Glyphosate-Resistant Junglerice (*Echinochloa colona*) from Mississippi and Tennessee: Magnitude and Resistance Mechanisms

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Abstract

Recently, several incidents of glyphosate failure on junglerice [*Echinochloa colona* (L.) Link] have been reported in the midsouthern United States, specifically in Mississippi and Tennessee. Research was conducted to measure the magnitude of glyphosate resistance and to determine the mechanism(s) of resistance to glyphosate in *E. colona* populations from Mississippi and Tennessee. ED_{50} (dose required to reduce plant growth by 50%) values for a resistant MSGR4 biotype, a resistant TNGR population, and a known susceptible MSGS population were 0.8, 1.62, and 0.23 kg ae ha^{-1} of glyphosate, respectively. The resistance index calculated from the these ED_{50} values indicated that the MSGR4 biotype and TNGR population were 4- and 7-fold, respectively, resistant to glyphosate relative to the MSGS population. The absorption patterns of [^{14}C]glyphosate in the TNGR and MSGS populations were similar. However, the MSGS population translocated 13% more [^{14}C]glyphosate out of the treated leaf compared with the TNGR population at 48 h after treatment. *EPSPS* gene sequence analyses of TNGR *E. colona* indicated no evidence of any point mutations, but several resistant biotypes, including MSGR4, possessed a single-nucleotide substitution of T for C at codon 106 position, resulting in a proline-to-serine substitution (CCA to TCA). Results from quantitative polymerase chain reaction analyses suggested that there was no amplification of the *EPSPS* gene in the resistant populations and biotypes. Thus, the mechanism of resistance in the MSGR population (and associated biotypes) is, in part, due to a target-site mutation at the 106 loci of the *EPSPS* gene, while reduced translocation of glyphosate was found to confer glyphosate resistance in the TNGR population.

Introduction

Echinochloa spp., including barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.), junglerice [*Echinochloa colona* (L.) Link], and rice barnyardgrass [*Echinochloa phyllopogon* (Stapf) Koso-Pol.] are important weeds in rice (*Oryza sativa* L.) production systems and other agronomic crops across the world (Bakkali et al. 2007; Holm et al. 1991). Herbicides are the main tool available to control *Echinochloa* spp. and have been in use for several decades. As a result, *Echinochloa* spp. have evolved resistance to several herbicides. For example, *E. colona* populations in Argentina, Australia, Bolivia, Colombia, Costa Rica, Egypt, El Salvador, Guatemala, Honduras, Iran, Nicaragua, Panama, the United States, and Venezuela have been confirmed resistant to one or more herbicide mechanisms of action, including acetyl-CoA carboxylase inhibitors, acetolactate synthase inhibitors, photosystem II inhibitors, synthetic auxins (cellulose inhibitors), and 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) inhibitor (glyphosate) (Heap 2018).

Since 2014, several incidents of failure of glyphosate on *E. colona* have been reported in the midsouthern U.S. states of Mississippi and Tennessee. *Echinochloa colona* plants, from populations in Tunica County, MS, that survived one or more field applications of glyphosate at 0.84 kg ae ha^{-1} (1X rate in Mississippi) were collected from the field and grown in a greenhouse in 2015. Progeny from these plants were screened with glyphosate at 0.84 and

$1.68\,\mathrm{kg\ ha^{-1}}$. Eight plants survived the $0.84\,\mathrm{kg\ ha^{-1}}$ rate and produced seed (first generation), while none of the plants survived glyphosate at $1.68\,\mathrm{kg\ ha^{-1}}$. A second series of screening experiments revealed that all progeny of first-generation plants were resistant to the recommended field rate of glyphosate and produced viable seed (second generation), indicating resistance trait heritability. Henceforth, this population is labeled as MSGR.

Preliminary glyphosate dose–response studies under greenhouse conditions indicated that a putative resistant *E. colona* population from Tennessee, hereafter referred to as TNGR, was only controlled 55% and 84% with glyphosate at 1.92 and $3.85\,\mathrm{kg}$ $\mathrm{ha^{-1}}$, respectively, while a susceptible population (TNGS) was controlled 91% and 99% with 0.86 and $1.72\,\mathrm{kg\ ha^{-1}}$ rates, respectively (Steckel et al. 2017). TNGR plants treated with glyphosate at $\leq 1.72\,\mathrm{kg\ ha^{-1}}$ produced viable seed. Progeny from TNGR plants that were treated with glyphosate at 0.86 or $1.72\,\mathrm{kg}$ $\mathrm{ha^{-1}}$ were grown out to 15-cm height and treated with $1.72\,\mathrm{kg}$ $\mathrm{ha^{-1}}$. All of the treated plants survived to produce seed, providing evidence of heritability of the glyphosate-resistance trait in TNGR.

