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Tolerance and Accumulation of Shikimic Acid in Response to Glyphosate Applications in Glyphosate-Resistant and Nonglyphosate-Resistant Cotton (*Gossypium hirsutum* L.)

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Measurement of shikimic acid accumulation in response to glyphosate inhibition of 5-enolpyruvylshikimate-3-phosphate synthase is a rapid and accurate assay to quantify glyphosate-induced damage in sensitive plants. Two methods of assaying shikimic acid, a spectrophotometric and a high-performance liquid chromatography (HPLC) method, were compared for their accuracy of recovering known amounts of shikimic acid spiked into plant samples. The HPLC method recovered essentially 100% of shikimic acid as compared with only 73% using the spectrophotometric method. Relative sensitivity to glyphosate was measured in glyphosate-resistant (GR) and non-GR cotton leaves, fruiting branches, and squares (floral buds) by assaying shikimic acid. Accumulation of shikimic acid was not observed in any tissue, either GR or non-GR, at rates of 5 mM glyphosate or less applied to leaves. All tissues of non-GR plants accumulated shikimic acid in response to glyphosate treatment; however, only fruiting branches and squares of GR plants accumulated a slight amount of shikimic acid. In non-GR cotton, fruiting branches and squares accumulated 18 and 11 times, respectively, more shikimic acid per micromolar of translocated glyphosate than leaf tissue, suggesting increased sensitivity to glyphosate of reproductive tissue over vegetative tissue. GR cotton leaves treated with 80 mM of glyphosate accumulated 57 times less shikimic acid per micromolar of translocated glyphosate than non-GR cotton but only 12.4- and 4-fold less in fruiting branches and squares, respectively. The increased sensitivity of reproductive structures to glyphosate inhibition may be due to a higher demand for shikimate pathway products and may provide an explanation for reports of fruit abortion from glyphosate-treated GR cotton.

KEYWORDS: Glyphosate; shikimic acid; herbicide resistance; transgenic crops; cotton; reproductive tolerance

INTRODUCTION

The herbicide glyphosate inhibits the biosynthesis of the aromatic amino acids tryptophan, tyrosine, and phenylalanine in sensitive plant species (*1*). Glyphosate competes with the substrate phosphoenolpyruvate (PEP) for a binding site on the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme [EC 2.5.1.19]. EPSPS is encoded in the nucleus and imported to plastids where it converts shikimate-3-phosphate and PEP into 5-enolpyruvylshikimate-3-phosphate (*1*). Besides inhibiting aromatic amino acid biosynthesis in sensitive plants, the interaction between glyphosate and EPSPS interferes with the production of secondary compounds derived from aromatic amino acids. The biosynthesis of proteins, auxins, pathogen defense compounds, phytoalexins, folic acid, precursors of lignins, flavonoids, plastoquinone, and hundreds of other phenolic and alkaloid compounds may all be affected by EPSPS inhibition due to the inhibition of aromatic amino acid biosynthesis (*2*).

Upon EPSPS inhibition by glyphosate, shikimic acid, the metabolic precursor of shikimate 3-phosphate, has been reported to increase rapidly in sensitive plants (*3*−*5*). Accumulation of shikimic acid to high levels may be the result of a loss of feedback control of the shikimic acid pathway by a downstream product that regulates the activity of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) synthase. A lack of DAHP synthase regulation may cause an unregulated flow of carbon to be diverted into intermediates upstream of the blocked EPSPS enzyme in the shikimic acid pathway, mainly shikimic acid (*6*).

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The immediate precursor to EPSP at the EPSPS blockage point is shikimic acid 3-phosphate, but Holländer-Czytko and Amrhein (*3*) reported that this compound is likely cleaved in the tonoplast or vacuole by a phosphatase enzyme, yielding shikimic acid.

Recently, methods of shikimic acid detection in glyphosate-treated plant tissues have been refined and may be used to rapidly determine glyphosate injury to sensitive plants as well as to evaluate the efficacy of different glyphosate formulations (*7*, *8*). A spectrophotometric method for determination of shikimic acid was adapted from Gaitonde and Gordon (*9*) by Singh and Shaner (*7*). The spectrophotometric method uses periodic acid to oxidize shikimic acid, producing *trans*-aconitic acid, which can then be identified at 380 nm. The high-performance liquid chromatography (HPLC) method quantifies the actual shikimic acid molecule at 219 nm using an UV−visible spectrum detector (*5*).

Glyphosate resistance has been conferred to several crop plants, including cotton, by the incorporation of a glyphosate-resistant (GR) CP4-EPSPS gene cloned from *Agrobacterium* sp. strain CP4 into their genomes (*10*−*12*). Singh and Shaner (*7*) found accumulation of shikimic acid in non-GR soybean (*Glycine max* Merr.L.) treated with glyphosate but not in GR *G. max* containing the CP4-EPSPS gene. This observation suggests that when sufficiently expressed, the CP4-EPSPS enzyme is capable of producing 5-enolpyruvylshikimate-3-phosphate in the presence of glyphosate with no observed accumulation of shikimic acid.

