

Evaluation of Glyphosate Resistance in Transgenic Lettuce

Russell T. Nagata,¹ Joan A. Dusky,¹ Robert J. Ferl, Antonio C. Torres,² and Daniel J. Cantliffe
*Horticultural Sciences Department, University of Florida, Institute of Food and Agricultural Sciences,
1251 Fifield Hall, P.O. Box 110690, Gainesville, FL 32611-0690*

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ABSTRACT. Six transgenic ‘South Bay’ lettuce lines (*Lactuca sativa* L.) with elevated levels of 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) were evaluated for tolerance to the herbicide glyphosate. The six lines were selected from ≈150 independent transformation events using an *Agrobacterium tumefaciens* system. Three assay methods were used to identify gene expression with regard to glyphosate resistance. Leaf disks of the transgenic lines were cultured on media containing 0 to 1280 μM glyphosate. Leaf disks of the control had lower dry weight (DW) at 40 μM and greater glyphosate than all the transgenic lines. The transgenic lines continued to grow even at 1280 μM. Plants 21 days old were sprayed in the greenhouse with rates of glyphosate at 0 to 35.84 kg·ha⁻¹. DW of all the lines were similar to the control, with a few exceptions, at glyphosate concentrations from 0 to 0.56 kg·ha⁻¹. At 2.24 to 8.96 kg·ha⁻¹ all of the transgenic lines had DW greater than the control, while at 17.92 and 35.84 kg·ha⁻¹ only B-32, B-33, C-3, and C-14 had DW greater than the control. The resistant line from the greenhouse experiment, B-32, grew normally in field trials at the highest glyphosate rate, 17.92 kg·ha⁻¹, while control plants died at 0.56 kg·ha⁻¹ glyphosate. Lines A-11 and C-3 had lower DW than B-32 at 2.24 kg·ha⁻¹ glyphosate and greater. While leaf disk assays can identify potential transformed lines expressing the EPSPS and glyphosate oxidase (GOX) gene, and greenhouse screening can evaluate seedling vigor after glyphosate application, field trials are necessary to evaluate plant growth and yield through the growing season. Chemical name used: N-(phosphono-methyl) glycine (glyphosate).

Weed control in lettuce can amount to a significant cost of lettuce (*Lactuca sativa*) production, ranging from \$500 to \$1,975 per hectare (Dusky and Stall, 1996). At present, chemical, manual and/or mechanical means are used individually or in combination for control of weeds in lettuce. Each method has its benefit and shortcomings. Chemical control procedures are easy to use and do little to disturb the roots of the growing lettuce plants. However, rates of the approved herbicides, which do not cause phytotoxicity in lettuce, usually will not provide 100% control of all weeds (Dusky et al., 1988; Dusky and Stall, 1996). Also, the narrow window of efficacy versus phytotoxicity of some chemicals now in use can result in severe crop loss (Dusky and Stall, 1995). Manual or mechanical weed control procedures disturb the soil surface and potentially the crop roots. This negatively affects lettuce growth resulting in reduced yields and head quality (Shreffler et al., 1994).

Currently, standard procedures to evaluate crops for tolerance to a particular herbicide include evaluating efficacy of weed control at various application rates and correlating this to crop phytotoxicity. When herbicide efficacy and crop tolerance are identified, registration of the herbicide for the crop may be pursued. Torres et al. (1999) incorporated a 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) gene which confers tolerance to the herbicide glyphosate into the iceberg lettuce cultivar ‘South Bay’. The herbicide glyphosate (Round-Up) was identified for its broad spectrum effectiveness to control weeds and its low environmental impact to nontarget organisms. These transgenic lines were not evaluated for field resistance to glyphosate nor the horticultural characteristics of the

mature head after application of glyphosate. Selection of expressed tolerances can be affected by age of the plant material being tested. Different tests might result in different interpretations of herbicide tolerance based on the method performed. Gene number and position effect can all affect expression of the character under question, thus, potentially altering gene expression and/or normal plant growth. This paper reports results of laboratory, greenhouse, and field tests to determine glyphosate tolerance in several transgenic lettuce lines.

