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Mechanisms and variability of glyphosate resistance in *Amaranthus palmeri* and *Ipomoea lacunosa*

By

Daniela Neves Ribeiro

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

Mississippi State, Mississippi

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2013

Mechanisms and variability of glyphosate resistance in *Amaranthus palmeri* and *Ipomoea lacunosa*

By

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The resistance of Palmer amaranth (PA) and the tolerance (natural resistance) of pitted morningglory (PM) to glyphosate have made these species among the most common and troublesome weeds in the southeastern U.S. since the adoption of glyphosate-resistant (GR) crops.

Populations of GR PA (R1 and R2) were identified in Mississippi. The inheritance of glyphosate resistance was examined in reciprocal crosses (RC) between glyphosate-resistant (R) and -susceptible (S) parents (Female-S × Male-R, S/R, and Female-R × Male-S, R/S), and second reciprocal crosses (2RC) (Female-S/R × Male-S/R, S/R//S/R, and Female-R/S × Male-R/S, R/S//R/S). Dose-response assays resulted in 17- to 4-fold resistance to glyphosate compared with S. Population S accumulated 325- and 8-times more shikimate at the highest glyphosate dose than in R1 and R2, respectively. cDNA sequence analysis of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene indicated no target site mutation. Genomes of R1, R2, RC, and 2RC contained from 1- to 59-fold more copies of *EPSPS* gene than S; *EPSPS* was highly expressed in R1 and R/S, but was poorly expressed in S, S/R, and R2. EPSPS activity was lower in S and S/R

than in R and R/S, glyphosate absent; all were inhibited by glyphosate. Western Blot analysis confirmed an increased EPSPS protein level to *EPSPS* copy number correlation. Thus, the level of resistance was decidedly influenced by the direction of the cross. R and S female plants were reproductively isolated and seed were still produced, suggesting that PA can produce seed both apomictically and sexually (facultative apomixis). This mode of reproduction determined the low copy number inheritance, as well as guaranteeing the GR trait stability in the R populations.

Dose-response assays resulted in 2.6-fold variability in tolerance to glyphosate between the most tolerant (MT) and the least tolerant (LT) PM populations. The level of tolerance positively correlated with the time of exposure to GR-crop system. Less shikimate was recovered in MT as compared to LT. Levels of aminomethylphosphonic acid (AMPA) were not different between populations and sarcosine was not present in either populations. Consequently, metabolism of glyphosate to AMPA or sarcosine is not a common factor in explaining natural resistance levels.

DEDICATION

"I can do all things through Christ, who strengthens me". (Philippians 4:13). I give glory and honor to Him for accomplishing this formidable academic task.

Thank you to my parents, Marcelo and Maria Cecília Ribeiro, you guided me in the right ways throughout. You lovingly put my joy in front of yours. You accepted the distance from your only daughter when I left the country to follow my career. The accolades and accomplishments I have achieved would be meaningless if I could not share them with you. Thank you for your love, for always believing in me, and for encouraging me to pursue my dreams. I love you and I hope that I can be there from now on for you, the way you have been there for me. Eu amo e para sempre irei amár-los!

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CHAPTER I

INTRODUCTION

The herbicide glyphosate was commercialized in 1974 and, since then, is considered the most important herbicide ever developed (Powles 2003; Perez-Jones et al. 2007). Glyphosate has become the principal postemergence, systemic, nonselective, broad-spectrum herbicide for the control of annual and perennial weeds (Baylis 2000; Perez-Jones et al. 2007). Although it was first used as a non-crop and plantation crop herbicide, now it is also used in non-tillage systems and in glyphosate-resistant (GR) crops (Owen and Zelaya 2005; Shaner 2000). Its development has led to improved yields, increases in conservation-tillage systems and higher quality agricultural products (Gianessi and Sankula 2004).

Glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EPSPS, EC 2.5.1.19) that catalyzes the conversion of shikimate-3-phosphate and phosphoenolpyruvate in to EPSP and inorganic phosphate in the shikimic acid pathway; hence affecting the chorismate pathway (Devine et al. 1993; Geiger and Fuchs 2002; Gruys et al. 1993; Steinrücken and Amrhein 1980). Inhibition of EPSPS results in shikimic acid accumulation and in reduction of biosynthetic processes, such as aromatic amino acids (phenylalanine, tyrosine and tryptophan), vitamins (K and E), proteins, alkaloids, lignin, flavonoids, coumarins, indole acetic acid (IAA), chlorophyll, carotenoids, benzoates and quinates (Amrhein et al. 1980; Anderson and Johnson 1990;

Arnaud et al. 1994; Bently 1990; Devine et al. 1993; Herrmann and Weaver 1999). These substances are essential to plant development (Devine et al. 1993). Moreover, the shikimic acid increase is related to decline in carbon fixation intermediates and reduction of photosynthesis (Duke et al. 2003).

Glyphosate was used worldwide for more than 20 years with no reports of evolved resistance in weed species (Bradshaw et al. 1997). Currently, reports involving glyphosate resistance are identified in 24 species of weeds in the world (Heap 2012). The first related case was with rigid ryegrass (*Lolium rigidum* Gaudin) in Australia (Powles et al. 1998; Pratley et al. 1999), followed by goosegrass [*Eleusine indica* (L.) Gaertn.] in Malaysia (Lee and Ngim 2000; Tran et al. 1999), horseweed [*Conyza canadensis* (L.) Cronquist] in the United States of America (Koger et al. 2004; VanGessel 2001), Italian ryegrass [*Lolium perenne* L. ssp. *multiflorum* (Lam.)] in Chile (Perez and Kogan 2003), hairy fleabane [*Conyza bonariensis* (L.) Cronquist] in South Africa (Urbano et al. 2005), buckhorn plantain (*Plantago lanceolata* L.) in South Africa (Heap 2012), common ragweed (*Ambrosia artemisiifolia* L.) in the United States of America (Sellers et al. 2005), giant ragweed (*Ambrosia trifida* L.) in the United States of America (Heap 2012), ragweed parthenium (*Parthenium hysterophorus* L.) in Colombia (Heap 2012), Palmer amaranth (*Amaranthus palmeri* S. Watson) in the United States of America (Culpepper et al. 2006), common waterhemp [*Amaranthus tuberculatus* (Moq.) JD Sauer (*syn. A. rudis*)] in the United States of America (Zelaya and Owen 2005), Johnsongrass [*Sorghum halepense* (L.) Pers.] in Argentina (Heap 2012), sourgrass [*Digitaria insularis* (L.) Mez ex Ekman] in Paraguay (Heap 2012), *Euphorbia heterophylla* in Brazil (Vidal et al. 2007), junglerice [*Echinochloa colona* (L.) Link] in Australia (Heap 2012), kochia

[*Bassia scoparia* (L.) A. J. Scott (*syn. Kochia scoparia*)] in United States of America (Heap 2012), liverseedgrass (*Urochloa panicoides* P. Beauv.) in Australia (Heap 2012), perennial ryegrass (*Lolium perenne* L.) in Argentina (Heap 2012), gramilla mansa (*Cynodon hirsutus* Stent) in Argentina (Heap 2012), sumatran fleabane [*Conyza sumatrensis* (Retz.) E. Walker] in Spain (Heap 2012), Australian fingergrass (*Chloris truncata* R. Br.) in Australia (Heap 2012), tropical sprangletop [*Leptochloa virgata* (L.) P. Beauv.] in Mexico (Heap 2012), annual bluegrass (*Poa annua* L.) in the United States of America (Heap 2012), ripgut brome (*Bromus diandrus* Roth) in Australia (Heap 2012), and spiny amaranth (*Amaranthus spinosus* L.) in the United States of America (Heap 2012).

The adoption of transgenic herbicide-resistant crops has increased dramatically in the last decade (Owen and Zelaya 2005). The results of this unprecedented change in agricultural practice have been many, but perhaps most dramatic is the simplification of weed control tactics and, consequently the change of weed communities (Owen and Zelaya 2005). The adoption of herbicide-resistant crops will result in greater selection pressure on the weed community due to a limited number of different herbicides used (Powles and Preston 2006). Selection pressure imparted by herbicide tactics can result in weed shifts attributable to the natural resistance (tolerance) of a particular species to the herbicide or the evolution of herbicide resistance within the weed population (Dill 2005; Owen and Zelaya 2005). This way, herbicide resistance in weeds is a natural phenomenon that preexists in those populations; therefore, the herbicide not being the causal agent but the selector of resistant individuals that were in low initial frequency (Christoffoleti et al. 1994).

Herbicides are very intense selective agents and evolution of herbicide resistance can be fast when genetic variability for herbicide occurs in weed populations (Diggle et al. 2003). In particular, several *Amaranthus* and *Ipomoea* species have tremendously high genetic variability in their response to herbicides (Smeda, personal communication; Poston, personal communication). The probability and rate of herbicide resistance evolution depends on the interaction between the population dynamics and population genetics of weed populations (Diggle and Neve 2001; Jasieniuk et al. 1996; Maxwell and Mortimer 1994). Important evolutionary factors include the intensity of selection, the frequency of resistant traits in natural populations, the mode of resistance inheritance, the relative fitness of susceptible and resistant biotypes in the presence and absence of herbicides, the intrinsic dynamics of weed population (seedbank), gene flow within and between populations, and in the *Amaranthus* genus gene flow between related species (Diggle et al. 2003; Mortimer et al. 1993; Trucco et al. 2007). Once resistance is significantly frequent within a population, it might spread quickly to other populations by pollen or seed, and potentially can be transmitted to other species via hybridization (Owen and Zelaya 2005; Rieger et al. 2002; Wetzel et al. 1999).

The weed's insensitivity to herbicide can be conferred by different mechanisms including reduced herbicide absorption, reduced translocation of herbicide from the site of absorption to the target-site, enhanced metabolic detoxification of the herbicide, sequestration or compartmentalization of the herbicides away from the target site, target-site mutations, and gene amplification/overexpression (Devine and Eberlein 1997; Gaines et al. 2010; Koger and Reddy 2005; Nandula 2010; Perez-Jones and Mallory-Smith 2010; Preston and Wakelin 2008). Weed species can be resistant to glyphosate by one or more

of these mechanisms (Koger and Reddy 2005; Nandula et al. 2012). Most of the related cases concern the reduced translocation of herbicide from the site of absorption to the target-site and target-site mutation (Baerson et al. 2002; Feng et al. 2004; Lorraine-Colwill et al. 2003; Michitte et al. 2005; Nandula et al. 2008, 2012; Ng et al. 2003; Perez-Jones et al. 2005; Preston and Wakelin 2008; Simarmata and Penner 2004; Wakelin and Preston 2006). Nowadays, the most frequently detected glyphosate degradation product is aminomethylphosphonic acid (AMPA). Consequently, it is assumed that glyphosate can be metabolized by plants via two pathways; one involves oxidative cleavage of the C-N bond and the other breaking of C-P bond (Duke 2011; Reddy et al. 2008). Recently, Gaines et al. (2010, 2011) proposed that the molecular basis of glyphosate-resistance in *Amaranthus palmeri* is due to gene amplification leading to multiple copy numbers of *EPSPS* and increased production of EPSPS protein. Moreover, the authors proposed that this mechanism of resistance is heritable. Chandi et al. (2012) studying a GR Palmer amaranth population from North Carolina found that inheritance of glyphosate resistance was incompletely dominant, nuclear inherited, and might not be consistent with a single gene mechanism of inheritance. Sosnoskie et al. (2012) found that GR can be transferred via pollen movement in Palmer amaranth. Trucco et al. (2007) observed the production of hybrid progeny resulting from crosses between Palmer amaranth and common waterhemp. Hence, the resistant trait tends to stay in equilibrium in the population indefinitely, unless the selection forces the population out of equilibrium (Fry and Rausher 1997). In natural populations, very few herbicide-resistant plants are found unless repeated applications of the herbicide were made continually in past years (Perez-Jones et al. 2007).

Therefore, herbicide-resistant weeds have become an increasing global hazard to agriculture, creating an urgency to understand the basis of resistance; likewise the genomics of weediness (Basu et al. 2004; Marshall 2001; Yuan et al. 2006). Weed science has given us a thorough knowledge of weedy traits, but we are largely ignorant about the functional genomics underlying these (Basu et al. 2004). Using the available tools of genomics, we can improve our understanding of weed resistance by finding and characterizing genes that might play a role in fitness, competitiveness and adaptations of weeds in the herbicide-applied agroecosystems (Weller et al. 2001). Given the inevitability of evolved herbicide resistance, it is important to consider tactics to prevent or delay the development of resistant populations. Regardless, it is important to evaluate the situation and make appropriate adjustments in weed management tactics to keep weed shifts and the herbicide resistance evolution from becoming an economic problem (Powles 2003).

The use of herbicides in agriculture, therefore, should be conditioned to scientific knowledge to manage the resistance and tolerance to herbicides. This way, it is necessary that the resistance and tolerance to herbicides be understood by the people directly or indirectly involved with the management of weeds seeking the adoption of appropriate and rational measures of management of resistant and tolerant weeds, making possible the continuous exploitation of agricultural areas with this problem, in ways to maximize the productivity without needing expansion of agricultural frontiers.

Resistance and tolerance to glyphosate in weed species is a major challenge for the sustainability of glyphosate use in crop and non-crop systems. GR Palmer amaranth populations have been identified in Mississippi. Variability in the level of tolerance to

glyphosate in pitted morningglory populations have been identified in Mississippi. The objectives of the research reported in the following chapters were to investigate: (1) the molecular mechanism conferring resistance to glyphosate in Palmer amaranth populations from Mississippi, (2) the mode of inheritance of resistant trait in these populations, (3) the variability in tolerance to glyphosate among morningglory accessions from U.S., (4) if the variability in glyphosate tolerance levels are correlated with the length of time exposed to GR systems, (5) if differential metabolism of glyphosate to AMPA and/or sarcosine is the underlying mechanism for differential tolerance to glyphosate among pitted morningglory populations from Mississippi.

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CHAPTER II

APOMIXIS INVOLVEMENT IN EPSPS GENE AMPLIFICATION INHERITANCE IN GLYPHOSATE-RESISTANT *Amaranthus palmeri*

2.1 Abstract

Glyphosate-resistant (R) Palmer amaranth populations (R1 and R2) have been identified in Mississippi. The inheritance of glyphosate resistance was examined by reciprocally crossing R maternal parents with susceptible (S) paternal parents (R/S) and crossing S maternal parent with R paternal parents (S/R) to generate reciprocal crosses (RC). Individuals from the RC populations were submitted to glyphosate dose-response assays resulting in a range of phenotypes from R to S. The response to glyphosate was more similar to the R than S parent when the female parent was R. Conversely, the response to glyphosate was more similar to the S parent when R was used as pollinator. Sequence comparisons of the predicted 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) mature protein from R1, R2, and S did not identify a target site mutation known to confer resistance in R populations. EPSPS activity was lower in S and S/R plants than in R and R/S plants in the absence of glyphosate; all were inhibited by the presence of glyphosate. Genomic estimation of *EPSPS* gene copy number relative to acetolactate synthase (*ALS*) using quantitative PCR showed that R and R/S contain more copies of EPSPS than S and S/R. Western Blot analysis confirmed that increased EPSPS protein levels were correlated with *EPSPS* copy number. Quantitative real-time PCR on cDNA

revealed that *EPSPS* was highly expressed in R1 and R/S, but was poorly expressed in S, S/R, and R2. Thus, the level of resistance was strongly influenced by the direction of the cross as demonstrated in all assays. This led us to hypothesize a facultative apomictic reproduction. The involvement of apomixis in glyphosate resistance inheritance was studied using 44 S, 36 R1 and 38 R2 reproductively isolated female individuals. In all cases seed were produced, with the exception of one R1 plant. Depending on the population, 60 to 100% of plants produced 1 to 1,000 seed, but some individuals produced up to 6,000 seed, suggesting that Palmer amaranth can produce seed both apomictically and sexually (facultative apomixis), with apomixis the determinant of low copy number inheritance in S/R population. Moreover, facultative apomixis would guarantee the glyphosate resistant trait stability in R populations.

2.2 Introduction

The herbicide glyphosate (*N*-(phosphonomethyl)glycine) was commercialized in 1974 and has established itself as the leading postemergence, systemic, nonselective, broad-spectrum herbicide for the control of annual and perennial weeds and volunteer crops in a wide range of different situations (Baylis 2000; Bradshaw et al. 1997; Franz et al. 1997; Perez-Jones et al. 2007; Powles 2003). Glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, EC 2.5.1.19), an enzyme of the shikimate pathway, thereby preventing the biosynthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan (Siehl et al. 1997; Steinrücken and Amrhein 1980). Glyphosate-resistant (GR) crop technology has been globally adopted, totaling 102 million ha in 2008, reducing herbicide use by 17 million kg and saving U.S. farmers

$1.2 billion per year (Gianessi 2005, 2008; James 2008; Padgette et al. 1996; Shaner 2000).

Although strong arguments were made against the likelihood of weeds developing resistance to glyphosate (Bradshaw et al. 1997), the first GR weed was confirmed after a lag time of about twenty years following the introduction of the herbicide glyphosate (Heap 2012; Powles et al. 1998; Pratley et al. 1999). Subsequent to GR rigid ryegrass (*Lolium rigidum* Gaudin) being reported in 1996, twenty-three other GR weed species have been confirmed (Heap 2012). Glyphosate resistance has recently been reported in Palmer amaranth (*Amaranthus palmeri* S. Wats.) populations, a troublesome agronomic weed with known multiple herbicide resistance (Culpepper et al. 2006; Vencill et al. 2008).

Palmer amaranth is a tall, upright, and dioecious summer annual that is native to the Mexican states of Sonora and Baja California, as well as parts of southern Arizona and California (Ehleringer 1983). Palmer amaranth was first described by Sereno Watson in 1877, working from specimens collected from San Diego County, CA, and from along the banks of the Rio Grande River (Watson 1877). The species has since spread into the southeastern United States, where it is a common and competitive weed in row crop production. Interference from Palmer amaranth affects the growth and yield of corn (*Zea mays* L.) (Massinga et al. 2001), soybean [*Glycine max* (L.) Merr.] (Bensch et al. 2003; Klingaman and Oliver 1994), peanut (*Arachis hypogaea* L.) (Burke et al. 2007), and cotton (*Gossypium hirsutum* L.) (Morgan et al. 2001; Rowland et al. 1999).

Currently, GR Palmer amaranth infests more than two million ha in 15 states (Alabama, Arizona, Arkansas, California, Georgia, Illinois, Louisiana, Michigan,

Missouri, Mississippi, New Mexico, North Carolina, Ohio, Tennessee, and Virginia) (Heap 2012). GR Palmer amaranth became the single greatest threat to the economic sustainability of cotton production, largely due to the lack of control provided by available POST herbicides. Mayo et al. (1995) reported that Palmer amaranth was more difficult to control with various herbicides than were other *Amaranthus* species.

The steady increase in weeds with evolved resistance to glyphosate has been exacerbated with the introduction and widespread adoption of GR crops (Duke and Powles 2009; James 2010) that has resulted in glyphosate being used more extensively than any other herbicide worldwide. In evolved resistance, single base pair mutations of the gene for EPSPS, the target site of glyphosate action; have not provided a high level of resistance (Perez-Jones and Mallory-Smith 2010). Alteration of more than one codon, as with site-directed mutagenesis to produce the GA21 version of maize EPSPS, was used to produce commercial GR maize with a much higher level of resistance (Green 2009). GA21-type mutations have apparently not occurred in nature. The most recent evolved weed populations have other mechanisms of resistance based on gene amplification of the *EPSPS* gene(s) (Gaines et al. 2010) or sequestration of glyphosate in the vacuole (Ge et al. 2010).

Gene amplification of target site genes has been documented as a resistance mechanism for insecticides (Bass and Field 2011) and fungicides (Selmecki et al. 2008). In the laboratory, step-wise increases in glyphosate concentrations of plant cell cultures selected for gene amplification of *EPSPS* (Pline-Srnic 2006). Amplification of the *EPSPS* gene has been found as a mechanism for field-evolved glyphosate resistance in Palmer amaranth (Gaines et al. 2010), Italian ryegrass [*Lolium perenne* L. ssp. *multiflorum*

(Lam.) Husnot] (Salas et al. 2012) and burningbush (*Kochia scoparia* (L.) Schrad.) (Westra et al. 2012). Glyphosate is the only herbicide for which there is field-evolved amplification of a target gene known to confer resistance.

In the case of Palmer amaranth, glyphosate resistance correlates with increase in the copy number of *EPSPS* genes, transcripts, protein content, and enzymatic activity (Gaines et al. 2011). These correlations indicate that between 30 and 50 copies endow for resistance to the normal field rate of glyphosate (0.5 to 1 kg ha^{-1}). These extra copies are found throughout the genome, on every chromosome (Gaines et al. 2010). Studies by Gaines and co-workers were all conducted on Palmer amaranth population that evolved in the southeastern USA. More recently, GR populations of this specie were found in the western alluvial plain of Mississippi (Heap 2012; Nandula et al. 2012).

GR Palmer amaranth is a problematic weed that economically affects southern U.S. agricultural systems. The current evolved mechanisms of resistance in Palmer amaranth is based on gene amplification of the *EPSPS* gene(s) (Gaines et al. 2010). This is a novel mechanism of resistance to herbicide in weeds and very little is understood about the mode of inheritance of this trait. The objectives of this research were to investigate the molecular mechanism of resistance to glyphosate in Palmer amaranth populations from Mississippi in light of the recent discovery made by Gaines et al. (2010) and to better understand some remaining conundrums regarding the mode of inheritance of this novel mechanism of resistance.

2.3 Materials and Methods

2.3.1 Plant Material and General Experimental Conditions

Seed of suspected GR (R) populations of Palmer amaranth were collected from 35 GPS site coordinates across the northwestern region of Mississippi, USA, in 2007 (Nandula et al. 2012). These row crop sites had been intensively treated with glyphosate during the last 10 years, with at least one in-season application each year of glyphosate at 0.84 g ae ha^{-1}. Each location corresponded to a population, with each population defined as a group of seed collected from 10 to 20 randomly selected female plants (Nandula et al. 2012) from seed collection at Mississippi State University's Delta Research and Extension Center, Stoneville, MS, maintained by Dr. Vijay K. Nandula. A known susceptible (S) population, collected in Washington County, MS, was included for comparison in all experiments. Seed were stored at 10 C until further use (Appendix A).

Experiments were carried out as described by Nandula et al. (2012). Briefly, germination, transplantation, growth, and treatment of all plants were conducted under the following conditions unless otherwise described. Seed were planted at 1-cm depth in 50-cm by 20-cm by 6-cm plastic trays with holes containing a commercial potting mix (Metro-Mix 360, Sun Gro Horticulture, Bellevue, WA 98008). Two weeks after emergence, Palmer amaranth plants were transplanted into 6-cm by 6-cm by 6-cm pots containing potting mix. Plants were fertilized with a nutrient solution (Miracle-Gro, The Scotts Company, Marysville, OH 43041) containing 200 mg L^{-1} each of N, P_2O_5, and K_2O one week after transplanting and then once per month; subirrigated as needed. Plants were greenhouse grown under 30/20 C day/night temperature with a 14-h photoperiod. Plants were sprayed at the 4- to 6-leaf stage with glyphosate (Roundup WeatherMAX,

Monsanto Company, St. Louis, MO 63167) using an 8002E nozzle and overhead compressed-air sprayer calibrated to deliver 140 L ha^{-1} at 280 kPa.

In addition, MS (Murashinge and Skoog 1962) tissue culture medium was tested in Palmer amaranth micropropagation, but this methodology was not used due to the extensive callus formation, especially in GR Palmer amaranth populations (Figure 2.12). This observation is interesting as it demonstrates a hormonal imbalance in Palmer amaranth, particularly in the resistant populations; unknown factors may play a role in sex-modifying hormones (section 2.4.7).

Nandula and co-workers (2012) conducted a preliminary screening with 840 g glyphosate ha^{-1} and confirmed 12 population accessions to be GR based on percentage of control (visible estimate of injury on a scale of 0 [no injury] to 100 [complete death]) and mortality (percentage of plants surviving [evidence of shoot regrowth at time of evaluation] in relation to total number of plants treated) measured 2 weeks after treatment (WAT) (Appendix A). Two populations with the greatest level of glyphosate-resistance (C1 and T4) were selected for subsequent research.

2.3.2 Development of Genetic Populations

Controlled crosses (Female-R × Male-R, R/R) were developed by treating C1 and T4 populations with 840 g glyphosate ha^{-1}, as previously described. Palmer amaranth is dioecious, i.e. male and female flowers develop on different plants. Consequently, the sex of the surviving treated plants was identified and at least 100 individuals of each gender were grown together in isolation from other populations in different greenhouses to ensure genetic purity of each controlled cross. Pollen from the male plants was physically spread on the female plants every morning over a period of two months. Palmer amaranth

inflorescence spikes were hand-harvested when the majority of the seed possessed coats that were black in color (seed maturity). Palmer amaranth seed were air-dried in a greenhouse (25/20 C day/night, 12-h photoperiod under natural sunlight conditions) for 7 days, cleaned, and stored at 10 C until further use. Resistance was confirmed by the application of glyphosate at the labeled rate to a sub-set of whole-plant first R/R controlled cross progeny (data not shown).

Controlled crosses (R/R) were then developed using the first R/R controlled crosses. Seed from the first controlled crosses were grown, sex identified and at least 100 individuals of each gender were grown together in isolation from other first R/R controlled cross to ensure genetic purity of each second generation. Plants were shaken daily to ensure adequate pollination, as described above. Mature seed spikes from the female plants were harvested, processed, and seed stored as mentioned earlier. A sub-set of second R/R controlled cross plants were grown and confirmed to be resistant to glyphosate (data not shown) following the previously described procedure. These controlled crosses were defined as parents to generate subsequent genetic populations, hereafter referred to as R1 and R2.

Reciprocal crosses (Female-S × Male-R, S/R, and Female-R × Male-S, R/S) were made by growing S females in isolation with each R male (R1 and R2), and each R female (R1 and R2) with S male in four separated greenhouses with the aim of generating four reciprocal crossed (RC) populations, hereafter referred to as S/R1, S/R2, R1/S, and R2/S. Plants were pollinated as previously described and resistance confirmed by the application of glyphosate at low rate (420 g ae ha^{-1}) to a sub-set of the RC generations. The majority ($\geq$ 85%) of S/R1 and S/R2 cross plants treated with glyphosate at the

described rate were controlled (data not shown) indicating homozygous susceptible individuals.

Second reciprocal crosses (Female-S/R × Male-S/R, S/R//S/R, and Female-R/S × Male-R/S, R/S//R/S) were made by growing each RC female in isolation with each RC male in four separated greenhouses with the aim of creating four second reciprocal crossed (2RC) populations, hereafter referred to as S/R1//S/R1, S/R2//S/R2, R1/S//R1/S, and R2/S//R2/S. Plants were pollinated as previously described and resistance confirmed by the application of glyphosate at low rate (420 g ae ha^{-1}) to a sub-set of the 2RC generations. The majority (≥70%) of plants of S/R1//S/R1 and S/R2//S/R2 populations were controlled, some plants of all four 2RC populations presented intermediate level of injury, and the majority (≥80%) of plants of R1/S//R1/S and R2/S//R2/S were not controlled (data not shown).

2.3.3 Glyphosate Dose-Response Bioassay

Plants from parental lines (R1 and R2) and from first and second reciprocal crosses were submitted to a dose-response experiment by applying glyphosate at 0, 52, 105, 210, 420, 840, 1,680, 3,360, and 6,720 g ha ae ha^{-1} at three- to four-leaf growth stage. Two WAT shoots were clipped at soil level and fresh weight was determined. Biomass data are reported as percentage of the nontreated control. There were three replications arranged in a completely randomized design. The experiment was conducted two times.

2.3.4 Shikimate Bioassay

Shikimate assays on Palmer amaranth populations were conducted following a previously reported protocol (Shaner et al. 2005). Seed from parental lines (R1 and R2) and S were grown as described until reaching 4- to 6-leaf stage. Plants of each population were sampled for one leaf disc (4-mm diameter) per population from the 3- to 4- leaf stage with a cork borer. The leaf discs were placed in 96-well microtiter plates, one disc per well, and added to each well 100 µL 10 mM ammonium phosphate (pH 4.4) plus 0.1% (v/v) Tween 80 surfactant solution and five concentrations of glyphosate-isopropylammonium (purity > 95%, Chem Service, West Chester, PA 19380) (0, 10, 33, 100, and 333 µM). Microtiter plates were then placed in a controlled environment chamber equipped with fluorescent bulbs (120 µmol m^{-2} s^{-1}) for 16 h at 22 C. Immediately after the 16-h incubation period, plates were placed in a -80 C freezer until the solution was frozen. The plates were thawed at 65 C for 30 min. Soon thereafter, 25 µL 1.25 N HCl was added to each well, and the plates were incubated at 65 C for 15 min. The leaf discs turned gray, indicating complete penetration of leaf by the acid. An extract aliquot of 25 µL was added to a new microtiter plate with 100 µL 0.25% periodic acid/0.25% sodium(meta)periodate solution. The plate was incubated at room temperature (25 C) for 90 min to allow shikimate oxidation. After incubation, the samples were mixed with 100 µL 0.6 N sodium hydroxide/0.22 M sodium sulfite. The optical density was measured spectrophotometrically at 380 nm within 30 min in a PowerWave XS microplate reader (Biotek, Winooski, VT 05404). Shikimate in µg mL^{-1} was determined based on a standard curve. The standard curve was determined using

nontreated plants and known concentrations of shikimate (Sigma-Aldrich, Saint Louis, MO 63103). Two experiments were conducted with four replications per population.

