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*Research Article*

# Physiology of Glyphosate-Resistant and Glyphosate-Susceptible Palmer Amaranth (*Amaranthus palmeri*) Biotypes Collected from North Carolina

**Jared R. Whitaker,[1] James D. Burton,[2] Alan C. York,[3] David L. Jordan,[3] and Aman Chandi[3]**

[1] *Department of Crop and Soil Sciences, University of Georgia, P.O. Box 8112, Statesboro, GA 30460, USA*
[2] *Department of Horticultural Science, North Carolina State University, Box 7609, Raleigh, NC 27695, USA*
[3] *Department of Crop Science, North Carolina State University, Box 7620, Raleigh, NC 27695-7620, USA*

Correspondence should be addressed to David L. Jordan; david_jordan@ncsu.edu

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Glyphosate-resistant (GR) biotypes of Palmer amaranth are now commonly found across the southern United States. Experiments were conducted to characterize physiological differences between a GR biotype and a glyphosate-susceptible (GS) biotype from North Carolina. The GR biotype had an 18-fold level of resistance based upon rates necessary to reduce shoot fresh weight 50%. Shikimate accumulated in both biotypes following glyphosate application, but greater concentrations were found in GS plants. Absorption and translocation of $^{14}$C-glyphosate were studied in both biotypes with and without an overspray with commercial glyphosate potassium salt (840 g ae ha$^{-1}$) immediately prior to $^{14}$C-glyphosate application. Greater absorption was noted 6 h after treatment (HAT) in GS compared with GR plants, but no differences were observed at 12 to 72 HAT. Oversprayed plants absorbed 33 and 61% more $^{14}$C by 48 and 72 HAT, respectively, than plants not oversprayed. $^{14}$C distribution (above treated leaf, below treated leaf, roots) was similar in both biotypes. Together, these results suggest that resistance in this biotype is not due to an altered target enzyme or translocation but may be in part due to the rate of glyphosate absorption. These results also are consistent with resistance being due to increased gene copy number for the target enzyme.

## 1. Introduction

Palmer amaranth is the most troublesome weed for cotton (*Gossypium hirsutum* L.) and soybean (*Glycine max* (L.) Merr.) producers in much of the southern United States [1]. Glyphosate has traditionally been effective in controlling Palmer amaranth [2, 3], and excellent control has been achieved in glyphosate-only systems [4–6]. Growers rapidly adopted GR crop technology for reasons discussed by Culpepper and York [4], and 73, 80, and 93% of the US corn (*Zea mays* L.), cotton, and soybean crops were planted to GR cultivars and hybrids in 2012 [7].

In the late 1990s, weed resistance to glyphosate was considered unlikely because of unique properties of the herbicide, such as its mechanism of action, absence of metabolic degradation in plants, and lack of residual activity in soil [8]. However, with widespread planting of GR crops and extensive reliance on glyphosate, resistant biotypes evolved. Today, resistance to glyphosate has been confirmed in 24 weed species [9]. The first confirmation of resistance to glyphosate in an *Amaranthus* species occurred with Palmer amaranth in Georgia in 2005 [10]. By 2012, GR Palmer amaranth had been confirmed in Alabama, Arizona, Arkansas, California, Delaware, Georgia, Illinois, Kansas, Louisiana, Michigan, Mississippi, Missouri, New Mexico, North Carolina, Ohio, Tennessee, and Virginia [9].

Resistance to glyphosate can be due to both target site and nontarget site mechanisms, and this has been reviewed recently [11, 12]. Target site resistance to glyphosate is due to altered herbicide interaction with the target enzyme, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, E.C.2.5.1.19). This can result from gene-sequence changes that reduce sensitivity of the expressed enzyme [11]. Alternatively, target site resistance to glyphosate can

result from increased enzyme levels due to either transient overexpression of mRNA or an increase in gene copy number [12]. Increased gene copy number was first identified in GR Palmer amaranth from Georgia [13] and later identified as a possible mechanism in GR Palmer amaranth from North Carolina [14] and GR Italian ryegrass (*Lolium perenne* spp. *multiflorum* (Lam.) Husnot) from Arkansas [15].

