Functional Plant Science and Biotechnology ©2011 Global Science Books

Leaf Roll Disc: An Explant Source for *in Vitro* Propagation and Genetic Transformation of Sugarcane

Karuna Mulleegadoo* • **Asha Dookun-Saumtally**

Mauritius Sugar Industry Research Institute, 1 Moka Road, Réduit, Mauritius

Corresponding author: * **nita.mulleegadoo@msiri.mu**

ABSTRACT

The use of leaf roll discs for *in vitro* propagation and genetic transformation of sugarcane was investigated. For *in vitro* propagation, three varieties namely, M 3035/66, S 17 and R 570 were used, and the number of shoots produced from leaf roll discs was compared to that of apical buds. No shoots were regenerated from apical buds of variety S 17 following a culture period of 24 weeks, as they were all either contaminated or oxidized. With M 3035/66 and R 570, only few shoots were obtained. On the other hand, using leaf roll discs, contamination and oxidation were minimal resulting in more than 70% of the cultured explants forming shoots. Prolific shoot regeneration occurred in all three varieties with a total of 400 and 280 shoot clumps produced in varieties M 3035/66 and S 17 respectively, and 250 clumps in R 570. For genetic transformation, leaf roll discs of varieties M 292/70 and M 3035/66, pre-cultured for 10 days on callus induction medium, were bombarded with plasmid pAHC25 containing the *gus* and *bar* genes using the Bio-Rad PDS-1000/He Particle Delivery System. After selection on bialaphos, 66 and 24 plants of M 3035/66 and M 292/70 were respectively regenerated and transferred to the glasshouse. Fifty-eight well-established plants of M 3035/66 and 17 of M 292/70 were sprayed with 0.1% herbicide Basta® four weeks later. Two plants of the latter variety were highly resistant to the herbicide whilst one displayed moderate tolerance. Upon analysis by PCR, all three plants were found to contain the *bar* gene. For M 3035/66, seven plants with a functional *bar* gene were obtained. Gene integration was confirmed by Southern blot analysis.

Keywords: micropropagation, apical buds, leaf roll discs, shoot regeneration, genetic transformation
Abbreviations: 2,4-D, 2,4-dichloro-phenoxyacetic acid; **MS**, Murashige and Skoog; **NAA**, α-naphthaleneacetic acid

INTRODUCTION

Sugarcane (*Saccharum* spp. hybrid) is a crop of major worldwide economic importance, with an estimated sugar production of 169.8 million tonnes in 2007-2008 (FAO 2011). It is also a prime candidate as a fuel crop because of its high biomass. In order to increase its rate of multiplication compared to conventional sett-based propagation method, tissue culture techniques have been investigated and conclusive results obtained. For instance, Hendre *et al*. (1983) reported that it was possible to produce some 260,000 shoots from a single shoot tip in four months out of which some 200,000 could be rooted within another two months. Using shoot tip culture also, Lee (1987) obtained more than 78,000 plantlets in three months whilst Lal and Singh (1994) found that the multiplication rate of shoots increased by 18.5-fold in a 17-20 day culture on modified MS medium containing kinetin and 6-BAP. In Brazil, a monthly average production of 50,000 plantlets has been reported (Uchoa *et al*. 1995) whereas in Mauritius, more than 165,000 plantlets were produced in 1999 (MSIRI 1999). Large scale production of sugarcane through micropogation has also been reported by Ali *et al*. (2008) using shoot apical meristems as explants. Similarly, a high throughput propagation system for production of disease-free planting stocks of sugarcane using apical meristem culture has been reported by Sharma *et al*. (2010).

Different explants can be used for *in vitro* propagation of sugarcane. In the early days, micropropagules derived from calli were used for establishing disease-free nurseries but nowadays the preferred method is by meristem or bud proliferation where the risk of somaclonal variation is negligible. Bud culture, however, has some disadvantages. For some varieties, intrinsic contamination in the bud cultures, especially axillary buds, can be a major problem (Taylor and Dukic 1993; Mulleegadoo and Dookun 1999) and sometimes not detectable until the second or third subculture, whilst for others, shoot proliferation from a single bud can be slow. Oxidation can also be a problem hindering shoot development (Mulleegadoo and Dookun 1999).

