In vitro generation of somaclonal variant plants of sugarcane (*Saccharum* spp*.* hybrids) for tolerance to toxins produced by *Fusarium sacchari*

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In vitro generation of somaclonal variant plants of sugarcane (*Saccharum* spp*.* hybrids) for tolerance to toxins produced by *Fusarium sacchari*

by

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

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Abstract

The fungus *Fusarium sacchari* (Butler) Gams causes stem rot in sugarcane especially in association with the stem borer *Eldana saccharina* Walker. Sugarcane plants tolerant to *F. sacchari* PNG40 were obtained by chemical mutagenesis and *in vitro* selection during somatic embryogenesis and plantlet regeneration on media containing *F. sacchari* culture filtrates (CF), followed by selection in the greenhouse. Somaclonal variants tolerant to *F. sacchari* PNG40 CF were established by treatment of calli with ethyl methanesulphonate (EMS) and various selection treatments. Investigations were conducted to test the effect of varying CF concentrations and the culture developmental stages (embryo maturation, embryo germination and plantlets) that were most effective in screening calli and plants. Incorporation of CF (0-100 ppm) in the media, at either embryo maturation or germination stages, resulted in significant callus necrosis, and consequent decreased plantlet yield. The highest callus necrosis of 95.55 ± 0.9 % and the lowest plant yield of 1.4 ± 0.45 plants/0.2 g were obtained after inclusion of 100 ppm CF in the germination medium compared with 61.5 ± 3.8 % and 43.8 ± 5.6 plants/0.2 g in the maturation medium, respectively. Exposure of whole plants with trimmed roots to 0-1500 ppm CF resulted in inhibition of root re-growth, with the 1500 ppm CF treatment having the greatest negative effect. Subsequent treatments involved immersing *in vitro* plantlets in varying concentrations of *F. sacchari* conidial suspensions. This resulted in 33.3 % and 100 % mortality with 10^3 and 10^5 conidia/ml treatments, respectively. Control and EMS-treated calli and potentially tolerant regenerated plants were selected using the established CF and inoculation treatments. Plants from EMS treatments displayed more varying root length. More plants with increased root growth, in the presence of CF, were produced from these treatments than from non-EMS treatments, indicating the ability of EMS to induce somaclonal variation. These putative tolerant plants were inoculated with PNG40 and those selected using CF *in vitro* were symptomless whilst the positive controls (plants unexposed to CF) were symptomatic. Re-isolation of *Fusarium* from the inoculated plants and identifying isolates as PNG40 using ISSR analysis confirmed tolerance of the asymptomatic plants and the fungus as the causal agent of the observed symptoms. This confirmed that tolerance to CF correlates to tolerance to *F. sacchari* PNG40. Future work includes testing stability of tolerance in the field and after sexual reproduction, and use of this protocol to produce plants that permit endophytic PNG40 colonisation towards biological control of *E. saccharina.*

FACULTY OF SCIENCE AND AGRICULTURE

DECLARATION 1 - PLAGIARISM

I, **Tendekai Mahlanza**, declare that

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

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Preface

The experimental work described in this thesis was carried out in the Biotechnology Department of the SAn Sugarcane Research Institute (SASRI), Mount Edgecombe, Durban, SA from January 2010 to December 2011, under the supervision of Prof. Paula Watt (UKZN), Dr Sandra Jane Snyman (SASRI and UKZN) and Dr Stuart Rutherford (SASRI).

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

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List of abbreviations

2,4-D	2,4-dichloro-phenoyacetic acid
4- FPA	4-fluorophenoxyacetic acid
A	adenine
AFLP	amplified fragment length polymorphism
ANOVA	analysis of variance
BAP	benzylaminopurine
C	cytosine
CF	culture filtrate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetatic acid
EIM	embryo initiation medium
EGM	embryo germination medium
EMM	embryo maturation medium
EMS	ethyl methanesulfonate
FAO	Food and Agriculture Organisation
FDA	fluorescein diacetate
G	guanine
H	hour(s)
IAEA	International Atomic and Energy Agency
ISSR	inter simple sequence repeats
KZN	Kwazulu-Natal
Min	minute(s)
MS	Murashige and Skoog medium
NAA	naphthaleneacetic acid
PCNB	pentachloronitrobenzene

PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
RAPD	random amplified polymorphic DNA
REML	residual maximum likelihood
RFLP	restriction fragment length polymorhsim
SAMPL	selective amplified microsatellite polymorphism length
SASRI	South African Sugarcane Research Institute
SCMV	sugarcane mosaic virus
S	second(s)
SrMV	sorghum mosaic virus
T	thymine
TBE	tris-borate-EDTA
UV	ultra-violet

1. Rationale and introduction to the study

Sugarcane (*Saccharum* spp*.* hybrids) is an important crop from which the principal sweetener, sugar, is produced. Commercial production of sugar from sugarcane began in India and China approximately 2500 years ago and spread to Western Europe in the eighteenth century (James, 2004). Today, in addition to sugar production, sugarcane and its by-products are used as raw materials in the production of fuel, chemicals, bio-fertilisers, paper and pulp (Almazan *et al.,* 1998) with the possibility of more products being produced through bio-refining (Arruda, 2011). In SA, the sugarcane industry employs 350 000 people and caters for the livelihood of one million people (Maloa, 2001). However, production of the crop is threatened by a number of factors such as pests and diseases.

Sugarcane is negatively affected by diseases largely due to its vegetative propagation through the use of stem sections (setts) and cultivation in large adjacent fields, resulting in the easy spread of disease (Bailey, 2004). Diseases range from bacterial, viral and fungal, as well as pests such as stem borers (Rott *et al.,* 2000). *Fusarium* is one of the fungi that infect sugarcane and causes Fusarium stem rot and Pokkah boeng (Croft, 2000). Fusarium stem rot, caused by *F. sacchari* (Butler) Gams, causes a reddish-brown discolouration of the stem tissue and eventually results in wilting of the plant (Whittle and Irwan, 2000). *Fusarium* is also associated with infestation of sugarcane by the pest (Lepidoptera: Pyralidae) as the borings made by the stem borer allow the fungus access to the stem tissue (Bourne, 1961; McFarlane *et al.,* 2009). Furthermore, *E. saccharina* is a major sugarcane pest, causing significant losses in SA (Downing *et al.,* 2000; Keeping, 2006). Cultivation of resistant varieties is seen to be the best control measure for *Fusarium* spp*.* (Bourne, 1961; Rutherford *et al.,* 1993; Keeping, 2006).

McFarlane *et al*. (2009) studied the relationship between *Fusarium* spp*.* and *E. saccharina* in sugarcane where it was observed that some *Fusarium* isolates were beneficial to *E. saccharina* larval growth and survival. However, they also found that other isolates negatively affected larval growth and survival, including an isolate of *F. sacchari*, PNG40. This antagonistic effect of *F. sacchari* PNG40 on *E. saccharina* suggests that it may be possible to use this fungus as a biological control agent (McFarlane *et al.,* 2009). *Fusarium* spp*.* produce a range of toxins, some of which are known to have insecticidal properties (Logrieco *et al.,* 1998). Nevertheless, as

mentioned previously, some of these toxins are responsible for the pathogenicity of *Fusarium* to sugarcane, e.g. fusaric acid causes wilting (Bourne, 1961). The use of *F. sacchari* PNG40 as a biological control agent is, therefore, limited due to its negative effectson sugarcane. One option to overcome this limitation is to develop sugarcane lines tolerant to *F. sacchari* PNG40, while permitting endophytic colonisation by the fungus. This could allow the use of PNG40 as a biological control agent, without causing damage to the sugarcane.

The generation of genetic variation from which desirable traits can then be selected is one of the prerequisites of any plant breeding program (Brown and Thorpe, 1995). Conventional breeding of disease tolerant varieties has been conducted by crossing lines with desirable agronomic traits with those with disease tolerance (Ming *et al.,* 2006). However, this approach takes 12-15 years to release a new variety due to the complex polyploid nature of sugarcane, which may result in desired genes being masked by alleles at the same locus in other chromosome sets (Butterfield *et al.,* 2001) and by the production of large numbers of clones that need to be screened over long periods (Barnes, 1964). Genetic engineering is another approach that can be utilised to control *Fusarium* spp.(Munkvold *et al.,* 1997; Funnell and Pedersen 2006; Makandar *et al.* 2006; Ramgareeb and Rutherford, 2006), but limitations in the form of transgene silencing (Manners and Casu, 2011) and legislation (Burnquist, 2006) hamper the use of this approach in sugarcane. Another approach is the use of *in vitro* culture techniques to generate somaclonal variation, which has the advantage of enabling selection of plants with desirable traits over a relatively short period of time (Duncan and Widholm, 1990).

In vitro culture techniques have been widely applied in micropropagation (Lee, 1987; Baksha *et al.,* 2002), virus elimination (Snyman *et al.*, 2005; Ramgareeb *et al.,* 2010), genetic transformation (Snyman, 2004; Shah *et al.,* 2009), and germplasm preservation (Taylor and Dukic 1993; Watt *et al.,* 2009). Larkin and Scowcroft (1981) described genetic variation as a result of spontaneous mutations occurring during *in vitro* culture (somaclonal variation) and realised its potential application in crop improvement. Since then, somaclonal variation has been successfully used in crop improvement (Breiman *et al.,* 1987, Van den Bulk, 1991; Lakshmanan, 2006; Snyman *et al.,* 2011). Further, the exposure of cultured cells to physical or chemical mutagens can be used together with *in vitro* culture to increase mutation frequency, thus enhancing somaclonal variation (Maluszynski *et al.,* 1995; Snyman *et al.,* 2011). An *in vitro* screening protocol

utilising an appropriate selecting agent can then be used to obtain disease tolerant plants (Van den Bulk, 1991; Chandra *et al.,* 2010).This approach allows the screening of large numbers of plants in a limited space over a short period of time (Ahloowalia and Maluszynski, 2001;Patade *et al*., 2008; Suprassana *et al.,* 2009). The purified toxins or pathogen culture filtrates containing toxins involved in disease development, are suitable selection agents for use *in vitro* (Daub, 1986; Svabova and Lebeda, 2005). However, it is important to expose the plants selected *in vitro* to the pathogen in order to confirm tolerance (Thakur *et al*., 2002; Tripathi *et al.,* 2008; Sengar *et al.,* 2009). Several studies have confirmed a correlation between tolerance of plants to toxins or culture filtrates and that obtained by inoculating with the pathogen (Gengenbach *et al.,* 1977; Arcioni *et al.,* 1987; Borras *et al.,* 2001; Chen and Swart, 2002).

It is, therefore, possible to use *in vitro* culture induced mutagenesis and fungal toxin-mediated screening, to select somaclonal variant sugarcane cells and subsequently plants that are tolerant to *F. sacchari* PNG40. Additionally, this strategy may allow endophytic colonisation and such plants will enable further development of biological control strategies for *E. saccharina*.

Towards this end, this study aimed to:

1) **determine conditions for *in vitro* screening of calli and plants for tolerance to *F. sacchari* PNG40 toxins using *Fusarium* culture filtrate.**
   Investigations were performed to test the effect of varying concentrations of *F. sacchari* PNG40 culture filtrates (CF) and to determine the developmental stage (embryo maturation, embryo germination, plantlet) at which they were most effective.

2) **select somaclonal variant sugarcane plants produced by chemical mutagenesis for tolerance to *F. sacchari* toxins.**
   The chemical mutagen, ethyl methanesulphonate (EMS), was used to induce mutations in embryogenic calli of the cultivar NCo376 which is susceptible to *F. sacchari* and *E. saccharina*. Calli and subsequently regenerated plants with tolerance to PNG40 CF were selected using the established CF treatments.

3) **confirm tolerance of somaclonal variants selected using the CF selection protocol by inoculation with *F. sacchari* PNG40.**

   The putative tolerant plants were exposed in the glasshouse to *F. sacchari* PNG40 in order to confirm their tolerance to the fungus.

2. Literature review

2.1 Background and importance of sugarcane

Sugarcane is a perennial, tropical or subtropical crop grown worldwide, within 30˚ of the equator, for its high sucrose accumulation (Ming *et al*., 2006). Commercially, the crop is established by means of seed cane and ratoons, when the bud and root primordia of the stool left after harvesting produce a stubble (James, 2004). Sugarcane grows well in medium to heavy, slightly alkaline, soils with good drainage, high organic matter (Anon., 2003) and an annual water supply of 1200-1500 mm (Tarimo and Takamura, 1998). Sugarcane has one of the most efficient photosynthetic mechanisms, capable of fixing 2-3 % radiant solar energy and achieves a high CO_2 coefficient (Almazan *et al*., 1998).

Sugarcane belongs to the genus *Saccharum* L., a part of the *Andropogoneae* tribe of the family *Poaceae* (grasses) (Selman-Housein *et al.,* 2000). Among the recognised species are S. *officinarum, S. spontaneum, S. sinense, S. edule, S. barberi and S. robustum* (Tarimo andTakamura*,* 1998). According to Grivet *et al*. (2004), sugarcane genetic resources can be divided into three groups:

(i) traditional cultivars: these are the noble cultivars which have brightly coloured stalks and are rich in sugar e.g. *S. officinarum* L. and the North Indian and Chinese cultivars which have thinner stalks, flatter colours and lower sugar content, e.g. *S. barberi;*

(ii) wild relatives: related to the traditional cultivars, they are informally grouped into the '*Saccharum* complex', have little or no sugar and have diverse morphological and ecological adaptations, e.g. *S. spontaneum* L.;

(iii) modern cultivars: created by Dutch breeders in Java in the early 1900s (Burnquist, 2001); these are hybrids of traditional cultivars and *S. spontaneum* L. and replaced the traditional cultivars during the 20th century.

The modern sugarcane cultivars are highly polyploid and aneuploid, originating from crosses between *S. officinarum* L. (2n = 80) and *S. spontaneum* L. (2n = 40 – 128) and backcrossing the interspecific hybrids with the *S. officinarum* L. parent (Stevenson, 1965; Sreenivasan *et al*., 1987; Butterfield *et al*., 2001; Lakshmanan *et al.,* 2005; Ming *et al*., 2006; Singh *et al*., 2010). In some of these cultivars, 10% of the chromosomes are inherited entirely from *S. spontaneum*, 80 % entirely from *S. officinarum* and 10 %

results from recombination of chromosomes from the two ancestral species (D'Hont *et al.,* 2008). These crosses introgressed disease resistance, vigour and adaptability into sugarcane lines leading to a combined interspecific genome that makes it the most complex of all the important crops (Ming *et al*., 2006). The complex cytology of sugarcane makes it extremely difficult to predict the resulting characteristics of hybrids obtained by cross pollination of members of the genus *Sacchurum*, thus the difficulties in breeding sugarcane (Barnes, 1964; D'Hont*et al*., 2008). In most crops, pest and disease resistance are regulated by both dominant and recessive genes, but in polyploids such as sugarcane, the recessive genes are obscured by homologous alleles, making them ineffective for breeding (Butterfield *et al*., 2001). Further, the effect of dominant genes in polyploids is not similar to that in diploids, due to the interaction of multiple alleles at a single locus, making it difficult to determine phenotype (Butterfield *et al.,* 2001).

Approximately 75% of the world's sugar is obtained from sugarcane and 25 % from sugar beet (*Beta vulgaris* L.) (Ming *et al*., 2006). Although over 100 countries cultivate sugarcane, the bulk of the world's production is produced by a few countries, including South Africa (SA) (Fischer *et al.,* 2009). In SA, a total of 19.26 million tonnes of sugarcane was produced in the 2008/2009 season (Sinderlar and Esterhuizen, 2010), but due to a severe drought in the 2010/11 season, production declined to 16.02 million tonnes and is expected to decline further to 15 million tonnes in the 2011/2012 season due to residual effects of the drought (Keamer and Esterhuizen, 2011). The sugar industry makes a vital contribution to rural economic activity in SA's sugarcane growing areas of Kwazulu-Natal (KZN), Mpumalanga and the Eastern Cape (Maloa, 2001) (Fig. 1).

The main by-products of sugar production are molasses, used as stock feed and in the production of ethanol (Zuurbier and Van de Vooren, 2008), and bagasse. The fibrous residue left after the juices are extracted from the cane, are used as fuel and in the production of cardboard, fibre board, furfural and wall board (Almazan *et al.,* 1998). Mohan *et al*. (2005) also used bagasse as an alternative to agar in apple micropropagation.

Despite its economic importance, sugarcane is attacked by various pests and diseases, causing significant losses in production (Allsopp and Manners*,* 1997), with over 50 different pathogens attacking sugarcane in SA (Rott *et al*., 2000). The crop is relatively susceptible to diseases as a result of being vegetatively propagated and

cultivated over large contiguous areas (Bailey, 2004). The former allows for spread and build-up of systemic diseases in planting material (Bailey, 2004) and the latter for disease build-up and spread in the field. This disease susceptibility is a major constraint in developing high sugar yielding varieties for use in the SA Sugar Industry (Butterfield *et al*., 1996). Infection by phytopathogenic fungi causes devastating epidemics on crop plants leading to persistent and significant crop yield losses (Knogge, 1996). The fungus *Fusarium* infects sugarcane seed cuttings and growing stalks following borer injury, causing Fusarium sett and stalk rot (Bourne, 1961).

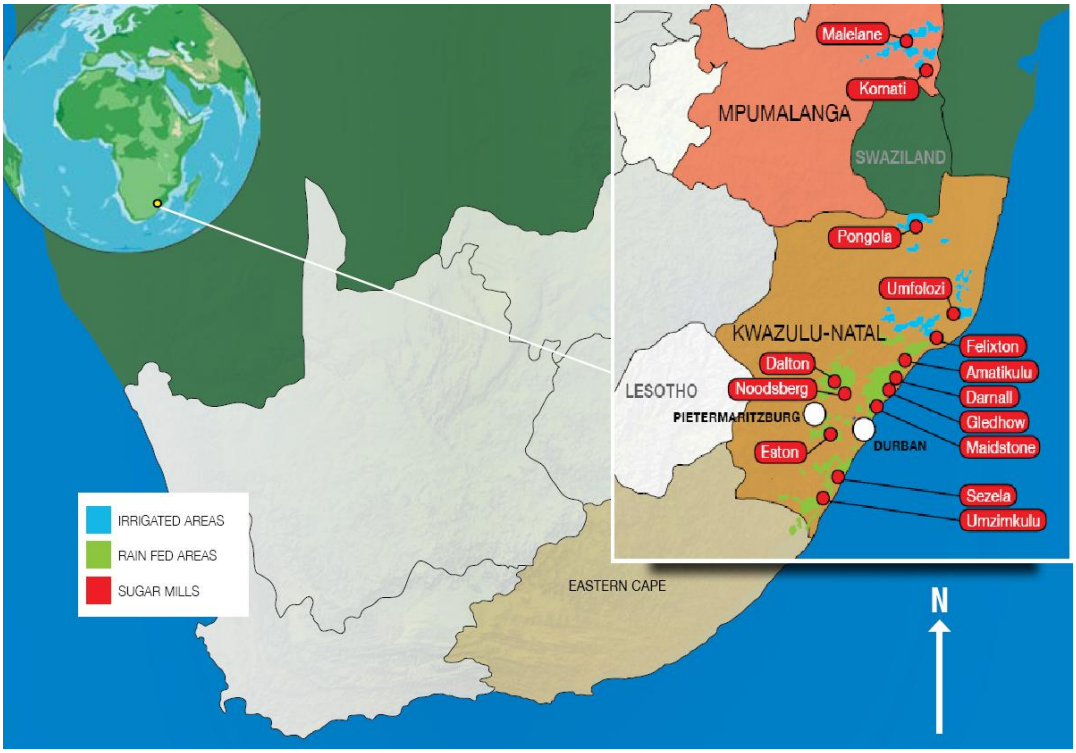


Figure 1: Sugarcane growing areas and mills in SA (From SA Sugar Industry Directory- 2010-2011)

2.2 *Fusarium* spp. and Fusarium stalk-rot

2.2.1 The pathogen

The genus *Fusarium* is a member of the order *Hypocreales,* which belongs to the class *Ascomycetes* (Seifert, 1996). The current classification system of this genus has 16 sections, 65 species and 77 subspecific varieties and forms (Leslie and Summerell, 2006). The genus is identified by the production of three types of asexual spores called conidia (Ohara *et al*., 2008). Macroconidia are large, slender, septate and canoe-

shaped and are produced in fruiting bodies called sporodochia; microconidia are markedly different and are produced on aerial mycelium and chlamydopores are produced by some species (Seifert, 1996). *Fusarium* spp. are commonly found together with higher plants and are prevalent in terrestrial ecosystems (Ploetz, 2005). Their infection of sugarcane leads to development of Fusarium sett and stalk rot and Pokkah boeng, the latter being mainly characterised by distortion of the upper stalk and leaf cholrosis (Bourne, 1961; Sivanesan and Waller, 1986; Croft, 2000). The species that causes Fusarium sett and stem rot was initially named *Fusarium moniliforme* Sheldon (Anamorph: *Gibberella moniliforme* [Sheldon] Wineland) (Bourne, 1961). However, Whittle and Irwan (2000) reported that two different races of *F. moniliforme* might be the cause of Fusarium sett and stem rot and Pokkah boeng in sugarcane.

Identification of *Fusarium* spp. by observing colony morphology features is difficult and unreliable (Rahjoo *et al.,* 2008). Molecular approaches are more dependable and these include DNA sequencing (Geiser *et al.,* 2004; McFarlane *et al.,* 2009), species-specific PCR (Rahjoo *et al.,* 2008), Random Amplified Polymorphic DNA (RAPD) (Sabir, 2006) and Amplified Fragment Length Polymorphism (AFLP) (Abdel-Satar *et al.,* 2003). However, the most widely used molecular technique for detecting variation amongst *Fusarium* spp. is Inter Simple Sequence Repeats (ISSRs) (Mishra *et al.,* 2006; Gurjar *et al.,* 2009; McFarlane *et al.,* 2009; Baysal *et al.,* 2010; Dinolfo *et al.,* 2010; Vitale *et al.,* 2011).

The taxonomy of the genus has been problematic (Kruger, 1989; Thrane, 1989) due to the inconsistency of the features used in identification of different species, thus leading to erroneous identification of some species (Edgerton, 1955; Nelson, 1991). *Fusarium moniliforme* was described as the only species in section *Liseola* (Snyder and Toussoun, 1965). However, *F. moniliforme* was renamed *F. verticillioides* Sacc. (Marasas *et al.,* 2001). Consequently, there was doubt on the identity of those isolates initially identified as "*F. moniliforme*" as they were not *F. verticillioides* (Leslie and Summerell, 2006). Based on the microconidial morphology, Nirenberg (1989) described 6 species, namely *F. anthophilum* (Braun) Wollenw, *F. fujikoroi* Nirenberg, *F. proliferatum* (Matsush.) Nirenberg, *F. sacchari* (Butler) Gams, *F. succisae* (Schroter) Sacc. and *F. verticillioides* Sacc in the section *Liseola*. Leslie and Summerell (2006) stated that strains initially identified as *F. moniliforme* that were not *F. verticillioides*, would probably be called other species, e.g. *F. fujikori* from rice, *F. thapsinum* from sorghum and *F. sacchari* in sugarcane. However, using RFLP analysis, McFarlane and

Rutherford (2005) identified *F. sacchari*, *F. verticillioides, F. proliferatum,* and *F. subglutinans* Wollenw. and Reink. in sugarcane stalks. In subsequent work with the aid of direct sequencing, isolates from sugarcane were identified as mainly *F. sacchari* and some as *F. pseudonygamai* O'Donnell & Nirenberg and *F. verticillioides* (McFarlane *et al.,* 2009).

2.2.2 Toxins produced by *Fusarium* spp.

Fungi produce compounds that are both toxic to animals (mycotoxic) and to plants (phytotoxic) (Desjardins and Hohn, 1997). In plants, they are involved in host-pathogen interaction and the development of disease (Amusa, 2005). The indicators of toxin involvement in plant pathogenesis include the presence of the toxin in diseased tissue (Yoder, 1980), similar host specificity and symptom induction as the pathogen (Amusa, 2005). Although toxins obtained from fungal cultures were found to be toxic on plants (Desjardins and Hohn, 1997), it has been difficult to establish their role in the development of plant disease (Yoder, 1980). Through the advent of biochemical and genetic analyses in the early 1960s, some species of the fungus *Cochliobolus* were found to produce toxins significant in causing plant disease (Desjardins and Hohn, 1997). Other fungal species that have been found to produce toxins include *Alternaria, Aspergillus*, *Claviceps, Fusarium* and *Penicillium* (Slavov, 2005).

Fusarium species produce secondary metabolites that interfere with the metabolic processes of plants and animals (Nadubinska and Ciamporova, 2001; Bennet and Klich, 2003), and the toxicity of these compounds on various crops has been well documented in the literature (Toyoda *et al.*, 1984; Remotti *et al.,*1997; Hidalgo *et al.,* 1998; Borras *et al.,* 2001; Chen and Swart, 2002; Khan, *et al.,* 2004; Leslie *et al.*, 2004). The phytotoxic effects of the toxins can be observed through necrosis, chlorosis and wilting of the plant (Slovov, 2005). The toxins occur widely in cereal grains infected by *Fusarium* spp., leading to contamination of food and stock feed (Bottalico, 1998). Examples of such compounds isolated from different cereals infected by *Fusarium* spp. are shown in Table 1. Some *Fusarium* species, *viz*. *F.sacchari*, *F. verticillioides, F. proliferatum* and *F. subglutinans,* have been isolated from sugarcane (McFarlane and Rutherford, 2005). The toxins they produce include fumonisins, fusaric acid, moniliformin and fusarin C, which have been isolated from *Fusarium*-infected cereals (Table 1).

Fusaric acid (5-butylpicolinic acid) (Fig. 2) is a wilt-toxin produced by many *Fusarium* species (Gaumann, 1957). It initially damages the stem and then spreads to the leaves, where it causes wilting (Ludwig, 1960). The movement of the compound up the xylem, in its undissociated state, results in faster movement up the xylem because it is uninterrupted by the negatively charged components of the xylem walls (Bourne, 1961). The compound permeates through the cell walls of the vascular bundle parenchyma without difficulty, thereby permitting the fungus access to the vascular bundles where it elicits the most damage (Bourne, 1961). Non-dialyzable pectic enzymes are thought to block the xylem vessels leading to wilting (Scheffer and Walker, 1953; Baayen *et al.,* 1997).

Fumonisin B_1 ($C_{34}H_{59}NO_{15}$) (Fig. 2) is the most prevalent fumonisin (Marasas *et al.*, 2000). Although not much is known about its phytotoxicity (Munkvold and Desjardins, 1997), it was shown to reduce chlorophyll synthesis significantly in duckweed (*Lemma minor)* (Vesonda *et al.,* 1992). Nelson *et al*. (1993) reported that only strains of *F. verticillioides* that produced high levels of fumonisins induced symptoms of stem rot in maize. Pure fumonisins were also reported to have caused necrosis in maize and tomato seedlings (Abbas *et al.,* 1994). Fumonisins are carcinogenic and it is suspected that their toxicity and carcinogenicity are due to their interruption of sphingolipid metabolism (Munkvold and Desjardins, 1997; Marasas *et al*., 2000). Sphingolipids are components of cell membranes (Munkvold and Desjardins, 1997), structurally similar to fumonisin B_1 (Marasas *et al.,* 2000) and are thought to be involved in signal transduction, membrane stability, programmed cell death and host-pathogen interaction in plants (Christie, 2010).

Moniliformin was isolated in maize infected by moniliformin-producing *Fusarium* species (Rabie *et al.,* 1982). Abbas *et al.* (1994) found pure moniliformin to be toxic to jimsonweed foliage (*Datura stramonium* L.). Its mechanism of action is thought to be through the inhibition of the mitochondrial oxidative enzyme, pyruvate dehyrogenase (Gathercole *et al.,* 1986), affecting the entry of carbon into the Krebs cycle during plant respiration (Schuller *et al.*, 1993).

Fusarins are 2-pyrrolidones with a methylated, polyunsaturated side chain (Desjardins and Proctor, 2007). Fusarin C (Fig. 2) was isolated from highly mutagenic culture filtrates of *F. verticillioides*, and was found to be mutagenic (Gelderblom *et al.,* 1984; Farber and Scott, 1989).

Table 1: Examples of toxins isolated by from different crops infected by *Fusarium* species

Toxin	Crop	Species	References
Trichothecenes	wheat, rye, oats, rice, maize, barley	*F. acuminatum, F. equiseti, F. graminearum, F. solani*	Desjardins and Hohn (1997)
Fusarin C	maize	*F. verticilliodes*	Farber and Scott (1989)
Zearalenone	wheat	*F. culmorum, F. graminearum*	Miedaner (1997)
Moniliformin	maize	*F. proliferatum*	Rabie*et al.* (1982)
Fumonisins	maize	*F. verticillioides, F. proliferatum, F. subglutinans*	Munkvold and Desjardins (1997)
Fusaric acid	maize, rice, sorghum	*F. moniliforme, F. subglutinans*	Bacon *et al.* (1996)
Beauvaricin	maize	*F. subglutinans*	Logrieco *et al.* (1996)

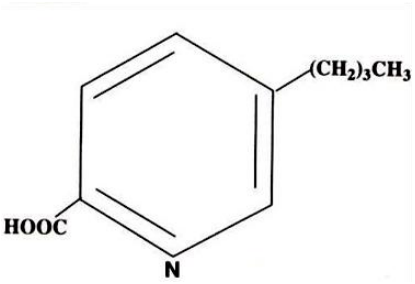
Fusarium spp. also produce gibberellic acid (Bryden *et al.,* 2001), fusaproliferin (Logrieco *et al.,* 1996) and beauvericin (Logrieco *et al.,* 1998). Fusaproliferin and beauvericin have been found to be toxic to insects (Gupta *et al.,* 1991; Logrieco *et al.,* 1996; McFarlane *et al.,* 2005). Dowd (1999) reported that beauvericin and fusaric acid have insecticidal properties due to their inhibition of the enzyme, phenoloxidase, which is responsible for insect pathogen control by means of encapsulation.

2.2.3 The disease and its control

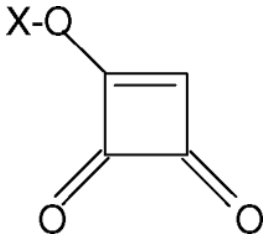
a) Disease symptoms

Fusarium sett and stem rot were first encountered in Barbados in 1922 (Cook, 1981). It is characterised by setts that have a purplish-red discolouration of the internal tissues, progressing from the cut ends or damaged rind to the inner parenchyma cells (Holliday, 1980). Infection spreads faster and discolouration is more pronounced in the vascular bundles than in the surrounding tissues (Croft, 2000). The buds of infected setts may swell and fail to germinate. These setts may split, revealing a purple-red discolouration of the vascular bundles and reddish-brown discolouration of the parenchyma (Bourne, 1961). In those setts that germinate, the young infected shoots turn purplish-red and decay (Sivanesan and Waller, 1986). Roots may fail to develop, but if they do, they become discoloured and decay (Cook, 1981).