2.3.5 DNA, RNA and cDNA Isolation

Total DNA and RNA were extracted from frozen 2- to 4- leaf stage tissue of S, R1, R2, RC, and 2RC plants. Genomic DNA was extracted using DNeasy Plant Mini kit (Qiagen, Valencia, CA 91355), quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE 19810), and checked for quality by gel electrophoresis. DNA concentrations were adjusted to 2 ng μL^{-1} in HPLC-grade water. Total RNA was isolated with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA samples were treated with the RNase-Free DNase (Qiagen) and then purified using the RNeasy Plant Mini Kit (Qiagen). The concentration of total RNA was determined spectrophotometrically and quality of purified total RNA was established by TAE agarose gel electrophoresis. The cDNA was synthesized from 2 µg of total RNA in a 20 µL reaction volume according to the manufacturer's instructions (iScript cDNA Synthesis Kit, Bio-Rad Laboratories, Hercules, CA 94547).

2.3.6 *EPSPS* cDNA Sequencing

Total RNAs for cDNA cloning were isolated from S, R1 and R2 populations as previously described. First strand cDNA synthesis was then performed using 1 µg total RNAs and M-MuLV reverse transcriptase (New England BioLabs, Ipswich, MA 01938) in a final volume of 20 µL according to the manufacturer's instructions. A pair of primers (sense: 5'-TGGCTCAAGCTACTACCATCAAC-3'; antisense: 5'-ATATAGCTACTCAATGCTTGGCGAAC-3') were designed based on the EPSPS

coding sequence from Palmer amaranth (GenBank accession number FJ861242) (Gaines et al. 2010). PCR reaction contained 1 µL cDNA; 0.1 mM each of forward and reverse primers; 0.2 mM each of dATP, dCTP, dGTP, and dTTP; 2 mM $MgCl_2$; and 1 U of proof-reading PfuUltra high-fidelity DNA polymerase (Stratagene, La Jolla, CA 92037) with a 1× concentration of supplied buffer in a final volume of 50 µL. The cycling conditions were 2 min at 95 C followed by 30 cycles of 20 sec at 95 C, 20 sec at 55 C, and 1 min at 72 C, with a final extension of 3 min at 72 C. PCR products were ligated into pCR Blunt TOPO vector using Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Carlsbad, CA 92008). Ligations were transformed into *Escherichia coli* TOP10 cells and plated out on selection media. Single colonies of six transformants of R1 and R2, and 11 transformants from S were cultured overnight in liquid LB media for plasmid extraction. Plasmid DNAs were isolated for sequencing using the M13F and M13R primers performed at GeneWiz (South Plainfield, NJ 07080). Sequences for each population were assembled using Lasergene v. 10.0 SeqMan (DNASTAR, Madison, WI 53705). Multiple DNA sequence alignments of EPSPS, including sequences from both R and S Palmer amaranth populations (Gaines et al. 2010), GenBank accession numbers FJ861242 and FJ861243, were constructed using ClustalW v. 10.0 (DNASTAR). RNA extraction and amplification of the *EPSPS* gene was performed on two S plants, one R1, and one R2 plants.

2.3.7 Quantitative PCR

Quantitative real-time PCR (qRT-PCR) was used to measure *EPSPS* genomic copy number relative to *ALS* (acetolactate synthase) and cDNA expression level of *EPSPS* relative to *ALS* in S, R1, R2, RC, and 2RC Palmer amaranth plants according to

previously described procedures (Gaines et al. 2010). The *ALS* gene was used as a low-copy control gene with known monogenic inheritance in other *Amaranthus* species (Trucco et al. 2005). The primer EPSPS F (5'-ATGTTGGACGCTCTCAGAACTCTTGGT-3') and EPSPS R (5'-TGAATTTCCTCCAGCAACGGCAAC-3') were used to amplify the *EPSPS* gene of Palmer amaranth. ALS primers ALS F (5'-GCTGCTGAAGGCTACGCTCG-3') and ALS R (5'-GCGGGACTGAGTCAAGAAGTGC-3') were used as an internal standard to normalize the samples for differences in the amounts of DNA. The optimal annealing temperature was assessed using gradient PCR. The specificity of the qPCR assay was verified on agarose gel. All primer pairs generated a single band (data not shown).

Briefly, triplicate genomic DNA templates (10 ng) or triplicate cDNA templates (10 ng) were amplified in a 25 µL reaction volume using Syber-Green master mix (Bio-Rad) by the following thermoprofile on a MiniOpticon System (Bio-Rad): 94 C for 10 min, and then 40 cycles of 94 C for 15 sec and 60 C for 1 min, followed by increasing the temperature by 0.5 C every 5 sec to access the product melt-curve (to 94 C). Negative controls consisting of template with no primers and primers with no template were included. Threshold cycles (Ct) were calculated using CFX Manager 2.0 (Bio-Rad). Data were analyzed by relative quantification using $2^{-\Delta\Delta Ct}$ equation and *EPSPS* was calculated as ΔCt = (Ct, *ALS* – Ct, *EPSPS*), being expressed as $2^{\Delta Ct}$ fold increase in *EPSPS* copy number or expression relative to *ALS*.

This study was divided into two experiments. The first experiment measured *EPSPS* genomic copy number and expression level in the population. Consequently, collected samples consisted of a bulk of leaf material from at least 10 individuals per

population studied (S, R1, R2, RC, and 2RC). Two experiments were conducted with ten replications per population. The second experiment measured *EPSPS* genomic copy number by individuals of each population. Therefore, 30 individuals were sampled per population (S, R1, R2, and RC).

2.3.8 Protein Extraction and EPSPS Enzyme Activity Assay

Protein was extracted from 2- to 4- leaf stage tissue of S, R1, R2, RC, and 2RC plants. Protein extraction and EPSPS assay were conducted following the procedures of Sammons et al. (2007) and Webb (1992). This method assays EPSPS specific activity in a continuous inorganic phosphate release assay allowing an estimation of the inhibition constant for glyphosate by determining the I_{50}. The enzyme purine nucleotide phosphorylase (PNP) scavenges phosphate to phosphorylyze the nucleoside bond of 2-amino-6-mercapto-7-methyl-purine riboside (MESG) to create an increase in absorbance at 360 nm due to the release of the modified purine. Maintaining an excess of the coupling enzyme PNP, allows the rate of phosphate produced in the EPSPS reaction to be determined.

A quantity of 10 g of leaf tissue was ground to fine powder in a chilled mortar. Powdered tissues were homogenized in 50 mL of cold extraction buffer (100 mM MOPS, 5 mM EDTA, 10% glycerol, 50 mM KCl, and 0.5 mM benzamidine, pH 7.0) with 1% polyvinylpolypyrrolidone and 10 mM β-mercaptoethanol using a Polytron (PT 3100, Brinkmann Instruments, Westbury, NY 11590). An amount of 500 µL protease inhibitor (Sigma-Aldrich) was added to the extract and gently mixed. Subsequently, the extract was centrifuged for 20 min at 18,000 × g (Sorvall RC 6 Plus, Thermo Electron Corporation, Asheville, NC 28801) at 4 C. The supernatant was decanted through a

cheesecloth into a cold beaker. Powdered ammonium sulfate was slowly added to continuously stirred supernatant to make 45% w v^{-1} concentration and centrifuged for 20 min at 30,000 × g (Sorvall RC 6 Plus) at 4 C. Protein extracts were precipitated out of solution by slowing adding ammonium sulfate to a concentration of 70% (w v^{-1}) with gentle stirring, and then centrifuged for 20 min at 30,000 × g (Sorvall RC 6 Plus) at 4 C. Pellets were dissolved in about 3 mL of extraction buffer and dialyzed (Slide-A-Lyzer 10K Dialysis Cassettes, Thermo Scientific, Rockford, IL 61101) overnight in 2 L of dialysis buffer (100 mM MOPS and 5 mM EDTA, pH 7.0) mixed with 10% glycerol and 5 mM β-mercaptoethanol, at 4 C on a stir plate. Protein concentrations were determined using a Bradford assay kit (Bio-Rad).

Specific activities of EPSPS from plants were determined in the presence and absence of glyphosate using EnzCheck phosphate assay kit (Invitrogen). The assay buffer consisted of 100 mM MOPS, 1 mM $MgCl_2$, 10% glycerol, 2 mM sodium molybdate, and 200 mM NaF. The following reagents were added to a cuvette in the following order: 600 µL 2 × assay buffer, 300 µL of ultrapure (phosphate free) water, 0.17 mM MESG, 1 unit PNP, 1.07 mM phosphoenolpyruvate (PEP), 25 µL EPSPS sample extract and glyphosate concentrations. The S EPSPS protein extract was not diluted, but R1, R2, RC, and 2RC extracts were diluted to adjust total soluble protein (TSP) to a linear relationship between absorbance and time. Each sample was assayed in three replicates at glyphosate concentrations of 0, 0.3, 1, 3, 10, 33, 100, 333 and 1,000 µM to obtain the enzyme activity inhibition curve. The solution was allowed to react for 20 min to deplete phosphate contaminants before starting the EPSPS reaction. After obtaining a background phosphate release level, the final step was to add 0.41 mM shikimate-3-

phosphate. Phosphate release above background level was measured for 10 min at 360 nm in a UV-3101 spectrophotometer (Shimadzu North America, Columbia, MD 21046). The slope was calculated to determine the amount of phosphate (μmol) released per microgram of TSP per min (μmol Pi/μg TSP/min). Two experiments were conducted with three replications per population. The collected samples consisted of a bulk of leaf material from at least 10 individuals per population studied.

2.3.9 Western blot analysis for the detection of EPSPS protein

Total cellular protein was isolated from 2- to 4- leaf stage tissue of S, R1, R2, RC, and 2RC plants. A quantity of 0.25 g of leaf tissue was ground to fine powder in a chilled mortar. Powdered tissues were homogenized in 500 μL cold extraction buffer (100 mM MOPS, 5 mM EDTA, 10% glycerin, and 50 mM KCl, pH 7.0) with freshly added 0.05 tablet protease inhibitor (Roche Applied Science, Indianapolis, IN 46250). The samples were thawed at room temperature and vortexed for 10 sec. The mixtures were kept cold and placed on a Geno/Grinder mechanical shaker (SPEX SamplePrep, Metuchen, NJ 08840) for 1 min at 1,750 stroke per min. This step was repeated three times. Subsequently, the extract was centrifuged for 5 min at 18,00 × g. The supernatant was transferred to a new vial and it was centrifuged for 5 min at 18,400 × g. This step was repeated one more time and extract was stored at -80 C. Protein concentrations were determined using a Bradford assay kit (Bio-Rad).

Western blot analysis for the detection of EPSPS protein were performed diluting soluble protein in 950 μL Laemmli premixed protein sample buffer (Bio-Rad) and 0.71 M β-mercaptoethanol to reach a final concentration of 15 μg μL^{-1}. The sample was transferred to a heating block at 90 C for 4 min and then allowed to cool to room

temperature. The soluble protein was separated on a 12% Tris-HCl precast polyacrylamide gel (Bio-Rad). After separation, the proteins were blotted onto 0.45 μM Immobilon-FL PVDF membranes (EMD Chemicals, San Diego, CA 92121) and the membrane was equilibrated with transfer buffer (0.2% methanol and 1x Tris/Glycine buffer, Bio-Rad). The membrane was washed with 1× Tris-buffered saline (TBS) (Bio-Rad) and incubated overnight at 4 C with 3% gelatin from cold water fish skin (Sigma-Aldrich). Western blot was probed with an EPSPS specific antibody (2 mg mL^{-1}) developed against recombinant maize EPSPS (Monsanto Company) at dilution 1:2,000 and re-probed against Alexa Fluor 635 goat anti-rabbit (Invitrogen) at dilution 1:2,000. Within 20 min of the final wash with 1 × TBS buffer and Tween-20, the array was scanned and analyzed on a Pharos FX Plus Molecular Imager (Bio-Rad) system equipped with an external 635 nm laser (Bio-Rad). Western blots were quantified by densitometric analysis using Quantity One analysis software (Bio-Rad) and represented as CNT (counts mm^{-2}). The collected samples consisted of a bulk of leaf material from at least 10 individuals per population studied with three replications per population.

2.3.10 Pollen Grain and Spermatic Cells Isolation

A large proportion of monogenic *EPSPS* progeny resulted from controlled crosses between Female-S x Male-R (R1 and R2). Consequently, differences in transmission rates between male and female gametes were proposed as an explanation for this phenomenon. To test it pollen grain were isolated following the procedures of Becker et al. (2003) with minor modifications, briefly described below. S, R1, and R2 plants were grown as previously described; about 10 inflorescences per plant were cut from the plants and placed in a humid chamber (90% humidity) for 2 h to ensure complete hydration of

pollen grains. The flower heads were then agitated in 10 mL of pollen sorting buffer (PSB: 10 mM $CaCl_2$, 1 mM KCl, 2 mM MES, 5% sucrose, pH 6.5, in double-distilled water). The pollen solution was filtered through a 30 µm nylon mesh. In a second filtration step using a 10 µm nylon mesh, pollen and other components larger than 10 µm were retained on the filter. Palmer amaranth pollen grain has a diameter of 20 µm (Franssen et al. 2001). They were washed from the filter in 10 mL of PSB and centrifuged at 450 × g (Sorvall RC 6 Plus) for 2 min. The supernatant was removed and the washing step was repeated twice. The pellet was re-suspended in 5 mL of PSB. The pollen was allowed to settle for 30 min and the supernatant, including small impurities, was removed. The pollen pellets, approximately 100 mg wet weight, were frozen in liquid nitrogen and DNA was extracted as previously described (section 2.3.5). Quantitative RT-PCR was used to measure *EPSPS* genomic copy number relative to *ALS* in pollen and respective leaf tissue as described above (section 2.3.7). The expected *EPSPS* gene copy for pollen sample would be a half of respective tissue sample. A few drops of the pollen:PSB mixture were placed on a scanning electron microscope (SEM) scrubbed, smeared, and allowed to dry. The pollen samples then were coated with palladium, viewed, and photographed with an SEM (Jeol JSM-5600, Jeol USA, Peabody, MA 01961) to estimate yield and confirm purity. Image was generated by Dr. Franck Dayan and J'Lynn Howell (USDA/ARS).

Pollen isolation was conducted following the procedures of Russell (1986), briefly described below. S, R1, and R2 plants were grown as previously described to isolate their sperm cells. Pollen was collected from flowers onto weighing paper by rolling the inflorescence between the thumb and forefinger. The aim of isolating the

spermatic cell was to study the pollen-mediated dispersal of *EPSPS* genomic copy number relative to *ALS*, as the grains of pollen contain a haploid vegetative cell and a haploid generative cell, together these cells form the microgametophyte (Hesse 2009; Tanaka 1993).The generative cell divides by mitosis to form the two sperm cells (haploids) completely enclosed within the vegetative cell cytoplasm either before pollen is shed (tricellular pollen) or within the pollen tube (bicellular pollen) (Borges et al. 2008; Hesse 2009; Kato 2001; Nagata et al. 1997).). The collected pollen grains were immersed into a 1.5 mL solution of 20% sucrose (w/v) and allowed to burst for 20 min. The solution was filtered with vacuum through a 10 µm nylon filter to remove the pollen cell wall and an additional 0.5 mL of 20% sucrose were used to rinse the filter. The 2.0 mL solution that passed through the filter is then layered over a chilled 1 mL 30% sucrose (w/v) solution in a 15 mL centrifuge tube, allowed to cool in an ice bucket for 5 min, and centrifuged at 3600 g (Sorvall RC 6 Plus) for 15 min at 4 C. The sperm cells were selectively separated into the 30% sucrose layer. Light microscopy of the sperm cells was conducted using a Nikon eclipse E600 microscope (Nikon, Tokyo, Japan) equipped with interference contrast microscopy and fluorescence microscopy (Nikon). The condition of the isolated sperm cells was evaluated using blue-fluorescent DAPI (4', 6-diamidino-2-phenylindole) nucleic acid stain (Life Technology, Grand Island, NY 14072), which will preferentially stain dsDNA, fluorescence emission of ~ 460 nm, and by staining with FITC (fluorescein isothiocyanate) (Sigma-Aldrich) with is reactive towards nucleophiles, fluorescence emission of ~521 nm.

2.3.11 Facultative Apomixis Hypothesis

A large proportion of monogenic *EPSPS* progeny resulted from controlled crosses between Female-S x Male-R (R1 and R2). Consequently, facultative apomixis was proposed as an elucidation for this phenomenon. To test apomixis in Palmer amaranth parent (S, R1 and R2) populations, pollination bags were placed over main inflorescences prior to emergence and were examined daily to determine their sex. The male plants were discarded and the female plants were isolated in different geographically located greenhouses to ensure repeatability of the apomixis test and exclude external sources of pollen contamination. A total of 44 S individuals were grown, Summer/Spring 2011, in Oxford, MS, 36 R1 individuals in Starkville, MS, and 38 R2 individuals in Abbeville, MS. Palmer amaranth inflorescence spikes were hand-harvested when the majority of the seed possessed coats that were black in color (seed maturity). Palmer amaranth seed were air-dried, cleaned, and stored at 10 C as previously described. Seed, which passed through the 30 mesh screen, but were sufficiently large to not pass through the 20 mesh screen, were counted by an electronic seed counter (Model 850-2, The Old Mill Company, Savage, MD 20763) with small seed bowl at maximum sensitivity. This experiment was repeated two more times without using different geographically located greenhouses and performing a visual (not quantitative) evaluation of presence vs. absence of seed formation.

Samples of approximately 100 seed per 10 apomictically produced population per parental population were planted on moistened commercial potting soil in plastic trays as previously described, covered with a thin layer of additional soil and placed in 10 C cold room for seven days. The trays were transferred to germination chambers for overnight at

25 C. Germination was stimulated by exposing the trays to two cycles of the following temperature regime: 18 C for 6 h night and 30 C for 6 h, 42 C for 6 h, and 30 C for 6 h, along with 18 h light (Gaines 2009). After germination, trays were kept under 25/20 C light/dark temperature with a 12-h photoperiod and plants were grown until sex segregation ratio was determined.

This study was divided in two experiments. The first experiment quantified the amount of seed produced apomictically by 44 S, 36 R1, and 38 R2 individuals and determined the female : male ratio of apomictically produced progeny. The second experiment qualitatively verified the presence or absence of apomictic seed produced by 10 individuals per population and it was repeated twice.

2.3.12 Intraspecific Genetic Diversity and Relationships

A genetic marker method that would confirm apomixis in Palmer amaranth was selected based on information from available literature (Burgos et al. 2012; Chan and Sun 1997; Chandi et al. 2012a; Giacomini et al. 2012; Lanoue et al. 1996; Lee et al. 2009; Ma et al. 2008; Popa et al. 2010; Wassom and Tranel 2005; Wetzel et al. 1999; Xu et al. 2011). RAPD (random-amplified polymorphic DNA) and ISSR (inter-simple sequence repeat, microsatellite) methods were used because they can provide up to a population level of identification of intraspecific variation.

DNA extraction was carried out as previously described from three different plants of R1 population (*n* = 3) and diluted to 2 ng μL^{-1}. RAPD analyses were performed using 12 decamer primers (Table 2.1) randomly selected from a list of 100 primers (NAPS Unit, University of British Columbia, Biotechnology Laboratory, Vancouver, BC, Canada). ISSR analyses were performed using 15 primers (Table 2.1) randomly selected

from a list of 100 primers (NAPS Unit, University of British Columbia) and five of Natascha Techen (National Center for Natural Products, University of Mississippi, USA) design. PCR reaction contained 10 ng DNA; 0.1 μM each of forward and reverse primers; 200 μM each of dATP, dCTP, dGTP, and dTTP (Promega, Madison, WI 53593); 1.5 mM $MgCl_2$; and 1 unit of Platinum Taq DNA polymerase (Invitrogen) with a 1 × concentration of supplied buffer in a final volume of 13.6 μL. The cycling conditions included 3 min at 94 C followed by 45 cycles of 30 sec at 94 C, 30 sec at 50 C, and 3 min at 72 C, with a final extension of 7 min at 72 C in a thermal cycler (PTC-225, MJ Research, Waterton, MA 02472). PCR products were analyzed by electrophoresis on a 2% TAE agarose gel stained with ethidium bromide and visualized under UV light using Quantity One (Bio-Rad, version 4.3.0). The sizes of the PCR products were compared to the molecular size standard 1 kb plus DNA ladder (Invitrogen).

2.3.13 Statistical Analysis

The experiments discussed in sections: 2.3.6 (*EPSPS* cDNA sequencing), 2.3.10 (pollen grain and spermatic cells isolation), 2.3.11 (facultative apomixis hypothesis), and 2.3.12 (intraspecific genetic diversity and relationships), were not statistically evaluated due to the dependent variables evaluated. All other data were analyzed by ANOVA via the PROC GLM statement using SAS software (version 9.3, SAS Institute, Cary, NC 27513) to determine the main effects and interactions of the factors at $P < 0.05$. No significant experiment effect was observed in repeated experiments; therefore, data from those experiments were pooled.

2.3.13.1 Glyphosate Dose-Response Bioassay and EPSPS Enzyme Activity Assay

Where ANOVA indicated significant differences between treatments, treatment means were separated using Fisher´s Protected LSD test at P = 0.05 using SAS software. In addition, non-linear regression was applied using a log-logistic model (Seefeldt et al. 1995) for the glyphosate dose-response assay (Equation 2.1) and for the EPSPS enzyme activity assay a three parameter log-logistic model was applied where the *L* parameter was excluded from equation 2.1.

$$y = L + \frac{U - L}{\left[1 + \left(\frac{D}{GR_{50}}\right)^{s}\right]} \tag{2.1}$$

Where: For glyphosate dose-response assay, y represents shoot fresh weight reduction as compared to nontreated plants in percentage at herbicide rate D, L is the mean response at very high herbicide rate (lower limit), U is the mean response when the herbicide rate is zero (upper limit), s is the slope of the line at GR_{50}, and GR_{50} is the herbicide rate required for 50% growth reduction; for EPSPS enzyme activity assay, y represents EPSPS enzyme activity (μmol Pi μg^{-1} TSP min^{-1}) at glyphosate concentration D, U is the mean inhibition when the glyphosate concentration is zero (upper limit), s is the slope of the line at IC_{50}, and IC_{50} is glyphosate concentration that reduced enzyme activity by 50%. The level of resistance was determined by calculating the ratio of GR_{50} of the R, RC and 2RC populations to the one of the S population for the glyphosate dose-response assay. The IC_{50} ratio of the R and RC populations to the one of the S population for the EPSPS enzyme activity assay was calculated.

Experience shows that usually a logistic dose-response curve reasonably describes what happens in crops and weeds in response to dose of a herbicide (Ritz and Streibig 2006). The estimate of the regression parameters was obtained using Sigma Plot (version 11, Systat Software, San Jose, CA 95110) and tested for significance using the *t*-test method ($P < 0.05$). For each parameter, the null hypothesis, H_0: parameter = 0, was tested against the alternative hypothesis, H_a: parameter ≠ 0. Moreover, three and four parameters log-logistic models were tested for lack-of-fit using R software (version 2.15.2, R Foundation for Statistical Computing, Vienna, Austria) using package drc, drm and modelFit function.

The whole plant dose-response assay and the EPSPS enzyme activity assay, then was used to determine any differences in potency between populations and the susceptible population at the respective GR_{50} or IC_{50} effect level is different according to *t*-Student test at $P < 0.05$. The null hypothesis, H_0: GR_{50} or IC_{50} populations / GR_{50} or IC_{50} susceptible population = 1, was tested against the alternative hypothesis, H_a: GR_{50} or IC_{50} populations / GR_{50} or IC_{50} susceptible population ≠ 1. This test was performed using the open-source R software using package drc, drm function, and the comparisons were given by means of the selectivity index (SI) function.

The data from EPSPS enzyme activity assay for populations S, R1, R2 and first reciprocal crosses were regressed against *EPSPS* relative copy number and *EPSPS* cDNA relative expression level.

2.3.13.2 Shikimate Bioassay, *EPSPS* Gene Copy Number and expression, and EPSPS Protein Quantification

Data variance was visually inspected by plotting residuals to confirm homogeneity of variance prior to statistical analysis. Where ANOVA indicated significant differences between treatments, means separation were performed using Fisher's protected least significant difference (LSD) test at P = 0.05 using SAS software. The data from *EPSPS* relative copy number were regressed against *EPSPS* cDNA relative expression level.

2.4 Results and Discussion

2.4.1 Glyphosate Dose-Response Bioassay

The F-test in the ANOVA was significant (P < 0.0001) for the pairing of dependent variable (fresh weight reduction) with main effect terms (populations and glyphosate dose) and interaction terms. The fresh weight reduction means by population were separated in eight groups (A, B, C, D, E, F, G, and H) using LSD test at P = 0.05 (2.42). The GR parents (R1 and R2) had the lowest fresh weight reduction (group A), followed in increasing order of reduction by R1/S (group B), R1/S//R1/S (group C), R2/S and S/R1//S/R1 (group D), R2/S//R2/S (group E), S/R1 (group F), S/R2//S/R2 and S/R2 (group G), and S (group H). Consequently, a pattern was observed where the reciprocal crosses generated by Female-S × Male-R (S/R) and by Female-S/R × Male-S/R (S/R//S/R) had the highest fresh weight reduction. This variability may be attributed to maternal effect, as the direction of the cross affected the level of resistance, GR_{50} ratio (Table 2.2). Chandi et al. (2012b) studied reciprocal crosses (R × S and S × R) progenies of Palmer amaranth and found that glyphosate resistance was not fully dominant over

susceptibility. Moreover, Sosnoskie et al. (2012) found that GR trait can be transferred via pollen movement in Palmer amaranth, but they found no GR offspring when the only source of pollen within 600 m was resistant male plants at the center of a 30-ha cotton field.

Shoot biomass in each Palmer amaranth population decreased as glyphosate rate increased (Table 2.2, Figure 2.1). However, there were different dose-responses between genetic populations, enabling differentiation among the majority of the genetic populations with the exception of S/R1 vs. S/R2//S/R2 and R2/S vs. S/R1//S/R1. This was accomplished by comparing the relative potencies among accessions at GR_{50} response level (SI) (Table 2.2). Also, the parameters for the lower limits were not different from zero according to t-Student test at $P < 0.05$ for dose-response analysis of populations S/R1, R2/S, S/R2, S/R2//S/R2, and S (Table 2.2). Consequently, data for those populations could be described by a model with zero as the lower limit (Ritz and Streibig 2006). A test for lack-of-fit was not significant ($P = 0.74$), indicating that the four parameters log-logistic model (Seefeldt et al. 1995) is appropriate to describe the data.

The GR_{50} estimates from the log-logistic response model for S population was 94 g ae ha^{-1} glyphosate, while GR_{50} for R1 was 17-fold (1,623 g ha^{-1}) greater and 14-fold (1,369 g ha^{-1}) greater for R2. These estimates are similar to 1,520, 1,300, and 90 g ae ha^{-1} glyphosate estimated for C1 and T4 (GR), and S populations of Palmer amaranth from Mississippi by Nandula et al. (2012). This level of glyphosate resistance is higher than that reported for a GR Palmer amaranth population from Georgia that had a GR_{50} of 1,200 g ha^{-1} and was eight-fold more resistant than a susceptible population with a GR_{50}

of 150 g ha^{-1} (Culpepper et al. 2006). A common occurrence in resistant populations was the stimulation of axillary growing points and continued growth at high glyphosate rates (1,680 and 3,360 g ha^{-1}). The same pattern was observed by Culpepper et al. (2006) and Nandula et al. (2012).

