EPIGENETIC PRIMING AND *in vitro* MUTAGENESIS IN SUGARCANE (SACCHARUM SPP. HYBRIDS) FOR RESISTANCE TO *Fusarium* SPECIES AND *Eldana saccharina* (LEPIDOPTERA: PYRALIDAE)

Eshani Govender

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By

ESHANI GOVENDER

Submitted in fulfilment of the academic requirements for the degree of Master of Science (MSc) in the Discipline of Biological Sciences, School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu Natal, Durban, South Africa.

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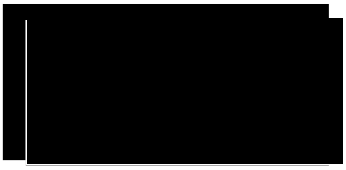
Preface

The experimental work described in this dissertation was carried out (part-time) in the Biotechnology Department of the South African Sugarcane Research Institute (SASRI), Mount Edgecombe, Durban, SA from January 2015 to February 2022, under the supervision of Prof. Paula Watt (UKZN), Dr Sandra Jane Snyman (SASRI) and Dr R 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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FACULTY OF SCIENCE AND AGRICULTURE

DECLARATION 1 - PLAGIARISM

I, Eshani Govender, declare that

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

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

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

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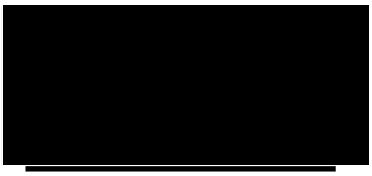

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Abstract

In the South African sugar industry, there have been substantial economic losses of R1 billion/annum caused by the indigenous pyralid borer, *Eldana saccharina* (Lepidoptera: Pyralidae). To develop control measures for *E. saccharina* in sugarcane, it is important to understand the interactions between the stalk borer and *Fusarium* spp. In previous studies, *in vitro* assays have shown that *Fusarium* strains may be antagonistic (e.g., *F. sacchari* PNG40) or beneficial (e.g., *F. pseudonyamai* SC17). *F. pseudonyamai* SC17 is a potential endophytic indicator of *E. saccharina* infestation, as the association between borer infestation and infection by the fungus causes *Fusarium* stalk rot in sugarcane. Studies have reported that the presence of endophytic fungi may have several benefits to the host plant, e.g., the production of phytohormones such as indole-3-acetic acid (IAA), which promotes plant development.

The study aimed to: 1) choose a suitable resistance priming agent between hexanoic acid (Hx) and cis-jasmone (CJ); 2) determine an appropriate culture filtrate (CF) concentration for *in vitro* screening of calli and plantlets for tolerance to *F. pseudonygamai*; 3) develop a protocol (epigenetic priming and mutagenesis) to generate mutants: primed only (0.6 mM Hx), a combination of priming and mutagenic agents (100 µM 5-AzaC + 16 mM EMS-induced); 4) screen for indole-3-acetic acid production by *F. pseudonygamai*; and 5) characterise *in vitro* selected mutants for *E. saccharina* and *F. pseudonygamai* resistance by comparing the levels of resistance between unprimed, primed, and primed + mutagenic plantlets through *ex vitro* screening. When *cis*-jasmone (CJ) and hexanoic acid (Hx) were investigated for their effect on priming for pathogen resistance, 0.6 mM Hx was selected as the optimum priming agent concentration for both the callus and plantlet regeneration stages.

At the highest CF concentration (100 ppm) at the embryo germination stage, the number of plantlets was greatly reduced to 58 and 98 plantlets/0.1 g of callus, for cultivars 88H0019 and N41 respectively, compared to more than 600 plantlets/0.1 g of callus in the no CF control. Unexpectedly, in the plantlet regeneration stage all the tested CF concentrations had a significant positive effect on the percentage of plantlets that re-rooted compared with the control. Both cultivars showed a 95 - 100 % rooting ability of the plantlets, which was significantly higher than the percentage of plantlets that rooted in the embryo germination media (EGM1) containing no CF (60 - 70 %) ($p < 0.001$). Likewise, all the concentrations of the CF had a positive effect on the root length of plantlets, with 1500 ppm CF resulting in the highest root length of 31.5 mm ± 4.3 for 88H0019 and 34.05 mm ± 3.9 for N41. Hence, *F. pseudonygamai* SC17 could not be used as an *in vitro* selection agent in a root re-growth

assay.

Due to the enhanced effect of *F. pseudonygamai* SC17 CF on root growth, the fungal isolate's potential to produce indole acetic-3-acid (IAA) was assessed. *F. pseudonygamai* produced the highest IAA concentration (743.1 nM) in the presence of L-tryptophan than in the treatment without L-tryptophan (457.2 nM). This suggests that the observed enhanced root growth may be due in part to the production of auxin (IAA) in the *F. pseudonygamai* SC17 CF.

Acclimatised *in vitro* plantlets (8-9 months old) were inoculated only with *F. pseudonygamai* SC17 or dual inoculated: firstly, with *F. pseudonygamai* SC17, then 1-2 2nd instar *E. saccharina* larvae that were placed into the leaf whorls 2 weeks later. To confirm tolerance of the putative mutants, fungal isolations were performed on the stem sections above the inoculation lesion from symptomatic and asymptomatic plants. The results revealed that the putative mutant plants that were primed with Hx only and treated with a combination of mutagens (EMS and 5-AzaC) and priming agent exhibited a significant decrease in lesion severity as compared with controls. For both treatments, a mild lesion severity rating was recorded for plants inoculated with only SC17 for cultivars N41 and 88H0019. For the plants that were dual inoculated there was a significant difference in the lesion severity ratings between the two treatments ($p < 0.001$). The lesion severity rating was moderate for cultivar 88H0019 (primed with Hx) and mild for cultivar N41 (primed with Hx). Plants from the combined treatment for both cultivars resulted in a mild lesion severity rating.

This protocol could be valuable in generating commercially important cultivars that are tolerant and resistant to *F. pseudonygamai* SC17 and possibly other sugarcane pathogens. Planting resistant cultivars is recommended as an economical and the best method for controlling diseases and pests. This approach used in this study will have the least impact on the environment and increase yields without the need for expensive chemical applications and labour.

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I would like to express my since gratitude to the following people in no specific order:

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Dedication

This dissertation is dedicated to my loving son, Kian Saiyuran Govender – may you never stop learning

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List of abbreviations

2,4-D - 2,4-dichloro-phenoxyacetic acid
ABA - Abscisic acid
AZA - Azelaic acid
AFLP - Amplified fragment length polymorphism
ANOVA - Analysis of variance
AOC - Allene oxide cyclase
AOS - Allene oxide synthase
AUX - Auxins
AZAC - Azacytidine
BABA - β-aminobutyric acid
BABA-IR - β-aminobutyric acid-induced immunity
BEA - Beauvericins
CF - Culture filtrate
CK - Cytokinins
DNA - Deoxyribonucleic acid
EGM - Embryo germination media
EIM - Embryo initiation media
EMM - Embryo maturation media
EMS - Ethane methanesulfonate
ENN - Enniatin
ET - Ethylene
ETI - Effector-triggered immunity
FA - Fusaric acid
FB1 – Fumonisin B1
GA - Gibberellins
GM - Genetic modification
HF - Hydrogen fluoride
HR - Hypersensitive responses
Hx - Hexanoic acid
IAA - Indole-3-acetic-acid
IR - Induced resistance
ISR - Induced systemic resistance
ISSR - Inter simple sequence repeats
JAs - Jasmonates
JA - Jasmonic acid

JA-ACC - Jasmonoyl ACC
JA-Ile - Jasmonoyl isoleucine
KZN - Kwa Zulu Natal
LOX - Lipoxygenase
LSR - Lesion severity rating
MAPK - Mitogen-activated protein kinase
MeJAs - Methyl jasmonates
MMS - Methyl methanesulfonate
MNU - N-methyl-N-nitrosourea
MON - Moniliformin
MS - Murashige and Skoog
OPDA - 12- oxo-phytodienoic acid
PCD - Programmed cell death
PDB - Potato dextrose broth
PEMs - Pro-embryogenic masses
PR - Pathogenesis-related
PPFD - Photon flux density
R - Resistance
RFLP - Restriction fragment length polymorphism
SA - Salicylic acid
SA - South Africa
SAR - Systemic acquired resistance
SASRI - South African Sugarcane Research Institute
SE - Somatic embryogenesis
UV – Ultraviolet

SI units

Centimetre – cm

Degree Celsius - °C

Gram per litre – g l^{-1}

Grams – g

Hours – hrs

Micromolar - µM

Milligrams per litre – mg l^{-1}

Millilitre -ml

Millilitre per litre – ml l^{-1}

Millimetre – mm

Millimolar – Mm

Minutes – min

Nanomolar - nM

Parts per million – ppm

Percentage - %

Revolutions per minute – rpm

Volume per volume – v/v

1. Introduction and rationale of the study

Sugarcane (*Saccharum* spp. hybrid) is a monocotyledonous crop that belongs to the Poaceae family (Patade and Suprasanna, 2008; Suprasanna et al., 2011; Hailu et al., 2018). It is significant due to its exceptional global and agro-economic value as a well-established multipurpose crop (Naz, 2003; Tolera et al., 2014). Sugarcane is grown in tropical and subtropical regions, and it contributes almost 75 % of the sugar production worldwide, the remaining 25 % being made from sugar beet (*Beta vulgaris)*. Hence, a considerable amount of research has focused on sugarcane crop improvement through conventional plant breeding, and in more recent time's modern tools of biotechnology and genetic engineering have been used (Baksha et al., 2002; Tolera et al., 2014; Shabbir et al., 2021).

One of the main challenges faced by the South African sugar industry is the infestation of sugarcane stalks by the indigenous borer *Eldana saccharina (*Lepidoptera*:* Pyralidae). It is regarded as a major pest and causes direct and indirect losses of up to R900 million per annum in the industry (Conlong, 1994a; Knogge, 1996; Keeping et al., 2014). Several species of lepidopteran stem borers have been found to significantly reduce yields in many crops such as sorghum, maize, sugarcane, and rice (Kleynhans et al., 2017; Péné et al., 2018). They consist of several species belonging to the genera *Sesamia, Diatraea, Chilo*, and *E. saccharina* (Potgieter et al., 2016; VanWeeldan et al., 2016; Péné et al., 2018)

A close association has been found between *E. saccharina* and *Fusarium* spp. in maize (Schulthess et al., 2002; Péné et al., 2018) and sugarcane (McFarlane et al., 2009; Mahlanza et al., 2013; Rutherford et al., 2021). The *E. saccharina* larvae bore into the sugarcane stalk tissue and create larval galleries around the infected tissue (Goebel and Way, 2003; Mahlanza et al., 2013; Péné et al., 2018). *Fusarium* species are initially present as symptomless endophytes within the sugarcane stalk (Mahlanza et al., 2013; Péné et al., 2018). *E. saccharina* damages the stem tissues which later leads to infection by the *Fusarium* species (McFarlane et al., 2009; Mahlanza et al., 2013; Péné et al., 2018; Rutherford et al., 2021). This is characterized by a dark-red discoloration around the affected areas (McFarlane et al., 2009; Péné et al., 2018). Some *Fusarium* strains have been found to be beneficial to the insect by accelerating development and increasing survival of the *E. saccharina* larvae in artificial diet (McFarlane et al., 2009). However, other isolates negatively affected larval growth and survival (McFarlane et al., 2009; Mahlanza et al., 2014; Rutherford et al., 2021). McFarlane et al. (2009) studied the relationship between various *Fusarium* spp. and *E. saccharina* in sugarcane where it was observed that the *F. pseudonygamai* SC17 isolate was beneficial to

E. saccharina growth and survival. *Fusarium* isolates may provide several benefits to *E. saccharina*, such as plant defence suppression by the production of fumonisins, auxins, and cytokinins (De La Torre-Hernandez et al. 2010; Dafoe et al. 2013; Zhang et al. 2015), and antimicrobial inhibition of entomopathogens (Sondergaard et al. 2016; Rutherford et al., 2021). Many studies have demonstrated that endophytic fungi can produce phytohormones, especially indole acetic acid (IAA) and gibberellins, as a direct system of plant growth promotion (Fu et al., 2015; Turbat et al., 2020).

There are many control measures that may be implemented to minimise sugarcane yield losses caused by diseases and pests. *Fusarium* stem rot can be controlled by using well maintained, disinfected and clean equipment that prevents unnecessary damage when cutting seed cane (Croft, 2000; Zhang and Jeyakumar, 2018). Plant diseases caused by *Fusarium* spp. can be controlled by using the correct phosphate, nitrogen, and potassium fertilizers, and using resistant cultivars (Mahlanza et al., 2013; Shabbir et al., 2021). Good farming practices are important but do not always eradicate infections (Zhang and Jeyakumar, 2018). Hence, the extensive damage caused by these pests have resulted in the need for developing *E. saccharina* and *Fusarium* species resistance. To overcome the above-mentioned limitations, it is hypothesised that sugarcane lines tolerant to *F. pseudonygamai* SC17 can be produced, which could still allow endophytic colonisation by the fungus, but resist transition to a necrotrophic phase. Planting resistant cultivars is recommended as an economical and the best method for controlling diseases and pests. This approach will have the least impact on the environment and increase yields without the need for expensive chemical applications and labour (Butterfield et al., 2001; Zhang and Jeyakumar, 2018; Shabbir et al., 2021).

Variety improvement in sugarcane has been achieved mainly by conventional breeding for cultivars with desired characteristics, such as increased yield and disease resistance (Snyman et al., 2011; Zhou, 2013; Dlamini, 2021). However, there are many limitations with regards to sugarcane conventional breeding, as this approach takes up to 10 to 15 years for selection to be completed and it could take much longer to commercially release a cultivar (Snyman et al., 2011; Ming et al., 2006; Tolera et al., 2014; Yadav et al., 2020; Shabbir et al., 2021). The complex genome of sugarcane due to polyploidy (Butterfield et al., 2001; Zhang and Jeyakumar, 2018) and adverse South African climatic conditions to produce pollen limits the progress of conventional breeding (Zhou, 2013; Dlamini, 2021). Over the last decades, various biotechnological approaches have been found to be successful in producing genotypes with improved traits in a variety of crops, including sugarcane. Genetic engineering can also be used to control *Fusarium* spp. by producing plants with improved resistance-conferring genes that target the fungal pathogens (Munkvold et al*.,* 1997; Wang et al., 2021), but the limitations

based on legislative rules affects the use of this approach in sugarcane (Mertens, 2008; Key et al., 2008; Kumar et al., 2020).

In vitro tissue culture techniques have been widely used to generate genetic variation in crops (Liang et al., 2019; Diehdhiou et al., 2021). Somaclonal variation is a result of spontaneous mutations that occur during *in vitro* culture systems (Larkin and Scrowcroft, 1981; Lakshmanan, 2006; Snyman et al*.,* 2011) and has been successfully utilized in genetic crop improvement (Heinz, 1973; Diehdhiou et al., 2021). Induced mutagenesis, somatic embryogenesis and organogenesis are *in vitro* techniques that can be used to induce genetic variation for crop improvement.

Genetic variability can be induced by irradiation (Mba et al., 2012; Suhesti et al., 2021), and treatment with physical (Suprasanna et al., 2009; Mir et al., 2020) and chemical mutagens (Koch et al., 2012; Forster and Shu, 2012; Holme et al., 2019; Mullins et al., 2021). This is known as induced mutagenesis. The targeting of mutations conferring desirable traits by imposing a selective pressure *in vitro* is an approach that has already been shown to be successful in sugarcane e.g., to obtain plants tolerant to the herbicide imazapyr (Koch et al., 2012; Munsamy et al., 2013; Rutherford et al., 2017), salt (Luan et al., 2007), drought (Masoabi et al., 2018), *F. sacchari* (Mahlanza et al., 2013) and Al^{3+} (Purnamanisingh and Hutami, 2016; Snyman et al., 2019). The present study will focus on chemical mutagenesis to produce plants resistant to both *F. pseudonygamai* SC17 and *E. saccharina*.

Recently, the effects of fungal culture filtrates or toxins on resistant and susceptible plants to assess disease resistance, by using *in vitro* techniques have been investigated (Mahlanza et al., 2013; Svabova and Lebeda, 2005; Suthar et al., 2021). The purified toxins or pathogen culture filtrates that contain toxins involved in plant disease development, are suitable for use as *in vitro* selection agents (Svabova and Lebeda, 2005; Suthar et al., 2021). Many studies have confirmed the positive correlation between the tolerance of plants to culture filtrates or toxins and those that are inoculated by the pathogen (Gray et al., 1986; Connell et al., 1990; Suthar et al., 2021).

Fusarium spp. are known to produce an extensive array of toxins that vary quantitatively and qualitatively between strains (Zhang et al., 2006; Aly et al., 2011; Wu et al. 2015; Lugtenberg et al., 2016). Infection by some endophytic fungi are regarded as symptomless since they can colonise their host plants in an asymptomatic state (Saikkonen et al. 2004; Yuan et al. 2010; Navarro-Meléndez and Heil, 2014). Endophytic fungi are symptomatic or asymptomatic inside plant tissues, and they share either a symbiotic or antagonistic interaction with the host plant

(Fontana et al., 2021). In a symbiotic interaction the fungal endophytes benefit from nutrition, shelter, and protection in the host plant, whilst the endophyte assists its hosts by stimulating its development, growth, stress tolerance, and adaptation (Saikkonen et al., 2004; Wang et al., 2015; Fontana et al., 2021). Symptomless endophytism is linked with increased jasmonate (JA) and suppressed salicylate (SA) signaling, which suggests that defence against necrotrophic pathogens and insect pests is heightened (many endophytic fungi have the potential to become necrotrophic pathogens) (Navarro-Meléndez and Heil, 2014; Kou et al., 2021). Jasmonates are involved in resistance to necrotrophs and chewing insects such as *E. saccharina* and appear to be involved in maintaining the non-symptomatic state of the endophyte (Glazebrook, 2005; Thaler et al., 2012; Ballare, 2014; Navarro-Meléndez and Heil, 2014; Kou et al., 2021). Necrotrophic colonisation occurs when the fungus produces certain toxins that activate the salicylate pathway (SA) and induces cell death and oxidative stress (De la Torre- Hernandez et al. 2010; Sanchez-Rangel et al. 2012; Arias et al., 2012; Blacutt et al., 2018). The increase in SA levels in a plant intensifies the susceptibility to necrotrophic pathogens (Ghozlan et al., 2020).

In the current study, it was hypothesised that the SA and JA pathways are antagonistic, and SA induction by *Fusarium* spp. likely acts to suppress the JA pathway. The *Fusarium* strains that are beneficial to *E. saccharina* (e.g., *F. pseudonygamai* SC17) (McFarlane et al., 2009) could be suppressing JA-induced defence responses which favour both *E. saccharina* and the fungus (Mahlanza et al., 2013; Ghozlan et al., 2020).

Research hypotheses

A previous study by Mahlanza et al. (2013) focused on the development of sugarcane mutants resistant to the mycotoxins produced by the detrimental strain *Fusarium sacchari* PNG40 (McFarlane et al., 2009) that reduced the suppression of the host plant defence and increased rates of symptomless endophytism. Mahlanza et al. (2013) used ethylmethansulfonate (EMS) and 5-Azacytidine (5-AzaC) to induce mutations, and very few mutant plants were produced. As mentioned above, the presence of valuable endophytic fungi may have some benefits for the host plants. Hence, in the current study, it was hypothesised that the development of plants resistant to the mycotoxins produced by the beneficial *Fusarium* strain, will also have reduced *E. saccharina* infestation. A protocol will be developed to test the above hypotheses. Given the role of JA in maintaining endophytes in a symptomless state, an epigenetic primed and mutagenic protocol had to be established. The established protocol should be able to 'fix' a favourable epigenetic state (e.g., Hx primed) for both *E. saccharina* and *Fusarium* resistance. The selection of putative mutants resistant to mycotoxins (able to induce SA responses and

inhibit JA responses) should result in sugarcane mutant cultivars with enhanced *E. saccharina* and *F. pseudonygamai* resistance. It was hypothesised that the *E. saccharina* beneficial *Fusarium* strain SC17 may be stimulating root growth by the auxin indole-3-acetic acid (IAA) production or increased activity of the enzyme ACC deaminase. An experiment will be conducted to quantify the amount of IAA produced by *F. pseudonygamai* SC17.

Aims and Objectives

This research aimed to develop an epigenetic primed and mutagenic protocol to generate and characterise mutant sugarcane cultivars that are resistant to both *E. saccharina and F. pseudonygamai* SC17. The cultivars chosen were N41 and 88H0019 which are intermediate-resistant and susceptible to *E. saccharina*, respectively. Once the protocol has been developed, it can be used to generate resistance to *E. saccharina and F. pseudonygamai* in sugarcane commercial lines. This was done by using the following *in vitro* techniques: somatic embryogenesis, chemical mutagenesis and priming followed by *ex vitro* screening. Hence, the main objectives of the study were as follows:

- to establish the most suitable priming agent between hexanoic acid and cis-jasmone.
- to determine an appropriate culture filtrate concentration for *in vitro* screening of calli and plantlets for tolerance to *F. pseudonygamai* (SC17).
- to generate additional mutants: primed only (Hx), and primed and mutagenic (5-AzaC + EMS-induced).
- to screen for indole-3-acetic acid production by isolating *F. pseudonygamai* SC17.
- to characterize *in vitro* selected mutants for *E. saccharina* and *Fusarium* resistance by comparing the levels of resistance between un-primed, primed, primed + mutagenic plantlets through *ex vitro* screening.

2. Literature Review

2.1 Commercial sugarcane cultivation and its importance worldwide

Sugarcane belongs to the genus *Saccharum L.,* which consists of three cultivated species (*Saccharum officinarum*, *S. sinesis and S. barbari*) and two wild species (*S. spontaneum and S. robustum*) (Kochhar, 1998; Mahmud *et al*., 2016). It is a large perennial grass cultivated worldwide in subtropical and tropical areas (Ming et al., 2006) for its high content in sucrose (Brumbley et al., 2008; Goebel et al., 2018; Péné et al., 2018) and ethanol production (D'Hont et al., 2008; Tolera et al., 2014). Approximately 110 countries generate sugar from either sugarcane or beet, in which sugarcane is used to produce most of the world's sugar, and the rest is produced from sugar beets (*Beta vulgaris L.)* (Grivet and Arruda, 2001; Lakshmanan et al., 2005; Ming et al., 2006; Mirajkar et al., 2019). Nearly 75 % of global sugar production is obtained from sugarcane, and the remaining 25 % is made from sugar beet (Kroger et al., 2006; Shabbir et al., 2021). The top ten producing countries are Brazil, India, China, Thailand, The United States, Pakistan, Mexico, France, Russia, and Australia (Iqbal et al., 2020; Shabbir et al., 2021).

Although sugarcane is primarily required for the food industry for its sucrose content, sugarcane is usually considered to be one of the most efficient and significant sources of biomass for biofuel production (D'Hont et al., 2008; Brumley et al., 2008). The by-products of sugar refining such as molasses, bagasse, furfuryl alcohol, furfural, diacetyl and dextran have many beneficial uses (O'Reilly, 1998; Brumley et al., 2008). The fibrous residue after sugar extraction, which is called bagasse, can be used to produce electricity for the power system, to fuel boilers in the sugar mills, as a livestock feed and to manufacture disposable packaging (Almazan *et al.,* 2001; Martinez-Hernandez et al., 2018). Molasses is used as a substrate for ethanol production as well as in syrups and animal feed (Brumbley et al., 2008).

In South Africa in 1852, the first sugar produced was derived from varieties of noble cane (*S. officinarum*) (Zhou, 2013). The modern sugarcane cultivars (*Saccharum* spp.) originated from crosses between *S. spontaneum L.* (wild sugarcane) and *S. officinarum L.* (sweet sugarcane), followed by backcrossing the interspecific hybrids with the noble parent plant, *S. officinarum* (Roach and Daniels, 1987; Brumbley et al., 2008; Singh et al., 2010). However, a recent study by Evans and Joshi (2016) described *S. cultum* as a novel species of *Saccharum*. Molecular phylogenetic studies have revealed the need to divide *Saccharum* spp. into four species: *S. spontaneum, S. officinarum, S. narenga* and the family of recent hybrid cultivars that has been formally named *Saccharum cultum*, originating from a cryptic founder species (Evans and

Joshi, 2016). The basic concept of plant breeding includes the improvement and combination of high sucrose content from *S. officinarum* and disease resistance from *S. spontaneum (*D'Hont et al., 2008).

Since ancient eras, sugarcane cultivation has gone through many changes into contemporary noble cane (D'Hont et al., 2008; Mirajkar et al., 2019). At first, the selection of interspecific hybrids and desirable clones introduced many agronomically beneficial traits into the cultivated species. Wild related species has played a significant part as the donor through gene introgression for most of the desirable traits (Brumbley et al., 2008; Singh et al., 2010). The development of intergeneric hybrids and pre-breeding strategies has played a significant role in the production of sugarcane cultivars with high yields (Brumbley et al., 2008; Singh et al., 2010; Mirajkar et al., 2019). Cultivated sugarcane has been improved with useful traits such as high fermentable sugar, high fiber and biotic and abiotic stress tolerance. Sugarcane is a complex crop due to the extreme level of polyploidy, however despite its genomic complexity, long breeding and selection cycles, and hybridization barriers within the genus, notable improvement has been attained to produce a varied range of cultivars, mutants, and hybrids appropriate for dissimilar agroclimatic conditions (Mirajkar et al., 2019).

2.2 The main challenges faced by the South African sugar industry

In most of the tropical and sub-tropical regions lepidopteran stem borers have been found to significantly reduce yields of a few crops such as sugarcane, sorghum, rice and corn, thus they are regarded as economically important pests (Kleynhans et al., 2017; Péné et al., 2018). In the South African sugarcane industry, there has been substantial yield losses caused by the indigenous pyralid borer *Eldana saccharina Walker* (Lepidoptera: Pyralidae) (Conlong, 1994a; Knogge, 1996; Keeping et al., 2014). *E. saccharina* has been regarded as a major pest of sugarcane and there was an initial outbreak in 1939 (Dick, 1945; Walton and Conlong, 2016), a second in 1970 (Carnegie, 1974) and the pest has been persistent since then (Fig. 1) (Walton and Conlong, 2016).

Sugarcane has great economic importance and plays major role in contributing to the sugar industry of South Africa. Sugarcane is still very much susceptible to several diseases and pests, causing substantial losses in sugarcane production. Cultivation over large adjacent fields and vegetative propagation of sugarcane make it relatively vulnerable to diseases such as rust and mosaic virus (Dick, 1945; Bailey, 2004). The development of high sugar yielding cultivars in the SA Sugar Industry is limited by the major pest *E. saccharina* (Butterfield and Thomas, 1996; Conlong, 2001). The larvae infest mature cane and their borings provide

access for the fungus *Fusarium* which infects sugarcane growing stalks and seed cuttings following stalk-borer injury, causing *Fusarium* stem rot (Bourne, 1961; McFarlane et al., 2009; Péné et al., 2018).

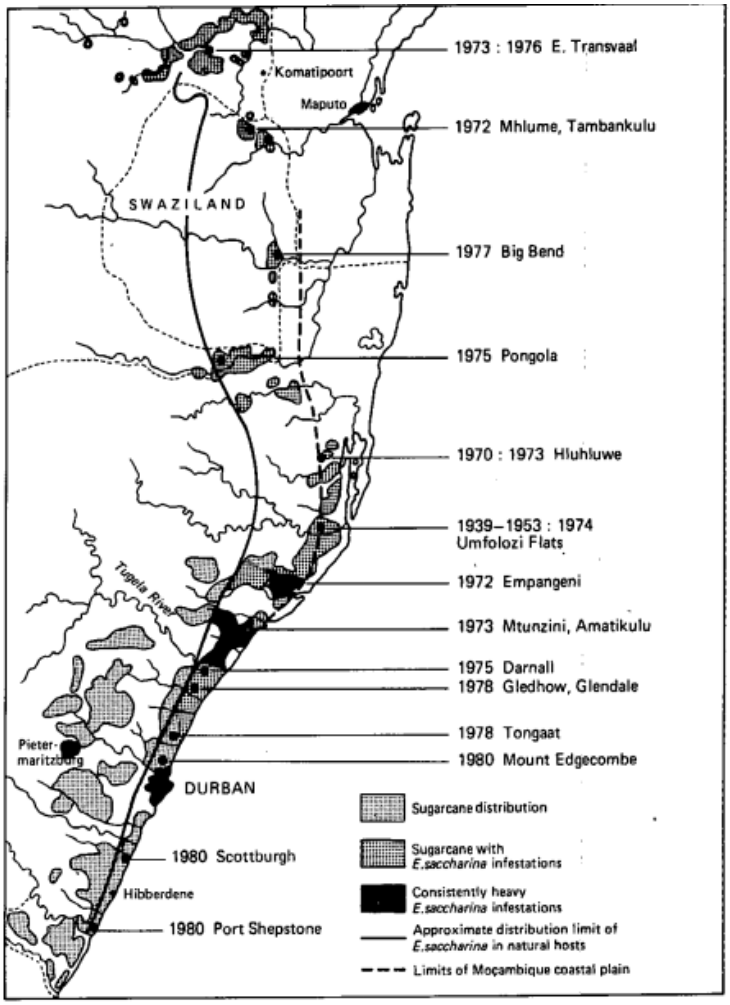


Figure 1: A map illustration of reported *E. saccharina* outbreaks in South Africa and Swaziland (Atkinson et al., 1981).

2.3 The history of *E. saccharina* in the sugarcane industry

2.3.1 History and distribution

The African stalk borer *E. saccharina* Walker (Lepidoptera: Pyralidae) was described over many years ago from specimens obtained from sugarcane in West Africa (Carnegie, 1974). *E. saccharina* is a stalk borer native to Africa which has been found to attack graminaceous crops (e.g., sugarcane, sorghum, and maize) and on various wild sedges and grasses (Waiyaki et al., 1968; Conlong 1994b; Walton and Conlong, 2016). An initial outbreak of *E. saccharina* occurred in 1939 at the Umfolozi Flats, Kwa-Zulu-Natal (Carnegie, 1974; Atkinson et al., 1981). *E. saccharina* was first observed in sugarcane in Sierra Leone, West Africa in

the late 1800's (Betbeder-Matibet, 1981; Walton and Conlong, 2016) and was later reported from sugarcane in East Africa (Girling, 1972; Conlong, 2001; Walton and Conlong, 2016). In 1939, after the first outbreak of *E. saccharina* in South Africa as stated by Dick (1945), the pest disappeared and re-emerged in the 1970s (Carnegie, 1974; Walton and Conlong, 2016). Thereafter *E. saccharina* spread both south and north of Hluluwe, becoming a destructive pest in Pongola, Mpumalanga, Swaziland and in the lower altitudes of KZN (Fig. 1) (Carnegie et al., 1974; Atkinson, 1979). The distribution of stalk borers is reported to be dependent on temperature, rainfall, altitude, and availability of food (Haile and Hofsvang, 2001). However, in recent years, *E. saccharina* has been observed further south, towards the higher altitudes and in colder climates (e.g Midlands North and South), extending to the Limpopo province and observed at the Boskop dam in the North-West province (Assefa et al., 2008). It has been reported that the initial colonisation of sugarcane by *E. saccharina* may have resulted from the removal of *E. saccharina* natural host plants (grasses and sedges) and the subsequent planting of sugarcane in these wetland areas (Conlong, 1994a; Conlong, 2001; Govender et al., 2010).

2.3.2 *E. saccharina* and borer-associated *Fusarium* stem rot

E. saccharina associated damage and early crop harvesting are major factors limiting sugarcane productivity (Leslie, 2004; Goebel et al., 2005; Mokhele et al., 2009, Keeping et al., 2014; Potgieter et al., 2016; Van Weelden et al., 2016; Péné et al., 2018). As a result of *E. saccharina* infestation due to secondary microbial degradation, sucrose is converted to glucose, thereby lowering the sucrose yields (Geobel, 2005). In sugarcane, the larva enters the stalk by boring through the bud, root primordia or cracked internodes and they then forages on the inner stalk tissue (Mokhele et al., 2009; Potgieter et al., 2016; Van Weelden et al., 2016; Péné et al., 2018). Frass (excrement) expelled from borings (as shown in Fig. 2), is an indication of insect infestation (Girling, 1972; Chinheya et al., 2009). The association between infection by the fungus (*Fusarium* spp.) and borer infestation can result from the wounds created by *E. saccharina* on the stalk, which provide the fungus access to the inner stalk issue, if not already present as a symptomless endophyte (McFarlane et al., 2009; Péné et al., 2018). The infected sugarcane stalks are characterised by a reddish discolouration because of secondary infestation by *Fusarium* spp. an opportunistic fungus which further reduces sugar content, and spreads to the undamaged nodes (Fig. 2) (Bourne, 1961; McFarlane et al., 2009; Chinheya et al., 2009; Péné et al., 2018).

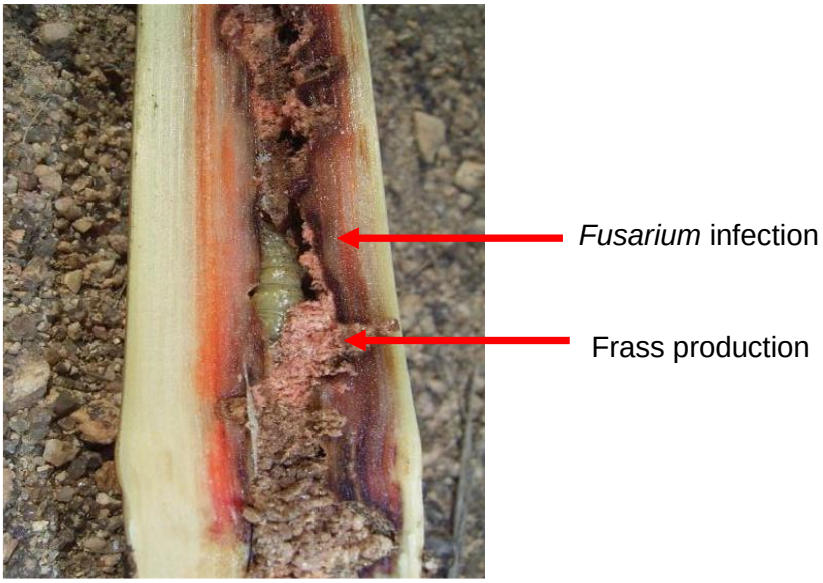


Figure 2: *Fusarium* infection in the stem seen by red colour associated with *E. saccharina* infestation (source: SASRI entomology department)

It has been reported that symptomless endophytic colonisation by *Fusarium verticilliodes* in maize is related to greater *E. saccharina* damage and infestation in contrast with those plants arising from seeds treated with a fungicide (Schulthess et al., 2002). This suggests a favourable association between the stalk-borer and the fungus (Schulthess et al., 2002). A range of volatiles such as alcohols, aldehydes and esters, which are responsible for insect attraction, were found to be produced by certain *Fusarium* isolates (e.g., *F. verticillioides*) (McFarlane and Rutherford, 2005). *F. verticillioides* has been shown to promote *E. saccharina* development and survival in maize (Ako et al., 2003). In sugarcane, McFarlane et al. (2009) found that although certain endophytic *Fusarium* isolates were beneficial to *E. saccharina* survival and growth rate, other isolates (e.g., *F. sacchari* isolate PNG40) were detrimental. The antagonistic effects of those *Fusarium* strains could possibly be due to the mode of action of metabolites produced by *Fusarium* spp., such as fusaproliferin (Logrieco et al., 1996; Munkvold et al., 2009), beauvericin (Logrieco et al., 2002), and fusaric acid (Ismaiel and Papenbrock, 2015), which are insecticidal toxins.

