Selection of an Ametryn tolerant sugarcane cellular line[1,2]

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ABSTRACT

Calli derived from sugarcane *Saccharum* sp (cultivar V64-10) were used to select and characterize an ametryn tolerant cellular line. The isolated cellular line (tolerant calli), their subsequent regenerated plants and the calli newly induced from these were analyzed and characterized for degree of tolerance. The concentration of 40 mg/L of ametryn produced growth inhibition of 58% in calli and 60% in cell suspensions. The tolerant calli tolerated a 6.25-fold higher ametryn concentration than the sensitive calli. The tolerant regenerated plants were regenerated from tolerant calli. The tolerant regenerated plants tolerated a 5.35-fold higher ametryn concentration than the sensitive regenerated plants. The calli derived from the tolerant regenerated plants tolerated a 4.3-fold higher ametryn concentration than those derived from sensitive regenerated plants. Consequently, the tolerance persisted throughout the differentiation into plants and dedifferentiation into calli. This performance suggests that an *in vitro* selection of pre-existing variability has taken place.

Key words: Sugarcane, *Saccharum* sp, cell suspensions, callus, regeneration, ametryn tolerance

RESUMEN

Selección de una línea celular de caña de azúcar tolerante a ametrina

Callos derivados de caña de azúcar, *Saccharum* sp (cultivar V64-10), se usaron para seleccionar y caracterizar una línea celular tolerante a ametrina. La línea celular aislada (callos tolerantes), sus subsecuentes plantas regeneradas y el nuevo callo inducido de éstas se analizaron y caracterizaron por el grado de tolerancia. La concentración de 40 mg/L de ametrina produjo 58% de inhibición del crecimiento en callos y 66% de inhibición del crecimiento en suspensiones celulares. Los callos tolerantes soportaron una concentración de ametrina 6.25 veces mayor que los callos sensibles. Plantas regeneradas tolerantes se regeneraron de callos resistentes. Las plantas regeneradas tolerantes resistieron 5.35 veces más concentración

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de ametrina que las plantas regeneradas sensibles. Los callos derivados de plantas regeneradas tolerantes soportaron una concentración 4.3 veces mas alta que aquellos derivados de plantas regeneradas sensibles. En consecuencia, la tolerancia persiste a través de la diferenciación en plantas y la desdiferenciación en callos. Este comportamiento sugiere que ha ocurrido una selección *in vitro* de variabilidad pre-existente.

INTRODUCTION

Sugarcane (*Saccharum* sp), a large grass of the family Gramineae, is one of the most important crops in tropical and subtropical countries. It produces about 65% of the sugar of the world. One of the factors that affects crops in the field is weeds, and the more effective way of controlling them is through the use of herbicides. In sugarcane the usual recommendation is the combination of ametryn and 2,4-D (2,4-dichlorophenoxyacetic acid), which has given excellent control but causes different degrees of phytotoxicity among commercial cultivars in Venezuela (Rincones y Gómez, 1983). Ametryn [2-(ethylamino)-4-(isopropylamino)-6-(methylthio)-s-triazine] is a triazine herbicide, with residual effect. Ametryn blocks electron transport from QA to QB during photosynthesis by interacting with the electron transport carrier B of photosystem II. Triazines also induce alterations in metabolic processes other than photosynthesis (Duke, 1987).

Recently an evaluation methodology has been established for the reaction of cell cultures to herbicides. The results of the technique in sugarcane show a high correlation with field tests. The establishment of cell, tissue and organ culture techniques has made easier the screening of sugarcane for sensitivity and tolerance to herbicides (Murashige and Skoog, 1962; Crocomo et al., 1981; Hughes, 1983; Mazur and Falco, 1989). This work reports the selection and characterization of an ametryn tolerant cellular line of sugarcane.

MATERIALS AND METHODS

Callus induction: Sugarcane cultivar V64-10 was selected for this study. Calli were initiated from immature leaf explants cultured as previously described (Ho and Vasil, 1983). Six-month-old plant material was used as a source for the explants. This was taken from the spindle leaf, just above the meristematic apex. The outer leaves were removed from mature shoots after one minute of surface sterilization with 95% ethanol, and then dipped into a 5% sodium hypochlorite solution for 20 minutes. After being washed with cold sterilized water, 3 × 4-mm explants were cut and transferred into solid MS medium (Murashige and Skoog, 1962) modified by Liu (1984). This medium was made up of MS

mineral salts supplemented by 100 mg/L myo-inositol, 1 mg/L thiamine HC1, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine, 2mg/L glycine, 50 mg/L arginine, 3 mg/L 2,4-D, 400 mg/L casein hydrolizate, 20g/L sucrose and 7 g/L agar. The pH was adjusted to 5.8. Calli were maintained by subculture; they were placed in fresh medium every three weeks. These were maintained in complete darkness during the first two months at 25°C.

