

Molecular Basis of Evolved Resistance to Glyphosate and Acetolactate Synthase-Inhibitor Herbicides in Kochia (*Kochia scoparia*) Accessions from Montana

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The rapid evolution and spread of glyphosate-resistant (GR) kochia in the Northern Great Plains is an increasing threat to GR cropping systems and conservation tillage practices common in this region. GR kochia accessions with 4.6- to 11-fold levels of resistance to glyphosate have recently been reported in Montana. Those GR kochia accessions were also suspected to be resistant to acetolactate synthase (ALS) inhibitors, i.e., multiple herbicide-resistant (MHR) kochia. In this research, the level of resistance to the ALS-inhibitor herbicides (sulfonylureas) and the molecular mechanisms conferring resistance to glyphosate and ALS-inhibitor herbicides in MHR kochia was investigated. On the basis of whole-plant dose–response assays, MHR kochia accessions (GIL01, JOP01, and CHES01) were 9.3- to 30-fold more resistant to premixed thifensulfuron methyl + tribenuron methyl + metsulfuron methyl than the susceptible (SUS) accession. In an *in vivo* leaf-disk shikimate assay, MHR plants accumulated less shikimate than the SUS plants at a discriminate dose of 100 μ M glyphosate. Sequencing of the conserved region of *EPSPS* revealed no target-site mutation at Thr₁₀₂ or Pro₁₀₆ residue. MHR kochia accessions had increased relative *EPSPS* gene copies (\sim 4 to 10) compared with the SUS accession (single copy). Furthermore, MHR kochia accumulated higher EPSPS protein compared with the SUS plants. Resistance to the ALS-inhibitor herbicides was conferred by Pro₁₉₇ amino acid substitution (proline to glutamine). *EPSPS* gene amplification and a single target-site mutation at Pro₁₉₇ in *ALS* gene confer resistance to glyphosate and ALS-inhibitor herbicides, respectively, in MHR kochia accessions from Montana. This is the first confirmation of occurrence of MHR kochia in Montana.

Nomenclature: Glyphosate; metsulfuron methyl; thifensulfuron methyl; tribenuron methyl; Kochia, *Kochia scoparia* (L.) Schrad.

Key words: *ALS* gene mutation, ALS-inhibitor resistance, *EPSPS* gene amplification, glyphosate resistance, multiple herbicide resistance.

Glyphosate is a systemic, nonselective, POST herbicide widely used for weed control in cropland and noncropland worldwide. Glyphosate has been described as a once-in-a-century herbicide because of its broad-spectrum efficacy on weeds, low mammalian toxicity, and environmentally benign nature (Duke and Powles 2008). Glyphosate inhibits the biosynthesis of aromatic amino acids (phenylalanine, tyrosine, and tryptophan) by inhibiting the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19) enzyme in the shikimate pathway in plants, ultimately causing growth inhibition and plant death (Duke and Powles 2008; Powles 2010). Glyphosate usage has increased dramatically after the introduction of

glyphosate-resistant (GR) crops in mid-1990s. In addition, this led to an increased adoption of conservation tillage by growers (Powles 2010). The exclusive use of glyphosate for weed control in GR cropping systems has been reported, with multiple applications per growing season (Norsworthy et al. 2007). Nevertheless, the increased selection pressure from glyphosate in GR crops resulted in evolution of GR weed biotypes. To date, glyphosate resistance has been reported in 31 weed species globally (Heap 2015).

Kochia, a monoecious C_4 diploid ($2n = 18$), is among the most problematic summer annual broadleaf weeds in the Northern Great Plains (NGP) of the United States, including Montana (Eberlein and Fore 1984; Forcella 1985; Friesen et al. 2009). Kochia possesses unique biological attributes, including early seedling emergence, rapid growth, tolerance to abiotic stresses (heat, salt, drought), prolific seed production ($> 50,000$ seeds plant⁻¹), low seed dormancy (1 to 2 yr), and long-distance seed dispersal by the tumble behavior of the

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mature plants (Baker et al. 2010; Christoffoleti et al. 1997; Friesen et al. 2009; Schwinghamer and Van Acker 2008). Furthermore, the protogynous nature of kochia flowering enforces a high degree of outcrossing and pollen-mediated gene flow (Mengistu and Messersmith 2002; Stallings et al. 1995). Consequently, there is a high genetic diversity within and among kochia populations (Stallings et al. 1995). All these biological traits make kochia a difficult-to-control weed species, with rapid development and spread of herbicide resistance. Kochia has evolved resistance to several herbicide mechanisms of action, including photosystem II inhibitors (atrazine), acetolactate synthase (ALS) inhibitors (sulfonylurea and imidazolinone herbicides), and synthetic auxins (dicamba and fluroxypyr) in the NGP (Heap 2015). In 2007, kochia accessions resistant to glyphosate were first detected in Kansas (Waite et al. 2013). Since then, GR kochia has emerged in nine other states in the NGP, including Montana, and in three provinces of Canada (Beckie et al. 2013; Heap 2015; Kumar et al. 2014). The increased occurrence of GR kochia is a potential threat to GR cropping systems such as corn (*Zea mays* L.), soybean [*Glycine max* (L.) Merr.], and sugar beet (*Beta vulgaris* L.), and to no-till wheat (*Triticum aestivum* L.)–fallow rotation common in this region.

Five major mechanisms of glyphosate resistance have been characterized in weed species so far, e.g., target-site *EPSPS* mutation, *EPSPS* gene amplification, active vacuolar sequestration, reduced translocation, and rapid necrosis response (Sammons and Gaines 2014). *EPSPS* gene amplification is a novel mechanism of glyphosate resistance, first documented in Palmer amaranth (*Amaranthus palmeri* S. Wats.) (Gaines et al. 2010). Increased *EPSPS* gene copies in GR Palmer amaranth was positively correlated with *EPSPS* protein abundance (Gaines et al. 2010). This resistance mechanism has also emerged in other GR weed species, such as tall waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] (Lorentz et al. 2014; Tranel et al. 2011), spiny amaranth (*Amaranthus spinosus* L.) (Nandula et al. 2014), and Italian ryegrass [*Lolium perenne* ssp. *multiflorum* (Lam.) Husnot] (Salas et al. 2012). Reduced absorption or translocation has not been associated with glyphosate resistance in GR kochia in the U.S. Great Plains (Waite et al. 2013; Wiersma et al. 2015).