It is important to understand the interactions between the stalk borer *E. saccharina* and *Fusarium* spp. since this will help develop control measures for this lepidopteran pest in sugarcane. *Fusarium* isolates that are beneficial to the stalk borer may help in reducing damage so it can be used to control plant infection, whilst harmful strains to the pest may be used as a biological control agent against the pest (Schulthess et al., 2002; Mahlanza et al., 2013).

2.4. The interaction between insect pests and *Fusarium* spp. in various economically important crops

2.4.1 The pathogen and/or endophyte

Fusarium spp. are phytopathogenic fungi that belong to a large complex genus, which are known as worldwide plant pathogens that colonise and infect several crops such as sugarcane, rice, maize, oats and wheat in semi-tropical and temperate areas (Hsuan et al., 2010; Shi et al., 2017). This genus is diverse, and the current classification system has 65 species, 55 varieties, and 22 forms in 16 sections (Leslie and Summerell, 2006, Summerell, 2019). *Fusarium* spp. are widespread in terrestrial ecosystems and are usually found together with higher plants (Ploetz, 2005). Sugarcane (*Saccharum* hybrids), rice (*Oryza Sativa*), bananas (*Musa* spp.) and maize (*Zea mays*) are amongst the most important agricultural crops in several countries (Hsuan et al., 2010; Rebouças et al., 2021). They are commonly infected by plant pathogenic fungi which consist of *Fusarium* species (Leslie and Summerell, 2006; Hsuan et al., 2010). Their infection of sugarcane results in *Fusarium* stem rot and Pokkah boeng (Bourne, 1961; Croft, 2000; Costa et al., 2019). Pokkah boeng in sugarcane is caused by species of the *Fusarium fujikuroi* species complex, characterized by deformation of the top of the plant or stem rot, and is regarded as an important fungal disease (Costa et al., 2019).

Endophytes are microorganisms that, during a variable period of their life cycle colonise plant tissues whilst being symptomless in their host (Carroll, 1988; Stone et al., 2004; Rodriguez et al., 2009; Numponsak et al., 2018). Colonisation of plants by endophytes can occur when host defence mechanisms and pathogen virulence are in balance, so that neither is negatively affected by the association (Bae et al., 2009; Pineda et al., 2010; Numponsak et al., 2018). These plant-endophyte interactions are mutualistic relationships in which the host obtains defence and growth promotion from biotic and abiotic stress, while the fungus acquires its nutrients and habitation from hostile environmental factors and competitors on the plant's exterior surface (Schultz and Boyle, 2005; Rigobelo and Baron, 2021). In previous studies, it has been reported that endophytic fungi have the potential to produce bioactive compounds that builds protection against microbial influencers in the host plant and promotes plant growth in a volatile and non-volatile environment (Morath et al., 2012; Suwannarach et al., 2015; Numponsak et al*.,* 2018). Numerous studies have reported that the secretion of secondary metabolites (phytohormones e.g., auxins, gibberellins, ethylene, siderophore and cytokinins) that are produced by endophytic fungi may be associated with plant growth promotion, as well as their ability of the to move insoluble phosphate and provide their host plants with nitrogen (Dai et al., 2008; Khan et al., 2012; Khan et al., 2015; Numponsak et al., 2018).

A varied range of endophytic fungi has been isolated from various plant species worldwide and found to be the cause of different diseases (Table 1). Most *Fusarium* strains are pathogenic to many crops and cause various diseases such as *Fusarium* wilt (Viswanathan et al., 2017), stalk rot (Mughogho and Rosenberg, 1984; Afolabi et al., 2008; Dela Cueva et al., 2019), and *Fusarium* head blight (Burrows et al., 2008; Shi et al., 2017) while others are endophytic (Bacon and Hinton, 1996; Macia-Vicente et al., 2008; Mahlanza et al., 2013).

2.4.2 *Fusarium*-produced mycotoxins in plant-pathogen interactions

Fusarium species produce an extensive range of secondary fungal metabolites known as mycotoxins (Bennett and Bentley, 1989; Marin et al, 2013; Ji et al., 2019), that are either harmful or phytotoxic to plant pathogens and insects, which allows them to act as biological control agents of diseases and pests (McFarlane et al., 2009; Ploetz et al., 2015; Zakaira et al., 2017). *Fusarium* species commonly found as disease causal agents (Table 1) also produce mycotoxins in the field or in storage (Table 2). A wide range of phytotoxic compounds that are chemically different are produced by members of the genus *Fusarium*, such as fumonisins (fumonisin B1, FB1), enniatin (ENN), fusaric acid (FA), beauvericins (BEA), trichothecenes and moniliformin (MON) (Desjardins, 2006; Ismaiel and Papenbrock, 2015; Zakaira, 2017; Dinolfo et al., 2017) (Table 2). Many studies have shown that these secondary metabolites hinder the metabolic processes of animals and plants (Bennet and Klich, 2003; Ismaiel and Papenbrock, 2015), and that they are toxic to several crops (Borras et al., 2001; Leslie et al., 2004; Ismaiel and Papenbrock, 2015). These phytotoxic compounds display various biological activities that cause physiological, metabolic and morphological effects and can be observed through chlorosis, necrosis, wilting, growth inhibition, seed germination inhibition and effects on calli (Desjardins and Hohn, 1997; Ismaiel and Papenbrock, 2015; Suthar et al., 2021).

Several studies have reported on the role of fumonisin B1 (FB1), a mycotoxin produced by *Fusarium verticillioides* that causes serious disease symptoms in maize and other grains (Gilchrist, 1997, 1998; Zhang et al., 2015). Fumonisin B1 has been found to be a strong elicitor of programmed cell death (PCD) in plants (Xing et al., 2013; Zhang et al., 2015). Hypersensitive response (HR) is a form of PCD, which occurs during incompatible plant-biotrophic-pathogen reactions. It also takes place when plant cells around the invasion site(s) rapidly and actively die to limit pathogen growth and stop the nutrient supply, and in doing so prevents the disease from spreading (Coll et al., 2011; Zhang et al., 2015). The sphingolipid biosynthetic pathway is inhibited by FB1, by hindering the ceramide synthase enzyme, which can result in cell death (Stone et al., 2000; Zhang et al., 2015). Additionally, FB1 can induce other HR-like responses including phenolic compounds, callose deposition, reactive oxygen

species (ROS) production and expression of pathogenesis-related (PR) proteins (Wolpert et al., 2002; Zhang et al., 2015). The PR proteins are a group of various proteins that are induced by defense-related signaling molecules and phytopathogens (Ali et al., 2018).

Table 1: Examples of *Fusarium* spp. commonly found as causal disease agents in economically important crops

Crop	*Fusarium* spp.	Disease	References
Cotton, *Gossypium* spp.	*F. oxysporum*	*Fusarium* wilt	Davis et al., 2006
Maize, *Zea mays L.*	*F. graminearum* *F. verticillioides* *F. proliferatum*	*Fusarium* stalk rot *Fusarium* cob and root rot	Rahjoo et al., 2008 Hsuan et al., 2010
Pineapple *Ananus comosus*	*F. guttiforme*	Fusariosis	Ploetz, 2003
Rice *Oryza sativa*	*F. moniliforme*	Bakanal disease	Iqbal et al., 2011
Sugarcane *Saccharum* hybrids	*F. moniliforme*	Pokkah boeng	Sanghera et al., 2018
Wheat *T. aestivum*	*F. graminaearum*	*Fusarium* head blight	Burrows et al., 2008 Li et al., 2019

Many studies have reported on several factors that may influence the production of mycotoxins. Among the factors are colonisation of the plant host and duration of fungal growth, temperature, host and substrate composition, water activity and moisture content (Ciegler, 1978; Ashiq, 2015; Zakaira, 2017). Stress factors including water shortage and insect/pest infestation might play a role in mycotoxin production (Sanchis and Magan, 2004; Milani, 2013; Zakaira, 2017). According to Atanda et al. (2013) humid and hot conditions are the two most critical factors that promote fungal growth and development including mycotoxin production. Hence, there are many interacting factors involved in promoting mycotoxigenic fungal growth

and mycotoxin production. The latter is dependent on the species of the mycotoxigenic fungi since different species have their own requirements for optimal growth and mycotoxin production (Cast, 2003; Zakaira, 2017).

Table 2: Examples of toxic compounds isolated from various crops infected by *Fusarium* spp.

***Fusarium* spp.**	**Mycotoxin**	**Crop**	**Reference**
F. moniliforme	Fusarins	Maize, soybean, rye	Bacon et al., 1996 Gai et al., 2018
F. equiseti, *F. sambicinum,* *F. graminearum*	Trichothecenes	Sorgum, wheat, maize	Brian et al., 1961 Wang et al., 2006 Shi et al., 2017
F. graminearum *F. culmorum* *F. equiseti*	Zearalenone	Corn, barley, wheat, oats, sorgum	Bennett and Klich, 2003 Ji et al., 2019
F. verticillioides *F. subglutinans*	Fusaric Acid	Wheat, barley	Brown et al., 2012 Zhang and Jeyakumar, 2018
F. verticillioides *F. proliferatum* *F. nygamai*	Fumonisin B1	Soybeans, maize	Thiel et al., 1992 Rheeder et al., 2002, Zhang and Jeyakumar, 2018
F. proliferatum	Fusaproliferin	Rice	Desjardins, 2006
F. sacchari	Moniliformin	Maize	Leslie and Summerell, 2006
F. oxysporum	Beauvericin		Zakaria et al., 2017

Mycotoxigenic *Fusarium* are responsible for causing mycotoxin contamination of agricultural crops which may occur in direct and indirect ways. Toxigenic fungi were further characterised into two groups namely: “field” fungi and “storage” fungi (Desjardins et al., 1996; Bennett and Klich, 2003; Zakaira, 2017). Mycotoxigenic *Fusarium* spp. that are categorised as field fungi are commonly known to infect crops in the field and contaminate them. These fungi are regarded as the initial phytopathogen that infects the crops and may produce a mycotoxin under suitable conditions as it grows within the crop (Desjardins, 1996; Bennett and Klich,

2003; Zakaira, 2017). There are several mycotoxigenic *Fusarium* spp. that are also phytopathogenic and can infect the plant host (Desjardins, 1996; Bennett and Klich, 2003; Zakaira, 2017). When agricultural products are stored, the mycotoxigenic fungi grows saprophytically on the stored products (Glenn, 2007; Zakaira, 2017).

2.4.3 Plant growth regulators produced by *Fusarium* spp.

The capacity of plants to resist pathogen attack is regulated through various mechanisms which include insect biological control agents (Compant et al., 2005; Petti et al., 2012). A plant's ability to tolerate abiotic and biotic stress is determined by the production of phytohormones (Bari and Jones, 2009; Dinolfo et al., 2017). These are naturally occurring compounds produced by plants that are crucial for reproduction, plant growth regulation, development, and survival (Adie et al., 2007; Bari and Jones, 2009; Dinolfo et al., 2017). There are several phytohormones, which include auxins (AUX), abscisic acid (ABA), cytokinins (CK), gibberellins (GA), jasmonates (JA), ethylene (ET), salicylic acid (SA), brassinosteroids, and peptide hormones which alter their levels during infection as a strategy to prevent colonisation of the pathogen (Adie et al., 2007; Bari and Jones, 2009; Dinolfo et al., 2017). They are responsible for signal transduction/perception, gene expression and cellular homeostasis (Peleg and Blumwald, 2011; Petti et al., 2012; De Vleesschauwer et al., 2013; Pozo et al., 2015; Spence and Bais, 2015; Chanclud and Morel, 2016). Therefore, they play a vital role in plant responses to, and resistance against disease (Peleg and Blumwald, 2011; Petti et al., 2012; De Vleesschauwer et al., 2013; Pozo et al., 2015; Spence and Bais, 2015; Chanclud and Morel, 2016).

The developmental processes in a plant, such as cell division, organ formation and differentiation (Oka et al., 1999; Vanneste, 2005; Benjamins and Scheres, 2008; Chanclud and Morel, 2016), and senescence (Kim et al., 2011; Chanclud and Morel, 2016) involves the use of auxins, which are indole-derived hormones. Auxins control the biotic and abiotic stress responses in plants (Peleg and Blumwald, 2011; Chanclud and Morel, 2017). In bacteria, tryptophan is a pre-cursor for auxin production, which is converted using tryptophan-2-monooxygenase enzymes into indole-3-acetamide (Zhao, 2010; Chanclud and Morel, 2016). Indole-3-acetic acid (IAA) is formed when indole-3-acetamide has been hydrolysed and is also the major active auxin form in plants even though it is produced through dissimilar biosynthesis pathways. Studies have shown that *Fusarium* spp. are involved in the production of fungal toxins (Tsavkelova et al., 2012; Chanclud and Morel, 2016). The phytohormone(s) salicylic acid (SA), jasmonates, abscisic acid and ethylene play an important role protecting plants against fungal pathogens such as *Fusarium oxysporum* (Dempsey and Klessig, 2012; Hinsch et al., 2016; Vrabka et al., 2019).

Gibberellins are diterpenoid acids that are biologically derived from tetracyclic diterpenoid hydrocarbons which are produced by micro-organisms (Srivastava et al., 2003). Gibberellins are essential endogenous hormones found in fungi and plants which control plant development by regulating various physiological mechanisms (Hooley, 1994; Miceli et al., 2019). These phytohormones play a role in stimulating root and stem elongation, flowering, leaf expansion, seed germination, fruit senescence or dormancy (Hedden and Sponsel, 2015). Gibberellic acid (GA_3) is the dominant product of gibberellins isolated from bacteria and fungi (Muddapur et al., 2015).

2.5 Phytohormone production by fungal endophytes

2.5.1 Phytohormones and endophytes

The plant cell wall creates a strong physical barrier that provides protection to the cell against microbial infection (Nafisi et al., 2015). It is made up of four main constituents, cellulose, pectins, hemicelluloses and glycopectins (Carpita and Gibeaut, 1993; Nafisi et al., 2015). There are dramatic chemical and structural changes that occur in the cell wall during pathogen infection (Vorwerk et al., 2004; Voigt, 2014; Nafisi et al., 2015), including deposition of callose (Luna et al., 2012; Nafisi et al., 2015), lignification (Zhao and Dixon, 2014; Nafisi et al., 2015), cell wall protein cross-linking (Bradley et al., 1992; Nafisi et al., 2015), accumulation of antimicrobial compounds (such as phytoalexins) and reactive oxygen species (Franke et al., 2005; O'Brien and Benkova, 2013; Nafisi et al., 2015), which may work to limit the infection and avoid additional pathogen progression. Plants have the ability to sense the cell wall integrity and, when compromised, intracellular events can be activated which involves phytohormone signalling cascades that can trigger defence mechanisms (Hamann, 2012; Nafisi et al., 2015). Plasmamembrane receptor(s) can sense the degradation of cell wall constituents, mainly by necrotrophic pathogens (Hamann, 2012; Nafisi et al., 2015). As a result, defence signalling cascades and subsequent inducible defence responses are activated (Monaghan and Zipfel, 2012; Nafisi et al., 2015).

The ecological adaptability of a plant is enhanced by colonisation of endophytic fungi, or it does so by improving plant tolerance against the abiotic and biotic stresses (Schultz and Boyle, 2005; Mehmood et al., 2018). There is a wide range of endophytic fungi, which does not cause damage when colonising the living plant tissue. Several benefits are provided to the host plants in different ways by endophytic fungi, such as supplementing absorption of minerals, promotion of growth and protection against pests and diseases (Sieber et al., 2002; Mehmood et al., 2018). Fungal endophytes can produce bioactive metabolites which are

responsible for promoting the plant endophyte interaction (Strobel, 2003; Mehmood et al., 2018). The most important effect of fungal symbiosis is plant growth promotion (Hassan et al., 2013), and it occurs via various secondary metabolites, including ammonia and plant growth regulators, particularly indole-3-acetic acid (IAA) (Sieber et al., 2002; Khan et al., 2012; Mehmood et al., 2018) and enzymatic acitivities such as 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase which plays a role in plant growth (Nascimento et al., 2014; Hossain and Sultana, 2020). The ethylene precursor, ACC is converted into α-ketobutyrate and NH_3 (ammonia), by the ACC deaminase enzyme (Nascimento et al., 2014; Hossain and Sultana, 2020). Plant growth is regulated when the ACC deaminase enzyme cleaves ACC produced by plants and thus reduces the ethylene levels in the plant (Glick et al., 2007; Hossain and Sultana, 2020).

2.5.2 IAA in fungal - plant interactions

Indole acetic acid is a renowned plant growth regulator of the auxin class, is produced by plants and microorganisms (Yuan et al., 2008; Fu et al., 2015; Ismail et al., 2021). In plants, it is responsible for the regulation of various aspects of their development and growth (Fu et al., 2015). Roots have been found to be very sensitive to fluctuations in IAA levels, which is essential for both primary and lateral root initiation (Khan et al., 2011; Fu et al., 2015; Turbat et al., 2020). IAA stimulates dose-dependent increase in the formation of lateral roots, adventitious roots and length of epidermal-derived root hairs (Overvoorde et al., 2010; Fu et al., 2015). Additionally, IAA is involved in plant-pathogen interactions such as pathogenesis and defence mechanisms (Kazan and Manners, 2009; Fu et al., 2015; Turbat et al., 2020). It has been suggested by Fu et al. (2015) that in various plant-fungus interaction systems, the fungi may use the fungal produced IAA and related compounds to interrelate with plants for symbiotic strategies or pathogenesis, resulting in promotion of plant growth and basal plant defence mechanism modification.

Fu et al. (2015), reviewed the interaction between plants and rhizosphere-associated microorganisms. Several organic compounds namely sugars, vitamins and organic acids are produced by roots, and these organic compounds have been found to be used as nutrients or signals by fungal populations. Fungi are known to enhance plant growth by increasing the availability of nutrients to their host. They do this by releasing volatile compounds, siderophores and phytohormones. Root formation and root hair development can be induced by fungal-produced IAA. The promotion of root development and growth allows the associated plant to boost its nutrient intake.

2.6 Crop improvement strategies for inducing tolerance to biotic and abiotic stress.

2.6.1 Conventional plant breeding

Conventional plant breeding is used to develop improved cultivars with specific characteristics suited to the needs of farmers and consumers (Manshardt, 2004; Cheavegatti-Gianotto et al., 2011; Getnet, 2017). Plant breeding is the recombination of desired genes from crop cultivars and closely related species by sexual hybridization (Manshardt, 2004; Cheavegatti-Gianotto et al., 2011; Getnet, 2017). Vegetative or asexual propagation through stem cuttings have been used in modern commercial sugarcane cultivation (Nalawade et al., 2018). Plant breeding programs are based on the development of a segregating population with genetic variability and follows several stages of selection and cloning of the superior genotypes (Gazaffi et al., 2014). There are several factors that determine the success of a breeding program, of which some are the suitable choice of parents to maximize the selection regime, experimental design for good accuracy and the correct choice of desired traits and the assessment period (Cheavegatti-Gianotto et al., 2011; Gazaffi et al., 2014).

Sugarcane has major limitations in terms of conventional breeding due to its polyploidy nature. This means that one cross can result in many progenies that vary in traits, such as yield, agronomic characters, and disease resistance (Berding et al., 2005). Sugarcane breeding programs are time consuming and labour intensive, consequently, it can take 10 - 12 years to develop and release an elite sugarcane cultivar (Gazaffi et al., 2014).

2.6.2 Genetic engineering

Genetic modification (GM) is defined as the insertion of specific genes into a genome, to develop plants expressing the desired traits (Birch, 1997; Scott et al., 2016; Kumar et al., 2020). The genes are artificially inserted into the plants rather than using sexual reproduction (Lakshmanan et al., 2005; Kumar et al., 2020). Genetic modification has become an essential biotechnological tool which is used to produce sugarcane with enhanced traits to survive biotic and abiotic stresses as reviewed by Lakshmanan et al. (2005) (Table 3).

While plant breeding results in hybridization between parents with desired characteristics, single traits are usually difficult to target (Scott et al., 2016). However, GM enables gene-by-gene introduction of well-defined characteristics into breeding programmes, and this also allows for the access of genes from a greatly extended range of organisms since hybridization is not reliant on compatibility. As a result, the pool of variation is greatly increased and a much better accuracy is possible when each desirable trait is introduced, without the presence of

undesirable genes (Scott et al., 2016). Furthermore, through genetic engineering the release of unwanted variation that is an inherent feature of hybridization is avoided. In addition, the time required to breed cultivars with improved traits of interest is shortened (Ledford, 2015; Scott et al., 2016).

The first genetically transformed crop was planted in 1996, where 1.7 million hectares of crop were planted (James, 2015; Scott et al., 2016). By 2018, there were over 191.7 million hectares of transgenic crops in 26 countries, mainly of the four crops which include soybean, maize, oilseed rape (canola) and cotton (Scott et al., 2016; ISAAA, 2018, Sieradzki et al., 2021). Some of the genetic traits introduced into transformed crops until 2015 were resistance to insects from *Bacillus thuringiensis* and tolerance to the herbicide glyphosate. In addition to the four main crops others such as beans, eucalyptus, papaya, sugar beet and apple are currently being genetically modified with desirable characteristics such as, disease resistance, salt tolerance, drought tolerance, nitrogen-use efficiency, improved nutritional quality and storage quality (Scott et al., 2016).

Genetic engineering is a great technique for crop improvement, since the resultant genetically modified crop may possess one or more traits of interest, such as disease resistance, herbicide tolerance, insect resistance, abiotic stress tolerance and nutritional improvement (Kumar et al., 2020). The implementation of transgenic technology has been shown to improve crop yields, reduce insecticide and pesticide use, and reduce the cost of crop production (Kumar et al., 2020). However, the possibility of GM for crop improvement has caused much controversy. One of the many concerns raised is about the impact of GM on the environment, also the fact that these transgenic crops carry foreign genes may be a limitation due to concerns of allergenicity and potential toxicity to humans (Mertens, 2008; Key et al., 2008; Lovei and Bohn, 2010; Kumar et al., 2020). Limitations of genetically engineered crops include inadequate knowledge about inheritance of transgene and transgene slicing (Lakshmanan et al., 2005, Kumar et al., 2020). Approaches that can achieve a similar outcome as genetic transformation systems, and do not incorporate foreign genes into the plant, would notably aid in overcoming public and regulatory judgement issues (Arruda, 2011; Kumar et al., 2020).

Table 3: Examples of reported applications of genetic modifications for sugarcane.

Trait	Gene of interest	References
Herbicide tolerance: Glufosinate ammonium	*pat*	Leibbrandt and Snyman, 2003
Glyphosate	*cp4 epsps*	Snyman et al., 2001 Wang et al., 2017 Nogueira et al., 2019
Virus resistance: Sugarcane Mosaic Virus (SCMV)	SCMV coat protein	Meyer and Snyman, 2011 Yao et al., 2017
Insect resistance: *E. saccharina*	cry1A (c), cry1Ab	Meyer and Snyman, 2011 Wang et al., 2017 Gianotto et al., 2019

2.6.3 Tissue culture systems as an integral step in crop improvement

In vitro tissue culture is defined as the culture of plant cells, tissues and organs in a nutritive culture media and under a controlled aseptic environment (Thorpe et al., 2007; Trigiano and Gray, 2016; Espinosa-Leal et al., 2018; Diehdhiou et al., 2021). Such techniques are valuable for the rapid multiplication of genotypes of interest, production of disease-free plants, production of plant-derived metabolites of commercial importance and plant genome transformations (Debnath et al., 2006; Altpeter et al., 2016; Espinosa-Leal et al., 2018). Through *in vitro* tissue culture the totipotency of plant cells is manipulated to form a whole plant with a combination of auxins and cytokinins in the culture media (Singh, 2006; Getnet, 2017; Liang et al., 2019; Diehdhiou et al., 2021).

In sugarcane, as for most plant species, there are two individual developmental pathways that can be followed (Fig. 3), somatic embryogenesis and organogenesis, and both have well-proven protocols. The regeneration of plants through organogenesis can be successfully accomplished directly from their apical meristem with no intervening callus phase (Lee, 1987; Grisham and Bourg, 1989; Siddiqui et al., 1994; Lorenzo et al., 1998; Saini et al., 2004; Singh et al., 2006; Ramgareeb et al., 2010; Snyman et al., 2011), or adventitious organs emerging from callus originating from the explant (Grisham and Bourg, 1989; Burner and Grisham, 1995; Gill et al., 2006; Lakshmanan et al., 2006; Ali et al., 2007b; Snyman et al., 2011; Espinosa-Leal et al., 2018). Plant regeneration through direct organogenesis, is the preferred choice for

true-to-type clones as somaclonal variation is minimised (Lakshmanan et al., 2005). Indirect organogenesis is n ot commonly used because indirect somatic embryogenesis protocols are well established, as discussed below.

Somatic embryogenesis is a developmental process whereby a plant somatic cell dedifferentiates into a totipotent embryogenic stem cell that then, through cell division and differentiation gives rise to an embryo. Somatic embryogenesis can be obtained using a variety of explants and is usually practiced by exposing them to a medium with plant growth regulators, generally the synthetic auxin 2, 4-dichlorophenoxyacetic acid (2,4-D) (Heinz and Mee, 1969; Nadar et al., 1978; Ho and Vasil, 1983; Lee, 1987; Snyman et al., 2001; Franklin et al., 2006; Behera and Sahoo, 2009; Snyman et al., 2011; Feher, 2015; Horstman et al., 2017).

Direct embryogenesis is categorized by the absence of an intermediate callus stage and the embryos are formed directly on the explant (Williams and Maheswaran, 1986; Snyman et al., 2001; Horstman et al., 2017). Indirect somatic embryogenesis is characterised by the presence of an intermediate callus stage, an organized mass of initially vacuolated cells which show vast degrees of compactness and is regarded as the most common pathway (Snyman et al., 2001; Ikeuchi et al., 2013; Horstman et al., 2017; Espinosa-Leal et al., 2018). The production of embryogenic callus follows the formation of pro-embryogenic masses (PEMs) within or on the surface of the callus mass, from which cell clusters or single cells grow into embryos (Halperin, 1966; Toonen et al., 1994; Horstman et al., 2017). The exposure to auxins, especially 2,4-D promotes callus and PEM initiation and proliferation, however, for histogenesis and elongation of the embryo the auxin needs to be removed (Horstman et al., 2017).

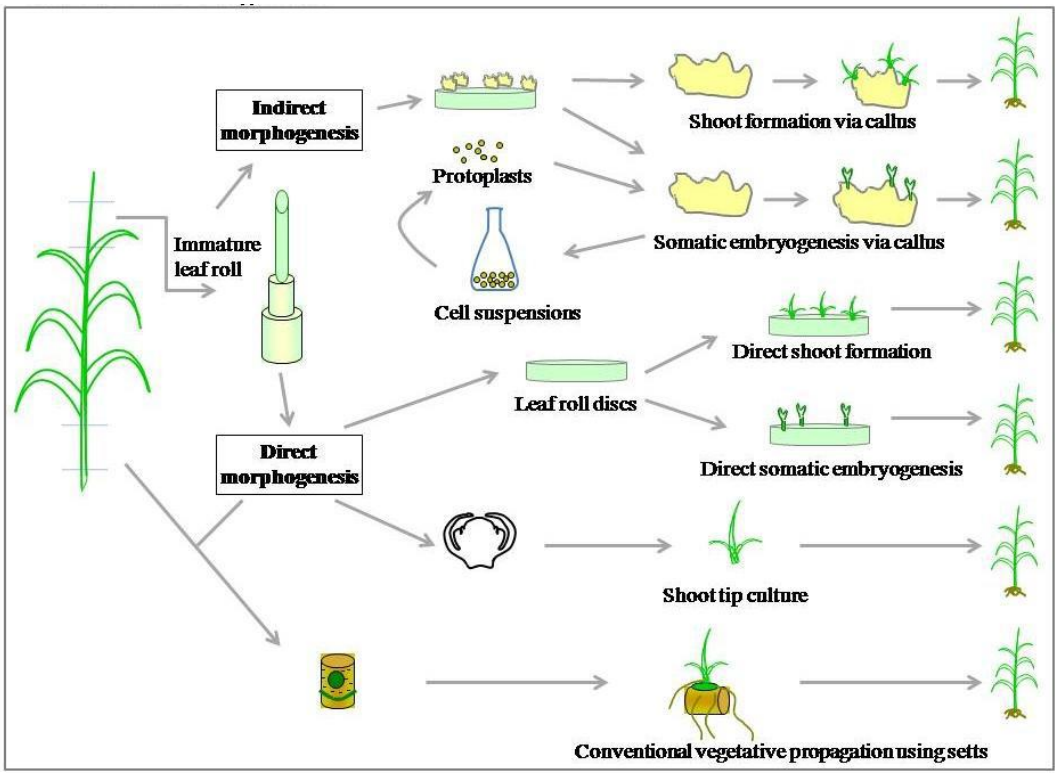


Figure 3: A depiction of the different routes of somatic embryogenesis and organogenesis by which sugarcane plants can be regenerated *in vitro* (Snyman, 2004).

2.6.4 *In vitro* induced somaclonal variation

Plant tissues that are cultured on medium supplemented with high concentrations of auxins, spend long periods in culture or many repeated subcultures may result in an undifferentiated mass of cells called callus, and later develops into plantlets, as mentioned in section 2.6.3. Plants regenerated via one of the two indirect routes have been reported to show a high frequency of variations (Bairu et al., 2011; Currais et al., 2013; Manchanda et al., 2018). There are several factors that may be responsible for inducing variability *in vitro* such as age of donor plant, explants source, genotype, *in vitro* culture environment (Silvarolla, 1992; Rastogi et al., 2015; Krishna et al., 2016), medium composition and plant growth regulator concentration (Skirvin, 1978; Silvarolla, 1992; Rastogi et al., 2015; Manchanda et al., 2018). Physiological variations are temporary, in response to a stimulus and disappear when it is removed (Rastogi et al., 2015). These induced modifications are best known as somaclonal variation and are defined as epigenetic/genetic changes that are observed among the plantlets regenerated under *in vitro* conditions (Larkin and Scrowcroft, 1981; Ramos et al., 1996; Rani and Raina, 2000; Rastogi et al., 2015; Manchanda et al., 2018). In 1981, Larkin and Scrowcroft were the first to describe somaclonal variation in *in vitro* cultured plants, after observing the resistance of *in vitro* plants that were previously susceptible to the toxin produced by *Helminthosporium sacchari* Butler that causes eyespot disease in sugarcane.

Somaclonal variation may be of three types, namely genetic, epigenetic, or physiological (Rastogi et al., 2015). Genetic variation occurs because of mutations or other changes in the DNA of the cell tissue, these are heritable in the next generation and essential for crop improvement (Larkin and Scrowcroft, 1981; Rastogi et al., 2015; Manchanda et al., 2018). Genetic variations are most useful to plant breeders since these variations are permanent, heritable, and stable during the sexual cycle (Larkin and Scrowcroft, 1981; Manchanda et al., 2018). Epigenetic variations are the physiological changes that are temporarily reversible (plants 'revert' to normal phenotype) and non-heritable (Silvarolla, 1992; Kaeppler et al., 2000; Rastogi et al., 2015; Manchanda et al., 2018). An epigenetic variation is also known as a developmental variation as it is induced during the various stages involved in the tissue culture process (Evans et al., 1984; Manchanda et al., 2018). These changes are non-heritable phenotypic variation.

Somaclonal variation has useful applications in sugarcane genetic improvement. Heinz (1973) was the first to report on *in vitro* screened somaclones of commercial sugarcane resistant to Fiji disease (Gill et al., 2007; Rastogi et al., 2015). Whilst somaclonal variation is undesirable when true-to-type clones are required (Jain, 2001), it may be used for plant improvement (Patade et al., 2006; Rutherford et al., 2014). As previously mentioned, breeding programmes take several years to produce plants with beneficial traits (Lakshmanan et al., 2005) (section 2.4). However, somaclonal variation has many advantages in helping conventional breeding by producing genotypes with desired traits much quicker (Raja et al., 2014; Rastogi et al., 2015). Somaclonal variation occurs by DNA methylation, point mutations, chromosomal rearrangement, and recombination, and altered sequence copy number, these changes usually take place during callus formation (Silvarolla, 1992; Jain, 2001; Khan et al., 2017, Manchanda et al., 2018). Hence, somaclonal variation allows for the production of genetic variants that may be exploited to generate phenotypes with valuable traits (Rutherford et al., 2014; Manchanda et al., 2018).

2.7 *In vitro* induced mutagenesis – Principles, types and applications of mutagens

Induced mutagenesis is defined as the exposure of chemicals or radiation to seeds and various plant explants to generate mutants with desirable traits (Mir et al., 2020). Mutations may be induced, or they may arise spontaneously (Jabeen and Mirza, 2004). It is regarded as one of the most useful techniques used in crop improvement to generate abiotic stress tolerant and disease resistant lines (Novak et al., 1992; Oladosu et al., 2016; Yasmeen et al., 2017; Mir et al., 2020) (Table 4). The limitations in case of traditional breeding, such as narrow gene

pool, low vigour, reduced fertility, complex genomes, and the lengthy breeding/selection cycle together hinders plant improvement and can be overcome via induced mutagenesis (Suprasanna et al., 2012; Penna and Jain, 2017). It is a cheap, simple and effective technique used to induce various mutations at the loci which controls economically important traits and/or eliminates undesirable genes present in elite breeding lines (Konzak, 2001; Yasmeen et al., 2017; Penna, 2017). More than a few desirable traits (e.g., high yield, quality, plant architecture, disease resistance and abiotic stress tolerance) can be found in a single induced mutant (Bughio et al., 2007; Penna and Jain, 2017; Andrew-Peter-Leon et al., 2021).

Mutagenesis can be carried out using various types of plant material, e.g., whole plantlets, seedlings, *in vitro* cultured cells like stem and leaf explants, tissues, organs, anthers, cell cultures, ovules, microspores, and protoplasts (Oladosu et al., 2016). The induction of mutations can be done on different plant material which are vegetative cuttings, *in vitro* cultured tissues like stem and leaf explants, anthers, cell cultures, calli, ovules, microspores, protoplasts (Oladosu et al., 2016).

