SURVEY FOR HERBICIDE RESISTANCE IN PALMER AMARANTH AND WATERHEMP IN TEXAS

A Thesis

by

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ABSTRACT

The distribution of herbicide resistance of Palmer amaranth and waterhemp to glyphosate, atrazine, tembotrione, fomesafen, and dicamba are unknown in the State of Texas. A random, semi-stratified survey targeting 6 farming regions where these species would most likely be found was conducted. Seeds from the two species were collected from 15 to 20 individuals to evaluate the sensitivity of these two species to the aforementioned herbicides. A total of 125 Palmer amaranth samples arriving from four regions, High Plains, Central TX, Gulf Coast, and Rio Grande Valley, were screened with the 5 herbicides applied POST at a recommended field rate. For waterhemp, a total of 115 samples originating from the two regions, Upper Gulf Coast and Central TX, were screened with these herbicides. At 21 days after application, a visual rating of survival (yes/no) and injury (0-100%) was taken as compared to a non-treated check and known susceptible/resistant standards. Results showed that resistance to glyphosate is widespread in the High Plains and Upper Gulf Coast, in Palmer amaranth and waterhemp, respectively. An EPSPS gene copy number analysis has revealed that the injury ratings were highly and negatively correlated with gene copy numbers, suggesting that EPSPS gene amplification is an important mechanism that endows resistance in the tested populations. Palmer amaranth resistance to atrazine was the greatest in the Upper High Plains where corn-based cropping systems are predominant. Likewise, atrazine resistant waterhemp was also found widespread in the Upper Gulf Coast region where corn and grain sorghum and widely grown. No resistance was observed for tembotrione,

but when ammonium sulfate was not added to the tank-mix (as recommended by the label), it revealed the regional differences in the level of sensitivity to this herbicide. Upper High Plains and the Upper Gulf Coast regions had the greatest number of Palmer amaranth and waterhemp populations, respectively that showed reduced sensitivity to tembotrione. Resistance to fomesafen and dicamba were not detected in the surveyed populations. However, at least one waterhemp population and two Palmer amaranth populations showed reduced sensitivity to reflex and dicamba, respectively. Results revealed that herbicide resistance is an emerging issue in Texas. Best management practices need to be implemented to manage existing resistant populations and also to reduce the risk of resistance evolution to future herbicide options.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a thesis committee consisting of Dr. Muthukumar Bagavathiannan (Chair), and Drs. Gaylon Morgan and Clark Neely (Department of Soil and Crop Sciences), and Dr. Thomas Isakeit (Department of Plant Pathology and Microbiology).

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

Herbicide resistance is a serious agricultural issue that threatens the sustainability of world food production. Reports of the evolution of herbicide resistance go back as early as the 1950s (Switzer 1957), long before the widespread use of herbicides and the adoption of herbicide-resistant crops. To date, herbicide resistance has been reported in 217 weed species with more than 450 unique cases (species x herbicide mechanism of action (MOA) combinations) in about 670,000 farms worldwide (Heap 2017), and continues to rise. Resistance has been reported to most major known herbicide mechanisms of action and no new herbicide with a unique mechanism of action has been marketed since 1991 (Duke 2012).

Herbicides remain the cornerstones for effective weed management in commercial production agriculture, but over-reliance on few herbicide MOA has resulted in the evolution of herbicide-resistant weeds. Glyphosate-resistant Palmer amaranth is the predominant issue in the Southern US, because of its competitive nature and propensity to develop resistance to multiple MOAs. Palmer amaranth is an annual forb native to the area encompassing northwestern Mexico and southern California to New Mexico and Texas (Sauer 1957). Palmer amaranth started to spread beyond its original range in the early 20th century, probably because of human activity transporting seeds or creating new habitats through agricultural expansion. This species was first reported in Virginia in 1915, Oklahoma in 1926, and South Carolina in 1957 (Sauer 1957). The first case of herbicide resistance (resistant to glyphosate) in Palmer amaranth

was reported in Macon County, Georgia (Culpepper et al. 2006) and was observed through-out the Southern US in a few years. Herbicide resistant Palmer amaranth has now infested more than 25 US states (Heap 2017). Surveys indicate that Palmer amaranth is one of the most problematic herbicide-resistant weeds causing great losses to cotton, corn, and soybean production in the Southern US (Morgan, et al. 2001, Riar et al. 2013; Webster and Nichols 2012). An important concern is the evolution of resistance to more than one herbicide MOA. The first Palmer amaranth population with multiple resistance to glyphosate and pyrithiobac-sodium (an ALS-inhibitor) was documented in 2006 (Sosnoskie et al. 2011). Palmer amaranth resistance to other important herbicide groups such as Triazines and Hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitors has also been documented in the Midwestern States (Thompson 2012; Jhala et al. 2014). As of now, Palmer amaranth has evolved resistance to at least six different herbicide MOA, including those above (Heap 2017).

Glyphosate resistance in Palmer amaranth is known to have evolved by over production of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme (Gaines 2010). In a resistant population a 40 to 100-fold increase in EPSPS copy number has been observed (Gaines 2010). Amplification of the EPSPS gene causes it to act as a molecular sponge to absorb glyphosate, allowing the uninhibited EPSPS to function normally after an application (Powles 2010). Target-site mutation and non-target site mechanisms may also contribute to glyphosate resistance in this species. Non-target site resistance to glyphosate through reduced absorption and translocation has also been reported (Chahal 2015).

Waterhemp, another amaranth species, is the most prominent and troublesome weed in agronomic crops in the Midwestern and Central US states, including Missouri, Iowa, and Illinois (Bradley et al. 2007). Its extended period of germination, rapid growth rate, and prolific seed production characteristics have enabled this weed to be persistent and dominant in crop fields (Hartzler et al. 2004; Sauer 1957). In Texas, this species is a significant weed issue in row-crop production systems in certain regions. Herbicide resistance has been widespread in this species as well. As of now, numerous waterhemp populations within the United States have been reported to be resistant to inhibitors of EPSPS, ALS, PPO, PSII, HPPD, and growth regulator herbicides (Heap 2017). Research in Illinois has revealed 10 to 35-fold resistance in waterhemp to the HPPD inhibitor mesotrione (Hausman et. al. 2011). McMullan and Green (2011) have reported similar results with a population found in Iowa with an 8-fold resistance to mesotrione, 10-fold resistance to atrazine, and a 28-fold resistance to thifensulfuron. In Texas, glyphosate-resistant waterhemp was confirmed in 2013 (Heap 2017).

Glyphosate resistance in waterhemp has been conferred through EPSPS gene amplification, a Pro106Ser amino acid substitution in the EPSPS enzyme, and a non-target-site mechanism (Bell et al. 2013; Chatham et al. 2013; Nandula et al. 2013). Resistance to PPO-inhibiting herbicides is highly conserved and is conferred by a codon deletion at amino acid 210 in the PPX2 gene (Patzoldt et al. 2006). PSII-inhibitor herbicide resistance may be conferred by a Ser264Gly amino acid mutation in the psbA enzyme (Foes et al. 1998; Merchant et al. 2008), but non-target-site resistance to atrazine is also common in waterhemp (Patzoldt et al. 2003). In a HPPD-inhibitor resistant

waterhemp population from Illinois, Ma et al. (2013) found no alterations in the HPPD sequence or HPPD expression. Rather, the mechanism of resistance was determined to be enhanced oxidative metabolism.

CHAPTER II

SURVEYING THE DISTRIBUTION OF HERBICIDE RESISTANCE IN PALMER AMARANTH ACROSS ROW CROP PRODUCTION SYSTEMS IN TEXAS

Introduction

Palmer amaranth (*Amaranthus palmeri* S. Wats.*)* is native to the Americas (Sauer 1957) and is one of the most troublesome, and economically challenging weeds throughout the southern United States (Ward et al. 2013). Palmer amaranth is dioecious (male and female flowers are found on separate plants) and is very competitive because of its ability to produce large amounts of seeds, rapid growth potential, extended emergence periodicity, water use efficiency, and survival under adverse conditions (Jha et al. 2006; Ward et al. 2013). These characteristics enable Palmer amaranth to effectively compete with crops for nutrients, water, light, and space, causing significant yield losses in crops such as cotton, corn, grain sorghum, and soybean (Bensch et al. 2003; Fast et al. 2009; Massinga et al. 2001; Moore et al. 2004; Morgan et al. 2001).

Herbicides have been heavily relied upon for the control of Palmer amaranth and this species appears to be a high-risk species for the evolution of herbicide resistance. In the southern US, Palmer amaranth resistance to glyphosate and acetolactate synthase (ALS)-inhibiting herbicides is prevalent (Culpepper et al. 2006; Sosnoskie et al. 2011; Wise et al. 2009). The first case of ALS-inhibitor resistant Palmer amaranth was reported in Kansas in 1993; resistance was observed throughout the southern US in a short period. However, the commercialization of glyphosate-resistant crop technology had facilitated the growers with an alternative herbicide option to effectively control

ALS-inhibitor resistant Palmer amaranth. Unfortunately, heavy reliance on glyphosate has led to the evolution of glyphosate resistance in Palmer amaranth. In 2004, the first case of glyphosate-resistant Palmer amaranth was confirmed in Macon County, Georgia (Culpepper et al. 2006). Over the past decade, glyphosate-resistant Palmer amaranth has been observed throughout the southern US and beyond. Currently, glyphosate-resistant Palmer amaranth is confirmed to occur in more than 25 US states (Heap 2017).

Glyphosate resistance in weeds is known to have evolved by a mutation in the target-site (Bostamam et al. 2012; Kaundan et al. 2008, 2011) or over production of the target-site enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Gaines et al. 2010), though the latter is most commonly reported in Palmer amaranth. In a resistant Palmer amaranth population, a 40 to 100-fold increase in EPSPS gene copy number has been observed (Gaines et al. 2010). Amplification of the EPSPS gene causes it to act as a molecular sponge to absorb glyphosate, allowing the uninhibited EPSPS to function normally after an application (Powles 2010). Non-target site mechanisms (reduced absorption, translocation, and/or metabolic degradation) may also contribute to glyphosate resistance in weeds. Non-target site resistance to glyphosate endowed in part by reduced herbicide translocation has been reported in Italian ryegrass (Bostamam et al. 2012; Gonzalez-Torralva 2012).

The practice of shifting to an alternative herbicide following resistance to a previously used herbicide has only perpetuated the problem by favoring the evolution of multiple herbicide resistance. For example, the first Palmer amaranth population with multiple resistance to glyphosate (EPSPS-inhibitor) and pyrithiobac-sodium

(ALS-inhibitor) was documented in 2006 (Sosnoskie et al. 2011). Palmer amaranth multiple resistance to other important herbicide sites of action (SOAs) such as Photosystem-II (PS-II)-inhibitors (e.g. triazines) and hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitors such as mesotrione and tembotrione has been documented in Nebraska (Jhala et al. 2014). More recently, Palmer amaranth with multiple resistance to ALS-, EPSPS-, and protoporphyrinogen oxidase (PPO)-inhibitor herbicides has been confirmed in the Mississippi Delta region (Heap 2017). The evolution of resistance to more than one herbicide SOA is of great concern as it limits the herbicide options available for effective weed control. Currently, Palmer amaranth is resistant to six SOAs (different levels of multiple resistance), including ALS-, EPSPS-, HPPD-, PS-II, mitosis-, and PPO-inhibitors (Heap 2017). Crops with engineered resistance to auxin herbicides such as 2,4-D and dicamba are currently under commercial cultivation and are viewed as effective tools for managing multiple herbicide resistant Palmer amaranth. However, a lack of management diversity could rapidly lead to the evolution of resistance to auxinic herbicides as well (Tehranchian et al. 2017).

The relative importance of independent evolution of resistance versus spread of resistance across landscapes is unknown. However, a study conducted by Bagavathiannan and Norsworthy (2016) has revealed that multiple resistance ALS-inhibitors and glyphosate is widespread in roadside Palmer amaranth populations in the Mississippi Delta region, suggesting the role of propagule movement through agricultural activities in the spread of herbicide resistance. Regardless of the source of

resistance, knowledge of the current status and distribution of herbicide resistance across agricultural regions will be invaluable in developing and implementing effective resistance management practices.

In Texas row-crop production, farmers have been frequently reporting Palmer amaranth control failures with certain herbicides. As of now, two cases of herbicide-resistant Palmer amaranth (resistance to atrazine in 1993 and to glyphosate in 2011) have been documented in Texas (Heap 2017). Anecdotal observations suggest that herbicide resistance could be widespread in this species across the state (Baumann 2013; McGinty et al. 2015). However, to date no systematic survey has been carried out in Texas to document the prevalence of herbicide resistance in Palmer amaranth. The objectives of this study were to: 1) survey the level of sensitivity of Palmer amaranth to important herbicides used in row crop production systems in Texas, and 2) examine the presence of molecular target-site mechanisms, if any, endowing glyphosate resistance in Palmer amaranth populations surveyed in Texas.

Materials and Methods

Field survey

Field surveys were conducted during late summer/fall 2014 to 2016 to collect Palmer amaranth samples throughout Texas for resistance evaluation. In order to obtain a representative sampling across important row-crop production areas in Texas, the survey was focused on six distinct regions: High Plains (from south of Lubbock to the Oklahoma border in the north), Rio Grande Valley (far south of Texas bordering Mexico), Coastal Bend (areas east and west of Corpus Christi, and towards San Antonio

in the north), Upper Gulf Coast (from Houston towards Victoria in the west and towards Columbus in the north), Central Texas (areas from San Antonio to Uvalde in the west and to Austin in the north), and Blacklands (from Temple to Tyler) (Fig. 1). Surveys were conducted following a semi-stratified survey methodology previously optimized and utilized by Bagavathiannan and Norsworthy (2016). The survey sites were randomly selected on a Google® map without any prior knowledge of the survey sites, using the ITN Converter software (version 1.88; Benichou Software). In each sub-region, about 50 random sites were marked. This software allows for optimizing the survey sites based on the most efficient travel route. The files were exported to a portable global positioning system (GPS) device (TomTom International, BV) that facilitated the navigation to the pre-determined survey sites. If Palmer amaranth was not present in the pre-determined survey site, the first population found along the route to the next survey site was collected. The survey specifically targeted uncontrolled Palmer amaranth escapes in row-crop production fields (cotton, corn, grain sorghum, and soybean). In each site, seed heads were harvested from about 15 random female Palmer amaranth plants, bagged, and dried in a hot-air oven at 50 C for 72 hours. Seed heads were mechanically thrashed, cleaned, and stored in glass vials prior to use in herbicide assays. A total of 220 samples were collected, of which 150 were randomly selected for the assays.

Herbicide assays

Herbicide assays were conducted at the Norman Borlaug Center for Southern Crop Improvement Greenhouse Research Facility at Texas A&M University. The

greenhouse was maintained at 30/26 C day/night temperature regime and 14 hr photoperiod. Resistance evaluations were carried out for five different herbicides each pertaining to different SOAs: glyphosate, atrazine, tembotrione, fomesafen, and dicamba. Glyphosate (EPSPS-inhibitor) has been widely used in glyphosate-resistant (Roundup Ready®) crops such as cotton, corn, and soybean throughout the state. Atrazine (PS-II inhibitor) has been an important herbicide historically in weed management programs of corn and grain sorghum. Tembotrione (HPPD-inhibitor) is labeled in corn and its use is expected to increase in the near future with the anticipated commercialization of HPPD-tolerant lines of cotton and soybean. Fomesafen (PPO-inhibitor) is labeled for use in cotton and soybean and has been an important pre-emergence herbicide in these crops. Dicamba (synthetic auxin) has been traditionally used in burndown applications prior to planting the row crops. Its use is expected to increase with the cultivation of dicamba-resistant (Roundup Ready® Xtend) cotton and soybean. The goal of this study is to establish resistance profiles for herbicides that have been already used widely in the state as well as the ones that are expected to be used more frequently in the near future.

All herbicide applications were made at the recommended field rates (Table 1). All herbicides were applied as postemergence to the weed (POST), but Palmer amaranth populations that showed resistance to POST applications of atrazine, tembotrione, or fomesafen were subjected to preemergence (PRE) evaluations. Seedlings were established in plastic growth trays (15 cm x 15 cm), with two replications and two experimental runs for each treatment. For POST assays, the trays were filled with potting

soil mix (LC1 Sunshine professional mix). Palmer amaranth seed were broadcast planted in each tray and thinned to approximately 30 to 40 seedlings at the 1-leaf stage. A known susceptible standard as well as a non-treated check were included for comparison for all herbicide treatments. The PRE herbicide evaluations were carried out using the growth trays (15 cm x 15 cm) filled with field soil collected from the Texas A&M Research Farm near Snook, TX in Burleson County (Belk Clay, pH 7.8). Non-treated controls were maintained for each of the population tested with PRE applications.

Herbicide applications were made using an automated spray chamber mounted with a TeeJet XR8002 nozzle calibrated to deliver 140 L ha^{-1} of spray liquid at an operating speed of 4.8 kmph and a pressure of 276 kPa. The POST applications were made at the 2- to 3-leaf seedling stage, whereas the PRE applications were made immediately after planting. After the PRE treatment, the pots were watered immediately to activate the herbicide. Evaluations were carried out 21 days after herbicide application (DAT). Injury ratings were conducted on a scale of 0 to 100%, in increments of 5, with 0 representing no visible injury compared to non-treated control and 100 indicating complete plant death. Additionally, survival frequency was recorded as the number of seedlings survived the herbicide application out of the total number of seedlings treated.

