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James C. Walker

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Resistance mechanism, control, and characterization of glyphosate-resistant giant
ragweed (*Ambrosia trifida* L.) in Mississippi

By

James C. Walker

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Weed Science
in the Department of Plant and Soil Sciences

Mississippi State, Mississippi

May 2019

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James C. Walker
2019

Resistance mechanism, control, and characterization of glyphosate-resistant giant
ragweed (*Ambrosia trifida* L.) in Mississippi

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Glyphosate-resistant (GR) giant ragweed (*Ambrosia trifida* L.) has been confirmed in several states across the mid-western and mid-southern U.S. Greenhouse and lab studies were conducted to investigate possible mechanism of glyphosate resistance in a suspect population from Monroe County, Mississippi. Translocation of ^{14}C -glyphosate in the susceptible biotype was 77%, compared to 12% in the resistant biotype at 120 hours after treatment, suggesting that the glyphosate resistance mechanism for this giant ragweed biotype is reduced translocation.

Dose response studies were conducted to confirm and characterize glyphosate resistance in suspect biotypes from Mississippi (MS-R) and Tennessee (TN-R). The ED_{50} for MS-R and TN-R were 3.9- and 6.3-fold higher than a susceptible biotype. Results from a fallow field study conducted in 2016 in Monroe County revealed PRE and POST treatments containing dicamba and mesotrione alone and in various combinations provided effective control of GR giant ragweed.

Studies were conducted to measure fitness, phenotypic, and genetic variation among GR biotypes from MS-R, TN-R, and Ohio (OH-R). Non-destructive

measurements of plants over an eight-week period revealed rapid early growth of two GR accessions from MS in the absence of glyphosate. However, no differences in vegetative biomass were recorded after eight weeks with the exception of OH-R biotype which exhibited lower biomass due to photoperiod sensitivity. Vegetative biomass and fecundity were similar. Multivariate and PCA analysis of traits grouped biotypes based on state of origin. Groupings by state of origin can be significant as managers could design similar methods of control to address giant ragweed in these areas. Simple sequence repeat (SSR) markers were used to record genetic diversity among and within biotypes. Genetic diversity values were high at 0.514, 0.502, and 0.525 within biotypes from MS, TN, and OH, respectively. However, genetic diversity did not differ due to glyphosate response or level of glyphosate resistance. High levels of genetic variation can be an indicator of the ability of giant ragweed biotypes to adapt to changing environments and conditions.

DEDICATION

This dissertation is dedicated to my father, James C. Walker, Jr. My dad passed away in 2016 while I was conducting my research for this project. He was extremely proud of the fact that I had chosen to continue my education at 46 years of age to pursue a doctoral degree. During his hospitalization, he shared with his medical staff on multiple occasions about my project and how proud he was of his son. I know he would have been proud that I stayed the course and finished. I miss him every single day.

ACKNOWLEDGEMENTS

I would like to begin by acknowledging my family. I could not have completed this project without the continued love and support of my wife, Cat, our sons, John Robert and Silas, and my daughters, Britton and McKenna. Family is everything.

I would like to sincerely thank my major professor, Dr. David R. Shaw, for allowing me the opportunity to pursue and complete my degree in a non-traditional manner. I am extremely grateful for his guidance, patience, and support as I navigated this process. It was truly an honor to have Dr. Shaw serve as my major professor.

I would also like to thank my committee members, Dr. Charles Bryson, Dr. Clifford H. Koger, Dr. Shien Lu, Dr. Daniel B. Reynolds, and Dr. Te-Ming (Paul) Tseng, for their support and guidance during the completion of this degree. I want to specifically recognize Dr. Koger for pushing me to pursue a doctoral degree; I would not have started without his encouragement. And also, a special thank you to Dr. Tseng, for molding an agronomist into a serviceable molecular scientist.

I would like to thank those from the Plant and Soil Sciences department for their support during the completion of this degree, including Dr. Reynold's graduate students and hourly employees; and fellow graduate students, Gourav Sharma, Shandrea Stallworth, Swati Shresta, and Zimming Yue for their coaching and assistance with various lab procedures.

Pursuing a doctoral degree while working full-time was tough. My sincerest gratitude is extended to Syngenta for allowing me the flexibility to pursue my degree. I would like to specifically thank Michael Boden, Jeff Mink, Mason Bennett, and my Syngenta South Delta team members. They offered continuous support and patience throughout this entire process.

Finally, thank you Mack and Jane Skelton, for hiring me as a cotton scout many years ago and launching my career in agricultural. I am eternally grateful.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Biology and Ecology

Giant ragweed (*Ambrosia trifida* L.) is an annual broadleaf weed in the Asteraceae or Sunflower family (Bryson and Defelice 2009) and is widely distributed across North America and Canada as a native species (Bassett and Crompton 1982). This weed is also found in Europe, Asia, and South America, prefers moist soil environments associated with floodplains, and is found along stream banks and drainage ditches. It is also found in cultivated fields, fence rows, waste areas, and low-lying pastures (Bassett and Crompton 1982).

Reproduction occurs from seeds, with plants producing up to 5,100 seeds per plant (Johnson et al. 2007) but typical seed production is approximately 275 seeds per plant (Abul-Fatih and Bazzaz 1979b). This represents relatively low fecundity compared to other annual weed species. Seeds are protected by a woody hull or involucre and have projections near seed tops that resemble points of a crown (Johnson et.al 2007). Schutte et al. (2012) described the involucre as embryo-covering structures that inhibit germination and produce covering structure-enforced (CSE) dormancy. Dormancy is also due to physiological dormancy of the embryo due to physiological mechanisms within the embryo (Bewley and Black 1994). Seeds are large and average 5.0 to 10.0 mm long and 2.0 to 3.0 mm wide (Bryson and Defelice 2009). Seed mortality can range from 20 to

90% (Abul-Fatih and Bazzaz 1979b). Low seed survival is caused by several factors identified in the literature. Pre-dispersal seed losses are caused by nonviable seed as well as seed infested with fruit fly larvae (*Diptera: Tephritidae*). Harrison et al. (2001) reported 62% of collected giant ragweed seeds were nonviable with an additional 19% damaged by insects. Post-dispersal losses also result from predation by birds, rodents, insects, and decay (Abul-Fatih and Bazzaz 1979b).

Giant ragweed seeds require a period of dormancy before germination can occur. Abul-Fatih and Bazzaz (1979b) used stratification to aide in breaking dormancy and observed seeds going through a primary and secondary dormancy before germinating. Seedlings emerge in early March and continue through July. The beginning of its emergence is earlier than most summer annuals (Johnson et al. 2007). Emergence can occur over a wide range of soil temperatures and seeding depths (Abul-Fatih and Bazzaz 1979b). The lowest recorded temperature for successful giant ragweed germination was 8 C, with 20 C being the optimum temperature for germination (Abul-Fatih and Bazzaz 1979b). Seeds can experience “self-burial” due to winter rainfall and cryoturbation (frost churning). This type of interaction produces shallow germination from 0.5 cm to 2 cm (Harrison et al. 2007). Shallow burial of giant ragweed seed can also be seen as a result of earthworm foraging (Harrison et al. 2003). Germination and emergence have been recorded as deep as 16 cm, with optimum emergence occurring from 2 to 5 cm (Abul-Fatih and Bazzaz 1979b; Harrison et al. 2007). The size of the giant ragweed seed does impact its germination and subsequent emergence. Larger seeds (> 6.6 mm diameter) were more successful at germinating and emerging from deeper seeding depths (10 cm)

than small seeds (< 4.8 mm diameter) (Abul-Fatih and Bazzaz 1979b; Harrison et al. 2007).

Seedlings are easily identified by large spoon-shaped cotyledons (Johnson et al. 2007). After the second pair of true leaves, leaves are opposite and palmately lobed with 3 to 5 lobes. Leaves are 7 cm to 30 cm long and 1 to 10 cm wide, and are attached to long petioles which range from 5 to 15 cm in length (Bryson and Defelice 2009). Giant ragweed stems are erect, rough, and pubescent (Bryson and Defelice 2009), exhibiting an aggressive growth habit reaching heights of 6 m (Abul-Fatih and Bazzaz 1979a; Bassett and Crompton 1982; Johnson et al. 2007). It is not uncommon for giant ragweed to grow 0.3 to 1.5 m taller than competing crops (Johnson et al. 2007).

Giant ragweed is monoecious, with separate male and female flowers that occur on the same plant. Male flowers occur as slender racemes at the upper terminal of plants with female flowers appearing in clusters at the leaf axils oriented below male flowers (Bryson and Defelice 2009). Flowering occurs from July through October (Bassett and Crompton 1982). A single giant ragweed plant can produce 10 million pollen grains per day and more than a billion throughout its life cycle (Johnson et al. 2007), with pollen traveling up to 60 m (Volenberg et al. 2005). Excessive pollen dispersed by wind allows giant ragweed plants to cross-pollinate. This contributes to the genetic diversity of giant ragweed populations and increases the potential for herbicide resistance (Johnson et al. 2007). Transfer of pollen among plants facilitates hybridization with other ragweed species such as common ragweed (*Ambrosia artemisiifolia* L.) (Bassett and Crompton 1982). Hybrid progeny are typically sterile and produce non-viable seed (Vincent et al. 1988). Humans can have allergic reactions to giant ragweed pollen. Wodehouse (1971)

suggested that perhaps pollen from giant ragweed and common ragweed could account for more seasonal allergic reactions than the combination of all other plants.

Giant ragweed is one of the most competitive summer annual weed species. Its early emergence, rapid growth, and large leaf area give it a competitive advantage. At an Urbana Illinois site, Abul-Fatih and Bazzaz (1979a) recorded giant ragweed with a leaf area index of 5 and light density reduction of 95% beneath plants. Soybean [*Glycine max* (L.) Merr.] yields were reduced 46 and 52%, respectively, with 2 giant ragweed plants per 9 m of row and 92 and 85%, respectively, with densities of 16 plants per 9 m of row in two years of field studies (Baysinger and Sims 1991). Yield losses of 13% were reported in corn (*Zea mays* L.) with 1.7 giant ragweed plants per 10 m² and weeds emerging simultaneously with the corn. Yield reductions in corn were 60% with 14 giant ragweed plants per 10 m² (Harrison et al. 2001).

1.2 History of Control

Giant ragweed has adapted over the last century to human manipulation of claimed lands for agricultural purposes (Bassett and Terasmae 1962). Shallow tillage destroys small giant ragweed plants but would also distribute giant ragweed seeds at an optimum seeding depth and promote emergence (Johnson et al. 2007). Deep tillage such as moldboard plowing can bury seeds deeper than the optimum germination zone. However, seed survival has been recorded at 20 cm after 9 years of burial (Harrison et al. 2007). Subsequent tillage trips following deep tillage could prolong germination by introducing seeds closer to the surface (Harrison et al. 2007). No-till cropping systems would reduce seed burial and expose giant ragweed seeds to predation (Harrison et al. 2003). The wide-scale planting of glyphosate-resistant crops (GRC) across the landscape

has caused a fundamental shift favoring reduced or no-till cropping systems. Results from a 2009 survey of growers from six states (Illinois, Indiana, Iowa, Nebraska, North Carolina, and Mississippi) revealed a large shift to reduced tillage systems after adoption of GRC. Respondents reported 82% had adopted either reduced-till or no-till systems since the initiation of GRC (Givens et al. 2009). Giant ragweed populations expanded in these no-till environments. Various tillage systems have produced mixed results in controlling giant ragweed. However, tillage can be a part of a plan to manage giant ragweed in combination with herbicides and other cultural means such as crop rotation.

1.2.1 Control Options in Corn

Herbicide options for controlling giant ragweed in corn are more numerous than in soybean or cotton. Effective pre-plant (PP), pre-emerge (PRE), and post-emerge (POST) herbicide options are available in corn. Due to giant ragweed's early emergence, it is imperative that early flushes be controlled prior to planting crops. This could be accomplished with tillage or the use of PP burndown herbicide applications. Broad spectrum non-selective herbicides such as glyphosate, glufosinate, or paraquat are good options but should not be used alone. Tank mixes including atrazine, dicamba, and or 2,4-D provide more consistent control (Johnson et al. 2007).

Giant ragweed is most competitive in corn after emergence through the V5 stage of corn development. Harrison et al. (2001) found that a four-week delay in giant ragweed emergence equated to a 4- to 8-fold reduction in competition with corn. These findings confirm the importance of an effective PRE herbicide in order to manage giant ragweed. There are multiple herbicide options for PRE applications in corn. PRE treatments including atrazine, atrazine + metolachlor, and atrazine + metolachlor +

mesotrione exhibited 48, 58, and 89% control of giant ragweed, respectively (Loux et al. 2011). Loux et al. (2011) found as the number of modes of action increased so did the level of control. Studies have also shown a synergistic effect when tank mixing a PS II inhibitor such as atrazine with mesotrione (Woodyard et al. 2009). PRE trials resulted in control levels of 52 and 63% with atrazine and saflufenacil + dimethenamid, respectively. However, greater levels of control were recorded with isoxaflutol + atrazine, dicamba, mesotrione + atrazine, saflufenacil, and dicamba + atrazine providing 77 to 83% control. Dicamba treatments controlled giant ragweed with the most consistency at 83% (Soltani et al. 2011). In general, PRE treatments gave inconsistent control of giant ragweed, emphasizing the need to follow PRE applications with POST treatments. Giant ragweed's prolonged emergence pattern or elasticity makes it difficult to gain adequate control with a single herbicide application. Greatest success in managing giant ragweed populations with herbicides involves combining PRE and POST herbicide applications and utilizing multiple modes of action (Johnson et al. 2007).

In field experiments where PRE applications were followed with POST applications in a two-pass program, the levels of giant ragweed control were 80% or greater. A PRE treatment of atrazine + metolachlor + mesotrione followed by a POST treatment of glyphosate provided 97% giant ragweed control (Loux et al. 2011). Soltani et al. (2011) evaluated numerous POST treatments and found dicamba + atrazine was the most consistent POST treatment, providing 82 to 94% control of giant ragweed. Giant ragweed control in corn is best obtained when a PRE is followed by a POST rather than a single POST application. However, when utilizing POST applications, they should be timed before giant ragweed reach 15 cm in height or yield loss will occur (Loux et al.

2011). When considering POST options, glufosinate has provided 95% control of giant ragweed and is an effective treatment where glufosinate-resistant crops are utilized (Barnett et al. 2013).

1.2.2 Control Options in Soybean

Giant ragweed control in soybean can be challenging, with fewer POST herbicide options as compared to corn. PP burndown scenarios will be similar in soybean with the exception of atrazine as a tank-mix partner with non-selective herbicides such as glyphosate, glufosinate, and paraquat. When a giant ragweed population is not acetolactate synthase (ALS)-resistant, the addition of chlorimuron or cloransulam to PP applications enhances control (Johnson et al. 2007). Saflufenacil alone and in combination with glyphosate has provided 90% control of emerged giant ragweed when applied PP (Vink et al. 2012). Prior to the introduction of GRCs, chlorimuron and imazquin provided control of giant ragweed in soybean (WSSA 1994). In 1998, a new ALS-inhibitor, chloransulam-methyl, was introduced and it proved to be the most effective ALS herbicide for controlling giant ragweed with less soybean injury when applied following PPI herbicides (Askew et al. 1999). Cloransulam can be applied PP, PRE, and POST. Yield losses from giant ragweed can be avoided if control is maintained for the first eight to ten weeks after soybean emerge (Baysinger and Sims 1991). This further emphasizes the competitive nature of giant ragweed and the importance of controlling early-emerging giant ragweed prior to planting and utilizing a PRE to eliminate early weed competition. Other PRE herbicides which have activity on giant ragweed in soybean include flumioxazin, imazquin, metribuzin, and sulfentrazone. None of these compounds applied PRE provided season long control and required follow up

POST treatments (Johnson et al. 2007). POST herbicides that provide adequate control of giant ragweed in soybean or cotton are limited (Steckel 2007).

Glyphosate-resistant (GR) soybean was introduced in 1996 and provided growers with an effective POST option for giant ragweed control (Wiesbrook et al. 2001). Its continuous use and reliance on a single mode of action (glyphosate) has led to reduced effectiveness as GR biotypes have been identified (Heap 2014). Norsworthy et al. (2011) observed the following when evaluating POST treatments in soybean. ALS-inhibitors chlorimuron and imazquin controlled giant ragweed less than 88%, while cloransulam exceeded 96% control. This confirms earlier examples of the effectiveness of cloransulam in non-ALS-resistant populations of giant ragweed. Norsworthy et al. (2010) observed more than 89% control of giant ragweed when evaluating the PPO inhibitors carfentrazone, flumioxazin, and fomesafen on two-node GR giant ragweed. Fomesafen provided 87 to 96% control of an ALS-resistant biotype of giant ragweed (Taylor et al. 2002). Control with PPOs is dependent upon weed's size. Six-node giant ragweed control was 66% with carfentrazone or fomesafen (Norsworthy et al. 2010). Glufosinate is the most consistent POST herbicide option providing more than 98% control of giant ragweed when applied sequentially (Wiesbrook et al. 2001). Spaunhorst et al. (2014) evaluated various combinations of flumioxazin, dicamba, chlorimuron, sulfentrazone, and glyphosate PP followed by POST treatment combinations of either dicamba, fomesafen, or cloransulam with glyphosate and observed 91% or greater control of GR giant ragweed.

