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Source: Weed Science, 59(3) : 299-304

Published By: Weed Science Society of America

URL: https://doi.org/10.1614/WS-D-10-00150.1

Weed Science 2011 59:299–304

Glyphosate Resistance in a Johnsongrass (*Sorghum halepense*) Biotype from Arkansas

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Johnsongrass is one of the most troublesome weeds of the world and is listed as a noxious weed in Arkansas. Reduced johnsongrass control with the recommended application rate of glyphosate (840 g ae ha^{-1}) was reported in a continuous soybean field near West Memphis, AR, in the fall of 2007. A greenhouse study was conducted (1) to confirm and characterize glyphosate resistance in the johnsongrass biotype from West Memphis and (2) to determine whether resistant and susceptible biotypes have differential glyphosate absorption or translocation. Dose–response studies revealed that the resistant biotype was five- to seven-fold less sensitive to glyphosate than the susceptible biotype. Glyphosate absorption was similar in resistant and susceptible biotypes at 72 h after treatment (HAT). However, the treated leaf of the resistant biotype retained 28 percentage points more absorbed ^{14}C glyphosate compared to the susceptible biotype at 72 HAT. Additionally, the resistant biotype had less ^{14}C glyphosate translocated to the aboveground tissue below the treated leaf and to roots compared to the susceptible biotype at 24 and 72 HAT. Reduced translocation and increased retention of glyphosate in treated leaves is a probable mechanism of resistance in this glyphosate-resistant johnsongrass biotype.
Nomenclature: Glyphosate; johnsongrass, *Sorghum halepense* (L.) Pers.
Key words: Herbicide resistance mechanism, noxious weed, perennial weed, weed control.

Johnsongrass ($2N = 2X = 40$), an invasive perennial grass species of Mediterranean origin, is a problematic weed of the southeastern United States (McWhorter 1989) and is listed as a noxious weed in the state of Arkansas (Arkansas State Plant Board [ASPB] 2006). Johnsongrass has a wide ecological amplitude (latitude extending from 55°N to 45°S) and infests 30 crops across 53 different countries (Holm et al. 1991). After introduction to the United States in the late 1700s, johnsongrass extensively spread through commercial sale of seed for fodder crops, Civil War cavalry movements, post–Civil War planting for erosion control, flooding, and contaminated seedlots, hay, or machinery (McWhorter 1971).

Enormous seed (28,000 seeds $plant^{-1}$) (Horowitz 1973) and rhizome (40 to 90 m $plant^{-1}$) (McWhorter and Jordan 1976) production per season, along with high biomass accumulation (as typical to C_4 plants), makes johnsongrass an effective competitor of sugarcane (Saccharum officinarum L.), soybean [*Glycine max* (L.) Merr], and cotton (*Gossypium hirsutum* L.) (Black et al. 1969; Millhollon 1995). Bridges and Chandler (1987) reported 70% cotton yield loss with 32 johnsongrass plants 9.8 m row^{-1}. Johnsongrass reduced the yield of six soybean cultivars by 23 to 43% (McWhorter and Hartwig 1968), and 88% soybean yield reduction resulted from full-season interference with 32 johnsongrass culms 10 m^{-2} (Williams and Hayes 1984).

