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Characterization of Glyphosate-Resistant Amaranthus Palmeri (Palmer Amaranth) Tolerance to ALS- and HPPD-Inhibiting Herbicides

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Characterization of Glyphosate-Resistant *Amaranthus Palmeri* (Palmer Amaranth) Tolerance to ALS- and HPPD-Inhibiting Herbicides

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ABSTRACT

Palmer amaranth is a principal weed problem across the United States and is resistant to several herbicide modes of action. By 2008, Palmer amaranth in Arkansas was reported to be resistant to both ALS- and EPSPS-inhibitors, but the predominant resistance mechanisms are yet to be explored. Herbicide options with different modes of action are needed to provide effective Palmer amaranth control and HPPD-inhibitors (e.g. mesotrione) are among these. The goal of this research was to elucidate the resistance profile of Palmer amaranth in Arkansas to ALS herbicides and glyphosate (EPSPS-inhibitor) as well as evaluate the differential tolerance of Palmer amaranth to mesotrione. This research aimed to (1) evaluate the response of Palmer amaranth populations to the full dose of glyphosate and mesotrione; (2) determine if tolerance to mesotrione is heritable; (3) determine the mechanism of resistance to glyphosate in selected accessions; and (4) verify the target-site as the mechanism of resistance in ALS-resistant Palmer amaranth. For objective 1, a total of 119 accessions were collected from crop fields in Arkansas between 2008 and 2014. Overall, 55% of the accessions (115) were glyphosate-resistant (GR). Mesotrione controlled 74% of the accessions (119); the remaining accessions had survivors with high injury (61%-90%). For objective 2, low level of tolerance to mesotrione (3- to 5-fold) was observed in four recalcitrant accessions. For objective 3, 20 accessions were selected. GR accessions had ED_{50} 494 g ha^{-1} to 1355 g ha^{-1} and for susceptible accessions ED_{50} ranged from 28 g ha^{-1} to 207 g ha^{-1}. *EPSPS* gene amplification was the primary mechanism of resistance. For objective 4, Palmer amaranth accessions were cross-resistant to pyrithiobac and trifloxysulfuron. Out of 20 accessions, 19 showed 21- to 56-fold resistance to trifloxysulfuron than the susceptible. Four and seven increased *ALS* copies were observed in a single plant from White and Mississippi counties, respectively, indicating the elevated *ALS* copies as potential

mechanism of resistance in these accessions. Although, all accessions but susceptible had Trp574Ser mutation along with Ala122Thr, Pro197Ala and Ser653Asn present in a few plants, confirming mutations at the target-site as the main mechanism of resistance to ALS-inhibitors.

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DEDICATION

I dedicate this work to the two gems of my life, daughter (Anshika Singh) and husband (Vijay Singh). Also, I would thank and pay my gratitude to both my parents and in-laws for their love, support and confidence in me throughout the program. Especially, I would take this opportunity to thank my dear husband for always being there, encouraging and motivating me to complete this program.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER III

CHAPTER IV

CHAPTER V

LIST OF FIGURES

CHAPTER III

CHAPTER IV

CHAPTER V

LIST OF SUBMITTED ARTICLE

CHAPTER I

INTRODUCTION

Introduction

Palmer amaranth (*Amaranthus palmeri* S. Wats) belongs to the *Amaranthaceae* family, which consists of approximately 75 species. Of these, 10 form a subgroup of dioecious species, which includes Palmer amaranth (Steckel 2007). It is native in the US with an ecological range stretching from northwestern Mexico to southern California, New Mexico, and Texas. Its broad range is facilitated by agriculture-related activities. Palmer amaranth was reported in Virginia in 1915, Oklahoma in 1926, and South Carolina in 1957 (Sauer 1957). Palmer amaranth is one of the most common, troublesome and economically challenging weeds of the southern US (Ward et al. 2013). The traits that make this species problematic are: small seed size (Sauer 1955), fast growth rate (Jha et al. 2008), high fecundity (Keeley et al. 1987), good light interception, and high water use efficiency (Ehleringer 1983). Palmer amaranth has a C_4 photosynthesis system (Ehleringer 1983), which gives it further advantage over C_3 crops. Palmer amaranth is drought- and shade-tolerant (Ehleringer 1983 and Jha et al. 2008). The competitiveness of Palmer amaranth results in high yield losses in agronomic crops such as cotton (92%) and soybean (68%) (Klingaman and Oliver 1994; Rowland et al. 1999). In grain sorghum, grain yield was reduced by 1.8% - 3.5% with 1 weed per 15 m of row (Moore et al. 2004). Palmer amaranth is dioecious; therefore, it is an obligate outcrosser (Franssen et al. 2001) and can hybridize with other *Amaranthus* species such as spiny amaranth (*Amaranthus spinosus*) (Tranel et al. 2002; Wassom and Tranel 2005). It has been reported that the pollen of Palmer amaranth can travel up to 46 km and at a distance of 300 m pollen from a glyphosate-resistant Palmer amaranth was transferred to glyphosate-susceptible Palmer amaranth (Sosnoskie et al. 2012). Palmer produces voluminous, tiny seeds, allowing it to spread extensively; thus, making it difficult to contain. Due to an extended seed emergence pattern, Palmer amaranth can germinate late in the season and not be controlled chemically because of crop stage constraints. These late-emerged plants, or

the escapes from herbicide applications, cause mechanical interference during harvest and contamination of the harvested product (Keeley et al. 1987; Jha et al. 2006). Sauer (1957) observed: ‘‘Of all the dioecious amaranths, *A. palmeri* has been by far the most successful as a weedy invader of artificial habitats, whether they were prepared by primitive or modern technology.’’ This exceptional ability to survive and thrive in the toughest conditions has challenged the ability of farmers and scientists to find ways to control it. Controlling Palmer amaranth has become a challenge because chemical control options are constrained by rapid resistance evolution in this species. To date, Palmer amaranth has evolved resistance to ALS (acetolactate synthase)-, EPSPS (5-enolpyruvylshikimate-3-phosphate synthase)-, microtubule assembly-, PSII (photosystem II)-, HPPD (p-hydroxyphenylpyruvate dioxygenase)-, and PPO (protoporphyrinogen oxidase) inhibitors (Heap 2017).

ALS inhibitors were introduced in 1982. When the ALS or AHAS enzyme is inhibited, the production of branched-chain amino acids isoleucine, leucine, and valine stops, which ultimately results in plant death. There are five chemical families of ALS herbicides; sulfonylurea (SU), imidazolinone (IMI), triazolopyrimidine (TP), pyrimidinyl(thio)benzoate (PTB), and sulfonylaminocarbonyl-triazolinone (Tranel and Wright 2002). The ALS herbicide characteristics of being broad spectrum, effective at low rates, and with moderate residual activity have made these herbicides popular among the growers in the United States (Tranel and Wright 2002). These herbicides have been used extensively to control Palmer amaranth (Gaeddert et al. 1997; Ward et al. 2013). Resistance to ALS-inhibiting herbicides was reported within five years of commercialization, with prickly lettuce (*Lactuca serriola* L.) and kochia [*Kochia scoparia* (L.) Shrad] being the first cases (Mallory-Smith et al. 1990; Primiani et al. 1990). Resistance to this group of herbicides is widespread. Today, 158 species are reported to

be resistant to ALS-inhibiting herbicides (Heap 2017). In the United States, 51 species, including Palmer amaranth, are ALS-resistant.

Glyphosate has been used extensively by producers to control different weed species, resulting in widespread evolution of resistance to this herbicide. The use of glyphosate increased with the introduction of glyphosate-resistant crop technology in 1990s. Before the adoption of HR (herbicide-resistant) crop technology, farmers used many herbicides with different modes of action (Foresman and Glasgow 2008; Gustafson 2008). The conventional weed management program was affected greatly by the technology (Green 2009). The general conventional practices such as tillage, inter- row cultivation, stale seedbed technique, flooding (in rice), and crop rotation were practiced by farmers as part of weed management strategies along with using herbicides with different modes of action. According to Benbrook (2012), generally, a 239 million kilogram increase in usage of herbicides was due to increased reliance on glyphosate. The dependence on glyphosate only in glyphosate-resistant corn, cotton, and soybean led to increasing evolution of glyphosate-resistant weeds in the United States between 1996 and 2011. In the glyphosate-resistant crop production system, crop rotations are not restricted and weed control is easier as glyphosate can control both broad- and narrow-leaved weeds and can be sprayed over the crop (Cerdeira and Duke 2006). However, farmers generally practice monoculture; very few rotate crops. One of the major reasons for the rapid and widespread adoption of glyphosate-resistant crops by growers was the reduction in expenses as glyphosate is relatively inexpensive compared to other herbicide programs, and the simplicity of the technology as only one herbicide is needed to control weeds (Gianessi 2008).

The first case of glyphosate-resistant Palmer amaranth was reported in Georgia in 2005 (Culpepper et al. 2006). Today, 27 states have reported the presence of glyphosate-resistant

Palmer amaranth (Heap 2017). Palmer amaranth is an obligate outcrosser; therefore, cross-pollination accelerated the spread of resistance. Many Palmer amaranth populations also have been selected already for resistance to ALS herbicides; hence, it is expected that many glyphosate-resistant Palmer amaranth are also resistant to ALS herbicides. Many cases of multiple resistances in Palmer amaranth have been reported. In Georgia, glyphosate-resistant populations were also reported to have resistance to pyrithiobac (Sosnoskie et al. 2011). Similarly, in Mississippi Palmer amaranth populations, which were resistant to glyphosate were also found to be resistant to ALS inhibitors (Nandula et al. 2012). Evolution of resistance to these two groups of herbicides has limited the option of sole reliance on a single mode of action in HR crops. Therefore, knowing the response of Palmer amaranth populations to alternative herbicides in HR crops will improve herbicide recommendations for resistance management.

A relatively recent group of herbicides used in corn and wheat is the HPPD inhibitors. In the biosynthesis process of plastoquinone and tocopherol, HPPD catalyzes the conversion of 4-hydroxymethylpyruvate to homogentisate (Grossmann and Ehrhardt 2007). The inhibition of HPPD by herbicides leads to photooxidative destruction of chlorophyll and destruction of membranes of photosynthetic organelles in emerging shoot tissue, resulting in a characteristic bleaching of new leaf tissues. HPPD inhibitors are divided into three families: isoxazoles (e.g. isoxaflutole and pyrasulfotole), pyrazolones (e.g. topramezone), and triketones (e.g. mesotrione and tembotrione) (Heap 2017). Herbicides belonging to the triketone family are more widely used than the others and are recommended for corn, sorghum, berries and asparagus. Broadleaf weed control and excellent crop tolerance are some of the characteristics of this group, which has made it an integral part of weed management program in corn production systems (Beaudegnies et al. 2009). In the southern US corn, cotton, and soybean are major crops and corn has inherent

tolerance to HPPD inhibitors. After the development of glyphosate- and glufosinate-resistant crop technology, scientists are now working towards the development of new combinations of HR crops to combat the evolution of resistance in weeds. Soybean and cotton with resistance to HPPD-inhibiting herbicides will be commercialized soon that would enable new uses for HPPD inhibitors. Launching this technology would have a significant impact on weed management practices because HPPD-inhibiting herbicides have some soil residual activity and could help control some troublesome weed species such as *Amaranthus* spp., *Solanum* spp., and *Polygonum* spp. etc. (Allen et al. 2012). To date, resistance to mesotrione is rare as only the *Amaranthus* species (*A. Palmeri* and *A. tuberculatus*) have been reported to develop resistance to it. Mesotrione-resistant waterhemp (*A. tuberculatus*) populations have been reported in Iowa, Illinois, Nebraska and Kansas in 2009 and resistant biotypes of Palmer amaranth were found in Kansas and Nebraska in 2009 and 2014, respectively (Heap 2017). Mesotrione and other HPPD inhibitors have been reported to be potent in controlling ALS- and PSII-inhibitor resistant weed biotypes (Sutton et al. 2002). Therefore, effective herbicides must be sustained as new herbicide resistance traits are being stacked in crops to control weeds.

This research aimed to evaluate the level of resistance in Palmer amaranth from Arkansas to ALS-inhibitors and glyphosate and their tolerance level to mesotrione. The research focus was also to determine the mechanism by which certain populations were able to survive the application of these herbicides. The objectives were to: (1) evaluate the differential tolerance of glyphosate-resistant Palmer amaranth populations to mesotrione; (2) determine the mechanism(s) conferring resistance to glyphosate; and (3) determine the level of resistance and mechanism(s) conferring resistance to ALS inhibitors among Palmer amaranth populations from Arkansas.

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CHAPTER II

REVIEW OF LITERATURE

Review of Literature

Crop yield losses due to *Amaranthus* infestation

In general, fast germination and rapid growth rate are attributes of a competitive plant (Badosevich and Holt 1984). To support its rapid growth and high biomass production, Palmer amaranth is a very strong competitor for water and nutrients resulting in huge crop yield losses depending on the density and duration of interference. In a study over two years and three locations in Oklahoma, it was reported that an increase of 1 Palmer amaranth row^{-1} resulted in 5-12% reduction in cotton lint yield (Rowland et al. 1999). An experiment conducted at College Station, TX revealed that Palmer amaranth infestation up to 10 plants 9 m^{-1} row decreased biomass of cotton by more than 50% at 8 weeks and season-long interference reduced the cotton yields from 13-54% (Morgan et al. 2001). Fast et al. (2009) reported that the interference of Palmer amaranth up to 63 days can cause 77% yield losses in cotton. In peanut, interference of Palmer amaranth at 1 plant m^{-2} of row resulted in a yield loss of up to 28% (Burke et al. 2007). In sweet potato, Palmer amaranth density of 0.5- 6.5 plants m^{-1} resulted in yield loss from 56% - 94%, respectively (Meyers et al. 2010). In Kansas, a competition study between corn and Palmer amaranth was conducted for 3 years when corn was planted in 10 m by 76 cm plots at a density of 75,000 plants ha^{-1}. Palmer amaranth at 0.5 to 8 plants m^{-2} emerged with corn significantly reduced grain yield from 11 to 91% (Massinga et al. 2001). Palmer amaranth in crop fields at harvest can damage a combine and reduces harvest efficiency. The time to harvest cotton ranged from 79 min ha^{-1} in weed-free plots to more than 90 min ha^{-1} at the highest weed density (3260 plants ha^{-1}).

Chemical control and evolution of resistance

Palmer amaranth can be controlled by making timely applications of pre-emergence (PRE) and post-emergence (POST) herbicides. Some of the PRE herbicides labeled on different crops for Palmer amaranth control are diuron (e.g. corn, cotton, grain sorghum), fluometuron (e.g. cotton, sugarcane), fomesafen (e.g. soybean, cotton), pendimethalin (e.g. corn, cotton, soybean, and many other crops), pyrithiobac (cotton), pyroxasulfone (e.g. corn, cotton, soybean, wheat), saflufenacil (e.g. corn, cotton, alfalfa), *S*-metolachlor (e.g. corn, cotton, soybean, and many other crops), and tembotrione (e.g. corn) (York and Culpepper 2009). Labelled postemergence herbicides for Palmer amaranth control include atrazine (e.g. corn, sorghum), dicamba (e.g. corn, sorghum), fomesafen (e.g. soybean), glufosinate (e.g. LibertyLink crops), glyphosate (e.g. Roundup Ready crops), and mesotrione (e.g. corn, sorghum) (Norsworthy et al. 2008). Excessive use of herbicides has resulted in the evolution of resistance in weeds. So, when a plant evolves resistance to an herbicide, the herbicide is no longer lethal because of: 1) changes in the target-site or 2) nontarget-site changes which include reduced absorption and translocation or enhanced metabolism (Powles and Yu 2010). Currently, there are 478 unique cases of herbicide-resistant weeds among 252 species worldwide (Heap 2017). In Palmer amaranth, resistance has been confirmed to six modes of action: EPSPS-, ALS-, microtubule assembly-, PSII-, HPPD- and PPO-inhibitors (Heap 2017).

Resistance to ALS- inhibitors

The ALS enzyme catalyzes two reactions: condensation of two pyruvate molecules to produce CO_2 and acetolactate, a precursor of valine and leucine; and condensation of pyruvate and α-ketobutyrate to form CO_2 and 2-acetohydroxybutyrate, a precursor of isoleucine. Hence, the inhibition of ALS enzyme leads to plant starvation of essential amino acids valine, leucine

and isoleucine resulting in plant death (Duke 1990). In 1982, chlorsulfuron was introduced as the first ALS-inhibiting herbicide (Tranel and Wright 2002). Since then, 57 ALS herbicides have been used widely as a chemical tool for weed control due to favorable characteristics such as; high margin of crop safety, efficacy at very low rates, broad-spectrum weed control, soil residual activity, and low level of toxicity to mammals (Mazur and Falco 1989; Tranel and Wright 2002). Due to over usage and the resulting intense selection pressure, resistant weeds were reported in less than five years of commercialization of ALS herbicides. In 1987, the first chlorsulfuron-resistant prickly lettuce (*Lactuca serriola* L.) and kochia (*Kochia scoparia* L.) were reported (Mallory-Smith et al. 1990; Primiani et al. 1990). In the United States, the first case of resistance to ALS-inhibiting herbicides in Palmer amaranth was reported in 1993 in Kansas State and in 1994, cross-resistance to five ALS herbicides was documented in Arkansas (Heap 2017). In general, Palmer amaranth is resistant to acetolactate synthase (ALS)-inhibitor throughout the United States. As a result, ALS-inhibiting herbicides are no longer effective on ALS-resistant populations of Palmer amaranth.

Target-site mechanism of resistance can occur by point mutations resulting in amino acid substitutions. Generally, a nucleotide in the DNA sequence of the gene is replaced by another. If this mutation results in an amino acid change in the protein sequence in a conserved region, the mutation alters the conformation of the substrate binding site, which reduces the herbicide binding affinity. Resistance to ALS-inhibiting herbicides mostly results from a single amino acid substitution in a catalytic domain of the ALS enzyme. Target-site resistance to ALS-inhibiting herbicides has been attributed to a change in one of eight amino acids located in various regions across the gene including Ala122, Pro197, Ala205, Asp376, Arg377, Trp574, Ser653, and Gly654 (Yu and Powles 2014). Mutations in one of these amino acids alter herbicide binding and

results in an herbicide-resistant plant (Corbett and Tardiff 2006). The specific mutation determines to which ALS inhibitor family the weed will be resistant. For instance, mutation in Pro197 results in high resistance to the sulfonylureas (SUs) and low resistance to imidazolinones (IMIs), Ala122 results in resistance to only the IMIs whereas Trp574 confers resistance to both SUs and IMIs (Yu and Powles 2014). The difference in resistance patterns associated with different mutations indicates that herbicides in different ALS families bind to different areas of the binding site (Powles and Yu 2010). The first detected *ALS* mutation Pro197His was in prickly lettuce which conferred resistance to sulfonylurea herbicide (Eberlein et al. 1997). Tribenuron-methyl resistance was due to Pro197Ser substitution in wild mustard (*Sinapis arvensis* L.) (Cruz-Hipolito et al. 2013). The substitution of Pro197 with Ala, Ser, or Gln is responsible for resistance in seven sulfonylurea-resistant biotypes of *Lindernia* spp. in rice (Uchino and Watanabe 2002). Also, a Pro197 substitution by Ala, His, Ser, and Thr was reported in eight wild radish (*Raphanus raphanistrum* L.) populations resistant to chlorsulfuron (20- to 160-fold), metosulam (10- to 46-fold), and metsulfuron (3- to 8- fold) (Yu et al. 2003).

In *Amaranthus* spp., the first case of target-site mutation (Trp574Leu) was reported in common waterhemp (*A. rudis* (Moq.) Sauer) from Illinois which was resistant to imazethapyr (1000- fold) and cross-resistant to thifensulfuron and flumetsulam (Foes et al. 1998). In Illinois, Trp574Leu, Ser653Asp, and Ser653Thr mutations were identified in ALS-resistant tall waterhemp (*A. tuberculatus* (Moq.) Sauer) biotypes resistant to imazethapyr, but only the biotype with a Trp574Leu mutation was resistant to thifensulfuron. This mutation has been found also in prostrate pigweed (*A. blitoides*) (Sibony and Rubin 2003), redroot pigweed (*A. retroflexus* L.), Powell amaranth (*A. powellii*), and smooth pigweed (*A. hybridus*) (Heap 2017). A spiny amaranth and Palmer amaranth hybrid was confirmed resistant to many ALS-inhibitors including

imazethapyr, nicosulfuron, pyrithiobac, and trifloxysulfuron (Molin et al. 2016). Sequencing of *ALS* gene revealed the presence of resistance-conferring mutation, Trp574Leu. A mutation at Asp376Glu in a smooth pigweed (*A. hybridus*) population from Pennsylvania conferred resistance to SU, IMI, PTB, and TP chemical families and exhibited 60- to 3200-fold resistance to all four ALS-inhibiting herbicide families (Whaley et al. 2007). In a similar case, *A. powellii* population from Canada showing high resistance to imazethapyr (25-fold), flumetsulam (9-fold) and flucarbazone (85-fold) contained Asp376Glu mutation (Ashigh et al. 2009). In another case from Ontario, Canada, *A. retroflexus* and *A. powellii* populations were resistant to imazethapyr and thifensulfuron. The amino acid substitutions at Ala122Thr, Ala205Val, and Trp574Leu were found in *A. retroflexus* whereas Ala122Thr, Trp574Leu, and Ser653Thr were confirmed in *A. powellii* (McNaughton et al. 2005). Mutation at Pro197 has been detected only in two of the *Amaranthus* spp. i.e. *A. retroflexus* and *A. blitoides* from Israel (Sibony et al. 2001; Sibony and Rubin 2003).

Non-target-site resistance (NTSR) mechanisms minimizes the amount of herbicide reaching the target site (Powles and Yu 2010). This includes decreased herbicide absorption and translocation, increased herbicide metabolism and sequestration. Herbicides acts as the substrates for enzymes involved in the detoxification process. Cytochrome P450 monooxygenase or glutathione S-transferases are the enzymes that conduct the process of detoxification (Delye 2013). Metabolism-based resistance to ALS inhibitors has been reported in only a few species, including rigid ryegrass (*Lolium rigidum*), blackgrass (*Alopecurus myosuroides*), rigid brome (*Bromus rigidum*), wild oat (*Avena fatua*), late watergrass (*Echinochloa phyllopogon*) and wild mustard (*Sinapis arvensis*) (Yu and Powles 2014). The resistance of wild mustard to sulfonylurea herbicides was due to enhanced metabolism. At 72 h after treatment only 17% of radioactivity

was recovered in the resistant biotype compared to 73% in susceptible plants (Veldhuis et al. 2000). In *E. phyllopogan*, resistance to bispyribac-sodium, was due to detoxification by cytochrome P450s (Yun et al. 2005). In *E. crus-galli* populations from Arkansas (AR2) and Mississippi (MS1), the addition of malathion to penoxsulam showed > 94% reduction in the dry weight of AR2 and MS1 populations suggested that P450 inhibition by malathion could be the reason for resistance to penoxsulam (Riar et al. 2012). Enhanced metabolism conferred cross resistance to four chemical families of ALS-inhibitors in a tall waterhemp population from Illinois (Guo et al. 2015). In general, non-target mechanism cause low level of resistance compared to target site mechanism (Tranel and Wright 2002; Yu and Powles 2014). After the first report of resistance to ALS-inhibitors in 1987 (Mallory-Smith et al. 1990), 159 weed species including 62 monocots and 97 dicots, have been documented with resistance to one or multiple ALS-inhibiting herbicide classes (Heap 2017). Because of the widespread occurrence of ALS-resistant weed species, glyphosate became the tool for Palmer amaranth control (Starke and Oliver1998; Bond et al. 2006).

