BSES Limited

FINAL REPORT SRDC PROJECT BSS242

A SUGARCANE TISSUE CULTURE SYSTEM

FOR MASS PROPAGATION AND TRANSFORMATION

by

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SUMMARY

Tissue culture and associated sugarcane transformation requires the use of the synthetic plant growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D) to induce and maintain the cells in an undifferentiated state (callus). Furthermore, during transformation, callus remains in culture for a substantial amount of time (6-7 months) before transgenic plantlets can be selected and regenerated. Both 2,4-D and the duration of culture are considered to contribute to somaclonal variation, a collection of heritable genetic changes generated by mutagenic factors in the tissue culture used prior to and during the transformation process. Our aim was to develop a new direct plant regeneration system for both mass propagation applications and for sugarcane genetic engineering that minimizes somoclonal variation and that is more rapid and cost effective.

An efficient direct plant regeneration system for sugarcane (named SmartSett®) was developed and optimized; plantlets can be regenerated from sugarcane tissues via a novel combination of plant growth regulators in 6-8 weeks. The system significantly minimizes somoclonal variation due to two factors: the time frame through tissue culture is short (32 weeks for the current callus system down to 6-8 weeks), and no callus phase is involved in this new system.

A strong IP position with regard to both the tissue culture and transformation systems has been established. Patent applications covering both of these techniques have been granted in Australia and submitted in Europe, USA and Canada. The first patent essentially covers the SmartSett® plant production and propagation technology for sugarcane. The second patent protects the new SmartSett® system-based sugarcane transformation method. In addition, the name SmartSett® has been registered as a Trade Mark for 10 years.

A series of replicated trials comparing SmartSett® plants to parental clones for performance and morphological traits was performed with selected genotypes proved the utility of this tissue culture system for mass propagation, and, by inference, genetic engineering. The system was used to rapidly deliver a new cultivar to the Mackay region to replace cultivars susceptible to orange rust.

A system to transform, regenerate and select transgenic plants from explant material (both leaf roll and inflorescence) using SmartSett® and microprojectile transformation has also been developed. At this stage, the system is not as efficient as the callus transformation system, but it is sufficient to generate a reasonable number of plants for molecular evaluation. Over 50 non-chimeric, transgenic plants expressing GFP are currently growing in the field at BSES Meringa. These plants were regenerated from explant material co-transformed with the NPTII gene construct and were selected on geneticin-supplemented media.

Agrobacterium-mediated transformation of sugarcane was tested, but with little success. This strategy is now being explored as an activity of the CRC for Sugar Industry Innovation through Biotechnology (CRC-SIIB).

The project also successfully demonstrated that sorghum and wheat plantlets could be regenerated directly from the leaf tissues without going through callus phase. This

implies that the SmartSett® system is applicable to other monocot species and provides a base plant production method that is rapid and minimizes the incidence of somaclonal variation. Using this method transgenic sorghum and wheat plants may be produced by both biolistic and *Agrobacterium*-mediated methods.

The development and potential applications of SmartSett® system has been presented to the Australian sugar industry through a wide range of forums. A paper on the technology was awarded the President's Medal at the 2003 ASSCT Conference.

The system is under use within CRC-SIIB and at the South African Sugarcane Research Institute. The Mauritius Sugar Industry Research Institute (MSIRI), Mauritius has contacted BSES to sign an agreement to transfer SmartSett® Technology for transformation and mass propagation programs.

The horticultural industry has contacted BSES to exploit SmartSett® system for ornamentals. BSES has undertaken a very limited exploratory investigation on rapid multiplication of new *Agapanthus* cultivars, but the results so far are not encouraging.

A BSES-University of Queensland (UQ) joint project is testing the utility of SmartSett® system in recovering plants infected with Fiji leaf gall virus and sugarcane mosaic virus.

1.0 BACKGROUND

The first successful demonstration of sugarcane transformation was carried out upon embryogenic callus (Bower & Birch, 1992, *Plant Journal* 2: 409-416), but this method requires the use of the synthetic plant growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D) to induce and maintain the cells in an undifferentiated state (callus). Furthermore, during transformation, callus remains in culture for a substantial amount of time (6-7 months) before transgenic plantlets can be selected and regenerated. Both 2,4-D and the duration of culture are considered to contribute to somaclonal variation, a collection of heritable genetic changes generated by mutagenic factors in the tissue culture used prior to and during the transformation process (Larkin & Snowcroft 1981, *Theor. Appl. Genetics* 60: 197-214).

Although transgenes could be successfully introduced and expressed in transgenic sugarcane, the impact of this genetic improvement was largely negated by the effects of somaclonal variation. Somaclonal variation is believed to be responsible for the undesirable traits observed in extensive field trials of untransformed sugarcane (Lourens & Martin 1987, *Crop Sci.* 27: 793-796; Burner & Grisham 1995, *Crop Sci.* 35: 875-880) or those transformed with commercially useful genes using the callus-based method (Vickers *et al.* 2004a,b, *Crop Sci.* in press). The vegetative propagation of sugarcane prevents the application of a backcrossing strategy commonly used in other crops to minimise the impact of deleterious traits caused by transformation.

Previous research at BSES demonstrated that sugarcane meristems are suitable targets for transformation, but the system needed further research to develop it into a practically useful system (Gambley *et al*. 1993, *Plant Cell Reps* 12: 343-346). Meristematic regions were originally chosen for transformation because of reports of low incidence of somaclonal variation in plants derived from meristems as compared to those generated from callus (Irvine *et al.* 1991, *Plant Cell Tissue Organ Cult.* 26: 15-25). However, a major limitation of this method was the difficulty in obtaining large numbers of shoot apical meristems required for transformation. An earlier finding in a BSES-CSIRO joint research project (BSS209) on sugarcane meristem transformation that shoot meristems can be produced in high frequency directly from young sugarcane leaves prompted us to explore its potential to develop a new sugarcane transformation technology. A significant technical hurdle, which could not be overcome at the time, was the means to select transformed cells from non-transformed ones. With the application of the green fluorescent protein (GFP) for *in vivo* visual selection of transgenic sugarcane (Elliott *et al.* 1998, *Aust J. Plant Physiol*. 25:739-743), excision and recovery of transformed sectors of meristems and axillary buds became a practical reality.

Meristem-based plant production and transformation methods have several advantages. It requires only short culture period to produce plants, and, often, strong growth regulators such as 2,4-D are not required. It thus has significant potential to both minimise somaclonal variation and, in conjunction with GFP, allow visual selection of transformed sectors relatively easily. This system may also possess a high *in vitro* multiplication rate and thus could be exploited for rapid mass propagation of new cultivars and transgenic plants. Thus, the primary aims of the project were to develop a new direct plant regeneration system for both mass propagation applications and for sugarcane genetic engineering via microprojectile bombardment and/ or by an *Agrobacterium*-based system.

2.0 OBJECTIVES

The core research strategy we conceived was to develop the initial observation of direct shoot regeneration in sugarcane leaf tissues to a generic and efficient plant production technology for sugarcane and probably for other major monocot crops, and then use that system to develop a novel method for sugarcane transformation.

The main objectives of this project were:
1. Protect identified IP by patent;
2. Develop an efficient direct plant regeneration system for sugarcane and determine clonal stability of plants produced by this method (now referred to as the SmartSett® system);
3. Develop microprojectile and/or *Agrobacterium*-mediated transformation systems based on SmartSett®;
4. Establish the utility of SmartSett® to other monocot crops;
5. Technology transfer to other groups.

These objectives were met.

Objective 1 - Protect identified IP by patent.

We developed novel systems for mass propagation and rapid transformation of sugarcane and a strong IP position with regard to both the tissue culture and transformation systems has been established. Patents covering both of these techniques have been granted in Australia and applications are currently under review in Europe, USA and Canada.

The first patent essentially covers the SmartSett® plant production and propagation technology for sugarcane, and other monocots, and has been awarded.

The second patent protects the new SmartSett® system-based sugarcane transformation method.

In addition, the name SmartSett® has been registered as a Trade Mark for 10 years.

Objective 2- Develop an efficient direct plant regeneration system for sugarcane and determine clonal stability of plants produced by this method (now referred to as the SmartSett® system).

An efficient direct plant regeneration system for sugarcane was developed and optimized; plantlets can be regenerated from sugarcane tissues via a novel combination of plant growth regulators in 6-8 weeks. The system significantly minimizes somoclonal variation due to two factors: the time frame through tissue culture is short (32 weeks for the current callus system down to 6-8 weeks), and no callus phase is involved in this new system.

A series of replicated trials comparing SmartSett® plants to parental clones for performance and morphological traits was performed with selected genotypes proved the utility of this tissue culture system for mass propagation, and, by inference, genetic

engineering. The system was used to rapidly deliver a new cultivar to the Mackay region to replace cultivars susceptible to orange rust.

Objective 3- Develop microprojectile and/or* Agrobacterium*-mediated transformation systems based on SmartSett®.

A system to transform, regenerate and select transgenic plants from explant material (both leaf roll and inflorescence) using SmartSett® and microprojectile transformation has been developed. At this stage, the system is not as efficient as the callus transformation system, but it is sufficient to generate a reasonable number of plants for molecular evaluation. Over 50 non-chimeric, transgenic plants expressing GFP are currently growing in the field at BSES Meringa. These plants were regenerated from explant material co-transformed with the NPTII gene construct and were selected on geneticin-supplemented media.

Agrobacterium-mediated transformation of sugarcane was tested, but with little success. This strategy is now being explored as an activity of the CRC for Sugar Industry Innovation through Biotechnology (CRC-SIIB).

Objective 4 - Establish the utility of SmartSett® to other monocot crops.

We successfully demonstrated that sorghum and wheat plantlets could be regenerated directly from the leaf tissues without going through callus phase. This implies that the SmartSett® system is applicable to other monocot species and provides a base plant production method that is rapid and minimizes the incidence of somaclonal variation. Using this method transgenic sorghum and wheat plants may be produced by both biolistic and *Agrobacterium*-mediated methods.

Objective 5 - Technology transfer to other groups.

The development and potential applications of SmartSett® system has been presented to the Australian sugar industry through a wide range of forums. A paper of the technology was awarded the President's Medal at the 2003 ASSCT Conference.

The system is under use within CRC-SIIB and at the South African Sugarcane Research Institute. The Mauritius Sugar Industry Research Institute (MSIRI), Mauritius has contacted BSES to sign an agreement to transfer SmartSett® Technology for transformation and mass propagation programs.

The horticultural industry has contacted BSES to exploit SmartSett® system for ornamentals. BSES has undertaken a very limited exploratory investigation on rapid multiplication of new *Agapanthus* cultivars, but the results so far are not encouraging.

A BSES-University of Queensland (UQ) joint project is testing the utility of SmartSett® system in recovering plants infected with Fiji leaf gall virus and sugarcane mosaic virus.

3.0 PROTECTION OF IDENTIFIED IP BY PATENT

We developed novel systems for mass propagation and rapid transformation of sugarcane within the project. A strong IP position with regard to both the tissue culture and transformation systems has been established. Patent applications covering both of these techniques have been submitted in Europe, USA and Canada.

The first patent, "PLANT REGENERATION" (WO01/82684; Applicants: Sugar Research and Development Corporation, BSES Limited and Commonwealth Scientific and Industrial Research Organisation) http://www.wipo.int/cgi-pct/guest/ifetch5?ENG+PCT-ALL.vdb+14+1094674-REVERSE+0+0+383603+BASICHTML-ENG+1+1+1+25+SEP-0/ and the second patent "MONOCOTYLENOUS PLANT TRANSFORMATION" (WO02/37951; Applicants: Sugar Research and Development Corporation, BSES Limited and Commonwealth Scientific and Industrial Research Organisation) http://www.wipo.int/cgi-pct/guest/ifetch5?ENG+PCT-ALL.vdb+14+1095133-REVERSE+0+1+52682+BASICHTML-ENG+1+1+1+25+SEP-0/HITNUM,B,,,,,,,+WO02%2f37951 have been awarded in Australia and are now being examined in the USA. These patents protect the new SmartSett® system-based sugarcane transformation method.

