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Glyphosate Tolerance Mechanism in Italian Ryegrass (*Lolium multiflorum*) from Mississippi

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A threefold glyphosate tolerance was identified in two Italian ryegrass populations, T1 and T2, from Mississippi. Laboratory experiments were conducted to characterize the mechanism of glyphosate tolerance in these populations. The T1 population absorbed less ^{14}C-glyphosate (43% of applied) compared to the susceptible (S) population (59% of applied) at 48 h after treatment (HAT). The T2 population absorbed ^{14}C-glyphosate at levels (56% of applied at 48 HAT) that were similar to both T1 and S populations, but tended to be more comparable to the S population. The amount of ^{14}C-glyphosate that remained in the treated leaf was significantly higher in both T1 (67% of absorbed) and T2 (65% of absorbed) populations compared to the S population (45% of absorbed) at 48 HAT. The amount of ^{14}C-glyphosate that moved out of treated leaf to shoot and root was lower in both T1 (25% of absorbed in shoot and 9% of absorbed in root) and T2 (25% of absorbed in shoot and 11% of absorbed in root) populations compared to the S population (40% of absorbed in shoot and 16% of absorbed in root) at 48 HAT. There were no differences in epicuticular wax mass among the three populations. Treating a single leaf with glyphosate solution at the field use rate (0.84 kg ae ha^{-1}) as 10 1-µl droplets killed the S plant but not the T1 and T2 plants (33 and 55% shoot fresh-weight reduction, respectively). Shikimic acid accumulated rapidly at higher levels in glyphosate-treated leaf segments of the S population compared to the T1 population up to 100 µM glyphosate. However, above 500 µM glyphosate, the levels of shikimate were similar in both the S and T1 populations. Furthermore, shikimic acid content was three- to sixfold more in whole plants of the S population treated with 0.22 kg ae ha^{-1} glyphosate compared to the T1 and T2 populations. No degradation of glyphosate to aminomethylphosphonic acid was detected among the tolerant and susceptible populations. These results indicate that tolerance to glyphosate in the T1 population is partly due to reduced absorption and translocation of glyphosate and in the T2 population it is partly due to reduced translocation of glyphosate.

Nomenclature: Glyphosate; Italian ryegrass, *Lolium multiflorum* Lam.

Key words: Absorption, glyphosate resistance, herbicide resistance, metabolism, shikimate, translocation, weed resistance.

Glyphosate is a nonselective, broad-spectrum, systemic, POST herbicide that has been used extensively throughout the world over the past three decades. It inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (EC 2.5.1.19), thus inhibiting biosynthesis of aromatic amino acids. This leads to several metabolic disturbances, including the inhibition of protein and secondary product biosynthesis (Franz et al. 1997) and the deregulation of the shikimate pathway, leading to general metabolic disruption (Duke et al. 2003). Since its commercialization in 1974, glyphosate has been used extensively in both crop and noncrop lands. Because of its lack of selectivity, glyphosate use was initially limited to preplant, postdirected, and postharvest applications for weed control. With the introduction of glyphosate-resistant (GR) crops in the mid-1990s, glyphosate is now widely used for weed control in GR crops without concern for crop injury. GR crops are currently grown in several countries, with particularly strong adoption in North America, Argentina, and Brazil.

The widespread adoption of GR crops has not only caused weed species shifts in these crops, but it has also resulted in evolution of GR weed biotypes. To date, 13 weed species are reported to be resistant to glyphosate (Heap 2007). They are rigid ryegrass (*Lolium rigidum* L.) in Australia (Powles et al. 1998; Pratley et al. 1999) and the United States (Simarmata et al. 2003), goosegrass [*Eleusine indica* (L.) Gaertn.] in Malaysia (Lee and Ngim 2000; Tran et al. 1999), horseweed [*Conyza canadensis* (L.) Cronq.] in the United States (Koger et al. 2004; Mueller et al. 2003; VanGessel 2001), Italian ryegrass (*Lolium multiflorum* L.) in Chile (Perez and Kogan 2003) and the United States (Nandula et al. 2007; Perez-Jones et al. 2005), common waterhemp (*Amaranthus rudis* Sauer) in the United States (Heap 2007; Owen and Zelaya 2005), common ragweed (*Ambrosia artemisiifolia* L.) in the United States (Sellers et al. 2005), Palmer amaranth (*Amaranthus palmerii* S. Wats) in the United States (Culpepper et al. 2006), hairy fleabane (*Conyza bonariensis* L.) in South Africa (Heap 2007) and Spain (Urbano et al. 2005), buckhorn plantain (*Plantago lanceolata* L.) in South Africa (Heap 2007), wild poinsettia (*Euphorbia heterophylla* L.) in Brazil (Heap 2007), giant ragweed (*Ambrosia trifida* L.) in the United States (Heap 2007), johnsongrass [*Sorghum halepense* (L.) Pers.] in Argentina (Heap 2007), and junglerice [*Echinochloa colona* (L.) Link] in Australia (Heap 2007).