ALS-inhibitor-resistant kochia is widely spread across Montana and other NGP states, primarily through the selection of mutations in the *ALS* gene

(Heap 2015; Primiani et al. 1990). In particular, three highly conserved amino acids (Pro₁₉₇, Asp₃₇₆, and Trp₅₇₄) in the *ALS* enzyme are sensitive to alteration by recurrent selection from ALS-inhibitor herbicides in kochia (Beckie et al. 2013; Tranel and Wright 2002; Tranel et al. 2015). An alteration in any of these amino acids confers a high level of resistance to sulfonylurea or imidazolinone herbicides in kochia (Tranel and Wright 2002; Tranel et al. 2015).

Winter-wheat–fallow rotation dominates > 90% of the dryland cropping systems of this region, including Montana. The average precipitation in Montana is < 30 cm, which is often a limiting factor for continuous cropping. The purpose of chemical fallow in wheat–fallow rotation is to prevent soil erosion and to conserve soil moisture for successful establishment of winter wheat planted in the fall (Lenssen et al. 2007). Glyphosate is the most commonly used herbicide to control kochia and other weeds in chemical fallow or in postharvest wheat stubble (Donald and Prato 1991; Kumar and Jha 2015a,b), and a typical chemical–fallow field receives three to four applications of glyphosate (0.870 kg ae ha⁻¹) before planting of winter wheat in a year. Because of enhanced selection pressure from repeated use of glyphosate, GR kochia accessions with 4.6- to 11-fold levels of resistance to glyphosate have evolved in wheat–fallow fields in northern Montana (Kumar et al. 2014). With historical reports on widespread occurrence of kochia resistant to ALS inhibitors in Montana and other adjoining states (Primiani et al. 1990; Sivakumaran et al. 1993), these confirmed GR kochia biotypes from Montana were also suspected to be resistant to ALS inhibitors (sulfonylurea), i.e., multiple herbicide-resistant (MHR) kochia. The objectives of this research were (1) to elucidate the mechanism conferring resistance to glyphosate and (2) to characterize the level of resistance to ALS-inhibitor herbicides (sulfonylureas) and determine if target-site mutation(s) in *ALS* might confer resistance to ALS inhibitors in MHR kochia accessions from Montana.

Materials and Methods

Plant Material. Three GR kochia accessions (GIL01, JOP01, and CHES01) collected in fall of 2012 from chemical–fallow fields (wheat–fallow rotation) in Hill and Liberty counties of northern Montana were investigated. The glyphosate-susceptible (SUS) kochia accession was collected

from a field used for long-term organic trials near Huntley, MT. Preliminary greenhouse dose–response experiments indicated that the SUS population was susceptible to glyphosate and ALS-inhibitor herbicides (data not shown). Plants from the dose–response study (Kumar et al. 2014) that survived at 1.68 kg ae ha⁻¹ were transplanted into 5-L plastic pots containing a commercial potting mix (VermiSoil™, Vermicrop Organics, 4265 Duluth Avenue, Rocklin, CA) in a greenhouse at the Montana State University Southern Agricultural Research Center (MSU-SARC) near Huntley, MT. The greenhouse was maintained at 25/23 C ± 3 C day/night temperatures and 16/8-h day/night photoperiods supplemented with metal halide lamps (400 μmol m⁻² s⁻¹). Kochia plants were watered once daily to avoid moisture stress, and fertilized (Miracle-Gro water-soluble fertilizer [24–8–16], Scotts Miracle-Gro Products Inc., 14111 Scottslawn Road, Marysville, OH) once a week to maintain good growth. Kochia plants (60 to 70 cm tall) were covered with pollen bags (DelStar Technologies, Inc., 601 Industrial Drive, Middletown, DE 19709) to avoid cross-pollination, and allowed to produce F1 seed. Two pollen bags (each of 50.8 by 45.7 cm size) were glued together lengthwise to fully cover each kochia plant. The F1 generation was selfed to obtain the F2 seed. Screening of F2 plants of the GR populations indicated that > 95% of individuals survived the 1.68 kg ha⁻¹ rate of glyphosate (data not shown). Similarly, F2 seeds of the SUS accession were obtained by selfing a group (to prevent inbreeding depression) of F1 plants under pollen isolation conditions as described previously, in a separate greenhouse. The F2 seeds of SUS and GR kochia accessions were used for subsequent experiments.

Shikimate Accumulation. Three plants per kochia accession (JOP01, GIL01, CHES01, and SUS) were tested for shikimate accumulation using an in vivo leaf-disk assay (Shaner et al. 2005). Three technical replicates (5-mm-diam leaf disks) from each young kochia plant (8 to 10 cm tall) per accession were sampled, and the assay was repeated in time. On the basis of preliminary experiments, 100 μM glyphosate (discriminating dose) was chosen to differentiate the shikimate accumulation between GR and SUS kochia plants. The excised leaf disks were placed into the wells of a 96-well microtiter plate containing 7.7 mM ammonium phosphate and glyphosate (molecular grade) at doses of 0, 100, or 1,000 μM. The samples were incubated in light

for 16 h at room temperature, frozen (–20 C) and thawed (60 C), and subjected to the extraction procedure described by Shaner et al. (2005) and Wiersma et al. (2015). Shikimate levels were recorded at 380 nm on a 96-well plate reader (BioTek™ Synergy™ 2 multi-mode microplate reader, Winooski, VT). A shikimate standard curve was developed to quantify shikimate accumulation (ng shikimate μl⁻¹) in the experimental samples (Shaner et al. 2005). Experiments were conducted in a completely randomized design. Data were subjected to ANOVA using PROC MIXED in SAS 9.2 (SAS Institute, Inc., SAS Campus Drive, Cary, NC 27513). Means were separated using Fisher's Protected LSD test at P < 0.05. The interaction of experimental run with accession, glyphosate dose, or accession by glyphosate dose was nonsignificant; therefore, data were pooled over experimental runs.

Sequencing of *EPSPS* Thr₁₀₂ and Pro₁₀₆ Codons.

