INHERITANCE OF GLYPHOSATE RESISTANCE IN *KOCHIA SCOPARIA*

by

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Abstract

Extensive, often exclusive, use of glyphosate in crop production has resulted in evolved glyphosate resistance in several weed species globally. Kochia is a competitive summer annual weed, well adapted to the North American Great Plains and has recently evolved resistance to glyphosate by gene amplification of 5-enolpyruvyl shikimate 3-phosphate synthase (*EPSPS*), the target-site of glyphosate. The overall objective of this research was to investigate the genetic basis of glyphosate resistance in kochia, specifically to study 1) the inheritance of glyphosate resistance and 2) determine the chromosomal distribution of *EPSPS* gene copies. Homozygous resistant (R) and susceptible (S) parental lines of kochia were identified. Using these parents, reciprocal crosses were performed to produce F_1 progeny. As expected for a nuclear encoded EPSPS gene, F_1 plants from both crosses survived various doses of glyphosate application. However, F_1 plants showed intermediate shikimate accumulation and *EPSPS* gene copies (relative to *ALS* reference gene) compared to parents. F_2 progeny were produced by selfing F_1 plants. In response to 870 g ae ha^{-1} glyphosate, F_2 plants (n=115) segregated into 3:1 (R:S) implying a Mendelian monogenic segregation of glyphosate resistance in kochia. Additionally, relative *EPSPS* gene copies ranged from 1-10 in the F_2 progeny (n=51) with a genotypic segregation of 40:11 (plants with 3 or more *EPSPS* gene copies: plants with 1 *EPSPS* gene copy). In F_2 dose-response, a correlation between the level of resistance and relative *EPSPS* gene copies was observed. Genomic organization of the amplified copies using fluorescent in situ hybridization (FISH) displayed a single and larger hybridization site of the *EPSPS* gene on one pair of homologous chromosomes in R compared to a faint hybridization site in S samples of kochia. These results suggest possibility of amplification of *EPSPS* gene mediated via unequal recombination leading to the evolution of the glyphosate resistance in kochia.

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Chapter 1 - Review of Literature

Kochia – A Problematic Weed of the US Great Plains

Kochia [*Kochia scoparia* (L.) Schrad.], a summer annual broadleaf weed, is commonly found in 42 of the contiguous United States and in southern Canada (Friesen et al. 2009). Although kochia grows all over North America, it is well adapted to the arid to semi-arid environments of the Great Plains and Canadian Prairies (Friesen et al. 2009) (Figure 1.1). Kochia is capable of tolerating extreme environments including drought, hot and cold temperatures, as well as saline soils (Friesen et al. 2009). Kochia is able to withstand such conditions because it is a C_4 plant (Friesen et al. 2009). C_4 plants can adapt to harsh environments because of more efficient water conservation and CO_2 fixing via the C_4 pathway. Typically, kochia germinates early in spring and continues to emerge throughout the season (Dille et al. 2012). Usually, flowering of kochia plants starts approximately 8 to 10 weeks after emergence (Thill and Mallory-Smith, 1996). Kochia is a short-day plant and will initiate flowering if exposed to a short photoperiod of less than 12 hours of light (Eberlein and Fore, 1984). A single, small flower is formed at each axial leaf along the branches. Kochia bears protogynous flowers, in which the stigmas protrude and are receptive one week prior to dihiscence of anthers on the same flower. Because of its protogynous nature, kochia is prone to outcrossing. Mulugeta et al. (1994) found that 99.9% of kochia pollen was deposited within 154 meters of the source plant and that pollen was viable for 1-12 days depending on prevailing temperature and humidity. Post senescence, or upon maturity, an abscission layer forms at the base of the plant and the above ground portion detaches from the roots. Once detached, the plants tumbles along with the direction of wind, dispersing seed along the landscape. Kochia is considered a prolific seed producer, with average seed production reported of 15,000 to 25,000 seeds per plant (Friesen et al. 2009). Kochia is a diploid species with a chromosome number of 2n=18 (Friesen et al. 2009).

Kochia is an aggressive competitor with crops because of its early and continual emergence, extreme environment tolerance, growth habit and high seed production. Studies have shown that kochia densities of 0.5 and 0.2 kochia plants m^{-1} reduce sugarbeet yields up to 32 and 18%, respectively (Schweizer, 1973; Mesbah et al. 1994). Season long competition of kochia in soybean has been shown to reduce yield up to 30% (Forcella 1985). Wicks et al. (1993)

documented that for every kg ha^{-1} of kochia dry weight, corn grain yields decreased 0.33 kg ha^{-1}. Futhermore, in a three year study, it was found that grain sorghum yields were reduced between 11-38% and as high as 85% in one season with above average rainfall because of kochia compeition (Wicks et al. 1994).

Herbicide Use for Kochia Management

Typically, kochia can effectively be managed by tillage or use of herbicides. Tillage controls kochia by uprooting and killing emerged plants and buring seed in the seedbank farther within the soil profile. Schwinghamer and Van Acker (2008) found a 48% reduction in kochia emergence when seed was buried 2 mm and 73% emergence reduction when buried 20 mm in the soil profile. Conversely, no-till practice can increase kochia emergence by four-fold (Anderson and Nielsen, 1996). In Kansas, a predominate concern is the prescence of kochia in winter wheat stubble and summer fallow (Godar, 2014). These systems are traditionally no-till systems which allow ample time for kochia to regrow and set seed after small grain harvest (Mickleson et al. 2004); therefore, growers heavily rely on herbicides for kochia control. Both pre-emergence (PRE) and post-emergence (POST) herbicide applications have traditionally been effective in controlling kochia. Several different herbicide modes of actions are available for PRE and POST control of kochia. Acetolactate synthase (ALS), protoporphyringogen oxidase (PPO) and hydroxyphenyl-pyruvate-dioxygenase (HPPD) inhibitors can be applied as both PRE and POST herbicides (Thompson et al. 2011). Additionally, seedling shoot inhibitors as well as photosynthesis (PSII) inhibitors can be applied PRE; whereas, plant growth regulators, such as dicamba, and glyphosate, 5-enolypyruvyl-shikimate-3 phosphate synthase (EPSPS)-inhibitors, are applied POST (Thompson et al. 2011). Kochia has been shown to exhibit some natural tolerance to 2,4-D and is not always controlled by a reccommended field use rate of 2,4-D; however, 99% of kochia was controlled with POST mixtures consisting of: atrazine, carfentrazone, fluroxypyr, bromoxynil plus MCPA, nicosulfuron plus dicamba, and nicosulfuron plus dicamba plus atrazine when applied late August to early Septmenber (Nandula and Manthey, 2002). Mickleson et al.(2004) found that early September applications of 631 g ae ha^{-1} glyphosate reduced kochia seed production 92-97% while a mixture of 631 g ae ha^{-1} glyphosate plus 561 g ae ha^{-1} 2,4-D reduced seed production 64-99%.

Glyphosate and Glyphosate-Resistant Crops

Glyphosate (N-(phosphonomethyl)glycine) is a non-selective, broad-spectrum herbicide that was introduced as Roundup™ in the early 1970's (Baylis, 2000). Glyphosate targets *EPSPS* in the shikimic acid pathway in plants (Cobb and Reade, 2010) (Figure 1.2). Glyphosate competitively occupies the binding site for phosphoenolpyruvate (PEP), which then results in the accumulation of shikimic acid upstream in the pathway. The EPSPS enzyme is important for synthesis of the aromatic amino acids: tyrosine, tryptophan, and phenylalanine. In addition, secondary plant metabolites including vitamins, flavonoids, and lignins are also synthesized in the shikimic acid pathway (Cobb and Reade, 2010). Mammals do not have the shikimic acid pathway, and therefore, human toxicity to glyphosate is a minimal concern (Cobb and Reade, 2010). Environmentally, glyphosate shows rapid soil binding and biodegradation (Pline-Srnic, 2006). When first introduced, glyphosate was primarily used to control vegetation in ditches, fallow fields, and rights-of-ways, but was not commonly used in agriculture (Nandula, 2010). Glyphosate was not only lethal to crops, but also extrememly expensive at that time.

Introduction of Roundup Ready™-canola and -soybeans in 1996 increased glyphosate's popularity in agriculture because the crops could withstand typically lethal glyphosate applications. By 1998, glyphosate-resistant (GR) corn and cotton were also available for cultivation. GR crops were created by identifying a glyphosate-insensitive CP4 *EPSPS* and inserting this transgene into crops (Bradhaw et al. 1997; Dill et al. 2008). In order to increase the level of resistance in the crops, CP4 EPSPS was paired with glyphosate-degrading enzymes such as glyphosate oxioreductase (GOX) or glyphosate acetyltransferase (GAT) (Bradshaw et al. 1997; Nandula et al. 2010).

GR cropping systems provided farmers with an option for reduced- and no-till practices (Young, 2006). Farmers that have adopted no-till practices rely on glyphosate as a burndown treatment, as opposed to mechanical weed control techniques. No-till practices reduce soil erosion and improve soil moisture retension. In addition to soil conservation, no-till has also reduced labor and fuel requirements compared to traditional tillage. Givens et al. (2009) found that among farmers who used conventional tillage prior to GR crops, 25 and 31% transitioned into no-till and reduced-till systems, respectively, after adopting GR crops. Therefore, GR crops were quickly adopted by growers and by 2006, over 96% of soybeans varieties planted in the U.S. were glyphosate-resistant (Dill et al. 2008). The use of GR crops continued to increase with

nearly 77% of combined corn (*Zea mays*), cotton (*Gossypium hirsutum*) and soybean (*Glycine max*) acres planted in 2008 in the US were GR (Nandula, 2010). However, integrated weed management practices, such as tillage and herbicide/crop rotations, were negetively impacted due to adpotion of GR technology and reliance on glyphosate as a single weed management tactic increased tremendously (Johnson et al. 2009).

Evolution of Glyphosate-Resistant Weeds

Weed Science Society of America (WSSA) defines herbicide resistance as the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type. Repeated and continuous use of glyphosate created selection pressure on weed species resulting in the evolution of resistance to glyphosate. Rigid ryegrass (*Lolium rigidum)* was the first weed species to evolve glyphosate resistance in 1996 in Austraila and was also identified in the US in 1998 (Powles et al. 1998; Heap, 2014). To date, there are 28 different weeds species that have evolved resistance to glyphosate worldwide, and 14 of which are present in the US (Heap, 2014). Several weed species in the US were identified as resistant to glyphosate soon after GR cropping systems were accepted and utilized. Horseweed (*Conyza canadensis*), was the second species to evolve resistance to glyphosate in the US (VanGessel, 2001; Heap, 2014). By 2005, five more weed species had evolved resistance to glyphosate including: Italian ryegrass (*Lolium multiflorum*), common ragweed (*Ambrosia artimisiifolia)*, giant ragweed (*Ambrosia trifida*), Palmer amaranth (*Amaranthus palmeri*), and tall waterhemp (*Amaranthus tuberculatus*) (Heap, 2014).

