The interaction between endophytic *Fusarium* species and *Eldana saccharina* (Lepidoptera) following *in vitro* mutagenesis for *F. sacchari* tolerance to control the borer in sugarcane

by

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Submitted in fulfilment of the academic requirements for the degree of

Doctor of Philosophy

in the College of Agriculture, Engineering and Science,

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Durban, South Africa

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February 2015

ABSTRACT

Eldana saccharina is a major pest in the South African sugar industry. Stalk damage by this borer and infection of bored tissue by opportunistic fungi result in loss of biomass and sucrose content, respectively. Amongst integrated management approaches, the best is employing *E. saccharina*-resistant genotypes. Resistance is attributed to physical stalk traits that impede boring and biochemical defences via nitrogen-based antiherbivory compounds. Further, *in vitro* assays have shown that *Fusarium* strains may be beneficial (e.g. *F. pseudonygamai* SC17) or antagonistic (e.g. *F. sacchari* PNG40) to the insect.

The first objective of this study was, therefore, to establish the effect of sugarcane stalk traits and infection by *Fusarium* spp. on resistance to *E. saccharina*. In the first of two glasshouse trials, mature and immature stalk internodes of seven cultivars of known *E. saccharina* resistance ratings were inoculated with 2nd instar larvae via nodal wounds. Stalk rind hardness was greatest in both mature (42.2 units) and immature internodes (25 units) of the resistant cultivar N33. The softest of both mature and immature stalk regions were from the very susceptible N11 (32 units) and susceptible NCo376 (17.7 units), respectively. Percent fibre content in mature internodes was highest in the resistant N33 and N17 (12.8 - 14.2%) and lowest in the susceptible N11 and NC0376 (10.9 - 11.2%) cultivars. In all but one cultivar, % nitrogen content/dry mass was higher in immature internodes (0.65 - 1.2 %) than mature ones (0.36 - 0.91%) and lower in stalks of the resistant N41, N29 and N33 (0.36 - 0.75%) than in those of the susceptible NCo376 and N41 (0.48 - 1.27%) cultivars. Damage and mass gain by larvae retrieved from stalks were not entirely consistent with the cultivar resistance ratings, probably because the inoculation method by-passed the rind; N29 and N33 were unaffected by lack of rind protection. Hence, the tested stalk traits may contribute to *E. saccharina* resistance to varying extents in different sugarcane cultivars. In another trial, immature and mature stalks of NCo376 and N41 were inoculated with SC17 and PNG40 and then with *E. saccharina* larvae. The stalk area discoloured by *Fusarium* infection was smaller in the immature (6.1 - 7.1 cm^2) than the mature (12.3 – 17.8 cm^2) internodes. The smallest stalk length bored was in PNG40-infected NCo376 (3.3 cm) and N41 (1.7 cm) mature internodes, whilst NCo376 stalks colonised by SC17 (8.2 cm) were the most damaged. Hence, the proposal that *Fusarium* strains affect *E. saccharina* differently thereby impacting cultivar resistance/susceptibility to the borer, is supported. The *in vivo* activity of *F. sacchari* PNG40 against *E. saccharina* was also established, corroborating its potential as a biological control agent against the borer. As this application of PNG40 is impeded by the fungus being the causal agent of Fusarium stem rot in sugarcane, *F. sacchari*-tolerant plants were then produced via induced mutagenesis.

Embryogenic calli of NCo376 and N41 were exposed to 32 mM ethyl methanesulphonate (EMS) for 4h. They were then placed on 100 ppm *F. sacchari* PNG40 culture filtrate (CF) at embryo maturation, germination or both stages, where 30.7 - 86.9% of the calli became necrotic and plantlet yield decreased by 59.2 - 99.2%. Roots of the regenerated plants were trimmed and placed on 1500 ppm CF. Plantlets with roots that regrew on CF medium beyond the 10 mm established threshold were deemed putatively tolerant (26.6 – 47.6% for EMS treatments, 5-24% for controls). These plants were acclimated and inoculated with PNG40 in the glasshouse. After 8 weeks, absence of symptoms, low lesion severity, re-isolation of PNG40 from the lesion and molecular identity of the isolates, confirmed some as PNG40 resistant. Re-isolation of PNG40 from undamaged tissue above the lesion, in plants with low lesion severity and no symptoms, confirmed endophytic colonisation and tolerance to the fungus in the mutants. Polymorphisms were detected in some mutants, using 24 RAPD primers.

The use of the tolerant mutants in *F. sacchari* PNG40-mediated control of *E. saccharina* was then investigated. Stalks of five tolerant mutants and parents of each NCo376 and N41 cultivars were inoculated with PNG40 and with *E. saccharina* larvae, 3 weeks later. The length bored was less (1.0 - 4.7 cm) in stalks of PNG40 infected-mutants and parents than in the controls (3.9 - 9.0 cm). However, the % stalk discoloured area due to PNG40 infection was less in the mutants (10.6 - 22.0%) than in the parents (N41 - 28.9% and NCo376 - 30.2%). Re-isolation of PNG40 from undamaged tissue, within the inoculated internode and that above it, confirmed endophytic colonisation and fungal spread across internodes. Amongst stalks inoculated with PNG40, one mutant of NCo376 and two of N41 displayed limited boring (1 - 2 cm) and % discoloured area (10.6 - 15.1%), and the highest % of endophytically colonised stalk sections (50 - 75%) in the internodes immediately above those inoculated. There were no differences between the mutants and their respective parents in stalk rind harness, fibre and nitrogen contents. This work, therefore, resulted in the production of *F. sacchari*-tolerant mutants, demonstrated the toxicity of *F. sacchari* PNG40 against *E. saccharina in vivo*, and the ability of the PNG40-tolerant mutants to support endophytic colonisation by the fungus. Demonstration of these *Fusarium - E. saccharina* interactions in the mutants under field conditions will lead to the application of biological control of *E. saccharina* using PNG40, as part of integrated management approaches for the pest.

Dedication

To my wife Nothando,

my parents Grace and Henry,

and sisters Vimbai and Ruvimbo.

Thank you for the love and support.

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DECLARATION 1 - PLAGIARISM

I, **Tendekai Mahlanza**, declare that

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

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

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

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DECLARATION 2 – PUBLISHED ARTICLES AND SUBMITTED MANUSCRIPTS

I, **Tendekai Mahlanza**, declare that I authored the published articles and the manuscript submitted for publication contained in this thesis, with assistance with proofreading from my supervisors.

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PREFACE

The experimental work described in this thesis was carried out in the Biotechnology Department of the South African Sugarcane Research Institute (SASRI), Mount Edgecombe, Durban, SA from January 2010 to November 2014, under the supervision of Prof. Paula Watt (UKZN), Dr Sandra Jane Snyman (SASRI and UKZN) and Dr Stuart Rutherford (SASRI).

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

ACKNOWLEDGEMENTS

Firstly, I thank the Almighty God for the strength, wisdom and guidance He granted me throughout this study.

I would like to express my sincere gratitude to the following people in no particular order:

- my supervisors Dr S. J. Snyman, Prof. M.P. Watt and Dr S. Rutherford for their guidance, support and encouragement throughout the course of this study;
- The SASRI Biotechnology staff for their encouragement and assistance with the tissue culture work;
- Sharon McFarlane and the SASRI Plant pathology staff for their assistance and knowledgeable advice about *Fusarium*;
- Malcom Keeping, Nelson Muthusamy and the SASRI entomology staff for their assistance with insect rearing;
- Nikki Sewpersad for assisting with the statistical analysis conducted in the study;
- Sheila Mhlongo, Innocent Mkhize and the SASRI technical team for their assistance with stalk and insect collection and the glasshouse work;
- Dr Bernard Potier for advice and proof reading write-up drafts;
- Dr Sumita Ramgareeb for support and guidance during the early stages of the study;
- Terence Mhora for the advice, support and encouragement throughout the course of the study;
- Fellow countrymen Philemon Sithole, Yeukai Manatsa and Dr Marvellous Zhou for the encouragement when I was home sick;
- The South African Sugarcane Research Institute (SASRI) and the National Research Foundation, South Africa for the financial support.
- My family for their constant support and prayers; and
- Finally, my fiancée Nothando Mafu for being a pillar of strength to me during this study.

TABLE OF CONTENTS

LIST OF TABLES

Chapter 2

Chapter 3

Chapter 4

Chapter 5

LIST OF FIGURES

Chapter 2

Chapter 3

Chapter 4

Chapter 5

LIST OF ABBREVIATIONS

2,4-D	2,4-dichloro-phenoyacetic acid
4- FPA	4-fluorophenoxyacetic acid
A	adenine
AFLP	amplified fragment length polymorphism
ANOVA	analysis of variance
BAP	benzylaminopurine
C	cytosine
CF	culture filtrate
cm	centimetre(s)
CRISPR	clustered regularly interspaced short palindromic repeats
DIBOA	2,4-dihydroxy-2H-1,4-benzoxazin-3-one
DIMBOA	2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetatic acid
EGM	embryo germination medium
EIM	embryo initiation medium
EMM	embryo maturation medium
EMS	ethyl methanesulfonate
FAO	Food and Agriculture Organisation
FDA	fluorescein diacetate
FT-NIR	fourier transform near-infrared
G	guanine
g	gram(s)
h	hour(s)
H_2SO_3	sulphourous acid
H_2SO_4	sulphuric acid
HNO_3	nitric acid
IAEA	International Atomic and Energy Agency
IPM	integrated pest management
ISSR	inter simple sequence repeats
JA	jasmonic acid
K	potassium
KZN	Kwazulu-Natal

L	litre(s)
m	metre(s)
mg	milligram(s)
min	minute(s)
mm	millimetre(s)
MS	Murashige and Skoog medium
N	nitrogen
NAA	naphthaleneacetic acid
NCE	non-clavicipitaceous endophytes
P	phosphorus
PCNB	pentachloronitrobenzene
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
R	resistant
RAPD	random amplified polymorphic DNA
RAPD	random amplified polymorphic DNA
REML	residual maximum likelihood
RFLP	restriction fragment length polymorhsim
S	second(s)
S	susceptible
SAMPL	selective amplified microsatellite polymorphism length
SASRI	South African Sugarcane Research Institute
SCMV	sugarcane mosaic virus
SE	standard error
SrMV	sorghum mosaic virus
T	thymine
TALEN	transcription factor-like effector nucleases
TBE	tris-borate-EDTA
UV	ultra-violet
VR	very resistant
VS	very susceptible
µl	microlitre(s)

CHAPTER 1

INTRODUCTION

1 Introduction

The South African (SA) sugar industry generates R12 billion in direct income annually and creates 79 000 direct and 350 000 indirect jobs translating to approximately one million people who depend on the industry for their livelihood (Sasa, 2014). Cultivated mainly for sugar, sugarcane is increasingly becoming an important crop for renewable energy production as worldwide interest in ethanol biofuels (Goldemberg, 2007; Chum *et al*., 2014) and electricity cogeneration from biomass (Guerra *et al*., 2014; Lora *et al*., 2014) grows. As the SA sugar industry continues efforts to obtain higher sugar yields (Zhou, 2013) and considers cogeneration (Smithers, 2014), sustainable sugarcane production through effective control of pests and diseases and development of genetically improved cultivars is critical.

The African sugarcane stalk borer *Eldana saccharina* Walker (Lepidoptera: Pyralidae) is a major pest of sugarcane in the SA sugar industry with damage inflicted by the insect causing annual losses of up to US$ 82 million (Black, 2014). The insect bores sugarcane stalk tissue, especially in the mature stage of the crop, thereby reducing valuable biomass (Atkinson, 1980; Goebel and Way, 2003). This damage is compounded by drought stress as physiological plant defence mechanisms against the insect are compromised (Keeping *et al.,* 2012). Borings provide opportunistic *Fusarium* spp. entry into the inner stalk, thus resulting in an association between *E. saccharina* damage and Fusarium stalk rot (McFarlane *et al.,* 2009). The *Fusarium* spp. convert sucrose to glucose resulting in lower sugar yields (Way and Goebel, 2003). Such damage has led to *E. saccharina* resistance being a priority for the SA sugar industry, with screening of borer-resistant genotypes being conducted in resource-intensive pot trials in the later stages of the breeding programme (Keeping, 2006). As a result, indirect losses are incurred as only a limited number of promising lines can be screened, and high sucrose genotypes may be discarded if susceptible to the borer (Butterfield and Thomas, 1996). Further, losses are sustained through early harvesting at 12 months instead of the economically viable age of 15-18 months in order to curtail *E. saccharina* damage in the mature crop (Keeping *et al*., 2014). *Eldana saccharina* damage also results in infection by other fungi such as *Colletotrichum falcatum* Went. (McFarlane and Bailey, 1996). Consequently, an integrated pest management (IPM) approach that entails use of insecticides, pre-trashing, destruction of infested stalks and limited application of nitrogen fertilisers, has been adopted to control *E. saccharina* (Webster *et al*., 2005). However, improvement of current control strategies and development of new ones is important as the borer continues to adapt to areas thought previously to be unfavourable for *E. saccharina* development (Kleynhans *et al.,* 2014).

The use of resistant cultivars is the best measure for *E. saccharina* control (Keeping, 2006). Sugarcane stalk rinds and fibre impede boring and digestibility of tissue, respectively, thereby contributing to resistance. However, fibre content, which is positively correlated with rind hardness, is negatively associated with sucrose recovery from the stalk rendering both stalk characteristics undesirable mechanisms for borer resistance (Keeping and Rutherford, 2004). Nonetheless, plants produce metabolites that are repellent, unpalatable or toxic to herbivores and thus play a role in insect resistance (Howe and Jander, 2008). Insect feeding on plant tissue elicits an induced acquired response facilitated by jasmonic acid and its derivatives (Reymond and Farmer, 1998), which trigger expression of defence genes responsible for synthesis of proteinase inhibitors, anti-nutritional compounds, signalling molecules and repair proteins (Leon *et al*., 2001). This physiological reaction to herbivory is a preferred mode of *E. saccharina* resistance to physical mechanisms as it does not negatively impact sucrose recovery. Hence, understating the role of physical and biochemical mechanisms of resistance in sugarcane genotypes will aid in enhancing selection strategies for *E. saccharina* resistance. Nevertheless, developing additional control measures to combat the borer such as fungus-mediated biological control, may also improve IPM of the pest.

Studies in maize have shown that endophytic *Fusarium verticillioides* Sacc. (Nirenberg) exacerbate *E. saccharina* damage (Schulthess *et al*., 2002) and fecundity (Ako *et al*., 2003). In sugarcane, *in vitro* dietary and olfactory choice bioassays demonstrated the beneficial and antagonistic effects of *Fusarium* isolates on *E. saccharina* (McFarlane *et al*., 2009). These findings suggest that *Fusarium* spp. may influence *E. saccharina* damage and, therefore, the management of borer-beneficial strains may improve control of the insect. However, the impact of *Fusarium* strains on *E. saccharina* damage and performance is yet to be determined *in vivo*. Furthermore, the negative *in vitro* effect of Fusarium strains, e.g. *F. sacchari* Butler and Khan) Gams PNG40, on *E. saccharina* indicates the potential of the fungus in biological control of the lepidopteran. *Fusarium* strains produce insecticidal compounds (Gupta *et al*., 1991, Logrieco *et al*., 1996; Guo *et al*., 2014) and their pathogenicity to insects pests has established their potential in biological control in various crops (Majumdar *et al.*, 2008; Mikunthan and Manjunatha 2008; Wenda-Piesik *et al.*, 2009; Batta 2012). However, the phytotoxicity of *E. saccharina*-antagonistic *Fusarium* strains, such as *F. sacchari* PNG40 which causes stem rot in sugarcane, limits their value in insect control. Hence, the development of *Fusarium*-tolerant sugarcane genotypes may aid in controlling Fusarium stem rot. Tolerance permits endophytic colonisation of plant tissue by the fungus thereby enabling use of PNG40 in biological control of the borer.

Conventional breeding of improved sugarcane genotypes is complicated by seed sterility, unsynchronised flowering and the polyploid and aneuploid genome of the crop, taking 12-15

years to release a new cultivar (Butterfield *et al*, 2001; Ming *et al*., 2006). Transgenic approaches in sugarcane are limited by technological, legislation and marketing concerns (Lakshmanan *et al*., 2005; Burnquist, 2006; Birch, 2014). However, well established *in vitro* culture systems in sugarcane (Nickel, 1964; Lee, 1987; Snyman, 2004; Mekonnen *et al*., 2014) provide an alternative strategy for crop improvement via *in vitro* mutagenesis and selection strategies (Rutherford *et al*., 2014). Chemical mutagens such ethyl methanesulphonate are effective at inducing point mutations in cells with minimal deleterious effects (Weil and Monde, 2009). Mutant cells and plants expressing desired traits may be screened under well-defined conditions by incorporating appropriate selection agents in the culture media (Novak and Brunner, 1992; Lebeda and Svabova, 2010). This approach has been employed for development of sugarcane genotypes with superior agronomic traits, disease, herbicide and salt tolerance (reviewed by Rutherford *et al*., 2014).

The present study aimed to investigate the contribution of physical and biochemical sugarcane stalk characteristics to *E. saccharina* resistance, determine the influence of *Fusarium* strains on borer damage and performance *in vivo,* and establish possible use of the *E. saccharina*-antagonistic strain *F. sacchari* PNG40 in endophytic biological control against the insect. This study also sought to produce *F. sacchari*-tolerant genotypes via *in vitro* mutagenesis and test the utility of such mutants in control of *E. saccharina* and associated Fusarium stem rot.

CHAPTER 2

LITERATURE REVIEW

2.1 Background and importance of sugarcane

Sugarcane is a perennial, tropical or subtropical crop grown worldwide, within 30˚ of the equator, for its high sucrose accumulation (Ming *et al.*, 2006; Moore *et al*., 2014). Commercially, the crop is established by means of seed cane and ratoons, when the bud and root primordia of the stool left after harvesting produce a stubble (Bonnet, 2014). Sugarcane grows well in medium to heavy, slightly alkaline soils with good drainage, high organic matter (Anon., 2003) and an annual water supply of 1200-1500 mm (Tarimo and Takamura, 1998). The crop has one of the most efficient photosynthetic mechanisms, capable of fixing 2-3 % radiant solar energy and achieves a high CO_2 coefficient (Almazan *et al.*, 1998; Sage *et al*., 2014).

