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Susceptible and Glyphosate-Resistant Palmer Amaranth (*Amaranthus palmeri*) Response to Glyphosate Using C^{14} as a Tracer: Retention, Uptake, and Translocation

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Abstract

The foliar retention, absorption, translocation, and diffusion of glyphosate in glyphosate resistant-(R) and susceptible (S)-Palmer amaranth populations from seed collected in Georgia in 2007 were examined. The R population of Palmer amaranth had an elevated copy number of the EPSPS gene conferring the mechanism of resistance. When applications of ^{14}C-glyphosate to a single leaf followed entire plant treatment with glyphosate, the distribution percentages were similar for R and S for the above and below treated leaves when harvested at 1, 6, 12, 24, and 48 hours after treatment (HAT). There were initially no differences between R and S at 1 HAT with an average of 8% absorption for both biotypes. However, data indicated that glyphosate absorption increased for R-Palmer amaranth reaching 41% within 6 HAT and was significantly different (P = 0.01) from the 28% absorbed by S-Palmer amaranth. Glyphosate resistant and susceptible Palmer amaranth averaged 44% ^{14}C-glyphosate absorption by 24 HAT. There were no differences for ^{14}C-glyphosate Bq/mg of plant tissue between R and S for the above the treated leaf and below the treated leaf portions of plants at 1, 6, 12, 24, or 48 HAT. However, root accumulation of ^{14}C-glyphosate in plant tissue was significantly greater by 12 HAT for the roots of R (1.21 Bq/mg) than for S (0.51 Bq/mg). The treated leaf of the R-Palmer amaranth plants exhibited greater translocation of ^{14}C-glyphosate in Bq/mg of tissue than the susceptible over time, indicating no detrimental effect or cost of fitness due to EPSPS gene amplification. Additionally, there were no differences in glyphosate retention in leaf discs assays between R and S biotypes. In spite of an average of 6.5 Bq efflux out of R and S leaf discs after 15 minute, only 0.4 Bq was retained after 150 minutes. Glyphosate was not retained over time in the leaf discs for R and S, and there

were no biotype differences within bathing times. However, the rate of efflux (the slope of the curves) was greater for the R biotype. These data support the reported gene amplification non-target site glyphosate resistance mechanism in Palmer amaranth.

Keywords

Amaranthus palmeri S. Wats, Absorption, Becquerel's, Glyphosate-Resistance, Herbicide Resistance, Translocation

1. Introduction

The use of glyphosate as a tool for weed control has become a standard practice for large scale glyphosate resistant crop production and vegetation management around the world. But since 1996 the incidence of glyphosate resistant weed species worldwide has gone from a low of zero reported in 1995, to 42 total in 2018 [1]. Many of these weed resistances are associated with the overuse of glyphosate in non-crop areas and glyphosate resistant crops.

The extensive application of glyphosate for glyphosate-resistant crop weed control promoted selection pressure for the occurrence of glyphosate-resistant (R) Palmer amaranth to appear in Georgia in 2004 [2], [3], subsequently occurring throughout the Southern United States [4]. It became the most troublesome weed of cotton in the region by 2009 [5] and 2013 [6]. The glyphosate-resistant Palmer amaranth reported in Georgia possesses a different mechanism of resistance than glyphosate-resistant horseweed and rigid ryegrass biotypes [2]. The mechanism of resistance is novel and attributed to increased copies of the gene required for production of the enzyme 5-enol-pyruvylshikimate-3phosphate synthase (EPSPS), with reports of up to 100 copies occurring in this population of R-Palmer amaranth [7].

As noted by Gaines *et al.* [7], varying herbicide mechanism-of-action and using different agronomic practices such as crop rotation may reduce glyphosate selection intensity. The fitness penalty associated with EPSPS gene amplification could cause the frequency of resistance to decrease for the glyphosate R-Palmer amaranth populations over time. There were no differences for ^{14}C-glyphosate absorption or translocation by the glyphosate-resistant Georgia biotype of Palmer amaranth verses a susceptible biotype. However, absorption and translocation of ^{14}C-glyphosate for this study was measured only at 48 hours after treatment (HAT) [2]. Differential absorption and mobility can vary over time between biotypes. California rigid ryegrass (*Lolium rigidum,* Gaudin) exhibited no differences in absorption or distribution of ^{14}C-glyphosate between susceptible and glyphosate resistant biotypes 1 to 3 days after treatment [8]. However, more glyphosate was present in treated leaves.