Mutation breeding employs three approaches namely physical, chemical or biological agent for the improvement of agronomic traits in crops (Kharkwal and Shu, 2009; Forster and Shu, 2012; Roychowdhury and Tah, 2013; Oladosu et al., 2016; Penna, 2017; Penna and Jain, 2017). As stated by Mba (2013), it is notable that the dosage and rate of exposure or administration of the mutagens is directly related to the types and frequency of mutations (Oladosu et al., 2016).

Ionising radiation can be used to induce mutations, which occurs by the transfer of energy via ionisation or excitation, to sites present within or near the genetic material. This results in increased chemical reactivity of those sites (Sikora et al., 2011; Rutherford et al., 2014). There are various types of radiation used which include high-energy forms such as ultraviolet (UV) light, X-rays, gamma rays and fast-moving neutrons (Waugh et al., 2006, Sikora et al., 2011; Rutherford et al., 2014; Suprasanna et al., 2). There is direct damage since UV light is absorbed by pyrimidines in DNA, and this results in the formation of pyrimidine dimers (TT and CC). This is responsible for causing point mutations (Waugh et al., 2006; Rutherford et al., 2014). X-rays and Gamma rays can penetrate tissue and ionize molecules, radicals are formed, which cause breaks in phosphodiester bonds, resulting in point mutations and deletions (Sikora et al., 2011; Waugh et al., 2006; Rutherford et al., 2014). Nikam et al. (2015) used gamma radiation to generate mutants and salt-tolerant lines in sugarcane and some of which were characterized for various agro-morphological traits and some exhibited improved sugar yield and number of millable canes.

Chemical mutagens are known for inducing mostly point mutations, which are ideal for producing missense and nonsense mutations, thus resulting in a series of change-of-function mutations (Talebi et al., 2012; Purnamaningsih and Hutami, 2016). The most widely used chemical mutagens are sodium azide, EMS, hydrogen fluoride (HF), methyl methanesulfonate (MMS), hydroxylamine and N-methyl-N-nitrosourea (MNU) (Waugh et al., 2006; Sikora et al., 2011; Khalil et al., 2018). EMS (an alkylating mutagen) has been reported to be the most efficient and powerful mutagen in plants, since it creates a high frequency of point mutations and a low frequency of chromosome irregularity (van Harten, 1998; Purnamaningsih and Hutami, 2016; Espina et al., 2018). The alkylation of guanine to form O^2- ethylguanine which is then able to pair with thymine instead of cytosine is induced by EMS (Waugh et al., 2006). This results in the errors during DNA repair with the A-T pair replacing G-C, particularly during DNA replication (Waugh et al., 2006; Khalil et al., 2018). EMS mutagenesis produces base pair substitutions that result in altered forms of the triplet codon sequence within a protein sequence. This simply changes the physiological characteristics of the protein without stopping its function (Rutherford et al., 2014; Khalil et al., 2018; Dlamini et al., 2021).

In sugarcane, induced mutagenesis has been achieved by exposing embryogenic calli to EMS for a few hours because DNA replication is most rapid at this stage, therefore ensuring the highest probability of incorrect DNA repair (Rutherford et al., 2014). Of relevance to the present study, chemical mutagens were used to treat banana shoot tips to produce variants that were tolerant to *Fusarium* wilt (Jain 2010; Purnamaningsih and Hutami, 2016). Arici et al. (2017) had recently reported on disease resistance of *Fusarium* dry rot by *F. avenaceum*, this study tested the effect of EMS against *F. avenaceum* in potato.

5-Azacytidine (5-AzaC) is a known nucleotide analogue of 5-cytosine, which acts as a demethylating agent. This results in gene expression and activates silenced genes, thus increasing the frequency of somaclonal variation (Christman, 2002; Issa and Kantarjian, 2009; Grzybkowska et al., 2018). Methylation is important in controlling DNA and gene expression (Grant-Downton and Dickinson, 2005; Munsamy et al., 2013; Us-Camas et al., 2014). This takes place by adding a methyl group at the 5’ position of the pyrimidine ring of cytosine in DNA (Us-Camas et al., 2014). A gene becomes silenced or is no longer expressed when it has been methylated (Grant-Downton and Dickinson, 2005; Us-Camas et al., 2014). Mutations in DNA may also be created by chromosomal changes because of changes in methylation (Pooggin, 2013; Us-Camas et al., 2014). As sugarcane has a polyploid genome, a change in its methylation activity results in an increase in the frequency of somaclonal variation to obtain traits of interest (Munsamy et al., 2013). Many studies have shown that 5-AzaC behaves strongly as an epimutagen, as a result the frequency of changes induced by 5-AzaC is greater

than sequence-based DNA mutations (Fieldes and Amyot, 1999; Munsamy et al., 2013; Sun et al., 2021).

In mutation breeding the process of classifying individuals with a target mutation is the key point, which involves two main steps: mutant screening and confirmation (Forster and Shu, 2012; Oladosu et al., 2016). When individuals are selected from a large, mutated population that meet a specific criterion, e.g., disease resistance, and early flowering compared with the parent, this process is known as mutant screening (Mba, 2013; Oladosu et al., 2016). However, these are known as false or putative mutants. Mutant confirmation is defined as the process of re-evaluating the putative mutants under a precise and replicated environment using many samples. Many putative mutants are shown to be false mutants through this process. The mutations that are significant for the enhancement of crops usually involve single bases, which may sometimes alter protein synthesis (Mba, 2013; Oladosu et al., 2016). As summarized in Table 4, there has been success using induced mutagenesis to generate sugarcane mutants with desirable traits. Variations can be generated by using different types of mutagens, which induces several forms of mutations (Rutherford et al., 2014; Oladosu et al., 2016).

Sugarcane mutant plants regenerated and multiplied using *in vitro* techniques are acclimatised before being planted out in the field for comparison and assessment of specific beneficial agronomic features (e.g., tiller number, stalk height, fibre content and sucrose content) (Gilbert et al., 2005; Rutherford et al., 2014). However, comparison and assessment can be done on well-developed plants. Molecular and phenotypic assessments of mutant plants are required to confirm if the phenotypic traits of interests are enhanced and positively expressed whilst other traits have not been negatively impacted. To determine if plants obtained *in vitro* for clonal propagation are acceptable for commercial cultivation, the phenotypic traits are further evaluated by *ex vitro* screening trials (Mahlanza et al., 2013; Rutherford et al., 2014; Oladosu et al., 2016).

2.8 Epigenetic priming for pest resistance

2.8.1 Induced responses in plants to pest attack

Lepidopterans are known to be nocturnal insects that exploit the volatile chemical cues from the plants which allow them to orient themselves towards the host plants (Renwick, 1989; Renwick and Chew, 1994; Zakir, 2011). The preference of the female insects during the selection of appropriate host plants for oviposition is usually important for the survival of offspring in lepidopteran herbivores. The larvae of Lepidopterans are relatively immobile and

do not have a choice to find alternate sources of food, after hatching on plants selected by the female moths (Renwick, 1989; Renwick and Chew, 1994; Zakir, 2011). Immediately after hatching, the larvae begin to feed on the green leaves and stalks of the selected host plant robustly often causing a substantial loss of plant biomass (Pare and Tumlinson, 1996; Kost and Heil, 2008).

Secondary metabolites are produced efficiently by some plant species in response to feeding damage by herbivorous arthropods, e.g., both non-volatile and volatile chemicals. Several studies have shown these secondary metabolites as a requirement for increasing plant resistance against herbivores both at below-ground and above-ground levels (Pare and Tumlinson, 1996; Heil, 2004; Kost and Heil, 2006; Kost and Heil, 2008). When plants are exposed to a range of biotic and abiotic environmental factors it affects their development and growth unfavourably (Rasmann et al., 2012; Bertini et al., 2019). Consequently, plants have evolved sophisticated adaptation and defence mechanisms to protect themselves against a few of the harmful insects and pathogens (Rasmann et al., 2012; Pastor et al., 2014; Lämke and Bäurle, 2017; Bertini et al., 2019). Plants have developed various defence strategies, which are constitutive, but most are specific to a certain type of attack as they are induced in response to a stimulus (Frost et al., 2008; Lämke and Bäurle, 2017; Bertini et al., 2019).

Table 4: Examples of the use of induced mutagenesis for desirable traits in sugarcane

Trait	Characteristic	Mutagen used	References
Physiological	Drought tolerance	EMS	Dlamini et al., 2021
	Salt tolerance	EMS	Mallikarjuna et al., 2018
	Salt tolerance	Gamma radiation	Nikam et al., 2015
	Increased aluminium-tolerance	EMS	Purnamaningsih and Hutami, 2016
Disease resistance	Red rot disease	Sodium azide	Ali et al., 2007a
	Fusarium sacchari resistance	EMS, 5-AzaC	Mahlanza et al., 2013, 2014,
	Yellow leaf disease resistance	Sodium azide, sodium nitrite, EMS	Kona et al., 2019
Herbicide tolerance	Imazaypr tolerance	EMS	Koch et al., 2012
			Koetle et al., 2018
	Imazypr and smut tolerance	5-AzaC	Munsamy et al., 2013

Most plants respond to herbivory by releasing protective compounds which are lethal to the attacker. The plant's innate immunity controls these inducible defences (Jones and Dangl, 2006; Pastor et al., 2013; Bertini et al., 2019). Recent studies have revealed that plants may be driven to enter a post-challenged state, which allows for an effective and quicker defence response to abiotic or biotic stressors (Conrath et al., 2006; Bertini et al., 2019). Priming is a resistance mechanism, which results in a physiological state that triggers the plants to respond fast and/or robustly upon subsequent pathogen attack and various abiotic stresses, is known as priming (Conrath, 2011; Aranega-Bou et al., 2014, Martinez-Medina et al., 2016; Bertini et al., 2019). This 'primed state' can be stimulated by a pre-exposure of the plants to low doses of biotic and abiotic stress inducers either synthetic or natural compounds (Aranega-Bou et al., 2014; Conrath et al., 2015; Bertini et al., 2019). Hence, in addition to microbe-specific molecules, referred to as pathogen – (PAMPS), microbe – (MAMPS), and host-associated molecular patterns (HAMPS), endogenous plant signalling metabolites can be used to mimic priming. These metabolites are salicylic acid (SA), jasmonic acid (JA), hexanoic acid (Hx) and their useful analogues (Aranega-Bou et al., 2014; Conrath et al., 2015; Bertini et al., 2019). Gene induction is associated with no or minimal priming-increased alertness (Slaughter et al., 2012; Bertini et al., 2019) and may have a long-term effect (Pastor et al., 2013).

Plants are protected by the pattern-triggered immunity (PTI) against the common potentially destructive micro-organisms. However, a smaller group of infectious plant pathogens, have developed mechanisms to suppress PTI, by using defence-suppressing effector molecules that deregulate the signalling pathways that control plant innate immunity (Jones and Dangl, 2006; Llorens et al., 2017). At early stages of attack, plants have developed the capability to recognise virulent pathogens and counter a suitable defence response, as a co-evolutionary response (Pastor et al., 2013, Pritchard and Birch, 2014; Llorens et al, 2017). Effector-triggered immunity (ETI) is a familiar example of co-evolved plant defence, which allows the plant to detect the presence or activity of pathogen effectors, also known as gene for gene resistance (Boyd, 2006; Qi et al., 2011). Effector-triggered immunity requires resistance (R) genes that are found in the host plant, which can render specific virulent pathogens a-virulent (Boyd, 2006; Llorens et al, 2017). Pest and pathogen attacks are reduced by stimulating downstream responses that may result in a systemic and local induced resistance through PTI and ETI. These inducible defence responses include cell wall reinforcements through lignification and callose deposition, the accumulation of pathogenesis-related (PR) proteins, and the production of secondary antimicrobial compounds (Shah, 2003; Boyd, 2006).

2.8.2 Priming for resistance *in vitro*

The most decisive stage for a plant is to first identify that it is being attacked (Nimchuk et al., 2003; Pastor et al., 2014). The time it takes for the plant to sense and accurately recognise a specific stress(or) defines how successful and applicable its reaction will be. In case of pathogen attack, the failure to provide an apt response will result in colonisation of the host tissues and thereafter disease (Ebel and Cosio, 1994; Jones and Takemoto, 2004; Pastor et al., 2014). The colonisation process is slowed down by this basal immunity of the plant, however this may not be able to effectively prevent disease as it is too weak (Nürnberger and Lipka, 2005; Pastor et al., 2014). The level of basal resistance of a plant can be improved by the treatment of a suitable stimulus. This is known as induced resistance (IR) (Hammerschmidt, 2009; Pastor et al., 2014; Mladenov et al., 2021). Plants have attained the ability to extensively enhance their defence responses against a wide range of pathogens including fungi, viruses, bacteria, and oomycetes toward which they are genetically vulnerable (Hammerschmidt, 2009; Llorens et al., 2017). Plants are treated with several agents including plant extracts, cell wall fragments and synthetic chemicals, which has been found to induce resistance to subsequent pathogen attack both systemically and locally (Hammerschmidt, 2009; Walters and Fountaine, 2009; Llorens et al., 2017). Induced resistance can be divided into two main types, induced systemic resistance (ISR) and systemic acquired resistance (SAR) (Pieterse et al., 2014; Llorens et al., 2017). Some studies have shown that phytohormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), cytokinin, abscisic acid (ABA), auxins and brassinosteroids are the main role players in directing signalling networks that play a role in the adaptive response of plants to its biotic/abiotic environment (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012; Bertini et al., 2019).

Plants have established an alternate approach to cope with various virulent pathogens (Pastor et al., 2013). They have developed the ability to acclimatise to the antagonistic conditions in their environment by alerting their immune system in response to threatening signals. The preliminary phase of resistance induction, in which the plant prepares for a forthcoming attack, but has not yet been challenged by a pathogen, is known as the priming phase (Conrath et al*.,* 2002; Martinez-Medina et al., 2016; Mauch-Mani et al., 2017; Mladenov et al., 2021). Whilst in the primed state, the primed plant may react faster or more robustly to the triggering stress cue when compared with that of a plant in the naïve (unprimed) state (Fig. 4) (Conrath, 2009; Aranega-Bou et al., 2014, Hilke et al., 2016; Kerchev et al., 2020). There are no changes in the DNA sequence, since priming acts at the phenotypic level and is therefore reversible eventually (Conrath, 2009; Aranega-Bou et al., 2014; Hilke et al., 2016; Lämke and Bäurle, 2017). The plant must create and store information that will allow it to utilize a quicker response to stress during this time interval (Pastor et al., 2014, Lämke and Bäurle, 2017). A few stimuli

such as insect pests, avirulent pathogens, synthetic substances, metabolic disturbances of the plant and abiotic stressors, and microbe- and host-derived molecules have been found to induce the priming state (Conrath et al., 2006; Aranega-Bou et al., 2014; Lämke and Bäurle, 2017). This great variety in priming triggers suggests that several strategies may lead to the initiation of the primed state. Studies have shown some of these stimuli to target epigenetic mechanisms (Bruce et al., 2007). Epigenetic mechanisms play a significant role in the regulation of gene expression in response to environmental stress in plants (Boyko and Kovalchuk, 2008; Pontvianne et al., 2010; Sudan et al., 2018). The relation between epigenetic changes and priming is further supported by the occurrence of transgenerational priming, in which the progeny of primed plants show an enhanced defence response (Luna et al., 2012. Martinez-Medina et al., 2016; Mauch-Mani et al., 2017; Mladenov et al., 2021). The de-methylation patterns at the coding region of stress-responsive genes which regulates their expression can be modified by abiotic factors like temperature, day length, water, salt, UV and oxidative stress in plants (Beck et al., 2004; Pecinka et al., 2009; Boyko et al., 2010; Zemach et al., 2013; Fang et al., 2014; Xie and Yu, 2015; Sudan et al., 2018). These epigenetic modifications can be very specific to species, tissue, organelle, or age of an organism. To generate plants that have an insignificant effect to a stress stimuli, the mechanisms involved need to be examined carefully (Bhutani et al., 2011; Sudan et al., 2018).

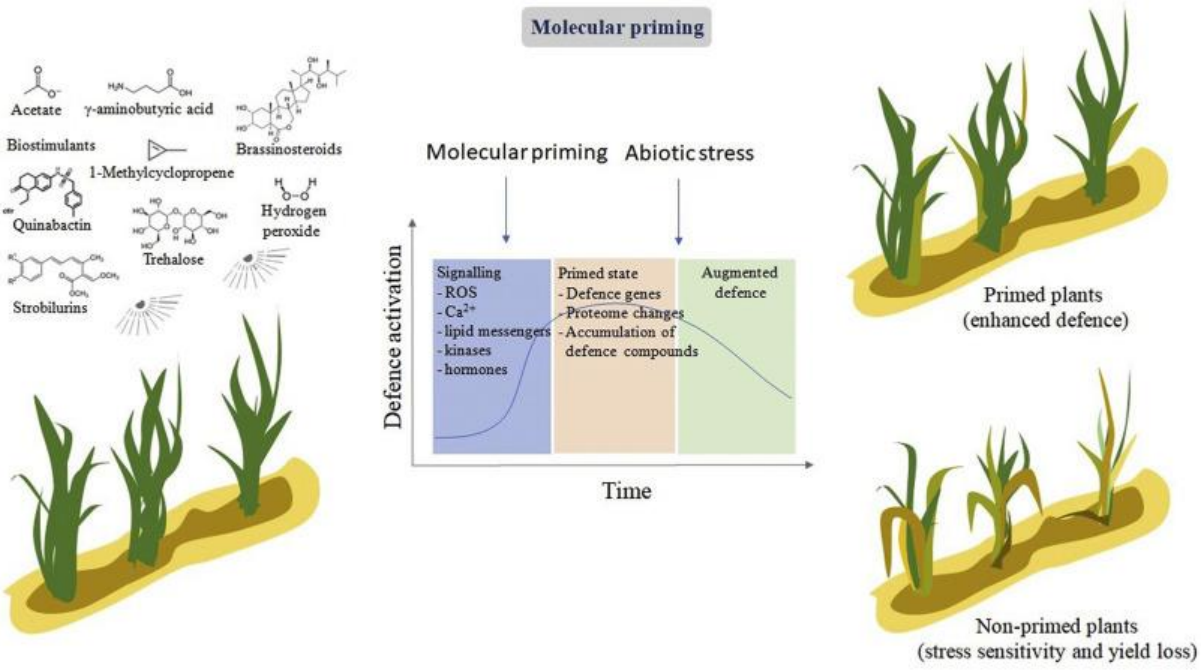


Figure 4: Illustration of plant defence activation by priming (Kerchev et al., 2020).

2.8.3 Priming-induced signals provide broad-spectrum plant protection

Plants are constantly challenged with a range of various pathogens and pests. These possible attackers apply various tactics to resist the plant defensive system (Balmer et al., 2012, Lämke and Bäurle, 2017). Pathogenic fungi can enter the host cell by breaking the cell walls, bacteria can enter plants through natural openings such as wounds or stomata, and insect herbivores use enzymes to reduce plant toxins (Balmer et al., 2012; Lämke and Bäurle, 2017). In plants, defence priming is activated by signals that indicate forthcoming attack by herbivores or pathogens (Balmer et al., 2012; Lämke and Bäurle, 2017). These defence-priming processes include systemic acquired resistance (SAR). This type of resistance is induced by localized necrotizing pathogen attack and initiates systemic priming of salicylic acid (SA) and pipecolic acid (PA)-inducible defence mechanisms (Jung et al., 2009; Pastor et al., 2013; Conrath et al., 2015). The defence-priming processes also comprises of induced systemic resistance (ISR), which is activated by fungi in the rhizophere and growth-promoting bacteria, the changes are associated with modulations of the jasmonate (JA) and ethylene (ET) pathways (Conrath et al., 2015). Hence, ISR is typically effective against pathogens that are controlled by JA-inducible defences (Ton et al., 2002; Pastor et al., 2013).

Most chemicals that induce priming are functional analogues or endogenous plant compounds. Hence, in response to biotic stress signals such as JA (Frost et al., 2008), SA (Kauss and Jeblick, 1995) and azelaic acid (Jung et al., 2009), the chemicals are synthesised by the plant (Pastor et al., 2013). Presently, is it thought that the resistance conferred by arbuscular mycorrhizal fungi is also linked to defence priming (Cameron et al., 2013; Conrath et al., 2015), wound-induced resistance (Chassot et al*.,* 2008) and β-aminobutyric acid-induced immunity (BABA-IR) (Jakab et al., 2001). β-aminobutyric acid (BABA) is a plant xenobiotic compound. It is a non-protein amino acid which can induce resistance in various plant species against a remarkably wide range of abiotic stresses (Pastor et al*.,* 2013). Moreover, BABA acts in an enantiomer-specific manner and is active at low concentrations (Cohen, 2002; Pastor et al*.,* 2013). Based on these characteristics, it has been suggested that BABA either ectopically activates an endogenous signalling compound that regulates several immune responses simultaneously, or that it simulates an endogenous plant signalling compound (Pastor et al., 2013).

2.9 Jasmonate signalling and manipulation by insects and pathogens

2.9.1 Signals in plant-microbe interactions

Multiple defence responses can be stimulated by a single priming-inducing treatment (Pastor et al., 2013). The response pathways of the central regulatory system of plant defence can be targeted by primed treatments, such as SA and JA (Conrath, 2011; Li et al., 2019). In general,

JA and ethylene (ET) signalling pathways are required for plant defence activation against herbivorous insects and necrotrophic pathogens (Penninckx et al., 1998; Li et al., 2019), whilst SA is a key regulator of defence against biotrophic and hemi-biotrophic pathogens (Pieterse et al., 2012; Campos et al., 2014; Caarls et al., 2015; Zhang et al., 2017). Plants encounter attacks by insects and pathogens with diverse modes of action, and they depend on crosstalk between various hormone signalling pathways to adjust suitable immune responses in contrast to these pathogens (Pieterse et al., 2012; Caarls et al., 2015; Zhang et al., 2017). The SA-JA antagonistic interaction has progressed as a significant strategy for plants to adjust their immune responses depending on the type of attack they experience (Pieterse et al., 2012; Zhang et al., 2017).

Jasmonates are produced from lipid components and sensed by protein receptors that trigger signal transduction pathways (Wasternack and Hause, 2013, Zhang et al., 2017; Wu and Ye, 2020). JA-induced plant defences affect insect herbivores, this includes leaf-eating insects, such as beetles and caterpillars, also piercing-sucking insects, such as leafhoppers, thrips and spider mites (Howe and Jander, 2008; Campos et al., 2014; Lu et al., 2015; Goossens et al., 2016; Zhang et al., 2017). Jasmonate signalling mediates plant defence against necrotrophic pathogens, fungal pathogens such as *Fusarium oxysporum, Plectosphaerella cucumerina, Botrytis cinereal, Alternaria brassicicola*, as well as the bacterial pathogen *Pectobacterium atrosepticum* (Campos et al., 2014; Yan and Xie, 2015; Zhang et al., 2017). In addition to the important role JA plays in plant defence, it also provides a significant role in plant reproduction and other developmental and growth processes, this includes adventitious and lateral root formation, leaf senescence, seed germination, nectaries, and resin ducts, and glandular trichomes formation (Wasternack and Hause, 2013; Campos et al., 2014; Kazan, 2015; Wasternack and Strnad, 2016; Zhang et al., 2017; Wu and Ye, 2020). However, these glandular trichomes, nectaries and resin ducts can generate compounds that are either directly or indirectly involved in plant defence, which connects the dual roles of JA in defence and development (Dicke and Baldwin, 2010; Campos et al., 2014; Zhang et al., 2017).

Jasmonate derivatives have been found to induce the biosynthesis of different defensive chemicals and proteins to counter pathogens and herbivores by functioning as chemical triggers (Wu and Ye, 2020). It is extensively accepted that herbivore and pathogen attacks are linked to a variety of herbivore-associated molecular patterns (HAMPs) such as insect secretions, microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) such as flagellin, and damage-associated molecular patterns (DAMPs) such as systemin or systemin-like peptides and plant cell wall-derived oligogalacturonides (Felton and Tumilson, 2008; Mithöfer and Boland, 2008; Hogenhout and Bos, 2011; Yamaguchi and Huffaker, 2011;

Campos et al., 2014; Heil and Land, 2014; Zhang et al., 2017). The plant pathogen recognition receptors (PRRs) that are positioned at the plant plasma membrane can recognise this attacker associated patterns and thus initiate JA signaling-dependent resistance (Qi et al., 2006; Song et al., 2006; Yamaguchi et al., 2006; Brutus et al., 2010; Yamaguchi et al., 2010; Mousavi et al., 2013; Choi et al., 2014; Zhang et al., 2017; Shi et al., 2019; Wu and Ye, 2020). In numerous genome-wide transcriptome studies, a substantial overlap of gene expression and genes involved in defence hormone signalling was detected across PAMP, HAMP, and DAMP responses (Campos et al., 2014; Zhang et al., 2017). Following the perception of cellular calcium flux and damage signals, the mitogen-activated protein kinase (MAPK) cascades are activated which intensifies the signaling from local to systemic leaves and from the cytosol into the nucleus. The sets of defensive gene expression is transcriptionally reprogrammed at this site JA and its derivatives (Wu and Ye, 2020).

The oxylipin biosynthesis pathway synthesizes JA and the pathway starts with α-linolenic acid that is released in the chloroplastic membranes following an insect or pathogen attack (Wasternack, 2007; Gfeller et al., 2010). This results in catalysis, the α-linolenic acid is processed by allen oxide synthase (AOS) and lipoxygenase (LOX), allene oxide cyclase (AOC) and generated in the chloroplast. The 12-oxo-phytodienoic acid (OPDA) is then transported into the peroxisome, where (+)-7-iso-JA is synthesized because of several cycles of β-oxidation (Wasternack and Hause, 2013; Larrieu and Vernoux, 2016; Zhang et al., 2017). This is followed by secretion into the cytosol, thereafter (+)-7-iso-JA is coupled with the amino acid isoleucine (Ile) to produce JA-Ile, known as the most bioactive form of JA (Fonseca *et al*., 2009; Zhang *et al*., 2017). The JA cytosolic pool is converted into JA metabolites, through carboxylation and/or hydroxylation, to attenuate JA signalling (Kitaoka et al., 2011; Koo et al., 2011; Koo and Howe, 2012; Zhang et al., 2017).

2.9.2 Activation and suppression of JA signalling for fungal pathogens

JA production is a standard feature in many plant-interacting fungal pathogens or symbionts, (Gimenez-Ibanez et al., 2016; Goossens et al., 2016; Zhang et al., 2017). For example, 22 JA and JA-related compounds were found in the *F.oxysporum (Fo)* f. sp *mattiole* culture filtrate (Miersch et al., 1999a, Zhang et al., 2017), and JA biosynthesis has been noted in *Aspergillus niger*, *Laccaria laccata*, *Pisolithus tinctorius* and *Lasiodiplopdia theobromae* (Miersch et al., 1999a; Miersch et al., 1999b; Tsukada et al., 2010; Zhang et al., 2017). However, the production of JA has only been described in plant-interacting fungi and this indicates that these fungi may have developed the ability to produce JA over time to colonise its host plants (Goossens et al., 2016; Zhang et al., 2017). A few of the fungal pathogens found in *Arabidopsis*, namely *F. oxysporum* f. sp. *matthioli* (Fomt) and *F. oxysporum* f. sp *conglutinanas*

(Focn) produce JA, JA- Leu and JA-II, and exhibit reduced virulence in the coi1 mutant (Cole et al., 2014; Zhang et al., 2017), this indicates that Fo infection is promoted by JA signaling (Zhang et al., 2017).

In contrast to hemi-biotrophic (a parasitic organism that invades living cells before tranisitioning to a necrotrophic state) and biotrophic pathogens (fungi that require their nutrients from a living host), necrotrophic pathogens (fungi that secretes toxins to kill the host plant and use the dead plant tissue as a source of nutrients) (Rajarammohan, 2021) and chewing insects suppress JA signaling for their success in host plants. One of the strategies used by the fungal pathogens is to decrease JA accumulation following infection, either by accelerating JA catabolism or by blocking JA biosynthesis. Otherwise, SA-JA antagonism may be utilised for suppression of JA-mediated defence (Zhang et al., 2017). A few fungal species has evolved the ability to break down JA (Patkar et al., 2015; Zhang et al., 2017). It has been reported that, for example the rice blast fungus *Magnaporthe oryzae* produces the antibiotic biosynthesis monooxygenase (Abm) and converts both fungal and plant-derived JA into 12OH-JA to facilitate host colonisation and indicate JA signaling (Patkar et al., 2015; Zhang et al., 2017).

Just as the pathogenic bacteria found in biotrophs and hemi-biotrophs activate JA signalling to inhibit SA signalling, necrotrophic pathogens can manipulate SA-JA antagonism to suppress JA-mediated defence responses. The fungus *Botrytis cinerea* produces an exopolysaccharide (β-(1,3) (1,6)-D-glucan) that stimulates the accumulation of SA and antagonistically suppresses JA-response gene expression, including that of proteinase inhibitors I and II (PI I and PI II) (El Oirdi et al., 2011; Zhang et al., 2017). Proteinaceous effectors are also secreted by pathogens and symbionts to suppress JA signaling. For example, the necrotrophic pathogen *Sclerotinia sclerotiorum* produces an intergrin-like (SSITL) protein, which is responsible for suppressing JA-ET signaling mediated resistance at the initial stage of infection (Zhu et al., 2013; Zhang et al., 2017).

2.9.3 Cis-jasmone and hexanoic acid applications for insect resistance

Jasmonates and its by-products, collectively known as jasmonates (JAs), are pervasive plant regulators (Ahmad et al., 2016). Apart from JAs and methyl jasmonates (MeJAs), other JAs particularly jasmonoyl ACC (JA-ACC), cis-jasmone, and jasmonoyl isoleucine (JA-Ile) with numerous biological functions have been mentioned (Ahmad et al., 2016). Additionally, a wide range of JA-induced plant growth, physiological and developmental activities has been reported. This includes biotic and abiotic stress tolerance, fertility, root elongation, storage organ formation, interaction with other hormones, and oxidative defence (Cipollini, 2010, Nafie

et al., 2011). Various other physiological functions related to JA responses include the accumulation of storage proteins, flowering, seed development (Wasternack et al., 2012), herbivory (Erb et al., 2012), wounding (Erb et al., 2012) and systemic resistance (Pieterse et al., 2002, 2012). Various studies have reported on its role in gene expression in different plants such as *Arabidopsis* (Sasaki et al., 2001), sugarcane (Nogueira et al., 2005), rice (Liu et al., 2012), tomato (Boter et al., 2004), which results in defence against environmental stresses (Ahmad et al., 2016).

Necrotrophic pathogen infection leads to the activation of JA dependent defence responses (Avanci et al., 2010). Plants treated with jasmonate have shown improved resistance against pests. Studies have shown that tomato plants treated with JA exhibit reduced numbers of *Frankliniella occidentalis* (thrips), aphids and flea beetles, which were attributed to an increase in the activities of proteinase inhibitors and polyphenol oxidase (Thaler et al., 2001). As reported in *Arabidopsis* and spinach (McConn et al., 1997; Schmelz et al., 2002). Studies on strawberry reported on the reduction of the adverse effects of oxidative and drought stress by MeJA (Wang, 1999). Barley seeds that were pre-treated with MeJA have shown reduced membrane damage than that of the non-treated plants (Bandurska et al., 2003).

According to Kravchuk *et al.* (2011), treatment with Hx was found to protect *Arabidopsis* plants against necrotrophic fungi *B. cinerea.* Similar findings were reported on tomato plants, the roots were treated with Hx, and resulted in protection against *B. cinerea* (Aranega-Bou et al*.,* 2014). The 4-week-old plants root treatment with Hx at concentrations below 1 mM for 48 h before infection considerably reduced the incidence of the disease, as did other non-natural (BABA) and natural (SA) compounds (Vicedo et al., 2009; Aranega-Bou et al*.,* 2014). These concentrations of Hx have shown no antimicrobial effect on the fungi, reduced conditioning times were not suitable for protection of the plant against this pathogen. In addition, it was suggested that plant protection may result from interactions with plant defense systems since there was no accumulation of Hx in the aerial part of the plant (Vicedo et al., 2009; Aranega-Bou et al., 2014). Callose accumulation was induced by Hx treatment upon infection by *B. cinerea* (Kohler et al., 2002; Aranega-Bou et al*.,* 2014). Cell wall fortification by the deposition of callose is an important component of resistance induced by various chemical inducers like benzo (1,2,3) thiadiazole-7-carbothioicacid *S*-methylester (BTH) and BABA (Aranega-Bou et al*.,* 2014).

Improved resistance against necrotrophs *Alternaria alternata* in Fortune mandarin (Llorens et al., 2013; Aranega-Bou et al., 2014; Llorens et al., 2016) and *A. brassicicola* in *Arabidopsis* (Kravchuk et al., 2011; Aranega-Bou et al*.,* 2014) has been noted with the use of Hx. In this

case, both callose priming and JA-signaling were necessary for Hx-IR. In addition, a rapid accumulation of abscisic acid (ABA) was observed, which could act as a positive regulator of callose deposition, thus supporting the fact that both the JA-signaling pathway and enhanced physical barriers are involved in Hx-IR against necrotrophic pathogens (Aranega-Bou et al., 2014). Hexanoic acid has also been used for inducing defence responses in tomato plants against *Botrytis cinerea*, tomato plants against *Pseudomonas syringae* (Leyva et al., 2008; Vicedo et al., 2009; Scalschi et al., 2013), tomato plants against *Phytophthora citrophthora* and *Alternaria solani* (Flors et al., 2003), and *Cucumis melo* against Melon necrotic ringspot virus (Fernández-Crespo et al., 2017). There is increasing evidence that focuses on seed priming, foliar spraying and hydrophonic solutions to induce priming (Levya et al., 2008; Scalschi et al., 2013; Llorens et al., 2016)

2.10 Molecular and phenotypic assessment of mutated plants

A wide variety of strategies are available for the characterization and detection of somaclonal variants which are based on the differences in morphological traits (Nhut et al., 2013; Krishna et al., 2016), biochemical (Kar et al., 2014, Krishna et al., 2016), molecular DNA markers (Martínez-Estrada et al., 2017) or their combinations (Dey et al., 2015; Krishna et al., 2016). Somaclonal variation can be achieved by point mutations, changes in chromosome number, somatic crossing over, somatic gene rearrangement, DNA methylation, histone modifications and RNA interference and epigenetic variation (Krishna et al., 2016). Molecular markers (DNA and protein based) are consistent and advantageous as they recognise internal changes that contain a genetic origin (Idrees, 2014). Some examples of DNA markers used for analysis include Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP), Inter Simple Sequence Repeats (ISSR) and Random Amplified Polymorphic DNA (RAPD) (Jiang, 2013; Nadeem et al., 2018).

