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Genetic Resources

In Vitro Selection of a Glyphosate-Tolerant Sugarcane Cellular Line

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Abstract. Cell suspensions derived from *Saccharum* spp. varieties (PR62258, V64-10, V71-51) were used to study the effects of glyphosate on cell suspensions of commercial cultivars of sugarcane in order to select and develop a glyphosate-tolerant cellular line. A liquid medium with 0.8 mM/L glyphosate was used for selection. The results showed growth inhibition of approximately 50% for cell suspensions in mediums with 0.8 mM/L glyphosate. The results obtained in vitro were consistent with the results reported from in vivo tests. Plants were regenerated from sensitive cell suspensions and tolerant cell suspensions from cultivar V71-51. Tolerant cell suspensions tolerated a concentration that was 12.5-fold higher than that of the sensitive cell suspensions. Plants regenerated from tolerant cell suspensions tolerated a glyphosate concentration that was 6-fold higher than that of the plants regenerated from sensitive cell suspensions. Cell suspensions derived from tolerant regenerated plants tolerated a glyphosate concentration that was 5-fold higher than that of those derived from sensitive regenerated plants. The RAPD patterns obtained with OPA-07 revealed a 564-bp band that can be used to characterize the tolerant cellular line. This band is not present in the sensitive cellular line. Consequently, the tolerance persisted throughout the differentiation into plants and dedifferentiation into cell suspensions. This performance suggests that an in vitro selection of pre-existing variability has taken place.

Key words: cell suspensions, herbicide tolerance, plants regenerated, RAPD, *Saccharum* spp.

Abbreviations: EPSP synthase, 5-enolpyruvyl-shikimate-3-phosphate synthase; RAPD, random amplification of polymorphic DNA.

Introduction

Sugarcane (*Saccharum* spp.), a large grass of the Gramineae family, is highly heterozygous and heterogeneous clonally propagated and is characterized by a high degree of polyploidy. Sugarcane is a crop of major importance, providing about 65% of the sugar produced in the world. Relatively few pesticides are used on sugarcane crops. Herbicides are now used as the main method for weed control (Moore, 1987).

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Glyphosate (N-[phosphonomethyl]glycine) is a broad-spectrum, non-selective herbicide that has little residual soil activity and is nontoxic to animals. Glyphosate inhibits aromatic amino acid synthesis. It is a potent inhibitor of 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSP synthase, EC. 2.5.1.19) (Duke, 1987). Glyphosate applied in sugarcane field experiments caused a 44% yield reduction under applications of 0.2-0.4 kg/ha (Richard, 1991).

Induction of resistance to diseases, herbicides, and pesticides through tissue culture has provided potentially useful strains of plants with specific capability to endure the test compounds (Hughes, 1983). Plant cell cultures tolerant to glyphosate have been previously isolated by means of in vitro selection in different species, including carrot (Nafziger et al., 1984), *Corydalis sempervirens* (Smart et al., 1985), tobacco (Singer and McDaniel, 1985; Dyer et al., 1988), tomato (Smith et al., 1986), petunia (Steinrucken et al., 1986), and *Cichorium intybus* (Sellin et al., 1992). Tolerant plants have been regenerated from tobacco (Singer and McDaniel, 1985; Jones et al., 1996), *Cichorium intybus* (Sellin et al., 1992), and soja (Padgette et al., 1997).

Various molecular marker types can be used to detect genetic changes related to tolerance. We chose random amplification of polymorphic DNA (RAPD) (Williams et al., 1990) because it allows random amplification of DNA sequences throughout the entire genome. RAPD polymorphisms result from a nucleotide base change, i.e., an insertion or deletion that alters the primer-binding site (Williams et al., 1993). These products of amplification can be polymorphic and are used as genetic markers (Tingey and del Tufo, 1993).

Here we report the effects of glyphosate on cell suspensions of commercial cultivars of sugarcane to select development and characterize a glyphosate-tolerant cellular line.