Sugarcane belongs to the genus *Saccharum* L., a part of the *Andropogoneae* tribe of the family *Poaceae* (grasses) (Azevedo *et al.*, 2011). Among the recognised species are *S*. *officinarum* Linnaeus*, S. spontaneum* Linnaeus*, S. sinense* Roxb*, S. edule* Hassk*, S. barberi* Jeswiet *and S. robustum* Brandes and Jeswiet (Tarimo and Takamura*,* 1998; Moore *et al*., 2014). The wild forms of sugarcane are thought to have evolved from Papua New Guinea and other Melanesian islands (James, 2004). According to Grivet *et al.* (2004), sugarcane genetic resources can be divided into three groups:

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

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

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

The modern sugarcane cultivars are highly polyploid and aneuploid, originating from crosses between *S. officinarum* (2n = 80) and *S. spontaneum* (2n = 40 – 128) and from backcrossing the interspecific hybrids with the *S. officinarum* parent (Stevenson, 1965; Sreenivasan *et al.*, 1987; Butterfield *et al.*, 2001; Ming *et al.*, 2006; Moore *et al*., 2014). In some of these cultivars, 10% of the chromosomes are inherited entirely from *S. spontaneum*, 80% from *S. officinarum* and 10% results from recombination of chromosomes from the two ancestral species (D'Hont *et al.,* 2008; Zhang *et al*., 2014). These crosses introgressed disease resistance, vigour and

adaptability into sugarcane lines leading to a combined interspecific genome that makes it the most complex of all the economically important crops (Ming *et al.*, 2006). The complex cytology of sugarcane makes it extremely difficult to predict the resulting characteristics of hybrids obtained by cross pollination of members of the genus *Saccharum*, thus the difficulties in breeding sugarcane (Barnes, 1964; D'Hont *et al.*, 2008). In most crops, pest and disease resistance are regulated by both dominant and recessive genes, but in polyploids such as sugarcane, the recessive genes are obscured by homologous alleles, making them ineffective for breeding (Butterfield *et al.*, 2001). Further, the effect of dominant genes in polyploids is not similar to that in diploids, due to the interaction of multiple alleles at a single locus, making it difficult to determine phenotype (Butterfield *et al.,* 2001). Transcriptome analysis and functional genomics studies in sugarcane are emerging and will aid molecular breeding of complex traits (Manner and Casu, 2011; De Setta *et al*., 2014; Zhang *et al*., 2014).

Sugarcane produces large amounts of biomass whilst accumulating high concentrations of sucrose (Manners and Casu, 2011). This justifies sugarcane's status as the world's most industrialised tropical crop (Moore and Ming, 2011). Approximately 75 % of the world's sugar is obtained from sugarcane and 25 % from sugar beet (*Beta vulgaris* Linneaus) (Ming *et al.*, 2006). Although over 100 countries cultivate the crop, the bulk of the world's sugarcane is produced by a few countries, including South Africa (SA) (Fischer *et al.,* 2009), which is rated amongst the top 15 cost-competitive sugar industries (Potgieter *et al.,* 2013). The sugar industry makes a vital contribution to rural economic activity in SA's sugarcane-growing areas of Kwazulu-Natal (KZN), Mpumalanga and the Eastern Cape (Esterhuizen, 2012) (Fig. 1). In SA, a total of 16.80 million metric tonnes (MMT) of sugarcane (2.0 MMT sugar) was produced in the 2012/2013 season and 18.10 MMT (2.1 MMT sugar) is forecast for the 2013/14 season (Kreamer and Esterhuizen, 2013), improvements after production had declined to 16.02 MMT due to a severe drought in the 2010/11 season (Esterhuizen, 2012).

The main products of industrial processing of sugarcane are sugar and ethanol, whilst by-products include molasses, bagasse, vinasse and filter cake (Gómez-Merino *et al.,* 2014). The production of sugar yields molasses, which is used as stock feed and in the manufucture of ethanol (Zuurbier and Van de Vooren, 2008). Bagasse, the fibrous biomass left after the juices are extracted from the cane, is used as fuel and in the production of cardboard, fibre board, furfural and wall board (Almazan *et al.,* 1998; Pippo and Luengo 2013). Mohan *et al.* (2005) also used bagasse as an alternative to agar in apple micropropagation. Vinasse and the filter cake, residues left over after extraction of sucrose, are utilised as fertiliser and stock feed (Cheavegatti-Gianotto *et al.*, 2011).

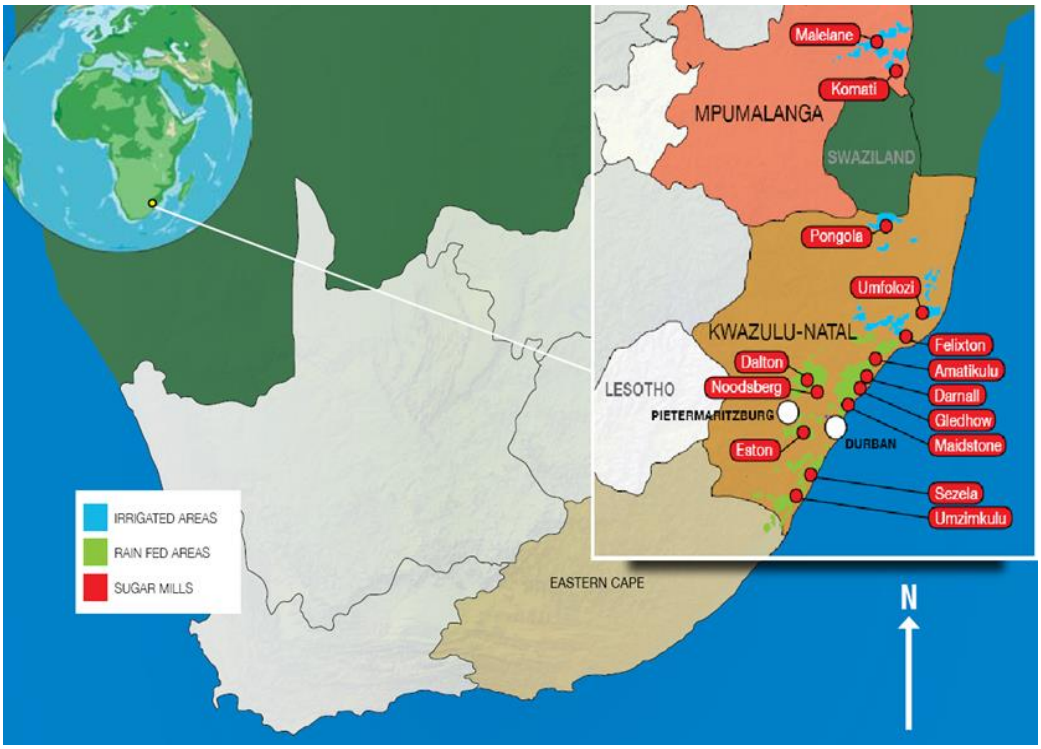

Figure 1: Sugarcane growing areas and mills in SA (SASA, 2014).

In recent years, economic interest in sugarcane has increased due to its potential to expedite sustainable energy production (Cheavegatti-Gianotto *et al.,* 2011; Botha and Moore, 2014). Despite its economic importance, sugarcane is attacked by various pests and diseases, causing significant losses in production. The crop is relatively susceptible to pests and diseases as a result of being vegetatively propagated and cultivated over large contiguous areas (Dick, 1945; Bailey, 2004). Pests like white grubs (*Hypopholis sommeri* Burm and *Schizonycha affinis* Boh) (McArthur and Leslie, 2004), the spotted sugarcane stalk borer [*Chilo sacchariphagus* (Bojer) (Lepidoptera:Crambidae)] (Rutherford and Conlong, 2010) and sugarcane thrips [*Fulmekiola serrata* (Kobus)] (Way *et al.,* 2010) are threats to the South African sugarcane crop. However, of greater significance than these is the stalk borer *Eldana saccharina,* which has been the most economically important pest in the South African sugar industry since the 1970s (Atkinson *et al.,* 1981; Way, 1994; Conlong, 2001; Leslie, 2013).

2.2 *Eldana saccharina*

2.2.1 Nature of damage

The African sugarcane stalk borer *E. saccharina* is an insect found naturally in sedges (Cyperaceae), e.g. *Cyperus immensus* Clarke and *C. papyrus* Linnaeus, and is a pest of sugarcane, maize and sorghum (Atkinson, 1980; Schulthess *et al.,* 2002). In sugarcane, the

larvae enter the stalk by boring through the bud, root primordia and cracks in the internodes and feed on the inner stem tissue (Dick, 1945; Leslie, 1993). The larvae can also bore into the stubble that remains after harvest, which becomes a source of infestation for subsequent ratoons (Girling, 1972). Cracks or borings in the stalk rind just above the node from which frass (excrement) is expelled (Fig. 2a), indicate attack by the insect (Girling, 1972; Carnegie, 1974). Splitting the infested stalks longitudinally reveals tunnels created by feeding larvae (Fig. 2b). This damage is compounded by opportunistic infections by fungi such as *Fusarium* spp. (Bourne, 1961; McFarlane *et al.,* 2009) characterised by reddish-brown discolouration of the bored tissue (Fusarium stalk rot) (Fig. 2b). These fungi are unable to breach the stalk rind unaided, therefore, they exploit the borer-inflicted wounds for access into the stalk. This collective damage has resulted in *E. saccharina* causing devastating damages to the South African sugarcane crop.

Figure 2: *Eldana saccharina* damage. a) Frass ejected from a crack in the stalk; and b) longitudinally split stalk revealing feeding larvae and fungal infection of the bored tissue (Photos from SASRI picture gallery.

2.2.2 Biology

All life cycle stages of *E. saccharina,* i.e. adult moth, eggs, larvae and pupae, can be present concurrently (Carnegie, 1974). The adult moths (Fig. 3a) have brown wings with a wingspan of 30-35 mm and live for approximately 7 days during which the male and females mate

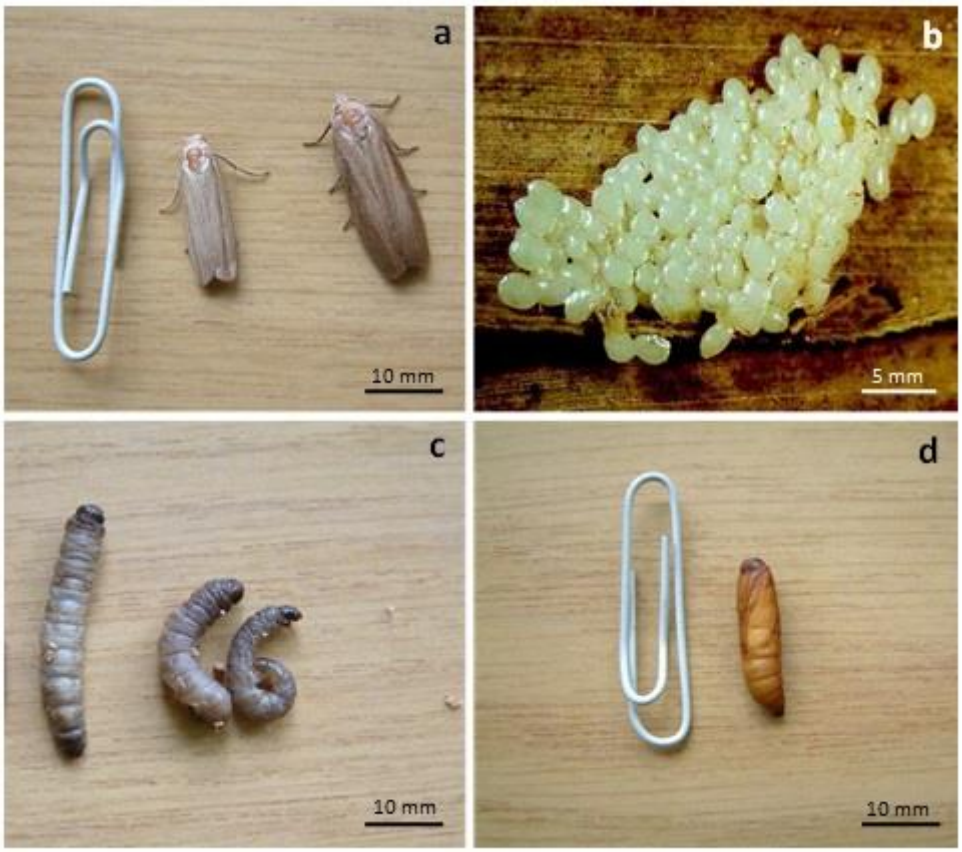

Figure 3: *E. saccharina* a) adults; b) eggs; c) larvae; and d) pupae.

(Carnegie, 1974). During courtship, the males appeal to the females by flapping their wings rapidly and outspreading hairs found on their abdomen into a round brush, a behaviour called displaying (Atkinson, 1981). After mating, oviposition occurs on the underside of dead leaf sheaths or in the space between the stalk and the soil, with each mated female laying up to 450 eggs (Carnegie, 1974). Atkinson (1980) observed that dry plant material was preferred over green leaves for oviposition, thus more eggs were found in older than in young green sugarcane. The eggs (Fig. 3b) hatch 8-10 days after oviposition and the neonate larvae are approximately 1.5 mm in length, increasing to 25-35 mm when fully grown (Dick, 1945). They forage on the sugarcane leaves for a few days and disperse from the oviposition sites seeking soft tissue on the stem, which they bore to gain access into the inner stalk tissue for feeding (Leslie, 1993). This larval stage (Fig. 3c) period varies from 20-60 days depending on temperature, with warmer conditions resulting in faster development (Dick, 1945; Carnegie, 1974). In addition, Atkinson and Nuss (1989) reported that larval survival and growth were promoted by the presence of nitrogen, both *in vitro* and in the field, thereby suggesting that the development of intensive farming practices, which incorporated application of nitrogen fertilisers, may have encouraged infestation of the sugarcane crop by *E. saccharina*. The

larvae moult 5-7 times, with the females having more larval instars than the males (Atkinson, 1980; Way, 1995). The final instar larva constructs a cocoon, which is either embedded in frass within the stalk or attached outside the stalk, and pupates (Fig. 3d) for approximately 10 days (Carnegie, 1974). Thereafter, eclosion takes place following sunset with males emerging first (Dick, 1945; Atkinson, 1981). The moths proceed with courtship and mating within 2-4 days of eclosion.

2.2.3 Distribution and economic importance

Although *E. saccharina* was reported in sugarcane in West Africa in the 1800s (Carnegie, 1974), the first outbreak in the SA sugar industry occurred in September 1939 in sugarcane fields on the Umfolozi Flats in KwaZulu-Natal (Dick, 1945) (Fig. 4). The introduction of resistant varieties into the industry temporarily solved the problem (Atkinson *et al.,* 1981). However, in 1970 another outbreak occurred at Hluhluwe, followed by more in the subsequent 2 years at Empangeni, Mtunzini, Amatikuku and also in Swaziland (Atkinson *et al.,* 1981) (Fig. 4). Since then, *E. saccharina* has been a consistent constraint to sugarcane production in South Africa with the borer being distributed along the sugarcane belt of KwaZulu-Natal province (Atkinson and Carnegie, 1989; Way and Goebel, 2003; Kleynhans *et al*., 2014). Atkinson (1979) noted that *E. saccharina*'s presence was limited to Richards Bay in the north and Port Shepstone in the south of the sugarcane belt. This confinement of the pest to sugarcane-producing areas along the coast was attributed to relatively lower inland temperatures than those ideal for *E. saccharina* reproduction (Way, 1994). However, Way (1994) reported presence of the borer in the Midlands region of KwaZulu-Natal, an inland area that was previously regarded too cold for the insect's development. Further, Conlong (2001) stated that *E. saccharina* distribution in the south had extended to Mkambati Nature Reserve, Eastern Cape and Assefa *et al.* (2008) then reported that the stalk borer had been found in Thohoyandou, Limpopo in the north. Potchefstroom, North West Province, a maize producing area, was announced as the new western limit for *E. saccharina* presence, thus raising concerns of introduction of the pest into the SA maize crop (Assefa *et al.,* 2008). These changes were ascribed to increases in temperatures in these areas, thus providing conducive conditions for the stalk borer's development (Way, 1994; Assefa *et al.,* 2008). Kleynhans *et al.* (2014) reported that evolved thermal tolerance in *E. saccharina* may impact phenology and distribution of the insect.

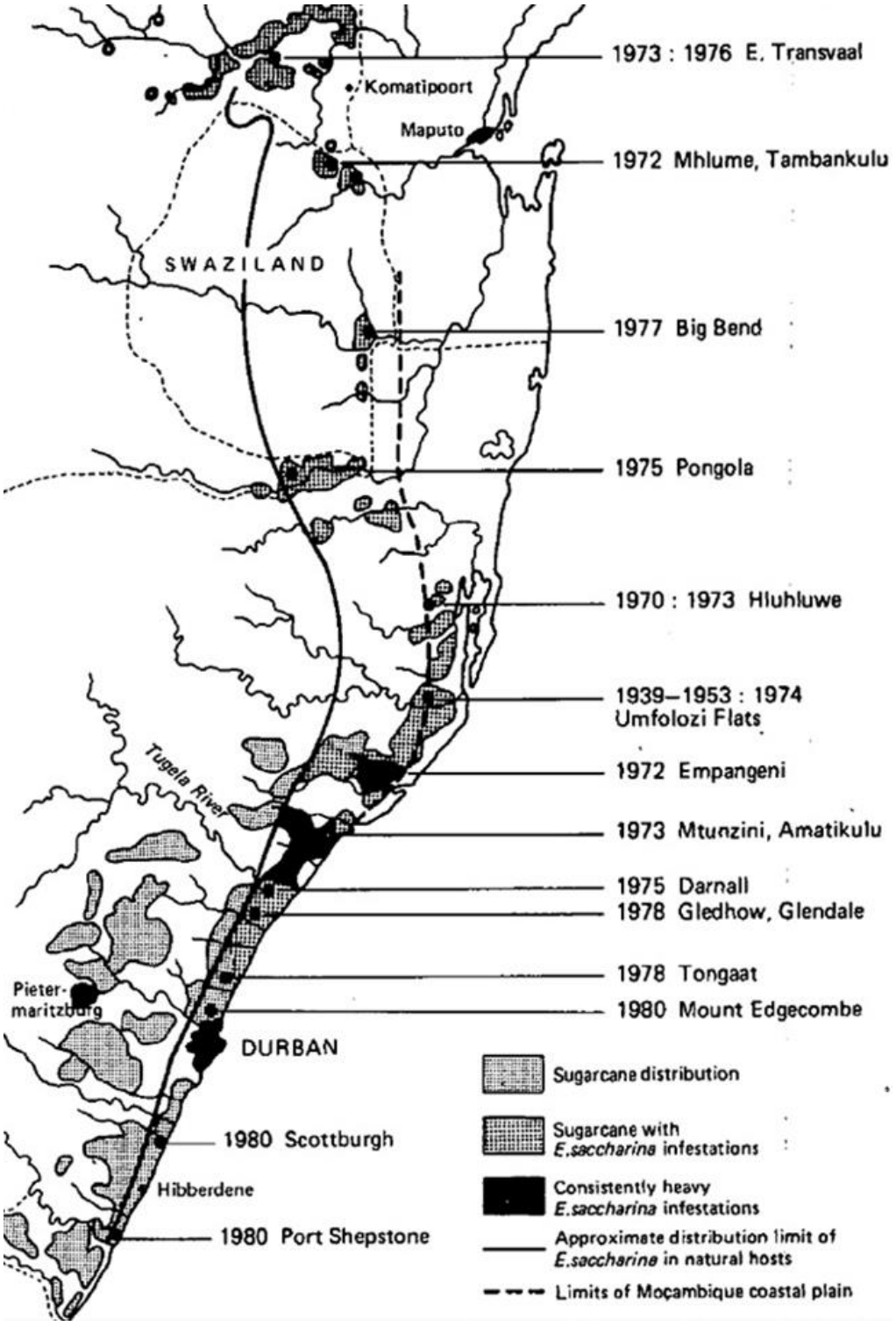

Figure 4: History of *Eldana saccharina* outbreaks in the South African and Swazi sugar industries. (From Atkinson *et al*., 1981).