Plant species that have been generated through somaclonal variation have been successfully released with improved traits including disease resistance, appearance, yield, abiotic stress tolerance and plant architecture (Anil et al., 2018). Some examples of plant species include maize (*Zea mays* L.) with improved grain quality and, a wheat variant (*Triticum aestivum* L.) with high yield (Anil et al., 2018). A sweet potato cultivar generated through somaclonal variation is comparable to the parent cultivar in disease resistance and yield, however it shows a more desirable darker and more stable skin colour (Moyer and Collins, 1983; Anil et al., 2018).

3. Materials and Methods

3.1 Plantlet regeneration using indirect somatic embryogenesis

3.1.1 Plant collection and preparation of material

Sugarcane plants of cultivars N41 and 88H0019 were harvested from the field at the South African Sugarcane Research Institute (SASRI), in Mount Edgecombe, Kwa-Zulu Natal (coordinates: 30 °S, 31 °E). They were selected based on their field ranking for *E. saccharina* tolerance, with 88H0019 and N41 being susceptible and resistant, respectively (Zhou, 2013). The preparation procedures for plant collection followed Snyman et al. (2006). The immature leaf rolls of the sugarcane stalk were surface decontaminated by wiping with 100 % (v/v) ethanol and a 30 cm section was cut transversely on the adaxial end. Thereafter, they were placed in a beaker with 70 % (v/v) ethanol prior to culture initiation in the laminar flow.

3.1.2 Callus initiation and embryo maturation

Callus initiation followed the protocol described by Snyman et al. (2006). The outer leaf layers of the 30 cm stalk apices were aseptically removed using a sterile scalpel until the inner immature leaf roll was visible (Fig. 5a). After each leaf sheath was removed the leaf rolls were swabbed with 70 % (v/v) ethanol. The inner leaf roll was cut transversely into thirty discs (approximately 2 mm thick) which were placed into Petri plates containing liquid embryo initiation medium (EIM) to remove phenolic exudes from the cut ends. Embryo initiation medium contained MS basal salts and vitamins (Murashige and Skoog 1962, Duchefa, Belgium), 20 g l^{-1}sucrose, 3 mg l^{-1} 2,4-D, pH 5.6 - 5.8, autoclaved at 121 ºC for 20 minutes. Thereafter, 10 leaf discs were cultured per 90 mm Petri dish (Concorde Plastics, Pty Ltd, RSA) with the adaxial surface in contact with semi-solid EIM [25ml semi-solid EIM (as described above) with 8 g l^{-1} agar (Neogen, Acumedia, USA)] (Fig. 5b). The plates were sealed with parafilm (Lasec SA) and maintained in the dark at 26 ± 1 °C for 3 weeks, with daily observation for microbial contamination. The cultures were sub-cultured on to fresh medium every 2 weeks.

After 8 weeks on EIM (Fig. 5b), 0.2 g of embryogenic calli were weighed using sterile aluminium weighing boats and placed on to Petri dishes containing 25 ml semi-solid embryo maturation medium (EMM) (Fig. 5d). The media composition for EMM was full strength MS with vitamins, 20 g l^{-1} sucrose, 1 mg l^{-1} 2,4-D and 8 g l^{-1} agar. The plates were sealed with parafilm and incubated in the dark at 26 - 27 °C for 3 weeks.

3.1.3 Embryo germination and plantlet regeneration

The embryogenic calli were transferred to 90 mm Petri dishes containing 25 ml embryo germination media (EGM1; EIM without 2,4-D) (Fig. 5e). The pieces of calli were evenly spread on the media, sub-cultured twice monthly, and incubated for 4 - 8 weeks at 16 h light (26 °C), 200 µm m^{-2} s^{-1} photon flux density (PPFD) and 8 h dark (23 °C) photoperiod. The regenerated plantlet clusters were divided and single rooted plantlets of about 20 mm in height were transferred to Magenta® vessels (Sigma-Aldrich, USA) with 100 ml EGM2 (20 plants per vessel). Embryo germination medium (EGM2) contained half strength MS basal salts and vitamins (Murashige and Skoog 1962, Duchefa, Belgium), 10 g l^{-1}sucrose, 8 g l^{-1} agar and pH 5.6 - 5.8, autoclaved at 121 ºC for 20 minutes.

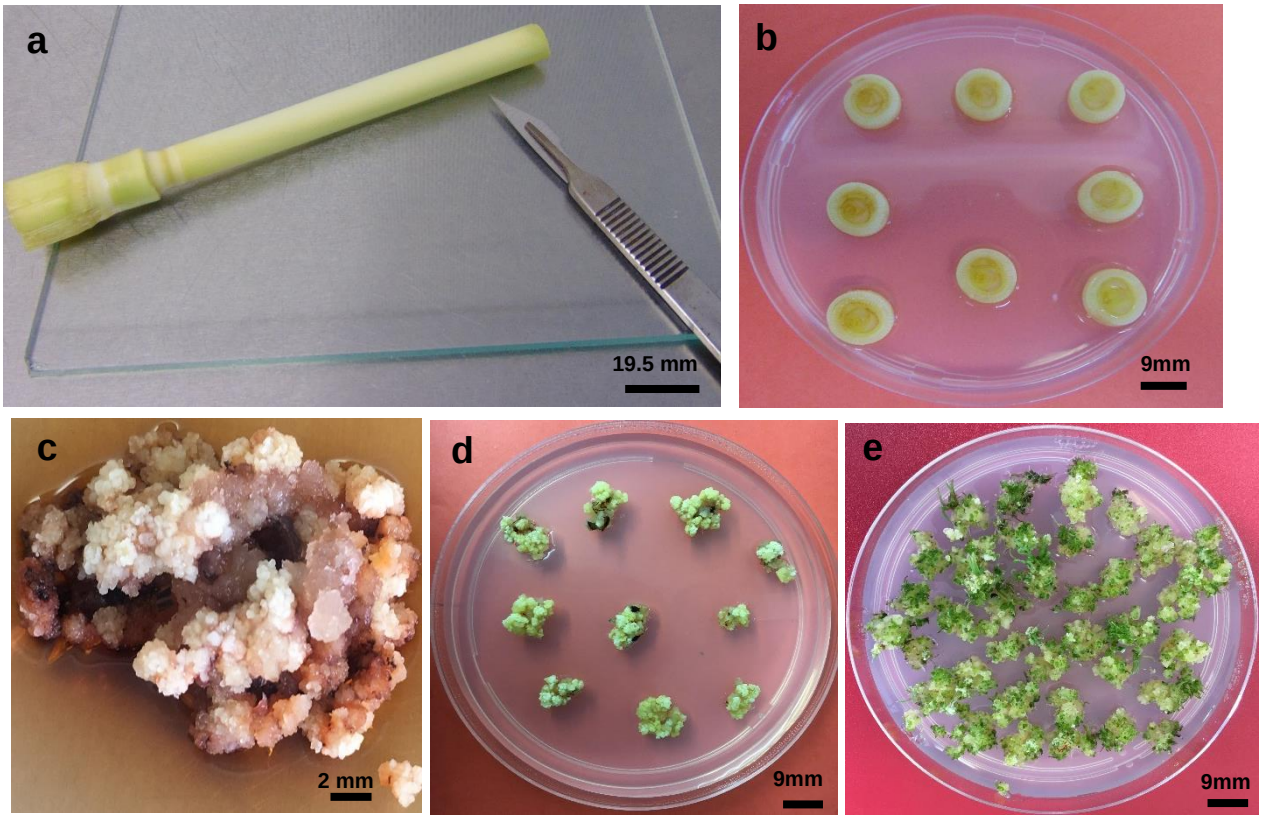


Figure 5: Photographs showing plantlet regeneration via indirect somatic embryogenesis from embryo initiation to germination stages: a) sugarcane leaf roll, b**)** leaf sections on embryo initiation medium (EIM), c) white compact embryogenic callus after 8 weeks, d) embryogenic callus on embryo maturation medium (EMM), and e) plantlet regeneration on embryo germination medium (EGM1).

3.1.4 Acclimatisation

Dead leaves were removed from *in vitro* plantlets and the remaining green leaves trimmed to above the growing point and placed into Magenta® vessels with 80 ml semi solid media (Fig. 6a). The plantlets were rinsed with distilled water to remove the media. The control plants and putative *Fusarium* mutants were hardened off *ex vitro* by planting in polystyrene seedling trays [(670 x 330 mm; (Hygrotech, Pretoria, SA)] containing a mix of vermiculite (Coastal Farmer's Co-op, Umhlali, SA) and peat moss (Nirom, Alberta, Canada) (1:1 v/v) (Fig. 6b). The plants were placed in the glasshouse at 20/34 °C (night/day temperature) and were watered using overhead sprinklers for 1 min twice a day and fertilized every 2 weeks with either N:P:K 5:1:5 pellets (Coastal Farmer's Co-op, Umhlali, SA) or Trelmix, 1 ml l^{-1} (Hubers (Pty) Ltd, Howick, SA) alternating bi-monthly for 5 - 6 months.

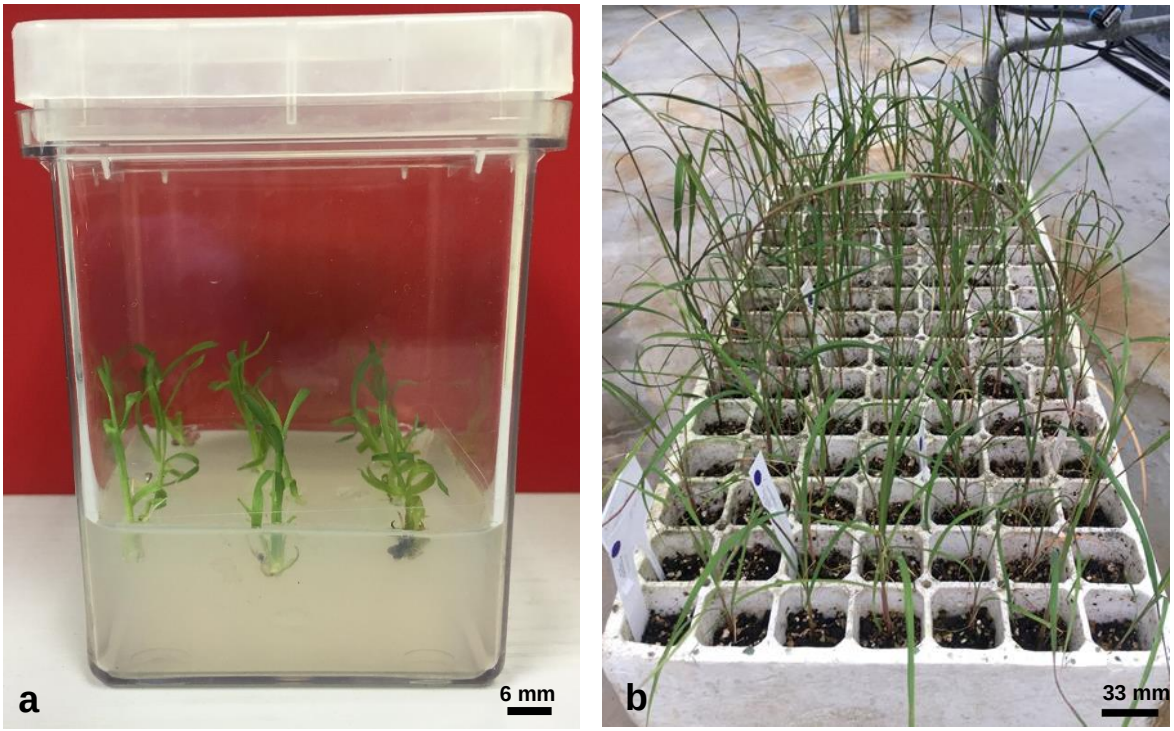


Figure 6: Photographs showing plantlet acclimatisation. a) Plantlet growth in Magenta® vessel, and b) Plantlet acclimatisation in seedling tray.

3.2 Establishment of suitable concentrations of priming agents at the callus and plantlet stages for plantlet production

After 8 weeks, embryogenic calli were exposed to two priming agents: hexanoic acid and cis-jasmone at the embryo maturation and germination stages to determine an effective priming agent of the two, and a suitable concentration at each stage. For the control, embryogenic calli were cultured on media without priming agents.

The embryogenic calli were produced as mentioned previously (section 3.1.2). At the embryo maturation stage, EMM (section 3.1.2) was supplemented with various concentrations of *cis*-jasmone (0, 0.2, 1, 5 or 10 µM) or hexanoic acid (0, 0.2, 0.6, 2 and 6 mM) in 90 mm Petri dishes for 3 weeks. The concentration range for cis-jasmone and hexanoic acid were according to Nieves et al. (2001) and Kravchuk et al. (2011) respectively. The cultures were incubated in the dark at 26 - 27 °C. Embryogenic calli were then transferred on EGM1 (section 3.1.3) containing the above-mentioned concentrations for the priming agents. The cultures were placed in the photoperiod growth room (section 3.1.3) for 4 - 12 weeks, sub-culturing every 2 weeks on EGM1 containing priming agents. The proportion (%) callus necrosis and number of plantlets produced per 0.2 g calli were recorded between 8 - 12 weeks. The most suitable priming agent at its effective concentration was selected for the ensuing *in vitro* mutagenesis and priming manipulations for *E. saccharina* tolerance.

3.3 *In vitro* mutagenesis and priming for *E. saccharina* tolerance

3.3.1 *In vitro* protocols that involved chemical mutagenesis and epigenetic priming for *E. saccharina* resistance

The experimental design for the exposure of plant tissue from two sugarcane varieties to the two tested mutagens, 5-AzaC (Sigma-Aldrich, St Louis, USA) and EMS (Sigma-Aldrich), and to the priming agents hexanoic acid (Sigma-Aldrich, St. Louis, USA) and cis-jasmonic acid (Sigma-Aldrich, St. Louis, USA) is illustrated in Fig. 7. The mutagenic protocol for the exposure of calli to EMS and 5-AzaC was that reported by Mahlanza et al. (2013). Hexanoic acid was selected as the most effective priming agent at 0.6 mM than cis-jasmone, in terms of callus production and plantlet yield.

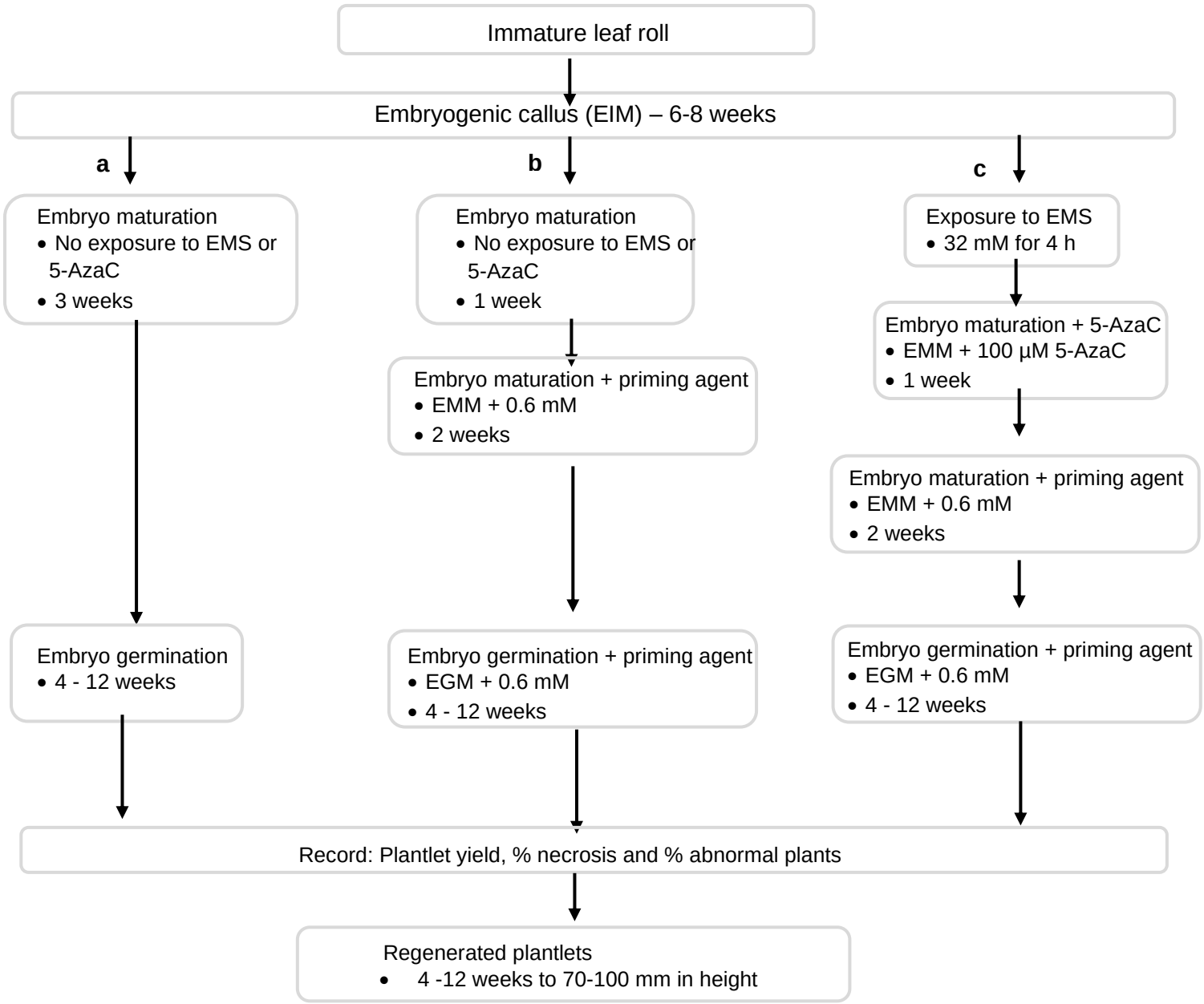


Figure 7: Experimental design for exposure of calli to priming agents, ethyl methanesulfonate (EMS), and 5- Azacytidine (5-AzaC)

a) 5-Azacytidine treatment of calli

A 100 µM stock solution of 5-AzaC was prepared and filter-sterilized using a 0.8/0.2 µm Acrodisc PF syringe filter (Pall Corporation, USA) in a fume-hood cupboard (Munsamy et al., 2013). The pieces of callus (0.2 g) obtained after 3 weeks on EIM as mentioned in section 3.1.2 were transferred to EMM containing 100 µM 5-AzaC (4 pieces per Petri dish). The medium was maintained at 45 °C before pouring the media and to avoid 5-AzaC degradation it was used immediately after preparation. After 1 week, the 0.2 g pieces of calli were transferred to standard EMM containing either one of the priming agents as mentioned below (section 3.3) for 2 weeks for callus recovery.

b) Treatment of embryogenic calli with ethyl methanesulfonate

A 1M EMS stock solution was prepared in the fume hood in a 50 ml Corning tube (Corning, Massachusetts, USA). Under aseptic conditions, the stock solution was filter sterilised by using a 0.22 µm syringe filter (Pall Corporation, Michigan, USA). The stock solution was used to prepare 10 ml of a 32 mM EMS solution (Koch et al., 2012) in 15 ml Corning tubes by diluting with liquid EMM. White embryogenic calli (0.2 g) were placed into Corning tubes containing the EMS solution for 4 hours (Fig. 7), and the tubes were gently inverted every 30 - 45 min. For the control, calli were transferred to liquid EMM with no EMS for 4 hours. Thereafter, the EMS solution was decanted and the calli were rinsed three times using liquid EMM and then placed on sterile filter paper to soak away the excess media.

c) Combination of EMS, 5-AzaC and Hx treatments

In the first protocol (Fig. 7a), embryogenic calli were established for 6 - 8 weeks, as described previously (section 3.1.2). Small pieces of white embryogenic calli were weighed out in the laminar flow and 0.2 g of calli were placed (4 pieces/Petri plate) on 25 ml of EIM for 3 weeks. There was no exposure to EMS or 5-AzaC. The calli were incubated in the dark at 26 ± 1 °C for 2 weeks. After 3 weeks, the calli were then cultured on EGM1 media without priming agents, as described in section 3.1.3 for each treatment. The cultures were placed in the photoperiod growth room (section 3.1.3) for 4 - 12 weeks, sub-culturing every 2 weeks. The number of plantlets per 0.2 g callus was recorded after 4 - 12 weeks because plant regeneration only took place after 4 weeks in culture. Percentage callus necrosis (a piece of callus was considered necrotic if > 50 % appeared brown/black) was recorded for all treatments.

In the second protocol (Fig. 7b) the embryogenic calli were placed on EMM media without mutagens for 1 week (Fig. 7). The calli were then exposed to the priming agent at both the callus maturation and the germination stages. The white embryogenic calli (0.2 g of callus) were then cultured on Petri plates containing 25 ml EMM (section 3.1.2) with 0.6 mM hexanoic acid for 2 weeks. The calli were incubated in the dark at 26 ± 1 °C for 2 weeks. The calli were then transferred to EGM1 containing the same concentration of hexanoic acid. The cultures were placed in the photoperiod growth room, as described in section 3.1.3 for 4 - 12 weeks, sub-culturing every 2 weeks on EGM1 containing the priming agent. The number of plantlets produced and callus necrosis per 0.2 g calli were recorded between 8 - 12 weeks.

In the third protocol (Fig. 7c) the embryogenic calli obtained after 6-8 weeks were exposed to both mutagens. White embryogenic calli (0.2 g of callus) were exposed to 32 mM EMS for 4 hours and then placed on embryo maturation media containing 100 µM 5-AZA for 1 week, as described in section 3.2. The calli were then cultured on Petri plates containing 25 ml EMM (section 3.1.2) with 0.6 mM Hx for 2 weeks. The calli were incubated in the dark at 26 ± 1 °C for 2 weeks. The surviving calli were then cultured onto EGM1 containing the same concentration of Hx. The cultures were placed in the photoperiod growth room (section 3.1.3) for 4 - 12 weeks, sub-culturing every 2 weeks. Callus necrosis and number of plants per 0.2 g callus were recorded after 12 weeks.

3.4 *Fusarium pseudonygamai* culture and filtrate preparation

3.4.1 Fungal culture and storage conditions

Cultures of *F. pseudonygamai* SC17 were obtained from stock cultures from the Pathology laboratory at SASRI. A colony from each of SC17 and PNG40 were grown on potato dextrose agar (PDA; Biolab, Wadeville, RSA) to produce cultures for storage. After 5 days, mycelial squares (5 x 5 mm) were transferred to Eppendorf microfuge tubes containing 1 ml of 15 % (v/v) glycerol solution (Merck, Wadeville, SA) and stored at - 80 °C. To prepare starter cultures for each isolate, a thawed mycelial square stored in 15 % (v/v) glycerol was placed on PDA for 3 days at 30 °C in an incubator. A 5 x 5 mm mycelial square was excised from the leading edge of the resultant fungal colony and transferred to Erlenmeyer flasks each containing 250 ml of potato dextrose broth (PDB) (Fluka, St Louis, USA). To allow for culture growth, the neck of the flask was plugged with a cotton wool bung, covered with aluminium foil (autoclaved prior to use) and agitated in a shaking incubator at 145 rpm and at 28 - 30 °C for 7 days.

3.4.2 Culture filtrate preparation

The culture filtrate preparation protocol was done according to Mahlanza et al. (2013). The 7-day-old liquid fungal culture was transferred into a sterile 250 ml centrifuge tube and centrifuged at 12 000 rpm for 5 min (Heraeus Megafuge 40R, Thermo Scientific). The supernatant was filtered through a sterile muslin cloth to collect the mycelia, the fresh mass of the mycelia was recorded immediately, and its dry mass was determined after incubation at 80 °C for 24 h. The culture filtrate was sequentially filtered through Whatman No. 1 filter paper, 0.45 µM membrane filter (Sartorius Stedim Biotech, Germany), a sintered glass filter unit (Millipore) using a vacuum pump and a 0.2 µM syringe filter (Millipore, Ireland). The culture filtrate (CF) was stored for a maximum of 24 h at 4 °C (Mahlanza et al., 2013). The CF concentration for each batch was expressed as fungal dry mass/volume of PDB used in the *F. pseudonygamai* SC17 liquid culture.

3.5 Establishment of culture filtrate selection treatments

3.5.1 Selection media with *F. pseudonygamai* SC17 CF

F. pseudonygamai SC17 CF was obtained as described in section 3.3.1. The selection media for calli cultures were prepared by adding the appropriate volume of CF stock solution to the autoclaved EIM medium to obtain the required CF concentrations. The 500 ml Schott bottle containing 350 ml of the medium was swirled gently to allow the media and CF to mix thoroughly before dispensing into Petri dishes. Fresh CF was prepared on a weekly basis, the embryogenic calli were cultured every week onto fresh embryo germination media containing CF.

3.5.2 Exposure of embryogenic callus to medium containing culture filtrate

To determine the effect of CF during the embryo germination stage, embryogenic calli were cultured on selection media (EGM1 containing 0 - 100 ppm CF) (section 3.4.1). For the control, embryogenic calli were exposed to EGM1 without CF.

At the embryo initiation stage (6 - 8 weeks), the calli were cultured on EIM without CF. After 8 weeks, the embryogenic calli (0.1 g per replicate) were then cultured on EMM supplemented with 0, 4, 20, 50 and 100 ppm CF. The cultures were placed in the photoperiod growth room for 4 - 12 weeks, and sub-culturing weekly on EGM1 containing fresh CF. The number of plantlets and percentage callus necrosis were recorded after 6 - 12 weeks. The percentage

callus necrosis (a piece of callus was considered necrotic if <50 % appeared brown/black) was recorded for each treatment.

3.5.3 Plantlet rooting in medium with culture filtrate

The root growth with culture filtrate protocol was done according to Mahlanza et al. (2013). *In vitro* plantlets (70 - 100 mm in height) that were maintained on EGM2 in the photoperiod growth room were used for this experiment. All the leaves were trimmed just above the ligule and the roots were trimmed to less than 1 mm, using a pair of forceps and a scalpel (Mahlanza et al., 2013). These trimmed plantlets were transferred to Magenta® vessels (5 plants/vessel), containing EGM2 with 0, 500, 1000 and 1500 ppm CF and incubated in the photoperiod growth room for 3 weeks, as mentioned in section 3.1.3. Root growth was determined after 3 weeks by measuring root length and the percentage of plantlets that rooted.

3.6 Screening for indole-acetic acid production using a colorimetric assay

3.6.1 Indole-3-acetic acid production

Quantification of indole-3-acetic acid (IAA) produced by *F. pseudonygamai* SC17 was conducted using a colorimetric assay as described by Khan et al. (2016). Due to the enhancing root growth effect by isolate SC17, an experiment was conducted to determine if this enhanced effect was due to the additional levels of IAA in the plants or in the culture filtrate.

The fungus was cultured in 100 ml PDB supplemented with and without 0.1 g l^{-1} L-tryptophan (Sigma-Aldrich), and both flasks were placed in a shaking incubator at 30 °C and 200 rpm for 7 days in the dark. This was used for culture filtrate preparation as explained earlier (section 3.4.2). Uninoculated broth was used as a negative control and the experiment was conducted in triplicate for each fungus. A volume of 40 ml of the culture filtrate was taken and adjusted to pH 2.8 by adding 1 N HCl. Ethyl acetate (15 ml) was added three times to the CF and vortexed. The solution was left to separate into two layers after which the top layer of ethyl acetate was transferred to a 100 ml rotary evaporator flask. The ethyl acetate fractions were combined and evaporated under vacuum at 45 °C in a rotary evaporator (Rotavapor-R, Büchi, USA). The residue was re-suspended in 2 ml of 50 % (v/v) methanol and 100 µl of the re-suspended extract was mixed with the Salkowski reagent [200 µl; 0.2 ml 0.5 mol l^{-1} FeCl3, 4.9 ml water and 4.9 ml 70 % (v/v) perchloric acid] in an ELISA plate (96 well) and kept in the dark for 30 mins (Gordon and Weber, 1951). The resultant solution turned reddish in colour and was measured at an absorbance reading of 530 nm using a plate reader (Synergy-HT, Bio-

Tek, USA). The amount of IAA (mg ml^{-1}) was estimated using a standard curve of pure IAA (Sigma- Aldrich, USA).

3.6.2 The effect of indole-3-acetic acid on plantlet regeneration

The leaves and roots of plantlets (70 - 100 mm in height) were trimmed, as described in 3.5.3. They were then placed in Magenta® vessels (5 plants/vessel), containing 80 ml of EGM1 with 0, 150, 300, and 460 nm IAA. The plants were incubated in the photoperiod growth room conditions for 3 weeks, as described in section 3.1.3. The root length of the individual plants from all treatments were recorded.

3.7 *Ex vitro* selection of primed and EMS-exposed *in vitro* plantlets and *E. saccharina* screening

In vitro plantlets were produced using the established mutagenic protocols, as described earlier (Fig. 7). The *F. pseudonygamai* SC17 CF did not inhibit callus growth so it could not be used as a selection agent in this study. The plants were divided into three categories: a) control, b) primed only with Hx, and c) primed with Hx and the use of mutagens EMS and 5-AzaC (Fig 7). Fifty plantlets were randomly selected per 'category' or 'plant family', planted in seedling trays and acclimatised for 8 - 9 months. The plants were placed in the glasshouse at 20/34 °C (night/day temperature) and were watered using overhead sprinklers twice a day for 1 min and fertilized every 2 weeks with either N:P:K 5:1:5 pellets (Coastal Farmer's Co-op, Umhlali, SA) or Trelmix, 1 ml l^{-1} [Hubers (Pty) Ltd, Howick, SA]. These plantlets were used for *ex vitro* screening, and to test their response to *E. saccharina* damage and tissue colonisation by *F. pseduonygamai* SC17.

3.7.1 Inoculation of plants using *F. pseudonygamai* culture filtrate

To confirm tolerance of the putative mutants to *F. pseudonygamai* and *E. saccharina*, the plants were acclimatised in the glasshouse and inoculated (Fig. 8). Plants with 1-2 internodes were transferred to pots (100 mm diameter, 90 mm in height) and inoculated by the toothpick inoculation method (McFarlane, pers.comm). Plants were inoculated with only SC17, and dual inoculated with SC17, and after 2 weeks, with 1-2 2nd instar *E. saccharina* larvae were placed within the leaf whorl. This was done to demonstrate the effect of the fungus only and both the fungus and eldana larvae on the putative mutant plants. The toothpicks were sterilised in boiling water for 2 h, with the water was being replaced at 30 min intervals and autoclaved them twice with 48 h between autoclaving. PDB was prepared in McCartney bottles (6 ml/bottle) and the toothpicks were immersed in the media (15 toothpicks/bottle) before it was

autoclaved. The media was inoculated with *F. pseudonygamai* by adding a 5 x 5 mm mycelial square from a 3 - day old colony cultured on PDA. The McCartney bottles were placed in a shaking incubator at 145 rpm for 3 weeks at a temperature of 28 - 30 °C. To inoculate the plants, the stems of the plants were stabbed 2-3 cm above the soil surface with *F. pseudonygamai* SC17-colonised toothpicks. The control plants were stabbed with either uncolonised or colonised toothpicks. To avoid secondary pathogen infection, prior to stabbing, the stem was swabbed with 70 % (v/v) ethanol, and the protruding toothpick was cut and parafilm wrapped around the wound after inoculation.

3.7.2 Detection and re-isolation of the presence of *F. pseudonygamai*

After 7 - 8 weeks of toothpick inoculation, the stems of the live and dead putative mutant plants (some with chlorotic, crinkled, and necrotic leaves) were removed and placed in Petri dishes. Using aseptic techniques, the leaves and stems were transferred separately to 50 ml Corning tubes (Corning, Greiner Bio-one, SA) and surface sterilised by submerging them sequentially in 95 % (v/v) ethanol for 2 min followed by 10 % (v/v) sodium hypochlorite for 5 min. The stems and leaves were rinsed twice in sterile water to remove the excess sodium hypochlorite, after which they were dried for 5 min by placing them on a sterile paper towel in the laminar air flow cabinet. The surface of the leaves and stems were pressed on PDA to confirm the removal of surface contaminants.

The stems of all the putative mutants were cut into longitudinal segments and the inoculation fungal lesion severity for each individual stem were visually rated on a scale of minimum 0 to a maximum of 3 (0 = no lesions, 1 = mild, 2 = moderate, 3 = severe). Fungal re-isolation from the undamaged area 20 - 30 mm above it or the inoculation lesion, was done by cutting longitudinal stem sections and placing these sections on Nash and Snyder (1962) medium and incubated for 5 days at 28 - 30 °C. The Nash and Snyder semi-selective medium for *Fusarium* spp. consists of 15 g l^{-1} peptone, 1 g l^{-1} potassium dihydrogen phosphate (KH_2PO_4), 0.5 g l^{-1} magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), 1 g l^{-1} Pentachloronitrobenzene (PNCB), 20 g l^{-1} agar, 0.1 % (w/v) streptomycin and 0.012 % (w/v) neomycin, pH 5.5 - 6.5, autoclaved at 121 °C for 20 mins. Once the fungus grew around the stem sections, mycelia was picked off from the colonies with an inoculating loop and cultured on Nash and Snyder medium for 3 days at 28 - 30 °C prior to DNA extraction. *F. pseudonygamai* SC17 mycelial squares from the stock culture were placed on Nash and Snyder media and used as positive controls. Colonies of the isolates were compared visually with SC17 and were subjected to molecular analyse for confirmation of fungal isolate identity.