Selective johnsongrass control in soybean has been achieved by dinitroaniline (McWhorter 1977), acetyl-coenzymeA carboxylase (ACCase)–inhibitor (Langemeier and Witt 1986), and acetolactate synthase (ALS)–inhibitor herbicides (Riley and Shaw 1988). Nevertheless, evolution of resistance to ALS-inhibitor (Heap 2011), ACCase-inhibitor (Burke et al. 2006; Smeda et al. 1997), and dinitroaniline herbicides (Heap 2011) has reduced the options for selective johnsongrass control in soybean. Glyphosate-resistant (GR) soybean technology became available to growers in the United States in 1996. Because of better weed management, cost savings, and simplicity of use, GR soybean has been widely adopted by growers in the United States and Argentina and currently represents more than 90% of the area planted to soybean (reviewed in Duke and Powles 2009). On the negative side, the overuse of this single weed management technology has led to the evolution of 21 GR weed species worldwide (Heap 2011). At least one GR weed species is present in 16 different countries. Important GR weed species infesting GR crops in North and South America are common ragweed (*Ambrosia artemisiifolia* L.), giant ragweed (*Ambrosia trifida* L.), hairy fleabane [*Conyza bonariensis* (L.) Cronq.], horseweed [*Conyza canadensis* (L.) Cronq.], Italian ryegrass (*Lolium multiflorum* Lam.), johnsongrass, Palmer amaranth (*Amaranthus palmeri* S. Wats.), rigid ryegrass (*Lolium rigidum* Gaud.), waterhemp [*Amaranthus tubuerculatus* (Moq.) Sauer], and wild poinsettia (*Euphorbia heterophylla* L.) (reviewed in Duke and Powles 2009). Among these GR weed species, johnsongrass is the most recent weed to evolve glyphosate resistance in the United States. The first report of GR johnsongrass was from Argentina in 2005 (Heap 2011). Vila-Aiub et al. (2007) reported 3.5- to 10.5-fold resistance of a johnsongrass biotype from Argentina to glyphosate. In 2007, glyphosate failed to control johnsongrass in West Memphis, AR, in a field that had been in continuous GR soybean for at least 6 yr, with glyphosate being the only herbicide used for weed control.

The mechanism of glyphosate resistance varies among weed species. Reduced glyphosate translocation to the young leaves of resistant biotypes is the most common mechanism of resistance and has been reported to be the partial mechanism of resistance in horseweed (Feng et al. 2004; Koger and Reddy 2005), Italian ryegrass (Michitte et al. 2007), and rigid ryegrass (Yu et al. 2009). Reduced absorption and increased vacuolar sequestration of glyphosate is another mechanism of glyphosate resistance in Italian ryegrass (Michitte et al. 2007) and horseweed (Ge et al. 2009), respectively. Mutations in glyphosate target-site gene, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19) at pro 106 imparted glyphosate resistance to goosegrass [*Eleusine indica* (L.) Gaertn.] (Baerson et al. 2002), Italian ryegrass (Jasieniuk

DOI: 10.1614/WS-D-10-00150.1
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et al. 2008), and rigid ryegrass (Wakelin and Preston 2006). In contrast, glyphosate resistance in common ragweed was neither reduced glyphosate absorption and translocation nor insensitive target site (Brewer and Oliver 2009). Gaines et al. (2010) reported EPSPS gene amplification as the reason for glyphosate resistance in Palmer amaranth. A study was conducted with objectives (1) to confirm and to characterize the level of glyphosate resistance and (2) to determine if reduced absorption and/or translocation are the mechanism of resistance in glyphosate-resistant johnsongrass.

Materials and Methods

Plant Material and Growth Conditions. Seeds of putative resistant and known susceptible johnsongrass biotypes were collected from a field near West Memphis, AR, following multiple glyphosate applications and from a field at the University of Arkansas Research Station in Fayetteville, respectively, during the fall of 2008. The field in West Memphis had been in continuous soybean production for at least 6 yr, and glyphosate had been the only herbicide used for weed control, often at lower than recommended rates. For all experiments, seeds from the putative resistant and susceptible biotypes were planted in separate 555 by 265 by 55–mm[3] plastic trays with commercial potting media[1] in the greenhouse under conditions of 30/20 ± 3 C day/night temperature and 16-h photoperiod. Following emergence, individual plants at the two-leaf stage were transplanted to 15-cm-diam plastic pots. Pots were watered daily and fertilized[2] once weekly to maintain good plant growth.

