In vitro culture and genetic transformation of selected ancestral and commercial sugarcane germplasm

by

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As the candidate's supervisor I have/~~have not~~ approved this dissertation for submission

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Abstract

Sugarcane is an economically important crop and its high demand has necessitated the use of biotechnology methods to produce and accelerate the production of desirable genotypes. One such method is genetic transformation. However, as sugarcane is a highly polyploid crop, which originated from interspecific crosses between *Saccharum spontaneum* and *S. officinarum*, efforts to transform it are inhibited by transgene promoter silencing. As ancestral lines have a simpler genetic makeup than modern varieties, they may be useful to test promoter function. Intrinsic to the generation of transgenic plants is the ability to produce plants from specific species and varieties, for which an indirect method of regeneration is needed. Consequently, the first objective of this study was to determine a high yielding protocol for somatic embryogenic calli. The second was to transform such calli and produce regenerated plants to assess transgene expression.

A preliminary study was conducted using eight ancestral varieties to determine which were the most responsive in culture. Leaf roll disks were cultured on 5 $mg.l^{-1}$ 2, 4-D and callus production was assessed. Based on these results and the availability of plant material, *S. spontaneum* Nigeria 1, *S. spontaneum* Nigeria 2, *S. spontaneum* Coimbatore, *S. officinarum* NG 77-69, and *S. officinarum* Black Cheribon and the commercial polyploid variety NCo376 were selected and tested on 11 different callus induction media. The *S. spontaneum* variety that generated the highest percentage of leaf disks that produced callus and plant yield was Nigeria 1 (61 % and 259 plants/10 disks, respectively), whilst the *S. officinarum* variety was Black Cheribon (75 % and 90 plants/10 disks, respectively). The best media for both comprised of MS salts and vitamins, 20 $g.l^{-1}$ sucrose, 0.5 $g.l^{-1}$ casein hydrolysate 5 $mg.l^{-1}$ 2, 4-D and 8 $g.l^{-1}$ agar. NCo376 produced the most amount of callus (93 %) when cultured on media containing 3 $mg.l^{-1}$ 2, 4-D and gave a final yield of 450 plants/10 disks.

Based on the yields obtained above and the availability of plant material, the varieties *S. spontaneum* Nigeria 1 and *S. officinarum* NG77-69 were selected for genetic transformation studies. Calli of these varieties as well as that of NCo376 were microprojectile bombarded with either pEmuKN + pAHC27 or pEmuKN + $pR_{11}F^{-}$. Following bombardment, the calli were cultured onto paromomycin-containing (1 $ml.l^{-1}$) selection media and regenerated plants were obtained after 8-12 weeks. Transgene integration into the plant genome was assessed using PCR and qPCR techniques, and indicated that all NCo376 plantlets contained the GUS and *npt II* transgenes. However, only 4 out of 5 and 2 out of 3 *S. officinarum* NG77-69 plants transformed with pAHC27 and $pR_{11}F^{-}$ respectively, and 6 out of 10 *S. spontaneum* Nigeria 1 plants transformed with $pR_{11}F^{-}$ contained these transgenes. The transformation efficiencies achieved

for NCo376, for the constructs pAHC27 and $pR_{11}F^-$ was 0.27 and 0.33 transgenic plants/blast, respectively. For NG77-69 it was 0.27 and 0.13 transgenic plants/blast, whilst that of Nigeria 1 was 0.20 and 0.40 transgenic plants/blast. Stable transgene expression in acclimatized plants was then assessed using a histochemical GUS assay and none of the plants expressed the GUS gene.

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DECLARATION 1 - PLAGIARISM

I, **Ellisha Pillay**, declare that

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Preface

The experimental work described in this thesis was carried out in the University of KwaZulu Natal, School of Life Science Building as well as in the Biotechnology Department of the South African Sugarcane Research Institute (SASRI), Mount Edgecombe, Durban, SA from January 2011 to December 2012, under the supervision of Prof. M.P. Watt (UKZN) and Dr S.J. Snyman (SASRI and UKZN).

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 in the text.

Table of contents

List of tables

List of Figures

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List of abbreviations

2, 4-D	2, 4-dichlorophenoxyacetic acid
SADC	South African Development Community
PCR	Polymerase chain reaction
qPCR	Qualitative real time PCR
GUS	β-glucuronidase
KZN	KwaZulu Natal
SASRI	South African Sugarcane Research Institute
PGR	Plant growth regulator
BAP	Benzylaminopurine
NAA	Naphthaleneacetic acid
IAA	Indole-3-acetic acid
GA	Gibberellic acid
IBA	Indole-3-butyric acid
MS	Murashige and Skoog
EMS	Ethyl methanesulfonate
5Azac	Sodium azide
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
Ti	Tumour inducing
CaMV	Cauliflower mosaic virus
AdhI	Alcohol dehydrogenase

npt II	Neomycin phosphotransferase
hpt	Hygromycin phosphotransferase
pat	Phosphinothricin-N-acetyltransferase
als	Acetolactatesynthase
MUG	Methyl umbelliferyl glucuronide
X-gluc	5-bromo-4-chloro-3-indolyl-glucuronide
gfp	Green fluorescent protein
PFP	Phosphofructokinase
LUC	Luciferase
PTGS	Post-transcriptional gene silencing
CIM	Callus induction media
EGM	Embryo germination medium/media
PEM	Plantlet establishment media
Ubi	Ubiquitin
LB	Luria-Bertani broth
LA	Luria-Bertani agar plates
PIG	Particle inflow gun
TTC	Triphenyltetrazolium chloride
ANOVA	Analysis of variance
NTC	Non-template control
Cq	Quantification cycle
TMV35S	Tobacco mosaic virus 35S
CP4	5-enolpyruyl-shikimate-3-phosphate synthase

CpG	Cytosine-phosphate-quanine site
TGS	Transcriptional gene silencing
PTGS	Post-transcriptional gene silencing

1. Introduction and rationale for the study

Sugarcane is a perennial grass of the family *Poaceae* (Daniels and Roach, 1987) and is an important source of sugar in most parts of the world (Anon., 2011a). It is grown in tropical and sub-tropical regions and South Africa is an important part of the global sugarcane industry (Anon., 2011c). In South Africa sugarcane farming affords employment for many, as well as providing important products such as sugar, ethanol and molasses (Anon., 2011a; 2011b; 2011c). With the constant increase in the global population and limited land available for agriculture there is a need to create novel genotypes and plants that yield more products per area planted out (Kern, 2002).

Conventional methods of producing sugarcane involve vegetative propagation through setts, but this is a long process spanning many years (8-15) that also carries the risk of disease transmission across the genetically identical plantation (Lee, 1987; Basnayake *et al*., 2011; Snyman *et al*., 2011). *In vitro* culture and its manipulations can be used to produce novel genotypes and accelerate their production (George, 1993; Snyman *et al*., 2011). It involves utilizing a single explant to produce many genetically identical plants, in a time frame that is much shorter than is possible with conventional propagation (George, 1993). *In vitro* culture also has many applications such as germplasm conservation (Chen *et al*., 1988; Watt *et al*., 2009), virus elimination (Ramgareeb *et al*., 2010), and production of novel genotypes through mutagenesis (Khan *et al*., 2009; Khan and Khan, 2010; Koch *et al*., 2012) and genetic transformation (Snyman *et al.*, 1996; 2000; 2001; 2006; van der Vyver, 2010; Basnayake *et al*., 2011; Taparia *et al*., 2012).

The most routinely employed method of *in vitro* propagation for commercial sugarcane cultivars is indirect somatic embryogenesis (Chen *et al*., 1988; Snyman *et al*., 1996; 2000; 2001; Patade *et al*., 2008; van der Vyver, 2010; Basnayake *et al*., 2011), although other morphogenic routes have been used (Ho and Vasil, 1983; Aftab and Iqbal, 1999; Franklin *et al*., 2006; Behera and Sahoo, 2009). Somatic embryogenesis is also the morphogenic route most commonly used for genetic transformation of commercial sugarcane cultivars as it involves the production of embryogenic calli (calli that can be regenerated into plantlets) (Snyman *et al*., 1996; Arencibia *et al*., 1998; Snyman *et al*., 2000; Khalil, 2002; Snyman *et al*., 2001; Basnayake *et al*., 2011; Taparia *et al*., 2012).

Genetic transformation comprises the introduction of transgenes into plant cells in an attempt to increase the agronomic value of a variety (Birch, 1997; Deo *et al*., 2010). Transgenes are genes that are obtained from exogenous sources and introduced into the genome of an organism/plant

in which these genes do not occur naturally (Birch, 1997). For sugarcane transformation, microprojectile bombardment is commonly-used for this purpose (Birch and Franks, 1991; Bower and Birch, 1992; Gambley *et al*., 1993; Sun *et al*., 1993; Snyman *et al*., 2001; Khalil, 2002; Taylor and Fauquet, 2002; Snyman *et al*., 2006; van der Vyver, 2010; Taparia *et al*., 2012). As this method accelerates DNA coated microprojectiles into plant cells, it allows for the direct penetration and uptake of the transgenes into the plant cytoplasm and nucleus (Birch and Franks, 1991; Finer *et al*., 1992; Taylor and Fauquet, 2002).

The origin of sugarcane can be traced back to interspecific crosses between the ancestral lines *Saccharum spontaneum* and *S. officinarum* (Daniels and Roach, 1987; D'Hont *et al*., 1996; Irvine, 1999; Butterfield *et al*., 2001; Grivet and Arruda, 2001). As a result, modern sugarcane cultivars have a limited genetic pool and aneuploid genomes with asymmetric chromosome inheritance and varied chromosome numbers (2n = 99 – 190) (Brumbley *et al*., 2008). This makes it difficult to breed varieties that are resistant/tolerant to pests and diseases (Lee, 1987; Irvine, 1999; Butterfield *et al*., 2001; Snyman *et al*., 2011).

Genetic transformation can be used as means to produce such varieties but, as sugarcane is highly polyploid, it experiences a high incidence of transgene silencing (Hansom *et al*., 1999; Ingelbrecht *et al*., 1999; Kapoor *et al*., 2005; Brumbley *et al*., 2008; Potier *et al*., 2008b; Mudge *et al*., 2009; Graham *et al*., 2011). During transformation events, the transgene integrates randomly into the genome and may be inserted multiple times which results in a high copy number (Birch, 1997; Hansom *et al*., 1999 Meng *et al*., 2003; Potier *et al*., 2008b; Mudge *et al*., 2009; Graham *et al*., 2011). This high copy number and positional effects can trigger epigenetic responses that result in hypermethylation of the promoter elements (transcriptional gene silencing) or coding regions of the transgene (post-transcriptional gene silencing) so that they are silenced (Birch, 1997; Hansom *et al*., 1999; Ingelbrecht *et al*., 1999; Meng *et al*., 2003; Potier *et al.*, 2008b; Mudge *et al*., 2009; Graham *et al*., 2011). The transgenes may also be integrated into regions of the genome that are transcriptionally repressed, thus hindering efforts to transform commercial sugarcane cultivars (Hansom *et al*., 1999; Ingelbrecht *et al*., 1999; Meng *et al*., 2003; Kapoor *et al*., 2005; Potier *et al*., 2008b; Mudge *et al*., 2009; Graham *et al*., 2011).

Imperative to the successful transformation of any plant variety is the development of reporter, selectable marker and, of particular interest to this study, functional promoter genes (Brumbley *et al*., 2008). The *gus* (Bower and Birch, 1992; Sun *et al*., 1993; Harinath and Nerker, 2012), luciferase (Basnayake *et al*., 2012) and anthocyanin (Snyman *et al*., 1996; McCallum *et al*.,

1998) reporter genes, as well as the *npt II* (Bower and Birch, 1992; Sun *et al*., 1993; van der Vyver, 2010; Basnayake *et al*., 2011) and bar (Witcher *et al*., 1998; Ingelbrecht *et al*., 1999) selectable marker genes have been used successfully in sugarcane transformation. However, transgenic sugarcane plants experience a high incidence of promoter silencing (Hansom *et al*., 1999; Ingelbrecht *et al*., 1999; Kapoor *et al*., 2005; Brumbley *et al*., 2008; Potier *et al*., 2008b; Mudge *et al*., 2009; Graham *et al*., 2011) which hinders attempts to produce novel genotypes through genetic transformation. As such, the development of functional promoters that are not silenced is of interest to the sugarcane industry. A promoter is the region to which RNA polymerase binds and starts the process of DNA transcription (Chawla, 2002). These need to be created for specific genes and species, and some also need to be tissue specific so that the transgene will only be expressed in a particular tissue type (e.g. root specific promoters will only allow expression of transgenes in root tissue). It is important, therefore, to test newly developed promoters to elucidate if they are functional. However, the experienced transgene silencing makes it difficult to determine if new promoters developed for sugarcane are non-functional or merely silenced. Consequently there is a need to develop a system that can be used to assess promoter activity.

The ancestral sugarcane varieties *S. spontaneum* and *S. officinarum* are polyploid, with 8 - 10 copies of the genome, respectively (Daniels and Roach, 1987; D'Hont *et al*., 1996; Irvine, 1999; Butterfield *et al*., 2001; Grivet and Arruda, 2001). However, they still have a simpler genetic makeup than modern commercial sugarcane cultivars. Hence, it is proposed that these varieties may demonstrate a reduced incidence of transgene silencing compared with commercial cultivars.

Several varieties of ancestral sugarcane were available for the present study, *viz*. *S. spontaneum* Nigeria 1, *S. spontaneum* Nigeria 2, *S. spontaneum* IK76-46, *S. spontaneum* Mandalay, *S. spontaneum* Coimbatore, *S. officinarum* Badilla, *S. officinarum* Black Cheribon and *S. officinarum* NG77-69. However, with the exception of van der Vyver (2010), no protocols were available for the plant regeneration via indirect somatic embryogenesis, and for the genetic transformation of these ancestral varieties. In contrast, such protocols for the commercial cultivar NCo376 have been optimized and are routinely used by the South African Sugarcane Research Institute (SASRI). Hence, this variety was used to compare the tested *in vitro* responses of the selected ancestral varieties.

Consequently, the objectives of this study were to:

1. **Establish a high yielding somatic embryogenesis protocol focussing on embryo production for the selected ancestral varieties.**

   This was achieved by investigating the effect of 11 different callus induction media on the % explants forming callus, the amount of embryogenic callus formed and plantlet regeneration of all tested ancestral varieties. The yields obtained from the *in vitro* culture of these varieties were compared with that obtained for NCo376 using the high yielding protocol established at SASRI.

2. **Assess transgene expression in transformed plants from the ancestral sugarcane varieties for which an indirect somatic embryogenesis protocol was established.**

   Using the established best callus induction medium, embryogenic calli of selected ancestral varieties and of NCo376 were generated and genetically transformed using microprojectile bombardment. These calli were then regenerated and the produced plantlets were acclimatized. Genomic DNA from these putatively transformed plants was extracted and used in PCR and qPCR analyses to determine if the transgenes were integrated into the genome. A histochemical GUS assay was then performed on leaf and root material of ancestral and commercial lines. This was undertaken to test for GUS expression as a comparison of promoter functionality in different genetic backgrounds and the findings may assist in the choice of genotype used in future studies.

2. Literature review

2.1 Background and economic importance of sugarcane

Sugarcane is an economically and agriculturally important crop that accounts for approximately 75 % of the world's sugar production (Lakshmanan, 2006a; James, 2008; Anon, 2011a). The South African sugar industry produced 1.9 million tons of saleable sugar in the 2010/2011 season (Anon, 2011b) and, as a member of Southern African Development Community (SADC), is one of the top five sugar exporters to world markets (Anon, 2011c). Consequently, sugarcane farming and milling is an important part of the South African economy. However, an increasing population translates into a greater demand for sugarcane and its products, which in turn places pressure on the sugar industry to supply the demand (Kern, 2002; Sharma *et al*., 2005).

Currently 70 % of the world's sugarcane is grown in tropical and subtropical regions of the southern hemisphere where there is limited arable land available for plantations (Snyman *et al*., 2011; Anon, 2012a). Profitable crop farming is dependent on ideal (or near ideal) biotic and abiotic factors (Kern, 2002). In South Africa, most of the sugarcane farms can be found within the KwaZulu Natal (KZN) and Mpumalanga regions (Figure 1), where the prevailing conditions are less than ideal for sugarcane farming (Anon, 2011d); this is also the southernmost region in which sugarcane is grown (Snyman *et al*., 2008). Land gradients, rainfall of less than 1000mm/annum and less than ideal soil conditions are some abiotic factors that negatively affect the annual yield of KZN cane plantations (Snyman *et al*., 2008; Anon, 2011d). Biotic factors such as pests and diseases also impact the yields obtained from sugarcane farms (Suman *et al*., 2000; Mukunthan and Nirmala, 2002; Singh *et al*., 2008).

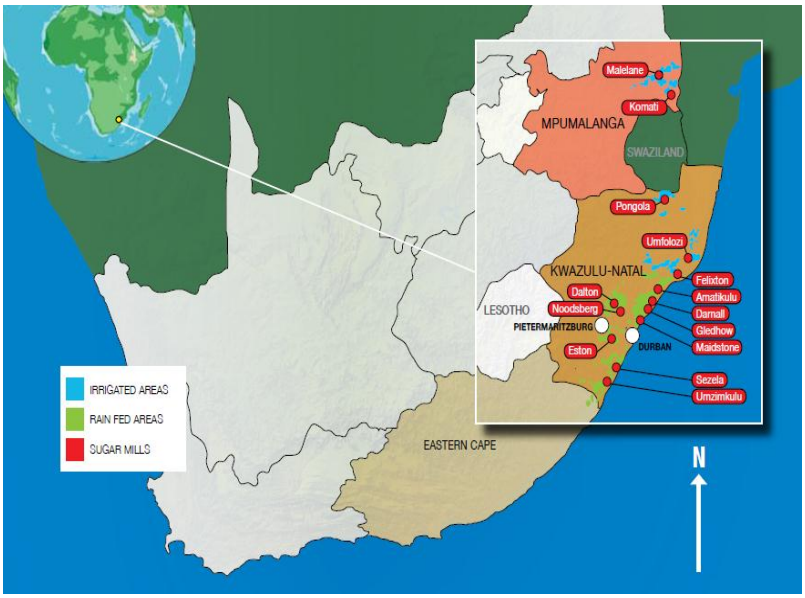

Figure 1. A map showing the distribution of the sugarcane growing areas, mills and SASRI research stations in the KwaZulu Natal and Mpumalanga regions. (From Anon, 2012a).

2.1.1 Origin and genetics of sugarcane

Sugarcane is a member of the *Poaceae* family of the *Andropogoneae* tribe and is a perennial grass with a characteristic sucrose rich stalk (Daniels and Roach, 1987; Screenivasan *et al*., 1987; D'Hont *et al*., 1996; Irvine, 1999; Butterfield *et al*., 2001; Grivet and Arruda, 2001; Anon, 2004). Modern commercial sugarcane cultivars can be traced back to interspecific crosses between *Saccharum officinarum* L. and *S. spontaneum* L. and then backcrossing with *S. officinarum* in a process referred to as nobilization (Daniels and Roach, 1987; Screenivasan *et al*., 1987; D'Hont *et al*., 1996; Irvine, 1999; Butterfield *et al*., 2001; Grivet and Arruda, 2001; Anon, 2004). As a result, commercially available modern sugarcane cultivars are complex polyploids and aneuploids (Daniels and Roach, 1987; Screenivasan *et al*., 1987; D'Hont *et al*., 1996; Irvine, 1999; Butterfield *et al*., 2001; Grivet and Arruda, 2001; Anon, 2004; Snyman *et al*., 2011).

The species *S. officinarum* (2n = 80), also referred to as noble cane, accounts for 80 percent of the genetic makeup of modern cultivars of sugarcane and confers characteristics of stalk thickness, high sucrose content and low fiber and starch content of the stalks (Daniels and Roach, 1987; Screenivasan *et al*., 1987; D'Hont *et al*., 1996; Irvine, 1999; Nair *et al*., 1999;

Butterfield *et al*., 2001; Lakshamanan *et al*., 2005). *S. spontaneum* (2n = 40-130) contributes 10 percent to the genetic makeup of modern sugarcane cultivars and confers robust and adaptable characteristics, as well as improved disease resistance (Daniels and Roach, 1987; Screenivasan *et al*., 1987; D'Hont *et al*., 1996; Irvine, 1999; Nair *et al*., 1999; Butterfield *et al*., 2001; Lakshamanan *et al*., 2005).

The ancestral lines *S. spontaneum* and *S. officinarum* have a basic chromosome number of 8 and 10, respectively (Daniels and Roach, 1987; Screenivasan *et al*., 1987; D'Hont *et al*., 1996; Butterfield *et al*., 2001; Grivet and Arruda, 2001). The interspecific cross between them has resulted in modern sugarcane cultivars being highly polyploid with 10-12 copies of the genome and a chromosome number ranging from 2n = 99 to 2n = 130 (Irvine, 1999; Butterfield *et al*., 2001; Brumbley *et al*., 2008). Their subsequent propagation via vegetative means has also resulted in a limited pool of genetic variation (Irvine, 1999; Butterfield *et al*., 2001; Brumbley *et al*., 2008; Snyman *et al*., 2011).

In order for the sugarcane industry to remain lucrative, there needs to be a constant supply of new varieties that are resistant to herbicides, plant diseases and pests, and that are able to grow in areas with poor soil quality or low rainfall (Kern, 2002; James, 2008; Snyman *et al*., 2011). However, this is limited by the narrow gene pool (e.g. absence of certain pest and disease resistance genes), the complex polyploidy of sugarcane, the long breeding and selection time, as well as the inability to predict the outcome of crosses (Ming *et al*., 1998; Irvine, 1999; Butterfield *et al*., 2001; Brumbley *et al*., 2008; van der Vyver, 2010; Snyman *et al*., 2011).

2.1.2 Sugarcane breeding

Currently, at SASRI, as well as in other parts of the world, new sugarcane varieties are being developed through integrated breeding programs that also make use of the germplasm of ancestral lines (Nair *et al*., 1999; Najarajan *et al*., 2000; Botha, 2007; Snyman *et al*., 2011). These varieties are then propagated asexually via stem segments called setts to create plantations of genetically identical sugarcane. However, these programs take a long time (8-15 years) and carry the risk that disease transmission can devastate entire plantations (Lee, 1987; Pathak *et al*., 2009; van der Vyver, 2010; Basnayake *et al*., 2011; Snyman *et al*., 2011). As an alternative to conventional breeding and propagation, new genotypes can be produced using *in vitro* interventions (Gambley *et al*., 1993; Snyman *et al*., 1996; Arencibia *et al*., 1998; McCallum *et al*., 1998; Snyman *et al*, 2000; Geetha and Padmanabhan, 2001; Snyman *et al*.,

2001; Khalil, 2002; Snyman *et al*., 2006; Patade *et al*., 2008; Khan *et al*., 2009; Khan and Khan, 2010; Ramgareeb *et al*., 2010; van der Vyver, 2010; Basnayake *et al*., 2011; Snyman *et al*., 2011; Taparia *et al*., 2012).

2.2. *In vitro* culture systems

According to George, 1993; Bhojwani and Razdan, 1996; Hartmann *et al*., 1997and George *et al*., 2008, micropropagation is the end result of a collection of *in vitro* culture systems resulting in the mass production of a single genotype, usually one with traits of interest (elite/superior). It is based on the plants' ability to regenerate whole plants from single cells (totipotency, usually, under the influence of exogenous plant growth regulators [PGRs]). Manipulating the PGRs and the nutrients in the culture media, as well as other environmental conditions, can produce the desired development in the plant. This is achieved through two possible routes of *in vitro* morphogenesis, *viz*. organogenesis and somatic embryogenesis and protocols exist for the micropropagation of sugarcane through both these routes (Table 1).

2.2.1 Organogenesis

Organogenesis is a morphogenic route that results in the formation of plant organs (George, 1993; Bhojwani and Razdan, 1996; Hartmann *et al*., 1997; George *et al*., 2008). This can be attained either through direct organ formation from an explant or through an intervening callus stage from which organs and plantlets can be regenerated (George, 1993; Bhojwani and Razdan, 1996; Hartmann *et al*., 1997; George *et al*., 2008). Both routes require the addition of exogenous PGRs in order to achieve the desired development in the plant. During direct organogenesis, multiplication of shoots is done under the influence of both auxins and cytokinins, whilst rooting is usually conducted under the influence of auxin alone (George, 1993; Mamum *et al*., 2004; Khan *et al*., 2006; Ali *et al*., 2008; George *et al*., 2008).

For shoot multiplication, a combination of auxin and cytokinin is required, of which the most commonly employed cytokinin is benzylaminopurine **(**BAP**)** (Lee, 1987; Gambley *et al*., 1993; Lorenzo *et al*., 2001; Huang *et al*., 2003; Khan *et al*., 2006; Ali *et al*., 2008) (Table 2). The optimum concentration of BAP differs among different sugarcane varieties and ranges from 0.5 to 2 $mg.l^{-1}$ (Mamum *et al*., 2004; Khan *et al*., 2006; Ali *et al*., 2008; Khan *et al*., 2008; Pathak *et al*., 2009), with 1.5 $mg.l^{-1}$ being the most commonly used (Mamum *et al*., 2004; Khan *et al*., 2006; Ali *et al*., 2008). Shoot production is usually improved when BAP is used in combination

with 6-furfuryaminopurine **(**kinetin**)**, the optimum concentration of which also varies amongst different varieties (0.1-0.5 mg.l^{-1}) (Lee, 1987; Khan *et al*., 2006; Ali *et al*., 2008; Pathak *et al*., 2009). As part of the direct organogenesis pathway, the auxins used during shoot multiplication include naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA) (Huang *et al*., 2003; Mamum *et al*., 2004; Khan *et al*., 2006; Khan *et al*., 2008; Khan *et al*., 2009) (Table 2) whilst the auxins most routinely used in the rooting of shoots are NAA (0.5-5 mg.l^{-1}) and indole-3-butyric acid (IBA**)** (0.5-1 mg.l^{-1}) (Mamum *et al*., 2004; Khan *et al*., 2006; Ali *et al*., 2008; Khan *et al*., 2008; Behera and Sahoo, 2009; Khan *et al*., 2009; Pathak *et al*., 2009) (Table 2).