The objectives of this research were to measure the magnitude of resistance and determine the mechanism(s) of resistance to glyphosate in *E. colona* populations. Whole-plant glyphosate dose–response studies, $[^{14}C]$glyphosate absorption and translocation studies, and *EPSPS* gene amplification, expression, and sequencing studies were conducted.

Materials and Methods

Echinochloa colona Populations

Glyphosate-resistant (GR) populations included in this research were MSGR and TNGR. Biotypes derived or selected from the second-generation MSGR population are designated as MSGR4, MSGR27, MSGR34, MSGR36, MSGR44A, MSGR44B, MSGR44C, and MSGR49. Populations susceptible to glyphosate are designated as KSGS and MSGS, which originated from Kansas and Mississippi, respectively.

Planting and Growth Conditions

Stoneville, MS

MSGR and MSGS *E. colona* seeds were germinated and 2-wk-old seedlings were transplanted into 6 by 6 by 6 cm pots containing a commercial potting mix (Metro-Mix® 360, Sun Gro Horticulture, Bellevue, WA 98008). Pots were maintained in a greenhouse set to $25/20 \pm 3$ C day/night temperature and a 13-h photoperiod that was provided by high-pressure sodium lights ($400\,\mathrm{\mu mol\ m^{-2}}$ $\mathrm{s^{-1}}$). Plants were fertilized once with a nutrient solution (Miracle-Gro®, Scotts Company, Marysville, OH 43040) 1 wk after transplanting and subirrigated as needed thereafter.

Manhattan, KS

Seed from nine *E. colona* accessions (MSGR4, MSGR27, MSGR34, GR44A, MSGR44B, MSGR44C, TNGR, KSGS, and MSGS) were germinated on trays (25 by 15 by 2.5 cm) containing a potting mix (Pro-Mix® Ultimate, Hummert International, Topeka, KS 66618). At 10 d after germination, seedlings ($1\,\mathrm{pot^{-1}}$) at the 2-leaf stage (~4- to 5-cm tall) were transplanted into 6.4 by 6.4 by 7.6 cm pots containing the same potting mix. The seedlings were grown in a greenhouse maintained at 25/19 C (day/night) temperatures, $60 \pm 5\%$ relative humidity, and 16/9-h day/night

photoperiod supplemented with $120\,\mathrm{\mu mol\ m^{-2}\ s^{-1}}$ illumination provided with sodium-vapor lamps.

Herbicide Application

Stoneville, MS

All glyphosate (Roundup WeatherMax®, Monsanto, St Louis, MO 63167) treatments were applied with a moving nozzle sprayer (Research Track Sprayer, Generation III, De Vries Manufacturing, Hollandale, MN 56045) equipped with 8002E nozzles (Spraying Systems, P.O. Box 7900, Wheaton, IL 60139) delivering $190\,\mathrm{L}$ $\mathrm{ha^{-1}}$ at 280 kPa to *E. colona* plants that were 10-cm tall and at the 3- to 4-leaf stage. At 3 wk after treatment, injury was visually assessed for each plant on a scale of 0% to 100%. A rating of 0% indicated no injury, and 100% indicated plant death, with values in between assessing the degree of injury and growth inhibition.

Manhattan, KS

Ten- to twelve-centimeter tall (~4-leaf stage) uniformly sized seedlings ($1\,\mathrm{pot^{-1}}$) were treated with glyphosate (Roundup WeatherMax®) at doses of 0.84 and $1.68\,\mathrm{g\ ae\ ha^{-1}}$ with 2% (g $\mathrm{L^{-1}}$) ammonium sulfate using the sprayer described earlier equipped with a flat-fan nozzle tip (80015LP) delivering $168\,\mathrm{L}$ $\mathrm{ha^{-1}}$ at 222 kPa at 4.8 km $\mathrm{h^{-1}}$ for resistance confirmation before conducting molecular studies.

Glyphosate Dose Response

Stoneville, MS

The plants of MSGR4 biotype and TNGR and MSGS populations were treated with glyphosate at 0, 0.21, 0.42, 0.84, 1.68, and $3.36\,\mathrm{kg\ ae\ ha^{-1}}$. To ensure enough data points for the dose–response curves, a higher rate of $6.72\,\mathrm{kg\ ha^{-1}}$ was also applied to MSGR4 and TNGR, while a lower rate of $0.11\,\mathrm{kg\ ha^{-1}}$ glyphosate was included for MSGS. There were three replications per treatment, a replication being $1\,\mathrm{pot^{-1}}$, and the experiment was performed three times. Dose–response work for the other MSGR biotypes was conducted only once and is not reported here.