Dose–Response Experiment. *Seedling Johnsongrass.* Resistant and susceptible johnsongrass plants at the five- to six-leaf stage were treated with seven glyphosate rates ranging from 26 to 1,680 g ae ha^{-1}. MON 78623 (potassium salt of glyphosate) was applied with 0.25% v/v nonionic surfactant (NIS). The treatments with the lowest and highest glyphosate application rates corresponded to 1/32 and 2 times, respectively, the recommended glyphosate rate of 840 g ha^{-1}. Two additional treatments of 3,360 and 6,720 g ha^{-1} glyphosate were included for the putative resistant biotype. Glyphosate treatments were applied in an automated spray chamber with a boom containing two flat fan 80-0067 nozzles calibrated to deliver 93.5 L ha^{-1}. After treatment, plants were returned to the greenhouse with environmental conditions as during early growth. The experimental layout was a completely randomized design with 20 replications for each glyphosate dose. The experiment was repeated in time. Treatment effect with regard to plant survival or death was recorded at 28 d after treatment (DAT). Mortality data were subjected to probit analysis with the use of PROC PROBIT in SAS[3] to determine the lethal dose needed to kill 50% (LD_{50}) of each biotype. Differential of biotypes to glyphosate was determined by confidence intervals of 95%.

Rhizomatous Johnsongrass. An additional experiment was conducted to determine the dose response of rhizomatous johnsongrass. Glyphosate-resistant and -susceptible johnsongrass plants were grown from 5- to 10-cm-long rhizome fragments under greenhouse conditions similar to the seedling johnsongrass dose–response experiment. At approximately 6 mo after transplanting rhizome fragments, aboveground shoots were trimmed to a height of 10 cm and allowed to regrow, resulting in plants of uniform height. When plants were 60 cm tall, each biotype was sprayed with glyphosate at 0, 250, 500, 1,000, 2,000, 4,000, and 8,000 g $ha^{-1}$for resistant plants and 0, 125, 250, 500, 1,000, and 2,000 g ha^{-1} for susceptible plants. Application conditions were similar to the seedling johnsongrass experiment. The experiment was laid out in a completely randomized design with four replications for each glyphosate dose and repeated in time. Aboveground biomass was harvested at 28 DAT, oven dried at 60 C for 3 d, and weighed. Plant mortality was determined based on presence or absence of regrowth and visual confirmation of rhizome death at 6 wk after harvesting the aboveground biomass (10 wk after treatment).

Probit analysis was performed as in the seedling johnsongrass dose–response experiment to determine LD_{50} for both biotypes. The dry-weight data were converted to a percentage of the nontreated control and were subjected to ANOVA using PROC MIXED in SAS. Homogeneity of variance was tested using PROC UNIVARIATE in SAS. Sums of squares were partitioned to test the linear, quadratic, and higher-order polynomial effects of percentage dry-weight reduction over glyphosate rates (Draper and Smith 1981). There was a nonsignificant trial by treatment interaction. Thus, data were pooled over the experiments. ANOVA indicated higher-order polynomials effects. Thus, percentage dry weight data were modeled with the use of the logistic function:

$$y = b \Big/ \left[1 + x/(\mathrm{GR}_{50})^{d}\right] \qquad [1]$$

where y is percent dry weight at a glyphosate dose x (g ae ha^{-1}), b is upper asymptote (constrained to 100), GR_{50} is glyphosate dose that reduces plant dry weight by 50%, and d is slope of the curve at the GR_{50}.

Absorption and Translocation of ^{14}C glyphosate. The experiment was arranged in a split-split plot design with four harvest timings as main plots, resistant and susceptible biotypes as sub-plots, and four plant portions as sub-subplots. The middle 5-cm portion of the second fully expanded leaf of individual johnsongrass plants at five- to six-leaf stage was marked with a permanent marker and covered with aluminum foil. Formulated glyphosate[4] was mixed with deionized water and applied to the johnsongrass plants at a dose of 870 g ha^{-1} with the use of the setup for the dose–response experiments. A small amount of the spray volume was then spiked with non-formulated, ^{14}C-phosphonomethyl-labeled glyphosate[5] (specific activity of 2.0 GBq $mmol^{-1}$) to make a stock solution with specific activity of 0.37 kBq μl^{-1}. As soon as plants dried (10 to 15 min), 4 µl of herbicide solution containing 1.48 kBq of ^{14}C glyphosate was applied to the marked portion of leaves on the adaxial side. A 50-µl microsyringe[6] equipped with a repeating dispenser to deliver 1- µl droplets was used to apply the ^{14}C-glyphosate solution. Plants were harvested 2, 8, 24, and 72 HAT by cutting them at the soil surface and sectioning them into four parts: treated leaf blade (treated leaf), tissue above treated leaf blade (above treated tissue), aboveground tissue below treated leaf blade (below treated tissue), and roots. Treatments were replicated four times, and the experiment was conducted twice.

