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A Novel Approach To Determine the Glyphosate Tolerant Trait in Soybeans

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The ability of soybean breeders to accurately, economically, and rapidly determine the transfer of the CP4 gene, the gene which confers soybean tolerance to the herbicide glyphosate, to elite soybean lines is essential to development of new glyphosate tolerant soybean (GTS) cultivars. This research focused on a simple greenhouse screening procedure to replace large, costly, and laborious field screening. Non-GTS seed was determined to be susceptible to soaking in a 1% glyphosate solution for 4 h. This process is quicker, more efficient, and as reliable as field screening for determination of glyphosate susceptibility in soybean seed. Furthermore, this research clearly demonstrates that the metabolic pathway of glyphosate activity, the shikimate acid pathway, is active, and the target enzyme of glyphosate, 5-enol-pyruvyl-shikimate-3-phosphate synthase, is present during seed germination.

KEYWORDS: EPSPS; glyphosate; glyphosate tolerant soybeans; glyphosate screening; soybean breeding

INTRODUCTION

The introduction and widespread adoption of glyphosate tolerant soybean (GTS) technology has revolutionized the production of soybeans from a weed management perspective. Nearly 81% of all soybean hectares grown in the United States are glyphosate tolerant (*1*). Producers are using GTS cultivars that are best adapted for their production area. A restraint to the rapid development of new GTS cultivars is the resources and time required to screen large numbers of breeding lines for transfer of the CP4 gene that confers glyphosate tolerance by traditional breeding methods. The ability to detect the presence of the CP4 gene at the seedling stage will contribute to enhanced genetic gains in the early stages of a breeding program.

Confirmation of CP4 gene transfer in the first filial (F_1) and backcross (BC) generations of seed during germination would optimize space and other resources for plants that need to be grown in a greenhouse. A common procedure in plant breeding is the genetic transfer, via backcrossing, of an important gene (such as CP4) to the genome of an established elite line or cultivar. Identification of specific individuals that contain the CP4 gene at each back cross generation will reduce the number of attempted pollinations that a breeder must make to have a reasonable chance of successful transfer of the CP4 gene to the subsequent generation. Moreover, screening of the F_2 generation, the first selfing generation following a cross, ensures the elimination of the susceptible recessive single plant individuals that do not contain the CP4 gene.

Furthermore, breeding programs are employing new techniques to accelerate recurrent parent genome recovery, including the transfer of glyphosate tolerance (*2*−*4*). This process uses molecular genetic markers to determine which backcross individuals have the most genome in common with the elite recurrent parent. To complete this task, DNA needs to be collected during rapid mitosis of young developing plants. A process to screen the seedlings would eliminate the need to collect DNA from 50% of the population, saving valuable time and resources. Currently, seed from plants derived by a cross of non-GTS × GTS are planted in the field, grown to the V3 stage, and sprayed with glyphosate to determine which plants contain the CP4 gene. The same procedure is required for each backcross, or selfing generation. A method to quickly and accurately screen seedlings can save time in determining which single-plant should be carried forward to the field for continued genetic improvement. Furthermore, if 25−50% of the lines can be eliminated by seedling screening, savings in land, personnel, and financial resources can be recognized.

Glyphosate has a unique mode of action in plants (*5*−*7*). Glyphosate inhibits aromatic amino acid biosynthesis, leading to blockage of protein and secondary metabolite production (*5*, *8*). It works by competitive inhibition of the enzyme 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS), which catalyzes an essential step in the aromatic amino acid biosynthetic pathway. EPSPS catalyzes the reaction of shikimate-3-phosphate and phosphoenolpyruvate to yield 5-enolpyruvylshikimate-3-phosphate (EPSP) and inorganic phosphate. EPSP is a precursor to chorismate formation, the base molecule for all aromatic amino acid formation. Thus, glyphosate blocks the formation of aromatic amino acids, resulting in plant death approximately 7−10 days after treatment with glyphosate.

Soybean seeds need to imbibe with water prior to initiation of germination. We postulate that if soybeans were soaked in solution containing glyphosate, germination of non-GTS would possibly be inhibited, while GTS would germinate normally.

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This research provides an examination of the expression of the CP4 gene in germinating soybeans as elucidated by changes in whole plant response to glyphosate absorption at the seed germination stage.

