Relative ecological fitness of glyphosate-resistant kochia from western Kansas

by

Omobolanle Adewale Osipitan

B.S., University of Agriculture, Abeokuta, Nigeria, 2006

M.S., Federal University of Agriculture, Abeokuta, Nigeria, 2011

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Agronomy

College of Agriculture

KANSAS STATE UNIVERSITY

Manhattan, Kansas

2016

Abstract

Kochia (*Kochia scoparia* L. Schrad.), one of the most problematic weeds in the Great Plains of United States, has evolved resistance to some herbicides including glyphosate (5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitor) which was first reported in western Kansas in 2007. The objectives of this research were to (1) characterize six kochia populations from western Kansas on the basis of glyphosate resistance or multiple herbicide resistance, (2) determine germination characteristics of the populations and evaluate their growth and fecundity in the field, and (3) determine if EPSPS gene amplification responsible for glyphosate resistance in kochia was associated with growth and fecundity cost in the plants. Six kochia populations were from Scott, Finney, Thomas, Phillips, Wallace, and Wichita counties. Based on shikimate assay for glyphosate and recommended field rates for four tested herbicides, three kochia populations (Scott (SC-R), Finney (FN-R), and Thomas (TH-R)) were grouped into glyphosate-resistant (GR) and three populations (Phillips (PH-S), Wallace (WA-S) and Wichita (WI-S)) were grouped into glyphosate-susceptible (GS). All populations were resistant to dicamba (synthetic auxin) and chlorsulfuron (acetolactate synthase inhibitor), however, atrazine (PS II inhibitor) resistance in FN-R was noted as exceptional among the GR populations. Across the three germination temperatures (5, 10 and 15 C), the GR populations consistently had less total cumulative germination and at 15 C, they consistently required more time to attain 50% of maximum cumulative germination than the GS populations. Both the field study and evaluation of relationship between EPSPS gene amplification and plant performance showed that differences in plant height, biomass accumulation and fecundity among populations were not in respect to glyphosate resistance but rather, differences in their inherent ability to grow and produce seeds in the presence or absence of neighbors. This research suggests that fitness

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Major Professor
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Acknowledgements

My profound gratitude goes to my Major Supervisor, Prof. J. Anita Dille for giving me this opportunity to further my study in Weed Science, her immense support, encouragement, and productive criticisms throughout the various stages of this research and my program. I would like to let you know that every bit of your contribution towards completion of this program are highly appreciated. I acknowledge with thanks the contributions of the members of my supervisory committee; Profs. Phillip Stahlman, David Hartnett and Allan Fritz for their sacrifices, advise and material support. Thanks to USDA-NIFA-Foundational Grant Program for providing funds for my research. I would also like to thank Mr. Russell Dille, Kevin Ascher and Cathy Minihan for their significant contributions to the completion of the field trials. Great thanks to Dr. Mithila Jugulam for allowing me to use her weed physiology laboratory during my research. I acknowledge with thanks Dr. Paul St. Amand of USDA CGCC laboratory for his help with genomic DNA extraction and gel quantification. Also appreciated is the assistance of Amar Godar for providing me enormous support necessary at various stages of this study. My sincere gratitude also goes to Dr. Timothy Todd and Yared Assefa for their suggestions and assistance in the data analysis. Thanks to Vijay Varanasi, Aruna Varanasi, Andrew Esser, Kindsey Niehues, George Mahama, Vuyiswa Bushula, Gared Shaffer, Chelsea Ahlquist, Samida Khadka, Edwin Akley, Iddirisu Yahaya, Kayode Ajewole and all my weed science folks for their inputs in different forms toward the completion of my PhD program. I greatly appreciate the moral support of my mother, Alhaja Nurat Osipitan. May God grant you long life and prosperity. Also appreciated is my wife, Azeezat Kikelomo for her love, understanding and support throughout my PhD program. Gratitude to my elder brothers Dr. Leke Osipitan and Engr. Waahid Kolawole

as well as other members of my family. To everyone, the best reward for good is good. Thank you all and God bless you.

Chapter 1 - Literature Review

Biology, Ecology and Management of Kochia

Kochia (*Kochia scoparia* (L.) Schrad.), a summer annual erect C_4 weed of the Chenopodiaceae family is native to central and eastern Europe and Asia, and was likely introduced to North America as an ornamental in the mid to late 1800's; it escaped from cultivation and spread throughout western US and Canada during the 1930's (Friesen et al. 2009; Scoggan 1957). Kochia is grown as an ornamental due to its dense and conical shape as well as its bright red color in fall. A review from 1880 to 1980 showed that kochia had remarkable adaptations and it had the highest rate of spread among invasive species in the western US (Forcella 1985). Currently, kochia occurs throughout most of the states in the US and the range of kochia is expanding northward in Canada (Friesen et al. 2009). High genetic diversity in kochia (both between and within populations) is maintained through substantial levels of gene flow irrespective of evolutionary processes such as selection pressure (Mengistu and Messersmith 2002). Kochia is particularly adapted to arid and semi-arid regions but can be found in a wide range of climatic regions throughout the world (Friesen et al. 2009).

Kochia produces protogynous flowers where the stigmas emerge before anther development. The stigmas usually emerge few days before pollen is shed, are receptive to foreign pollen during that time, and deteriorate before anther dehiscence which prevents self-pollination within the same flower (Stallings et al. 1995). This mechanism necessitates outcrossing. Kochia pollen can travel 30 to 50 m and remain viable for 1 to 12 days depending on the environmental conditions (Stallings et al. 1995). Kochia may commence flowering between July to September depending on the biotype and photoperiod (Eberlein and Fore 1984). Flowers are either perfect with 3 to 5 stamens or imperfect by being pistillate, but both types have two stigmas (Friesen et al. 2009). Kochia has utricle fruits with an oval, brown to black seed. It has been estimated that an isolated kochia plant can produce as many as 330,000 seeds (Esser 2014). Reports also showed that more viable seeds were found on lower parts of the plant than upper parts (Esser 2014). This may be attributed to more reception of pollen by the stigmas on the lower parts of the plant than those on the upper of the plant. In the presence of wind, the dried kochia plant severs from the root system and tumbles across the landscape spreading seed wherever it rolls (Becker 1978). Forcella (1985) reported that kochia was one of the fastest spreading weeds in the United States and Canada, and this was largely attributed to its tumbling method of seed dispersal.

Longevity of kochia seed in the soil is averaged at 1.5 years as seed viability can decline to 1% after 3 years (Zorner et al. 1984). Kochia commences germination in early spring and germination events can occur multiple times till the late summer (Dille et al. 2012). At the end of the season, kochia is capable of germinating more than 90% of its total viable seed in the seedbank (Schwinghamer and Van Acker 2008). However, emergence significantly decreases with increase in soil depth because of small seed size. Seedlings were not able to emerge from a depth greater than 80 mm (Schwinghamer and Van Acker 2008).

The competitive growth habit and high seed production promote the persistence of kochia (Friesen et al. 2009). Kochia is considered as one of the most problematic weeds in cultivated fields including soybean (*Glycine max*), sorghum (*Sorghum bicolor*), corn (*Zea mays*), wheat (*Triticum spp.*) and sunflower (*Helianthus annuus*) (Kumar and Jha 2015; Wolf et al. 2000). Kochia interference in a crop can reduce grain yield and quality (Manthey et al. 1996) and obstruct mechanical harvest. For example, kochia competition reduced sunflower yield by 76% (Lewis and Gulden 2014), competition from 70 kochia plants m^{-2} of row decreased wheat yield by 58% (Friesen et al. 1991), and density of 1.5 kochia plants m^{-1} of row reduced sugarbeet (*Beta vulgaris*) yield by 64% (Mesbah et al. 1994).

As reviewed by Waite (2010), tillage is one of the most effective practices for kochia control. Tillage operation before crop planting can remove most kochia that would emerge throughout the growing season. Herbicides have become the preferred method for weed control due to wide adoption of no-till systems. Several soil-applied herbicides applied before or at planting may effectively control kochia (Kumar and Jha 2015; Tonks and Westra 1997; Wicks et al.1993). In many cropping systems, it is common to control kochia before planting by using POST nonselective herbicide such as glyphosate (Donald and Prato 1991). Glyphosate is also used as a POST selective herbicide in resistant crops such as soybean and corn (Kumar and Jha 2015; Wolf et al. 2000). However, the conventional methods of using glyphosate as a selective herbicide may no longer be successful due to occurrence of glyphosate-resistant kochia.

Herbicide Resistance in Weeds

Continuous use of an herbicide mode of action over a long period of time may result in selection pressure that evolves populations of weed species with herbicide resistant traits. Over the last 45 years, herbicide resistance has increased rapidly worldwide. The first cases of evolved herbicide resistance were to one of the first commercialized synthetic herbicides, 2,4-D. Wild carrot (*Daucus carota*) and spreading dayflower (*Commelina diffusa*) were both reported to have evolved resistance to 2,4-D in 1957, around 10 years after use of 2,4-D had become widespread (Heap 2016). Because few synthetic herbicides were available during the early days of 2,4-D use, it was widely used for control of broadleaf weeds. The next herbicide mode of action group to have weeds evolve resistance to it was that of photosystem II (PS II) inhibitors. The first case of evolved herbicide resistance was not confirmed until 1970 when simazine-resistant common groundsel (*Senecio vulgaris*) was reported (Ryan 1970). After this, occurrence of other PS II inhibitor-resistant weed species began to increase rapidly. Resistance has been reported in about 200 plant species and in 22 of the 25 herbicide mode of action groups (Heap 2016). About 158 weed species have developed resistance to acetolactate synthase (ALS) inhibitor herbicides alone. Synthetic auxins and glyphosate each have about 32 weed species that have developed resistance (Heap 2016). A relatively newer herbicide mode of action, hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitor, currently has the fewest number of herbicide resistance cases with only two reported (Heap 2016).

Mechanisms of herbicide resistance in weed populations can be target site- or non-target site-based. Herbicides have distinct target protein sites where they act to disrupt biochemical processes leading to plant death. In the case of target site-based resistance, as herbicide arrives at the target site in the plant, there is less effect either due to an alteration in the target enzyme affinity for the herbicide or a greater number of target sites, thereby allowing the normal metabolic process in plant to occur (Friesen et al. 1996; Holt et al. 1993).

Herbicide must be translocated to the site of action in order to be phytotoxic, otherwise the herbicide will be ineffective. Non-target site-based resistance mechanisms reduce the amount of herbicide getting to the target site. These include reduced uptake and/or translocation, sequestration into parts of the plant with little or no target site, and enhanced metabolism of the herbicide molecule to an inactive molecule (Sammons and Gaines 2014). Weeds can also exhibit cross and multiple resistance to herbicides. Cross resistance occurs when a single resistance mechanism confers resistance to several herbicides of the same mode of action. Multiple resistance occurs when two or more resistance mechanisms evolved in a plant to more than one mode of action herbicide, often due to sequential selection by different herbicide modes of action (Heap et al. 2001).

Herbicide Resistance in Kochia

Kochia has been reported to have developed resistance to four herbicide modes of action: PS II-inhibitors (Foes et al. 1999; Peterson 1999), ALS- inhibitors (Primiani et al. 1990), synthetic auxins (Bell et al. 1972; Cranston et al. 2001) and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitor (Heap 2016). A contributing factor for selection of herbicide-resistant individuals in kochia is the high genetic diversity among and within populations (Mengistu and Messersmith 2002) which are usually facilitated by wind-mediated pollen movement and outcrossing (Guttieri et al. 1998). Varanasi et al. (2015) reported a case of a single kochia population with multiple resistance to these four modes of action, namely glyphosate (EPSPS inhibitor), atrazine (PS II inhibitor), chlorsulfuron (ALS inhibitor) and dicamba (synthetic auxin) herbicides.

Photosystem II inhibitor resistance was the first reported case of resistance in kochia and this was in a corn field in 1976 (Heap 2016). The most common mechanism of triazine resistance is a mutation in quinone B (QB) binding domain of D1 protein in PS II, thereby reduces efficiency of PS II (Gronwald 1997; Varanasi et al. 2015). Up to now, at least 100 plant species have developed resistance to triazine (Heap 2016).

Kochia was one of the first weed species to develop resistance to ALS-inhibiting herbicides (Primiani et al. 1990). The first cases of kochia resistance to an ALS-inhibitor (chlorsulfuron) was reported from cropland and in a wheat field in the US in 1987 (Primiani et al. 1990; Saari et al. 1990; Thompson et al. 1994). Target-site mutation of the ALS gene has been widely reported to be responsible for plant resistance to ALS-inhibiting herbicides. The most common target-site mutations in ALS gene sequences are Pro197 and Trp574 (Heap 2016). Target-site mutations at Pro197 with Thr, Ser, Arg, Leu, Glu, and Ala substitutions were reported for chlorsulfuron-resistant kochia populations from Kansas (Guttieri et al. 1992, 1995). A Trp574Leu mutation of the ALS gene was identified in ALS-inhibitor–resistant populations from Illinois (Foes et al. 1999) and the Czech Republic (Salava et al. 2004).

In 1994, populations of kochia not controlled by field use rates of dicamba (70 g ha^{-1}) were discovered in a corn field in Nebraska and in wheat fields in Montana and since then, producer reports of poor kochia control with dicamba have increased at a slow but steady pace (Cranston et al. 2001). Up to now, at least 25 plant species have developed resistance to synthetic auxin herbicides around the world (Heap 2016). The mechanisms of weed resistance to synthetic auxins are still being investigated. However, kochia resistance to dicamba in a Montana population was not due to differential absorption, translocation, or metabolism (Cranston et al. 2001). More recently, in 2007, glyphosate-resistant (GR) kochia was confirmed in Kansas in multiple cropping systems such as corn, cotton, and soybeans (Godar et al. 2015; Waite et al. 2013).

Glyphosate Resistance in Weeds and Kochia

Glyphosate resistance has been reported in at least 32 weed species and the mechanism of glyphosate resistance is still being revealed. Major reported mechanisms of GR were reduced translocation in johnsongrass [*Sorghum halepense* (Vila-Aiub et al. 2012)], giant ragweed [*Ambrosia trifida* (Robertson 2010)], and ryegrass [*Lolium rigidum* (Wakelin and Preston 2006)]; sequestration in horseweed [*Conyza canadensis* (Ge et al. 2010)], and ryegrass (Ge et al. 2012)]; target –site-based EPSPS pro106 amino acid mutation in goosegrass [*Eleucine indica*

(Yu et al. 2015)], ryegrass (Wakelin and Preston 2006)], and tall waterhemp [*Amaranthus tuberculatus* (Nandula et al. 2013)]; and EPSPS gene amplification in Palmer amaranth [*Amaranthus palmeri* (Gaines et al. 2010)], tall waterhemp (Tranel et al. 2011), and kochia (Jugulam et al. 2014).

Worldwide, GR kochia was first reported in western Kansas in 2007, and kochia plants were selected primarily in GR corn, cotton and soybean fields (Heap 2016). Field-evolved resistance of kochia to glyphosate is due to amplification of one of two EPSPS alleles (genes) with no effects on other pathway genes (Jugulam et al. 2014; Wiersma et al. 2015). When a GR kochia plant amplifies the number of EPSPS genes, it leads to overproduction of EPSPS enzyme, such that even in the presence of glyphosate there is an abundant supply of uninhibited EPSPS enzyme (Wiersma et al. 2015). In a GR kochia plant, there can be 3 to 10 times more functional copies of the gene encoding EPSPS (Gaines et al. 2016), and the more copies of the EPSPS gene in kochia, the greater its resistance to glyphosate (Godar et al. 2015; Jugulam et al. 2014). Amplified EPSPS genes in GR kochia are in tandem arrangement on one chromosome in which segregation of EPSPS copies followed single locus inheritance (Jugulam et al. 2014). This is different from GR Palmer amaranth in which relatively greater amplification of the EPSPS genes (>120 copies) were randomly dispersed throughout the genome (Gaines et al. 2010), and likely mediated by transposable elements (Gaines et al. 2013). The inheritance of EPSPS gene amplification is more complex than most herbicide resistance mechanisms (Sammons and Gaines 2014). Inheritance of EPSPS gene copy appeared to not be stable and followed non-Mendelian patterns (Giacomini et al. 2013; Jugulam et al. 2014).

Fitness Cost of Herbicide Resistance

The relative fitness of resistant and susceptible genotypes has a powerful influence on the population dynamics of mixed resistant and susceptible plant populations (Gressel and Segel 1982). Under normal field conditions, resistant and susceptible plants compete with each other and the crop. Since many mutations in plants have deleterious, pleiotropic effects on its likelihood of survival and/or reproduction (Roles and Conner 2008), it is generally assumed that herbicide resistance mutations will be associated with an initial cost to a plant's fitness (Vila-Aiub et al. 2015). The assumption that resistant plants are less fit than susceptible plants in the absence of herbicide application is mostly based on early studies of triazine resistance. These studies indicated a marked reduction in the reproductive success of triazine-resistant biotypes relative to susceptible biotypes of most weed species in the absence of triazine herbicide application (as reviewed by Jasieniuk et al. 1996). The most common mechanism for triazine resistance, mutation in the quinone B (QB) binding domain of D1 protein in PS II, decreases the binding affinity for triazine molecules to the protein (Devine and Shukla 2000). This mutation reduces efficiency of PS II, which results in reduced plant fitness. Although, fitness cost has been widely detected in triazine-resistant plants, this cannot be generalized for resistance to other herbicide modes of action. No fitness cost has been observed in GR populations of Palmer amaranth (Giacomini et al. 2014; Vila-Aiub et al. 2014), horseweed (Shrestha et al. 2010; Davis et al. 2009), asthmaweed (*Conyza bonariensis*) (Travlos and Chachalis 2013), ryegrass (Pedersen et al. 2007) or kochia (Kumar and Jha 2015). In a few cases, there were reports of fitness cost such as less seed germination (Debban et al. 2015) and reduced seed quality (Van Etten et al. 2016) in tall morningglory (*Ipomoea purpurea*) and decreasing frequency of GR phenotypes over time in a segregating ryegrass population (Preston and Wakelin 2009).

Accurate quantitative estimates of the relative fitness of resistant and susceptible plants in the absence of herbicide have been difficult to obtain (Jasieniuk et al. 1996; Van Etten et al. 2016). A more appropriate comparison to estimate differential fitness of resistant and susceptible plants is to compare plants within the same weed population (Pedersen et al. 2007) or use of isogenic lines (Vila-Aiub et al. 2015). However, studies have generally compared the fitness of resistant and susceptible plants from very different populations (Stowe and Holt 1988; Holt 1990; Jacobs et al. 1988; Shrestha et al. 2010; Thompson et al. 1994; Van Etten et al. 2016), and of these, some compared any resistant and any susceptible population (Marshall et al. 1993; Mortimer et al. 1992; Shrestha et al. 2010). To control confounding effects of plant response to resistance or susceptible traits and genetic background resulting from environmental experience, care must be taken to select populations from the same geographic area and growing conditions (Jasieniuk et al. 1996), and several resistant and susceptible populations should be compared (Cousens et al. 1997; Strauss et al. 2002; Van Etten et al. 2016). A comparative study of one resistant and one susceptible population is unlikely to be truly representative.

Measuring fitness throughout a plant's life cycle has also been recommended (Van Etten et al. 2016; Vila-Aiub et al. 2015). Examining a single growth stage (seed germination, emergence rate, growth rate, phenology, or seed production) of the life cycle may not be sufficient to identify differential fitness for resistance traits. Studies that examine multiple stages are more likely to detect fitness differences between herbicide-resistant and -susceptible populations (Vila-Aiub et al. 2015).

Research Direction

Fitness trade-off as a result of evolution of glyphosate resistance in kochia populations from western Kansas will be evaluated at three life cycle stages: germination, growth, and seed production. Six kochia populations (three suspected GR and three expected GS) will be used, with five collected in 2012 and one in 2007. Research will be conducted in a greenhouse to characterize the populations through assay of their resistance to label rate of glyphosate and to increased rates as discriminatory doses. Populations will also be assayed for resistance to multiple herbicide modes of action, *viz-a-viz*, a PS II inhibitor (atrazine), an ALS-inhibitor (chlorsulfuron), and a synthetic auxin (dicamba). The reason to assess the multiple herbicide resistance of these populations will be to ensure that any fitness cost (or perhaps benefit) in the kochia population will not be confounding or based on resistance to an herbicide mode of action other than EPSPS inhibition (glyphosate). A molecular assay, i*n vivo* shikimate accumulation in leaf disks of representative plants from the kochia populations will be conducted to complement results from the glyphosate discriminatory dose assay. Also, a germination study will be conducted in growth chambers to evaluate the germination dynamics of these populations at three different temperatures (5, 10 and 15 C).

A field study will be conducted to determine differential ecological fitness between the GR and GS kochia populations in respect to their growth and fecundity under a competition gradient using a target plant-neighborhood design. The influence of intra- (kochia) and inter- (corn) specific neighbors on the kochia target populations will also be evaluated, as in reality, kochia plants are not expected to occur in isolation in their natural environment. Appropriate descriptive variable of plant growth, such as growing degree days (GDD), will be explored to explain increase in plant height and stem diameter of the kochia populations over time.

The relationship between the genetic basis of glyphosate resistance (EPSPS gene amplification) in these kochia populations and the populations' fitness variables (growth and seed production) will be evaluated to determine the fitness cost or benefit associated with glyphosate resistance. Target plants from the field study were cloned in the greenhouse to measure EPSPS gene amplification. Corresponding fitness variables from each target plant will be correlated to their level of gene amplification.

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Chapter 2 - Characterization and Seed Germination of Kochia Populations from Western Kansas

Abstract

A greenhouse study evaluated single and multiple herbicide resistance in six kochia populations from western Kansas to glyphosate (EPSPS inhibitor), atrazine (PS II inhibitor), chlorsulfuron (ALS inhibitor) and dicamba (synthetic auxin). Kochia seed were collected from suspected glyphosate-resistant (-R) and –susceptible (-S) populations in Scott, Finney, Phillips, Wallace and Wichita Counties in 2012 and from Thomas County in 2007. The germination dynamics of these populations were evaluated at three constant temperatures of 5, 10 and 15 C. Three populations (Scott (SC-R), Finney (FN-R), and Thomas (TH-R)) were confirmed to be resistant to a field use rate (0.84 kg ae ha^{-1}) of glyphosate while about 12 to 30% of the individuals showed resistance to double the field use rate. Populations from Phillips (PH-S), Wallace (WA-S) and Wichita (WI-S) Counties were susceptible to the field use rate of glyphosate. Five populations were atrazine-susceptible while 90% of the FN-R individuals were resistant to atrazine. At least 38% of individuals in each population were resistant to dicamba and chlorsulfuron. An i*n vivo* shikimate accumulation assay showed that the SC-R, FN-R and TH-R accumulated less shikimate compared to PH-S, WA-S and WI-S by 16 h after incubation. Maximum cumulative germination for all populations increased as temperature increased from 5 to 15 C, while time required to reach 50% of maximum germination was shorter as temperature increased. The GR populations consistently had less total cumulative germination across the three temperatures and at 15 C, took more time to attain 50% of maximum cumulative germination than the GS populations. The GS population would have a competitive advantage

for limited resources through early and greater emergence by occupying the biological space before GR seedlings could emerge, in the absence of glyphosate application.