In Mauritius, these problems have been recurrent for bud cultures initiated from old ratoon crops and the production of tissue-culture plantlets has been very difficult for some varieties. For instance, a recent attempt to initiate apical bud cultures of three sugarcane varieties, M 3035/66, S 17 and R 570, that have been growing in the field for about 14 years, has failed as a result of high contamination. These varieties have been extensively cultivated but their yields have declined over the years (MSIRI 2004). Consequently, it was proposed to set up new trials from tissue-culture plantlets for yield comparison. In the present study, an alternative approach for the production of *in vitro* plantlets of sugarcane using leaf roll discs as explants was investigated and compared with apical bud culture. A similar approach, the SmartSett™ technology, has been used in Australia and has given good results. Shoot regeneration was prolific with a 20- to 125-fold increase, depending on the cultivar, over the number of plants produced from a stalk (Geijskes *et al*. 2003).

Genetic transformation has been identified as a useful tool for sugarcane improvement because breeding advances by conventional methods are slow due to the genetic complexity of the plant and its low fertility. Sugarcane has been transformed in several laboratories for a range of agronomic traits including resistance to herbicides (Gallo-Meagher and Irvine 1996; CTC 1997-1998; Snyman *et al*. 1998; MSIRI 1999), viruses (Joyce *et al*. 1998), bacteria (Zhang *et al*. 1999) and insects (Smith *et al*. 1999). More recently, novel traits such as bioplastic production (Brumbley *et al*. 2003) and increased sugar content (Wu and Birch 2007) have been

Received: 20 April, 2011. Accepted: 12 October, 2011.

Original Research Paper

introduced into sugarcane.

Embryogenic callus has been the preferred recipient material for DNA delivery into sugarcane using microprojectile bombardment. Production of callus is, however, a labour-intensive process that requires regular subcultures. Moreover, depending upon varieties, about three to four months are required before sufficient callus is obtained, thereby increasing the time for production of transgenic plants. In order to overcome these constraints in transformation programmes, alternative explant sources and regeneration pathways have been investigated. An approach using leaf roll discs as targets for the production of transgenic sugarcane (Snyman *et al*. 2001; Mulleegadoo and Dookun-Saumtally 2005) has given promising results. However, in the latter study, a high percentage of escapes was obtained. The aim of the present study was, therefore, to further investigate the leaf roll disc bombardment system and selective regeneration scheme in order to improve the transformation efficiency. The effect of modifying the regeneration pathway to include a selective callus phase prior to regeneration was determined. Two varieties, M 292/70 and M 30335/66, were used in this study.

MATERIALS AND METHODS

Micropropagation

1. Apical bud culture

Three commercial varieties namely, M 3035/66, S 17 and R 570 were used. Cane tops from 14 year-old ratoon crops were surface-sterilized in a six-fold dilution commercial bleach containing 3.25% active chlorine for 15 min and rinsed in three changes of sterile distilled water. Enclosing leaf sheaths were removed and young buds were dissected and cultured on initiation medium (Mulleegadoo and Dookun 1999) in a 16-h photoperiod. Regenerated shoots were micropropagated in liquid multiplication medium (Mulleegadoo and Dookun 1999) and the total number of shoots regenerated after 24 weeks were counted. Six cane tops were used for each variety and a total of 24 and 22 apical buds were initiated for varieties M 3035/66 and S 17 respectively. For variety R 570, 26 buds were cultured.

2. Leaf roll disc culture

For leaf roll disc culture, cane tops were sterilized as for apical bud culture. Outermost leaf sheaths were aseptically removed and segments of young leaf roll discs about 2 mm thick were cultured in continuous darkness at 26°C on callus induction medium -CI_3 (Gallo-Meagher and Irvine 1996). Six cane tops were used for each variety and 20 leaf roll discs per cane top were cultured.