During indirect organogenesis, the explant is placed onto media that allows for the formation of callus, which is formed in the presence of high levels of auxin and cytokinin. When sufficient calli are produced they can be regenerated to produce plant organs and plantlets in the same manner as through direct organogenesis (George, 1993; Bhojwani and Razdan, 1996; Hartmann *et al*., 1997). The auxin 2.4-dichlorophenoxyacetic acid (2, 4-D) is routinely used for callus induction and approximately 3 mg.l^{-1} is reported to give the best callus induction in commercial varieties of sugarcane (Mamum *et al*., 2004; Behera and Sahoo, 2009; Khan *et al*., 2009) (Table 2).

The nutrient formulation most commonly used for the organogenesis protocols is that of Murashige and Skoog (1962) (Ho and Vasil, 1983; Lorenzo *et al*., 2001; Mamum *et al*., 2004; Behera and Sahoo, 2009; Ramgareeb *et al*., 2010) and sucrose in the range of 20-30 g.l^{-1} is the most commonly used carbon source (Lorenzo *et al*., 2001; Huang *et al*., 2003; Ali *et al*., 2008; Behera and Sahoo, 2009; Mordocco *et al*., 2009; Ramgareeb *et al*., 2010). These components have in certain cases been supplemented by coconut water (Ho and Vasil, 1987; Mamum *et al*., 2004), coconut milk (Lee, 1987), myo-inisitol (Lee, 1987; Lorenzo *et al*., 2001), arginine and thiamine (Lee, 1987).

Table 1. Examples of studies employing different morphogenic routes of sugarcane micropropagation and their applications.

Route of Morphogenesis	Application	Reference
Direct organogenesis	Micropropagation	Lee, 1987
	Genetic transformation	Gambley *et al*., 1993
	Micropropagation	Lorenzo *et al*., 2001
	Micropropagation	Huang *et al*., 2003
	Micropropagation	Khan *et al*., 2006
	Micropropagation	Ali *et al*., 2008
	Micropropagation	Khan *et al*., 2008
	Micropropagation	Pathak *et al*., 2009
	Virus elimination	Ramgareeb *et al*., 2010
Indirect organogenesis	Micropropagation	Mamum *et al*., 2004
	Micropropagation	Behera and Sahoo, 2009
	Mutagenesis	Khan *et al*., 2009
	Virus elimination	Ramgareeb *et al*., 2010
Direct somatic embryogenesis	Micropropagation	Aftab and Iqbal, 1999
	Genetic transformation	Snyman *et al*., 2000, 2001, 2006
	Micropropagation	Geetha and Padmanabhan, 2001
	Minimal growth and storage/germplasm preservation	Watt *et al*., 2009
	Genetic transformation	van der Vyver, 2010
	Genetic transformation	Taparia *et al*., 2012
Indirect somatic embryogenesis	Culture storage/germplasm preservation	Chen *et al*., 1988
	Genetic transformation	Snyman *et al*., 1996, 2001
	Genetic transformation	Arencibia *et al*., 1998
	Genetic transformation	McCallum *et al*., 1998
	Genetic transformation	Khalil, 2002
	Mutagenesis/trait selection	Patade *et al*., 2008
	Mutagenesis	Khan and Khan, 2010
	Genetic transformation	van der Vyver, 2010
	Genetic transformation	Basnayake *et al*., 2011

Table 2. A summary of the various plant growth regulators used during the *in vitro* culture stages of organogenesis in sugarcane. Growth regulators used include 6-firfuryaminopurine (kinetin), benzylaminopurine (BAP), naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), gibberellic acid (GA) and 2, 4-dichlorophenoxyacetic acid (2, 4-D).

Route of Morphogenesis	Callus induction	Multiplication	Rooting	Reference
Direct organogenesis	-	Kinetin, BAP	-	Lee, 1987
	-	Kinetin, BAP	-	Gambley *et al*., 1993
	-	BAP	-	Lorenzo *et al*., 2001
	-	BAP and NAA	-	Huang *et al*., 2003
	-	Kinetin, IBA, IAA and BAP	IBA and NAA	Khan *et al*., 2006
	-	Kinetin, BAP	-	Lakshmanan *et al*., 2006b
	-	BAP, kinetin, GA	NAA and IBA	Ali *et al*., 2008
	-	BAP and GA	NAA and IBA	Khan *et al*., 2008
	-	BAP, kinetin and NAA	NAA and IBA	Pathak *et al*., 2009
	-	BA, kinetin and NAA	-	Ramgareeb *et al*., 2010
Indirect organogenesis	2, 4-D	BAP, IBA and NAA	IAA, NAA and IBA	Mamum *et al*., 2004
	2, 4-D	BAP, kinetin, IBA and NAA	IBA, NAA and IAA	Behera and Sahoo, 2009
	2, 4-D	IBA, IAA and kinetin	IBA	Khan *et al*., 2009
	2, 4-D	BA, 2, 4-D	-	Ramgareeb *et al*., 2010

2.2.2 Somatic embryogenesis

Somatic embryogenesis involves the formation of embryos from somatic cells which then germinate to form plants (George, 1993; Bhojwani and Razdan, 1996; Hartmann *et al*., 1997; Snyman *et al*., 2001; Lakshmanan *et al*., 2006a; Deo *et al*., 2010). They resemble their zygotic counterparts in structure and have similar developmental stages (George, 1993; Bhojwani and Razdan, 1996; Hartmann *et al*., 1997; Snyman *et al*., 2001; Lakshmanan *et al*., 2006a; Deo *et al*., 2010). Consequently, the embryos' development can be tracked by assessing the shape of the embryoids (Ho and Vasil, 1983; George, 1993; Bhojwani and Razdan, 1996; Hartmann *et al*., 1997; Snyman *et al*., 2001; Lakshmanan *et al*., 2006a; Deo *et al*., 2010). In monocotyledonous plants such as sugarcane, globular shaped embryos elongate as they progress through the scutellar, coleoptilar and torpedo stages, before forming plantlets (Ho and Vasil, 1983; George, 1993; Deo *et al*., 2010). As with organogenesis, somatic embryogenesis can be achieved either directly or indirectly through a callus stage (George, 1993; Bhojwani and Razdan, 1996; Hartmann *et al*., 1997; George *et al*., 2008).

a) Direct somatic embryogenesis

Immature leaf rolls (Snyman *et al*., 2000; Geetha and Padmanabhan, 2001; Snyman *et al*., 2001; Snyman *et al*., 2006; Taparia *et al*., 2012) and immature inflorescences (Desai *et al*., 2004; Snyman *et al*., 2006) have been reported as explants for direct embryogenesis of sugarcane and the response of the explant in culture has been found to be genotype-dependent. The production of somatic embryos in direct somatic embryogenesis is usually achieved by exposing the explant to low levels of an auxin (usually 2, 4-D) (Snyman *et al*., 2000; Geetha and Padmanabhan, 2001; Snyman *et al*., 2001; 2006; Taparia *et al*., 2012), thereby avoiding the formation of callus. As direct somatic embryogenesis does not require high levels of or prolonged exposure to 2, 4-D (Snyman *et al*., 2000; 2001; 2006; Taparia *et al*., 2012) it has a reduced risk of somaclonal variation (Larkin and Scowcroft, 1981).

b) Indirect somatic embryogenesis

Indirect somatic embryogenesis is routinely employed for sugarcane micropropagation as the callus obtained through this route has many applications, *viz*. genetic engineering (Snyman *et al*., 1996; McCallum *et al*., 1998; Snyman *et al*., 2001; Khalil, 2002; van der Vyver, 2010; Basnayake *et al*., 2011), germplasm conservation (Chen *et al*., 1988), mutagenesis (Patade *et*

al., 2008; Khan and Khan, 2010; Koch *et al*., 2012) and micropropagation (Snyman *et al*., 2001) (Table 1).

Ho and Vasil (1983) observed that calli formed on sugarcane explants undergoing indirect somatic embryogenesis were not uniform in appearance and comprised of morphogenically dissimilar regions. According to their classification, type 1 callus is white and nodular in appearance and is embryogenic with a high level of regeneration (Ho and Vasil, 1983). Type 2 callus is yellow, soft and friable and may be embryogenic, and type 3 callus is mucilaginous and shiny in appearance and is non-embryogenic (Ho and Vasil, 1983).

In contrast, Taylor *et al.* (1992) classified type 1 callus as "semi-translucent callus containing loose, large, elongated cells", type 2 as mucilaginous, grey-yellow calli, type 3 as yellow-white compact and nodular callus and type 4 as yellow and friable callus. Those authors considered type 1 and 2 callus as non-embryogenic callus, type 3 as embryogenic and type 4 as organogenic (plantlets that are produced from this type of callus, form through organogenesis and not embryogenesis as there are no somatic embryos present) (Taylor *et al*., 1992).

Although the classifications of Ho and Vasil (1983) and Taylor *et al*. (1992) differ (Table 3), both stated that embryogenic callus was white and nodular with cells that are round and densely cytoplasmic. As embryogenic callus is required for many *in vitro* manipulations, these descriptions aid in its selection. At present, the classification system proposed by Taylor *et al*. (1992) is favored and is used in many genetic modification studies (Bower and Birch, 1992; Snyman *et al*., 1999; Basnayake *et al*., 2011).

Table 3. Callus classifications and descriptions made by Ho and Vasil (1983) and Taylor *et al*. (1992).

Type	Callus description	
	Ho and Vasil (1983)	Taylor *et al*. (1992)
1	White, nodular, highly embryogenic	Semi-translucent, non-embryogenic
2	Yellow, friable, can be embryogenic	Grey-yellow, mucilaginous, non-embryogenic
3	Greyish, mucilaginous, non-embryogenic	Yellow-white, nodular, highly embryogenic
4	Not applicable	Yellow, friable, organogenic

Callus has been obtained from many varieties of sugarcane (Arencibia *et al*., 1998; Khalil, 2002; Lakshmanan *et al*., 2006a; van der Vyver, 2010) and immature leaf rolls and inflorescence have been reported to produce the greatest amount of embryogenic callus (Desai *et al*., 2004; Lakshmanan *et al*., 2005; Lakshmanan, 2006a; Snyman *et al*., 2006; Snyman *et al*., 2011). Consequently, leaf disks are the most commonly-used explant for indirect somatic embryogenesis (Snyman *et al*., 2000; Geetha and Padmanabhan, 2001; Snyman *et al*., 2001; Khalil *et al*., 2002; Franklin *et al*., 2006; Snyman *et al*., 2006; Taparia *et al*., 2012). The amount of embryogenic callus obtained differs amongst varieties (Chen *et al*., 1988; Snyman *et al*., 1996; van der Vyver, 2010) and explant sources (Heinz and Mee, 1969; Desai *et al*., 2004; Lakshmanan *et al*., 2005; Snyman *et al*., 2006), and is affected by the age of the explant (Deo *et al*., 2010; Taparia *et al*., 2012), culture media and growth conditions.

As mentioned previously, the induction of callus requires an exogenous auxin as it promotes cell division whilst inhibiting cell differentiation (George, 1993; Bhojwani and Razdan, 1996; Hartmann *et al*., 1997; Deo *et al*., 2010). High levels of auxin in the culture media result in embryo formation and removing the auxin results in embryo maturation and plantlet development (George, 1993; Bhojwani and Razdan, 1996; Hartmann *et al*., 1997; Chen *et al*., 1988; Khalil, 2002; Snyman *et al*., 2006; Basnayake *et al*., 2011). The most-commonly reported media for callus induction of sugarcane contains MS with vitamins, sucrose as a carbon source and 2, 4-D (Chen *et al*., 1988; Snyman *et al*., 2001; Khalil, 2002; Basnayake *et al*., 2011; Snyman *et al*., 2011) (Table 4).

One of the first studies on sugarcane regeneration through callus culture was conducted by Heinz and Mee (1969) using the auxin 2, 4-D and this has since become the most routinely employed auxin to induce callus formation for somatic embryogenesis in sugarcane (Chen *et al*., 1988; McCallum *et al*., 1998; Snyman *et al*., 2000; Geetha and Padmanabhan, 2001; Snyman *et al*., 2001; Khalil, 2002; Lakshmanan, 2006a; Patade *et al*., 2008; Snyman *et al*., 2011) (Table 4). 2, 4-D is a synthetic auxin and its optimal concentration for callus induction differs amongst different varieties but 3-4 $mg.l^{-1}$ is reported to be the most effective concentration for commercial varieties (McCallum *et al*., 1998; Khalil, 2002; Basnayake *et al*., 2011). Other auxins found to generate callus in sugarcane are picloram (Chengalrayan *et al*., 2005) and 4-flourophenoxyacetic acid (Brisisbe *et al*., 1994; Lakshmanan, 2006a).

It is generally accepted that the yield and regenerative potential of somatic embryos declines with the length of time the calli are kept in culture (Chen *et al*., 1988; George, 1993; Bhojwani and Razdan, 1996; Franklin *et al*., 2006; Taparia *et al*., 2012). Chen *et al*. (1988) were able to

maintain the regenerative potential of calli for 30 months by alternating between high and low 2, 4-D concentrations (Chen *et al*., 1988). However, they found that cultures younger than 12 months were still capable of somatic embryogenesis, whereas older cultures were only regenerable through organogenesis (Chen *et al*., 1988). When trying to improve on this, Franklin *et al*. (2006) found that a 3 $mg.l^{-1}$ 2, 4-D pretreatment of leaf roll explants enhanced the regeneration capacity of the cultures.

Similar to organogenesis, MS formulation with vitamins is routinely used in somatic embryogenesis media for sugarcane, but this has been supplemented with other components including coconut water (Khalil *et al*., 2002; Patade *et al*., 2008), malt extract (Patade *et al*., 2008), myo-inositol (Arencibia *et al*., 1998; Patade *et al*., 2008) and the amino acids glycine (Fitch and Moore, 1993) and proline (Fitch and Moore, 1993) (Table 4). Sucrose is the most used carbon source for somatic embryogenesis protocols (Arencibia *et al*., 1998; Aftab and Iqbal, 1999; Geetha and Padmanabhan, 2001; Khalil, 2002; Snyman *et al*., 2006; Taparia *et al*., 2012) although others have been used viz. fructose, glucose, glycerol, lactose, maltose, mannitol and sorbitol (Brisisbe *et al*., 1994; Gill *et al*., 2004; Singh *et al*., 2008).

2.2.3 Comparison of the different morphogenic routes

In conclusion, it is clear from the literature that successful micropropagation of commercial sugarcane is undertaken through both direct organogenesis and indirect somatic embryogenesis (Tables 1 and 2). Lee (1987) found that plants obtained via direct organogenesis were more phenotypically similar to the parent plant than those obtained via indirect somatic embryogenesis. Further, 78400 plantlets were achieved after a five month period using direct somatic embryogenesis, whereas only 900 plantlets were produced through indirect somatic embryogenesis in the same time frame (Lee, 1987). Snyman *et al*. (2000; 2001) found direct embryogenesis to be the fastest and most cost-efficient route of morphogenesis, which impacts on the final cost of plantlets and might reduce the risk of somaclonal variation, as discussed below. However, the indirect route is preferred for genetic transformation studies (Snyman *et al*., 1996; McCallum *et al*., 1998; Snyman *et al*., 2000; 2001; Khalil, 2002; van der Vyver, 2010; Basnayake *et al*., 2011; Taparia *et al*., 2012) as it increases the chances of obtaining transgenic plants since there are more cells present.

Table 4. Examples of the media constituents in callus induction and plant regeneration media during indirect somatic embryogenesis in sugarcane. Growth regulators used include 2, 4-D, IBA, NAA, FPA (flourophenoxyacetic acid) and kinetin.

Media	Carbon source*	PGR	Nutrients, amino acids and vitamins	Reference
Callus initiation	Sucrose	2, 4-D, NAA	MS, casein hydrolysate, kinetin, thiamine HCI, nicotinic acid, pyridoxine HCI, arginine, coconut milk	Chen *et al*., 1988
	Maltose	2, 4-D, NAA, FPA	MS	Brisisbe *et al*., 1994
	Sucrose	2, 4-D	MS, casein hydrolysate	Snyman *et al*., 1996
	Sucrose	2, 4-D	MS, casein hydrolysate	Snyman *et al*., 2001
	Sucrose	2, 4-D	MS, coconut water and casein hydrolysate	Khalil, 2002
	Sucrose, maltose	2, 4-D, kinetin	MS	Gill *et al*., 2004
	Sucrose	2, 4-D	MS, glutamine, casein hydrolysate, coconut water	Patade *et al*., 2008
	Sucrose	2, 4-D	MS, nicotinic acid, coconut water, glycine, thiamine, pyridoxine-HCl	Basnayake *et al*., 2011
Plant regeneration	Sucrose	-	MS	Chen *et al*., 1988
	Sucrose	-	MS	McCallum *et al*., 1988
	Sucrose	-	MS	Snyman *et al*., 1996
	Sucrose	Kinetin	MS	Snyman *et al*., 2001
	Sucrose	-	MS, myo-inositol	Khalil, 2002
	Sucrose	-	MS, casein hydrolysate	Khan and Khan, 2010
	Sucrose	-	MS, casein hydrolysate	van der Vyver, 2010
	Sucrose	-	MS	Basnayake *et al*., 2011

2.2.4 Somaclonal variation

According to Larkin and Scowcroft (1981); George (1993); Bhojwani and Razdan (1996); Kaeppler *et al*. (2000); Singh *et al*. (2008); Khan *et al*. (2009) and Khan and Khan (2010) somaclonal variation refers to the genetic variation that exists amongst some somatic cells and it is due to differences in genetic and epigenetic gene regulation. It occurs naturally through alterations in mitotic cell division, mutations and errors in transcription. Consequently, a high degree of somaclonal variation can be expected in indirect morphogenic routes that require the intervening callus stage. Such routes require that cultures are maintained for longer periods of time than through direct routes, and are exposed to high concentrations of auxin which results in an amplified rate of cell division. All of these manipulations increase the incidence of transcriptional errors and can lead to a variation in the genomes of cells that arise from the same parent cell (Larkin and Scowcroft, 1981; George, 1993; Bhojwani and Razdan, 1996; Kaeppler *et al*., 2000; Singh *et al*., 2008; Khan *et al*., 2009; Khan and Khan, 2010).

In cases where true-to-type cultures are required, indirect morphogenic routes should, therefore, be avoided as they increase the occurrence of somaclonal variation. In contrast, by exploiting it, is possible to create new/novel genotypes of agronomic importance (e.g. herbicide and pest tolerance, disease resistance) (Larkin and Scowcroft, 1981; George, 1993; Bhojwani and Razdan, 1996; Hartmann *et al*., 1997; Zambrano *et al*., 2003; Singh *et al*., 2008). The production of new genotypes is especially important in species with limited gene pools such as sugarcane (Larkin and Scowcroft, 1981; D'Hont *et al*., 1996; Irvine, 1999; Butterfield *et al*., 2001; Grivet and Arruda, 2001; Lakshmanan *et al*., 2005; Brumbley *et al*., 2008). The target tissues are intentionally treated with mutating agents, and examples of mutagens used in sugarcane include gamma radiation (Patade *et al*., 2008; Khan *et al*., 2009; Khan and Khan, 2010), sodium azide (Ali *et al*., 2007), and ethyl methanesulfonate (EMS) (Kenganal *et al*., 2008; Koch *et al*., 2012; Mahlanza *et al*., 2012). This is done on the premise that after exposure to a mutagen, some cells would have mutated and will divide and confer characteristics of interest (e.g. herbicide or disease tolerance) (Zambrano *et al*., 2003; Patade *et al*., 2008; Singh *et al*., 2008; Khan *et al*., 2009; Khan and Khan, 2010; Koch *et al*., 2012; Mahlanza *et al*., 2012). However, rigorous screening is required to distinguish and select those cells that have the desired mutation from those that do not (Larkin and Scowcroft, 1981; George, 1993; Bhojwani and Razdan, 1996; Hartmann *et al*., 1997). The mutated target tissue is placed onto selection media (e.g. media containing the herbicide under study) - if the cells have undergone the appropriate mutation they will survive and divide and produce plants with desirable traits (Larkin and Scowcroft, 1981; George, 1993; Bhojwani and Razdan, 1996; Hartmann *et al*.,

1997; Zambrano *et al*., 2003; Patade *et al*., 2008; Singh *et al*., 2008; Khan *et al*., 2009; Khan and Khan, 2010; Koch *et al*., 2012; Mahlanza *et al*., 2012).

2.3. Genetic engineering

The increasing global demands for sugarcane and its products require the sugarcane industry to provide a large number of plants to the sugarcane farmers that are robust and can withstand unfavourable environmental conditions. As previously mentioned, such plants are usually from varieties that are the product of complex breeding programs which can take between 8 to 15 years (Snyman *et al*., 1996; Lakshmanan *et al*., 2005; Pathak *et al*., 2009; Basnayake *et al*., 2011; Snyman *et al*., 2011). These breeding programs involve the random combination of genes where the transfer of a desired gene is not guaranteed due to the aneuploid nature of the sugarcane genome (Snyman *et al*., 1996; McCallum *et al*., 1998). Genetic engineering can be a more directed alternative to conventional plant breeding (Gambley *et al*., 1993; Snyman *et al*., 1996; McCallum *et al*., 1998; Snyman *et al*., 2000; 2001; 2006; Deo *et al*., 2010; Taparia *et al*., 2012). During this process, specific gene(s) of interest is identified and isolated, and the exogenous DNA is introduced into a target organism or cell(s) (Newell, 2000; Chawla, 2002; Brumbley *et al*., 2008; Deo *et al*., 2010). The introduction of exogenous DNA into a plant can be achieved through various means and is usually employed to introduce genes of interest or value into an organism (e.g. herbicide tolerance) (Newell, 2000; Chawla, 2002; Brumbley *et al*., 2008; Deo *et al*., 2010).

2.3.1 Current methods of plant transformation

The method chosen for sugarcane plant transformation is dependent on the type of tissue being modified. Embryogenic calli (Snyman *et al*., 1996; Ingelbrecht *et al*., 1999; Groenewald and Botha, 2001; Snyman *et al*., 2001, van der Vyver, 2010, Taparia *et al*., 2012), protoplasts (Arencibia *et al*., 1995), immature inflorescence (Snyman *et al*., 2006) and apical meristems (Gambley *et al*., 1993) have all been used in sugarcane transformation studies.

Totipotent cells are particularly favoured in transformation studies as they are capable of producing embryogenic callus (Birch and Franks, 1991; Christou, 1996; Hansen and Wright, 1999; Chawla, 2002). As embryogenic callus [type 3 callus (Taylor *et al*., 1992), section 2.2.1.4] forms close to the surface of the explant, it is easily accessible to transgene delivery (Christou, 1996; Hansen and Wright, 1999; Deo *et al*., 2010). Such calli are also highly

regenerable and, therefore, are the preferred explant for transformation studies (Snyman *et al*., 1996; Ingelbrecht *et al*., 1999; Groenewald and Botha, 2001; Snyman *et al*., 2001, van der Vyver, 2010, Taparia *et al*., 2012). The different methods used to genetically engineer or transform sugarcane are microprojectile bombardment (Birch and Franks, 1991; Bower and Birch, 1992; Sun *et al*., 1993; Snyman *et al*., 1996, Ingelbrecht *et al*., 1999; Khalil, 2002; Snyman *et al*., 2006, van der Vyver, 2010; Kim *et al*., 2012; Taparia *et al*., 2012), *Agrobacterium*-mediated transformation (Arencibia *et al*., 1998; Elliot *et al*., 1998), electroporation (Arencibia *et al*., 1995; Rakoczy-Trojanowska, 2002) and polyethylene glycol treatment (Chen *et al*., 1987; Aftab and Iqbal, 2001). Of these, microprojectile bombardment and *Agrobacterium*-mediated transformation are the most commonly-employed.

a) Electroporation and polyethylene glycol treatment

Electroporation involves passing an electric pulse through the plant cells that are to be transformed. It works on the premise that subjecting plant cells to an electric field causes the temporary formation of pores (Newell, 2000; Rakoczy-Trojanowsha, 2002), through which the genes of interest can enter the plant cell. Electroporation has the advantages of being fast and easy to use, as well as causing minimal cell toxicity (Newell, 2000; Rakoczy-Trojanowsha, 2002).

The polyethylene glycol treatment (Chen *et al*., 1987; Newell, 2000; Aftab and Iqbal, 2001) allows for the binding and precipitation of exogenous DNA (genes of interest) to the plant cells' membrane. As it also stimulates endocytosis, it allows for the precipitated DNA on the cells surface to be taken up by the cell (Newell, 2000).

Both electroporation and polyethylene glycol have been used successfully in plant transformation studies (Chen *et al*., 1987; Arencibia *et al*., 1995; Aftab and Iqbal, 2001), but as their application is best suited to protoplasts and cell suspension, they are not widely used for sugarcane transformation.

b) *Agrobacterium*-mediated transformation

Agrobacterium tumefaciens is a gram-negative bacterium that infects plants (De Cleene and Deley, 1976; de la Riva *et al*., 1998; Hansen and Wright, 1999; Newell, 2000; Zupan *et al*., 2000) and causes the formation of a tumour or abnormal tissue on the plant, known as crown

gall tumour (De Cleene and Deley, 1976; de la Riva *et al*., 1998; Zupan *et al*., 2000). This bacterium contains a tumour inducing (Ti) plasmid that integrates into the host cells' DNA and induces the formation of the tumours (de la Riva *et al*., 1998; Zupan *et al*., 2000). The portion of the Ti plasmid that is inserted into the plants' DNA is known as T-DNA and has two types of genes, the oncogenic genes that code for the synthesis of auxins and cytokinins (which cause the tumour), and the genes that code for the formation of opines (food source for the bacterium) (de la Riva *et al*., 1998; Hansen and Wright, 1999; Zupan *et al*., 2000). Also in the Ti plasmid are virulence genes that are responsible for the integration of the T-DNA into the host's DNA (de la Riva *et al*., 1998; Hansen and Wright, 1999; Zupan *et al*., 2000).