$[^{14}C]$Glyphosate Absorption, Translocation, and Phosphorimaging

Stoneville, MS

Three-leaf-stage TNGR and MSGS *E. colona* plants were transferred from the greenhouse to a growth chamber 7 d before $[^{14}C]$ glyphosate application for acclimatization. The growth chamber was maintained at 25/20 C with a 13-h photoperiod ($300\,\mathrm{\mu mol}$ $\mathrm{m^{-2}\ s^{-1}}$) provided by fluorescent and incandescent bulbs. Plants were left in the growth chamber until harvest. Plants were not treated with nonradioactive glyphosate. Overspray with glyphosate is recommended when differences between resistant and susceptible weed biotypes are expected to be significant (Shaner 2009). While treatment with nonradioactive glyphosate has been reported before (Lorraine-Colwill et al. 2003), it has also been omitted elsewhere (Koger and Reddy 2005).

A solution containing glyphosate at a final concentration of 0.84 kg in $190\,\mathrm{L\ ha^{-1}}$ was made using $[^{14}C]$glyphosate ($[^{14}C]$ methyl labeled with 2.0 GBq $\mathrm{mmol^{-1}}$ specific activity; American Radiolabeled Chemicals, 101 Arc Drive, St Louis, MO 63146), a commercial potassium salt formulation of glyphosate, and distilled water. A 10-μl volume of the solution was applied to the adaxial surface of the second fully expanded leaf blade in the form of tiny droplets with a micro-applicator. Each plant received

approximately 2.08 kBq of [^{14}C]glyphosate in a total volume of 10 μl. Plants were harvested at 1, 4, 24, and 48 h after treatment (HAT). Thereafter, standard procedures to measure absorption and translocation (Nandula and Vencill 2015; Nandula et al. 2013) of [^{14}C]glyphosate in *E. colona* plants were followed as described below.

At each harvest, the treated leaf (TL) was removed and rinsed in 10 ml of 10% methanol for 20 s to remove the nonabsorbed [^{14}C]glyphosate from the leaf surface. Two 1-ml aliquots of the leaf wash were mixed with separate 10-ml scintillation cocktail (Ecolume, ICN, Costa Mesa, CA 92626) volumes to measure nonabsorbed [^{14}C]glyphosate. After the TL was removed, each plant was further divided into shoot above treated leaf (SATL), shoot below treated leaf (SBTL), and roots for measuring translocation. The four plant parts were dried and combusted in a biological oxidizer (Packard Instruments, Downers Grove, IL 60515), and the evolved $^{14}CO_2$ was trapped in a scintillant cocktail. Radioactivity from leaf washes and oxidations was quantified using liquid scintillation spectrometry (Packard Tri-Carb 2100TR, Packard Instruments). The average recovery of applied [^{14}C]glyphosate was 95%, based on the sum of the radioactivity measured in all plant parts (absorption, expressed as percent of applied ^{14}C) and leaf washes. The total radioactivity recovered in all plant parts except the TL was designated as translocated ^{14}C and expressed as percent of absorbed. There were three replications (1 pot^{-1}) per HAT per population, and the experiment was repeated once.

A separate set of plants from both populations was treated with [^{14}C]glyphosate as described earlier and used for phosphorimaging analysis. At similar HAT time points, the TLs from the plants were removed to wash off unabsorbed radioactivity and set aside. The rest of the plant, including the aboveground parts and roots, was mounted between layers of plain white paper and paper towels, frozen, dried, and cooled to room temperature. The plant was placed in a 20 by 40 cm exposure cassette (GE Healthcare Bio-Sciences, Piscataway, NJ 08854) and brought into contact with a storage phosphor screen (BAS IPSR 2025 E, GE Healthcare Bio-Sciences) under diffused lighting. The apparatus was placed in a dark cabinet for 24 h. A phosphorimager (Typhoon FLA 7000, GE Healthcare Bio-Sciences) was used to detect distribution of [^{14}C]glyphosate and develop an image. There were two replications (a replication being 1 pot^{-1}) per harvest time per population, and the experiment was repeated once.