Three plants from each kochia accession were sampled, and the experiment was repeated in time. A 100-mg sample of young leaf tissue was flash frozen with liquid nitrogen and ground to a fine powder. The genomic DNA (gDNA) extraction was performed using the Qiagen DNeasy Plant mini kit. The DNA quality and concentration was determined using a Bio-Rad SmartSpect™ Plus spectrophotometer. The conserved region of the *EPSPS* gene encompassing Pro₁₀₆ and Thr₁₀₂ codons was amplified by polymerase chain reaction (PCR). The primer set previously designed for Palmer amaranth *EPSPS* sequence (200 base pairs [bp]) was utilized (Gaines et al. 2010); the forward and reverse primer sequences were 5'-ATGTTG-GACGCTCTCAGAACT-3' and 5'-TGAATTTCCTCCA GCAACGGC-3', respectively. Each PCR reaction contained 10 μl of GoTaq® Green Master Mix (containing *Taq* DNA polymerase, deoxynucleotide triphosphates [dNTPs], MgCl₂, and reaction buffers), 1 μl each of forward and reverse primers (5 μM), and 5 μl of gDNA (2 ng μl⁻¹). The thermocycle conditions for PCR were: initial denaturation at 98 C for 1 min, followed by 30 cycles of denaturation at 98 C for 15 s, primer annealing at 50 C for 30 s, and product extension at 72 C for 45 s. A final extension cycle of 10 min at 72 C was included, and the reaction was terminated and held at 4 C. PCR products were separated on 1% agarose gel, stained with ethidium bromide, and isolated from the gel using the GENECLEAN® II Kit (MP Biochemical™) by following manufacturer's protocol. Sanger sequencing of purified PCR

fragments was performed using an ABITM 3130×L genetic analyzer. CLC genomics workbench software was utilized to analyze the sequence reads, and the *EPSPS* sequence of kochia samples was aligned to a reference glyphosate-susceptible Palmer amaranth *EPSPS* sequence to determine substitutions at Pro₁₀₆ or Thr₁₀₂ codon.

***EPSPS* Genomic Copy Number.** Real-time quantitative PCR (qPCR) experiments on genomic DNA were performed to determine relative *EPSPS*:*ALS* genomic copy number in the kochia accessions tested. Three plants per accession were sampled. The *ALS* gene was chosen as a reference gene because of the stability of *ALS* gene expression across kochia populations (Wiersma et al. 2015). For all samples, the *ALS*plant1 : *ALS*plant2 relative gene copy number was equal to 1, also reported by Wiersma et al. (2015). The gDNA extracted for *EPSPS* gene sequencing was used for determining the gene copy number because of its high quality (260/280 ratio of ≥ 1.8). Primer sequences specific to kochia *EPSPS* and *ALS* gene were similar to those previously reported (Wiersma et al. 2015). The primer set for the *EPSPS* gene (*EPSPF1*: 5'-GGC CAA AAGGGCAATCGTGGAG-3' and *EPSPR1*: 5'-CATTGCCGTTCCCGCGTTTCC-3') produced an approximately 102-bp product, and the *ALS* primer set (*ALSF1*: 5'-ATGCAGA CAA TGTTGGATAC-3' and *ALSR1*: 5'-TCAACCA-TCG ATA CGAACAT-3') amplified 159-bp PCR products. Each qPCR reaction contained 2 μ l of gDNA (2 ng μ l⁻¹) template, 1 \times Perfecta SYBR Green Supermix, and 250 nM each of forward and reverse primers (Giacomini et al. 2014). Each qPCR reaction was performed with a final reaction volume of 12.5 μ l on a 96-well PCR plate. The qPCR thermo profiles were: 95 C for 15 min, 40 cycles of 95 C for 30 s, and 60 C for 1 min, followed by melt-curve analysis. Negative controls containing 10 μ l of Mastermix, 250 nM each of forward and reverse primers, and 2.5 μ l of high-performance liquid chromatography water with no gDNA template were also included. Standard curves for each primer pair were developed using a 10-fold serial dilution of the genomic DNA, and primer efficiency was 102% for *EPSPS* and 101% for *ALS*. Threshold cycles (C_t) were recorded by the Bio-Rad CFX96TM Real Time System C 1000 TouchTM thermal cycler. The qPCR reaction efficiency was 102% (R^2 of 0.99 and a slope of -3.215). There was a linear relationship between C_t values and log DNA concentration, indicating that the C_t

values were a reliable estimate of the relative *EPSPS* gene copy number. The relative *EPSPS* gene copy number was calculated as $2^{-\Delta C_t}$, where $\Delta C_t = C_{t, EPSPS} - C_{t, ALS}$ (Gaines et al. 2010). Each sample was run in triplicate to calculate the mean and standard error of the increase in relative *EPSPS* copy number. Correlation analysis between relative *EPSPS* copy number and shikimate accumulation or glyphosate resistance level (50% growth inhibition [I_{50}] or 50% shoot dry weight reduction [GR_{50}] values) was performed using PROC CORR in SAS 9.2.

***EPSPS* Protein Abundance.** To further confirm the *EPSPS* gene amplification in the GR kochia accessions, the relative abundance of the *EPSPS* protein was determined using a western blot. A protein assay was conducted by following a previously established protocol (Bolt and Mahoney 1997) with slight modification. Three plants per kochia accession were sampled, and the experiment was repeated. Young leaf tissue (100 mg) was ground to a fine powder in liquid nitrogen. Total protein was extracted using a 1 : 4 ratio of leaf tissue to laemmli buffer (10% β -mercaptoethanol, 60% sodium dodecyl sulfate [SDS] [10% w/v], 20% glycerol, 10% H₂O). Protein samples were then boiled for 5 min and centrifuged for 5 min at 16,900 $\times g$. Total protein in 15- μ l aliquots were loaded in each lane and resolved by 10% (w/v) SDS-polyacrylamide gel electrophoresis at 25 mA per gel for 1 h. Gel was stained with Ponceau S before the membrane transfer to visually confirm that each lane had the same amount of protein loaded. Protein of each sample was transferred to nitrocellulose membrane (Osmonics Nitrobind nitrocellulose transfer membranes, GE Water and Process Technologies, 4636 Somerton Road, Feasterville-Trevoze, PA 19053) at 300 mA for approximately 1 h. The nitrocellulose membrane was blocked for 12 h at 4 C in TBST (20 mM Tris base [pH 7.5], 0.5 M NaCl, 0.05% Tween-20) (Sigma-Aldrich Co. LLC., 3050 Spruce Street, St. Louis, MO 63103) containing 5% milk powder. Membrane was incubated at 20 C for 1 h with primary *EPSPS* antibody (Monsanto Co., St. Louis, MO) that was diluted 1 : 2,000 in TBST milk solution. Membrane was rinsed three times in TBST, and incubated at 20 C for 1 h with secondary antibody (goat anti-rabbit) conjugated with horseradish peroxidase (ImmunoPure Antibody; Thermo Fischer) using a dilution of 1 : 5,000 in TBST milk solution. Membrane was again rinsed three

times in TBST milk solution, and an activator 24 solution (SuperSignal West Pico chemiluminescent substrate from Thermo Fischer) was applied to the membrane to activate chemiluminescence. The protein bands (signals) were detected using a Bio-Rad ChemiDoc™ XRS imager. One susceptible and two confirmed GR and Palmer amaranth accession from Georgia (Gaines et al. 2010) and a confirmed GR kochia accession from Colorado (Wiersma et al. 2015) were included for comparison (positive controls). The selected positive controls of the GR weeds were known to have high EPSPS protein abundance (Gaines et al. 2010; Wiersma et al. 2015), and were used for qualitative assessment of EPSPS protein in the GR vs. SUS kochia accessions tested.

