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Studies on the Rapid Clonal Propagation of *Saccharum officinarum*

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Abstract: A protocol for rapid *in vitro* propagation of *Saccharum officinarum* using meristem and axillary buds as explants was developed. Murashige and Skoog (MS) medium supplemented with 20 g l^{-1} sucrose, 2 g l^{-1} gelrite and various combination of kinetin (Kn) and gibberrellic acid (GA_3) was used as a culture initiation medium. Thirty three percent of the apical buds and 20% of the axillary buds grew into shoots within 15 days on media containing 1 mg l^{-1} Kn and 0.1 mg l^{-1} GA_3. Transferring the shoots after they were 5cm long on the liquid medium for 15 days led to the maximum multiplication of the shoots. Plantlets with profuse roots were obtained 10 days after the shoots were removed off and planted on the liquid medium containing 60 g l^{-1} of sucrose. More then 95% of the plantlets survived after one month when they were transplanted in the vermiculite.

Key words: *In vitro*, sugarcane, explant, growth regulators

INTRODUCTION

Sugarcane plays a significant role in sugar industry and is grown on 1093.4 thousand hectares with production of 52.517 million tones (Anonymous 2002). Improvement of sugarcane is hampered by the intricate flowering behavior under the existing climatic conditions. Breeding programme in the country involves only the import of fuzz and selection of exotic lines. Rapid multiplication is a serious problem in sugarcane breeding programme. It takes 6-7 years of field multiplication for the release of variety/exotic line on commercial scale. During that period degradation of variety/exotic line starts due to infestation of different systemic diseases (Hoy *et al*., 2001). *In vitro* micropropagation provides an opportunity for the rapid multiplication of sugarcane. By this technology nearly 1000,000 healthier, virus-free, true to type sugarcane plantlets can be produced from a single plant in one year (Lee, 1987). Plant tissue culture has been used with success in rapid clonal propagation of a number of economically important plants. There have been several research papers on *in vitro* propagation of sugarcane (Noguera *et al*., 2002; Pawar *et al*., 2002). Singh *et al*., 2001 described the micropropagation of sugarcane from shoot tip explants. The effect of explant source and genotype on growth of sugarcane *in vitro* was examined by Mulleegadoo and Dookun (1999). By micropropagation over 1.5 million plants can be produced form a single shoot tip in six months (Anita *et al*., 2000). In this paper, we report a micropropagation protocol of *S. officinarum* using apical and axillary buds as explants.

MATERIALS AND METHODS

Plant material: Three sugarcane cultivars CO-975, CP-77-400 and HSF-240 were used in this study. The stems of sugarcane were collected from Phaalia Sugar Mills during April 2003. Agricultural Biotechnology Programme, NARC, provided the experimental facilities. Apical meristem and axillary buds were cultured to study micropropagation. The size of apical meristem was 4-5 mm and that of axillary bud 4-8 mm in diameter.

Decontamination and culture of explants: Apical meristem and axillary buds were taken from plants grown in the field and dipped in a solution of ascorbic acid 100 mg l^{-1} and citric acid 150 mg l^{-1} for one hour. The explants were surface decontaminated by an initial dipping in 70% ethanol (v/v) for 2 min followed by treatment with 20% Clorox (commercial bleach containing 5% v/v sodium hypochlorite for 20min. There after the plant material was rinsed three times in sterilized distilled water. The explants were cut under aseptic conditions into 4-8mm segments and planted onto sterilized tissue culture media in culture tubes (24 x 100 mm). When the shoots developed from the apical and lateral buds were upto 3-5cm long, they were transferred to shoot multiplication medium. Clumps with 5-10 well grown shoots were transferred onto rooting medium.

Culture medium and conditions: The basal medium (BM) was consisted of Murashige and Skoog (1962) salts with macro, microelements and vitamins. Culture

Table 1: Effects of Kn and GA_3 on the growth of apical and axillary buds of sugarcane on culture initiation medium

	No. of buds inoculated		No. of buds forming shoots		%age of buds forming shoots		Average shoot height (cm)	
Hormonal level (mg l^{-1})	Apical	Axillary	Apical	Axillary	Apical	Axillary	Apical	Axillary
0.0	15	30	-	-	-	-	-	-
Kn 0.1	15	30	-	-	-	-	-	-
Kn 0.5	15	30	1	-	6.6	-	3±0.0	-
Kn 1.0	15	30	2	1	13.3	3.3	2±0.2	2±0.0
Kn 1.5	15	30	1	1	6.6	3.3	2±0.0	2±0.0
Kn 2.0	15	30	-	-	-	-	-	-
Kn 0.1+GA 1.0	15	30	-	-	-	-	-	-
Kn 0.5+GA 0.5	15	30	2	3	13.3	10.0	6±0.6	6±0.4
Kn 1.0+GA 0.1	15	30	5	6	33.3	20.0	5±0.3	4±0.7
Kn 1.5+GA0.05	15	30	2	4	13.3	13.3	3±0.2	2±0.5
Kn 2.0+GA 0.01	15	30	1	-	6.6	-	3±0.0	-
GA 1.0	15	30	-	-	-	-	-	-
GA 0.5	15	30	-	-	-	-	-	-
GA 0.1	15	30	-	-	-	-	-	-
GA 0.05	15	30	-	-	-	-	-	-
GA 0.01	15	30	-	-	-	-	-	-