Among these weeds, the first evidence of evolved resistance to glyphosate was reported in rigid ryegrass (Powles et al. 1998). A rigid ryegrass population from an orchard in Australia (Orange, New South Wales), exposed to two to three glyphosate applications per year for 15 yr, exhibited a seven- to 11-fold greater resistance compared to a susceptible population. In a second report on glyphosate-resistant rigid ryegrass from Australia, a ryegrass biotype selected from a population (Echuca, Northern Victoria) exposed to glyphosate applications for 15 yr was nearly 10-fold more resistant compared to a susceptible biotype (Pratley et al. 1999). Italian ryegrass populations from a fruit orchard in Chile exposed to three glyphosate applications annually for 8 to 10 yr, evolved two- to fourfold greater resistance compared to susceptible population (Perez and Kogan, 2003). An Italian ryegrass

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population selected from an orchard in Oregon, where glyphosate effectively controlled weeds during the previous 15 yr, exhibited a fivefold greater tolerance to glyphosate compared to a susceptible population (Perez-Jones et al. 2005). This population was previously reported to survive glyphosate at 1.68 kg ae ha^{-1} (Perez-Jones et al. 2004).

We have previously reported a threefold glyphosate tolerance in two Italian ryegrass populations from Mississippi (Nandula et al. 2007). In this research, we examined the role of absorption and translocation of glyphosate in the mechanism of glyphosate tolerance in these populations. The specific objectives of the research were to (1) determine absorption, translocation, and metabolism patterns of glyphosate in two T and one S Italian ryegrass populations, (2) compare epicuticular wax content for T and S populations, (3) determine efficacy of single-leaf–treated glyphosate on the whole plant control, and (4) compare shikimic acid accumulation patterns in response to glyphosate application in T and S populations.

Materials and Methods

Plant Material and Growing Conditions. Two glyphosate-tolerant Italian ryegrass populations, Tribbett and Fratesi, hereafter referred to as T1 and T2, respectively, and Elizabeth, an S population, were included in this research. Seed selection, storage, germination, transplanting, fertilization, and irrigation procedures used were as described by Nandula et al. (2007). Transplanted Italian ryegrass plants (5 to 8 cm tall) of the T1 and T2 populations were treated with a commercial formulation of glyphosate (potassium salt)[1] at 0.22 kg ae ha^{-1} with a moving-nozzle sprayer equipped with 8002E nozzles[2] delivering 190 L ha^{-1} at 280 kPa to eliminate susceptible plants. Similar conditions were used in the glyphosate metabolism experiment. Plants were grown in a greenhouse 25/20 C (± 3 C) day/night temperature under natural light. For ^{14}C-absorption and translocation experiments, plants were transferred from the greenhouse to a growth chamber 2 d prior to ^{14}C-glyphosate application for acclimatization. The growth chamber was maintained at 25/20 C with a 13-h photoperiod (300 μmol m^{-2} s^{-1}) provided by fluorescent and incandescent bulbs. Plants were left in the growth chamber until harvest.

^{14}C-glyphosate Absorption and Translocation. ^{14}C-glyphosate (^{14}C-methyl labeled with 2.0 GBq $mmol^{-1}$ specific activity, 99.5% radiochemical purity in an aqueous stock solution of 7.4 MBq ml^{-1}) was mixed with commercial potassium salt formulation of glyphosate to obtain a final concentration of 0.84 kg ae ha^{-1} (1× field rate) in 190 L of water. Each plant received approximately 5.0 kBq of ^{14}C-glyphosate in a total volume of 10 μl. Treatment solutions were applied with a microsyringe[3] to the adaxial surface of the third fully expanded leaf blade of 10- to 15-cm-tall (four leaves, two to three tillers) Italian ryegrass plants as 10 1-μl droplets. Plants were not sprayed with commercial glyphosate prior to application of ^{14}C-glyphosate to minimize stress during the exposure period.

