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Developmental and hormonal regulation of direct shoot organogenesis and somatic embryogenesis in sugarcane (*Saccharum* spp. interspecific hybrids) leaf culture

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Abstract Rapid and efficient in vitro regeneration methods that minimise somaclonal variation are critical for the genetic transformation and mass propagation of commercial varieties. Using a transverse thin cell layer culture system, we have identified some of the developmental and physiological constraints that limit high-frequency regeneration in sugarcane leaf tissue. Tissue polarity and consequently the orientation of the explant in culture, size and developmental phase of explant, and auxin concentration play a significant role in determining the organogenic potential of leaf tissue in culture. Both adventitious shoot production and somatic embryogenesis occurred on the proximal cut surface of the explant, and a regeneration gradient, decreasing gradually from the basal to the distal end, exists in the leaf roll. Importantly, auxin, when added to the culture medium, reduced this spatial developmental constraint, as well as the effect of genotype on plant regeneration. Transverse sections (1–2 mm thick) obtained from young leaf spindle rolls and orienting explants with its distal end facing the medium (directly in contact with medium) are critical for maximum regeneration. Shoot regeneration was observed as early as 3 weeks on MS medium supplemented with α-naphthalenencetic acid (NAA) and 6-benzyladenine, while somatic embryogenesis or both adventitious shoot organogenesis and somatic embryogenesis occurred on medium with NAA and chlorophenoxyacetic acid. Twenty shoots or more could be generated from a single transverse section explant. These shoots regenerated roots and successfully established after transplanted to pots. Large numbers of plantlets can be regenerated directly and rapidly using this system. SmartSett®, the registered name for this process and the plants produced, will have significant practical applications for the mass propagation of new cultivars and in genetic modification programs. The SmartSett® system has already been used commercially to produce substantial numbers of plants of orange rust-resistant and new cultivars in Australia.

Keywords Direct regeneration · *Saccharum* · SmartSett® · Somaclonal variation · Somatic embryogenesis · Sugarcane · Thin cell layer culture

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Introduction

Commercial sugarcane (Poaceae), mainly the complex aneuploid polyploid interspecific hybrids of *Saccharum officinarum* and S. *spontaneum* (Irvine 1999), is a major field crop accounting for about 75% of sugar production worldwide (World Sugar Statistics 2005). It is one of the most efficient biomass crops with adaptability to both tropical and subtropical conditions, and is now being targeted as a biofactory with potential for the integrated production of sugar and other industrial and high-value products (Nonato et al. 2001; McQualter et al. 2004; Lakshmanan et al. 2005).

Sugarcane is vegetatively propagated for commercial planting by stem cuttings, called billets. In Australia, a major sugar exporter, over 1.2 billion new sugarcane seedlings are planted each year. Production of disease-free seedlings in such large numbers during the planting season is laborious and time consuming. In addition, it requires a substantial quantity of cane, more than 1 Mt annually, that otherwise could be used for sugar production. Development of more efficient methods for large-scale production of pathogen-free planting material would contribute significantly to the overall productivity of the sugar industry.

Tissue culture offers an opportunity to mass produce disease-free planting material and is now used to supplement commercial sugarcane propagation in many countries including Brazil, the United States, India and Cuba. The two most commonly used methods for sugarcane micropropagation are shoot tip culture (Hendre et al. 1983; Lee 1987; Burner and Grisham 1995) and callus culture (Ho and Vasil 1983; Liu 1993; Chengalrayan and Gallo-Meagher 2001). Although these techniques have been successfully used in many crop species, micropropagated sugarcane plants often show considerable levels of somaclonal variation (Lourens and Martin 1987; Burner and Grisham 1995). Elimination or substantial reduction of somaclonal variation is, therefore, critical for any practical application of tissue culture technology for sugarcane improvement and propagation.

Rapid regeneration of plants directly from explants presents an effective strategy to avoid or substantially reduce somaclonal variation as it minimises culture duration and eliminates or minimises callus formation in culture. However, reliable methods for rapid, high-frequency direct plant regeneration are limited in monocotyledonous plants, especially for species in the *Poaceae*. As part of our research to establish an efficient, commercially useful sugarcane regeneration and transformation system that minimises somaclonal variation, we have investigated the biological constraints that limit rapid in vitro regeneration in sugarcane. Using the concept of thin cell layer culture (Tran Thanh Van 1973; Lakshmanan et al. 1995) we identified some developmental and hormonal constraints that affect in vitro morphogenesis of sugarcane and successfully exploited this knowledge to develop a rapid and efficient direct organogenesis or somatic embryogenesis system that could be used for both mass production and genetic transformation. Here, we report on the development of the regeneration system and discuss the role of developmental and hormonal factors that regulate shoot organogenesis and somatic embryogenesis in leaf cultures of sugarcane.

Materials and methods

Plant material

The sugarcane cultivar Q165^A, which was readily available throughout the year, was used for most of the experiments reported here. Sugarcane cultivars Q117, Q156, Q157, Q167^A, Q172^A, Q185^A, Q188^A, Q190^A, Q196^A, Q197^A, Q200^A, Q205^A, Q216^A and Q222^A were also used.