Cotton varieties resistant to glyphosate have been commercially available since 1997. Since that time, growers and agronomists have expressed concern over instances of decreased boll retention and pollination problems in glyphosate-treated GR cotton. Previous research has demonstrated translocation and accumulation of ^{14}C-glyphosate in reproductive structures, reductions in pollen viability, and abnormal floral anatomy and boll loss in glyphosate-treated GR cotton (*13*−*16*).

The current study investigates whether the reports of fruit abscission and pollination problems due to glyphosate treatments to GR cotton can be explained by innate differences in glyphosate sensitivity between reproductive and vegetative organs. Shikimic acid accumulation was used as a measure of glyphosate sensitivity. If the CP4-EPSPS gene was poorly expressed in reproductive tissues, the magnitude of difference in shikimic acid accumulation between glyphosate sensitive and resistant tissues may be lower upon treatment with glyphosate than in tissues where it is sufficiently expressed. Shikimic acid may also accumulate in GR tissues if there was insufficient GR CP4-EPSPS present. In this scenario, the native EPSPS present would be inhibited by glyphosate allowing shikimic acid to accumulate because little noninhibited EPSPS (either native or CP4-EPSPS) enzyme would be available to convert the shikimic acid to 5-enolpyruvylshikimate-3-phosphate. Differences in glyphosate sensitivity among tissues may provide an explanation for the observed increased fruit abscission and developmental abnormalities upon glyphosate treatment in GR cotton.

MATERIALS AND METHODS

Measurement of Shikimic Acid Accumulation in Glyphosate-Treated Tissues. *Plant Material.* Two isogenic Delta Pine and Land cotton varieties were used for all studies. "DP 5415RR" (GR) and "DP 5415" (non-GR) cotton were planted in 30 cm pots containing Metro-Mix 360 (Scotts-Sierra Horticultural Products Co., 14111 Scottslawn Rd., Marysville, OH 43041). Pots were thinned to one plant per pot at emergence and were grown in a plastic greenhouse maintained at 25 ± 2 °C constant temperature where natural sunlight was supplemented 4 h daily with mercury halide lights providing a total of a 16 h day length. The leaf subtending the first position square on the first fruiting branch (dedicated reproductive branch bearing fruit) was treated with glyphosate 5 days before anthesis. A 9 cm^2 area of the leaf surface was covered with 43 μL of herbicide solution containing an 80, 40, 10, 5, 1, or 0 mM solution of glyphosate (Roundup Ultra, Monsanto Company, 700 Chesterfield Parkway North, St. Louis, MO 63198). These rates, per 9 cm^2 area, corresponded to field rates of 8.96, 4.48, 1.12, 0.560, 0.112, or 0 kg ae/ha glyphosate. To quantify the percent of total applied glyphosate accumulating in treated leaves, squares, and fruiting branches, each treatment included 3.3 kBq ^{14}C-glyphosate (Sigma Co., 11542 Fort Mims Dr., St. Louis, MO 63146-3510). Plants were harvested 72 h after treatment. The treated leaf was washed with 10 mL of 1:1 water:methanol + 0.25% (v/v) nonionic surfactant (Induce nonionic low foam wetter/spreader adjuvant contains 90% nonionic surfactant (alkylarylpolyoxyalkane ether and 2-propanol), free fatty acids, and 10% water. Helena Chemical Co., Suite 500, 6075 Poplar Avenue, Memphis, TN 38137). A 1 mL subsample from each leaf rinse was counted using liquid scintillation spectrometry to determine the amount of nonabsorbed herbicide. Plants were divided into the following parts: treated leaf, square subtending to treated leaf, and fruiting branch to which leaf and square were attached before separation. Plant parts were put on ice immediately and transported to the laboratory where they were stored at −30 °C until analysis.

Shikimic Acid Analyses. For shikimic acid measurements in reproductive stage plants, both the HPLC procedure of Harring et al. (*8*) and the spectrophotometric procedure of Singh and Shaner (*7*) were used and compared. Samples were ground in liquid nitrogen and extracted with 4 mL of 0.01 M H_2SO_4 g^{-1} tissue for 60 min on a shaker. One milliliter of 0.4 M $NaHCO_3$ was added, and samples were centrifuged at 10 000*g* for 10 min at 4 °C. The supernatant was stored at −30 °C until analysis. For HPLC analysis, the sample was diluted 5-fold and was analyzed according to the methods of Lydon and Duke (*5*) with a 65 μL injection volume separated on an Allsphere ODS-1 column (Alltech Associates, 2051 Waukegan Road, Deerfield, IL 60015) using a Water's (34 Maple Street, Milford, MA 01757) Lambda Max model 481 UV−visible spectrum detector. For the spectophotometric analysis, 20−50 μL of the nondiluted sample was analyzed according to the methods of Singh and Shaner (*7*) using a Perkin-Elmer (45 William Street, Wellesley, MA 02481-4078) UV/Vis Lambda 10 Spectrometer.

