Micro propagation of sugarcane (*Saccharum officinarum* L.) through auxiliary buds

P S Warakagoda, S Subasinghe, D L C Kumari and T S Neththikumara
Department of Crop Science, Faculty of Agriculture, University of Ruhuna, Mapalana, Kamburupitiya

Abstract
Sugarcane (Saccharum officinarum L.) *is an economically important plantation crop in Sri Lanka. There is a need to identify new vegetative propagation methods for mass scale production of commercial varieties of sugarcane to maintain clonal uniformity. In this study experiments were carried out to find the proper surface sterilization procedure, proliferation and rooting media and proper acclimatization process to complete the micro-propagation cycle for sugarcane auxiliary buds.*
Three different concentrations of Clorox (5.25% NaOCl) (15% (v/v), 20% (v/v), and 25% (v/v) with three different time durations (10, 15, 20 min.) were tested for survival percentage of in- vitro cultured auxiliary buds of sugarcane. Explants were established on MS (Murashige and Skoog, 1962) medium transferred to proliferation media with four different concentrations of BAP (Benzine Amino Purine) (0.5, 1.0, 1.5 and 2 mg/l) and 0.4 mg/l IBA (Indol Butyric Acid). To reduce browning, incorporation of 0.1 g/l Poly Vinyl Pyrolidone (PVP) to culture media was also tested. To induce in- vitro rooting, addition of 1.0 g/l activated charcoal to MS medium with four different concentrations of IBA (0.5, 1.0, 1.5 and 2.0 mg/l) with 0.2 mg/l BAP were tested. Suitable acclimatization procedure was tested with different combinations of sterilized sand and coir dust (1:0, 0:1, 1:1, 2:1 and 1: 2) as potting media for the acclimatization of in vitro derived plantlets.
Results revealed that 25% (v/v) Clorox for 20 min exposure time was the best surface sterilization procedure for sugarcane auxiliary buds. MS medium with 1.0 mg/l BAP and 0.4 mg/l IBA gave the highest proliferation rate (1:10) after eight weeks. The incorporation of 0.1 g/l PVP to the same medium reduced browning that occurred in the medium with out effects on shoot proliferation and growth. Highest root initiation rate and higher number of roots were observed in MS medium with 1 mg/l IBA, 0.2 mg/l BAP and 1 g/l activated charcoal. The most suitable potting medium for acclimatization of in vitro raised plantlets was found to be sand: coir dust at 1:2 ratio in which 100% survival rate was observed.
Keywords: acclimatization, auxiliary buds, clonal uniformity, ex-plants, *in-vitro*, micro-propagation, proliferation, surface sterilization

Introduction

Sugarcane is an economically important plantation crop in Sri Lanka. The main product of sugarcane is sugar and the other value added byproducts based on sugar manufacture are alcohol, vinegar, yeast, spirits, rum, fiber boards and acids. Cane tops, molasses and other derivatives could be used for formulating livestock feed (www. sugarres.gov.lk/highlights.htm). Currently there are about 9,000 ha under sugarcane cultivation in Sri Lanka. The cultivation is concentrated in Moneragala and Ampara districts and the average yield is 62 Mt/ha (Central Bank of Sri Lanka, Annual Report 2004). With the increasing demand for high quality sugarcane products, establishment of homogeneous plantations is a vital requirement. Propagation of sugarcane is done by asexual propagation and in commercial scale cultivation, stem cuttings is the main propagation method. The amount of planting material provided by this method is limited and there is a high risk of spreading diseases. The cost of planting materials is also high. Tissue culture techniques could be used as a tool for rapid clonal propagation and

production of disease-free, cost effective planting materials. However there are some limitations in surface sterilization of explants resulting in contaminations and browning due to secretion of polyphenols.

The main objective of this study was to develop a suitable protocol for *in-vitro* propagation of sugarcane through auxiliary buds. It is useful not only for rapid clonal propagation, but also for preservation of germplasm as well. The specific objectives were:

- to develop a protocol for surface sterilization of auxiliary buds
- to select an appropriate hormone combination for shoot proliferation
- to select an appropriate medium for root initiation and
- to identify proper acclimatization procedures for *in-vitro* derived plantlets.

Materials and methods

A series of experiments were conducted at the tissue culture laboratory, Faculty of Agriculture, University of Ruhuna. Auxiliary buds at the base of the leaf sheath of healthy sugarcane plants were used as explants. All the planting materials were washed thoroughly through running tap water for about one hour with a few drops of Teepol and dipped in 0.05 mg/l of fungicide (Thiram) solution for removal of fungal propagules.