MATERIALS AND METHODS

Research was conducted during the spring and summer of 2002 to determine if groups of soybean seed containing the CP4 gene (GTS) could be identified from groups of seed not having this trait. Studies were conducted using TN01-299-RR soybean seed as the GTS line, and 5601T was utilized as the non-GTS line (9). Both soybean lines were developed by the University of Tennessee soybean breeding program and had similar germination characteristics and vigor in early growth. Homogeneous populations of either GTS seed or non-GTS seed were used for the first studies. The glyphosate formulation used was an isopropylamine salt at 444 g of acid equivalent (ae)/L (1.95 mol/L) containing a tallowamine based surfactant.

Glyphosate concentrations examined in the preliminary study were 0, 0.5, 2, and 5% v/v (0, 0.01, 0.04, and 0.1 M). Soaking time was 0, 4, or 16 h. After soaking for the designated interval, 10 seeds from each treatment combination were planted 2 cm deep into soilless potting medium, placed in a greenhouse, and allowed to germinate and develop for two weeks. Plants were grown under supplemental metal halide lighting (400 microeinsteins/cm) with 16-h light and 8-h dark periods. Average daily high and low temperatures in the greenhouse were 32 and 16 °C, respectively. The planting medium was throughly watered the evening prior to planting soybean seeds and not watered soon after planting, so as not to dilute the herbicide. Watering was resumed 24 h after planting treated soybean seeds, and plants were watered twice daily for the duration of the study. Each treatment combination was replicated four times in a completely randomized design, and the study was conducted twice. Results from the preliminary research indicated that revisions in the glyphosate concentration and soaking duration may provide greater discernment between GTS and non-GTS soybean seed.

The next study investigated glyphosate concentrations of 0, 0.5, and 1% v/v (0, 0.01, and 0.02 M) with soaking durations of 1, 2, and 4 h. After soaking for the appropriate time period, 10 seeds from each treatment combination were planted and grown as previously described. Each treatment was replicated 4 times in a completely randomized design, and the study was conducted twice. Data collected included plant count, percent germination, and fresh weight 14 days after germination. Germination normally occurred 5 days after planting.

The next series of experiments determined if the soaking procedure would be able to select GTS seed from non-GTS seed from populations containing both types of seed and to determine if the proposed screening procedure would produce results similar to those from traditional field studies where postemergence applications are used to determine the GTS trait. To accomplish this examination, seed lots were counted out with the following ratios of GTS: non-GTS, 100:0, 75:25, 50:50, 25:75, and 0:100. A 1% (0.02 M) glyphosate solution and soak time of 4 h was used. Greenhouse studies used 12 total seeds per treatment combination.

Field comparisons were initiated by hand-mixing populations at the same ratios as the greenhouse study to total 150 seeds/plot and subsequently planted in the field. When the plants in the field reached the V3 stage of development, glyphosate was applied at 1.68 kg ae/ha to select GTS plants from non-GTS plants. Applications in the field were made using a CO_2-pressurized backpack sprayer with 8002 nozzles operated at 255 kPa that delivered 190 L/ha of the same glyphosate formulation used in the greenhouse studies. Data collected for the greenhouse study included plant count, percent germination, and fresh weight 14 days after germination. Data collected for field comparisons included plant count prior to glyphosate application and number of plants not affected by glyphosate application. The plant count data was used to calculate percent soybean mortality from the glyphosate treatment. The seed lots were examined in a completely randomized design in both the greenhouse and field study. Each hand mixed population was replicated 4 times, and each study was conducted twice.

Data were subjected to analysis of variance and means were separated by Fisher's Least Significant Difference Test ($P = 0.05$). No treatment by study interactions was detected; therefore, data from replicate studies were combined prior to ANOVA. Mixed population studies were subjected to Chi-square test for goodness of fit.

Table 1. Germination and Fresh Weight of Soybean Seed as Affected by Concentration of Glyphosate Soaking Solution and Duration of Soaking[a]

genotype	glyphosate solution	treatment time	plant count	germination	fresh weight
	%	hours		%	grams
TN01-299-RR (GTS)	0	4	8	80	11.3
		16	8	80	12.5
	0.5	4	8	80	10.2
		16	8	80	12.4
	2	4	8	80	12.2
		16	4	40	6.3
	5	4	8	80	12.5
		16	3	30	5.8
TN5601T (non-GTS)	0	4	9	90	16.5
		16	8	80	10.4
	0.5	4	4	40	5.4
		16	0	0	0
	2	4	0	0	0
		16	0	0	0
	5	4	0	0	0
		16	0	0	0
LSD (0.05)			2	20	2.0

[a] Means separated according to Fisher's Protected LSD test $P = 0.05$.