SmartSett® has been registered as Trade Mark, No 931708 in the Register of Trade Marks for a period of 10 years starting from 23 October 2002 for the goods and services coming from this technology/patents.

4.0 DEVELOPMENT OF AN EFFICIENT DIRECT PLANT REGENERATION SYSTEM FOR SUGARCANE AND DETERMINATION OF THE CLONAL STABILITY OF PLANTS PRODUCED BY THE SMARTSETT® SYSTEM

A detailed description of relevant experimental methods and results related to the development of the sugarcane SmartSett® system are given below. Similar strategies were also employed to test the SmartSett® system in other monocot species, such as sorghum, wheat and banana (Section 6). Experimental results obtained with these species are also presented in this report, but further details can be found in Plant Regeneration patent.

4.1 General development

4.1.1 Methodology

Sugarcane experimental plant material

The sugarcane cultivar Q165$^{\phi}$, which was readily available throughout the year, was used for most of the experiments reported here. Sugarcane clones Q117, Q152, Q156, Q157, Q167$^{\phi}$, Q172$^{\phi}$, Q185$^{\phi}$, Q188$^{\phi}$, Q190$^{\phi}$, Q196$^{\phi}$, Q197$^{\phi}$, Q200$^{\phi}$, Q205$^{\phi}$, 84N538 and 92S280 were also used for some experiments in this investigation.

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 (tissue used for culturing). After appropriate sterilisation of shoot tops, a 10-15 cm long basal portion of leaf rolls, starting from the leaf base just above the apical meristem, was excised and used as the source of tissue for culture initiation. The sterile leaf rolls were then cut sequentially into 1.5-2 mm thick transverse sections (TS explants), beginning from the basal end of the roll. We prepared 10-30 TS from each leaf roll depending on the objective of the experiment.

For all experiments Murashige and Skoog 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 (12x2 cm) with their distal ends in contact with the medium. All plant growth regulators and antioxidants (ascorbic acid, 150 mg/L and citric acid, 100mg/L) used were filter-sterilised and added to the autoclaved media. The pH of all media was adjusted to 5.7 ± 0.1 before autoclaving at 110 kPa for 20 min at 120°C. Except where liquid cultures were tested, all shoot regeneration experiments were performed with semisolid media gelled with Difco agar (7.5 g/L). All cultures were incubated at 25 ± 1°C under 16:8 h photoperiod provided by cool white fluorescent tubes with a photon flux density of 30 μmol m^{-2} s^{-1} at the culture level.

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

Role of tissue polarity, plant growth regulators, explant size and genotype on shoot regeneration

The role 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, the optimised shoot regeneration medium (SRM) for the cultivar Q165$^{\phi}$, with its proximal or distal surface in direct contact with the medium. In addition, 10 mm long leaf roll segments were placed horizontally on SRM, allowing regeneration to occur on both ends.

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

To evaluate the influence 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.

In another experiment, TS explants of 12 sugarcane clones (listed in section 4.1.1) with very diverse genetic backgrounds were cultured on SRM for 4 weeks and subsequently on BM for another 4-6 weeks to assess the effect of genotype on regeneration efficiency of the procedure developed for Q165$^{\Phi}$ in this study.

In a further experiment, all 12 cultivars tested with SRM were cultured for 4 weeks on BM supplemented with 10 μM NAA and 10 μM 2-chlorophenoxyacetic acid (CPA) followed by a period of 6-8 weeks growth on BM.

Spatial distribution of shoot regeneration potential in leaf rolls and its manipulation by auxins

In this experiment, 30 TS explants were harvested from each immature leaf roll by sequential transverse-sectioning beginning from the basal end of the leaf roll. They were divided into three distinct groups: the first 10 TS from the basal end of the leaf roll form the 'proximal segments', the next 10 TS constitute the 'middle segments' and the following 10 TS represent the 'distal segments'. These three groups of explants were cultured on SRM or BM enriched with 4 μM BA and various concentrations of IAA or NAA for 8 weeks and their shoot regeneration response was compared.

Membrane raft culture and shoot regeneration

The effect of liquid medium on shoot regeneration was studied by culturing TS explants in baby food jars or in Erlenmeyer flasks (100 mL) containing 30 mL liquid regeneration medium (SRM without agar), which was agitated continuously on a gyratory shaker set at 120 rpm. Cultures were also prepared by placing explants on membrane rafts (Sigma, USA) floated on liquid SRM in GA7 vessels (Magenta Corp., Chicago, USA). Explants were placed with its distal end in contact with the membrane and were transferred to fresh medium every week for the first 4 weeks of culture and 2-weekly 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 (12x2 cm) for 2-3 weeks for further growth and development. For root development, 20-30 mm tall shoots were cultured in baby food jar containing BM for 2-3 weeks. Plantlets with at least 2-3 cm long roots were transplanted to seedling trays filled with perlite:peat moss:sand (1:1:1 v/v) mixture and acclimatised for 7-10 days under high humidity before they were transferred to a nursery.

To assess the potential of ex vitro rooting to reduce the duration of plants in culture, 110 Q165$^{\Phi}$ shootlets (30-40 mm tall) were transplanted to seedling trays containing perlite:peat moss:sand (1:1:1 v/v) mixture and maintained in a glasshouse for 6 weeks. These transplants were irrigated at least twice weekly and fertilised with Thrive® (an all-purpose water-soluble plant food, at 10-day intervals throughout the experiment.

General

All *in vitro* experiments were repeated at least once. Each treatment had 8-10 replicates and 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 presented are means ± standard deviations.

4.1.2 Results

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

Leaf explants cultured in all media 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 due to the production of phenolic compounds. The initial experiments showed that wound-induced phenolic exudation and tissue discolouration, which occurred soon after excision, could be greatly reduced by preparing explants in liquid BM with antioxidants ascorbic acid (150 mg/L) and citric acid (100 mg/L) (data not given). However, a similar result was also obtained with liquid BM without antioxidants and, hence, for all subsequent experiments explants were excised in liquid BM.

Table 1 **Shoot production in thin leaf explants (1-2 mm) of sugarcane cultivar Q165$^{\Phi}$ after 8 weeks of culture on MS medium supplemented with different concentrations of 6-benzyladenine (BA), kinetin (KIN) and α-naphthaleneacetic acid (NAA). Explants were oriented randomly without considering whether proximal or distal end was in contact with the medium. The data presented are the average of at least two experiments.**

Treatment			% of explants producing shoots	% of explants producing different numbers of shoots		
BA (μM)	KIN (μM)	NAA (μM)		1-10	11-20	>20
0	0	0	0	0	0	0
4	0	10	45.5	11.5	17.0	16.5
4	0	20	50.9	10.4	20.4	20.0
4	0	40	55.5	16.5	17.5	20.5
0	4	10	32.8	15.0	10.7	7.1
0	4	20	37.3	20.6	8.6	7.3
0	4	40	43.5	21.7	12.9	8.8

Tissue polarity, explant size and plant growth regulators determine shoot regeneration in TS culture

Results from preliminary experiments where tissue polarity (explant orientation) was not considered during culture showed that supplementing BM with KIN, BA or NAA alone is insufficient to induce shoot production in sugarcane leaf tissue. However, sporadic shoot formation was observed when the medium contained both an auxin and a cytokinin at least at 4 μM level (data not presented). The percentage of explants producing shoots and the rate of shoot production however increased substantially with increasing the level of auxin in the medium (Table 1). For instance, nearly half of the explants cultured on BM enriched with 4 μM BA and 10 μM NAA produced shoots, with the majority of them having more than 10 shoots within 7 weeks of culture (Table 1). However, the total number of explants producing shoots was reduced when BA was replaced with kinetin. Increasing the ratio of auxin to cytokinin (NAA to BA or kinetin) from 2.5 to 10 had no

significant effect in enhancing shoot production. Indeed, increasing the level of NAA inhibited shoot growth and caused excessive root production in all the tested media irrespective of the type of cytokinin employed. Because of this, BM supplemented with 4 μM BA and 10 μM NAA was used for further experiments. Notably, in all the regenerating cultures shoots were produced directly on the cut surface of the explant without callus production.

The ability of leaf tissue to form shoot buds was greatly influenced by the tissue polarity and to a considerable extent by the size of the explant (Table 2). When TS explants were cultured with their proximal ends in direct contact with the medium, only about 8% of the explants produced shoots. Most of these explants produced only a few shoots, less than five per explant, and usually they were formed from the edge of the cut surface of the distal end. In contrast, almost 80% of the TS explants cultured with their distal ends in direct contact with the medium regenerated shoots within 8 weeks (Table 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 each at the end of the experiment.

Increasing the explant size resulted in a marked reduction in shoot formation in leaf tissue (Table 2). In cultures where 5-6 mm long leaf roll segments were used as explants, only 33% of them produced shoots, even when they were grown with their distal end in contact with the medium (Table 2). Again, in these cultures, the number 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. Significantly, when the explant orientation was reversed (i.e. the proximal end in contact with the medium), the proportion of larger leaf roll segments (5-6 mm) producing shoots was almost three times that from TS explants.

Table 2 **Influence of explant size and orientation on shoot production in thin leaf explants (1-2 mm) of sugarcane cultivar Q165$^{\phi}$ after 8 weeks of culture on MS medium supplemented with 4 μM 6-benzyladenine (BA) and 10 μM α-naphthaleneacetic acid (NAA). Explants were oriented with their distal or proximal ends in contact with medium. Data were subjected to AOV; in each column, values followed by the same letter are not significantly different (P < 0.05).**

Explant		% of explants producing shoots	% of explants producing different numbers of shoots		
Size (mm)	Orientation (distal end)		1-10	11-20	>20
1-2	up	8.2c	8.2a	0.0a	0.0a
1-2	down	79.0b	14.7a	20.9b	43.8b
5-6	up	22.1c	10.0a	12.1c	2.0a
5-6	down	32.7c	10.0a	10.5c	12.0a

Based on the knowledge about the role of polarity on shoot regeneration, an experiment examined the effect of different concentrations and ratios of plant growth regulators on shoot regeneration. Increasing the concentration of BA and NAA did not cause any

significant change in the total number of explants producing shoots, as 80-90% of the cultured explants produced shoots regardless of the amount of growth regulators in the medium (Table 3). Nonetheless, the percentage of explants with more than 20 shoots was considerably reduced when the level of BA was increased to 8 μM. In addition, higher amounts of both BA and NAA caused considerable growth and morphological changes in the cultured tissue. In particular, BA at 8 μM produced shorter shoots and inhibited root development, which was more pronounced in media with lower levels (10 and 20 μM) of NAA. Higher concentrations of NAA also had a pronounced inhibitory effect on shoot elongation, but root growth was promoted with increased amount of auxin.

Table 3 **Shoot production in thin leaf explants (1-2 mm) of sugarcane cultivar Q165$^{\phi}$ after 8 weeks of culture on MS medium supplemented with different concentrations of 6-benzyladenine (BA) and α-naphthaleneacetic acid (NAA). Explants were oriented with their distal ends in contact with medium. The data presented are the average of at least two experiments.**

Treatment		% of explants producing shoots	% of explants producing different numbers of shoots		
BA (μM)	NAA (μM)		1-10	11-20	>20
0	0	0	0	0	0
4	10	94.4	3.3	8.8	82.2
4	20	85.4	4.5	8.1	72.7
4	40	80.8	6.6	11.6	62.5
4	60	92.5	0.0	8.3	84.1
8	10	84.4	4.4	14.4	65.5
8	20	78.1	8.1	17.2	52.7
8	40	85.0	3.3	18.1	65.0
8	60	89.1	6.6	15.0	67.5

Table 4 **Relative efficiency of various auxins on shoot production in thin leaf explants (1-2 mm) of sugarcane cultivar Q165$^{\phi}$ after 8 weeks of culture on MS medium containing 6-benzyladenine (BA). Explants were oriented with its distal end in contact with medium. Data were subjected to AOV; in each column, values followed by the same letter are not significantly different ($P < 0.05$).**

Treatment				% of explants producing shoots	% of explants producing different numbers of shoots		
BA (μM)	NAA (μM)	IAA (μM)	IBA (μM)		1-10	11-20	>20
0	0	0	0	0.0a	0.0a	0.0a	0.0a
4	10	0	0	88.1b	10.0a	14.5b	66.3b
4	0	10	0	10.0a	9.3a	0.0a	0.0a
4	0	0	10	3.1a	3.1a	0.0a	0.0a

Among the three different auxins tested (NAA, IAA and IBA), NAA was the most efficient in regenerating shoots in sugarcane TS explants (Table 4). Replacing BA with

zeatin did not produce shoots in any of the concentrations tested. Presence of TDZ in the medium, with or without BA, caused callus formation after 4-6 weeks of culture. TDZ-induced callus was mostly non-organogenic.

