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Micropropagation of Elite Sugarcane Planting Materials from Callus Culture in Vitro

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Abstract: Many plants including sugarcane (*Saccharum* spp.) has been successfully regenerated from callus culture *in vitro* worldwide and a varying range of explants were used. Presented in this paper is a procedure in which segments of the young innermost rolled leaves (2–3 cm) of four sugarcane cultivars (Q77N1232, Cadmus, Co997 and a local cutltivar (Lae1) cultured onto Murashige and Skoog (MS) medium supplemented with $3\,g\,L^{-1}$ 2,4-D, $0.5\,g\,L^{-1}$ PVP, $2\,ml\,L^{-1}$ BAP, $100\,ml\,L^{-1}$ coconut water, $30\,g\,L^{-1}$ sucrose and $8\,g\,L^{-1}$ agar (MSC3). Using this procedure, successful initiation of large amounts of contaminant-free, embryogenic callus ranging from 80–90 per cent was obtained which were potentially capable of efficient plantlet regeneration. Plantlets were regenerated by transferring the embryogenic callus onto regeneration media of same composition as MSC3 but without auxin (2,4-D). Adequate multi-shooting was achieved using no more than $2\,ml\,L^{-1}$ BAP and prior to acclimatization, rooting was achieved by sub-culturing the plantlets onto medium lacking cytokinin (BAP). Ninety to ninety five percent of the plantlets were successfully acclimatized in the green house and field planted after 6 weeks. There was no indication of morphological deviation from the parents.

Keywords: Tissue culture, embryogenic callus, micropropagation, elite planting materials

INTRODUCTION

Man has utilized 3,000 species of plants for food, of which 150 have entered world commerce. However, only 16 crops feed the World's population, among them being sugarcane (*Saccharum* hybrids) (Chaudhary, 1994). This crop is vegetatively propagated and is grown in many different countries across the World. Sugarcane provides about 65–70% of sugar produced in the World (Adam et al. 1995) and 90% in Papua New Guinea (Wagih and Musa, 2000).

The importance of this crop is demonstrated by the bulk volume produced, amount of energy provided, income generated and the type and number of people involved in sugarcane production. In PNG, sugarcane is used for commercial production as well as co-staple food together with other root and tuber crops. A significant portion, however, is commercially-based production of sugarcane. In the future, consumption of sugarcane is expected to increase sharply as the population increases; hence there will be an increase in production and demand for elite and improved planting materials.

Despite the importance of sugarcane, seed multiplication and propagation through cuttings by conventional method is slow, present risks of pest and disease transmission, and takes some years before a new cultivar is released and cultivated fully on a commercial basis (Dookun et al. 1996). Additionally, the time spent is considered as an economic loss and requires huge investment in improving elite propagative planting materials from the few parent stocks available. Sugarcane micropropagation has been reported by many workers using callus culture (Barba et al. 1977), bud culture (Wagih et al. 1995) and culture segment of young leaves (Alam et al. 1995). Among the many *in vitro* propagation techniques for sugarcane, Hendre et al. (1983) reported that it was possible to produce some 260,000 shoots from single shoot tips in four months and Comstock (1988) reported that about 20,000 plantlets could be multiplied weekly using shoot apex.

The latest advances in plant tissue culture and biotechnology, and the ability to regenerate improved planting materials from any portion of a plant using plant tissue culture techniques, means that there is now the potential to make propagation improvements much more quickly than by conventional methods. Such rapid sugarcane improvement could be very impor-

tant in PNG today where the population is growing at a fast rate. For this to be successful, sugarcane plants must be manipulated at the tissue level, and thus an understanding of the aims and techniques of sugarcane tissue culture is therefore essential. This paper describes an efficient and rapid method of micro-propagating elite sugarcane planting materials using callus culture *in vitro*.

MATERIALS AND METHODS

Field Practices and Sources of Material

Plant material

Three varieties of sugarcane (*Saccharum* spp. hybrids), Q77N1232, Cadmus, Co997 and a local cutltivar (Lae1) of unknown origin but widely cultivated by locals in Lae were grown at the Experimental Farm of the University of Technology, Lae, PNG, located at an altitude of 54 m above sea level at 147° 00' E and 6° 45' S. Canes were planted at a spacing of 1.5 × 0.5 m. Young shoots 10±2 months old were used as the source of explant. Tops stalks of these varieties were cut at the third node from the top visible dewlap, collected in paper bags and taken to the laboratory for immediate use.