Materials and Methods

All transgenic lines were derived from ‘South Bay’ lettuce via an *Agrobacterium tumefaciens* mediated transfer of the 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) and glyphosate oxidase (GOX) genes using the procedure described by Torres et al. (1993). Two different *A. tumefaciens* vectors, pMON 17194 and pMON 17204 (Monsanto Chemical Co, St. Louis, Mo.) were used. Vector pMON 17194 (Kanamycin resistant gene, glyphosate oxidase, and EPSPS activity) was used in group A and C, while vector pMON 17204 (Kanamycin resistant gene, β-glucuronidase, glyphosate oxidase, and EPSPS activity) was used for group B. The numbered lines A-11, A-13, B-32, B-33, C-3, and C-14 were selected following preliminary screening procedures for resistance to glyphosate and the homozygous presence of the EPSPS and GOX genes (Torres et al., 1999). Nontransformed ‘South Bay’ was used as the control for all experiments reported in this paper. The source of glyphosate was Rodeo (Monsanto Chem. Co.), which is manufactured without a surfactant added. When a surfactant was required, Induce (Helena Chemical Co., Memphis, Tenn.) was added at the rate of 0.25% (v/v) to each solution.

LEAF DISK ASSAY. The fifth and/or sixth fully expanded leaf of 4-week-old ‘South Bay’ control plants and the six transgenic lettuce lines were excised near the stem, rinsed under running tap water and disinfected in 0.5% (v/v) sodium hypochlorite solution containing 0.05% (v/v) Tween 20 for 10 min. The leaves were

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¹University of Florida, Institute of Food and Agricultural Sciences, Everglades Research and Education Center, P.O. Box 8003, Belle Glade, FL 33430.

²Current address: EMBRAPA/CNPq, P.O. Box 218, Brasilia-DF, Brazil 70359-970.

Table 1. Dry weight of 28-d-old leaf disks of 'South Bay' (SB) lettuce (control) and six transgenic lettuce lines grown on MS media supplemented with varying rates of glyphosate herbicide. Data for all five experiments pooled.

Glyphosate (μM)	Transgenic line [dry wt (mg)]							
	SB	A-11	A-13	B-32	B-33	C-3	C-14	LSD ²
0	87	92	86	92	98	100	83	26
10	83	73	85	79	91	98	74	23
20	62	96	85	83	92	100	86	25
40	34	57	58	61	75	63	72	22
80	16	74	78	75	80	92	76	24
160	15	76	86	88	85	90	78	27
320	8	54	58	54	69	76	53	19
500	9	46	54	45	59	72	49	19
640	9	47	50	46	55	66	64	16
1000	8	63	60	79	70	77	66	18
1280	8	51	53	66	64	68	70	17

²Mean separation within columns by LSD, $P = 0.05$.

rinsed with sterile deionized water three times using enough water to cover the leaves in the beaker. A single row of leaf disks were cut with a 1-cm-diameter cork hole borer from the margins of individual leaves. Three leaf disks, each from a different leaf on the same line were placed into a 90-mm petri dish containing 20 ml media with 0, 10, 20, 40, 80, 160, 320, 500, 640, 1000, or 1280 μM active ingredient (a.i.) of glyphosate amine salts. The media also contained MS salts (Murashige and Skoog, 1962); Gamborg's B-5 vitamins (Gamborg et al., 1968); sucrose at 30.0 $\text{g}\cdot\text{L}^{-1}$; agar at 7.0 $\text{g}\cdot\text{L}^{-1}$; indoleacetic acid at 0.876 $\text{mg}\cdot\text{L}^{-1}$; and benzyladenine at 0.495 $\text{mg}\cdot\text{L}^{-1}$. Media pH was adjusted to 5.8 with 0.5 N HCl or KOH solution as needed. A stock solution of glyphosate was filter sterilized and added to the media just prior to filling the petri dishes. Individual petri dishes were sealed with parafilm to reduce the potential for contamination of the cultures.

The petri dishes containing the leaf disks were incubated at 26 ± 2 °C with a daily 14-h photoperiod of 2 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ provided by cool-white fluorescent lamps. After 4 weeks, the individual leaf disks were weighed, dried at 70 °C for 72 h and reweighed. The experiment was repeated a total of five times with two replications in each repeat. Data were subjected to analysis of variance procedures and means separated by Fisher's LSD (SAS Institute, Inc., 1996).