Dose-response assays

Dose-response assay was conducted on a highly resistant sample for each herbicide. The herbicide doses used on the susceptible populations were 1/32, 1/16, 1/8, 1/4, 1/2, 1, and 2X the recommended field rate. For the putative resistant populations, the herbicides were applied at 1/2, 2, 4, 8, 16, and 32X rates. Each treatment included

three replications and two experimental runs; each replication consisted of 6 seedlings established in a six-cell growth tray (POST) or a 15 cm x 15 cm flat (PRE) planted with 25 seeds. The trays were filled with potting soil media (POST applications) or with field soil (PRE herbicides). Herbicide applications were made at the 2- to 3-leaf seedling stage (POST) or immediately after planting (PRE) as described earlier. Visual observations were carried out at 21 DAT. For POST dose-response assays, survival (%) was recorded as the number of surviving seedlings out of the total number of seedlings sprayed. For PRE assays, survival (%) was scored as the number of surviving seedlings in the treated trays compared to the non-treated control maintained for each population.

EPSPS target-site analysis

Population selection

Palmer amaranth populations with varying levels of resistance to glyphosate from the four regions where this species was prevalent (High Plains, Rio Grande Valley, Central Texas, and Coastal Bend) were selected for EPSPS gene copy number analysis. For this purpose, populations were selected based on the injury ratings observed for glyphosate in greenhouse screening. A total of seven populations were selected from each region: three highly resistant populations (0-20% injury), three populations with reduced sensitivity (50-80% injury) and a susceptible population (90-100% injury). Leaf tissues were collected from three random surviving plants in each population, which served as replicates for each population. Leaf tissue samples for the susceptible population were obtained prior to herbicide application.

DNA extraction

Total genomic DNA was extracted using a modified hexadecyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle 1990). Briefly, 0.05 g of leaf tissue was placed in 2-ml microtubes (Qiagen, Germantown, MD) containing two stainless steel beads (Qiagen, Germantown, MD). To each microtube, 500 µl of the CTAB extraction buffer (containing 100 mM Tris-HCl, 20 mM EDTA, 2 M NaCl, 2% CTAB, 2% polyvinylpyrrolidone-40, 1 mM phenanthroline, and 0.3% β-mercaptoethanol) was added. The sample was then homogenized using a Retsch Mixer Mill MM400 (Verder Scientific Inc., Newtown, PA) at 30 Hz for 2 min. After adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) to each tube, the mixture was incubated at 55 C for 60 min, followed by centrifugation at 12,000 rpm for 10 min. The supernatant was transferred to a new 1.5 ml centrifuge tube (Eppendorf North America, Hauppauge, NY) containing an equal volume of absolute isopropanol, mixed by gently inverting the tubes, and incubated overnight at -20 C. The DNA was then pelleted by centrifugation at 12,000 rpm for 10 min. The DNA pellet was washed with absolute ethanol, air dried, and re-suspended in 30 µl of 1x TE (containing 10 mM Tris-HCl, and 1 mM ethylenediaminetetraacetic acid [EDTA]). The genomic DNA was quantified using a NanoDrop 2000c V. 1.0 spectrophotometer (NanoDrop Technologies, Wilmington, DE), diluted to 1 ng μl^{-1} with deionized water, and the diluted samples were stored at 4 C for further analysis.

Quantification of EPSPS gene copy number

The EPSPS gene copy number in each sample was determined using a real-time quantitative polymerase chain reaction (RT-qPCR). The ALS gene was used as a positive control for these reactions. The primer sets for the EPSPS gene, EPSF1 (5′-ATGTTGGACGCTCTCAGAACTCTTGGT-3′) x EPSR8 (5′-TGAATTTCCTCCAGCAACGGCAA-3′) and the ALS primer sets, ALSF2 (5′-GCTGCTGAAGGCTACGCT-3′) × ALSR2 (5′- GCG GGACTGAGTCAAGAAGTG-3′) were designed based on sequences from Palmer amaranth (Gaines et al. 2010). For the qPCR reactions, 25 µL reaction mixes were prepared using 12.5 µL of SYBR green supermix and 2.5 µL of genomic DNA. Each sample was run for each primer set in triplicate. The following thermoprofile was used: 95 C for 3 min for initial denaturation, 40 cycles of 30s at 95 C for denaturation, and 60 C for 60s for annealing/extension. This program was then followed by a melt curve analysis at 55 C for 30s. The cycle threshold (Ct) values were calculated using the BioRad iQ5 thermocycler software.

Determination of EPSPS target-site mutation

For the glyphosate-resistant populations that did not show elevated EPSPS gene copy numbers, subsequent experiments were conducted to determine if mutations were present at the herbicide target site. In this regard, conserved regions of the EPSPS gene were sequenced. Forward (EPSPSF- 5'CCAAAAGGGCAGTCGTAGAG 3') and reverse (EPSPSR- 5'ACCTTGAATTTCCTCCAGCA 3') primers (Varanasi et al. 2015) were used. The 25 µl PCR reaction mix consisted of 12.5 µl of 2x PCR master mix

(Takara Bio USA, Inc.), 2.5 µl of each primer (5 µM), 4 µl of the genomic DNA (50 ng μl^{-1}), and 3.5 µl of water. The thermoprofile used in the PCR reaction was as follows: 95 C for 3 min followed by 40 cycles of denaturation at 95 C for 30s, annealing at 53.5 C for 45s, and final extension at 72 C for 7 min. After the reaction, the plates were maintained at 4 C until further processing. The PCR products were separated using the electrophoresis procedure in 1% agarose gel and were purified using a NucleoSpin® Gel and PCR Clean up kit (Takara Bio USA, Inc.). The samples were sequenced at the Institute for Plant Genomics & Biotechnology, Texas A&M University, College Station, TX. The nucleotide sequences obtained were aligned using the Bioedit software (version 7.2.6, NCSU) to determine potential single nucleotide polymorphisms conferring herbicide resistance. The sequences were aligned using known EPSPS sequences available on the National Center for Biotechnology Information (NCBI) database.

Statistical Analyses

Spatial distribution of Palmer amaranth sensitivity to various herbicides across Texas was mapped using ArcGIS (version 10.5; ESRI). Kernel density analysis was used to illustrate the distribution densities of Palmer amaranth across different regions of Texas (Fig. 2). Interpolation analysis was implemented to predict the distribution of Palmer amaranth sensitivity to herbicides across a spatial scale using the limited number of sample data points obtained in the study (e.g. see Fig. 4). Two different spatial maps were generated for each herbicide to illustrate a) the level of sensitivity to the given herbicide (e.g. Fig. 4a), and b) the frequency of survivors in a given population to show the stage of advancement of resistance (e.g. Fig. 4b).

Plant survival (%) was regressed against herbicide dose using a 3-parameter logistic regression equation, which provided the best fit to the data. All regression analyses were carried out using the SigmaPlot software (version 13, Systat Software, Inc., San Jose, CA). The amount of herbicide that caused 50% mortality of the test population (LD_{50}) was estimated from the regression equations. Resistance ratio (R/S) was computed as the LD_{50} value of the resistant population divided by the LD_{50} of the susceptible standard. The relative EPSPS gene copy number estimates for the Ct data were calculated using the following formulas:

$$(1)\ \Delta\mathrm{Ct} = \mathrm{Ct\ of\ target}\ (EPSPS) - \mathrm{Ct\ of\ reference\ (ALS)}$$

$$(2)\ \Delta\Delta\mathrm{Ct} = \Delta\mathrm{Ct\ of\ Treatment\ Individual} - \Delta\mathrm{Ct\ of\ Control}$$

$$(3)\ \mathrm{Copy\ number} = 2^{-\Delta\Delta\mathrm{Ct}}$$

A Pearson correlation analysis was performed, using SigmaPlot (version 13, Systat Software, Inc., San Jose, CA), to determine the strength of association between the EPSPS gene copy number estimates and the injury levels obtained.

Results and Discussion

Regional distribution of Palmer amaranth

Targeted surveys in important row-crop production areas across Texas have revealed the level of distribution (area and density of infestation) of Palmer amaranth in these regions (Fig. 2). In particular, Palmer amaranth infestations were the greatest in the High Plains, followed by Rio Grande Valley, Coastal Bend, and Central Texas regions

(particularly in areas west of San Antonio), whereas very little Palmer amaranth infestation was observed in the Upper Gulf Coast and Blacklands regions. These infestations were consistent with the major cotton, corn, and grain sorghum producing regions of Texas (except for the Upper Gulf Coast and Blacklands) (Fig. 3).

Response to glyphosate

Of the 140 Palmer amaranth populations evaluated in this study, 32% of the samples were resistant (0-49% injury) and 18% were less sensitive (50-89% injury) for glyphosate (Fig 4a; Table 2). Texas High Plains had the greatest number of non-susceptible (resistant or less sensitive) populations compared to other regions for glyphosate. Sixty two percent of all Palmer amaranth populations evaluated from the High Plains region were resistant to glyphosate (0-49% injury), followed by 19% in Central Texas and 13% each in Rio Grande Valley and Coastal Bend regions (Table 2). The Central Texas region had the highest frequency (81%) of susceptible populations compared to other regions. Dose-response assay on one of the resistant populations (TX-GR7) revealed 30-fold resistance to glyphosate in this population compared to the susceptible standard (TX-S29) (Fig. 5; Table 3). The LD_{50} values of the glyphosate-resistant and susceptible populations were 3429 and 113 g ai ha^{-1}, respectively.

The frequency of seedling survival to glyphosate in each tested population followed a very similar trend with the regional-scale distribution of resistance. The Palmer amaranth populations collected from the Texas High Plains region had the

highest number of survivors, followed by Rio Grande Valley and Coastal Bend regions (Fig. 4b). The survival frequency indicates the stage of advancement of resistance in a given population. In early stages of evolution, resistance could not be noticed by growers since the resistant individuals are present at low frequencies and the survivors are usually mistaken for weed escapes caused by application and/or environmental factors. Growers typically start noticing resistance when plant survival is approx. >20%. Glyphosate resistance is already in the advanced stages of evolution in the majority of Palmer amaranth populations in Texas High Plains, indicating that growers in this region have failed to recognize the issue soon enough and implement more diversified practices. The Texas High Plains region is characterized by intensive cultivation of glyphosate-resistant cotton and corn, often with limited crop and/or herbicide diversity. In some pockets of this region, monoculture cotton is common. These practices have likely led to frequent applications of glyphosate and high selection pressure for the evolution of resistance to this herbicide. Frequent use of glyphosate in glyphosate-resistant crops has influenced the evolution of glyphosate-resistant weeds elsewhere (Culpepper et al. 2006; Norsworthy et al. 2008; Steckel et al. 2008). The survival frequency analysis has also revealed that glyphosate resistance is in the early stages of evolution in Palmer amaranth populations in Central Texas and Coastal Bend regions. With adequate measures, farmers in these regions can address the glyphosate-resistance issue before it becomes noticeable, at which point it will be too late. Norsworthy et al. (2012) have outlined a number of best management practices (BMPs) that can be utilized to delay/manage herbicide resistance in weeds.

Response to atrazine

Palmer amaranth resistance to atrazine (POST applications) was confirmed in 17% of the 135 populations tested, with about 7% of the populations showing resistance and 10% showing less sensitivity to this herbicide (Table 2). Atrazine resistance is particularly prevalent in the Texas High Plains (Fig. 6a), with about 16 and 22% of the populations classified as resistant and less sensitive, respectively (Table 2). About 4% of the Palmer amaranth collected from the Coastal Bend region was resistant to atrazine, whereas no resistance was detected among the samples obtained from Central Texas and Rio Grande Valley regions. Dose-response assay (POST) conducted on a resistant population (TX-AR16) showed >32-fold resistance to atrazine compared to a susceptible standard (TX-S29) based on the LD_{50} values (Fig. 7; Table 3). The TX-AR16 atrazine resistant population could not be killed at 32X the recommended field rate, which was the highest dose tested in this study. Further, resistance to atrazine is in its advanced stages of evolution (>50% seedling survival) in the High Plains, especially in the Upper High Plains region (Fig. 6b) compared to the other areas. In this region, corn and grain sorghum are widely grown (Fig. 3), where atrazine has been an important herbicide historically. Palmer amaranth resistance to atrazine is in its very early stages of evolution in few pockets of the Rio Grande Valley and Coastal Bend regions, whereas resistance was not detected in the Central Texas region. Implementation of herbicide resistance BMPs is vital to delay the evolution of Palmer amaranth resistance to atrazine in these areas. A subset of the Palmer amaranth populations that showed resistance to POST atrazine applications were tested for their response to atrazine PRE. Results showed that

the POST atrazine resistant populations from the Coastal Bend region were susceptible to PRE applications, whereas approx. 50% of the populations from the Upper High Plains were also resistant to atrazine PRE and resistance was present at noticeable frequencies (Fig. 8; Table 2). Multiple resistance to both glyphosate and atrazine (POST) was also common in Palmer amaranth collected in the Texas High Plains, with about 31% of the tested populations from this region showing resistance or less sensitivity to both of these herbicides.

Response to tembotrione

Palmer amaranth response to tembotrione generally followed the trend for glyphosate and atrazine in terms of regional variability in response (Fig. 9 a,b; Table 2). It is important to note that the tembotrione applications did not include ammonium sulfate (AMS) to the mix as required by the Laudis® label, thus representing a reduced efficacy/application rate. None of the tested populations showed resistance to tembotrione. Results, however, revealed the regional-scale variability in sensitivity to tembotrione in tested Palmer amaranth populations under these potentially reduced rates. About 39, 23, and 17% of the tested populations, respectively, from the High Plains, Rio Grande Valley, and Coastal Bend regions showed reduced sensitivity to tembotrione at this application rate (Table 2).

About 18% of the tested populations from the High Plains region that showed resistance or reduced sensitivity to atrazine also showed reduced sensitivity to tembotrione. Rapid metabolic degradation is an important mechanism of crop selectivity to HPPD-inhibitor herbicides such as tembotrione (Schulte and Kocher 2009)

as well as the PS-II inhibitor atrazine (Shimabukuro 1967). In a HPPD-inhibitor (mesotrione)- and atrazine-resistant waterhemp population documented in Illinois, Ma et al. (2013) have found that rapid metabolism through distinct detoxification mechanisms was responsible for resistance. Application of cytochrome P450 monooxygenase inhibitors such as malathion and tetcyclacis increased HPPD-inhibitor activity in the resistant seedlings, suggesting the potential role of cytochrome P450 family of genes in metabolic degradation of the herbicide. Given this evidence, it is likely that the prevalence of atrazine resistance in Palmer amaranth in the Texas High Plains is predisposing some of the populations with decreased sensitivity to tembotrione at reduced rates. In particular, higher frequencies of seedlings showed reduced sensitivity to tembotrione in populations collected from the Upper High Plains. This region also had the most resistant populations for atrazine POST as well as PRE applications (Fig. 6b, 8). Botha et al. (2014) conducted a survey for sensitivity to tembotrione in Palmer amaranth populations collected across Arkansas and reported that Palmer amaranth survival to this herbicide ranged from 2 to 51% in the tested populations, with injury levels ranging from 80-99%. These findings support the inherent variability in tolerance to tembotrione in Palmer amaranth, likely influenced by prior exposure to this and other herbicides.

It has been well established that weed escapes resulting from sub-lethal herbicide applications can accumulate minor resistance alleles through outcrossing and gradually increase the level of resistance (i.e. polygenic resistance) in subsequent generations (Busi et al. 2013; Gressel 1995a; Neve and Powles 2005). Additional

research will be carried out to evaluate Palmer amaranth response to tembotrione with the addition of AMS to the tank mix. Nevertheless, current results illustrate the potential risks associated with reduced rate applications of this herbicide. In addition to intentionally cutting application rates, low dose scenarios may occur with less than optimal application considerations (e.g. lack of an adjuvant), inefficient equipment and calibration, and/or poor environmental conditions. Thus, it is imperative to pay close attention to the factors affecting herbicide efficacy and minimize the risk for the evolution of polygenic resistance; high herbicide efficacies will select away from polygenic resistance in weed populations (Gressel 1995b).

Response to fomesafen

None of the tested Palmer amaranth populations showed any resistance to the field rate of fomesafen (Fig 10a; Table 2). PPO-inhibitor herbicides have been used historically in cotton and soybean production in Texas. Considering the prevalence of Palmer amaranth in the majority of cotton production areas in the state, it is likely that they have been exposed to PPO-inhibitor herbicides used in cotton fields. Recently, PPO-inhibitor herbicide resistance has been widely reported in Palmer amaranth populations in Arkansas and Tennessee (Heap 2017; Salas et al. 2016). This possibly has resulted from heavy reliance on PPO-inhibitor herbicides for controlling multiple ALS-inhibitor- and glyphosate-resistant Palmer amaranth populations in the Mississippi Delta region. The absence of resistance to PPO-inhibitor herbicides in our screening does not completely exclude the possibility of PPO resistance occurring in Texas. In fact, some populations had few survivors, though severely injured and stunted (Fig.

10b). However, results indicate that PPO-inhibitor resistance is not currently a concern on a regional scale in Texas, but it is imperative that growers continue to preserve the utility of this herbicide SOA through the implementation of proactive resistance management strategies.

Response to dicamba

Following the reports of glyphosate-resistant Palmer amaranth, management programs in the southern US have actively utilized dicamba (auxinic herbicide) for spring burndown applications (Norsworthy et al. 2007). The frequency of use of dicamba on Palmer amaranth is expected to increase with the cultivation of dicamba-resistant crops. In the current survey conducted in Texas, none of the Palmer amaranth populations survived dicamba applications (Fig. 11a). However, two populations had few individuals with reduced sensitivity to this herbicide at 21 DAT. While it is unlikely that these plants will regrow to produce seed under greenhouse conditions, variable field conditions could allow some of these survivors to perpetuate and increase the risk of resistance through recurrent selection, as previously described. Tehranchian et al. (2017) have shown under greenhouse conditions that recurrent selection using sub-lethal doses of dicamba can reduce the susceptibility of Palmer amaranth to this herbicide.