Experiment 1: Identification of a suitable sterilization procedure for the collected explants

Explants were dipped and shaken in Clorox solutions with different concentrations and different exposure time periods (Table 1.1). Explants were thoroughly washed using sterilized distilled water to remove the sterilent before culturing. Survival rates and colour of the explants or media were observed at two weekly intervals.

Table 1.1 Treatment combinations used in surface sterilization procedure

Exposure time (minutes) / Clorox concentration (v/v)	10	15	20
15%	T1	T2	T3
20%	T4	T5	T6
25%	T7	T8	T9

Experiment 2: Identification of a suitable proliferation medium for initiated explants

Establishment of explants was done in MS medium and the cultures with initiated shoots were transferred to MS medium having different concentrations of BAP (Table 1.2) with 0.4 mg/l IBA. The aims were to detect the applicability of BAP alone without the combination of Kinetine with an auxine (IBA) instead of previously tested auxins as IAA and NAA to get the maximum number of shoots from auxiliary buds. (www.publish.csiro.au/paper/BT9950135.htm), (www.cababstractsplus.org).

Table 1.2 Different concentrations of BAP in four different proliferation media

Treatment	BAP concentration (mg/l)
T1	0.5
T2	1.0
T3	1.5
T4	2.0

Each treatment was repeated after adding 0.1 g/l PVP to the culture media, to detect its effect on browning. Height of the plants and number of new shoots formed in the cultures were recorded at weekly intervals.

Experiment 3: Selection of the suitable rooting medium

MS medium with four different IBA concentrations and 0.2 mg/l BAP were used with and without incorporation of 1.0 g/l activated charcoal (Table 1.3). Here the attempts were to detect the applicability of IBA and activated charcoal instead of NAA to initiate *in-vitro* rooting (www.cababstractsplus.org). To enhance further multiplication BAP was also used. Time taken for rooting and length of the roots were recorded at weekly intervals.

Table 1.3 Treatment combinations used to derive *in-vitro* roots

IBA concentration (mg/l)	With charcoal	Without charcoal
0.5	T1	T 5
1.0	T 2	T 6
1.5	T 3	T7
2.0	T 4	T 8

8g/l agar was used as the solidifying agent for the used cultures and 23^0C temperature, > 50% relative humidity, 16 hour photo period and 1220 Lux light intensity were maintained as culture conditions.

Experiment 4: Selection of a suitable acclimatization procedure for in vitro derived plantlets

Five different potting media with different ratios of sand and coir dust were tested for the acclimatization of plantlets prior to establishing in field conditions (Table1.4). 60% relative humidity was maintained inside the propagator where the pots were placed for the first two weeks and then transferred to the plant house. Survival percentages of plants and their growth performances were recorded at 2-weekly intervals.

Table 1.4 Sand and coir dust ratios used in different potting media

Potting media	Sand: Coir dust ratio
T 1	1:0
T 2	0:1
T 3	1:1
T 4	2:1
T 5	1:2

All experiments were carried out using a completely randomized design (CRD) with 20 replicates. The recorded data were statistically analyzed.

Results and discussion

Experiment 1: Identification of a suitable sterilization procedure for the collected ex-plants

According to the analyzed data, interaction of Clorox concentration and exposure time significantly affected the survival rates ($P< 0.05$) after four weeks. Survival rate of T8 treatment (25% Clorox for 15 minutes) was observed as a higher value (40%), after 2 weeks (Fig.1.1). After 4 weeks, the same treatment showed 50% survival percentage with contamination percentage of 50% (Fig. 1.2). At this time T9 treatment (25% (v/v) Clorox for 20 minutes) showed 70% survival with only 30% contamination rate. Therefore this treatment was selected as the most suitable. Here, contamination occurred randomly

within the first 2 week period and after that, the survival rate was increased with the increase of Clorox concentration and exposure time.

