Field-evolved resistance to four modes of action of herbicides in a single kochia (Kochia scoparia L. Schrad.) population

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Abstract

BACKGROUND: Evolution of multiple herbicide resistance in weeds is a serious threat to weed management in crop production. Kochia is an economically important broadleaf weed in the US Great Plains. This study aimed to confirm resistance to four sites of action of herbicides in a single kochia (Kochia scoparia L. Schrad.) population from a crop field near Garden City (GC), Kansas, and further determine the underlying mechanisms of resistance.

RESULTS: One-fourth of the GC plants survived the labeled rate or higher of atrazine [photosystem II (PSII) inhibitor], and the surviving plants had the Ser-264 to Gly mutation in the psbA gene, the target site of atrazine. Results showed that 90% of GC plants survived the labeled rate of dicamba, a synthetic auxin. At least 87% of the plants survived up to 72 g a.i. ha\(^{-1}\) of chlorsulfuron [acetolactate synthase (ALS) inhibitor], and analysis of the ALS gene revealed the presence of Pro-197 to Thr and/or Trp-574 to Lue mutation(s). Most GC plants also survived the labeled rate of glyphosate [5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitor], and the resistant plants had 5–9 EPSPS gene copies (relative to the ALS gene).

CONCLUSION: We confirm the first case of evolution of resistance to four herbicide sites of action (PSII, ALS and EPSPS inhibitors and synthetic auxins) in a single kochia population, and target-site-based mechanisms confer resistance to atrazine, glyphosate and chlorsulfuron.

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Keywords: multiple herbicide resistance; Kochia scoparia; mechanism of resistance; psbA gene; EPSPS gene; ALS gene; dicamba

1 INTRODUCTION

Use of herbicides is critical for weed control and to increase food grain production worldwide, especially in developed countries. However, continuous and indiscriminate use of herbicides has resulted in the evolution of herbicide resistance in a number of weeds. To date, 431 cases (species \(\times\) site of action) of herbicide resistance have been documented to 22 of the 25 known herbicide sites of action.1 Weeds resistant to herbicides with multiple sites of action (SOAs) are of greater concern, as they reduce the herbicide rotation options and the effectiveness of herbicides, as well as increasing the cost of weed control. Multiple herbicide resistance has been confirmed in a number of economically important weeds across the globe, including Palmer amaranth (Amaranthus palmeri S. Wats.),2 waterhemp (A. tuberculatus Sauer),3 rigid ryegrass (Lolium rigidum Gaudin)4 and kochia (Kochia scoparia).5

Kochia is an annual broadleaf invasive weed that disperses by a tumbling mechanism and infests both crop and range lands. It is a problem weed in the North American Great Plains and has become a dominant weed species in several counties of western Kansas and neighbouring states. Kochia can germinate and emerge early in the growing season and has the ability to survive under severe heat, drought and saline conditions. It is a competitive weed causing substantial yield losses in field crops.6 The outcrossing nature of kochia, combined with its prolific seed production, results in genetically diverse populations that facilitate the evolution of multiple herbicide resistance mechanisms. In the past, biotypes of kochia with evolved resistance to photosystem II (PSII) inhibitors,7 acetolactate synthase (ALS) inhibitors8 and synthetic auxins1 were documented. Recently, several populations of kochia were also confirmed as being resistant to glyphosate.9 Nonetheless, evolved resistance to these four herbicides in a single population has not been reported.

In the summer of 2012, some kochia biotypes in a population were found to be uncontrolled by labeled rates of dicamba, glyphosate and metribuzin in a field near Garden City (GC), Kansas. The field had been sprayed with several herbicides with different SOAs in the past (Table 1). The overall goal of this research was to confirm herbicide resistance to PSII (atrazine),...
ALS (chlorsulfuron) and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (glyphosate) inhibitors and synthetic auxins (dicamba), as well as to determine the mechanisms of resistance to these herbicides in this single kochia population.

2 MATERIALS AND METHODS

2.1 Field history and seed collection

Kochia plants that had survived field recommended rates of dicamba, glyphosate and metribuzin (Table 1) were potted and transferred to a greenhouse. The plants were allowed to cross among themselves, and, upon maturity, seeds were harvested from these plants. A known kochia biotype from Manhattan, Kansas, susceptible (S) to atrazine, dicamba, chlorsulfuron and glyphosate, was used for comparison.