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 harvested from the lowermost portion of leaf roll (designated into three groups: proximal, middle and distal) was determined by their position in the leaf roll as well as the medium composition in which they were grown (Table 5). Clearly explants from proximal and middle segments were more prolific than those from distal segment. The percentage of proximal and middle segment explants producing shoots was not significantly different in all the tested media, except for the proximal segments grown on medium with the lowest level of NAA (5 μM). In contrast, increasing the NAA level from 5 to 10 μM increased the percentage of distal segment explants producing shoots from 18 to 45 within 8 weeks of culture, but no further improvement was evident at higher auxin levels (Table 5). Similarly, a remarkable increase in the proportion of explants with more than 20 shoots was observed when medium NAA content was increased from 5 to 10 μM.

Table 5 **Spatial distribution of shoot regeneration in thin leaf explants (1-2 mm) of sugarcane cultivar Q165$^{\phi}$ after 8 weeks of culture on MS medium containing 6-benzyladenine (BA) and α-naphthaleneacetic acid (NAA). Explants were oriented with its distal end in contact with medium. Data were subjected to AOV and means were compared with LSD. In each column, for a given medium, values followed by the same letter are not significantly different (P < 0.05).**

Medium		Leaf explant source*	% of explants producing shoots	% of explants producing different numbers of shoots		
BA (μM)	NAA (μM)			1-10	11-20	>20
4	5	PS	54.0a	17.0a	23.0a	14.0a
4	5	MS	69.0a	18.0a	24.0a	28.0b
4	5	DS	18.1b	13.6a	4.5b	0.0c
4	10	PS	75.4a	5.4a	29.0a	40.9a
4	10	MS	89.0a	10.0a	22.0a	57.0a
4	10	DS	45.3b	15.3a	16.9b	13.0b
4	20	PS	79.0a	6.0a	17.0a	57.0a
4	20	MS	75.0a	5.8a	16.6a	52.5a
4	20	DS	41.6b	8.3a	17.5a	15.8b
4	40	PS	76.3a	10.9a	24.5a	42.7a
4	40	MS	82.2a	6.6a	24.4a	51.1a
4	40	DS	52.5b	7.5a	17.5a	27.5b

*PS=Proximal segment; MS=Middle segment; DS=Distal segment

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

To assess the efficiency of regeneration procedure developed with Q165$^{\phi}$ in inducing shoot formation in other sugarcane genotypes, eight cultivars with different genetic backgrounds were grown on SRM. Although all but one cultivar regenerated plants on

SRM, the frequency of shoot production was highly variable within the cultivars tested (Table 6). For instance, almost half of the explants from cultivars Q96 and Q117 produced shoots, whilst regeneration was recorded only in 4% of the explants from Q200$^{\phi}$.

Table 6 **Effect of genotype on shoot production in thin leaf explants (1-2 mm) of sugarcane cultivars cultured on shoot regeneration medium (SRM) for 8 weeks. Explants were oriented with its distal end in contact with medium. Data presented are means ± SDs.**

Genotype	% of explants producing shoots	% of explants producing different numbers of shoots	
		1-10	>10
Q96	47.6 ± 19.8	25.2 ± 13.7	22.3 ± 10.9
Q117	50.6 ± 15.6	18.7 ± 10.2	31.8 ± 10.4
Q152	26.0 ± 19.5	26.0 ± 19.5	0.0
Q157	15.7 ± 15.0	15.7 ± 15.0	0.0
Q165$^{\phi}$	72.1 ± 18.4	22.8 ± 9.1	49.2 ± 14.3
Q188$^{\phi}$	37.5	37.5	0.0
Q200$^{\phi}$	3.6	3.8	0.0
Q205$^{\phi}$	28.0	26.4	1.6

Table 7 **A comparative analysis of shoot production in thin leaf explants (1-2 mm) of sugarcane cultivars cultured on SRM1 (4 μM BA and 10 μM NAA) and SRM2 (10 μM NAA and 10 μM CPA) for 8 weeks. Explants were oriented with its distal end in contact with the medium. Data presented are means ± SDs.**

Genotype	% of explants producing shoots		% of explants producing different numbers of shoots in SRM2		
	SRM1	SRM2	1-10	10-20	>20
Q156	1.0±2.8	51.7±34.5	46.7±32.0	5.0±7.9	0.0±0.0
Q165$^{\phi}$	88.1	100			
Q167$^{\phi}$	0.0±0.0	50.0±16.9	26.3±15.9	15.0±9.2	8.7±8.3
Q172$^{\phi}$	0.0±0.0	49.1±23.0	30.0±20.0	16.4±19.6	2.7±4.6
Q185$^{\phi}$	0.0±0.0	23.1±25.6	10.7±17.0	4.6±6.6	7.7±9.4
Q188$^{\phi}$	37.5±28.9	67.0±30.8	46.2±16.5	11.0±10.5	9.8±12.2
Q190$^{\phi}$	0.0±0.0	25.8±26.4	25.8±26.4	0.0±0.0	0.0±0.0
Q196$^{\phi}$	0.0±0.0	88.8±11.2	46.3±23.8	38.8±30.9	3.7±4.6
Q197$^{\phi}$	3.3±11.5	49.1239.6	41.7±33.2	5.8±6.6	1.7±2.8
Q200$^{\phi}$	3.6±8.0	35.0 ±29.4	34.0±22.7	10.0±14.9	16.0±32.0
Q205$^{\phi}$	28.1±23.1	60.0±29.4	34.0±22.7	10.0±14.9	16.0±32.0
84N538	0.0±0.0	22.3±22.4	21.5±21.1	0.8±2.7	0.0±0.0
92S280	0.0±0.0	14.2±10.8	10.0±8.5	3.3±6.5	0.8±2.8

The effect of genotype on regeneration was reduced remarkably by the substitution of BA with CPA in the culture medium (Table 7). In this experiment, only 5 of 12 clones (in addition to Q165$^{\phi}$) cultured on SRM1 produced shoots, and, more significantly, three of these cultivars (Q156, Q197$^{\phi}$ and Q200$^{\phi}$) responded extremely poorly with just 1-3% of

explants being organogenic. However, all these 12 clones cultured on BM supplemented with 10 μM NAA and 10 μM CPA (SRM2) produced shoots within 8 weeks of culture. In addition, under the same culture condition, at least 50% of the explants from seven genotypes regenerated shoots (Table 7). Presence of CPA in the medium caused microcallus formation on the cut surface of the explants, which turned out to be highly morphogenic in many of the 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 SRM1 or SRM2 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 (Figure 1). Separated shootlets (2-3 cm long) cultured on BM grew well and almost 95% of them produced at least 3-4 roots (5-6 cm long) within 6 weeks of incubation (data not presented).

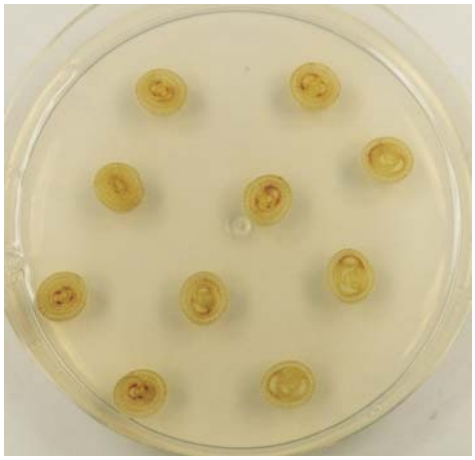
Nearly 90% of the shootlets (3-4 cm long) transferred to perlite:peat moss:sand mixture in seedling trays, and kept under high humidity and shaded conditions for the first 5-7 days, rooted and developed into well established plantlets ready for field planting within 6-8 weeks (data not presented). Almost all the transplanted seedlings in the field established and grew well, but provision of irrigation was essential during the initial stage (first 2-3 weeks) of establishment.

4.2 Further refinement of the SmartSett® system

As part of the SmartSett® system development, considerable research effort has been directed to define methods that minimise the occurrence of somaclonal variation in monocot crops, including sugarcane, during tissue culture and transformation. This effort led to the identification of most morphogenically responsive explants, use of growth regulators that are less mutagenic, prevention of or substantial reduction in callus formation, and a significant decrease in culture duration, which collectively formed the basis of methods for rapid regeneration of sugarcane from leaf and inflorescence explants.

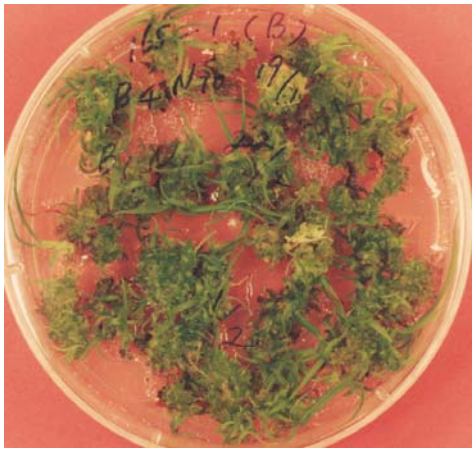
To further enhance the quality of sugarcane plants produced by tissue culture, we continued our research effort to improve the direct regeneration method from two different perspectives: achieving a substantial reduction in the duration of plant growth regulator application, and reducing the time required to regenerate fully grown plantlets without compromising the productivity of the system.

This improvement is based on the finding that light, at least in part, plays a regulatory role in the induction and development of sugarcane shoot meristem in culture. Light appears to have an inhibitory effect on some specific developmental stages of meristem formation. The effect of light on meristem development is very striking, in that a remarkable enhancement in plant regeneration was obtained with the incubation of cultures in dark during the early period of meristem differentiation.

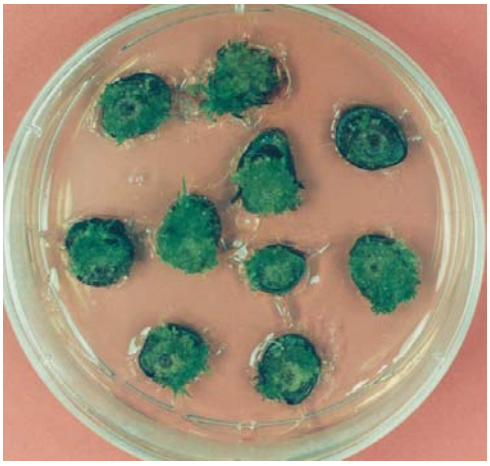


Sugarcane leaf sections immediately after culture

3-week-old cultures showing the emergence of shootlets



6-week-old cultures showing further shoot development



10-week-old cultures with well-developed plantlets

Figure 1 Different stages of SmartSett® plant production

4.2.1 Methodology and results

1-2 mm thick transverse sections of immature leaf tissues cultured on medium supplemented with 4 μM BA and 10 μM NAA could produce double the number of plantlets when they were grown in the absence of light for the first 30 days as compared to those maintained under light throughout the growth period. In dark-incubated cultures, nearly 90%of the explants were highly morphogenic, producing more than 20 shoots each at the end of 60 days of culture (Tables 8 and 9). Taken together, the results of the initial experiments of this invention showed that high frequency plant production can be achieved with just 30 days of growth regulator application alone.