Plants were harvested at 24 and 48 HAT and divided into treated leaf, shoot (including tillers), and roots. Treated leaf was immersed in 10 ml 10% methanol in a glass vial and gently shaken for 20 s to remove nonabsorbed ^{14}C-glyphosate remaining on the leaf surface. The leaf wash repeated with additional 10 ml of 10% methanol. Two 1-ml aliquots of each leaf wash were mixed with 10 ml scintillation cocktail.[4] The plant parts were wrapped in a single layer of tissue paper,[5] placed in a glass vial, and oven dried at 60 C for 48 h. Oven-dried plant samples were combusted in a biological oxidizer[6] and the evolved $^{14}CO_2$ was trapped in 10 ml Carbosorb E[7] and 12 ml Permaflour E+.[8] Radioactivity from leaf washes and oxidations was quantified using liquid scintillation spectrometry.[9] The sum of ^{14}C present in the two leaf washes and oxidized plant parts per plant (a single replication) represented 98% recovery of ^{14}C-glyphosate. The experiment had five replications per treatment.

Wax Extraction. Twenty-five fully expanded leaves from greenhouse-grown Italian ryegrass plants (75 d old, 30 to 40 cm tall, five to six leaves, four to eight tillers) were chosen for wax extraction. Total fresh weight and leaf area of selected leaves was recorded. Epicuticular wax was extracted using the procedure described previously (Chachalis et al. 2001; Koger and Reddy 2005). Wax was extracted by immersing leaves in 400 ml high pressure liquid chromatography (HPLC)-grade chloroform in a glass beaker at room temperature for 20 s in a sonicator. The chloroform–wax solution was filtered into a preweighed beaker. Chloroform was evaporated to dryness under a fume hood for 96 h. Wax mass was expressed as wax mass per unit leaf area and wax mass per unit leaf fresh weight. Three replicates of each population—T1, T2, and S—were analyzed.

Efficacy of Leaf-Treated Glyphosate on Whole Plant. Italian ryegrass plants with four leaves, two to three tillers, and that were 10 to 15 cm tall were used in the study. Treatment solution was prepared using a commercial potassium salt formulation of glyphosate at 0.84 kg ae ha^{-1} (1× field rate) in 190 L of water. Ten microliters of glyphosate solution was placed on the adaxial surface of a third fully expanded leaf as 10 droplets. Plants were harvested 3 wk after treatment and weight of green shoot biomass was recorded and expressed as percentage of shoot fresh-weight reduction compared with respective nontreated plants. There were three replications per treatment.

Shikimic Acid Bioassay with Leaf Segments. Shikimic acid assay was conducted following protocols described previously (Perez-Jones et al. 2005; Singh and Shaner 1998). Italian ryegrass plants from populations T1 and S were used. A fully expanded third leaf was excised from 10- to 15-cm-tall (four leaves, two to three tillers) plants and the lowest 2.5-cm portion of the leaf divided into 0.5-cm leaf segments. A single leaf segment per well was placed in 96-well plates holding 100 μl of glyphosate treatment solutions (0, 0.9, 1.9, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, and 1,000 μM) prepared with 10 mM $NH_4H_2PO_4$ (pH 4.4). The leaf segments were left in the wells for 16 h under laboratory fluorescent lights at 25 C. The reaction plates were then frozen at −20 C and defrosted at 25 C causing rupture of the leaf tissue. Thereafter, 25 μl of 0.25 N HCl was added to each well and plates were placed at 60 C for 20 min, which caused complete tissue digestion. Twenty-five microliters of well contents were added to a different plate containing 100 μl of 0.25% periodic acid/0.25% sodium(meta)periodate solution.

The plate was incubated for 1 h at 25 C to facilitate oxidation of shikimic acid. Then 100 µl of 0.6 N NaOH/0.22 M Na_2SO_3 solution was added to the samples and optical density at 380 nm was measured.[10] Shikimic acid content was expressed in µg g^{-1} fresh weight determined with a standard curve generated from known concentrations of shikimic acid. There were four replications per treatment.

Shikimic Acid Bioassay with Whole Plants. Italian ryegrass plants (four leaves, two to three tillers, and 10 to 15 cm tall) from all populations were treated with a commercial potassium salt formulation of glyphosate at 0.22 kg ae ha^{-1} in 190 L of water. Aboveground shoot biomass was harvested 1 wk after treatment, washed with running water, rinsed with distilled water to remove glyphosate–potassium remaining on the leaf surface, blotted dry with paper towels, and air dried. There were five replications per treatment with eight plants per replication. Shikimic acid values represent average of duplicate samples per replication.

