In vitro Assay Selection of Glyphosate Resistance in Lettuce

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ABSTRACT. Glyphosate-resistant plants of 'South Bay' lettuce (*Lactuca sativa* L.) were produced by using *Agrobacterium tumefaciens* containing a plasmid carrying glyphosate oxidase and EPSPS gene. An in vitro assay was performed to determine the sensitivity of 'South Bay' leaf discs and seedling explants to varying glyphosate concentrations. The I_{50} for glyphosate leaf discs was 53.8 μM and for glyphosate seedlings 7.6 μM . There was a high correlation between the response of leaf discs and seedlings to glyphosate based on dry weight. These findings will allow identification of glyphosate-resistant transformants in an early stage of plant development, saving time and reducing the cost in generating an improved cultivar with the glyphosate resistance trait.

Glyphosate is a systemic, nonselective, postemergence herbicide that is rapidly translocated from foliage to the roots, rhizomes, and apical tissue of treated plants (Franz et al., 1997). Once inside the plant, glyphosate interferes with aromatic amino acid biosynthesis by inhibiting the activity of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a key enzyme of the shikimate pathway (Amrhein et al., 1980; Bradshaw et al., 1997; Franz et al., 1996; Herrmann, 1995; John, 1997; Mousdale and Coggins, 1984; Wang et al., 1991).

The development of glyphosate-resistant crops could provide an effective tool to nonselectively control problem weeds without injuring the crop species. Further, glyphosate breaks down in the soil, does not bioaccumulate and has a very low level of toxicity in mammals, birds, and fish. It is, therefore, considered to be environmentally safe (Franz et al., 1997). Simple selection of plants on glyphosate, with or without mutagenesis agents, has not been successful in generating new glyphosate-resistant crops (Bradshaw et al., 1997). However, the ability to transform plants using *Agrobacterium tumefaciens* (Smith and Townsend) Ti plasmids has made it possible to increase plant tolerance to glyphosate by incorporating an EPSPS gene in the nuclear genome of the host as well as amplification of the gene already in place (Comai et al., 1985; Fillatti et al., 1987; John, 1997; Jordan and McHughen, 1988; Shah et al., 1986; Wang et al., 1991).

Effective, inexpensive weed control in lettuce remains a major goal for lettuce produced on organic soil (Dusky et al., 1988). A fast, efficient and reproducible system for lettuce transformation has been developed (Torres et al., 1993). In this report we determined the sensitivity of 'South Bay' lettuce to glyphosate in order

Fig. 1. In vitro response of glyphosate on leaf disc and seedling explants of lettuce. (A) The inner rings are nontransformed leaf discs. The outer discs are from line 60 showing a glyphosate resistance putative transformant. The leaf discs growing at 800 μM glyphosate were from line 60 only. (B) 72-h-old nontransformed seedlings growing in glyphosate. Numbers represent glyphosate concentration in μM .

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to develop a nondestructive biotest for selection of lettuce transformants tolerant to glyphosate in their early stages of plant development.

Materials and Methods

SENSITIVITY OF LETTUCE EXPLANTS TO GLYPHOSATE. Leaf discs excised from 30-d-old plants grown under greenhouse conditions ($25 \pm 3^\circ\text{C}$ and 11 h natural daylength light) were used as explants. The explants were disinfected with 0.5% sodium hypochlorite (10 $\times$ diluted bleach) containing two drops of Tween 20 per 100 mL solution. After disinfection, the solution was decanted and the explants were rinsed three times with sterile distilled water. Next, explants were inoculated in 90-mm petri dishes containing callus induction medium consisting of MS (Murashige and Skoog, 1962) salts and (in $\text{mg}\cdot\text{L}^{-1}$): sucrose 30,000; i-inositol 100; thiamine-HCl, 1.0; pyridoxine-HCl, 0.05; nicotinic acid, 0.05; glycine, 2.0; naphthaleneacetic acid (NAA), 0.05; benzyladenine (BA), 0.2 and Phytagar (Sigma) 7,000. Glyphosate was cold sterilized using Pro-X 0.22 μm membrane filtration and was then added to the medium at the following μM concentrations: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 300, and 400. The cultures were illuminated 16 h per day with $62\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of light at 25°C . The experiment was arranged as a randomized complete block design with four replications and repeated twice. Each replication consisted of a single 90-mm petri dish containing five leaf discs. The discs were

allowed to form callus and the callus was harvested at 28 d. Callus fresh weight was recorded. Callus was then dried for 72 h at 70°C and dry weight was measured.