Genomic DNA Extraction and EPSPS Gene Sequencing

Manhattan, KS

To determine whether any of the known glyphosate resistance–conferring amino acid substitutions at position 102 or 106 of the EPSPS protein were present in the MSGR or TNGR populations, a segment of the *EPSPS* gene was sequenced. The genomic DNA (gDNA) from MSGR, TNGR, MSGS, and KSGS plants (3 plants per biotype/population) was isolated using Plant DNAzol™ Reagent (Invitrogen™, Thermo Fisher Scientific, 168 Third Avenue, Waltham, MA 02451) according to the manufacturer's protocol. The quality of gDNA was determined by gel electrophoresis, and the quantity was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE 19810). To amplify the *EPSPS* gene, a polymerase chain reaction (PCR) was performed using a T100™ Thermal Cycler (Bio-Rad, Hercules, CA 94547) on a 50-μl total reaction mixture containing

100 ng of gDNA (2 μl), 0.5 μM each (5 μl) of forward primer (AWF-5′-AACAGTGAGGAYGTYCACTACATGCT-3′) and reverse primer (EC2R1-5′-CCATGAAGGTTTTTCTGCGACT--3′) (Alarcón-Reverte et al. 2015; Latasa 2014; Nguyen et al. 2016), 25 μl of Taq 2X PCR Master Mix (Promega, Fitchburg, WI 53711), and 13 μl nuclease-free water to make up the final volume. The following PCR conditions were used: initial denaturation at 95 C for 7 min, followed by 32 cycles of denaturation at 95 C for 45 s, annealing at 58 C for 30 s and extension at 72 C for 1 min, and then a final extension at 72 C for 10 min. The PCR product was purified using a Thermo Scientific™ GeneJET™ PCR Purification Kit following the manufacturer's guidelines. The purified gene fragment was Sanger sequenced by Genewiz (South Plainfield, NJ 07080). The gene sequences were analyzed and aligned using SnapGene® software (GSL Biotech, Chicago, IL 60615; available at snapgene.com) and MultAlin software (Corpet 1988).

Stoneville, MS, and Urbana-Champaign, IL

Procedures like those described earlier were used in Stoneville, MS, and Urbana-Champaign, IL, to extract genomic DNA, amplify the *EPSPS* gene, and sequence amplicons. Briefly, leaf tissue was collected, freeze-dried, and ground to a fine powder. DNA was extracted using a Maxwell A Maxwell 16™ (Promega) automated DNA-isolation machine employing Promega AS1030 Tissue DNA Purification Kits. Agarose gel–purified amplicons were cloned using a TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA 92008) and sequenced at the USDA-ARS Southeast Area Genomics Facility at Stoneville, MS. Sequences were analyzed using Sequencher (v. 5.4.5, Gene Codes, Ann Arbor, MI 48108) software. Partial *EPSPS* gene of *E. colona* plants from MSGR (first generation; see "Introduction") and MSGS populations was amplified using a forward primer (EleuEPSPSF-5′-GCGGTAGTTGTTGGCTGTGGTG-3′) and reverse primer (EleuEPSPSR-5′-TCAATCCGACAACCAAGTCGC-3′) (Han et al. 2016). The primers were intended to amplify the regions covering the Pro-106 codon in susceptible and resistant plants. The same primers were also used for analyses of second-generation MSGR biotypes, TNGR, and MSGS populations.

Quantitative PCR to Determine Relative EPSPS Copy Number

Manhattan, KS

To determine whether any amplification of the *EPSPS* gene is present in MSGR or TNGR populations, real-time quantitative PCR (qPCR) was performed. The gDNA extracted from all GR or GS *E. colona* (the same samples described earlier) was used to perform a quantitative PCR (StepOnePlus™ real-time detection system, Life Technologies, Grand Island, NY 14072) with β-tubulin as a reference gene (Godar et al. 2015) in a 96-well microtiter plate containing a Master Mix of 8 μl of Power SYBR Green (Life Technologies), 2 μl each of forward and reverse primers (5 μM), 2 μl of gDNA (16 ng μl^{-1}), for a total reaction volume of 14 μl. The gene-specific forward primer (*EPSPS* LOLF-5′-CTGATGGCTGCTCCTTTAGCTC-3′) and reverse primer (*EPSPS* LOLR-5′-CCCAGCTATCAGAATGCTCTGC-3′) (Salas et al. 2012) that are expected to amplify a 136-bp fragment were used. The following primer sequences for the reference gene β-tubulin (forward: -5′ATGTGGGATGCCAAGAACATGATGTG3′; and reverse: -5′-TCCACTCCACAAAGTAGGAAGAGTTCT-3′) were used. A minimum of three technical replicates of each sample was used in each experiment, and each experiment was repeated. The

following qPCR conditions were maintained: initial denaturation at 94 C for 10 min, followed by denaturation at 94 C for 30 s and annealing at 60 C for 45 s repeated for 40 cycles. To determine the primer specificity in the reaction, a melt-curve analysis was included at the end of the procedure. Single curves were observed for both the *β-tubulin* and *EPSPS* primers. The relative copy number of *EPSPS* was determined by the comparative Ct method (as $2^{-\triangle Ct}$) (Schmittgen and Livak 2008), where $\triangle Ct = [Ct\ EPSPS - Ct\ \beta\text{-}tubulin]$. *β-tubulin* was used as a reference gene for normalizing the copy number data (Pfaffl 2001). The glyphosate-susceptible (MSGS1) sample, which has a single copy of *EPSPS*, was used for calibrating the copy number. The copy number was averaged across the replications, and the standard deviation was calculated for each plant sample.