Preparation of explants, culture conditions and shoot regeneration experiments

Shoot tops of 4 to 8-month-old field-grown sugarcane plants were used as the source of explant. After removing the outer mature leaves, shoot tops were thoroughly washed with water, sprayed with 70% ethanol and, under aseptic conditions, additional outer leaves were removed to isolate immature leaf rolls (about 1 cm diameter) formed by the innermost 5–6 tightly furled spindle leaves. Since the young developing leaves are very tightly furled, the innermost leaves will be free from microbial contamination. An 8–10 cm long basal portion of the leaf roll, starting from the leaf base just above the apical meristem, was excised and placed in liquid Murashige and Skoog medium (MS; Murashige and Skoog 1962). Each portion was then cut sequentially into 1–2 mm thick transverse sections (TS), beginning from the basal end of the roll. Ten to 30 TS explants were prepared from each leaf roll depending on the objective of the experiment.

For all experiments MS mineral formulation supplemented with 30 g/L sucrose was used as the basal nutrient medium (BM). Unless otherwise specified, for shoot regeneration experiments TS explants were cultured in petri dishes (90 mm × 25 mm) with the distal end in contact with the medium. All plant growth regulators and antioxidants (ascorbic acid [AA], 150 mg/L and citric acid [CA], 100 mg/L) were filter-sterilised and added to the autoclaved media. The pH of media was adjusted to 5.7 ± 0.1 before autoclaving. Except where liquid cultures were tested, all shoot regeneration experiments were performed with semi-solid media gelled with Davis J3 grade agar (8 g/L). All cultures were sealed with a single layer of 3 M Micropore^{TM} tape and incubated at $26 \pm 1^{\circ}$C under 16 h photoperiod provided by cool white fluorescent tubes with a photon flux density of 30 μmol m^{-2} s^{-1} at the culture level. Cultures were transferred to fresh medium once every 3 weeks, or more frequently if necessary, and maintained for 8–12 weeks for different experiments.

Preliminary experiments to assess the potential of direct shoot regeneration from sugarcane leaf tissue were performed with cultivars Q117 or Q165^A. Leaf TS explants of Q117 were cultured on BM supplemented with different concentrations and combinations of α-naphthaleneacetic acid (NAA; 10–50 μM), 6-furfurylaminopurine (kinetin; 4–20 μM) and 6-benzyladenine (BA; 4–12 μM). Based on the preliminary results obtained with Q117, TS explants of Q165^A were cultured on BM supplemented with 10–40 μM NAA and 4 μM BA and/or 4 μM kinetin (KIN) for 8 weeks to determine the optimal growth regulator requirement for consistent high-frequency direct shoot regeneration. Since the role of tissue polarity/explant orientation (whether the proximal or distal end of the leaf section is in direct contact with the medium) on shoot regeneration was not considered

in these experiments, explants were oriented randomly on culture medium.

Tissue polarity, plant growth regulators, explant size and genotype effects on shoot regeneration

The effect of tissue polarity on shoot bud regeneration was examined by culturing TS explants and 5–6 mm long leaf roll segments on BM supplemented with 4 μM BA and 10 μM NAA. This shoot regeneration medium (SRM) was optimised for Q165^A. Explants were placed either with the proximal or distal surface in direct contact with the medium (facing the medium).

Besides NAA, BA and kinetin, the effects of other commonly used plant growth regulators on shoot bud regeneration were determined by culturing TS explants on BM supplemented with different concentrations and combinations of BA, indole-3-acetic acid (IAA: 5–20 μM), indole-3-butyric acid (IBA: 5–20 μM), 2-chlorophenoxyacetic acid (CPA: 5–20 μM), 4-hydroxy-3-methyl-*trans*-2-butenylanminopurine (zeatin: 4–8 μM), and 1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea (thidiazuron: 2–8 μM) for 6–8 weeks.

To evaluate the effect of explant size on shoot morphogenesis, immature leaf rolls were cut transversely into 1–2 and 5–6 mm long segments and cultured on SRM for 6 weeks and then on BM for another 6–8 weeks. To ascertain the overall utility of this system for sugarcane propagation TS explants of 12 commercially cultivated sugarcane cultivars with diverse genetic backgrounds (Q156, Q167^A, Q172^A, Q185^A, Q188^A, Q190^A, Q196^A, Q197^A, Q200^A, Q205^A, Q216^A and Q222^A) were cultured on SRM for 8 weeks and subsequently on BM for another 4–6 weeks. The response of these genotypes to this new system was further investigated by culturing them for 8 weeks on an embryogenic medium (EM, which contains MS minerals and nutrients, 10 μM NAA and 10 μM CPA) followed by 6–8 weeks growth on BM. Preliminary experiments showed that medium containing CPA and NAA induces embryogenic cultures in sugarcane.

Spatial distribution of shoot regeneration potential in leaf roll and its manipulation by auxin

Thirty TS explants were harvested from each immature leaf roll by sequential transverse sectioning beginning from the basal end of the leaf roll and they were divided equally into three distinct groups; the proximal, middle and distal segments relative to the basal end of the leaf roll. These three groups of explants were cultured on SRM or BM enriched with 4 μM BA and 5–40 μM NAA for 8 weeks and shoot regeneration response was measured.