This wide distribution of *E. saccharina* in the SA sugar industry has resulted in devastating economic losses. Infestation by the lepidopteran pest results in lower sucrose levels; lower stalk length and mass; and higher fibre, which leads to extraction of less juice (Goebel and Way, 2003). Opportunistic fungi (e.g. *Fusarium* spp.) that infect the stem tissue as a result of borer-inflicted damage, metabolise sucrose to glucose, leading to less sugar being obtained from affected stalks (Way and Goebel, 2003). The larvae are ravenous feeders and as manyas 12 can be found in a stalk, capable of hollowing it out and also spreading to the roots (Carnegie, 1974). This corporate damage translates into losses in valuable revenue for the SA sugar industry. Baker (2014) reported that losses due to *E. saccharina* damage was estimated to be US $89 million per annum. Additionally, as screening for *E. saccharina* resistance is carried out during the later stages of the breeding programme, high sugar yielding genotypes selected during the earlier phases are discarded due to susceptibility to the borer, thus hampering development of new improved commercial varieties (Butterfield and Thomas, 1996; Zhou, 2013a). Such losses continue to persist in the SA sugar industry and justify *E. saccharina* as one of the major priorities in pest and disease management efforts of the SA sugar industry.

2.2.4 Control

E. saccharina is cryptic with some life cycle stages concealed from control measures (Leslie, 1993). However, after the eggs hatch, the neonate larvae are unprotected as they disperse from the oviposition sites. The adult moths are also exposed during dispersal, mating and oviposition (Leslie, 1993). This renders these two stages most vulnerable to control strategies. An Integrated Pest Management (IPM) approach which involves use of chemicals (Leslie, 2003), crop management (Webster *et al.,* 2005) and use of resistant varieties (Rutherford *et al.,* 1993; Keeping and Rutherford, 2004; Keeping, 2006), has been employed to control *E. saccharina* in the SA sugar industry.

Crop management practices that form part of the IPM strategy comprise of early harvesting when the cane is 12 months-old; destruction of all infested stalks and stubble; limited application of nitrogen fertilisers; and pre-trashing, i.e. removal of dry leaves from stalks to restrict oviposition (Webster *et al.,* 2005). In chemical control, the insecticide α-cypermethrin (Fastac®) has been employed successfully to curb the stalk borer in sugarcane (Leslie, 2006). The application of this insecticide represses the population of *E. saccharina* during periods when infestation is usually at its peak, thereby allowing harvesting at a more economically viable age of 15-18 months than at 12 months-old (Leslie, 2009). However, as borers reside deep within stalk tissue, the efficacy of insecticide application against borers is limited as they may be inaccessible to the chemicals (Srikanth *et al.,* 2011). Moreover, the use of insecticides

is undesirable as it may be harmful to human health and negatively impact the environment (Aktar *et al.,* 2009). A more attractive approach is the use of resistant varieties as it provides an inherent control mechanism in the plant.

2.3 Insect resistance in sugarcane

Resistance to insects in sugarcane cultivars is attributed to physical and chemical mechanisms. Physical characteristics of the stalk such as rind and fibre content impede insect boring of the stalk (Keeping and Rutherford, 2004). Rinds provide a tough barrier that prevents or delays penetration of the stalk by larvae, thereby exposing them to mortality factors e.g. predation by ants, insecticides and unfavourable weather conditions, on the exterior of the plant (Mabulu, 2013). The sugarcane rind is composed of lignocellulosic fibres containing parenchyma cells and vascular bundles with thick cell walls, which give them a high tensile strength (Han and Wu, 2004). This structural feature imposes a mechanical challenge for insect mandibles to cut (Kvedaras *et al.,* 2007). Keeping and Rutherford (2004) reported a negative correlation between rind hardness of 72 sugarcane cultivars and internodes bored by *E. saccharina*, borer numbers and borer mass per stalk. Additionally, the surface of the rind may have epicuticular stalk waxes, which contain C30 alcohol, C30 alderhyde and triacontanol, may be involved in larval antixenosis (Rutherford and Van Staden, 1996). Once larvae penetrate the rind, stalk fibre (composed of cellulose, hemicelluloses and lignin) (Santiago *et al*. 2013), is the main physical resistance mechanism as high fibre plant tissue is difficult for insects to digest. Lignification of cell walls as the plant matures increases the fibre content of the stalk (Gibson *et al*. 2009) and may lead to increased resistance. However, though high fibre content in sugarcane promotes borer resistance, it is undesirable as it negatively affects sucrose recovery and complicates milling (Singh *et al*., 2013a). Further, the positive correlation between fibre and rind hardness also renders the latter unattractive (Keeping and Rutherford, 2004). Silicification of plant cell walls by applying silicon to the soil can also increase the impenetrability of the rind and indigestibility of tissue by insects, thus contributing to resistance (Kvedaras and Keeping, 2007; Keeping *et al*., 2014).

Insect herbivores elicit an induced acquired response (IAR) facilitated by jasmonic acid (JA), its derivatives and ethylene (Reymond and Farmer, 1998). Damage to plant tissue as a result of herbivory elicits a wound response pathway which is mediated by jasmonates (Howe and Schaller, 2008). Upon wounding of the plant (e.g. insect boring), the action of systemin, a signalling polypeptide, in the damaged cell membranes leads to release of linoleic acid, an intermediate of the JA signalling pathway (Farmer and Ryan, 1992). Studies in solanaceaous crops have indicated that systemin also serves as a long-range signal transported via the phloem to undamaged tissues where it induces systemic defence responses in the plant by

induction of JA pathway (Ryan, 2000). The accumulation of JA and its intermediates activates expression of wound-inducible defence genes responsible for synthesis of proteinase inhibitors, anti-nutritional compounds, signalling molecules and repair proteins (Leon *et al.,* 2001). These inducible biochemicals are largely nitrogen-based which include proteinase-inhibiting benzoxazinoids 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one (DIMBOA) and its derivative 2,4-dihydroxy-2H-1,4-benzoxazin-3-one (DIBOA), anti-nutritional polyphenol oxidases and phenylpropanoid polyamine conjugates (Rutherford, 2014).

Producing sugarcane genotypes that express these physical and biochemical defences via conventional breeding methods is time consuming due to the complex cytogenetics of the crop (Butterfield *et al.,* 2001; Ming *et al.,* 2006). Additionally, *E. saccharina* resistance obtained via conventional breeding has been found to be inversely related to resistance to sugarcane smut (Heinze *et al.,* 2001), a fungal disease that is capable of causing severe sugarcane yield losses. Transgenic sugarcane exhibiting resistance to stem borers via expression of *cry1A* genes from *Bacillus thuringiensis* Berliner*,* has also been attempted (Arencibia *et al.,* 1997; Weng *et al.,* 2011; Srikanth *et al.,* 2011). However, transgenic sugarcane is yet to be approved for commercial production worldwide (Meyer and Snyman, 2013). These deterrences warrant additional strategies to complement existing management approaches for controlling *E. saccharina* in sugarcane.

2.4 Biological control of insect pests

Biological control is defined as the deliberate use of insects, entomopathogenic nematodes and microorganisms to manage pest populations (Mahr *et al.,* 2001; Pal and Gardner, 2006). Several ecological relationships occur between insects, fungi and their plant hosts, e.g. mutualism, parasitism, commensalism and neutralism (Pal and Gardner, 2006). Whilst some of these interactions may be detrimental to the host, some are beneficial. Various fungal and insect species that occur in plants have been found to be natural enemies of pests (Faria and Wraight, 2001; Vega *et al.*, 2009). Conlong (2001) reported that *Schembria eldana* Barraclough (Diptera:Tachinidae), *Syzeuctus* sp., *Goniozus garoue* (Risbec) (Hymenoptera: Bethylidae), *Actia* sp., *Beauveria bassiana* (Balsamo) Vuillemin and *Iphiaulax* sp. obtained from sugarcane, maize and sedges, were parasitiods of *E. saccharina*. Such associations may be manipulated in biological control strategies against insect pests. This approach has advantages over use of insecticides which pose a threat to human health, non-target organisms and the environment via residue contamination of soils and water bodies, and are also prone to redundancy when the insects attain resistance to the chemicals (Mahr *et al.,* 2001). Along with the use of insect parasitiods, the utilisation of entomopathogenic fungi–mediated biological control as part of the integrated pest management may contribute towards

environmentally friendly and cost-effective control of pests such as *E. saccharina.* However, unlike insect parasitiods, endophytic entomopathogens may also benefit the plant through growth enhancement, disease resistance and drought tolerance (Kaldau and Bacon, 2008). Identifying a plant – fungus relationship in which the microorganism protects the host from the pest without the microbe causing disease, i.e. endophytism, is important in employing entomopathogens as biological control agents.

2.4.1 Endophytes

Endophytes are microorganisms that colonise plant tissues for part of their life cycle without causing apparent symptoms in their host (Saikkonen *et al.,* 1998; Azvedo *et al.,* 2000; Schultz and Boyle, 2005; Porras-Alfaro and Bayman, 2011). A diverse range of fungal endophytes has been isolated from different plant species worldwide (Schultz *et al.*, 1993; Hoff *et al.,* 2004; Crozier *et al.,* 2006; Kim *et al.,* 2007; Macia-Vicente *et al.*, 2008; Gazis and Chaverri, 2010). In a study of 12 plant species, Schultz *et al.* (1993) isolated 16 different endophytic fungal strains from each of the 11 species. Mehnaz (2013) reviewed a range of non-pathogenic fungal species isolated from sugarcane. These plant - endophyte interactions are mutualistic associations in which the host obtains growth promotion and defence from biotic and abiotic stresses, whilst the fungus gains nutrients and habitation from competitors and unfavourable environmental factors on the exterior of the plant (Schultz and Boyle, 2005).

Fungal endophytes are capable of colonising the plant host systemically or locally in roots, stem and leaf tissues (Saikkonen *et al.,* 1998). They can grow in intercellular spaces of plant tissues where they benefit from nutrients released into the apoplast (Clay and Schardl, 2002; Kaldau and Bacon, 2008), whilst others occur intracellularly (Rodriguez *et al.,* 2009). The endophyte aids the plant by producing secondary metabolites which exhibit antimicrobial (Danielsen and Jensen, 1999; Gao *et al.,* 2010), insecticidal (Azevedo *et al.,* 2000; Vega *et al.,* 2008) and growth-enhancing (Zhi-lin *et al.*, 2007; Machungo *et al.*, 2009) activities. Hence, the losses the plant incurs in supporting the endophyte are compensated by the microorganism's contribution to host fitness (Backman and Sikora, 2008). Schultz *et al.* (1999) stated that the outcome of a plant – microorganism interaction (i.e. disease development or endophytic colonisation) depends on the virulence of the microbe, its adaption to the host, defence responses of the host and environmental conditions. Those authors proposed that endophytic colonisation of a host plant occurs when the virulence of the microorganism and the defence mechanisms of the host are at an equilibrium such that neither is negatively impacted by the association. Elements that may disturb this balance, e.g. environmental factors that stress the host, can result in disease development (Schultz and Boyle, 2005).

Based on evolutionary history, taxonomy, plant hosts and ecology, fungal endophytes are categorised into clavicipitaceous and non-clavicipitaceous classes (Table 1) (Rodriguez *et al.*, 2009). The clavicipitaceous fungi (Class 1) (order - Hypocreales, family - Clavicipitaceae) are classified into over 33 genera and 800 species (Eriksson, 2006), including *Cordyceps*, *Balansia*, *Epichloe*, *Claviceps*, (Bacon and White, 2000) and *Neotyphodium* spp. (Kaldau and Bacon, 2008). They exclusively occupy above ground parts of numerous grasses with colonisation levels increasing from the basal towards the apical regions (Kaldau and Bacon, 2008). Whilst certain species colonise a range of grasses, some are host specific (Saikkonen *et al.*, 1998). Clavicipitaceous fungi produce plant growth regulators (e.g. indole acetic acid) (De Battista *et al.*, 1990), loline alkaloids that are involved in drought tolerance, peramine alkaloids which display antiherbivory activities (Bush *et al.*, 1997) and antifungal compounds (e.g. indole derivatives and sesquiterpene) (Yue *et al.*, 2000). Kaldau and Bacon (2008) reviewed a range of insects and nematodes that are inhibited by *Epichloe* spp. and *Neotyphodium* spp. These attributes of clavicipitaceous fungi have resulted in their use in the production of endophyte-enhanced turf (Bacon *et al.*, 1997) and pasture (Easton *et al.*, 2001) grasses. However, some species have been implicated in livestock toxicosis due to their production of ergot alkaloids in colonised pastures, e.g. tall fescue and rye grass (Looper *et al.*, 2012; Young *et al.*, 2012).

Rodriguez *et al.* (2009) distinguished non-clavicipitaceous endophytes (NCE) into three functional classes based on ecological interaction with the host (Table 1). Class 2 NCE are a group of fungi belonging to either the Ascomycota or Basidiomycota and include some *Fusarium* and *Colletotrichum* spp. (Rodriguez *et al.*, 2008). They are notable for their extensive presence in roots, rhizome and shoots, occupying host tissue in intra- and intercellular spaces and achieving levels of colonisation of up to 100% (Rodriguez *et al.*, 2009). Class 2 NCE are transmitted to other individual plants horizontally or vertically via the seed coats (Redman *et al.*, 2002). They also are reported to produce metabolites that enhance growth (Tudzynski and Sharon, 2002), induce disease (Schultz *et al.*, 1999) and drought (Rodriguez *et al.*, 2008) tolerance. Class 3 NCE (Table 1) mainly belong to the Ascomycota, subphyla Pezizomycotina and Saccharomycotina (Higgins *et al.*, 2007), and are differentiated by their localised occurrence mainly within above ground parts (Rodriguez *et al.*, 2009). They are a highly diverse group with numerous species colonising a small area of the host tissue such that individual leaves of the same plant may house conglomerates of endophytes (Arnold *et al.*, 2000). Class 4 NCE (Table 1) occur only in the roots and are characterised by dark melanised

Table 1: Characteristics of clavicipitaceous and non-clavicipitaceous endophytes (modified from Rodriguez *et al.*, 2009)

Criteria	Clavicipitaceous	Non-clavicipitaceous		
	Class 1	Class 2	Class 3	Class 4
Host range	Narrow	Broad	Broad	Broad
Tissue(s) colonised	Shoot and rhizome	Shoot, root and rhizome	Shoot	Root
In planta colonisation	Extensive	Extensive	Limited	Extensive
In planta biodiversity	Low	Low	High	Unknown
Transmission	Vertical and horizontal	Vertical and horizontal	Horizontal	Horizontal
Examples	*Epichloe* spp. *Cordyceps* spp. *Claviceps* spp. *Balansia* spp	*Fusarium culmorum (*Smith) Sacc, *Fusarium oxysporum* Snyder and Hansen, *Colletotrichum magna* Jenkins & Winstead	*Ustilago maydis* Corda*,* *Phyllosticta* spp.	*Chloridium paucisporum* Wang & Wilcox*, Leptodontidium orchidicola* Sigler & Currah

septate hyphae and microsclerotia, which occupy intra- and intercellular spaces (Rodriguez *et al.*, 2009). The group constitutes of 320 genera and 114 families, which are found in 587 plant species located in various ecosystems including high-stress environments (Rodriguez *et al.*, 2009). Their presence in the rhizosphere may serve to decrease the carbon available to pathogenic microorganisms, whilst synthesis of melanin by the endophytes may be involved production of antiherbivory metabolites (Mandyam and Jumpponen, 2005).

2.4.2 Endophyte-mediated biological control of insects

A number of studies in various crops have documented the negative effect of endophytic fungi on insect pests. Bing and Lewis (1993) reported that *B. bassiana* reduced the population of the European cornborer, *Ostrinia nubilalis* (Hiibner) (Lepidoptera: Crambidae) by 31-60% in maize. In that study, 100% of the larvae mycosed by *B. bassiana* were obtained from plants endophytically colonised by the fungus. In a study by Prestidge and Gallagher (1988), the tunnel length bored by the Argentine stem weevil *Listronotus bonariensis* (Kuschel) (Coleoptera: Curculionidae) in ryegrass was reduced by infecting plants with the endophyte

Acremonium lolii Latch, Christensen, and Samuels. In sugarcane, McFarlane *et al.* (2009) observed minimal damage by *E. saccharina* in stalks endophytically colonised by certain *Fusarium* strains. In addition, those *Fusarium* strains reduced larval mass and survival of the stalk borer in dietary inclusion assays and repelled the insect in olfactory choice assays.

Endophytes produce various secondary metabolites that are detrimental to insects via different modes of action. For example, antibiosis of insect larvae was reported to occur as a result of production of bioactive volatile organic compounds, e.g. alcohols, esters and ketones, by endophytes (Lacey *et al.*, 2009). Alkaloids produced by endophytes can act as feeding deterrents by rendering plant tissue unpalatable by insects (Carroll, 1988). For instance, Clay (1988) reported that armyworm (*Spodoptera* sp.) larvae consumed less ergot alkaloid-treated maize leaf tissue than those that fed on untreated leaf tissue. Some metabolites produced by endophytes are also toxic to insects, e.g. lolitrem B is a neurotoxin produced by some clavicipitaceous fungi and has been observed to result in slower development rates and higher mortality in stem weevil (*Listronotus* sp.) larvae feeding on diet containing the alkaloid (Gaynor and Rowan, 1986). Also, some *Fusarium* spp. produce fusaproliferin and beauvericin (Gupta *et al.*, 1991; Logrieco *et al.*, 1996), compounds reported to be toxic to insects through inhibition of phenoloxidase, an enzyme which acts against entomopathogens by means of encapsulation (Dowd, 1999). In addition to production of secondary metabolites, the presence of endophytes in host tissues may also trigger plant defences (via jasmonate signalling pathway) against insects, thereby inducing resistance to pests (Backman and Sikora, 2008). Endophytic entomopathogenic fungi may, therefore, be introduced into crops as biological control agents against insect pests (Shah and Pell, 2003).

2.4.3 *Fusarium* spp. as endophytic biological control agents

The genus *Fusarium* is a member of the order Hypocreales, which belongs to the class Ascomycetes (Seifert, 1996). *Fusarium* spp. are commonly found together with higher plants and are prevalent in terrestrial ecosystems (Ploetz, 2005), colonising a wide range of plant species, e.g. *F. verticillioides* infects over 1000 species (Bacon and Yates, 2006). Most strains are pathogenic to various crops causing wilts (Baayen *et al.*, 1997; Akkopru and Demir, 2005; Sharma and Muehlbauer 2007; Muthomi *et al.,* 2012) and rots (Mughogho and Rosenberg, 1984; Croft, 2000; Akinsanmi *et al.*, 2004; Afolabi *et al.*, 2008), whilst others are endophytic (Bacon and Hinton, 1996; Bacon and Yates, 2006; Macia-Vicente *et al.*, 2009; Zakaria and Rahman, 2011). Endophytic *Fusarium* strains have been isolated from maize (Bacon and Yates, 2006), barley (Macia-Vicente *et al.*, 2008), wheat (Larran *et al.*, 2007) and sugarcane (McFarlane *et al.*, 2009). They have potential as biological control agents of pests and diseases as they produce a wide array of compounds that are harmful to insects and

pathogens. For example, Athman *et al.* (2006) and Zum Felde *et al.* (2006) reported that inoculation of banana roots with endophytic *Fusarium* resulted in lower numbers of the nematode *Radopholus similis* (Cobb) Thorne than in uninoculated plants. Endophytic *Fusarium verticillioides* was also shown to reduce maize smut disease symptoms induced by *Ustilago maydis* (Lee *et al.*, 2009; Estrada *et al.*, 2012). Kidane and Laing (2010) documented the negative effect an endophytic strain of *F. oxysporum* on its pathogenic counterpart *F. oxysporum* f. sp. *cubense* (E.F. Smith) Snyder and Hansen which causes wilt in banana. Navarro-Meléndez and Heil (2014) reported that endophytic *Fusarium* spp. experimentally introduced into Lima bean plants resulted in elevated levels of jasmonic acid, a plant signalling metabolite responsible for plant response against herbivory, as previously discussed.