Statistical Analysis

All experiments were conducted using a completely randomized design. Data from all experiments, with the exception of the *EPSPS* sequence analysis and gene copy number, were analyzed by ANOVA via the PROC GLM statement using SAS software (v. 9.2, SAS Institute, 100 SAS Campus Drive, Cary, NC 27513). Data from repeated experiments were pooled due to a nonsignificant experiment effect. Nonlinear regression analysis was applied to fit a sigmoidal log-logistic curve of the form:

$$y = a / \{1 + \exp[-(x - x_0) / b]\} \qquad [1]$$

where a is an asymptote, x and x_0 are the upper and lower response limits with the latter approaching 0, and b is the slope of the curve around x_0, to relate the effect of glyphosate dose on *E. colona* control. Equation parameters were computed using SigmaPlot (v. 11.0, Systat Software, 1735 Technology Drive #430, San Jose, CA 95110). Treatment means in selected experiments were separated using Fisher's protected LSD at P = 0.05.

Results and Discussion

Whole-Plant Response to Glyphosate

Stoneville, MS

Response of *E. colona* biotypes and populations to glyphosate dose is presented in Figure 1. ED$_{50}$ (dose required to reduce plant growth by 50%) values for the MSGR4 biotype, TNGR, and MSGS populations were 0.8, 1.62, and 0.23 kg ha^{-1} of glyphosate, respectively. The resistance index calculated from the above ED$_{50}$ values indicated that the MSGR4 biotype and TNGR population were 4- and 7-fold resistant, respectively, relative to the MSGS population. The resistance levels reported here are higher than those reported from northeastern Australia (2- to 2.5-fold) (Han et al. 2016), but lower than those reported from northwestern Australia (8.3-fold) (Gaines et al. 2012). *Echinochloa colona* accessions from a corn (*Zea mays* L.) field in California were 6.6-fold (Alarcón-Reverte et al. 2013) and 4- to 9-fold (Alarcón-Reverte et al. 2015) resistant to glyphosate. Response of *E. colona* to glyphosate has been shown to be dependent on temperature and growing conditions (Han et al. 2016; Nguyen et al. 2016). Therefore, the level of resistance to glyphosate in *E. colona* is better measured under conditions similar to the natural environment when *E. colona* emerges and is at the correct growth stage for labeled applications of glyphosate.
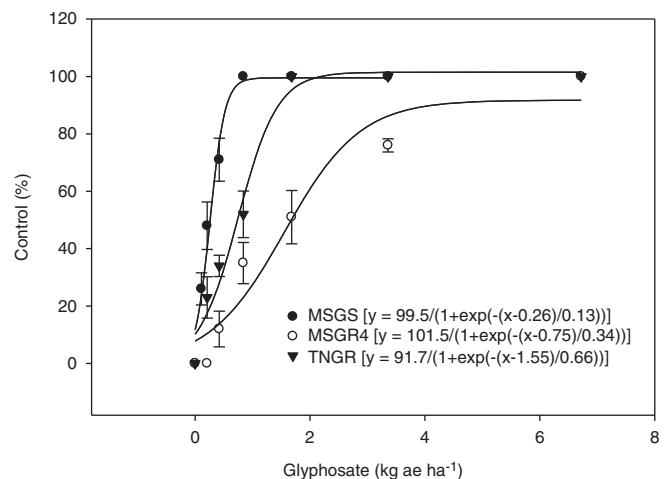

Figure 1. Glyphosate dose response on control of glyphosate-resistant (TNGR population and MSGR4 biotype) and glyphosate-susceptible (MSGS) *Echinochloa colona* populations 3 wk after treatment. Vertical bars represent standard error of mean.

[^{14}C]Glyphosate Absorption, Translocation, and Phosphorimaging

Stoneville, MS

The absorption patterns of [^{14}C]glyphosate in the TNGR and MSGS populations were similar throughout the time course of the experiment. About 55% and 53% of applied [^{14}C]glyphosate was detected at 48 HAT for the TNGR and MSGS biotypes, respectively (Table 1). Nguyen et al. (2016) reported similar levels of [^{14}C]glyphosate absorption between resistant and susceptible *E. colona* populations from Australia within each of two temperature regimes. Further, there were no differences in [^{14}C]glyphosate uptake between resistant and susceptible *E. colona* plants from California (Alarcón-Reverte et al. 2013).