1.2.3 Control Options in Cotton

Giant ragweed control in cotton (*Gossypium hirsutum* L.) is challenging, as there are few POST options that provide adequate control. Norsworthy et al. (2010) observed prometryn and diuron providing effective control of GR giant ragweed. Diuron controlled giant ragweed 100% at various growth stages. These products can be applied PRE or POST directed. Herbicides are POST directed by using specialized spray equipment to direct the herbicide toward the base of crops. This type of application allows the herbicide to contact weeds more effectively while minimizing contact with cotton plants. The ALS-inhibitors trifloxysulfuron and pyrithiobac provided more than 90% control for two-node stage but decreased to 55% for six-node stage giant ragweed. The PPOs fomesafen, carfentrazone, and flumioxazin controlled giant ragweed 89% for two-node stage but control decreased to 66% for six-node stage plants. Glufosinate and MSMA provided the most consistent control of giant ragweed at the 90% level. Many of these treatments do require that cotton be large enough to post-direct herbicides.

1.3 Herbicide Resistance

Herbicide resistance can be defined as the inherited ability of a plant to survive and produce offspring after exposure to a herbicide at a rate considered lethal in the wild type. Resistance may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis (WSSA 2013). The International Survey of Herbicide-Resistant Weeds lists the following criteria for listing a herbicide-resistant biotype: suspected biotype meets requirements as defined by WSSA for herbicide resistance, resistance must be confirmed with data generated from approved scientific methods, resistance must be heritable, suspected biotype must

have proven problematic under commercial field conditions, and resistance must be derived naturally and not through artificial selection (Heap 2018).

The first reporting of herbicide resistance occurred in 1957 in Ontario. A population of wild carrot (*Daucus carota* L.) was confirmed resistant to the synthetic auxin 2,4-D (Switzer 1957). Since that time, the rate of evolved herbicide resistance has increased exponentially. As of January 2019, there have been 497 resistant biotypes among 255 different weed species confirmed globally (Heap 2019). The continued use of several different ALS-inhibiting herbicides to control giant ragweed in soybean throughout the 1990's led to the development of ALS resistance (Taylor et al. 2002). The first occurrence was confirmed in Indiana in 1998 and to date seven states have confirmed ALS-resistant giant ragweed biotypes (Heap 2019). ALS resistance in giant ragweed has been documented due to the substitution of a single amino acid tryptophan 574 to leucine (Patzoldt and Tranel 2002).

Glyphosate resistance in giant ragweed was first confirmed in 2004 in Ohio. Since that time, a total of 12 states have reported GR biotypes (Heap 2019). Giant ragweed was actually the first weed to confirm glyphosate resistance in Canada in 2008 (Heap 2014). The advent of herbicide resistance adds to the difficulty of managing giant ragweed populations in cropping systems and will force producers to incorporate other methods of control.

New cotton and soybean cultivars with resistance to dicamba, glyphosate, glufosinate, 4-hydroxyphenyl-pyruvate dioxygenase (HPPD), and 2,4-D are being developed (Green 2014). New technologies do provide additional opportunities. However, the focus remains on utilizing herbicides as the primary means of control.

Managers must utilize all available technologies including non-herbicide methods such as crop rotation to promote more sustainable weed control systems.

1.3.1 Mechanisms of Glyphosate Resistance

Powles and Preston (2006) grouped the mechanism for glyphosate resistance as either target site or non-target site based. Target site resistance was described as resistance where a gene mutation induces a change to a target site enzyme disabling a herbicide's ability to inhibit proper enzyme function. A herbicide's target site is the specific enzyme that is targeted by a particular herbicide. That enzyme for glyphosate resistance is 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Powles and Preston 2006). The first weed documented with glyphosate resistance due to mutated EPSPS enzyme was goosegrass [*Eleusine indica* (L.) Gaertn.] (Lee and Ngim 2000). This population developed glyphosate resistance due to a point mutation in EPSPS enzyme as a result of the substitution of the amino acid proline at site 106 (Pro106) for either serine or threonine (Ng et al. 2003). There have been additional reports of substitutions at the Pro106 position in rigid ryegrass (*Lolium rigidum* Gaud.); proline to alanine substitution (Pro106Ala) (Simarmata et al. 2008), proline to threonine substitution (Pro106Thr) (Wakelin and Preston 2006), and proline to leucine (Pro106Leu) (Kaundun et al. 2011). Devine and Preston (2000) found another amino acid substitution at position Gly101 with alanine being substituted. Finally, studies in goosegrass discovered a point mutation found to occur in combination with Pro106Ser, with a substitution of threonine to isoleucine (Thr102Ile) at a new position 102 (Jalaludin et al. 2013). Glyphosate resistance due to EPSPS Pro106 mutations is inherited by a single, semi-dominant, nuclear gene (Ng et al. 2003). The studies indicating EPSPS gene mutations utilize deoxyribonucleic

acid (DNA) sequencing to detect mutations that might occur as insertions or deletions of nucleotides, single nucleotide changes (point mutations), or inversion of sequences (Slotta 2008). One measure of target site sensitivity is shikimate levels in glyphosate-treated plants. Research has indicated that inhibition of EPSPS resulting from glyphosate applications causes shikimate accumulation in treated plant tissues. Mueller et al. (2003) saw shikimate levels rise in both resistant and susceptible horseweed (*Conyza canadensis* L.) biotypes initially, with levels decreasing in resistant plants at 4 days after treatment (DAT), while levels continued to increase in susceptible plants.

Non-target site mechanism of glyphosate resistance can involve reduced translocation of glyphosate, reduced uptake or absorption, vacuolar sequestration of glyphosate, and over-expression of EPSPS enzyme. Changes in the pattern of translocation within plants can confer glyphosate resistance. For example, glyphosate might accumulate near leaf tips in GR plants and fail to translocate to growing points and roots. This translocation failure results in the plant surviving the glyphosate application (Powles and Preston 2006). Lorraine-Colwill et al. (2003) found GR rigid ryegrass accumulated ^{14}C -glyphosate at leaf tips, whereas susceptible plants accumulation occurred at root tips. Reduced translocation of glyphosate has also been found in horseweed (Feng et al. 2004; Koger and Reddy 2005). However, recent work with GR giant ragweed found no differences in ^{14}C -glyphosate translocation between resistant and susceptible biotypes (Norsworthy et al. 2011). Similar results were discovered in common ragweed (*Ambrosia artemisiifolia* L.) (Brewer and Oliver 2009) and in Palmer amaranth (*Amaranthus palmeri* S. Watson) (Culpepper et al. 2006).

Reduced uptake and absorption of glyphosate as a mechanism for glyphosate resistance has been documented in Italian ryegrass [*Lolium perrene* L.ssp. multiflorum (Lam.) Husnot] (Michette et al. 2007) and johnsongrass [*Sorghum halepense* (L). Pers.] (Vila-Aiub et al. 2012). Differences were seen in resistant plants as compared to susceptible with higher contact angles of leaves, thicker cuticles of leaves, and composition of epicuticular wax on leaf surfaces.

Horseweed has been documented as GR due to increased levels of glyphosate in the vacuoles of resistant biotypes (Ge et al. 2010). Once glyphosate has been sequestered in vacuoles, it is no longer in the cytoplasmic pool and available to be moved to sensitive plant tissues.

Gaines et al. (2009) utilized quantitative polymerase chain reaction (PCR) of genomic DNA and fluorescent in-situ hybridization analysis to determine a GR population of Palmer amaranth contained a high degree of target site duplication as a mechanism for glyphosate resistance. The resistant biotype exhibited 5 to 160 times more copies of EPSPS gene distributed throughout its genome.

1.3.2 Giant Ragweed's Differential Response to Glyphosate

The typical response of sensitive plants following a glyphosate application include retarded growth and leaf chlorosis within 7 days. Above-ground plant tissues become necrotic after 21 days (Singh and Shaner 1998). However, GR giant ragweed has exhibited two different responses to glyphosate. Brabham et al. (2011) described a rapid necrosis response in a GR biotype from Indiana. Following glyphosate applications, mature leaves became necrotic within 24 hours of application. However, young leaves and meristematic tissues were unaffected and continued to grow normally. This type of

response would suggest that there is a possibility that glyphosate is held in mature leaves and fails to translocate due to a reduced translocation mechanism (Brabham et al. 2011). In contrast, Vink et al. (2012) recorded plants from a Leamington, Ontario population surviving a glyphosate screening dose of 1,800 g ae ha⁻¹. Survivors exhibited symptoms similar to susceptible plants with gradual chlorosis of meristematic tissue within 7 days. However, unlike susceptible plants resistant individuals did show stunting but began to regrow after one to two weeks.

1.3.3 Fitness Cost Associated with Herbicide Resistance

Plant fitness is a degree of survivorship impacted by competitiveness and fecundity, which contributes to subsequent generations (Harper 1977; Warwick and Black 1981). Evolutionary theory predicts that as plants are exposed to new environments there are costs associated with their adaptation to these environments (Purrington 2000). Orr and Betancourt (2001) described these adaptations as either new mutations or standing genetic variation. Traits that appear after the application of selection pressure were considered new mutations, whereas those occurring prior to selection were natural or resulting from standing genetic variation. A population of weeds can have herbicide-resistant individuals occurring prior to the use of a herbicide. This assumption can be made based on the theory of natural selection. Herbicide resistance derived from natural mutations can result in fitness costs either from pleiotropic effects directly related to the resistance gene or can be linked to the resistance gene with one or multiple loci responsible for the fitness cost (Mithila et al. 2011). Herbicide-resistant individuals were documented in rigid ryegrass populations that had not been treated with a herbicide (Preston and Powles 2002). However, evolved herbicide resistance more frequently

results from selection pressure occurring through herbicide usage and the subsequent development of herbicide-resistant alleles.

Herbicide-resistant biotypes that are impacted by fitness penalty would decrease over time due to their lack of competitiveness in the absence of selection by that herbicide (Anderson et al. 1996, Holt and Thill 1994). However, in some herbicide-resistant accessions the mutation responsible for resistance does not carry a fitness cost (Holt and Thill 1994; Sibony and Rubin 2003). Where fitness costs occur due to herbicide-resistant alleles, a recent review by Vila-Aiub et al. (2009) gave three possible reasons. Fitness cost may occur when mutations in target enzymes reduce plant functions such as metabolism (Powles and Preston 2006; Vila-Aiub et al. 2009; Powles and Yu 2010). Werck-Reichart et al. (2000) reported when herbicide resistance is due to over-production of cytochrome P450 enzymes, resources are diverted away from growth and reproduction of resistant plants. Finally, fitness costs can occur due to changes in ecological interactions. For example, a resistant phenotype might become less attractive to pollinators (Salzmann et al. 2008).

A population of common groundsel (*Senecio vulgaris* L.) that was not controlled by atrazine was documented in 1968 (Ryan 1970). Triazine resistance mechanism was found to be due to a mutation in the chloroplastic psbA gene where a serine 264 to glycine substitution occurs in the photosystem II D1 protein. This mutation confers resistance due to the reduced affinity of the binding site (Trebst 1996). Triazine-resistant plants experienced fitness costs expressed as reduced photosynthetic capacity, suppressed biomass accumulation, and reduced seed production when compared to susceptible plants (Conrad and Radosevich 1979; Warwick and Black 1981). Parks et al. (1996) found

triazine-resistant plants to be more easily controlled with PS II inhibiting herbicides such as bentazon when compared to susceptible plants. The 1980's and 90's saw wide adoption of both ACCase and ALS-inhibiting herbicides. Resistance to these classes was recorded within 5 years of their introduction (Shaner 1992). Resistance mechanism for both classes of herbicides was due to reduced sensitivity in the target site. Eight amino acid substitutions are currently documented to confer ACCase resistance (Vila-Aiub et al. 2009). Several fitness studies have been conducted evaluating some but not all of the amino acid substitutions conferring ACCase resistance and results were varied. Fitness traits were measured in rigid ryegrass and black-grass (*Alopecurus myosuroides* Huds.) known to carry the Ile1781Leu substitution and no penalty was recorded (Vila-Aiub et al. 2005). However, one population of rigid ryegrass with the Ile1781Leu mutation did express increased seed dormancy influenced by the environment (Vila-Aiub et al. 2005). Under drought conditions, blackgrass with the Asp2078Gly mutation showed reduced growth when compared to susceptible plants (Menchari et al. 2008). Studies also recorded reduced ACCase activity in Asp2078Gly, Ile2041Asn, and Cys2088Arg mutations (Delye et al. 2003, 2005; Yu et al. 2007). ALS resistance has been documented due to 21 amino acid substitutions (Powles and Yu 2010). Fitness studies have only been conducted on a few of the mutations. Initial fitness studies revealed little to no fitness penalty related to field evolved ALS resistance (Holt and Thill 1994; Tranel and Wright 2002). However, negative effects have been documented in resistant weeds containing mutations at Pro197 and Trp574 (Tardiff et al. 2006; Yu et al. 2007; Tranel et al. 2008). Several weed species have shown ALS resistance resulting from 11 different amino acid substitutions for Pro197 (Yu et al. 2007, Powles and Yu 2010). A resistant biotype of

prickly lettuce (*Lactuca serriola* L.) containing the Pro197His substitution showed a 15% reduction in biomass under competitive conditions when compared to susceptible plants (Alocer-Ruthling et al. 1992). Powell's amaranth (*Amaranthus powellii* S. Watson) populations with the Trp574Leu mutation were shown to have severe changes in morphology resulting in reduced competitiveness. Resistant plants had thinner stems and roots. Vegetative biomass was decreased 67% and seed production was also reduced when compared to susceptible plants (Tardiff et al. 2006).

Widespread usage of glyphosate on GRCs throughout the mid-1990's to the present resulted in many populations of GR weeds. There are currently 17 species of weeds with confirmed resistance to glyphosate in the United States (Heap 2018). Resistance mechanism responsible for glyphosate resistance is often due to sub-lethal rates of glyphosate reaching the target site because of reduced translocation of the herbicide (Powles and Preston 2006). A population of rigid ryegrass, confirmed GR due to reduced translocation, was evaluated and exhibited normal growth patterns under competitive conditions with wheat when compared to susceptible accessions (Pederson et al. 2007). However, that same population, when exposed to low densities of wheat, did produce fewer but larger seeds (Pederson et al. 2007). Fitness studies were conducted on a population of Palmer amaranth conferring glyphosate resistance due to overexpression of EPSPS gene. Vila-Aiub et al. (2014) found no correlation between EPSPS gene copy number and vegetative or reproductive biomass regardless of competition level. Studies conducted in 2006 and 2008 were designed to measure growth and phenology of GR and susceptible horseweed. In non-competitive scenarios, resistant biotypes grew faster, flowered earlier, and consequently set seeds as much as 25 days earlier than susceptible

accessions (Shrestha et al. 2010). However, there were no differences in the two biotypes in seed production. Early flowering was also recorded in a population of GR common lambsquarters (*Chenopodium album* L.) that initiated inflorescence four to six weeks prior to a susceptible biotype but there were no differences in seed production (Westhoven et al. 2008).

1.4 Glyphosate

1.4.1 Mode of Action

Glyphosate is a non-selective herbicide and provides control of both grasses and broadleaf weeds while having little negative impact on the environment due to no soil activity (Powles and Preston 2006). The mode of action of glyphosate involves the inhibition of EPSPS producing 5-enolpyruvylshikimate-3-phosphate (EPSP) from shikimate-3-phosphate and phosphoenolpyruvate in the shikimic acid pathway. EPSP inhibition leads to the depletion of the aromatic amino acids tryptophan, tyrosine, and phenylalanine, all needed for protein synthesis (WSSA 2007). After treatment, plants will stop growing followed by chlorosis and necrosis within 4 – 7 days for susceptible grasses and 10 – 20 days for less susceptible species. Glyphosate is translocated from shoots and leaves to the roots of treated plants (Ross 2009). The target site for glyphosate resistance is the chloroplastic enzyme EPSP (Powles and Preston 2006).

1.4.2 Pattern of Use

Glyphosate was introduced in 1974 by Monsanto. Its primary use at that time was in non-agricultural applications to control a wide spectrum of weeds along roadsides or rights-of-way (Powles and Preston 2006). The introduction of GR soybean in 1996, corn

in 1997, and cotton in 1998 totally changed use patterns of glyphosate, as growers were then able to make applications in-season (Young 2006). Prior to the availability of GRCs, glyphosate was primarily used in crops before emergence as a burndown treatment (Young 2006). By 2001, GR cotton hectares accounted for 56% of planted hectares in the United States. GR soybean had risen to over 70% with corn at 10% of planted hectares (Zelaya and Owen 2005). These numbers have continued to escalate. In 2014, the percentage of planted hectares represented by herbicide-tolerant cultivars of cotton, soybean, and corn were 91, 94, and 90%, respectively (Fernandez-Cornejo 2018). This type of broad adaptation of GRCs allowed glyphosate to become the single largest herbicide used globally (Duke and Powles 2008). Soybean producers traded tillage and multiple mode of action herbicide chemistries to control weeds for a single herbicide with only one mode of action. This placed extreme selection pressure on weeds for glyphosate resistance (Young 2006).