2.2 Herbicide treatments

Multiple-herbicide-resistant (R) and S seeds of kochia were planted in separate pots in the greenhouse for whole-plant herbicide treatment experiments. Plants were grown under a 15/9 h day/night photoperiod, supplemented with 250 μmol m⁻² s⁻¹ of illumination provided by sodium vapor lamps. Twenty-four plants (10–12 cm tall) of R kochia were treated separately with several rates of herbicides: atrazine (AAtrex® 4L at 0, 2240, 4480 and 8960 g a.i. ha⁻¹), chlorsulfuron (Glean® XP at 0, 9, 18, 54 and 108 g a.i. ha⁻¹; 0.25% v/v crop oil concentrate), chlorpyrifos (Roundup Weathermax® at 0, 2240 g a.e. ha⁻¹; 1% v/v crop oil concentrate), chlorsulfuron (Glean® XP at 0, 9, 18, 36 and 72 g a.i. ha⁻¹; 0.25% v/v nonionic surfactant), glyphosate (Roundup Weathermax® at 0, 868, 1736 and 3472 g a.e. ha⁻¹; 2% w/v ammonium sulfate) and dicamba (Clarity® at 560, 1120 and 2240 g a.e. ha⁻¹) with recommended adjuvants. The field recommended rates of atrazine, chlorsulfuron, glyphosate and dicamba are 2240 g a.i. ha⁻¹, 18 g a.i. ha⁻¹, 868 g a.e. ha⁻¹ and 560 g a.e. ha⁻¹ respectively. However, S kochia plants (n = 12 to 24) were treated separately with only the lowest dose (used in this study) of herbicides. Thus, a total of 68 S and 312 R kochia plants were used to treat with herbicides. All treatments were applied with a moving single-nozzle bench-type sprayer equipped with a flat-fan nozzle tip delivering 168 L ha⁻¹ at 222 kPa in a single pass at 3.2 km h⁻¹. The sprayer was cleaned thoroughly after each herbicide treatment with ammonia (1%). Plant survival (dead or alive) was assessed 4 weeks after treatment (WAT).

2.3 Genomic DNA isolation and target-site specific primer design

Fresh leaf tissue was collected from individual plants, flash frozen and stored at −80 °C for genomic DNA (gDNA) isolation. gDNA was extracted from frozen leaf tissue (100 mg) using DNeasy Plant Mini kit (Qiagen Inc., Valencia, CA), following the manufacturer’s instructions. DNA was quantified on a NanoDrop spectrophotometer. About 10 μL of the purified gDNA product (25 ng μL⁻¹) was sequenced using an ABI 3730 DNA analyzer (Life Technologies, Carlsbad, CA).

2.4 Polymerase chain reaction (PCR) and gene sequencing

PCR was performed in a T100 thermal cycler (Bio-Rad Inc., Hercules, CA) using PCR master mix (Promega, Madison, WI). The 25 μL reaction volume consisted of 12.5 μL of PCR master mix (2X), 2.5 μL of forward primer (5 μM), 2.5 μL of reverse primer (5 μM), 3 μL of gDNA template (15 ng μL⁻¹) and 4.5 μL of nuclelease-free water. For amplification of the psbA gene, the following PCR conditions were used: 95 °C for 3 min, 45 cycles of 95 °C for 30s, 55 °C for 30 s and 72 °C for 1 min, followed by 72 °C for 5 min. The ALS gene was amplified using the following PCR conditions: 95 °C for 3 min, 45 cycles of 95 °C for 30 s, 55 °C for 45 s and 72 °C for 2 min, followed by 72 °C for 8 min. PCR conditions for the EPSPS gene were 95 °C for 3 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, followed by 72 °C for 5 min. The PCR products were run on 1% agarose gel with 500 and 100 bp markers to confirm amplicon size.

PCR products were purified using GeneJet PCR purification kit (Thermo Fisher Scientific) and quantified using a NanoDrop spectrophotometer. About 10 μL of the purified PCR product (25 ng μL⁻¹) was sequenced using an ABI 3730 DNA analyzer (Life Technologies, Carlsbad, CA).

2.5 Target-site DNA sequence analysis

Nucleotide sequences of psbA, EPSPS and ALS genes were aligned using MultAlin software to analyze the presence of any known target-site mutation(s) that confer resistance to the respective herbicide. Further, the homozygous or heterozygous nature of the point mutations was detected by analyzing chromatograms of the sequences using FinchTV 1.4.0 software (Geospiza, Inc., Seattle, WA). Overlapping double peaks of similar heights were considered to be heterozygote point mutations.