Dicamba-resistant Palmer amaranth has not been documented till date in the US, but populations with reduced sensitivity to this herbicide has been reported recently in Tennessee (Steckel 2017).

Documented field resistance to synthetic auxin herbicides is among the lowest in comparison to other herbicide SOA (Heap 2017). This could be attributed to the

complex mode of action of synthetic auxins, presence of rare alleles endowing resistance in nature, and potential fitness penalties associated with resistance imparting alleles (Jugulam et al. 2011). Nevertheless, at least 29 weed species have evolved resistance to auxinic herbicides worldwide (Heap 2017). Bernards et al. (2012) reported at least 10-fold less sensitivity to 2,4-D (synthetic auxin herbicide) in a waterhemp population in Nebraska. This waterhemp population has also exhibited reduced sensitivity to dicamba. Field evidence indicates that continuous use of 2,4-D for more than 10 consecutive years has favored auxin resistance in this population (Bernards et al. 2012). More recently, Shergill et al. (2017) confirmed 2,4-D resistance in a waterhemp population in Missouri, which was already resistant to glyphosate, chlorimuron (ALS-inhibitor), atrazine, and fomesafen. What is evident is that potential resistance of Palmer amaranth to the auxin herbicides will leave the growers with very few alternative options for effectively controlling this species. Proper stewardship programs must be in place to prolong the utility of the auxin-resistant crop technologies.

EPSPS target-site analysis

EPSPS gene copy number and plant injury ratings to glyphosate were highly correlated (r = - 0.96), suggesting that elevated EPSPS gene copy numbers is likely the primary cause of glyphosate resistance in tested populations (Fig. 12). This trend was similar for all the three regions (High Plains, Rio Grande Valley, and Central Texas) investigated for the EPSPS target-site analysis (Fig. 13). The amplification of the EPSPS gene produces an abundant supply of the EPSPS enzyme, which ultimately reduces the

impact of glyphosate on the resistant plants (Gaines et al. 2010; Salas et al. 2012). Gene amplification has been reported as one of the most prominent mechanisms of glyphosate-resistance in several weed species. In the present study, EPSPS gene copy numbers ranged from 1 to 158 across the glyphosate-resistant populations. Gaines et al. (2011) suggested that 30 to 50 EPSPS genomic copies are necessary to provide resistance to glyphosate at rates ranging from 0.5 to 1 kg ai ha^{-1}. However, the number of EPSPS gene copies required for evading the effect of glyphosate may vary depending on the species and presence of other resistance mechanisms. Moreover, inheritance of the elevated copy numbers to subsequent generations is often unpredictable (Chandi et al. 2012).

Though EPSPS gene amplification appears to be the major mechanism responsible for glyphosate resistance in the Palmer amaranth populations evaluated in Texas, there were at least two resistant populations (TX-GR25 and TX-GR27) that did not exhibit elevated copy numbers. Thus, mutation at the EPSPS gene or a non-target-site mechanism (reduced absorption, translocation, and/or metabolic detoxification) is likely responsible for glyphosate resistance in these populations. To determine the presence of known target-site mutation, a small region of the *EPSPS* gene was sequenced from the genomic DNA extracted from these two populations. The partial EPSPS sequence of the resistant plants did not reveal any previously known mutation at the sites of Thr_{102} or Pro_{106} (data not shown). Salas et al. (2012) have reported a similar scenario where some of the glyphosate-resistant Italian ryegrass populations neither correlated with gene copy numbers nor did they have any known mutation at the

glyphosate target-site. It is likely that these populations possess non-target site mechanisms and require further investigation.

Table 1. Details of the herbicides used in the evaluations

Common name	Trade name	Site of Action (Group)[a]	Rate (g ai/ae ha^{-1})	Adjuvant[c]	Manufacturer
Glyphosate	Roundup Powermax®	EPSPS-inhibitor (9)	868[b]	None	Monsanto Company, St. Louis, MO
Atrazine	Aatrex®	PSII-inhibitor (5)	1,115	1% v/v COC	Syngenta Crop Protection, Greensboro, NC
Tembotrione	Laudis®	HPPD-inhibitor (27)	93	1% v/v MSO	Bayer CropScience, Research Triangle Park, NC
Dicamba	Clarity®	Synthetic auxin (4)	284[b]	1% v/v COC	BASF Corporation Agricultural Products, Research Triangle Park, NC
Fomesafen	Reflex®	PPO-inhibitor (14)	213	1% v/v COC	Syngenta Crop Protection, Greensboro, NC

[a]Abbreviations: EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; HPPD, 4-hydroxyphenylpyruvate dioxygenase; PPO, protoporphyrinogen oxidase; PSII, photosystem II

[b]Values represent acid equivalency (g ae ha^{-1})

[c]COC – Crop Oil Concentrate; MSO – Methylated Seed Oil

[d]Laudis® applications did not include ammonium sulfate (AMS)

Table 2. Herbicide resistance profile in Palmer amaranth samples evaluated from across four different sub-regions of Texas

Herbicide	Sub-region	Resistant[a]	Less sensitive[a]	Susceptible[a]	Total[b]
		----------% of populations ----------			
Glyphosate	High Plains	62	21	17	52
	Central Texas	19	0	81	32
	Coastal Bend	13	25	63	24
	Rio Grande Valley	13	25	63	32
Atrazine (POST)	High Plains	16	22	62	50
	Central Texas	0	0	100	32
	Coastal Bend	4	9	87	23
	Rio Grande Valley	0	3	97	30
Tembotrione[c]	High Plains	0	39	61	49
	Central Texas	0	0	100	32
	Coastal Bend	0	17	83	24
	Rio Grande Valley	0	23	77	30
Fomesafen (POST)	High Plains	0	0	100	50
	Central Texas	0	0	100	32
	Coastal Bend	0	0	100	24
	Rio Grande Valley	0	0	100	28
Dicamba	High Plains	0	3	97	30
	Central Texas	0	0	100	14
	Coastal Bend	0	0	100	14
	Rio Grande Valley	0	7	93	15

[a]Resistant - 0 to 49% injury, less sensitive - 50 to 89% injury, and susceptible - 90 to 100% injury

[b]Total number of populations evaluated for each herbicide in each region

[c]Tembotrione applications did not include ammonium sulfate (AMS) adjuvant.

Table 3. *LD*50[a] values and resistance ratios for the highest resistant Palmer amaranth populations sampled across Texas for glyphosate and atrazine

Herbicide	Population	Regression equation	R^2	RMSE	LD_{50} (g ae/ai ha^{-1})	SE	R/S[b]
Glyphosate	TX-GR	$Y = 140/[1+e^{0.0005(x-2156)}]$	0.97	9	3429	477	30
	TX-GS	$Y = 106/[1+e^{-0.020(x-107)}]$	0.96	10	113	18	
Atrazine	TX-AR16	-	-	-	-	-	>32[c]
	TX-AS	$Y = 3598027/[1+e^{0.0023(x+4614)}]$	0.92	13	339	79	

[a]LD_{50} is the herbicide rate (g ae ha^{-1} for glyphosate and g ai ha^{-1} for atrazine) that caused 50% plant mortality at 21 days after treatment

[b]R/S (resistance ratio) was calculated based on the LD_{50} values of the resistant population relative to the susceptible standard

[c]The regression equation could not be developed on this atrazine-resistant population because complete mortality could not be achieved even at the highest rate tested (32X)

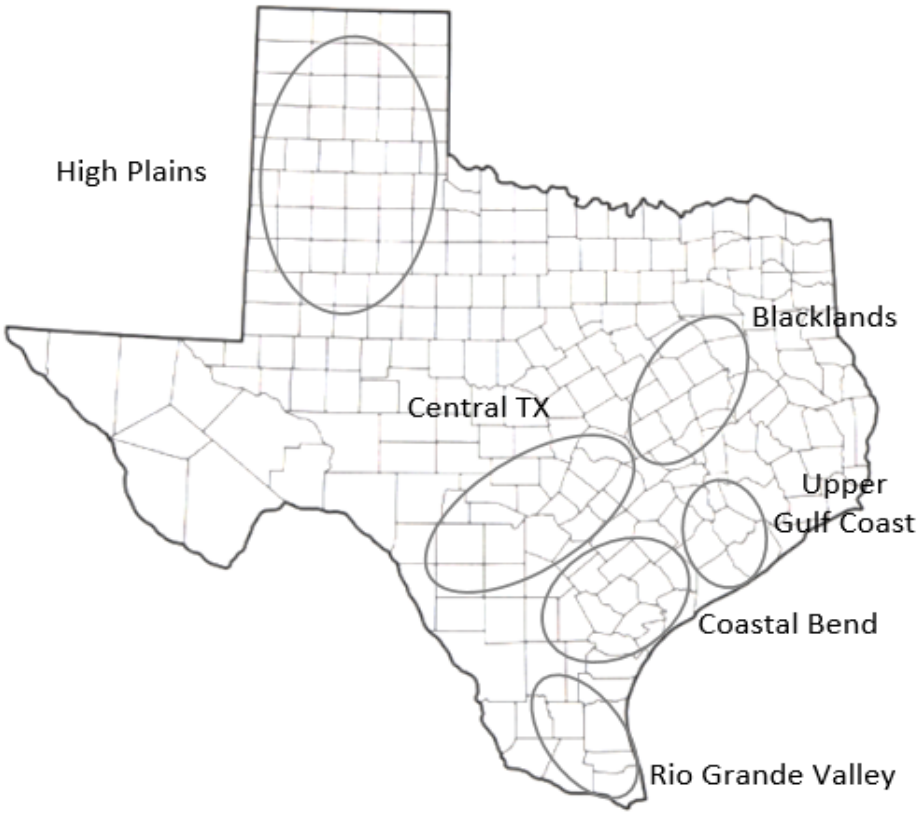

Figure 1. Sub-regions of Texas where survey for Palmer amaranth was carried out in this study

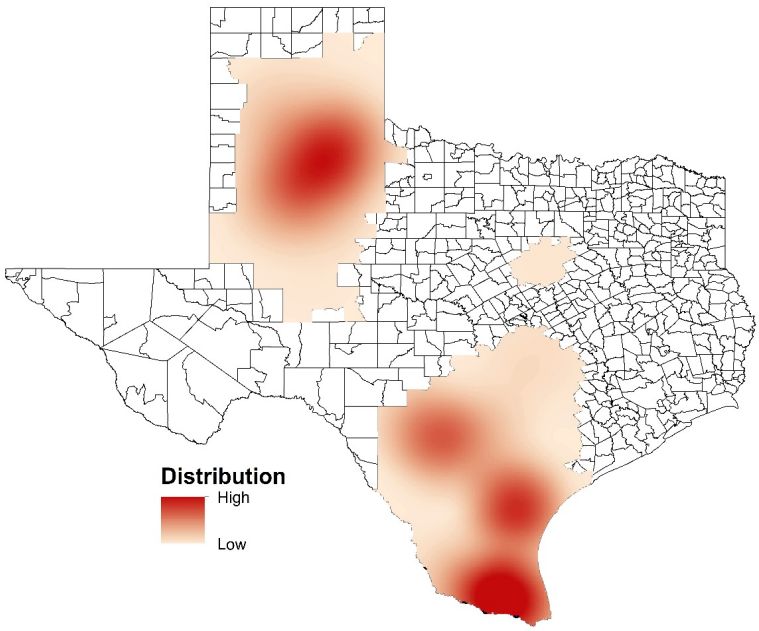

Figure 2. Distribution gradient of Palmer amaranth across different regions of Texas. Dark red represents areas with high infestation of Palmer amaranth, whereas light shaded areas represent areas with low distribution of Palmer amaranth.

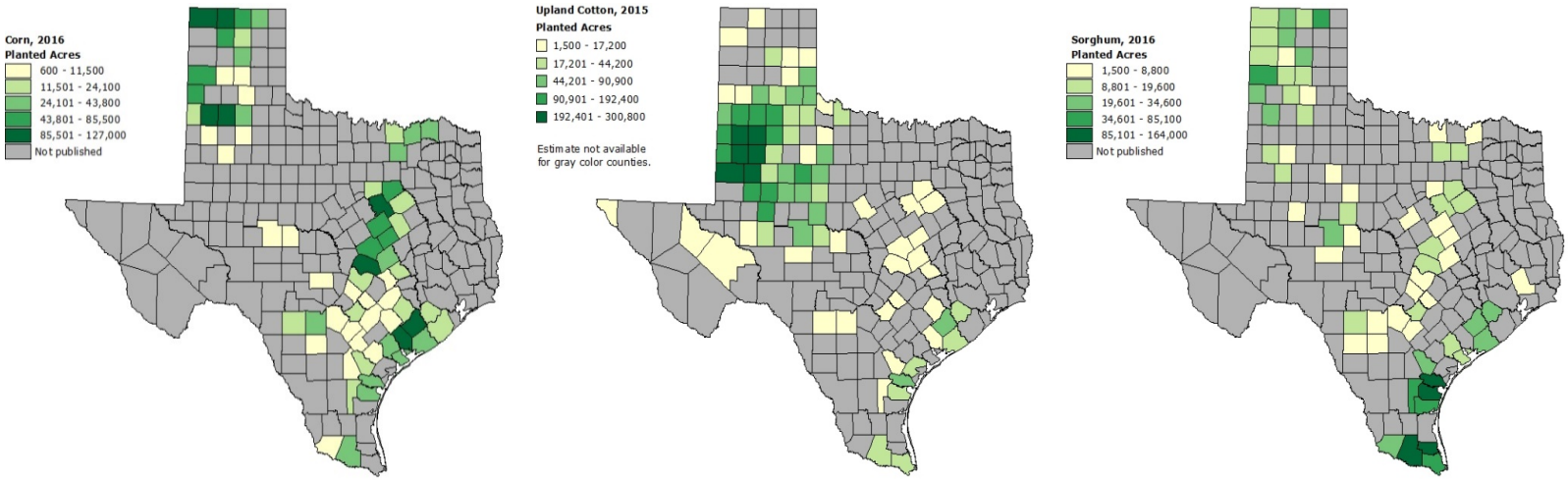

Figure 3. Distribution of corn, cotton and sorghum producing acres across Texas (Source: USDA-NASS 2016)

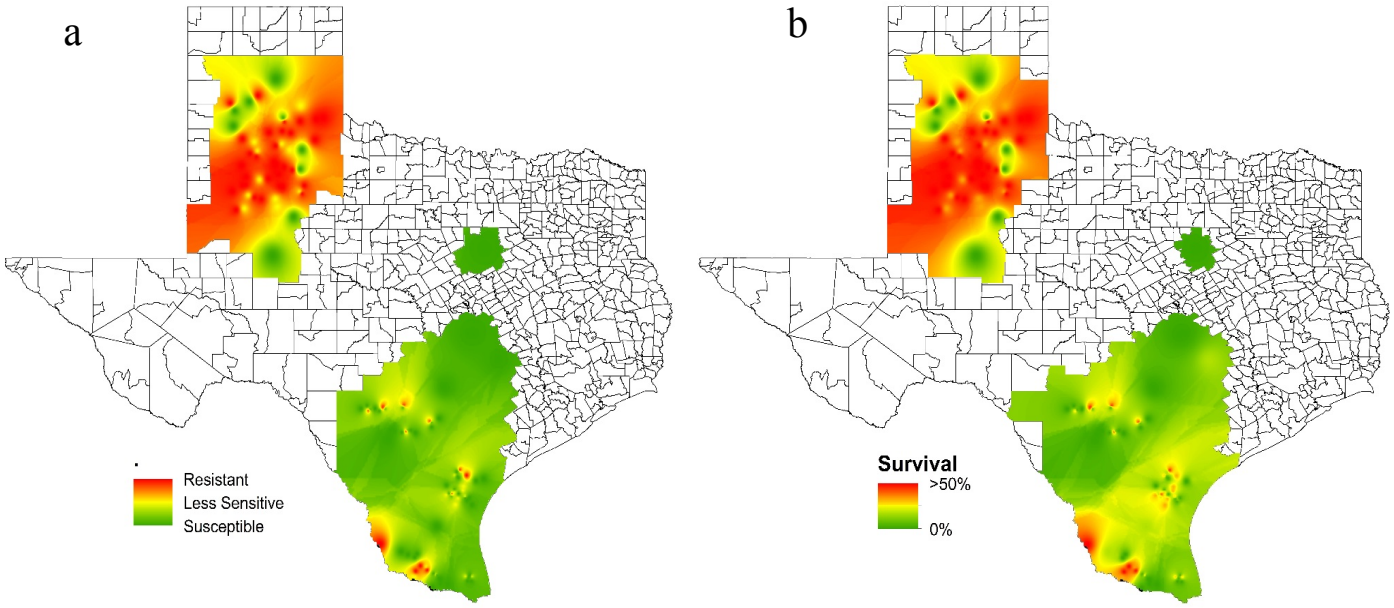

Figure 4. Regional-scale distribution of Palmer amaranth response to glyphosate based on injury (a) and frequency of survivors (b). Resistance levels: resistant - 0 to 49% injury, less sensitive - 50 to 89% injury, and susceptible - 90 to 100% injury. Frequency of survival indicates the stage of advancement of resistance in a given production field. For instance, 50% survival indicates that about half of the individuals in the population are already resistant and that resistance is highly noticeable in the field.