Powdered leaf sample (0.5 g) placed in an extraction cell with 12.5 ml of deionized water (pH 2, adjusted with HCl) was vortexed for 2 min, then extracted using MARS Xpress* Ultra-High Throughput Microwave Digestion System[11] for 20 sec at 100 watts. The sample was filtered through a 0.45-µm filter, and a 100-µl aliquot was analyzed for shikimic acid by HPLC[12] using a 250 × 4.6-mm, 5-µm Gemini C_{18} column[13]. The mobile phase consisted of 98% H_2O (pH 3.0) and 2% MeOH in H_2O-eluted isometrically with a flow rate 1 ml per min. The sample injection volume was 10 µl. Shikimic acid, with a retention time of 4.5 min, was monitored at 212 nm. The limit of quantitation for aminomethylphosphonic acid (AMPA) is 3.7 pg on the column. There were five replications per treatment with eight plants per replication. Shikimic acid values represent average of duplicate samples per replication.

Glyphosate Metabolism. Plant growth stage, herbicide treatment, and shoot harvesting conditions were similar to those described in the 'Shikimic Acid with Whole Plants' section. AMPA extraction and derivatization were performed according to published procedures (Reddy et al. 2004). AMPA was derivatized with 2,2,3,3,4,4,4-heptafluoro-1-butanol and trifluoroacetic anhydride (1:2 v/v mixture) and analyzed by gas chromatography–mass spectrometry. There were five replications per treatment with eight plants per replication. AMPA values represent average of duplicate samples per replication.

Statistical Analysis. All experiments were conducted using a completely randomized design and repeated, except the shikimic acid assay with whole plants and the glyphosate metabolism experiment, both of which were performed once. All data were analyzed by ANOVA. No significant experiment effect was observed in all repeated experiments, except the shikimic acid assay with leaf segments; therefore, data from experiments were pooled. Treatment means were separated using Fisher's Protected LSD test at P = 0.05. Data from the shikimic acid assay with leaf segments are presented separately for each trial of the experiment. Nonlinear regression analysis determined the effect of glyphosate concentration on enzyme activity, based on shikimic acid formation. A sigmoidal log-logistic model was used to relate shikimic acid levels (y) to herbicide concentration (x).

$$y = c + \langle d - c/1 + \exp\{b[\log(x) - \log(e)]\}\rangle \quad [1]$$

In this equation, the parameter e is also denoted I_{50} and it is herbicide concentration producing a response halfway between the upper limit, d, and lower limit, c. The parameter b is the relative slope of the curve around e. The regression parameters for the above equation were computed using R software (Ritz and Streibig 2005).

Table 1. Absorption of ^{14}C-glyphosate in three Italian ryegrass populations.

Population	^{14}C-glyphosate absorption	
	24 HAT[a]	48 HAT
	% of applied[b]	
T1	43 a	43 a
T2	52 ab	56 ab
S	56 b	59 b

[a] Abbreviations: HAT, h after treatment; S, susceptible; T, tolerant.
[b] Means within the column followed by the same letters are not different (P = 0.05) according to Fisher's Protected LSD test.

Results and Discussion

^{14}C-glyphosate Absorption and Translocation. There were differences in the extent of ^{14}C-glyphosate absorption among the three Italian ryegrass populations (Table 1). The T1 population absorbed less ^{14}C-glyphosate (43 and 43% of applied) compared to the S population (56 and 59% of applied) at 24 and 48 HAT, respectively. The T2 population absorbed ^{14}C-glyphosate at levels (52 and 56% of applied at 24 and 48 HAT, respectively) that were similar to both T1 and S populations, but tended to be more comparable to the S population. In contrast to these results, other researchers have observed no differences in uptake of glyphosate between resistant and susceptible populations of rigid ryegrass (Feng et al. 1999; Simarmata et al. 2003) and Italian ryegrass (Perez et al. 2004).