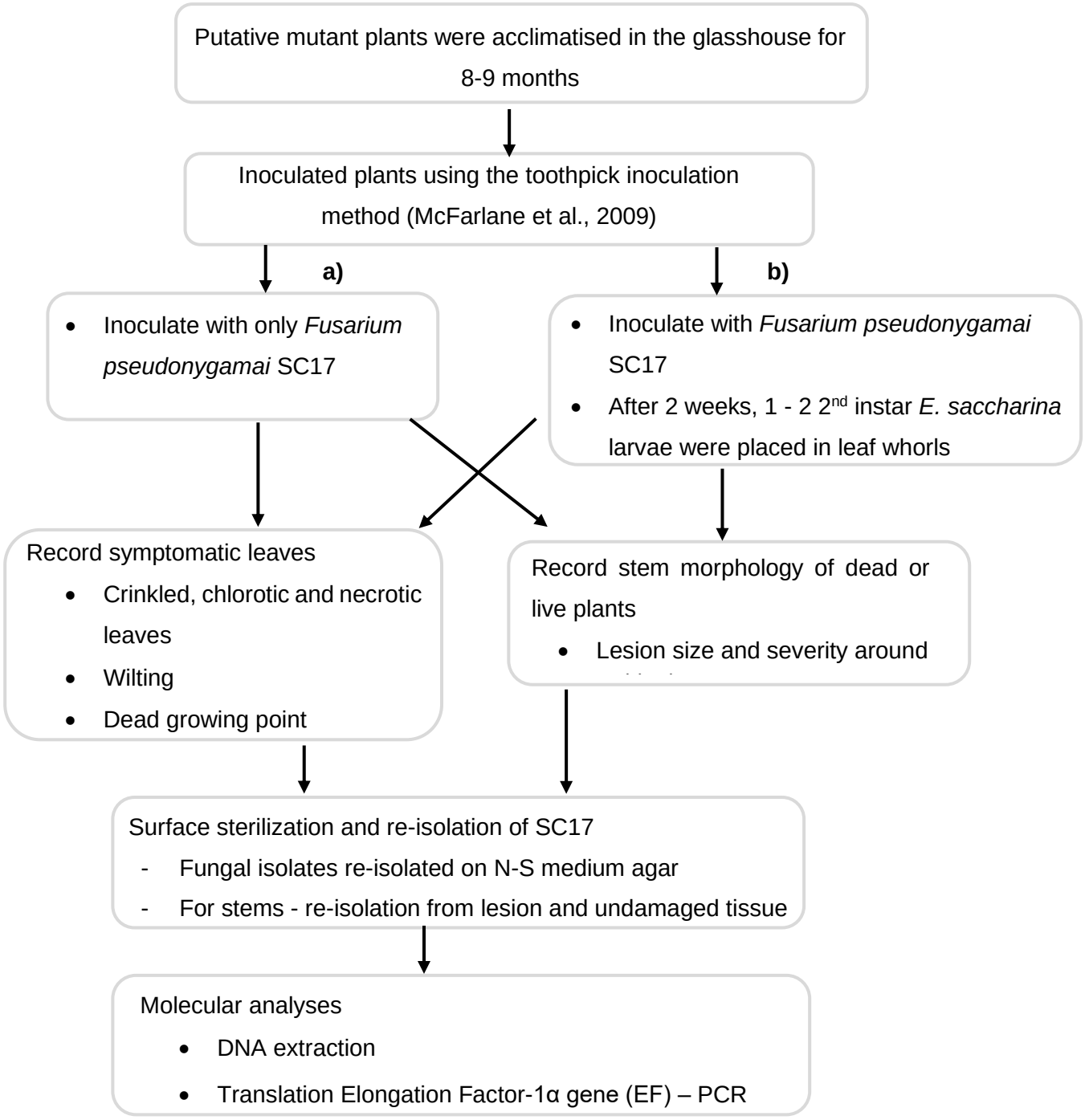


Figure 8: Summary of manipulations undertaken to confirm tolerance of plants to *Fusarium pseudonygamai* (SC17).

3.8 Molecular analyses of *Fusarium* isolates that were retrieved from the putative mutants

3.8.1 DNA extraction

The PrepMan® Ultra Sample (Applied Biosystems, California, USA) protocol was used to conduct fungal DNA extractions. The *F. pseudonygamai* SC17 isolate was grown on corn meal agar and incubated for 3 - 5 days at 28 - 30 °C. The resultant mycelia were aseptically transferred from the fungal colonies to sterile 1.5 ml microfuge tubes, using an inoculating loop. For the DNA extraction process 100 - 200 µl PrepMan® Ultra Kit sample preparation reagent (Applied Biosystems, California, USA) was added into the sterile 1.5 ml microfuge tubes (Quality Scientific Plastics, California, USA) containing 0.3 mm glass beads. The tubes were vortexed for 1 minute and the mycelia were ground using sterile plastic pestles. The tubes were then placed in a heating block at 100 °C for 30 min to release the DNA. The tubes were centrifuged (Centrifuge 5415R, Eppendorf, Hamburg, Germany) at 12 000 rpm for 5 min and the supernatant was transferred to clean microfuge tubes. The DNA was purified by adding a 10 % (v/v) of 3 M sodium acetate and 250 % (v/v) of absolute ethanol to the supernatant, which was incubated at - 20 °C and vortexed at 30 min intervals for 2 h. The tubes were centrifuged at 13000 rpm for 20 min for the precipitated DNA to form a pellet. The supernatants were discarded, and the pellets were washed by adding 700 µl of 70 % (v/v) ethanol and centrifuged at 13 000 rpm for 20 mins. The pellets were dried in the laminar flow and then re-suspended by adding 30 µl PrepMan® elution buffer and the solutions were vortexed. The concentrations of the DNA samples were determined using a spectrophotometer (NanoDrop Technologies, Delaware, USA).

3.8.2 Translation Elongation Factor-1α gene (EF) – PCR

The re-isolated fungal isolates were inoculated after 3 days on PDA and the extraction of DNA (section 3.8.1) was conducted using the resultant colonies. The elongation factor (EF) primers were used to test the fungal isolates that were retrieved from the putative mutant plants. The PCR was conducted on the resultant colonies. The PCR reactions were done using a PCR kit (Kapa Biosystems, Massachusetts, USA) in 0.2 ml MicroAmp 8-strip PCR tubes (Applied Biosystems, California, USA) containing a final volume of 30 µl composed of 19.85 µl PCR water (Promega, USA), 4 µl Taq buffer with $MgCl_2$ (1.5 mM), 0.45 µl dNTPs (10 mM), 1 µl primer (EF1) (10 µm), 1 µl primer (EF2) (10 µm), 0.2 µl Taq polymerase and 2 µl DNA template. PCR cycling condition were as follows: initially 3 minutes of denaturation at 95 °C then 35 cycles of denaturation at 94

°C for 30 s, 1 min of primer annealing at 50 °C and extension for 1 min 30 s at 72 ºC, and a final extension step at 72 °C for 10 min. The PCR products were visualised using a 1.2 % agarose gel.

3.9 Statistical Analyses

To analyse the data, the Genstat statistical package 18th edition (VSN International, Hemel Hempstead, UK) was used. The Shapiro-Wilk test was initially used to test the data for normality. In some instances, before conducting the suitable statistical tests, data had to be transformed (log10), as reported in the results section.

4. Results

4.1 Establishing a suitable priming agent concentration at the callus initiation and plantlet regeneration stages for plantlet production

This study aimed to establish a suitable concentration of the selected priming agents, cis-jasmone (CJ) and hexanoic acid (Hx) at the callus initiation and the plantlet regeneration stage. The cultivar N41 is known to be intermediate-resistant to *E. saccharina*, whilst cultivar 88H0019 is known to be susceptible to *E. saccharina* in the field (Zhou et al., 2013). The effect of priming agents on callus production, callus necrosis, and plantlet regeneration was investigated.

4.1.1 The effect of priming agents (CJ or Hx) supplied at the callus initiation stage

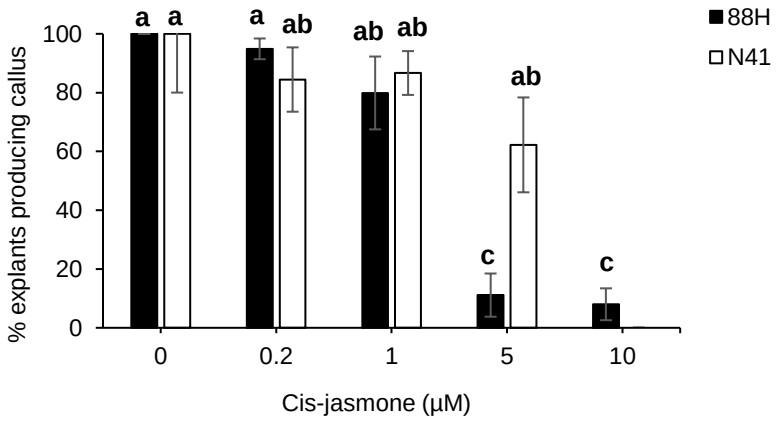
To determine an appropriate priming agent concentration at the callus initiation and plantlet regeneration stage, embryogenic calli (0.2 g per replicate) were transferred to embryo maturation medium (EMM) consisting of CJ (0, 0.2, 1, 5 and 10 µM) and Hx (0, 0.2, 0.6, 2 and 6 mM) for 2 weeks.

After 8 weeks, a significant difference in the percentage of explants producing callus amongst the CJ treatments was detected for both cultivars ($p < 0.001$) (Fig. 9a). There was a significant decrease in the percentage of explants producing callus for cultivar 88H0019 with increasing concentrations of CJ. For cultivar N41, there were no significant differences in the percentage of explants producing callus between the control, 0.2, 1.0, and 5 µM CJ treatments. For cultivar 88H0019, there was a significant difference between 5 µM CJ (11 % ± 7.35) and 10 µM CJ (8 % ± 5.42) compared with the control and other treatments. These two treatments inhibited callus production in cultivar 88H0019 compared with the other treatments that resulted in 60 - 100 % callus production ($p < 0.001$, Fig. 9a). For cultivar N41, there was no callus production recorded at 10 µM CJ.

After 8 weeks for each cultivar, there were significant differences in the percentage of explants producing callus when compared with the control and all the tested Hx concentrations at the end of the study ($p < 0.001$, Fig. 9b). The percentage of explants producing callus was significantly lower at 6 mM Hx for cultivar 88H0019 (24 % ± 8.63) and N41 (10 % ± 2.22) than the control and the other treatments ($p < 0.001$). For cultivar N41, there were no significant differences in the percentage of explants producing callus amongst the control, 0.2, 0.6, and 2 mM Hx treatments. For 88H0019 and N41, there was a significant decrease in the percentage of explants producing

callus with an increase in Hx supplied (Fig. 9b). The 6 mM Hx treatment significantly inhibited the percentage callus production by the explants of both cultivars. Also, callus exposure to the higher priming agents resulted in more severe necrosis than the lower concentrations of both priming agents (Fig. 10a, b). These results indicated that the percentage of explants producing callus by both N41 and 88H0019 explants were positively affected by the tested priming agents concentrations, except for the 5 and 10 µM CJ (Fig. 9a, b).

a)



b)

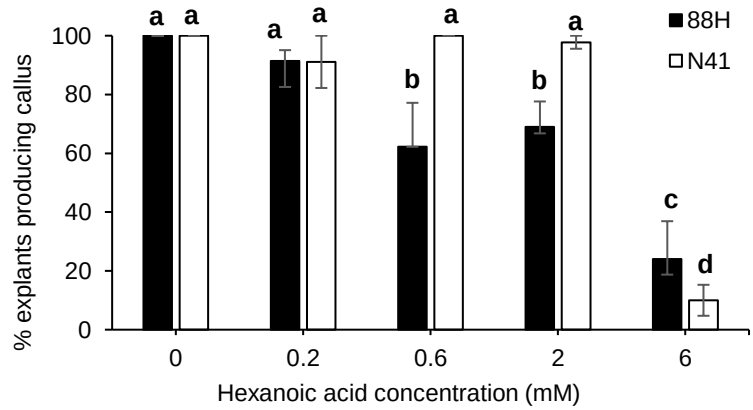
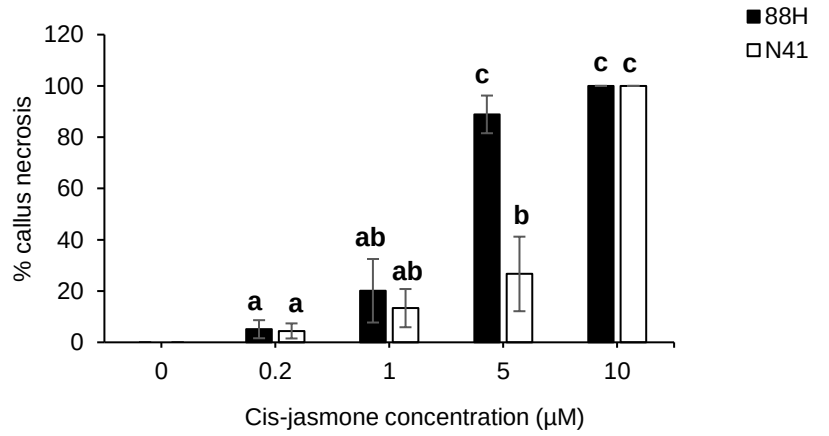


Figure 9: The effect of priming agent concentration on % explants producing callus in two sugarcane cultivars after 8 weeks on embryo maturation medium containing either a) cis-jasmone or b) hexanoic acid. Dissimilar alphabet characters denote a statistically significant difference between the cultivars for each concentration. Data sets were analysed by One-way ANOVA, Duncan's multiple range test, $p < 0.001$, $n = 9$, mean ± SE.

In addition to callus production, callus necrosis was also selected as a parameter to test the effect of the priming agents and select an appropriate concentration for callus production and plantlet regeneration in subsequent studies to establish a protocol for the treatment with selected priming agents. Callus necrosis was recorded if at least 50 % of a callus piece turned brown or black after 3 weeks in culture (Mahlanza et al., 2013). Callus necrosis for each treatment was recorded after 8 weeks in the dark on embryo maturation media containing either CJ or Hx (Fig. 10a, b). No callus necrosis was observed in the control calli of both cultivars (Fig. 10a). For both cultivars, there was a significant difference in callus necrosis between the controls and the CJ treatments ($p < 0.001$), with increasing CJ concentrations significantly increasing necrosis. For both cultivars, the highest negative effect (100 %) on callus necrosis was recorded on media containing 10 µM CJ (Fig. 10a). There were no significant differences in callus necrosis amongst the control and the 0.2 and 1 µM CJ treatments ($p > 0.001$). There were only significant differences in percentage explants producing callus amongst the 5 µM CJ (89 % ± 7.35 for 88H0019; 27 % ± 14.53 for N41) and the 10 µM CJ (100 % ± 0 for 88H0019; 100% ± 0 for N41) treatments compared with the control ($p < 0.001$) (Fig. 10a).

No callus necrosis was recorded for the control plantlets (Fig. 10b). For cultivar 88H0019, there was a significant increase in callus necrosis with an increase in Hx exposure. The media containing 6 mM Hx had the highest negative effect (67 %) on callus necrosis for both cultivars (Fig. 10b). There were no significant differences in callus necrosis observed amongst the 0, 0.2 (88H0019 and N41), 0.6 (N41), and 2 mM (N41) Hx treatments. For 88H0019, there were significant differences in callus necrosis recorded for cultivar 88H0019 between the 0.6 (38 % ± 14.95), 2 (31 % ± 8.24), and 6 mM Hx treatments (67 % ± 16.67) compared with the control. There was a significant negative effect on callus necrosis by 6 mM Hx (87 % ± 5.53), compared with the control and the other Hx treatments ($p < 0.001$) (Fig. 12 b). In conclusion, the highest tested concentration of CJ (10 µM) and Hx (6 mM) resulted in the lowest callus production and highest callus necrosis for both cultivars (Fig. 10a, b).

a)



b)

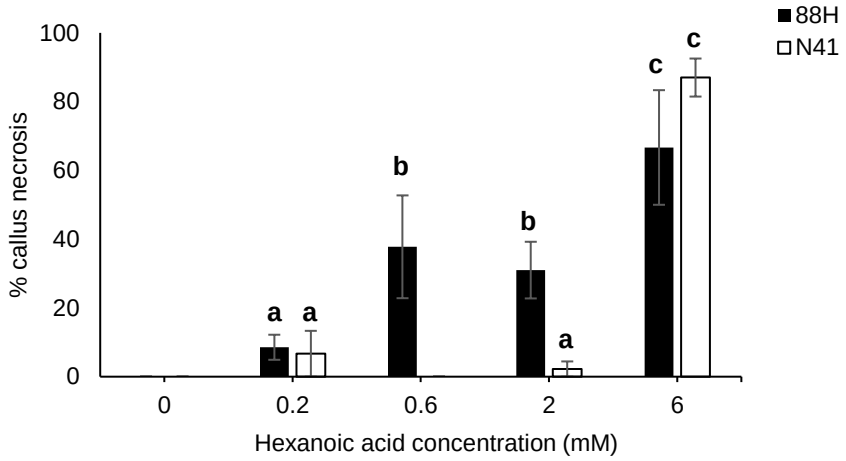


Figure 10: The effect of priming agent concentration on % callus necrosis on embryo maturation medium containing either a) cis- jasmone or b) hexanoic acid. Dissimilar alphabet characters denote a statistically significant difference between the cultivars for each concentration. Data sets were analysed by One-way ANOVA, Duncan's multiple range test, $p < 0.001$, $n = 9$, mean ± SE.

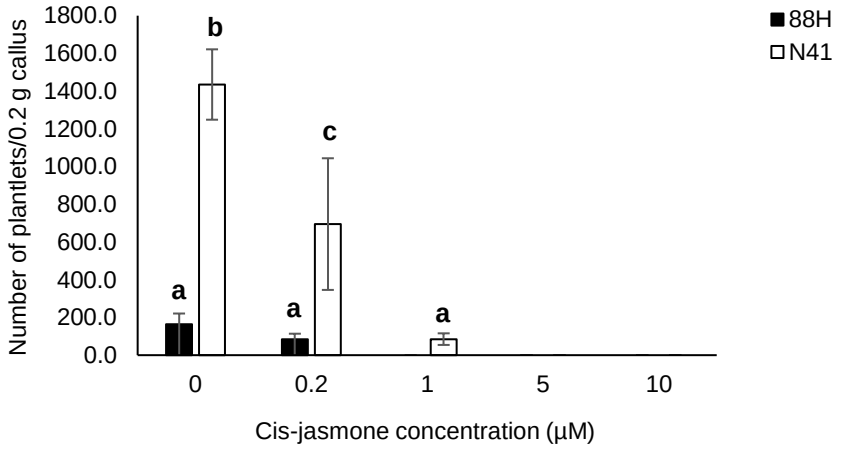
4.1.2 Comparison of plantlet yield for both priming agents

Embryogenic calli were produced on EIM without priming agents for 8 weeks, after which they were transferred to EMM for 1 week. They were then cultured on EGM containing 0, 0.2, 1, 5, and 10 µM CJ or 0, 0.2, 0.6, 2, and 6 mM Hx, and number of plants/ 0.2 g callus was recorded after 6 - 12 weeks. Plantlet yield on both the CJ and Hx treatments was negatively affected by high concentrations of the priming agents for the two cultivars (Fig. 11a, b). The plantlet yield of N41

supplied with CJ significantly decreased in the 0.2 (695 plantlets/0.2 g callus ± 349.0) and 1 µM CJ treatment (85 plantlets/0.2 g callus ± 30.79), when compared with the control (1435 plantlets/0.2 g callus ± 186.57). For both cultivars, there were no plantlets produced when exposed to the 5 and 10 µM CJ treatments and the results indicated a negative effect of the increasing supply of CJ concentrations ($p < 0.001$) (Fig. 11a). There was no significant difference in the number of plants/ 0.2 g of callus produced for cultivar 88H0019 across all the tested concentrations of CJ ($p > 0.001$).

In the Hx treatments, there was no significant difference in plantlet yield between the control and all the treatments for cultivar 88H0019 (Fig. 11b). There was a significant decrease in the number of plantlets/0.2 g of callus amongst the control (1015 plantlets/0.2 g callus ± 207.1), 0.2 (915 plantlets/0.2 g callus ± 71.82), 0.6 (785 plantlets/0.2 g callus ± 272.93) and 2 mM Hx treatments (61 plantlets/0.2 g callus ± 24.95) for N41 ($p < 0.001$). The callus of cultivar N41 produced significantly more plantlets than that of 88H0019 at the 0.2 and 0.6 mM Hx treatments ($p < 0.001$).

a)



b)

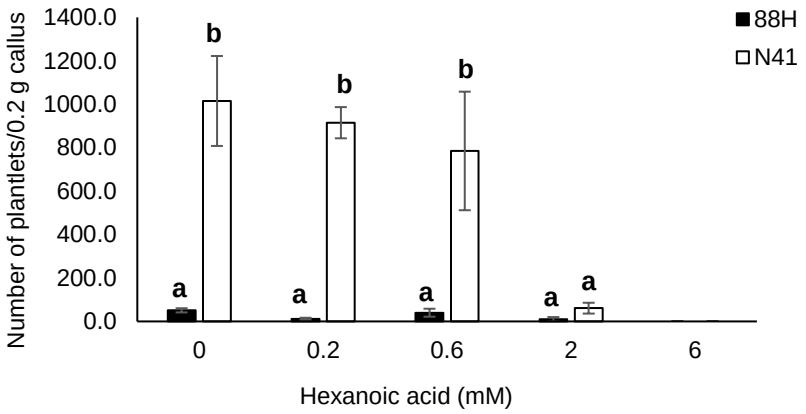


Figure 11: The effect of priming agent concentration on the number of plantlets produced/0.2 g of callus on embryo germination medium containing either a) cis- jasmone or b) hexanoic acid. Dissimilar alphabet characters denote a statistically significant difference amongst cultivars for each concentration. Data sets were analysed by One-way ANOVA, Duncan's multiple range test, $p < 0.001$, mean ± SE n = 4.

As expected, the number of plantlets/ 0.2 g of callus is inversely proportional to percentage callus necrosis for both cultivars (Fig. 10, 11). The treatments that showed a high percentage of callus necrosis produced low plantlet yields (5 and 10 µM CJ treatments; 2 and 6 mM Hx treatments). The research findings from this investigation showed that Hx resulted in better callus production and plantlet yield than CJ. Hence, of the two priming agents tested, 0.6 mM Hx allowed sufficient callus production and plantlet yield, whilst still likely having a priming effect, and was chosen for further studies.

From the two priming agents tested, more plants were produced using Hx as a priming agent (Table 5). The percentage of plantlets for 88H0019 and N41 were expressed as a percentage of the plants produced in the control. For cultivar N41, the highest plantlet yield was 90 % recorded at 0.2 mM Hx, and for cultivar 88H0019 78 % plantlet yield was recorded at 0.6 mM Hx. For both the callus and plantlet production stages, 0.6 mM Hx was selected as the suitable concentration since it was the only concentration that produced greater than 75 % plantlet yield for both cultivars. The research findings from this investigation showed that Hx produced improved callus production and plantlet yield compared with CJ, and therefore was chosen as a priming agent for further studies.

Table 5: Table showing the percentage of plantlets produced for each priming agent at different concentrations for cultivars 88H0019 and N41.

Priming agent	Treatment CJ (µM) Hx (mM)	Plantlet yield for 88H0019 (% plants)	Plantlet yield for N41 (% plants)
CJ	0 (control)	100	100
	0.2	51	48
	1	0	6
	5	0	0
	10	0	0
HX	0 (control)	100	100
	0.2	24	90
	0.6	78	77
	2	20	6
	6	0	0

4.2 Establishing callus and *in vitro* plantlet screening conditions using *F. pseudonygamai* culture filtrate

This investigation aimed to establish: the concentration of *F. pseudonygamai* SC17 culture filtrate at which somatic embryos could be screened for resistance to the selected *Fusarium* strain at the embryo germination stage; and a suitable culture filtrate concentration for screening putative mutant plantlets at the end of the plantlet production stage.

4.2.1 Determining a suitable *F. pseudonygamai* culture filtrate concentration for screening somatic embryos at the embryo germination stage

In this study, different concentrations of *F. pseudonygamai* SC17 CF were incorporated in the embryo germination media to determine the most appropriate one for screening embryos for resistance to the *Fusarium* strain SC17. The embryogenic calli was not treated with priming and mutagenic agents prior to exposure to the CF.

Embryogenic calli were exposed to different concentrations (0, 4, 20, 50, and 100 ppm) of *F. pseudonygamai* CF at the embryo germination stage to determine which CF concentration resulted in callus necrosis, and if it also inhibited embryo germination. The embryogenic callus was produced after 6 - 8 weeks on EIM without CF, which was then transferred to EMM without CF for 3 weeks. Then, embryogenic calli (0.1 g per replicate) was cultured on EGM1 supplemented with 0, 4, 20, 50, and 100 ppm CF. Callus necrosis was measured when at least 50 % of a callus piece had browned, and more calli pieces became necrotic with increasing CF levels in the media (Fig. 12a - e). A significant difference in percentage callus necrosis was recorded between the control (13 % ± 2) and 100 ppm CF treatment (68 % ± 10) for cultivar N41 ($p < 0.001$). The only recorded significant difference in percentage callus necrosis between both cultivars was with the 50 ppm CF exposure (88 % ± 8 for 88H0019; 21 % ± 9 for N41) ($p < 0.001$; Fig. 13a). After 3 weeks on embryo germination media, some necrotic calli, especially from cultivar 88H0019 that were exposed to 50 - 100 ppm CF, developed root hairs. The percentage callus necrosis for calli transferred to EGM medium containing 4 (73 % ± 8 for 88H0019; 38 % ± 7 for N41), 20 (73 % ± 6 for 88H0019; 35 % ± 0 for N41) and 50 ppm CF (88 % ± 8 for 88H0019; 21% ± 9 for N41) was higher than that of the control (13 % ± 2) (Fig. 13a). Based on the results obtained, 88H0019 was susceptible to the culture filtrate and produced the highest percentage callus necrosis compared with N41.

The incorporation of CF in EGM1 resulted in decreased plantlet yield for both cultivars as compared with the EGM1 without CF (Fig. 13b). For cultivar 88H0019, there were no significant differences in the number of plantlets/0.1 g of callus between the control and the 4, 20, 50 and 100 ppm CF. However, the highest CF treatment resulted in significantly fewer plants than the control ($p < 0.001$; Fig. 13b). In cultivar N41, plantlet yield decreased significantly at 100 ppm CF (98 plantlets/0.1 g of callus ± 29) compared with the control (1020 plantlets/0.1 g of callus ± 97) and ($p < 0.001$; Fig. 13b). As expected, the results indicate an inverse proportional relationship between the number of plantlets/0.1 g of callus and percentage callus necrosis (Fig. 13a and b). The CF stress at the embryo germination stage resulted in most callus pieces eventually turning black/necrotic, but there was greening of the non-necrotic areas within each callus piece on EGM1 + CF and plantlet yield was recorded by week 12 (Fig. 13b). Plantlets were still produced at the highest CF concentration (100 µM) that was tested, however a greater CF concentration should have been analysed. Hence, *F. pseudonygamai* SC17 could not be used as an *in vitro* selection agent at the callus and regeneration stage.

The number of abnormal plantlets/0.1 g of callus from each treatment was also recorded at the end of the embryo germination stage (Fig. 13c). During the germination stage, the CF had a positive effect on the number of abnormal plants/0.1 g of callus produced. There was a significant difference in the number of abnormal plants/0.1 g of callus between both cultivars ($p < 0.05$). In cultivar 88H0019, the number of abnormal plants/0.1 g of callus was significantly higher at 50 ppm CF (21 abnormal plants/0.1 g of callus ± 10), whereas the 4, 50, and 100 ppm CF treatments did not cause visible abnormalities in N41 plantlets.

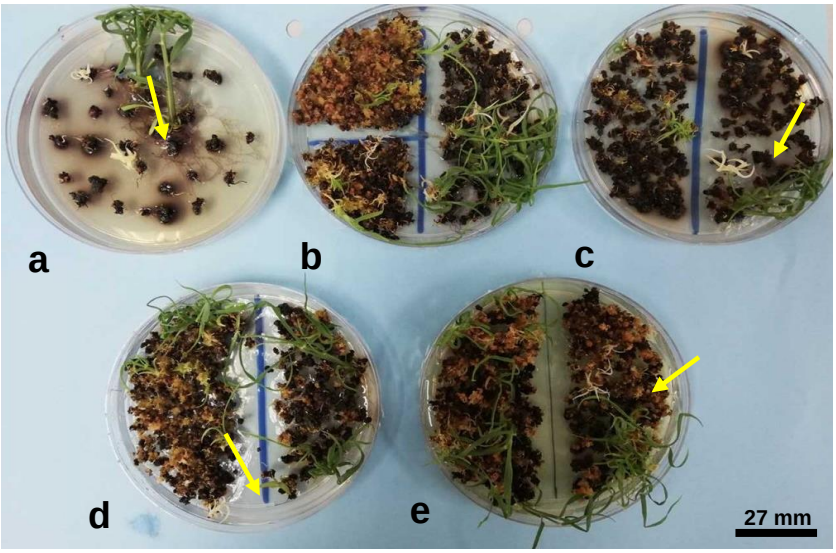
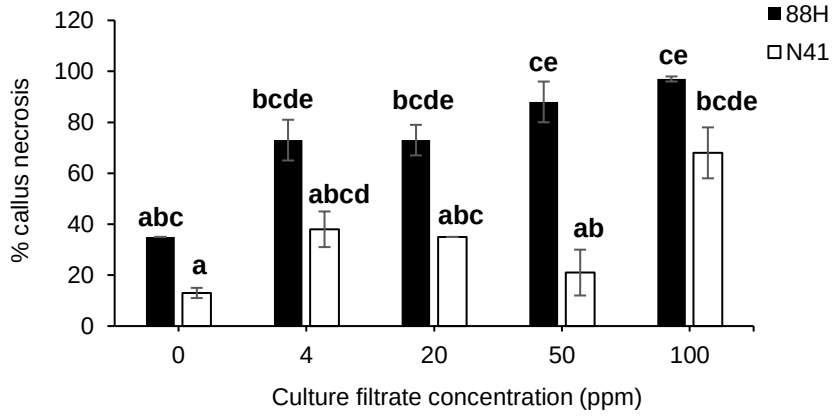


Figure 12: The effect of *F. pseudonygamai* CF on callus proliferation and plantlet regeneration in embryo germination media after 7 - 8 weeks with weekly sub-culturing. Embryogenic calli were cultured on EMM-CF for 3 weeks without sub-culturing, before culturing on EGM1 supplemented with: a) 0, b) 4, c) 20, d) 50, and e) 100 ppm. The arrows indicate albino plantlets.

a)

d



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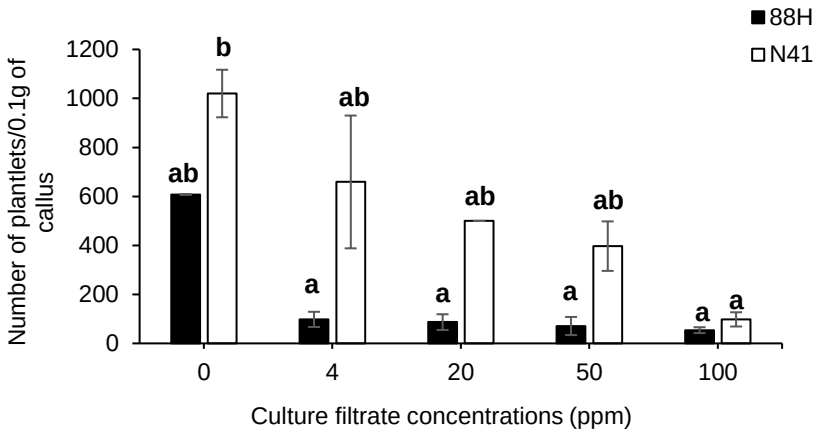


c)

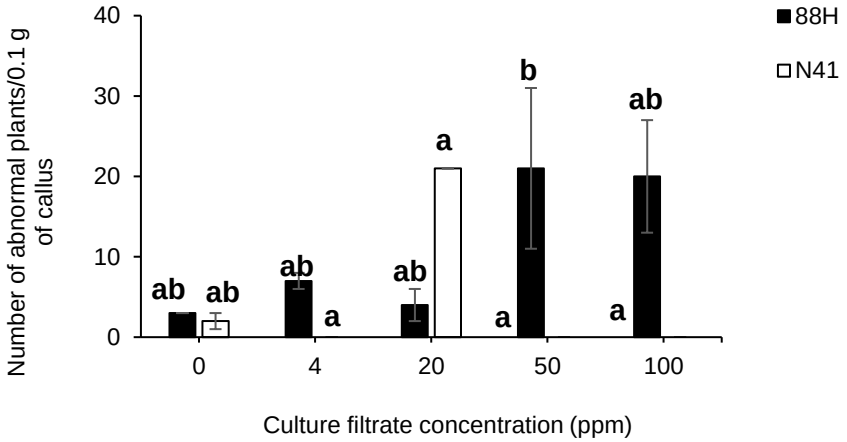
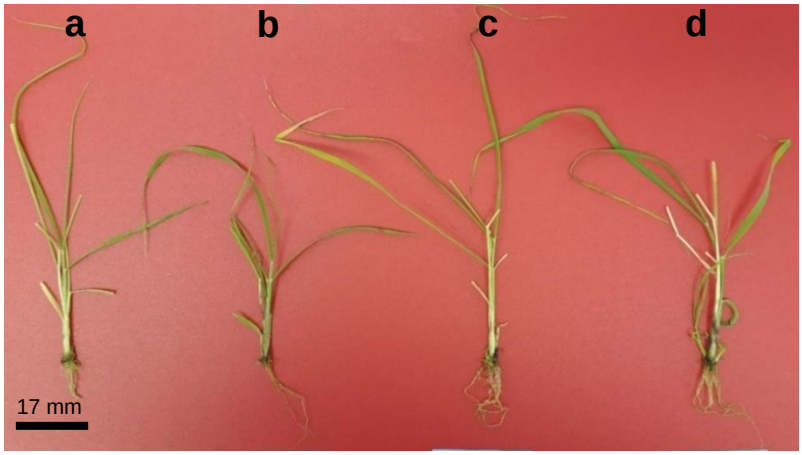


Figure 13: The effect of culture filtrate concentration on callus proliferation and plantlet regeneration in embryo germination medium after 4-8 weeks with weekly subculturing. Embryogenic calli were cultured on EMM-CF for 3 weeks without subculturing, before culturing on EGM+CF. a) Percentage callus necrosis; b) Number of plants/ 0.1 g of callus; and c) Number of abnormal plants/0.1 g of callus. Dissimilar alphabet characters denote a statistically significant difference amongst cultivars for each concentration. Data sets were analysed by One-way ANOVA and Sidak post hoc test. $P \geq 0.05$, $n = 4$, mean ± SE.

4.2.2 Establishing a culture filtrate concentration for screening putative mutant plantlets

In this investigation, the development of a protocol for selecting plantlets produced from the somatic embryos that survived the CF selection pressure at the embryo germination stage was attempted. This investigation was based on the rooting ability and root length of the plants, under the various treatments, after their roots were trimmed to less than 1mm in length. Mahlanza et al. (2013) found that CF from the fungal isolate *F. sacchari* PNG40 inhibited root growth of the plants that were trimmed. Those results indicated that improved root growth for mutant plants could be used to select for resistance to toxins present in the CF (Mahlanza et al., 2013).

However, in this study, the addition of different concentrations (0, 500, 1000, 1500 ppm) of CF from the *Fusarium* isolate SC17 to EGM1 enhanced the rooting ability of plantlets (Fig. 14). Plantlets were cultured on EGM1 + CF after their roots were trimmed to less than 1 mm and then transferred to EGM1 containing 0, 500, 1000, and 1500 ppm CF. Root growth was determined after 3 weeks for both cultivars, plants from the CF treatments had an increased root length and slight discoloration at the base of the stem compared with the untreated controls (Fig. 14). Although there was no significant interaction observed between treatment versus cultivar, there was a significant difference in the root length of plantlets amongst the treatments after 3 weeks ($p < 0.001$) (Fig. 15). For both cultivars, root re-growth increased significantly ($p < 0.001$) across all tested CF concentrations. On average, for both cultivars, the controls had significantly shorter root lengths than all other treatments ($p < 0.001$). Plant root length (mm) was significantly higher for cultivar N41 compared with 88H0019 ($p < 0.001$). The results show that all the concentrations of the CF affected the root length of plantlets, with the 1500 ppm CF resulting in the highest root length (31.5 mm ± 4.3, for 88H0019) and (34.05 mm ± 3.9, for N41).



