Establishment of *in vitro* shoot multiplication and short-term embryo storage protocols for single genetic modification events in sugarcane

By

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ABSTRACT

Sugarcane (*Saccharum* spp. hybrids) is an important economic crop that accounts for more than 80% of world sugar production. Genetic improvement and selection to produce cultivars with traits of interest is difficult in sugarcane because of its complex polyploid and aneuploid genome. Genetic transformation and *in vitro* mutagenesis techniques are, therefore, being investigated for such purposes. However, it takes a long period to multiply plants regenerated from such events as: 1) the *in vitro* plants have to establish well *in vitro*; 2) then they need to acclimatize in the greenhouse and 3) then multiplied using setts, before they can be tested for traits of interest. Furthermore, once a plant is lost in culture, the whole genetic event is lost, which can occur due to lack of labour resources at the time of subculture. The aim of this study was, therefore, two-fold: 1) to develop a protocol to multiply single shoots regenerated from single transformation or mutation events *in vitro*; and 2) to establish a strategy for short term storage of somatic embryos developed from such events.

Preliminary investigations were undertaken using sugarcane varieties NCo310 and NCo376. Two approaches were employed for shoot multiplication, *viz*. either multiply the shoots when they were well developed or multiply them immediately after embryo germination. In the former, shoots (2 cm in height), produced via indirect somatic embryogenesis, were cultured on six different media, each containing full strength MS salts and vitamins, 20 g l^{-1} sucrose and different combinations and concentrations of plant growth regulators (PGRs), *viz*. M1 (no PGRs), M2 (0.1 mg l^{-1} BAP and 0.015 mg l^{-1} kinetin), M3 (6 mg l^{-1} BAP and 1 mg l^{-1} kinetin), M4 (0.5 mg l^{-1} BAP and 0.25 mg l^{-1} kinetin), M5 (1 mg l^{-1} BAP, 0.1 mg l^{-1} kinetin and 1 mg l^{-1} NAA) and M6 (1 mg l^{-1} IBA, 1 mg l^{-1} kinetin and 0.5 mg l^{-1} GA3). The shoots were subcultured two or three times onto same medium. At 6 w, the total shoot yield (no. shoot/original shoot explant) was significantly highest on medium M5 for both NCo376 (12.7 ± 4.0) and NCo310 (7.2 ± 3.4). Similarly, this medium resulted in the highest total number of shoots per embryo at 6 w (11.0 ± 2.0) when applied to germinating embryos of NCo376. Modifications of this medium were investigated but none was found to be better than M5 ($P < 0.05$). After multiplication, the shoots were transferred to rooting liquid medium containing ½ strength MS salts and vitamins, 20 g l^{-1}sucrose and 1 mg l^{-1} IBA. More than 80% of the shoots from each medium rooted within 3 w.

The developed protocol was then applied to varieties N41, N50 and transgenic lines of NCo376. The results confirmed that M5 can be used for high yielding shoot multiplication for those varieties producing 6.6 ± 0.9 and 4.3 ± 1.3 shoots per shoot at 6 w for N41 and N50, respectively. Subculturing for a further 2 w increased the shoot yields to 18.6 ± 2.3 for N41 and 8.0 ± 0.3 for N50. Transgenic shoots multiplied using the developed protocol, were used to investigate the stability of the transgene in *in vitro* culture. This was done by testing for the presence of the gene in those shoots using end point PCR. The results showed the presence of the transgene in all the transgenic shoots indicating the protocol did not have a negative effect on the stability of the transgene.

To establish a protocol for slow growth storage of somatic embryos, mature embryos of variety NCo376 were encapsulated in alginate beads and placed on semi-solid medium containing ½ strength MS salts and vitamins, 5 g l^{-1} sucrose and 9 g l^{-1} agar. The cultures were kept in the dark at room temperature for a month and at 18 ^{o}C for 1, 2 and 3 months. The embryos were assessed for germination capacity at the end of each period by transferring them to medium containing full strength MS salts and vitamins, 20 g l^{-1} sucrose, 0.5 g l^{-1} casein hydrolysate and 8 g l^{-1} agar. Embryos that were stored at 18 ^{o}C for 1 month had the highest survival percentage (66 ± 5.7% germination) compared with the other treatments and control (53.3 ± 6.7% germination).

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DECLARATION 1 - PLAGIARISM

I, **Edwin Mupanehari**, declare that

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PREFACE

The experimental work described in this thesis was conducted at the University of Kwazulu-Natal, School of Life Sciences and at the South African Sugar Research Institute, Biotechnology Department, under the supervision of Professor M. P. Watt and Dr S. J. Snyman.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged.

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LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
AFLP	amplified fragment length polymorphism
ANOVA	analysis of variants
BAP	6-benzylaminopurine
$CaCl_2$	calcium chloride
$CaCl_2.2H_2O$	calcium chloride dihydrate
CIM	callus induction medium
CP	coat protein
d	day
DNA	deoxyribonucleic acid
EGM	embryo germination medium
EMS	ethylmethanesulphonate
FAO	Food and Agriculture Organization
FDV	Fiji disease virus
FP	forward primer
$g\ l^{-1}$	grams per litre
$g\ v^{-1}$	grams per volume
GA3	gibberellic acid
GM	genetic modification
h	hour
IAEA	International Atomic Energy Agency

IBA	indole-3-butyric acid
Kinetin	N-6furfuryladenanine
LSD	least significance difference
mg l^{-1}	milligrams per litre
MgCa	magnesium calcium
$MgCl_2$	magnesium chloride
$MgCl_2.6H_2O$	magnesium chloride hexahydrate
min	minute
ml	millilitre
mm	millimetre
MS	Murashige and Skoog, 1962
NAA	1-napththylacetic acid
ng	nanograms
NI	neutral invertase
PCR	polymerase chain reaction
PFP	pyrophosphate: fructose 6-phosphate 1-phosphotransferase
PGRs	plant growth regulators
RAPD	random amplified polymorphic DNA
RP	reverse primer
rpm	revolutions per minute
SASA	South African Sugar Association
SASRI	South African Sugarcane Research Institute

SCMV	sugarcane mosaic virus
SCYLV	Sugarcane yellow leaf virus
SrMv	sorghum mosaic virus
T-DNA	transfer-DNA
TDZ	thiadiazuron
$v\ v^{-1}$	volume per volume
w	week
%	percent
^{o}C	degrees celcius
µl	microlitre
µM	micromolar
$\mu mol\ m^{-2}\ s^{-1}$	micromole per meter per second

1. INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is an important cash and industrial crop and is widely cultivated in the tropical and subtropical countries of the world (Grivet and Arruda, 2001; Lakshmanan, 2006; Sengar et al., 2011; Snyman et al., 2011b; Mnisi and Dhlamini, 2012). According to Menossi et al. (2008), most of the commercial sugarcane plants are able to accumulate sucrose levels up to 0.7 M in mature internodes and the crop is the most important sources of sweetening in the world (80% of world sugar) (IllovoSugar, 2013). In addition, it is also used as a raw material for the production of ethanol, bagasse and molasses (Singh and Solomon, 1995; Menossi et al., 2008; Khamrit et al., 2012; Mnisi and Dhlamini, 2012; Raza et al., 2012). The latter two are largely used for energy co-generation at the mill and for the production of animal feed, which further increase the value of the crop (Singh and Solomon, 1995; Menossi et al., 2008; Malabadi et al., 2011). In South Africa, sugarcane is the third most important agricultural crop with 2.5 million tons of sugar being produced per annum, resulting in an annual average income of ZAR8 billion (SASA, 2013). According to South African Sugar Association (SASA), 60% of the sugar is marketed in the Southern African Customs Union and the rest is exported to markets in Africa, Asia and the Middle East.

Sugarcane belongs to the genus *Saccharum* L. and modern hybrids of sugarcane are a result of interspecific hybridization of one or more species of *S. officinarum* and *S. spontaneum* (Daniels and Roach, 1987; Grivet and Arruda, 2001; Menossi et al., 2008; Sengar et al., 2011; Snyman et al., 2011b). Consequently, commercial sugarcane varieties (the hybrids of such crosses) are highly polyploid and aneuploid (Roach, 1989; Grivet and Arruda, 2001; Lakshmanan et al., 2005; Snyman et al., 2008; Snyman et al., 2011b). As a result of, and in addition to their complex genome, the hybrids are associated with problems such as poor fertility, lack of genetic diversity, long breeding cycle and difficulty in targeting specific traits in the progeny (Roach, 1989; Grivet and Arruda, 2001; Lakshmanan et al., 2005; Snyman et al., 2008; Sengar et al., 2011; Snyman et al., 2011b). Due to this, breeding and selection for new traits of interest in sugarcane are difficult and slow. For example, back-crossing for the introduction of specific traits is difficult because of the complex genome and consequent random genetic rearrangement occurring at meiosis (Falco et al., 2000).

South Africa is the southernmost part of the world were sugarcane is grown (Snyman et al., 2008). As a result, the South African sugarcane industry is faced with numerous challenges, which include low annual rainfall levels and unique pests and diseases (Snyman et al., 2008). Consequently, commercial varieties must be produced to withstand these challenges (Snyman et al., 2011b). However, the production of such varieties takes long owing to the complex genome of sugarcane and the fact that it is traditionally propagated vegetatively through stem cuttings, which is labour intensive and slow (Blackburn, 1984; Lakshmanan et al., 2005; Lakshmanan, 2006; Sengar et al., 2011; Snyman et al., 2011b). Hence, the need to explore new approaches other than asexual reproduction such as *in vitro* propagation for precision breeding to improve varieties for specific traits, as well as for quick multiplication of such varieties.

Different transformation techniques such as particle bombardment (Bower and Birch, 1992; Bower et al., 1996; Gallo-Meagher and Irvine, 1996; Leibbrandt and Snyman, 2003), A*grobacterium*-mediated gene transfer (Enriquez-Obregon et al., 1998; Enriquez-Obregon et al., 2000), polyethylene glycol and electroporation (Arencibia et al., 1999) have, therefore, been developed for the genetic modification (GM) of sugarcane. These have resulted in the production of sugarcane cultivars with herbicide tolerance (Gallo-Meagher and Irvine, 1996; Enriquez-Obregon et al., 1998), disease resistance (Joyce and McQualter, 1998; Ingelbrecht et al., 1999; Sooknandan et al., 2003) and improved sucrose content (Ma et al., 2000; Botha et al., 2001). Chemical and physical mutagens have also been used to produce *in vitro* sugarcane plants with disease resistance (Khairwal et al., 1984; Ali et al., 2007b), drought tolerance (Wagih et al., 2004) and salt tolerance (Patade and Suprasanna, 2008; Patade and Suprasanna, 2009).

While the GM and mutagenesis techniques mentioned above have the potential to improve the efficiency of sugarcane crop improvement compared with conventional methods (Bower and Birch, 1992; Bower et al., 1996; Gallo-Meagher and Irvine, 1996; Taparia et al., 2012), each event is unique and results in plants which are genetically different from those produced from other events. Conventional vegetative multiplication of such few plants produced from the GM and induced mutagenesis events has low multiplication rates and results in only approximately 8-10 new plants per annum per bud (Blackburn, 1984; Snyman et al., 2011b). This means that

several cycles are required to multiply one event to obtain enough material for further studies in the laboratory and in the field. Hence, there is need to speed up this process.

Preservation of germplasm also plays an important role in breeding programs of many plant species, including sugarcane, as it enables storage of important material from the program (Snyman et al., 2011b). Conventional methods of storage such as conservation stands and greenhouse collections are currently being used for this purpose (Snyman et al., 2011b). However, these require land and facilities which are labour intensive and expensive to maintain. Furthermore, under these conditions, there is a high risk of germplasm loss through natural disasters, pests and diseases (Taylor and Dukic, 1993; Snyman et al., 2011b). Consequently, *in vitro* strategies such as cryopreservation (González-Arnao et al., 1999; Martinez-Montero et al., 2008) and slow growth storage (Paul and Duvik, 1993; Taylor and Dukic, 1993; Sarwar and Siddiqui, 2004; Watt et al., 2009) are now being explored for many plants including sugarcane, to alleviate some of the challenges resulting from the use of conventional methods (Snyman et al., 2011b). Furthermore, for material obtained from GM and induced mutagenesis events, it is important not to lose the initial modified material (cells, somatic embryos or plants) developed from such events. This is because, once the embryo or plant is lost, the whole event is lost.

A method for slow growth storage of sugarcane somatic embryos, which uses naked (non-encapsulated) embryos, has been published (Watt et al., 2009). However, according to Singh and Chand (2010), in addition to protection of explants from desiccation and mechanical injury, encapsulation is an alternative method which can be used because it allows for ease of handling. For this reason, this method was investigated in this study.

Consequently, the main aims of this study were:

1. **To develop a protocol to multiply *in vitro*, single shoots regenerated from single transformation or mutation events.**

   Objective 1: To establish a medium that results in the production of high shoot yield when applied to different sugarcane varieties.

This was achieved by testing different media for shoot multiplication of well-developed shoots (approximately 2 cm in height) of sugarcane varieties NCo376 and NCo310. The medium that resulted in the production of significantly the highest shoot yield of both varieties, from a single shoot, was then tested on newly released sugarcane varieties N41 and N50. The medium was also tested in the semi-solid state on young shoots (immediately after embryo germination) of variety NCo376 to produce shoots more rapidly and minimize the time required in culture.

Objective 2: To investigate the effect of the established protocol on the genetic stability of multiplied transgenic shoots.

The established protocol was applied to well-developed *in vitro* transgenic shoots of NCo376 lines to test, using end point polymerase chain reaction (PCR) and gel electrophoresis, if the protocol resulted in loss of the transgene in the multiplied shoots.

2. **To establish an *in vitro* strategy for short-term storage of encapsulated somatic embryos developed from single genetic transformation and induced mutagenesis events.**

Objective: To find a suitable slow-growth conditions for storage of encapsulated NCo376 somatic embryos.

Somatic embryos of sugarcane variety NCo376 were separated immediately before they germinated and individually encapsulated using a sodium alginate solution. After encapsulation, the alginate beads with the embryos were stored at room temperature and 18 ^{o}C for 1, 2 and 3 months. At the end of each of these periods, the embryos were retrieved from storage and subcultured on normal regeneration medium. The percentages of embryos that germinated from each of those stored at 18 ^{o}C were compared with that from the control (room temperature).

2. LITERATURE REVIEW

2.1 Background and economic importance of sugarcane

Sugarcane (*Saccharum* spp. hybrids) accounts for approximately 80% of the world's sugar production and ranks among the ten most planted crops in the world (Suprasanna et al., 2011; IllovoSugar, 2013). It is grown in all tropical and subtropical regions, on both sides of the equator (Lakshmanan et al., 2005; Snyman et al., 2011b; Suprasanna et al., 2011; Khamrit et al., 2012; Mnisi and Dhlamini, 2012). The plants are perennial grasses that form stools of stout, jointed and fibrous stalks or culms that can be several meters in length and are juicy, with high concentrations of sucrose (Cheavegatti-Gianotto et al., 2011; Mnisi and Dhlamini, 2012).

In 2010, the Food and Agriculture Organization (FAO) estimated that sugarcane was cultivated on about 23.8 million hectares, in more than 90 countries, with a worldwide harvest of 1.69 billion tons, with Brazil being the largest producer. A similar trend was reported in 2007, when, in terms of the world's total tonnage production, the main sugarcane producing countries were Brazil (33%), India (23%), China (7%), Thailand (4%), Pakistan (4%), Mexico (3%), Colombia (3%), Australia (2%), the United States (2%) and the Philippines (2%) (FNP, 2009). During the 2010/11 season, Brazil harvested about 625 million tons of the sugarcane in a cultivated area of just over eight million hectares. The average yield was 77 tons/ha, higher than the corn yield in the United States (9.3 tons/ha) and sweet sorghum in China (60 tons/ha) (Dal-Bianco et al., 2012). The crop is also commercially grown in South Africa with the sugar industry estimated to produce an annual average income of ZAR8 billion. (SASA, 2013).

Globally, an estimated total of about 45 million farmers, their dependants, and a large number of agricultural labourers are involved in sugarcane cultivation, harvesting and ancillary activities, 7.5% of whom are rural populations (Mnisi and Dhlamini, 2012). Singh and Solomon (1995) reported that sugarcane is one of the most efficient crops in the world in terms of transforming solar energy into sugars and many cellulosic and non-cellulosic products. Processing of sugarcane is, therefore, an agro-industry that is a valuable contributor of both food and energy. In India, the sugar industry has been reported to be the focal point for socio-economic development in the rural areas by mobilizing rural resources, generating employment and higher income, and developing transport and communication facilities (Mnisi and Dhlamini, 2012).

As a highly productive C4 grass, sugarcane is not only used as the main source of sugar, but more recently to produce ethanol, a renewable transportation fuel (Cunff et al., 2008). To this end, there has been increased interest in this crop due to the impending need to decrease the dependency on fossil fuels (Altpeter and Oraby, 2010).

Bagasse, the fibrous residue of the cane stalk after crushing and extraction of the juice is used in the production of paper, newsprint, particle and fibre boards and as a source of energy in the sugar factory (Singh and Solomon, 1995; Malabadi et al., 2011). Molasses, the mother liquor left over after the crystallization of sucrose, is used in the production of ethyl alcohol, animal feed as well as the production of yeast (Singh and Solomon, 1995).

Sugarcane belongs to the family *Poaceae* (Lakshmanan et al., 2006; Behera and Sahoo, 2009) and tribe *Andropogenae* (Grivet and Arruda, 2001). The genus *Saccharum* is traditionally regarded to have six species (Daniels and Roach, 1987; Jackson, 2005). Two of the species, *S. spontaneum* and *S. robustum*, are found growing in the wild, while *S. officinarum*, *S. barberi*, *S. sinense* and *S. edule* are generally found in cultivation (Jackson, 2005).

Up until the end of the 19th century, the most cultivated sugarcane plants were clones of the high sucrose-producing *S. officinarum* (2n = 80), also known as one of the 'noble' canes (Jackson, 2005; Lakshmanan et al., 2005). Sugarcane breeders made a breakthrough in increasing yield and disease resistance by crossing *S. officinarum* to *S. spontaneum* (2n = 40-130), which is a wild and vigorous relative (Grivet and Aruda, 2001). A series of backcrossing, in a process known as 'nobilization' (Grivet and Arruda, 2001), to *S. officinarum*, which has a high sugar content, resulted in cultivars with increased yields, improved ratooning ability and disease resistance ((Daniels and Roach, 1987; D'Hont et al., 1998; Lakshmanan et al., 2005). The cultivated sugarcane plants grown today are, therefore, highly heterozygous and have complex polyploid genomes because they were produced through interspecific hybridization involving three or four species of *Saccharum*. These were *S. officinarum* and *S. spontaneum* with contributions from *S. robustum*, *S. sinense* and related grass genera *Miscanthus*, *Eriatnthus* and *Narenga* (Altpeter and Oraby, 2010). The genetic component of *S. spontaneum* is reduced in commercial hybrids that are grown today, and of the chromosomes in such commercial hybrid cultivars, approximately 80% are derived from *S. officinarum* and 10% are from *S. spontaneum*, with the remainder being chromosomes from the two species produced by natural process of synapsis during meiosis

(Daniels and Roach, 1987; Lakshmanan et al., 2005). Only a few clones of *S. officinarum* and *S. spontaneum* are thought to have been involved in the development of the early hybrids (Roach, 1989). Thus, there is a narrow genetic base in sugarcane breeding programs for the improvement of traits of interest (Jackson, 2005).