To remove nonabsorbed glyphosate, the treated 5-cm portion of the treated leaf was rinsed for 15 s with 1 ml of a methanol:water (1:1 v/v) solution containing NIS at 0.25% v/v. The rinsate was collected in a 20-ml scintillation vial, mixed with 10 ml of scintillation cocktail, and radio assayed by liquid

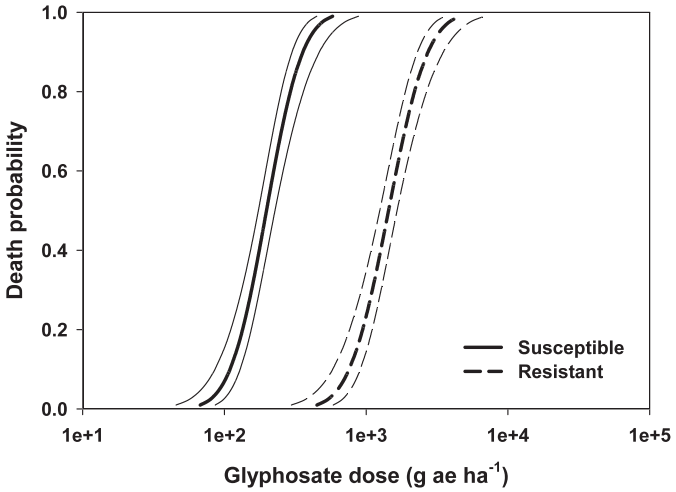

Figure 1. Probit analysis to predict the lethal glyphosate dose required to kill seedling glyphosate-susceptible and -resistant johnsongrass biotypes (thick lines). Thin lines represent 95% confidence intervals (CI) for each biotype.

scintillation spectroscopy[7] (LSS) to determine the amount of nonabsorbed ^{14}C. Following rinsing of the treated portion of the treated leaf and dissection, all plant parts were dried for 48 h at 50 C. Individual plant parts were oxidized with the use of a biological oxidizer,[8] and evolved CO_2 was trapped in 15 ml of scintillation cocktail and radio assayed with the use of LSS.

Absorption data were expressed as a percentage of ^{14}C glyphosate applied. However, translocation data were expressed as a percentage of radioactivity (^{14}C) recovered in plants. Data were tested for normality with the use of the PROC UNIVARIATE procedure in SAS. To improve homogeneity of variance, data were subjected to arcsine square-root transformation prior to ANOVA. The sums of squares were partitioned to reflect a split-split-plot treatment structure and trial effects with the use of the MIXED procedure in SAS. Data from two trials were pooled because of nonsignificant trial by biotype and trial by harvest interval interactions. Fisher's LSD test at $P \leq 0.05$ was used to perform mean separations as appropriate (Steel and Torrie 1980).

Results and Discussion

Dose–Response Study. *Seedling Johnsongrass.* The glyphosate LD_{50} values of resistant and susceptible johnsongrass grown from seed were 1440 and 200 g ha^{-1}, respectively (Figure 1 and Table 1). Based on the R/S ratio calculated from LD_{50} values, the resistant biotype was seven times less sensitive to glyphosate than the susceptible biotype (Table 1). The recommended application rate of glyphosate in soybean is 840 g ha^{-1}; thus, 1.7 times the normal field rate of glyphosate was needed to kill 50% of the resistant biotype plants. Conversely, the LD_{50} value of the susceptible biotype was even less than half of the field rate of glyphosate.