Two weeks later, the leaf roll discs were transferred to regeneration medium in a 16-h photoperiod. That medium was similar to CI_3 medium except that it lacked 2,4-D and contained 2 mg l^{-1} kinetin and 5 mg l^{-1} NAA. After 4-6 weeks, the cultures were transferred to hormone-free solid MS (Murashige and Skoog 1962) medium. When the shoots reached two to three cm in height, they were transferred to liquid MS medium. Subculture onto fresh medium was effected every 2-3 weeks and each clump of shoots was split into individual plants or smaller clumps for further multiplication. As for apical bud culture, shoot multiplication was monitored over a 24-week period.

Genetic transformation

1. Target tissue

Immature leaf rolls of sugarcane varieties, M 292/70 and M 3035/66 obtained from field-grown plants were used. Cane tops were surface-sterilised as described earlier and leaf roll discs were cultured on CI_3 medium for 10 days. Prior to bombardment, leaf roll discs were arranged in a circle, 2.5 cm in diameter, in a Petri dish containing an osmotic medium - CI_3 medium supplemented with 0.2 M mannitol and 0.2 M sorbitol. They were osmotically treated for 4 h prior to and 4 h after bombardment. A total of 20 Petri dishes were bombarded for each variety.

2. Microprojectile bombardment

Plasmid pAHC25 (Christensen and Quail 1996) containing the *ß-glucuronidase* (*gus*) and *bar* genes both under the control of the maize ubiquitin promoter was precipitated onto gold microcarriers as described previously (Mulleegadoo and Dookun-Saumtally 2005). The *bar* gene encodes the enzyme phosphinothricin acetyl transferase which detoxifies phosphinothricin (PPT) and its derivatives which are the active ingredients of the herbicide Basta®.

The Bio-Rad PDS-1000/He Particle Delivery System was used to accelerate the DNA-coated microcarriers onto the leaf roll discs. The cultures were placed at a distance of 6 cm from the macrocarrier launch assembly and were bombarded at 1550 psi under 27 in. Hg vacuum. Controls consisted of non-bombarded tissues. Four hours after bombardment, the leaf rolls were transferred from the osmotic medium to CI_3 medium.

3. Histochemical GUS assay

Two days after bombardment, GUS activity was histochemically assayed in randomly selected leaf rolls following the protocol described by Jefferson *et al*. (1987) using X-GLUC as substrate.

4. Selective regeneration

Six days after bombardment, leaf roll discs of variety M 3036/66 were transferred onto selective callus medium containing 6 mg l^{-1} bialaphos, and they were subcultured onto fresh medium every two weeks. Six weeks later, they were transferred onto regeneration medium containing 5 mg l^{-1} NAA, 2 mg l^{-1} Kinetin (Irvine and Benda 1985) and 2 mg l^{-1} bialaphos, in a 16-h photoperiod. For variety M 292/70, which was observed to be more susceptible to bialaphos in preliminary experiments, the concentration of the selective agent used in the callus and regeneration media was 3 mg l^{-1} and 1 mg l^{-1} respectively. Shoots were rooted on medium containing 4 mg l^{-1} NAA and 2 mg l^{-1} bialaphos. Plantlets with a well-established root system were transferred to the glasshouse.

5. Herbicide test

Four weeks after transfer to the glasshouse, all established plants were sprayed with a 0.1% solution (v/v) of the herbicide Basta®, a commercial formulation of glufosinate ammonium (140 g l^{-1}). The plants were scored for herbicide tolerance 10 days later.

6. DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was extracted from leaf tissue of resistant plants using a CTAB (hexadecyltrimethylammonium bromide) extraction procedure based on Doyle and Doyle (1987). The presence of the *bar* gene in putative transformants was determined by PCR using primers 5′-TCAGATCTCGGTGACGGGCA-3′ and 5′-CCATGA GCCCAGAACGACGCCCG-3′ which amplify an internal 560-bp fragment of the gene. Thermocycling conditions were as follows: 94°C for 5 min, 55°C for 1 min and 72°C for 2 min followed by 30 cycles at 94°C for 45 s, 55°C for 45 s and 72°C for 2 min. The final extension step was for 7 min at 72°C. PCR products were analysed on 1% agarose gel.