2.5 *Fusarium* spp. – *Eldana saccharina* interactions

The wounds inflicted by *E. saccharina* on plants provide *Fusarium* spp access to the inner stalk tissues, thus resulting in an association between borer infestation and infection by the fungus. Moreover, studies in maize and sugarcane revealed that *Fusarium* spp. impact the biology of the lepidopteran during this interaction. For instance, Schulthess *et al.* (2002) reported that in maize, stalks infected by endophytic strains of *F. verticilloides* showed greater damage by *E. saccharina* than those treated with a fungicide. In addition, Ako *et al.* (2003) observed that *E. saccharina* oviposited approximately four times more on maize stems infected with *F. verticillioides* than on the uninoculated controls. Those studies in maize indicated that *F. verticillioides* promotes *E. saccharina* survival and development. Similarly, findings from studies in sugarcane by McFarlane *et al.* (2009) revealed that some endophytic *Fusarium* strains were beneficial to *E. saccharina* growth and survival in *in vitro* assays. Bartlet and Wicklow (1999) identified volatile alcohols aldehydes, esters and phenolics produced by *F. verticillioides*, which were responsible for attraction of sap beetles (Coleoptera: Nitidulidae) in bioassays. Ako *et al.* (2003) reported that these compounds are also known to attract lepidopterous stem borers. However, McFarlane *et al.* (2009) also reported that some *Fusarium* isolates exhibited harmful effects on *E. saccharina* larval weight and survival in dietary inclusion assays. Olfactory choice assays carried out in that study also indicated that these *Fusarium* isolates repelled the borer. These antagonistic effects of the *Fusarium* isolates on the pyralid may be due to action of metabolites such as beauvericin, fusaproliferin (Gupta *et al.*, 1991; Logrieco *et al.*, 1996) and fusaric acid (Dowd, 1999), which are insecticidal compounds known to be produced by *Fusarium* spp.

The interactions between *Fusarium* spp. and *E. saccharina* have implications on control approaches for the lepidopterous pest in sugarcane. Curbing plant infection by *Fusarium* strains beneficial to *E. saccharina* may aid in reducing damage by the borer. More importantly,

Fusarium strains harmful to the lepidopteran may be employed as biological control agents against the pest. However, the latter approach is impeded by the susceptibility of sugarcane to Fusarium stem rot.

2.6 Fusarium stem rot

2.6.1 The pathogen

Fusarium stem rot was first encountered in sugarcane in Barbados in 1922 (Cook, 1981). The species that causes the disease was initially named *Fusarium moniliforme* Sheldon (Anamorph: *Gibberella moniliforme* [Sheldon] Wineland) (Bourne, 1961). However, the taxonomy of the genus *Fusarium* has been problematic (Kruger, 1989; Thrane, 1989) due to inconsistency of the features used in identification of different species, thus leading to erroneous identification of some species (Edgerton, 1955; Nelson, 1991). The current classification system of this genus has 16 sections, 65 species and 77 subspecific varieties and forms (Leslie and Summerell, 2006). The genus is identified by the production of three types of asexual spores called conidia (Ohara *et al.*, 2008). Macroconidia are large, slender, septate and canoe-shaped and are produced in fruiting bodies called sporodochia; microconidia are markedly different and are produced on aerial mycelium and chlamydopores are produced by some species (Seifert, 1996). *Fusarium moniliforme* was described as the only species in section Liseola (Snyder and Toussoun, 1965). However, *F. moniliforme* was renamed *F. verticillioides* Sacc. (Marasas *et al.*, 2001). Consequently, there was doubt on the identity of those isolates initially identified as "*F. moniliforme*" as they were not *F. verticillioides* (Leslie and Summerell, 2006). Leslie and Summerell (2006) stated that strains initially identified as *F. moniliforme* that were not *F. verticillioides*, would probably be called other species, e.g. *F. fujikori* from rice, *F. thapsinum* from sorghum and *F. sacchari* in sugarcane. However, using RFLP analysis, McFarlane and Rutherford (2005) identified *F. sacchari, F. verticillioides, F. proliferatum*, and *F. subglutinans* Wollenw. and Reink. in sugarcane stalks. In subsequent work with the aid of direct sequencing, isolates from sugarcane were identified as mainly *F. sacchari* and some as *F. pseudonygamai* O'Donnell and Nirenberg and *F. verticillioides* (McFarlane *et al.*, 2009).

2.6.2 The disease

Infection of sugarcane by *Fusarium* occurs in stems that have been injured or damaged by borers *Diatraea saccharalis* Fabricius (Holliday, 1980) or *E. saccharina* (McFarlane *et al.*, 2009). The disease is characterised by red-brown discolouration of the parenchyma, which is darker in the vascular tissues. The fungus spreads in the xylem (Sivanesan and Waller, 1986)

resulting in the longitudinal splitting of the stalks, which reveals symptoms extending across internodes (Cook, 1981). Those symptoms are frequently found to spread rapidly, more towards the base than towards the top of the stem (Cook, 1981). The leaves wilt, turn yellow and dry up (Croft, 2000).

Bourne (1961) reported that wilting in infected plants is probably induced by the wilting agent fusaric acid, which is produced by the fungus. The movement of the compound up the xylem in its undissociated state, results in faster advancement up the xylem because it is uninterrupted by the negatively charged components of the xylem walls (Bourne, 1961). The compound permeates through the cell walls of the vascular bundle parenchyma without difficulty, thereby permitting the fungus access to the vascular bundles where it elicits the most damage (Bourne, 1961). In addition, necrosis of infected plant tissue may be due to the action of fumonisins, which are phytotoxic compounds produced by *Fusarium* spp. (Nelson *et al.*, 1993; Marasas *et al.*, 2000; Nishiuchi, 2013) that interrupt sphingolipid metabolism (Munkvold and Desjardins, 1997; Marasas *et al.*, 2000; Torre-Hernandez *et al*., 2010). Sphingolipids are components of cell membranes (Munkvold and Desjardins, 1997), structurally similar to fumonisin B_1 (Marasas *et al.*, 2000) and are thought to be involved in signal transduction, membrane stability, programmed cell death and host-pathogen interaction in plants (Christie, 2010). Monoliformin, also produced by *Fusarium* spp., may cause disease by inhibiting the mitochondrial oxidative enzyme, pyruvate dehyrogenase, affecting the entry of carbon into the Krebs cycle during plant respiration (Schuller *et al.*, 1993; Nishiuchi, 2013). Other phytotoxins produced by *Fusarium* spp. which may be involved in disease development in plants include trichothecenes (Desjardins and Hohn, 1997; Menke, 2012), zearalenone (Miedaner, 1997; Logrieco *et al.,* 2002) and fusarins (Desjardins and Proctor, 2007).

Whilst *Fusarium* is mainly reliant on stalk borer damage for access into sugarcane stalks, it can also be transmitted via the cut ends of setts, immature adventitious roots, nodal leaf scars of stems planted in infected soils and the use of cane cuttings obtained from infected stems (Holliday, 1980). The fungus grows on decaying plant material and produces a large number of conidia (Bourne, 1961) that are spread by wind and rain (Croft, 2000). In the SA sugar industry, Fusarium stem rot is mainly a problem in association with *E. saccharina* damage where tissue surrounding the borer tunnels is discoloured, thus compounding damage caused by the pest. Production of sugarcane cultivars tolerant to *Fusarium* may aid in reducing such damage and also enable control of *E. saccharina* via endophytic biological control methods using insecticidal strains of the fungus.

2.7 Development of genotypes tolerant to *Fusarium* spp.

2.7.1 Conventional breeding approaches

In conventional breeding, carefully selected parents are crossed to reproduce offspring that exhibit specific characteristics that meet human requirements, based on sexual genetic inheritance of parental traits by the progeny according to Mendelian genetics (Acquaah, 2007). The variation generated in offspring is a result of gene recombination, varying chromosome number and mutations (Poehlman and Sleper, 1995). In this way, and for centuries, plant breeders have been developing crops with superior growth, yields and pest and disease resistance compared with their wild relatives (James, 2004; Ming *et al.,* 2006; Todd *et al*., 2014).

Sugarcane breeders aim to produce varieties with high yield, high sucrose content, good ratoonability, low fibre levels and pest and disease resistance (Jackson, 2005; Srikanth *et al.*, 2011; Zhou, 2013a). The commercial sugarcane cultivars used today resulted from crosses of *S. officinarum* and *S. spontaneum* (Stevenson, 1965; Sreenivasan *et al.*, 1987; Butterfield *et al.*, 2001; Ming *et al.*, 2006; Singh *et al.*, 2010; Todd *et al*., 2014). However, the reproductive biology and complex genome of sugarcane complicate breeding of genetically-improved varieties by conventional means (Selman-Housein *et al.*, 2000; Gill *et al.,* 2004; Ming *et al.,* 2006). For instance, flowering in male and female parent plants does not coincide (Selman-Housein *et al.*, 2000) and pollen production varies between varieties causing variation in crossing and selfing (James, 2004). In addition, pollen viability is short-lived, thus making it difficult for sugarcane breeders to carry out intended crosses (Anon, 2004). Offspring of parents are surveyed in a number of crosses and promising genotypes are then selected (Stafne *et al.,* 2001; Tai *et al.,* 2003; Berding *et al.,* 2004). Due to the polyploidy of sugarcane, a single cross can produce large numbers of offspring that vary in a range of features which include size, yield and disease resistance (Barnes, 1964; Olaoye, 2001; Berding *et al.,* 2004). Furthermore, sugarcane genotypes differ in fertility and produce small seed that is fertile under specific conditions (Poehlman and Sleper, 1995; James, 2004). Hence, sugarcane breeding is a laborious and time-consuming process, with developement of new superior clones taking 12-15 years (Burnquist 2001; Butterfield *et al*, 2001; Lakshmanan, 2005). Nevertheless, some *Fusarium*-tolerant genotypes have been produced through conventional breeding in sugarcane (Lyrene *et al.*, 1977), maize (Kozhukhova *et al.*, 2007; Afolabi, 2008; Tembo *et al.,* 2013) and wheat (Jansen *et al.,* 2005; Lv *et al*., 2014).

Biotechnological tools can be used to assist conventional breeding and reduce the time taken in producing desired genotypes (Selman-Housein *et al.*, 2000; Wang *et al.,* 2005; Garcia and Mather, 2014). For example, marker-assisted selection (MAS) has been utilised to assist

breeders to select for certain genes in crops (Wang *et al.,* 2005, Singh *et al.,* 2013b). Genetic maps that show the position of certain genes on the chromosomes have been constructed for various crops, aiding plant breeders in breeding programs (Poehlman and Sleper, 1995; Bohra *et al*., 2014). This approach, called molecular breeding (Wang *et al.*, 2005), has been widely used in breeding programs of cereals and other crops (Butterfield *et al.,* 2001; Korzun, 2003; Pan *et al.,* 2003; Wang *et al.*, 2005, Singh *et al.,* 2013b; Garcia and Mather, 2014). However, due to the polyploid nature of sugarcane, the link between the genes and alleles present in the genotype and their expression in the phenotype is complicated by silencing and differential expression of gene copies (Butterfield *et al.,* 2001, Manners, 2011). Current advancements in elucidating sugarcane sequences will enable the utilisation genomic information resources in breeding strategies for the crop (De Setta *et al.,* 2014).

2.7.2 Genetic engineering

Genetic transformation is the insertion of specific genes into a genome where the inserted gene is expressed (Poehlman and Sleper, 1995). Crops that have been transformed and are commercially available include canola, cotton, maize, tomato and soybean (ISAAA, 2013). In 2012, there were 17.3 million farmers in 28 countries cultivating transgenic crops under 170.3 million hectares, which increased to 175 million hectares in 2013 (ISAAA, 2013). Sugarcane transformation started in the 1980s (Chen *et al.,* 1987) and particle bombardment has been the main method used to introduce genes into sugarcane cells (Allsopp and Manners, 1997; Snyman *et al.,* 2000; Kaur *et al.*, 2007; Van der Vyver *et al.,* 2013; Joyce *et al.,* 2014). Cell electroporation (Arencibia *et al.,* 1999) and *Agrobacterium tumefaciens*-mediated transformation (Dong *et al*., 2014; Kumar *et al.*, 2014), have also been used.

The high chromosome numbers and genomic complexities of sugarcane makes expression of inserted genes complicated (Lakshmanan *et al.,* 2005; Xue *et al.,* 2014). However, strategies for the development of disease resistant transgenic sugarcane have been established. They involve insertion of genes capable of degrading or inactivating pathotoxins, producing polypeptide signals that induce expression of protease inhibitors and producing enzymes that enhance the toxicity of antibiotics produced by plants (Allsopp and Manners, 1997), expression of untranslatable virus coat proteins (Zhu *et al.,* 2011) and RNA inference of virus coat proteins (Zhuo *et al.,* 2014). Resistance has been achieved by genetic transformation for diseases which include sorghum mosaic virus (SrMV) (Ingelbrecht *et al.,* 1999), sugarcane leaf scald (Zhang and Birch, 2000), sugarcane rust (*Puccinia melanocephala* Syd. and Syd.) (Enriquez *et al.,* 2000), Fiji disease virus (McQualter *et al.,* 2004), sugarcane yellow leaf virus

(ScYLV) (Gilbert *et al.,* 2009; Zhu *et al.,* 2011) and sugarcane mosaic virus (ScMV), (Gilbert *et al.,* 2005; Zhuo *et al.,* 2014). Other traits that have been introduced into sugarcane by genetic transformation include increased sucrose content, suppressed flowering and resistance to sugarcane borers *D. saccharalis* (Burnquist, 2006), *Proceras venosatus* Walker (Weng *et al.,* 2011), herbicide (van der Vyver *et al.,* 2013), drought (Reis *et. al.,* 2014) and salinity (Kumar *et al.,* 2014) tolerance. However, none of these is available commercially.

Makandar *et al.* (2006) obtained resistance to Fusarium head blight in wheat (caused by *F. graminearum*) by inserting the *NRP1* gene from *Arabidopsis thaliana.* In maize, *F. moniliforme* infection was reduced by controlling the European corn borer by inserting genes coding for the endotoxin *cryIA(b)* produced by *B. thuringienesis,* which resulted in lower levels of fumonisins that cause symptoms of Fusarium ear rot (Munkvold *et al.,* 1997). Funnell and Pedersen (2006) inserted genes that lowered the lignin levels in sorghum, which resulted in resistance to *F. moniliforme.* Gaspar *et al.* (2014) obtained resistance to *F. oxysporum* in cotton by transforming calli to express the NaD1, a defensin that has antifungal properties.

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

Despite research and development being carried out since the 1980s (Chen *et al*., 1987), the first transgenic sugarcane variety in the world was only approved for commercialisation in 2013 in Indonesia (www.thejakartapost.com). This slow adoption of genetically modified sugarcane is due to limitations which include transgene silencing, inadequate knowledge about inheritance of transgenes (Lakshmanan *et al.*, 2005), legislation (Burnquist, 2006, Arruda, 2011; Meyer and Snyman, 2013) and intellectual property issues (Birch, 2014). Further, transformation of monocotyledons is limited by inefficient transformation systems and low cell competence (Sood *et al.,* 2011).

2.8 *In vitro* culture systems

In vitro culture refers to the culture of plant cells, tissues and organs, under controlled sterile laboratory conditions that allow them to regenerate into whole plants (Jain, 2006; Thorpe, 2007). The process manipulates the cells' ability to regenerate into whole plants (totipotency) (George, 1993; Litz and Gray, 1995). Since its discovery in the 1930-1940s, plant cell culture has been an essential part in plant improvement (Sangwan *et al.*, 1997), with a wide application in plant physiology and biotechnology strategies (Karp, 1995, Birch, 2014).

Sugarcane culture was first successfully carried out by Nickel (1964), who produced calli which later developed roots. Whole plant regeneration was then achieved by Heinz and Mee (1969). Since then, sugarcane culture has had various applications, e.g. micropropagation (Lee, 1987; Baksha *et al.,*2002; Pawar *et al.,* 2002; Cheema and Hussain, 2004; Meyer *et al.,* 2007; Behera and Sahoo, 2009; Kaur and Sandhu, 2014), virus elimination (Irvine and Benda, 1985; Parmessur *et al.,* 2002; Snyman *et al.*, 2005; Ramgareeb *et al.,* 2010, Neelamathi *et al.,* 2014), genetic transformation (Snyman *et al.,* 2000; Snyman, 2004; Lakshamanan *et al.,* 2005; Shah *et al.,* 2009, Joyce *et al*., 2014), improvement via somaclonal variation (Krishnamurthi and Tlaskal, 1974; Liu and Chen, 1978; Peros *et al.,* 1994; Patade *et al.,* 2005; Singh *et al.,* 2008) and germplasm preservation (Gnanapragasam and Vasol, 1990; Taylor and Dukic, 1993; Watt *et al.,* 2009; Nogueira *et al.,* 2013).

Conventionally, sugarcane is vegetatively propagated by means of stem cuttings (known as setts) with 2-3 nodes (Behera and Sahoo, 2009) which results in a low rate of plant multiplication, *viz.* 10-20 plants being produced per stalk (Geijskes *et al.,* 2003). The planting material also causes spreading of diseases (Hoy *et al.,* 2003). Consequently, the distribution of new cultivars to farmers is time consuming. In comparison, Geijskes *et al.* (2003) showed that micropropagation is up to 35 times more productive than the conventional approach. At the SASRI, Snyman *et al.* (2008) found that 32-600 plants per stalk could be obtained from different SA sugarcane varieties. Sugarcane micropropagation is, therefore, a highly beneficial technique for the rapid production of good quality planting material (Bailey and Brechet, 1989; Karim *et al.,* 2004; Roy and Kabir, 2007; Ali *et al.,* 2008; Khan *et al.,* 2008; Behera and Sahoo, 2009; Kaur and Sandhu, 2014).