Resistance to EPSPS-inhibitor (Glyphosate)

Glyphosate [*N*-(phosphonomethyl) glycine] inhibits essential aromatic amino acids (phenylalanine, tyrosine and tryptophan) which are required for protein synthesis (Steinrucken and Amrhein 1980). The inhibition of 5-enolpyruvylshikimate-3-phophate synthase (EPSPS) by glyphosate leads to reduced feedback inhibition of the pathway, resulting in a massive flow of carbon to shikimate-3-phosphate, which is then converted into high levels of shikimate (Duke 1990). The high levels of shikimate accumulation in glyphosate-treated plant tissues helped to discover EPSPS as the molecular target of glyphosate (Steinrucken and Amrhein 1980).

Eventually it was demonstrated that glyphosate interferes indirectly with plant photosynthesis, respiration, and membrane permeability (Geiger et al. 1986) and ultimately the plant dies of a cascade of physiological malfunctions.

Glyphosate, introduced by Monsanto in 1974, is a systemic, non-selective, post-emergence herbicide with low mammalian toxicity (Franz et al. 1997; Baylis 2000; Woodburn 2000). It lacks soil activity, does not leach to ground water, and poses no risk to crops planted after application (Baylis 2000; Duke and Powles 2008). With the introduction of genetically modified glyphosate-resistant crops in the 1990s, the use of glyphosate significantly increased. The level of glyphosate usage in 1996 was 13%; after 6 years of commercialization of GR cotton glyphosate usage increased to 70% (NASS 1997, 2001, 2004). By 2007, 91% of cotton in the US was glyphosate-resistant. The rapid adoption of the glyphosate-resistant technology and continued use of glyphosate imposed a massive selection pressure on weed populations and led to the evolution of glyphosate-resistant Palmer amaranth. Today, a total of 37 weed species including 20 dicots and 17 monocot weed species have evolved resistance to glyphosate (Heap 2017). The following mechanisms of evolved glyphosate resistance have been reported (1) reduced glyphosate translocation; (2) increased glyphosate sequestration; (3) rapid necrosis response; (4) an altered *EPSPS* target-site; and (5) *EPSPS* gene amplification (Sammons and Gaines 2014).

Glyphosate translocates easily within the plant to the growing points. Translocation of glyphosate from the source leaves to sink tissues following sucrose movement takes place in the phloem (Gougler and Geiger 1981; McAllister and Haderlie 1985) and it can also be taken up through the roots via the xylem vessels (Sprankle et al. 1975). The reduced transport of glyphosate to physiologically active meristematic tissues has been described as the resistance

mechanism in the majority of glyphosate-resistant weeds. In Australia, the first case of glyphosate resistance in rigid ryegrass (*L. rigidum*) populations was due to reduced translocation of the herbicide from treated leaves to other parts (Powles et al. 1998; Pratley et al. 1999; Lorraine-Colwill et al. 2002). Glyphosate resistance in horseweed *(Conyza Canadensis)* was due to reduced translocation (Feng et al. 2004).

Altered-target site confers lower resistance levels (2- to 4-fold) to glyphosate than nontarget-site resistance (Dinelli et al. 2006; Sammons et al. 2007; Kaundun et al. 2008). Among weedy species, point mutations in amino acid sequence at Thr102 and Pro106 in *EPSPS* has resulted in resistance to glyphosate. The point mutation of Pro106Ser in the *EPSPS* gene was identified as molecular basis of glyphosate resistance in goosegrass (*Eleusine indica)* in Malaysia (Lee and Nigm 2000; Baerson et al. 2002) and the Philippines (Kaundun et al. 2008), rigid ryegrass (*L. rigidum)* in California (Simarmata and Penner 2008), Italian ryegrass in Chile (Perez-Jones et al. 2007) and in California (Jasieniuk et al. 2008), *A. tuberculatus* in Mississippi (Nandula et al. 2013), and *Echinochloa colona* in California (Alarcón-Reverte et al. 2013). A different amino acid substitution at this same site, Pro106 Ala, was implicated in the resistance of Italian ryegrass and rigid ryegrass to glyphosate in South Africa (Yu et al. 2007; Jasieniuk et al. 2008). Recently, in Malaysia, target-site resistance to glyphosate in goosegrass (*E. indica*) has been associated with co-existing substitutions at Thr102Ile and Pro106Ser residues also called TIPS (Yu et al. 2015). This double amino acid substitutions in the *EPSPS* gene in glyphosate-resistant individuals results in high level of resistance (>180-fold). This TIPS mutation was found first in the glyphosate-tolerant EPSPS in corn. The de novo TIPS mutants of *E. indica* were 32-fold more resistant to glyphosate than those harboring the single mutation Pro 106 Ser (Yu et al. 2015).

One of the lesser known mechanisms of resistance is gene amplification. Only a few cases involving amplification of the target gene has been reported as the basis for resistance in weedy plants. A thorough study on the first glyphosate-resistant Palmer amaranth populations did not show any changes in glyphosate absorption and translocation and there were no mutations found at the target-site in *EPSPS* (Gaines et al. 2010). Repeated application of glyphosate for seven years resulted in increase in *EPSPS* gene in Palmer amaranth. Resistant plants had 77-fold more copies of the *EPSPS* gene. This mechanism has conferred 40-fold resistance to glyphosate (Gaines et al. 2010). The mechanism was also observed in tall waterhemp (*A. tuberculatus*) (Tranel et al. 2011). In Italian ryegrass (*L. perenne*) up to 25 more copies of *EPSPS* gene than the susceptible plants were found (Salas et al. 2012). Recently, *EPSPS* gene amplification has also been reported in several other glyphosate-resistant weed species including *A. spinosus* (Nandula et al. 2014), *B. diandrus* (Malone et al. 2016), *Chloris truncata* (Ngo et al. 2017), and *Kochia scoparia* (Wiersma et al. 2015).

Resistance to HPPD-inhibitor (Mesotrione)

Mesotrione is a selective herbicide, which can be used for pre- and post-emergence control of a wide range of broadleaf and grass weeds in maize (*Zea mays*). It was introduced in 2001 in the US markets as Callisto. Mesotrione competitively inhibits the HPPD enzyme in the cytoplasm of the chloroplasts (Dan 2008). Chemically, mesotrione [2-(4-mesyl-2-nitrobenzoyl) cyclohexane-1, 3-dione] belongs to the triketone family and is derived from natural chemicals (phytotoxin and leptospermone) secreted by the red bottlebrush (*Callistemon citrinus Stapf*.) plant (Cornes 2005). In 1977, a Zeneca scientist accidently noticed empty spaces around the red bottlebrush plants. Further investigation confirmed the presence of an allelochemical,

leptospermone, which can control some broadleaf- and grassy weeds (Hellyer 1968; Mitchell et al. 2001). Further research resulted in the development of the benzoylcyclohexane-1, 3-dione (triketones) herbicide chemical family (Mitchell et al. 2001; Duke et al. 2002). Mesotrione is environmentally safe as it has low toxicity and is degraded quickly by microorganisms (Cornes 2005).

The presence of HPPD target site in plants has been confirmed *in vivo* (Schultz et al. 1985; Prisbylla et al. 1993). HPPD is a critical enzyme in the pathway that converts tyrosine to plastoquinone and α-tocopherol (Shultz et al. 1985; Mitchell et al. 2001; Wakabayashi and Boger 2002). Inhibition of the HPPD enzyme results in the depletion of plastoquinone levels which consequently reduces the amount of carotenoids (Lee et al. 1997). Thus, a decrease in carotenoid levels results in bleaching and necrosis caused by destruction of chlorophyll in plants. Norsworthy et al. (2008) reported that mesotrione provided 97 to 98% control of Palmer amaranth from Arkansas; thus, mesotrione could be another tool for controlling glyphosate-resistant biotypes. There are only six cases of resistance to mesotrione reported to date including: *A. tuberculatus* in 2009 (multiple resistance to 3 sites of action in seed corn cropping system) and in 2011 (multiple resistance to 4 sites of action in corn and soybean cropping systems), Iowa; *A.tuberculatus* and *A. palmeri* in 2011, Nebraska; *A. palmeri* in 2009, Kansas; and *A. tuberculatus* in 2009, Illinois (Heap 2017). Herbicide detoxification mediated by cytochrome P450 monooxygenases conferred mesotrione resistance in tall waterhemp (Ma et al. 2013).

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CHAPTER-III

DIFFERENTIAL TOLERANCE OF GLYPHOSATE–RESISTANT PALMER AMARANTH (*AMARANTHUS PALMERI*) TO MESOTRIONE IN ARKANSAS, USA

Abstract

Palmer amaranth is the number one weed problem in the southern United States. The objectives of this study were to evaluate the differential tolerance of glyphosate-resistant Palmer amaranth to mesotrione and quantify the level of tolerance in recalcitrant accessions and their offspring. Seeds were collected from crop fields across Arkansas between 2008 and 2014. In a greenhouse study, seedlings (7-10 cm tall) were treated with the field use rate of glyphosate at 840 g ae ha^{-1} or mesotrione at 105 g ai ha^{-1}. The bioassays were conducted twice with two replications (50 seedlings per replication). Overall, 55% of the accessions (115) were resistant to glyphosate with injury ranging from 14-92% and 58% survivors. Almost 20% of the accessions were highly resistant with 93% survivors and incurred injury 2-86%. The majority of survivors from glyphosate application incurred between 31-60% injury. Mesotrione killed 74% of the accessions (119); the remaining accessions had survivors with injury ranging from 61%-90%. The accessions with survivors showing lower injury levels were selected for estimation of tolerance level. Dose response assays were conducted with four recalcitrant tolerant populations and their F1 progeny. The average effective dose (ED_{50}) for the parent accessions and F1 progeny was 21.5 g ai ha^{-1} and 27.5 g ai ha^{-1}, respectively. Low level of tolerance (3- to 5-fold) was observed in recalcitrant Palmer amaranth populations.

Introduction

Palmer amaranth (*Amaranthus palmeri*) is one of the most common and troublesome weeds in corn (*Zea mays*), cotton (*Gossypium hirsutum* L.), and soybean [*Glycine max* (L.) Merr.] in the southern United States (Webster 2005). In Palmer amaranth, the male and female inflorescences are present on separate plants (Keeley et al. 1987). High seed production (0.6 million per plant), fast growth (Klingaman and Oliver 1994; Norsworthy et al. 2008a), extended emergence (Jha et al. 2006) and tall structure (Culpepper et al. 2006) make Palmer amaranth highly competitive with crops. Palmer amaranth can grow 3.5 cm d^{-1} and reach a final height of up to 2 m (Norsworthy et al. 2008a) and quickly makes a canopy over crops. Ten Palmer amaranth plants m^{-2} can reduce soybean yield up to 68% (Klingaman and Oliver 1994) and 0.9 plants m^{-2} can reduce cotton lint yield up to 92% (Rowland et al. 1999). Palmer amaranth obstructs cotton harvest. At a density of 0.3 Palmer m^{-2} reduced cotton yield by 22% and reduced mechanical harvesting efficiency by 2.4% (Smith et al. 2000). In corn, Palmer amaranth density of 0.5 to 8 plants m^{-1} of row can reduce corn yield from 11 to 91% (Massinga et al. 2001; Massinga and Currie 2002).

Controlling Palmer amaranth is a major challenge because chemical control options are limited by rapid resistance evolution in this species. Today, Palmer amaranth has evolved resistance to ALS (acetolactate synthase)-, EPSPS (5-enolpyruvyl-shikimate-3-phosphate synthase)-, microtubule assembly-, PS II (photosystem II)-, HPPD (4-hydroxyphenylpyruvate dioxygenase)-, and PPO (protoporphyrinogen oxidase) inhibitors (Heap 2017a). In general, a good and effective weed management program is a balance of conventional and modern management practices including minimum tillage, cultivation, herbicide application, and crop rotation. After the widespread evolution of resistance to ALS inhibitors, glyphosate became the

primary tool to control Palmer amaranth (Bond et al. 2006; Starke and Oliver 1998). Glyphosate was introduced in 1974 and revolutionized weed management. Glyphosate is a non-selective herbicide with a unique mode of action, minimal or no metabolism in plants, and no residual activity in soil (Bradshaw et al. 1997). Its use increased with the introduction of glyphosate-resistant (GR) crop technology in the 1990s and resulted in the selection of Palmer amaranth resistance to glyphosate. Palmer amaranth is an obligate outcrosser, therefore, allowing herbicide resistance to spread rapidly (Steckel 2007). Sosnoskie et al. (2012) reported that the glyphosate resistance trait was transferred across a distance of 300 m through pollen flow. Hence, apart from high fecundity and patch expansion, resistance to glyphosate is also spreading through long distance wind-pollination and movement of tiny seeds resulting in wide-spread glyphosate resistance. Currently, 27 states have reported GR Palmer amaranth (Heap 2017a). Many cases of multiple resistances to different herbicide modes of action in Palmer amaranth have been reported also. Therefore, knowing the response of Palmer amaranth to alternative herbicides in herbicide-resistant (HR) crops is important to make effective herbicide recommendations for resistance management.

4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors belong to a group of herbicides used mainly in corn, sorghum and wheat. HPPD is the target of several herbicide families-isoxazoles, triketones, and pyroxazoles. Its inhibition results in the depletion of the plant plastoquinone and vitamin E pools, leading to bleaching symptoms. These herbicides are very potent for pre- and postemergence control of a wide range of broadleaf and grass weeds. Effective control of annual broadleaf (*Amaranthus* spp., *Ipomoea* spp., *Solanum* spp., *Polygonum* spp. etc.) and grass (*Urochloa platyphylla*, *Digitaria* spp., *Setaria* spp. etc.) weeds and excellent crop tolerance are some of the characteristics of this group, which has made it an integral part of

weed management programs in corn, sorghum and wheat production systems (Beaudegnies et al. 2009). Their efficacy raised interest in the development of resistant transgenic crops. Recently, transgenic soybeans tolerant to mesotrione, tembotrione and isoxaflutole have been developed (Siehl et al. 2014). To date, resistance to HPPD- inhibitors (e.g. mesotrione) is rare. Mesotrione-resistant tall waterhemp (*Amaranthus tuberculatus*) populations have been reported in Iowa, Illinois, Nebraska, and Kansas in 2009 and resistant biotypes of Palmer amaranth were found in Kansas and Nebraska in 2009 and 2011, respectively (Heap 2017a). It is important to use this chemistry wisely to delay the evolution of resistance to this herbicide. Also, it is important that the efficacy of herbicides that still work is sustained, especially when crops with multiple stacked traits are commercialized. We evaluated the differential tolerance of GR Palmer amaranth accessions from Arkansas to mesotrione and quantified the level of tolerance in recalcitrant accessions and their offspring.

Methods and Materials

Plant materials and bioassays with glyphosate and mesotrione. Palmer amaranth plants that remained in the fields at the end of the growing season were sampled in late summer between 2008 and 2014 across Arkansas (Figure 1). A total of 115 accessions were tested with glyphosate and 119 accessions were tested with mesotrione. Each field was represented by 10 to 20 plants that were sampled separately and were threshed manually. To conduct the herbicide bioassays, a composite seed sample from each field (hereafter referred to as an accession) was prepared by mixing 500 mg of seed per plant. Composite seeds were planted in 50-cell trays (Redwayfeed Garden and Pet supply, 290 Briceland Rd, Reedway, CA 95560) filled with Sunshine®potting medium (Sunshine premix #1®, Sun Gro Horticulture, 15831 NE 8th Street, Suite 100, Bellevue,

WA 98008). The experiment was set up as randomized complete block design with two replications and two runs (50 plants per replication) where each tray was a replication with a single seedling per cell. Thus, a total of 200 plants (7-10 cm tall) representing a field were treated with recommended doses of glyphosate at 840 g ae ha^{-1} (Roundup PowerMax®, Monsanto) or mesotrione at 105 g ai ha^{-1} (Callisto® 480 SL, Syngenta Crop Protection, Inc.). Mesotrione was applied with 1% COC (crop oil concentrate) and 2.5% liquid AMS (ammonium sulfate). The plants were sprayed in a spray chamber using a boom fitted with two flat-fan nozzle delivering 187 L ha^{-1} at 269 kPa. At 21 d after treatment (DAT), each plant was evaluated visually for injury relative to the non-treated control. Injury was recorded on a scale of 0 to 100% where 0 had no injury and 100% was dead. Data were analyzed using ANOVA in JMP Pro v12. Hierarchal clustering of accessions was done using injury and mortality data.

Mesotrione dose-response bioassay. Out of 119 accessions tested in the mesotrione bioassay, four accessions (parent) which had the most survivors with injury ranging from 11-60% were investigated further to determine if the offspring of survivors would be as tolerant or more tolerant than the parent accession. Survivors from the four recalcitrant accessions were grown to produce seeds (F1 progeny). In this process survivor plants from the same accession were grown together and were separated from other accessions at a minimum distance of 20 m. In dose response assay, seeds of the parent accession and F1 progeny were planted in 11 x 11 cm square pots filled with Sunshine Mix LC1 potting soil (Sun Gro Horticulture Canada Ltd., Vancouver, British Columbia, Canada). A susceptible Palmer amaranth accession (CRW09-A) as well as susceptible tall waterhemp (TW-S) and resistant tall waterhemp (TW-R) accessions were also included in the study as out-group checks. Seedlings were thinned to five per pot and sprayed, when 7-10 cm tall. The recalcitrant Palmer amaranth and resistant TW-R accessions were treated

with 0, 13.25, 26.25, 52.5, 105, and 210 g ai ha^{-1} mesotrione. The susceptible accessions were sprayed with 0, 3.28, 6.56, 13.25, 26.25, 52.5 and 105 g ai ha^{-1} of mesotrione. The herbicide was applied with 1% COC and 2.5% v/v liquid AMS as described previously. The experiment was conducted in a randomized complete block design with 6 replications and 5 plants per replication. At 21 DAT, visible injury and the number of survivors were recorded. Injury ratings were based on visual estimations of bleaching, necrosis, and plant vigor on a scale of 0 (no effect) to 100 (plant death). Data were analyzed using SigmaPlot v.13. Data were subjected to non-linear regression analysis using a three parameter log-logistic equation (1) to determine the mesotrione dose causing 50% control.

$$y= c/[1+e^{-a\,(x-b)}] \quad [1]$$

where Y is the % injury of the nontreated control; a is the asymptote; b is the growth rate; c is the inflection point; and x is the mesotrione dose.

Results and Discussion

Palmer amaranth response to glyphosate. In Arkansas, the majority of Palmer amaranth has been reported to be resistant to glyphosate (Norsworthy et al. 2008b). This current research revealed inter- and intrapopulation variation on injury and mortality of 115 Palmer amaranth accessions treated with the recommended dose (840 g ae ha^{-1}) of glyphosate. The accessions differentiated into four clusters based on mortality and levels of injury of survivors (Figure 2 and Table 1). The first cluster consisted of 31 accessions out of 115 (27%) with 96% mortality. The survivors (4%) incurred 99% injury. This was the susceptible group. The second cluster consisted of 34 accessions with 64% mortality and survivors with an average injury of 88%. Several survivors showed some tolerance, with injury from 61-89%. This group was classified

slightly resistant. The third cluster was composed of 21 accessions with an average mortality and injury of 6% and 21%, respectively. Approximately 50% of the survivors in this cluster incurred 0-10% injury and 31% of the survivors showed 11-30% injury. This cluster was classified as highly resistant. The fourth cluster constituted of 29 accessions with an average injury of 52% and mortality of 13%. The majority of survivors in this cluster incurred 31-60% injury. This group was classified resistant to glyphosate. Overall, 73% of the sampled fields in Arkansas had Palmer amaranth with various levels of resistance to glyphosate (clusters 2, 3, and 4). Eighteen percent of the sampled fields had highly resistant populations (cluster 3).

The extent of variability in response to glyphosate is shown in Figure 3. The 2008 accessions had 0%-99% mortality with an average of 52%. In 2009, the range of mortality also was 0%-98%, but the mean was 15%. Another statewide sampling in 2011 showed a narrower range of mortality (52% to 100%), indicating that the frequency of glyphosate resistance was higher across the state relative to earlier samplings. The accessions collected in 2012 and 2013 represented fields infested with Palmer amaranth that were uniformly resistant, with 12 and 8% average mortality, respectively. These were targeted samplings of problem fields in response to requests by growers through Extension Agents. The follow-up sampling across the state in 2014 showed highly variable response to glyphosate, similar to the variability observed among accessions collected in 2008 and 2009. In 2014, the average mortality was 64%, just slightly higher than that of 2008. The distribution of samples between 2008 and 2014 was similar; thus, we can say that resistance to glyphosate across the state increased only slightly in six years. This is probably a reflection of mitigation practices adopted by most farmers, primarily including the use of residual herbicides and application of multiple modes of action postemergence.

Glyphosate-resistant Palmer amaranth is widespread in the United States (Figure 4) (Heap 2017a). The first case of GR Palmer amaranth was confirmed in 2005 in Georgia where GR cotton was planted in the same field for approximately seven years and has used only glyphosate three times a season for weed control (Culpepper et al. 2006). In the same period (2005), in Tennessee, Palmer amaranth escapes were reported in a GR cotton field and eventually confirmed resistant by Steckel et al. (2008). In Arkansas, GR Palmer amaranth from a soybean field in Mississippi county was reported also in 2005 (Norsworthy et al. 2008b). This population survived two applications of a full dose of glyphosate (840 g ae ha^{-1}). The spread of GR Palmer amaranth in the Southern United States was very rapid. Three years after detecting the first case of GR Palmer amaranth, 49 counties in the southern United States were reported to have at least one GR population in 2008. In one more year (2009) the number increased to 93 counties (Nichols et al. 2009). The evolution of resistance to glyphosate became a concern because a reliable and most affordable chemical tool to control weeds postemergence lost its utility. Consequently, Liberty Link® technology and other modes of action are promoted to growers to manage GR Palmer amaranth.

