Analysis of historical flowering data, investigations into aspects of pollen biology and selected biotechniques to complement sugarcane breeding in South Africa.

by

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ABSTRACT

Sugarcane breeding through sexual reproduction is conducted at the South African Sugarcane Research Institute (SASRI) where difficulties have been encountered including: (a) asynchronous flowering between desired parental genotypes; (b) low pollen viability and no pollen storage options; and (c) the assessment of hybridity of progeny in introgression breeding.

The first aim of the study was to evaluate flower synchronisation within controlled facilities at SASRI [glasshouse (G) and photoperiod (P) house] using data obtained over 19 years. These were subjected to analysis of variance using the Statistical Analysis System as a completely randomised design and the mean comparisons of the variables were done using Fisher's least significant difference. Since sugarcane pollen reportedly remains viable for only 20 min and the stigma is receptive for 7 days, it is desirable that the pollen donors should emerge later than the pollen receptors and that flowering should overlap by no more than 7 days. Data showed significant ($p < 0.0001$) differences among photoperiod treatments for time of flowering and pollen viability for 16 genotypes. The genotypes in P treatments produced more fertile pollen and flowered later (53 - 64 % pollen viability and 179 - 188 days to flowering, respectively) than those in the G treatments (39 - 51 % pollen viability and 158 - 183 days to flowering). Although partial flower synchronisation among genotypes was achieved, with desired genotypes flowering from 6 to 21 days apart, photoperiod conditions could be fine-tuned to reduce the latter to allow improved planning of desired crosses and to enhance the production of hybrids.

Methods for *in vitro* pollen germination, pollen viability, anthesis time and pollen storage were then evaluated using two pre-released *Saccharum* hybrid genotypes (06B1187 and 11K1617). Pollen germination was determined *in vitro* in liquid media containing sucrose (0, 100, 200, 300, 400 and 500 g/l), boric acid (0.1 g/l), calcium nitrate (0.3 g/l) and magnesium sulphate (0.1 g/l). Furthermore, pollen germination was tested in five media formulations published for the Poaceae and the best medium was as above with sucrose (300 g/l) and agar (10 g/l). The identification of an easy, fast (minutes) and accurate viability stain to use during cross-pollination was assessed using starch-iodine, aniline blue, fluorescein diacetate, acetocarmine and 2,3,5-triphenyltetrazolium chloride (MTT). Compared with *in vitro* pollen germination, all stains over-estimated viability (31.6 % vs 37.07 to 82.8 %, respectively, r values = < 0.4), but MTT was the best as the percentage viability was closely correlated with *in vitro* pollen germination and it distinguished viable from non-viable pollen grains. Anthesis time determination showed that viability of pollen collected at 07h00 was high compared with times 09h00 to 13h00 (26.23 ± 2.9 % vs 14.63 ± 4.1 % and 0, respectively). Short-term storage of sugarcane pollen was demonstrated

at 9 °C for 10 days which will be useful to produce hybrids when desired parental genotypes flower at asynchronous times.

Selected biotechnological methods were evaluated for their application in supporting conventional sugarcane breeding at SASRI. Protoplasts were isolated from *in vitro* leaf mesophyll tissue of cultivar NCo376 with the best of the tested media being sorbitol (109.3 g/l), KH_2PO_4 (0.14 g/l), $CaCl_2$ (0.11 g/l), $MgCl_2$ (0.1 g/l), pectinase (2 g/l) and cellulase (4 g/l) ($5.4 \times 10^5 \pm 0.40$ protoplasts/g f. mass with 91.53 ± 0.55 % viability with Evan's blue). *In vitro* inflorescence production was attempted from embryogenic callus cultured on Murashige and Skoog medium supplemented with 2,4-dichlorophenoxyacetic acid (0.003 g/l), polyvinylpyrrolidone (0.1 g/l), thiamine hydrochloride (0.001 g/l), myo-inositol (0.1 g/l), sucrose (30 g/l), agar (9 g/l) and proline (0, 0.04 and 0.06 g/l). No *in vitro* inflorescence induction was observed after 6 months. In order to identify progenies from bi-parental crosses, molecular screening of seedlings was carried out using two previously identified simple sequence repeat (SSR) primers. Seven crosses were made by the SASRI breeders and 60 progenies were screened. Hybrids can be identified by the presence of SSR amplified amplicons from both the pollen donor and pollen receptor. Results showed that the amplicon from the pollen donor (*Erianthus arundinaceus*; 475 bp) was absent in all of the progenies tested hence no hybrid was detected.

PREFACE

The experimental work described in this thesis was carried out in the Plant Breeding crossing office and Pathology Laboratory of the South African Sugarcane Research Institute (SASRI), Mount Edgecombe, Durban, from January 2014 to May 2016, under the supervision of Dr. Sandy Jane Snyman (SASRI and UKZN) and Prof. Paula Watt (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 acknowledge in the text.

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Dr. Sandy Jane Snyman

Signed

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Prof. Paula Watt

DECLARATION 1

I, **NONSIKELELO YVONNE MHLONGO**, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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DECLARATION 2: PUBLICATIONS

Chapter 3

Mhlongo N.Y., Zhou M.M., Snyman S.J., Watt M.P., 2016. Assessment of photoperiod treatments on flowering and pollen production in a sugarcane breeding programme in South Africa. Poster presentation at the South African Association of Botanists (SAAB)/ Southern African Society for Systematic Biology (SASSB) joint congress (University of Free State, South Africa, 10th to 13th January, 2016).

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Table of Contents

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

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ABBREVIATIONS AND SYMBOLS

♂	pollen donor (male)
♀	pollen receptor (female)
%	percent
°C	degrees Celsius
2,4-D	2,4-dichlorophenoxyacetic acid
ANOVA	analysis of variance
$CaNO_3$	calcium nitrate
cm	centimetre
cv %	coefficient of variation
F pr.	F probability
g/kg	grams per kilogram
g/l	grams per litre
h	hours
H_3BO_3	boric acid
HCl	hydrochloric acid
kg/cm	Pascal
$kg/m^2/s^3$	Watt
KOH	potassium hydroxide
LSD	Fisher's least significant differences
m	metres
min	minutes
mg	milligram
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
mm	millimetre
MS	Murashige and Skoog salts and vitamins
NDTF	natural date to flowering
p-value	probability value
PCR	polymerase chain reaction
PEG	polyethylene glycol
R^2	coefficient of determination
RH	relative humidity
rpm	revolutions per minute
s	seconds

SACU	South African Customs Union
SASA	South African Sugar Association
SASRI	South African Sugarcane Research Institute
SE	standard error
SSRs	simple sequence repeats
µl	microliter
µm	micrometre
USDA-ARS	United States Department of Agriculture, Agricultural Research Service

CHAPTER 1: GENERAL INTRODUCTION

Sugarcane is an economically important crop that is cultivated world-wide, on more than 20 million hectares in tropical and sub-tropical regions, producing up to 1.3 million metric tons of crushable stalks (D'Hont *et al*. 2008; Henry 2010; Fageria *et al*. 2013; Moore *et al*. 2014a). In South Africa, sugarcane is the main source of sugar production for local consumption and exports, with 2,3 million tons of sugar produced in each season (SASA 2015). In other countries such as Brazil, it has been used for bioethanol production and generation of electricity (Macedo *et al*. 2008). The South African sugar industry is one of the world's leading cost-competitive producers of high quality sugar and makes an important contribution to employment, sustainable development and the national economy (SASA 2015). At the South African Sugarcane Research Institute (SASRI), sugarcane improvement through breeding is geared towards developing improved cultivars with desirable traits such as high sucrose content and yield, drought tolerance, resistance from pest and diseases, good ratooning ability and adaptation to various agro-climatic regions (Berding *et al*. 2007).

Sugarcane belongs to the genus *Saccharum*, which is classified under the tribe Andropogoneae in the grass family Poaceae, with other members such as maize and sorghum (D'Hont *et al*. 2008). Modern sugarcane cultivars (commercial-type hybrids) are interspecific hybrids of domesticated cane *Saccharum officinarum* (2n = 8x = 80) and wild species of *S. spontaneum* (2n = 5x = 40 to 2n = 16x = 128). The former has high sugar content and the latter has low sugar content but has valued agronomic characteristics such as disease resistance and tolerance to biotic and abiotic stress (D'Hont *et al*. 1998; D'Hont *et al*. 2008). Backcrossing of initial hybrids to *S. officinarum* clones followed by intensive selection lead to the development of more productive varieties, with good ratooning ability and increased resistance to biotic and abiotic stresses (D'Hont *et al*. 2008). This resulted in modern cultivars being highly polyploid (8 - 14x) with a large complex genome (Butterfield 2005; D'Hont *et al*. 2008), thereby making sugarcane a difficult crop to work with at genetic and molecular levels (Henry 2010).

Breeding forms the backbone of variety improvement and since the start of the breeding programme in the 1940s at SASRI, there has been great success of new cultivar release (Brett 1947; Zhou 2013). It takes about 11 to 15 years to complete a sugarcane breeding cycle, starting with crossing elite parental genotypes followed by several stages of testing and selection, and concluding with a new released variety (Zhou 2013). However, in recent years, genetic diversity within the breeding population has reached a plateau as a result of repeated cycles of backcrossing

modern cultivars, which has led to extensive allelic losses (M Zhou, SASRI, personal communication 2015). Hence, there is a focus to broaden the SASRI germplasm gene pool and numerous attempts have been made to introgress genes from wild species (e.g. *S. spontaneum*) and related genera (e.g. *Erianthus arundinaceus* and *Miscanthus*) which have agronomic traits such as ratoonability and vigour, tolerance to environmental stresses, and disease resistance that could further contribute to sugarcane improvement (Piperidis *et al*. 2000; Kennedy 2001; James 2007; Sundaram *et al*. 2010; Gao *et al*. 2015). The aim of an introgression breeding programme is to introduce novel genetic material, which can be a single gene or a quantitative trait locus from a low-productivity genotype (donor) into a productive (recipient) genotype that lacks that particular trait (Santchurn 2010; Brozynska *et al*. 2016). One of the challenges of introgression breeding is asynchronous flowering experienced between the commercial-type sugarcane varieties at SASRI and the wild germplasm.

Sugarcane has poor and variable flowering with no fertile pollen production in temperate and sub-tropical sugar industries such as South Africa, which is a major impediment for plant improvement programmes (Berding *et al*. 2007). The optimal temperatures for inflorescence development and pollen fertility are 28 °C during the day and 23 °C at night (Horsley and Zhou 2013). It is well documented that temperatures below 23 °C delay inflorescence development and reduce pollen fertility, while day-time temperatures above 31 °C and night-time temperatures below 18 °C are detrimental for flowering and fertile pollen production (Clements and Awada 1965; Brett and Harding 1974; Moore and Nuss 1987; Horsley and Zhou 2013; Zhou 2013; Melloni *et al*. 2015). In South Africa, low flowering and pollen sterility occur due to low night-time temperatures (below 18 °C), thereby negatively influencing the ability to make crosses (Brett 1951; Moore and Nuss 1987; Moore and Berding 2013; Zhou 2013). Hence, this was improved by establishing heated growth chambers to provide defined conditions that are favourable to artificially induced flowering and increase pollen fertility (Nuss 1982; Zhou 2013). Since flowering is also regulated by day-length, photoperiod treatments are used to enhance pollen viability and induce, synchronise and distribute flowering across the pollination season at the South African Sugarcane Research Institute (SASRI) (Horsley and Zhou 2013). For successful hybridisation, flowering must be synchronous to create specific combinations during cross-pollination. So, this is an important criterion for sugarcane breeding at SASRI (Horsley and Zhou 2013).

Pollen viability testing is also essential to categorise plants as either pollen donors or pollen receptors during cross-pollination. At SASRI, the starch-iodine stain is currently used for pollen

viability testing and is based on staining starch black when present within the pollen grain. However, the validity of this stain has been criticised among researchers as it cannot distinguish between viable and non-viable pollen grains (Wang *et al*. 2004; Melloni *et al*. 2013). Finding a more precise and faster method for testing pollen viability during crossing is important in reducing erroneous classification of the inflorescences as pollen donors or pollen receptors. Pollen viability has been evaluated by various staining techniques (e.g. tetrazolium salts to detect dehydrogenase activity, aniline blue to detect callose in pollen walls and pollen tubes, iodine to determine starch content, fluorescein diacetate and propidium iodide to determine esterase activity and the intactness of the plasma membrane), *in vitro* and *in vivo* germination techniques and seed-set analysis (Dafni and Firmage 2000; Wang *et al*. 2004). The time of pollen shedding and pollen storage are also critical in addressing the issue of asynchronous flowering due to the short life-span of pollen (thought to be about 20 min in sugarcane after anther dehiscence) (Amaral *et al*. 2013). Pollen storage has been investigated for many Poaceae spp. and has been found to be an effective approach used to prolong pollen viability and overcome hybridisation barriers between desirable parental plants that have different flowering times (Tai 1989; Wang *et al*. 2004; Ge *et al*. 2011; Amaral *et al*. 2013). Conditions of storage for the retention of viability varies, from drying to exposure to low temperatures and short-term storage where temperatures range from 4 to -20 °C (Towill 1985). To date, sugarcane pollen in Brazil has been successfully stored at -20 °C for 30 days (Amaral *et al*. 2013).

As reviewed by Burris *et al*. (2015), the incorporation of biotechnological methods into the conventional sugarcane breeding programmes has assisted plant breeders in producing improved crops. *In vitro* flowering is advantageous and could be of great potential for breeding to reduce the juvenility stage of the genotypes, allow for flower synchronisation and to study the physiology of flowering (Kiełkowska and Havey 2011; Murthy *et al*. 2012), thereby increasing the chances of achieving desired cross-combinations. The transition of vegetative to reproductive growth *in vitro* is widely known to be regulated by an array of internal and external factors such as plant growth regulators, nutrients, pH of the culture medium and light (Castello *et al*. 2016). *In vitro* flowering has been achieved for various Poaceae such as switchgrass (Alexandrova *et al*. 1996), pearl millet (Devi *et al*. 2000) and maize (Kranz and Lorz 1993). The promotion of *in vitro* flowering by exogenous proline from immature inflorescence and juvenile explants has been well documented in many plant species (Virupakshi *et al*. 2002; Glowacka *et al*. 2010). In sugarcane, only Virupakshi *et al*. (2002) managed to induce *in vitro* flowering from embryogenic callus.

Even when synchronisation of flowering is achieved through *in vitro* flowering, incompatibility between species and related genera could be a challenge. Advances in biotechnology through isolation, culture and regeneration of protoplasts has created the potential to cross barriers and cultivate new varieties of plants (Yousuf *et al*. 2015). The first step towards somatic fusion is the isolation of high quality and quantity of protoplasts (generally between 5×10^4 to 1×10^6 viable protoplasts/ml) and multiple studies on Poaceae spp. have reported similar approaches (Ahuja 1982; Durieu and Ochatt 2000; Davey *et al*. 2005), where leaves and cell cultures are the preferred sources of protoplast isolation as large numbers of protoplasts can be obtained. An enzymatic method is commonly used to isolate protoplasts and a number of suitable enzymes such cellulase, pectinase or hemi-cellulase have been used (Davey *et al*. 2005; Vasil and Vasil 2012). Hybrid plants obtained through protoplast manipulations can be used in breeding programmes to develop new cultivars (Yousuf *et al*. 2015). Since sugarcane is polyploid in nature and is vegetatively propagated, protoplast-associated techniques may hold promise in creating variation using somatic fusion (Aftab and Iqbal 1999; Khan *et al*. 2001).

Following cross-pollination or somatic fusion, confirmation of the transfer of genetic material in hybrids needs to be assessed. Currently, the use of simple sequence repeats (SSRs) markers in sugarcane is a routine practice offering plant breeders an accurate tool for identifying gene transfer which can facilitate variety development and crop improvement (Pan 2010). Microsatellite or SSRs are usually the markers of choice in hybridity screening because they are abundant, co-dominantly inherited, and highly reproducible (Piperidis *et al*. 2000; Pan 2010; Gao *et al*. 2015). To date, verification of the introgression of wild germplasm into commercial sugarcane cultivars using SSRs has been successful (Piperidis *et al*. 2000; Pan *et al*. 2006).

In order to support the activities at SASRI, this study focused on: (a) understanding the flowering trends of selected parental genotypes to enable better planning of desired cross-combinations; (b) finding methods for accurate pollen viability testing, time of day for pollen collection and methods for pollen storage and; (c) evaluating biotechnological methods (such as *in vitro* inflorescence production, protoplast isolation and progeny identification using SSR markers) for supporting conventional breeding.

2 CHAPTER 2: LITERATURE REVIEW

2.1 COMMERCIAL IMPORTANCE OF SUGARCANE

Sugarcane is one of the most valued agricultural commodities worldwide (Moore *et al*. 2014a) and it grows naturally in tropical and sub-tropical regions. There are about 176 million metric tonnes (raw value) of sugar produced from commercial plantations each year (USDA 2014/2015). The crop is of economic value in many countries such as South Africa, Brazil, Australia, India, China, Thailand, Pakistan, Mexico, Cuba, Columbia, United States of America (USA), Philippines, Argentina, Myanmar, and Bangladesh (D'Hont *et al*. 2008).

In South Africa, sugarcane is grown in 14 cane-producing areas extending from Northern Pondoland in the Eastern Cape Province through the coastal belt and KwaZulu-Natal Midlands to the Mpumalanga Lowveld (Figure 2.1). There are six milling companies consisting of 14 sugar mills within the cane-growing regions. On average, South Africa produces 2.3 million tonnes of sugar per season, with 76 % being marketed in the South African Customs Union (SACU) and the remainder exported to markets in Africa, Asia and the USA (SASA 2015).

The sugarcane crop has three main uses, *viz*. sugar production, bioethanol production and generation of electricity. Sugar is the third highest source of plant-derived nutrients, estimated to be about 152 Kcal/capita/day (Moore *et al*. 2014a). Initially, it is extracted from raw cane at sugarcane mills and further refined to produce white sugar for consumption in food and beverages (Moore 1987). In addition to sucrose production, sugarcane is increasingly used as a renewable feedstock for biofuel and electricity production for two reasons. Firstly, due to the need to reduce CO_2 emissions to overcome the impact of climate change and, secondly due to the constant decline on availability of non-renewable petroleum feed stocks (Moore 1987; Waclawovsky *et al.* 2010).

Sugarcane has become the basis of first-generation renewable feedstock for the production of the biofuel ethanol through fermentation from extracted sugars and biomass (D'Hont *et al*. 2008; Waclawovsky *et al*. 2010; Manners 2011). For over a decade, countries such as Brazil have been producing bioethanol from sugarcane to meet the demand for internal ethanol consumption utilised by cars and other automobiles (Pessoa-Jr *et al*. 2005; D'Hont *et al*. 2008; Waclawovsky *et al*. 2010).

Developing new varieties with high biomass is drawing interest in several countries (Waclawovsky *et al*. 2010). This is done to meet the electricity demands which is carried out through the conversion of cellulosic residues such as bagasse (D'Hont *et al*. 2008). Bagasse is a

fibrous portion left after sugarcane juice extraction and when harvested, it is burnt in fire boilers for producing steam (Paturau 1989). The steam is then used as a source of power in sugar mills and running turbines in power stations (Moore 1987; Cheavegatti-Gianotto *et al*. 2011). In this way, future generations around the world may benefit through cogeneration of electricity and the production of electricity for the national grid. The sugarcane crop is also used for the production animal feed (Franchi *et al*. 2002), paper (Paturau 1989; Heinz *et al*. 1994), bio-plastics (Arruda 2011), and bio-chemicals (Cherubini 2010).

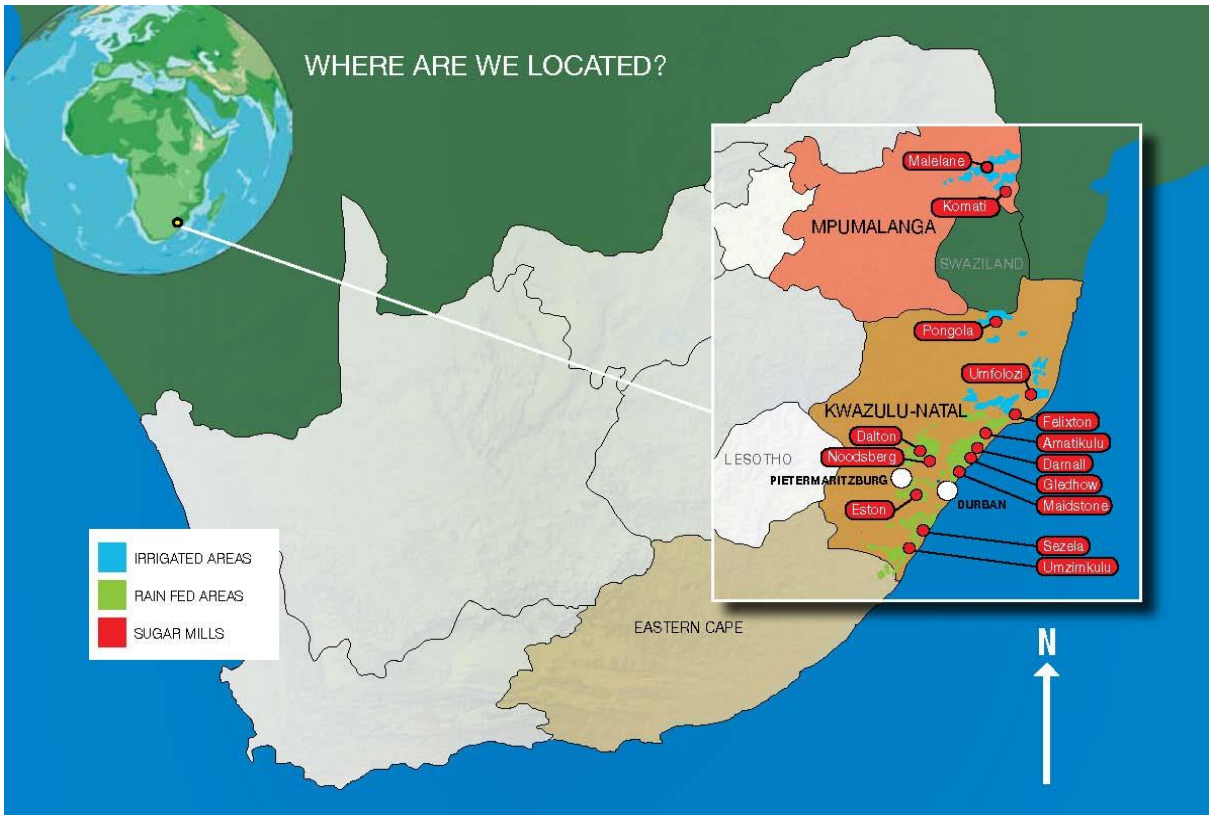


Figure 2.1: Areas of growing sugarcane and mills in South Africa (copied from South African Sugar Industry Directory, 2013/2014).

2.2 IMPROVEMENT OF MODERN SUGARCANE CULTIVARS THROUGH INTROGRESSION BREEDING

Introgression breeding, also referred to as 'nobilisation' or 'base broadening', is the crossing of noble cane (*S. officinarum*) and its wild relatives (various species of *Saccharum* and related genera), followed by repeated backcrosses of the resultant hybrids to noble canes (Figure 2.2) (Santchurn 2010). The first nobilisation breeding practice occurred in Java which produced POJ 2725 and POJ 2878 (Heinz *et al.* 1994) through the interspecific hybridisation derived from *S. officinarum* and *S. spontaneum* (James 2007). The main objective of that hybridisation was to

develop disease resistance but it further provided increased yields, improved ratoonability, and adaptability under unfavourable conditions (Roach 1972).

The key event in sugarcane breeding was the production of the nobilised cultivar, POJ 2878, of Proefstation Oost, Java in 1921 (Ming *et al.* 2006). This variety has become an important cultivar across the world and is an ancestor of most modern cultivars today (Jackson 2005). Modern cultivars are comprised of 15 - 20 nobilised cultivars that can be traced back to the initial nobilised genetic base developed in Java and India (Roach 1989; Moore *et al.* 2014a).

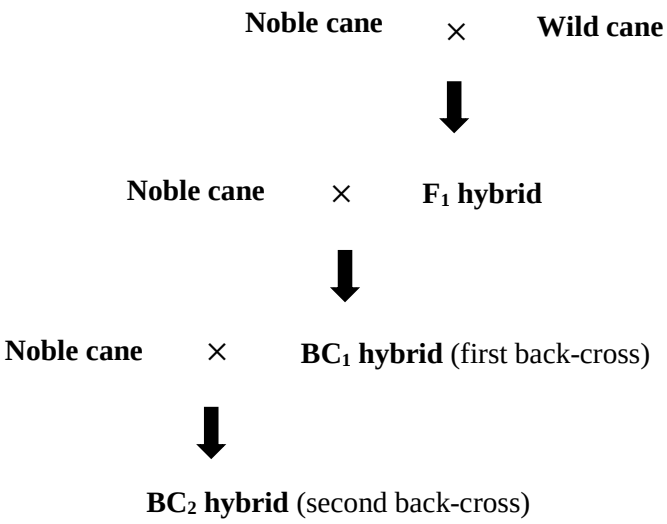


Figure 2.2: The nobilisation process. Noble cane includes *S. officinarum* or commercial-type hybrids with high sucrose content. Wild cane includes *S. spontaneum*, *Erianthus* and *Miscanthus* (Santchurn 2010).

In South Africa, the N (Natal) varieties also originated from the POJ 2878 nobilised cultivar but, due to multiple events of backcrossing over the years with elite modern hybrids, their genetic diversity has become limited. Some countries, including South Africa, have tried a base-broadening programme by crossing wild canes (e.g. *S. spontaneum*, *Erianthus* and *Miscanthus*) with the commercial-type hybrids with an aim of incorporating deficient traits (James 2007; Piperidis *et al.* 2010). However, none of the efforts made had long-term benefits. A reason for this could be the inability to trace the transfer of genes into the gene pool of breeding programmes which has led to failure of base-broadening programmes around the world (Moore *et al.* 2014a).

There is large genetic variation within the *Saccharum* genus (Tai and Miller 2002; Moore *et al.* 2014a). According to Gao *et al.* (2015) intergeneric hybridisation between *Saccharum* and *Erianthus* has been challenging possibly due to pollen-pistil incompatibility. However, in recent

years in China, a successful intergeneric cross was achieved between *S. spontaneum* and *Erianthus* which produced fertile F_1 hybrids when backcrossed to the modern cultivars (Gao *et al.* 2015).

2.3 PLANT BREEDING PRACTICES IN SOUTH AFRICA

2.3.1 Historical background

Sugarcane breeding is of fundamental importance to the sugar industry and is focused towards increasing sucrose yield. Although sugarcane breeding has been practiced over the years, there remains an unlimited opportunity to improve the varieties available for various uses and, in some cases to modify their characters (Barnes 1964; Ming *et al.* 2006). As reviewed by Cheavegatti-Gianotto *et al.* (2011) in Brazil, introduction of improved hybrids has increased productivity and disease resistance in varieties released for commercial cultivation by farmers.

From 1852, the South African sugarcane industry operated solely on naturally-occurring varieties of *S. officinarum* ('noble canes') species and was dependent on regular imports of new varieties as foreign varieties became susceptible to local diseases, such as the mosaic virus (Brett 1950). In 1883, one variety of the *Saccharum* species, named 'Uba' (*S. sinensis*), was found to be resistant to sugarcane mosaic virus and soon became of great interest to growers (Barnes 1964). However, after 30 years of commercial production, Uba was discovered to be susceptible to the streak virus, thereby leaving the sugarcane industry with no varieties to replace it (Barnes 1964). During 1925, the South African Sugarcane Experimental Station (now the South African Sugarcane Research Institute, SASRI) was established in Mount Edgecombe with the aim of importing, testing and releasing new varieties.

Initially, it was believed that no fertile pollen could be produced in sugarcane in South Africa which meant that no sexual crosses could be made (Brett 1947). In 1946, the variety Co301 was assessed for pollen fertility and seed production by placing it in a controlled facility (greenhouse) with increased night temperatures (Barnes 1964). Soon after, it was observed that the number of seedlings produced had increased considerably (Barnes 1964). Studies conducted by Brett (1953) demonstrated that the natural night temperatures in South Africa contributed to the low pollen fertility and he concluded that artificial induction was essential for the production of viable pollen. Controlled photoperiod facilities, such as the glasshouse and photoperiod house, were then constructed at SASRI in 1966 and 1971, respectively. This approach has been adopted by other sugar industries such as Argentina, China, Florida, Louisiana and Taiwan (James 2007). The advantages of such photoperiod facilities are that the crossing activities can be planned and

flowering of clones can be manipulated selectively to ensure that desired combinations are achieved (Berding *et al.* 2004; James 2007). This led to successful cross-pollination and production of large numbers of seedlings within the breeding programmes in many countries (Bischoff and Gravois 2004; Zhou 2013; Melloni *et al.* 2015).

There are three main conventional breeding aspects within the SASRI breeding programme, *viz.* parental variety collection and evaluation; cross-pollination; and selection of desired varieties, all of which will be discussed below.

2.3.2 Germplasm collection

The collection of the germplasm with traits of interest is the first step towards starting any sugarcane breeding programme (James 2007). The world's sugarcane germplasm collection is maintained by the United States Department of Agriculture, Agricultural Research Service (USDA-ARS), Florida, and by the Sugarcane Breeding institute at Cannanore, India (Comstock *et al.* 1995; Balakrishnan *et al.* 2000; Fageria *et al.* 2013). At SASRI, 1800 germplasm lines have been developed with desirable traits such as high cane yield, high sucrose content and disease resistance since the 1970s (Zhou 2013).

2.3.3 Conventional breeding

Parental variety collection and evaluation

As previously illustrated, the South African sugar industry is divided into three agro-climatic zones, *viz*. northern irrigated, coastal and midlands (the latter two are rain-fed) (Table 2.1). However, climatic conditions in South Africa are not ideal for the production of fertile pollen of the sugarcane crop (Brett 1950). For that reason, controlled facilities (glasshouse and photoperiod house) are required to induce sugarcane flowering. In Africa, only South Africa has the facilities for breeding and development of sugarcane varieties and most countries in Africa grow South African-bred 'N' varieties.

Table 2.1: Agro-climatic zones of the South African growing regions for which the SASRI plant breeding programme caters (Zhou and Joshi 2012; Zhou 2013).

Conditions represented§	**Breeding research stations**	**Altitude (m)**	**Latitude**	**Age at harvest (months)**
Northern irrigated[1]	Pongola	308	27°24´	12
	Mpumalanga	170	25°33´	12
	Empangeni	102	28°43´	12
Coastal rainfed[2]	Gingindlovu	93	29°01´	12 - 18
	Kearsney	241	29°17´	16 - 18
Midlands[2]	Bruyns Hill	1 012	29°25´	24
	Glenside	997	29°25´	24

§ **Different geographical regions**:

[1] Irrigated areas – low rainfall, low relative humidity, moderate temperatures.

[2] Rain-fed regions – moderate rainfall, high relative humidity, high temperatures.