Table 8 **Influence of light during plant growth regulator treatment on sugarcane plant (Q165$^{\Phi}$) regeneration after 60 days of culture. Cultures were grown on growth regulator-containing medium for 30 days and were subsequently transferred to MS medium. The data are the average of at least two experiments.**

Light	Growth regulator			Explants producing shoots (%)	% of explants producing different numbers of shoots		
	BA (μM)	NAA (μM)	KIM (μM)		1-10	11-20	>20
From dark	4	10	0	95.0	5.0		90.0
	0	10	10	17.5		7.5	10.0
	0	0	0	0			
From light	4	10	0	47.5		6.5	41.0
	0	10	10	5.0		1.0	4.0
	0	0	0	0			

Table 9 **Influence of light during plant growth regulator treatment on sugarcane plant (Q165$^{\Phi}$) regeneration after 60 days of culture. Cultures were grown on growth regulator-containing medium for 30 days and were subsequently transferred to EM (embryo growth) medium. The data are the average of at least two experiments.**

Light	Growth regulator			Explants producing shoots (%)	% of explants producing different numbers of shoots		
	BA (μM)	NAA (μM)	CPA (μM)		1-10	11-20	>20
From dark	4	10	0	100	5		95
	0	10	10	100	26	6	68
	0	0	0	0			
From light	4	10	0	77	16	9	52
	0	10	10	92	42		50
	0	0	0	0			

More notably, especially from the perspective of minimising somaclonal variation, the outcomes of further experimentation resulted in the development of a method to produce sugarcane plants with very short periods of plant growth regulator treatment. For instance, a large proportion of explants, nearly 70%, can be induced to produce shoots by

treating them with 4 μM BA and 10 μM NAA just for 1 day. Almost 90% of the explants, however, could be induced to produce shoots by culturing for 8-10 days on the same medium. Significantly, the number of highly morphogenic explants also increased substantially during this period (Tables 10 and 11).

Table 10 Determination of the minimum duration of growth regulator requirement for shoot induction in sugarcane (Q165$^{\Phi}$) leaf culture. Cultures were maintained in dark for the first 14 days. The data are the average of at least two experiments.

Number of days on growth-regulator containing medium	Explants producing shoots (%)	% of explants producing different numbers of shoots		
		1-10	11-20	>20
0	0	0	0	0
3	100	30	20	50
6	100	18	11	71
8	100	31	6	63
10	100	31	17	52
14	100	26	11	63
30	100	30	7	63

Table 11 Determination of the minimum duration of growth regulator requirement for shoot induction in sugarcane (Q165$^{\Phi}$) leaf culture. Cultures were maintained in dark for the first 14 days. The data are the average of at least two experiments.

Number of days on growth-regulator containing medium	Explants producing shoots (%)	% of explants producing different numbers of shoots		
		1-10	11-20	>20
0	0	0	0	0
1	71	49	6	16
2	93	36	23	34
4	98	48	6	44
6	96	45	0	51
30	100	47	5	48

In brief, this new approach presents a method for large-scale production of sugarcane plants that ensures the highest level of clonal fidelity of propagules than by the methods described earlier. Following regeneration, shootlets could be readily separated, rooted and established as seedlings suitable for commercial crop production. This method of plant production has been successfully demonstrated with the commercial cultivars such as Q165$^{\Phi}$, Q196$^{\Phi}$ and Q205$^{\Phi}$.

4.2.2 Conclusions

The two important findings from experiments conducted to further refine the SmartSett® system were:

- Avoidance of light during growth regulator treatment dramatically enhances shoot production in sugarcane leaf tissues under conditions defined above;
- The efficiency of plant production can be significantly improved by a short exposure of leaf tissues to growth regulators in culture.

4.3 Field evaluation to determine the genetic fidelity of plants produced by the SmartSett® system

We established that plantlets can be regenerated from sugarcane tissues via the novel combination of plant growth regulators in 6-8 weeks. We believed that the potential for somaclonal variation is significantly minimised in this tissue culture system due to two factors. Firstly, the time frame through tissue culture is short (32 weeks for the current callus system down to 6-8 weeks) and no callus phase is involved in this new system. However, it was necessary to rigorously test this belief. This was achieved by optimising the tissue culture conditions and subsequent acclimitisation for field growth with nine sugarcane genotypes. Two replicated trials comparing SmartSett® plants to parental clones for performance and morphological traits were established with selected genotypes to prove the utility of this tissue culture system for mass propagation, and, by inference, genetic engineering.

4.3.1 Methodology

Field trials were established at BSES Meringa to determine the extent of somaclonal variation in micropropagated plants and their progeny. Trial 1 compared Q96 and Q117 in the plant crop (Trial 1A) and in the first ratoon (Trial 1AR); Trial 1B was a plant-crop comparison of the same varieties established with setts from Trial 1A. Trial 2A compared nine genotypes - 90N876, Q96, Q114, Q117, Q124, Q152, Q157, Q165$^{\phi}$, and Q170$^{\phi}$ - in the plant crop.

Plantlets derived from tissue culture and stem cuttings (billets) were raised as potted plants in the nursery for 8 weeks prior to field planting. The trials were established with a total of 500 propagules of micropropagated and single-eye billet plants randomly assigned to different plots in the experimental field. The experimental design was a randomised-block design of six replicates of each variety with two treatments (tissue culture propagules and single-eye billet propagules) in six blocks. Each block of four plots contained plants representative of each treatment. Each plot consisted of three rows at 1.5 m by 19 propagules at 0.5 m spacing, with 1.0 m spacing between plots. All plots were fertilised with commercial fertiliser Crop King 66 (365 kg/ha) and urea (120 kg/ha). Plants were harvested after about 12 months and stalk number, stalk height, stalk diameter at fifth node, number of nodes, length of nodes, number of tillers, leaf length, leaf width, stool weight, commercial cane sugar (CCS) and fibre content were measured. Data were subjected to analysis of variance.

4.3.2 Results

Overall, there was more variation in the tissue cultured plants than the control one-eye sett plants. Some of the differences between parameters were small, and are only apparent in trials conducted with this degree of precision. Of most interest are the harvest statistics. In trial 1AR (Table 12) there was no statistical difference between the tissue culture and sett plants at harvest in CCS, its components or stool weight. This is extremely encouraging and suggests that the differences in other characters measured during the season are not important and the key industry parameter, tonnes sugar per hectare, is not influenced by propagation method by first ratoon. In trial 1B (plant crop), there was no reduction in the variation amongst propagules from the tissue culture relative to variation within the plants single-eye setts.

The results of Trial 2A are more interesting, as more diverse genotypes, 90N876, Q96M, Q114, Q117, Q124, Q152, Q157, Q165$^{\phi}$ and Q170$^{\phi}$, were included. Morphological data were collected at three times, April, July (Table 13) and December 2003 (Table 14). This trial was harvested in December, and stool weight, CCS, brix, fibre moisture content and Pol reading were recorded at that time. No significant difference was found in any of the commercially significant harvest parameters in this trial (Table 14), indicating the usefulness of the SmartSett® propagation method for commercial seedling production.

4.4 Application of SmartSett® for production of commercial planting material

4.4.1 Methods

The micropropagation system was tested on a large scale in May 2001 when BSES used the technique to increase the amount of sugarcane cultivar Q196$^{\phi}$ released in the Central District to combat a devastating outbreak of orange rust (*Puccinia keunii*) in the dominant cultivar Q124. One hundred and fifty sugarcane tops were used in the micropropagation system to initiate approximately 55,000 plants. The process from initiation to replanting of the progeny into plastic punnets (filled with a mixture of sand and potting mix) took about 3 months.

From the 50,000 SmartSett® seedlings destined for the Central District, 45,000 survived the initial 3-4 week growing and hardening period in Brisbane glasshouses before being sent to Mackay by road transport in three separate batches.

Before delivery of the seedlings, an existing poly tunnel at BSES Mackay measuring 6.5 m by 24 m with 3 m sides was modified to accommodate the large numbers of seedling punnets. Wire ‘shelves’ were constructed along the length of the poly tunnel. Each shelf was made from three lengths of 8-gauge high-tensile wire with individual strainers attached to each wire. A single punnet delivered inside a plastic tray measuring 30 cm by 36 cm containing 42 cells could be placed onto the three wires. This modification enabled the poly tunnel to house 696 punnets (on a single level), giving the poly tunnel a total capacity of 29,232 seedlings. The poly tunnel roof was made from a waterproof material (Solar Ultra) that cuts out 100% UV and allows in 85% light.

Table 12 **Field evaluation of agronomic and yield characteristics of micropropagated Q96 and Q117 sugarcane plants (Field Trial 1). Data were subjected to analysis of variance and mean and lsd values are presented (P < 0.05). OES = plants propagated from one-eye sett (control). TC= plants propagated by thin section culture. 1A = First plant crop where plantlets produced by micropropagation and conventional one eye setts were used for planting. 1B = Second cycle of plant crop using planting material obtained from trial 1A. 1AR = First-ratoon crop of trial 1A. ND = not determined.**

Trait	Propagation method	1A		1B		1AR	
		Q96	Q117	Q96	Q117	Q96	Q117
Stalk length (cm)	OES	259.18	241.19	308.31	269.45	232.27	210.65
	TC	233.14	236.72	307.43	270.96	216.28	198.08
	lsd	9.87		10.08		10.90	
Number of stalks	OES	ND	ND	4.97	4.08	6.04	3.86
	TC	ND	ND	6.08	4.04	7.58	3.85
	lsd			0.32		0.44	
Number of nodes	OES	18.30	18.97	21.03	22.76	12.25	12.70
	TC	18.40	18.95	22.38	23.35	12.32	12.31
	lsd	-		0.64		-	
Node length (cm)	OES	14.28	12.85	14.57	11.88	19.3	16.94
	TC	12.83	12.61	13.76	11.67	17.99	16.47
	lsd	0.67		0.28		1.78	
Stalk (5^{th} node) diameter (mm)	OES	ND	ND	24.92	27.56	20.45	24.48
	TC	ND	ND	21.77	25.41	17.45	21.89
	lsd			0.94		0.73	
Leaf length (cm)	OES	184.41	176.30	170.71	161.36	175.47	165.75
	TC	176.24	167.21	166.80	155.91	167.88	160.24
	lsd	3.85		6.36		3.74	
Total tillers	OES	ND	ND	7.76	5.66	7.72	4.16
	TC	ND	ND	9.26	5.69	10.45	4.51
	lsd			0.48		0.86	
Stool weight (kg)	OES	5.64	5.54	6.06	5.36	4.82	3.88
	TC	4.52	4.38	6.01	5.01	4.60	3.25
	lsd	0.56		0.42		0.43	
CCS(%)	OES	13.45	17.01	15.28	19.39	14.04	19.97
	TC	12.83	16.05	14.93	18.92	14.15	17.44
	lsd	0.29		0.22		0.41	
Brix (º)	OES	21.94	24.39	20.80	24.21	19.88	23.18
	TC	21.32	23.55	20.44	23.55	19.82	22.66
	lsd	0.32		0.26		0.47	
Fibre (%)	OES	15.38	11.73	14.70	12.44	14.01	11.91
	TC	15.53	13.06	15.04	12.67	14.24	12.24
	lsd	0.32		0.22		0.50	

Table 13 **Summary of means and least significant differences for stalk, sucker and total tiller numbers counted in April and July 2003. Data presented as a 2 x 2 matrix of propagation method (PM) x cultivar (C), with least significant differences, marginal means for clones and propagation methods, and a general mean for Trial 2A**

Character	Statistic	PM	90N876	Q96	Q114	Q117	Q124	Q152	Q157	Q165$^{\phi}$	Q170$^{\phi}$	$\bar{x}_{PM}$
April												
# Stalks		**OES**	6.14	6.59	5.70	5.91	6.35	7.67	7.85	7.24	7.25	6.75
		SR	7.38	7.41	6.69	6.21	8.65	10.32	8.86	9.18	8.72	8.16
	$\bar{x}_C$		6.76	7.00	6.20	6.06	7.50	9.00	8.36	8.21	7.98	7.45
	$lsd_{0.05}$ C x PM		-									
	$lsd_{0.05}$ C		0.92									
	$lsd_{0.05}$ PM		0.36									
# Suckers		**OES**	0.35	0.11	0.65	0.46	0.04	0.83	0.04	0.30	0.14	0.33
		SR	0.61	0.63	0.39	0.60	0.17	1.15	0.32	0.33	0.17	0.49
	$\bar{x}_C$		0.48	0.37	0.52	0.53	0.10	0.99	0.18	0.31	0.16	0.41
	$lsd_{0.05}$ C x PM		-									
	$lsd_{0.05}$ C		0.39									
	$lsd_{0.05}$ PM		0.15									

Table 13 (cont.)

