL EXPERIENCE

**North Dakota State University**

- **Global Food Systems Study Abroad Program – China** May 2013
  - Visited three Chinese agriculture universities, toured nine diverse agricultural businesses and gained a broad understanding of the agricultural problems China faces
- **Bioenergy Crops in Europe Study Abroad Tour – Germany, Austria, Italy** June 2013
  - Visited two bioenergy facilities in Austria, toured three university research farms, one in Germany, Austria and Italy, and studied at the University of Bologna for a week on crop physiology and bioenergy plant characteristics

**National FFA Organization**

- **International Leadership Seminar – Argentina** January 2013
  - Toured a number of farms that specialized in either grain, sheep or dairy production; visited a Bunge soybean crushing facility in Rosario, and got a firsthand experience of the stockyards in Buenos Aires

## HONORS AND AWARDS

- **2018 M.S. Student Oral Contest 3rd Place** *Weed Science Society of America*
- **2018 Graduate Student Travel Award** *Weed Science Society of America*
- **2017 Weeds Contest 1st Place Graduate Student** *North Central Weed Science Society*
- **2017 Weeds Contest 2nd Place Graduate Team Member** *North Central Weed Science Society*
- **2016 Weed Biology, Ecology, and Management 1st Place Paper** *North Central Weed Science Society*
- **2016 J. Fielding Reed Scholarship** *American Society of Agronomy*
- **2016 Summa Cum Laude Distinction** *North Dakota State University*
- **2016 Top Ten Senior Award** *NDSU College of Agriculture, Food Systems, & Natural Resources*
- **2016 Outstanding Senior in Agronomy, Crop, Soil & Environmental Sciences** *American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America*
- **2015 Messersmith Teaching Fellowship** *NDSU Department of Plant Sciences*
- **2014 Agronomy Society of America Crops Contest High Individual**
- **2014 Young Leader Scholarship Program Recipient** *Mid-America CropLife Association*
- **2013 American FFA Degree** *National FFA Organization*
  - The American FFA Degree is awarded to members who have demonstrated the highest level of commitment to FFA and made significant accomplishments in their supervised agricultural experiences.

## CLUBS AND PROFESSIONAL AFFILIATIONS

- **Agriculture Future of America Alliance** *Lifetime Member*
- **Agronomy Society of America, Soil Science Society of America, Crop Science Society of America** *Member 2014 – Present*
- **National FFA Organization**
  - 2011-12 North Dakota State FFA President
- **NDSU Gamma Sigma Delta,** *The Honor Society of Agriculture, Inducted 2015*
- **North Central Weed Science Society** *Member 2016 - Present*
- **Purdue University Botany and Plant Pathology Graduate Student Organization**
  - 2016-17 Vice President
- **Weed Science Society of America** *Member, 2017 - Present*

## UNIVERSITY SERVICE

**NDSU Career Center** April 2014 – June 2014

- Search Committee for Career Specialist, Alumni Relations, Undergraduate Representative

**NDSU Department of Agribusiness and Applied Economics** May 2015 – September 2015

- Search Committee for the Assistant Director of Center for Agricultural Policy and Trade Studies, Undergraduate Representative

**NDSU Department of Plant Sciences**

- 2014-Present Curriculum Committee, Undergraduate Representative
- 2013 Social Media Committee, Undergraduate Representative

## PROFESSIONAL MEDIA

McCauley C, Boe JE, Winklepleck C, Steppig NR, Young BG (2017) Nozzle selection and boom height: Two factors that affect herbicide particle drift. https://www.youtube.com/watch?v=IHWBlHn