The quantity of ^{14}C-glyphosate that remained in the treated leaf at 48 HAT was higher in both T1 (67% of absorbed) and T2 (65% of absorbed) populations compared to the S population (45% of absorbed) (Table 2). In other words, less ^{14}C-glyphosate was moved out of the treated leaf of the T1 (33% of absorbed) and T2 (35% of absorbed) populations compared to the S population (55% of absorbed). Furthermore, the amount of ^{14}C-glyphosate that accumulated in the shoot and root was lower in both T1 (15 to 25% of absorbed in shoot and 6 to 9% of absorbed in root) and T2 (18 to 25% of absorbed in shoot and 6 to 11% of absorbed in root) populations at 24 and 48 HAT compared to the S population (22 to 40% of absorbed in shoot and 10 to 16% of absorbed in root). As with absorption data, these results are different from those reported earlier for rigid ryegrass biotypes (Feng et al. 1999; Simarmata et al. 2003) and Italian ryegrass populations (Perez et al. 2004) where there were no differences between resistant and susceptible plants in distribution patterns of ^{14}C-glyphosate. The higher accumulation of ^{14}C-glyphosate in roots of the S population compared to the T1 and T2 populations is in agreement with earlier reports of preferential accumulation of glyphosate in the roots of susceptible rigid ryegrass plants, whereas more glyphosate accumulated in the leaf tips of resistant plants (Lorraine-Colwill et al. 2003). This differential glyphosate buildup was concluded to be due to an alteration to the cellular transport of glyphosate conferring resistance.

Table 2. Distribution of ^{14}C-glyphosate in three Italian ryegrass populations.[a]

	^{14}C-glyphosate distribution					
	Treated leaf		Shoot		Root	
Population	24 HAT	48 HAT	24 HAT	48 HAT	24 HAT	48 HAT
	% of absorbed[b]					
T1	79 a	67 a	15 a	25 a	6 a	9 a
T2	76 a	65 a	18 ab	25 a	6 a	11 a
S	68 b	45 b	22 b	40 b	10 b	16 b

[a] Abbreviations: HAT, h after treatment; S, susceptible; T, tolerant.
[b] Means within the column followed by the same letters are not different (P = 0.05) according to Fisher's Protected LSD test.

Leaf Wax Mass. We hypothesized that the differences in ^{14}C-glyphosate absorption and translocation between the tolerant (T1 and T2) and susceptible (S) Italian ryegrass populations are related to differences in leaf epicuticular wax content. There were no differences in epicuticular wax mass among the three Italian ryegrass populations (Table 3). The amount of wax ranged between 36 to 40 μg cm^{-2} leaf area and 1,314 to 1,413 μg g^{-1} leaf fresh weight in three populations, which was similar to levels reported for other weed species. Koger and Reddy (2005) reported 67 to 80 μg wax cm^{-2} leaf area in glyphosate-resistant and -susceptible biotypes of horseweed from Mississippi and Chachalis et al. (2001) measured 14 to 57 μg cm^{-2} leaf area in ivyleaf morningglory [*Ipomoea hederacea* (L.) Jacq.] and smallflower morningglory [*Jacquemontia tamnifolia* (L.) Griseb.]. The differences in ^{14}C-glyphosate absorption in the three populations in this study, despite similar leaf wax content may be due to differences in the composition of the epicuticular wax and not total mass per se.

Efficacy of Leaf-Treated Glyphosate on Whole Plant. Treating a single leaf with the 1× glyphosate rate, 0.84 kg ha^{-1}, as 10 1-μl droplets resulted in the death of S plants at 3 wk after treatment. In contrast to S plants, the T1 and T2 plants survived with 33 and 55% reduction in shoot fresh weight, respectively (Table 4). The T1 plant recovered better compared to the T2 plant, with the latter exhibiting a higher degree of chlorosis (Figure 1). These results corroborate diminished translocation of ^{14}C-glyphosate in T1 and T2 populations compared to the S population (Table 2). Similar results were reported for horseweed by Koger and Reddy (2005). In horseweed, treating two leaves (10 μl as several droplets on each leaf) with 1× glyphosate solution resulted in 100% control of susceptible biotypes and 38 to 58% control of resistant biotypes (Koger and Reddy 2005).

Shikimic Acid Assay with Leaf Segments. Shikimic acid accumulated at higher levels in glyphosate-treated leaf segments of the S population compared to the T1 population

Table 3. Epicuticular wax mass of leaves of three Italian ryegrass populations.[a]

	Epicuticular wax mass	
Population	μg cm^{-2} leaf area[c]	μg g^{-1} leaf fresh weight[c]
T1[b]	38 a	1,413 a
T2	40 a	1,314 a
S	36 a	1,317 a

[a] Plants were 75 d old (30 to 40 cm tall, five to six leaves, four to eight tillers).
[b] Abbreviations: S, susceptible; T, tolerant.
[c] Means within the column followed by the same letters are not different (P = 0.05) according to Fisher's Protected LSD test.