2.2 Propagation of sugarcane

2.2.1 Conventional strategies

Sugarcane varieties are propagated vegetatively. This is achieved by stem cuttings of mature canes known as 'setts' or billets with two to three lateral buds (or dormant eyes) (Lakshmanan, 2006; Slater et al., 2008; Behera and Sahoo, 2009; Cheavegatti-Gianotto et al., 2011; Snyman et al., 2011b). Setts are prone to fungal attack (due to their high sugar content) and are routinely treated with fungicide to protect them until germination (Bull, 2000). According to Cheavegatti-Gianotto et al. (2011), the cuttings are usually taken from the upper third of the stalk of 8–12 months old plant canes or from 6-8 months old ratoons. Setts are usually 300-450 mm in length and are planted in trenches. Ploughing is 300 mm deep and the furrows are cut to a depth of 200-300 mm. Rows are spaced at distances varying from 800 to 1500 mm and are planted with 8-12 tons of planting material per hectare. Stalks are distributed in furrows in pairs against the upper part of the other. After the stalks are distributed in the furrow, they are sectioned into two to three node pieces to interrupt apical dominance that exists in the intact stalk.

After planting, the buds develop into primary and secondary stalks and gradually form a dense homogeneous tuft, known as a stool (Bull, 2000). The cuttings are carefully sorted in order to eliminate those that are misshapen or have already started to sprout. Sugarcane varieties differ in their degree of temperature sensitivity but, in general, germination of the buds is slow at temperatures less than 18 ^{o}C and will be increasingly rapid up to 35 ^{o}C (Bull, 2000).

Sugarcane breeding programmes rely on extensive crossing of elite cultivars and involve cross pollination (Selman-Housein, 2000). This is performed by bringing the arrows of the female and male together in isolation to allow for natural cross pollination. Alternatively, the pollen is manually dusted onto the flowering arrow of the female clone (Sleper and Poehlman, 2006). The process of breeding sugarcane through traditional ways usually takes between 12–15 years (Barba et al., 1978; Pathak et al., 2009). This is due to the limited gene pool, complex polyploid

and aneuploid genome, mentioned above, which makes breeding and field selection for superior traits difficult and generally slow.

Once new genotypes have been produced and selected via breeding programs, they are propagated vegetatively by nodal cuttings (Cheavegatti-Gianotto et al., 2011; Snyman et al., 2011b), as discussed above. This method of propagation is widely considered as being a slow process (Soodi et al., 2006; Roy and Kabir, 2007; Sengar et al., 2011). Another disadvantage is that pathogens accumulate generation after generation, which reduces the yield and quality of the sugarcane (Lee, 1987; Soodi et al., 2006; Roy and Kabir, 2007; Pathak et al., 2009). These issues can be addressed (Lee, 1987; Chengalryan and Gallo-Meagher, 2001; Snyman et al., 2011b) with *in vitro* culture techniques. Snyman et al. (2006) reported that tissue culture can increase the vegetative propagation potential of sugarcane by 20-35 times. In 9½ months, it is possible to produce enough planting material from one spindle to plant a hectare using mass propagation by tissue culture, whereas only about 100 seed pieces are available by the end of 9½ months by conventional methods (Lee, 1987). In addition, tissue culture produced plants, particularly those from meristem culture are pathogen free (Sengar et al., 2011).

2.2.2 *In vitro* culture systems for sugarcane

There are two primary morphogenic pathways leading to whole plant regeneration *in vitro* and these are somatic embryogenesis and organogenesis (Phillips, 2004; George et al., 2008; Slater et al., 2008). Both developmental pathways can occur either directly from the explant or indirectly following an unorganized callus stage.

a) Organogenesis

Organogenesis is the formation of organs (shoot or root) from a plant tissue (George et al., 2008; Slater et al., 2008). It can occur either directly, where buds and shoots are produced from a tissue, or indirectly through a callus phase. According to those authors, the process relies on the inherent plasticity of the plant tissue and is regulated by altering the components of the medium. In particular, it is the auxin to cytokinin ratio of the medium that determines the developmental pathway of regeneration (George et al., 2008). Shoot formation is normally induced by increasing the cytokinin to auxin ratio of the culture medium.

According to Sugiyama (1999), organogenesis occurs in three phases that are recognized on the basis of temporal requirement for specific balance of phytohormones. In the first phase, cells in the explants acquire 'competence', which is the ability to respond to hormonal signals. In the second, the dedifferentiated cells are canalized and determined for specific organ formation in response to exogenous phytohormones and in the third, morphogenesis proceeds independently of the exogenously supplied phytohormones.

b) Somatic embryogenesis

Somatic embryogenesis is defined as a morphological pathway in which a bipolar structure resembling a zygotic embryo develops from a non-zygotic cell without vascular connection with the original tissue (Anold et al., 2002; George et al., 2008). It occurs through an orderly series of characteristic embryological stages from a somatic cell rather than fusion of gametes (Jimenez, 2001), either directly on the explant or indirectly after a callus phase (Franklin et al., 2006; Namasivayam, 2007; George et al., 2008). As with organogenesis, the process is feasible because plants possess cellular totipotency meaning that, individual somatic cells can regenerate into whole plants (Namasivayam, 2007; George et al., 2008).

Somatic embryogenesis is conventionally divided into two main stages, *viz*. induction and expression (Jimenez, 2005). In the former, somatic cells acquire embryogenic characteristics by means of a complete reorganization of the cellular state, including physiology, metabolism and gene expression (Jimenez, 2005). It is usually after a change in one or more culture conditions (e.g. culture medium, composition of plant growth regulators [PGRs], carbohydrate source and osmotic potential) that the induced cells reach the expression stage in which they display their embryogenic competence and differentiate into somatic embryos (Jimenez, 2005). The induction of somatic embryogenesis must then consist of the termination of the current gene expression and its replacement with the embryogenic gene expression programme (George, 1993; Anold et al., 2002; George et al., 2008). Anold et al. (2002) further stated that PGRs and stress play a central role in mediating the signal transduction cascade leading to the reprogramming of the gene expression. This results in a series of cell divisions that either induce unorganized callus growth or polarized growth leading to embryogenesis.

Anold et al. (2002) summarized plant regeneration via somatic embryogenesis as follows: 1) initiation of embryogenic cultures by culturing the primary explant on medium supplemented with PGRs, mainly auxin but often also cytokinin; 2) proliferation of embryogenic cultures on solidified medium or in liquid medium supplemented with PGRs, similar as under initiation; 3) prematuration of somatic embryos in medium lacking PGRs, which inhibits proliferation and stimulates somatic embryo formation and early development; 4) maturation of somatic embryos by culturing on medium supplemented with abscisic acid (ABA) and or reduced osmotic potential and 5) regeneration of plants on medium lacking PGRs.

Consequently, to produce plants via somatic embryogenesis, a number of critical physical and chemical treatments should be applied with proper timing (Anold et al., 2002; George et al., 2008). Various culture treatments can be manipulated to optimize the frequency and morphological quality of the somatic embryos (Phillips, 2004; George et al., 2008). Typical treatment factors include the PGR source and concentration (especially the auxin), choice of explant, nutrient medium composition (e.g. inorganic versus organic nitrogen sources, carbohydrate sources and concentrations), culture environment (e.g. liquid or semi-solid, pH, humidity, light quality and quantity or absence of light, temperature and gaseous environment) and osmotic potential (Phillips, 2004; George et al., 2008). A high auxin signal, often via the addition of 2,4-dichlorophenoxyacetic acid (2,4-D) to the culture medium is usually important to induce somatic embryogenesis.

Several protocols for somatic embryogenesis have been developed for sugarcane using various explants, examples of which are listed in Table 1. Almost all were established on Murashige and Skoog (MS) (1962) medium supplemented with different PGRs. Immature leaf rolls have been the most common explants type used for plant regeneration (Brisibe et al., 1994; Burner and Grisham, 1995; Falco et al., 1996; Lakshmanan et al., 2006). Snyman et al. (2006) reported that developing inflorescence can also be used for rapid production of embryogenic callus in sugarcane. The other explants that have been used for plant regeneration through both direct and indirect somatic embryogenesis are apical meristems (Ali et al., 2007a; Biradar et al., 2009), spindle leaves (Barba et al., 1978; Ali et al., 2007a), pith parenchyma (Ali et al., 2007a), leaf mid rib segments (Franklin et al., 2009) and mature seeds (Chengalrayan et al., 2005).

From published reports (Table1), explants have been primarily initiated and established on semi-solid MS medium supplemented with PGRs, while liquid medium has been mainly used for shoot multiplication and rooting (Lorenzo et al., 1998; Chengalrayan et al., 2005; Pathak et al., 2009). In most of the studies somatic embryogenesis was induced in the presence of the auxin 2,4-D and in some cases picloram and thiadiazuron (TDZ) (Chengalryan and Gallo-Meagher, 2001; Chengalrayan et al., 2005). Other PGRs, for example 1-naphthylacetic acid (NAA) and 6-benzylaminopurine (BAP), have been used mainly for shoot multiplication (Burner and Grisham, 1995; Falco et al., 1996; Lorenzo et al., 1998; Ikram-ul-Haq and Memon, 2012) and rooting (Chengalrayan et al., 2005; Ikram-ul-Haq and Memon, 2012).

Most of the published reports (Table 1) omit some important information such as the number of subcultures, the number of shoots obtained per plant, details of acclimatization and field trials.

c) Applications

Micropropagation is used for clonal multiplication of plants using meristematic or non meristematic cells or tissues as the explant *in vitro* (George et al., 2008; Sengar et al., 2011). During the last thirty years, *in vitro* techniques that result in micropropagation have become widely used in commercial horticulture and agriculture for mass propagation of crop plants (George, 1993; George et al., 2008; Sengar et al., 2011), including sugarcane (Table 1). These techniques also form the basis of various other technologies such as genetic engineering and induced mutation on breeding as discussed in the next sections.

d) Genetic fidelity

Despite the advantages of *in vitro* propagation, phenotypic instability has been observed in micropropagated plants of many species, including sugarcane (Devarumath et al., 2007). Consequently, one of the most crucial concerns in *in vitro* propagation is, to retain the genetic fidelity of plants produced *in vitro* with respect to the mother plants (Devarumath et al., 2007).

The occurrence and degree of somaclonal variation, as will be discussed in the ensuing sections, in *in vitro*-produced plants depend upon a number of factors including the type and source of the explant and method of propagation (Devarumath et al., 2007; Senapati et al., 2012).

Several strategies such as morphological descriptions (differences in plant stature, leaf morphology and pigmentation abnormality), physiological/biochemical descriptions (e.g. response to hormones and light), field assessments and molecular studies (genomic DNA analysis) have been developed to assess the genetic purity of tissue culture raised clones (Trigiano and Gray, 2005; Tawar et al., 2008; Bairu, 2011). However, some of these techniques have limitations, e.g. morphological, physiological and field assessment methods are subject to environmental effects while biochemical assessments vary with the developmental stages of the plant (Senapati et al., 2012). Ideally, a thorough molecular and phenotypic assessment of *in vitro*-derived plants is necessary. This has resulted in molecular techniques being the commonly preferred methods todate.

Molecular techniques including polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), rapid amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) are used for detecting and characterizing variation at DNA level. Out of these available techniques, RAPD and SSR are the most commonly used techniques to measure polymorphism in sugarcane (Orepeza et al., 1995; Shahid et al., 2011; Pandey et al., 2012). These two techniques have proven to be reliable, reproducible, easy to regenerate, inexpensive and use a versatile set of markers that relies on repeated amplification of the DNA sequence using single primers (Isabel et al., 1993; Senapati et al., 2012). However, some changes are epigenetic in nature (Lourens and Martin, 1987) and phenotypic assessment over several cycles of field ratoons is necessary (Irvine et al., 1991).

2.3 Genetic manipulation

As mentioned before, genetic improvement of elite sugarcane clones by conventional breeding is difficult due to its highly complex polyploid and aneuploid nature, poor fertility and the long period (over 10 years) required for field selection of new cultivars. In this regard, genetic manipulation via genetic engineering and *in vitro* mutagenesis are valuable tools to introduce commercially-important traits into sugarcane germplasm.

Table 1: Examples of different protocols developed to regenerate sugarcane plants via somatic embryogenesis. PGRs 2,4-D = 2,4-dichlorophenoxyacetic acid, BA or BAP = 6-benzylaminopurine, GA3 = gibberellic acid, IBA = indole-3-butyric acid, TDZ = thiadiazuron, NAA = 1-napththylacetic acid and kinetin = N-6-furfuryladenanine.

Route of morphogenesis	Explant	PGR	Type of media	Variety	Reference
Indirect somatic embryogenesis	Spindle tissue	2,4-D	Semi-solid	Not specified	Barba et al. (1978)
	Immature leaf roll	2,4-D, IBA	Semi- solid	NCo310	Brisibe et al. (1994)
	Immature leaf roll	2,4-D, kinetin, BAP	Semi- solid (initiation), liquid (elongation and rooting)	CP 74-383	Burner and Grisham (1995)
	Immature leaf segments	2,4-D, BAP, kinetin, zeatin, NAA	Liquid (cell suspension), semi-solid (plant regeneration)	SP79-1011	Falco et al. (1996)
	Immature leaf sheath	2,4-D, NAA, TDZ	Semi-solid	CP4-1198	Gallo-Meagher et al. (2000)
	Mature seed	2,4-D, picloram, TDZ, IBA	Semi-solid (initiation) and liquid (rooting)	CP84-1198	Chengalrayan et al. (2005)
	Immature leaf roll	BAP, kinetin, NAA	Semi-solid	Q117, Q156, Q157, Q167, Q172, Q185, Q188, Q190, Q196, Q197, Q200, Q205, Q216, Q222	Lakshmanan et al. (2006)

Table 1 continued

Route of morphogenesis	**Explant**	**PGR**	**Type of media**	**Variety**	**Reference**
	Immature leaf roll	2,4-D	Semi-solid	88H0019, NCo376, N12, N27	Snyman et al. (2006)
	Shoot apical meristem, spindle leaves, pith parenchyma	2,4-D, BAP, kinetin	Semi-solid	CP 77 400, BL-4	Ali et al. (2007a)
	Immature leaf roll	2,4-D, IBA, amino acid	Semi-solid	SP-241	Asad et al. (2009)
	Immature leaf tips	2,4-D, BAP, kinetin, NAA, IBA	Semi-solid (initiation and regeneration), liquid (rooting)	CPF-237	Ikram-ul-Haq and Memon (2012)
Direct somatic embryogenesis	Immature leaf roll	2,4-D, NAA	Not specified	H50-7209, H57-5026, H59-2159, H60-8489, H64-5472, H64-6336, H68-7480.	Nadar and Heinz (1977)
	Spindle leaf	2,4-D, BAP, NAA	Semi-solid	RB7335275	Lee (1987)
	Sugarcane meristems	Paclobutrazol, GA_3	Semi-solid, liquid	C-1051-73	Lorenzo et al. (1998)

Table1 continued

Route of morphogenesis	Explant	PGR	Type of media	Variety	Reference
	Immature leaf roll	2,4-D	Semi-solid	N12, N19	Snyman et al. (2000)
	Immature leaf roll	2,4-D	Semi-solid	N12, N19	Snyman et al. (2001)
	Mid rib segments	2,4-D, BAP, NAA	Semi-solid	COC-671	Franklin et al. (2006)
	Shoot apical meristem, spindle leaves, pith parenchyma	2,4-D, BAP, kinetin	Semi-solid	CP 77 400, BL-4	Ali et al. (2007a)
	Apical meristem	BAP	Semi-solid	COC-671	Biradar et al. (2009)
	Shoot tip	BAP, kinetin, NAA	Semi-solid (initiation), liquid (shoot multiplication and rooting)	CoS99259, COSe01235	Pathak et al. (2009)
	Apical meristem	2,4-D, BAP, kinetin	Semi-solid	Q117, Q135, Q200,Q209, Q157, Q158, Q185, Q186, Q208, Q174, Tellus, Q138, Q170, Q183, KQ228, Q204	Shiromani et al. (2011)

Table 1 continued

Route of morphogenesis	Explant	PGR	Type of media	Variety	Reference
	Shoot apical Meristem	2,4-D, BAP, IBA	Semi-solid	Phil 66-07	Khamrit et al. (2012)
Organogenesis	Shoot apices, leaves and inflorescences	2,4-D, kinetin, IAA	Semi-solid	H50-7209, H37-1933	Heinz and Mee (1969)
	Shoot tip	GA3, IBA, kinetin, BAP	Liquid	CP74-383	Burner and Grisham (1995)
	Immature leaf segments	2,4-D, BAP, kinetin, zeatin, NAA	Liquid (cell suspension)	SP79-1011	Falco et al. (1996)
	Meristem	2,4-D, kinetin, BAP, NAA, IBA	Semi-solid	Q61, Suphan, Co622, K84-200, POJ288, ROC	Visessuwan et al. (1999)
	Immature leaf roll	TDZ	Semi-solid	CP84-1198	Chengalryan and Gallo-Meagher (2001)
	Immature shoot tips	BAP, IBA, kinetin, IAA, NAA,	Semi solid	Lsd28	Baksha et al. (2002)

Table 1 continued

Route of morphogenesis	Explant	PGR	Type of media	Variety	Reference
	Apical meristem, apical bud, axillary bud	BAP, kinetin	Semi-solid	HSF-240, SPF-213, SPF-234, CP77/400, CPF237	Cheema and Hussain (2004)
	Seeds	TDZ, 2,4-D, picloram	Semi-solid	CP84-1198	Chengalrayan et al. (2005)
	Leaf spindle	2,4-D, NAA, kinetin, IBA, BAP	Liquid	CoJ88	Singh et al. (2008)

2.3.1 *In vitro* mutagenesis

Agronomically-improved sugarcane varieties tolerant to biotic and abiotic stresses are highly beneficial as unfavourable environmental factors can challenge cultivation and crop productivity (Suprasanna et al., 2011). Although such crops have been produced by traditional breeding programmes, fast tracking their release is essential in developing improved varieties. As a result, biotechnological approaches which include somaclonal variation and *in vitro* mutagenesis, followed by exposure to selection agents, are being applied for the isolation of agronomically useful mutants (Jain, 2005), including in sugarcane (Khan et al., 1998; Ali et al., 2007b; Patade et al., 2008; Koch et al., 2012; Mahlanza et al., 2013).