The Ti plasmid is exploited in plant transformation studies, whereby a gene of interest in added within the T-DNA boundaries, and the oncogenes are usually removed and replaced with genes that allow for the selection of transformed cells (marker genes, such as genes for antibiotic resistance) (de la Riva *et al*., 1998; Hansen and Wright, 1999; Newell, 2000; Zupan *et al*., 2000). *Agrobacterium*-mediated transformation has the advantage of reduced transgene silencing (de la Riva *et al*., 1998; Hansen and Wright, 1999; Zupan *et al*., 2000) and has been reported in both monocotyledonous and dicotyledonous plants. Initially thought to work only on dicotyledonous plants, as *A. tumefaciens* does not naturally infect monocotyledonous species (De Cleene and Deley, 1976; de la Riva *et al*., 1998), this transgene delivery system has been used successfully in both groups (Arencibia *et al*., 1998; Elliot *et al*., 1998, Anderson and Birch, 2012).

c) Microprojectile bombardment

Microprojectile bombardment involves accelerating DNA-coated microprojectiles into the tissue to be transformed (Birch and Franks, 1991; Finer *et al*., 1992; Hansen and Wright, 1999; Newell, 2000). There are ultimately two different methods of microprojectile bombardment, the gene gun and the particle inflow gun (Figure 2) (Birch and Franks, 1991; Finer *et al*., 1992; Hansen and Wright, 1999; Newell, 2000; Deo *et al*., 2010). The former makes use of a macrocarrier and microcarriers (Birch and Franks, 1991; Finer *et al*., 1992; Newell, 2000; Taylor and Fauquet, 2002; Deo *et al*., 2010). A pressurized gas (usually helium) is used to accelerate the macrocarrier, which has DNA coated microcarriers dried onto its surface, into the target tissue (Birch and Franks, 1991; Finer *et al*., 1992; Newell, 2000; Taylor and Fauquet, 2002; Deo *et al*., 2010). The particle inflow gun method differs in that there is no macrocarrier, rather it is the DNA-coated microprojectiles that are accelerated into the plant tissues (Birch and

Franks, 1991; Finer *et al*., 1992; Newell, 2000; Taylor and Fauquet, 2002; Deo *et al*., 2010). Although the gene gun allows for a gentler and more uniform delivery to the target tissue than the particle inflow gun, the latter is less expensive and easier to use (Taylor and Fauquet, 2002; Deo *et al*., 2010).

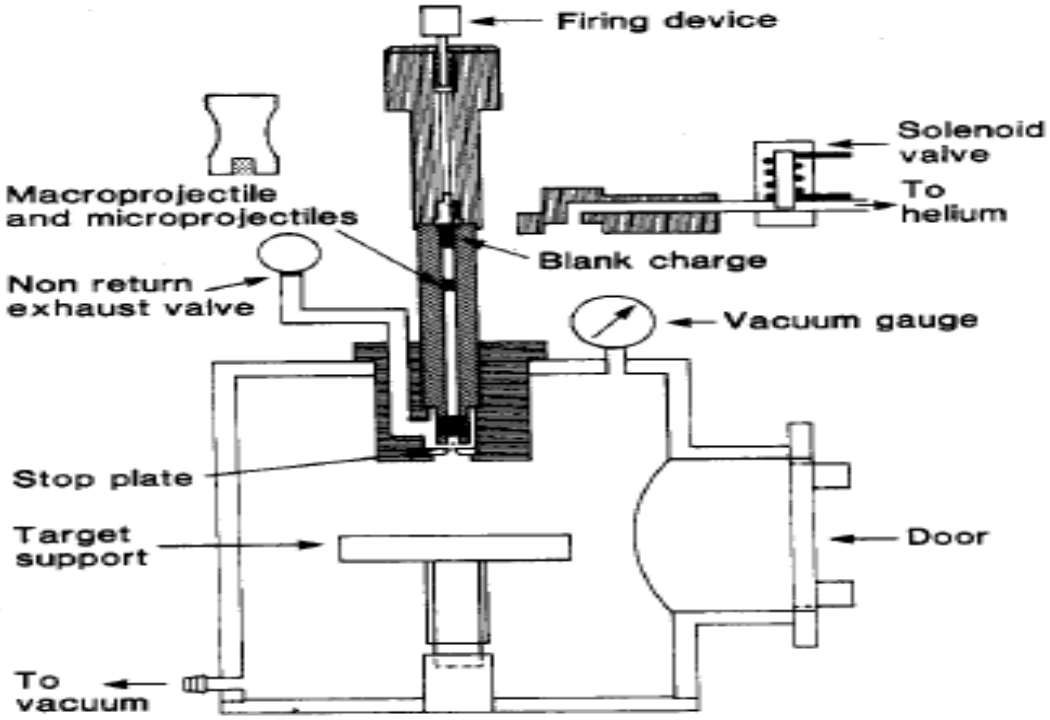

Figure 2: Diagrammatic representation of a microprojectile bombardment system. Plant species that are not amendable to *Agrobacterium*-mediated transformation are transformed through this method. A pressurized gas, usually helium, is used to accelerate DNA coated microprojectiles into target tissue (taken from Birch and Franks, 1991).

Microcarriers are particles on to which the DNA is coated, for example tungsten (Birch and Franks, 1991; Chamberlain *et al*., 1994, Snyman *et al*., 1996; 2006; van der Vyver, 2010; Taparia *et al*., 2012) and gold (Birch and Franks, 1991; Sun *et al*., 1993; Emani *et al*., 2002, Taparia *et al*., 2012). In plant cell transformation studies, gold particles are the preferred microcarriers due to their uniformity in shape, which cause less cell damage than tungsten, and

do not incur toxicity and DNA degradation (Hansen and Wright, 1999; Deo *et al*., 2010; Taparia *et al*., 2012). Of the two, tungsten is much more readily available and is a more cost-effective microcarrier than gold (Deo *et al*., 2010). The diameter of the microprojectile/microcarrier has been shown to play a role in cell survival after bombardment and the amount of transient gene expression (Hansen and Wright, 1999; Deo *et al*., 2010) Taparia *et al*. (2012) observed that 0.3 µm particles resulted in five times higher transformation efficiency than with 1 µm particles.

Prior to bombardment, the target tissue is placed onto a conditioning medium [such as osmoticum (Vain *et al*., 1993)] that will allow easy penetration of the microcarriers into the cells and their nuclei. Treating the target tissue with an osmoticum causes plasmolysation of the cells which reduces damage to the cell membrane and allows for direct penetration of the exogenous DNA into the nucleus (Deo *et al*., 2010). The success of the transformation is dependent on: 1) the penetration of the DNA into the nuclei of the target tissue (Hansen and Wright, 1999; Deo *et al*., 2010); 2) the integration of the transgene into the genome of the target tissue (Southgate *et al*., 1995; Hansen and Wright, 1999; Deo *et al*., 2010); 3) the expression of the transgene and (Southgate *et al*., 1995; Hansen and Wright, 1999; Deo *et al*., 2010) 4) the correct selection of cells that have been transformed (Southgate *et al*., 1995; Hansen and Wright, 1999; Deo *et al*., 2010).

2.3.2 Plasmid vectors

A plasmid vector is the 'vehicle' that carries the gene of interest (transgene) into recipient cells. As bacteria have naturally occurring plasmids, it is these plasmids that are isolated and genetically modified so that they carry genes of interest into a target organism (Christou, 1996; Hansen and Wright, 1999; Newell, 2000; Chawla, 2002; Wang *et al*., 2009). A number of restriction sites along the length of the plasmid sequence allow for the removal of 'unwanted' genes and their replacement with transgenes (Hansen and Wright, 1999; Chawla, 2002; Wang *et al*., 2009). These include: 1) the gene(s) of interest; 2) a promoter that allows for the expression of the gene of interest; 3) selectable marker genes; 4) reporter genes and; 5) a terminator. Plasmid vectors must also be readily accepted into prokaryotic (usually *Escherichia coli*) hosts so that they are rapidly multiplied (cloned) (Newell, 2000; Chawla, 2002; Wang *et al*., 2009).

a) Promoter elements

Promoter sequences are vital to the expression of genes. They are sequences of DNA to which RNA polymerase binds before beginning to transcribe all the genes downstream of the promoter into RNA; this RNA is then translated into protein(s) and expressed by the cell(s) (Chawla, 2002). Promoters can be constitutive or specific, in which case only a certain tissue and cell type would express the gene of interest (Potenza *et al*., 2004; Damaj *et al*., 2010).

Constitutive promoters can be of viral (e.g. cauliflower mosaic virus [CaMV]) or of plant origin (e.g. ubiquitin) and are used to drive the expression of the transgene in all cells, regardless of type (Potenza *et al*., 2004). Different constitutive promoters have been used in sugarcane transformation studies (Table 5) but the maize ubiquitin, the synthetic Emu and the CaMV 35S promoters are the most frequently-used (Bower and Birch, 1992; Snyman *et al*., 1996; Potenza *et al*., 2004; Basnayake *et al*., 2011; Damaj *et al*., 2012) (Table 5). The maize ubiquitin promoter allows for the expression of downstream genes constitutively and, therefore, provides a high level of transient transgene activity (Meng *et al*., 2003). The CaMV 35S promoter was reported to have a limited capability to initiate transcription of transgenes (Viaplana *et al*., 2001). The Emu promoter is a recombinant promoter that produces a high level of transgene expression in cereals and other monocotyledonous plants (Last *et al*., 1991; Chamberlain *et al*., 1994). Last *et al*. (1991) tested the function of different promoters linked to the β-glucuronidase gene in monocotyledonous plants. They found that when compared with CaMV 35S and the maize alcohol dehydrogenase (AdhI) promoter, the Emu promoter gave a 10-50 fold greater β-glucuronidase expression (Last *et al*., 1991). A similar finding was made by Chamberlain *et al*. (1994), who concluded that the high levels of neomycin phosphotransferase (NPT II) enzyme retrieved from their transgenic plant cells was due to linking the *npt II* gene to the Emu promoter. However, the disadvantage of using constitutive promoters is that the expression of the transgene in all cells can negatively affect plant metabolism and development (Potenza *et al*., 2004; Damaj *et al*., 2010).

Specific or regulated promoters can be used to regulate gene expression spatially and temporally and gene expression can be limited to a particular time in the plants development and a particular cell type (Potenza *et al*., 2004; Damaj *et al*., 2010). As this allows for only specific cells to receive an increase in metabolism with minimal effects on non-targeted cells (Potenza *et al*., 2004; Damaj *et al*., 2010), the development of tissue or cell-specific promoters is of interest to plant transformation studies (Potenza *et al*., 2004; Damaj *et al*., 2010). Sugarcane stalks are

rich in sucrose and promoters that are specific for stalk/stem cells are of interest to sugarcane researchers (Potier *et al*., 2008a; Damaj *et al*., 2010; Mudge *et al*., 2013). Damaj *et al*. (2010) identified and isolated sugarcane promoters from the *dirigent* and *o-methyltransferase* genes (both of which are highly expressed in stalk cells) and fused them with the *gus* reporter gene. Those authors found that under the control of these promoters, GUS was successfully expressed, both transiently and stably, and was limited to the stalk (no GUS expression in roots or leaves) (Damaj *et al*., 2010). A similar finding was made by Mudge *et al*. (2013) where 75 – 80 % of transgenic sugarcane plants expressed LUC driven by stalk specific promoters. Potier *et al.* (2008a) isolated stalk specific promoters from maize and sorghum plants and combined them with the *gus* reporter gene. However, they reported that the complex ploidy of sugarcane hinders the development tissue or cell-specific promoters for sugarcane (Potier *et al*., 2008a; Damaj *et al*., 2010).

Table 5. Transgenes and their respective promoters introduced into plant cells through microprojectile bombardment.

Gene*	Promoter	Engineered trait	Reference
gus, npt II	Emu	GUS activity, kanamycin resistance	Bower and Birch, 1992
gus	Emu	GUS activity	Gambley *et al*., 1993
gus, npt II	Maize Adh 1, CaMV 35S	GUS activity, Kanamycin resistance	Sun *et al*., 1993
gus, npt II	Emu	GUS activity, kanamycin resistance	Chamberlain *et al*., 1994
Anthocyanin	CaMV 35S	Anthocyanin expression	Snyman *et al*., 1996
gus, bar gene	Maize ubiquitin promoter, TMV 35S	GUS activity, bialaphos resistance	Witcher *et al*., 1998
Bar gene, *npt II*	Maize ubiquitin promoter	Bialaphos resistance Geneticin resistance,	Ingelbrecht *et al*., 1999
pfp, npt II	Maize ubiquitin promoter	PFP, geneticin resistance	Groenewald and Botha, 2001
npt II, CP4	Emu	Kanamycin resistance, glyphospate resistance	Snyman *et al*., 2006
gfp, npt II	Maize ubiquitin	GFP activity, geneticin resistance	van der Vyver, 2010
luc, npt II	Maize ubiquitin	LUC activity, geneticin resistance	Basnayake *et al*., 2011

* GUS – β-glucuronidase, NPT II - neomycin phosphotransferase, PFP – phosphofructokinase, LUC – luciferase, CP4 – 5-enolpyruyl-shikimate-3-phosphate synthase, GFP – green fluorescent protein

b) Selectable marker and reporter genes

Following the introduction of the gene of interest into the target tissue, certain measures need to be taken in order to ensure that only those cells that have successfully taken up the transgene survive (Hansen and Wright, 1999; Newell, 2000; Chawla, 2002; Finer, 2010). In order to accomplish this, it is necessary to co-transform the target tissue with the gene of interest and a selectable marker (Hansen and Wright, 1999; Finer, 2010). The latter is a gene that encodes resistance to an external agent, usually antibiotics and herbicides (Chamberlain *et al*., 1994; Hansen and Wright, 1999; Snyman *et al*., 2000; 2001; Chawla, 2002, Snyman *et al*., 2006; Rosellini, 2011). These are incorporated into the *in vitro* culture medium and, if the cells have

been successfully transformed, they will express the gene conferring the resistance and survive on it (Chamberlain *et al*., 1994; McCallum *et al*., 1998; Hansen and Wright, 1999; Chawla, 2002, Rosellini, 2011). There are many selectable markers that are used in plant transformation experiments, the most common of which are listed in Table 6. If, however, cells have not been transformed then they will be killed by the antibiotics/herbicide in the media, allowing easy differentiation between successfully and unsuccessfully transformed cells (Hansen and Wright, 1999).

The neomycin phosphotransferase (*npt II*) (Sun *et al*., 1993; McCallum *et al*., 1998; Snyman *et al*., 2000; 2001; Potier *et al*., 2008b) and the hygromycin phosphotransferase (*hpt*) (Bower and Birch, 1992; Chamberlain *et al*., 1994) genes are commonly-used selectable markers that confer resistance to the aminoglycoside antibiotics kanamycin/geneticin and hygromycin, respectively. The bar, phosphinothricin-N-acetyltransferase (*pat*), and the acetolactatesynthase (*als*) genes confer resistance to the herbicides bialaphos, phosphinothricin and sulfonylurea, respectively. These have all been successfully used as selectable marker genes in plant transformation studies (Bower and Birch, 1992; Sun *et al*., 1993; Chamberlain *et al*., 1994; McCallum *et al*., 1998; Witcher *et al*., 1998; Ingelbrecht *et al*., 1999; Snyman *et al*., 2000; 2001; Groenewald and Botha, 2001; Snyman *et al*., 2006; Potier *et al*., 2008b; van der Vyver, 2010; Basnayake *et al*., 2011; Taparia *et al*., 2012).

A reporter gene is one that allows for the detection of cells that have been successfully transformed. It can be used to assess both transient and stable gene expression and is therefore included in the plasmid vector (Chawla, 2002). Reporter genes work on the premise that the gene construct has been taken up by the cell (whether it is integrated in the genome or merely in the cytoplasm) and that the addition of an agent that corresponds to the reporter will produce a visible effect on the transformed cell(s) (Chawla, 2002). Some reporter genes produce histochemical reactions when combined with a substrate that the encoded gene can catalyze, such as in the case of β-glucuronidase (*gus*) (Jefferson, 1987) or generate a visible light, such as luciferase (Basnayake *et al*., 2011).

GUS is an enzyme isolated from *E. coli* and is useful in plant transformation studies because it is not present in higher plants (Jefferson, 1987). It is versatile in that it can be tested in histochemical, spectrophotochemical and fluorometric assays (Jefferson, 1987). It is a highly stable fusion protein and does not compromise the expression of other proteins. However, as the assay is destructive to the transformed tissue it cannot be employed in all instances (Jefferson,

1987). Fluorometric GUS assays utilize 4-methyl umbelliferyl glucuronide (MUG) as the substrate (Jefferson, 1987; Last *et al*., 1991, Potier *et al*., 2008b), and offer higher detection sensitivity than the spectrophotochemical GUS assay (Jefferson, 1987). Histochemical GUS assays are easy to perform and require incubating the transformed tissue with 5-bromo-4-chloro-3-indolyl-glucuronide (X-gluc) (Jefferson, 1987). Hence, it is routinely employed in sugarcane transformation studies to assess transient gene expression (Bower and Birch, 1992; Gambley *et al*., 1993; Sun *et al*., 1993; Chamberlain *et al*., 1994; Witcher *et al*., 1998; Viaplana *et al*., 2001; Potier *et al*., 2008b; van der Vyver, 2010; Harinath and Nerker, 2012) (Table 6).

Another reporter gene that is use in plant transformation studies is the green fluorescent protein (GFP). In this case, the transformed cells fluoresce when illuminated with light at a certain wavelength, indicating that the gene is being expressed (Elliot *et al*., 1998; Kavita and Burma, 2008). Both GUS and GFP have been used in sugarcane transformation and offer an indication of gene expression (Bower and Birch, 1992; Gambley *et al*., 1993; Sun *et al*., 1993; Chamberlain *et al*., 1994; Elliot *et al*., 1998; Witcher *et al*., 1998; Viaplana *et al*., 2001; Kavita and Burma, 2008; Potier *et al*., 2008b; van der Vyver, 2010; Harianth and Nerker, 2012) (Table 6). A study by Kavita and Burma (2008) on *Brassica juncea* found a discrepancy in the amount of time a promoter is active and the time that the reporter genes' products persist. When comparing the reporter genes *gus* and *gfp*, they found that GUS persisted in the cells after the promoter activity had stopped, whilst GFP expression followed promoter activity and ceased when the promoter activity had stopped (Kavita and Burma, 2008). Their findings indicate that the choice of gene is dependent on the nature of the study being conducted, with *gfp* being better than *gus* to temporal expression profile studies (Kavita and Burma, 2008).

Table 6. Selection strategies used in plant transformation studies and the respective marker and reporter genes.

Selective marker gene	Selection strategy	Reporter gene	Reference
npt II	Geneticin and kanamycin resistance	*gus*	Bower and Birch, 1992
-	-	*gus*	Gambley *et al*., 1993
npt II	Kanamycin resistance	*gus*	Sun *et al*., 1993
npt II	Kanamycin resistance	*gus*	Chamberlain *et al*., 1994
-	-	Anthocyanin	Snyman *et al*., 1996
npt II	Geneticin resistance	Anthocyanin	McCallum *et al*., 1998
Bar gene	Bialaphos resistance	*gus*	Witcher *et al*., 1998
Bar gene, *npt II*	Bialaphos resistance, Geneticin resistance	-	Ingelbrecht *et al*., 1999
npt II	Geneticin resistance	-	Groenewald and Botha, 2001
npt II, CP4	Kanamycin resistance, glyphospate resistance	-	Snyman *et al*., 2006
npt II	Kanamycin resistance	*gus*	Potier *et al*., 2008b
npt II	Geneticin resistance	*gfp*	Van der Vyver, 2010
npt II	Geneticin resistance	*luc*	Basnayake *et al*., 2011

2.3.3 Difficulties involved in sugarcane transformation

Transgene silencing remains one of the biggest problems facing plant transformation (Brumbley *et al*., 2008; Graham *et al*., 2011). This is especially true in plants with complex genomes, such as the polyploid sugarcane (Hansom *et al*., 1999; Ingelbrecht *et al*., 1999; Brumbley *et al*., 2008; Potier *et al*., 2008a; Mudge *et al*., 2009; Graham *et al*., 2011). Gene silencing is the result of DNA methylation where a methyl group ($-CH_3$) is bound to the CpG islands of the gene (Adams, 1990; Vanyushin, 2005). The binding of the methyl group either: 1) signals histones to bind to the DNA and degrade it, preventing the transcription of the RNA and the translation of the coded protein; or 2) prevent RNA polymerase from accessing that region of the gene (Adams, 1990; Vanyushin, 2005).

Gene silencing can be divided into two types, *viz*. transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) (Ingelbrecht *et al*., 1999; Yu and Kumar, 2003; Kapoor *et al*., 2005; Potier *et al*., 2008b; Graham *et al*., 2011). Although both are the result of DNA methylation, the type of silencing is dependent on the region in which the methylation occurs (Ingelbrecht *et al*., 1999; Yu and Kumar, 2003; Kapoor *et al*., 2005; Potier *et al*., 2008b; Graham *et al*., 2011). In transcriptional gene silencing the promoter region of the construct is methylated and may involve changes in chromatin structure, whilst in post-transcriptional gene silencing it is specific coding sequences that are methylated and degraded (Ingelbrecht *et al*., 1999; Yu and Kumar, 2003; Kapoor *et al*., 2005; Potier *et al*., 2008b; Graham *et al*., 2011).

Whilst epigenetic gene silencing is recognized to be an important part of gene regulation (defensive mechanism against viral attacks) (Voinnet, 2002; Yu and Kumar, 2003), it becomes problematic during genetic transformation studies when the transgene is silenced (Kapoor *et al*., 2005; Graham *et al*., 2011). When a transgene is introduced into the transformant, it is integrated randomly into the genome and its integrated position can determine whether the transgene is silenced or not (Meng *et al*., 2003; Graham *et al*., 2011). Another important factor that plays a role in transgene silencing is copy number (Meng *et al*., 2003; Graham *et al*., 2011), which refers to the number of times to which the transgene is inserted into the genome (Meng *et al*., 2003; Graham *et al*., 2011). As mentioned above, sugarcane transformation is difficult because of its genetic complexity and high ploidy (Daniels and Roach, 1987; Hansom *et al*., 1999; Ingelbrecht *et al*., 1999; Irvine, 1999; Butterfield *et al*., 2001; Kapoor *et al*., 2005; Brumbley *et al*., 2008; Potier *et al*., 2008b; Mudge *et al*., 2009; Graham *et al*., 2011). The high number of chromosome pairs can result in an increased incidence of the transgene being inserted many times in the genome, it also increases the incidence of the transgene being integrated into a region of importance, resulting not only in the transgene being silenced but also compromising the expression of essential genes (Kapoor *et al*., 2005). These difficulties in sugarcane transformation have slowed the progress of producing transgenic lines of economic importance. Hence, there is a need to produce a promoter that does not experience transgene silencing and to create a system that can be used to test promoter function.

3. Materials and methods

3.1 *In vitro* culture studies on selected ancestral sugarcane germplasm

3.1.1 Selection of ancestral varieties for the study

Field-grown sugarcane stalks from the variety NCo376 (a commercial variety) and the ancestral varieties *S. spontaneum* Nigeria 1, *S. spontaneum* Nigeria 2, *S. spontaneum* IK76-46, *S. spontaneum* Mandalay, *S. spontaneum* Coimbatore, *S. officinarum* Badilla, *S. officinarum* Black Cheribon and *S. officinarum* NG77-69, kindly provided by SASRI (Mount Edgecombe, KwaZulu Natal), were all used in this preliminary study. Explants were prepared as mentioned below (3.1.2) and plated onto callus induction media (CIM) containing MS (Murashige and Skoog, 1962) with vitamins, 20 $g.l^{-1}$ sucrose, 0.5 $g.l^{-1}$ casein hydrolysate, 5 $mg.l^{-1}$ 2,4-D and solidified with 8 $g.l^{-1}$ agar agar (pH 5.8). The cultures were kept in the dark at 26 ± 1 °C and subcultured onto fresh media every three weeks for six weeks. After six weeks on CIM, the callus was transferred onto embryo germination media [(EGM) (20 $g.l^{-1}$ sucrose, 0.5 $g.l^{-1}$ casein hydrolysate and 8 $g.l^{-1}$ agar agar (pH 5.8)]. Callus was subcultured onto fresh EGM every three weeks for six weeks, during which time the percentage of explants that formed callus was recorded.

Based on the results obtained, six varieties of sugarcane were selected for subsequent use *viz.* NCo376, *S. spontaneum* Nigeria 1, *S. spontaneum* Nigeria 2, *S. spontaneum* Coimbatore, *S. officinarum* Black cheribon and *S. officinarum* NG77-69.

3.1.2 Explant preparation and callus induction

Sugarcane stalks were collected from the fields at SASRI (Figure 3). The outermost leaves were excised before the leaf rolls were decontaminated by swabbing them with 70 % ($v.v^{-1}$) ethanol. The leaf rolls were transferred to the laminar flow where the outermost leaf sheaths were aseptically removed to reveal the inner leaf roll. They were then sliced into 30 disks, each of which was 2 mm thick, in liquid MS medium (CIM without agar agar). They were then plated onto the tested semi-solid media with a total of 10 disks per 90mm petri dish (30 ml of CIM per plate) (Table 7).

All media were autoclaved at 121 °C for 20 minutes. In the instance of temperature sensitivity, as in the case of proline and the antibiotic paromomycin (Sigma-Aldrich, MO, USA), the component was filter sterilized (0.2 µm pore diameter) (Acrodisc® 25 mm PF syringe filter, Pall Corporation, MI, USA) and added to the sterile media after it had cooled to approximately 50 °C.

