EFFECT OF SORBITOL PRETREATMENT ON YIELD AND VIABILITY OF PROTOPLASTS ISOLATED FROM ETIOLATED SHOOTS OF THREE CULTIVARS OF SORGHUM BICOLOR

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ABSTRACT

This study aimed at improving Sorghum protoplasts isolation procedures and maximizing yield using etiolated shoots of three cultivars (MH51, CH9 and CVX) for Sorghum bicolor (L) Moench. Hypocotyl sections were pretreated in sorbitol for varying periods of time and protoplast isolated in medium (pH 5.7) containing 3% cellulase (ONOZUKA R-10), 0.5% macerozyme (R-10) and 0.1% pectolyase (Y-23) in protoplast wash solution. Pretreatment time of 3.5 hours in sorbitol was found to be optimal for protoplast yield and viability. Optimal protoplast yields of 9.22 x 10^3, 8.10 x 10^3 and 6.80 x 10^3 were obtained per gram fresh weight of hypocotyl tissue in cultivars MH51, CH9 and CVX respectively, at 2.5 hour digestion time. This low enzyme digestion time decreased the toxic effects of enzyme preparations and consequently enhanced viability up to 75%. Results show that etiolated seedlings can provide a rapid and high-yielding source of viable Sorghum protoplasts. Efforts should, therefore, be geared towards improving the plating efficiency and regeneration capacity of protoplasts.

Keywords: Sorghum bicolor, protoplasts, etiolated shoot, sorbitol, yield, viability.

INTRODUCTION:

Plant protoplasts have become indispensable materials in several crop-breeding programmes as they are used for mutant selection, somatic hybridization, genetic transformation and plant regeneration (Galun, 1981). Such cellular genetic manipulations offer new possibilities for developing new varieties of Sorghum, an important crop used as food, fibre, fodder and fuel in the tropical and subtropical regions. Most of the processes require efficient methods for protoplasts isolation, culture and plant regeneration. Although several reports on Sorghum plant regeneration from cell calli have been described (Sharma et al., 1989; Cai and Butler, 1990), little has been reported on isolation, culture and regeneration from protoplasts (Chin and Scott, 1979; Wei and Xu, 1990).

The few recorded (Chourey and Sharp, 1985; Murphy and Cocking, 1988; Wei and Xu, 1989, 1990; Guo and Liang, 1991; Hagio et al., 1991) successes in Sorghum protoplasts isolation and culture have been achieved through the use of cell suspension cultures obtained from calli of different ex-plant sources, mostly immature embryos. The process of obtaining protoplast from cell suspension cultures takes at least 3 months and requires 14-16 hours exposure to enzyme mixture (Wei and Xu, 1990). The use of etiolated tissue offers several advantages over the cell suspension culture method.

These include easy manipulation, rapid growth and rapid isolation.

No record is available on the etiolated tissue method in Sorghum protoplasts isolation. Also, data on protoplast yield and viability in Sorghum are scarce in literature. This work was, therefore, carried out to determine the effect of sorbitol pretreatment on protoplast yield and viability in etiolated shoots of some Sorghum bicolor cultivars.

MATERIALS AND METHODS

(a) Sources of Sorghum bicolor cultivars.

Three cultivars of Sorghum bicolor (CH9, MH51 and CVX) were used in this study. The first two cultivars (CH9 and MH51), which are commercial hybrids, were supplied by seed production unit of Spic Science Foundation, Madras, India. The third cultivar (CVX) was supplied by Swaminathan Research Foundation, Madras, India.

(b) Germination.

Seeds of the three cultivars were surface-sterilized by soaking in 2% sodium hypochlorite for 10 minutes. Thereafter, they were rinsed four times with sterile distilled water and further surface-sterilized by soaking in 0.1% HgCl2 solution for 7 minutes. The double sterilization technique used was to ensure that the seeds were completely sterile before germination. The seeds were again rinsed four times with sterile distilled water and were germinated in sterile magenta boxes at 25°C in a dark cupboard.

(c) Protoplast Isolation and Purification

Ten-day old etiolated seedlings were used for protoplast extraction. Hypocotyl sections measuring 3 cm in length were cut twice longitudinally and
soaked in 600 mM sorbitol for varying periods of time (0.1, 3.5, 5 and 7 hours) prior to enzymic treatment. The protoplast isolation medium (Frearsen et. al., 1973) contained 3% cellulase (Onozuka RS), 0.5% macerozyme (R-10) and 0.1% pectolyase (Y-23) dissolved in protoplast wash solution. The pre-treated hypocotyl sections were incubated in the enzyme mixture (pH 5.7) at 27°C for varying periods of time (0.1, 2.5, 3.5 and 5 hours) in complete darkness but interrupted by gentle manual agitation at 20 mins intervals. Protoplasts were separated from undigested tissue by sieving through 30μm steel mesh and purified by sedimentation method recommended by Millam et. al., 1991. Complete digestion of cell wall was confirmed in the purified protoplasts using calcofluor white (Negata and Takebe, 1970).

(c) Protoplast Yield and Viability Test
Protoplasts were counted using a Fush-Rosenthal haemocytometer and yield determined per gram fresh weight of hypocotyl tissue. Ten counts were made per replicate. The counts were subject to analyses of variance tests and their means separated using the Duncan's multiple range tests. Viability of protoplasts was determined using the fluorescein diacetate (FDA) technique (Widholm, 1971; Bengoechea and Dodds, 1986).