Somaclonal variation has been defined as variation originating in cell and tissue culture (Larkin and Scowcroft, 1981; Anastassopoulos and Keil, 1996; Trigiano and Gray, 2005; Tawar et al., 2008; Bairu, 2011). According to Trigiano and Gray (2005) and Tawar et al. (2008), this type of variation is a result of three major factors, *viz*. physiological (variation as a result of habituation to PGRs in culture and culture conditions), genetic (variation as a result of alterations at the chromosomal level, e.g. a deletion, duplication and somatic recombination) and biochemical (e.g. alterations in carbon metabolism leading to lack of photosynthetic ability).

In perennial crops such as sugarcane that are asexually propagated, somaclonal variation offers an excellent opportunity to add new genotypes to the gene pool (Trigiano and Gray, 2005) increasing variability (Bairu, 2011). Genetic variation through mutations can occur naturally in culture as mentioned above or can be induced by specific treatments with chemical (ethyl methanesulfonate, sodium azide and sodium nitrate) (Table 2) and physical (gamma rays and ion beams) (Table 3) mutagens.

According to Patade and Suprasanna (2008), *in vitro* mutagenesis has several advantages compared with classical mutation (e.g. conventional mutagenesis using seeds and or vegetative propagules) techniques. These are high mutation frequency, uniform mutagen treatment and application of selective agents to homologous cell populations, use of single cell systems in comparison with the organized complexity of whole plants and seeds, occurrence of dominant mutations, requirement of lesser space to handle large populations within a short span of time and keeping the material free of diseases.

Table 2. Examples of traits in sugarcane mutants obtained with chemical mutagens.

Mutagen	Trait	Variety	Reference
Sodium azide	Red rot disease resistance	CO 1148	Khairwal et al. (1984)
	Colletotrichum falcatum resistance	CP 77,400	Ali et al. (2007b)
Sodium nitrite	Drought tolerance	Q77N1232, Co6519, Cadmus	Wagih et al. (2004)
Ethyl methanesulfonate	Red rot disease resistance	CO 1148	Khairwal et al. (1984)
	Salt tolerance	Co 86032	Kenganal et al. (2008)
	Herbicide (imazapyr) tolerance	N12	Koch et al. (2012)
	Brown rust resistance	B4362	Oloriz et al. (2012)
		CP48-103, CP58-14	Sadat and Hoveize (2012)
	Fusarium sacchari tolerance	NCo376	Mahlanza et al. (2013)

In sugarcane, many somaclones with desirable agronomic characteristics have been produced to date, including those with Fiji and downy mildew disease resistance (Leu, 1978; Liu and Chen, 1978), eye spot resistance (Hoy et al., 2003; Suprasanna et al., 2006), smut resistance (Boer and Roa, 1991; Jalaja et al., 2006), red rot disease resistance (Jalaja et al., 2006; Singh et al., 2008; Kumar et al., 2012), rust resistance (Yasuda et al., 1982), yield improvement (Korneva and Maribona, 1984; Doule, 2006), elevated sucrose content (Hoy et al., 2003; Doule, 2006; Suprasanna et al., 2006) and increased sugar recovery (Doule, 2006).) These examples demonstrated that the combination of *in vitro* culture and induced mutagenesis is relatively inexpensive, simple and efficient (Ahloowalia, 1998; Van Harten, 1998; Suprasanna et al., 2011).

Table 3. Examples of traits in sugarcane mutants obtained with gamma irradiation. * = plant height, plant girth, number of stalks per stool, weight per stool, sucrose %, commercial cane sugar %, fibre %, cane yield, sugar yield.

Trait	**Variety**	**Reference**
Salt tolerance	CoC-671	Patade et al. (2006)
	CoC-671	Patade et al. (2008)
	Co86032	Patade and Suprasanna (2009)
Red rot disease resistance	CO 1148	Khairwal et al. (1984)
	Lsd-2/54, Nagarbari, Latarijab	Majid et al. (2001)
	CP 77 400	Ali et al. (2007b)
Chlorophyll mutations	NIA-98, NIA-2004, BL4	Khan et al. (2009)
Improved agronomic traits *	NIA-98, NIA-2004, BL4	Khan et al. (2007)
Increased yield	CP4/33	Khan and Khan (2010)
Increased yield and sucrose content	AEC 81, BL4	Khan et al. (1998)
	CP-43/33	Khan et al. (2000)
Delayed flowering	Lsd-2/54, Nagarbari, Latarijab	Majid et al. (2001)
Resistance to water logging	Lsd-2/54, Nagarbari, Latarijab	Majid et al. (2001)

2.3.2 Genetic engineering

Genetic transformation refers to the transfer of foreign genes (transgenes) from other non-related plants, fungi, viruses and animals into plant cells (Newell, 2000; Sengar et al., 2011; Rivera et al., 2012). The genetic transformation of plants, including sugarcane, is being used as a way to hasten the production of plants with improved agronomic traits that would normally have taken a long time using conventional methods (Bower and Birch, 1992; Newell, 2000; Sengar et al., 2011; Rivera et al., 2012).

Different transformation techniques have been developed to introduce genes of interest in sugarcane callus. The most commonly-used ones includes microprojectile DNA bombardment (Bower and Birch, 1992; Bower et al., 1996; Lakshmanan et al., 2005; Rivera et al., 2012), electroporation or polyethylene glycol (PEG) treatment (Arencibia et al., 1995; Rivera et al., 2012) and *Agrobacterium*-mediated genetic transformation (Arencibia et al., 1998). In the first, the target tissue is bombarded with microprojectiles coated with the foreign DNA, in the second, electric pulse induces membrane permeabilisation providing a local driving force for ionic and molecular transport through the pores and in the third, a disarmed pathogenic bacterium introduces a plasmid carrying the gene of interest into the target organism. These techniques have been used to achieve a range of new characteristics such as herbicide resistance, virus resistance, insect resistance, and altered sucrose enzyme regulation (Table 4).

The first transgenic cells of sugarcane were obtained with electroporation or PEG treatment of sugarcane protoplasts (Chen et al., 1987). However, this approach did not receive so much attention due to severe difficulties with plant regeneration from the protoplasts of the tested cultivars (Bower and Birch, 1992; Lakshmanan et al., 2005). More transgenic sugarcane plants were then successfully produced via biolistic gene transfer (Bower and Birch, 1992). Following that report, genetic transformation of sugarcane using electroporation (Arencibia et al., 1995) and *Agrobacterium*-mediated gene transfer (Arencibia et al., 1998) were also reported. Sugarcane cultivars with improved agronomic characteristics, amongst others, e.g. borer resistance, enhanced metabolic system, herbicide tolerance and *Puccinia melanocephala* resistance were then produced (Table 4).

Of all the techniques, microprojectile DNA bombardment-mediated transformation is the most commonly-used for sugarcane transformation (Table 4) as it is highly reproducible, adaptable to new explant types, less genotype dependent and allows introduction of multiple unlinked expression cassettes for stacking of traits (Bower and Birch, 1992; Altpeter et al., 2005). According to Newell (2000), the other reason why microprojectile DNA bombardment-mediated transformation is commonly used is that it is species independent and avoids the complex interaction between bacterium and plant tissue, with the result that the DNA to be introduced does not need to contain the sequence necessary for T-DNA replication and transfer, as is the case with *Agrobacterium*-mediated gene transfer.

2.3.3 Characterisation of modified plants

Transgenic plants are now widely used in both basic and applied studies in plant biology (Bhat and Scrinivansan, 2002). However, variability in agronomic traits of transformed clones due to somaclonal variation and transformation procedures often occurs necessitating the need for both field and laboratory evaluation (Arencibia et al., 1999; Gilbert et al., 2005; Gilbert et al., 2009). According to Gilbert et al. (2005) and Arencibia et al. (1999), somaclonal variation caused by tissue culture procedures may produce undesirable field characteristics in genetically transformed sugarcane that are not readily identifiable in the laboratory or greenhouse. Hence, it is crucial to conduct agronomic analyses in the field across several generations to ensure stability of transgene expression (Table 4).

Molecular techniques such as polymerase chain reaction (PCR), Southern, Northern and Western hybridization are the most common techniques employed in laboratories to assess the integration and expression of the introduced gene (Bhat and Scrinivansan, 2002). Bioassays, if available, can also provide functionality of the transgene product. PCR amplification of the marker gene is often taken as an initial indication of the transgenic status of the regenerants (Potrykus, 1991; Bhat and Scrinivansan, 2002). However , according to Bhat and Scrinivansan (2002), Southern analysis is essential to prove the integration of the foreign gene into the host genome and to assess the number of independent insertions of the transgenes, or copy number.

In sugarcane, Random Amplified Polymorphic DNA (RAPD), Amplified Fragment length Polymorphism (AFLP) and Simple sequence repeats (SSR) markers have been used to identify genetic variations following regeneration of embryogenic callus (Gilbert et al., 2009; Watt et al., 2009; Snyman et al., 2011a; Rutherford et al., 2013).

Table 4. Examples of traits obtained in sugarcane using genetic transformation methods [modified from Watt et al. (2010)]. SCMV = Sugarcane Mosaic Virus, SrMv = Sorghum Mosaic Virus, CP = Coat Protein, SCYLV = Sugarcane Yellow Leaf Virus, PFP = Pyrophosphate: Fructose 6-phosphate 1-phosphotransferase, NI = Neutral invertase.

Trait	Gene	Transformation method	Variety	Field trial	Reference
Antibiotic resistance	Neomycin phosphotransferase (npt-11) (selectable marker gene)	Microprojectile	Pindar	No	Bower and Birch (1992)
Herbicide tolerance					
Bialaphos	*bar*	Microprojectile	NCo376	Yes	Gallo-Meagher and Irvine (1996)
Phosphinothricin	*bar*	*Agrobacterium*	Ja60-5	Yes	Enriquez-Obregon et al. (1998)
Glufosinate ammonium	*pat* gene	Microprojectile	NCo310	Yes	Leibbrandt and Snyman (2003)
Disease resistance					
Sugarcane mosaic virus (SCMV)	*SCMV-CP*	Microprojectile	Q155	No	Joyce and McQualter (1998)
Sorghum mosaic virus (SrMV)	*SrMV-CP*	Microprojectile	CP72-121, CP65-357	No	Ingelbrecht et al. (1999)
Sugarcane leaf scald	albD	Microprojectile	Q63, Q87	Yes	Zhang et al. (1999)

Table 4 continued

Trait	Gene	Transformation method	Variety	Field trial	Reference
Puccinia melanocephala	Glucanase chittanase and *ap24*	*Agrobacterium*	B4362	No	Enriquez-Obregon et al. (2000)
Sugarcane Yellow leaf Virus (SCYLV)	SCYLV-CP	Microprojectile	CC84-75	No	Rangel et al. (2003)
SCMV	*SCMV-CP*	Microprojectile	NCo310	Yes	Sooknandan et al. (2003)
Fiji leaf gall	FVS9 ORF1	Microprojectile	Q124	No	McQualter et al. (2004a)
Fungal damage resistance	Chitinase	Microprojectile	Phill 66-07	No	Khamrit et al. (2012)
Pest resistance					
Sugarcane stem borer	Cry1A	Electroporation	Ja60-5	Yes	Arencibia et al. (1999)
Sugarcane cane grub resistance	*Gna or pin11*	Microprojectile	Q117	No	Nutt et al. (1999)
Mexican rice borer	*gna*	Microprojectile	CP65-357	Yes	Legaspi and Mirkov (2000)
Sugarcane borer and Mexican rice borer	*gna*	Microprojectile	CP65-357	Yes	Setamou et al. (2002)

Table 4 continued

Trait	Gene	Transformation method	Variety	Field trial	Reference
Metabolic engineering, alternative products					
Fructo oligosaccharide oxidase	lsdA	*Agrobacterium*	B4362	No	Enriquez-Obregon et al. (2000)
Sucrose accumulation	Antisense soluble acid invertase	Microprojectile	H62-4671	No	Ma et al. (2000)
Sucrose accumulation	Soluble acid invertase	Microprojectile	Q117	No	Botha et al. (2001)
Polyhydroxybutyrate	*phaA, phaB, phaC*	Microprojectile	Q117	No	Brumbley et al. (2003)
p-Hydroxybenzoic acid	*hchl, cpl*	Microprojectile	Q117	No	McQualter et al. (2004b)
Polyphenol oxidase	*ppo*	Microprojectile	Q117	Yes	Vickers et al. (2005)
UDP-glucose dehydrogenase	UDPG-DH	Microprojectile	NCo310	No	Bekker (2007)

Table 4 continued

Trait	Gene	Transformation method	Variety	Field trial	Reference
Pyrophosphate: Fructose 6-phosphate 1-phosphotransferase (PFP)	PFP	Microprojectile	NCo310	Yes	Groenewald and Botha (2007)
Neutral invertase (NI)	*NI*	Microprojectile	NCo310	No	Rossouw et al. (2007)
H+ translocating vacuolar pyrophosphatase (VPPase)	*VPPase*	Microprojectile	NCo310	No	Swart (2007)

2.4 *In vitro* germplasm storage

The use of *in vitro* tissue culture techniques is also important for germplasm collection, storage and multiplication of vegetatively propagated species such as sugarcane (Englemann, 1991). Conservation in the field presents major drawbacks because diseases and environmental disasters threaten the safety of plant genetic resources conserved in this way (Withers and Engels, 1990; Muhammad and Siddique, 2004). As a result, a number of *in vitro* storage techniques have been developed including minimal growth storage and cryopreservation.

2.4.1 Cryopreservation

Cryopreservation is referred to as the conservation of plant propagules at very low temperatures usually below -150 ^{o}C (Gonzalez-Benito et al., 2004; Gonzalez-Arnao and Engelman, 2006; Barraco et al., 2011). At such ultra-low temperatures, metabolic activities are virtually stopped thereby enabling theoretically unlimited storage durations (Gonzalez-Arnao and Engelman, 2006; Barraco et al., 2011). Different types of plant cell, tissues and organs can be cryopreserved including cell suspensions, pollen, embryogenic cultures, somatic and zygotic embryos and shoot apices or meristems. Cryopreservation, the storage of material under liquid nitrogen at -196 ^{o}C is regarded as the only technique that is currently available for safe and cost-effective long-term conservation of genetic resources of vegetatively propagated plant species such as sugarcane, which cannot be stored in the form of dehydrated seeds in seed banks (Gonzalez-Arnao and Engelman, 2006; Barraco et al., 2011).

For vegetatively propagated species such as sugarcane, the most widely used organs for storage are shoot apices excised from *in vitro* plants (Gonzalez-Arnao et al., 1993; Paulet et al., 1993; Gonzalez-Benito et al., 2004). However, cryopreservation protocols have also been developed for sugarcane cell suspensions (Ulrich et al., 1984; Gnanapragasam and Vasil, 1990), embryogenic calli (Eksomtramage et al., 1992; Martninez-Montero et al., 1998), somatic embryos (Martinez-Montero et al., 2008) and shoot tips (Gonzalez-Arnao et al., 1993; Paulet et al., 1993). With shoot tips, encapsulation-dehydration, a technique first developed by Dereuddre et al. (1990), was successfully applied to 15 sugarcane varieties (Gonzalez-Arnao, 1996).

Barraco et al. (2011), also successfully cryopreserved two sugarcane varieties, PVS2 and PVS3 using encapsulation and droplet-vitrification.

2.4.2 Minimal growth storage

Minimal growth (slow growth) storage refers to direct ways of restricting growth and morphogenesis *in vitro* (Englemann, 1991; Scocchi and Maroginski, 2004; Watt et al., 2009; Engelmann, 2012). Slow growth storage techniques are routinely used for medium-term conservation of numerous plant species, both from temperate and tropical regions including crops, trees, endangered species and medicinal plants (Ashmore, 1997; Engelmann, 2012).

The aim for short and medium term storage of plants under minimal growth conditions is to reduce the growth and to increase the intervals between subcultures (Englemann, 1991). Physical conditions of incubation and or modifications of the culture media, which reduce the growth rate of tissues stored *in vitro,* are usually employed for the preservation of plant germplasm (Scocchi and Maroginski, 2004). According to Engelmann (1991; 2012) and Watt et al. (2009), the most commonly-employed techniques to achieve slow growth include temperature reduction, altering the composition of the culture medium (e.g. by lowering the content of mineral elements and or sucrose concentration), the addition of osmotic growth inhibitors (e.g. mannitol) or growth inhibitors (e.g. abscisic acid), physiological stage of the explants (i.e. the presence of the root system increases the survival capacities of some species), modification of the gaseous environment (e.g. lowering oxygen level by covering tissues with mineral oil) and encapsulation using cryoprotectant materials like hydrogel, alginate gel, ethylene glycol and dimethylsulphoxide.

In vitro storage of sugarcane plants under slow growth conditions has been developed at the Sugarcane Breeding Institute Coimbatore, India, and the French International Aid Organization, CIRAD, in Montpellier, France (Taylor and Dukic, 1993). At CIRAD, *in vitro* sugarcane plants were established from apical meristem tissue and axillary buds (buds growing in the upper leaf axil of stalks) and maintained at 18 ^{o}C for up to two years (Paul and Duvik, 1993; Paulet et al., 1993). At the Bereau of Sugarcane Experiment Stations in Brisbane, Australia, an *in vitro* germplasm collection was also established from apical meristems for over 200 *Saccharum*

species hybrid cultivars which were conserved under slow growth conditions (Paul and Duvik, 1993). In India, *in vitro* sugarcane plants established from apical meristems were maintained at 25 ^{o}C and subcultured after six months (Paul and Duvik, 1993). In South Africa, Watt et al. (2009) developed a protocol for slow growth storage of a local variety 88H0019. The protocol involves storage of non-encapsulated globular somatic embryos at 18 on regeneration medium with 0.6 mg l^{-1} 2,4-D and storage of whole plantlets at 18 ^{o}C on a medium containing half-strength MS basal salts and vitamins (Murashige and Skoog, 1962), 10 g l^{-1} sucrose and 30 g l^{-1} sorbitol.

3. MATERIALS AND METHODS

3.1 Plant material collection and preparation

Sugarcane plant stalks of varieties NCo310 and NCo376 were collected from the South African Sugar Research Institute (SASRI) in Mount Edgecombe, KwaZulu-Natal, South Africa, and used to produce shoots *in vitro* through indirect somatic embryogenesis. SASRI supplied *in vitro* shoots of N41, N50 and of transformed lines of NCo376 (NUE5, NUE9, NUE21, NUE23 and NUE43). Shoots from all the varieties were used for shoot multiplication, while embryogenic calli of NCo376 were used for slow growth storage investigations.

For callus production, the uppermost portion of the stalk for NCo310 and NCo376, which is the portion containing the shoot meristem and immature leaf whorls, was removed. Approximately 30 mm of the leaf roll was decontaminated in 70% (v v^{-1}) ethanol for 5 min. The leaf rolls were then transferred to the laminar flow-hood for callus induction.

3.2 Experimental design

A summary of the experimental design for the different stages followed to produce somatic embryos and plants via indirect somatic embryogenesis and the establishment of a protocol for shoot multiplication of NCo376 and NCo310 is shown in Figure 1. Eight media were tested for shoot multiplication of well-developed shoots (20 mm in height), the best of which was tested on young shoots. These media were then tested on varieties N41, N50 and the transformed lines of NCo376.