Character	Statistic	PM	90N876	Q96	Q114	Q117	Q124	Q152	Q157	Q165$^{\phi}$	Q170^{A}	$\bar{x}_{PM}$
April												
Total tillers		**OES SR**	6.50	6.70	6.35	6.37	6.39	8.50	7.89	7.55	7.39	7.07
			7.99	8.04	7.08	6.81	8.82	11.47	9.19	9.51	8.88	8.64
	$\bar{x}_C$		7.24	7.37	6.72	6.59	7.60	9.99	8.54	8.53	8.14	7.86
	$lsd_{0.05}$ C x PM	-										
	$lsd_{0.05}$ C	0.90										
	$lsd_{0.05}$ PM	0.36										
July												
# Stalks		**OES SR**	5.75	6.02	4.96	5.52	5.91	7.13	6.37	6.66	6.47	6.09
			7.06	7.22	6.17	6.14	7.77	10.37	7.45	8.33	7.92	7.61
	$\bar{x}_C$		6.40	6.62	5.57	5.83	6.84	8.75	6.91	7.50	7.20	6.85
	$lsd_{0.05}$ C x PM	1.54										
	$lsd_{0.05}$ C	0.84										
	$lsd_{0.05}$ PM	0.33										

Table 13 (cont.)

Character	Statistic	PM	90N876	Q96	Q114	Q117	Q124	Q152	Q157	Q165$^{\phi}$	Q170$^{\phi}$	$\bar{x}_{PM}$
July												
# Suckers		**OES**	0.29	1.19	0.74	0.39	1.48	4.87	2.61	1.18	1.78	1.62
		SR	1.24	2.07	1.64	0.61	3.21	7.71	4.04	1.92	2.00	2.72
	$\bar{x}_C$		0.77	1.63	1.19	0.50	2.35	6.29	3.32	1.55	1.89	2.17
	$lsd_{0.05}$ C x PM		1.65									
	$lsd_{0.05}$ C		0.90									
	$lsd_{0.05}$ PM		0.36									
Total tillers		**OES**	6.04	7.20	5.70	5.91	7.39	12.00	8.97	7.84	8.25	7.70
		SR	8.31	9.30	7.82	6.75	10.98	18.08	11.49	10.25	9.93	10.32
	$\bar{x}_C$		7.17	8.25	6.76	6.33	9.19	15.04	10.23	9.04	9.09	9.01
	$lsd_{0.05}$ C x PM		2.59									
	$lsd_{0.05}$ C		1.41									
	$lsd_{0.05}$ PM		0.56									

Table 14 **Summary of means and least significant differences for stalk, sucker and total tiller numbers counted in December 2003. Plot weight, CCS, Brix °, Fibre %, % moisture and Pol reading measured 2-10 December presented as a 2 x 2 matrix of propagation method [1] (PM) x cultivar (C), with least significant differences, marginal means for clones and propagation methods and a general mean for Trial 2A**

Character	Statistic	PM	90N876	Q96	Q114	Q117	Q124	Q152	Q157	Q165$^{\phi}$	Q170$^{\phi}$	$\bar{x}_{PM}$
December												
# Stalks		**OES**	4.27	5.26	3.33	4.63	3.91	5.87	2.78	5.38	4.30	4.41
		SR	5.12	5.93	4.32	4.81	5.54	7.05	3.71	6.45	6.17	5.45
	$\bar{x}_C$		4.70	5.59	3.83	4.72	4.72	6.46	3.21	5.88	5.23	4.93
	$lsd_{0.05}$ C x PM		-									
	$lsd_{0.05}$ C		1.72									
	$lsd_{0.05}$ PM		0.68									
# Suckers		**OES**	0.30	0.50	0.76	1.24	1.26	1.94	1.33	1.07	0.74	1.01
		SR	0.60	1.65	0.88	0.54	1.67	2.87	2.06	0.80	1.23	1.36
	$\bar{x}_C$		0.45	1.07	0.82	0.89	1.46	2.41	1.68	0.92	0.99	1.19
	$lsd_{0.05}$ C x PM		-									
	$lsd_{0.05}$ C		1.34									
	$lsd_{0.05}$ PM		-									

Table 14 (cont.)

Character	Statistic	PM	90N876	Q96	Q114	Q117	Q124	Q152	Q157	Q165$^{\phi}$	Q170$^{\phi}$	$\bar{x}_{PM}$
# Dead		**OES**	1.01	0.70	1.52	1.00	1.84	1.22	2.44	1.55	0.91	1.36
		SR	1.35	0.82	1.76	1.06	1.63	1.31	3.26	1.04	1.81	1.56
	$\bar{x}_C$		1.18	0.76	1.64	1.03	1.74	1.27	2.86	1.30	1.36	1.46
	$lsd_{0.05}$ C x PM	-										
	$lsd_{0.05}$ C	0.96										
	$lsd_{0.05}$ PM	-										
Total tillers		**OES**	4.57	5.76	4.08	5.88	5.17	7.82	4.12	6.44	5.04	5.42
		SR	5.73	7.57	5.20	5.35	7.21	9.92	5.77	7.26	7.40	6.81
	$\bar{x}_C$		5.15	6.67	4.64	5.62	6.19	8.87	4.90	6.80	6.22	6.12
	$lsd_{0.05}$ C x PM	-										
	$lsd_{0.05}$ C	2.12										
	$lsd_{0.05}$ PM	0.84										

Table 14 (cont.)

Character	Statistic	PM	90N876	Q96	Q114	Q117	Q124	Q152	Q157	Q165$^{\phi}$	Q170$^{\phi}$	$\bar{x}_{PM}$
Weight (kg)		**OES**	5.97	5.33	4.23	4.71	4.87	4.98	3.80	4.80	4.87	4.82
		SR	5.53	4.97	4.54	4.42	5.36	5.04	3.32	4.47	4.48	4.68
	$\bar{x}_C$		5.75	5.15	4.31	4.55	5.10	5.01	3.56	4.63	4.67	**4.75**
	$lsd_{0.05}$ C x PM		-									
	$lsd_{0.05}$ C		1.11									
	$lsd_{0.05}$ PM		-									
CCS		**OES**	18.80	17.34	17.39	19.09	18.64	17.71	17.66	18.88	17.36	18.10
		SR	19.17	17.26	18.21	19.30	19.11	17.14	17.76	18.99	17.34	18.25
	$\bar{x}_C$		18.99	17.30	17.81	19.20	18.86	17.43	17.71	18.94	17.35	**18.17**
	$lsd_{0.05}$ C x PM		-									
	$lsd_{0.05}$ C		1.10									
	$lsd_{0.05}$ PM		-									

Table 14 (cont.)

Character	Statistic	PM	90N876	Q96	Q114	Q117	Q124	Q152	Q157	Q165$^{\phi}$	Q170$^{\phi}$	$\bar{x}_{PM}$
Brix		**OES SR**	25.19	23.27	23.64	24.82	24.14	23.87	24.05	24.62	23.44	24.12
			25.28	22.98	24.21	24.77	24.44	23.64	24.67	24.69	23.63	24.26
	$\bar{x}_C$		25.23	23.12	23.94	24.80	24.30	23.76	24.36	24.66	23.54	24.19
	$lsd_{0.05}$ C x PM		-									
	$lsd_{0.05}$ C		0.79									
	$lsd_{0.05}$ PM		-									
Fibre %		**OES SR**	12.39	14.07	11.78	11.48	11.16	12.10	12.33	12.38	13.10	12.31
			12.83	13.73	12.11	11.86	11.34	12.85	12.68	13.02	13.04	12.61
	$\bar{x}_C$		12.61	13.90	11.95	11.67	11.26	12.47	12.51	12.70	13.07	12.46
	$lsd_{0.05}$ C x PM		-									
	$lsd_{0.05}$ C		0.69									
	$lsd_{0.05}$ PM		0.27									

Table 14 (cont.)

Character	Statistic	PM	90N876	Q96	Q114	Q117	Q124	Q152	Q157	Q165$^{\phi}$	Q170$^{\phi}$	$\bar{x}_{PM}$
% Moisture		**OES**	66.43	66.16	68.49	67.54	68.31	67.60	67.48	66.67	67.00	67.29
		SR	65.84	66.60	67.61	66.95	67.78	67.12	66.93	66.05	67.01	66.88
	$\bar{x}_C$		66.14	66.38	68.03	67.25	68.04	67.36	67.20	66.35	67.01	67.08
	$lsd_{0.05}$ C x PM		-									
	$lsd_{0.05}$ C		1.08									
	$lsd_{0.05}$ PM		-									
Pol reading (Z °)		**OES**	102.00	94.17	94.83	102.07	99.32	96.28	96.77	101.65	94.12	97.93
		SR	103.79	93.17	99.01	102.66	101.31	94.16	98.84	102.41	94.91	98.92
	$\bar{x}_C$		102.89	93.67	97.04	102.37	100.30	95.22	97.81	102.03	94.51	98.43
	$lsd_{0.05}$ C x PM		-									
	$lsd_{0.05}$ C		4.72									
	$lsd_{0.05}$ PM		-									

The first batch of seedlings arrived on 7 September 2001. This batch consisted of 250 trays with approximately 10,500 seedlings. The seedlings remained in the poly tunnel for 14 d, where they were watered twice daily; morning and evening for 15 min with a fine spray on an automatic timer. They were also fertilised at weekly intervals with a solution of one measured spoon of Thrive® per 9 L of water. There was substantial variability in the height of the seedlings from tray to tray, with the height of the seedlings from base to the first exposed dewlap ranging from 2 to 14 cm. Weekly trimmingof the more advanced seedlings enabled the smaller ones to catch up (and provided more uniform sized seedlings for planting into the field). After 2 weeks in the poly tunnel, the seedlings were transferred to outside benches and exposed to direct sunlight for another 14 d. Once outside, twice daily watering, and weekly fertilising and trimming of the seedlings continued (at the same rates as inside the poly tunnel). All seedlings from Batch 1 successfully survived this 4-week hardening process and grew to a size suitable for transplanting.

The second batch of plants, consisting of 480 trays with approximately 20,000 seedlings, arrived on 12 October 2001. Batch 2 received the same treatment as Batch 1 and again 100% survived.

The third batch of 450 trays arrived on 6 November 2001. This batch contained around 15,000 seedlings. . The reduction from Batch 2 capacity was due to an estimated loss of around 20% of the seedlings during transplanting from the agar medium into punnets in Brisbane. The majority of Batch 3 seedlings received the same treatment as previous batches and were moved to outside benches 14 d after arriving in Mackay. A small proportion of Batch 3 was used to conduct two minor experiments to gain information on hardening techniques and transplant shock. Some seedlings were placed outside on benches in direct sunlight, while some remained in the waterproof enclosure with 100% UV protection. These experiments are detailed Jackson (2003, *BSES Project Report* PR03004).

The first batch was planted into block D2 at the Mackay Sugar Experiment Station on 3-4 October 2001, 25 d after arriving. Plant spacing within rows was 45 cm and row spacing was 1.5 m. Planting was done with a water-wheel vegetable planter with enough water applied to fill the hole formed by the wheel spike. The planting rate was about 8,000 seedlings per normal working day. The ground was prepared in the traditional way and was left flat after receiving a final rotary hoeing and being marked out for row position.