Introduction

Kochia [*Kochia scoparia* (L.) Schrad.] of the Chenopodiaceae family is a summer annual erect forb. Kochia can be found in arid and semi-arid regions; these include but not limited to Great Plains region of the United States (Friesen et al. 2009). It is self-compatible and produces protogynous flowers where the stigmas emerge before anther development. The stigmas usually emerge about one week before pollen is shed, are receptive to foreign pollen during that time, and deteriorate before anther dehiscence which prevents self-pollination within the same flower (Stallings et al. 1995). This mechanism necessitates outcrossing and promotes genetic diversity among species. It was estimated that an isolated kochia plant can produce as many as 330,000 seed (Esser 2014). In the presence of wind, the dried kochia plant severs from the root system and tumbles across the landscape spreading seed wherever it rolls (Becker 1978). Forcella (1985) reported that kochia was one of the fastest spreading weeds in the United States and Canada, and this was largely attributed to its tumbling method of seed dispersal. Longevity of kochia seeds in the soil is averaged at 1.5 years (Schwinghamer and Van Acker 2008) as seed viability can decline to 1% after 3 years (Zorner et al. 1984). Viable kochia seeds germinate rapidly under favorable environmental conditions (Zorner et al. 1984). The temperature for rapid and highest total germination of kochia seed is between 15 and 25 C (Romo and Haferkam 1987; Sbatella and Wilson 2010). Seeds are able to germinate under high stress conditions, such as very low soil moisture (Romo and Haferkamp 1987), high salinity up to 1000 mM NaCl (Khan et al. 2001), and at temperatures as low as 5 C (Dille et al. 2012; Sbatella and Wilson 2010). Germination of kochia seed was not affected by alternating versus constant temperatures and light was not required as the percentage of kochia seeds that germinate in constant darkness was not different from those exposed to light (Romo and Haferkam 1987; Thompson et al. 1994). Kochia exhibits early germination in the spring (as early as March in western Kansas) and germination can occur multiple times (flushes) till the late summer, enabling it to take advantage of the available moisture (Dille et al. 2012). About 70 to 90% of seedlings emerged within the first 2 weeks of favorable conditions while a few still emerged into summer (Dille et al. 2012). From spring to late summer, the percentage cumulative germination of kochia can be more than 90% of its total viable seeds found in the shallow seedbank (Schwinghamer and Van Acker 2008).

Introduction of glyphosate-resistant (GR) crops in the mid-1990s has significantly increased the use of glyphosate as a post-emergence (POST) herbicide after planting in no-till cropping systems. This introduction has led to exceptional adoption of glyphosate by growers leading to total use of approximately 825 million kg worldwide in 2014 from about 406 million kg 10 years before (Benbrook 2016). Consequently, due to intensive and repeated use of glyphosate for weed control, the evolution of GR kochia through selection pressure has occurred. The first cases of GR kochia were reported in western Kansas in 2007 (Godar 2014, Waite et al. 2013). In a survey of western Kansas crop consultants conducted in 2012, almost all respondents reported presence of GR kochia in at least a few fields, with a majority reporting GR kochia in more than 20% of the fields (Godar 2014).

Kochia was identified to have developed resistance to other herbicides much earlier to discovering its resistance to glyphosate (Heap 2016). These include resistance to photosystem II (PS II) inhibitors (Foes et al. 1999; Peterson 1999), acetolactate synthase (ALS) inhibitors (Primiani et al. 1990), and synthetic auxins (Bell et al. 1972; Cranston et al. 2001). A contributing factor for selection of herbicide-resistant individuals in kochia is the high genetic diversity among and within populations (Mengistu and Messersmith 2002) facilitated by wind-mediated pollen movement and outcrossing (Guttieri et al. 1998). Varanasi et al. (2015) reported a kochia population with multiple resistance to glyphosate (EPSPS inhibitor), atrazine (PS II inhibitor), chlorsulfuron (ALS inhibitor) and dicamba (synthetic auxin). This suggests the importance of evaluating resistance of biotypes to multiple herbicide modes of action in a study aimed at assessing the biological cost of resistance evolution to a particular herbicide. This will help to rightly attribute a likely biological trade-off or benefit (if at all) to the appropriate mode of action resistance.

Herbicide selection pressure can also influence selection for adaptive traits for a given environmental condition (Sbatella and Wilson 2010). The influence of temperature on rate of weed seed germination has been widely explored to explain the differences between herbicide-resistant and -susceptible weed biotypes (Elahifard and Mijani 2014; Park et al. 2004; Tang et al. 2015; Thompson et al. 1994; Vila-Aiub et al. 2005). For example, ALS-inhibitor resistant biotypes of kochia (Dyer et al. 1993; Thompson et al. 1994), downy brome [*Bromus tectorum* (Park et al. 2004)] and wild mustard [*Sinapis arvensis* (Topuz et al. 2015)] achieved greater total germination and reached 50% germination earlier than susceptible biotypes at suboptimum temperatures. However, in the case of triazine resistance, Mapplebeck et al. (1982) reported that the atrazine-resistant biotype of field mustard (*Brassica campestris*) could only achieve 25% of total germination compared to susceptible biotype. Triazine resistance is widely known to have a negative impact on plant growth and survival (Bergelson and Purrington 1996; Holt and Thill 1994; Jansen and Pfister 1990). Similarly, evaluation of seed germination in acetyl-CoA carboxylase (ACCase) resistant weed biotypes (Vila-Aiub et al. 2005 in *Lolium rigidum;* Tang et al. 2015 in *Polypogon fugax)* showed that resistant individuals consistently had lower seed germination at suboptimal temperature and required more time to reach 50% germination than the susceptible individuals. These studies suggest that the biological implication for resistance to herbicide depends on (but not limited to) the herbicide mode of action and plant species.

Understanding germination characteristics can help determine when and how herbicide-resistant and -susceptible kochia establish. Also, understanding the germination potential of herbicide-resistant and -susceptible individuals in a weed population will help determine or predict the persistence of the subpopulations (resistant or susceptible) for future generations.

The objectives of the study were (1) to screen six kochia populations from western Kansas for glyphosate (EPSPS inhibitor) resistance at field use rate and double field use rate, as well as screen for multiple herbicide resistance to atrazine (PS II inhibitor), dicamba (synthetic auxin) and chlorsulfuron (ALS inhibitor) at field use rates and (2) to evaluate the germination characteristics of these six kochia populations in response to three constant temperatures.

Materials and Methods

Six kochia populations were collected from different fields in western Kansas. Kochia seed was collected from one population in Thomas County in 2007, and seeds from five populations were collected in 2012, including suspected GR populations in Scott and Finney Counties and suspected GS populations from Phillips, Wallace and Wichita Counties (Figure 2.1). Seed from most fields were harvested from more than 10 mature plants per site and bulked in separate bags for each population. Seeds were stripped off plants and cleaned using an air column separator. To minimize loss of seed quality, the collected seeds were stored in cold (≤ 2

C) until herbicide screening in spring 2014 and kept at room temperature about a year before germination study in fall 2015 and spring 2016.

Herbicide Resistance Screening.

Seeds of each kochia population were germinated in 28 by 6 by 8 cm flats filled with moisture control potting mix (Miracle Gro, Marysville, OH) and grown in the Department of Agronomy - Weed Science greenhouse at Kansas State University, Manhattan, KS. The greenhouse was maintained at 25/20 C day/night, photoperiod of 15/9 h day/night enhanced with 120 µmol m^{-2} s^{-1} illumination provided by sodium vapor lamps, and about 60% relative humidity. Once 2 to 3 cm tall, seedlings were transplanted into 8.5 by 8.5 by 7 cm plastic pots filled with the same potting mix. Five different herbicide treatments were applied to individual plants when they reached 8 to 10 cm in height, including glyphosate (Roundup WeatherMax, Monsanto Company, St. Louis, MO) at a field use rate (1X, 0.84 kg ae ha^{-1}) and double the field use rate (2X, 1.68 kg ae ha^{-1}) along with 2% v/v liquid ammonium sulfate. Other treatments included field use rate (1X) of atrazine (Aatrex 4L, Syngenta Crop Protection Inc., Houston, TX) (2.24 kg ai ha^{-1}) plus 1% (v/v) crop oil concentrate, dicamba (Clarity, BASF Corporation, 26 Davis Drive, NC) (0.56 kg ae ha^{-1}) plus 0.25% (v/v) non-ionic surfactant (NIS), and chlorsulfuron (Glean XP, DuPont, Company, Wilmington, DE) (0.018 kg ai ha^{-1}) plus 0.25% (v/v) NIS. Herbicides were applied in a cabinet spray chamber (Research Track Sprayer, De Vries Manufacturing, P.O. Box 184, Hollandale, MN). The spray chamber had a flat-fan nozzle tip (80015LP TeeJet tip, Spraying Systems Co., P.O. Box 7900, Wheaton, IL) that delivered a spray volume of 187 L ha^{-1} using a pressure of 138 kpa with a speed of 3.5 kmph. Herbicide treatments were replicated 10 and 7 times for first and second runs, respectively. There were also five untreated plants for each population as controls in each run. The study was conducted twice, laid out in complete randomized design on experimental benches in the greenhouse.

Plant injury score, survival, and dry weight of plants were recorded at 4 wk after herbicide treatment. Plant injury score was on the scale of 1 to 9, where 1 represented no injury and 9 represented completely injured or dead plants. Survival was measured as percentage of living plants compared to total plants sprayed. To determine the dry weight, plants were cut at ground level, bagged individually and placed in the oven at 60 C for 72 h, then weighed. Injury score and shoot dry weight response of populations to herbicide treatment were tested with a one-way ANOVA in R version 3.2.3 (R Core Team 2015) and Tukey's Honestly Significant Difference (α= 5%) was used to compare injury score and shoot dry weight means among populations.

Shikimate Accumulation Assay.

The six kochia populations were assessed for shikimate accumulation to further validate the characterization of GR and GS populations. In plants, glyphosate inhibits production of the aromatic amino acids (tryptophan, phenylalanine and tyrosine) in the shikimic acid pathway causing a build-up of shikimate-3-phosphate, a substrate of EPSPS and its dephosphorylated state, shikimate (Shaner et al. 2005). Both GR and GS plants are expected to accumulate shikimate after exposure to glyphosate but the levels are much higher in GS plants (Wiersma et al. 2015). A measure of shikimate accumulation can be determined following the procedure developed by Shaner et al. (2005) and modified by Godar (2014). Plants of each population were grown in the greenhouse in a similar method and condition described above. Ten representative plants that were10 cm tall were selected from each population. Four 5-mm leaf disks were collected from two young fully-expanded leaves from each plant. Three leaf disks (as three technical replicates) from each plant were placed in transparent 96-well microtiter plates containing 100 µL of 100 µM of glyphosate (Glyphosate PESTANAL®, analytical standard, Sigma-Aldrich Co. LLC, St. Louis, MO) solution to differentiate the shikimate accumulation between GR and GS kochia plants. One leaf disk was placed in 100 µL buffer without glyphosate as non-treated control. The plates were immediately wrapped with clear plastic to reduce evaporation and incubated at room temperature for 16 h under continuous light (200 µmol m-^{2} s^{-1}). The plates were frozen at -20 C for 20 min and thawed at 60 C for 20 min. Twenty-five microliters of 1.25 N HCl was added to each well and plates were wrapped with clear plastic and incubated at 60 C until the entire leaf disk turned brown (approx. 20 to 25 min). Twenty-five microliters of aliquot from each well was transferred to corresponding wells in a new plate and was oxidized by adding 100 µL of reaction buffer (periodic acid (0.25% v/v)/sodium-m-periodate solution (0.25 v/v)) and incubated at 40 C for 20 min. After oxidation, the solution was quenched by adding 100 µL of 0.6 M NaOH/0.22 M Na_2SO_3 solution to each well. Optical density at a wavelength of 380 nm was measured within three to five minutes using a spectrophotometer (Epoch Micro-Volume Spectrophotometer System, BioTek, Winooski, VT. Values were converted to shikimate concentration (ng µL^{-1}) by using a standard curve generated from a known shikimate solution (Godar 2014).

The quantity of extracted shikimate was estimated by subtracting the concentration of shikimate in the treated wells from the concentration in their respective control wells. Shikimate accumulation in plants from the six kochia populations were analyzed using one-way ANOVA to test if there was difference among populations and the means were compared using Tukey's Honestly Significant Difference (α= 5%).

Germination Study.

Germination rates of the six kochia populations were evaluated at three constant temperatures of 5, 10, and 15 C in 24 h in darkened growth chambers, replicated three times, and the experiment was repeated. The temperature in each of the growth chambers was constantly monitored with an internal thermometer. Thirty seed from each population were placed in petri dishes (100 by 15 mm) containing one filter paper (Whatman No. 2) soaked with about 3 ml of distilled water and moistened with additional water as needed throughout the study. A seed was considered germinated when the radicle was about 2 mm long. Germination counts were done every 12 h for the first 144 h (6 d) and subsequent germination counts were done every 24 h for a total of 21 d. Germinated seeds were removed after each count. The initial 12 h observation interval was to capture the lag and rapid germination stages, while the 24 h observation interval would capture remaining germination. After the final germination count, un-germinated seeds in the petri dishes were re-wetted seven days later. The viability of the re-wetted seeds was tested through finger press for hardness after 78 h at 24 C (a modified procedure of Davis et al. 2005). Relatively hard seeds were considered viable while easily ruptured seeds were considered unviable. Both germinated seeds and hard seeds after wetting accounted for the total viable seeds.

A non-linear three parameter logistic regression model was used to describe germination dynamics fit in R version 3.2.3 (R Core Team 2015):

$$y = d / [1+(t/t_o)^b] \qquad [2.1]$$

where y is cumulative germination (%) at time t, d is maximum cumulative germination, t_o is time required to reach 50% of maximum cumulative germination, and b is slope of function around t_o. Analysis of variance was conducted to determine the effect of temperature on parameter estimates among populations and parameter estimates were ranked based on Tukey's Honestly Significant Difference (α= 5%) in R V 3.2.3 (R Core Team 2015).

Results and Discussion

Confirmation of Glyphosate and Multiple Herbicide Resistance.

The six populations from western Kansas were diverse in their response to glyphosate and other herbicide modes of action (Table 2.1). Based on visual assessment of survival at 4 wk after herbicide application, all kochia plants from Scott (SC-R) and Thomas (TH-R) populations survived and 77% of kochia plants from Finney (FN-R) population survived the field use rate of glyphosate (Table 2.1). One out of 17 plants from the Wichita County population survived while no plants survived from the Phillips and Wallace County populations at the field use rate of glyphosate. The average injury score corresponded to the survival rating for each of these populations (Table 2.1). Thus, it was confirmed that the kochia populations from Scott, Finney and Thomas Counties were identified as GR, while kochia populations from Phillips, Wallace, and Wichita Counties were identified as GS in response to the field use rate of glyphosate. When treated with double the field rate of glyphosate, all kochia plants from the three GS populations did not survive, while 12, 30, and 13% of kochia plants from the SC-R, FN-R and TH-R populations survived, respectively (Table 2.1). This suggests that the resistant populations were still segregating for glyphosate resistance. At field use rate of atrazine 90% of FN-R population survived, while no individuals in any of the other kochia populations survived (Table 2.1). This result was corroborated by analysis of injury score in which all individuals in all populations except FN-R were dead in response to atrazine (Table 2.1).

Kochia population response to other herbicide modes of action showed that at least 38% of individuals survived dicamba application and at least 59% survived chlorsulfuron in all populations (Table 2.1). The average injury score showed that the populations were equally injured by dicamba and chlorsulfuron except for FN-R which was not injured as severely by dicamba when compared to the other populations (Table 2.1).

Shoot dry weight of the treated plants relative to the untreated control showed that at 4 wk after glyphosate application at field use rate, individuals in the three GR populations had more dry weight than that of the GS populations (Table 2.2). Application of twice the field use rate of glyphosate further reduced shoot dry weight of all populations, yet the GS populations were more affected than GR populations. At 4 wk after atrazine application, the relative shoot dry weight of FN-R was greater when compared to other populations indicating its resistance to atrazine (Table 2.2). All populations' shoot dry weights were similarly reduced by dicamba and chlorsulfuron except for FN-R with higher relative shoot dry weight than most of the populations after application of dicamba.

Overall, these results confirm that three of the kochia populations were truly resistant to glyphosate (GR) including those from SC, FN and TH counties, while the other three populations from PH, WI, and WA counties were susceptible to glyphosate (GS). All populations have some level of resistance to dicamba and chlorsulfuron. Among the GR populations, FN-R was also confirmed to be atrazine resistant, and thus any assessment of biological cost of glyphosate resistance should also consider atrazine resistance in FN-R.

Shikimate Accumulation Assay.

The average shikimate accumulation in each GS population (43 ng μL^{-1}) was significantly greater than each of the GR populations (18 ng μL^{-1}) as shown in Figure 2.2. Generally, at a discriminating dose of 100 µM, it is expected that there will be differential shikimate accumulation between GR and GS individuals (Gaines et al. 2010; Shaner et al. 2005). The use of shikimate accumulation had been previously used as a rapid nondestructive method for characterization of GR and GS kochia biotypes (Godar et al. 2015; Jugulam et al. 2014; Kumar et al. 2015).

Germination Rate

The regression model (Equation 2.1) for each population fit the germination data as all parameter estimates were significantly different from zero ($P < 0.001$) on the basis of *t* tests (data not shown) and coefficient of determination (R^2) was sufficient (0.56 to 0.94) except when there was poor germination (Table 2.3). Maximum cumulative seed germination increased with increase in temperature from 5 to 15 C while time required to reach 50% of the maximum cumulative germination was shorter with increase in temperature for all populations (Figure 2.3, Table 2.3). None of the GR populations germinated at 5C, while the GS populations had maximum cumulative germination ranging from 4 to 16% of total seed or 6 to 19% of viable seeds (Figure 2.3A and D; Table 2.3). At 10 and 15 C, the three GR populations had greater maximum cumulative germination than the GS populations (Figure 2.3B, C, E and F; Table 2.3). The maximum germination in respect to percent of total seeds for PH-S was the least (44% for 10 C and 56.3% for 15 C) compared to other GS populations but the maximum germination was not different among the GS populations in respect to percent of viable seeds. At 5 C, the time required to reach 50% of the maximum cumulative germination could not be estimated for the GR populations due to very poor germination, however, among the GS populations there was no difference in required time to attain 50% germination. At 10 C, the difference in time required to reach 50% of maximum germination among the GR and GS populations were more obvious when germination characteristics was measured in respect to total viable seeds, and in this case, the GR populations needed more time to germinate. At 15 C, the GR populations consistently required more time to reach the maximum germination than the GS.

The influence of temperature on speed and level of germination of GR and GS kochia populations is important in agronomy, as it relates to cohort establishment (periodic emergence of plants of the same species per unit land area in a growing season) and optimum time of weed control. Since the GR populations have delayed germination at constant low temperatures, an early season management strategy with no effective supplemental weed control for subsequent cohorts will likely increase the proportion of GR individuals within a kochia population over time. Conversely, when both GR and GS are left uncontrolled, it is ecologically expected that species with early emergence would have competitive advantage over those with delayed emergence as rapid occupation of biological space by seedlings with an annual growth habit (in particular) is crucial to capture light and avoid shading from competitors (neighbors), and especially when soil resources are limited. The GR populations not only have delayed time to achieve 50% of their maximum germination but also possess lower ability to germinate at constant temperatures. In this study, the difference in total germination among GR and GS populations during the period of observation may be attributed to either differential seed viability or dormancy. It was observed that the GR populations had more nonviable seeds than the GS populations (data not shown). Noting that this study was conducted three years after the seeds were collected (except for TH-R which was collected earlier) suggests that the GR population seeds may lose viability sooner than GS populations. Preston and Wakelin (2008) reported that at the end of three years, there was an observable decrease in proportion of surviving GR individuals in seeds of a segregating population of ryegrass (*Lolium rigidum*) from a cross between GR and GS parents. Most recently, Van Etten et al. (2016) reported a negative correlation between glyphosate resistance and seed quality or seed germination in a study that examined 43 naturally occurring populations of tall morningglory (*Ipomoea purpurea*) that varied in their resistance to glyphosate. This was similar to an earlier report by Debban et al. (2015) that GR tall morningglory had more nonviable seeds than GS lines. The relatively less longevity or viability of GR seeds may not be unconnected with the postulation that metabolic energy cost associated with high or over production of EPSPS protein can be detrimental to other protein production since protein production or repair is known to be important to long term seed viability, particularly when seed are stored in dry state (Shen-Miller 2002).

Conclusion

These six kochia populations from western Kansas can be clearly grouped into GR and GS biotypes as all populations were resistant to a synthetic auxin and an ALS-inhibitor herbicide, however, PS II resistance in FN-R was noted as unique among the GR populations. This result suggests that it is important to evaluate existence of multiple herbicide resistance among several populations in a study aimed at assessing the biological cost of evolution of resistance to a particular herbicide. This will help to rightly attribute a likely biological trade-off or benefit (if at all) to the mode of action that the population is resistant to.

The differential germination speed observed between GR and GS populations is expected to give the GS population competitive advantage for limited resources through early seedling emergence with subsequent early canopy cover and root growth. The early canopy cover and root growth may reduce the growth and consequently seed production of GR individuals in the absence of glyphosate. Also, differential total germination under ideal temperature (15 C) is an indication of differences in seed viability. Consequently, in a segregating population or a seed mixture of GR and GS kochia individuals, the frequency of the GR to GS may decrease over time in the absence of glyphosate application. Thus, any weed management strategy that is timely to remove both early and later emerging kochia seedlings, as well as strategy that delays the germination of seeds to another growing season, can help reduce the frequency of GR individuals within a population on the field. One such strategy may include a residual soil-applied herbicide that would remove earlier germinating GS seedlings, and continue to be present when later germinating GR seedling emerge. Another strategy is the use of tillage to bury kochia seed to a soil depth that impedes seedling establishment, such that even when seed are repositioned (through tillage) to soil depth conducive for germination in a subsequent farming season, many of the GR seeds could have lost their viability.

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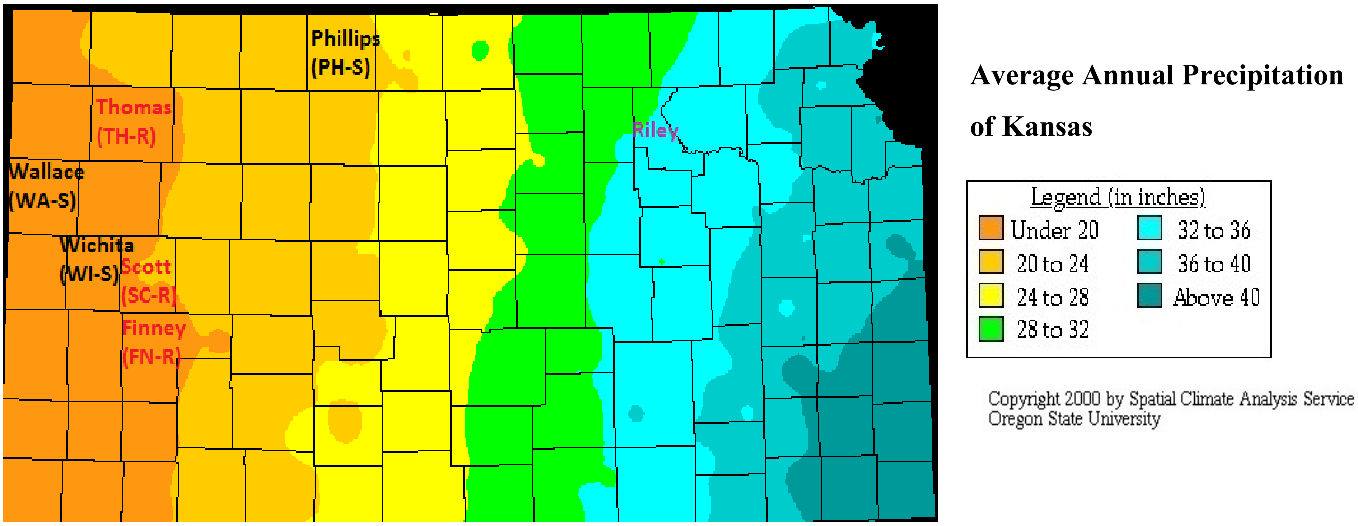

Average Annual Precipitation of Kansas

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Figure 2.1: Precipitation of the locations where *Kochia scoparia* populations were collected for field study. The glyphosate-resistant populations are in red while the susceptible populations are in black text.