For *in vitro* storage of somatic embryos (not shown on Figure 1), four storage protocols were tested on the embryos before germination (section 3.7).

3.3 Somatic embryogenesis standard culture procedures

3.3.1 Stage 1: Callus induction

The indirect somatic embryogenesis protocol of Snyman (2004) was employed. The leaf rolls were swabbed with 70% (v v^{-1}) ethanol in the laminar flow hood. Leaf sheaths on the outer surface of the stalk were removed aseptically using sterile forceps. The inner leaf rolls were sliced transversely into 30 disks (approximately 1-2 mm thick) in liquid medium containing full

strength MS basal salts and vitamins (Murashige and Skoog, 1962), 20 g l^{-1} sucrose, 0.5 g l^{-1} casein hydrolysate and 3 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), pH 5.6-5.8. The leaf disks were placed with their adaxial surfaces facing downwards on semi-solid callus induction medium (CIM) (10 disks/90 mm Petri dish with 30 ml medium) containing full strength MS basal salts and vitamins (Murashige and Skoog, 1962), 20 g l^{-1} sucrose, 0.5 g l^{-1} casein hydrolysate, 3 mg l^{-1} 2,4-D and 8 g l^{-1} agar, pH 5.6-5.8. The cultures were kept in the dark at 25±1°C. The explants were transferred to fresh CIM twice after 3 w.

3.3.2 Stage 2: Embryo germination

After 6-8 w, embryogenic calli were subcultured on embryo germination medium (EGM1) (CIM without 2,4-D) (10 pieces/90 mm Petri dish with 30 ml medium). Following embryo germination, calli with shoots approximately 5-10 mm in height were transferred to semi-solid regeneration medium (EGM2) (4 pieces/culture bottle with 20 ml medium) containing ½ strength MS salts and vitamins (Murashige and Skoog, 1962), 5 g l^{-1} sucrose and 9 g l^{-1} agar. Calli with shoots less than 5 mm in length were separated and transferred back to EGM1.

3.4 Shoot multiplication

3.4.1 Multiplication of well-developed shoots

Shoots, approximately 2-3 cm long, were transferred from EGM2 and placed in six liquid media (M1-M6) (1shoot/culture tube with 5 ml medium), each containing full strength MS salts and vitamins (Murashige and Skoog, 1962), 20 g l^{-1} sucrose and different concentrations and combinations of plant growth regulators (PGRs) (Table 5). The cultures were subcultured onto the same media every 3 w.

After 6 w, five different shoot clumps from medium M5 were subcultured in the same fresh medium (1 shoot clump/Magenta vessel with 30 ml medium) for 2 w. Then, the medium M5 was modified (M5A and B) to test other concentrations and combinations of the PGRs, as shown in Table 6. The modified media, together with M5, were tested for shoot multiplication of sugarcane varieties NCo376, N41 and N50.

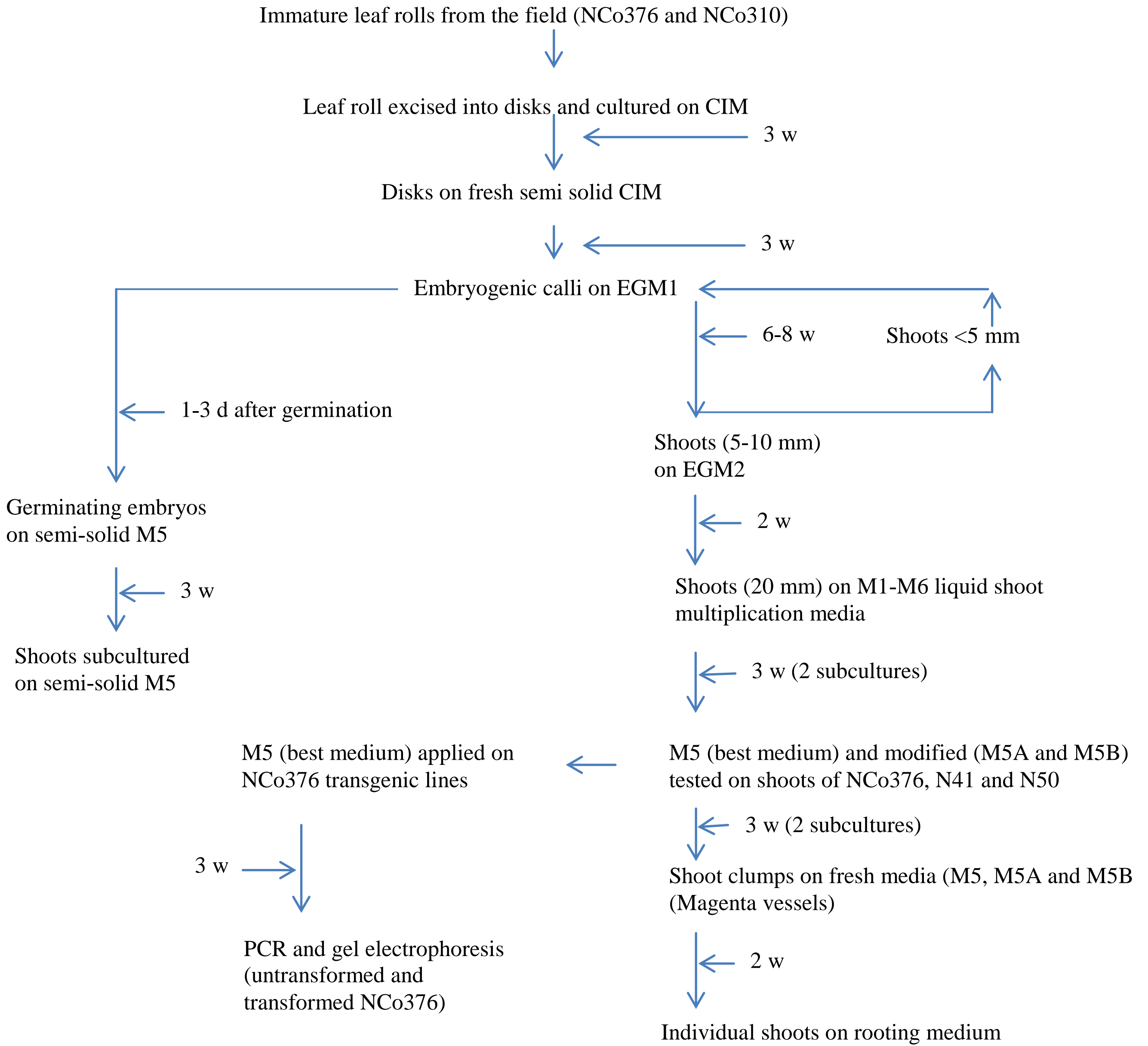


Figure 1. A summary of the experimental design and stages of indirect somatic embryogenesis standard culture procedures followed to establish *in vitro* shoot multiplication protocol. CIM (section 3.3.1), EGM1 and 2 (section 3.3.2), T1 and T2 (section 3.4.2) and M1-M6 (Table 5).

Table 5. Different media formulations used for shoot multiplication of NCo376 and NCo310. All media had full strength MS salts and vitamins and 20 g l^{-1} sucrose. BAP = 6-benzylaminopurine, GA_3 = gibberellic acid, IBA = indole-3-butyric acid, NAA = 1-napththylacetic acid, Kinetin = N-6-furfuryladenanine.

Medium components	**Media**					
(mg l^{-1})	M1	M2	M3	M4	M5	M6
BAP	0	0.1	6	0.5	1	0
Kinetin	0	0.015	1	0.25	0.1	1
NAA	0	0	0	0	1	0
GA_3	0	0	0	0	0	0.5
IBA	0	0	0	0	0	1

Table 6. Formulations of modified media used for shoot multiplication. All media had full strength MS salts and vitamins and 20 g l^{-1} sucrose. IAA = Indoleacetic acid. Other PGRs as in Table 5.

Medium components	**Media**		
(mg l^{-1})	M5	M5A	M5B
BAP	1	1	2
Kinetin	0.1	0.1	0.1
NAA	1	0	1
IAA	0	1	0

3.4.2 Multiplication of shoots during germination

After 3-5 w on EGM1, somatic embryos of NCo376 were separated and individual embryos were transferred (5 embryos/20 mm Petri dish with 3 ml medium) to EGM1 and semi-solid M5 (M5 with 9 g l^{-1} agar).

3.5 Rooting

After 2 w in Magenta vessels, four individual shoots from each medium were separated from the shoot clumps and each placed (1 shoot/culture tube with 5 ml medium) on rooting medium containing ½ strength MS salts and vitamins, 20 g l^{-1} sucrose and 1 mg l^{-1} IBA for 3 w.

3.6 Environmental conditions

All cultures, except those at the callus induction stage and on slow growth storage, were incubated at 25 ^{o}C during the 16 h light and at 23 ^{o}C during the 8 h dark, respectively, with the light period at 200 µmol m^{-2} s^{-1} photon flux density. All media were at pH 5.6-5.8 and were autoclaved for 20 min at 121°C.

3.7 *In vitro* storage of somatic embryos

Somatic embryos of cultivar NCo376 were used as explants for encapsulation in sodium alginate beads. To achieve encapsulation, a matrix consisting of sodium alginate and calcium chloride solutions was prepared as per Katouzi et al. (2011) and Nor Asmah et al. (2011).

Two 1 mM solutions each of $MgCl_2$ and $CaCl_2$ were prepared using $MgCl_2.6H_2O$ and $CaCl_2.2H_2O$. The two solutions were used to make 1 µM MgCa by adding 1 ml of 1 mM $CaCl_2$ into a litre of 1 mM $MgCl_2$. Then, 1 g of sodium alginic acid was dissolved in 100 ml of 1 µM MgCa and the solution was autoclaved for 20 min at 121°C to sterilize and thicken it. For polymerization, 0.1 M $CaCl_2$ was prepared.

About 20 ml each of 0.1 M $CaCl_2$ and alginic acid were each added to 50 ml beakers. Somatic embryos were separated and put in a beaker containing alginic acid. The embryos where then pipetted individually from the alginic acid using a sterile pipette (with tip cut off) and dropped into the beaker containing 0.1 M $CaCl_2$. The droplets, each containing one explant, were then

maintained in this medium for 30 min with slow agitation on a rotary shaker to achieve polymerization of the sodium alginate. Firm beads that formed around each embryo were recovered by decanting the $CaCl_2$ and blotted dry on sterile 90 mm filter paper.

The sodium alginate beads were placed on semi-solid medium (5 beads/20 mm Petri dish with 3 ml medium) containing ½ strength MS salts and vitamins (Murashige and Skoog, 1962), 5 g l^{-1} sucrose and 9 g l^{-1} agar. The Petri dishes were incubated at two temperatures (18 and 25 ^{o}C), for 3 months in the dark. The individual treatments, which were a combination of temperature and time, included T1 (25±1 ^{o}C for 1 month), T2 (18±1 ^{o}C for 1 month), T3 (18±1 ^{o}C for 2 months) and T4 (25±1 ^{o}C for 3 months).

3.8 Molecular analysis

3.8.1 Genomic Deoxyribonucleic Acid (DNA) quantification

DNA was extracted from both the untransformed (NCo376) and transformed NCo376 lines (TG1, TG2, TG3, TG4 and TG5) following the procedure provided in the Qiagen DNeasy Plant Mini kit (Qiagen, Germany). The transgenic lines of NCo376 were produced at SASRI following particle bombardment of calli with the gene of interest (GO1).

Samples of young leaves were collected from both the untransformed and transformed shoots and finely ground in liquid nitrogen in a mortar and pestle. About 150 mg of the finely ground leaf tissue were transferred to Eppendorf tubes. Then 400 µl buffer AP1 and 4 µl RNase A were added individually, without mixing, to the individual samples which were then vortexed and incubated for 10 min at 65°C. The tubes were inverted 2 to 3 times during the incubation period. The buffer AP2 (130 µl) was added to the samples and then mixed well before they were incubated on ice for 5 min and then centrifuged for 5 min at 14 000 rpm.

Following the centrifugation, the lysate was pipetted into a QIAshredder Mini spin column in a 2 ml Eppendorf tube and centrifuged for 5 min at 14 000 rpm. The flow-through fraction was transferred into a new tube without disturbing the pellet, before 1.5X volume buffer AP3/E was added. Mixing was done by pipetting and then 650 µl of the mixture was transferred into a DNeasy Mini spin column in a 2 ml microfuge tube and centrifuged for 1 min at 8000 rpm. The flow-through fraction was discarded and the procedure was repeated with the remaining sample.

The spin column was then placed into new tubes, centrifuged for 1 min at 8000 rpm after the addition of 500 ml buffer AW. At this stage the liquid was discarded, 500 µl buffer AW were added, and then the column was centrifuged for 2 min at 14 000 rpm. The spin column was removed carefully and transferred to a new 1.5 ml tube, after which 40 µl buffer AE was added, then the sample was incubated for 5 min at room temperature and centrifuged for 1 min at 8000 rpm. A nanodrop spectrophotometer (ND-1000, NanoDrop Technologies, Delaware, USA) was used to quantify the genomic DNA.

3.8.2 Polymerase Chain Reaction (PCR)

The genomic DNA samples were diluted with water (1:5) A total of 8 µl of water were added to each sample. For each sample, 2 µl were added individually into a 25 µl PCR reaction (Table 7). Water, reverse (GO1-1057) and forward (GO1-542) primers were used as negative controls and no DNA template controls while a sample of the plasmid (25 ng) was used as the positive control (Table 7). DNA amplification was done using a thermal cycler (Biorad MyCycler). Denaturation of the DNA was achieved by one cycle at 98 ^{o}C and 30 cycles at 95 ^{o}C. This was followed by 30 cycles for annealing or extension at 62 ^{o}C for 30 seconds, 72 ^{o}C for 1 min and one cycle at 72 ^{o}C for 4 min.

3.8.3 Agarose gel electrophoresis

After the PCR reactions, the products were separated by loading 10 µl of the each of the PCR products mixed with 3 µl GelRed^{TM} onto a 1.2% (g v^{-1}) agarose gel. A 1 kb Plus DNA (Fermentas O'GeneRuler^{TM} lader) molecular weight size marker was used. The mixtures were pipetted into the wells of the agarose gel. The DNA was allowed to move from the wells along the length of the gel which was set at 100 V for 5 min and then 85 V for about 45 min before it was viewed using a transluminator.

3.9 Photography

All photographs were taken using a Nikon E4500 camera.

Table 7. Components of the cocktails that were prepared for genomic DNA analysis using polymerase chain reaction (PCR). RP = reverse primer (GO1-1057), FP = forward primer (GO1-542) and positive control = GO1 plasmid.

Component	**Quantity (µl per reaction)**				
	Genomic DNA samples	FP control	RP control	no template	Positive control
KAPA Taq Buffer A	2.5	2.5	2.5	2.5	2.5
2 mM dNTP	2.5	2.5	2.5	2.5	2.5
6 µM FP	0.2	0.2	-	0.2	0.2
6 µM RP	0.2	-	0.2	0.2	0.2
5U/µl KAPA Taq	0.2	0.2	0.2	0.2	0.2
Ambion water	17.4	19.1	19.1	17.4	17.4
DNA sample (25 ng)	2.0	2.0 (plasmid)	2.0 (plasmid)	-	2.0 (plasmid)
Total (µl)	25.0	25.0	25.0	25.0	25.0

3.10 Data collection and statistical analysis

Following callus induction, the number of leaf disks with callus per Petri dish and a qualitative estimate of the amount of white embryogenic callus produced per plate were recorded. After plant regeneration and transfer of individual shoots on shoot multiplication media, the number of shoots produced per shoot was recorded at 3, 6 and 8 w. The numbers of replicates for each treatment were 15 for multiplication and rooting and 5 for plantlet establishment (8 w).

The number of shoots produced per individual embryo was recorded after 3 and 6 w in culture and at 3 w after retrieval from storage, percentage embryos survival was also recorded.

All data were analysed using IBM SPSS statistics version 21 by performing Analysis of Variance (ANOVA) followed by Least Significance Difference (LSD) test to separate significantly different means ($P<0.05$).

4. RESULTS

4.1 *In vitro* somatic embryogenesis in sugarcane

As per previous reports on the same and other sugarcane varieties (Gallo-Meagher et al., 2000; Jimenez, 2001; Snyman et al., 2005; Ikram-ul-Haq and Memon, 2012; Khamrit et al., 2012), callus induction on leaf disks of the sugarcane varieties NCo310 and NCo376 was successful, with 90 to 100% of the leaf disk explants producing callus. The different stages which were followed to produce somatic embryos are shown in Figure 2.

To induce callus production, the leaf disks were place on semi-solid medium containing full strength MS salts and vitamins (Murashige and Skoog, 1962), 20 g l^{-1} sucrose, 0.5 g l^{-1} casein hydrolysate and 3 mg l^{-1} 2,4-D. Browning of the leaf disks due to phenolic production was observed during the first week of initiation. However, most leaf disks developed white nodular calli by the end of 6 w. A considerable difference in terms of quantity of callus produced per disk was noted, with leaf disks of NCo376 producing the most calli. Then calli were subcultured on the same medium, without plant growth regulators (PGRs), for embryo germination. It took 6-8 w for the embryos to develop shoots. Pieces of calli (approx. 10 mm in diameter) with germinating embryos were subcultured (4 pieces/culture bottle with 20 ml medium) after 6-8 w on medium containing ½ strength MS salts and vitamins, 5 g l^{-1} sucrose and 9 g l^{-1} agar for shoot growth. NCo376 produced more shoots (55.0 ± 3.5 shoots/disk) than NCo310 (30.0 ± 4.0 shoots/disk), which relates to the amount of callus that the disks formed.

4.2 Establishment of a protocol for *in vitro* shoot multiplication using sugarcane varieties NCo376 and NCo310

The preliminary investigations to establish a high yielding shoot multiplication protocol were undertaken with the varieties NCo376 and NCo310. Individual shoots (20 mm in height), produced from germinated somatic embryos, were placed in six liquid media (M1-M6). Phenolic substances developed during the first week of culture (Table 8) resulting in the media turning brownish in colour. However, the shoots did not die as a result of phenolics (Table 8). The percentage of shoots that survived in the presence of phenolic substances was high in most cases with those on M1, M2 and M5 for NCo376 having a 100% survival while the rest had more than 80% survival (Table 8).