Whole plants can be regenerated *in vitro* via somatic embryogenesis or organogenesis (Fig. 5) and each of the two morphogenic routes has wide applications (Table 2). Organogenesis involves the regeneration of plants either directly from tissues (e.g. shoot tips) or indirectly from callus, an undifferentiated mass of cells (George, 1993). Regeneration via direct organogenesis, i.e. without the callus stage, reduces the chance of obtaining variant plants through somaclonal variation (Lakshmanan *et al.,* 2006). In sugarcane, the manipulation of plant growth regulators, i.e auxins and cytokinins in the medium, results in the formation of shoots and roots from callus (Lee, 1987; Karim *et al.,* 2004; Behera and Sahoo, 2009; Dibax *et al.,* 2013), shoot tips (Fitch *et al.,* 2001; Baksha *et al.,* 2002; Pawar *et al.,* 2002; Ali *et al.,* 2008; Sughra *et al.,* 2014) and auxillary buds (Cheema and Hussain, 2004; Mekonnen *et al.,* 2014). In somatic embryogenesis, somatic cells form bipolar embryos that are similar to those formed from zygotic cells (Ahloowalia and Maretzki, 1983; Litz and Gray, 1995; Ali *et al.,*

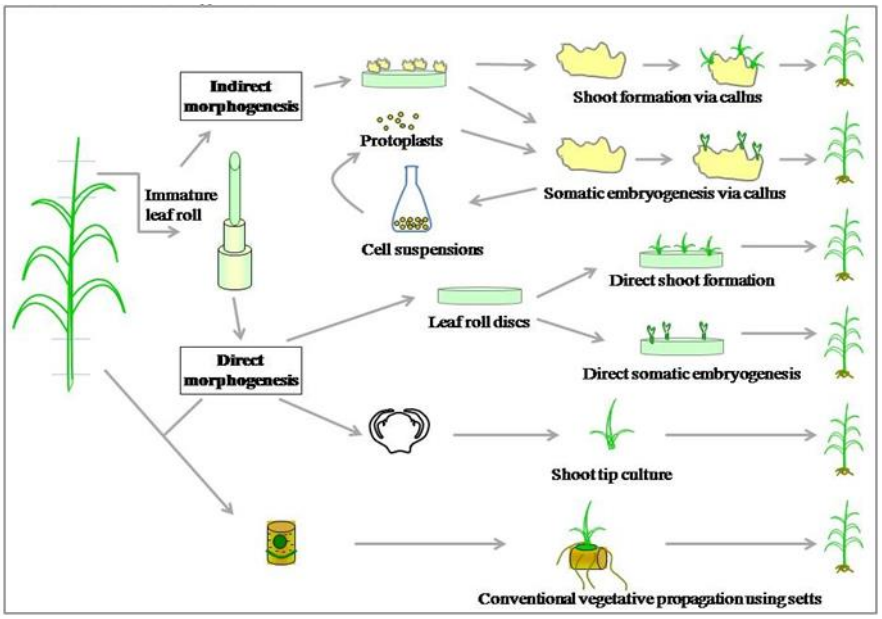

Figure 5: Illustration of indirect and direct morphogenesis routes in sugarcane plantlet regeneration (from Snyman, 2004).

2007a). Somatic embryo formation, similar to zygotic embryo development, is characterised by the development of cells into globular, heart-shaped and finally torpedo-shaped stages in dicotyledons (Terzi and Loschiavo, 1990; Zimmerman, 1993; Litz and Gray, 1995; Dodeman *et al.,* 1997; Malabadi *et al.,* 2011) or globular, scutellar and coleoptilar stages in monocotyledons (Gray *et al.,* 1995). Burrieza *et al.* (2012) demonstrated the accumulation and nuclear localisation of dehydrins (proteins usually expressed late in zygotic embryogenesis) in sugarcane embryos, thus indicating their involvement in induction and maintenance of somatic embryogenesis.

As with organogenesis, somatic embryos can be produced directly from cells of the explant (e.g. leaf roll), i.e. direct somatic embryogenesis (Snyman, 2004) or indirectly via a callus stage, i.e. indirect somatic embryogenesis (Ho and Vasil, 1983; Snyman, 2004; Malabadi *et al.,* 2011). Sugarcane produces compact embryogenic callus, friable non-embryogenic callus and mucilaginous non-embryogenic callus (Ho and Vasil, 1983; Guiderdoni and Demarly, 1988; Lakshmanan, 2006; Rae *et al*., 2014). The ability of sugarcane leaf segments to produce calli of different embryogenic potential was demonstrated by Guiderdoni and Demarly (1988). Those authors reported that the innermost sheath produces white compact embryogenic callus, the intermediate produces friable non-embryogenic callus and the outer produces mucilaginous non-embryogenic callus. Sugarcane embryos can be produced directly from leaf

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

Application	Morphogenesis route	Reference
Micropropagation	Direct organogenesis	Baksha *et al.* (2002)
	Indirect organogenesis	Meyer *et al.* (2007)
	Direct somatic embryogenesis	Behera and Sahoo (2009)
	Direct organogenesis	Kaur and Sandhu (2014)
Pathogen elimination		
Sugarcane mosaic virus	Direct organogenesis	Irvine and Benda (1985)
Yellow leaf syndrome (YLS), sugarcane yellow leaf virus	Indirect somatic embryogenesis and direct organogenesis	Parmessur *et al.* (2002)
Ratooning Stunting disease, sugarcane mosaic virus, sugarcane yellow leaf virus, sugarcane leaf yellows phytoplasma	Direct somatic embryogenesis	Snyman *et al.* (2005)
Sugarcane mosaic virus, sugarcane yellow leaf virus	Indirect somatic embryogenesis, direct and indirect organogenesis	Ramgareeb *et al.* (2010)
Genetic transformation	Direct and indirect somatic embryogenesis	Snyman *et al.* (2000)
	Direct somatic embryogenesis	Snyman *et al.* (2006)
	Direct organogenesis	Kumar *et al*. (2014)
Breeding		
Fiji disease resistance	Indirect organogenesis	Krishnamurthi and Tlaskal (1974)
Performance and yield	Indirect organogenesis	Liu and Chen (1978)
Sugarcane rust and yield	Direct and indirect organogenesis	Peros *et al.* (1994)
Salinity and drought tolerance	Indirect somatic embryogenesis	Patade *et al.* (2005)
Red rot, yield, height	Indirect somatic embryogenesis	Singh *et al.* (2008)
Drought tolerance	Indirect somatic embryogenesis	Rao and Ftz (2013)
Germplasm preservation		
Cryopreservation	Indirect somatic embryogenesis	Gnanapragasam and Vasil (1990)
Cryopreservation	Indirect somatic embryogenesis	Chanprame *et al.* (1993)
	Direct organogenesis	Taylor and Dukic (1993)
Slow growth	Indirect somatic embryogenesis	Watt *et al.* (2009)

discs and indirectly from callus on media containing low and high concentrations of 2,4-D (2,4-Dichlophenoxyacetic acid), respectively (Snyman *et al.*, 2000; Laskshmanan, 2006; Sharma *et al.,* 2007) and no callus forms in the absence of 2,4-D (Ho and Vasil, 1983). Other plant growth regulators used in sugarcane somatic embryogenesis include benzylaminopurine (BAP), kinetin (Gill *et al.,* 2004) 3,6-dichloro-O-anisic acid (dicamba), naphthaleneacetic acid (NAA) and 4-fluorophenoxyacetic acid (4-FPA) (Brisibe *et al.,* 1994). Chengalrayan *et al*. (2005) produced callus from sugarcane seeds on media containing picloram. Embryo formation in sugarcane is also dependent on the genotype with different varieties requiring media with varying levels of auxins, sugar and amino acids (Ozias-Akins *et al*., 1992; Ito *et al.,* 1999; Gill *et al.,* 2004; Onay *et al.,* 2007; Birch, 2014). Embryo germination generally occurs in media with no auxins (Snyman *et al.,* 2000; Parmessur *et al.,* 2002; Ramgareeb *et al.*, 2010).

2.8.1 *In vitro* culture-induced variation

Somaclonal variation in *in vitro* cultured plants was first described by Larkin and Scowcroft (1981) when they observed the resistance of previously susceptible *in vitro* plants to the toxin produced by *Helminthosporium sacchari* Butler*,* which causes eyespot in sugarcane. They called this spontaneous genetic change somaclonal variation, and defined it as heritable genetic variation that results from *in vitro* culture. However, variations in *in vitro* cultured sugarcane had been observed before by Heinze and Mee (1969). Since then, somaclonal variation has been utilized vastly in crop improvement, and is known to occur in many plant species (reviewed by Bairu *et al.,* 2011), including barley (Bregitzer *et al.,* 2002), maize (Vasconcelos *et al.,* 2008), petunia (Abu-Qaoud *et al.,* 2010), olives (Peyvandi *et al.,* 2010), potato (Ehasanpour *et al.,*2007), rice (Ngezahayo *et al.,* 2007), sorghum (Raveendran *et al.,* 1998), strawberry (Mohamed, 2007), sugarcane (Larkin and Scowcroft, 1983; Burner and Grisham, 1995; Snyman *et al.,* 2011; Rutherford *et al.,* 2014) and wheat (Abouzied, 2011).

Alterations in a cell's genome may result from stress induced on cells when they are exposed to new environments (McClintock, 1984). When cells are cultured *in vitro*, they are exposed to conditions of high sucrose, nitrogen, salt concentrations and osmotic potential different to those of soils. In addition, culture media usually contain plant growth regulators, which induce stress on the cells (Desjardins *et al.,* 2009; Lebeda and Svabova, 2010). Consequently, the cell's control mechanisms may break down leading to changes in the genome through different processes (Phililps *et al.,* 1994; Campbell *et al.,* 2011). For example, changes in DNA methylation patterns can affect gene expression by changing the structure of chromatin resulting in breaking of chromosomes due to delayed DNA replication (Kaeppler and Philips, 1993; Stelpflug *et al.,* 2014). DNA methylation has also been shown to result in the transposition of genetic elements in genomic DNA (Brown, 1989; Wang *et al.,* 2013). As a

result, increased DNA methylation leads to decreased gene expression and conversely, reduced DNA methylation enhances gene expression (George, 1993; Zhao and Chen, 2014). Variation also occurs due to activation of transposable elements as a result of *in vitro* culture (McClintock, 1984; Hirochika *et al.,* 1996; Kaeppler *et al.,* 2000; Peschke *et al.,* 2000; Zhang *et al.,* 2014). The activated transposable elements cause a change in the DNA sequence that can lead to a change in gene expression (Rossi *et al.,* 2001; Zhang *et al*., 2014). In addition, when cells are in stressful conditions, the number of copies of a specific gene within the genome can increase during cell differentiation, leading to an increase in mRNA synthesis and higher levels of the respective protein, which can manifest in the phenotype (Larkin and Scowcroft, 1981; Teaster and Hoagland, 2014).

Changes in chromosome structure can also occur during cell division due to stress of the culture environment, through inversion, deletion, fusion and duplication of sections of the chromosomes (Larkin *et al.*, 1989, Acanda *et al.,* 2013). Further, single base pair changes in the DNA sequence can occur due to the breakdown of systems that control the base sequencing (Philips *et al.,* 1994). Larkin and Scowcroft (1981) reported that the different mechanisms by which somaclonal variation may occur seem to be applicable to situations where variation already exists in the explant whilst others apply when cells are in culture. PontaroliI and CamadroII (2005) proposed that pre-existent ploidy variation within the explant may be a source of somaclonal variation. However, some of the variation observed in culture is epigenetic, i.e. it is reversible and cannot be passed on sexually to the next generation (George, 1993; Kaeppler *et al.*, 2000; Joyce *et al.,* 2003; Patade *et al.,* 2005). These epigenetic effects are due to changes in gene expression regulating mechanisms and not changes in the genetic sequence of the gene, which may be expressed in divided cells after mitosis, but not in the offspring of the regenerated plants after sexual reproduction (Chaleff, 1983). For instance, Sun *et al.* (2013) observed diminished pollen viability in regenerants torenia (*Torenia fournieri* Lind.) after one to nine sub-cultures. However, after sexual crosses, the pollen viability was recovered suggesting that epigenetic, and not genetic, factors such as DNA methylation were responsible the observed variation.

The extent of variation in cells also depends on the type of explant used (George, 1993) with variation likely to be greater in older and more differentiated material (Karp, 1995; Wang and Wang, 2012). Interestingly, Wang and Wang (2012) also reported that in some cases older cultures may exhibit less somaclonal variation. Genetic differences between the parent and the somaclones are less when plants are obtained from axillary meristems as opposed to regeneration via a callus stage (Hanna *et al.,* 1984; Ali *et al.,* 2008). Through molecular analysis of somaclones in sugarcane, Zuchhi *et al.* (2002) found that some genotypes are more prone to somaclonal variation than others. This may be due to varying ploidy levels

amongst species, with variation being expressed more in haploids and diploids than in polyploids (Karp, 1995). In addition, as different genotypes differ in genetic stability they may differ in susceptibility to mutations (Joyce *et al.,* 2003). Inclusion of plant growth regulators, auxins and cytokinins, singly or in combination, in the medium can result in cells mutating (George, 1993). For example, Bairu *et al.* (2006) showed that growth regulators increase somaclonal variation by increasing cell division in bananas. Other commonly used media constituents, e.g. yeast extract, coconut milk, kinetin and micronutrient metals, have also been shown to alter the ploidy level of cells and cause chromosome damage (George, 1993). For these reasons, the length of time cells are in culture affects the degree of variation (Burner and Grisham, 1995; Sun *et al.,* 2013).

Somaclonal variation is undesirable when true-to-type plants are required, e.g. during micropropagation (Litz and Gray, 1995; Bouman and De Klerk, 2001; Kour *et al.,* 2012) and in transgenic plants (Joyce *et al.,* 2014). In such cases, molecular studies to detect variants are necessary (Khoddamzadeh *et al.,* 2013; Bello-Bello *et al.,* 2014). However, somaclonal variation is also a source of variant plants that can be utilised for plant improvement (Patade *et al.,* 2005; Rutherford *et al.,* 2014). New traits, which conventional breeding may be unable to develop, can be obtained through screening large numbers of somaclonal variants (Jain, 2001). Despite the discovery of somaclonal variation in the 1940s, its application to crop improvement only started to be utilised in the 1970s (Thorpe, 2007). Table 3 shows examples from sugarcane in which somaclones have been screened for disease resistance. Other traits which have been developed in sugarcane through somaclonal variation include increased yield and performance (Liu and Chen, 1978), low fibre content, longer internode lengths (Rajeswari *et al.,* 2009), drought tolerance (Rao and Ftz, 2013) and sugar yield (Raza *et al.,* 2014).

2.8.2 Induced mutagenesis

a) Principles and types of mutagens

Mutagenesis refers to the artificial induction of genetic variation via the use of physical or chemical mutagens (Drake and Koch, 1976; Anderson, 1995). It was first carried out using X-rays in the fruit fly *Drosophila* spp. by Muller in 1927 (Van Harten, 1998). In plants, various methods which include heat treatment, centrifugation and ageing of seeds, were initially carried out in an attempt to induce mutations (Van Harten, 1998). Ionizing radiation, X-rays, gamma rays and thermal neutrons were later used, but the first attempts resulted in low mutation frequencies and lethal effects on the plants, which were resolved by improving treatment conditions (Novak and Brunner, 1992; Brunner, 1995). Mutagens that have been

Table 3: Examples of pest and disease resistance traits obtained via somaclonal variation in sugarcane

Disease/pest	References
Fiji disease virus	Krishnarmurthi and Tlaskal (1974)
Eyespot (*H. sacchari*)	Larkin and Scowcroft (1983)
Sugarcane borer (*D. saccharalis*)	White and Irvine (1987)
Red rot (*C. falcatum*)	Singh *et al.* (2008); Sengar *et al.* (2009)
Brown rust (*Puccinia melanocephala* Syd. & Syd.)	Litardo *et al.* (2011)
Sugarcane mosaic virus	Khan *et al.* (2013)

used in sugarcane include sodium azide, ethyl methanesulphonate, 5-azacytidine and gamma rays (reviewed by Rutherford *et al.,* 2014). The mechanisms that result in mutations during induced mutagenesis are similar to those that result in spontaneous mutations during *in vitro* culture (Jain *et al.,* 1998). However, the frequency of mutagen-induced mutations is higher than that of spontaneous mutations in *in vitro* culture (Novak and Brunner, 1992). Obtaining desired mutations through the use of mutagens is based on chance and may also result in lethal effects that can disrupt normal plant development (Roane, 1973; Nair *et al.,* 2014).

The use of physical mutagens in mutation breeding in plants dates back to the early 20th century with the use of X-rays and later, gamma and neutron radiation (Novak and Brunner, 1992). They have been used in mutation breeding of sugarcane and many other crops (Van Harten, 1998, Nawaz and Shu, 2014). Mutation efficiency of physical mutagenic agents depends on the dose, dose rate, dose distribution and exposure time (Brunner, 1995; Suprassana *et al.,* 2009). The establishment of these parameters relies upon radiation type, radiation facilities and the type of material to be exposed to the radiation (Brunner, 1995). X-rays and gamma rays can penetrate deep into the tissue due to limited scattering and concentration of the ion beam on the plant tissue leading to high mutation frequency compared with UV-light and neutron radiation (Suprasanna *et al.,* 2009). Furthermore, X-rays and gamma rays cause the formation of radicals that break DNA strands (Waugh *et al.,* 2006) and ionize nitrogenous bases, especially during DNA replication, leading to heritable errors in the base sequence (Medina *et al.,* 2005). UV-light causes covalent bonding between neighbouring pyrimidines resulting in the formation dimers that alter DNA replication (Waugh *et al.,* 2006). Physical mutagens are less hazardous and are easier to handle compared to chemical mutagens (Suprasanna *et al.,* 2009), but are relatively expensive due to the equipment required (Poelhman and Sleper, 1995).

Chemical mutagens used in mutagenesis include hydroxylamine, methyl methanesulfonate (MMS), N–methyl-N–N–nitrosoguanidine (MNNG), ethyl methanesulfonate (EMS), nitrous acid and N-methyl-N-nitrosourea (MNU) (Inoue, 2006; Shu *et al.,* 2012). The mechanisms by which they effect changes in DNA include base analog, intercalation and base modification (Waugh *et al.,* 2006), which result in different types of mutations (Table 4). Mutation frequency is dependent on the concentration, temperature and pH of the mutagen (Van Harten, 1998) and access of cells to the mutagen in the cell-mutagen suspension (Durand, 1990; Chen *et al.,* 2013).

Ethyl methanesulfonate (EMS) has been widely used in mutagenesis of many plant species that include palm (Omar and Novak, 1990), wheat (Masrizal *et al.*, 1991), *Arabidopsis* (Jander *et al.,* 2003), sweet potato (Luan *et al.*, 2007), soyabean (Van *et al.,* 2008), banana (Chen *et al.,* 2012) and rice (Serrat *et al*., 2014). In sugarcane, this mutagen has been used in mutation breeding for various traits including high sugar content (Khairwal *et al.,* 1984), salt tolerance (Kengenal *et al.,* 2008), herbicide tolerance (Koch *et al.,* 2012) and disease resistance (Mahlanza *et al*., 2013). It is a popular mutagen because of its ability to induce high point mutation frequencies without causing lethal abnormalities to the chromosomes (Waugh *et al.,* 2006; Weil and Monde, 2009; Nair *et al.,* 2014). Ethyl methanesulfonate is an alkylating agent that induces the alkylation of guanine to form O^2-ethylguanine which is capable of pairing with thymine instead of cytosine (Kim *et al.,* 2006; Waugh *et al.,* 2006). This results in errors during DNA repair with the A-T pair replacing G-C (transition mutation) (Anderson, 1995; Davies *et al.,* 1999), especially during DNA replication (Durand, 1990). The methylation inhibitor 5-azacytidine has also been used in sugarcane to obtain variants with tolerance to the *Ustilago scitaminea* Syd. and Syd. and the herbicide imazapyr (Munsamy *et al.,* 2013).