Establishment of cell suspension: Cell suspension was established from callus induced through the methodology described by Ho and Vasil (1983). The calli-covered explants were transferred to 50 ml liquid medium in 250-ml Erlenmeyer flasks to initiate cell suspensions. The liquid medium composition was the same as that of the callus-inducing medium except that 500 mg/L casein hydrolizate and 30 g/L sucrose was added, and Arginine and Agar were omitted. The flasks were placed on a gyratory shaker at 25°C, with a photoperiod of 16-h light intensity (90-100 E/m^{2}s), to induce chlorophyllaceous cell, and 8-h darkness. The medium was changed every five days.

Herbicide testing: Calli (500 mg) and cell suspensions (of approximately 5,000,000 cells per milliliter) were inoculated into the same medium that were used for the callus induction and establishment of cell suspensions, respectively, except that 0, 10, 20, 30, 40 and 80 mg/L ametryn in 0.5 N hydrochloric acid was added. The pH was adjusted to 5.8. Calli and cell suspensions were incubated to 25°C with a photoperiod of 16-h light intensity (90-100 μE/m^{2}s) and 8-h darkness. Every seven days for four weeks, culture media were changed, and fresh and dry weight was evaluated, in milligrams of calli and in milligram per milliliter of cell suspensions.

Selection of ametryn tolerant callus and cell suspension: Calli (500 mg) and cell suspensions (of approximately 5,000,000 cells per milliliter) were inoculated into the same medium that was used for the callus induction and establishment of cell suspensions supplemented or non-selective medium (NSM) with 40 mg/L ametryn in 0.5 N hydrochloric acid selective medium (SM). They were cultured at 25°C with a photoperiod of 16-h light intensity (90-100 μE/m^{2}s) and 9-h darkness. Calli were subcultured every month. Cell suspensions were subculture every five days.

Plant regeneration: Calli and cell suspensions developed in NSM and SM were transferred to medium described for callus induction except that this medium had 1 mg/L less of 2,4-D, and 0.5 mg/L more of benzyl amino purine (BAP). These were maintained in a growth chamber with environmental conditions of 25°C, photoperiod of 16-h light intensity 90-100 μE/m^{2}s) and 8-h darkness, and subcultured every these weeks. Shoots of 4 to 5 cm were transferred to medium for root-

ing. This medium was made up of MS mineral salts supplemented by 3 mg/L naphthalene acetic acid (NAA), 100 mg/L myo-inositol, 1 mg/L thiamine HC1, 20 g/L sucrose and 7 g/L agar. The pH was adjusted to 5.8. The aforementioned growth conditions were maintained.

Assessment of ametryn tolerance: Ametryn effects on cell growth were determined by transferring calli (500 mg) to callus-inducing medium either containing or not containing various ametryn concentrations. After four weeks at 25°C, photoperiod of 16-h light intensity 90-100 E/m^2s) and 8-h darkness, the growth rate was assessed as dry weight increase. Tests were carried out on cell suspensions, generated plants (R1) and calli derived from leaves issued from regenerated plants. All experiments were conducted with the randomized complete design, five replications.

RESULTS

Herbicide testing: The deleterious effect of ametryn on the cellular growth of callus and cell suspension of the sugarcane cultivar V64-10 was confirmed in the 4th week when there was a comparison with the control (0 mg/L of ametryn). The degree of inhibition in growth was similar at 20 and 30 mg/L concentrations of ametryn, whereas at the 10 mg/L concentration it was less. The concentration of 40 mg/L of ametryn produced 58% growth inhibition in callus and 66% growth inhibition in cell suspension, whereas at the 80 mg/L concentration, growth inhibition was 95% in callus and 100% in cell suspension.

Plant Regeneration: Shoot regeneration was induced in the absence of ametryn. More than 70% of the sensitive calli regenerated buds after two subcultures, whereas three subcultures were necessary for the tolerant calli to obtain similar results. The growth for plants regenerated from sensitive and tolerant calli was similar. The performance of the ametryn tolerant regenerated plants growing in the SM was as good as that of the sensitive regenerated plants growing in the NSM. It was not possible to obtain a reasonable quantity of regenerated plants from cell suspensions.