The estimated GR_{50} for each RC was different from each parent but closer to its maternal parent than the midpoint, with the R1/S, S/R1, R2/S, and S/R2 values of 1,138, 464, 759, and 363 g ha^{-1}, respectively (Table 2.2 and Figure 2.1). The 2RC dose-response was intermediate between those of the R and S populations (R1/S//R1/S: 976.44, S/R1//S/R1: 664, R2/S//R2/S: 570, and S/R2//S/R2: 433 g ha^{-1}), containing both highly susceptible and highly resistant individuals and a range of intermediate phenotypes (Figure 2.1). These results were expected on the basis of the previous screening studies (glyphosate at low rate, 420 g ae ha^{-1}) at the sub-set of the population as previously described (section 2.3.2), suggesting that the mode of inheritance of GR trait was maternally affected. This pattern was previously observed by other authors when studying the inheritance of glyphosate resistance in Palmer amaranth (Chandi et al. 2012b; Gaines 2009; Gaines et al. 2011; Giacomini et al. 2011). Inheritance of glyphosate resistance was suggested to be incompletely dominant, nuclear inherited, and might follow a polygenic additive pattern in populations of Palmer amaranth from Georgia and North Carolina (Chandi et al. 2012b; Gaines 2009). Although, both authors found an unpredictable behavior in the inheritance of glyphosate resistance in some genetic populations studied. Gaines (2009) findings stimulated us to study the mechanism of resistance and the mode of inheritance of evolved resistance in GR Palmer amaranth populations from Mississippi.

2.4.2 Shikimate Bioassay

The F-test in the ANOVA was significant ($P < 0.0001$) for the pairing of dependent variable (shikimate level) with main effect terms populations ($P < 0.0001$) and glyphosate concentration ($P < 0.0029$), and interaction terms ($P < 0.0016$). The shikimate level means by population were grouped using LSD test at $P = 0.05$ (2.94). The S biotype had the highest shikimate accumulation mean among glyphosate concentrations (14.51 $\mu g\ mL^{-1}$) differing from the grouped R2 (1.12 $\mu g\ mL^{-1}$) and R1 (- 0.06 $\mu g\ mL^{-1}$) populations. In addition, the increase in shikimate production with glyphosate concentration was only observed at S population ($P < 0.0001$), indicating that the level of shikimate accumulation was not different among glyphosate concentrations for R1 ($P < 0.9999$) and R2 ($P < 0.9427$) with the increment of glyphosate concentration.

Plants from the S population accumulated shikimate at all four glyphosate doses, whereas plants from the R1 population did not accumulate shikimate at 10 and 33 µM glyphosate (Figure 2.2). There was some shikimate accumulation in the R2 population at all doses and in R1 at the two highest doses tested (Figure 2.2). Nandula et al. (2012) conducted a similar study using higher doses of glyphosate (500 and 1,000 µM) and populations C1 and T4 (Appendix A), but these populations went through a different process to yield their second generation resistant populations (C1B1 and T4B1). They found that the T4B1 population pattern of shikimate accumulation followed a similar trend as S population. The authors suggested that the difference of accumulation of shikimate in the C1B1 and T4B1 populations could be due to a different mechanism of resistance. Other authors reported shikimate detection in glyphosate-treated, glyphosate-susceptible Palmer amaranth populations from Georgia, but none or some in resistant

populations (Culpepper et al. 2006; Gaines 2009; Gaines et al. 2011). However, shikimate accumulation was documented in both glyphosate-resistant and -susceptible populations from Tennessee (Steckel et al. 2008).

2.4.3 *EPSPS* cDNA Sequencing

There was little to no difference among sequences from two R1 (six clones) and two R2 (six clones) individuals, consequently the consensus sequence represents residues common to all glyphosate-resistance sequences (R_ consensus) (Figure 2.3). Alignment of consensus sequences from R and S individuals showed several polymorphism found in all S sequences in the alignment contig when compared with the reference S sequence (FJ861242, Gaines et al. 2010) (Figure 2.3). This could have been due to the bulking of seed from several plants at the time of initial collection of this population in Mississippi.

The cDNA sequence analysis of the *EPSPS* gene in both GR (R1 and R2) populations revealed several nucleotide substitutions resulting in silent mutations when comparing with the S population sequence. When aligned, the R and S sequences were very similar to the Palmer amaranth reference sequences, FJ861242 (S) and FJ861243 (R) (Gaines et al. 2010). However there were three single nucleotide polymorphisms (SNP) between S and R clones (adenine/thymine - contig position 36, cytosine/guanine - contig position 72, and guanine/adenine - contig position 866, respectively) resulting in a glutamine to histidine, histidine to glutamine, and arginine to lysine amino acid substitution, respectively (Figure 2.3 and 2.4). The two SNP at contig position 36 and 72 were part of the amino acid sequence of transpeptidase, consequently the only SNP (contig position 866) that resulted in a non-synonymous mutation was an arginine to

lysine amino acid substitution at position 215, based on the maize mature EPSPS numbering system (Figure 2.4).

The arginine : lysine at 215 SNP was not observed when aligning R with the S and R reference sequences (FJ861242 and FJ861243) (Figure 2.4), suggesting that they do not confer resistance. None of these SNPs have been shown previously to confer resistance to glyphosate (Perez-Jones and Mallory-Smith 2010; Powles and Preston 2006). Moreover, no mutation was observed in the R cDNA at the proline 106 residue recognized to confer glyphosate resistance in other weed species (Figure 2.4).

2.4.4 *EPSPS* Gene Amplification Correlates with *EPSPS* Gene Copy Number and Level of Glyphosate Resistance

EPSPS genomic copy number and expression level at the population level (bulked samples of genomic DNA and cDNA from several plants per population) was determined. *EPSPS* genomic copy number of individuals of each population S, R1, R2, and RC (30 individuals sampled per population) were also determined. Bulked samples of genomic DNA from several alfalfa plants per population were used as templates in PCR reaction to rapidly estimate genetic relatedness among populations (Yu and Pauls 1993).

In the bulked sample experiment, the F-test in the ANOVA was significant ($P < 0.0001$) for the pairing of dependent variable (*EPSPS* relative copy number) with main effect term populations. The copy number means by population were separated in eight groups (A, B, C, D, E, F, G. and H) using LSD test at $P = 0.05$ (12.61). The GR parent (R1) and R1/S//R1/S had the highest *EPSPS* relative copy number (group A), followed in decreasing order by R1/S//R1/S and R1/S (group B), R1/S and R2 (group C), R2 and

R2/S (group D), R2/S and S/R1 (group E), S/R1 and R2/S//R2/S (group F), R2/S//R2/S, S/R2//S/R2, and S/R1//S/R1 (group G), and S/R2//S/R2, S/R1//S/R1, S/R2, and S (group H). Genomic estimation of *EPSPS* gene copy number relative to *ALS* using qRT-PCR showed that R1 (59 relative copies) and R2 (33 relative copies) populations contained multiple copies of the *EPSPS* gene, but the S population contained a single copy of the *EPSPS* gene (Figures 2.5 and 2.6). Gaines et al. (2010) studied a GR population from Georgia in which their genomes contained from 5-fold to more than 160-fold copies of the *EPSPS* gene than their susceptible plant. Chandi et al. (2012b) performed a similar study in a resistant population from North Carolina and found 22 to 63 relative copies of *EPSPS* gene. The *EPSPS* copy number observed for each bulked RC was different from each parent but closer to its maternal parent than the midpoint, with the R1/S, S/R1, R2/S, and S/R2 values of 43, 19, 30, and 1, respectively (Figure 2.5). Giacomini et al. (2011) indicated a wide range, from 1 to 80, in *EPSPS* copy number in the majority of their reciprocal crosses (R × S and S × R) of GR Palmer amaranth populations studied. Gaines et al. (2011) observed a range of *EPSPS* genomic copy numbers from a single copy to 39 relative copies in six individuals from the S × R cross. The 2RC genomic copy number varied greatly and was an intermediate value between those of the R and S populations (R1/S//R1/S: 53, S/R1//S/R1: 5, R2/S//R2/S: 15, and S/R2//S/R2: 6), containing both highly susceptible and highly resistant individuals and a range of intermediate genotypes (Figure 2.5). Gaines et al. (2010) observed one individual Palmer amaranth (S/R × S/R) that had a higher relative copy number than the sum of their relative copy number from both parents. However, the S × R plants were verified to be resistant by treatment with 400 g ha^{-1} glyphosate, thus probably not selecting

apomictically-generated individuals. Consequently, the pattern observed at the dose-response study (section 2.4.1) was also observed when quantifying the number of copies of the *EPSPS* gene; the reciprocal crosses generated by Female-S × Male-R (S/R) and by Female-S/R × Male-S/R (S/R//S/R) had lower number of relative copies then the reciprocal crosses generated by Female-R × Male-S (R/S) and by Female-R/S × Male-R/S (R/S//R/S) (Figure 2.5).

Quantitative RT-PCR on cDNA revealed that the single copy of *EPSPS* gene was proportionally expressed, with a 1:1 correlation to *EPSPS* gene copy:*EPSPS* transcript richness (Figure 2.5 and 2.6). The *EPSPS* expression level relative to *ALS* showed a strong and positive correlation ($r = 0.94$, $P < 0.0001$, excluding R2 data) between *EPSPS* relative copy number and *EPSPS* relative expression (Figure 2.6). Those correlations were previously observed by Gaines et al. (2010). Moreover, the F-test in the ANOVA was significant ($P < 0.0001$) for the pairing of dependent variable (*EPSPS* expression level) with main effect term populations. The expression level means by population were separated in five groups (A, B, C, D, and E) using LSD test at $P = 0.05$ (14.61). The GR parent (R1) had the highest *EPSPS* expression level (group A), followed in decreasing order by R1/S//R1/S (group B), R1/S, R2/S, and S/R1//S/R1 (group C), R2/S, S/R1//S/R1, R2/S//R2/S, and R2 (group D), and S/R1//S/R1, R2/S//R2/S, R2, S/R2//S/R2, S/R2, S/R1, and S (group E). The *EPSPS* copy number was positively correlated with the gene expression level, consequently the pattern previously described was observed where the reciprocal crosses generated by Female-S × Male-R (S/R) and by Female-S/R × Male-S/R (S/R//S/R) had lower *EPSPS* expression level then the reciprocal crosses generated by Female-R × Male-S (R/S) and by Female-R/S × Male-R/S (R/S//R/S)

(Figure 2.5). An exception was R2 population that had a positive correlation between *EPSPS* gene copy and *EPSPS* expression level, but not in the same fashion ($r = 0.87$, $P < 0.0001$, including R2 data) as the other populations studied (Figure 2.6). This result indicates that another mechanism of resistance may be involved in the R2 population in addition to the increased *EPSPS* gene copy. Nandula et al. (2012) generated a second generation resistant populations (C1B1 and T4B1) using the populations C1 and T4. They found that the T4B1 pattern of shikimate accumulation followed a similar trend as that of S population, whereas C1B1 accumulated negligible shikimate levels; suggesting that the difference of accumulation of shikimate in the C1B1 and T4B1 could be due to a different mechanism of resistance. The T4B1 absorbed less ^{14}C-glyphosate in comparison with the C1B1 and their S populations at 24 HAT. Furthermore, more absorbed glyphosate accumulated in the root of the T4B1 than that of C1B1 and their S populations at 48 HAT. Another possibility would be the interference of siRNA (small interfering RNAs) in the expression of the multiple copies of *EPSPS* gene in R2 population (Kittler and Buchholz 2003).

In the second experiment, the F-test in the ANOVA was significant ($P < 0.0001$) for the pairing of dependent variable (*EPSPS* copy number) with main effect term population. The *EPSPS* genomic copy number means by population were separated in four groups (A, B, C, and D) using LSD test at $P = 0.05$ (11.47). The GR parent (R1) had the highest copy number (group A, 72 relative copies), followed in decreasing order by R2 and R1/S (47 and 38 relative copies, respectively) (group B), R1/S and R2/S (38 and 27 relative copies, respectively) (group C), and S/R2, S/R1 and S (7, 7, and 1 relative copies, respectively) (group D) (Figure 2.7). Consequently, a pattern was observed where

the reciprocal crosses generated by Female-S × Male-R (S/R) had the lowest *EPSPS* genomic copy number, statistically regressing to the number of copies of the S population (Figures 2.5, 2.6, and 2.7). This observation follows the pattern observed at 2.4.1 section and in the first experiment (bulk sample) where the direction of the cross affected the level of resistance and number of multiple copies of the *EPSPS* gene (Figures 2.1, 2.5, 2.6, and 2.7). Furthermore, 73% of S/R1 and 70% of S/R2 individuals analyzed had a single copy of the *EPSPS* gene (Figure 2.7). Based on this experiment, we hypothesized that the mode of inheritance of GR trait and the stability of *EPSPS* gene amplification transmission across generations was partly due to facultative apomixis reproduction (discussed below in section 2.4.7). This phenomenon was previously observed in Palmer amaranth, as non-hybrid progeny resulted from crosses between Palmer amaranth and common waterhemp (*Amaranthus rudis* Sauer) (Trucco et al. 2007). This would explain the great variation in *EPSPS* gene copy number in plants from the R1 and R2 populations and the regression to a single copy of the gene in reciprocal crosses generated by Female-S × Male-R (S/R). Greater variability in *EPSPS* copy numbers in GR populations was observed in similar studies (Chandi et al. 2012b; Gaines et al. 2010); likewise there was greater variability in susceptibility in RC progeny (Gaines 2009; Gaines et al. 2011). Consequently, facultative apomixis could function to maintain the accumulated large number of gene copies in the population (Gaines et al. 2010) and explain the observation of a greater number of *EPSPS* single copied gene individuals in S/R RC.

The higher number of copies of the *EPSPS* gene resulted in over-production of EPSPS and, consequently, higher levels of resistance, confirming the occurrence of gene

amplification as the mechanism conferring resistance to glyphosate in two populations of Palmer amaranth from Mississippi.

2.4.5 EPSPS Protein Activity and Quantity Correlates with *EPSPS* Gene Copy Number and Level of Glyphosate Resistance

EPSPS specific activity in each Palmer amaranth population was inhibited by glyphosate concentrations (Table 2.3, Figure 2.8). The amounts of glyphosate needed to reduce the EPSPS activity by 50% (I_{50}) were similar in all samples analyzed, ranging from 5.5 to 55 μM glyphosate (Table 2.3). Similar results were obtained in GR Italian ryegrass (5.5 μM glyphosate) and Palmer amaranth (24 μM) (Gaines et al. 2010; Salas et al. 2012). However, there were different dose-responses between genetic populations, enabling differentiation among S vs. R1 (P = 0.03), S vs. R1/S (P = 0.04), and S vs. S/R1 (P = 0.03). This was accomplished by comparing the relative potencies among populations at IC_{50} response level (SI) (Table 2.3). Although, statistical analyses of the IC_{50} on the specific activity indicate that are some significant differences, these differences do not account for the differences in the level of resistance. For example, the IC_{50} of S population is greater than all the other populations; this is probably due to the greater differences in the overall EPSPS specific activity in the population with multiple copies in comparison with S population. Moreover the IC_{50} ratio for all populations was smaller than 1. A test for lack of fit was significant (P = 0.0007) indicating that the 3-parameter log-logistic model was not the most appropriate to describe the data for all populations, suggesting that another model may be more appropriate mainly for populations S, S/R1 and S/R2 that were inhibited by lower glyphosate concentrations and showed a more linear relationship. A 3-parameter log-logistic model was used to be

consistent with other reports on glyphosate resistance (Gaines 2009, Gaines et al. 2010, Salas et al. 2012), allowing the comparison between their studies and ours.

The F-test was performed separately for the relationship between EPSPS enzyme activity and populations in the absence of inhibitor (Figure 2.9), glyphosate, because the EPSPS enzyme activity was inhibited by glyphosate at different concentrations in all populations studied (Figure 2.8). This occurs due to the same interaction between EPSPS enzyme and inhibitor (glyphosate), as demonstrated above (section 2.4.3). The F-test in the ANOVA was significant ($P < 0.0001$). The EPSPS enzyme activity means by population were separated in seven groups (A, B, C, D, E, F, and G) using LSD test at $P = 0.05$ (0.01). The GR parent (R1) had the highest enzyme activity in the absence of glyphosate (group A), followed in decreasing order by the other GR parent (R2) (group B), R1/S//R1/S (group C), R1/S (group D), R2/S and R2/S//R2/S (group E), S/R1//S/R1, S/R2//S/R2, S/R2, and S/R1 (group F), and S/R1 and S (group G). The EPSPS specific activity showed a solid and positive correlation between *EPSPS* relative copy number ($r = 0.87$, $P < 0.0001$, including and excluding R2 data) and *EPSPS* expression level relative to *ALS* ($r = 0.97$, $P < 0.0001$, excluding R2 data; $r = 0.84$, $P < 0.0001$, including R2 data) (Figure 2.10).

In the absence of glyphosate, the specific activity of EPSPS in the R2 and R1 populations ranged from 0.12 to 0.19 μmol μg^{-1} protein min^{-1}, respectively, while that of the S plants was 0.02 μmol μg^{-1} protein min^{-1} (Figure 2.9). A similar specific enzyme activity was observed in glyphosate-resistant and -susceptible Palmer amaranth from Georgia (Gaines et al. 2010) and Italian ryegrass from Arkansas (Salas et al. 2012). R1 and R2 plants had a nine to six-fold increase, respectively, in EPSPS enzyme activity

relative to the enzyme activity in S plants. Gaines et al. (2010) found a 16-fold increase in specific activity between glyphosate-resistant and -susceptible populations of Palmer amaranth. Moreover, Salas et al. (2012) found on average six-fold higher basal enzyme activity in Italian ryegrass resistant to glyphosate in comparison with the susceptible one.

EPSPS protein quantity was measured with immunoblotting. The F-test in the ANOVA was significant (P < 0.0001) for the pairing of dependent variable (CNT) with main effect terms populations (P < 0.0001). The CNT means by population were separated in five groups (A, B, C, D, and E) using LSD test at P = 0.05 (6,531.2). The GR parents (R1 and R2) and R1/S had the highest CNT mean among populations (group A, ranging from 30,900 to 36,900 CNT), followed in decreasing order by S/R1, R2/S, and R1/S//R1/S (group B, ranging from 14,000 to 17,500 CNT), R1/S//R1/S, R2/S//R2/S, and S/R1//S/R1 (group C, ranging from 8,400 to 14,000 CNT), R2/S//R2/S, S/R1//S/R1, S/R2//S/R2, and S/R2 (group D, ranging from 5,900 to 9,800 CNT), and S/R1//S/R1, S/R2//S/R2, S/R2, and S (group E, ranging from 2,900 to 8,400). The EPSPS signal in plants increased with *EPSPS* relative copy number (Figure 2.9). The EPSPS protein quantity and EPSPS specific activity were positively correlated with the *EPSPS* gene copy number (Figure 2.9). Gaines et al. (2010), studying a Palmer amaranth GR population from Georgia, observed a positive correlation between the level of saturation of EPSPS signal in plants with increased *EPSPS* relative copy number.

Consequently, the pattern observed at the dose-response study (section 2.4.1) and *EPSPS* relative number of copies and cDNA expression (section 2.4.4, Figures 2.5-2.7) was also observed when studying the specific activity of the EPSPS enzyme and when quantifying the EPSPS protein (Figure 2.8-2.10); the reciprocal crosses generated by

Female-S × Male-R (S/R) and by Female-S/R × Male-S/R (S/R//S/R) had lesser EPSPS quantity and specific enzyme activity than the reciprocal crosses generated by Female-R × Male-S (R/S) and by Female-R/S × Male-R/S (R/S//R/S).

2.4.6 Pollen Grain and Spermatic Cells Study

A large proportion of monogenic *EPSPS* progeny resulted from controlled crosses between Female-S × Male-R (R1 and R2). Differences in transmission rates between male and female gametes could explain this phenomenon. For that, qRT-PCR was used to measure *EPSPS* genomic copy number relative to *ALS* in pollen and respective leaf tissue. The hypothesis was that expected *EPSPS* gene copy for pollen sample would be half of respective leaf tissue sample. However, the *EPSPS* relative copy number from isolated pollen and leaf tissue did not different. Moreover, SEM analysis demonstrated poor yield rate and purity of the pollen grains isolated per sample with the protocol used (Figure 2.11).

Spermatic cell were isolated to study the pollen-mediated dispersal of *EPSPS* genomic copy number relative to *ALS*. The protocol described above (section 2.3.10) was performed several times with minor modifications suggested by Dr. Scott D. Russell (University of Oklahoma) with the goal of adjust it to a higher yield rate and quality of isolated sperm cells. The methodologies used never reached a level of isolating an amount of sperm cells that could be used at a qRT-PCR study (Figure 2.12). One possible explanation is that Palmer amaranth is predominantly bicellular and, consequently, the generative cell mitosis and formation of the two sperm cells may be formed only after pollen germination (Russel 1991). Russell (1991) reviewed the literature and found that, of 243 families surveyed, 137 families were bicellular (56%), 55 were tricellular (23%)

and 51 families had both types of pollen (21%). Our study did not culture Palmer amaranth in order to grow the pollen tubes, trigger mitosis, and obtain the sperm cells. A next step would be to do this extra step and verify if Palmer amaranth is a bicellular species and, thus enable the isolation of sperm cells for study the pollen-mediated dispersal of *EPSPS* genomic copy number involvement in EPSPS gene amplification inheritance in GR Palmer amaranth.

2.4.7 Facultative Apomixis and Intraspecific Genetic Diversity

Involvement of apomixis in glyphosate resistance inheritance was confirmed two times by the verification of seed production in reproductively isolated female plants (Figure 2.12). In other experiment 44 S, 36 R1 and 38 R2 reproductively isolated female individuals were studied. In all cases seed were produced, with the exception of one R1 plant. From 60 to 100% (depending on the population) of individuals studied produced 1 to 1,000 seed, and some S individuals produced as many as 6,000 seed (Figure 2.13). This amount is small when compared with normally reported seed production per female plants of 200,000 to 600,000 seed (Keeley et al. 1987), but it would be sufficient to maintain the resistant trait in the population, and replenish the seed bank and to spread geographically.

To confirm apomixis in Palmer amaranth we use RAPD and ISSR genetic markers. The initially screened RAPD and ISSR primers produced clear and scorable amplification products ranging in size from 400 to 2,000 bp and 500 to 2,000 bp, respectively (Figures 2.14 and 2.15). RAPD primers 305, 313 and 332, and the ISSR primer UBC 845 produced no clear bands. However, among the 12 RAPD primers tested only two (312 and 327) produced polymorphic bands that allowed the differentiation

among the three R1 individuals; a 17% efficacy of intraspecific differentiation (Figure 2.14). In addition, among the 15 primers tested only six, UBC 812, UBC 817, UBC 825, UBC 842, (AAC)6K and (GGGGT)3M, produced polymorphic bands that allow the differentiation among the three R1 individuals; a 40% efficacy of intraspecific differentiation (Figure 2.15).

One of the primers (UBC 808) was previously tested with maternal parent of R and S Palmer amaranth populations from Georgia and their 18 reciprocal crosses (Female-S × Male-R and Female-R × Male-S) (Giacomini et al. 2011). All plants had distinctly different bands than the maternal plant when using two set of ISSR markers, UBC 808 and 850 (Giacomini et al. 2011). Our results showed a lower efficacy of intraspecific differentiation among the 27 primers tested, including the UBC 808, using two different types of molecular markers (RAPD and ISSR). Consequently, no RAPD and ISSR polymorphism would be expected in apomictic produced seed (clones) when using those primers to test for apomixis. This assumption is based on our results and previous reports of genetic stability of *in vitro* derived plants (Chandrika et al. 2010; Lata et al. 2010, 2011)

While the AFLP (amplified fragment length polymorphism) method can provide intraspecific identification level of variant in populations, previous research studying 15 accessions of Palmer amaranth grouped them in a single cluster (Wassom and Tranel 2005). Chandi et al. (2012a), studying four glyphosate-resistant and four glyphosate-susceptible accessions, found that the vast majority of genetic variation always resided among rather than within populations. Burgos and co-workers (2012) also utilized

microsatellite markers and verified that in 70% of the populations studied, all their individuals belonged to the same cluster.

Wetzel et al. (1999) rejected the use of ribosomal DNA internal-transcribe-spacer (ITS) polymorphisms as an effective molecular marker system to study *Amaranthus* hybrid lines. Moreover, the authors studied the transfer of ALS resistance trait from Palmer amaranth to common waterhemp found several unique bands in the Palmer amaranth × waterhemp hybrid when DNA analysis was performed using AFLP. Chan and Sun (1997) using 30 isozyme loci found no allozyme variation at the intraspecific level within 60 accessions representing 23 crop and wild species of *Amaranthus*, including Palmer amaranth. Moreover, the authors used 27 primers in RAPD analysis generated a total of 900 bands (loci) and intraspecific accessions exhibited higher levels of genetic similarity. The two accessions of Palmer amaranth studied, from Mexico and Senegal, had a 30.5% Jaccard similarity. Lanoue et al. (1996) examined relationships among 28 wild and cultivated *Amaranthus* species based upon restriction-site variation in two chloroplast DNA regions and in a nuclear DNA region. They detected 11 potentially informative restriction-site mutations and seven length-polymorphism, although a low level of interspecific variation was generated which generated poorly resolved trees.

The involvement of apomixis in glyphosate resistance inheritance was confirmed three times in this research. Data from section 2.4.1-2.4.5 and from other researchers (Chandi et al. 2012b; Gaines 2009; Gaines et al. 2010, 2011; Giacomini et al. 2011; Sosnoskie et al. 2012) strongly suggest that Palmer amaranth can produce seed both apomictically (facultative apomixis), and sexually. This would support the theory that apomictic seed production by the S mother may be a determinant of low copy number

inheritance in S/R offspring. Moreover, facultative apomixis would guarantee the GR trait stability in R populations. Gustafsson (1947) stated “the apomictic mode of reproduction guarantees a protracted existence to the individual genotypes, over long periods and over wide areas”. Moreover, prior evaluations observed the production of non-hybrid progeny resulting from crosses between Palmer amaranth and common waterhemp, supporting the occurrence of agamospermy in these species (Franssen et al. 2001; Steinau et al. 2003; Trucco et al. 2007; Wetzel et al. 1999). Wetzel et al. (1999), studying the transfer of ALS resistance trait from Palmer amaranth to common waterhemp, found that when Palmer amaranth was used as the female in the cross, the hybrid plant had morphological characteristics similar to Palmer amaranth. Sosnoskie and co-workers (2012) found that the GR trait can be transferred via pollen movement in Palmer amaranth and that 40 to 50% (1 and 5 m distances) and 60 to 80% (further distances until 300 m) of the offspring were not resistant to glyphosate even though the only source of pollen in 600 m was resistant male plants at the center of a 30 ha cotton field. The authors conclude that an external pollen provider or the effect of either autopollination or agamospermy resulted in the generation of the sensitive offspring. Because the level of apomixis is unknown, inheritance of additional *EPSPS* gene copies from parents to progeny can be highly unpredictable.

The female : male ratio of the apomictically produced offspring was close to 50:50. Trucco et al. (2007) characterized the Palmer amaranth by common waterhemp non-hybrid offspring and revealed that they possessed DNA content values similar to those of the female parent and were all female in gender. Moreover, Sosnoskie et al. (2012) evaluated the male to female ratio of the offspring of randomly selected Palmer

amaranth susceptible plants restricted to GR male source of pollen and showed that there were no statistical bases towards one gender. In our study, the apomictically generated offspring were female and male in gender. That was unexpected as it is assumed that agamospermatic offspring are clones of the mother plants. However, McKone and Tonkyn (1986) found great intrapopulation variability in sex expression of the monoecious common ragweed (*Ambrosia artemisiifolia* L.), from all-female to approximately 78% male. They concluded that the non-random distribution of gender in the field could be a response to any of a number of conditions that vary spatially or had a genetic basis. In addition, it was observed spatial variation in the ratio of male to female flowers varies among populations of wild rice (*Zizania aquatica*) (Willson and Ruppel 1984). Several studies have demonstrated that the spatial segregation of the sexes in populations of dioecious plants is controlled by environmental variables (Bierzychudek and Eckhart 1988, Dodson 1962, Doust and Cavers 1982, Grant and Mitton 1979). Though, few data exist on the extent and causes of gender variation in plants. Moreover, very little is known about apomixis phenomenon in plants, and especially in our target plant of study, Palmer amaranth. This species does not have a heteromorphic sex chromosome (Grant 1959a), and the factors that determine sex are unknown at this time. It is possible that the sex determination factors are present in both female and male dioecious plants and that unknown factors may play a role in sex expression or sex-modifying hormones. Reversion of sex through chemical treatment in unisexual species with homomorphic chromosomes indicates a delicate balance between sex-determining genes and physiological conditions in such species (Chattopadhyay and Sharma 1991). The genes that affect the sexual expression of flowers were analyzed and their sequences

indicate that they are involved in hormone metabolism (Lebel-Hardenack and Grant 1997). Emerson (1924, 1932a) and Jones (1934) predicted that the many gene mutations which affect sex in maize are the building blocks that could lead to the development of a different type of sex expression in this plant. In fact, Emerson (1932b) and Jones (1932, 1934) have produced dioecious strains of maize by the proper combinations of two genes. A trangenerational plasticity via hormones was previously observed in shaded Palmer amaranth plants, where maternal environmental stress induced changes in abscisic acid (ABA) content of their seed (Jha et al. 2010). Additionally, the current understanding of sex determination in dioecious species suggests that the change from unisexuality to bisexuality had been a short step in evolution (Chattopadhyay and Sharma 1991). Franssen et al. (2001) studied pollen morphological differences in *Amaranthus* species and found differences between the monoecious and dioecious *Amaranthus* species except Palmer amaranth, whose pollen was similar to that of the monoecious species. Grant (1959b) performed cytological studies in four dioecious *Amaranthus* species and found that since haploid numbers of 16 and 17 are found in both monoecious and dioecious species, it would seem that the aneuploidy condition (monoecious) in *Amaranthus* arose early and hybridization within the genus has resulted in promoting the gene condition with has been necessary for the expression of the dioecious condition. Consequently, the sex determination apparatus defining unisexuality may still be present in dioecious plants.