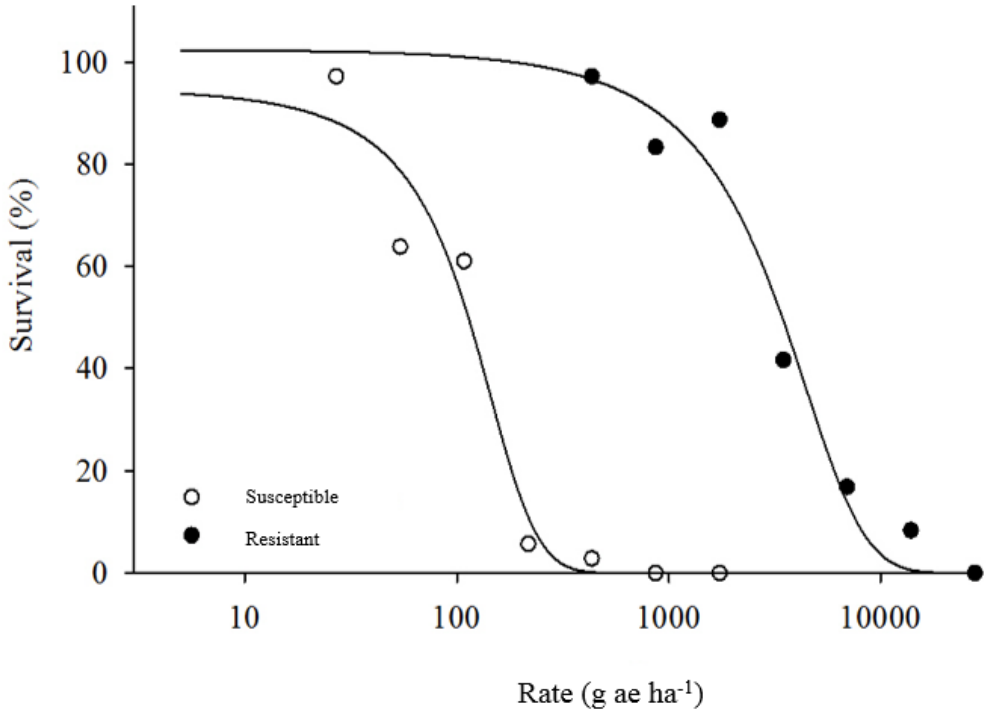

Figure 5. Dose-response analyses of resistant (TX-GR7) and susceptible (TX-S29) Palmer amaranth populations to glyphosate (recommended field rate of glyphosate = 868 g ae ha^{-1})

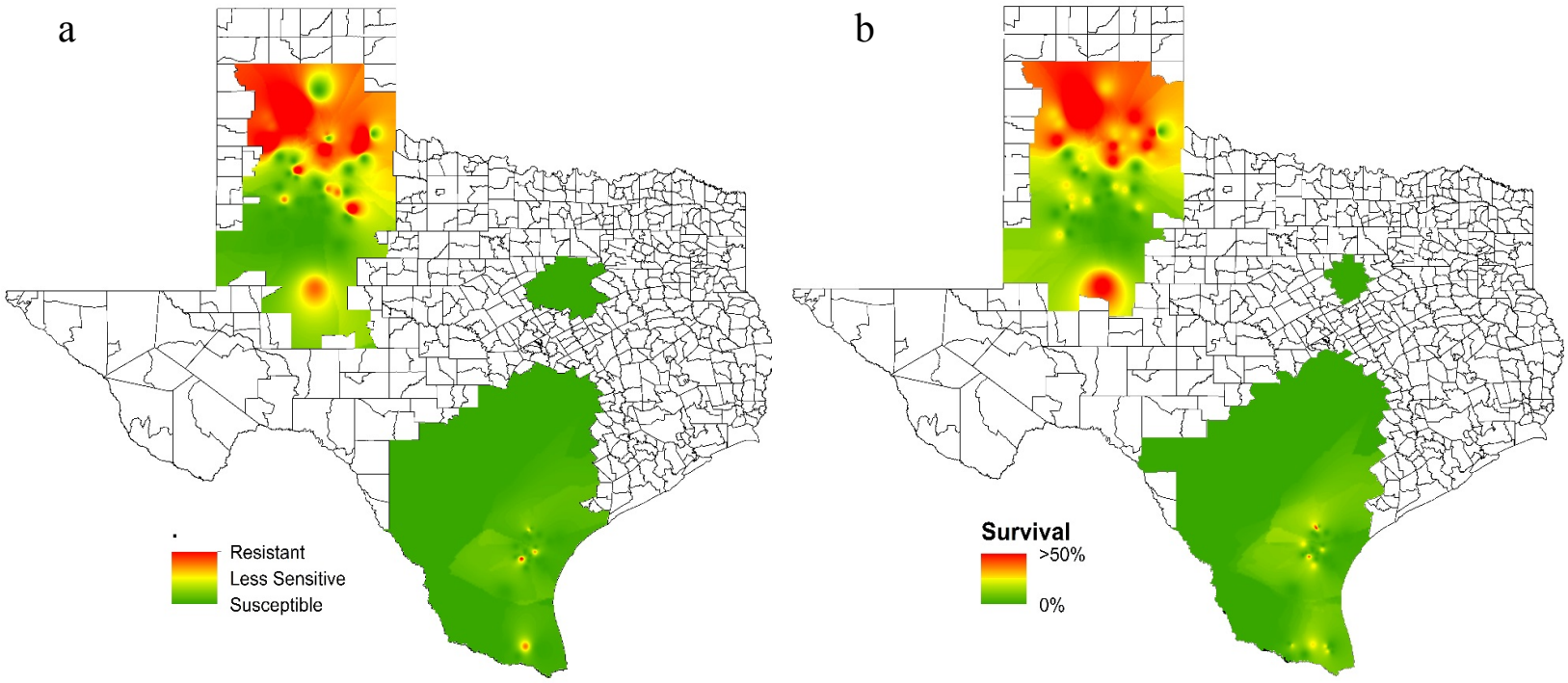

Figure 6. Regional-scale distribution of Palmer amaranth sensitivity to atrazine (applied postemergence) based on injury (a) and frequency of survivors (b). Resistance levels: resistant - 0 to 49% injury, less sensitive - 50 to 89% injury, and susceptible - 90 to 100% injury. Frequency of survival indicates the stage of advancement of resistance in a given production field. For instance, 50% survival indicates that about half of the individuals in the population are already resistant and that resistance is highly noticeable in the field.

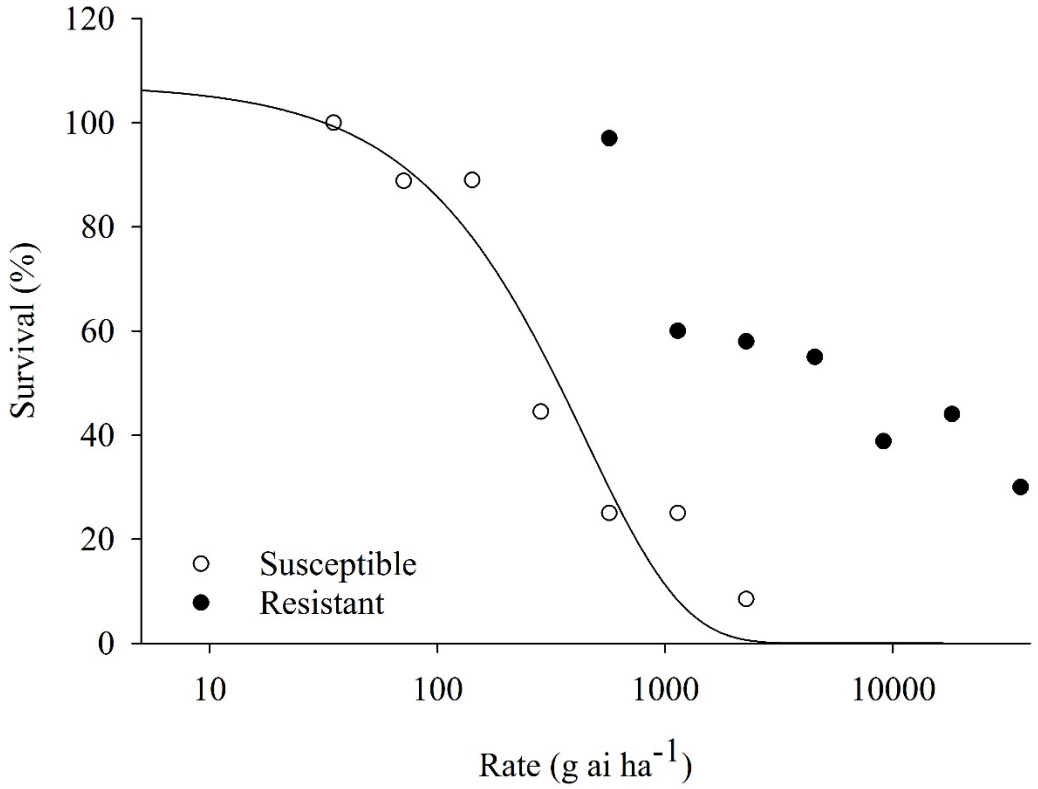

Figure 7. Dose-response analysis of resistant (TX-AR16) and susceptible (TX-S29) Palmer amaranth populations for atrazine (recommended field rate of atrazine = 1,115 g ai ha^{-1})

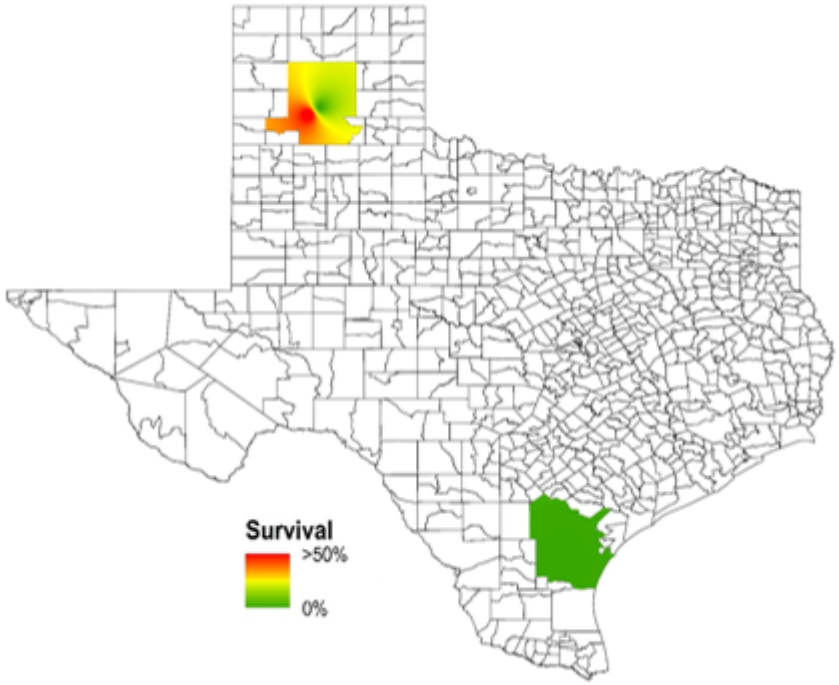

Figure 8. Regional-scale distribution of Palmer amaranth sensitivity to atrazine (applied preemergence) based on survival estimates compared to non-treated control. Survival estimates (%) >0 indicates the presence of resistance to atrazine preemergence and also the stage of advancement of resistance in a given production field. For instance, 50% survival indicates that about half of the individuals in the population are already resistant and that resistance is highly noticeable in the field.

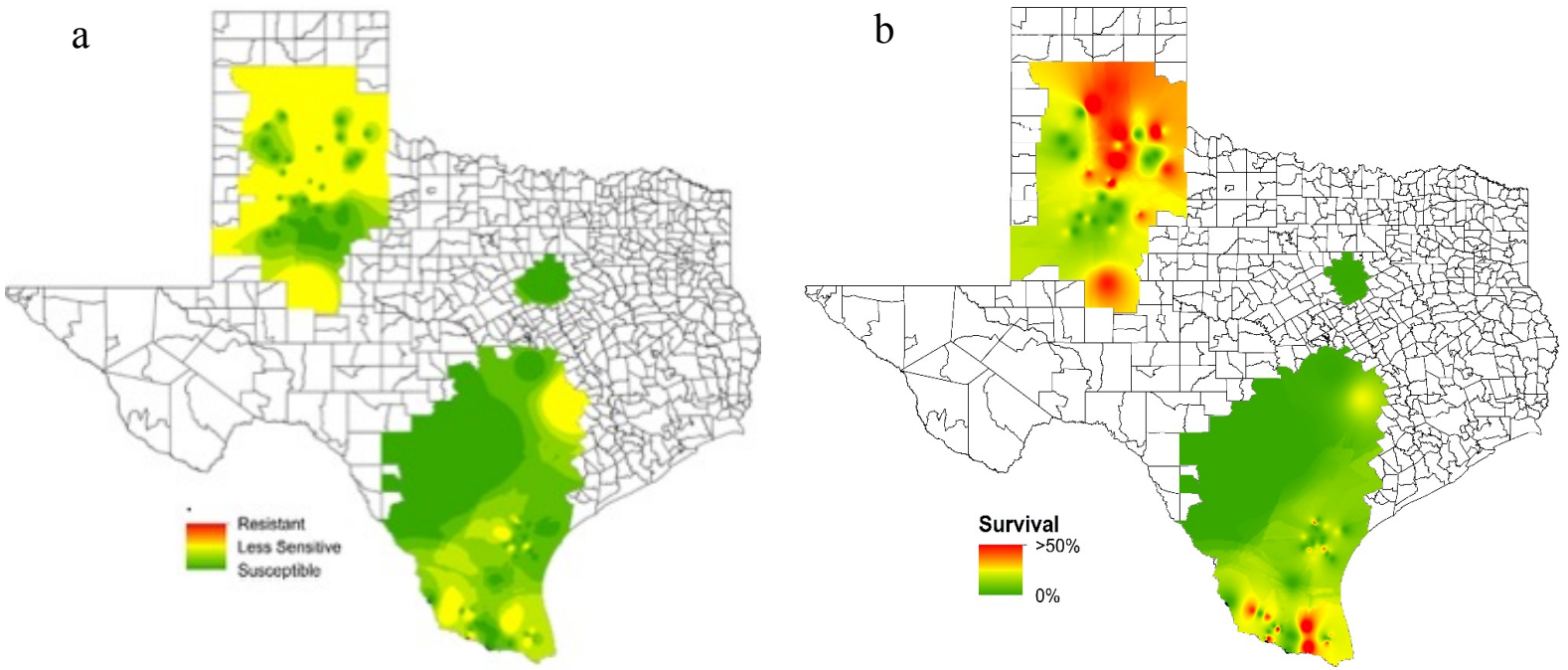

Figure 9. Regional-scale distribution of Palmer amaranth sensitivity to tembotrione based on injury (a) and frequency of survivors (b). Resistance levels: resistant - 0 to 49% injury, less sensitive - 50 to 89% injury, and susceptible - 90 to 100% injury. Frequency of survival indicates the stage of advancement of resistance in a given production field. For instance, 50% survival indicates that about half of the individuals in the population are already resistant and that resistance is highly noticeable in the field. Tembotrione applications did not include ammonium sulfate (AMS) to the mix.

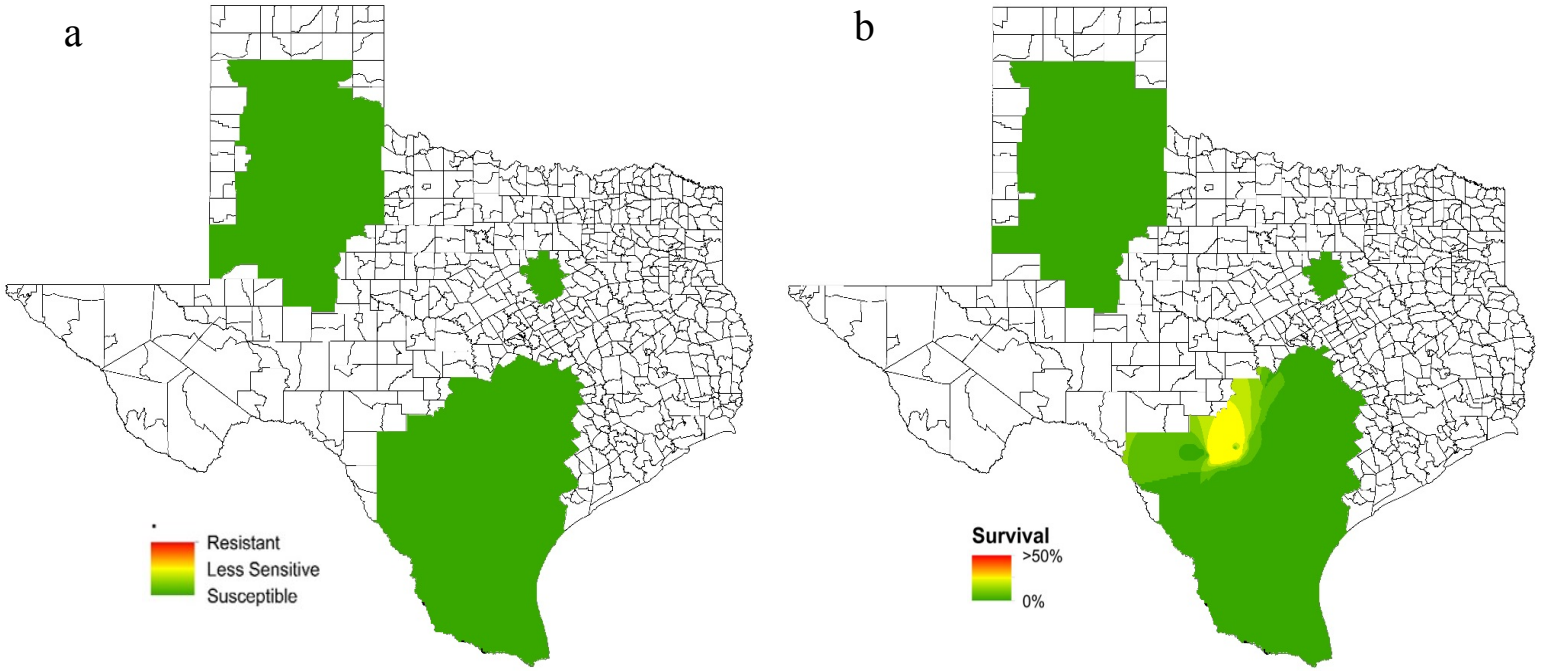

Figure 10. Regional-scale distribution of Palmer amaranth sensitivity to fomesafen based on injury (a) and frequency of survivors (b). Resistance levels: resistant - 0 to 49% injury, less sensitive - 50 to 89% injury, and susceptible - 90 to 100% injury. Based on the scoring scale, all tested populations were susceptible to fomasefen. However, some populations had few individuals surviving fomesefen applications 21 days after treatment, though injury was >90% and the plants did not regrow to produce seed.