Analysis of variance was used to determine if there were interactions between glyphosate concentrations across each experiment, so data for the two experiments could be combined. Data were transformed to percent inhibition compared to a 0 μM glyphosate control and regression analysis was used to determine the concentration of glyphosate which caused a 50% reduction in growth (I_{50}). The data were linearized by plotting percent inhibition versus the $\log_e$ of treatment concentration (Bewick et al., 1990). Linear and quadratic models were developed. The most appropriate regression model was selected based on R^2 values and plots of residuals. A 95% confidence interval (CI) for I_{50} was established according to previously published methodology (Draper and Smith, 1981).

Sensitivity of lettuce seedlings to glyphosate. ‘South Bay’ lettuce seeds were germinated aseptically on a filter paper platform in a sterile 250-mL Erlenmeyer flask with the base of the platform immersed in 25 mL of sterile nutrient solution containing MS salts (Murashige and Skoog, 1962). The cultures were illuminated 16 h per day with $62\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light and exposed to constant 25°C temperature. Two-day-old seedlings were harvested and transferred to medium containing MS salts, vitamins, 3% sucrose, 0.7% Phytagar (Sigma) and glyphosate at 0, 20, 40, 60, 80, 100, 200, 400, 600, 800, and 1000 μM .

Table 1. Effects of glyphosate concentration on inhibition of leaf and seedling dry weight. These data were used to calculate Pearson correlation coefficient.

Glyphosate concn (μM)	Leaf dry wt (mg)	Inhibition (%)	Seedling dry wt (mg)	Inhibition (%)
0	360.6	100.0	220.8	100.0
20	222.1	61.6	100.1	45.4
40	213.5	59.2	67.4	30.5
60	180.4	50.0	52.9	24.0
80	153.2	42.5	35.5	16.1
100	155.7	43.2	48.5	22.0
200	54.9	40.2	29.2	13.2
400	26.0	7.2	22.2	10.1
600	13.1	3.6	23.4	10.6
800	14.6	4.1	16.9	7.7
1000	11.5	3.2	20.4	9.3

Correlation analysis

Variable	N	Mean	SD	Sun
Leaf	11	37.70545	30.88643	414.7600
Seedlings	11	26.24364	26.94672	288.6800
		Minimum		Maximum
Leaf		3.20000		100.0000
Seedling		7.65000		100.0000

Pearson correlation coefficient/ $P > H_0$: $\text{Rho} = 0/N = 11$

	Leaf	Seedling
Leaf	1.000	0.87110
	0.0	0.0005
Seedling	0.87119	1.0000
	0.0005	0.0

Pearson correlation coefficient = 0.87

Table 2. The I_{50} concentration of glyphosate (in μM) that causes 50% of growth inhibition in callus formation from leaf disc of GUS positive transgenic lettuce.

Plant no.	I_{50}
3	129 (109–304) ^z
12	26 (1–78)
44	852 (752–997)
60	958 ^y

^zNumbers in parentheses denote 95% confidence interval limits.

^yConfidence interval could not be calculated because the most appropriate model was a quadratic with a plateau (SAS/STAT).

Fig. 2. Staining of GUS-positive R_1 seedlings from selfed R_0 line 60 plant.

Table 3. Segregation of GUS staining and glyphosate resistance in R_1 seedlings.

R_0 plant no.	GUS ⁺ , glyphosate resistant	GUS ⁻ , glyphosate sensitive	χ^2	p
44	23	7	0.040	0.75–0.90
60	40	12	0.1025	0.50–0.75

The response of ‘South Bay’ leaf tissue to the presence of glyphosate in the callus medium was correlated to the response of newly germinated ‘South Bay’ seedlings (2-d-old) grown with glyphosate in the germination medium (MS salts, 3% sucrose and 0.7% phytagar). The experiment was arranged in a randomized complete-block design with four replications and was repeated twice. Each replication consisted of 5 seedlings growing in a single 90-mm petri dish. The cultures were illuminated 16 h per day with $62 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light and exposed to constant 25 °C temperature. The seedlings were maintained 30 d on glyphosate medium, when fresh and dry weights were recorded.