In order to establish the differences in absorption and translocation of glyphosate in resistant and susceptible Palmer amaranth at the time of its identifi-

cation, studies were conducted to examine differences between the biotypes. Experiments were conducted with glyphosate resistant and susceptible Palmer amaranth over time to compare glyphosate foliar retention, absorption, translocation *in vivo* and efflux *in vitro* using leaf discs assays.

2. Materials and Methods

Palmer amaranth plants for experiments. Seed from an F2 generation of known glyphosate resistant Palmer amaranth that had been treated with glyphosate were collected at plant maturity. Seed from a known Palmer amaranth susceptible population were also collected. Mature seed were harvested from female plants, cleaned, and then chilled at 1 C for at least 3 weeks before planting in a greenhouse. The greenhouse was maintained at 32˚C ± 5˚C, and natural light was supplemented for 12 hours each day by metal halide lamps (400 μE m^{-2} s^{-1}), with relative humidity ranging from 30% to 70%. Seed of the resistant and susceptible biotypes of Palmer amaranth were planted separately into round pots (15 cm diameter, 15 cm deep) containing commercial potting media. Seedlings were thinned to one plant per pot within 2 days after emergence. Plants were watered by drip irrigation and were fertilized as needed to maintain good growth. Plants were grown in the greenhouse for 7 to 14 days.

^{14}C-glyphosate whole plant absorption and translocation. Glyphosate-resistant (R) and susceptible (S) Palmer amaranth were grown in the greenhouse with the above described conditions. Plants were 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}, with a 12 hour photoperiod. Plants were allowed to acclimate for 2 days before treatment with glyphosate. The study was conducted as 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 in time.

The second fully expanded Palmer amaranth leaf [9] [10] was covered with polyethylene film before over-spraying 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 the radiolabeled solution using a microapplicator. The spotting solution was prepared by mixing 0.5 ml of the spray solution with ^{14}C-labeled glyphosate (100:1, v:v). Technical grade phosphono-methyl-^{14}C-glyphosate with 10,942 kBq mg^{-1} specific activity and 99% radiochemical purity was used. Ten 1-μl droplets of ^{14}C-glyphosate solution were placed on the adaxial leaf surface approximately 2 mm away from the center vein, beginning at the base of the leaf and moving toward the center. Total specific activity applied contained approximately 2 kBq per plant of radioactivity. Plants were returned to the growth chamber immediately after spotting.

Beginning at hour 1, then at 6, 12, 24, and 48 HAT, plants were harvested. Research on common waterhemp [*Amaranthus tuberculatus* (Moq.) J.D. Sauer]

indicated maximum glyphosate absorption at 26 to 50 HAT [10]. Plants were cut at the soil line and sectioned into four parts: treated leaf, tissue above the treated leaf, tissue below the treated leaf, and roots. Soil was removed by washing the roots over a wire grid. Treated leaves were rinsed twice for 15 seconds with 5 ml of methanol:deionized water (1:1, v:v) to remove non-absorbed ^{14}C-glyphosate [10]. A 1-ml aliquot of the combined rinsates was added to 10 ml of scintillation fluid, and radioactivity was quantified by liquid scintillation spectrometry (LSC). All plant parts were dried for 48 hours at 45˚C, weighed, and combusted with a biological sample oxidizer to recover absorbed ^{14}C-glyphosate as CO_2. Radioactivity in the oxidized samples was quantified by LSC. The amount of herbicide absorbed was calculated as the total radioactivity recovered from oxidation of the four plant parts and expressed as a percent of the total radioactivity applied. Distribution of ^{14}C-glyphosate in various plant parts was expressed as the percentage of total absorbed radioactivity, or as Bq mg^{-1} of tissue dry weight. Recovery of $^{14}CO_2$ was 77 to 99% (**Table 1**).