According to Zhou (2013), the criteria for selection of desired traits at SASRI include high sucrose content, good ratooning ability and resistance to pests and diseases. A desired parental genotype has to have the ability to transmit high sucrose yield and favourable traits to their progeny during the crossing process. As sugarcane flowering is limited, a facility providing temperature and day-length controls is needed to initiate flowering. Six photoperiod treatments are being used by the SASRI breeders, three in the glasshouse and three in the photoperiod house (Horsley and Zhou 2013) (Table 2.2). These facilities have been found to stimulate flowering in shy-flowering varieties, and also to increase fertility in other genotypes (Horsley and Zhou 2013). Temperatures are kept above 21 °C, as suggested by Brett and Harding (1974), to improve pollen fertility. Flowering induction commences in February for the flowering season that occurs during the period of May to August. The time for inflorescence emergence varies between 90 and 110 days after initiation (Brunkhorst *et al.* 2000; Zhou 2013). Single-budded setts (sections of the stalk) are planted in canisters filled with river sand in September every year and replicated across both facilities (Brunkhorst 2003; Zhou 2013). The cane is watered daily and fertilised well, but the amount of nitrogen applied to the plants is reduced six weeks before the photoperiod treatments start (Brunkhorst 2003).

Table 2.2: Photoperiod treatments used for inducing flowering at SASRI [copied from Horsley and Zhou (2013)].

Facility	Treatment	Day-length (h)	Rate of decline of day-length¥
Glasshouse	G1	12.30	Constant dawn at 05h45
	G2	12.30	Constant dawn at 05h30
	G3	13.00	Constant day-length for 60 d, then 60 s decline for 10 d, then 90 s decline
Photoperiod	P1	12.35	30 s
house	P2	12.30	30 s
	P3	12.30	30 s

¥Rate of decline: in the glasshouse, extending the natural day-length is achieved artificially by reducing the rate at which it declines naturally. In the photoperiod house, manipulating the day-length by reducing each artificial light treatment by 30 s each day from the start of initiation of flowering (February each year).

Cross-pollination

Cross-pollination is the principal method used to create new genetic variability in sugarcane (James 2007). Before crosses can be made, a census is conducted to determine which parental varieties have emerging inflorescences. An inflorescence is a cluster of florets arranged on a stem that is composed of a main axis as illustrated in Figure 2.3. Each spikelet is a hermaphrodite inflorescence, both male (androecium) and female (pistil) parts in the same floret. In the androecium, there are three stamens and each stamen has one anther (Blackburn 1984). The anthers are bilobed with filaments being attached to the connective base between the lobes and may be bright yellow to purple in colour (Moore 1987). In the pistil, the stigma can be red to purple appearing as a purple inflorescence (Amaral *et al.* 2013). Upon the opening of the spikelets, flowering begins at the top progressing to the base.

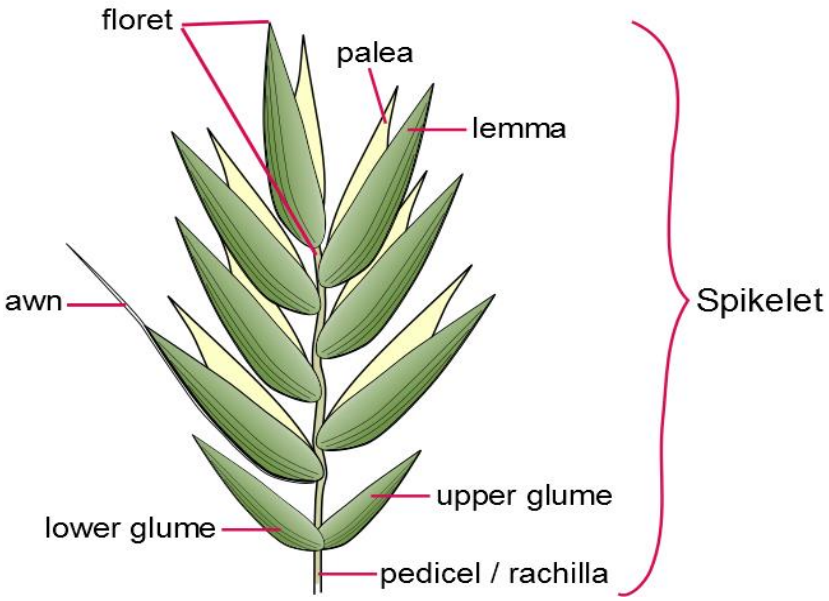
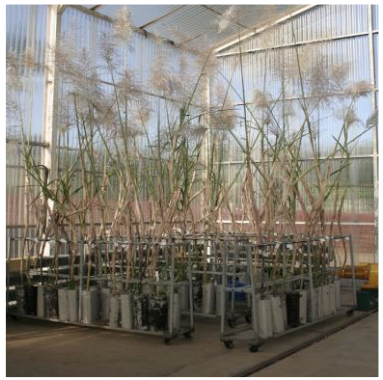


Figure 2.3: Diagram of a typical inflorescence from the Poaceae family (Hargreaves 2016).

At SASRI, the crossing programme consists of various steps as illustrated in Figure 2.4. The protocol used is as described by Zhou (2013). Once the number of emerged inflorescence are counted on a daily basis, they are moved to the glasshouse for laboratory testing to determine pollen viability. A sample of anthers is taken from each inflorescence, the pollen is tested for viability using the starch-iodine test (Mulugeta *et al.* 1994) and percentage viability is calculated. If viability is less than 30 %, that variety is used as a pollen receptor whereas if pollen viability is greater than 30 %, that variety is used as a pollen donor. In some instances, emasculation by hot water treatment (50 °C, 3 min) is carried out to eradicate fertile pollen from a variety when it is required as a pollen receptor during crossing. After fertility classification of the varieties, the critical process of deciding which combination to cross is done. The selected parental lines are set up to make either a bi-parental cross or a poly-parental cross and the crosses are separated by compartments within the glasshouse which aid in preventing contamination (Figure 2.4). The glasshouse is thermostatically controlled and conditions are kept above 21 °C and relative humidity at 70 % to allow for optimum fertilisation and seed-set. Fertilisation is allowed to proceed for approximately 14 days until pollen shedding stops. The selected pollen donor is discarded and only the pollen receptor plant is sent to the ripening area for seed-set. The inflorescence then forms ‘fluff’, indicating that the seed is ready to be collected, a process that takes 3 - 5 weeks (Tew and Cobill 2008).



(A) Sugarcane varieties in the glasshouse ready for crossing

(B) Laboratory pollen viability testing (starch-iodine stain)

(C) Emasculation of the inflorescence by hot water treatment (50 °C, 3 min)

(D) Bi-parental and poly-parental crosses set up in the glasshouse

(E) Sugarcane 'fuzz' developing for future seed harvest

(F) Sugarcane seedlings growing in the trays

Figure 2.4: Steps involved in artificial crosses performed in the SASRI breeding programme.

As discussed later in this review, the storage of seeds under optimum conditions is critical as crossing is labour intensive and, hence, expensive to undertake (James 2007). Long-term storage at low temperatures (-20 °C) is used as minimal loss of seed viability has been shown under such conditions (Copeland and McDonald 1995). The seed is a dry, one-seeded caryopsis, formed from a single carpel with the ovary wall (pericarp) being united with the seed coat or testa (Nuss *et al.* 1999). It is very small (0.5mm), seen as a yellow-brown in colour and has an ovate shape (Heinz 2013). Collectively, the seeds are referred to as fuzz or fluff (Nuss *et al.* 1999). After collection, the matured seed is stored in a 30 °C oven for 24 h. A sample is taken, weighed and a seed germination test is performed in duplicate. This is done in a Petri dish layered with moist filter paper. Seed germination takes about 6 days. Breeders decide on how much seed to use for planting based on results from the germination test. Then the remaining seed is labelled and placed in a vacuum-sealed plastic bag with silica blue to reduce the moisture content within the seeds. Rao (1980) demonstrated that sugarcane fuzz is short-lived, losing 90 % viability in 80 days at 28 °C if not desiccated. Once packaged, seeds are stored at -20 °C until required for use. The seeds are viable for a period of 10 years in storage (Brunkhorst *et al.* 2000). The seeds are used to raise seedlings for the selection programmes and are sown annually in seed boxes. The seedlings are planted outdoors into clay bricks on concrete slabs (terraces) so that the seed-cane is available for planting in the field (Brunkhorst *et al.* 2000).

2.4 SUGARCANE FLOWERING

2.4.1 Synchronisation of flowering

Sugarcane breeding programmes in the early years relied on natural hybridisation between varieties that had synchronised flowering times in the field. This occurred commonly within species and among similar varieties. It was therefore impossible to create crosses with divergent types such as late-flowering, *S. officinarum* and early-flowering, *S. spontaneum* (Tai *et al*. 1991). This limitation is referred to as asynchronous flowering, when the desired parental varieties emerge at different time periods thereby reducing/preventing the formation of particular crosses during cross-pollination (Moore and Nuss 1987). Inflorescence from sugarcane varieties can emerge up to eight weeks apart during the crossing season (Nuss 1982), making it difficult to achieve desired crosses by breeders especially between early- and late-flowering varieties. It is well documented that sugarcane varieties can be distinguished as early-, to intermediate- to late-flowering (El-Manhaly *et al.* 1980). Moore and Nuss (1987) showed that the ability to synchronise flowering is vital for the improvement of sugarcane. There are two possible ways to overcoming this limitation, *viz*. manipulation of photoperiod treatments (as described below) or through the storage of sugarcane pollen (as described in section 2.9). Manipulation of photoperiod treatments

requires a treatment protocol to be developed through the understanding of: (a) how the various plant and environmental factors control the flowering process; (b) which factors are most important in the particular location; and (c) the cost of modifying the controlling factors to achieve synchronisation.

Artificial photoperiod has gained recognition not only in temperate climates where it is necessary, but also in tropical/subtropical climates where sugarcane flowering occurs naturally (Srivastava *et al.* 2006). For that reason, knowledge of the factors that regulate flowering is valuable to the plant breeder who must be able to control the timing of flowering with precision (LaBorde 2007).

2.4.2 Factors affecting flowering

In commercial production, flowering is a disadvantage since it can reduce the sucrose yields if flowered fields are not managed properly (Moore *et al.* 2014b). However, in plant breeding, the occurrence of flowering is essential for developing new varieties (Coleman 1959). In the context of this review, sugarcane flowering as an important characteristic for plant breeding is emphasised.

Flowering is both genetically and environmentally controlled (Durai *et al.* 2014). Most sugarcane-growing countries have conducted research around factors affecting the flowering process in order to improve sugarcane breeding (Coleman 1959). Studies have shown that flower initiation, the transition from vegetative to reproductive growth, requires the correct combination of external factors such as photoperiodism and day-length, temperature, soil moisture content, relative humidity, nutrition, and age of the plant (Blackburn 1984; Moore and Nuss 1987), as described below.

Photoperiodism and day-length

In many species, floral induction occurs in response to photoperiodism which, in the context of artificial breeding, is the alteration of day-length for plants to distinguish between seasons (Moore *et al.* 2014b). In sugarcane breeding, it is generally accepted that the leaves are the organs that perceive the day-length and produce a signal where the floral primordium is differentiated (Moore *et al.* 2014b). As observed in other plants, young sugarcane leaves are most effective in producing the flowering signal (Moore *et al.* 2014b).

Photoperiodism is regarded as one of main factors responsible for controlling the conversion from vegetative to reproductive growth in grasses and legumes (Aamlid *et al.* 1999). Particularly in the

Poaceae, studies have shown that photoperiodism is related to the timing of other exogenous factors such as temperature, relative humidity, rainfall and day-length, which also contribute to the inhibition of seed formation (Loch *et al.* 1999; Moore *et al.* 2014b). In subtropical sugarcane growing areas, such as South Africa, the control of day-length is achieved through the use of artificial photoperiod regimes (LaBorde 2007). These treatments are not only geared at initiating flowering but also to increase pollen fertility, induce flowering in shy-flowering varieties and to synchronise flowering to expand the number of crosses to be made per season. Although sugarcane has generally been accepted as an intermediate-day plant, i.e. the inflorescence emerges only when the photoperiod falls within the relatively narrow range of 12 h to 12.5 h of light, it responds progressively to increased night lengths (Coleman 1962; Bischoff and Gravois 2004). Good flowering was obtained even for shy-flowering genotypes by using photoperiods of 12.5 and 12.75 h, shortened by 30 seconds per day (Julien 1971; Brett and Harding 1974). Coleman (1959) showed that sugarcane inflorescence emergence can be induced through alternating from long days to shortened days.

Temperature and relative humidity

In addition to photoperiod, sugarcane flowering has certain temperature minima, optima and maxima for completion (Moore *et al.* 2014b). Flowering, therefore, may be adversely affected in subtropical and temperate areas where the daily night-time are below a certain critical temperature (LaBorde 2007). Temperatures below 21 °C at night-time have been found to prevent initiation of flowering and delayed the emergence of the inflorescence in sugarcane (Moore and Nuss 1987; Moore *et al.* 2014b). Maximum temperatures also have an inhibitory effect (Edwards and Paxton 1979; LaBorde 2007). The optimum temperature during the day is about 28 °C and it has been reported that inflorescence emergence is reduced when temperatures are above 31 °C (Moore and Nuss 1987). High temperatures are generally associated with cloudless skies, lack of rainfall and low humidity which may lead to water deficiency and drought stress known to inhibit inflorescence emergence (Moore *et al.* 2014b).

Soil moisture content

Soil moisture content only plays a role at the onset of flowering (Moore and Nuss 1987). In general, soils must be well drained and should possess a reasonable moisture-holding capacity as a buffer against fluctuations in rainfall (LaBorde 2007). At low levels of soil moisture the rate of flowering is reduced [Alexander, 1942 as cited by Moore *et al.* (2014)]. Hence, adequate soil moisture content and plant water status is vital not only for induction and rate of development, but for timing inflorescence emergence, anthesis and regulating seed set (Lundqvist 1961).

Nutrition

In floral induction, the nutritional status of a plant is important since the development of the flowering parts is dependent on food availability and translocation (Copeland and McDonald 1995; LaBorde 2007). Sugarcane produces inflorescences under a wide range of nutritional conditions, although this does not mean that specific nutrients do not affect flowering (Brunkhorst 2003). For maximum flowering to occur, sugarcane must be grown vigorously before induction (LaBorde 2007).

Nitrogen is the essential nutrient required in the largest amount for growth but is the most limited in the soil (Singh 2013). For this reason, the requirements of the crop must be met by external application of nitrogen to achieve satisfactory crop yield. High levels of nitrogen, especially during initiation, may reduce or delay flowering, while too little nitrogen may negatively affect flowering intensity, inflorescence size and seed set (Brunkhorst 2001; LaBorde 2007). A difference in age, variety and the availability of water affects the extent to which nitrogen inhibits flowering (Nuss *et al.* 1999). Nuss *et al.* (1999) found that sugarcane flowering in South Africa was delayed by 25 days due to excessive concentrations of nitrogen in the soil. In addition, in Australia doubling the dosage of nitrogen reduced the emergence and development of the inflorescences (Berding *et al.* 2004). As nitrogen plays a central role in flowering, defined quantities of fertilizer must to be applied.

Age of the plant

The time of planting influences the time of flowering of the sugarcane crop and this is taken into consideration in an attempt to synchronise the flowering periods of different varieties (Brett 1953; El Manhaly *et al.* 1984; Moore and Nuss 1987). Sugarcane has a juvenile phase of development during which induction of inflorescence emergence is impossible (Lundqvist 1961; LaBorde 2007).

Varieties that have inflorescence emergence often are said to have shorter juvenile phases compared with those that rarely emerge (Moore and Nuss 1987). As reported by Jones and Senft (1985), the minimum age of the sugarcane plant for the initiation of flowering induction is 12 - 16 weeks. Optimum aged plants are classified as having three to four visible internodes (Coleman 1969). For older plants (> 16 weeks), they will have reduced flowering and are characterised as being in a senility stage (too old for abundant flowering) (Lundqvist 1961; Moore and Nuss 1987).

2.5 POLLEN DEVELOPMENT, VIABILITY AND STORAGE OF POLLEN

As there is only limited information regarding reproductive biology in sugarcane, Poaceae systems will be reviewed.

2.5.1 Pollen development

Pollen plays a vital role in the flow of genes in plants (Ellstrand 1992). It is a specialised male gametophyte that develops within the anthers of flowering plants such as sugarcane (Copeland and McDonald 1995). When the pollen has matured, it undergoes dehiscence and is released from the anther to the external surroundings. It can be transported via different vectors such as the wind or insects, depending on the species. However, in sugarcane, no insect or animal vectors are known. Once transported, the viable pollen settles on a compatible stigma and it will germinate as it takes up water and begins to swell (Heslop-Harrison 1992). The vegetative cell is triggered and grows out a pollen tube. In mature angiosperms, the rate of germination differs based on the type of pollen grain, either bi- or tri-cellular (Brewbaker 1959). Bi-cellular pollen grains are dehydrated prior to release and become metabolically inactive and contain one generative and one vegetative cell (Bots and Mariani 2005). The generative cell undergoes a second mitosis after pollen tube growth has already started thereby making germination and the rate of metabolism relatively slow (Bots and Mariani 2005). Tri-cellular pollen grains, on the other hand, are partially hydrated and are metabolically active upon release. They contain one vegetative and two sperm cells, and the second mitosis stage occurred during development in the anther thus allowing for rapid pollen tube formation (Heslop-Harrison 1992; Nepi *et al.* 2001; Bots and Mariani 2005). Sugarcane pollen has a short half-life of 12 min, and remains viable for only 35 min under ambient conditions (26 °C and 67 % relative humidity) (Amaral *et al.* 2013).

The viability of pollen is influenced at different stages of development by two main factors which are relative humidity and temperature as described below (Bots and Mariani 2005).

Relative humidity

The response of pollen to high or low humidity may differ amongst species and is usually associated with the intrinsic hydration state of the pollen at dehiscence (Nepi *et al.* 2001). During dehiscence, the pollen of the Poaceae family contain more than 30 % water (Franchi *et al.* 2002). At low relative humidity in the environment, the pollen is highly sensitive as loss of water is rapid. For example, in *Zea mays* L. at 20 % RH viability is lost after 50 min, whereas at 75 % RH viability is lost after 4 h (Aylor 2004). In *Sorghum biocolor* L., pollen desiccated at 50 - 55 % RH

lost viability after 30 min (Lansac *et al.* 1994). In addition, low relative humidity during anthesis in sugarcane has been found to lead to poor seed-set (Nuss 1979).

Temperature

Temperature can affect pollen grains caused by transportation by the pollinator, germination on the stigma and/or during the development in the anther (Bots and Mariani 2005). After dehiscence, temperature stress results in severe concerns for pollen viability in both cold and heat conditions depending on the species. For example, in maize pollen, temperatures above 32 °C have been shown to cause a critical reduction in pollen germination during the pollination stages of anthesis (Herrero and Johnson 1980). However, the most critical period for heat stress is between 7 - 15 days prior to anthesis, which corresponds to the developmental stage observed in cereal crops (Sato *et al.* 2002).

In sugarcane, low pollen viability is associated with low temperature during the flowering process (Nuss 1980). Temperatures below 15 °C before or during flowering have an unfavourable effect on the production of fertile pollen and the rate of development of the inflorescence (Berding 1981).

2.5.2 Pollen viability and fertility

Pollen viability is defined as "the capacity to live, grow, germinate or develop", but viable pollen only germinates under optimal conditions (Beyhan and Serdar 2008). Viability testing gives an indication of whether or not the pollen grain has the potential to transfer sperm cells to the embryo sac during the process of fertilisation. It is one of the critical stages in artificial pollination and sugarcane breeding programmes (Rodriguez-Riano and Dafni 2000), since pollen must be viable at the time of pollination for seed-set to occur. Viability techniques provide a means of evaluating the potential of the pollen to germinate on a compatible stigma. Finding a reliable technique for testing sugarcane pollen viability during crossing is critical in reducing erroneous classification of the inflorescences as pollen donors or pollen receptors (Melloni *et al.* 2013). In addition, the success of any storage programme is dependent of the breeder's ability to distinguish between viable and non-viable pollen grains. There are several methods that can be used for evaluation of pollen viability: (a) staining techniques; (b) *in vitro* and *in vivo* germination tests and; (c) analysing seed-set data.

Staining techniques

Staining techniques are commonly used to determine viability of the pollen grains, as they are relatively quick to perform compared with other viability techniques. Although numerous staining techniques have been developed to determine pollen viability, no single one is ideal for all species as indicated in Table 2.3. In addition to the techniques listed in Table 2.3, Rodriguez-Riano and Dafni (2000) proposed the use of heat-killed (80 ℃) pollen as a control to investigate the effectiveness of the stain when determining pollen viability.

Pollen germination (*in vitro* and *in vivo*)

Germination tests determine the ability of pollen to germinate and form pollen tubes. The tests are based on an assumption that if pollen is capable of germinating, it is fertile or viable (Barrow 1983). There are two major ways of assessing germination - *in vivo* and *in vitro*.

In vivo germination involves the germination of pollen on an un-pollinated stigma on either a whole plant or the stigma grown in agar medium (10 g/l agar and 200 g/l sucrose) (Heslop-Harrison 1992). Both methods are time-consuming and may lead to overestimated of viability if the pistil is over-pollinated (Bots and Mariani 2005). The stigma plays an important role in the fertilisation process as it allows for the adhesion and hydration of pollen grains in the presence of various enzymes (Chaudhary 2014). The receptivity of the stigma for pollen varies depending on the species and it can persist for 1 h to several days (Heslop-Harrison 2000). Results can be obtained in a few days (Heslop-Harrison 1992).

Different types of *in vitro* pollen media composition have been used for pollen viability of flowering plants (Table 2.4). *In vitro* germination involves the use of artificial media and is used to determine viability of fresh or stored pollen samples (Hauser and Morrison 1964). Studies have shown that this is a more accurate method than the staining techniques (Bots and Mariani 2005). However, optimisation of components from the medium need to be evaluated since the use of sub-optimal medium may underestimate viability (Bots and Mariani 2005). The medium consists of essential components such as calcium, magnesium, sucrose and boric acid (Brewbaker and Kwack 1963; Horsley *et al.* 2007).

Table 2.3: Examples of staining techniques commonly used for pollen of flowering plants (angiosperms).

Stains used	Mechanism of staining	Advantage	Disadvantage	Reference/s
Iodine and potassium iodide	Stains starch.	Easily stained. Shows good colour contrast between viable and non-viable pollen grains.	Does not distinguish between viable and non-viable pollen grains.	Mulugeta *et al*. (1994); Huang *et al*. (2004); Ge *et al*. (2011); Melloni *et al*. (2013)
Aniline blue in lactophenol	Has affinity for the cytoplasm of viable pollen grains and detects callose in pollen walls and tubes.	Easily stained.	Shows poor colour contrast between viable and non-viable pollen grains. Cannot distinguish between viable and non-viable pollen grains.	Asghari (2000); Wang *et al*. (2004); Ge *et al*. (2011)
Acetocarmine	Stains nuclei and weakly stains the cytoplasm of viable pollen grains.	Convenient for assessing cell development from pre-meiotic stages of mature pollen.	Overestimates viability compared with *in vitro* germination.	Heslop-Harrison (1992); Gaaliche *et al*. (2013)
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Detects the presence of mitochondrial succinate dehydrogenase.	Results closely correlated to the *in vitro* germination.	Variability in colour tonalities making it difficult to distinguish between viable and non-viable pollen grains.	Rodriguez-Riano and Dafni (2000)
2,3,5-triphenyl-2H-tetrazolium chloride (TTC)	Detects the presence of mitochondrial succinate dehydrogenase.	Can distinguish between viable and non-viable pollen grains. Results closely correlated to *in vitro* germination.	-	Huang *et al*. (2004); Gaaliche *et al*. (2013)
Fluorescein diacetate	Indicates the integrity of the vegetative cell plasma membrane by the presence of a non-specific esterase in the cytoplasm.	Highly sensitive. Simple to use. Strongly correlated to *in vitro* germination and seed-set. Convenient as slides can be prepared and counted at least one week later.	Requires a fluorescent microscope.	Jones and Senft (1985); Heslop-Harrison (1992); Kalkar and Neha (2012)
Propidium iodide	Intercalates DNA and RNA by penetrating the membranes of dead or non-viable cells.	Convenient as slides can be prepared and counted at least one week later.	Requires a fluorescent microscope.	Jones and Senft (1985)

Sucrose has been found to maintain osmotic pressure, acts as a substrate for pollen metabolism and serves as a source of carbohydrate (Visser 1955; Shivanna and Johri 1985). Boric acid has the ability to make complexes with sugar and this sugar-borate complex is known to be capable of better translocation than non-borate, non-ionized sugar molecules (Sidhu and Malik 1986). Calcium and magnesium both aid in enhancing germination (Pfahler and Linskens 1972). The media is often adjusted to pH 5 - 8 depending on the species. Krishnamurthi (1980) found that sugarcane pollen germinated successfully in media containing sucrose, boric acid, calcium nitrate, magnesium sulphate and water.

Seed set

Seed-set is an indicator of reproductive success and the formation of seed (Owens *et al.* 1991). However, it is laborious and hand-pollination could lead to over-estimation of viability if the pistil is over-pollinated. Un-pollinated receptive stigmata are pollinated lightly with the pollen samples (Heslop-Harrison 1992). Too much pollen may prevent hydration whereas little quantities of the pollen could lead to unsuccessful pollination. This test examines every step of compatibility between the pollen donors and pollen receptors used during fertilisation (Heslop-Harrison 1992; Gao *et al.* 2015).

Table 2.4: Examples of *in vitro* media composition for pollen germination of angiosperms. Components are in g/l.

Species	Agar /Liquid	Sucrose	Boric acid	Calcium nitrate	Magnesium sulphate	Temperature (°C), pH and RH	Additional components			Reference/s
							Manganese sulphate	Polyethylene glycol	Potassium nitrate	
Non-Poaceae species										
Abelmoschus esculentus Moench.	Agar (10)	150	0.1	-	-	23-25 pH 5.6	-	100 (6000 grade)	-	Patil *et al.* (2013)
Agave spp.	Agar (5)	102.7	0.02	0.2	-	25 pH 5.8	-	-	-	Díaz and Garay (2008)
Amaryllidaceae spp.	Liquid	100	0.1	0.3	0.2	25	-	-	0.1	Papenfus *et al.* (2014)
Cajanus cajan (L.) Millsp.	Agar (10)	375	0.25	0.3	0.2	20.5 ± 2	-	150 (4000 grade)	0.1	Jayaprakash and Sarla (2001)
Chrysanthemum spp.	Liquid	120	-	-	-	pH 6	-	160 (4000 grade)	-	Yang and Endo (2005)
Crocus sativus L.	Liquid	100	1	-	-	-	-	-	-	Grilli Caiola *et al.* (2011)
Eucalyptus spp.	Liquid	300	0.00015	-	-	-	-	-	-	Horsley *et al.* (2007)
Ficus carica L.	Agar (10)	50	0.005	-	-	-	25	-	-	Gaaliche *et al.* (2013)
Gossypium spp. L.	Agar (20)	300	0.01	-	0.01	32 50 % RH	0.05	-	0.005	Kakani *et al.* (2005)
Malus domestica Borkh.	Liquid	68.46	0.02	0.3	-	pH 6 - 7	-	-	-	Calzoni *et al.* (1979)
Ziziphus jujube M.	Agar (10)	150	0.1	-	-	-	25	-	-	Rouhakhsh *et al.* (2014)

Table 2.4 (cont.)

Species	Agar/ Liquid	Sucrose	Boric acid	Calcium nitrate	Magnesium sulphate	Temperature (°C), pH and RH	Additional components			Reference/s
							Manganese sulphate	Polyethylene glycol	Potassium nitrate	
Poaceae species										
Panicum virgatum L. and *Festuca arundinacea*	Agar (10)	273.8	0.08	0.21	-	24-36 80-99% RH	-	-	-	Wang *et al.* (2004); Ge *et al.* (2011)
Sorghum bicolor (L.) Moench	Liquid	150	-	2	-	-	2	-	300 (20 000 grade)	Lansac *et al.* (1994)
Zea mays L.	Agar (6)	150	0.1	0.3	-	100% RH	-	-	-	Pfahler and Linskens (1972)
Saccharum spp.	Liquid	300	0.1	0.06	0.1	-	-	-	-	Amaral *et al.* (2013)
Saccharum spp.	Agar (10)	300	-	-	-	22-30	-	-	-	Sartoris (1942)
Saccharum spp.	Agar (10)	300	-	-	-	25 95% RH	-	-	-	Melloni *et al.* (2013)
Saccharum spp.	Liquid	300	0.1	0.3	0.1	-	-	-	-	Krishnamurthi (1980); Singh (2013)

2.5.2 Storage of pollen

Conservation/storage of pollen can be used as an attempt to address asynchronous flowering (Amaral *et al.* 2013), for maintaining germplasm collections and potentially to avoid insect and disease pests when importing foreign germplasm. It has become of great value to breeders and geneticists in eliminating time and space problems encountered in artificial crossing (Khosh-Khui *et al.* 1976; Kalkar and Neha 2012). In sugarcane, some studies have reported successfully storage of pollen (Kopp *et al.* 2002; Amaral *et al.* 2013). However, pollen storage conditions differ based on the type of species.

Pollen storage can be affected by two main factors, *viz.* temperature and moisture content (Towill 1985), although other factors such as atmospheric composition and oxygen pressure are also known to affect pollen viability (Bots and Mariani 2005). The latter factors are rarely manipulated to achieve optimum storage condition, except in the case of freeze- or vacuum-dried pollen (Hanna 1994). Pollen storage has been well documented and is known to be maintained at low temperatures (Towill 1985). Table 2.5 summarises some reports on the short-term storage (up to 39 days) at low temperatures for pollen of the Poaceae family.

For many species within the Poaceae family, temperatures for short-term storage of pollen ranges from 4 °C and -20 °C and pollen viability has been maintained for a few days to a year (Towill 1985). Desiccation prior to storage is an important element to consider. Pollen from the Poaceae is recalcitrant, i.e. sensitive to dehydration, and is generally known for poor storability as a result of this. Nevertheless, pollen from some grasses can be dehydrated to an extent. For example, maize pollen can be dehydrated to such low moisture content that freezable water is removed while still retaining viability (Hoekstra 1995). A moisture content of above 20 % resulted in deleterious formation of ice crystals which pierced the cellular membranes during storage (Towill 1985; Hoekstra 1995). According to Hoekstra (1995), a moisture content of below 20 % within the pollen grains is recommend as only tightly bound water is present. This provides possibilities for cryogenic storage in the range 10 - 20 % moisture content. A useful storage technique is needed to collect mature pollen from the plant and maintain the normal functioning of the pollen grain during the dormant stage. The effectiveness of a storage method should be assessed by testing pollen viability before use (Amaral *et al.* 2013) which can be determined using direct (seed-set analysis) and indirect techniques (Shivanna and Johri 1985; Kalkar and Neha 2012). In addition, more than one technique could be used to prevent under- and over-estimation of these results (Amaral *et al.* 2013).

Table 2.5: Summary of published methods for the short-term pollen storage for some Poaceae species.

Species	Storage conditions		Duration (days)	Reference
	Temperature (°C)	Relative humidity (%)		
Lolium multiflorum Lam.	-20	-	39	Nitzsche (1970)
Pennisetum americanum (L.) Leeke	4	-	7	Pokhriyal and Mangth (1979)
P. typhoides	-18	-	36	Chaudhury and Shivanna (1986)
Saccharum spp.	5-13	85	12	Dutt 1929 as cited by Moore and Nuss (1987)
Saccharum spp.	4	100	14	Moore and Nuss (1987)
Saccharum spp.	-18	100	30	Amaral *et al.* (2013)
Sorghum biocolor L.	4	75	1	Patil and Goud (1980)
Zea mays L	-20	-	12	Kalkar and Neha (2012)
Zea mays L.	4	-	4	Kalkar and Neha (2012)

2.6 SOME APPLICATIONS OF BIOTECHNOLOGY METHODS IN CONVENTIONAL SUGARCANE BREEDING

Sugarcane breeders around the world are making use of biotechnological methods to improve their conventional breeding practices. Biotechnological tools are powerful in obtaining genetic information and in increasing genetic variation among parental genotypes that have been insufficient to improve the key traits of the crop (Pan 2010). Hence, for the purpose of this study, *in vitro* inflorescence production, protoplast isolation and molecular progeny identification using single sequence repeats (SSRs) were of interest.

