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*Full Length Research Paper*

# Response of sugarcane (*Saccharum sp*.) varieties to embryogenic callus induction and *in vitro* salt stress

**Ch. Gandonou, J. Abrini, M. Idaomar and N. Skali Senhaji***

Laboratoire de Biologie Cellulaire et Moléculaire, Faculté des Sciences de Tétouan
Université Abdelmalek Essaâdi, B.P. 2121 Tétouan, Morocco.

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**Response of three varieties of sugarcane (*Saccharum* sp.) to callus induction, embryogenic callus production and *in vitro* salt tolerance was studied. For callus induction and embryogenic callus production, leaf bases segments were subjected to *in vitro* culture on Murashige and Skoog (MS) medium supplemented with 3 mg $l^{-1}$ 2,4 Dichlorophenoxyacetic acid for 4 weeks. To evaluate salt tolerance of the varieties, growing calli were exposed after two subsequent subcultures (4 weeks each) to different concentrations of NaCl (0, 17, 34, 68 and 102 mM) added to the culture medium for 4 weeks. Comparision of genotypes was based on callus induction percentage, embryogenic callus production percentage and relative fresh weight growth (RFWG). For salt tolerance, necrosis percentage and relative fresh weight growth of callus were used. The three varieties responded well to callus induction with a percentage of induction about 82, 84 and 100% for CP70-321, NCo310 and CP65- 357, respectively. The high percentages of embryogenic callus obtained for the three varieties indicated that these varieties have a high capacity for embryogenic callus production. Relative fresh weight growth of callus was about 1.076, 1.282 and 0.925 for CP70-321, NCo310 and CP65-357, respectively. NaCl effect resulted in calli necrosis and a reduction of their growth. However, growing calli derived from varieties CP70-321 and NCo310 showed less necrosis percentages and less relative fresh weight growth reduction under salt stress. They appeared to be more salt tolerant *in vitro* than CP65-357.**

**Key words:** Callus induction, embryogenic callus culture, *in vitro* salt tolerance, *Saccharum* sp.

## INTRODUCTION

Selections of favorable somaclonal variant strains from callus culture are supplementary tools to traditional breeding for production of stress-resistant plants (Larkin and Scowcroft, 1981; Dix, 1993; Ashraf, 1994). The introduction of a given genotype in *in vitro* selection program depends on its aptitude to *in vitro* culture, particularly to callus induction and embryogenic callus production. In fact, for several species, studies have shown that genotype affects plants *in vitro* culture response (Abe and Futsuhara, 1984; Mikami and Kinoshita, 1988; Van Sint Jan et al., 1990; Arzani and Mirodjagh, 1999).

Salinity is a major factor limiting the crop productivity in the arid and semi-arid areas of the world (Ashraf, 1994). Plant tissue culture techniques provide a promising approach to develop salt-tolerant plants. *In vitro* selection of salt tolerant cell lines and regenerated plants has been reported in several species such as potato (Sabbah and Tal, 1990), rice (Lutts et al, 1999); Hordeum (Sibi and Fakiri, 2000), wheat (Barakat and Abdel-Latif, 1996) and sunflower (Alvarez et al., 2003). This suggests that tissue culture selection can be used to improve salt tolerance of plants.

*Corresponding author. E-mail: senhajin@hotmail.com; Fax: 00212 39 99 45 00.

In sugarcane, little is known about the importance of genotype on *in vitro* culture ability. Burner (1992), showed the genotype effect on callus production and plant regeneration by analysing the *in vitro* response of three sugarcane varieties to callus production from mature caryopses and by measuring their regeneration rate. He revealed a high difference among varieties in their callus production ability and in plant regeneration rate. Studies concerning sugarcane *in vitro* salt tolerance are rare. Gonzalez et al. (1995) reported that NaCl stress reduces cell survival rate in four sugarcane genotypes using cell suspensions and found that genotypes respond differently when confronted with NaCl in their culture medium. The objectives of this study were to determine, for the first time, the response of three sugarcane varieties to callus induction and embryogenic callus production using leaf explants, and to evaluate *in vitro* salt tolerance of these varieties. Thus, the purpose of this study was to screen varieties in order to identify those which can be used for *in vitro* salt tolerance selection program.