1.4.3 Development of Glyphosate Resistance

The heavy adoption of GRCs and the ability to make multiple applications of glyphosate during the course of a growing season has contributed to the development of GR weeds (Zelaya and Owen 2005). In 1996, the first reported case of glyphosate resistance occurred in rigid ryegrass in Australia. GR rigid ryegrass was documented two years later in the United States in California. The first dicot recorded with glyphosate resistance in North America was horseweed in 2000. Italian ryegrass was discovered with glyphosate resistance later in the United States in 2004. The first Palmer amaranth population with glyphosate resistance was confirmed in Georgia in 2006 and has since been recorded in 23 states, making Palmer amaranth one of the most prolific GR weed

species. As previously discussed, GR giant ragweed was first observed in Ohio in 2004 and in more recent years has been documented in southern growing regions including populations in Arkansas, Tennessee, Missouri, and Mississippi (Heap 2014). Additional control failures of glyphosate have been reported in giant ragweed from multiple locations in Mississippi.

1.5 Dicamba

1.5.1 Mode of Action

Dicamba is a member of the synthetic auxin family of herbicides and more specifically a subgroup of the benzoic acids. Benzoic acids are chlorinated derivatives of benzoic acids (Ross 2009). Auxins that occur naturally in plants regulate cell division and elongation and developmental processes such as vascular tissue and meristem differentiation, leaf initiation, phyllotaxy, senescence, apical dominance and root formation. Indole-3-acetic acid (IAA) is the main naturally-occurring auxin in plants (Grossman 2009). The mode of action of dicamba involves mimicking IAA by acidifying the cell wall and stimulating the activity of a membrane-bound ATPase proton pump. The reduction in the apoplastic pH induces cell elongation by increasing the activity of enzymes responsible for cell wall loosening. Low concentrations of auxin-mimicking herbicides also stimulate ribonucleic acid (RNA) polymerase, resulting in subsequent increases in RNA, DNA, and protein biosynthesis. Abnormal increases in these processes lead to uncontrolled cell division and growth, which results in vascular tissue destruction. High concentrations of auxin-mimicking herbicides inhibit cell division and growth, usually in meristematic tissues that accumulate photosynthate assimilates and herbicide from the phloem. Auxin-mimicking herbicides stimulate ethylene evolution which can

produce the epinastic response often seen from exposure to these herbicides (WSSA 2007). Typical symptomology seen in treated plants includes twisting and curling of stems and petioles (epinasty), stem swelling and elongation, and leaf cupping. Symptoms are followed by chlorosis at the growing points, growth inhibition, wilting, and necrosis. At low applications rates, the tips of new leaves may develop into narrow extensions of the midrib, and puckering of the young leaves may develop (WSSA 2007). Dicamba does have soil activity and is mobile in the soil. Dicamba can be leached out of the zone of control in three to twelve weeks. However, dicamba may persist longer in dry soil conditions but has a half-life of more than 14 days under favorable conditions for rapid metabolism (WSSA 2007).

1.5.2 Pattern of Use

The synthetic auxins were discovered in the 1940's and included 2,4-D and MCPA. These compounds were the first synthetic herbicides developed and led to additional active ingredients developed in the 1950's including dicamba and others (Ross 2009). Dicamba exhibits selective activity on broadleaf weeds and is primarily used in monocot cropping systems. Dicamba is labeled PP, PRE, and POST in corn; POST in grain sorghum [*Sorghum bicolor* (L.) Moench ssp. Bicolor], small grains, pasture/rangeland, grasses/fallow, turfgrasses, and asparagus (*Asparagus officinalis* L.). Dicamba also has PP applications for other crops but soil activity requires crop specific restrictions (Anonymous 2010). Dicamba-resistant (DR) cultivars of cotton and soybean have recently been developed and are currently available (Green 2014). DR cotton and soybean cultivars will provide producers broad adaptability with this technology and an effective option for controlling herbicide-resistant biotypes of broadleaf weeds such as

giant ragweed and Palmer amaranth. New formulations of synthetic auxins have been designed with less volatile salts and improved adjuvant systems to minimize off-target movement and volatility (Li et al. 2013).

1.5.3 Development of Dicamba Resistance

There are currently four species of weeds in North America with resistance to the synthetic auxins. In 1990, charlock or California rape (*Sinapis arvensis* L.) was documented resistant to dicamba in Manitoba Canada. In 1995, a dicamba-resistant biotype of tumbleweed (*Kochia scoparia* L.) was documented in Montana and in North Dakota in the United States. Since this discovery, dicamba-resistant kochia biotypes have also been discovered in Idaho, Colorado, and Nebraska. A dicamba-resistant population of hemp nettle (*Galeopsis tetrahit* L.) was identified in Alberta Canada. More recently, in 2007 a dicamba-resistant biotype of prickly lettuce (*Lactuca serriola* L.) was documented in Washington State (Heap 2014). Of those weed species that do confer resistance to dicamba, the mechanism for resistance is unknown at this time. At the present, there are no known populations of giant ragweed resistant to dicamba.

1.6 Mesotrione

1.6.1 Mode of Action

Mesotrione is a member of the benzoylcyclohexane-1,3-dione or triketone family of herbicides. The mode of action is the inhibition of the enzyme HPPD affecting carotenoid biosynthesis (WSSA 2007). HPPD is a component of the biosynthetic pathway that converts tyrosine to plastoquinone and α -tocopherol. Plastoquinone is a critical cofactor for phytoene desaturase, a component of the carotenoid biosynthetic

pathway. Thus, the depletion of plastoquinone levels by inhibition of HPPD results in depletion of carotenoids, leading to bleaching symptoms followed by necrosis (Mitchell et al. 2001). Mesotrione is absorbed through the shoot and can be absorbed through the root following a POST application. It is absorbed through the seed and emerging root and shoot following PRE applications. It is translocated in the xylem and phloem and moved throughout the targeted plants. Corn rapidly metabolizes mesotrione to inactive metabolites. Mesotrione is rapidly broken down in the soil by microorganisms. The average half-life is from 5-15 days. The potential for leaching is negligible (WSSA 2007).

1.6.2 Pattern of Use

Mesotrione is labeled PRE and POST in corn and sugarcane (*Saccharum officinarum* L.). However, mesotrione can only be used as a PRE in grain sorghum and sweet corn (*Zea mays* L. var. *rugosa*). Mesotrione provides effective control of many broadleaf weeds as well as large crabgrass [*Digitaria sanguinalis* (L.) Scop.] and broadleaf signalgrass [*Urochloa palyphylla* (Munro ex C. Wright) R.D. Webster] (Anonymous 2014). Mesotrione is seldom used as a stand-alone treatment and is typically tank mixed with either atrazine or metolachlor or both to improve the spectrum and length of weed control. The synergistic effect when tank mixing atrazine with mesotrione was mentioned previously. This strategy also incorporates multiple modes of action to reduce selection pressure and prevent the development of herbicide resistance.

1.6.3 Development of Mesotrione Resistance

There are currently two species of *Amaranthus* documented as mesotrione-resistant. In 2009, both tall waterhemp (*Amaranthus tuberculatus* L.) and Palmer amaranth were identified as mesotrione-resistant in Iowa and Kansas, respectively. Both of these populations were also confirmed to be cross-resistant to atrazine and the ALS-inhibitors. In 2011, tall waterhemp populations in Iowa were also found to be GR. Mesotrione-resistant biotypes were added in Nebraska for tall waterhemp and palmer amaranth in 2011. At this time, the mechanism for mesotrione resistance is unknown. There are no known mesotrione-resistant biotypes of giant ragweed to date (Heap 2014).

1.7 Variation in Weed Species

1.7.1 Phenotypical Variation and Adaptation

Bradshaw (1965) defined phenotypic plasticity as the degree to which expression of an individual genotype is manipulated by its environment. These changes can be exhibited morphologically or physiologically. Baker (1974) provided a list of characteristics to describe the ideal weed. Several characteristics dealt with the ability of a plant to adapt to ranges in environmental conditions: germination achieved under variety of environments, continuous seed production while conditions allow, seed production in both favorable and non-favorable environs, seed adaptations to promote both short and long-range dispersal, survival of perennials through vegetative adaptations (brittleness and regeneration from fragments), and ability to compete through specialized methods (rosette, smothering growth, allelochemicals). Success of major weeds can be further explained through the combination of multiple adaptations over time (Baker 1974).

There are many examples of weeds adapting to manipulations by humans. Development of civilization involved forest clearing practices that favored species exhibiting increased seed dispersal capability, high reproductive rates, and compressed life cycles (Anderson 1952). Grasslands with long histories of grazing have selected for grass ecotypes reproducing from rhizomes or stolons (Mack and Thompson 1982). Weeds that have adapted along roadways or areas of high traffic exhibit low growth habits, leaves form a rosette, stems become more flexible, and seeds adapt adhesive methods to facilitate dispersal via wheels or feet (Frenkel 1970).

Weeds that occur in annual crops grown in dense stands often exhibit a more erect, columnar growth habit, and delayed flowering. False flax [*Camelina sativa* (L.) Crantz] is an annual species that has a branching growth habit. However, where it occurs in dense stands of flax it appears as an erect non-branching agroecotype (Sinskaia and Beztuzheva 1931). Mimetic forms of weeds can develop when the weed closely resembles the specific crop. Barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.] is native to Asia and is a serious problem in cultivated rice (*Oryza sativa* L.). Due to selection through hand weeding, *E. crus-galli* var. *oryzicola* has become a crop mimic. The growth habit, leaf orientation, and leaf color are similar to cultivated rice (Barrett 1983). Selection pressure where herbicides are reducing susceptible races within weed species is widely documented in the literature. Resistant phenotypes can then be separated or described by varying degrees of tolerance or response to a particular herbicide.

Giant ragweed exhibits phenotypic variation most notable through leaf morphology. Leaves typically have three lobes but can vary from a single lobe to as many

as seven lobes. Leaves are most often opposite but can occur alternate (Bassett and Crompton 1982). Giant ragweed produces a wide range of seed sizes. The variation in seed sizes promotes better survival under a range of environmental conditions (Abul-Fatih and Bazzaz 1979b, Schutte et al. 2008a). Giant ragweed has adapted its emergence pattern to a more prolonged emergence where environments are disturbed such as agricultural production fields (Hartnett et al. 1987). Schutte et al. (2008b) recorded emergence from March to July. High levels of embryo dormancy delay germination until soil temperatures exceed 20 C. Schutte et al. (2012) found seeds from successional populations emerged in a synchronous pattern that occurred early in the season. In contrast, giant ragweed seeds from agricultural areas emerged over a longer period. The occurrence of later-emerging ecotypes could have resulted from selection pressure via agricultural practices that controlled the early emerging biotypes (Schutte et al. 2012). There have been two phenotypes of giant ragweed identified based on differing responses to glyphosate. One expressing a rapid necrosis response (Brabham et al. 2011) and one having a slow response (Vink et al. 2012). Although the glyphosate resistance mechanism has not been determined in giant ragweed, this differential growth response following glyphosate applications might suggest a different mechanism of resistance among these two phenotypes.

1.7.2 Genetic Variation

Weedy plant species by their nature have evolved successfully due in part to natural selection aided by heritable variation within populations (Barrett 1982). Fisher (1958) proved that a population's ability to gain competitiveness is directly correlated to its degree of genetic variation in fitness. Barrett (1982) listed several factors that impact

genetic variation: variables that influence recombination; chromosome number, frequency of crossing over, sterility and fertility barriers, mating system, system of pollination, seed dispersal system, and life history strategy of species. There are also evolutionary and ecological factors that can influence genetic variation. One factor is founder effect often referred to as bottlenecks, can be defined as decreased genetic diversity where populations arise from a small group of colonizing ancestors (Mayr 1963). An additional factor is genetic drift or change in the frequency of a gene in a population due to random sampling of individuals (Lacy 1987). Finally, environmental heterogeneity or the support of higher species richness in natural habitats by allowing niche differentiation and species co-existence can also impact genetic variation (Stein et al. 2014). Gene flow can occur in giant ragweed through both seed and pollen. Giant ragweed is an open or cross-pollinated species. Cross-pollinated species experience a more rapid change from a genetic standpoint than self-pollinated species (Baker 1974). Open-pollinated species exhibit higher levels of genetic variation than self-pollinated species (Barrett 1982).

Genetic variability among weedy plants such as giant ragweed provides significant obstacles when attempting to develop management plans (Sterling et al. 2004). Native plant populations exhibit greater genetic variability than shown in exotic invasive species (Sterling et al. 2004). Weeds encountered in production areas are more adapted genetically and exhibit greater phenotypic plasticity as well as increased seed production (Radosevich et al. 2007). This variation among weed populations can also be expressed as differences in growth patterns (Bond and Oliver 2006) and susceptibility to herbicides (Burgos et al. 2001; Gossett et al. 1992; Horak and Peterson 1995).

Assessment of genetic variation within a given population of weeds could provide valuable information when managing herbicide resistance (McRoberts et al. 2005). Success of chemical and biological control methods can be impacted by occurrence of resistant biotypes resulting from genetic diversity (Sterling et al. 2004). Ultra-specific biological methods can be ineffective where high degrees of genetic diversity occur in the target weed. However, DNA markers can be used to better understand genetic variation within a target species (Nissen et al. 1995). Molecular markers can be used to identify genome hybridization among related species that can lead to herbicide resistance, gene flow, or transfer of other traits that can lead to specie decline such as seed shattering (Gu et al. 2006). There have been a number of studies utilizing markers to measure genetic diversity among various weed species. Marker types used in most recent studies include: random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR), intersimple sequence repeats (ISSR), DNA sequences, and amplified fragment length polymorphism (AFLP) (Chandi et al. 2013; Meyer et al. 2017; Ray and Roy 2009; Slotta et al. 2006). Molecular markers are derived from DNA sequence polymorphisms. Due to molecular markers ability to reveal Mendelian inheritance, markers can be utilized to document the evolutionary history of a species by phylogenetic characteristics. Markers can also be used to analyze genetic structures, population structures, and develop gene maps of a species (Hoshino et al. 2012). Slotta et al. (2006) used microsatellite markers to document genetic diversity and gene flow in Canada thistle [*Cirsium arvense* (L.) Scop.], also a member of the Asteraceae family. More recently Meyer et al. (2017) developed SSR markers to measure the genetic diversity of common ragweed. Of the markers evaluated in this study, 40% were transferable to giant ragweed.

1.8 Statement of Objectives

The objectives of this study are to (1) confirm and characterize glyphosate resistance among giant ragweed biotypes from Mississippi and Tennessee, (2) investigate possible mechanism of glyphosate resistance in giant ragweed, (3) determine if there are fitness costs associated with glyphosate resistance in giant ragweed, (4) utilize biological characterization and genetic markers to measure the levels of phenotypic and genetic variation among the various accessions within each biotype and across states, and (5) determine the effectiveness of various PRE and POST herbicide treatments containing both dicamba and mesotrione in controlling GR giant ragweed.

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CHAPTER II

INVESTIGATIONS INTO POSSIBLE RESISTANCE MECHANISM OF
GLYPHOSATE-RESISTANT GIANT RAGWEED
(*Ambrosia trifida* L.) IN MISSISSIPPI

2.1 Abstract

Glyphosate-resistant (GR) giant ragweed (*Ambrosia trifida* L.) was reported in Mississippi in 2014. Herbicide resistance to multiple classes of herbicides has allowed giant ragweed to become a more widespread problem in crop fields. Crop losses due to uncontrolled populations of giant ragweed can be devastating. The mechanism responsible for glyphosate resistance in giant ragweed has not been determined. A study was conducted in 2016 to confirm glyphosate resistance and investigate possible mechanism of resistance in a suspect population from Monroe County. Shikimate accumulations following an application of glyphosate at 420 g ae ha⁻¹ at 1, 2, and 5 days after treatment (DAT) were 4.4, 5.3, and 4.9 times higher in susceptible biotypes. Absorption of ¹⁴C-glyphosate did not differ between susceptible and resistant biotypes at 120 hours after treatment (HAT). However, translocation of ¹⁴C-glyphosate out of treated leaf in susceptible biotypes was higher (77%) compared to resistant biotype (12%), at 120 HAT. This would suggest the mechanism conferring glyphosate resistance in this particular giant ragweed biotype is due to reduced translocation. Sequencing of the conserved region of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) containing

the Pro106 location did not reveal any mutations in the target site. This provides critical information for managers when selecting specific herbicides for control of GR giant ragweed.

2.2 Introduction

Giant ragweed is a problematic annual weed species belonging to the Asteraceae family (Bryson and Defelice 2009). Native to North America, giant ragweed is often found along alluvial floodplains and has adapted well to disturbed lands such as agricultural fields (Bassett and Crompton 1982). Locally adapted giant ragweed ecotypes have thrived in agricultural settings due to extended germination patterns and aggressive growth habits (Harrison et al. 2001). Crop losses due to uncontrolled giant ragweed populations can be severe (Fickett et al. 2013a, b).