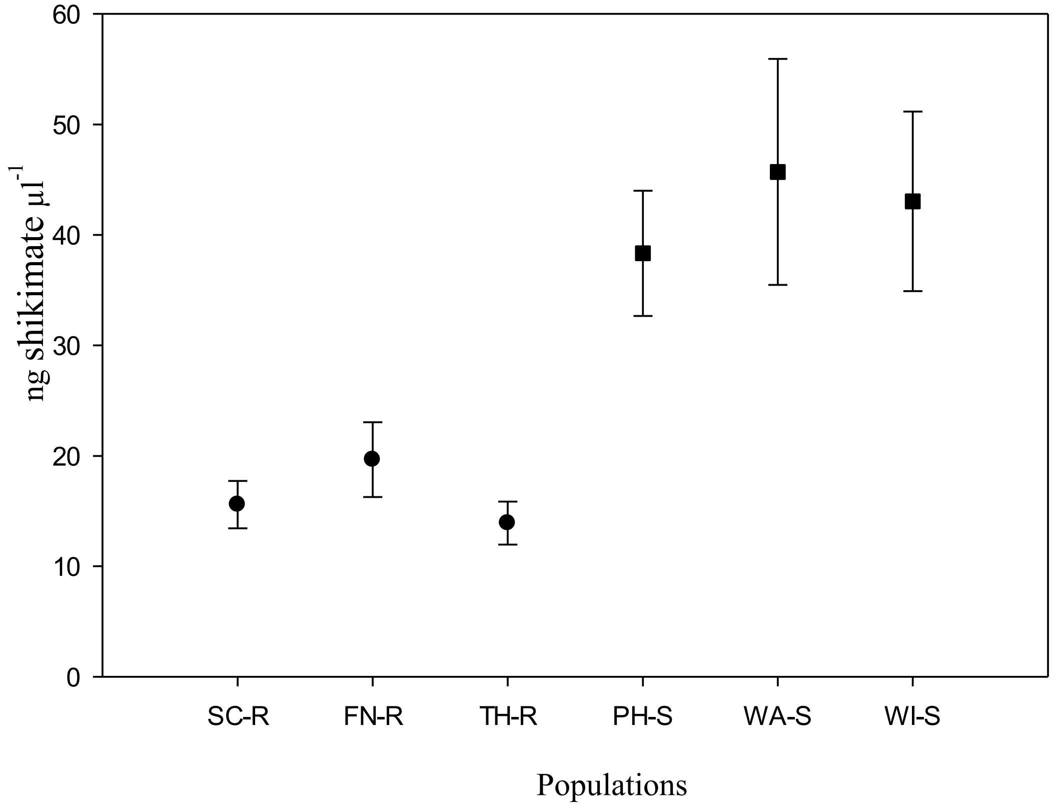

Figure 2.2: Mean (and standard error) for shikimate accumulation (ng ul^{-1}) of 10 representative plants for each of six kochia populations. Suspected glyphosate-resistant populations were from Scott (SC-R), Finney (FN-R), and Thomas Counties (TH-R) while suspected glyphosate-susceptible populations were from Phillips (PH-S), Wallace (WA-S) and Wichita Counties (WI-S) in western Kansas.

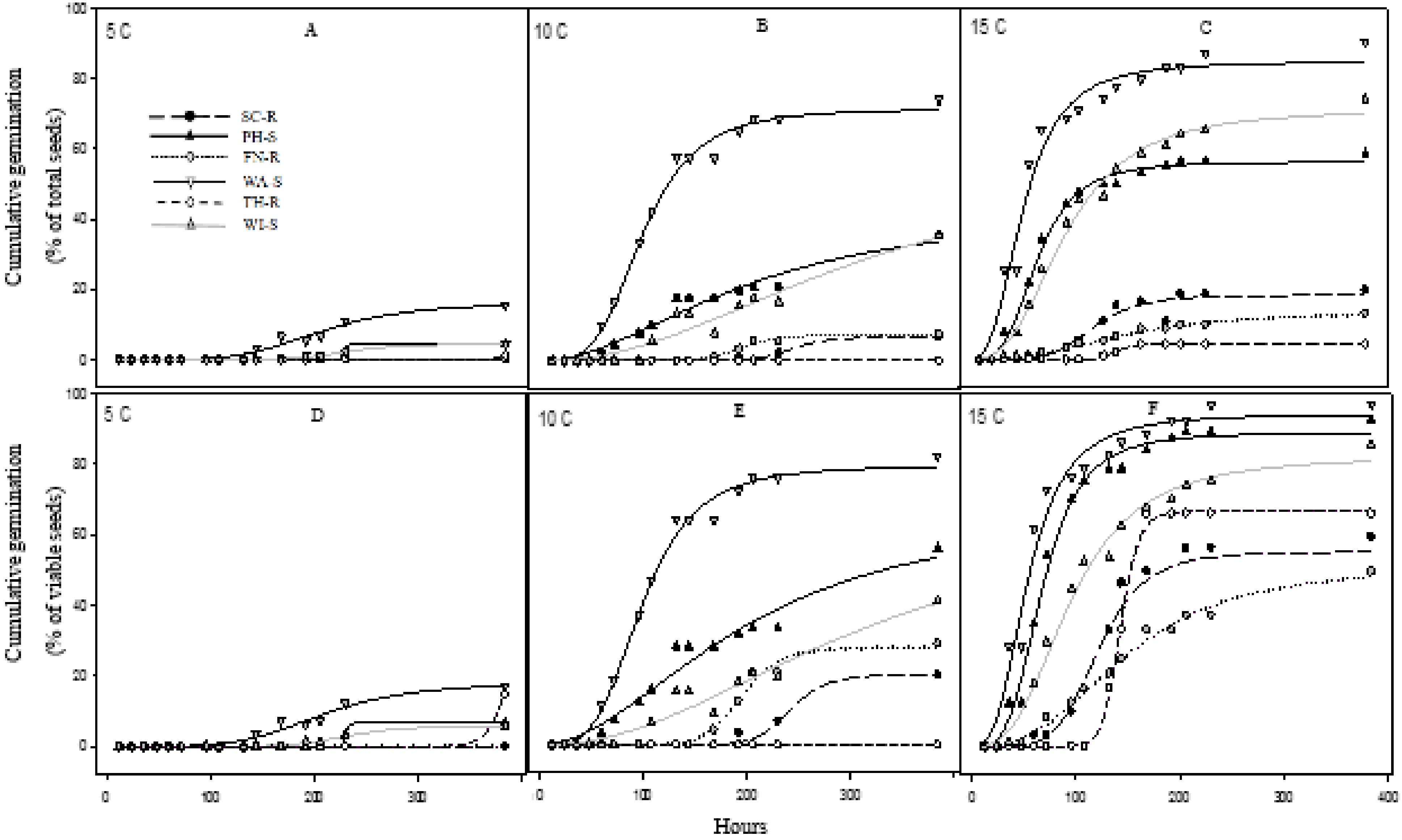
Cumulative germination
(% of total seeds)
Cumulative germination
(% of viable seeds)
5 C
A
10 C
B
15 C
C
5 C
D
10 C
E
15 C
F
SC-R
PH-S
FN-R
WA-S
TH-R
WI-S
100
80
60
40
20
0
0
100
200
300
400
Hours

Figure 2.3: Cumulative germination as a percentage of 30 seed (A, B, C) and as a percentage of viable seed (D, E, F) at one of three constant temperatures of 5, 10 and 15 C. Germination dynamics of glyphosate-resistant and -susceptible kochia populations are modeled with Equation 2.1. Parameter estimates for each model are included in Table 2.3. Plots with solid lines and triangle symbols represent the GR populations while those with broken lines and circle symbols represents the GS populations.

Table 2.1: Survival (% of total individuals) and injury score at 4 wk after herbicide treatment of six kochia populations from western Kansas. Injury score was on the scale of 1 to 9, where 1 represents no injury and 9 represents completely injured (or dead). Total number of individuals in each population from two trials were 17.

Herbicide[b]	Mode of action	Rate	Survival					
			SC-R[a]	FN-R	TH-R	PH-S	WA-S	WI-S
		kg ha^{-1}	…………………..%					
Glyphosate[c]	EPSPS inhibitor	0.84 ae	100	77	100	0	0	7
		1.68 ae	12	30	13	0	0	0
Atrazine[d]	PS II inhibitor	2.24 ai	0	90	0	0	0	0
Dicamba[e]	Synthetic auxin	0.50 ae	69	91	58	46	69	38
Chlorsulfuron[e]	ALS inhibitor	0.018 ai	67	59	66	83	100	64
			………………Injury score..........................					
Glyphosate	EPSPS inhibitor	0.84 ae	1.1 B	2.0 B	1.0 B	8.9 A	8.9 A	8.8 A
		1.68 ae	6.0 AB	6.6 AB	3.6 B	9.0 A	9.0 A	9.0 A
Atrazine	PS II inhibitor	2.24 ai	8.9 A	2.5 B	9.0 A	9.0 A	8.5 A	9.0 A
Dicamba	Synthetic auxin	0.50 ae	6.1 A	3.0 B	5.0 AB	6.6 A	5.8 A	6.0 A
Chlorsulfuron	ALS inhibitor	0.018 ai	6.6 A	5.7 A	3.3 A	3.7 A	6.7 A	2.1 A

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[a] Abbreviations: SC-R, FN-R and TH-R were suspected glyphosate resistant populations from Scott, Finney, and Thomas Counties, respectively, while PH-S, WA-S and WI-S were expected

glyphosate susceptible populations from Phillips, Wallace and Wichita Counties, respectively in western Kansas.

[b] Herbicide treatments were applied to 8- to 10-cm tall kochia plants.

[c] with ammonium sulfate at 2% v/v.

[d] with crop oil concentrate at 1% v/v.

[e] with nonionic surfactant at 0.25% v/v.

[f] Injury score followed by the same letter within a row (among populations) are not significantly different based on Tukey's honestly significant difference at $P < 0.05$.

Table 2.2: Relative shoot dry weight (% of untreated) at 4 wk after herbicide treatment of six kochia populations from western Kansas. Total number of individuals in each population from two trials was 17.

Herbicide[b]	Mode of action	Rate	Relative shoot dry weight					
			SC-R[a]	FN-R	TH-R	PH-S	WA-S	WI-S
		kg ha^{-1}	………………% of untreated					………………….
Glyphosate[c]	EPSPS inhibitor	0.84 ae	48.6 A	49.6 A	26.7 A	1.7 B	6.7 B	6.3 B
		1.68 ae	7.9 A	10.1 A	8.8 A	0.5 B	5.5 AB	1.3 B
Atrazine[d]	PS II inhibitor	2.24 ai	1.1 B	71.3 A	0.2 B	1.2 B	5.5 B	1.7 B
Dicamba[e]	Synthetic auxin	0.50 ae	18.1 B	55.6 A	21.8 B	10.6 B	12.6 B	43.4 AB
Chlorsulfuron[e]	ALS inhibitor	0.018 ai	12.5 A	25.7 A	65.0 A	26.1 A	17.6 A	48.4 A

[a] Abbreviations: SC-R, FN-R and TH-R were suspected glyphosate resistant populations from Scott, Finney, and Thomas Counties, respectively, while PH-S, WA-S and WI-S were expected glyphosate susceptible populations from Phillips, Wallace and Wichita Counties, respectively in western Kansas.

[b] Herbicide treatments were applied to 8- to 10-cm tall kochia plants.

[c] with ammonium sulfate at 2% v/v.

[d] with crop oil concentrate at 1% v/v.

[e] with nonionic surfactant at 0.25% v/v.

[f] Shoot dry weight followed by the same letter within a row (among populations) are not significantly different based on Tukey's honestly significant difference at $P < 0.05$.

Table 2.3: Parameter estimates obtained from logistic regression model (Equation 2.1) to describe the germination dynamics in kochia populations.

	Parameter estimates								
	b			D			t_0		
	5 C	10 C	15 C	5 C	10 C	15 C	5 C	10 C	15 C
Cumulative germination (% of total seeds)				…………%.................			…………...h………….		
SC-R[a]	n	-14.1	-5.5	n	6.6 A	18.4 C	n	243 A	122 A
FN-R	n	-10.6	-2.7	n	7.4 C	13.7 C	n	195 A	148 A
TH-R	n	n	-16.8	n	n	4.4 C	n	n	143 A
PH-S	-2.6	-1.9	-3.6	4.4	43.8 B	56.3 B	1092	200 A	68 C
WA-S	-5.3	-3.5	-2.9	16.1	71.4 A	84.8 A	214	99 B	54 D
WI-S	-2.4	-2.2	-2.9	4.2	57.5 A	71.2 A	976	313 A	95 B
Cumulative germination (% of viable seeds)									
SC-R	n	-17.2	-5.5	n	19.8 B	55.1 B	n	240 A	122 AB
FN-R	n	-10.7	-2.7	n	27.7 B	51.5 B	n	195 A	149 A
TH-R	n	n	-16.4	n	n	66.8 B	n	n	143 A
PH-S	-3.1	-1.9	-3.7	6.6	67.9 A	88.9 A	1951	199 A	68 C
WA-S	-5.4	-3.8	-3.2	18.7	76.5 A	90.5 A	213	99 B	52 C
WI-S	-2.4	-2.1	-2.9	5.8	69.5 A	85.8 A	848	315 A	97 B

[a] Abbreviations: SC-R, FN-R and TH-R were glyphosate resistant populations from Scott, Finney, and Thomas county respectively while PH-S, WA-S and WI-S were glyphosate susceptible populations from Phillips, Wallace and Wichita county respectively. b is

the slope of the regression function, d is the maximum cumulative germination, and t_o is the time required to reach 50% of maximum cumulative germination.

[n] Parameters could not be estimated due to very poor germination

[c] Within a column (among populations), ranked parameter estimates without a similar letter were significantly different ($P < 0.05$) while estimates were left unranked if there was no significant different among populations. Comparison of estimates among population was conducted using Tukey's honestly significant difference test ($\alpha = 5\%$)

Chapter 3 - Effect of Neighbors on Relative Fitness of Glyphosate-Resistant Kochia (*Kochia scoparia*) Populations

Abstract

Understanding the ecological fitness of a population with resistance traits is important in describing the evolutionary trajectory of the population. It was hypothesized that glyphosate resistance in kochia due to overproduction of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme and the consequent metabolic cost of overproduction will be a trade-off for plant fitness. A field study was conducted near Manhattan, KS in 2014 and 2015 to evaluate the ecological fitness of six kochia populations from western Kansas. Three populations were glyphosate-resistant (GR) and three were glyphosate -susceptible (GS). Ecological fitness was evaluated using a target-neighborhood competition design. In 2014 and 2015, one individual target kochia plant from each population was surrounded by one of three densities of 12, 35 and 70 kochia plants m^{-2}. In 2015, corn as neighbor was also studied with 12 and 35 corn plants m^{-2}. There were no differences in rate of increase in plant height and in number of days required to reach 50% of final height among the kochia populations. At the end of growing season, differences in plant height, biomass and seed production among the populations were magnified by high neighbor density. Competitive response, a measure of the ability of populations to continue their growth with increasing neighbor biomass, was only significant for plant height and biomass with corn neighbors. In general, there was no strong evidence that these differences were due to glyphosate resistance. Corn neighbors reduced kochia populations' plant height, biomass and seed production more than kochia neighbors. Overproduction of EPSPS did not translate into reduced fitness. This implies that GR kochia are likely to persist in field

populations, even in the absence of glyphosate application, thereby making weed management more difficult.

Introduction

Plants with heritable resistance to environmental stresses may have an ecological disadvantage compared to susceptible plants when the selective force or stress is absent (Vila-Aiub et al. 2009a). A population is said to be resistant to an herbicide when resistant biotypes make up at least 20% of the total population (Neve et al. 2011) either in form of seeds or plants. Resistant weeds are able to withstand the phytotoxicity of herbicides because of the presence of resistance alleles originating from random DNA mutations (Powles and Yu 2010). These resistance alleles regulate a number of highly efficient, constitutive defense mechanisms that prevent herbicides from inhibiting key metabolic pathways. Since many mutations in plants have deleterious, pleiotropic effects on its likelihood of survival and/or reproduction (Roles and Conner 2008), it is generally assumed that herbicide resistance mutations will be associated with an initial cost to a plant’s fitness (Vila-Aiub et al. 2009b).

Fitness can be defined as the average success in producing offspring contributing to the next generation by a particular biotype relative to another biotype (Vila-Aiub et al. 2015). Fitness is important in describing the evolutionary advantage of a biotype, such as greater survivorship, faster growth rate, greater fecundity, and stronger competitive ability compared to another biotype (Shrestha et al. 2010; Sibony and Rubin 2002). Survival is a broad term and can be attributed to several different stages in the life cycle such as seed viability, seedling vigor, competitive ability, and percentage of reproductive plants, among others. In regards to a weed population with herbicide resistance, the ability to establish, grow, survive, and reproduce successfully is one of the most important factors influencing the appearance and persistence of herbicide-resistant biotypes. The relative fitness of resistant and susceptible biotypes has a powerful influence on the dynamics of a mixed resistant and susceptible population by shifting the proportion or frequency of biotypes in the population (Gressel and Segel 1982).

Plant growth versus defense predicted by the resource-based allocation theory (Herms and Mattson 1992) suggests that there is a trade-off between allocation of resources for plant growth and for plant defense against environmental stress. In this study, a case of herbicide resistance conferred by high or overproduction of protein (enzymes) was examined. The mechanism responsible for glyphosate resistance in kochia has been reported to be amplification of 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) gene which translates to high or overproduction of EPSPS enzyme in plant. If overproduction of EPSPS enzyme conferred glyphosate resistance in kochia (Godar et al. 2015; Jugulam et al. 2014), it is expected that more metabolic energy, compared to normal EPSPS production in a susceptible individual, will be required for enzyme production at the detriment of other plant functions or growth processes.

Investigations have shown that some of the physiological and genetic processes that resulted in herbicide resistance are associated with reduced ecological fitness of the resistant population. Triazine-resistant weed species such as redroot pigweed (*Amaranthus retroflexus*) (Conard and Radosevich 1979; Sibony and Rubin 2003), common groundsel (*Senecio vulgaris*) (Conard and Radosevich 1979), and black nightshade (*Solanum nigrum*) (Kremer and Kropff 1999) showed reduction in biomass when subjected to competition with the susceptible biotype in the absence of herbicide. The most common mechanism for triazine resistance in weeds is a result of mutation in the quinone B (QB) protein in photosystem II (PSII), which decreased the binding affinity for triazine molecules to the protein (Devine and Shukla 2000). This mutation reduces efficiency of photosystem II, which results in reduced plant fitness.

Acetolactate synthase (ALS)-inhibitor herbicides such as sulfonylurea, imidazolinone, triazolopyrimidine, pyrimidinyl thio and oxy-benzoate products inhibit the ability of ALS enzyme to catalyze the first step in biosynthesis of branched chain amino acids (Devine and Shukla 2000; Zabalza et al. 2007). There was no difference in competitive ability between ALS-resistant (ALS-R) and -susceptible (ALS-S) biotypes for prostrate pigweed (*Amaranthus blitoides*) (Sibony and Rubin 2002), horseweed (*Conyza canadensis*) (Park et al. 2004), field mustard (*Brassica rapa*) *(*Butler 2012), watergrass (*Echinochloa phyllopogon*) (Boddy et al. 2012) and kochia (Légère et al. 2013) on the basis of shoot dry weight and seed production. In many cases the ALS-R trait was not associated with growth penalty in either noncompetitive or competitive conditions. In the absence of application of ALS-inhibiting herbicides, the ALS-R biotype would remain at a similar frequency in a mixed population of resistant and susceptible biotypes in the field.

Efforts are still being made to identify how development of glyphosate resistance in weeds can result in differential fitness and reports so far have shown that fitness differences between GR and GS cannot be generalized for weed species. Pedersen et al. (2007) reported fitness cost in terms of seed production of GR rigid ryegrass (*Lolium rigidum*) when compared to susceptible (GS) biotype under wheat competition, although the amount of difference in seed production of both GR and GS biotypes was reduced with increased competition. Davis et al. (2009) reported that GR horseweed biotypes produced similar amounts of biomass and seeds compared to GS biotypes, while the work of Shrestha et al. (2010) showed that a GR biotype of horseweed accumulated more dry matter, and had more flowers and seeds per plant than the GS horseweed in mixed populations under increasing levels of competition and limited resources. Kumar and Jha (2015) reported that no differences in growth and reproductive parameters were evident between the tested GR and GS kochia biotypes across an intraspecific competition gradient.

In some cases, evaluation of resource competitive interactions between herbicide-resistant versus -susceptible weed biotypes provide evidence for the expression of fitness costs associated with evolved herbicide-resistant traits. Such fitness costs impact the ecology and evolutionary trajectory of resistant populations negatively by reducing the proportion of the resistant biotypes over time in the absence of herbicide application (Vila-Aiub et al. 2009b). Various studies to determine differences in ecological fitness among biotypes or populations have been carried out under competitive and non-competitive situations. Under normal field conditions however, resistant and susceptible plants are usually under competition with each other and / or with crop for available resources. Thus, evaluating the weed–weed and weed–crop interactions are essential to predict the evolutionary trajectories of herbicide-susceptible and resistant weed species.

Density or crowding is important as it modifies interactions between plants through competition for resources. Many research studies have used densities or crowding to subject plant biotypes to competition in order to measure their fitness through their vegetative and reproductive responses. Two main competition designs used to evaluate differential growth and/or fecundity of resistant and susceptible weed populations include replacement series (Légère et al. 2013; Shrestha et al. 2010; Travlos and Chachalis 2013) and target-neighborhood design (Vila-Aiub et al. 2005, 2009a). The advantage of target-neighborhood over replacement series design seems to be the ability of the former to allow the study of competition effect or response under varying densities and direct comparison of two or more biotypes under the effects of different densities. In this study, target-neighborhood design was used to compare six populations of kochia. Although some studies (Pedersen et al. 2007; Shrestha et al. 2010; Travlos and Chachalis 2013) only compared two populations, this study compared three GR and three GS kochia populations collected from fields in western Kansas to ensure that what is observed is not due to environment from where the populations were collected. The fitness of these populations was evaluated in a field condition which is very different from greenhouse or partial field (where plants were placed in pots in an open field) studies used in most ecological fitness experiments (Kumar and Jha 2015; Shrestha et al. 2010; Vila-Aiub et al. 2009a; Wang et al. 2014).

High or overproduction of EPSPS enzyme has endowed glyphosate resistance in kochia and the metabolic cost (energy) of production of this enzyme may be a trade-off for plant growth and fecundity. This fitness cost may likely be more evident when plants are in competition with neighbors. In cases where the GR populations are relatively less fit, this may translate into an evolutionary advantage for the GS biotypes in the absence of glyphosate application. The objectives of this study were (a) to evaluate the relative fitness between GR and GS kochia populations from western Kansas under varying neighbor densities in the absence of glyphosate application, and (b) to evaluate how type of competition gradient (intra- or interspecific) influences the difference in growth and fecundity among the GR and GS kochia populations.