Comparison of HPLC and Spectrophotometric Shikimic Acid Assays. HPLC and spectrophotometric methods were directly compared by adding either 435.5, 54.4, 6.8, 0.9, or 0.1 μg shikimic acid standard mL^{-1} plant supernatant from multiple nonglyphosate-treated plants (Sigma). Identical samples containing known shikimic acid concentrations were analyzed by either the HPLC or the spectophotometric methods. A standard curve was developed using pure shikimic acid standards with known concentrations for each method. The μg shikimic acid mL^{-1} plant supernatant from all plants was determined by comparison with the standard curves for each respective method.

Measurement of ^{14}C-Glyphosate Translocation in Reproductive Stage Plants. The remaining plant residue, after extraction, was force-air-dried at 50 °C and combusted using a Harvey^9 biological oxidizer to recover any nonextracted ^{14}C-glyphosate (model OX500, J. Harvey Instrument Corporation, 123 Patterson Street, Hillsdale, NJ 07642). A 100 μL sample of the supernatant was analyzed for ^{14}C-glyphosate content using liquid scintillation spectrometry. The sum of ^{14}C-glyphosate from the extracted and nonextracted fractions was divided by the total amount of ^{14}C-glyphosate applied to determine translocation. The percent translocation was then multiplied by the concentration of glyphosate applied to determine the amount of glyphosate translocated to each organ.

Experimental Design and Statistical Analysis. Studies were arranged in a randomized complete block design with four replications (blocks). Each study was repeated three times. Data were subjected to analysis of variance using SAS, version 8.0 (SAS Institute, Inc., SAS Campus Drive, Cary, NC 27513-2414). Data from the three repeated experiments were combined due to nonsignificant run interactions. Glyphosate translocation means were separated using Fisher's Protected LSD test at $\alpha = 0.05$. Data for shikimic acid accumulation over glyphosate accumulation and comparison of shikimic acid assays were subjected

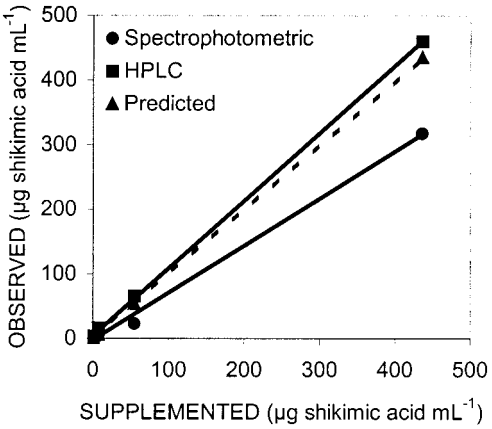

Figure 1. Comparison of spectrophotometric and HPLC methods for determining shikimic acid. Plant extracts were spiked with known amounts of shikimic acid and analyzed by both methods. Expected vs observed recovery of shikimic acid (μg shikimic acid mL^{-1}) was plotted for both methods. Linear equations for each method are as follows: spectrophotmetric method, $y = 0.73x - 2.15$ ($R^2 = 0.997$); HPLC method, $y = 1.04x + 3.80$ ($R^2 = 0.999$); predicted, $y = 1x$.

to regression analysis. For shikimic acid accumulation at various glyphosate rates, standard errors for each mean are reported.

RESULTS AND DISCUSSION

Comparison of HPLC and Spectrophotometric Analyses of Shikimic Acid. Because shikimic acid is commonly detected by either a spectrophotometric or an HPLC assay, the two methods were compared for detection efficiency. Shikimic acid accumulation as detected by the HPLC and spectrophotometric assays differed in samples of plant extract spiked with known amounts of shikimic acid (**Figure 1**). The slope of the supplemented vs observed micrograms shikimic acid mL^{-1} line from HPLC samples was 1.04, indicating high correlation between expected and observed shikimic acid at all concentrations (**Figure 1**). In contrast, the slope of the line from spectrophotometric samples was 0.73, suggesting that shikimic acid concentration was underestimated. The percent deviation from the expected amount of shikimic acid did not depend on shikimic acid concentrations, although the actual numerical difference increased with increasing shikimic acid concentrations. These data suggest that the spectrophotometric method may be less precise in its estimation of shikimic acid concentration than the HPLC method. Singh and Shaner (*7*) reported greater than 80% recovery of spiked shikimic acid in crude extracts; however, recovery in the spectrophotometric method in this study reached a maximum at 73%. In plant samples with high amounts of shikimic acid (as determined by the HPLC method), 20 μL of sample was used for spectrophotmetric analysis instead of 50 μL. Even by measuring smaller sample volumes (thus diluting samples), maximum values of shikimic acid accumulation only reached 65−80% of those from the HPLC method (**Figure 3a−c**). The concentration of shikimic acid where detection was saturated for both the HPLC and the spectrophotometer was similar. The HPLC method reached a detection plateau at 14.1 μg shikimic acid per sample, while the spectrophotometric method plateaued at 13.6 μg shikimic acid. The proximity of the detection saturation points of the two methods cannot account for the lower recovery observed with the spectrophotometric method. The two methods actually quantify different compounds, at different wavelengths. The spectrophotometric method measures *trans*-aconitic acid at 380 nm, while the HPLC method measures actual shikimic acid at 219 nm (*5*). The detection of two different compounds at two different wavelengths may explain some of the differences between the two methods. Although both methods may show relative differences in shikimic acid accumulation, these data suggest that the spectrophotometric method may underestimate the true amount of shikimic acid in each sample, especially in samples with high amounts of shikimic acid.