2.6.1 *In vitro* inflorescence induction in pollen recalcitrant plants

The applications of modern biotechnological methods for the development of transgenic plants and exploitation of somaclonal variation require optimisation of *in vitro* culture techniques (Zhong *et al.* 1998; Ali *et al.* 2015). At SASRI, tissue culture research was initiated in the late 1980s and it has been integral in the development of genetically modified (GM) plants as an alternative method for sugarcane improvement (Snyman *et al.* 2008). Tissue culture has also been used in sugarcane for rapid *in vitro* multiplication of elite sugarcane clones, germplasm storage and improvements through somaclonal variation and mutation breeding (Snyman *et al.* 2008; Birch 2013; Ali *et al.* 2015). *In vitro* culture is used to produce inflorescences for various crops e.g. switchgrass (Alexandrova *et al.* 1996), pearl millet (Devi *et al.* 2000), maize (Kranz and Lorz 1993) and sugarcane (Virupakshi *et al.* 2002). Different explant sources have been utilised to investigate the influence of culture medium, plant growth regulators and photoperiod on inflorescence production *in vitro* (Devi *et al.* 2000; Kiełkowska and Havey 2011; Castello *et al.* 2016).

The production of inflorescence *in vitro* using tissue culture protocols could serve as an important tool in studying inflorescence induction, initiation and the floral developmental process (Castello *et al.* 2016). *In vitro* inflorescence production can reduce the influence of environmental factors and can explain the key influences affecting the flowering process by controlling environmental factors (Zhang *et al.* 2008). The induction of flowering is not only dependent on the environment but also on the interaction between culture medium composition, *viz*. plant growth regulators, auxin-cytokinin equilibrium, nutrients and pH of the medium (Castello *et al.* 2016). Investigations have showed that the application of exogenous hormones to the culture medium stimulated *in vitro* inflorescence production in many plant species. For example, in switchgrass (*Panicum virgatum* L.), nodal segments from tillers were cultured *in vitro* on MS in full at first mention, in vitro on full strength MS (1962) medium supplemented with 6-benzylaminopurine (BAP) and

after ± 5 weeks in culture, inflorescences were produced with fully developed spikelets and perfect terminal florets (2 - 7 mm in length) with 200 - 700 spikelets per inflorescence (Alexandrova *et al.* 1996). Moreover in pearl millet (*Pennisetum glaucum* L.), shoot apices were cultured on MS medium supplemented with 6-benzyladenine (BA) at concentrations of 2 and 4 mg/l and *in vitro* inflorescence production was observed at an 80 % frequency after 22 weeks in culture (Devi *et al.* 2000).

The direct inflorescence production from *in vitro* cultures could also be used for *in vitro* fertilisation and the production of hybrids between genera or species that are difficult to cross normally (Singh *et al.* 2013). In sugarcane, only Virupakshi *et al.* (2002) managed to successfully produce sugarcane inflorescences using juvenile explants via a callus phase. The addition of proline was presumed to inactivate the polyphenol oxidase and caused an increase in the differentiation of morphogenic callus (Glowacka *et al.* 2010).

2.6.2 Protoplast isolation, fusion and culture

Both somatic hybridisation and transformation technologies have provided reliable approaches for combining interspecific and intergeneric traits of sexual incompatible plants (Durieu and Ochatt 2000; Aftab *et al.* 2002; Davey *et al.* 2005). Somatic hybridisation by protoplast fusion in sugarcane became of interest in the 1980s prior to the development of GM technologies. Maretzki and Nickell (1973) were the first to report the isolation of sugarcane protoplasts. Since then a number of reports have appeared on their isolation and fusion (Krishnamurthi 1976; Larkin 1981; Tabaeizadeh *et al.* 1986; Taylor *et al.* 1992; Khan *et al.* 2001). In sugarcane, there have been challenges in producing good-quality and high yields of protoplasts and in regenerating plants from protoplasts (Taylor *et al.* 1992). There are various factors that contribute towards these limitations *viz*. (a) selection of enzymes to facilitate cell wall degradation; (b) genotype; (c) growth regulators; and (d) establishment of protoplast culture conditions.

Protoplast work fell out of vogue due to difficulties in isolating and culturing protoplast when new technologies such as *Agrobacterium* mediated gene transfer and microprojectile bombardment emerged using highly regenerable callus cultures. In recent years, there has been a renewed interest in protoplasts for various crops where significant progress has been made in the regeneration of protoplasts into whole plants which will allow for the establishment of plants without the need for GM technologies as reviewed by Burris *et al*. (2015). Direct transformation of protoplasts was reported for a number of plant species such as ryegrass (Wang *et al.* 1997), rice (Zhang and Wu 1988), maize (Burris *et al.* 2015) and switchgrass (Merrick and Fei 2015). According to Potrykus (1991), direct transfer of genes using protoplast has been found to allow

for high frequency of DNA uptake and the integration of exogenous DNA into the cells. Regenerable cultures have been initiated from immature embryos, seeds, seedlings, shoot meristems, young leaves, and immature inflorescences (Robacker and Corley 1992). Hence, somatic hybridisation using protoplasts could offer an opportunity for sugarcane crop improvement.

Fusion of plant protoplasts is not species-specific and can be carried out routinely between two species, irrespective of taxonomic relationships (Davey *et al.* 2005). Fusion can be induced in two ways, *viz*. chemical or electrical (Ahuja 1982; Davey *et al.* 2005). Chemical fusion involves the use of polyethylene glycol (PEG) whereas the electrical technique uses a short electric pulse of sufficient voltage to cause reversible membrane breakdown, to fuse the protoplasts (Bates *et al.* 1983; Aftab *et al.* 2002). Somatic hybridisation by chemical means has been reported in sugarcane (commercial-type hybrid x commercial-type hybrid) (Evans *et al.* 1980; Khan *et al.* 2001).

With the difficulties in manipulating protoplast cultures for plant species of the Poaceae family, the use of embryogenic cell suspension cultures could potentially yield totipotent protoplasts that can produce into whole plants (Aftab and Iqbal 1999). Somatic hybridisation for producing useful variation in sugarcane holds promise since it is a polypoid and a vegetatively propagated crop (Aftab *et al.* 2002). Therefore, there is scope for further studies to be conducted.

Numerous studies have used anther culture as an attempt to generate haploid plants (Wernicke *et al.* 1979; Narayanaswamy 1994). According to Narayanaswamy (1994), successful anther cultures require microspores to produce callus tissue or embryos where anthers are cultured at a specific development stage and the culture medium requirements are fulfilled. For the Poaceae species, there are very low yields of haploid plants and only occasionally have the techniques been used to bring parental lines to homozygosity (Narayanaswamy 1994).

Somatic hybridisation by protoplast fusion has been used widely in attempts to transfer nuclear or cytoplasmic traits from one species to another (Birch 2013). One essential criterion for applying this is that the isolated protoplasts should be able to regenerate into plants. Chen *et al.* (1988) produced sugarcane plants from protoplasts derived from embryogenic cell suspension cultures but their protocol was not repeatable. Protoplasts of graminaceous species have been difficult to culture, although callus formation has been obtained from protoplasts derived from cell cultures of sugarcane, rice and barley as reviewed by Evans *et al.* (1980).

2.6.3 Molecular markers in breeding

Despite the difficulties associated with breeding due to the genetic complexity of sugarcane, there is new technology that allows for exploiting information at a molecular level which will make breeding more efficient (Butterfield 2005; Molina *et al.* 2013). For example, the use of molecular markers can reveal unknown genetic make-up found within chromosome regions that contribute to the inheritance of important disease and yield traits. In sugarcane there are a few markers that have been discovered to assist breeders, e.g. R12H16 (Le Cunff *et al.* 2008; Molina *et al.* 2013) which is associated with the Bru1 gene that has two alleles where one is dominant and confers resistance to the fungal disease, brown rust (Molina *et al.* 2013). The use of the R12H16 maker has improved the efficiency of the screening process for brown rust resistance in sugarcane breeding (Costet *et al.* 2012; Racedo *et al.* 2013).

Molecular markers have many applications such as determining the hybrid nature of progenies derived from introgression programmes, germplasm evaluation, variety identification and protection, and identifying the pollen donor of a clone derived from polycrosses (Costa *et al.* 2014; Xavier *et al.* 2014). According to Pan (2010), in all these applications, the most commonly used molecular markers are the DNA microsatellite markers or simple sequence repeats (SSRs). SSRs are short DNA fragments that contain various numbers of tandem repeat units of di-, tri-, or tetra-nucleotide motifs (Pan 2010; Tew and Pan 2010; Singh *et al.* 2014). More than 200 SSRs have now been isolated from sugarcane and have been used to progress mapping the sugarcane genome (Aitken *et al.* 2005). At SASRI, microsatellite markers have been used to implement a DNA fingerprinting database as a quick and accurate approach for variety identification (Joshi and Albertse 2013). Through introgression breeding, the insertion of desired traits into the sugarcane genome will allow for improvement in productivity, nutritional quality and development of parental stock which will lead to the development of new resistant varieties against environmental factors (Pan 2010; Singh *et al.* 2014). Hence, the identification of the transfer of genes is vital in tracking the advancement of these programmes.

There are two detection methods for SSRs, *viz*. (a) a manual gel electrophoresis system using polyacrylamide or agarose gels for separation and autoradiography or silver stain for fingerprint images; and (b) an automatic genetic analyser based on a capillary electrophoresis system (Piperidis *et al.* 2001; Pan *et al.* 2006; Sivapragasam *et al.* 2015).

In recent years, microsatellite markers have been used to screen resultant progenies from cross-pollinations. In a typical bi-parental cross, it is expected that the F_1 progeny will inherit half the

set of chromosomes from the pollen donor and the other half from the pollen receptor, so the DNA markers will be contained on chromosomes. Unfortunately, it is not possible to predict the frequency of inheritance in the progeny from the cross-combinations (Pan *et al.* 2006). A progeny is considered to be a hybrid if it inherits the SSR alleles specific to both the pollen donor and pollen receptor (Tew and Pan 2010). If the only alleles detected were from the pollen receptor, it is scored as a progeny from self-pollination. If alleles are not found in either the pollen receptor or pollen donor, they are classified as 'contaminants' (Pan *et al.* 2006).

CHAPTER 3: HISTORICAL SUGARCANE FLOWERING TRENDS AND IMPLICATIONS FOR INTROGRESSION CROSSING

3.1 INTRODUCTION

Breeding programmes form an integral part of most sugarcane industries around the world in order to create increased genetic variation (e.g. improved yield, pest and disease resistance) in sugarcane progenies. Various parental genotypes (such as wild sugarcane species or related genera) are needed when creating bi- and poly-parental crosses to enhance the incorporation of desirable and new genetic material into the breeding gene pool (Moore *et al.* 2014b). However, parental lines frequently have different flowering times (Melloni *et al.* 2015), which limit the crosses that can be made, and an understanding of the variability in time of flowering will provide guidance in attempts to synchronise desired parental genotypes to produce the required crosses.

Early sugarcane breeders in tropical countries relied on hybridisation of genotypes that flowered during the same time-frame in the field. According to Moore *et al.* (2014), variability in flowering within and over the years has a direct effect on genotype improvement programmes. This limitation has resulted in the inability to create desirable crosses among parental genotypes with high breeding value and those of unknown breeding value. Thus, practical and accurate methods are needed to delay early-flowering genotypes and advance late-flowering genotypes. Also, it is crucial to increase pollen fertility which would ultimately allow breeders to extend the crossing range to develop new sugarcane varieties. Out of season flowering activities were first started in 1939 in Florida where flowering was induced by controlled photoperiod treatments (reviewed by Brett 1962). This allowed *Saccharum spontaneum* of the Turkestan genotype to be successfully crossed with commercial-type sugarcane hybrids (Brett 1962).

Sugarcane breeders want to make intergeneric crosses, for example between commercial-type *Saccharum* spp. hybrids and *Erianthus spp.* Such crosses would serve to broaden the genetic base of modern sugarcane cultivars by increasing characteristics such as fibre content and disease resistance (Melloni *et al.* 2015). However, as there are different flowering time ranges between the genera, it is difficult to synchronise them (Nuss 1982; Moore and Nuss 1987; Tai *et al.* 1991; Gao *et al.* 2015). Further, the wild species of sugarcane, such as *S. spontaneum*, are early-flowering genotypes (Tai *et al.* 1991) which also limits the number of interspecific crosses that can be made in an introgression breeding programme.

The ability to delay flowering in early- and intermediate-flowering genotypes is a practical method to use in overcoming asynchronous flowering and has been utilised in many breeding

programmes around the world (James 1969; Julien 1971; Moore and Heinz 1971; Midmore 1980; Moore and Nuss 1987; Rizk *et al.* 2007). There have been numerous strategies to delay flowering by researchers worldwide and five of these strategies have been successful, *viz.* (a) inducing flowering in immature cane by altering planting dates (Moore *et al.* 2014b); (b) using constant day-length at the beginning of the flowering cycle and declining day-lengths later (Horsley and Zhou 2013); (c) starting with declining day-lengths and stopping the process by introducing constant day-lengths (Horsley and Zhou 2013); (d) exposing the cane to cooler ambient temperatures (Moore *et al.* 2014b); and (e) applying nitrogen before flower initiation or 20 days after initiation process (Moore *et al.* 2014b). The most effective approach to delay flowering is the prevention of natural floral induction by a night light break regime for a specific duration followed by the exposure to extended day-length (Midmore 1980; Moore *et al.* 2014b).

In countries such as Brazil, sugarcane flowering occurs naturally and fertile pollen is produced in its northern regions (Melloni *et al.* 2015). However, in South Africa and other sub-tropical countries, flowering is limited, highly variable and the pollen is sterile or fertility is very low (Brett 1962; Berding *et al.* 2004; Horsley and Zhou 2013). Many of the desirable parental clones cannot be used for cross-pollination as a result of these unfavourable conditions. For this reason, managed photoperiod facilities have been established in South Africa (Brett and Harding 1974; Zhou 2013), and other countries such as Australia (Berding 1981; Berding *et al.* 2010) and Louisiana, USA (Bischoff and Gravois 2004) to provide temperature and day-length control. At SASRI, the first photoperiod facility was constructed in 1971 where approximately 800 plants were exposed to five different photoperiod treatments each year (Nuss 1982). At present, the facility is still essential for: (a) increasing pollen fertility; (b) inducing flowering in shy-flowering plants; and (c) synchronising early- and late-flowering genotypes (Brett and Harding 1974; Nuss 1980; Nuss 1982; Horsley and Zhou 2013). As previously described (section 2.4.3 a.), there are six photoperiod treatments that have been used each year and these are pre-arranged to synchronise flowering of all genotypes intended to be used as either a pollen donor or a pollen receptor in the breeding programme.

At SASRI, synchronism of flowering genotypes is targeted between treatments G1+P1 (1st combination), G2+P3 (2nd combination), and G3+P2 (3rd combination), where G represents the glasshouse facility and P the photoperiod house (Horsley and Zhou 2013). These combinations are intended to: (a) synchronise flowering in parental genotypes; (b) provide a good spread in flowering times to extend the flowering season; and (c) give rise to genotypes with high pollen

fertility in the photoperiod house treatments to be used as pollen donors, and genotypes from the glasshouse treatments to be used as pollen receptors during crossing.

The use of photoperiod regimes requires critical analyse of flowering data over several years to identify trends in order to refine the treatments. This information provides the means to plan photoperiod treatments in order to synchronise flowering, taking into account the effect of particular treatments on pollen viability. In addition, sugarcane breeders would have more control over pollen fertility of the genotypes as being selected as either a pollen donor or a pollen receptor in making bi- or poly-parental crosses (Horsley and Zhou 2013). Using data from the plant breeding database for 1995-2014 crossing seasons the objectives of this study were to: (a) evaluate flower synchronisation information of important parental genotypes; and (b) evaluate photoperiod treatments and seasonal effects (i.e. over time) on flowering and pollen viability of selected genotypes.

3.2 MATERIALS AND METHODS

3.2.1 Variables extracted from the SASRI database

The data used for this study were from the SASRI crossing database [stored in Oracle® (California, United States)] for flowering seasons 1995 to 2014, a period of 19 years. The data selected consisted of three variables, *viz*. (a) natural date to flowering; (b) the stage of inflorescence opening; and (c) percentage pollen viability.

3.2.2 Data analyses

The data were extracted from the Oracle® database in Excel (Microsoft Office) spreadsheets and subjected to analysis of variance using the Statistical Analysis System (SAS Version 9.2 2009; Cary, NC, USA). Data were analysed as a completely randomised design and the mean comparisons of the variables were done using Fisher's least significant difference (LSD).

The following linear mixed model (Searle, 1971) was used for this study:

$$Y_{ijk} = P_i + V_j + PV_{ij} + Y_k + PY_{ik} + VY_{jk} + PVY_{ijk} + Error_{ijk}$$

Where,

$Yijk$ = observed response of the i^{th} photoperiod treatments to j^{th} genotypes for k^{th} number of years;
Pi = is the random effect of the i^{th};
Vj = is the random effect of the j^{th};

PV*ij* = is the random interaction effect of the i^{th} photoperiod treatments and j^{th} genotypes;
Y*k* = is the random effect of k^{th} number of years;
PY*ik* = is the random interaction effect of the i^{th} photoperiod treatments and k^{th} number of years;
VY*jk* = is the random interaction effect of the j^{th} genotypes and k^{th} number of years;
PVY*ijk* = is the random interaction effect of the i^{th} photoperiod treatments and the j^{th} genotypes in k^{th} number of years;
Error*ijk* = normally distributed experimental residual error.

3.2.3 Summary of the experimental work conducted in 1995 - 2014 to generate the data in the Oracle® database

The experimental protocols and measurements described below were taken from consecutive years between 1994 and 2014 for the genotypes planted in each crossing season. They were undertaken by the SASRI Plant Breeding team (Brett and Harding 1974; Nuss 1980; Nuss 1982; Brunkhorst *et al*. 2000; Brunkhorst 2001; Brunkhorst 2003; Anonymous 2013; Horsley and Zhou 2013; Zhou 2013) and are described here as the background for the analyses performed in the present study.

3.2.3.1 Study site

The study was done at SASRI located in Mount Edgecombe, Durban, South Africa (29° 42' 24.5585”S, 31° 02' 45.1735”E).

3.2.3.2 Data collection

The data were collected three times a week on a Monday, Wednesday and Friday.

a) Natural date to flowering (NDTF)

In both glasshouse (G) and photoperiod (P) facilities, all the plants were checked for flower/inflorescence emergence on a daily basis. Once flowering was detected, those plants were removed from the photoperiod treatments and placed in a designated area within the glasshouse. Upon removal of each flowering plant, the natural date to flowering (NDTF) was captured as the date, month and year (e.g. 25 June 2000). It was then expressed on a quantitative basis by converting the number of days from the beginning of that particular year (from the 1^{st} of January) to the day when the flower emerged during the inspection days.

b) Stage of inflorescence opening

After identifying the plants that were flowering, the stage of inflorescence opening was recorded. The stage of inflorescence opening refers to the stage at which the inflorescence was sampled prior to pollen viability testing, as shown in Figure 3.1. As the florets opened, the inflorescences were graded based on the amount of pollen shed and assessing how far along the inflorescence the anthers and stigmas protruded from the florets (Anonymous 2013). It should be noted that the inflorescence opens from the top downwards and is divided into five equal portions (Figure 3.1) (Anonymous 2013). The inflorescences were classified into one of these five stages, *viz*. stage 1 (20 % flowering), stage 3 (40 % flowering), stage 5 (60 % flowering), stage 7 (80 % flowering) and stage 9 (100 % flowering). At stage 1, the first set of anthers and stigmas were exposed from the tip of the flower. Stages 3 - 7 reflect more anthers and stigmas opening sequentially. At Stage 9, the flower was fully open and the last set of anthers and stigmas were exposed. It takes about 7 - 14 days for the whole flower to open. Sampling of the anthers was not done at one specific stage of inflorescence opening however, this was dependant on what was available on the inspection days and the number of flowered genotypes. For example, if the genotype was graded as stage 1 and there was no desirable genotype to create a particular cross then it could be left for the next crossing day and possibly more genotypes would have flowered. The data were captured as one of the stages e.g. S1, S3, S5, S7 or S9.

c) Percentage pollen viability

After grading the flowers, samples of a few florets with mature anthers were collected and taken to the crossing laboratory for microscopic examination. A pollen viability test was done using the starch-iodine stain (1 g/l iodine and 2 g/l potassium iodide in water) (Mulugeta *et al.* 1994). Different florets, taken from various parts of the flower, were placed on a clean microscopic slide and one or two drops of the stain was added. After the addition of the stain, the anthers were isolated from the florets by pulling apart using dissecting needles and were lightly squashed by using a sharp scalpel and a dissecting needle to release the pollen grains. The pollen grains in the stain were covered with a coverslip to prevent displacement. After 1 min, the slides were examined under a light microscope (Zeiss Axioskop at 100 X magnification). Percentage pollen viability was calculated using the proportion of stained versus unstained pollen grains. This was used to determine the fertility/sterility of the flowers where fertile pollen grains were seen as black/dark brown and infertile pollen grains as yellow in colour (Mulugeta *et al.* 1994). Five random microscopic fields were selected and counted as total pollen grains per field of view and then percentage viability was determined (Zhou M, SASRI, Personal communication, 2015).

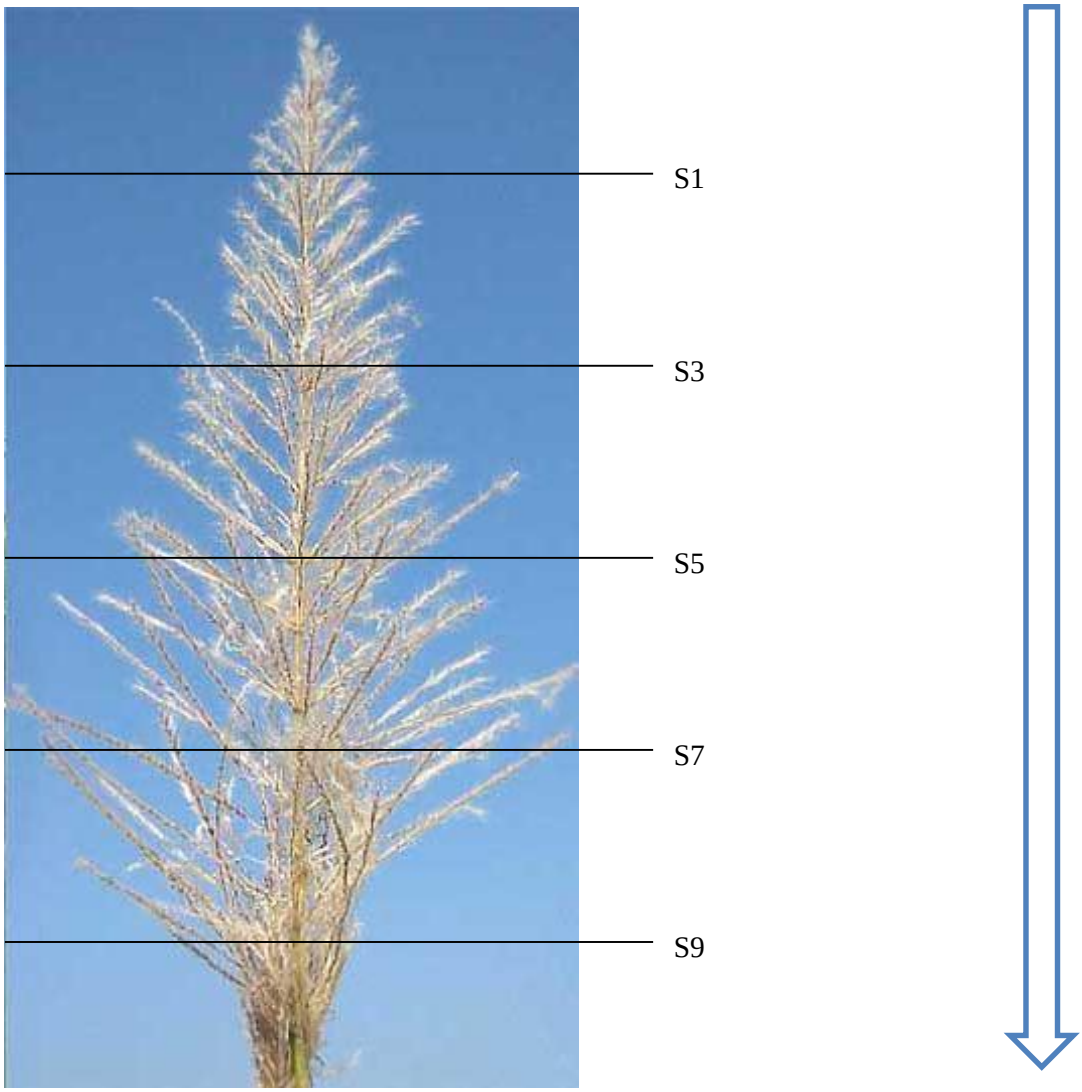


Figure 3.1: Diagram of a sugarcane inflorescence showing five stages (S1, S3, S5, S7 and S9) of inflorescence opening from the top downwards (Source: Yadav 2009).

3.2.3.3 Plant management

The stalks intended for flower initiation were cut into single budded setts which were planted in germination trays (45.5 cm × 23.5 cm) with planting media made up of concrete stones, Umgeni river sand and peat moss (1:3:1 ratio). After planting, the trays were kept in a glasshouse under controlled temperatures of 30 °C (day and night) and were watered daily (approximately 500 ml/tray) until germination occurred. After six weeks, each plantlet was transplanted into river sand in metal canisters (45.8 cm × 13.4 cm) and the canisters were placed on trolleys mounted on railway lines (Figure 3.2). Thereafter they were all kept under natural ambient conditions (± 18 - 35 °C, 30 - 60 % RH) until the month of February when the plants were ready for exposure to photoperiod treatments.

(A)  (B) 

Figure 3.2: Maintenance of plants for inflorescence production. (A) Plants transplanted into canisters after six weeks and mounted on the trolleys and (B) mature plants ready for inflorescence emergence (Anonymous 2013).

3.2.3.4 Fertilizer and irrigation

The setts were fertilized in weeks 1 and 2 with NutriFeed powder (5 ml per canister; 1 g/l [Starke Ayres, South Africa]). Then in week 3 and up to 16 weeks, there was a weekly application of LAN (limestone ammonium nitrate, Coastal Farmers' Co-operative Ltd., KwaZulu-Natal) as 5:1:5 (N:P:K) (3.5 g per canister). For the next three months (January until March), there was a two weekly application of a non-nitrogenous (NN) mixture (1 kg potassium chloride and 4 kg superphosphate; 3.5 g per canister). Thereafter, the weekly applications were alternated between LAN and NN fertilizer throughout the crossing season. An automated drip irrigation system was used to water the plants and it was fitted with a timer that was set to provide water (approximately 500 ml) three times a day (8h00, 12h00 and 15h00) for 3 min time periods.

3.2.3.5 Genotypes used

The genotypes used during crossing were collected from the SASRI germplasm collection where the parental material for the breeding programme is grown within the surrounding fields. Flowering data from 16 genotypes were included in this study (Table 3.1). There were nine commercial-type hybrids (*Saccharum* hybrids), one first generation introgression cross (F_1 hybrid), one *S. spontaneum*, two *S. robustum* and three *E. arundinaceus* lines. The genotypes were chosen based on desired cross combinations between commercial-type hybrids and wild species or F_1 hybrids. The commercial-type hybrids were intended to be used as pollen receptors (♀) while the F_1 hybrid, *S. spontaneum*, *S. robustum* and *E. arundinaceus* were intended to be

used as pollen donors (♂). The genotypes intended to be used as pollen donors were of interest for introgression breeding based on their desirable characteristics.

Table 3.1: List of genotypes used for the study

Genotype	**SASRI code**	**Number of lines per species group**
Commercial-type hybrids	06G0127, 07U1552, 06B0697, CO285, 06B1187, 07U0537, 06S0746, 95L0828, N28	9
†F_1 hybrid	04X0016	1
S. spontaneum	Taiwan11	1
S. robustum	IM76-227, IK76-417	2
E. arundinaceus	IS76-220, IK76-22, IS76-205	3

†First generation introgression cross

3.2.3.6 Glasshouse and photoperiod house

The two photoperiod facilities used for the experiments were the glasshouse and photoperiod house which have controlled environments (Figure 3.3). The glasshouse had a transparent structure with roof vents, doors and windows whereas the photoperiod house was a completely enclosed structure.

The light treatments were set up prior to floral induction to match the desired flowering treatment and were controlled separately. Both fluorescent and incandescent lights were used and the light intensities were fixed at 58 $kg/m^2/s^3$ and 80 $kg/m^2/s^3$, respectively. The temperature was maintained within the range of 18 to 32 °C using pipes containing circulated, heated water. The relative humidity (RH) was controlled by the use of humidifiers (fine sprays) in maintaining humid surroundings of 70 to 100 % RH. Hydrothermographs and climastats were used to monitor both temperature (> 30 °C) and relative humidity (> 70 % RH) to maintain conditions that were optimal for flower development and flowering for each genotype.

(A)  (B) 

Figure 3.3: The SASRI facilities used to initiate the photoperiod treatments based in Mount Edgecombe (KwaZulu-Natal). (A) glasshouse and (B) photoperiod house (Anonymous 2013).

3.2.3.7 Photoperiod treatments

The photoperiod house and glasshouse were each divided into three compartments, each with manually-controlled individual time switches. The photoperiod regimes began during early February before the cold season commenced and the regime continued until the end of August. Six photoperiod regimes were structured, as described previously in (Table 2.2). In the glasshouse, natural day-length was extended by reducing the rate at which it declined naturally whereas in the photoperiod house, day-length was reduced by 30 s per day. In treatments G1 and G2 the lights were switched on daily at 05h45 and 05h30, respectively with constant day-lengths of 12.5 h. In treatment G3, there was a constant day-length of 13 h initially and then the day-length was decreased by 60 s and 90 s per day for 60 and 10 days, respectively. For the P treatments, there were decreasing day-lengths by 30 s per day where P1 started at 12.35 h and P2 and P3 started at 12.5 h. The treatments differed by the amount of 'darkness'/night time that the genotypes received.

3.3 RESULTS

Pollen viability, flowering time (i.e. natural date to flowering, NDTF) and stage of inflorescence opening for each of the 16 genotypes were compared over 19 years for 6 different treatments (3 photoperiod house treatments and 3 glasshouse treatments) in order to gains insights into pollen production, flowering synchronisation and times of flowering, all of which are needed for making decisions about introgression crossing.

The coefficient of determination (R^2) value and the coefficient of variation (CV %) for three different traits, *viz.* pollen viability, natural date to flowering, and stage of inflorescence opening for a 19 year period are summarised in Appendix 1. Pollen viability ($R^2 = 0.708654$) and natural date to flowering ($R^2 = 0.804154$) both showed significantly high R^2 values compared with stage of inflorescence opening ($R^2 = 0.348098$). This indicates that both datasets (pollen viability and natural date to flowering) best fitted the model compared with the data for stage of inflorescence opening. Stage of inflorescence opening showed a higher CV % when compared with pollen viability and natural date to flowering, indicating that there was a high degree of variation among the data.