Approximately 8,000 seedlings were planted on 3 October 2001 into the 0.56 ha north D2 block with the water-wheel planter. Problems encountered with the water wheel were minimal, with the exception of soil build-up on the wheels, with dry powdery soils sticking to the wet surfaces. The build up of soil around the spike blocked water access to the formed hole, which was often not deep enough to push the seedling into due to lack of water. This was overcome to some extent by attaching a self-cleaning mechanism that consisted of two flat metal pieces that scraped the flat surface of the wheel either side of the centralised spike. Spray-line irrigation was set up on the same day and approximately 30 mm was applied to the newly replanted seedlings that evening.

On 4 October 2001, the remaining seedlings from Batch 1 were planted into the south D2 block using the Crop Improvement Group's planter. This forms a furrow where the plant

is placed, then filled slightly. This method allowed the seedlings in this block to receive flood irrigation immediately after planting.

On 13 November 2001, 7,000 seedlings from Batch 2 were planted at Proserpine. A further 8,000 seedlings were planted in the same block on 19 November 2001 (the delay between the two plantings was due to heavy rain falling during the night of 13 November 2001). The ground where the seedlings were planted was prepared and finished with a mound-forming operation. The plants were then delivered straight into the raised mound with the water-wheel planter. Approximately 30 mm of water were applied to the seedlings with a travelling-gun irrigator immediately following the completion of all of the planting at the Proserpine block.

The remaining 5,000 seedlings from Batch 2 were planted at Graham Davies' farm at Munburra, southwest of Mackay, on 22 November 2001, some 36 d after arriving in Mackay from Brisbane. Each individual plant had to be pre-pulled before trays were placed on the planter, as the plants had become firmly attached to the punnets by the extensive root growth. The ground at the Munburra site was prepared as normal, but left flat after the final rotary hoeing. Mark-out tines were used to form planting rows. Plants were delivered straight into the flat ground with the water-wheel planter.

The 15,000 seedlings from Batch 3 (including the seedlings used in the hardening experiment) were also planted at Graham Davies' farm at Munburra on 3-4 December 2001. These plants were also planted into flat ground using the water-wheel planter. The sandy soil site had an application of mill mud before planting. The centre-pivot irrigator applied 30 mm of water 5 d after planting, helping to ensure a greater than 95% survival of the seedlings.

4.4.2 Results

Seedlings established very well and survived transplant shock under hot field conditions. The hardening process did not affect establishment survival rate. Seedlings hardened in full sun from arrival in November established just as well as those that remained in the poly tunnel for the full 4 weeks. However, keeping seedlings in a waterproof enclosure for at least 2 weeks after arrival to protect them from heavy wind or rain is recommended.

The 3-4-week growing period in the Brisbane glasshouses provided much-needed early hardening. Seedlings were protected with shade cloth under high humid conditions for 1 full week with the removal of shade cloth for the following 2-3 weeks during this period. The preferred height of the seedlings on arrival would be about 5-7 cm to the first exposed dewlap. Trimming of more advanced seedlings whilst in the Brisbane glasshouses would help keep seedlings at more consistent height on arrival. Some tended to be very elongated and thin, bending over and almost breaking off at the growing point, whilst others were very small with just a couple of leaf blades showing.

Seedlings became root bound after 5 weeks from arrival, with a root ball starting to develop at the bottom of the seedling punnet. Seedlings could be ready for planting in 3-4 weeks after arrival. If plants are held longer than 5 weeks before planting, then

transplanting into jiffy pots made out of peat is recommended. Here, they can continue to grow for another month or so and be maintained by trimming.

Planting into a furrow, which is gradually filled in then hilled up, proved to be the most effective planting procedure. This gave the seedling more rooting depth and more support than the option of planting straight into flat ground. The perceived smaller root system in comparison to normal plant cane and standard seedling tended to cause the plant to become water stressed earlier due to the bulk of the roots being in the top 10 cm of the soil profile.

Seedlings planted with water easily survived for 5 days before irrigation, but it would be recommended to irrigate within 2 days after planting. Seedlings should be treated more like horticultural crops with more regular watering with lesser amounts. Irrigating every 4-5 days with 25 mm for at least five irrigations proved to be quite effective. This will vary with soil type and it pays to monitor the moisture status of the top 10 cm of soil. Tensiometers could also be used in this role.

Options for most appropriate fertilizer application needs more attention. Problems arose in Proserpine where urea was placed on top of the soil then washed in with 25 mm of irrigation. This caused root burn and death to some seedlings. Seedlings had only been growing in the field for approximately two weeks. The other method of fertilisation consisted of drilling a ratoon mixture 15 cm either side of the plant at 10-15 cm depth when the plants were approximately 5 weeks old. The plant could not access this band of fertiliser very readily for quite some time. Options such as fertilizer delivered at planting either into the furrow or with the water, or foliar fertilizers applied just after planting, with a side dressing applied once the root system is more advanced need to be assessed.

Options for most appropriate weed control also need to be assessed. Ideally a pre-emergent product that can be sprayed over the crop just after planting and watered in with the first irrigation, with minimal crop phytotoxicity, would be the answer. This option with Dual® and Atrazine® gave good results at the Davies' site. Another possible option is Stomp® and Atrazine®. Maintaining weed control through cultivation can be quite risky if wet weather eventuates, weeds would flourish under these circumstances with no way of controlling them. Losses from weed competition need to be avoided at the early stages of growth, especially if the cost of production for seedlings is high per hectare.

As the crop progressed and started to stool out after about 3 months the stand started to look sprawly with thin stalks at the December planted sandy site of Graham Davies at Munburra. The sprawling got worse over time until most of the crop had lodged. This was a concern as the crop was not large. A number of agronomic, genetic or tissue culture factors could be responsible for this trait. Agronomic factors could include depth of the root mass at planting, or timing of planting, that is, late planted hot conditions versus early planted cool conditions and there effect on stool development. The accessibility to fertiliser could also play a part in root development. The Davies' site was flat planted and not hilled up. Plants would have suffered moisture stress more regularly and for longer periods, especially during the very dry summer period of 2001/2002. The base of the stool would have had less support and stalk contact would have been reduced for further root development. The lack of water could also have induced less strength at the base of each stalk due to lack of stalk thickening. The stool itself produced a high number of

stalks (16-18), which were concentrated into a small area at the base of the plant, that is, they converged to a central point at the soil surface.

The seedlings planted at BSES Mackay were planted earlier, in the first week of October and were monitored very closely so water stress was not a major factor in their development. These plants were also provided with a deeper rooting depth, some were planted into a furrow and hilled up and some were planted flat then hilled up. The resulting crop was a lot healthier looking crop with numerous (8-15) stalks per stool of good comparable thickness to conventional plant cane. Though the crop was a good size it still succumbed to lodging and sprawling and would not be considered the ideal presentation for plant cutting, that is, an erect crop. Other factors affecting the crop at the experiment station could be due to excess nitrogen as the block had been fallowed for 2 years and had a ratoon mix applied to the crop.

Genetic factors such as varietal susceptibility to lodging could play a part, as Q196$^{\phi}$ has been prone to lodging. The tissue culture process itself (hormone treatments) could be responsible for root development being less vigorous. The fact that the roots start to ball up in the plastic punnets after 4 weeks could also be a factor. This may also be due to the type of planting receptacle rather than the tissue culture process.

It has been suggested that crops with smaller root systems, with the bulk of the roots being closer to the surface could play a large part in crop lodging: "The development of a high proportion of surface roots may also have some bearing on the tendency of crops to lean and lodge, a feature which is detrimental to Queensland harvesting methods" (*Manual of Cane-growing* 2nd ed. 1965). However, no documented evidence has been found in the literature to support this speculation.

5.0 MICROPROJECTILE AND *AGROBACTERIUM*-MEDIATED TRANSFORMATION

There are two methods for gene delivery into sugarcane tissues currently available. As part of CC027, it was demonstrated that sugarcane callus can be transformed by *Agrobacterium* (Elliott *et al.* 1998, *Aust. J. Plant Physiol*. 25: 739-743). Microprojectile transformation using sugarcane callus is the method of choice and is being routinely used in almost all the sugarcane biotechnology laboratories worldwide. To eliminate the undesirable aspects of callus-based transformation, we have successfully adapted the SmartSett® system as the base plant-production technology for sugarcane transformation. As part of this research, we needed to establish that regenerable tissues could be transformed and that non-chimeric plants can be effectively regenerated in an appropriate time frame. Reporter genes such as GFP and selectable genes such as NPT were used to achieve this objective.

All the details of transformation experiments performed and results obtained are included in the patent 'Plant Transformation'.

5.1 Microprojectile transformation

5.1.1 Methodology

Essentially the SmartSett® cultures were initiated as described in section 4.2. Four days after initiation, explants were transformed with expression constructs containing genes encoding GFP (a visible selection marker) (*pGEM.Ubi*) and neomycin phosphotransferase (an enzyme conferring kanamycin resistance; *pUbi.KN*) in transformed cells using a Particle Inflow Gun. Both genes were driven by the maize ubiquitin promoter (*Ubi*) and the *nos* 3'region as the terminator. Transformation was carried out at 3000-6000 kPa pressure. 6-10 days post-bombardment, explants were transferred to selection medium containing 100-125 mg/L paromomycin to select the putative transgenic plants. Experiments were performed with cultivars Q117, Q165$^{\phi}$ and Q205$^{\phi}$.

Several experiments were performed to understand/compare the effect of different DNA carriers such as tungsten and gold particles, the method of DNA preparation, the influence of explant age, various aspects antibiotic selection, and transformation pressures on the efficiency of gene integration and expression in both leaf and inflorescence tissues. Experiments were also conducted to improve the plant regeneration system to recover fully transformed lines from primary chimeric clones using GFP section.

5.1.2 Results

GFP fluorescence microscopy was performed to select transgenic shoots regenerated in the bombarded tissues. Both leaf and inflorescence tissues produced GFP-positive shoots (Figure 2), but most were chimeric. However, leaf whorl and inflorescence explants were different in that regeneration from inflorescence explants was embryogenic. This mode of regeneration resulted in the production of GFP positive plantlets directly in the inflorescence tissue, rather than the shoot organogenesis typical of leaf tissues. Using the procedure described above, we have produced more than 70 independent transgenic clones (Q165$^{\phi}$) expressing GFP and resistant to paramomycin

The presence and integration of GFP gene in the transgenic lines was confirmed by polymerase chain analysis (PCR) (Fig. 3) and Southern hybridisation (Fig. 4). PCR amplified the expected 750bp GFP coding region in all the 50 lines tested. Southern hybridisation data Fig) clearly proved the integration of GFP gene in transformed clones. The presence of different hybridisation bands indicate multiple insertions of gene, which is typical of plants obtained by microprojectile transformation.