Rhizomatous Johnsongrass. Resistant and susceptible johnsongrass plants grown from rhizomes differed in mortality response to glyphosate dose (Figure 2 and Table 1). The LD_{50} value of the resistant biotype (1,830 g ha^{-1}) was five times more than the LD_{50} value of the susceptible biotype (350 g ha^{-1}). More than two times the field recommended rate of glyphosate was needed to kill 50% of the resistant rhizomatous johnsongrass plants. In addition, the glyphosate-resistant rhizomatous johnsongrass required five times more glyphosate than the susceptible biotype to reduce aboveground biomass by 50% (Table 1). Glyphosate-resistant rhizomatous johnsongrass required 2.8 times more glyphosate (2350 g ha^{-1}) than the field-use rate of 840 g ha^{-1} to reduce aboveground biomass by 50%.

A 3- to 10-fold level of glyphosate resistance has been reported in other plant species including horseweed (Dinelli et al. 2006), hairy fleabane (Dinelli et al. 2008), Italian ryegrass (Perez-Jones et al. 2007), and rigid ryegrass (Wakelin et al. 2004). Glyphosate-resistant johnsongrass in Argentina had a 3.7- to 6.9-fold level of resistance based on LD_{50} values and 3.5- to 10-fold resistance level based on GR_{50} values (Vila-Aiub et al. 2007). Dose–response studies with Arkansas and Argentina populations of glyphosate-resistant johnsongrass displayed similar level of resistance to glyphosate.

Absorption and Translocation of ^{14}C glyphosate. More than 90% of the applied ^{14}C glyphosate was recovered from both resistant and susceptible biotypes. Absorption of ^{14}C glyphosate was similar between biotypes at all harvest intervals and increased from 28 to 30% at 2 HAT to 56 to 59% at 72 HAT (Figure 3). Of the total glyphosate absorbed, more than 75% was absorbed by 8 HAT in both biotypes. Reduced absorption was observed in glyphosate-resistant Italian ryegrass (Michitte et al. 2007), but was not the resistance mechanism in horseweed (Feng et al. 2004; Koger and Reddy 2005) and rigid ryegrass (Yu et al. 2009) nor does it appear to

Table 1. Glyphosate dose required to reduce the growth of johnsongrass biotypes by 50% (GR_{50}) or kill 50% of plants (LD_{50}).

Biotype	Dose (g ae ha^{-1})	95% confidence interval	R/S ratio[a]
		Seedling LD_{50}[b]	
Resistant	1,440	1,250–1,660	7.3
Susceptible	200	170–230	
		Rhizomatous LD_{50}	
Resistant	1,830	1,240–2,730	5.2
Susceptible	350	190–560	
		Rhizomatous GR_{50}[c]	
Resistant	2,350	1,780–2,920	5.0
Susceptible	470	350–590	

[a] R/S ratio was calculated by dividing the GR_{50} or LD_{50} dose (g ae ha^{-1}) of resistant biotype by the GR_{50} or LD_{50} dose of susceptible biotype.
[b] LD_{50} was determined by conducting probit analysis in SAS.
[c] GR_{50} was calculated with the use of logistic dose-response model $y = b/(1 + x/GR_{50})^d$, where y is the plant dry weight (percent of nontreated) at glyphosate dose x, b is the upper asymptote (constrained to 100), GR_{50} is the glyphosate dose required to reduce plant dry weight by 50%, and d is the slope of curve (1.33 and 1.67 for susceptible and resistant biotypes, respectively).