Characterization of ametryn tolerant callus: Few small sectors of fresh callus (tolerant calli) growth appeared over the brown callus inoculated into SM after two to three months. Figure 1 shows a comparison of the growth kinetics of sensitive calli (SC) and tolerant calli (TC) in a medium containing increasing concentrations of ametryn. Growth of the sensitive calli was 50% inhibited by about 32 mg/L, whereas 200 mg/L ametryn was required to exert the same effect on the tolerant calli. These tolerated a concentration 6.25-fold higher than the sensitive calli.

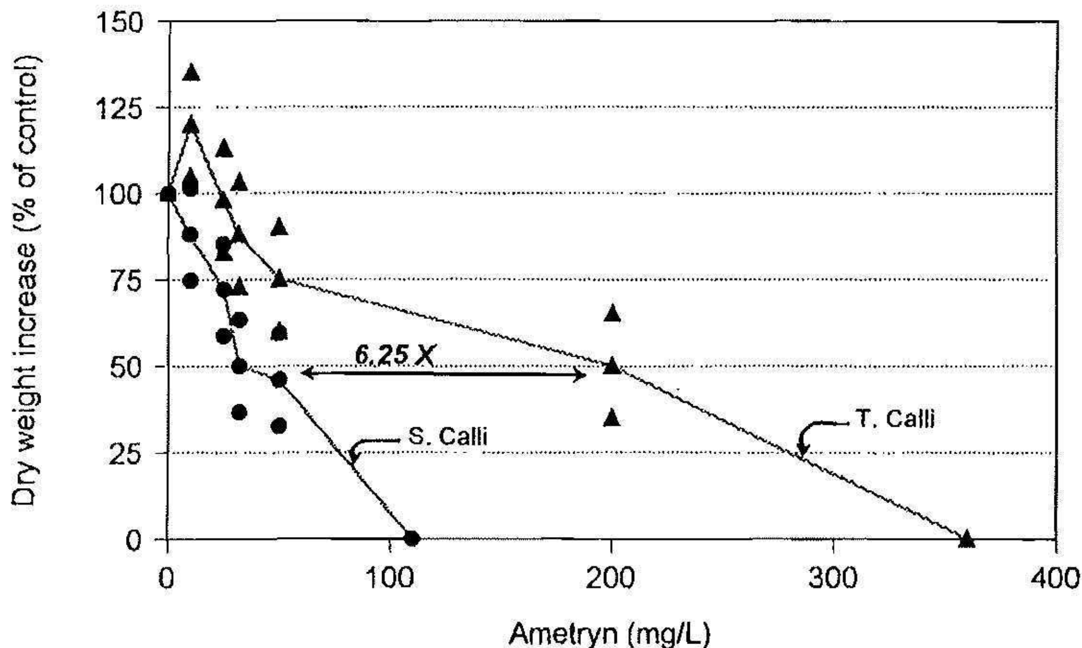

FIGURE 1. Effect of Ametryn on sensitive (●) and tolerant (▲) sugarcane calli growth.

Characterization of ametryn tolerant cell suspension: Cell suspensions proliferated a little after one subcultured at SM. There was rapid death of the majority of the cells. The others (tolerant cell suspensions) had a slow growth after three subcultures in SM. Figure 2 shows a comparison of the growth kinetics of sensitive cell suspensions (SS) and tolerant cell suspensions (TS) in a medium containing increasing concentrations of ametryn. Growth of the sensitive cell suspensions was 50% inhibited by about 25 mg/L, whereas 125 mg/L ametryn was required to exert the same effect on the tolerant cell suspensions. These tolerated a concentration 5-fold higher than the sensitive cell suspensions.

Characterization of ametryn tolerant regenerated plants: The survival of the sensitive regenerated plants (SR1) and tolerant regenerated plants (TR1) in media containing increasing concentrations was compared (Figure 3). The survival of the sensitive regenerated plants was 50% inhibited by about 28 mg/L ametryn, whereas 150 mg/L was required to exert the same effect on tolerant regenerated plants. These tolerated a concentration 5.35-fold higher than the sensitive regenerated plants.