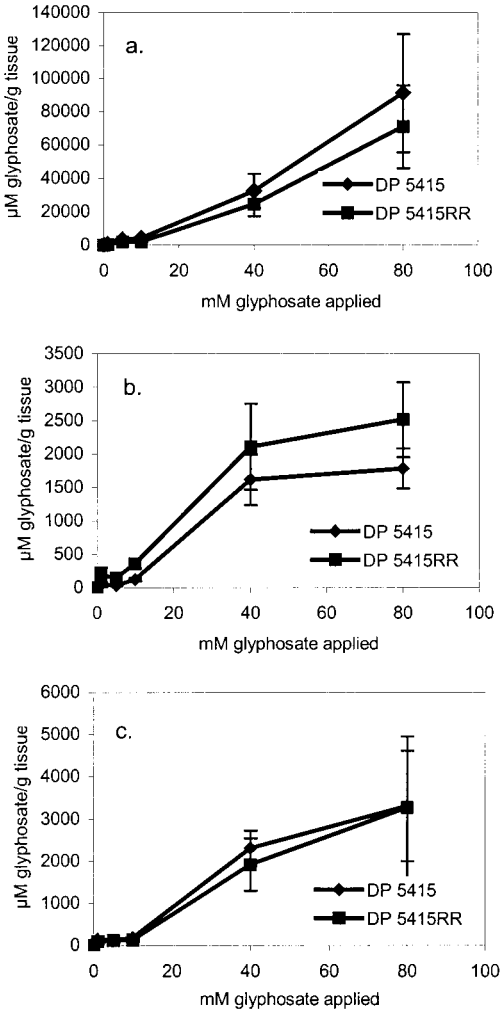

Figure 2. Accumulation of glyphosate (μM g^{-1} fresh weight) per millimolar of applied glyphosate in DP 5415 and DP 5415RR cotton tissues. Treated leaves (a), fruiting branches (b), and squares (c). Bars represent the standard error of each mean. Where bars are absent, they are obscured by the data symbol.

Effect of Rate and Variety on Glyphosate Translocation. Translocation of glyphosate from the point of application to a nontreated tissue determines the glyphosate dose present in a tissue, and thus the amount of glyphosate injury. ^{14}C-glyphosate applied to the treated leaf of reproductive stage cotton plants was subsequently translocated to fruiting branches and squares. The percent of applied glyphosate remaining in the treated leaf did not vary significantly by glyphosate rate or by variety (DP 5415 or DP 5415RR) (**Table 1**). Approximately 60% of the applied glyphosate remained in the treated leaf. Translocation to fruiting branches was dependent upon both variety and rate. The greatest percent of glyphosate translocation occurred at the 1 mM glyphosate rate in DP 5415RR, with 8.1% of the applied glyphosate accumulating in the fruiting branch. At the 1 and 5 mM glyphosate rates, translocation to fruiting branches was significantly greater in DP 5415RR than in DP 5415 (**Table 1**). These data may suggest that a greater amount of glyphosate

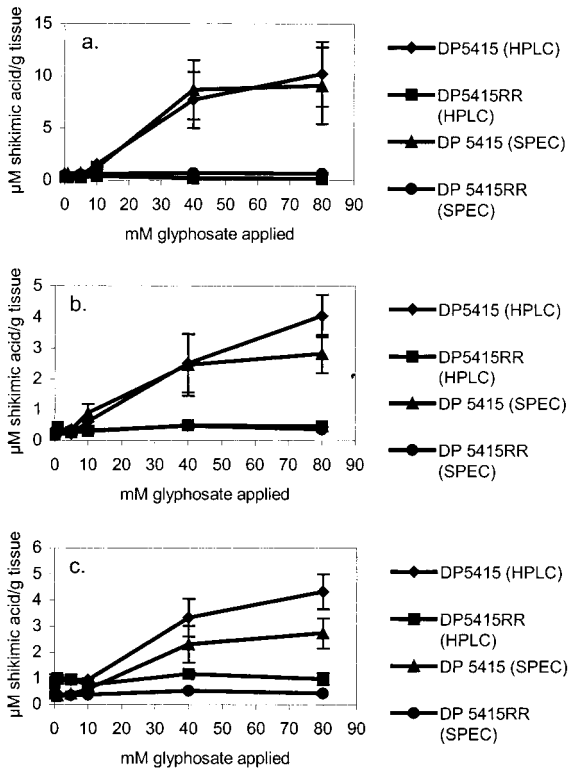

Figure 3. Accumulation of shikimic acid (μM g^{-1} fresh weight) per millimolar of applied glyphosate in DP 5415 and DP 5415RR cotton tissues using the HPLC method or the spectrophotometric method. (a) Treated leaves, (b) fruiting branches, and (c) squares. Bars represent the standard error of each mean. Where bars are absent, they are obscured by the data symbol.