Nontarget site resistance to glyphosate can include altered absorption, translocation, or metabolism. Resistance to glyphosate due to altered translocation within the plant is reported to be the most common mechanism of resistance, and this mechanism has been documented in several weed species [11, 16]. Recent research demonstrated that the mechanism of altered movement in GR horseweed (*Coynza Canadensis* (L.) Cronq.) and *Lolium* spp. was associated with vacuolar sequestration of glyphosate, resulting in reduced cytoplasmic concentration [17, 18]. Vacuolar sequestration is also associated with restricted glyphosate movement into the phloem and reduced translocation throughout the plant. The authors concluded that there was a tonoplast transporter present in the GR biotypes that moved glyphosate out of the cytoplasm, but this transporter was not present or active in GS biotypes [17, 18].

Glyphosate resistance is often associated with more than one mechanism. Reduced herbicide absorption has been often associated with resistance, but it is rarely the only mechanism. Until recently, glyphosate metabolism had not been detected, and thus metabolism was not thought to be a means of resistance [12]. However, glyphosate metabolism has been suggested but not confirmed as part of the mechanism of resistance [19, 20].

Culpepper et al. [10] suggested that altered absorption and translocation are not associated with glyphosate resistance in a Georgia biotype of Palmer amaranth when compared 48 h after treatment with $^{14}$C label. In susceptible plants, glyphosate competes with the substrate phosphoenolpyruvate for a binding site on EPSPS, resulting in unregulated flow of carbon into the shikimate pathway and a characteristic accumulation of shikimate in sensitive tissues [21]. The Georgia GS Palmer amaranth biotype accumulated shikimate after exposure to glyphosate, compared with no accumulation in the GR biotype [10]. Gaines et al. [22] reported a 60- to 120-fold increase in gene copy number in the Georgia GR biotype, and the increase in gene copy number was correlated with the level of resistance. Resistance levels differ considerably among biotypes of Palmer amaranth collected in North Carolina and Georgia. For example, GR biotypes from Georgia have levels of resistance ranging from 3- to 8-fold higher than susceptible biotypes, whereas GR biotypes from North Carolina range from 3- to 22-fold higher [23].

Comparing absorption and translocation between resistant and susceptible biotypes can be important in defining the possible mechanism of resistance for glyphosate. Additionally, methodology can vary among researchers and can contribute to variation in conclusions used in developing plausible explanations of mechanisms of resistance. Therefore, research was conducted to determine the level of resistance in a North Carolina GR Palmer amaranth biotype, to evaluate possible differences in shikimate accumulation in GR and GS biotypes following glyphosate application, and to compare absorption and translocation of $^{14}$C-glyphosate in the GR biotype and a GS biotype in the presence and absence of an overspray with a commercial formulation of glyphosate.

## 2. Materials and Methods

*2.1. Seed Collection and Growing Conditions.* Experiments were conducted in a greenhouse maintained at 32 ± 5°C with natural lighting supplemented for 14 h by metal halide lamps (Hubbell Lighting, Inc., Greenville, SC) delivering 400 $\mu$mol m$^{-2}$s$^{-1}$ photosynthetic photon flux density. Plants were grown in a commercial potting medium (Metro Mix 300, Sun Gro Horticulture, Bellevue, WA) and were irrigated four times daily with an automated irrigation system to maintain optimum soil moisture. Plants were fertilized with a water soluble fertilizer (Peters Professional Water Soluble 20-20-20 Fertilizer, Scotts Company, Marysville, OH) as needed to maintain good growth. Glyphosate was applied using a track sprayer equipped with a single even-spray flat-fan nozzle (TeeJet TP8002E Even Flat Spray Tips, TeeJet Technologies, Wheaton, IL) delivering 140 L ha$^{-1}$ at 275 kPa. Following herbicide application, plants were returned to the greenhouse where irrigation was withheld for 24 h.

Palmer amaranth seeds were collected from a known GS population near Clayton, NC. Seeds for the GR biotype were collected near Parkton, NC, from plants that survived multiple applications of glyphosate during the 2006 growing season. Seedlings from the Parkton collection were grown in the greenhouse in pots 25 by 28 cm (depth by diameter) and treated when plants were 7 to 10 cm tall with glyphosate potassium salt (Roundup WEATHERMAX herbicide, 540 g ae glyphosate per liter, Monsanto Company, St. Louis, MO) at 1200 g ae ha$^{-1}$. Preliminary research determined that glyphosate at 280 g ha$^{-1}$ was completely effective on the Clayton GS biotype. Greater than 95% of plants from the initial Parkton collection survived glyphosate application in the greenhouse. Plants from the Parkton collection surviving glyphosate application were allowed to cross-pollinate. Flowering was induced by covering 40 to 60 cm tall plants with black plastic for 14 h for five consecutive nights. Male plants were interspersed among female plants, and cross-pollination was facilitated by shaking the inflorescences of male plants daily during pollination. Mature seed were collected, and the process, including glyphosate application, was repeated two additional times to obtain an F4 population which was used in all experiments. Seed were stored at 1°C until use.