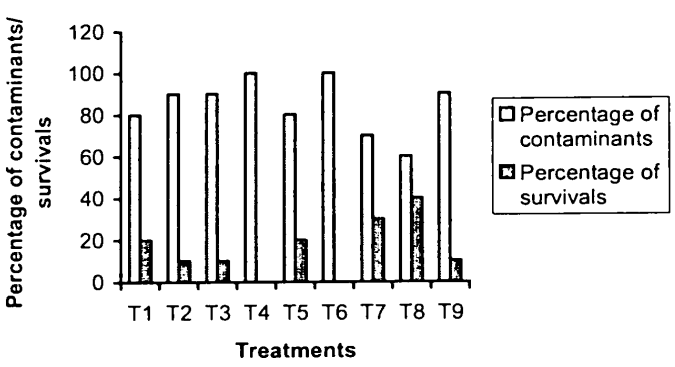

Fig. 1.1 Percentages of fungal /bacterial contaminants after 2 weeks

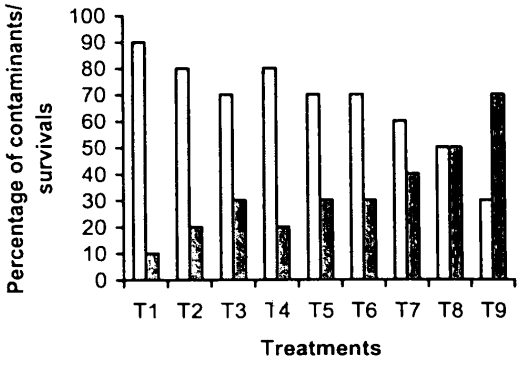

Fig. 1.2 Percentages of fungal/bacterial contaminants after 4 weeks

Experiment 2: Identification of a suitable proliferation medium for initiated ex-plants

BAP concentrations had a significant effect ($P < 0.05$) on heights of formed shoots and when mean height per plant was evaluated, T1 treatment (MS medium with 0.5 mg/l BAP, 0.5 mg/l IBA) and T2 treatment (MS medium with 1.0 mg/l BAP, 0.5 mg/l IBA) showed higher values during the 4 weeks time period (Fig.1.3). According to Fig. 1.4, T_2 shows the highest mean values for number of shoots at weekly intervals (1.5, 1.7, 1.8, and 2.3). Therefore BAP 1.0 mg/l and IBA 0.5 mg/l, was the most appropriate hormone combination for proliferation. The effect of applying 0.1 mg/l PVP to culture medium was zero for height of the plants and number of shoots formed in addition to reducing the browning of the culture medium that occurred after two weeks from culture establishment. PVP acts as an absorbent, which reduced the browning of culture media. Browning reduces the growth performances of plantlets by inhibiting the nutrient absorption from culture media (Kumar U, 2001).