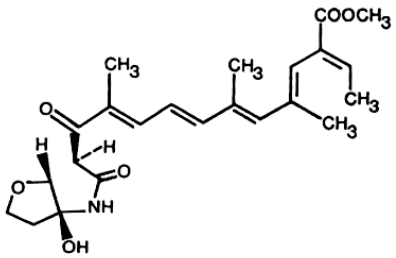
In mature standing cane stalks, infection occurs in stems that have been injured or damaged by cane borer *Diatraea saccharalis* (Holliday, 1980). *Fusarium* spp. have also



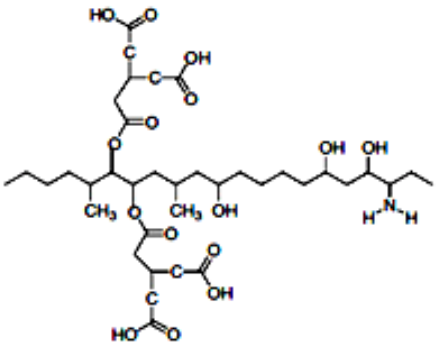
Fusaric acid



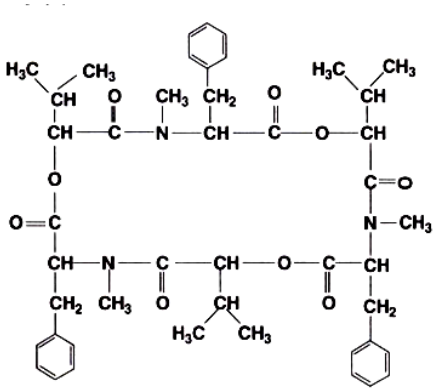
Moniliformin (X= Na K)



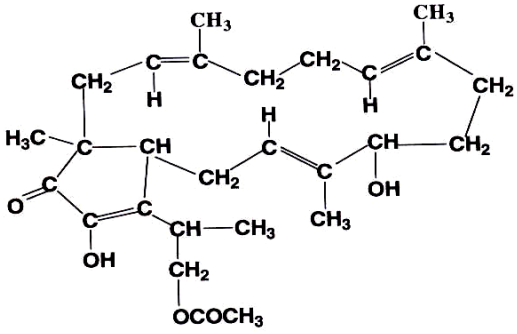
Fusarin C



Fumonisin B_1



Beauvericin



Fusaproliferin

Figure 2: The chemical structures of fusaric acid, moniliformin, fusarin C, fumonisin B_1, beauvericin and fusaproliferin (Bryden *et al.,* 2001).

been isolated from sugarcane stalk tissue damaged by the stalk borer, *Eldana saccharina* (McFarlane *et al.,* 2009). The purple discolouration of the parenchyma, as observed in the setts, is spread out and the red discolouration of the vascular tissues is darker (Fig. 3). The fungus spreads in the xylem (Sivanesan and Waller, 1986) resulting in the longitudinal splitting of the stalks, which reveals symptoms extending across internodes (Cook, 1981). Those symptoms are frequently found to spread rapidly, more towards the base than towards the top of the stem (Cook, 1981). The leaves wilt, turn yellow and dry up (Croft 2000). This wilting is probably induced by the wilting agent, fusaric acid, produced by the fungus (Bourne, 1961).

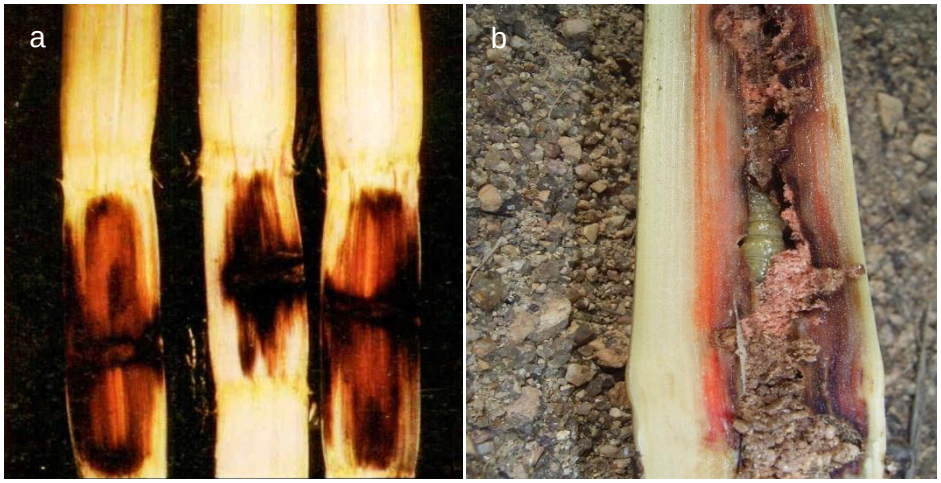


Figure 3:a) Fusarium stem rot in sugarcane (Bourne, 1961) b) *Fusarium* infection associated with *Eldana saccharina* infestation (courtesy of SASRI Entomology Department).

b) Disease transmission and epidemiology

Fusarium sett and stem rot can be transmitted through the cut ends of setts, immature adventitious roots, nodal leaf scars of stems planted in infected soils and the use of cane cuttings obtained from infected stems (Holliday, 1980). In India, the disease was reported to damage sugarcane by reducing germination of setts, wilting and drying up of immature shoots during mid-year and wilting of the stalks towards the end of the year (Bourne, 1961). In 1951-1956, Fusarium stem rot caused significant losses in Florida, (U.S.A.), as a result of heavy winds, excessive rainfall and vast damage by the stem borer at the pre-ripening stage (Bourne, 1961). The fungus also infects other grass crops, such as maize (Szoke *et al.,* 2009) and sorghum (Tesso *et al.,* 2004). The fungus *F. sacchari* grows on decaying plant material and produces a large number of conidia (Bourne, 1961) that are spread by wind and rain (Croft, 2000). The stem borer

D. saccharalis was shown to carry the fungus from plant to plant in different locations, and provide access of conidia in the wind and rain to the inner stem, through their damage made to the stalk (Bourne, 1961).

Sugarcane infestation by the stem borer *E.* saccharina is a major problem in the SA Sugar Industry (Keeping, 2006). The lepidopteran's infestation of sugarcane by boring the stalk rind permits *Fusarium* species access to the stem tissue. As a result, *E. saccharina* infestation is usually associated with *Fusarium* infection (Fig. 3b) (McFarlane *et al.,* 2009). Further, some endophytic *Fusarium* spp*.* have been found to attract *E. saccharina* and other lepidopterans in maize (Schulthess *et al*., 2002). However, McFarlane *et al.* (2009) reported that whilst some *Fusarium* isolates also encourage growth of *E. saccharina* larvae in sugarcane, some (including endophytic isolates of *F. sacchari*) inhibit growth of the larvae and thus may be useful in biological control of the lepidopteran pest.

c) Control of *Fusarium*

The disease can be controlled by using clean, well maintained, equipment that prevents unwarranted damage when cutting seed cane (Croft, 2000). Fungicides are also a means of control of *Fusarium* in sugarcane and wheat (Bourne, 1961; Halley *et al.,* 2008). Dipping setts in mercurial fungicides, before planting, has also been used to control Fusarium sett and stem rot in sugarcane (Bourne, 1961). Doses of ethyl mercury acetate and phenyl mercury acetate were reportedly effective in Louisiana, U.S.A. (Bourne, 1961). However, some fungal species might develop resistance to fungicides through mutation and reduce the efficacy of the fungicide (Georgopoulos, 1977). Furthermore, *Fusarium* spp*.* prevalence in the soil can be affected considerably by crop rotation practices (Menon and Williams, 1957). Although the use of resistant varieties is the best means of control, some strains have been found to overcome resistance and the once-resistant varieties were reported to be susceptible (Bourne, 1961).

2.3 Development of genotypes resistant to *Fusarium* spp*.*

2.3.1 Conventional breeding approaches

Conventional breeding refers to the artificial mating of selected parents and the selection of the progeny, such that evolution is biased towards producing crops with

specific characteristics that meet human requirements (Manshardt, 2004). It is based on sexual genetic inheritance of parental traits by the offspring according to Mendelian genetics. Plants with the desired traits are self- or cross-pollinated and the obtained progeny produced inherits the desired characteristics. The variation generated in offspring is a result of gene recombination, varying chromosome number and mutations (Poehlman and Sleper, 1995). In this way, and for centuries, plant breeders have been developing crops with superior growth, yields and pest and disease resistance compared with their wild relatives (James, 2004; Ming *et al.,* 2006).

Sugarcane breeders aim to produce varieties with high yield, high sucrose content, good ratooning ability, low fibre levels and pest and disease resistance (Jackson, 2005). The commercial sugarcane cultivars used today resulted from crosses of *S. officinarum* and *S. spontaneum* (Stevenson, 1965; Sreenivasan *et al*., 1987; Butterfield *et al*., 2001; Ming *et al*., 2006; Singh *et al*., 2010). However, as already mentioned, breeding genetically-improved varieties by conventional means, utilising Mendelian genetics, is difficult due to the complex nature of the sugarcane genome (Selman-Housein *et al.*, 2000; Gill *et al.,* 2004; Ming *et al.,* 2006). Offspring of crossed parents are surveyed in a number of crosses and promising varieties are selected as clones (Stafne *et al.,* 2001; Tai *et al.,* 2003; Berding *et al.,* 2004). However, due to the polyploidy of sugarcane, a single cross can produce large numbers of offspring that vary in a range of features which include size, yield and disease resistance (Barnes, 1964; Olaoye, 2001; Berding *et al.,* 2004). Furthermore, sugarcane genotypes differ in fertility and produce small, unusable seed (Poehlman and Sleper, 1995; James, 2004). Although it was once thought that sugarcane seed was sterile, it was discovered in the late 1800s that sugarcane produced fertile seeds in favourable environmental conditions (James, 2004).

Sugarcane is largely cross-pollinated by wind (Poehlman and Sleper, 1995). Pollen production varies between varieties causing variation in crossing and selfing (James, 2004). Moreover, flowering in sugarcane plants does not coincide (Selman-Housein *et al.*, 2000) and pollen viability is short-lived, thus making it difficult for sugarcane breeders to carry out intended crosses (Anon, 2004). Above all, sugarcane breeding is time consuming, with new superior clones taking 12-15 years to be produced (Burnquist 2001; Butterfield, 2001; Lakshmanan, 2005). Nevertheless, some *Fusarium* resistant clones have been produced through conventional breeding in sugarcane

(Lyrene *et al.*, 1977), maize (Kozhukhova *et al*., 2007; Afolabi, 2008) wheat and barley (Jansen *et al.,* 2005).

Biotechnological tools can be used to assist conventional breeding in producing desired genotypes faster (Selman-Housein *et al.*, 2000; Wang *et al.,* 2005). For example, marker-assisted selection (MAS) has been utilised to assist breeders to select for certain genes in crops (Wang *et al.,* 2005). Genetic maps that show the position of certain genes on the chromosomes have been constructed for various crops, aiding plant breeders in breeding programs (Poehlman and Sleper, 1995). This approach, called molecular breeding (Wang *et al.*, 2005), has been widely used in breeding programs of cereals and other crops (Butterfield *et al.,* 2001; Korzun, 2003; Pan, 2003; Wang *et al.*, 2005). However, due to the polyploid nature of sugarcane, the link between the genes and alleles present in the genotype and their expression in the phenotype is complicated by silencing and differential expression of gene copies (Butterfield *et al.,* 2001).

2.3.2 Genetic engineering for resistance

Genetic transformation is the insertion of specific genes into a genome (Birch, 1997) where the inserted gene is expressed (Poehlman and Sleper, 1995). Crops that have been transformed include canola, cotton, maize, tomato and soybean (Burnquist, 2006). In 2010, there were 15.4 million farmers cultivating transgenic crops under 148 million hectares in 29 countries (Anon, 2011). Sugarcane transformation started in the 1980s (Chen *et al.,* 1987) and particle bombardment has been the main method used to introduce genes into sugarcane cells (Allsopp and Manners, 1997; Snyman *et al.,* 2000; Kaur *et al.*, 2007). *Agrobacterium tumefaciens*-mediated transformation (Enriquez *et al.,* 2000) and cell electroporation (Arencibia *et al.,* 1999) have also been used.

The high chromosome numbers and genomic complexities of sugarcane makes expression of inserted genes complicated (Lakshmanan *et al.,* 2005). However, strategies for the development of disease resistant transgenic sugarcane have been established. They involve insertion of genes capable of degrading or inactivating pathotoxins, producing polypeptide signals that induce expression of protease inhibitors and producing enzymes that enhance the toxicity of antibiotics produced by plants (Allsopp and Manners, 1997). Resistance has been achieved by genetic transformation for diseases which include sugarcane mosaic virus (ScMV) (Gilbert *et*

al., 2005), sorghum mosaic virus (SrMV) (Ingelbrecht *et al.,* 1999), sugarcane leaf scald (Zhang and Birch, 2000) and sugarcane rust (*Puccinia melanocephala* Syd. and Syd.) (Enriquez *et al.,* 2000). Other traits that have been introduced into sugarcane by genetic transformation include herbicide tolerance, resistance to sugarcane borer (*D. saccharalis*), increased sucrose content and suppressed flowering (Burnquist, 2006).

Makandar *et al.* (2006) obtained resistance to Fusarium head blight in wheat (caused by *F. graminearum*) by inserting the *NRP1* gene from *Arabidopsis thaliana.* In maize, *F. moniliforme* infection was reduced by controlling the European cob borer by inserting genes coding for the endotoxin *cryIA(b)* produced by *Bacillus thuringienesis,* which resulted in lower levels of fumonisins that cause symptoms of Fusarium ear rot (Munkvold *et al.,* 1997). Funnell and Pedersen (2006) inserted genes that lowered the lignin levels in sorghum, which resulted in resistance to *F. moniliforme.*

Ramgareeb and Rutherford (2006) found antifungal peptides that are potent against *Fusarium* and smut in sugarcane, of which the ponericin PONG1, was the most effective. Furthermore, its activity was shown to be enhanced by an indolicidin REV4, when the two were used in combination. Genes that code for these peptides could be inserted into the sugarcane genome to control *Fusarium* and smut.

However, transgenic sugarcane is yet to be produced commercially despite research and development being carried out in a number of countries (Burnquist, 2006). This is due to limitations which include transgene silencing, inadequate knowledge about inheritance of transgenes (Lakshmanan *et al*., 2005) and legislation (Burnquist, 2006, Arruda, 2011). Further, transformation of monocotyledons is limited by inefficient transformation systems and low cell competence (Sood *et al.,* 2011).

2.4 *In vitro* culture systems

In vitro culture refers to the culture of plant cells, tissues and organs, under controlled sterile laboratory conditions (Jain, 2006; Thorpe, 2007) that allow them to regenerate into whole plants (Poehlman and Sleper, 1995). The process manipulates the cells' ability to regenerate into whole plants (totipotency) (George, 1993; Litz and Gray, 1995). Since its discovery in the 1930-1940s, plant cell culture has been an essential part in plant improvement (Sangwan *et al.*, 1997), with a wide application in plant biotechnology strategies (Karp, 1995). Sugarcane culture was first successfully carried out by Nickel (1964), who produced calli which later developed roots. Whole plant

regeneration was then achieved by Heinz and Mee (1969). Since then, sugarcane culture has had various applications, e.g. micropropagation (Lee, 1987; Baksha *et al.,*2002; Pawar *et al.,* 2002; Cheema and Hussain, 2004; Meyer *et al.,*2007; Behera and Sahoo, 2009), virus elimination (Irvine and Benda, 1985; Parmessur *et al.,* 2002; Snyman *et al.*, 2005; Ramgareeb *et al.,* 2010), genetic transformation (Snyman *et al.,* 2000; Snyman, 2004; Lakshamanan *et al.,* 2005; Shah *et al.,*2009), improvement via somaclonal variation (Krishnamurthi and Tlaskal, 1974; Liu and Chen, 1978; Peros *et al.,* 1994; Patade *et al.,* 2005; Singh *et al.,* 2008) and germplasm preservation (Gnanapragasam and Vasol, 1990; Taylor and Dukic, 1993; Watt *et al.,* 2009).

Conventionally, sugarcane is vegetatively propagated by means of stem cuttings with 2-3 nodes (Behera and Sahoo, 2009) which results in a low rate of plant multiplication, *viz.* 10-20 plants being produced per stalk (Geijskes *et al.,* 2003). The planting material also causes spreading of diseases (Hoy *et al.,* 2003). Consequently, the distribution of new cultivars to farmers is time consuming. In comparison, Geijskes *et al.* (2003) showed that micropropagation is up to 35 times more productive than the conventional approach. At the South African Sugarcane Research Institute (SASRI), Snyman *et al.* (2008) found that 32-600 plants per stalk could be obtained from different SA sugarcane varieties. Sugarcane micropropagation is, therefore, a highly beneficial technique for the rapid production of good quality planting material (Bailey and Brechet, 1989; Karim*et al.,* 2004; Roy and Kabir, 2007; Ali *et al.,* 2008; Khan *et al.,* 2008; Behera and Sahoo, 2009).

Whole plants can be regenerated *in vitro* via somatic embryogenesis or organogenesis (Fig. 4) and each of the two morphogenesis routes has wide applications (Table 2). Organogenesis involves the regeneration of plants either directly from tissues (e.g. shoot tips) or indirectly from callus, an undifferentiated mass of cells (George, 1993). Regeneration via direct organogenesis, i.e. without the callus stage, reduces the chance of obtaining variant plants through somaclonal variation (Lakshmanan *et al.,* 2006). In sugarcane, the manipulation of plant growth regulators, i.e auxins and cytokinins in the medium, results in the formation of shoots and roots from callus (Lee, 1987; Karim *et al.,* 2004; Behera and Sahoo, 2009), shoot tips (Fitch *et al.,* 2001; Baksha *et al.,* 2002; Pawar *et al.,* 2002; Ali *et al.,* 2008) and auxillary buds (Cheema and Hussain, 2004). In somatic embryogenesis, somatic cells form bipolar embryos that are similar to those formed from zygotic cells (Ahloowalia and Maretzki, 1983; Litz and Gray, 1995; Ali *et al.,* 2007a). Somatic embryo formation, similar to zygotic embryo

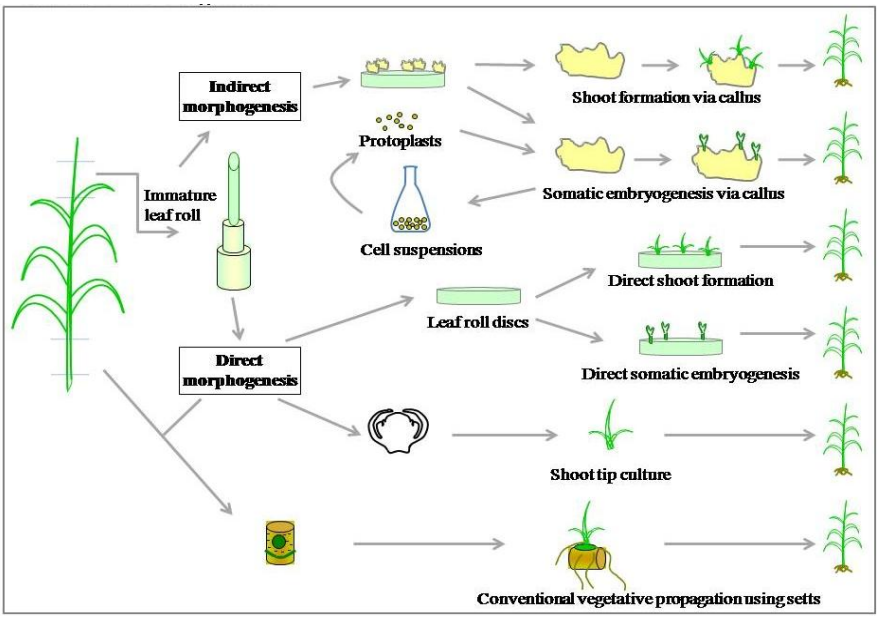


Figure 4: Illustration of indirect and direct morphogenesis routes in sugarcane plantlet regeneration (modified from Snyman, 2004).

development, is characterised by the development of cells into globular, heart-shaped and finally torpedo-shaped stages in dicotyledons (Terzi and Loschiavo, 1990; Zimmerman, 1993; Litz and Gray, 1995; Dodeman *et al.,* 1997) or globular, scutellar and coleoptilar stages in monocotyledons (Gray *et al.,* 1995)

As with organogenesis, somatic embryos can be produced directly from cells of the explant (e.g. leaf roll), i.e. direct somatic embryogenesis (Snyman, 2004) or indirectly via a callus stage, i.e. indirect somatic embryogenesis (Ho and Vasil, 1983; Sakar, 2009; Snyman, 2004). Sugarcane produces compact embryogenic callus, friable non-embryogenic callus and mucilaginous non-embryogenic callus (Ho and Vasil, 1983; Guiderdoni and Demarly, 1988; Lakshmanan, 2006). The ability of sugarcane leaf segments to produce calli of different embryogenic potential was demonstrated by Guiderdoni and Demarly (1988). Those authors reported that the innermost sheath produces white compact embryogenic callus, the intermediate produces friable non-embryogenic callus and the outer produces mucilaginous non-embryogenic callus. Sugarcane embryos can be produced directly from leaf discs and indirectly from callus on media containing low and high concentrations of 2,4-D (2,4-Dichlophenoxyacetic acid), respectively (Snyman *et al*., 2000; Laskshmanan, 2006; Sharma *et al.,* 2007) and no callus forms in the absence of 2,4-D (Ho and Vasil, 1983). Embryo germination

Table 2: Examples of applications of sugarcane *in vitro* culture via different morphogenesis routes

Application	Morphogenesis route	Reference
Micropropagation	Direct organogenesis	Baksha *et al*. (2002)
	Indirect organogenesis	Behera and Sahoo (2009)
	Direct organogenesis	Cheema and Hussain (2004)
	Direct somatic embryogenesis	Meyer *et al.* (2007)
	Direct organogenesis	Pawar *et al.* (2002)
	Direct organogenesis	Geijskes *et al.* (2003)
Pathogen elimination		
Sugarcane mosaic virus	Direct organogenesis	Irvine and Benda (1985)
Yellow leaf syndrome (YLS), sugarcane yellow leaf virus	Indirect somatic embryogenesis and direct organogenesis	Parmessur *et al.* (2002)
Ratooning Stunting disease, sugarcane mosaic virus, sugarcane yellow leaf virus, sugarcane leaf yellows phytoplasma	Direct somatic embryogenesis	Snyman *et al*. (2005)
Sugarcane mosaic virus, sugarcane yellow leaf virus	Indirect somatic embryogenesis, direct and indirect organogenesis	Ramgareeb *et al*. (2010)
Genetic transformation	Direct and indirect somatic embryogenesis	Snyman *et al*. (2000)
	Direct somatic embryogenesis	Snyman *et al.* (2006)
Breeding		
Fiji disease resistance	Indirect organogenesis	Krishnamurthi and Tlaskal (1974)
Performance and yield	Indirect organogenesis	Liu and Chen (1978)
Sugarcane rust and yield	Direct and indirect organogenesis	Peros *et al.* (1994)
Red rot, yield, height, nodal length.	Indirect somatic embryogenesis	Singh *et al.* (2008)
Salinity and drought tolerance	Indirect somatic embryogenesis	Patade *et al.* (2005)
Germplasm preservation		
	Direct organogenesis	Taylor and Dukic (1993)
Slow growth	Indirect somatic embryogenesis	Watt *et al.* (2009)
Cryopreservation	Indirect somatic embryogenesis	Gnanapragasam and Vasil (1990)
Crypreservation	Indirect somatic embryogenesis	Chanprame *et al.* (1993)

occurs in media without auxins (Snyman *et al.,* 2000; Parmessur *et al.,* 2002; Ramgareeb *et al*., 2010).Other plant growth regulators used in sugarcane somatic embryogenesis include benzylaminopurine (BAP), kinetin (Gill *et al.,* 2004) 3,6-dichloro-O-anisic acid (dicamba), naphthaleneacetic acid (NAA) and 4-fluorophenoxyacetic acid (4-FPA) (Brisibe *et al.,* 1994). Chengalrayan *et al.* (2005) produced callus from sugarcane seeds on media containing picloram. Embryo formation in sugarcane is also dependent on the genotype and in different varieties on media with different levels of auxins, sugar and amino acids (Ozias-Akins *et al.,* 1992; Ito *et al.,* 1999; Gill *et al.,* 2004; Onay *et al.,* 2007).

2.4.1 *In vitro* culture-induced variation

Somaclonal variation in *in vitro* cultured plants was first described by Larkin and Scowcroft (1981) when they observed the resistance of previously susceptible *in vitro* plants to the toxin produced by *Helminthosporium sacchari* Butler*,* which causes eyespot in sugarcane. They called this spontaneous genetic change somaclonal variation, and defined it as heritable genetic variation that results from *in vitro* culture. However, variations in *in vitro* cultured sugarcane had been observed before by Heinze and Mee (1969). Since then, somaclonal variation has been utilized vastly in crop improvement, and is known to occur in many plant species, including barley (Bregitzer *et al.,* 2002), maize (Vasconcelos *et al.,* 2008), olives (Peyvandi *et al.,* 2010), petunia (Abu-Qaoud *et al.,* 2010), potato (Ehasanpour *et al.,*2007), rice (Ngezahayo *et al.,* 2007), sorghum (Raveendran *et al.,* 1998), strawberry (Mohamed, 2007), sugarcane (Larkin and Scowcroft, 1983; Burner and Grisham, 1995; Snyman *et al.,* 2011) and wheat (Abouzied, 2011).

Alterations in a cell's genome may result from stress induced on cells when they are exposed to new environments (McClintock, 1984). When cells are cultured *in vitro*, they are exposed to conditions of high sucrose, nitrogen, salt concentrations and osmotic potential different to those of soils. In addition, culture media usually contain plant growth regulators, which induce stress on the cells (Desjardins *et al.,* 2009; Lebeda and Svabova, 2010). Consequently, the cell's control mechanisms may break down leading to changes in the genome through different processes (Phililps *et al.,* 1994). For example, changes in DNA methylation patterns can affect gene expression by changing the structure of chromatin resulting in breaking of chromosomes due to

delayed DNA replication (Kaeppler and Philips, 1993). DNA methylation has also been shown to result in the transposition of genetic elements in genomic DNA (Brown, 1989). As a result, increased DNA methylation leads to decreased gene expression and decreased DNA methylation enhances gene expression (George, 1993). Variation also occurs due to activation of transposable elements as a result of *in vitro* culture (McClintock, 1984; Hirochika *et al.,* 1996; Kaeppler *et al.,* 2000; Peschke *et al.,* 2000). The activated transposable elements cause a change in the DNA sequence that can lead to a change in gene expression (Rossi *et al.,* 2001). In addition, when cells are in stressful conditions, the number of copies of a specific gene within the genome can increase during cell differentiation, leading to an increase in mRNA synthesis and higher levels of the respective protein, which can manifest in the phenotype (Larkin and Scowcroft, 1981).

Changes in chromosome structure can also occur during cell division due to stress of the culture environment, through inversion, deletion, fusion and duplication of sections of the chromosomes (Larkin *et al*., 1989). Further, single base pair changes in the DNA sequence can occur due to the breakdown of systems that control the base sequencing (Philips *et al.,* 1994). Larkin and Scowcroft (1981) reported that the different mechanisms by which somaclonal variation may occur seem to be applicable to situations where variation already exists in the explant whilst others apply when cells are in culture. However, some of the variation observed in culture is epigenetic, i.e. it is reversible and cannot be passed on sexually to the next generation (George, 1993; Kaeppler *et al*., 2000; Joyce *et al.,* 2003; Patade *et al.,* 2005). These epigenetic effects are due to changes in gene expression regulating mechanisms and not changes in the genetic sequence of the gene, which may be expressed in divided cells after mitosis ,but not in the offspring of the regenerated plants after sexual reproduction (Chaleff,1983).

The extent of variation in cells also depends on the type of explant used (George, 1993) with variation likely to be greater in older and more differentiated material (Karp, 1995). Genetic differences between the parent and the somaclones are less when plants are obtained from axillary meristems as opposed to regeneration via a callus stage (Hanna *et al.,* 1984; Ali *et al.,* 2008). Zuchhi *et al.* (2002) through molecular analysis of somaclones in sugarcane, found that some genotypes are more prone to somaclonal variation than others. This may be due to varying ploidy levels between

species, with variation being expressed more in haploids and diploids than in polyploids (Karp, 1995). In addition, as different genotypes differ in genetic stability they may differ in susceptibility to mutations (Joyce *et al.,* 2003). Inclusion of plant growth regulators, auxins and cytokinins, singly or in combination, in the medium can result in cells mutating (George, 1993). For example, Bairu *et al*. (2006) showed that growth regulators increase somaclonal variation by increasing cell division in bananas. Other commonly used media constituents, e.g. yeast extract, coconut milk, kinetin and micronutrient metals, have also been shown to alter the ploidy level of cells and cause chromosome damage (George, 1993). For these reasons, the length of time cells are in culture affects the degree of variation (Burner and Grisham, 1995).

Somaclonal variation is undesirable when true-to-type plants are required, e.g. during micropropagation (Litz and Gray, 1995; Bouman and De Klerk, 2001). In such cases, rigorous field testing is necessary to eliminate variants (Arya *et al.,* 2009). However, somaclonal variation is a source of variant plants that can be utilised for plant improvement (Patade *et al.,* 2005). New traits, which conventional breeding may be unable to develop, can be obtained through screening large numbers of somaclonal variants (Jain, 2001). Despite the discovery of somaclonal variation in the 1940s, its application to crop improvement only started to be utilised in the 1970s (Thorpe, 2007). Table 3 shows sugarcane and other crops from which somaclones have been screened for disease resistance. Other traits which have been developed in sugarcane through somaclonal variation include increased yield and performance (Liu and Chen, 1978), low fibre content and longer stem and internode lengths (Rajeswari *et al.,* 2009).

Table 3: Examples of crops in which somaclonal variation has been used to develop disease resistance

Crop	Trait	References
Maize	*Helminthosporium maydis* resistance	Brettell *et al.* (1980)
Rice	*Helminthosporium oryzae*	Ling *et al.* (1985)
Sugarcane	Rust resistance	Peros *et al.* (1994)
	Eyespot resistance	Larkin and Scowcroft (1983)
	Fiji disease resistance	Krishnarmurthi and Tlaskal (1974)
	Red rot resistance	Singh *et al.* (2008)
	Sugarcane borer resistance	White and Irvine (1987)
Barley	*Rhynchosporium secalis* resistance	Pickering (1989)

2.4.2 Induced mutagenesis

a) Principles and types of mutagens

Mutagenesis refers to the artificial induction of genetic variation via the use of physical or chemical mutagens (Drake and Koch, 1976). It was first carried out using X-rays in the fruit fly, *Drosophila* spp., by Muller in 1927 (Van Harten, 1998). In plants, various methods which include heat treatment, centrifugation and ageing of seeds, were initially carried out in an attempt to induce mutations (Van Harten, 1998). Ionizing radiation, X-rays, gamma rays and thermal neutrons were later used, but the first attempts resulted in low mutation frequencies and lethal effects on the plants, which were resolved by improving treatment conditions (Novak and Brunner, 1992; Brunner, 1995). The mechanisms that result in mutations (as discussed in 3.4.1) during induced mutagenesis are similar to those that result in spontaneous mutations during *in vitro* culture (Jain *et al.,* 1998). However, the frequency of mutagen-induced mutations is higher than that of spontaneous mutations in *in vitro* culture (Novak and Brunner, 1992). Obtaining desired mutations through the use of mutagens is based on chance and may also result in lethal effects that can disrupt normal plant development (Roane, 1973).

The use of physical mutagens in mutation breeding dates back to the early 20th century with the use of X-rays and later, gamma and neutron radiation (Novak and Brunner, 1992). They have been used in mutation breeding of sugarcane and many other crops (Table 4). Mutation efficiency of physical mutagenic agents depends on the dose, dose rate, dose distribution and exposure time (Brunner, 1995; Suprassana *et al.,* 2009). The establishment of these parameters relies upon radiation type, radiation facilities and the type of material to be exposed to the radiation (Brunner, 1995). X-rays and gamma rays can penetrate deep into the tissue due to limited scattering and concentration of the ion beam on the plant tissue leading to high mutation frequency compared with UV-light and neutron radiation (Suprasanna *et al.,* 2009). Furthermore, X-rays and gamma rays cause the formation of radicals that break DNA strands (Waugh *et al.,* 2006) and ionize nitrogenous bases, especially during DNA replication, leading to heritable errors in the base sequence (Medina *et al.,* 2005). UV-light causes covalent bonding between neighbouring pyrimidines resulting in the formation dimers that alter DNA replication (Waugh *et al.,* 2006). Physical mutagens are less hazardous and are easier to handle compared to chemical mutagens (Suprasanna *et al.,* 2009),

but are relatively expensive due to the equipment required (Poelhman and Sleper, 1995). Gamma rays have been commonly used for sugarcane mutagenesis (Table 4).

