

Full Length Research Paper

Regeneration of plantlets under NaCl stress from NaN_3 treated sugarcane explants

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Accepted 31 October, 2011

In this experiment, mutation induction in explants using NaN_3 and subsequently, callus growth and plant regeneration under NaCl stressed conditions was assessed in some sugarcane (*Saccharum officinarum* L.) cultivars (Thatta-10, CPF-237 and SPHS-19). Immature bases of leaf tips were cultured on MS_{2n} (MS salts, 3.0 mg L^{-1} ; 2,4-D, 0.5% NaN_3) for 6 days then sub-cultured on MS_2 (MS salts, 3.0 mg L^{-1} 2,4-D), MS_{2a} (MS_2 , 25 mol m^{-3} NaCl), MS_{2b} (MS_2 , 50 mol m^{-3} NaCl) and MS_{2c} (MS_2 , 75 mol m^{-3} NaCl) media in dark condition. After 6 weeks, callus growth was observed to be significantly higher ($72.34 \pm 3.70\%$) in CPF-237 in the control (MS_2), and lowest ($57.66 \pm 4.34\%$) in SPHS-19 in MS_{2d} culture. Somatic embryos were induced in proliferated calluses on MS_3 (MS salts, 0.5 mg L^{-1} ; BAP, 0.4 mg L^{-1} ; kin, 0.3 g L^{-1} ; casein hydrolysate, 3% sucrose) medium under dark condition for 2 weeks. These calluses were sub-cultured on MS_4 (MS, 0.3 mg L^{-1} ; BAP, 0.2 mg L^{-1} ; kin, 3% glucose), MS_{4a} (MS_4 , 25 mol m^{-3} NaCl), MS_{4b} (MS_4 , 50 mol m^{-3} NaCl) and MS_{4c} (MS_4 , 75 mol m^{-3} NaCl) media. Maximum of 8.41 ± 0.36 plantlets callus⁻¹ were regenerated in MS_4 (control) culture of Thatta-10, and 4.94 ± 0.05 plantlets of CPF-237 in 25 mol m^{-3} NaCl stressed plant regeneration (MS_{4a}) medium. Plant regeneration on MS_{4b} (2.21 ± 0.17 plantlets callus⁻¹) was observed in CPF-237 only. Regenerated plantlets were rooted and considered as salt tolerant in comparison to its parent cultivars.

Key words: Kinetin, somatic embryos, regenerated plantlets, *Saccharum officinarum*.

INTRODUCTION

Today, about 65% sugar is contributed by sugarcane (*Saccharum* spp.) of the world (Alam et al., 1995). It is a cash as well as domestic food source. Unfortunately, a number of disease and abiotic factors such as cold, drought and salinity have been reducing its vegetative production significantly. Improvement of this crop against these stresses has gained great importance. Sugarcane is heterozygous crop naturally. *In vitro* selection is important for superior somaclones. It is a supplementary tool for the development of stress resistant plants through

traditional breeding (Dix, 1993; Ashraf, 1994).

Among aseptic plant cultures, plant regeneration through callus development and somatic embryogenesis has got initial prime step for manipulation of crops by using modern biotechnological techniques (Saharan et al., 2004). This technique is also important to exploit the induced as well as existed somaclonal variations among the cultured explants or multiplied calluses (Islam et al., 2011). Aseptic plant cell selection of relative salt tolerant genotypes or cell lines is possible and it has been reported in many plant species (Alvarez et al., 2003; Gandonou et al., 2005; Lutts et al., 1999; Haq et al., 2011). *In vitro* plant regeneration has always remained an excellent tool for diagnosis of correlation for plant morphogenesis against salt stress.

Sodium azide is an important and most powerful chemical mutagen for crop plants. It affects plant physiology significantly and decreases cyanide resistant plant respiration among the callus cultures (Wen and

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Abbreviations: MS, Murashige and Skoog basal salts; NaN_3 , sodium azide; 2,4-D, 2,4-dichlorophenoxyacetic acid; kin, kinetin; BAP, benzyleaminopurine; NaCl, sodium chloride; F Wt, fresh weight; D Wt, dry weight.