3.3.1 The effect of photoperiod treatments on pollen viability, time of flowering and stage of inflorescence opening

The treatments within the two facilities, *viz.* glasshouse (G treatments: G1, G2 and G3) and photoperiod house (P treatments: P1, P2, P3, P5) allowed plants to have high pollen viabilities (39.3 - 67.1 %) when compared with the untreated control treatment (30.37 %) [Table 3.2; ($p = 0.0001$, ANOVA, Appendix 1)]. The highly significant ($p = 0.0001$) photoperiod effect for pollen viability indicated that inflorescences derived from the different photoperiod treatments produce different amounts of pollen. This is important because higher amounts of pollen are required in a plant selected as a pollen donor than in one selected as the pollen receptor. Significantly higher pollen viabilities were recorded in G1, P1, P2 and P3 when compared with G2, G3 and the untreated control. However, at SASRI any plant with a pollen viability of > 30 % can be used for crossing and is classified as a pollen donor (Zhou 2013).

The natural date to flowering (NDTF) had values ranging from 157 to 196 days [Table 3.2; ($p = 0.0001$, ANOVA, Appendix 1)]. The highly significant ($p = 0.0001$) photoperiod effect on natural date to flowering indicates that different photoperiod treatments induce genotypes to produce inflorescences at different times, which is a pre-requisite for a successful breeding programme. Photoperiod treatments are designed to spread the period of flowering from May to August so that sufficient space is available for crossing in the glasshouse facilities. From a logistic and planning point of view, it is important to note that there are 160 cubicles in the glasshouse, sufficient to hold 160 cross combination at a time for a period of pollination before transfer to ripening area. The P treatments resulted in late-flowering times ranging from 178 to 196 days compared with those of the G treatments with flowering times ranging from 158 to 183 days. Collectively, the plants in all of the P treatments (i.e. P1, P2 and P3) flowered later with an average time to flowering of 184 days when compared with the G treatments which had an average time

to flowering of 171 days. For the P treatments, plants in P5 had longer natural dates to flowering compared with P1, P3 and P2 (196, 179, 183, and 189 days, respectively). The order of flowering dates in the chambers was P5, P2, P3 and then P1. In the glasshouse treatments, NDTF was higher in G3 compared with G1 and G2 (183, 158 and 173 days, respectively). The order of flowering dates in the chambers was G3, G2 and then G1. The time to flowering was significantly higher in P3 ($P > 0.05$) compared with P1 and that in G3, and it was significantly lower than P2 and P5 ($p < 0.05$).

Inflorescence stage (i.e. proportion of the inflorescence opening) was not significantly different among the different photoperiod treatments ($p = 0.6237$, ANOVA, Appendix 1). For all the treatments, excluding treatment P5, the same average inflorescence opening stage of 3 was recorded (Table 3.2). This means that sampling of the pollen to determine viability can be done at any stage of inflorescence opening.

The photoperiod treatments have been designed to achieve flower synchronisation among the plants. This was done by pairing the treatments to make up three combinations (i.e. G1+P1 for early-flowering, G2+P3 for intermediate-flowering and G3+P2 for late-flowering). For the first combination between treatments G1+P1, there were no differences observed in pollen viability percentage ranging from 51.23 to 53.74 % (Table 3.2, $P > 0.05$). This means that plants from both treatments could be used as either a pollen donor or a pollen receptor during crossing. However, within this combination, there was a flowering range of 158 to 179 days where genotypes in the G1 treatment flowered 21 days earlier than the P1 genotypes. This indicates that the plants subjected to the G1 and P1 treatments had a wide time range (21 days) apart, and flower synchronisation in the early period of crossing was not achieved.

For the second combination of treatments G2+P3, the P3 treatment resulted in significantly higher pollen viability ($p < 0.05$) compared with the G2 treatment (59.5 % vs 42.4 %; Table 3.2). This indicates that plants in the G2 treatment could potentially be used as pollen receptors, while plants in the P3 treatment could potentially be used as pollen donors. There was a flowering time range of 173 to 183 days where genotypes in the G2 treatment flowered 10 days earlier than the P3 genotypes. This means that the plants from the G2 and P3 treatments flowered within the acceptable flowering time range (≤ 10 days), therefore synchronisation was achieved.

For the third combination of treatments, G3+P2, the P2 treatment resulted in significantly higher ($p < 0.05$) pollen viability compared with the G3 treatment (64.7 vs 39.3 %, respectively). There

was a flowering time range of 183 to 189 days. Genotypes in the G3 treatment flowered 6 days earlier than the P3 genotypes. This means that the plants from the G3 and P2 treatments also flowered within the acceptable flowering time range (≤ 10 days) thereby achieving the necessary synchronisation for crossing purposes.

3.3.2 The effect of genotype on pollen viability, flowering time and inflorescence opening stage

The genotypes were grouped according to species type, as shown in Table 3.1. The percentage pollen viability values ranged from 21.73 to 92.56 % [Table 3.3; ($p < 0.0001$, ANOVA, Appendix 1)]. Genotype N28 showed the lowest percentage pollen viability while 06G0127 showed the highest (21.73 and 92.56 %, respectively). On average, the commercial-type hybrids (CH) were found to have higher pollen viability percentages compared with *Erianthus* spp. (ES), *S. spontaneum* (SS), *S. robustum* spp. (SR) and the first generation introgression hybrid (F_1). The order of percentage pollen viability was commercial-type hybrids < *Erianthus* spp. < *S. robustum* < F_1 hybrid < *S. spontaneum* for the different species. Nevertheless, the *Erianthus* spp., *S. robustum* spp., *S. spontaneum* and F_1 hybrid still showed pollen viabilities greater than 30 % and according to the SASRI classification, the genotypes could be used as pollen donors during crossing.

Table 3.2: A comparison of pollen viability, natural date to flowering and stage of inflorescence opening at different photoperiod treatments for sixteen genotypes over 19 years. a-e indicates significant differences [ANOVA and t-test (Fisher's LSD); mean ± SD; n = 429].

Photoperiod treatment[1]	**Pollen viability (%)**	**Natural date to flowering (days)**	**Average stage of inflorescence opening (S1-S9)**
†P5	67.11±8.39^{a}	196.00±25.11^{a}	5.22±2.54^{a}
P2	64.75±23.10^{a}	188.74±11.60^{b}	2.61±1.84^{bc}
P3	59.50±21.01^{ab}	183.28±11.10^{c}	2.43±1.43^{c}
P1	52.74±19.81^{b}	178.84±22.73^{c}	3.41±2.21^{b}
G1	51.23±27.23^{b}	157.63±9.98^{f}	2.77±2.10^{bc}
G2	42.39±27.68^{c}	173.00±16.42^{d}	3.20±2.33^{bc}
G3	39.34±32.94^{c}	182.76±14.90^{c}	2.76±1.77^{bc}
Control	30.37±32.72^{d}	162.48±21.43^{e}	2.73±1.82^{bc}
P Total	59.00	183.62	2.82

Table 3.2 (cont.)

G Total	44.32	171.13	2.91
Total means	48.91	176.38	2.94
LSD $_{(0.05)}$	8.3424	4.4699	0.8631

†Treatment (P5) was used for experimental purposes however, was later discontinued as similar results were obtained as the P3 treatment (Zhou M, SASRI, Personal communication, 2015).

[1]G1 = commencing day-length 12.30, constant dawn at 05h45

G2 = commencing day-length 12.30, constant dawn at 05h30

G3 = commencing day-length 13.00, constant day-length for 60 days, 60 s decline for 10 days then 90 s decline until inflorescence emergence

P1 = commencing day-length 12.35, declination 30 s per day

P2 = commencing day-length 12.30, declination 30 s per day

P3 = commencing day-length 12.30, declination 30 s per day

The natural date to flowering had values ranging from 162 to 202 days [Table 3.3; ANOVA, ($p < 0.0001$, Appendix 1)]. Genotype 07U0537 had the shortest number of days to flowering while 06G0127 had the longest (162.60 and 202.28 days, respectively). The genotypes differed significantly in flowering times and flowering was classified into three periods: early-flowering was between 120 to 151 days, intermediate-flowering was between 152 to 181 days and late-flowering was between 182 and 213 days. This categorisation was based on which month each flowering time occurred e.g. May, June or July. Within these, there were 10 intermediate-flowering and 6 late-flowering genotypes. The intermediate-flowering genotypes consisted of some commercial-type hybrids, the F_1 hybrid, the *S. spontaneum* and an *Erianthus* spp. (IS76-220) with time of flowering ranging from 162 to 181 days. The *S. robustum* and some *Erianthus* spp. (IK76-22 and IS76-205) were found to be late-flowering genotypes with time of flowering ranging from 181 to 199 days. This indicates that less cross-combinations could be done using *S. robustum* and *Erianthus* spp. genotypes.

The proportion of inflorescence opening (i.e. inflorescence stage of opening) had values ranging from stage 2 to stage 6 indicating the stage at which the inflorescence was sampled prior pollen viability testing [Table 3.3; ($p < 0.0001$, ANOVA, Appendix 1)]. The inflorescence stage was not dependent on the species as differences were observed across the various species groups. Based on pollen viability percentages and flowering times, there were desirable introgression cross-combinations that could be made. For example, CO285 (♀) with pollen viability of 71.35 % and flowering time of 174 days could be matched with IS76-220 (♂) having 58.33 % pollen viability and flowering time of 181 days. Genotype 95L0828 (♀) with pollen viability of 43.08 % and

flowering time of 171 days could be matched with Taiwan11 (♂) having 42.11 % pollen viability and flowering time of 173 days.

Table 3.3: A comparison of pollen viability, flowering time and stage of inflorescence opening for different genotypes over 19 years. a-i indicates significant differences [ANOVA and t-test (Fisher's LSD); mean ± SD; n = 429].

Genotype	Pollen viability (%)	Natural date to flowering (days)	Average stage of inflorescence opening (S1-S9)
06G0127	92.56±8.07^{a}	202.28±13.38^{a}	2.78±1.52^{e}
07U1552	88.33±7.64^{a}	192.67±16.17^{bc}	3.00±2.00^{cde}
06B0697	79.00±20.25^{ab}	162.60±9.90^{i}	2.00±1.41^{e}
CO285	71.35±14.21^{bc}	173.98±12.12^{efg}	3.19±1.94^{bcde}
06B1187	67.76±28.62^{bcd}	172.03±16.09^{fgh}	2.79±2.09^{de}
07U0537	60.67±24.95^{cde}	162.33±8.24^{i}	6.33±2.07^{a}
‡‡ IS76-220	58.33±25.17^{cdef}	180.67±6.43^{de}	4.33±1.15^{bcd}
‡‡‡‡ IM76-227	55.46±9.23^{def}	198.64±11.40^{ab}	3.00±2.37^{cde}
‡‡ IK76-22	54.71±17.51^{defg}	190.71±16.86^{bc}	2.41±1.97^{e}
06S0746	46.40±24.22^{efgh}	165.60±12.33^{hi}	4.50±2.67^{bc}
‡ 04X0016	45.24±16.40^{fgh}	178.30±15.33^{ef}	2.67±1.47^{e}
‡‡‡‡ IK76-417	43.17±21.28^{gh}	193.48±13.67^{bc}	2.39±1.85^{e}
95L0828	43.08±22.68^{gh}	170.77±16.69^{fgh}	3.00±2.06^{cde}
‡‡‡ TAIWAN11	42.11±32.25^{gh}	173.27±12.58^{efgh}	1.81±1.91^{e}
‡‡ IS76-205	37.50±34.01^{h}	187.08±16.44^{cd}	4.69±2.81^{b}
N28	21.73±28.53^{i}	169.35±27.36^{ghi}	3.09±2.04^{cde}
LSD $_{(0.05)}$	8.3424	4.4699	0.8631

‡ F_1 hybrid cross; ‡‡ *Erianthus spp.*; ‡‡‡ *S. spontaneum*; ‡‡‡‡ *S. robustum*

3.3.3 The interaction between photoperiod treatments and genotypes with regards to pollen viability, flowering time and stage of inflorescence opening

There were no significant differences in pollen viability [Table 3.4; (p = 0.0661, ANOVA, Appendix 1)] amongst genotypes regardless of photoperiod treatments. Collectively in treatments G1, P3 and P5, genotypes had pollen viability percentages > 30 %, indicating that all the genotypes could be used as pollen donors during crossing according to the SASRI classification. Among the species desired to be used as pollen donors i.e. F_1 hybrid, *S. spontaneum*, *Erianthus*,

and *S. robustum*, it was evident that some treatments resulted in genotypes with high pollen viability while the others gave low pollen viability. For example, in treatment P2, 04X0016 (F_1 hybrid) showed a higher pollen viability percentage compared with treatment G3 (53.12 % vs 26.07 %, respectively). In treatment G1, Taiwan11 (*S. spontaneum*) showed a higher pollen viability percentage (62.5 %) compared with treatment P2 (25 %). In treatment P1, IK76-417 (*S. robustum*) showed a higher pollen viability percentage (54.92 %) than treatment G3 (21.75 %). This indicated that genotypes in treatments that gave < 30 % pollen viability should be excluded for the next crossing season thereby opening space for other possible parental genotypes to be planted.

The natural date to flowering for treatments G and P ranged from 149 - 209 and 154 - 216 days, respectively [Table 3.4, ($p = 0.0353$, ANOVA, Appendix 1)]. Flowering has been categorised into three time periods, *viz.* early-flowering (from 120 to 151 days), intermediate-flowering (from 152 to 181 days) and late-flowering (from 182 to 212 days). The majority of the *Erianthus* (♂) and *S. robustum* (♂) plants were late-flowering genotypes which flowered during the month of July (182 to 212 days). On the other hand, the commercial-type hybrids (♀) mostly flowered during the month of June (152 to 181 days) which were intermediate-flowering genotypes. This indicated that less cross-combinations could be made during the late-flowering period over the years thereby minimising the chances of creating interspecific and intergeneric hybrids.

Nevertheless, within all the treatments, there were possible cross combinations among the *Saccharum* spp. (*S. spontaneum* and *S. robustum*), related genera (*Erianthus* spp.) and the F_1 hybrid (04X0016). For example, the wild spp. (*S. robustum, S. spontaneum* and *Erianthus* spp.) and F_1 hybrid could be used as pollen donors and the commercial-type hybrids as the pollen receptors. This was based on the observation that plants which had similar flowering dates with a limit of $\leq$ 10 days apart can be cross-pollinated. These possible cross-combinations are highlighted in Appendix 2. From the possible combinations, there were more possible chances to create crosses during the month of June (152 to 181 days) compared with any of the other months. For example, Taiwan11 (♂) in treatment G1 could be matched with any one of the following commercial-type hybrids (♀) such as 06B0697 (G2), 06S0746 (G2) and 95L0828 (P1) (Appendix 2). The total number of cross-combination that could be made for each genotypes were: (1) *S. spontaneum* - 25, (2) the F_1 hybrid - 21, (3) the *Erianthus* spp. - 20 and (4) the *S. robustum* spp. - 11 (Appendix 2).

Table 3.4: A comparison of pollen viability, flowering time and stage of inflorescence opening between the photoperiod treatments and genotypes. Mean values are presented [ANOVA, and t-test (Fisher's LSD), means values ± SD, n = 429].

Photoperiod treatment[1]	Genotype	Pollen viability (%)	Natural date to flowering (days)	Average stage of inflorescence opening (S1-S9)
Control	CO285	68.27±14.87	170.47±8.33	2.47±1.19
Control	N28	17.73±26.69	159.82±23.76	2.82±1.99
G1	‡ 04X0016	38.17±18.50	148.90±5.84	2.00±1.41
G1	06B0697	73.33±33.29	162.00±8.19	2.33±2.31
G1	06B1187	47.44±33.97	157.00±8.22	2.56±1.67
G1	07U0537	46.00±12.68	159.00±8.29	6.00±2.58
G1	CO285	59.67±12.91	160.17±5.88	2.33±1.63
G1	‡‡ IK76-22	41.00±24.60	171.60±2.51	2.60±0.89
G1	‡‡‡ TAIWAN11	62.50±36.98	155.83±13.64	3.00±3.10
G2	‡ 04X0016	36.10±16.73	160.75±3.02	3.17±1.59
G2	06B0697	71.25±10.31	154.00±2.00	2.00±1.15
G2	06B1187	55.00±31.89	158.50±1.73	4.00±2.58
G2	06G0127	95.00±10.00	185.50±14.46	2.50±1.00
G2	06S0746	37.80±26.28	155.40±2.76	4.80±3.05
G2	95L0828	29.52±25.94	171.04±14.67	2.57±2.20
G2	CO285	63.17±11.75	174.00±21.38	4.33±3.01
G2	‡‡‡‡ IK76-417	32.00±14.35	186.50±7.84	2.00±1.67
G2	‡‡‡‡ IM76-227	52.14±6.89	195.43±10.63	1.86±2.27
G2	‡‡ IS76-205	37.50±34.01	187.08±16.44	4.69±2.81
G2	‡‡ IS76-220	58.33±25.17	180.67±6.43	4.33±1.15
G2	N28	14.40±13.72	176.00±1.41	*
G2	‡‡‡ TAIWAN11	45.00±30.62	172.67±11.68	*
G3	‡ 04X0016	26.07±18.83	182.67±7.30	3.20±1.42
G3	06B1187	86.25±11.09	166.75±3.40	5.00±2.83
G3	06G0127	97.00±2.83	208.50±3.54	3.00±2.83
G3	07U1552	88.33±7.64	192.67±16.17	3.00±2.00
G3	95L0828	30.25±19.69	185.11±12.04	2.56±1.46
G3	‡‡‡ IK76-417	21.75±23.92	186.50±5.32	1.50±1.00
G3	N28	19.17±29.73	170.50±29.66	2.33±2.07

Table 3.4 (cont.)

G3	‡‡‡ TAIWAN11	*	183.00±3.61	1.00
P1	06S0746	55.00±19.58	175.80±9.07	4.20±2.35
P1	07U0537	85.00	169.00	7.00
P1	95L0828	54.87±11.09	154.48±9.51	3.52±2.11
P1	‡‡ IK76-22	60.42±10.25	198.67±13.26	2.33±2.31
P1	‡‡‡‡ IK76-417	54.92±15.38	198.85±15.43	2.85±2.08
P1	‡‡‡‡ IM76-227	61.25±10.90	204.25±11.90	5.00
P1	N28	9.67±23.68	179.00±13.05	3.33±2.34
P2	‡ 04X0016	53.13±8.18	191.54±5.12	2.50±1.59
P2	06B1187	81.08±14.88	189.58±2.91	1.83±1.34
P2	06G0127	89.86±8.47	209.86±7.86	3.29±1.80
P2	95L0828	53.25±16.50	191.00±13.64	3.00
P2	CO285	81.69±7.26	182.44±4.23	3.75±1.91
P2	N28	65.00	223.00	3.00
P2	‡‡‡ TAIWAN11	25.00±21.55	175.85.25	1.62±2.22
P3	‡ 04X0016	52.59±9.12	182.09±9.11	2.56±1.32
P3	06B0697	95.00±5.00	174.67±1.15	1.67±1.16
P3	06G0127	92.60±7.99	202.60±10.31	2.20±1.10
P3	07U0537	95.00	169.00	7.00
P3	95L0828	42.00±25.15	176.60±1.34	2.60±1.67
P3	N28	40.00	211.00	3.00
P3	‡‡‡ TAIWAN11	*	181.17±3.13	1.33±0.82
P5	95L0828	63.25±9.88	170.50±6.40	5.50±3.00
P5	N28	70.20±6.38	216.40±7.77	5.00±2.45

*Missing values

‡ F_1 hybrid cross; ‡‡ *Erianthus*; ‡‡‡ *S. spontaneum*; ‡‡‡‡ *S. robustum*

[1]G1 = commencing day-length 12.30, constant dawn at 05h45

G2 = commencing day-length 12.30, constant dawn at 05h30

G3 = commencing day-length 13.00, constant day-length for 60 days, 60 s decline for 10 days then 90 decline until inflorescence emergence

P1 = commencing day-length 12.35, declination 30 s per day

P2 = commencing day-length 12.30, declination 30 s per day

P3 = commencing day-length 12.30, declination 30 s per day

3.3.4 Genotype effect over time on pollen viability, time of flowering and stage of inflorescence opening

The interaction between genotypes vs the number of years (flowering seasons) showed no significant differences in pollen viability ($p = 0.9620$, ANOVA, Appendix 1) and the stage of inflorescence opening ($p = 0.3224$, ANOVA, Appendix 1) (Table 3.5). The percentage pollen viability among the commercial-type hybrids, *Erianthus* spp., *S. robustum* spp., F_1 hybrid and *S. spontaneum* ranged from 13 to 93 %, 5 to 58 %, 11 to 59 %, 34 to 58 % and 33 to 78 %, respectively. The stage of inflorescence opening gave a similar range from 1 to 6 across the different species.

Natural dates to flowering were 154 to 209 days ($p = 0.0001$, ANOVA, Appendix 1). The genotypes flowered from early June (152 to 181 days) to late July (182 to 213 days). *S. spontaneum* and the F_1 hybrid mostly flowered in June whereas the *S. robustum* spp. and *Erianthus* spp. mostly flowered in July. The flowering times of the commercial-type hybrids were distributed between the two months (June and July) but mostly concentrated within the month of June of which is the peak period of the crossing season.

When comparing the genotypes across the years for all the treatments, there was year to year inconsistency where the same cross-combination could not be created in the sequential years. Nevertheless, there were still possible cross-combinations that could be matched each year. For example, a cross combination between an *Erianthus* spp. and a commercial type hybrid: in 2013, IS76-22 (♂) with 40 % pollen viability and a flowering time of 172 days could be crossed with 07U0537 (♀) having 90 % pollen viability and a flowering time of 169 days. However, in the following year 2014, IS76-22 had a flowering time of 195 days while 07U0537 had a flowering time of 159 days and could not be crossed. The closer the flowering times of genotypes ($\leq$ 10 days apart) to be used as either a pollen donor or a pollen receptor allows for an increase in chances to facilitate successful cross-fertilisation and thereby increasing seed-set.

Table 3.5: A comparison of pollen viability, flowering time and inflorescence opening stage between genotypes and the number of years. Mean values are presented [ANOVA, and t-test (Fisher's LSD), means values ± SD, n = 429].

Genotype	Year	Pollen viability (%)	Natural date to flowering (days)	Average stage of inflorescence opening (S1-S9)
04X0016¤	2007	53.08±5.22	183.73±10	2.20±1.26
04X0016	2008	57.65±7.10	181.06±7.76	2.67±1.03
04X0016	2009	44.44±10.32	177.77±17.43	2.94±1.59
04X0016	2010	33.76±24.74	175.05±20.47	2.50±1.82
04X0016	2014	34.56±14.38	172.78±11.44	2.89±1.27
06B0697†	2014	79.00±20.25	162.60±9.90	2.00±1.41
06B1187†	2014	67.76±28.63	172.03±16.09	2.79±2.09
06G0127†	2014	92.56±8.07	202.28±13.38	2.78±1.52
06S0746†	2014	46.40±24.22	165.60±12.33	4.50±2.67
07U0537†	2013	90.00±7.07	169.00	7.00
07U0537	2014	46.00±12.68	159.00±8.29	6.00±2.58
07U1552†	2014	88.33±7.64	192.67±16.17	3.00±2.00
95L0828†	2000	62.89±11.34	178.33±9.90	5.67±1.41
95L0828	2001	67.13±7.90	154.38±17.79	5.25±2.25
95L0828	2006	41.00±26.65	170.71±15.21	2.71±1.38
95L0828	2007	44.00±21.04	168.63±9.27	2.25±1.49
95L0828	2008	32.52±19.93	170.06±16.47	2.39±1.78
95L0828	2009	45.00±5.00	169.38±11.61	2.50±1.41
95L0828	2010	26.67±23.63	194.67±28.29	2.33±1.15
95L0828	2013	*	175.00	1.00
95L0828	2014	48.50±2.12	191.50±14.85	1.00
CO285†	1996	78.82±11.45	177.82±10.97	4.09±1.64
CO285	2002	70.00±14.63	170.63±9.38	2.50±1.35
CO285	2003	60.33±8.50	164.33±5.86	2.33±2.31
CO285	2014	68.00±16.05	187.40±17.80	5.00±3.16
IK76-22♯	1996	55.71±4.15	188.29±3.73	2.43±2.23
IK76-22	2013	40.00	172.00	1.00
IK76-22	2014	55.56±23.91	194.67±22.16	2.56±1.94
IK76-417♯	1996	50.67±9.17	189.11±5.78	2.33±1.73

Table 3.5 (cont.)

IK76-417	2003	11.00±15.56	182.00±1.41	1.00
IK76-417	2014	42.92±24.26	198.67±16.75	2.67±2.06
IM76-227§	1996	52.50±3.67	200.33±5.39	3.33±2.66
IM76-227	2014	59.00±12.94	196.60±16.71	2.60±2.19
IS76-205§	2011	5.00	196.00±19.80	5.00
IS76-205	2012	25.00±12.25	172.67±5.01	6.33±3.011
IS76-205	2014	54.00±41.59	200.80±9.76	2.60±1.67
IS76-220≠	2014	58.33±25.17	180.67±6.43	4.33±1.15
N28†	1995	13.09±26.71	151.67±21.32	2.39±2.03
N28	1997	51.13±32.20	202.75±20.08	4.25±2.12
N28	1998	*	165.78±16.761	3.22±2.33
N28	2003	31.44±21.93	183.63±15.98	4.13±1.26
N28	2014	51.67±10.41	209.33±4.73	1.67±1.15
TAIWAN11¥	2011	*	182.00±3.36	1.11±0.47
TAIWAN11	2012	32.67±27.18	165.80±13.25	2.20±2.48
TAIWAN11	2014	77.50±25.98	162.00±10.49	3.50±2.52

*Missing values; ¤F_1 hybrid; †commercial hybrid; §*S. robustum*; ≠Erianthus; ¥*S. spontaneum*

3.3.5 Photoperiod treatment effect over time on pollen viability, time of flowering and stage of inflorescence opening

Pollen viability for treatments G and P ranged from 5 to 62.95 % and 25 to 87.56 %, respectively [Table 3.6; (p = 0.001, Appendix 1)]. On average, the P treatments showed a higher pollen viability over the years compared with the G treatments. This indicates that the P treatments enhanced the production of fertile pollen among the genotypes.

The distribution of flowering times of the G and P treatments ranged from 141 to 172 days and 138 to 216 days, respectively for natural date to flowering (p = 0.0001, ANOVA, Appendix 1). Treatment P3 in 2010 had the longest time to flowering of 207 days and treatment G1 showed the shortest time of 141 days across all treatments and the years. The flowering times were grouped as early (130 to 160 days), intermediate (161 to 180 days) and late (181 to 210 days) and were plotted as in Figure 3.4, which assisted in visualising trends in synchronous flowering between the planned combinations: G1+P1, G2+P3 and G3+P2. For each of the three combinations, similar trends were observed in that inflorescences from the genotypes were found to emerge progressively over time showing a good spread through the crossing seasons for each year. For the G1+P1 combination, there was early-flowering, the G2+P3 combination allowed for

intermediate-flowering and the G3+P2 combination facilitated late-flowering. Crossing combinations were planned by SASRI breeders based on the flowering times thereby matching genotypes allocated in the G treatments with those genotypes allocated in the P treatments. The G treatments (G1, G2 and G3) showed earlier flowering times compared with that of the P treatments (P1, P2 and P3) and this was evident across the years (Figure 3.4).

There was year- to -year inconsistency observed in flowering times amongst the different photoperiod treatments used (Figure 3.4). Over the years between the executed combinations, some of the flowering times were close together while in other years synchronised flowering was not achieved. A narrow time range for flowering increases the chances of creating more possible cross-combinations within that particular period of that crossing season. Conversely, a wide flowering time range limits/prevents cross-combinations that can be made. For example, for the first combination (G1+P1) in 2013 there were flowering times between these two treatments of 172 to 169 days. Genotypes in the G1 treatment flowered 3 days later than the P1 treatment. In 2009 there were flowering times of 141 to 158 days where genotypes in the G1 treatment flowered 17 days earlier than the P1 genotypes. For the second combination (G2+P3), in year 2008 there were flowering times between these two treatments of 169 to 176 days where genotypes in the G2 treatment flowered 10 days earlier than the P3 genotypes. In 2009, there were flowering times of 159 to 193 days where genotypes in the G2 treatment flowered 34 days earlier than the P3 genotypes. For the third combination (G3+P3), in 2014 there were flowering times between the two treatments of 188 to 194 days where genotypes in the G3 treatment flowered 6 days earlier than the P3 genotypes. In 2010, there were flowering times of 187 to 199 days where genotypes in the G3 treatment flowered 12 days earlier than the P2 genotypes.

Table 3.6: A comparison of pollen viability, flowering time and inflorescence opening stage between photoperiod treatments and the number of years. Mean values are presented [ANOVA, and t-test (Fisher's LSD), mean values ± SD, n = 429].

Photoperiod treatment[1]	**Year**	**Pollen viability (%)**	**Natural date to flowering (days)**	**Average stage of inflorescence opening (S1-S9)**
Control	1995	13.09±26.71	151.67±21.32	2.39±2.03
Control	2002	68.27±14.87	170.47±8.33	2.47±1.19
Control	2003	30.50±23.06	182.25±13.73	4.00±1.35
G1	2002	59.00±18.52	156.00	2.33±1.15
G1	2003	60.33±8.50	164.33±5.86	2.33±2.31

Table 3.6 (cont.)

G1	2009	15.00±7.07	140.67±1.15	1.67±1.15
G1	2010	49.75±4.11	152.431±1.51	2.14±1.57
G1	2012	47.50±36.63	154.00±16.83	2.50±3.00
G1	2013	40.00	172.00	1.00
G1	2014	53.68±30.73	160.91±8.60	3.36±2.28
G2	1996	53.25±10.26	187.25±21.86	3.00±2.62
G2	1997	*	176.00	3.00
G2	2000	66.67±8.57	182.50±9.65	5.67±1.03
G2	2003	24.00±5.57	176.00±2.00	5.00
G2	2006	6.00±8.49	167.50±13.44	1.00
G2	2007	10.00	160.25±3.50	1.50±1.00
G2	2008	16.30±8.15	168.58±13.10	1.67±1.78
G2	2009	50.00±50	158.71±2.36	3.00±1.15
G2	2010	12.50±15.55	173.75±24.85	3.00±2.83
G2	2011	5.00	187.17±11.74	2.33±2.07
G2	2012	28.57±19.52	170.00±5.98	5.00±3.16
G2	2014	52.90±27.58	172.33±17.86	3.25±2.28
G3	1998	*	151.50±2.89	3.00±2.31
G3	2003	11.00±15.56	182.00±1.4	1.00
G3	2008	21.00±19.49	185.92±13.25	3.00±1.48
G3	2009	42.00±5.70	177.89±4.46	3.00±1.41
G3	2010	13.33±15.38	186.67±8.00	3.00±1.79
G3	2011	*	183.00±3.61	1.00
G3	2014	62.95±30	188.47±15.95	2.84±2.12
P1	1996	54.06±5.10	188.65±4.01	2.76±1.99
P1	1997	58.00	188.00	3.00
P1	1998	*	177.20±13.74	3.40±2.61
P1	2000	55.33±14.22	170.00±1.73	5.67±2.31
P1	2001	71.00±2.83	138.25±2.06	5.00±1.63
P1	2006	53.50±4.95	153.50±3.54	4.00±1.41
P1	2008	50.83±9.00	155.67±3.60	2.50±1.93
P1	2009	47.50±3.54	157.50±2.12	3.00
P1	2013	85.00	169.00	7.00
P1	2014	59.77±18.74	197.14±21.04	3.64±2.42
P2	1996	85.38±2.07	184.13±2.10	4.00±1.51

Table 3.6 (cont.)