The introduction and widespread adoption of GRC has dominated the farm landscape since the mid-1990's (Young 2006). Consequently, the intense selection pressure placed on the herbicide glyphosate has resulted in evolved herbicide resistance. Resistance to glyphosate in giant ragweed was first confirmed in Ohio in 2004 (Heap 2014). Since that time, glyphosate resistance has been confirmed in 12 states (Heap 2018). Once considered more of a problem weed across the central corn-belt, GR giant ragweed has been confirmed in Arkansas, Mississippi, and Tennessee (Norsworthy et al. 2011; Nandula et al. 2015; Norsworthy et al. 2010).

Recent studies have confirmed two phenotypes of giant ragweed exhibiting different responses to glyphosate. Brabham et al. (2011) described a GR accession from Indiana showing a rapid necrosis response. Mature leaves become necrotic 24 HAT with glyphosate while younger meristematic tissues show no symptoms and continue to grow

normally. This type of response might indicate the resistance mechanism can be related to glyphosate being held in mature tissues and therefore not translocating. The second phenotype from Wisconsin was described by Glettner and Stoltenberg (2015) as having a slow response to glyphosate. Investigations of this population did not show any differences in absorption and translocation of ^{14}C -glyphosate. Dose response studies revealed the target site was 4.5 to 5.4 times less sensitive in the slow response accession than a susceptible accession. However, at high rates of glyphosate (1,000-2,000 μM) the differential response of glyphosate was overcome, indicating a sensitive target site.

Studies designed to confirm glyphosate resistance mechanism test for the possibility of both target site and non-target site-based mechanisms. Dose response studies utilize titrated rates of glyphosate in treating both a resistant and susceptible population. The relationship when comparing resistant to susceptible accessions for both ED_{50} and LD_{50} rates of glyphosate characterizes the level of resistance and therefore the levels of sensitivity in the 5-enolpyruvylshikimate-3-phosphate (EPSP) target site (Glettner and Stoltenberg 2015). Glyphosate's mode of action is the inhibition of the enzyme EPSPS. This essentially blocks a required step in the aromatic amino acid synthesis pathway (Mueller et al. 2003). The result is higher accumulations of shikimic acid in susceptible plants (Pline et al. 2002). Studies designed to measure shikimic acid accumulations in glyphosate-treated susceptible and resistant plants can characterize the sensitivity level of the EPSP enzyme. Sequencing of the conserved region of EPSPS containing the Pro106 location can be conducted to detect mutations in the target site (Wakelin and Preston 2006). Glyphosate is taken up through leaves and stems then translocated to the roots of treated plants (Ross 2009). Mechanisms that interfere with the

uptake and movement of glyphosate active to the target site are considered non-target site (Powles and Preston 2006). One method to evaluate reduced uptake and translocation of a herbicide such as glyphosate involves monitoring the movement of ^{14}C -radiolabeled glyphosate in both resistant and susceptible plants (Lorraine-Colwill et al. 2003).

The resistance mechanism to glyphosate in giant ragweed has yet to be determined (Sammons and Gaines 2014). However, a recent study confirmed glyphosate resistance in a giant ragweed accession from Mississippi (Nandula et al. 2015). Absorption patterns were similar for both resistant and susceptible biotypes. However, the susceptible biotype translocated more ^{14}C -glyphosate at 48 and 96 HAT of 71% and 77%, respectively, compared to 44% and 68% in the resistant biotype out of the treated leaf (Nandula et al. 2015). This data suggests glyphosate resistance in this particular giant ragweed biotype is related to reduced translocation.

The objectives of this study are to confirm GR and determine possible mechanism of glyphosate resistance in giant ragweed biotype from Mississippi.

2.3 Materials and Methods

2.3.1 Plant Material

A population of giant ragweed surviving multiple in-season applications of glyphosate was sampled in the fall of 2014. The population was collected near Aberdeen, MS (33°48'24" N, 88°34'39" W). A susceptible population was also collected from a non-crop area near Aberdeen, MS (33°47'49" N, 88°43'52" W). Fifteen to twenty plants were harvested from each site. Plants were allowed to dry naturally and seeds were collected and bulked from each population with populations kept separate. Seeds were stored at 4 C until needed. After removal from cold storage, seeds were placed in mesh

bags and buried in layers of damp sand. Containers of stratified seed were placed in an incubator at 10 C for eight to twelve weeks to alleviate dormancy. After incubation, seeds were removed and placed in lidded germination trays lined with damp filter paper and placed in a growth chamber and subjected to 30/20 C day/night temperatures with 16-hour photoperiod. Germinated seeds were transplanted into six celled trays (6 cm-diameter cells) containing a 2:1 ratio of Sun Gro[®] professional potting mix (Sun Gro[®] Horticulture) to sand and placed in greenhouse with 30/20 C day/night temperatures with 16-hour photoperiod. Trays were fertilized with Osmocote[®] Plus Smart-Release Plant Food 15-9-12 (The Scotts Company) and sub-irrigated as needed.

2.3.2 Shikimate Accumulation

Methods to quantify shikimate levels in giant ragweed were developed from Singh and Shaner (1998) and Mueller et al. (2003). Plants from both a glyphosate-susceptible (GS) (MS-S) and GR (MS-R) biotype were established as described above. Plants were allowed to reach the two to three-node growth stage (10-15 cm). Glyphosate was applied at 420 g ae ha⁻¹ with a stationary spray chamber with boom having two flat fan TeeJet 80067 nozzles delivering 94 L ha⁻¹. The formulation of glyphosate used in the study was Roundup PowerMax[®], containing the potassium salt of glyphosate. After treatment, plants were placed in a growth chamber with 30/20 C temperatures and 12-hour diurnal photoperiod and maintained with adequate water and nutrients until harvest timing was achieved. Plants were harvested above ground at 24, 48, and 120 HAT with glyphosate. An untreated control plant was included for each treatment. Three plants were harvested for each timing. Plant tissues were weighed at each timing to record biomass. All tissues were stored at -80 C after harvest until all harvest timings had been

completed. Samples were weighed and placed in 2 mL tubes containing four, 2.8-mm ceramic beads and processed for 2 minutes with Precellys® Evolution (BERTIN Corp. MD, USA) homogenizer at 6,708 g. HCL (0.25 N) was added to each sample at a ratio of 6 ml HCL per 1 g of plant tissue. Samples were incubated on a rotary shaker for 24 hours. Samples were removed and centrifuged at 6,708 g for 5 minutes. The aliquot was pipetted off the upper layer of each sample and filtered through a 0.2-µm nylon filter and into a 2 ml high pressure HPLC vial.

Analytical-grade shikimate was used to create a standard calibration curve (Fig. 2.1). Shikimate concentrations were recorded by injection of a 10 µl diluted sample into HPLC. HPLC containing photodiode array detector with wavelength setting at 212 nm. Column was 250 by 4.6 mm. Mobile phase was 0.1% formic acid with a flow rate of 0.3 ml min⁻¹. Run time was 5 minutes, where shikimate was recorded at 3.5 minutes.

Data for each biotype by timing were subjected to separate ANOVA. Mean comparisons were made using Fisher's protected LSD test at $\alpha \leq 0.05$.

2.3.3 Absorption and Translocation of ¹⁴C-glyphosate

MS-R and MS-S giant ragweed plants at the 4-node growth stage (15 cm) were treated with glyphosate at 840 g ae ha⁻¹ with a stationary spray chamber. The spray boom was equipped with two flat fan TeeJet 800067 nozzles calibrated to deliver 94 L ha⁻¹. The third fully expanded leaf was covered with aluminum foil. A solution was prepared that consisted of glyphosate at a concentration equivalent to 840 g ae ha⁻¹ in 140 L using ¹⁴C-glyphosate (¹⁴C-phosphonomethyl-labeled glyphosate with specific activity of 2.0 GBq mmol⁻¹ in an aqueous stock solution of 7.4 MBq mL⁻¹, American Radiolabeled Chemicals, Inc. St. Louis, MO USA) and distilled water. The aluminum foil was removed

and a 10 μ l volume sample of the solution containing 5 kBq of ^{14}C -glyphosate was applied to the upper surface of the unsprayed leaf of each plant using a micro applicator. After treatment, plants were placed in a growth chamber with 30/20 C temperatures and 12-hour diurnal photoperiod until harvest. Plants were harvested above ground at 24, 48, and 120 HAT with glyphosate. At each harvest timing, the treated leaf was removed and placed in a glass vial containing 10 mL of 10% methanol and shaken for 20 seconds to remove unabsorbed ^{14}C -glyphosate from the leaf surface. This process was repeated with an additional 10 mL 10% methanol solution. Two 1-ml aliquots from each leaf rinse were mixed with 10 mL scintillation cocktail (Ecolume, ICN Costa Mesa, CA USA) to record non-absorbed ^{14}C -glyphosate.

Plants were harvested at each timing and separated into treated leaf (TL), leaf above treated leaf (ATL), leaf below treated leaf (BTL), and root crown (RC). Harvested plant parts were wrapped in a single tissue paper (Kimwipes, Kimberly-Clark Corp. Roswell, GA USA). Samples were then placed in glass vials and oven dried at 60 C for 24 hours. Dried plant samples were combusted in a biological oxidizer (Packard Instruments Company, Downers Grove, IL) and the $^{14}\text{CO}_2$ was collected in 10 mL Carbosorb E (Packard BioScience Company, Meridan, CT USA) and 10 mL Permafluor E+ (Packard BioScience). Radioactivity from leaf rinsates and oxidations was quantified utilizing liquid scintillation analyzer (Tri-Carb 2900TR, PerkinElmer Life and Analytical Sciences, Shelton, CT USA). The average recovery of ^{14}C -glyphosate was 90% calculated as the sum of counts per minute (CPM) for all plant parts, absorption (expressed as percent of recovered ^{14}C), and leaf rinses. Total radioactivity collected in all plant parts except treated leaf was designated as translocated ^{14}C and presented as

percentage of absorbed. There were three replicates per harvest timing and biotype and experiment was conducted once.

Data for each biotype by timing were subjected to separate ANOVA. Mean comparisons were made using Fisher's protected LSD test at $\alpha \leq 0.05$.

2.3.4 EPSPS Sequence Analysis

2.3.4.1 Ribonucleic Acid (RNA) Isolation

Establishment of plant materials for each biotype was described previously. Leaf tissues were excised from four plants each of MS-R and MS-S three-node growth stage giant ragweed plants for RNA extraction. TRIZOL RNA isolation protocol was followed (Chomczynski and Sacchin 1987). Leaf tissue (0.125 g) was placed in 2 mL microtube (Qiagen) containing four 2.8-mm ceramic beads. The samples were processed for 2 minutes with Precellys[®] Evolution (BERTIN Corp. MD, USA) homogenizer at 6,708 g. Samples were incubated for five minutes at room temperature. TRIZOL (Invitrogen) (500 μ l) was added to each tube and mixed by inverting. Samples were incubated again for five minutes at room temperature. After incubation, an additional 500 μ l of TRIZOL was added to each tube and centrifuged at 9,660 g for ten minutes. Supernatant was transferred to a new sterile 2 mL tube and 200 μ l of chloroform was added to each tube. Samples were vortexed for 15 seconds and incubated for 2-3 minutes at room temperature then centrifuged at 9,660 g for 15 minutes. Supernatant was transferred to a new 2 mL tube and 500 μ l of isopropanol was added and mixed by inverting tubes. Samples were incubated for ten minutes at room temperature then centrifuged at 9,660 g for ten minutes. Supernatant was discarded and tubes inverted on a paper towel and allowed to dry. Ethanol (75%) was added at a volume of 500 μ l to tubes and vortexed

briefly to dislodge the RNA pellet. Samples were centrifuged at 6,708 g for five minutes. Supernatant was discarded and tubes were inverted and allowed to air-dry for ten minutes. RNA pellet was dissolved in 25 µl of DPEC-treated water (Ambion) by passing solution several times through a pipette. RNA was quantified using NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies). DNase treatment contained the following: 1 µg of RNA sample, 1 µl 10X DNase reaction buffer, 1µl DNase (ampl. grade), and DPEC-treated water was added to bring the total volume to 10 µl and then placed the DNase sample in a RNase-free 0.5 mL polymerase chain reaction (PCR) tube. Samples were incubated for fifteen minutes at room temperature. DNase sample was inactivated by adding 1 µl of 25 mM EDTA and incubated for ten minutes at 65°C.

2.3.4.2 Reverse Transcription/cDNA Synthesis

Promega protocol was used for this procedure. Reverse transcription sample was prepared by adding the following: 4 µl of MgCl₂ 25mM, 2 µl reverse transcription 10X buffer, 2 µl dNTP mixture 10 mM, 0.5 µl recombinant RNasin® ribonuclease inhibitor, 1 µl AMV reverse transcriptase, Oligo (dT)₁₅ primer, 1 µg RNA sample, and DPEC-treated water was added to bring total volume to 20 µl in a RNase-free 0.5 mL PCR tube. Samples were incubated at 42 C for fifteen minutes, at 95 C for five minutes, and at -4 C for five minutes. After incubation, samples were stored at -20 C. cDNA samples were diluted with DPEC-treated water to 100 µl total volume, and quantified using NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies).

2.3.4.3 PCR Reaction

Primer sequences used were EPSPS_AtFt-5' ACATGCTTGGGGCTCAAGAA 3' and EPSPS_AtRt-5' TTGAATTACCAGCAGCGGT 3' (Nandula et al. 2015). PCR 25 µl reactions were prepared in RNase-free 0.5 mL PCR tubes containing 12.5 µl Taq 2X Master Mix DNA polymerase, 1 µl forward primer, 1 µl reverse primer, 2 µl complimentary DNA (cDNA) (100 ng µl⁻¹), and 8.5 µl deionized water. PCR profile starts with 94 C for 1 minute followed by 30 cycles denaturation at 94 C for 30 seconds, annealing at 56 C for 30 seconds, and extension at 72 C for 1 minute. A final extension at 72 C for 5 minutes completed the reaction. PCR products were electrophoresed to test for quality.

2.3.4.4 PCR Product Preparation

Wizard® SV Gel and PCR Clean-Up System (Promega Corp.) was used for this process. Membrane binding solution (25 µl) was added to the PCR product. PCR product was transferred into a sterile SV mini-column assembly and incubated for one minute at room temperature. After incubation, samples were centrifuged at 9,660 g for one minute. Flow-through was discarded and the mini-column was reinserted into the collection tube, and 700 µl of membrane wash solution was added to the collection tube. Samples were centrifuged at 9,660 g for one minute. Flow-through was discarded and the mini-column was reinserted into a new collection tube. This step was repeated with 500 µl of membrane wash solution and centrifuged at 9,660 g for five minutes. Mini-column was transferred to a sterile 1.5 mL tube and 25 µl of DPEC-treated water was added to the mini-column. Samples were incubated at room temperature for one minute then

centrifuged at 9,660 g for a minute. Finally, the mini-column was discarded and processed samples were stored at -20 C until ready for sequencing.

2.3.4.5 Sequencing

Purified PCR products were quantified using NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies). Quantifications revealed all samples were within the desired concentration range of Bp 10-20 ng μl^{-1} . An 8 μl sample of purified PCR product was combined with 4 μl each of Forward and Reverse primers for a total volume of 12 μl and placed into bar coded microtubes. Samples were then sequenced by Eurofins Genomics, Louisville KY. EPSPS sequence results were analyzed using software to test for the presence or absence of mutations of target site in MS-R biotype.

2.4 Results and Discussion

2.4.1 Shikimate Accumulation

The untreated plants accumulated low levels of shikimate. Shikimate accumulations in control plants did not exceed 48 $\mu\text{g g}^{-1}$ fresh weight (Data not shown). MS-S biotype had higher shikimate accumulations than the MS-R biotype resulting from an application of glyphosate (420 g ae ha^{-1}) at 1, 2, and 5 days after treatment (DAT) (Table 2.1). A similar observation was made in sunflower (*Helianthus annuus* L.) treated with 420 g ae ha^{-1} of glyphosate, where shikimate accumulation increased in the plant until 7 DAT (Henry et al. 2007). Shikimate accumulation at 1, 2, and 5 DAT were 4.4, 5.3, and 4.9, respectively, times higher in MS-S biotype when compared to MS-R biotype. Norsworthy et al. (2010) recorded similar results from Tennessee with

susceptible giant ragweed plants exhibiting 3.3- and 3.8-fold higher shikimate at 1 and 3 DAT, respectively, in comparison to resistant plants.

The largest difference in shikimate levels between the MS-R and MS-S biotypes occurred at 2 DAT. Fresh weight of the susceptible biotype ($2,242 \mu\text{g g}^{-1}$) was 5.3 times higher than resistant biotype ($425.9 \mu\text{g g}^{-1}$). Mueller et al. (2003) documented similar levels of shikimate accumulations in GR horseweed (*Conyza canadensis* L.) ranging from 2,000-2,500 $\mu\text{g g}^{-1}$ at 2 DAT. These results indicate that a simple bioassay can be utilized to confirm glyphosate resistance in suspect biotypes.