Materials and Methods

A field study was conducted at the Kansas State University Department of Agronomy Ashland Bottoms Experiment Field (39.12577N 96.6365W) near Manhattan, KS in 2014 and

2015. The two locations were within 1 km of each other. The soil series of the field was Kennebec, which is a moderately well drained silt loam soil formed in alluvium (NRCS 2016). In 2014, results of soil analysis of the experimental field were documented for soil pH (7.1), nitrogen (17 ppm), phosphorus (26.5 ppm), potassium (339 ppm), organic matter (2.5%) and cation exchange capacity (13.1 meq 100 g^{-1}). In 2015, the soil pH (6.2), nitrogen (78.9 ppm), phosphorus (47.1 ppm), potassium (529 ppm), organic matter (2.5%), and cation exchange capacity (13.1 meq 100 g^{-1}) were recorded.

The six kochia populations used in this experiment were originally collected from different fields in western Kansas; these include confirmed GR populations from Scott (SC-R), Finney (FN-R), and Thomas (TH-R) Counties and GS populations from Phillip (PH-S), Wallace (WA-S) and Wichita (WI-S) Counties. All the seeds were collected in 2012 except those of TH-R which were collected in 2007. Seed from most fields were harvested from more than 10 mature plants per site and bulked in separate bags for each population. The classification of these populations into GR or GS was based on single discriminatory dose (Chapter 2).

The density treatment design was a modified form of target-neighborhood method used by Vila-Aiub et al. (2009a). In 2014, one target plant from each of the six kochia populations was surrounded by neighboring kochia densities equivalent to 12 (low), 35 (moderate), or 70 (high) plants m^{-2}. The two treatment factors were six kochia populations and three neighbor densities. Treatments were in a randomized complete block design with 10 replications in 2014. Individual plot size was 0.6 m by 0.6 m and spacing between plots was 1 m. Seeds were coated with Laponite RD® gel of a rate of 0.012 g ml^{-1} of water (Nordby and Hartzler 2004) to prevent displacement of the shallowly-sown kochia seeds by wind and to create a moist environment for seed germination. A 12 ml syringe was used to place 10 ml of solution containing approximately

10 kochia seeds on the soil surface. After kochia germination, seedlings were thinned to the respective proportions of target plant to neighbor densities. For the 12 and 35 neighbor plants m^{-2} treatment, the distance from target to neighbor was 15 cm while for the 70 neighbors m^{-2} treatment, the distance from target to the immediate neighbor and between neighbor and neighbor was about 10 cm.

In 2015, five kochia populations were used as targets excluding the WA-S population. The WA-S population was excluded to allow efficient management of plots as there were more treatment combinations than the previous year. Four kochia neighbor density treatments were evaluated, by adding a no neighbor (0) treatment. A complementary study in 2015 included corn as a neighbor at densities of 0, 12, and 35 corn plants m^{-2} to evaluate effect of crop density on the target plants. The levels of the two treatment factors in the kochia-only study were five kochia populations and four kochia neighbor densities while in the corn-neighbor study, the levels of the two factors were five kochia populations and three corn densities. Both studies were conducted within the same randomized complete block design with seven replications. Individual plot size was 0.6 m by 0.6 m and spacing between plots was 3 m. In 2015, seedlings were grown in the greenhouse and transplanted into the experiment rather than direct seeding. In the greenhouse, kochia seeds were shallowly sown in 25 by 15 by 2.5-cm plastic trays filled with commercial potting mixture (Miracle Gro, Marysville, OH). The trays were placed in a no-hole runoff catchment tray and water was added to the tray for sub-irrigation. The greenhouse was maintained at 25/20 C day/night, 60% relative humidity, and 15/9 h day/night photoperiod. Kochia seedlings of about 5 cm tall were transplanted into the field in their respective target plot. In the case of corn as neighboring plant, corn seeds were directly sown in the field and the respective kochia seedlings (about 5 cm tall) as target plants were transplanted at corn emergence. For the 12 and 35 plants m^{-2} neighbor densities, the target to neighbor distant was about 15 cm while for the 70 plants m^{-2} neighbor densities, the distance from target to the immediate neighbor and between neighbor and neighbor was about 10 cm.

The neighbor densities at the commencement and end of each trial were recorded, and the neighbor biomass was collected at the end of the season. Kochia target plant height, stem diameter at base of the plant, and plant canopy width (as a measure of the widest canopy) were collected biweekly starting from 3 weeks after planting or at transplanting in 2014 and 2015, respectively. Days to first flowering as a phenological variable was recorded. Target plants were harvested at about 130 and 120 days after establishment in the field in 2014 and 2015, respectively. Fresh and dry weight of the harvested target plants were recorded. The freshly harvested plants were placed in oven at 40 C for about 72 h and weighed. Seeds were collected by stripping them off the plants and cleaned using an air column separator. Total and 1000-seed weight per plant were taken. Total seed number (TS) was calculated for each plant:

$$TS = (SW_T/SW_{1000}) * 1000 \qquad [3.1]$$

where SW_T is total seed weight (g $plant^{-1}$) and SW_{1000} is weight of 1000 kochia seed (g). Growing degree days (GDD) for each day after sowing of seeds were calculated as recommended by Schwinghamer and Van Acker (2008) for kochia:

$$GDD_{daily} = [(T_{max} + T_{min})/2] - T_{base} \qquad [3.2]$$

$$\text{Cumulative GDD} = \sum_{i=1}^{n} \text{GDDdaily} \qquad [3.3]$$

where T_{max} is the maximum daily air temperature, T_{min} is the minimum daily air temperature, and T_{base} is the base temperature at which plant growth and development was deemed not to occur (0 C); n is the number of days elapsed from sowing date, and GDD_{daily} was a nonnegative value (daily GDD values that were negative were replaced by 0). A base temperature of 0 C seemed reasonable given that kochia has been known to emerge early in the spring (Dille et al. 2012) and 0 C has been used previously as a biologically justifiable base temperature for modeling the germination and emergence of kochia (Schwinghamer and Van Acker 2008).

Growth of kochia populations over time was modeled using plant height as a function of cumulative GDD with the following three parameter sigmoid function:

$$H= a/[1+\exp(-(x-c)/b)] \qquad [3.4]$$

where H is the target plant height (cm) at cumulative GDD (x), while parameter a is the maximum height (cm), parameter c is the cumulative GDD required to attain 50% of the maximum height, and parameter b is the slope of the curve at the inflection point (and near 50% cumulative GDD). The growth regression curve and analysis were done using SigmaPlot V.12.3 (Systat Software, Inc).

Due to changes in the neighbor density between the commencement and end of the field trial, analysis of covariance (ANCOVA) was used to analyze the effects of the factors (neighbor densities and populations) on the fitness variables where density at the end of the season was considered at the random effect using the generalized linear mixed model (GLMM) in GLIMMIX procedure of SAS 9.4 software (SAS Institute Inc., Cary, NC), which did not necessarily assume normal distribution of data, was found suitable for the collected fitness data and was therefore used for ANCOVA.

The effects of neighbor biomass on target plant height, biomass, seed weight per plant, and seed number per plant across competition gradient were evaluated using exponential decay function in SigmaPlot V.12.3. This function consistently gave the highest coefficient of determination (r^2) compared to other models (linear, power and hyperbolic) tested and it has been used and recommended for competition studies by Freckleton and Watkinson (2001):

$$Y = a*\exp(-b*x) \qquad [3.5]$$

where Y is target plant height, biomass, seed weight or seed number per plant, parameter *a* is the maximum target biomass, seed weight or seed number per plant when neighbor biomass is low or zero; parameter *b* is the slope of the function and measures the "competitive response" as the degree of target response to neighbor biomass, and x is the neighbor biomass (kg m^{-2}). The response for target kochia biomass to increasing biomass of corn as the neighbor plant was not described by exponential decay nor hyperbolic models, thus a linear regression was fit:

$$Y= a-bx \qquad [3.6]$$

where x is corn neighbor biomass (kg m^{-2}). The parameter estimates of the models were compared using ANOVA (Vila-Aiub et al. 2009a).

Results and Discussion

Effect of neighbor densities on target plant height over time.

The three-parameter sigmoid model (Equation 3.4) adequately fit the data for plant height over time with the least P-value of 0.005 for all populations. The rate of change in plant height over time as measured by the slope (b estimate) of the sigmoid growth model was not different among populations under the influence of low or high kochia neighbor densities in either year (Figure 3.2 and 3.3; Table 3.1 and 3.2). Similarly, there was no difference in the rate of change in stem diameter or canopy spread among populations over time (data not shown).

At low neighbor density, the estimated final plant height (parameter 'a') was different among populations in 2014 (Figure 3.2; Table 3.1) but not different in 2015 (Figure 3.3; Table 3.1). At high neighbor density in both years, there were differences in estimated final plant height among populations (Figure 3.2 and 3.3; Table 3.2), however, ANCOVA showed that there was an interaction between populations and densities for the observed final plant height (Table 3.3). The ranking of observed final plant heights among the populations at low neighbor density (Table 3.4) were similar to those estimated (parameter ‘a’) by the sigmoid model for 2015 data, and ranking of observed final plant height among the populations at high neighbor densities (Table 3.4) were similar to the those of parameter ‘a’ for both 2014 and 2015 data.

The recorded differences in final plant height were not consistent among GR and GS populations. For instance, in competition with low neighbor density, averaged over years, TH-R was the shortest among populations at the end of the season and was about 23% of the tallest recorded population (SC-R) (Figure 3.2 and Table 3.1). While in competition with high neighbor density averaged over years, TH-R and WI-S plants were about 22 and 30% of the average height attained by SC-R (130 cm), and 35 and 29% of the average height attained by PH-S (124 cm), while three populations (SC-R, PH-S and FN-R) had similar heights at harvest (Figure 3.3 and Table 3.2).

It is important to note that if just two populations were compared for plant height, such as SC-R versus WI-S or TH-R versus PH-S, the differences could have been erroneously attributed to evolution of glyphosate resistance in the populations. The results highlight the importance of evaluating more than just two populations for fitness comparison. It is obvious that factor(s) other than GR trait resulted in differences of final plant height among these populations. These differences could be as a result of their genetic background (Giacomini et al. 2014; Mengistu and Messersmith 2002) or adaptive features that evolved due to their original environmental conditions (Jasieniuk et al. 1996).

Phenology estimate in the model showed that thermal time, cumulative GDD required to attain 50% of maximum height, was not different among populations and the cumulative GDD of each population falls between 1265 to 1894 GDD in 2014 and 1422 to 2006 GDD in 2015 across neighbor densities (Table 3.1 and 3.2). Based on these estimates, there was no evidence that temperature required for increase in height differed among the populations.

Effect of neighbor density on target plant biomass and fecundity.

Analysis of covariance of growth and reproductive measurements at the end of season showed that there were interactions between the two main factors of kochia population and kochia neighbor density for target plant biomass in both years, as well as for seed weight and number of seeds produced per plant in 2015 (Table 3.3). The differences among populations were significant at high neighbor density for these measurements (Table 3.5 and 3.6), however, the differences among populations were not in respect to glyphosate resistance. Across populations, as neighbor density increased, plant biomass decreased by 80% in 2014 and 85% in 2015 (Table 3.5) while total seed weight and number per plant were reduced by 71 and 80% respectively (Table 3.6).

Target plant biomass at harvest showed no differences among populations at low neighbor density but there were differences at high neighbor density but these differences were not consistent among GR and GS populations (Table 3.5). Similarly, the effect of kochia neighbor density on total seed weight and seed number per plant was significant at high density where SC-R and PH-S had similar total seed weights (13.1 to 14.8 g plant^{-1}) and these were greater than the seed weight of FN-R, TH-R, and WI-S (Table 3.6). The impact of high neighbor density compared to low neighbor density on plant biomass and seed production among populations might have been exaggerated. At low density, the proximity of target to neighbor was 15 cm while at high density it was only 10 cm, hence there was confounding effect of target to neighbor distance and number of neighbors at each density level. However, the evaluation of proximity effect may not be necessary for this study, as the crowding treatment for all populations was similar and this study was more concerned about the response of each population to the crowding level.

The effect of type of neighbor (intra- and inter-specific) was significant on target plant height, biomass, seed weight, and seed number (Table 3.7). Corn as neighbor consistently had more suppressive effect than kochia as neighbor on these variables. The differential suppressive level of type of neighbor on the populations was not influenced by the density of neighbor (Table 3.7).

In general, the cases of differences among populations at varying level of neighbor competition were not clearly related to glyphosate resistance, but rather the differences showed some consistency in direction of inherent ability of the populations to capture resources in the presence of neighbors. This is evident in the ANCOVA of pooled populations of GR or GS, the P-value (>0.05) showed that there was no difference between GR and GS pooled populations for plant height, stem diameter, plant width, days to flowering, seed weight, 1000-seed weight, number of seed per plant and reproductive effort (Table 3.8). Results were consistent with findings of Kumar and Jha (2015) who reported that there was no difference in plant growth and reproductive indices between GR and GS kochia populations in a greenhouse study.

Effect of neighbor biomass on target plant height, biomass and fecundity.

The exponential decay regression model (Equation 3.5) adequately explained ($P < 0.001$) variations in plant height, seed weight and seed number responses of target plants to increasing biomass accumulation of the two types of neighboring plants and target plant biomass response to increasing kochia neighbors' biomass, while target plant biomass response to increasing corn neighbors' biomass could only be fit (*P* <0.001) with a linear model (Equation 3.6). Only 2015 data could adequately be explained by these two models. Each model provided two estimates, that is productivity of target plants (parameter **a**) in the absence of neighbor competition and competitive response (parameter **b**) to increasing neighbor biomass. Competitive response of target plants to increasing neighbor biomass evaluated a populations' response to competition by comparing estimates of the regression slopes (parameter **b**); the smaller the value of the slope, the stronger the competitive response to increasing neighbor biomass (Goldberg and Landa 1991; Weiner 1982). An initial analysis showed that neighbor biomass was strongly correlated (r = 0.83) with increasing density of neighbors. In general, increasing neighbor biomass had negative impact on target plant height, biomass accumulation and seed production (*P* < 0.001).

When grown as isolated plants in 2015, there were differences in kochia plant height (Figure 3.4), biomass accumulation (Figure 3.5), and seed weight or number per plant (Figures 3.6 and 3.7) among the populations. These differences were not in respect to glyphosate resistance, but rather the differences were related to inherent differences in biological productivity for each population. In all measurements, TH-R consistently was the shortest with low biomass and seed production while SC-R and PH-S consistently were the tallest with greatest biomass and seed production. This suggests that the populations have different potential productivity or fitness when resources are not limited.

Impact of kochia neighbor biomass.

Competitive response of plant height to increasing biomass of neighboring plants as measured by the slope was different among populations; however, this was not related to being GR or GS (Figure 3.4). In this case, SC-R, PH-S, and FN-R showed superior competitive response compared to TH-R and WI-S. This shows that the populations have different abilities to respond to increasing competition imposed by increasing intraspecific neighbor biomass. Competitive response of target plant biomass (Figure 3.5), total seed weight (Figure 3.6) and seed number (Figure 3.7) were not different among populations to increasing biomass of kochia neighbor**.**

Impact of corn neighbor biomass.

There was differential competitive response of plant height among kochia populations to increasing corn neighbor biomass (Figure 3.8) but was not in respect of being GR or GS. The competitive response of target plant's biomass accumulation to increasing biomass of corn was different among populations (Figure 3.9). The GR populations (SC-R, FN-R and TH-R) consistently showed superior competitive responses (smaller regression slopes) than the GS populations (PH-S and WI-S) to increasing biomass of corn neighbors. This is similar to a report that differential fitness (vegetative growth) between an ACCase herbicide susceptible and resistant rigid ryegrass (endowed by enhanced rates of cytochrome P450 enzyme activity) was magnified under inter-specific neighbor (wheat) than intra-specific neighbors (Vila-Aiub et al. 2009a). In this study, however, the differential biomass accumulation among resistant and susceptible populations in competition with corn neighbors may be insufficient evidence for differential fitness between GR and GS as other measured parameters such as plant height (Figure 3.8), stem diameter (data not shown), and seed production (Figures 3.10 and 3.11) were not different. It may be important to add that the observed differences in kochia response to intra-specific (kochia) and inter-specific (corn) neighbors may not be dissociated with differences in species-specific resource accumulation and allocation strategy of the type of neighbor (Weigelt et al. 2002) and plants with bigger seeds and larger size such as corn tends to have greater competitive effect (Goldberg and Fleetwood 1987). In general, these results suggest that the productivity of kochia populations in the absence of neighbors (as estimated by parameter a) and competitive response of populations to increasing competition by neighbors (as estimated by parameter b) are independent of each other.

Conclusions

Comparisons among multiple populations provide a better assessment of plant fitness than just a single pair comparison between herbicide-resistant and -susceptible populations. These results clearly show that a single pairwise comparison would not be sufficient and may be misleading, hence, it is important when conducting fitness studies to compare multiple pairs of biotypes for a resistant trait to check for consistency and allow validity.

Use of pure-lines or isogenic lines collected from inbreeding of several filial generations has been recommended and has been previously used as reliable plant material for fitness studies (Giacomini et al. 2014; Kumar and Jha 2015; Vila-Aiub et al. 2009b). Many studies used a single pair comparison between resistant and susceptible individuals of such lines to arrive at conclusion on fitness studies. This study did not evaluate such inbred lines. It may be important to evaluate the sufficiency of this single pairwise approach on fitness studies, by conducting comparisons among several pairs of resistant and susceptible inbred lines developed from different populations.

If the overproduction of EPSPS enzyme that endowed glyphosate resistance in kochia and the consequent metabolic cost of production of this enzyme at the expense of other protein production needed for plant growth and fecundity will be a trade-off for plant fitness, then it is expected that this trade-off will be more obvious under competition with neighbors for resources. In this field study, however, metabolic cost of overproduction of EPSPS did not translate into reduced growth rate and less fecundity even under competition imposed by intra- and inter-specific plant neighbors. This is similar to other previously reported studies as they could not associate fitness cost to glyphosate resistance endowed by overproduction of EPSPS in plant species such as kochia (Kumar and Jha 2015) and Palmer amaranth (Giacomini et al. 2014; Vila-Aiub et al. 2014). This implies that GR kochia plants due to overproduction of EPSPS enzyme are likely to persist in field populations, even in the absence of glyphosate application. And over time, glyphosate-based selective and non-selective weed control of a weed community with kochia may be completely discouraging.

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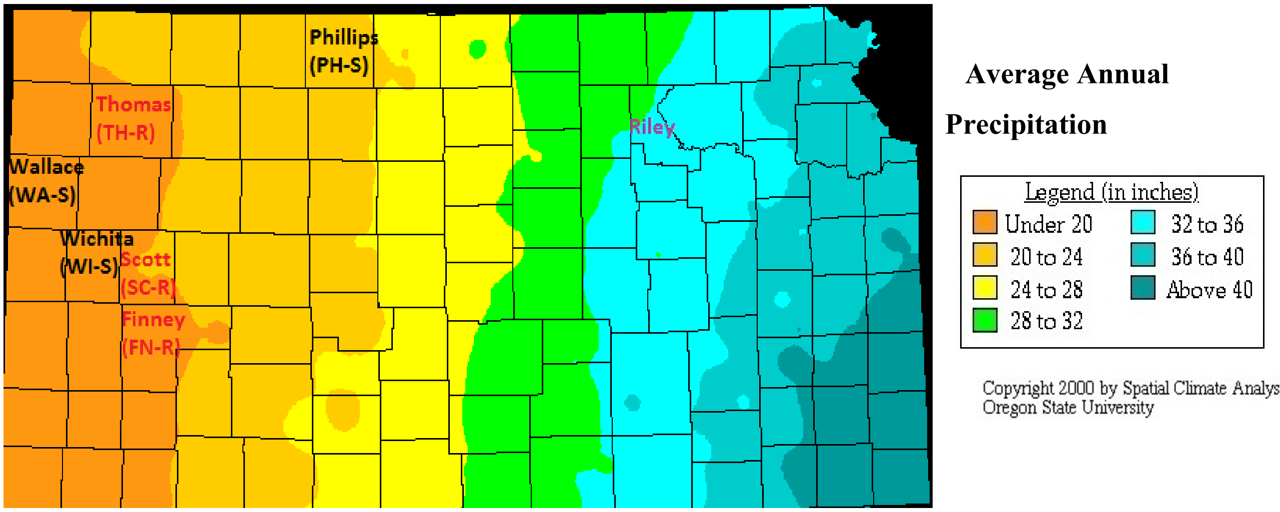

Average Annual Precipitation

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Figure 3.1: Precipitation of the locations where *Kochia scoparia* populations were collected for field study. The glyphosate-resistant populations have abbreviations that end with R while the susceptible populations have abbreviations that end with S. The field experiment was conducted in Riley County in 2014 and 2015, and served as a 'common garden' to minimize the effect of environmental variation among populations.