Membrane raft culture and shoot regeneration

Experiments were also conducted with explants cultured on membrane rafts (Sigma, USA) floated on liquid SRM in GA7 vessels (Magenta Corp., Chicago, USA). Explants were placed with the distal end in contact with the raft membrane and were transferred to fresh medium every week for the first 4 weeks of culture and every 2 weeks thereafter.

Shoot growth, in vitro and ex vitro rooting, and seedling establishment

Clusters of shootlets, 5–15 mm tall, were excised and cultured on BM with or without BA (1–2 μM) in Petri dishes (90 mm × 25 mm) for 2–3 weeks. For root development, 20–30 mm tall shoots were cultured in 175 mL glass culture vessels (Sigma V0633) fitted with vented Magenta B-caps (Sigma B3031) containing BM for 2–3 weeks. Plantlets with roots at least 20–30 mm long were transplanted to seedling trays filled with a perlite, peat moss and sand (1:1:1v/v) and acclimatised for 7–10 days under high humidity before transferring to a nursery.

To assess the potential of ex vitro rooting to reduce the duration of plants in culture, Q165^A shootlets (30–40 mm tall) were transplanted to seedling trays containing perlite: peat moss: sand (1:1:1 v/v) and maintained in a glasshouse for 6 weeks. These transplants were irrigated at least twice weekly and fertilised with Thrive® (Arthur Yates and Co Ltd., Homebush, Australia), a commercial all-purpose water-soluble plant nutrient formulation, once every 10 days.

Statistical analysis

All experiments were repeated and each treatment had 6–8 replicates. Each replicate consisted of at least 10 TS explants or 10–15 shootlets depending on the experiment. Morphogenic responses of explants were evaluated after 6–8 weeks of culture. Data were analysed using analysis of variants (AOV) and Tukey's HSD multiple rage test was used for comparison of means.

Results

Initial observations on leaf TS culture; phenolic exudation is a significant problem

Leaf explants cultured in all media except those containing CPA enlarged considerably and turned green within 7 days of culture. However, nearly half of the explants turned brown and died within 2 weeks of culture apparently due to excessive production of phenolic compounds. The initial experiments showed that wounding-induced phenolic exudation and tissue discolouration, which occurred soon after excision, could be greatly reduced by preparing explants in liquid BM with the antioxidants ascorbic acid (150 mg/L) and citric acid (100 mg/L) (data not shown). However, a similar result was also obtained with liquid BM without

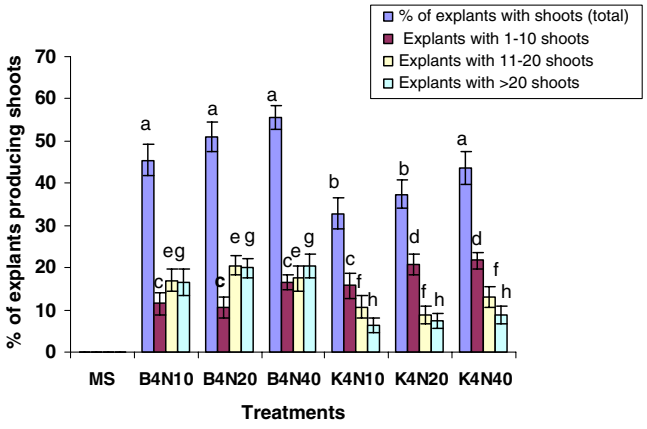

Fig. 1 Shoot production in thin leaf explants (1–2 mm) of sugarcane cultivar Q165^A after 8 weeks of culture on MS medium supplemented with 4 μM 6-benzyladenine (B) or kinetin (K) and 10–40 μM α-naphthaleneacetic acid (N). Explants were oriented randomly without considering whether the proximal or distal end was in contact with the medium. Data shown are the mean of 14 replicates ± standard error. In each category, values with the same letter are not significantly different ($P<0.05$)

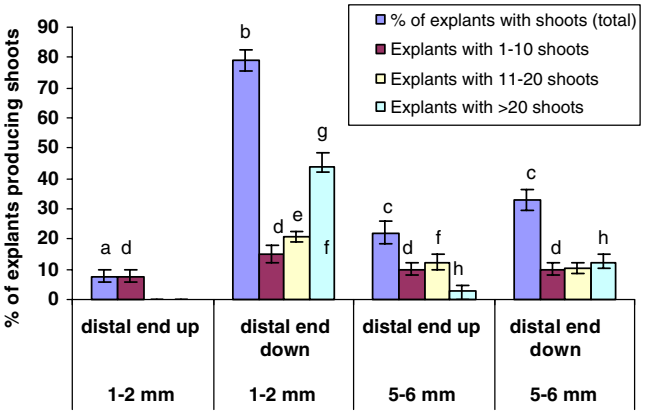

Fig. 2 Influence of explant size and orientation on shoot production in thin leaf explants (1–2 mm) of sugarcane cultivar Q165^A after 8 weeks of culture on MS medium supplemented with 4 μM 6-benzyladenine and 10 μM α-naphthaleneacetic acid. Explants were oriented with either the distal or proximal end in contact with the medium. Data shown are the mean of 14 replicates ± standard error. Values with the same letter are not significantly different ($P<0.05$)

antioxidants and, hence, for all subsequent experiments explants were excised in liquid BM.