Mutagenesis can be carried out using parent material or *in vitro* cultures (Suprassana *et al.,* 2009). Axillary and adventitious buds, apical meristems (Ahloowalia and Maluszynski, 2001), anthers (Mulwa and Mwanza, 2006) and seeds (Rahman *et al*., 2013) can be used. Plants produced from mutated embryogenic callus cells can be chimeric as a result of mutations occurring unevenly amongst the diploid cells (Van Harten, 1998; Datta and Chakrabarty, 2009, Shu et al., 2012). Consequently, the use of haploid cell cultures (e.g. microspores) is favoured over diploid cultures due to the expression of recessive genes without being masked by dominant genes after crossing (Swanson *et al.,* 1989; Suprassana *et al.,* 2009). The haploid plants that result can be inbred to produce diploid plants with the desired traits, making selection easier and less time consuming (Mulwa and Mwanza, 2006). In addition, the

Table 4: Examples of chemical mutagens and the damage they induce in DNA (Inoue, 2006)

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

production of double haploids from mutagenized microspores or anthers, assists in preventing formation of chimeras leading to the regeneration of plants with homozygous alleles (Maluszynski *et al.,* 1995; Sugihara *et al.,* 2013). Hence, the generation of double haploids makes selection of mutants more efficient (Griffing, 1975; Huang *et al.,* 2014). However, the difficulty of the technique and complexity of sugarcane genetics, renders use of haploids in improvement of the crop a challenging approach (Palmer *et al.,* 2005).

b) Selection of variant cells and plants

The development of effective strategies for selection of desirable traits is an important step in plant breeding programmes (Roane, 1973; Van den Bulk, 1991; Novak and Brunner, 1992; Lebeda and Svabova, 2010). Conventionally, selection of traits of interest is carried out in the field, but this is laborious and time-consuming compared with *in vitro* selection techniques (Novak and Brunner, 1992; Jain, 2001; Patade *et al.,* 2008). This is because a selection pressure can be applied to *in vitro* cultured cells and/or to the regenerated plants in the culture medium and subsequently to the field plants (Maluszynski *et al.,* 1995; Chandra *et al.,* 2010). Rutherford *et al*. (2014) reviewed studies in which *in vitro* and *ex vitro* screening were used to obtained sugarcane somaclonal variants with resistance to biotic and abiotic stresses and desirable agronomic traits. This approach allows for selection of a large number of mutant cells and plants in a small space and provides a specific and controlled environment that is free from biotic and abiotic factors that might negatively influence selection (Chaleff, 1983; Duncan and Widholm, 1990; Clemente and Cadenas, 2012). Cells can be exposed to herbicides (Koch *et al.,* 2012), water stress (Rao and Ftz, 2013), high salt concentrations (Al-

Rawahy and Farooq, 2014) and fungal toxins (Mahlanza *et al*., 2013; Vedna and Kumar, 2014). In addition, the technique allows for the introduction of a pathogen in a controlled environment, negating the need for strict quarantine if carried out *ex-vitro* (Chandra *et al.,* 2010).

However, limitations of *in vitro* selection are that traits expressed at the cellular level might not be expressed at the plant level (Daub, 1986). Furthermore, the technique cannot be used to select certain phenotypic traits (e.g. agronomic traits), which require cell differentiation and organisation (Chaleff, 1983; Rai *et al.,* 2011). When the desired traits are dominant and homozygous recessive, resistant cells and plants can be selected immediately, but crossing is necessary in cases of heterozygous plants in order to obtain plants with recessive traits (Allard, 1999). The traits expressed in cells as a result of epigenetic variation may not be expressed in the progeny of the plants, as the epigenetic effects are reversed by meiosis during sexual reproduction (Chaleff, 1983; George, 1993; Suprassana *et al.,* 2009).

To apply a selection pressure *in vitro*, the concentration of the selection agent that kills or inhibits the growth of cells, has to be established for incorporation into the selection medium (Mahlanza *et al.,* 2013). Exposure of cells to the selection agent can either be single-step with 2-3 times the lethal dose of the agent, or multiple-step where the concentration of the selection agent is gradually increased, starting at the lethal concentration (Suprassana *et al.,* 2009). Screening for disease resistance involves the use of a selection agent known to be involved in pathogenicity and ensuring uniform exposure of each cell, such that susceptible cells are killed and the resistance ones survive and regenerate into plants (Daub, 1986; Lebeda and Svabova, 2010). The pathogen, its toxins or culture filtrates, can be used in selecting lines that are disease resistant.

i) Use of pathogens in selection

The pathogen responsible for causing a disease can be used as an *in vitro* selection agent for resistance (Daub, 1986; Van den Bulk, 1991; Lebeda and Svabova, 2010) (Table 5). Fungal conidia can be inoculated onto shoot cultures and these visually monitored for resistance to the fungus, provided there is a correlation with the effect of the fungus *in vivo* (George, 1993; Devnarain, 2010). Factors that may influence the expression of resistance include the concentration of the inoculum, temperature and the composition of the medium (Xue and Hall, 1992; Bertetti *et al.,* 2009), which may lead to inconsistent results being obtained (Daub, 1986). Moreover, this option has limitations including: 1) uneven exposure of the cells to the pathogen; 2) whether resistance can be expressed in *in vitro* cultured cells; and 3) the overgrowth of the pathogen on the cells and medium, which makes it difficult to make observations (Daub, 1986; Slavov, 2005).

ii) Use of toxins and culture filtrates in selection

Fungi secrete toxins as a mode of protection against a host plant's defences, enabling them to kill host cells and in the process induce disease symptoms (Nishiuchi, 2013). These toxins cause wilting, necrosis and chlorosis of plants (Chandra *et al.,* 2010). Over 250 fungal and bacterial phytotoxins have been extracted and characterised (Lebeda and Svabova, 2010). They can, therefore, be used as *in vitro* selection agents (Chandra *et al.,* 2010) (Table 5). This strategy allows uniform exposure of the cells to the selection pressure by culturing them on media containing the toxin (Daub, 1986). A prerequisite for the use of a toxin is to determine that it contributes to pathogenesis, i.e. that it is a pathotoxin (Van den Bulk, 1991; Slavov, 2005). To determine this, various approaches can be undertaken, *viz.*:1) the phytotoxin can be extracted from the infected plant; 2) the phytotoxin's presence at a crucial stage of the disease can be tested; and 3) the phytotoxin's ability to induce symptoms on the plant can be assessed (Yoder, 1980; Slavov, 2005). Further, the gene(s) responsible for the synthesis of the toxin can be made dysfunctional and pathogenesis of the mutated fungus can then be assessed (Desjardins and Hohn, 1997). In this strategy, it is postulated that cells resistant to the phytotoxins will also be resistant to the pathogen (Daub, 1986; Van den Bulk, 1991; Desjardins and Hohn, 1997; Chandra *et al.,* 2010). Consequently, initial tests should be conducted to establish the effect of the toxin or filtrate on the plant tissue cultures to determine a suitable concentration of the toxin or filtrate that can be used in selection (Lebeda and Svabova, 2010; Grzebelus *et al.,* 2013). However, due to the conditions provided *in vitro*, the concentration of toxins produced is likely to be greater than that produced by the fungus *in vivo* (Yoder, 1980, Sharma *et al.,* 2010). This might result in a weak correlation between the amount of toxin *in vitro* and virulence of the fungus *in vivo* (Yoder, 1980; Tripathi *et al.,* 2008).

The purified toxins can be used in selection strategies (Remotti *et al.,* 1997; Khan *et al.,* 2004; Slavov, 2005). They can be purified from culture filtrates (Mayama *et al.*, 1990; Alvi and Iqbal, 2014) or acquired from commercial suppliers (Desjardins and Hohn, 1997; Remotti, 1997; Horacek *et al*., 2013). El Hadrami *et al.* (2005) reviewed purified toxins from different fungal pathogens that have been used to select for disease resistance *in vitro*. Gengenbach *et al.* (1977) used the purified toxin produced by *Helminthosporium maydis* Nisik. and Miyake, which induces southern corn leaf blight in maize, to select for cells that were resistant to the disease. Ali *et al.* (2007b) partially purified a toxin produced by *C. falcatum* and used it to select mutants resistant to red rot in sugarcane. Eyespot disease resistant sugarcane genotypes have also been selected by using a toxin produced by *H. sacchari* (Chaleff, 1983; Prasad and Naik, 2000).

Table 5: Examples of selection studies for disease resistance in sugarcane using different selection agents.

Pathogen	Selection agent	Reference
Fiji disease virus	Pathogen	Krishnamurthhi and Tlaskal (1974)
Helminthosporium sacchari	Toxin	Larkin and Scowcroft (1983)
Puccinia melanocephala	Pathogen	Peros *et al.* (1994)
Helminthosporium sacchari	Toxin	Leal et al. (1996)
Colletotrichum falcatum	Culture filtrate	Mohanraj et al. (2003)
Colletotrichum falcatum	Pathogen	Singh *et al.* (2008)
Colletotrichum falcatum	Purified culture filtrate	Ali *et al.* (2007b)
Colletotrichum falcatum	Culture filtrate	Kumar *et al.* (2012)
Fusarium sacchari	Culture filtrate	Mahlanza *et al.* (2013)

Culture filtrates can be used when there is no reliable description of the toxins produced by the fungus. The fungal CF are prepared by passing the liquid culture through a series of filters in order to remove the mycelia and conidia (Sengar *et al.,* 2009; Mahlanza *et al.,* 2013). This is an easy and effective method as culture filtrates have been shown to be phytotoxic (Suprasanna *et al.,* 2009, Chandra *et al.,* 2010; Grzebelus *et al.,* 2013). Hidalgo *et al.* (1998) reported that culture filtrates of *F. subglutinans* were toxic to pineapple calli and leaves. *F. oxysporum* culture filtrates incorporated into tissue culture media were also reported to have an inhibitory effect on the growth of *Amaranthus hybridus* Linnaeus plantlet roots (Chen and Swart, 2002). Thakur *et al*. (2014) selected *Zingiber officinale* Rosc. plants resistant to *F. oxysporum* by exposing calli to fungal culture filtrate in the culture medium. However, although culture filtrates contain the toxins, their effect on callus or plants can be due to interaction of the toxins with other compounds present in the filtrate, which may not be important in pathogenesis (Van den Bulk, 1991; Sharma *et al*., 2010).

Tolerance to toxins or culture filtrates expressed by somaclonal variants should correlate to tolerance to the pathogen (Van den Bulk, 1991; Svabova and Lebeda, 2005; Grzebelus *et al*., 2013). Hence, the toxin-tolerant plants should be inoculated with the pathogen to confirm tolerance (Chen and Swart, 2002; Mahlanza *et al.,* 2013). According to Koch's postulates (Parry, 1990), plants susceptible to the pathogen should exhibit symptoms similar to those displayed by diseased plants from which the pathogen was initially isolated. The tolerant plants should display no or minimal symptoms in the presence of the pathogen in the plant tissue (Gengenbach *et al.,* 1977; Arcioni *et al.,* 1987; Botta *et al.,* 1994; Grzebelus *et al*., 2013). Since inoculation is usually carried out in non-sterile environments and there is, therefore, potential

for secondary infection by other pathogens, it is important to confirm that the inoculated pathogen is the causal agent of observed symptoms (Harris *et al.,* 1999). This can be achieved by re-isolation of the pathogen onto appropriate culture media and identification of the isolates (Chen and Swart, 2002; Abdel-Monaim *et al.,* 2011; Mahlanza *et al.,* 2013).

c) Molecular analyses of variants

Analysis of the changes that occur at the DNA level resulting from culture-induced somaclonal variation and mutagenic treatments are important to understand the resulting variation (Hoezel and Green, 1998; Rasheed *et al.,* 2005; Rutherford *et al.,* 2014). Evaluation of variation based on visible traits is not reliable as they are dependent on the environment and age of plants (Kunert *et al.,* 2003). Molecular markers (DNA and protein based) are more reliable as they identify variations that have a genetic origin (Kunert et al., 2003; Talve *et al.,* 2014). DNA marker systems used in analysis of such variation include Amplified Fragment Length Polymorphism (AFLP) (Chuang *et al.,* 2009; Landey *et al.,* 2014), Restriction Fragment Length Polymorphism (RFLP) (Patzak, 2003) and Random Amplified Polymorphic DNA (RAPD) (Rasheed *et al.,* 2005; Thakur and Ishii, 2014). Flow cytometry has also been used utilised to assess variation due to changes in ploidy (Acanda *et al*., 2013; Shilpha *et al.* 2014).

AFLPs involve the following steps: 1) digestion of genomic DNA; 2) attachment of small DNA segments called adapters to the digested fragments; 3) PCR amplification of the fragments using primers specific for the adapters and 4) separation of the PCR products (Saunders *et al.,* 2001, Chuang *et al.*, 2009). The technique requires no prior knowledge of the genomic DNA sequence, as they generate a large number of polymorphic bands and results are reproducible (Yang *et al.,* 2005). Munsamy *et al*. (2013) used AFLP analyses to detect polymorphism in sugarcane calli trested with 5-azacytidine. In the RFLP method, genomic DNA is digested using restriction enzymes and the resulting fragments are separated by gel electrophoresis. A radioactive-labelled DNA probe is used to identify a fragment with the desired sequence (Liu, 2007). Difficulties in handling and storage of the radioactive reagents make RFLP an unfavourable technique (Nakazato and Gastony, 2006). RAPD is a simple and time efficient technique compared to RFLP (Garcia *et al.,* 2004). It results in amplification of few random segments of DNA, allowing for variation in length and number of amplified segments when the sequence of the segments is altered (Hoezel and Green, 1998). RAPDs have been used widely for analysis of genetic variation in sugarcane (Shahid *et al*., 2011; Pandey *et al*., 2012; Shahid *et al*., 2014).

d) Phenotypic evaluation of variants

In somaclonal variant plants, the expression of the desired trait has to be accompanied by important agronomic features (Singh *et al*., 2008) as certain traits cannot be determined *in vitro*. In sugarcane, transferring *in vitro* plants to the field is, therefore, necessary in order to enable observation of agronomic features such as cane height, number of nodes, stalk weight, internodal length and sucrose content, which determine yield in the crop (Liu and Chen, 1978; Dalvi *et al.,* 2012; Nikam *et al.,* 2014). These traits can be assessed and a comparison made between the somaclonal variants and vegetatively propagated plants (Krishnamurthi and Tlaskal 1974; Shkvarnikov and Kulik, 1975; Song *et al*., 1994; Watt *et al*., 2009). Song *et al*. (1994) compared brown spot disease-resistant soyabean plants obtained by *in vitro* screening with their parents and selected those with similar or superior agronomic traits. Krishnamurthi and Tlaskal (1974) developed *in vitro* sugarcane lines that were resistant to Fiji disease virus through somaclonal variation and selected lines that had retained the high yield that characterised the parents. Nikam *et al*. (2014) produced salinity-tolerant sugarcane mutants via gamma radiation of calli and some of these genotypes expressed higher sugar yield, percent brix and number of millable stalks.

Aims of the study

This study aimed to investigate the influence of stalk characteristics and associated *Fusarium* spp. on *E. saccharina* resistance, and the use of *Fusarium*-tolerant sugarcane mutants to manipulate the insect-fungus relationship in endophytic biological control of the borer. As physical resistance mechanisms to *E. saccharina* resistance are not ideal and nitrogen-based metabolites are essential in biochemical defences, Chapter 3 describes the contribution of stalk rind hardness, fibre and nitrogen content of mature and immature stalk internodes on *E. saccharin*a resistance in seven sugarcane cultivars of varying borer resistance ratings, towards improving resistance screening strategies. Further, due to previous demonstrations of the beneficial and harmful effects of *Fusarium* spp. on *E. saccharina in vitro*, the influence of *Fusarium* spp. infecting sugarcane stalks on *E. saccharina* resistance was investigated. This established the negative effect of *F. sacchari* PNG40 on *E. saccharina* damage and performance, thus highlighting the potential of the fungus in biological control. As *E. saccharina* damage is associated with Fusarium stem rot, thereby impeding *Fusarium*-mediated control of the borer, Chapter 4 describes development of a protocol for production of *Fusarium*-tolerant sugarcane mutants (cultivar NCo376) by *in vitro* mutagenesis using ethyl methanesulphonate and selection using fungal culture filtrates and the pathogen. The usefulness of the produced *Fusarium*-tolerant mutants (cultivars N41 and NCo376) in the control of *E. saccharina* and associated Fusarium stem rot, was tested in a glasshouse trial. In Chapter 5, the impact of mutagenesis on stalk rind hardness, fibre and nitrogen content and the ability of the mutants to support endophytic colonisation, were determined.

CHAPTER 3

Eldana saccharina (Lepidoptera: Pyralidae) resistance sugarcane in (*Saccharum* sp.): Effects of *Fusarium* spp., stalk rind fibre and nitrogen content

Eldana saccharina (Lepidoptera: Pyralidae) resistance in sugarcane (*Saccharum* sp.): effects of *Fusarium* spp., stalk rind, fibre and nitrogen content

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Sugarcane cultivar resistance to *Eldana saccharina* is the best strategy to control the stalk borer. The present study aimed to establish the influence of stalk rind hardness, fibre and nitrogen content, and the effect *Fusarium* species associated with *E. saccharina* infestation, on borer damage and performance in different sugarcane cultivars. In two glasshouse trials, larvae were inoculated into 7-month-old stalks of seven cultivars with different *E. saccharina* resistance ratings *via* wounds created in the rind. In one of the trials, *E. saccharina*-beneficial fungal strain *Fusarium pseudonygamai* SC17 and *E. saccharina* antagonistic *F. sacchari* PNG40 were inoculated into the stalk of N41 and NCo376 three weeks before larval inoculation. Rind hardness and fibre content were higher in resistant cultivars and mature stalk parts, whilst nitrogen content was lower in these genotypes and in older tissues. However, *E. saccharina* survival, damage and growth indicators were not entirely consistent with known resistance ratings of cultivars, possibly due to the absence of the rind effect owing to mechanical wounding and differential effects of fibre and nitrogen in the cultivars. The absence of rind protection did not affect resistance in N29 and N33 upper internodes. *Fusarium* infection was higher in the bottom of the stalk compared to the top in both N41 and NCo376. The least length bored was exhibited in the bottom internodes of NCo376 (3.3 cm) and N41 (1.7 cm) infected with PNG40, whilst SC17-colonized NCo376 stalks experienced the most damage (8.2 cm). The contribution of stalk rind hardness, fibre and nitrogen content to *E. saccharina* resistance in sugarcane may vary in different cultivars and stalk parts, whilst biochemical defences possibly play a larger role in some genotypes. Infection by *Fusarium* species, especially in lower internodes, may have variable effects on resistance to the borer depending on the fungal strain present.

Key words: *Eldana saccharina*, fibre, *Fusarium* sp., nitrogen, resistance, stalk rind hardness, sugarcane.