2.6 Quantitative real-time PCR (qPCR)

A qPCR reaction was performed using a CFX96™ real-time detection system (Bio-Rad) to determine the EPSPS gene copy number in glyphosate-resistant kochia from GC. The qPCR reaction mix consisted of 8 μL of SYBR Green master mix (Bio-Rad), 2 μL each of forward and reverse primers (5 μM) and 2 μL of gDNA (15 ng μL⁻¹) to make the total reaction volume up to 14 μL. The EPSPS gene copy number was measured relative to the ALS gene (reference gene). PCR conditions were 95 °C for 15 min and 40 cycles of 95 °C for 30 s and 60 °C for 1 min. A melt curve profile was included following the thermal cycling protocol to determine the specificity of the qPCR reaction. The following primer sequences were used: EPSPS F 5’-GGCCAAAAAGGGCAATCGTGGAG-3’ and EPSPS R 5’-CATTGCCGTCCGCCTTCCC-3’; ALS F 5’-CCGGTCT TCCCTTACCTCTT-3’ and ALS R 5’-GGAAGGTGTGAGTGAAGTT TG-3’. The EPSPS gene copy number was measured in five GC plants and two S plants with three technical replicates. The gene copy number was determined using the 2⁻ΔΔCt method, where Ct is the threshold cycle and ΔCt = Ct_target gene (EPSPS) − Ct_reference gene (ALS).14

3 RESULTS

3.1 Resistance to atrazine

Results showed that 25% of GC plants survived atrazine at 2240 g a.i. ha⁻¹ (labeled rate) or higher, whereas none of the S plants survived the labeled rate of atrazine (Fig. 1A). Atrazine did not cause any visible injury on GC plants that survived, indicating very high levels of resistance (data not shown). A mortality of 75% of the GC plants with the labeled rate of atrazine indicated triazine mutants at a low frequency in the GC population.

Sequence comparison of atrazine-resistant GC plants with the known S plants revealed a single mutation in the psbA gene, involving substitution of amino acid serine (AGT) with glycine (GGT) at position 264 (the nucleotide position number is based on the amino acid sequence from Arabidopsis), indicating target-site resistance to atrazine (Fig. 1B). There was no valine (GTA) to isoleucine (ATA) mutation at position 219 in GC kochia (Fig. 1B).
3.2 Resistance to dicamba

Dicamba at 560 g a.e. ha\(^{-1}\) (labeled rate) killed all the S kochia plants. Results showed that 90% of the GC plants survived dicamba application at the labeled rate (Fig. 1C). Survival of the GC plants decreased with increasing rates: 75 and 20% at 1120 and 2240 g a.e. ha\(^{-1}\) respectively.

3.3 Resistance to chlorsulfuron

At least 87% of the GC kochia survived 9 g a.i. ha\(^{-1}\) (half the labeled rate) or higher rates of chlorsulfuron (Fig. 2A). Plants of S kochia did not survive 9 g a.i. ha\(^{-1}\) of chlorsulfuron. The GC plants developed varying levels of injury to chlorsulfuron that did not correlate with applied rates (data not shown).

Nucleotide sequence analysis of the ALS gene in GC kochia revealed point mutations involving substitution of proline (CCG) with threonine (ACG) at position 197 and/or tryptophan (TGG) with leucine (TTG) at position 574 (Fig. 2B). The Pro-197 to Thr mutation was the most common and was present in both homoygous and heterozygous forms. The Trp-574 to Leu mutation was present in few plants, and only two plants carried both mutations. The absence of correlation of injury with chlorsulfuron rates is likely due to plants carrying different mutations and their zygosity.

4 DISCUSSION

Kochia resistance to triazines, ALS inhibitors, glyphosate and dicamba has been reported previously in independent populations. Also, multiple resistance to triazines and ALS inhibitor herbicides in kochia was reported in Indiana and Illinois in 1995, and more recently (2013) multiple resistance to ALS inhibitors and glyphosate has been reported in Montana. Resistance to dicamba was first reported in 1995 in Montana and North Dakota. This is the first report of the presence of resistance to four herbicide SOAs in a single kochia population after over 30 years of using different herbicide modes of action in this field.