^{14}C-glyphosate uptake and efflux by leaf discs. A leaf discs experiment was conducted to examine the efflux of glyphosate uptake by R and S Palmer amaranth, similar to Chase *et al*. [11]. All plants were grown in the same fashion as described in the absorption and translocation studies.

Leaf disc 14*C-glyphosate loading*. The uptake buffer solution consisted of 5 mM monobasic potassium phosphate (KH_2PO_4), pH 5.44, 1.5% surfactant (Berol 907), and 2.2 *u*M (0.11 *u*Ci) ^{14}C-glyphosate. For the experiment, 1.1 ml of the ^{14}C-glyphosate uptake buffer was added to a beveled-edge watch-glass. Seven-millimeter wide leaf discs were used in uptake and efflux experiments. The discs were cut from fully expanded leaves using cork-borers, taking care to avoid the midrib and main veins. The discs were rinsed three times with distilled water to clean the surfaces and to remove debris from the cut edge. Discs were then allowed to float on distilled water until needed. The specific leaf disc weight was determined on 10 sets of three discs for each biotype prior to use in the uptake experiments. Leaf discs were blotted dry on filter paper, weight taken, and specific leaf weight was expressed as g/m^2. Three leaf discs were plotted dry and then placed in the buffer, lower leaf surface down, and the watch-glass covered with a Petri-dish cover to reduce evaporation. The covered watch-glass was then transferred to the growth chamber for three hours, where lighting was provided by fluorescent and incandescent lamps at 450 μE m^{-2} s^{-1} at 30˚C. Since the mechanism of resistance is gene amplification, no glyphosate metabolism would occur for the S and R biotypes [7]. No sampling was done during the influx period.

Leaf discs efflux of ^{14}C-glyphosate. For the efflux study, a second buffer consisted of 5 mM monobasic potassium phosphate (KH_2PO_4), pH 5.3, with no herbicide or radioactivity. For efflux examination, 300 *u*L of the second buffer was placed into micro-centrifuge tubes (1 ml) with a single leaf disc, it was then shaken by hand and washed for 3 seconds to remove any ^{14}C-glyphosate remaining

Table 1. Dry weight biomass and distribution of applied ^{14}C-glyphosate over time in glyphosate resistant and susceptible Palmer amaranth.[a,b]

HAT	Resistant	Susceptible	Pigweed within time	
		% of applied		
		Wash		
1	91	89	0.6424	NS
6	48	55	0.1932	NS
12	42	49	0.1154	NS
24	39	44	0.3409	NS
48	34	39	0.3038	NS
		Absorbed		
1	8	7	0.9159	NS
6	41	28	0.0108	*
12	47	38	0.0886	NS
24	45	43	0.6966	NS
48	43	49	0.1647	NS
		Total recovery		
1	99	96	0.5708	NS
6	90	82	0.1614	NS
12	88	87	0.7858	NS
24	84	87	0.5906	NS
48	77	89	0.0152	*
		Plant weight (mg/plant)		
1	464	569	0.5181	NS
6	542	847	0.0815	NS
12	786	749	0.8245	NS
24	791	759	0.8483	NS
48	995	1192	0.2311	NS

[a]Abbreviations: HAT, hours after treatment; NS, *, **, ***, Not significant or significant at $P \leq 0.05$, 0.01 and 0.001 levels, respectively. [b]Numbers indicate the percent distribution of the applied ^{14}C glyphosate.

on the surface. The rinse was performed twice, then combined and quantified by LSC. Individual disc were then placed into different micro-centrifuge tubes containing 300 *u*L of the second buffer. All tubes were placed onto a rotary shaker set at 100 rpm. Time-dependent efflux was then performed at 15, 30, 45, 60, 75, 90, 105, 120, 135, and 150 minutes. At the end of each efflux interval, the disc were again rinsed (which was collected for quantification), then moved to another micro-centrifuge tube containing 300 *u*L of the second buffer and continued replaced on the shaker till the next sample time. This continued until

reaching 150 minutes. The amount of ^{14}C-glyphosate contained in each aliquot was determined by LSC, and quantified based on a mass balance. Then each disc was oxidized as previously described to quantify any remaining ^{14}C-glyphosate. The experimental design was repeated-measures and the treatments were replicated 5 times. The experiment was conducted twice and the data combined for analysis.