Tissue polarity, explant size and auxin determine shoot regeneration and embryogenesis in TS culture

Results of preliminary experiments where tissue polarity (explant orientation in culture) was not considered showed that supplementing BM with KIN, BA or NAA alone is insufficient to induce shoot production in sugarcane leaf tissues. However, sporadic shoot formation was observed when BM was enriched with an auxin and a cytokinin, at least at 4 μM level (data not shown). Nearly half of the explants cultured on BM enriched with 4 μM BA and 10 μM NAA produced shoots within 8 weeks of culture (Fig. 1). However, the proportion of explants producing shoots was significantly lower, particularly those producing >20 shoots per explant, when BA was replaced with kinetin. Increasing the ratio of auxin to cytokinin (NAA to BA or KIN) from 2.5 to 10 had no significant effect in enhancing shoot production. Indeed, increasing the level of NAA inhibited shoot elongation growth and caused excessive root production in all the tested media irrespective of the type of cytokinin employed. Thus, BM supplemented with 4 μM BA and 10 μM NAA (SRM) was used for further experiments. Notably, shoots were produced directly on the cut surface of the explant without callus production in all growth regulator-supplemented media tested (Fig. 6a,b).

The potential of leaf tissue to form shoot buds was greatly influenced by both the tissue polarity and the size of the explant (Fig. 2). When TS explants from 1–2 mm long leaf rolls were cultured with their proximal end in direct contact with the medium, only about 8% of them produced shoots. Most of them produced only a few shoots, generally less than 5 per explant, and usually they were formed from the edge of the cut surface of the distal end. In contrast, almost 80% of TS explants from 1–2 mm long leaf rolls cultured with their distal end in direct contact with the medium regenerated shoots within 8 weeks (Fig. 2). In these explants shoot production was almost always restricted to the proximal end of the explant and shoot primordial growth was visible within 2 weeks of culture. Nearly half of the organogenic explants produced more than 20 shoots within 8 weeks in culture.

Increasing the explant size resulted in a marked reduction in shoot formation in leaf tissue (Fig. 2). In cultures where 5–6 mm long leaf roll segments were used, only 33% of those grown with their distal end in contact with the medium produced shoots (Fig. 2). Again, in these cultures, the proportion of explants producing more than 20 shoots was also reduced to about one-third of the total organogenic explants as opposed to nearly 50% obtained with TS explants.

Increasing the concentration of BA and NAA beyond 4 and 10 μM, respectively, did not significantly change the proportion of explants producing shoots as 80–90% of the cultured explants produced shoots regardless of the increased concentration of growth regulators in the medium (Fig. 3). Higher amounts of both BA and NAA caused considerable growth and morphological changes in the regenerating tissues. In particular, BA at 8 μM resulted in shorter shoots and also inhibited root development, which was more pronounced in media with lower levels (10 and 20 μM) of NAA (data not shown). Higher concentrations of NAA also had a pronounced inhibitory effect on shoot elongation, but root growth was promoted with increased auxin concentration (data not shown).

Of the three auxins tested, NAA was the most effective in inducing shoot regeneration in TS explants (Fig. 4). Replacing BA with zeatin did not produce shoots in any of the concentrations tested. The presence of TDZ in the medium,

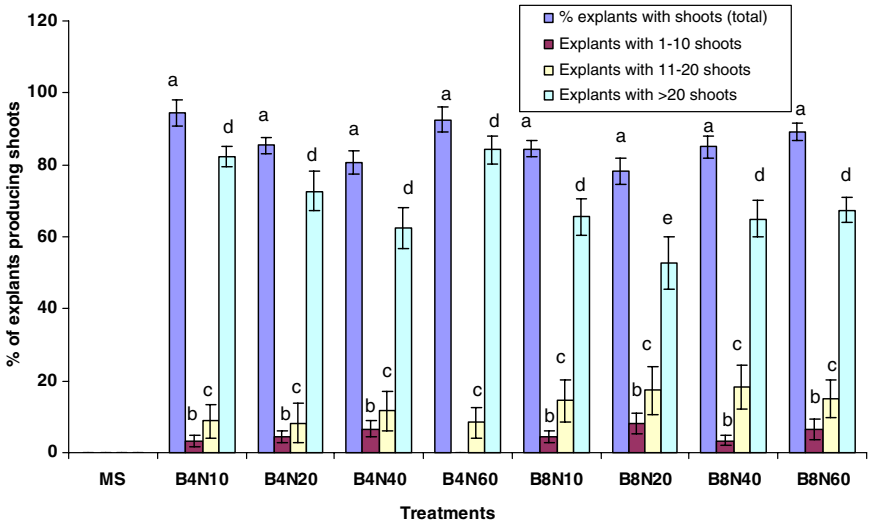

Fig. 3 Shoot production in thin leaf explants (1–2 mm) of sugarcane cultivar Q165^A after 8 weeks of culture on MS medium supplemented with 4–8 μM 6-benzyladenine (B) and 10–60 μM α-naphthaleneacetic acid (N). Explants were oriented with the distal end in contact with the medium. Data shown are the mean of 12 replicates ± standard error. In each category, values with the same letter are not significantly different ($P<0.05$)

with or without BA, caused extensive callus formation after 4–6 weeks of culture.