P2	2002	79.83±5.49	178.33±3.20	2.67±1.97
P2	2003	65.00	223.00	3.00
P2	2006	56.00±19.05	184.33±3.51	3.00
P2	2007	55.00±4.08	198.50±1.91	2.00±1.15
P2	2008	52.50±6.89	190.67±3.50	2.67±0.82
P2	2009	49.50±4.97	187.40±2.22	2.80±2.20
P2	2010	58.00±14.40	199.20±7.09	2.20±1.10
P2	2011	*	181.80±3.35	1.00
P2	2012	25.00±21.55	172.13±9.95	2.00±2.83
P2	2014	83.19±13.73	196.19±11.02	2.71±1.93
P3	2007	52.31±6.96	178.00±3.85	2.47±1.41
P3	2008	60.45±5.68	176.25±3.47	2.67±1.15
P3	2009	44.38±5.63	192.88±4.26	3.00±1.51
P3	2010	35.00	207.00	1.00
P3	2011	*	181.17±3.13	1.33±0.82
P3	2013	47.50±67.18	172.00±4.24	4.00±4.24
P3	2014	87.56±18.91	194.22±16.61	2.11±1.054
P5	1997	70.20±6.38	216.40±7.77	5.00±2.45
P5	2001	63.25±9.88	170.50±6.40	5.50±3.00

*Missing values

[1]G1 = commencing day-length 12.30, constant dawn at 05h45

G2 = commencing day-length 12.30, constant dawn at 05h30

G3 = commencing day-length 13.00, constant day-length for 60 days, 60 s decline for 10 days then 90 s decline until inflorescence emergence

P1 = commencing day-length 12.35, declination 30 s per day

P2 = commencing day-length 12.30, declination 30 s per day

P3 = commencing day-length 12.30, declination 30 s per day

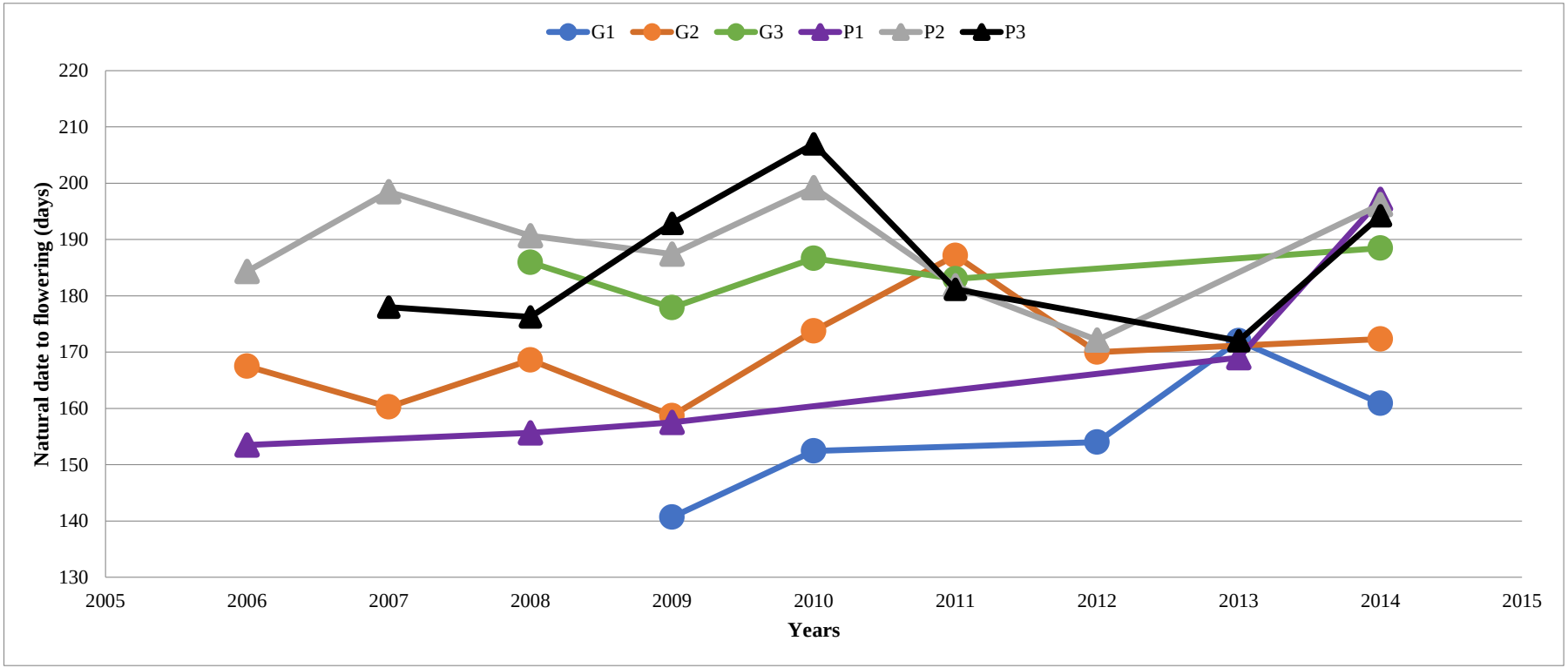


Figure 3.4: Trends in natural date to flowering from 2006 to 2014 in six photoperiod treatments (G1, G2, G3, P1, P2 and P3), where day one represents 01 January and not the day on which photoperiod treatments started. The control and P5 treatment for years 1995 to 2005 were excluded from the above results as data were insufficient to observe the trends. Each shape and colour represents the same group aimed to achieve synchronous using the planned combinations, *viz.* G1+P1, G2+P3 and G3+P2 (G = glasshouse, P = photoperiod house). Data obtained from Table 3.6.

3.4 DISCUSSION

3.4.1 Artificial conditions required for the production of viable pollen

At high latitudes, field conditions are not ideal for either flowering or pollen production (Moore *et al.* 2014b). However, flowering is essential for sugarcane breeders to develop new varieties and for that reason, environmental conditions have to be manipulated by using two artificial facilities, *viz.* glasshouse and photoperiod house. Manipulating conditions such as increased temperatures, relative humidity and day-lengths have been found to increase pollen viability across a number of genotypes (Brett 1947; Horsley and Zhou 2013). According to Durai *et al.* (2015), pollen viability percentage of a parental genotype is the deciding factor for its use as a pollen donor or pollen receptor during cross-pollination.

The production of viable pollen in the 16 genotypes was assessed in six photoperiod treatments to determine the proportions of viable pollen. Genotypes in the photoperiod house treatments were found to produce large proportions of viable pollen (52.74 - 67.11 %, Table 3.2) compared with genotypes from the glasshouse treatments (39.34 - 51.23 %, Table 3.2). In each of the six photoperiod treatments, the plants produced different amounts of pollen and therefore plants in the P treatments would most likely be used as pollen donors whereas plants in the G treatments would be used as pollen receptors. This is in agreement with findings made by Horsley and Zhou (2013) where genotypes in the glasshouse (G) treatments produced less viable pollen that the photoperiod house (P) treatments. The reason for this could be that in the photoperiod house was entirely closed and there was better control in temperature (18 - 32 °C) and relative humidity (70 - 100 %) using pipes containing circulated, heated water and humidifiers, respectively. In the glasshouse, on the other hand, there was a high rate of temperature fluctuations resulting in extreme temperatures of $\geq$ 32 °C which could have led to overheating during inflorescence emergence and led to low pollen viability (Horsley and Zhou 2013). For that reason, the glasshouse could be used to generate pollen receptors during crossing. Sato *et al.* (2002) has shown that heat stress prior to anthesis (pollen shedding) affects the developmental stages of pollen production. This may also affect the rate of pollen shed, pollen viability, pollen tube growth in the style, the fertility of the ovule or the growth of the fertilized ovule to ultimately produce seed (Nuss 1979; Sato *et al.* 2002).

3.4.2 Effect of photoperiod treatment on time of flowering/inflorescence emergence

The time of flowering of 16 genotypes was assessed in six photoperiod treatments to determine flowering patterns influenced by the different photoperiod treatments. The characteristics of the

managed photoperiod treatments have been proven to be optimal at a day-length of 12.30 h and this is within the range of other investigations reporting on successful artificially-induced inflorescence emergence (Nuss 1980; Nuss *et al.* 1999; Srivastava *et al.* 2006; Hamdi *et al.* 2010). The time to flowering has been found to be controlled by time of initiation of photoperiod treatments, rate of decline in day-length and temperature during the growth of the initial inflorescence (Moore 1974; Brett *et al.* 1975; Nuss 1980; Moore *et al.* 2014b). From the data for the six photoperiod treatments used at SASRI, the G1 and G2 treatments (constant day-lengths) analysed in this study were found to produce earlier flowering dates compared with the P treatments (decreasing day-lengths). This contradicts the findings reported by Brett and Harding (1974) and Edwards and Paxton (1979) that constant day-lengths gave late-flowering dates compared with decreasing day-lengths. Delayed flowering in treatments P2 and P3 were observed where they differed in the amount of 'darkness'/night time that the genotypes received. Different responses were observed in relation to pollen viability as treatment P3 resulted in less viable pollen than treatment P2 (Table 3.2). Nevertheless, it appears as though decreasing the day-length by 30 s could be used to delay flowering of early-flowering genotypes. In addition, it was found that beginning with constant day-lengths and later commencing with declining day-lengths (treatment G3) could be another effective method of delaying flowering.

The overall P treatments showed late-flowering dates compared with the G treatments (178 - 196 days and 158 - 183 days, respectively). It is interesting to note that although the photoperiod treatments stimulated different responses on the genotypes, the flowering times did not overlap, as expected. This observation limits cross-combinations in the early, intermediate and late crossing periods. The implication that asynchronous flowering will have on breeding will be the limited chances of promoting cross-pollination thereby affecting hybrid creation.

3.4.3 Genotypic response regarding pollen viability and time of flowering over 19 years and within the six photoperiod treatments

The 16 genotypes under investigation varied greatly in flowering response regarding pollen viability and time of flowering across the years. These results were similar to observations reported by Pratap and Singh (2003) and Rizk *et al.* (2007), where flowering varied from clone to clone among the related genera and within the *Saccharum* species. Percentage pollen viability of genotypes intended to be used as pollen donors (wild spp. and F_1 hybrid) were found to be greater than 30 %, although less than 60 %, which indicated that these genotypes could still be used as pollen donors (♂) but there is still room for improvement in increasing pollen viability among the genotypes. At SASRI, those genotypes with pollen viability greater than 30 % but

intended to be used as pollen receptors, are emasculated (e.g. the use of hot-water treatment at 50 °C for 3 min) to eliminate chances of self-pollination and promote outcrossing. Genotypes intended to be used as pollen donors (F_1 hybrid, *S. spontaneum*, *Erianthus* spp. and *S. robustum* spp.) responded differently among the different photoperiod treatments with regards to pollen production. There were some treatments that were superior in pollen production than others among each genotype (Table 3.5). For example: 04X0016: P2 = 53.12 % vs G3 = 26.07 %; Taiwan11: G1 = 62.5 % vs P2 = 25 % and IK76-417: P1 = 54.92 % vs G3 = 21.75 %.

The following genotypes should be excluded in these particular treatments for the next successive seasons because they are intended to be used as male donors however, they have low pollen fertility (< 30 %): (a) F_1 hybrid (04X0016) allocated in treatment G3, (b) *S. spontaneum* (Taiwan11) allocated in treatment P2 and (c) *S. robustum* (IS76-417) allocated in treatment G3.

The flowering response of the different genotypes were variable through the successive seasons. It is well known that different genotypes behave differently in relation to the number of days required to allow for flower stimulation (Berding and Hurney 2005; Junejo *et al.* 2012). For the 19 year period, the *Erianthus* spp. and *S. robustum* spp. were found to be late-flowering (182 - 213 days) genotypes. On the other hand, the commercial-type hybrids mostly emerged in the month of June (between 152 to 181 days). Therefore breeders will make less cross-combinations between the commercial-type hybrids and *Erianthus* or *S. robustum* spp. during the late period of the crossing season. This emphasises difficulties in attempting to make crosses between commercial-type hybrids and wild species in the breeding programme. Breeders from other countries such as Brazil, Australia and India have attempted to bridge flowering times through the use of pollen storage. In Brazil, sugarcane pollen was found to remain viable for 30 days at -20 °C (Amaral *et al.* 2013) and would potentially provide a way to overcome asynchronous flowering.

3.4.4 Seasonal effect on flowering stimulated by the different photoperiod treatments

The efficacy of the photoperiod treatments have been proven to be an important contribution towards spreading the crossing season over a longer time period across the years. Search data can predict the number of cross-combinations, particularly for introgression breeding, that could be made and the extracted information could help in the selection of desirable genotypes targeted to achieve specific crosses. Genotypes were classified based on their flowering times as early (May), intermediate (June) and late (July) in each season (Figure 3.2). The mean flowering dates for the different photoperiod treatments (G1, G2, G3, P1, P2 and P3) gave added confirmation of the

general trends where the first combination (G1+P1) allowed for early-flowering, the second combination (G2+P3) allowed for intermediate-flowering and the third combination (G3+P2) allowed for late-flowering (Horsley and Zhou 2013).

To achieve the desirable cross combinations for sugarcane breeding, flowering times need to be synchronised. According to Nuss (1982), inflorescence emergence of sugarcane genotypes may occur up to eight weeks apart in the crossing season which creates a major barrier in cross-pollination. Sugarcane pollen is only viable for < 35 min under ambient conditions (Amaral *et al.* 2013) while the stigma is receptive for 5 - 7 days (Heslop-Harrison 1992; Singh *et al.* 2009). This emphasises the importance of choosing genotypes where the pollen donors (♂) flower at least 10 days later than the pollen receptors (♀) to give the 5 - 7 day period for cross-pollination to occur. The results from this study show wide flowering times that exist between desirable genotypes needed to be used for cross-pollination. It is expected that genotypes from the G treatments should flower before the genotypes from the photoperiod house (Figure 3.4). The number of possible cross combinations that could be made each year were highly variable with respect to the flowering genotypes. The results further point to the importance of bridging the gap between the flowering times the need to further manipulate the photoperiod regimes to increase the chances of creating hybrids (Table 3.6).

CHAPTER 4: OPTIMISING POLLEN VIABILITY ASSESSMENT AND STORAGE METHODS

4.1 INTRODUCTION

As discussed previously in Chapter 3, many sugarcane improvement programmes are constricted by sexual recombination among modern cultivars (*Saccharum spp.*), wild spp. and related genera, due to inconsistent flowering of genotypes, pollen sterility and incompatibility (Krishnamurthi 1980; Singh *et al.* 2009; Alarmelu and Shanthi 2011). For breeders, it is essential to re-evaluate the crossing techniques with an aim of achieving good seed sett and the creation of interspecific/intergeneric hybrids.

Pollen viability testing is one important component in cross-pollination activities (Rodriguez-Riano and Dafni 2000). It provides knowledge of the ability of pollen grains to germinate on receptive stigmas present on an inflorescence chosen as a pollen receptor during crossing. In the crossing programme at SASRI, the inflorescences are classified as either pollen donors or pollen receptors on the basis of pollen viability and the opening of the anthers, with the pollen donor having medium to high level of viable pollen (> 30 %) and the pollen receptor having no or low levels of viable pollen (< 30 %) (Zhou 2013). For example, a typical bi-parental cross will comprise one genotype as a pollen donor (♂) while the other as a pollen receptor (♀) (McIntyre and Jackson 2001; Cheavegatti-Gianotto *et al.* 2011; Melloni *et al.* 2013). In sugarcane, the most commonly employed method for establishing pollen viability is the starch-iodine stain (Melloni *et al.* 2013). The advantages of using this method is the ease and rapidity of classifying the sugarcane inflorescences prior crossing (Pedersen *et al.* 2004). However, some researchers have found the starch-iodine stain to over-estimate viability (Rodriguez-Riano and Dafni 2000; Melloni *et al.* 2013). The lack of a reliable pollen viability technique has limited genetic improvement in various crops such as sugarcane, maize and sorghum (Vieira *et al.* 2015). Low seed germination would most likely be due to the incorrect classification of the inflorescence as a pollen donor or pollen receptor (Melloni *et al.* 2013). Therefore, according to Rodriguez-Riano and Dafni (2000), for a detailed determination of pollen viability, more than one assessment should be used (staining techniques, *in vitro* germination or *in vivo* germination), to avoid under or overestimation of viability.

In vitro pollen germination has been regarded as a more accurate technique to use for pollen viability studies than the staining methods (Bots and Mariani 2005; Soares *et al.* 2008). Various compositions of culture media have been used and changes in sucrose and boron concentrations have usually been necessary for optimal germination (Towill and Walters 2000). Numerous

researchers have found that sugarcane pollen grains are highly sensitive to temperature, relative humidity and light as a result of the very thin porous cell walls that surrounds them (Krishnamurthi 1980; Berding 1981; Tai 1989). They rapidly lose viability soon after pollen shedding and as previously mentioned have a life-span of approximately 20 - 35 min under ambient conditions (26.5 ℃ and 67 % RH) (Anonymous 2008). Pollen storage across various crops has been found to be an effective method used to extend pollen viability and overcome hybridisation barriers between plants that flower at different times, thereby contributing to the generation of variability obtained from artificial crosses and increasing the efficiency of breeding programmes (Kalkar and Neha 2012; Amaral *et al.* 2013; Moura *et al.* 2015). Appropriate pollen conservation strategies, especially in sugarcane breeding, could aid in generating improvement by introgressing new genetic traits into desired species (Vieira *et al.* 2015). Several protocols have been tested with no success (Krishnamurthi 1980; Moore and Nuss 1987), until recently in Brazil where it was reported that pollen could be stored at -20 °C for 30 days (Amaral *et al.* 2013). There have been no reports on pollen viability testing, time of pollen shedding and pollen storage of South African sugarcane genotypes.

The specific aims of this study were to:

a) select an appropriate culture medium for *in vitro* pollen germination;
b) find an easy, accurate and fast (minutes) method for testing pollen viability during crossing;
c) determine the optimum time of day to collect pollen; and
d) enhance short-term storage of pollen to facilitate use in controlled cross-pollination.

4.2 MATERIALS AND METHODS

4.2.1 Genotypes used in the study

Fresh pollen samples were collected from the South African Sugarcane Research Institute (Mount Edgecombe, South Africa; 29° 42' 24.5585”S, 31° 02' 45.1735”E) during the period of May to July for years 2014 and 2015. Three genotypes were selected: 06B1187 (commercial C-type hybrid 1), 11K1617 (C-type hybrid 2) and 06G0127 (C-type hybrid 3).

4.2.2 Pollen collection

The plants which had emerged inflorescences were kept in the glasshouse in separate compartments. Pollen was collected at 7h00 on the day of the experiment by dusting the inflorescence into a Petri plate (Corning, 35 mm x 10 mm). The glasshouse temperature at the

time of collection was ± 25 °C and the relative humidity (RH) was ± 50 %. After collection, the samples were transported on Petri plates to the laboratory. Approximately 10 anthers per treatment were removed from the inflorescence for pollen viability testing. Pollen collected in this manner was used for assessment of pollen viability, storage and optimum time of day for collection (Figure 4.1).

4.2.3. *In vitro* pollen germination technique development: optimising sucrose concentration and comparing five media formulations

There were five sucrose concentrations (100 g/l, 200 g/l, 300 g/l, 400 g/l and 50 g/l) used for determining pollen germination compared with a control medium (without sucrose). The medium consisted of boric acid (0.1 g/l), calcium nitrate (0.3 g/l) and magnesium sulphate (0.1 g/l) (Krishnamurthi 1980). In addition, five media formulations were used by altering certain components from published protocols of the Poaceae family (Table 4.1).

Table 4.1: Five different media formulations assessed for pollen germination to determine the optimal medium for pollen viability testing.

Composition (g/l)	**Medium 1**	**Medium 2**	**Medium 3**	**Medium 4**	**Medium 5**
Sucrose	100 - 500†	300	300	300	300
Boric acid	0.1	-	0.1	0.1	0.1
Calcium nitrate	0.3	-	0.3	0.06	0.3
Magnesium sulphate	0.1	-	0.1	0.1	0.1
Agar	-	10	10	-	-
Polyethylene glycol	-	-	-	-	150*
Reference (s)	Krishnamurthi (1980); Singh (2013)	Melloni *et al.* (2013)	Modified from Krishnamurthi (1980)	Amaral *et al.* (2013)	Modified from Krishnamurthi (1980)

*volume by volume concentration (150 ml/l).

†optimum sucrose optimisation using five concentrations (100 g/l, 200 g/l, 300 g/l, 400 g/l and 500 g/l) for Medium 1.

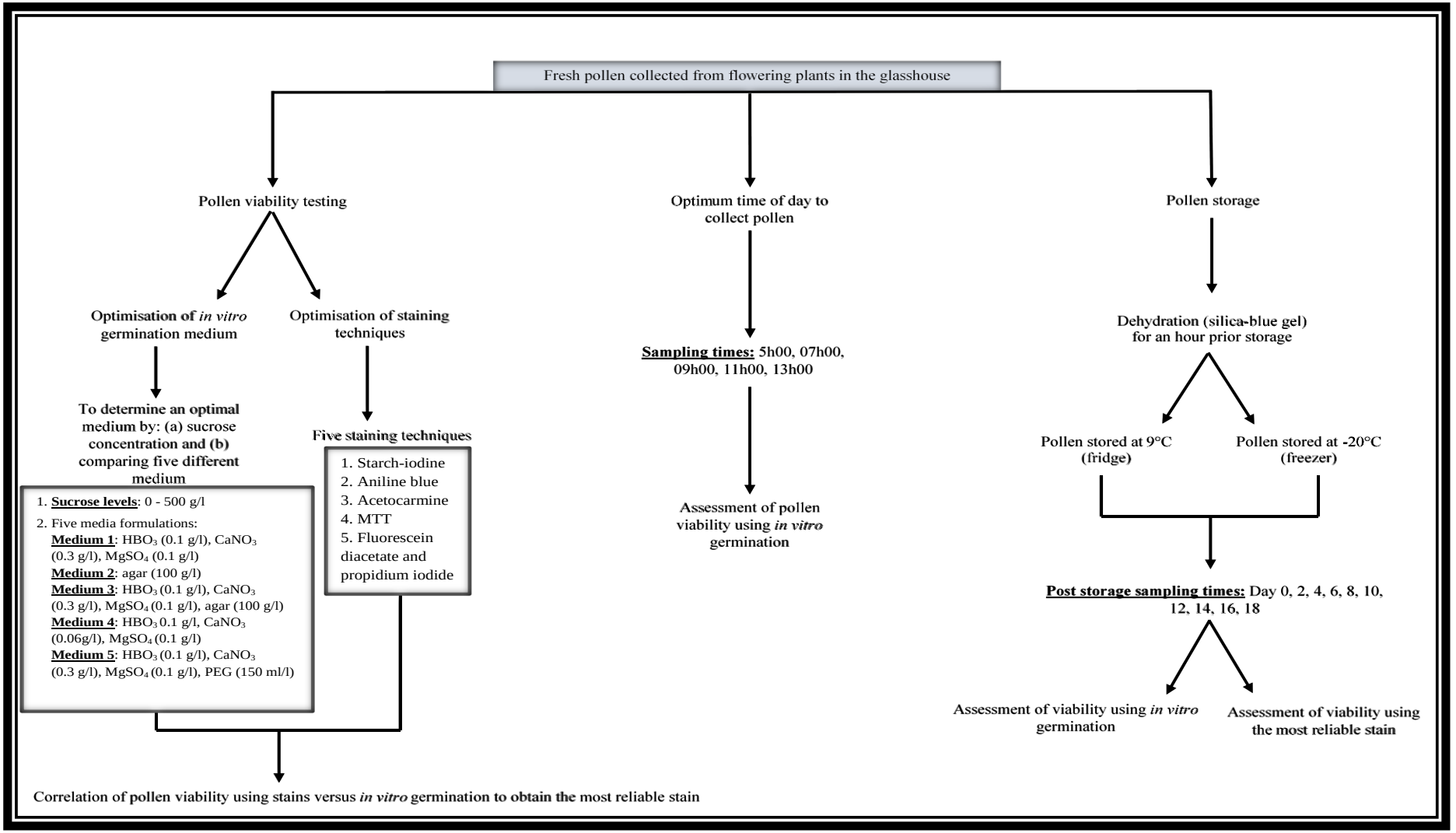


Figure 4.1: Outline for sugarcane pollen studies through pollen viability testing, anthesis time determination (optimum time of day to collect pollen) and pollen storage.

All media were autoclaved at 121 °C, at a pressure of 1 kg/cm^2 for 20 min without sucrose. The pH was adjusted to 6.9 using HCl or KOH. After autoclaving, sucrose was added and Media 1, 4 and 5 were stored at 4 °C (refrigerator) until use. Media 2 and 3 were poured into Petri plates, in a laminar flow chamber and were placed at room temperature (± 23 °C) to solidify. The plates were then stored at 4 °C (refrigerator) until use.

The collected pollen samples were dusted on the surface of the agar plate (Media 2 and 3). For Media 1, 4 and 5, an amount of 40 µl of pollen in liquid media was placed on clean microscopic slides. Here, detached anthers were mixed with the medium and were squashed using a clean scalpel, thereby releasing pollen into the media. From the slides, the mixtures were pipetted into a 96-well polymerase chain reaction (PCR) plate and were kept uncovered at high humidity (> 70 % RH) in a moist chamber (sealed container with moistened tissue paper). A control treatment (without sucrose) was used for comparative purposes for all media formulations. The Petri plates and the 96-well PCR plates were incubated in a germination chamber (Scientific, series 2000 incubator) with a controlled temperature of 30 ± 1 °C for 6 h. Pollen grains were distinguished based on the pollen tube length, "when the pollen tube is greater than the diameter of the pollen grain" (Tuinstra and Wedel 2000). Data were scored as % pollen germinating and % pollen bursting. The length of the pollen tubes was also measured for samples in the five media formulations

4.2.4 Pollen staining techniques

A number of staining techniques were assessed for determining pollen viability. Dead pollen controls were treated at 100 °C for 8 h.

a) Fluorescein diacetate (FDA) and propidium iodide (PI)

The FDA/PI staining technique used was done according to Jones and Senft (1985). A stock solution of fluorescein diacetate (FDA; Sigma-Aldrich) was prepared by dissolving it in acetone at a concentration of 2 mg/ml. Five to ten drops of a sucrose solution (600 g/l) was added to the FDA stock solution before use until the mixture turned milky/cloudy. The precipitate was allowed to settle at room temperature (23 °C) for 30 min. A stock solution of propidium iodide of 0.02 mg/ml was prepared by adding distilled water. The stains were then mixed in a 3:1 (FDA: PI). For pollen viability testing, the pollen grains were stained for ± 5 min and viable pollen emitted bright reddish-green fluorescence while non-viable pollen grains emitted brown-red fluorescence.

b) Aniline blue in lactophenol (ABL)

The aniline blue is a lactophenol stain (Asghari 2000) and was prepared by mixing together liquefied phenol (20 ml), glycerine (40 ml) and distilled water (40 ml). Thereafter, aniline blue di-ammonium salt (1 g; Sigma-Aldrich) was added. The pollen grains were stained for ± 30 min and viable pollen was dark-blue while non-viable pollen grains was light blue/clear.

c) Iodine and potassium iodide (IKI) / starch-iodine

Iodine and potassium iodide stain (Mulugeta *et al.* 1994) also referred to as the 'Lugol' solution were made up with iodine (1 g; Merck, South Africa) and potassium iodide (2 g; Merck, South Africa) dissolved in distilled water (100 ml). The pollen grains were stained for ± 5 min and viable pollen was black/dark brown while non-viable pollen grains was light brown/yellow.

d) Acetocarmine (AC)

The acetocarmine stain (Heslop-Harrison 1992) was done by preparing an acetic acid solution (450 ml/l) using distilled water. The acetic acid solution was heated until boiling and carmine (1 g; Sigma-Aldrich) was carefully added while stirring for a few minutes. The mixture was allowed to cool and was filtered using Whatman® filter paper #1. The pollen grains were stained for ± 30 min at 37 ± 1 °C and viable pollen was darkred while non-viable pollen was lightred/clear.

e) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

The MTT solution was prepared by dissolving MTT (1g; Sigma-Aldrich) and sucrose (5 g) in distilled water (100 ml) the pollen samples were mixed with one drop of the MTT solution and were incubated at 30 °C for 5 min. Pollen grains that were stained light red to red were considered viable and non-coloured/black pollen grains were considered non-viable.

4.2.5 Anthesis time determination (time of pollen shedding)

The inflorescence was completely enclosed the night before collection by using a brown paper bag. Pollen was collected in ± 2 h intervals from 5h00 to 13h00.The pollen grains were collected in the bag by tapping the inflorescence and viability was assessed using the determined optimal medium (adapted from Singh *et al.* 2009). A hygrometer (Electronic Temperature Instruments Limited®) was used to assess the temperature and relative humidity at each time period.

4.2.6 Pollen storage treatments

a) Dehydration in pre-storage stage

For dehydration in the pre-storage stage, silica blue gel was used. The pollen collected in Petri plates were placed uncovered in a vacuum desiccator under silica blue gel (1000g; Sigma-Aldrich) for an hour in the fridge (9 °C) (Figure 4.2).

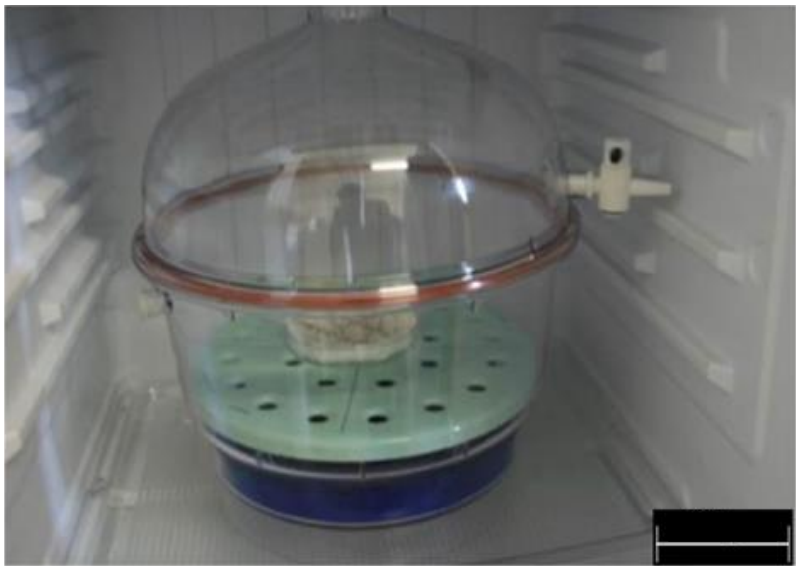


Figure 4.2: Dehydrated samples of sugarcane pollen in silica blue gel in a plastic desiccator at 9 °C. Scale bar = 100 mm.

b) Storage

Immediately after pollen dehydration, the Petri plates were covered, sealed with parafilm and transferred to the two storage conditions, *viz.* fridge (9 °C) and freezer (-20 °C), respectively.

c) Assessment of viability in stored pollen

To assess the viability of the pollen grains, the samples were removed from the storage conditions and subjected to rehydration for 10 min in a moist chamber (layered with wet paper towels to achieve high RH) at ± 30 °C. Sampling was done in two day intervals from day 0 to day 18. Viability was assessed by culturing in the best artificial medium from section 4.2.3 and the MTT stain from section 4.2.4 (e).