Material and Methods

Establishment of cell suspensions

Cultivated sugarcane varieties V64-10, PR62258, and V71-51 were used in this study. Calli were established from immature leaf explants that were cultured as described above (Zambrano et al., 1999). To initiate cell suspensions, the calli-covered explants were transferred to 50 mL of liquid medium in 250-mL Erlenmeyer flasks. The liquid medium composition was the same as the callus-inducing medium, except that 500 mg/L hydrolyzed casein and 30 g/L sucrose were added and arginine and agar were omitted. The flasks were placed in a gyratory shaker at 25°C with a photoperiod of 16 h light (30-40 $\mu E/m^2 s$) and 8 h dark. The medium was changed every 5 days.

Herbicide testing

Cell suspensions of approximately 5×10^6 cell/mL from the 3 cultivars under study were inoculated into the same liquid medium that was used for the establishment of cell suspensions, except that hydrolyzed casein and 2.4-D were omitted and 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mM/L glyphosate were added. Cell suspensions were incubated to 25°C with a photoperiod of 16 h light

(90-100 μE/m^2s) and 8 h dark. The growth was measured after 3 weeks of incubation.

Selection of glyphosate-tolerant cell suspensions

Cell suspensions from sugarcane cultivar V71-51 were selected for this study. These were inoculated into the same liquid medium that was used for the herbicide testing, supplemented with 0.8 mM/L glyphosate (selective liquid medium [SM]), or without herbicide (nonselective liquid medium [NSM]). The aforementioned growth conditions were maintained.

Plant regeneration

Cell suspensions developed into NSM (cell suspension sensibles [SS]) and SM (cell suspension tolerant [TS]) were inoculated for 3 weeks into NSM, except that 500 mg/L hydrolyzed casein was added. These suspensions were transferred to the same solid medium as for callus induction, but the 2.4-D was diminished to 1 mg/L and 0.5 mg/L benzyl amino purine was added. The suspensions were maintained in complete darkness during the first 3 weeks and then transferred to a growth chamber with environmental conditions of 25°C and a photoperiod of 16 h light (90-100 μE/m^2s) and 8 h dark. They were subcultured every 3 weeks. Shoots of 4-5 cm were transferred to medium for rooting. This medium was made up of MS mineral salts supplemented with 3 mg/L naphthaleneacetic acid, 100 mg/L myoinositol, 1 mg/L thiamine HCl, 20 g/L sucrose, and 7 g/L agar. The pH was adjusted to 5.8. The aforementioned growth conditions were maintained.

Assessment of glyphosate tolerance

Glyphosate effects on cell growth were determined by inoculating cell suspensions (SS, TS) into cell suspension medium containing or not containing various glyphosate concentrations. After 4 weeks in 25°C and a photoperiod of 16 h light (90-100 μE/m^2s) and 8 h darkness, the growth rate was assessed as dry weight increase. Tests were performed on regenerated plants (R1) and cell suspensions initiated from calli derived from leaves issued from regenerated plants. All experiments were conducted with the randomized complete design and 3 replications.

DNA analysis

Genomic DNA was isolated from fresh young tissue of sugarcane leaves according to Hoisington et al. (1994). PCR was performed according to Zambrano et al. (2003) in a total volume of 25 μL, containing 10 mM of Tris-HCl (pH 9), 50 mM of KCl, 2.5 mM of $MgCl_2$, 200 μM of each dNTP, 0.2 μM of primer, 25 ng of genomic DNA, and 1 U of *Taq* DNA polymerase (Promega). The reaction mixture was overlaid with 15 μL of mineral oil. For amplification, 15 primers from Operon technologies were used: OPA01, OPA04, OPA07, OPB04, OPB07, OPK03, OPK05, OPK15, OPM04, OPM10, OPM14, OPM16, OPM17, OPM18, and OPM20.