For the ^{14}C-glyphosate efflux experiments, regression analysis was performed using nonlinear regression. The intent was to determine if the response could be described by using the exponential decay equation [12]

$$y = B_o e^{-B_1(x)} \quad (1)$$

where y is ^{14}C-glyphosate concentration in Becquerel's, B_0 is the initial concentration in solution after 15 minutes, B_1 is efflux rate, and x is time in minutes after treatment. Data for the exponential decay equations were subjected to ANOVA using the general linear models procedures with mean separation of parameter estimates using 95% asymptotic confidence intervals. Data were graphed in Sigmaplot 14 (Systat Software, San Jose, CA) (**Figure 1**).

3. Results and Discussion

Leaf disc study. There were no significant interactions when comparing ^{14}C-glyphosate in the rinses, bathing solutions, or retention by the leaf discs between R and S Palmer amaranth over the 150 minute study (**Figure 1**). Although there were no statistical differences between the R and S Palmer amaranth biotypes parameter estimates (Data not shown), time did effect the amount of

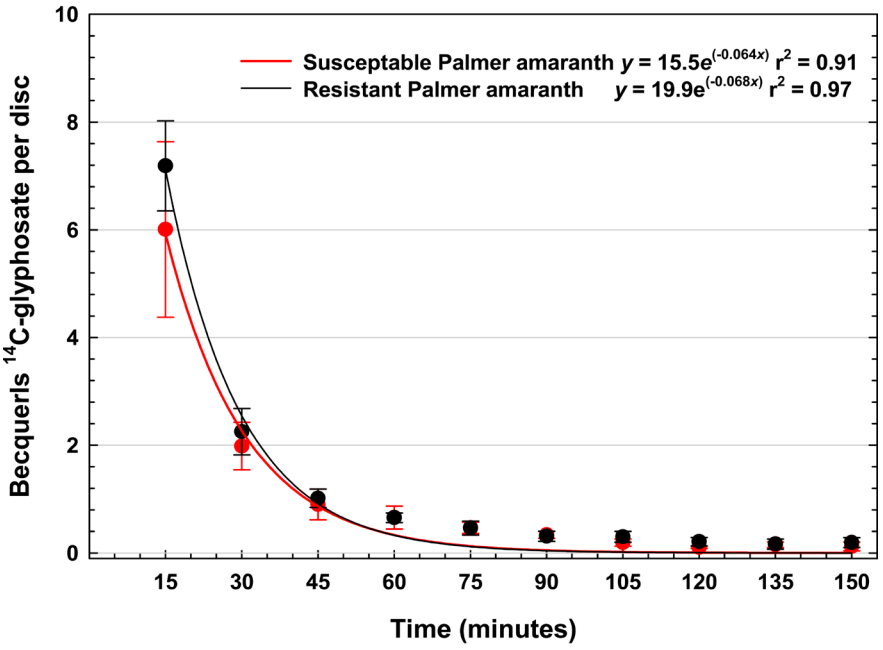

Figure 1. Comparative efflux of ^{14}C-glyphosate from leaf discs of glyphosate susceptible- and glyphosate resistant-*Amaranthus palmeri* after a 3-hour loading period. Each experimental unit contained 30 leaf discs.