Callus culture

Embryogenic callus (EC) was established as described by Wagih et al. (1998). The spindle cylinder of the innermost young rolled leaves just above the meristem tips (approximately 3 cm long) were surface-sterilized by dipping ino ethanol and flaming, then dipping in 1.5% sodium hypochlorite (NaOCl) for 15 minutes and washing three times with distilled water. A sterile scalpel blade removed the outer rolled leaves and the edge of the cylinder. The remainder of the cylinder (about 2 cm long) was cut transversally into four equal segments and cultured onto callus induction medium (MSC3). This medium consisted of MS (Murashige and Skoog, 1962), supplemented with 3 mg L^{-1} 2,4-D, 0.5 g L^{-1} polyvinylpyrrolidone (PVP), 0.5 ml L^{-1} BAP, 100 ml L^{-1} coconut water, 30 g L^{-1} sucrose; 8 g L^{-1} agar (Sigma Products) was used as the gelling agent. The pH was adjusted to 5.8 and a manual dispenser was used to dispense 20 ml into 7 cm plastic vials, then autoclaved at 15 psi (121°C) for 15 minutes.

Cultured vials were incubated in the dark at 26±2°C and routinely sub-cultured 2 times in the first week of culture (Bhojwani and Razdan, 1983) onto fresh medium to avoid toxicity of leached phenolics. It was then sub-cultured at 3 week intervals for further callus induction. Selected ECs were sub-cultured twice a month until abundant calli were obtained. ECs for plantlet regeneration were transferred onto the same media lacking 2,4-dichlorophenoxyacetic acid (2,4-D) (MSC) and incubated under photoperiods of 16 hours and 30 mol μE $m^{-2}s^{-1}$ of illumination at 28±2°C. Fifty percent of the shoots about 3–5 cm long with small roots were singled out and placed in 1.5 cm diameter test tubes with sterile distilled water under natural daylight (Wagih, 1999) and 50% were sub-cultured onto media lacking cytokinin (BAP) (Anderson, 1975) until abundant roots developed. Plantlets were then transferred to the green house for acclimatization (hardening) prior to field planting.

Acclimatization of Tissue Cultured Plants in the Greenhouse

Hardening of tissue cultured plants

Rooted plantlets were washed under running tap-water and transferred into a substrate mixture of sterile soil, 1 part farmyard manure, 1 part sand and 1 part top soil (ratio 1:1:1) in germinating trays. As the humidity is constantly high in Lae, no cover was provided to minimize transpiration. Careful watering twice a day was performed, ensuring that the soil was damp, but not wet and water logged up to 2 weeks and allowed to grow for 3–4 weeks until they reached a favourable height. At this age, the plants were watered once a day.

Four week old plants were then taken outside the greenhouse under shade, had their older roots and leaves trimmed once and transplanted into new soil of the composition; performance was monitored based on plant growth charac-

teristics under greenhouse conditions. There was no artificial light, both at night and in the day. This condition was considered an analogue of the field situation where the plants receive sunlight only during the day and less or nothing at night. Watering was done twice (8 am and 4 pm) a day, and adequate watering was carried every Friday afternoon to cater for requirements during weekends.

Growth Performances of the in vitro Regenerated Plants in the Greenhouse

Monitoring of plant growth

The growth performances of 100 plants in greenhouse conditions were monitored by assessing the development of leaves and roots, increase in height, tillering ability and phenotypic variations. Total number of leaves and roots per hill were and measured. The leaf lengths were measured from the base of leaf zero (first rolled leaf) to the tip of the longest leaf. The tillering ability was assessed manually by counting the tillers per hill. These plants were then uprooted, gently rocked to remove access soils and the roots washed under tap water. The roots per hill were counted and the longest roots measured. Broken roots were not considered for measurement and average data are presented in Table 2. The data collected were analyzed using the analytical software package *Statistix*.