Characterization of ametryn tolerant calli derived from leaves issued from regenerated plants: Calli were initiated once again from leaves issued from regenerated plants to evaluate the tolerance of the regenerated plants. The dry weight gain of calli derived

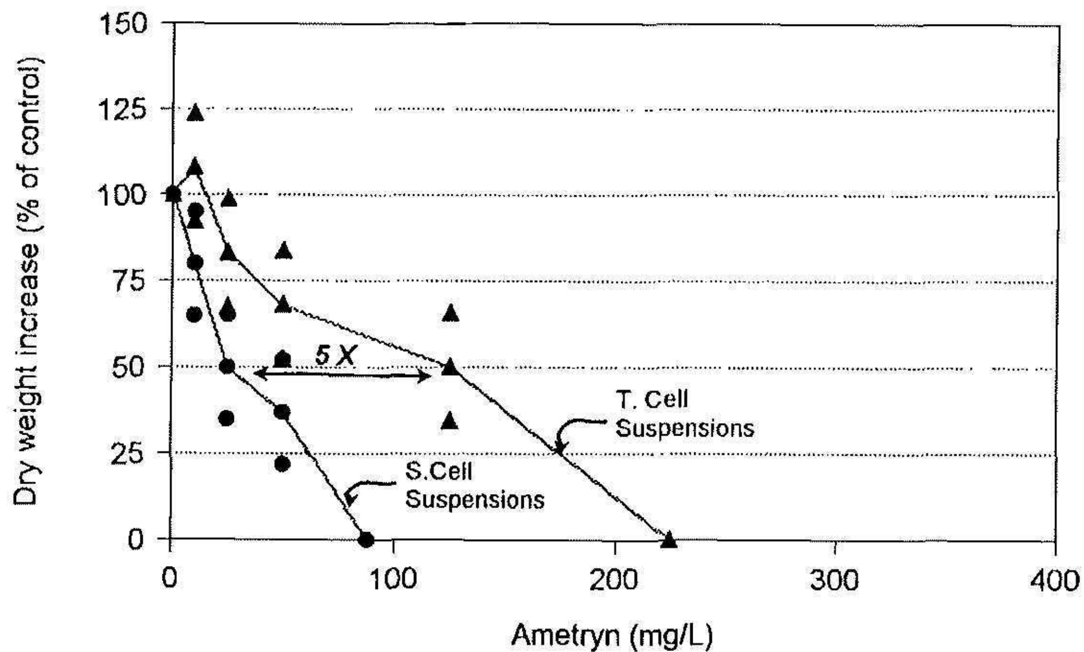

FIGURE 2. Effect of Ametryn on sensitive (●) and tolerant (▲) sugarcane cell suspension growth.

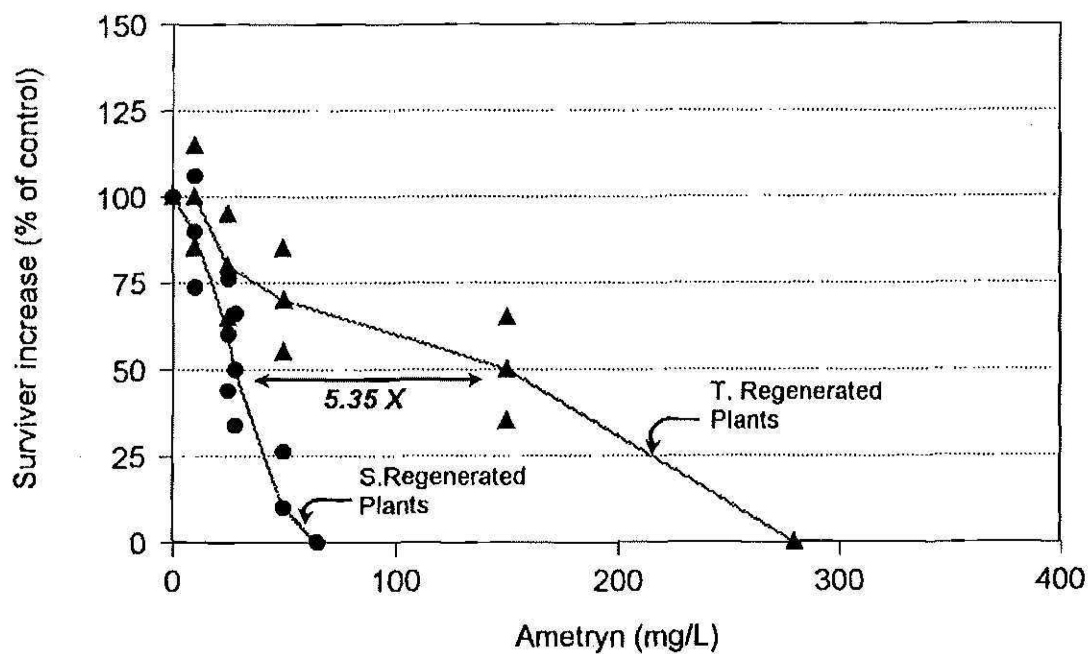

FIGURE 3. Effect of Ametryn on sensitive (●) and tolerant (▲) sugarcane regenerated plant survival.

from tolerant regenerated plants and from sensitive regenerated plants was similar when both were cultivated without ametryn. Figure 4 shows a comparison of the growth performance of the calli derived from leaves of tolerant regenerated plants (CTR1) and the sensitive regenerated plants (CSR1) growing in medium containing increasing concentrations of ametryn. The growth of the calli from SR1 was 50% inhibited by 22 mg/L ametryn, whereas 95 mg/L was required to exert the same effect on the calli derived from tolerant regenerated plants. These tolerated a concentration 4.3-fold higher than the calli from sensitive regenerated plants.