Preventative approaches are still the most effective and economical programs for managing GR Palmer amaranth. These include the use of soil residual herbicides with different modes of action, including protox inhibitors (e.g., fomesafen, flumioxazin), dinitroanilines (e.g., pendimethalin, trifluralin), triazines (e.g., atrazine, simazine), chloroacetamides (e.g., alachlor, pyroxasulfone, *S*-metolachlor), and substituted ureas (e.g., diuron, fluometuron). The integrated approaches could be the adoption of alternative herbicide-resistant technology (i.e. Liberty Link®, Enlist™ etc.) and crop rotation (i.e. GR-cotton or GR-soybean followed by non-GR corn,

or other crops) that provide greater chemical weed management options (Givens et al. 2009; Culpepper et al. 2010).

Palmer amaranth response to mesotrione. Palmer amaranth in Arkansas has evolved resistance to major herbicide modes of action including EPSPS- (Norsworthy et al. 2008a), ALS- (Burgos et al. 2001), and PPO- (Salas et al. 2016) inhibitors. A comparatively newer chemistry of herbicides that inhibit HPPD (e.g. mesotrione, tembotrione) has been used to control Palmer amaranth. Mesotrione is applied pre- or postemergence in corn, sorghum and wheat to control annual broadleaf weeds including Palmer amaranth. Resistance to mesotrione in Palmer amaranth has not been observed yet in Arkansas, but it has been reported in Kansas (Thompson et al. 2012) and Nebraska (Jhala et al. 2014).

The Palmer amaranth response to mesotrione differed within and among accessions. Overall, 74% of the accessions were controlled completely with 105 g ha^{-1} mesotrione. Analysis of injury and mortality grouped the accessions into four clusters (Figure 5 and Table 2). The first cluster was comprised of 88 accessions (74%) that were sensitive to mesotrione. These 88 accessions showed an average mortality and injury of 94% and 98%, respectively. The second cluster was slightly tolerant to mesotrione. It constituted of 15 accessions with 54% mortality and wherein 64% of the survivors incurred 61-89% injury. The third cluster was moderately tolerant where 14 accessions (12%) had low average mortality (22%), but with survivors incurring high levels of injury. The fourth group consisted of only two accessions that were more tolerant to mesotrione than all the others. The accessions in cluster 4 incurred an average mortality and injury of 6% and 46%, respectively. The survivors in this cluster showed a minimum injury of 30% and maximum injury of 88%. Therefore, some Palmer amaranth populations in Arkansas are more difficult to control with mesotrione than others. An important

aspect to note is the variability in sensitivity to mesotrione within and among populations (Figure 6).

The 2008 accessions had an average mortality of 89%, but the range of mortality among accessions was 30%-100%. The accessions sampled in 2009, 2011, 2012 and 2013 showed an average mortality of 96%, 93%, 63% and 95%, respectively. Generally, these accessions were highly sensitive to mesotrione. Evaluating a large sample size in 2014, from across the state, revealed also high variability in accession responses to mesotrione. The average mortality was 92%, confirming that the field populations across the state were generally susceptible to mesotrione. Among all accessions from 2008–2014, two accessions, CRW09-B (2009) and PHI13-C (2013) were outliers. The former showed 70% mortality and the latter showed 88% mortality, which were noticeably lower than those of the other accessions. Accessions with high survivors and low injury are high-risk accessions and are expected to be more prone to evolution of resistance. The mesotrione-resistant Palmer amaranth population from Nebraska can be controlled only 55% with a full dose of mesotrione (Jhala et al. 2014). Another *Amaranthus* species, tall waterhemp (*A. tuberculatus*), which is most commonly found in the northern United States has evolved resistance to mesotrione ahead of Palmer amaranth. The first case was in Illinois where a full dose of mesotrione could control the resistant tall waterhemp population only 40% (Hausman et al. 2011). Mesotrione-resistant tall waterhemp was also reported in Iowa and Nebraska in 2009 and 2011 (Heap, 2017a). Resistance to mesotrione among *Amaranthus* spp. has been reported in states with large areas of corn production including Illinois, Iowa, Kansas and Nebraska. The combined area under corn production in these four states was 16 million ha in 2016, representing 43% of the total corn production area in the United States (USDA 2017).

This current research showed that more than 50% of Palmer amaranth accessions were controlled completely by mesotrione. Most others had live plants 3 WAT, but these were barely alive. However, some accessions (PHI08-A, STF08-A, CRI12-B and PHI12-A) were noteworthy because of having low mortality (<25%) and survivors with relatively low level of injury (31-60%). This extensive screening of Palmer amaranth with the field recommended dose (105 g ai ha^{-1}) of mesotrione revealed the existence of tolerant biotypes. Usually, resistant weedy plants go unnoticed until the population size becomes large enough to cause economic loss. It is important to detect recalcitrant populations, or fields with some tolerant individuals, so that the management approach is adjusted to control such type of populations. If not, resistance would evolve sooner among these recalcitrant populations. These relatively tolerant plants can be controlled with the addition of another mode of action in the spray mixture, or a sequential application of another herbicide.

It is also important to know the herbicide response profiles of troublesome weed species to inform the discovery new technologies to combat resistant weeds. Currently, agro-chemical companies (Monsanto Agrochemical Co., Dow AgroSciences, Bayer CropScience, Syngenta Crop Protection Inc. and BASF Chemical Co.) have stacked multiple herbicide resistance traits in crops in addition to glyphosate resistance. These trait combinations are as follows: glyphosate + glufosinate (soybean, corn, cotton); glyphosate + ALS inhibitors (soybean, corn, canola); glyphosate + glufosinate + 2,4-D (soybean, cotton); glyphosate + glufosinate + dicamba (soybean, corn, cotton); glyphosate + glufosinate + HPPD inhibitors (soybean and cotton); glyphosate + glufosinate + 2,4-D + ACCase inhibitors (corn); and glufosinate + dicamba (wheat) (Green 2016). Multiple herbicide traits such as GlyTol Liberty Link (glyphosate + glufosinate), Xtend Flex® (glyphosate + glufosinate + dicamba), Roundup Ready®Xtend (glyphosate +

dicamba), Enlist™ (glyphosate + 2,4-D) and Enlist E3™ (glyphosate + glufosinate + 2,4-D) are already available in the market. These multiple-herbicide-resistant crops will provide new options for resistant weed management.

Mesotrione dose response bioassay. Survivors from four tolerant (field-collected) accessions (Table 3) identified in the general screening were grown to produce seeds (F1 progeny) (Figure 7 and Table 4). The parent accessions and the F1 progeny were evaluated to determine the level of tolerance to mesotrione. Along with Palmer amaranth susceptible standard (CRW09-A), TW-R and TW-S standards were also included as reference. The ED_{50} value for CRW08-A was 9 g ai ha^{-1} and the ED_{50} values for recalcitrant parent Palmer amaranth populations ranged from 20 to 23 g ai ha^{-1}, showing 2- to 3-fold level of tolerance. The F1 population, being partially purified, was expected to have elevated levels of tolerance to mesotrione relative to the parent. On the contrary, the response of F1 progeny to mesotrione was similar to that of the parent accessions. The effective dose to control 50% of the F1 progeny was 28 g ai ha^{-1} for PHI08-A, 24 g ai ha^{-1} for STF08-A, 29 g ai ha^{-1} for CRI12-B and 29 g ai ha^{-1} for PHI12-A. These were similar to the ED_{50} values for the parent accessions: 22 g ai ha^{-1} for PHI08-A and STF08-A; 23 and 20 g ai ha^{-1} for CRI12-B and PHI12-A, respectively. The TW-R and TW-S accessions were controlled 50% at 122 g ai ha^{-1} and 7 g ai ha^{-1}. Thus, the recalcitrant Palmer amaranth accessions and their F1 progenies were 4- to 5-fold less tolerant than TW-R, but 3- to 4-fold more tolerant to mesotrione than the susceptible standards CRW09-A and TW-S. Theoretically, a two-fold increase in tolerance to an herbicide should be considered as evolving resistance. Heap (2017b) suggested that any resistance value less than 10-fold indicates low-level resistance or partial resistance. These recalcitrant populations will most likely be the harbingers of evolved resistance.

Response of glyphosate-resistant Palmer amaranth accessions to mesotrione. The response of 50 GR accessions (clusters 3 and 4) including four recalcitrant accessions to mesotrione were analyzed (Table 1). After treatment with 105 g ai ha^{-1} mesotrione, 14 accessions from the GR clusters (3 and 4) showed <60% mortality whereas only 5 accessions from the glyphosate–susceptible clusters (1 and 2) had <60% mortality. It is interesting to note that out of four putative mesotrione-tolerant accessions three (CRI12-B, PHI12-A, and STF08-A) were highly resistant to glyphosate with 5, 5, and 16% mortality, respectively. This seemed to indicate that increased tolerance to mesotrione would occur more frequently among GR populations than among susceptible ones. Further research is needed to support this hypothesis.

This research showed that although resistance to glyphosate is widespread, a large proportion (57%) of the populations is still susceptible to glyphosate. This means that several growers are in a good position to stay ahead of the resistance quagmire. The glyphosate-resistant populations are at different levels of purification. Researchers in the private sector and academia have been searching actively for options. The triketones, including mesotrione, are effective on Palmer amaranth, but these and other alternative herbicides need to be used judiciously. The risk of escapes from recalcitrant populations is high and will be aggravated by suboptimal factors related to plant growth (primarily size), herbicide application, and the environment. The high-tolerance phenotype is not yet stable and tolerance to mesotrione did not increase in the F1 progeny. However, the evolution of resistance starts here.

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Table 1. Cluster analysis of Palmer amaranth accessions from Arkansas, USA, sprayed with 840 g ae ha^{-1} glyphosate.

Cluster	No. of accessions	Mortality (%)	Injury (%)		Mean frequency (N) of survivors at various levels of injury (%)[a]				Overall frequency (N) of survivors	Resistance category[b]
		Mean	Mean	Range	0-10	11-30	31-60	61-89	Mean	
1	31	96	99	66-100	0	0	1	5	2	S
2	34	64	88	24-100	9	3	8	41	15	SR
3	21	6	21	02-86	95	62	22	8	47	HR
4	29	13	52	14-92	17	30	89	35	43	R

[a]Average number of survivors based on levels of injury
[b]S= susceptible; SR= slightly resistant; R= resistant; HR= highly resistant

Table 2. Cluster analysis of Palmer amaranth accessions from Arkansas, USA, sprayed with 105 g ai ha^{-1} mesotrione.

Cluster	No. of accessions	Mortality (%)	Injury (%)		Mean frequency (N) of survivors at various levels of injury (%)[a]				Overall frequency (N) of survivors	Resistance category[b]
		Mean	Mean	Range	0-10	11-30	31-60	61-89	Mean	
1	88	94	98	72-100	0	0	1	11	3	S
2	15	54	83	10-100	2	6	25	59	23	ST
3	14	22	71	25-100	0	3	66	87	39	MT
4	2	6	46	30-88	0	42	131	16	47	T

[a]Average number of survivors based on levels of injury

[b]S= susceptible; ST= slightly tolerant; MT= moderately tolerant; T= tolerant

Table 3. Differential tolerance of glyphosate-resistant Palmer amaranth accessions to mesotrione (105 g ai ha^{-1}) in Arkansas, USA

	Injury (%)[b]		Mean frequency (N) of survivors at various levels of injury (%)[c]						
Accession[a]	Mean	Range	HT	T	MT	ST	S	Mortality (%)	Survivor (%)
CRA08-B	97	10-100	1	0	1	16	182	89	11
LON08-A	100	60-100	0	0	2	0	198	99	1
MIS08-C	99	70-100	0	0	0	6	194	97	4
PHI08-A	95	50-100	0	0	8	27	165	83	17
STF08-A	67	10-100	2	6	100	32	60	30	70
STF08-C	84	20-100	0	8	26	48	118	58	43
JEF09-B	97	40-100	0	0	5	18	177	89	11
LIN09-A	99	70-100	0	0	0	2	198	97	3
LIN09-B	94	10-100	12	0	2	2	184	91	9
LIN09-C	99	70-100	0	0	0	8	192	96	4
WHI09-A	98	40-100	0	0	1	11	188	94	7
CLA12-A	86	10-100	3	5	13	62	117	58	42
CLA12-B	88	5-100	5	1	2	65	127	63	37
CRI12-A	86	5-100	1	2	10	83	104	52	49
CRI12-B	79	5-100	6	13	41	14	126	63	37
PHI12-A	78	20-100	0	33	13	47	107	53	48
PHI12-C	87	5-100	4	0	41	18	137	67	33
PHI12-D	95	25-100	0	1	2	32	165	82	18
LON13-A	100	-	0	0	0	0	100	100	0
LON13-B	100	-	0	0	0	0	100	100	0
LON13-C	99	70-100	0	0	0	4	96	96	4
LON13-E	100	-	0	0	0	0	100	100	0
PHI13-B	100	80-100	0	0	0	2	98	96	4

Table.3 (Cont.)

	Injury (%)[b]		Mean frequency (N) of survivors at various levels of injury (%)[c]						
Accession[a]	Mean	Range	HT	T	MT	ST	S	Mortality (%)	Survivor (%)
PHI13-C	97	60-100	0	0	1	11	88	88	12
CLA14-A	73	55-100	0	0	14	176	10	5	95
CRI14-C	69	40-100	0	0	68	108	24	12	88
GRE14-C	63	30-100	0	2	118	40	40	20	80
LAW14-C	100	-	0	0	0	0	200	100	0
LEE14-A	70	40-100	0	0	94	50	56	28	72
LEE14-C	99	85-100	0	0	0	4	196	98	2
LEE14-D	97	65-100	0	0	0	18	182	91	9
LEE14-H	100	-	0	0	0	0	200	100	0
LEE14-J	69	45-100	0	0	58	136	6	3	97
LEE14-K	81	10-100	2	0	0	160	38	19	81
MIS14-C	72	55-100	0	0	56	114	30	15	85
MIS14-H	71	55-100	0	0	46	132	22	11	89
WHI14-A	63	35-100	0	0	128	40	32	16	84

[a]Accessions were resistant to 840 g ae ha^{-1} glyphosate (< 70% injury and < 20% mortality).

[b]Plants were sprayed at 7-10 cm tall. Data were recorded 21 days after herbicide application.

[c]Survivors were categorized based on visible injury, where S=sensitive (90-100% injury), ST= slightly tolerant (61-89% injury), MT= moderately tolerant (31-60% injury), T= tolerant (11-30% injury), and HT= highly tolerant (0-10% injury).

Table 4**.** ED_{50} values of recalcitrant parent and F1 progeny of Palmer amaranth accessions sprayed with mesotrione, Arkansas, USA

	Parent				F1[b]			
Accession[b]	ED_{50}[a] (g ai ha^{-1})	Regression Equation	Lower 95%	Upper 95%	ED_{50}[a] (g ai ha^{-1})	Regression Equation	Lower 95%	Upper 95%
PHI08-A	22 (2)[d]	$Y = 101/[1+e^{-0.068(x-21.88)}]$	18	25	28 (1)[c]	$Y = 100/[1+e^{-0.080(x-27.34)}]$	26	31
STF08-A	22 (3)	$Y = 101/[1+e^{-0.011(x-22.38)}]$	16	28	24 (1)	$Y = 100/[1+e^{-0.058(x-26.67)}]$	22	27
CRI12-B	23 (3)	$Y = 101/[1+e^{-0.044(x-22.94)}]$	17	28	29 (1)	$Y = 101/[1+e^{-0.07147(x-27.45)}]$	26	32
PHI12-A	20 (3)	$Y = 101/[1+e^{-0.048(x-20.22)}]$	15	25	29 (1)	$Y = 101/[1+e^{-0.080(x-27.07)}]$	26	32
TW-R[c]	122 (144)	$Y = 139/[1+e^{-0.011(x-175.4)}]$	95	149	118 (10)	$Y = 85/[1+e^{-0.013(x-88.35)}]$	92	146
TW-S[c]	7 (1)	$Y = 102/[1+e^{-0.358(x-6.82)}]$	6	7	7 (1)	$Y = 102/[1+e^{-0.388(x-6.81)}]$	6	7
CRW09-A	9 (1)	$Y = 97/[1+e^{-0.341(x-8.45)}]$	8	9	8 (1)	$Y = 98/[1+e^{-0.293(x-7.88)}]$	7	9

[a]ED_{50} is the herbicide concentration that could effectively control 50% of the plants at 3 WAT.

[b]Putative tolerant accessions (parent and F1) were treated at 7-10 cm with 5 doses plus check (0, 13.25, 26.25, 52.5, 105, and 210 g ai ha^{-1}); the susceptible standards (TW-S and CRW09-A) were treated with 6 doses plus check (0, 3.28, 6.56, 13.25, 26.25, 52.5 and 105 g ai ha^{-1}) of mesotrione. COC (1%) and AMS (2.5% v/v) were added to the spray mix.

[c]Tall waterhemp, resistant (TW-R) and tall waterhemp, susceptible (TW-S)

[d]Std error = standard error of the estimate

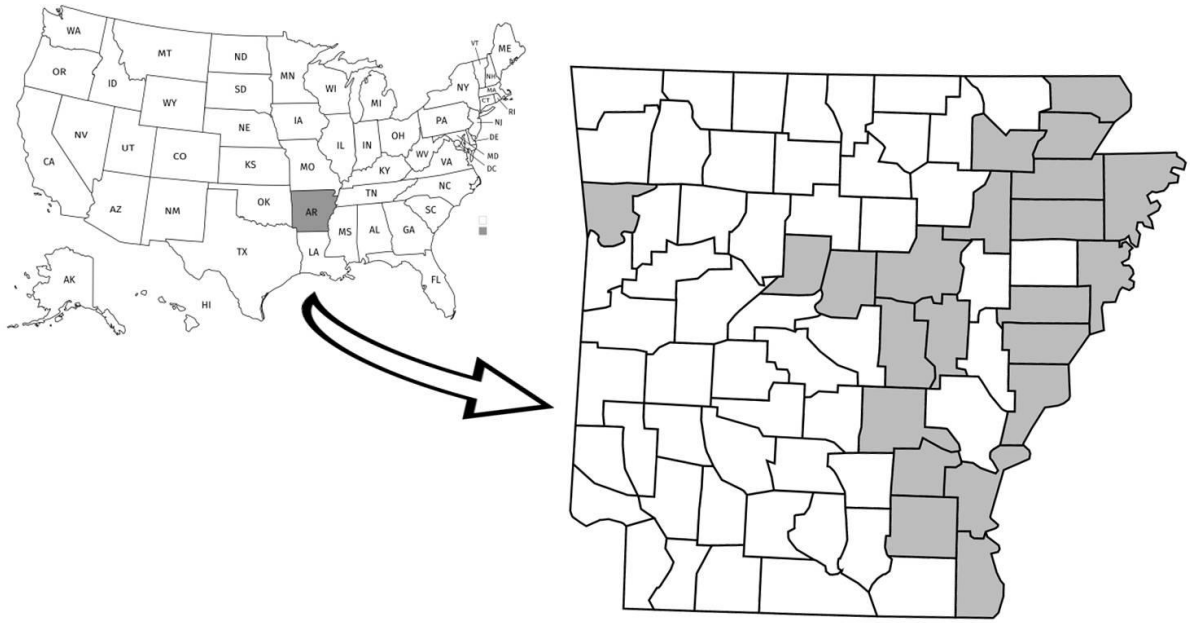

Figure 1. Map of Arkansas showing counties from where the Palmer amaranth accessions were collected between 2008–2014.

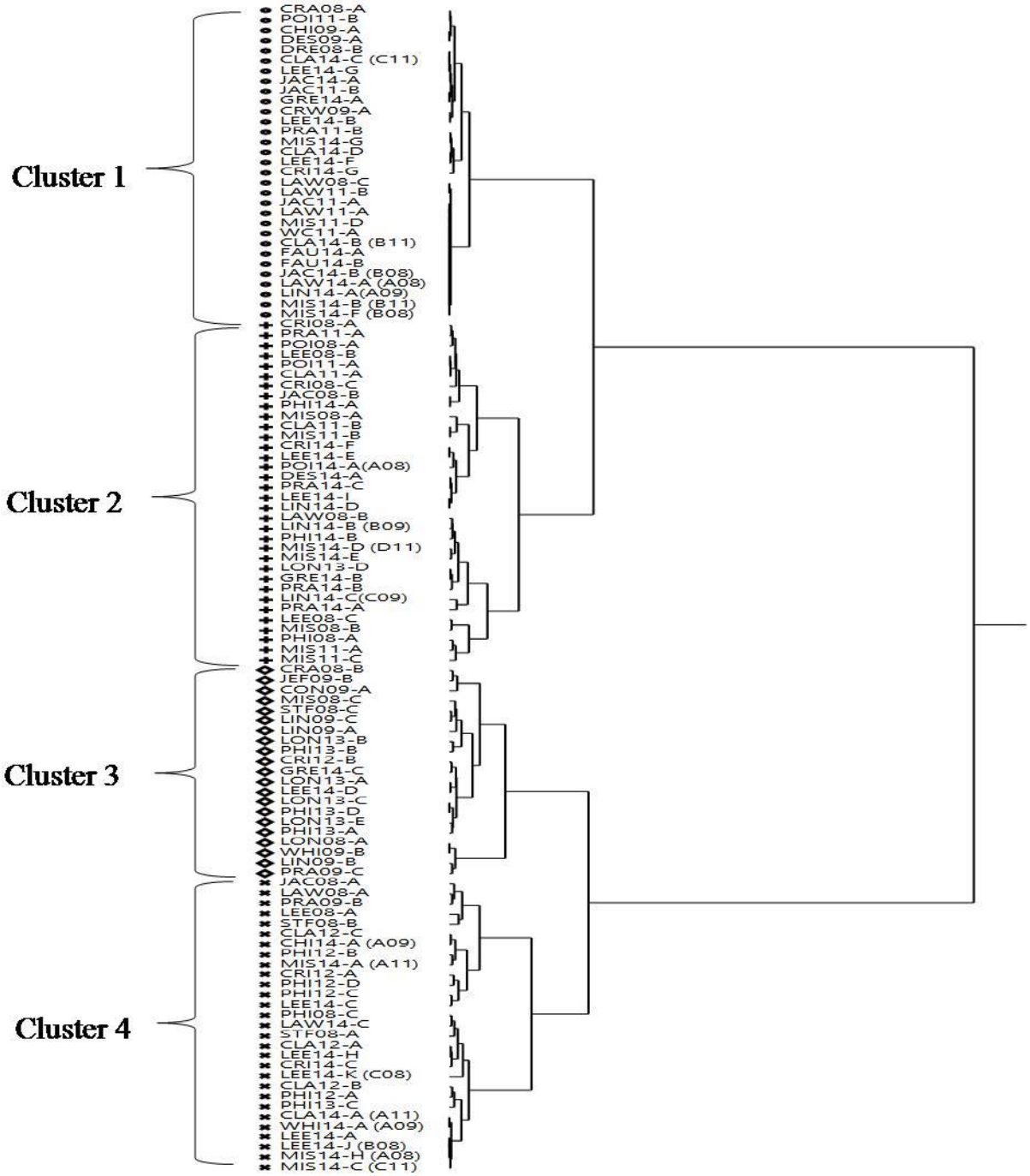

Figure 2. Hierarchal cluster analysis of Palmer amaranth accessions sprayed with 840 g ae ha^{-1} glyphosate based on % injury and mortality at 21 days after treatment. Glyphosate was applied to 7-10 cm-tall seedlings. Cluster 1 (n= 31 accessions; sensitive), cluster 2 (n= 34 accessions; slightly resistant), cluster 3 (n= 21 accessions; highly resistant), cluster 4 (n= 29 accessions; resistant).