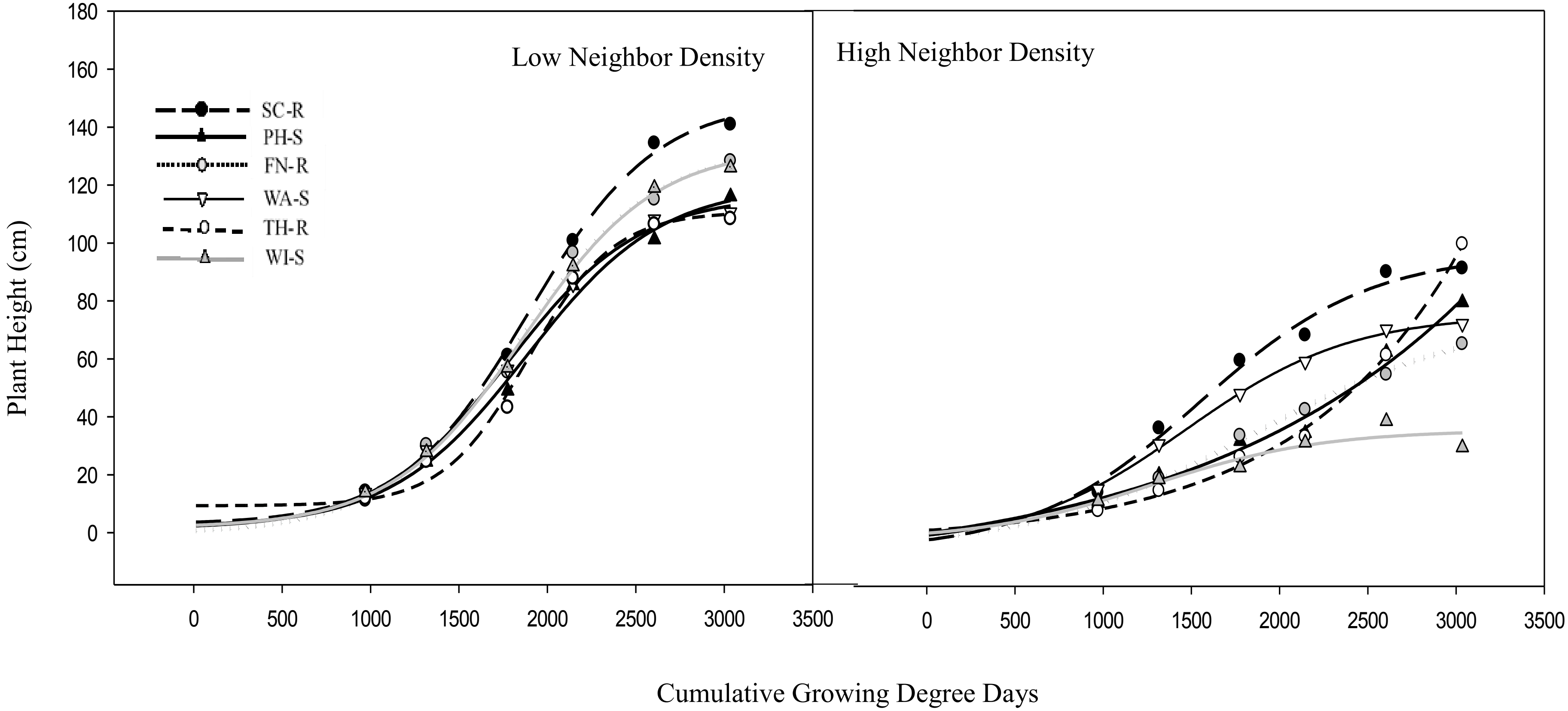
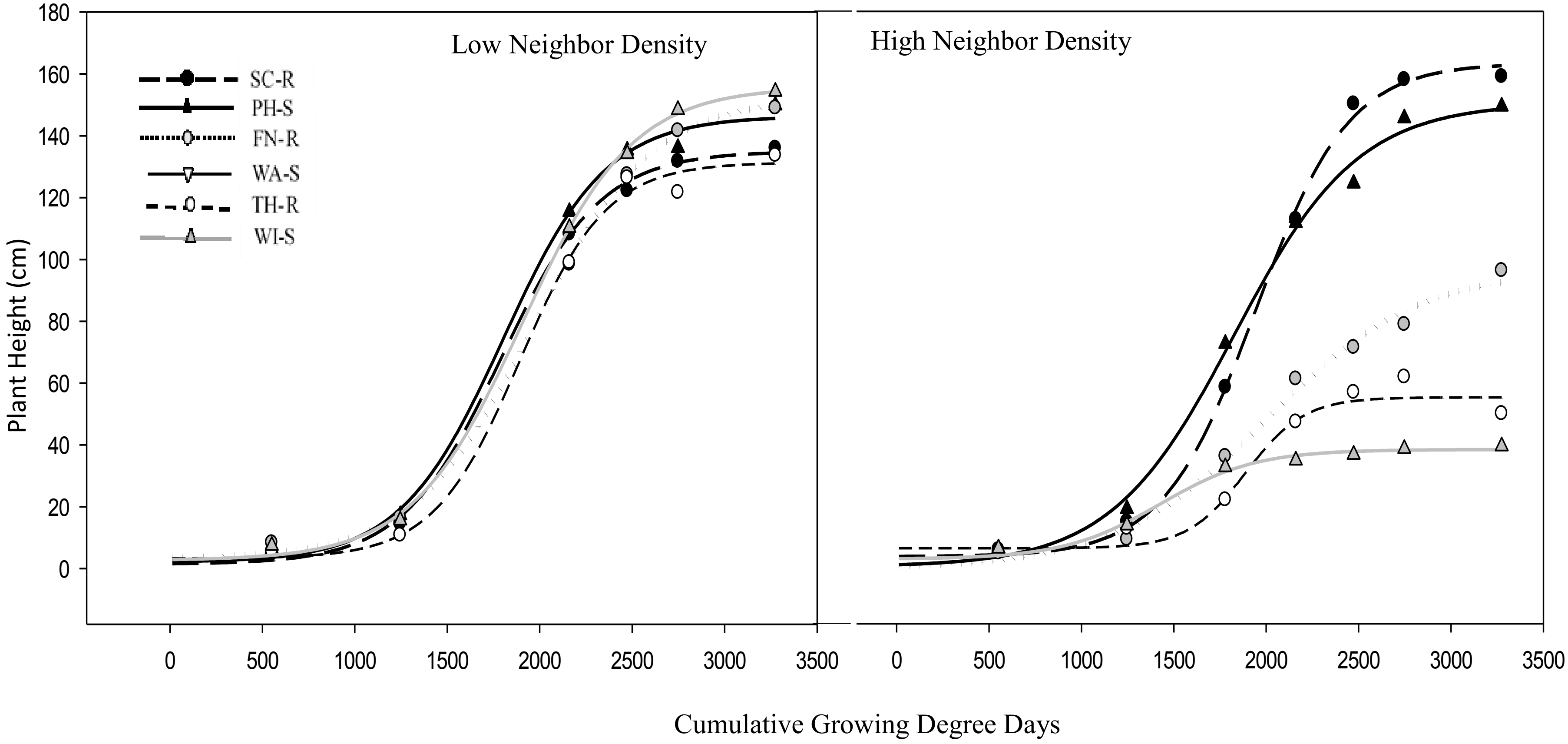


Figure 3.2: Increase in kochia plant height (cm) as a function of cumulative growing degree days (Tbase = 0 C, from the date of planting) at low (10 plants m^{-2}) and high (70 plants m^{-2}) kochia neighbor density using a three parameter sigmoid model (Equation 3.4) in 2014. Resistant populations are represented by broken lines and mean values in circles while the susceptible populations are represented by solid lines and mean values in triangles. Table 3.1 and 3.2 shows the parameter estimates of the model.



Low Neighbor Density
High Neighbor Density
SC-R
PH-S
FN-R
WA-S
TH-R
WI-S
Plant Height (cm)
180
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
3000
3500
Cumulative Growing Degree Days

Figure 3.3: Increase in kochia plant height (cm) as a function of cumulative growing degree days (Tbase = 0 C, from the date of planting) at low (10 plants m^{-2}) and high (70 plants m^{-2}) kochia neighbor density using a three parameter sigmoid model (Equation 3.4) in 2015. Resistant populations are represented by broken lines and mean values in circles while the susceptible populations are represented by solid lines and mean values in triangles. Table 3.1 and 3.2 shows the parameter estimates of the model

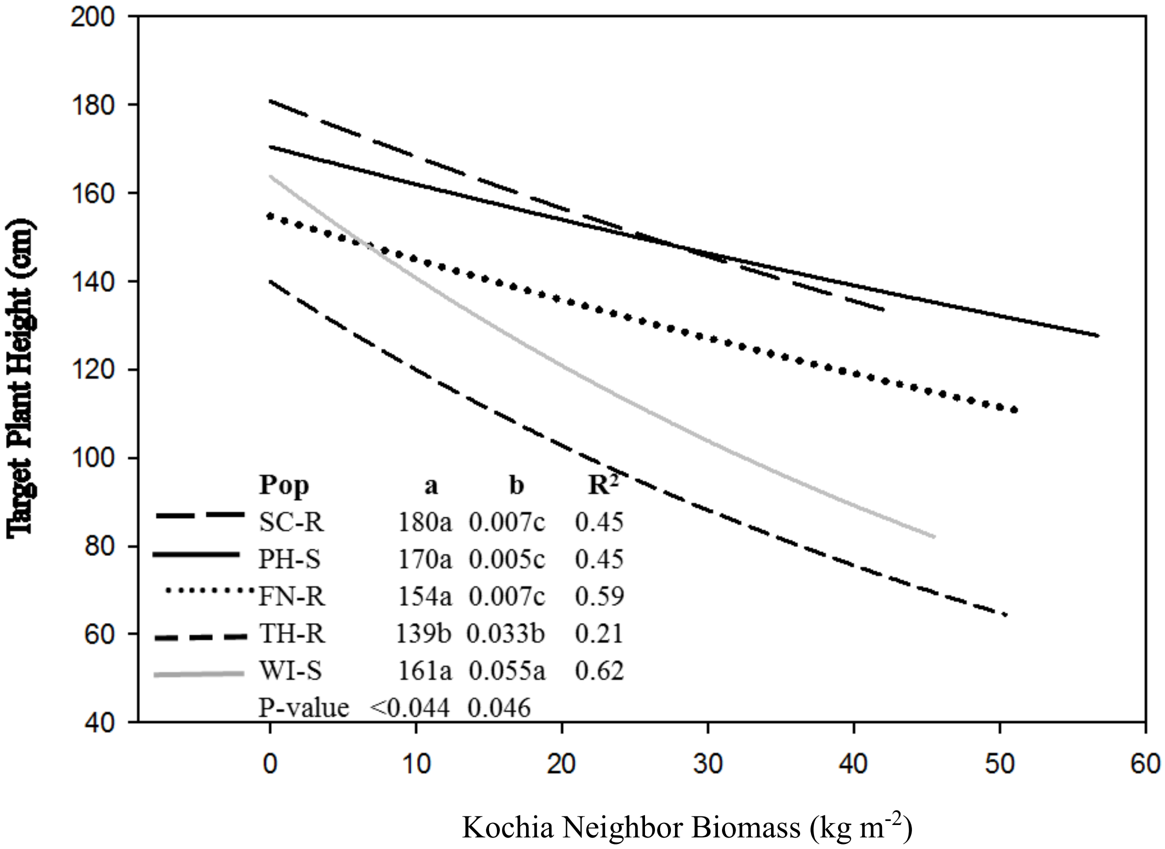


Figure 3.4: Response of target plant height (cm) to increasing kochia neighbor biomass (kg m^{-2}). Glyphosate-resistant populations are represented by broken lines while the glyphosate-susceptible populations are represented by solid lines. Equation 3.5 was fit to the data. Parameter **a** is the estimated target plant height in the absence of competition, **b** is the slope of the model which measures the competitive response of the target plant height and $\mathbf{R^2}$ is the coefficient of determination. Parameter estimates within a column with different letters are different (α= 0.05).

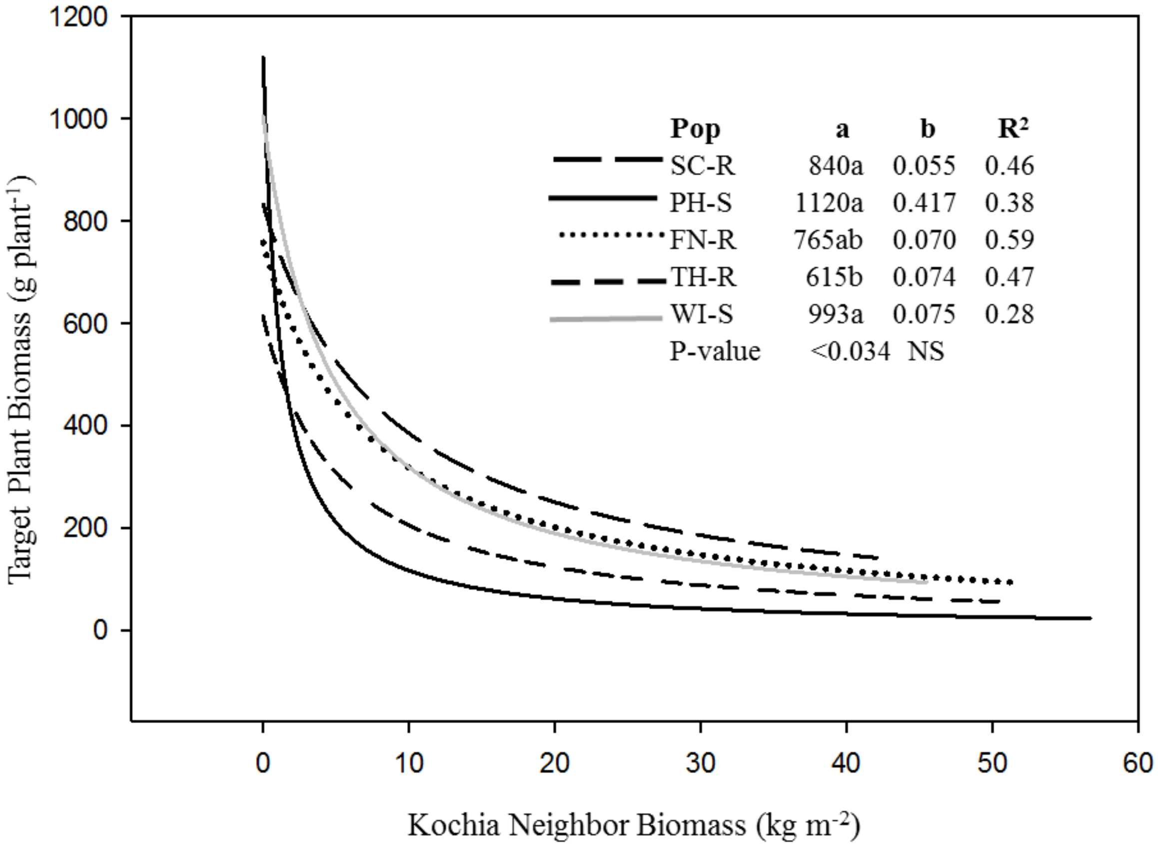


Figure 3.5: Response of target plant biomass (g plant^{-1}) to increasing kochia neighbor biomass (kg m^{-2}). Glyphosate resistant populations are represented by broken lines while the glyphosate susceptible populations are represented by solid lines. Equation 3.5 was fit to the data. Parameter **a** is the estimated target plant biomass in the absence of competition, **b** is the slope of the model which measures the competitive response of the plant biomass, and **R²** is the coefficient of determination. Parameter estimates within a column with different letters are different (α= 0.05).

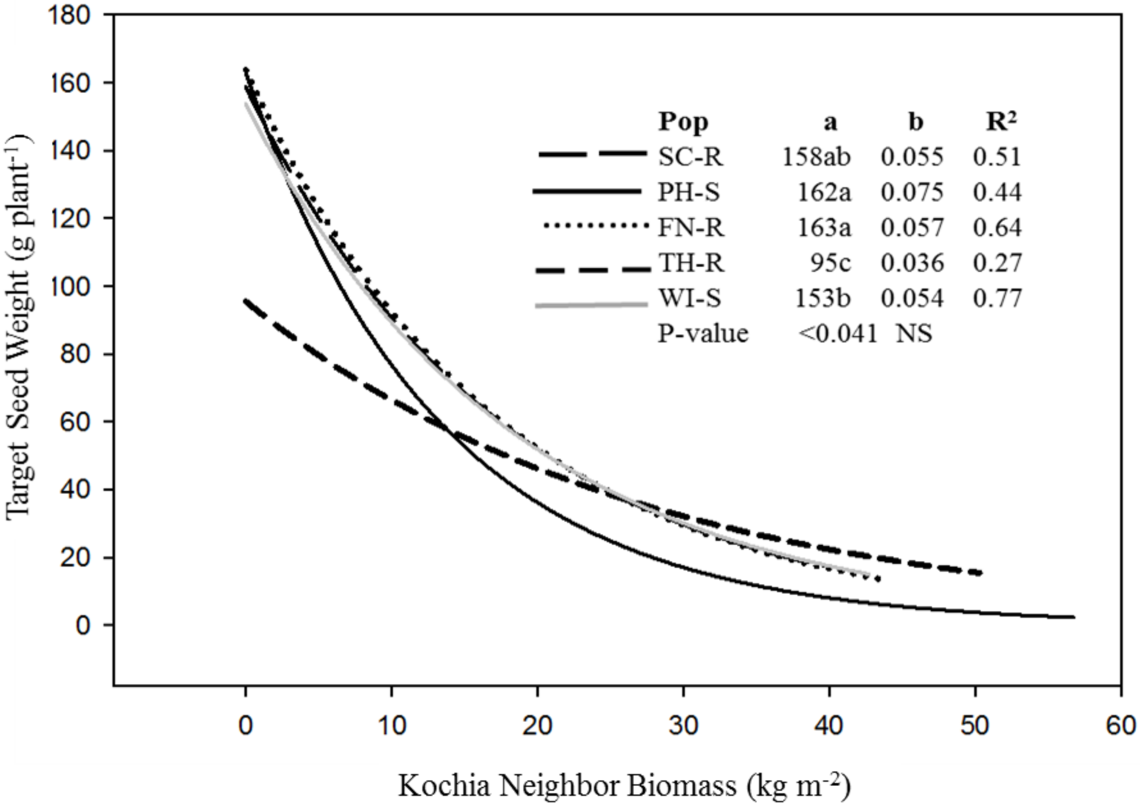


Figure 3.6: Response of target plant seed weight g plant^{-1}) to increasing kochia neighbor biomass (kg/m^{2}). Glyphosate resistant populations are represented by broken lines while the glyphosate susceptible populations are represented by solid lines. Equation 3.5 was used to fit the data. Parameter **a** is the estimated target plant seed weight in the absence of competition, **b** is the slope of the model which measures the competitive response of the target plant seed weight, x is the kochia neighbor biomass, and **R^{2}** is the coefficient of determination. Parameter estimates within a column with different letters are different (α= 0.05).

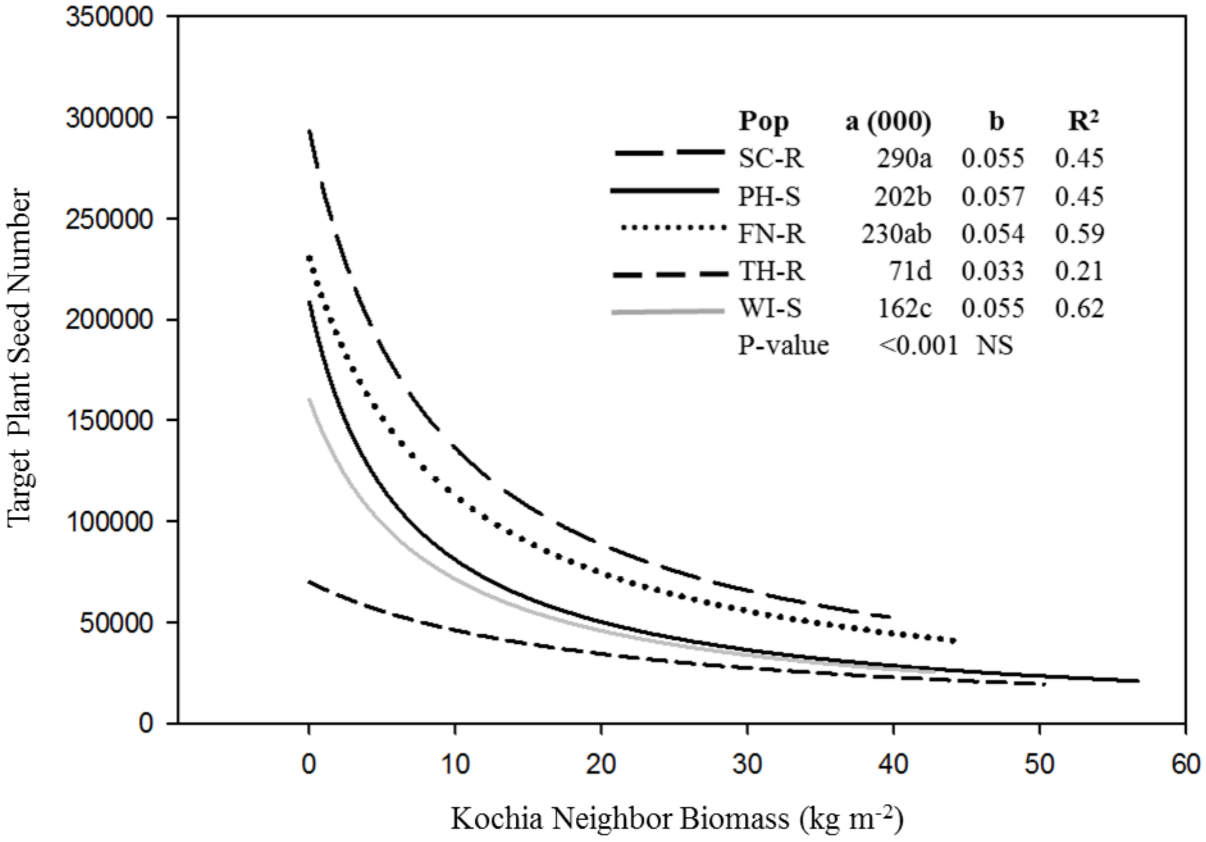


Figure 3.7: Response of target plant seed number to increasing kochia neighbor biomass (kg m^{-2}). Glyphosate resistant populations are represented by broken lines while the glyphosate susceptible populations are represented by solid lines. Equation 3.5 was used to fit the data. Parameter **a** is the estimated target plant seed number in the absence of competition, **b** is the slope of the model which measures the competitive response of the target plant seed number, x is the kochia neighbor biomass, and $\mathbf{R^2}$ is the coefficient of determination. Parameter estimates within a column with different letters are different ($\alpha = 0.05$).

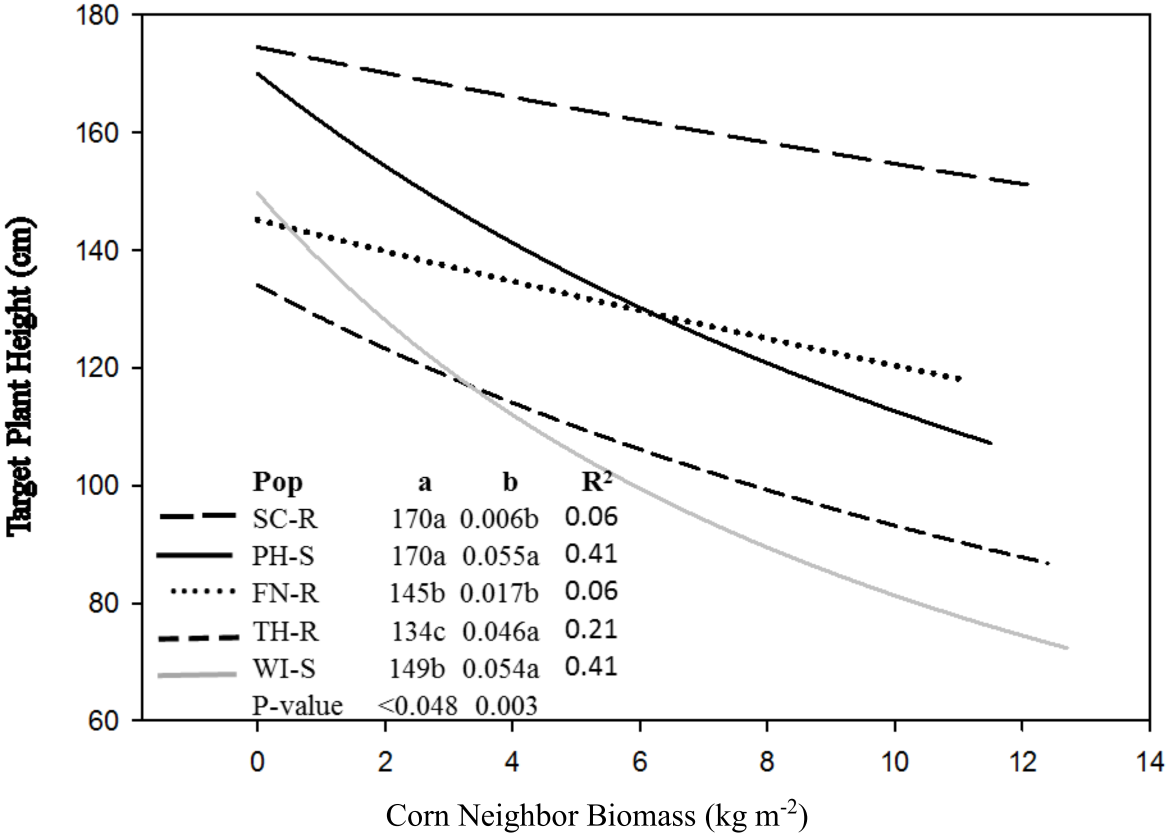


Figure 3.8: Response of target plant height (cm) to increasing corn neighbor biomass (kg m^{-2}). Glyphosate resistant populations are represented by broken lines while the glyphosate susceptible populations are represented by solid lines. Equation 3.5 was used to fit the data. Parameter **a** is the estimated target plant height in the absence of competition, **b** is the slope of the model which measures the competitive response of the target plant height, x is the corn neighbor biomass, and **R^2** is the coefficient of determination. Parameter estimates within a column with different letters are different (α= 0.05).