*2.2. Glyphosate Dose Response and Shikimate Assay.* Seedlings of both the GR and GS biotypes were grown as previously described in pots 10 by 12 cm (diameter by depth) and were thinned to one seedling per pot. Glyphosate at 0, 50, 100, 500, 1000, and 5000 g ha$^{-1}$ was applied when plants were 7 to 10 cm tall using the track sprayer previously described. Shikimic acid content of the plants was determined 5 DAT (days after treatment), and fresh weight

of live shoot tissue was recorded 21 DAT. The experimental design was a randomized complete block with treatments replicated six times, blocking against plant size, and the experiment was repeated once.

Shikimic acid content was determined using a method modified from Shaner et al. [24]. Two leaf discs (4 mm diameter) per plant were excised from the youngest, fully expanded leaves using a cork borer. Each disc was placed in an individual well of a 96-well microtiter plate that contained 100 $\mu$L of buffer of 10 mM ammonium phosphate (pH 4.4) and 0.1% (v/v) nonionic surfactant (Tween 80 surfactant, Fisher Scientific, Pittsburg, PA). Plates were covered with a lid, sealed with a strip of Parafilm (Pechiney Plastic Packaging Co., Chicago, IL), placed in a plastic freezer bag, and stored at −20°C. For the analysis, plates were thawed at 60°C for 30 min., and then 25 $\mu$L of 1.25 N HCl was pipetted into each well. After incubation at 60°C for 15 min, 25 $\mu$L aliquot was transferred from each well to a separate microtiter plate, 100 $\mu$L of 0.25% (w/v) periodic acid/0.25% (w/v) m-periodate solution was added to each well, and the plate was incubated at 25°C for 90 min. The reaction was terminated by adding 100 $\mu$L of 0.6 N sodium hydroxide/0.22 M sodium sulfite to each well, and the optical density was measured at 380 nm within 30 min using a spectrophotometer ($\mu$Quant Microplate Spectrophotometer, BioTek Instruments, Inc., Winooski, VT). Background optical density was determined from wells containing the control discs (0 glyphosate rate) and was subtracted from each of the glyphosate treatments. A shikimic acid standard curve was developed by adding known amounts of shikimic acid to extracts from control leaf discs, and results are reported as micrograms shikimic acid per milliliter.

*2.3. Absorption and Translocation.* Seed of GR and GS biotypes were planted separately in pots containing a sandy soil with low organic matter. Plants 10 to 14 cm tall with 9 to 11 leaves were selected for the experiment. The experiment was conducted as a randomized complete block design with five treatments replicated six times, blocking against plant size, and the experiment was repeated once. Four treatments had a factorial arrangement based on biotype (GR and GS) and glyphosate overspray (not oversprayed or oversprayed with the previously described commercial formulation of glyphosate potassium salt at 840 g ha$^{-1}$ immediately before $^{14}$C-glyphosate application). Entire plants were oversprayed using the track sprayer described previously. The uppermost fully expanded leaf was then spotted with 10 $\mu$L of $^{14}$C-glyphosate solution using a microapplicator. Technical grade phosphonomethyl-$^{14}$C-glyphosate (Sigma Chemical Co., St. Louis, MO) with 2.035 GBq/mmol specific activity and 99% radiochemical purity was used. The spotting solution contained 330 $\mu$L of $^{14}$C-glyphosate diluted in 920 $\mu$L of deionized water with 0.125% (v/v) nonionic surfactant (Induce, Helena Chemical Company, Collierville, TN). Glyphosate dose from 10 $\mu$L of spotting solution equaled 140 g ha$^{-1}$ based on a 10-cm$^2$ leaf (approximate size of treated leaf) and contained 6.5 kBq of radioactivity.