Whole-Plant Dose–Response for ALS-Inhibitor Herbicide.

Whole-plant dose–response experiments were conducted in the greenhouse at the MSU-SARC near Huntley, MT to quantify the level of resistance to premixed thifensulfuron methyl (27.30% by wt) + tribenuron methyl (13.60% by wt) + metsulfuron methyl (10.90% by wt) (Ally® Extra SG, DuPont Company, Wilmington, DE 19898) in the GR kochia accessions (GIL01, JOP01, and CHES01), referred to as MHR, compared with the SUS accession. The experiment was conducted under similar growth conditions as previously described. Single kochia seedlings were transplanted into 10-cm-diam pots containing the same potting mix. Experiments were arranged in a randomized complete block design with eight replications (one plant per pot), and repeated two times. Actively growing 8- to 10-cm-tall kochia plants were sprayed with the premixed ALS-inhibitor herbicide at doses of 0, 0.0025, 0.0045, 0.009, 0.018, 0.036, 0.072, 0.144, 0.288, and 0.648 kg ai ha⁻¹. Methylated seed oil at 1% (v/v) was included. Herbicide applications were made inside a stationary cabinet spray chamber equipped with a flat-fan nozzle tip (TeeJet 8001XR, Spraying System Co., Wheaton, IL) calibrated to deliver 94 L ha⁻¹ of spray solution at 276 kPa. After the herbicide application, plants were returned to the greenhouse, watered daily to avoid moisture stress, and fertilized (as previously described). Kochia plants were harvested at the soil level at 3 wk after treatment and dried at 60 C for 3 d to determine shoot dry weight (g plant⁻¹).

Shoot dry-weight data from the whole-plant dose–response experiments were subjected to ANOVA using PROC MIXED procedure in SAS.

Residual analyses were performed using PROC UNIVARIATE in SAS. Data were combined across experimental runs because of nonsignificant interaction of experimental run by treatment. Pooled data for each kochia accession were regressed over doses of premixed thifensulfuron methyl + tribenuron methyl + metsulfuron methyl herbicide using the four-parameter log-logistic model (Seefeldt et al. 1995)

$$Y = C + \{D - C / 1 + \exp[B(\log X - \log E)]\} \quad [1]$$

where Y refers to shoot dry weight (g plant⁻¹), C is the lower limit, D is the upper limit, B is the slope, E is the herbicide dose required for 50% reduction in shoot dry weight referred to as GR₅₀, and X is the herbicide dose. A lack-of-fit test showed the accuracy of the nonlinear model ($P = 0.516$). Parameter estimates, standard errors, and GR₉₀ values (dose of the herbicide required for 90% reduction in shoot dry weight) of the dose–response curves were determined using the *drc* package in R software (Knezevic et al. 2007). Resistance index (referred to as R/S ratio) for each kochia accession was estimated by dividing the GR₅₀ value of a resistant accession by the GR₅₀ value of the SUS accession.

ALS Gene Sequencing. The gDNA was extracted from three kochia plants per accession using the Qiagen DNeasy Plant mini kit, and the experiment was repeated. The quality and concentration of gDNA for each sample was determined using a Bio-Rad SmartSpect Plus spectrophotometer. From previously published research (Beckie et al. 2013; Warwick et al. 2008), a primer set (*ALSFI*: 5'-ATGGCGTCTACTG TGCAAATCCC-3' and *ALSRI*: 5'-AACTT GTTCTTCCA TCACCTTC G-3') was designed to amplify almost the entire *ALS* gene (1,800 bp). Each PCR reaction contained 8.75 μl of iTaq™ DNA polymerase master mix (containing iTaq DNA polymerase, dNTPs, MgCl₂, and reaction buffers), 0.5 μl each of forward and reverse primers (25 μM), and 2.5 μl of DNA template (30 ng μl⁻¹). PCR was performed using a Bio-Rad T100™ thermal cycler under the following conditions: initial denaturation at 95 C for 3 min, 30 cycles of 30 s at 95 C, 30 s at 66 C, and 90 s at 72 C followed by 10 min at 72 C. The reaction was terminated and held at 4 C. The PCR fragment was purified using QIAquick PCR purification kit following the manufacturer's protocol. The Sanger sequencing of purified PCR fragments was performed. Samples were sequenced

with the same primer set used for PCR amplification and another internal primer set (*ALSF2*: 5'-GAC GGATGCTTTTCAG GAG-3' and *ALSR2*: 5'-TTGGCGAGGGTTTCTGTAC-3'). The sequence reads of experimental samples were analyzed using the MultAlin software, and aligned to a reference *ALS* gene from SUS kochia. The aligned nucleotide sequences were analyzed for any mutation at the single nucleotide position. The derived amino acid positions were based on the amino acid sequence of the *ALS* gene in *Arabidopsis* (Sathasivan et al. 1990).

Results and Discussion

Shikimate Accumulation. No difference in shikimate accumulation was observed between the nontreated (0 μM glyphosate) GR and SUS kochia accessions (Table 1). However, at a discriminate dose of 100 μM glyphosate, the SUS kochia plants accumulated 19.83 ng of shikimate μl^{-1} , which was 6- to 17-fold higher than the shikimate accumulated by GR plants of JOP01, CHES01, and GIL01 accessions. No differences in shikimate accumulation were observed among kochia accessions at 1,000 μM glyphosate (data not shown). Similarly, GR plants of *Amaranthus* species and Italian ryegrass accumulated less shikimate compared with the susceptible plants at a glyphosate dose of 100 μM , and levels of shikimate did not differ between susceptible and GR plants at 500 and 1,000 μM glyphosate (Gaines et al. 2010; Nandula et al. 2008, 2012). Shikimate, a dephosphorylated substrate of the EPSPS enzyme, accumulates in plants when treated with glyphosate (Singh and Shaner 1998). This assay can reliably be used to confirm resistance to glyphosate, also reported for several other GR weed species (Gaines et al. 2010; Nandula et al. 2014; Shaner et al. 2005).