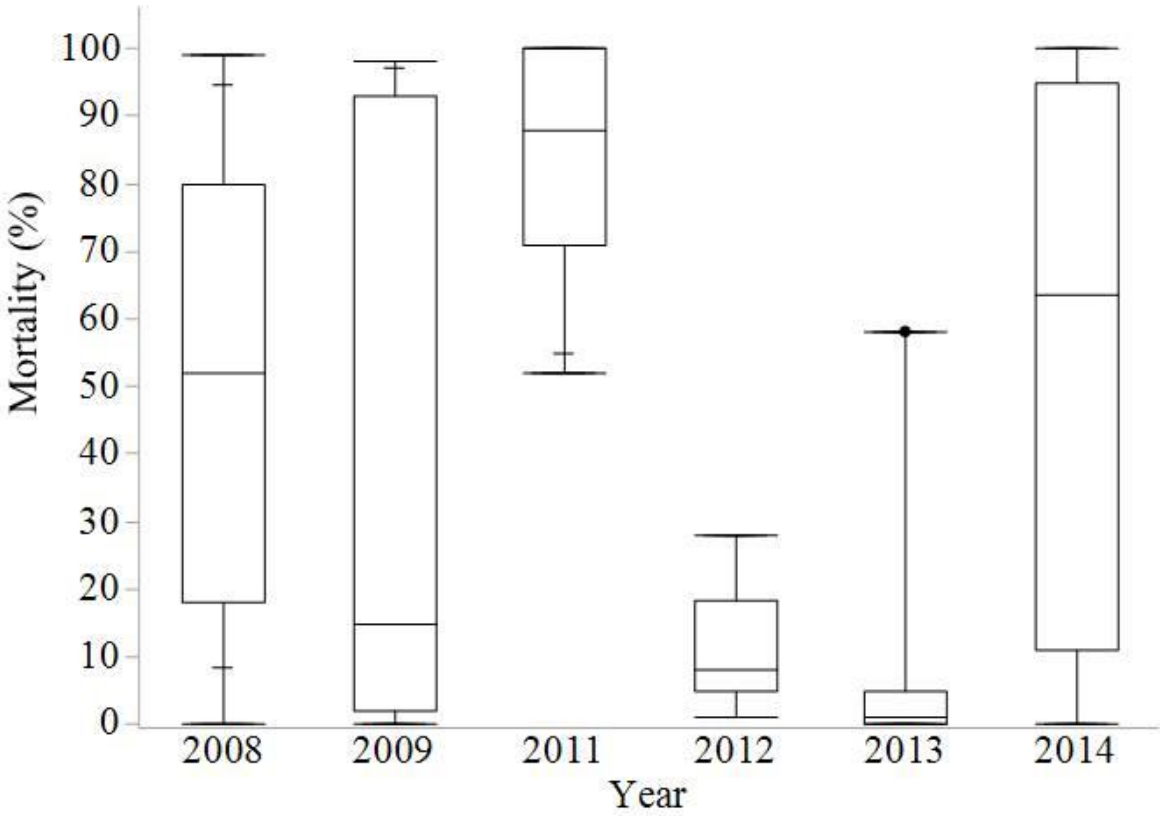

Figure 3. Variability in response to glyphosate (840 g ae ha^{-1}) among Palmer amaranth accessions collected between 2008 and 2014. Glyphosate was applied to 7-10 cm tall seedlings. Mortality was recorded 21 days after treatment. Box plot shows median values (horizontal line inside the box), first and third quartile values (box-outlines), minimum and maximum values (whiskers), and outlier values (closed circles).

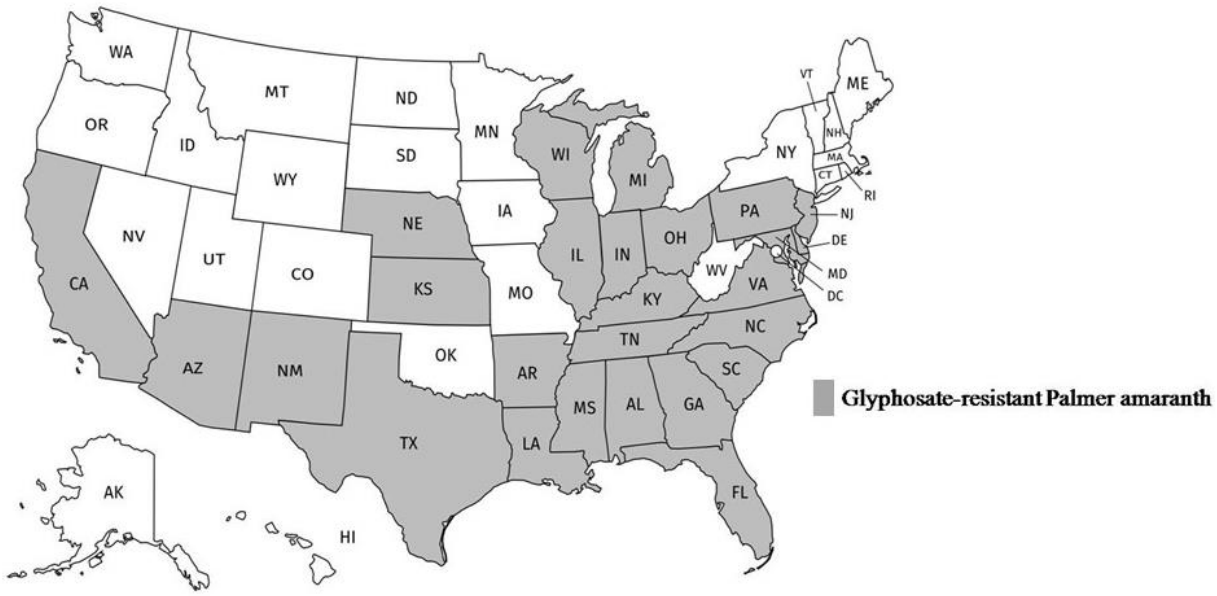

Figure 4. The occurrence of glyphosate-resistant Palmer amaranth across the United States (Heap 2017a).

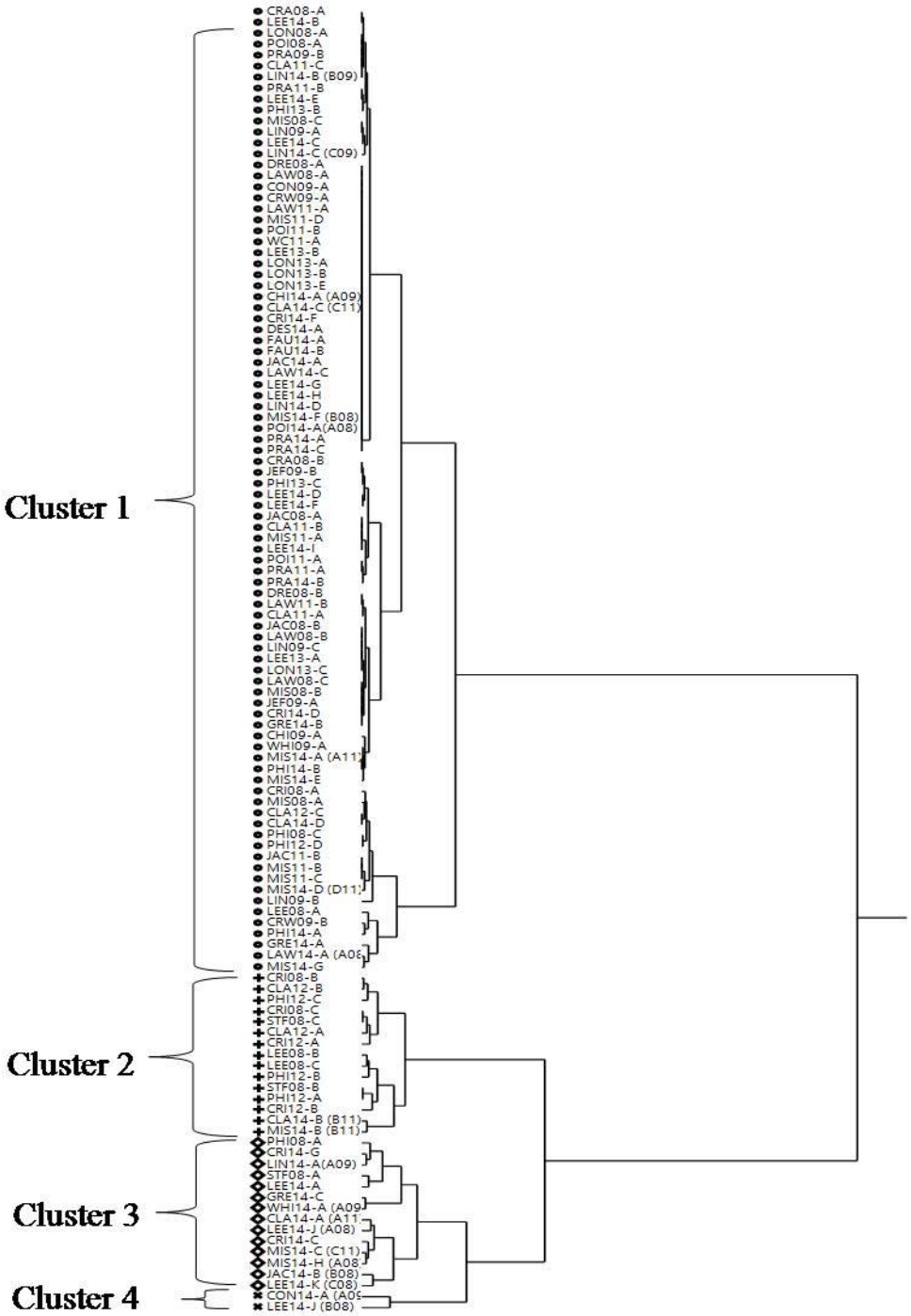

Figure 5. Hierarchal cluster analysis of Palmer amaranth accessions sprayed with 105 g ai ha^{-1} mesotrione based on % injury and mortality at 21 days after treatment. Mesotrione was applied with 1% COC and 2.5% liquid AMS v/v to 7-10 cm-tall seedlings. Cluster 1 (n= 88 accessions; sensitive), cluster 2 (n= 15 accessions; slightly tolerant), cluster 3 (n= 14 accessions; moderately tolerant), cluster 4 (n= 2 accessions; tolerant).

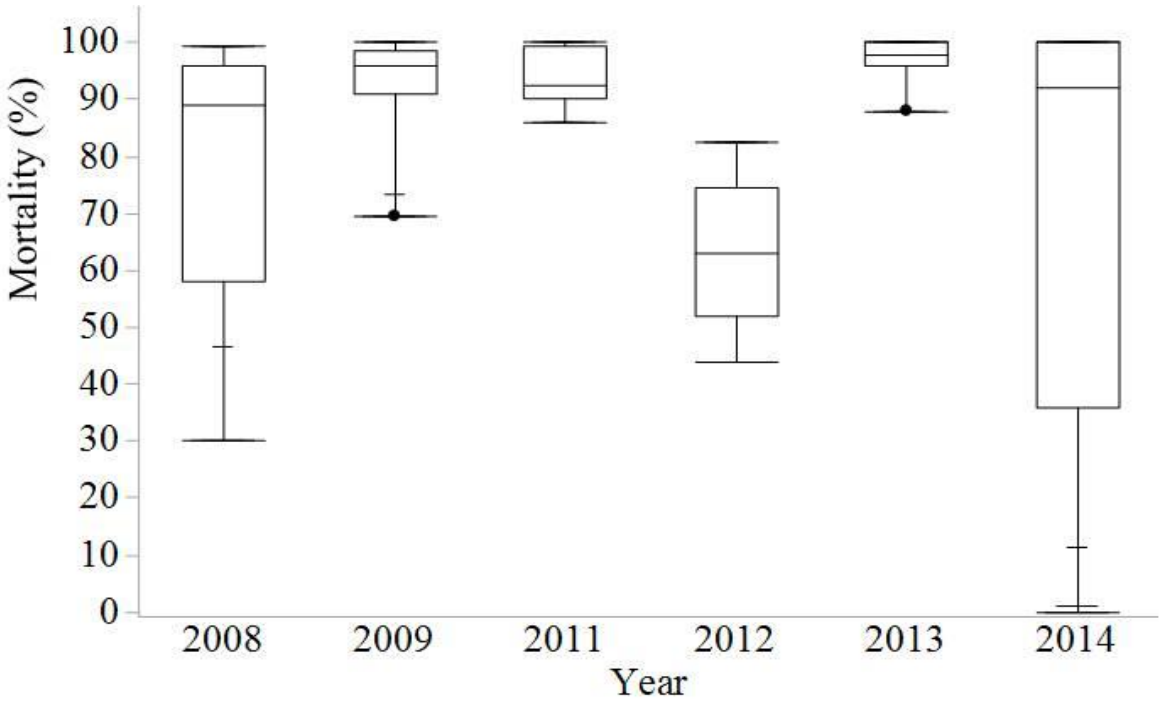

Figure 6. Variability in response to mesotrione (105 g ai ha^{-1}) among Palmer amaranth accessions collected between 2008 and 2014. Mesotrione was applied to 7-10 cm tall seedlings and plants were evaluated for mortality % at 21 days after treatment. Box plot shows median values (horizontal line inside the box), first and third quartile values (box-outlines), minimum and maximum values (whiskers), and outlier values (closed circles).

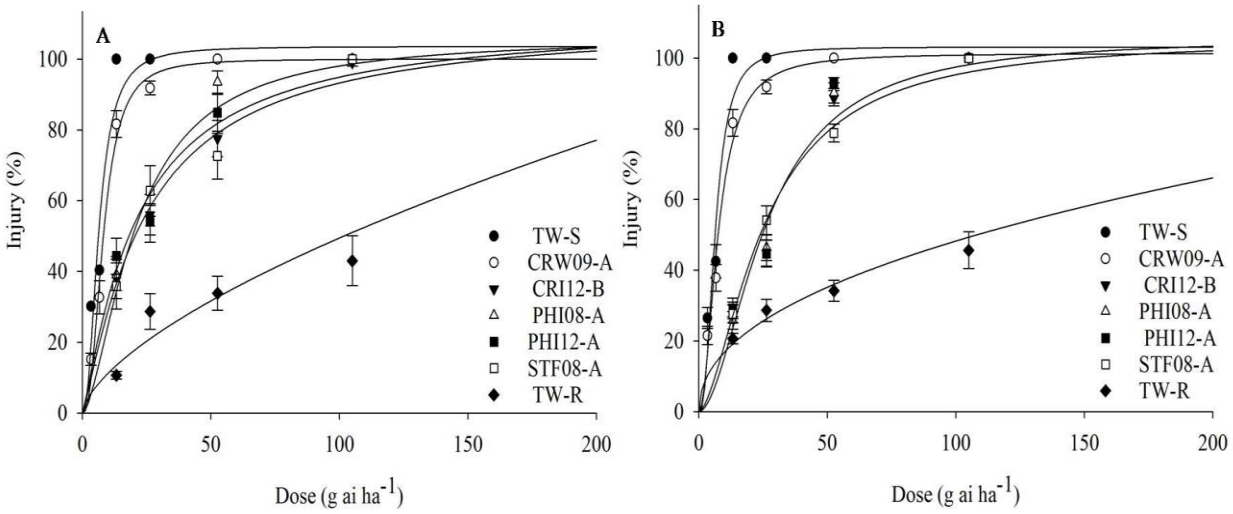

Figure 7. Dose response analysis curves of recalcitrant Palmer amaranth accessions-(A) parent accessions; (B) F1 progeny. Recalcitrant parent accessions and the F1 progeny of survivors were sprayed at 7-10 cm tall (0, 13.25, 26.25, 52.5, 105, and 210 g ai ha^{-1}). The susceptible standard (TW-S and CRW09-A) were treated with 0, 3.28, 6.56, 13.25, 26.25, 52.5 and 105 g ai ha^{-1} of mesotrione. COC (1%) and AMS (2.5% v/v) were added in the spray mix. Resistant tall waterhemp (TW-R) and susceptible tall waterhemp (TW-S) were used as out-groups for comparison. The X axis shows the dose (g ai ha^{-1}) and Y axis shows % injury in comparison to non-treated control plants. Data were recorded 21 days after treatment.

CHAPTER-IV

EPSPS AMPLIFICATION PRIMARILY CONFERS GLYPHOSATE RESISTANCE, BUT OTHER MECHANISMS ALSO OCCUR AMONG PALMER AMARANTH (*AMARANTHUS PALMERI*) POPULATIONS

Abstract

Resistance to glyphosate is widespread in the United States, especially, in Palmer amaranth (*Amaranthus palmeri*). This research was conducted to determine if resistance to glyphosate among Palmer amaranth populations in Arkansas, USA was due solely to increased *EPSPS* copy number and if copy number is correlated with level of resistance to glyphosate. One hundred-fifteen Palmer amaranth accessions were sprayed with the full rate (840 g ae ha^{-1}) of glyphosate. Twenty Palmer amaranth accessions representing different agroecological zones were used. Seven of the accessions were controlled completely with this dose; the rest were resistant. The glyphosate-resistant (GR) accessions had effective dose to control 50% (ED_{50}) values ranging from 494 g ae ha^{-1} to 1355 g ae ha^{-1}, a 3- to 48-fold resistance level compared to the susceptible standard (SS). The ED_{50} for SS was 28 g ae ha^{-1} and ED_{50} values for the other susceptible accessions were 84 g ae ha^{-1} to 207 g ae ha^{-1}. The 5-enolpyruvylshikimate- 3-phosphate synthase (*EPSPS*) copy number was determined for all 20 accessions with four plants per accession. In 13 GR accessions (CLA11-A, CON09-A, JAC11-B, LEE08-A, MIS08-B, MIS11-A,-B,-C, PHI08-A, POI08-A, POI11-B, STF08-A and WHI11-A) all plants (52) were resistant to glyphosate. Eight GR accessions (45% of plants tested) had 19 to 224 more *EPSPS* copies than SS. In GR accessions, the level of injury on the plants and *EPSPS* copy number were strongly, negatively correlated (r= -0.78). A 4% decline in injury was observed with every additional *EPSPS* copy. Meaning, highly resistant plants had more *EPSPS* copies. ED_{50} values were strongly correlated with *EPSPS* copy number. The highly resistant accession MIS11-B had ED_{50} 1355 g ae ha^{-1} and 150 gene copies. Partial sequencing of *EPSPS* from the remaining five GR accessions did not show any of the known resistance-conferring ($Thr_{102}Ile$ or $Pro_{106}Ser$) mutations. We conclude that *EPSPS* gene amplification is the primary mechanism for glyphosate resistance among Palmer amaranth from Arkansas. However, about 40% of GR accessions

harbor other mechanisms besides *EPSPS* amplification and target site mutation, possibly non-target site resistance mechanisms.

Introduction

Glyphosate (N-(phosphonomethyl) glycine), a non-selective herbicide, is the most popular herbicide worldwide, due to its use in GR (glyphosate-resistant) crops (Duke and Powles 2008). Glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme of the shikimate pathway, which inhibits the production of essential aromatic amino acids phenylalanine, tyrosine and tryptophan (Steinrücken and Amrhein 1980). In the 1990s, it was speculated that resistance to glyphosate was unlikely because of its unique target (EPSPS) and minimal degradation in plants (Bradshaw et al. 1997). However, with persistent broad-scale use, resistance to glyphosate has evolved in 36 weed species including common ragweed (*Ambrosia artemisiifolia* L.), hairy fleabane (*Conyza bonariensis* L.), horseweed (*C. canadensis* L.), goosegrass (*Elusine indica* L.), Italian ryegrass (*Lolium perenne* ssp. multiflorum Lam.), rigid ryegrass (*L. rigidum*), kochia (*Kochia scoparia* L.), tall waterhemp (*Amaranthus tuberculatus* (Moq.) Sauer.) and Palmer amaranth (*A. palmeri* S. Wats.) in North America (Heap 2017). This questions the dependability on glyphosate to control resistant weeds in general, and Palmer amaranth in specific. Palmer amaranth is highly competitive weed considering its ability to produce large amounts of seeds, rapid growth, extended emergence, and survival under adverse conditions (Jha et al. 2006; Ward et al. 2013). These characteristics empowers it to compete with crops for nutrients, water, light, and space (Monks and Oliver 1988) and thus, significantly reduces the crop yield. A recent survey by the Weed Science Society of America showed Palmer amaranth as the number one most troublesome and difficult-to-control weed in the United States (WSSA 2016).

GR weeds have evolved resistance due to: (i) mutations at the target site (Baerson at al. 2002), (ii) restricted glyphosate absorption and translocation (Lorraine-Colwill et al. 2002), (iii) increased glyphosate sequestration (Ge et al. 2012), (iv) *EPSPS* amplification (Gaines et al.

2010), and (v) *EPSPS* expression (Dinelli et al. 2006). The first GR Palmer amaranth population exhibited no changes in glyphosate uptake and translocation (Culpepper et al. 2006**)** and did not contain mutations in the target (*EPSPS*) gene associated with glyphosate resistance but was reported to have increased *EPSPS* copies (Gaines et al. 2010). So far, amplification of the *EPSPS* gene has been reported to confer resistance in many weed species such as, *A. tuberculatus* (Chatham et al. 2015), *A. spinosus* (Nandula et al. 2014), *Bromus diandrus* (Malone et al. 2016), *L. perenne* L. ssp. *multiflorum* (Salas et al. 2012) and *K. scoparia* (L.) Schrad. (Wiersma 2012). GR Palmer amaranth is widespread in Arkansas but the mechanism (s) involved is not yet known. This study was conducted to 1) survey the occurrence of *EPSPS* gene amplification and target-site mutation(s) among GR Palmer amaranth accessions from Arkansas and 2) determine the correlation of *EPSPS* copy number with resistance level to glyphosate. Developing a novel method to manage resistance based on physiological or molecular mechanisms hinges on a thorough understanding of resistance mechanisms occurring across weed populations.