Table 4. Efficacy of glyphosate on single-leaf–treated Italian ryegrass populations.[a]

Population	Shoot fresh weight reduction
	%[c]
T1[b]	33 a
T2	55 b
S	100 c

[a] Plants (10 to 15 cm tall, four leaves, two to three tillers) were treated with 10 μl of a solution containing a commercial formulation of glyphosate (potassium salt) at a concentration of 0.84 kg ae ha^{-1} in 190 L of water. Ten 1-μl droplets of treatment solution were applied with a microsyringe on the adaxial surface of the third fully expanded leaf blade.
[b] Abbreviations: S, susceptible; T, tolerant.
[c] Means within the column followed by the same letters are not different (P = 0.05) according to Fisher's Protected LSD test.

(Figure 2). However, levels of shikimate were similar in the S and T1 populations at 500 and 1,000 μM glyphosate. Similar results were reported by Perez-Jones et al. (2005) in glyphosate-resistant and susceptible Italian ryegrass biotypes from Oregon. The glyphosate I_{50} values for the S population were 12.3 and 19.6 μM compared to 112.6 and 87.1 μM in T1 population in trials 1 and 2, respectively (Table 5).

Shikimic Acid Assay with Whole Plants. There were no differences in shikimic acid levels between nontreated Italian ryegrass plants of the three populations (Table 6). However, glyphosate-treated plants from the S population accumulated more shikimic acid (3,886 μg g^{-1} shoot tissue) than those from the T1 (1,356 μg g^{-1} shoot tissue) and T2 (630 μg g^{-1} shoot tissue) populations (Table 6). Similar results of shikimic acid accumulation at higher levels in susceptible biotypes compared to their resistant biotypes have been reported in whole plants of Italian ryegrass (Perez-Jones et al. 2005), ryegrass (Simarmata et al. 2003), and goosegrass (Tran et al. 1999).

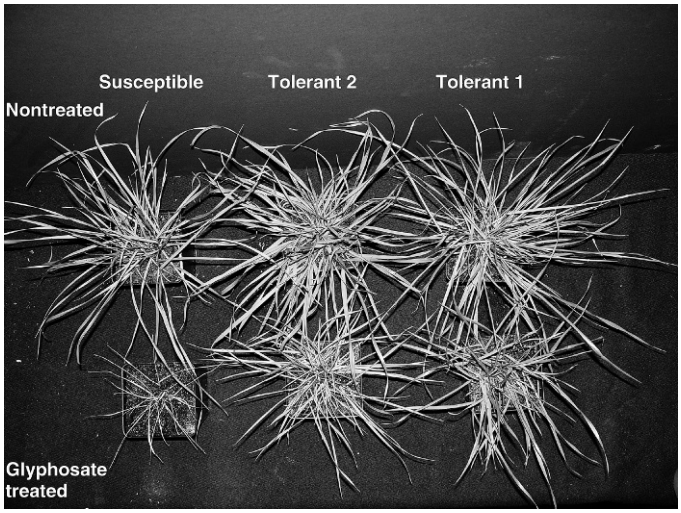

Figure 1. Glyphosate-susceptible (S, left column) and glyphosate-tolerant (T2, middle column and T1, right column) Italian ryegrass populations from Mississippi. Upper and bottom rows represent nontreated and treated plants, respectively. A solution containing a commercial formulation of glyphosate (potassium salt) at a concentration of 0.84 kg ae ha^{-1} in 190 L of water was applied with a microsyringe to the adaxial surface of the third fully expanded leaf blade of 10- to 15-cm-tall (four leaves, two to three tillers) Italian ryegrass plants as 10 1-μl droplets. Three weeks after treatment, the S plant was killed, and the T1 and T2 plants survived with some growth reduction from glyphosate. A color version of this figure is available in the online journal.