The level of apomixis in Palmer amaranth is unknown. The expected increase, maintenance or decrease of R alleles in the population with the continued use of glyphosate would be affected by the mode of inheritance of the resistant trait. The mode of inheritance of the resistant trait is being affected by the apomictic mode of

reproduction observed in this plant. Facultative apomixis could function to maintain the stability of high levels of *EPSPS* gene copy number in GR Palmer amaranth populations, as additional copies may be gained during genetic recombination. Likewise, in the absence of glyphosate selection, it could dilute to exclude this trait from the population by apomictic seed production by the S mother in S/R crosses. In different GR Palmer amaranth populations, *EPSPS* gene copy number varied greatly between plants (Chandi et al. 2012b; Gaines et al. 2010, 2011; Ribeiro et al. 2011, 2012). A deeper understand regarding the apomictic trait and sex determination factors in Palmer amaranth populations is essential for understanding the stability of multiple *EPSPS* gene copy in populations.

2.4.8 Conclusion

The mechanism of resistance to glyphosate in Palmer amaranth populations from Mississippi is due to multiple copies of the *EPSPS* gene, in addition to differential absorption and/or translocation of glyphosate (Nandula et al. 2012), leading to a positively correlated gene amplification, protein quantity, enzyme activity, and level of resistance. No target site mutation was observed as mechanism of resistance. The R2 population had a positive correlation between *EPSPS* gene copy and *EPSPS* expression level, but not in the same fashion as the other populations studied, indicating that another mechanism of resistance may be involved in the R2 population in addition to the increased *EPSPS* gene copy. The response of progeny to glyphosate was more similar to the R than the S parent when the female parent was R. Conversely, when R was used as the pollinator the response in progeny to glyphosate was more similar to the S parent. Thus, the level of resistance in progeny was strongly influenced by the direction of the

cross. This led us to hypothesize and demonstrate that facultative apomictic mode of reproduction in Palmer amaranth is involved in the mode of inheritance of the resistant trait. This mode of reproduction determined the low copy number inheritance, as well as guaranteeing the glyphosate resistant trait stability in resistant populations.

In light of these new evidences, we examine earlier data regarding the incompletely dominant or additive and monogenic or polygenic inheritance of glyphosate resistance in Palmer amaranth, and we suggest that some prior conclusions may be premature as the mode of inheritance would be influenced by the level of apomixis in the population. In agreement is the nuclear inheritance of the resistant trait as resistance was inherited both maternally and paternally.

Table 2.1 NAPS Unit[a] list of RAPD (random-amplified polymorphic DNA) and ISSR (inter-simple sequence repeat, microsatellite) primers used to study intrapopulation variability of Palmer amaranth.

RAPD Primer	Sequence	SSR Primer	Sequence
305	5′-GCTGGTACCC-3′	UBC 807	5′-AGAGAGAGAGAGAGAGT-3′
308	5′-AGC GGCTAGG-3′	UBC 808	5′-AGAGAGAGAGAGAGAGC-3′
312	5′-ACG GCG TCAC-3′	UBC 812	5′-GAGAGAGAGAGAGAGAA-3′
313	5′-ACG GCA GTGG-3′	UBC 817	5′-CACACACACACACACAA-3′
322	5′-GCC GCT ACTA-3′	UBC 825	5′-ACACACACACACACACT-3′
327	5′-ATACGGCGTC-3′	UBC 834	5′-AGAGAGAGAGAGAGAGYT-3′
329	5′-GCGAACCTCC-3′	UBC 835	5′-AGAGAGAGAGAGAGAGYC-3′
331	5′-GCCTAGTCAC-3′	UBC 842	5′-GAGAGAGAGAGAGAGAYG-3′
332	5′-AACGCGTAGA-3′	UBC 845	5′-CTCTCTCTCTCTCTCTRG-3′
335	5′-TGGACCACCC-3′	UBC 856	5′-ACACACACACACACACYA-3′
349	5′-GGAGCCCCCT-3′	(GGC)6W	5′-GGCGGCGGCGGCGGCGGCW-3′
354	5′-CTAGAGGCCG-3′	(AAC)6K	5′-AACAACAACAACAACAACK-3′
		(AAG)6Y	5′-AAGAAGAAGAAGAAGAAGY-3′
		(GGAT)4H	5′-GGATGGATGGATGGATH-3′
		(GGGGT)3M	5′-GGGGTGGGGTGGGGTM-3′

[a] RAPD analyses were performed using 12 decamer primers randomly selected from a list of 100 primers (NAPS Unit, University of British Columbia, Biotechnology Laboratory, Vancouver, BC, Canada). ISSR analyses were performed using 15 primers randomly selected from a list of 100 primers (NAPS Unit, University of British Columbia) and five of Natascha Techen (National Center for Natural Products, University of Mississippi, USA) design.

Table 2.2 Glyphosate dose-response parameters and variables in the log-logistic model[a] estimates for parents, reciprocal crosses and second reciprocal crosses of Palmer amaranth[b] at 14 days after treatment.

Population code[b, **]	R^2	L[c]	U[c]	s[c]	GR_{50}[c]	GR_{50} ratio[c]
		---------- % Fresh weight reduction (SE)[d] ---------			g ae ha^{-1} (SE)[d]	
R1	0.99	16.56* (2.59)	100.24* (1.18)	3.42* (0.51)	1623.25* (68.45)	17.20
R1/S	0.99	8.05* (2.07)	97.92* (1.21)	3.70* (0.41)	1138.08* (44.71)	12.06
S/R1	0.99	3.69 (2.29)	100.75* (1.90)	1.69* (0.16)	464.17* (29.14)	4.92
R1/S//R1/S	0.98	6.63* (2.74)	97.98* (1.41)	2.20* (0.24)	976.44* (56.73)	10.35
S/R1//S/R1	0.99	7.08* (2.01)	98.02* (1.48)	2.65* (0.32)	663.64* (29.93)	7.03
R2	0.98	16.85* (2.72)	100.67* (1.23)	2.85* (0.37)	1368.81* (71.33)	14.51
R2/S	0.99	3.54 (2.12)	97.85* (1.48)	2.35* (0.23)	758.88* (36.49)	8.04
S/R2	0.99	1.78 (1.79)	99.89* (1.88)	2.02* (0.18)	363.03* (18.73)	3.85
R2/S//R2/S	0.99	4.81* (1.91)	98.65* (1.51)	2.49* (0.25)	569.67* (26.50)	6.04
S/R2//S/R2	0.99	2.08 (1.55)	97.65* (1.53)	3.35* (0.41)	433.11* (14.79)	4.59
S	0.99	1.07 (1.37)	98.86* (2.58)	1.75* (0.16)	94.36* (5.59)	-

[a] Model proposed by Seefeldt et al. (1995): y [fresh weight (% of untreated control)] = $L + \{(U - L)/[1 + (D/GR_{50})^s]\}$.

[b] Resistant parents (R1 and R2), susceptible parent (S), reciprocal crosses (Female-S x Male-R, S/R, and Female-R x Male-S, R/S), second reciprocal crosses (Female-S/R x Male-S/R, S/R//S/R, and Female-R/S x Male-R/S, R/S//R/S).

[c] The parameter estimates are L, lower limit of response; U, upper limit of response; s, slope of the curve around the point of inflexion (GR_{50}); GR_{50}, glyphosate dose required to cause a 50% reduction in plant growth and GR_{50} dose was estimated using responses to nine glyphosate doses (0, 52, 105, 210, 420, 840, 1,680, 3,360, and 6,720 g ha^{-1}); and GR_{50} ratio, GR_{50} populations / GR_{50} susceptible population.

[d] SE represents the standard error of the mean where $n = 6$ (polled data from two experiments).

[*] Estimated parameters of the log-logistic model are different according to t-Student test at $P < 0.05$; accept alternative hypothesis, H_a: parameter $\neq 0$.

[**] Relative potencies between populations and susceptible population at GR_{50} response level are different according to t-Student test at $P < 0.05$; accept alternative hypothesis, H_a: relative potency $\neq 1$. The two exceptions were the relative potencies of S/R1 vs. S/R2//S/R2 and R2/S vs. S/R1//S/R1 at GR_{50} response level are not different according to t-Student test at $P < 0.05$; accept null hypothesis, H_0: relative potency = 1.

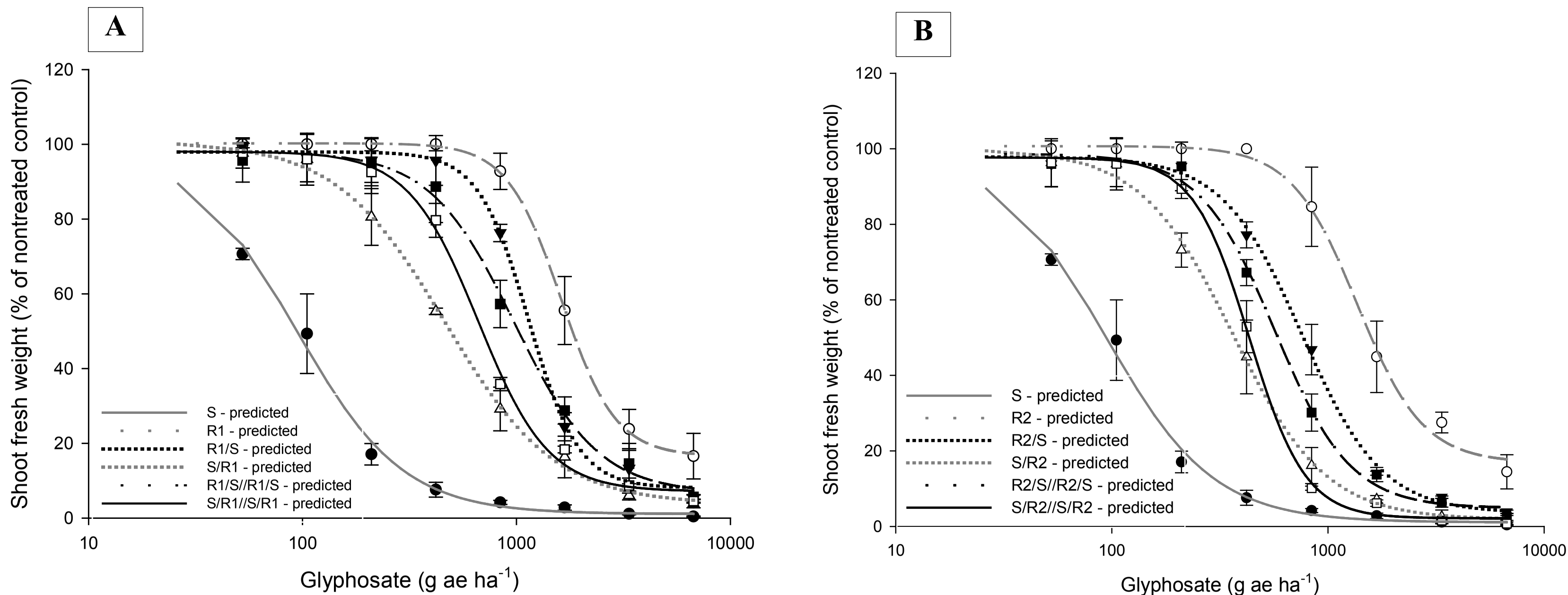

Figure 2.1 Response of glyphosate-susceptible, -resistant, and first and second reciprocal crosses of Palmer amaranth populations to glyphosate dose.

Response of glyphosate-susceptible (S), -resistant (R1 and R2), and first (R/S and S/R) and second (R/S//R/S and S/R//S/R) reciprocal crosses of Palmer amaranth populations in the three- to four-leaf growth stage to glyphosate dose (log scale) 2 weeks after treatment. A, parents, first, and second reciprocal cross for glyphosate-resistant (R1) and -susceptible (S) parents; S, dark circle and gray solid line; R1, open circle and gray dashed line; R1/S, dark triangle and black dotted line; S/R1, open triangle and gray dotted line; R1/S//R1/S, dark square and black dashed line; S/R1//S/R1, open square and black solid line. B, parents, first and second reciprocal cross for glyphosate-resistant (R2) and -susceptible (S) parents; S, dark circle and gray solid line; R2, open circle and gray dashed line; R2/S, dark triangle and black dotted line; S/R2, open triangle and gray dotted line; R2/S//R2/S, dark square and black dashed line; S/R2//S/R2, open square and black solid line. Vertical bars represent ± standard error of the mean (*n* = 6).

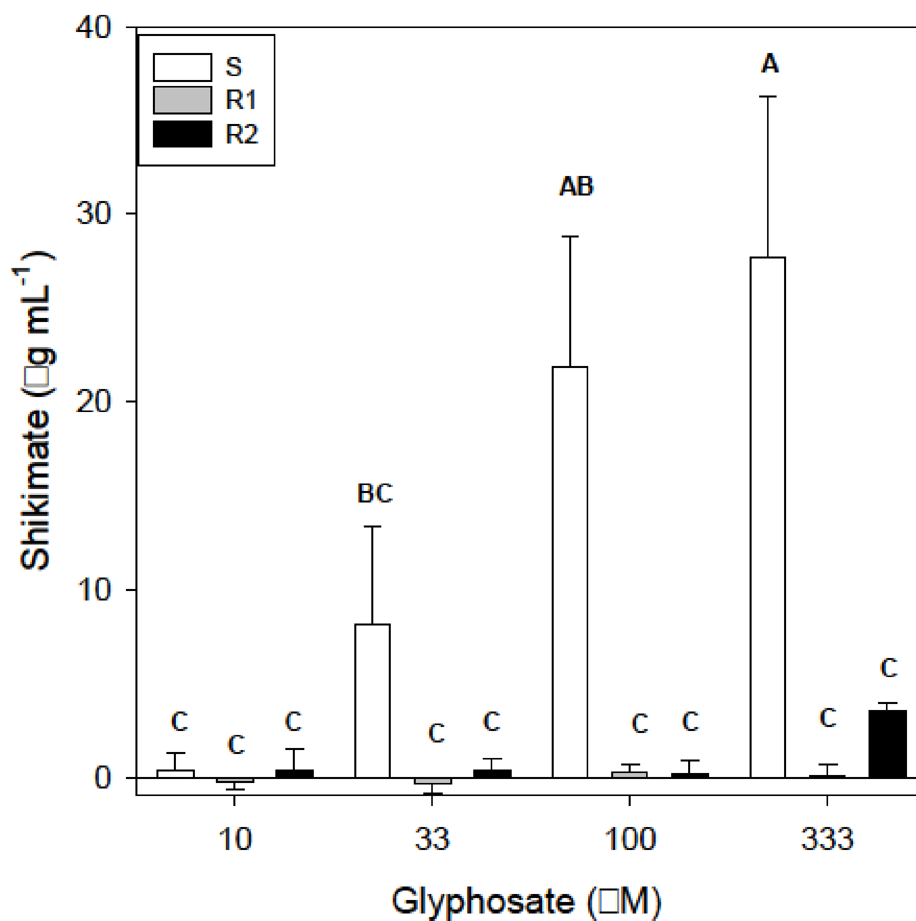

Figure 2.2 Effect of glyphosate concentration on shikimate levels in excised leaf discs of glyphosate-resistant and -susceptible Palmer amaranth populations.

Means of shikimate level followed by the same letter are not significantly different by Fisher's LSD test at 0.05. Vertical bars represent ± standard error of the mean (n = 8).

```
R_consensus   ATGGCTCAAGCTACTACCATCAACAATGGTGTCCATACTGGTCAATTGCACCATACTTTA 60
S_consensus   ATGGCTCAAGCTACTACCATCAACAATGGTGTCCAAACTGGTCAATTGCACCATACTTTA 60
FJ861243      ATGGCTCAAGCTACTACCATCAACAATGGTGTCCATACTGGTCAATTGCACCATACTTTA 60
FJ861242      ATGGCTCAAGCTACTACCATCAACAATGGTGTCCATACTGGTCAATTGCACCATACTTTA 60
              *********************************** ************************

R_consensus   CCCAAAACCCAGTTACCCAAATCTTCAAAAACTCTTAATTTTGGATCAAACTTGAGAATT 120
S_consensus   CCCAAAACCCACTTACCCAAATCTTCAAAAACTCTTAATTTTGGATCAAACTTGAGAATT 120
FJ861243      CCCAAAACCCAGTTACCCAAATCTTCAAAAACTCTTAATTTTGGATCAAACTTGAGAATT 120
FJ861242      CCCAAAACCCAGTTACCCAAATCTTCAAAAAYTCTTAATTTTGGATCAAACTTGAGAATT 120
              *********** ******************* ****************************

R_consensus   TCTCCAAAGTTCATGTCTTTAACCAATAAAAGAGTTGGTGGGCAATCATCAATTGTTCCC 180
S_consensus   TCTCCAAAGTTCATGTCTTTAACCAATAAAAGAGTTGGTGGGCAATCTTCAATTGTTCCC 180
FJ861243      TCTCCAAAGTTCATGTCTTTAACCAATAAAAGAGTTGGTGGGCAATCATCAATTGTTCCC 180
FJ861242      TCTCCAAAGTTCATGTCTTTAACCAATAAAAGAGTTGGTGGGCAATCATCAATTGTTCCC 180
              *********************************************** ************

R_consensus   AAGATTCAAGCTTCTGTTGCTGCTGCAGCTGAGAAACCTTCATCTGTCCCAGAAATTGTG 240
S_consensus   AAGATTCAAGCTTCTGTTGCTGCTGCAGCTGAGAAACCTTCATCTGTCCCAGAAATTGTG 240
FJ861243      AAGATTCAAGCTTCTGTTGCTGCTGCAGCTGAGAAACCTTCATCTGTCCCAGAAATTGTG 240
FJ861242      AAGATTCAAGCTTCTGTTGCTGCTGCAGCTGAGAAACCTTCATCTGTCCCAGAAATTGTG 240
              ************************************************************

R_consensus   TTACAACCCATCAAAGAGATCTCTGGTACTGTTCAATTGCCTGGGTCAAAGTCTTTATCC 300
S_consensus   TTACAACCCATCAAAGAGATCTCTGGTACTGTTCAATTGCCTGGGTCAAAGTCTTTATCC 300
FJ861243      TTACAACCCATCAAAGAGATCTCTGGTACTGTTCAATTGCCTGGGTCAAAGTCTTTATCC 300
FJ861242      TTACAACCCATCAAAGAGATCTCTGGTACTGTTCAATTGCCTGGGTCAAAGTCTTTATCC 300
              ************************************************************

R_consensus   AATCGAATCCTTCTTTTAGCTGCTTTGTCTGAGGGCACAACAGTGGTCGACAACTTGCTG 360
S_consensus   AATCGAATCCTTCTTTTAGCTGCTTTGTCTGAGGGCACAACAGTGGTYGACAACTTGCTG 360
FJ861243      AATCGAATCCTTCTTTTAGCTGCTTTGTCTGAGGGCACAACAGTGGTCGACAACTTGCTG 360
FJ861242      AATCGAATCCTTCTTTTAGCTGCTTTGTCTGAGGGCACAACAGTGGTCGACAACTTGCTG 360
              *********************************************** ************
R_consensus   TATAGTGATGATATTCTTTATATGTTGGACGCTCTCAGAACTCTTGGTTTAAAAGTGGAG 420
S_consensus   TATAGTGATGATATTCTTTATATGTTGGACGCTCTCAGAACTCTTGGTTTAAAAGTKGAG 420
FJ861243      TATAGTGATGATATTCTTTATATGTTGGACGCTCTCAGAACTCTTGGTTTAAAAGTGGAG 420
FJ861242      TATAGTGATGATATTCTTTATATGTTGGACGCTCTCAGAACTCTTGGTTTAAAAGTGGAG 420
              ******************************************************** ***

R_consensus   GATGATAGTACAGCCAAAAGGGCAGTCGTAGAGGGTTGTGGTGGTCTGTTTCCTGTTGGT 480
S_consensus   GATGATAGTACAGCCAAAAGGGCAGTCGTAGAGGGTTGTGGTGGTCTGTTTCCTGTTGGT 480
FJ861243      GATGATAGTACAGCCAAAAGGGCAGTCGTAGAGGGTTGTGGTGGTCTGTTTCCTGTTGGT 480
FJ861242      GATGATAGTACAGCCAAAAGGGCAGTCGTAGAGGGTTGTGGTGGTCTGTTTCCTGTTGGT 480
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Figure 2.3 Alignment of full-length consensus 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) sequences from glyphosate resistant (R[a]) and susceptible (S) Palmer amaranth individuals from cDNA clones and reference sequences (FJ861242[b] and FJ861243[c]).

[a] There was little to no difference among sequences from six R1 and six R2 clones; the consensus sequence represents residues common to all glyphosate-resistant sequences.
[b] Glyphosate-susceptible Palmer amaranth from Georgia (Gaines et al. 2010) sequence reference, sequence information can be found at http://www.ncbi.nlm.nih.gov/nuccore/
[c] Glyphosate-resistant Palmer amaranth from Georgia (Gaines et al. 2010) sequence reference, sequence information can be found at http://www.ncbi.nlm.nih.gov/nuccore/
Asterisks indicate nucleotides in all sequences are the same. ATG (start) and TGA (stop) codons are indicated with a box. The gray highlight is a single nucleotide polymorphism.

```
R_consensus   AAAGATGGAAAGGAAGAGATTCAACTTTTCCTTGGTAATGCAGGAACAGCGATGCGCCCA 540
S_consensus   AAAGATGGAAAGGAAGAGATTCAACTTTTCCTTGGTAATGCAGGAACAGCGATGCGCCCA 540
FJ861243      AAAGATGGAAAGGAAGAGATTCAACTTTTCCTTGGTAATGCAGGAACAGCGATGCGCCCA 540
FJ861242      AAAGATGGAAAGGAAGAGATTCAACTTTTCCTTGGTAATGCAGGAACAGCGATGCGCCCA 540
              ************************************************************

R_consensus   TTGACAGCTGCGGTTGCCGTTGCTGGAGGAAATTCAAGTTATGTGCTTGATGGAGTACCA 600
S_consensus   TTGACAGCTGCGGTTGCCGTTGCTGGAGGAAATTCAAGTTATGTGCTTGATGGAGTACCA 600
FJ861243      TTGACAGCTGCGGTTGCCGTTGCTGGAGGAAATTCAAGTTATGTGCTTGATGGAGTACCA 600
FJ861242      TTGACAGCTGCGGTTGCCGTTGCTGGAGGAAATTCAAGTTATGTGCTTGATGGAGTACCA 600
              ************************************************************

R_consensus   AGAATGAGGGAGCGCCCCATTGGGGATCTGGTAGCAGGTCTAAAGCAACTTGGTTCAGAT 660
S_consensus   AGAATGAGGGAGCGCCCCATTGGGGATYTGGTAGCAGGTCTAAAGCAACTTGGTTCAGAT 660
FJ861243      AGAATGAGGGAGCGCCCCATTGGGGATCTGGTAGCAGGTCTAAAGCAACTTGGTTCAGAT 660
FJ861242      AGAATGAGGGAGCGCCCCATTGGGGATCTGGTAGCAGGTCTAAAGCAACTTGGTTCAGAT 660
              *************************** ********************************

R_consensus   GTAGATTGTTTTCTTGGCACAAATTGCCCTCCTGTTCGGGTCAATGCTAAAGGAGGCCTT 720
S_consensus   GTTGACTGTTTTCTTGGCACAAATTGCCCTCCTGTTCGGGTCAATGCTAAAGGAGGCCTT 720
FJ861243      GTAGATTGTTTTCTTGGCACAAATTGCCCTCCTGTTCGGGTCAATGCTAAAGGAGGCCTT 720
FJ861242      STAGATTGTTTTCTTGGCACAAATTGCCCTCCTGTTCGGGTCAATGCTAAAGGAGGCCTT 720
               * ** ******************************************************

R_consensus   CCAGGGGGCAAGGTCAAGCTCTCTGGATCGGTTAGTAGCCAATATTTAACTGCACTTCTC 780
S_consensus   CCAGGGGGCAAGGTCAAGCTCTCTGGATCAGTTAGTAGCCAATATTTAACTGCACTTCTC 780
FJ861243      CCAGGGGGCAAGGTCAAGCTCTCTGGATCGGTTAGTAGCCAATATTTAACTGCACTTCTC 780
FJ861242      CCAGGGGGCAAGGTCAAGCTCTCTGGATCGGTTAGTAGCCAATATTTAACTGCACTTCTC 780
              ***************************** ******************************

R_consensus   ATGGCTACTCCTTTGGGTCTTGGAGACGTGGAGATTGAGATAGTTGATAAATTGATTTCT 840
S_consensus   ATGGCTACTCCTTTGGGTCTTGGAGACGTGGAGATTGAGATAGTTGATAAATTGATTTCT 840
FJ861243      ATGGCTACTCCTTTGGGTCTTGGAGACGTGGAGATTGAGATAGTTGATAAATTGATTTCT 840
FJ861242      ATGGCTACTCCTTTGGGTCTTGGAGACGTGGAGATTGAGATAGTTGATAAATTGATTTCT 840
              ************************************************************

R_consensus   GTACCGTATGTTGAAATGACAATAAAGTTGATGGAACGCTTTGGAGTATCCGTAGAACAT 900
S_consensus   GTACCGTATGTTGAAATGACAATAAGGTTGATGGAACGCTTTGGAGTATCCGTAGAACAT 900
FJ861243      GTACCGTATGTTGAAATGACAATAAAGTTGATGGAACGCTTTGGAGTATCCGTAGAACAT 900
FJ861242      GTACCGTATGTTGAAATGACAATAAAGTTGATGGAACGCTTTGGAGTATCCGTAGAACAT 900
              ************************* **********************************

R_consensus   AGTGATAGTTGGGACAGGTTCTACATTCGAGGTGGTCAGAAATACAAATCTCCTGGAAAG 960
S_consensus   AGTGATAGTTGGGACAGGTTCTACATTCGAGGTGGTCAGAAATACAAATCTCCTGGAAAG 960
FJ861243      AGTGATAGTTGGGACAGGTTCTACATTCGAGGTGGTCAGAAATACAAATCTCCTGGAAAG 960
FJ861242      AGTGATAGTTGGGACAGGTTCTACATTCGAGGTGGTCAGAAATACAAATCTCCTGGAAAG 960
              ************************************************************

R_consensus   GCATATGTTGAGGGTGATGCTTCAAGTGCTAGCTACTTCCTAGCCGGAGCCGCCGTCACT 1020
S_consensus   GCATATGTAGAGGGGGACGCTTCTAGTGCTAGCTACTTCCTAGCAGGAGCCGCCGTCACT 1020
FJ861243      GCATATGTTGAGGGTGATGCTTCAAGTGCTAGCTACTTCCTAGCCGGAGCCGCCGTCACT 1020
FJ861242      GCATATGTTGAGGGTGATGCTTCAAGTGCTAGCTACTTCCTAGCCGGAGCCGCCGTCACT 1020
              ******** ***** ** ***** ******************** ***************

R_consensus   GGTGGGACTGTCACTGTCAAGGGTTGTGGAACAAGCAGTTTACAGGGTGATGTAAAATTT 1080
S_consensus   GGTGGGACTGTGACTGTCAAGGGTTGTGGAACAAGCAGTTTACAGGGTGATGTAAAATTT 1080
FJ861243      GGTGGGACTGTCACTGTCAAGGGTTGTGGAACAAGCAGTTTACAGGGTGATGTAAAATTT 1080
FJ861242      GGTGGGACTGTCACTGTCAAGGGTTGTGGAACAAGCAGTTTACAGGGTGATGTAAAATTT 1080
              *********** ************************************************
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Figure 2.3 (Continued)

