Desiccation of callus enhances somatic embryogenesis and subsequent shoot regeneration in sugarcane
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Callus cultures were established in three commercial sugarcane varieties, viz., CoJ 64, CoJ 83 and CoJ 86, from spindle leaf segments on Murashige and Skoog (MS) medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D; 4 mg/L) and 6-benzylaminopurine (BAP; 0.5 mg/L). The calli were sub-cultured on MS+2,4-D (2 mg/L)+BAP (0.5 mg/L)+agar (0.8% w/v) medium (control) and MS+2,4-D (2 mg/L)+BAP (0.5 mg/L)+agar (1.6% w/v) medium (treatment) to study the effect of desiccation caused by double agar on somatic embryogenesis. Per cent somatic embryogenesis observed in treatment-calli of sugarcane varieties CoJ 64, CoJ 83 and CoJ 86 was 90, 90.63 and 89.66, respectively; while in control-calli the corresponding figures were 66.67, 64.52 and 63.33. Likewise, shoot regeneration from desiccated calli on MS+BAP (0.5 mg/L) medium was also higher over non-desiccated control, i.e., 84.27, 86.52 and 83.13% as compared to 54.35, 56.25 and 50.38%, respectively in CoJ 64, CoJ 83 and CoJ 86. Thus, this fairly simple double agar medium provided an alternative method for improving somatic embryogenesis and, hence, regeneration frequency of sugarcane callus.

Keywords: Callus, desiccation, regeneration, Saccharum officinarum L., somatic embryogenesis, sugarcane

Sugarcane (Saccharum spp., 2n = 100-205) is an important sugar crop of India. Annually, it accounts for nearly 70% of the total sugar produced worldwide. Conventional propagation rate through sets of sugarcane is very slow, which is a major problem in the rapid spread of newly developed varieties. Moreover, during vegetative propagation, the pathogens keep on accumulating generation after generation, which result in the decline of a variety. In this regard, the innovative approaches of cell and tissue culture possess a significant promise for the creation of new genetic variability for the improvement of a crop and quick multiplication of the varieties.

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Induction of somatic embryos is an important route for the regeneration of plants on a large-scale from cell cultures. Somatic embryogenesis is a process by which haploid or diploid somatic cells develop into differentiated plants through characteristic embryological stages without the fusion of gametes. Frequency of plant regeneration from callus cultures depends directly on the frequency of somatic embryogenesis. A number of factors (genotype, medium constitution, auxins, sugars, amino acids, growth retardants, desiccation, etc.) influences the process of somatic embryogenesis and plant regeneration. Desiccation has been reported to promote somatic embryo differentiation and development in soybean, grape vines, wheat, spruce, cassava and rice. Therefore, the present study was conducted to determine the effect of desiccation on somatic embryogenesis and subsequent shoot regeneration in sugarcane.

Healthy apical portions (10-15 cm long) were taken from 6-month-old field-grown plants of three commercial varieties of sugarcane, viz., CoJ 64, CoJ 83 and CoJ 86. Outer mature leaves were removed till a spindle of 5-6 cm length and about 1.0 cm diameter was obtained. Spindles were surface-sterilized using mercuric chloride (0.1%) for 8 min and washed thrice with sterile water. After removing outer two to three whorls of leaves, the innermost whorls of young leaves were cut into small segments (1.0-1.5 cm) to serve as explants. These were then aseptically cultured on Murashige and Skoog (MS) medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D; 4 mg/L), 6-benzylaminopurine (BAP; 0.5 mg/L) and agar (0.8% w/v) for callus induction. The obtained calli were sub-cultured on two different media, viz., MS+2,4-D (2 mg/L)+BAP (0.5 mg/L)+agar (0.8% w/v), which served as control, and MS+2,4-D (2 mg/L)+BAP (0.5 mg/L)+agar (1.6% w/v) (treatment) to observe the effect of desiccation caused by double agar on somatic embryogenesis. The embryogenic calli were transferred to MS+BAP (0.5 mg/L)+agar (0.8% w/v) medium for shoot regeneration. The cultures were incubated at 25±2°C and 70-80% humidity. Primary and secondary callus cultures were kept in total dark. For shoot regeneration, an illumination of 5000 lux
was given with 16/8 h (light/dark) regimes. Observations were recorded on per cent callus induction, somatic embryogenesis and shoot regeneration. The experiments on somatic embryogenesis and shoot regeneration were replicated twice and the data were analyzed using completely randomized design.

The explants cultured on callusing medium [MS+2,4-D (4 mg/L)+BAP (0.5 mg/L)+agar (0.8% w/v)] exhibited swelling after 2-3 d of incubation, followed by subsequent unwhorling of the leaves. This was attributed to rapid cell elongation primarily caused by auxin 2,4-D. The callus proliferation was first seen at the cut ends of second and third inner leaves after 7-8 d of incubation. This is because the cut surfaces absorb more nutrients leading to rapid cell division and subsequent callus formation. The primary explants of all the three varieties cultured on the callusing medium exhibited browning of the medium. In general, browning caused by the reaction of phenolic compounds, secreted from cut ends of cultured explants, with the medium constituents is an undesirable phenomenon as it affects the nutrient uptake by blocking cut ends of the explants. However, in the present case, there was little browning and it did not inhibit the callus growth. Browning has been reported in sugarcane cultures in some previous studies also.\(^{11,12}\) The callus induction medium induced excellent callusing (Fig. 1a) in all the three varieties, viz., 92.88, 93.11 and 91.10% in CoJ 64, CoJ 83 and CoJ 86, respectively. This implies that genotypic differences among the sugar cane varieties were little with respect to callus induction.