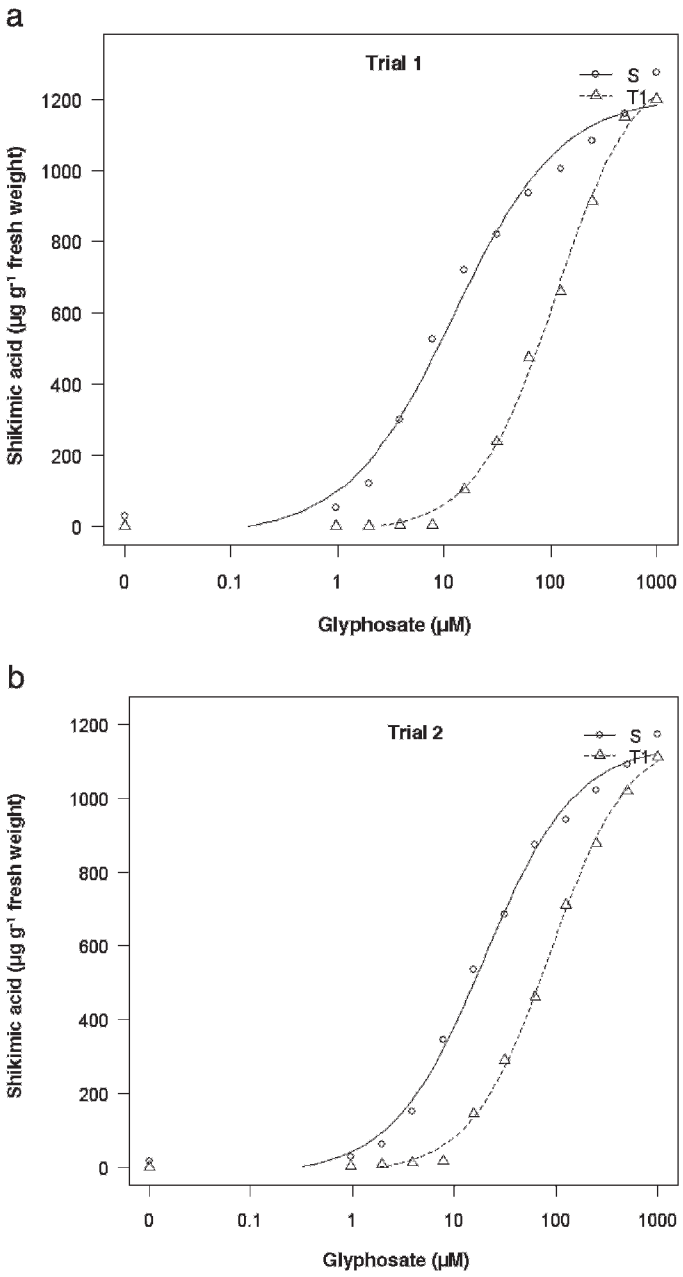

Figure 2. Levels of shikimic acid in 0.5-cm leaf segments of glyphosate-tolerant (T1) and glyphosate-susceptible (S) Italian ryegrass populations after treatment with glyphosate in trial 1 and trial 2. Mean values are plotted.

Table 5. I_{50} and sigmoidal model parameter estimates based on shikimic acid accumulation in 0.5-cm leaf segments of two Italian ryegrass populations after treatment with glyphosate.[a]

	Population			
	Trial 1		Trial 2	
Model parameters[b]	S[b]	T1[b]	S	T1
b	−0.87	−1.14	−0.96	−1.12
c	−24.37	−16.89	−19.83	−14.47
d	1,209.71	1,316.79	1,147.12	1,173.02
e/I_{50}[b] (µM)	12.31	112.61	19.56	87.05

[a] Abbreviations: I_{50}, herbicide concentration producing a response half-way between the upper limit, *d*, and lower limit, *c*; S, susceptible; T, tolerant.

[b] Model parameter estimates are for sigmoidal log-logistic model described in the text.

Table 6. Accumulation of shikimic acid in glyphosate-treated Italian ryegrass populations.[a]

	Shikimic acid	
Population	Nontreated	Glyphosate treated
	µg g^{-1} shoot tissue[c]	
T1[b]	308 a	1,356 a
T2	467 a	630 a
S	742 a	3,886 b

[a] Plants (10 to 15 cm tall, four leaves, two to three tillers) were treated with a commercial formulation of glyphosate (potassium salt) at 0.22 kg ae ha^{-1} in 190 L of water. Aboveground shoot biomass was harvested and analyzed 1 wk after treatment.

[b] Abbreviations: S, susceptible; T, tolerant.

[c] Means within the column followed by the same letters are not different (P = 0.05) according to Fisher's Protected LSD test.

Glyphosate Metabolism. No degradation of glyphosate to AMPA was detected in these tolerant and susceptible Italian ryegrass populations (data not shown). Previously, ^{14}C-glyphosate metabolism comparisons between the resistant and susceptible rigid ryegrass biotypes revealed no differences (Feng et al. 1999; Lorraine-Colwill et al. 2003).