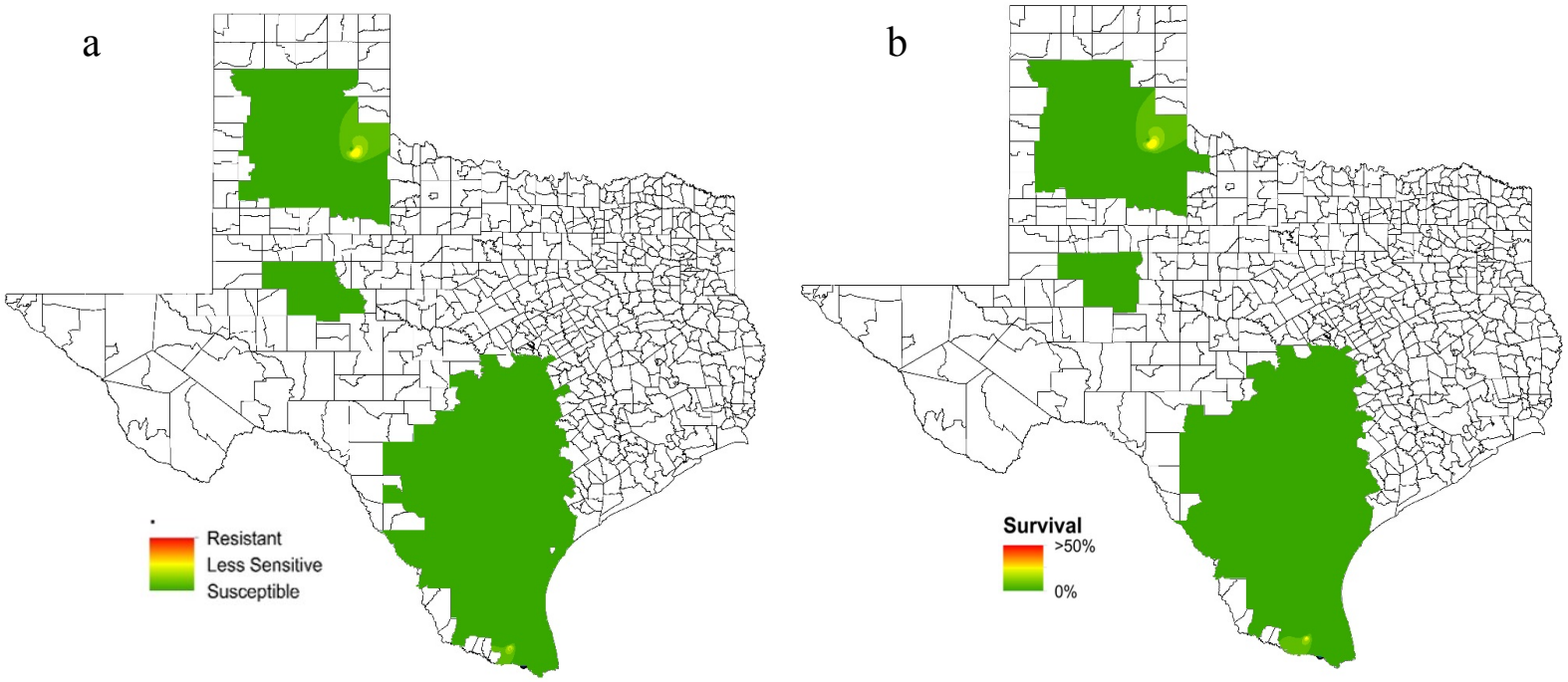

Figure 11. Regional-scale distribution of Palmer amaranth sensitivity to dicamba based on injury (a) and frequency of survivors (b). Resistance levels: resistant - 0 to 49% injury, less sensitive - 50 to 89% injury, and susceptible - 90 to 100% injury. Frequency of survival indicates the stage of advancement of resistance in a given production field. For instance, 50% survival indicates that about half of the individuals in the population are already resistant and that resistance is highly noticeable in the field.

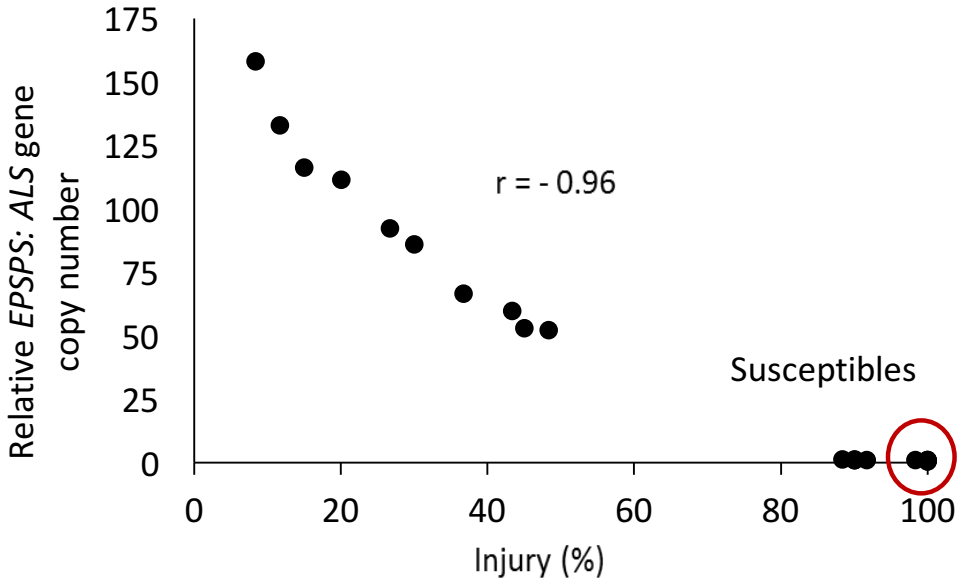

Figure 12. Correlation of relative *EPSPS* gene copy number with the injury (%) of selected glyphosate-resistant as well as susceptible Palmer amaranth populations collected from Texas

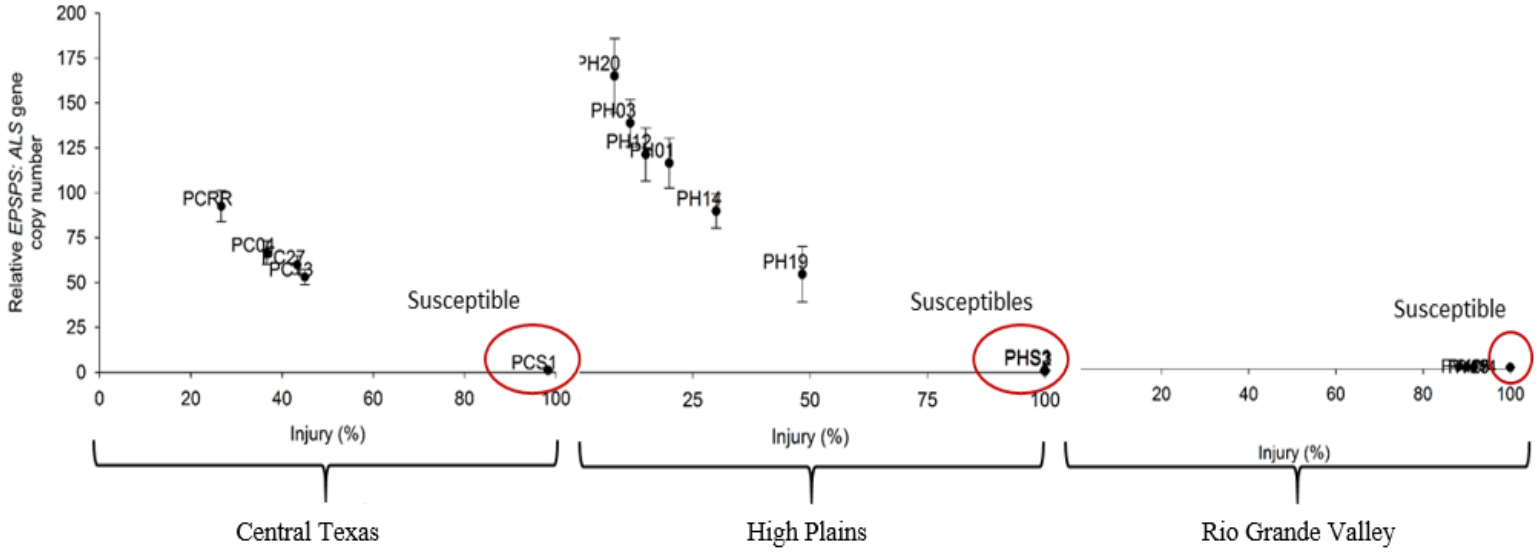

Figure 13. Relative *EPSPS* gene copy number of selected resistant as well as susceptible Palmer amaranth populations from three different sub-regions in Texas

CHAPTER III

SURVEYING THE DISTRIBUTION OF HERBICIDE RESISTANCE IN WATERHEMP (*Amaranthus tuberculatus*) ACROSS ROW CROP PRODUCTION SYSTEMS IN TEXAS

Introduction

Waterhemp (*Amaranthus tuberculatus*) has emerged as one of the most problematic weeds in the US, especially in the US corn and soybean belt (Hager et al. 1997; Hinz and Owen 1997). In Texas, this species is known to be problematic in row-crop fields (corn, cotton, grain sorghum, and soybean) in the southeastern region (Cline 2013). Waterhemp is a highly competitive species and can produce in excess of 100,000 seeds per plant, thus establishing large soil seedbanks (Hager et al. 1997; Battles et al. 1998). It is a dioecious, obligate outcrosser with a high potential for interspecific hybridization (Wetzel et al. 1999; Franssen et al. 2001). Waterhemp can emerge over a long period and escape management interventions (Hartzler et al. 1999). It has been reported that heavy infestations of waterhemp can reduce corn and soybean yields up to 74 and 56%, respectively (Bensch et al. 2003; Steckel and Sprague 2004). Severe waterhemp infestations not only impact crop yields, but can also interfere with mechanical harvesting and reduce harvest efficiency as well as product quality. Controlling waterhemp has become a challenge in recent times due to the inconsistency in control with several important herbicides.

There has been numerous reports of waterhemp resistance to several herbicide sites of action (SOA) (Heap 2017). As of today, waterhemp has evolved resistance to

ALS (acetolactate synthase)-, PS II (photosystem II)-, EPSPS (5-enolpyruvyl-shikimate-3-phosphate synthase)-, HPPD (4-hydroxyphenylpyruvate dioxygenase)-, and PPO (protoporphyrinogen oxidase)- inhibitors, as well as synthetic auxins (Heap 2017). Resistance to the acetolactate synthase (ALS)-inhibiting herbicides have been reported more than two decades ago, which previously were able to provide effective control of waterhemp (Hinz and Owen 1997; Sprauge et al. 1997). The first case of waterhemp resistance to atrazine was documented in 1990 in a field corn production field in Nebraska (Schleufer et al. 1992). Field surveys conducted by Anderson et al. (1996) in that region had revealed that 61% of all surveyed populations were resistant to atrazine. Glyphosate resistance is prevalent in this species across the country, confirmed to occur in at least 18 different states from Texas to North Dakota (Heap 2017). EPSPS gene amplification is a common mechanism of resistance in about 91% of the glyphosate-resistant waterhemp populations tested in Illinois by Chatham et al. (2015). HPPD-inhibitor-resistant waterhemp populations were confirmed in 2009 in seed corn production fields in Illinois (Hausman et al. 2011) and Iowa (McMullan and Green 2011).

PPO-inhibitor resistance is an emerging concern in this species. The first PPO-inhibitor resistant waterhemp populations were documented in 2009 in soybean-corn production systems in Iowa and Illinois (Heap 2017). The vast majority of the PPO-inhibitor resistant waterhemp populations reported till date are only resistant to foliar applications and still controlled by PRE applications (e.g. Wuerffel et al. 2015). Waterhemp resistance to the synthetic auxin herbicide 2,4-D was first confirmed in a

native grass-seed production field in Nebraska (Bernards et al. 2012). This 2,4-D-resistant population has also exhibited three-fold less sensitivity to dicamba based on I_{50} values. Additional 2,4-D resistant populations have been documented in Illinois (Heap 2017) and Missouri (Shergill et al. 2017). The evolution of multiple herbicide resistance is of particular concern in waterhemp. As of now, waterhemp resistance to five different herbicide SOA namely ALS-, PS-II, PPO-, and HPPD-inhibitors, and synthetic auxins (Heap 2017) or ALS-, EPSPS-, PS-II-, and PPO-inhibitors, and synthetic auxins (Shergill et al. 2017) were reported in Illinois and Missouri, respectively. The evolution of multiple resistance severely limits the number of herbicide options available for effective control.

In Texas, inconsistent control or control failure of waterhemp has been often reported by growers and crop consultants for some of the commonly used herbicides (Bradshaw, Personal Communications). Glyphosate-resistance was documented in two waterhemp populations in Texas (Light et al. 2011). These two populations collected in 2006 and 2008 in southeast Texas showed up to 3-fold resistance to glyphosate. However, field evidence suggests that herbicide resistance could be more prevalent in this species in Texas (Cline 2013). While it is imperative to understand the background level of weed resistance to important herbicide options in order to design effective management programs, knowledge on this aspect is limited for waterhemp populations infesting row-crop production fields throughout Texas. Therefore, the objectives of this study were to: 1) survey the level of sensitivity of waterhemp to important herbicides used in row-crop production systems in Texas, and 2) examine the presence of molecular target-site

mechanisms, if any, endowing glyphosate resistance in waterhemp populations surveyed in Texas.

Materials and Methods

Field survey

Field surveys were conducted during 2014 to 2016 to sample waterhemp populations throughout Texas for evaluating the background level of waterhemp response to various herbicide options. In order to obtain a representative sampling across important row-crop production areas in Texas, the survey was focused on six distinct regions: High Plains, Rio Grande Valley, Central Texas, Coastal Bend, Upper Gulf Coast, and Blacklands region (Fig. 14). The field surveys were conducted each summer/ fall (2014 to 2016) following a semi-stratified survey methodology previously optimized and utilized by Bagavathiannan and Norsworthy (2016). The survey sites for each region were randomly selected on a Google® map using the ITN Converter software (version 1.88; Benichou Software) and further loaded on a GPS device (TomTom International, BV) to navigate to the survey sites. If waterhemp was not present in a pre-determined survey site, the first population along the survey route leading to the next survey site was used for sampling. In each site, waterhemp seed heads were collected from about 15 random female plants. The coordinates of the actual sites from where the plant samples were collected were recorded using a Garmin etrex® 10 (Garmin International Inc.) handheld GPS system. The samples were dried in an oven at 50 C for 72 hours. Seed heads were mechanically thrashed, cleaned, and stored in glass vials at room temperature prior to further processing. A total of about 160 populations were collected during the

surveys, of which 127 populations were randomly selected for conducting herbicide screenings.

Herbicide assays

The herbicide assays were conducted at the Norman Borlaug Center for Southern Crop Improvement Greenhouse Research Facility located at Texas A&M University, College Station, TX. The greenhouse was maintained at 30/26 C day/night temperature regime and 14 hr photoperiod. The samples were screened for five different herbicides with distinct SOAs: glyphosate (EPSPS-inhibitor), atrazine (PS-II-inhibitor), tembotrione (HPPD-inhibitor), fomesafen (PPO-inhibitor), and dicamba (synthetic auxin). All herbicides were tested to determine waterhemp resistance to POST applications. However, populations that showed resistance to POST applications of atrazine, tembotrione, or fomesafen were subsequently screened for PRE applications of these herbicides. Glyphosate, atrazine, and fomesafen have been widely used in row-crop production in the region. The use of tembotrione is expected to increase, given the anticipated commercialization of the HPPD-inhibitor-resistant crop technology in the near future. Further, the use of synthetic auxins have already increased with the cultivation of dicamba-resistant (Roundup Ready® Xtend) cotton and soybean. Thus, this experiment is designed to establish resistance profiles of waterhemp populations collected in Texas to herbicides that have widespread historical use as well as the ones that will likely be used frequently in the near future.

Herbicide evaluations were carried out using plastic growth trays (15 x 15 cm) filled with potting soil mix (LC1 Sunshine mix) for POST applications or with field soil (Belk Clay, pH 7.8) collected from Texas A&M research farm near Snook, TX (Burleson County) for PRE applications. For each treatment, two replications and two experimental runs were established. Known susceptible standards as well as non-treated checks were also maintained alongside for comparison. Initial germination tests revealed a high level of seed dormancy in waterhemp. A cold treatment at -13 C for 21 d and then at room temperature for 7 d was effective in breaking dormancy and improving seed germination. The seed were broadcast planted in each tray and at the 1-leaf stage the seedlings were thinned to provide a uniform density of about 30 to 40 seedlings per tray for POST treatments. No thinning was applicable to the PRE treatments.