Materials and Methods

Plant Material. Palmer amaranth plants, which remained in the fields at the end of the growing season, were sampled in late summer between 2008 and 2014 across Arkansas. Inflorescences of at least 10 plants per field were collected, air-dried, and threshed manually. A composite seed sample from each field (hereafter referred to as accession) was prepared by mixing 500 mg of seed per plant. A total of 115 accessions were tested with glyphosate (data not shown). Out of 115 accessions 20 were selected from 13 counties (Figure 1) to represent a broad range of responses to glyphosate. These accessions included the susceptible standard (SS) Palmer

amaranth population, which was collected from a field historically planted mostly with watermelons and vegetables.

General testing for resistance to glyphosate. The composite seed samples were planted in 50-cell trays (Redwayfeed Garden and Pet supply, 290 Briceland Rd, Reedway, CA 95560) filled with potting medium (Sunshine premix #1®, Sun Gro Horticulture, 15831 NE 8th Street, Suite 100, Bellevue, WA 98008). The experiment was set up as randomized complete block design with two replications (50 plants per replication) where each tray was a replication with a single seedling per cell. The bioassay was repeated. Thus, a total of 200 plants representing a field were treated with the recommended dose of glyphosate at 840 g ae ha^{-1} (Roundup Powermax®, Monsanto). Seedlings, 7-10 cm tall, were sprayed in a spray chamber using a boom fitted with two flat-fan nozzles delivering 187 L ha^{-1} at 32 psi. Plants were labeled and leaf tissues were collected before herbicide treatment for use in succeeding experiments. At 21 d after treatment (DAT), each plant was evaluated visually for injury relative to the non-treated control. Injury was recorded on a scale of 0-100% where 0 had no injury and 100% was dead. Data were analyzed using ANOVA in JMP Pro v12. Hierarchal clustering of accessions was done using injury and mortality data.

Evaluation of resistance level to glyphosate. Twenty Palmer amaranth accessions were used in this study. Seeds of each accession were planted in 11- x 11-cm square pots filled with Sunshine Mix LC1 potting soil (Sun Gro Horticulture Canada Ltd., Vancouver, British Columbia, Canada). Seedlings (5 per pot, 7-10 cm tall) of resistant accessions were sprayed with eight doses of glyphosate from 0, 110, 220, 420, 840, 1680, 3360 and 6720 g ae ha^{-1} and the susceptible accessions with 0, 27.5, 55, 110, 220, 420, 840, and 1680 g ae ha^{-1}. Glyphosate was applied following the procedure used in the previous section. The experiment was conducted in a

randomized complete block design with six replications. The number of survivors and injury were recorded at 21 DAT. Data were analyzed using SigmaPlot v.13. Dose response data were subjected to non-linear regression analysis and fitted with a three parameter log-logistic equation (1) to determine the glyphosate dose that would cause 50 and 90% control

$$y= c/[1+e^{-a\,(x-b)}] \quad [1]$$

where Y is the % injury; a is the asymptote; b is the slope of the line; c is the inflection point; and x is the glyphosate dose.

***EPSPS* gene copy number determination**. Leaf tissues from four plants per accession were collected. After herbicide application (840 g ae ha^{-1} of glyphosate), tissues from surviving plants with injuries ranging from 10- to 70%, were collected at 21 DAT to determine the *EPSPS* copy number and for susceptible accessions tissues collected before spray were used. Leaf tissues were frozen immediately in liquid nitrogen, and stored at -80 C until processed. Genomic DNA was extracted from approximately 100 mg of leaf tissue using a modified CTAB protocol (Doyle and Doyle 1990), quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE), and checked for quality by gel electrophoresis.

Quantitative real-time polymerase chain reaction (qPCR) was used to determine the *EPSPS* copy number relative to *A36*. *A36* (*Celosia trigina* PI649298) is a RNA dead-box helicase. A different reference gene was used because the Palmer amaranth populations were also resistant to ALS (acetolactate synthase) herbicides and any *ALS* amplification could result in faulty data interpretation. The *A36* primers were designed using Biolign and Primer 3 softwares from sequences of the *Amaranthus* genus: A36_F244 (5'TTGGAACTGTCAGAGCAACC3') and A36_R363 (5'GAACCCACTT CCACCAAAAC3'). To amplify the *EPSPS* gene, the primer sets EPSF1 (5'ATGTTGGACGCT CTCAGAACTCTTGGT3') and EPSR8

(5'TGAATTTCCTCCAGCAACGGCAA3') designed by Gaines et al. (2010) were used. For the qPCR, 25-µL reactions were made using 12.5 µL of Bio-Rad iQ SYBR Green Supermix, 1 µL of the forward and reverse primers (10 µM), and 10 ng gDNA. The thermoprofile consisted of 15 min denaturation at 95 C, 40 cycles of 95 C for 30 s, and 60 C for 60 s. This program was followed by a melt curve analysis of 81 cycles at 55 C for 30 s (Chandi et al. 2012). A negative control consisting of primers with no template DNA was included. No amplification products were observed in negative control reactions. Data were analyzed using a modification of the 2-ΔΔCt method to express genomic copy number of *EPSPS* relative to *A36* as ΔCt = (Ct, *A36* - Ct, *EPSPS*). The relative increase in genomic *EPSPS* copy number was expressed as 2ΔCt (Gaines et al. 2011). Each population had four biological replicates and each sample was run in triplicate (for each primer pair) to calculate the mean and standard error of the increase in *EPSPS* copy number.

Partial sequencing of *EPSPS*. A small fragment of the *EPSPS* gene was sequenced from five, GR *A. palmeri* accessions (4 plants per accession), which did not show any increase in *EPSPS* copy number. The susceptible standard *EPSPS* was also sequenced. The purpose was to determine if any of the known resistance-conferring amino acid substitutions at Thr_{102} or Pro_{106} were present. Genomic DNA was used to amplify a short sequence (150 bp) of *EPSPS*. Forward and reverse primers (EPSPSF- 5'CCAAAAGGGCAGTCGTAGAG 3'; EPSPSR-5'ACCTTGAATTTCCTCCAGCA 3') designed by Varanasi et al. (2015) were used. The 25-µl PCR reaction consisted of 12.5 µl 2x PCR master mix (Takara Bio USA, Inc.), 2.5 µl of both the forward and reverse primers (5 µM), 4 µl gDNA (50 ng μl^{-1}), and 3.5 µl of water. The PCR was performed with the following conditions: initial denaturation at 95 C for 3 min, followed by 40 cycles of denaturation at 95 C for 30 s, annealing at 53.5 C for 45 s, final extension at 72 C for 7

min and infinite hold at 4 C. The PCR product was run on a 1% agarose gel to verify the fragment size, was purified using a NucleoSpin® Gel and PCR Clean up kit (Takara Bio USA, Inc.). The purified DNA was sequenced with the same primers used for PCR and were prepared with ABI's BigDye® Terminator v3.1 for sequencing at IPGB (Institute for Plant Genomics and Biotechnology), Texas A&M University, College Station, TX. The nucleotide sequences were aligned using Bioedit software. The sequences from both SS and GR plants were aligned based on the available *EPSPS* sequences *A. palmeri* (FJ861242.1 and FJ861243.1) and *A. tuberculatus* (FJ869881.1) at GeneBank.

Results and Discussion

Response of Palmer amaranth to glyphosate. Palmer amaranth is among the most resistance-prone dicots and have been confirmed resistant to six herbicide modes of action in the United States (Heap 2017). Intensive use of glyphosate has resulted in the evolution of GR Palmer amaranth in 30 states including Arkansas. The Palmer amaranth accessions evaluated differentiated into three clusters based on frequency of survivors and levels of injury from treatment with 840 g ae ha^{-1} glyphosate (Table 1). The first cluster consisted of 7 susceptible accessions with 98% mortality and 99% injury on the remaining plants 3 WAT. Three of these had a few survivors with injury ranging from 61-89%. The susceptible standard, which was in this cluster, was killed 100% at this dose. The second cluster constituted of 6 accessions with 63% mortality and an average injury of 80%. Several survivors showed some tolerance, with injury from 6-80%. This group was slightly resistant. The third cluster was composed of 7 accessions with an average mortality of 32% and an average injury of 50%. The majority of survivors in this cluster incurred 0-10% injury. This was classified as resistant. Of the 200 plants

per accession sprayed with the 1X rate of glyphosate the most resistant survivors (0 – 10% injury) were from Conway (112 plants), Mississippi (107), Lee (87), and St. Francis (70) counties (Table 2). One accession from Mississippi county had the most number of survivors of all accessions with the lowest injury (5-10%).

The commercialization of GR crop technology aided the growers with a substitute to control ALS-inhibitor resistant Palmer amaranth but unfortunately, overflow of glyphosate has prompted the evolution of glyphosate resistance in Palmer amaranth. The first GR Palmer amaranth population from Georgia was documented in 2005 (Culpepper et al. 2006). Over the past decade, GR Palmer amaranth has been observed throughout the United States, such as, in 2005 in North Carolina; 2006 in Arkansas, South Carolina and Tennessee; 2007 in New Mexico; 2008 in Alabama and Mississippi; 2010 in Illinois, Kentucky, Louisiana, and Ohio; 2011 in Kansas, Michigan, Virginia, and Texas; 2012 in Arizona, California, Delaware, and Indiana; 2013 in Florida, Pennsylvania, and Wisconsin; 2014 in Maryland, and New Jersey; and 2016 in Nebraska (Heap 2017).

Resistance level to glyphosate. The glyphosate doses that caused 50% and 90% mortality (ED_{50} and ED_{90}) of the susceptible standard (SS) were 28 (±5) g ha^{-1} and 193 (±16) g ha^{-1}, respectively (Table 3). The ED_{90} for SS was very low, about one-fourth of the field use rate. The ED_{50} for other accessions ranged from 84 (±18) to 1355 (±78) g ha^{-1}. Based on ED_{50} values, these accessions had 3- to 48-fold resistance relative to the SS. The ED_{90} values for accessions CRA08-A, JAC08-B, JAC11-A, LAW11-A, MIS11-D, and PRA11-B ranged from 211 g ha^{-1} to 797g ha^{-1}, indicating that these accessions could be controlled with the field dose of glyphosate. The ED_{50} values of accessions LEE08-A, POI08-A, JAC11-B, POI11-B and WHI11-A ranged from 969 g ha^{-1} to 1459 g ha^{-1}. These were resistant to glyphosate. CLA11-A, CON09-A, MIS11-

A,-B,-C, MIS08-B, PHI08-A and STF08-A were highly resistant as the highest dose could not attain 90% control of these accessions. Previously, Norsworthy et al. (2008) reported 115-fold resistance in a sample from Mississippi county whereas the susceptible ones had ED_{50} values ranging from 24.4 to 35.5 g ae ha^{-1}. The ED_{50} values for GR Palmer amaranth in Arkansas were similar to those reported previously for other GR *A. palmeri* (Gaines et al. 2010; Ribeiro et al. 2014), and *A. tuberculatus* (Nandula et al. 2013). Resistance to glyphosate is generally either due to target site resistance (TSR) or non-target site resistance (NTSR) (Shaner et al. 2012; Fernández-Moreno et al. 2016). NTSR mechanisms with respect to glyphosate include reduced absorption and translocation (Vila-Aiub et al. 2012; Fernández-Moreno et al. 2017), increased vacuolar sequestration (Ge et al. 2012), and degradation to non-toxic compounds (Rojano-Delgado et al. 2012; de Carvalho et al. 2012), all of which result in lesser glyphosate translocation in plants. Reduced glyphosate translocation is the most commonly reported resistance mechanism which occurred in *C. canadensis* (Feng et al. 2004), *C. bonariensis* (Dinelli et al. 2008), *L. multiflorum* (Perez et al. 2004) and *L. rigidum* (Wakelin et al. 2004). Reduced translocation results in a higher level of resistance (7- to 11-fold) than the level of resistance (2- to 3-fold) afforded by *EPSPS* mutations in resistant species (Preston and Wakelin 2008). On the other hand, TSR mechanisms confer resistance to glyphosate as a result of either changes in the herbicide-binding site in the *EPSPS* gene (Yu et al. 2015; Fernández-Moreno et al. 2016), or overexpression of the EPSPS protein by gene amplification (Gaines et al. 2010; Salas et al. 2012; Ribeiro et al. 2014).

EPSPS* genomic copy number relative to *A36. In Palmer amaranth accessions from Arkansas, the susceptible population had a single copy of *EPSPS* (Figure 2). Out of 20 accessions tested, 13 were resistant and survivor plants from 8 GR accessions CLA11-A, CON09-A, LEE08-A,

MIS08-B, MIS11-A, MIS11-B, MIS11-C, and POI11-B (cluster 2 and 3) had 19 to 224 maximum copies of *EPSPS*, with a mean of 6 to 149 copies. The *EPSPS* copies varied within and among resistant populations. For instance, the relative copy number in MIS11-A ranged from 78 to 109, in MIS11-B 61 to 224 and in MIS11-C from a single copy to 182 copies with a standard deviation of 15, 75, and 84, respectively (Figure 2). In a resistant plant, gene amplification produces abundant EPSPS enzymes which counterbalance any depletion of the enzyme because of inhibition by glyphosate and therefore, even after treatment with glyphosate resistant plants are still able to produce aromatic amino acid and survive. Cases of *EPSPS* gene amplification seems to be a common resistance mechanism in GR Palmer amaranth. Gaines et al. (2010) reported the first case of *EPSPS* amplification in Palmer amaranth from Georgia. In that population, the resistant plants had up to 160 *EPSPS* copies that resulted in 40-fold overexpression of the gene. GR Palmer amaranth from North Carolina had 22 to 63 *EPSPS* copies (Chandi et al. 2012); in Kansas, 50 to 140 copies (Varanasi et al. 2015); in Mississippi, two GR populations had 33 and 59 copies (Ribeiro et al. 2014); in New Mexico, up to 8 copies (Mohseni-Moghadam et al. 2013); and recently, in Nebraska, 32 to 105 copies (Chahal et al. 2017). It has been documented that <30 *EPSPS* copies result in resistance to field recommended rates of glyphosate (Gaines et al. 2011). In our research, the level of injury from glyphosate application was negatively strongly correlated (r= -0.78) with genomic *EPSPS* copy number (Figure 3). In other words, increase in *EPSPS* copy resulted in resistance to glyphosate. Seven susceptible accessions (including SS) which showed higher injury >60% had only one copy whereas those with <60% injury (resistant) had higher *EPSPS* copy numbers. The highly resistant populations MIS11-A, MIS11-B, and MIS11-C with an average of 30, 31 and 26% injury at 1X rate had 87, 149, and 118 gene copies, respectively. Another interesting aspect was

that with each additional copy of *EPSPS* a 4% decline in injury was observed. A correlation between *EPSPS* copy number and ED_{50} of the GR accessions (r= 0.72) validates the involvement of *EPSPS* amplification in imparting glyphosate resistance. For instance, 62% of the GR accessions had 10 or more *EPSPS* copies, and survived the field rate of glyphosate (840 g ae ha^{-1}). The *EPSPS* gene duplication mechanism has been extensively studied. An increase in *EPSPS* copies (30-50) resulted in increased level of resistance to glyphosate (0.5-1 kg ha^{-1}) when compared to susceptible plants with lesser *EPSPS* copy number (Gaines et al. 2010). After, 2010, gene amplification as a mechanism of resistance to glyphosate was also reported in various weed species. For instance, *A. spinosus* showed up to 37 more copies and a five-fold increase in resistance to glyphosate (Nandula et al. 2012) and *A. tuberculatus* had five- to seven-fold glyphosate resistance and up to eight extra copies of *EPSPS* than the susceptible (Lorentz et al. 2014). *Bromus diandrus* from Australia showed five-fold resistance and enhanced *EPSPS* copies ranged from 10-30 (Malone et al. 2015). Out of three resistant accessions of *Eleusine indica*, one showed 28 more copies of *EPSPS*, however other two accessions had mutations at the target-site (Chen et al. 2015). *Kochia scoparia* populations showed *EPSPS* amplification with three to eight copies (Weirsma et al. 2015) and *L. perenne* ssp. multiflorum was found to be 13-fold more resistant than the susceptible biotype and had up to 30 gene copies (Salas et al. 2012). This indicates that *EPSPS* gene amplification mechanism has been vastly adopted across weed species in order to confer resistance to glyphosate. In our study, analysis of multiple accessions collected from different agricultural areas of Arkansas shows the variability within and among field populations in terms of level of resistance and r mechanisms involved. It is a fact that resistance to glyphosate in Palmer amaranth is rampant in Arkansas. Nonetheless, 30% of the populations can still be controlled with glyphosate and should be managed proactively to delay resistance

evolution. Large-scale surveys are informative in making appropriate management decisions. The current study revealed that only 62% of the GR accessions carry the *EPSPS* amplification resistance mechanism. It is also possible that not all resistant plants in the population harbor the same resistance mechanism. Therefore, novel non-chemical control approaches (i.e. gene silencing) that target this specific mechanism will not control resistant plants harboring other mechanisms.

Partial *EPSPS* gene sequencing. Resistance-conferring point mutations at Pro_{106} and Thr_{102} have been documented in GR weed species. Substitutions of Pro_{106}Ala in *L. rigidum* in Australia (Yu et al. 2007), Pro_{106}Leu in *L. rigidum* from South Africa (Kaundun et al. 2011), Pro_{106}Ser in *E. indica* from Malaysia (Baerson et al. 2002), or Pro_{106}Thr in *E. indica* from Malaysia (Ng et al. 2004) all resulted in low-level resistance to glyphosate. TIPS *EPSPS* was used to produce the first commercial varieties of GR maize. A high resistance to glyphosate has been reported in the presence of *Zea mays EPSPS* multiple mutant T_{102}I and P_{106}S (Eichholtz et al. 2001). The same TIPS double mutation was discovered in *E. indica* resulting in Thr_{102}Ile and Pro_{106}Ser was reported and provided high-level resistance to glyphosate (Yu et al. 2015). In our study, 38% of GR accessions JAC11-B, PHI08-A, POI08-A, STF08-A and WHI11-A did not show gene amplification (up to 2 copies). Partial gene sequencing (4 plants per accession) was conducted to see if T_{102} or P_{106} mutations is responsible for glyphosate resistance. The gene region sequenced is highly conserved. The partial SS-*EPSPS* sequence was identical to the susceptible *EPSPS* sequences from GeneBank. The GR-*EPSPS* did not contain any changes in the amino acid sequence in the conserved region (Figure 4). Some silent mutations were observed in plants POI08-A1, STF08-A1 and STF08-A4 at P_{106} (CCA to CCG) and in five plants from PHI08-A1, PHI08-A3, STF08-A1, STF08-A3 and WHI11-A1 at L_{107} (TTG to TTA) (Figure 4 and 5).

Therefore, none of the known target site mutations were involved in resistance to glyphosate in these accessions.

In conclusion, resistance to glyphosate among Palmer amaranth populations studied here was conferred by amplification of the *EPSPS* gene among 62% of the GR populations. The GR plants not showing *EPSPS* amplification nor mutations indicate the putative involvement of NTSR mechanisms, which requires further investigation.

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Table 1. Cluster analysis of 20 Palmer amaranth accessions from Arkansas, USA, sprayed with 840 g ae ha^{-1} glyphosate.

		Injury (%)[b]			Mean frequency of survivors[c]					
Cluster	No. of Accessions[a]	Mean	Min	Max	HR	R	MR	SR	S	Mortality (%)
1	7	99	66	100	0	0	0	4	196	98
2	6	80	6	100	15	6	17	37	126	63
3	7	50	3	100	80	14	14	26	66	32

[a]Accessions were selected from different counties of Arkansas to show variability across populations.
[b]Plants were sprayed at 7-10 cm tall. Data were recorded 21 days after herbicide application.
[c]Survivors were categorized based on visible injury, where S=sensitive (90-100% injury), SR=slightly resistant (61-89% injury), MR=moderately resistant (31-60% injury), R=resistant (11-30% injury), and HR=highly resistant (0-10% injury).

Table 2. Response of *Amaranthus palmeri* accessions to glyphosate (840 g ae ha^{-1}), Arkansas, USA.

	Injury (%)[b]			Mean frequency of survivors[c]					Mortality (%)	Survivor (%)
Accession[a]	Mean	Min	Max	HR	R	MR	SR	S		
CRA08-A	98	0	100	0	0	0	8	192	96	4
JAC08-B	96	30	100	0	0	0	10	190	95	5
PHI08-A	81	0	100	15	8	14	30	133	67	33
POI08-A	83	0	100	10	1	4	29	156	78	22
LEE08-A	52	0	100	87	3	2	19	89	45	55
MIS08-B	65	0	100	59	3	5	18	115	58	42
STF08-A	46	0	100	70	22	20	44	44	16	84
CON09-A	36	0	100	112	7	12	14	55	28	72
JAC11-A	100	100	100	0	0	0	0	200	100	0
LAW11-A	100	100	100	0	0	0	0	200	100	0
MIS11-D	100	100	100	0	0	0	0	200	100	0
PRA11-B	97	70	100	0	0	0	10	190	95	5
CLA11-A	85	5	100	12	4	8	20	156	78	22
JAC11-B	76	15	100	17	12	30	41	100	50	50
MIS11-C	74	5	100	24	10	24	38	104	52	48
WHI11-A	80	10	100	10	0	19	64	107	54	46
MIS11-A	52	10	100	66	24	19	48	43	22	78
MIS11-B	60	5	100	91	30	15	10	54	27	73
POI11-B	42	5	100	75	10	24	31	60	30	70
SS[d]	100	100	100	0	0	0	0	200	100	0

[a]Accessions were selected to represent different levels of resistance.
[b]Plants were sprayed at 7-10 cm tall. Data were recorded 21 days after herbicide application.
[c]Survivors were categorized based on visible injury, where S=sensitive (90-100% injury), SR=slightly resistant (61-89% injury), MR=moderately resistant (31-60% injury), R=resistant (11-30% injury), and HR=highly resistant (0-10% injury).
[d]Susceptible standard accession.

Table 3. ED_{50} and ED_{90} values for glyphosate-resistant Palmer amaranth accessions (7-10 cm tall) from Arkansas, USA.