Membrane raft culture

The hypothesis that the use of a membrane raft culture (MRC) supported by liquid medium would reduce tissue browning and promotes shoot regeneration proved to be incorrect. Indeed, the effect of phenolic exudates was greater in static MRC and it dramatically reduced shoot production (Fig. 5). The results indicate that MRC is not suitable for shoot induction, but it may be useful for improving shoot growth in explants where shoot production has already started.

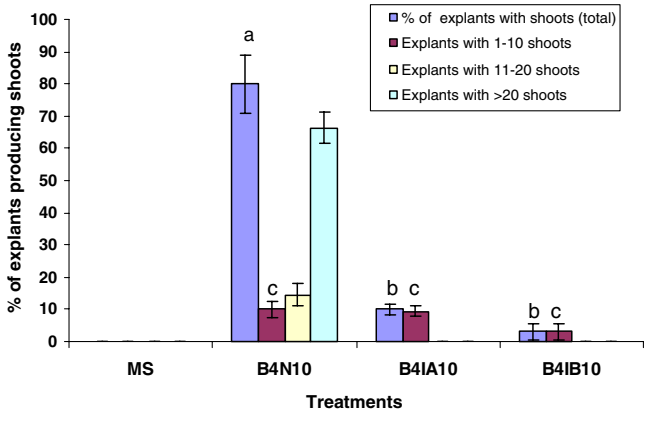

Fig. 4 Relative efficiency of various auxins on shoot production in thin leaf explants (1–2 mm) of sugarcane cultivar Q165^A after 8 weeks of culture on MS medium containing 4 μM 6-benzyladenine (B) and either 10 μM α-naphthaleneacetic acid (N), indoleacetic acid (IA) or indolebutyric acid (IB). Explants were oriented with the distal end in contact with the medium. Data shown are the mean of 12 replicates ± standard error. In each category. values with the same letter are not significantly different ($P<0.05$)

Shoot regeneration potential of TS explant is dependent on its position (developmental stage) in the leaf roll and can be manipulated by auxin

The organogenic potential of TS explants originating from the leaf roll (devided into three groups: basal [proximal], middle and apical [distal)] relative to the basal end of the leaf roll which is closer to the shoot apical meristem) was determined by both their position in the leaf roll as well as the composition of the medium in which they were grown (Table 1). Explants from basal (proximal) and middle segments were significantly more prolific than those from the apical (distal) segment (Table 1). The proportion of proximal and middle segment explants producing shoots was not significantly different in all the tested media except

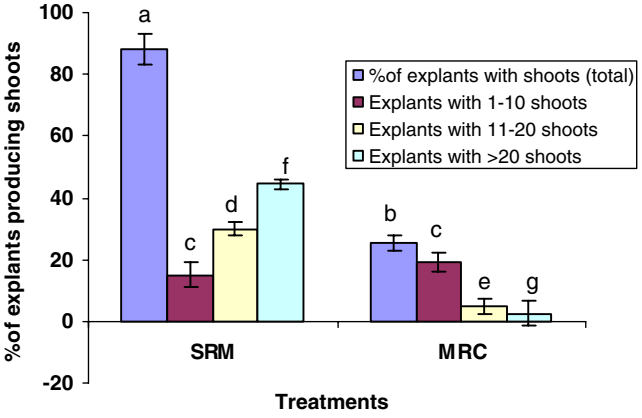

Fig. 5 Comparison of shoot production on semi-solid agar (medium, SRM) and membrane raft culture (MRC; medium, liquid SRM). Data presented are mean of 15–20 replicates ± standard error. In each category, values with the same letter are not significantly different ($P<0.05$)

Table 1 Spatial distribution of shoot regeneration in thin leaf explants (1–2 mm) of sugarcane cultivar Q165^A after 8 weeks of culture on MS medium containing 6-benzyladenine (BA) and α-naphthaleneacetic acid (NAA)

Medium		Leaf explant[a]	Percentage of explants producing shoots	% of explants producing 1–10 (+), 11–20 (++) and >20 (+++) shoots		
BA (μM)	NAA (μM)			+	++	+++
4	5	BS	54.0 ± 6.5b	17.0 ± 3.7a	23.0 ± 4.4a	14.0 ± 4.0b
4	5	MS	69.0 ± 6.9a	18.0 ± 4.1a	24.0 ± 5.6a	28.0 ± 6.9b
4	5	AS	18.1 ± 5.9c	13.6 ± 4.2a	4.5 ± 4.0b	0.0b
4	10	BS	75.4 ± 6.8a	5.4 ± 2.6a	29.0 ± 3.1a	40.9 ± 8.6a
4	10	MS	89.0 ± 5.4a	10.0 ± 4.2a	22.0 ± 2.9a	57.0 ± 9.3a
4	10	AS	45.3 ± 8.1d	15.3 ± 5.6a	16.9 ± 3.5a	13.0 ± 3.4b
4	20	BS	79.0 ± 6.4a	6.0 ± 2.6a	17.0 ± 3.0a	57.0 ± 8.4a
4	20	MS	75.0 ± 5.5a	5.8 ± 3.0a	16.6 ± 2.6a	52.5 ± 5.9a
4	20	AS	41.6 ± 6.0bd	8.3 ± 2.2a	17.5 ± 3.3a	15.8 ± 3.6b
4	40	BS	76.3 ± 5.6a	10.9 ± 3.6a	24.5 ± 4.5a	42.7 ± 6.5a
4	40	MS	82.2 ± 5.7a	6.6 ± 3.7a	24.4 ± 3.3a	51.1 ± 6.5a
4	40	AS	52.5 ± 7.3b	7.5 ± 2.9a	17.5 ± 2.2a	27.5 ± 5.5b