Table 1. Translocation of Glyphosate from Treated Leaf to Fruiting Branch and Squares of DP 5415 and DP 5415RR Reproductive Stage Cotton Plants as Affected by Glyphosate Application Rate

	% of applied glyphosate								
	fruiting branch					squares		treated leaf	
mM glyphosate	DP 5415		DP 5415RR			DP 5415 and DP5415RR		DP 5415 and DP5415RR	
1	1.9	a[a]	8.1	a	*[b]	12.4	a	60.0	a
5	0.3	a	2.5	b	*	1.9	b	65.0	a
10	1.1	a	2.0	b	NS	1.4	b	44.0	a
40	1.9	a	2.9	b	NS	6.2	b	64.0	a
80	1.4	a	2.2	b	NS	4.7	b	76.0	a

[a] Means followed by the same letter in a column are not significantly different at $\alpha = 0.05$ according to Fisher's protected LSD. [b] Means of fruiting branch from DP 5415 and DP 5415RR are significantly different (*) or are not significantly different (NS) at a particular rate of glyphosate according to student's *t*-test at $\alpha = 0.05$.

is able to translocate out of the treated leaf in DP 5415RR because glyphosate-induced cellular damage to leaf tissue does not occur to the same extent as in DP 5415. In leaves treated with 10, 40, or 80 mM of glyphosate, visual injury at the area of application was observed in DP 5415 leaves but not in DP 5415RR leaves 72 h after treatment. Field studies of GR cotton have reported no observable foliar injury due to glyphosate treatment (*16*).

Translocation of glyphosate to squares was dependent upon glyphosate rate but not on variety. At 1 mM glyphosate, 12.4% of applied glyphosate was translocated to square tissue in both DP 5415 and DP 5415RR (**Table 1**). At higher rates, however, translocation ranged from 1.4 to 6.2% of applied glyphosate. Pline et al. (*13*) showed that 0.2% of applied glyphosate translocated to squares when applications were made to the upper-most fully expanded main stem leaf at the 12 leaf growth stage. The higher rate of translocation to squares in the current study is a result of the fact that glyphosate was applied to the subtending leaf of the square, which is in closer proximity to the squares than the upper-most fully expanded main stem leaf. Benedict and Kohel (*17*) reported that subtending leaves are a major source of photoassimilate for developing bolls. Glyphosate translocation generally follows the path of photoassimilates in plants (*18*−*20*). Therefore, it is not unexpected that developing squares would accumulate more glyphosate than fruiting branches, which may not be as strong of a sink as reproductive squares, even though fruiting branches are in closer proximity to the glyphosate-treated subtending leaf.

The amount of applied glyphosate per gram of tissue generally followed the pattern of percent of applied glyphosate translocation (**Table 1** and **Figure 2a**−**c**). Treated leaves retained 1.86 mM g^{-1} of glyphosate at the 1 mM rate and reached 91 mM g^{-1} at the 80 mM rate (**Figure 2a**). Fruiting branches from DP 5415RR accumulated more glyphosate than those from DP 5415 at the 1, 5, and 10 mM rates of glyphosate but do not differ at the 40 and 80 mM rates (**Figure 2b**). Accumulation in fruiting branches reaches a maximum at the 80 mM rate with 2510 μM glyphosate g^{-1}. In squares, accumulation reaches a maximum at the 80 mM rate of 3220 μM glyphosate g^{-1} (**Figure 2c**). Thus, translocation of glyphosate from the point of application to other tissues resulted in different doses of glyphosate, which can subsequently be correlated with shikimic acid accumulation.

Shikimic Acid Accumulation in Reproductive Stage Plants. Estimations of shikimic acid in samples differed between the HPLC and the spectrophotometer methods (**Figure 3a**−**c**). In samples with low amounts of shikimic acid, estimations from both methods are comparable; however, in samples with higher amounts of shikimic acid, the spectrophotometric method estimates of shikimic acid were 15−35% less than the HPLC method. Because of these differences and the greater accuracy of the HPLC method (**Figure 1**), only HPLC data will be described when comparing shikimic acid accumulation.