## MATERIALS AND METHODS

### Plant materials

The three sugarcane (*Saccharum sp*.) varieties were obtained from the Technical Center of Sugar's Cultures (CTCS), Morocco. CP70-321 and CP65-357 are American varieties (Canal Point) but are largely cultivated in Morocco while NCo310, South African variety (Natal Coimbatore) is not cultivated in Morocco. Stalk segments were surface disinfected with ethanol 70% sown in pots containing biosol in greenhouse. After germination, sugarcane plants were grown in these conditions until approximately 7 months.

### Callus induction

The explants used for callus induction are cylinders of leaf provided from the sheath of the youngest sheets. The basal part of the stem (constituted by the sheath of leaves) is surface sterilized for 10 min in 0.03% mercuric chloride supplemented with Tween 80, followed by three changes of sterile distilled water (10 min each). After drying on sterile filter paper, leaf cylinders were aseptically placed on MS (Murashige and Skoog, 1962) medium supplemented with 3 mg $l^{-1}$ 2,4-D (2.4 dichlorophenoxyacetic acid) and 30 g $l^{-1}$ sucrose. The pH was adjusted to 5.8 with 0.1 N NaOH and all media were solidified with 8 g $l^{-1}$ agar before autoclaving during 20 min at 120°C. Five explants were cultivated per jar and cultures were kept in darkness at 25±1°C. Callus induction percentage was determined after 4 weeks.

### Embryogenic callus evaluation

Distinction between embryogenic and no-embryogenic callus was carried out on the basis of callus external aspect. Embryogenic calli are of glossed aspect, compact, characterized by their yellow colour and their globular structure, while no-embryogenic callus are of wet aspect, translucent and of colour more brownish (Van Sint Jan et al., 1990). After 4 weeks of culture, the number of embryogenic calli was recorded for each cultivar. These data were transformed into percentages expressed as a percentage of embryogenic calli per total number of calli obtained.

### Callus growth

After two subcultures of 4 weeks each, calli obtained were used for callus growth study. Calli were weighed before their transfer to fresh callus induction medium ($W_0$). They were weighed again after 4 weeks of culture ($W_1$). Relative fresh weight growth (RFWG) of callus was calculated as $(W_1 - W_0)/ W_0$.

### *In vitro* salt treatment

After two subcultures, the growing calli were transferred to callus culture medium containing 0, 17, 34, 68 and 102 mM NaCl. Calli were maintained on their respective treatments for 4 weeks. For each medium, callus necrosis percentage was determined visually as percentage of necrotic callus and relative fresh weight growth of no- necrotic calli was calculated as above.

### Statistical analysis

Number of explants that induced callus and embryogenic callus number were analysed as binomial-distribution variates with a number of explants ranging between 35 and 55. One way analysis of variance (ANOVA) was used for RFWG study with 4 replicates per variety. In the case of salt treatment, two independent experiments were carried out with similar results. Data presented here correspond to that of one of the two experiments. For each experiment, 30 to 35 calli were used (5 per jar). Callus necrosis percentages were analyzed as binomial distribution variates while a two ways ANOVA (salinity and genotype) was used for RFWG analysis with 4 replications for each NaCl concentration and each genotype. Analysis was carried out using SAS program (SAS Institute, 1992).

## RESULTS AND DISCUSSION

### Callus induction

The three varieties showed high percentages of callus induction greater than 84% (Table 1). Significant difference ($p < 0.05$) was observed among varieties (Table 1). Based on this parameter, CP65-357 presented the best response compared to the two other genotypes which showed similar response. These results showed that callus induction capacity is genotype dependent as found in rice (Mikami and Kinoshita, 1988, and Van Sint Jan et al., 1990) and in sugarcane (Burner, 1992).

### Embryogenic calli production

Distinction between embryogenic and non-embryogenic callus was carried out on the basis of callus external aspect (Figures 1 and 2). In our experiments, in addition to these two previous types, we observed an intermediar

**Table 1.** Callus induction percentages, embryogenic callus percentages and callus relative fresh weight growth (RfWG) of three sugar cane varities.

| Varieties | Callus induction (%) | Embryogenic callus (%) | Callus RFWG |
|---|---|---|---|
| CP65-357 | 100 [a] | 94.74 [a] | 0.925 [b] |
| NCo310 | 84.44 [b] | 94.74 [a] | 1.282 [a] |
| CP70-321 | 81.82 [b] | 95.55 [a] | 1.076 [ab] |

Means within columns followed by the same letter do not differ significantly ($p < 0.05$).