^{14}C-glyposate retained in the leaf discs. Regardless of the amount of ^{14}C-glyphosate that initially in fluxed into the leaf discs from the treatment solution, the amount of efflux back into the buffer solution decreased over time. By 150 minutes, only 0.4 Bq ^{14}C-glyphosate were retained by R and S leaf discs (after oxidation). The amount retained by both biotypes was 6 or more Bq of ^{14}C-glyphosate at 15 minute, and 2 and 1 Bq of ^{14}C-glyphosate at 30 and 45 minute, respectively. At 60 minute and greater, less than 1 Bq of ^{14}C-glyphosate was retained. Thus, the longer the discs were floating on the buffer solution the more of the ^{14}C-glyphosate that had initially in fluxed into the tissue, efflux out. If the average Bq ^{14}C-glyphosate 6.5 in the R and S biotypes at 15 minute is compared to the average of 0.4 at 150 minutes, then 94% of the glyphosate that initially moved into the tissue efflux out. This could have been caused by the large diffusion gradient between the treated leaf discs and the buffer-bathing solution. Although very little ^{14}C-glyphosate was retained over time and there were no differences between biotypes within bathing times, the rate of efflux (*i.e.*, the slope of the curves) was greater for the R biotype from 15 to 45 minute. This could indicate that the R Palmer amaranth biotypes initially facilitated a transient albeit larger diffusion gradient due to glyphosate/enzyme interaction. Gene amplification of EPSPS in the R biotype would result in more absorbed glyphosate complexing with the target enzyme compared to the S biotypes. These data support gene amplification as the resistance mechanism and not differential movement into the leaf discs, retention and/or partitioning [2] [13] [14].

Absorption and Translocation. Across experiments 77 to 99% of total applied radioactivity was recovered from leaf washes and oxidation of plant parts (**Table 1**). There were no plant weight differences between biotypes during the study; however, both biotypes continued to grow (**Table 1**). There were no biotypes differences in ^{14}C-glyphosate recovered from the washes for any of the exposure times. However, the percent of ^{14}C-glyphosate recovered in the washes decreased over time for both biotypes (data not shown). The R and S biotypes absorbed 43 and 49% of the applied glyphosate after 48 HAT, respectively. Li *et al.* [10] reported 40% to 65% ^{14}C-glyphosate absorption by common waterhemp 26 to 50 HAT. At 12, 24 and 48 HAT there were no absorption difference between the R and S biotype. However, 6 HAT, R Palmer amaranth biotypes absorbed 13% more glyphosate than susceptible plants (41% verses 28% of the applied herbicide for R and S biotypes, respectively).

This ephemeral difference in glyphosate absorption could have been caused by the EPSPS gene amplification resistance mechanism reported for Palmer amaranth [2] [13] [14]. The over expression of the target enzyme in the R Palmer amaranth biotype effectively decreases the concentration of glyphosate in the tissue due to herbicide/target enzyme interaction, thus maintaining a higher diffusion gradient in the R biotype compared to S biotype which facilitated additional absorption. Glyphosate that is interacting with the target enzyme is effectively not influencing the tissue concentration gradient. Consequently, because

the S biotypes has orders of magnitude less available EPSPS synthases there is a higher concentration of free glyphosate in the tissue solution and a correspondently lower concentration gradient and less diffusion.

Although only 8% and 7% for the R and S Palmer amaranth biotypes of applied ^{14}C-glyphosate had been absorbed 1 HAT (Table 1), respectively, 82 and 90% remained in the treated leaf (Table 2). After 6 HAT, there was significantly greater mass of ^{14}C-glyphosate absorbed in the R treated leaf as compared to the S biotype: 13.5 verses 8.1 Bq, respectively with P = 0.0063 (Table 2). Although

Table 2. Distribution of ^{14}C-glyphosate over time in glyphosate resistant and susceptible Palmer amaranth as a percentage of material absorbed and Becquerel's per mg of plant tissue.[a,b]