Shikimic acid accumulation was dependent on the tissue measured, the rate of glyphosate applied, and the cotton variety. Shikimic acid accumulation in DP 5415RR was generally independent of rate, with very little accumulation in any tissue, even up to the 40 and 80 mM glyphosate rates (**Figure 3a**−**c**). Singh and Shaner (*7*) reported that GR soybean treated with 0.75 kg ai/ha glyphosate contained only 0.2 μM shikimic acid g^{-1} fresh weight. Because shikimic acid is a metabolic intermediate in the shikimate pathway, there is likely some low background level present at all times in the plant. This concentration may differ in different tissues. Our data show a background level of 0.2 μM shikimic acid g^{-1} fresh weight in the leaves and fruiting branches of nontreated cotton but 0.8 μM shikimic acid g^{-1} fresh weight in squares from nontreated cotton (**Figure 3a**−**c**). Background levels of shikimic acid did not differ among the two varieties. Different levels of endogenous shikimic acid may reflect differences in tissue function or may suggest a generally slower incorporation of shikimic acid into downstream products, thus resulting in slightly elevated levels in comparison to other tissues. To our knowledge, there are no reports to date comparing endogenous levels of shikimic acid in vegetative and reproductive tissues.

In contrast to DP 5415RR, shikimic acid accumulation in DP 5415 cotton increased with increasing rates of glyphosate. At

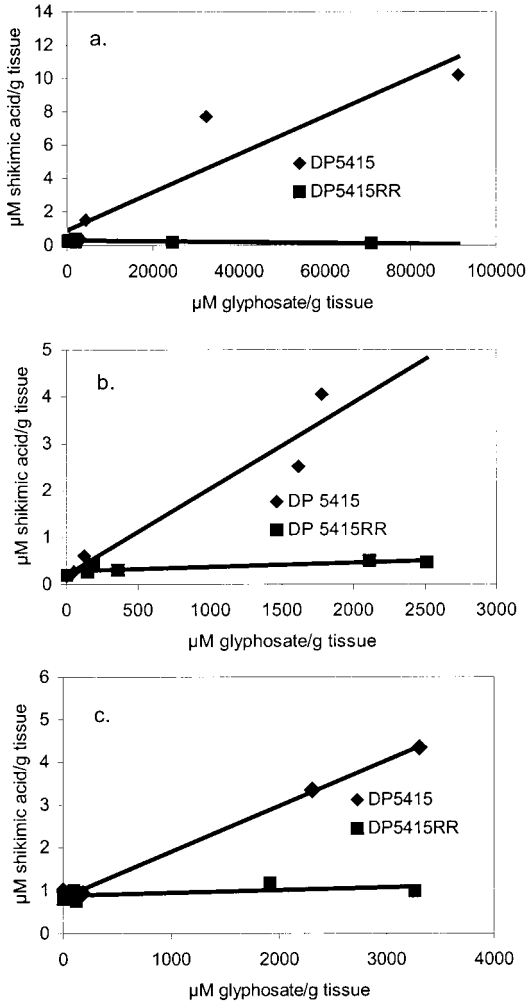

Figure 4. Accumulation of shikimic acid (μM g^{-1} fresh weight) per micromolar of accumulated glyphosate g^{-1} fresh weight in DP 5415 and DP 5415RR cotton tissues. Linear equations were fit to all data (R^2 values range from 0.87 to 0.99). Treated leaf (a): DP 5415RR, $y = -0.000\,002x + 0.3$; DP 5415, $y = 0.0001x + 0.9$. Fruiting branch (b): DP 5415RR, $y = 0.0018x + 0.2$; DP 5415 $y = 0.000\,08x + 0.3$. Square (c): DP 5415RR, $y = 0.000\,06x + 0.9$; DP 5415, $y = 0.0011x + 0.8$.

the 1 and 5 mM glyphosate rates, elevated shikimic acid was not evident in any tissues; however, at the 10, 40, and 80 mM glyphosate rates, shikimic acid increased. Shikimic acid reached 10.1 μM g^{-1} fresh weight at the 80 mM glyphosate rate in treated leaves (**Figure 3a**). In the fruiting branch, accumulation peaked at 4.04 μM shikimic acid g^{-1} fresh weight and in the square at 4.33 μM shikimic acid g^{-1} fresh weight (**Figure 3b**,**c**). These upper levels of shikimic acid accumulation are in general agreement with those reported in other sensitive plants. Harring et al. (*8*) reported 18−21 μM of shikimic acid g^{-1} fresh weight after a whole plant treatment of 1 kg ai/ha glyphosate in *Brassica napus* L. Corn plants treated with a whole plant application of 0.75 kg ai/ha glyphosate reached a maximum of 12 μM of shikimic acid g^{-1} fresh weight 10 days after treatment (*7*). However, the amount of glyphosate reaching the analyzed tissue is not known in either of these studies.