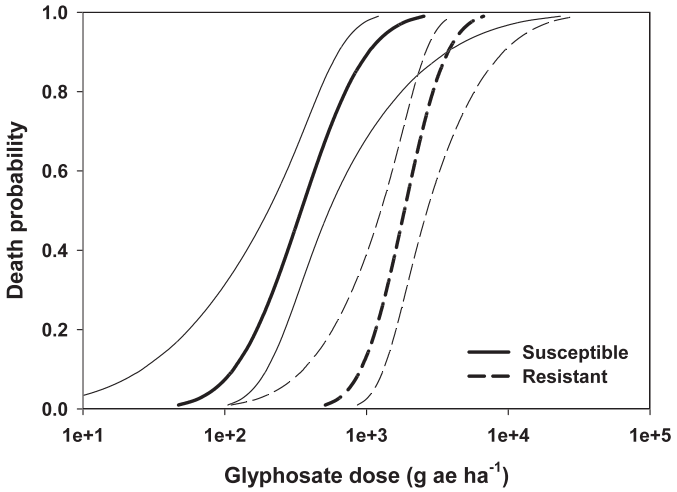

Figure 2. Probit analysis to predict the lethal glyphosate dose required to kill rhizomatous glyphosate-susceptible and -resistant johnsongrass biotypes (thick lines). Thin lines represent 95% confidence intervals (CI) for each biotype.

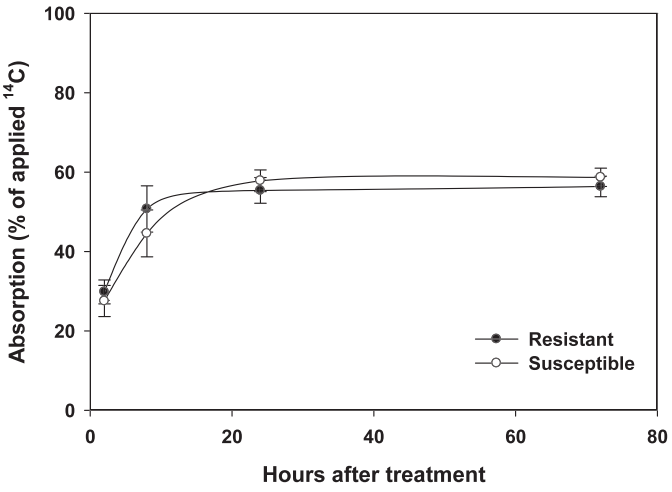

Figure 3. Absorption of ^{14}C glyphosate through adaxial surface of glyphosate-resistant and -susceptible johnsongrass biotypes. Bars indicate standard error of mean.

be the resistance mechanism of the glyphosate-resistant johnsongrass biotype from Arkansas.

The distribution of radioactivity as a percentage of recovered ^{14}C in different plant parts of glyphosate-resistant and -susceptible johnsongrass biotypes is presented in Figure 4. More than 50% of the absorbed radioactivity remained in the treated leaf of resistant and susceptible biotypes at all harvest intervals (Figure 4A). Percent radioactivity in the treated leaves of both biotypes was