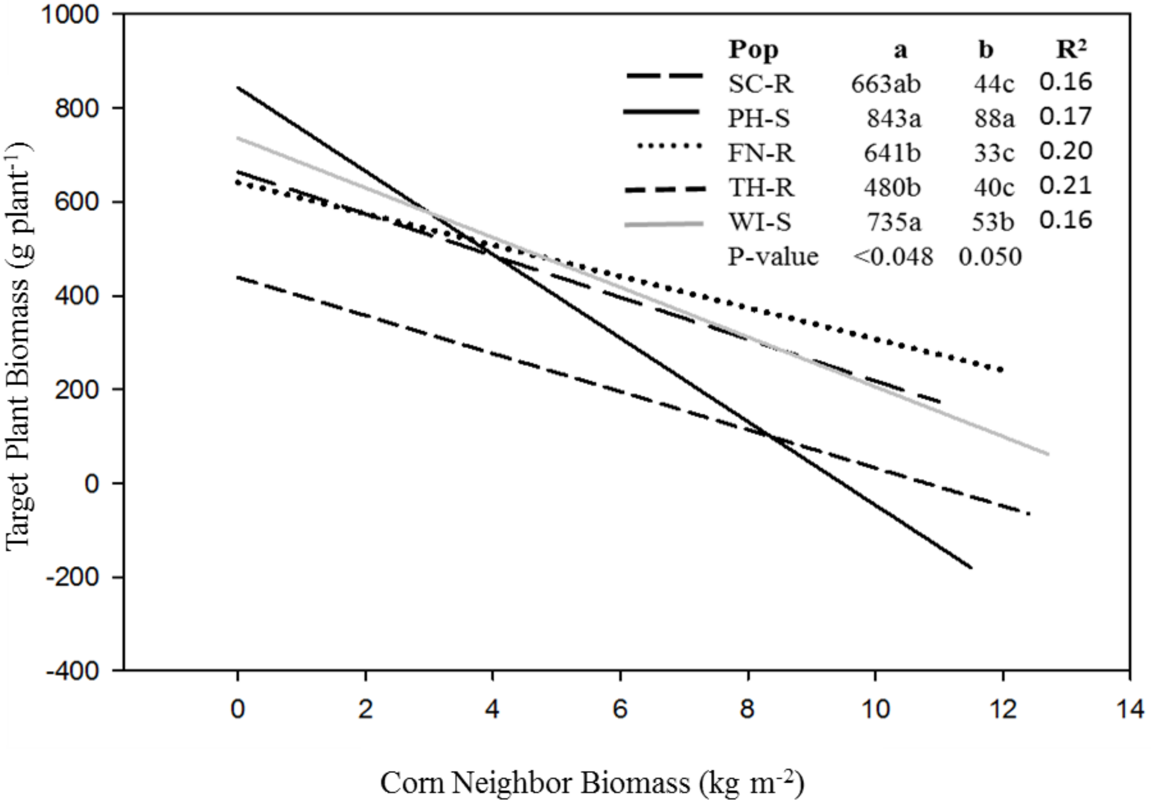


Figure 3.9: Response of target plant biomass (g plant^{-1}) to increasing corn neighbor biomass (kg m^{-2}). Glyphosate resistant populations are represented by broken lines while the glyphosate susceptible populations are represented by solid lines. Equation 3.6 was used to fit the data. Parameter **a** is the estimated target plant biomass in the absence of competition, **b** is the slope of the model which measures the competitive response of the target plant biomass, x is the corn neighbor biomass, and **R^2** is the coefficient of determination. Parameter estimates within a column with different letters are different (α= 0.05).

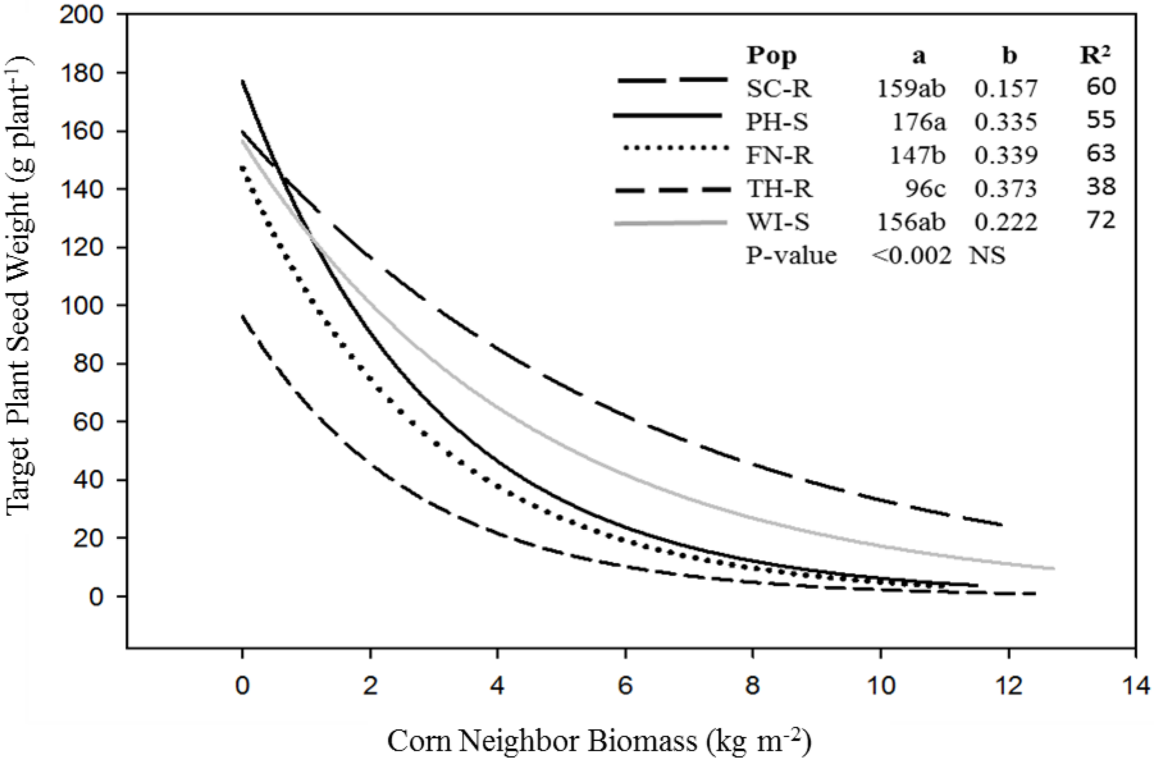


Figure 3.10: Response of target plant seed weight (g plant^{-1}) to increasing corn neighbor biomass (kg m^{-2}). Glyphosate resistant populations are represented by broken lines while the glyphosate susceptible populations are represented by solid lines. Equation 3.5 was used to fit the data. Parameter **a** is the estimated target plant seed weight in the absence of competition, **b** is the slope of the model which measures the competitive response of the target plant seed weight, x is the corn neighbor biomass, and **R^2** is the coefficient of determination. Parameter estimates within a column with different letters are different (α= 0.05).

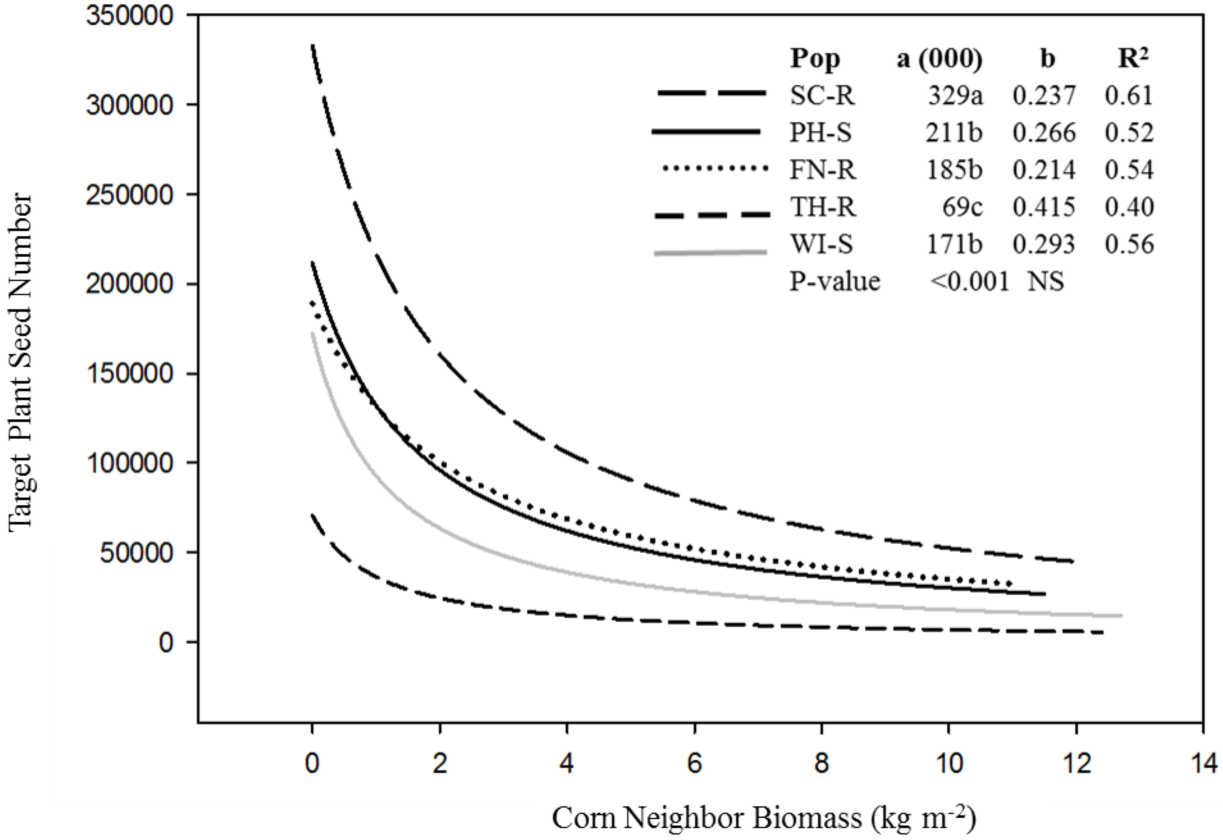


Figure 3.11: Response of target plant seed number to increasing corn neighbor biomass (kg m^{-2}). Glyphosate resistant populations are represented by broken lines while the glyphosate susceptible populations are represented by solid lines. Equation 3.5 was used to fit the data. Parameter **a** is the estimated target plant seed number in the absence of competition, **b** is the slope of the model which measures the competitive response of the target plant seed number, x is the corn neighbor biomass, and $\mathbf{R^2}$ is the coefficient of determination. Parameter estimates within a column with different letters are different (α= 0.05).

Table 3.1: Parameter estimates (SE) and coefficient of determination (R^2) for plant height of each population and year in response to low neighbor density when Equation 3.4 was fit to data. Parameter **a** represents plant height at harvest, **b** represents the slope of the curve and **c** represents the cumulative growing degree days at 50% of the maximum height.

		Parameter estimates (±SE)			
Year	Population	a	b	c	R^2
		cm		CGDD	
2014	SC-R	146 (21)	360 (112)	1,894 (108)	0.80
	PH-S	118 (21)	381 (138)	1,881 (133)	0.73
	FN-R	133 (36)	381 (220)	1,841 (223)	0.61
	WA-S	115 (26)	371 (176)	1,779 (180)	0.58
	TH-R	102 (15)	242 (106)	1,891 (107)	0.67
	WI-S	131 (30)	373 (186)	1,864 (183)	0.63
	P-value	0.038	NS	NS	
2015	SC-R	133 (11)	268 (79)	1,793 (84)	0.89
	PH-S	144 (12)	278 (72)	1,802 (77)	0.85
	FN-R	149 (26)	323 (146)	1,961 (155)	0.68
	TH-R	128 (10)	233 (67)	1,893 (72)	0.84
	WI-S	153 (8)	298 (46)	1,904 (49)	0.94
	P-value	NS	NS	NS	

CGDD = Cumulative growing degree days

NS = Not significant ($P > 0.05$)

Estimates with different letters are significantly different ($\alpha = 0.05$) within a column

Table 3.2: Parameter estimates (SE) and coefficient of determination (R^2) for plant height of each population and year in response to high neighbor density when Equation 3.4 was fit to data. Parameter **a** represents plant height at harvest, **b** represents the slope of the curve and **c** represents the cumulative growing degree days at 50% of the maximum height.

Year	Population	Parameter estimates (±SE)			R^2
		a	b	c	
		cm		CGDD	
2014	SC-R	102 (28)	4817 (244)	1545 (244)	0.59
	PH-S	99 (28)	1576 (662)	1629 (600)	0.45
	FN-R	80 (77)	634 (902)	1866 (826)	0.29
	WA-S	79 (38)	469 (417)	1466 (425)	0.34
	TH-R	37 (24)	927 (213)	1276 (787)	0.48
	WI-S	38 (13)	483 (311)	1265 (294)	0.53
	P-value	0.016	NS	NS	
2015	SC-R	159 (17)	251 (81)	1944 (89)	0.82
	PH-S	149 (21)	335 (121)	1827 (128)	0.74
	FN-R	96 (26)	411 (235)	2006 (247)	0.63
	TH-R	48 (14)	158 (198)	1889 (227)	0.24
	WI-S	35 (13)	260 (313)	1422 (366)	0.31
	P-value	<0.001	NS	NS	

CGDD = Cumulative growing degree days

NS = Not significant (P > 0.05)

Estimates with different letters are significantly different (α= 0.05) within a column

Table 3.3: P-value of analysis of covariance for fitness variables of populations at varying kochia neighbor densities. Not significant at p>0.05.

Fitness variables	Population		Density		Population by Density	
	2014	2015	2014	2015	2014	2015
Height at harvest (cm)	NS	0.004	0.001	0.001	0.021	0.002
Biomass at harvest (g)	NS	0.025	0.001	0.001	0.012	0.003
Stem diameter at harvest (cm)	NS	NS	NS	NS	NS	NS
Plant width at harvest (cm)	-	NS	-	0.001	-	NS
Days to flowering	0.004	0.017	0.032	NS	NS	NS
Total seed weight (g plant^{-1})	NS	0.048	0.001	0.001	NS	0.013
1000 seed weight (g)	NS	NS	NS	NS	NS	NS
Total seed number (plant^{-1})	NS	0.001	0.001	0.001	NS	0.016
Reproductive Effort	NS	NS	NS	NS	NS	NS

NS = Not significant

Table 3.4: Means of target plant height at harvest (cm) at different levels of kochia neighbor densities.

		Plant height at harvest			
		Kochia neighbor densities			
Year	Population	Low	Moderate	High	P-value
		-------------------- cm ----------------------			
2014	SC-R	140	89 a	91 a	NS
	PH-S	116	119 a	80 a	NS
	FN-R	128	87 a	65 a	NS
	WA-S	111	102 a	72 a	NS
	TH-R	108	43 b	79 a	0.004
	WI-S	126	81 a	30 b	0.002
	P-value	NS	0.02	0.03	
2015	SC-R	170	135	151 a	NS
	PH-S	150	145	149 a	NS
	FN-R	149	150	96 a	NS
	TH-R	108	114	45 b	0.003
	WI-S	154	134	39 b	0.001
	P-value	NS	NS	0.01	

NS = Not significant

Estimates with different letters are significantly different (α= 0.05) within a column

Table 3.5: Means of target plant biomass (g plant^{-1}) at different levels of kochia neighbor densities.

		Biomass at harvest			
		Kochia neighbor densities			
Year	Population	Low	Moderate	High	P-value
		--------------------- g plant^{-1} -----------------------			
2014	SC-R	1556	86	87 a	0.002
	PH-S	1077	561	71 a	NS
	FN-R	1430	389	127 a	0.01
	WA-S	1118	254	318 a	NS
	TH-R	561	161	211 a	NS
	WI-S	2302	57	17 b	0.001
	P-value	NS	NS	0.04	
2015	SC-R	315	181	66 a	NS
	PH-S	354	162	46 a	0.04
	FN-R	577	121	39 b	0.001
	WA-S	-	-	-	-
	TH-R	241	218	33 b	0.001
	WI-S	477	141	19 b	0.001
	P-value	NS	NS	0.001	

NS = Not significant

Estimates with different letters are significantly different (α= 0.05) within a column

Table 3.6: Means of seed weight (g $plant^{-1}$) and seed number ($plant^{-1}$) at different levels of kochia neighbor densities in 2015.

	Seed weight; 2015				Seed number; 2015			
	Kochia neighbor densities				Kochia neighbor densities			
Population	Low	Moderate	High	P-value	Low	Moderate	High	P-value
SC-R	63.50	37.40	13.1a	0.16	146,112	47,663	16,074a	0.02
PH-S	82.50	34.51	14.8a	0.05	113,074	70,713	16,323a	0.01
FN-R	120.57	50.72	1.7b	0.001	199,970	74,123	3,037b	0.001
TH-R	65.31	65.32	1.32b	0.001	41,086	62,626	1,105b	0.001
WI-S	74.60	39.01	0.82b	0.001	80,591	22,358	1,294b	0.001
P-value	NS	NS	0.001		NS	NS	0.001	

NS = Not significant

Estimates with different letters are significantly different (α= 0.05) within a column

Table 3.7: Analysis of covariance for target response under intra-specific (kochia) and interspecific (corn) neighbors for 10 and 35 plants m^{-2} neighbor densities.

	Type of neighbor			Type of neighbor by Density
Fitness variables	Corn	Kochia	P-value	P-value
Plant height at harvest (cm)	128	143	0.02*	NS
Biomass at harvest (g $plant^{-1}$)	239	292	0.04*	NS
Seed weight (g $plant^{-1}$)	29.9	63.9	0.02*	NS
Seed number ($plant^{-1}$)	45,300	85,703	0.006*	NS

* Significant difference at $\alpha \leq 0.05$.

NS = Not significant

Table 3.8: P-values of the analysis of covariance for pooled populations (Resistant (R) versus Susceptible (S)), neighbor density and interaction.

	RvsS		Density		RvsS by Density	
Fitness variables	2014	2015	2014	2015	2014	2015
Plant height at harvest (cm)	NS	NS	0.001**	0.001**	0.02*	NS
Biomass at harvest (g plant^{-1})	NS	NS	0.001**	0.001**	NS	NS
Stem diameter at harvest (cm)	NS	NS	0.001**	NS	NS	NS
Days to flowering	NS	NS	0.03*	NS	NS	NS
Total seed weight (g plant^{-1})	NS	NS	0.001**	0.001**	NS	NS
1000 seed weight (g)	NS	NS	NS	NS	NS	NS
Total seed number	NS	NS	0.001**	0.001**	NS	NS
Reproductive Effort	NS	NS	NS	NS	NS	NS

* Significant (≤ 0.05)

** Highly significant (≤ 0.01)

Chapter 4 - Relationship between 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) Gene Amplification and Ecological Fitness of *Kochia scoparia*

Abstract

Mechanism for glyphosate resistance in kochia is reported to be gene amplification of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Reports have shown that level of glyphosate resistance in kochia increases with increase in EPSPS gene copies. A field study was conducted near Manhattan, KS in 2014 and 2015 to evaluate the ecological fitness of six kochia populations from western Kansas. Initial discriminatory dose assay in the greenhouse showed that populations from Finney (FN-R), Scott (SC-R) and Thomas (TH-R) counties were glyphosate-resistant (GR) while populations from Phillips (PH-S), Wallace (WA-S) and Wichita (WI-S) counties were glyphosate-susceptible (GS). One individual target kochia plant from each of the populations was surrounded by one of three densities of neighbor kochia plants, equivalent to 12, 35 and 70 plants m^{-2} or one of two densities of neighbor corn plants, equivalent to 12 and 35 plants m^{-2}. EPSPS gene copy assay of each population was done with their respective genomic DNA using real time polymerase chain reaction (qPCR). Shikimate accumulation assay was also conducted to complement the qPCR results. Result showed that at least 75% of the individuals from the three GR populations had high gene copy numbers ranging from 3 to 16 while the three GS populations had gene copy numbers that were ≤ 2. About 25% of the individuals in the GR populations had EPSPS gene copy number as low as those found in GS populations. These further confirmed our results from discriminatory dose response in classification of these populations into GR or GS and that the GR populations were still segregating for glyphosate resistance. Shikimate accumulation assay corroborated the classification of the populations into GR and GS. Regardless of population and competition gradient, there was no relationship between EPSPS gene copy and fitness variables, except for 1000-seed weight which showed an increase with increase in EPSPS gene copy. Lack of associated fitness cost for glyphosate resistance endowed by amplified functional copies of EPSPS genes suggests that kochia plants with high EPSPS gene copy numbers are likely to persist in field populations, even without selection pressure, potentially leading to widespread of glyphosate-resistant kochia.

Introduction

Glyphosate is the most widely used agricultural herbicide globally (Woodburn 2000). Use of glyphosate to control weeds was the biggest single factor in promoting soil and moisture conserving no-till crop production systems in North America, as US alone accounts for 19% of global glyphosate use (Benbrook 2016). Rapid adoption of glyphosate-resistant (GR) crops since mid 1990s significantly increased the total volume of glyphosate herbicides applied and this was due to easy-to-use high level of in-crop weed control glyphosate provided (Benbrook 2016). This method of weed control has helped growers achieve more stable crop production under highly variable growing conditions. However, the increased use of glyphosate for weed control has led to rapid rise in evolution of glyphosate resistance in weeds in the US, Argentina, and Brazil (Heap 2016) where GR crops have been massively adopted (James 2014).

Through repeated imposition of the same herbicide, susceptible biotypes of a weed species in a population are controlled while herbicide-resistant biotypes of the same weed species if they are present, are left behind to reproduce and spread, leading to evolution of populations with increasing numbers of herbicide-resistant individuals. At least 32 weed species have developed resistance to glyphosate in the US (Heap, 2016). 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) is a key enzyme in the shikimate pathway which is crucial for biosynthesis of three aromatic amino acids (phenylalanine, tyrosine, and tryptophan). A variety of mechanisms confer resistance to glyphosate in plants including target site modification by mutation of EPSPS pro106 codon [Baerson et al. 2002 in goosegrass (*Eleucine indica*)], altered or reduced translocation of glyphosate suggested to be caused by vacuolar sequestration [(Koger and Reddy 2005 in horseweed (*Conyza canadensis*)], and EPSPS gene amplification [(Gaines et al. 2010 in Palmer amaranth (*Amaranthus palmeri*)].

In Kansas and Colorado populations, field-evolved resistance of kochia to glyphosate was because of amplification of one of two EPSPS alleles (genes) (Jugulam et al. 2014; Wiersma et al. 2015). When a glyphosate-resistant (GR) kochia plant has amplified the number of EPSPS genes, it leads to overproduction of EPSPS enzyme, such that even in the presence of glyphosate there would be an abundant supply of uninhibited EPSPS enzyme (Wiersma et al. 2015). In a GR kochia plant, there can be 3 to 10 times more functional copies of the gene encoding EPSPS compared to susceptible kochia plant (Gaines et al. 2016), and the more copies of the EPSPS gene in kochia, the greater its resistance level to glyphosate (Godar et al. 2015; Jugulam et al. 2014). Amplified EPSPS gene in GR kochia are in tandem arrangement on one chromosome in which segregation of EPSPS copies followed single locus inheritance (Jugulam et al. 2014). In GR Palmer amaranth, copies of the EPSPS genes were randomly dispersed throughout the genome with many more copies than kochia (Gaines et al. 2010), and likely mediated by transposable elements (Gaines et al. 2013).