Herbicide applications were made at recommended field use rates (Table 4), using an automated spray chamber mounted with a TeeJet XR8002 nozzle calibrated to deliver 140 L ha^{-1} of spray liquid at a speed of 4.8 kmph and a pressure of 276 kPa. The herbicide applications were made immediately after planting (PRE treatments) or at the 2- to 3-leaf seedling stage (POST treatments). The PRE treated trays were watered immediately after the herbicide application to activate the herbicides. Observations were carried out on treated plots at 21 days after herbicide application (DAT) to document seedling injury rating and survival. Injury ratings were carried out on a scale of 0 to 100% (0 = no visible injury compared to non-treated control; 100 = complete plant death). Survival frequency was documented as the number of seedlings surviving herbicide applications, divided by the total number of treated seedlings.

Dose-response assays

Dose-response assays were conducted on a highly resistant population for each herbicide. The herbicide doses used were: 1/32, 1/16, 1/8, 1/4, 1/2, 1, and 2X the recommended field rate for the susceptible populations and 1/2, 2, 4, 8, 16, and 32X rates for the putative resistant populations. The plant material for POST treatments were established using potting soil media, whereas the PRE treatments were applied on field soil. The seedlings were established in six-cell growth trays (POST) with a single healthy seedling in each cell or 15 cm x 15 cm trays (PRE) planted with 25 seeds. For each treatment, three replications and two experimental runs were conducted. POST herbicide applications were made at the 2- to 3-leaf seedling stage, whereas the PRE herbicides were applied immediately after planting and irrigated right after herbicide application to activate the herbicide. The weed response to herbicide applications was evaluated at 21 DAT. Specifically, frequency of seedling survival (i.e. survival %) was recorded as the number of seedlings survived the herbicide application divided by the total number of seedlings treated with the herbicide.

EPSPS target-site analysis

Population Selection and DNA extraction

Four glyphosate-resistant waterhemp populations each were selected from the two regions (Central Texas and Coastal Bend) where glyphosate resistance is widespread, for EPSPS target-site analysis. Populations were selected based on injury ratings recorded in initial screening, with at least three resistant populations (0-20%

injury), three populations with reduced sensitivity (50-80% injury), along with a susceptible control (90-100% injury). These plants were grown for 4 weeks in a greenhouse. Leaf tissues were collected from three random plants of each population (i.e. three replications). Susceptible plant samples were collected prior to herbicide application, whereas leaf tissues of resistant populations were collected upon confirmation of resistance after herbicide treatment.

Total genomic DNA was extracted using a modified hexadecyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1990). The genomic DNA was quantified using a NanoDrop 2000c V. 1.0 spectrophotometer (NanoDrop Technologies, Wilmington, DE,), diluted to 1 ng μl^{-1} and 50 ng ul^{-1} with deionized water for gene copy number and target-site mutation analysis, respectively. The diluted samples were stored at 4 C until further analysis.

Determination of EPSPS gene copy number

The *EPSPS* gene copy number was determined using a real-time quantitative polymerase chain reaction (RT-qPCR), using the *ALS* gene as the positive control. Sequences from Palmer amaranth (*A. palmeri*) (Gaines et al. 2010) were used for designing the primer sets for the EPSPS gene [EPSF1 (5′-ATGTTGGACGCTCTCAGAACTCTTGGT-3′) x EPSR8 (5′-TGAATTTCCTCCAGCAACGGCAA-3′)] and the ALS gene [ALSF2 (5′-GCTGCTGAAGGCTACGCT-3′) × ALSR2 (5′- GCG GGACTGAGTCAAGAAGTG-3′)]. For the RT-qPCR reaction, 25 µl reactions were made using 12.5 µl of SYBR green supermix and 2.5 µl of genomic DNA. Each sample was run three times for each primer

set with the following thermoprofile: 95 C for 3 min for initial denaturation, 40 cycles of 30s at 95 C for denaturation, and 60s at 60 C for annealing/extension. A melt curve analysis was subsequently carried out at 55 C for 30 s. The BioRad iQ5 Thermocycler software was used to calculate the cycle threshold (Ct) values.

Determination of EPSPS target-site mutation

Conserved regions of the *EPSPS* gene were sequenced to identify the presence of any known mutations in the EPSPS target-site for the resistant accessions that did not show *EPSPS* gene amplification. In this regard, the primers designed by Varanasi et al. (2015) were used [

forward (EPSPSF- 5'CCAAAAGGGCAGTCGTAGAG 3'); reverse (EPSPSR-5'ACCTTGAATTTCCTCCAGCA 3')]. A 25 µl PCR reaction mix was prepared, consisting of 12.5 µl of 2x PCR master mix (Takara Bio USA, Inc.), 2.5 µl of each primer (5 µM), 4 µl of the genomic DNA (50 ng μl^{-1}), and 3.5 µl of water. The following thermoprofile was used in the PCR reactions: 95 C for 3 min followed by 40 cycles of denaturation at 95 C for 30s, annealing at 53.5 C for 45s, and final extension at 72 C for 7 min. The plates were maintained at 4 C upon completion of the reaction. Agarose (1%) gel electrophoresis was used to separate the PCR products, which were subsequently purified using a NucleoSpin® Gel and PCR Clean up kit (Takara Bio USA, Inc.). The samples were sequenced at the Institute for Plant Genomics & Biotechnology, Texas A&M University, College Station, TX. To determine potential single nucleotide polymorphisms conferring glyphosate resistance, the nucleotide sequences were aligned

using the Bioedit software (version 7.2.6, NCSU) based on the known EPSPS sequences available on the National Center for Biotechnology Information (NCBI) database.

Statistical Analyses

Spatial maps were developed using ArcGIS (version 10.5; ESRI) to illustrate spatial distribution of waterhemp sensitivity to various herbicides across Texas. The distribution densities of waterhemp across different regions of Texas are shown using kernel density analysis (Fig. 15). Further, the distribution of waterhemp sensitivity to herbicides across a spatial scale was predicted using the interpolation analysis technique (e.g. see Fig. 17). The spatial maps show a) the level of sensitivity of waterhemp to the given herbicide (e.g. Fig. 17a), and b) the frequency of survivors in a given population, indicating the stage of advancement of resistance within the population (e.g. Fig. 17b). A 3-parameter logistic regression equation provided the best regression fit for the dose-response data, and was carried out using the SigmaPlot software (version 13, Systat Software, Inc., San Jose, CA). The regression equations were used to calculate the amount of herbicide that caused 50% mortality of the test population (LD_{50}). The LD_{50} value of the resistant population divided by the LD_{50} of the susceptible standard provided the resistance ratio (R/S) values. The relative EPSPS gene copy number estimates for the Ct data were calculated using the following formulas:

$$(1)\ \Delta\mathrm{Ct} = \mathrm{Ct\ of\ target}\ (EPSPS) - \mathrm{Ct\ of\ reference\ (ALS)}$$

$$(2)\ \Delta\Delta\mathrm{Ct} = \Delta\mathrm{Ct\ of\ Treatment\ Individual} - \Delta\mathrm{Ct\ of\ Control}$$

$$(3)\ \mathrm{Copy\ number} = 2^{-\Delta\Delta\mathrm{Ct}}$$

To determine the association between the EPSPS gene copy number estimates and the glyphosate injury levels obtained, a Pearson correlation analysis was performed using SigmaPlot (version 13, Systat Software, Inc., San Jose, CA).

Results and Discussion

Regional distribution of waterhemp in Texas

Waterhemp is widely distributed in the Upper Gulf Coast and Coastal Bend (Combined as Gulf Coast) as well as the Blacklands region (including parts of Central Texas), whereas this species is rarely present in the High Plains and Rio Grande Valley (Fig. 15). It appears that waterhemp has a specific ecological niche and regional adaptation within Texas. Both waterhemp and Palmer amaranth co-occurred in a narrow geographical range in the Coastal Bend and Central Texas regions (data not shown). Apart from this, these two species have exhibited distinct regional preference within Texas. Waterhemp generally prefers moist, wet environment (Nordby et al. 2007), which is common in southeastern Texas. This region is often characterized by high rainfall (>100 cm/year) and wet conditions, which may explain the dominance of waterhemp in this geography. The distribution of waterhemp observed in this study is also consistent with previous reports (Light et al. 2011; Cline et al. 2013).

Response to glyphosate

Of the 112 waterhemp populations evaluated for glyphosate, 27% of them were resistant and 20% were less sensitive to this herbicide (Fig 17; Table 5), illustrating the prevalence of insufficient waterhemp control with glyphosate. The first case of

glyphosate-resistant waterhemp was reported in 2005 from Missouri (Legleiter and Bradley 2008), and soon it was confirmed across 18 US states and in Ontario, Canada (Heap 2017). This unprecedented increase in glyphosate-resistant waterhemp has been due to heavy reliance on glyphosate for weed control in glyphosate-resistant (Roundup Ready®) crops. Dose-response assay for a high glyphosate-resistant waterhemp population (TX-GR25) has indicated 9-fold resistance compared with a susceptible standard (TX-S15) (Fig. 18; Table 6). The population evaluated by Legleiter and Bradley (2008) in Missouri had 19-fold resistance compared to a susceptible standard. Sarangi et al. (2015) indicated 3- to 39-fold glyphosate resistance in different waterhemp populations originating from Nebraska. Likewise, several folds of resistance to glyphosate has been reported in waterhemp populations collected from Illinois, Iowa and Missouri (Smith and Hallett 2006).

As a dioeceous species, waterhemp has high outcrossing potential and can also hybridize with other *Amaranthus* species such as Palmer amaranth (*A. Palmeri*) (Franssen et al. 2001). The high potential for outcrossing improves species diversity and also increases the likelihood of pollen-mediated transfer of herbicide resistance from resistant to susceptible populations (Sarangi et al. 2017). Among the regions within Texas, Gulf Coast had 46% resistant and 28% of less sensitive populations, whereas the Blacklands region had 9 and 12% waterhemp populations that were resistant or less sensitive to glyphosate, respectively.

Response to atrazine

In the current study, several Gulf Coast waterhemp populations showed resistance or reduced sensitivity to atrazine applied POST. Of the 55 populations evaluated from this region, 15% of them were resistant and 27% of them were less sensitive to atrazine POST (Fig. 19; Table 5). The Gulf Coast region is characterized by intensive corn production where atrazine has been heavily used for weed control for long time. However, atrazine resistance is relatively less prevalent in the waterhemp populations collected in the Blacklands region, suggesting the practice of more diversified tactics in this region.

Atrazine has been heavily used in corn and grain sorghum production for the control of waterhemp and other annual dicot weeds in the US Midwest and other parts. Atrazine-resistant waterhemp has been widely documented in Illinois, Iowa, Kansas, and Missouri (Heap 2017). Cost-effectiveness along with season long broad-spectrum weed control has made atrazine a reliable POST tankmix partner with a number of other herbicides; the importance of atrazine as a tankmix partner has been well documented (Johnson et al. 2002; Armel et al. 2005; Abendroth et al. 2006). Given this importance, resistance to atrazine would limit the weed control options available in corn and grain sorghum. Dose-response bioassay of the high atrazine-resistant waterhemp population (TX-AR31) from the current survey has revealed >64-fold resistance compared with a susceptible standard (TX- S15) (Fig. 7; Table 3). The resistant sample did not reach 50% mortality even at the highest rate tested (32X). Foes et al. (1998) reported 185-fold resistance to atrazine in a waterhemp population, required >20 kg ha^{-1} of this herbicide

to inhibit 50% growth (Foes et al. 1998). Further, profiling of atrazine resistant populations especially in the Gulf Coast region indicated the presence of atrazine resistance in advanced stages of in the majority of waterhemp populations.

Response to tembotrione

The HPPD-inhibiting herbicides are among the few alternative herbicides available for the control of glyphosate- and atrazine-resistant *Amaranthus* spp. in corn fields (Sutton et al. 2002). The HPPD-inhibiting herbicides, tembotrione and mesotrione in particular, are currently used extensively in corn production due to their broad spectrum weed control activity and high crop tolerance (Bollman et al. 2008). In the current survey, there appears to be a trend of reduced sensitivity to tembotrione in atrazine-resistant populations (Figs. 19a, 21a). McMullan and Green (2011) have reported that that resistance to atrazine can contribute to the evolution of resistance to HPPD-inhibiting herbicides. Atrazine and HPPD-inhibiting herbicides are commonly applied together, and resistance to atrazine would increase the intensity of the selection pressure on the HPPD-inhibiting herbicide, as if it had been applied alone (McMullan and Green 2011). In the current study, high frequency of waterhemp survivors were recorded in populations collected from the Gulf Coast region. About 35% of the populations from this region were less sensitive to tembotrione (Fig 21; Table 5). However, no resistance to tembotrione has been observed in this survey. It is important to note that AMS has not been added to tembotrione herbicide mix in current study, thus results should be interpreted accordingly. However, results revealed the presence of high variability in tolerance to tembotrione across different regions.

Response to dicamba

None of the waterhemp populations tested in this study were resistant to dicamba (Table 5). To this date, only two cases of 2,4-D-resistant waterhemp have been documented elsewhere (Bernards et al. 2012; Evans 2016), but resistance to synthetic auxin type herbicides in other weed species have been reported, such as, wild radish (*Raphanus raphanistrum*) (Walsh et al. 2004), wild mustard (*Brassica kaber*), prickly lettuce (*Lactuca serriola*) (Burke et al. 2009), and globe fringebrush (*Fimbristylis miliacea*) (Karim et al. 2004). The evolution of resistance to synthetic auxin herbicides is alarming as it could affect the utilization of newer crop technologies. Proper stewardship of these new technologies is critical to maintain their effectiveness.

Response to fomesafen

No case of fomesafen resistance has been observed in the waterhemp populations evaluated in this study. However, one population from the Blacklands region showed reduced sensitivity to this herbicide (Fig. 22). Resistance to fomesafen and other PPO-inhibiting herbicides have been widely documented in waterhemp populations in corn production areas (Heap 2017; Patzoldt et al. 2005; Shoup et al. 2003). The first case of PPO-inhibitor resistance was documented in waterhemp in Kansas in 2000 (Shoup et al. 2003). The population evaluated by Shoup et al. (2003) had 34-, 82-, 8-, and 4-fold resistant compared to a susceptible standard for acifluorfen, lactofen, fomesafen, and sulfentrazone, respectively. In Illinois, a waterhemp population was resistant to three different herbicide families that inhibit PPO, which

include diphenylethers (acifluorfen, fomesafen, and lactofen), N-phenyl-phthalimides (flumiclorac and flumioxazin), and triazolinone (sulfentrazone) (Patzoldt et al. 2005). The levels of resistance to each of the PPO inhibitors were relatively similar, between 2.2-fold and 6.2-fold, with the exception of lactofen, which was 23-fold when compared with a susceptible waterhemp population (Patzoldt et al. 2005). PPO-inhibitor resistance is becoming a widespread phenomenon in this species and efforts are critical to prevent the evolution of waterhemp resistance to PPO-inhibitors in Texas.

EPSPS target-site analysis

Relative EPSPS gene copy number and injury ratings of waterhemp were highly correlated (r = - 0.93), indicating that EPSPS copy numbers are the primary cause of glyphosate resistance in tested populations (Fig. 23). The amplification of the EPSPS gene produces an abundant supply of EPSPS to absorb glyphosate, and reduces its negative effects (Gaines et al. 2010; Salas et al. 2012). EPSPS gene amplification has been reported as one of the most prominent mechanism of glyphosate-resistance in several weed species. However, findings of Light et al. (2011) have indicated that EPSPS gene amplicons in case of glyphosate-resistant waterhemp were not predictive of variable copy numbers as generally observed in Palmer amaranth. In contrast, Chatham et al. (2015) reported EPSPS gene amplification as the primary mechanism responsible for glyphosate resistance in waterhemp populations originating from four (Illinois, Kansas, Missouri, and Nebraska) out of five locations.

In the current study, glyphosate-resistant waterhemp populations showed up to 11 gene copies and the level of resistance increased with the number of copies (Fig. 23). Similar findings have been reported by Chatham et al. (2015), where up to 12 EPSPS gene copies were detected in glyphosate-resistant waterhemp populations from Illinois. Further, EPSPS gene copy numbers varied by location. However, the number of gene copies observed in glyphosate-resistant waterhemp (up to 12) were significantly lower than the ones observed for glyphosate-resistant Palmer amaranth (up to 155, data not shown). Our findings support Chatham et al. (2015) who also documented fewer EPSPS gene copies in waterhemp compared to Palmer amaranth.