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R_consensus   GCCGAAGTTCTTGAGAAGATGGGTTGCAAGGTCACCTGGACAGAGAATAGTGTAACTGTT 1140
S_consensus   GCCGAAGTTCTTGAGAAGATGGGTTGCAAGGTCACCTGGACAGAGAATAGTGTAACTGTT 1140
FJ861243      GCCGAAGTTCTTGAGAAGATGGGTTGCAAGGTCACCTGGACAGAGAATAGTGTAACTGTT 1140
FJ861242      GCCGAAGTTCTTGAGAAGATGGGTTGCAAGGTCACCTGGACAGAGAATAGTGTAACTGTT 1140
              ************************************************************

R_consensus   ACTGGACCACCCAGGGATTCATCTGGAAAGAAACATCTGCGTGCTATCGACGTCAACATG 1200
S_consensus   ACTGGACCACCCAGGGATTCATCTGGAAAGAAACATCTGCGTGCTATCGACGTCAACATG 1200
FJ861243      ACTGGACCACCCAGGGATTCATCTGGAAAGAAACATCTGCGTGCTATCGACGTCAACATG 1200
FJ861242      ACTGGACCACCCAGGGATTCATCTGGAAGGAAACATCTGCGTGCTATCGACGTCAACATG 1200
              **************************** *******************************

R_consensus   AACAAAATGCCAGATGTTGCTATGACTCTTGCAGTTGTTGCCTTGTATGCAGATGGGCCC 1260
S_consensus   AACAAAATGCCAGATGTTGCTATGACTCTTGCAGTTGTTGCSTTGTATGCAGATGGGCCC 1260
FJ861243      AACAAAATGCCAGATGTTGCTATGACTCTTGCAGTTGTTGCCTTGTATGCAGATGGGCCC 1260
FJ861242      AACAAAATGCCAGATGTTGCTATGACTCTTGCAGTTGTTGCCTTGTATGCAGATGGGCCC 1260
              ***************************************** ******************

R_consensus   ACCGCCATCAGAGATGTGGCTAGCTGGAGAGTGAAGGAAACCGAACGGATGATTGCCATT 1320
S_consensus   ACCGCCATCAGAGATGTGGCTAGCTGGAGAGTGAAGGAAACSGAACGGATGATTGCCATY 1320
FJ861243      ACCGCCATCAGAGATGTGGCTAGCTGGAGAGTGAAGGAAACCGAACGGATGATTGCCATT 1320
FJ861242      ACCGCCATCAGAGATGTGGCTAGCTGGAGAGTGAAGGAAACCGAACGGATGATTGCCATT 1320
              ***************************************** *****************

R_consensus   TGCACAGAACTGAGAAAGCTTGGGGCAACAGTTGAGGAAGGATCTGATTACTGTGTGATC 1380
S_consensus   TGCACAGAACTGAGAAAGCTTGGGGCAACAGTTGAGGAAGGATCTGATTACTGTGTGATC 1380
FJ861243      TGCACAGAACTGAGAAAGCTTGGGGCAACAGTTGAGGAAGGATCTGATTACTGTGTGATC 1380
FJ861242      TGCACAGAACTGAGAAAGCTTGGGGCAACAGTTGAGGAAGGATCTGATTACTGTGTGATC 1380
              ************************************************************

R_consensus   ACTCCGCCTGAAAAGCTAAACCCCACCGCCATTGAAACTTATGACGATCACCGAATGGCC 1440
S_consensus   ACTCCGCCTGAAAAGCTAAACCCCACCGCCATTGAAACTTATGACGATCACCGAATGGCC 1440
FJ861243      ACTCCGCCTGAAAAGCTAAACCCCACCGCCATTGAAACTTATGACGATCACCGAATGGCC 1440
FJ861242      ACTCCGCCTGAAAAGCTAAACCCCACCGCCATTGAAACTTATGACGATCACCGAATGGCC 1440
              ************************************************************

R_consensus   ATGGCATTCTCTCTTGCTGCCTGTGCAGATGTTCCCGTCACTATCCTTGATCCGGGATGC 1500
S_consensus   ATGGCATTCTCTCTTGCTGCCTGTGCAGATGTTCCCGTCACTATCCTTGATCCGGGATGC 1500
FJ861243      ATGGCATTCTCTCTTGCTGCCTGTGCAGATGTTCCCGTCACTATCCTTGATCCGGGATGC 1500
FJ861242      ATGGCATTCTCTCTTGCTGCCTGTGCAGATGTTCCCGTCACTATCCTTGATCCGGGATGC 1500
              ************************************************************

R_consensus   ACCCGTAAAACCTTCCCGGACTACTTTGATGTTTTAGAAAAGTTCGCCAAGCATTGA 1557
S_consensus   ACCCGTAAAACCTTCCCGGACTACTTTGATGTTTTAGAAAAGTTCGCCAAGCATTGA 1557
FJ861243      ACCCGTAAAACCTTCCCGGACTACTTTGATGTTTTAGAAAAGTTCGCCAAGCATTGA 1557
FJ861242      ACCCGTAAAACCTTCCCGGACTACTTTGATGTTTTAGAAAAGTTCGCCAAGCATTGA 1557
              *********************************************************
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Figure 2.3 (Continued)

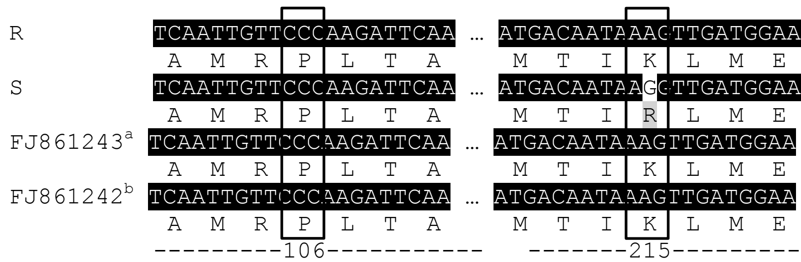

Figure 2.4 Partial and deduced amino acid sequence alignment of the 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene of glyphosate-susceptible (S) and glyphosate-resistant (R) Palmer amaranth populations.

a FJ861243, glyphosate-resistant Palmer amaranth from Georgia (Gaines et al. 2010) sequence reference, sequence information can be found at http://www.ncbi.nlm.nih.gov/nuccore/
b FJ861242, glyphosate-susceptible Palmer amaranth from Georgia (Gaines et al. 2010) sequence reference, sequence information can be found at http://www.ncbi.nlm.nih.gov/nuccore/
The boxed codon shows the substitutions of arginine (R) to lysine (K) at amino acid 215 and no substitution at proline (P) 106, when using the maize mature EPSPS numbering system.

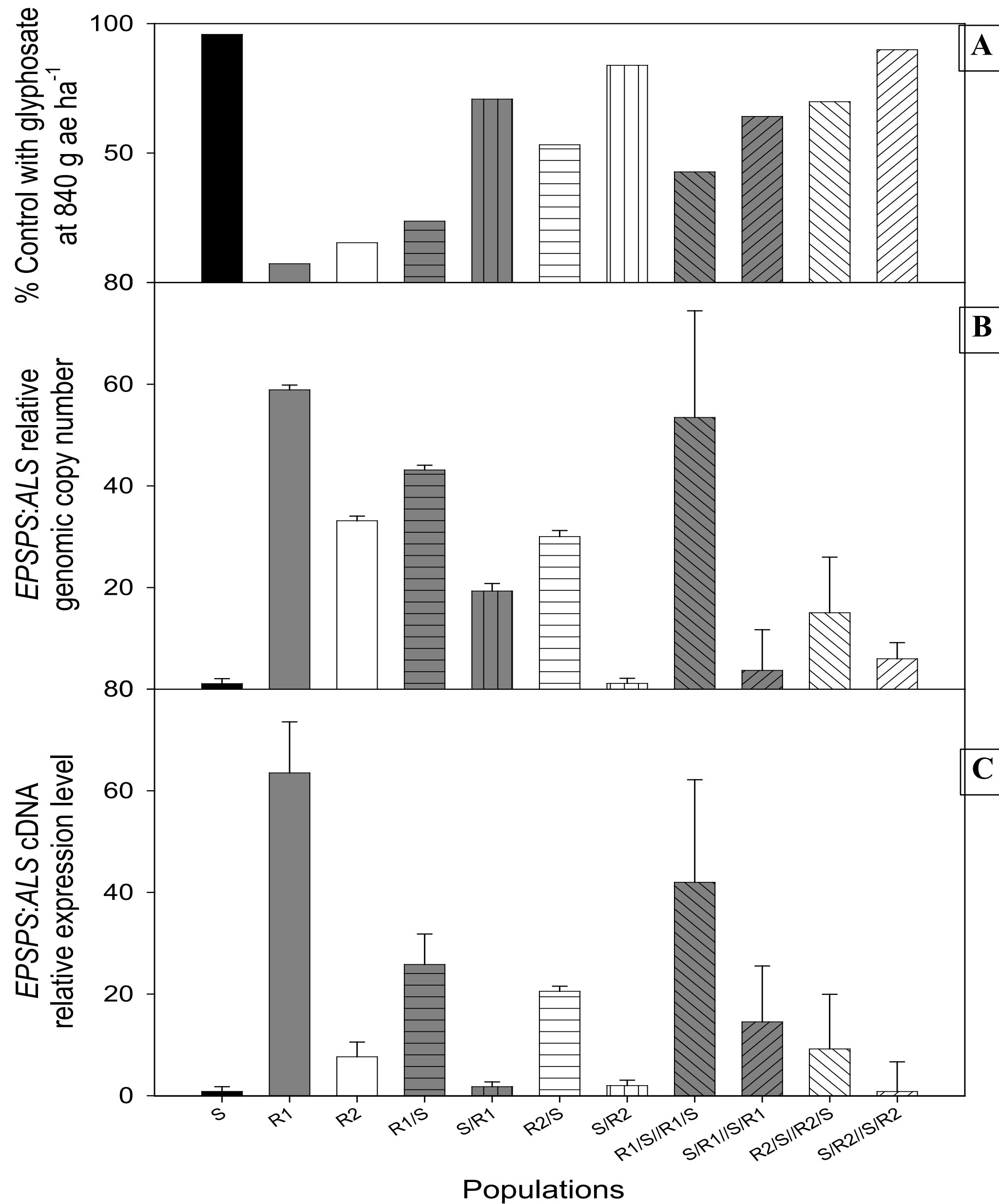

Figure 2.5 Glyphosate-susceptible, -resistant, and first and second reciprocal crosses of Palmer amaranth populations control, genomic copy number and cDNA expression level of 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*).

Glyphosate-susceptible (S), -resistant (R1 and R2), and first (R/S and S/R) and second (R/S//R/S and S/R//S/R) reciprocal crosses of Palmer amaranth populations control (% of control) at 840 g ae ha^{-1} glyphosate (field dose) (A), genomic copy number (B) and cDNA expression level (C) of 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) relative to acetolactate synthase (*ALS*) gene. Vertical bars represent ± standard error of the mean (n = 20)

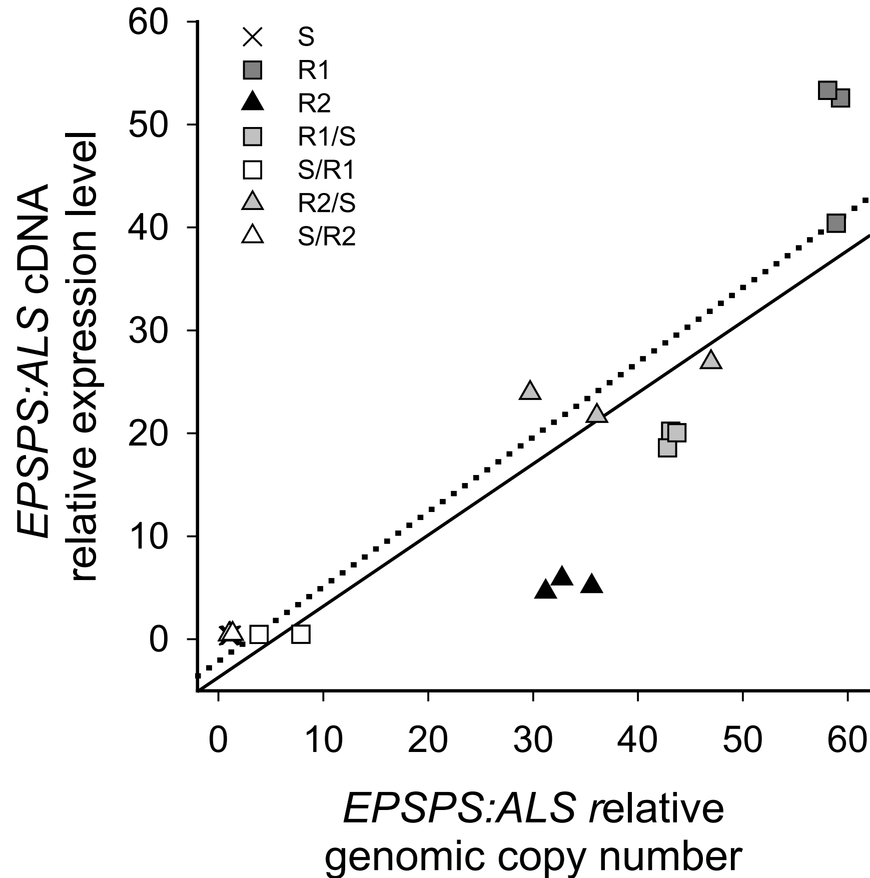

Figure 2.6 Positive correlation between increase in 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) genomic copy number with increase in *EPSPS* cDNA expression levels in glyphosate-susceptible, -resistant, and first reciprocal crosses of Palmer amaranth populations.

Positive correlation between increase in 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) relative to acetolactate synthase (*ALS*) genomic copy number with increase in *EPSPS : ALS* cDNA expression levels in glyphosate-susceptible (S), -resistant (R1 and R2), and first reciprocal crosses (R/S and S/R) of Palmer amaranth populations. Regression of the entire dataset indicated a good relationship between mRNA levels and copy numbers (solid line, $r = 0.87$, $P < 0.0001$). The R2 population did not fit the model as well as the other populations. Excluding this population from the dataset improved the strength of that relationship (dotted line, $r = 0.94$, $P < 0.0001$).

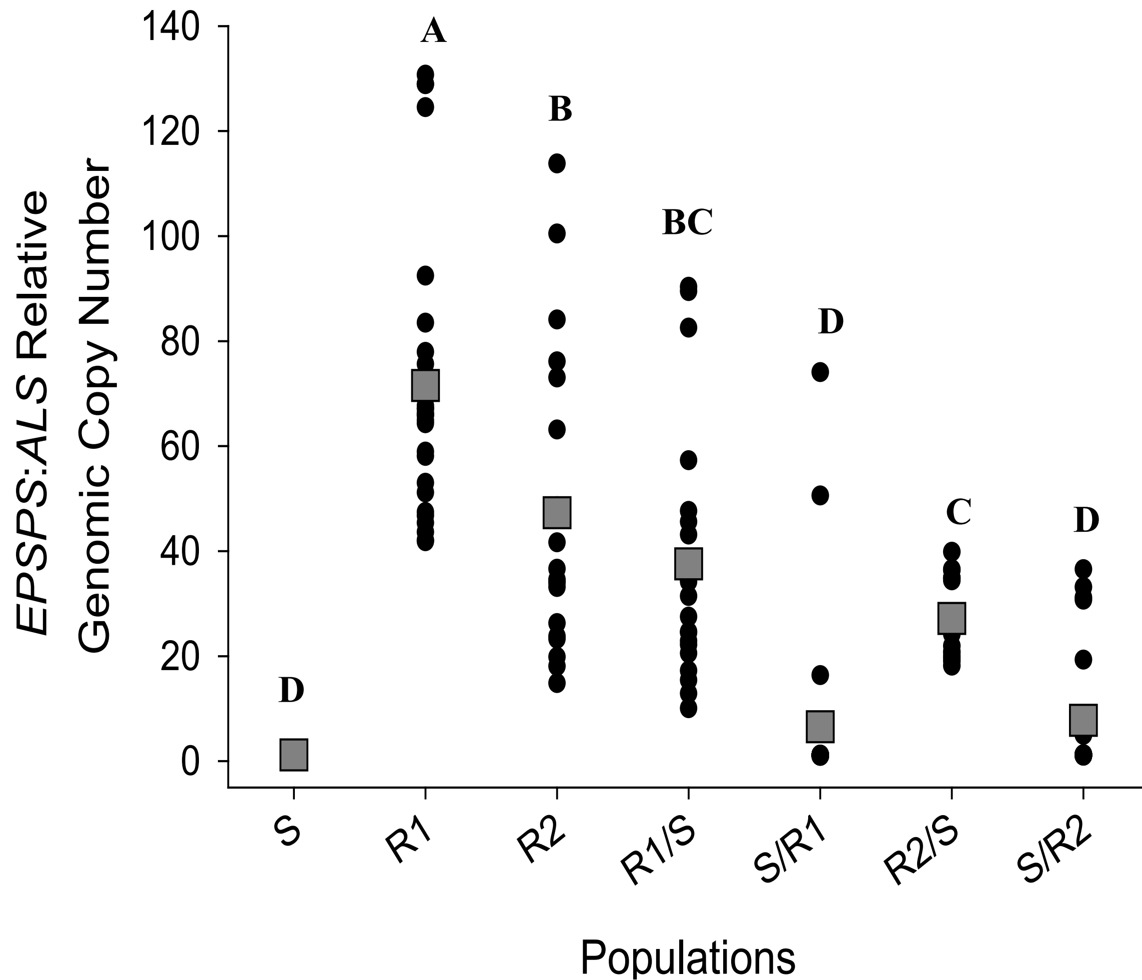

Figure 2.7 Genomic copy number of 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) genomic copy of thirty sampled individuals per Palmer amaranth population.

Genomic copy number of 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) relative to acetolactate synthase (*ALS*) genomic copy of thirty (n = 30) sampled individuals per Palmer amaranth population (dark circle), glyphosate-susceptible (S), -resistant (R1 and R2), and first (R/S and S/R) reciprocal crosses. Means of *EPSPS* relative gene copy number followed by the same letter are not significantly different by LSD test at 0.05. The *EPSPS* copy number segregation pattern observed in Figures 2.5 and 2.6, when bulked sample population, is observed when averaging sampled individuals (dark gray square).

Table 2.3 EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) specific activity dose response parameters and variables in the log-logistic model[a] estimates for parents and first reciprocal crosses of Palmer amaranth[b].

Population code[b, **]	U^c % relative to control, μmol Pi μg^{-1} TSP min^{-1} (SE)[d]	s^c	IC_{50}[c] μM (SE)	IC_{50} ratio[c]
R1	100.08* (3.88)	0.96* (0.14)	15.87* (2.97)	0.29
R1/S	93.74* (4.94)	0.88* (0.15)	15.48* (4.08)	0.28
S/R1	100.06* (4.73)	0.84* (0.11)	6.62* (1.54)	0.12
R2	102.44* (3.60)	1.03* (0.14)	20.55* (3.57)	0.37
R2/S	98.26* (4.97)	0.82* (0.13)	5.54* (1.31)	0.10
S/R2	90.41* (2.91)	1.48* (0.27)	25.20* (3.57)	0.46
S	103.13* (4.14)	0.54* (0.07)	55.14* (14.88)	-

[a] Three parameter log-logistic model: y [μmol Pi μg^{-1} TSP min^{-1} (% of untreated control)] = $\{U/[1 + (D/IC_{50})^s]\}$. Pi, inorganic phosphate; TSP, total soluble protein.

[b] Resistant parents (R1 and R2), susceptible parent (S), and reciprocal crosses (Female-S x Male-R, S/R, and Female-R x Male-S, R/S).

[c] The parameters estimates are U, upper limit of response; s, slope of the curve around the point of inflexion (IC_{50}); IC_{50}, glyphosate concentration that reduced enzyme activity by 50% and IC_{50} concentration was estimated using responses to nine glyphosate concentrations (0, 0.3, 1, 3, 10, 33, 100, 333 and 1,000 μM); and IC_{50} ratio, IC_{50} populations / IC_{50} susceptible population.

[d] SE represents the standard error of the mean where $n = 6$ (polled data from two experiments).

* Estimated parameters of the log-logistic model are different according to t-Student test at $P < 0.05$; accept alternative hypothesis, H_a: parameter ≠ 0.

** Relative potencies between S vs. R1, S vs. R1/S, and S vs. S/R1 populations and susceptible population at IC_{50} response level are different according to t-Student test at $P < 0.05$; accept alternative hypothesis, H_a: relative potency ≠ 1. The other populations did not differ the relative potencies at IC_{50} response level according to t-Student test at $P < 0.05$; accept null hypothesis, H_0: relative potency = 1. Although, statistical analyses of the IC_{50} on the specific activity indicate that are some significant differences, these differences do not account for the differences in the level of resistance. For example, the IC_{50} of S population is greater than all the other populations; this is probably due to the greater differences in the overall EPSPS specific activity in the population with multiple copies in comparison with S population. Moreover the IC_{50} ratio for all populations was smaller than 1.

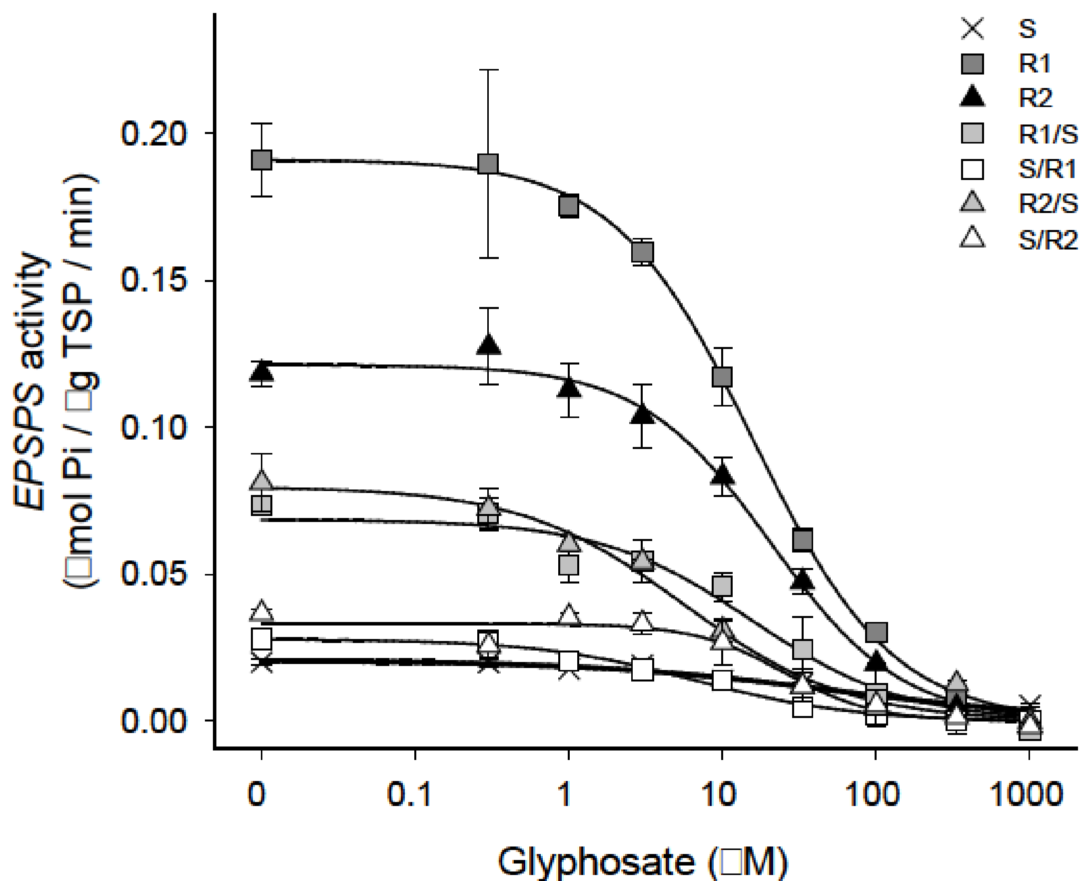

Figure 2.8 Dose-response of glyphosate against EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) enzyme activity of glyphosate-susceptible, -resistant, and first reciprocal cross of Palmer amaranth populations.

Dose-response of glyphosate against EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) enzyme activity of glyphosate-susceptible (S), -resistant (R1 and R2), and first reciprocal cross (R/S and S/R) of Palmer amaranth populations. Glyphosate inhibition assays were normalized for total soluble protein (TSP) quantity. S: black cross, 1 relative *EPSPS* copy, IC_{50} (glyphosate concentration that reduced enzyme activity by 50%) = 55 µM; R1: dark gray square, 59 relative copies, IC_{50} = 16 µM; R2: black triangle, 33 relative copies, IC_{50} = 21 µM; R1/S: light gray square, 43 relative copies, IC_{50} = 15 µM; S/R1: open square, 19 relative copies, IC_{50} = 7 µM; R2/S: light gray triangle, 30 relative copies, IC_{50} = 6 µM; S/R2: open triangle, 1 relative copy, IC_{50} = 26 µM. Pi, inorganic phosphate. Vertical bars represent ± standard error of the mean (n = 6).

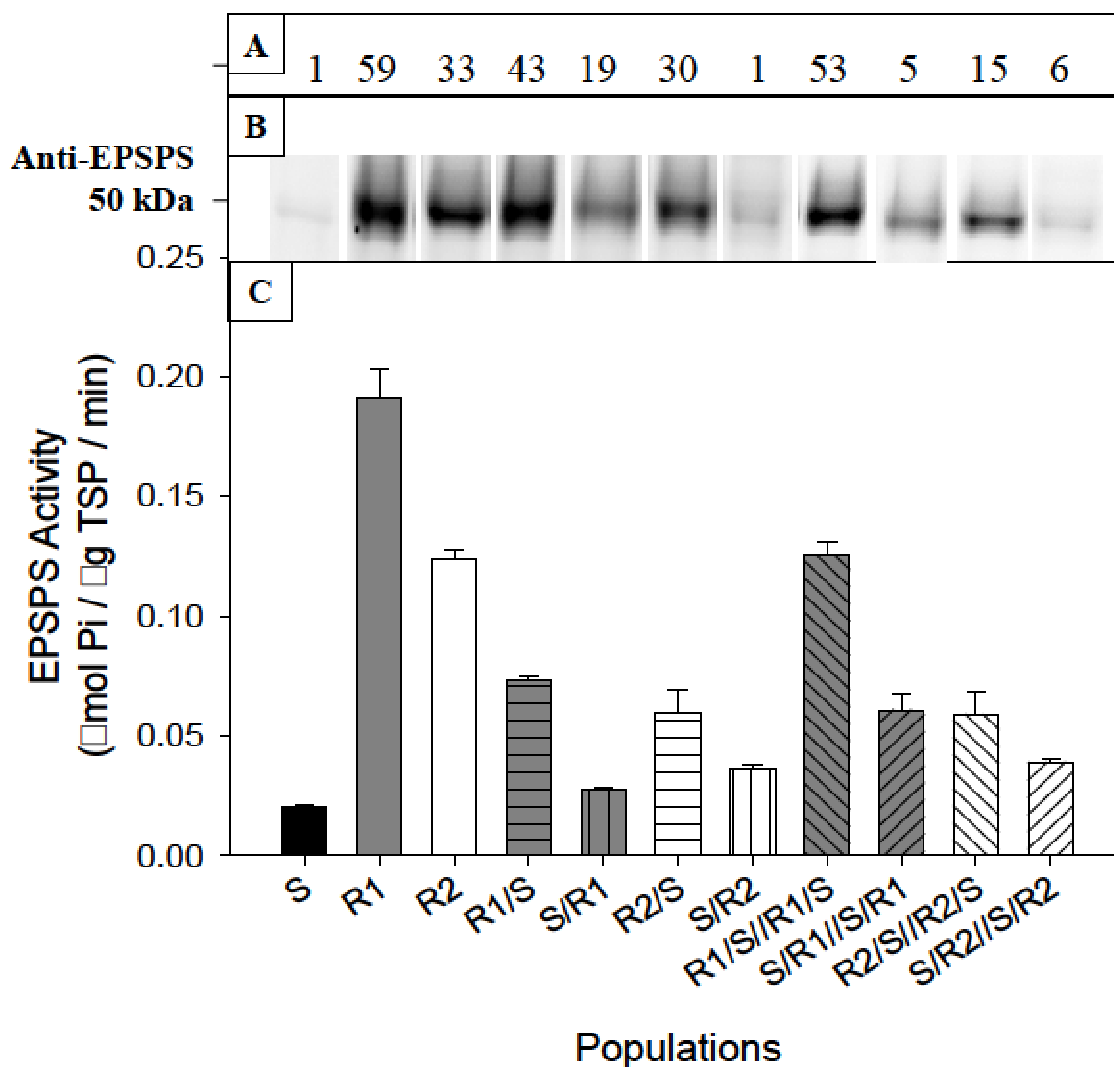

Figure 2.9 Positive correlation among *EPSPS* (5-enolpyruvylshikimate-3-phosphate synthase) genomic copy number, EPSPS quantity, and specific activity of EPSPS enzyme of Palmer amaranth populations.