Liang, 1995). It is a well known prominent mutagenic in several plants and animals (Grant and Salamone, 1994). Sodium azide is functionally mutagenic chemical in various organisms, but not in *Drosophila* (Kamra and Gallopudi, 1979; Arenaz et al., 1989) and *Arabidopsis*. Actually, mutagenicity in living systems is mediated through biosynthesis of organic metabolite of azide compound (Owais and Kleinhofs, 1988). This metabolite creates point mutation in DNA when entering into the nucleus (Gichner and Veleminsky, 1977).

Aseptic plant regeneration has been an incremental tool for mutation induction, while totipotency of a single cell is a useful work for the establishment of pure form of species. It can facilitate the development of several numbers of its new genotypes (Mandal et al., 2000; Barakat et al., 2010; Al-Qurainy and Khan, 2010). Similarly, indirect plant regeneration in sugarcane has been established for some local sugarcane cultivars by Haq et al. (2011). This protocol could be utilized successfully for the improvement of sugarcane through *in-vitro* mutagenesis to develop more selected and unique relevant traits of this crop.

The aim of the present study was to induce mutation in initial explants of sugarcane (*Saccharum officinarum* L.) cultivars, after which they are subjected to callus growth and then subsequent plant regeneration under different saline stressed cultures. Cell lines are selected on the basis of growth behaviors under NaCl stressed conditions. Regenerated plantlets under NaCl stressed conditions may be involved in inducing salt tolerance. Developed plantlets may be resistant sources against saline stressed culture conditions.

MATERIALS AND METHODS

Explants sterilization and NaN₃ treatment

Immature bases of leaf tips (0.8 to 1.2 cm) were excised. These tips were sterilized by washing with 90% ethanol for 2 min. They were stirred in 30% commercially available Robin Bleach[®] (5.25% v/v NaOCl) for 30 min. Sterilized explants were treated with 0.5% NaN₃ for 6-days that was supplied in callusing medium (MS₂). The pH of medium was adjusted to 4.6 to 4.7.

Callus induction and its proliferation

After the treatment of NaN₃, explants were sub-cultured on NaN₃ free medium, MS₂ [MS basal salts (Murashige and Skoog, 1962); vitamins B 5 complex (Gamborg et al., 1968); 2% sucrose and solidified with 8% agar medium supplemented with 3.0 mg L⁻¹ 2,4-dichlorophenoxy acetic acid (MS₂).

Somatic embryogenesis and plant regeneration

For somatic embryo induction, well proliferated calluses (>6-weeks old) on MS₂ medium were sub-cultured on MS₃ medium supplemented with kin (0.4 mg L⁻¹), BAP (0.5 mg L⁻¹) and casein hydrolysate (0.3 g L⁻¹) in dark conditions. After 3-weeks, culture was sub-cultured on MS₄ (0.3 mg L⁻¹ BAP, 0.2 mg L⁻¹ kin, 3%

glucose) medium for plant regeneration under dark conditions. When plant regeneration started, cultures were shifted to light conditions for further development.

Application of NaCl treatments

Four levels of salt (NaCl) stress were maintained at callusing stage that is MS₂ (control), MS_{2a} (MS₂, 25 mol m⁻³ NaCl), MS_{2b} (MS₂, 50 mol m⁻³ NaCl) and MS_{2c} (MS₂, 75 mol m⁻³ NaCl). Cultures were incubated under dark conditions for 6-weeks. At plant regeneration stage, calluses from MS₂, MS_{2a}, MS_{2b} and MS_{2c} were sub-cultured on MS₄, MS_{4a} (MS₄, 25 mol m⁻³ NaCl) and MS_{4b} (MS₄, 50 mol m⁻³ NaCl) and MS_{4c} (MS₄, 75 mol m⁻³ NaCl) media after somatic embryo induction (MS₃) for 6-weeks also.