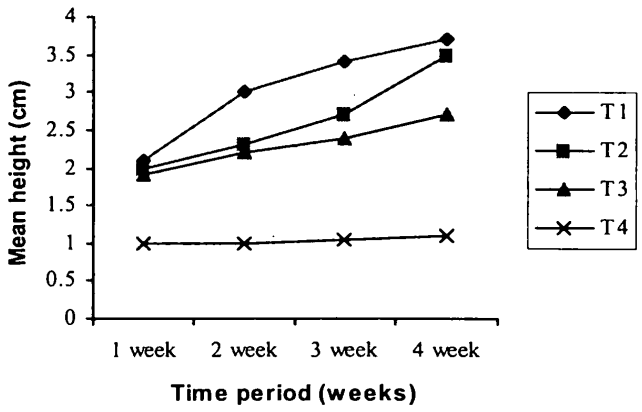

Fig. 1.3 Effect of different hormone combinations on height

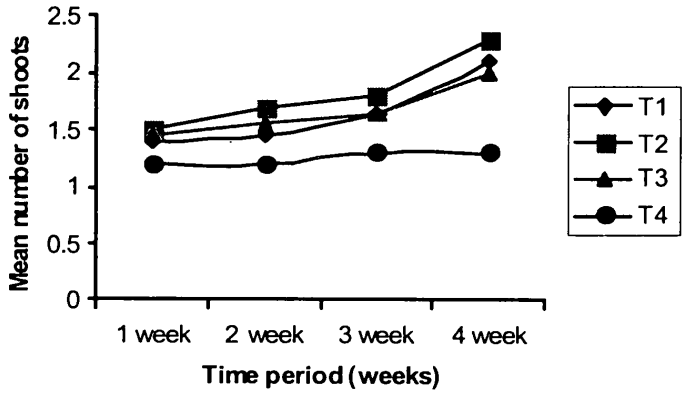

Fig. 1.4 Mean number of shoots under different hormone combinations

Experiment 3: Selection of suitable rooting medium

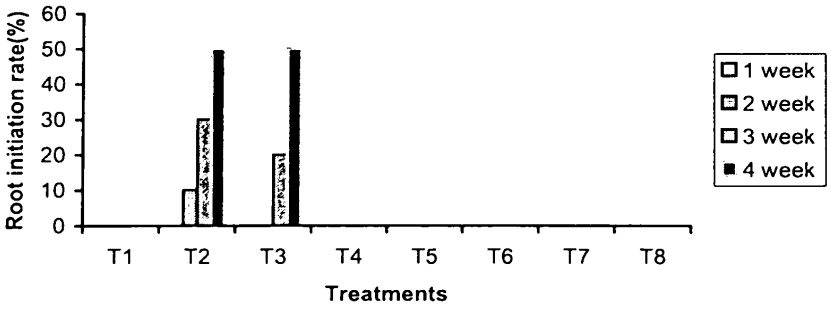

Fig. 1.5 Root initiation rates under different hormone combinations

Both IBA concentration and presence of activated charcoal had a significant ($P< 0.05$) effect on rooting, resulting in an interaction effect. None of the treatments produced roots during the first week. According to Fig 1.5, roots were observed only in T2 treatment (MS medium with 1.0 mg/l IBA, 0.2 mg/l BAP and 1 mg/l activated charcoal) during 2nd week. 50% of shoots produced roots, both in T2 treatment and in T3 treatment (MS medium with 1.5 mg/l IBA, 0.2 mg/l BAP and 1.0 mg/l activated charcoal) after 4 weeks. Among the 8 treatments, T2 and T3 are suitable for the rooting. MS medium with 1.0 mg/l IBA, 0.2 mg/l BAP and 1 mg/l activated charcoal (T2) was the best rooting medium because of early initiation of roots. Within 4 weeks time period, plantlets in media without activated charcoal failed to form roots.

Experiment 4: Selection of a suitable acclimatization procedure for in- vitro derived plantlets

100% survival rate was observed in T_5 treatment where sand: coir dust (1:2 ratio) was applied (Fig. 1.6). Plants did not survive on sand (T1) or coir dust (T2) alone, after 4 weeks time (zero survival rate). After a month 60% of plants in T5 (sand: coir dust ratio 1:2) formed new shoots (Fig. 1.7) whereas 40% plants in T3 (sand: coir dust ratio 1:1) and 30% plants in T4 (sand: coir dust 2:1 ratio) formed new shoots.

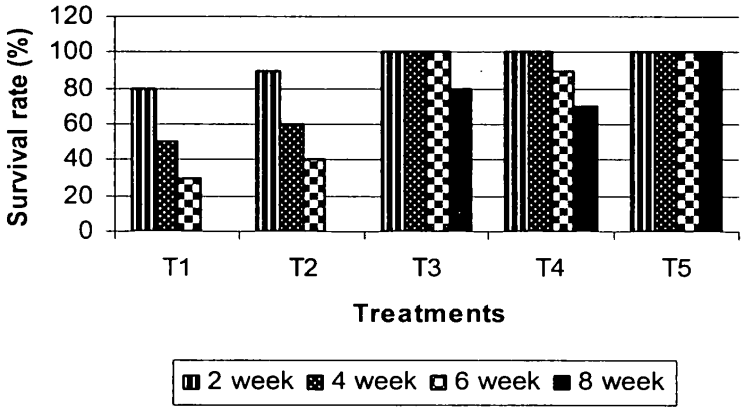

Fig. 1.6 Survival rates of plants in different potting media (after a month)

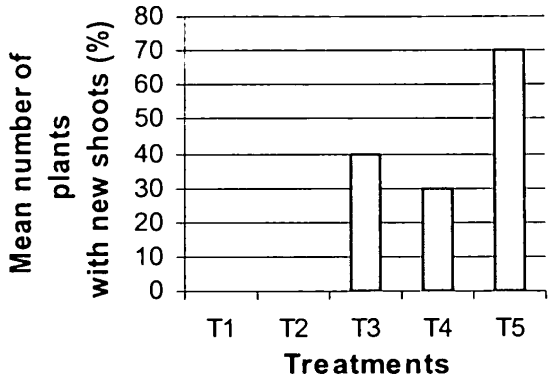

Fig. 1.7 Mean number of plants with new shoots

Conclusions

Auxiliary buds of sugarcane can be successfully surface sterilized using Clorox (25% for 20 minutes). MS medium with 1.0 mg/l BAP, 0.4 mg/l IBA and 0.1 g/l PVP was chosen as the best medium for shoot multiplication, based on the mean height per plant and mean number of shoots per explant. MS medium with 1.0 mg/l IBA, 0.2 mg/l BAP and 1.0 g/l activated charcoal was selected as the rooting medium. Sugarcane plants can be acclimatized in a potting mixture of sand and coir dust (1:2) and maintained under 60% relative humidity for two weeks.

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