4.2.7 Microscopy

The percentage viability was calculated from the total viability in a microscopic field of vision over the total number of pollen grains present within that microscopic field. Approximately three hundred (300) pollen grains were evaluated per treatment. Two microscopes were used to observe

viability at a magnification of 200 X, *viz.* light microscope (Nikon eclipse 50i; Zeiss) for the IKI, ABL, AC and MTT stains as well as pollen germination, and for the FDA-PI stain, the fluorescent microscope (Axioskop, Zeiss) was used. The images were captured using a Zeiss AxioCam imaging system and were saved on a PC-compatible computer through the AxioVision™ software.

4.2.8 Statistical analyses

Data were analysed based on five replicates and three sub-replicates (each which is a whole microscopic field of vision). The pollen samples were equally divided into five slides or agar plates and the sub-replicates correspond to three random microscopic fields of vision. The data were analysed using analysis of variance (ANOVA) and means (± SE) were compared using the Student's t-test at a 5 % probability level (GenStat® Release 8.1).

4.3 RESULTS

4.3.1. *In vitro* pollen germination technique development: optimising sucrose concentration and comparing five media formulations

The assessment of *in vitro* pollen germination medium involved the following: (a) optimisation of sucrose concentration; and (b) comparison of five media formulations. The best sucrose concentration was evaluated using genotypes, C-type hybrid 1 (06B1187) and C-type hybrid 3 (06G0127) while the comparison of five media formulations was evaluated using genotypes, C-type hybrid 1 (06B1187) and C-type hybrid 2 (11K1617) to obtain the best germination medium for high germination without the pollen grain membrane rupturing.

There were highly significant differences in % pollen germination and % pollen bursting for sucrose concentration ($p < 0.001$, Appendix 3A and 3B). There were no significant differences in % pollen germination and % pollen bursting among genotypes ($p = 0.615$) and the interaction between each genotype and sucrose concentration ($p = 0.902$, Appendix 3A and 3B).

The highest pollen germination (43.97 ± 2.17 %) was observed in medium containing 300 g/l sucrose whereas the lowest pollen germination was in the control treatment (0 g/l) (Table 4.2). The highest pollen bursting % was seen in the control treatment (59.39 %) whereas the lowest % of pollen bursting was observed for medium containing 500 g/l sucrose (2.33 %, Table 4.2). There was an inversely proportional relationship between pollen germination and pollen bursting, where

an increase in sucrose concentration caused a decrease in pollen bursting for all the five sucrose levels tested.

Table 4.2: Mean values for the percentage pollen germinated and pollen burst amongst five different levels of sucrose concentrations used for *in vitro* germination of pollen from genotypes, C-type 1 and C-type 3. Letters (a-e) indicate statistical significant differences ($p < 0.05$), where means indicated by the same letter are not significantly different (n = 5, mean ± SE, ANOVA).

Sucrose concentration (g/l)	Pollen germination (%)	Pollen bursting (%)
0 (Control)	0^{e}	59.39 ± 1.50^{a}
100	10.61 ± 0.96^{d}	38.03 ± 1.80^{b}
200	34.5 ± 2.33^{b}	13.17 ± 2.21^{c}
300	43.97 ± 2.17^{a}	3.28 ± 0.96^{d}
400	16.53 ± 2.04^{c}	3.28 ± 0.84^{d}
500	11.28 ± 2.67^{d}	2.33 ± 0.88^{d}

A maximum pollen tube length of 1172.79 ± 7.36 µm was observed in pollen on *in vitro* germination media containing sucrose concentration of 300 g/l. At higher concentrations (400 g/l and 500 g/l sucrose) the pollen tube length was 707.32 ± 2.57 and 474.66 ± 3.29 µm, respectively. These findings are supported by several other researchers who also found that sugarcane pollen germinates optimally at a sucrose concentration of 300 g/l (Krishnamurthi 1980; Singh *et al.* 2009; Amaral *et al.* 2013). Without sucrose, there was a high degree of pollen bursting (59.39 ± 1.50 %) and pollen integrity deteriorated with the internal contents leaching into the media. This indicates that sucrose plays a significant role in pollen germination. Several reports have suggested that sucrose acts as an osmoticum and an energy source for pollen germination (Shivanna and Johri 1985; Geetha *et al.* 2004; Devrnja *et al.* 2012) and it is critical to determine an optimal concentration as inhibitory effects on pollen tube growth have also been reported (Geetha *et al.* 2004).

In vitro germination of pollen grains is a common technique used for pollen viability studies (Soares *et al.* 2008). It is critical to determine an optimal medium to use for the germination to accurately estimate pollen viability for cross-pollination. There were significant differences in pollen germination and pollen bursting between all five media formulations as well as among genotypes ($p < 0.05$, Appendix 4A and 4B). The highest germination was evident in Medium 3 (Figure 4.3) while the lowest was in Medium 2 (30.33 % and 0 %, respectively; Figure 4.3A). On

the other hand, the highest pollen bursting figures were observed in Medium 2 (Figure 4.3) while the lowest pollen bursting occurred in Medium 3 and 5 (35.87 ± 2.39 % and 0 %, respectively; Figure 4.3B).

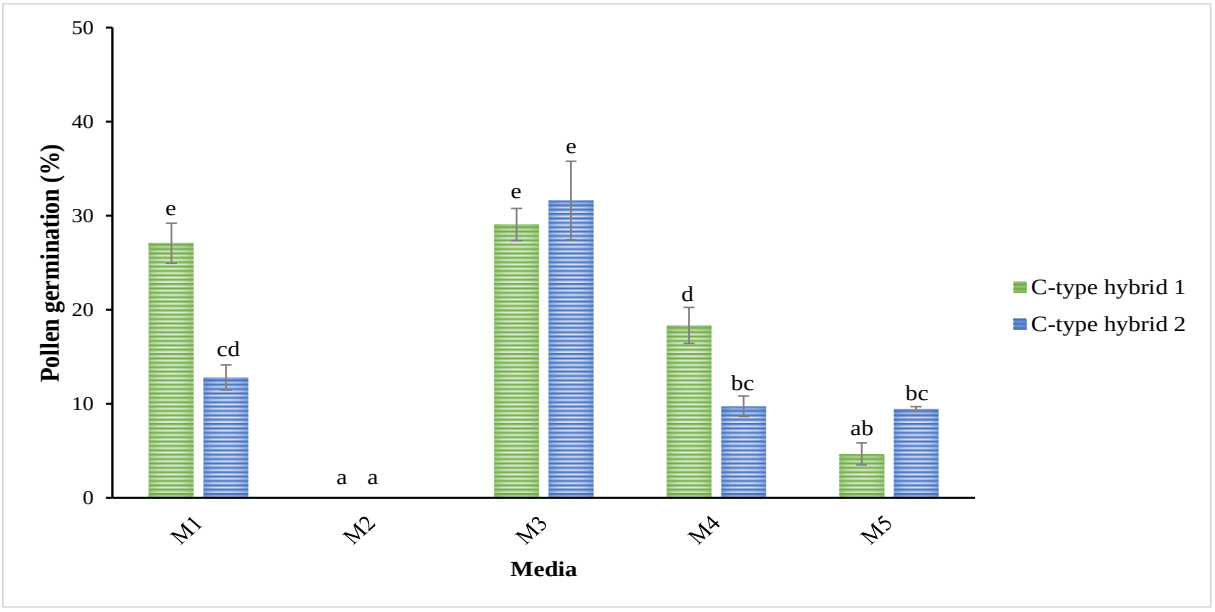
In Medium 3, pollen germination was 29.07 ± 1.71 % and 31.6 ± 4.17 % for the C-type hybrid 1 and C-type hybrid 2, respectively, and no pollen bursting was observed (Figure 4.3). This indicates that Medium 3 facilitated optimal germination without causing the membranes of the pollen grains to rupture. No pollen bursting was evident in Medium 5 (Figure 4.3A and B), however the percentage germination was low compared to Medium 3 which could be due to the addition of polyethylene glycol. Therefore, Medium 3 was used for further pollen germination tests.

4.3.2 Comparison of staining techniques and in vitro pollen germination method

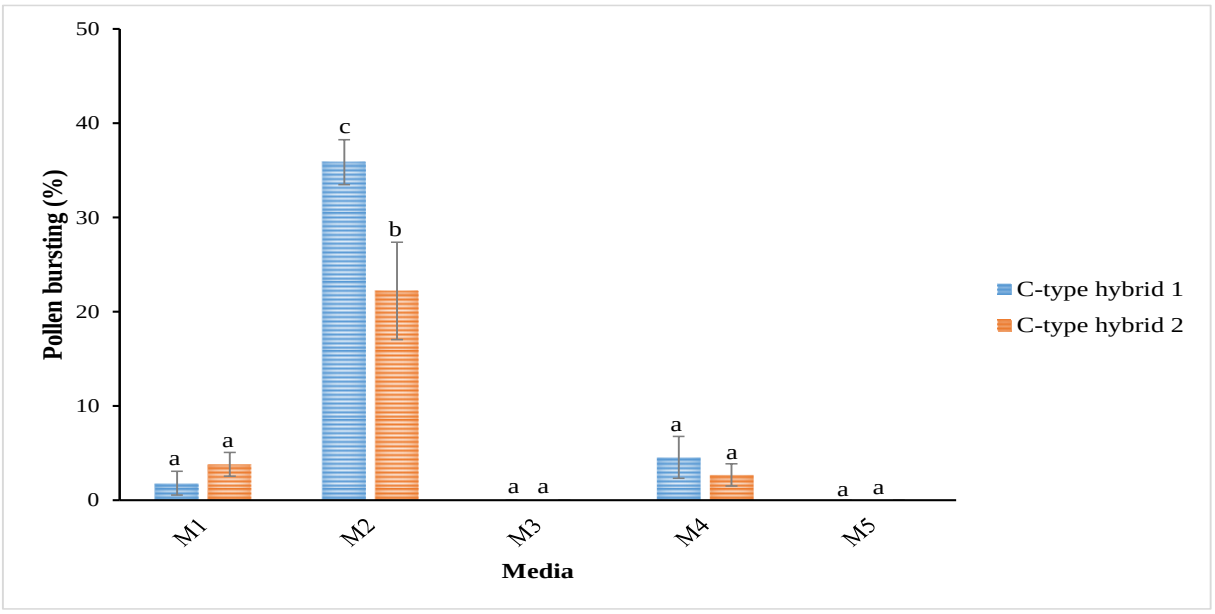
Pollen viability was studied using six techniques, *viz.* starch-iodine (IKI), aniline blue (ABL), acetocarmine (AC), fluorescein diacetate and propidium iodide (FDA-PI), (MTT) and *in vitro* pollen germination using genotypes C-type hybrid 1 and C-type hybrid 2. All the staining techniques were compared with *in vitro* pollen germination results (as the more accurate estimate of viability).

A comparison of stains with the *in vitro* pollen germination method showed significant effects for all the variables evaluated (genotype and viability tests), as well as the interaction among them ($p < 0.001$, Appendix 5). For C-type hybrid 1, all the staining tests (IKI, ABL, AC, FDA-PI and MTT) showed significant ($p < 0.05$) differences when compared with *in vitro* germination indicating an overestimation of viability (Table 4.3). The IKI stain detected the highest viability (82.07 % ± 1.39) while *in vitro* pollen germination showed the lowest viability (29.07 % ± 1.71). For C-type 2, IKI, ABL and AC showed significant ($p < 0.05$) differences whereas FDA-PI and MTT showed no significant ($p > 0.05$) differences when compared with *in vitro* pollen germination. The AC stain showed the highest viability (68 % ± 1.91) while *in vitro* pollen germination showed the lowest viability (31.6 % ± 4.17). The interaction between MTT and FDA-PI stains showed no significant ($p > 0.05$, Table 4.3) differences in pollen viability for C-type hybrid 1 and C-type hybrid 2 indicating similarity in viability determination. Moreover, these stains could distinguish between viable and non-viable pollen grains.

(A)



(B)



M1 (Medium 1) - sucrose (300 g/l), boric acid (0.1 g/l), calcium nitrate (0.3 g/l), magnesium sulphate (0.1 g/l)

M2 (Medium 2) - sucrose (300 g/l), agar (10 g/l)

M3 (Medium 3) - sucrose (300 g/l), boric acid (0.1 g/l), calcium nitrate (0.3 g/l), magnesium sulphate (0.1 g/l), agar (10 g/l)

M4 (Medium 4) - sucrose (300 g/l), boric acid (0.1 g/l), calcium nitrate (0.06 g/l), magnesium sulphate (0.1 g/l)

M5 (Medium 5) - sucrose (300 g/l), boric acid (0.1 g/l), calcium nitrate (0.3 g/l), magnesium sulphate (0.1 g/l), polyethylene glycol (150 ml/l)

Figure 4.3: Assessment of five media formulations (M1 - M5) to determine an optimal medium for *in vitro* pollen germination amongst genotypes 06B1187 (C-type hybrid 1) and 11K1617 (C-type hybrid 2). Results were scored as percentage (A) pollen germinating and (B) pollen bursting. Letters (a-e) indicate statistical significant differences ($p < 0.05$), where treatments indicated by the same letter are not significantly different (n = 5, means ± SE, ANOVA).

Correlation tests were conducted to compare all the staining techniques with *in vitro* pollen germination. All correlation coefficients (r) indicated that there was no significant ($p > 0.05$) difference between each of the stains and *in vitro* pollen germination (Table 4.4). For C-type hybrid 1, the *r* values were from 0.0626 to 0.3835 indicating that there was a low correlation between each stain and the *in vitro* percentage pollen germination (Table 4.4). For C-type hybrid 2, the *r* values were from were 0.1104 to 0.2940 indicating a low correlation between IKI, ABL AC and MTT and *in vitro* pollen germination. However, the 'r' value between FDA-PI and *in vitro* pollen germination showed a strong linear correlation (0.5270) indicating similarities in pollen viability.

The viability techniques varied in the detection of viable and non-viable pollen grains (Figure 4.4). It was found that stains IKI, ABL and AC gave false positive results (Table 4.5). These stains did not accurately discriminate between viable and non-viable pollen grains leading to overestimation of viability. The MTT and FDA-PI stains could distinguish between viable and non-viable pollen grains thus showing an improved degree of accuracy when compared with the other stains.

Table 4.3: Comparison of pollen viability from two sugarcane genotypes using different staining techniques and *in vitro* pollen germination. Letters (a-g) indicate statistical significance ($p < 0.05$), where treatments indicated by the same letter are not significantly different (n = 5, means ± SE, ANOVA).

Viability techniques	**Pollen viability (%)**	
	C-type hybrid 1 (06B1187)	**C-type hybrid 2 (11K1617)**
In vitro pollen germination	29.07 ± 1.71^{a}	31.6 ± 4.17^{a}
MTT	45.13 ± 2.03^{bc}	37.07 ± 1.25^{ab}
Fluorescein diacetate and propidium iodide	49.27 ± 1.58^{cd}	37.13 ± 2.24^{ab}
Starch-iodine	82.07 ± 1.39^{g}	56.33 ± 2.07^{d}
Aniline blue	76.67 ± 1.36^{fg}	58.27 ± 2.02^{de}
Acetocarmine	82.0 ± 1.77^{g}	68 ± 1.91^{ef}

Table 4.4: Correlation coefficients between staining techniques and *in vitro* pollen germination for two genotypes. Level of significance was 5 % assessed using Pearson correlation coefficient.

Tests	C-type hybrid 1		C-type hybrid 2	
	P value	**Correlation coefficients**	**P value**	**Correlation coefficients**
Starch-iodine	0.8500	0.1181	0.7789	0.1745
Aniline blue	0.9201	0.0628	0.6592	0.2710
Acetocarmine	0.5869	0.3306	0.6312	0.2940
FDA-PI	0.5239	0.3835	0.3872	0.5034
MTT	0.5396	0.3703	0.8597	0.1104

Table 4.5: Viability techniques tested against a control treatment (heated at 100 °C for 6 h)

Viability techniques	C-type 1		C-type 2	
	Means ± SE	**Means ± SE (Control)**	**Means ± SE**	**Means ± SE (Control)**
In vitro pollen germination	29.07 ± 1.71	0	31.6 ± 4.17	0
MTT	45.13 ± 2.03	0	37.07 ± 1.25	0
Fluorescein diacetate and propidium iodide	49.27 ± 1.58	0	37.13 ± 2.24	0
Starch-iodine	82.07 ± 1.39	83 ± 1.8	56.33 ± 2.07	52.80 ± 3
Aniline blue	76.67 ± 1.36	77.2 ± 2.5	58.27 ± 2.02	47 ± 4.2
Acetocarmine	82.8 ± 1.77	82.6 ± 1.7	68 ± 1.91	55.2 ± 3

4.3.3 Anthesis time determination

The time of pollen shedding was assessed by sampling in two hour intervals from 5h00 to 13h00 and viability was tested using *in vitro* pollen germination for genotypes C-type hybrid 1 and C-type hybrid 2 to determine at what optimal time period/s pollen should be sampled for viability determination.

There were highly significant interactions among genotypes, time of pollen shedding and an interaction between genotypes and time of collection ($p < 0.001$, Appendix 6). At 07h00, the highest pollen viability of 26.23 ± 2.9 % was recorded compared with times 09h00, 11h00 and 13h00 (14.63 ± 4.1 % , 0 % and 0 %, respectively).

For C-type hybrid 1 and C-type hybrid 2, time 07h00 showed the highest pollen viability of 23.33 ± 0.93 % and 29.60 ± 1.42 %, respectively (Figure 4.5). No significant ($p > 0.05$) differences were observed between time periods 05h00 and 07h00 indicating that pollen is best collected between these times. After 4 h, viability dropped for both genotypes by 45 % and 63 %, respectively (Figure 4.6), indicating that it reduced at a faster rate as temperatures increased during the day (Figure 4.6). The results obtained from this experiment showed that after the period of 07h00, pollen viability decreased as a result of an increase in temperature and a drop in relative humidity.

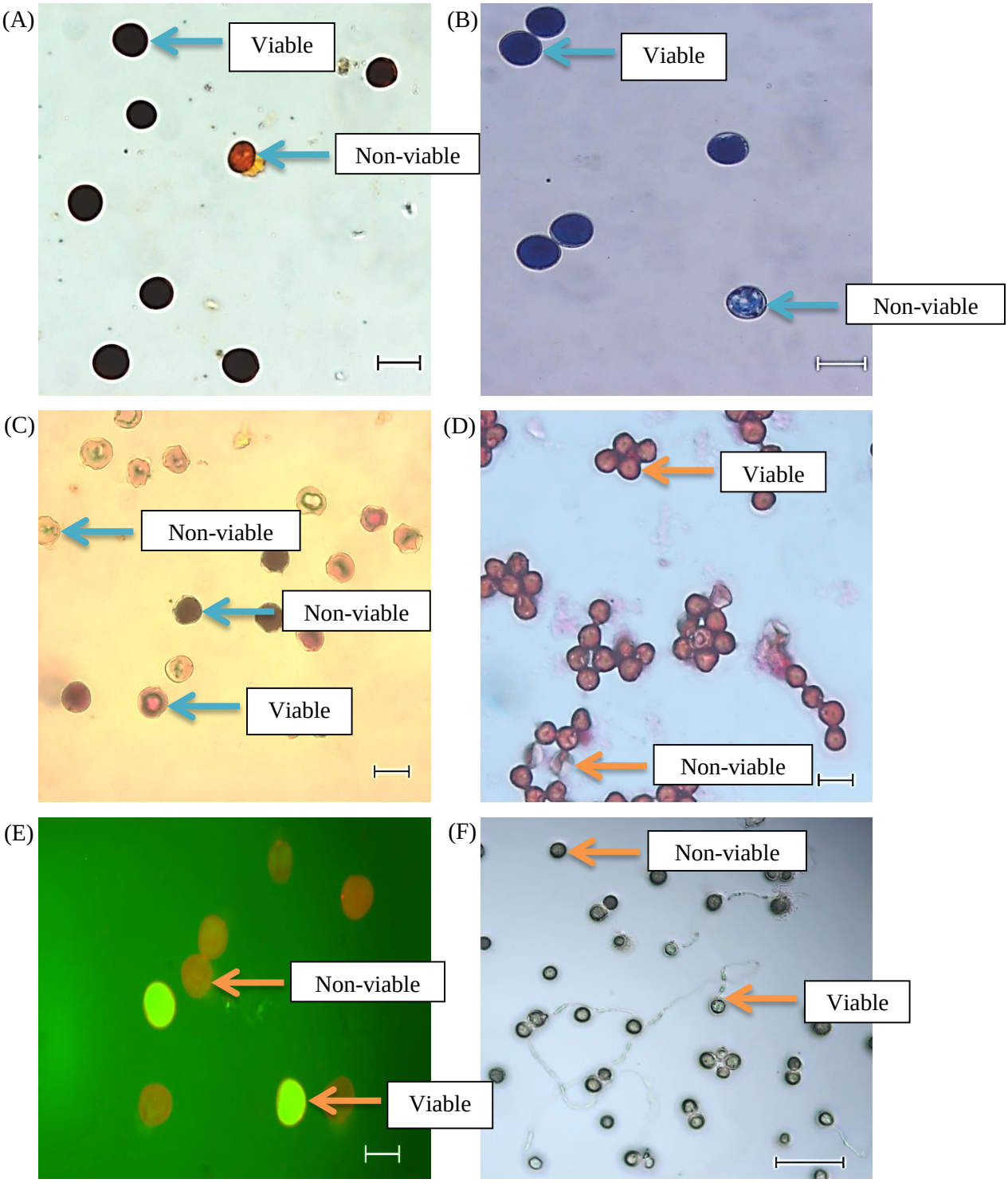


Figure 4.4: Microscopic images of pollen samples tested using six viability techniques. (A) starch-iodine (IKI), (B) aniline blue (ABL), (C) MTT, (D) acetocarmine (AC), (E) fluorescein diacetate and propidium iodide (FDA-PI) and (F) *in vitro* pollen germination. Scale = 50 µm (a-e) and 100 µm (f).

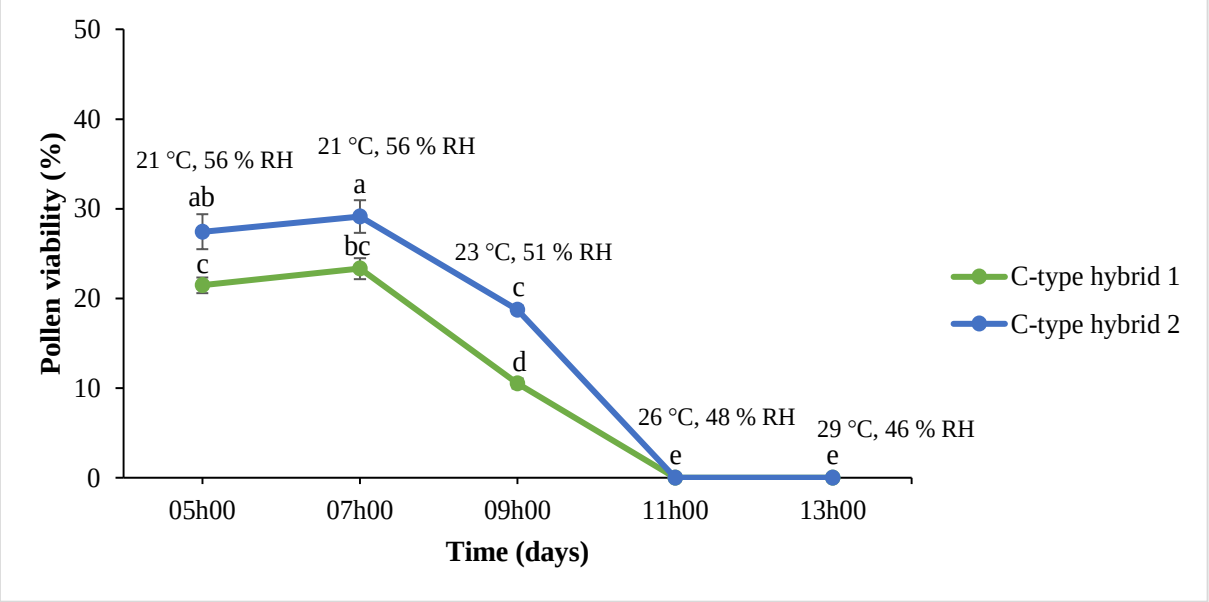


Figure 4.5: Anthesis time determination of two genotypes (C-type hybrid 1 and C-type hybrid 2) sampled at two hour intervals (from 05h00 – 13h00). Viability was assessed using the *in vitro* pollen germination method. Letters (a-e) indicate statistical significance ($p < 0.05$), where treatments indicated by the same letter are not significantly different (n = 5, means ± SE, ANOVA). Temperature (°C) and relative humidity (RH) above each time interval.

4.3.4 Storage of sugarcane pollen

In an attempt to assess if pollen viability could be prolonged by storage, samples were subjected to two storage regimes, *viz.* 9 °C (fridge) and -20 °C (freezer). Pollen viability was tested at two day intervals using *in vitro* pollen germination and the MTT stain for genotypes C-type 1 hybrid and C-type 2 hybrid.

Pollen germination percentage of the two genotypes was significantly affected by storage temperature and storage time duration (Figure 4.6; $p < 0.001$, Appendix 7A and B). The highest pollen viability was observed at day 0 (pre-storage) for C-type hybrid 1 and C-type hybrid 2 (46.4 ± 1.67 % and 30.4 ± 1.36 %, respectively; Figure 4.6). Germination ceased at day 16 for C-type hybrid 1 and at day 12 for C-type hybrid 2. Viability ceased at day 18 for both genotypes. The results indicated that pollen viability percentage decreased with increase in storage duration at 9 °C. However, on average, according to *in vitro* germination, sugarcane pollen (8.07 ± 0.36 and 9.2 ± 0.33) can be stored for at least 10 days and according to the MTT stain (6.4 ± 0.38 and 5.33 ± 0.27), pollen can be stored for at least 16 days at 9 °C after one hour dehydration for C-type hybrid 1 and 2, respectively. Storage at -20 °C was not successful as no viability was detected

after 2 days of storage indicating that sugarcane pollen cannot be kept at this storage temperature to prolong viability (results not shown).

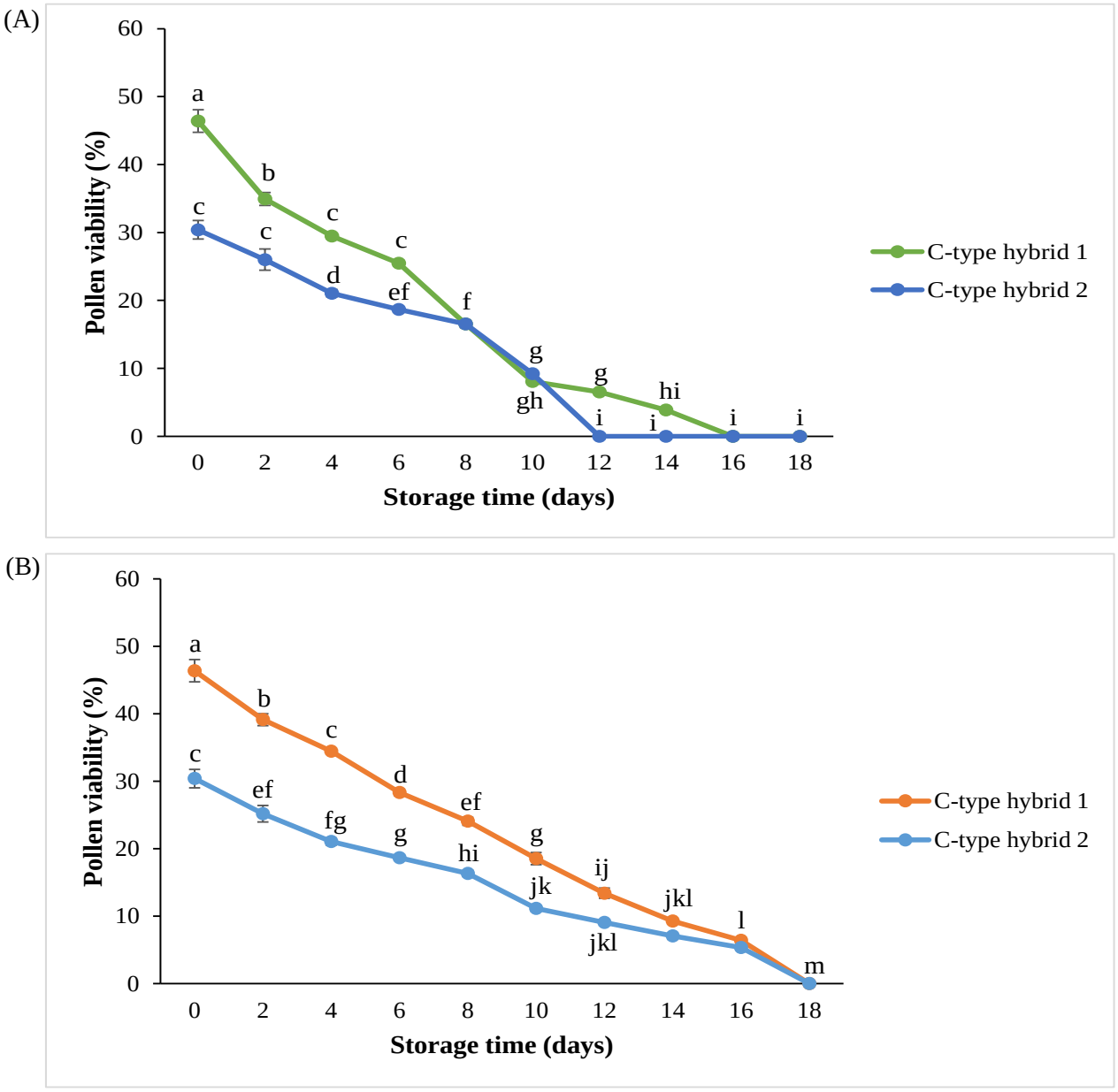


Figure 4.6: A comparison of *in vitro* pollen germination and the MTT stain to assess pollen viability of sugarcane pollen stored at 9 °C over time. (A) *In vitro* pollen germination and (B) MTT stain. Samples were dehydrated for an hour using silica-blue gel prior exposure to the storage temperatures. Letters (a-m) indicate statistical significance ($p < 0.05$), where treatments indicated by the same letter are not significantly different (n = 5, means ± SE, ANOVA).

4.4 DISCUSSION

Pollen is essential for seed production and serves as primary means of gene flow among desirable genotypes. The knowledge of the viability status of pollen is required to perform crosses, and pollen viability can be reduced by factors that act during development or during the transport from the anther to stigma. However, the availability of data is limited in understanding pollen viability and storing sugarcane pollen. The currently used pollen viability test (starch-iodine stain) in most sugarcane breeding programmes has been found to have a major drawback i.e. it cannot distinguish between viable and non-viable pollen (Melloni *et al.* 2013). Prior to pollen viability determination, it is also vital to obtain the optimal time of day to collect pollen to prevent under- or over-estimation of viability during crossing. As asynchronous flowering is still a challenge for sugarcane breeders, pollen storage could potentially be an efficient method to overcome barriers to hybridisation between plants flowering at different times or growing in different regions. For those reasons, this study focused on three aspects, *viz*. (a) finding an accurate estimate of viability; (b) finding an optimal time of day of pollen shed; and (c) prolonging pollen viability.