Weeds can evolve berbicide resistance via target-site and/or non-target site based mechanisms. Target-site resistance typically results from a mutation that induces an nucleotide/amino acid change in the enzyme preventing the herbicide from binding to the target-site (Powels and Yu, 2010). Overexpression of the target enzyme, conferred by gene amplification or changes in the promoter, is also considered a target-site based mechanism of herbicide resistance (Powels and Yu, 2010). Other mechanisms such as reduced absorption/translocation, enhanced metabolism, and vacuole seqestration are considered as non-target-site-based mechanisms (Delye et al. 2013). Known mechanisms of glyphosate resistance in weeds include target-site alterations, limited or reduced translocation, increased EPSPS gene copy number, vacuole sequestration and rapid necrosis response (Sammons and Gaines, 2014).

Rigid ryegrass species were found to have reduced translocation as the primary mechanism of resistance (Wakelin et al. 2004). In some biotypes of rigid ryegrass and goosegrass (*Eleusine indica*), an altered target-site, in which the amino acid proline at position 106 in the EPSPS gene is changed to serine or threonine, also results in glyphosate resistance (Wakelin and Preston, 2006; Baerson et al. 2002). Recently, Palmer amaranth (*Amaranthus palmeri*), Italian ryegrass, common waterhemp (*Amaranthus tuberculatus*) and kochia were found to exhibit increased EPSPS gene copy number as means of resistance to glyphosate (Gaines et al. 2010; Salas et al. 2012; Tranel et al. 2011; Wiersma, 2012). Gaines et al. (2010) identified a single nucleotide polymorphism (SNP) at amino acid position 316 in which lysine was substituted for arginine in glyphosate-resistant Palmer amaranth; however, susceptible Palmer amaranth also had this mutation indicating it was unlikely to be the source of resistance. Using quantitative PCR, it was found that GR Palmer amaranth plants had increased *EPSPS* gene copy numbers ranging from 5 to 160. Fluorescent in situ hybridization (FISH) mapping was done to determine the chromosomal locations of the *EPSPS* gene copies in Palmer amaranth and showed that the duplicated genes were randomly spread across the entire genome.

Gene Amplification as a Means of Resistance

Gene amplification can occur via chomosome duplication, unequal crossing over, or transposable elements (Zhang, 2003). When a duplicated gene sequence contains transcription sequence or inserted behind a promotor, increased mRNA and protein levels are observed (Sammons and Gaines, 2014). Gene amplification leading to insecticide resistance has been documented in the aphid (*Myzus persicae)* and mosquito (*Culex quinquefasciatus*) (Field et al. 1989; Mouches et al. 1986). In the case of plants, aluminum tolerance in corn was found to be associated with increased MATE1 gene copies, in which tandem triplication was found to provide tolerance to typically lethal concentrations of aluminum (Maron et al. 2013). In the case of Palmer amaranth, genetic mobile elements, specifically transposons, are the most probable cause of the multiple *EPSPS* gene copies located across the entire genome (Gaines et al. 2010; Gaines et al. 2013). Increased *EPSPS* gene copies also tend to positively correlate with the level of glyphosate resistance in Palmer amaranth (Gaines et al. 2013).

Genetic Basis of Herbicide Resistance in Weeds

The genetic basis for herbicide resistance varies much like the mechanisms of herbicide resistance. In general, herbicide resistance in weeds is inherited as a nuclear trait; however, cytoplasmic inheritance, or inheritance via maternal DNA, has been documented in some triazine-resistant weed species (Jasieniuk et al. 1996). Nuclear-inherited resistance tends to spread more rapidly than cytoplasmic-inherited resistance due to enrichment of resistant alleles within a population as resistance can be transmitted via seed and pollen as opposed to only seed in a cytoplasmic-inherited resistance mechanism (Mithila and Godar, 2013). Additionally, herbicide resistance in the majority of weeds tends be a domiant or semi-dominant trait. In wild mustard (*Brassica* kaber), resistance to picloram, 2,4-D, and dicamba result from single, dominant nuclear genes (Jugulam et al. 2005; Jasieniuk et al. 1995). MCPA resistance in wild radish (*Raphanus raphanistrum*) is governed by a single incompletely dominant nuclear gene (Mithila et al. 2013). In kochia, dicamba resistance was found to be controlled by a single nuclear trait with a high degree of dominance (Preston et al. 2009). Nonetheless, few cases of recessive gene inheritance of herbicide resistance have been documented. Trifluralin resistance in green foxtail (*Setaria viridis*), and clopryalid resistance in yellow starthistle (*Centura solstitialis*) are examples of resistance conferred by a single nuclear recessive gene (Jasieniuk et al. 1994; Sabba et al. 2003). Inheritance of glyphosate resistance has been reported in some weed species. Using traditional breeding methods, glyphosate resistance in rigid ryegrass was found to be a nuclear, incompletely dominate trait (Lorrain-Colwill et al. 2001; Simarmata et al. 2005). Similarly, glyphosate resistance in goosegrass and horseweed was also found to be a semi-dominant trait (Ng et al. 2004; Zelaya et al. 2004). However, glyphosate resistance in Palmer amaranth populations from North Carolina and New Mexico were found to exhibit polygenic inheritance as opposed to a single gene (Chandi et al. 2012; Mohseni-Moghadam et al. 2013).

Evolution of Herbicide Resistance in Kochia

Kochia is prone to evolving resistance to herbicides with different modes of actions. Kochia has evolved resistance to photosystem II-, ALS- and auxin-inhibiting herbicides (Heap, 2014). Photosystem II-inhibitor (e.g. atrazine) resistance in kochia was first reported in 1976 in Kansas. Subsequently, ALS-inhibitor and auxinic herbicide resistances were reported in other US states in 1987 and 1995, respectively (Morrison and Devine, 1994; Cranston et al. 2001).

Primiani et al. (1990) collected kochia biotypes from wheat stubble fields in Kansas that were resistant to both pre-plant and post-emergence applications of ALS-inhibitor herbicides. The level of resistance to ALS-inhibitos ranged from 3-75 and 3-30 fold for PRE and POST applications of several diferent ALS-inhibitor herbicides, respectively. A dicamba resistant kochia biotype from Henry county, NE was found to be 30 times more resistant than a susceptible biotype (Preston et al. 2009). It has been estimated that more than 90% of kochia populations across the prairies are ALS-inhibitor resistant (Beckie et al. 2011). This sizable estimation of ALS-inhibitor resistance in kochia populations adds to the concerns of evolution of multiple herbicide resistance in kochia. In Alberta, Canada, GR kochia populations were screened for ALS-inhibitor and dicamba resistance; however, only ALS resistance was confirmed in those GR populations (Beckie et al. 2013).

Glyphosate-Resistant Kochia

Kochia was first confirmed resistant to glyphosate in Kansas in 2007; since then GR kochia has been reported and confirmed in six additional US states and three Canadian provinces (Waite et al. 2013; Heap, 2014; Beckie, 2014; Godar, 2104). Across western Kansas, it is estimated that approximately one-third of the kochia populations possess glyphosate resistance (Godar, 2014). Eight GR kochia populations were found to have an ED_{50} (effective dose to cause 50% mortality) ranging from 0.54-1.35 kg ae ha^{-1} compared to a glyphosate-susceptible (GS) population with an ED_{50} of 0.17 kg ae ha^{-1} which corresponded with a resistance index of 3.3-8.0 for GR populations previously tested (Godar, 2014).

Several studies have been conducted to identify the mechanism of glyphosate resistance in kochia. In four kochia populations that exhibited differential response to glyphosate, no significant difference between absorption and translocation of glyphosate was found (Waite et al. 2013; Godar 2014); and application of glyphosate at 870 g ae ha^{-1} resulted in 4-91% injury of those populations 3 weeks after treatment (Waite et al. 2013). Wiersma (2012) found increased *EPSPS* gene copies in several kochia populations that survived 870 g ae ha^{-1} or higher doses of glyphosate. Additionally, increased transcript and protein was positively correlated with increased *EPSPS* gene copies, suggesting that the *EPSPS* copies are functional in kochia. *EPSPS* sequence was also analyzed and no amino acid (Proline 106) mutations were found at the binding site.

The above studies suggest that glyphosate resistance in kochia is mediated by *EPSPS* gene amplification and not due to glyphosate uptake or translocation differences between GR and GS kochia; however, the genetic basis and the *EPSPS* copy location on the genome of kochia are unknown. Therefore, the overall goal of this research was to investigate the genetic basis of glyphosate resistance in kochia as well as to determine the chromosomal distribution of *EPSPS* gene copies.

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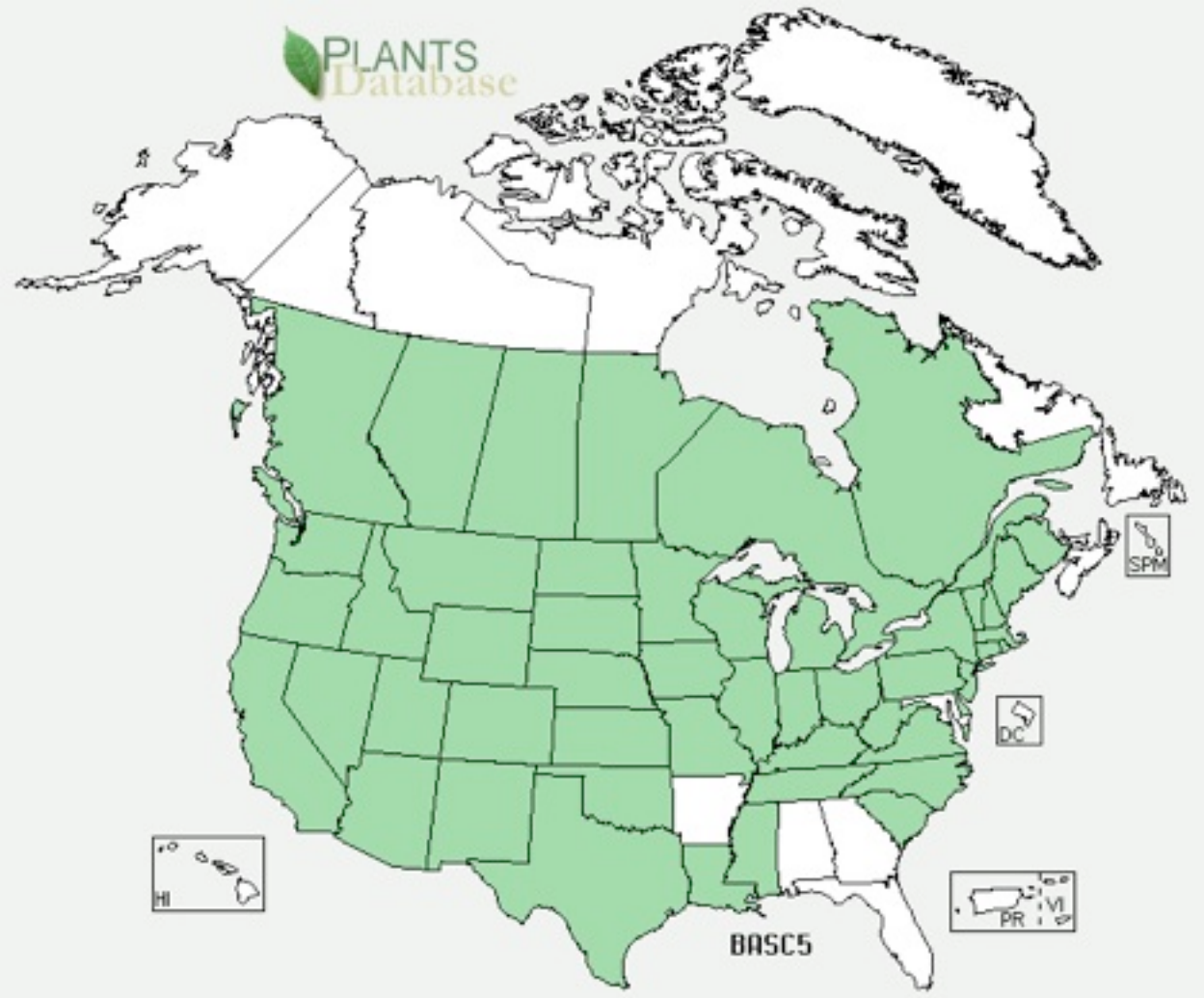

Figure 1.1 Distribution of kochia in the United States and Canada. Shaded areas represent states/provinces where kochia is present in cropland, non-cropland, or both.