Population	ED_{50} (g ae ha^{-1})[a]	R/S[b]	ED_{90} (g ae ha^{-1})	R/S[b]
CRA08-A	177 (± 14)[c]	6	797 (± 74)	4
JAC08-B	207 (± 15)	7	757 (± 61)	4
LEE08-A	522 (± 23)	19	1403 (± 152)	7
MIS08-B	1153 (± 72)	41	ND[d]	ND
PHI08-A	494 (± 18)	18	ND	ND
POI08-A	525 (± 20)	19	1459 (± 101)	8
STF08-A	834 (± 57)	30	ND	ND
CON09-A	1219 (± 60)	44	ND	ND
CLA11-A	761 (± 34)	27	ND	ND
JAC11-A	93 (± 6)	3	211 (± 17)	1
JAC11-B	322 (± 16)	12	1063 (± 155)	5
LAW11-A	100 (± 5)	4	225 (± 20)	1
MIS11-A	1305 (± 83)	47	ND	ND
MIS11-B	1355 (± 78)	48	ND	ND
MIS11-C	1204 (± 74)	43	ND	ND
MIS11-D	84 (± 18)	3	432 (± 39)	2
POI11-B	355 (± 18)	13	1264 (± 134)	7
PRA11-B	147 (± 20)	5	795 (± 76)	4
WHI11-A	392 (± 15)	14	1118 (± 545)[c]	6
SS[e]	28 (± 6)	-	193 (± 16)	-

[a]ED_{50} (effective dose to cause 50% of injury) and ED_{90} (effective dose to cause 90% of injury) was calculated with non-linear logistic 3 parameters; regression equation: $y= c/[1+e^{-a\,(x-b)}]$ where Y is the % injury; a is the asymptote; b is the slope; c is the inflection point; and x is the glyphosate dose.

[b]Resistance levels (R/S) calculated using the dose of the resistant accession relative to the susceptible standard.

[c]Standard error.

[d]ND (not determined) the highest rate applied resulted in less than 90% control of the accession.

[e]Susceptible standard accession.

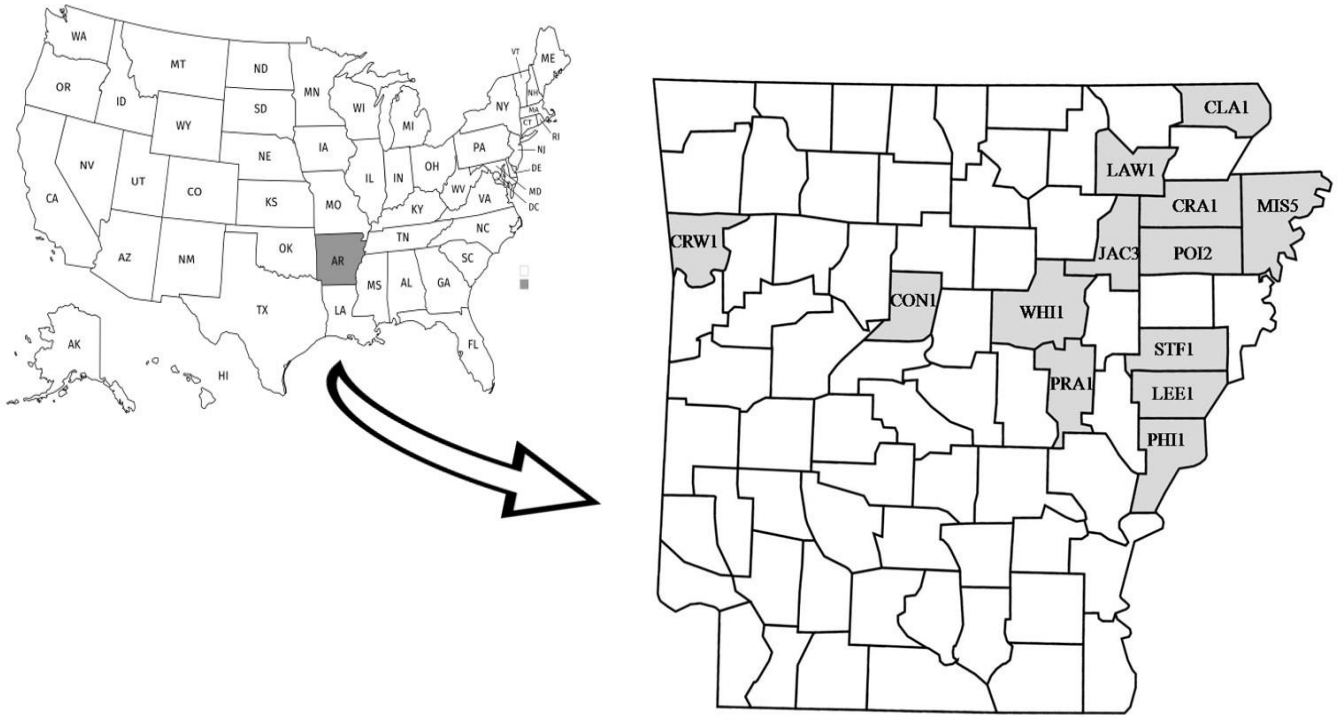

Figure 1. Arkansas map with highlighted counties from which 20 Palmer amaranth accessions were collected between 2008 and 2011. County names are CLA (Clay), CON (Conway), CRA (Craighead), CRW (Crawford), JAC (Jackson), LAW (Lawrence), LEE (Lee), MIS (Mississippi), PHI (Phillips), POI (Poinsett), PRA (Prairie), STF (St Francis), and WHI (White). Name of each county is followed by the number of accessions collected.

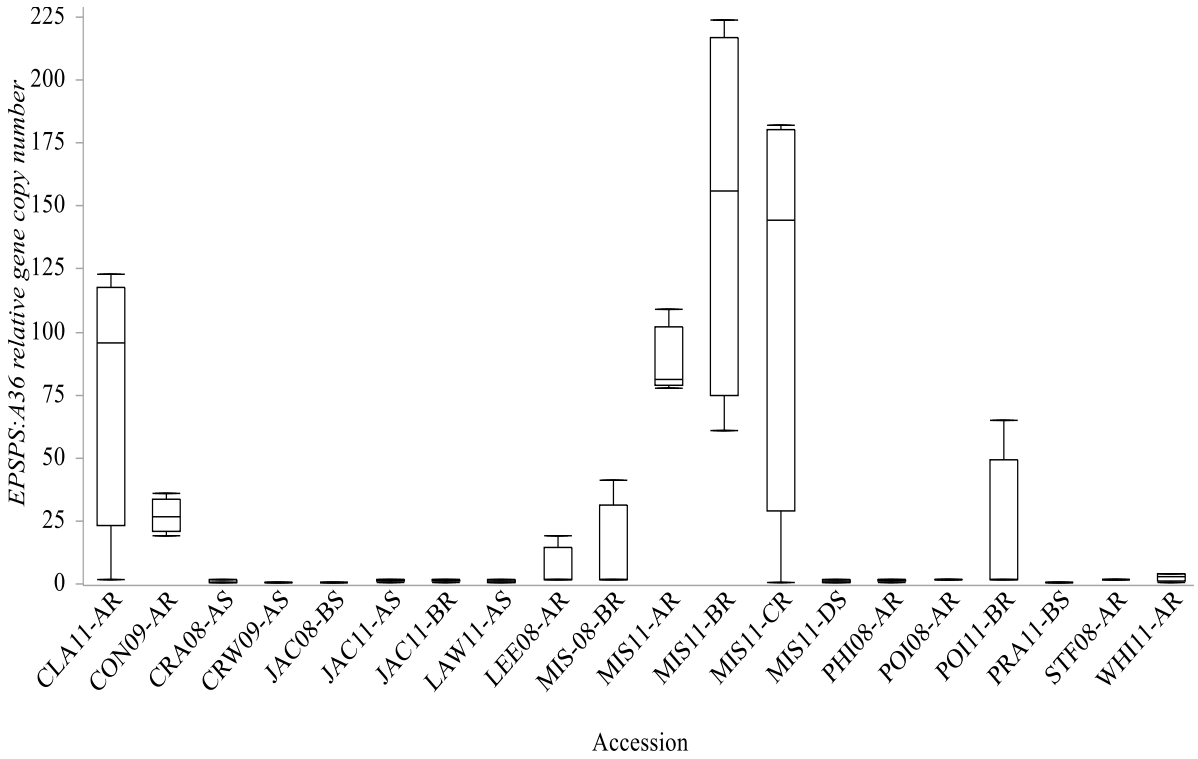

Figure 2. Variability in relative *EPSPS: A36* gene copy number among susceptible (S) and resistant (R) Palmer amaranth accessions. Box plot shows median values (horizontal line inside the box), first and third quartile values (box-outlines), minimum and maximum values (whiskers).

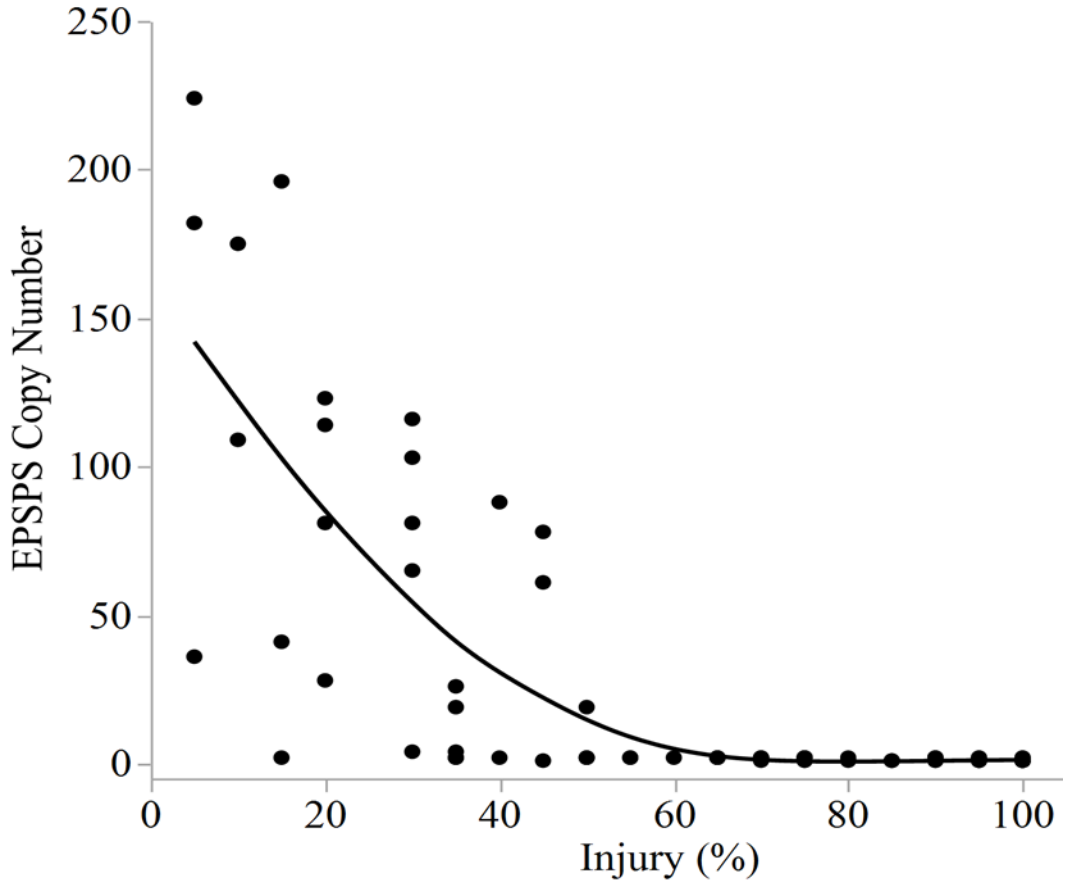

Figure 3. *EPSPS* genomic copy number versus the level of injury in glyphosate-resistant and –susceptible Palmer amaranth. Data was subjected to quadratic regression equation a+b* injury + c*injury^2 where, a= intercept, b= slope of the line, c= quadratic. The correlation between percent injury (x-axis) and *EPSPS: A36* relative genomic copy number (y-axis) was (r= -0.78).

Amino acid position	100	101	102	103	104	105	106	107	108	109	110
Amino acid code	Ala	Gly	Thr	Ala	Met	Arg	Pro	Leu	Thr	Ala	Ala
JAC11-B1	GCA	GGA	ACA	GCG	ATG	CGC	CCA	TTG	ACA	GCT	GCG
JAC11-B2	GCA	GGA	ACA	GCG	ATG	CGC	CCA	TTG	ACA	GCT	GCG
JAC11-B3	GCA	GGA	ACA	GCG	ATG	CGC	CCA	TTG	ACA	GCT	GCG
JAC11-B4	GCA	GGA	ACA	GCG	ATG	CGC	CCA	TTG	ACA	GCT	GCG
PHI08-A1	GCA	GGA	ACA	GCG	ATG	CGC	CCA	**TTA**	ACA	GCT	GCG
PHI08-A2	GCA	GGA	ACA	GCG	ATG	CGC	CCA	TTG	ACA	GCT	GCG
PHI08-A3	GCA	GGA	ACA	GCG	ATG	CGC	CCA	**TTA**	ACA	GCT	GCG
PHI08-A4	GCA	GGA	ACA	GCG	ATG	CGC	CCA	TTG	ACA	GCT	GCG
POI08-A1	GCA	GGA	ACA	GCG	ATG	CGC	**CCG**	TTG	ACA	GCT	GCG
POI08-A2	GCA	GGA	ACA	GCG	ATG	CGC	CCA	TTG	ACA	GCT	GCG
POI08-A3	GCA	GGA	ACA	GCG	ATG	CGC	CCA	TTG	ACA	GCT	GCG
POI08-A4	GCA	GGA	ACA	GCG	ATG	CGC	CCA	TTG	ACA	GCT	GCG
STF08-A1	GCA	GGA	ACA	GCG	ATG	CGC	**CCG**	**TTA**	ACA	GCT	GCG
STF08-A2	GCA	GGA	ACA	GCG	ATG	CGC	CCA	TTG	ACA	GCT	GCG
STF08-A3	GCA	GGA	ACA	GCG	ATG	CGC	CCA	TTG	ACA	GCT	GCG
STF08-A4	GCA	GGA	ACA	GCG	ATG	CGC	**CCG**	**TTA**	ACA	GCT	GCG
WHI11-A1	GCA	GGA	ACA	GCG	ATG	CGC	CCA	**TTA**	ACA	GCT	GCG
WHI11-A2	GCA	GGA	ACA	GCG	ATG	CGC	CCA	TTG	ACA	GCT	GCG
WHI11-A3	GCA	GGA	ACA	GCG	ATG	CGC	CCA	TTG	ACA	GCT	GCG
WHI11-A4	GCA	GGA	ACA	GCG	ATG	CGC	CCA	TTG	ACA	GCT	GCG
SUS Std	GCA	GGA	ACA	GCG	ATG	CGC	CCA	TTG	ACA	GCT	GCG
A. palmeri S (FJ861242.1)	GCA	GGA	ACA	GCG	ATG	CGC	CCA	TTG	ACA	GCT	GCG
A. tuberculatus S (FJ869881.1)	GCA	GGA	ACA	GCG	ATG	CGC	CCA	TTG	ACA	GCT	GCG
A. palmeri R (FJ861243.1)	GCA	GGA	ACA	GCG	ATG	CGC	TCA	TTG	ACA	GCT	GCG

Figure 4. Partial nucleotide sequence of glyphosate-resistant and -susceptible Palmer amaranth. The highlighted nucleotides coding for amino acid position 106 and 107 show polymorphism in some plants but the change did not result in mutations conferring resistance.

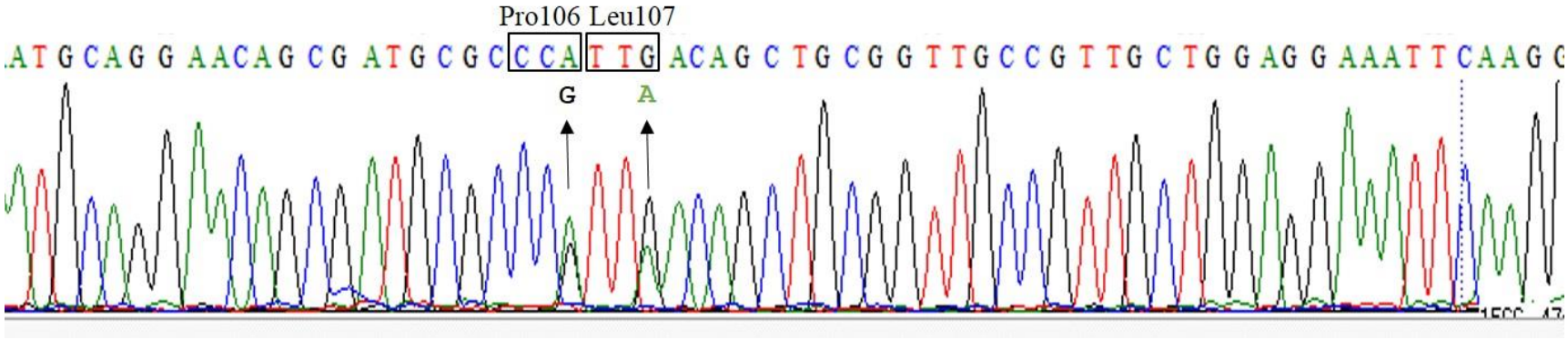

Figure 5. Chromatogram sequence of partial *EPSPS* gene showing polymorphism at amino acid position 106 and 107. At positon 106 a change in nucleotide CCA to CCG (both coding for proline) and at 107 a change in nucleotide TTG to TTA (both coding for leucine) was observed.

CHAPTER-V

ALTERED-TARGET SITE MECHANISM OF RESISTANCE IN ALS-RESISTANT PALMER AMARANTH POPULATIONS FROM ARKANSAS, USA

Abstract

BACKGROUND: Palmer amaranth (*Amaranthus palmeri*) is one of the most common and troublesome weeds in the United States. Palmer amaranth resistance to ALS-inhibitors has been documented in Arkansas but the mechanism of resistance is not yet known. Resistance to ALS-inhibitors is frequently attributed to target-site mutation(s). Therefore, experiments were conducted to (1) confirm cross-resistance to two ALS herbicides and (2) to unravel the resistance mechanism in 20 Palmer amaranth accessions from 13 counties in Arkansas.

RESULTS: All Palmer amaranth populations studied in this research are cross-resistant to pyrithiobac and trifloxysulfuron. The dose of trifloxysulfuron that caused 50% effective control were 21- to 56-fold greater for resistant accessions than susceptible (SS) ones. All but three accessions hadone or two copies of *ALS*. A maximum of seven copies were observed in a single plant from Mississippi county and four copies in a plant from White county. Trp574Ser mutation (cofers resistance to IMIs, SUs and TPs) occurred in all the resistant accessions along with Ala122Thr (results in resistance to IMIs), Pro197Ala (grants resistance to SUs) and Ser653Asn (causes resistance to IMIs) present in a few plants.

CONCLUSION: This study confirmed that mutations at the target-site is the mechanism of ALS resistance in Palmer amaranth populations from Arkansas.

1. INTRODUCTION

Acetolactate synthase (ALS)-inhibiting herbicides were commercialized in 1982 and was considered as an achievement in the history of weed science. These herbicides are used in very small quantities (grams per hectare) compared to high doses of many other herbicides, which reduced the total amount of herbicide active ingredient applied to crops (Bellinder et al. 1994). Many herbicides in this group have broad-spectrum weed control, soil residual activity, wide application windows, high margins of crop safety, and low mammalian toxicities (Mazur and Falco 1989). Acetolactate synthase is the first common enzyme in the biosynthetic pathway of the branched-chain amino acids, valine, leucine, and isoleucine (Durner et al. 1991). Acetolactate synthase-inhibiting herbicides consist of five chemical families: sulfonylureas (SUs), imidazolinones (IMIs), pyrimidinylthiobenzoates (PTBs), triazolopyrimidines (TPs), and sulfonylaminocarbonyltriazolinones (SCTs) (Heap 2017). Pyrithiobac (PTBs), imazaquin (IMIs), and trifloxysulfuron, and nicosulfuron (SUs) (Shaner 2014) are traditionally used to control Palmer amaranth (*Amaranthus palmeri* S. Wats.) in the major crops such as cotton, corn and soybean. The long-term, recurrent use of these chemistries has resulted in evolution of resistance across many weed species. Since the documentation of the first case of herbicide resistance in prickly lettuce (*Lactuca serriola* L.) in 1986 (Mallory-Smith et al. 1990), 158 weed species (both monocots and dicots) have been reported with resistance to ALS-inhibiting herbicides (Heap 2017). In general, *Amaranthus* species are among the most prone to evolve resistance to herbicides because of its high genetic variability (high mutation rates), high seed production (large population size), and continuous seed emergence pattern (high propensity to escape) (Lovell et al. 1996, Norsworthy et al. 2008). Since 1993, *Amaranthus* species including Palmer amaranth (*Amaranthus palmeri*) (Gaeddert et al. 1997), Powell amaranth (*Amaranthus powellii* S. Watson.) (McNaughton et al. 2005), common waterhemp (*Amaranthus rudis* (Moq.) Sauer)

(Hinz and Owen 1997), livid amaranth (*Amaranthus lividus* L.) (Manley et al. 1996), redroot pigweed (*Amaranthus retroflexus* L.) (McNaughton et al. 2005), have been confirmed to be resistant to ALS-inhibiting herbicides. ALS-inhibitor resistance in Palmer amaranth exists throughout the United States (Bond et al. 2006).

Broadly, two mechanisms of resistance to ALS-inhibitors in weed species have been elucidated: (1) resistance related to target site (ALS) mutations (Saari et al. 1994) and (2) processes (structural, biochemical, or physiological) external to the target site that result in reduced amount of herbicide reaching the site of action, or protection from phytotoxic effects of the herbicide (Menendez et al. 1997; Veldhuis et al. 2000). The latter mechanisms are collectively known as nontarget-site resistance (NTSR) among which are reduced absorption or translocation, increased herbicide detoxification, sequestration of herbicide in the vacuoles or cell walls, and increased production of antioxidants.

Many weed species have been documented to possess target-site based ALS resistance. Eight point mutations at Ala122, Pro197, Ala205, Asp376, Arg377, Trp574, Ser653 and Gly654 are known to confer resistance (Heap 2017). Cross-resistance patterns associated with an altered ALS occur among these herbicide classes: (1) SU and TP resistant, (2) IMI and PTB resistant, or (3) resistant to all classes (Powles and Yu 2010). Amino acid substitutions at Ala122 or Ser653 conferred resistance to IMI herbicides with low-level resistance to SUs (Bernasconi et al. 1995; Devine and Eberlein 1997), whereas substitution at Pro197 conferred resistance to SUs (Guttieri et al. 1992), but with low or no cross-resistance to IMIs. Asp376Glu substitution is the only mutation that confers resistance to all five chemical families of ALS-inhibitors (Whaley et al. 2007) and substitution at Trp574 confers resistance to IMIs, SUs and TPs. In *Amaranthus* species such as waterhemp and smooth pigweed (*A. hybridus* L.), mutations in the *ALS* at amino acid

positions Trp574 or Ser653 (Patzoldt and Tranel 2007) and Ala122, Ala205, Asp376, Trp574, or Ser653 (Whaley et al. 2007), respectively, are known to confer ALS-inhibitor resistance. Recently, resistance to ALS-inhibitors in Palmer amaranth was confirmed to be due to amino acid substitutions at Trp574, or Ser653 (Molin et al. 2016; Heap 2017). The objective of this study was to determine if resistance to ALS-inhibiting herbicides in various Palmer amaranth populations is due to gene amplification or target-site mutations. This will help predict which classes of ALS inhibitors may still be used for Palmer amaranth management.