2.4.2 Absorption and Translocation of ^{14}C -glyphosate

The total recovery of ^{14}C was greater than 90%. Absorption was higher in the MS-S biotype at both 24 and 48 HAT; however, by 120 HAT there was no difference in absorption between MS-S and MS-R giant ragweed biotypes (Fig. 2.2). This suggests that the resistance mechanism may not be related to reduced absorption. The amount of radioactivity translocated out of the treated leaf was lower in MS-R biotype at each timing. Overall translocation for MS-R biotype at 24, 48 and 120 HAT was 27, 39 and 12%, respectively, compared to 93, 99, and 77%, respectively for MS-S biotype. Nandula et al. (2015) showed similar results with susceptible giant ragweed biotype absorbing more ^{14}C -glyphosate than resistant biotype. Results of 48 and 96 HAT absorption were 71 and 76%, respectively for the resistant biotype, compared to 44 and 66%, respectively for the susceptible biotype. The distribution of ^{14}C -glyphosate in MS-R and MS-S giant ragweed biotypes is summarized in Table 2.2. ^{14}C -glyphosate levels retained in the treated leaf were higher for MS-R biotype at each timing. There were no differences in ^{14}C -glyphosate accumulation above the treated leaf (ATL) for either biotype. Higher ^{14}C -

glyphosate levels were detected below the treated leaf (BTL) at 24 and 48 HAT for MS-S (89 and 96%, respectively) compared to MS-R biotype (7 and 4%, respectively). MS-S had the highest ^{14}C -glyphosate accumulation in root crown (RC) (56%) compared to MS-R (7%). Distribution of radioactivity indicates that the majority of ^{14}C -glyphosate was retained in the TL and therefore failed to translocate to other plant parts in MS-R biotype. Koger and Reddy (2005) recorded reduced translocation of ^{14}C -glyphosate in resistant biotypes of horseweed when compared to susceptible biotypes. The MS-S biotype showed translocation progressing across timings from the TL to BTL and finally to RC. Glyphosate should translocate in this manner in susceptible plants (Ross 2009). These findings suggest that the glyphosate resistance mechanism in this particular MS-R biotype appears to be related to reduced translocation of glyphosate.

2.4.3 EPSPS Sequencing

Sequencing was conducted on the conserved region of EPSPS which contains the Pro106 location from four plants each of MS-R and MS-S giant ragweed biotypes. The results from sequencing confirmed that there was no mutation present at the Pro106 location (Figure 2.3). These findings indicate that the mechanism conferring glyphosate resistance in this MS-R giant ragweed biotype is not due to a point mutation. Nandula et al. (2015) generated similar results in a GR giant ragweed biotype from Tunica County.

Shikimate accumulations provided an effective means to confirm glyphosate resistance in a giant ragweed population from Monroe County, Mississippi. This particular biotype was found to exhibit a reduction in translocation of ^{14}C -glyphosate. Horseweed [*Conyza canadensis* (L.) Cronquist] which confers glyphosate resistance due to reduced translocation has been found to rapidly sequester glyphosate into vacuoles,

reducing the amount of glyphosate available to enter the phloem of treated plants (Ge et al. 2010). Future research should be conducted to investigate the possibility of vacuolar sequestration in GR giant ragweed. Properly elucidating the mechanism of glyphosate resistance can be helpful for managers to better understand the rate of inheritance of resistant trait in a given population of resistant weeds. Confirmation of glyphosate resistance in a Monroe County accession of giant ragweed indicates its expansion into the eastern portion of Mississippi. Growers will need to consider non-glyphosate herbicides and systems when considering chemical control options of giant ragweed to avoid control failures and further selection for glyphosate resistance. Weed control systems will need to be more diverse by incorporating the rotation of herbicide chemistries, maintaining effective herbicide rates, utilizing multiple effective modes of action, and adopting non-herbicide weed control methods such as tillage and crop rotation. Growers who do encounter GR giant ragweed, must focus on limiting late season escapes which can then contribute to the soil seedbank and sustained weed competition.

Figure 2.1 Shikimate standard calibration curve.

^a Abbreviations: mAu, milli-Absorbance units; min, minute.

Table 2.1 Shikimate accumulation in glyphosate-susceptible and glyphosate-resistant giant ragweed biotypes.

Shikimate at DAT with Glyphosate ^{a, b, c}			
Biotype	1 DAT	2 DAT	5 DAT
	$\mu\text{g g}^{-1}$ fresh wt		
Susceptible	1770.4 a	2242 a	2299.5 a
Resistant	396.6 b	425.9 b	467.8 b

^a Means followed by the same letter within a column are not different according to Fisher's Protected LSD at $P < 0.05$.

^b Glyphosate was applied at 840 g ae ha^{-1}

^c Abbreviation: DAT, sampling date, days after treatment.

Figure 2.2 Absorption and translocation of ^{14}C -glyphosate in glyphosate-susceptible and glyphosate-resistant giant ragweed biotypes.^{a, b}

^a Abbreviations: R, resistant; S, susceptible; TL, treated leaf; AL, above treated leaf; BL, below treated leaf; RC, root crown; h, hours after treatment.

^b Means within bars followed by the same lowercase letters are not significantly different based on Fisher's Protected LSD test at $\alpha = 0.05$.

Table 2.2 Distribution of ^{14}C -glyphosate in glyphosate-resistant and glyphosate-susceptible giant ragweed biotypes at 24, 48, and 120 hours after treatment.

	Hours after treatment											
	24	48	120	24	48	120	24	48	120	24	48	120
	% Absorbed											
	TL ^a			ATL			BTL			RC		
Biotype												
MS-R	73	61	88	11	4	3	7	4	2	9	10	7
MS-S	7	1	23	4	1	9	89	96	11	1	2	56
LSD (.05)	S	S	S	NS	NS	NS	S	S	NS	NS	NS	S

^a Abbreviations: MS-R, glyphosate-resistant; MS-S, glyphosate-susceptible; S, significant; NS, not significant; TL, treated leaf; ATL, above treated leaf; BTL, below treated leaf; RC, root crown.

Figure 2.3 Alignment of conserved region of EPSPS from glyphosate-resistant and glyphosate-susceptible giant ragweed biotypes. The codons highlighted with box reveal no mutation at the Pro106 position of EPSPS.^a

^a Abbreviations: EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; GR, glyphosate-resistant; GS, glyphosate-susceptible; MS, Mississippi

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CHAPTER III
CONFIRMATION AND CONTROL OF GLYPHOSATE-RESISTANT GIANT
RAGWEED (*Ambrosia trifida* L.) IN MISSISSIPPI

3.1 Abstract

Glyphosate-resistant (GR) giant ragweed (*Ambrosia trifida* L.) in Mississippi was reported in 2015. The use of herbicides to control resistant populations of giant ragweed become more difficult due to evolved herbicide resistance to multiple classes of herbicides including glyphosate and acetolactate synthase (ALS)-inhibiting compounds. Previous studies have confirmed improved levels of control when utilizing both pre-emergence (PRE) and post-emergence (POST) herbicide applications in a system. A study was conducted to confirm and characterize glyphosate resistance in a suspect population from Mississippi and to evaluate the efficacy of PRE and POST applied herbicides to control giant ragweed. Dose response studies revealed that the ED₅₀ for Mississippi GR (MS-R) and Tennessee GR (TN-R) biotypes was 3.9- and 6.3-fold higher than glyphosate-susceptible (GS) (MS-S) biotype. A fallow field study was conducted in 2016 to evaluate the control of GR giant ragweed in Monroe County, Mississippi. PRE and POST herbicide treatments containing dicamba and mesotrione alone and in various combinations were evaluated. At three weeks after treatment (WAT), PRE treatments of mesotrione, dicamba, S-metolachlor + metribuzin + mesotrione, and dicamba + mesotrione provided 77, 86, 86, and 90% control, respectively. At four WAT, POST

treatments of mesotrione, fomesafen, mesotrione + glyphosate, fomesafen + dicamba, dicamba, and fomesafen + dicamba + glyphosate provided 56, 60, 86, 98, 99, and 100% control, respectively. The highest levels of control were achieved with treatments containing multiple modes of action. These results confirm that dicamba and mesotrione are effective options for designing comprehensive herbicide programs to manage GR giant ragweed.

3.2 Introduction

Giant ragweed is an annual broadleaf weed and is a member of the Asteraceae family. A native of North America, giant ragweed is widely distributed and can be found in non-crop areas along streams and riparian areas (Bassett and Crompton 1982). In agricultural settings, giant ragweed can be introduced to fields from surrounding roadsides, hedgerows, fencerows, and drainage ditches (Baysinger and Sims 1991). Giant ragweed has become more prevalent in crop production due to its early and sustained emergence as well as its aggressive growth habit (Abul-Fatih and Bazzaz 1979a).

Responses from a recent survey of certified crop advisors from the U.S. Corn Belt and Ontario, Canada placed giant ragweed as one of the most difficult weeds to manage in 45% of counties responding (Regnier et al. 2016). There are a number of factors which have contributed to the rise in prevalence of giant ragweed. One noticeable change is a longer emergence pattern in giant ragweed rising from disturbed lands such as agricultural fields (Hartnett et al. 1987). Tillage practices can have a varied effect on giant ragweed emergence. While shallow tillage can promote emergence by placing seeds at optimum depths (0.5-2 cm), deep tillage or subsequent tillage can bury seeds at depths up to 20 cm that would reduce emergence (Harrison et al. 2007). However, the shift to

GR crops (GRC) also increased the amount of no-till acreage (Horowitz 2010).

Reduction in tillage would eliminate seed burial and expose seeds to predation (Harrison et al. 2001) and also encourage the presence of earthworms (*Lumbricus terrestris* L.) (Schutte et al. 2010). Regnier et al. (2008) recorded earthworms facilitating giant ragweed establishment due to burial of seed in their burrows. Another factor related to the widespread adoption of GRC was the shift or reliance in many cases on a total POST system (Young 2006). This coupled with prolonged emergence has selected for late-emerging giant ragweed ecotypes (Schutte et al. 2012).

Another factor influencing the management of giant ragweed with herbicides is evolved herbicide resistance. Giant ragweed accessions have confirmed herbicide resistance to glyphosate and ALS herbicides (Heap 2018). Options for developing herbicide systems to provide acceptable weed control among resistant populations of giant ragweed have become limited and crop losses due to uncontrolled giant ragweed can be devastating (Fickett et al. 2013 a, b). Recent research has focused on the efficacy of dicamba in response to dicamba-resistant (DR) cultivars of soybean [*Glycine max* (L.) Merr.] and cotton (*Gossypium hirsutum* L.) becoming available. Spaunhorst et al. (2014) found the addition of dicamba to glyphosate in DR soybean, regardless of POST timing, increased levels of giant ragweed control over glyphosate alone. Treatments containing dicamba provided in excess of 90% control of GR giant ragweed. In the same study, sequential applications of dicamba plus glyphosate reduced waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] density 90%, while glyphosate alone reduced waterhemp density 42%. In response to the increasing levels of resistance to multiple classes of herbicides in numerous weed species, seed companies have developed cultivars

containing multiple herbicide traits (Green and Castle 2010). These traits include tolerance to glyphosate, glufosinate, 2,4-D, dicamba, and 4-hydroxyphenyl-pyruvate dioxygenase (HPPD) herbicides in soybean and cotton cultivars (Green 2014). There are currently no confirmed cases of evolved herbicide resistance to either HPPD inhibitors or the synthetic auxins in giant ragweed (Heap 2018).

The objective of this study will be to confirm and characterize glyphosate resistance in a suspect population of giant ragweed from Mississippi and Tennessee, and to evaluate the efficacy of herbicide treatments including mesotrione and dicamba in controlling GR giant ragweed under fallow field conditions.

3.3 Materials and Methods

3.3.1 Greenhouse Dose Response

Seedlings at the two-leaf growth stage were transplanted from the greenhouse into larger pots (10 cm diameter). Six plants from each of the following biotypes: MS-S, Hemphill (MS-R), and TN-R were treated at the four-node growth stage (15 to 20 cm tall) with glyphosate + nonionic surfactant (NIS) 0.25% (v/v) at each of the following titration rates: 52, 105, 210, 420, 840, 1680, 3360, and 6720 g ae ha⁻¹. The lowest rate represents 1/16 of the recommended rate of glyphosate and the highest rate being eight times the recommended rate. The formulation of glyphosate used in the study was Roundup PowerMax[®], containing the potassium salt of glyphosate. A control of water plus NIS only was included for each treatment. The treatments were completely randomized with six replicates per treatment and experiment was conducted twice.

Applications were made in a stationary spray chamber containing a boom equipped with two flat fan TeeJet 800067 nozzles calibrated to deliver 94 L ha⁻¹. Treated

plants were returned to the greenhouse after treatment and supplied adequate water and nutrients for 28 days. Results were recorded after 28 days. Live plants were recorded if they had any green tissue 28 days after treatment (DAT). Plants were harvested, force-air dried at 30 C for 48 hours, and weighed. The effective dose required to reduce dry shoot biomass 50% (ED₅₀) relative to untreated plants and lethal dose or rate required to kill 50% (LD₅₀) for each treatment were determined using PROC PROBIT in SAS. Confidence intervals of 95% were utilized to determine if biotypes responded differently to titrated rates of glyphosate.

3.3.2 Field Evaluations of Dicamba and Mesotrione Systems

A field site was selected near Aberdeen, MS (33°48'23" N, 88°34'42" W) with consistent native populations of giant ragweed having survived glyphosate applications. This site had a soil type classified as a Houlka-Leeper-West Point series prairie bottomland soil. The site was planted to GR soybean in the summer of 2015 and had surviving giant ragweed plants at harvest. Soybean was harvested in fall of 2015 and no tillage was conducted prior to this field evaluation. Sources of materials and herbicide rates used in the experiment are listed in Table 3.1.

The experimental design was a randomized complete block with four replications conducted as a bare ground study. The herbicide treatments, timings, and rates are listed in Table 3.2 and 3.3. Herbicide treatments were applied PRE and post emergence (POST) as separate trials. The trial area received a nonselective burndown herbicide prior to application of PRE treatments. The POST trial area received no burndown prior to applications. Individual plot size was 3.8 m wide by 7.6 m long and the middle 1.9 m by 7.6 m area was treated. Herbicide treatments were applied using a hand-held boom and

backpack CO₂ pressurized sprayer. This system was calibrated to deliver 102 L ha⁻¹ at a pressure of 275 kPa using TeeJet DG 11002 flat fan nozzles.

Weed densities were determined by counting the number of giant ragweed plants in a 1 m² quadrant for each replication prior to POST applications. POST applications were made to 15-20 cm giant ragweed. Weed control evaluations were made at 21 DAT for PRE treatments, and at 14, 28, and 42 DAT for POST treatments and stated as percent control as compared to the untreated control at each replication. This study was to be conducted in the spring of 2015 and again in 2016. However, weed densities were not sufficient to conduct a trial in 2015. The PRE trial was conducted once whereas POST trial was conducted twice in 2016 at the same site. Data from POST trial was pooled for analysis.

3.4 Results and Discussion

3.4.1 Greenhouse Dose Response

ED₅₀ values were 3.9- and 6.3-fold greater in Hemphill (MS-R) and TN-R, respectively (386 g ae ha⁻¹; 625 g ae ha⁻¹), than MS-S (100 g ae ha⁻¹) (Fig. 3.1). Glyphosate resistance levels have been recorded in giant ragweed from Tennessee, Arkansas, and Mississippi of 5.3-, 2.3/7.2-, and 1.5-fold resistant, respectively when compared to susceptible biotypes (Norsworthy et al. 2010; Norsworthy et al. 2011; Nandula et al. 2015). LD₅₀ values for resistant biotypes were 1.16 and 1.35 times the recommended use rate for glyphosate (Fig. 3.2). These results confirm glyphosate resistance in Mississippi and Tennessee biotypes. As glyphosate resistance continues to expand in giant ragweed populations, managers will be forced to develop more

comprehensive weed management plans to provide acceptable control and reduce further selection of glyphosate resistance.

3.4.2 Field Evaluations of Dicamba and Mesotrione Systems

3.4.2.1 PRE Trials

Treatment effects were recorded among PRE herbicide treatments. Giant ragweed control ranged from 32 to 90% three WAT (Table 3.2). Metribuzin provided the lowest control level of 32%, while mesotrione + dicamba produced the highest control level of 90%. The following treatments: dicamba, S-metolachlor + metribuzin + mesotrione, and dicamba + mesotrione provided 86, 86, and 90% control, respectively. Treatments including mesotrione, S-metolachlor + metribuzin, and S-metolachlor + mesotrione provided lower levels of 78, 63, 63% control, respectively. Treatments containing dicamba and the combination of metribuzin (PS II inhibitor) with mesotrione (HPPD inhibitor) provided excellent control of giant ragweed. Synergistic effects of combining a PS II with an HPPD inhibitor herbicide have been documented in redroot pigweed (Hugie et al. 2008). As soybean cultivars become available with herbicide traits that will enable the use of dicamba and HPPD inhibitors such as mesotrione, these data suggest several effective PRE treatment options to manage GR giant ragweed.

