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Bioassay for detection of glyphosate or kanamycin resistance in lettuce plants.

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ABSTRACT

Transgenic plants of lettuce cv. 'South Bay' (tolerant to herbicide glyphosate and kanamycin) were produced by using *Agrobacterium tumefaciens*. A biotest was developed using aseptically grown seedlings in medium with glyphosate or kanamycin. Glyphosate and kanamycin did not affect germination of transgenic and non transgenic seeds. Transgenic seedlings had differentiation and growth of roots (tap and secondary roots). The process of root differentiation and growth in non-transgenic seedlings was inhibited. This assay provided an effective and inexpensive biotest for identification of seeds of transgenic lettuce resistant to either glyphosate or kanamycin.

***Keywords**: Lactuca sativa*; biotest; transgenic selection; GMO; transgenic test.

RESUMO

Bioensaio para detecção de plantas de alface resistentes ao glifosato e à canamicina.

Plantas transgênicas de alface cv. South Bay (tolerantes ao herbicida glifosato e à canamicina) foram produzidas utilizando *Agrobacterium tumefaciens.* Um bioteste foi desenvolvido utilizando-se plântulas crescidas em meio com glifosato ou canamicina. Glifosato e canamicina não afetaram a germinação das sementes transgênicas e não transgênicas. Plântulas transgênicas apresentaram diferenciação e crescimento das raízes (principal e secundárias). O processo de diferenciação e crescimento de raízes em plântulas não transgênicas foi inibido. Este bioensaio apresenta-se como um teste eficiente e de baixo custo para identificação de sementes de plantas transgênicas de alface resisitentes a glifosato e canamicina.

***Palavras-chave*:** *Lactuca sativa*; bioteste; seleção transgênica; OGM

Glyphosate is a systemic and non selective herbicide used to control weeds in several crop plants. Glyphosate interferes with aromatic 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; John, 1997; Singh and Shaner, 1998). Kanamycin is an antibiotic aminoglycoside produced by *Streptomyces kanamyceticus* (Windholz, 1983) and interferes with protein synthesis by binding to the 30S ribosomal subunit, which causes misreading of genetic code (Ellsworth *et* al., 1998). Glyphosate and kanamycin are generally used as selection markers for evaluation of transgenes in genetically modified organisms (GMO). The identification of transgenes is necessary in breeding programs and in selection of homozygous lines. In general, GMO identification tests are performed by DNA analysis using high technology laboratory, using trained personal and sophisticated equipment. In this article we describe an effective and inexpensive biotest useful for detection transgenic lettuce seeds resistant to either glyphosate or kanamycin.

MATERIAL AND METHODS

Lactuca sativa L. seeds cv. South Bay were disinfested by immerging in 0.5 % sodium hypochlorite solution, for 20 min. The seeds were then rinsed with autoclaved distilled water and aseptically germinated in medium containing MS salts (Murashige and Skoog, 1962), 0.2% phytagel, sucrose (0 or 3%) and glyphosate (0; 50; 100 or 200 μM). For kanamycin tolerance, the seeds were germinated in medium containing 0; 50 or 100 mg/L of this antibiotic. The pH of all media was adjusted to 5.7. The cultures were grown at 27°C, 16-hour photoperiod at a light intensity of 62 $\mu mol.m^{-2}.s^{-1}$. Seed germination, tap root length and number of secondary roots were evaluated at 13 days after sowing. The experiment was arranged as a randomized complete design with ten replications per treatment. Each replication consisted of a single 25X150 mm test-tube with two seeds in each. The number of germinated seeds was recorded. F test was performed for evaluation variances homogeneity then, specific mean comparisons between treatments (Transgenic X Non-transgenic) were analyzed using Student's test, at 5% of significance (Snedecor, 1946). Data on glyphosate or kanamycin concentration and genotype were subject to analysis of variance, in factorial model, using MSTATC guide for personal computers (Version 2.10, Michigan State University, MI). Adjusted means and LSD for root growth and number of secondary root were calculated.

RESULTS AND DISCUSSION

Effect of ghyphosate on germination and in root growth

Germination was normal and no differences were observed between non-transgenic and transgenic seedlings in different glyphosate concentrations (Table 1). These results are in agreement with those of Nascimento *et al*. (2000), where the number of germinated seeds of transgenic and non-transgenic *Glycine max* was not affected by glyphosate up 200 μM. Measurements of tap root length and the number of secondary root formed were taken to evaluate root development. These parameters estimate the ability of the root system to develop in a particular substrate and how this growth affects meristematic activity (Lynch, 1995). Variance analysis for tap root growth and number of secondary root (Table 2) showed interaction between glyphosate and genotype. In addition, no statistic difference in root growth between transgenic and non transgenic seedlings was observed in medium without glyphosate (Table 2).