RAPDs were amplified in an MJ Research PTC 200 thermalcycler. An initial denaturation cycle was done at 94°C for 5 min, followed by 45 cycles of amplification by denaturing at 94°C for 1 min, annealing at 36°C for 30 s, and extension at 72°C for 2 min. The final step was a single extension cycle at 72°C for 7 min. Amplification products were analyzed with electrophoresis in 1.4% agarose gels by using Tris-borate buffer, staining with ethidium bromide, and photographing under UV light. λ Phage DNA double digested with *Hin*d III and *Eco*R I endonucleases was used as a marker. The gels were digitized by using 1D Image Analysis software (Eastman Kodak Company, 1997).

Results and Discussion

Herbicide testing

The deleterious effect of glyphosate on cellular growth of cell suspension of the sugarcane cultivars V64-10, PR62258, and V71-51 was confirmed in the third week when the growth was compared with that of the control (0 mg/L glyphosate). Sugarcane cells incubated in 1.0 and 1.2 mM/L glyphosate showed growth inhibition above 65% in the 3 cultivars. The treatment with 0.8 mM/L glyphosate produced growth reduction below 50% in the 3 cultivars. The degree of growth inhibition was below 38% with 0.6, 0.4, and 0.2 mM/L glyphosate.

Plant regeneration

Shoot regeneration was induced in the absence of glyphosate. More than 30% of the SS from cultivar V71-51 regenerated, but only 20% of the TS did. Tolerant cell suspensions cannot be regenerated from cultivars V64-10 and PR62258. The regenerated plantlets and cell suspensions initiated from calli of leaves from regenerated plants of cultivar V71-51 were tested for resistance to glyphosate. The growth for sensitive regenerated plants (SR1) and tolerant regenerated plants (TR1) was similar. The performance of the tolerant regenerated plants growing in the SSM was as good as that of the sensitive regenerated plants growing in the NSSM. All sensitiva regenerated plants died in SSM.

Characterization of glyphosate-tolerant cell suspensions

Cell suspensions proliferated little after one subculture at SM, and rapid death occurred in most of the cells. The others (TS) had slow growth after 2 subcultures at SM. A comparison of the growth kinetics of SS and TS in a medium containing increasing concentrations of glyphosate is shown in Figure 1. Growth of the SS was inhibited by 50% at about 0.8 mM/L, whereas 10 mM/L glyphosate was required to exert the same effect on the TS. These tolerated a concentration 12.5-fold higher than the SS.

Characterization of glyphosate-tolerant regenerated plants

The survival rates of the tolerant regenerated plants (TR1) and the sensitive regenerated plants (SR1) on media containing increasing glyphosate concentration were compared (Figure 2). The survival rate of the SR1 was 50% at about 1 mM/L, whereas 6 mM/L glyphosate was required to exert the similar effect on the TR1. The tolerant regenerated plants tolerated a concentration 6-fold higher than that of the sensitive regenerated plants.

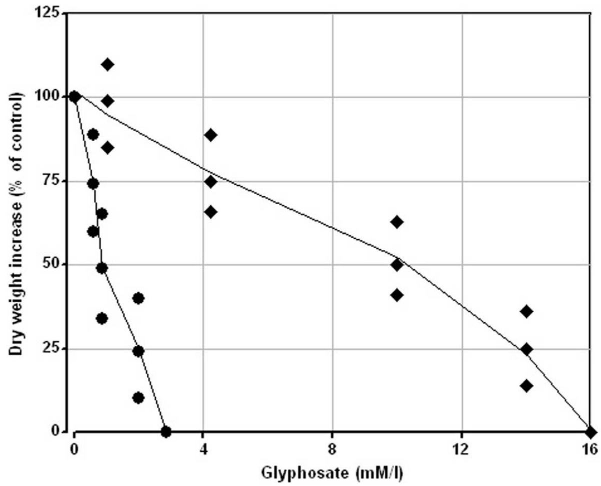

Figure 1. Effect of glyphosate on growth of sensitive (●) and tolerant (◆) sugarcane cell suspensions.