To compare relative differences in shikimic acid accumulation in response to increasing doses of glyphosate, the relationship between glyphosate accumulation and accumulated shikimic acid concentration was described using linear regression analysis. In DP 5415RR-treated leaves, shikimic acid did not accumulate in response to glyphosate (slope $= -2 \times 10^{-6}$; **Figure 4a**). In DP 5415RR fruiting branches and squares, however, a slight accumulation of shikimic acid was observed in response to glyphosate (slopes, 8×10^{-5} and 6×10^{-5}, respectively; **Figure 4b**,**c**). Although small, the difference in glyphosate accumulation among fruiting branches, squares, and leaves may explain why glyphosate is more injurious to squares and fruiting branches than to leaves of GR cotton. Harring et al. (*8*) showed a high correlation between accumulation of shikimic acid 48 h after treatment and percent plant death 14 d after treatment. Even low levels of shikimic acid accumulation corresponded to pronounced tissue injury. The accumulation of shikimic acid is not in itself toxic to plants; however, accumulation signals that EPSPS is inhibited, leading to perturbation of plant metabolic control (*1*).

Glyphosate-induced shikimic acid accumulation was much higher in DP 5415 than in DP 5415RR plants. In DP 5415-treated leaves, 0.1 nM shikimic acid per μM of glyphosate g^{-1} fresh weight accumulated (**Figure 4a**). However, in fruiting branches and squares, 1.8 and 1.1 nM shikimic acid accumulated per μM of glyphosate g^{-1} fresh weight (**Figure 4b**,**c**). This increase suggested that following glyphosate treatments, fruiting branches and squares accumulate 18- and 11-fold more shikimic acid per micromolar of glyphosate than treated leaves. The increase in shikimic acid accumulation in fruiting branches and squares as compared to the leaves was similar in DP 5415 and DP 5415RR. Becerril et al. (*21*) reported higher accumulation of shikimic acid in the flowers than in the leaf tissue of velvetleaf (*Abutilon theophrasti* L.), following glyphosate treatments. It was hypothesized that the ability of a flower to act as a terminal sink would allow it to continuously accumulate glyphosate, therefore increasing the amount of shikimic acid and glyphosate toxicity over time. The short duration of the current study (72 h) may thus underestimate the amount of glyphosate and shikimic acid that may accumulate in developing squares over a longer time period. Additionally, there has been little evidence of glyphosate metabolic degradation in higher plants; so, as glyphosate continues to translocate in the phytotoxic parental form, shikimic acid accumulation may reach even higher levels in squares after 72 h (*22*).

The ratio of DP 5415 to DP 5415RR accumulation of shikimic acid per micromolar glyphosate also reflects an innate difference in glyphosate sensitivity among tissues. At low rates of glyphosate (<10 mM), elevated shikimic acid is not evident in any tissue from either DP 5415 or DP 5415RR (**Figure 5**). However, at 40 and 80 mM glyphosate, treated leaves of DP 5415RR accumulated 30- and 60-fold less shikimic acid per micromolar glyphosate than treated leaves of DP 5415, respectively. However, fruiting branches of DP 5415RR accumulated only 6.6- and 12.4-fold less shikimic acid per micromolar glyphosate than fruiting branches of DP 5415, at 40 and 80 mM glyphosate, respectively. The magnitude of difference between accumulation in DP 5415 and accumulation in DP 5415RR squares was the least of all tissues. At 40 and 80 mM glyphosate, DP 5415RR squares accumulated only 2- and 4-fold less shikimic acid, respectively, than did squares from DP 5415. These differences would suggest that at 80 mM of glyphosate, treated leaves of DP 5415RR are 60-fold more resistant to glyphosate (accumulated 60-fold less shikimic acid) than those of DP 5415, while DP 5415RR fruiting branches and squares are only 13- and 4-fold more resistant, respectively.

Because an increase above background level of shikimic acid is often associated with glyphosate-induced injury (*4*), differences in accumulation among tissues may suggest relative differences in glyphosate tolerance between these tissues.

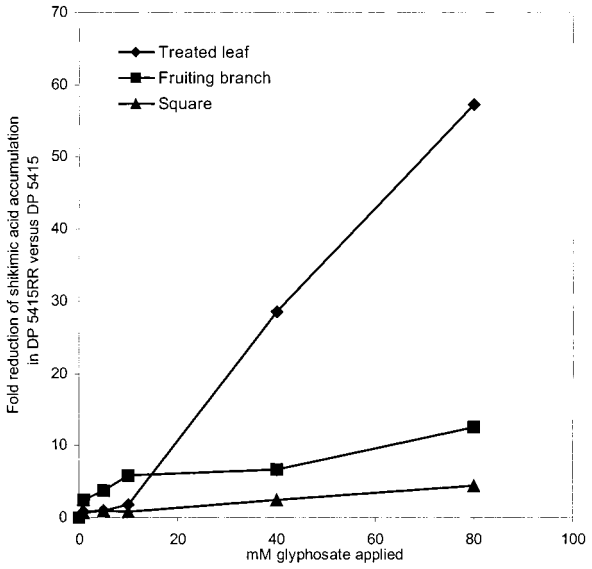

Figure 5. Fold reduction of μM shikimic acid/μM glyphosate g fresh weight^{-1} in DP 5415RR rather than DP 5415 treated leaves, fruiting branches, and squares from plants treated with 0, 1, 5, 10, 40, and 80 mM glyphosate.