SEEDLING ASSAY. Two seeds from each line were sown in a single width-wise row of a Todd Planter flat (No. 125) filled with Fafard

#2 growing medium (C. Fafard, Springfield, Mass). The flats were watered and kept in a glass greenhouse at days/nights of 32/18 °C. At 7 d postsowing, individual cells in the flat were thinned to a single plant. At 21 d postsowing, seedlings from individual flats were sprayed with glyphosate at 0, 0.018, 0.035, 0.07, 0.14, 0.28, 0.56, 1.12, 2.24, 4.48, or 8.96, plus 0.25% v/v surfactant as in Expt. 1. To establish a better kill curve for the transgenic lines, two additional glyphosate concentrations, 17.72 or 35.84 $\text{kg}\cdot\text{ha}^{-1}$ were added, while the 0.018 and 0.035 concentrations were omitted from Expts. 2 and 3. Application of glyphosate solutions were made with a CO₂ backpack sprayer operated at 2.07×10^5 Pa, equipped with T-jet 8004 flat fan nozzles and applied above the plant canopy at the rate of 332 $\text{L}\cdot\text{ha}^{-1}$. Two weeks after treatment, five plants from the center of each row were harvested individually at the soil surface level and dried at 40 °C for 5 d. Sample dry weight (DW) was then recorded. The planting, treatment and harvest dates were 20 Oct. 1994, 9 Nov. 1994, 22 Nov. 1994; 6 Dec. 1994, 21 Dec. 1994, 4 Jan. 1995, 17 Jan. 1995, 8 Feb. 1995, and 22 Feb. 1995, respectively, for the three runs.

The experiment was repeated a total of three times, with four replications each time the experiment was conducted. The flats were arranged in a randomized complete block design for each experiment. Data were analyzed by Fisher's LSD.

WHOLE-PLANT ASSAY. 'South Bay' and the transgenic lines A-11, B-32, and C-3 were used for Expt. 1 and 'South Bay' and line B-32 were used for Expt. 2. Line B-32 was selected for Expt. 2 due to its

Table 2. Dry weight of 'South Bay' (SB) lettuce (nontransgenic) and six transgenic lettuce lines 14 d after glyphosate treatment of 21-d-old lettuce plants grown in a greenhouse.² Data from Expt. 3.

Glyphosate ($\text{kg}\cdot\text{ha}^{-1}$)	Transgenic lines [dry wt (mg)]							
	SB	A-11	A-13	B-32	B-33	C-3	C-14	LSD ²
0	303	216	219	318	263	267	283	115
0.07	449	320	282	348	336	423	388	79
0.14	353	292	293	401	389	449	468	88
0.28	272	352	368	281	238	301	320	104
0.56	331	397	405	466	340	463	519	88
1.12	188	342	410	251	249	378	395	66
2.24	121	284	260	237	169	242	292	43
4.48	103	354	365	427	341	309	499	83
8.96	73	191	260	324	216	250	366	70
17.92	78	140	144	372	198	229	430	69
35.84	76	137	115	301	149	147	324	64

²Mean separation within columns by LSD, $P = 0.05$.

Table 3. Dry weight of field-grown 'South Bay' (SB) lettuce (nontransgenic) and three transgenic lettuce lines treated with glyphosate herbicide at 21 d postsowing and harvested. Data from Expt. 2.

Glyphosate (kg·ha ⁻¹)	SB	Transgenic lines [dry wt (g)]			
		A-11	B-32	C-3	LSD ²
0	15.5	14.5	27.0	23.2	10.6
0.07	17.2	14.0	28.1	21.3	6.6
0.14	3.5	11.2	24.1	26.6	10.7
0.56	---	9.4	26.5	22.7	6.4
1.12	---	11.9	28.1	23.5	12.3
2.24	---	11.5	30.2	17.7	7.4
4.48	---	7.3	28.2	15.8	11.3
8.96	---	---	28.7	9.3	3.8
17.92	---	---	21.5	---	---

²Mean separation within rows by LSD, $P = 0.05$.