Plants were harvested at 6, 12, 24, 48, and 72 HAT and divided into four regions: (1) treated leaf, (2) shoot above treated leaf, (3) shoot below treated leaf, and (4) roots. The treated leaf was removed at the point of attachment to the stem. This point of attachment was the basis for division of plant parts. Roots were washed over wire mesh to remove soil. Foliar absorption of glyphosate was determined by washing the treated leaf in 20 mL of 50 : 50 mixture of methanol and deionized water with 0.25% (v/v) nonionic surfactant for 1 min to remove herbicide remaining on the leaf surface. One mL aliquots of the leaf wash were added to 15 mL of scintillation cocktail (ScintiVerse BD cocktail, Scintanalyzed, Fisher Scientific, Fairlawn, NJ), and radioactivity was quantified with liquid scintillation spectrometry (Packard TRI-CARB 2100TR Liquid Scintillation Spectrometer, Packard Instrument Company, Downers Grove, IL) (LSS). Plant parts were dried for 72 h at 45°C, weighed, and combusted with a biological sample oxidizer (Model OX-500 Biological Material Oxidizer, R.J. Harvey Instrument Corp., Hillsdale, NJ). Radioactivity was quantified by LSS. Absorption was expressed as a percentage of total recovered $^{14}$C. Distribution of $^{14}$C in each plant part was expressed as a percentage of absorbed $^{14}$C.

*2.4. Statistical Analysis.* Shoot fresh weights from the dose response experiment were converted to percent reduction relative to the control (0 g ha$^{-1}$ glyphosate). Data for shoot fresh weight reduction and shikimate production were plotted versus $\log_{10}$ herbicide doses to develop a dose response curve using SIGMAPLOT 11.2 (SigmaPlot, version 11.0, Systat Software, Inc., San Jose, CA). An $I_{50}$ value for percent reduction in fresh weight was calculated from the regression equation. Data from the absorption and translocation experiment were subjected to ANOVA with sums of squares partitioned appropriately for a 2 (overspray treatments) by 2 (Palmer amaranth biotypes) by 6 (harvest date) factorial arrangement. Experimental run was considered a random effect. Significant interaction and main effect means were separated with Fisher's Protected LSD test at $P \leq 0.05$.

## 3. Results and Discussion

*3.1. Glyphosate Dose Response.* The $I_{50}$ parameter estimates for percent reduction in fresh weight were 18-fold higher for the GR biotype (2,565 g ha$^{-1}$) compared with the GS biotype (146 g ha$^{-1}$) (Figure 1). This is similar to the 20-fold difference reported by Whitaker [25] from other studies with the same seed. Levels of glyphosate resistance can vary among Palmer amaranth collections. Culpepper et al. [10] reported that GR Palmer amaranth in Georgia had a 6- to 8-fold level of resistance compared with a known GS biotype. In Arkansas, a GR biotype was reported to have between 79- and 115-fold level of resistance [26], while Steckel et al. [27] reported a 1.5- to 5-fold level of glyphosate resistance in Palmer amaranth collected in Tennessee. Although these results suggest that resistance likely evolved independently across geographical regions and that mechanisms of resistance may vary among populations, differences in resistance levels among states

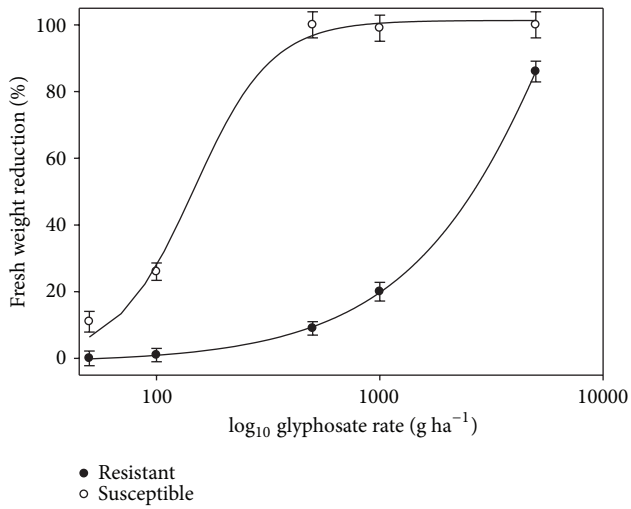


Figure 1: Percent reduction in Palmer amaranth fresh weight of glyphosate-resistant and glyphosate-susceptible biotypes 21 days after glyphosate application. Data are pooled over two experiments. Regression expressions are resistant biotype, $Y = 0.000001x^2 + 0.02x - 1.3$, $r^2 = 0.99$; susceptible biotype, $Y = 101/(1+(x/146)^{-2.5})$, $r^2 = 0.99$.