Positive correlation among *EPSPS* (5-enolpyruvylshikimate-3-phosphate synthase) genomic copy number (A), EPSPS quantity (B), and specific activity of EPSPS enzyme (absence of inhibitor glyphosate) in glyphosate-susceptible (S), -resistant (R1 and R2), and first (R/S and S/R) and second (R/S//R/S and S/R//S/R) reciprocal crosses of Palmer amaranth populations. Pi, inorganic phosphate; TSP, total soluble protein. Vertical bars represent ± standard error of the mean ($n = 6$).

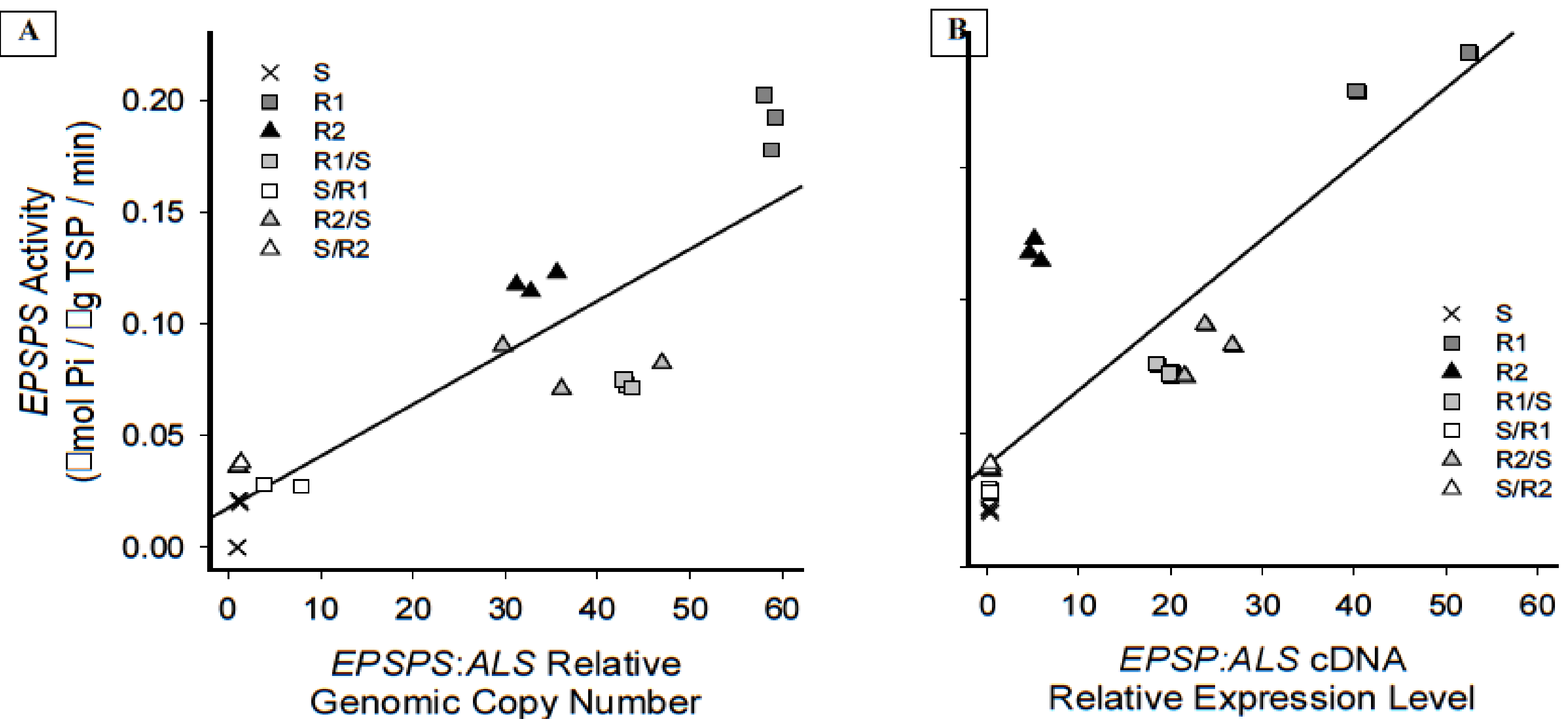

Figure 2.10 Positive correlation between specific activity of EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) enzyme and *EPSPS* genomic copy number and *EPSPS* cDNA expression levels of Palmer amaranth populations.

Positive correlation between specific activity of EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) enzyme and *EPSPS* relative to acetolactate synthase (*ALS*) genomic copy number (A) and *EPSPS* : *ALS* cDNA expression levels (B) in glyphosate-susceptible (S), -resistant (R1 and R2), and first reciprocal cross (R/S and S/R) of Palmer amaranth populations. A, regression of the entire dataset indicated a strong relationship between enzyme activity and copy number (solid and dotted line, $r = 0.87$, $P < 0.0001$). B, Regression of the entire dataset indicated a good relationship between mRNA levels and enzyme activity (dotted line, $r = 0.84$, $P < 0.0001$). The R2 population did not fit the model as well as the other populations. Excluding this population from the dataset improved the strength of that relationship (solid line, $r = 0.97$, $P < 0.0001$).

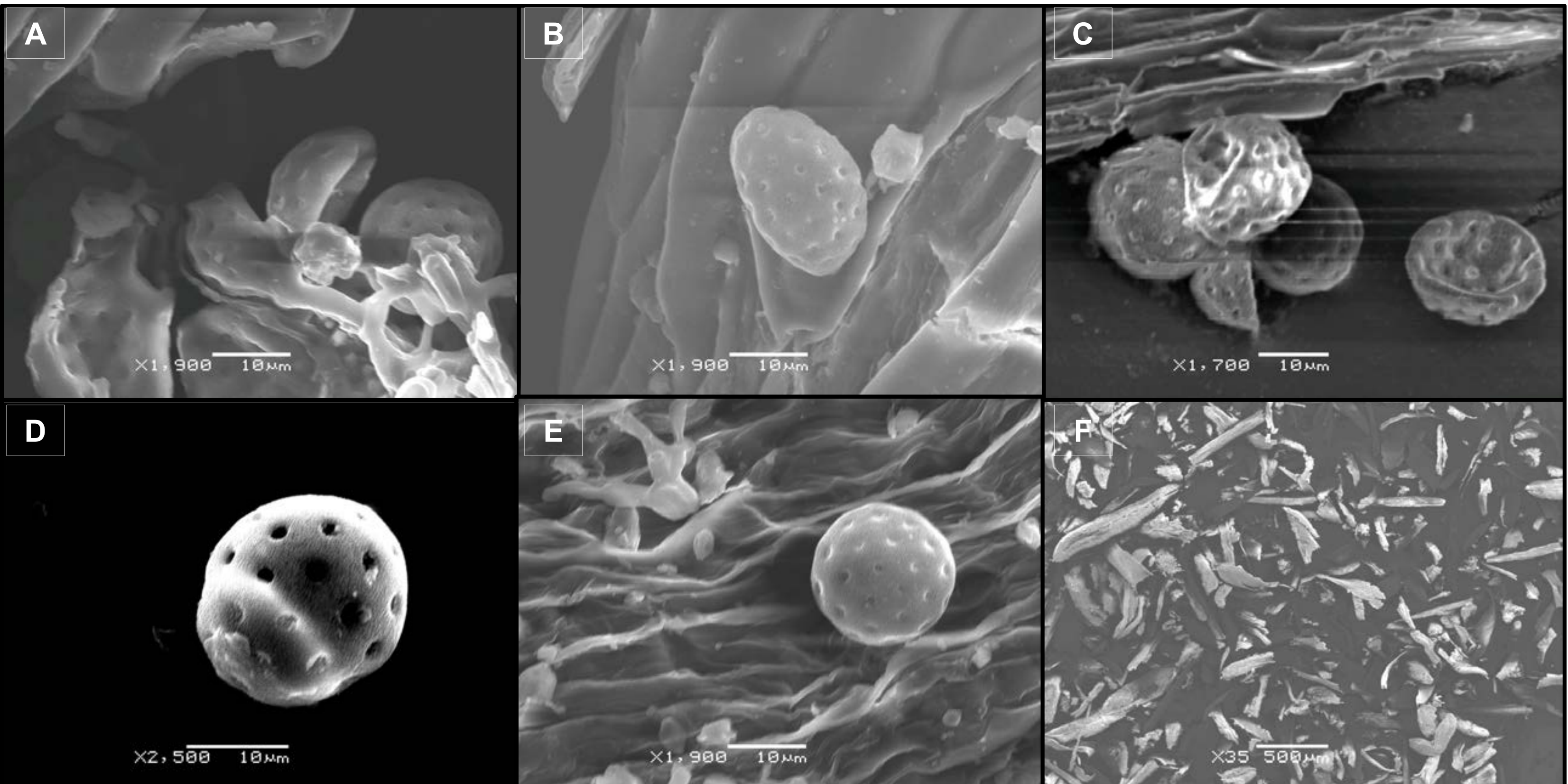

Figure 2.11 Scanning electron microscope images of the pollen isolated solution of Palmer amaranth populations.

A, B, C, E, and F shows that the pollen isolation protocol tested was unable to isolate pollen from other plant tissues isolated. D, pollen of Palmer amaranth isolated and intact. (Photo credit: Dr. Franck Dayan and J'Lynn Howell, USDA/ARS).

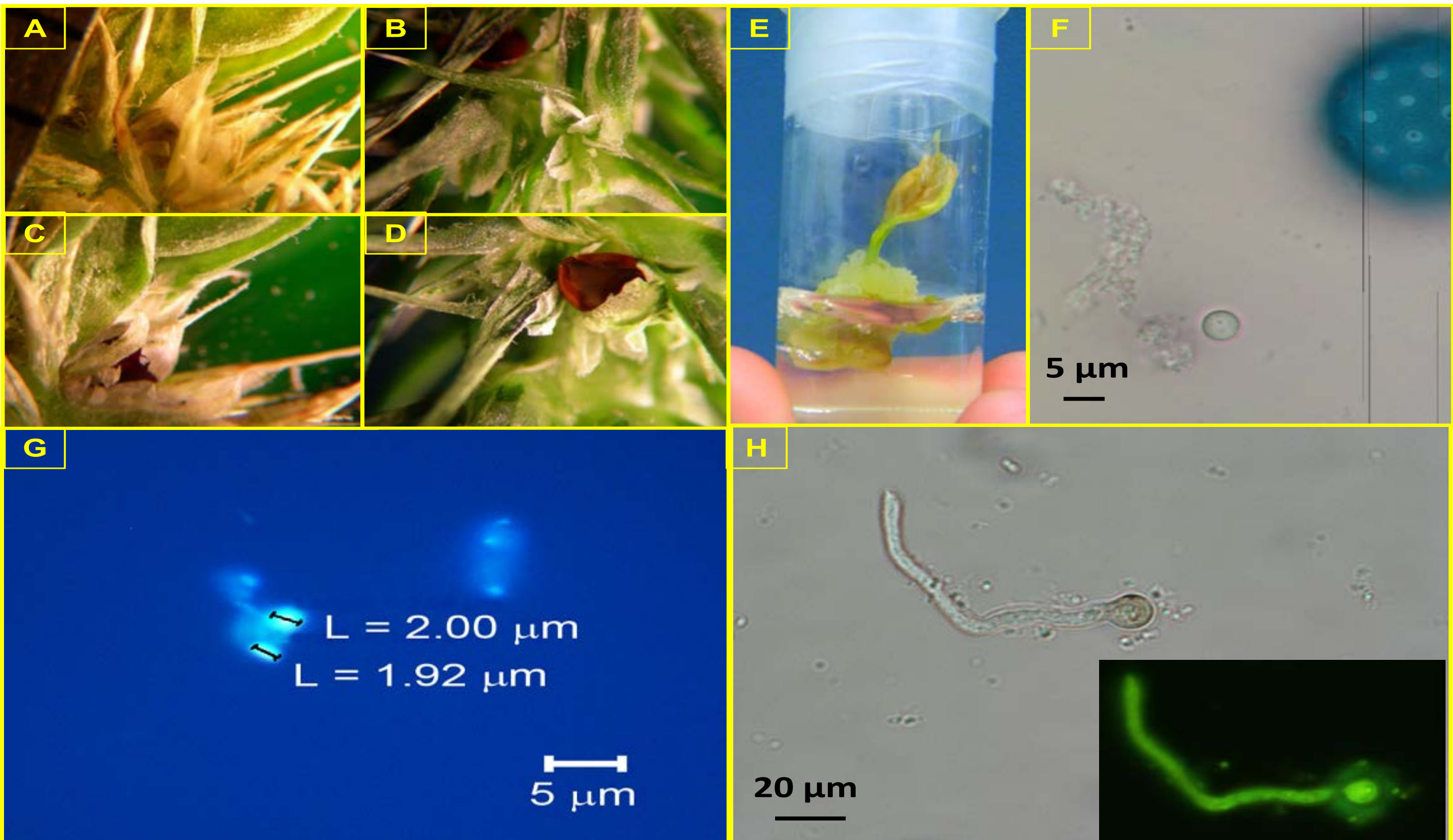

Figure 2.12 A panel of images from seed produced apomictically, callus formation, sperm cell and pollen grain of Palmer amaranth populations.

A panel of images from seed produced apomictically (A-D) by Palmer amaranth glyphosate-susceptible (A and C) and -resistant (B and D). Callus formation under regular MS tissue culture medium (Murashige and Skoog 1962) in glyphosate-resistant Palmer amaranth (E). Possibly isolated sperm cell and pollen grain of Palmer amaranth (F). Fluorescence microscopy in possibly isolated pair of sperm cells (G) and in possibly pollen grain with pollen tube germinated in sucrose medium (H).

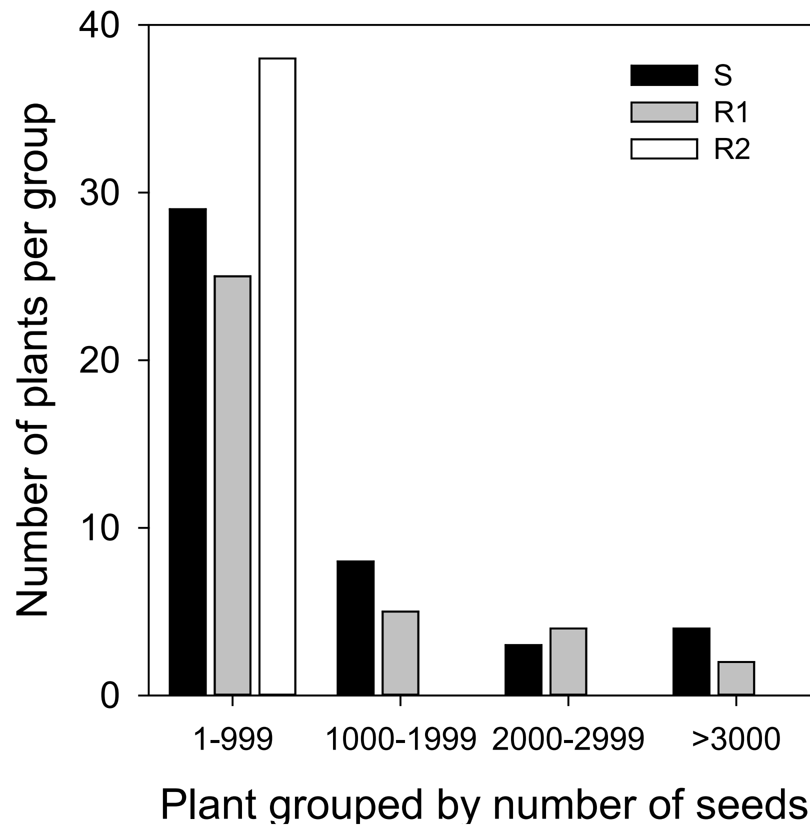

Figure 2.13 Seed production of reproductively isolated female plants due to the effect of agamospermy/apomixis of Palmer amaranth populations.

Seed production of reproductively isolated female plants due to the effect of agamospermy/apomixis of glyphosate-susceptible (S) and -resistant (R1 and R2) Palmer amaranth plants used as parents to generate first reciprocal crosses (R/S and S/R) studies.

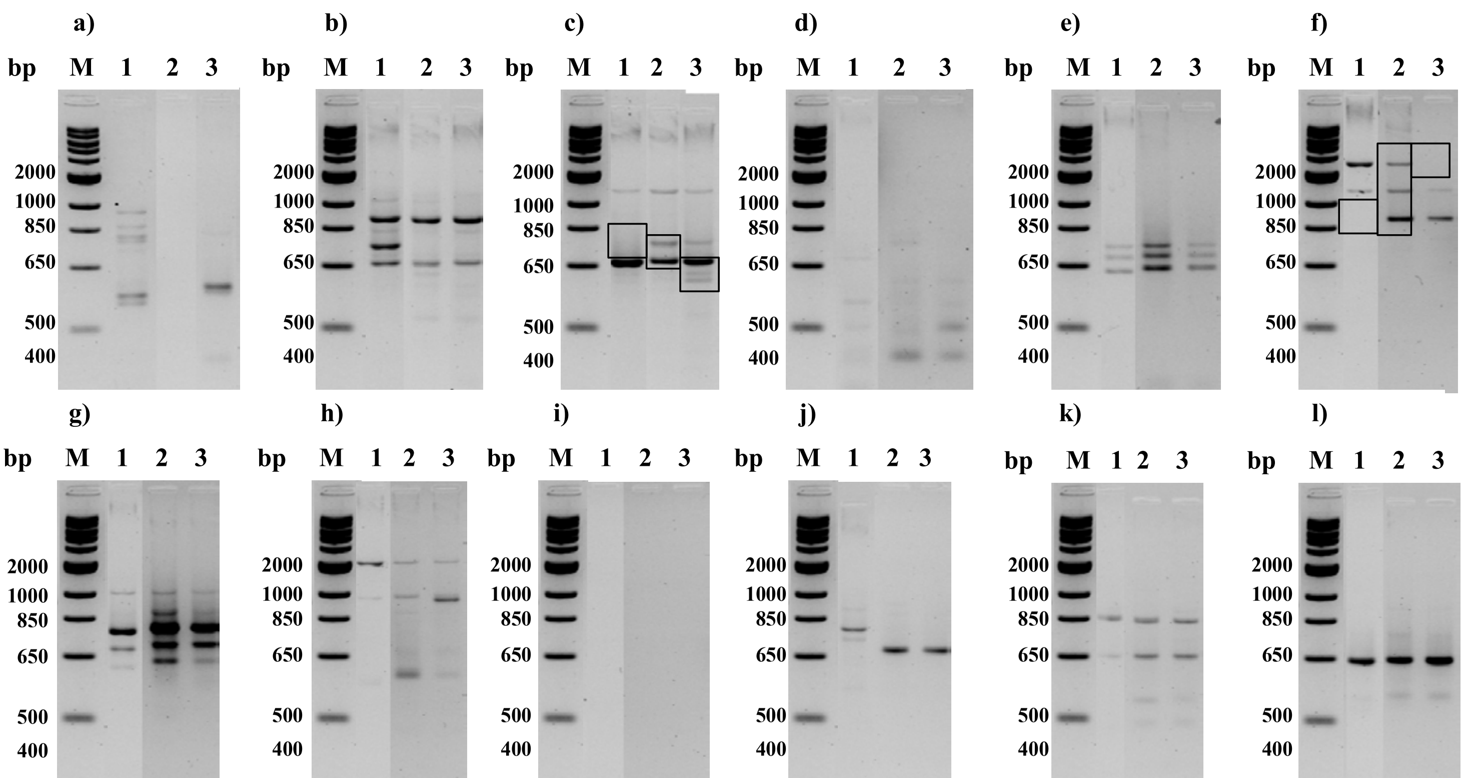

Figure 2.14 Random-amplified polymorphic DNA (RAPD) amplification pattern obtained for three samples from R1 population of Palmer amaranth.

Random-amplified polymorphic DNA (RAPD) amplification pattern obtained for three samples from R1 population of Palmer amaranth (1-3); using (a) primer 305, (b) primer 308, (c) primer 312, (d) primer 313, (e) primer 322, (f) primer 327, (g) primer 329, (h) primer 331, (i) primer 332, (j) primer 335, (k) primer 349, and (l) primer 354. RAPD analyses were performed using 12 decamer primers (Table 2.1) randomly selected from a list of 100 primers (NAPS Unit, University of British Columbia Biotechnology Laboratory, Vancouver, BC, Canada). M, 1-kb plus DNA ladder weight marker. Open square, polymorphic band

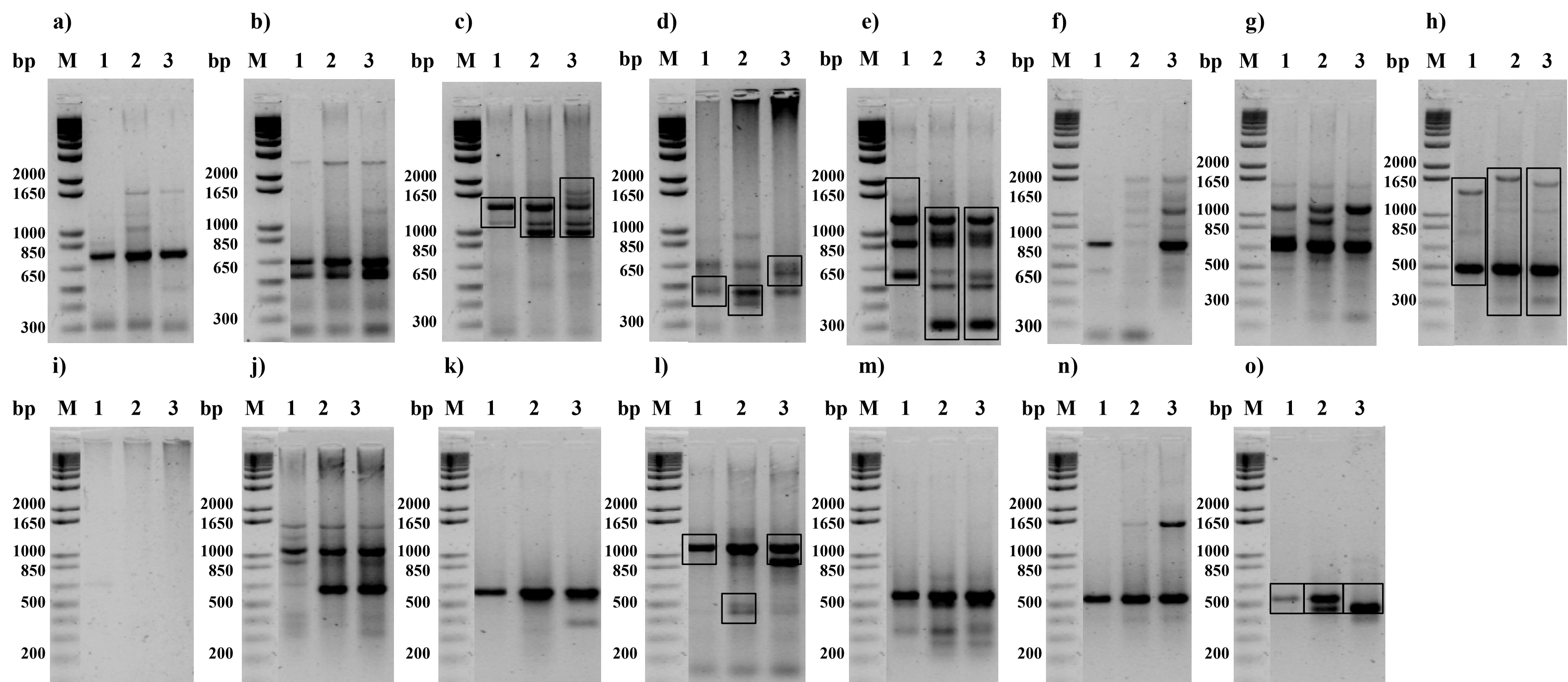

Figure 2.15 Inter-simple sequence repeat (ISSR, microsatellite) amplification pattern obtained for three samples from R1 population of Palmer amaranth.

Inter-simple sequence repeat (ISSR, microsatellite) amplification pattern obtained for three samples from R1 population of Palmer amaranth (1-3); using (a) primer UBC 807, (b) primer UBC 808, (c) primer UBC 812, (d) primer UBC 817, (e) primer UBC 825, (f) primer UBC 834, (g) primer UBC 835, (h) primer UBC 842, (i) primer UBC 845, (j) primer UBC 856, (k) primer (GGC)6W, (l) primer (AAC)6K, (m) primer (AAG)6Y, (n) primer (GGAT)4H, and (o) primer (GGGGT)3M. ISSR analyses were performed using 15 primers (Table 2.1) randomly chosen from a list of 100 primers (NAPS Unit, University of British Columbia Biotechnology Laboratory, Vancouver, BC, Canada) and five of Natascha Techen (National Center for Natural Products, University of Mississippi, USA) design. M, 1-kb plus DNA ladder weight marker. Open square, polymorphic band.

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CHAPTER III

VARIABLE TOLERANCE TO GLYPHOSATE IN PITTED MORNINGGLORY (*Ipomoea lacunosa*) ACCESSIONS

3.1 Abstract

Glyphosate is considered by many to be the most important herbicide ever developed. Aminomethylphosphonic acid (AMPA) is the most frequently detected metabolite of glyphosate in higher plants. The natural tolerance of morningglories (*Ipomoea* spp.) to glyphosate has made these plants among the most common and troublesome weeds in the southeastern U.S. since the adoption of glyphosate-resistant crops. Experiments were conducted to determine (1) the variability in tolerance to glyphosate among morningglories accessions, (2) if the variability in glyphosate tolerance levels is correlated with repeated exposure to glyphosate, and (3) if there is any correlation of metabolism of glyphosate to AMPA and/or sarcosine in pitted morningglory (*Ipomoea lacunosa* L.) populations and their natural level of tolerance to glyphosate. An initial glyphosate screening of 73 accessions of morningglories resulted in control ranging from -120 to 85% at 420 g ae ha^{-1} and from -25 to 100% at 840 g ae ha^{-1} glyphosate; pitted morningglory was relatively more tolerant than the other morningglory species. Consequently, fourteen pitted morningglory populations were selected for dose-response assays that resulted in GR_{50} values range from 59 to 151 g ae ha^{-1} glyphosate; a 2.6-fold variability in tolerance to glyphosate among the accessions. Moreover, a pattern

was observed where the populations that had a history of less exposure to glyphosate had smaller GR_{50} values. Subsequently, the most tolerant (MT) and the least tolerant (LT) populations where selected for a differential metabolism study. In one experiment, populations were both treated with 420 g ae ha^{-1} glyphosate and evaluated 14 days after treatment (DAT). Less glyphosate and shikimate was recovered in MT than in LT. However, AMPA was not different between populations. Moreover, the lowest glyphosate/AMPA ratio was observed in the MT, indicating that MT presented the highest metabolism ratio. In another experiment, populations were evaluated 1, 3, and 6 DAT with their GR_{50} rate so that metabolism could be evaluated at similar toxicity levels. More glyphosate was recovered in MT as time after treatment increased, but it was constant in LT. AMPA did not differ by population and evaluation time. The ratio of glyphosate degraded to AMPA was different between MT and LT, and LT at 3 and 6 DAT had the highest metabolism ratio. Lower levels of shikimate were observed for MT at 3 and 6 DAT and for LT at all harvesting times. Sarcosine was not present in either MT or LT in both experiments. Although some pitted morningglories were more tolerant to glyphosate than others, and there was considerable variation between populations in the glyphosate to AMPA ratio, metabolism of glyphosate to AMPA or sarcosine is a common factor in explaining natural resistance levels.

3.2 Introduction

Glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EPSPS) that catalyzes the conversion of shikimate-3-phosphate and phosphoenolpyruvate to EPSP and inorganic phosphate in the shikimic acid pathway (Devine et al. 1993; Geiger and Fuchs 2002; Gruys et al. 1993; Steinrücken and Amrhein

1980). Inhibition of EPSPS results in shikimic acid accumulation, reduction or accumulation of benzoates and quinates, and reduction of biosynthetic products, such as aromatic amino acids (phenylalanine, tyrosine and tryptophan), vitamins (K and E), proteins, alkaloids, lignin, flavonoids, coumarins, indole acetic acid (IAA), chlorophyll content, and carotenoids (Amrhein et al. 1980; Anderson and Johnson 1990; Arnaud et al. 1994; Bently 1990, Devine et al. 1993; Herrmann and Weaver 1999). Moreover, the shikimic acid increase relates to a decline in carbon fixation intermediates and reduction of photosynthesis (Duke et al. 2003a).