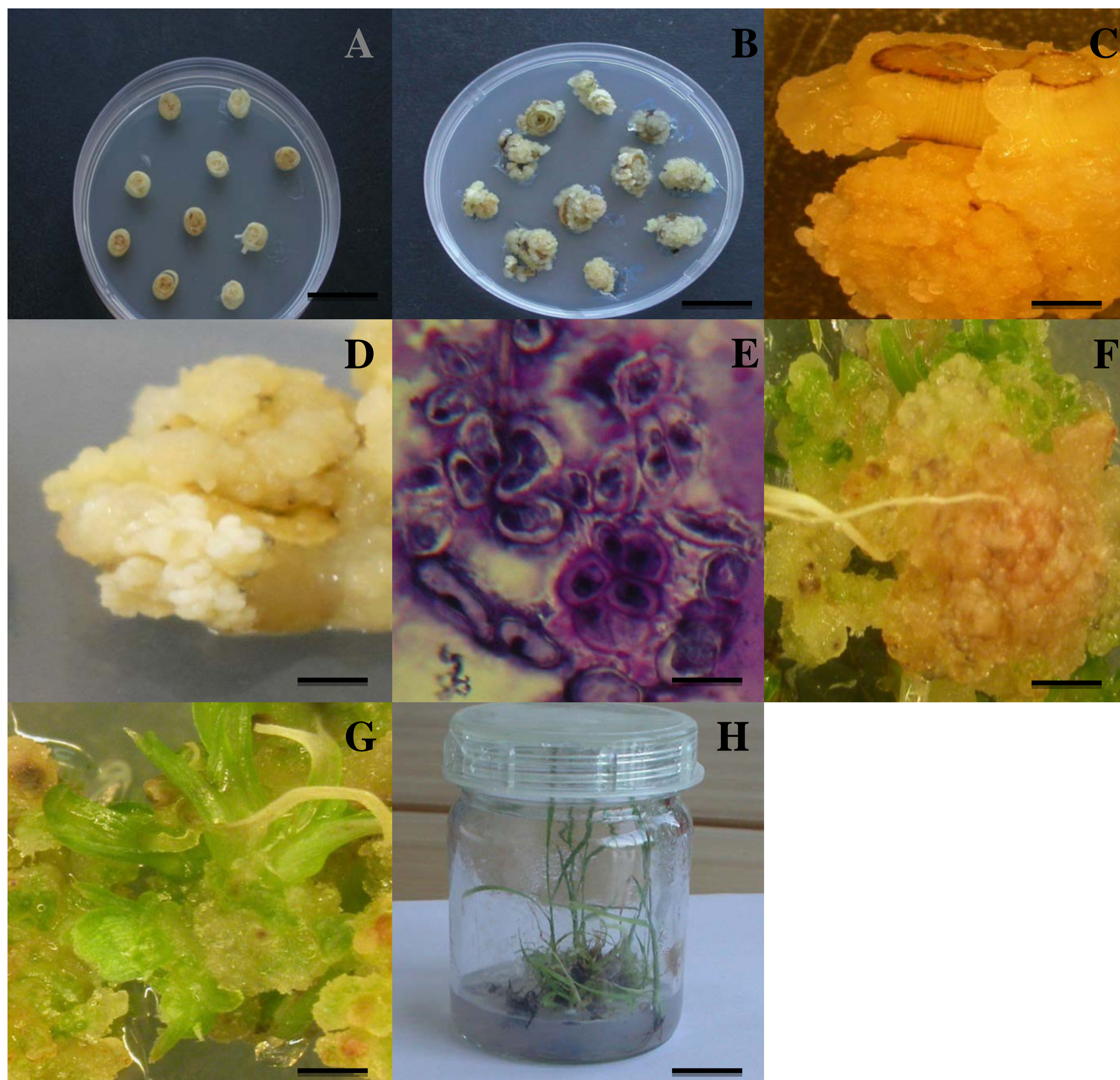


Figure 2. Different stages of indirect somatic embryogenesis recorded for NCo310 and NCo376. (A) immature leaf disks on callus induction medium (CIM), bar = 23 mm; (B) callus developing from the leaf disks on CIM, bar = 20 mm; (C) piece of non-embryogenic callus viewed with a dissecting microscope, bar = 4 mm; (D) piece of embryogenic callus, bar = 3.4 mm; (E) stained embryogenic cells viewed with a compound microscope, bar = 0.003µm; (F) germinating somatic embryos, bar = 3.2 mm; (G) germinating somatic embryos, bar = 3.2 mm; (H) shoots, bar = 12 mm.

Table 8. The effect of the presence of phenolics in the media on shoot multiplication of NCo376 and NCo310 after 3 and 6 w. Phenolics were observed a week after the shoots were cultured on the media M1-M6. Statistical analyses of data were carried out using ANOVA and mean separation using LSD test (n = 15). Different upper case (A-C) and lower case letters (a-c) indicate significant differences amongst corresponding means of the media at 3 and 6 w, respectively (P < 0.05).

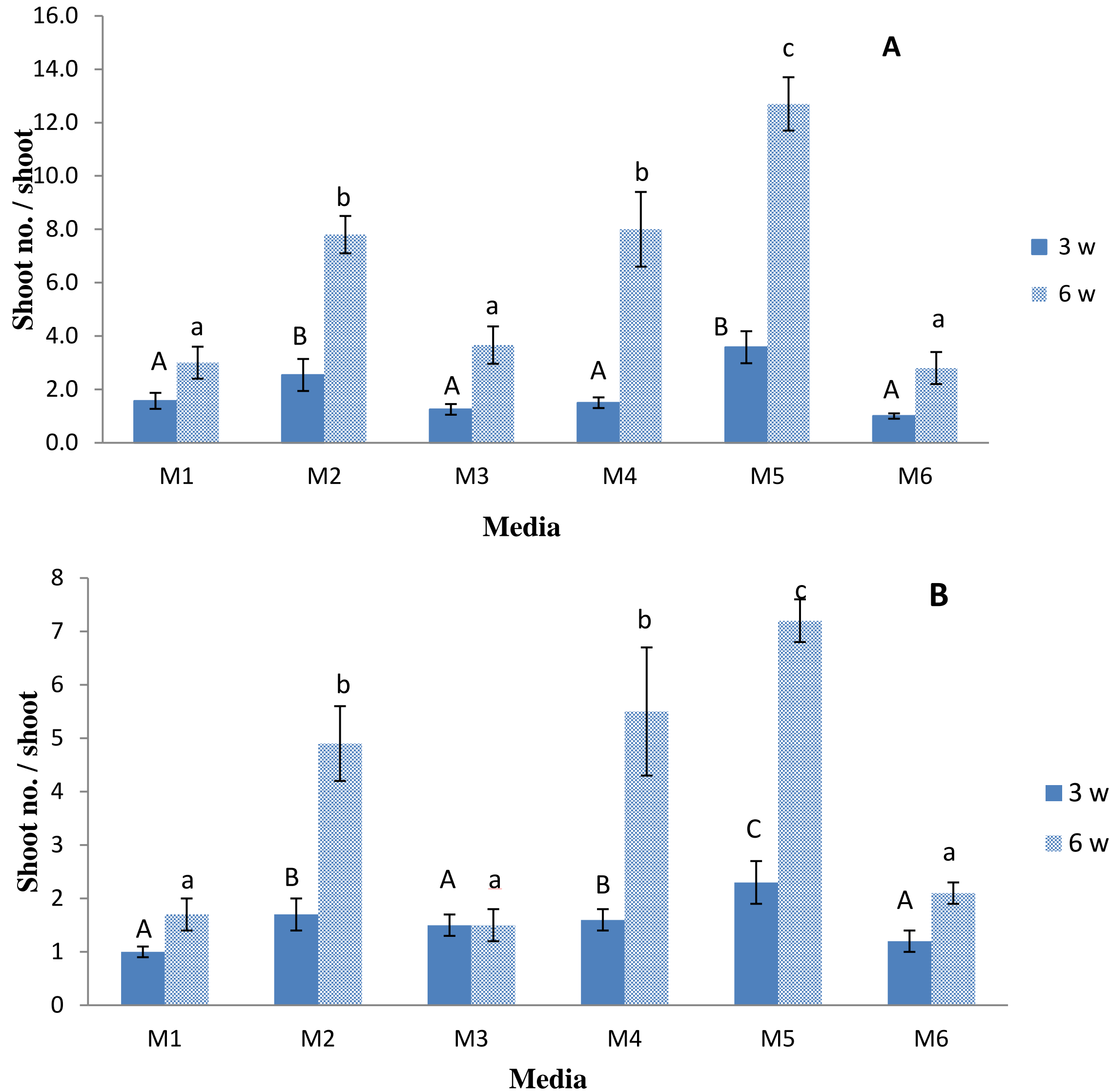
	NCo376						**NCo310**					
	% dead plants		**% dead with phenolics**		**% survival plants with phenolics**		**% dead plants**		**% dead with phenolics**		**% survival plants with phenolics**	
Media	3 w	6 w	3 w	6 w	3 w	6 w	3 w	6 w	3 w	6 w	3 w	6 w
M1	6.7^{A}	0.0^{a}	6.7^{A}	0.0^{a}	93.3^{A}	100.0^{a}	0.0^{A}	0.0^{a}	0.0^{A}	0.0^{a}	100.0^{A}	100.0^{a}
M2	0.0^{B}	0.0^{b}	0.0^{B}	0.0^{b}	100.0^{B}	100.0^{b}	0.0^{B}	6.7^{b}	0.0^{B}	6.7^{b}	100.0^{B}	93.3^{b}
M3	16.7^{A}	14.3^{a}	8.3^{A}	14.3^{a}	91.7^{A}	85.7^{a}	16.7^{A}	16.7^{a}	16.7^{A}	16.7^{a}	83.3^{A}	83.3^{a}
M4	6.8^{A}	6.7^{b}	6.7^{A}	6.7^{b}	93.3^{A}	93.3^{b}	0.0^{B}	6.7^{b}	0.0^{B}	6.7^{b}	100.0^{B}	93.3^{b}
M5	6.7^{B}	0.0^{c}	0.0^{B}	0.0^{c}	100.0^{B}	100.0^{c}	13.3^{C}	0.0^{c}	6.7^{C}	0.0^{c}	93.3^{C}	100.0^{c}
M6	16.7^{A}	6.7^{a}	13.3^{A}	6.7^{a}	86.7^{A}	93.3^{a}	6.7^{A}	6.7^{a}	6.7^{A}	6.7^{a}	93.3^{A}	93.3^{a}

Swelling of shoot bud primordia was observed within the first 3 d of culture, an observation similar to that made by Pathak et al. (2009). There were significant statistical differences ($P < 0.05$) amongst the media for the average number of shoots produced per shoot at 3 w for NCo376 (Figure 3A, Appendix 1). The number of shoots produced per shoot on media M2 and M5 was not significantly different. However, it was significantly higher compared with those produced on M1, M3, M4 and M6 (Figure 3A).

For NCo310, significant differences were also observed amongst the media in terms of shoot yield per shoot at 3 w (Figure 3B, Appendix 2). However, in contrast to NCo376, which produced the same number of shoots on media M2 and M5 ($P < 0.05$), NCo310 produced the highest number of shoots per individual shoot on M5, followed by M2 and M4. As shown in Figure 3B, the other media produced the lowest shoot yields.

After 3 w, the whole shoot clumps from each medium were subcultured onto the same fresh medium. Phenolic substances were still observed in the media but had no negative effect on shoot survival (Table 8). For both varieties, at 6 w in culture, shoots continued to multiply in the culture tubes. The dimensions of the proliferating clumps differed (Figures 4 and 5) depending on the ability of each medium to induce shoots.

At the end of 6 w, statistical analyses showed significant differences with respect to the number of shoots produced per individual shoot amongst the media for either varieties ($P < 0.05$) (Figure 3, Appendices 3 and 4). Medium M5 produced the highest number of shoots yielding 12.7 ± 4.0 and 7.2 ± 3.4 shoots per shoot for NCo376 and NCo310, respectively. There was no statistical difference between the number of shoots produced on M2 and M4 for both varieties at 6 w ($P > 0.05$). The two media produced 7.8 ± 2.0 and 8.0 ± 5.3 shoots per single shoot for NCo376, respectively, and 4.9 ± 2.8 and 5.5 ± 5.1, for NCo310, respectively (Figure 3). The other media (M1, M3 and M6) produced the lowest number of shoots for both varieties. Similar yields of shoots as those produced on M5 were obtained by Roy and Kabir (2009) using sugarcane cultivar lsd32.



M1 = no plant growth regulators (PGRs)

M2 = 0.1 mg l^{-1} BAP, 0.015 mg l^{-1} kinetin

M3 = 6 mg l^{-1} BAP, 1 mg l^{-1} kinetin

M4 = 0.5 mg l^{-1} BAP, 0.25 mg l^{-1} kinetin

M5 = 1 mg l^{-1} BAP, 0.1 mg l^{-1} kinetin, 1 mg l^{-1} NAA

M6 = 1 mg l^{-1} IBA, 1 mg l^{-1} kinetin, 0.5 mg l^{-1} GA3

Figure 3. The effect of plant growth regulator combinations on shoot multiplication of (A) NCo376 and (B) NCo310 shoots after 3 and 6 w on liquid media. All media had full strength MS salts and vitamins and 20 g l^{-1} sucrose. Shoots (approx. 20 mm in height) were used and subcultured onto the same medium after 3 w. Statistical analyses of data were carried out using Analysis of Variance (ANOVA) and mean separation, using Least Significance Difference (LSD) tests (n = 15). Different upper case (A-C) and lower case letters (a-c) indicates significant mean differences amongst the media at 3 and 6 w, respectively ($P < 0.05$).

The presence of the auxin NAA, at 1 mg l^{-1}, considerably increased the number of shoots produced resulting in M5 inducing the highest number of shoots for both varieties. In contrast to this, a high concentration of the cytokinin BAP (6 mg l^{-1}) (M3) and the combination of an auxin (IBA), a cytokinin (Kinetin) and GA_3 (M6) produced the lowest shoot yield for both varieties. Similar low yields of shoots were produced on M1,which had no plant growth regulators (Figure 3).

The shoot multiplication rate (Table 9) was determined from the results in Figure 3. The highest shoot multiplication rate at both 3 and 6 w was on M5 for both varieties: 2.3 and 9.1 for NCo376 and 2.3 and 4.9 for NCo310.

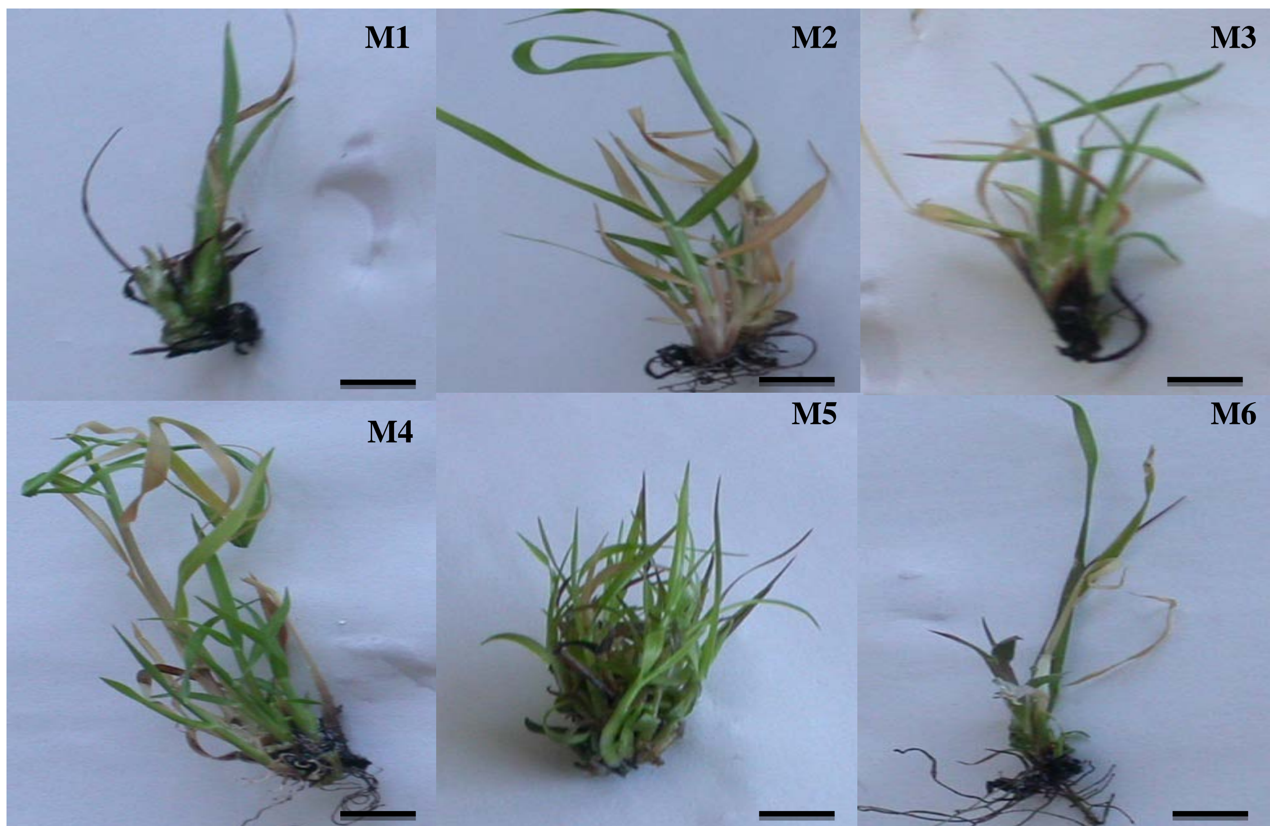


Figure 4. Visual representation of the effect of the tested media (M1-M6) on shoot multiplication of NCo376 after 6 w. Scale bars: M1 = 13.5 mm; M2 = 16.3 mm; M3 = 11 mm; M4 = 13.8 mm; M5 = 13.3 mm; M6 = 13.8 mm.