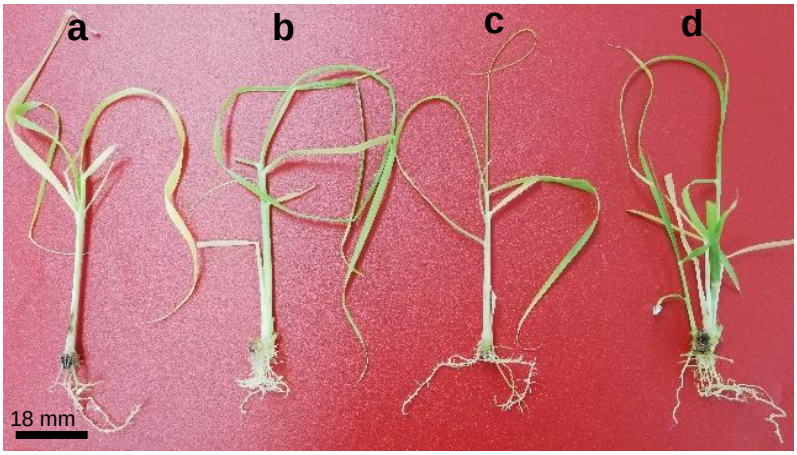


Figure 14: The effect of *F. pseudonygamai* culture filtrate (CF) on root growth of plantlets after 3 weeks for cultivars 88H0019 and N41 respectively. a) control, b) 500 ppm, c) 1000 ppm, and d) 1500 ppm CF.

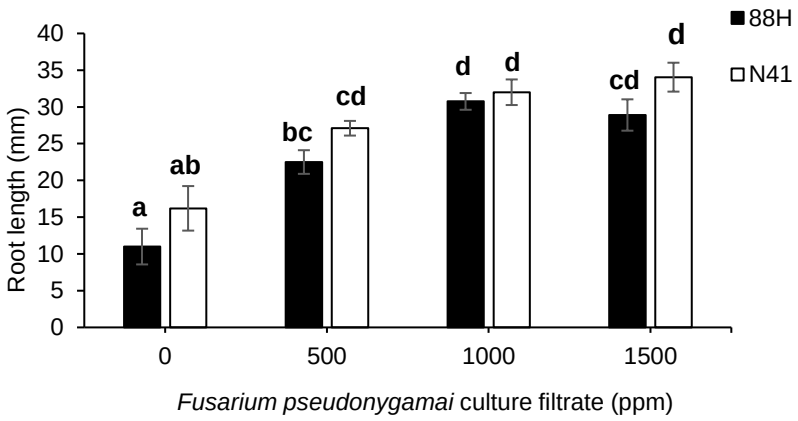


Figure 15: The effect of *F. pseudonygamai* culture filtrate (CF) on root growth using plantlets that had their leaves and roots trimmed before being cultured on media with 0 – 1500 ppm CF after 3 weeks. Root length (mm) was measured after 3 weeks. Dissimilar alphabet characters denote a statistically significant difference amongst cultivars for each concentration. Data sets were analysed by One-way ANOVA and Duncan's Multiple Range test, $P \geq 0.05$, mean ± SE, n = 4.

The results reported by Mahlanza et al. (2013) indicated that the roots of 50 % of mutant plantlets with trimmed roots cultured on media containing 1500 ppm CF re-regrew to at least 10 mm in length over 3 weeks. In his study he used 10 mm re-growth as the criterion for the selection of CF- tolerant plants. Consequently, this value was set as the criterion for the selection of CF-tolerant plants in the current study.

In this study, there was a significant positive difference in the percentage of plantlets that rooted between the control and all the tested CF concentrations ($p < 0.001$) (Fig. 16). The results recorded for both cultivars indicated a 95 - 100 % rooting ability of the plantlets in EGM1 + CF containing 500, 1000, and 1500 ppm CF, which was significantly higher than the percentage of plantlets that rooted in EGM1 containing no CF (60 - 70 %) ($p < 0.001$). For both cultivars, there was no significant difference between all the tested CF concentrations ($p > 0.001$). In conclusion, the results obtained from this investigation indicated that the *E. saccharina* beneficial strain *F. pseudonygamai* SC17 used in this study cannot be used as an *in vitro* selection agent because of its root promoting property. In many studies the phytotoxicity of *Fusarium* culture filtrates and inhibitory effect on root growth was assessed in crops such as sugarcane (Mahlanza et al., 2013), banana (Rebouças et al., 2021), and maize (Mirsam et al., 2021). Due to the enhanced root growth of the plantlets across all the tested CF concentrations, it was hypothesised that the *E. saccharina* beneficial *Fusarium* strain (SC17) is stimulating root growth by auxin (IAA) production or increased activity of the enzyme ACC deaminase and will be discussed later.

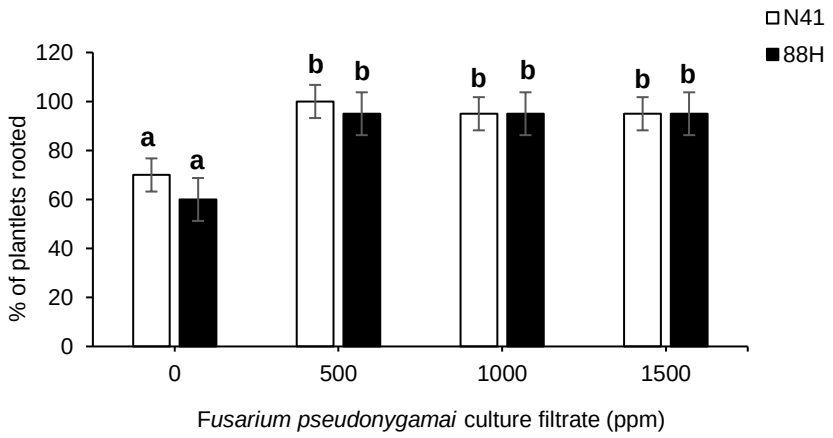


Figure 16: The effect of *F. pseudogynamai* culture filtrate (CF) in the re-rooting media on the % of plantlets that re-rooted. Root length (mm) was measured after 3 weeks. Re-rooting defined as roots ≥ 10 mm after root triiming to 1mm. Dissimilar alphabet characters denote a statistically significant difference amongst cultivars for each concentration. Data sets were analysed by (One-way ANOVA and Duncan's multiple range test, $P \geq 0.05$, mean ± SE, n = 4).

4.3 Screening for indole-3-acetic acid production from isolate *F. pseudonygamai*

Many studies have demonstrated that endophytic fungi can produce phytohormones, especially indole acetic acid (IAA) and gibberellins, as a direct system of plant growth promotion (Fu et al., 2015; Turbat et al., 2020). Therefore, these endophytes are of potential interest since their plant growth-regulating compounds can be used to enhance crop quality and yield. The compounds that are secreted by endophytes can rescue plant growth in a stressful environment and postively influence plant development (Khan et al., 2008; Khan et al., 2011; Khan et al., 2012; Turbat et al., 2020). The results shown in Figs. 15 and 16 (section 4.2.1) indicated a significant positive effect in root growth for cultivars 88H0019 and N41 when exposed to different concentrations of the *F. pseudonygamai* SC17 CF. Hence, an experiment was conducted to determine if this enhanced effect was due to indole-3-acetic acid production by the fungal endophyte by using trimmed plants and culturing them on EGM1 with 0, 150, 300, and 460 nM IAA, and then: a) quantifying the amount of indole-3-acetic acid produced by *F. pseudonygamai* SC17; b) determining the effect of an exogenous supply of indole-3-acetic acid on root growth after 3 weeks; and c) determining the effect of IAA on the fresh and dry mass of roots and shoots after 3 weeks.

4.3.1 To quantify the amount of indole-3-acetic acid produced by *F. pseudonygamai* SC17

The colorimetric method described by Khan et al. (2016) for the quantification of IAA production by microbes was used in this study. To quantify the amount of IAA produced by the *Fusarium* fungal isolate SC17. There was no significant difference in IAA production in the presence or absence of the precursor L-tryptophan by *F. pseudonygamai* ($p = 0.092$) (Fig. 17). However, the results obtained indicated that *F. pseudonygamai* produced the highest IAA concentration (743.1 nM) in the presence of L-tryptophan than in the treatment without L-tryptophan (457.2 nM). The amount of IAA produced from the CF in the rooting experiment was calculated using the concentration of the culture filtrate incorporated in the rooting media (Fig. 15) and the IAA concentration obtained in the treatment without L-tryptophan (Fig. 17) as seen in Table 6. The results indicated that the rooting media containing 1000 and 1500 ppm CF resulted in a significant increase in root length as compared with the control for both cultivars, these were equivalent to 298.2 nM and 447.4 nM IAA, respectively (Table 6).

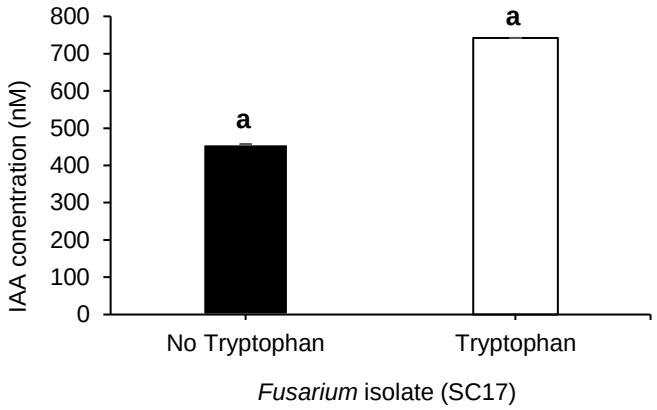


Figure 17: A comparison of the amount of IAA produced by the *F. pseudonygamai* SC17 after incubation for 7 days in potato dextrose broth supplemented with and without 0.1 g L^{-1} tryptophan. Dissimilar alphabet characters denote a statistically significant difference. Data sets were analysed by Two-sample T-test; Duncan's multiple range test; $p > 0.05$; n = 3, mean ± SE.

Table 6: Indole-3-acetic acid (IAA) production by fungal isolate *F. pseudonygamai* SC17.

F. pseudonygamai **CF concentration in rooting media (ppm)**	***F. pseudonygamai*** **CF volume (ml) Final volume of 350 ml**	**Calculated IAA concentration in diluted *F. pseudonygamai* CF (nM)**
0	0	0
500	114.3	149.1
1000	228.6	298.2
1500	342.9	447.4

4.3.2 The effect of exogenous indole-3-acetic acid on root growth after root trimming using *in vitro* plantlets

Indole-3-acetic acid is commonly used as a plant hormone to control growth and development in many crops (Cleland, 1987; Rayle and Cleland, 1992; Bunsangiam et al., 2021). Indole acetic acid produced by microbes improves the structure of roots and boosts the availability of nutrients to microbial endophytes (Soliman et al., 2020; Ismail et al., 2021). To determine the effect of IAA on root re-growth, root length, and on the fresh and dry weight of the roots and shoots, different concentrations of IAA (0, 150, 300, and 460 nM) were incorporated into the rooting medium for 3

weeks. The highest concentration of IAA used in this experiment simulates that produced in the 1500 ppm CF. Rooted plantlets were used for this investigation, and were prepared as for previous studies (i.e., roots trimmed to ± 1 mm, and leaves to just above the ligule as per Mahlanza et al. 2013).

The effect of IAA on the root growth and the percentage of plantlets rooted was recorded after 3 weeks. The results indicated a positive effect on root length of the plantlets that were supplied with exogenous IAA (0, 150, 300, and 460 nM) after 3 weeks in culture (Fig. 18). For cultivar 88H00019, the root length was significantly higher at 300 nM (34.17 mm ± 2.14) than 460 nM IAA treatment (21.45 mm ± 1.98) (Fig. 19). For cultivar N41, there was no significant difference in root length across all the tested IAA concentrations. However, there were significant differences in root length between both cultivars ($p < 0.001$). In cultivar 88H0019, root length was significantly higher at 300 nM IAA (34.17 mm ± 10) compared with the control (17.24 mm ± 7.95), 150 (20.47 mm ± 4.29), 300 (18.82 mm ± 3.76) and 460 nM IAA (18.97 mm ± 5.06) treatments for cultivar N41 (Fig. 19). For both cultivars, the results indicated that there were no significant differences in the percentage of plantlets that rooted across all the tested IAA concentrations ($p > 0.05$) (Fig. 20). However, the percentage of plantlets that rooted for cultivar 88H0019 was significantly higher at the 300 nM IAA (100 % ± 0.00) treatments than control ones (44 % ± 0.92) and 150 nM IAA (64 % ± 0.86) for cultivar N41 ($p = 0.008$).

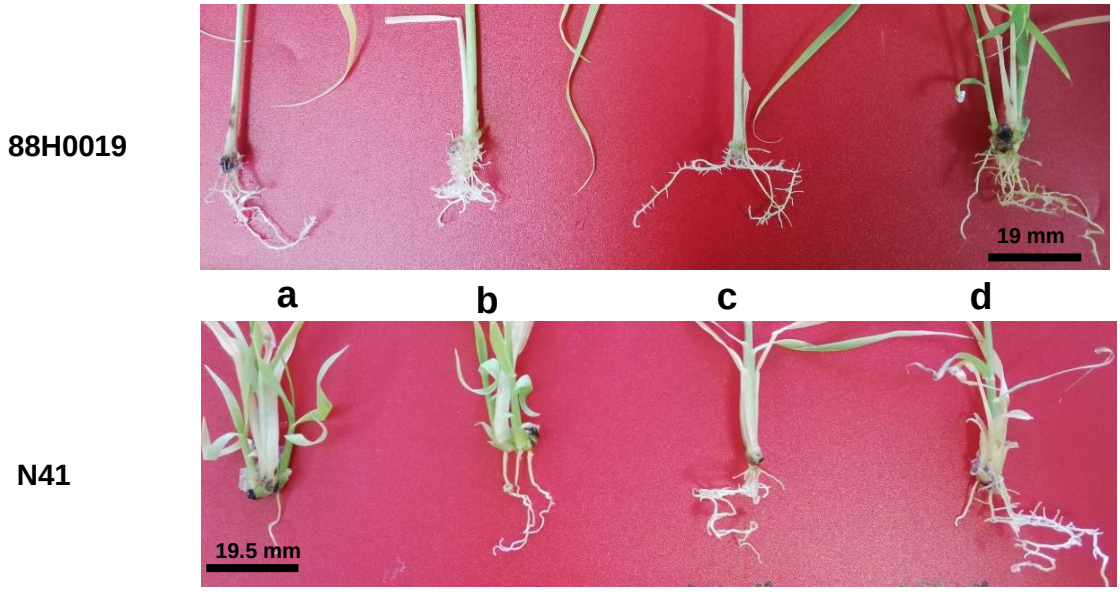


Figure 18: The effect of IAA on root growth of plantlets over 3 weeks for cultivars 88H0019 and N41. a) control; b) 150 nM, c) 300 nM, and d) 460 nM IAA.

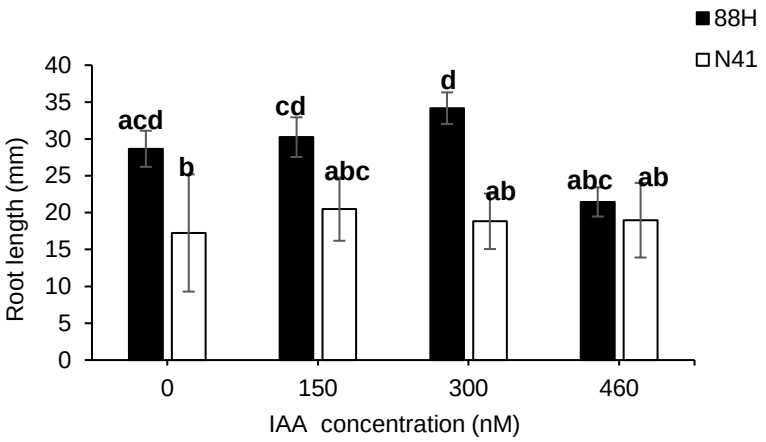


Figure 19: The incorporation of IAA (0 - 460 nM) in the rooting media and its effect on the root length of plantlets for cultivars 88H0019 and N41. The results were obtained after 3 weeks. Root length was only recorded for roots ≥ 10 mm. Dissimilar alphabet characters denote a statistically significant difference amongst cultivars for each concentration. Data sets were analysed by One-way ANOVA, Duncan's multiple range test. $p < 0.05$, $n = 5$, mean ± SE.

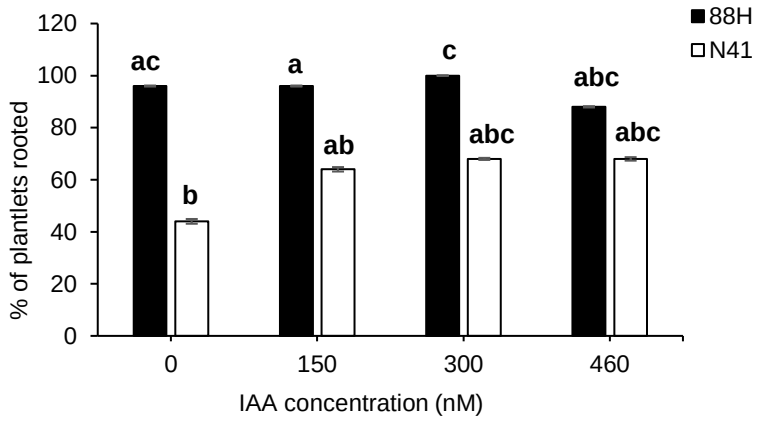


Figure 20: The incorporation of IAA (0 - 460 nM) in the rooting media and its effect on the % of plantlets rooted for cultivars 88H0019 and N41. The results were obtained after 3 weeks. Re-rooting is defined as roots ≥ 10 mm. Dissimilar alphabet characters denote a statistically significant difference amongst cultivars for each concentration. Data sets were analysed by One-way ANOVA, Duncan's multiple range test. $p \geq 0.05$, $n = 5$, mean ± SE.

In terms of root and shoot fresh mass for cultivar 88H0019, there were no significant differences in shoot fresh mass (g) amongst all the tested IAA concentrations (Fig. 21a). However, for cultivar 88H0019, the shoot fresh mass (g) at 300 nm IAA (0.363 g ± 0.059) was significantly higher than

all the tested IAA concentrations for cultivar N41; control (0.180 g ± 0.09), 150 (0.220 g ± 0.06), 300 (0.190 g ± 0.04) and 460 nM IAA (0.230 g ± 0.05) ($p = 0.004$). For cultivar N41, there was a significant increase in shoot dry mass (g), recorded at 460 nM IAA (0.140 g ± 0.08) when compared with the control and the other IAA treatments ($p = 0.009$) (Fig. 21b).

For cultivar N41, the root fresh mass (g) of plantlets was not significantly affected by the exogenous supply of IAA at any of the tested concentrations (Fig. 22a). However, for cultivar 88H0019 the results recorded indicated a significant increase in root fresh mass (g) for all the tested IAA concentrations ($p < 0.001$). For cultivar 88H0019, the root fresh mass (g) for 150 nM (0.091 g ± 0.011) and 300 nM IAA treatments (0.087 g ± 0.017) were significantly higher than the control (0.052 g ± 0.004) and 460 nM IAA treatments (0.056 g ± 0.010) ($p < 0.001$). For cultivar N41, there was no significant difference in root dry mass (g) for all the tested IAA concentrations (Fig. 24b). For cultivar 88H0019, the results indicate a significant decrease in root dry mass (g) across the tested IAA concentrations ($p < 0.001$) (Fig. 22b). The 150 nM IAA treatment (0.034 g ± 0.004) had a higher root dry mass (g) than the other IAA treatments (Fig. 22b). In conclusion, for both cultivars, the results obtained indicate that the exogenous application of IAA has no significant positive effect on *in vitro* plantlets in terms of root length and % rooting whereas the CF does, suggesting that the enhanced root growth could be due to auxin (IAA) production or ACC deaminase activity by the CF (Jaroszuk-Ścisel et al. 2019). The CF was not included as part of the selection protocol because of the confounding effects observed on the root characteristics of plantlets and callus proliferation after exposure to the *E. saccharina* beneficial *Fusarium* strain (SC17).

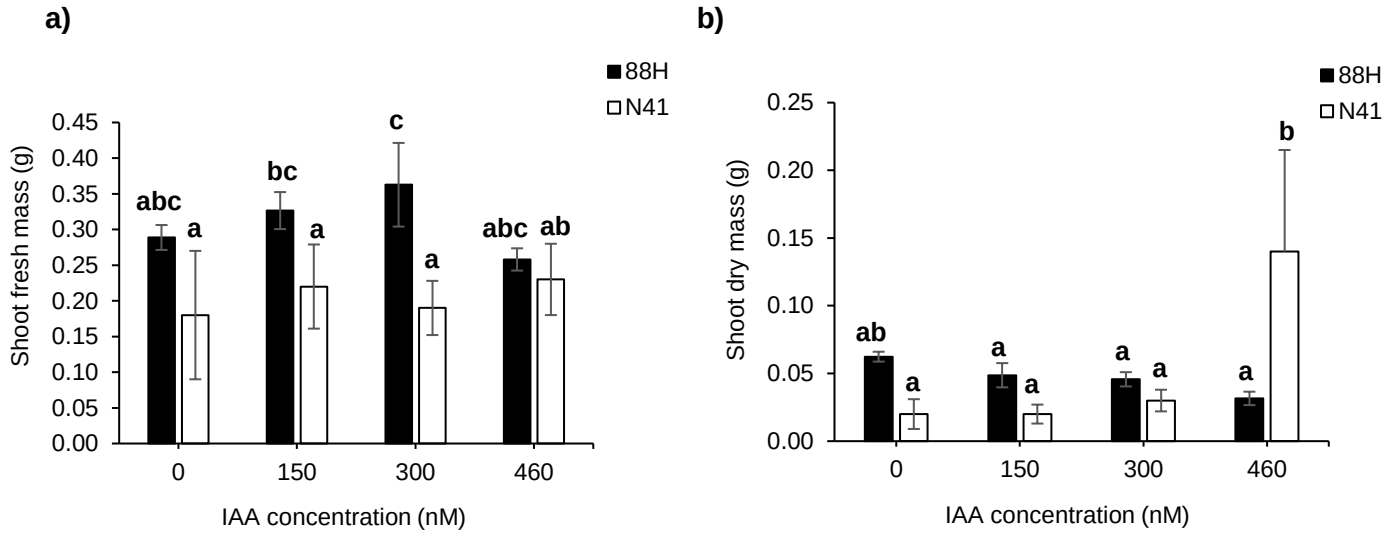
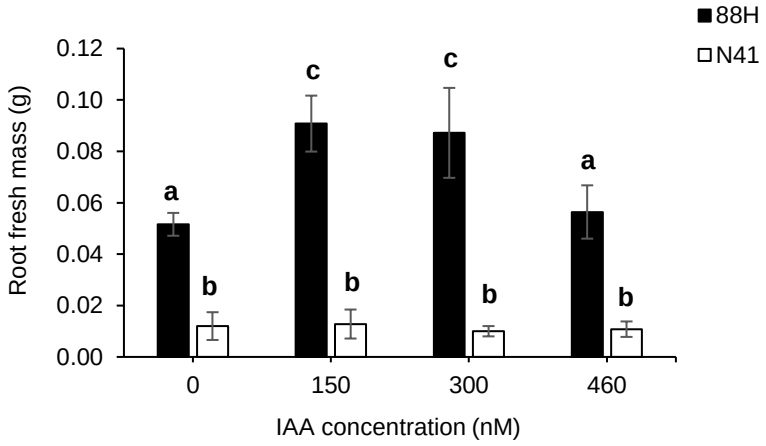


Figure 21: The incorporation of IAA (0 - 460 nM) in the rooting media and its effect on a) shoot fresh mass (g) and b) shoot dry mass (g) of *in vitro* plantlets for cultivars 88H0019 and N41. The results were obtained after 3 weeks in culture. Fresh and dry mass was recorded for plantlets that have re-rooted to ≥ 10 mm. Dissimilar alphabet characters denote a statistically significant difference amongst cultivars for each concentration. Data sets were analysed by (One-way ANOVA, Duncan's multiple range test, $p < 0.05$, $n = 5$, mean ± SE).

a)



b)

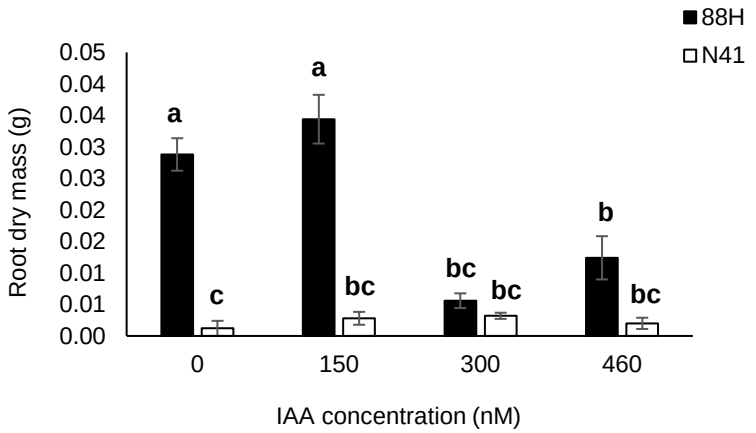


Figure 22: The incorporation of IAA (0 - 460 nM) in the re-rooting media and its effect on a) root fresh mass (g) and b) root dry mass (g) for cultivars 88H0019 and N41. The results were obtained after 3 weeks in culture. Fresh and dry mass was recorded for plantlets that have re-rooted to ≥ 10 mm. Dissimilar alphabet characters denote a statistically significant difference amongst cultivars for each concentration. Data sets were analysed by One-way ANOVA, Duncan's multiple range test, $p < 0.05$, mean ± SE, n = 5.

4.4 Mutagenesis and priming for *Fusarium pseudonygamai* and *E. saccharina saccharina* tolerance *in vitro*

The objective of this study was to establish a protocol for developing putative sugarcane mutants that had been exposed to a suitable priming agent and with/without a mutagenic treatment. The chemical mutagens that was used in this study were 16 mM ethyl methanesulfonate (EMS) and 100 µM 5-AzaCytidine (5-AzaC) according to Koch et al. (2012) and Munsamy et al. (2013) respectively. The purpose of using a combination of mutagens was to generate epigenetic (5-AzaC via demethylation; Grzybkowska et al., 2018) and genetic (EMS induces point mutations; Hoffmann, 1980; Lethin *et al*., 2020) variation in the cells. To supplement this approach, *in vitro* priming of the demethylated epigenetic state during remethylation (Hx primed) could further increase *E. saccharina* resistance both directly, through enhancing JA responses, and indirectly by reducing susceptibility to *Fusarium* mycotoxins (Ravensdale et al., 2014; Llorens et al., 2016).

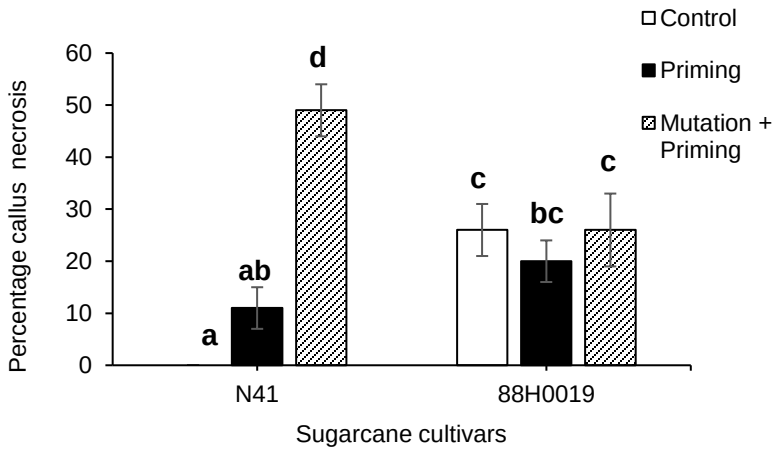
For this study, the priming agent Hx was selected based on the preliminary results obtained for determining the most effective concentration of the priming agent concentration at the embryo maturation and plantlet regeneration stages (section 4.1). To develop a protocol that combined

mutagenesis followed by priming or priming only, the following treatments were investigated: i) no priming and no mutagens (control); ii) primed only with hexanoic acid; and iii) primed with hexanoic acid and exposed to two mutagens, EMS and 5-AzaC. Due to technical difficulties the protocol development for using CF as a selection agent at the embryo germination and regeneration stage was not conducted. Hence, it was decided that mutated/primed plantlets would be produced (as mentioned in section 3.3) as unselected 'families' (not selected using CF *in vitro*) but rather screened with *Fusarium* SC17 and *E. saccharina* larvae *ex vitro* at the plantlet stage.

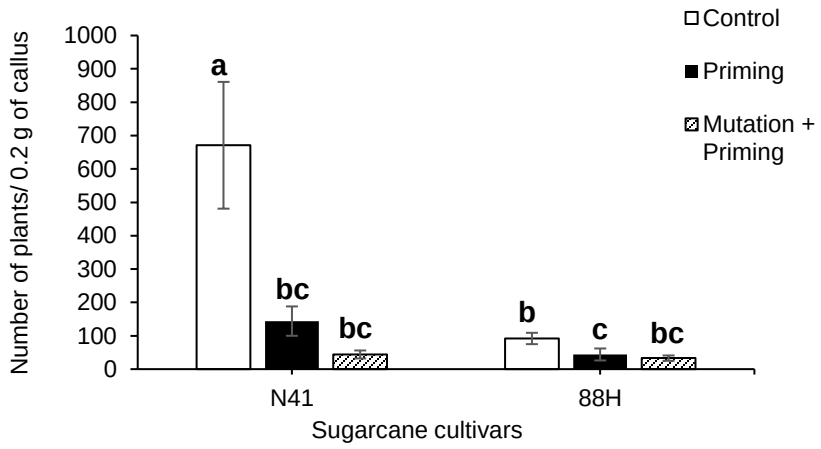
For the protocol that exposed calli to the priming agent only, eight-week-old embryogenic calli were cultured on EMM media with no exposure to mutagens for 1 week (Fig. 7a). The calli were exposed to the priming agent only at the embryo maturation and germination stages. The embryogenic calli (0.2 g) were cultured on EMM with 0.6 mM Hx for 2 weeks, cultured on EGM1, then cultured on EGM1 containing the same concentration of Hx as mentioned above. For the combined treatment (primed + mutated), the embryogenic callus produced after 8 weeks were exposed to both mutagens and the priming agent. Based on the results shown in Munsamy et al. (2013), the callus was exposed to 32 mM EMS for 4 hours and cultured on EMM with 100 µM 5-AzaC for 1 week. Thereafter, the calli was cultured on EMM with 0.6 mM Hx for 2 weeks. The surviving calli were cultured on EGM1 with the same concentration of Hx for 4 - 12 weeks and maintained in the light room. The cultures were then assessed in terms of % callus necrosis, the number of plants/0.2 g of callus, and the number of abnormal plants/0.2 g of callus. As before a piece of callus was considered necrotic if greater than 50 % appeared brown to black.

For both treatments, it was observed that each cultivar responded differently (Fig. 23a). For cultivar N41, there was no significant difference in callus necrosis between the control and the treatment that used a priming agent only. As expected, no callus necrosis was recorded for the control for cultivar N41 as they had not been exposed to the priming agent at the maturation and germination stages. However, for cultivar 88H0019 a low percentage of callus necrosis was recorded. The response of different sugarcane cultivars *in vitro* is variable, and this may the reason for the difference in callus necrosis observed between the controls for both cultivars. For cultivar 88H0019, there were no significant differences in callus necrosis for both treatments when compared with the control ($p > 0.001$) (Fig. 23a). There was a significant negative effect on callus necrosis by the combined treatment (mutagens + priming agent) (49 % ± 5) than control and the other treatment for both cultivars ($p > 0.001$).

a)



b)



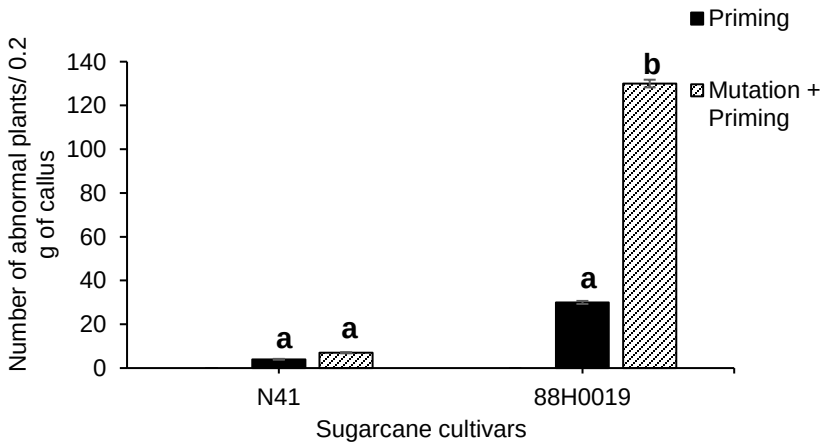


Figure 23: The effect of priming calli and *in vitro* plantlets with Hx and a combined treatment (5-AzaC, EMS, and Hx). Results were recorded after 8-12 weeks. a) percentage callus necrosis; b) number of plants/0.2 g of callus; and the number of abnormal plants/0.2 g of callus. Data sets were analysed by One-way ANOVA, Duncan's multiple range test, $p < 0.001$, $n = 20$, mean ± SE.

The surviving calli were then transferred on EGM1 containing the selected concentration of Hx (0.6 mM) and the number of plants/ 0.2 g callus was recorded (Fig. 23b). Plantlet production was recorded across all treatments and both cultivars. There was a significantly higher number of plants produced/0.2 g of callus for the control for cultivar N41 (671 plantlets/0.2 g of callus) than the other two treatments for both cultivars ($p < 0.001$). There were no significant differences in the number of plants produced/0.2 g callus between the primed and the combined treatment of 5-AzaC, EMS, and Hx for both cultivars ($p > 0.001$). As expected, there was an inverse proportional relationship between percentage callus necrosis and the number of plants/0.2 g callus, with the treatments that recorded the highest callus necrosis produced the lowest number of plants (Fig. 23a, b). In conclusion, the results indicated that the combined treatment (mutagens + priming) resulted in high callus necrosis and a small number of plants/0.2 g of callus.