Though EPSPS gene amplification was the major mechanism responsible for glyphosate resistance in the waterhemp populations evaluated in the current study, there were three populations that did not show elevated copy numbers as the cause of high level of resistance. To determine if there are any mutations in the glyphosate target-site, a small conserved region of the EPSPS gene from genomic DNA of the three glyphosate-resistant populations was sequenced. The partial EPSPS sequence of the resistant plants did not reveal any mutation at Thr_{102} and Pro_{106} (data not shown). Similar to our results, the known Pro106Ser mutation was not found in glyphosate-resistant populations from Illinois, Kansas, Missouri, and Nebraska that did not have elevated EPSPS copy numbers. Thus, it is possible that these populations have developed non-target-site resistance mechanisms (e.g. Nandula et al. 2013). Further research is necessary to characterize glyphosate resistance in these populations

Table 4. Details of the herbicides used in the evaluations

Common name	Trade name	Site of Action (Group)[a]	Rate (g ai/ae ha^{-1})	Adjuvant[c]	Manufacturer
Glyphosate	Roundup Powermax®	EPSPS-inhibitor (9)	868[b]	None	Monsanto Company, St. Louis, MO
Atrazine	Aatrex®	PSII-inhibitor (5)	1,115	1% v/v COC	Syngenta Crop Protection, Greensboro, NC
Tembotrione	Laudis®	HPPD-inhibitor (27)	93	1% v/v MSO	Bayer CropScience, Research Triangle Park, NC
Dicamba	Clarity®	Synthetic auxin (4)	284[b]	1% v/v COC	BASF Corporation Agricultural Products, Research Triangle Park, NC
Fomesafen	Reflex®	PPO-inhibitor (14)	213	1% v/v COC	Syngenta Crop Protection, Greensboro, NC

[a]Abbreviations: EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; HPPD, 4-hydroxyphenylpyruvate dioxygenase; PPO, protoporphyrinogen oxidase; PSII, photosystem II

[b]Values represent acid equivalency (g ae ha^{-1})

[c]COC – Crop Oil Concentrate; MSO – Methylated Seed Oil

[d]Laudis® applications did not include ammonium sulfate (AMS)

Table 5. Herbicide resistance profile in waterhemp samples evaluated from two different sub-regions of Texas

	Region	Resistant[a]	Less Sensitive[a]	Susceptible[a]	Total[b]
		----------% of populations----------			
Glyphosate	Blacklands	9	12	79	58
	Gulf Coast	46	28	26	54
Atrazine (POST)	Blacklands	2	0	98	54
	Gulf Coast	15	27	58	55
Tembotrione[c]	Blacklands	0	0	100	58
	Gulf Coast	0	35	65	54
Fomesafen (POST)	Blacklands	0	2	98	58
	Gulf Coast	0	0	100	52
Dicamba	Blacklands	0	0	100	14
	Gulf Coast	0	0	100	30

[a]Resistant - 0 to 49% injury, less sensitive - 50 to 89% injury, and susceptible - 90 to 100% injury
[b]Total number of populations evaluated for each herbicide in each region
[c]Tembotrione applications did not include ammonium sulfate (AMS) adjuvant.

Table 6. *LD*50[a] values and resistance ratios for the highest resistant waterhemp populations sampled across Texas for glyphosate and atrazine

Herbicide	Population[b]	R^2	RMSE	LD_{50} (g ae/ai ha^{-1})	SE	R/S[c]
Glyphosate	TX-GR25	0.89	17	2833	940	9
	TX-S15	0.95	11	304	62	
Atrazine	TX-AR31[d]	_	_	_	_	>64
	TX-S15	0.96	11	570	1	

[a]LD_{50} is the herbicide rate (g ae ha^{-1} for glyphosate and g ai ha^{-1} for atrazine) that caused 50% plant mortality at 21 days after treatment

[b]Best fit regression equation for glyphosate-resistant population (TX-GR25): 4-parameter logistic function, glyphosate susceptible (TX-S15): 3-parameter logistic function, atrazine susceptible population (TX-S15): quadratic function

[c]R/S (resistance ratio) was calculated based on the LD_{50} values of the resistant population relative to the susceptible standard

[d]The regression equation could not be developed on this atrazine-resistant population because complete mortality could not be achieved even at the highest rate tested (32X)

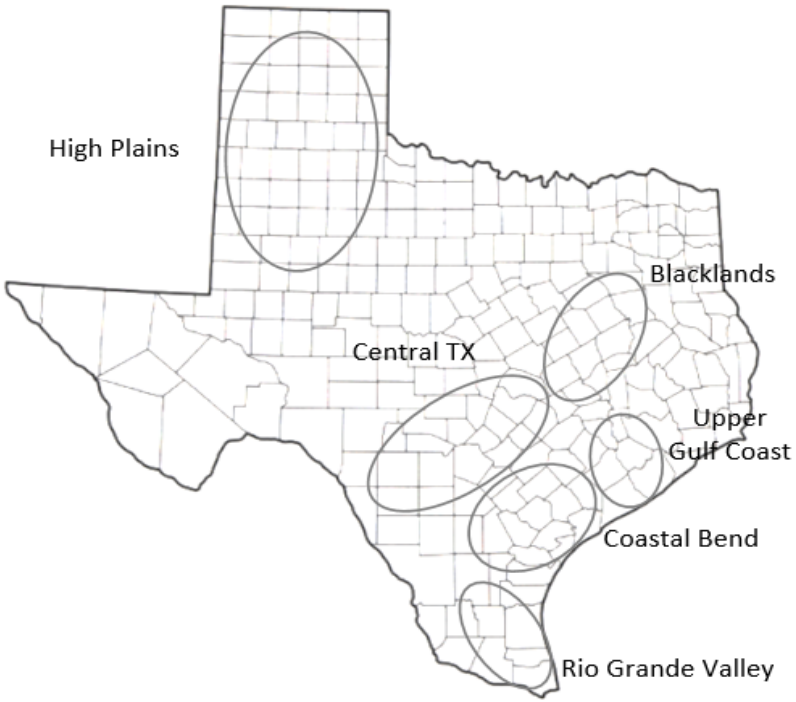

Figure 14. Sub-regions of Texas where survey for waterhemp was carried out in this study

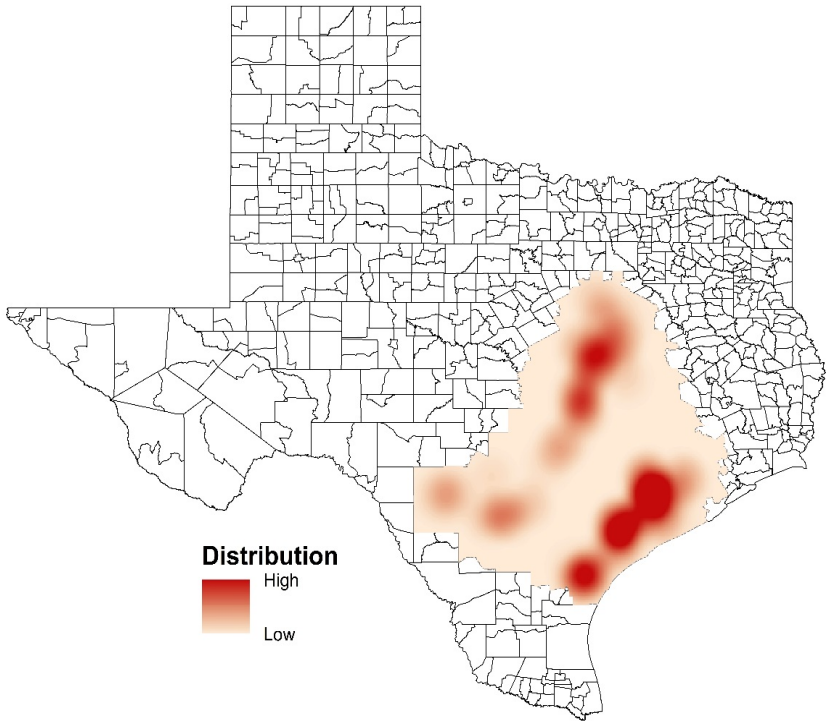

Figure 15. Distribution gradient of waterhemp across different regions of Texas. Dark red represents areas with high infestation of waterhemp, whereas light shaded areas represent areas with low distribution of waterhemp.

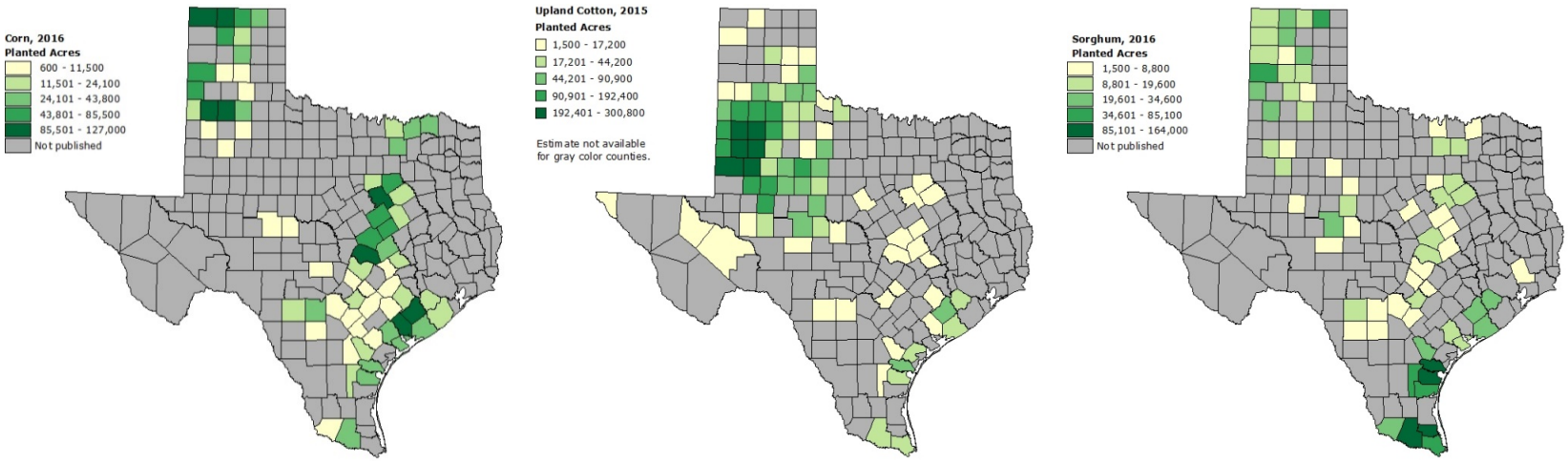

Figure 16. Distribution of corn, cotton and sorghum production acreage across Texas (Source: USDA-NASS 2016)

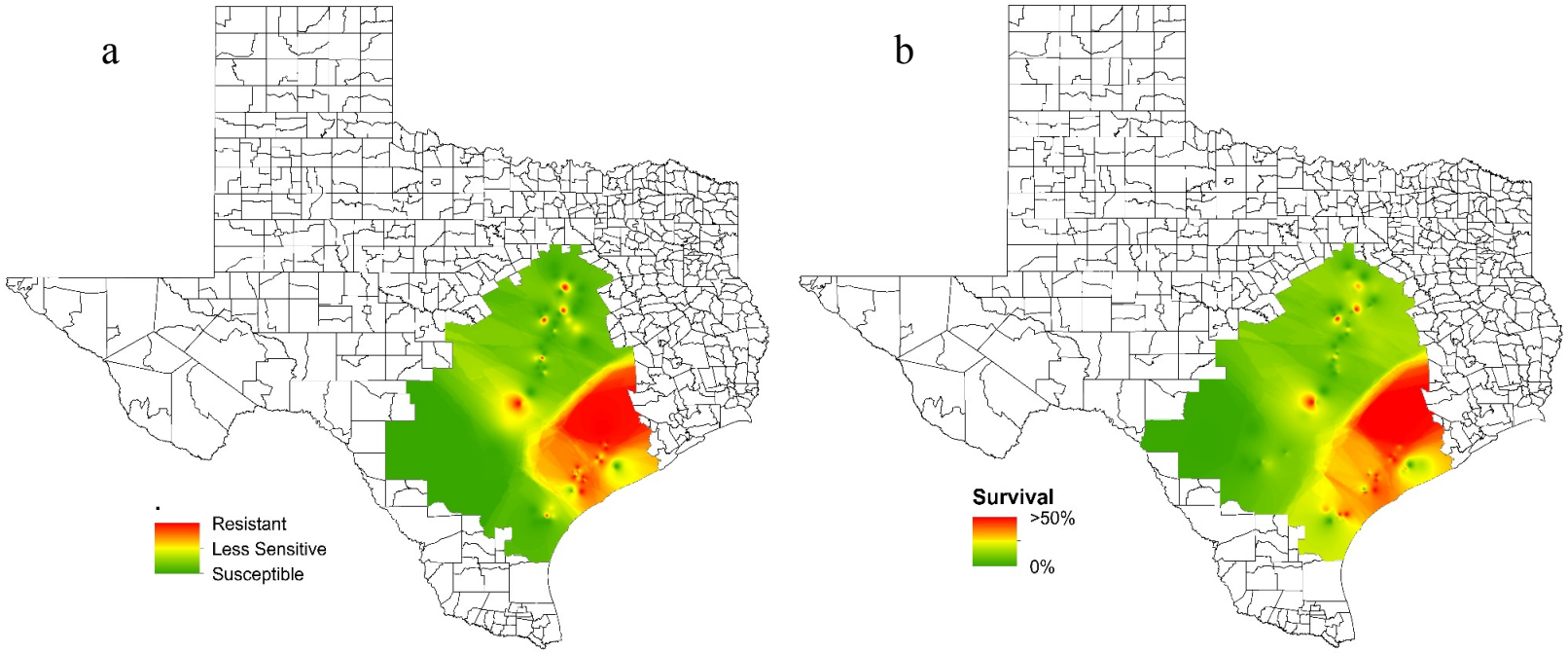

Figure 17. Regional-scale distribution of waterhemp response to glyphosate based on injury (a) and frequency of survivors (b). Resistance levels: resistant - 0 to 49% injury, less sensitive - 50 to 89% injury, and susceptible - 90 to 100% injury. Frequency of survival indicates the stage of advancement of resistance in a given production field. For instance, 50% survival indicates that about half of the individuals in the population are already resistant and that resistance is highly noticeable in the field.

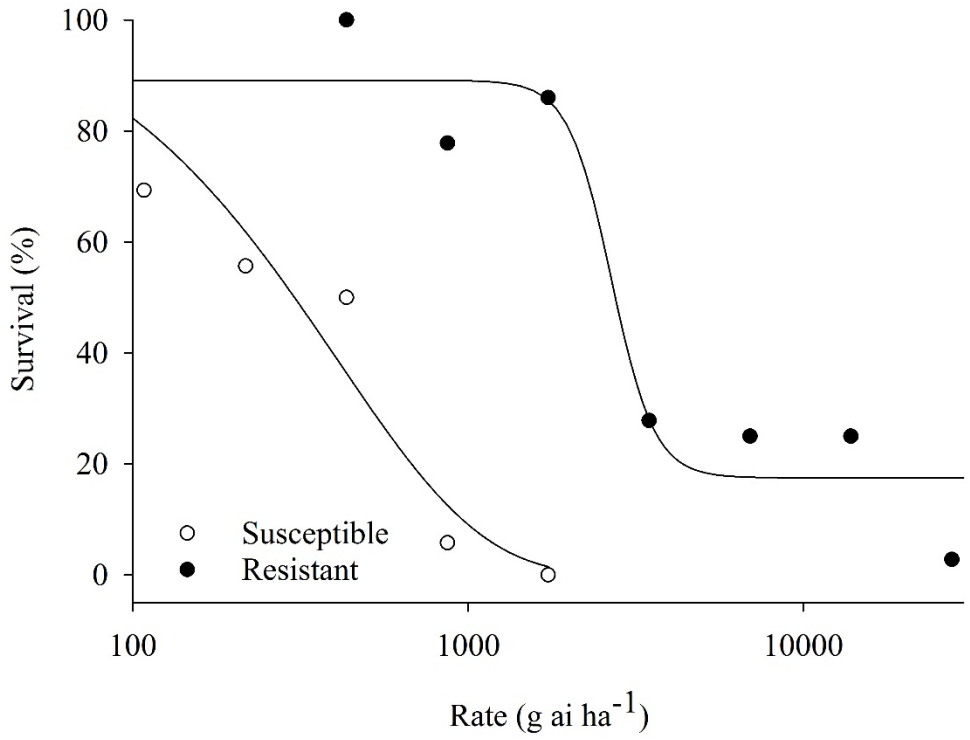

Figure 18. Dose-response analysis of glyphosate-resistant (TX-GR25) and –susceptible (TX-S15) waterhemp populations to glyphosate rates (1X = 868 g ae ha^{-1})

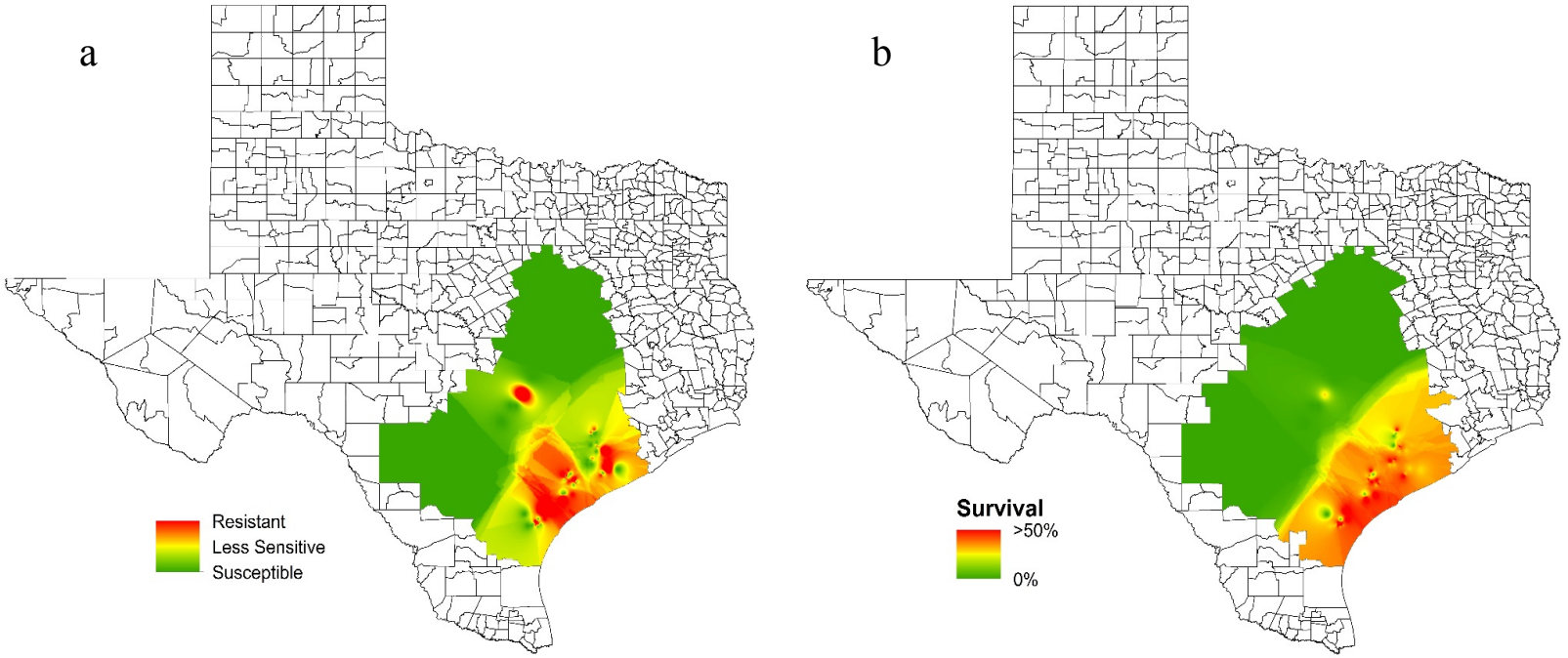

Figure 19. Regional-scale distribution of waterhemp sensitivity to atrazine (applied postemergence) based on injury (a) and frequency of survivors (b). Resistance levels: resistant - 0 to 49% injury, less sensitive - 50 to 89% injury, and susceptible - 90 to 100% injury. Frequency of survival indicates the stage of advancement of resistance in a given production field. For instance, 50% survival indicates that about half of the individuals in the population are already resistant and that resistance is highly noticeable in the field.