The translocation pattern of [^{14}C]glyphosate was similar between the TNGR and MSGS plants up to 24 HAT (Table 1). Thereafter, the MSGS population (30.2% of absorbed) translocated nearly 13% more [^{14}C]glyphosate out of the TL compared with the TNGR population (17.3% of absorbed) at 48 HAT. Alarcón-Reverte et al. (2013) reported no difference in translocation levels of [^{14}C]glyphosate between resistant and susceptible *E. colona* populations from California.

The above pattern is indicative of a mechanism of glyphosate resistance because of reduced translocation, which was reported in several resistant weed species such as hairy fleabane [*Erigeron bonariensis* L.] (Dinelli et al. 2008), horseweed (*Erigeron canadensis* L.) (Dinelli et al. 2006; Feng et al. 2004; Koger and Reddy 2005), Italian ryegrass [*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot] (Nandula et al. 2008; Perez-Jones et al. 2007), rigid ryegrass (*Lolium rigidum* Gaudin) (Lorraine-Colwill et al. 2003; Wakelin et al. 2004), and waterhemp [*Amaranthus tuberculatus* (Moq.) J. D. Sauer] (Nandula et al. 2013). The glyphosate translocation model proposed by Shaner (2009), which hypothesized the existence of a barrier at the cellular level preventing glyphosate loading into the phloem, may have a role in the resistant TNGR population. The glyphosate in the TNGR plants could possibly be loaded into vacuoles via a system akin to the sequestration mechanism described in *E. canadensis* (Ge et al. 2010) and *Lolium* spp. (Ge et al. 2012).

Distribution of absorbed [^{14}C]glyphosate in the TNGR and MSGS populations is summarized in Table 1. The quantity of

Table 1. Absorption, translocation, and distribution of [^{14}C]glyphosate in resistant and susceptible *Echinochloa colona* populations.[a,b]

Population	Harvest time	Absorption	Translocation[d]	Distribution[c]			
				TL	SATL	SBTL	Roots
	HAT	% of applied		——————————————% of absorbed——————————————			
TNGR	1	7a	1.7a	98.3a	0.67a	0.69a	0.34a
MSGS	1	4a	1.7a	98.3a	0.38a	0.72a	0.60a
TNGR	4	15a	7.8a	92.2a	0.90a	3.4a	3.50a
MSGS	4	15a	9.2a	90.8a	0.60a	2.7a	5.90a
TNGR	24	48a	17.7a	82.3a	2.10a	11.1b	4.50a
MSGS	24	47a	20.3a	79.7a	1.80a	7.5a	11.0b
TNGR	48	55a	17.3a	82.7b	1.70a	12.0a	3.60a
MSGS	48	53a	30.2b	69.8a	3.70b	10.7a	15.8b

[a]Abbreviations: HAT, h after treatment; SATL, shoot above treated leaf; SBTL, shoot below treated leaf; TL, treated leaf.
[b]Similar and different letters indicate no difference and significant difference, respectively, between population means for the same parameter (absorption, translocation, or distribution) within the same harvest time according to Fisher's LSD at 5% level of probability. For example, translocation of absorbed [^{14}C]glyphosate between the two populations is different at 48 HAT.
[c]Distribution represents partitioning of absorbed [^{14}C]glyphosate between the TL, SATL, SBTL, and roots.
[d][^{14}C]glyphosate outside of TL (SATL, SBTL, and roots) was considered as translocation.

[^{14}C]glyphosate that accumulated in various parts of the plant was similar between the TNGR and MSGS populations at respective harvest times after treatment, except for the TL, SATL, and roots at 48 HAT, and SBTL and roots at 24 HAT. At 48 HAT, the TNGR plants (82.7% of absorbed) had more [^{14}C]glyphosate remaining in the TL than the MSGS plants (69.8% of absorbed). Additionally, the level of [^{14}C]glyphosate that translocated to the SATL was lower in the TNGR population (1.7% of absorbed) compared with the MSGS population (3.7% of absorbed). It was intriguing to realize that the resistant TNGR population accumulated more [^{14}C]glyphosate in the SBTL tissues (11.1% of absorbed) than the MSGS population (7.5% of absorbed). This could have been due to an inherent machinery in the TNGR plants to divert glyphosate away from the growing point (part of SATL) and prevent its accumulation at phytotoxic levels. Whether the accumulated glyphosate was being loaded into vacuoles is unknown, but an interesting possibility. The MSGS roots acquired more [^{14}C]glyphosate than the TNGR roots, resulting in 11% and 15.8% of absorbed compared with 4.5% and 3.6% of absorbed at 24 and 48 HAT, respectively. Overall, the distribution data support the translocation data, in that the TNGR population translocated more [^{14}C]glyphosate at 48 HAT than the MSGS population, as reflected in the differences between the respective TL, SATL, and roots at 48 HAT.