Viability testing has a direct impact on the quality of the crosses because fertile pollen will increase the chance of cross-pollination, thereby resulting in the production of seed (Melloni *et al.* 2013). *In vitro* pollen germination is one of the most convenient and reliable methods used to test the viability of fresh or stored pollen in various crops (Rodriguez-Riano and Dafni 2000). It is a valuable tool for understanding the complexities underlying the germination process (Rodriguez-Riano and Dafni 2000). The media composition used for pollen germination varies according to the plant species (Wang *et al.* 2004). From the current results, Medium 3 consisting of sucrose (300 g/l), boric acid (0.1 g/l), calcium nitrate (0.3 g/l), magnesium sulphate (0.1 g/l) and agar (10 g/l) was the best medium for pollen germination (Figure 4.4). Similar findings were observed by Melloni *et al.* (2013). According to Patil *et al.* (2013), the addition of polyethylene glycol in Medium 5 could prevent membrane rupturing acting as osmoticum for the pollen grains. However, for this study it caused a decline in germination and in pollen tube growth.

Pollen staining techniques have been preferred for viability testing over *in vitro* and *in vivo* germination in many breeding programmes because they are quick and easy to use (Melloni *et al.* 2013). The comparison between the staining techniques and *in vitro* germination showed that all the stains tested over-estimated viability in comparison with *in vitro* germination (Table 4.3). These findings are in agreement with previously reported work (Rodriguez-Riano and Dafni 2000; Melloni *et al.* 2013). The results indicated that the MTT and FDA-PI stains have the ability to distinguish between viable and non-viable grains and they correlated closely to *in vitro*

germination (Table 4.4 and Appendix 4). As previously proposed by Melloni *et al.* (2013), the unreliability of the starch-iodine stain was supported as starch was detected in aborted/non-viable pollen grains after heated at 100 °C for 8 h. The MTT and FDA-PI stains showed potential as viability detectors as they were similar to the *in vitro* germination method (deemed to be an accurate determination of viability) (Melloni *et al.* 2013). The correlation tests only showed a strong interaction between FDA-PI and *in vitro* pollen germination for C-type 2 hybrid (Table 4.4). However, as the FDA-PI stain requires a fluorescence microscope, it is not suited for evaluating pollen viability for sugarcane breeding as the fast and simple criteria is not met. The MTT stain was classified as the second best stain based on accuracy and was a reliable staining technique for pollen viability determination in this study.

Anthesis time has a profound effect on pollen viability as pollen shedding occurs at specific times (Singh *et al.* 2009). The optimum time periods to collect pollen were to be between 5h00 and 7h00, when the temperature was 21 °C and RH was 56 % (Figure 4.6). These findings were similar to those of Singh *et al.* (2009). The decline in viability after 07h00 is thought to have been associated with water loss and the maintenance of the dehydration state under natural conditions (Melloni *et al.* 2013). For rice pollen, rapid loss of water leads to a sharp drop in viability, by nearly 50 % between 6 and 20 min, after anther dehiscence and pollen shedding (Coast *et al.* 2016). In this study, it is postulated that pollen can remain viable for up to 60 min under the conditions of the SASRI facilities. It is therefore, recommended that pollen should be collected at 1st (5h00) or 2nd (7h00) pollen shed where viability is the highest and the anthers are exposed.

Temperature and other factors like relative humidity are the major elements influencing pollen viability (Kalkar and Neha 2012). Since the development of a controlled pollination programme may be dependent on stored pollen, the third part of the study focused on determining the most suitable temperature regimes for short-term storage (30 days). The optimal *in vitro* medium and the MTT stain were used to determine the viability of pollen stored in the two storage regimes (9 and -20 °C). There was a rapid decline in the viability of pollen after 2 days of storage at -20 °C (Figure 4.7), suggesting the formation of ice-crystals which could have caused rupturing of the membranes thus leading to cell death (Towill and Walters 2000). However, the current findings of pollen showing no viability at -20 °C contradict with those made by Tai (1989) in the United States and Amaral *et al.* (2013) in Brazil who suggested that sugarcane pollen can be stored at -20 °C for short-term storage (30 days). In the current study, sugarcane pollen from the pollen donor can be preserved for a period of 10 days (Figure 4.7 A and B) at 9 °C after drying for an hour using silica blue gel. Freeze drying for pollen of crops such as maize has been successful in

maintaining viability at low temperatures (Kalkar and Neha 2012). Future studies on sugarcane pollen could include the assessment of effectiveness of such an approach.

CHAPTER 5: TOWARDS THE DEVELOPMENT OF BIOTECHNOLOGY METHODS TO SUPPORT CONVENTIONAL SUGARCANE BREEDING AT THE SOUTH AFRICAN SUGARCANE RESEARCH INSTITUTE (SASRI)

5.1 INTRODUCTION

As previously discussed (Chapter 1), modern sugarcane cultivars are derived from the interspecific hybridisation between *S. officinarum* and *S. spontaneum* (D'Hont *et al.* 1995). The high levels of polyploidy, the narrow gene pool of cultivars, problems with the production of fertile pollen and the long progeny selection cycle have imposed difficulties in sugarcane improvement using conventional breeding techniques (Seema *et al.* 2014). At SASRI, breeders have been interested in introgressing genes from wild species (e.g. *S. spontaneum*) and related genera (e.g. *E. arundinaceus*, known to possess a number of traits of agronomic importance including pest and disease resistance and tolerance to drought and water-logging conditions) in an attempt to increase genetic diversity among modern cultivars (Zhou 2013). In spite of numerous efforts towards interspecific/intergeneric hybridisation, little progress has been made, mainly due to variable flowering and cross-incompatibility among some sugarcane species (Zhou 2013). Therefore it is essential to study and adopt strategies to overcome the existing barriers for successful creating hybrids at SASRI.

There are a number of biotechniques that have the potential to address the challenges associated with introgression breeding for example: (a) *in vitro* flowering; (b) protoplast isolation; and (c) molecular detection of introgression hybrids. Initial attempts towards developing these techniques are the focus of this study.

As discussed in Chapters 2 and 3, sugarcane flowering is essential for breeding, particularly in genetic introgression programmes where synchronous inflorescence production is required in order to successfully cross pollinate. Low and variable flowering has been found to occur in temperate conditions such as South Africa. At SASRI, asynchronous flowering of desired parental genotypes is a major drawback in achieving the breeding objectives. Although *in vitro* flowering has only been reported once in sugarcane, the technique has been used in other Poaceae species (Virupakshi *et al.* 2002; Lin *et al.* 2003; Murthy *et al.* 2012). *In vitro* inflorescence production has become a valuable tool in assisting micropropagators to release new species and cultivars into the commercial market more rapidly as synchronised flowering and pollen production can be achieved (Murthy *et al.* 2012). Studies on the Poaceae have reported the reduction of flowering times to a few months by using *in vitro* culture systems (Virupakshi *et al.* 2002; Lin *et al.* 2003).

Another technique that has not been well investigated in sugarcane for creating hybrids is somatic fusion. Crosses between commercial-type hybrids x *Erianthus* are difficult to make and had very low success rates, which has been a serious limitation in the utilisation of the *Erianthus* genus as a potential parental genotype (Mohanraj and Nair 2014). Somatic fusion could be a potential approach in generating intergeneric hybrids. The isolation, culture and regeneration of protoplasts are important steps in somatic hybridisation of other economically valuable plants (Karamian and Ranjbar 2013). The *in vitro* fusion of plant protoplasts prior to regeneration of hybrid plants has been suggested as a technique for introducing greater diversity into plants for breeding (Kao and Michayluk 1974; Duquenne *et al.* 2007). Several papers have been published on sugarcane protoplast isolation and culture (Chen *et al.* 1987; Taylor *et al.* 1992; Aftab and Iqbal 1999; Aftab *et al.* 2002). However, only somatic embryos and not plantlets could be recovered after protoplast fusion (Tabaeizadeh *et al.* 1986) such as in the combination *S. officinarum* (sugarcane) × *Pennisetum americanum* (pearl millet).

After somatic fusion or the more conventional cross-pollination, the identification of successful gene transfer or hybridisation is essential before hybrid progenies can be used further in introgression studies (Cai *et al.* 2005). Molecular markers have been used in sugarcane breeding programmes, such as the R12H16 marker specific for the Bru1 (brown rust resistance) gene, in order to improve the efficiency of the screening process (D'Hont *et al.* 1995; Piperidis *et al.* 2001; Cai *et al.* 2005; Joshi and Albertse 2013). Identification of hybrids using morphological characteristics is inaccurate due to difficulties in distinguishing self-pollinated progeny or those derived from contaminated pollen (D'Hont *et al.* 1995). Simple sequence repeats (SSRs) have been molecular markers of choice to screen hybridity because they are abundant, co-dominantly inherited, and highly reproducible (Pan 2010). For those reasons, SSRs provide a reliable approach for identifying hybrids in sugarcane (D'Hont *et al.* 1995; Piperidis *et al.* 2000). The specific aims of the current study were to: (a) test a published protocol for *in vitro* inflorescence production; (b) establish a protocol for protoplast isolation; and (c) determine hybridity of SASRI introgression crosses using simple sequence repeats (SSRs) for early selection in introgression breeding.

5.2 MATERIALS AND METHODS

5.2.1 Callus initiation and *in vitro* inflorescence production

The immature leaf roll section from cultivar NCo376 was obtained from the SASRI field and embryogenic callus was initiated as described by Snyman (2004). The leaf whorls were washed and decontaminated in ethanol (100 %; v/v) and the outer 2 - 3 leaf sheath layers were removed.

Leaf roll discs (30 per immature leaf roll) 2 - 3 mm thick were cut from the apical meristem region (30 cm) and were aseptically inoculated in an inverted orientation on Murashige and Skoog (1962) (MS) medium (Highveld Biological, South Africa), supplemented with sucrose (20 g/l), 2,4-Dichlorophenoxyacetic acid (2,4-D) (0.003 g/l) and gelled with agar (9 g/l), pH 5.6. The Petri plates were incubated in the dark at ± 26 °C to allow for callus initiation. After three weeks, the embryogenic calli on leaf discs were transferred to MS medium (as above) and supplemented with polyvinylpyrrolidone (0.1 g/l), thiamine hydrochloride (0.001 g/l), myo-inositol (0.1 g/l) and proline (0, 0.04 and 0.06 g/l) (Virupakshi *et al.* 2002). The pH of the medium was adjusted to pH 5.8 prior to autoclaving (15 psi, 121 °C, 15 min) and was dispensed into glass culture vessels (62.4 mm × 62.4 mm × 95.8 mm). The transferred calli were maintained at room temperature (23 °C) under ambient light for 1 week before being transferred to a growth room with conditions set at a photoperiod treatment of 6 h light/18 h dark and temperature at 23 ± 2 °C as per the published protocol (Virupakshi *et al.* 2002). The vessels were kept for 180 days without sub-culturing and monthly observations were made, after which percentage callus mass increase (mass increase / original mass × 100) was calculated.

5.2.2 Protoplast isolation and viability testing

In vitro leaf material of the NCo376 cultivar obtained from the SASRI Biotechnology laboratory was used to isolate sugarcane protoplasts. The leaves (1 g fresh mass) were cut into approximately 1 mm^2 pieces using a surgical blade (size 10A, Lasec). The enzyme solution for the protoplast isolation consisted of cellulase (1 - 4 g/l; R-10; Duchefa Biochemie), pectinase R10 (2 g/l; Sigma-Aldrich), sorbitol (109.3 g/l), KH_2PO_4 (0.14 g/l), $CaCl_2$ (0.11 g/l) and $MgCl_2$ (0.1 g/l) at pH 5.6 (Snyman 1992). The leaf pieces in this medium were incubated in Petri dishes (60 mm × 15 mm) on a shaker (Sorvall®) at 50 rpm for 6 - 12 h at 25 ± 2 ºC. After the cell wall digestion, the cells and enzyme mixture were filtered through a 250 µm nylon sieve followed by centrifugation (Eppendorf®) in glass tubes for 10 min at 100 rpm. The pellets obtained were suspended in a washing solution (as above without the enzymes) and centrifuged twice at 100 rpm for 10 min each. Washing solution (0.5 ml) was added to re-suspend the pellet. The experiment was conducted in triplicate and yield and viability were determined.

The isolated protoplasts were observed using a light microscope (Nikon eclipse 50i; Zeiss) and counted using a Fuchs-Rosenthal counting chamber (0.0625 mm^2). The protoplasts were viewed at 200 X magnification and the number of protoplasts observed were recorded. Yields of protoplasts were calculated using the equation given as protoplast yield (1 g fresh mass) = total cells counted × dilution factor × 10 000 / number of squares counted.

Thereafter, the viability of protoplasts was determined using Evan Blue stain (0.5 g/l; Fisher Scientific) made in sorbitol (0.6 g/l) (Larkin 1976). Images were captured using the Zeiss AxioCam imaging system and were saved on a PC-compatible computer through the AxioVision™ software.

5.2.3 Hybridity analysis using Single Sequence Repeats (SSRs)

a) Crosses, seed germination and seedling transplanting

The cross-combinations were set-up in the SASRI glasshouse by breeders in cubicles as bi-parental crosses (consisting of one pollen donor and one pollen receptor genotype per cubicle). Fourteen days after the cross set-up, the pollen receptor (♀) was taken to the ripening area (21 - 30 °C, > 60 % RH) until mature (when the inflorescence started to wilt) while the pollen donor (♂) was discarded. Seed was harvested by detaching the inflorescence from the stalk and leaving it to dry in an incubator for 24 h at 30 °C. After drying, all the seeds were germinated on moist peat moss in seedling trays and kept in the glasshouse. They were watered daily (approximately 500 ml/tray), fertilized with LAN (limestone ammonium nitrate, Coastal Farmers' Co-operative Ltd., KwaZulu-Natal) and 5:1:5 (N:P:K) weekly and kept at temperatures of 30 °C (day and night). After six weeks, seedlings were transplanted in air-bricks in the nursery outside.

b) Plant material analysed

The plant material consisted of 60 progenies from 7 cross-combinations (between *Saccharum* and *Erianthus*) and a known hybrid obtained from Australia per kind favour N Piperidis (SRA) (Table 5.1).

Table 5.1: List of species and crosses included in the study.

Number	Crosses made (*Saccharum* × *Erianthus*)	Number of progenies germinated and analysed
1	06B0362 + IK76-22	4
2	06B0249 + IK76-22	1
3	87L0573 + IJ76-407	1
4	KF70190 + IJ76-332	12
5	06B0249 + IJ76-407	35
6	05T0245 + IJ76-407	1
7	N40 + IJ76-332	6

c) DNA extraction

The total genomic DNA was extracted from young seedlings (~10 cm high) from 3 mm^2 leaf pieces using two methods, *viz*. (a) DNeasy™ Plant Mini Kit (QIAGEN, Hilden, Germany); and (b) a crude extraction procedure as established and optimised at SASRI (Joshi and Albertse 2013). This procedure was carried out by grinding each leaf piece with a metal rod in an Eppendorf tube in 0.5 M NaOH (300 µl), followed by transferring the supernatant (20 µl) into 1 M Tris base (480 µl; pH 8.0). Extracted DNA was stored at -20 °C until processed for further analysis. The extraction using the kit was done according to the manufacturer's instructions.

The DNA quantity from both techniques was determined using the Nanodrop 1000 Spectrophotometer (Thermo Scientific, RSA). For quantification and purity determinations, absorbance values were measured at 260 and 280 nm wavelengths. The quality of DNA was visually assessed on an agarose (10 g/l) gel prepared in 1X TAE buffer containing SYBR® green I Nucleic Acid Gel Stain (0.5 µg/ml; Invitrogen™) and visualised under 302 nm ultraviolet light.

d) SSRs, PCR, GeneScan and data analysis

Hybrids were identified from the germinated seedlings using Single Sequence Repeats (SSRs) primers based on the length polymorphism of the 5S rDNA spacer between *Saccharum* and *Erianthus* (Piperidis *et al.* 2000). Conditions for amplification and electrophoresis of the 5S spacer region were essentially as described in D'Hont *et al.* (1995) with modifications (below).

The PCR was conducted in a final reaction volume of 25 µl [DNA template (25 ng), dNTP (0.2 mM of each), $MgCl_2$ (25 mM), Taq buffer (1.5 µM), BSA (20 µM), primer pair (6 µM of each), and 1 Unit Taq DNA polymerase]. The primer pair was used for this work were as described in

D'Hont *et al*. (1998) where the sequences were as follows: 5' GTGACC-TCC-TGC-GAA-GTC-CT 3' (forward primer) and 5' CCC-ATC-CGTGTA-CTA-CTC-TC 3' (reverse primer). Thermal cycling conditions consisted of one 5 min cycle at 94 °C followed by 35 cycles of 30 s at 94 °C, 15 s at 55 °C, and 30 s at 72 °C in a Gene Amp® PCR System 2700 thermocycler. A negative or no template control, in which DNA was omitted, was included in every PCR run. The PCR mixture was diluted 10-fold and 1 µl of the PCR dilution was mixed with GeneScan™ 600 LIZ® Size Standard (0.5 µl; Applied Biosystems) and HI DI™ Formamide (8.5 µl; Applied Biosystems). The fragment analysis mix was denatured at 95 °C for 5 min and flash cooled on ice. The fragment analysis mix was resolved on the ABI 3500 genetic analyser (Applied Biosystems). Electropherogram visualisation and analysis was performed using SoftGenetics GeneMarker™ software Version 2.4.0 (SoftGenetics LLC).

5.3 RESULTS

5.3.1 *In vitro* inflorescence production from callus cultures

The effect proline on *in vitro* inflorescence production from embryogenic callus of cultivar NCo376 was investigated medium with three different proline concentrations (1, 40 and 60 mg/l proline). No *in vitro* inflorescence production was observed after 180 days in culture for all proline concentrations tested. In all media formulations, the leaf roll discs formed two types of callus, *viz*. (a) embryogenic calli; and (b) non-embryogenic calli (Table 5.2). The formation of embryogenic callus was evident at the lower concentrations of proline (0 and 40 mg/l) whereas at the higher concentration (60 mg/l proline), non-embryogenic, mucilagenous callus was produced (Table 5.2). At the higher proline concentration, percentage callus mass increase over 6 months was the highest whereas the media without proline had the lowest (50.48 ± 12.36 and 17 ± 2.14, respectively; $p < 0.05$, Table 5.2).

Browning of the explants and surrounding media was visible 2 - 3 days after explants were initially transferred to the media regardless of their composition. Phenolic production was evident during the first 30 days in culture and ceased thereafter. Microbial contamination was minimal in all concentrations tested and out of the 60 leaf roll discs cultured, only 10 % had bacterial contamination after 6 months (Table 5.2). At 40 mg/l proline concentration, precocious embryogenic germination was observed in 5 % of the vessels after 90 days in culture. Due to time constraints no further studies were undertaken.

Table 5.2: A summary of the effect of proline on callus culture for the production of inflorescences *in vitro*. Scale bars = 10 mm

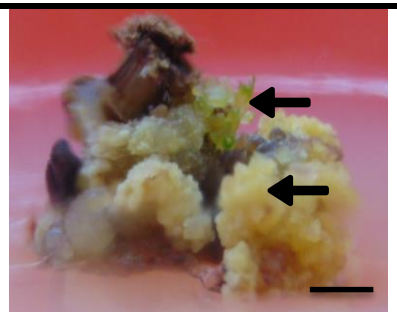
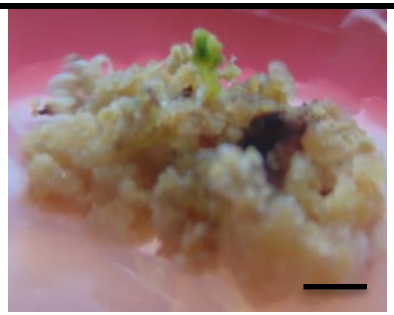
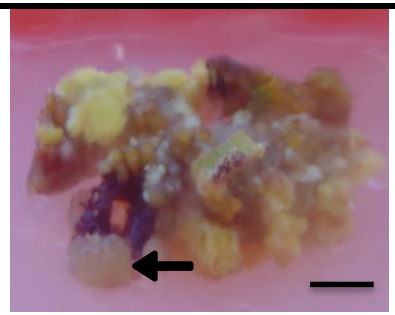
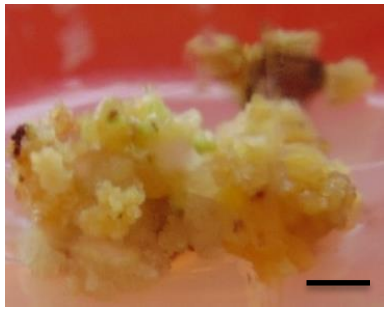
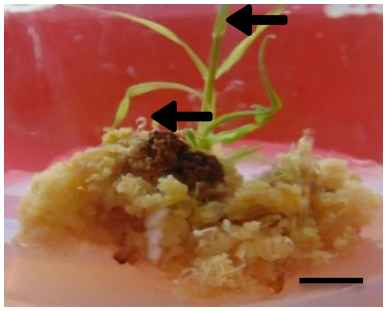
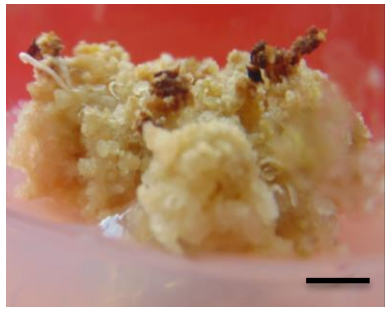
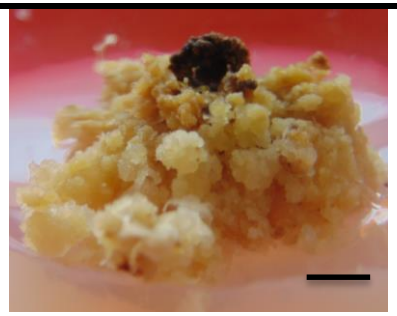
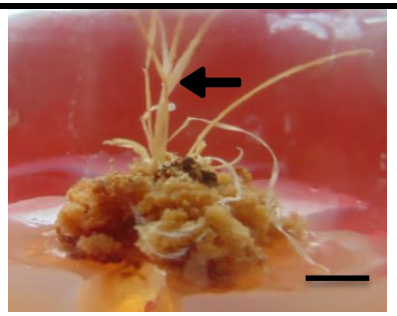
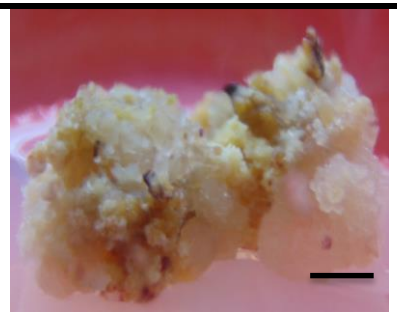
Parameters measured over 6 months	**Time (days)**	**Characteristics of callus over time**		
		Proline (mg/l)		
		0	**40**	**60**
Description of callus appearance	30	 New growth (green shoot, arrow), embryogenic callus (yellow, arrow)	 Embryogenic callus, new growth around edges of callus mass	 Non-embryogenic callus (mucilagenous, crystalline and opaque, arrow)
	90	 Increased proportion of embryogenic callus compared with previous observation, red spots evident, medium drying out and cracking	 White shoot-like structure (arrow), medium drying, red spots, one jar with a green shoot (5 % precocious embryogenic germination)	 Increased proportion of non-embryogenic callus compared with last observation (white and opaque), white shoot structures, medium drying out and cracking

Table 5.2 cont.

Parameters measured over 6 months	**Time (days)**	**Characteristics of callus over time**		
		Proline (mg/l)		
		0	**40**	**60**
	180	 Increased proportion of embryogenic callus compared with previous observation, medium drying out and cracking	 Dead shoot (from precocious germination, arrow), browning of the explant, medium drying out and cracking	 Increased porportion of non-embryogenic callus compared with previous observation (white and opaque), medium drying out and cracking
Percentage microbial contamination (%)	30	0	0	10 (white/pink bacterial)
	90	0	10 (white/pink bacterial)	5 (white/pink bacterial)
	180	0	5 (white/pink bacterial)	0
Average percentage fresh mass increase over time † (%)	-	17 ± 2.14	19.58 ± 2.11	50.48 ± 12.36

† means ± SE, n = 20

5.3.2 Protoplast isolation

In an attempt to isolate mesophyll protoplasts of high quantity and quality, four concentrations of cellulase (1, 2, 3 and 4 g/l) were assessed (Snyman 1992). The effect of cellulase concentration on protoplast yield and viability was monitored. Protoplasts from mesophyll tissue were spherical and rich in chloroplasts that were randomly distributed in the cytosol (Figure 5.1A). Protoplast size was between 24.9 to 30.39 µm (Figure 5.1A). Non-viable protoplasts were seen as a dark-blue colour when stained with Evans blue which penetrated the ruptured protoplast membrane (Figure 5.1B). The enzyme mixture digested the cell walls and released the protoplasts between 8 - 12 h (Khan *et al.* 2001) of incubation (Figure 5.1C). Figure 5.1D and E show isolated bundle sheath strands from the enzymatic degradation.

Protoplast yields ranged from $1.33 \times 10^5 \pm 0.12$ to $5.4 \times 10^5 \pm 0.40$ per gram fresh leaf material with viability percentages ranging from 70.16 ± 1.75 to 91.53 ± 0.55 (Table 5.3). By increasing the enzyme concentration from 1 to 4 g/l cellulase, there was an increase in the protoplast yield and viability (Table 5.3). High significant ($p < 0.001$) interactions were observed among protoplast yield and viability for the four cellulase concentrations tested. A cellulase concentration of 4 g/l resulted in the highest yield ($5.4 \times 10^5 \pm 0.40$ protoplasts/g f. weight) and at 1 g/l cellulase the lowest ($1.33 \times 10^5 \pm 0.12$ protoplasts/g f. weight) (Table 5.3). The viability percentage with Evans Blue with 4 g/l cellulase concentration was the highest (91.53 ± 0.55 %). It was observed that high concentrations of pectinase (> 3 g/l) resulted in low yields and viability (results not shown). Since the yield and viability of protoplasts were high, the medium composition was not modified.

Table 5.3: The effect of cellulase concentration (g/l) on the yield and viability of mesophyll protoplasts isolated from NCo376 leaf material grown *in vitro*. Viability was determined using the Evans Blue stain after 8 - 12 h. Letters (a - d) indicate statistical significance ($p < 0.05$), where treatments indicated by the same letter are not significantly different (means ± SE, n = 3, ANOVA).

Cellulase (g/l)	Protoplast yield/ g. fresh weight	Viability (%)
1	$1.33 \times 10^5 \pm 0.12^a$	70.16 ± 1.75^a
2	$2.17 \times 10^5 \pm 0.17^b$	70.65 ± 1.67^a
3	$4.67 \times 10^5 \pm 0.32^c$	86.29 ± 2.86^b
4	$5.4 \times 10^5 \pm 0.40^d$	91.53 ± 0.55^b

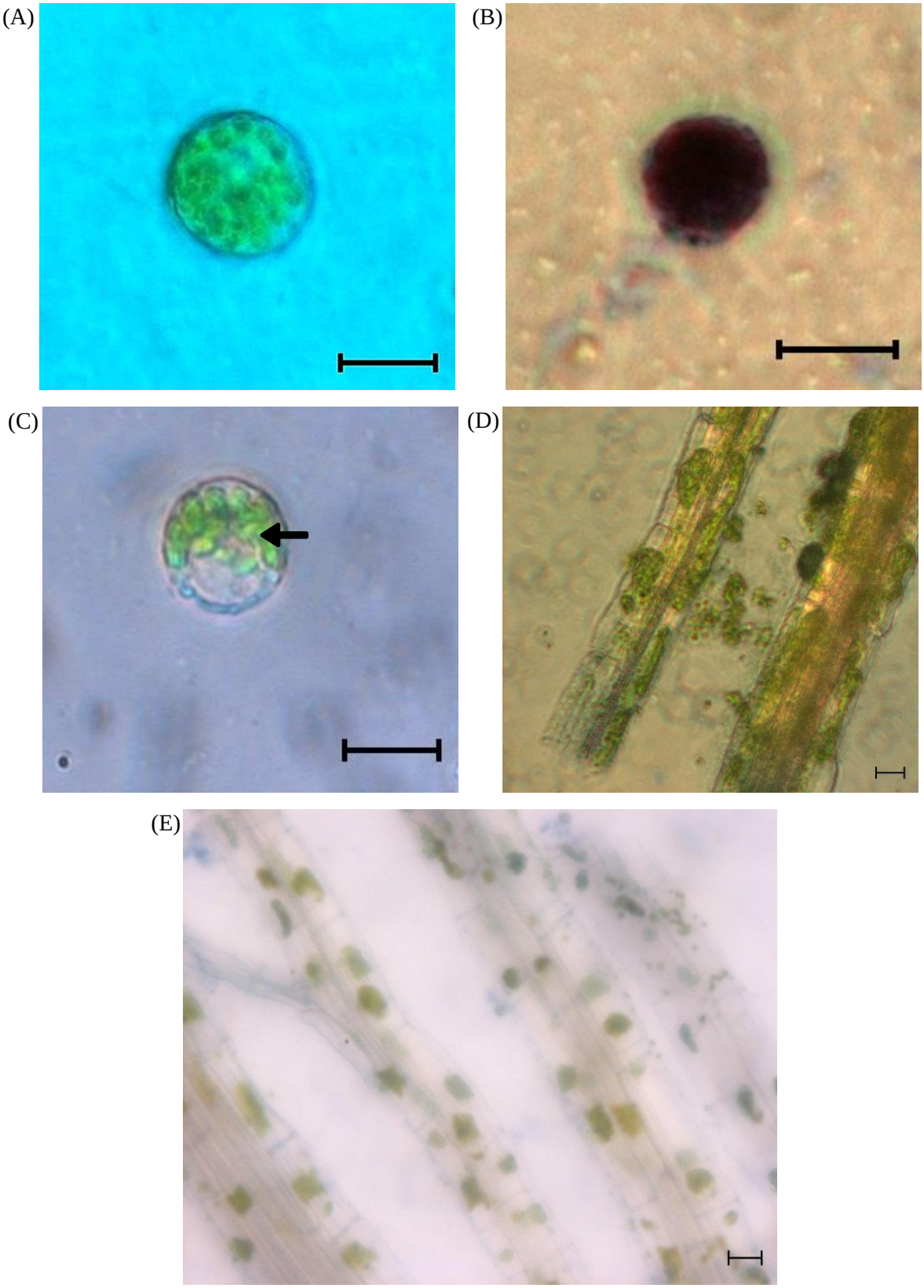


Figure 5.1: Microscopic images of sugarcane protoplasts isolated from *in vitro* leaf material stained with Evans blue. (A) viable protoplast, (B) non-viable protoplast, (C) protoplast with numerous chloroplasts in the cytosol (arrow), (D) digested bundle sheath strands, (E) 400X magnified isolated bundle sheath strands. Scale bar = 20 µm.

5.3.3 Hybridity screening

a) Comparison of DNA extracted using a crude method and a commercial kit

Two genomic DNA extraction methods were assessed, *viz*. crude and kit extraction to identify a simple method to be used for hybridity testing by determining the yield, purity and quality of the DNA using each method. The kit extraction method yielded more genomic DNA compared with the crude extraction method for all genotypes tested (175 - 330 ng/µl and 18.88 - 40.59 ng/µl, respectively) (Table 5.4). The kit extraction method resulted in genomic DNA of high purity and quality (Figure 5.2A). On the other hand, the DNA extracted from the crude method has some sheared DNA, shown as smears on the agarose gel (Figure 5.2B). The DNA shearing could indicate degradation of DNA or RNA/polysaccharide contamination (Wilkie *et al.* 1997).

The drawback with the kit extraction method for hybridity screening is that the method is expensive and time consuming compared with the crude method. Therefore, even though the crude extraction procedure produced low quality DNA, it was selected as a method for hybrid determination by SSRs as it is rapid, simple and cheap, and only requires 5 ng DNA per PCR reaction.