potential for tolerance to glyphosate and excellent horticultural qualities which were similar to the original 'South Bay'. Experiment 1 was conducted two times, each time with four replications, and was sown on 20 Oct. 1994 and 6 Feb. 1995. At 4 weeks postsowing, glyphosate treatments of 0, 0.14, 0.56, 1.12, 2.24, 4.48, 8.96, or 17.72 kg·ha⁻¹ were applied over the crop canopy. Application of herbicide solutions were made 30 cm above the plant canopy as described above. Five heads from the center of each plot were harvested 4 Jan. and 22 Apr. 1995, respectively, for each trial. The lettuce heads were trimmed to marketable size as defined by USDA standards (U.S. Dept Agr., 1973). The heads were chopped individually into 3 × 3 cm pieces or smaller and individual leaf layers separated by mixing. The samples were placed in brown paper bags and dried at 40 °C. The samples were mixed daily to promote faster drying of the leaves and prevent decay. When the leaves were dry (5 to 8 d), the samples were weighed.

Experiment 2 was conducted three times, with four replications each time it was conducted. Seeds were sown initially 2 Feb. 1996 and a second time 16 Feb. Experiment 2 was conducted similar to the first, except treatment rates of glyphosate were 0, 0.56, 1.12, 2.24, 4.48, or 17.72 kg·ha⁻¹ with 0.25% v/v surfactant. Spray timing and procedures were the same as mentioned previously.

All field plantings were conducted on a Pahokee muck (euic, hyperthermic Lithic Medisaprist) at the University of Florida, Institute of Food and Agricultural Sciences, Everglades Research and Education Center, Belle Glade. Guidelines for conducting field plantings of transgenic plant material were followed according to U.S. Dept. of Agriculture and University of Florida Institutional Biosafety Committee Guidelines. Culture and management of the lettuce crop followed standard practices for the Everglades Agricultural Area (EAA). Fertilizer inputs were based on preplant soil test recommendations (Hochmuth et al., 1994). All plantings were made on 15 cm raised beds on 90 cm centers. Each plot consisted of a 6.1 m section of bed. Two lettuce seeds were planted every 30 cm in a single row down the center of each bed by hand. The plots were arranged in a randomized complete block design. At the four-leaf stage the seedlings were thinned to a single plant. Pest and disease problems were controlled following recommendations in the Vegetable Production Guide for Florida (Maynard and Hochmuth, 1995).

Five heads from each plot were harvested 10 and 30 Apr. 1996 for run one and two, respectively, and trimmed to marketable heads (U.S. Dept. of Agriculture, 1973). FW (all above ground green leaves and head) and fresh trimmed marketable head weights were recorded as yield then the heads were dried and weighed as described previously.

Results and Discussion

LEAF DISK ASSAY. Leaf disk DWs were significantly reduced in the control at 20 μM glyphosate when compared to lines A-11, B-33, and C-3 (Table 1). Similar weight reductions in control disks continued at glyphosate levels >40 μM when compared to all the transgenic lines. Transgenic lines had only slight differences among the lines in DWs when leaf disks were grown at all glyphosate levels >40 μM.

For all transgenic lines, the highest glyphosate concentration (1280 μM) was not lethal to the leaf disks. Control leaf disks were killed at 320 μM glyphosate and higher. All transgenic lettuce leaf disks survived all levels of glyphosate with callus growth around the margins of the disks. At lower glyphosate concentrations (<320 μM) the callus mass covered the entire surface of the disk and shoots were seen growing from the callus mat. This indicated that a concentration of glyphosate >320 μM has inhibitory effects in organogenesis preventing shoot differentiation. However, callus growth continued in medium containing up to 1280 μM glyphosate. The differences in response of dry matter accumulation among those lines suggested distinct categories of transgene tolerance to glyphosate concentration.

SEEDLING ASSAY. Three experiments were conducted with seedlings grown in the greenhouse. Glyphosate levels in the second and third experiments were increased to 35.8 kg·ha⁻¹ glyphosate in an attempt to establish greater differences in DW among the transgenic lines. Since trends for all three experiments were similar, only the results from Expt. 3 containing the highest glyphosate concentration applied will be discussed (Table 2). Seedling DWs in the nontransgenic seedlings were less than those from lines C-3 and C-14 at 0.14 and 0.56 kg·ha⁻¹ glyphosate. At 1.12 kg·ha⁻¹ glyphosate, control DW was less than that from lines A-11, A-13, C-3, and C-14. At 2.24 to 8.96 kg·ha⁻¹ all transgenic lines had greater DWs than the control. At glyphosate levels above 8.96 kg·ha⁻¹ only the B and C lines maintained DWs greater than the control. The transgenic lines B-32 and C-14 retained the greatest DW values at the highest glyphosate concentration (35.8 kg·ha⁻¹).