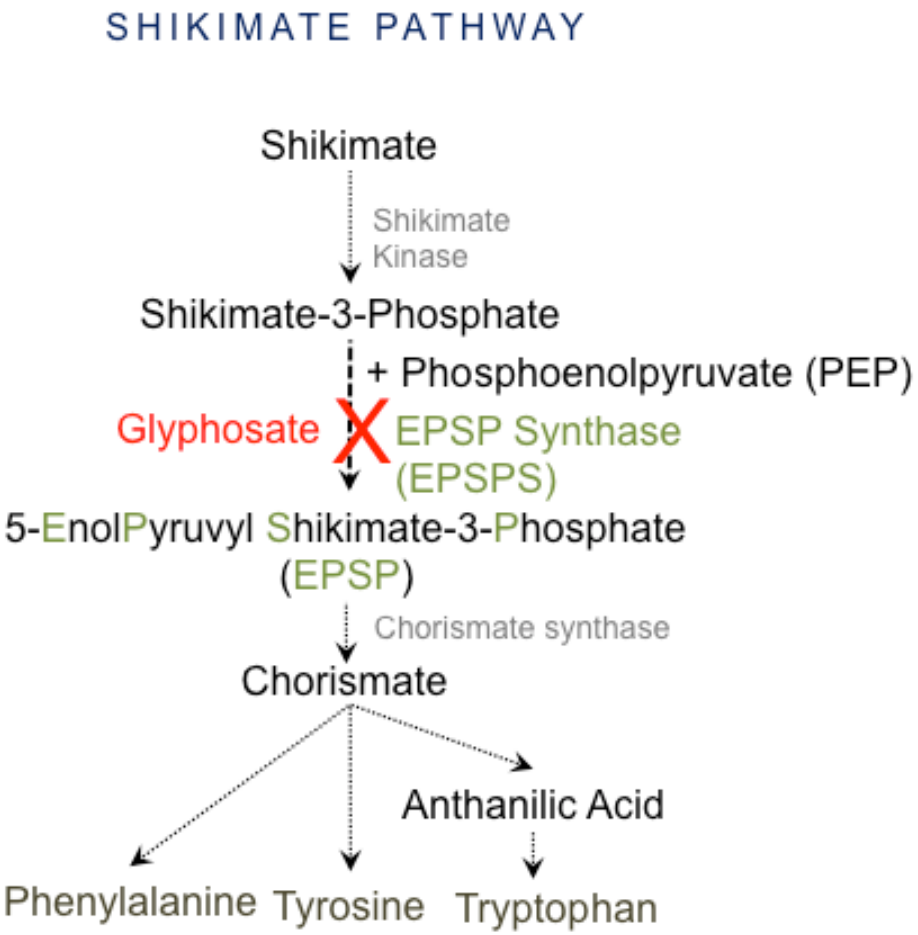

Figure 1.2 Shikimic acid pathway disrupted by glyphosate.

Chapter 2 - Inheritance of Glyphosate Resistance in Kochia

Abstract

Extensive, often exclusive, use of glyphosate in crop production has resulted in evolved glyphosate resistance in several weed species globally. Kochia is a competitive summer annual weed, well adapted to the North American Great Plains and has recently evolved resistance to glyphosate by gene amplification of 5-enolpyruvyl shikimate 3-phosphate synthase (*EPSPS*), the target-site of glyphosate. The overall objective of this research was to investigate the genetic basis of glyphosate resistance in kochia, specifically to study 1) the inheritance of glyphosate resistance and 2) determine the chromosomal distribution of *EPSPS* gene copies. Homozygous resistant (R) and susceptible (S) parental lines of kochia were identified. Using these parents, reciprocal crosses were performed to produce F_1 progeny. As expected for a nuclear encoded *EPSPS* gene, F_1 plants from both crosses survived various doses of glyphosate application. However, F_1 plants showed intermediate shikimate accumulation and *EPSPS* gene copies (relative to *ALS* reference gene) compared to parents. F_2 progeny were produced by selfing F_1 plants. In response to 870 g ae ha^{-1} glyphosate, F_2 plants (n=115) segregated into 3:1 (R:S) implying a Mendelian monogenic segregation of glyphosate resistance in kochia. Additionally, relative *EPSPS* gene copies ranged from 1-10 in the F_2 progeny (n=51) with a genotypic segregation of 40:11 (plants with 3 or more *EPSPS* gene copies: plants with 1 *EPSPS* gene copy).In F_2 dose-response, a correlation between the level of resistance and relative *EPSPS* gene copies was observed. Genomic organization of the amplified copies using fluorescent in situ hybridization (FISH) displayed a single and larger hybridization site of the *EPSPS* gene on one pair of homologous chromosomes in R compared to a faint hybridization site in S samples of kochia. These results suggest possibility of amplification of *EPSPS* gene mediated via unequal recombination leading to the evolution of the glyphosate resistance in kochia.

Introduction

Kochia [*Kochia scoparia* (L.) Schrad.] is a broadleaf weed that predominates in semi-arid environments of the U.S. Great Plains and Canadian Prairies. This annual weed creates substantial economic impact in the central Great Plains states and Canadian Prairies where it infests cropland and non-cropland areas including wheat, corn, sorghum, sugar beet, pastures, rangeland, waste areas, ditch banks, and roadsides (Friesen et al. 2009). Biological characteristics of kochia such as early germination that continues all season, and tolerance to hot and cold temperatures as well as drought make it an extremely competitive weed (Dille et al. 2012; Friesen et al. 2009). On average, kochia produces 15,000-25,000 seed per plant and upon maturity the seed gets dispersed via a tumbleweed mechanism in which wind blows the plant across the landscape scattering seed (Friesen et al. 2009). Kochia infestation is typically controlled by tillage and/or use of herbicides.

Glyphosate is a non-selective herbicide introduced in the early 1970's that targets the 5-enolpyruvylshikimate 3-phosphate synthase (*EPSPS*) enzyme in the shikimic acid pathway. (Baylis, 2000; Cobb and Reade, 2010). This pathway is responsible for synthesizing aromatic amino acids as well as vitamins and lignins (Cobb and Reade, 2010). Introduction of glyphosate-resistant crops in 1996 increased glyphosate use in agriculture. This technology provided farmers with opportunities to use reduced and no-tillage practices which were quickly adapted in addition to glyphosate burndown treatments (Young, 2006). Reduced tillage and no-till are used extensively in western Kansas because of soil moisture retention and reduced eroison benefits. Conversely, no-till practice can increase kochia emergence by four-fold; therefore, this necessitates use of herbicides to control kochia (Anderson and Nielsen, 1996).

Continuous and often exclusive use of glyphosate created selection pressure in weed species that resulted in glyphosate resistance. Worldwide there are 28 different weed species that have evolved gyphosate resistance, 14 of which are present in the US (Heap, 2014). In Kansas alone, six different weed species including horseweed (*Conyza canadensis*), tall waterhemp (*Amaranthus tuberculatus*), giant ragweed (*Ambrosia trifida*), common ragweed (*Ambrosia artimisiifolia)*, kochia, and Palmer amaranth (*Amaranthus palmeri*) have been confirmed as glyphosate-resistant (Heap, 2014). Glyphosate-resistant kochia was first identified in Kansas in

2007, but has since been reported and confirmed in six additional US states and three Canadian provinces (Waite et al. 2013; Heap, 2014; Beckie, 2014). Across western Kansas, it is estimated that approximately one-third of the kochia populations have evolved glyphosate resistance (Godar, 2014). Several studies have been conducted to identify the mechanism of glyphosate resistance in kochia. In four kochia populations that expressed differential responses to glyphosate, no significant differences in absorption or translocation were found among populations; however, a glyphosate application of 870 g ae ha^{-1} resulted in 4-91% injury among those populations 3 weeks after treatment (Waite et al. 2013). Wiersma (2012) found increased *EPSPS* gene copies in several different kochia populations that survived glyphosate applications. Additionally, increased transcript and protein abundance was directly correlated with increased *EPSPS* gene copies. *EPSPS* sequence was also analyzed and no amino acid (Proline 106) substitutions were found at the binding site.

Kochia is prone to evolve resistance to herbicides with different modes of action. In addition to glyphosate, kochia resistance to triazines, ALS-inhibitors and dicamba has been documented across the US. Photosystem II-inhibitor resistance in kochia was first reported in Kansas in 1976 followed by ALS- and auxinic-inhibitor resistances in 1987 and 1995, respectively, in the US (Morrison and Devine, 1994; Cranston et al. 2001). It has been estimated that more than 90% of kochia populations across the prairies are ALS-inhibitor resistant (Beckie et al. 2011). This estimation of ALS-inhibitor resistance already present in kochia populations adds to the concerns of evolution of multiple herbicide resistance in kochia. Understanding the genetic basis behind different herbicide resistances will help in implementing and maintaining successful weed management practices. Although there is evidence that glyphosate resistance in kochia is mediated by *EPSPS* gene amplification, the genetic basis of resistance and the location of *EPSPS* gene copies in the genome of kochia are unknown. Therefore, the overall goal of this research was to investigate the genetic basis of glyphosate resistance in kochia and determine the chromosomal distribution of *EPSPS* gene copies.

