PHYSIOLOGICAL CHARACTERIZATION OF GYLPHOSATE-RESISTANT PALMER AMARANTH (*AMARANTHUS PALMERI*)

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Abstract

Glyphosate resistance has been confirmed in a population of Palmer amaranth (*Amaranthus palmeri*) in central Georgia. The resistance/susceptible ratio are approximately 27 for this population. Uptake of foliar applied ^{14}C-glyphosate was examined in glyphosate-susceptible and –resistant biotypes. No differences in foliar uptake were observed. Leaf samples of from glyphosate-susceptible and –resistant biotypes were analyzed via atomic absorption spectrometry for differences in calcium concentration that could affect glyphosate activity. No differences were observed between the biotypes. A laboratory bioassay was conducted to determine the inhibition of EPSP via shikimate accumulation in glyphosate-resistant and – susceptible Palmer amaranth. In the glyphosate-susceptible biotype, shikimate accumulated at glyphosate concentrations above 40 µg glyphosate ml^{-1} at the lowest glyphosate concentration exposed (8.4 mg ae L^{-1}). In the glyphosate-resistant biotype, shikimate accumulation was not observed except at the highest glyphosate concentration of 84 mg L^{-1}. Translocation experiments were conducted to determine if glyphosate translocation out of the treated leaf was limited. Significant differences in glyphosate translocation out of the treated leaf were not observed between the glyphosate-susceptible and -resistant biotype. These data indicate that glyphosate-resistance for this Palmer amaranth biotype is based on a difference in the site of action rather than limited translocation.

Introduction

Palmer amaranth is among the three most troublesome weeds in Georgia cotton, peanut (*Arachis hypogaea* L.), and soybean [*Glycine max* (L.) Merr.] (Webster 2005). It is presently the most common *Amaranthus* species in Georgia agronomic crops, which is likely in response to its competitiveness and aggressive growth habit and prolific seed production.

Since commercialization of glyphosate-resistant cotton in 1997, some Georgia growers have produced this cotton in a monoculture system and have relied exclusively on glyphosate applied multiple times each season to manage Palmer amaranth. A cotton grower in Macon County, Georgia was unable to control Palmer amaranth with glyphosate in 2004. The objectives of this research were as follows to identify the mechanism(s) allowing this biotype to tolerate glyphosate at rates known to be lethal to glyphosate-susceptible Palmer amaranth.

Materials and Methods

Mature seeds from a single female Palmer amaranth plant surviving three glyphosate (0.84 kg ha^{-1}) applications were collected at one of the previously described Macon County, Georgia sites in the fall of 2004. The seeds (F1 generation) were hand-cleaned and stored in a refrigerator at 1 C until use. Seeds from a known glyphosate-susceptible population of Palmer amaranth were collected from the University of Georgia Ponder Farm Research Station in Worth County and stored in a similar manner.

^{14}C-Glyphosate Absorption. Plants were taken from the greenhouse experiment described above for analysis and were grown in a greenhouse with supplemental lighting by halide lamps at (400 µE m^{-2} s^{-1}) and temperature of 35/25 C) A commercial formulation of potassium salt of glyphosate at 0.84 kg ha^{-1} was mixed with ^{14}C-glyphosate (^{14}C-2-glycine, specific activity = 7.4 mCi $mmol^{-1}$; 99% purity). Ten 1-µl drops of herbicide solution containing a total of 3.4 kBq were applied uniformly to the upper surface of a mature leaf (2 cm length) when Palmer amaranth was 6 to 10 cm tall. The treated leaf was excised, petiole placed in 15 mL of distilled water in a 20 mL scintillation vial and treated with 3.4 kBq of 14C-glyphosate. After 24 h, the excised leaf was washed with two sequential applications of 1 ml of 70:30 methanol:water (v:v) for 10- 20 seconds. This wash was added to 18 ml of scintillation cocktail. in 2 ml of 70:30 methanol:water (v:v) mixed with 18 ml of scintillation cocktail. The ^{14}C in the leaf wash was quantified by liquid scintillation spectrometry. Absorbed herbicide, expressed as percent of applied, was calculated from the difference between applied ^{14}C and ^{14}C quantified in the leaf wash. Treatments were replicated three times, and the experiment was repeated once.

^{14}C-Glyphosate Translocation. Glyphosate-resistant and -susceptible Palmer amaranth were grown in the greenhouse as described and then moved into a growth chamber with a constant 28 C temperature and 50% relative humidity when they were 10 to 15 cm tall. Growth chamber lighting was provided by fluorescent and incandescent lamps at 450 µE m^{-2} s^{-1}. Plants were allowed to acclimate for 2 d before treatment with glyphosate. The study was a randomized complete block design with treatments arranged as a split plot and replicated five times. Whole plots were biotypes, and sub-plots were plant parts harvested. The study was repeated once.

