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Regeneration of plantlets under NaCl stress from NaN$_3$ treated sugarcane explants

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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$_2$ (MS salts, 3.0 mg L$^{-1}$; 2,4-D, 0.5% NaN$_3$) for 6 days then sub-cultured on MS$_3$ (MS salts, 3.0 mg L$^{-1}$ 2,4-D), MS$_{2b}$ (MS$_2$, 25 mol m$^{-3}$ NaCl), MS$_{2c}$ (MS$_2$, 50 mol m$^{-3}$ NaCl) and MS$_{2d}$ (MS$_2$, 75 mol m$^{-3}$ NaCl) media in dark condition. After 6 weeks, callus growth was observed to be significantly higher (72.34 ± 3.70%) in CPF-237 in the control (MS$_2$), and lowest (57.66 ± 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$_3$ (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
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 conditions. 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 NaN$_3$ 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% NaN$_3$ for 6-days that was supplied in callusing medium (MS$_2$). The pH of medium was adjusted to 4.6 to 4.7.

**Callus induction and its proliferation**

After the treatment of NaN$_3$, explants were sub-cultured on NaN$_3$ free medium, MS$_3$ [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 (MS$_2$)].

**Somatic embryogenesis and plant regeneration**

For somatic embryo induction, well proliferated calluses (>6-weeks old) on MS$_2$ medium were sub-cultured on MS$_3$ 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 MS$_4$ (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 MS$_2$ (control), 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). Cultures were incubated under dark conditions for 6-weeks. At plant regeneration stage, calluses from MS$_2$, MS$_{2a}$, MS$_{2b}$ and MS$_{2c}$ were sub-cultured on MS$_3$, MS$_{3a}$ (MS$_3$, 25 mol m$^{-3}$ NaCl) and MS$_{3c}$ (MS$_3$, 75 mol m$^{-3}$ NaCl) media after somatic embryo induction (MS$_3$) 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:

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

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$^+$ 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 $\frac{16}{24}$ 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 NaN$_3$ 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...
create mutation (Kleinhaus 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$_{2a}$) for 6-days (Figure 1). They were washed with liquid callusing medium and again sub-cultured on mutagen free callusing medium (MS$_3$). They were also cultured on NaCl stressed callus growth media such as MS$_{2b}$, 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±3.70%) rate was observed on MS$_2$ culture of CPF-237, while lower 7.48±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 callus 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$_{2b}$ in all cultivars. Similarly, plant regeneration was not observed on MS$_{2c}$ 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**

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Figure 2. Callusing in sugarcane (Saccharum officinarum L.) cv., CPF-237 after mutagen (NaN₃) treatment for 6-days on different NaCl stresses. a: Callusing on MS nutrient medium with 0 mM NaCl (MS₂); b: Callus proliferation on MS nutrient medium with 25 mM NaCl (MS₂a); c: Culture on 50mM NaCl (MS₂b); d: On 75mM NaCl culture (MS₂c).

Table 1. Some callus growth and plant regeneration related parameters of 6-days NaN₃ stressed sugarcane cultivars cultured on different NaCl stressed nutrient media.

<table>
<thead>
<tr>
<th>Name of cultivars</th>
<th>Medium</th>
<th>Thatta-10</th>
<th>CPF-237</th>
<th>SPHS-19</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sub-cultures of NaN₃ treated explants on different NaCl stressed cultures</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>a. Callus proliferation (%)</td>
<td>MS₂</td>
<td>67.18±3.26</td>
<td>72.34±3.70</td>
<td>57.66±4.34</td>
</tr>
<tr>
<td></td>
<td>MS₂a</td>
<td>49.07±3.93</td>
<td>38.33±3.69</td>
<td>38.57±1.50</td>
</tr>
<tr>
<td></td>
<td>MS₂b</td>
<td>42.27±2.16</td>
<td>17.03±2.32</td>
<td>20.48±1.09</td>
</tr>
<tr>
<td></td>
<td>MS₂c</td>
<td>28.61±3.62</td>
<td>34.15±1.08</td>
<td>7.48±0.85</td>
</tr>
<tr>
<td>b. Relative water contents (%)</td>
<td>MS₂</td>
<td>75.32±3.37</td>
<td>74.08±2.33</td>
<td>75.16±3.14</td>
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<td></td>
<td>MS₂a</td>
<td>71.44±2.69</td>
<td>63.37±2.69</td>
<td>64.97±3.44</td>
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<td>MS₂b</td>
<td>58.69±3.06</td>
<td>52.14±2.12</td>
<td>54.60±3.40</td>
</tr>
<tr>
<td></td>
<td>MS₂c</td>
<td>49.45±4.17</td>
<td>56.93±2.15</td>
<td>48.47±2.04</td>
</tr>
<tr>
<td><strong>Number of regenerated plantlets callus⁻¹ on NaCl stressed cultures and their heights</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. No. of plantlets callus⁻¹</td>
<td>MS₄</td>
<td>8.41±0.36</td>
<td>6.25±0.13</td>
<td>6.95±0.05</td>
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<tr>
<td></td>
<td>MS₄a</td>
<td>1.33±0.24</td>
<td>4.94±0.05</td>
<td>0.92±0.09</td>
</tr>
<tr>
<td></td>
<td>MS₄b</td>
<td>-</td>
<td>2.21±0.17</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MS₄c</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>b. Plant heights (cm)</td>
<td>MS₄</td>
<td>4.61±0.03</td>
<td>4.02±0.25</td>
<td>4.05±0.05</td>
</tr>
<tr>
<td></td>
<td>MS₄a</td>
<td>1.66±0.34</td>
<td>3.25±0.11</td>
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<td>MS₄b</td>
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