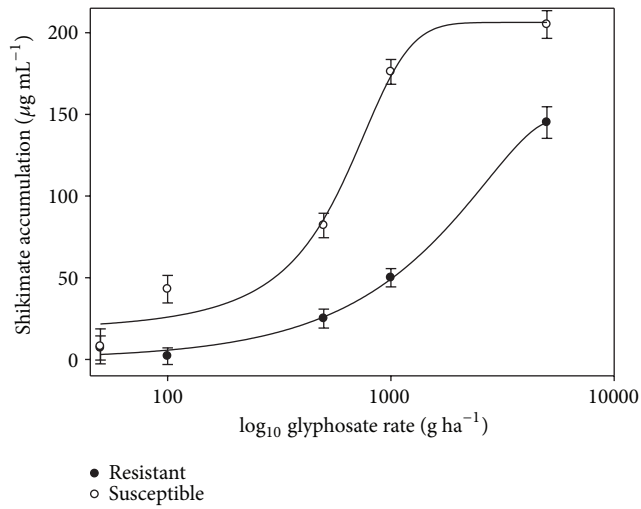


Figure 2: Shikimate accumulation by glyphosate-resistant and glyphosate-susceptible Palmer amaranth 5 d after glyphosate application. Data are pooled over two experiments. Regression expressions are resistant biotype, $Y = 0.000001x^2 + 0.01x - 0.36$, $r^2 = 0.99$; susceptible biotype, $Y = 145/(1 + e^{-((x-1246)/406)})$, $r^2 = 0.99$.

may also reflect differences in methodology. In Arkansas, resistance levels were based upon the amount of glyphosate needed to cause 50% mortality, while in Georgia, North Carolina, and Tennessee they were based on visible control estimates or shoot fresh weight reduction. Other factors, including the sensitivity of the GS biotypes and the methodology regarding GR seed selection, also may have played a role in determining the level of resistance. In the Arkansas study, GS Palmer amaranth seed was collected from a South Carolina field with no history of glyphosate use [26]. Seed of the GS biotype in North Carolina was collected from a field that had been treated with glyphosate at least once per year for several consecutive years, but glyphosate consistently controlled the Palmer amaranth completely in that field.

*3.2. Shikimate Assay.* The shikimate concentration was low ($<10\ \mu g\ mL^{-1}$) and similar in both biotypes treated with glyphosate at $50\ g\ ha^{-1}$ (Figure 2). Shikimate increased in both biotypes as glyphosate concentration increased, but the increase was greater in the GS biotype. At glyphosate rates of $100\ g\ ha^{-1}$ or more, shikimate concentration was always greater in the susceptible biotype. Shikimate accumulation in the GR biotype suggests unaltered EPSPS [28]. Steckel et al. [27] also reported that shikimate accumulated in both GR and GS biotypes of Palmer amaranth collected from Tennessee. Glyphosate resistance in a Georgia GR Palmer amaranth has been attributed to gene amplification and a resulting overexpression of EPSPS [29]. When inheritance of resistance was studied in this Georgia biotype, the F1 progeny had variable levels of shikimate accumulation, which was correlated with variable EPSPS copy number [29]. Chandi et al. [14] reported variable EPSPS copy number when comparing the same GR biotype used in the current study. With an increased EPSPS copy number, one would expect shikimate to accumulate but at a lesser rate than observed in the GS biotype.

*3.3. Absorption and Translocation.* The effect of application rate on glyphosate absorption and translocation has been studied in both GR and GS plants, and the results varied with the mechanism of resistance. In GR horseweed, where resistance was attributed to reduced translocation, the amount of glyphosate translocated in GR plants was less than in GS plants regardless of application rate [30]. When the mechanism of glyphosate resistance is due to an insensitive EPSPS, the translocation pattern is more complicated when the application rate changes [31]. Absorption and translocation were, therefore, observed over time in GR and GS Palmer amaranth biotypes at different application rates.