7. Southern blot analysis

Southern blot analysis was carried out using a digoxigenin-labelled probe. The probe was prepared using a Dig PCR Probe Synthesis Kit (Roche Products) using primers to amplify fragments internal to the *bar* coding region according to the manufacturer's instructions. Fifteen µg of DNA were restricted with *Eco*RI, electrophoresed overnight in a 0.8% agarose gel and transferred to a nylon membrane using standard procedures (Sambrook *et al*., 1989). Membranes were hybridized overnight, washed twice in 2X SSC, 0.1% SDS and twice in 0.5X SSC, 0.1% SDS. CDP-*Star* (Roche Products) was used as the chemiluminescent substrate and the light signals were detected on an X-Ray film.

RESULTS

Micropropagation

1. Apical bud culture

Using apical buds, shoot development did not occur or was very poor in all three varieties. For variety M 3035/66, out of the 24 buds cultured, only four developed into shoots after eight weeks, the rest being either contaminated or oxidized (**Table 1**). Shoot multiplication was also poor and after 24 weeks culture only 12 shoots were formed. In R 570, shoot development was even poorer. Out of 26 buds cultured, only two developed into shoots and after multiplication, a total of six shoots were obtained. With variety S 17, it was not possible to regenerate shoots as all 22 buds initiated were either contaminated with saprophytic organisms or oxidized (**Table 1**).

2. Leaf roll disc culture

On CI_3 medium, the leaf roll discs enlarged and a small amount of callus formed on the cut surface of some leaf roll discs. Upon transfer to regeneration medium, some callus formed in nearly all the leaf roll discs and in a few of them, small globular embryos were observed. One to two weeks after transfer to regeneration medium, small shoot primordia emerged from the callus in the three varieties. Shoots were transferred to hormone-free MS medium and subcultured onto fresh medium every two to three weeks.

Contamination and/or oxidation was minimal. Out of 120 leaf roll discs cultured per variety, fewer than 35 were contaminated or were oxidized. This is a significant improvement as in apical bud culture a very high rate of contamination/oxidation was obtained when explants were taken from similarly grown mother plants.

In variety M 3035/66, shoot clumps were formed five weeks after culture initiation (**Fig. 1**) and two weeks later, well-developed clumps were transferred to liquid MS medium for micropropagation. Tillering was highly prolific in this variety and 15 weeks after culture initiation, more than 275 shoot clumps were regenerated. Nine weeks later, 400 shoot clumps were obtained (**Table 1**). In varieties S 17 and R 570, although growth was slower and tillering not as vigorous as in M 3035/66, prolific shoot regeneration was also observed. Shoot clumps formed seven weeks after culture initiation in S 17 and five weeks later, 50 shoot clumps were transferred to liquid MS medium. Twenty-four weeks after culture initiation, 280 shoot clumps were obtained. In variety R 570, more than 100 shoot clumps were regenerated within 14 weeks of culture and 10 weeks later, 250 clumps were produced (**Table 1**). With an average production of five single shoots per shoot clump, between 1250 and 2000 single shoots were regenerated from leaf roll discs depending on the variety.

Genetic transformation

1. Transient GUS assay

Two days after bombardment, histochemical *gus* assays were performed on leaf rolls and several intense blue spots were observed, indicating efficient delivery of the plasmid to the target tissues (**Fig. 2**).

2. Regeneration

Six days after bombardment, the bombarded tissues were transferred to selective callus medium containing 3 or 6 mg/L bialaphos. They were cultured for a period of six weeks before transfer under light to regeneration medium containing 1 or 2 mg/L bialaphos. On CI_3 medium, callus formed on the cut surfaces of the leaf roll discs and four to six weeks after transfer to regeneration medium, green shoot primordia could be seen emerging from the callus. Most of the leaf roll discs were, however, no longer viable after exposure to the selective agent and this was probably due to the fact that light accelerates the phytotoxic effect of the herbicide (Casas *et al*. 1993). Twenty to twenty-six weeks after bombardment, 24 plantlets of M 292/70 and 66 of M 3035/66 with a well-established root system were transferred to the glasshouse.