DISCUSSION

Sugarcane in vitro growth was inhibited by ametryn as reported by other authors (Crocomo et al., 1981; Ochoa-Alejo and Crocomo, 1988). The results obtained in callus and cell suspension cultures from cultivar V-64-10 of sugarcane were in agreement with the results reported in field tests by Rincones and Gómez (1983). The concentration of 40 mg/L of ametryn produced 58% growth inhibition in calli and 66% growth inhibition in cell suspensions. Similar results have been reported by Ochoa-Alejo and Crocomo (1988) where sugarcane cells incubated in 40 mg/L of ametryn herbicide showed 75% growth inhibition. The isolated cellular line (tolerant calli), their subsequent

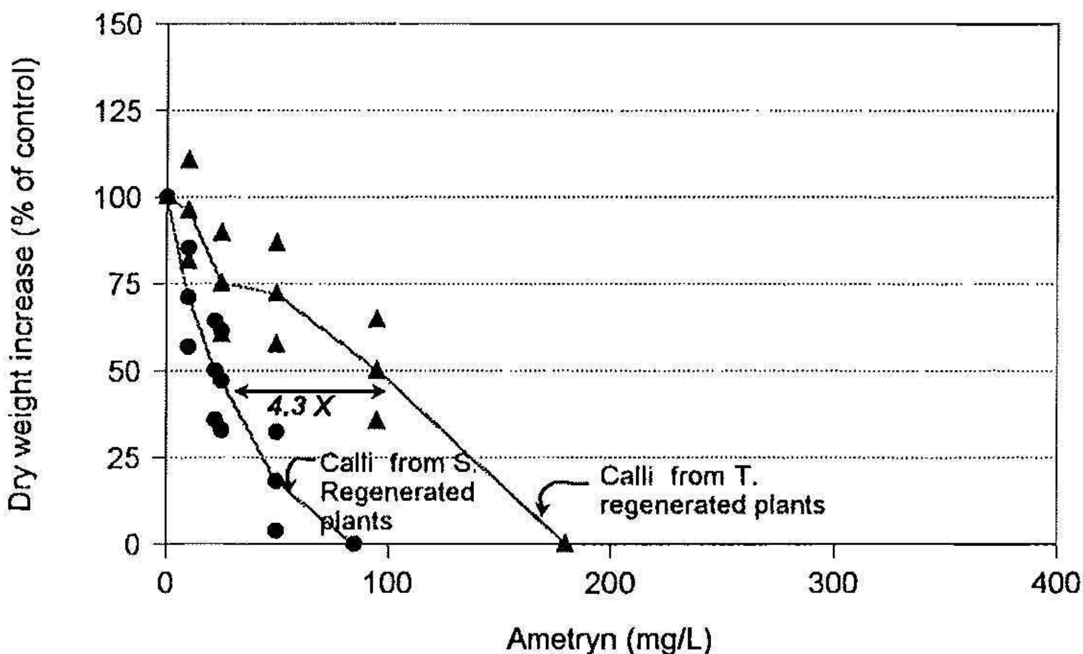

FIGURE 4. Effect of Ametryn on sensitive (●) and tolerant (▲) sugarcane calli from regenerated plant growth.

regenerated plants and the calli newly induced from these were analyzed and characterized for degree of tolerance. The tolerant calli tolerated a 6.25-fold higher ametryn concentration than the sensitive calli. The tolerant regenerated plants were regenerated from tolerant calli. The tolerant regenerated plants tolerated a 5.35-fold higher ametryn concentration than the sensitive regenerated plants. The calli derived from the tolerant regenerated plants tolerated a 4.3-fold higher ametryn concentration that those derived from sensitive regenerated plants. Consequently, the tolerance persisted throughout the differentiation into plants and dedifferentiation into calli as observed in soybean with atrazine by Wrather and Freytag (1991), and in tomato and in chicory with glyphosate by Smith et al. (1986) and Sellin et al. (1992), respectively. This performance suggests that an *in vitro* selection of pre-existing variability has taken place.

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