All treatments excluding CIM-A4 were subcultured onto fresh media every three weeks for six weeks. For treatment CIM-A4, leaf disks were plated onto media containing 5 $mg.l^{-1}$ IBA for one week, after which they were transferred to media containing 3 $mg.l^{-1}$ 2, 4-D. Thereafter, leaf disks with calli were subcultured onto fresh media every three weeks for six weeks. All cultures were kept in the dark at 26 ± 1 °C. During this time, % disks showing callus formation, % dead disks, % microbial contamination (and type of contaminant if applicable) were assessed per Petri dish every three weeks.

Figure 3. Plant material source. Field grown sugarcane stalks were harvested from SASRI (Mount Edgecombe, KwaZulu Natal) and used as explants for the experiments in this study.

Table 7. Constituents (and their concentrations) of semi-solid culture media used for callus induction of the selected sugarcane germplasm (NCo376, *S. spontaneum* Nigeria 1, *S. spontaneum* Nigeria 2, *S. spontaneum* Coimbatore, *S. officinarum* Black cheribon and *S. officinarum* NG77-69). All media contained MS with vitamins, 0.5 $g.l^{-1}$ casein hydrolysate and was solidified with 8 $g.l^{-1}$ agar agar.

CIM	Carbon source	Plant growth regulators	Other
A1 (Std. CIM*)	20 $g.l^{-1}$ sucrose	3 $mg.l^{-1}$ 2, 4-D	-
A2	20 $g.l^{-1}$ sucrose	5 $mg.l^{-1}$ 2, 4-D	-
A3	20 $g.l^{-1}$ sucrose	5 $mg.l^{-1}$ 2, 4-D + 0.5 $mg.l^{-1}$ BA	-
A4	20 $g.l^{-1}$ sucrose	5 $mg.l^{-1}$ IBA (1 week) then 3 $mg.l^{-1}$ 2, 4-D (3 weeks)	-
B1	20 $g.l^{-1}$ sucrose	3 $mg.l^{-1}$ picloram	-
B2	20 $g.l^{-1}$ sucrose	5 $mg.l^{-1}$ picloram	-
B3	20 $g.l^{-1}$ sucrose	3 $mg.l^{-1}$ 2, 4-D + 2 $mg.l^{-1}$ picloram	-
C1	20 $g.l^{-1}$ sucrose	3 $mg.l^{-1}$ 2, 4-D	0.3 $g.l^{-1}$ proline
C2	20 $g.l^{-1}$ sucrose	3 $mg.l^{-1}$ 2, 4-D	Double nitrogen of MS
C3	20 $g.l^{-1}$ sucrose	3 $mg.l^{-1}$ 2, 4-D	0.3 $g.l^{-1}$ proline + double nitrogen of MS
D	13 $g.l^{-1}$ sucrose + 1 $g.l^{-1}$ each of ribose, xylose, arabinose, glucose, mannose, galactose, fructose	3 $mg.l^{-1}$ 2, 4-D	-

*Std. CIM – standard CIM used by SASRI for the *in vitro* culture of NCo376.

3.1.3 Embryo germination and plantlet establishment

After six weeks on the tested callus induction media, calli were transferred to 90 mm Petri dishes containing embryo germination medium (EGM) [(CIM without auxin) (30 ml EGM per Petri dish)]. Cultures were maintained in a growth room at 26 ± 1 °C with a photoperiod of 16 hours light (35 µmol/m^2/s) and 8 hours dark and were subcultured every three weeks for six weeks. Shoots that reached 2 cm in height were then transferred into Sterivent® vessels (Duchefa, Netherlands) containing plantlet establishment media (PEM) (½ MS, 5 $g.l^{-1}$ sucrose, 0.5 $g.l^{-1}$ casein hydrolysate and 8 $g.l^{-1}$ agar agar (pH 5.8)). For callus induction ten leaf disks were initiated per plate and the calli formed by these disks were maintained separately throughout the culture process (callus induction, embryo germination and plantlet establishment). Consequently, plant yield was calculated as the number of plants obtained/original ten disks.

3.1.4 Acclimatization

After 4-6 weeks on PEM or until the shoots of the plantlets reached approximately 10 cm in height), the plantlets were transferred to polystyrene seedling trays (Grovida, RSA) containing 1:1 peat moss (Grovida, RSA) and vermiculite (Hygrotech, RSA). Plantlets in the seedling trays were placed in a glasshouse chamber at 80 % humidity for three days where they were watered with a spray of water for 5 minutes every 12 hours. They were then transferred to a general glasshouse chamber at 60 % humidity and watered for 5 minutes twice a day in summer and once a day in winter. After two weeks in the glasshouse, the seedling trays with the plantlets were transferred to a polycarbonate tunnel, at ambient temperatures, and allowed to grow.

3.2 Study to test transgene integration and expression in selected ancestral sugarcane varieties

3.2.1 Production of embryogenic callus

NCo376, *S. spontaneum* Nigeria 1 and *S. officinarum* NG77-69 immature leaf rolls were used to generate callus in this study, and only type 3 callus (Taylor *et al*., 1992) was used. Explants were prepared and maintained as in 3.1.2, plated on CIM-A2, and subcultured onto fresh media every three weeks until sufficient embryogenic callus was produced for transformation.

3.2.2 Plasmid constructs

The plasmids pAHC27, pEmuKN and $pR_{11}F^-$ were used in this study (Figure 4). The pAHC27 plasmid, (6.842 kB) contained the β-glucuronidase (GUS) coding sequence (isolated from *E. coli*) under the control of the maize ubiquitin (Ubi) promoter with the *A. tumefaciens* nos3 terminator sequence and the coding sequence for ampicillin resistance. The plasmid pEmuKN (5.645 kB) contained the coding sequence for the selectable marker gene neomycin phosphotransferase II (*npt II*) under the control of the Emu promoter (conferring resistance to the antibiotic kanamycin), with the nos3 terminator sequence (Last *et al*., 1990). The plasmid $pR_{11}F^-$ (approximately 6 kB) was kindly provided by B.A.M Potier (SASRI, Mount Edgecombe, KwaZulu Natal) and contained the GUS coding sequence driven by a root specific promoter.

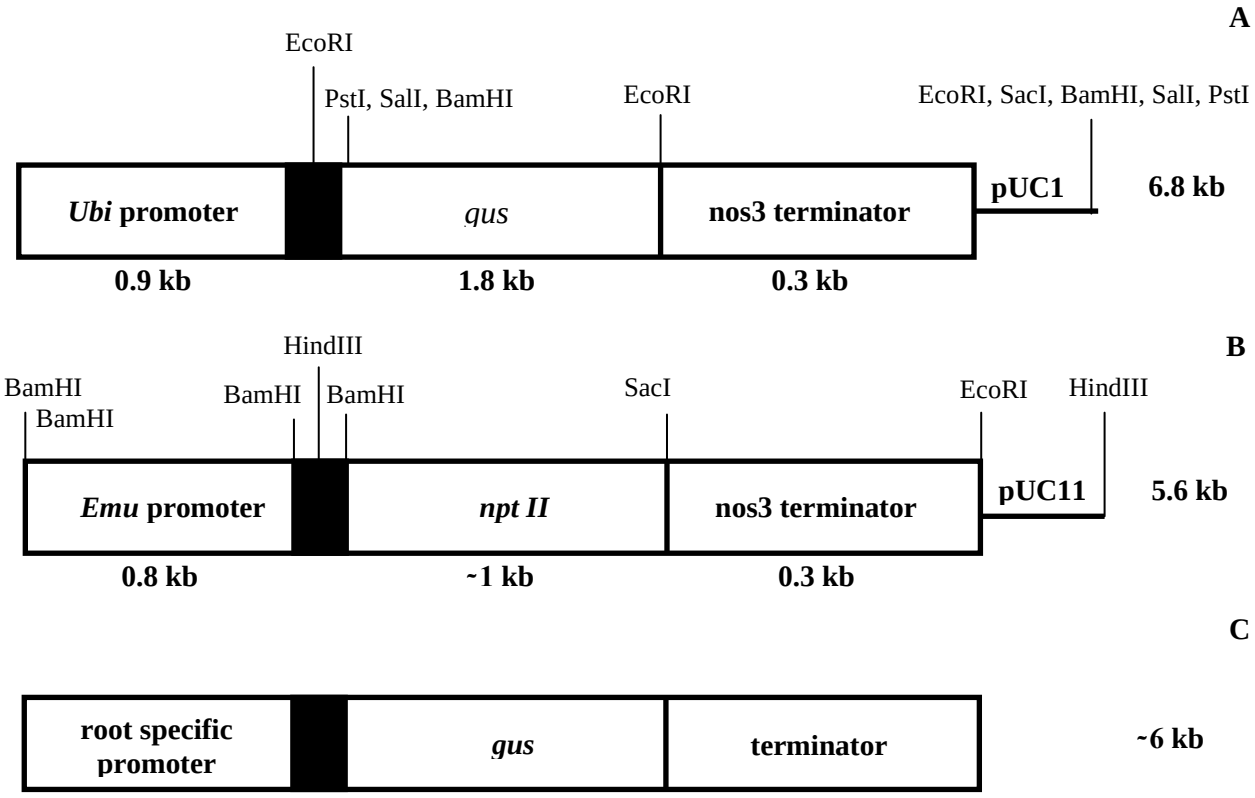

Figure 4. Simplified construct diagrams of the plasmids A) pAHC27, B) pEmuKN and C) $pR_{11}F^-$ showing sizes of selected sequences and restriction sites. The details of the plasmid $pR_{11}F^-$ are under the intellectual property of B.A.M Potier (SASRI).

3.2.3 Transformation of bacterial cells via electroporation

Competent *E. coli* cells (TOP 10 Electropcomp™ cells, Invitrogen, RSA) were grown in tubes containing 5 ml of Luria-Bertani broth (LB, Appendix 1) and 5 µl (100 $mg.ml^{-1}$) of the appropriate antibiotic for the plasmid used (ampicillin for pAHC27 and kanamycin for pEmuKN). Bacterial colonies were picked off Luria-Bertani agar plates (LA, Appendix 2), using sterile wooden toothpicks, and added to tubes containing the LB and antibiotic and were shaken at 300 rpm at 37 °C for one hour. Thereafter, 3 ml of the culture was added to 250 ml conical flasks containing 100 ml of LB and 100 µl of the respective antibiotic, returned to the shaker and allowed to grow overnight. An uninnoculated control was also included to rule out accidental microbial contamination of LB and antibiotic stocks.

Electrocuvettes were decontaminated by washing them with 1 M HCl; these were then sprayed with absolute ethanol and allowed to dry in a laminar flow. The plasmid solution (1 - 2 µl, 1-50 $ng.\mu l^{-1}$) was combined with 70 µl of freshly-grown competent cells and the mixture was left on ice for 10 minutes, before being transferred to the electrocuvettes. Immediately after electroporation, 450 ml LB was added to the electrocuvette and mixed, then the contents of the cuvette were transferred to Eppendorf tubes (1.5 ml) and placed into a shaking incubator for one hour at 37 °C and 300 rpm.

3.2.4 Plasmid extraction

Transformed competent cells were plated onto LA plates and incubated at 37 °C overnight. Plasmid extraction was undertaken using the QIAGEN Plasmid Purification Maxi Kit (100 ml) (Qiagen, Whitehead Scientific, RSA) and following the instructions in the handbook provided. The resultant DNA was quantified and assessed for purity at 260 nm using a Nanodrop spectrophotometer (ND-1000, NanoDrop Technologies, Delaware, USA).

A restriction digest of each plasmid was undertaken to determine DNA yield and quality (Figure 5). The restriction enzymes used were SalI, EcoRI, BamHI, SacI and PstI (Inqaba Biotech, RSA) for the plasmid pAHC27 and BamHI, SacI, EcoRI and HindIII (Inqaba Biotech, RSA) for pEmuKN. Extracted DNA was mixed with loading dye and GelRed™ nucleic acid gel stain (Biotium, California, USA) and separated by gel electrophoresis (SeaKem® LE agarose, Lonza, Maine, USA) along with a 1 kB plus O'Gene™ Ruler (Inqaba Biotech, RSA) molecular marker

at 85 v before being viewed with a transilluminator (AlphaImager™ 220, Alpha Innotech Corparation, California, USA).

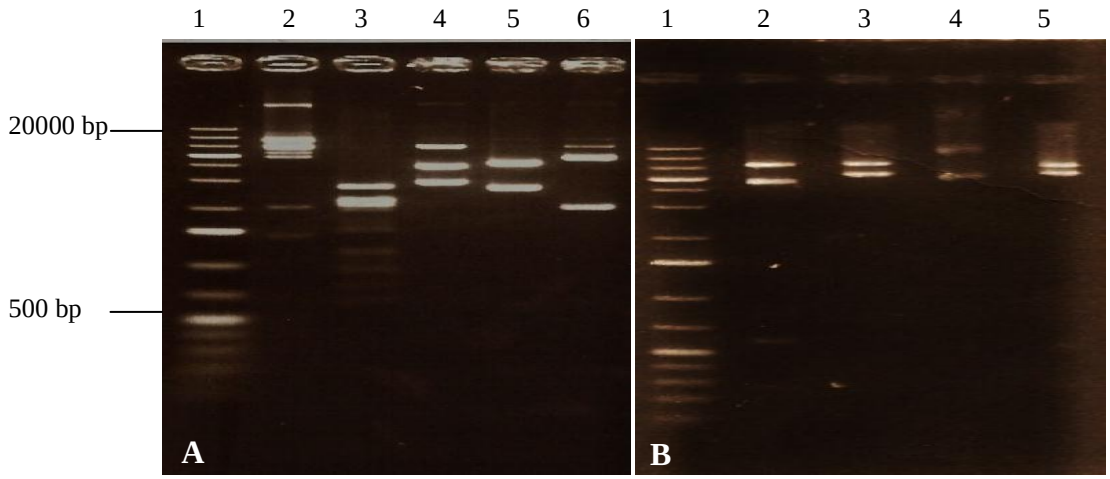

Figure 5. Agarose gel electrophoresis of restriction fragments of A) pAHC27 and B) pEmuKN. Extracted DNA was run on 2 % (m/v^{-1}) agarose gel at 85 v. 500-1000 ng of sample DNA was loaded in each lane.

A. lane 1 - 1 kb plus O'Gene™ Ruler (500 ng), lane 2 - SalI, lane 3 – EcoRI/BamHI, lane 4 - SacI, lane 5 – EcoRI, lane 6 – PstI.
B. lane 1 - 1 kb plus O'Gene™ Ruler (500 ng), lane 2 – BamHI, lane 3 – SacI, lane 4 – EcoRI, lane 5 – HindIII.

3.2.5 Microprojectile bombardment

Type 3 embryogenic calli were placed onto an osmoticum medium (CIM + 0.2 M mannitol and 0.2 M sorbitol) (Vain *et al.*, 1993) to cover a circle of 3 cm diameter and kept in the dark for four hours prior to bombardment. Callus was co-bombarded with either a combination of pEmuKN + pAHC27 or pEmuKN + $pR_{11}F^{-}$. The microprojectile used in this study was tungsten M - 10 (0.7 µm) (Biorad, California, USA). The tungsten particles were surface sterilized with absolute ethanol, followed with three rinses in sterile distilled water. Tungsten (50 µl per tube) was combined with 500 $ng.\mu l^{-1}$ of each plasmid to be co-bombarded, 50 µl 2.5 M calcium chloride (Appendix 3) and 20 µl 0.1 M spermidine (Appendix 4) and maintained on ice, to be used immediately. The spermidine and the calcium chloride were filter sterilized (0.2 µm pore

diameter) (Acrodisc® 25 mm PF syringe filter, Pall Corporation, MI, USA) before being included into the precipitation mix. The microprojectile bombardment was conducted under sterile conditions as per Snyman (2004). Approximately half an hour prior to bombardment, the calli were removed from the dark and allowed to air dry in the laminar flow. The particle inflow gun used (Figure 6) in the study was manufactured at SASRI, based on the design of Finer *et al*. (1992). In the laminar flow, 110 µl of the overlying aqueous solution was removed from the bombardment mix, the remaining solution was then agitated to suspend tungsten particles and 4 µl was placed onto a 13 mm Swinny filter (Millipore, Schwalbach, Germany) and screwed into place in the bombardment chamber (Snyman *et al*., 2006). The distance between the Swinny filter, metal grids and baffles were as per SASRI standard transformation protocols (Snyman *et al*., 2006). The callus to be bombarded was placed on a metal grid and covered with a baffle (1 mm^2 nylon mesh) that rested 1.5 cm above the callus (Snyman *et al*., 2006). These were then placed into the bombardment chamber 6 cm under the Swinny filter (Snyman *et al*., 2006). The chamber was evacuated to create a vacuum and the DNA-coated tungsten particles were accelerated onto the target callus microprojectiles using pressurized helium gas as the propellant. Following bombardment, the callus was placed back onto the osmoticum media to 'recover' for four hours in the dark and the callus was then transferred onto CIM for two days in the dark.

3.2.6 Selection of transformed cells

Subsequent to the CIM treatment, each callus piece was separated into 4 mm portions and plated onto a pre-punched medium (media that had been scored to increase the area of contact between calli and media) comprising of CIM + 150 $mg.l^{-1}$ of the antibiotic paromomycin (Sigma-Aldrich, MO, USA, Appendix 5) (dark selection media, Appendix 6). The cultures were maintained in the dark and were closely monitored; after 6 - 8 weeks, the calli that had increased in size were transferred to light selection media (EGM + 150 $mg.l^{-1}$ paromomycin, Appendix 7) and placed in a photoperiod growth room. Growth room conditions and photoperiod are as per 3.1.3. Plantlets that reached 2 cm in height were transferred into Sterivent® vessels (Duchefa, Netherlands) containing PEM + 150 $mg.l^{-1}$ paromomycin until they reached approximately 10 cm. Thereafter, the plantlets were acclimatized in a misting chamber and greenhouse, as described in section 3.1.4.

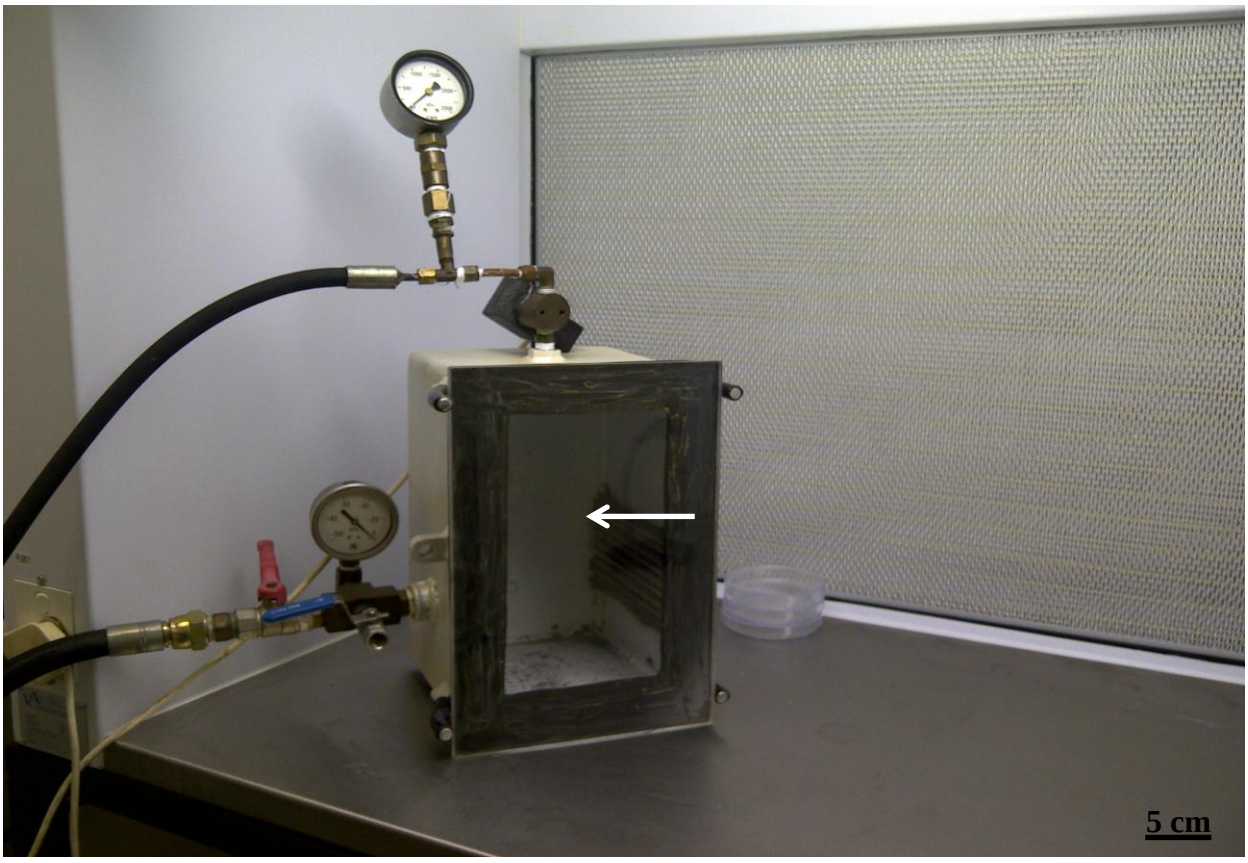

Figure 6. The Particle Inflow Gun (PIG) used for microprojectile bombardments in this study. This apparatus was manufactured at SASRI, based on the design by Finer *et al*. (1992). Calli were placed on a metal grid slotted 6 cm below the microprojectile source (indicated by the arrow) and covered with a protective nylon mesh. The microprojectiles were accelerated onto the callus using helium gas.

3.2.7 Viability assay

Bombarded calli that did not regenerate or produce plantlets were immersed in 0.8 % ($m.v^{-1}$) 2,3,5 triphenyltetrazolium chloride (TTC/tetrazolium red, Appendix 8) (Sigma-Aldrich, MO, USA) dissolved in a 0.05 M phosphate buffer (Appendix 9). After incubation in the dark at room temperature overnight, calli that turned red were considered to be alive.

3.2.8 Plant genomic DNA extraction and polymerase chain reaction (PCR) amplification

Genomic DNA from the leaves of putatively transformed plants was extracted using the QIAGEN DNeasy Plant Mini Kit and following the instructions provided (Qiagen, Germany). The DNA obtained was quantified and assessed for purity at 260 nm using a Nanodrop

spectrophotometer (ND-1000, NanoDrop Technologies, Delaware, USA). Polymerase chain reaction (PCR) amplification was carried out with the forward and reverse primers for *gus* and *npt II*. The primer sequences used are presented in Table 8.

Table 8. Primer sequences used for PCR amplification of the *gus* and *npt II* transgenes.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
GUS	GTGACAAAAACCACCCAAGC	AATAACGGTTCAGGCACAGC
npt II	CTCCTGCCGAGAAAGTATC	TCATCCTGATCGACAAGACC

The end-point PCR reactions were conducted using a PCR kit (Kapa Biosystems, Massachusetts, USA). To avoid multiplexing, each primer set was used in separate PCR reactions. The total reaction volume for each reaction was 25 µl and contained 2.5 µl PCR buffer (10 x buffer), 0.5 µl dNTP's (10 mM), 0.7 µl of each forward and reverse primer (6 mM), 0.13 µl taq polymerase, 1 µl magnesium chloride (25 mM), 14.47 µl PCR water (Ambion, Texas, USA) and 5 µl of the genomic DNA.

Following initial denaturation using a Biorad MyCycler thermal cycler at 94 °C for four minutes, the cycling parameters used were 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 30 seconds and initial extension at 72 °C for 30 seconds. Another extension cycle was then carried out at 72 °C for five minutes. The products of the PCR reaction (8 µl/reaction) were loaded onto a 2 % ($m.v^{-1}$) agarose gel with 2 µl of loading dye/GelRed™ nucleic acid gel stain mix. The DNA was allowed to migrate at 100 v for 3 minutes and then 85 v for 45 minutes before viewed using a transilluminator.

3.2.9 Quantitative Real-Time PCR (qPCR) analyses

The qPCR analyses were conducted on the *gus* and *npt II* coding sequences. A standard curve for both *gus* and *npt* II was produced using DNA containing these genes (i.e. DNA from an untransformed plant that had been 'spiked' with linearized plasmid DNA). Tubulin was used as a house-keeping/reference gene, therefore a standard curve was also set up for this gene.

Linearized plasmid DNA containing the *gus*, tubulin and *npt* II genes was made up to a 1/100, 1/1000 and 1/100 dilution respectively. A fivefold dilution series was set up for each of the

target genes and a volume of 0.65 µl for each forward and reverse primer was determined to be the most efficient for qPCR analysis.

The total volume for each reaction was 13 µl and contained 0.65 µl of each forward and reverse primer (6 mM stock), 6.25 µl SYBR Green Jumpstart® Taq ready mix (Sigma-Aldrich, Mo, USA), 3.45 µl PCR water (Ambion, Texas, USA) and 2 µl DNA. The primer sequences for *gus* and *npt II* are shown in Table 7, whilst the tubulin forward primer was 5' AGGCCCAACTACTCCAACCT 3' and the reverse was 5' TGAGGAGAGCATGAAGTGGA 3'. qPCR amplification was carried out in duplicate using the Biorad CFX96™ Real Time PCR Detection System (Biorad C1000 thermal cycler and the CFX96 Optical Reaction Module; Biorad, USA) and analyzed using Biorad CFX Manager™ software (Biorad, USA). An initial denaturation cycle at 95 °C for three minutes was followed by 38 cycles of denaturation at 95 °C for 10 seconds, annealing at 61.5 °C for 30 seconds and extension at 72 °C for 30 seconds. Subsequent to the establishment of the standard curves, DNA was extracted from transformed plantlets from each variety and amplified. Untransformed control DNA was also included.

The transformation efficiency was calculated by dividing the number of plantlets that were successfully transformed (presence of both *npt II* and *gus* genes) by the number of bombardments carried out for a particular construct and variety according to Taparia *et al*. (2012).