3. Herbicide test and molecular analysis

Four weeks after transfer to the glasshouse, 17 well-established plants of M 292/70 together with non-transformed controls were sprayed with a 0.1% solution of the herbicide Basta® and they were assessed for tolerance 10 days later. Two plants displayed high resistance to the herbicide with all leaves remaining green, one moderate resistance whereby some necrosed spots were observed while the others were as severely necrosed as the non-transformed controls. For M 3035/66, out of 58 well-established plants that were treated with the herbicide, seven were highly resistant whilst the others displayed severe necrosis.

PCR analysis was performed on the 10 resistant plants to detect the presence of the *bar* gene, and a band corresponding to the expected 560 bp amplifiable fragment of the gene was obtained in all plants (**Fig. 3**). Gene integration was confirmed by Southern blot analysis by the presence of a 1481 bp band, after digestion of genomic DNA with *Eco*RI and hybridization with a PCR Dig-labelled *bar* probe (results not shown).

DISCUSSION

The results in this study demonstrate that leaf roll discs can be successfully used for the production of *in vitro* plantlets from sugarcane varieties that have been in the field for many years. In comparison to apical bud culture whereby contamination and oxidation were major problems hindering shoot regeneration, in leaf disc culture more than 70% of the initiated explants formed shoots. Thus in variety S 17, no shoots were regenerated for apical bud culture as opposed to more than 275 shoot clumps obtained for leaf disc culture from the same number of cane tops. Similarly for varieties M 3036/66 and R 570, very few shoots were regenerated from apical bud culture compared to prolific shoot regeneration for leaf disc culture. In a previous study, Mulleegadoo and Dookun (1999) also reported that contamination was a serious problem in axillary bud cultures of sugarcane in the three varieties under study. For apical bud culture, although contamination was less severe, oxidation was a problem hindering shoot development. The higher level of contamination/ oxidation observed in this study

Table 1 Shoot regeneration from apical buds and leaf roll discs of three sugarcane varieties.

Variety	Explant source					
	Apical buds*			Leaf roll discs*		
	No initiated	% contamination / oxidation	Total number of shoots formed after 24 weeks	No initiated	% contamination / oxidation	Total number of shoot clumps formed after 24 weeks
M 3035/66	24	83	12	120	21	400
S 17	22	100	0	120	28	280
R 570	26	92	6	120	25	250

*Both apical buds and leaf roll discs were obtained from six cane tops

Fig. 1 Different stages in shoot regeneration from leaf roll discs of variety M 3035/66. (**A**) Dissection of leaf roll discs from sugarcane top; (**B**) Culture on callus induction medium; (**C**) Formation of shoot primordia after transfer to regeneration medium; (**D, E**) Shoot regeneration.

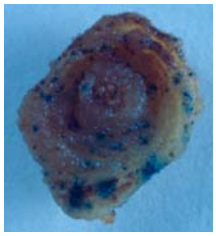

Fig. 2 Transient *gus* expression in leaf roll discs.

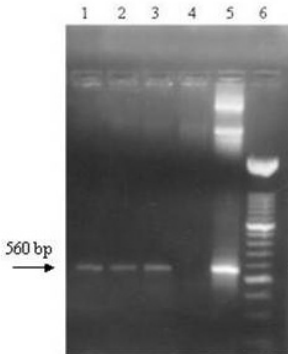

Fig. 3 PCR analysis for the detection of the *bar* gene in sugarcane plants. Lanes 1, 2, 3: transformed plants; lane 4: untransformed plant; lane 5: positive plasmid control; lane 6: molecular weight marker (100 bp ladder).

with apical buds was most probably due to the fact that buds were taken from old ratoon field-grown crops.