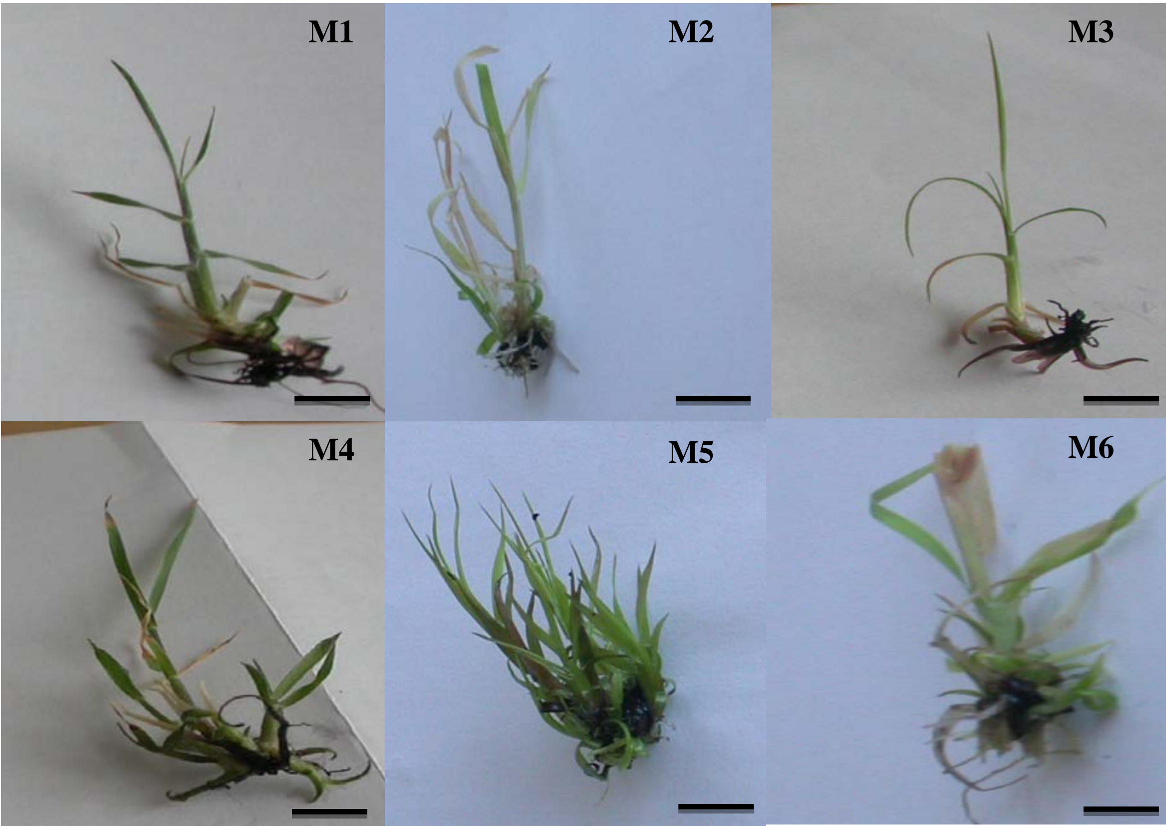
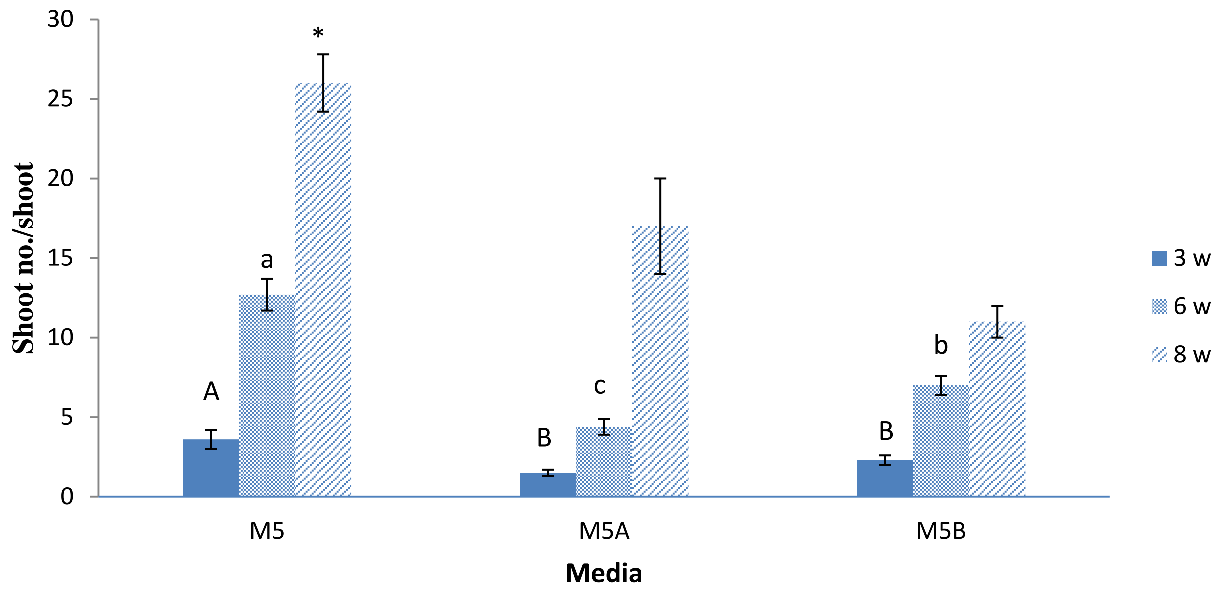


Figure 5. Visual representation of the effect of the tested media (M1-M6) on shoot multiplication of NCo310 after 6 w. Scale bars: M1 = 14.4 mm; M2 = 15.0 mm; M3 = 15.1 mm; M4 = 14.4 mm; M5 = 13.3 mm; M6 = 10.6 mm.

Table 9. The effect of media composition on the multiplication rate from each medium after each 3 w subculture for varieties NCo376 and NCo310. Media was the same as in Figure 3. S1 = subculture 1 and S2 = subculture 2. M1-M3 as in Figure 3.

Variety	**Multiplication rate/3 w**											
	Media											
	M1		M2		M3		M4		M5		M6	
	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2
NCo376	1.6	1.9	2.5	3.0	1.3	2.9	1.5	3.2	3.6	3.5	1.0	2.8
NCo310	1.0	1.7	1.7	2.8	1.5	1.0	1.6	3.0	2.3	3.1	1.2	1.8

As M5 promoted the highest shoot yields (Figure 3), this medium was then modified by altering the amount of PGRs and the modifications were tested with shoots of NCo376 (Figure 6). The medium was modified by increasing the concentration of BAP to 2 mg l^{-1} (M5B) and replacing NAA with another auxin, IAA, without changing the concentration (M5A). Further, at the end of the two 3 w periods (as before), the shoots were then subcultured for 2 w on same media in Magenta vessels.



M5 = 1 mg l^{-1} BAP, 0.1 mg l^{-1} kinetin, 1 mg l^{-1} NAA

M5A = 1 mg l^{-1} BAP, 0.1 mg l^{-1} kinetin, 1 mg l^{-1} IAA

M5B = 2 mg l^{-1} BAP, 0.1 mg l^{-1} kinetin, 1 mg l^{-1} NAA

Figure 6. The effect of modifications to Medium 5 on shoot multiplication of NCo376 after 3, 6 and 8 w on liquid media. All media had full strength MS salts with vitamins and 20 g l^{-1} sucrose. Shoots (approx. 20 mm in height) were cultured on the media with two 3 weekly subcultures followed by a 2 w subculture. Statistical analyses of data were carried out using ANOVA and mean separation, using LSD test (n = 5-15). Different upper case (A-B) and lower case letters (a-c) and * indicate significant mean differences amongst the media at 3, 6 and 8 w, respectively ($P < 0.05$).

At 3 w, significant mean differences with respect to the shoot multiplication were observed amongst the three media (Appendix 5). However, none resulted in the production of more shoots than M5 (Figure 6). Similarly, at 6 w, M5 had induced significantly the highest number of shoots followed by M5B, while M5A produced the lowest (Figures 6 and 7, Appendix 6). At 6 w, the shoot clumps produced on each of the media were then subcultured onto the same media in Magenta vessels (Figure 8) for a further 2 w. This was done to attempt to increase the shoot yield further.

The shoot multiplication rates at 3 and 6 w were determined from the results in Figure 6. The highest multiplication rates were observed on M5, which had 3.6 and 3.5 shoots/3 w (Table 10).

Table 10. The effect of modifications to M5 on shoot multiplication rate of each medium after each 3 w subculture for variety NCo376. Media was the same as in Figure 6. S1 = subculture 1 and S2 = subculture 2. M5, M5A and M5B as in Figure 6.

Media	**Multiplication rate/3 w**	
	S1	S2
M5	3.6	3.5
M5A	1.5	2.9
M5B	2.3	3.0

At 8 w, the media also resulted in significant differences in terms of total shoot yield with M5 producing a total average yield of 26 ± 1.8 shoots per shoot (Figure 6, Appendix 7). This was significantly higher compared with 17 ± 3.0 and 11 ± 1.0 shoots produced on M5A and M5B, respectively (Figure 6). There were no statistical differences between the total number of shoots per shoot produced on M5A and M5B at 8 w.

The shoots in the clumps that were produced after 8 w were then separated and individual shoots (approx. 6.5 cm in height) from each medium, were transferred to a rooting medium (Figures 9 and 10). There were no significant differences amongst the media with regard to rooting and more than 80% of the individual shoots from each medium rooted.

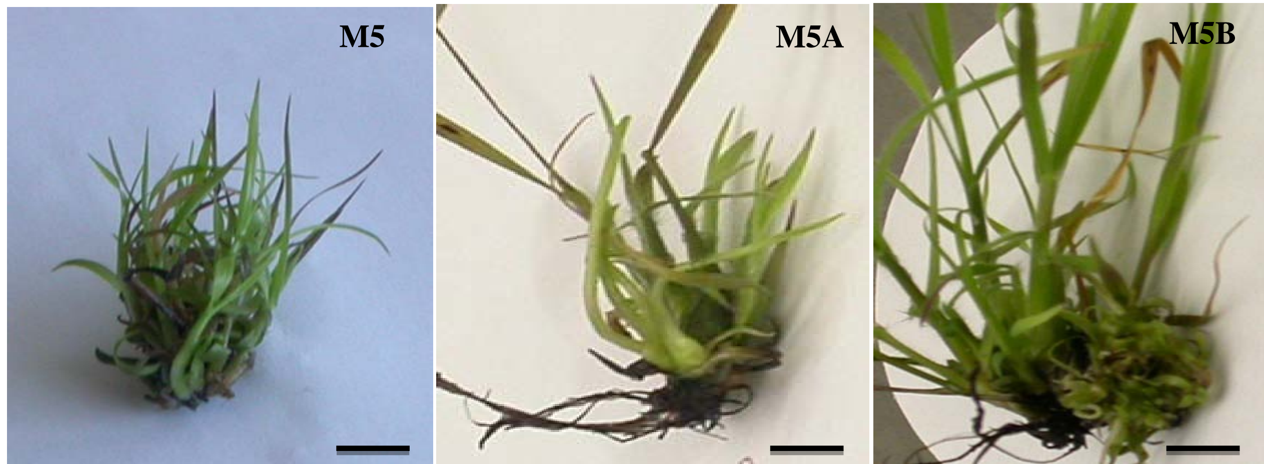


Figure 7. Visual comparison of the effect of M5 and the modified media on shoot multiplication of NCo376 after 6 w. Scale bars: M5 = 13.5 mm; M5A = 16.0 mm; M5B = 16.1 mm.

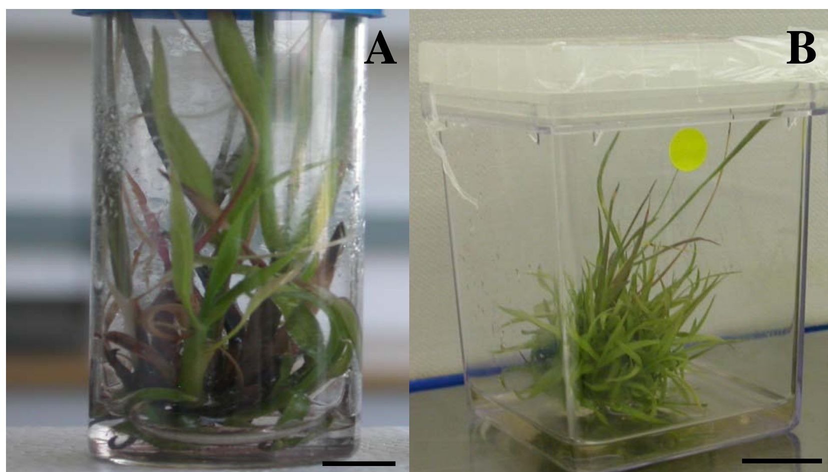


Figure 8. A visual comparison of shoot clumps at 6 w in culture tubes (A) and at 8 w in Magenta vessels (B). Scale bars: A = 16.5 mm and B =17.3 mm.

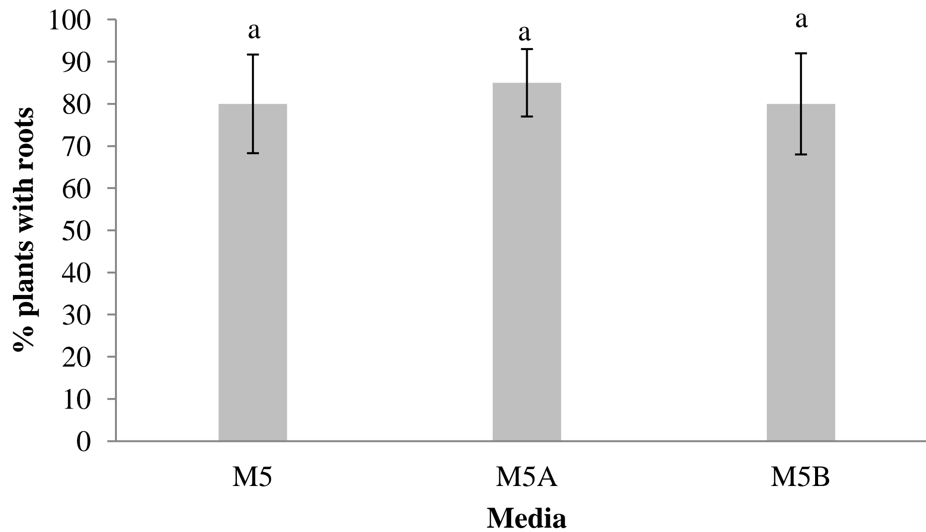


Figure 9. The effect of the composition of shoot multiplication media on rooting of NCo376. M5, M5A and M5B as in Figure 6. Individual shoots (approx. 65 mm shoot height) were separated from the shoot clumps and subcultured on rooting medium containing ½ strength MS salts and vitamins, 5 g l^{-1} sucrose and 1 mg l^{-1} IBA. Statistical analyses of data were carried out using ANOVA and mean separation using LSD test (n = 15). Similar lower case letters indicates no significant mean differences amongst the media at 3 w ($P > 0.05$).

Figure 10. A rooted shoot of variety NCo376. Bar = 11.0 mm.

4.3 Testing the protocol

4.3.1 Application to other varieties (N41 and N50) with respect to plantlet yield.

The previous studies showed that the best shoot multiplication protocol involved placing 20 mm long shoots on M5 for two 3 w periods (5 ml M5 in culture tubes), followed by a 2 w subculture in Magenta vessels (30 ml M5), and then rooting. This procedure was then tested on the sugarcane varieties N41 and N50, but the modifications to M5 were also tested in case there were genotypic differences.

Shoots (approx. 20 mm in height) were cultured on the media, as described previously. Phenolic substances were also observed in the media, but as observed before, did not have a negative effect on survival of the shoots. At 3 w, there were no significant differences in terms of the average number of shoots produced per individual shoot amongst the three tested media for either varieties (Figure 11, Appendix 8 and 9). There were also no significant differences at 6 w for N50 (Figure 11B, Appendix 11). However, for N41, at 6 w, M5 and M5B produced a significantly higher average shoot yield per shoot compared with M5B (Figure 11A, Appendix 10).

The shoot multiplication rates determined from the results in Table 11 showed M5 to promote the highest rate for both varieties at both 3 and 6 w *viz.* 2.1 and 3.6 for N41, respectively and 1.7 and 2.4 for N50, respectively (Table 13).

Table 11. The effect of M5 and modified media on multiplication rate after each 3 w subculture for varieties N41 and N50. S1 = subculture 1 and S2 = subculture 2. M5, M5A and M5B as in figure 6.

Variety	**Multiplication rate/3 w**					
	Media					
	M5		M5A		M5B	
	S1	S2	S1	S2	S1	S2
N41	2.1	3.6	1.3	2.8	1.7	3.2
N50	1.7	2.4	1.0	2.3	1.7	2.4

For variety N41, at 8 w, after transfer of the shoot clumps onto fresh medium in Magenta, there were significant differences in terms of total average shoot yield per clump (Figure 11A, Appendix 13). M5 and M5B produced significantly higher number of shoots than M5A for N41. For N50, at 8 w, M5 also produced significantly the highest total average number of shoots per clump followed by M5B, while M5A produced the lowest (Figure 11B, Appendix 14).

4.3.2 Application to shoots of newly-germinating embryos

In this study, the aim was to investigate if M5 could be used to multiply very young shoots, i. e. immediately after embryo germination. This was done to try and minimize the time required in culture.

Embryos for NCo376 were separated and individually cultured (5 embryos/20 mm Petri dish with 3 ml medium) on two semi-solid media (normal regeneration medium and M5). Shoots were observed on the germinating embryos between 1 and 2 w after culture initiation. The shoots were allowed to multiply for 3 w after which they were subcultured onto same medium.

At 3 w, the number of shoots produced per shoot on M5 using young shoots (soon after embryo germination) (5.0 ± 0.4) was significantly higher than those with older shoots (20 mm long) (3.6 ± 0.6). However, at 6 w, the shoot yield produced on M5 using young shoots (11.0 ± 2.0) was the same as that with older shoots (12.7 ± 0.4). Although the final shoot yield produced on M5 with both young and older shoots at 6 w (from the time the shoots were cultured on M5) was the same, usable shoots were obtained 6 w earlier using young shoots (from callus induction) (Figure 16; p 66).

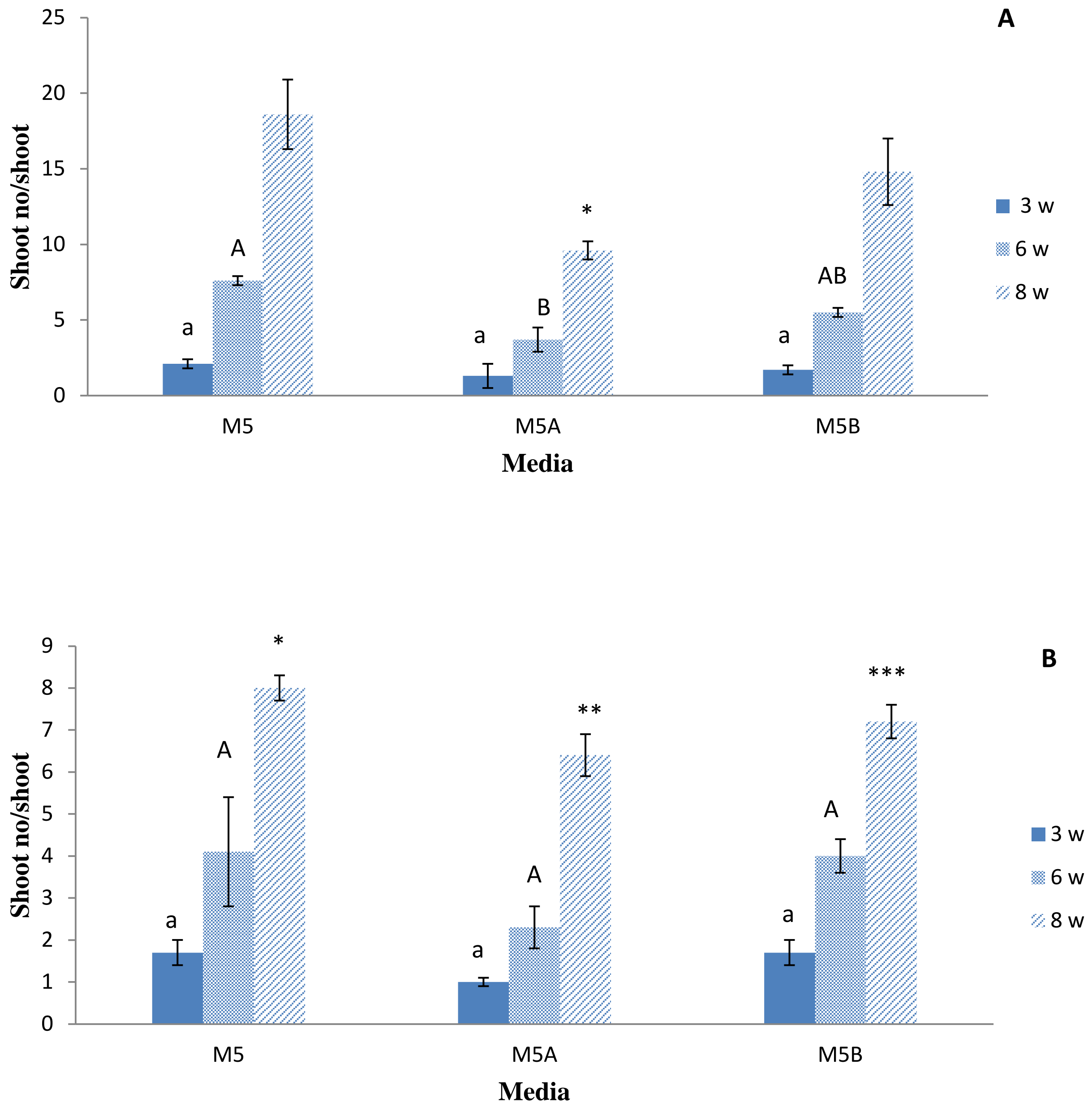


Figure 11. The effect of M5 and modified media on shoot multiplication of (A) N41 and (B) N50 after 3, 6 and 8 w. All media were the same as detailed in Figure 6. Shoots (approx. 20 mm in height) were cultured on the media for two 3 w subcultures, followed by a 2 w subculture. Statistical analyses of data were carried out using ANOVA and mean separation, using LSD test (n = 5-15). Different upper case (A-B), lower case letters (a-c) and * indicates significant mean differences amongst the media at 3, 6 and 8 w, respectively ($P < 0.05$).