2. MATERIALS AND METHODS

2.1 Plant material and general testing for resistance to ALS herbicides

Palmer amaranth inflorescences were collected from 20 fields representing 13 counties in Arkansas between 2008 and 2011. At least 10 plants were sampled per field. The samples were air-dried, threshed manually, and a composite seed sample was prepared by mixing equal amounts of seed per plant. This composite seed sample is referred to as an accession representing a field population. Composite seed from each accession were planted in 50-cell trays (Redwayfeed Garden and Pet supply, 290 Briceland Rd, Reedway, CA 95560) filled with Sunshine®potting medium (Sunshine premix #1®, Sun Gro Horticulture, 15831 NE 8th Street, Suite 100, Bellevue, WA 98008). The experiment was set up as randomized complete block design with two replications and two runs (50 plants per replication) where each tray was a replication with a single seedling per cell. Thus, a total of 200 plants (at 2-3 leaf stage) representing a field were treated with recommended doses of pyrithiobac at 73 g ai ha^{-1} (Staple®, DuPont Crop Protection) and trifloxysulfuron 8 g ai ha^{-1} (Envoke®, Syngenta Crop Protection, Inc). Both the treatments were applied with 0.25% v/v NIS (nonionic surfactant). Plants were

sprayed in a spray chamber using a boom fitted with two flat-fan nozzles delivering 187 L ha^{-1} at 32 psi. Plants were labeled and leaf tissues were collected before herbicide treatment for the succeeding experiments. At 21 d after treatment (DAT), each plant was evaluated visually for injury relative to the non-treated control. Injury was recorded on a scale of 0-100% where 0 had no injury and 100% was dead. Data were analyzed using ANOVA in JMP Pro v12. Hierarchal clustering of accessions was done using injury and mortality data.

2.2 Evaluation of resistance level to trifloxysulfuron

A dose response assay was conducted with 20 Palmer amaranth accessions showing different responses to trifloxysulfuron. Seeds were planted in 11- x 11-cm square pots filled with Sunshine Mix LC1 potting soil (Sun Gro Horticulture Canada Ltd., Vancouver, British Columbia, Canada). Five seedlings per pot were maintained and sprayed with eight doses of trifloxysulfuron from 0, 1, 2, 4, 8, 16, 32, and 64 g ai ha^{-1} for the resistant accessions. The susceptible (SS) accession was sprayed with 0, 0.5, 1, 2, 4, 8, and 16 g ai ha^{-1}. The herbicide application was made following the procedure used in the previous section. The experiment was conducted in a randomized complete block design with four replications. The number of survivors and injury were recorded at 21 DAT. Data were analyzed using SigmaPlot v.13. Non-linear regression analysis was conducted and the data was fitted with a three-parameter log-logistic model (equation 1) to determine the trifloxysulfuron dose that would cause 50 and 90% control

$$y= c/[1+e^{-a\,(x-b)}] \quad [1]$$

where Y is the % injury; a is the asymptote; b is the slope; c is the inflection point; and x is the trifloxysulfuron dose.

2.3 *ALS* gene copy number determination

Leaf tissues from four plants per accession were collected before and after herbicide application (in both the cases plants were labelled from which tissues were collected). After trifloxysulfuron application (8 g ai ha^{-1}), tissues from survivor plants were collected to determine the *ALS* gene copy number. Leaf tissues were frozen immediately in liquid nitrogen, and stored at -80 C until processed. Genomic DNA was extracted from approximately 100 mg of leaf tissue using a modified CTAB protocol (Doyle and Doyle 1990), quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE), and checked for quality by gel electrophoresis.

Quantitative real-time polymerase chain reaction (qPCR) was used to determine the *ALS* gene copy number relative to *A36*. *A36* (*Celosia trigina* PI649298) is a RNA dead-box helicase. The *A36* primers were designed using Biolign and Primer 3 softwares from sequences of the *Amaranthus* genus: A36_F244 (5'TTGGAACTGTCAGAGCAACC3') and A36_R363 (5'GAACCCACTT CCACCAAAAC3') (Lawton-Rauh A. pers. communication). To amplify the *ALS* gene, the primer sets ALSF2 (5'AGCTCTGGAACGTGAAGGC3') and ALSR2 (5'TCAATTAAAACCGGTCCGGG3') designed by Gaines et al. (2010) were used. For the qPCR, 25-µL reactions were made using 12.5 µL of Bio-Rad iQ SYBR Green Supermix, 1 µL of the forward and reverse primers (10 µM), and 10 ng gDNA. The thermoprofile consisting of 15 min denaturation at 95 C, 40 cycles of 95 C for 30 s, and 60 C for 60 s was used. This program was followed by a melt curve analysis of 81 cycles of 55 C for 30 s (Chandi et al. 2012). A negative control consisting of primers with no template DNA was included. No amplification products were observed in negative control reactions. Data were analyzed using a modification of the 2-ΔΔCt method to express genomic copy number of *ALS* relative to *A36* as ΔCt = (Ct, *A36* - Ct, *ALS*), and relative increase in genomic *ALS* copy number was expressed as 2ΔCt (Gaines et

al. 2011). Each population had four biological and three technical replicates (for each primer pair) to calculate the mean and standard error of the increase in *ALS* copy number relative to A36.

2.4 *ALS* gene sequencing

Genomic DNA was used to amplify the complete sequence of *ALS* gene. The PCR reaction consisted of 25 µl 2x PCR master mix (Takara Bio USA, Inc.), 2 µl of both the forward and reverse primers (10 µM), 4 µl gDNA (50 ng μl^{-1}), and 17 µl of water to make a 50-µl total volume. The following primers were used to sequence the full gene (2 kb): forward primer PAALS_F-5' ATGGCGTCCACTTCAACAAAC3', reverse primer PAALS_R-5'GGTGATGGAAGAAGGGCTTATTAG3'; and internal primers PAALS_F2-5' AGGATATTCCTAGAATTGTTAAGG3', PAALS_F3-5'ATGCGGTTGTAAGTACCGGTGT3', PAALS_R1-5'CCTGGACCTGTTTTGATTGATA3'. The PCR was performed with the following conditions: initial denaturation at 94 C for 5 min, followed by 30 cycles of denaturation at 94 C for 1 min, annealing at 67 C for 1.5 min, extension at 72 C for 2 min and the final extension at 72 C for 5 min. The PCR tubes were held at 4 C until processed. The PCR product was run on a 1% agarose gel to confirm the expected fragment size (2 kb). The PCR product was purified using a NucleoSpin® Gel and PCR Clean up kit (Takara Bio USA, Inc.) and was sequenced at IPGB (Institute for Plant Genomics and Biotechnology), Texas A&M University, College Station, TX. The nucleotide sequences were aligned using Bioedit software.

3. RESULTS AND DISCUSSION

3.1 Response of palmer amaranth to ALS herbicides

Palmer amaranth accessions evaluated in this study showed variable response to pyrithiobac (73 g ai ha^{-1}), trifloxysulfuron (8 g ai ha^{-1}) and a tank-mix of both pyrithiobac and trifloxysulfuron (Table 1 and 2). Each herbicide treatment was analysed and differentiated in three clusters (Table 1). For pyrithiobac, the first cluster consisted of two susceptible accessions (SS and CON09-A). At 3WAT, an average injury (90%) and mortality (88%) was observed. The second cluster constituted of seven accessions with 50% mortality and an average injury of 44%. Several survivors had a minimum injury of 16%. This group was resistant. The third cluster was composed of 11 accessions with an average mortality of 24% and an average injury of 42%. This was classified as highly resistant. For trifloxysulfuron, the first cluster consisted of two accessions with 84% mortality and the remaining plants showing 94% injury 3 WAT. This cluster was the susceptible group. The second cluster constituted of eight accessions with 51% mortality and an average injury of 54%. This group was resistant. The third cluster was composed of 10 accessions with an average mortality of 27% and an average injury of 35%. This was classified as highly resistant. All the accessions treated with pyrithiobac and trifloxysulfuron were in the same group or cluster of resistance except CLA11-A, LAW11-A, and WHI11-A. CLA11-A and LAW11-A were highly resistant (cluster 3) to pyrithiobac and resistant to trifloxysulfuron (cluster 2). On the contrary, WHI11-A was highly resistant (cluster 3) to trifloxysulfuron and resistant (cluster 2) to pyrithiobac. Both pyrithiobac and trifloxysulfuron are being used in cotton fields to control many broadleaved weed species with low level injury to the crop (Jordan et al. 1993). Pyrithiobac applied both as PRE and POST provided effective control of *Amaranthus* species (Dotray et al. 1996). Similarly, POST application of trifloxysulfuron controlled many weeds including smooth pigweed, and palmer amaranth (Porterfield et al. 2002).

Once otherwise effective herbicides now fail to provide the desired weed control, especially to Palmer amaranth as indicated in our results.

In a different experiment, a tank-mix of pyrithiobac and trifloxysulfuron was applied on the same 20 accessions to see any variation in the control of Palmer amaranth. The results were similar to the previous experiment. Palmer amaranth was not controlled by the tank-mix of both the herbicides, which confirmed cross-resistance to both herbicide families. The cluster analysis differentiated in three groups (Table 1). The two accessions in the first cluster were susceptible with 85% mortality and 90% injury 3 WAT. Seven accessions in the second cluster (resistant) had an average injury of 45% and 46% survivors. The highly resistant cluster (3) consisted of 11 accessions that had only 26% mortality. For all the three treatments 200 plants per accession were sprayed with the 1X rate of each treatment the most resistant survivors (0 – 10% injury) were from Clay, Mississippi, Phillips, St. Francis and White counties (Table 2). ALS-resistant Palmer amaranth is widespread across the United States in at least 12 states (Heap 2017). It was reported in Kansas in 1993, Arkansas in 1994, Tennessee in 1994, North Carolina in 1995, South Carolina in 1997, Georgia in 2000, Florida and Mississippi in 2008, Arizona in 2012, Illinois in 2013, Delaware and Maryland in 2014 (Heap 2017). Cross-resistance to multiple ALS herbicides is a common phenomenon in Palmer amaranth. In 2001, imazaquin-resistant Palmer amaranth accessions from Arkansas were reported to be cross-resistant to chlorimuron, diclosulam, and pyrithiobac (Burgos et al. 2001). In another study in Georgia, imazapic-resistant accessions were also resistant to chlorimuron, diclosulam, and pyrithiobac (Wise et al. 2009). Thus, Palmer amaranth has evolved cross-resistance to IMIs, PTBs and SUs. This recent study showed that cross-resistance to pryrithiobac and trifloxysulfuron in Palmer amaranth is the dominant pattern. The previous cross-resistance study in Arkansas included seven population from Lawrence

county only whereas this recent study covers 13 counties. Why no other ALS-inhibitor-resistance-conferring mutations occurred among Palmer amaranth populations in this region is not known. The best chemical control practice is the use of residual and foliar herbicides with different modes of action.

3.2 Resistance level to trifloxysulfuron

The susceptible accessions were completely controlled at recommended field rate (8 g ai ha^{-1}) of trifloxysulfuron. The herbicide dose that caused 50% mortality (ED_{50}) of the susceptible accessions SS and CON09-A was 0.80 g ai ha^{-1} and 2 g ai ha^{-1}. The ED_{50} for other accessions ranged from 17 to 44 g ai ha^{-1} (Table 3). On the basis of ED_{50} values, these accessions had 22- to 56-fold resistance relative to the SS standard. Compared to others, accessions from Clay, Mississippi, Philips and White counties showed more resistance to trifloxysulfuron. The ED_{90} values could only be calculated for SS (3 g ai ha^{-1}) and CON09-A (8 g ai ha^{-1}). Therefore, most likely these accessions could be controlled with the field-recommended dose of trifloxysulfuron. The remaining 18 accessions were highly resistant to trifloxysulfuron as the highest dose cannot achieve 90% control. Different levels of resistance to ALS herbicides have been documented. In a smooth pigweed population 5- to 7-fold resistance to chlorimuron was observed in comparison to susceptible population (Poston et al. 2001). In Kansas, a 2800-fold resistance to imazethapyr was reported in Palmer amaranth (Sprague et al. 1997). The GR_{50} (growth reduction) of the resistant Palmer amaranth was > 7000 g ai ha^{-1} whereas the GR_{50} of SS was very low (2.5 g ai ha^{-1}) compared to the recommended rate (70 g ai ha^{-1}). This high resistance was due to an insensitive ALS enzyme. Similarly, a high level of resistance (537-fold) to imazethapyr was confirmed in smooth pigweed conferred by a Ser653Asn mutation (Whaley et al. 2006). Cross-resistant Palmer amaranth population from Missisippi showed a 112-, 700-, and 150-fold

resistance to pyrithiobac, trifloxysulfuron, and nicosulfuron, respectively, resulted from Trp574Leu mutation (Molin et al. 2016).

3.3 *ALS* genomic copy number relative to *A36*

Resistance to herbicides is generally caused by two mechanisms: target-site and non-target-site (Yu and Powles 2014). Target-site resistance could be because of the amino acid substitution and gene amplification. Gene amplification was not so common in the plant species, however, in the recent years, it has been widely reported as one of the prominent mechanisms of resistance in glyphosate-resistant weed species. In most of the reports of *EPSPS* gene amplification, *ALS* gene has always been used as reference gene owing to its consistency of having single or low copies. However, it is always possible that ALS-resistant populations could have duplication of the *ALS* gene as observed with *EPSPS*. Our data showed that Palmer amaranth from Arkansas, whether susceptible or resistant to ALS inhibitors, had a 1- to 2-copies of *ALS* (Figure 1). There were a few exceptions at least one plant each from MIS08-B, MIS11-A and WHI11-A accessions had more *ALS* copies. A maximum of seven copies were observed in a single plant from Mississippi county and four copies in a plant from White county. These results are novel, but to establish gene amplification as a mechanism of resistance in ALS-resistant Palmer amaranth, further investigations are needed such as southern blotting or FISH (fluorescence *in situ* hybridization) as done in previous studies (Gaines et al. 2010; Dillon et al. 2016). Ploidy level should also be investigated. To establish strong power of prediction, more plants need to be analyzed per population. Recently in Japan, seven accessions of *Alopecurus aequalis* resistant to thifensulfuron-methyl had up to 4 *ALS* gene copy numbers in one of the accessions (Iwakam et al. 2017). The revelation of additional copies was based on the polymorphism observed in the sequenced gene. The presence of multiple copies was confirmed by cloning the gene of interest.

Although, resistance to thifensulfuron-methyl in these accessions was due to mutations at the target-site (substitution of Pro197 with Ser, Leu or Thr).

3.4 *ALS* sequence analysis

Of the 20 Palmer amaranth accessions, 18 were resistant to at least two ALS herbicides representing two families- pyrithiobac (PTB) and trifloxysulfuron (SU). In Arkansas, Palmer amaranth resistance to ALS-inhibitors was first reported in a population from Lawrence county in 1994 but the mechanism of resistance is not yet known. However, Burgos et al. (2001) indicated that ALS resistance in this population from Arkansas could be due to an altered target site. For ALS-inhibitors, mutation at the target-site is the primary mechanism of resistance reported so far. Therefore, it is highly likely that Palmer amaranth from Arkansas has evolved resistance due to mutations at the target-site. The *ALS* gene (approx. 2 kb) was amplified from 20 Palmer amaranth accessions (4 plants per accession) (Figure 2). Sequence data analysis revealed that Trp574Leu (Figure 3 and 4) was present in all the resistant accessions. This point mutation is known to confer resistance in various weed species to different families such as IMIs, SUs, and TPs of ALS-inhibitors (Heap 2017). This explains strong cross-resistance between trofloxysulfuron and pyrithiobac among ALS-inhibitor-resistant accessions. Trp574Leu mutation has also been found in other *Amaranthus* species such as prostrate pigweed (*A. blitoides* L.) (Sibony and Rubin 2003), tall waterhemp (*A. tuberculatus* (Moq.) Sauer (Foes et al. 1998), redroot pigweed, Powell amaranth (McNaughton et al. 2005), and smooth pigweed (*A. hybridus*) (Schmenk et al. 1997). Out of 80 plants for which *ALS* was sequenced, five plants (from CLA11-A, MIS08-B, MIS11-C, and STF08-A counties) had guanine to adenine substitution at position 653 resulting in a Ser653Asn mutation (Figure 3). The Ser653Asn mutation is known to confer resistance only to IMIs (Powles and Yu 2010) which makes it imperative to know the whole

plant response of these three accessions to IMIs. The plants which carried both Trp574Leu and Ser653Asn mutations incurred 30-40% injury to trifloxysulfuron and the accessions in general were resistant to trifloxysulfuron (Table 2). Recently, two mutations Trp574Leu and Ser653Asn were reported in two different Palmer amaranth plants from Mississippi, however, the first mutation was found more frequently (Molin et al. 2016). These resistant populations from Mississippi showed cross-resistance to IMIs, PTBs, and SUs. Similarly, in ALS-resistant Palmer amaranth population from Brazil, all plants had Trp574Leu mutation, while 10 of 24 plants had Ser653Asn (Küpper et al. 2017). The populations from Brazil were resistant to SUs but no information regarding cross-resistance to IMIs was available. Also, these mutations were not present in the same plant. In tall waterhemp, Trp574Leu provided high levels of resistance to IMIs, SUs and TPs whereas Ser653Asn caused resistance to IMIs (Patzoldt and Tranel 2007).

Mutation from Ala122Thr triggers resistance to IMIs but low resistance to SUs and opposite to that Pro197Ala confers resistance to SUs (Powles and Yu 2010). One plant from MIS11-A which had Pro197Ala incurred only 10% injury and the accession in whole plant response assay showed high level of resistance to trifloxysulfuron. Also, the presence of double mutation Pro197Ala and Trp574Leu could be the reason for this highly-resistant plant. On the other hand, a single plant from WHI11-A which had 30% injury harbored both Ala122Thr and Trp574Leu mutation. So far, this is the first case where these mutations (Pro197Ala and Ala122Thr) have been observed in Palmer amaranth, although it had been reported in other ALS-inhibitor-resistant species (Heap 2017). In this research, we found that 30% of the accessions had double mutations and all the mutations conform to the expected cross-resistance patterns. This is also the first time that the *ALS* mutations survey was conducted at such a vast level with multiple

populations from multiple counties of a state, which provides a comprehensive assessment of the resistance status to ALS inhibitors and prevailing resistance mechanisms.

In the present scenario where Palmer amaranth has evolved resistance to several modes of action, knowledge of the mechanisms of herbicide resistance is crucial for planning sustainable weed management practices. The consequences of having multiple target-site mutations in many *Amaranthus* species is detrimental to chemical weed management. Target-site mutations are heritable and ALS resistance is a dominant trait (Powles and Yu 2010), therefore, characteristics of Palmer amaranth such as high seed production, morphological dimorphism, and obligate outcrossing accelerate the process of spreading the resistance trait. Palmer amaranth may also hybridize with other species. For example, a hybrid between Palmer amaranth and spiny amaranth (*Amaranthus spinosus* L.) was found to possess Trp574Leu mutation which originally existed in Palmer amaranth and was the source of resistance to ALS-inhibitor in the hybrid (Molin et al. 2016). The hybrids thus produced would be more vigorous and difficult-to-control. Therefore, it is strongly recommended to judiciously integrate crop rotation and application of herbicides with different modes of action at PRE and POST to combat troublesome weeds.

In conclusion, this research documented that resistance to ALS-inhibitors in Palmer amaranth is widespread (95% of the accessions) in Arkansas. ALS-resistant populations are cross-resistant to PTBs and SUs. Resistance is primarily due to TSR mechanism involving Trp574Ser mutation, with a few cases of double mutations involving Ala122Thr, Pro197Ala or Ser653Asn.

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Table 1. Cluster analysis of 20 Palmer amaranth accessions from Arkansas, USA, sprayed with ALS herbicides.

Cluster	Pyrithiobac				Trifloxysulfuron				Pyrithiobac + Trifloxysulfuron			
	No. of Accessions[a]	Injury (%)[b]		Mortality (%)	No. of Accessions[a]	Injury (%)[b]		Mortality (%)	No. of Accessions[a]	Injury (%)[b]		Mortality (%)
		Mean	Min			Mean	Min			Mean	Min	
1	2	90	77	88	2	94	65	84	2	90	75	85
2	7	44	16	50	8	54	22	51	7	45	15	54
3	11	42	12	24	10	35	9	27	11	36	11	26

[a]Accessions were collected from different counties Arkansas.

[b]Plants were sprayed at 2-3 leaf stage with 20 GPA of pyrithiobac (73 g ai ha^{-1}), trifloxysulfuron (8 g ai ha^{-1}), and a tankmix of pyrithiobac and trifloxysulfuron. NIS (0.25% v/v) was added to all the treatments. Injury and mortality were recorded 21 days after herbicide application.

Table 2. Response of Palmer amaranth accessions to ALS-inhibitor herbicides, Arkansas, USA.

	Pyrithiobac (73 g ai ha^{-1})			Trifloxysulfuron (8 g ai ha^{-1})			Pyrithiobac + Trifloxysulfuron		
Accession[a]	Injury %[b]		Mortality %	Injury %		Mortality %	Injury %		Mortality %
	Mean	Min[c]		Mean	Min		Mean	Min	
CRA08-A	40	15	48	62	30	55	50	20	46
JAC08-B	38	5	51	58	15	37	47	15	51
LEE08-A	45	10	25	30	10	30	39	10	55
MIS08-B	25	5	15	45	15	22	30	5	38
PHI08-A	35	15	20	20	5	47	28	15	40
POI08-A	40	10	30	35	15	32	55	10	21
STF08-A	55	5	12	29	5	38	38	20	15
CON09-A	80	55	75	88	60	68	80	50	70
CLA11-A	42	15	18	46	20	35	52	10	58
JAC11-A	53	5	56	58	35	45	40	15	49
JAC11-B	47	20	46	61	10	31	40	20	47
LAW11-A	50	25	28	60	15	70	46	15	58
MIS11-A	35	5	30	45	5	18	31	5	27
MIS11-B	29	10	38	32	10	25	45	10	21
MIS11-C	46	20	22	51	5	12	38	20	33
MIS11-D	40	25	43	40	30	62	50	20	51
POI11-B	55	15	36	30	15	21	48	15	64
PRA11-B	49	25	60	50	20	74	39	10	65
WHI11-A	38	20	42	35	5	20	28	10	18
SS[d]	100	98	100	100	100	100	100	100	100

[a]Accessions were selected to represent different levels of resistance.

[b]Plants were sprayed with pyrithiobac (73 g ai ha^{-1}), trifloxysulfuron (8 g ai ha^{-1}) and a tank mix of pyrithiobac (73 g ai ha^{-1}) plus trifloxysulfuron (8 g ai ha^{-1}) at 2-3 leaf stage. Both the treatments were applied with 0.25% v/v NIS (nonionic surfactant). Data were recorded 21 days after herbicide application.
[c]Min= minimum injury on the survivor plants
[d]Susceptible standard accession.