The low level of contamination coupled with the prolific shoot regeneration obtained with leaf roll disc culture imply that far less resources as well as starting material would be required in the culture initiation process using these explants. Thus, leaf roll disc culture of sugarcane could replace apical bud culture when contamination and oxidation are major setbacks in the culture initiation process.

Our results have further demonstrated that leaf roll discs can be used as targets for the production of transgenic sugarcane. Previous studies (Snyman *et al*. 2001; Mulleegadoo and Dookun-Saumtally 2005) also reported on the use of primary explants of sugarcane as targets for microprojectile bombardment. Bombardment of primary explants has also been used in other monocotyledonous plants such as orchardgrass (Denchev *et al*. 1997), maize (Brettschneider *et al*. 1997), wheat (Barro *et al*. 1998), rice (Vain *et al*. 1998) and barley (Cho *et al*. 1999) whereby leaf tissue, scutellar tissue, immature embryos and endosperm have been used as targets.

The time lapse from bombardment to generation of transgenic sugarcane plants was between 20 and 26 weeks, as opposed to a period of seven to eight months reported in a recent study using embryogenic callus as targets (Mulleegadoo and Dookun-Saumtally 2009). Similarly, in their studies with leaf roll discs, Snyman *et al*. (2001) obtained transgenic plants within a period of 13 to 22 weeks compared to some 36 weeks using embryogenic callus as targets. Ingelbrecht *et al*. (1999) reported a lapse of about 24 weeks from bombardment to production of transgenic plants. The age of the callus was between 7 and 40 weeks. Our previous studies (results unpublished) have shown that callus, suitable for bombardment, was obtained 10 to 14 weeks after initiation, depending upon varieties. Regular subcultures, every three to four weeks, onto fresh medium were also required. Similarly, Taylor *et al*. (1992) found that sugarcane embryogenic callus was established after 70 to 84 days in culture. The above results with direct bombardment of leaf roll discs therefore indicate a substantial time saving for the production of transgenic plants.

Three transgenic herbicide-resistant plants of variety M 292/70 were produced using a selective callus phase with 3 mg l^{-1} bialaphos and regeneration on 1 mg l^{-1} bialaphos. The 14 herbicide-susceptible plants probably contained a silenced *bar* gene or were escapes produced from an inadequate selection regime. With variety M 3035/66, 7 out of 58 plants contained a functional *bar* gene. The transformation efficiencies expressed in terms of herbicide-resistant plants per bombardment was 0.15 and 0.35 for varieties M 292/70 and M 30305/66, respectively. In a previous study, two transgenic herbicide-resistant plants of variety M 292/70, out of a total of 17, were obtained using bombardment of leaf rolls and direct regeneration on 1 mg l^{-1} bialaphos (Mulleegadoo and Dookun-Saumtally 2005). These results indicate that a modification of the selection scheme to include a selective callus phase on high-bialaphos containing medium, prior to regeneration, was still inadequate as it did not reduce the escape rate. The high escape rate could actually be due to the selective agent used in the study as similar results have been reported by other authors. For instance, Gallo-Meagher and Irvine (1996) reported an escape rate of forty-one percent and transformation efficiency of 1.5 transgenic plants per bombardment with variety NCo 310 after selection for eight months on bialaphos-containing media. Bower *et al*. (1996) found that selection on phosphinotricin was problematic in cultivar Q 117 due to extreme variation in the concentration required to kill callus. On the other hand, selection on geneticin was reliable, and an escape rate of less than 5% and high transformation efficiency of 20 transgenic plants per bombardment was obtained. A high transformation efficiency, using geneticin, has also been reported by Falco *et al*. (2000) using bombardment of embryogenic callus. On the other hand, in our work, using conventional callus bombardment method and selection on bialaphos, an escape rate of 91% was obtained (Mulleegadoo and Dookun-Saumtally 2009).