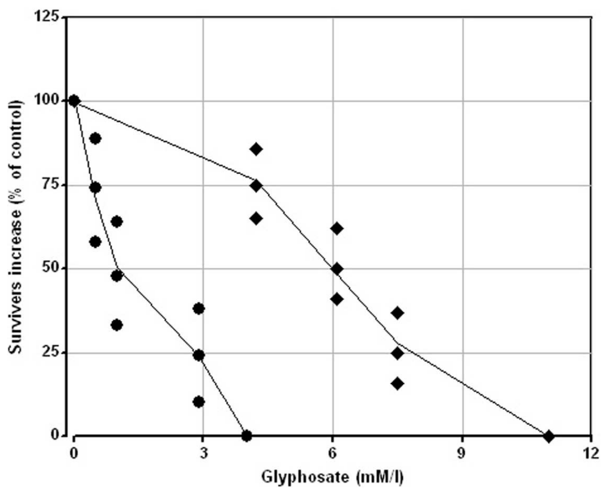

Figure 2. Effect of glyphosate on survival of sensitive (●) and tolerant (◆) sugarcane regenerated plants.

Characterization of glyphosate-tolerant cell suspensions derived from leaves issued from regenerated plants

Cell suspensions were established once again from calli of leaves from regenerated plants to evaluate the tolerance of the regenerated plants. The dry weight gain of cell suspensions derived from tolerant regenerated plants and from sensitive regenerated plants was similar when both were cultivated without glyphosate. A comparison of the growth performance of the cell suspension derived from calli from leaves of TR1 (TSR1) and the sensitive plants (SSR1) growing on medium

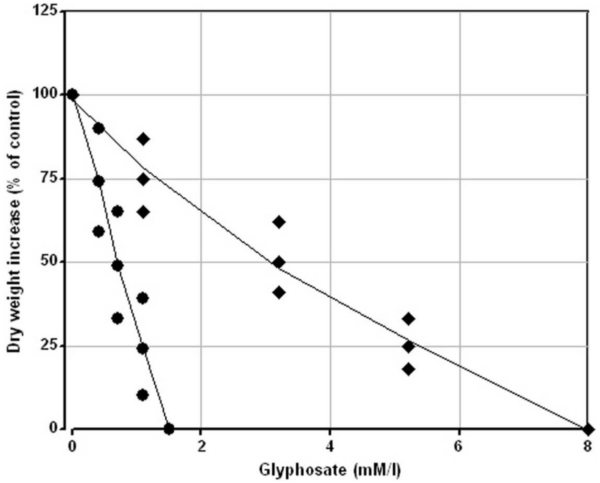

Figure 3. Effect of glyphosate on growth of sensitive (●) and tolerant (◆) sugarcane cell suspensions from regenerated plants.

containing increasing concentrations of glyphosate is shown in Figure 3. Growth inhibition of the SSR1 was 50% at 0.6 mM/L, whereas 3 mM/L glyphosate was required to exert the same effect on TSR1. The TSR1 tolerated a concentration 5-fold higher than that of the SSR1.

DNA analysis

We have tested sensitivity of the RAPD technique for detecting polymorphism among the cv. V71-51 from sugarcane (sensitive to glyphosate) and the cellular line (tolerant to glyphosate) selected in vitro. High polymorphism was observed with all primers, but of the 15 primers, only OPA-07 was useful for differentiating the tolerant cellular line.

The RAPD patterns obtained with OPA-07 (Figure 4) showed fragments smaller than 1130 bp in the 4 samples. These amplification products revealed a 564-bp band that can be used to characterize the tolerant regenerated plants (1, 2). This band is not present in the sensitive regenerated plants (3, 4), and a band of 585 that can be used to characterize the tolerant cell suspensions (5, 6) is not present in the sensitive cell suspensions (7, 8).