Materials and Methods

Identification of Homozygous Parental Lines

Glyphosate-resistant (R) kochia seed was obtained from fields in Lane, Russell and Phillips counties in Kansas. Seed of glyphosate-susceptible (S) was collected from Ellis county

Kansas. Seed was germinated in 28x6x8 cm flats filled with 2 kg Miracle-Gro moisture control potting mix in the Weed Science Greenhouse at Kansas State University. The greenhouse was maintained at 25/20 °C day/night and 15/9 photoperiod supplemented with 200 μmol m^{-2} s^{-1} photosynthetic photon flux provided with sodium vapor lamps. Once 2-3 cm tall, seedlings were transplanted into 5 cm plastic cone pots filled with 160 g Miracle-Gro moisture control potting mix. Plants 8-10 cm in height were then sprayed with formulated glyphosate (Roundup WeatherMAX, Monsanto) at a rate of 870 g ae ha^{-1} in 2% (v/v) ammonium sulfate (AMS) using a chamber bench-type sprayer calibrated to deliver 187 L ha^{-1} at 138 kPa. All subsequent glyphosate applications contained 2% (v/v) AMS solution. Also, in all treatments known glyphosate-resistant and -susceptible kochia plants were included as positive and negative controls.

Plants that survived glyphosate application were transplanted individually into 15-cm round pots filled with 400 g Miracle-Gro moisture control potting mix. Upon flowering, the plants were self-pollinated using microperforated bread bags to produce seed. Seed was collected separately from all plants of Lane, Russell and Phillips populations. About 50 seeds from each per plant were sown in the greenhouse as described earlier and progeny from each self-pollinated plant were grown. When plants were 8-10 cm tall, the plants were sprayed with formulated glyphosate at a rate of 870 g ae ha^{-1}. One week after treatment, plants susceptible to glyphosate showed chlorosis and subsequently died; whereas resistant plants showed slight chlorosis, recovered, and then continued to grow normally. Progeny from self-pollinated plants that showed presence of glyphosate resistant and susceptible plants were identified as heterozygous (segregating) and hence were not use for any genetic analysis experiments. Progeny that were all resistant to glyphosate without any susceptible individuals were labeled as homozygous resistant; whereas, those that were all found susceptible to glyphosate were considered as homozygous susceptible. Thus, the zygosity was determined based on phenotypic response to glyphosate since homogeneous response indicates whether or not alleles are segregating.

Production of F_1 and F_2 Progeny

Seed of homozygous R and S parents were planted in soil in the greenhouse as described above. Parental R and S plants were transplanted into 15-cm round pots filled with 400 g

Miracle-Gro moisture control potting mix when 10-12 cm tall. Reciprocal crosses of homozygous R and S plants were performed as follows. For clarification, RxS respresents a resistant female pollinated with susceptible pollen and vice versa for SxR. Kochia bears protogynous flowers, therefore the stigmas (two per flower) are receptive approximately one week before antheis of the same flower. Therefore, prior to stigma emergence, all the leaves and apical meristems were removed from a few randomly selected branches of R or S plants and covered with translucent white water-repellent paper bags (Lawson '217' Bag; Lawson Bags, Northfield, IL) . After stigma emergence, using sterile forceps, pollen from dehisced anthers of R or S (chosen as male parents) was transferred seperately onto the stigmas of the maternal flower. Immediately after pollination, the flowers were covered with the same pollination bags. Stigmas were dusted twice with pollen from the male parent, once when stigmas protruded, and the second time approximately 3 days after the first pollen dusting. After the second pollination, flowers were covered for 10 days. In one S plant, the entire plant was dusted daily with pollen from an R plant to ensure hybrid seed production. Mature F_1 seed were harvested separately from reciprocal crosses. The seed was germinated as described above. Seedlings were transplanted into 4.5x4.5x6 square pots filled with 60 g Miracle-Gro moisture control potting mix when 2-3 cm tall. Some F_1 plants were transplanted into 15-cm round pots filled with 400 g Micacle-Gro moisture control potting mix and self-pollinated to produce F_2 seed. Mature F_2 seed was harvested seperately from each F_1 plant and the family derived from each individual F_1 plant was designated as an F_2 family.

Whole-Plant Dose-Response Experiments

Parental lines, F_1 and F_2 progeny were all subjected to whole-plant dose-response to glyphosate. When plants reached 8-10 cm in height, plants were treated with formulated glyphosate (Roundup WeatherMAX, Monsanto; St. Louis, MO) in 2% (v/v) ammonium sulfate (AMS) using a chamber bench-type sprayer calibrated to deliver 187 L ha^{-1} at 138 kPa. Parental lines were treated with six different doses (0, 0.25, 0.5, 1, 2, and 3X; where X is 870 g ae ha^{-1}). At least four plants were included per dose. F_1 plants were subjected to only five glyphosate doses (0, 0.25, 0.5, 1, and 2X) due to limited availability of F_1 plants. Five F_1 plants were included in each dose, and 3 plants were used for biomass determination and two plants were used for self-pollination to produce F_2 seed. F_2 plants derived from F_1 plants from two different

families were used to determine segregation rates when treated with (0, 0.25, 0.5, 1, 2, 3, and 4X) doses of glyphosate. Visual observation of plant mortality to glyphosate treatment was recorded 3 weeks after treatment (WAT). Above ground biomass was harvested 3 WAT from all plants except from the F_1 plants that were maintained for F_2 seed production. The plant samples that were harvested were dried in at 60 °C in an oven for 72 hours. Dry weight was recorded and the data analyzed using the 'drc' package in R (Knezevic, 2007). The three-parameter non-linear log-logistic model (Equation 2.1) showed good fit, thus, the relationship between herbicide dose and mortality or aboveground biomass was described as

Equation 2.1 Three parameter non-linear regression model.

$$Y = \frac{d}{\{1 + \exp(b(\log(x) - \log(e)))\}}$$

where Y is aboveground biomass, e (also known as GR_{50}) denotes the herbicide dose that caused 50% response, d is the response upper limit, b denotes the relative slope around e, and x represents herbicide dose. The response lower limit was set equal to 0.

Eight F_2 families were subjected to 870 g ae ha^{-1} glyphosate treatment and response was recorded weekly for 3 WAT.

Shikimate Assay

Glyphosate inhibits production of the aromatic amino acids in the shikimic acid pathway causing a build-up of shikimate-3-phosphate, a substrate of EPSPS (Figure 1.2), and its dephosphorylated state-shikimate (Shaner et al. 2005). Thus, glyphosate susceptible plants will accumulate shikimate after exposure to glyphosate. A measure of shikimate accumulation can be determined following the procedure developed by Shaner et al. (2005). Six 6-mm leaf disks were collected from the top leaf of a single plant. Leaf disks were place in a 96-well microtiter plate with one disk per well. Individual leaf disks were subjected to either a buffer solution (0 µM gyphosate) or glyphosate solution. The buffer solution was comprised of 0.6902 g ammonium phosphate dissolved in 600 ml deionized water. In all treatments, 100 µM glyphosate was used, except in dose-response experiments. In shikimate dose-response assay glyphosate concentrations ranging from 0 µM to 1000 µM were used. After each disk was placed in a well containing buffer with or without glyphosate, the plates were wrapped with clear, plastic wrap and incubated under light for 16 hours. After the appropriate time had elsapsed, plates were frozen and thawed at -20 and 60 °C, respectively. Leaf disks were then treated with 1.25 N HCl

(25 µL) and incubated at 60 °C for 20 minutes. In a new 96-well microtiter plate, 25 µL of solution from the treated leaf disk was added to 100 µL of reaction buffer (periodic acid (0.25% v/v)/meta-periodate (0.25% v/v)). The plates were incubated at 23°C for 90 minutes and then 100 µL of quenching buffer (0.6 M sodium hydroxide/0.22 M sodium sulfite) was added to each well.

Shikimate accumulation was measured at OD_{380} using an Epoch Microplate Sprectrophotometer (BioTek Instruments, Inc.) equipped with Gen5 version 2.01 software. A shikimate accumulation standard curve was generated and used to calculate the shikimate accumulation in each well. The values for 0 µM glyphosate treatment were subtracted from the 100 µM glyphosate treatment (or whichever concentration of glyphosate was teseted) to determine the change or accumulation of shikimate in ng shikimate μL^{-1} solution. Each plant sample was done in triplicate and repeated twice.

Estimation of Relative EPSPS Gene Copy Number

Relative *EPSPS* gene copy number was determined by quantitative PCR (qPCR) using genomic DNA (gDNA). To account for variability, the acetolactase synthase (*ALS*) gene was used to normalize the relative copy number. *ALS* was chosen as a reference as there have been no reports of variation in *ALS* gene copies in plant species (Wiersma, 2012). EPSPS gene copy number was determined in parents, F_1 and F_2 plants. Fresh leaf tissue (100 mg) was collected in 1.5 mL microcentrifuge tubes, immediately frozen in liquid nitrogen and stored at -20 °C until DNA was extracted. Genomic DNA was extracted using a Quigen DNeasy kit. The protocol as outlined in the kit was followed for DNA extraction. The final concentration of DNA was diluted to 8 ng/µL and qPCR was performed using a Bio-Rad CFX-96 Touch for all experiments.

K. scoparia specific primers were used for qPCR and *EPSPS* forward and reverse sequences were 5’ GGCCAAAAGGGCAATCGTGGAG 3’ and 5’ CATTGCCGTTCCCGCGTTTCC 3’, respectively. *ALS* forward and reverse primers were 5’ ATGCAGACAATGTTGGATAC 3’ and 5’ TCAACCATCGATACGAACAT 3’, respectively. These primers produced products of 102 and 159 bp for *EPSPS* and *ALS*, respectively. qPCR was performed using 96-well microtiter plates with each well containing a master mix comprised of: 10 µL of iQ™ SYBR ® Green Super Mix (Bio-Rad), 1µL of each corresponding forward and reverse primer (5 µM), 16 ng of gDNA, and 4 µL of diH_2O. Each reaction was done in triplicate

and duplicated. Cycle parameters were set at 95 °C for 3 minutes for the initial denaturing, 95 °C for 10 seconds for denaturing, annealing and extension at 60 °C for 30 seconds and the denaturing/annealing steps repeated 39 times for a total of 40 cycles.

A threshold (C_T) was used to determine at which cycle the primers had reached a point of equal products. EPSPS copy number was normalized to the ALS reference gene ($\Delta C_T = C_T^{EPSPS} - C_T^{ALS}$) (Gaines et al. 2010). The ΔC_T method ($2^{-\Delta C_T}$ = relative gene copy number) was used to determine copy number in Microsoft Excel. Each plant sample was measured in triplicate and repeated twice. Replicates were averaged and standard deviation was calculated.

Sequencing the EPSPS Gene

Geneomic DNA was extracted as previously described and used to amplify and sequence the *EPSPS* binding site at the Proline 106 position. A 200 bp PCR product was amplified using the forward and reverse primers 5' CCAAAAGGGCAGTCGTAGAG 3' and 5' ACCTTGAATTTCCTCCAGCA 3', respectively. Each reaction was comprised of 2.5 µL of both forward and reverse primers (5 µM), 12.5 µL of Promega PCR Master Mix (Promega), 40 ng of gDNA and 2.5 µL of nuclease free water (Promega) for a total reaction volume of 25 µL. The initial PCR denaturation step was done at 95 °C for 3 minutes, followed by 40 cycles of denaturation at 95 °C for 30 seconds, primer- annealing at 60°C for 30 sec, and product extension at 72 °C for 1 minute. After 40 cycles, the reaction was completed and held at 4°C. The PCR product was separated on 1% agarose gel stained with ethidium bromide and bands were detected. The remaining PCR product was purified using GeneJET PCR Purification Kit (ThermoScientific #K0701) and separated on another agarose gel to ensure PCR product quality post purification. PCR products were sequenced at the Genomic and Sequencing Lab in the Plant Pathology Department at Kansas State University and aligned using Multalin software (Corpet, 1988).