Sequencing of *EPSPS* Thr₁₀₂ and Pro₁₀₆ Codons.

In general, target-site mutation in GR weed species involves amino acid substitution at the Pro₁₀₆ position (proline to serine, threonine, alanine, or leucine) (Sammons and Gaines 2014). However, a field-evolved population of GR goosegrass [*Elyusine indica* (L.) Gaertn.] has recently been identified in Australia with a double mutation at Thr₁₀₂ codon (threonine to isoleucine) and Pro₁₀₆ codon (proline to serine); which conferred a high level of glyphosate resistance in the GR goosegrass population (Yu et al. 2015). There was no point mutation at the Pro₁₀₆ residue of the derived amino acid sequences in any of the tested GR kochia accessions from Montana

Table 1. Shikimate accumulation in glyphosate-susceptible and glyphosate-resistant kochia accessions from Montana.

Accession ^b	Shikimate accumulation ^a	
	0 μM glyphosate ^c	100 μM glyphosate
	—ng μl^{-1} —	
SUS	2.06 a	19.83 a
JOP01	2.21 a	1.91 b
CHES01	1.79 a	3.30 b
GIL01	1.20 a	1.15 b

^a Leaf disks (5 mm diam) were excised from young (8 to 10 cm tall) actively growing kochia plants, and accumulation of shikimate was determined by in vivo assay.

^b Abbreviations: SUS, susceptible kochia accession, Huntley, MT; GIL01, glyphosate-resistant (GR) kochia accession from Gildford, MT; JOP01, GR kochia accession from Joplin, MT; CHES01, GR kochia accession from Chester, MT.

^c Means within the column followed by the same letters are not significantly different on the basis of Fisher's Protected LSD test at $P < 0.05$.

(Table 2). Furthermore, there was no mutation observed at the Thr₁₀₂ codon in the 200-bp *EPSPS* sequence of GR kochia (Table 2). Sequence alignment of tested individuals with reference *EPSPS* sequence of Palmer amaranth exhibited similar consensus at Pro₁₀₆ and Thr₁₀₂ (Table 2). Therefore, amino acid substitution at the Pro₁₀₆ or Thr₁₀₂ codon of *EPSPS* can be ruled out as a possible mechanism of glyphosate resistance in the GR kochia populations tested in this research. Although mutation at the Thr₁₀₂ position was not investigated, there was a lack of Pro₁₀₆ mutation in the *EPSPS* gene in GR kochia populations from Kansas and Colorado (Wiersma et al. 2015). A lack of target-site mutation exists in GR populations of other weed species, such as Palmer amaranth and spiny amaranth (Gaines et al. 2010; Nandula et al. 2014).

***EPSPS* Genomic Copy Number.** Results from the qPCR analysis revealed that plants of JOP01 accession had approximately 6 to 8, GIL01 had 4 to 9, and CHES01 had 6 to 10 relative copies of the *EPSPS* gene (Figure 1). The relative *EPSPS* copy number varied among plants within a GR accession, which may be expected in kochia, a cross-pollinated species with high level of genetic diversity (Mengistu and Messersmith 2002, Stallings et al. 1995). In contrast, all SUS kochia plants had only one relative *EPSPS* copy number. GR kochia accessions from Kansas and Colorado had three to eight relative *EPSPS*:*ALS* genomic copy numbers (Wiersma et al. 2015). *EPSPS* gene amplification (increased copy number) is a novel mechanism of resistance to glyphosate, also reported in other GR

Table 2. Nucleotide bases and derived amino acid sequence in the conserved region of the *EPSPS* gene from glyphosate-susceptible and glyphosate-resistant kochia accessions from Montana. The bold nucleotide codons encoded Thr₁₀₂ and Pro₁₀₆ residues.^a

Reference <i>EPSPS</i> gene sequence of Palmer amaranth	GGA	ACG	GCA	ATG	CGC	CCA	TTG	ACA	GCT	GCA	GTT
Amino acid positions	Gly	Thr	Ala	Met	Arg	Pro	Leu	Thr	Ala	Ala	Val
	101	102	103	104	105	106	107	108	109	110	111
SUS1	GGA	ACG	GCA	ATG	CGC	CCA	TTG	ACA	GCT	GCA	GTT
SUS2	GGA	ACG	GCA	ATG	CGC	CCA	TTG	ACA	GCT	GCA	GTT
SUS3	GGA	ACG	GCA	ATG	CGC	CCA	TTG	ACA	GCT	GCA	GTT
JOP011	GGA	ACG	GCA	ATG	CGC	CCA	TTG	ACA	GCT	GCA	GTT
JOP012	GGA	ACG	GCA	ATG	CGC	CCA	TTG	ACA	GCT	GCA	GTT
JOP013	GGA	ACG	GCA	ATG	CGC	CCA	TTG	ACA	GCT	GCA	GTT
GIL011	GGA	ACG	GCA	ATG	CGC	CCA	TTG	ACA	GCT	GCA	GTT
GIL012	GGA	ACG	GCA	ATG	CGC	CCA	TTG	ACA	GCT	GCA	GTT
GIL013	GGA	ACG	GCA	ATG	CGC	CCA	TTG	ACA	GCT	GCA	GTT
CHES011	GGA	ACG	GCA	ATG	CGC	CCA	TTG	ACA	GCT	GCA	GTT
CHES012	GGA	ACG	GCA	ATG	CGC	CCA	TTG	ACA	GCT	GCA	GTT
CHES013	GGA	ACG	GCA	ATG	CGC	CCA	TTG	ACA	GCT	GCA	GTT

^a Abbreviations: SUS1, SUS2, and SUS3, three kochia plants from glyphosate-susceptible (SUS) accession; JOP011, JOP012, and JOP013, three kochia plants from glyphosate-resistant (GR) JOP01 accession; GIL011, GIL012, and GIL013, three kochia plants from GR GIL01 accession; CHES011, CHES012, and CHES013, three kochia plants from GR CHES01 accession.

weed species. For instance, GR Palmer amaranth had high relative *EPSPS* gene copies of 40 to 100 in populations from Georgia (Gaines et al. 2010), 20 to 60 in populations from North Carolina (Chandi et al. 2012), and 33 to 59 in populations from Mississippi (Ribeiro et al. 2014). GR spiny amaranth from Mississippi had 26 to 37 copies, and Italian ryegrass from Arkansas had 15 to 25 relative copies of the *EPSPS* gene (Nandula et al. 2014; Salas et al. 2012). However, lower folds of *EPSPS* gene amplification (2- to 10-fold) have been reported in GR Palmer amaranth accessions from New Mexico (Mohseni-Moghadam et al. 2013). The range of relative *EPSPS* gene copies (4 to 10) observed in GR kochia accessions from Montana

was comparable with the range (three to eight relative *EPSPS* copies) reported recently in GR kochia accessions from Kansas and Colorado (Wiersma et al. 2015), and also with that observed in GR tall waterhemp (two to eight copies) (Lorentz et al. 2014).