Explants were oriented with the distal end in contact with the medium. Data shown are the mean of 12 replicates ± standard error. In each column, values followed by the same letter are not significantly different ($P<0.05$)

[a]BS, Basal (proximal) segment; MS, Middle segment; AS, Apical (distal) segment

for proximal segments grown on medium with the lowest concentration of NAA (5 μM). In contrast, increasing the NAA level from 5 to 40 μM increased the proportion of apical segment explants producing shoots from 18 to 53% within 8 weeks of culture. Similarly, a remarkable increase in the proportion of explants with more than 20 shoots was observed in basal and middle segments when the NAA concentration was increased from 5 to 10 μM, but no further improvement was evident at higher auxin levels (Table 1).

Genotype plays a significant role in controlling shoot regeneration but this influence can be minimised with auxin (CPA)

To assess the efficiency of the regeneration procedure developed with Q165^A, 12 commercial cultivars with different genetic backgrounds were grown on SRM. Five cultivars regenerated plants on SRM, but the frequency of shoot production was highly variable among genotypes (Table 2)

Preliminary experiments with Q117 showed that replacing BA with CPA (5–20 μM) induces somatic embryogenesis (SE) and the regeneration potential (SE) of explants was restricted to the proximal cut surface (Fig. 6c; data not presented). CPA and NAA at 10 μM concentration was sufficient to produce somatic embryos in 70–80% of the TS explants cultured and, hence, this medium was used as embryogenic medium (EM) for further experimentation with different genotypes. With EM the effect of genotype on regeneration was minimized substantially (Table 2). Only 5 of 12 different genotypes cultured on SRM produced shoots, and, more significantly, three of these regenerating varieties (Q156, Q197^A and Q200^A) responded extremely poorly with just 1–3% of explants being organogenic. However, all 12 genotypes cultured on EM produced somatic embryo-derived shoots (most of the embryos did not develop roots) and some plantlets within 8 weeks of culture. Furthermore, under the same culture condition, at least 50% of the explants from seven genotypes regenerated shoots (Table 2). The presence of CPA in the medium caused microcallus formation on the cut surface of the explants, which proved highly embryogenic in many of the genotypes tested. The original material used in these experiments were obtained from plants grown under extremely dry field conditions, and this presumably led to large variations in organogenic response among replicates was observed in all cultivars tested.

Growth regulator-free medium favours shoot growth and root development but rooting and seedling establishment can be readily achieved ex vitro

Explants with clusters of shoots left on SRM or EM for more than 6–8 weeks produced roots, but shoot growth was not uniform due to the apical dominance effect exerted by a few shoots in each cluster. For optimal growth and development, shoots were separated and cultured on BM for at least 4 weeks. Separated shootlets (20–30 mm long) cultured on BM grew well (Fig. 6d), and almost 95% of shootlets produced at least 3 roots (50–60 mm long) within 6 weeks of incubation (Fig. 6e).

Nearly 90% of the shootlets (30–40 mm long) transferred to seedling trays, and kept under high humidity and shaded conditions for the first 5–7 days, rooted and developed into well established plantlets ready for field planting within 6–8 weeks. Almost all the transplanted seedlings in the field established and grew well (Fig. 6f), but overhead irrigation was needed during the initial stage (first 2–3 weeks) of establishment.

Table 2 A comparative analysis of shoot production in thin leaf explants (1–2 mm) of sugarcane cultivars cultured on direct shoot regeneration medium SRM (4 μM BA and 10 μM NAA) or embryogenic medium EM (10 μM NAA and 10 μM CPA) for 8 weeks

Explants were oriented with the distal end in contact with the medium. Data presented are mean of 10–12 replicates ± standard error