Chemical mutagens used in mutagenesis include hydroxylamine, methyl methanesulfonate (MMS), N–methyl-N–N–nitrosoguanidine (MNNG), ethyl methanesulfonate (EMS), nitrous acid and N-methyl-N-nitrosourea (MNU) (Inoue, 2006). The mechanisms by which they effect changes in DNA include base analog, intercalation and base modification (Waugh *et al.,* 2006), which result in different types of mutations (Table 5). Mutation frequency is dependent on the concentration, temperature and pH of the mutagen (Van Harten, 1998) and access of cells to the mutagen in the cell-mutagen suspension (Durand, 1990).

Table 4: Examples of mutation breeding in different crops

Crop	Mutagen	Trait	References
Cassava	Gamma rays	Starch yield	Joseph *et al.* (2004)
Sugarcane	Gamma rays	Yield	Khan *et al.* (2007)
	Gamma rays	Salt tolerance	Patade *et al*. (2008)
	Gamma rays, Sodium azide	Red rot resistance	Ali *et al.* (2007b)
	EMS	Salt tolerance	Kenganal *et al.* (2008)
Wheat	X-rays, EMS, Hydroxylamine, Dimethyl sulphate, Gamma rays	Gluten content	Shkvarnikov and Kulik, (1975)
Rice	EMA, Sodium azide	Blast disease resistance	Sarma *et al.* (1999)
Sweet potato	EMS	Salt tolerance	Luan *et al.* (2007)

The chemical mutagen ethyl methanesulfonate (EMS) has been widely used in mutagenesis of many plant species that include *Arabidopsis* (Jander *et al.,* 2003), sweet potato (Luan *et al.*, 2007), wheat (Masrizal *et al*., 1991), palm (Omar and Novak, 1990) and soyabean (Van *et al.,* 2008; Hoffman *et al*., 2004). In sugarcane, this mutagen has been used in mutation breeding for various traits including high sugar content (Khairwal *et al.,* 1984), salt tolerance (Kengenal *et al.,* 2008) and herbicide tolerance (Koch *et al.,* in press). It is a popular mutagen because of its ability to induce high point mutation frequencies without causing lethal abnormalities to the

chromosomes (Waugh *et al.,* 2006; Well and Monde, 2009). EMS is an alkylating agent that induces the alkylation of guanine to form O^2-ethylguanine which is capable of pairing with thymine instead of cytosine (Kim *et al.,* 2006; Waugh *et al.,* 2006). This results in the errors during DNA repair with the G-C pair replacing A-T (Davies *et al.,* 1999), especially during DNA replication (Durand, 1990).

Mutagenesis can be carried out using the parent material or *in vitro* cultures (Suprassana*et al.,* 2009). Axillary and adventitious buds, apical meristems (Ahloowalia and Maluszynski, 2001), seeds, microconidia and anthers (Mulwa and Mwanza, 2006) can be used. The plants produced from mutated embryogenic callus cells can be chimeric as a result of mutations occurring unevenly amongst the diploid cells (Van Harten, 1998; Datta and Chakrabarty, 2009). Consequently, the use of haploid cell cultures (e.g. microspores) is favoured over diploid cultures due to the expression of recessive genes without being masked by dominant genes after crossing (Swanson *et al.,* 1989; Suprassana *et al.,* 2009). The haploid plants that result can be inbred to produce diploid plants with the desired traits, making selection easier and less time consuming (Mulwa and Mwanza, 2006). In addition, the production of double haploids from mutagenized microspores or anthers, assists in preventing the formation of chimeras leading to the regeneration of plants with homozygous alleles (Maluszynski *et al.,* 1995). Hence, the production of double haploids makes selection of mutants more efficient (Griffing, 1975). However, the use of haploids in sugarcane improvement is difficult due to the difficulty of the technique (Palmer *et al.,* 2005) and the crop's complex genetics (Moore and Fitch, 1990).

b) Selection of variant cells and plants

The development of effective strategies for selection of desirable traits is an important step in plant breeding programmes (Roane, 1973; Van den Bulk, 1991; Novak and Brunner, 1992; Lebeda and Svabova, 2010). Conventionally, selection of traits of interest is carried out in the field, but this is laborious and time-consuming compared with *in vitro* selection techniques (Novak and Brunner, 1992; Jain, 2001; Patade *et al.,* 2008). This is because a selection pressure can be applied to *in vitro* cultured cells and/or to the regenerated plants in the culture medium and subsequently to the field plants (Maluszynski *et al.,* 1995; Chandra *et al.,* 2010). This approach allows for selection of a large number of mutant cells and plants in a small space and provides a specific and controlled environment that is free from biotic and abiotic factors that might

Table 5: Examples of chemical mutagens and the damage they induce in DNA (Inoue, 2006)

Chemical mutagen	Mode of action	Mutation type
4-nitroquinoline 1-oxide (4-NQO), Diepoxybutane (DEB)	DNA adducts	Base-pair substitution
ICR-170	Intercalation	Frameshift
Mitomycin C (MMC), 1, 2, 7, 8- diepoxyoctane (DEO)	Interstrand cross-linking	Deletion
N-methyl- N'-nitro-N-nitrosoguanidine (MNNG), Ethyl methane sulfonate (EMS), Methyl methane sulfonate (MMS)	Alkylation	Base-pair substitution
Nitrous acid (NA), Hydroxylamine (HA)	Modification of bases	Base-pair substitution
2-amino purine (2AP)	Base analog	Base-pair substitution

negatively influence selection (Chaleff, 1983; Duncan and Widholm, 1990). Cells can be exposed to stress temperatures, herbicides, high salt concentrations and fungal toxins (George, 1993). In addition, the technique allows for the introduction of a pathogen in a controlled environment, negating the need for strict quarantine if carried out *ex-vitro* (Chandra *et al.,* 2010).

However, limitations of *in vitro* selection are that traits expressed at the cellular level might not be expressed at the plant level (Daub, 1986). Furthermore, the technique cannot be used to select certain phenotypic traits (e.g. agronomic traits), which require cell differentiation and organisation (Chaleff, 1983). When the desired traits are dominant and homozygous recessive, resistant cells and plants can be selected immediately, but crossing is necessary in cases of heterozygous plants in order to obtain plants with recessive traits (Van den Bulk, 1991). The traits expressed in cells as a result of epigenetic variation, may not be expressed in the progeny of the plants, as the epigenetic effects are reversed by meiosis during sexual reproduction (Chaleff, 1983; George, 1993; Suprassana *et al.,* 2009).

To apply a selection pressure *in vitro*, the concentration of the selection agent that kills or inhibits the growth of cells, has to be established for incorporation into the selection medium (Suprassana *et al.,* 2009). Exposure of cells to the selection agent can either be single-step with 2-3 times the lethal dose of the agent, or multiple-step where the

concentration of the selection agent is gradually increased, starting at the lethal concentration (Suprassana *et al.,* 2009). Selecting for disease resistance involves the use of a selection agent known to be involved in pathogenicity and ensuring uniform exposure of each cell to the selection agent, such that it kills the susceptible cells and the resistance ones survive and regenerate into plants (Daub, 1986; Lebeda and Svabova, 2010). The pathogen, its toxins or culture filtrates, can be used in selecting lines that are disease resistant.

i) Use of pathogens in selection

The pathogen responsible for causing the disease can be used as the *in vitro* selection agent for resistance (Daub, 1986; Van den Bulk, 1991; Lebeda and Svabova, 2010) (Table 6). Factors that may influence the expression of resistance include the concentration of the inoculum, temperature and the composition of the medium (Xue and Hall, 1992; Bertetti *et al.,* 2009), which may lead to inconsistent results being obtained (Daub, 1986). Moreover, this option has limitations including: 1) uneven exposure of the cells to the pathogen; 2) whether resistance can be expressed in *in vitro* cultured cells; and 3) the overgrowth of the pathogen on the cells and medium, which makes it difficult to make observations (Daub, 1986). Fungal conidia can be inoculated onto shoot cultures and these visually monitored for resistance to the fungus, provided there is a correlation with the effect of the fungus *in vivo* (George, 1993).

ii) Use of toxins and culture filtrates in selection

Fungi secrete toxins as a mode of protection against a host plant's defences, enabling them to kill host cells and in the process induce disease symptoms (Markham and Hille, 2001). These toxins cause wilting, necrosis and chlorosis of plants (Chandra *et al.,* 2010). Over 250 fungal and bacterial phytotoxins have been extracted and characterised (Lebeda and Svabova, 2010). They can, therefore, be used as *in vitro* selection agents (Chandra *et al.,* 2010) (Table 6). This strategy allows uniform exposure of the cells to the selection pressure by culturing them on media containing the toxin (Daub, 1986). A prerequisite for the use of a toxin is to determine that it contributes to pathogenesis, i.e. that it is a pathotoxin (Van den Bulk, 1991). To determine this, various approaches can be undertaken, *viz.*:1) the phytotoxin can be extracted from the infected plant; 2) the phytotoxin's presence at a crucial stage of the disease can be tested; and 3) the phytotoxin's ability to induce symptoms on the plant

Table 6: Examples of selection studies for disease resistance in crops using different selection agents

Crop	Pathogen	Selection agent	Reference
Pineapple	*F. subglutinans*	Culture filtrate	Borras *et al*. (2001)
	F. subglutinans	Culture filtrate	Hidalgo *et al*. (1998)
Amaranthus	*F. oxysporum*	Culture filtrate	Chen and Swart (2002)
Tomato	*F. oxysporum* f. sp. *lycospersici*	Culture filtrate	Toyoda *et al*. (1984)
Peter Pears	*F. oxysporum* f. sp. *gladioli*	Fusaric acid	Remotti *et al* (1997)
Chick pea	*F. oxtsporum* f. sp. *ciceris*	Purified culture filtrate	Khan *et al*. (2004)
Maize	*H. maydis*race T	Purified culture filtrate	Gengenbach *et al*. (1977)
Soybean	*Septoria glycines*	Purified culture filtrate	Song *et al*. (1994)
Sugarcane	*Puccinia melanocephala*	Pathogen	Peros *et al*. (1994)
	Fiji disease virus	Pathogen	Krishnamurthhi and Tlaskal (1974)
	Colletotrichum falcatum	Pathogen	Singh *et al*. (2008)
	Colletotrichum falcatum	Purified culture filtrate	Ali *et al*. (2007b)

can be assessed (Yoder, 1980). Further, the gene(s) responsible for the synthesis of the toxin can be made dysfunctional and pathogenesis of the mutated fungus can then be assessed (Desjardins and Hohn, 1997). In this strategy, it is postulated that cells resistant to the phytotoxins will also be resistant to the pathogen (Daub, 1986; Van den Bulk, 1991; Desjardins and Hohn, 1997; Chandra *et al.,* 2010). Consequently, initial tests should be conducted to establish the effect of the toxin or filtrate on the plant tissue cultures to determine a suitable concentration of the toxin or filtrate that can be used in selection (Lebeda and Svabova, 2010). However, due to the conditions provided *in vitro*, the concentration of toxins produced is likely to be greater than that produced by the fungus *in vivo* (Yoder, 1980). This might result in a weak correlation between the amount of toxin *in vitro* and virulence of the fungus *in vivo* (Yoder, 1980; Tripathi *et al.,* 2008).

The purified toxins can be used in selection strategies (Remotti *et al.,* 1997; Khan *et al.,* 2004). They can be either host specific or non-host specific (Markham and Hille, 2001) and can be purified from culture filtrates (Mayama *et al.*, 1990) or acquired from commercial suppliers (Desjardins and Hohn, 1997; Remotti, 1997). Purification of the

culture filtrates can be done using ultra filtration and fast protein liquid chromatography (Bailey, 1995). Gengenbach *et al.* (1977) used the purified toxin produced by *Helminthosporium maydis* Nisik. and Miyake, which induces southern corn leaf blight in maize, to select for cells that were resistant to the disease. Ali *et al.* (2007b) partially purified a toxin produced by *Colletotrichum falcatum* Went. and used it to select mutants resistant to red rot in sugarcane. Resistance to wildfire disease of tobacco caused by *Pseudomonas tabaci* (Wolf and Foster) Stevens was successfully obtained in plants selected using methyl sulfoximine (a compound functionally similar to tabtoxin), the toxin responsible for symptoms of wildfire. In sugarcane, plants resistant to eyespot disease have been selected by using a toxin produced by the *H. sacchari* (Chaleff, 1983; Prasad and Naik, 2000).

Culture filtrates can be used when there is no reliable description of the toxins produced by the fungus. The fungal CF are prepared by passing the liquid culture through a series of filters in order to remove the mycelia and conidia (Sengar *et al.,* 2009). This is an easy and effective method as culture filtrates have been shown to be phytotoxic (Suprasanna *et al.,* 2009, Chandra *et al.,* 2010). Hidalgo *et al*. (1998) reported that culture filtrates of *F. subglutinans* were toxic to pineapple calli and leaves. *Fusarium oxysporum* culture filtrates incorporated into tissue culture media were also reported to have an inhibitory effect on the growth of *Amaranthus hybridus* plantlet roots (Chen and Swart, 2002). Toyoda *et al.* (1984) used *F. oxysporum* f. sp. *lycopersici* (Sacc.) Snyder and Hansen culture filtrates to select for resistant tomato cells after exposure to the mutagen N-methyl-N-nitro-N-nitrosoguanidine (MNNG). However, although culture filtrates contain the toxins, their effect on callus or plants can be due to interaction of the toxins with other compounds present in the filtrate, which may not be important in pathogenesis (Van den Bulk, 1991). Further, cells should not be exposed to high levels of the toxin early in selection as this might affect the ability of resistant cells to regenerate (Gengenbach *et al.,* 1977).

Tolerance to toxins or culture filtrates expressed by somaclonal variants should correlate to tolerance to the pathogen (Van den Bulk, 1991; Svabova and Lebeda, 2005). Hence, the toxin-tolerant plants should be inoculated with the pathogen to confirm tolerance (Chen and Swart, 2002). According to Koch's postulates (Parry, 1990), plants susceptible to the pathogen should exhibit symptoms similar to those displayed by diseased plants from which the pathogen was initially isolated. The tolerant plants should display no or minimal symptoms in the presence of the pathogen

in the plant tissue (Gengenbach *et al.,* 1977; Arcioni *et al.,* 1987; Botta *et al.,* 1994). Since inoculation is usually carried out in non-sterile environments and there is, therefore, potential for secondary infection by other pathogens, it is important to confirm that the inoculated pathogen is the causal agent of observed symptoms (Harris *et al.,* 1999). This can be achieved by re-isolation of the pathogen onto appropriate culture media and identification of the isolates (Chen and Swart, 2002; Abdel-Monaim *et al.,* 2011).

c) Molecular analyses of variants

Analysis of the changes that occur at the DNA level resulting from culture-induced somaclonal variation and mutagenic treatments are important to understand the resulting variation (Hoezel, 1998; Rasheed *et al.,* 2005). Evaluation of variation based on visible traits is not reliable as they are dependent on the environment and age of plants (Kunert *et al.,* 2003). Molecular markers (DNA and protein based) are more reliable as they identify internal changes that have a genetic origin (Kunert *et al.,* 2003). DNA marker systems used in analysis of such variation include Amplified Fragment Length Polymorphism (AFLP) (Chuang *et al.,* 2009), Restriction Fragment Length Polymorphism (RFLP) (Patzak, 2003) and Random Amplified Polymorphic DNA (RAPD) (Rasheed *et al.,* 2005).

AFLPs involve the following steps: 1) digestion of genomic DNA; 2) attachment of small DNA segments called adapters to the digested fragments; 3) PCR amplification of the fragments using primers specific for the adapters and 4) separation of the PCR products (Saunders *et al.,* 2001, Chuang *et al*., 2009). The technique requires no prior knowledge of the genomic DNA sequence, as they generate a large number of polymorphic bands and results are reproducible (Yang *et al.,* 2005). Consequently, it has been used widely in analysis of somaclonal variation and mutations (e.g. Pontaroli and Camadro, 2005; Steinmacher *et al.,* 2007)

In the RFLP method, genomic DNA is digested using restriction enzymes and the resulting fragments are separated by gel electrophoresis. A radioactive-labelled DNA probe isused to identify a fragment with the desired sequence (Liu, 2007). Difficulties in handling and storage of the radioactive reagents make RFLP an unfavourable technique (Nakazato and Gastony, 2006).

RAPD is a simple and time efficient technique compared to RFLP (Garcia *et al.,* 2004). It results in amplification of few random segments of DNA, allowing for variation in

length and number of amplified segments when the sequence of the segments is altered (Hoezel and Green, 1998). RAPDs have been used widely for analysis of genetic variation (Afiah *et al.,* 2007; Ehsanpour *et al.,* 2007;Ngezahayo *et al.,* 2007) and Cuesta *et al.* (2010) recommended the use of RAPDs for analysis of somaclonal variation. Those authors also used ISSR, Selective Amplified Microsatellite Polymorphism Length (SAMPL) and AFLPs and found that only the RAPDs revealed differences between clones on pine. Ehsanpour *et al.* (2007) used RAPDs to detect variation in potato after exposure of calli to UV-C radiation.

d) Phenotypic evaluation of variants

In somaclonal variant plants, the expression of the desired trait has to be accompanied by important agronomic features (Singh *et al.,* 2008) as certain traits cannot be determined *in vitro*. In sugarcane, transferring *in vitro* plants to the field is, therefore, necessary in order to enable observation of agronomic features such as cane height, number of nodes, stalk weight, internodal length and sucrose content, which determine yield in the crop (Liu and Chen, 1978). These traits can be assessed and a comparison made between the somaclonal variants and vegetatively propagated plants (Krishnamurthi and Tlaskal 1974; Shkvarnikov and Kulik, 1975; Song *et al.,*1994; Watt *et al*., 2009). Song *et al.* (1994) compared brown spot disease resistant soyabean plants obtained by *in vitro* screening with their parents and selected those with similar or superior agronomic traits. Krishnamurthi and Tlaskal (1974) developed *in vitro* sugarcane lines that were resistant to Fiji disease virus through somaclonal variation and selected lines that had retained the high yield that characterised the parents.

In vitro induced-mutagenesis and subsequent selection using fungal culture filtrate is, therefore, an alternative approach to conventional breeding and genetic engineering for producing sugarcane plants tolerant to *F. sacchari.*

3. Materials and methods

3.1 *Fusarium sacchari* culture and filtrate preparation

3.1.1 *F. sacchari* – PNG40 isolation, culture and preservation

Fusarium sacchari PNG40 was isolated from aborted *E. saccharina* borings in sugarcane stalks from Pongola (McFarlane *et al.,* 2009). The fungus was grown on Nash and Snyder agar [15 g/l peptone, 1.0 g/l K_2HPO_4 (Sigma, St Louis, USA), 0.5 g/l Mg_2SO_4 (ACE Chemicals, Johannesburg, SA), 1.0 g/l pentachloronitrobenzene ($C_6Cl_5NO_2$) (PCNB), 20 g/l agar, 0.1 % (w/v) streptomycin (Sigma) , 0.012 % (m/v) neomycin (Sigma)], a selective medium for *Fusarium* spp*.* and the species was confirmed using Inter Sequence Simple Repeats (ISSRs) (McFarlane *et al.,* 2009). Once isolated, a PNG40 colony was transferred to Potato Dextrose Agar (PDA) (Biolab, Wadeville, SA) in a 90 mm Petri dish (Concorde Plastics, Johannesburg, SA). After 5 days, mycelial squares (5 x 5 mm) were cut using a scalpel and were transferred to 2 ml vials (Corning, Massachusetts, USA) containing 900 µl of 15 % (v/v) glycerol (Merck, Wadeville, SA) and were stored at -80 °C for preservation. These mycelial squares were used in future investigations to prepare starter cultures.

Two methods of preparing the starter and batch cultures were attempted. In the first procedure, a mycelial square was thawed and transferred to an Erlhenmeyer flask containing 100ml potato dextrose broth (PDB) (200 ml/l potato infusion, 10g/l glucose [Sigma] at pH 5.6 and autoclaved at 121 °C, 1.2 kg/cm^2 for 20 min). The flask was agitated in a shaking incubator (Labcon, Johannesburg, SA) at 145 rpm and 28-30 °C for 7 days. Thereafter, mycelia were sieved out by passing the culture through sterile cloth (Supa Wipes, Setsmart, Saltriver, SA) over a funnel into a sterile Schott bottle. The concentration of the conidial suspension was established using a haemocytometer (Hawksley, Sussex, UK) and was adjusted to 3 x 10^6 conidia/ml using sterile water. A batch culture was prepared by transferring 100 µl of the conidial suspension to 250 ml PDB in an Erlhenmeyer flask. The flask was plugged using cotton wool, sealed with aluminium foil and maintained in a shaking incubator at 145 rpm and 29 ˚C for 7 days. For this method, the colour and dry mass of the batch cultures were observed to be inconsistent from batch to batch probably due to variations in quantities of constituents of the potato infusion amongst the batches.

In an alternative procedure, a mycelial square from the culture stock was thawed and placed on PDA (Sigma, St Louis, USA) in a Petri dish using a sterile inoculating loop. The plate was sealed with parafilm (Pechiney Plastic Packaging, Wisconsin, USA) and incubated at 30 ˚C for 3 days. Thereafter, a mycelial square was cut from the leading edge of the *F. sacchari* colony (Fig. 5), transferred to 250 ml of PDB (6a) (Fluka, St Louis, USA) in an Erlhenmeyer flask and agitated at 145 rpm and28-30 ˚C. After 7 days, the culture was used for filtrate preparation, as described in section 3.1.2. As this approach resulted in better consistency between batches, it was used for all subsequent investigations. All procedures were carried out under aseptic conditions.

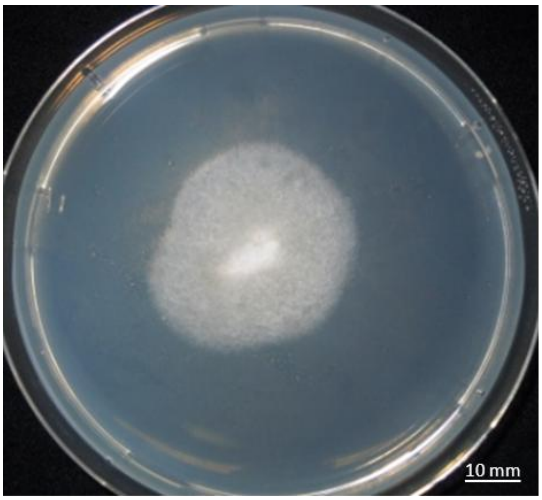


Figure 5: *Fusarium sacchari* colony on Potato Dextrose Agar (PDA) after 3 days.

3.1.2 *Fusarium sacchari* culture filtrate preparation

The 7 day-old culture (Fig. 6b) (prepared as described in section 3.1) was transferred to a sterile 250 ml centrifuge tube and centrifuged (Du Pont, Sorvall® RC 5C) at 12 000 rpm for 5 min. The supernatant was filtered through sterile cloth (Supa Wipes) into a sterile 250 ml centrifuge tube to eliminate the mycelia. The mycelia were caught in the cloth and the fresh mass was recorded immediately; the dry mass was recorded after drying the pellet at 80 °C for 24 h. The filtrate was filtered sequentially through 1) filter paper (Whattman No. 1) using a vacuum pump 2) 0.45 µm membrane filter (Millipore, Ireland) placed on a sintered glass filter (Millipore) using a vacuum pump 3) 0.2 µm syringe filter (Millipore). The filtrate (Fig. 6c) was stored at 4 °C until incorporation into selection media within 24 h. The concentration of each batch of CF was expressed as fungal dry mass/volume of PDB used in the *Fusarium* liquid culture. To ensure batch-to-batch consistency, the absorbance spectrum (200–700 nm) of each batch of CF

(Fig. 7) was recorded using a multi-mode microplate reader (Synergy HT, BioTek® Instruments, Vermont, USA) within an hour of filtration and compared with previous batches. A sample of each batch was diluted with distilled water (1:3) in order to measure absorbance at 250-300 nm.

Figure 6: A visual comparison of a) Potato Dextrose Broth (PDB); b) *Fusarium sacchari* culture in PDB; and c) *Fusarium sacchari* PNG40 culture filtrate after filtration through 0.22 µm filter.

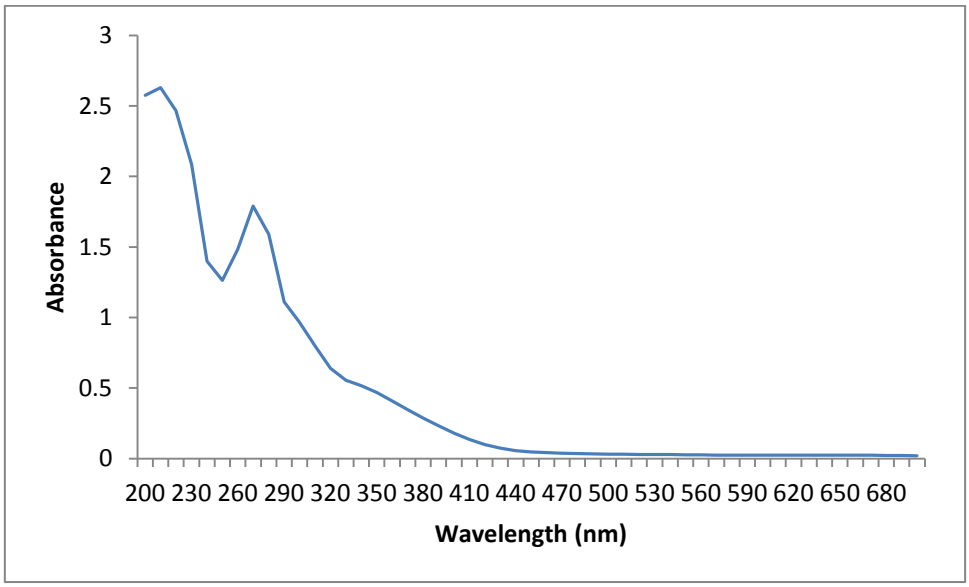


Figure 7: An absorbance spectrum typical of each batch culture used in the investigations.

3.2 Indirect somatic embryogenesis

A modified protocol of Snyman *et al.* (1996) was used in the investigations as the standard protocol for plant regeneration via indirect somatic embryogenesis.

3.2.1 Plant explant source, collection and preparation

The variety NCo376 was field-grown in a field at South African Sugarcane Research Institute (SASRI), Mount Edgecombe, Durban. Sugarcane stalks were harvested by cutting the stalk at the base using a pair of secateurs. The immature leaf roll was removed above the natural breaking point of the sugarcane stalk and leaves were pruned from the apical stalk sections. In the laboratory, the leaf roll was cut transversely on the upper end to 100-150 mm (Fig. 8a) and surface sterilised by swabbing with 95 % (v/v) ethanol. Thereafter, it was inverted and transferred to a beaker with 95 % (v/v) ethanol until initiation in the laminar flow.

3.2.2 Embryo initiation and embryo maturation

Under aseptic conditions, the outer leaf sheaths of the stalk apices were removed using a sterile scalpel until the inner immature leaf roll was exposed. The leaf rolls were swabbed with 70 % (v/v) ethanol after each sheath was removed with exception to the last leaf sheath. The leaf rolls were cut transversely 5-6 cm above the apical meristems and were placed in Petri dishes with liquid Embryo Initiation Medium (EIM) (Table 7) to wash off phenolic exudes from the cut ends. The leaf roll was sliced into 30 transverse sections, each approximately 2 mm thick, from the tip of the leaf roll towards the base of the apical meristem. The leaf roll sections were then placed onto semi-solid EIM in Petri dishes 10 sections per plate (Fig. 8b) with the ventral surface of each disk in contact with the medium. The plates were sealed with parafilm and incubated in the dark at 25-28 °C, subculturing on to fresh medium every 2 weeks. Embryogenic calli started forming after 4 weeks (Fig. 8c).

After 6-8 weeks, 0.2 g of the resulting callus containing both white compact (Fig. 8d) and yellow friable callus, were weighed using sterile aluminium weighing boats and transferred to a Petri dish containing approximately 30 ml semi-solid Embryo Maturation Medium (EMM) (Table 7) (Fig.8e). The plates were sealed with parafilm and incubated in the dark at 25-27 °C for 3 weeks without subculturing.

3.2.3 Embryo germination and plantlet establishment

After embryo maturation, the embryogenic calli were then transferred to Petri dishes with Embryo Germination Medium (EGM1) (Table 7). The pieces of calli were spread out evenly on the media. The plates were incubated in the growth room with 16 h light

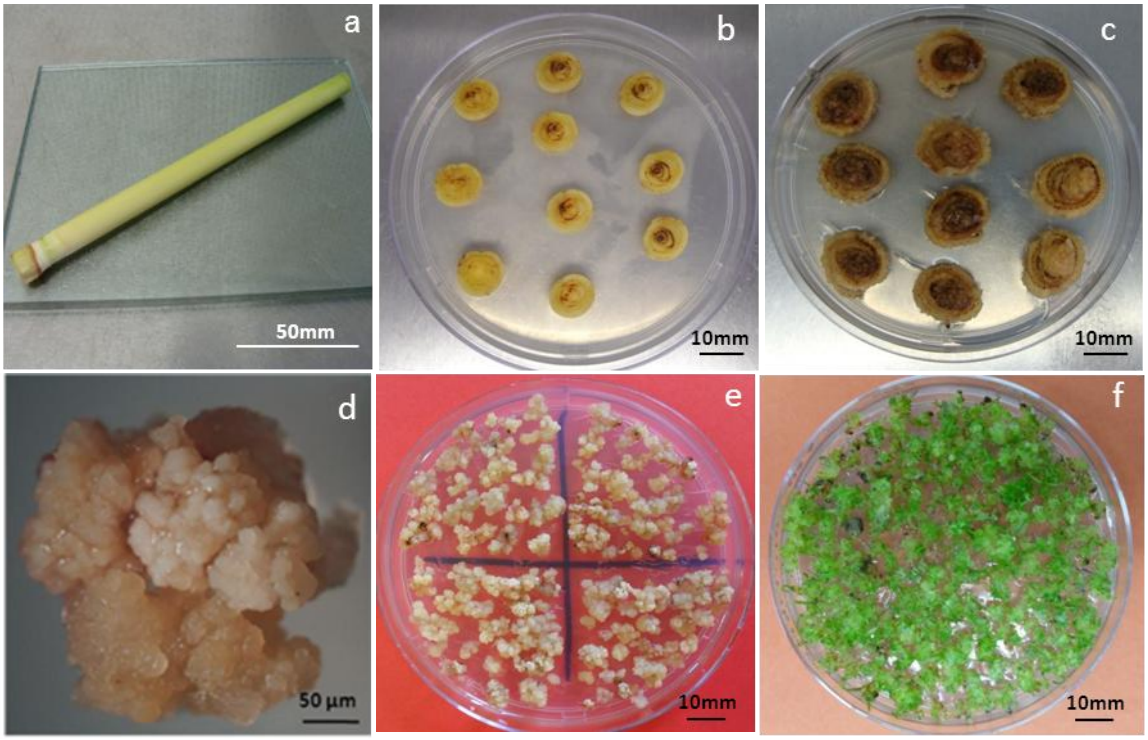


Figure 8: Illustrations of indirect somatic embrygenesis from initiation to embryo germination stages: a) sugarcane leaf roll; b) leaf sections on Embryo Initiation Medium (EIM); c) leaf roll section after 4 weeks; d) white compact embryogenic callus after 8 weeks; e) embryogenic callus on Embryo Maturation Medium (EMM); f) embryo germination on EGM1.

Table 7: Media composition for embryo maturation, germination and plantlet establishment stages

Constituents (g/l)	Medium*			
	EIM**	EMM***	EGM1****	EGM2
MS salts (Murashge and Skoog, 1962) *****	full	full	full	half
Casein hydrolysate (Sigma, USA)	0.5	0.5	0.5	-
Sucrose (Hullets, SA)	20	20	20	5
2,4-dichloro-phenoyacetic acid Sigma (USA)	3	1	-	-
Agar- agar (Biolab)	8	8	8	8

* pH for all media was adjusted to 5.8 using KOH and HCl and autoclaved for 20 min at 121 °C, 1.2 kg/cm^2 for 20 min.
**EIM- Embryo initiation medium ***EMM- Embryo maturation medium ****EGM- Embryo germination medium
***** MS salts were either full- or half-strength

(Philips fluorescent bulb, TL-D 90 de-luxe, Holland; 200 µm/m^2/s photon flux density) and 8 h dark at 26-30 ˚C for 4-8 weeks. The germinated plantlet clusters were separated and individual rooting plantlets of at least 20 mm in height were transferred to Sterivent vessels (110 x 100 x 80 mm) (Duchfa, Belgium) for 4-12 weeks. The vessels contained approximately 180 ml of EGM1, with 20 plants per Sterivent (Fig. 9a). These plantlets were used for further investigations.