Chromosome Location of the Amplified EPSPS Gene Copies

FISH work was performed in the Plant Pathology Department at Kansas State University using the outlined procedure below.

Chromosome preparation and FISH procedure

Somatic chromosome preparations of R and S kochia were done using the drop technique, direct probe labeling by nick translation, and the FISH procedure as described previously (Kato et al. 2004; Kato et al. 2006) with minor modifications. Root tips were collected from young plants and treated in a nitrous oxide gas chamber for 90 minutes, fixed on ice in cold 90% acetic acid for 10 minutes, washed and stored in 70% ethanol at −20 °C. For slide preparation, roots were washed in tap water for 10 minutes and then in KCl buffer 5 minutes (75 mM KCl, 7.5 mM EDTA, pH 4); 7 meristems (0.5–1 mm long) were placed in 20 µl of 4% cellulase Onozuka R-10 (Yakult, Japan, Tokyo cat # 201069), 1% pectolyase Y23 (Karlan cat # 8006) in KCl buffer, and incubated for 43 minutes at 37 °C. Digested meristems were washed for 5 minutes in ice-cold Tris–EDTA buffer, pH 7.6, then three times in 100% ethanol. Meristems were dispersed with a needle in 20 µL of ice-cold acetic acid - methanol mix (9:1) and immediately dropped on to 3 pre-cleaned glass slides placed in a humid chamber. Dried preparations were UV cross-linked, soaked in methacarn solution (methanol : chlorophorm : glacial acetic acid 6:3:1) during 1 minute, dried and used for hybridization on the same day. For labeling the nucleolus organizing region (NOR) rRNA loci, clone pTa71, containing a 9-kb insertion with 18S, 5.8S, and 26S rRNA wheat genes and intergenic spacers (Gerlach and Bedbrook 1979) was used as a probe. Five µl of probe mixture contained 200 ng of each *EPSPS* gene PCR product labeled with Texas red-5-dCTP and 160 ng of pTa71 labeled with Fluorescein-12-dUTP (PerkinElmer, cat # NEL413001EA and NEL426001EA). The mixture of probes and the slide preparation were denatured at 100°C separately. The rest of the FISH procedure and washes were the same as in Kato et al. (2006). Chromosome preparations were mounted and counterstained with 4', 6-diamidino-2-phenylindole solution (DAPI) in Vectashield (Vector Laboratories, cat # H-1200, H-1300). Images were captured with a Zeiss Axioplan 2 microscope using a cooled charge-coupled device camera CoolSNAP HQ2 (Photometrics) and AxioVision 4.8 software (Zeiss) and processed using the Adobe Photoshop software (Adobe Systems Incorporated, San Jose, CA, USA).

EPSPS FISH probe

Sequences of kochia *EPSPS* mRNA from the susceptible parent (Ellis county population) and the *Amaranthus palmeri EPSPS* gene (accession number JX56456) were used to develop the PCR primers. The *EPSPS* gene was amplified using kochia genomic DNA as a template isolated with Qiagen DNeasy Plant Mini kit (cat. # 69104). The PCR reaction included JumpStart

REDTaq ReadyMix (Sigma, Cat. P0982), 0.4 µM of each primer and 0.5-4 ng/µl of template DNA. PCR cycles consisted of 96 °C for 5 minutes for the initial denaturation, 35 cycles of annealing and extension: 96 °C for 30 seconds, 57 °C for 30 seconds, 72 °C for 4 minutes and a final extension at 72 °C for 15 minutes. PCR products were cut and eluted from agarose gel with Qiagen Gel Extraction kit (Cat. # 28706) and re-amplified using the same primers. PCR products were purified with Invitrogen PCR Purification kit (Cat. # K3100-01) and verified by sequencing (Genewiz). The sequence of amplified part of kochia EPSPS gene was submitted to NCBI GeneBank database with accession number KJ374721. Three PCR products were tested separately by FISH and products 1 and 3 showed no background staining on kochia chromosomes were used as a pooled FISH probe.

Results

Whole Plant Response to Glyphosate

Parental R and S plants were identified based on whole plant response to 870 g ae ha^{-1} glyphosate 3 WAT. Self-pollination of kochia plants from Lane (n=4), Phillips (n=2), and Ellis (n=2) were successful. Ellis populations are labeled as PSI in tables. Three WAT, progeny from both plants in the Phillips population survived 870 g ae ha^{-1} glyphosate while progeny from the Ellis county plants were susceptible (Figure 2.1). All plants from the Lane population segregated as glyphosate R or S (Table 2.1). Progeny from Phillips 88-4 and PSI-6 were selected as R and S parental lines, respectively. Whole-plant dose-response (0, 0.25, 0.5, 1, 2, 3X doses) of parental lines indicated that the R plants were 3.4 times more resistant than the S line (p-value 0.007). GR_{50} (effective dose to cause 50% growth reduction) was 367 and 1266 g ae ha^{-1} for S and R, respectively (Table 2.2. and Figure 2.2). Reciprocal crosses (RxS and SxR) were successful, approximately 10% of hand-pollinations set seed. Specifically, of 450 hand crosses made, 50 F_1 seed were produced and 45 seed germinated. Twenty-two SxR and 23 RxS (45 total) F_1 seedlings were produced. F_1 progeny were sprayed with various doses of glyphosate (0, 0.25, 0.50, 1 and 2X) and phenotypic data was collected for 3 WAT in comparison to parental R and S plants. F_1 plants from both RxS and SxR crosses survived all rates of glyphosate as expected from a nuclear encoded *EPSPS* gene (Figure 2.3).

A total of 115 F_2 plants representing a composite of 8 families were treated with a 1X rate of glyphosate. In response to the glyphosate application, the F_2 plants segregated 3

resistant:1 susceptible (Figure 2.4). Chi-square tests for goodness of fit to a 3:1 segregation (R:S) supported the null hypothesis; the observed frequencies (R or S) after herbicide treatment were in accordance with the expected frequencies for a 3:1 (R:S) segregation ratio. Plants from two F_2 families (derived from self-pollination of F_1 SxR or F_1 RxS) segregated 37:13 (R:S) and 48:17 (R:S). When response of all F_2 plants (n=115) to 1X rate of glyphosate was pooled, plants segregated into 85:30 (R:S) with a $\chi^2 = 0.076$ (Table 2.3, Table 2.4). Glyphosate dose response (0, 0.25, 0.5, 1, 2, 3, and 4X) of F_2 progeny, 3 WAT also demonstrated segregation of plants into 3:1 (R:S) at each dose (Table 2.5). Phenotypic variation in response to 1X glyphosate was observed in F_2 progeny within a family (Figure 2.4). The variability (plant size and branching) did not correlate with the number of EPSPS gene copies; therefore, the phenotypic variation observed could possibly be attributed to the vast genetic variability present in kochia.

However, after glyphosate treatment, two F_1 plants were found to be not true parental crosses, as progeny of one F_1 (SxR) did not have any plants survive glyphosate treatment and on the other hand, all plants in one F_2 family (derived from self-pollination of RxS) were resistant to glyphosate without R:S segregation; therefore, data from these crosses was not included in data analyses.

Shikimate Accumulation and Gene Copy Determination

Shikimate accumulation was significantly higher in susceptible parental plants compared to resistant plants (Figure 2.5). F_1 plants accumulated an intermediate level of shikimate compared to either parent at low doses; however, all plants showed higher shikimate accumulation at 500 and 1000 µM (Figure 2.5). In general, shikimate accumulation negatively correlated with *EPSPS* gene copies, with low accumulation corresponding to higher number of gene copies (Figure 2.6 A to C). Relative *EPSPS*:*ALS* gene copies were measured in parental and F_1 plants. Realtive *EPSPS* copies in parental resistant lines ranged from 6.5-7.5, while susceptible lines had 0.6-0.8 copies. Relative *EPSPS*:*ALS* copies for susceptible plants are less than one due to the size of the amplified product. *EPSPS* primers produce 159 bp product while *ALS* primers produced 102 bp product, which is approximately 65% of the *EPSPS* primer product. For this reason, and for simplifying presentation of copy number, relative *EPSPS*:*ALS* copies of 0.6-0.8 for S plants are considered as 1 and *EPSPS* copies in R plants are normalized accordingly. All F_1 progeny possessed 3.4 to 4.6 *EPSPS* copies, intermediate to either R and S

parent (Figure 2.6 A to C). In the F_2 progeny (n=51), relative *EPSPS*:*ALS* gene copies ranged from 1-10 with approximately 21.5, 51, and 27.5% plants possessing 1, 3-7, and 8-10 copies, respectively (Figure 2.7). Segregation of the F_2 plants (n=51) was 40:11 in which 40 plants had multiple *EPSPS* gene copies while 11 had only one gene copy. Chi-square analysis of F_2 relative *EPSPS* gene copy number segregation of plants with 3 or more *EPSPS* gene copies: plants with 1 *EPSPS* gene copy supported the null hypothesis; the observed frequencies (plants with more than 3 copies: plants with 1 copy) were in accordance with the expected frequencies for a 3:1 (R:S) segregation ratio base on *EPSPS* gene copy number (Table 2.6).

Sequencing the EPSPS Gene

Mutations in the *EPSPS* gene, specifically at amino acid 106 position, where a change from proline to serine or threonine, has been known to result in resistance to glyphosate in some weed species, such as rigid ryegrass and goosegrass (Wakelin and Preston, 2006; Baerson et al. 2002). The *EPSPS* gene in kochia was sequenced to determine if mutation at amino acid proline106 position was present in the R plants. The results indicated no amino acid change at the proline 106 site in either R or S parents, suggesting that a mutation in the *EPSPS* gene is not the basis for glyphosate resistance in these kochia populations (Figure 2.8).

Chromosomal Distribution of EPSPS Gene Copies

Analysis of FISH indicated a visible increase in *EPSPS* signal in R plants relative to S plants. In S plants, three chromosome pairs with nucleolus organizer region (NOR) sites were detected; one of which, with a minor NOR signal, had the *EPSPS* gene on the distal end (Figure 2.9 A). On prometaphase chromosomes and interphase nuclei, only a faint *EPSPS* signal was seen on each chromatid on S samples (Figure 2.9 B, C). On metaphase spreads of R kochia, the *EPSPS* probe detected much brighter signal on the same chromosome pair with a minor NOR site (Figure 2.9 D). On prometaphase chromosomes and interphase nuclei of R samples, 5-7 partially overlapping signals of the *EPSPS* probe can be distinguished at this location (Figure 2.9 E, F). Isolation of *EPSPS* probe localized on one chromosome is dissimilar than Palmer amaranth in which several *EPSPS* copies were dispersed throughout the entire genome, likely mediated by transposable elements (Gaines et al. 2010; Gaines et al. 2013).