After 6 wks of incubation, the primary calli obtained from the cultured spindle explants were excised and sub-cultured on MS+2,4-D (2 mg/L)+BAP (0.5 mg/L)+agar (0.8% w/v) medium, which served as control, and MS+2,4-D (2 mg/L)+BAP (0.5 mg/L)+agar (1.6% w/v) medium to study the effect of double agar (desiccation) on somatic embryogenesis. In response to the agar concentration in both the media, the secondary calli of all the varieties exhibited varied degree of somatic embryogenesis. The light yellow, nodular and friable calli were considered as embryogenic, whereas smooth, watery and whitish calli were considered as non-embryogenic (Fig. 1b).

It is clear from Table 1 that differences between varieties of sugarcane with respect to per cent somatic embryogenesis on both the media were non-significant. When concentration of agar in the medium was 0.8%, per cent somatic embryogenesis observed was 68.72, 68.18 and 66.16% in the calli of varieties CoJ 64, CoJ 83 and CoJ 86, respectively. Upon elevating agar concentration to 1.6%, a significant increase in per cent somatic embryogenesis was recorded in the calli of all varieties and the corresponding data were 91.42, 90.75 and 89.28%. Fig.1c shows calli of variety CoJ 83 sub-cultured on double agar medium. It has

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**Fig. 1 (a-d)—Induction of callus and subsequent plant regeneration in sugarcane:** (a) Callus proliferation from spindle explants of sugarcane variety CoJ 83 on MS+2,4-D (4 mg/L)+BAP (0.5 mg/L) medium after 3 wks of culture; (b) Stereomicrograph (16×) showing embryogenic (EC) and non-embryogenic (NEC) sectors in sub-cultured callus of CoJ 83; (c) Calli of CoJ 83 sub-cultured on MS+2,4-D (2 mg/L)+BAP (0.5 mg/L) medium containing 0.8% w/v agar (I) and 1.6% w/v agar (II); and (d) Shoot regeneration in CoJ 83 from non-desiccated calli (I) and desiccated calli (II) after 1 month of incubation.
been suggested that desiccation of embryos affected the endogenous abscisic acid (ABA) level\(^4\) which promoted somatic embryogenesis in wheat\(^13\). In rice calli, desiccation induced two proteins having mol wts 22 kDa and 26 kDa, which were either not detected or present in a very low quantity in the non-desiccated calli\(^9\).

The embryogenic calli from both the cultures (control & dessicated) were transferred on a single shoot regeneration medium [MS + BAP (0.5 mg/L) + agar (0.8% w/v)]. It is also clear from Table 1 that differences between varieties of sugarcane for per cent shoot regeneration from embryogenic calli of both the cultures were non-significant. The embryogenic calli from control culture showed 57.59, 63.23 and 55.62% shoot regeneration in varieties CoJ 64, CoJ 83 and CoJ 86, respectively. On the other hand, embryogenic calli from desiccated callus culture exhibited increased shoot regeneration, i.e., 87.50, 86.91 and 84.99% in varieties CoJ 64, CoJ 83 (Fig. 1d) and CoJ 86, respectively. The cultures which did not show regeneration were found to be non-embryogenic. The differences in per cent shoot regeneration from desiccated and control calli were significant. In an earlier study on sugarcane, shoot regeneration from embryogenic callus has been reported to be improved on 1.2 and 1.6% (w/v) agar\(^14\).

In the present study, desiccation of callus cultures was achieved on 1.6% (w/v agar) and thereafter desiccated and non-desiccated calli were cultured on a single shoot regeneration medium. As a result, desiccation improved somatic embryogenesis in sugarcane and the calli exhibited a greater regeneration frequency, which is highly desirable for genetic transformation experiments.

### Table 1—Mean per cent somatic embryogenesis on two different MS media and mean per cent shoot regeneration on MS + BAP (0.5 mg/L) + agar (0.8% w/v) medium in sugarcane varieties

<table>
<thead>
<tr>
<th>Medium</th>
<th>Mean % somatic embryogenesis</th>
<th>CoJ 64</th>
<th>CoJ 83</th>
<th>CoJ 86</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS + 2.4-D (2 mg/L) + BAP (0.5 mg/L) + agar</td>
<td>68.72</td>
<td>68.18</td>
<td>66.16</td>
<td></td>
</tr>
<tr>
<td>(0.8% w/v) [Control]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS + 2.4-D (2 mg/L) + BAP (0.5 mg/L) + agar</td>
<td>91.42</td>
<td>90.75</td>
<td>89.28</td>
<td></td>
</tr>
<tr>
<td>(1.6% w/v) [Treatment]</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Calli</th>
<th>Mean % shoot regeneration</th>
<th>CoJ 64</th>
<th>CoJ 83</th>
<th>CoJ 86</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-desiccated calli</td>
<td>57.59</td>
<td>63.23</td>
<td>55.62</td>
<td></td>
</tr>
<tr>
<td>Desiccated calli</td>
<td>87.50</td>
<td>86.91</td>
<td>84.99</td>
<td></td>
</tr>
</tbody>
</table>

CD for treatment means for somatic embryogenesis at 5% level of significance = 7.44

CD for varietal means for shoot regeneration at 5% level of significance = 14.18

References