Determination of morphological attributes

The 6-weeks old calluses multiplied under NaCl stressed conditions were fragmented into small pieces and weighed at an interval of 6-weeks. Callus proliferation rate was calculated by applying the formula below:

$$\text{Callus proliferation (\%)} = \frac{\text{Callus Final Wt} - \text{Callus Initial Wt}}{\text{Callus Final Wt}} \times 100$$

These fragmented calluses were dried after taking F Wt weight at 72°C for three days, and then D Wt was determined by electric balance. The relative water contents (RWC) were calculated (Robinson and Neil, 1985; Conroy et al., 1988).

After 12-weeks of plant regeneration culture, the number of regenerated plantlets callus⁻¹ and their heights was measured.

The pH of plant nutrient medium was adjusted (5.7-5.8) before sterilization. The cultures for callus growth and somatic embryo induction were incubated under dark conditions, while plant regeneration was also done under dark condition for initial 6-weeks; it was transferred to $\frac{16}{8}$ h day and light conditions (light intensity 15 μmol m⁻² s⁻¹) at 25°C±1.

Data analysis

Cultures for each treatment were maintained and comprised 7 replicates. Data significance of treatment was computed with COSTAT computer package (CoHort software, Berkeley, USA).

RESULTS AND DISCUSSION

The *In-vitro* culture based on mutational breeding is an important conventional method for crop improvement. This method is based on alterations of nucleotide sequence of DNA within a gene through exposure of vegetative or reproductive parts to physical as well as chemical mutagens. *In vitro* cultures are controlled phenomena that allow better treatment of mutagen and further vitrified tissues become easily permeable to mutagen (Ziv, 1991). The NaN₃ is water soluble that dissociates into hydrozoic ions. Its ionization capacity is very high, when dissolved in phosphate buffer at low pH (3.2), almost 19 times more than at pH 6.0. It penetrates through cell membrane and interacts with genome to

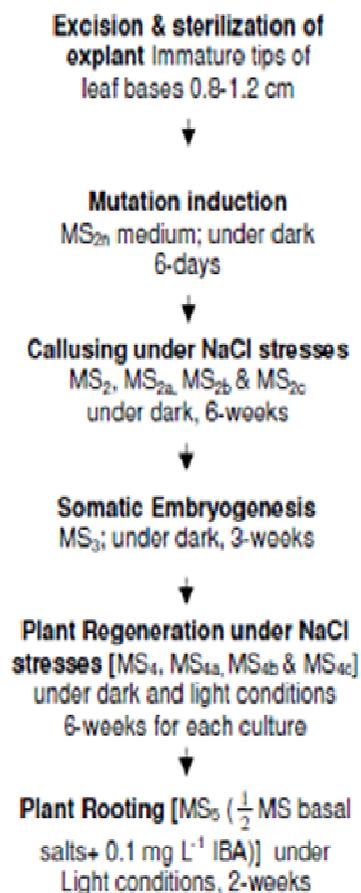


Figure 1. A schematic representation of aseptic plant regeneration of sugarcane cultivars from NaN_3 treated explants under different levels of NaCl stresses.

create mutation (Kleinhofs et al., 1974).

In general, normal plant regeneration in sugarcane through somatic embryogenesis is obtained within 20-weeks. During this study, mutagenic plantlets were regenerated, when 0.8-1.20 sized meristematic bases of young leaves were treated with 0.5% NaN_3 on callus induction medium as well as multiplication medium (MS_{2n}) for 6-days (Figure 1). They were washed with liquid callusing medium and again sub-cultured on mutagen free callusing medium (MS_2). They were also cultured on NaCl stressed callus growth media such as MS_{2a} , MS_{2b} and MS_{2c} . After 6-weeks of culture, calluses were excised from the explants and their F Wt was taken. These calluses were sub-cultured again on their respective medium for further multiplication (Figure 2) for 6-weeks under dark conditions. Final F Wt of calluses from each treatment was measured again. Maximum callus proliferation ($72.34 \pm 3.70\%$) rate was observed on MS_2 culture of CPF-237, while lower $7.48 \pm 0.85\%$ in SPHS-19 significantly (Table 1). After final F Wt, calluses

were dried, and then D Wt was taken. Relative water contents were also observed among the calluses that were higher in Thatta-10 (75.32 ± 3.37), but lower in NaCl stressed cultures significantly (Table 1). The continuous callus cultures on NaCl stressed medium causes induction of some characteristics such as to stop growth, abnormality or resistance in callus against salt stresses (Haq et al., 2011; Htwe et al., 2011).