Whole-plant herbicide treatment results indicated that a proportion of this GC population was resistant to all four herbicides tested (atrazine, chlorsulfuron, glyphosate and dicamba) (Figs 1 to 3). Sequencing of the psbA gene in GC plants revealed substitution of Ser-264 with Gly, but no changes in Val-219 or Ala-251 residues (Fig. 1B). Val-219 to Ile and Ala-251 to Val point mutations have been reported to confer resistance to metribuzine. The Ser-264 to Gly mutation results in several-fold resistance to atrazine compared with the wild type.

The most common mechanism of resistance to ALS inhibitors is the presence of point mutations spanning five highly conserved domains of the ALS enzyme in plants. GC kochia carried the known mutations of Pro-197 to Thr and Trp-574 to Leu, with both being found in two of the 15 plants analyzed (Fig. 2B). Previously, substitution of the amino acid Pro-197 with Thr, Ser, Arg, Leu, Glu and Ala were reported for chlorsulfuron-resistant kochia biotypes from Kansas. Accumulation of different ALS gene mutations in a single individual is possible owing to the outcrossing nature of GC kochia, plant mortality increased with the higher rates of glyphosate; only 30% of GC plants survived glyphosate at 3472 g a.e. ha\(^{-1}\). This indicates low to moderate levels of resistance to glyphosate. Similar levels of resistance have been reported in other glyphosate-resistant kochia populations from the region.

The EPSPS gene copy number was measured relative to an ALS gene in five plants of GC kochia. Increased EPSPS gene copy numbers ranging from 5 to 9 were observed in GC plants, suggesting gene amplification as a contributing mechanism to glyphosate resistance (Fig. 3B). None of the GC plants carried a mutation at proline 106 in their EPSPS gene according to the sequencing studies (Fig. 3C).
kochia and can occur either by sequential mutations or intragenic recombination between the amino acid 197 and 574 resistance alleles of the ALS gene.18

Weed resistance to glyphosate has been shown to have evolved as a result of altered translocation, EPSPS gene mutation, EPSPS gene amplification and vacuole sequestration.20 In GC kochia plants, low to medium levels of resistance were observed, and resistant plants possessed 5–9 EPSPS copies (relative to the ALS gene), and no mutation at Pro-106 in the EPSPS gene was present.

In the present study, 90% of the GC population survived the labeled rate of dicamba. Recently, Crespo et al.22 found that greater than 560 g a.e. ha⁻¹ (labeled rate) of dicamba was needed to achieve an 80% reduction in dry weight for all kochia accessions tested, and one accession required 3500 g a.e. ha⁻¹ of dicamba to achieve a 50% dry weight reduction. One earlier study reported the slow spread of dicamba-resistant kochia in the production fields of Montana, suggesting the quantitative

(Figs 3B and C). This mechanism of resistance has been reported in several other glyphosate-resistant kochia populations in the Central Great Plains of the United States.9,13,21

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nature of the trait. These studies are important in the context of the development of new dicamba-resistant soybean, corn and cotton to control glyphosate-resistant broadleaf weeds, possibly resulting in increased use of dicamba and selection for resistance. Previous research suggests that the dicamba resistance in kochia (populations from Montana) was not due to reduced herbicide uptake or altered translocation; however, the concentration of the primary metabolite of dicamba was significantly higher in dicamba-resistant kochia than in dicamba-susceptible kochia. Nonetheless, the mechanism of dicamba resistance in kochia is not known conclusively. Experiments are in progress in Jugulam’s lab to understand the mechanism of dicamba resistance in kochia from Kansas.

In conclusion, we report herbicide resistance to four SOAs and the underlying resistance mechanisms in a single kochia population from Kansas. Evolution of multiple herbicide resistance will reduce the herbicide options for effective control of kochia in numerous cropping systems. More importantly, the addition of one new SOA on top of these herbicides to weed management programs will not decrease the probability of selecting for resistance to that new SOA. It is not clear whether multiple herbicide resistance in kochia arose from accumulation of different resistance alleles (to specific herbicides) in a single individual from outcrossing or whether it resulted from sequential selection with different SOAs. Effective long-distance seed dispersal characteristics, as well as the outcrossing nature of kochia plants, may result in rapid accumulation of herbicide resistance alleles in a population. Kochia management strategies in the region should focus on minimizing herbicide selection pressure and precluding the spread of the resistance alleles to achieve sustainable benefit from the upcoming new herbicide-tolerant crop technologies.

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REFERENCES