Glyphosate was commercialized in 1974. Since then, the agricultural community has used it extensively in agriculture worldwide to become the most commercialized and important herbicide ever developed (Duke and Powles 2008; Perez-Jones et al. 2007; Powles 2003). First used as a non-crop, preplant, or orchard and vine crop herbicide, it is now also used in no-tillage systems and in glyphosate-resistant (GR) crops (Owen and Zelaya 2005; Shaner 2000).

The adoption of transgenic, herbicide-resistant crops, has increased dramatically in the last two decades (Duke and Cerdeira 2010; Owen and Zelaya 2005). This unprecedented change in agriculture has many effects. One of the highest impacts has been the simplification of weed-control tactics and the resulting changes in weed communities (Owen and Zelaya 2005). The adoption of herbicide-resistant crops results in greater selection pressure on the weed community due to a limited variety of herbicides used (Powles and Preston 2006). Selection pressure from herbicides can result in weed shifts attributable to the natural resistance (tolerance) of a particular species to the herbicide or the evolution of resistance within the weed population (Dill 2005; Owen

and Zelaya 2005). The evolution of herbicide defense traits in weedy species is possibly one of the best examples of rapid adaptation to a changing environment (Cousens and Mortimer 1995; Yuan et al. 2006).

Herbicides are very intense selective agents, and although glyphosate is considered a nonselective herbicide, several weed species show varying degrees of natural tolerance, such as common lambsquarters (*Chenopodium album* L.), giant ragweed (*Ambrosia trifida* L.), velvetleaf (*Abutilon theophrasti* Medik), jussieu [*Dicliptera chinensis* (L.) Juss.], common evening-primrose (*Oenothera biennis* L.), wild parsnip (*Pastinaca sativa* L.), common pokeweed (*Phytolacca americana* L.), field horsetail (*Equisetum arvense* L.), *Commelina* spp., and *Ipomoea* spp. (Owen 2008). In particular, pitted morningglory (*Ipomoea lacunosa* L.), one of the most common and troublesome weed species in southern U.S. row crops (Webster 2001, 2004, 2005), has considerable genetic variability in its response to glyphosate at typical GR crop application rates (Bryson et al. 2008; Burke et al. 2007, 2009; Chachalis et al. 2001; Koger and Reddy 2005a; Norsworthy et al. 2001; Norsworthy and Oliver 2002; Reddy and Whiting 2000; Reddy et al. 2008; Shaw and Arnold 2002; Webster et al. 1999). Differences in levels of tolerance to glyphosate in pitted morningglory have been attributed to several factors, glyphosate rate and spray coverage being the most important. In addition, its tolerance has also been attributed to limited absorption by Norsworthy et al. (2001) and Starke and Oliver (1998) and, controversially, not attributed to limited absorption and translocation by Koger et al. (2004) and Koger and Reddy (2005a).

The most frequently detected of glyphosate's degradation products is aminomethylphosphonic acid (AMPA) (Duke 2011); however, some researchers have

reported sarcosine as a degradation product (Sandberg et al. 1980; Sprankle et al. 1978). Consequently, some assume that glyphosate can be metabolized by plants via two pathways. One involves oxidative cleavage of the C-N bond and the other breaking of the C-P bond (Duke 2011; Reddy et al. 2008). Most plants do not metabolize glyphosate sufficiently to avoid its toxic effects, but researchers found that the following plants could metabolize glyphosate to AMPA: quackgrass [*Agropyron repens* (L.) Beauv.] (Coupland 1984), alligatorweed [*Alternanthera philoxeroides* (Mart.) Griseb.] (Eberbach and Bowmer 1995), Canada thistle [*Cirsium arvense* (L.) Scop.] (Sandberg et al. 1980), field bindweed (*Convolvulus arvensis* L.) (Sandberg et al. 1980; Sprankle et al. 1978), field horsetail (Marshall et al. 1987), tall morningglory [*Ipomoea purpurea* (L.) Roth] (Sandberg et al. 1980), sicklepod [*Senna obtusifolia* (L.) H. S. Irwin & Barneby], coffee senna [*Cassia occidentalis* (L.) Link], Illinois bundleflower [*Desmanthus illinoensis* (Michx.) MacM. ex B. L. Robins. & Fern.], kudzu [*Pueraria montana* var. lobata (Willd.) Maesen & S. M. Almeida], horseweed [*Conyza canadensis* (L.) Cronq.] (Reddy et al. 2008). Field bindweed (Sprankle et al. 1978) and tall morningglory (Sandberg et al. 1980) also accumulates sarcosine as a metabolite of glyphosate.

Transgenic GR soybean [*Glycine max* (L.) Merr.] and canola (*Brassica napus* L.) metabolizes glyphosate to AMPA (Duke 2011; Nandula et al. 2007a; Reddy et al. 2008). Resistance to glyphosate is conferred by two transgenes for glyphosate-insensitive EPSPS, the *cp4 epsps* gene from *Agrobacterium tumefaciens* strain CP4 and *zm-2mepsps* produced by site-directed mutagenesis of corn (*Zea mays* L.) *EPSPS*, and one transgene for metabolic degradation, from *Ochrobactrum anthropic* strain LBAA which encodes glyphosate oxidoreductase (GOX). Resistance to glyphosate is conferred by the *cp4 epsps*

gene in soybean and by *cp4 epsps* and *gox* genes in canola (Green 2009; McLaren and Copping 2011). Detection of AMPA following glyphosate treatment in soybean suggests that a plant GOX is responsible for this conversion (Reddy et al. 2008). However, nothing is known about the enzymology of glyphosate degradation to AMPA in plants. Moreover, AMPA is phytotoxic to plants, and its mode of action is apparently different from that of glyphosate (Reddy et al. 2004).

The objectives of this research were to investigate (1) the variability in tolerance to glyphosate among morningglory accessions, (2) if the variability in glyphosate tolerance levels are correlated with the length of time exposed to GR systems, (3) if the level of tolerance is inversely correlated with shikimate accumulation, and (4) if differential metabolism of glyphosate to AMPA and/or sarcosine is the underlying mechanism for differential tolerance to glyphosate among pitted morningglory populations.

3.3 Materials and Methods

3.3.1 Plant Material and General Experimental Conditions

During 2004 through 2006, seed from a total of 71 accessions (Appendix B) of ivyleaf morningglory (*Ipomoea hederacea* Jacq.), palmleaf morningglory (*I. wrightii* A. Gray), pitted morningglory, and purple moonflower (*I. turbinate* Lag.) were randomly collected at multiple locations across the U.S. that had or had not been exposed to glyphosate for several years to GR crop management systems (Burke et al. 2009). Seed of two populations of pitted morningglory, one population not exposed to a GR crop system and another exposed to four years of GR crop management, were collected in 1990 and 1999, respectively (Appendix B). Each seed sample is an accession from the herbarium

located at the U.S. Department of Agriculture, Agricultural Research Service, Southern Weed Science Research Unit in Stoneville, MS, under Dr. Krishna N. Reddy until 2011. Morningglory seed were stored at 10 C until used. Germination of seed, transplanting of seedlings, growth of plants, and all experiments were conducted under greenhouse (30/22 C day/night, 12-h photoperiod under natural sunlight conditions) growing conditions unless otherwise described. Seed were planted at 1-cm depth in 50-cm by 20-cm by 6-cm plastic trays with drain holes containing a commercial potting mix (Metro-Mix 360, Sun Gro Horticulture, Bellevue, WA 98008). Two weeks after emergence, seedlings were transplanted into 6-cm by 6-cm by 6-cm pots containing the soil mix mentioned before. Plants were watered as needed. Plants were fertilized once by sub-irrigating the pots with a nutrient solution (Miracle-Gro, The Scotts Company, Marysville, OH 43041) containing 200 mg L^{-1} of each N, P_2O_5, and K_2O at 4 weeks after transplanting. All herbicide treatments were applied with an air-pressurized indoor spray chamber equipped with an 8002E flat-fan nozzle (Spraying Systems Company, Wheaton, IL 60139) delivering 140L ha^{-1} at 280 kPa, made on plants of each morningglory accession at four- to five-leaf stage (beginning to vine).

3.3.2 Screening of Populations with Discriminating Glyphosate Doses

In order to detect any potential variability in tolerance to glyphosate among the accessions, a preliminary screening study was used so that extreme variance in level of tolerance would be the criteria for the populations selected for a dose-response study. Plants of each morningglory accession were treated with glyphosate (Roundup WeatherMAX, Monsanto Company, St. Louis, MO 63146) at 0, 420, and 840 g ae ha^{-1}. Percent control [visible estimate of injury on a scale of 0 (no injury) to 100 (complete

death)] was recorded two and three weeks after treatment (WAT), and aboveground shoot fresh weight (expressed in terms of nontreated control plants) was recorded at 3 WAT by cutting the plants at the soil surface level and fresh weight for each pot (including any dead leaf tissue) was recorded. A fresh weight reduction parameter was selected to include the effect of water stress-induced by glyphosate phytotoxicity (Burke et al. 2009). The experimental design consisted of four replications of each population at each dose, one plant per replication, in a completely randomized design, and the experiment was conducted two times.

3.3.3 Glyphosate Dose Response in Pitted Morningglory Accessions

Fourteen pitted morningglory accessions were selected based strictly on their response to preliminary screening in the above study, being the six most tolerant and the seven least tolerant accessions among the 73 initial populations (Table 3.1) and a population accession from Dr. Vijay K. Nandula, Mississippi State University, Research Center in Stoneville, MS. Experimental procedures were similar to those described in the above study except for glyphosate rate and harvesting time. Glyphosate applications at 0, 105, 210, 420, 840, and 1,680 g ha^{-1} were used to determine the dose response of each of the 14 accessions. Percent control ratings were recorded at 2 WAT, as separation of vines between control plants can later become problematic. Data were expressed as percent shoot fresh weight reduction as compared to nontreated plants. There were four replications per treatment, one plant per replication, in a completely randomized design, and the experiment was conducted twice.

3.3.4 Glyphosate Metabolism Study

The most tolerant (MS-WAS-8) and least tolerant (MS-YAZ-1) populations were selected based on the dose response assay described above and were analyzed for metabolism of glyphosate to AMPA and sarcosine, as well as shikimate, the precursor of aromatic amino acids. Two experiments were conducted. In the first, five plants were treated with glyphosate at 420 g ae ha^{-1} (0.5 × field rate) and harvested at two WAT, and the experiment was conducted twice. In the second, three plant replications were treated at their respective GR_{50} doses (MS-WAS-8: 151 g ae ha^{-1}; MS-YAZ-1: 59 g ae ha^{-1}) and at one, three, and six days after treatment (DAT) plants were harvested, and the experiment replicated over time. The GR_{50} rate for each plant accession was selected so that the two accessions would have the same level of phytotoxicity interfering with metabolism of glyphosate. There was one plant per replication, in a completely randomized design, and the experiments were conducted at different times. At harvesting, plants were excised at the soil surface, washed with running water, rinsed with distilled water to remove glyphosate remaining on the leaf surface, and blotted dry with paper towels. Each sample consisted of all leaves from each single plant (replicate) and leaves were pre-dried in a greenhouse and then oven dried at 80 C for two weeks, ground with a mortar and pestle, and analyzed for glyphosate, AMPA, shikimate and sarcosine.

Extraction and derivation were performed for glyphosate and AMPA analysis according to Alferness and Wiebe (2001) and Reddy et al. (2008), with modifications. Ground tissue (0.25 g) was extracted with 8 mL of water in a 20 mL scintillation vial, shaken, placed in a sonicating bath for 20 min, and then centrifuged (Sorvall RC 6 Plus, Thermo Electron Corporation, Asheville, NC 28801) at 5,000 × g, 20 C, for 20 min. The

supernatant was collected, and the remaining tissue sample pellet was extracted a second time by adding 4 mL of water, and procedures were performed as in the first extraction. The volume of the combined supernatant was measured and syringe-filtered (0.45 μm) into a new 20 mL plastic vial. Then, 45 μL of HCl was added to the supernatant and shaken. Four milliliters were transferred to a 20 mL scintillation vial with a Teflon-lined cap, shaken with 2 mL of CH_2Cl_2, and centrifuged (Savant speed vac, model SVC 200, Savant Instruments, Holbrook, NY 11741) at 300 × g, 25 C, for 10 min. A portion (1.8 mL) of the top water layer was taken, and 200 μL of acidic modifier [(16 g KH_2PO_4 : 160 mL H_2O) : 13.4 mL HCl] was added and vortexed. One mL was loaded to a cation exchange (CAX) resin column (AG 50W-X8 Resin 200-400 mesh, H+ 0.8 by 4 cm, Bio-Rad Laboratories, Hercules, CA 94547) previously equilibrated with two 5 mL portions of water. The sample was eluted until the column bed was reached. Seven hundred microliters of CAX mobile phase (160 mL H_2O : 40 mL MeOH : 2.7 mL HCl) was added, eluted, and discarded. Twelve milliliters of CAX mobile phase were again added to the column to elute the analytes. The eluate was collected in a 20 mL vial and evaporated to dryness using a Savant speed vac. To the dried sample was added 1.5 mL of CAX mobile phase, and then the vial was placed in a sonicating bath for 30 min. A 20 μL aliquot was syringe-filtered (0.2 μm) and added to 640 μL of a solution of 2,2,3,3,4,4,4-heptafluoro-1-butanol and trifluoroacetic anhydride (1:2) in a chilled 4 mL vial with a teflon-lined lid in a duplicate extraction experiment. The mixture was allowed to equilibrate at room temperature for 10 min. The vial was transferred to a heating block at 90 C for 1 h and then allowed to cool to room temperature. The solvent was evaporated

under a stream of nitrogen at 50 C, and the residue was dissolved in 80 µL of ethyl acetate containing 0.2% citral.

For the analysis of shikimate and sarcosine, a 1 g powdered sample was placed in a 20 mL scintillation vial and extracted with 15 mL water, shaken, placed in a sonicating bath for 20 min, and then centrifuged (Sorvall RC 6 Plus) at 5,000 × g, 20 C, for 20 min. A 4 mL aliquot of supernatant was removed to a new 20 mL vial. The tissue sample pellet was extracted a second time by adding 5 mL of water, and procedures were performed as in the first extraction. A 2 mL aliquot of supernatant was removed and combined with the previous 4 mL aliquot, totaling 6 mL of supernatant. Then 30 µL of HCl was added to supernatant and shaken. One half of the total supernatant was transferred to a tared vial, frozen and lyophilized. Dry weight was recovered and 5 mg of lyophilized extract was transferred to GC vial, in a duplicate extraction experiment, to be treated with 50 µL of N-O-bis-(trimethylsilyl)-trifluoroacetamide and N-N-dimethylformamide (1:1) and vortexed. The vial was transferred to a heating block at 70 C for 30 min, allowed to cool to room temperature, and centrifuged (Savant speed vac model SVC 200) at 300 × g, 25 C, for 10 min. Then 25 µL of clear liquid was transferred to a GC vial and analyzed by GC-MS.

Analysis of glyphosate and AMPA was performed by GC-MS (Agilent 6890 series GC coupled to a JEOL GCMateII mass spectrometer, JEOL USA, Peabody, MA 01960) using a DB-5 capillary column (Agilent Technologies, Foster City, CA 94404), 30 m length by 0.25 mm i.d. by 0.25 µm film. The GC temperature program was: initial, 80 C, held for 2.5 min, raised to 160 C at 30 C min^{-1} rate, raised to 270 C at 40 C min^{-1} rate, raised to 300 C at 35 C min^{-1} rate, and kept at this temperature for 1.5 min. The

carrier gas was ultrahigh purity helium, at 1 mL min^{-1} flow rate. The injection port was kept at 250 C, the GC-MS interface and the ionization chamber at 230 C. The volume of injection was 1 μL (splitless injection). The mass spectrum was acquired in the positive, low resolution, ion monitoring mode selected, and electron impact 70 eV. AMPA was monitored using m/z 571, 502, 446, 372 (retention time 5.97 min); glyphosate was monitored using m/z 611, 584, 486, 460 (retention time 6.77 min). Glyphosate and AMPA in the samples were quantitated from a calibration curve of the respective standards (glyphosate, purity 99.5%, Chem Service, West Chester, PA 19380; AMPA, purity 99%, Sigma-Aldrich, Saint Louis, MO 63103). Analysis was performed in duplicate. The LOD and LOQ for glyphosate were 19.9 and 160 pg on column (1 μL injection), respectively. The LOD and LOQ for AMPA were 4.16 and 12.61 pg on column (1 μL injection), respectively.

Analysis of sarcosine and shikimate was performed by GC-MS using the same conditions as in the analysis of glyphosate and AMPA, except the GC temperature program was: initial, 120 C, held for 2 min and raised to 300 C at 17 C min^{-1} rate, then held at this temperature for 0.5 min. Sarcosine was monitored using m/z 233, 218, 190, 160 (retention time 5.58 min); shikimate was monitored at m/z 462, 447, 372, 255 (retention time 8.67 min). Sarcosine and shikimic acid in the samples were quantitated from a calibration curve of the respective standards (shikimic acid, purity 99%, Sigma-Aldrich; sarcosine, Sigma-Aldrich). Analysis was performed in duplicate. Sarcosine was not detected in any of the samples. The LOD and LOQ for shikimate were 929.15 and 2,815.66 pg on column (1 μL injection), respectively.

3.3.5 Statistical Analysis

All data were analyzed by ANOVA via the PROC GLM statement using SAS software (version 9.3, SAS Institute, Cary, NC 27513) to determine the main effects and interactions of the factors at $P < 0.05$. No significant experiment effect was observed in repeated experiments; therefore, data from experiments were pooled.

3.3.5.1 Screening of Populations with Discriminating Glyphosate Dose

Data variance was visually inspected by plotting residuals to confirm homogeneity of variance prior to statistical analysis. Means separation were performed using Fisher's protected least significant difference (LSD) test at $P = 0.05$ using SAS software.

3.3.5.2 Glyphosate Dose Response in Pitted Morningglory Accessions

Where ANOVA indicated significant differences between treatments, non-linear regression was applied using a log-logistic model (Seefeldt et al. 1995) (Equation 3.1).

$$y = L + \frac{U - L}{\left[1 + \left(\frac{D}{GR_{50}}\right)^{s}\right]} \tag{3.1}$$

Where: y represents shoot fresh weight reduction as compared to nontreated plants in percentage at herbicide rate D, L is the mean response at very high herbicide rate (lower limit), U is the mean response when the herbicide rate is zero (upper limit), s is the slope of the line at GR_{50}, and GR_{50} is the herbicide rate required for 50% growth reduction.

Experience shows that usually a logistic dose-response curve reasonably describes what happens in the crop and weeds in response to different doses of herbicide (Ritz and Streibig 2006). The estimate of the four regression parameters was obtained using Sigma Plot (version 11, Systat Software, San Jose, CA 95110) and tested for significance using the *t*-test method ($P < 0.05$). For each parameter, the null hypothesis, H_0: parameter = 0, was tested against the alternative hypothesis, H_a: parameter ≠ 0. The remaining part is to find out if there is any difference in potency between accessions and the least tolerant accession at the GR_{50} effect level according to *t*-Student test at P < 0.05. The null hypothesis, H_0: GR_{50} accession / GR_{50} least tolerant accession = 1, was tested against the alternative hypothesis, H_a: GR_{50} accession / GR_{50} least tolerant accession ≠ 1. This test was performed using the open-source R software (version 2.15.2, R Foundation for Statistical Computing) using package drc, drm function, and the comparisons were given by means of the selectivity index (SI) function.

3.3.5.3 Glyphosate Metabolism Study

Data variance was visually inspected by plotting residuals to confirm homogeneity of variance prior to statistical analysis using SAS. The Shapiro-Wilk test was applied to verify if the data among populations were normally distributed, and Hartley's F_{max} test was applied to verify if different populations have a similar variance using SAS. Means separation was performed using Fisher's protected least significant difference (LSD) test at P = 0.05.

3.4 Results and Discussion

3.4.1 Screening of Populations with Discriminating Glyphosate Doses

The application of the ‘F’ test on variance analyses detected interaction between the three glyphosate doses and 73 populations of morningglories ($P < 0.0001$) on the percent of control (2 and 3 WAT) and percent of fresh weight reduction (3 WAT), indicating that all the morningglory populations have different levels of tolerance to glyphosate at 0, 420, and 840 g ae ha^{-1}.

Glyphosate injury in morningglories consisted of chlorosis of the newest leaves, epinastic response and, in some cases, necrosis of the growing point. The same symptomology was reported by Burke et al. (2009). The response to glyphosate application varied among morningglories accessions. All plants survived 420 g ha^{-1} of glyphosate and some accessions were killed with 840 g ha^{-1} of glyphosate (Table 3.1). The visual rating of control ranged from 20 to 80% at 420 g ha^{-1} glyphosate and 50 to 100% at 840 g ha^{-1} glyphosate at 2 WAT; at 3 WAT ranged from 48 to 85% and from 63 to 100%, respectively (Table 3.1). The percentage of fresh weight reduction ranged from -120 to 85% at 420 g ha^{-1} glyphosate and from -25 to 100% at 840 g ha^{-1} glyphosate (Table 3.1). The negative values of percentage of control were previously reported in literature as indicative of no response to glyphosate treatment (Burke et al. 2009) and of growth stimulation by subtoxic levels of glyphosate (Velini et al. 2008).

The four comparison species included in this study (ivyleaf morningglory, purple moonflower, palmleaf morningglory, and pitted morningglory) had consistent inherent variability in control by glyphosate between species and among germplasm accessions (Table 3.1). Pitted morningglory accessions were the least sensitive to glyphosate among

doses and types of evaluation (Table 3.1) and, consequently, 14 populations of pitted morningglory were selected to proceed with dose-response studies. The criteria used were the fresh weight reduction at 840 g ha^{-1} (3 WAT) evaluation, the germination rate observed (data not presented), and to restrict the accessions collected from Mississippi.

3.4.2 Glyphosate Dose Response in Pitted Morningglory Accessions

The F-test in the ANOVA was significant ($P < 0.0035$) for the pairing of dependent variable (fresh weight reduction) with main effect terms (populations and glyphosate dose) and interaction terms, indicating that the response to glyphosate rates varied among the fourteen morningglory populations. Shoot biomass in each pitted morningglory population decreased as glyphosate rate increased (Table 3.2). However, there were different dose-responses between accessions, enabling differentiation between the six most tolerant (MS-WAS-8, MS-VJ, MS-SCO-1, MS-PAN-1, MS-YAZ-2, and MS-COA-1) and the eight least tolerant (MS-ITA-1, MS-LEE-2, MS-WAS-2, MS-MAR-1, MS-99, MS-90, MS-QUI-1, and MS-YAZ-1) accessions. This was accomplished by comparing the relative potencies among accessions at the GR_{50} response level (SI) (Table 3.2).

The populations with less exposure to the GR crop management system were the ones with numerically smaller GR_{50} values (MS-99: 106, MS-90: 91, MS-QUI-1: 59, and MS-YAZ-1: 58 g ha^{-1} glyphosate). This variability could be attributed to potential glyphosate exposure to each accession (Table 3.2). Koger et al. (2004) acknowledged that pitted morningglory went from the fifth to the second most common weed in Mississippi soybean six years after the introduction of GR soybean. Therefore, it appears that the emergence of pitted morningglory as a major weed problem has coincided with the

widespread adoption of GR system. The tolerance of morningglory may be a consequence of mutations arising after the introduction of the herbicide, or they may predate the widespread use of the herbicide and were selected from genetic variation that already existed in the population, probably an exaptation (Baucom and Mauricio 2010). Also, localized adaptations have probably resulted in the evolution of several pitted morningglory ecotypes in North America.

Burgos et al. (2011) used ISSR (inter-simple sequence repeat) markers to study intraspecific population structure in pitted morningglory. They detected subpopulation differentiations in those accessions from proximal locations and clustered together populations with high similarity of agricultural environments, like Arkansas and Mississippi. On the other hand, intraspecific accessions almost always clustered together among various *Ipomoea* species (Huang and Sun 2000). Moreover, it is possible that the colonization of pitted morningglory in the southern U.S. started with only one genotype and evolved with time due to localized adaptations and hybridization with compatible species (Bryson et al. 2008). Consequently, the morphological and genetic variance of pitted morningglory in the southern U.S. may impact the efficacy of weed management strategies.

R/S ratios indicated a 2.6 fold difference between the least and most sensitive accessions of pitted morningglory. The MS-YAZ-1 accession had the lowest GR_{50} value and MS-WAS-8 accession had the highest. The 2.6 fold R/S ratio of glyphosate tolerance is more than that reported for other pitted morningglory accessions from the southern U.S., which was 1.9-fold less when comparing their most tolerant to their least tolerant populations (Burke et al. 2009). However, R/S ratio is lower than the resistance levels

reported in GR biotypes of other species, such as goosegrass [*Eleusine indica* (L.) Gaertn.] 2- to 12-fold (Baerson et al. 2002; Lee and Ngim 2000; Tran et al. 1999), horseweed 8- to 13-fold (Koger and Reddy 2005b; VanGessel 2001), hairy fleabane [*Conyza bonariensis* (L.) Cronq.] 2.9- to 10.5-fold (Urbano et al. 2007), rigid ryegrass (*Lolium rigidum* Gaudin) 3- to 14-fold (Powles et al. 1998; Pratley et al. 1999; Simarmata and Penner 2008; Wakelin and Preston 2006; Wakelin et al. 2004; Yu et al. 2007), Italian ryegrass [*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot] 2- to 15-fold (Jasieniuk et al. 2008; Nandula et al. 2007b; Perez and Kogan 2003; Perez-Jones et al. 2005), and Palmer amaranth (*Amaranthus palmeri* S. Wats.) 6- to 8-fold (Culpepper et al. 2006). Note that in weed species that have evolved glyphosate resistance, the resistance mechanisms thus far elucidated are target-site based and nontarget-site based (Perez-Jones and Mallory-Smith 2010; Powles and Preston 2006). The following processes have been reported to provide different levels of resistance to glyphosate: reduced glyphosate absorption (~ 3-fold) (Michitte et al. 2007; Nandula et al. 2008), impaired glyphosate translocation (~ 3- to 13-fold) (Dinelli et al. 2006, 2008; Feng et al. 2004; Koger and Reddy 2005b; Lorraine-Colwill et al. 2003; Nandula et al. 2008), sequestration to vacuole (2.9- to 5.6-fold) (Dinelli et al. 2008; Ge et al. 2010), *EPSPS* mutations (~ 2- to 15-fold) (Baerson et al. 2002; Jasieniuk et al. 2008; Perez-Jones et al. 2007; Tran et al. 1999; Simarmata and Penner 2008; Wakelin and Preston 2006) and overproduction of target enzyme (6- to 8-fold) (Gaines et al. 2010) in weedy species.

Since legume species have been reported to metabolize glyphosate (Duke et al. 2003b; Reddy et al. 2008), the different sensitivities to glyphosate in certain populations of pitted morningglory may be due to differences in the levels of degradation of

glyphosate to the much less phytotoxic metabolite of glyphosate, AMPA, or even sarcosine. To test this hypothesis, glyphosate, shikimate, AMPA and sarcosine levels were measured in leaves of the most (MS-WAS-8) and least (MS-YAZ-1) glyphosate tolerant populations, hereafter referred to as population MT and LT, respectively.

3.4.3 Glyphosate Metabolism Study

The F-test in the ANOVA was performed for both experiments. For the first experiment, the dependent variable amount of AMPA accumulated was not significant ($P < 0.1938$). On the other hand, the dependent variables of amount of glyphosate accumulated, glyphosate/AMPA ratio, and amount of shikimate accumulated were significant ($P < 0.0003$, 0.0001, and 0.0425, respectively). For the second experiment, the interaction between the GR_{50} glyphosate rate and two populations of pitted morningglory was not significant on the amount of AMPA accumulated ($P < 0.5678$), but it was significant on the amount of glyphosate accumulated ($P < 0.0045$), glyphosate/AMPA ratio ($P < 0.0259$), and shikimate accumulated ($P < 0.0113$). Therefore, variation in sensitivities to glyphosate in the MT and LT populations were not due to differences in the levels of degradation of glyphosate to AMPA. Sarcosine was not detected in pitted morningglory accessions in either experiment.

Two experimental designs were used. In the first, glyphosate, shikimate and AMPA concentrations were compared between populations, that both received 420 g ae glyphosate ha^{-1}. In second, glyphosate, shikimate and AMPA concentrations were compared at different times after treatment with glyphosate rates that would only affect growth by 50%. For the first experiment, there was less than half as much glyphosate in the MT than in the LT plants (Table 3.3). In the second experiment, the amount of

glyphosate accumulating was proportionally similar to the amount applied on the two accessions. For the second experiment, the MT at 1 and 3 DAT had lower glyphosate concentration than MT at 6 DAT; the glyphosate concentration did not change in the LT at 1, 3, and 6 DAT, indicating that glyphosate may be more slowly taken up in the most tolerant population.