3.2.4 Acclimation

Dead leaves were removed from 70-100mm plantlets and the remaining green leaves trimmed above the growing point. The roots were washed with tap water to remove excess media. The plantlets were planted in polystyrene speedling trays (Fig. 9b) containing a substrate composed of peatmoss (Nirom, Alberta, Canada) and vermiculite (Hygrotech, Pretoria, SA) (1:1) (v/v). A 10 kg mixture of the substrate was supplemented with 5 g dolmitic lime (Calmasil®, Middleburg, SA). The plants were incubated in a misting chamber and were watered using a fine mist sprayer (1.5 l/min, 1 min every 6 h) for 3 days. They were then transferred to a polytunnel, watered using automatic sprayers for 5 min (600 ml/min) twice a day and fertilised every 2 weeks (NPK 5:1:5, Profert, Noordsberg, SA) for 2 months.

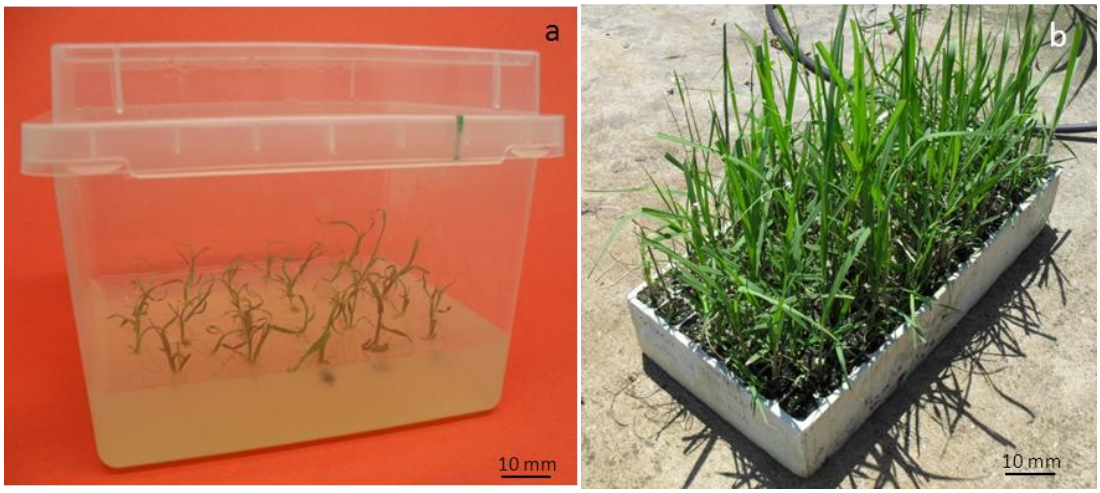


Figure 9: (a) Plantlet growth in sterivents. (b) Plantlet acclimation in speedling trays.

3.3 Establishment of culture filtrate-selection treatments

3.3.1 Selection media preparation

Fusarium sacchari PNG40 culture filtrate (CF) was obtained as described in section 3.1.2. To prepare the selection media containing 0-1500 ppm CF, the appropriate volume of CF stock solution was then added to autoclaved media (± 50 °C) prepared as stated in Table 7. The bottle was swirled gently to allow the CF and media to mix thoroughly before dispensing into Petri dishes for embryo maturation and germination, Magenta® vessels(65 x 65 x 95 mm) (Sigma) or Sterivents (plantlet stage). Fresh CF was used to for each batch of media.

3.3.2 Exposure of embryogenic callus to medium with culture filtrate

Embryogenic calli (Fig. 8d) were exposed to selection media (section 3.3.1) at the embryo maturation and germination stages (Fig. 10 I and II), to determine the most appropriate CF concentrations for selection at each stage. For the control, calli and plantlet regeneration were undertaken without CF in the media.

In one experiment (Fig. 10 I), embryogenic calli were established for 6-8 weeks (section 3.2.1) and 0.2g of calli per replicate were placed on EMM with 0, 20, 50 or 100 ppm CF in Petri dishes. The calli were incubated in the dark at 25-27 °C, subculturing weekly on fresh EMM containing CF. After 3 weeks, callus necrosis and fresh mass were recorded. Callus dry mass was recorded after drying the calli at 70 °C for 48 h. Embryo germination and plantlet growth were then carried out on media without CF, as described in section 3.2. The number of plants obtained per 0.2 g callus for each treatment were recorded after 8-12 weeks.

In another experiment (Fig 10, II), embryo initiation (6-8 weeks) and maturation (3 weeks) (section 3.2.1) were carried out on media without CF. The embryogenic calli were then transferred to Petri dishes containing EGM1 supplemented with 0, 4, 20, 50 and 100 ppm CF. The Petri dishes were placed in the photoperiod growth room for 4-12 weeks, subculturing weekly on EGM1 with fresh CF. Callus necrosis and number of plants per replicate were recorded after 4 and 12 weeks, respectively.

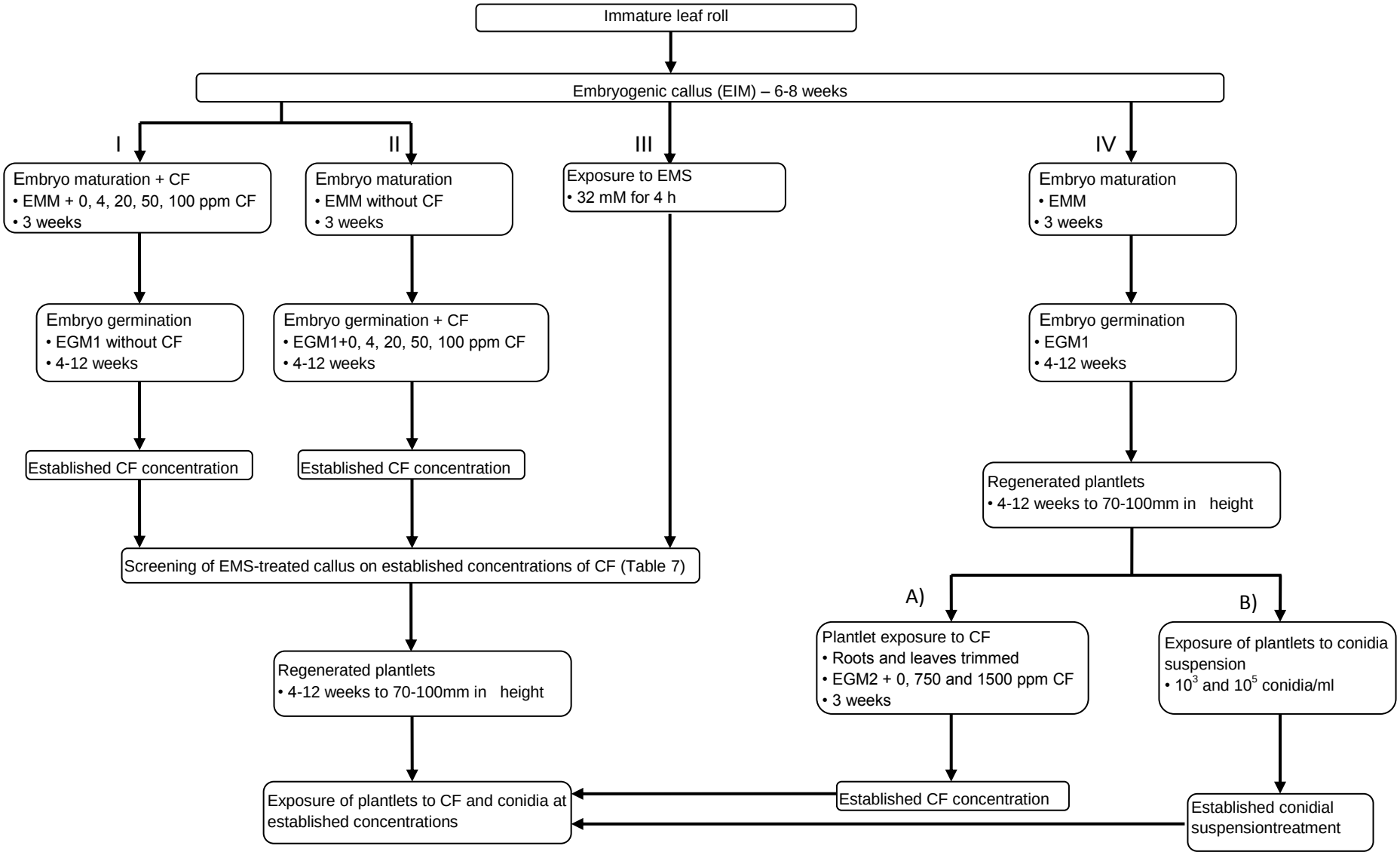


Figure 10: Experimental design for exposure of calli to ethyl methanesulphonate (EMS) and selection using *Fusarium sacchari* culture filtrate (CF) and inoculation with conidia. In the controls, calli were not treated with EMS and the subsequently regenerated plants were exposed to the CF.

3.3.3 Exposure of plantlets to medium with culture filtrate

Plantlets were produced as described in section 3.2. Plantlets, 70-100 mm in height, were placed on sterile paper towels and all the dead leaves at the base of the plantlet were removed using a pair of forceps and scalpel. Three treatments were then tested:

a) Culture of plantlets in media with culture filtrate

The plantlets were transferred to Sterivents (10 plants/Sterivent) containing EGM1 supplemented with 0, 4, 20, 50, 100, 250, 500, 750 and 1500 ppm CF. The plants were incubated in the growth room for 3 weeks (section 3.2.3). The procedure did not have an effect on the plantlets as a result alternative approaches were carried out.

b) *In vitro* dipping of plantlets in culture filtrate

Culture filtrate solutions 0, 10, 20, 100, 150, 300, 600, 1200, 1800, 2400 and 3000 ppm were prepared in 250 ml autoclaved beakers. The plantlets were held at the base of the stem with a pair of forceps and the shoots were immersed for 2-3 s in 250 ml of the tested CF solution. Excess CF was gently shaken off and the plants were immediately transferred to Sterivent vessels, 10 plants per Sterivent, containing EGM1 and incubated in the photoperiod growth room. The procedure did not have any effect on the plantlets as no symptoms developed after 4 weeks.

c) Plantlet root trimming and re-growth in medium with culture filtrate

Plantlets (70-100 mm) (Fig. 11a) were placed on autoclaved paper towels. The roots were trimmed to less than 1mm and all the leaves were trimmed just above the ligule (Fig. 11b) using a scalpel and a pair of forceps. Initially, the plantlets were transferred to Sterivent vessels, 12 plants per Sterivent, containing EGM1 with 750 ppm and 1500 ppm CF. However, due to contamination (incurred due to a mite infestation in the growth room) the plants were transferred to Magenta® vessels (5 plants/vessel)(Fig. 11c) containing EGM2 (Table7) instead of Sterivents with EGM1. The plants were incubated in the photoperiod growth room for 3 weeks. Thereafter, the plants were observed for root re-growth and root length of the individual plants from each treatment was recorded. This method was utilised for selection of plantlets obtained from EMS-treated calli (Fig. 10 lV A).

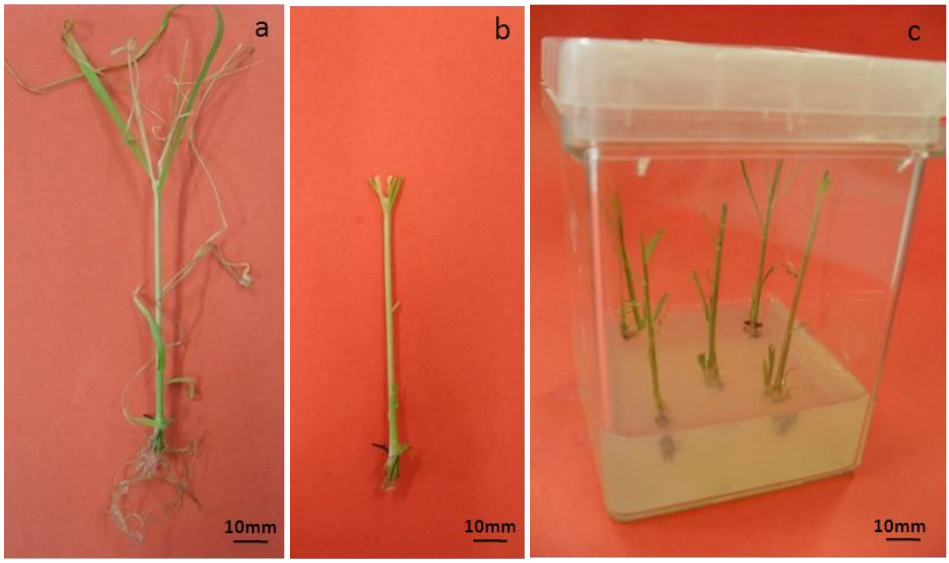


Figure 11: Illustrations of root and leaf trimming prior to culture: a) plantlet before trimming; b) plantlet after root and leaf trim; c) trimmed plantlets on EGM2 in a Magenta® vessel.

3.4 Exposure of plantlets to *F. sacchari* conidia

Plantlets produced as described in section 3.2 were inoculated with *F. sacchari* conidia (Fig. 10 IV B). Conidial suspensions (10^3 and 10^5 conidia/ml) were prepared from *F. sacchari* PNG40 cultures as described in section 3.1. Two different *in vitro* inoculation techniques were attempted. In the first method, 50-70 mm plantlets were placed on sterile paper towels and the dead leaves at the base of the stem were removed using a scalpel and pair of forceps. The leaves were trimmed just above the ligule and the plantlets were inoculated by immersing in 100ml of the conidial suspensions (shoots only) for 2-3 s. Excess liquid was shaken off prior to planting.

In the second method, the plantlets were placed on autoclaved paper towels and the leaves were trimmed with a pair of scissors to remove dead leaves. The plants were inoculated by dipping the scissors into the conidial suspension, trimming the leaves a second time.

Inoculated plantlets from both procedures were planted into Sterivent vessels (6 plants per vessel) containing moist sterile medium of peatmoss and vermiculite (2:1)(v/v)(autoclaved twice, 24 h between each autoclave) and watered with full strength MS salt solution using

a pipette immediately after planting. The Sterivent vessels were incubated in the growth room (section 3.2.3) until mycelia were observed growing on the plantlets. At this point, the Sterivents were transferred to a glasshouse (23-30 °C) and the lids were opened to allow plantlet acclimation. The plants were watered with approximately 80 ml/Sterivent every two days and plantlet mortality was recorded after 2 weeks. As little to no infection was obtained using the second inoculation method, the first method was adopted.

3.5 Embryogenic callus exposure to EMS

A 1M ethyl methanesulphonate (EMS) (Sigma, St. Louis, USA) stock solution was prepared in the fumehood in a 50 ml tube (Corning, Massachusetts, USA). Under aseptic conditions, the stock solution was filter sterilised by passing it through a 0.22 µm syringe filter (Pall Corporation, Michigan, USA). The stock solution was used to prepare 10 ml 32 mM solutions (Koch*et al.*, 2012) in 15 ml tubes (Corning, Massachusetts, USA) by diluting with liquid EMM. White, compact and yellow, friable calli were weighed (0.2 g) and placed into the 32 mM EMS solutions for 4 h (Fig. 10 lll), gently inverting the tubes every 30-45 min. For the controls, calli were transferred to liquid EMM with no EMS for 4 h. Thereafter, the EMS solutions were decanted and the calli were rinsed three times using liquid EMM and then placed on sterile filter paper to soak away the excess media. Appropriate safety precautions were taken during handling of the EMS solutions: spills were wiped with paper towels with 1M KOH and the EMS liquid waste was deactivated using 1M KOH and poured into a plastic bag with cat litter which was then incinerated.

3.6 Selection of EMS-exposed calli and regenerated plants

3.6.1 Callus and plantlet selection using culture filtrate

After exposure to EMS for 4 h, the calli were selected for tolerance using eight CF treatments (Table 8). The calli were transferred into Petri dishes containing EMM for 3 weeks and subsequently onto EGM1 for 4-8 weeks. In each of the selection treatments, 100 ppm CF (established as described in section 3.3.2) was incorporated into EMM and EGM1, as per treatments listed in Table 8. Callus necrosis, fresh and dry mass were recorded after 3 weeks on EMM. Callus necrosis and number of plants per 0.2 g of callus were also recorded after 4 and 12 weeks on EGM1.

Plantlets (20mm in height) that germinated from each treatment were transferred to Sterivent vessels until they were 70-100 mm tall. The roots and leaves of the plantlets were then trimmed and plantlets were transferred to Magenta® vessels containing EGM2 supplemented with 1500 ppm CF (established in section 3.3.3c). Some plantlets from treatment 1 were exposed to EGM2 only [i.e. treatment 1(-CF)] and some were exposed to EGM2 supplemented with 1500 ppm CF [i.e. treatment 1(+CF)]. Root re-growth was observed for each treatment and the root length was measured after 3 weeks. Plantlets that had a root length of at least 10 mm (established as described in section 3.3.3c) were selected for inoculation with *F. sacchari* conidia.

Table 8: Treatments used to select culture filtrate (CF)-tolerant calli after EMS exposure. Embryogenic calli (0.2g) produced on EIM were exposed to EMS (32mM) for 4 hours. n = 10- 8

Treatment	EMS* (32 mM,4 h)	Culture stages	
		Embryo maturation (3 weeks)	Embryo germination and plantlet establishment (4-8 weeks)
		CF** (100ppm)	CF (100ppm)
1	-***	-	-
2	+****	-	-
3	-	+	-
4	+	+	-
5	-	-	+
6	+	-	+
7	-	+	+
8	+	+	+

* EMS – Ethyl methane sulfonate ** CF – culture filtrate *** - – not included **** + – included

3.6.2 Inoculation of plantlets using *F. sacchari conidia*

Plantlets with a root length of at least 10 mm were inoculated with *F. sacchari* conidia in order to confirm tolerance to *F. sacchari* (Fig. 12 I). However, due to the mite infestation some of the plants were contaminated. Of these plants, those with at least 10 mm in root length were immediately acclimatised (section 3.2.3) and the uncontaminated ones were maintained on EGM2 until inoculation. As a result, two methods of inoculation were employed on the two batches of plants i.e. the uncontaminated plants (group 1) (Fig. 12 IA) and contaminated acclimatised plants (group 2) (Fig. 12 IB).

The group 1 plants were removed from culture and were acclimated (section 3.2.4) and, when they reached a height of 150-200 mm, they were inoculated by immersion in 500 ml of a 10^5 conidia/ml suspension for 2-3 s before planting in pots (100 mm diameter; 90 height). Transparent plastic bags were put over each plant and the pots were placed in the glasshouse at 20-34 °C (night-day temperatures). The plastic bags were removed once mycelia were observed growing on the plant. The plants were watered everyday for 2 weeks with approximately 50 ml per pot. The plants that survived were allowed to grow for 6 weeks and were then re-inoculated using the whorl inoculation method ([1]McFarlane, pers. comm.). Prior to this, the plants were stressed by not watering for 5 days. The leaves were held together around the growing region using parafilm to make a 'cup' and 200 µl of a 10^5 conidial/ml suspension was pipetted into the leaf whorl. Half of the plants from the control Treatment 1(-CF) (section 3.6.1) were inoculated with sterile water and the other half with the conidial suspension. The plants were kept in the glasshouse at 20-34 °C, watered using automatic sprayers for 5 min (600 ml/min) twice a day and fertilised every fortnight with NPK (5:1:5).

After 8 weeks, plants with 1-2 internodes were transferred to pots (200 mm diameter; 170 mm height) placed in troughs (200 x 40 cm) and were stressed by not watering for a week. They were inoculated by the toothpick inoculation method ([1]McFarlane, pers. comm). Wooden toothpicks were sterilised by boiling in water for 2 h, replacing the water every 30 min, and autoclaving them twice with 48 h between autoclaving. PDB was prepared in McCartney bottles (6 ml/ bottle) and the toothpicks were placed in the bottles (15 tooth picks/ bottle) prior to autoclaving the media. The media was then inoculated with *F. sacchari* mycelia from a 3 day-old colony on PDA using an inoculating loop. One bottle was left uninoculated as a control. The McCartney bottles were placed in a shaking incubator for 3 weeks at 145 rpm and 28-30 °C. The plants were inoculated by stabbing the stalk with the PNG40-colonised toothpicks 2-3 cm above the soil surface. The protruding toothpick was cut leaving a piece imbedded in the plant tissue. The stabbed area was swabbed with 70 % (v/v) ethanol and allowed to dry prior to stabbing and parafilm was wrapped around the wound to prevent secondary infection by other

[1]McFarlane. S.A.,2011 SAn Sugarcane Research Institute (SASRI), Private Bag X02, Mt. Edgecombe, Durban, 4300, SA.

pathogens. As controls, some treatment 1 (-CF) plants were stabbed with sterile toothpicks and some were stabbed with the PNG40-colonised toothpicks.

The group 2 plants were acclimated (section 3.2.4) and then transferred to pots (100 mm diameter; 90 height). The plants were inoculated by whorl inoculation and then stab inoculation, as described above. These plants were not inoculated by immersion as they were too big for that manipulation.

3.6.3 Detection of presence of *F. sacchari* in plant tissue

Stems of dead and live plants and symptomatic leaves (Fig. 12 II) observed 7-8 weeks after inoculation were placed in Petri dishes. Under aseptic conditions, the stems and leaves were transferred to 50 ml tubes (Corning, Massachusetts, USA) and surface sterilised by submerging in 95 % (v/v) ethanol for 2 min followed by 10 % (v/v) sodium hypochlorite for 5 min. Excess sodium hypochlorite was washed off by submerging the stems and the leaves in sterile water twice. They were then placed in sterile Petri dishes or sterile paper towel and left to dry in the laminar air flow cabinet. Thereafter, the surfaces of leaves and stems were pressed or rolled, respectively, on PDA in a 90 mm Petri dish to test the effectiveness of the surface sterilisation. The leaves were then cut across the leaf blade and the leaf sections were placed on Nash and Snyder medium (section 3.1.1) in another 90 mm Petri dish. The stems were split into longitudinal sections and lesion severity for individual stems was rated on a scale of 0-3 (0 = no lesions 1 = mild, 2 = moderate, 3 = severe). One of the two longitudinal sections was used for re-isolation of the fungus from the lesion and the undamaged area above it and the other was used for staining the stem tissue with lactophenol cotton blue stain (Sigma, St. Louis, USA) (Fig. 12 III).

a) Re-isolation of PNG40 from plant tissue

A longitudinal section from the stem of each dead or symptomless plant was further cut into transverse sections (3-5 mm). The sections from the lesion and the undamaged area above it were then placed on Nash and Snyder agar and incubated at 28-30°C until the fungus was observed growing around the stem sections. Precautions were taken to

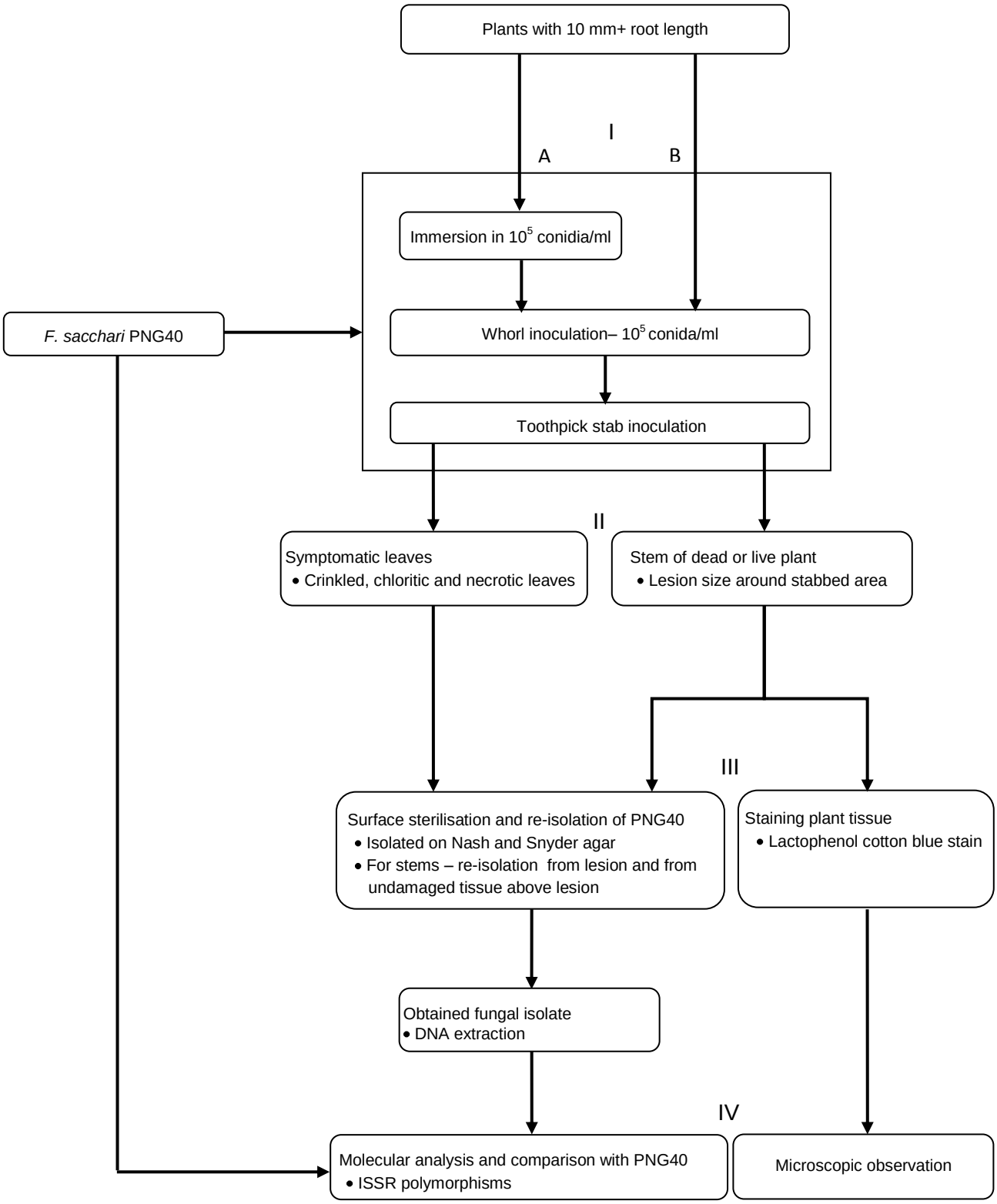


Figure 12: Summary of manipulations undertaken to confirm tolerance of plants to *Fusarium sacchari* PNG40.

prevent contamination from the lesion to the undamaged tissue. Mycelia from the colonies were picked using an inoculating loop and subcultured on Nash and Snyder medium for 3 days at 28-30 °C before DNA extraction. *Fusarium sacchari* PNG40 mycelial squares from the culture stock used initially to prepare the inoculum were placed on Nash and Snyder agar as positive controls. Colonies of the isolates were visually compared to the PNG40 colonies and were subjected to molecular analysis for further comparison.

b) Staining of plant tissue

Stem tissue from each dead or symptomless plant was cut into thin sections from the lesion and the undamaged tissue above it. The sections were transferred to 1.5 ml microfuge tubes containing 1ml of 5% (v/v) KOH. The tubes were placed in a water bath at 65 °C for 30 min. to leach-out pigments. The sections were rinsed with 1 % (v/v) HCl and then twice with distilled water. The microfuge tubes were then filled with 70% (v/v) lactophenol cotton blue stain solution (Sigma) diluted with lactophenol [25 % (v/v) lactic acid, 25 % (v/v) phenol, 50 % (v/v) glycerol] and then placed in a water bath at 65 °C. After 30 min, the stain solution was decanted and destaining of the plant tissue was performed by filling tubes with lactophenol and incubating them at 65 °C for 30 min. A wet mount of the stained tissue was prepared by placing the tissue sections in a lactic acid (98 %) (Sigma) and glycerol medium (1:1) (v/v) on a glass slide and placing a cover slip over the mounted specimen. The specimen was examined with a compound light microscope for fungal colonisation. In the event of the tissues being deeply stained, thus hindering microscopic examination, the sections were destained further using the procedure, as mentioned above.

3.6.4 Molecular analysis of isolated fungus

a) DNA extraction

DNA extraction was carried out by adding 100-200 µl PrepMan™ Ultra Kit sample preparation reagent (Applied Biosystems, California, USA) into sterile 1.5 ml microfuge tubes (Quality Scientific Plastics, California, USA) containing 0.2 g sterile 0.3 mm glass beads. Mycelia were scraped from 30-40 mm diameter fungal colonies, using an inoculating loop into the microfuge tubes. The tubes were vortexed and the mycelia were ground using sterile plastic pestles, after which, the tubes were placed in a block heater at

100 °C for 30 min to release the DNA. The tubes were then centrifuged (Centrifuge 5415R, Eppendorf, Hamburg, Germany) at 13 000 rpm for 5 min and the supernatant was transferred to new 1.5 ml microfuge tubes. The DNA was cleaned by adding a 10% (v/v) of 3M sodium acetate and 250 % (v/v) of absolute ethanol to the supernatant, vortexing and incubating at -20 °C for 2 h. Thereafter, the tubes were centrifuged at 13 000 rpm for 20 min to pellet the precipitated DNA. The supernatant was decanted and the pellet was washed by adding 700 µl of 70 % (v/v) ethanol and centrifuging at 13 000 rpm for 20 min. The pellet was dried in the laminar flow and then re-suspended by adding 30 µl PrepMan™ elution buffer and vortexing. DNA concentration was determined using a spectrophotometer (NanoDrop Technologies, Delaware, USA) and the concentration was adjusted to 200-250 ng /µl using an appropriate volume of elution buffer.

b) Inter simple sequence repeats (ISSR) – PCR

Regions between microsatellite markers were amplified using ISSR 1, ISSR 2, ISSR 4 and ISSR 8 primers (Table 9). The efficacy of these primers to discriminate *Fusarium* species and isolates was tested by performing ISSR-PCR using different *Fusarium* species and isolates (Table 10). Single spore culture stocks of these isolates obtained from the Plant Pathology Department at SASRI were inoculated on PDA and after 3 days, DNA extraction (section 7.2.3a) was carried out from the resulting colonies.PCR reactions were carried out for each primer using a PCR kit (Kapa Biosystems, Massachusetts, USA) in 0.2 ml PCR tubes containing a final volume of 19.5 µl composed of 13.42 µl PCR water (Ambion, Texas, USA), 2 µl Taq buffer with $MgCl_2$(15mM), 0.4 µl dNTPs (10 µm), 1.6 µl primer (10 µm), 0.5 units Taq polymerase and 2 µl DNA template.PCR amplification was carried out using a MyCycler Thermocycler (Biorad, Californina, USA) for ISSR 1 and ISSR 4 and a GeneAmp® PCR System 9700 (Applied Biosystems, California,USA) for ISSR 2 and ISSR 8. Cycling conditions for ISSR 1 commenced with 95 °C for 2 min, then followed by 32 cycles of denaturation at 94 °C for 30 s, primer annealing (temperature in Table 9) for 30 s, extension at 72 °C for 30 s, and a single final extension at 72 °C for 5 min. ISSR 2 , ISSR 4 and ISSR 8 conditions were an initial 94 °C for 5 min, followed by 32 cycles of 94 °C for 1 min, annealing (temperature for each primer in Table 9) for 1 min and 72 °C for 2 min,

Table 9: ISSR primer sequences and annealing temperatures used in discriminating *Fusarium* species and isolates

Primer	Sequence	Annealing temperature (°C)
ISSR 1	$CCCGCATCC(CA)_9$	57
ISSR 2	$CCCGGATCC(GA)_9$	55
ISSR 4	$(AG)_9G$	51
ISSR8	$(CCA)_5RY$*	45

* R = purine Y=pyrimidine

Table 10: *Fusarium* species and isolates used to determine efficacy of ISSR primers to distinguish different *Fusarium* species and isolates

Isolate No.	Species	Variety*	Location
4	*F. andiyazi*	98F	Pongola
6	*F. proliferatum*	98F	Pongola
7	*F. proliferatum*	98F	Pongola
8	*F. andiyazi*	98F	Pongola
9	*F. andiyazi*	N41	Gowar
PNG 40	*F. sacchari*	-	Pongola

*Sugarcane variety from which the *Fusarium* species were isolated

and a final extension at 72 °C for 7 min. PCR products were separated on a 2 % (m/v) SeaKem® LE agarose gel (Lonza, Maine, USA) in 1 % (m/v) Tris Boric EDTA (TBE) buffer. Loading dye [20 % (v/v) Ficoll 400, 0.13% (v/v) Bromophenolblue, 0.05 % (v/v) Xylene cyanol) incorporated with 0.6 % (v/v) GelRed™ nucleic acid gel stain (10 000X) (Biotium, California, USA)] was mixed with the PCR products prior to separation at 60 V, together with a 100 bp O'Gene™ Ruler DNA Ladder Mix (Fermentas, Maryland, USA) as the molecular weight marker. The gel was visualised and photographed using a AlphaImager™ 2200 gel doc system (Alpha Innotech Corparation, California, USA). The banding patterns of the *Fusarium* isolates were analysed and the differences were noted. DNA from re-isolated fungi from the dead plants was subjected to ISSR-PCR using ISSR

1, ISSR 2, ISSR 4 and ISSR 8 and the banding patterns were compared to those of PNG40 in order to confirm similarity between the inoculated *F. sacchari* PNG40 and the re-isolated fungus (Fig. 12 IV).