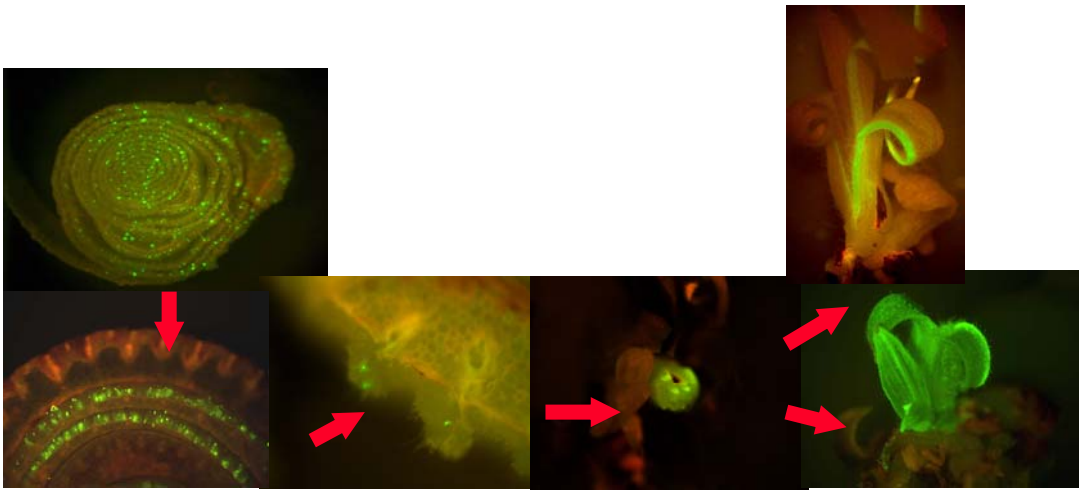


Figure 2 **Progressive stages of the development of a GFP-expressing sugarcane shoot from a transformed Q165$^{\Phi}$ leaf segment**

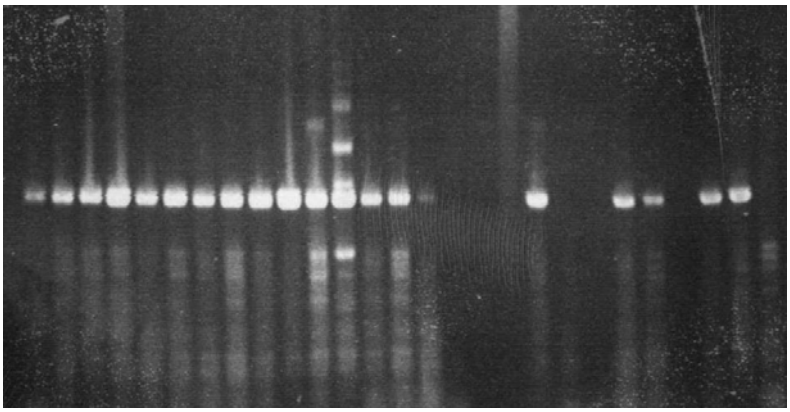


Figure 3 **Amplification of a 750 bp band confirming the presence of GFP gene in the putative transgenic sugarcane (Q165$^{\Phi}$)**

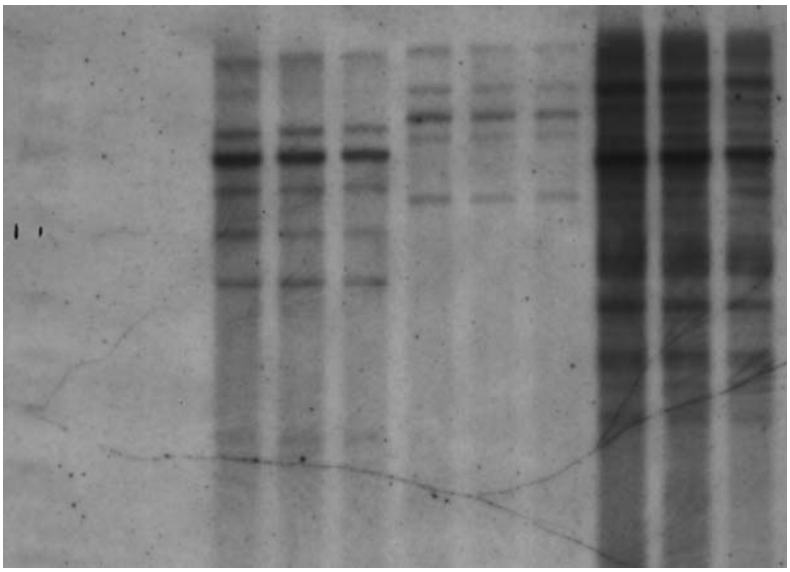


Figure 4 **Southern analysis of putative transgenic lines (Q165$^{\phi}$) showing different hybridising band sequences indicative of multiple insertion of GFP gene**

Comparative morphological evaluation of transgenic lines produced by the SmartSett® system showed a clear distinction between plants derived from callus-derived and those from the new method. Transgenic plants obtained from SmartSett® system (Fig. 5) were more close to the untransformed plants morphologically, with normal nodes and internodes, thick stems and healthy, normal-looking leaves. They also grew well and produced normal tillers. Billets from these plants were used for the transgenic field trial established in Meringa (section 4.3).

Figure 5 **Transgenic plants produced by callus-based (left) and SmartSett® system (middle). Untransformed control plants are shown in the right panel.**

5.1.3 Conclusions

A system to transform, regenerate and select transgenic plants from explant material (both leaf roll and inflorescence) has been developed. At this stage, the system is not as efficient as the callus transformation system, but it is sufficient to generate a reasonable number of plants for molecular evaluation.

Over 50 non-chimeric, transgenic plants expressing GFP are currently growing in the field at BSES Meringa. These plants were regenerated from explant material co-transformed with the NPTII gene construct and were selected on geneticin-supplemented media.

5.2 *Agrobacterium*-mediated transformation

Although it is the primary transformation method for dicot species, *Agrobacterium*-mediated transformation has been demonstrated for a number of monocot plants including many major crops such as maize, barley, rice, wheat and sugarcane. Gene delivery utilising *Agrobacterium* has some specific advantages over microprojectile bombardment for producing genetically engineered crops, including:

- Integration of transferred DNA is less complex.
- Fewer transgenic copies are inserted in recipient plant cells and multiple insertions at individual loci are less frequent.
- Fewer fragments of transgenes are inserted.
- Longer single DNA molecules can be transferred.
- Insertion is into transcriptionally active regions.

Our primary goal was to test the amenability of the sugarcane thin sections to *Agrobacterium*-mediated transformation. Different experimental strategies and many experiments were performed with regards to this, but transformation efficiencies for green leaf tissues and somatic embryos arising from thin sections of leaf tissues were much lower, and were not sufficient for us to obtain transgenic plants. With such low frequency, research has focus upon improving this to a level capable of yielding transgenic plants, rather than pursuing any transgenic plant directly.

5.2.1 Methodology and results

Briefly, young leaf sections (1-2 mm thick) were cultured with different *Agrobacterium* strains (LBA 4404, AGL0, AGL1) under normal, or those specifically used for monocot species, co-cultivation conditions for different duration. Based on the information available on monocot species, many aspects of *Agrobacterium*-mediated transformation were experimentally tested using sugarcane leaf segments and somatic embryos. These included:

- Different *Agrobacterium tumefaciens* strains used for cereal transformations tested;
- Varying length of co-cultivation period for survival of explants;
- Different temperature during co-cultivation;
- Presence of various chemical signals facilitating *Agrobacterium* infection;

- Presence of plant protein extracts from highly susceptible plants species to induce *Agrobacterium* virulence;
- Different co-cultivation media;
- Vacuum infiltration during inoculation;
- Presence of antioxidants in the medium;
- Agrolistics (shooting *Agrobacterium* at the tissues);
- Heat or cold-shock;
- Production of microcalli on the thin sections.

The production of microcalli on thin sections was investigated as sugarcane callus was shown to be more amenable to *Agrobacterium* infection. However, no increase in transformation efficiency was evident here.

During the course of extensive *Agrobacterium* testing, a method to detect and monitor the presence of *Agrobacterium* cells on the regenerating embryos using fluorescence was devised. Thus, good colonisation was demonstrated, but gene transfer did not eventuate. All *Agrobacterium* strains that have been beneficial for monocots to date were tested, including LBA4404, AGL0, AGL1, EHA101 and EHA105. A comparison of different sugarcane cultivars for susceptibility to *Agrobacterium* conducted in an earlier report (Elliott *et al.* 1998, *Aust. J. Plant Physiol*. 25: 739-43) showed little variation in susceptibility to *Agrobacterium*, as did a comparison of binary vectors utilised. In fact, the previous study identified that varying the transformation parameters gave the highest effect on transformation. Those parameters (the inclusion of 200 µM acetosyringone, presence of antioxidants in the tissue culture media, the omission of 2,4-D from the co-cultivation conditions, and temperature at 22-25°C during co-cultivation), identified as crucial in that study for *Agrobacterium*-mediated transformation of sugarcane were also tested in the recent experimentation, but with little success.

5.2.2 Conclusions

Agrobacterium- mediated sugarcane transformation has been reported by laboratories in Cuba, Australia and India. The Cuban techniques differ slightly to our own and were tested for application to thin sections, without any success. Very recently, an Indian group published a method which uses an entirely different target tissue, shoot apical or axillary meristem (Manicavasagam *et al*., 2004, *Plant Cell Reports* in press). This method appears to be simple and yielded the highest transformation efficiency ever obtained for sugarcane. Significant success in other areas in BSS242 has not enabled an increase in commitment to the *Agrobacterium* testing. However, the experiences and results generated in this research have put us in a good position if *Agrobacterium* is to be investigated further. This strategy is now being explored as an activity of the CRC for Sugar Industry Innovation through Biotechnology (CRC-SIIB).

6.0 DEMONSTRATION OF WIDER UTILITY OF THE SMARTSETT® SYSTEM TO OTHER MONOCOT SPECIES

We believe that this tissue-culture system may have significant practical application in other monocot crops such as the broadacre cereals, maize, sorghum and banana. Preliminary studies with sorghum, wheat and banana proved its potential utility in sorghum and wheat and IP protection has been extended to these and to other monocot species.

6.1 Sorghum

Briefly, thin section explants were taken from the basal end of leaf sheaths from young developing seedling of *Sorghum bicolor* cv. New Nugget and cultured on MS medium supplemented with various growth regulators, 3% sucrose and 7.5 g/L Davis J3 agar under conditions similar to that of sugarcane.

Of the different media tested, only those containing CPA alone or in combination with BA produced somatic embryos and shoots (Figure 6). In experiments with CPA (0-40 μM) and CPA with BA (2.5 μM), embryo-like structures could be clearly seen in cultures with several levels of BA within 3-4 weeks of culture. Shoots and plantlets were formed from embryos when they were placed onto basal MS medium without CPA. In these cultures shoot regeneration was enhanced by the addition of kinetin after the initial embryo formation. Shoots readily formed roots on basal MS medium. The time taken from culture initiation to plants in greenhouse was approximately 12 weeks. Experiments using the media optimized for sugarcane (BA 4 μM) and NAA (10 μM) did not produce any shoots even after extended periods of culture.

6.2 Wheat

Young wheat plants *Triticum aestivum* cv. Hartog, supplied by Grainco, Toowoomba were used for all experiments. Transverse sections (2 mm thick) of leaf base, which sometimes included immature developing floral material depending on where the sections were taken along the stem, were cultured under the same conditions as described for sorghum.

Regeneration was direct from the explants and usually from around the outer leafwhorl (Figure 7). Shoot bud development occurred within 3-4 weeks of culture and was restricted to media supplemented with 5-10 μM CPA. Shoot regeneration was organogenic and there was no evidence of somatic embryogenesis in any media tested. After the appearance of small shoots, plants were left to develop on media containing 5 μM zeatin, a natural cytokinin. Shoots (20 mm tall) readily rooted on half-strength MS basal medium.

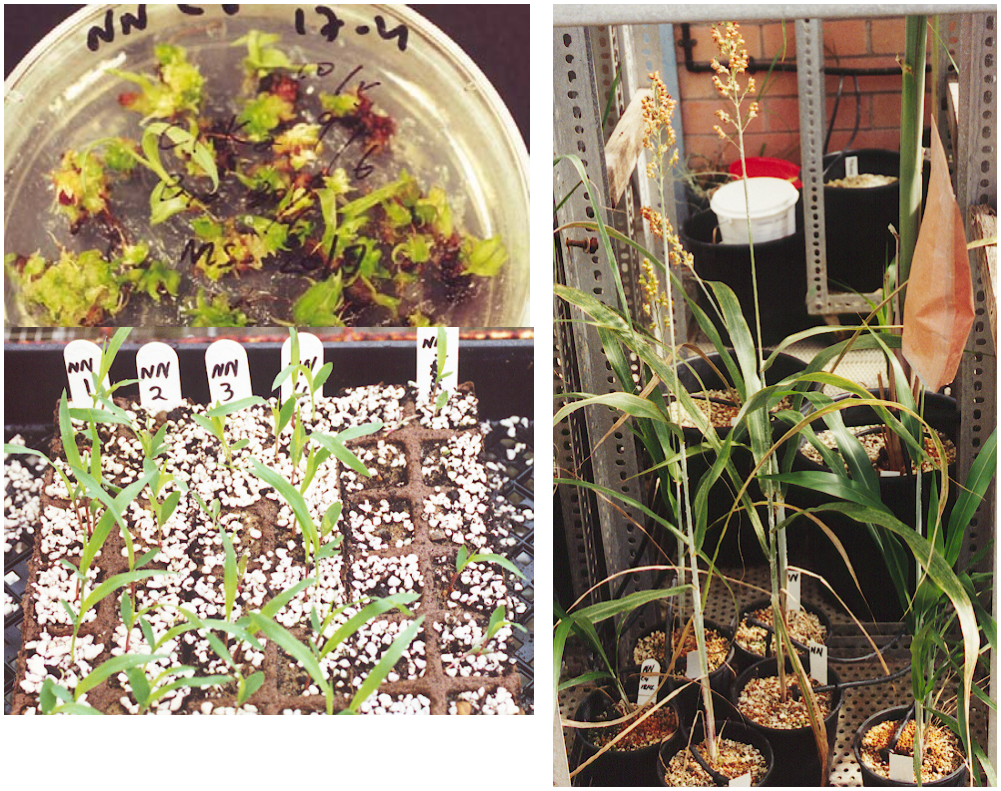


Figure 6. **(Clockwise from top left) *Sorghum bicolor* cv. New Nugget culture showing shoot regeneration from leaf base transverse sections (top left); regenerated plants with seeds (right); seedlings obtained from seeds produced by regenerated plants (bottom left).**

6.3 Banana

Using Cavendish banana cv. Williams we had partial success. Banana leaf segments and young developing inflorescence segments (all 1-2 mm thick) were used as test tissues. We were able to develop embryoids from inflorescence and leaf tissues, but they were extremely slow growing and did not develop further into complete plantlets. Clearly, inflorescence explants were more responsive than leaf tissues. Because of the resource limitation and increasing priority for the development of transformation systems, we could not explore more experimental strategies to develop banana SmartSett® system any further.