Shikimate levels ranged from 97.44 to 9,868 μg g^{-1} of tissue in all of the experiments (Table 3.3). When both accessions were treated with the same dose of glyphosate, the LT accumulated almost 15-fold more shikimate. In the second experiment, MT at 1 and 3 DAT accumulated the same amount of shikimate as the LT at 1 and 3 DAT. Moreover, LT at 1, 3, and 6 DAT had the same shikimate levels as MT at 3 and 6 DAT. Similar shikimate levels in the two accessions, each given its GR_{50} rate of glyphosate, indicated that the shikimate pathway was inhibited about the same in the two populations, even though the glyphosate dose varied considerably. By blocking EPSPS, glyphosate causes many-fold increases in shikimate levels in non-GR plants and, consequently, elevated shikimate levels are used as an early and highly sensitive indicator of glyphosate effects on glyphosate-sensitive plant tissue (Harring et al. 1998; Lydon and Duke 1988).

AMPA was present in both populations in both experiments, and its concentration did not differ within experiments, ranging from 0.29 to 3.38 μg g^{-1} of tissue (Table 3.3). Duke et al. (2003b) treating GR soybean with three different glyphosate treatments at two different locations, reported AMPA concentrations ranging from 0.49 to 25 μg g^{-1} of seed tissue. Arregui et al. (2004) monitored a field-grown GR soybean for three years for AMPA residues that ranged from 0.3-5.7 μg g^{-1} of plant tissue and from 0.4-0.9 μg g^{-1} of

grain. Reddy et al. (2008), treating several species with their respective GR_{50} rates, reported AMPA accumulation ranging from 0.12 to 4.8 µg g^{-1} of tissue at 7 DAT. In their previous study, 8 µg of AMPA g^{-1} of tissue was detected in GR soybean 7 DAT with glyphosate at 672 g ae ha^{-1} (Reddy et al. 2004). In addition, for the second experiment, AMPA levels appeared to increase from 1 to 6 DAT for both populations, but the increases were not statistically significant. Shikimate levels appeared to decrease from 1 to 6 DAT for both populations, but the only statistically significant change was for the MT population between 1 and 6 DAT. Under different growth conditions, Reddy et al. (2004) found that AMPA levels in treated leaves were highest 1 DAT and decreased over a period of 22 days. They found that glyphosate levels did not decrease as rapidly as those of AMPA. The same pattern was observed in our study, but the glyphosate level in MT increased from 1 to 6 DAT.

For the first experiment, the lowest glyphosate to AMPA ratio was observed in MT ($1.28E^{-5}$), indicating that even though the amount of AMPA accumulated was not different between populations, the ratio of glyphosate being degraded to AMPA was different with MT being the one with the highest metabolism ratio (Table 3.3). For the second experiment, MT at all harvesting times and LT at 1 DAT were not different in their glyphosate to AMPA ratio (Table 3.3). Moreover MT at 3 DAT was not different from LT at 1 and 3 DAT. However, LT at 3 and 6 DAT differ from the others and it was the highest metabolism ratio, but this population received the lowest glyphosate rate (Table 3.3). Although MT and LT were treated at the same rate at the first experiment, MT had a lower glyphosate concentration compared to LT, even though the concentration of AMPA was not different (Table 3.3). MT was less affected by glyphosate than LT,

consequently more biomass production in MT after glyphosate treatment may have resulted in dilution of glyphosate in the tissue. Another possibility is that AMPA may degrade and/or translocate more rapidly than glyphosate in green treated leaves (Duke 2011). Glyphosate translocates to roots from which some of it can be exuded into the soil (Coupland and Caseley 1979; Kremer et al. 2005; Reddy et al. 2008).

Our AMPA data do not support the theory that metabolism of glyphosate explains the relative sensitivities to glyphosate in the two pitted morningglory populations tested. Neither an isolated plant GOX enzyme nor a gene for it has been reported in plants (Nandula et al. 2007a). Moreover, there has been no conclusive evidence of metabolic degradation as an important mechanism of evolving resistance (Duke 2011). The fact that pitted morningglory populations with variable levels of tolerance accumulated the same amount of AMPA does not support the view that enhanced metabolism of glyphosate is involved in the tolerance of the MT morningglory accession. Gene mutation or amplification of plant genes for GOX-like enzyme activity or horizontal transfer of microbial genes for glyphosate-degrading enzymes could result in GR weeds (Duke 2011). This mechanism of tolerance could be combined with another, like differential absorption and/or translocation of glyphosate. This is supported by our results of increased glyphosate concentration from 1 to 6 DAT in the MT, suggesting that glyphosate may be slowly taken up in this population. This single or multiple mechanism of tolerance hypothesis should be investigated in future studies of absorption and/or translocation of glyphosate in the studied pitted morningglory populations. Likewise, AMPA may degrade and/or translocate more rapidly than glyphosate.

Table 3.1 Control of 73 morningglory accessions with glyphosate at 420 and 840 g ae ha^{-1} at 2 and 3 weeks after treatment (WAT).

species / accession code[a]	exposure to glyphosate[b]	420 g glyphosate ha^{-1}						840 g glyphosate ha^{-1}					
		fresh weight reduction[c]		control[d]				fresh weight reduction		control			
		3 WAT	(SE[e])	2 WAT	(SE)	3 WAT	(SE)	3 WAT	(SE)	2 WAT	(SE)	3 WAT	(SE)
		%						%					
AL-LAM-1	No	50	(9)	35	(6)	73	(5)	57	(13)	64	(5)	76	(5)
AL-MAR-1	Yes	44	(32)	60	(8)	73	(10)	74	(13)	75	(6)	80	(8)
AL-MAR-2	Yes	36	(9)	63	(3)	65	(6)	80	(5)	70	(7)	80	(0)
AL-PIC-1	Yes	53	(14)	64	(5)	69	(6)	68	(7)	69	(3)	78	(5)
AR-ASH-1	Yes	44	(15)	55	(7)	60	(0)	83	(12)	70	(20)	85	(16)
AR-ASH-2	Yes	48	(16)	64	(5)	64	(8)	70	(9)	78	(6)	80	(0)
AR-CHI-1	Yes	-48	(45)	40	(0)	60	(0)	49	(14)	68	(3)	65	(6)
AR-UNI-1	Yes	-37	(19)	20	(0)	50	(8)	43	(23)	50	(0)	68	(3)
LA-UNI-1	Yes	76	(20)	70	(5)	80	(10)	80	(21)	65	(22)	85	(32)
LA-WCA-1	Yes	45	(29)	73	(5)	70	(8)	81	(13)	84	(5)	84	(5)
MS-COA-1	Yes	-27	(57)	26	(17)	53	(10)	8	(28)	59	(9)	64	(5)
MS-COA-2	Yes	-120	(55)	38	(10)	53	(5)	-25	(25)	64	(5)	63	(5)
MS-COV-1	Yes	40	(19)	65	(9)	68	(10)	50	(30)	71	(9)	69	(9)
MS-FOR-2	Yes	4	(59)	66	(8)	74	(5)	65	(14)	75	(4)	78	(6)
MS-ISS-1	Yes	15	(23)	53	(6)	63	(3)	92	(2)	80	(0)	87	(3)
MS-ISS-2[f]	No	14	(23)	58	(6)	70	(4)	85	(4)	85	(6)	84	(3)
MS-ITA-1	No	30	(12)	70	(0)	66	(5)	46	(14)	70	(0)	78	(3)
MS-JON-3	No	-84	(66)	49	(17)	58	(5)	44	(30)	74	(5)	79	(6)
MS-LAU-1	No	52	(6)	64	(5)	70	(7)	74	(14)	75	(0)	78	(3)

Table 3.1 (Continued)

MS-LEE-1	Yes	65	(23)	60	(11)	63	(17)	89	(6)	81	(3)	81	(3)
MS-LEE-2	Yes	45	(10)	61	(3)	70	(4)	70	(8)	75	(4)	80	(0)
MS-LEF-1	Yes	68	(8)	74	(3)	70	(4)	80	(7)	79	(3)	80	(0)
MS-MAR-1	Yes	-24	(49)	56	(5)	60	(8)	1	(73)	71	(6)	65	(17)
MS-MAR-2	Yes	74	(19)	56	(15)	58	(21)	78	(12)	71	(6)	73	(5)
MS-PAN-1	Yes	-47	(36)	46	(11)	68	(6)	42	(21)	69	(6)	73	(3)
MS-QUI-1	No	27	(25)	63	(5)	60	(9)	89	(4)	95	(6)	96	(5)
MS-SCO-1	Yes	82	(5)	60	(0)	70	(0)	91	(4)	78	(5)	76	(3)
MS-SIP-2	Yes	56	(10)	66	(5)	61	(6)	77	(9)	74	(3)	75	(0)
MS-TUN-1	Yes	-15	(30)	43	(13)	63	(3)	76	(5)	80	(0)	79	(3)
MS-WAR-1	Yes	-21	(28)	56	(5)	50	(12)	54	(14)	75	(4)	80	(0)
MS-WAS-1	No	67	(5)	65	(4)	63	(10)	93	(2)	80	(0)	80	(0)
MS-WAS2-2[e]	Yes	77	(4)	65	(6)	70	(0)	96	(1)	90	(7)	95	(7)
MS-WAS-2	No	52	(20)	48	(5)	71	(3)	88	(5)	81	(3)	80	(0)
MS-WAS-3	Yes	1	(24)	50	(7)	50	(0)	54	(25)	75	(4)	76	(3)
MS-WAS-4	Yes	-9	(74)	55	(24)	71	(3)	42	(11)	75	(0)	78	(3)
MS-WAS-5	Yes	77	(8)	71	(3)	75	(4)	88	(4)	81	(3)	85	(0)
MS-WAS-6	Yes	3	(32)	50	(0)	65	(4)	47	(22)	70	(0)	74	(3)
MS-WAS-7	Yes	82	(3)	80	(0)	85	(0)	95	(2)	85	(0)	90	(0)
MS-WAS-8	Yes	-33	(40)	40	(0)	50	(0)	42	(16)	70	(0)	70	(0)
MS-YAL-1	Yes	-13	(45)	51	(6)	53	(5)	62	(19)	71	(5)	70	(4)
MS-YAZ-1	No	73	(8)	70	(0)	80	(0)	71	(13)	78	(3)	79	(6)
MS-YAZ-2	Yes	43	(18)	50	(0)	62	(10)	100	(0)	100	(1)	100	(2)
TN-FAY-1	No	19	(55)	40	(5)	60	(10)	50	(55)	60	(15)	85	(20)
TN-FAY-2[e]	No	57	(5)	80	(0)	70	(0)	55	(15)	76	(3)	80	(0)
TN-HAR-1	No	72	(22)	65	(13)	71	(10)	59	(15)	69	(3)	73	(3)

Table 3.1 (Continued)

TN-SHE-1	Yes	-8	(43)	43	(15)	54	(9)	85	(5)	70	(14)	80	(0)
SC-AND-1	No	-22	(34)	55	(6)	55	(10)	77	(9)	75	(0)	80	(0)
MS-99[g]	Yes	57	(19)	65	(0)	83	(5)	69	(26)	81	(4)	80	(0)
Payne-2[e]	Yes	27	(4)	50	(0)	63	(0)	72	(1)	70	(0)	76	(0)
Young-2[e]	Yes	13	(13)	39	(10)	53	(0)	45	(10)	60	(0)	70	(3)
Burdine-2[e]	Yes	-10	(23)	38	(8)	60	(5)	47	(8)	71	(5)	75	(0)
AL1-2[e]	Yes	30	(18)	55	(6)	64	(5)	67	(14)	75	(0)	79	(0)
AR1-2[e]	Yes	28	(46)	58	(0)	56	(15)	60	(14)	76	(3)	74	(4)
AR8-2[e]	Yes	-37	(7)	45	(6)	63	(3)	24	(14)	66	(5)	75	(4)
AR13-2[e]	Yes	18	(27)	45	(5)	63	(3)	52	(13)	73	(3)	75	(5)
AR14-2[e]	Yes	-32	(13)	40	(6)	60	(5)	53	(20)	65	(5)	74	(4)
AR17-2[e]	Yes	36	(12)	49	(6)	65	(10)	61	(15)	70	(4)	75	(6)
AR18-2[e]	Yes	-16	(20)	50	(8)	64	(7)	62	(13)	66	(4)	76	(3)
AR25-2[e]	Yes	-44	(11)	37	(6)	62	(6)	41	(21)	70	(7)	78	(4)
DE1-2[e]	Yes	3	(39)	63	(5)	59	(0)	60	(3)	73	(3)	76	(3)
GA1-2[e]	Yes	26	(32)	48	(12)	70	(3)	-4	(13)	63	(0)	73	(3)
KY1-2[e]	Yes	47	(11)	69	(3)	69	(9)	56	(10)	76	(3)	76	(3)
LA2-2[e]	Yes	85	(4)	79	(0)	70	(5)	89	(21)	75	(5)	84	(3)
LSU-2[e]	Yes	66	(19)	78	(6)	71	(6)	83	(24)	81	(5)	85	(5)
MO1-2[e]	Yes	-14	(6)	58	(3)	65	(0)	35	(4)	74	(3)	74	(0)
MO2-2[e]	Yes	28	(14)	63	(3)	71	(3)	61	(8)	75	(3)	76	(0)
NC2-2[e]	Yes	-27	(39)	50	(10)	63	(6)	47	(18)	68	(5)	79	(5)
OK1-2[e]	Yes	-79	(30)	33	(5)	68	(10)	73	(21)	76	(4)	79	(3)
TN1-2[e]	Yes	28	(33)	61	(0)	69	(3)	78	(5)	76	(3)	80	(3)
MS-90[h]	No	36	(76)	67	(3)	67	(5)	34	(9)	70	(3)	75	(3)
Ivyleaf morningglory	Yes	-71	(21)	20	(3)	48	(5)	24	(8)	69	(3)	66	(0)

Table 3.1 (Continued)

Moonflower	Yes	28	(39)	73	(12)	70	(8)	35	(20)	75	(0)	75	(9)
Palmleaf morningglory	Yes	66	(28)	70	(10)	70	(0)	98	(36)	100	(6)	100	(3)
LSD (0.05)		44		8		12		25		6		6	

[a] For collection location, see Table B.1. Geographic positions of morningglories accessions selected for the glyphosate screening study.
[b] Based on the collection location, each accession was rated on the likelihood the collection site was treated with glyphosate. In-crop collections were likely exposed to glyphosate (as indicated by "Yes"), while ditch bank and waste area collections have likely not been treated with glyphosate (as indicated by "No").
[c] Data are expressed as percent of shoot fresh weight reduction as compared to nontreated plants and represent mean of eight replications.
[d] Data are expressed as percent control visually estimation of injury on a scale of 0 (no injury) to 100 (complete death) and represent mean of eight replications.
[e] SE represents the standard error of the mean where $n = 8$ (polled data from two experiments).
[f] Second generation, plants of each accession were let to self-pollinated to generate a second generation of seed.
[g] Precise location of accession is unknown as this population was harvested in 1999, although it was collected at the Mississippi Delta region and it was under four year of glyphosate-resistant crops management system.
[h] Precise location of collection is unknown as this population was harvested in 1990, although it was collected at the Mississippi Delta region and it was not exposed to glyphosate-resistant crops management system.

Table 3.2 Glyphosate dose response parameters and variables in the log-logistic model[a] estimates for 14 pitted morningglory accessions at 14 days after treatment.

accession code[b]	R^2	L[c]	U[c]	s[c]	GR_{50}[c]	GR_{50} ratio[c]
		---------- % Fresh weight reduction (SE)[d]		-----------	g ae ha^{-1} (SE)	
MS-WAS-8**	0.93	7.24 (6.09)	88.06* (8.95)	3.18* (1.19)	151.44* (26.99)	2.59
MS-VJ[e]**	0.93	15.98* (6.73)	141.19* (10.79)	4.57* (1.33)	147.45* (15.96)	2.52
MS-SCO-1**	0.92	3.76 (4.65)	85.85* (7.65)	4.35* (1.77)	136.88* (18.77)	2.34
MS-PAN-1**	0.95	1.29 (9.33)	100.00* (7.65)	1.50* (0.62)	135.91* (30.99)	2.33
MS-YAZ-2**	0.82	10.94 (8.10)	100.32* (10.77)	3.10* (1.55)	131.85* (22.54)	2.26
MS-COA-1**	0.94	7.29 (6.06)	100.11* (7.64)	2.28* (0.88)	130.83* (20.80)	2.24
MS-ITA-1	0.97	10.04* (5.00)	126.17* (8.82)	3.25* (0.99)	125.07* (12.68)	2.14
MS-LEE-2	0.90	2.39 (4.77)	100.02* (7.66)	3.65* (1.51)	116.52* (12.14)	1.99
MS-WAS-2	0.91	-0.59 (16.88)	93.26* (7.69)	0.94 (0.57)	115.35* (51.81)	1.97
MS-MAR-1	0.94	3.11 (6.97)	112.25* (8.82)	1.86* (0.72)	113.45* (19.19)	1.94
MS-99	1.00	0.75 (0.36)	135.20* (23.27)	-9.67 (22.42)	106.42 (43.45)	1.82
MS-90	0.97	7.16 (9.64)	90.99* (8.83)	2.48 (2.73)	90.94* (26.64)	1.56
MS-QUI-1	0.95	1.91 (24.02)	99.99* (8.84)	1.10 (1.45)	59.11 (35.84)	1.01
MS-YAZ-1	0.98	3.10 (5.20)	128.32* (8.84)	2.60 (3.24)	58.43 (42.76)	-

[a] Model proposed by Seefeldt et al. (1995): y [fresh weight (% of untreated control)] = $L + \{(U - L)/[1 + (D/GR_{50})^s]\}$.

[b] For collection location, see Table B.1. Geographic positions of morningglories accessions selected for the glyphosate screening study.

[c] The parameters estimates are L, lower limit of response; U, upper limit of response; s, slope of the curve around the point of inflexion (GR_{50}); GR_{50}, glyphosate dose required to cause a 50% reduction in plant growth and GR_{50} dose was estimated using responses to six glyphosate doses (0, 105, 210, 420, 840, and 1,680 g ha^{-1}); and GR_{50} ratio, GR_{50} accessions / GR_{50} least tolerant accession.

[d] SE represents the standard error of the mean where $n = 8$ (polled data from two experiments).

[e] Germoplasm collection of Dr. Vijay K. Nandula, Mississippi State University Research Center in Stoneville, MS, it was obtained in 2002 from Azlin Seed Service, Leland, MS.

* Estimated parameters of the log-logistic model are different according to *t*-Student test at $P < 0.05$; accept alternative hypothesis, H_a: parameter $\neq 0$.

** Relative potencies between accessions and least tolerant accession (MS-YAZ-1) at GR_{50} response level is different according to *t*-Student test at $P < 0.05$; accept alternative hypothesis, H_a: relative potency $\neq 1$.

Table 3.3 Effect of glyphosate at 420 g ae ha^{-1} and GR_{50} rate on shikimate, glyphosate, aminomethylphosphonic acid (AMPA) concentration at 1, 3, 6 and 14 days after treatment in the most (MS-WAS-8) and least (MS-YAZ-1) tolerant pitted morningglory.

accession code[a]	glyphosate rate	DAT	shikimate (SE)[c, d]	glyphosate (SE)[d]	AMPA (SE)[d]	Ratio (SE)[d]
	------- ga ae ha^{-1} -------		----------------------------------	µg g^{-1} of tissue	----------------------------------	glyphosate/AMPA
MS-WAS-8	420	14	683.94 (554.37) b	$3.57E^{-5}$ ($2.02E^{-5}$) b	3.38 (1.77) a	$1.28E^{-5}$ ($0.46E^{-5}$) b
MS-YAZ-1			9868.30 (10793.31) a	$9.78E^{-5}$ ($3.63E^{-5}$) a	2.41 (0.91) a	$3.76E^{-5}$ ($1.11E^{-5}$) a
MS-WAS-8	151[b]	1	190.85 (16.87) A	14.87 (2.66) B	0.62 (0.10) A	23.55 (2.69) A
		3	148.16 (40.42) AB	11.55 (3.01) BC	0.72 (0.39) A	20.35 (6.02) AB
		6	97.44 (22.28) B	22.66 (7.48) A	0.84 (0.19) A	26.26 (9.06) A
MS-YAZ-1	58[b]	1	135.94 (34.93) AB	5.40 (2.62) C	0.29 (0.12) A	17.69 (5.61) AB
		3	133.35 (27.20) AB	5.19 (1.61) C	0.49 (0.17) A	10.82 (5.03) BC
		6	118.39 (40.23) B	4.77 (3.78) C	0.77 (0.39) A	6.91 (5.01) C

[a] For collection location, see Table B.1. Geographic positions of morningglories accessions selected for the glyphosate screening study.
[b] GR_{50}, glyphosate dose required to cause a 50% reduction in plant growth; 151 g ae ha^{-1} for the most tolerant population (MS-WAS-8) and 58 g ae ha^{-1} for the least tolerant population (MS-YAZ-1).
[c] SE represents the standard error of the mean where n = 10 (polled data from two experiments of glyphosate at 420 g ae ha^{-1}).and n = 6 (polled data from two experiments of glyphosate at GR_{50} rate).
[d] Means of glyphosate at 420 g ae ha^{-1} (lowercase) and GR_{50} rate (uppercase) within a column followed by the same letter are not significantly different at the 5% level as determined by the LSD test.

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APPENDIX A

GLYPHOSATE-RESISTANT PALMER AMARANTH ACCESSIONS

A.1 Collection of glyphosate-resistant Palmer amaranth by accession code and geographic location

Table A.1 Geographic locations, percentage of control and mortality of resistant Palmer amaranth populations from Mississippi 2 weeks after treatment with glyphosate at 840 g ae ha^{-1}, study performed by Nandula et al. (2012)[a].

Population	County	Control[b]	Mortality[b]
		--------------------- % ---------------------	
C1	Coahoma	50	7
T1	Tunica	30	8
T2	Tunica	10	16
T3	Tunica	20	2
T4	Tunica	10	6
T5	Tunica	30	24
T6	Tunica	10	36
T7	Tunica	10	3
T8	Tunica	30	7
T9	Tunica	30	1
T10	Tunica	30	45
T11	Tunica	20	8
Susceptible	Washington	100	100

[a] Nandula, V. K., K. N. Reddy, C. H. Koger, D. H. Poston, A. M. Rimando, S. O. Duke, J. A. Bond, and D. N. Ribeiro. 2012. Multiple resistance to glyphosate and pyrithiobac in Palmer amaranth (*Amaranthus palmeri*) from Mississippi and response to flumiclorac. Weed Sci. 60:179-188.

[b] Control indicates visible estimate of injury on a scale of 0 (no injury) to 100 (complete death) and mortality indicates percentage of plants surviving (evidence of shoot regrowth at time of evaluation) in relation to total number of plants treated.

APPENDIX B

DETERMINATION OF THE INHERENT VARIABILITY IN PITTED MORNINGGLORY CONTROL BY GLYPHOSATE

B.1 Collection of morningglories by accession code and geographic location

Table B.1 Geographic locations of morningglory accessions selected for the glyphosate screening study.

Species / Accession code	Geographic position and Harvest date
AL-LAM-1	Alabama. Lamar Co.: Kennedy, 21 Oct 2004
AL-MAR-1	Alabama. Marion Co.: Hamilton, 21 Oct 2004
AL-MAR-2	Alabama. Marion Co.: Hamilton, 21 Oct 2004
AL-PIC-1	Alabama. Pickens Co.: Reform, 21 Oct 2004
AR-ASH-1	Arkansas. Ashley Co.: Montrose, 6 Oct 2004
AR-ASH-2	Arkansas. Ashley Co.: Montrose, 6 Oct 2004
AR-CHI-1	Arkansas. Chicot Co.: Eudora, 6 Oct 2004
AR-UNI-1	Arkansas. Union Co.: Strong, 6 Oct 2004
LA-UNI-1	Louisiana. Union Par.: Marion, 6 Oct 2004
LA-WCA-1	Louisiana. West Carroll Par.: Pioneer, 6 Oct 2004
MS-COA-1	Mississippi. Coahoma Co.: Clarksdale, Oct 2003
MS-COA-2	Mississippi. Coahoma Co.: Lyon, 21 Sep 2004
MS-COV-1	Mississippi. Covington Co.: Seminary, 28 Sep 2004
MS-FOR-2	Mississippi. Forrest Co.: Hattiesburg, 28 Sep 2004
MS-ISS-1	Mississippi. Issaquena Co.: Fitler, Sep 2003
MS-ISS-2	Mississippi. Issaquena Co.: Fitler, second generation[a]
MS-ITA-1	Mississippi. Itawamba Co.: Tremont, 21 Oct 2004
MS-JON-3	Mississippi. Jones Co.: Laurel, 28 Sep 2004
MS-LAU-1	Mississippi. Lauderdale Co.: Meehan, 28 Sep 2004
MS-LEE-1	Mississippi. Lee Co.: Verona, 21 Oct 2004
MS-LEE-2	Mississippi. Lee Co.: Verona, 21 Oct 2004
MS-LEF-1	Mississippi. Leflore Co.: Sidon, Sep 2003
MS-MAR-1	Mississippi. Marshall Co.; Holly Springs, 26 Oct 2004
MS-MAR-2	Mississippi. Marshall Co.; Holly Springs Experiment Station, 26 Oct 2004
MS-PAN-1	Mississippi. Panola Co.: Batesville, Oct 2003
MS-QUI-1	Mississippi. Quitman Co.: Lambert, 21 Sep. 2004
MS-SCO-1	Mississippi. Scott Co.: Forrest, 28 Sep 2004
MS-SIP-2	Mississippi. Simpson Co.: D'Lo, 28 Sep 2004
MS-TUN-1	Mississippi. Tunica Co.: Dundee, 21 Sep 2004
MS-WAR-1	Mississippi. Warren Co.: Bovina, Oct 2003
MS-WAS-1	Mississippi. Washington Co.: Elizabeth, Sep 2003
MS-WAS-2	Mississippi. Washington Co.: Elizabeth, Sep 2003
MS-WAS2-2	Mississippi. Washington Co.: Elizabeth, second generation
MS-WAS-3	Mississippi. Washington Co.: Stoneville, Sep 2003
MS-WAS-4	Mississippi. Washington Co.: Stoneville, Sep 2003

Table B.1 (continued)

MS-WAS-5	Mississippi. Washington Co.: Stoneville, Sep 2003
MS-WAS-6	Mississippi. Washington Co.: Elizabeth, 1 Oct 2004
MS-WAS-7	Mississippi. Washington Co.: Elizabeth, 1 Oct 2004
MS-WAS-8	Mississippi. Washington Co.: Stoneville, 15 Oct 2004
MS-YAL-1	Mississippi. Yalobusha Co.: Coffeeville, Oct 2003
MS-YAZ-1	Mississippi. Yazoo Co.: Holly Bluff, Sep 2003
MS-YAZ-2	Mississippi. Yazoo Co.: Satartia, Sep 2003
TN-FAY-1	Tennessee. Fayette Co.: Moscow, 26 Oct 2004
TN-FAY-2	Tennessee. Fayette Co.: Moscow, second generation.
TN-HAR-1	Tennessee. Hardeman Co.: Grand Junction, 26 Oct 2004
TN-SHE-1	Tennessee. Shelby Co.: Memphis, 26 Oct 2004
SC-AND-1	South Carolina. Pendleton Co.: Clemson Univ. Pendleton, 16 Dec 2004
MS-99	Mississippi, 1999
Payne-2	Tennessee. Shelby Co.: Arlington, second generation
Young-2	Mississippi, second generation
Burdine-2	Mississippi, second generation
AL1-2	Alabama. Pickens Co., second generation
AR1-2	Arkansas. Washington Co., second generation
AR8-2	Arkansas. Crittenden Co., second generation
AR13-2	Arkansas. St. Francis Co., second generation
AR14-2	Arkansas. Lonoke Co., second generation
AR17-2	Arkansas. Desha Co., second generation
AR18-2	Arkansas. Miller Co., second generation
AR25-2	Arkansas. Ashley Co., second generation
DE1-2	Delaware. Sussex Co., second generation
GA1-2	Georgia. Colquitt Co., second generation
KY1-2	Kentucky. Daviess Co., second generation
LA2-2	Louisiana. Tensas Co., second generation
LSU-2	Louisiana. West Baton Rouge Parish Co.: LSU. Baton Rouge, second generation
MO1-2	Missouri. Knox Co., second generation
MO2-2	Missouri. Dunklin Co., second generation
NC2-2	North Carolina. Johnston Co., second generation
OK1-2	Oklahoma. Sequoyah Co., second generation
TN1-2	Tennessee. Madison Co., second generation
MS-90	Mississippi, 1990
Ivyleaf morningglory	Mississippi, 2004
Moonflower	Mississippi, 2004
Palmleaf morningglory	Mississippi, 2004

[a] Second generation, flowers of each plant accession were self-pollinated to generate a second generation of seed.