RESULTS AND DISCUSSIONS
Protoplasts were readily isolated from etiolated seedlings of the three Sorgum cultivars (Plates 1). The optimal pre-treatment time, which gave the highest yield and viability values in 600 mM sorbitol was 3.5 hours in all cultivars. Pre-treating the tissues for longer periods was detrimental to both yield and viability of protoplasts (Table 1, Fig. 1). In general, cultivar MH51 gave the highest proportion of viable protoplasts while cultivar X gave the least. The untreated tissues (control) gave yields which were significantly lower than (P<0.01) yields from pre-treated tissues for each cultivar (Table 1).

Protoplast viability for the untreated tissue did not differ significantly (P>0.05) from those pretreated for 1 hour in sorbitol but differed significantly (P<0.01) from those pretreated for longer periods. This was observed in all the cultivars which further consolidates reports from previous workers that pre-treating the tissues in sorbitol before enzyme digestion enhances protoplast viability and yield (Nishimura et. al., 1984; Wright, 1985; Theodoropolos and Roubelakis-Angelakis, 1990).

Protoplast reasee started within 2 hours of enzymic digestion but the optimal incubation time in the enzyme mixture was 2.5 hours (Fig. 2). Incubating beyond this period resulted in a considerable reduction in yield due to a high proportion of the protoplasts becoming lysed. Protoplast yield at optimal conditions (2.5 hours digestion in enzyme mixture following a 3.5 hour pre-treatment in sorbitol) was in the range of 6.8-9.22 x 10^5 per gram fresh weight of hypocotyl tissue (Table 1). The purified isolate did not show fluorescence when stained with calcofluor white indicating that cell wall digestion was complete. Cultivar MH51 gave the highest protoplast yield (P<0.05) and the best viability while the unimproved cultivar X gave the lowest yields.

Yields obtained in the present study were similar to those obtained from suspension cultures of other Sorgum cultivars reported by Wei and Xu (1990). Also, since etiolated seedlings synthesize cell wall with less pectate components (Bengoechea and Dodds, 1986; Tan et. al., 1987; Bellini et. al., 1990) enzyme treatment time was considerably lowered (2.5 hours) which in turn decreased the toxic effects of enzyme preparations thereby facilitating the production of viable protoplasts. Previous workers have reported 14-16 hours as optimal enzyme treatment time for protoplast isolation when suspension cells of some Sorgum cultivars were employed (Wei and Xu 1990).

Table 1: Protoplast yield (X ± S.E.) in three Sorgum cultivars following different periods of pre-treatment in sorbitol and 2.5 hrs digestion in enzyme mixture

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Pre-treatment Period (hrs)</th>
<th>Yield (per gm fresh weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MH51</td>
<td>0</td>
<td>701,000±48.9</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>740,000±52.2</td>
</tr>
<tr>
<td></td>
<td>3.5</td>
<td>922,000±64.7</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>890,000±43.2</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>820,000±39.0</td>
</tr>
<tr>
<td>CH9</td>
<td>0</td>
<td>680,000±32.8</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>840,000±47.2</td>
</tr>
<tr>
<td></td>
<td>3.5</td>
<td>810,000±84.3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>780,000±64.2</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>720,000±54.9</td>
</tr>
<tr>
<td>CVX</td>
<td>0</td>
<td>490,000±39.8</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>540,000±54.7</td>
</tr>
<tr>
<td></td>
<td>3.5</td>
<td>880,000±62.1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>580,000±43.2</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>590,000±44.0</td>
</tr>
</tbody>
</table>

* Figures followed by the same case letter are not significantly different from each other (P>0.05)

Plasmolyzing cells with alcoholic sugars (sorbitol or mannitol) or carbohydrates (sucrose and glucose) before enzyme treatment enhances the stability of

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Fig. 1: Viability of protoplasts in three cultivars of Sorgum bicolor under different pre-treatment periods
the protoplasts (Eriksson, 1985). It also improves protoplast yield (Nishimura et al., 1984; Wright, 1985; Theodoropoulos and Roubelakis-Angelakis, 1990). The results of this study show that 3.5 hours of 600mM sorbitol pre-treatment time was optimal for yield and viability of protoplasts. Keeping the tissues for longer periods in sorbitol adversely affected viability (Fig. 1) and yield (Table 1). The exact pretreatment time, however, depended on the thickness of the materials as well as the conditions under which they were raised. The latter determines the amount of cutin deposits on the cell walls. For example, whole vines of Vitis vinifera raised in the green house required 24 hours of pretreatment for optimal yield (Wright, 1985) while those grown in vitro required 72 hours for a significant increase in yield (Theodoropoulos and Roubelakis-Angelakis, 1990). Both groups of investigators used similar pre-treatment media.

In summary, the results of the present study showed that etiolated seedlings can provide a rapid and high-yielding source of viable Sorghum protoplasts.

Plate 1: Freshly isolated protoplasts of *Sorghum bicolor* following pretreatment in sorbitol and digestion in enzyme mixture. (Scale bar = 40 µ)

A - MH51
B - CH9
C - CVX
D - Protoplasts from untreated tissues (control)
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REFERENCES