Correlation of *EPSPS* Gene Copy Number with Glyphosate Resistance. The *EPSPS* gene copy number of SUS and GR kochia plants was correlated with the shikimate accumulation at the discriminating dose of glyphosate (Figure 2). The SUS plants with one relative *EPSPS* copy had higher shikimate accumulation compared with the GR kochia plants with 4 to 10 relative *EPSPS* copies. These results further support the hypothesis of overproduction of the EPSPS enzyme from increased copies of the *EPSPS* gene produced by GR kochia plants, thus allowing them to tolerate lethal doses ($> 1.26 \text{ kg ae ha}^{-1}$) of glyphosate.

The I_{50} and GR_{50} values (Figure 3) were obtained from our previously reported whole-plant dose–response curves of the selected GR and SUS accessions (Kumar et al. 2014). A strong positive linear relationship was observed between I_{50} and *EPSPS* gene copy number ($r = 0.92$) (Figure 3a), and between GR_{50} and *EPSPS* gene copy number ($r = 0.86$) (Figure 3b). This further suggests that 4- to 10-fold increased *EPSPS* gene copy numbers resulted in 4.6- to 11-fold levels of resistance (based on resistance index [R/S ratio]) to glyphosate in selected GR kochia accessions from Montana. It is

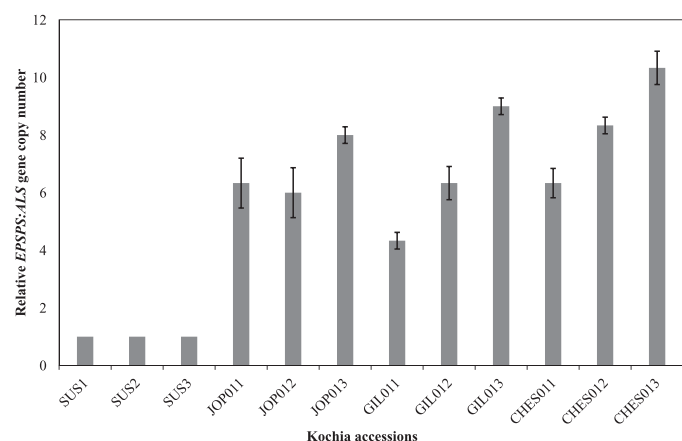


Figure 1. Relative *EPSPS* genomic copy number in glyphosate-resistant (JOP01, GIL01, and CHES01) and susceptible (SUS) kochia accessions from Montana. Vertical bars represent standard error of mean ($n = 6$).

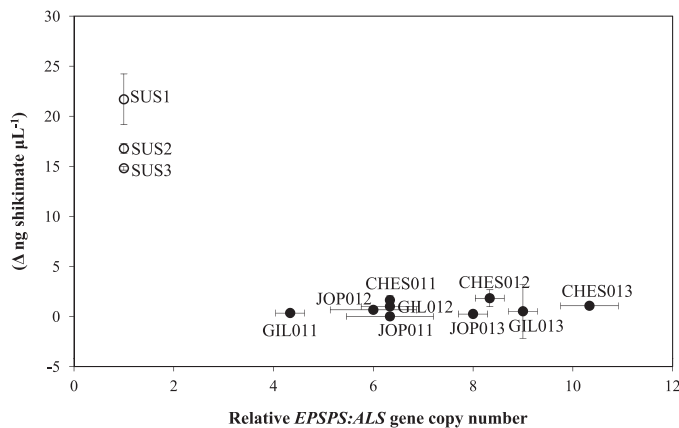


Figure 2. Correlation of relative *EPSPS* genomic copy number vs. shikimate accumulation in glyphosate-resistant (JOP01, GIL01, and CHES01) and susceptible (SUS) kochia accessions from Montana. Shikimate accumulation was measured after incubation of samples in 100 μM glyphosate in an in vivo leaf-disk assay. The Δ ng shikimate μL⁻¹ was calculated as the difference in shikimate accumulation between treated and untreated plants. Error bars indicate standard error.

likely that increase in selection pressure from glyphosate may select GR kochia individuals with higher copies of the *EPSPS* gene, conferring higher levels of glyphosate resistance.

EPSPS Protein Abundance. A representative image of EPSPS protein abundance of the tested kochia accessions (SUS vs. GIL01, JOP01, and CHES01) is presented in Figure 4. The protein assay with polyclonal anti-EPSPS antibody using western blot indicated that the GR kochia accessions from Montana had higher EPSPS protein abundance compared with the SUS kochia accession. To elaborate, the three GR kochia accessions (lanes 2, 3, and 4, respectively) with 4 to 10 relative copies of the *EPSPS* gene showed intense EPSPS protein signal, and the signal saturated at a molecular weight of 48 kDa (Figure 4). No signal was detected in lane 1 containing the SUS kochia accession. A comparable signal of the EPSPS protein extract was observed in a GR kochia accession (GR-CO) from Colorado (lane 5), with eight relative *EPSPS:ALS* gene copies. Similarly, a highly intense EPSPS signal (saturated the film) was observed in lanes 7 and 8 containing GR Palmer amaranth (GR1 and GR2, with approximately 50 and 100 relative copies of the *EPSPS* gene, respectively) protein extracts, whereas no signal was detected at 48 kDa in lane 6 containing glyphosate-susceptible (SUS-P) Palmer amaranth (one relative copy of *EPSPS:ALS* gene) protein extract. There is generally a positive correlation