3.2.10 Histochemical GUS assay

Histochemical GUS assays were conducted as per Jefferson (1987). Transgenic plants of the variety 88H0019 were used as a positive control for all GUS assays as they stably express the *gus* gene. Plant material (callus, leaf and root) were cut into 2 cm pieces and immersed (enough to submerge plant material) in the GUS substrate buffer [(5 bromo-4 chloro-3 indolyl-β glucuronic acid) (X-gluc, Appendix 10) (Sigma, RSA)] and incubated at 37 °C overnight. Enzyme activity and gene expression were confirmed by the presence of blue foci on the plant material. Subsequent to incubation, the GUS buffer was removed and replaced with 70 % ethanol, in order to extract chlorophyll from the tissue so that blue foci were easily recognized.

3.3 Microscopy and photography

In order to estimate the amount of embryogenic and non-embryogenic callus that was produced during induction, a sample of calli from each treatment was viewed using compound (Olympus venox AHBS3) and dissecting microscopes (Zeiss Stemi SVII). For enhanced contrast, the cells were stained with 1 % ($m.v^{-1}$) toluidine blue. Microscopic photographic records were collected at various stages of the embryo and plantlets' development using a Nikon camera (E4500); all other photographic recording was done using the Nikon L21 model.

3.4 Data analyses

The plant regeneration yields obtained during the study on somatic embryogenesis in NCo376 and selected ancestral varieties were based on the number of plants obtained per original 10 explants placed onto callus induction media.

Qualitative data were assessed visually and scored using the following system: '-' referred to no embryogenic callus formation, '+' denoted that less than 25 % of callus was embryogenic, '++' signified that more than 25 % of formed callus was embryogenic and '+++' represented that more than 50 % of formed callus was embryogenic. Quantitative data were analysed using the statistical package GraphPad Prism 6 (Version 6.01). The data were tested for normal distribution and significant differences between treatments using an analysis of variance (ANOVA) test and the Tukey's post-hoc test. A probability of $F \leq 0.05$ was considered significant.

4. Results

4.1 Somatic embryogenesis in NCo376 and selected varieties of *S. spontaneum* and *S. officinarum*

4.1.1 Somatic embryogenesis in the commercial sugarcane cultivar NCo376

With the exception of that of the work by van der Vyver (2010), no somatic embryogenesis protocols have been reported for the ancestral sugarcane varieties. In contrast, somatic embryogenesis protocols for the commercial cultivar NCo376 have been optimized. Hence, in the current study, the response of the ancestral varieties to the tested callus induction media were compared with that of NCo376 using its optimized protocol. Consequently, the developmental process and culture stages of the latter are described here so as to compare them with those of the selected ancestral varieties. Leaf rolls (Figure 7A) were decontaminated, cut into disks (Figure 7B) and established on standard CIM (Std. CIM). Swelling of the leaf disks was observed within 3-4 days after culture initiation and callus formation was noticeable after one week in culture (Figure 7C). Callus was transferred onto fresh Std. CIM every two weeks. After 5-6 weeks in culture type 1, 2 and 3 callus (Taylor *et al*., 1992) were observed. Type 3 embryogenic callus was recognized as yellow to white in colour with a nodular appearance (Figure 8A) (Taylor *et al*., 1992). Microscopic visualization (with a compound microscope) of type 3 callus showed that it was comprised mostly of embryogenic cells. These cells were distinguishable from non-embryogenic cells by their smaller size and prominent nuclei (Figure 8B). Type 1 callus was identified as semi-translucent and non-embryogenic (Figure 8C), whilst type 2 appeared yellow to grey in colour with a mucilaginous appearance (Figure 8D) (Taylor *et al*., 1992).

Ninety-three % of NCo376 explants produced callus, of which the predominant callus type as described by Taylor *et al*. (1992) was type 3 embryogenic callus. This was expected, as this somatic embryogenesis protocol has been previously optimized for this variety. After 6-8 weeks on Std. CIM, calli were transferred onto EGM and germination occurred within the next two weeks. The recorded yield for NCo376 was 450 plantlets/10 disks (i.e. number of plantlets/10 original explants). This yield is relatively high (more than double) when compared to 207 plants/10 disks obtained by Khalil (2002) with a commercial sugarcane cultivar.

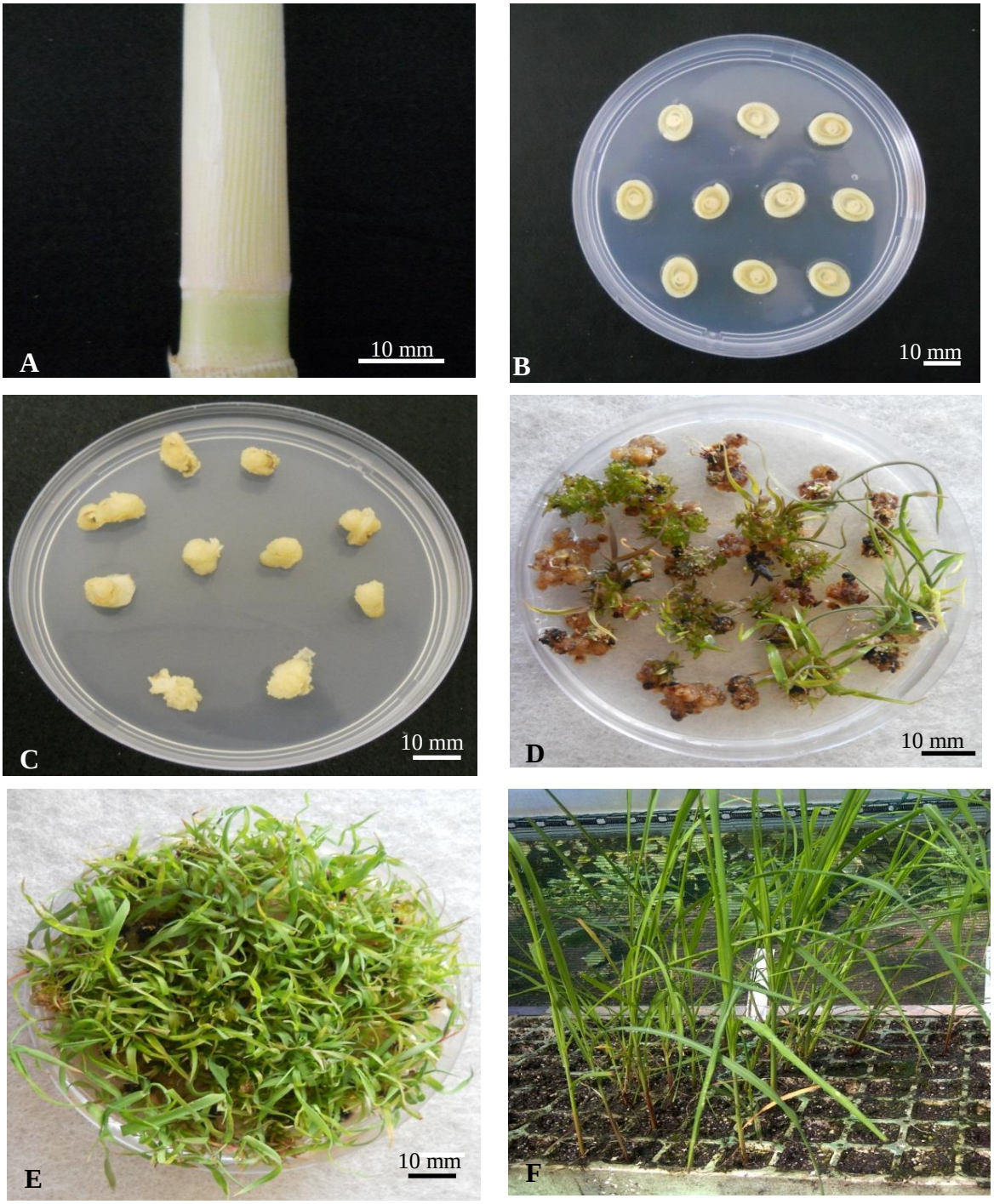

Figure 7. Stages involved in indirect somatic embryogenesis of the commercial sugarcane cultivar NCo376. (A) Field grown immature leaf rolls were prepared for use as explants, stalks were surface sterilized, (B) cut into leaf-roll disks and plated onto Std. CIM. After 3-4 days in the dark, leaf disks swelled in size and (C) after one week, began to form callus. (D and E) Following 6-8 weeks on Std. CIM, calli were transferred to EGM in the light and the embryos germinated and formed plantlets. (F) Once the shoots of the plantlets were approximately 10 cm in height they were planted out in seedling trays and moved to the glasshouse to be acclimatized.

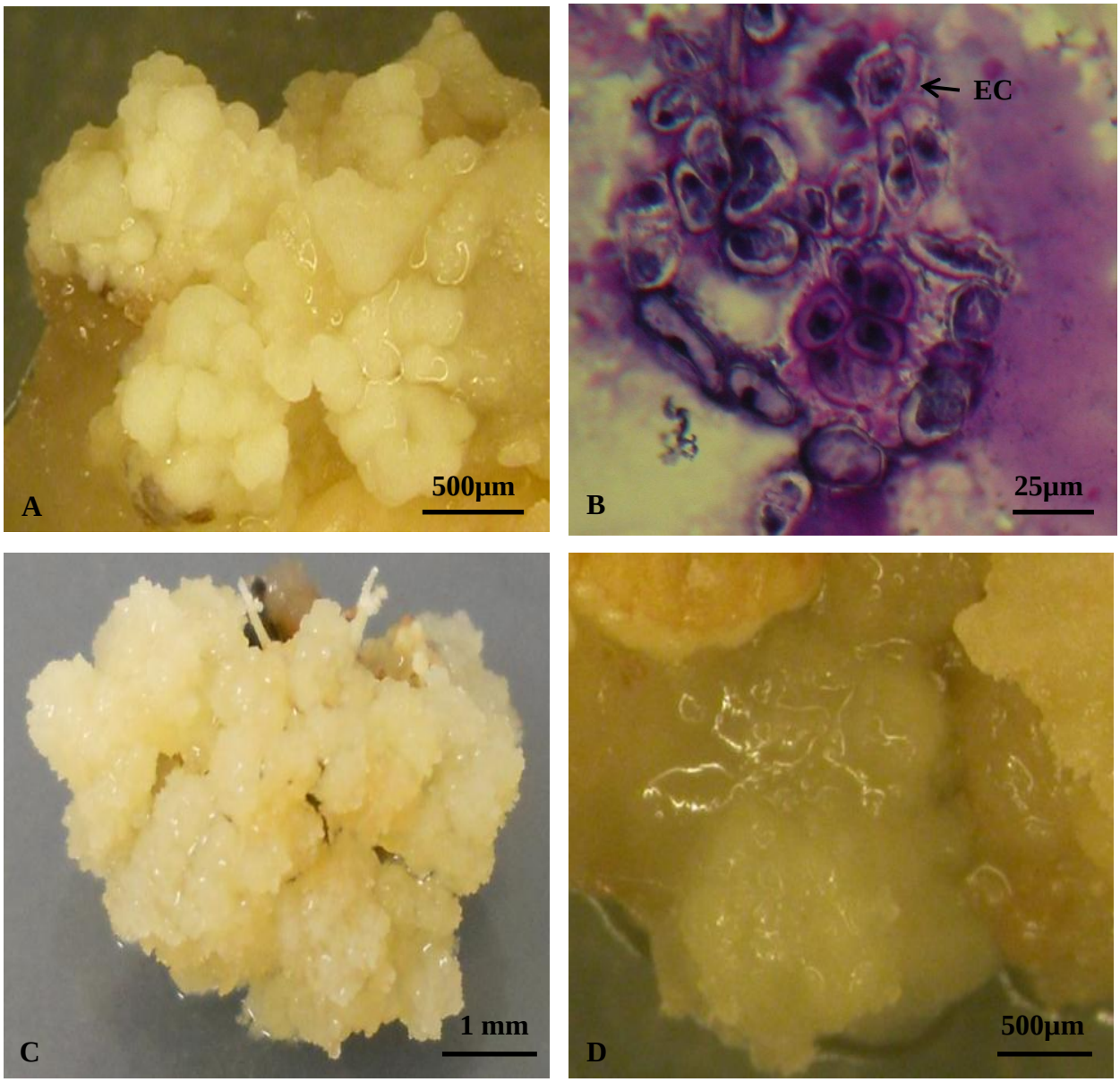

Figure 8. The types of callus produced by indirect somatic embryogenesis of NCo376. (A) Type 3 callus was yellow to white in colour, nodular in appearance and embryogenic (Taylor *et al.*, 1992). (B) Embryogenic cells were identified by microscopic examination of type 3 callus (B) (arrow denotes embryogenic cells [EC] with prominent nuclei). (C) Type 1 callus appeared semi-translucent. (D) Type 2 was yellow-to grey and mucilaginous (Taylor *et al.*, 1992).

4.1.2 Screening of ancestral sugarcane germplasm to determine the varieties to be used in subsequent studies

The *S. spontaneum* varieties Nigeria 1, Nigeria 2, IK76-46, Mandalay and Coimbatore and the *S. officinarum* varieties Badilla, Black Cheribon and NG77-69 were initially tested to determine which varieties responded the best to the callus induction medium (CIM) used for NCo376. Leaf discs were cultured onto CIM for six weeks and the % disks that produced callus was recorded. As a 2, 4-D concentration range of 3 to 4 $mg.l^{-1}$ was reported to be the most effective for callus production of commercial sugarcane cultivars (McCallum *et al*., 1998; Khalil, 2001; Basnayake *et al*., 2011), 5 $mg.l^{-1}$ 2, 4-D (instead of 3 $mg.l^{-1}$ as for NCo376) was used in this preliminary study to try and ensure cell division and promote callus formation.

As with NCo376 (Section 4.1.1, Figure 7) swelling of leaf disks of the tested varieties was observed 3-4 days after establishment on CIM. Calli was recorded in 87 % of Coimbatore, 80 % of Nigeria 1 and Nigeria 2 disks, and 70 % of Mandalay and IK76-46 disks (Figure 9), and there was no significant difference amongst the responses of the varieties. *S. spontaneum* Nigeria 1, Nigeria 2 and Coimbatore were then selected for subsequent studies based on the availability of plant material at SASRI, at the time of this study. Of the *S. officinarum* varieties, although Badilla was significantly more responsive to CIM (97 % of explants formed callus) than Black Cheribon (73 % of leaf disks with callus) (Figure 9), this variety could not be used in subsequent experiments due to limited plant material. Hence, Black Cheribon and NG77-69 (80 % of disks with callus) were selected to be used in subsequent investigations.

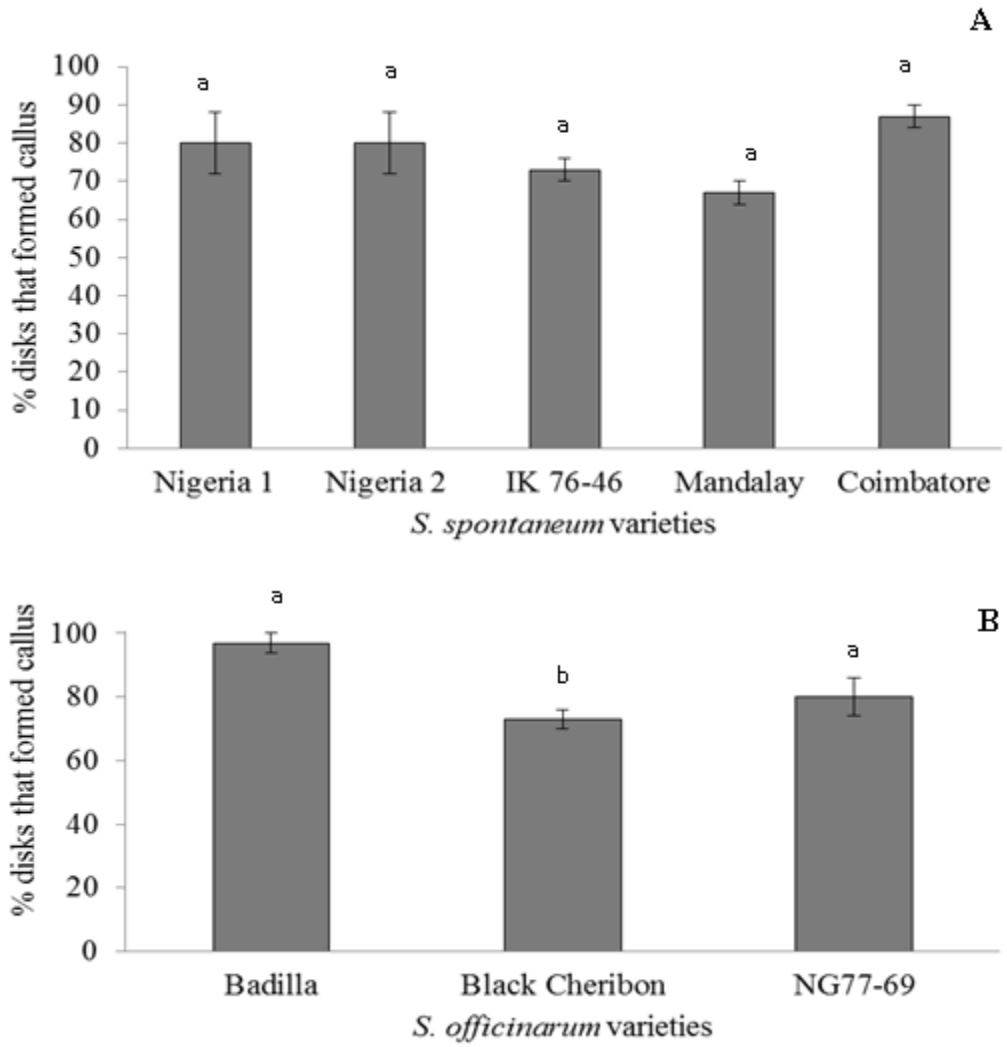

Figure 9. *In vitro* response of (A) S*accharum spontaneum* Nigeria 1, Nigeria 2, IK76-46, Mandalay and Coimbatore (B) and *S. officinarum* Badilla, Black Cheribon and NG77-69 sugarcane varieties to callus induction medium (CIM) containing 5 $mg.l^{-1}$ 2, 4-D after six weeks. n = 30, mean ± SE. Dissimilar alphabet characters denote statistical significant differences between varieties.

4.1.3 Determining callus induction media for selected varieties of ancestral sugarcane

Following the results shown in Figure 9, a total of 11 different media compositions were tested for each of the selected ancestral varieties in an attempt to improve their *in vitro* response to stage 1 of the protocol. Leaf disks were plated onto CIM, the % explants forming callus was recorded and a qualitative assessment of the amount of embryogenic callus and the callus type

were made. Following germination, the number of plantlets/10 disks was assessed. These results were compared with those obtained for NCo376 under the optimized protocol (CIM containing 3 mg.l^{-1} 2, 4-D) for this variety (Section 4.1.1).

Different concentrations of 2, 4-D were tested as this is the auxin routinely employed for sugarcane somatic embryogenesis (Chen *et al*., 1988; McCallum *et al*., 1998; Snyman *et al*., 2000; Geetha and Padmanabhan, 2001; Snyman *et al*., 2001; Khalil, 2002; Snyman *et al*., 2004; Lakshmanan, 2006a; Snyman *et al*., 2006; Patade *et al*., 2008; Behera and Sahoo, 2009). The concentrations and combinations used were 3 mg.l^{-1} 2, 4-D (CIM-A1), 5 mg.l^{-1} 2, 4-D (CIM-A2), 3 mg.l^{-1} 2, 4-D + 0.5 mg.l^{-1} BA (CIM-A3) and 5 mg.l^{-1} 2, 4-D for 1 week, which was followed by culture onto media with 3 mg.l^{-1} 2, 4-D (CIM-A4). The auxin picloram was included in this study as it was gave favourable results for sugarcane in a study by Chengalrayan *et al*. (2005). The concentrations used in the present study were 3 mg.l^{-1} (CIM-B1), 5 mg.l^{-1} (CIM-B2) and 2 mg.l^{-1} picloram in combination with 3 mg.l^{-1} 2, 4-D (CIM-B3). The amino acid proline into the CIM was also tested as it was successfully used in sugarcane by Fitch and Moore (1993) and Singh *et al*. (2008). The medium containing proline at 3 mg.l^{-1} was coded CIM-C1, that with double the nitrogen available of MS (2N of MS) was CIM-C2 and 3 mg.l^{-1} proline with double nitrogen of MS as C3. As simple sugars supply cell wall precursors for cytokinesis, CIM supplemented with ribose, xylose, arabinose, glucose, mannose, galactose and fructose (CIM-D) was tested to determine its effect on callus production and generation of embryogenic cells.

***S. spontaneum* Nigeria 1**

Swelling of *S. spontaneum* Nigeria 1 leaf disks was observed 3-4 days after initiation onto CIM and callus formation occurred within two weeks for all treatments. Nigeria 1 demonstrated the greatest % explants forming callus (61 %) when cultured on CIM-A2 (5 mg.l^{-1} 2, 4-D), although there was no significant difference between this and the % explants forming callus on CIM-A1 (3 mg.l^{-1} 2, 4-D; 53 %), CIM-C1 (CIM with proline; 57 %) and CIM-D (CIM with simple sugars; 47 %) (Table 9). Culture onto CIM-A2 also produced the greatest plantlet yield (259 plantlets/10 disks) of all tested CIM, but this was still less than half of that obtained with NCo376 on Std. CIM (450 plantlets/10 disks). This significant difference can be explained by the observation that more than 50 % of the callus obtained for Nigeria 1 was type 1 non-embryogenic and non-regenerable for all tested media.

The presence of the cytokinin BA (CIM-A3) and the auxin IBA (CIM-A4) in the CIM appeared to have a negative effect on both the % explants forming callus and the plantlet yield (Table 9). There was no significant difference in % explants producing callus and the plantlet yield amongst the different picloram experiments (CIM-B1, B2 and B3). These treatments produced mostly type 2 non-embryogenic callus and therefore, had low plant yields. Explants initiated onto CIM-C1 demonstrated significantly higher % explants forming callus than those on CIM-C3 (57 vs. 28 %) although there was no difference in the number of plantlets obtained/10 disks (Table 9). The % survival after acclimatization ranged from 60 – 100, with CIM-A2 giving the highest % survival (100 %). In conclusion, none of the tested callus induction media resulted in yields similar or close to that of NCo376.

***S. spontaneum* Nigeria 2**

The time taken for *S. spontaneum* Nigeria 2 leaf disks to swell (3-4 days) and produce callus (two weeks) was the same as that of Nigeria 1. The media compositions that gave the highest % explants forming callus were CIM-A1 (3 $mg.l^{-1}$ 2, 4-D; 21 %), CIM-A2 (5 $mg.l^{-1}$ 2, 4-D; 39 %), CIM-C2 (2N of MS; 21 %) and CIM-D (simple sugars; 35 %) (Table 10). However, the CIM that gave the greatest plantlet yields for Nigeria 2 were CIM-A2 and A3 (160 and 153 plantlets respectively). Both these media compositions produced approximately 50 % type 3 embryogenic callus.

As seen in Table 10, the use of the auxins IBA (CIM-A4) significantly reduced the % explants forming callus and explants established onto CIM containing picloram (CIM-B1, B2 and B3) experienced 100 % necrosis (Figure 10). There was no significant difference amongst explants cultured on CIM-C1, C2 and C3 with respect to % explants forming callus and plantlet yield; however, CIM-C2 and C3 formed more type 3 callus than CIM-C1. Post-acclimatization survival was high for all treatments that produced plantlets (80 to 100 %). Plantlets that were regenerated from calli formed on CIM-A1, A2, C2 and C3, all demonstrated 100 % survival after acclimatization.

Figure 10. *S. spontaneum* Nigeria 2 explants established onto CIM containing picloram, either alone or in combination with 2, 4-D, experienced 100 % leaf disk necrosis.

***S. spontaneum* Coimbatore**

Swelling of *S. spontaneum* Coimbatore 2 leaf disks was observed 3-4 days after initiation and callus was produced two weeks after establishment on CIM. Explants cultured on CIM-A2 (5 $mg.l^{-1}$ 2, 4-D) gave significantly higher % explants forming callus (57 %) than all other treatments (Table 11). The media compositions that produced the most amount of type 3 callus were CIM-A1, A2 and A3. Although grey mucilaginous callus (type 2) was also observed in cultures on CIM-A3, the higher proportion of embryogenic callus on this media, compared to that of others resulted in the significantly highest plantlet yield (100 plantlets/10 disks) achieved for this variety. However, this was significantly lower than the 450 plantlets/10 disks obtained with NCo376 on Std. CIM.

As observed with Nigeria 1 and Nigeria 2, explants established on CIM-A4 (CIM with IBA) produced lower % explants forming callus and plantlet yields than explants cultured on CIM with 2, 4-D alone, whilst those of CIM-B1,B2 and B3 did not form any callus. When explants were cultured onto CIM-A4, only 10 % formed callus and a plantlet yield of 37 plantlets/10 disks was obtained, whilst all CIM containing picloram resulted in 100 % leaf disk necrosis. The % survival after acclimatization observed for this variety was lower than that of the other *S. spontaneum* varieties, and ranged from 60 – 90%.

Table 9. Response of the ancestral sugarcane variety *S. spontaneum* Nigeria 1 to different callus induction media (CIM). Discs were cultured onto modified CIM for 6 - 7 weeks before being subcultured onto embryo germination medium for 2 – 6 weeks. All CIM contained MS, 0.5 $g.l^{-1}$ casein hydrolysate and 8 $g.l^{-1}$ agar agar. CIM–A, B and C also had 20 $g.l^{-1}$ sucrose. Once shoots had reached 2 cm in height they were transferred to plantlet establishment medium for 4 – 8 weeks. All treatments were analysed for statistical differences between treatments using ANOVA and Tukey post-hoc test ($P < 0.05$, n = 150, mean ± SE) and dissimilar alphabet characters denote significant difference between treatments.