In non-transgenic seedlings glyphosate inhibited root growth and the final length of tap roots decreased in glyphosate concentration ranging from 50 to 200 μM (Table 2). No secondary root formation was observed in medium with glyphosate at 10 days. At 30 days secondary root development was poor. Glyphosate has been reported to inhibit root growth in *Glycine max* seedlings (Nascimento *et al*., 2000). Also, Torres *et al*. (1999) showed repression of morphogenetic events by glyphosate using *in vitro* leaf disc assay. These authors observed an inhibition of callus growth in non transgenic *Lactuca sativa* leaf discs in glyphosate medium. In contrast, leaf discs from transgenic plants grew on glyphosate medium up to 800 μM.

Transgenic seedlings grew normally in medium containing glyphosate up to 200 μM. At 13-days, no statistical difference was observed in both length of tap root and the number of secondary roots (Table 2).

Addition of sucrose did not affect germination and no differences were observed between non-transgenic and transgenic seedlings in different glyphosate concentration (Table 1).

Usually, seed with high percent of germination and vigor is necessary to apply this bioassay since low vigor seeds affect seedling development and the interpretation of the data become obscure.

Effect of Kanamycin on germination and in root growth

Similar to the addition of glyphosate, germination of transgenic and non transgenic seeds was not affected by an exogenous supply of kanamycin (Table 3). After 13 days kanamycin inhibited tap root growth of non transgenic seedlings and no secondary root formation occurred (Table 4). Kanamycin has been used as selective marker for transformants selection of several plants species (Jin *et al*., 2000; Ko *et al*., 1998; Torres

et al., 1993, 2000). This substance has been established as having inhibitory effect in morphogenetic process on *in vitro* culture of susceptible cell, tissue or organ explant. In contrast, transgenic seedlings grew normally in medium with kanamycin. Both, the length of tap root and secondary root formation were not inhibited in medium with kanamycin up to 100 $mg.L^{-1}$ (Table 4)..

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Table 1. Seed germination of south bay lettuce transgenic and non-transgenic in media containing ms salts or ms salts with 3% sucrose at various glyphosate levels.

Glyphosate concentration (μM)	**Genotype**	Germination (%) in MS salts medium [1]	Germination (%) in MS salts medium with 3% Sucrose [1]
0	Non-transgenic	80 [ns]	80 [ns]
	Transgenic	90	90
50	Non-transgenic	90 [ns]	90 [ns]
	Transgenic	80	85
100	Non-transgenic	100 [ns]	100 [ns]
	Transgenic	90	90
200	Non-transgenic	95 [ns]	95 [ns]
	Transgenic	80	85

[1]t test, [ns] nonsignificant at 1%

Table 2. Variance analysis and least significant difference test for evaluation tap root length and number of secondary roots within each level of glyphosate of 13-day-old lettuce seedlings (transgenic and non-transgenic) growing in media containing ms salts.

Glysphosate concentration (μM)	**Genotype**	**Tap root length Cm**	**Number of secondary root**
0	Non-transgenic	1.760	1.220
	Transgenic	1.744	1.242
50	Non-transgenic	1.340	1.063
	Transgenic	1.685	1.224
100	Non-transgenic	1.257	1.000
	Transgenic	1.697	1.167
200	Non-transgenic	1.196	1.000
	Transgenic	1.649	1.213
	LSD (0.01)	0.09900	0.07484

LSD 1%

Table 3. Seed germination of south bay lettuce transgenic and non-transgenic in media containing ms salts at various kanamycin levels.

Kanamycin concentration (mg. l^{-1})	**Genotype**	Germination (%) [1]
0	Non- transgenic	100 [ns]
	Transgenic	75
50	Non- transgenic	95 [ns]
	Transgenic	90
100	Non- transgenic	75 [ns]
	Transgenic	90

[1] t test, [ns] nonsignificant at 1%

Table 4. Variance analysis and least significant difference test for evaluation tap root length and number of secondary roots within each level of kanamycin of 13-day-old lettuce seedlings (transgenic and non-transgenic) growing in media containing ms salts.

Glysphosate concentration (μM)	**Genotype**	**Tap root length**	**Number of secondary root**
0	Non-transgenic	1.676	1.354
	Transgenic	1.582	1.243
50	Non-transgenic	1.260	1.000
	Transgenic	1.587	1.256
100	Non-transgenic	1.289	1.000
	Transgenic	1.659	1.176
	LSD (0.01)	0.0844	0.0755

LSD at 1%