RESULTS AND DISCUSSION

For the four varieties, callus induction, plantlet regeneration, shoot and root development including contamination and plant growth performance and characteristics recorded show large variability. The influence of the two media types (MSC3 and MSC) on callus initiation and regeneration including phenotypic variations based on plant characteristics observed are discussed below.

Cultivar	TCT TW	Iw (g)	Fw (g)	CG (g)	Ga	SD	Va	C.V.
Q77N1232	10 1–3	453.92	460.31	6.39	14.58	8.95	80.16	61.39
	10 3–6	460.31	473.53	13.22				
	10 6–9	473.53	497.67	24.14				
	10 9–12	497.67	492.50					
Cadmus	10 1–3	459.17	466.41	7.24	15.69	9.32	86.90	59.42
	10 3–6	466.41	480.55	14.14				
	10 6–9	480.55	506.24	25.69				
	10 9–12	506.24	506.21					
Co997	10 1–3	461.79	469.37	7.59	16.52	10.05	101.09	60.86
	10 3–6	469.37	483.96	14.56				
	10 6–9	483.96	511.37	27.41				
	10 9–12	511.37	511.34					
Lae1	10 1–3	456.55	462.84	6.29	14.39	8.88	78.91	61.73
	10 3–6	462.84	475.83	12.99				
	10 6–9	475.83	499.72	23.89				
	10 9–12	499.72	253.61					

Table 1. Growth determination of EC on MSC3 media by fresh weights (g). Total number of culture tubes (TCT), time in weeks (TW), initial weights (Iw), final weight (Fw) and callus growth (CG) are given. All initial weights include the weight of the culture tubes and the media ($20\,\mathrm{ml\,L^{-1}}$). Grand average (Ga), standard deviation (SD), variance (Va) and coefficient of variation (C.V.) are also shown. The former three are the average weights of callus (g) and the four are calculated based on the CG figures. Grand average is sum of the average CG for each cultivar between 1–3, 3–6 and 6–9 weeks. Callus growth in the period 9–12 weeks was not considered for statistical analysis as weight differences were negative.

Characters	**Varieties**			
	Q77N1232	Cadmus	Co997	Lae1
Plant heights (cm)				
Range	7–8.0	6.5–7	7–8.5	7–80
Average	7.50	6.50	7.40	7.00
Mean	7.50	6.52	7.36	7.10
SD	0.35	0.19	0.47	0.63
Variances	0.13	0.04	0.22	0.40
C.V.	4.71	2.95	6.42	8.91
Leaves (no)				
Range	6.50	7.50	6.00	5.00
Average	6.00	7.00	7.50	6.50
Mean	5.98	6.98	7.48	6.46
SD	0.55	0.38	0.22	0.11
Variance	0.31	0.14	0.05	0.01
C.V.	9.27	5.40	2.90	1.77
Leaf length (cm)				
Range	12–15.8	17–20.6	16–20.4	14–22.3
Average	10–13.6	14–18.4	14–18.2	12–20
Mean	11.78	16.18	16.06	15.95
SD	1.74	1.51	1.53	2.66
Variances	3.01	2.28	2.34	7.05
C.V.	14.73	9.34	9.53	16.65
Roots (no)				
Range	6–80	4.5–6.4	8–10.2	6.8–10.4
Average	8–10	5.4–8.0	8–11.4	9–10.2
Mean	9.04	6.66	9.66	9.60
SD	0.86	0.89	1.28	0.39
Variances	0.73	0.79	1.63	0.16
C.V.	9.47	13.37	13.21	4.10
Root Length (cm)				
Range	6.0–8.0	8–10.4	8–10.6	8–12.8
Average	6.5–7.0	7–8.8	8–12.4	10–11.6
Mean	6.76	7.86	10.18	10.78
SD	0.21	0.63	1.57	0.94
Variances	0.04	0.40	2.46	0.88
C.V.	3.08	8.08	15.41	8.71

Table 2. Growth assessment of greenhouse-grown plants by growth characters. For the selected 100 plants (25 per cultivar), range was calculated by subtracting shortest from longest average lengths and the average was calculated by dividing the total number and lengths of parameters considered by 100. Standard deviation (SD) and coefficient of variation (C.V.) are calculated based on the average data obtained (full data not shown).