Genotype	Percentage of explants producing shoots		Percentage of explants producing 1–10 (+), 11–20 (++) and >20 (+++) shoots in EM		
	SRM	EM	+	++	+++
Q156	1.0 ± 2.8	51.7 ± 9.9	46.7 ± 9.2	5.0 ± 2.3	0 ± 0
Q167[A]	0.0 ± 0.0	50.0 ± 5.9	26.3 ± 5.6	15.0 ± 3.2	8.7 ± 2.9
Q172[A]	0.0 ± 0.0	49.1 ± 6.9	30.0 ± 6.0	16.4 ± 5.9	2.7 ± 1.4
Q185[A]	0.0 ± 0.0	23.1 ± 7.1	10.7 ± 4.7	4.6 ± 1.8	7.7 ± 2.6
Q188[A]	37.5 ± 28.9	64.0 ± 10.2	46.2 ± 5.5	11.0 ± 3.5	9.8 ± 4.0
Q190[A]	0.0 ± 0.0	25.8 ± 7.6	25.8 ± 7.6	0 ± 0	0 ± 0
Q196[A]	0.0 ± 0.0	88.8 ± 3.9	46.3 ± 8.4	38.8 ± 10.9	3.7 ± 1.6
Q197[A]	3.3 ± 11.5	49.1 ± 11.4	41.7 ± 9.6	5.8 ± 1.9	1.7 ± 0.8
Q200[A]	3.6 ± 8.0	36.0 ± 2.4	36.0 ± 2.4	0 ± 0	0 ± 0
Q205[A]	28.1 ± 23.1	60.0 ± 9.3	34 ± 7.1	10.0 ± 4.7	16.0 ± 10.1
Q216[A]	0.0 ± 0.0	22.3 ± 6.2	21.5 ± 5.8	0.8 ± 7.6	0 ± 0
Q222[A]	0.0 ± 0.0	14.2 ± 3.1	10.0 ± 2.4	3.3 ± 1.8	0.8 ± 0.8

Discussion

Sugarcane was one of the first plant species to be successfully cultured and regenerated in vitro (Barba and Nickell 1969; Heinz and Mee 1969). Since then, considerable effort has been expended to develop efficient procedures for sugarcane micropropagation (Ho and Vasil 1983; Lee 1987; Burner and Grisham 1995; Chengalrayan and Gallo-Meagher 2001). Unfortunately, micropropagation of sugarcane, even with meristem culture, results in somaclonal variation, causing marked morphological anomalies and yield penalties (Lourens and Martin 1987; Burner and Grisham 1995). In this investigation, we have identified some of the developmental and physiological factors that limit in vitro morphogenesis and exploited this new knowledge to develop a rapid and efficient sugarcane regeneration system that potentially minimises the incidence of somaclonal variation.

The application of the thin cell layer culture concept was critical in achieving this success. The original thin cell layer culture system or thin section culture system was developed primarily to understand the developmental, cellular and molecular regulation of in vitro morphogenesis (Tran Thanh Van 1973) and here we have successfully used it to elucidate the factors that regulate shoot organogenesis and embryogenesis in sugarcane. The TS culture system capitalises on certain morphogenic and developmental characteristics of cells in the explant. Previous studies with orchids (Lakshmanan et al. 1995), *Lilium longiflorum* (Nhut et al. 2001b) and *Brassica* (Cheng et al. 2001) showed

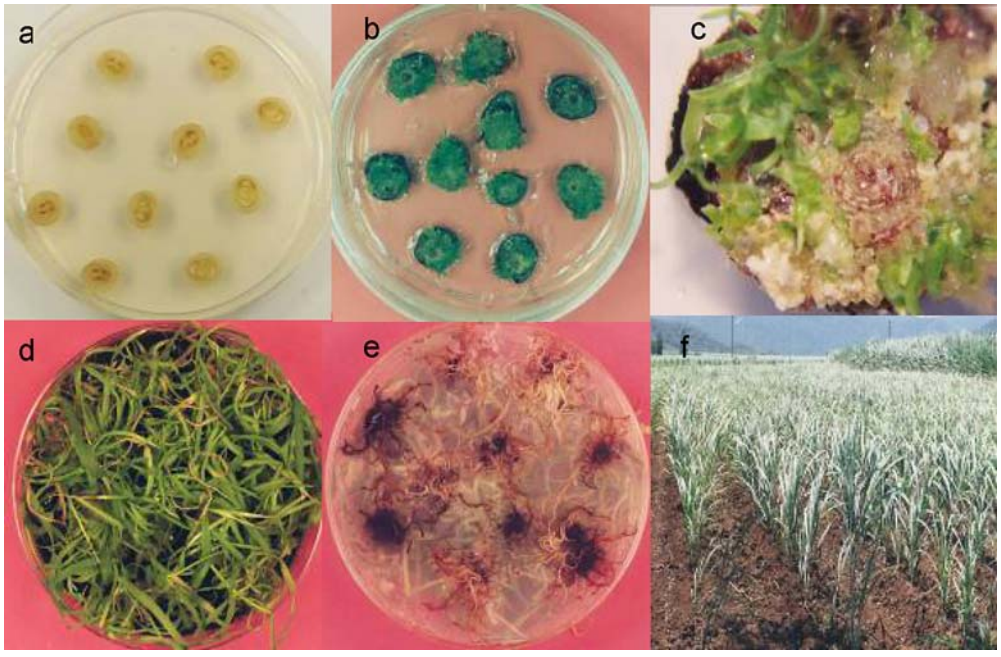

Fig. 6 Different stages of SmartSett® plant regeneration in sugarcane: **a** thin section (TS) explants from young leaf rolls; **b** shoot regeneration in ts explants; **c** somatic embroys in different stages of development including differentiation into shoots and plantlets; **d** plantets ready for transplantation; **e** root development; **f** field evaluation of SmartSett® seedlings

that TS explants are far superior to larger explants in regenerating plants and increasing productivity. In our study also, TS explants produced significantly more plants than the larger (5–6 mm long) leaf roll segments (Table 2). The exact reason for this difference in regenerative response is not known, but clearly the results indicate a major role for some explant-related factors to account for this outcome. It was also evident that organogenically competent cells are not limited in explants, as profuse regeneration occurred in TS explants under optimal conditions, suggesting some physical and/or physiological barriers limiting the morphogenic progression of cells in larger explants. It is well established that the morphogenic fate of a cell is determined to a great extent by its neighbouring cells and tissues—a phenomenon termed correlative inhibition (Lyndon and Francis 1992). Upon excision, cells are released from correlative controls and under appropriate culture conditions they express their morphogenic potential as observed here in sugarcane TS explants and in other species reported earlier (Lakshmanan et al. 1995; Gendy et al. 1996; Nhut et al. 2001a).