Apparently, well proliferated calluses in callusing control (MS_2) as well as NaCl stressed cultures were sub-cultured on somatic embryogenesis medium (MS_3) for three weeks separately. After somatic embryo induction, calluses were sub-cultured on plant regeneration medium (MS_4) as well as on NaCl stressed plant regeneration medium on the basis of their respective already developed medium (control and salt stressed). Maximum plant regeneration was observed in control (MS_4) plant regeneration cultures of each cultivar. Plant regeneration efficiency was decreased with the increase in NaCl stress. No plant regeneration was observed in MS_{4c} in all cultivars. Similarly, plant regeneration was not observed on MS_{4b} cultures of Thatta-10 and SPHS-19, while 2.21 ± 0.17 plantlets callus⁻¹ was regenerated in CPF-237 sugarcane cultivar (Table 1, Figure 3). Plant height of the regenerated plantlets of CPF-237 was less than other cultivars on each of control as well as NaCl stressed cultures.

Meanwhile, NaN_3 remained a potential mutagen under *in vitro* that induces biochemical mutant regeneration (Hibberd et al., 1982; Bhagwat and Duncan, 1998). Although, this mutagen causes gene alterations or mutations at gene level, it also causes chromosomal aberration mostly (El-Den, 1993; Del Campo et al., 1999). Morphologically, immediate effects of NaN_3 on cultured meristematic cells appear to be blocked in callus induction or its proliferation. This is because of blackening action of genome separation and its multiplication at S-phased during cell cycle. Further, it is reported that some induced mutant biochemical causes inhibition of respiratory chains as well as electron chain (Johnsen et al., 2002). Through multidisciplinary actions of NaN_3 , it is impossible to detect its action in explants. In this study, mutagenic plantlets were mostly abnormal, while abnormal plantlets were removed and normal plantlets were allowed to grow in NaCl stressed plant regeneration medium. These regenerated plantlets in CPF-237 were expected to be salt tolerant in comparison to plantlets regenerated in control (MS_4) medium in open air soil conditions.

ACKNOWLEDGEMENT

The authors are thankful to the Pakistan Agriculture Research Council (PARC), Islamabad, Pakistan for providing funds under ALP research program to carry out this present research work.