Discussion

F_1 phenotypic response to glyphosate from both SxR and RxS crosses supported the nuclear inheritance of glyphosate resistance in kochia. Similarly, glyphosate resistance in rigid ryegrass, goosegrass, and horseweed was reported to be inherited via nuclear genes (Lorrain-Colwill et al. 2001; Simarmata et al. 2005; Ng et al. 2004; Zelaya et al. 2004). In this research it was found that three *EPSPS* copies were sufficient to provide resistance to a field use dose of glyphosate in kochia; however, shikimate dose-response data indicate that with increase in glyphosate concentration (100, 250, and 500 μM), variation in shikimate accumulation primarily depended on the number of *EPSPS* copies present in resistant plants. These data suggested that R plants with few *EPSPS* copies will accumulate more shikimate at higher glyphosate rates compared to those with higher copy numbers. F_2 whole-plant dose-response data suggest that regardless of glyphosate dose, the progeny segregated 3:1 (R:S), demonstrating that glyphosate resistance in kochia behaved like a Mendelian monogenic trait. Futhermore, F_2 plants also segregated 3:1 for *EPSPS* gene copy number as well (plants with 3 or more *EPSPS* gene copies:plants with 1 *EPSPS* gene copy). Although a phenotypic and genotypic segregation ratio of 3:1 (R:S) was observed in F_2 progeny, genotypic segregation based on *EPSPS* gene copy number appears to be more complicated; as the possibility of recombination during meiosis at the location, where *EPSPS* copies are present may result in variation in copy number.

Gene amplification as a means of glyphosate resistance was first reported in Palmer amaranth (Gaines et al. 2010). Interestingly, it was found that *EPSPS* copies in glyphosate-resistant Palmer amaranth were randomly distributed throughout the entire genome, possibly mediated by transposable elements (Gaines et al. 2010; Gaines et al. 2013). However, in kochia, FISH results indicated a cluster of *EPSPS* gene copies located at one location on two homologous chromosomes (Figure 2.9). Based on the chromosomal location of *EPSPS* gene copies, it is clear that the gene amplification mechanism resulting in glyphosate resistance in kochia evolved differently than that of Palmer amaranth. FISH analysis also indicated that the *EPSPS* copies were arranged in tandem in kochia, as all copies are localized on a single chromosome. Tandem arrangement of *EPSPS* copies suggest that glyphosate resistance in kochia may have evolved possibly via unequal recombination or unequal crossing over. This physical exchange of chromosome segments between homologous chromatids typically occurs during prophase of meiosis I; however, in a rare event, recombination is possible during mitosis as well

(Brooker, 2009). In addition to gene duplication, unequal crossing over can also lead to gene inversion or deletion (Hurles, 2004). Once a duplicated gene becomes fixed, the gene may become degraded (nonfunctionalization), embark a new function (neofunctionalization), or compliment the original gene (subfunctionalization) (Hurles, 2004). Based on the positive correlation between number of *EPSPS* copies and level of glyphosate resistance in kochia, it can be assumed that the gene copies are fixed as subfunctionalization genes (Hurles, 2004). This hypothesis needs to be tested.

In the context of gene amplification, cytogenetic arrangement of the amplified genes and the number *EPSPS* copies may largely determine the inheritance of glyphosate resistance in kochia. Since the *EPSPS* copies were present in tandem at the distal end of homologous chromosomes, there is a likelihood that the copies may tend to inherit as a single gene. In the case of kochia, 3:1 (R:S) segregation of F_2 progeny provides evidence that the *EPSPS* copies are inherited as a single Mendelian trait. Genetic diversity can results from natural events, such as gene deletion or duplication; however, this occurs at a very low frequency in nature. Intense selection pressure from glyphosate may have selected individuals with duplicated *EPSPS* genes and over repeated selection the *EPSPS* copies may have increased due to unequal recombination/crossing over.

Nuclear monogenic inheritance of glyphosate resistance in kochia will likely spread more rapidly across populations and geographies as the resistance can be transmitted via pollen and seed. Presence of only three copies of the *EPSPS* gene are sufficient to provide resistance to the field use rate of glyphosate. The positive correlation of resistance level and *EPSPS* gene copies suggested an additive effect of copies on glyphosate resistance. Furthermore, in phenotypic response to field rates (870 g ae ha^{-1}), glyphosate resistance trait may migrate across geographies at frequencies similar to a completely dominant trait as plants with 3-10 copies will all survive a 1X rate of glyphosate. However, from a genotypic view, *EPSPS* gene copy number is unlikely to be a completely dominant trait as different number of copies may be present and inherited on different chromosomes in progeny.

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Figure 2.1 Response of Phillips 88-4 (left) and PSI-6 (right) progeny to 870 g ae ha^{-1} glyphosate 3 WAT.

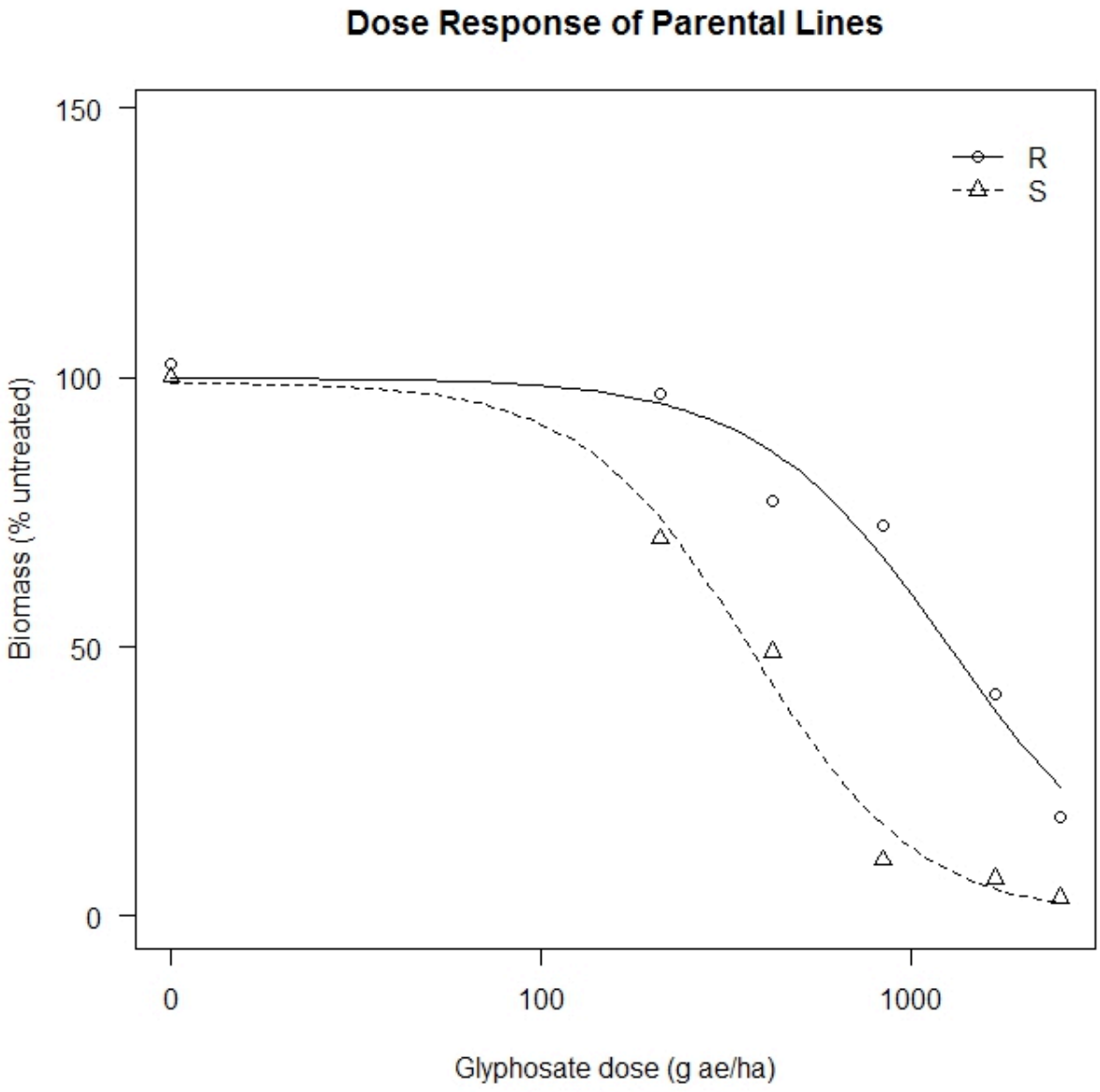

Figure 2.2 Whole-plant dose-response of parental lines as aboveground dry biomass presented as percent of untreated control.

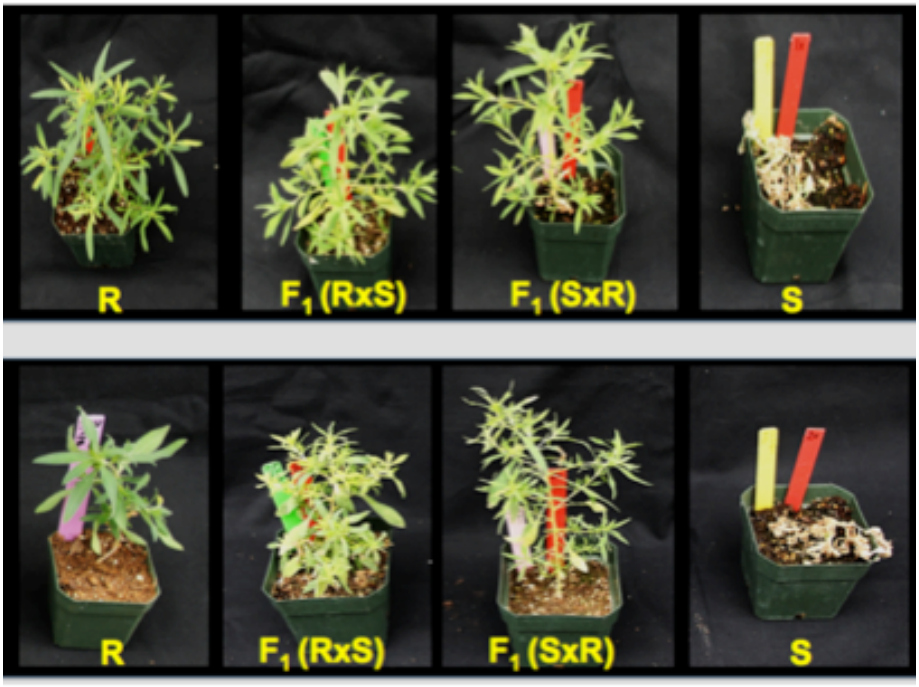

Figure 2.3 Whole-plant response of F_1 reciprocal crosses and parental lines in response to glyphosate 3 WAT.