Linkages between the genetics or physiology of weed resistance to herbicides and plant fitness are necessary to understand and predict the persistence, spread and possible elimination of herbicide-resistant individuals from a weed population. A mutation resulting in herbicide resistance can have negative pleiotropic effect on normal plant metabolism and this can reduce quality or quantity of the resources required for plant growth and reproduction; consequently, the mutation confers fitness costs on the herbicide-resistant individuals (Preston et al. 2006; Vila-Aiub et al. 2009). Cases of fitness cost as a result of evolution of herbicide resistance have been reported and the most common example in plants was in triazine resistance. Fitness cost has been widely attributed to triazine-resistant plants, where the chloroplastic *psbA* gene mutation that encodes for a serine-264 to glycine (Ser-264–Gly) amino acid substitution in the photosystem II (PSII) D1 protein causes a reduction in physiological activity, growth rates, resource competitive ability and fecundity, thus plants that are less fit than triazine-susceptible plants (Bergelson and Purrington 1996; Holt and Thill 1994; Jansen and Pfister 1990). As a consequence of reduced photosynthetic ability and reduced resource allocation, triazine-resistant biotypes were also shown to have reduced resistance to folivory and fungus infection (Gassmann and Futuyma 2005; Salzmann et al. 2008).

The study of ecological fitness is important in describing the evolutionary advantage of a biotype, which is based on its survivorship, fecundity and competitive ability (Shrestha et al. 2010; Sibony and Rubin 2002). Ecological fitness is better assessed in the presence of plant competition because in reality, plants are usually competing with each other in the field. The question is whether the genetic mechanism (EPSPS gene amplification) responsible for glyphosate resistance in kochia will result in a fitness cost for the resistant population in the absence of glyphosate application. If EPSPS gene amplification causes a fitness cost, do these costs increase steadily with the increased level of resistance caused by increase in the number of EPSPS gene copies?

Several researchers have studied whether glyphosate resistance in plants has an associated ecological fitness trade-off. Results of these studies suggest that fitness of GR plants varied by weed species, by mechanism of glyphosate resistance, by genetic background, and environmental stress such as competition for resources. When glyphosate resistance was conferred by reduced translocation in ryegrass (*Lolium rigidum)*, seeds produced by the GR individuals were less than those of the susceptible plants, and there was a decrease in the proportion of resistant individuals in the population from 45 to 11% after 3 years of no glyphosate use (Preston and Wakelin 2008). Similarly, Van Etten et al. (2016) reported that GR tall morningglory (*Ipomoea purpurea*) populations had lower germination rates, shorter roots and smaller aboveground size compared to their susceptible counterpart. Meanwhile, Wang et al. (2014) suggested that overexpression of a native rice EPSPS gene lead to fitness advantages for the GR lines, even without exposure to glyphosate. Similarly, Shrestha et al. (2010) reported that a GR biotype of horseweed accumulated more dry matter, had a greater number of flowers per plant, and more seeds per plant than the glyphosate susceptible (GS) horseweed grown in pots with increasing levels of neighbor competition and limited resources. Contrarily, no difference in growth or fecundity was reported in GR horseweed compared to susceptible biotype in a no-competition condition in the field (Davis et al. 2009). Brabham et al. (2011) reported that GR giant ragweed (*Ambrosia trifida*) displayed rapid, early season growth, but produced 25% less seed than the GS biotype. Kumar and Jha (2015) reported that EPSPS gene amplification-induced glyphosate resistance in kochia in Montana did not translate into a fitness penalty for the GR kochia biotypes across competition gradient in a study conducted in the greenhouse.

Similarly, Giacomini et al. (2014) showed that there was no significant effect of EPSPS gene copy number on biomass and seed production of GR Palmer amaranth. There were cases of fitness trade cost and benefits attributed to glyphosate resistance, but this cannot be generalized for glyphosate resistance.

Glyphosate-resistant kochia was first reported in 2007 from fields in western Kansas (Godar et al. 2015; Waite et al. 2013). There has been a paucity of information on the ecological fitness of EPSPS gene amplification-induced glyphosate resistance in plants, and this has not been done in the field environment. At the commencement of this study in 2014, there were no publications on the effect of EPSPS gene amplification on growth and reproductive fitness of GR kochia populations.

The presence and level of glyphosate resistance of individual plants in a kochia population can be determined non-destructively through molecular assay of EPSPS gene amplification, such that the plant can be available for other assessments (Giacomini et al. 2014; Godar et al. 2015; Jugulam et al. 2014; Wiersma, et al. 2015). Chatham et al. (2015) reported that this method successfully detected 91% of known GR individuals in a tall waterhemp (*Amaranthus tuberculatus*) population. There was strong correlation between EPSPS gene copy number and EPSPS protein abundance or shikimate accumulation in kochia (Godar et al. 2015; Kumar and Jha 2015; Wiersma et al. 2015). EPSPS gene copy number and protein abundance were elevated three- to ten-fold in GR kochia individuals relative to GS individuals (Gaines et al. 2016). Glyphosate-resistant kochia individuals with increased EPSPS gene copy numbers had consistently lower shikimate accumulation in leaf disks treated with 100 μM glyphosate (Wiersma et al. 2015).

When quantifying the relative fitness of a resistant population against a susceptible population, a comparison between just two populations (a susceptible and resistant) may not accurately estimate fitness costs associated with the resistance trait (Vila-Aiub et al. 2015). This comparison assumes that if the resistance allele was removed, the resistant biotype would behave exactly like the susceptible (Giacomini et al. 2014). In reality, large amount of natural genetic variation in most weed species, such as common sunflower (*Helianthus annuus*) (Lawson et al. 1994), tall waterhemp (Waselkov and Olsen 2014), Palmer amaranth (Chandi et al. 2013) and kochia (Mengistu and Messersmith 2002). This inherent genetic variation results in wide variation in fitness-related traits such as seed production and seed viability, even among individuals collected from the same field population (Giacomini et al. 2014). Comparative fitness between a pair of resistant and susceptible populations may erroneously attribute fitness cost to resistant trait whereas cost might have been as a result of genetic background (Jasieniuk et al. 1996) or segregation at fitness-related loci unrelated to the resistance trait (Giacomini et al. 2014). Kochia was reported to have high genetic diversity among and within populations (Mengistu and Messersmith 2002). To evaluate the relationship between EPSPS gene amplification and kochia fitness, populations with a range of EPSPS gene copy numbers would be beneficial (Preston et al. 2009). Resource competition driven by weed–weed and weed–crop competitive interactions could amplify fitness costs that are not greatly expressed in the absence of plant competition (Jordan 1999).

The objectives of this study were (1) to quantify the EPSPS gene copy number in the six kochia populations, (2) to relate EPSPS gene copy number and shikimate accumulation in order to further classify populations into GR and GS, and (3) to evaluate the relationship between

EPSPS gene copy number and kochia vegetative growth or reproductive fitness traits under intra- and inter-specific competition gradients in field.

Materials and Methods

The procedures described below were for two trials (2014 and 2015) except shikimate accumulation assay which was only done in 2014.

Cloning of Target Populations.

At 8 weeks after establishing the target kochia plants in the field as described in chapter 3, cuttings of 4 cm were taken from an actively growing branch from each target plant, placed in 250 ml water in miniature plastic pots and transported to the greenhouse while still fresh. In the greenhouse, each of the cuttings was reduced to a three-leaf segment that was 2 cm in length. Stem was cut at an angle at the bottom-most node and rooting hormone powder [Bontone, RootingPowder (Indole-3-butyric acid 0.10%) Bonide Products Inc, 6301 Suttiff Rd, Oriskany, NY] was added to the cut point. Cuttings were planted into 0.8 L plastic pots filled with potting soil (Miracle Gro, Marysville, OH) and the pots were covered with 18-cm humidity domes and sealed with duct tape to maintain humidity and minimize evapotranspiration. The greenhouse was maintained at 25/20 C day/night, photoperiod of 15/9 h day/night enhanced with 120 μmol m^{-2} s^{-1} illumination by sodium vapor lamps, and about 60% relative humidity. After 14 days in the humidity dome, the cloned plants appeared well established. They were uncovered and allow to grow to 7 cm in height under the greenhouse condition before leaves were collected for further assays.

EPSPS Gene Copy Number Assay.

Five actively growing young leaves of about 1 cm long were collected from each cloned target plant and placed into 96-deep-well plates for genomic DNA (gDNA) extraction. Leaf tissues were freeze-dried for 48 to 72 hrs at -40 C and 150 mbar vacuum. The freeze-dried samples were ground using 4.5 mm zinc-coated steel beads in 96-deep-well plates in a mixer mill (Retsch, Model 400) at 30 cycles per second for 4 min. Genomic DNA was extracted from each sample using a CTAB-based (cetyltrimethyl ammonium bromide) method modified from Doyle and Doyle (1987). This protocol was originally designed for big bluestem (*Andropogon gerardi*) but added 4% polyvinylpolypyrrolidone, 11 mM tris(2-carboxyethyl) phosphine hydrochloride (TCEP), and 14 mM sodium diethyldithiocarbamate trihydrate (DIECA) to the standard CTAB extraction buffer (M. Galliart, L. Johnston, and P. St. Amand, personal communication). Extracted gDNA was re-suspended in 10 mM Tris-HCl and stored at -20 C. Determination of gDNA concentration and quality using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) showed that 96% of the gDNA samples were free from protein impurities as 260/280 ratios were greater than 1.8. All gDNA samples were standardized to 10 ng μL^{-1}. A qPCR reaction was performed using a CFX96TM real-time detection system (Bio-Rad Laboratories Inc., Philadelphia, PA) to determine the EPSPS gene copy number in the extracted gDNA samples. Each qPCR reaction mix consisted of 8 µL of SYBR Green Master Mix (Bio-Rad Laboratories Inc., Philadelphia, PA), 2 µL each of forward and reverse primers (5 µM) and 2 µL of gDNA (10 ng μL^{-1}) to make the total reaction volume of 14 µL. The EPSPS gene copy number was measured relative to the ALS gene (endogenous gene). Primer sequences used for the EPSPS and ALS genes of kochia were previously reported (Godar et al. 2015; Varanasi et al. 2015). The ALS gene was selected as an endogenous gene due to its stability across kochia populations and the ALS gene copy number was expected to be low (Wiersma et al. 2015). The initial denaturation step was 95 C for 15 min, followed by 39 cycles of denaturation at 95 C for 30 s, and a combined annealing/extension step at 60 C for 1 min. Each reaction was done in three replicates. The EPSPS genomic copy number relative to ALS gene was quantified by the ΔCt method. The relative increase in the EPSPS gene copy number was calculated as $2^{\Delta Ct}$, where *C*t is the threshold cycle and Δ*C*t = *C*t target gene (*EPSPS*) – *C*t endogenous gene (*ALS*) (Gaines et al. 2010).

Shikimate Accumulation Assay.

The same set of cloned plants used for EPSPS gene copy assay in 2014 were used for shikimate accumulation assay using an *in vivo* leaf-disk assay (Shaner et al. 2005). From each cloned plant, four leaf disks of equal size (4-mm diameter) were obtained using a perforator from two representative leaves. The leaf disks were placed in a transparent 96-well microtiter plate with one disk per well. Three leaf disks were treated with 100 µL of 100 µM of glyphosate (Glyphosate PESTANAL®, analytical standard, Sigma-Aldrich Co. LLC, St. Louis, MO) while one leaf disk was treated with 100 µL of buffer (0.6902 g ammonium phosphate dissolved in 600 ml deionized water) as the no-glyphosate non-treated control. Then, plate was wrapped with clear, plastic wrap and incubated under light (200 µmol $m^{-2}s^{-1}$) for 16 h at room temperature. Plate was then frozen and thawed at -20 and 60 C, respectively, for 20 mins each. Leaf disks were then treated with 1.25 N HCl (25 µL) and incubated at 60 C for 20 mins. Twenty-five microliters of this new solution from each well was added to 100 µL of reaction buffer (periodic acid (0.25% v/v)/meta-periodate (0.25% v/v)) in a new transparent 96-well microtiter plate. The plate was incubated at 40 C for 20 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 optical density of OD380 using an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT). A shikimate standard curve was developed to convert the OD380 values to ng shikimate μL^{-1}. The value for non-treated samples was subtracted from their corresponding 100 µM glyphosate-treated samples to determine the change or accumulation of shikimate in ng shikimate μL^{-1} solution of each glyphosate-treated sample and average shikimate accumulation was calculated for each plant. These values were used to correlate EPSPS gene copy number with shikimate accumulations in classifying each kochia population as either GS or GR.

Field Fitness Variables.

The plant materials, field conditions, imposed treatments and fitness variables for this study were previously described (Chapter 3). The data from the field trials were used to evaluate the relationship between EPSPS gene copy number and fitness variables for each population. The competition design was target-neighborhood with three levels (10, 35, and 70 plants m^{-2}) for kochia neighbor (intraspecific) competition in both 2014 and 2015 and two levels (10 and 35 plants m^{-2}) for corn neighbor (interspecific) competition added in 2015. There was also a no-competition treatment added as control in 2015. The target kochia plant was in center of each level of competition. All treatments were arranged in a randomized complete block design. Plant height, stem diameter at base of the plant, plant width (as a measure of the widest canopy) were collected biweekly starting from 3 weeks after planting. Days to first flowering was documented. When target kochia plants reached physiological maturity (about 20 weeks after planting), plants were clipped at soil surface, bagged and dried at 40 C for 72 hr and weighed. Seeds from each plant were stripped off the plants and cleaned using an air column separator. Total and 1000-seed weight per plant were measured. Total seed number (TS) was calculated for each plant by dividing total seed weight (SW_T) by seed weight of 1000 seeds (SW_{1000}) multiplied by 1000. Reproductive effort was also calculated by dividing total seed weight (g plant^{-1}) of target plant by its respective plant biomass (g plant^{-1}).

Initial analysis of variance (ANOVA) showed that there was no interaction between year of trial and treatments for final plant height, final stem diameter, final biomass, 1000-seed weight, total seed number and seed weight per plant, hence, data were combined across years. Analysis of covariance (ANCOVA), a combination of linear regression and analysis of variance, was conducted using PROC Mixed procedure of SAS version 9.4 (SAS Institute, Cary, NC) to evaluate the relationship between EPSPS gene copy number on fitness variables such as final plant height, final stem diameter, final biomass, 1000-seed weight, total number of seeds, and reproductive effort. The regression aspect of the ANCOVA separated the effect of EPSPS gene copy number from the effect of population on the fitness variables. EPSPS gene copy number was the covariate in the PROC Mixed procedure while population was the independent variable for each of the fitness variables. Evaluation of interaction effects between EPSPS gene copy number and either population or neighbor density on fitness variables was also performed. The direction and strength of relationship between EPSPS gene copy number and fitness variables of each of the three GR populations (SC-R, FN-R and TH-R) and pooled GS populations (Gly-S) was conducted for each intraspecific neighbor density and across interspecific neighbor density (as there was no interaction between EPSPS gene copy number and interspecific competition gradient) using Pearson correlation analyses in SAS version 9.4 (SAS Institute, Cary, NC).

Graphical presentation of results was done using graphical packages in R V.3.2.3 (R Core Team 2015).

Results and Discussion

EPSPS Gene Copy Number and Shikimate Accumulation.

The EPSPS gene copy number of individuals in the confirmed GR populations ranged from 1 to 8 copies in SC-R and from 1 to 16 copies in FN-R and TH-R (Figure 4.1). The mean EPSPS gene copy number for SC-R, FN-R and TH-R was 5, 6 and 5, respectively, while the GS kochia populations had EPSPS gene copy numbers less than two (Figure 4.1). These results confirmed the characterization of the six populations as to being GR or GS based on the previous discriminatory dose assay and the shikimate assay (Chapter 2). Similar method of identification of GR individuals has been used in several other studies (Chatham et al. 2015; Giacomini et al. 2014; Godar et al. 2015; Kumar and Jha 2015; Wiersma et al. 2015). These results show that about 25% of individuals in each GR population had EPSPS gene copy numbers as low as those found in GS populations (Figure 4.1). This clearly demonstrated that the three GR kochia populations were still segregating for resistance as they have a wide range of EPSPS gene copy numbers. Few as three copies of the EPSPS gene were sufficient to confer resistance to glyphosate in kochia (Wiersma et al. 2015). The increase in EPSPS gene copy number resulted in an increase in EPSPS protein abundance (Wiersma et al. 2015) and consequently, an increase in phenotypic resistance to glyphosate in kochia (Godar et al. 2015; Jugulam et al. 2014; Kumar et al. 2015).

The GS populations accumulated 33.8 to 45.7 ng shikimate μl^{-1} while the GR populations accumulated 13.9 to 19.6 ng shikimate μL^{-1} for 16 hr incubation period (Figure 4.2).

Leaf disks of plants with few EPSPS gene copies accumulated more shikimate than plants with more EPSPS gene copies. Both GR and GS plants accumulated shikimate after glyphosate application but shikimate accumulation was expected to be less in GR than GS individuals (Pline et al. 2002). It has also been reported that there was negative correlation between EPSPS gene copy number and shikimate accumulation (Godar et al. 2015; Kumar and Jha 2015; Wiersma et al. 2015). Assessment of EPSPS gene copy and shikimate accumulation in plants provided rapid non-destructive identification of GR or GS populations. The shikimate accumulation results further validated the initial classification of these populations into GR and GS.

Relationship between EPSPS Gene Copy Number and Kochia Growth and Fecundity.

The ANCOVA showed that interaction between EPSPS gene copy and neighbor density was only significant for 1000-seed weight (Table 4.1). Increase in EPSPS gene copy number was positively correlated with an increase in 1000-seed weight at moderate neighbor density while no correlation in 1000 seed weight was observed with low and high neighbor densities (Figure 4.3). Interaction between EPSPS gene copy number and population was significant for final plant height, final stem diameter, and 1000-seed weight fitness variables (Table 4.1). A further analysis showed that the relationship between EPSPS gene copy number and these variables was only significant for TH-R, which was a negative correlation between EPSPS gene copy and plant height and stem diameter but a positive correlation for 1000-seed weight (Figure 4.4). The ANCOVA showed that regardless of population and neighbor density, EPSPS gene copy number had no relationship with growth or reproductive measurements except for 1000-seed weight (Table 4.1), which showed a significant increase with increase in EPSPS gene copy (Figure 4.5). The significant relationship between EPSPS gene copy number and 1000-seed weight may not be solely attributed to EPSPS gene amplification associated with glyphosate resistance, but because the GR populations were still segregating. Giacomini et al. (2014) reported that an initially revealed fitness cost associated with some variables in pooled GR families in Palmer amaranth were later found to be as a result of segregation of fitness-related loci other than EPSPS gene copy number. Similarly, McCloskey and Holt (1991) reported that differential response of triazine-resistant and -susceptible biotypes to the effect of growth temperature in biomass production was because of differential genetic background (nuclear or chloroplast genome) rather than differences in triazine resistance alleles. In this study, the kochia plants were from different populations; it was not unexpected that their environmental experience and genetic background may confound their response to any factor including EPSPS gene amplification. This suggests that in a fitness study using only one resistant biotype to measure fitness cost relative to a susceptible counterpart or within its segregating self may be misleading. In an outcrossing species, high genetic diversity across all loci are expected (Chandi et al. 2013) making it difficult to pinpoint the true causative sources of fitness differences (Giacomini et al. 2014). In kochia, genetic diversity (both between and within populations) is very high and maintained through substantial levels of gene flow through outcrossing (Mengistu and Messersmith 2002). Without examining several populations or biotypes, erroneous conclusions can be made on fitness cost as it relates to glyphosate resistance in kochia, particularly when using a non-isogenic line. As shown in this study, a GR population (TH-R) was showing fitness costs for final plant height, final stem diameter and reproductive effort, and a fitness benefit for 1000-seed weight under moderate kochia neighbor density; this could have been wrongly attributed only to EPSPS gene copy number if other populations (SC-R and FN-R) were not evaluated.

Across interspecific competition imposed by corn, ANCOVA (data not shown) coupled with correlation analysis (Figure 4.6) showed that there was no relationship between EPSPS gene copy number and kochia growth or reproductive measurements in this field study.

Conclusion

The three segregating GR kochia populations used for this study provided the opportunity to assess individual plant ecological fitness response across a wide range of EPSPS gene copies. However, no fitness cost could be attributed to amplification of EPSPS gene in these populations even when intraspecific or interspecific competition with neighbors occurred in a field situation. Lack of associated fitness cost for glyphosate resistance endowed by amplified functional copies of EPSPS genes suggests that kochia plants with high EPSPS gene copy numbers are likely to persist in field populations, even without selection pressure, potentially leading to widespread of GR kochia plants.

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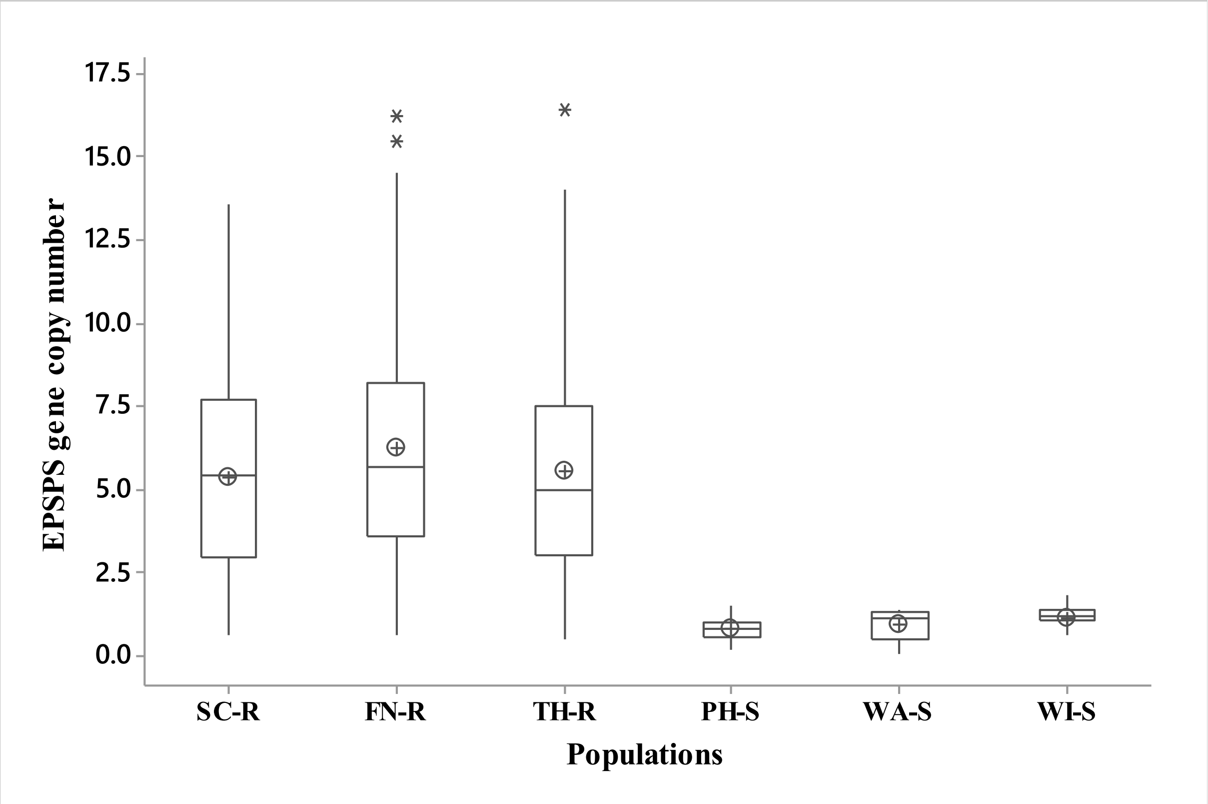

Figure 4.1: Box plots describing the distribution of EPSPS gene copy number of each kochia population; includes the mean (symbol), median (line), and quartiles (first 25, 50 and 75%). These populations were three supposed glyphosate-resistant kochia biotypes (SC-R, FN-R and TH-R) and three suspected glyphosate-susceptible kochia biotypes (PH-S, WA-S and WI-S). The EPSPS gene copy was estimated in relative to EPSPS gene copy (defaulted to be one) of a known glyphosate susceptible kochia plant.