3.4.2.2 POST Trials

Pre-treatment counts ranged from 12 to 540 giant ragweed plants per 1 m² quadrant. Sufficient densities of giant ragweed were present to evaluate herbicide treatments. Glyphosate applications were not effective as control levels did not exceed 18%, further confirming the resistance levels of giant ragweed present at this site. Control

levels at 14 DAT ranged from 3 to 56% (Table 3.3). Dicamba treatments did provide the highest levels of control at 14 DAT. All treatments that contained dicamba controlled giant ragweed more than 90% at 28 DAT, with mesotrione + glyphosate controlling 86%. The increase in control levels of dicamba treatments from 14 DAT to 28 DAT is an indicator that the mode of action of dicamba is slow to develop and requires more than 14 days to provide adequate control levels. Similar levels of control were recorded at 42 DAT. Spaunhorst et al. (2014) recorded control of giant ragweed increased to more than 90% when dicamba was added to glyphosate treatments in DR soybean. Soltani et al. (2011) found dicamba treatments to be the most consistent when evaluating PRE treatments in corn, control levels containing dicamba PRE were from 60 to 83%. Treatments containing fomesafen did not exceed 60% control. Giant ragweed plants averaged 15 to 20 cm at the time of application. However, plants ranged from just emerged to 30 cm. Effective control of giant ragweed with PPO herbicides is size-dependent. In one study, six-node giant ragweed control was 66% with carfentrazone or fomesafen (Norsworthy et al. 2010). There was no change in giant ragweed control from 28 to 42 DAT evaluation for most treatments. However, mesotrione alone did show a slight increase in control. This could be attributed to soil activity reducing additional emergence of giant ragweed. Results from this trial did identify that dicamba is an effective POST option for control of GR giant ragweed. Glyphosate + mesotrione also provided effective control levels. This confirms the ability to utilize dicamba and mesotrione in herbicide systems to manage GR giant ragweed. Newly developed cultivars with dicamba and HPPD resistance will provide managers another tool to manage

difficult weeds including those exhibiting evolved herbicide resistance to multiple herbicide classes.

In summary, GR populations of giant ragweed continue to expand. However, results from these field trials suggest several effective PRE and POST herbicide treatments for control of GR giant ragweed. PRE treatments containing either dicamba or mesotrione + metribuzin provided excellent control. POST treatments of glyphosate alone did not provide acceptable levels of control. However, POST treatments containing dicamba alone or in tank mixes with fomesafen or glyphosate provided greater than 90% control. Although this trial did not provide PRE followed by POST treatments, the value of PRE and POST treatments evaluated in this trial could be conceptualized in a two-pass system. Additional value can be realized from treatments such as mesotrione that have both contact and residual control when applied POST. This would also allow producers to incorporate multiple effective modes of action when selecting herbicides that have more than one mode of action as a formulated premix or as a tank mix partner. This strategy also reduces late-season escapes that not only contribute to the soil seedbank but also can select for later emerging ecotypes. These findings suggest that the use of dicamba in DR crops will provide growers with a tool for managing herbicide-resistant weeds. As HPPD-resistant cultivars of soybean and cotton become available, the use of herbicides such as mesotrione will also be a viable option for weed management systems. However, for weed control systems to be sustainable a combination of residual herbicides will be required in support of herbicide-tolerant crops to reduce selection pressure from herbicides as well as incorporating various cultural practices.

Figure 3.1 Biomass reduction in response to glyphosate rate in glyphosate-susceptible and glyphosate-resistant giant ragweed biotypes 28 DAT.^{a, b}

^a Abbreviation: DAT, days after treatment; MS (S), Mississippi susceptible; TN, Tennessee

^b ED₅₀ denoted by black line.

Figure 3.2 % Mortality in response to glyphosate rate in glyphosate-susceptible and glyphosate-resistant giant ragweed biotypes 28 DAT.^{a, b}

^a Abbreviation: DAT, days after treatment; MS (S), Mississippi susceptible; TN, Tennessee.

^b LD₅₀ denoted by black line.

Table 3.1 Source of the materials used in the experiments.

Common Name	Trade Name	Rate ^{a, b}	Manufacturer
		kg ai or ae ha ⁻¹	
dicamba	Clarity	0.56	BASF Corporation, RTP, NC; www.basf.com
mesotrione	Callisto	0.105-0.175	Syngenta Crop Protection, LLC., Greensboro, NC; www.syngenta.com
metribuzin	Tricor	0.3175	United Phosphorous, Inc., King of Prussia, PA; www.upi-usa.com
S-metolachlor	Dual Magnum	1.06	Syngenta Crop Protection, LLC., Greensboro, NC; www.syngenta.com
glyphosate	Roundup Powermax	1.05	Monsanto Corporation, St. Louis, MO; www.monsanto.com
fomesafen	Flexstar	0.42	Syngenta Crop Protection, LLC., Greensboro, NC; www.syngenta.com

^aAbbreviations: ai, active ingredient; ae, acid equivalent.

^bActive ingredient rate used for mesotrione, metribuzin, S-metolachlor, and fomesafen. Acid equivalent rate used for dicamba and glyphosate.

Table 3.2 Control of glyphosate-resistant giant ragweed three weeks after PRE treatments.

Treatment	Timing ^a	Rate ^b	Control ^c
		kg ai or ae ha ⁻¹	%
dicamba	PRE	0.56	86
mesotrione	PRE	0.175	78
metribuzin	PRE	0.3175	33
dicamba + mesotrione	PRE	0.56 + 0.175	90
S-metolachlor + metribuzin	PRE	1.06 + 0.3175	63
S-metolachlor + metribuzin + mesotrione	PRE	1.06 + 0.3175 + 0.175	86
S-metolachlor + mesotrione	PRE	1.06 + 0.175	63
LSD (.05)			15.7

^a Abbreviations: PRE, pre-emergence (references weed emergence); ai, active ingredient, ae, acid equivalent.

^b Active ingredient rate used for mesotrione, metribuzin, and S-metolachlor. Acid equivalent rate used for dicamba and glyphosate.

^c Giant ragweed control rated using a scale of 0 to 100 (0 = no injury and 100 = plant death).

Table 3.3 Control of glyphosate-resistant giant ragweed 14, 28, and 42 DAT with POST treatments.

Treatment ^a	Timing ^b	Rate ^c	14 DAT ^d	28 DAT	42 DAT
		kg ai or ae ha ⁻¹		% Control	
glyphosate	POST	1.05	3	7	18
mesotrione	POST	0.105	15	57	65
dicamba	POST	0.56	55	100	100
fomesafen	POST	0.42	27	60	57
fomesafen + dicamba + glyphosate	POST	0.42 + 0.56 + 1.05	34	100	100
fomesafen + dicamba	POST	0.42 + 0.56	56	99	99
dicamba + glyphosate	POST	0.56 + 1.05	45	99	100
mesotrione + glyphosate	POST	0.105 + 1.05	30	86	79
fomesafen + glyphosate	POST	0.42 + 1.05	40	41	42
LSD (.10)			22.6	26.6	20.9

^a NIS (non-ionic surfactant) included with all treatments at 0.25 % (v/v).

^b Abbreviations: DAT, days after treatment; POST, post-emergence; ai, active ingredient; ae, acid equivalent.

^c Active ingredient rate used for fomesafen and mesotrione. Acid equivalent rate used for dicamba and glyphosate.

^d Giant ragweed control rated using a scale of 0 to 100 (0 = no injury and 100 = plant death).

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CHAPTER IV
FITNESS, PHENOTYPIC, AND GENETIC VARIATION IN GLYPHOSATE-
RESISTANT AND GLYPHOSATE-SUSCEPTIBLE
GIANT RAGWEED (*Ambrosia trifida* L.)

4.1 Abstract

Glyphosate-resistant (GR) giant ragweed (*Ambrosia trifida* L.) has been confirmed in several states across the mid-western U.S. and also in the mid-southern U.S. Large degrees of phenotypic variation have been recorded among giant ragweed populations including prolonged emergence, growth patterns, and susceptibility to herbicides. Previous fitness studies have shown a GR biotype from Indiana exhibited rapid early growth, early flowering, but lower fecundity when compared to susceptible accessions. These variations can be attributed to local adaptations or levels of genetic diversity. The ability to gain information of local and regional phenotypes, fitness, and genetic diversity in populations can be useful in understanding evolutionary shifts, population structure, distribution, and predictability of the development of herbicide resistance. A study was conducted to measure fitness, phenotypic, and genetic diversity of six GR and glyphosate-susceptible (GS) giant ragweed biotypes from Mississippi (MS), Tennessee (TN), and Ohio (OH). Non-destructive measurements of plants over an eight-week period showed rapid early growth of several Mississippi GR (MS-R) accessions in the absence of glyphosate. However, no differences in vegetative biomass

were observed after eight weeks. One exception was a GR biotype from Ohio (OH-R) which had lower measurements for both vegetative and reproductive traits. These results suggest lower production and earlier flowering in this case were due to the photoperiod sensitivity of northern accessions, as a GR biotype from Tennessee (TN-R) had intermediate values for several traits. Vegetative biomass and fecundity were similar among biotypes regardless of response to glyphosate. Therefore, glyphosate resistance among these biotypes did not carry a fitness penalty. A multivariate and PCA analysis of all biotypes by trait identified three groups or clusters. Groupings were identified by state of origin, as the MS biotypes shared many of the same traits. OH-R was separate and in its own group. Genetic diversity was measured using seven simple sequence repeat (SSR) markers to generate genetic diversity, Shannon's index, and genetic distance among and within six biotypes. The greatest diversity was among biotypes, at 0.5324. Genetic diversity within populations by state for MS, TN, and OH were 0.514, 0.502, and 0.525, respectively. Shannon's index showed similar trends and ranged from 0.806 to 0.852. Genetic diversity due to response to glyphosate was similar; resistant (0.515) and susceptible (0.508). Diversity was not reduced due to glyphosate resistance. Genetic distance matrix indicated MS-R Hemphill was the closest related to TN-R. In contrast, OH-R was the most dissimilar or unrelated to MS-R Hayes biotype. A dendrogram generated from the genetic distance matrix revealed three clusters of biotypes. MS-S was identified as separate and distinct from all resistant biotypes. These results revealed locally adapted ecotypes produced higher vegetative and reproductive biomass than northern U.S. accessions from TN and OH. Studies revealed phenotypic and genetic variation among and within GR and GS giant ragweed biotypes.

4.2 Introduction

Giant ragweed is an annual weed that prefers early succession and has become widely distributed across North America (Bassett and Crompton 1982). Early and sustained emergence pattern along with its aggressive growth habits make it one of the most competitive weeds among agricultural crops (Harrison et al. 2001). Substantial yield losses have been documented in important agronomic crops. Yield losses of 13% were reported in corn (*Zea mays* L.) with 1.7 giant ragweed plants per 10 m² and weeds emerging simultaneously with corn. Where giant ragweed densities increased to 14 giant ragweed plants per 10 m², corn yields were reduced 60% (Harrison et al. 2001). Soybean [*Glycine max* (L.) Merr.] yields were reduced 45 to 77%, respectively, with season long interference from giant ragweed densities of 1 plant per m² (Webster et al. 1994).

Management of giant ragweed is difficult due to prolonged emergence, diversity of seed sizes, and high levels of embryo dormancy (Schutte et al. 2008a; Abul-Fatih and Bazzaz 1979a; Schutte et al. 2012). Giant ragweed persists in both conventional tilled and no-till systems (Loux and Berry 1991). Giant ragweed has been recorded in no-till fields for 15 years after tillage ceased (Hartnett et al. 1987).

Chemical methods are still the primary means for controlling giant ragweed. However, evolved herbicide resistance has provided complications and limited herbicide options. The first reporting of a herbicide-resistant giant ragweed population occurred in Indiana with resistance to acetolactate synthase (ALS) herbicides in 1988. There have since been seven additional states reporting ALS-resistant giant ragweed (Heap 2014). The first case of glyphosate resistance in giant ragweed occurred in Ohio in 2004. To date, a total of 12 states have reported GR giant ragweed (Heap 2018). In the mid-

southern U.S. region, GR biotypes of giant ragweed from Arkansas, Mississippi, and Tennessee exhibited 7.2-, 1.5-, and 5.3-fold levels of resistance, respectively when compared to susceptible biotypes (Norsworthy et al. 2011; Nandula et al. 2015; Norsworthy et al. 2010) Giant ragweed populations from Minnesota, Missouri, and Ohio have demonstrated cross resistance to both ALS and glyphosate (Heap 2018). Cross resistance creates a serious issue when choosing management strategies and further restricts herbicide options to manage herbicide resistance.

There are a number of factors that impact the evolution of a resistance trait in the environment: the rate in which a resistance gene has mutated, initial level of resistance trait, fecundity, gene flow, and fitness (Jasieniuk et al. 1996). Knowledge of fitness penalties is crucial in predicting intensity and degree to which herbicide resistance will advance. The occurrence of fitness penalties associated with herbicide resistance would suggest that when selection pressure is removed, resistant individuals would eventually decline (Anderson et al. 1996, Holt and Thill 1994).

Fitness studies have been conducted on several species of GR weeds and results were varied. A resistant biotype of rigid ryegrass (*Lolium rigidum* Gaud.), grown in low densities of wheat, produced fewer but larger seeds than susceptible biotypes (Pederson et al. 2007). Vila-Aiub et al. (2014) recorded no differences in vegetative or reproductive biomass between susceptible and resistant biotypes of Palmer amaranth (*Amaranthus palmeri* S. Watson.) regardless of stand density. Under non-competitive conditions, resistant horseweed (*Conyza canadensis* L.) grew more rapidly and reached reproductive growth stages 25 days earlier than susceptible accessions. However, there were no differences in seed production (Shresta et al. 2010).

Brabham et al. (2011) designed studies to measure fitness in GR giant ragweed in the absence of glyphosate, as a field study, and in the presence of glyphosate, as a greenhouse study. Results from the field study indicated the resistant biotype exhibited more aggressive early growth. However, 50 days after planting there were no differences in height, shoot weight, or leaf area. Reproductively, the resistant biotype flowered earlier but produced 25% fewer seeds than the susceptible biotype. Plant materials for the greenhouse study were as follows: GR and GS collected from homozygous parents; two additional lines generated from field collected seeds from open pollinated GR and GS plants described as GR X Q and GS X Q (Q unknown pollen donor due to open pollination in field). Three- to five-node plants were treated with glyphosate at 840 g ae ha⁻¹ followed by 2,520 g ae ha⁻¹ 14 days later. An outcrossing rate between GR and GS of 31% was detected due to the fact that 61% of the offspring were tolerant to the 840 g ae ha⁻¹ of glyphosate. The second application of glyphosate at 2,520 g ae ha⁻¹ provided complete control of GS plants. However, several heterozygous and homozygous GR plants did survive to produce seed. These findings would indicate that in the presence of glyphosate the resistance trait would continue to exist and potentially spread in agricultural systems.

In a similar study conducted in Wisconsin on GR giant ragweed that did not exhibit the rapid necrosis response to glyphosate, results indicated there were no differences between susceptible and resistant biotypes through vegetative growth stages under non-competitive conditions in the absence of glyphosate. However, the resistant biotype exhibited higher fecundity with 812 seeds per plant compared to 425 seeds per plant from the sensitive biotype (Glettner and Stoltenberg 2015). These findings would

support the increase of the resistance trait even when selection by glyphosate is removed. Results from fitness studies have been varied. However, a clear understanding of fitness is critical when determining the dynamics of evolved herbicide resistance.

Due to the wide genetic variation among weed species, studies designed to measure genetic diversity have become more prevalent. Weeds by their nature have a higher probability of encountering individuals containing a resistant allele due to their genetic variation (Mangolin et al. 2012). Marker assisted studies can provide an effective method for documenting levels of diversity among and within weed biotypes. Molecular markers are used to detect levels of polymorphism. Polymorphism is a term used to describe multiple forms of a gene in an individual or within a given population. AFLP markers were used successfully to confirm high levels of polymorphism in foxtail millet [*Setaria italica* (L.) Beauv.] and green foxtail millet [*Setaria viridis* (L.) Beauv.] (d'Ennequin et al. 2000). In the study of millets, high degrees of variation were recorded among biotypes. However, little variation was found within biotypes due to the fact that millet is self-pollinating. Ray and Roy (2009) conducted genetic diversity studies of six *Amaranthus* species collected from eight different regions of the Indo-Gangetic Plain. Amaranths have become a desirable cultivated species in India due to biomass production, nutritional value, drought tolerance, and pest resistance (Stallknecht and Schultz-Schaeffer 1993). Varying environmental and site conditions in this region have caused the *Amaranthus* species to develop several different ecotypes. Levels of diversity were described using pair-wise genetic similarity values expressed on a scale from 0 to 1. The value of 1 would indicate the most genetically similar and 0 the least. Genetic similarity values among *Amaranthus* species ranged from 0.16 to 0.97, confirming

genetic diversity. Cluster analysis grouped populations from the same species. These findings can be useful when attempting to identify and preserve a desirable ecotype. Chandi et al. (2013) used AFLP markers to measure genetic diversity of Palmer amaranth. Eight Palmer amaranth populations from North Carolina (NC) and Georgia (GA) were collected for this study. Half of the populations were GR. Genetic similarity value was low at 0.34 for all individuals and populations revealing a high degree of diversity. The authors hypothesized that continued use of glyphosate in the resistant populations would result in lower diversity than susceptible populations. However, genetic similarity values when grouped by response to glyphosate were similar with 0.34 for resistant and 0.35 for susceptible. Overall, variation was most impacted by grouping within populations accounting for 77% of total variation. From a weed management perspective, these results might suggest that management practices could be effective across all species regardless of response to glyphosate or geographical location.