Note: culture time was 15 days ± represent standard deviation of the mean

initiation medium was solid BM containing 0-2 mg l^{-1} of Kn and 0-1 mg l^{-1} of GA_3 (Table 1). Shoot multiplication medium was same as shoot initiation but liquid containing 1 mg l^{-1} of Kinetin and 0.1 mg l^{-1} of GA_3. Rooting medium was liquid BM with 6% sucrose without growth regulators. Shoot initiation medium was enriched with 20 g l^{-1} sucrose and solidified with 2 g l^{-1} of gelrite. The pH was adjusted to 5.8 prior to autoclaving at 121 °C for 20 min. All the cultures were inoculated under 40 $\mu molm^{-2}s^{-1}$ light provided by white cool fluorescent tube lamps at a photoperiod of 14 h at 25±1 °C.

Plantlet transplanting: Once rooted, the plants were carefully extracted from the tissue culture vessels and washed gently in water to remove excess medium and sucrose traces to discourage infection by fungal contamination. The plantlets were blotted lightly on filter papers and than planted into pots containing vermiculite in green house where relative humidity was 80-85%, light intensity was about 30% of natural light and temperature ranged from 25-30°C. The plantlets were sprayed with water 2-3 times every day in the first month and fertilized with the solution MS macro elements every 7-10 days.

RESULTS AND DISCUSSION

Surface sterilization: Surface sterilization of field grown plants could not be standardized despite several disinfection treatments used to increase the number of aseptic cultures. Explants either died or were heavily contaminated and showed bacterial contamination within 3 days of culture initiation.

Shoot initiation and multiplication: Not all the explants produced shoots on culture initiation medium. The effect of different medium combinations on culture establishment and growth is shown (Table 1). The explants on medium without growth regulators (control) failed to grow. However, shoot initiation was observed in cultures supplemented with plants growth regulators (Fig. 1a). Growth responses were better with apical buds than axillary buds. The Table 1 shows that 20% of the apical buds and 33% of the axillary buds developed into shoots. No significant difference in shooting response was detected between axillary buds and shoot apices. The length of the shoot was also similar in both types of explants. These results are in corroboration with Mulleegadoo and Dookun (1999).

The effect of the presence of two cytokinens types GA_3 and Kn in the MS medium was examined during this phase of propagation. GA_3 alone was less favorable for shoot initiation than Kn. With the progressive increase in Kn concentration from 0-2 there was a steady decrease in shoot length (6-2 cm). On the other hand, a gradual increase in GA_3 concentration in the same medium significantly stimulates the shoot initiation and marginally increases the shoot length. Higher and lower concentrations of both cytokinens were not able to support shoot initiation. Among all the concentrations tested, the best response (33%; shoot length 6cm) was achieved using 1 mg l^{-1} Kn in combination with 0.1 mg l^{-1} GA_3.

Single combination of Kn (1 mg l^{-1}) and GA_3 (0.1 mg l^{-1}) was used for the multiplication of three different cultivars. Different varieties showed different behavior for a single combination of cytokinens. The variety CP-77-400 was the most responsive for multiplication (25 shoots per culture) (Fig. 1b). On the other hand the recalcitrant variety was HSF-240 as it grows well in drought condition. The results reveal that HSF-240 requires some