3.7 Microscopy and photography

Stereo images of specimens were observed and photographs were taken using either a Nikon SMZ 1500 stereoscopic zoom microscope attached to a Nikon DS-Fi1 camera (Nikon, New York, USA) or a Leica MZ125 stereoscopic zoom microscope (Leica, Heerbrug, Swizterland) attached to a AxioCam camera (Carl Zeiss, Jena, Germany). Light microscopic observation of specimens was carried out using a Nikon Eclipse 50i light microscope (Nikon, New York, USA). Other images of the specimens were taken using a Canon 400D or Nikon Coolpix L21 camera.

3.8 Statistical analysis

Data were analysed using Genstat statistical package 13th edition (VSN International, Hemel Hempstead, UK). Data were initially tested for normality using the Shapiro-Wilk test and for homogeneity using the Bartlett test. In some cases, data were either arcsine or $\log_{10}$ transformed prior to carrying out appropriate statistical tests, as noted for each set of results in the following section.

4. Results

4.1 Establishment of callus and plantlet screening conditions

This investigation served to establish the following: 1) the concentration of *F. sacchari* culture filtrate (CF) at which somatic embryos could be screened during embryo maturation and embryo germination stages; 2) the effect of CF on the regenerative potential of somatic embryos; and 3) a method for screening germinated plantlets using CF and *F. sacchari* PNG40 conidia.

4.1.1 Determination of *F. sacchari* culture filtrate concentration for screening of somatic embryos

In two studies, varying concentrations of *F. sacchari* CF were incorporated at either the embryo maturation or embryo germination stages in order to establish the appropriate concentration for screening embryos at each stage. The specific details of statistical analyses are given in Appendices 1-7.

a) Establishment of culture filtrate concentration for screening somatic embryos at the maturation stage

To determine CF concentration for screening at embryo maturation, embryogenic calli (0.2g per replicate) were transferred to embryo maturation medium (EMM) containing 0, 20, 50 and 100 ppm CF for 3 weeks. A piece of callus was recorded as necrotic if at least 50 % of the piece was brown, eventually turning black (Fig. 13b - d). No necrosis was observed in the control (Fig. 13a), but percentage necrotic callus increased significantly as the concentration of CF increased, with the highest value (61.5%) being obtained from the 100 ppm CF treatment (Figs. 13 and 14). The extent of necrosis (50 – 100 % of the callus being brown – black) varied from one piece of callus to another (Fig. 13c) and some calli from higher CF concentrations were not necrotic (Fig. 13d) when the data were recorded. Some necrotic calli, especially those exposed to 100 ppm CF, turned mucilaginous and developed root hairs after 3 weeks. The fresh and dry mass of each callus was recorded after 3 weeks of exposure on EMM containing CF. Subculturing was carried out weekly on media with freshly-prepared CF. Despite the significant differences in callus necrosis, no significant differences in fresh and dry mass were detected amongst the treatments (Fig 15).

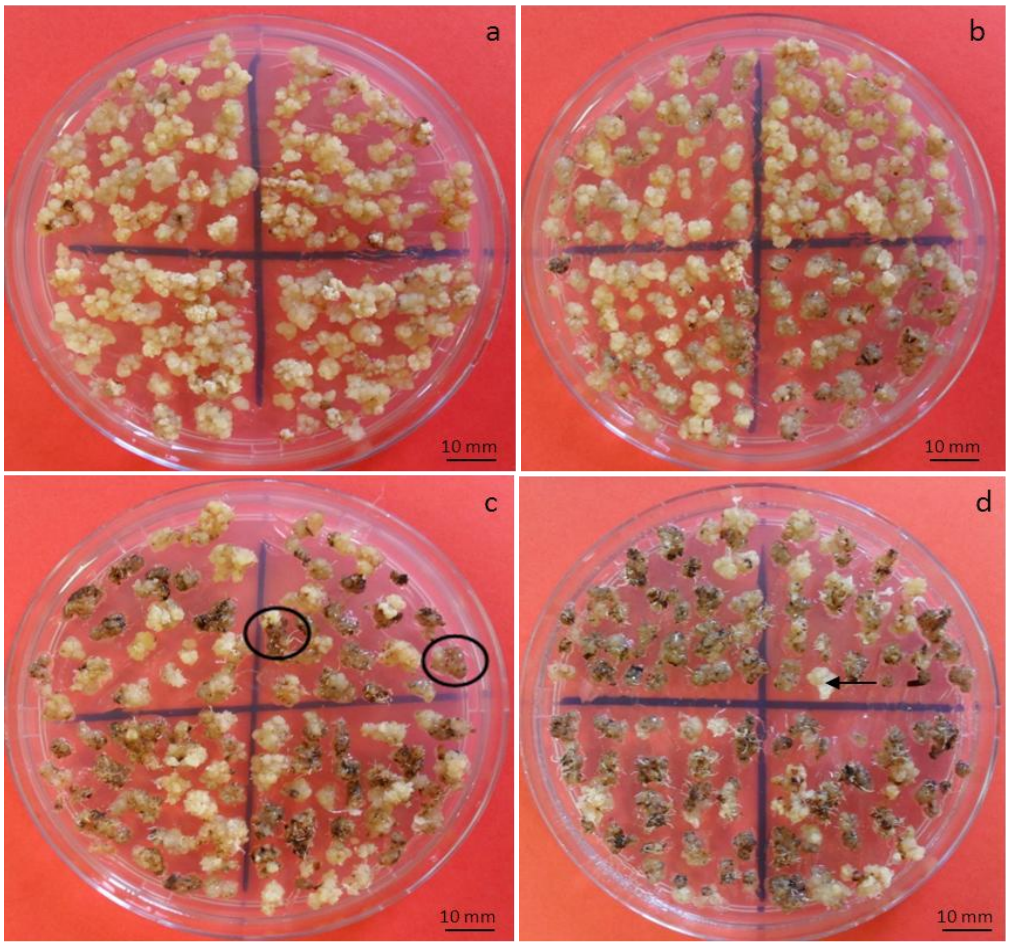


Figure 13: The visual appearance of embryogenic calli exposed to culture filtrate (CF) during embryo maturation for 3 weeks. Embryogenic calli (0.2g/replicate in each quadrant) were cultured on embryo Maturation Medium (EMM) supplemented with (a) 0, (b) 20, (c) 50 and (d) 100 ppm CF for 3 weeks with weekly subculture. The encircled calli (15c) show varying extents of necrosis in the same replicate. The solid arrow (15d) indicates a piece of callus completely free of necrosis from replicate with mostly necrotic calli.

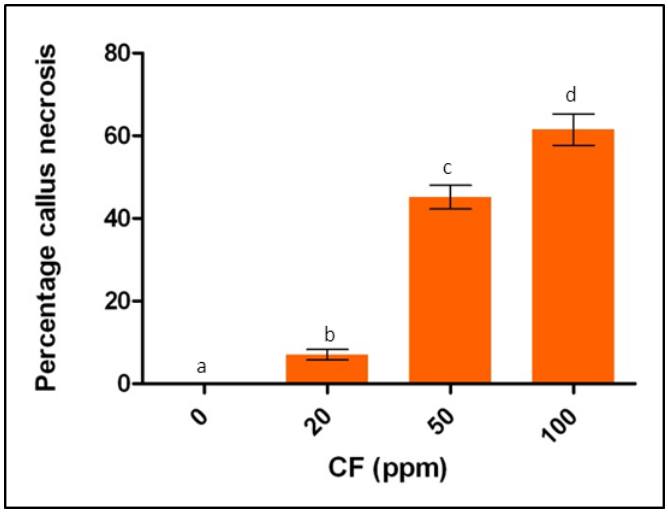
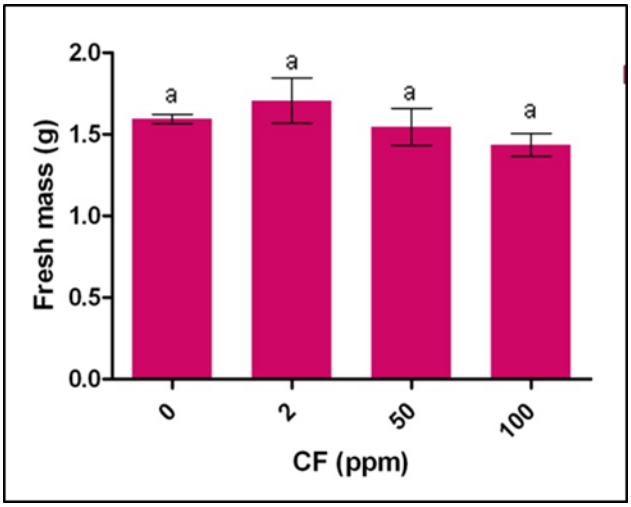


Figure 14: Percentage callus necrosis during embryo maturation on medium containing culture filtrate. The culture filtrate was incorporated in EMM and results were recorded after 3 weeks of culture. Dissimilar alphabet characters denote a statistical significance (REML variance components analysis analysis and Holm-Sidak test; data was arcsine transformed, but untransformed data is presented here; F pr< 0.001, n=12-21, mean ± SE, Appendix 1)

The effect of CF incorporated into the media during embryo maturation on subsequent embryo germination was determined by transferring calli exposed to EMM+CF to EGM1-CF for 4 - 12 weeks, subculturing every 4 weeks. The embryos started germinating after 1 week on EGM1. The embryos from the control (Fig.16a), and most from the 20 ppm treatment, germinated normally (Fig. 16b). However, some non-necrotic calli only produced roots (Fig. 16c) and those calli that were brown eventually turned black (Fig. 16d). The number of germinated plants per 0.2 g of callus from each treatment was recorded after 4-12 weeks (Fig. 17). No significant differences in number of plants were observed between the control and 20 ppm, and between the 50 and 100 ppm CF treatments. However, the latter produced significantly fewer plants than the control (no CF in medium), but not compared with the 20 ppm CF treatment. Although there were no statistically significant differences with respect to plantlet yield amongst the tested CF concentrations (20, 50 and 100 ppm) (Fig. 17), there was a trend indicating an inhibiting effect of the CF. Hence, 100 ppm was chosen as the selection treatment at the embryo maturation stage. It was noted that, although fewer plants survived at the highest CF concentrations, those that did, developed faster than those produced on a medium without CF and at the lowest CF concentrations (Fig. 16a-d). Embryos from the former germinated in the first week and those from the latter 3-4 days later.

(a)



(b)

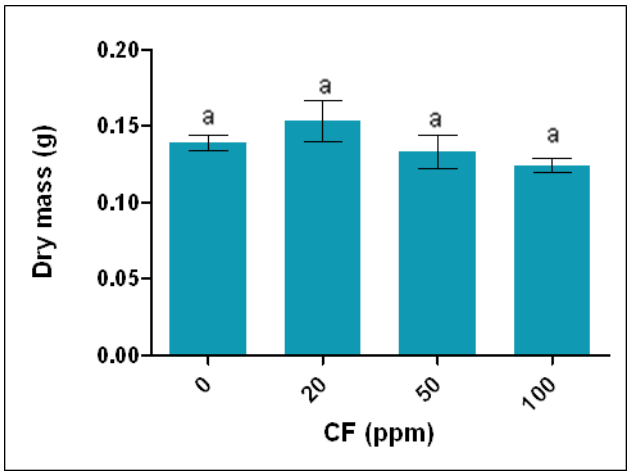


Figure 15: The effect of culture filtrate on callus (a) fresh mass and (b) dry mass. Embryogenic calli were cultured on EMM+CF for 3 weeks with weekly subculture, after which callus fresh and dry mass were recorded. Dissimilar alphabet characters denote a statistical significance (One-way ANOVA; Fresh mass F pr. = 0.312, Appendix 2, Dry mass F pr. = 0.221 Appendix 3; n = 5, mean ± SE).

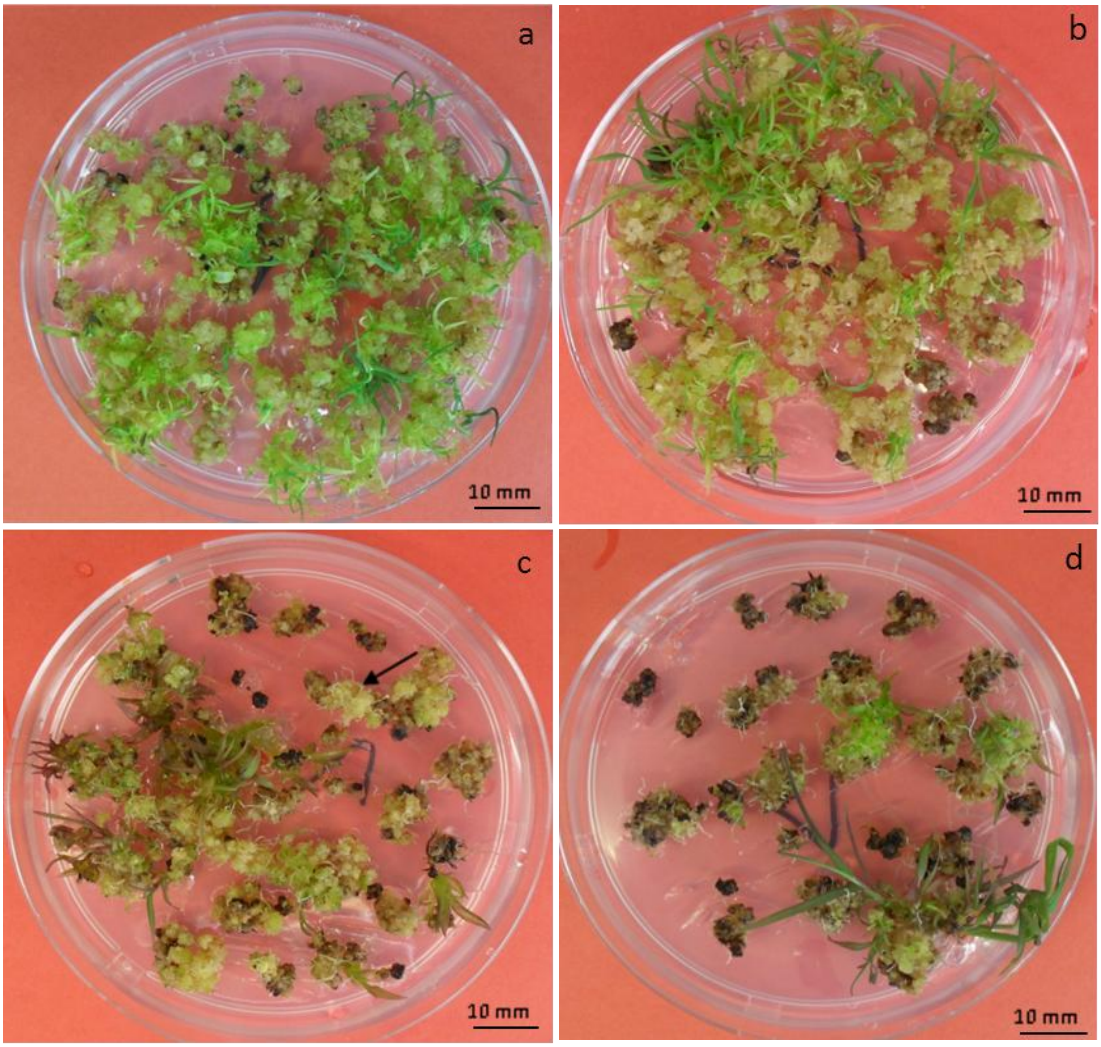


Figure 16: The visual appearance of germinating embryos on EGM1-CF after 4 weeks following culture on EMM+CF for 3 weeks. Embryogenic calli were cultured to EMM supplemented with (a) 0, (b) 20, (c) 50 and (d) 100 ppm CF for 3 weeks prior to placement on EGM1. Arrow (4c) indicates un-necrotic callus producing root hairs.

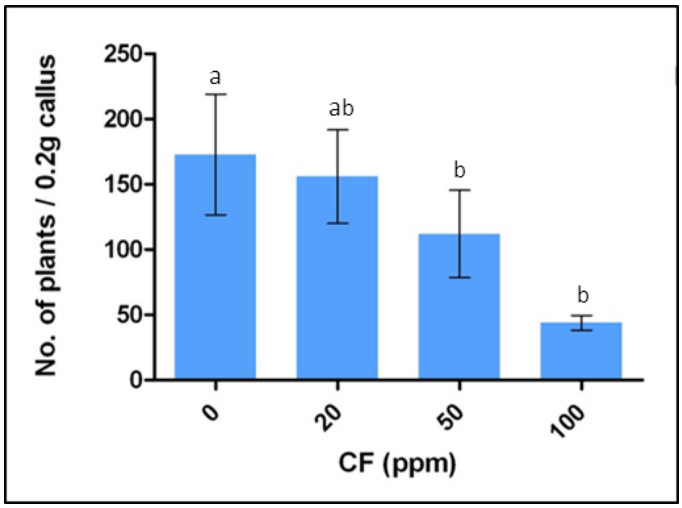


Figure 17: Plantlet yield after exposure of embryos to culture filtrate during the maturation stage. Embryogenic calli were cultured on EMM+CF for 3 weeks and number of plantlets per 0.2 g callus was recorded after 4-12 weeks of germination on EGM1 - CF, subculturing after every 4 weeks. Dissimilar alphabet characters denote a statistical significance (REML variance components analysis and Holm-Sidak test, data was Log_{10} transformed, but untransformed data is presented here, F pr. < 0.001, n = 12-21, mean ± SE, Appendix 4).

b) Establishment of culture filtrate concentration for screening somatic embryos at the germination stage

Embryogenic calli were cultured on EMM-CF for 3 weeks without subculturing, after which they were transferred to EGM1 containing 0-100 ppm CF for 4-12 weeks. The calli started exhibiting signs of necrosis within the first week on the CF-containing medium but callus necrosis was recorded only after 4 weeks (Fig. 18). The percentage callus necrosis was significantly higher in the CF treatments than in the control (Fig. 19). No significant differences were detected amongst the 20, 50 and 100 ppm CF treatments; however, significant differences were observed between each of these treatments and the 4 ppm treatment.

The calli started greening within the first week on EGM1+CF and the number of plants per 0.2 g was recorded after 4-8 weeks (Fig. 20). Those produced from 0.2 g embryogenic callus decreased by at least 50 % from treatment to treatment, as CF concentration increased. The yield of plantlets from the control was significantly higher than that from all the CF treatments; the 4 ppm CF treatment produced significantly more plants than the 20 ppm one and the significantly lowest yields were obtained from the 50 and 100 ppm CF

treatments. Although there were no significant differences in plantlet yield between the 50 and 100 ppm treatments, the latter was chosen as the selection treatment for screening callus at the embryo germination stage.

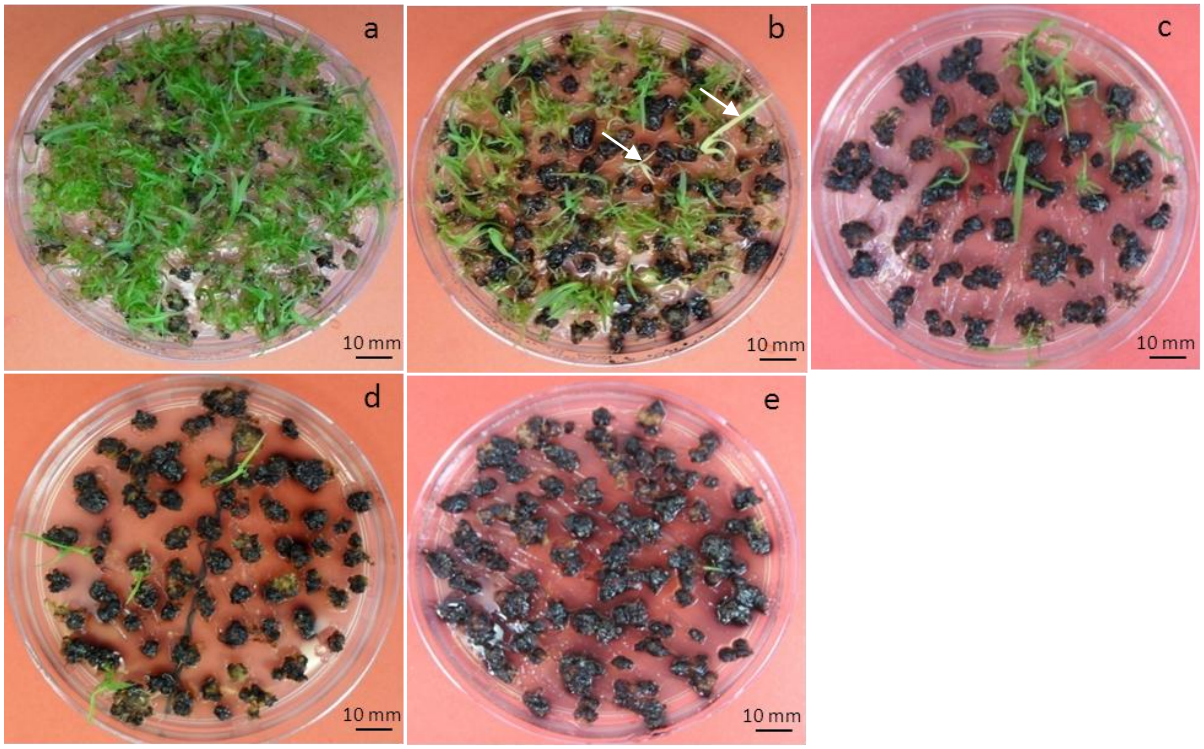


Figure 18: The visual appearance of the effect of culture filtrate incorporated in EGM1 on embryo germination after 4 weeks with weekly subculturing. Embryogenic calli were cultured on EMM-CF for 3 weeks without subculturing, prior to culture on EGM1 supplemented with (a) 0; (b) 4; (c) 20; (d) 50 and (e) 100 ppm. Arrows indicate albino plants.

As expected, there was an inverse relationship between percentage callus necrosis and plantlet yield with the treatments recording the highest necrosis producing the lowest number of plants (Figs. 19 and 20). Also, callus exposure to CF at the embryo germination stage resulted in more severe callus necrosis than at the embryo maturation stage (Fig. 14 and 19) with the highest percentage necrosis recorded at each stage being 95.5 ± 0.9 % and 61.6 ± 3.9 %, respectively. Further, the necrotic calli on EGM1+CF were mostly black in colour compared to brown exhibited by those exposed to EMM+CF (Fig. 13 and 18). Consequently, fewer plants were produced from the former than from the latter (Fig. 19 and 20).

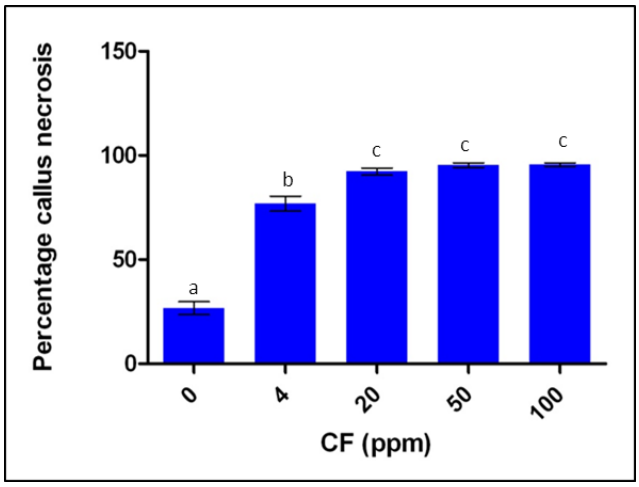


Figure 19: Percentage callus necrosis after embryo germination on medium containing culture filtrate. The embryogenic calli were cultured on EMM-CF for 3 weeks without subculturing prior to embryo germination on EGM1+CF. Dissimilar alphabet characters denote a statistical significance (REML variance components analysis and Holm-Sidak test, data was arcsine transformed, but untransformed data is presented here, F pr. < 0.001, n = 13 - 21, mean ± SE, Appendix 5).

The number of abnormal plants per 0.2 g of callus produced from each treatment after incorporation of CF during either maturation or germination was also recorded (Fig. 21). During the maturation stage, the CF concentration did not have an effect on the number of abnormal plants produced. However, when calli were exposed to CF during germination, significantly fewer abnormal plants were produced from the 20 and 100 ppm treatments compared to the control, but the result from the 50 ppm treatment was the same as the control. At the same CF concentration, there was no significant difference in abnormal plantlet yield between exposure of calli during maturation and germination (Fig. 21). The highest percentage of abnormal plants was recorded for the 50 ppm treatment in the germination stage and no abnormal plants were produced from calli exposed to 100 ppm CF at either tested stage.

4.1.2 Establishment of culture filtrate treatment for plantlet screening

This investigation served to establish a protocol for selecting plantlets produced from the somatic embryos that survived the CF selection pressure. Plantlets were obtained via the standard plantlet regeneration protocol after 17-21 weeks after initiation on embryo initiation medium. Their roots and leaves were trimmed to less than 1 mm and the plantlets

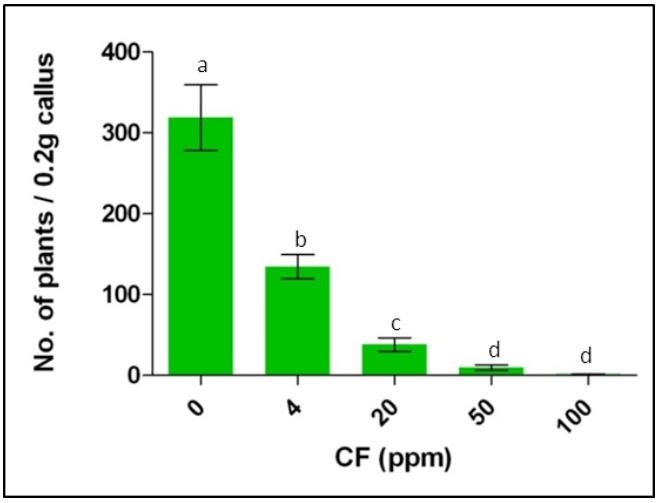


Figure 20: The effect of culture filtrate on embryo germination after 4-8 weeks with weekly subculturing. Embryogenic calli were cultured on EMM-CF for 3 weeks without subculturing, prior to embryo germination on EGM1+CF. Dissimilar alphabet characters denote a statistical significance (REML variance components analysis and Holm-Sidak test, data was Log_{10} transformed, but untransformed data is presented here, F pr. < 0.001, n = 13 -21, mean ± SE, Appendix 6).

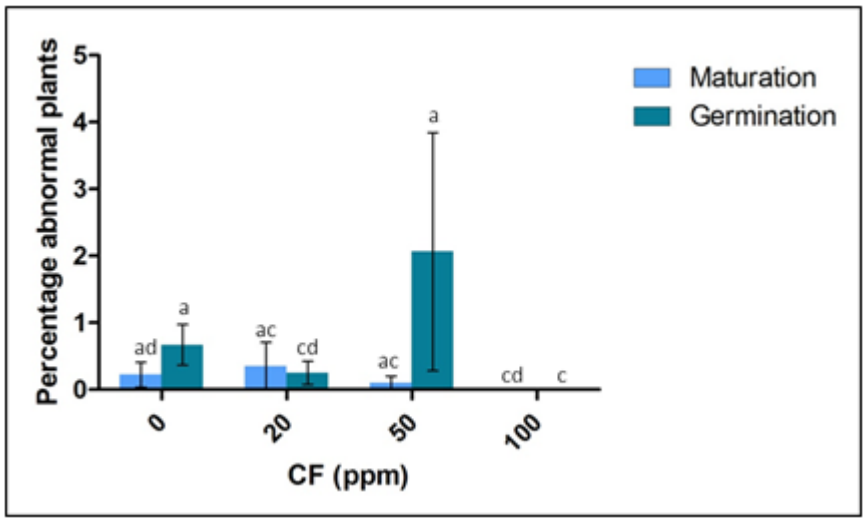


Figure 21: A comparison of number of abnormal plants germinated after incorporation of culture filtrate at either embryo maturation or embryo germination stage. In one experiment, calli were cultured on EMM+CF for 3 weeks then on EGM-CF and in another experiment calli were cultured on EMM-CF then on EGM+CF. Number of abnormal plants per gram callus were recorded after 4-12 weeks on EGM (Kruskal and Wallis one-way ANOVA and Mann-Whitney U test, Chi square pr. = 0.013, n = 13-21, mean ± SE).

were transferred to EGM2 containing 0, 750 and 1500 ppm CF. When root re-growth was determined after 3 weeks, plants from the CF treatments showed stunted root growth and discolouration at the base of the stem (Fig. 22). Significant root re-growth inhibition was observed in the 750 ppm and 1500 ppm treatments with plants with root lengths of 17.8 ± 1.7 mm and 8.5 ± 2 mm, respectively, compared with 39.4 ± 2.1 mm in the control plants (Fig. 23). As the root re-growth in plants from the 1500 ppm treatment was significantly the most inhibited, that concentration was chosen as suitable for screening and selection of CF-tolerant plantlets. Since the average root length at 1500 ppm was 8.5 mm ± 2, it was decided to set 10 mm as the minimum root length threshold to be achieved by potentially tolerant plants exposed to 1500 ppm. The leaves re-grew to approximately the original length in all the treatments, although they were slightly pale green in the 750 and 1500 ppm CF treatments compared with those of the control.

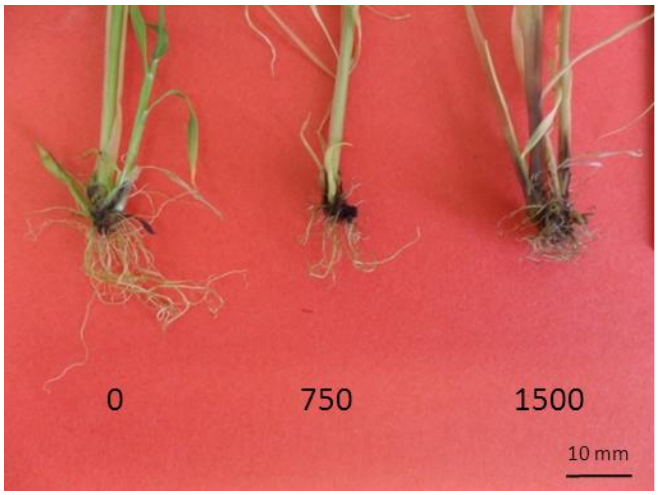


Figure 22: The visual appearance of the inhibitory effect of culture filtrate on root re-growth after root trimming. The roots were trimmed to less than 1mm and the leaves were trimmed just above the natural growing point prior to culture on EGM2 supplemented with 0, 750 and 1500 ppm CF for 3 weeks.