4.3.3 Application to transgenic shoots

To test that the transgene was stably maintained in each shoot during multiplication using the developed multiplication protocol, transgenic shoots of NCo376 lines with a known transgene were used.

Shoots of the transgenic lines were cultured on the medium for 3 w. There were no significant differences amongst the lines in terms of the number of shoots produced per shoot at 3 w (Figure 12) and the multiplication rate was an average of 3 shoots/3 w. This shoot multiplication rate was lower than that for untransformed NCo376 (3.6 shoots/3 w) and higher than that for N41 and N50, which produced 2.1 and 1.7 shoots/3 w, respectively, when tested on well-developed shoots on the same medium

After 6 w on multiplication medium, genomic DNA was extracted from young leaves of untransformed NCo376 and the transformed lines. The DNA was amplified using end point Polymerase Chain Reaction (PCR) after which the products were separated using agarose gel electrophoresis (Figure 13). The presence of the transgene in all the transgenic lines (lanes 3-10) was confirmed while the untransformed NCo376 did not have the gene (lanes 13 and 14) (Figure 13).

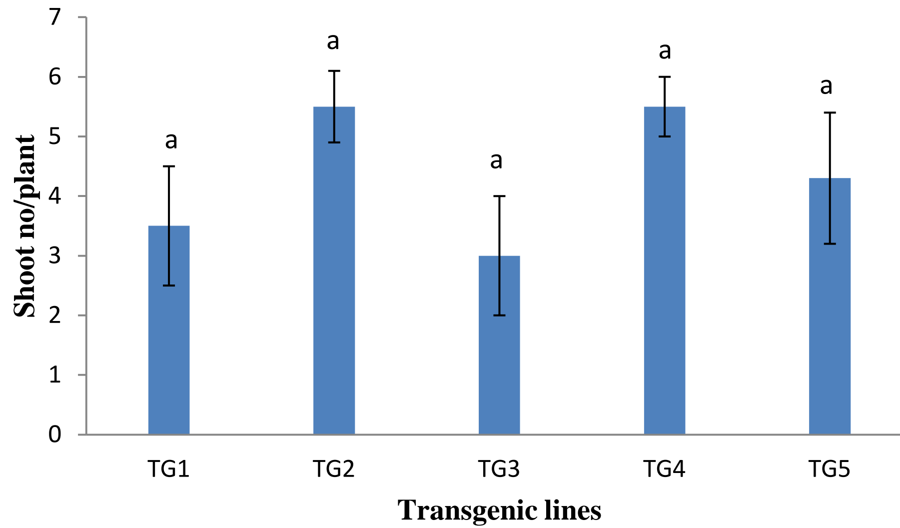
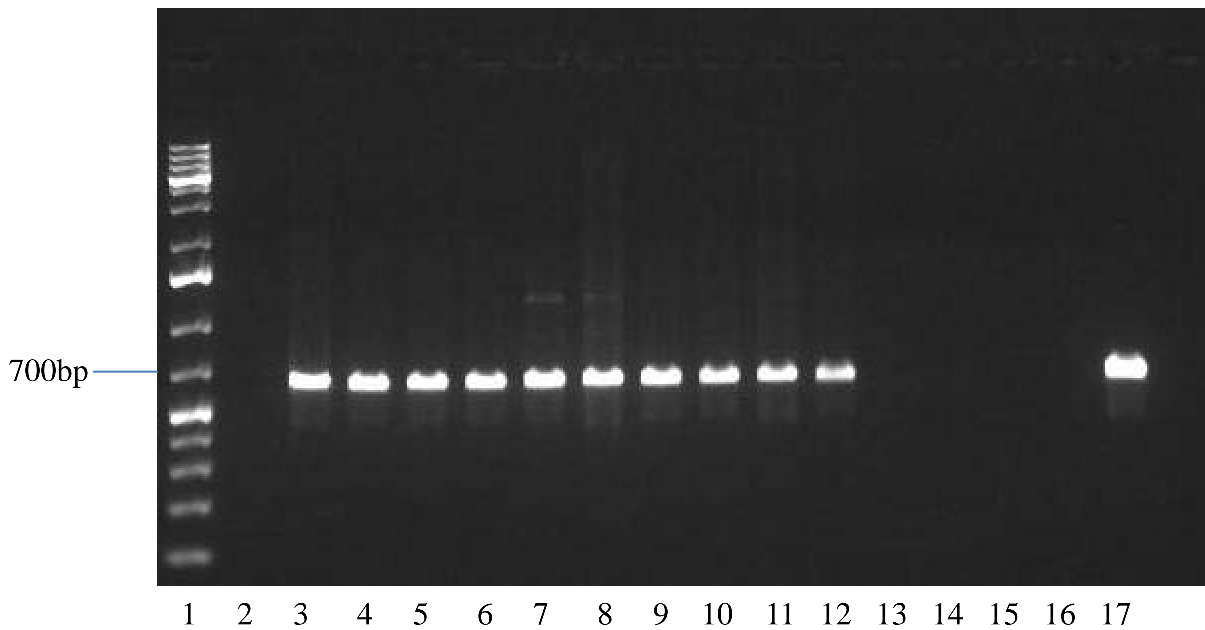


Figure 12. Shoot multiplication of different transgenic lines of NCo376 on medium M5 at 3 w. Single transgenic shoots (approximately 20 mm in height) were used and subcultured onto fresh the same fresh medium after 3 w. Statistical analyses of data were carried out using ANOVA and mean separation using LSD test ($n = 4$). A similar lower case letter indicates no significant mean differences amongst transgenic lines ($P > 0.05$).



PCR products

Figure 13. Agarose gel electrophoresis of end point PCR products of DNA extracted from transformed and untransformed shoots of variety NCo376. The PCR products for both the untransformed and transformed lines were loaded onto a 1.2% agarose gel. bp = base pair, lane 1 = 1 kb plus O'Gene™ Ruler, lane 2 = no template control, lanes 3 and 4 = TG1, lanes 5 and 6 = TG2, lanes 7 and 8 = TG3, lanes 9 and 10 = TG4, lanes 11 and 12 = TG5, lanes 13 and 14 = untransformed NCo376, lane 15 = forward primer control, lane 16 = reverse primer control and lane 17 = positive plasmid control.

4.4 *In vitro* storage of somatic embryos

Another objective was to establish a protocol for the short-term storage of sugarcane somatic embryos. According to previous reports (Paul and Duvik, 1993; Paulet et al., 1993; Watt et al., 2009), such a method could prove useful when there is a need for short-term storage of somatic embryos developed from genetic modification events.

Somatic embryos of the variety NCo376 were encapsulated in sodium alginate beads and stored at room temperature and 18 ^{o}C for 3 months in the dark on a medium containing ½ strength MS salts and vitamins (Murashige and Skoog, 1962), 5 g l^{-1} sucrose and 9 g l^{-1} agar.

At the end of each month, the embryos were removed from storage and subcultured on normal regeneration medium. At room temperature, the embryos did not survive after 1 month. For all treatments, those that did not die during storage germinated, and produced shoots within a week upon transfer to the regeneration medium (Figure 15). There were significant differences amongst the treatments in terms of percentage embryo germination per treatment (Appendix 16), with embryos that were stored at 18 ^{o}C for 1 month having significantly the highest percentage germination (65 ± 6.7%), but not different from the control (Figure 14). There were no statistical differences between percentage embryo germination for control (53.3 ± 6.7%) and 2 months at 18 ^{o}C (40.0 ± 5.8%), but these were significantly higher compared with 3 month storage (20 ± 5.6%).

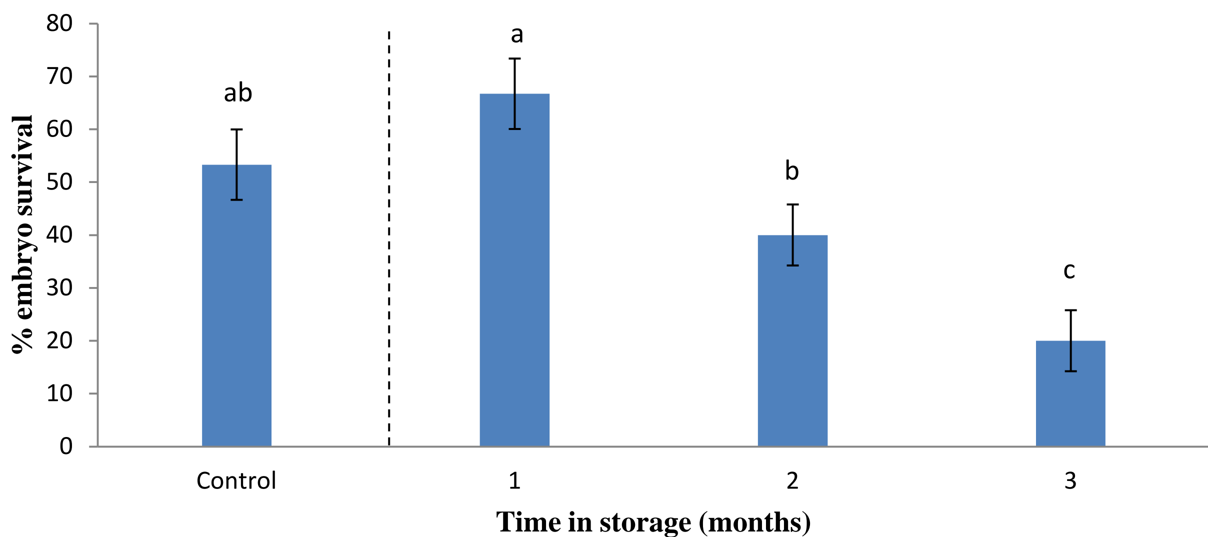


Figure 14. The effect of storage time (months) at 18 ^{o}C on survival of somatic embryos of NCo376. Control was stored for 1 month at 25 ^{o}C. Results for embryo germination were determined after 3 w on regeneration medium. Statistical analyses of data were carried out using ANOVA and mean separation using LSD test (n = 15). Different lower case letters (a-c) indicates significant differences amongst the treatments ($P < 0.05$).

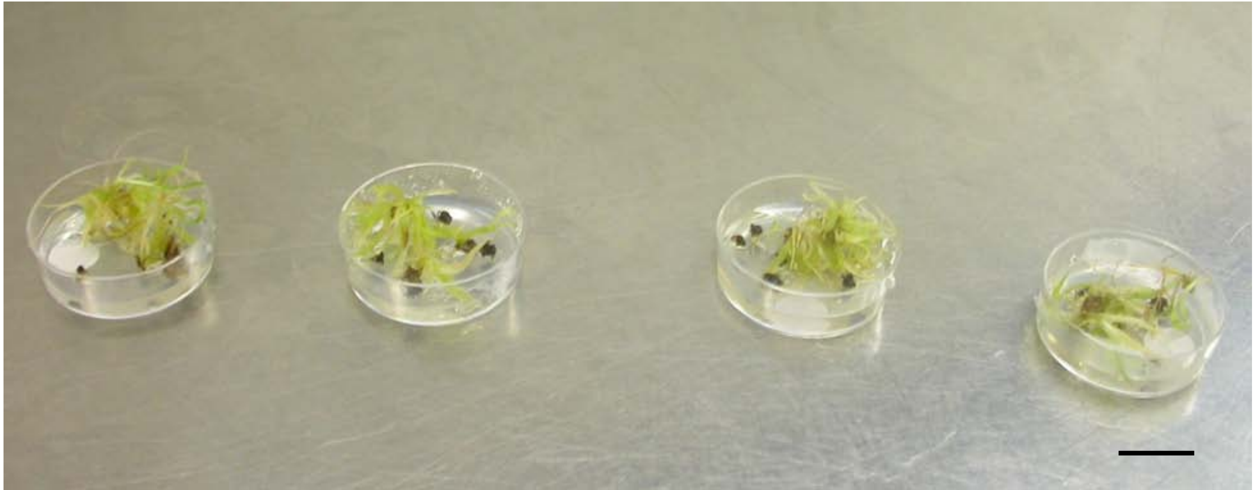


Figure 15. Well-developed shoots from germinated embryos of NCo376 1 month after retrieval from storage at 18 ^{o}C. Scale bar = 4.6 mm.

5. DISCUSSION

5.1 Establishment of *in vitro* shoot multiplication protocol

As emphasized previously, genetic improvement and selection to produce cultivars with traits of interest is difficult in sugarcane because of its complex polyploid and aneuploid genome (Roach, 1989; D'Hont et al., 1998; Jackson, 2005; Lakshmanan et al., 2005). Techniques for genetic transformation (Bower and Birch, 1992; Arencibia et al., 1995; Bower et al., 1996; Arencibia et al., 1998; Lakshmanan et al., 2005; Sengar et al., 2011; Rivera et al., 2012) and *in vitro* mutagenesis (Majid et al., 2001; Ali et al., 2007b; Khan et al., 2007; Patade and Suprasanna, 2008; Patade et al., 2008; Khan et al., 2009; Bairu, 2011) are, therefore, being explored for sugarcane. However, according to the literature (Bower and Birch, 1992; Bower et al., 1996; Gallo-Meagher and Irvine, 1996; Behera and Sahoo, 2009; Kim et al., 2012; Taparia et al., 2012), very few plants (1-3) are obtained per single transformation event.

Furthermore, as the plants produced from these transformation events are presently multiplied through conventional means, it takes a long time (Figure 16) to bulk up and get enough material for genetic and phenotypic analysis (Lee, 1987; Chengalryan and Gallo-Meagher, 2001; Singh et al., 2006; Snyman et al., 2006; Snyman et al., 2011b). This is because the *in vitro* plants have to establish well *in vitro*, followed by acclimatization in the greenhouse and then multiplication using setts, before there are a sufficient number of clones for field testing for the traits of interest. Establishment of an efficient shoot multiplication protocol to multiply and get sufficient material for analysis in a relatively short period of time is, therefore, necessary. Such was one of the objectives of this study.

Indirect somatic embryogenesis protocols are well established (Snyman et al., 2000; Snyman, 2004; Snyman et al., 2006) and are routinely used for plantlet regeneration after genetic transformation (Bower and Birch, 1992; Bower et al., 1996; Gallo-Meagher and Irvine, 1996; Joyce and McQualter, 1998; Rangel et al., 2003; Sooknandan et al., 2003), induced mutagenesis (Ali et al., 2007b; Kenganal et al., 2008; Patade and Suprasanna, 2009; Khan and Khan, 2010; Koch et al., 2012; Munsamy et al., 2013) and conservation of genotypes of potential interest (Paul and Duvik, 1993; Taylor and Dukic, 1993; Watt et al., 2009) of sugarcane. In this study,

callus was successfully induced on immature leaf explants on a medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) while plantlet regeneration was achieved on the same medium without any plant growth regulators (PGRs). According to Chengalryan and Gallo-Meagher (2001), rapid callus formation has been obtained mostly from young expanding leaves (leaf disks) or immature inflorescences. In most studies involving the production of plants via indirect somatic embryogenesis, callus was induced in the presence of 2,4-D (Chengalryan and Gallo-Meagher, 2001; Ikram-ul-Haq and Memon, 2012) while plant regeneration was reported on medium with reduced auxin concentration or no auxin (Ahloowalia and Meretzki, 1983; Bower et al., 1996; Watt et al., 2009; Shiromani et al., 2011; Koch et al., 2012). The present study followed a similar approach resulting in a total of 55.0 ± 3.5 and 30.0 ± 4.0 shoots/leaf disk for NCo376 and NCo310, respectively. As total number of shoots regenerated for NCo376 was approximately two-fold more than those produced by NCo310, it suggests strong genotypic differences. Hence, the somatic embryogenesis protocol for NCo310 needed to be optimized by, for example, testing different concentrations of 2,4-D or other auxins (Table 1).

The use of liquid medium for shoot multiplication has been reported in a number of plant species including sugarcane (Ali et al., 2008; Biradar et al., 2009; Pathak et al., 2009; Yadav et al., 2012). It is commonly used for shoot multiplication in sugarcane because it allows for more rapid growth and development of plants (Watad et al., 1996; Lorenzo et al., 1998). The other advantages of a liquid substrate over a semi-solid one include reduced cost of media preparation, lack of impurities from the solidifying agent and greater efficiency in transferring plantlets to the *ex vitro* environment (Watad et al., 1996; Lorenzo et al., 1998; Hung et al., 2006; Mehrotra et al., 2007; Snyman et al., 2011a).

In this study, six liquid media with different combinations and concentrations of plant growth regulators were tested on well-developed shoots (approximately 20 mm in height) of both varieties NCo376 and NCo310. The best shoot yield was obtained on medium M5, which contained full strength MS salts and vitamins (Murashige and Skoog, 1962), 20 g l^{-1} sucrose,1 mg l^{-1} BAP, 0.1 mg l^{-1} kinetin and 1 mg l^{-1} NAA (Figure 3). This medium resulted in the production of 12.7 ± 4.0 and 7.2 ± 3.4 shoots per shoot for NCo376 and NCo310, respectively, which was significantly higher than the yield obtained with the other media tested (Figure 3).