Plantlets that were derived from the primed, and mutation + primed treatment-derived calli were developed slowly during the germination stage, as it took 10 - 12 weeks to get shoots (approximately 15 mm in height) compared with those from the control treatment which took 8 weeks. For both cultivars, the controls did not produce abnormal plants. A small percentage of abnormal plants, appearing albino and chimeric/dwarf-like were recorded for both treatments (Fig. 23c). There were no significant differences across the treatments for cultivar N41. However, for cultivar 88H0019 the number of abnormal plants produced from the mutation + primed treatment (130 abnormal plantlets ± 1.76) was significantly higher than the priming treatment ($p < 0.001$). The results obtained indicate that EMS exposure inhibits callus proliferation and development. Kona et al. (2019) reported on the effect of high concentrations of EMS and 2,4-D, in which higher concentrations inhibited callus proliferation.

4.5 Selection of plants tolerant to only *F. pseudonygamai* inoculation, dual inoculation with *E. saccharina* larvae and *F. pseudonygamai* using an *ex vitro* screening method

To evaluate the best protocol to achieve *E. saccharina* resistant mutants, chemical mutagenesis followed by priming or priming only was conducted on embryogenic calli and plantlets. Thereafter, the putative mutants were screened *ex vitro* by inoculating the plants with *F. pseudonygamai* SC17 and *E. saccharina* larvae. Plants used for screening were produced by exposing embryogenic calli to 5-AzaC and EMS, and then primed with Hx or exposed to the priming agent only using the established protocol. From each treatment, fifty *in vitro* plantlets were selected

randomly for acclimatisation. Plantlets were acclimatised for 8 - 9 months before they were used for *ex vitro* screening. For each treatment, plants were divided equally depending on the number of plants that survived acclimatisation. These plants were then tested for their response to *E. saccharina* and *F. pseudonygamai* SC17 inoculations in the glasshouse.

The objective of this study was to determine response to tissue colonisation by *Fusarium pseudonygamai* SC17 and combined fungal and *E. saccharina* larval inoculations in sugarcane cultivars N41 and 88H0019. Hence, this investigation focussed on two approaches to test for tolerance *E. saccharina* and *F. pseudonygamai.* Half of the *in vitro* plants were inoculated only with *Fusarium* SC17, using the toothpick inoculation method. The other half of the plants were dual inoculated: once with *Fusarium* SC17, using the toothpick inoculation method and 2 weeks later with 1-2 2nd instar *E. saccharina* larvae that were placed into the leaf whorls. To confirm tolerance of the putative mutant plants to *E. saccharina* and *F. pseudonygamai* SC17, fungal isolations were performed on the stem sections from above the inoculation lesion from symptomatic and asymptomatic plants. Lesion severity ratings were recorded for these plants.

4.5.1 Re-isolation of *F. pseudonygamai* SC17 from putative-tolerant plants

The presence of *F. pseudonygamai* SC17 was confirmed in stems of asymptomatic and symptomatic plants two months after inoculation, by surface sterilizing and culturing transverse sections of the stem on selective Nash and Snyder (1962) medium (Fig. 24a - c). To confirm the effectiveness of the surface sterilization, stem sections were pressed on PDA media to test for microbial growth, of which all samples were negative. *Fusarium*-like colonies grew from the lesions of both the asymptomatic and symptomatic stem sections. There was no growth observed from the stem sections of the non-inoculated plants. *F. pseudonygamai* could not be re-isolated from undamaged tissue above the inoculation lesion in two of the fifteen asymptomatic plants from all treatments (Table 7 and 8), for both cultivars that were only inoculated with only SC17. The fungus was retrieved from the other thirteen asymptomatic plants. *F. pseudonygamai* could not be re-isolated from the undamaged tissue in five of the eight asymptomatic plants for N41 and 88H0019 from all treatments for putative mutants that were dual inoculated with the fungus and *E. saccharina* larvae (Table 9 and 10). The fungus was retrieved from the stem sections of both symptomatic and asymptomatic plants.

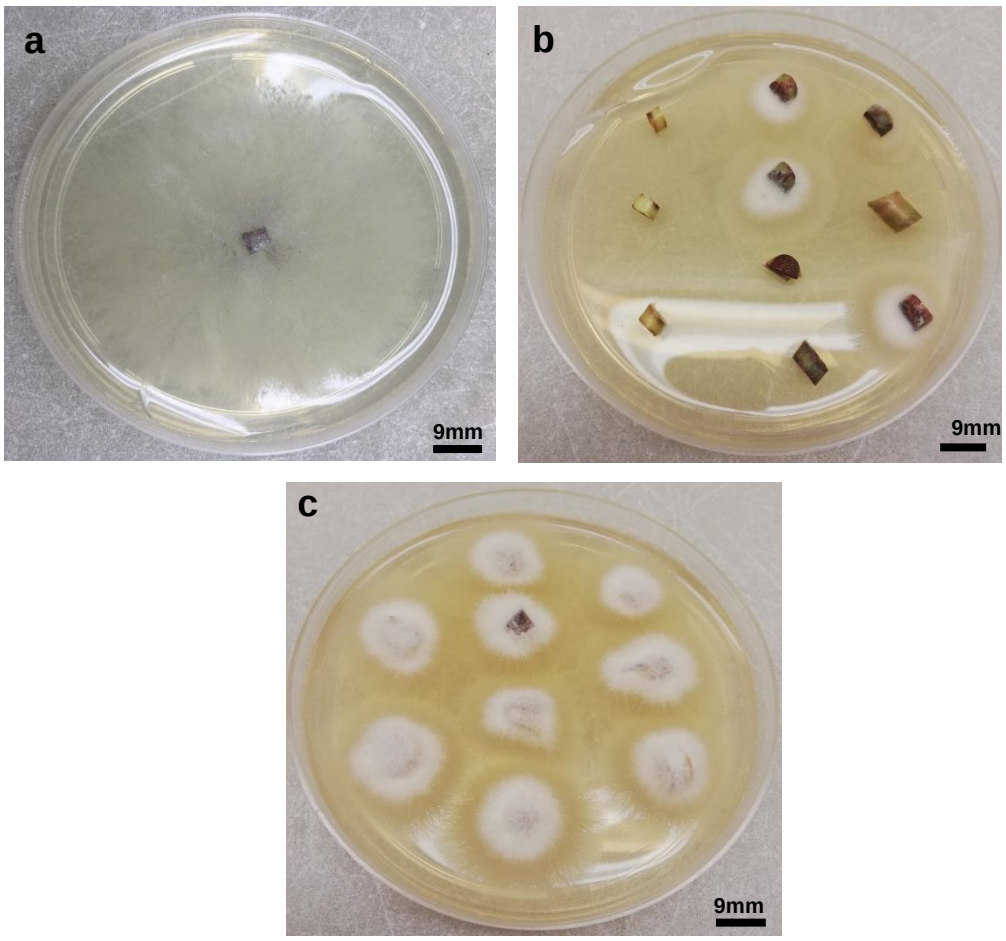


Figure 24: A visual comparison of *F. pseudonygamai* SC17 and the fungal isolates that were retrieved from the inoculated plants, after two months. a) SC17; b) fungus re-isolated from damaged stem sections from an inoculated plant treated with only the priming agent Hx (cultivar N41) and c) fungus re-isolated from damaged stem sections from an inoculated control plant (cultivar 88H0019).

4.5.2 Detection and confirmation of the identity of *F. pseudonygamai*

To confirm the identity of the fungal isolates obtained from asymptomatic, symptomatic, and dead plants, fungal genomic DNA was extracted. Molecular analysis was conducted using elongation factor (EF) primers and banding patterns were compared with that of *F. pseudonygamai* SC17 (positive control) to confirm their identity. For both cultivars, the banding patterns generated from all the retrieved fungal isolates were like SC17 (positive control) for all the treatments (Fig. 25). However, some fungi that were re-isolated from a few plants for both cultivars could not be

identified. These isolates that were retrieved and could not be confirmed were from the following treatments: a) Lane 25 - combined mutagenic and primed treatment for cultivar N41; b) Lane 38 - primed with hexanoic acid treatment for cultivar N41; c) Lane 45 - 46 – cultivar 88H0019 control plants; and d) Lane 53 - combined mutagenic and primed treatment for cultivar 88H0019 (Fig. 25).

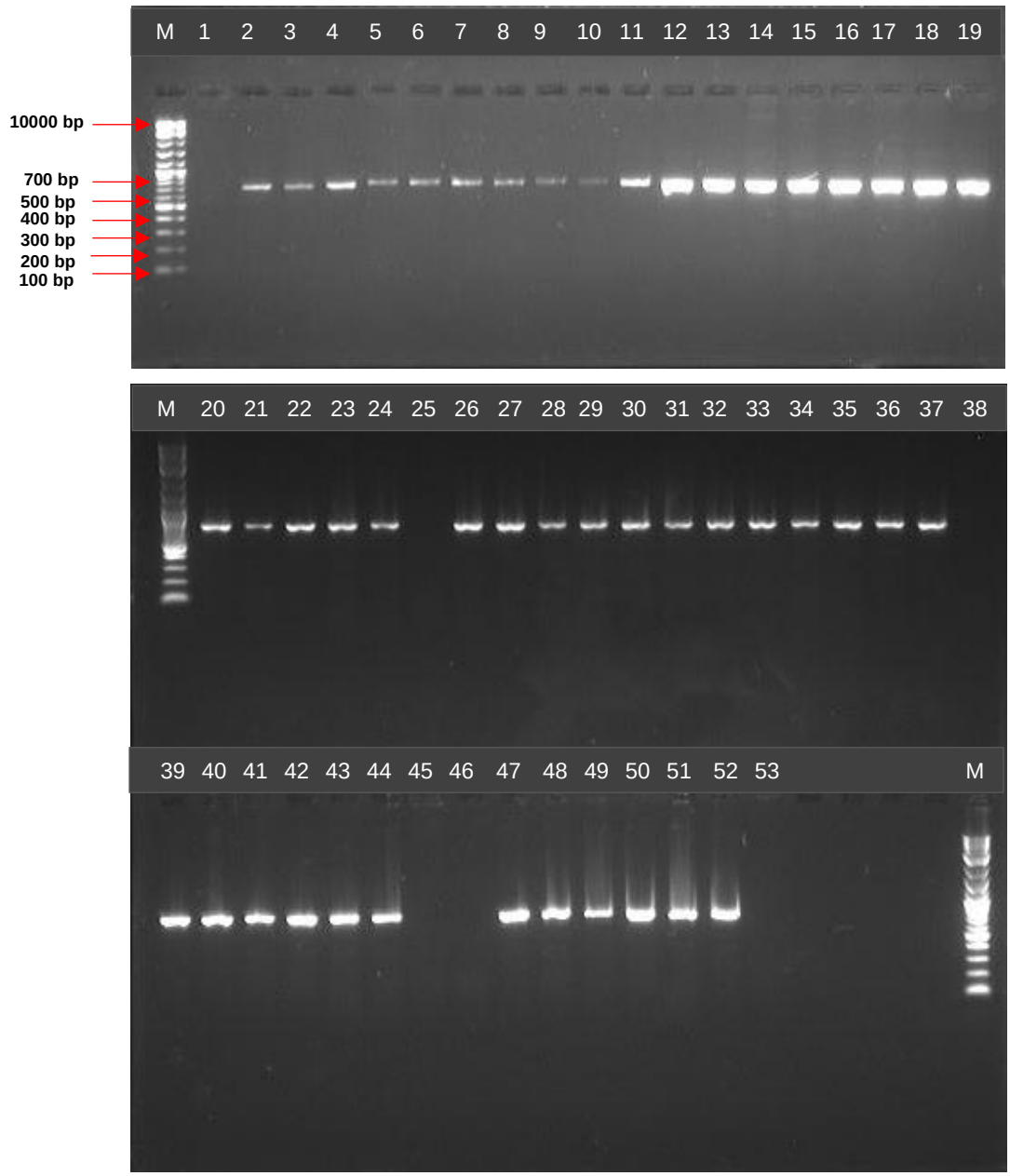


Figure 25: A comparison of banding patterns of the fungal isolates obtained from the inoculated plant tissue and *F. pseudonygamai* SC17 using the elongation factor primers. The isolates were retrieved from symptomatic plants, dead plants and asymptomatic plants by surface sterilizing

leaves and stems and placing cut sections on Nash and Snyder agar. 1kb plus DNA ladder, Lane 1 - negative control (water), 2 - SC17 isolate

4.5.3 *Ex vitro* screening of plantlets using *F. pseudonygamai* SC17 and *E. saccharina* larvae

The infected plants exhibited symptoms after being inoculated with only F. *pseudonygamai* and dual inoculated with *F. pseudonygamai* SC17 and *E. saccharina* (Fig. 26a - d). The reaction to SC17 and *E. saccharina* of the putative mutant plants was confirmed by re-isolating the fungus as well as assessing the lesion severity of the stalks. Lesion severity is a parameter of plant disease intensity that is usually required for the comparison of phenotypes for disease resistance, understanding yield loss, and assessing the effects of different treatments on disease (Kranz, 1988; Bock et al., 2010; Bock et al., 2021). Lesion severity ratings were recorded as 0= no lesions, 1= mild, 2= moderate and 3= severe (Fig. 27a - d).

For cultivar N41, four of the seven control plants and all the 88H0019 control plants, which were inoculated with SC17 colonised toothpicks displayed symptoms. These results are per the current *E. saccharina* rating [N41: intermediate-resistant to *E. saccharina* (Zhou, 2013) and 88H0019: susceptible to *E. saccharina*]. These control plants were regarded as susceptible with a mean lesion severity rating (LSR) of 2. There was only 1 dead plant for cultivar 88H0019 after 2 months. Three plants from the control for cultivar N41 were asymptomatic and showed a mean LSR of 2, these were regarded as resistant. There were no adverse effects seen on the plants that were stabbed with sterile toothpicks (negative control) (Table 7 and 8). For cultivar N41, nine out of the twenty-one putative mutants were asymptomatic and exhibited a mean LSR 1, 8 weeks after toothpick stab inoculation with only the *Fusarium pseudonygamai* SC17 culture filtrate. For cultivar 88H0019, three of the eleven putative mutants were asymptomatic. For both cultivars, re-isolation of the fungal isolates from the lesion area and/or undamaged tissue from all the asymptomatic plants were conducted. Re-isolation of *Fusarium* from the undamaged tissue from seven of these plants indicated that they permitted potential endophytic colonisation by *F. pseudonygamai* SC17 and were regarded as being tolerant to the fungus (Table 7). There was one asymptomatic plant from N41 in which the fungus was re-isolated from the lesion only, with no lesion severity. As a result, this plant was classified as resistant to the fungus as it appeared to limit *F. pseudonygamai* SC17 proliferation and growth (Table 7). All six control plants for 88H were susceptible with a mean LSR of 2 (Table 8).

Figure 26: Illustration showing dead plants and plants with symptoms after inoculation with *Fusarium pseudonygamai*. a) dead growing point; b) dead plant 7-8 weeks after inoculation; c) chlorosis and necrosis; d) crinkling and chlorosis 3-7 weeks after stabbing stems with *Fusarium pseudonygamai* colonised toothpick

Some of the putative mutant plants were dual inoculated, i.e., were inoculated with *F. pseudonygamai* SC17 and 2 weeks later with 1-2 2nd instar larvae that were placed into the leaf whorls the plants. The tolerance and resistance of these *in vitro* mutated plants were confirmed using the same method for the plants inoculated with only *F. pseudonygamai* SC17. For both cultivars, there were no plants from the control that were asymptomatic (Table 9 and 10). For cultivar N41, two months after the dual inoculation, seven out of the twenty inoculated plants from both treatments were asymptomatic and exhibited a mean LSR of 1. The fungal isolate was isolated from the undamaged tissue of three of these plants and was regarded as being tolerant to the fungus (Table 9). The other five symptomless plants were regarded as resistant since the fungus was only re-isolated from the inoculated lesions, this includes treatments 1-2 for N41 (Table 9). For 88H0019, there was one out of nine plants for both treatments that were asymptomatic with an LSR of 2, this was regarded as resistant. (Table 10). All the 88H0019 control plants exhibited severe symptoms and were regarded as susceptible.

Table 7: A summary of the inoculated plants and their responses to tissue colonisation by *F. pseudonygamai* SC17 in sugarcane cultivar N41, 2 months after toothpick stab inoculation method. Plants were inoculated with only *Fusarium* SC17. The plants that were used for *ex vitro* screening were exposed to chemical mutagens followed by priming (treatment 1) or exposed to only priming (treatment 2). The external symptoms were recorded as: 0- no symptoms, 1- crinkled leaves, 2- chlorotic leaves, 3- necrotic leaves, 4- dead growing point, 5- wilting, and 6- all symptoms. Lesion severity ratings were assessed using a scoring system: 0- no lesion, 1- mild lesion, 2- moderate lesion, and 3- severe lesion.

Treatment	Line number	External symptoms	Severity of lesion	Dead (D) or Alive (A)	Re-isolation on NS agar from lesion	Re-isolation on NS agar from undamaged areas above lesion	Tolerant, Susceptible or Resistant
Control (uninoculated)	#1	0	0	A	N	N	-
	#2	0	0	A	N	N	-
	#3	0	0	A	N	N	-
Control (inoculated)	#5	2	2	A	Y	N	S
	#9	0	2	A	Y	N	R
	#9	2	1	A	Y	N	S
	#10	0	2	A	Y	N	R
	#13	2	3	A	Y	N	S
	#15	2	2	A	Y	N	S
	#15	0	3	A	Y	N	R
Treatment 1 *(In-vitro mutagenesis* with Azac and EMS + in-vitro priming with hexanoic acid)	#6	2	0	A	N	Y	S
	#6	2	0	A	N	Y	S
	#8	2	0	A	N	Y	S
	#11	0	0	A	Y	Y	T
	#11	2	0	A	N	Y	S
	#11	0	1	A	Y	Y	T
	#12	0	1	A	Y	Y	T
	#12	0	0	A	Y	Y	T

	#16	2	1	A	Y	N	S
	#16	0	0	A	Y	N	R
	#17	2	3	A	Y	N	S
	#17	2	1	A	Y	N	S
	#17	2	3	A	Y	N	S
	#17	2	0	A	Y	N	S
Treatment 2	#8	0	0	A	N	Y	T
(*In-vitro priming* with hexanoic acid)	#9	0	0	A	N	Y	T
	#10	0	0	A	Y	Y	T
	#10	0	0	A	Y	Y	T
	#10	2	3	A	Y	N	S
	#13	1,2,3,5	3	A	Y	N	S
	#17	2	0	A	N	Y	S

Table 8: A summary of the inoculated plants and their responses to tissue colonisation by *F. pseudonygamai* SC17 in sugarcane cultivar 88H0019, 2 months after toothpick stab inoculation method. Plants were inoculated with only *F. pseudonygamai* SC17. The plants that were used for *ex vitro* screening were exposed to chemical mutagens followed by priming (treatment 1) or exposed to only priming (treatment 2). The external symptoms were recorded as: 0- no symptoms, 1- crinkled leaves, 2- chlorotic leaves, 3- necrotic leaves, 4- dead growing point, 5- wilting, and 6- all symptoms. Lesion severity ratings were assessed using a scoring system: 0- no lesion, 1- mild lesion, 2- moderate lesion, and 3- severe lesion.

Treatment	Line	External symptoms	Severity of lesion	Dead (D) or Alive (A)	Re-isolation on NS agar from the lesion	Re-isolation on NS agar from undamaged areas above the lesion	Tolerant, Susceptible, Resistant
Control (inoculated)	#16	1,2	0	A	N	N	S
	#18	1,2,3,4	3	A	Y	N	S
	#14	2	3	A	Y	N	S
	#18	1,2,3,5	1	A	N	N	S
	#17	2	3	A	Y	N	S
	#14	3,5	3	D	N	N	S
Treatment 1 (*In vitro* priming with hexanoic acid + in-vitro mutagenesis with 5-AzaC and EMS)	#21	0	2	A	Y	Y	T
	#1	1,2,3	2	A	N	N	S
	#4	2,3	1	A	N	Y	S
	#1	1,2,3	2	A	N	N	S
Treatment 2 (*In vitro* priming with hexanoic acid)	#7	1,2	1	A	N	Y	S
	#7	1,2	1	A	N	Y	S
	#17	0	2	A	Y	Y	T
	#11	0	1	A	Y	Y	T
	#19	2	3	A	Y	N	S
	#11	3,4	2	D	Y	N	S
	#7	2	0	A	N	N	S

Table 9: A summary of the inoculated plants and their response to tissue colonisation by *F. pseudonygamai* SC17 in sugarcane cultivar N41, 2 months after toothpick stab inoculation method. Plants were inoculated with *F. pseudonygamai* SC17, and, 2 weeks later, with 1-2 2nd instar *E. saccharina* larvae that were placed into the leaf whorls the plants. The plants that were used for *ex vitro* screening were exposed to chemical mutagens followed by priming (treatment 1) or exposed to only priming (treatment 2). The external symptoms were recorded as: 0- no symptoms, 1- crinkled leaves, 2- chlorotic leaves, 3- necrotic leaves, 4- dead growing point, 5- wilting, and 6- all symptoms. Lesion severity ratings were assessed using a scoring system: 0- no lesion, 1- mild lesion, 2- moderate lesion, and 3- severe lesion.

Treatment	Line	External symptoms	Severity of lesion	Dead (D) or Alive (A)	Re-isolation on NS agar from the lesion	Re-isolation on NS agar from undamaged areas above the lesion	Tolerant, Susceptible, or Resistant
Control (uninoculated)	#1	0	0	A	N	N	-
	#2	0	0	A	N	N	-
	#3	0	0	A	N	N	-
Control (inoculated)	#9	6	2	A	Y	N	S
	#1	6	3	A	Y	N	S
	#6	1,2,3,5	2	A	Y	N	S
	#11	6	3	A	Y	N	S
	#1	1,2,3	3	A	Y	N	S
	#13	1,2,3,5	2	A	Y	N	S
	#10	1,2,3,5	3	D	N	N	S
	#5	6	3	D	N	N	S
	#15	1,2,3	2	D	N	N	S
	#5	1,2,3,4	2	A	Y	N	S
	#6	6	3	D	N	N	S
Treatment 1 (*In vitro* mutagenesis with 5-AzaC and EMS + *in vitro* priming with hexanoic acid)	#8	1,3,4	3	A	Y	N	S
	#8	1,2,4,5	2	A	Y	N	S
	#6	2,5	0	A	N	N	S
	#16	0	1	A	Y	N	R
	#17	0	1	A	Y	N	R
	#16	0	1	A	Y	Y	T
	#6	1,2	2	A	Y	N	S
	#1	0	1	A	Y	Y	T

	#4	2,4	2	A	Y	N	S
Treatment 2	#13	1,2,3	0	A	N	Y	S
(*In vitro* priming	#17	0	1	A	Y	N	R
with hexanoic	#9	0	1	A	Y	N	R
acid)	#8	1,2,5	2	A	Y	N	S
	#13	2	2	A	Y	N	S
	#9	2	2	A	Y	N	S
	#17	2	1	A	N	N	S
	#2	2,4	0	A	N	Y	S
	#17	1,2,3	0	A	N	Y	S
	#17	0	3	A	Y	N	R
	#9	1,2,3	2	A	Y	N	S

Table 10: A summary of the inoculated plants and their response to tissue colonisation by *F. pseudonygamai* SC17 in sugarcane cultivar 88H0019, 2 months after toothpick stab inoculation method. Plants were inoculated with *F. pseudonygamai* SC17, and, 2 weeks later, with 1-2 2nd instar *E. saccharina* larvae that were placed into the leaf whorls the plants. The plants that were used for *ex vitro* screening were exposed to chemical mutagens followed by priming (treatment 1) or exposed to only priming (treatment 2). The external symptoms were recorded as: 0- no symptoms, 1- crinkled leaves, 2- chlorotic leaves, 3- necrotic leaves, 4- dead growing point, 5- wilting, and 6- all symptoms. Lesion severity ratings were assessed using a scoring system: 0- no lesion, 1- mild lesion, 2- moderate lesion, and 3- severe lesion.

Treatment	Line	External symptoms	Severity of lesion	Dead (D) or Alive (A)	Re-isolation on NS agar from the lesion	Re-isolation on NS agar from undamaged areas above the lesion	Tolerant, Susceptible, Resistant
Control (inoculated)	1	1,2	0	A	Y	N	S
	3	6	3	D	Y	N	S
	1	6	3	D	N	N	S
Treatment 1 (*In vitro* priming with hexanoic acid + in-vitro mutagenesis with 5-AzaC and EMS)	3	1,2	3	A	Y	N	S
	#8	0	2	A	Y	N	R
	#4	1,2,3	2	A	Y	N	S
	#10	1,2,3	0	D	N	N	S
	#3	2	1	A	Y	N	S
Treatment 2 (*In vitro* priming with hexanoic acid)	#7	1,2,3,4	0	D	N	N	S
	#3	1,2,3,4	3	A	N	N	S
	#15	1,2,3,4	3	A	N	N	S
	#17	1,2,3,5	2	D	N	N	S
	#19	1,2,3,4	3	D	N	N	S

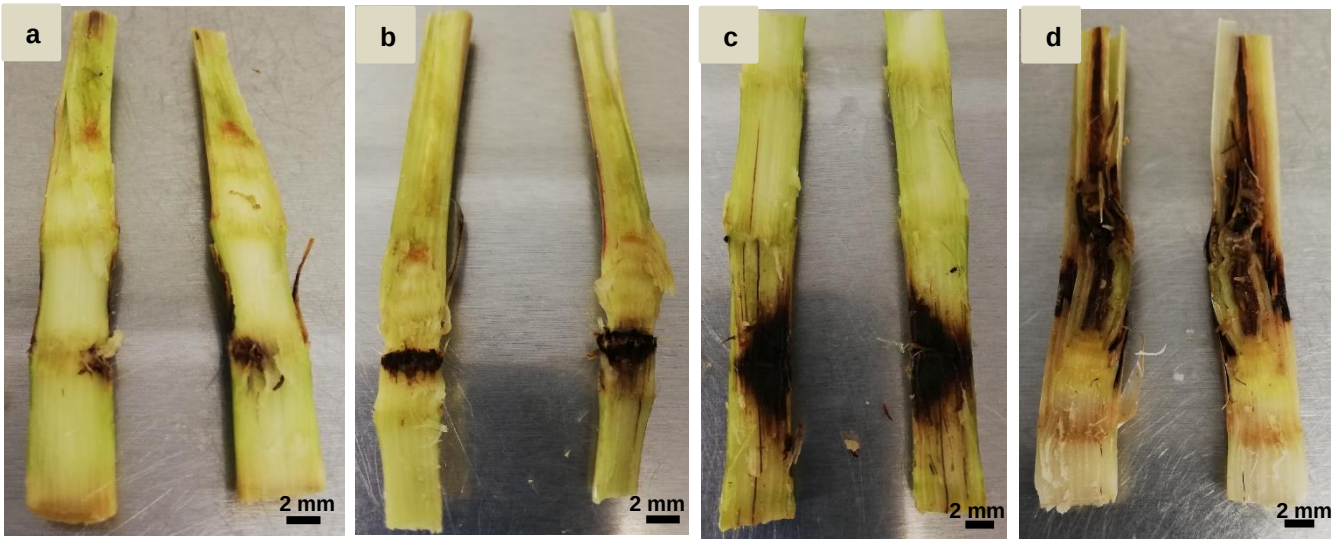


Figure 27: A comparison of lesion severity in the inoculated stems sections of the plants: a) no lesion; b) mild lesion; b) moderate lesion, and d) severe lesion. Plants were stabbed 2-3 cm from the bottom of the stalk with toothpicks colonised with *F. pseudonygamai* SC17. Some plants (not shown) were dual inoculated after 2 weeks with 2nd instar *E. saccharina* larvae, stems were collected after 7 weeks for re-isolation.

As mentioned above, the tolerance and resistance to *F. pseudonygamai* SC17 and *E. saccharina* of the putative mutant plants was confirmed by re-isolating the fungi and by assessing lesion severity of the stalks. For cultivar 88H0019, there was a significant decrease in the lesion severity ratings of the putative mutant plants that were inoculated with only SC17 for the primed and combined treatment compared with the control ($p = 0.002$) (Fig. 28). For cultivar N41, there was a significant decrease in the lesion severity ratings of the putative mutant plantlets that were inoculated with only SC17 for the primed and combined treatment compared with the control ($p = 0.002$). However, there was no significant difference in the lesion severity ratings between both cultivars across the treatments.

For cultivar 88H0019, there was a significant decrease in the lesion severity ratings of the putative mutant plants that were dual inoculated with SC17 and the 2nd instar *E. saccharina* larvae, between the control and mutation + primed treatment ($p < 0.001$) (Fig. 29). For cultivar N41, there was a significant decrease in the lesion severity ratings for the primed and combined treatment when compared with the control ($p < 0.001$). In the primed treatment, cultivar N41 showed a significant decrease in lesion severity ratings when compared with cultivar 88H0019 ($p < 0.001$). In conclusion, these results indicated that the putative mutant plants that were primed with Hx only and treated with a combination of mutagens (EMS and 5-AzaC) and the priming agent Hx exhibited a significant decrease in lesion severity.

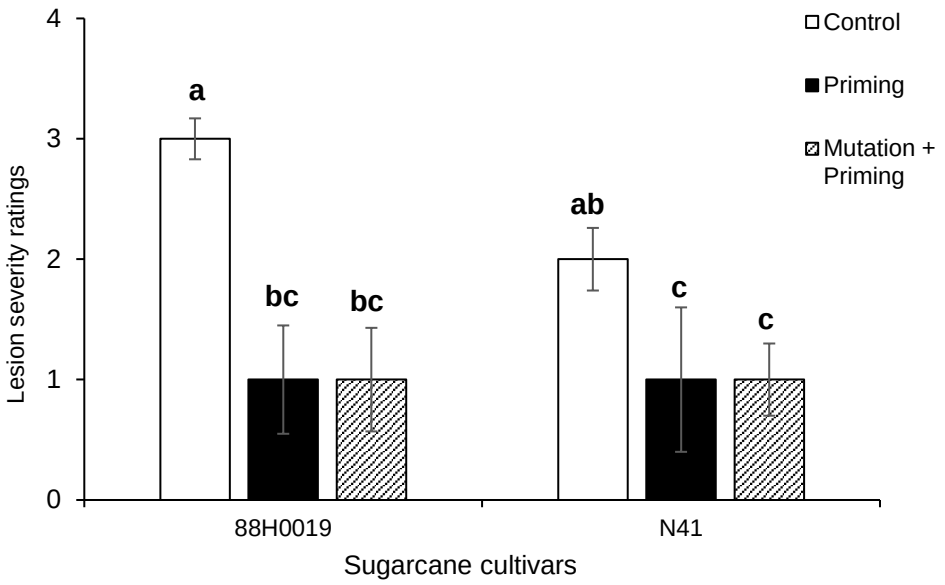


Figure 28: The lesion severity ratings of plantlets that were inoculated with only *Fusarium* SC17 for cultivars 88H0019 and N41**.** Dissimilar alphabet characters denote a statistically significant difference amongst treatments for each concentration. Data sets were analysed by One-way ANOVA and Duncan's multiple range test. P ≥ 0.05, n = 6 - 13, mean ± SE.

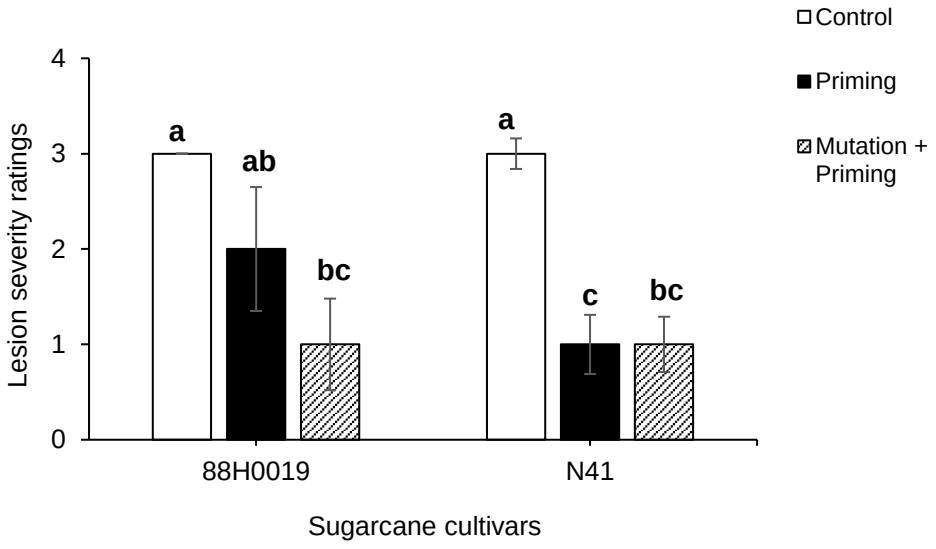


Figure 29: The lesion severity ratings of plantlets that were dual inoculated with SC17 and 2 weeks later with 2nd instar *E. saccharina* larvae, after 2 weeks for cultivars 88H0019 and N41. Dissimilar alphabet characters denote a statistically significant difference amongst treatments for each treatment. Data sets were analysed by One-way ANOVA and Duncan's multiple range test. P ≥ 0.10, n = 11 - 14, mean ± SE.

5. Discussion

5.1 The effect of priming agents on callus production and plantlet yield

Plants have evolved to survive a wide variety of biotic and abiotic stressors by developing inducible defence mechanisms, many of which are triggered by pathogen attack (Scalschi et al., 2013; Lamke and Baurle, 2017; Bertini et al., 2018; Anderson and Kim, 2021). Plant immunity against microbial presence relies on general elicitors, which signal the presence of potential pathogens (Bertini et al., 2018; Anderson and Kim, 2021). Recent studies in plant defence show that plants can be primed, which allows for a rapid and effective defence response to biotic and abiotic stressors (Conrath et al., 2006; Martinez-Medina et al., 2016; Bertini et al., 2018; Anderson and Kim, 2021). Enhanced resistance to biotic and abiotic stressors by using chemical biostimuli or biological organisms to induce molecular priming has been reported (Thakur and Sohal, 2013; Westman et al., 2019; Kerchev et al., 2020). Plants may activate different defence pathways depending on the type of pathogen attack (Garcia-Brugger et al., 2006; Thakur and Sohal, 2013; Anderson and Kim, 2021). The treatment of plants with various chemical elicitors has been found to activate different biosynthetic pathways. Necrotrophs are commonly known to initiate jasmonic acid (JA) and ethylene-dependent responses, while biotrophic pathogens activate salicylic acid (SA) dependent responses (Thakur and Sohal, 2013; Anderson and Kim, 2021).

Priming agents can be applied to whole plants, roots and seeds by means of seed priming (Luna et al., 2012; Thapa et al., 2020), foliage sprays, or addition to a hydroponic culture medium (Levya et al., 2008; Westman et al., 2018). Seed priming with chemicals that induce resistance has emerged as a novel strategy to protect crops against herbivory and disease attacks (Worrall et al., 2012; Luna et al., 2012; Paudel et al*.,* 2020; Thapa et al., 2020). Studies on rice (Bertini et al., 2018), tomato (Luna et al., 2016; Llorens et al., 2020), broccoli and *Arabidopsis* (Venegas-Molina et al., 2020), sugarcane (Patade et al., 2012; Duarte et al., 2018), and citrus (Caccalano et al., 2021) have demonstrated the effective use of several chemical elicitors to induce defence priming.