Approximately 86% of the total applied $^{14}C$-label was recovered from leaf washes and oxidized plant parts. Interactions of Palmer amaranth biotype by harvest time and glyphosate overspray by harvest time were significant. Maximum absorption was observed in the GS and GR biotypes at 6 and 12 HAT, respectively (Table 1). The GS plants absorbed 67% more glyphosate than GR plants 6 HAT. However, absorption by both biotypes was similar at 12 to 72 hours (25 to 35%). Glyphosate absorption in this experiment was similar to that previously observed in Palmer amaranth. Culpepper et al. [10] reported 31 to 36% absorption 48 HAT, while Grey et al. [32] reported 44% absorption 24 HAT. The role of a slower rate of glyphosate absorption in GR versus GS was surprising based on previous research, and the impact of this finding on better understanding of the mechanism or mechanisms of glyphosate resistance in Palmer amaranth is not known.

Table 1: Absorption of $^{14}$C-glyphosate as affected by Palmer amaranth biotype and glyphosate overspray[a].

| Harvest time | Biotype[b] | | Glyphosate overspray[c] | |
|---|---|---|---|---|
| | Resistant | Susceptible | Not oversprayed | Oversprayed |
| h | % | | | |
| 6 | 18$^{c}$ | 30$^{ab}$ | 22$^{d}$ | 25$^{cd}$ |
| 12 | 35$^{a}$ | 30$^{ab}$ | 36$^{ab}$ | 29$^{bcd}$ |
| 24 | 32$^{ab}$ | 25$^{bc}$ | 26$^{cd}$ | 31$^{abc}$ |
| 48 | 32$^{ab}$ | 31$^{ab}$ | 26$^{cd}$ | 36$^{ab}$ |
| 72 | 32$^{ab}$ | 33$^{ab}$ | 25$^{cd}$ | 40$^{a}$ |

[a]Absorption expressed as percentage of total $^{14}$C recovered. Means within the effect of biotype or glyphosate overspray followed by the same letter are not different according to Fisher's Protected LSD at $P \leq 0.05$.
[b]Data pooled over glyphosate overspray options.
[c]Data pooled over glyphosate-resistant and glyphosate-susceptible biotypes. Glyphosate applied at 840 g/ha.

Overspraying plants with glyphosate affected $^{14}$C-glyphosate absorption. Absorption was similar during the first 24 hours regardless of overspray (Table 1). However, plants oversprayed with glyphosate absorbed 38 and 60% more $^{14}$C-glyphosate 48 and 72 HAT, respectively, than plants not oversprayed. This difference in absorption was likely associated with the adjuvant package in the commercial glyphosate formulation used to overspray compared to 0.125% (v/v) nonionic surfactant included in the $^{14}$C-glyphosate spotting solution. Li et al. [33] reported that glyphosate absorption by common waterhemp (*Amaranthus rudis* Sauer) was affected by glyphosate formulation. Differences in absorption and translocation observed when plants were oversprayed versus plants receiving only treatment of a single leaf also may have been associated with changes in physiology caused by glyphosate within the first few days after treatment. The experimental procedure was insufficient to delineate between formulation and adjuvant contributions to $^{14}$C absorption and translocation versus overall changes in physiology caused by glyphosate mode of action.

Main effects and interactions for biotype, glyphosate overspray, and harvest time were not significant for $^{14}$C distribution in the plants. In both biotypes, 40 to 43% of $^{14}$C applied remained in the treated leaf, while 30 to 31, 22, and 5 to 7% were found in shoot tissue above the treated leaf, shoot tissue below the treated leaf, and roots, respectively (data not shown).

## 4. Conclusions

Results of this experiment indicate that glyphosate resistance in the North Carolina Palmer amaranth biotype examined is not due to limited absorption or translocation of glyphosate. Both glyphosate absorption into the treated leaf and translocation to meristematic areas in the GR biotype were similar to or greater than in the GS biotype by 12 HAT. The role of a slower rate of glyphosate absorption in the GR biotype compared with the GS biotype 6 HAT is not known with respect to resistance mechanisms. Shikimate accumulation at higher rates of glyphosate suggests that resistance is not due to an altered EPSPS. Together, these results support a mechanism of increased EPSPS gene copy number, an observation reported by Chandi et al. [14] for this biotype.

## Conflict of Interests

None of the authors has a conflict of interests in terms of the products mentioned in the paper.

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