Phosphorimaging results (Figure 2) also corroborate the translocation and especially the distribution results at 48 HAT. The phosphorimages of the TNGR (Figure 2A and B) and MSGS plants (Figure 2E and F) were similar at 1 and 4 HAT. At 24 HAT, [^{14}C] glyphosate had accumulated throughout the MSGS plant (Figure 2C), whereas the TNGR plant exhibited movement of glyphosate from the TL to SBTL and roots only. At 48 HAT, the TNGR plant (Figure 2H) clearly had restricted movement of [^{14}C] glyphosate compared with the MSGS plant (Figure 2D), where even the leaf and root tips seemed to have gathered glyphosate.

EPSPS Gene Sequencing

Manhattan, KS

Single-nucleotide mutation(s) at residues 102 or 106 in the EPSPS protein resulting in the substitution of amino acids has been reported to reduce glyphosate binding at the target site,

conferring low- or high-level glyphosate resistance, respectively (Powles and Preston 2006; Yu et al. 2015). The *EPSPS* gene sequence analyses of TNGR *E. colona* (NCBI accession number: JN004269.1, JN004268.1) indicated no evidence of any point mutation(s) at 102 or 106 (Figure 3). However, in all MSGR biotypes, a single-nucleotide substitution of T for C at codon 106 position was identified, conferring a predicted proline-to-serine substitution (CCA to TCA) (Figure 3).

Urbana-Champaign, IL, and Stoneville, MS

Similar results were obtained in Urbana-Champaign, where the Pro-106-Ser change was detected in the MSGR4, MSGR27,
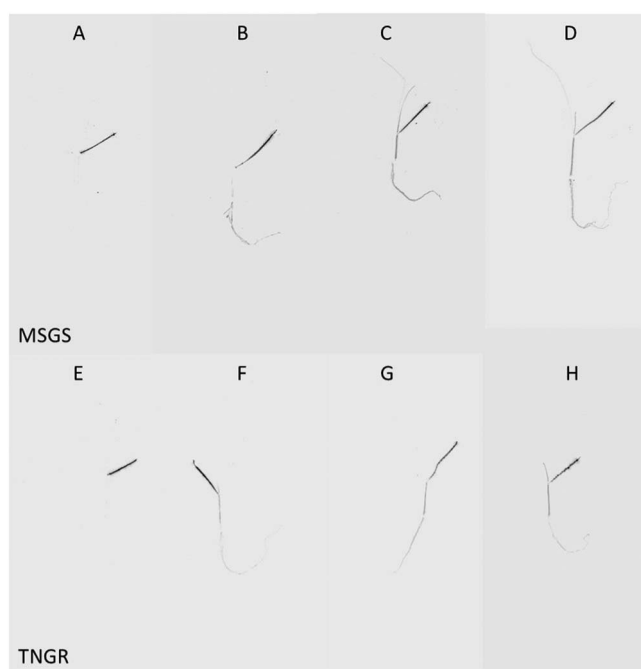

Figure 2. Phosphorimages of *Echinochloa colona* plants from the susceptible MSGS (top row) and resistant TNGR (bottom row) populations treated with [^{14}C]glyphosate at 1 (A, E), 4 (B, F), 24 (C, G), and 48 (D, H) HAT (left to right columns). The darkest areas indicate the treated area of the second fully expanded leaf.

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MSGR4-1    CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR4-2    CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR4-3    CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR27-1   CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR27-2   CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR27-3   CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR34-1   CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR34-2   CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR34-3   CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR36-1   CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR36-2   CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR36-3   CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR44A-1  CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR44A-2  CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR44A-3  CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR44B-1  CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR44B-2  CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR44B-3  CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR44C-1  CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR44C-2  CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR44C-3  CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR49-1   CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR49-2   CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG
MSGR49-3   CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG TCA CTG

TNGR1-1    CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG CCA CTG
TNGR1-2    CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG CCA CTG
TNGR1-3    CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG CCA CTG
TNGR2-1    CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG CCA CTG
TNGR2-2    CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG CCA CTG
TNGR2-3    CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG CCA CTG
TNGR3-1    CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG CCA CTG
TNGR3-2    CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG CCA CTG
TNGR3-3    CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG CCA CTG