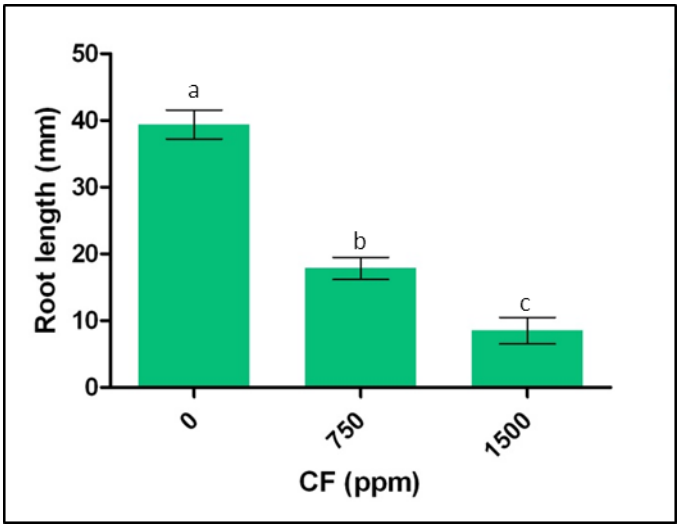


Figure 23: The effect of culture filtrate on root re-growth after root trimming. The leaves were trimmed just above the growing point and roots were trimmed to less than 1 mm prior to culture on EGM2 with culture filtrate and root length was recorded after 3 weeks. Dissimilar alphabet characters denote a statistical significance (One-way ANOVA and Holm-Sidak, $P< 0.05$, n = 5-6, mean ± SE, Appendix 7).

4.1.3 Establishment of *in vitro F. sacchari* spore inoculation treatment for sugarcane plantlets

To determine the concentration of *F. sacchari* conidia for *in vitro* plantlet inoculation, shoots of plantlets produced via the standard regeneration protocol after 8 - 12 weeks of embryo germination were immersed in 0, 10^3 and 10^5 conidia/ml *F. sacchari* conidial suspensions (12 plants/treatment) for approximately 5 s, after which the plantlets were planted in sterile soil *in vitro*. After 2 days, mycelia grew on the plants inoculated with the conidial suspensions, after which sterivent lids were opened to prevent the fungus from over growing and the infected plants wilted, dried and died after 2 weeks (Fig. 24). No mortality was observed in the control, but 43.4 and 100% plants died due to immersion in the 10^3 and 10^5 conidia/ml treatments, respectively (Fig. 25, no statistical analysis was performed). Further, the fungus proliferated on the soil surface and after 2 weeks, it was more noticeable in the 10^5 than in the10^3 conidia/ml treatment.

Figure 24: The visual effect of *Fusarium sacchari* conidia on sugarcane plantlets 14 days after inoculation. Plantlets were dipped in (a) sterile deionised water; (b) 10^3; and (c) 10^5 conidial/ml**.**

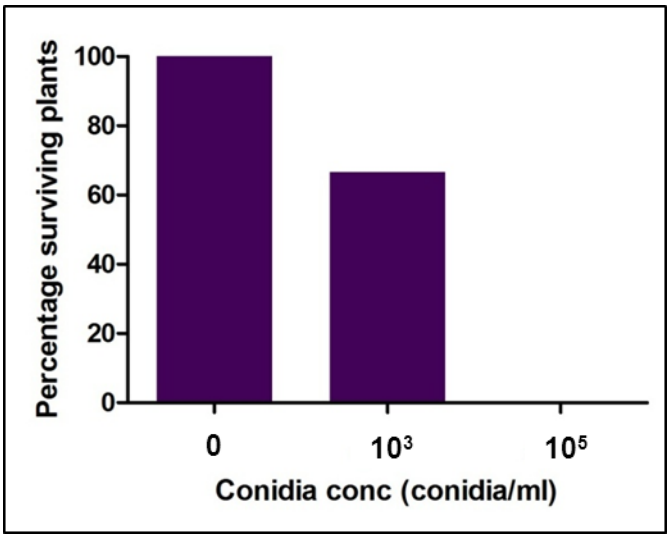


Figure 25: Mortality of plantlets 14 days after inoculation with *Fusarium sacchari* conidial suspensions. The shoots of a total of 12 plantlets/treatment were immersed in conidial suspensions and planted on sterile peatmoss and vermiculite in sterivents. The lids were opened as soon as mycelia started growing on the plantlets.

4.2 Selection of calli and plants tolerant to *F. sacchari* culture filtrate and conidia

The objective of this investigation was to expose embryogenic calli to ethyl methanesulfonate (EMS) to induce mutations, and then subject those calli and the regenerated plants to the various selection treatments established (section 4.1). The treatment conditions for EMS exposure were established by Koch *et al.* (2012) and were

deemed to permit mutations in sugarcane embryos whilst allowing normal embryo germination (Koch *et al.,* 2012). The specific details of statistical analyses are given in Appendices 8-11.

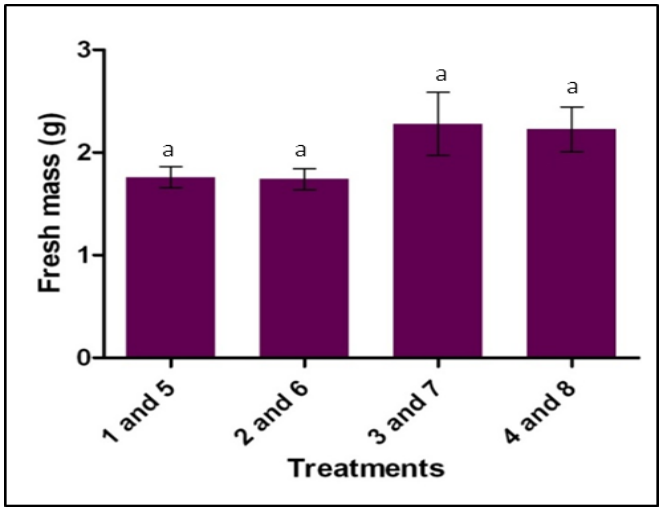
4.2.1 Screening of EMS - treated callus using culture filtrate

Embryogenic calli were exposed to the mutagenic agent EMS and subsequently to CF in three selection treatments (Table 8). These included exposure of the calli to 100 ppm CF (section 4.1.1 a and b) incorporated in either embryo maturation (treatment 4, Fig. 26) or embryo germination medium (treatment 6, Fig. 27), and in both media (treatment 8, Fig. 26). Treatments 3, 5 and 7, were the controls for the respective selection treatments and such calli were not treated with EMS. Treatment 1 was the control for which there was neither EMS treatment nor CF exposure; treatment 2 included EMS treatment and no CF exposure.

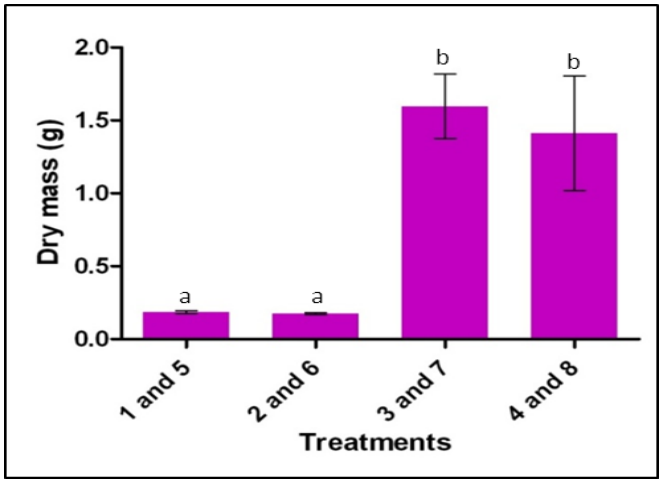
Callus fresh and dry mass were recorded after 3 weeks of embryo maturation (Fig. 26); the treatments are presented in pairs since, for each, their conditions were similar up to when the data were recorded. Callus fresh mass was not significantly different amongst all treatments (Fig. 26a). However, calli cultured on EMM+CF (treatments 3, 4, 7 and 8) had significantly higher dry masses than those on EMM-CF (treatments 1, 2, 5 and 6) (Fig. 26b). This may be due to a mutation that resulted in increased cell division in calli cultured on EMM+CF, thus suggesting the mutagenic effect of the CF. No significant differences in callus fresh and dry mass were observed between EMS-treated calli (treatments 2, 4, 6 and 8) and calli not treated with EMS (treatments 1, 3, 5, and 7).

Callus necrosis was recorded after 3 weeks of embryo maturation in the dark (Fig. 27) and only calli cultured on EMM+CF (treatments 3, 4, 7 and 8) were found to be necrotic. The percentage callus necrosis was not significantly different between the EMS-treated (treatments 4 and 8) and the non-treated calli (treatments 3 and 7). As expected, no necrosis was observed in calli from treatments 1, 2, 5 and 6 as they had not been exposed to CF during the embryo maturation stage.

(a)



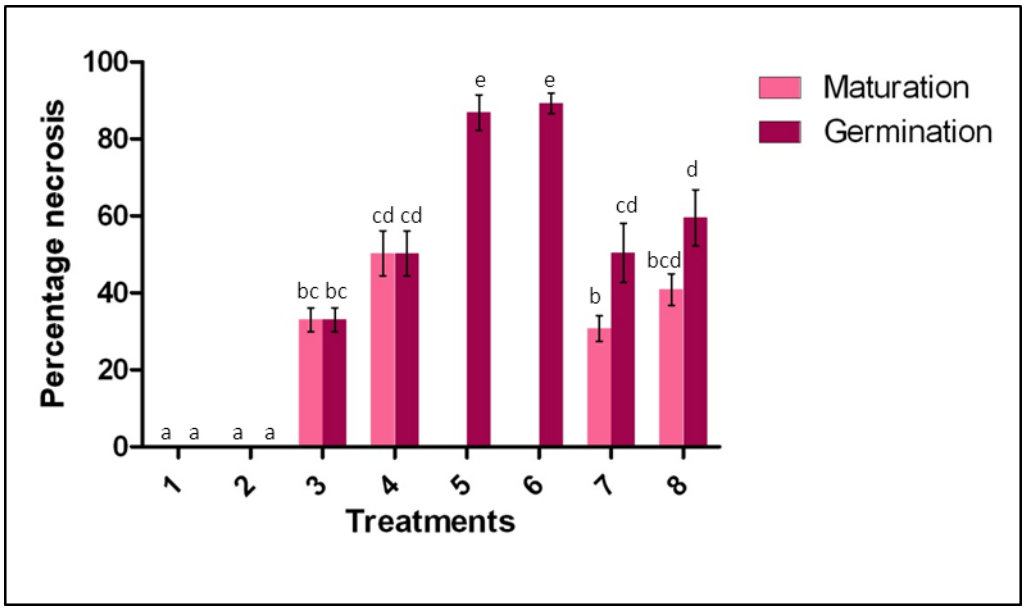
(b)



Key

	EMS	CF (Maturation)	CF (Germination)
Treatment 1:	-	-	-
Treatment 2:	+	-	-
Treatment 3:	-	+	-
Treatment 4:	+	+	-
Treatment 5:	-	-	+
Treatment 6:	+	-	+
Treatment 7:	-	+	+
Treatment 8:	+	+	+

Figure 26: The effect of culture filtrate incorporated during embryo maturation on fresh and dry mass of ethyl methanesulphonate (EMS) -treated calli. The embryogenic calli were exposed to 32 mM EMS for 4 h and cultured on EMM+CF for 3 weeks, after which fresh and dry mass were recorded. The table indicates details of each treatment. Dissimilar alphabet characters denote a statistical significance. Fresh mass (REML variance components analysis, F pr. = 0.114, n=4-6, mean ± SE, Appendix 8). Dry mass (REML variance components analysis and Holm- Sidak test, F pr. < 0.001, n = 4-6, mean ± SE, Appendix 9).



Key

	EMS	CF (Maturation)	CF (Germination)
Treatment 1:	-	-	-
Treatment 2:	+	-	-
Treatment 3:	-	+	-
Treatment 4:	+	+	-
Treatment 5:	-	-	+
Treatment 6:	+	-	+
Treatment 7:	-	+	+
Treatment 8:	+	+	+

Figure 27: The effect of culture filtrate on percentage callus necrosis of EMS-treated calli. Embryogenic calli were exposed to 32mM EMS for 4 h and then to 100 ppm CF incorporated in either EMM for 3 weeks or EGM1 for 4-12 weeks, and in both. Percentage callus necrosis was recorded after 3 weeks of embryo maturation on EMM+CF and after 4 weeks of embryo germination on EGM1+CF. The table indicates details of each treatment. Dissimilar alphabet characters denote a statistical significance (REML variance components analysis and Holm-Sidak test, F pr. < 0.001, n = 10-12, mean ± SE, Appendix 10).

After embryo maturation, calli from treatments 1 - 4 were transferred to EGM1-CF and those from treatments 5 - 8 to EGM1+CF, for 4 - 12 weeks. The calli started exhibiting signs of necrosis during the first week of embryo germination (Fig. 28a) and percentage callus necrosis was recorded after 4 weeks (Fig. 28d). The calli produced roots probably due to the stress environment (Fig. 28a - d) (Remotti *et al.,* 1997). Amongst the calli exposed to CF during embryo maturation, those from treatments 3 and 4 were not exposed to CF during embryo germination and as a result, percentage callus necrosis remained constant at 33 % ± 3.11 and 50.3 % ± 5.8, respectively. However, the calli that

were brown during embryo maturation eventually turned black after 4 weeks on EGM1-CF. The calli from treatments 5 and 6 were exposed to CF during the germination stage only and had the two highest percentage necrosis values of 86.9 % ± 4.6 and 89.2 % ± 2.6, respectively, despite not being exposed to CF during embryo maturation. Treatments 5 and 6 resulted in significantly higher percentage necrosis than treatments 3 and 4, which involved calli exposure to CF during embryo maturation only. The calli from treatments 7 and 8 were treated with CF during both maturation and germination stages. For treatment 7, the percentage callus necrosis increased significantly from the maturation stage to the germination stage, but for treatment 8, no significant differences were obtained between the 2 stages (Fig. 27). Callus necrosis recorded during embryo maturation and germination for calli from EMS treatments 4, 6 and 8 were not significantly different from those exhibited by calli from the respective non-EMS control treatments 3, 5 and 7.

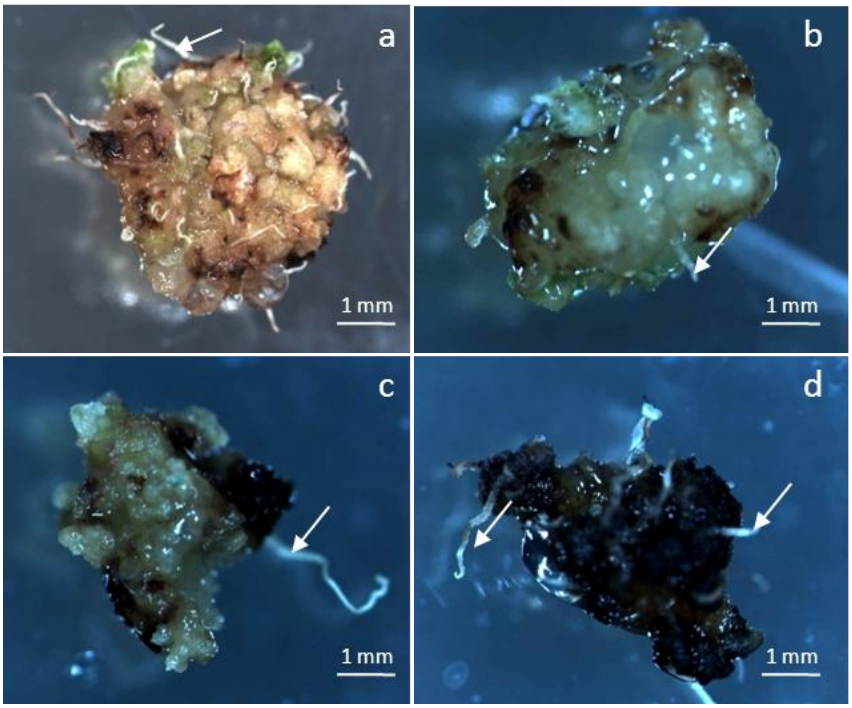


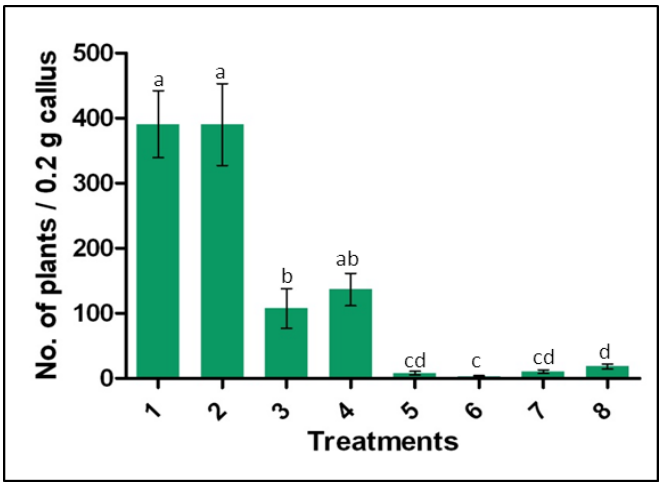
Figure 28: The progression of necrosis in EMS-exposed calli on EGM1 containing 100 ppm culture filtrate after (a) 1 week; (b) 2 weeks; (c) 3 weeks and (d) 4 weeks. The calli were subcultured on to EGM1 with fresh culture filtrate every week. The arrows indicate root hairs protruding from the callus as a result of exposure of the callus to stress conditions.

The number of plants produced from the initial 0.2 g of callus from the selection treatments and controls were also recorded (Fig. 29). It was observed that the plantlet yield generally decreased with an increase in treatment severity. As previously recorded (section 4.1.1 a and b), the number of plants produced from calli exposed to CF during the germination stage (treatments 5, 6, 7 and 8) were significantly fewer than those from calli cultured on medium with CF during maturation (treatments 3 and 4). Despite treatments 7 and 8 having CF pressure in both stages, and treatments 5 and 6 in germination only, no significant differences with regards to plantlet yield were obtained amongst them. However, treatment 8 produced significantly more plants than treatment 6. Although not significantly different, the EMS selection treatments 4 and 8 yielded more plants than their respective controls (treatments 3 and 7). The number of plants produced from the EMS selection treatment 4 was significantly higher than from the other EMS selection treatments (6 and 8). Plantlet yield from treatments 1 and 2 was the same; further, as they did not involve a CF pressure at either developmental stage, both produced significantly higher numbers of plants than all the other tested treatments, except for treatment 4. A relationship was again observed between number of plants and percentage callus necrosis, with treatments that exhibited high percentage callus necrosis producing low plant yields (Fig. 29).

The calli from all the treatments (1 - 8) produced abnormal plants and their percentage per 0.2 g callus was recorded for each treatment. The abnormalities included albino and chimeric individuals (Fig. 30a-c). Although not significantly different, probably due to the small sample size (n = 7-12), the treatments that included EMS-treated calli (treatment 2, 6 and 8) yielded a higher percentage number of abnormal plants than their respective controls (treatments 1, 5 and 7). The exception was treatment 4 that resulted in a lower percentage of abnormal plants than treatment 3 (Fig. 31).

4.2.2 *In vitro* screening of germinated plantlets using culture filtrate

In this investigation, the plants that survived the CF selection pressure during embryo maturation and germination stages (section 4.2.1) were screened further for tolerance to CF at the plantlet stage. The roots of such plants were trimmed to less than 1 mm and plants were cultured on EGM2 incorporated with 1500 ppm CF (section 4.1.2). Contamination by a mite infestation in the growth room reduced the number of plants from



Key

	EMS	CF (Maturation)	CF (Germination)
Treatment 1:	-	-	-
Treatment 2:	+	-	-
Treatment 3:	-	+	-
Treatment 4:	+	+	-
Treatment 5:	-	-	+
Treatment 6:	+	-	+
Treatment 7:	-	+	+
Treatment 8:	+	+	+

Figure 29: Plantlet yield from EMS-treated calli after incorporation of culture filtrate at either embryo maturation or germination stages. The embryogenic calli (0.2g/replicate) were exposed to 32 mM EMS for 4 h and then to 100 ppm CF incorporated in either EMM for 3 weeks or EGM1 for 4-12 weeks, and in both. Number of plants produced per 0.2 g of callus was recorded after 4-12 weeks on EGM1. The table indicates details of each treatment. Dissimilar alphabet characters denote a statistical significance (REML variance components analysis and Holm-Sidak test, data was Log_{10} transformed, but untransformed data is presented here, F pr. < 0.001, n = 7-12, mean ± SE, Appendix 11).

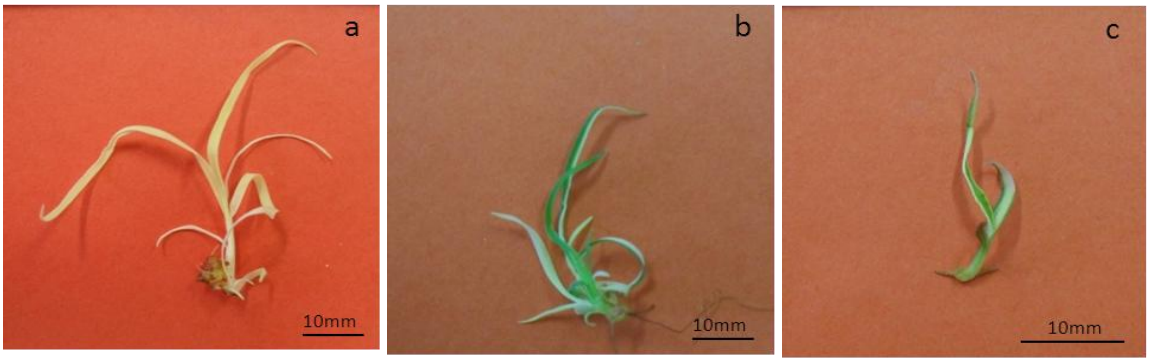
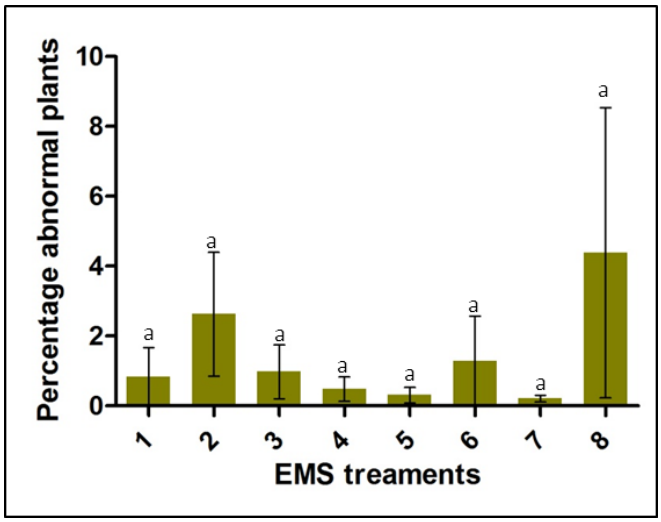


Figure 30: Visually abnormal plantlets produced from EMS-exposed calli. (a) albino, (b) and (c) chimeric plantlets



Key

	EMS	CF (Maturation)	CF (Germination)
Treatment 1:	-	-	-
Treatment 2:	+	-	-
Treatment 3:	-	+	-
Treatment 4:	+	+	-
Treatment 5:	-	-	+
Treatment 6:	+	-	+
Treatment 7:	-	+	+
Treatment 8:	+	+	+

Figure 31: Percentage abnormal plants produced from EMS-treated calli. Embryogenic calli were exposed to 32 mM EMS for 4 h and then to 100 ppm culture filtrate incorporated in either EMM for 3 weeks or EGM1 for 4-12 weeks, and in both. Percentage number of abnormal (albino and chimeric) plants produced per 0.2g of callus for was recorded after 4-12 weeks on EGM1. The table indicates details of each treatment. Dissimilar alphabet characters denote a statistical significance (Kruskal and Wallis one-way ANOVA, Chi square pr. = 0.769, n = 7-12, mean ± SE).

each treatment from the desired 50 plants to 6-45 plants reported per treatment. After 3 weeks, plants with a root length of at least 10 mm were selected (Fig. 32a) for further screening by inoculation with *F. sacchari* and the rest were discarded (Fig. 32b). As previously observed (section 4.1.2, Fig. 23) plants, in the absence of EMS displayed an average root length of 8.5 ± 2 mm, 3 weeks after root trimming and placement on EGM2 containing 1500 ppm. Hence, in subsequent studies, plants produced from EMS-treated calli with a root length of at least 10 mm, were considered to be potentially tolerant to the CF.

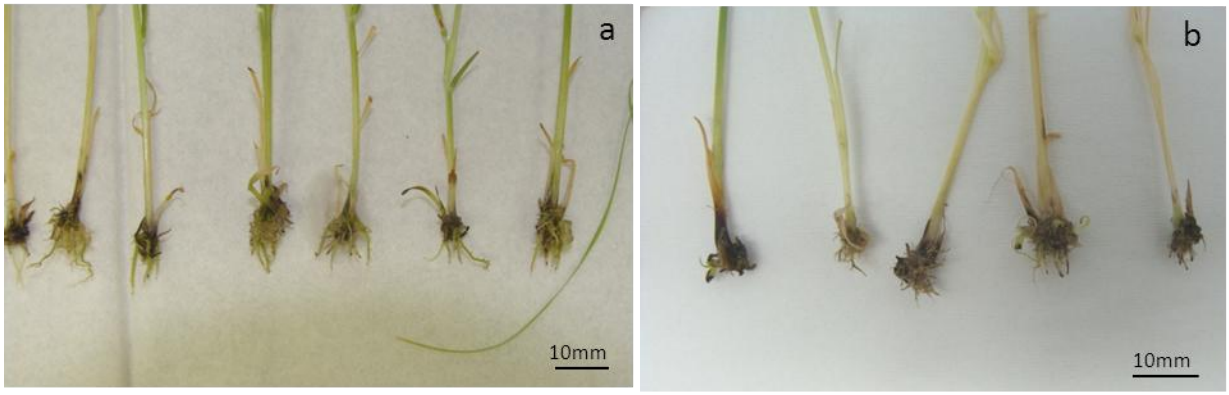
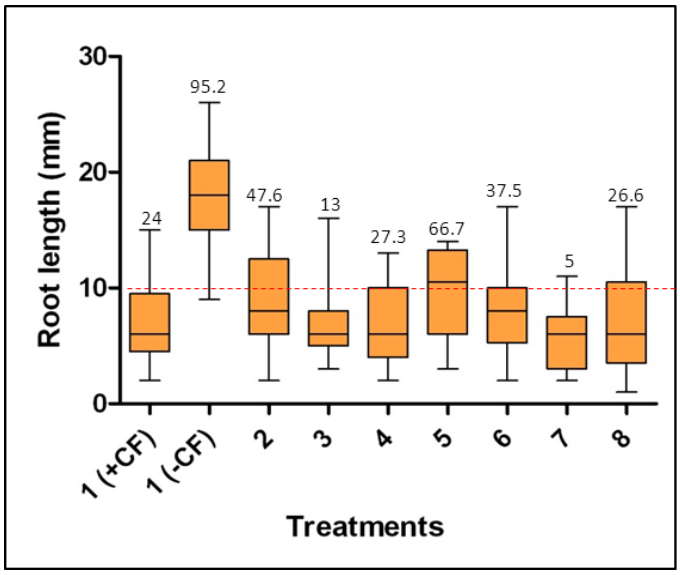


Figure 32:A visual comparison of the appearance roots after root trimming and re-growth on EGM2+CF. Roots of plantlets germinated from EMS-exposed calli were trimmed and plantlets were cultured on EGM2 supplemented with 1500 ppm culture filtrate for 3 weeks. Plantlets with roots of at least 10 mm in length were selected for further screening and those with less were discarded. (a) Roots from selected plantlets and (b) from discarded plantlets.

The root lengths for plants from treatments with EMS-treated calli (treatments 2, 4 and 8) displayed wider interquartile ranges (distance between the 25th percentile and 75th percentile) than their corresponding controls [1 (+CF), 3 and 7, respectively] except for treatment 6 that had a narrower interquartile range than its control (treatment 5) (Fig. 33). In addition, EMS treatments 2, 6 and 8 had wider total ranges than their respective controls (1, 5 and 7), except for treatment 4 that had a narrower range than treatment 3. The wider ranges reflected greater variation in root length in the EMS than in the non-EMS treatments. Furthermore, the EMS treatments had more plants with at least 10 mm in root length than the non-EMS treatments (with the exception of treatment 6) (Fig. 33). This suggests that there may have been mutations that enhanced the plant's ability to overcome root re-growth inhibition by the CF. The root length data from treatment 5 were divergent from those of the other non-EMS treatments probably due the low sample size as a result of contamination by mites as previously mentioned. As expected, treatment 1 (-CF), the negative control, had the highest percentage number of plants with at least a 10 mm root length (95.2 %).

4.2.3 *Ex vitro* screening of plantlets using *F. sacchari*

The plants selected from section 4.2.2 were inoculated with *F. sacchari.* A total of 22 plants (group 1) were exposed sequentially to *F. sacchari* by immersing the shoots in a 10^5



Key

	EMS	CF (Maturation)	CF (Germination)
Treatment 1:	-	-	-
Treatment 2:	+	-	-
Treatment 3:	-	+	-
Treatment 4:	+	+	-
Treatment 5:	-	-	+
Treatment 6:	+	-	+
Treatment 7:	-	+	+
Treatment 8:	+	+	+

Figure 33: The effect of EMS on root re-growth after trimming roots and culture on EGM2+CF. The leaves of plantlets were trimmed and the roots were trimmed to 1 mm before culture on EGM2 + 1500 ppm CF. Plantlets whose roots were at least 10 mm (threshold represented by dashed line) were selected for further investigations. The box represents the interquartile range and the lower, middle and upper limits of the box represent the 25th percentile, median and 75th percentile, respectively. The lower and upper bars represent lowest and highest values recorded, respectively. The numbers above the bars indicate the percentage number of plants with a root length above the 10 mm threshold. n = 6-45, mean ± SE. The table indicates details of each treatment.

conidia/ml suspension, inoculating the leaf whorls with 10^5 conidia/ml and stabbing the stems with toothpicks colonised with *F. sacchari*. Those plants that survived each inoculation step were used for the subsequent one. Another group of 20 plants (group 2) was exposed only to *F. sacchari* by whorl inoculation followed by stabbing with *F. sacchari*-colonised toothpicks as these plants were too big to immerse in the conidial suspension. Of the 22 plants from group 1 inoculated by immersion, mycelia grew on 4

plants from treatment 3 and 4 (2 plants from each treatment) (Fig. 33) after 2 days and the plants eventually wilted, dried and died by day 16 (Fig. 34a). However, no plants from the positive control (treatment 1) died. Two months after whorl inoculation, no mortality or symptoms were observed in all whorl-inoculated plants (groups 1 and 2). The positive controls of both groups exhibited leaf crinkling, chlorosis and necrosis 3-4 weeks after stabbing the stems with *F. sacchari*-colonised toothpicks (Fig. 34b and c) and, after 6-7 weeks, the shoot growing point dried and died in some of these positive controls (Fig. 34d and e; Table 11). Wilting leaves and dead shoot growing point were also observed in some plants from treatment 8 and a single plant from treatment 3 exhibited necrosis and chlorosis (Table 11). Longitudinal sections of stems from dead and live plants from all treatments revealed lesions of varying severity progressing from the stabbed area (Fig. 35). Mortality, symptoms and lesion severity ratings for individual plants from treatments 1 - 8 were recorded (Fig. 35a-d; Table 11). Dead plants were observed in the positive control and treatment 8 only with 42.9 and 40 % mortality (group 1 and 2 combined), respectively. Lesion severity for symptomless plants ranged from 1-2 whilst it was 2-3 in the symptomatic ones. For each individual plant, one of the two longitudinal sections was used for microscopic examination after staining and the other was used to re-isolate PNG40.