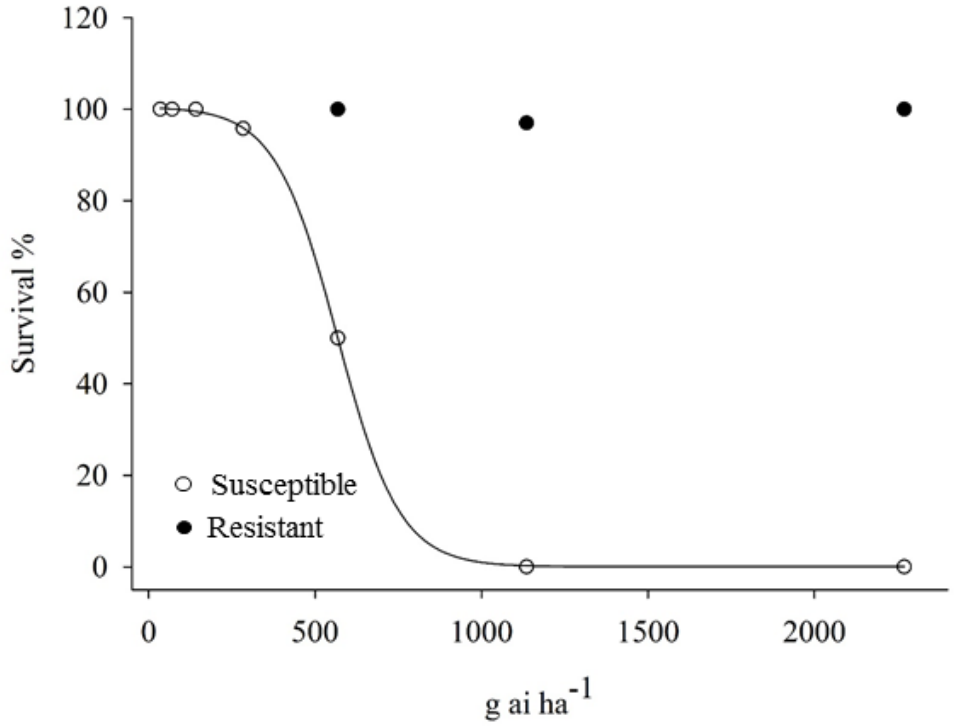

Figure 20. Dose-response analysis of resistant (TX-AR31) and susceptible (TX-S15) waterhemp populations for atrazine (recommended field rate of atrazine = 1,115 g ai ha^{-1})

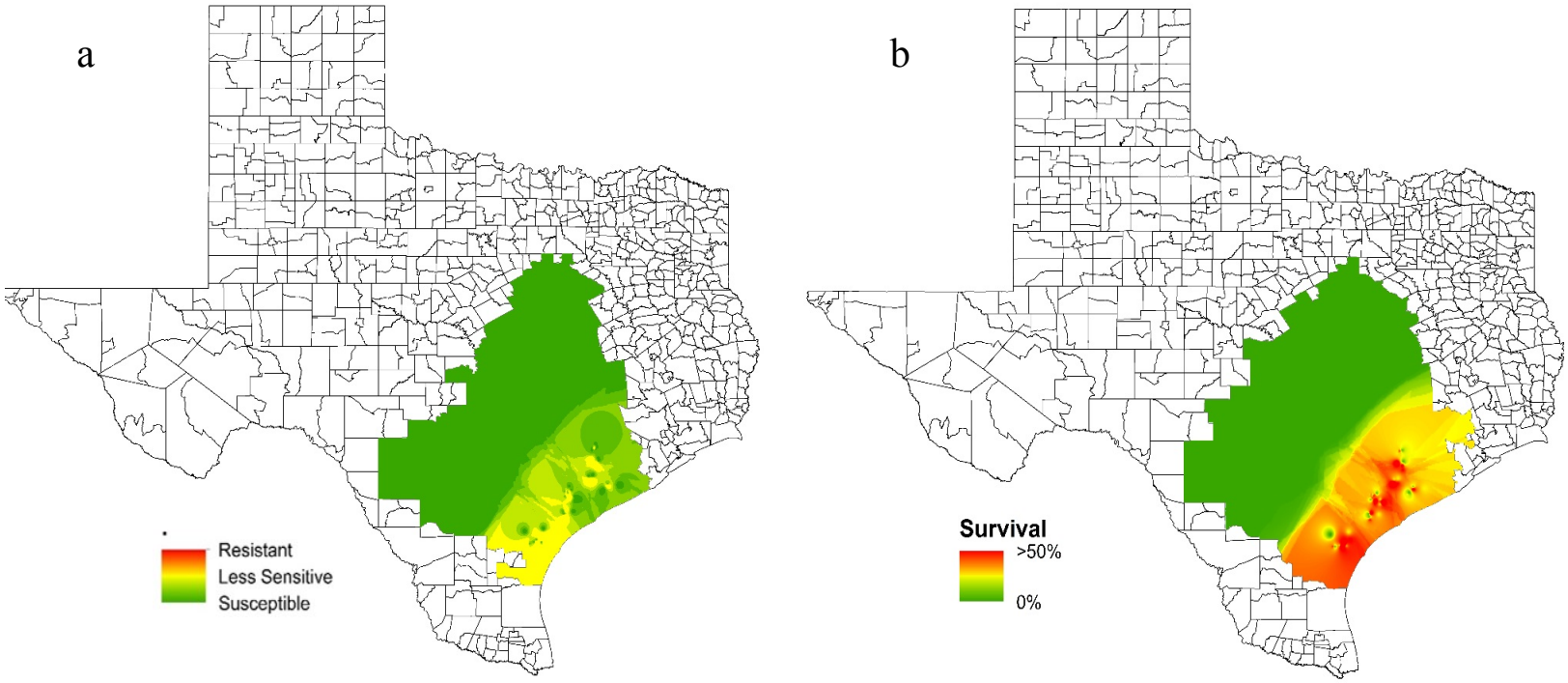

Figure 21. Regional-scale distribution of waterhemp sensitivity to tembotrione based on injury (a) and frequency of survivors (b). Resistance levels: resistant - 0 to 49% injury, less sensitive - 50 to 89% injury, and susceptible - 90 to 100% injury. Frequency of survival indicates the stage of advancement of resistance in a given production field. For instance, 50% survival indicates that about half of the individuals in the population are already resistant and that resistance is highly noticeable in the field. Tembotrione applications did not include ammonium sulfate (AMS) to the mix.

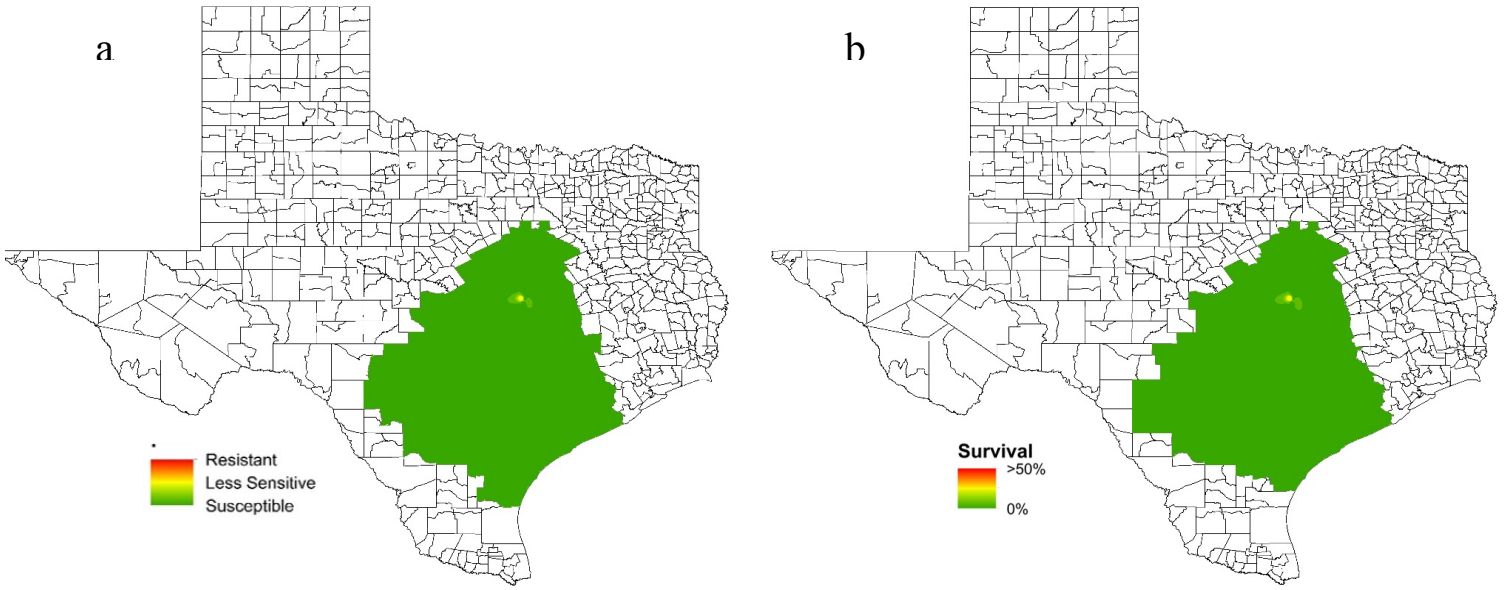

Figure 22. Regional-scale distribution of waterhemp sensitivity to fomesafen based on injury (a) and frequency of survivors (b). Resistance levels: resistant - 0 to 49% injury, less sensitive - 50 to 89% injury, and susceptible - 90 to 100% injury. Based on the scoring scale, all tested populations were susceptible to fomasefen. However, some populations had few individuals surviving fomesefen applications 21 days after treatment, though injury was >90% and the plants did not regrow to produce seed.

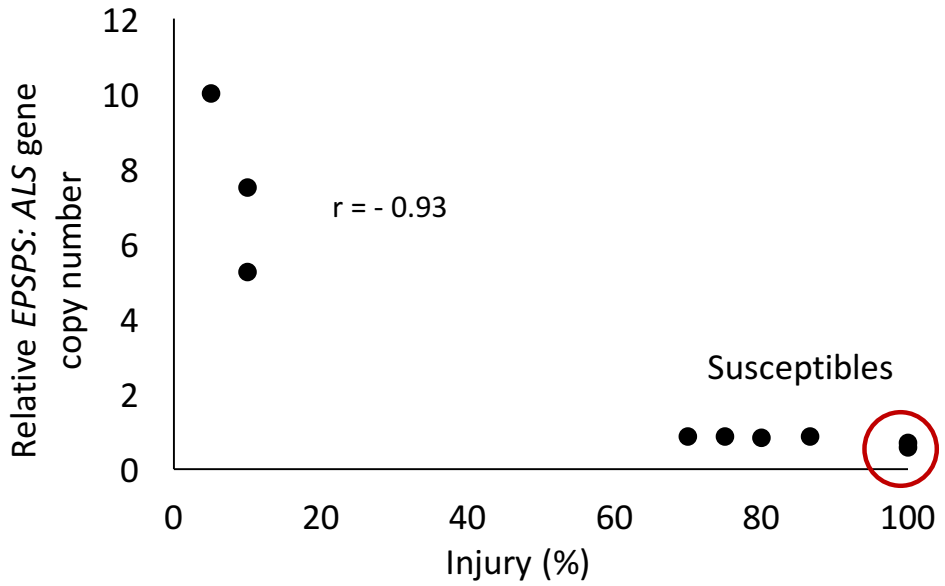

Figure 23. Correlation of relative *EPSPS* genomic copy number with the injury (%) of selected glyphosate-resistant waterhemp populations and susceptible accessions collected from Texas.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Findings from this study clearly illustrate the current status of Palmer amaranth and waterhemp response to various important herbicides across spatial scales in Texas. Until now, no such regional-scale surveys have been conducted in Texas. While the first glyphosate- as well as atrazine-resistant Palmer amaranth and waterhemp populations have been confirmed long back in Texas, the spread of resistance across regional scales has been unknown. Growers and crop consultants have frequently reported control failures with these two herbicides. Field control failures can be attributed to a myriad of causes such as sub-optimal application conditions, poor coverage, environmental factors, or herbicide resistance. Controlled greenhouse evaluations in this study has confirmed the presence of resistance to glyphosate and atrazine in several Palmer amaranth and waterhemp populations.

The Texas High Plains region has the most Palmer amaranth resistance issue, with multiple resistances to glyphosate and atrazine being prevalent. This trend could be attributed to the lack of management diversity utilized for controlling Palmer amaranth in the region. Cotton monocropping is common in the lower and central High Plains. In these areas, glyphosate-resistant cotton cultivars have been grown repeatedly; weed management in this system relies heavily on repeated applications of glyphosate without sufficient management diversity. Further north in the Upper High Plains, corn production is intensive and atrazine has long been a backbone of weed management

programs in the region. Palmer amaranth resistance to atrazine POST does not always endow resistance when applied PRE. However, at least two populations in the Upper High Plains showed resistance to both PRE and POST. In relative terms, Palmer amaranth resistance to glyphosate is more widespread compared to that of atrazine. This is possibly due to fact that other herbicides have been used in the corn system in addition to atrazine for weed management, whereas glyphosate has been the sole herbicide used for weed management in cotton in many producer fields. For waterhemp, resistance to glyphosate and atrazine are prevalent in the Upper Gulf Coast region. In particular, multiple resistance to glyphosate and atrazine is common. The Upper Gulf Coast region is characterized by widespread cultivation of glyphosate-resistant crops, where the selection pressure imposed by glyphosate has been immense. Further, corn and grain sorghum are commonly grown in this region with frequent applications of atrazine for weed management. As a result, resistances to glyphosate and atrazine are common. In other regions, resistance to glyphosate and atrazine in Palmer amaranth as well as in waterhemp is in the early stages of evolution for various herbicides, as evidenced by the relatively low frequencies of survivors in these populations. It is critical that growers in these regions act quickly to ensure the continued utility of these herbicides through implementation of BMPs.

While no resistance has been detected for the HPPD-inhibitor tembotrione in Palmer amaranth or waterhemp, there was high variability in the level of sensitivity across the regions. Specifically, the High Plains region as well as the Upper Gulf Coast region had many populations that showed reduced sensitivity to this herbicide. It has

been shown that HPPD-inhibitor herbicides have been rapidly metabolized in tolerant plants and the ability for enhanced metabolism in these populations could be contributing to reduced sensitivity. It is important to note that the populations with reduced sensitivity to tembotrione came from regions where glyphosate and atrazine resistance is common.

Resistance to the PPO-inhibitor fomesafen or the auxin herbicide dicamba has not been detected in the populations tested in this study. In regions where resistance is widespread to glyphosate and atrazine, growers will heavily use alternative options. The PPO-inhibitor herbicides as well as the upcoming auxin-resistant crop technologies will help manage existing resistance issues in these species, but a lack of diversity in management can lead to resistance to these herbicide SOA as well. Considering the evidence of multiple herbicide resistances involving HPPD-inhibitors, PPO-inhibitors and synthetic auxins elsewhere, it is only a matter of time before these currently effective tools become ineffective in Texas row-crop production systems. It is extremely imperative that growers and weed management practitioners understand the importance of proactive tactics for herbicide resistance management (as outlined in Norsworthy et al. 2012) and prolong the utility of available herbicide options. Diversification of weed management options including chemical and non-chemical tools is of paramount importance. Additional research and extension efforts are necessary in developing and delivering sound BMPs for herbicide resistance management.

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