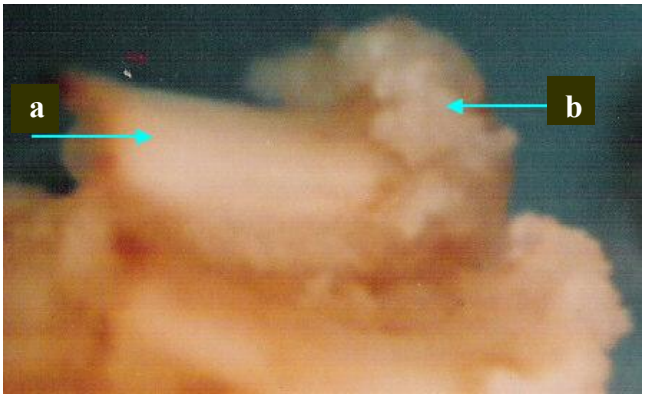


**Figure 1**. Embryogenic callus induced from leaf explant of sugarcane (Cv. CP65-357) after 4 weeks of culture (a = explant ; b = callus).

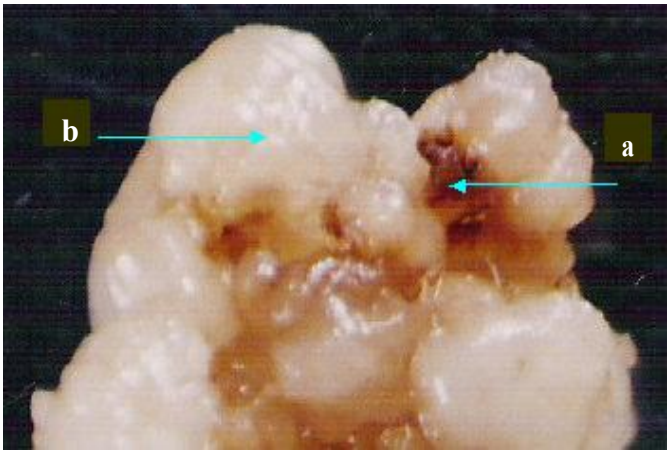


**Figure 2**. No-embryogenic callus induced from leaf explant of sugarcane (Cv. NCo310) after 8 weeks of culture ( a = mains of explant; b = callus).

type with a non-embryogenic tissue covered by an embryogenic tissue. This type of callus had been already observed for sugarcane (Guiderdoni, 1986) and sorghum (Mackinnon et al., 1986). For embryogenic calli percentage determination, we classified the intermediar type as embryogenic because, in further subcultures, the embryogenic tissue grows faster than the non-embryogenic tissue. The three varieties showed high embryogenic callus percentages (about 95%) but no significant difference was observed among them (Table 1).

## Callus growth

The relative fresh weight growth (RFWG) of callus after the 4 weeks of culture were 0.925, 1.076 and 1.282 for CP65- 357, CP70-321 and NCo310, respectively. Thus, callus intial biomass pratically doubled after 4 weeks of culture. Significant differences were observed among genotypes (Table 1). NCo310 showed a high growth while CP65-357 presented the weakest. These results indicated that callus growth was genotype dependent which is in agreement with the data found in rice (Van Sint Jan et al., 1990), durum wheat (Bommineni and Jauhar, 1996; Ozgen et al., 1996; Arzani and Mirodjagh, 1999) and in bread wheat (Hess and Carman, 1998), where a high effect of genotype on callus growth capacity was observed.

## *In vitro* salt treatment

In the absence of NaCl, none of the calli from CP70-321 or from NCo310 showed necrosis while 2.86% of CP65-357 calli were necrosed. The addition of NaCl to culture medium caused an increase in calli necrosis for all varieties (Table 2) and significant difference in calli necrosis was observed among genotypes. No callus of CP70-321 shows necrosis below 68 mM NaCl, while for NCO310, the first callus necrosis was observed at 34 mM NaCl. For NCo310 calli, the effect of NaCl was significant ($p < 0.05$) only at the highest dose of NaCl used (102 mM) while this effect was significant ($p < 0.05$) at 68 mM for CP65- 357 calli (Table 2). These results revealed significant differences ($p < 0.05$) among varieties for callus necrosis percentages, and are in agreement with the data reported by Karadimova and Dambova (1993) in durum wheat. These authors observed that higher concentrations of NaCl caused brown coloration and apparent necrosis and reduced callus growth. Similar results were reported for other genotypes of durum wheat

**Table 2.** Necrosis percentages of callus obtained from three sugarcane varieties (NCo310, CP70-321 and CP65-357) as affected by different concentrations of NaCl.