RESULTS AND DISCUSSION

Glyphosate concentrations of 0.5, 2, and 5% (0.01, 0.04, 0.1 M) prevented the development of non-GTS seed, while GTS seed developed normally (4 h soak time). Seeds soaked in control solutions, ones without glyphosate, developed normally for both soaking periods (**Table 1**). Percent germination for GTS seed was 80% for most glyphosate rates and 4 h soak intervals (**Table 1**). Fresh weight and percent germination declined for GTS seed soaked in 2 and 5% glyphosate solutions for 16 h. Non-GTS seed soaked in solutions containing glyphosate failed to develop (0% germination) except for seed soaked in 0.5% glyphosate solution for 4 h (40% germination). Affected non-GTS seedlings developed a 1−2 cm radical, which became necrotic and did not elongate. Results from this study indicated that soaking non-GTS seed in glyphosate solution prevented germination.

For glyphosate concentrations of 0.5−1% and soak times of 1, 2, and 4 h, all GTS seeds germinated and developed normally (**Table 2**). This characteristic is vitally important to the development of this procedure to ensure that GTS seeds are readily identified and only recessive individuals are killed. Non-GTS seed treated with 0.5% glyphosate solution displayed 80, 60, and 40% germination for 1, 2, and 4 h soak time, respectively. Fresh weight of GTS seedlings was not effected by glyphosate concentration or soak time. Non-GTS seed treated with 1% (0.02 M) glyphosate solutions for 1, 2, and 4 h displayed 70, 20, and 0% germination, respectively. A decline similar to percent germination in non-GTS seedling fresh weight was also observed.

Mixed populations of GTS and non-GTS responded to glyphosate application as expected (**Table 3**). Populations that were made up of seed that were glyphosate tolerant survived (100%) a 1.68-kg ae/ha glyphosate application. Non-GTS populations were completely killed (100%) from the same

Table 2. Germination and Fresh Weight of Soybean Seed as Affected by Concentration of Glyphosate Soaking Solution and Duration of Soaking[a]

genotype	glyphosate solution	treatment time	plant count	germination	fresh weight
	%	hours		%	grams
TN01-299-RR (GTS)	0	1	8	80	16.6
		2	9	90	18.4
		4	9	90	18.0
	0.5	1	8	80	18.5
		2	7	70	13.3
		4	8	80	16.9
	1	1	8	80	17.4
		2	8	80	17.0
		4	8	80	19.1
TN5601T (non-GTS)	0	1	9	90	19.7
		2	9	90	20.4
		4	9	90	20.3
	0.5	1	8	80	8.6
		2	6	60	5.5
		4	4	40	5.7
	1	1	7	70	6.1
		2	2	20	4.0
		4	0	0	0
LSD (0.05)			1	14	2.0

[a] Means separated according to Fisher's Protected LSD test $P = 0.05$.

Table 3. GTS and Non-GTS Mixed Populations Segregated in the Field by Application of Glyphosate at 1.68 kg ae/ha[a]

seed ratio	seed ratio	plants living	plants killed	% living	% killed	χ^{2b}
GTS	non-GTS					
100	0	130	0	100	0	0.00
75	25	97	36	73	27	0.21
50	50	64	71	47	53	0.36
25	75	32	101	24	76	0.05
0	100	0	131	0	100	0.00
LSD (0.05)[c]		8	7	5	5	

[a] GTS, glyphosate tolerant soybean; non-GTS, non-glyphosate tolerant soybean. [b] Uncorrected Chi-square goodness-of-fit test for segregation. None of the Chi-square values are significant at the 95% confidence level ($\chi^2_{0.05,\ 1d.f.} = 3.84$). [c] Means separated according to Fisher's Protected LSD test $P = 0.05$.

glyphosate application. Results for other mixed populations (GTS/non-GTS) were 3:1 (73% survival), 1:1 (53% survival), and 1:3 (24% survival). Data for all populations fit expectations (Chi-square > 0.05).