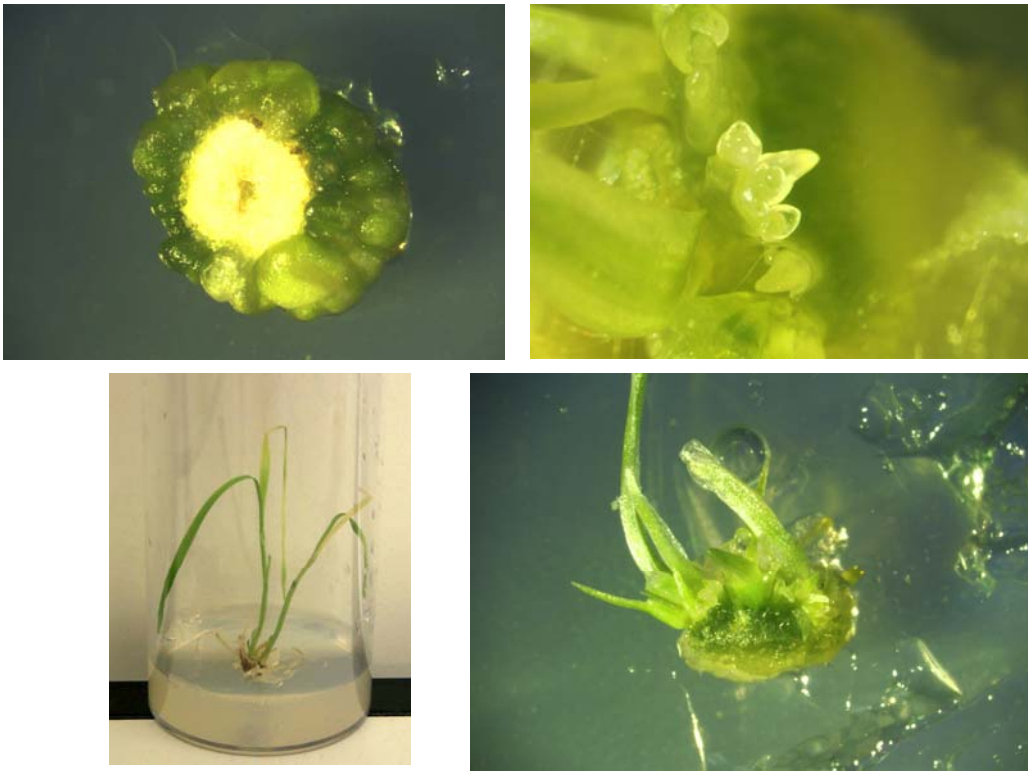


Figure 7 **(Clockwise from top left) *Triticum aestivum* cv. Hartog leaf base section cultures showing shoot primordial development (top left); further development of shoot primordial into shootlets (top and bottom right); well developed plantlets (bottom left)**

6.4 Conclusions

Successful demonstration of sorghum and wheat experiments showed that, as with sugarcane, plantlets could be regenerated directly from the leaf tissues without going through callus phase. This implies that the SmartSett® system is applicable to other monocot species and provides a base plant production method that is rapid and minimizes the incidence of somaclonal variation. Using this method transgenic sorghum and wheat plants may be produced by both biolistic and *Agrobacterium*-mediated methods.

7.0 TECHNOLOGY TRANSFER TO OTHER SUGARCANE GENETIC-ENGINEERING PROJECTS AND GROUPS

The development and potential applications of SmartSett® system has been presented to the Australian sugar industry through the BSES-initiated *Activate* industry forum, BSES Station Field Days and through the ASSCT conference. In addition, this work has been presented internationally at the 10th International Association of Plant Tissue Culture and Biotechnology Congress held at Orlando, Florida in June 2002, and will be presented in

the forthcoming ISSCT conference at Guatemala in 2005. A paper of the technology was awarded the President's Medal at the 2003 ASSCT Conference/

In 2003, BSES has signed a technology transfer agreement with South African Sugarcane Research Institute SASRI) and Dr Sandy Snyman, a scientist from SASRI, spent more than 1 month at BSES to obtain first hand knowledge/practical experience of the technology. Dr Snyman apparently has using SmartSett® technology to produce virus-free plant material from infected clones.

Recently, the Mauritius Sugar Industry Research Institute (MSIRI), Mauritius has contacted BSES to sign an agreement to transfer SmartSett® Technology for transformation and mass propagation programs. BSES and MSIRI have agreed on the terms and conditions and an agreement to this effect will be signed by both parties soon.

Besides the sugar industry researchers and institutions, people from the Australian horticultural industry also contacted BSES to exploit SmartSett® system for ornamentals. BSES has undertaken a very limited exploratory investigation on rapid multiplication of new *Agapanthus* cultivars, but the results so far are not encouraging.

In 2003, BSES researchers have initiated a BSES-University of Queensland (UQ) joint project to facilitate the use of this technology in other areas of plant research. As a consequence of this development, BSES and UQ started a Master's project to evaluate the utility of SmartSett® system in recovering plants infected with Fiji leaf gall virus and sugarcane mosaic virus.

Within BSES, this direct regeneration system has been used to engineer *Nicotiana alata* protease inhibitor gene into Q165(ϕ) to generate canegrub resistant canes. These plantlets, however, did not produce any detectable amounts of protease inhibitors.

With the advent of the CRC for Sugar Industry Innovation through Biotechnology, the bulk of the transformation projects are now encapsulated and centered at BSES. We believe that this will facilitate the transfer of technology associated with this project.

8.0 OUTPUTS

The beneficial outputs from this project are very clear. The major achievements of this project are the development of SmartSett®, a system for mass production of sugarcane, and novel strategies and practically useful methods to overcome limitations associated with the callus-based tissue culture and transformation systems. These technological advancements are protected by the two patents:

- Plant Regeneration PCT - WO01/82684;
- Monocotyledonous Plant Transformation PCT - WO0237951.
- Full electronic versions of these patents can be obtained by going to http://www.wipo.int/cgi-pct/guest/ifetch5?ENG+PCT-ALL.vdb+14+1094674-REVERSE+0+0+383603+BASICHTML-ENG+1+1+1+25+SEP-0/ (for PCT - WO01/826) and http://www.wipo.int/cgi-pct/guest/ifetch5?ENG+PCT-ALL.vdb+14+1095133-REVERSE+0+1+52682+BASICHTML-

ENG+1+1+1+25+SEP-0/HITNUM,B,,,,,,,+WO02%2f37951 (for PCT - WO0237951).

These patents and technologies have led to the following new research collaborations and projects:

- Sugarcane plastid transformation: BSES and University of Central Florida, Orlando, USA;
- Manipulation of gibberellin metabolism in plants: BSES and Long Ashton Research Station, Bristol, UK;
- Manipulation of sugarcane bud germination and plant architecture: BSES and University of Queensland;
- Further development of SmartSett® to improve efficiency, and to address the issues related to public perception and regulatory requirements: CRC-SIIB (involving BSES, QUT, UQ and Farmacule).

Production and delivery of seedlings of Q196$^{\phi}$, Q205$^{\phi}$ and other advanced clones (Q208$^{\phi}$ and 85N1205) for the central region to expedite planting of varieties to replace Q124.

In addition, the development of SmartSett® system led to signing of an agreement between BSES and South African Sugar Research Institute in 2003 for joint transgenic sugarcane research activities in the future.

Research outcomes of this project were published in industry journals and proceedings of relevant scientific meetings (see section 13).

The project also contributed to expanding and establishing a strong research base with specialized skills and expertise for transgenic sugarcane program in Brisbane.

9.0 INTELLECTUAL PROPERTY

The intellectual property developed during the course of this project has been protected through the patents applications in Europe, USA and Canada. Details are presented in section 3.

Besides the two patents, SmartSett® has been registered as Trade Mark, No 931708 in the Register of Trade Marks for a period of 10 years starting from 23 October 2002 for the goods and services coming from this technology/patents.

10.0 EXPECTED OUTCOMES

The successful outputs of this project are likely to have a significant impact upon the Sugar Industry. The expected outcomes are:

- Production of sufficient seed canes for early release of new cultivars: SmartSett® system being a rapid method for large-scale production, it could be integrated into breeding/selection program to expedite the release of new cultivars. It is expected

that with SmartSett® system, new cultivars could be released at least 2 years ahead of normal schedule. This has already demonstrated with the production of advanced clones such as Q208(ϕ and 85N1205.

- Provision of replacement cultivars for immediate, large-scale replanting: It is highly likely that there will not be sufficient planting material to distribute should a cultivar need to be replaced rather urgently due to disease outbreak or similar situations. This has happened recently in the Australian sugar industry with the orange rust outbreak in the Central Region.
- Recovery of pathogen-free plants form diseased plants: The unexpected discovery that the SmartSett® method developed in this project could be used to produce pathogen-free plants from those infected with YLS prompted us to evaluate the potential of this technique to eliminate Fiji leaf gall, ratoon stunting disease, leaf scald and sugarcane mosaic in diseased clones. The results obtained showed that SmartSett® method can eliminate Fiji leaf gall from 60%, mosaic from 33% and ratoon stunting disease from 100% of infected parent plants.
- The SmartSett® transformation system is expected to play an ongoing role in sugarcane transgenic research, especially in the CRC-SIIB programs. Clear demonstration of the minimal impact of the transformation process upon key agronomic parameters in sugarcane will clear the way to the production of novel canes with genes to confer disease and pest resistance as well as the production of novel value-added products.

11.0 FUTURE RESEARCH NEEDS

Practical, commercially useful transgenic technology and genetically modified (GM) products need to satisfy a growing number of regulatory requirements, such as integration of one or a few copies of transgene, minimal integration of non-gene vector DNA, non-antibiotic selection and elimination of virus DNA as part of transgene or integrating vector backbone. Clearly, the future research of SmartSett® system should address all these aspects of a practical sugarcane transformation system. In addition, it is also critical that the efficiency and the genotype-range of SmartSett® system should be improved.

From the propagation perspective, much further effort needs to be directed to develop the SmartSett® into a semi-automated or highly automated system.

12.0 RECOMMENDATIONS

- Integrate SmartSett® into the BSES/CSIRO new variety release program.
- Use this technology as a leverage to initiate research programs that are useful to the Australian sugar industry.
- Conduct more research to improve the efficiency and the genotype-range of SmartSett® transformation system.
- Enhance SmartSett® transformation system with respect to regulatory and public acceptance requirement.

13.0 PUBLICATIONS

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