The most important observation in our study is the regulatory effect of developmental polarity on organogenesis. Both shoot regeneration and SE were dramatically lower when TS explants were cultured with their proximal end in direct contact with the medium (proximal end facing the medium). Regeneration rarely occurred only on the distal end of the explant, irrespective of the orientation or size of the explant. Auxin plays a major role in establishing developmental polarity in plants (Leyser and Day 2003), and previous studies indicate that polar transport of auxin establishes polar cell proliferation and organogenesis (Paterson 1983; Sachs 1991; Liu et al. 1993). Hence, it is likely that the endogenous auxin in the explants was probably transported polarly within the cut segments so that the critical auxin concentration necessary to induce organogenesis is found only at the proximal end of the explant. Clearly, results from some of our experiments (Tables 1 and 2) suggest that auxin is a significant determinant of in vitro organogenesis in sugarcane.

While the polar regeneration is possibly due to polar auxin transport, it does not fully explain the poor regeneration observed in explants with the proximal cut surface in direct contact with the medium (proximal end facing down). One logical explanation would be a possible oxygen tension developing in the meristematic cells at the explant-medium interface. Oxygen is needed for cell division and meristem differentiation and the poor regeneration observed could, at least in part, be due to a potential oxygen deficiency at the proximal cut surface of the explants embedded in semi-solid medium. This is further corroborated by the lack of regeneration observed in those explants that were completely submerged in the agar medium.

Regeneration of shoots, as our results suggest, was largely dependant on the availability of an auxin in the medium, although an auxin and cytokinin combination also induced shoot production in sugarcane leaf culture. Where a cytokinin was included in the medium, auxin concentration should be significantly higher than that of cytokinin to achieve high-frequency shoot production. This high auxin requirement is an interesting observation as often a cytokinin alone or both an auxin and a cytokinin are needed to effect shoot induction in culture. Monocotyledonous plants, especially the members of Poaceae, are recalcitrant, and young meristematic tissues such as immature leaf, developing inflorescence and the basal plate of the leaf are the only organogenically responsive explants (Liu 1993; Nhut et al. 2001a). Due to the meristematic nature of these tissues, they often contain relatively high levels of cytokinins to support cell proliferation. As shoot meristem formation rests on the balance of cytokinin to auxin, it is conceivable that these tissues may require an external supply of auxin to induce competent cells to develop into shoot meristem.

Another important finding of this study was the spatial distribution of regenerability in the leaf roll. The basal (proximal) and middle segments (up to 20) are highly regenerative, and the regeneration potential decreased in the successive distal segments (Table 1). A similar observation has been made in other plant species (Lakshmanan et al. 1997), yet we do not know how a cell acquires organogenic competence when it is young and why this potential disappears during cell maturation. Previous studies with tobacco (McDaniel et al. 1992) and mangosteen (Lakshmanan et al. 1997) revealed that cells are morphogenically primed by the action of a cytokinin, while in another species, alfalfa, petiole cells acquired competence in the presence of an auxin (Finstad et al. 1993). These studies also revealed that only specific target cells primed to respond to morphogenic stimuli, such as plant hormones, could enter into an organogenic process. In maturing tissues such as the distal segment TS explants (e.g. those from apical segments (Table 1), these target cells could exist with different levels of organogenic competence and require either different duration or different levels of exposure to morphogenic stimuli for meristem development. It is, therefore, possible that the morphogenically competent cells in the poorly regenerating distal segment TS explants may be fewer or at a reduced level of competence and consequently a higher level of inductive signal (auxin treatment) is required to make them organogenic. This view is reinforced with the finding that auxin significantly reduces the genotype effect on organogenesis (Table 2).

In summary, our study demonstrated that tissue polarity and consequently the orientation of the explant in culture, size and developmental stage of explant, genotype and auxin concentration determine the organogenic potential of sugarcane leaf explants in culture. More importantly, we have demonstrated that auxin could reduce the developmental and genetic constraints on organogenesis. This has considerable practical significance and has been successfully used to develop a rapid plant production system for sugarcane. Using this plant production system as many as 2000 plantlets could be generated from a single mother plant in 4 months as opposed to 10–15 plantlets by conventional multiplication through setts (stem cuttings) over a year. This new in vitro propagation method, SmartSett®, requires only leaf material for plant propagation, leaving the entire harvestable cane

stalks for sugar production. SmartSett® has already been commercially used for mass production and rapid adoption of new cultivars (Geijskes et al. 2003; Fig. 1f).

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