Top row: Phenotypic response to 1X rate of glyphosate 3 WAT.

Bottom row: Phenotypic response to 2X rate of glyphosate 3 WAT.

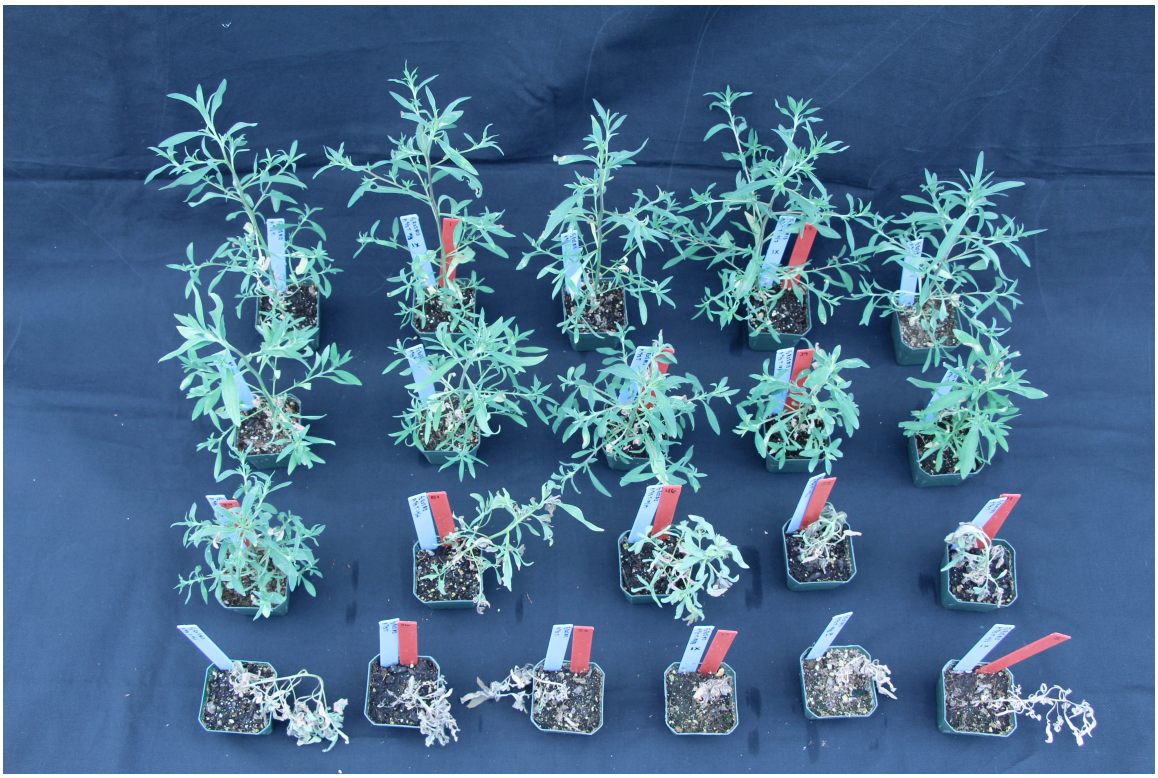

Figure 2.4 Segregation of a single F_2 RxS family 3 WAT to 870 g ae ha^{-1} glyphosate. Image shows 13:8 (R:S) segregation (n=21, χ^2 = 1.921).

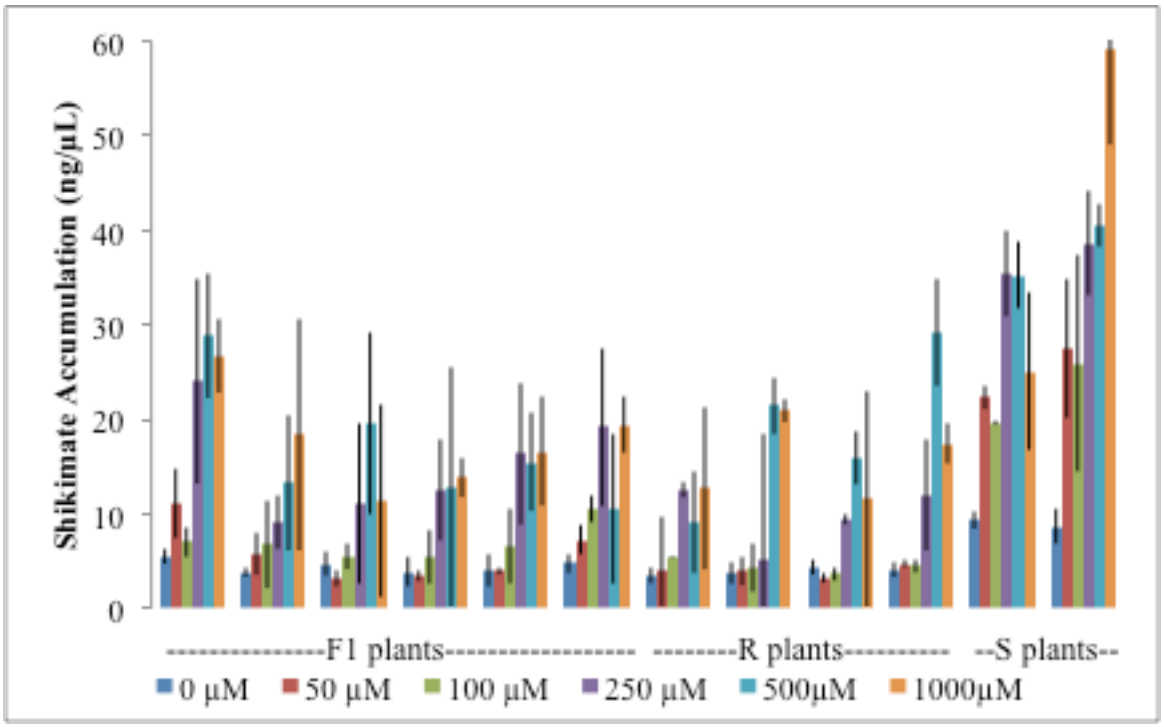

Figure 2.5 Dose-response shikimate accumulation of F_1 progeny and parental R and S plants to six different glyphosate concentrations and vertical lines within bars represent ±1 standard error.

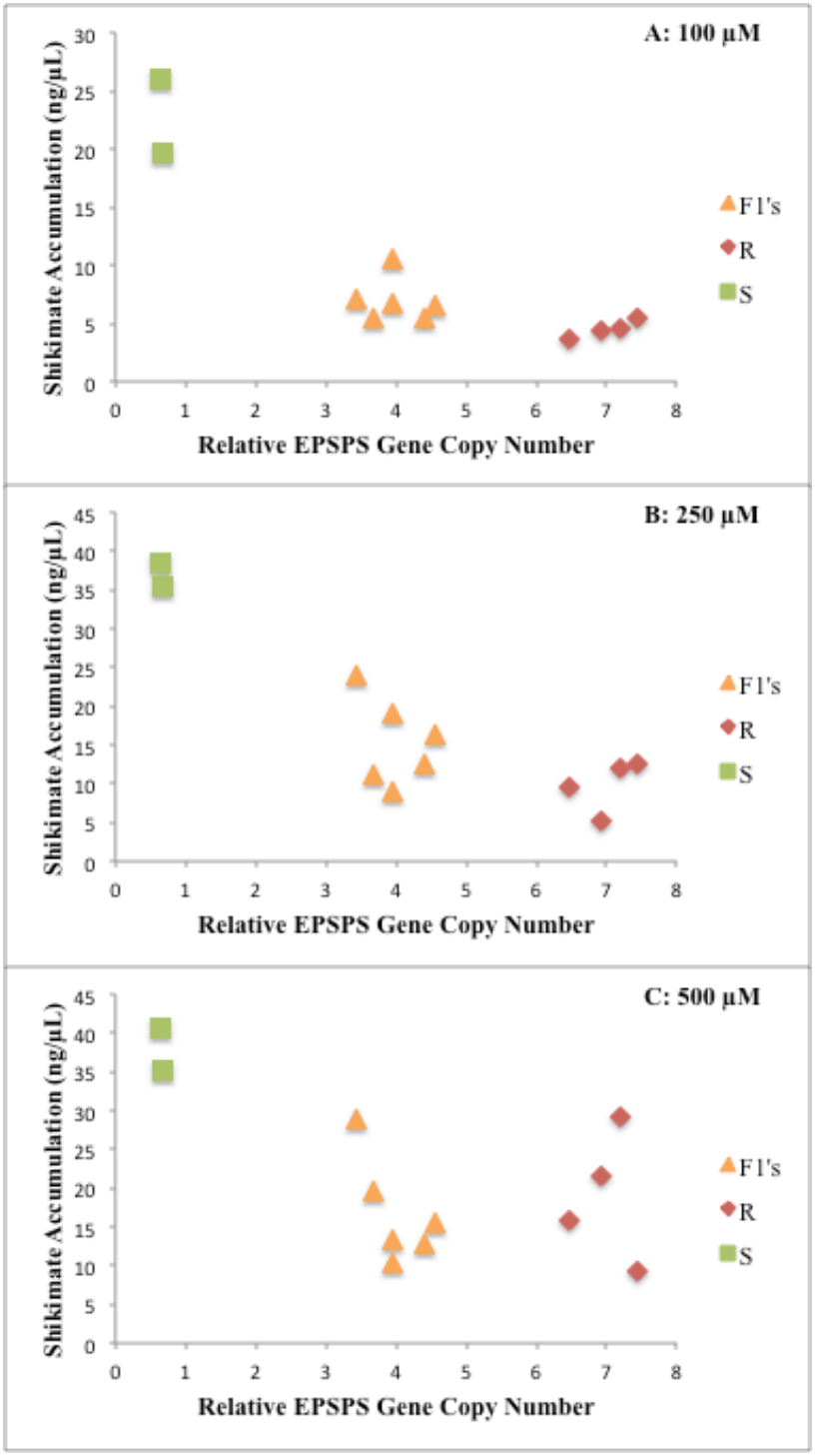

Figure 2.6 A) Relative *EPSPS* gene copy number and shikimate accumulation for parental lines and F_1 progeny in 100 µM glyphosate. B) Relative *EPSPS* gene copy number and shikimate accumulation for parental lines and F_1 progeny in 250 µM glyphosate. C) Relative *EPSPS* gene copy number and shikimate accumulation for parental lines and F_1 progeny in 500 µM glyphosate.