HAT	Resistant	Susceptible	Pigweed within time		Resistant	Susceptible	Pigweed within time	
	% of absorbed				Bq/mg tissue			
Above treated leaf								
1	5	3	0.5203	NS	0.13	0.08	0.9225	NS
6	6	7	0.7382	NS	1.0	0.6	0.4097	NS
12	12	12	0.9225	NS	2.1	1.6	0.3165	NS
24	13	12	0.6877	NS	1.5	1.3	0.7001	NS
48	28	15	<0.0001	***	2.0	1.5	0.2418	NS
Below treated leaf								
1	9	5	0.1851	NS	0.04	0.02	0.8312	NS
6	6	8	0.5409	NS	0.15	0.09	0.5077	NS
12	13	10	0.4034	NS	0.25	0.20	0.4782	NS
24	18	14	0.1427	NS	0.32	0.33	0.9222	NS
48	19	25	0.0280	*	0.40	0.34	0.3962	NS
Roots								
1	5	3	0.3914	NS	0.07	0.03	0.8481	NS
6	2	4	0.4091	NS	0.23	0.16	0.7509	NS
12	15	6	0.0002	**	1.21	0.51	0.0020	**
24	13	8	0.0147	*	0.81	0.48	0.1417	NS
48	20	8	<0.0001	***	1.03	0.61	0.0511	NS
Treated leaf								
1	82	90	0.1448	NS	2.9	2.8	0.9345	NS
6	85	80	0.3728	NS	13.5	8.1	0.0063	**
12	60	72	0.0322	*	9.3	8.1	0.5146	NS
24	56	67	0.0395	*	7.4	10.5	0.0996	NS
48	33	52	0.0005	***	3.0	7.1	0.0288	*

[a]Abbreviations: HAT, hours after treatment; NS, *, **, ***, Not significant or significant at P ≤ 0.05, 0.01 and 0.001 levels, respectively. [b]Numbers indicate the percent distribution of ^{14}C-glyphosate among the four sectioned portions of each plant.

the S biotype had absorbed a higher mass of glyphosate at 24 and 48 HAT than the R biotype (**Table 2**), these plants were probably moribund at these times while the R biotype plants were healthy [15] [16]. Although the rapidity of response to glyphosate in susceptible species varies, Geiger *et al.* [16] reported disruption of the C3 plant cycle within minutes and a concomitant decline in translocation. Thus, the lower amounts of glyphosate (based on percent absorbed at 12, 24 and 48 HAT and Bq ^{14}C-glyphosate at 48 HAT) in the R biotype treated leaves was probably due to movement via translocation to untreated tissue. The more compelling data is the 13.5 to 3.0 Bq decrease in amount of glyphosate from 6 to 48 HAT in the treated leaf of the R biotype (**Table 2**), verses no change over the same time frame in the S biotype of 8.1 to 7.1 Bq, respectively.

The only statistical differences in percent of absorbed ^{14}C-glyphosate re-distributed from treated to non-treated tissue (*i.e.*, leaves above and below the treated leaf and roots) was at 48 HAT in shoot tissue and roots at 12, 24 and 48 HAT (**Table 2**). Almost twice as much ^{14}C-glyphosate (28 verses 15% absorbed) had been acropetally translocated to tissue above the treat leaf in the R biotype. In shoot tissue below the treated leaf, the slightly higher (25 verses 19% of absorbed) amount of ^{14}C-glyphosate in the S biotype at 48 HAT, was probably due to continued basipetal translocation in the heathy R biotype tissue verses moribund S biotype tissue. In root tissue at 12, 24 and 48 HAT, there was 15 verses 6 Bq, 13 verses 8 Bq, and 20 verses 8 Bq, more of the absorbed glyphosate translocated by the R verses S biotypes, respectively. Twelve HAT, the amount of glyphosate translocated to the roots was greater in the R biotype verses the S:1.21 verses 0.51 Bq. The root data also supports the conclusion that there was the continuation of robust translocation in the R biotype.

Differential translocation between the biotypes was also manifested using shoot/root ratios (**Figure 2**). Based on Bq/mg tissue, shoot/root ratios for the S biotype were 1.3 (100 verses 44), 0.9 (19 verses 10), 1.3 (25 verses 11) and 3 (15 verses 5) times higher than the R biotype at 1, 12, 24 and 48 HAT, respectively. There was not a difference in the ratios at 6 HAT. The higher shoot/root ratios in the S biotype reflects a lack of glyphosate translocation. Glyphosate self-limits translocation quickly in treated susceptible plants [15] [16]. Once absorbed into susceptible plants, glyphosate directly and indirectly inhibits processes that affect translocation and thus redistribution.