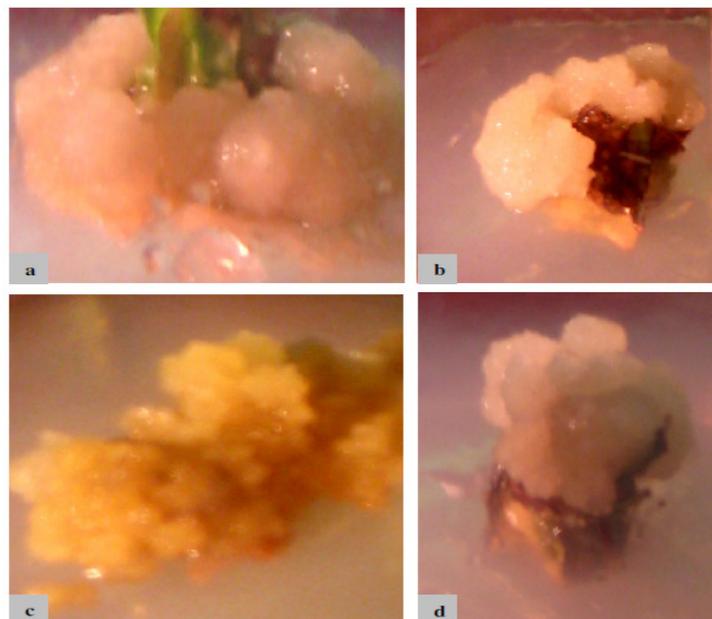


Figure 2. Callusing in sugarcane (*Saccharum officinarum* L.) cv., CPF-237 after mutagen (NaN_3) treatment for 6-days on different NaCl stresses. a: Callusing on MS nutrient medium with 0 mM NaCl (MS_2); b: Callus proliferation on MS nutrient medium with 25 mM NaCl (MS_{2a}); c: Culture on 50mM NaCl (MS_{2b}); d: On 75mM NaCl culture (MS_{2c}).

Table 1. Some callus growth and plant regeneration related parameters of 6-days NaN_3 stressed sugarcane cultivars cultured on different NaCl stressed nutrient media.

Name of cultivars	Medium	Thatta-10	CPF-237	SPHS-19
Sub-cultures of NaN_3 treated explants on different NaCl stressed cultures				
a. Callus proliferation (%)	MS_2	67.18±3.26	72.34±3.70	57.66±4.34
***	MS_{2a}	49.07±3.93	38.33±3.69	38.57±1.50
	MS_{2b}	42.27±2.16	17.03±2.32	20.48±1.09
	MS_{2c}	28.61±3.62	34.15±1.08	7.48±0.85
b. Relative water contents (%)	MS_2	75.32±3.37	74.08±2.33	75.16±3.14
**	MS_{2a}	71.44±2.69	63.37±2.69	64.97±3.44
	MS_{2b}	58.69±3.06	52.14±2.12	54.60±3.40
	MS_{2c}	49.45±4.17	56.93±2.15	48.47±2.04
Number of regenerated plantlets callus⁻¹ on NaCl stressed cultures and their heights				
a. No. of plantlets callus ⁻¹	MS_4	8.41±0.36	6.25±0.13	6.95±0.05
	MS_{4a}	1.33±0.24	4.94±0.05	0.92±0.09
	MS_{4b}	-	2.21±0.17	-
	MS_{4c}	-	-	-
b. Plant heights (cm)	MS_4	4.61±0.03	4.02±0.25	4.05±0.05
	MS_{4a}	1.66±0.34	3.25±0.11	2.02±0.03
	MS_{4b}	-	2.10±0.05	-
	MS_{4c}	-	-	-

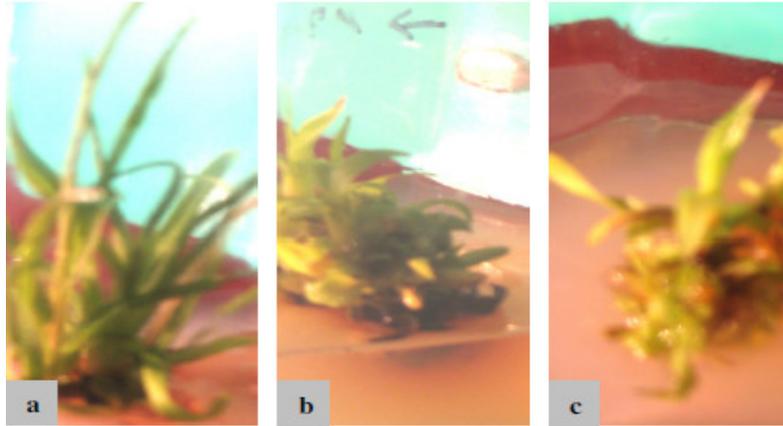


Figure 3. Plant regeneration in sugarcane (*Saccharum officinarum* L.) cv., CPF-237 under different NaCl stresses. a: Shoot multiplication on MS nutrient medium with 0 mM NaCl (MS₄); b: Plant multiplication on MS nutrient medium with 25 mM NaCl (MS_{4a}); & c: Culture on 50mM NaCl stressed culture (MS_{4b}).

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