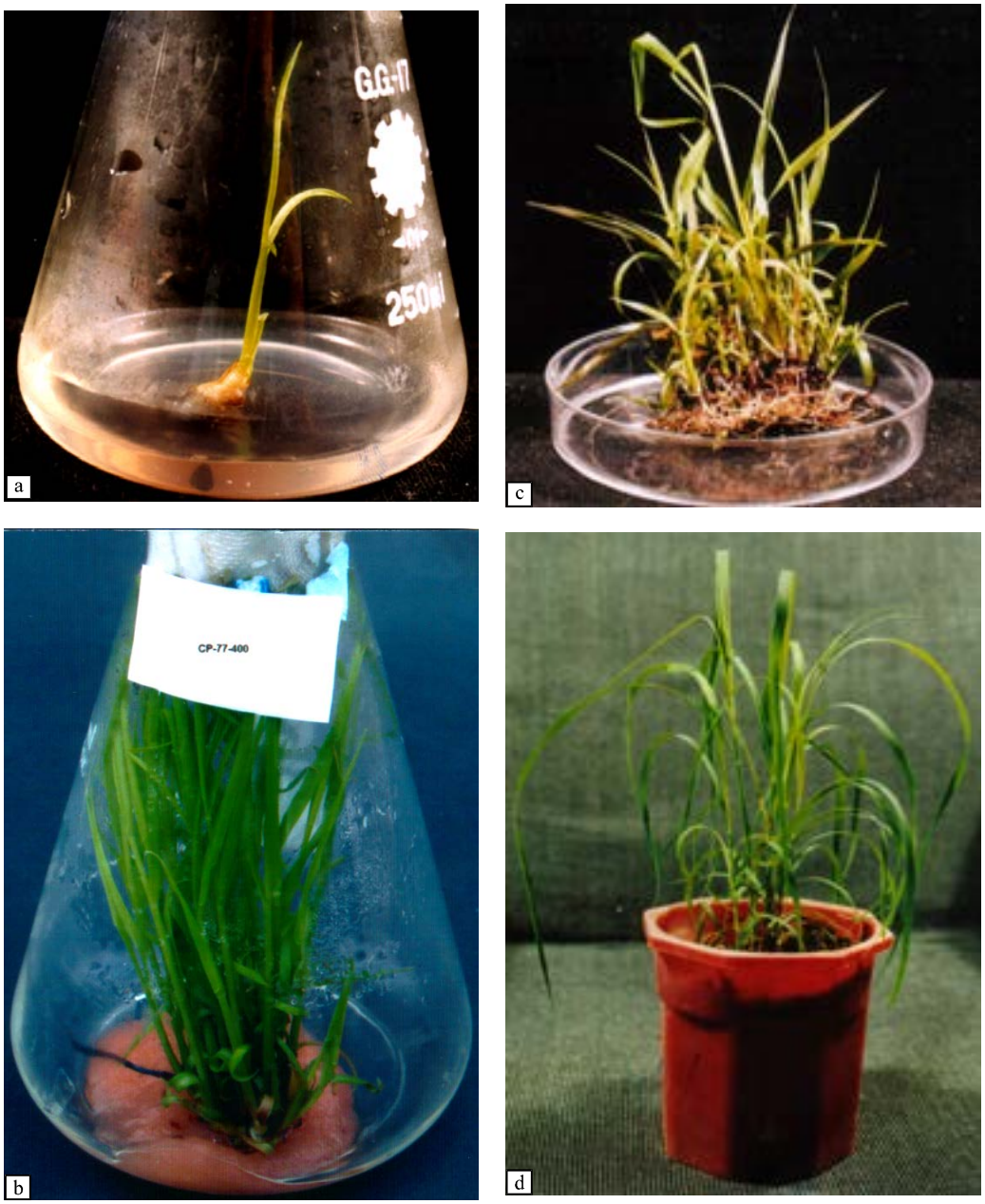

Fig. 1: Micropropagation of sugarcane
a) shoot initiation on culture initiation medium after 15 days
b) multiplication of sugarcane on liquid media after 15 days of culture
c) profuse rooting of sugarcane on PGR-free medium containing 6% sucrose after 10 days
d) acclimatization after 15 days in the green house

modifications in the medium. The plant remained greenish and grew well. Significant differences in the multiplication rates and the number of shoots formed by per plant were observed between different varieties. Shoot height was also good reflecting the role of kinetin. Patel *et al.* (2001) recorded highest multiplication on 1.5 mg l^{-1} Kn length of main shoot and no. of leaves on the main shoot. Chattha *et al.* (2001) demonstrated rapid and maximum multiplication on MS with GA_3 and BA containing medium. Shoots were well grown, strong enough and easily separable. There was no similar degree of shoots multiplication. These results are contrary to Singh *et al.* (2001).

Rooting and acclimatizing: Rooting and acclimatization were the most labor-intensive stages of this protocol as

the plants of S. *officinarum* did not always respond favorably especially the later stage. In some plant species rooting *in vitro* is largely genotype-dependant with shoots rooting naturally during propagation, thereby obviating the inclusion of a separate rooting stage. In other species a PGR-free medium encourages root formation. However, root formation is often inhibited by cytokinens, which are important for shoot multiplication (George and Sherrington, 1984). PGR-free medium containing 6% sucrose was the ideal for the protocol to be financially viable as 100% rooting was observed. Root initiation was noted after 4 days and within 10 days profuse rooting was observed (Fig. 1c). For rooting ½ strength MS medium with NAA and 60 g l^{-1} sucrose resulted in 75% rooting as reported by Singh *et al.* (2001) after 50 days.

Numerous futile attempts were made to acclimatize rooted S. *officinarum* plants that had been transplanted to vermiculite. A fully functional root system is harder to induce *in vitro* than *ex vitro* (Rayns, 1993). Frequently, the roots of the former lack root hairs and are prone to damage during planting out. The acclimated plantlets with profuse roots showed obvious growth 5 days after they were transplanted in vermiculite in green house (Fig. 1d).

Spraying water had strong positive effects on the survival of the plantlets. One month after transplanting the survival rate of the plantlets was higher than 95% and the young plantlets grew vigorously. Each plant formed 3-4 tillers 6 cm in length on its base after 10 days. In conclusion, we report an efficient and easy to handle protocol for micropropagation of sugarcane. This protocol provides a successful rapid technique that can be used for rapid propagation. The application of this protocol can help minimize the variety assessment period and contribute to the rapid propagation of high yielding cultivars of sugarcane.

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