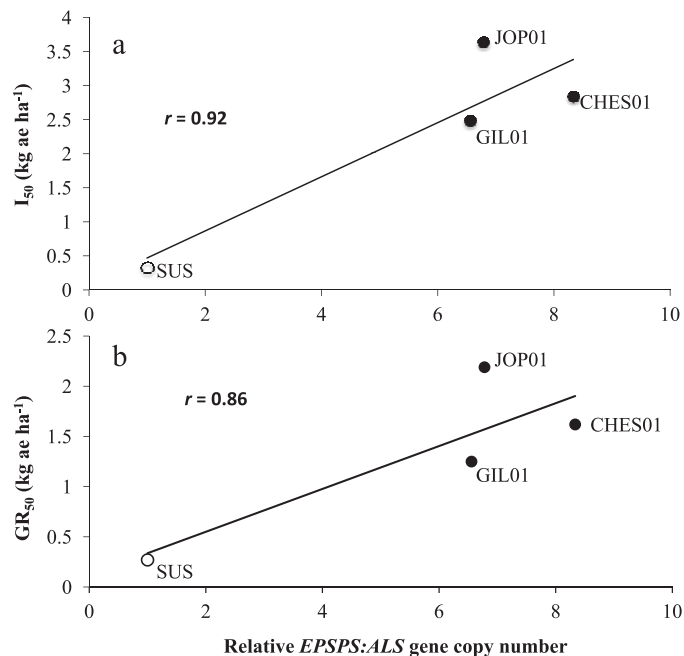


Figure 3. (a) Correlation of relative *EPSPS* genomic copy number with the amount of glyphosate needed for 50% control (I_{50} value) of glyphosate-resistant (JOP01, GIL01, and CHES01) and susceptible (SUS) accessions from Montana. (b) Correlation of relative *EPSPS* genomic copy number with the amount of glyphosate needed for 50% shoot dry weight reduction (GR₅₀ value) of glyphosate-resistant (JOP01, GIL01, and CHES01) and susceptible (SUS) kochia accessions from Montana. The I_{50} and GR₅₀ values were obtained from the glyphosate dose–response study on the selected accessions (Kumar et al. 2014).

between thicker EPSPS protein bands and higher *EPSPS* gene copy numbers (Gaines et al. 2010; Wiersma et al. 2015). The qualitative protein assay indicated that GR kochia accessions from Montana with increased *EPSPS* gene copies accumulated higher EPSPS protein.

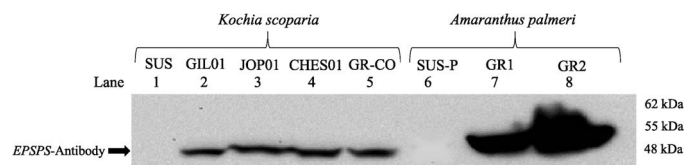


Figure 4. A representative image of nitrocellulose membrane showing EPSPS protein bands probed with anti-EPSPS polyclonal antibody in a western blot. Lane 1, glyphosate-susceptible kochia accession (SUS) from Montana; lanes 2 to 4, glyphosate-resistant kochia accessions (GIL01, JOP01, and CHES01, respectively) from Montana; lane 5, GR kochia accession (GR-CO) from Colorado (eight copies of *EPSPS*); lane 6, glyphosate-susceptible (SUS-P) Palmer amaranth accession from Georgia; lane 7 to 8, glyphosate-resistant Palmer amaranth accessions (GR1 and GR2 with ~ 50 and 100 copies of *EPSPS*, respectively) from Georgia. A total of three plants per kochia accession was sampled in each experimental run.

Table 3. Regression parameters (Equation 1: D = upper limit, C = lower limit, B = slope) for whole-plant dose response based on shoot dry weight (g plant^{-1}) of multiple herbicide-resistant (MHR) and susceptible (SUS) kochia accessions from Montana treated with thifensulfuron methyl + tribenuron methyl + metsulfuron, methyl.

Accession ^a	Regression parameters (\pm SE)				R/S ^c	GR ₉₀ ^b
	D	C	B	GR ₅₀ ^b		
SUS	4.24 (0.06)	0.14 (0.02)	1.8 (0.09)	0.003 (0.0001)	—	0.01 (0.007)
JOP01	4.17 (0.05)	0.56 (0.04)	0.5 (0.06)	0.090 (0.004)	30	5.06 (0.46)
CHES01	3.57 (0.06)	0.89 (0.03)	0.4 (0.07)	0.028 (0.001)	9.3	2.86 (0.35)
GIL01	3.57 (0.06)	0.73 (0.03)	0.6 (0.08)	0.087 (0.004)	29	3.75 (0.39)

^a Abbreviations: SUS, susceptible kochia accession, Huntley, MT; GIL01, multiple herbicide-resistant (MHR) kochia accession from Gildford, MT; JOP01, MHR kochia accession from Joplin, MT; CHES01, MHR kochia accession from Chester, MT.

^b GR₅₀ is the effective dose (kg ai ha^{-1}) of thifensulfuron methyl + tribenuron methyl + metsulfuron methyl required for 50% shoot dry weight reduction; GR₉₀ is the effective dose (kg ai ha^{-1}) of thifensulfuron methyl + tribenuron methyl + metsulfuron methyl required for 90% shoot dry weight reduction.

^c R/S is calculated as a ratio of GR₅₀ of a MHR accession to GR₅₀ of the SUS kochia accession.

Whole-Plant Dose–Response for ALS-Inhibitor Herbicide.

On the basis of shoot dry weight, GR₅₀ values for JOP01, CHES01, and GIL01 MHR kochia accessions were 0.090, 0.028, and 0.087 kg ha^{-1} , respectively, compared with the GR₅₀ value of 0.003 kg ha^{-1} for the SUS accession (Table 3). The R/S ratios (resistance index) indicate that the MHR kochia accessions were 9.3- to 30-fold more resistant to the ALS-inhibitor herbicide premix (thifensulfuron methyl + tribenuron methyl + metsulfuron methyl) compared with the SUS population. Also, the GR₉₀ values of MHR kochia accessions were 159 to 281 times the field-use rate (0.018 kg ai ha^{-1}) of the herbicide (Table 3). This is expected because of continuous use of sulfonylurea herbicides for > 8 yr in dryland wheat–fallow fields in northern Montana from where the MHR kochia accessions were collected. Furthermore, the MHR kochia accessions showed differential response to the ALS-inhibitor herbicide, and the hierarchy of resistance was: JOP01 = GIL01 > CHES01 on the basis of pair-wise t -test of the GR₅₀ values (data not shown). Kochia accessions from Kansas reported by Saari et al. (1990) had 12- to 28-fold levels of resistance to sulfonylurea herbicides. Similarly, Primiani et al. (1990) reported 8- to 30-fold levels of resistance to chlorsulfuron, metsulfuron, and sulfometuron herbicides in kochia accessions collected from wheat fields in Kansas. With continued use of sulfonylurea herbicides, there is a widespread occurrence of ALS-inhibitor-resistant kochia in the cereal production regions of the U.S. Great Plains and in Canada (Beckie et al. 2013; Heap 2015).