CIM	Media composition	% explants forming callus	Predominant callus type (> 50 % of callus)	Amount of type 3 (embryogenic) callus	No. of plantlets / original 10 disks	% survival after acclimatization
A1	3 $mg.l^{-1}$ 2, 4-D	53 ± 8^{a}	1	+++	211 ± 7^{a}	90
A2	5 $mg.l^{-1}$ 2, 4-D	61 ± 10^{a}	1	+++	259 ± 11^{b}	100
A3	5 $mg.l^{-1}$ 2, 4-D + 0.5 $mg.l^{-1}$ BA	23 ± 5^{bc}	1	++	145 ± 7^{c}	60
A4	5 mg.l-1 IBA then 3 $mg.l^{-1}$ 2, 4-D*	15 ± 5^{b}	1	++	102 ± 7^{d}	80
B1	3 $mg.l^{-1}$ picloram	18 ± 3^{bc}	2	+	15 ± 2^{e}	80
B2	5 $mg.l^{-1}$ picloram	23 ± 4^{bc}	2	+	13 ± 2^{e}	70
B3	3 $mg.l^{-1}$ 2, 4-D + 2 $mg.l^{-1}$ picloram	16 ± 3^{b}	2	+	11 ± 2^{e}	90
C1	3 $mg.l^{-1}$ 2, 4-D + 0.3 $g.l^{-1}$ proline	57 ± 2^{a}	2	++	57 ± 4^{f}	90
C2	3 $mg.l^{-1}$ 2, 4-D + 2N of MS	42 ± 2^{ac}	2	++	43 ± 4^{f}	90
C3	3 $mg.l^{-1}$ 2, 4-D + 0.3 $g.l^{-1}$ proline + 2N of MS	28 ± 2^{bcd}	1	++	44 ± 6^{f}	100
D	13 $g.l^{-1}$ sucrose + 7x 1$g.l^{-1}$ simple sugars**	47 ± 4^{a}	1	++	13 ± 1^{e}	70

* Explants were cultured onto CIM containing 5 $mg.l^{-1}$ IBA for one week and then transferred onto CIM with 3 $mg.l^{-1}$ 2, 4-D for three weeks.

** Explants were cultured onto CIM containing 13 $g.l^{-1}$ sucrose + 1 $g.l^{-1}$ each of ribose, xylose, arabinose, glucose, mannose, galactose and fructose.

Table 10. Response of the ancestral sugarcane variety *S. spontaneum* Nigeria 2 to different callus induction media. Culture conditions and media compositions are as described in Table 9. All treatments were analysed for statistical differences between treatments using ANOVA and Tukey post-hoc test ($P < 0.05$, n = 150, mean ± SE) and dissimilar alphabet characters denote significant difference between treatments.

CIM	% explants forming callus	Predominant callus type (> 50 % of callus)	Amount of type 3 (embryogenic) callus	No. of plantlets /original 10 disks	% survival after acclimatization
A1	21 ± 7^{ac}	1	++	79 ± 7^{a}	100
A2	39 ± 9^{b}	1	+++	160 ± 13^{b}	100
A3	7 ± 4^{c}	1	+++	153 ± 15^{b}	80
A4	9 ± 3^{c}	1	++	8 ± 1^{c}	88
B1	0 ± 0^{c}	-	-	0^{c}	-
B2	0 ± 0^{c}	-	-	0^{c}	-
B3	0 ± 0^{c}	-	-	0^{c}	-
C1	19 ± 4^{ac}	2	+	16 ± 2^{c}	80
C2	21 ± 2^{ab}	2	++	12 ± 1^{c}	100
C3	14 ± 3^{ac}	2	++	12 ± 1^{c}	100
D	35 ± 3^{b}	1	+	16 ± 2^{c}	90

Table 11. Response of the ancestral sugarcane variety *S. spontaneum* Coimbatore to different callus induction media. Culture conditions and media compositions are as described in Table 9. All treatments were analysed for statistical differences between treatments using ANOVA and Tukey post-hoc test ($P < 0.05$, n = 150, mean ± SE)and dissimilar alphabet characters denote significant difference between treatments.

CIM	% explants forming callus	Predominant callus type (> 50 % of callus)	Amount of type 3 (embryogenic) callus	No. of plantlets /original 10 disks	% survival after acclimatization
A1	39 ± 5 [a]	1	++	61 ± 4 [a]	60
A2	57 ± 8 [b]	1	++	67 ± 2 [a]	80
A3	7 ± 5 [c]	2	+++	100 ± 0 [b]	60
A4	10 ±2 [c]	2	+	37 ± 7 [c]	90
B1	0 ± [c]	-	-	0 ± 0 [d]	-
B2	0 ± 0 [c]	-	-	0 ±0 [d]	-
B3	0 ± 0 [c]	-	-	0 ± 0 [d]	-
C1	19 ± 1 [ad]	2	+	18 ± 2 [d]	90
C2	0 ± 0 [c]	-	-	0 ± 0 [d]	-
C3	7 ± 2 [cd]	2	+	9 ± 1 [d]	89
D	23 ± 4 [ad]	1	+	46 ± 4 [c]	80

***S. officinarum* NG77-69**

The time taken for *S. officinarum* NG77-69 leaf disks to swell was 3-4 days after establishment onto CIM and callus formation was observed two weeks after initiation. For this variety, the media composition that gave the significantly highest % explants forming callus was CIM-A2 (5 mg.l^{-1} 2, 4-D; 50 %). This medium together with CIM-A1 (3 mg.l^{-1} 2, 4-D) also resulted in the highest plantlet yields obtained (94 and 85 plantlets respectively) (Table 12). These yields were still approximately five times less than that of NCo376 (450 plantlets). The predominant callus type observed was type 1 (non-embryogenic callus), but CIM-A1 and A2 also produced type 3 (embryogenic) callus.

As previously observed for *S. spontaneum* Nigeria 2 and Coimbatore, no callus was produced on CIM that contained picloram (CIM-B1, B2 and B3) and resulted in 100 % leaf disk necrosis. The same result was obtained when explants where cultured on CIM that had 2N of MS (CIM-C2). There was no significant difference in % explants producing callus amongst CIM-A1 (14 %), CIM-A4 (3 %), CIM-C1 (13 %), CIM-C3 (9 %) and CIM-D (16 %). All plantlets produced from the different treatments, except that of CIM-C1 (90 % survival after acclimatization), were successfully acclimatized.

***S. officinarum* Black Cheribon**

As there were limited *S. officinarum* Black Cheribon stalks available, the results presented are based on lower sample numbers than those for the ancestral varieties discussed previously. Swelling of Black Cheribon disks was observed after 3-4 days and callus formation was noted after three weeks in the dark. The medium that resulted in the highest % explants forming callus (75 %) was CIM-A2 (5 mg.l^{-1} 2, 4-D). Explants cultured onto this medium also produced the highest plantlet yield (90 plantlets/10 disks), although this was not significantly different to the yields obtained from CIM-A1 (75 plantlets) CIM-C1 (75 plantlets) and CIM-C2 (75 plantlets) (Table 13).

The presence of BA (CIM-A3) and IBA (CIM-A4) in the callus induction media resulted in no callus production and therefore no plantlets. The same result was obtained when explants where established onto CIM-B3 (CIM with 2, 4-D + proline). There was no significant difference between CIM-A1, CIM-A2, CIM-C1 and CIM-C2 with respect to % explants forming callus and plantlet yield. Explants cultured onto CIM with simple sugars produced 14 % explants forming callus and 13 plantlets/10 disks. The % survival after acclimatization ranged from 75 – 100 % across all tested media compositions for this variety, with plants from CIM-A2 and B2 having 100 % successful acclimatization.

Table 12. Response of the ancestral sugarcane variety *S. officinarum* NG77-69 to different callus induction media. Culture conditions and media compositions are as described in Table 9. All treatments were analysed for statistical differences between treatments using ANOVA and Tukey post-hoc test ($P < 0.05$, n = 150, mean ± SE) and dissimilar alphabet characters denote significant difference between treatments.

CIM	% explants forming callus	Predominant callus type (> 50 % of callus)	Amount of type 3 (embryogenic) callus	No. of plantlets /original 10 disks	% survival after acclimatization
A1	14 ± 5^{a}	1	++	94 ± 2^{a}	100
A2	50 ± 5^{b}	1	++	85 ± 5^{a}	100
A3	1 ± 1^{c}	1	-	0 ± 0^{b}	-
A4	3 ± 1^{ac}	1	+	13 ± 1^{b}	100
B1	0 ± 0^{c}	-	-	0 ± 0^{b}	-
B2	0 ± 0^{c}	-	-	0 ± 0^{b}	-
B3	0 ± 0^{c}	-	-	0 ± 0^{b}	-
C1	13 ± 2^{a}	2	+	28 ± 3^{c}	90
C2	0 ± 0^{c}	-	-	0 ± 0^{b}	-
C3	9 ± 2^{ac}	2	++	18 ± 2^{c}	100
D	16 ± 3^{a}	1	+	22 ± 2^{c}	100

Table 13. Response of the ancestral sugarcane variety *S. officinarum* Black Cheribon to different callus induction media. Culture conditions and media compositions are as described in Table 9. All treatments were analysed for statistical differences between treatments using ANOVA and Tukey post-hoc test ($P < 0.05$, n = 30 - 90, mean ± SE) and dissimilar alphabet characters denote significant difference between treatments.

CIM	% explants forming callus	Predominant callus type (> 50 % of callus)	Amount of type 3 (embryogenic) callus	No. of plantlets /original 10 disks	% survival after acclimatization
A1	33 ± 19^{a}	1	++	75 ± 18^{ad}	80
A2	75 ± 14^{a}	1	++	90 ± 22^{a}	100
A3	0 ± 0^{bc}	-	-	0 ± 0^{bc}	-
A4	0 ± 0^{b}	-	-	0 ± 0^{c}	-
B1	27 ± 7^{bc}	1	+	13 ± 2^{bc}	80
B2	50 ± 4^{bc}	1	+	18 ± 2^{b}	100
B3	0 ± 0^{b}	-	-	0 ± 0^{c}	-
C1	50 ± 8^{a}	1	++	75 ± 7^{a}	80
C2	52 ± 4^{ac}	1	++	75 ± 5^{a}	90
C3	30 ± 4^{bcd}	1	+	48 ± 4^{d}	90
D	14 ± 4^{ad}	1	+	13 ± 1^{bc}	75

4.1.4 Determining the effect of leaf disk position on callus formation

During the preparation and establishment of sugarcane cultures, each leaf roll was sliced into 30 leaf disks. These disks were plated on CIM with a total of 10 leaf disks/Petri-dish. Sugarcane grows from the nodes upwards (Moore, 1987) and the ten leaf disks closest to the node would be more meristematic than those further away. Hence, it was postulated that the ten leaf disks closest to the node (Zone 1) would give a higher % callus formation than those furthest away (Zone 3) (the ten disks in the middle were referred to as Zone 2). Statistical analyses were undertaken to determine if there was a significant difference in the % explants forming callus amongst the different leaf disk zones (positions). The results from these analyses showed that the position of the leaf disk within the leaf roll, had no significant effect on the % explants forming callus (Table 14).

4.1.5 Comparison of the final yields obtained for the selected ancestral varieties

The yields of the CIM that resulted in the highest number of plantlets/10 disks for each of the ancestral varieties were compared with NCo376 and each other (Table15). The CIM that contained the auxin 2, 4-D, either alone (CIM-A1 and A2), or in combination with BAP (CIM-A3), gave the highest plant yields for the tested ancestral varieties (60 – 259 plantlets/10 disks). However, these were significantly lower than the yield of NCo376 (450 plantlets/10 disks, Table 15). Also, the number of plantlets/10 disks on the above mentioned media differed amongst the varieties and ranged from 60 – 259 plantlets/10 disks (Table 15). The highest plant yield obtained with 2, 4-D (CIM-A2, 5 $mg.l^{-1}$ 2, 4-D) was 259 plantlets/10 disks with *S. spontaneum* Nigeria 1, but this was only about half of that obtained with NCo376 on Std. CIM (450 plantlets/10 disks). In terms of plant yields, *S. spontaneum* varieties (100 – 259 plantlets/10 disks) performed better than *S. officinarum* varieties (90 – 94 plantlets/10 disks).

The commercial cultivar NCo376 and the ancestral varieties Nigeria 1, Nigeria 2, NG77-69 and Black Cheribon, all showed 100 % survival post-acclimatization. Hence, the final plant yields attained for these varieties were equal to the number of plantlets obtained/10 disks (NCo376 – 450 plants, Nigeria 1 – 259 plants, Nigeria 2 – 160 plants, NG77-69 – 94 plants and Black Cheribon – 90 plants) (Table 15). However, the Coimbatore variety demonstrated 60 % survival after acclimatization, this translates into a final yield of 60 plants (Table 15) which is lower than those obtained with all other tested germplasm.

Although the % explants forming callus for Nigeria 2, Coimbatore, NG77-69 and Black Cheribon varied between 7 to 75 %, the number of plantlets obtained/10 disks were 90 – 160 plants/10 disks (Table 15). This indicated that plant regeneration (or the conversion of embryos to plants) was good and that it was the callus induction stage (stage 1 of indirect somatic embryogenesis, i.e. the formation of type 3 embryogenic callus) that was limiting the attainable plant yields.

Table 14. Comparison of the % explants forming callus amongst the different leaf disk zones (positions) within the sugarcane stalk, for the selected ancestral varieties. Culture conditions as described in Table 9. All zones were analysed for statistical differences between them for all treatments using ANOVA and Tukey post-hoc tests ($P < 0.05$, n = 30 - 150, mean ± SE) and dissimilar alphabet characters denote significant difference amongst disk zones.

		Variety				
Treatment	Zone	Nigeria 1	Nigeria 2	Coimbatore	NG77-69	Black Cheribon
A1	1	40 ± 18 [a]	20 ± 11 [a]	24 ± 6 [a]	13 ± 10 [a]	35 ± 35 [a]
	2	60 ± 12 [a]	14 ± 7 [a]	34 ± 10 [a]	16 ± 8 [a]	0 ± 0 [a]
	3	60 ± 9 [a]	22 ± 17 [a]	28 ± 12 [a]	10 ± 6 [a]	60 ± 0 [a]
A2	1	65 ± 19 [a]	28 ± 17 [a]	66 ± 9 [a]	57 ± 2 [a]	75 ± 25 [a]
	2	74 ± 15 [a]	54 ± 15 [a]	62 ± 14 [a]	46 ± 12 [a]	100 ± 0 [a]
	3	60 ± 9 [a]	36 ± 17 [a]	42 ± 18 [a]	46 ± 12 [a]	50 ± 0 [a]
A3	1	22 ± 8 [a]	5 ± 5 [a]	10 ± 10 [a]	0 ± 0 [a]	0 ± 0 [a]
	2	16 ± 8 [a]	8 ± 8 [a]	10 ± 10 [a]	2 ± 2 [a]	0 ± 0 [a]
	3	28 ± 12 [a]	6 ± 6 [a]	0 ± 0 [a]	0 ± 0 [a]	0 ± 0 [a]
A4	1	8 ± 5 [a]	16 ± 6 [a]	10 ± 4 [a]	7 ± 3 [a]	0 ± 0 [a]
	2	10 ± 4 [a]	2 ± 2 [a]	14 ± 4 [a]	0 ± 0 [a]	0 ± 0 [a]
	3	27 ± 12 [a]	2 ± 2 [a]	6 ± 4 [a]	0 ± 0 [a]	0 ± 0 [a]
B1	1	60 ± 4 [a]	24 ± 8 [a]	26 ± 7 [a]	18 ± 4 [a]	60 ± 20 [a]
	2	58 ± 2 [a]	20 ± 6 [a]	18 ± 6 [a]	12 ± 4 [a]	40 ± 20 [a]
	3	52 ± 4 [a]	12 ± 4 [a]	14 ± 4 [a]	8 ± 4 [a]	50 ± 0 [a]
B2	1	46 ± 4 [a]	18 ± 4 [a]	0 ± 0 [a]	0 ± 0 [a]	55 ± 5 [a]
	2	42 ± 4 [a]	24 ± 4 [a]	0 ± 0 [a]	0 ± 0 [a]	60 ± 0 [a]
	3	38 ± 4 [a]	20 ± 3 [a]	0 ± 0 [a]	0 ± 0 [a]	40 ± 0 [a]
B3	1	34 ± 2 [a]	18 ± 4 [a]	10 ± 3 [a]	14 ± 4 [a]	30 ± 10 [a]
	2	24 ± 2 [a]	14 ± 4 [a]	4 ± 2 [a]	8 ± 4 [a]	35 ± 5 [a]
	3	26 ± 4 [a]	10 ± 3 [a]	6 ± 4 [a]	6 ± 2 [a]	25 ± 5 [a]
C1	1	26 ± 6 [a]	0 ± 0 [a]	0 ± 0 [a]	0 ± 0 [a]	27 ± 15 [a]
	2	18 ± 6 [a]	0 ± 0 [a]	0 ± 0 [a]	0 ± 0 [a]	27 ± 15 [a]
	3	12 ± 2 [a]	0 ± 0 [a]	0 ± 0 [a]	0 ± 0 [a]	20 ± 12 [a]
C2	1	28 ± 6 [a]	0 ± 0 [a]	0 ± 0 [a]	0 ± 0 [a]	53 ± 7 [a]
	2	20 ± 7 [a]	0 ± 0 [a]	0 ± 0 [a]	0 ± 0 [a]	53 ± 3 [a]
	3	20 ± 5 [a]	0 ± 0 [a]	0 ± 0 [a]	0 ± 0 [a]	43 ± 12 [a]
C3	1	24 ± 2 [a]	0 ± 0 [a]	0 ± 0 [a]	0 ± 0 [a]	0 ± 0 [a]
	2	14 ± 5 [a]	0 ± 0 [a]	0 ± 0 [a]	0 ± 0 [a]	0 ± 0 [a]
	3	10 ± 5 [a]	0 ± 0 [a]	0 ± 0 [a]	0 ± 0 [a]	0 ± 0 [a]
D	1	50 ± 6 [a]	42 ± 6 [a]	32 ± 4 [a]	16 ± 7 [a]	22 ± 7 [a]
	2	34 ± 2 [a]	32 ± 4 [a]	10 ± 6 [a]	16 ± 7 [a]	10 ± 6 [a]
	3	58 ± 9 [a]	32 ± 6 [a]	28 ± 8 [a]	16 ± 4 [a]	10 ± 4 [a]

Table 15. Comparison of the highest final yields obtained for each tested variety and the media composition used. Culture conditions are as described in Table 9. All treatments were analysed for statistical differences between varieties using ANOVA and Tukey post-hoc test ($P < 0.05$, n = 30 - 150, mean ± SE) and dissimilar alphabet characters denote significant difference amongst varieties.

Cultivar/variety	CIM	% explants forming callus	No. of plantlets /10 disks	% survival after acclimatization	Final yields (No. of plantlets/10 disks)
NCo376	A1	93 ± 2^{a}	450 ± 6^{a}	100	450
S. spontaneum Nigeria 1	A2	61 ± 10^{b}	259 ± 11^{b}	100	259
S. spontaneum Nigeria 2	A2	39 ± 9^{bd}	160 ± 13^{c}	100	160
S. spontaneum Coimbatore	A3	7 ± 5^{c}	100 ± 0^{cd}	60	60
S. officinarum NG77-69	A1	14 ± 5^{d}	94 ± 2^{d}	100	94
S. officinarum Black Cheribon	A2	75 ± 14^{ab}	90 ± 22^{d}	100	90

4.2 Transformation of the commercial cultivar NCo376 and the ancestral varieties *S. spontaneum* Nigeria 1 and *S. officinarum* NG77-69

For this study, the ancestral varieties *S. spontaneum* Nigeria 1 and *S. officinarum* NG77-69 and the commercial cultivar NCo376 were used. The objective was to determine the proportion of plants expressing the *gus* transgene in the selected ancestral sugarcane varieties compared with NCo376, to test the hypothesis that genotypes with a simpler genome may be less prone to transgene silencing.

4.2.1 Transient expression of microprojectile bombarded callus

Type 3 (embryogenic) callus (Taylor *et al*., 1992) for each of the cultivar/varieties NCo376, *S. spontaneum* Nigeria 1 and *S. officinarum* NG77-69 were selected from cultures and plated onto osmoticum medium (Vain *et al*., 1993) four hours prior to and after bombardment. Following bombardment with the *gus* and *npt II* genes, calli were transferred onto standard callus induction medium (Std. CIM) for two days to 'recover'. A sample of bombarded calli from each variety and construct were assessed for transient transgene expression using a histochemical GUS assay and all assayed calli were found to have regions positive for transient GUS expression (Figure 11). However, transient expression was limited to 3 – 5 blue foci per calli, regardless of variety and plasmid construct tested.

4.2.2 Selection of transformed cells

Subsequent to recovery on Std. CIM, calli were transferred onto selection media (CIM + paromomycin) and maintained in the dark for 6-8 weeks, with fortnightly subculture. During this period, cells that were not successfully transformed died (due to lack of resistance to paromomycin). Calli comprising such cells were seen as dark brown to black with a 'shiny' appearance (Figure 12) and were approximately 20 % of the total number of bombardments. The putatively transformed cells (those that survived) divided and formed calli that were observed as yellow to white and nodular in appearance. These were transferred onto embryo germination media (EGM) containing paromomycin (to continue the selection process) and maintained in a photoperiod growth room to facilitate embryo germination. Shoot growth was first observed 3 - 4 weeks after establishment on EGM, root development was slower than that of the shoots, with root formation occurring 4 – 5 weeks after transfer onto EGM. After 8 - 12 weeks the regenerated plantlets were acclimatized in a glasshouse and used in subsequent

studies. It must be noted that some calli which survived the selection process did not regenerate into plantlets when transferred onto EGM. Although these calli did not turn black or demonstrate any necrotic symptoms, they were deemed dead after a TTC viability test was conducted on them.

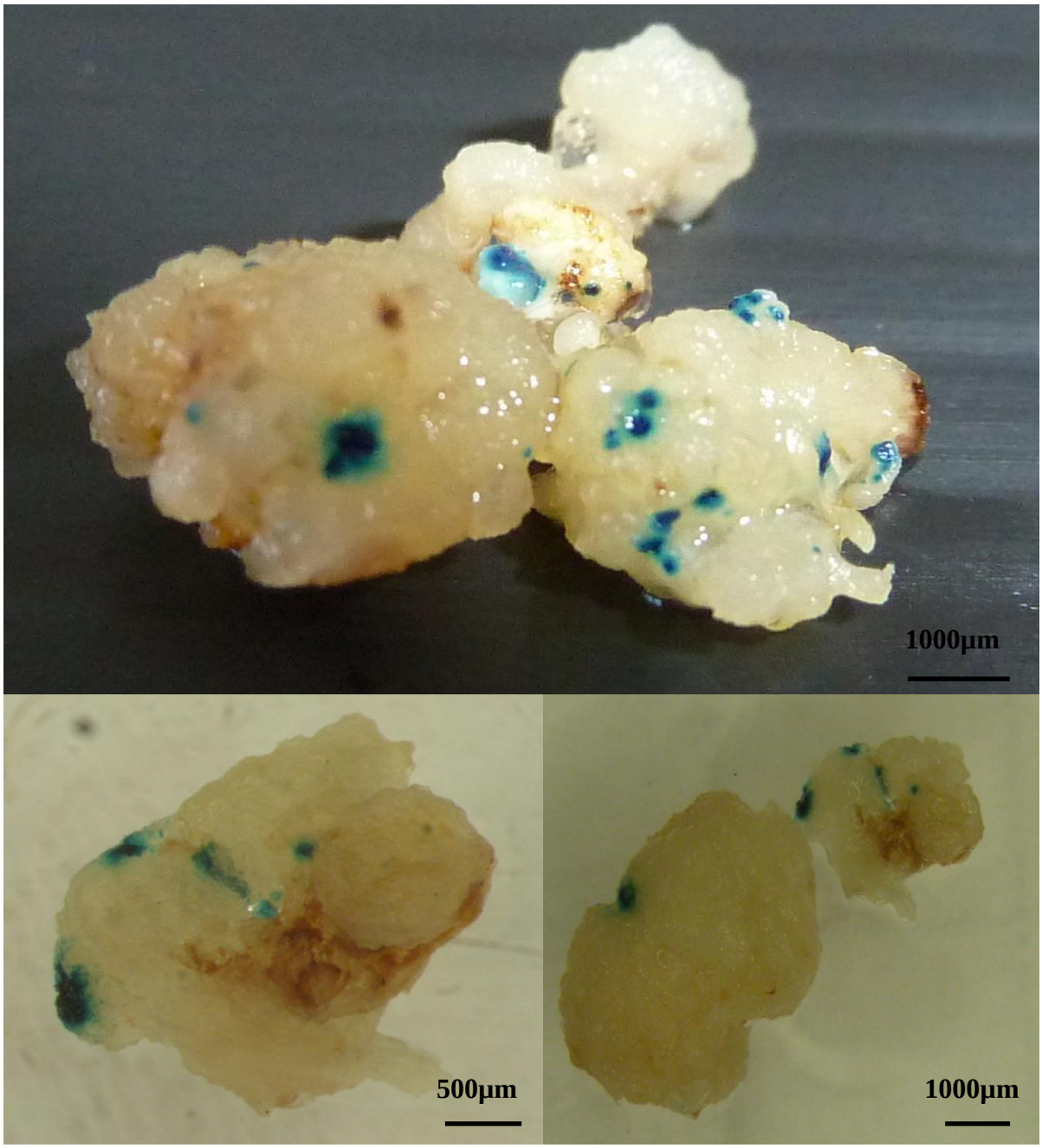

Figure 11. A sample of microprojectile bombarded sugarcane calli that were positive for transient transgene (*gus*) expression. Blue foci indicated regions in which the *gus* gene was transiently expressed.