| NaCl concentrations (mM) | Varieties | | |
|---|---|---|---|
| | CP65-357 | NCo310 | CP70-321 |
| 0 | 2.86 $^{ab}$ | 0 $^{a}$ | 0 $^{a}$ |
| 17 | 8.57 $^{ab}$ | 0 $^{a}$ | 0 $^{a}$ |
| 34 | 13.33 $^{bc}$ | 10.00 $^{ab}$ | 0 $^{a}$ |
| 68 | 40 $^{cd}$ | 6.67 $^{ab}$ | 2.86 $^{ab}$ |
| 102 | 43.33 $^{d}$ | 16.67 $^{bcd}$ | 8.57 $^{ab}$ |

Means followed by the same letter are not significantly different at $p < 0.05$.

**Table 3.** Analysis of variances (ANOVA) for relative fresh weight growth (RFWG) of callus obtained from three sugarcane varieties (NCo310, CP70-321 and CP65-357) as affected by different concentrations of NaCl.

| Source of variation | Degrees of freedom | Sum of squares | Mean squares | F value |
|---|---|---|---|---|
| Genotype | 2 | 2.3391 | 1.1695 | 78.62*** |
| Salinity | 4 | 2.0826 | 0.5206 | 35.00*** |
| Genotype x salinity | 8 | 0.1439 | 0.0179 | 1.21$^{ns}$ |
| Sampling error | 45 | 0.6694 | 0.0148 | |

***: Significant at $p < 0.001$; $^{ns}$: not significant

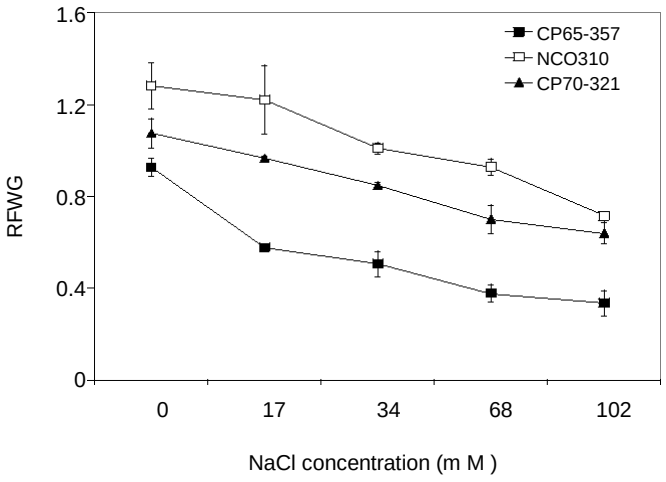


**Figure 3.** Relative fresh weight growth (RFWG) of callus obtained from three sugarcane varieties (NCo310, CP70-321 and CP65-357) as affected by different concentrations of NaCl.

(Arzani and Mirodjagh, 1999). In other four sugarcane genotypes, using cell suspension *in vitro,* Gonzalez et al. (1995) have shown that NaCl stress reduces cell survival rate in all genotypes tested and found significant differences among them.

Callus RFWG decreased as the concentration of NaCl increased in the culture medium (Figure 3). This decrease was more important for CP65-357 in comparison with the two other varieties. For example, at 17 mM NaCl (the lowest NaCl concentration used), RFWG were about 1.219, 0.966 and 0.578 respectively for NCo310, CP70-321 and CP65-357, corresponding to 95%, 90% and 63%, respectively, to that of the control. At highest NaCl concentration used (102 mM), callus RFWG for NCo310, CP70-321 and CP65-357 were 0.715, 0.64 and 0.334, which correspond to 56%, 59% and 37% of control, respectively. Analysis of variance for RFWG presented in Table 3 showed significant differences ($p < 0.001$) among the different NaCl concentrations and among varieties. In rice (Lutts et al., 1996; Basu et al., 2002) and sunflower (Alvarez et al., 2003), it was shown that NaCl reduces callus growth and that genotypes respond differently to this stress. Thus NCo310 and CP70-321 appeared to be more tolerant to NaCl than CP65-357.

The finding of superior genotypes CP70-321 and NCo310 and inferior one CP65-357 for salt tolerance together with their high potential for embryogenic callus induction lead us to suggest these varieties as a good model to study physiological mechanisms associated with *in vitro* salt tolerance and *in vitro* selection for salt tolerance in sugarcane. Further studies on the whole plant response of CP70-321 or NCo310 and CP65-357 to salinity in a greenhouse hydroponic system and in the field are underway.

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