WHOLE-PLANT ASSAY. For weed control using glyphosate in a commercial lettuce field at the three to four leaf stage, it is estimated that application rates of 0.28 to 0.56 kg·ha⁻¹ are needed for control of annual weeds and 0.56 to 1.12 kg·ha⁻¹ for perennial weeds. Plant vigor before glyphosate application measured as accumulated DW was similar among lettuce lines A-11, C-3, and the control (Table 3). DWs of control plants were unaffected by glyphosate application at the 0.14 kg·ha⁻¹ rates compared to lines A-11 and C-3. Line B-32 retained significantly greater DWs than all the other lines at 0 and

Table 4. Fresh weight, fresh trim, and trim dry weight of 'South Bay' lettuce (control) and transgenic lettuce line B-32 at harvest maturity treated with different rates of the herbicide glyphosate. Plants treated 21 d postsowing.

Glyphosate (kg·ha ⁻¹)	Fresh wt (g)			Trim wt (g)			Dry wt (g)		
	SB	B-32	LSD	SB	B-32	LSD	SB	B-32	LSD
0.0	753.5 ^z	672.8	68.1	531.2	506.0	47.7	21.5	21.6	1.9
0.56	11.8	730.5	39.7	NA ^y	537.3		NA	21.4	
1.12	Dead	681.5		NA	482.4		NA	19.4	
2.24	Dead	723.2		NA	514.0		NA	21.3	
4.48	Dead	763.6		NA	559.1		NA	21.7	
17.92	Dead	765.9		NA	563.6		NA	23.0	

^zMeans separation within columns by LSD, *P* = 0.05.

^yNot available.

0.07 kg·ha⁻¹ glyphosate. All control plants were killed at glyphosate rates \geq 0.56 kg·ha⁻¹ and higher. Transgenic lettuce line B-32 was unaffected by glyphosate applications as high as 17.92 kg·ha⁻¹. DWs of line A-11 at the 0.14 to 1.12 kg·ha⁻¹ rates were less than those of the B-32 and C-3 lines, and at glyphosate rates of 8.96 and 17.92 kg·ha⁻¹, A-11 plants were killed.

In the second field experiment, line B-32 and 'South Bay' were compared directly for total DW, marketable trimmed head weight, and DW of marketable trimmed heads. Control plants were killed by all levels of glyphosate applied, while the B-32 line survived all glyphosate levels up to 17.92 kg·ha⁻¹ (Table 4). Fresh and trim weights of B-32 treated with glyphosate at 4.48 or 17.92 kg·ha⁻¹ were in fact, greater than the control. None of the plants treated with glyphosate appeared morphologically different than the nontreated control plants.

Torres et al. (1999) have reported the usefulness of the leaf disk assay for screening glyphosate resistance in transgenic lettuce lines. In the present work, DWs of control leaf disks were reduced by glyphosate while several transgenic lines, namely B-32 and C-3, were unaffected by fairly high levels of glyphosate. Growth of A-line leaf disks was generally adversely affected by glyphosate in the media. These same comparisons were observed from the greenhouse seedling assay and in the field assays. Although better than the control, the vigor and glyphosate resistance of the A lines were generally less than the B and C lines. The A and C lines had the same vector, pMon 17194.

Thus, initial screening for glyphosate resistance in transgenic lines can be accomplished by leaf disk assay. However, in order to better predict field performance, the greenhouse screen can give further evidence to seedling vigor as well as survival of the transgenics. After using the proper selection protocol, the more costly field trials must be conducted in order to ascertain yield and quality traits of the new transgenic lines, as well as efficacy of the glyphosate plant herbicide resistance system. In the present work, glyphosate-tolerant lines of lettuce with the horticultural characteristics of the common commercial cultivar 'South Bay' for the EAA were identified.

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