Reduced reproductive tolerance to glyphosate has been reported in transgenic GR tobacco (*Nicotinia tabacum* L.) plants containing different constructs of the CP4-EPSPS gene encoded in either the chloroplast or the nuclear genomes (*23*). In all constructs, the amount of glyphosate needed to reach 50% injury (50% reduction in seed set) of reproductive tissues was less than that for vegetative tissues. Western blot analysis of tobacco plants containing these constructs indicated that CP4 protein levels were 10-fold lower in flower petals and 50-fold lower in immature anthers and ovaries than in mature leaf tissue (*23*). The reduced reproductive organ EPSPS expression was explained to be due to lower plastid numbers in these cells.

Differing levels of native EPSPS expression have been reported to occur in different organs of wheat (*Triticum aestivum* L.), petunia, and tomato (*Lycopersicon esculentum* L.). In wheat plants, developed leaves contained 43% of the total EPSPS plant enzyme, while roots contained 5−40% (*24*). In mature petunia plants, EPSPS expression is very high in petals but barely detectable in mature leaves (*25*). Expression of EPSPS in tomato was shown to be the highest in roots and flowers, lower in stems, and lowest in leaves and cotyledons (*26*). Differences in native EPSPS expression between vegetative and reproductive organs of cotton are likely to exist as well. Demand is high for shikimate pathway intermediates in reproductive organs where gametogenesis, sporopollenin production during pollen biogenesis, and organ growth would require high rates of protein synthesis and high concentrations of phenylpropanoids (*27*). The greater glyphosate sensitivity of square tissue over leaf tissue in cotton may be due to higher metabolic activity and demand for proteins and phenylpropanoids in developing squares than in leaves. Thus, upon glyphosate inhibition of EPSPS, shikimic acid in squares may accumulate to a higher level than in leaves at an equal amount of glyphosate, because the shikimic acid pathway is more active in reproductive tissues. Greater amounts of carbon would therefore be invested in the pathway due to the lack of feedback inhibition of DAHP synthase, thus causing increased production of shikimic acid (*6*).

Because demand for shikimate pathway products may be greater in reproductive tissues than in vegetative tissues, crops that express a GR EPSPS gene may need greater expression in reproductive tissues than vegetative tissue in order to meet the plant's demand for shikimate products in the presence of glyphosate. In the presence of glyphosate, the native glyphosate-sensitive EPSPS would be inhibited, leaving only the GR EPSPS to convert shikimate 3-phosphate into 5-enolpyruvylshikimate-3-phosphate and produce downstream products. If there was insufficient GR EPSPS present, the developing reproductive organ may be starved for necessary aromatic amino acids or other shikimate pathway products. This starvation may then lead to reported developmental abnormalities or abortion in pollen, ovaries, or other floral organs, which lead to reduced seed count in bolls from treated plants (*14*, *15*) or increased fruit abscission (*16*, *28*). Fruit retention and pollen viability in nontreated GR cotton is similar to that of non-GR cotton isolines (*14*), suggesting that insufficient shikimate pathway products only occur in the presence of glyphosate. Shikimic acid may not necessarily accumulate in these reproductive organs if there was some GR CP4-EPSPS present to metabolize shikimate 3-phosphate, therefore not allowing the unregulated flow of carbon into the shikimate pathway by DAHP deregulation and a subsequent buildup of shikimic acid. The level of CP4-EPSPS needed to prevent shikimic acid accumulation may be much less than the level needed to fulfill an organ's need for shikimate pathway products.

In summary, this work has demonstrated that of the two methods tested for shikimic acid determination, the HPLC method provided a more accurate estimate of shikimic acid. The rates of glyphosate accumulation in the treated leaves and squares of GR and non-GR cotton were similar; however, the fruiting branches of GR cotton accumulated more glyphosate at low application rates than its non-GR counterpart. Shikimic acid did not accumulate in response to glyphosate treatment in the treated leaves of GR cotton plants but increased slightly in squares and fruiting branches. All tissues of non-GR cotton accumulated shikimic acid in response to glyphosate rates of 10 mM or greater. As compared to the treated leaves, non-GR cotton accumulated 18- and 11-times more shikimic acid in the fruiting branches and squares, respectively. Leaves from GR cotton plants treated with 80 mM of glyphosate accumulated 57 times less shikimic acid than those from non-GR cotton but only 12.4- and 4-fold less for fruiting branches and squares, respectively, suggesting increased sensitivity of reproductive tissues to glyphosate. The increased glyphosate sensitivity in reproductive organs may be due to higher demand for shikimate pathway products. Research comparing the level of native and GR CP4-EPSPS expression in various tissues of cotton plants is needed. If expression of the CP4-EPSPS was not sufficient in reproductive tissues, the reported increases in fruit abortion in glyphosate-treated GR cotton may be explained.

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