Callus Development and the Influence of MSC3 Media

The protocol used in this project was found to yield large amounts of contamination-free callus with a high regeneration rate. After 11 days of sub-culture, callus initiation began with small white clusters of callus with creamy surfaces. After 6–9 weeks of cultivation, a considerable mass of white, compact and yellow, fast-growing, friable callus had accumulated on all the explants cultured (Figure 1). The rate of contamination of explants cultured due to the incidence of microbes, media, culture tubes or non-aseptic techniques was low (Figure 1) except during the first three weeks of sub-culture when 20–30% of the explants were lost. This was mainly due to leaching of browning substances onto the culture media. The incidence of grey, non-embryogenic mucilaginous callus was negligible except when cultures were prolonged.

At the end of the second sub-culture, more than 95% of the explants were contamination-free and had produced a considerable mass of callus. Observation has shown that callus proliferation was not restricted to the areas close to cut surfaces, but was observed where a wound has occurred. When calli were sub-cultured at three week intervals on MSC3 medium for 3–6 weeks, callus growth remained vigorous. It was found that continued sub-culturing for 6–9 weeks and beyond resulted in dehydration, a decreases in callus growth and loss of regenerative ability (Figure 2). Only 3–6 weeks old callus had a high regeneration potential and germination was detected 12 days after transfer to the MSC media. Analysis of variance showed that observed variation among mean callus growth of the four cultivars was highly significant ($p = 0.99$). The mean callus growth of the cultivars was 14.58 (Q77N1232), 15.7 (Cadmus), 16.5 (Co997) and 14.4 g (Lae1), respectively (Table 1).

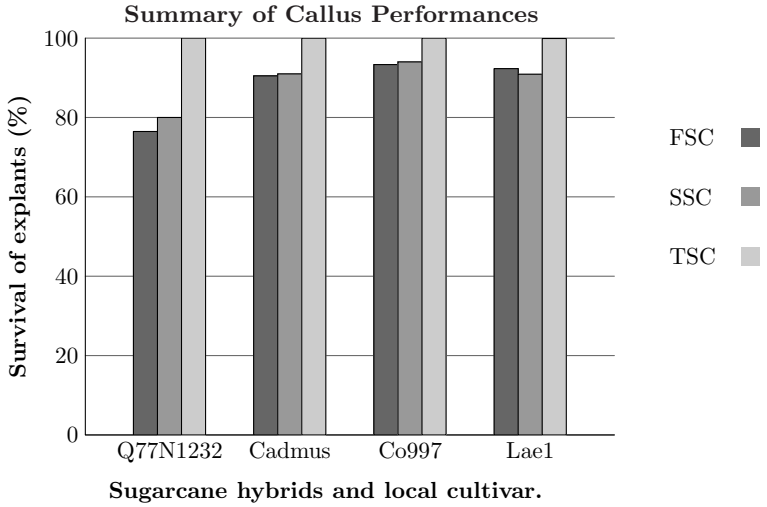

Figure 1. Survival of explants as percentage from first, second and third subcultures (FSC, SSC, TSC) at 3, 6 and 9 weeks. Percentage calculations are based on contaminated explants versus the total number cultured.

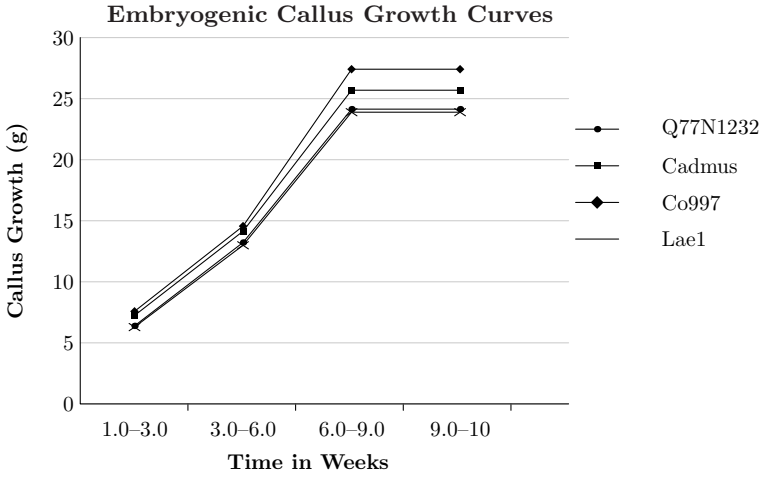

Figure 2. Callus growth of the four sugarcane cultivars at 3, 6 and 9 weeks. There was no further callus growth after 9 weeks of culture.