MSGS1-1    CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG CCA CTG
MSGS1-2    CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG CCA CTG
MSGS1-3    CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG CCA CTG

KSGS1-1    CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG CCA CTG
KSGS1-2    CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG CCA CTG
KSGS1-3    CTC TTC TTG GGG AAT GCT GGA ACT GCA ATG CGG CCA CTG
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Threonine 102   Proline 106 Serine

Figure 3. Nucleotide sequence alignment of *EPSPS* gene fragment from glyphosate-susceptible (KSGS and MSGS) and glyphosate-resistant (MSGR and TNGR) *Echinochloa colona*. Known resistance-conferring mutations at codons 102 and 106 are indicated (codon numbering based on the *Arabidopsis thaliana EPSPS* gene sequence). Three plants from each population were used for sequence analysis. ACT, threonine; CCA, proline; TCA, serine.

MSGR34, MSGR36, MSGR49, and MSGR44A biotypes (unpublished data). Further, in Stoneville, several first-generation accessions were found to have one or more alleles corresponding to the *EPSPS* gene with the serine replacement at the 106 position (unpublished data). These results suggest that the EPSPS enzyme may not be sensitive to glyphosate in MSGR biotypes, confirmable by an EPSPS assay, and that another mechanism of resistance to glyphosate is in play in the TNGR population. The first case of a Pro-106-Ser target-site mutation associated with glyphosate resistance in *E. colona* was reported from California (Alarcón-Reverte et al. 2013). In a newer report, two mutations, Pro-106-Ser and Pro-106-Thr, were reported in an *E. colona* population, also from California (Alarcón-Reverte et al. 2015). Han et al. (2016) confirmed Pro-106-Thr and Pro-106-Leu in *E. colona* from Australia. It is interesting that our report and all other previously documented Pro-106 substitutions have occurred in the past 5 yr.

EPSPS Gene Copy Number

Manhattan, KS

The results of qPCR analyses suggest that there is no variation in relative *EPSPS* gene copies between resistant and susceptible *E. colona* (Figure 4). Amplification of the *EPSPS* gene does not confer resistance to glyphosate in these populations.

In summary, *E. colona* populations from Mississippi and Tennessee have been confirmed to be 4- and 7-fold resistant to
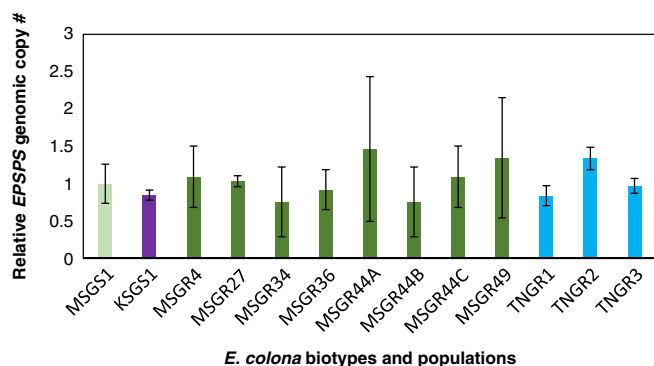
Figure 4. The *EPSPS* gene copy numbers of glyphosate-resistant (MSGR and TNGR) and glyphosate-susceptible (MSGS1 and KSGS1) *Echinochloa colona*. The relative *EPSPS* gene copy numbers were calculated using sample MSGS1. Error bars represent the standard error of the mean ($n = 3$ technical replicates). The *β-tubulin* gene was used as a reference to normalize the qPCR data.

glyphosate, respectively. The mechanism of resistance in the MSGR population (and associated biotypes) is, at least in part, due to a mutation at the 106th loci of the EPSPS protein, resulting in replacement of proline with a serine residue. Other glyphosate-resistance mechanisms such as sequestration and differential translocation could have a role but were not investigated in the MSGR population. The TNGR population exhibited a reduced translocation mechanism of resistance to glyphosate. An *E. colona* population from Mississippi was recently reported to be resistant to herbicides spanning four unique mechanisms of action, but not glyphosate (Wright et al. 2016, 2018), thereby indicating the expanding problem of resistance to a broad spectrum of herbicides in *E. colona* populations from the midsouthern U.S. states of Mississippi, Tennessee, and Arkansas. Han et al. (2016) observed that a field use rate of glyphosate at $0.45\,\mathrm{kg\,ha^{-1}}$ controlled *E. colona* plants resistant to glyphosate and carrying two *EPSPS* mutations under day/night temperatures of 25/20 C, but not at 35/30 C, wherein 68% of mutant resistant plants survived. The reports cited and research results presented here indicate the necessity of developing *E. colona* management strategies that include chemical, cultural, and mechanical tools.

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