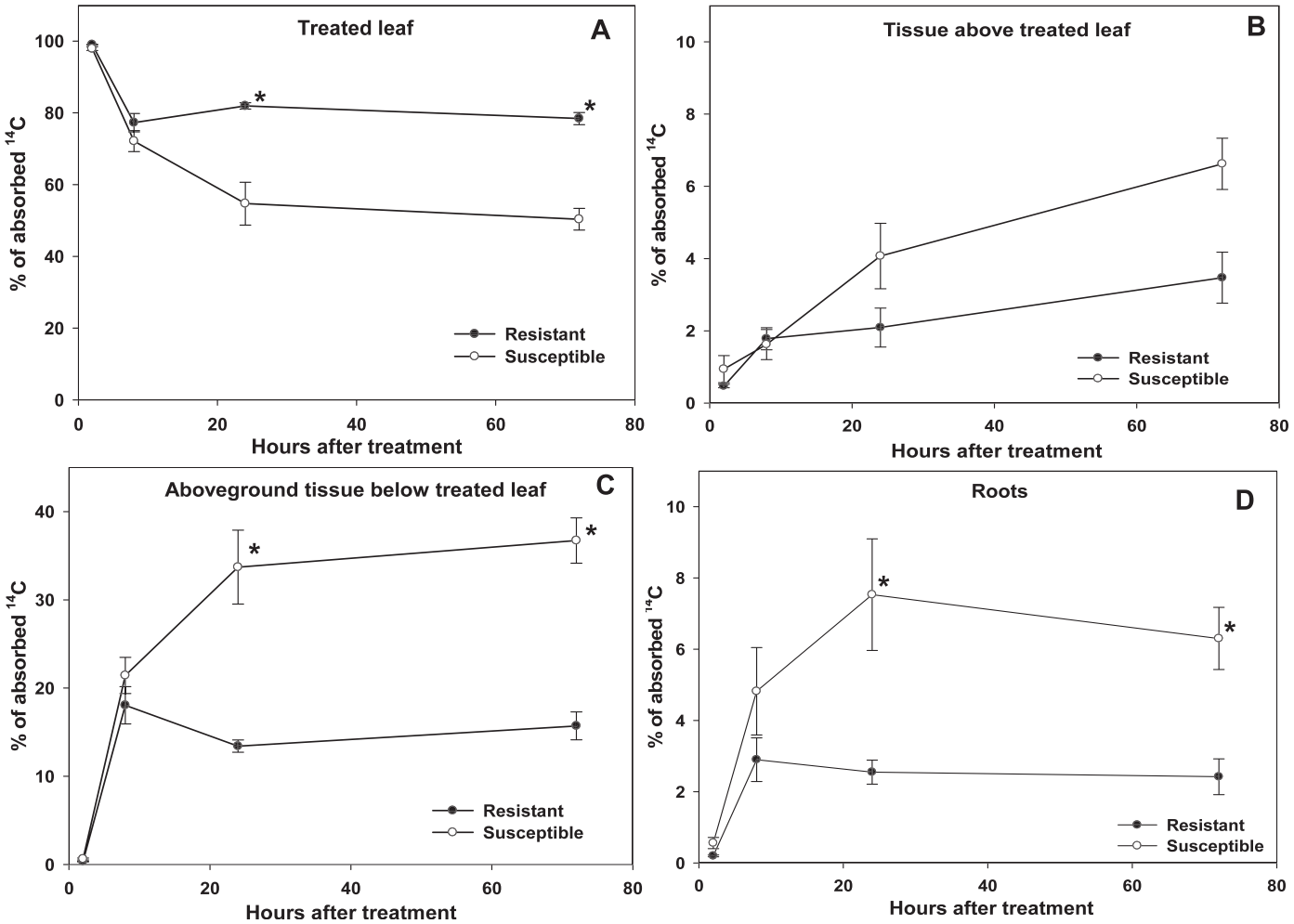

Figure 4. Distribution of absorbed radioactivity in different plant parts (treated leaf [A], tissue above treated leaf [B], aboveground tissue below treated leaf [C], and roots [D]) of glyphosate-resistant and -susceptible johnsongrass biotypes. The asterisk indicates significant difference ($P \leq 0.05$) between resistant and susceptible biotypes at respective harvest intervals. Bars indicate standard error of mean.

similar until 8 HAT (77 and 72% in resistant and susceptible biotype, respectively). However, the treated leaf of the resistant biotype had 27 and 28 percentage points more ^{14}C than the susceptible biotype at 24 and 72 HAT, respectively. Greater glyphosate retention in the treated leaves of the resistant biotype than in the susceptible biotype was also reported for Italian ryegrass (Perez-Jones et al. 2007) and horseweed (Koger and Reddy 2005).

In the glyphosate-susceptible johnsongrass biotype, total translocation out of the treated leaf increased from 1% at 2 HAT to 50% at 72 HAT (data not shown). However, the maximum translocation out of the treated leaf of the resistant biotype was only 23% at 8 HAT. At all harvest intervals, similar amounts of ^{14}C were recovered in the above-treated tissue of resistant and susceptible biotypes (Figure 4B). Below-treated tissue of the resistant and susceptible biotypes had similar amounts of radioactivity up to 8 HAT. Thereafter, differences were evident. Compared to the susceptible biotype, the resistant biotype translocated almost 20 percentage points less radioactivity to the aboveground tissue below the treated leaf at 24 and 72 HAT (Figure 4C), which would increase the likelihood of regrowth of treated plants. Furthermore, the resistant biotype translocated less radioactivity to the roots than the susceptible biotype at 24 and 72 HAT (Figure 4D). Reduced glyphosate translocation in glyphosate-resistant johnsongrass is in accordance with the reduced translocation observed in glyphosate-resistant horseweed (Feng et al. 2004; Koger and Reddy 2005), Italian ryegrass (Michitte et al. 2007), and rigid ryegrass (Yu et al. 2009). Glyphosate-resistant johnsongrass accessions from Argentina with a similar level of glyphosate resistance as in the Arkansas accession also had reduced glyphosate translocation out of treated leaf (reviewed in Powles and Yu 2010).