The second fully expanded Palmer amaranth leaf was covered with polyethylene film before overspraying with potassium salt of glyphosate at 0.84 kg ha^{-1} mixed with deionized water. The film was then removed and the leaf was spotted with a radiolabeled solution. The spotting solution was prepared by mixing the spray solution with ^{14}C-labeled glyphosate (100:1, v:v) Technical grade phosphono-methyl-^{14}C-glyphosate[9] with 10,942 kBq mg^{-1} specific activity and 99% radiochemical purity was used. Five 1-µl droplets of ^{14}C-glyphosate were placed approximately 2 mm away from the center vein, beginning at the leaf's petiole end moving toward the leaf center, on the

adaxial surface of the leaf. Total specific activity applied contained approximately 2 kBq of radioactivity. Plants were returned to the growth chamber immediately after spotting.

Plants were removed from soil 48 h after treatment and sectioned into meristematic treated leaf. Treated leaves were rinsed twice for 15 s with 5 ml of methanol:deionized water (1:1, v:v) to remove non-absorbed ^{14}C-glyphosate (Li et al. 2005). A 1-ml aliquot of the combined rinsates was added to 10 ml of scintillation fluid, and radioactivity was quantified by liquid scintillation spectrometry. The treated leaf was then further divided by dissecting a 3-mm wide zone completely around the outer edge to remove the meristematic tissue. All plant parts were dried for 48 h at 45 C, weighed, and combusted with a biological sample oxidizer. Radioactivity in the oxidized samples was quantified by liquid scintillation spectrometry. The amount of herbicide absorbed by plants was calculated as the total radioactivity recovered from the rinsate and oxidized tissues. Recovery efficiency was greater than 90%.

In Vivo Shikimate Assay. Glyphosate-resistant and -susceptible plants were grown in the greenhouse as previously described. Shikimate was determined according to a modification of the method of Gaitonde and Gordon (1958), Koger et al. (2005), and Shaner et al. (2005). Six leaf discs (3 mm dia.) per plant from the youngest fully expanded leaf of each biotype were excised and placed in a 1-ml solution containing 8.4, 42, or 84.5 mg L^{-1} of potassium salt of glyphosate[2] for 16 h at 25 C under supplemental light (400 µE m^{-2} s^{-1}). Leaf discs were then placed in 0.4 mL of 0.25 N HCl for 60 min after which a 100-µl aliquot was mixed with 0.4 ml of a 0.25% periodic acid with 0.25% metaperiodate solution for 60 min. After the periodic acid/metaperiodate reaction, a 0.4-ml aliquot of 0.6 M sodium hydroxide with 0.22 M sodium sulfite solution was added. Optical density of the solution at 380 nm was determined using a spectrophotometer. A shikimate standard curve was developed by adding known amounts of shikimate to vials containing leaf discs not exposed to glyphosate. Shikimate levels are reported as µg shikimate ml^{-1} HCl solution. Treatments were replicated three times, and the study was repeated three times.

Calcium Analysis. Glyphosate-resistant and -susceptible Palmer amaranth were grown in the greenhouse as described in the absorption experiment and foliage (1 g of leaves from the youngest leaves present) was harvested from plants at the 6- to 8-leaf stage. Three replicates consisting of 1 g dry weight each were used for each biotype. Plant material was analyzed for calcium content as a percent of total dry weight using an atomic adsorption spectrometer[14]. Treatments were replicated three times, and the study was repeated once.

Ploidy Determination*:* Nuclear DNA content of developing leaves of greenhouse-grown glyphsoate-resistant and -susceptible Palmer amaranth was measured by flow cytometry. Samples were prepared following the methods outlined by Morgan et al. (1998). Leaf tissue was chopped at room temperature using a razor blade in 0.5 ml of isolation medium (high-resolution DNA kit solution A, type T: DNA isolation) [16]. The suspension was filtered through a 40-µm filter and mixed with 4- to 5-fold volume of staining solution (high-resolution DNA kit solution B, type T: staining) with DAPI as the

DNA-specific fluorochrome. The nuclear suspension was analyzed on a PAS-III flow cytometer with 100-W high pressure mercury lamp; KG1, BG38, UG1, OG515 filters; TK 560 mirror; and GG 435 as barrier filter. Eleven thousand nuclei per plant sample were analyzed.

Statistical Analysis. All data were subjected to ANOVA using the general linear models of SAS (1999). Within each experiment, data were combined for analysis because there were no run interactions. In laboratory experiments, means were separated by Fisher's Protected LSD test at the 0.05 probability level and the standard error of the mean was calculated. For the shikimate assay, standard error of the means and a linear regression of the resulting shikimate values verses glyphosate concentration were computed for the susceptible biotype. Shikimate was not detectable in the glyphosate-resistant biotype, hence standard error and R^2 values are not reported for this biotype.