4. Conclusions

The data presented in this study support previous research demonstrating that the glyphosate resistance mechanism in Palmer Amaranth is gene amplification of 5-eno-pysuvylshikimate-2-phosphate synthase [2] [13] [14]. This resistance mechanism is different from two additionally reported glyphosate resistance mechanisms [17] [18] [19] [20]. An interesting difference in two of the known glyphosate resistance mechanisms is that with gene amplification higher rates of

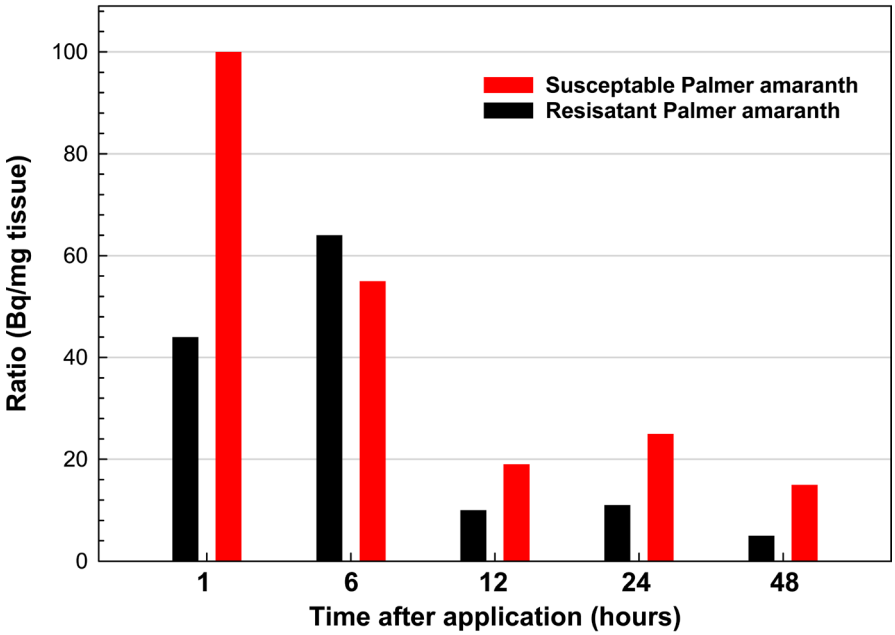

Figure 2. Shoot to root ratio for the mass of ^{14}C-glyphosate expressed as Bq/mg tissue for susceptible- and glyphosate resistant-*Amaranthus plameri* over time.

glyphosate will control the R biotype whereas with target site resistance even excessively high rates will not provide any control [2] [14]. This phenomena is because no matter how much glyphosate is applied to an altered site biotype, EPSPS is not inhibited. With EPSPS gene amplification, the wildtype form of EPSPS is inhibited by glyphosate so if enough is applied, some level of control will be achieved [2].

With gene amplification, the form of EPSPS is the same in both biotypes, but more is produced in the R biotype. Thus, absorbed glyphosate continues to inhibit EPSPS in both biotypes, but because the R biotype has orders of magnitude greater enzyme, treated plants are not susceptible. Gene amplification was first noted in tissue culture research examining glyphosate resistance in crop species [21]. The higher amount of wild type (*i.e.* normal inhibition by glyphosate) EPSPS in the R Palmer amaranth biotype was manifested in leaf discs as short term higher rates of retention. The lower short term efflux rates in the R biotype leaf discs (15 to 45 minute) could have resulted from more of the absorbed glyphosate complexing with EPSPS. The *in vivo* studies demonstrated that the R biotype had greater rates of absorption and translocation. Because of the R biotype complexes more of the absorbed glyphosate, a higher diffusion gradient is maintained resulting in higher initial influx. Over the study period, the R biotype gene amplification of EPSPS allowed for the continuation of glyphosate redistribution through normal acropetal and basipetal translocation. The over expression of EPSPS in the R biotype allows more glyphosate to be absorbed and translocated. As long as the stoichiometry favors significantly greater EPSPS than glyphosate molecules, normal aromatic amino acid biosynthesis of tryptophan, tyrosine, and phenylalanine will proceed and ultimately normal growth.

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Conflicts of Interest

The authors declare no conflicts of interest.

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