ALS Gene Sequencing. There was a point mutation causing amino acid substitution at Pro₁₉₇

residue in the *ALS* gene sequence (1,800 bp) of MHR kochia accessions (JOP01, CHES01, and GIL01). Compared with the SUS accession with CCG codon at Pro₁₉₇, there was a change from CCG to CAG codon in all MHR kochia plants (Table 4). This point mutation resulted in an amino acid substitution from Pro₁₉₇ to Gln₁₉₇, which conferred high level of resistance (up to 30-fold) to the ALS-inhibitor sulfonylurea herbicide tested (Table 3). Although multiple amino acid substitutions (Pro₁₉₇, Asp₃₇₆, and Trp₅₇₄ sites) have been reported in ALS-inhibitor-resistant kochia populations (Beckie et al. 2013; Tranel et al. 2015), we did not observe any amino acid substitution at Asp₃₇₆ or Trp₅₇₄ position of the *ALS* gene (almost the entire gene was sequenced) in the tested kochia populations. Among all previously known mutations in *ALS* gene, substitution of proline at position 197 with threonine, arginine, leucine, glutamine, serine, or alanine is known to confer high level of resistance to sulfonylurea herbicides in kochia (Beckie et al. 2013; Tranel and Wright 2002; Tranel et al. 2015).

In conclusion, this research serves as a first report on the molecular mechanisms of resistance to glyphosate and ALS-inhibitor herbicides in MHR kochia from Montana. There was no mutation found at the Pro₁₀₆ codon of the *EPSPS* gene, but the MHR kochia accessions had 4 to 10-fold amplification of the *EPSPS* gene compared with a single relative *EPSPS* genomic copy in the SUS accession. MHR kochia plants also accumulated higher EPSPS protein compared with the SUS plants. Substitution of glutamine for proline at position 197 of the *ALS* gene conferred 9.3- to 30-fold levels of resistance to the ALS-inhibitor herbicide in MHR kochia.

Table 4. Nucleotide bases and derived amino acid sequences of a fragment of *ALS* gene from susceptible and multiple herbicide-resistant kochia accessions from Montana, showing a single nucleotide mutation (bold and underlined codon) at Pro₁₉₇ residue.^a

Reference <i>ALS</i> gene sequence of kochia ^b	ACG	GGG	CAG	GTG	CCG	CGG	CGA	ATG	ATT	GGG	ACG
Amino acid positions	Thr	Gly	Gln	Val	Pro	Arg	Arg	Met	Ile	Gly	Thr
	193	194	195	196	197	198	199	200	201	202	203
SUS1	ACG	GGG	CAG	GTG	CCG	CGG	CGA	ATG	ATT	GGG	ACG
SUS2	ACG	GGG	CAG	GTG	CCG	CGG	CGA	ATG	ATT	GGG	ACG
SUS3	ACG	GGG	CAG	GTG	CCG	CGG	CGA	ATG	ATT	GGG	ACG
JOP011	ACG	GGG	CAG	GTG	<u>CAG</u>	CGG	CGA	ATG	ATT	GGG	ACG
JOP012	ACG	GGG	CAG	GTG	<u>CAG</u>	CGG	CGA	ATG	ATT	GGG	ACG
JOP013	ACG	GGG	CAG	GTG	<u>CAG</u>	CGG	CGA	ATG	ATT	GGG	ACG
GIL011	ACG	GGG	CAG	GTG	<u>CAG</u>	CGG	CGA	ATG	ATT	GGG	ACG
GIL012	ACG	GGG	CAG	GTG	<u>CAG</u>	CGG	CGA	ATG	ATT	GGG	ACG
GIL013	ACG	GGG	CAG	GTG	<u>CAG</u>	CGG	CGA	ATG	ATT	GGG	ACG
CHES011	ACG	GGG	CAG	GTG	<u>CAG</u>	CGG	CGA	ATG	ATT	GGG	ACG
CHES012	ACG	GGG	CAG	GTG	<u>CAG</u>	CGG	CGA	ATG	ATT	GGG	ACG
CHES013	ACG	GGG	CAG	GTG	<u>CAG</u>	CGG	CGA	ATG	ATT	GGG	ACG

^a Almost the entire *ALS* gene was sequenced (1,800 base pairs).

^b Abbreviations: SUS1, SUS2, and SUS3, three kochia plants from susceptible (SUS) accession; JOP011, JOP012, and JOP013, three kochia plants from multiple herbicide-resistant (MHR) JOP01 accession; GIL011, GIL012, and GIL013, three kochia plants from MHR GIL01 accession; CHES011, CHES012, and CHES013, three kochia plants from MHR CHES01 accession.

Evolution of glyphosate- and ALS-inhibitor MHR kochia in wheat–fallow fields would be a potential concern for growers in the NGP. Wind-mediated tumble mechanism of seed dispersal coupled with pollen-mediated gene flow through outcrossing would ensure rapid spread of MHR kochia. In separate studies, we evaluated the effectiveness of various alternative PRE and POST herbicides to be used for kochia control in wheat, corn/grain sorghum, soybean, chemical–fallow, and postharvest wheat stubble (Kumar et al. 2014; Kumar and Jha 2015a,b). Growers should adopt those effective alternative modes-of-action herbicides and prevent seed production from MHR kochia plants in their production farms. Reduced absorption/translocation and metabolism were ruled out as mechanisms of glyphosate resistance in kochia accessions from Kansas (Waite et al. 2013); however, the possibility of multiple mechanisms of resistance to glyphosate in the selected GR kochia accessions needs to be considered. We are investigating the inheritance pattern of amplified *EPSPS* gene copies and fitness cost (if any) associated with increase in relative *EPSPS* gene copies in GR kochia in the presence and absence of glyphosate selection pressure. A random field survey was conducted in 2013 and 2014 to collect kochia populations (approximately 200) from cropland and noncropland areas across north-central Montana. The populations are under investigation for possible multiple resistance to three different

herbicide groups (glyphosate, ALS inhibitors, and dicamba) and for determining the frequency and geographical distribution of MHR kochia in Montana. These key research questions will provide insights into the strategies to prevent further spread of MHR kochia in this region.

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