Currently, genetic diversity studies of giant ragweed are limited. However, a recent study conducted in France utilized SSR markers to measure genetic diversity in common ragweed (*Ambrosia artemisiifolia* L.). Comparisons were made between North American (native range) and European (invasive range) accessions. Mating system was found to be similar for both populations. High levels of within-population genetic diversity were recorded for both populations. Genetic structure patterns suggested that the European population evolved from two main introduction events with secondary colonizing events occurring later. Of the SSR markers utilized for this study, 40% were found to be transferable to giant ragweed and this species of *Ambrosia* was more phylogenetically distant to common ragweed than Cuban ragweed (*Ambrosia*

psilostachya DC.) or slimleaf bur ragweed (*Ambrosia tenuifolia* Spreng.) (Meyer et al. 2017).

The objectives of this study were to determine if glyphosate resistance carries a fitness penalty in giant ragweed, and to measure the phenotypic and genetic diversity among and within giant ragweed biotypes from Mississippi, Tennessee, and Ohio.

4.3 Materials and Methods

4.3.1 Plant Material

Three indigenous populations of giant ragweed having each survived multiple in-season applications of glyphosate were sampled in the fall of 2014. Two populations were located near Aberdeen, MS (Hayes; 33°49'04" N, 88°43'08" W and Younger; 33°48'24" N, 88°34'39" W). One population was collected near Slate Springs, MS (Hemphill; 33°45'09" N, 89°27'25" W). A susceptible (MS-S; 33°47'49" N, 88°43'52" W) population was also collected from a non-crop area near Aberdeen, MS. Fifteen to twenty plants were harvested from each site. Plants were allowed to dry naturally and seed collected and bulked from each population with populations kept separate. GR giant ragweed seeds from Tennessee and Ohio were obtained from university cooperators (University of Tennessee; Jackson, TN and Ohio State University; Columbus, OH). Seeds were stored at 4 C until needed. Seeds were placed in mesh bags and buried in layers of damp sand. Containers of stratified seed were placed in an incubator at 10 C for eight to twelve weeks to alleviate dormancy. After incubation, seeds were placed in lidded germination trays lined with damp filter paper and placed in a growth chamber and subjected to 30/20 C day/night temperatures with 16-hour photoperiod. Germinated seeds were transplanted into six celled trays (6 cm-diameter cells) containing a 2:1 ratio of Sun

Gro[®] professional potting mix (Sun Gro[®] Horticulture) and sand and placed in greenhouse with 30/20 C day/night temperatures with 16-hour photoperiod. Trays were fertilized with Osmocote[®] Plus Smart-Release Plant Food 15-9-12 (The Scotts Company) and sub-irrigated as needed.

4.3.2 Glyphosate Resistance Screening

Six cotyledon giant ragweed plants from each biotype were transplanted from the greenhouse into 10 cm diameter pots containing a 2:1 ratio of Sun Gro[®] Professional potting mix and sand. Pots were fertilized with Osmocote[®] Plus Smart-Release Plant Food 15-9-12 and sub-irrigated as needed. Giant ragweed plants (10-15 cm) were treated with 840 g ae ha⁻¹ of glyphosate. The formulation of glyphosate used in the study was Roundup PowerMax[®], containing the potassium salt of glyphosate.

Applications were made in a stationary spray chamber with a spray boom equipped with two flat fan TeeJet 800067 nozzles calibrated to deliver 94 L ha⁻¹. Treated plants were returned to the greenhouse after treatment and supplied adequate water and nutrients for 21 days. Results were recorded after 21 days. Live plants were recorded if they had any green tissue 21 days after treatment (DAT).

4.3.3 Biological Characterization

A biological characterization study was conducted in 2015 and 2016 at the R. R. Foil Plant Science Center in Starkville, MS (33°28'09" N, 88°47'00" W). Six untreated plants were selected from each of the MS biotypes from the procedure discussed above as well as the TN and OH biotypes. Individual seedlings were transplanted at the six-leaf growth stage into larger sub-irrigated planters (SIP) (Fig 4 .1.) SIPs were filled with Sun

Gro[®] Professional Mix and 453.6 g of 13-13-13 analysis granular fertilizer was placed around the inside rim of each SIP. The SIP was then covered with 6 mm plastic sheeting and secured with cable ties. An incision was made through the plastic sheeting to accept transplants. SIPs containing seedlings were placed outside in a randomized complete block design spaced 60 cm apart on a concrete slab and subjected to natural day/night temperatures. In 2015 and 2016, plants were transplanted on August 5 and July 22, respectively. The average day and night temperatures for July/August for R. R. Foil Plant Science Center in Starkville, MS were 32 C and 21 C, respectively (Anonymous 2018). A border was established around the perimeter of the six biotypes with giant ragweed plants in SIPs to eliminate edge effects. Plants were maintained with a drip watering system designed to water at a rate of 1.9 liters per hour for one hour twice each day for eight weeks.

The following measurements were recorded weekly: number of nodes, number of main stem leaves, and height (ground to apical meristem). After eight weeks plants were harvested and leaf area measured with a leaf area meter (LI-3100, LI-COR Inc, Lincoln, NE 68504-1357). Plants were force-air dried at 30 C for 72 hours. Dried plants were separated into the following components: stems, leaves, inflorescence, and seeds. Each component was weighed separately to produce dry weights.

4.3.3.1 Data Analysis

Analysis was performed in JMP for Windows (version 10.0; SAS Institute, Cary, NC). ANOVA was conducted for each trait using a randomized complete block design with 6 replicates. Differences within and among ecotypes in plant traits were calculated using one-way ANOVA and means were separated by Fischer's Protected LSD test at the

5% significance level. Principal component analysis (PCA) was conducted with all plant traits, and a multi-variate clustering was performed to group the giant ragweed biotypes by trait.

4.3.4 Genetic Diversity

4.3.4.1 DNA Extraction

Plant materials were established as previously discussed and plants were maintained in a greenhouse with 30/20 C day/night temperatures with 16-hour photoperiod. One new fully expanded leaf was collected from six- to eight-node plants from each of the six biotypes. Ten plants were sampled from each biotype. Sampled leaves were placed in aluminum foil, labeled, and immediately submerged in liquid nitrogen. Tissue samples were stored at -8 C until use. Deoxyribonucleic acid (DNA) from harvested leaves were isolated using CTAB method (Doyle and Doyle 1990). Fresh leaf tissue (0.125 g) was placed in 2-mL microtube containing four 2.8-mm ceramic beads. CTAB extraction buffer (500 µl) (100mM Tris-HCl, 20 mM EDTA, 2 M NaCl, 2% CTAB, 2% polyvinylpyrrolidone-40, 1 mM phenanthroline, and 0.3% b-mercaptoethanol) was added to each tube. The samples were processed for 2 minutes with Precellys[®] Evolution (BERTIN Corp. MD, USA) homogenizer at 6,708 g. Chloroform: isoamyl alcohol (500 µl) was added to each tube. The mixture was then incubated at 55 C for 45 minutes in a water bath. After incubation the tubes were centrifuged at 9,660 g for 10 minutes. The supernatant was removed from tubes and placed into a fresh 1.5 mL tube. Isopropyl alcohol (250 µl) was added to each tube and inverted to mix contents. After mixing, the tubes were placed in -80 C freezer and stored overnight. Samples were removed from the freezer and allowed to thaw at room

temperature. Tubes were then centrifuged at 9,660 g for 10 minutes. Isopropyl alcohol was then carefully poured out of tubes not to discard the DNA pellet. Ethanol (500 μ l) was added to tubes to wash the DNA pellet and again carefully poured out of tubes. Tubes were then inverted and allowed to air dry. The DNA pellet was then resuspended in 50 μ l of Tris-EDTA (TE) buffer containing 10 mM Tris-HCL, and 1 mM EDTA. Genomic DNA was quantified using NanoDrop[®] ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies). DNA was diluted to 100 ng μ l⁻¹ with deionized water, and used as template in polymerase chain reaction (PCR).

4.3.4.2 Microsatellite DNA Amplification

PCR reactions were conducted using 7 SSR primers (Table 4.2). PCR 25 μ l reactions contained 12.5 μ l Taq 2X Master Mix DNA polymerase, 1 μ l forward primer, 1 μ l reverse primer, 1 μ l DNA (100 ng μ l⁻¹), and 9.5 μ l deionized water. PCR profile starts with 94 C for 1 minute followed by 30 cycles denaturation at 94 C for 30 seconds, annealing at 56 C for 30 seconds, and extension at 72 C for 1 minute. A final extension at 72 C for 5 minutes completed the reaction. The PCR products were electrophoresed in a 6% denaturing polyacrylamide gel (PAGE) at 180V for 40 minutes. Gels were stained with ethidium bromide and bands were photographed.

4.3.4.3 Data Analysis

Bands produced with SSR primers were scored as binary data using Cross Checker 2.91 (Buntjer 1999); 1 for presence, and 0 for absence of the band. Data matrix was then utilized to generate allele number, number of alleles per marker, genetic distance (D), Shannon's index (I), and Nei's genetic diversity (h), using POPGENE

software version 1.32 (Yeh et al. 1996). Genetic distance values were utilized to generate cluster analysis with UPGMA algorithm. A dendrogram was created with POPGENE software. A one-way ANOVA was conducted to compare gene diversity of SSR markers among and within giant ragweed accessions and groups. Means were separated by Fischer's Protected LSD test at the 5% significance level.

4.4 Results and Discussion

4.4.1 Glyphosate Resistance Screening

Results from this trial confirmed that the suspect giant ragweed biotypes were resistant to glyphosate (Table 4.1). Visual ratings 21 DAT for suspected resistant biotypes ranged from 18 to 32% control, while susceptible plants were controlled 100%. However, resistant biotypes did exhibit some chlorosis and stunting from glyphosate application. Based on these findings, the suspect resistant biotypes (Hayes, Hemphill, Younger, TN and OH) will be referred to as GR.

4.4.2 Biological Characterization

4.4.2.1 Vegetative Characteristics

Experiment by treatment interactions were not different, so data from 2015 and 2016 were pooled for analysis. Growth patterns were similar through the first four weeks of growth (Figure 4.2). Height measurements for Hayes (MS-R) and Hemphill (MS-R) were higher than other biotypes at the fifth week of measurement. However, by the eighth week biotypes were not different in height with the exception of OH-R biotype which was shorter at 78 cm with mean height at final measurement of 118 cm. Brabham et al. (2011) reported rapid early growth patterns in GR giant ragweed in comparison to GS

accessions. However, at 50 days after planting biotypes showed no difference in height, shoot weight, and leaf area. Similar trends were recorded with number of nodes.

Hemphill (MS-R) having the highest number of nodes at harvest 15 and OH-R fewest at 9 with a mean of 13 (Fig. 4.2). Number of main stem leaves varied over the eight-week period. However, there was some difficulty maintaining accuracy with some leaf shed occurring as plants approached final measurement. Hemphill (MS-R) had the highest number of main stem leaves at harvest of 26 with Hayes (MS-R), OH-R, and Younger (MS-R) having fewer main stem leaves of 17, 15, and 15, respectively, with mean of 20 (Fig 4.2). Leaf areas revealed similar results. Hemphill (MS-R) had the highest leaf area at 12010 cm² and OH-R had the lowest leaf area at 1916 cm² with mean of 7590 cm² (Table 4.3). Similar leaf areas were reported by Glettner and Stoltenberg (2015) of 7730 and 7580 cm² for resistant and susceptible giant ragweed, respectively. Dry stem weights revealed Hemphill (MS-R), Hayes (MS-R), and MS-S all having higher stem weights of 167 g, 158 g, and 146 g, respectively. OH-R had the lowest stem weight 29 g with mean stem weight of 108 g (Table 4.3). Similar stem weights were reported in field grown populations of GR and GS giant ragweed 200 g and 224 g, respectively, in the absence of glyphosate with no difference (Brabham et al. 2011). Overall growth based on vegetative characteristics was not different when comparing resistant to susceptible biotypes with the exception of OH-R which had the lowest values for each characteristic measured. It is possible that lower vegetative biomass of OH-R could result from it having evolved from higher latitudes. Similar results have been seen in switchgrass (*Panicum virgatum* L.). A study was designed to quantify latitudinal adaptations of twenty switchgrass populations consisting of both northern and southern U.S. populations. Northern populations

experienced reductions in biomass averaging 4.5% per degree of latitude change southward out of their hardiness zone (Casler et al. 2004). These findings confirm photoperiod sensitivity in switchgrass. Sanderson et al. (1999) showed several effects of bringing northern populations of switchgrass south. These latitudinal changes caused earlier flowering and a reduction of 0.72 Mg ha⁻¹ of biomass per degree of latitude change south.

4.4.2.2 Reproductive Characteristics

Seed yields were only calculated from 2015. In 2016, at the prescribed harvest timing all biotypes had not produced mature seed. In 2015 and 2016 plants were transplanted on August 5 and July 22, respectively. Although plants were transplanted 14 days sooner in 2016, there were actually 292 more growing degree units accumulated in 2016 versus 2015 (Anonymous 2018) (Fig.4.3). Maximum temperatures were also similar the week following transplant in both years. Therefore, issues related to potential transplant shock did not differ in either year. Plants were maintained under similar protocols prior to transplant in the greenhouse and were transplanted at the 6-leaf growth stage in each trial year. Dry seed weights ranged from 70 to 11 g with a mean of 52 g. Seed dry weights were lowest in OH-R biotype at 11 g. However, there were no differences in seed dry weights among the other biotypes (Table 4.4). Reproductive ratios were calculated for each biotype and indicates the proportion of total reproductive biomass dedicated to seed production. Younger (MS-R) and TN-R had the highest ratios of 49 and 46%, respectively, and OH-R had the lowest ratio of 6% with a mean of 36% (Table 4.4). Although seed data was not collected in 2016 due to the lack of maturity among biotypes, inflorescence dry weights were collected in both 2015 and 2016. Due to

the fact that the giant ragweed flower structure contains both male and female flower parts, the inflorescence dry weights would still provide information regarding potential reproductive capacity even with immature seeds contained in the 2016 data. Inflorescence dry weights were combined for analysis. Inflorescence dry weights ranged from 15 to 48 g with a mean of 32 g (Table 4.4). OH-R produced lower inflorescence weights of 15 g, while inflorescence dry weights were similar among the other biotypes. Number of days to initiate flowering were variable among biotypes. Days to flower ranged from 49-62 with a mean of 56 (Table 4.4) OH-R biotype had the earliest flowering date among all biotypes of 49. However, this was not different than Younger (MS-R). Days to flower among the other MS biotypes was similar regardless of response to glyphosate. This indicates the photoperiod sensitivity of giant ragweed. OH-R biotype has been impacted by moving southward. MS biotypes flowered on average 8 days later than OH-R. The early flowering was also a factor in growth pattern with OH-R. By reaching reproductive growth stages sooner, vegetative characteristics reflected lower values when compared to southern biotypes. In contrast, the southern biotypes flowered later and were able to take advantage of longer growing conditions of their native environment.

4.4.2.3 Multivariate Cluster Analysis

Cluster analysis was conducted on six giant ragweed biotypes. Data generated from biological characterization study conducted in 2015 and 2016 were used to generate clusters of the biotypes based on nine different characteristics. The largest cluster is indicated by the red lines on the dendrogram includes Hayes (MS-R), Hemphill (MS-R) and MS-S biotypes (Fig. 4.4). The accessions from the red cluster had high values for height (132 cm), leaf area (10,654 cm²), stem dry weight (157 g), number of nodes (14),

leaf dry weight (56 g), and number of main stem leaves (22). Red cluster had intermediate values for days to flower (59), inflorescence weight (37 g), and seed dry weight (54 g). The green cluster, as indicated by its green lines on the dendrogram, contains Young (MS-R) and TN-R biotypes. Accessions in this cluster had intermediate values for height (116 cm), leaf area (5,843 cm²), stem dry weight (75 g), number of nodes (12), leaf dry weight (29 g), number of main stem leaves (18), days to flower (55), and inflorescence weight (32 g) (Fig. 4.4). Seed dry weights were highest for the green cluster at 68 g. The blue cluster as indicated by the blue line on the dendrogram contains only one biotype, OH-R. OH-R had the lowest values for all characteristics: height (78 cm), number of main stem leaves (15), number of nodes (9), leaf area (1,916 cm²), leaf dry weight (14 g), stem dry weight (29 g), inflorescence dry weight (15 g), seed dry weight (11 g), and days to flower (50) (Fig. 4.4).

4.4.2.4 Principal Component Analysis

A PCA was utilized to describe giant ragweed biotypes based on nine traits. The first two axes of the PCA described 92.9% of the variation with component 1 and component 2 explaining 78.4% and 14.8%, respectively. The PCA correlated well with cluster analysis and separated biotypes into three groups (Figure 4.5). The first group indicated by red circle included Hayes (MS-R), Hemphill (MS-R), and MS-S biotypes. The second group in the green circle includes Younger (MS-R) and TN-R biotypes. The final group in the blue circle consists of only one biotype OH-R.