Minimizing the Effects of Browning Substances Leached into the Culture Media

Dookun et al. (1996) have detoxified phenols by either modifying the redox potential, inactivating phenolase enzymes, reducing phenolase activativation or substrate availability. Adams et al. (1995) reported that light activated polyphenoloxidases to form oxidates; less than 4% browning occurred under a light intensity of 150 Lux and 18% at 500 Lux with cultured garlic meristem clone explants. Given limited resources, an addition of 0.5–1 g L^{-1} of polyvinylpyrrolidone (PVP) to cultures exposed to 16 hours light and 8 hours darkness and continuous subcultures (cultures in the dark) has proven to be a cheap and efficient means of reducing tissue browning.

The degree of browning was found to be variety-specific and in more than 5% of the explants necrosis started from the younger rolled leaves rather than older ones. This indicated that using older explants (2–3 years old) would be advisable to avoid the leaching of phenols. Additionally, it was obvious that necrosis occurred more frequently in the early days of the initial culture, both in explants, calli, young shoots and rooted plants. This indicated that phenols were present wherever there was active plant growth (the meristematic regions of shoots and roots), except in the mature part of a plant such as the stem.

Shoot Regeneration, Root Development and Dedifferentiation on MSC Media

Following the transfer of EC onto MSC, numerous embryoids or miniature shoots (green-purplish) started to develop on callus surfaces. Most callus turned purple to purplish-green and within 3 to 4 weeks the entire EC surfaces were covered with green and healthy shoots. Four week old isolated shoots on MSC media developed a few or no roots and special effort had to be made to stimulate root development. For these shoots, the method of Wagih and Musa (2000) was adopted. Shoots without any forms of roots were transferred to 2 mL of sterile distilled water and within 14 days developed abundant roots. Rooting in MSC media in the presence of 2 ml L^{-1} BAP resulted in certain root development.

Genetic Variation on Regenerated Plantlets

Individual plantlets showed great variation with such differing phenotypic characters as pale green, wide and thick and hairy or arrow leaves. Dwarf plantlets and albino plantlets as reported

by Wagih and Musa (2000) were obvious in many cases. Many researchers, including Wagih and Adkins (1989) and Liu and Chen (1974), have reported similar types of variation in their work with sugarcane tissue cultures. An attempt to pin point the cause of these variations was impossible at the time of this project, but current work on sugarcane tissue culture at the University of Technology Biotechnology Centre is addressing this problem. Greenhouse-grown plants have shown no variations like those observed *in vitro* and the plants were clones of their mother plants.

Multiplication of Shoots in Vitro

So far as lack of planting materials is concerned, induction of multi-shoots is all that was required. Induction at a BAP concentration of $2\,\mathrm{ml\,L^{-1}}$ resulted numerous plantlets, which were potentially capable of growing into mature plants. In this project, a total of 750 plantlets on average per month were regenerated and would have regenerated as many as 9000–10000 in 12 months. This demonstrates that thousands of plants can be micro-propagated for commercial sugarcane production from a few available parent stocks.

Assessment of Plant Growth and Development

Growth as indicated by leaf development, root formation, multishoots and height increase of sub-cultured plantlets was favorable, and there was numerous multiplication of shoots with huge variation in leaf length, color and venation, thickness and plant height. Determination of actual leaf and root lengths, shoot heights and multishoots while in the culture tubes was impossible and Table 2 shows some of the variation of 100 randomly selected plants in the greenhouse. Some 95–100% of the plants grown successfully reached maturity. One-way analysis of variance showed that all the plant parameters used for assessing growth and development of glasshouse grown plants (Table 2) were significantly different with p-values of the plant parameters heights (0.02), leaves (0.00), leaf length (0.01), root (0.00) and root length (0.00). The observed variations had probability values of less than 1 percent.

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