4.2.4 Detection and re-isolation of *F. sacchari* PNG40 from potentially resistant plantlets.

Tissues from one of the longitudinal stem sections of each dead or asymptomatic plant (section 4.2.1), were stained with lactophenol blue and examined microscopically in order to detect endophytic fungal colonization of the plant tissue. Dead cells in the lesions of the dead and symptomless plants damaged by the inoculation treatment were brown in colour, and blue-stained hyphae were observed in between cells surrounding the lesions (Fig. 36). However, in asymptomatic plants no colonization was observed in undamaged tissue above the lesion. In the dead plants, lesions extended to the growing region. No hyphal growth was observed in the negative control.

Prior to cutting stem longitudinal sections, stems and symptomatic leaves were surface sterilised and pressed or rolled on PDA to test the effectiveness of the sterilisation. No fungi grew on PDA plates indicating that surface sterilisation was effective. The cut longitudinal section of each dead or asymptomatic plant and the symptomatic leaves, were

Figure 34: Dead plants and symptoms after inoculation with *Fusarium sacchari.* a) Dead plant 16 days after immersion of shoots in 10^5 conidia/ml suspension; b) leaf necrosis, chlorosis and crinkling; c) chlorosis and necrosis; d) dead growing point ; e) wilted plant 3-7 weeks after stabbing stems with *Fusarium sacchari*-colonised toothpicks.

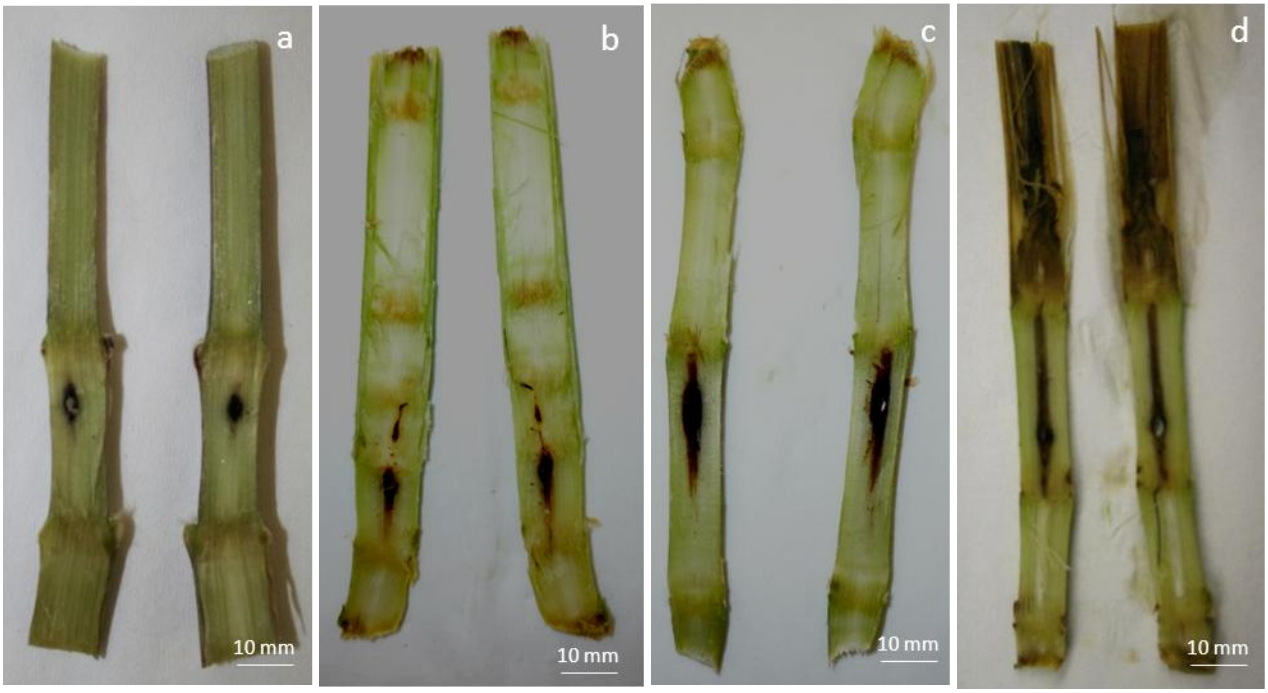


Figure 35: A comparison of lesion severity in inoculated stems. a) no lesion; b) mild lesion; c) moderate lesion; and d) severe lesion. Plants were stabbed 2-3 cm above the soil surface with toothpicks colonized with *Fusarium sacchari* PNG40 and stems were collected after 7 weeks.

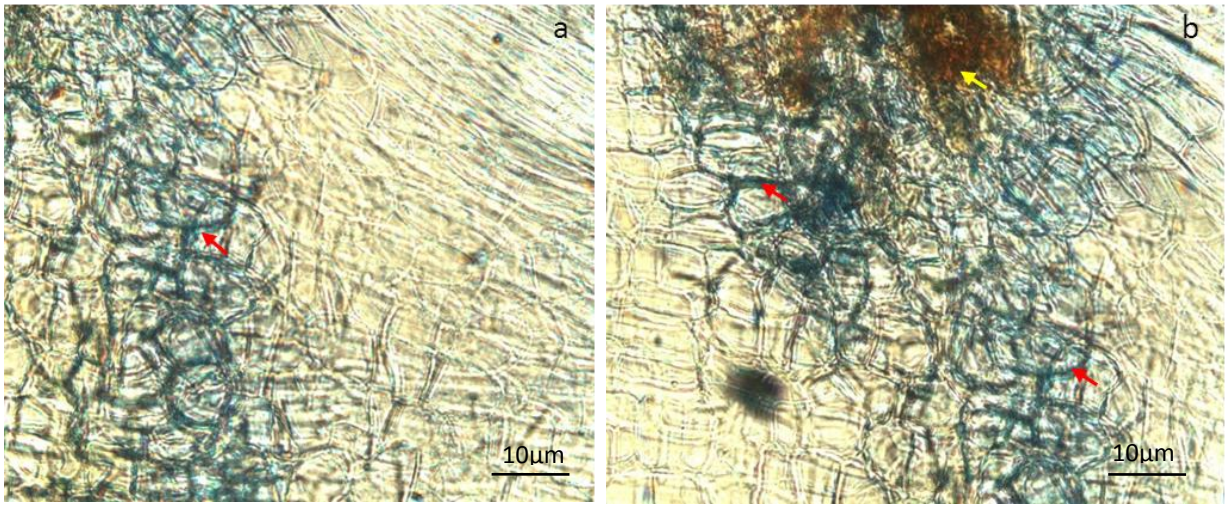


Figure 36: Stained tissue of inoculated plants showing fungal colonisation. Red arrows indicate blue-stained hyphae growing in between cells and the yellow arrow indicates dead stem tissue.

cut into transverse sections and placed on *Fusarium*-selective Nash and Snyder agar in order to re-isolate PNG40 from the plant tissues. *Fusarium*-like colonies grew from the lesions of the dead plants, asymptomatic plants and the leaf sections. No growth was observed from the stem sections from uninoculated plants (Fig. 37a-d). *Fusarium* could not be re-isolated from undamaged tissue above the lesion in most plants; however, the fungus was retrieved from some plants in all treatments except for treatment 5 (Table 11).

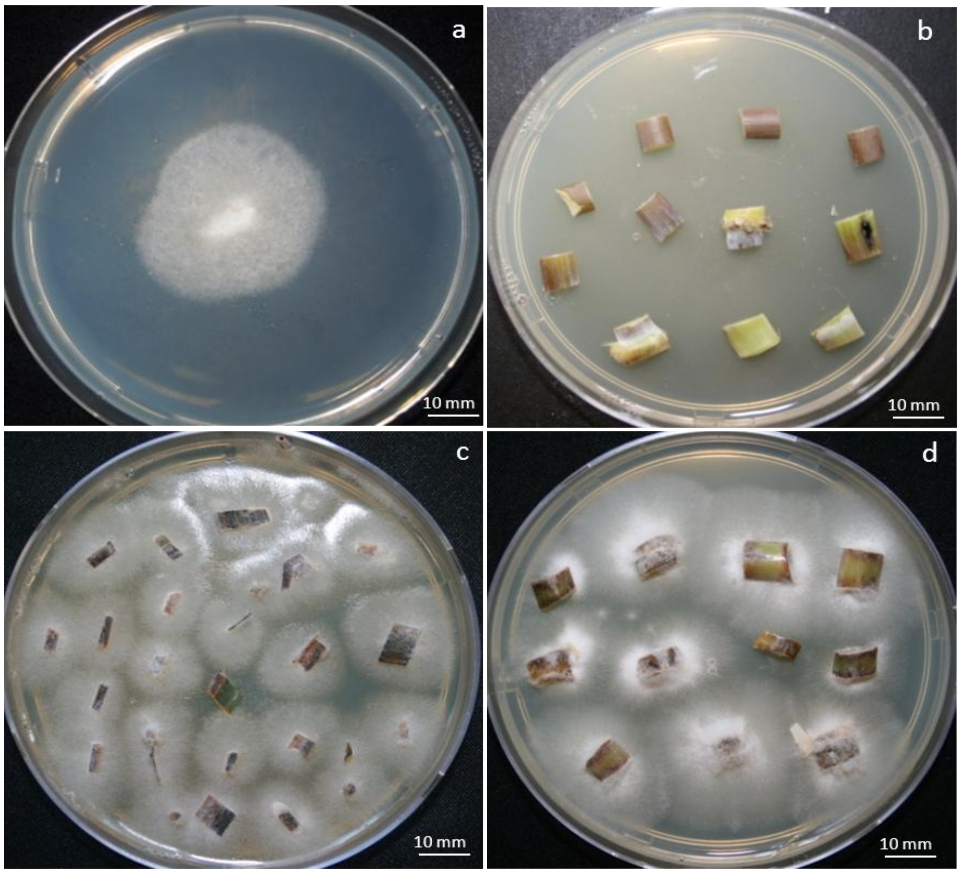


Figure 37: A visual comparison of a) PNG40; b) stem sections from uninoculated control plant; c) fungus re-isolated from damaged leaves; and d) stem of dead plant. Plant material was surface sterilised and surfaces of the plant leaves or stems were gently pressed or rolled on potato dextrose agar to test effectiveness of surface sterilisation before cutting them and placing on Nash and Snyder medium.

4.2.5 Confirmation of identity of retrieved fungal isolates

Genomic DNA was extracted from the fungal isolates obtained from dead plants, damaged leaves and asymptomatic plants. Molecular analysis was conducted using ISSR primers and banding patterns were compared to PNG40 in order to confirm their identity. The effectiveness of the primers ISSR 1, ISSR 2, ISSR 4 and ISSR 8 to distinguish between different *Fusarium* isolates and species was tested by subjecting six different *Fusarium* isolates to ISSR-PCR using these primers. The number of monomorphic, polymorphic bands and unique banding patterns obtained from the *Fusarium* isolates using each primer was recorded (Table 12). ISSR 1 discriminated 4 different genotypes from the 6 isolates, distinguishing between isolates 1 and 4, but was not able to differentiate between isolates 2 and 3, and between isolates 5 and 6 (Fig. 38a). However, ISSR 4 discriminated 5 genotypes, revealing different banding patterns between isolates 1 and 4, and between 2 and 3 (Fig 38c). Further, ISSR 8 detected differences between isolate 5 and 6. ISSR 2 discriminated only 3 genotypes, showing similar banding patterns for isolates 1 and 4, 2 and 3, and between 5 and 6. Hence, a combination of primers ISSR 1, ISSR 4 and ISSR 8 was able to separate each of the 6 different *Fusarium* isolates (Fig 38a, c and d).

The fungal isolates retrieved from the plants were then subjected to ISSR analysis in order to confirm their identity as PNG40. The primers ISSR 1, ISSR 4 and ISSR 8 were used since as discussed above, their combination allowed discrimination of *Fusarium* species and isolates. The banding patterns generated from all the isolates were similar to PNG40 (Fig. 39a-c), hence confirming them as PNG40 (Table 11). Due to time constraints, some of the isolates were not analysed using ISSRs; however, the colony morphology in all of these isolates was similar to that of *Fusarium* spp.

4.2.6 *F. sacchari* PNG40-tolerant plants

All the 15 plants from treatments 2, 4, 5, 6 and 7 tested by toothpick stab inoculation were asymptomatic and exhibited lesion severity ratings (LSR) ranging from 1-2 after two months (except one plant from treatment 5 with a LSR of 3) (Table 11); these were regarded as tolerant. One of the three plants from treatment 3 was symptomatic and displayed a LSR of 2 and exhibited no symptoms and LSRs of 1 and 2. Treatment 8 had 10 plants tested (the highest amongst the treatments) and four of these displayed

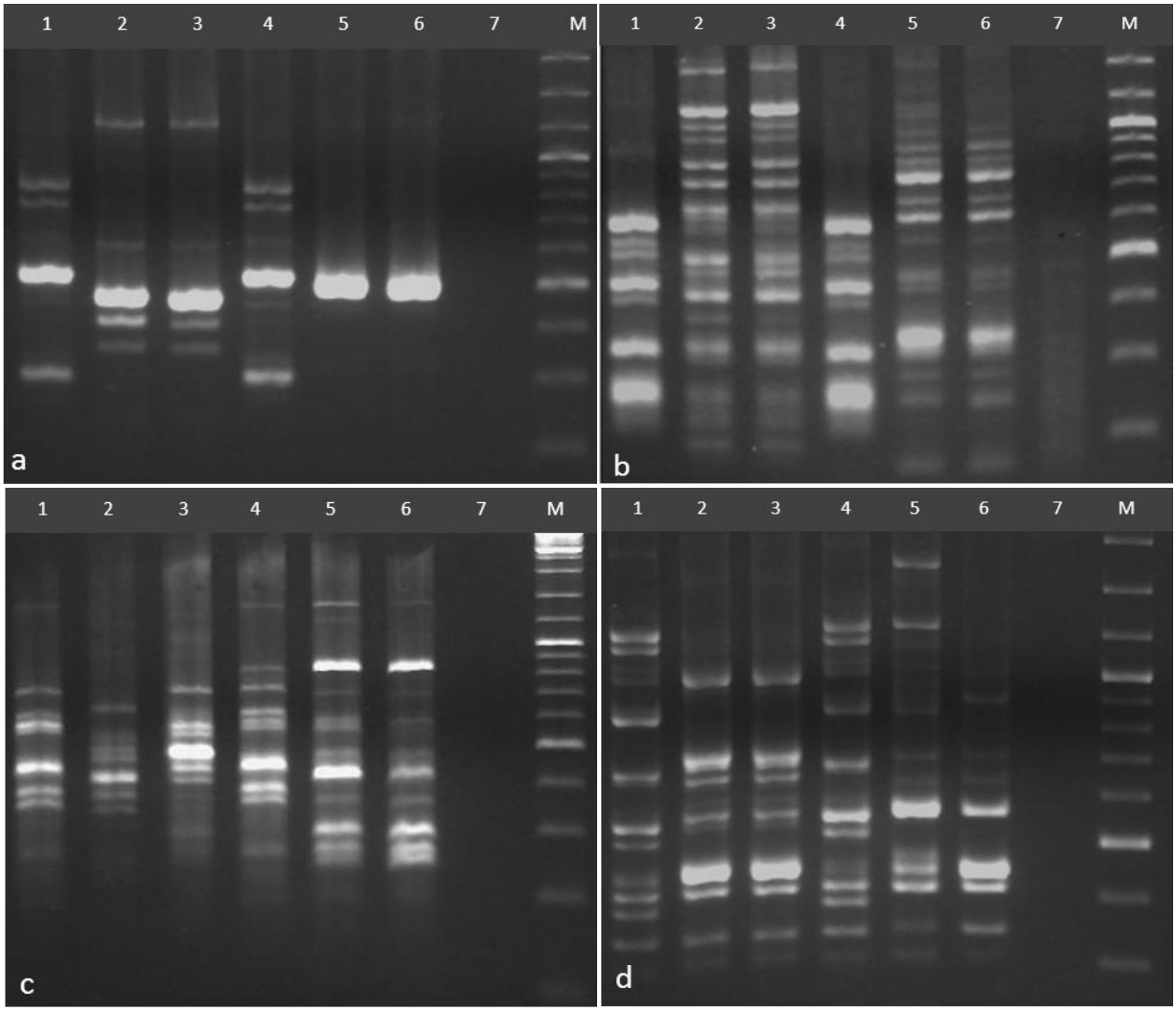


Figure 38: Differentiation of different *Fusarium* species and isolates using ISSR polymorphisms. Four different primers a) ISSR 1; b) ISSR 2; c) ISSR 4; and d) ISSR 8, were used on 6 different isolates: Lane 1- *F. andiyazi 98F4*; 2 - *F. proliferatum 98F6*; 3- *F. proliferatum* 98F7; 4- *F. andiyazi* 98F8; 5- *F. andiyazi* N419; 6-*F. sacchari* PNG40; 7- Water and M- 100 bp marker.

symptoms and died (40% mortality) and a LSR of 3 was recorded from stems of these dead plants. The other 6 plants from treatment 8 were asymptomatic and LSR ranged from 1-2. Plants from treatment 1 stabbed with sterile toothpicks displayed a LSR of 0. Six of the seven plants from treatment 1 inoculated with PNG40-colonised toothpicks displayed symptoms; 3 of them died and a LSR that ranged from 2 – 3 was recorded. A total of 26 tolerant plants were obtained from all the eight treatments and 10 of these permitted endophytic colonisation (Table 11).

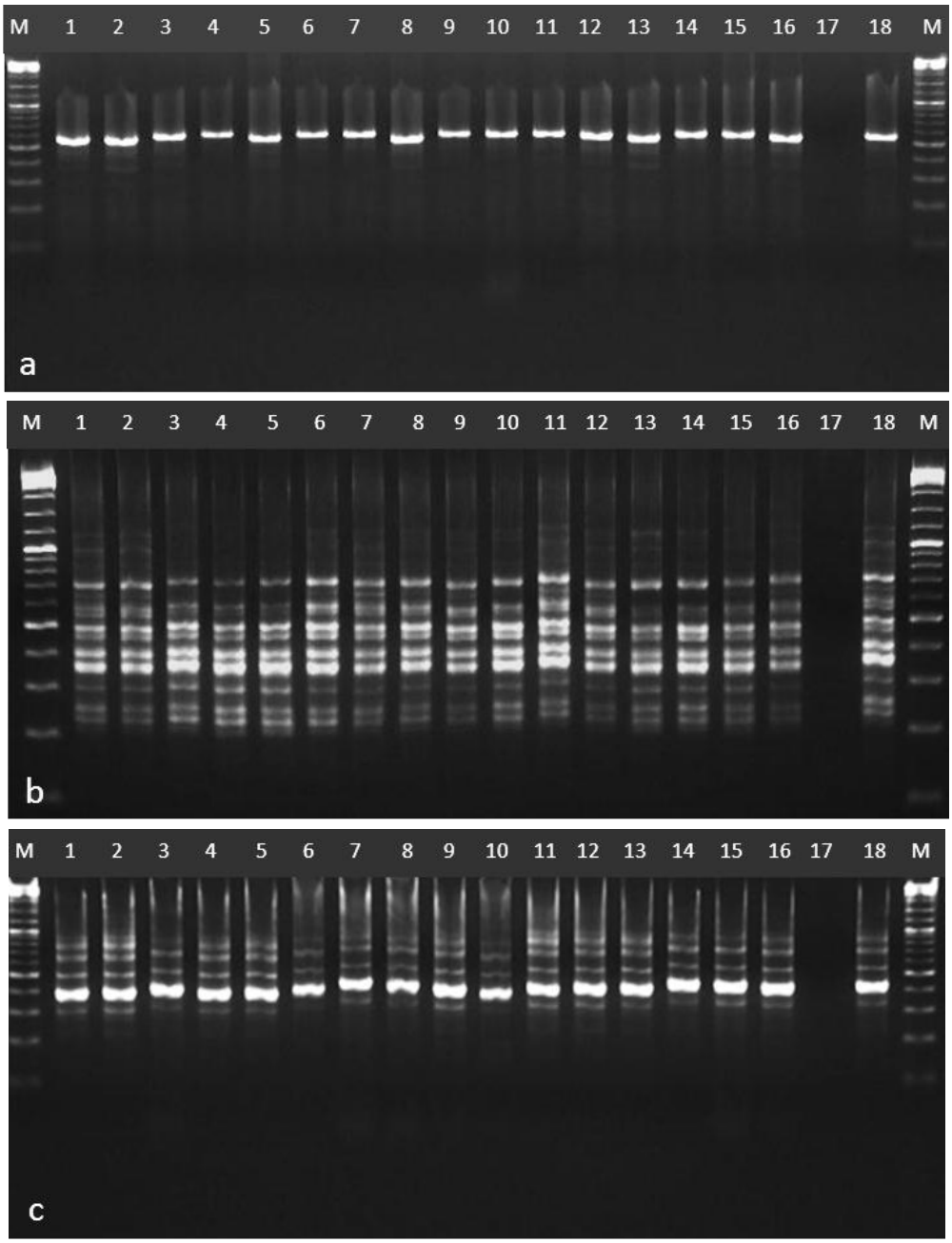


Figure 39: A comparison of banding patterns of the fungal isolates obtained from plant tissue and PNG40 using primers: a) ISSR 1; b) ISSR 4 and c) ISSR 8. The isolates were retrieved from damaged leaves (lanes 1-2), dead plants (lanes 3-7) and asymptomatic plants (lanes 8-16) by surface sterilising leaves and stems and placing cut sections on Nash and Snyder agar. Lanes 17- negative control (water), 18-PNG40 and M-100 bpmarker.

Table 11: Disease severity in plants produced from treatments 1-8 (Fig. 33) after inoculation with *Fusarium sacchari*. Plants were inoculated either by immersion in 10^5 conidia/ml, whorl inoculation and toothpick-stab inoculation, sequentially or whorl inoculation and toothpick-stab inoculation. Symptoms, mortality and lesion severity ratings were recorded 3-7 weeks after stab inoculation and re-isolation of PNG40 from the lesions and the undamaged area above the lesion was carried out. ISSR analysis was used to compare the isolates to PNG40

Treatment	Mortality (%)	Plant	External symptoms***	Severity of lesion****	Dead (D) or alive (A)	Re-isolation on NS agar from lesion	ISSR comparison of isolate to PNG40	Re-isolation on NS agar from undamaged area above lesion	ISSR comparison of isolate to PNG40
T1	0	C7	0	0	A	N*	nd	N	nd
(sterile		C8	0	0	A	N	nd	N	nd
toothpick)		C10	0	0	A	N	nd	N	nd
T1	42.9	C1	1,2,3,4,5	3	D	Y**	+	N	nd
(Inoculated		C2	2,3,4,5	3	D	Y	+	N	nd
with		C3	2,3	2	A	Y	nd	N	nd
PNG40)		C4	0	2	A	Y	nd	Y	nd
		C5	3,4	3	A	Y	+	N	nd
		C6	3,4	3	A	Y	+	N	nd
		C9	3,4,5,	3	D	Y	nd	N	nd
T2	0	2a	0	1	A	Y	nd	Y	nd
		2b	0	2	A	Y	nd	Y	nd
		2c	0	2	A	Y	nd	N	nd
		2d	0	2	A	Y	nd	Y	nd
T3	0	3c	0	1	A	Y	nd	Y	nd
		3g	0	2	A	Y	nd	Y	nd
		3j	2,3	2	A	Y	nd	Y	nd
T4	0	4a	0	1	A	Y	+	N	nd
		4h	0	1	A	Y	nd	Y	+
		4l	0	2	A	Y	nd	N	nd

Table 11 continued …

Treatment	Mortality (%)	Plant	External symptoms***	Severity of lesion****	Dead (D) or alive (A)	Re-isolation on NS agar from lesion	ISSR comparison of isolate to PNG40	Re-isolation on NS agar from undamaged area above lesion	ISSR comparison of isolate to PNG40
T5	0	5a	0	2	A	Y	+	N	nd
		5b	0	2	A	Y	nd	N	nd
		5c	0	3	A	Y	nd	N	nd
		5e	0	2	A	Y	+	N	nd
		5f	0	1	A	Y	+	N	nd
T6	0	6b	0	2	A	Y	nd	Y	nd
		6d	0	1	A	Y	nd	N	nd
T7	0	7a	0	1	A	Y	nd	Y	nd
		7b	0	1	A	Y	nd	N	nd
T8	40	8a	0	2	A	Y	+	N	nd
		8f	4,5	3	D	Y	+	N	nd
		8g	0	1	A	Y	nd	Y	nd
		8i	4,5	3	D	Y	nd	N	nd
		8j	0	2	A	Y	nd	N	nd
		8k	0	2	A	Y	+	N	nd
		8n	0	1	A	Y	nd	N	nd
		8r	4,5	3	D	Y	+	N	nd
		8s	0	1	A	Y	+	N	nd
		8t	4,5	3	D	Y	nd	N	nd

*****N=No,**Y= Yes; nd= not determined

*******external symptoms: 0 = no symptoms, 1 = leaves crinkling, 2 = leaves chlorotic, 3 = leaves necrotic, 4 = dead growing point, 5 = wilting

****lesion severity rating: 0 = no lesions, 1= mild, 2= moderate, 3 = severe

Table 12: Effectiveness of primers ISSR 1, ISSR 2, ISSR 4 and ISSR 8 to distinguish between 6 different *Fusarium* isolates. *Fusarium* species and isolates; *F. andiyazi* 98F4, *F. proliferatum* 98F6, *F. proliferatum* 98F7, *F. andiyazi* 98F8, *F. andiyazi* N419 and *F. sacchari* PNG40 from culture stocks were cultured on PDA and genomic DNA was extracted from the fungi. ISSR- PCR was performed and the unique banding patterns were noted.

Primer	Number of bands	Number of monomorphic bands	Number of polymorphic bands	Unique banding patterns
ISSR 1	12	0	12	4
ISSR 2	24	0	24	3
ISSR 4	19	0	19	5
ISSR 8	18	3	15	4
Total no. of bands	73	3	70	-

5. Discussion

The objective of this study was to produce somaclonal variant sugarcane plants *in vitro* through induced mutagenesis and select plants tolerant to *F. sacchari* PNG40. The approach was to generate variation in embryogenic cells via somatic embryogenesis and mutagenesis using ethyl methanesulphonate (EMS), as per the protocol of Koch *et al*. (2012). The investigation established 100 ppm *F. sacchari* PNG40 culture filtrate (CF) as a suitable treatment for screening sugarcane somatic embryos at maturation and germination stages; 1500 ppm CF was the appropriate treatment for *in vitro* selection of tolerant plants. Tolerant plants were selected by applying the determined CF selection pressure to EMS-treated calli cultured at either embryo maturation or germination and to the subsequently regenerated plants. A further selection by inoculation with *F. sacchari* PNG40 was conducted to confirm tolerance of the *in vitro* regenerated and selected plants, a procedure that revealed that these plants were tolerant to the fungus.

5.1 Evaluation of *Fusarium sacchari* culture filtrate as an *in vitro* selecting agent for tolerance to *Fusarium*

According to Daub (1986), selecting agents used in screening for disease tolerance should play a role in pathogenesis and demonstrate a negative effect on plant cells, tissues, organs or whole plants. The selecting agent used in the present study (*F. sacchari* PNG40 CF) was found to have all such effects. The incorporation of the CF into *in vitro* culture media had a negative impact on the number of regenerated plants by causing callus necrosis at embryo maturation and germination (Figs. 13, 14, 18 and 19), reducing the number of germinated embryos (Figs. 17 and 20) and on germinated plants by inhibiting root growth (Figs. 22 and 23). The greatest effects on somatic embryos were observed when the CF was included during embryo germination rather than on maturation.

Cellular necrosis (e.g. brown, black callus) *in vitro* has been found to be indicative of cell death using cell viability stains such as fluorescein diacetate (FDA) (Toyoda *et al.,* 1984). Those authors also worked on *in vitro* screening for tolerance to *Fusarium,* but in tomato, and they observed that cell viability was reduced as the CF concentration in the medium increased. Although FDA was not used in the present investigation, visual assessment of callus necrosis revealed a similar effect of CF on cell viability between that study and the

current one (Figs 13, 14, 18 and 19). That observation was also made in studies using CF to obtain carnation (Thakur *et al*., 2002), pineapple (Hidalgo *et al.,* 1998) and *Amaranthus* (Chen and Swart, 2002) plants tolerant to *Fusarium* spp*.* In the present study, due to the reduced cell viability, fewer embryos were formed and as a result higher callus necrosis caused fewer embryos to germinate (Figs. 17 and 18). This is consistent with results of a study by Sengar *et al.* (2009), which showed that fewer plants germinated from sugarcane embryos exposed to *Colletotrichum falcatum* CF compared with the control. It may be possible that the non-significance in plantlet yields amongst the CF treatments was due to the small sample number used (n=12-21), and this will be addressed in future studies. It was also observed in the current study that some calli did not produce shoots, but produced roots only. Ho and Vasil (1983) reported that sugarcane calli derived from older leaf sheaths failed to form embryoids only and produced roots. Further, the exposure of calli to *in vitro* stress, e.g. pesticides (Sammaiah *et al.,* 2011), mutagen (Koch *et al.,* in press) and toxins (Remotti *et al.,* 1997) have been reported to result in the production of only roots by the calli. In the current study, it is possible that the uneven exposure of embryoids to CF resulted in the damage of the shoot meristem but not of the root meristem.

The regeneration of plants from some necrotic calli indicated that a piece of callus may consist of tolerant cells, as well as some that may not have come into adequate contact with the CF. This could be due to the 3 dimensional nature of the calli and consequent death of the cells on the surface of the callus pieces and not those in deeper layers, which were less exposed to the CF. This would allow inner cells to divide, thereby resulting in the non-significant differences in fresh and dry mass amongst calli from the all treatments. This shows that at the time of recording the data callus necrosis was a better indicator of the toxic effect of the CF on calli than fresh and dry mass. Unfortunately, survival of embryogenic cells due to insuffcient exposure to CF pressure may result in the regeneration and selection of plants germinated from susceptible embryos (escapes).

In a study to select tomato plants tolerant to *Fusarium* CF, Toyoda *et al.* (1984) noted that it is critical to ensure infiltration of the CF to deeper callus cell layers in order to avoid escapes. In that study, adequate exposure to *F. oxysporum* f. sp. lycopersici CF was achieved by submerging tomato calli placed on semi-solid medium incorporated with CF, in liquid medium also containing the CF (liquid-on-agar method). In another approach, Jin

et al. (1996) incorporated *F. solani* CF in soya bean embryogenic suspension cultures to select CF-tolerant embryos. Incorporation of CF in liquid media during culture using methods such as suspension or temporary immersion cultures, together with use of smaller callus pieces can, therefore, ensure adequate exposure of callus cells to the CF. However, work in our laboratory (unpublished) has shown that sugarcane calli do not survive such immersion treatments.

Studies in carnation (Buiatti *et al.,* 1985), pineapple (Hidalgo *et al.,* 1998), wheat (Ahmed *et al.,* 1996) and tomato (Matsumoto *et al.,* 2010) used changes in callus fresh mass to indicate stress induced by the CF. Fresh mass is not a reliable parameter to determine cell division as it is due to the accumulation of dry matter and water. Dry mass is a result of active metabolism leading to cell elongation, division and cellular dry matter deposition. In the present study, the inclusion of CF during embryo maturation did not result in significant differences in callus fresh and dry mass amongst the CF treatments (Fig. 15). In contrast, necrosis was found to be a relatively good indicator of the effect of the CF on the calli.

Fungal culture filtrates contain phytotoxic metabolites (Daub, 1986). Jin *et al.* (1996) isolated a protein from *F. solani* CF which, when incorporated in soyabean callus media, induced necrosis in the calli. In another study, maize calli displayed necrosis when cultured on media containing *F. moniliforme* toxins fumonisin B_1 and moniliformin (Van Asch *et al.,* 1992). Moniliformin inhibits the mitochondrial oxidative enzyme, pyruvate dehyrogenase (Gathercole *et al*., 1986), which affects the entry of carbon into the Krebs cycle during plant respiration (Schuller *et al.,* 1993). Fumonisins are reported to have caused necrosis in maize and tomato seedlings (Abbas *et al*., 1994). Furthermore, Bailey (1995) found that *F. oxysporum* produced a necrosis-inducing peptide Nep1 and Bae *et al*. (2006) also reported that Nep1 extracted and purified from *F. oxysporum* f. sp. *erythroxyli* CF caused cell membrane damage, production of reactive oxygen species, ethylene synthesis and cell death in *Arabidopsis* seedlings. It is possible, therefore, that these compounds, or others with similar effect, were present in *F. sacchari* PNG40 CF.