In conclusion, our results have shown that micropropagation of sugarcane can be substantially enhanced using leaf roll discs, and would be an advantage in varieties where contamination and oxidation of apical buds hinder the culture initiation process. Leaf roll discs have also been found to be potential targets for genetic transformation. However, in order to effectively compare the transformation efficiencies of leaf roll discs as opposed to embryogenic callus bombardment systems, in terms of number of transgenic plants recovered per bombardment, time taken and input of resources for callus production, both systems need to be further evaluated concurrently using geneticin as the

selective agent.

ACKNOWLEDGEMENTS

We would like to thank Drs P. Quail and A. Christensen for providing the plasmid pAHC25.

REFERENCES

Ali A, Naz S, Siddiqui FA, Iqbal J (2008) An efficient protocol for large scale production of sugarcane through micropropagation. *Pakistan Journal of Botany* **40 (1)**, 139-149

Barro F, Cannell ME, Lazzeri PA, Barceló P (1998) The influence of auxins on transformation of wheat and tritordeum and analysis of transgene integration patterns in transformants. *Theoretical and Applied Genetics* **97**, 684-695

Bower R, Elliot AR, Potier BA, Birch RG (1996) High efficiency, microprojectile-mediated cotransformation of sugarcane using visible or selectable markers. *Molecular Breeding* **2**, 239-249

Brettschneider R, Becker D, Lorz H (1997) Efficient transformation of scutellar tissue of immature maize embryos. *Theoretical and Applied Genetics* **94**, 737-748

Brumbley SM, Petrasovits LA, Bonaventura PA, O'Shea MJ, Purnell MP, Nielsen LK (2003) Production of polyhydroxyalkanoates in sugarcane. In: *International Society of Sugar Cane Technologists IV Molecular Biology Workshop*, Montpellier, France, p 31 (Abstract)

Casas AM, Kononowicz AK, Zehr UB, Tomes DT, Axtell JD, Butler LG, Bressan RA, Hasegawa PM (1993) Transgenic sorghum plants via microprojectile bombardment. *Proceedings of the National Academy of Sciences USA* **90**, 11212-11216

Christensen AH, Quail PH (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Research* **5**, 213-218

Cho M-J, Choi HW, Buchanan BB, Lemaux PG (1999) Inheritance of tissue-specific expression of barley hordein promoter-*uidA* fusions in transgenic barley plants. *Theoretical and Applied Genetics* **98**, 1253-1262

CTC (Centro de Tecnologia Copersucar) (1997/1998) *Annual Report*, p 31

Denchev PD, Songstad DD, McDaniel JK, Conger BV (1997) Transgenic orchardgrass (*Dactylis glomerata*) plants by direct embryogenesis from microprojectile bombarded leaf cells. *Plant Cell Reports* **16**, 813-819

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**, 11-15

Falco MC, Tulmann Neto A, Ulian EC (2000) Transformation and expression of a gene for herbicide resistance in a Brazilian sugarcane. *Plant Cell Reports* **19**, 1188-1194

FAO (2011) Available online: http://www.fao.org/docrep/011/ai474e/ai474e08.htm

Gallo-Meagher M, Irvine JE (1996) Herbicide resistant transgenic sugarcane plants containing the *bar* gene. *Crop Science* **36**, 1367-1374

Geijskes RJ, Wang L, Lakshmanan P, Mckeon MG, Berding N, Swain RS, Elliot AR, Grof CPL, Jackson JA, Smith GR (2003) Smartsett™ seedlings: Tissue cultured seed plants for the Australian sugar industry. *Sugar Cane International May/June*, 13-17

Hendre RR, Iyer RS, Kotwal M, Khuspe SS, Mascarenhas AF (1983) Rapid multiplication of sugarcane by tissue culture. *Sugarcane* **1**, 5-8

Ingelbrecht IL, Irvine JE, Mirkov TE (1999) Posttranscriptional gene silencing in transgenic sugarcane. Dissection of homology-dependent virus resistance in a monocot that has a complex polyploid genome. *Plant Physiology* **119**, 1187-1197

Irvine JE, Benda GTA (1985) Sugarcane mosaic virus in plantlets regenerated from diseased leaf tissue. *Plant Cell, Tissue and Organ Culture* **5**, 101-106