INTRODUCTION

The African sugarcane stalk borer, *Eldana saccharina* Walker (Lepidoptera: Pyralidae), is a notorious pest in sugarcane (*Saccharum* spp.) where its larvae feed ravenously on internal tissue (Dick 1945; Leslie 2009), especially in the lower regions of the stalk (Mazodze & Conlong 2003). This damage is compounded by fungal infection of wounded tissue by *Fusarium* spp., characterized by reddish-brown discolouration of stem tissue (Fusarium stem rot) surrounding borings (McFarlane *et al.* 2009). Consequently, susceptibility of sugarcane lines to this borer negatively impacts on the development of improved commercial varieties. *Eldana saccharina* resistance screening is conducted during the final stages of the South African sugarcane breeding programme due to the laborious, costly and time-consuming nature of the screening trials. Early in the breeding programme, clones are selected for high sucrose content and sugar yield but are discarded if susceptible to the borer in the final selection stage (Butterfield & Thomas 1996). Hence, time, labour and funds are wasted by carrying *E. saccharina*-susceptible clones through the selection process.

An Integrated Pest Management (IPM) approach employing a range of control practices has been implemented in the South African sugar industry to curb *E. saccharina* (Conlong & Rutherford 2008). Early harvesting, destruction of infected stalks and limited nitrogen fertilizer use, restrict the development and spread of the insect (Webster *et al.* 2005). In addition, the insecticide α-cypermethrin (Fastac®) has been successful in suppressing borer populations during summer periods when populations are on the increase (Leslie 2009). However, plant-based resistance to *E. saccharina* is

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the most attractive strategy since resistant genotypes express innate plant defence mechanisms, *e.g.* stalk rind hardness, fibre content, nutritional value and insecticidal metabolites, against the stalk borer.

Stalk rinds can provide a tough physical barrier which is difficult for larvae to penetrate. This results in prolonged exposure of insects to mortality factors on the exterior of the plant. Artificially inoculated pot trials have revealed that rind hardness was positively correlated with *E. saccharina* resistance as both low numbers of internodes bored and recovered larvae were associated with cultivars with harder rinds (Keeping & Rutherford 2004). High stalk fibre content, a character directly correlated with rind hardness, also correlates with *E. saccharina* resistance (Rutherford *et al.* 1993). Fibre, which is composed of cellulose, hemicellulose and lignin deposited in cell walls, complicates insect feeding as it is difficult to cut and digest fibrous plant tissue (Santiago *et al.* 2013). While rind hardness and fibre may act as defence mechanisms against *E. saccharina,* they have negative effects on the milling process such as reduced sucrose recovery from the stalk (Keeping & Rutherford 2004).

Beyond the physical impediments to *E. saccharina* boring, the nutritional content and physiologically mediated defence responses of different genotypes play a role in resistance. Plant nitrogen is of nutritional value to insects as it is essential for protein synthesis (Mattson 1980). High stalk nitrogen content was found to result in increased growth and survival of *E. saccharina* in susceptible varieties (Atkinson & Nuss 1989). Cultivars or plant parts with low nitrogen content may therefore be unattractive to the pest. On the other hand, insect attack may trigger the synthesis of plant defence components that contain nitrogen, *e.g.* proteinase inhibitors, benzoxazinoids and phenylpropanoid polyamine conjugates (Rutherford 2014). Recently released cultivars N29, N39 and N41 have moderate rind hardness and fibre content, but exhibit resistance to *E. saccharina* (Keeping & Rutherford 2004). This departure from the established direct correlation between rind hardness and *E. saccharina* resistance may be due to enhanced expression of biochemical and protein resistance mechanisms in these cultivars.

However, whilst sugarcane cultivars may possess constitutive and physiologically reactive defences against *E. saccharina*, stalks are often colonized by endophytic fungi (McFarlane & Rutherford 2005). Endophytes can produce insecticidal compounds, *e.g.* ergot alkaloids (Clay 1988), beauvaricin and fusaproliferin (Logrieco *et al.* 1996), and also insect-attracting volatiles (Bartelt & Wicklow 1999). The presence of endophytes in sugarcane may therefore influence *E. saccharina* resistance. McFarlane *et al.* (2009) isolated 71 endophytic *Fusarium* isolates from 400 stalks of 14 different sugarcane cultivars collected from 11 geographical regions of KwaZulu-Natal province, South Africa. In that study, some endophytic *Fusarium* isolates promoted larval growth and survival in dietary inclusion assays and attracted the borer in olfactory choice assays. However, these authors also found that some isolates had a negative effect on the borer. These *Fusarium–E. saccharina* interactions have implications on *E. saccharina* resistance of sugarcane cultivars. For example, resistance to the stalk borer varies within the relatively resistant cultivar N41. The isolation of *F. pseudonygamai* Nirenberg & O'Donnell SC17 (a strain beneficial to *E. saccharina*) from borings in N41 stalks suggested that colonization by such fungi may increase borer damage (McFarlane *et al.* 2009). By contrast, *Fusarium sacchari* (E.J. Butler & Hafiz Khan) W. Gams PNG40 was isolated from an aborted *E. saccharina* boring in stalks of the susceptible cultivar N30 exhibiting limited borer damage, and *in vitro* assays revealed that the fungus was antagonistic to the insect (McFarlane *et al.* 2009). It is therefore possible that *E. saccharina* resistance of a cultivar may be influenced by the type of *Fusarium* strain, *i.e.* beneficial or antagonistic, colonizing the stalk. Furthermore, strains harmful to the pest, *e.g. F. sacchari* PNG40 could be employed as endophytic biological control agents.

The objectives of this study were, therefore, to establish the influence of stalk rind hardness, fibre and nitrogen content of internodes of different maturity on *E. saccharina* resistance in seven sugarcane cultivars. The study also aimed to determine the impact of *E. saccharina*-beneficial *F. pseudonygamai* SC17 and antagonistic *F. sacchari* PNG40 colonizing sugarcane tissue, on stalk borer damage, survival and growth.

MATERIAL AND METHODS

Plant collection, marcotting and establishment

Seven-month-old stalks of seven cultivars with varying *E. saccharina* resistance ratings (Table 1) were obtained from the South African Sugarcane

Table 1. *Eldana saccharina* resistance ratings of seven cultivars as established by the South African Sugarcane Research Institute (Keeping 2006).

Cultivar	*E. saccharina* resistance rating	Abbreviation[a]
N11	Very susceptible	$N11_{VS}$
NCo376	Susceptible	$NCo376_{S}$
N12	Semi-resistant	$N12_{SR}$
N17	Semi resistant	$N17_{SR}$
N41	Semi-resistant	$N41_{SR}$
N29	Resistant	$N29_{R}$
N33	Resistant	$N33_{R}$

[a]Subscript indicates the resistance rating of the cultivar. VS = very susceptible, S = susceptible, SR = semi-resistant, R = resistant.

Research Institute, Kearsney Research Farm, KwaZulu-Natal. The stalks were inspected for *E. saccharina* infestation and only undamaged stalks were collected. Marcotting was carried out within 2 h of cutting the stalks. Each stalk was placed in a cylindrical metal canister (6 l) allowing approximately 20 cm of the cut end to protrude through an opening in the base of the vessel. The cut end of the stalk was submerged in a marcotting solution composed of H_2SO_3 (1.8 mM), H_3PO_4 (0.77 mM), H_2SO_4 (0.38 mM) and HNO_3 (0.60 mM) (James 1980). The canister was filled with a substrate consisting of peat moss, vermiculite and riversand (3:3:1), which covered the third to fifth nodes from the cut end of the stem. Ten stalks per cultivar were arranged in a complete randomized block design in a glasshouse (21/33 °C, day/night), watered with approximately 1 l every second day and transferred to fresh marcotting solution every fourth day. After two weeks, the first to third leaves were still viable and the stalks had established roots in the covered nodes. The plants were removed from the marcotting solution and the submerged stalk ends were cut and removed. The cut ends of the stalk were sealed with molten wax to avoid infection by pathogens. Thereafter, the plants were fertilized once every week with 5 g of NPK (4:1:1) and watered for 3 min twice a day using low pressure drippers (4 l/h) connected to an automatic watering system for 3 weeks in order to 'flush out' residual acid from the plant tissue before inoculation.

Eldana saccharina *collection, rearing and inoculation*

Eldana saccharina larvae (second to sixth instars) were collected from sugarcane stalks in commercial farms in Sezela, KwaZulu-Natal. The larvae were reared on an artificial diet (Gillespie 1993) in 30 ml ventilated vials until pupation and subsequent eclosion of moths. To facilitate mating, the female and male moths (10–15 each) were transferred to oviposition boxes (30 × 30 × 15 cm) lined with paper towel on the inside. The female moths started ovipositing on the paper towels after a day and eggs were collected daily for 2–3 days. The eggs were incubated at 24 °C until they hatched and neonate larvae were transferred to a diet and reared at 27 °C until they reached the second instar stage. A modified version of a method by Kvedaras & Keeping (2007) was used to inoculate plants with *E. saccharina* larvae. Briefly, the reared second instar larvae were weighed and placed singly into modified 1.5 ml Eppendorf tubes. The adapted tubes permitted ventilation through the cap *via* a wire mesh and an outlet for larvae to a restricted site on the stalk *via* a cut opening in the base of the tube. Inoculation was conducted on two sites on each stalk, namely second nodes from the base and the growing point of the stalk. The rind was breached by drilling the stalk approximately 5 mm into the node at a 90° angle using a hand drill. The cut openings of the tubes containing the larvae were placed over the wounds in the stalk and secured using Prestick®. Plants were assessed for the production of frass at the wounds after 24 h to establish success of infestation.

Trial harvest

A randomly chosen stalk from each cultivar was cut, the larvae were collected and their development was assessed each week for 2 and 3 weeks after larval inoculation. The trial was harvested after 3 weeks as larvae had achieved fifth to sixth instar stages. The stalks were split longitudinally and larvae and pupae were collected and weighed within 2 h. The number of larvae and pupae recovered were indications of survival. The length bored in top and bottom sections of the stalk was measured.

Stalk rind hardness measurements, fibre and nitrogen content determinations

Twelve stalks per cultivar were obtained from 7-month-old field-grown plants. Two segments of different maturity, namely three internodes just below the growing region (immature) and three from the base of stalk (mature), were cut from each

stalk for rind hardness, fibre and nitrogen content determinations. Rind hardness was measured using a durometer (Model 1600, RexGauge Company, Glenview, IL, U.S.A.). Three readings were taken from the middle of each of the three internodes of stalk segments. The stalk sections were then shredded using a disintegrator and mixed thoroughly before fibre content was measured using a fourier transform near-infrared (FT-NIR) spectrometer (Matrix F, Bruker, Bryanston, South Africa). The shredded cane was dried for 24 h at 70 °C, milled (C and M Laboratory Mills, Baltimore, MD, U.S.A.), passed through a 1 mm sieve and the fine material obtained was analysed for nitrogen content using the TruSpec Carbon and Nitrogen Determinator (Leco Corporation, St. Joseph, MI, U.S.A.).

Fungal culture and inoculation

Fungal isolates *F. sacchari* PNG40 and *F. pseudonygamai* SC17 were obtained from sugarcane stalks by McFarlane *et al.* (2009). For each isolate, a starter culture was prepared by placing a mycelial square (stored in 15 % glycerol) onto potato-dextrose-agar (PDA) for 3 days at 30 °C. Thereafter, a 5 × 5 mm mycelial square was excised from the leading edge of the colony and transferred to 250 ml of potato-dextrose-broth (PDB) in an Erlhenmeyer flask. The flask was agitated at 145 rpm at 28–30 °C for 7 days, after which the culture was passed through sterile cheese cloth to remove mycelia. The conidial suspension was centrifuged at 12 000 rpm for 5 min and the supernatant was discarded. The conidial pellet was washed with sterile water, the suspension centrifuged and supernatant decanted. The inoculum was prepared by re-suspending the conidial pellet in sterile water and adjusting the suspension to 10^5 conidia/ml using a haemocytometer (Hawksley, Sussex, U.K.). Stalks of cultivars N41 and NCo376 were marcotted as described above. The stalks were bored through approximately half the diameter using a hand drill at a 45° angle at two locations, namely second nodes from both the base and growing region. The nodes and the drill bit were disinfected with 70 % ethanol prior to inoculation. The conidial suspensions (200 µl) of the two isolates were pipetted into the wounds of different stalks of each variety and sealed with Parafilm®. Sterile water was used for the controls of each cultivar. Three treatments were established for each cultivar, namely *F. sacchari* PNG40 and *F. pseudonygamai* SC17 and controls with 10 plants per treatment arranged in a complete randomized block design.

Eldana saccharina *inoculation of* Fusarium*-infected plants and trial harvest*

Three weeks after inoculation with the *Fusarium* strains, the plants were inoculated with 2nd instar *E. saccharina* larvae as described above. The Eppendorf tubes containing the larvae were positioned over the wounds created during *Fusarium* inoculation. The plants were harvested three weeks after *E. saccharina* inoculation. At harvest, 40 cm segments were cut from each stalk 20 cm below and above the inoculation point. Under aseptic conditions, each segment was surface-decontaminated by swabbing with 70 % ethanol and then flaming using a burner. The stalk segments were carefully split longitudinally and larvae and pupae were collected and weighed within an hour. The length of the borings inflicted by the larvae was measured. Images of the longitudinal sections were captured using a digital camera (Canon 400D) to assess stalk tissue discolouration due to infection by the *Fusarium* spp. The area discoloured in each segment was obtained by analysing the images using Assess© software for plant disease quantification (version 2.0, American Phytopathological Society, St. Paul, MN, U.S.A.). To confirm PNG40 or SC17 as the causal agent of the discolouration, sections of discoloured tissue (5 × 5 mm^2) were aseptically excised from the stalk segments and placed on Nash and Snyder agar and incubated for 5 days at 30 °C. The colony morphologies of the isolates were compared to that of either PNG40 or SC17, depending on the strain used to inoculate the plant from which each isolate was retrieved. To confirm the identity of isolates that were morphologically similar to either PNG40 or SC17, molecular analyses using inter-simple sequence repeats (ISSRs) were conducted according to the method by Mahlanza *et al.* (2013).

Statistical analyses

All analyses were conducted using Genstat statistical package (14th edition, VSN International, Hemel Hempstead, U.K.). Data were analysed for normality using the Shapiro-Wilk test. Analysis of variance (ANOVA) was used to analyse rind hardness, fibre and nitrogen content data. *Eldana saccharina* survival data were analysed using the

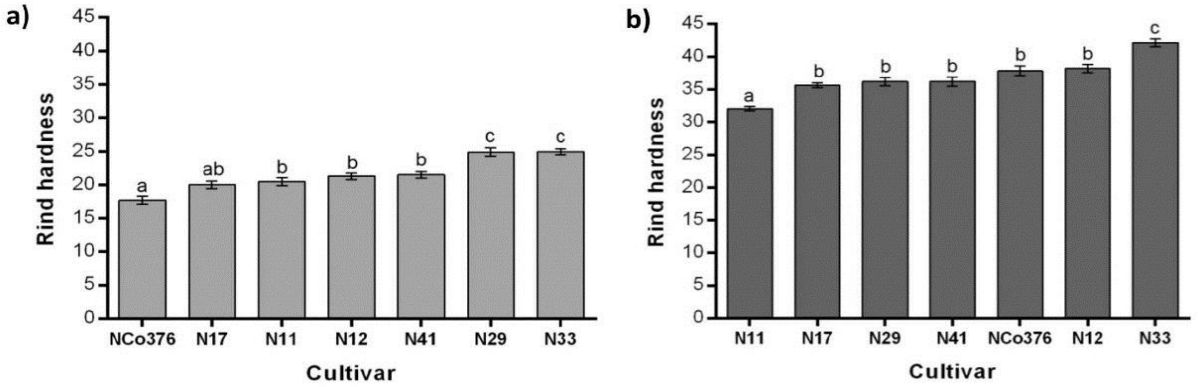

Fig. 1. Stalk rind hardness in (**a**) top ($P \leq 0.001$) and (**b**) bottom ($P \leq 0.001$) sections of seven cultivars presented in order of increasing hardness. Different letters above the bars indicate statistical significance ($P \leq 0.05$). Mean ± S.E., $n = 12$.

generalized linear mixed model with a binomial distribution and a logarithmic link function, and Fisher's protected least significant difference test was used as the *post hoc* test. All other data were analysed using restricted maximum likelihood (REML) method. The Sidak test was used as the *post hoc* analysis for all data except that for survival. Detected differences were significant at $P \leq 0.05$.

RESULTS

Stalk rind hardness, fibre and nitrogen content of the seven cultivars

Stalk rinds of top and bottom internodes were generally harder in cultivars resistant to *E. saccharina*. In the top internodes, resistant N29 ($N29_R$) and N33 ($N33_R$) exhibited significantly harder rinds than the rest of the cultivars, whilst the susceptible NCo376 ($NCo376_S$) possessed the softest ($P \leq 0.001$) (Fig. 1a). Cultivar $N33_R$ also displayed the hardest rinds in the bottom internodes amongst the seven cultivars and the softest rind was observed in the very susceptible N11 ($N11_{VS}$) ($P \leq 0.001$) (Fig. 1b). Similarly, fibre content in the upper stalk regions was highest in $N33_R$ and $N29_R$, whereas semi-resistant N17 ($N17_{SR}$) and $NCo376_S$ exhibited the lowest ($P = 0.018$) (Fig. 2a). However, the lower stalk regions of $N17_{SR}$ had the highest fibre content followed by $N33_R$. Cultivars $N11_{VS}$ and $NCo376_S$ displayed the least fibre content ($P = 0.028$) (Fig. 2b). As expected, both rind hardness and fibre content where higher in the bottom than top internodes (Figs 1, 2). Nitrogen content in the top internodes was highest in $N11_{VS}$, followed by $NCo376_S$, $N17_{SR}$, $N33_R$, $N12_{SR}$ and $N29_R$, with the lowest recorded in $N41_{SR}$ ($P = 0.002$) (Fig. 3). In the bottom stalk regions, percentage nitrogen decreased from $N17_S$, $NCo376_S$, $N12_{SR}$, $N41_{SR}$,

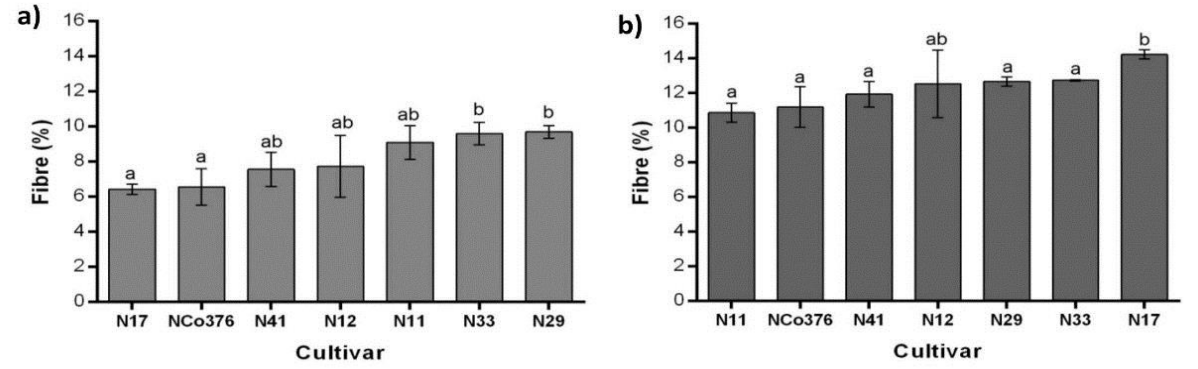

Fig. 2. Fibre content in (**a**) top ($P \leq 0.018$) and (**b**) bottom ($P \leq 0.024$) sections of seven cultivars presented in order of increasing percentage fibre. Different letters above the bars indicate statistical significance ($P \leq 0.05$). Mean ± S.E., $n = 12$.