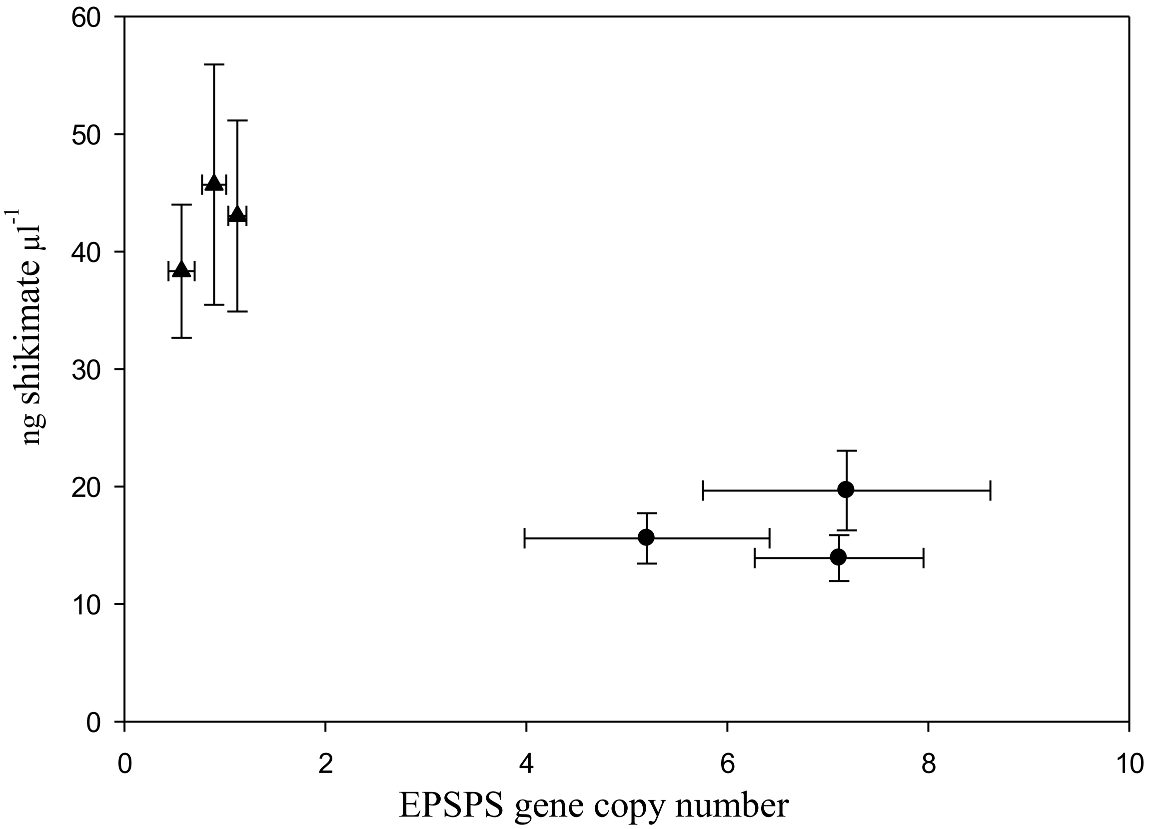

Figure 4.2: EPSPS gene copy number versus shikimate accumulation in glyphosate-resistant (SC-R, FN-R and TH-R) and -susceptible (PH-S, WA-S and WI-S) kochia populations. Shikimate accumulation was measured after incubation of samples in 100 μM glyphosate in an *in vivo* leaf-disk assay for 16 hr. The amount of shikimate in non-treated check was subtracted from glyphosate-treated leaf disks of each population. Error bars indicate standard error.

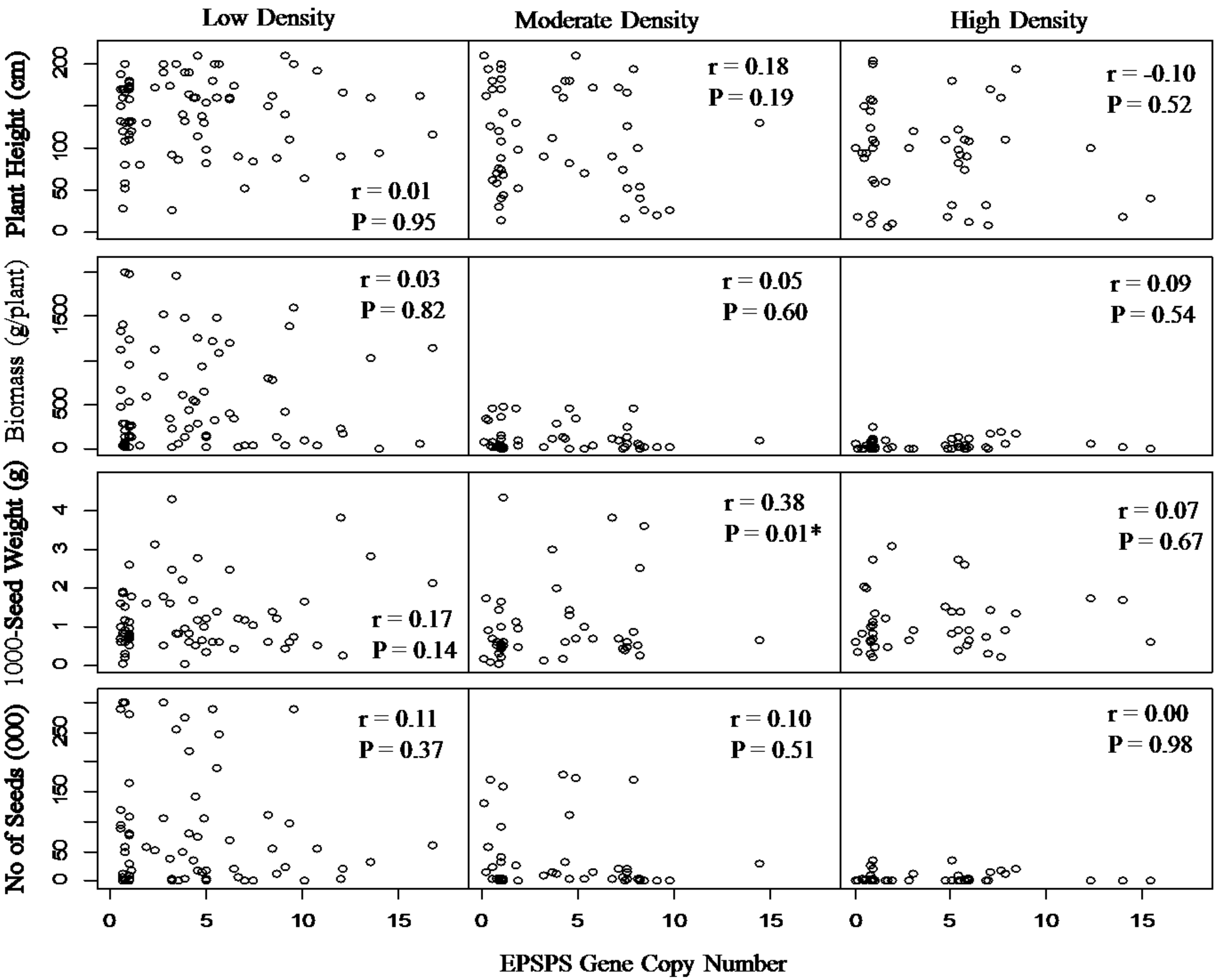
Low Density
Moderate Density
High Density
Plant Height (cm)
Biomass (g/plant)
1000-Seed Weight (g)
No of Seeds (000)
r = 0.01
P = 0.95
r = 0.18
P = 0.19
r = -0.10
P = 0.52
r = 0.03
P = 0.82
r = 0.05
P = 0.60
r = 0.09
P = 0.54
r = 0.17
P = 0.14
r = 0.38
P = 0.01*
r = 0.07
P = 0.67
r = 0.11
P = 0.37
r = 0.10
P = 0.51
r = 0.00
P = 0.98
0
50
100
200
500
1500
1
2
3
4
150
250
5
10
15
EPSPS Gene Copy Number

Figure 4.3: Relationship between EPSPS gene copy number and fitness variables of matured plant height, final biomass, 1000-seed weight (g), and number of seeds per plant for each target plant when grown at low (10 plants m^{-2}), moderate (35 plants m^{-2}), and high (70 plants m^{-2}) kochia neighbor densities.

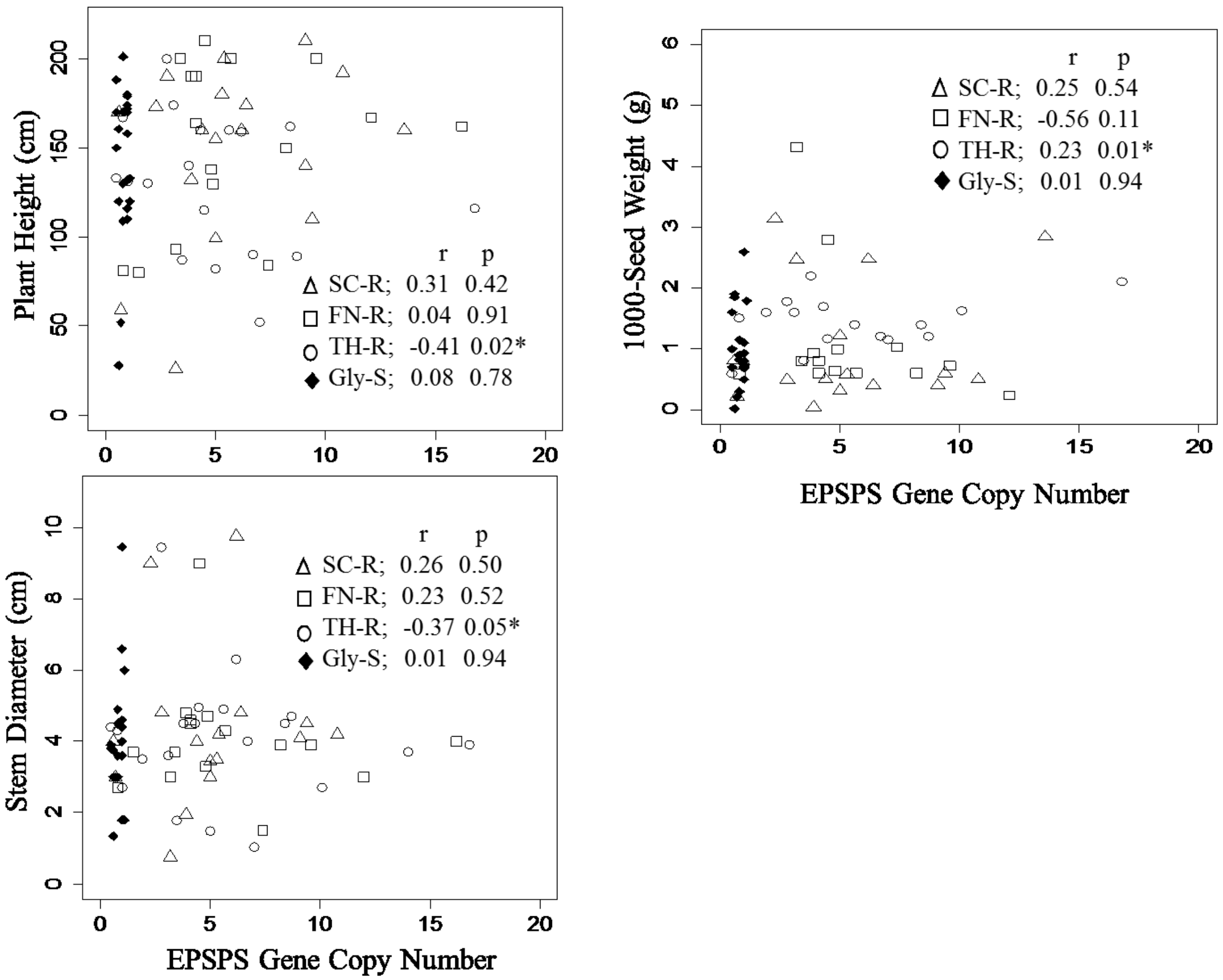

Figure 4.4: Relationship between EPSPS gene copy number and fitness variables of plant height, stem diameter and 100-seed-weight for each of the kochia populations. SC-R, FN-R and TH-R are segregating glyphosate resistant kochia populations while Gly-S represents the pooled glyphosate susceptible kochia populations. r is the correlation coefficient and p is the P-value. P ≤0.05 is significant

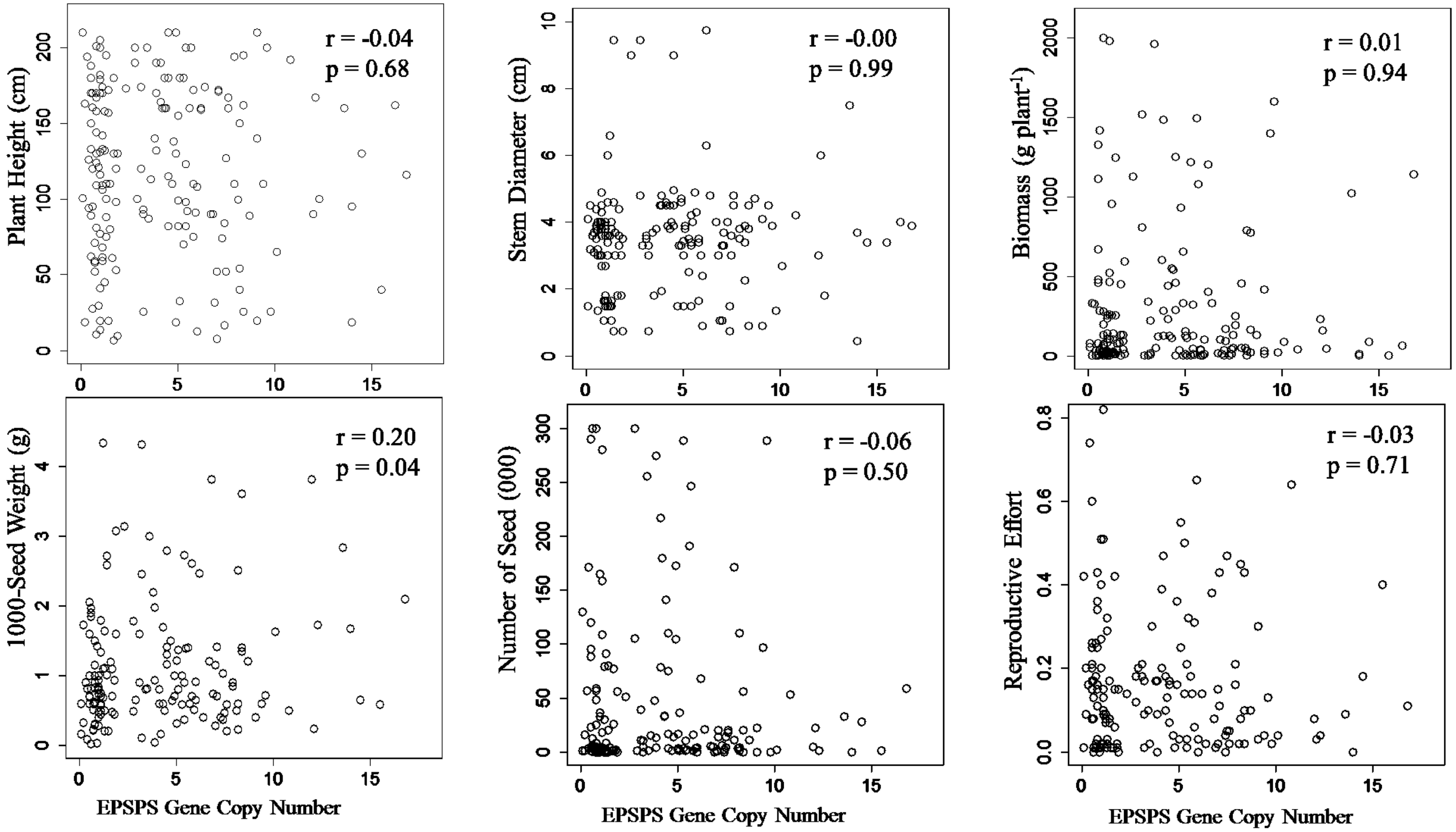

Figure 4.5: Relationship between EPSPS gene copy number and fitness variables for pooled kochia populations and intraspecific (kochia) neighbor densities. r is the correlation coefficient and p is the P-value. P ≤0.05 is significant

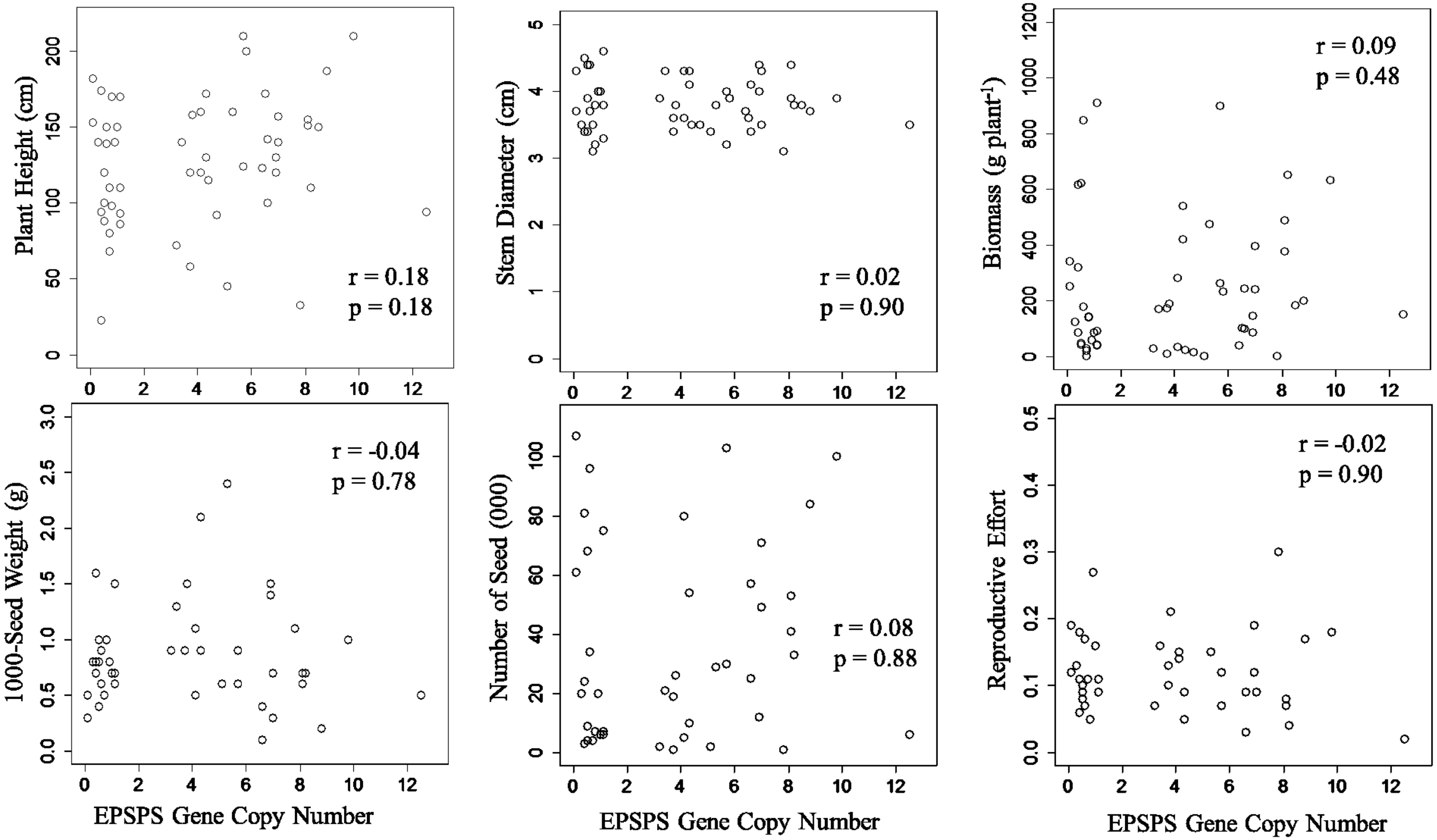

Figure 4.6: Relationship between EPSPS gene copy number and fitness variables for pooled kochia populations and interspecific (corn) neighbor densities. r is the correlation coefficient and p is the P-value. $P \leq 0.05$ is significant

Table 4.1: P-values of the ANCOVA on the effect of EPSPS gene copy number, and interaction between EPSPS gene copy number and kochia population or neighbor density on vegetative and reproductive variables.

Fitness variables	EPSPS copy	EPSPS copy*Population	EPSPS copy*Density
Plant height (cm)	0.522	0.042*	0.870
Stem diameter (cm)	0.814	0.021*	0.930
Plant width (cm)			
Biomass (g $plant^{-1}$)	0.656	0.623	0.842
Days to flowering	0.480	0.758	0.444
Total seed weight (g $plant^{-1}$)	0.587	0.532	0.709
1000-seed weight (g)	0.019*	0.025*	0.039*
Seed number ($plant^{-1}$)	0.458	0.860	0.876
Reproductive Effort	0.433	0.138	0.671

*Significant at α = 0.05

Chapter 5 - General Conclusions

This study showed that the kochia populations from western Kansas which were either suspected to be resistant or susceptible to glyphosate also showed some level of resistance to other herbicides with different mode of actions such as ALS-inhibitor, PS II-inhibitor, and synthetic auxins. This result suggests the importance of evaluating biotypes resistance to multiple herbicide modes of action in a study aimed at assessing the biological cost of evolution of resistance to a particular herbicide so that any biological trade-off or benefit are attributed to the appropriate mode of action resistance.

Despite our expectations, literature reviews have shown that fitness costs as a result of evolution of herbicide resistance in plant are not wide-ranging. This may not be unconnected with the differences in methodology and interpretation of studies that attempt to quantify fitness costs or evolutionary trade-offs associated with evolved resistance to herbicide. It is also very likely that fitness costs vary based on weed species, the mechanism of resistance involved, and the genetic background through which resistance is expressed. Nevertheless, conducting these studies to identify differences in life history traits had shown a great potential in detecting fitness cost.

Of the life history stages measured, fitness difference between the glyphosate resistant and susceptible kochia populations was consistently found in their germination characteristics. The GR populations showed less seed longevity, slower germination rate and less total germination than the GS populations. While plant growth rate, days to flowering, and seed production were not necessarily different between GR and GS population. Also, there was no major correlation between genetic basis of glyphosate resistance (EPSPS gene amplification) and plant growth and reproduction in kochia.

In general, this study suggests that any weed management strategy that delays the germination of seeds can help reduce the frequency of GR individuals in a kochia population in the field. Otherwise, once the GR individuals germinate and become seedling, they grow, reproduce and spread even under intense competition with either kochia neighbors or interspecific plant neighbor. Thus, agronomic practices that would take advantage of differences in the seed biology of GR and GS should be explored to help minimize or reverse the evolution of glyphosate resistance in kochia populations.

To gain further knowledge of the potential fitness costs associated with glyphosate resistance, future fitness studies of resistant weed species should examine fitness costs at various life history stages and across many populations. These findings should be incorporated in weed population models to explore the consequences of fitness penalties when a weed population evolve herbicide resistance and to identify potential management strategies to prevent, delay or reverse the evolution of resistance.