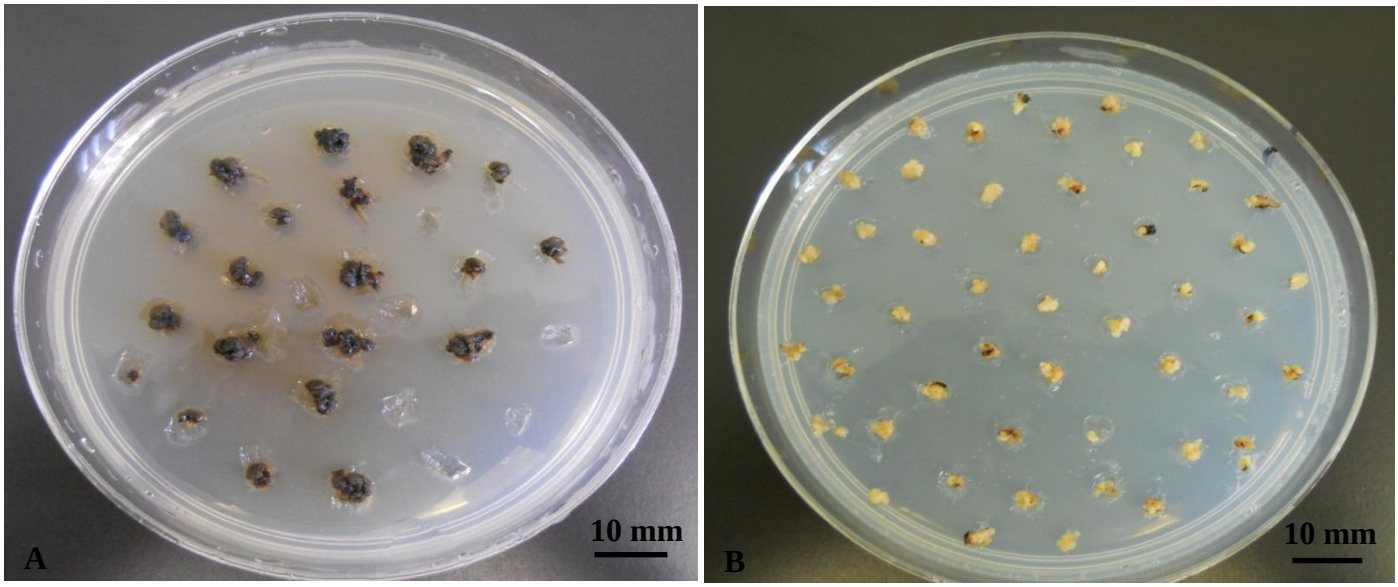

Figure 12. Selection of transformed sugarcane calli. (A) Calli that did not take up the *npt II* gene died when plated onto selection media and were observed as brown to black calli. (B) Growing calli containing cells that were successfully transformed.

4.2.3 Assessment of stable gene integration using end-point PCR

Bombarded calli produced embryos that germinated into plants (putatively transformed) that were acclimatized successfully (Figure 13). A PCR reaction was carried out in order to determine if the transgenes (*gus* and *npt II*) were successfully integrated into the plant genome. DNA was extracted from leaf material of *S. spontaneum* Nigeria 1 plants, transformed with either pAHC27 + pEmuKN or $pR_{11}F^{-}$ + pEmuKN, and used in a PCR reaction. The products of the PCR were then run on an agarose gel (2 % $m.v^{-1}$) with positive (plasmid DNA), negative (DNA from the leaves of an untransformed plant) and no template (water) controls. Analysis of the gel image revealed banding patterns in all lanes including those that contained the water (lanes 2 and 18) and negative (lanes 3 and 4) controls (Figure 14). Attempts to eliminate this background contamination included cleaning and decontaminating micropipettes and work area, using a new water stock, primer stocks and reagents. However, these efforts were unsuccessful as background contamination persisted. Nevertheless, a band in the positive control (plasmid DNA) lane is clearly visible (Figure 14, lane 19), indicating that the PCR reaction for the primer sets used was successful and the plasmids used contained the genes of interest.

Figure 13. Putatively transformed sugarcane plants produced in this study. Calli that survived selection on media containing paromomycin were regenerated into plantlets that were acclimatized and maintained in a greenhouse.

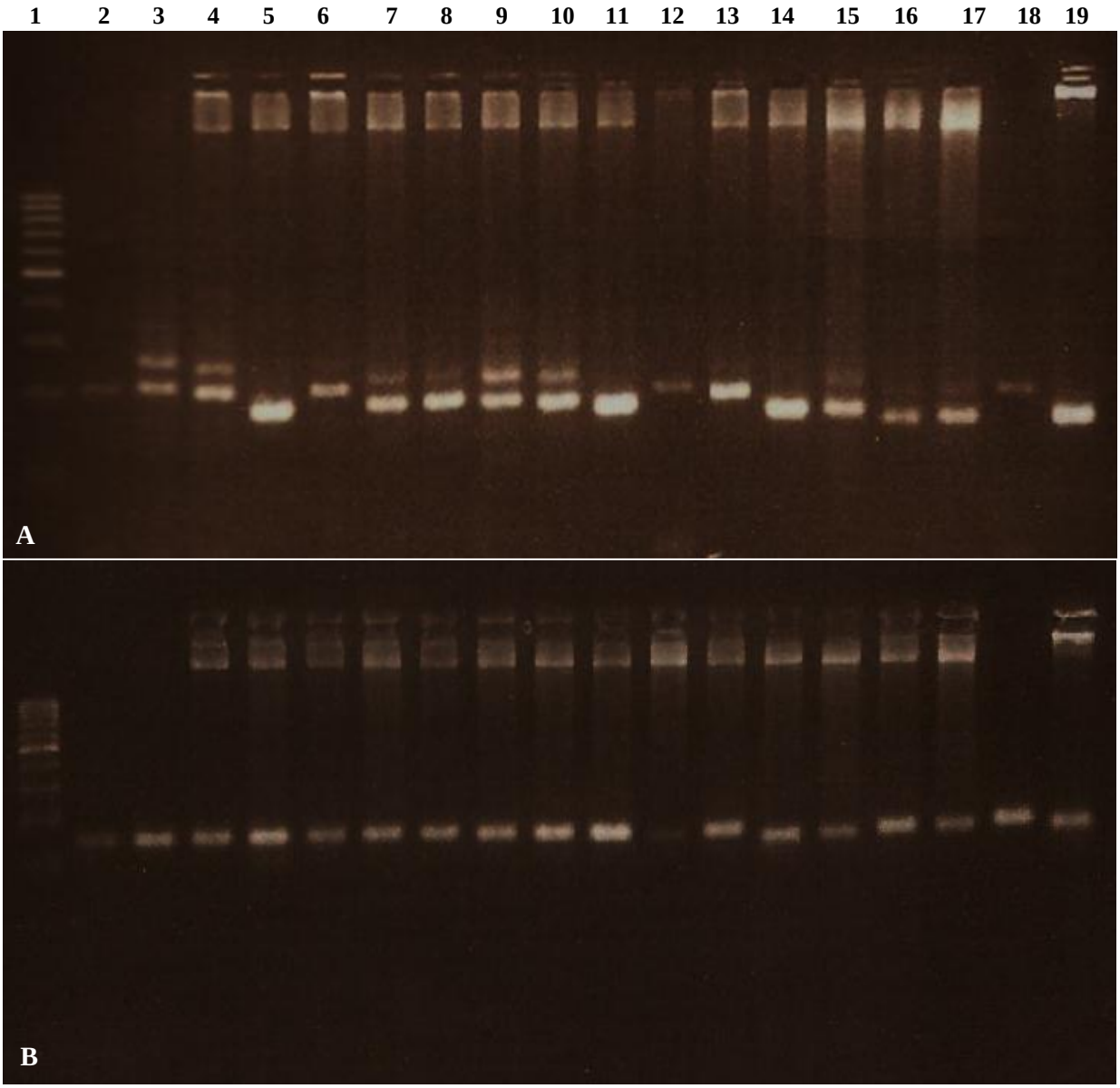

Figure 14. Agarose gel electrophoresis of end-point PCR products in order to test for the presence of the transgenes A) *gus* and B) *npt II* using genomic DNA extracted from shoots of putatively transformed *S. spontaneum* Nigeria 1 plants. Lane 1 - molecular marker (100 bp), lane 2 – no template (water) control 1, lane 3 – negative (untransformed) control 1, lane 4 – negative (untransformed plant) control 2, lane 5-17 – transformed plantlets 1-13, lane 18 – no template control 2, lane 19 – positive plasmid control.

4.2.4 Quantitative real-time PCR (qPCR) assessment of transgene integration

As a result of the background contamination that was observed in the products of the end-point PCR reactions of putatively transformed plant DNA, qPCR was used to determine if the transgenes *gus* and *npt II* were successfully integrated into the plant genome. Tubulin was used as the house-keeping/reference gene for all qPCR analysis and a standard curve was set up for the *gus* (Appendix 11), *npt II* (Appendix 12) and tubulin (Appendix 13) genes, to determine optimum qPCR conditions and to validate a range of DNA concentrations. A five-fold dilution and a primer concentration of 0.65 µl for each gene were found to give the best qPCR efficiencies.

The DNA from leaves of putatively transformed plants was analysed, as was that of an untransformed plant and a non-template control (NTC). Fold expression values were generated by the software used in the qPCR analyses. Although these provide information on the expression of a gene of interest, in the current study they were used to indicate if the *gus* and *npt II* transgenes were successfully integrated into the genome of the samples. qPCR analyses revealed that some samples had very low fold expression values (< 0.1) (Table 16). Therefore, samples with a difference of five quantification cycle values (Cq) or more than the untransformed control were considered to have successful transgene integration. Transformation efficiency was determined by dividing the number of successfully transformed plants (presence of both *gus* and *npt II*) by the number of microprojectile bombardments for each variety and construct. The products of selected qPCR reactions for *gus*, *npt II* and tubulin were run on an agarose gel and confirmed that the products obtained were the correct size (Figure 15).

All plantlets of the commercial sugarcane cultivar NCo376 that were bombarded with either pAHC27 + pEmuKN or $pR_{11}F^-$ + pEmuKN demonstrated successful transgene integration of *gus* and *npt II*. The ancestral variety *S. spontaneum* Nigeria 1 produced more transgenic plants, when bombarded with both plasmid constructs (a total of 13 plants), compared with NCo376 (9 plants) and *S. officinarum* NG77-69 (8 plants) and gave the highest transformation efficiencies (Table 17). All (100 %) *S. spontaneum* Nigeria 1 plants that were bombarded with pAHC27 + pEmuKN contained the *gus* and *npt II* transgenes, but only 6 out of 10 plants that were bombarded with $pR_{11}F^-$ + pEmuKN showed *gus* and *npt II* integration (Table 17). Of the *S. officinarum* NG77-69 variety, only 4 out of 5 plants transformed with pAHC27 + pEmuKN, and 2 out of 3 plants transformed with $pR_{11}F^-$ + pEmuKN, were positive for the presence of *gus* and *npt II*. Although NCo376 did not produce the highest number of transgenic plants, 100 % of the plants had successful integration of the *gus* and *npt II* genes.

Table 16. Fold expression values for the *gus* and *npt II* transgenes for each transformed variety. 'Expression' of transgenes was normalized with the house-keeping gene tubulin. NCo376 samples 1 – 5 were bombarded with $pR_{11}F^-$; NCo376 samples 6 – 9 with pAHC27; *S. spontaneum* Nigeria 1 samples 1 – 10 with $pR_{11}F^-$; Nigeria 1 samples 11 – 13 with pAHC27; *S. officinarum* NG77-69 samples 1 – 3 with $pR_{11}F^-$ and NG77-69 samples 4 – 8 with pAHC27.

Sample	Cultivar/variety					
	NCo376		Nigeria 1		NG77-69	
	gus	*npt II*	*gus*	*npt II*	*gus*	*npt II*
1	786	1552	2202	196	13358	2530
2	706	1950	1427	153	6746	1387
3	99	231	11	3	2	0.3
4	42	103	7	1	10478	1948
5	109	208	4	1	2	0.2
6	84	191	3	3	7408	1671
7	76	197	7	< 0.1	5336	831
8	1148	2849	1	< 0.1	5220	1250
9	1000	2000	< 0.1	< 0.1	-	-
10	-	-	7	< 0.1	-	-
11	-	-	15	< 0.1	-	-
12	-	-	2	< 0.1	-	-
13	-	-	3	< 0.1	-	-

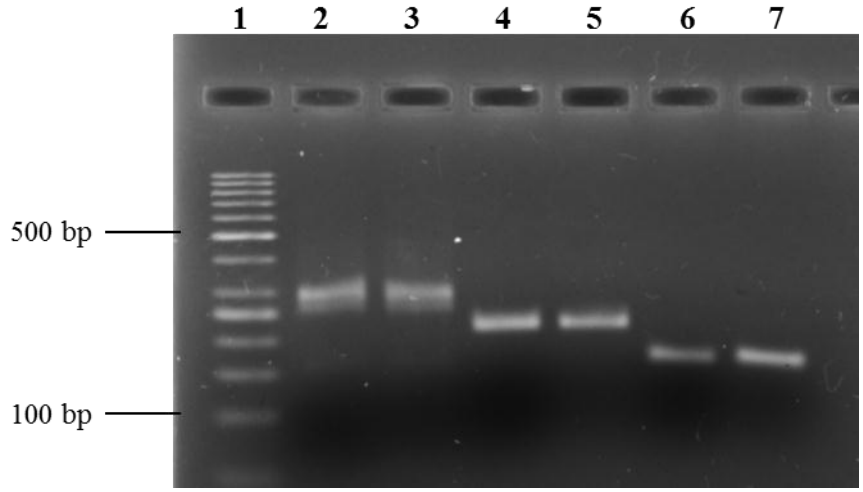

Figure 15. Agarose gel electrophoresis of tubulin, *gus* and *npt II* qPCR products. Lane 1 – molecular marker (50 bp), lanes 2 and 3 – tubulin, lanes 4 and 5 –*gus*, lanes 6 and 7 – *npt II*. The banding patterns obtained confirmed that the products obtained were the correct size.

4.2.5 Assessment of stable transgene expression

As the results from the qPCR analysis indicated that the majority of transformed plants contained the *gus* transgene, a histochemical GUS assay was performed on all transformed plants to determine gene expression. The sugarcane variety 88H0019 stably expresses the GUS transgene and was used as a positive control for GUS expression in the current study. A constitutive and a root specific promoter were used during the transformation of the *S. spontaneum* Nigeria 1 and *S. officinarum* NG77-69, as such both shoot and root material from these varieties as well as 88H0019 were assayed.

Subsequent to incubation in GUS buffer, all root and leaf material harvested from 88H0019 plants turned blue, indicating stable GUS expression (Figure 16). All transformed plants of the commercial cultivar NCo376 and the ancestral varieties *S. spontaneum* Nigeria 1 and *S. officinarum* NG77-69 were negative for stable expression of GUS, regardless of the constructs used in transformation (Table 17), indicating possible transgene silencing.

Table 17. Transformation efficiency of NCo376, *S. spontaneum* Nigeria 1 and *S. officinarum* NG77-69 plantlets transformed with the constructs pAHC27 + pEmuKN and $pR_{11}F^-$ + pEmuKN. For each construct combination and each variety, 15 bombardments were carried out. A histochemical GUS assay was conducted on a sample of calli from each variety and construct combination, and all calli tested were positive for transient expression.

Cultivar/variety	Construct	No. of putative transgenic plantlets (plantlets that survived selection)	No. of plantlets with stable gene integration (as determined by qPCR)		Transformation efficiency (no. of transgenic plants/blast)	No. of plantlets with stable expression of GUS (histochemical assay)
			npt II	*gus*		
NCo376	pAHC27	4	4	4	0.27	0
	$pR_{11}F^-$	5	5	5	0.33	0
S. spontaneum Nigeria 1	pAHC27	3	3	3	0.20	0
	$pR_{11}F^-$	10	6	6	0.40	0
S. officinarum NG77-69	pAHC27	5	4	4	0.27	0
	$pR_{11}F^-$	3	2	2	0.13	0

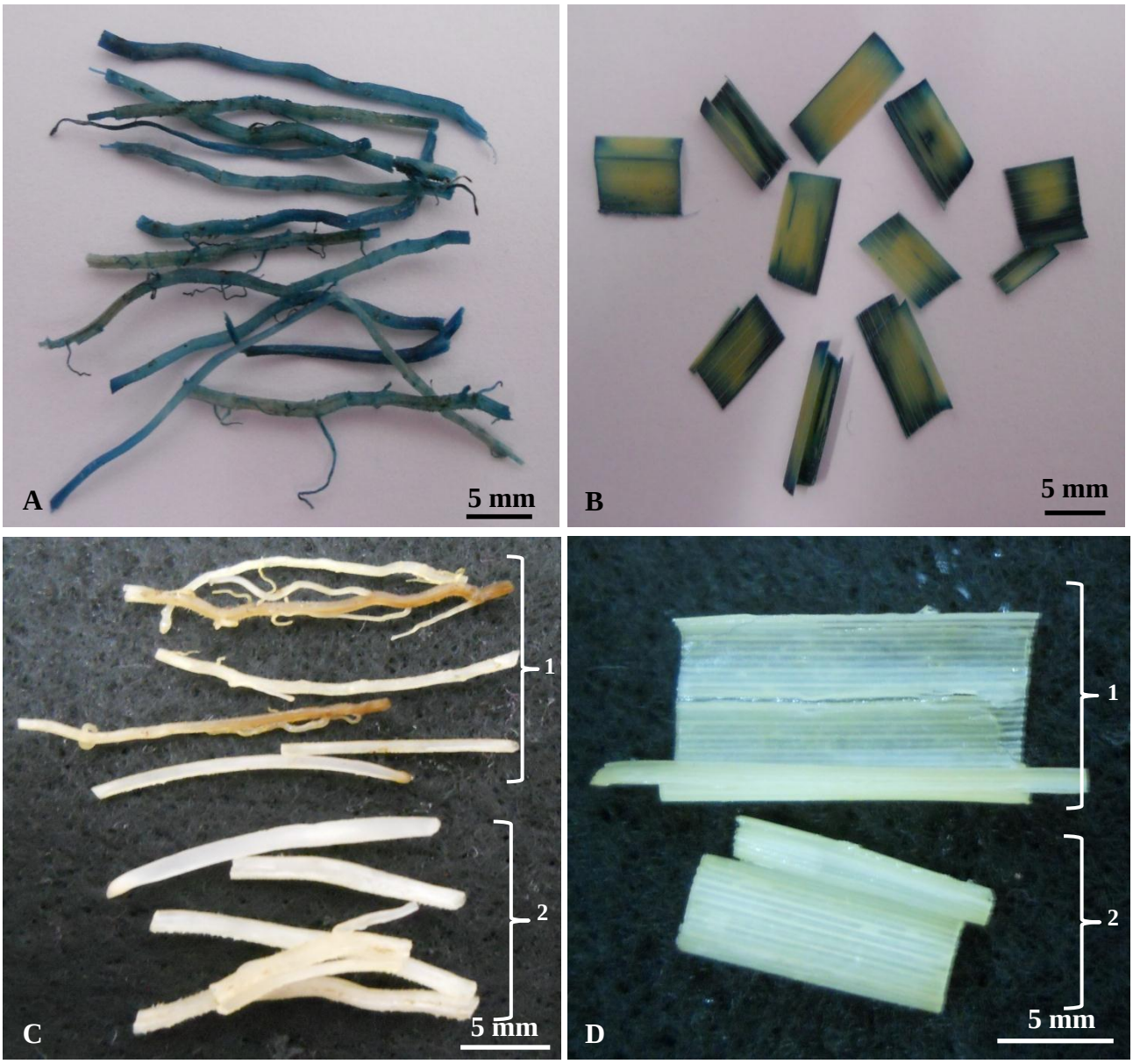

Figure 16. Assessment of stable transgene expression in selected sugarcane lines. A histochemical GUS assay was performed on the roots and leaves of sugarcane varieties/cultivar 88H0019 (positive control), *S. spontaneum* Nigeria 1, *S. officinarum* NG77-69 and NCo376. Plant material from the control cultivar turned blue when incubated in GUS buffer, whilst that from the other sugarcane lines did not. Blue regions indicate stable GUS expression. A – 88H0019 roots, B – 88H0019 leaves, C – Nigeria 1 (1) and Nigeria 2 (2) roots and D – Nigeria 1(1) and Nigeria 2 (2) leaves.

5. Discussion

5.1 Determining a callus induction medium composition for selected varieties of ancestral sugarcane that is high yielding for somatic embryos and plants

It is generally accepted that plant yields decline after transformation (Snyman *et al*., 1996). Consequently, there is a need to establish a micropropagation protocol that gives the highest yields of plants possible so that, following transformation, an adequate transgenic plant yield is obtained for subsequent analysis. Transformation efficiency represents the number of plantlets obtained per transformation event. As the highest transformation efficiencies obtained thus far for commercial sugarcane cultivars are low and range from 2 to 2.6 transgenic plants per transformation event (Bower *et al*., 1996; Gallo-Meagher and Irvine, 1996; Kim *et al.*, 2012; Taparia *et al*., 2012), a somatic embryogenesis protocol that produces a high plant yield would increase the amount of transgenic plants obtained. Consequently, this study sought to establish a somatic embryogenesis protocol for the ancestral varieties *S. spontaneum* Nigeria 1, *S. spontaneum* Nigeria 2, *S. spontaneum* Coimbatore, *S. officinarum* NG77-69 and *S. officinarum* Black Cheribon, for such subsequent manipulations. According to the literature, for most commercial sugarcane cultivars the media used for plantlet regeneration (or stage 2) of indirect somatic embryogenesis are very similar as they usually lack PGRs (Bower and Birch, 1992; Snyman *et al*., 1996; Arencibia *et al*., 1998; Chen *et al*., 1988; McCallum *et al*., 1998; Snyman *et al*., 2001; Patade *et al*., 2008; Khan and Khan, 2010; van der Vyver, 2010; Basnayake *et al*., 2011; Malabadi *et al*., 2011) (Table 4). However, the composition of the callus induction media (CIM), especially the type and concentration of PGRs used, varies for different cultivars and varieties (Bower and Birch, 1992; Snyman *et al*., 1996; Arencibia *et al*., 1998; Chen *et al*., 1988; McCallum *et al*., 1998; Snyman *et al*., 2001; Patade *et al*., 2008; Khan and Khan, 2010; van der Vyver, 2010; Basnayake *et al*., 2011; Malabadi *et al*., 2011) (Table 4). This indicates that for commercial sugarcane cultivars there is a genotype dependent response to the CIM used in indirect somatic embryogenesis.

The composition of culture media plays a crucial role in the *in vitro* morphogenesis of a plant (George, 1993; Bhojwani and Razdan, 1996; Hartmann *et al*., 1997). In indirect somatic embryogenesis, this has an effect on the production of embryogenic calli and their subsequent regeneration into plantlets (George, 1993; Bhojwani and Razdan, 1996; Hartmann *et al*., 1997). Further, as discussed above, it seems that media composition needs to be established for each genotype and to-date there has been much work done on formulating and optimizing indirect somatic embryogenesis protocols for commercial sugarcane varieties (Lee, 1987; Snyman *et al*.,

1996; McCallum *et al*., 1998; Snyman *et al*., 2000; Snyman *et al*., 2001; Khalil, 2002; Mamum *et al*., 2004; Behera and Sahoo, 2009; Basnayake *et al*., 201l; Malabadi *et al*., 2011). However, there are limited reports of *in vitro* culture protocols for the ancestral sugarcane varieties *S. spontaneum* and *S. officinarum*. Notable exceptions are the studies by van der Vyver (2010) who worked with two varieties of *S. officinarum* (Black Cheribon and Badilla) and by Xu *et al*. (2008) who used the Badilla variety. As information on yields of ancestral varieties is lacking and yields obtained by SASRI for this cultivar are considered adequate for subsequent transformation work, this cultivar was used as a measure of desired yields for the tested ancestral varieties.

The preliminary investigation of the response of the selected ancestral varieties to CIM containing 5 $mg.l^{-1}$ 2, 4-D, revealed that the % explants forming callus ranged from 67 to 97 % (Figure 9) but these results are based on a small sample number (n = 30). In order to improve the % explants forming callus, so that a plantlet yield similar to that obtained with NCo376 could be achieved, the response of selected ancestral varieties to different callus induction media, with respect to % explants forming callus, the amount of embryogenic callus produced and subsequent plantlet yields, was investigated.

Of all the tested CIM compositions, CIM-A1 and CIM-A2 (3 and 5 $mg.l^{-1}$ 2, 4 –D, respectively) gave the highest plantlet yields for *S. spontaneum* Nigeria 1 (259 plantlets), *S. spontaneum* Nigeria 2 (160 plantlets), *S. officinarum* NG77-69 (94 plantlets) and *S. officinarum* Black Cheribon (90 plantlets) (Table 15). Of the tested media compositions, these most closely resembled Std. CIM (i.e. CIM that contained MS with vitamins, casein hydrolysate and the auxin 2, 4-D without any other PGRs, nutrients and amino acids), which is used for the culture of the commercial cultivar NCo376. Previous work done on commercial sugarcane cultivars found that a 2, 4-D concentration range of 2 to 5 $mg.l^{-1}$ resulted in high level of callus formation (20 – 100 % of explants with callus formation) (Taylor *et al*., 1992; Mamum *et al*., 2004; Behera and Sahoo, 2009) and plantlet yields of 124 – 207 plants/10 disks (Khalil, 2002; Behera and Sahoo, 2009). The results in the current study support this as a concentration range of 3 to 5 $mg.l^{-1}$ 2, 4-D produced the greatest % explants forming callus (14 – 75 %) and number of plantlets (90 – 259 plantlets/10 disks) for most of the tested varieties (Table 15). A similar observation was made by van der Vyver (2010) who found that 3 $mg.l^{-1}$ 2, 4-D gave the greatest amounts of embryogenic callus formation for the ancestral variety *S. officinarum* Black Cheribon. *S. spontaneum* Coimbatore gave the highest final yield when cultured onto CIM-A3 (5 $mg.l^{-1}$ 2, 4 –D + 0.5 $mg.l^{-1}$ BA; 60 plantlets). There is a genotype-dependent response to the different media compositions and combinations of PGRs, and the optimum concentration of

2, 4-D is dependent on the genotypes. The results obtained in the current study, and those of van der Vyver (2010), show that this is not only true for commercial cultivars, but also for ancestral varieties.