In a number of studies, e.g. in alfalfa (Arcioni *et al.,* 1987; Binarova *et al.,* 1990), gladiolus (Remotti *et al.,* 1997), carnation (Thakur *et al.,* 2002), onion (Tripathi *et al.,* 2008) and rose geranium (Saxena *et al.,* 2008), the CF or toxin was added during embryogenic callus culture and only the surviving calli were then transferred to the regeneration medium

without CF. However, although no literature could be found on studies where CF was included in the embryo germination medium, in the present study, all calli (necrotic and non-necrotic) were transferred to the regeneration medium. It is significant, therefore, that results from the current study indicate that the CF was more toxic when incorporated during embryo germination than in maturation as indicated by resulting levels of necrosis (Figs. 14 and 19) and lower plantlet yields (Figs. 14 and 18). A further indication of the differences between the effect of the CF on embryo maturation and germination was the fact that 20 and 100 ppm CF in the germination medium resulted in reduced abnormal yield. Prolonged exposure of rice somatic embryos to light has been reported to lead to necrosis (Doung, 2003). (Kevers *et al.,* 1995) reported necrosis due to low levels of photoprotector carotenoids in sugarbeet calli cultured in the light. In the current study, such necrosis in sugarcane embryos may have occurred during the photoperiod, thereby facilitating more damage by toxins in the CF during embryo germination than in maturation. Further, Asai *et al.* (2000) reported that fumonisin B_1-induced cell death in *Arabidopsis* protoplasts was light dependent. Hence, it is also possible that in the current study fumonisin B_1, known to be produced by *Fusarium* spp. (Munkvold and Desjardins, 1997), might have also contributed to the high necrosis observed in the germination stage. In addition, as different morphogenic and physiological processes occur in embryogenic calli and germinating embryos, it is not unexpected that CF affects them differently. For example, Guiderdoni *et al.* (1990) reported a protein specific to sugarcane embryogenic calli and Blanco *et al.* (1997) detected a soluble protein associated with the regenerative ability of sugarcane embryogenic calli. The inhibition of such metabolites specific to embryo germination more than those at embryo maturation by toxins in the CF could, therefore, result in more cell damage during germination.

The non-significance in abnormal plant yield obtained from applying CF pressure during maturation may be the result of the small sample size used (n=13 - 21) (Fig. 21). Although *Fusarium* CFs contain mutagenic compounds (Matsumoto *et al*. (2010), the lethal effect of the 100 ppm treatment as evidenced by the low total plantlet yield, may have killed any abnormal cell that may have developed. The 50 ppm treatment might have been able to induce mutations, but might not have been sufficient to kill abnormal cells produced whilst the 4 and 20 ppm treatments may have been too low to either induce mutations or kill abnormal cells.

It is important for tolerance expressed at the cellular level to also be exhibited at the whole plant level (Daub, 1986). *Fusarium* CFs have been reported in other studies to inhibit root growth. For example, Khan *et al.* (2004) reported inhibition of roots of chickpea seedlings by *F. oxysporum* f. sp. *ciceris* CF*.* The root growth of *s*usceptible *Amaranthus* seedlings was inhibited by *F. oxysporum* CF and the resistant ones exhibited improved root growth in the presence of the CF (Chen and Swart, 2002). In addition, Baker *et al.* (1981) extracted metabolites from *F. solani* CF that inhibited lemon seedling root growth and identified these compounds as fusarubin, javanicin and anhydrofusarubin. Bae *et al.* (2006) also demonstrated that exposure of *Arabidopsis* seedlings to the peptide Nep1 purified from *F. oxysporum* CF drastically reduced root growth. In the present study, 1500 ppm CF was found to result in the greatest inhibitory effect on root re-growth of sugarcane plantlets with trimmed roots (Figs. 22 and 23).

Other studies in alfalfa (Arcioni, 1987), carnation (Buiatti *et al.,* 1985; Arai and Takeuchi, 1993), wheat (Ahmed *et al.,* 1996) and pineapple (Hidalgo *et al.,* 1998) showed that responses to CFs, with regards to parameters such as callus mass, necrosis and root length, vary from variety to variety and the effect of the CF on different species varieties correlates with their known levels of tolerance to *Fusarium*. The present results, therefore, apply only to the tested sugarcane variety and similar work needs to be done to expand the application of this approach.

In conclusion, the investigations discussed above demonstrated that, as *F. sacchari* PNG40 CF reduces the regenerative potential of sugarcane somatic embryos and inhibits root growth, it can be used to select plants tolerant to *Fusarium*. Although in most studies that focused on selection for tolerance to fungal diseases (Arcioni *et al.,* 1987, Binarova *et al,* 1990; Koike *et al.,* 1991; Ahmed *et al.,* 1996; Sengar*et al.,* 2009) CFs were incorporated during the first stage of embryogenic callus culture, in sugarcane *Fusarium* CF was found to be more lethal during the germination stage. However, a higher CF concentration than that used in the germination stage might be used during maturation to achieve a more stringent selection pressure than that tested.

5.2 Application of the *in vitro* screening protocol in the selection of EMS-induced variants tolerant to *F. sacchari*

As the frequency of mutagen-induced mutations is higher than spontaneous mutations *in vitro* (Novak and Brunner, 1992), embryogenic calli were treated with EMS in order to increase the mutation frequency and enhance somaclonal variation. Subsequent selection of *F. sacchari*-tolerant plants was conducted *in vitro* using the established CF selection treatments.

Studies have shown that EMS induces mutations (Jabeen and Mirza, 2002; Lee *et al.,* 2003; Hoffmann *et al.,* 2004), thus generating variation amongst plants produced from the treated calli. Further, several studies have reported on EMS treatments of calli and subsequent selection of plants with desired traits using appropriate selection protocols. These traits include herbicide resistance (Jander *et al.,* 2003; Koch *et al.,* in press), salt tolerance (Luan *et al.,* 2007; Kenganal *et al.,* 2008) and disease resistance (Imelda *et al.,* 2000; Purwati and Sudarsorno, 2007; Matsumoto *et al.,* 2010). In the present study, more variable root growth was obtained in treatments which included EMS-treated calli than those with non-treated controls (Figs. 32 and 33). In addition, it appeared that embryos from EMS treatments increased the number of abnormal plants, but this was not statistically significant (Figs. 30 and 31).

From the variants generated by the mutagenic treatment, CF-tolerant plants were obtained by selection of the EMS-treated embryogenic calli and selection of the subsequent regenerated plants using CF at the established CF concentrations. Although not statistically significant, the EMS treatments 4 and 8 resulted in higher plantlet yield compared to their respective non-EMS control treatments 3 and 7 (Fig. 29). As induced mutagenesis is random, it is possible that the treatment of embryogenic calli with EMS resulted in only a few more variant plants with the desired mutation than the non-treated ones. The use of a larger sample size in future may result in significantly more number of plants from the EMS treatments than non-EMS treatments. In addition, the display of improved root re-growth by more plants from EMS treatments 2, 4 and 8 than their respective non-EMS controls treatments [1(-CF), 3 and 7] indicates that the EMS treatment enhanced the plants' ability to overcome root re-growth inhibition, thus

increasing the production of tolerant plants. Further, the former showed no disease symptoms and had low lesion severity ratings when inoculated with PNG40, except for some plants from treatment 8 (Table 11). Purwati and Sudarsorno (2007) reported that the treatment of embryogenic calli with EMS followed by selection with CF and fusaric acid increased the chances of obtaining Abaca variant plants tolerant to *F. oxysporum* f. sp. *cubense* compared to culture without *in vitro* screening and selection.

The results also indicated that treatment of calli with 32 mM EMS for 4 hrs was able to induce variation whilst allowing normal embryo germination. This is evidenced by similar plantlet yield between treatment 1 and 2 despite calli from treatment 2 being exposed to EMS (Fig. 29). Waugh *et al.* (2006) reported that EMS is a popular mutagen due to its ability to induce point mutations without casing lethal abnormalities to the chromosomes. Koch *et al.* (in press) recorded differences in total plantlet yield from calli treated with 0 and 32 mM EMS for 4 h, but obtained similar number of normal plants. This difference between that study and the present one may be due to the differences in number of replicates and the sugarcane varieties used, as Van *et al.* (2008) demonstrated that response to treatment with EMS is genotype-dependant. In another study, Kenganal *et al*. (2008) reported that treatment of sugarcane calli with 40 mM EMS for 2.5 h permitted maximum plantlet regeneration. Koch *et al.* (in press) also observed similar fresh mass between calli treated with 0 and 32 mM EMS after 3 weeks, but differences were observed only after 8 weeks. However, the similarities in fresh mass may have been due to accumulation of water, as previously discussed. In the current study, no significant differences in both fresh and dry mass were obtained between calli from the EMS and non-EMS treatments recorded after 3 weeks (Fig. 26). This suggests that the EMS treatment either did not affect cell elongation and division or the effects were simply not evident after 3 weeks.

The incorporation of CF into the media during embryo maturation, germination and both stages negatively affected the EMS-treated embryogenic calli by causing necrosis (Figs. 27 and 28) and reducing embryo regenerative potential (Fig. 29). It also affected the germinated plants by inhibiting root growth (Figs. 32 and 33). As expected from previous investigations (section 4.1 a and b), callus necrosis was more severe in treatments in which the CF was included only during germination (treatments 5 and 6) than if included in maturation medium (treatments 3 and 4) (Fig. 27). Consequently, the former produced

fewer plants than the latter (Fig. 29). Susceptible calli and plants were screened out and the tolerant ones were selected for further testing by the action of the CF on the three developmental stages (maturation, germination and plantlets) (treatments 2-8). This was evidenced by the absence of symptoms in plants selected using CF after inoculation with PNG40 whilst those unexposed to CF (treatment 1) displayed symptoms and high mortality (Table 11).

The inclusion of CF at both embryo maturation and germination stages (treatments 7 and 8) resulted in lower necrosis of embryogenic calli than predicted as it was significantly lower than in treatments when plants were only exposed during germination (treatments 5 and 6) (Fig. 27). As a consequence, treatment 8 produced significantly more plants than treatment 6 (Fig. 29). There are no reports in the literature of incorporation of CF in both embryo maturation and germination stages. However, it is possible that the effects of the CF in treatments 7 and 8 may be due to the prolonged exposure to the mutagenic toxins in the CF. *Fusarium* liquid cultures have been reported in a number of studies to contain mutagenic compounds. Bjeldanes and Thomson (1979) and Lu and Jeffery (1993) using the Ames test for mutagenicity demonstrated that culture extracts of *F. moniliforme* were mutagenic and the latter identified fusarin C as one of the mutagenic compounds in the extracts. Further, Gelderblom *et al*. (1984) and Bacon *et al*. (1989) reported that different *Fusarium* species and isolates produced fusarin C. It is possible, therefore, that in the current study fusarin C and other unknown mutagenic compounds were present in the *F. sacchari* CF and induced mutations leading to survival and germination of more embryos observed in treatment 8.

Hence, the present study revealed that EMS can be used to induce variation and increase somaclonal variants tolerant to *F. sacchari* PNG40, which can then be selected using the established CF screening protocol. Exposure of the EMS-treated calli to CF selection pressure is effective in selecting tolerant embryos and plants and the CF during embryo germination is more effective than during the maturation stage. Further, assessment of root growth in the presence of the CF is effective in selecting tolerant plants, as demonstrated by the subsequent inoculation of putative-tolerant plants to PNG40 (Fig. 33 and Table 11)

5.3 Confirmation of tolerance by inoculation with *F. sacchari*

In several studies in maize (Gengenbach *et al.,* 1977), alfalfa (Arcioni *et al.,* 1987), pineapple (Borras *et al.,* 2001), *Amaranthus* (Chen and Swart, 2002), carnation (Thakur *et al*., 2002) and sugarcane (Sengar *et al.,* 2009) disease tolerant plants obtained by *in vitro* selection strategies (without mutagenic agents) were challenged with the pathogen in order to confirm tolerance. Further, according to Afolabi (2008), who assessed tolerance to *Fusarium* in maize lines, artificial inoculation provides disease pressure that allows discrimination of genotypes with different levels of tolerance. In the current study, putative-tolerant plants (albeit of one genotype only, before mutagenesis) were inoculated with *F. sacchari PNG40.* Microscopic observation of the fungus in the plant tissue (Fig. 36), re-isolation of the fungus from symptomatic and asymptomatic plants (Fig. 37 and table 11) and confirmation of the identity of the retrieved fungus as PNG40 using molecular analysis (Fig. 39 and Table 11), attested to the tolerance of the symptomless plants to *F. sacchari* PNG40. Although some plants from treatment 8 exhibited symptoms and died and one of the three plants from treatment 3 was also symptomatic, the rest of the obtained plants did not display symptoms. Further, dead plants displayed a lesion severity rating of 3 and ratings ranged from 1 - 2 in the asymptomatic plants (Table 11). This tolerance exhibited by the asymptomatic plants indicates a correlation between tolerance exhibited to the CF *in vitro* and that displayed to the whole organism (*F. sacchari)*. This concurs with studies in which tolerance to CFs of *H. maydis* (Gengenbach *et al.,* 1977), *F. oxysporum* f. sp. *medicaginis* (Arcioni *et al.,* 1987), *F. eumartii* (Carp.) Snyder and Hansen (Botta *et al.,* 1994), *F. subglutinans* (Borras *et al.,* 2001), *F. oxysporum* (Chen and Swart, 2002) and *C. falcatum* (Sengar *et al.,* 2009) correlated with tolerance to the respective fungal pathogen. The high mortality observed in plants from the control (treatment 1) (Table 11) is probably due to those plants not being subjected to CF pressure during *in vitro* culture (sections 4.2.1 and 4.2.2). In this case, the susceptible plants were not screened out leading to symptoms and high mortality observed in these plants when they were exposed to PNG40. This is in contrast to plants regenerated from the other treatments (2 – 8) which were exposed to CF pressure at either embryo maturation, germination, both stages (Fig. 29) and/or at plantlet level (Fig. 33). This approach screened out the susceptible plants and selected the potentially tolerant ones for further testing by inoculation with PNG40.

Selection for disease resistance requires a reliable inoculation method in order to assess resistance to the pathogen (Miedaner, 1997). In the current study, inoculation by stabbing with the *F. sacchari*-colonised toothpicks proved to be most effective compared with dipping in the conidial suspension and inoculating the leaf whorls with the suspension. Although immersion in the conidial suspension resulted in some plants dying, this manipulation was limited by the size of the plants that could be tested in this way, without causing damage. The whorl inoculation procedure did not result in any visible disease symptoms. In contrast, 'stab inoculation' not only resulted in symptoms but it also allowed for disease assessment by rating the lesion size in the stem tissue resulting from PNG40 infection. Afolabi (2008) used the stab inoculation method to assess tolerance to *F. verticillioides* in various maize lines and they reported that the level of tolerance of each line was consistent when the plants were inoculated in different seasons. Other studies also indicated that an inoculation method that introduces stalk rot-causing fungi (e.g. *Fusarium* spp*.* and *Colletotrichum* spp*.*) directly into the stalk tissue of sugarcane, maize and sorghum is effective in infecting the plants and is convenient as it allows disease severity rating on which level to tolerance can be based. The reports in the literature give evidence of two methods used to inoculate plants. The first involves injecting stalks with spore suspensions which has been carried out in maize (Ledencan *et al.,* 2003) and sorghum (Tesso *et al.,* 2004, Tesso*et al.,* 2005; Tesso *et al*., 2009) using *Fusarium* spp*.* and maize (Carson and Hooker, 1981) and sugarcane (Sengar *et al.,* 2009) using *Colletotrichum* spp*.* The other method entails stabbing the stalks with toothpicks colonised with *Fusarium* which has been reported for maize (Gilbertson, 1985; Dickman and Partridge, 1989; Clements *et al.* 2003) and *Amaranthus* (Chen and Swart, 2002). In all these studies, disease severity ratings were based on the lesion size induced by infection of the stalk.

Fusarium infection of sugarcane leading to Fusarium stalk rot is facilitated by *E. saccharina* stem borings that allow the fungus access to the stem tissue (McFarlane *et al*., 2009). In the current study, this was mimicked by stabbing the stem with toothpicks thereby carrying the fungus into the stalk tissue. In contrast, the other two tested methods (immersion and whorl inoculation) may not have facilitated access of the fungus to the stalk tissue. Further, the negative results from the whorl inoculation method were also

probably due to the leaf whorl being tight and not allowing infiltration of the conidial suspension into the stalk.

Re-isolation of the inoculated pathogen from symptomatic plants is necessary as it confirms the introduced pathogen as the causal agent for the observed symptoms (Abdel-Monaim *et al.,* 2011). For example, Chen and Swart (2002) stabbed *Amaranthus* stalks with *F. oxysporum* colonised toothpicks and retrieved the fungus from susceptible and resistant plants by placing tissue from the stem lesions onto PDA. In this study, the re-isolation of PNG40 from symptomatic plants indicated that the symptoms were due to infection by this fungus. Further, retrieval of the pathogen from symptomless plants suggested that the plants were able to prevent the pathogen from inducing symptoms, i.e. they were tolerant. PNG40 was re-isolated from the undamaged tissue above the lesion in some plants, but was absent in others. This presence of PNG40 in the undamaged tissue was indicative of endophytic colonisation of the stalk tissue by the fungus. As McFarlane *et al*. (2009) reported the toxic effect of PNG40 on *E. saccharina*, plants that allow endophytic colonisation by *F. sacchari* may be useful in developing biological control strategies for *E. saccharina.* The absence of the fungus in undamaged tissue as observed in some plants may have been due to complete tolerance to the fungus such that the plants did not permit endophytic colonisation.

In most reported studies (Swart *et al.,* 1999; Chen and Swart, 2002; Tahmatsidou *et al.,* 2006), the identity of isolates retrieved after re-infection was confirmed based on the morphology of the fungal colony. However, since different *Fusarium* spp. are difficult to distinguish using colony morphology, it is beneficial and more accurate to use molecular methods to determine their identity (Vitale *et al.,* 2011). In current study, ISSR analysis was conducted on some of the retrieved isolates and the banding patterns generated using 3 primers ISSR1, ISSR4 and ISSR8 matched those from PNG40 (Fig 40). ISSRs have been used widely to detect variation amongst *Fusarium* isolates (Mishra *et al.,* 2006; Gurjar *et al.,* 2009; McFarlane, *et al.,* 2009; Baysal *et al.,* 2010; Dinolfo *et al.,* 2010; Vitale *et al.,* 2011) and are effective in distinguishing different isolates and species, as was also observed in the present study (Fig. 38 and Table 11). In another approach, Jecz and Korbin (2010) carried nested PCR on DNA extracted from strawberry plants inoculated with *Verticillium dahlia* using *V. dahlia*-specific primers in order to detect the presence of the fungus in the plant tissue.

In conclusion, tolerance to *F. sacchari* PNG40 of somaclonal variant plants selected using the CF *in vitro* selection protocol was confirmed by inoculation with the fungus. This established a correlation between selection using CF and using PNG40. The study also demonstrated that inoculation by stabbing stalks with PNG40-colonised toothpicks is effective as it allows the fungus entry to the stalk tissue and permits disease assessment. Further, molecular analysis of fungal isolates retrieved from inoculated plant tissue can accurately confirm the identity of the isolates.

5.4 Concluding remarks and future work

In vitro culture and induced mutagenesis are potentially-useful complementary approaches to conventional breeding for developing somaclonal variant plants exhibiting disease tolerance. The findings from the present study indicate that the treatment of calli (variety NCo376) with EMS in combination with selection of tolerant cells and plants using PNG40 CF incorporated in the culture media, facilitated the production of plants tolerant to *F. sacchari*. In this regard, putative tolerant plants were produced by exposure of embryogenic calli to 32 mM EMS for 4 h and selection by inclusion of 100 ppm CF in the culture media during embryo germination. As in other studies using *Fusarium* CF (Chen and Swart, 2002; Khan *et al.,* 2004), testing of whole plants by assessing root re-growth *in vitro* in the presence of 1500 ppm CF for 3 weeks was successful as a subsequent step in selecting *F. sacchari* PNG40-tolerant plants. A final selection was undertaken by inoculating the potentially-tolerant plants with *F. sacchari* and assessing disease development, and asymptomatic plants exhibiting mild to moderate lesion severity (2 months after toothpick stab inoculation) were considered tolerant to *F. sacchari.*

However, further testing is necessary to establish the response of these NCo376 variants to PNG40 under field conditions. Future work will be carried out by transferring plants to the field and inoculating 7-8 month old plants with *F. sacchari* PNG40. In addition, as the origin of the observed tolerance could be epigenetic (Joyce *et al.,* 2003; Patade *et al.,* 2005), the stability of tolerance over subsequent generations will, therefore, be tested by sexual crosses. Once stability of tolerance is established, these plants may be used to introgress tolerance to *Fusarium* into commercial sugarcane varieties. In addition, and as discussed previously, the response of calli and plants to EMS and CF may be genotype-

dependant, as found by Ahmed *et al*. (1996) working with CF to obtain *Fusarium*-tolerant plants from different varieties of wheat and Van *et al.* (2008) who regenerated plants from EMS-treated soybean calli. To obtain desired traits in more commercially important varieties than the one used in the present study, it is, therefore, necessary to test the developed protocol in those varieties. This will include variety N41, for the reasons discussed below. This attainment of tolerance to *F. sacchari* in sugarcane varieties will, therefore, enable control of Fusarium stalk rot and increase yield.

A particularly interesting application of the developed protocol is the potential use of those plants permitting endophytic colonisation by *F. sacchari* PNG40 in developing biological control strategies for *E. saccharina. Eldana saccharina* is a lepidopteran pest that has caused damage and economic losses in the South African sugarcane crop since the 1970's (Mokhele *et al.,* 2009). Infestation by the stem borer results in lower sucrose levels, higher fibre and lower stalk length and mass (Goebel and Way, 2003). Such damage by the borer has been reported to lead to yield losses worth US$10 million per annum (Black *et al.,* 1995). Control strategies involve an integrated use of chemicals (Leslie, 2003), resistant varieties (Rutherford *et al,* 1993; Keeping and Rutherford, 2004; Keeping, 2006), crop management (Webster *et al.,* 2005) and biological control (Conlong, 2001).

The variety NCo376 was used in this study as it is susceptible to *Fusarium* and *E. saccharina* and is amenable to tissue culture. *Fusarium sacchari* causes Fusarium stem rot in sugarcane, a disease that causes by reddish-brown discolouration of the stalk tissue leading to reduced cane quality and failure of setts to germinate. Its impact is largely dependent on the susceptibility of sugarcane to *E. saccharina*, as the stem borer damage enables entry of *F. sacchari* into the stalk tissue (McFarlane *et al.,* 2009; Govender *et al.,* 2010). The association between the stem borer and *F. sacchari* is characterised by reddish-brown discolouration of the stem tissue with *E. saccharina* borings (Way and Goebel, 2003; McFarlane *et al.* 2009). Further, McFarlane *et al.* (2009) reported that in an *in vitro* assay *Fusarium* species isolated from *E. saccharina* borings in sugarcane stalks were either beneficial or antagonistic to the stalk borer's survival and development. In that study, amongst the *Fusarium* species isolated from *E. saccharina* borings and undamaged tissue, *F. sacchari* was the most dominant. Further, isolates from this species were the most toxic to *E. saccharina* with up to 80 % mortality of larvae with PNG40 being the most

antagonistic isolate to the stem borer in an *in vitro* assay (McFarlane *et al.,* 2009). It may be, therefore, possible to use PNG40 to control *E. saccharina*.

However, because *F. sacchari* PNG40 is the causal agent of stalk rot, the potential application of the fungus in the biological control of *E. saccharina* is limited. The PNG40-tolerant plants obtained in the present study may be used to overcome this problem. It is hypothesised that PNG40 endophytically colonising NCo376 variants can negatively affect *E. saccharina* survival and development, and thus be used as a biological control agent of the stem borer. To test this hypothesis, PNG40-colonised NCo376 variants obtained from the *in vitro* selection protocol will be bulked by planting single-budded stem cuttings from each plant. PNG40 will be introduced into stem tissue of the plants by stabbing with PNG40-colonised toothpicks. Plants endophytically colonised by PNG40 will be inoculated with *E. saccharina* eggs according to the protocol by Keeping (2006). This procedure involves inoculating stalks of 7-8 month-old pot trial plants with *E. saccharina* eggs. The toxicity of the endophytic PNG40 on the stem borer will be assessed by counting the number of larvae, pupae and pupal cases per stalk and recording their fresh mass and comparing with NCo376 (non-variant) controls without PNG40 colonisation. Re-isolation of PNG40 from *E. saccharina* borings will establish if the fungus was responsible for any observed inhibition of development or mortality of *E. saccharina.*

NCo376 is a variety that was widely grown in SA from 1955-2000 (Anon, 2006). However, due to its susceptibility to disease, e.g. smut and sugarcane mosaic virus, and *E. saccharina* it is being replaced by the newer 'N' varieties. N41 is one such variety and it is of more commercial importance in SA. It has been found that N41 exhibits variable resistance to *E. saccharina* in the field and is also colonised by different *Fusarium* strains (Rutherford, 2011, pers. comm.). An isolate of *F. pseudonygamai,* SC17, was isolated from N41 and the fungus was found to be beneficial to *E. saccharina* in an *in vitro* assay (McFarlane *et al.* (2009). It is hypothesised that the colonisation of N41 by different *Fusarium* spp. is responsible for the variable resistance to *E. saccharina* observed in this variety. To test this, N41 (non-variant) plants will be inoculated separately with PNG40 and SC17. The fungus-colonised plants will then be inoculated with *E. saccharina* and survival and development of the stem borer will be compared between the PNG40- and SC17-

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colonised plants. It is expected that N41 plants colonised by PNG40 will exhibit better resistance to *E. saccharina* than those with SC17, thus may be used to improve the resistance of N41 to the stem borer. However, *Fusarium* spp. frequently cause disease in N41 with plants often showing symptoms of Pokkah boeng, a disease also caused by *Fusarium* strains. This hampers the use of the *Fusarium* strains in the control of *E. saccharina* in N41. To overcome this, N41 calli will be treated with EMS and exposed to CF and resultant PNG40-tolerant plants selected as per protocol developed for NCo376 in the present study. The N41 variants endophytically colonised by PNG40 will then be inoculated with *E. saccharina* in order to assess the effectiveness of endophytic PNG40 against *E. saccharina*. An additional result of such a study is that it allows for testing the current protocol on a commercially important variety, thereby giving an indication of the usefulness of the protocol on different genotypes.

This use of endophytic PNG40 as a biocontrol agent of *E. saccharina* may also have additional benefits. Endophytic colonisation of plant tissue may alter the physiology of the plant and change levels of plant bioactive compounds, e.g. alkaloids, polyphenols, lignins and flavonoids (Zhi-lin *et al.,* 2007; Tang *et al.,* 2011). In addition to producing insecticidal compounds (Logrieco *et al.,* 1996; Logrieco *et al.,* 1998), endophytes are also known to produce plant growth regulating (Carrol, 1988; Machungo *et al.,* 2009; Yong *et al.,* 2009) and antimicrobial (Ding *et al.,* 2010) metabolites. As a result, endophytes may enhance growth and increase plant yield and also have negative effects on other plant pathogens (Danielsen and Jensen, 1999; Narisawa *et al.,* 2002; Park *et al.,* 2003). Lee *et al.* (2009) reported that endophytic *F. verticillioides* inoculated simultaneously with the smut pathogen *Ustilago maydis* reduced smut disease severity and also enhanced plant growth in maize. Further, Estrada *et al.* (2011) simultaneously inoculated *F. verticillioides* and *U. maydis* onto media and using liquid chromatography–mass spectrometry found that *F. verticillioides* produced antimicrobial metabolites that reduced *U. maydis* biomass. It may be possible that a similar relationship occurs between *Fusarium* and *U. scitamenea*, the smut pathogen in sugarcane. It is, therefore, hypothesised that endophytic *F. sacchari* produces metabolites that enhance plant growth and also compounds that are toxic to both *E. saccharina* and fungal pathogens (e.g. smut), which may result in resistance to both *E. saccharina* and fungal diseases. To test this hypothesis, changes in smut resistance will be assessed in *F. sacchari* PNG40-colonised plants (NCo376 and N41 variants) exhibiting

tolerance to *E. saccharina* compared with uncolonised variants. Conventionally, resistance to *E. saccharina* in sugarcane is inversely related to smut resistance and it is difficult to obtain both in the same plant. This work is a novel approach to overcome this barrier and obtain both *E. saccharina* and smut resistance. Further, metabolic profiling using High Performance Liquid Chromatography and Mass Spectrophotometry (HPLC-MS) will be conducted to determine nature and levels of plant and fungal secondary metabolites in endophytically colonised plants.

In summary, future work will, therefore, be aimed at 1) testing the stability of tolerance to PNG40 obtained in the current study in the field and after sexual reproduction; 2) carrying out a pot trial to determine the effectiveness of endophytic PNG40 in NCo376 variants in biological control of *E. saccharina;* 3) comparing the survival and growth of *E. saccharina* in N41 (non-variant) plants colonised by PNG40 and SC17 and using the current protocol (or a modified one, if required) to obtain PNG40-tolerant N41 variant plants and test their response to *E. saccharina*; and 4) assessing changes in smut resistance and levels of plant and fungal secondary metabolites in stalk tissue of NCo376 and N41 variants colonised by endophytic PNG40.

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Appendices

Statistical analysis of data

Appendix 1

F pr. value used to determine percentage callus necrosis as a result of culture of callus on EMM incorporated with CF for 3 weeks using REML variance component analysis.

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
CF concentration	533.15	3	177.72	47	<0.001

Appendix 2

F pr. value used to determine effect of CF on callus fresh mass after culture on EMM incorporated with CF for 3 weeks with using One-way ANOVA.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
CF concentration	3	0.15054	0.05018	1.33	0.312
Residual	12	0.45433	0.03786		
Total	15	0.60487			

Appendix 3

F pr. value used determine effect of CF on callus dry mass after culture on EMM incorporated with CF for 3 weeks with using One-way ANOVA.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
CF concentration	3	0.001776	0.000592	1.7	0.221
Residual	12	0.00419	0.000349		
Total	15	0.005966			

Appendix 4

F pr. value used to determine the effect of incorporation of CF at the embryo maturation stage on subsequent embryo germination using REML variance component analysis.

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
CF concentration	380.71	4	95.18	78	<0.001

Appendix 5

F pr. value used to determine percentage callus necrosis as a result of culturing calli on EGM1 with CF using REML variance component analysis.

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
CF concentration	21.96	3	7.32	32	<0.001

Appendix 6

F pr. value used to determine the effect of CF in EGM1 on embryo germination using REML variance component analysis.

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
CF concentration	270.99	4	67.75	67	<0.001

Appendix 7

F pr. value used to determine the effect of CF on root re-growth using One-way ANOVA.

Source of variation	d.f	s.s.	m.s.	v.r.	F pr.
CF concentration	2	2690.23	1345.12	64.15	<.001
Residual	14	293.53	20.97		
Total	16	2983.76			

Appendix 8

F pr. values used to determine the effect of CF on fresh mass of EMS-treated calli using REML variance component analysis.

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
CF concentration	33.7	3	11.23	5	<0.001

Appendix 9

F pr. values used to determine the effect of CF on dry mass of EMS-treated calli using REML variance component analysis.

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
CF concentration	7.03	3	2.34	15	0.114

Appendix 10

F pr. values used to determine effect of CF on percentage callus necrosis of EMS-treated calli using REML variance component analysis.

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Stage	174.95	1	174.95	157	<0.001
Treatment	454.69	7	64.96	157	<0.001
Stage.Treament	366.29	7	52.33	157	<0.001

Appendix 11

F pr. values used to determine effect of CF on number of plants produced from EMS-treated calli using REML variance component analysis.

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
CF concentration	380.37	7	54.34	74	<0.001