Sugarcane in vitro growth was inhibited by glyphosate as reported in carrot (Nafziger et al., 1984), tomato (Smith et al., 1986), tobacco (Dyer et al., 1988), and chicory (Sellin et al., 1992). The 12.5-fold increase in tolerance of tolerant cell suspensions is in the range of those reported (between 10- and 100-fold) for other species by different authors (Nafziger et al., 1984; Sellin et al., 1992). Plants were regenerated from sensitive cell suspensions and tolerant cell suspensions. Plants regenerated from tolerant cell suspensions tolerated a 6-fold higher glyphosate concentration than the plants regenerated from sensitive cell suspensions. The cell suspensions derived from the tolerant regenerated plants tolerated a glyphosate concentration that was 5-fold higher than those derived from

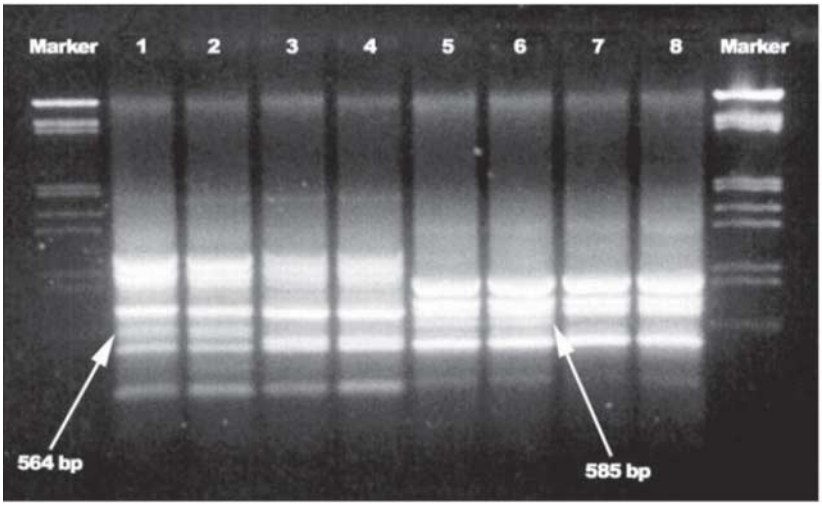

Figure 4. RAPD polymorphisms of sugarcane-sensitive cellular line (V71-51) and sugarcane-tolerant cellular line to glyphosate with primers OPB-07. Molecular marker, λ DNA / *Hin*d III–*Eco*R I.

sensitive regenerated plants. Plants regenerated showed little or no variation of tolerance.

RAPD analysis with arbitrary oligonucleotide primers efficiently differentiated sugarcane-tolerant cellular line by their genetic changes due to in vitro selection of pre-existing variability. In sugarcane, RAPD technology has been used to study the genetic stability in plants obtained by means of in vitro culture (Tingey and del Tufo, 1993), to detect the genetic variability among cultivars (Harvey and Botha, 1996), to analyze diversity and phylogeny in the genera *Saccharum* (Vijayan et al., 1999), and to identify resistant clones to SCMV generated to induce mutation (Zambrano et al., 2003).

This performance suggests that an in vitro selection of pre-existing variability has occurred. Similar results have been reported by Vasil (1988), Shenoy and Vasil (1992), and Chowdhury and Vasil (1993). Consequently, the tolerance persisted throughout the differentiation into plants and dedifferentiation into cell suspensions is observed in tobacco (Jones et al., 1996), chicory (Sellin et al., 1992), and soybean (Padgette et al., 1997). To our knowledge, no analysis of glyphosate tolerance inheritance of plants obtained from cell suspension is available. Two main hypotheses have been developed—the tolerance to glyphosate is inherited as a recessive trait or as an epigenetic variant (Sellin et al., 1992). In the epigenetic case, tolerance can be maintained only vegetatively.

By using in vitro selection, we have obtained a glyphosate-tolerant cellular line as obtained in carrot by Nafziger et al. (1984), *Corydalis sempervirens* by Smart et al. (1985), tobacco by Singer and McDaniel (1985) and Dyer et al. (1988), tomato by Smith et al. (1986), petunia by Steinrucken et al. (1986), and *Cichorium intybus* by Sellin et al. (1992). Tolerant plants have been regenerated from tobacco by Singer and McDaniel (1985). Consequently, the tolerance persisted throughout the differentiation into plants and dedifferentiation into cell suspensions as observed in tomato (Smith et al., 1986) and chicory (Sellin et al., 1992).

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