Although 2, 4-D, either alone (CIM-A1 and A2) or with BA (CIM-A3) gave the highest plant yields for all ancestral varieties (60 – 259 plantlets/10 disks), they were significantly lower than that of NCo376 (450 plantlets/10 disks) (Table 15). As the plant yield is related to the amount of embryogenic callus produced, callus induction media containing different PGRs, nutrients and amino acids were then tested in an attempt to improve this. In contrast to 2, 4-D, the other tested auxins (*viz*. picloram and IBA) produced little or no embryogenic callus (Table 9, 10, 11, 12 and 13). However, it should be noted that although culture on IBA (CIM-A4) produced less embryogenic callus and lower plantlet yields than 2, 4-D for each variety, the yield obtained for Nigeria 1 on IBA (102 plantlets/10 disks) was still greater than the highest final yields obtained with the Coimbatore, NG77-69 and Black Cheribon varieties (60, 94 and 90 plantlets/10 disks, respectively; Table 15). The observation that IBA and picloram produced less embryogenic callus than 2, 4-D supports that made by Behera and Sahoo (2009), who found that the presence of IBA in the callus induction media did not produce callus and plantlets of a significant amount in the tested commercial sugarcane cultivar (*S. officinarum* Nayana). However, this finding is in opposition to those of Chengalrayan *et al*. (2005), working with a commercial sugarcane cultivar. Those authors found that increased picloram concentrations increased the amount of embryogenic callus obtained from sugarcane seed explants (the hybrid variety CP 84-1198). For the Nigeria 2, Coimbatore and NG77-69 varieties no callus was formed (Tables 10, 11 and 12) and 100 % of explants displayed necrosis (Figure 10). At high concentrations picloram is known to have phytotoxic effects, (George *et al*., 2008) and it is possible that for the above mentioned varieties the concentration of picloram used was unsuitable. The differences in results between the above mentioned authors and the current study is due to a genotype-dependent response of the varieties to picloram.

Proline is an amino acid and can be used by plants as a reduced nitrogen source or for protein synthesis (Bhojwani and Razdan, 1996; George *et al*., 2008). Hence, the effect of the addition of proline or the increase of supplied nitrogen in the CIM, on the % explants forming callus, amount of embryogenic callus and plantlet yields was investigated. Singh *et al*. (2008) found that CIM containing proline produced similar amounts of callus to CIM with 8 $mg.l^{-1}$ 2, 4-D, but in the current study proline had no significant effect on the % explants forming callus (7 – 57 %) or the amount of type 3 (embryogenic) callus produced, and consequently, it had no significant effect on number of plantlets obtained for all tested ancestral sugarcane varieties (0 –

75 plantlets/10 disks, Table 9, 10, 11, 12 and 13). For Nigeria 1, Nigeria 2 and Black Cheribon there was no significant difference between % explants forming callus on Std. CIM or CIM containing proline or 2N of MS. These results indicate that sufficient nitrogen was provided to the leaf disks on Std. CIM through the incorporation of MS salts and vitamins and casein hydrolysate in the culture media. However, for the Coimbatore and NG77-69 varieties culture onto CIM with 2N of MS did not produce any callus. For these varieties, the additional nitrogen supplied by doubling the amount of nitrogen available in MS was inhibitory to callus formation and consequently plant regeneration.

Another attempt to improve the amount of type 3 callus produced involved the use of simple sugars into the callus induction media. The predominant callus types observed from experiments with 2, 4-D, picloram, proline and nitrogen were type 1 and type 2, both of which are non-embryogenic (Taylor *et al*., 1992). Microscopic evaluation of non-embryogenic cells showed that they were multi-nucleate, indicating that only karyokinesis occurred. As simple sugars are the precursors of cell wall formation and play an important role in cytokinesis, they have been used successfully in somatic embryogenesis protocols for the grass *Digitaria eriantha* (Watt *et al*., 1988). Based on the work done by those authors, the effect of simple sugars in the CIM on the embryogenic callus and plantlets yield of ancestral sugarcane varieties was investigated. In the current study, the inclusion of simple sugars to the CIM did not make a significant difference to the amount of embryogenic callus produced or the number of plantlets obtained (Nigeria 1 – 13 plantlets, Nigeria 2 – 16 plantlets, Coimbatore – 46 plantlets, NG77-69 – 22 plantlets and Black Cheribon – 13 plantlets) (Table 9, 10, 11, 12, and 13). This may be due to an insufficient/low simple sugar concentration (1 $mg.l^{-1}$ for each of the seven simple sugars used) used to encourage cytokinesis and the formation of embryogenic callus, which directly affected the plant yield obtained. The finding that the inclusion of simple sugars to the CIM did not make a significant difference to the amount of embryogenic callus formed supports that of Ramgareeb *et al*. (2001), were there was no significant difference between the effect of sucrose and simple sugars in the callus induction medium on the % of explants of the grass *Cynodon dactylon* forming callus.

When immature leaf rolls are cut into disks and established in culture, they are cut from the most meristematic regions. As 30 disks are cut from a single stalk, there is a difference amongst disks in terms of their position in the meristematic region. This should have had an effect on the % explants producing callus, with the most meristematic disks possibly producing higher % of explants forming callus. However, a statistical comparison amongst the first, second and third set of 10 disks excised from the stalk revealed that the position of the leaf disks made no

significant difference to the % explants forming callus and the subsequent plant yields (Table 14). The observed results may be due to the fact that the most meristematic region of the sugarcane stalk was used to establish cultures. Although each leaf disk differed in terms of proximity to the node (area of growth), they were all cut from the most meristematic region of the stalk and were therefore, 'equally meristematic'.

Across all tested ancestral lines, the *S. spontaneum* varieties (100 – 259 plants/10 disks) generally produced higher plant yields than the *S. officinarum* varieties (90 – 94 plants/10 disks) (Table 15). The reason for this is unknown, but *S. spontaneum* plants are known to be more robust and adaptable than *S. officinarum* (Daniels and Roach, 1987).

The acclimatization success for the highest yielding CIM for all tested varieties, except *S. spontaneum* Coimbatore, was 100 % (Table 15). However, for the Coimbatore variety, only 60 % of plants survived acclimatization, which affects the final yields obtained for this variety. As previously mentioned, the low plant yields obtained are related to the low % explants forming callus. The exceptions to this were *S. spontaneum* Coimbatore and *S. officinarum* NG77-69 (Table 15). Compared with the other ancestral varieties, both of these had significantly lower % explants forming callus but produced similar plant yields, but the reason for this is unknown. From the highest yielding CIM, a wide range in the % explants forming callus was observed. However, subsequent to regeneration, a plantlet yield for the Nigeria 2, Coimbatore, NG77-69 and Black Cheribon varieties ranged from 90 – 160 plantlets/10 disks (Table 15). This shows that the conversion of embryos to plantlets is good and confirms that it is indeed the callus induction stage that is the critical stage in the somatic embryogenesis of sugarcane. Consequently, it is this stage that needs to be refined for each genotype.

5.2 Transformation of the commercial cultivar NCo376 and the ancestral varieties *S. spontaneum* Nigeria 1 and *S. officinarum* NG77-69.

As commercial sugarcane cultivars experience a high incidence of promoter methylation and transgene silencing (Hansom *et al*., 1999; Ingelbrecht *et al*., 1999; Brumbley *et al*., 2008; Potier *et al*., 2008b; Mudge *et al*., 2009; Graham *et al*., 2011) the ancestral sugarcane varieties *S. spontaneum* Nigeria 1 and *S. officinarum* NG77-69 were transformed so that transgene expression, particularly promoter functionality, in ancestral varieties could be assessed. Sugarcane transformation is routinely done using microprojectile bombardment (Birch and Franks, 1991; Bower and Birch, 1992; Snyman *et al*., 2001; 2006; van der Vyver 2010; Taparia *et al*., 2012) and this method of genetic transformation was followed in the current study.

Transient GUS expression in transformed calli was assessed using a histochemical assay, where blue foci indicated cells that took up the transgenes. A high number of cells that demonstrate transient expression is desirable as it indicates that the transgene was successfully delivered. In the current study, blue foci were observed across all sugarcane lines bombarded but the level of transient expression was limited to a few (3-5) blue loci per piece of callus (Figure 11). This is very low, particularly when compared with the transient expression levels (2200 red foci/cm^2) obtained by McCallum *et al*. (1998) using the anthocyanin reporter gene system on transformed commercial sugarcane cultivars. Also, in contrast to the *gus* reporter system, the anthocyanin reporter system has the advantage of being non-destructive to cells (Snyman *et al*., 1996), and assayed calli can be maintained and regenerated into transgenic plantlets.

Transgene integration of *gus* and *npt II* into the genome was initially assessed using traditional end-point PCR analyses, but due to persistent background contamination these analyses had to be abandoned. In comparison with end-point PCR, real-time quantitative PCR (qPCR) amplification products are measured by the amount of fluorescence after each round of amplification. Consequently, this technique was employed to assess transgene integration in the putatively transformed plants. qPCR analyses revealed that not all plantlets that developed from bombarded calli contained the *gus* and *npt II* transgenes (Table 17). As selection of transformed cells was based on resistance to the aminoglycoside antibiotic paromomycin, plantlets regenerated on selection media were expected to contain the *npt II* transgene. These results indicated that escapes are possible when using paromomycin as the selective agent for the tested varieties. Hauptmann *et al*. (1988) reported that grass species, such as sugarcane, have a natural resistance to aminoglycoside antibiotics and their use as selective agents may result in escapes. The low number of regenerated plants obtained in the current study could also be improved by optimizing the selective agent used for ancestral varieties. Other selection strategies used in sugarcane transformation studies that could be tested, include the use of geneticin (Bower and Birch, 1992; McCallum *et al*., 1998 van der Vyver, 2010; Basnayake *et al*., 2012; Taparia *et al*., 2012), kanamycin (Khalil, 2002), hygromycin (Bower and Birch, 1992) and a combination of geneticin and paromomycin (Kim *et al*., 2012).

McCallum *et al*. (1998), using the commercial sugarcane variety NCo376 and the geneticin selection strategy, were able to obtain 12 discreet transgenic plantlets (the amount of transformed callus or number of blasts is not reported). This was higher than the number of transgenic NCo376 plants produced in the present study (0.27 plants/blast transformed with pAHC27 and 0.33 plants/blast transformed with $pR_{11}F^-$). Other studies on commercial sugarcane varieties yielded 2.2 transgenic plants/blast (Kim *et al*., 2012; Taparia *et al*., 2012), the

transformation efficiency achieved by those authors was almost eight times greater than observed with NCo376 in the present study. The only other study conducted on transformation of ancestral sugarcane varieties was by van der Vyver (2010) were the *S. officinarum* variety Badilla yielded a transformation efficiency of 0.6 transgenic plants per blast. This was also higher than the efficiencies obtained with *S. spontaneum* Nigeria 1 (0.20 transgenic plants/blast for the construct pAHC27 and 0.40 plants/blast for $pR_{11}F^-$) and *S. officinarum* NG77-69 (0.27 transgenic plants/blast for the construct pAHC27 and 0.13 plants/blast for $pR_{11}F^-$) (Table 17).

The type and size of the microprojectile used can affect the transformation efficiency. Taparia *et al*. (2012) found that the highest amount of discreet transgenic sugarcane plants was obtained when four week old calli were bombarded with 0.3 µm gold microprojectiles and selected on geneticin (Taparia *et al*., 2012). Although tungsten is cheaper than gold microprojectiles, the latter is uniform in shape and does not result in high tissue damage (Hansen and Wright, 1991; Deo *et al*., 2010). Similarly, smaller sized microprojectiles cause less tissue damage than larger microprojectiles. Also, as most transformation protocols take into consideration the mass of the microprojectiles rather than the size, smaller sized particles will be able to bind more DNA and carry it into plant tissue, resulting in a higher transformation efficiency (Taparia *et al*., 2012). In the current study, tungsten microprojectiles that were 0.7 µm in size were used in all transformation experiments and this may have affected the transformation efficiencies obtained. Hence, the effect of gold or smaller sized tungsten microprojectiles on the transformation efficiencies obtained for ancestral varieties should be investigated.

The transgenic plant yields obtained for the ancestral varieties used in the current study (0.13 – 0.4 transgenic plants/blast) and by van der Vyver (2010) (0.6 transgenic plants/blast) are lower than those reported for commercial cultivars (2 – 2.6 transgenic plants/blast) (Bower *et al*., 1996; Gallo-Meagher and Irvine, 1996; Kim *et al*., 2012; Taparia *et al*., 2012). This is linked to the plantlet yields obtained with the *in vitro* protocol used to generate material for transformation. In contrast to protocols for ancestral lines, somatic embryogenesis protocols have been optimized for the commercial cultivars. The low yield of transgenic plants obtained further highlight the need to develop high yielding somatic embryogenesis protocols for ancestral varieties.

A histochemical GUS assay was conducted to determine if transgenic plantlets expressed the *gus* transgene. All transgenic NCo376 plantlets were negative for stable GUS expression, indicating that gene silencing occurred. These results support those of Sun *et al*. (1993), who found that although bombarded calli were positive for GUS expression, the regenerated plantlets were not. In contrast, Witcher *et al*. (1998) working with maize and the GUS transgene driven

by the ubiquitin promoter, reported high levels of stable GUS expression in the transformed plants. Other transformation studies in sugarcane have either assessed expression in calli (Khalil, 2002; Basnayake *et al*., 2011), or have used ELISA to measure transgene expression (Bower and Birch, 1992; Kim *et al*., 2012).

As previously mentioned, ancestral sugarcane varieties have a simpler genome than commercial cultivars. Hence, it was hypothesized that the former would experience less transgene silencing. However, all transgenic *S. spontaneum* Nigeria 1 and *S. officinarum* NG77-69 plantlets were negative for GUS expression (Table 17). Although fewer than commercial cultivars, ancestral sugarcane varieties still have many copies of the genome (Daniels and Roach, 1987; D'Hont *et al*., 1996; Irvine, 1999; Butterfield *et al*., 2001; Grivet and Arruda, 2001) which may have resulted in the *gus* transgene being silenced. Other factors that may have caused the observed silencing are the position in which *gus* was integrated in the plant genome and its copy number (Meng *et al*., 2003; Graham *et al*., 2011). However, as no sequencing or southern hybridization analyses were conducted in the current study, the effect of these factors on transgene silencing was not determined.

5.3 Conclusion

To-date the protocol that results in the highest plant yields for the Nigeria 1, Nigeria 2, NG77-69 and Black Cheribon ancestral varieties is as follows: 1) leaf disks are established onto CIM containing MS salts and vitamins, 0.5 g.l-1 casein hydrolysate, 20 $g.l^{-1}$ sucrose and 2, 4-D, either at 3 $mg.l^{-1}$ (NG77-69) or 5 $mg.l^{-1}$ (for Nigeria 1, Nigeria 2 and Black Cheribon). For the Coimbatore variety, establishment of disks onto CIM containing 3 $mg.l^{-1}$ 2, 4-D + 0.5 $mg.l^{-1}$ BA resulted in the highest plant yields. 2) The callus obtained from culture on CIM is then regenerated on EGM (CIM without 2, 4-D); 3) and the resultant plantlets are transferred to PEM (½ strength MS, 0.5 $g.l^{-1}$ casein hydrolysate and 5 $g.l^{-1}$ sucrose); 4) this is then followed by acclimatization. Although in the current study this is the protocol that gave the highest number of plantlets (60 – 259 plantlets/10 disks), if one compares with what is obtained with NCo376 (450 plantlets/10 disks), it is not high yielding. These yields are particularly low for the Coimbatore, NG77-69 and Black Cheribon varieties (60 – 94 plantlets/10 disks). The plant yields attained are related to the amount of embryogenic callus formed as the predominant callus type was non-embryogenic.

Furthermore, the position of the leaf disks within the leaf roll did not have a significant effect on the % explants forming callus, nor did the various tested combinations of nutrients, PGRs, carbon sources and amino acids in the CIM. Of note, CIM that contained IBA and picloram negatively affected leaf disk survival and resulted in leaf disk necrosis.

The response of the tested varieties to CIM is genotype dependent and the best plant yields were obtained with Nigeria 1 and Nigeria 2 (259 and 160 plantlets, respectively). However, the regeneration of callus into plantlets was found to be relatively good for all tested varieties. This confirms that it is the callus induction stage, particularly the formation of embryogenic callus that directly affects the plant yield obtained. Consequently, there is a need to investigate this further and possible modifications include thidiazuron and Dicamba as both PGRs have been reported to be effective in inducing embryogenic callus formation in sugarcane (Brisisbe *et al*., 1994; Malabadi *et al*., 2011). Also the use of disaccharides in the CIM should be investigated as bioltose and cellibiose were found to improve the amount of embryogenic cells in barley cultures (Cai *et al*., 1992).

The hypothesis that ancestral varieties would show a lower incidence of transgene silencing than commercial cultivars as they have a simpler genome, was not supported by the results obtained in the current study. One of the main reasons for this is that transformation efficiencies were low when compared with other commercial cultivars (2 to 2.6 transgenic plants per transformation event) (Bower *et al*., 1996; Gallo-Meagher and Irvine, 1996; Kim *et al*., 2012; Taparia *et al*., 2012). For NCo376, 0.27 and 0.33 transgenic plants/blast for the constructs pAHC27 and $pR_{11}F^{-}$ respectively, was obtained. For Nigeria 1, a transformation efficiency of 0.20 and 0.40 transgenic plants/blast was achieved whilst that of NG77-69 was 0.27 and 0.13 transgenic plants were observed/blast, for the constructs pAHC27 and $pR_{11}F^{-}$, respectively. Secondly, this was a preliminary study with a limited number of bombardments (15 bombardments per construct per cultivar). Thirdly, the embryogenic capacity of cells from ancestral clones was not fully exploited. Further optimization of the *in vitro* protocols used to generate embryogenic callus could be undertaken for future studies.

Whilst some transgene silencing was expected, histochemical GUS assays of both roots and leaves of plants that contained the GUS transgene as determined by real-time PCR revealed no expression in any of the transgenic plants, which was unexpected. According to the literature, the use of the ubiquitin promoter in sugarcane transformation studies results in transgene expression in almost all transformants (Potier *et al*., 2008a; Mudge *et al*., 2013). Possible explanations for the lack of expression could be: a) promoter integrity had been compromised either at bombardment or during plasmid preparation; b) post-transcriptional gene silencing.

Further molecular characterization of putative transgenic plants using techniques such as inverse-PCR and Southern blot analysis could have been carried out but due to time constraints this was not done.

Other optimization techniques could include the use of different sources of microprojectiles. As the size and type of the microprojectile used have been shown to affect the transformation efficiencies achieved (Southgate *et al*., 1995; Taparia *et al*., 2012), future work could consider using gold microprojectiles for the transformation of ancestral varieties as they have been shown to give higher transformation efficiencies than tungsten (Southgate *et al*., 1995; Taparia *et al*., 2012). Another route for future transformation studies is the use of demethylating agents such as azacytidine as part of the transformation protocol (Tyunin *et al*., 2012). Transgene silencing, whether transcriptional or post-transcriptional, involves the methylation of DNA sequences. Subsequent to transformation and prior to regeneration, calli could be treated with different concentrations of azacytidine and tested for transgene silencing. Although the results obtained in the present study did not support the hypothesis of using ancestral germplasm with a 'simpler' genome organization than commercial hybrids to test the functionality of promoters, the establishment of a high yielding somatic embryogenesis and transformation protocol for the ancestral varieties is worth pursuing.

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Appendices

Appendix 1

Luria-Bertani (LB) broth

Component	Concentration (per liter)
Bacto-Tryptone	10 g
Bacto-Yeast extract	5 g
NaCl	10 g

Appendix 2

Luria-Bertani (LB) agar

Component	Concentration (per liter)
Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Agar agar	15 g

Appendix 3

Calcium chloride solution (2.5 M)*

Component	Concentration (per 10 ml)
Calcium chloride	3.8 g
Sterile distilled water	10 ml

*Filter sterilize and store -20 °C.

Appendix 4

Spermidine solution (0.1 M)*

Component	Concentration (per 10 ml)
Spermidine	145 mg
Sterile distilled water	10 ml

*Filter sterilize and store -20 °C.

Appendix 5

Paromomycin stock*

Component	Concentration (per 20 ml)
Paromomycin salt	4 g
Distilled water	20 ml

*Filter sterilize and store at 4 °C for 21 days or -80 °C.

Appendix 6

Dark selection media (pH 5.8)

Component	Concentration (per liter)
MS salts + vitamins	4.4 g
Sucrose	20 g
Casein hydrolysate	0.5 g
2, 4-D	3 mg
Paromomycin stock*	1 ml
Agar agar	9 g

*Add filter sterilized paromomycin stock to media once it cools to ~50 °C and swirl.

Appendix 7

Light selection media (pH 5.8)

Component	Concentration (per liter)
MS salts + vitamins	4.4 g
Sucrose	20 g
Casein hydrolysate	0.5 g
Paromomycin stock*	1 ml
Agar agar	9 g

*Add filter sterilized paromomycin stock to media once it cools to ~50 °C and swirl.

Appendix 8

Tetrazolium red buffer (TTC – 2, 3, 5 Triphenyltetrazolium chloride)*

Component	Concentration (per liter)
TTC	8 g
Phosphate buffer (0.05 M)	1000 ml

*Store in a dark bottle at 4 °C.

Appendix 9

Phosphate buffer (0.1 M)

Component	Concentration (per liter)
Sodium phosphate monobasic solution (1 M)	68.4 ml
Sodium phosphate dibasic solution (1 M)	31.6 ml
Distilled water	900 ml

Phosphate buffer (0.05 M)

Component	Concentration (per liter)
Phosphate buffer (0.1 M)	500 ml
Distilled water	500 ml

Appendix 10

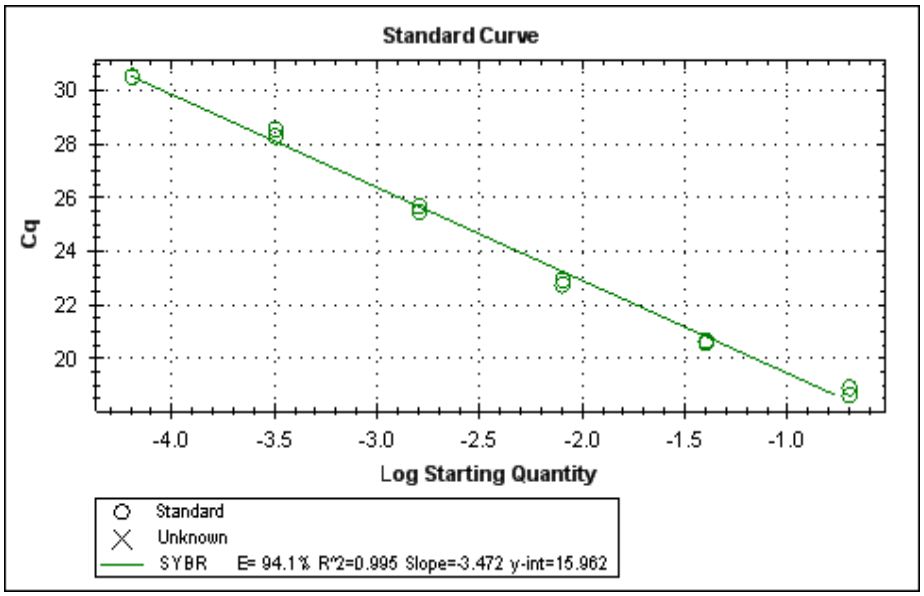
GUS (beta-glucuronidase) buffer*

Component	Concentration (per 100 ml)
Phosphate buffer (0.05 M)	10 ml
EDTA (0.01 M)	2 ml
Triton X-100	100 µl
Sterile distilled water	987.4 ml
X-gluc	80 mg
Dimethyl formamide	500 µl

*Store in a dark bottle at 4 °C.

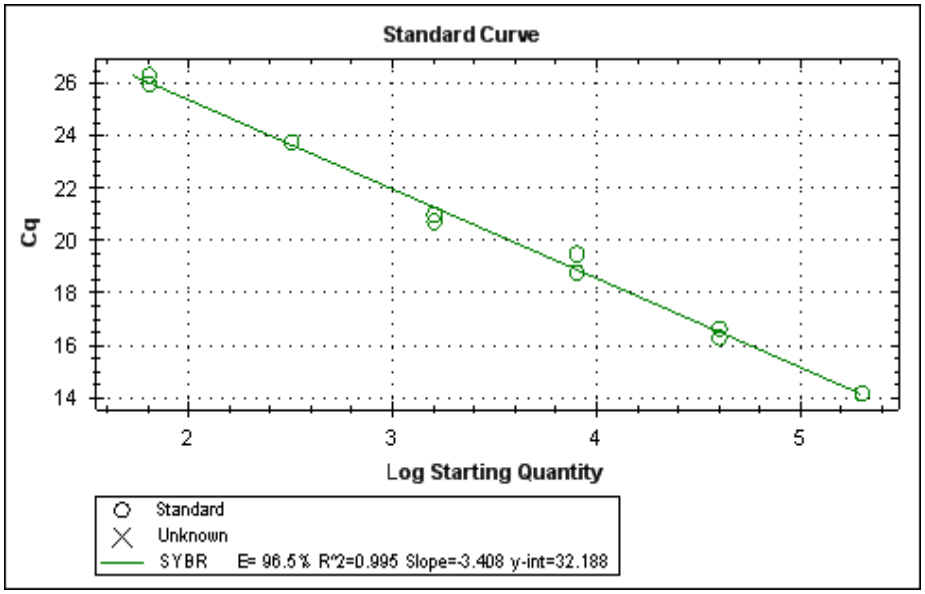
Appendix 11

GUS standard curve



Appendix 12

npt II standard curve



Appendix 13

Tubulin standard curve