Results from these studies indicate a high degree of variation in phenological traits among giant ragweed biotypes studied. Overall, the majority of the variation can be attributed to geographical regions of origin among the biotypes. In general, the MS

biotypes regardless of response to glyphosate trended toward the highest values for each of the traits measured. Therefore, there were no fitness costs associated with evolved glyphosate resistance in MS biotypes. The GR biotypes had comparable to or greater days to flower, vegetative biomass, and reproductive biomass when compared to GS biotype. MS biotypes took advantage of growing conditions and generated high values in vegetative traits. The next group of biotypes [Younger (MS-R) and TN-R] produced intermediate values across most traits. One explanation for the Younger (MS-R) biotype pairing with TN-R lies within a local farming interaction. Immediately north of the farm where the Younger (MS-R) biotype was collected, a producer from Tennessee also farms acreage. It is a strong possibility that this producer inadvertently introduced GR giant ragweed seed through contaminated farm machinery. This has been documented throughout the literature. The likelihood is these two populations have similar genetics based on their degree of similarities in the traits measured. The cluster analysis/PCA was accurate in describing this relationship. One trait that this group produced higher results was in seed production. The reproductive ratios also indicated this for both Younger (MS-R) and TN-R. It is possible that this slight move south from their native hardiness zone is actually creating a more conducive environment for seed production. Although, this might provide a competitive advantage as to seed production, the locally adapted MS biotypes would be more competitive from a vegetative standpoint. MS biotypes grew taller and produced more leaf area that would shade and potentially reduce the ability of other less adapted (northern) biotypes to produce comparable amounts of seed if occurring in a mixed population.

4.4.3 Genetic Variation

4.4.3.1 Marker Analysis

A total of 667 bands were generated using seven primer combinations. Primers utilized produced polymorphic bands or alleles at a rate of 85.7%. The number of bands scored per primer ranged from 80 to 165 with an average of 101. The primer producing the highest average number of amplified alleles was SSR-10. Data indicated SSR-26 produced a monomorphic pattern and was not able to differentiate variation among biotypes. Nei described genetic diversity as the average expected heterozygosity or the expectancy that a pair of alleles occurring at the same loci will be different (Nei 1978). Genetic diversity is expressed in values ranging from 0 to 1. As values approach 1, the higher the degree of genetic diversity. Primer EST-69 produced the highest level of genetic diversity ($h = 0.665$, Table 4.5). The lowest level of genetic diversity was detected at locus EST-89 ($h = 0.491$, Table 4.5).

4.4.3.2 Genetic Diversity

Gene diversity and Shannon's index were utilized to record levels of genetic variation among and within six giant ragweed biotypes. Shannon's index indicates the level of ambiguity in identifying which species to assign an individual when randomly picked from a collection of species. Higher values approaching 1 would indicate higher genetic diversity. High levels of genetic diversity were recorded among and within giant ragweed biotypes. Overall genetic diversity among biotypes was ($h = 0.5324$, $I = 0.8616$, Table 4.5). Genetic diversity within biotypes ranged from 0.502 to 0.526 (Table 4.6). The TN-R biotype exhibited the lowest genetic diversity and Shannon's index values ($h = 0.502$, $I = 0.806$). Younger MS-R biotype revealed the highest genetic diversity and

Shannon's index values ($h = 0.526$, $I = 0.852$). Genetic diversity values when considering location or state of origin ranked from lowest to highest were TN ($h = 0.502$, $I = 0.806$), MS ($h = 0.514$, $I = 0.834$), and OH-R ($h = 0.525$, $I = 0.849$, Table 4.6). There were only slight differences in genetic diversity when considering response to glyphosate. Genetic diversity values for resistant and susceptible biotypes were ($h = 0.515$, $I = 0.834$) and ($h = 0.508$, $I = 0.818$), respectively. This would suggest that as the levels of glyphosate resistance become more prevalent in a population that genetic diversity does not decrease. Similar differences were recorded between susceptible and resistant palmer accessions from NC and GA with genetic similarity values of 0.35 and 0.34, respectively (Chandi et al. 2013).

4.4.3.3 Genetic Distance

Genetic distance between two individuals or groups can be described as the proportion of alleles that the two individuals or groups do not share. Two similar individuals have the minimum value of distance and simultaneously the maximum value of similarity (Nei 1978). Table 4.7 depicts a genetic distance matrix. All giant ragweed biotypes are listed as groups along the top and left-hand side of the matrix. Genetic distance values are recorded in cells that correspond with the biotype or group along the top and the appropriate group along the left side of matrix. Therefore, the value in a given cell reflects the genetic distance between the two corresponding groups. Groups are more closely related with the smallest genetic distance values. Genetic distance was lowest between TN-R and MS-R Hemphill ($D = 0.189$). The groups with the highest genetic distance were OH-R and MS-R Hayes ($D = 0.828$). Figure 4.6 expresses genetic distance values between biotypes as a dendrogram. There are three clusters identified through this

depiction. The MS-S group has the greatest overall genetic distance and the dendrogram indicates the MS-S group as separate and distinct from the other biotypes. The second group contains MS-R Hemphill, TN-R, and MS-R Hayes with similar genetic distance values. The final group consists of OH-R and MS-R Younger biotypes.

High degrees of phenotypic and genetic variation further confirm how adaptive giant ragweed has become. However, differences due to changes in latitudinal cline were unexpected. Further research is needed to include additional sites with varying latitudes and longitudes to better understand those relationships. Overall genetic variation was confirmed using 7 SSR markers. This set of markers identified 667 different alleles from 60 giant ragweed individuals among the six biotypes. Obviously, more data points could be generated by increasing the number of molecular markers and individuals. However, markers developed for specific traits would provide more information. For example, genes determining seed dormancy would provide more specific data regarding success of giant ragweed ecotypes evolving from agricultural fields. Future research could involve developing specific markers to isolate genes related to seed dormancy. Lefebvre et al. (2006) conducted a study of two genes (NCED6 and NCED9) responsible for the synthesis of abscisic acid (ABA). ABA is involved in the induction of embryo dormancy. Similar genes could be identified for giant ragweed.

Table 4.1 Control of glyphosate-susceptible and glyphosate-resistant giant ragweed 21 DAT with glyphosate screening dose at 840 g ae ha⁻¹.

Biotype ^a	Glyphosate Response	% Control ^b
MS-S	Susceptible	100
Hemphill (MS)	Resistant	32
Hayes (MS)	Resistant	20
Younger (MS)	Resistant	18
TN-R	Resistant	20
OH-R	Resistant	28

^a Abbreviations: DAT, days after treatment; MS-S, Mississippi susceptible; MS, Mississippi; TN-R, Tennessee resistant; OH-R, Ohio resistant.

^b Giant ragweed control rated using a scale of 0 to 100 (0 = no injury and 100 = plant death).

Figure 4.1 Diagram of sub-irrigated planter (SIP). Image by Gamezeyaaaur-Own work, CCO.

Table 4.2 List of seven SSR markers used for DNA amplification.

Marker	Forward	Reverse	Reference
	5' to 3'	5' to 3'	
SSR-10	CGTCAATGGACGATGAAGAA	CCACGTCTTCAAGAATAACAAA	Meyer et al. 2017
SSR-17	GAACATGGATTATGAAGATGCAG	GATTAAGGTTGTCAATAAGGATTGG	Meyer et al. 2017
SSR-26	TCAAGAAATTGATTTAGAACCAAGG	GGAGAACTTGCGCTCGTATT	Meyer et al. 2017
EST-69	TGTGAAAACGAGGGTTAGGG	AAGCAACGCAAACCAAAGAG	Meyer et al. 2017
EST-73	GACTCATGCATATGGAACACG	CCAAATGGTCTACCTCCTGC	Meyer et al. 2017
EST-74	TCGATGACAATCTCGCAGAC	AACGGTTCAGACACTCCACC	Meyer et al. 2017
EST-89	AGCATTGGTGAGTTTGGTCC	TTAGGTCGATCGAGTTGCC	Meyer et al. 2017

^a Abbreviations: DNA, deoxyribonucleic acid; SSR, simple sequence repeats; EST, expressed sequence tag.

Figure 4.2 Trend lines of weekly height, number of leaves, and number of nodes of glyphosate-resistant and glyphosate-susceptible giant ragweed recorded over an eight-week period in 2015 and 2016 at the R. R. Foil Plant Science Center in Starkville, MS. Shaded areas indicate 95% confidence interval limits of the standard error of the mean for each trend line.^a

^a Abbreviations: R, resistant; MS, Mississippi; OH, Ohio, and TN, Tennessee.

Table 4.3 Vegetative biomass measurements of glyphosate-resistant and glyphosate-susceptible giant ragweed in the absence of glyphosate in 2015 and 2016 at the R. R. Foil Plant Science Center in Starkville, MS.

Biotype ^b	Leaf area ^a	Leaf weight	Stem weight
Hemphill (MS-R)	12010 a	66 a	167 a
MS-Susceptible	9981 a	58 ab	146 a
Hayes (MS-R)	9945 ab	43 bc	158 a
Younger (MS-R)	6055 bc	26 cd	69 bc
TN-R	5630 c	31 c	82 b
OH-R	1916 d	14 d	29 c
Mean	7590	40	108
Prob > F	<.0001	<.0001	<.0001

^a Means followed by the same letter in the same column are not significantly different at $P \leq 0.05$.

^b Abbreviations: MS, Mississippi; R, resistant; TN, Tennessee; OH, Ohio.

Figure 4.3 Trend lines of accumulated growing degree units recorded over an eight-week period in 2015 and 2016 at the R. R. Foil Plant Science Center in Starkville, MS.^{a, b}

^a Weather data sourced from National Oceanic & Atmospheric Administration.

^b Abbreviation: GDU, growing degree units.

Table 4.4 Reproductive biomass measurements and days to initiation of flowering of glyphosate-resistant and glyphosate-susceptible giant ragweed in the absence of glyphosate in 2015 and 2016 at the R. R. Foil Plant Science Center in Starkville, MS.

Biotype ^c	Inflor weight ^a	Seed weight	Days to flower	Rep Ratio ^b
Hemphill (MS-R)	33 a	53 a	63 a	37
MS-Susceptible	30 ab	50 a	58 ab	35
Hayes (MS-R)	48 a	59 a	56 b	42
Younger (MS-R)	30 ab	70 a	55 bc	50
TN-R	33 a	67 a	55 b	46
OH-R	15 b	11 b	50 c	6
Mean	31.58	51.56	56.27	35.85
Prob > F	0.0294	0.0015	<.0001	

^a Means followed by the same letter in the same column are not significantly different at $P \leq 0.05$.

^b Reproductive ratios were calculated by the formula [(total seed wt/plant wt (seed + stem dry wt)].

^c Abbreviations: Inflor, inflorescence; Rep, reproductive; MS, Mississippi; R, resistant; TN, Tennessee; OH, Ohio.

Figure 4.4 Multivariate Cluster Analysis of traits of glyphosate-resistant and glyphosate-susceptible giant ragweed in the absence of glyphosate in 2015 and 2016 at the R. R. Foil Plant Science Center in Starkville, MS.^{a, b}

^a Blue indicates lower values, red indicates higher values, and grey indicates intermediate values.

^b Abbreviations: R, resistant; MS, Mississippi; TN, Tennessee; OH, Ohio; Wt., weight in grams; Flwr, flower; Inflor, inflorescence; #, number.

Figure 4.5 Principal Component Analysis (PCA) of traits of glyphosate-resistant and glyphosate-susceptible giant ragweed in the absence of glyphosate in 2015 and 2016 at the R. R. Foil Plant Science Center in Starkville, MS.^{a, b, c}

^a Red diamonds indicate individual traits.

^b Abbreviations: OH-R, Ohio resistant; TN-R, Tennessee resistant; Y-R, Younger Mississippi resistant; HA-R, Hayes Mississippi resistant; HE-R, Hemphill Mississippi resistant; MS-S, Mississippi susceptible; Flwr, flower; Wt., weight; Inflor, inflorescence; sq cm, centimeter squared; #, number.

^c Red, Blue, and Green circles indicate clusters of biotypes.

Table 4.5 Allele number, gene diversity (h), and Shannon's index (I) of seven SSR markers.

Marker ^a	Allele #	Gene Diversity (h)	Shannon's Index (I)
SSR-10	165	0.663	1.093
SSR-17	91	0.662	1.034
SSR-26*	60	0	0
EST-69	80	0.665	1.096
EST-73	88	0.647	1.069
EST-74	95	0.639	1.054
EST-89	88	0.491	0.684
Mean	95.3	0.5324	0.8616

^a * denotes monomorphic pattern

^b Abbreviations: SSR, simple sequence repeats; EST, expressed sequence tag.

Table 4.6 Gene diversity (h), and Shannon's index (I) within six giant ragweed biotypes.

Group ^a	Populations per group	Gene Diversity	Shannon's Index
		(h)	(I)
MS-S	10	0.508	0.818
Hemphill (MS-R)	10	0.518	0.843
TN-R	10	0.502	0.806
Hayes (MS-R)	10	0.503	0.821
Younger (MS-R)	10	0.526	0.852
OH-R	10	0.525	0.849

^a Abbreviations: MS, Mississippi; S, susceptible; R, resistant, TN, Tennessee; OH, Ohio.

Table 4.7 Genetic distance (D) among six giant ragweed biotypes.

Biotype	MS-S	MS-R Hemp	TN-R	MS-R Hayes	MS-R Younger	OH-R
MS-S	N/A					
MS-R Hemp	0.0477					
TN-R	0.0695	0.0189				
MS-R Hayes	0.0623	0.026	0.0359			
MS-R Younger	0.0621	0.0218	0.0288	0.0486		
OH-R	0.0402	0.0352	0.0425	0.0828	0.0234	N/A

^a Abbreviations: MS, Mississippi; S, susceptible; R, resistant, TN, Tennessee; OH, Ohio; N/A, not available.

Figure 4.6 Dendrogram of genetic distance (D) among six giant ragweed biotypes.^a

^a Abbreviations: MS-S, Mississippi susceptible; MS-R(He), Mississippi resistant Hemphill; TN-R, Tennessee resistant; MS-R(Ha), Mississippi resistant Hayes; MS-R(Y), Mississippi resistant Younger; OH-R, Ohio resistant.

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CHAPTER V

CONCLUSIONS

Glyphosate-resistant (GR) giant ragweed (*Ambrosia trifida* L.) continues to expand its range as new resistant populations were confirmed with these findings in Monroe County, MS. The mechanism for glyphosate resistance appears to be related to reduced translocation for the accession from Monroe County. However, the actual mechanism is still not clearly understood (Sammons and Gaines 2014).

Producers will need to focus more on non-chemical methods of control to manage GR giant ragweed populations. Methods to manage late-season escapes might include hand weeding to eliminate contributions to the soil seed bank and also to reduce elasticity in populations evolving from agricultural fields. Greater attention needs to be placed on managing giant ragweed along field borders such as drainage ditches, creek banks, old fencerows, and woodlands to reduce introduction from these areas adjacent to agricultural fields. A more diverse weed control system should also include, crop rotation, fallowing, cover crops, tillage, and proper sanitation of equipment. However, where herbicides are incorporated into a system including dicamba-resistant (DR) crops, dicamba applied pre-emergence (PRE) or post-emergence (POST) provided excellent control of GR giant ragweed. As 4-hydroxyphenyl-pyruvate dioxygenase (HPPD)-resistant cultivars of cotton (*Gossypium hirsutum* L.) and soybean [*Glycine max* (L.) Merr.] become available, products such as mesotrione can also be incorporated both PRE and POST to provide another mode

of action as well as an additional herbicide trait to manage GR giant ragweed. There are currently no known cases of herbicide resistance to either dicamba or HPPDs in giant ragweed. The incorporation of DR and HPPD-resistant crops into a system will allow the mixture of these two herbicide modes of action. Herbicide mixtures can be effective tools in reducing the selection for herbicide resistance (Beckie 2006). However, these mixtures will not serve as stand-alone solutions and should be supported with other control methods to provide a comprehensive approach to overall weed control. These types of strategies will be required to protect the herbicide-tolerant traits.

Glyphosate resistance in giant ragweed populations studied did not appear to carry a fitness penalty. However, results were based on a small sample size ($n=6$) from populations with unknown frequency of GR alleles. Future research to evaluate fitness should be conducted on populations from controlled crosses of both resistant and susceptible individuals (Menchari et al. 2008).

Results from the biological characterization revealed large amounts of phenotypic variation among giant ragweed biotypes. Multi-variate cluster analysis and principal component analysis (PCA) accurately grouped individuals by state of origin. From a weed control standpoint, these findings might suggest that when giant ragweed are encountered in similar geographical areas, effective control methods can be adopted to address them as a group. Reduced vegetative and reproductive biomass values for northern biotypes does suggest that individuals can be negatively impacted when moved beyond their hardiness zones (Casler et al. 2004). This is further evidence of the ability of giant ragweed to evolve into a more locally adapted ecotype or the “general purpose genotype” (Baker 1974).

Molecular markers that were selected for the genetic diversity study were effective at detecting high degrees of genetic variation within and among giant ragweed biotypes. Although genetic diversity did not decline as a result of response to glyphosate, the high levels of genetic variation recorded can be an indicator of the ability of giant ragweed biotypes to more readily adapt to changing environments (Barrett and Schluter 2008). Better understanding of evolutionary genetics can be critical in predicting the rate at which herbicide-resistance might occur. Evolution of glyphosate resistance could be further examined in model organisms such as *Arabidopsis thaliana* (Jander et al. 2003).

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