Hexanoic acid (Hx) has been used for inducing defence responses in *Arabidopsis* and tomato plants against *Botrytis cinerea*, tomato plants against *Pseudomonas syringae* (Leyva et al., 2008; Vicedo et al., 2009; Kravchuk et al., 2011; Scalschi et al., 2013), tomato plants against *Phytophthora citrophthora* and *Alternaria solani* (Flors et al., 2003), *Cucumis melo* against Melon necrotic ringspot virus (Fernández-Crespo et al., 2017), and citrus against *Xanthomonas citri* and *Alternaria alternata* (Llorens et al., 2013, 2015b; Llorens et al., 2016; Caccalano et al., 2021). However, the use of Hx on callus and plantlets is a novel approach

since published studies have mostly investigated seed priming (Borges et al., 2019; Ben Youssef et al., 2021), hydrophonic culture systems (Scalschi et al., 2013), foliar spraying (Levya et al., 2008) and soil drench applications (Vicedo et al., 2009; Llorens et al., 2013; Llorens et al., 2015) to induce defence priming (Levya et al., 2008; Scalschi et al., 2013; Llorens et al., 2016). However, Djami-Tchatchou et al. (2017) reported on the comparison of priming-related responses in *Nicotiana tobacum* callus cells triggered by Hx and Azelaic acid (Aza). That study revealed that exposure to Hx and Aza were able to induce many genes of importance to priming, signaling and defence-related responses in plants. There was a significant up-regulation in the *PR-1a* expression in the cells treated with Aza and Hx. *PR-1a* and *Defensin* are important gene transcripts that have been found to be induced after treatment with resistance inducing agents (Shah and Zeier, 2013; Djami-Tchatchou et al., 2017). PR-1 proteins have been used as molecular markers of SA-dependent systemic acquired resistance (SAR), these proteins contribute to increased pathogen resistance by causing harmful effects to microbial attackers directly during SAR (Djami-Tchatchou et al., 2017).

To assess the effectiveness of Hx and CJ as priming agents in the current study, Hx and CJ were applied to calli and regenerated plantlets *in vitro.* Sugarcane leaf discs and embryogenic calli were first exposed to various concentrations of the priming agents to select an appropriate one that induced calli proliferation and plantlet regeneration. The findings revealed that both sugarcane cultivars, 88H0019 and N41 pre-treated with Hx, resulted in better calli production and plantlet yield compared with the CJ treatments. The effect of the priming agents on calli production and plantlet yield was tested using embryogenic calli (0.2 g per replicate) that was transferred to embryo maturation medium (EMM) consisting of CJ (0, 0.2, 1, 5 and 10 µM) and Hx (0, 0.2, 0.6, 2 and 6 mM) for 2 weeks. The number of plantlets/ 0.2 g of callus was inversely proportional to percentage callus necrosis for both cultivars, as expected (Fig. 10, 11). The treatments that showed a high percentage of callus necrosis produced low plantlet yields (5 and 10 µM CJ treatments; 2 and 6 mM Hx treatments). The percentage of plantlets for 88H0019 and N41 were expressed as a percentage of the plants produced in the control. For cultivar N41, the highest plantlet yield of 90 % was recorded using 0.2 mM Hx, and cultivar 88H0019 recorded a 78 % plantlet yield using 0.6 mM Hx. For both the calli and plantlet regeneration stages, 0.6 mM Hx was selected as an appropriate concentration since it was the only one that produced a greater percentage of plantlets for both cultivars.

Many studies reported on the application of Hx in dicotyledonous plants such as tomato (Scalschi et al., 2013; Scalschi et al., 2014) and citrus (Llorens et al., 2016; Caccalano et al., 2021) to induce resistance against pathogens. Hexanoic acid has also been used to effectively

protect *Arabidopsis* plants (monocotyledonous) against pathogens (Kravchuk et al., 2011; Venegas-Molina et al., 2020). Those studies used 0.6 - 25 mM Hx to induce resistance against different pathogens. Hexanoic acid has been applied using different techniques such as hydroponic culture systems (Scalschi et al., 2013), foliar spray (Levya et al., 2008), and soil drench applications (Vicedo et al., 2009; Llorens et al., 2013; Llorens et al., 2015) to four-week-old tomato, citrus, and *Arabidopsis* plants. The results obtained in the present study revealed Hx at 0.6 mM to be a suitable priming agent in terms of calli production and plantlet regeneration. Hence, this concentration was chosen as a priming agent for further studies.

5.2 *Fusarium pseudonygamai* SC17 culture filtrate as a selection agent at the callus and plantlet regeneration stages

The association between fungi and host plants has been discussed in many studies (e.g., Shen et al., 2019; Fadiji and Babalola, 2020; Rigobelo and Baron, 2021). Endophytes have been found to improve plant growth by secreting phytohormones. They also help in improving nutrition using bidirectional nutrient transfer and enhancing plant health by protecting them against phytopathogens (Andreozzi et al., 2019; Shen et al., 2019; Fadiji and Babalola, 2020). The ability of endophytes to enhance host defence responses against diseases and reduce damage caused by pathogen attack is widely acknowledged (Ganley et al., 2008; Mejía et al., 2008; Fadiji and Babalola, 2020). This endophytic interaction is known as balanced antagonism (Schulz et al., 2015), i.e., the endophyte requires the activation of virulence mechanisms for colonisation and the triggering of host defences by these events to recognise the plant as a host. The fungus benefits by surviving off the nutrients from the host plant and, in return, provides benefits to the plants, including tolerance to abiotic and biotic stresses (Bamisile et al., 2018; Rigobelo and Baron, 2021).

Researchers have been investigating the effects of fungal mycotoxins or culture filtrates on resistant and susceptible genotypes of crop plants to assess disease resistance using *in vitro* selection techniques for many years (e.g., Binarova et al., 1990; Mahlanza et al., 2013; Suthar et al., 2021). The evaluation of resistance using *in vitro* techniques is dependent upon a positive relationship between *in vitro* culture filtrate resistance and whole plant disease resistance. The selection for tolerant lines using *Fusarium* culture filtrates and purified toxins has been widely used in callus cultures of sugarcane (*Saccharum* hybrids) (Mahlanza et al., 2013), cumin (*Cuminum cyminum)* (Suthar et al., 2021), turmeric (*Curcuma longa L*) (Gayatri et al., 2005) and sugar beet *(Beta vulgaris L.)* (Yerzhebayeva et al., 2019). *Fusarium* culture filtrates have been used as a selection agent *in vitro* shoot clumps for banana (*Musa* spp.)

(Rebouças et al., 2021) and vanilla plants (*Vanilla planifolia Jacks.*) (Ramírez-Mosqueda et al., 2019).

Programmed cell death is an important biological process which occurs under stress conditions during normal growth and development (Lam, 2004; Dickman and Fluhr, 2013; Zhang et al., 2015). Hypersensitive response (HR) is an extensively studied form of PCD in plants, which takes place during incompatible plant-pathogen interactions. Reaction oxygen species (ROS) that are associated with PCD of host cells are generated by the plant during the HR response when an oxidative burst occurs (Howlett, 2006). This form of PCD also follows when plant cells around the invasion site(s) rapidly and actively die to limit pathogen growth and prevent nutrient supply and, consequently, preventing the disease from spreading through the whole plant (Coll et al., 2011; Zhang et al., 2015).

There are two major pathways required for plant innate immunity, the jasmonic acid (JA) and salicylic acid (SA) pathway, induced by necrotrophs and biotrophs, respectively (Thomma et al., 1998; Glazebrook, 2005; Klemme et al., 2019). In the case of biotrophs, the plant responds to these pathogens via the SA pathway, inducing SA-specific pathogen response genes that result in HR which inhibits pathogen growth and development (Shah, 2003; Klemme et al., 2019). In a resistant plant genotype, necrotrophic fungi have been found to activate the JA signaling pathways, which triggers the JA-specific pathogen response genes that block the hypersensitive response (HR) (Van Loon and Van Strien, 1999; Clarke et al., 2000; Mur et al., 2006; Klemme et al., 2019). However, the pathogens find a way to avoid activation of the JA signaling pathway (responsible for promoting plant resistance) by increasing SA which inhibits the JA pathways (Klemme et al., 2019). The SA pathway has been induced to stimulate HR, this allows the necrotrophic fungal pathogens to secrete toxins in the host plant, promoting cell death, to access nutrition from the dead host tissue (Howlett, 2006; Zhang et al., 2015). In a susceptible plant, *Fusarium* spp. can use this mechanism by producing fumonisins since this mycotoxin causes programmed cell death (PCD) (Zhang et al., 2015; Daou et al., 2021).

There are numerous species of known fungi, although only a few of them, namely *Penicillium* spp., *Fusarium* spp., and *Aspergillus* spp. have been found to produce most of the mycotoxins that significantly affect agricultural crops (Kabak, 2009; Freire and de Rocha, 2017; Daou et al., 2021). The mycotoxin fumonisin B1 (FB1) produced by *Fusarium verticilliodes (moniliforme)* is a strong sphingolipid-like programmed cell death (PCD) elicitor that causes severe disease symptoms in maize and other grain crops (Gilchrist 1997, 1998; Zhang et al., 2015; Daou et al., 2021), all monocotyledonous species such as sugarcane. The sphingolipid biosynthetic pathway is inhibited by FB1 which hinders the production of ceramide synthase,

which results in cell death (Stone et al., 2000; Desai et al., 2002, Daou et al., 2021). Chivasa et al. (2005) stated that in *Arabidopsis* fumonisin B1 mediates cell death by altering the abundance of specific intracellular proteins in *Arabidopsis* by depleting extracellular adenosine triphosphate (ATP). The exogenous application of FB1 to germinated maize seeds resulted in a dose-dependent increase of SA, which was associated with an increase in sphingoid bases and inhibition of ceramide synthase (De La Torre-Hernandez et al., 2010; Blacutt et al., 2018). As a result, FB1 increased maize PCD and necrotrophic fungal colonisation of the plant by activating the SA pathway (Arias et al., 2012; Blacutt et al., 2018). Williams et al. (2007) also stated that in maize plants there was a dose-dependent reduction in root mass that was inversely correlated with fumonisin B1.

Since no work on sugarcane has been conducted to determine the effect *F. pseudonygamai* SC17 on calli proliferation and plantlet production, it was necessary to determine a suitable CF concentration for screening somatic embryos at the embryo germination stage and plantlets at the plantlet regeneration stage. The effect of different concentrations (0, 4, 20, 50 and 100 ppm) of *F. pseudonygamai* SC17 CF on calli production, plantlet yield and production of abnormal plantlets was recorded after 3 weeks. The incorporation of *F. pseudonygamai* SC17 CF in the EGM1 culture media resulted in necrosis of the embryogenic calli but plantlets were still obtained at all the tested concentrations of the CF (Figs 13a, b and c). There was a dose-dependent effect on callus necrosis and decrease in plantlet yield. For cultivar N41, there was a significant decrease in plantlet yield between the control (1020 plantlets/0.1 g of callus) and 100 ppm CF (98 plantlets/0.1 g of callus). There were no significant differences in plantlet yield observed for cultivar 88H0019 between the control and all the tested CF concentrations. Similarly, Mahlanza et al. (2013) reported an increase in callus necrosis and a decrease in plantlet yield when sugarcane callus cultures were placed in medium containing increasing concentrations of *F. sacchari* PNG40 CF. Similar results were also found in callus cultures of cumin (Suthar et al., 2021) and shoot clumps of banana (Rebouças et al., 2021). According to Daub (1986), selection agents used for *in vitro* screening for disease tolerance played a role in pathogenesis and showed a negative effect on plant tissues, cells, organs or whole plants. The selection agent used in the current study, *F. pseudonygamai* SC17, did not result in complete necrosis at the embryo germination stage as compared with the fungal strain *F. sacchari* PNG40 used in the study of Mahlanza et al. (2013).

Mahlanza et al. (2013) also stated that the production of sugarcane plants from the calli exposed to CF during embryo germination may have been due to light-dependent activity of mycotoxins (Asai et al., 2000), or different effects of the CF on the various physiological and biochemical processes that occur at the embryo germination stage. It is assumed that the

toxicity of the CF during the embryo germination stage under light conditions could likely be due to secondary metabolites (e.g., fumonisins) produced by *Fusarium spp.* which cause cell death. In a study established by Asai et al. (2000) an *Arabidopsis* protoplasts model was used to understand plant cell death signalling. Programmed cell death (PCD) was induced in wild-type protoplast by the toxin. When the protocol was established to understand plant cell death, the results indicated that FB1 induced PCD is light-dependent and it required the jasmonate, salicylate, and ethylene dependent signalling pathways (Williams et al., 2007; Asai et al., 2000). This explains the plants need for light during the oxidative burst to produce reactive oxygen (Govin and Levin, 2002; Zhang et al., 2015).

In the current study, greatly reduced numbers of plantlets were obtained at the highest CF concentration (100 µM) for cultivars 88H0019 and N41, 58 and 98 plantlets/0.1 g of callus, respectively compared to more than 600 plantlets/0.1 g of callus in the no CF control. Unexpectedly, all the tested CF concentrations had a significant positive effect on the percentage of plantlets that re-rooted compared with the control. Plantlets were still produced at the highest CF concentration (100 µM) that was tested, however a greater CF concentration should have been analysed. Hence, *F. pseudonygamai* SC17 could not be used as an *in vitro* selection agent in a root re-growth assay.

When the protocol for determining a suitable CF concentration for screening somatic embryos was established, it was then necessary to determine a suitable CF concentration for screening putative mutant plants at the end of the regeneration stage under *in vitro* conditions. Mahlanza et al. (2013) reported on the inhibition of root growth by *F. sacchari* PNG40 CF and this indicated that root re-growth can be used for selection of putative mutant lines *in vitro*. Many researchers doing studies on maize (Yoder, 1973), *Amaranthus hybridus* (Chen and Swart, 2002), and winter cherry (*Withania somnifera*) (Chakraborty et al., 2020) found that culture filtrates have an inhibitory effect on callus or root growth and proved to be a reliable and quick technique to produce resistant lines against biotic stresses.

A root re-growth test was established to screen putative mutant lines at the whole plant level. This test has been used by Mahlanza et al. (2013) and demonstrated that the roots of 50 % of the mutant plantlets with trimmed roots cultured on media containing 1500 ppm CF re-regrew to at least 10 mm in length over 3 weeks. In his study he used 10 mm re-growth as the criterion for the selection of CF- tolerant plants. Subsequently, this value was set as the criterion for the selection of CF- tolerant plants in the current study. The results obtained from this investigation indicated that the eldana beneficial strain *F. pseudonygamai* SC17 could not be used as an *in vitro* selection agent because the presence of CF in the culture media

promoted root growth for cultivars N41 and 88H0019 instead of inhibiting growth. The addition of different concentrations (500, 1000, 1500 ppm) of *F. pseudonygamai* SC17 CF to the EGM2 medium significantly enhanced the rooting ability of plantlets compared with the controls for both cultivars. The results revealed that all the tested CF concentrations had a significant positive effect on the percentage of plantlets that re-rooted between the control and all the tested CF concentrations (Fig. 16). The results recorded for both cultivars indicated a 95 - 100 % rooting ability of the plantlets in EGM1 + CF containing 500, 1000, and 1500 ppm CF, which was significantly higher than the percentage of plantlets that rooted in EGM1 containing no CF (60 - 70 %).

Although there was no significant interaction observed between treatment versus cultivar, there was a significant difference in the root length of plantlets amongst the treatments after 3 weeks (Fig. 15). The results show that all the tested concentrations of the CF affected the root length of plantlets, with the 1500 ppm CF resulting in the highest root length (31.5 mm ± 4.3, for 88H0019) and (34.05 mm ± 3.9, for N41). The root growth of *in vitro* plantlets were inhibited in the presence of 750 ppm and 1500 ppm *F. sacchari* CF in sugarcane (Mahlanza et al., 2013). Liao et al. (2017) reported that the use of the insect-pathogenic fungus *Metarhizium robertsii* culture filtrate promoted lateral root growth and root hair development of *Arabidopsis* seedlings.

In the present study, due to the percentage of plants that re-rooted and root length of the plantlets across all the tested CF concentrations, *F. pseudonygamai* SC17 could not be used as an *in vitro* selection agent because of its root promoting property. As previously mentioned Williams et al. (2007) reported on a dose-dependent (increasing fumonisin = decreasing root growth) reduction in root mass of maize plants that was inversely correlated with fumonisin B_1, sphingoid base 1-phosphate in roots, and sphingoid bases. Therefore, *F. pseudonygamai* SC17 CF may not contain fumonisins since it promoted root growth.

It was then hypothesised that the *E. saccharina* beneficial strain *F. pseudonygamai* SC17 CF stimulated root growth by auxin (IAA) production and/or through the presence of active ACC deaminase in the CF. Assays were conducted to screen the *F. pseudonygamai* SC17 fungal isolate for IAA production. For future work a toxicological profile for *F. pseudonygamai* SC17 will be required to determine the toxins present that inhibits plantlet regeneration at the callus and regeneration stages.

5.3 Fungal endophytes and plant growth promotion *in vitro*

The association between the host plant and some endophytic fungi is mutualistic (Khan et al., 2015; Rigobelo and Baron, 2021). The inner part of the host plant is a protected niche for endophytes since this contains the essential nutrients for fungal growth and survival, consequently the fungi improve the plant's fitness by several mechanisms (Khan et al., 2015; Lugtenberg et al., 2016; Chitnis et al., 2020; Rigobelo and Baron, 2021). The positive aspects of endophytic colonisation in host plants are well documented (Berg, 2009; Rigobelo and Baron, 2021). The direct benefits of endophytic colonisation include an increase in the production of phytohormones and acquisition of nutrients, which are associated with the improved root development, plant height and increase in biomass production (Bamisile et al., 2018). On the other hand, the indirect ones is the activation of systemic resistance, production of secondary metabolites and antibiotics, production of siderophores and protection against biotic and abiotic stresses (Rigobelo and Baron, 2021).

Indole-3-acetic acid (IAA) is the main auxin produced by endophytic fungi that plays a significant role in plant growth and development (e.g., Jaroszuk-Ściseł et al. 2014; Fadiji and Babalola, 2020; Fontana et al., 2021; Rigobelo and Baron, 2021). Low levels of IAA have been found to stimulate root elongation, whilst high concentrations result in the formation of adventitious and lateral roots (Jaroszuk-Ścisel et al., 2019). Some plant growth-promoting fungi (PGPF) also play an important role in plant growth by producing the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Nascimento et al., 2014; Hossain and Sultana, 2020). The ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) is converted into α-ketobutyrate and NH_3 (ammonia), by the ACC deaminase enzyme (Nascimento et al., 2014; Hossain and Sultana, 2020). Plant growth is regulated when the ACC deaminase enzyme cleaves ACC produced by plants and thus reduces the ethylene levels in the plant (Glick et al., 2007; Hossain and Sultana, 2020). The production of ACC deaminase has been reported in some fungi, such as *Phytophthora sojae* (Jia et al., 1999; Singh and Kashyap, 2012), *Fusarium graminearum* (Svoboda et al., 2019), and *Issatchenkia occidentalis* (Palmer et al., 2007). Jaroszuk-Ścisel et al. (2019) reported on the ability of the mycoparasitic *Trichoderma* (DEMTkZ3A0) strain to synthesize phytohormones GA, IAA and the enzyme-ACC deaminase.

Due to the observed enhanced root growth of the plantlets across all the CF concentrations tested in the present study, it was hypothesised that the beneficial *Fusarium* strain (SC17) stimulated root growth by auxin (IAA) production and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity. To test the former, a quantitative colourimetric was conducted to screen for IAA production by the *F. pseudonygamai* fungal isolate SC17 (Khan et al., 2016).

The *F. pseudonyagamai* isolate was able to synthesise IAA in both the presence and absence of L-tryptophan. The isolated *Fusarium* endophyte SC17 produced 743.1 nM and 457.2 nM IAA in the presence and absence of L-tryptophan, respectively (Fig. 19). The addition of 0.1 g L^{-1} L-tryptophan enhanced the IAA production by the isolate. Similar findings on the production of IAA by fungi isolated from sugarcane (Asrul et al., 2020), coffee (Numponsak et al., 2018), *Arabidopsis* (Liao et al. 2017), *Sophora flavescens* (Turbat et al., 2020), maize (Vrabka et al., 2019), and rice (Kuswinanti et al., 2015; Restu and Payangan, 2019) have been reported. Meents et al. (2019) reported on the production and accumulation of IAA in the mycelia of two beneficial endophytic fungi, *Mortierella hyaline* and *Piriformospora indica* found in *Arabidopsis* roots. Contreras-Cornejo et al. (2009) reported on the direct correlation between lateral root development in *Arabidopsis* seedlings inoculated with *T. virens* and increased levels of fungal IAA. Depending on growth conditions, microbial endophytes can produce IAA in varying quantities. The results recorded in those studies support the hypothesis that the observed enhanced root growth in the plantlets could have been due to the production of auxin (IAA) by the SC17 CF. However, as only screening for IAA production by the *F. pseudonyagamai* isolate SC17 was conducted, the activity of ACC deaminase will need to be determined in future studies. Plant growth-promoting fungi (PGPF) also play an important role in plant growth by producing the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Nascimento et al., 2014; Hossain and Sultana, 2020).

5.4 *Ex vitro* screening of putative mutant sugarcane plants for tolerance to *F. pseudonygamai* and *Eldana saccharina*

Plants have the ability to develop an enhanced state of resistance, known as induced resistance (IR), in addition to basal resistance (Llorens et al., 2015; Llorens et al., 2020). Basal resistance also known as innate immunity, is the first line of inducible defences that protect plants against attack by pathogens (Freeman and Beattie, 2008; Llorens et al., 2015; Llorens et al., 2020). Many attempts have been made to enhance stress tolerance in crops by inducing stress memory progressively. The most promising example of improving stress tolerance is by activating priming responses and targeting epigenome modifications (Martinez-Medina et al., 2016; Mauch-Mani et al., 2017; Mladenov et al., 2021). Defence priming is triggered by a priming stimulus, which is also responsible for inducing a persistent primed state of enhanced plant defence readiness (Martinez-Medina et al., 2016; Mauch-Mani et al., 2017; Mladenov et al., 2021). Priming is a state in which, during a second attack, the plant displays a quicker and more vigorous response in contrast to the initial one. Consequently, this robust response increases the plants chances of survival (Kinoshita and Seki, 2015; Mladenov et al., 2021). Priming occurs at the phenotypic level and does not involve changes to the DNA sequence and is therefore reversible (Conrath et al., 2015; Mladenov et al., 2021). Defence priming can

be induced using a diverse-range of mechanisms such as, a mild abiotic stress, treatment with synthetic or natural chemicals, infection by pathogens and colonisation of roots by beneficial microbes (Mauch-Mani et al., 2017; Balestrini et al., 2018; Ashapkin et al., 2020; Ioannou et al., 2020; Mladenov et al., 2021).

When a plant encounters a priming stimulus, a period of stress memory follows, which induces altered transcriptional regulation (Balmer et al., 2015; Mladenov et al., 2021). This modification results in sustained changes in gene expression or an improved transcriptional reaction to a secondary stimulus and is one possible indicator of stress memory (Oberkofler et al., 2021; Mladenov et al., 2021). Depending on the type of initial stimulus and the pathosystem involved there are a diverse set of defence mechanisms used by primed plants against pathogen attack (Balmer et al., 2015; Mladenov et al., 2021). It has been observed that defence mechanisms depend strongly on the priming state, which has been divided into a 'priming phase, a 'post-challenge primed state', and a 'transgenerational primed state' (Gamir et al., 2014; Pastor et al., 2014; Balmer et al., 2015). A priming stimulus triggers the priming phase which lasts until the plant is exposed to a challenging stress. During the primed phase, the levels of several primary and secondary metabolities, hormones, enzymes and other molecules are slightly changed, which puts the plant in a standby state (Gamir et al., 2014; Pastor et al., 2014; Balmer et al., 2015). When exposed to a challenging stress, the plant moves into the post-challenged primed state in which suitable reactions to combat the given stressor are induced quickly. The transgenerational primed state is the heritability of transcriptional changes from a parent to its progeny (Gamir et al., 2014; Pastor et al., 2014; Balmer et al., 2015; Martinez-Medina et al., 2016).

The use of Hx to enhance resistance to pathogens has been effectively demonstrated in *Arabidopsis* against *B. cinerea* (Kravchuk et al., 2011) and tomato plants against *P. syringe* and *B. cinera* (Leyva et al., 2008; Vicedo et al., 2009; Scalschi et al., 2013). Some studies have reported on the effect of Hx against the necrotrophic fungus *A. alternata* in *Arabidopsis* (Venegas-Molina et al., 2020) and 'Fortune' mandarin (Llorens et al., 2015).

In this study, Hx was used in combination with the chemical mutagen 5-AzaC. 5-Azacytidine has been widely used in many studies which generates epigenetic changes by inhibiting DNA methylation (Munsamy et al., 2013; Kakoulidou et al., 2021). Epigenetic mechanisms such as histone modifications and DNA methylation may be carriers of stress memory and trigger immune responses, therefore has been confirmed as key factors responsible for inducing resistance to biotic/abiotic stresses (Espinas et al., 2016; Lämke and Baurle, 2017; Ashapkin et al., 2020; Turgat-Kara et al., 2020). There is increasing experimental evidence that suggests the involvement of epigenetic modification with defence priming against stressors (He and Li,

2018; Mladenov et al., 2021). Epigenetic modifications have been found to cause prolonged stress memory, specifically in seed priming (Sani et al., 2013; Mladenov et al., 2021). A few studies have reported that defence priming can be inherited across sexual generations, a phenomenon referred to as 'transgenerational priming' (Martinez-Medina et al., 2016). It has been suggested that DNA methylation regulates priming by contributing to transgenerational priming (Luna et al., 2012; Martinez-Medina et al., 2016). Kim et al. (2017) reported on the involvement of DNA methylation in improving drought tolerance in primed seedlings exposed to a cycle of mild drought and re-watering treatments. Martinez-Medina et al. (2016) has shown the significance and dynamic involvement of epigenetic mechanisms, this indicated the possibility that epigenetic changes could be the main factor in establishing a priming effect. Several molecular markers were found to be useful for detecting the primed state, in *Arabidopsis.* These molecular markers include high levels of pathogen-recognition receptors, increased expression of transcription factor genes, enhanced accumulation of the mitogen-activated protein kinases MPK3 and MPK6, specific modifications to histones and DNA hypomethylation (Conrath et al., 2015; Martinez-Medina et al., 2016).

A protocol was established to develop putative mutant sugarcane lines that were primed with only Hx, or treated with a combination of Hx, mutagens EMS, and 5-AzaC. Following the establishment of the protocol for chemical mutagenesis and priming, embryogenic calli were exposed to, (32 mM EMS for 4 hrs, 100 µM 5-AzaC in embryo maturation media (EMM) for 1 week, and EMM containing 0.6 mM Hx for 2 weeks, followed by a sub-culture on embryo germination media (EGM1) containing 0.6 mM Hx for 4-12 weeks), and (no exposure to EMS and 5-AzaC, sub-cultured in EMM containing 0.6 mM Hx for 2 weeks, followed by a sub-culture onto EGM1 containing 0.6 mM Hx for 4-12 weeks). From each treatment, 50 *in vitro* plantlets were randomly selected for acclimatisation for 8 - 9 months, before they were used for *ex vitro* screening. To confirm tolerance of putative mutant lines, plants were inoculated with only *F. pseudonygamai* SC17 and dual inoculated with *F. pseudonygamai* SC17 and, after 2 weeks, 2nd instar eldana larvae were placed inside the leaf whorls. The main reason for using a combination of mutagens was to generate epigenetic (5-AzaC; Grzybkowska et al., 2018) and genetic (EMS; Lethin et al., 2020) variations in calli. It was hypothesised that *in vitro* priming with Hx could increase resistance to eldana and *F. pseudonygamai* SC17 whilst mutation (epimutagenesis in particular) could 'fix' this resistance during subsequent cycles of clonal propagation and chimera dissolution.

In this study, for N41 and 88H0019, embryogenic calli that were primed with Hx, and exposed to the mutagens + Hx, resulted in increased calli necrosis and a lesser number of plants/0.2 g of callus than the control. For cultivars N41 and 88H0019, there were no significant differences

in the number of plants produced/0.2 g callus between the primed and the combined treatment of 5-AzaC, EMS, and Hx. For the primed only treatment, there were 144 and 44 plants produced/0.2 g of callus for N41 and 88H0019, respectively. For the combined treatment, there were 44 and 33 plants produced/0.2 g of callus for N41 and 88H0019, respectively.

Lesion severity is an effective parameter of plant disease intensity for disease resistance (Bock et al., 2010; Bock et al., 2021), hence it was used in the present study to assess tolerance to *E. saccharina* and *F. pseudonygamai*. Lesion severity was recorded 2 months after inoculation, and the results obtained indicated that the putative mutant plants from both treatments exhibited a significant decrease in fungal lesion severity when compared with the controls for both N41 and 88H0019 (Figs 28 and 29). The lesion severity rating was as follows: 0- no lesion; 1- mild lesion; 2- moderate lesion; and 3- severe lesion. For both treatments, a mild lesion severity rating was recorded for plants inoculated with only SC17 for cultivars N41 and 88H0019. For the plants that were dual inoculated there was a significant difference in the lesion severity ratings between the two treatments. The lesion severity rating was moderate for cultivar 88H0019 (primed with Hx) and mild for cultivar N41 (primed with Hx). Plants from the combined treatment for both cultivars resulted in a mild lesion severity rating.

Similar findings have been recorded in previous studies, whereby exposure to Hx via soil drench, foliar spray or hydroponic conditions have resulted in reduced lesion severity caused by various pathogens in *Arabidopsis* (Kravchuk et al., 2011; Venegas-Molina et al., 2020), tomato (Levya et al., 2008; Scalschi et al., 2013), and citrus (Llorens et al., 2013, 2015). Novel studies in citrus ('Fortune' mandarin) demonstrated that Hx applied as a soil drench reduced the incidence of the necrotrophic fungus *Alternaria alternata* by 50 % (Llorens et al., 2013; Llorens et al., 2017). Scalschi et al. (2013) reported that Hx treatments protected tomato plants against *Pseudomonas syringe* in a concentration-dependent manner in all tomato cultivars analysed when Hx was applied by soil drench or in hydroponic culture systems. Four-week-old tomato plants that were treated with 0.6 mM Hx in hydroponic conditions showed reduced symptoms by 50 % (Scalschi et al. 2013). Several studies have reported on the enhancement of resistance mechanisms using Hx, and it is known that this priming agent induces resistance against necrotrophic pathogens by activating the JA pathway (Vicedo et al., 2009; Llorens et al., 2013; Venegas-Molina et al., 2020). These results are supported by previous studies on the association between JA-signaling pathways and attack by necrotrophic pathogens in different plant species. For example, Llorens et al. (2016) reported on the alteration in metabolic pathways after treatment with Hx, inoculation with *A. Alternata*, and inoculation with *A. Alternata* and treatment with Hx. Published literature suggests that treatment with Hx can induce the JA pathway against necrotrophic pathogens and increase compounds such as 12-

oxo-phytodienoic acid (OPDA), jasmonic acid (JA) or jasmonic-isoleucine (JA-IIe) (Vicedo et al., 2009; Camañes et al., 2015; Llorens et al., 2016). The results obtained in the current study have proven that *in vitro* priming with Hx only or in combination with the mutagens 5-AzaC and EMS produced plants that were resistant to *E. saccharina* and *F. pseudonygamai* SC17.

6. Conclusion

A protocol for producing sugarcane cultivars resistant to *F. pseudonygamai* SC17 and *E. saccharina* using two approaches was achieved. These two approaches were epigenetic priming (Hx) and chemical mutagenesis (EMS, 5-AzaC) or priming only (Hx). The established protocol for selecting a suitable priming agent concentration was accomplished. The relevant established concentration was 0.6 mM Hx that was used at both, the calli production and plantlet regeneration stages. To generate sugarcane lines tolerant to *F. pseudonygamai* and *E. saccharina*, *in vitro* plantlets produced from the established mutagenic/priming protocol needed to be selected on media containing the selection agent (i.e., *F. pseudonygamai* CF). The use of *in vitro* screening techniques using selective agents is an important strategy to select putative mutants before *ex vitro* screening and molecular analysis. The *F. pseudonygamai* SC17 CF was incorporated in the embryo germination media to determine its effect as a selection agent at the calli and plantlet regeneration stages. Root growth was enhanced by the increasing levels of the CF for cultivars 88H0019 and N41. As mentioned previously, fungal endophytes produce several secondary metabolites and toxins that are beneficial or antagonistic to pests or host plants. Hence, it was important to determine the plant growth-promoting activities of fungal pathogens.

In addition, this study revealed that there was an increase in root growth after 3 weeks, when plants were trimmed and transferred to semi solid embryo germination media (EGM2) containing increasing CF concentrations. It was suggested that the increase in root growth (1500 ppm CF) was likely due to the production of IAA or the regulation of the ACC deaminase enzyme by the endophyte. However, only screening for IAA production by the *F. pseudonyagamai* fungal isolate SC17 was conducted and detecting the activity of ACC deaminase still needs to be done. To determine if the fungal culture filtrate produced IAA, assays were conducted to screen *F. pseudonyagamai* SC17 for plant growth-promoting traits. The *F. pseudonyagamai* isolate was able to synthesise IAA in both the presence and absence of L-tryptophan. *F. pseudonygamai* CF produced higher levels of IAA in the presence than in the absence of L- tryptophan. The established protocol produced putative mutants that were randomly selected and acclimatised *ex vitro* for 8 - 9 months. The putative mutants that were

only primed with Hx and the combined treatment (mutagens + Hx) resulted in decreased lesion severity ratings upon infection with only SC17, and dual inoculated with SC17 and 2nd instar eldana larvae.

In summary, the sugarcane putative mutants were randomly selected from each treatment without *in vitro* selection, this was due to the enhanced root growth caused by *F. pseudonygamai* in the study. The plant-growth promoting traits of the fungal isolate was assessed and it was found to produce IAA. The use of Hx to induce resistance against the necrotrophic pathogen *F. pseudonygamai* was effective in sugarcane. It would be valuable to investigate the different changes in transcript levels of JA/ET and SA-responsive marker genes in response to Hx. The established protocols can be used to assess resistance to *F. pseudonygamai* and *E. saccharina* in other commercially important sugarcane cultivars.

Further studies on the following should be conducted:

- Develop an *in vitro* selection protocol using *F. pseudonygamai* CF at higher concentrations than those used here during plantlet regeneration.
- A toxicological profile for *F. pseudonygamai* is required.
- Determining the activity of ACC deaminase of the fungal isolate *F. pseudonygamai.*

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