Similar results were obtained by Pathak et al. (2009) who reported that liquid MS medium supplemented with BAP, kinetin and NAA (0.5 mg l^{-1} each) was most suitable for producing the optimum number of shoots for sugarcane variety CoS99259 (23.5 ± 2.6 shoots per 2-3 shoots). In the same study, those authors further reported that BAP and kinetin, used individually, induced fewer numbers of shoots than when used in combination. Those findings are also similar to the results obtained in this study where BAP and kinetin were used in combination at different concentrations (M2 and M3) (Figure 3) although none produced better yields than M5. The addition of NAA to BAP and kinetin (M5) significantly increased the shoot yield for both varieties (Figure 3). The same observations were also made by Yadav et al. (2012) whose results showed that MS medium supplemented with BAP, kinetin and NAA (0.5 mg l^{-1} each) was the best for shoot initiation and culture establishment using shoot tip and meristem explants of sugarcane. Other studies involving the use of BAP and NAA reported these PGRs as best shoot inducers when used in combination or individually depending on the concentration (Irvine et al., 1991; Lakshmanan et al., 2006; Ali et al., 2008; Biradar et al., 2009; Mustafa and Khan, 2012).

Further attempts were made to improve the supply of PGRs, to increase the production of shoots. This was done by modifying M5, the resulting media of which were tested on varieties NCo376, N41 and N50. The different varieties were used to see if the established protocol could be used for shoot multiplication of several sugarcane varieties. Singh et al. (2006), working with eight Indian sugarcane varieties, reported that different genotypes have varied sensitivities to various PGRs, thus a single combination of PGRs may not suit all varieties. Individual shoots of the tested varieties were, therefore, multiplied on M5 and modifications to this medium, for a period of 6 w with a further 2 w subculture. The results showed M5 to be the best as it resulted in the production of the highest final shoot yields at 8 w: 26.0 ± 4.0, 18.6 ± 0.3 and 8.0 ± 0.3 shoots per shoot for NCo376, N41 and N50, respectively (Figure 6). These results indicate that, although the final shoot yield obtained with N50 was significantly lower than that of the other varieties, this protocol can be used for shoot multiplication of these varieties to bulk up or produce enough shoots for genetic and phenotypic analysis. This study also showed that, increasing the concentration of BAP from 1 mg l^{-1} (M5) to 2 mg l^{-1} (M5B) in an attempt to improve the PGR supply did not result in an increase in the shoot yield compared with that of M5. However, this was in contrast to the results obtained by Biradar et al. (2009) who reported that BAP at 2 mg l^{-1}

used alone in liquid media could produce the highest shoot yield directly from shoot tip culture, but the shoots produced were weak, tiny and non-separable making them difficult to manage.

Due to the best response in terms of shoot yield obtained on M5 (Figure 3), further investigations were carried out using this medium in semi-solid state to multiply young shoots of NCo376 soon after embryo germination. Semi-solid medium was used instead of liquid medium because sugarcane embryos do not survive liquid immersion (Munsamy et al., 2013). The aim was to shorten the time required to produce a single shoot in culture, which would then be subsequently multiplied. It was hypothesized that this shoot multiplication route would result in faster production of shoots ready for use for both genetic and phenotypic analysis. At 6 w, the final shoot yield in terms of average number of shoots per shoot, obtained using the protocol on well-developed shoots (12.7 ± 0.4) (Figure 3) was the same as that on individual embryos (11.0 ± 2.0). These results suggest that, although the yield was the same, less time is taken in culture to produce shoots of a suitable size (20 mm) for further multiplication, when the protocol is applied to young shoots of NCo376 than the well-developed ones.

5.2 Determining the effect of the established protocol on genetic stability of the multiplied transgenic shoots

The protocol was used to multiply transgenic shoots of NCo376 lines with the aim of testing for stability of the transgene in the multiplied shoots as an indication of the effect of the protocol on possible somaclonal variation. The presence of the transgene was tested using end point polymerase chain reaction (PCR). Previous reports have reported incidences of genetic changes arising in *in vitro*-produced plants including sugarcane (Irvine et al., 1991; Devarumath et al., 2007; Senapati et al., 2012). These changes are known as somaclonal variation. Several factors including physiological (genetic changes induced by habituation to PGRs and culture conditions) and genetic (changes as a result of chromosomal rearrangements such as a deletion, duplication and somatic recombination) have been reported to cause genetic changes in *in vitro* culture (Trigiano and Gray, 2005). This has resulted in production of plants that are genetically dissimilar from the parent plants. Trigiano and Gray (2005) further stated that the frequency of genetic variation from tissue culture-derived plants is high. However, it is perceived that the

incidence of somaclonal variation is higher in callus cultures than in shoot cultures where production of adventitious shoots is via direct organogenesis (Rutherford et al., 2013).
In this study, transgenic shoots multiplied using the established protocol, were tested for the presence of the transgene using end point PCR. This technique has been used in previous studies to detect the presence of known genes in many plant species in including sugarcane. The results of this study revealed that all the samples of the multiplied shoots tested positive for the GO1 (Figure 14) while those from the non-transformed tested negative. Although the tests targeted only one gene and not the whole genome, these results indicate that the transgene remained stable during shoot multiplication using the established protocol. The bands produced from the PCR products as shown on Figure 13 suggests that the transgene in the multiplied shoots was not negatively affected by the protocol.

5.3 Determination of slow growth storage protocol

Minimal growth (slow growth) refers to direct ways of restricting growth and morphogenesis *in vitro* as one or a combination of reduced temperature, reduced nutrient supply, presence of osmotically-active additives, reduced oxygen and reduced light (Englemann, 1991; Paul and Duvik, 1993; Watt et al., 2010). Losses of breeding germplasm maintained in field collections, which occur due to environment and biological hazards have necessitated the development of *in vitro* storage strategies (Paul and Duvik, 1993; González-Arnao et al., 1999; Martinez-Montero et al., 2008; Watt et al., 2009). According to Watt et al. (2009), because of greenhouse and labour constrains, which are often encountered at times when plantlets reach a desirable stage for transplanting, it is necessary to hold back material in culture. Furthermore, for varieties improved through genetic manipulation techniques, which includes genetic engineering and induced mutagenesis, it is critical not to lose somatic embryos developed from these events. Consequently, it is necessary to develop a protocol to enable short-term storage of somatic embryos developed from the single transformation events.

Somatic embryos have been successfully encapsulated for *in vitro* storage in several plant species including sugarcane (Nieves et al., 2003; Martinez-Montero et al., 2008). According to published reports (Gonzalez-Arnao et al., 1993; Martninez-Montero et al., 1998; González-Arnao et al., 1999; Nieves et al., 2003; Martinez-Montero et al., 2008), encapsulation-

dehydration is the most-commonly used method for *in vitro* storage of sugarcane germplasm. However, all this work has been done for cryopreservation purposes. For slow growth storage, a method exists (Watt et al., 2009), but it uses naked embryos. According to Singh and Chand (2010) and Kikowska and Thiem (2011), encapsulation has several advantages over the methods such as the ability to protect the explants from desiccation and mechanical injury and the possibility of adding useful material (e.g. PGRs, fungicides, pesticides and antibiotics required during germination and for protection against pathogens into the encapsulating matrix). In addition, plantlets can be regenerated directly without any induction of a callus phase, avoiding somaclonal variation among the regenerated plants (Maqsood et al., 2012). Therefore, this method was tried using sodium alginate beads.

Somatic embryos of variety NCo376 were encapsulated immediately before germination and stored at 18 and 25 ^{o}C for 1, 2 and 3 months. The results of this study showed that out of the four tested conditions (Figure 14), storage for a 1 month at 18 ^{o}C resulted in the highest percentage embryo survival (65 ± 6.7%) (Figure 14). The results also revealed that, storage of the embryos at 18 ^{o}C for a further 2 months resulted in reduced % embryo survival (20 ± 5.6%). This suggests that the embryos were able to sustain chilling injury during the first two months after which viability loss occurred. According to a review by Englemann (1991), most tropical plant species are generally cold sensitive and are often susceptible to physiological damages caused by chilling injury, which include various changes in the metabolism, protein content and composition and functioning of the membranes. This may explain why the somatic embryos used in this study were able to withstand low temperatures for one month beyond which survival rate became very low (Figure 14). This study differed from that of Watt et al. (2009) in that those authors did not encapsulate the embryos. Furthermore, they were able to suppress growth of the immature somatic embryos for three months on normal regeneration medium at 18 ^{o}C. The time period achieved by those authors is longer than that in this current study possibly because their embryos were not encapsulated and were stored on nutrient medium. Nevertheless, in practice, it is possible to store and, therefore, hold back further development of somatic embryos for 1 to 2 months in cases when space and labour are not available, as discussed above.

5.4 Conclusions

a) Shoot multiplication

The results of this study demonstrated that the best protocol for multiplication of shoots of NCo376 and NCo310 resulting in 12.7 ± 4.0 and 7.2 ± 3.4 shoots/shoot, respectively, in 6 w, involved placing 20 mm long shoots in 5 ml liquid medium containing full strength MS salts and vitamins (Murashige and Skoog, 1962), 20 mg l^{-1} sucrose, 1 mg l^{-1} BAP, 0.1 mg l^{-1} kinetin and 1 mg l^{-1} NAA in culture for 6 w with 3 w subcultures, then 2 w for shoot growth, followed by rooting on liquid medium containing ½ strength MS salts and vitamins (Murashige and Skoog, 1962), 5 mg l^{-1} sucrose and 1 mg l^{-1} IBA.

In addition, the results demonstrated that this protocol can be used for high yielding shoot multiplication of two other sugarcane genotypes, N41 and N50 resulting in a total final yield per shoot of 18.6 ± 2.3 and 8.0 ± 0.3, respectively, which are adequate for molecular and phenotypic analyses. Further, molecular studies indicated that the protocol can be applied to well-developed shoots of transgenic lines of a sugarcane variety, NCo376, without adversely affecting the presence of the transgene in the multiplied shoots.

The results also showed that a modification of the protocol (3 ml medium with 8 g l^{-1} agar) can be applied to young shoots of NCo376 to obtain shoots 6 w earlier than if bigger shoots (20 mm long) are used. However, in this regard, further investigations need to be carried out to test if the protocol with agar can be applied for the same purpose to other sugarcane genotypes.

b) *In vitro* storage of somatic embryos

The results of this study also demonstrated that it is possible to store encapsulated sugarcane somatic embryos immediately before germination as follows: 1) produce somatic embryos via normal somatic embryogenesis from immature leaf rolls; 2) encapsulate individual embryos in alginate beads; 3) place the beads on normal regeneration medium and; 4) then store at 18 ^{o}C for 1-2 months. This strategy can be used for short term storage of germplasm material which is not immediately required or cannot be used due to labour and or storage limitations. However work done by Watt et al. (2009) show that it is possible to keep the embryos for periods longer than 1 to 2 months without encapsulating them. However, the advantages of encapsulation are that the

explants are protected from desiccation and mechanical injury. In addition, useful material required during germination and for protection against pathogens can be included in the encapsulating matrix. Consequently, further investigations to increase the yield and storage time should be pursued.

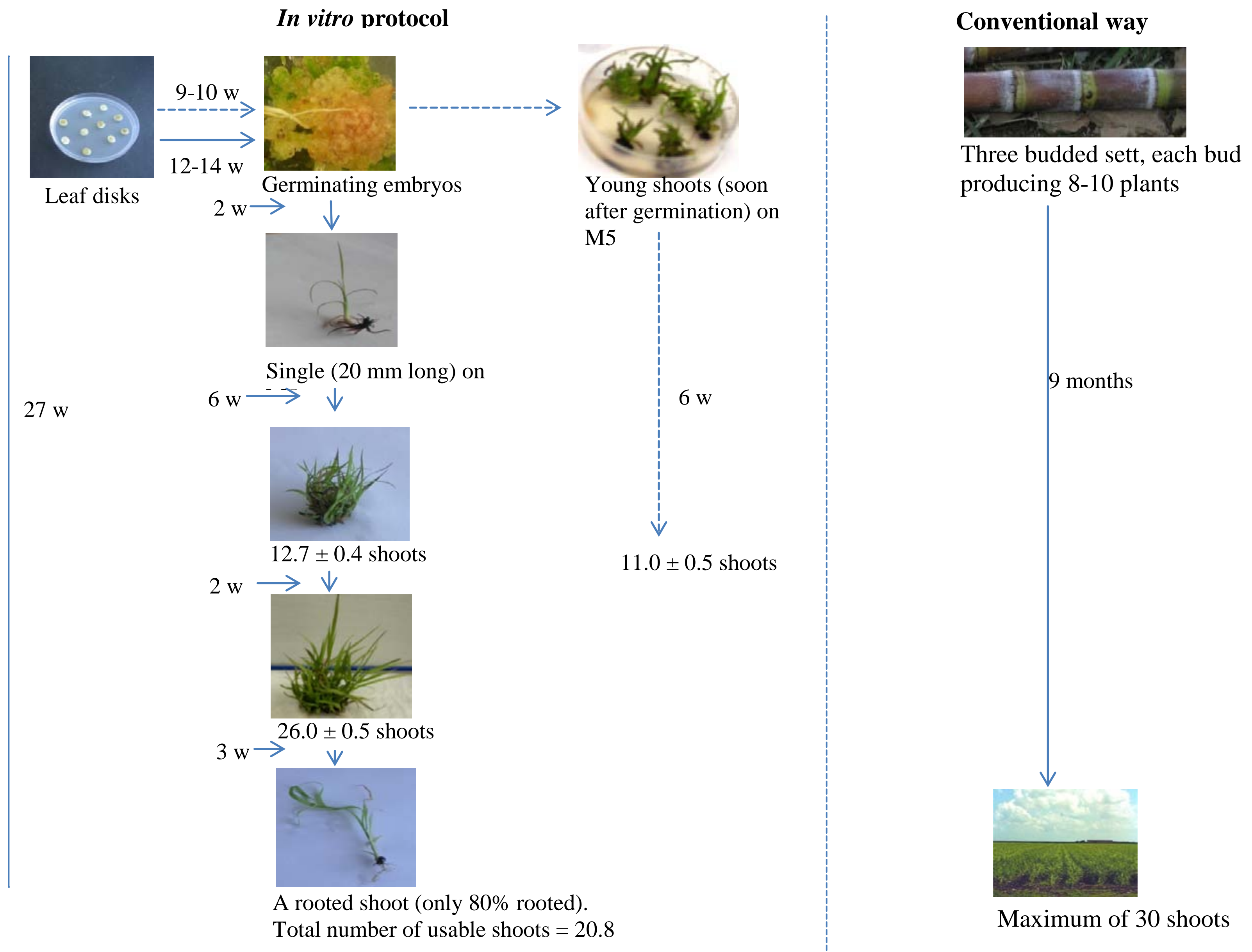


Figure 16. A comparison of shoot yields obtained using the *in vitro* shoot multiplication protocol versus the conventional method.

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APPENDICES

Appendix 1

F pr. values used to determine effect of different concentrations and combinations of plant growth regulators on shoot multiplication of sugarcane variety NCo376 after 3 w in liquid media. d.f = degrees of freedom, s.s = sum of squares, m.s = mean square, F = F-test statistic value and Sig. = significance

Source of variation	d.f.	s.s.	m.s	F	Sig.
Media	5	72.9	14.6	6..8	0.001
Residual	84	180.8	2.2		
Total	89	253.7			

Appendix 2

F pr. values used to determine effect of different concentrations and combination of plant growth regulators on shoot multiplication of sugarcane variety NCo310 after 3 w in liquid media

Source of Variation	d.f	s.s	m.s	F	Sig.
Media	5	12.6	2.5	3.1	0.001
Residual	84	67.2	0.8		
Total	89	79.8			

Appendix 3

F pr. values used to determine effect of different concentrations and combination of plant growth regulators on shoot multiplication of sugarcane variety NCo376 after 6 weeks in liquid media

Source of variation	d.f.	s.s.	m.s	F	Sig.
Media	5	995.6	199.1	15.5	0.001
Residual	69	884.1	12.8		
Total	74	1879.7			

Appendix 4

F pr. values used to determine effect of different concentrations and combination of plant growth regulators on shoot multiplication of sugarcane variety NCo310 after 6 weeks in liquid media

Source of variation	d.f	s.s	m.s	F	Sig.
Media	5	305.8	61.1	10.2	0.001
Residual	76	453.8	6.0		
Total	81	759.5			

Appendix 5

F. pr. values used to determine the effect of modifications to Medium 5 on shoot multiplication of NCo376 after 3 w on liquid media.

Source o variation	d.f	s.s.	m.s.	F	Sig.
Media	2	40.2	20.1	8.5	0.001
residual	41	96.5	2.4		
Total	43	136.7			

Appendix 6.

F pr. values used to determine the effect of modifications to Medium 5 on shoot multiplication of NCo376 after 6 w on liquid media.

Source of variation	d.f.	s.s	m.s.	F	Sig.
Media	2	535.0	267.5	37.7	0.001
Residual	41	291.0	7.1		
Total	43	826.0			

Appendix 7

F pr. values used to determine the effect of modifications to Medium 5 on shoot multiplication of NCo376 after 8 w on liquid media.

Source of variation	d.f	s.s	m.s	F	Sig.
Media	2	570.0	285.0	12.7	0.001
Residual	12	270.0	22.5		
Total	14	840.0			

Appendix 8

F pr. values used to determine the effect of M5 and modified media on shoot multiplication of N41 after 3 w.

Source of variation	d.f.	s.s	m.s	F	Sig.
Media	2	2.8	1.4	1.8	0.200
Residual	24	18.4	0.7		
Total	26	21.2			

Appendix 9.

F pr values used to determine the effect of M5 and modified media on shoot multiplication of N50 after 3 w.

Source of variation	d.f.	s.s.	m.s.	F	Sig.
Media	2	3.0	1.5	2.8	0.100
Residual	26	14.2	0.5		
Total	28	17.2			

Appendix 10.

F pr values used to determine the effect of M5 and modified media on shoot multiplication of N41 6 w.

Source of variation	d.f	s.s	m.s	F	Sig.
Media	2	49.6	24.8	4.5	0.001
Residual	16	88.6	5.5		
Total	18	138.1			

Appendix 11.

F pr values used to determine the effect of M5 and modified media on shoot multiplication of N50 after 6 w.

Source of variation	d.f.	s.s	m.s	F	Sig.
Media	2	11.1	5.5	1.8	0.200
Residual	15	45.3	3.0		
Total	17	56.4			

Appendix 12.

F pr values used to determine the effect of M5 and modified media on shoot multiplication of N50 after 6 w.

Source of variation	d.f	s.s	m.s.	F	Sig.
Between Groups	2	213.7	106.9	11.1	0.000
Within Groups	12	115.2	9.6		
Total	14	328.9			

Appendix 13.

F pr values used to determine the effect of M5 and modified media on shoot multiplication of N41 8 w.

Source of variation	d.f	s.s	m.s	F	Sig.
Media	2	6.4	3.2	9.6	0.001
Residual	12	4.0	0.3		
Total	14	10.4			

Appendix 14

F pr values used to determine the effect of storage time (months) at 18 ^{o}C on survival of somatic embryos of NCo376.

Source of variation	d.f	s.s.	m.s	F	Sig.
Media	3	3566.7	1188.9	10.2	0.001
Residual	8	933.3	116.7		
Total	11	4500.0			