Table 3. Nonlinear regression parameters and herbicide dose required for 50% control of ALS-susceptible and –resistant Palmer amaranth accessions from Arkansas, USA.

Accession	Regression equation	R^2	LL (g ai ha^{-1})[b]	ED_{50} (g ai ha^{-1})[a]	UL (g ai ha^{-1})[c]	R/S[d]
CRA08-A	$Y = 53 / [1+e^{-0.207(x-7.878)}]$	0.95	9	22	35	28
JAC08-B	$Y = 53 / [1+e^{-0.139(x-9.981)}]$	0.94	13	30	47	38
LEE08-A	$Y = 63 / [1+e^{-0.094(x-15.460)}]$	0.94	21	30	40	38
MIS08-B	$Y = 60 / [1+e^{-0.184(x-8.168)}]$	0.92	12	17	23	22
PHI08-A	$Y = 59 / [1+e^{-0.102(x-12.261)}]$	0.94	18	29	40	37
POI08-A	$Y = 55 / [1+e^{-0.133(x-10.342)}]$	0.94	15	27	39	35
STF08-A	$Y = 61 / [1+e^{-0.148(x-10.577)}]$	0.93	14	21	28	26
CON09-A	$Y = 100 / [1+e^{-0.397(x-2.391)}]$	0.99	2	2	3	3
CLA11-A	$Y = 52 / [1+e^{-0.094(x-11.758)}]$	0.96	7	44	81	56
JAC11-A	$Y = 55 / [1+e^{-0.171(x-9.045)}]$	0.94	13	23	33	29
JAC11-B	$Y = 75 / [1+e^{-0.270(x-5.180)}]$	0.95	11	19	27	24
LAW11-A	$Y = 56 / [1+e^{-0.130(x-9.321)}]$	0.96	15	26	37	33
MIS11-A	$Y = 50 / [1+e^{-0.189(x-8.312)}]$	0.94	18	32	102	41
MIS11-B	$Y = 57 / [1+e^{-0.079(x-14.143)}]$	0.97	22	39	56	50
MIS11-C	$Y = 60 / [1+e^{-0.084(x-13.757)}]$	0.95	21	35	48	44
MIS11-D	$Y = 52 / [1+e^{-0.192(x-8.147)}]$	0.94	8	24	40	31
POI11-B	$Y = 60 / [1+e^{-0.073(x-17.236)}]$	0.94	25	39	53	50
PRA11-B	$Y = 54 / [1+e^{-0.171(x-8.870)}]$	0.94	13	22	31	28
WHI11-A	$Y = 56 / [1+e^{-0.127(x-11.787)}]$	0.89	17	29	41	37
SS[e]	$Y = 101 / [1+e^{-0.794(x-1.341)}]$	0.99	0.6	0.8	1.0	1.0

[a]ED_{50} (effective dose to cause 50% of injury) was calculated with non-linear logistic 3 parameters; regression equation: $y = c/[1+e^{-a\,(x-b)}]$ where Y is the % injury; a is the asymptote; b is the slope; c is the inflection point; and x is the trifloxysufluron dose.
[b]LL= lower limit of 95% confidence interval.
[c]UL= upper limit of 95% confidence interval.
[d]Resistance levels (R/S) calculated as the ratio of the ED_{50} value for the putative resistant accession relative to the susceptible standard.
[e]Susceptible standard accession.

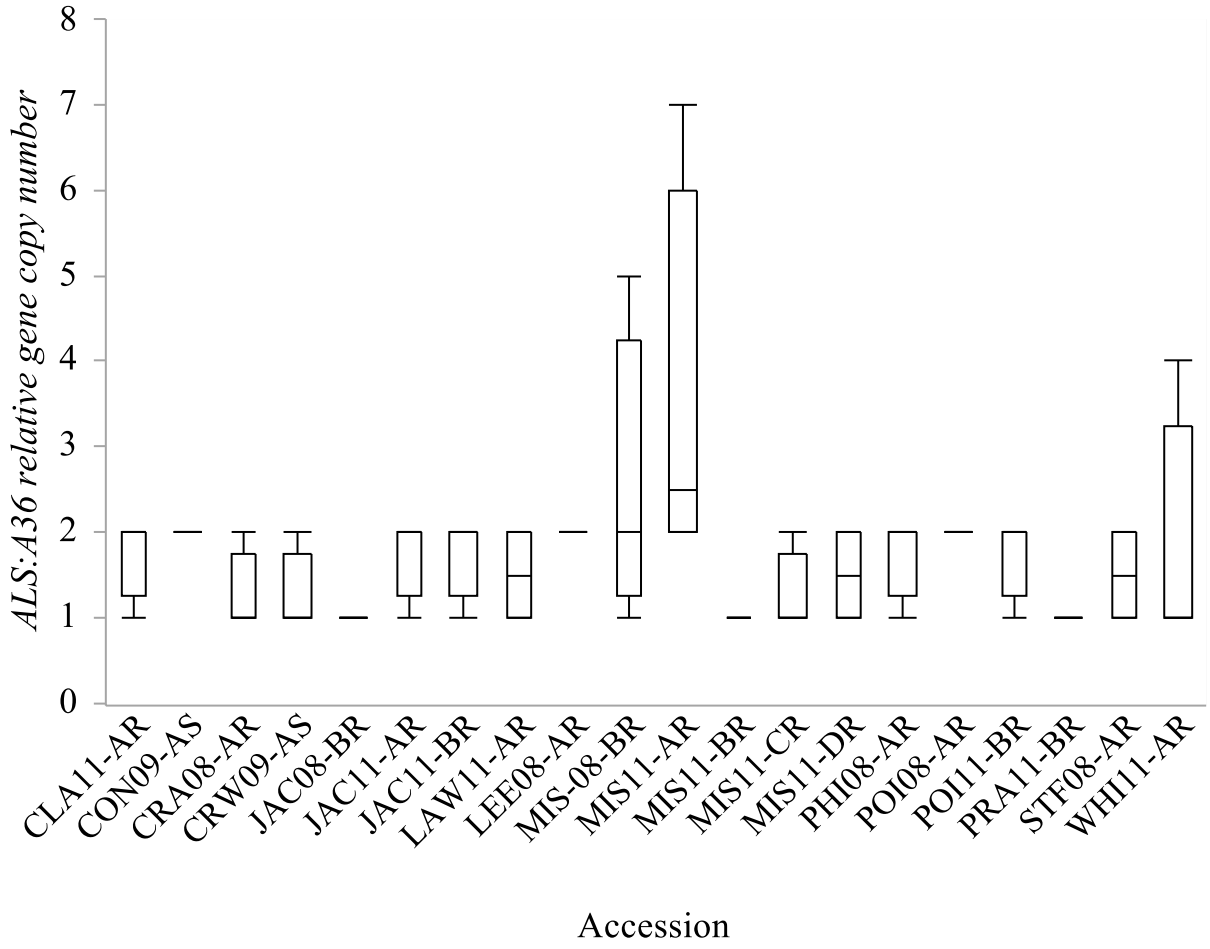


Figure **1**. Variability in relative *ALS: A36* gene copy number among susceptible (S) and resistant (R) Palmer amaranth accessions (four plants per accession). Box plot shows median values (horizontal line inside the box), first and third quartile values (box-outlines), minimum and maximum values (whiskers).

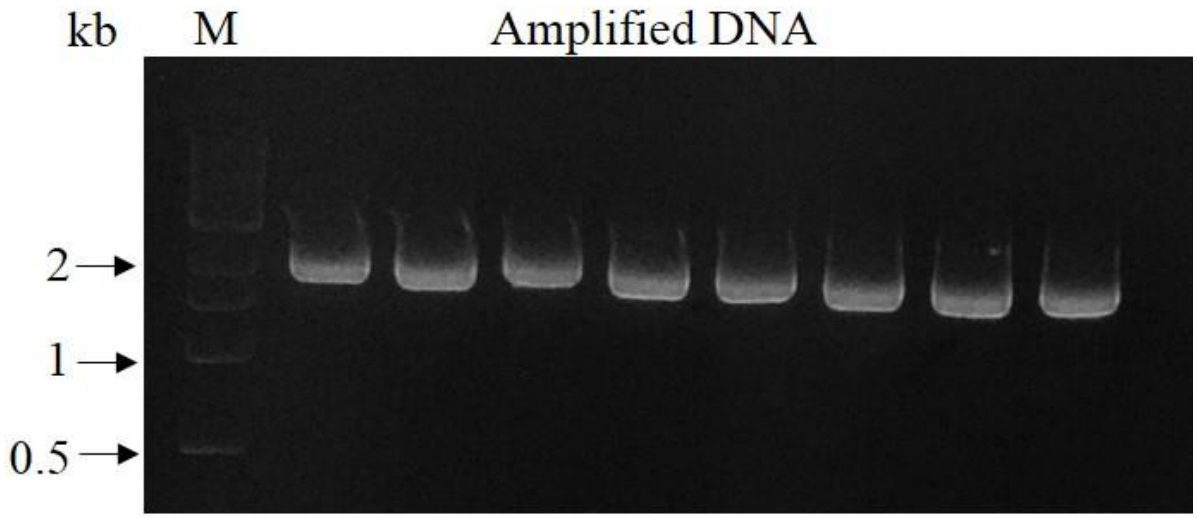
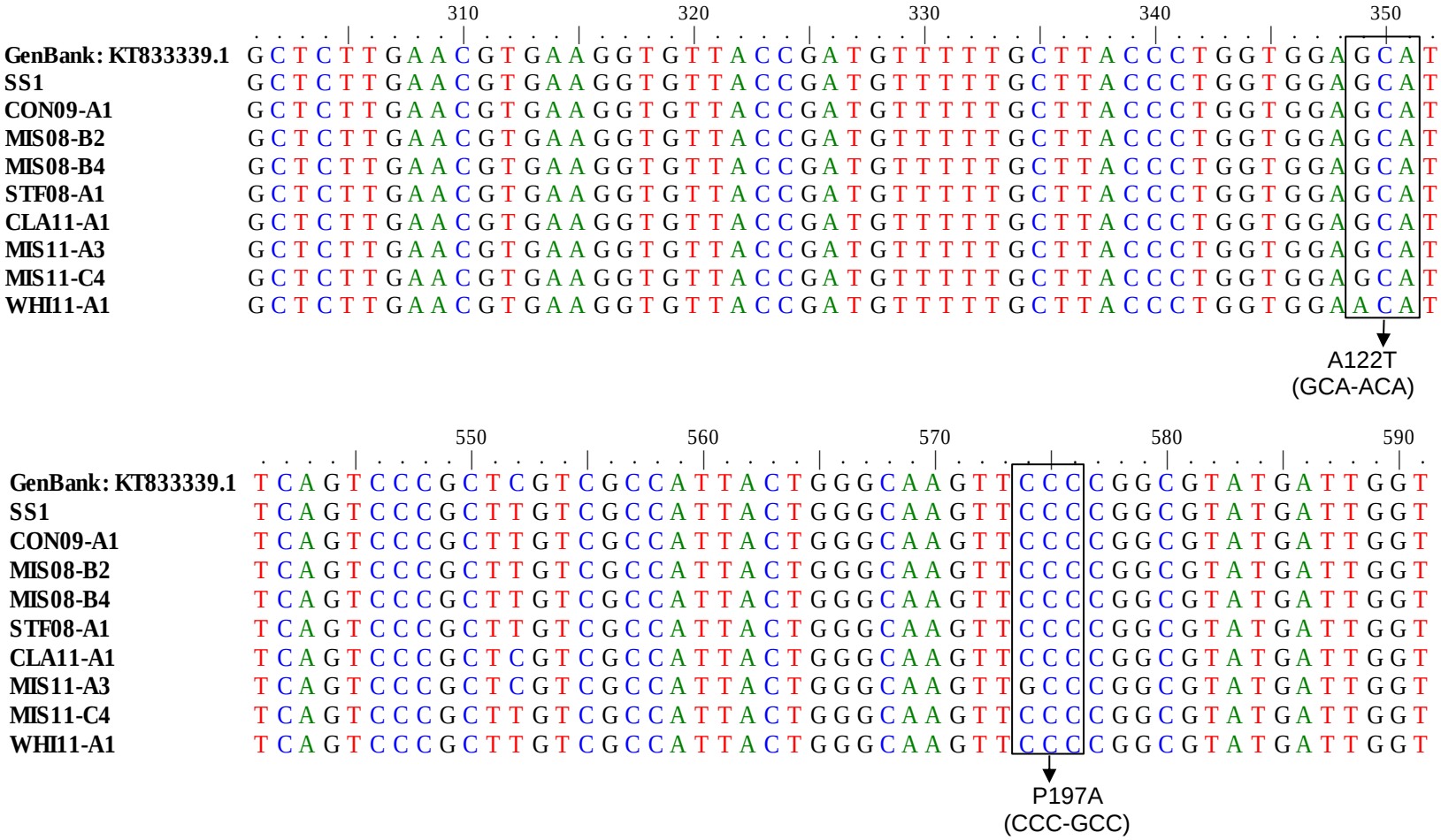


Figure **2**. Gel image of amplified *ALS* gene (~2kb) from a few ALS-resistant Palmer amaranth accessions on 1% agarose gel. Sample ID from left to right: (1) M (marker), (2) MIS11-A, (3) MIS11-B, (4) MIS11-C, (5) STF08-A, (6) LEE08-A, (7) PRA11-B, (8) CLA11-A and (9) SS.

Accession	ED_{50} (g ai ha^{-1})	Ala122 Thr	Pro197Ala	Ser653Asn	Trp574Leu
CRA08-A	22				
JAC08-B	30				
LEE08-A	30				
MIS08-B	17				
PHI08-A	29				
POI08-A	27				
STF08-A	21				
CON09-A	2				
CLA11-A	44				
JAC11-A	23				
JAC11-B	19				
LAW11-A	26				
MIS11-A	32				
MIS11-B	39				
MIS11-C	35				
MIS11-D	24				
POI11-B	39				
PRA11-B	22				
WHI11-A	29				
SS	0.8				

Figure **3**. A graphic presentation of resistance-conferring ALS mutations found in ALS-resistant Palmer amaranth accessions with their corresponding ED_{50} values for trifloxysulfuron, Arkansas.


310
320
330
340
350
GenBank: KT833339.1 G C T C T T G A A C G T G A A G G T G T T A C C G A T G T T T T T G C T T A C C C T G G T G G A G C A T
SS1 G C T C T T G A A C G T G A A G G T G T T A C C G A T G T T T T T G C T T A C C C T G G T G G A G C A T
CON09-A1 G C T C T T G A A C G T G A A G G T G T T A C C G A T G T T T T T G C T T A C C C T G G T G G A G C A T
MIS08-B2 G C T C T T G A A C G T G A A G G T G T T A C C G A T G T T T T T G C T T A C C C T G G T G G A G C A T
MIS08-B4 G C T C T T G A A C G T G A A G G T G T T A C C G A T G T T T T T G C T T A C C C T G G T G G A G C A T
STF08-A1 G C T C T T G A A C G T G A A G G T G T T A C C G A T G T T T T T G C T T A C C C T G G T G G A G C A T
CLA11-A1 G C T C T T G A A C G T G A A G G T G T T A C C G A T G T T T T T G C T T A C C C T G G T G G A G C A T
MIS11-A3 G C T C T T G A A C G T G A A G G T G T T A C C G A T G T T T T T G C T T A C C C T G G T G G A G C A T
MIS11-C4 G C T C T T G A A C G T G A A G G T G T T A C C G A T G T T T T T G C T T A C C C T G G T G G A G C A T
WHI11-A1 G C T C T T G A A C G T G A A G G T G T T A C C G A T G T T T T T G C T T A C C C T G G T G G A A C A T
A122T
(GCA-ACA)
550
560
570
580
590
GenBank: KT833339.1 T C A G T C C C G C T C G T C G C C A T T A C T G G G C A A G T T C C C C G G C G T A T G A T T G G T
SS1 T C A G T C C C G C T T G T C G C C A T T A C T G G G C A A G T T C C C C G G C G T A T G A T T G G T
CON09-A1 T C A G T C C C G C T T G T C G C C A T T A C T G G G C A A G T T C C C C G G C G T A T G A T T G G T
MIS08-B2 T C A G T C C C G C T T G T C G C C A T T A C T G G G C A A G T T C C C C G G C G T A T G A T T G G T
MIS08-B4 T C A G T C C C G C T T G T C G C C A T T A C T G G G C A A G T T C C C C G G C G T A T G A T T G G T
STF08-A1 T C A G T C C C G C T T G T C G C C A T T A C T G G G C A A G T T C C C C G G C G T A T G A T T G G T
CLA11-A1 T C A G T C C C G C T C G T C G C C A T T A C T G G G C A A G T T C C C C G G C G T A T G A T T G G T
MIS11-A3 T C A G T C C C G C T C G T C G C C A T T A C T G G G C A A G T T G C C C G G C G T A T G A T T G G T
MIS11-C4 T C A G T C C C G C T T G T C G C C A T T A C T G G G C A A G T T C C C C G G C G T A T G A T T G G T
WHI11-A1 T C A G T C C C G C T T G T C G C C A T T A C T G G G C A A G T T C C C C G G C G T A T G A T T G G T
P197A
(CCC-GCC)

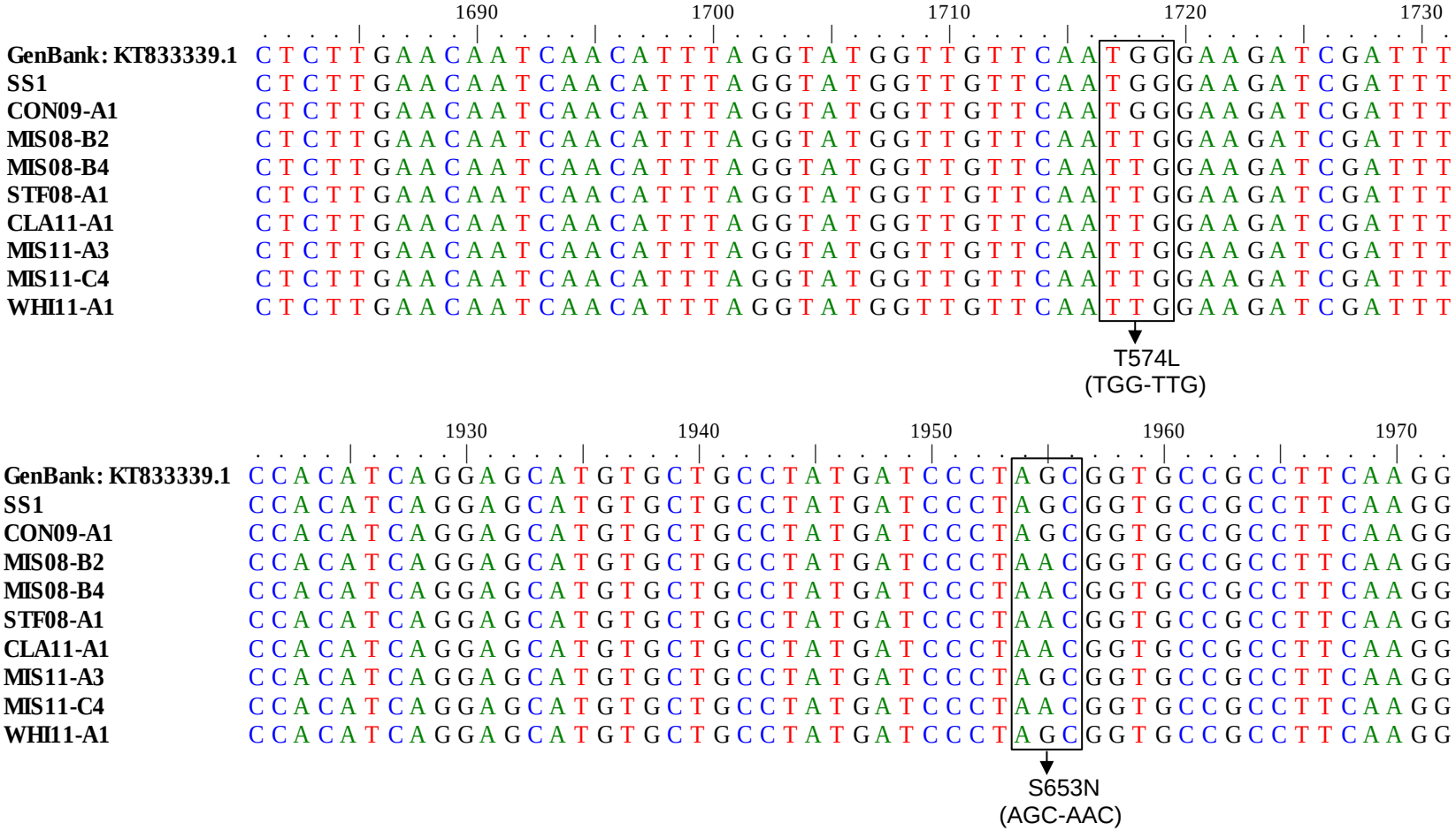


Figure **4**. Alignment and analysis of a portion of *ALS* gene sequence from susceptible (SS1, CON09-A1 and GenBank: KT833339.1) and resistant (MIS08-B2-B4, STF08-A1, CLA11-A1, MIS11-A3, MIS11-C4 and WHI11-A1) Palmer amaranth accessions. A few sequences are presented here to show the site of four *ALS* mutations found in Palmer amaranth accessions from Arkansas. Amino acid numbering refers to the *Arabidopsis thaliana*, *ALS* gene sequence.

CONCLUSIONS

Palmer amaranth populations in the United States have evolved resistance to several modes of action. So far in Arkansas, Palmer amaranth populations have been documented to evolve resistance to ALS-, EPSPS- and PPO-inhibitors. Palmer amaranth accessions were found to be cross-resistant to two ALS-inhibitors: pyrithiobac and trifloxysulfuron. Trp574Ser mutation occurred in all the resistant accessions and a few plants also had Ala122Thr, Pro197Ala and Ser653Asn mutations. Approximately, 55% of the total 119 accessions that were collected between 2008 and 2014 were resistant to glyphosate and majority of the accessions had *EPSPS* gene amplification as the mechanism of resistance. However, in some of the accessions the presence of non-target site mechanism is a possibility. Alternative herbicides are required to control this otherwise troublesome weed. Mesotrione, is an alternative to combat ALS- and EPSPS-resistant accessions. Mesotrione controlled 74% of the total accessions and remaining accessions survived with high injury (61%-90%). Resistance to multiple modes of action in Palmer amaranth emphasize the need for adoption of integrated weed management strategies to minimize herbicide usage and eventually, delay the evolution of herbicide-resistant weeds.