In the field and greenhouse experiments, which utilized a series of glyphosate rates, data were subjected to non-linear regression in addition to ANOVA. Visible Palmer amaranth control and fresh weight, expressed as a percent of the non-treated control, were regressed against the $\log_{10}$ of the glyphosate rate (SAS 1999). The intent was to determine if the response could be described by the log-logistic dose-response curve (equation [1]), where C = lower limit, D = upper limit, b= slope, and I_{50} = dose giving 50% response (Seefeldt et al. 1995).

$$y = C + \frac{D - C}{1 + (x / I_{50})^{(b)}} \qquad [1]$$

The log-logistic dose-response curve, commonly referred to as a sigmoid curve, is typical in dose-response studies where the dose (i.e. rate) ranges from no effect to complete death (Seefeldt et al. 1995). Constants generated by SAS® (SAS 1999) allowed the equation to be solved, and the glyphosate rates required to produce 50% visible control and 50% fresh weight reduction were determined. For presentation, parameters were fitted with a sigmoid response curve which had been previously generated.

Results and Discussion

Uptake, Translocation , Absorption and Translocation. No differences in ^{14}C absorption were noted following ^{14}C-glyphosate application to glyphosate-resistant and -susceptible Palmer amaranth in either the absorption or translocation experiment. Resistant and susceptible plants absorbed 36 and 31%, respectively, of the applied ^{14}C 48 h after application in the absorption study. In the translocation study, differences in absorption in resistant and susceptible plants were not observed. Similar results were noted with glyphosate-resistant and -susceptible horseweed (Feng et al. 2004; Koger and Reddy 2005) and rigid ryegrass (Wakelin et al. 2004).

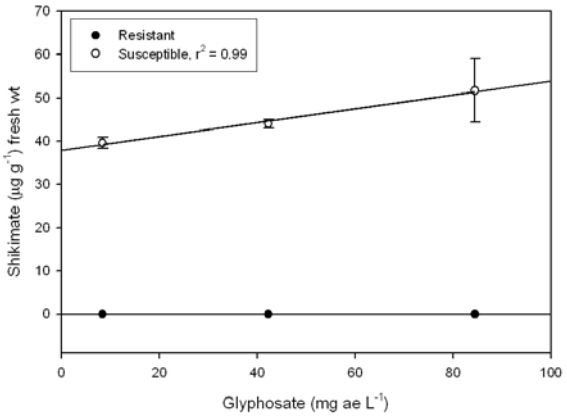
Translocation of ^{14}C out of the treated leaf and distribution of ^{14}C throughout the plant also did not differ between glyphosate-resistant and -susceptible Palmer amaranth biotypes. Forty-two percent and 34% of the applied ^{14}C was translocated out of the treated leaves of resistant and susceptible plants, respectively. These data indicate that

neither reduced absorption nor reduced translocation of herbicide is the basis for resistance.

Ploidy. Higher numbers of chromosomes are often correlated with increased plasticity of a plant in relation to a stress. If glyphosate-resistant Palmer amaranth had higher ploidy levels relative to glyphosate-susceptible Palmer amaranth, then greater chromosome numbers could be related to herbicide resistance. That was not the case in this study as glyphostate-resistant and -susceptible Palmer amaranth had similar ploidy levels.

In Vivo Shikimate Assay. Shikimate was detected in leaf tissue of glyphosate-susceptible Palmer amaranth at the lowest concentration of glyphosate examined (8.4 mg ae L^{-1}), and shikimate concentration increased linearly as glyphosate concentration increased (Figure 1). Shikimate was not detected in leaf tissue of glyphosate-resistant Palmer amaranth regardless of the glyphosate concentration.

Figure 1. Effect of glyphosate concentration on shikimate levels from leaf discs from glyphosate-resistant and susceptible Palmer amaranth biotypes. Error bars indicate standard error of the mean.



Summary

Our results suggest that the glyphosate-resistant Palmer amaranth biotype from central Georgia possesses a different mechanism of resistance than glyphosate-resistant horseweed biotypes that have thus far been described. We observed no differences in glyphosate absorption and translocation between glyphosate-resistant and -susceptible biotypes. This is in contrast to results with horseweed and rigid ryegrass, where limited translocation of glyphosate out of treated leaves was observed with glyphosate-resistant biotypes (Feng et al. 2004; Koger and Reddy 2005; Wakelin et al. 2004).The level of resistance to glyphosate in this Palmer amaranth biotype (6- to 8-fold in whole plants) is less than that often observed in biotypes resistant to other modes of herbicide action. It is, however, similar to that in other species confirmed to be resistant to glyphosate (HRAC 2005). Regardless of the level of resistance, a grower's ability to manage this biotype of Palmer amaranth in the field with glyphosate no longer exists.

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