Analysis of variance was used to determine interactions between glyphosate concentration and each experiment. Regression analysis was conducted as before, an I_{50} calculated, and 95% CI was constructed. Callus dry weight from the previous experiment was correlated to seedling dry weight.

SENSITIVITY OF TRANSFORMED GLYPHOSATE-RESISTANT PLANTS TO GLYPHOSATE. *Agrobacterium tumefaciens* ABI carrying plant transformation vector pMON 17204 containing NPT II, β -glucuronidase (GUS), glyphosate oxidase, and EPSPS activity was used to produce transgenic lettuce. The transformation procedure was the same described by Torres et al. (1993). Putative transformants

for glyphosate resistance were selected based on positive expression of GUS. Standard callus medium containing glyphosate was prepared as before. The glyphosate concentrations used in these experiments were: 0, 250, 400, 500, 600, 800, and 1000 μM . Data were analyzed as previously described. An I_{50} with 95% CI was calculated for each transformant, when possible.

Results and Discussion

Glyphosate inhibited callus growth in nontransformed lettuce leaf discs (Fig. 1A, inner rings). No growth was observed at 250 μM of glyphosate and cellular necrosis appeared around the cut surface of the leaf discs. In contrast, the transformed discs (outer ring) grew on glyphosate medium up to 1000 μM of glyphosate while control discs died. Growth of nontransformed seedlings decreased with increasing glyphosate concentration up to 100 μM (Fig. 1B), thereafter growth stopped. At 200 μM of glyphosate the cotyledons lost green color (chlorophyll) and started dying.

The sensitivity of lettuce explants and 2-d-old seedlings to glyphosate was measured in terms of the predicted concentration of glyphosate (in μM) that caused a 50% growth inhibition (I_{50}). For ‘South Bay’ lettuce leaf discs the I_{50} was 53.8 μM glyphosate. The 95% CI was 46.8 to 61.2 μM . The I_{50} for glyphosate calculated for ‘South Bay’ seedlings was only 7.6 μM . The 95% CI was 1.2 to 16.7 μM . Although there was a substantial difference between the I_{50} ’s for leaf discs and seedlings, 53.8 and 7.6 μM , respectively, there was a high correlation between the response of leaf discs and seedlings to glyphosate based on dry weight (0.87) calculated from the results presented in Table 1. This indicated that the leaf disc assay portrayed an accurate prediction of the response of ‘South Bay’ seedlings to glyphosate. Because of this correlation transformants could be tested for relative glyphosate resistance, in an early stage of development, using the leaf disc assay.

Four putative transformants expressing the GUS gene were selected for in vitro determination of glyphosate resistance. Results based from the I_{50} biotest gave rise to at least two categories of transformants (Table 2). The first included GUS positive and glyphosate sensitive plants. The appearance of the GUS reporter gene in plant 12 was not indicative of cotransformation with the glyphosate resistance gene. This finding indicated that the expression of GUS activity and kanamycin resistance was not always accomplished by the expression of the presence of the glyphosate resistance trait in the same T-DNA. This could be due to gene silencing resulting from transcriptional or post-transcriptional inhibition (Jelenkovic and Billings, 1998; Ko et al., 1998). The second group of plants were GUS positive and glyphosate resistant, i.e., plants 3, 44, and 60. A molecular procedure should be developed to positively demonstrate the incorporation, transcription and/or translation of the glyphosate resistance transgene in the future before the costly and time consuming process of progeny evaluation is begun.

The in vitro assay described here also provided a tool for ranking transformed plants in relation to glyphosate resistance. The higher the I_{50} value, the higher is the level of tissue tolerance (Escorial et al., 1996). Of the four plants described, only plants 44 and 60 would be selected for further greenhouse and field evaluation. R_1 progeny from those plants had GUS activity (Fig. 2) that was linked to glyphosate resistance. The chimeric construct was genetically stable and the segregation pattern suggested inheritance of GUS and glyphosate genes as a single dominant trait as shown in Table 3.

A further benefit of such an assay is the ability to predict how a transformant might potentially respond to a given concentration

of glyphosate. Transformants were compared at the I_{50} level because the accuracy of the prediction at this point. Once the regression model is selected, the concentration of glyphosate that will cause inhibition at other levels can be predicted. This information then might be useful in order to rank independent transgenic lines then to field test only the top performers.

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