Greenhouse results that utilized a 1% glyphosate solution/ 4 h soak interval produced similar results to the field tests (**Table 4**). Populations that were GTS survived the soaking treatment (93%). Non-GTS populations were completely killed (100%) from the same glyphosate treatment. Results for mixed populations (GTS:non-GTS) were 3:1 (69% survival), 1:1 (43% survival), 1:3 (18% survival). Data for all populations fit expectations (Chi-square > 0.05) for the soaking treatment. The percentage of soybean seeds surviving the soaking treatment was lower due to variability of inherent germination of the soybean seeds. In the field study, 150 seeds were planted per row and counted after germination. This number was used as the total population number for percentage killed calculations. In the greenhouse study, 12 seeds were used in each population. It is possible that not all 12 seeds were viable, thus leading to lower germination numbers for the greenhouse study. However,

Table 4. GTS and Non-GTS Mixed Populations Segregated in Greenhouse by Soaking Seed in a 1% (0.02M) Glyphosate Solution for 4 Hours[a]

seed ratio	seed ratio	plants living	% germination	χ^{2b}
GTS	non-GTS			
100	0	11	93	0.08
75	25	8	69	0.44
50	50	5	43	0.33
25	75	2	18	0.44
0	100	0	0	0.00
LSD (0.05)[c]		1	9	

[a] GTS, glyphosate tolerant soybean; non-GTS, non-glyphosate tolerant soybean. [b] Uncorrected Chi-square goodness-of-fit test for segregation. None of the Chi-square values are significant at the 95% confidence level ($\chi^2_{0.05,\ 1d.f.} = 3.84$). [c] Means separated according to Fisher's Protected LSD test $P = 0.05$.

results from both studies fit Chi-square expectations. Only 12 seeds were used in the greenhouse study to simulate a breeder's approach to this screening procedure. Single plant harvest for development of individual breeding lines produce a limited seed supply, and only a small portion of the harvested seeds can be spared for screening to save as many seeds as possible for field increase.

Comparison of the two selection methods indicated that the greenhouse screening procedure was more efficient. The soaking method utilized four, 32 cell float trays (1.36 m^2 total), whereas the field study required 112 m^2 to collect the same data. Furthermore, the soaking screening method required approximately 19 days after planting to determine seed susceptibility to glyphosate. The field study took nearly 6 weeks (twice as much time) from planting to glyphosate application at the V3 growth stage, and then glyphosate did not fully manifest symptomology for an additional 10 d. The soaking method not only required less land resources and time but also determined which seed lines to plant in the field, saving valuable land resources where non-GTS seed would have been planted.

Results from this research demonstrate that EPSPS is clearly active during seed germination, because germination did not occur with non-GTS seed. Research conducted on glyphosate applications to non-GTS plants indicated subsequent seeds displayed reduced hypocotyl and primary root lengths and reduced shoot weight in 1 month old seedlings (10). Glyphosate applied to parent plant vegetative tissue translocated to the metabolic sink for seed production and remained active in the seed after harvest. In a separate study, 11 plant species were subjected to glyphosate application to determine the effect on developing seed. Effects from glyphosate application was apparent on 7 of the 11 species tested (11). Results from these studies combined with our results indicate that glyphosate is not only active at seed germination but is also stable inside seed from previous application to parent plant tissue.

A possible drawback to the greenhouse screening method is that seedling mortality induced by glyphosate could be confounded by less than complete germination of soybean seed lots. However, non-GTS seed can be examined after planting to determine if seeds were viable. Additionally, with the described method, viable non-GTS soybean seed will extend a radical prior to displaying affects of glyphosate treatment, while nonviable seed will not extend a radical. This indicated that nonviable seed can be differentiated from glyphosate affected non-GTS seed with this method.

This research conclusively demonstrates that transfer of the glyphosate tolerance gene in soybean can be detected in seed

by using a rapid economical greenhouse method. Deployment of this technique will ultimately lead to enhanced genetic gains in soybean breeding. This process will allow breeders to rapidly (1) confirm transfer of glyphosate tolerance gene in F_1 seed, (2) eliminate individuals not expressing the glyphosate tolerance gene in F_2, or subsequent BC inbreeding generations, (3) reduce the amount of tissue samples needed for molecular marker testing, and (4) reduce the amount of land resources required to develop and deploy a new GTS cultivar. This process is currently being used to help confirm CP4 gene activity in molecular marker assisted recurrent parent genome recovery of a GTS version of 5601T.

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