The primary target site for glyphosate activity in plants is EPSPS located in plastids such as chloroplasts (Hetherington et al. 1998). The precursor of EPSPS (pre-EPSPS) is located in the cytosol and is also sensitive to glyphosate (Della-Cioppa et al. 1986). Thus, any mechanism sequestering glyphosate out of the cytosol can impart resistance (Hetherington et al. 1998). Potential mechanisms to keep glyphosate away from the cytosol are inhibition of active glyphosate uptake, active pumping of glyphosate into apoplast, sequestration into the vacuole, and/or inhibition of glyphosate uptake or retention in chloroplast (Shaner 2009). Absorption of glyphosate was similar in glyphosate-resistant and -susceptible johnsongrass biotypes. Thus, inhibition of uptake does not seem to be the mechanism of glyphosate resistance. However, glyphosate retention in the treated leaf was more and translocation out of the treated leaf was less in the glyphosate-resistant than in the glyphosate-susceptible johnsongrass biotype. Yuan et al. (2007) reported that glyphosate upregulates the adenosine triphosphate-binding cassette (ABC) transporters: membrane-bound proteins that can sequester glyphosate in the vacuole or can actively pump glyphosate out of the cell into the apoplast. With nuclear magnetic resonance studies, Ge et al. (2009) revealed that vacuolar sequestration is the mechanism of resistance in glyphosate-resistant horseweed, and a similar mechanism may be possible in johnsongrass based on the pattern of ^{14}C-glyphosate translocation. Future research is needed to evaluate if target site resistance (insensitivity and/or over expression of EPSPS) complements the non-target site resistance.

This research confirms the first documented glyphosate-resistant johnsongrass biotype in the United States. To ensure the long-term sustainability of glyphosate for the control of johnsongrass, resistance management strategies must be implemented at the grower level at once. As a result of finding this glyphosate-resistant johnsongrass biotype in eastern Arkansas, efforts are currently underway to determine the extent of spread of the resistant biotype from the infested field, as well as evaluation of progeny from johnsongrass samples collected from fields in the fall throughout eastern Arkansas.

Sources of Materials

[1] Professional growing mix, LC1 Mix. Sun Gro Horticulture Distribution Inc., 15831 N.E. 8th St., Suite 100, Bellevue, WA 98008.

[2] Miracle-Gro® Water Soluble All Purpose Plant Food, Scotts Miracle-Gro Products, Inc., P.O. Box 606, Marysville, OH 43040.

[3] PROC PROBIT, Statistical Analysis Systems, version 9.1, SAS Institute Inc., SAS Campus Drive, Cary, NC 27513.

[4] Formulated glyphosate, RoundupPowerMax™, 48.7% glyphosate,N-(phosphonomethyl)glycine potassium salt. Monsanto Co., 800 North Lindbergh Blvd., St. Louis, MO 63167.

[5] Glyphosate-(phosphonomethyl-^{14}C), American Radiolabeled Chemicals, Inc., 101 Arc Drive, St. Louis, MO 63178.

[6] Microliter™ syringe. Hamilton Co., 4970 Energy Way, Reno, NV 89502.

[7] Packard Tri-Carb 2100TR Liquid Scintillation Spectrometer, Packard Instrument Co., 220 Warrenville Rd., Downers Grove, IL 60515.

[8] Biological Oxidizer OX500, R.J. Harvey Instrument Corporation, 11 Jane St., Tappan, NY 10983.

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Received October 14, 2010, and approved March 4, 2011.