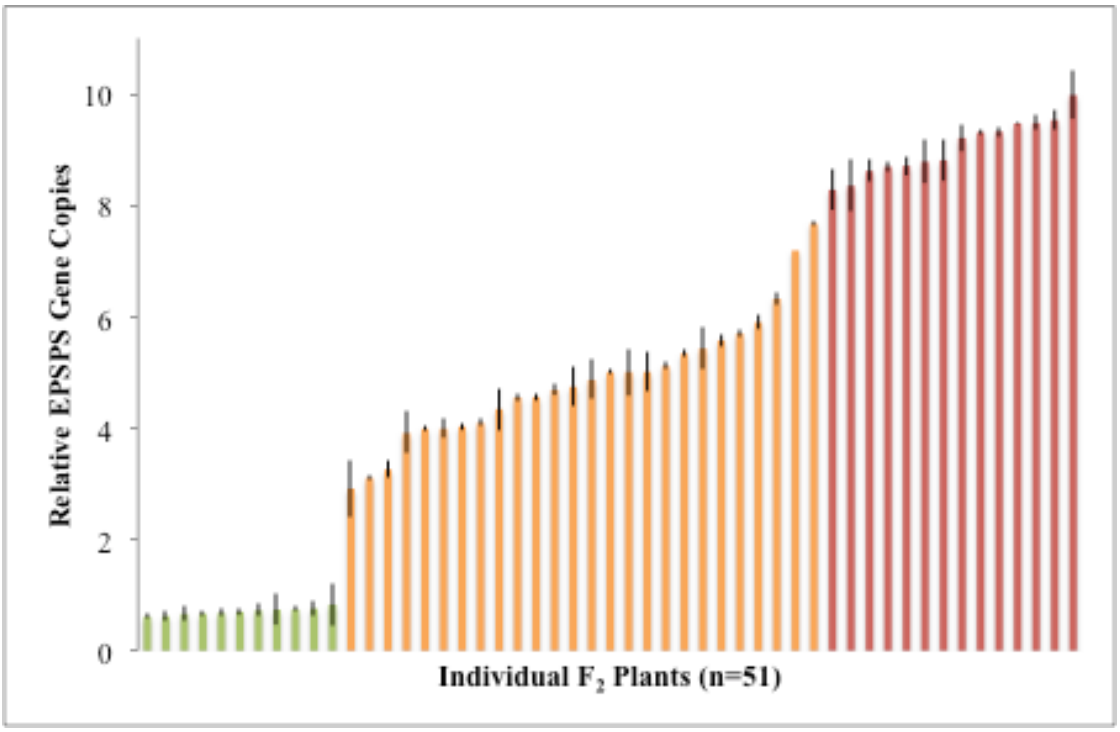

Figure 2.7 Relative *EPSPS*:*ALS* gene copies for F_2 progeny (n=51) ranged from 1-10 with 22.5% having 1 copy, 51% having 3-7 copies, and 27.5% having 8-10 copies. Vertical bars are ± 1 standard error.

Figure 2.8 Sequence comparison of R and S kochia *EPSPS* coding regions known to have mutations (Proline 106) conferring resistance to glyphosate in other species (e.g. rigid ryegrass and goosegrass).

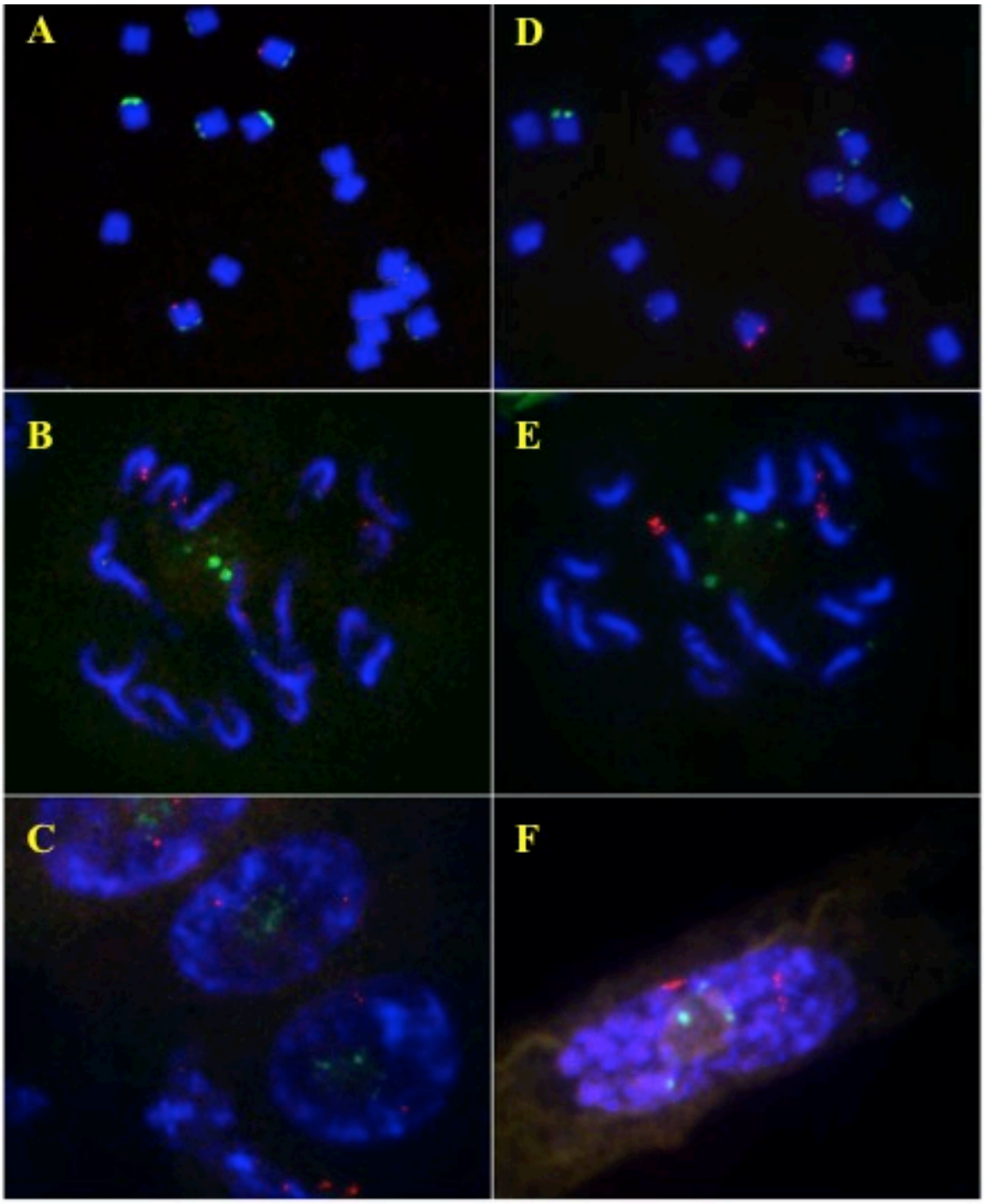

Figure 2.9 FISH images of glyphosate-resistant and -susceptible kochia.

Figures A-C are images of glyphosate-susceptible (PSI) parent and D-F are of glyphosate-resistant (Phillips 88) parent. Red signal of the *EPSPS* probe can be seen on somatic metaphase chromosomes (top row), prometaphase chromosomes (middle row) and interphase nuclei (bottom row).

Table 2.1 Identification of parental lines based on phenotypic response to 870 g ae ha^{-1} glyphosate.

Populations	1 WAT		2 WAT		3 WAT		
	Dead	Alive	Dead	Alive	Dead	Alive	Total
PSI-4	9	7	13	3	16	0	16
PSI-6	20	9	25	4	29	0	29
Lane 101-3	3	18	4	17	4	17	21
Lane 101-6	0	13	2	11	2	11	13
Lane 101-9	0	18	1	17	2	16	18
Lane 101-10	2	26	2	26	3	28	31
Phillips 88-4	0	22	0	22	0	22	22
Phillips 88-6	0	17	0	17	0	17	17

*Plants that survived 3 WAT were classified as glyphosate-resistant and plants that were dead were classified as glyphosate-susceptible.

Table 2.2 Growth reduction from glyphosate and resistance index for parental lines.

Population	GR_5	GR_{50}	GR_{90}	RI
	----------kg ae ha^{-1} ----------			
S	0.08 (0.04)	0.37 (0.06)	1.16 (0.32)	1
R	0.22 (0.14)	1.27 (0.23)	4.71 (1.59)	3.44**

GR_5: glyphosate dose required to cause 5% growth reduction; GR_{50}: glyphosate dose required to cause 50% growth reduction; GR_{90} glyphosate dose required to cause 90% growth reduction. Values in parenthesis are ±1 standard error.

**; RI value significantly greater than 1 at $p < 0.01$.

Table 2.3 Phenotypic segregation of F_2 families to 870 g ae ha^{-1} glyphosate 3 WAT.

Family	Alive (R)	Dead (S)	Total	χ^2
SxR #1	5	2	6	0.222
SxR #2	11	8	19	2.965
SxR #5	4	2	6	0.222
SxR #8	17	2	19	2.123
RxS #3	16	5	21	0.016
RxS #9	5	1	6	0.222
RxS #12	14	3	17	0.490
RxS #13	13	8	21	1.921
All F_2 Plants	85	30	115	0.072

Chi square goodness of fit (df = 1, $\alpha = 0.05$) testing for 3:1 (R:S) segregation with a critical value $\chi^2 = 3.84$. A calculated $\chi^2 < 3.84$ indiciates the observed segregation is in accoradance with the expected segregation (3:1).

Table 2.4 Phenotypic segregation of F_2 progeny 3 WAT to 870 g ae ha^{-1}.

	Alive (R)	Dead (S)	Total	χ^2
F_2 Plants (SxR)	37	13	50	0.027
F_2 Plants (RxS)	48	17	65	0.046
Pooled F_2 Plants	85	30	115	0.072

Chi square goodness of fit (df = 1, $\alpha = 0.05$) testing for 3:1 (R:S) segregation with a critical value $\chi^2 = 3.84$. A calculated $\chi^2 < 3.84$ indiciates the observed segregation is in accoradance with the expected segregation (3:1).

Table 2.5 Phenotypic response of F_2 progeny to various rates of glyphosate 3 WAT.

Glyphosate Dose (g ae ha^{-1})	F_2 Progeny Response			
	Alive (R)	Dead (S)	Total	$\chi 2$
0	4	0	4	1.333
218	3	1	4	0.000
435	2	2	4	1.333
870	2	2	4	1.333
1740	9	3	12	0.000
2610	7	5	12	1.778
3480	10	6	16	1.333
Pooled	37	19	56	2.381

Chi square goodness of fit (df = 1, $\alpha = 0.05$) testing for 3:1 (R:S) segregation with a critical value $\chi^2 = 3.84$. A calculated $\chi^2 < 3.84$ indiciates the observed segregation is in accoradance with the expected segregation (3:1).

Table 2.6 Chi-square analysis of relative *EPSPS* gene copy number segregation in the F2 progeny.

	Plante with 3 or more *EPSPS* Gene Copies (R)	Plants with One *EPSPS* Gene Copy (S)	Total	χ^2
F_2 Plants (SxR)	11	4	15	0.022
F_2 Plants (RxS)	29	7	36	0.592
Pooled F_2 Plants	40	11	51	0.320

Chi square goodness of fit (df = 1, $\alpha = 0.05$) testing for 3R:1S segregation based on *EPSPS* gene copy number with a critical value $\chi^2 = 3.84$. A calculated $\chi^2 < 3.84$ indiciates the observed segregation is in accoradance with the expected segregation (3:1).