Taken together, the above results indicate that tolerance to glyphosate in the T1 population is partly due to reduced absorption and translocation of glyphosate compared to the S population. Similarly, reduced translocation of glyphosate has contributed to tolerance of the T2 population. Although no differences were detected in leaf epicuticular mass among the populations, the composition of wax may have a role in reducing glyphosate absorption and translocation in the T1 and T2 populations. Treating one leaf vs. spraying the whole plant with glyphosate provided valuable information on the role of translocation in tolerance to glyphosate in the T1 and the T2 populations. These two populations had similar herbicide dose values required to cause a 50% reduction in plant growth, 0.66 kg ha^{-1} (Nandula et al. 2007). Although glyphosate translocation patterns were similar in the T1 and the T2 populations, apparent differential absorption rate could have resulted in lesser injury to the T1 population compared to the T2 population, with the latter exhibiting enhanced chlorosis (Figure 1). If the mechanism of glyphosate resistance is due to differential translocation, similar levels of shikimic acid accumulation at high glyphosate rates in both glyphosate-resistant and glyphosate-susceptible plants can be expected (D. Shaner, personal communication). Furthermore, differential accumulation of shikimic acid in susceptible and resistant plants exposed to low glyphosate concentrations is expected. Results from the shikimic acid assays seen in this and earlier studies (Perez-Jones et al. 2005) confirm the above observation. No AMPA, a metabolite of glyphosate, was detected in these tolerant and susceptible populations.

In previous studies, reduced glyphosate absorption and/or translocation had not been detected in resistant biotypes of rigid ryegrass (Feng et al. 1999; Simarmata et al. 2003) and Italian ryegrass (Perez et al. 2004). However, Lorraine-Colwill et al. (2003) demonstrated accumulation of glyphosate in the roots of susceptible rigid ryegrass plants, whereas more glyphosate accumulated in the leaf tips of resistant plants. In addition, no differences in glyphosate metabolism existed between the tolerant and susceptible populations as reported earlier (Feng et al. 1999; Lorraine-Colwill et al. 2003). Perez-Jones et al. (2005) did not detect any amino acid changes at the *epsps* gene, which encodes for EPSPS, in a resistant Italian

ryegrass biotype. Further, Baerson et al. (2002a) did not attribute a threefold *epsps* gene amplification in a resistant rigid ryegrass biotype to resistance. On the other hand, an amino acid change at position 106 from Pro to Ser in the *epsps* gene conferring glyphosate resistance was reported in goosegrass (Baerson et al. 2002b). Additionally, a second substitution at position 106 leading to Thr from Pro was identified in the resistant goosegrass biotype (Ng et al. 2003), which was also reported in rigid ryegrass (Wakelin and Preston 2006). The role of potential changes in the *epsps* gene in providing tolerance to glyphosate in the T1 and T2 populations warrants further investigation.

Sources of Materials

[1] Potassium salt of glyphosate, Roundup WEATHERMAX®, Monsanto Company, 800 North Lindbergh Boulevard, St. Louis, MO 63167.

[2] Teejet 8002E nozzle, Spraying Systems Company, P.O. Box 7900, Wheaton, IL 60189.

[3] Hamilton syringe, Hamilton Company, 4990 Energy Way, Reno, NV 89520.

[4] EcoLume, ICN, 3300 Hyland Avenue, Costa Mesa, CA 92626.

[5] Kimwipes EX-L, Kimberly-Clark Corporation, 1400 Holcomb Bridge Road, Roswell, GA 30076.

[6] Packard oxidizer 306, Packard Instruments Company, 2200 Warrenville Road, Dowers Grove, IL 60515.

[7] Carbosorb E, Packard BioScience Company, 800 Research Parkway, Meridian, CT 06450.

[8] Permafluor E^+, Packard BioScience Company, 800 Research Parkway, Meridian, CT 06450.

[9] Tri-carb 2500TR liquid scintillation analyzer, Packard BioScience Company, 800 Research Parkway, Dowers Grove, IL 60515.

[10] KC4 software, BioTek Instruments, Inc., 100 Tigan Street, Winooski, VT 05404.

[11] MARS Xpress* ultrahigh throughput microwave digestion system, CEM Corporation, 3100 Smith Farm Road, Matthews, NC 28104.

[12] HPLC, Agilent Technologies, Inc., 5301 Stevens Creek Boulevard, Santa Clara, CA 95051.

[13] Gemini C_{18} column, Phenomenex, 411 Madrid Avenue, Torrance, CA 90501.

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