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO Jounal* **6**, 3901-3907

Joyce PA, McQualter RB, Handley JA, Dale JL, Harding RM, Smith GR (1998) Transgenic sugarcane resistant to sugarcane mosaic virus. *Proceedings of the Australian Society of Sugar Cane Technologists* **20**, 204-210

Lal N, Singh HN (1994) Rapid clonal multiplication of sugarcane by tissue culture. *Plant Tissue Culture* **4 (1)**, 1-7

Lee TSG (1987) Micropropagation of sugarcane (*Saccharum* spp.). *Plant Cell, Tissue and Organ Culture* **10**, 47-55

MSIRI (1999) Mauritius Sugar Industry Research Institute. *Annual Report*, p 55

MSIRI (2004) Mauritius Sugar Industry Research Institute. *Annual Report*, p 54

Mulleegadoo KD, Dookun A (1999) Effect of explant source and genotype on growth of sugarcane *in vitro*. *Revue Agricole et Sucrière de Ile Maurice* **78 (2)**, 35-39

Mulleegadoo KD, Dookun-Saumtally A (2005) Genetic transformation of sugar cane by microprojectile bombardment of young leaf rolls. In: Hogarth DM (Ed) *Proceedings of the XXV International Society of Sugar Cane Technologists Congress* **2**, 579-584

Mulleegadoo K, Dookun-Saumtally A (2009) Genetic transformation of two Mauritian sugarcane varieties for resistance to the herbicide Basta®. *Sugar Cane International* **27 (2)**, 65-69

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual* (2nd Edn), Cold Spring Harbor Laboratory Press, pp 9.31-9.58

Sharma V, Acuna GM, Damaj M, El-Hout N (2010) Title. Available online: http://a-c-s.confex.com/crops/2010am/webprogram/Paper59974.html

Smith GR, Joyce PA, Nutt KA, McQualter RB, Taylor GO, Allsopp PG (1999) Evaluation of transgenic sugarcane plants engineered for resistance to mosaic and canegrubs. In: Singh V, Kumar V (Eds) *Proceedings of the XX111 International Society of Sugar Cane Technologists Congress* **2**, 291-297

Snyman SJ, Leibbrandt, NB, Botha FC (1998) Buster resistant sugarcane. *Proceedings of the South African Sugar Technologists' Association* **72**, 138-139

Snyman SJ, Huckett BI, Watt MP, Botha FC (2001) A comparison of direct and indirect somatic embryogenesis for the production of transgenic sugarcane (*Saccharum* spp. hybrids). *Acta Horticulturae* **560**, 105-108

Taylor PWJ, Ko H-L, Adkins SW, Rathus C, Birch RG (1992) Establishment of embryogenic callus and high protoplast yielding suspension cultures of sugarcane (*Saccharum* spp. hybrids). *Plant Cell, Tissue and Organ Culture* **28**, 69-78

Taylor PWJ, Dukic S (1993) Development of an *in vitro* culture technique for conservation of *Saccharum* spp. hybrid germplasm. *Plant Cell, Tissue and Organ Culture* **34**, 217-222

Uchoa PEA, Gerald LTS, Nogueira Junior P, Neto JAP (1995) Sugarcane biofactory: achievements and problems. In: Cock JH, Brekelbaum T (Eds) *Proceedings of the XX11 International Society of Sugar Cane Technologists Congress* **2**, 441-444

Vain P, Worland B, Kohli A, Snape JW, Christou P (1998) The green fluorescent protein (GFP) as a vital screenable marker in rice transformation. *Theoretical and Applied Genetics* **96**, 164-169

Wu L, Birch RG (2007) Doubled sugar content in sugarcane plants modified to produce a sucrose isomer. *Plant Biotechnology Journal* **5**, 109-117

Zhang L, Xu J, Birch RG (1999) Engineered detoxification confers resistance against a pathogenic bacterium. *Nature Biotechnology* **17**, 1021-1024