Table 5.4: A comparison of the yield of DNA using the kit versus the crude extraction. DNA concentration was determined using the NanoDrop spectrophotometer.

Genotypes	**DNA yield (ng/µl)**	
	Kit extraction	Crude extraction
IK7622	221.5	18.88
06B0362	310.51	34.5
Progeny 1	321	24.99
Progeny 2	175	23.81
Progeny 3	330	40.59
Progeny 4	276	27.5

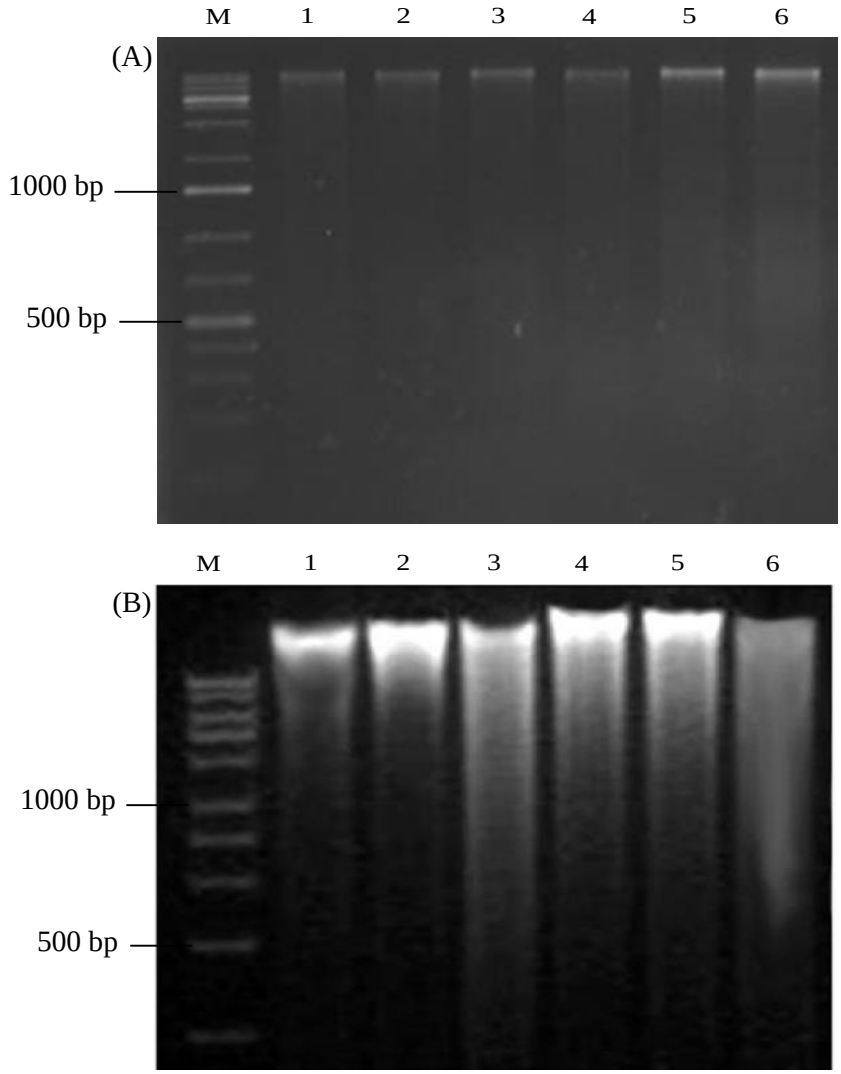


Figure 5.2: A comparison of DNA extraction using two methods, *viz.* (A) DNeasy™ Plant Mini Kit and (B) crude extraction method used at SASRI. M = 100 bp marker, lane 1 = pollen donor, lane 2 = pollen receptor and lanes 3 - 6 = potential hybrid-progenies.

b) DNA fragment analysis using SSRs

There were seven cross-combinations of intergeneric crosses between commercial-type hybrids and wild type *E. arundinaceus* that were made during the 2014 crossing period. One SSR primer pair was used for screening to determine whether cross-pollination had successfully occurred (D'Hont *et al.* 1995). In the present study, the positive hybrid control contained both alleles and was therefore identified as a hybrid (Figure 5.3A). This proves that current method is reliable and reproducible and could be further used for early selection of introgression progenies in the plant breeding programme. From the 60 seedlings from crosses between commercial-type hybrids and *E. arundinaceus*, no intergeneric hybrids were identified as both alleles, one from the pollen donor

(475 bp) and the other from the pollen receptor (303 bp) were not present in all progenies tested (Figure 5.3) thus these were classified as self-pollinated progenies.

Table 5.5: The number of seedlings and the number of hybrids retrieved from intergeneric crosses between commercial-type species and *E. arundinaceus*.

Pollen receptor (*Saccharum* hybrid)	**Pollen donor (*Erianthus* spp.)**	**Number of seedlings screened**	**Number of hybrids detected**
06B0362	IK76-22	4	0
06B0249	IK76-22	1	0
87L0573	IJ76-407	1	0
KF70190	IJ76-332	12	0
06B0249	IJ76-407	35	0
05T0245	IJ76-407	1	0
N40	IJ76-332	6	0
Genotype A*	Genotype B*	1	1

*Control - a known hybrid obtained from Australia per kind favour, N Piperidis (SRA)

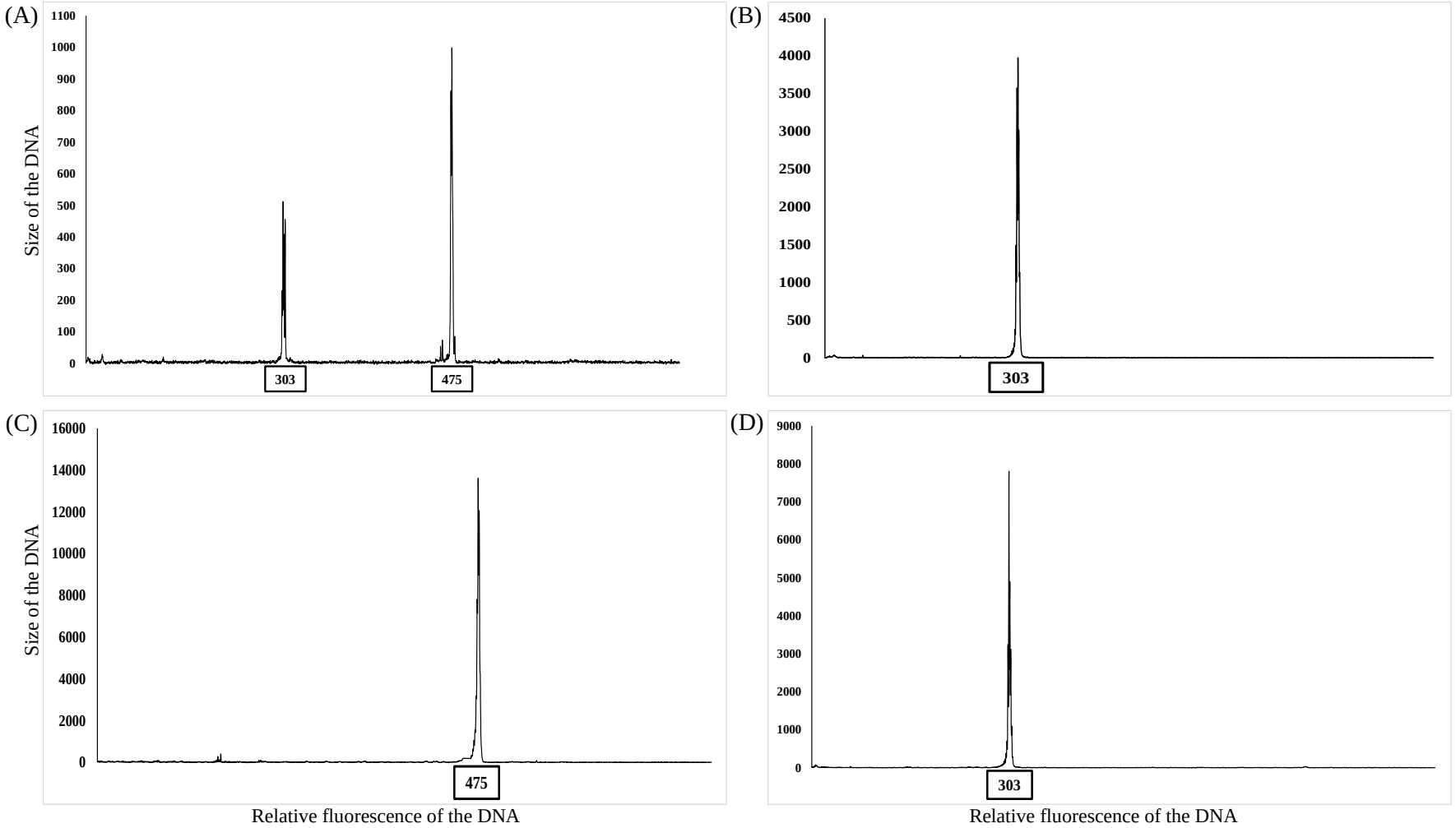


Figure 5.3: Electropherograms showing SSR amplicons from parental genotypes and selected progeny. (A) positive control (hybrid) showing 2 amplicons, one from *Saccharum* spp. and one from *Erianthus* spp., (B) *Saccharum* spp. (303 bp amplicon), (C) *Erianthus* spp. (475 bp amplicon) and (D) resultant non-hybrid-progeny (303 bp amplicon).

5.4 DISCUSSION

As discussed throughout, attempts by sugarcane breeders are being made in order to achieve flower synchronism. An alternative biotechnology approach could be that of the production of inflorescences *in vitro* using sugarcane tissue culture systems even though success has been limited to a single report by Virupakshi *et al*. (2002). In the current study, no *in vitro* inflorescence production was observed after 6 months of callus cultured on MS medium supplemented with proline as per the published protocol.

The induction of flowering is a complex process and there is no universal combination of plant growth hormones that induce flowering in tissue culture (reviewed by Murthy *et al.* 2012). From the media tested in this study, the addition of proline was found to stimulate an increase in callus production over time (Table 5.2) but had no effect on inducing *in vitro* inflorescence production. Future work could include optimising media components such as growth regulators 6-benzylaminopurine (Mudoi *et al.* 2013), thidiazuron (Murthy *et al.* 2012) and 1-naphthaleneacetic acid (Devi *et al.* 2000) and light exposure (Murthy *et al.* 2012) to induce flowering as an approach to overcome asynchronous flowering.

Given that sugarcane flowering is variable in South Africa (Horsley and Zhou 2013), an efficient protocol for *in vitro* flowering will be beneficial for breeding to overcome these barriers. *In vitro* flowering in various crops including sugarcane could offer a reduction of the breeding cycle in terms of time and would allow for synchronised flowering of desired parental genotypes in order to achieve breeding objectives. A study conducted by Nandagopal and Ranjitha Kumari (2006) reported that *in vitro* flowering of *Cichorium intybus* L. (belonging to the Asteraceae family) was initiated after 45 days in culture, a process that would have taken about 521 days under the normal breeding cycle. If such a breakthrough was to be developed for sugarcane, it could reduce labour costs and optimise space required for sugarcane crossing (Goldman *et al.* 2010). Once *in vitro* methods are standardized for obtaining flowering, this technology can be used for attempting hybridisation between sugarcane species and related genera (e.g. *Erianthus*).

The process of protoplast isolation is the first step to establish for protocols for somatic fusion culture (Piwowarczyk and Pindel 2015). In the current study, a method was established to determine the optimum enzyme concentration needed to isolate a sufficient number of viable mesophyll protoplasts for further protoplast culture research. Results showed that a cellulase concentration of 4 g/l was ideal to obtain high yields and viability ($5.4 \times 10^5 \pm 0.40$ protoplasts/g f. weight and 91.53 ± 0.55 %, respectively; Table 5.3) of mesophyll protoplasts. According to

Davey *et al*. (2005), protoplasts are usually cultured at an initial plating density of 5×10^4 to 1×10^6 protoplasts/ml indicating that the protoplast yield obtained from the current study would be sufficient to be used further for protoplast fusion and subsequent culture. Similar results were reported by Khan *et al.* (2001) where a mean yield of 1.5 to 2×10^5 protoplasts/ml from sugarcane and Sun *et al.* (2013) 1 to 7×10^6 protoplasts/ml from maize, wheat and rice.

Successful protoplast fusion and plant regeneration will permit gene transfer between sexually incompatible genotypes (Harris *et al.* 1988; Davey *et al.* 2005). Protoplasts can be induced to divide and regenerate into plants with a high efficiency over a broad range of osmotic conditions (Merrick and Fei 2015). High yields of viable protoplasts will allow for protoplast fusion and regeneration to potentially improve the genetic make-up of the hybrid plant. As technical improvements have been made in hybrid formation, more interspecific and intergeneric fertile hybrids among the Poaceae species through somatic fusion and subsequent hybrid regeneration have been reported (Xia *et al.* 2003; Ge *et al.* 2006; Vasil and Vasil 2012).

Numerous studies have reported on successful protoplast fusion for Poaceae species (Tabaeizadeh *et al.* 1986; Falco *et al.* 1996; Durieu and Ochatt 2000; Aftab *et al.* 2002). Durieu and Ochatt (2000) demonstrated that chemical fusion using polyethylene glycol (PEG) is more efficient and reproducible compared with electrofusion for pea (*Pisum sativum*) and grass pea (*Lathyrus sativus*). In a study conducted by Xia *et al.* (2003), it was reported that protoplasts from *Triticum aestivum* L (wheat) and *Agropyron elongatum* (tall wheatgrass) were fused using PEG to produce fertile intergeneric somatic hybrid plants that were propagated in successive generations. In sugarcane, Aftab *et al*. (2002) reported protoplast electrofusion between two commercial cultivars of *Saccharum* spp. hybrids (CoL-54 and CP-43/33) which formed microcallus. However, the limitation was the inability of the hybridised microcallus to form plants.

Plant regeneration from protoplasts has also been demonstrated for Poaceae species (Davey *et al.* 2005). Harris *et al*. (1988) reported the regeneration of plantlets from cultured wheat (*T. aestivum* L.) protoplasts isolated from anthers. Similarly, Ge *et al.* (2006) showed that 108 plantlets were produced from protoplasts between common wheat (*T. aestivum* L.) and Italian ryegrass (*Lolium multiflorum* Lam.), but only 14 survived until maturity. Both protoplast fusion and regeneration for sugarcane could offer an opportunity to optimise parameters such as medium composition and physical factors (light and dark exposure) in order to maximise plantlet formation from protoplasts.

Once a hybrid has been created, be it from conventional cross-pollination or somatic fusion, it needs to be confirmed as containing genetic material from both parental genotypes. There are difficulties in identifying hybrids from self-progenies or progenies arising from using pollen contamination using morphological methods. Simple sequence repeats (SSRs) have been implemented to accurately screen the putative progenies for hybridity (Singh *et al.* 2014). In the current study where crossings were made between commercial-type hybrids and *Erianthus*, potential hybrids were screened using the method of Piperidis *et al.* (2000). All tested progenies showed the presence of only one amplicon, specific to identifying a *Saccharum* spp. hence, the resulting progenies were from self-pollinated seed (Figure 5.3).

There were two possible reasons for the failed cross-combination between *Saccharum* and *Erianthus*, *viz*. (a) pollen-pistil incongruity of the two genera (D'Hont *et al.* 1995); or (b) pollen sterility (Piperidis *et al.* 2000). These observations emphasize the importance of using molecular marker technology to validate hybrid creation from attempts to cross-pollinate commercial-type hybrids and wild species/related genera for sugarcane breeding.

Therefore this present study demonstrated that the SSR molecular approach to identify hybridity i.e. the presence of *Saccharum*- and *Erianthus*-specific DNA marker, could be applied in sugarcane breeding for identification of interspecific or intergeneric crosses.

CHAPTER 6 CONCLUDING REMARKS AND FUTURE WORK

6.1 FLOWERING TRENDS AND POLLEN VIABILITY METHODS FOR INTROGRESSION BREEDING

In South Africa, there is a low incidence of flowering and infertile pollen is produced under field conditions (Zhou 2013). This resulted in the establishment of heated growth chambers with photoperiod treatments. For the analyses of 19 years of observations and experiments in the present study, it was found that heated facilities (glasshouse and photoperiod house) in South Africa have contributed significantly towards increasing the production of fertile pollen and flowering through artificial photo-induction. Photo-induction within the facilities has been done by using six treatments, three in the glasshouse (G) and three in the photoperiod house (P). Our findings showed that the genotypes in P treatments produced more fertile pollen and flowered later in the year (53 - 64 % viability and 179 - 188 days to flowering) than in the G treatments (39 - 51 % viability and 158 - 183 days to flowering). Since sugarcane pollen is only viable for 20 min, it is desirable that genotypes to be used as pollen donors should flower later than genotypes used as pollen receptor as stigma receptivity generally lasts for 7 days.

The historical data study highlighted the importance of the photoperiod treatments 'spreading' the crossing season from April to July but synchronisation of desirable parental genotypes has not been well established. There were some potential introgression crosses that could be made between the commercial-type hybrids and *S. spontaneum* / *Erianthus* / *S. robustum* / F_1 hybrid based on their flowering times and pollen fertility. Genotypes chosen as pollen receptors emerged earlier than the pollen donors and the trend was consistent over the years. The major finding that has negative consequences for the crossing programme is that the flowering times between the desired pollen receptors and pollen donors were wide apart (> 10 days) thus limiting the chances of successful cross-pollination using wild (*S. spontaneum*) and related genera (*Erianthus* spp.) relatives. For example, some crosses between commercial-type hybrids and *S. spontaneum* or *Erianthus* species were difficult to achieve as commercial-type hybrids were late-flowering genotypes (181 to 199 days) while *S. spontaneum* and *Erianthus* were intermediate-flowering genotypes (162 to 181 days). This limitation severely hampers progress in introgression crossing.

Although the analyses of historical data provided valuable information regarding the type and the number of cross-combinations that could be achieved, there are still barriers that exist in flower synchronisation of parental genotypes. The information generated in the present study on pollen viability and flowering attributes will be very useful for the sugarcane breeders to efficiently plan and execute the crossing programme by allocation of genotypes in the appropriate photoperiod

treatments. The management of the factors that govern flowering i.e. day-length, relative humidity and temperature can enable breeders to obtain flowering genotypes at any period of the year, thus promoting flowering synchronism between desired parental genotypes and allowing a better planning of ideal crosses.

An optimal solution for maximum *in vitro* germination of sugarcane pollen was investigated in order to evaluate percentage pollen germination as an indicator of pollen viability. In the current study, the best *in vitro* pollen germination medium was found to contain sucrose (300 g/l), boric acid (0.1 g/l), calcium nitrate (0.3 g/l), magnesium sulphate (0.1 g/l) and agar (10 g/l). This medium was then used to test the viability of both fresh and stored pollen of the genotypes used in the present investigation, thereby allowing generalised procedures for pollen viability testing. Pollen staining techniques have been preferred for viability testing over *in vitro* and *in vivo* germination because they are quick and easy to use in many breeding programmes. Our findings suggested that pollen viability testing in sugarcane breeding should be assessed using the MTT stain as it closely correlated to *in vitro* pollen germination. In addition, the MTT stain is easy to carry out and takes up to 5 min for colour development at 30 °C. The current starch-iodine stain which is commonly used in sugarcane breeding was found to overestimate pollen viability and could not distinguish between viable and non-viable pollen grains. From this observation, the starch-iodine stain is an inaccurate estimation of pollen viability. Inaccurate results obtained from pollen viability testing will result in incorrect classification of genotypes as pollen donors or pollen receptors leading to low seed-set. Future research for pollen viability testing should be investigating the proportions of resultant seed from crosses made after pollen viability was tested using the MTT stain compared with the conventionally used starch-iodine stain.

Sugarcane pollen should be collected at 5h00 or 7h00 prior to pollen viability testing as viability was the highest (~30 %) during these time periods. After these time periods, pollen viability declines rapidly due to an increase in temperature and a decrease in relative humidity. Based on the results obtained in the current study, a general recommendation for pollen storage can be made: storage at 9 °C can be useful in the SASRI sugarcane breeding programme where the pollen of the pollen donor can be stored for 10 days before pollinating the pollen receptor. An optimised protocol for sugarcane pollen storage is useful for overcoming asynchronous flowering. However, differences in pollen viability among genotypes in relation to time of the day for collection could be observed in their performance in low temperature conditions.

Collectively, future studies could include: (a) altering of the photoperiod treatments (e.g. day-lengths) to synchronise flowering; (b) establishing other techniques such as pollen storage in order manage pollen availability for making desired crosses and; (c) *in vivo* pollination using the stored pollen from different donors and the resultant plants could be analysed with SSR markers to confirm the hybridity of the crosses.

6.2 BIOTECHNIQUES WITH POTENTIAL TO SUPPORT CONVENTIONAL BREEDING

Conventional breeding requires supplementary methods in order to achieve the breeding objectives of increasing genetic diversity among breeding populations. Apart from mutation breeding, *in vitro* methods also can enable the creation of genetic variation among species, for example *in vitro* inflorescence production, protoplast isolation and hybridity screening using SSRs.

In vitro flowering has provided an alternative method for overcoming asynchronous flowering for a number of crops. In the present study, no *in vitro* inflorescences were produced after 6 months in the callus cultures. For pearl millet, *in vitro* flowering was achieved on MS medium supplemented with high levels (4 mg/l) of 6-benzylaminopurine (Devi *et al.* 2000). An optimised protocol for *in vitro* flowering is important to obtain for sugarcane as it could provide a means to synchronised flowering, a reduction in the normal breeding cycle and it could facilitate an understanding the physiology of flowering. In future, *in vitro* flowering of sugarcane research could include: (a) manipulation and optimisation of the concentration of plant growth regulators (such as 6-benzylaminopurine, thidiazuron and 1-naphthaleneacetic acid); and (b) light exposure of 16 h light and 8 h dark conditions based on previous studies to successfully induce floral development for sugarcane genotypes.

Isolated protoplasts are the first step in facilitating new recombinants to be obtained by protoplast fusion and subsequent plantlet regeneration. In the present study, protoplast yield ($5.4 \times 10^5 \pm 0.4$ protoplasts/g f. weight) and viability (91.53 ± 0.55 %) at 4 g/l cellulase was found to be ideal based on similar findings from literature, thus the medium will be suitable for future protoplast fusion and regeneration research. After isolating sugarcane protoplast using the current mentioned protocol, chemical fusion (Mishra *et al.* 2015) using polyethylene glycol or electrofusion (Aftab *et al.* 2002) could be attempted. Subsequently, the suspensions could then be cultured in an optimised medium for the formation of hybrid plants of incompatible sugarcane genotypes. For

example, Nayak and Sen (1991) reported successful formation of plantlets derived from protoplast suspensions of *Paspalum scrobiculatum* L. (kodo millet).

Another technique investigated in this study was that of SSRs because they have been described as an effective diagnostic tool for hybrid identification among interspecific and intergeneric crosses (Padmanabhan *et al.* 2015). In the present study, no hybrid was identified in the 60 progenies tested using the 5S rDNA primer pair (D'Hont *et al.* 1995). The problems of hybrid identification has now been overcome with the use of a simple and efficient PCR on intact leaf tissue to allow for hybrid seedlings to be identified within six weeks of germination (Piperidis *et al.* 2000). The PCR-based primers have been developed which target 5s rDNA sequences shown to be polymorphic between the *Saccharum* and *Erianthus* genomes (D'Hont *et al.* 1995; Harvey *et al.* 1998). In the current study, the PCR-based technique is advantageous as it uses small pieces of leaf material as a source of genomic DNA thus is less time consuming. In addition, the non-hybrids can be discarded immediately thereby allowing for savings in resources, especially time and space. Despite the numerous efforts of plant breeders, intergeneric crosses between *Saccharum* and *Erianthus* have been difficult to produce because of pollen-pistil incompatibility of the two genera (D'Hont *et al.* 1995). For example, a study conducted by Piperidis *et al.* (2000), showed that out of 808 seedlings from crosses between commercial-type hybrids and *E. arundinaceus*, no hybrid was identified. Further, out of 520 seedlings from crosses between *S. officinarum* and *E. arundinaceus*, 37 hybrids were identified and only 19 survived (Piperidis *et al.* 2000). Future investigation could include: (a) testing crosses between *S. officinarum* and *E. arundinaceus* created at SASRI using the rDNA primer pair and (b) upon obtaining hybrids, they could be added to field trials for further analysis of incorporated genetic traits.

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APPENDICES

APPENDIX 1

Summary of F-values and probability (Pr > F: F-probability), (ANOVA, n = 429) for pollen viability, natural date to flowering and stage of inflorescence opening.

	Pollen viability (%)		**Natural date to flowering (days)**		**Stage of inflorescence opening (S1-S9)**	
Source	F Value	Pr > F	F Value	Pr > F	F Value	Pr > F
✠PT	7.52	<.0001**	39.66	<.0001**	0.76	0.6237
Genotype	7.20	<.0001**	19.56	<.0001**	3.07	0.0001**
PT*Genotype	1.67	0.0661	1.81	0.0353*	1.55	0.0905
Year	4.82	<.0001**	13.91	<.0001**	1.99	0.0130**
PT*Year	2.67	0.0010**	3.23	<.0001**	0.45	0.9682
Genotype*Year	0.20	0.9620	4.56	<.0001**	1.16	0.3224
PT*Genotype*Year	0.19	0.6629	0.18	0.6688	0.30	0.5819
R^2 value	0.708654		0.804154		0.348098	
CV %	35.44006		5.439622		63.02883	

✠PT - Photoperiod treatments *significant differences at 5% level

**high significant differences at 1% level

APPENDIX 2

Possible cross combinations for introgression (PT by genotype)

Taiwan11 (*S. spontaneum*) ♂		
Photoperiod treatment	**Natural date to flowering**	**Pollen receptors**
G1	155	06B0697 (G2), 06S0746 (G2), 95L0828 (P1)
G2	172	CO285 (BP and G1), 06B0697 (G1), 95L0828 (G2 and P5), 06B1187 (G3), N28 (G3), 07U0537 (P1 and P3)
G3	183	CO285 (G2 and P2), 95L0828 (G2), N28 (G2), 06S0746 (P1), 06B0697 (P3), 95L0828 (P3)
P2	175	CO285 (BP and G2), 95L0828 (G2 and P5), 06B1187 (G3), N28 (G3), 07U0537 (P1 and P3)
P3	181	CO285 (G2), N28 (G2 and G3), 06S0746 (P1), 06B0697 (P3), 95L0828 (G2, P3 and P5)
04X0016 (F_1 hybrid) ♂		
Photoperiod treatment	**Natural date to flowering**	**Pollen receptors**
G1	148	-
G2	160	CO285 (G1), 06B0697 (G2), 06B1187 (G1 and G2), N28 (BP), 07U0537 (G1), 06S0746 (G2), 95L0828 (P1)
G3	182	CO285 (G2 and P2), 06B0697 (P3), N28 (G2 and P1), 06S0746 (P1), 95L0828 (P3)
P2	191	CO285 (P2), 06B1187 (P2), 06G0127 (G2), 95L0828 (G3)
P3	182	CO285 (G2 and P2), 06B0697 (P3), N28 (G2 and P1), 06S0746 (P1), 95L0828 (P3)
IK76-22 (*E. arundinaceus*) ♂		
Photoperiod treatment	**Natural date to flowering**	**Pollen receptors**
G1	171	CO285 (BP), 06B0697 (G1), 95L0828 (P5), 06B1187 (G3), N28 (G3), 07U0537 (P1 and P3)

P1	198	06B1187 (P2), 07U1552 (G3), 95L0828 (P2)

IS76-205 (*E. arundinaceus*) ♂

Photoperiod treatment	Natural date to flowering	Pollen receptors
G2	187	06B0697 (P3), N28 (P1), 06G0127 (G2), 06S0746 (P1), 95L0828 (G3 and P3)

IS76-220 (*E. arundinaceus*) ♂

Photoperiod treatment	Natural date to flowering	Pollen receptors
G2	180	CO285 (BP and G2), 95L0828 (G2), N28 (G2 and G3), 06S0746 (P1), 06B0697 (P3), 95L0828 (P3 and P5)

IK76-417 (*S. robustum*) ♂

Photoperiod treatment	Natural date to flowering	Pollen receptors
G2	186	N28 (G2 and P1), 06G0127 (G2), 06S0746 (P1), 95L0828 (G3 and P3)
G3	186	N28 (G2 and P1), 06G0127 (G2), 06S0746 (P1), 95L0828 (G3 and P3)
P1	198	06B1187 (P2), 07U1552 (G3), 95L0828 (P2)

IM76-227 (*S. robustum*) ♂

Photoperiod treatment	Natural date to flowering	Pollen receptors
G2	195	06B1187 (P2), 06G0127 (G2), 95L0828 (G3 and P2)
P1	204	06G0127 (P3)

APPENDIX 3A

Percentage germination assessed using fresh pollen to determine an optimal sucrose concentration for *in vitro* germination medium (ANOVA)

Source of variation	Degrees of freedom	Mean square	F probability
Genotype	1	382.72	0.030
Sucrose concentration	5	3280.23	<.001
Genotype × Sucrose concentration	5	428.16	<.001
Residual	55	77.14	

APPENDIX 3B

Percentage bursting assessed using fresh pollen to determine an optimal sucrose concentration for *in vitro* germination medium (ANOVA)

Source of variation	Degrees of freedom	Mean square	F probability
Genotype	1	11.68	0.614
Sucrose concentration	5	6673.25	<.001
Genotype × Sucrose concentration	5	14.25	0.902
Residual	55	45.31	

APPENDIX 4A

Percentage germination assessed using fresh pollen to determine an optimal media formulation for *in vitro* germination (ANOVA)

Source of variation	Degrees of freedom	Mean square	F probability
Genotype	1	120.642	0.001
Sucrose concentration	4	1364.202	<.001
Genotype × Sucrose concentration	4	161.687	<.001
Residual	36	9.968	

APPENDIX 4B

Percentage bursting assessed using fresh pollen to determine an optimal media formulation for *in vitro* germination (ANOVA)

Source of variation	Degrees of freedom	Mean square	F probability
Genotype	1	91.576	0.004
Sucrose concentration	4	1531.576	<.001
Genotype × Sucrose concentration	4	98.520	<.001
Residual	36	9.878	

APPENDIX 5

Comparison of staining techniques and *in vitro* pollen germination to determine a reliable stain for pollen viability testing

Source of variation	Degrees of freedom	Mean square	F probability
Genotype	1	2444.82	<.001
Sucrose concentration	5	3424.59	<.001
Genotype × Sucrose concentration	5	230.29	<.001
Residual	44	18.85	

APPENDIX 6

Anthesis time determination to identify the optimal time to collect sugarcane pollen (ANOVA)

Source of variation	Degrees of freedom	Mean square	F probability
Genotype	1	199.556	<.001
Time of day	4	1617.495	<.001
Genotype × Time of day	4	35.495	<.001
Residual	36	4.901	

APPENDIX 7A

Short-term storage of pollen assessed using *in vitro* germination (ANOVA)

Source of variation	Degrees of freedom	Mean square	F probability
Genotype	1	665.167	<.001
Day	8	1823.469	<.001
Genotype × Time	8	72.110	<.001
Residual	68	3.547	

APPENDIX 7B

Short-term storage of pollen assessed using the MTT stain (ANOVA)

Source of variation	Degrees of freedom	Mean square	F probability
Genotype	1	1433.884	<.001
Day	9	1522.894	<.001
Genotype × Time	9	80.252	<.001
Residual	76	3.630	