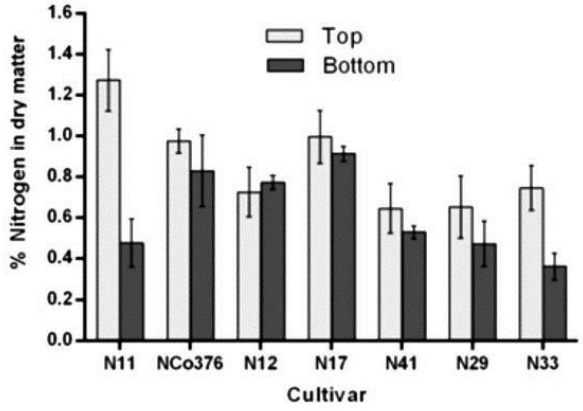

Fig. 3. Nitrogen content in top and bottom stalk regions of the seven cultivars. Mean ± S.E.

$N11_{VS}$ to $N29_{R}$, with the lowest being displayed in $N33_{R}$ (Fig. 3). The top internodes had more nitrogen that the lower stalk regions ($P \leq 0.001$), and a significant interaction was detected between cultivar and stalk region ($P = 0.019$) (Table 2).

E. saccharina survival, damage and growth in the seven cultivars

Survival, stalk damage and weight gain of the borer after inoculation of stalks of seven cultivars *via* openings in the rind did not entirely reflect their known *E. saccharina* resistance ratings. Survival of larvae inoculated in top ($P = 0.698$) and bottom ($P = 0.907$) internodes was not significantly different amongst the seven cultivars (Fig. 4). However, stalk damage by larvae in the top internodes indicated that $N33_{R}$ displayed the least length bored (4.4 cm) although this was not significantly less than $NCo376_{S}$ (6.8 cm), $N17_{SR}$ (7.9 cm) and $N29_{R}$ (8 cm) (Fig. 5a). However, $N33_{R}$ exhibited significantly less damage than the very susceptible $N11_{VS}$ (8.75 cm) and semi-resistant N41 ($N41_{SR}$) (9.2 cm) and N12 ($N12_{SR}$) (10.5cm) ($P = 0.027$). No differences in stalk damage in the top internodes were revealed amongst cultivars $N29_{R}$, $N12_{SR}$, $N17_{SR}$, $N41_{SR}$, $NCo376_{S}$ and $N11_{VS}$. Stalk length bored in bottom internodes did not indicate any significant differences amongst the cultivars ($P = 0.568$) (Fig. 5b). Six of the seven cultivars exhibited 15–52.4 % less stalk damage in the bottom (4.5–7.2 cm) than the top internodes (4.4–10.5 cm) with the exception of $N33_{R}$.

Larvae retrieved from the top internodes of $N29_{R}$ (0.079 g) gained significantly less weight than $N12_{SR}$ (0.133 g), $N11_{VS}$ (0.134 g), $NCo376_{S}$ (0.165 g) and $N17_{SR}$ (0.175 g) ($P = 0.001$) (Fig. 6a). Mass gain in larvae from $N17_{SR}$ and $N41_{SR}$ (0.100 g) were not significantly different from those in susceptible $NCo376_{S}$ and $N11_{VS}$. Furthermore, larvae from $N17_{SR}$ weighed significantly more than $N41_{SR}$ with those from the former exhibiting the highest weights amongst the cultivars. As no larvae were retrieved from $N33_{R}$, larval weights could not be obtained. Weights of larvae retrieved from the bottom internodes indicated that larvae from $NCo376_{S}$ (0.117 g) and $N17_{SR}$ (0.118 g) gained significantly more weight than the other cultivars, with those from $N12_{SR}$ (0.053 g) gaining the least ($P = 0.014$) (Fig. 6b). Larvae from the top internodes the cultivars gained more (0.079–0.157 g) than those from the bottom (0.053–0.118 g).

In vivo effect of *Fusarium* spp. on *E. saccharina* damage and performance

Inoculation of cultivars $N41_{SR}$ and $NCo376_{S}$ in the top and lower internodes of the stalk revealed red-brown discolouration typical of stalk rot, which was used as an indicator of infection by *Fusarium* sp. Fungal re-isolation confirmed the presence of *Fusarium* in the discoloured tissue and comparison of ISSR profiles of the isolates and inoculated strains confirmed the identity of retrieved fungi as either PNG40 or SC17. Data from stalks from which other fungi were isolated were not used. There were no significant differences among the controls and stalks inoculated with PNG40 and SC17 in the upper internodes ($P = 0.156$) (Fig. 7a). In the bottom stalk regions,

Table 2. Two-way ANOVA for nitrogen content in top and bottom stalk regions of the seven varieties.

Source of variation	d.f.	s.s.	m.s.	v.r.	*F* pr.
Cultivar	6	1.08	0.18	4.87	0.002
Stalk region	1	0.60	0.60	16.01	<0.001
Cultivar × stalk region	6	0.70	0.12	3.15	0.019
Residual	26	1.00	0.037		
Total	41	3.40			

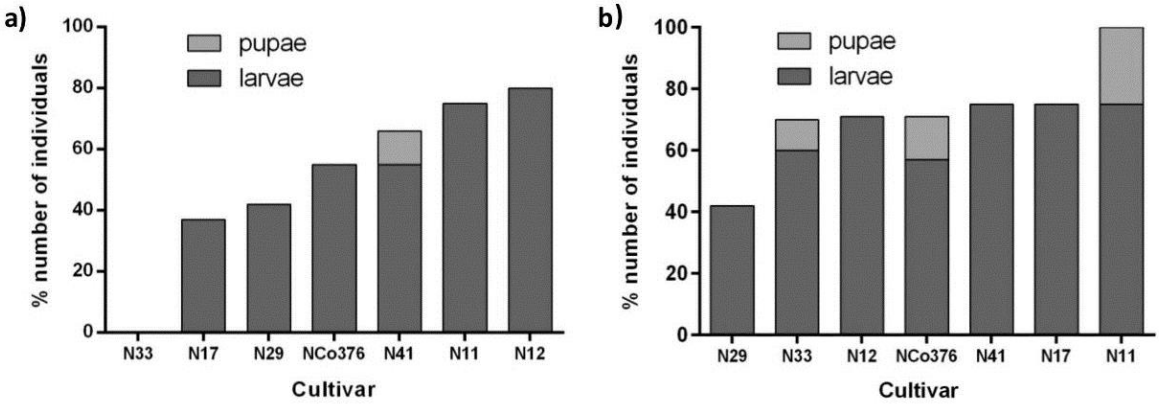

Fig. 4. Survival of *Eldana saccharina* inoculated into nodes of (**a**) top and (**b**) bottom regions of stalks of seven cultivars presented in order of increasing percentage number of individuals retrieved. $n = 6–9$.

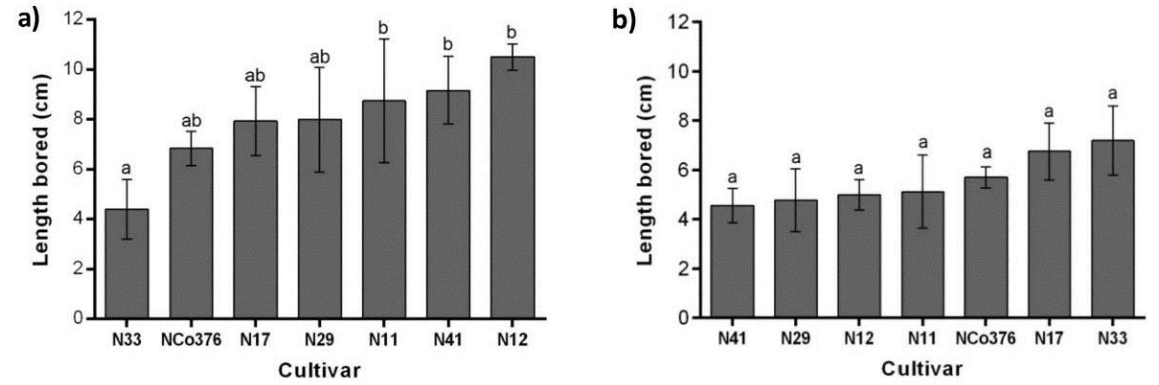

Fig. 5. Stalk damage by larvae in (**a**) top and (**b**) bottom internodes of seven varieties presented in order of increasing length bored. Different letters above the bars indicate statistical significance ($P \leq 0.05$); Top ($P = 0.047$), bottom ($P = 0.468$). Mean ± S.E., $n = 6–10$.

the discoloured area in N41$_{SR}$ and NCo376$_S$ stalks inoculated with PNG40 and SC17 were significantly larger than in their respective uninoculated controls ($P = 0.002$) (Fig. 7b). There were no significant differences in stalk area discoloured by PNG40 between NCo376$_S$ and N41$_{SR}$. However, stalk discolouration by SC17 was significantly greater in NCo376$_S$ than N41$_{SR}$ (Fig. 7b).

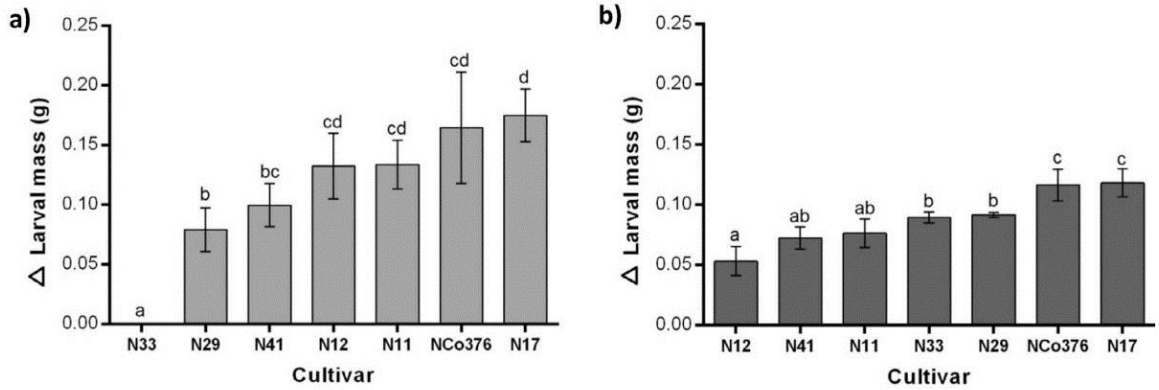

Fig. 6. Weight gained by *Eldana saccharina* larvae inoculated in (**a**) top and (**b**) bottom internodes of seven varieties presented in order of increasing change in larval mass. Different letters above the bars indicate statistical significance ($P \leq 0.05$); Top ($P = 0.011$), bottom ($P = 0.002$). Mean ± S.E., $n = 4–7$.

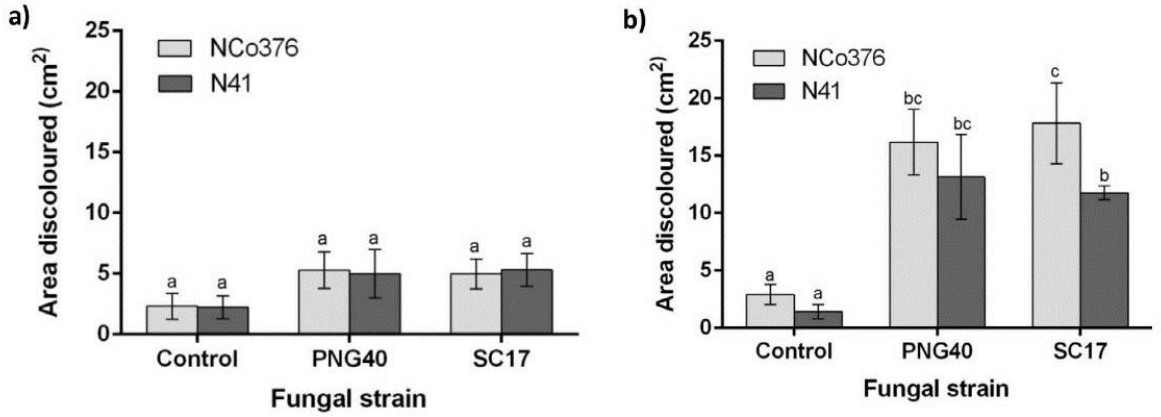

Fig. 7. Stalk tissue discolouration after inoculation of (**a**) top (P = 0.156) and (**b**) bottom (P = 0.002) regions of NCo376$_S$ and N41$_{SR}$ stalks with PNG40 and SC17. Different letters above the bars indicate statistical significance (P < 0.05). Mean ± S.E., n = 6–10.

Survival of larvae inoculated in the top internodes of NCo376$_S$ and N41S$_R$ stalks was not significantly different between treatments, namely uninoculated, infected with PNG40 and infected with SC17 (P = 0.206) (Fig. 8a,c). Survival in the bottom internodes of NCo376 was significantly different amongst all three treatments ($P \leq 0.001$). All the individuals inoculated into NCo376$_S$ stalks infected by SC17 were recovered with some pupae being obtained, whilst 60 % survival was recorded from those colonized by PNG40 and no pupae were recovered (Fig. 8b). Significantly more larvae were obtained in uninoculated stalks than in PNG40-infected ones, but survival from the controls was significantly less than in plants colonized by SC17. In N41$_{SR}$, survival in SC17-colonized plants was higher than in those infected by PNG40 ($P \leq 0.001$) (Fig. 8d).

Stalk borer damage in top internodes of NCo376$_S$ and N41$_{SR}$ was not significantly different amongst the control PNG40 and SC17 treatments of each cultivar (Fig. 9a). However, in both cultivars, borer damage in stalks colonized by PNG40 was significantly lower than in the controls ($P \leq 0.001$) (Fig. 9b). In NCo376$_S$, stalks colonized by SC17 showed significantly more damage than uninoculated ones, but stalk length bored in N41 stalks infected by the fungus was similar to the controls (Fig. 9b). Furthermore, SC17-colonized NCo376$_S$ stalks displayed significantly more damage than those of N41$_{SR}$ infected by the same fungus. Although NCo376 controls and PNG-colonized stalks showed more damage than those from N41$_{SR}$, these differences were not significant.

Mass gained was not significantly different among larvae retrieved from uninoculated, PNG40- and SC17-inoculated upper stalk regions of NCo376$_S$ and N41$_{SR}$ (P = 0.089) (Fig. 10a). Larvae from the lower internodes of control plants and those infected by PNG40 were not significantly different. However, larvae from stalks colonized by SC17 gained significantly more weight than those from controls and PNG40-infected plants (P = 0.017) (Fig. 10b). In N41$_{SR}$ plants, infection by PNG40 resulted in larvae gaining less weight than in stalks colonized by SC17 and the uninoculated controls. Larvae obtained from NCo376$_S$ plants infected by either PNG40 or SC17 gained more weight than those from N41$_{SR}$ stalks colonized by the same strains (Fig. 10b).

DISCUSSION

Plant resistance to insects is multifaceted with synergistic or antagonistic effects amongst the various mechanisms (Sadasivam & Thayumanayan 2003). The observations in the present study indicated that rind hardness, fibre and nitrogen content contribute to *E. saccharina* survival, damage and growth to varying extents in different varieties. In addition, the differences in rind hardness, fibre and nitrogen content between young and mature stalk parts were shown to influence borer damage and performance between these stalk regions.

Rind hardness and fibre content were generally greater in the resistant than the susceptible cultivars. Conventionally, *E. saccharina* resistance ratings are established using a resistance screening method in which eggs are inoculated into leaf sheaths in the lower stalk regions (Keeping 2006).

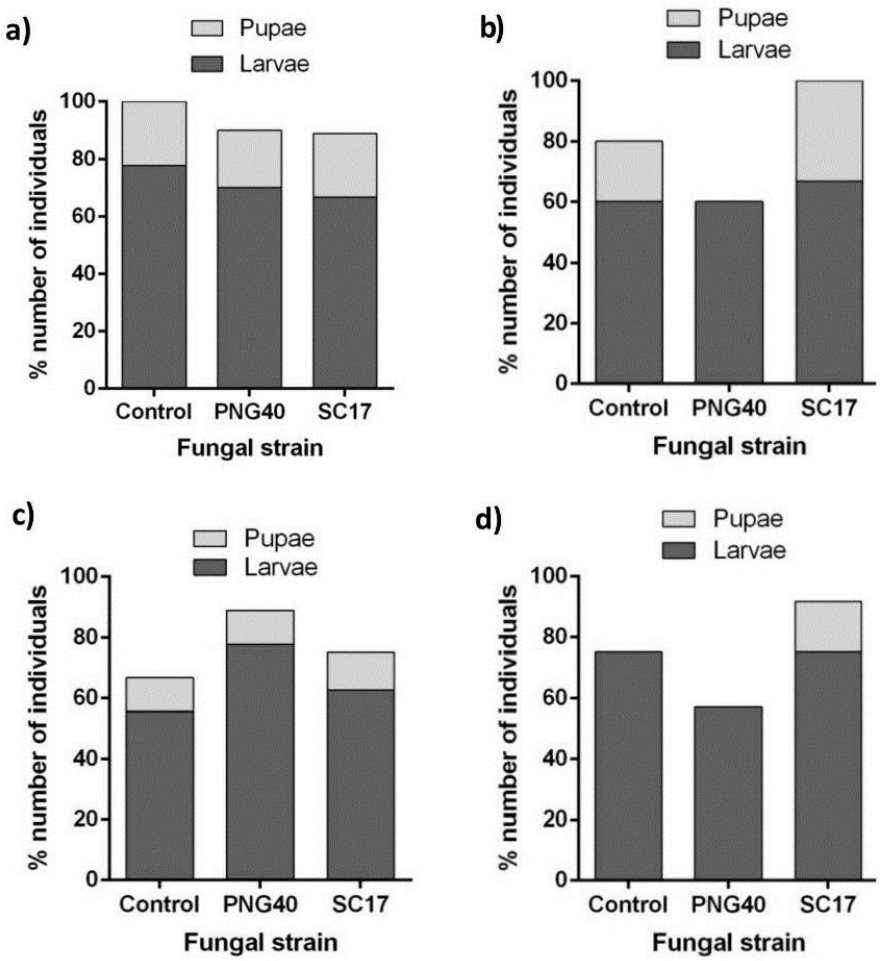

Fig. 8. The effect of PNG40 and SC17 on survival of *Eldana saccharina* in $NCo376_S$ (**a**) top and (**b**) bottom and $N41_{SR}$ (**c**) top and (**d**) bottom stalks regions, 3 weeks after inoculation of *Fusarium* the infected plants with larvae. $n = 6–10$.

In this method, the rind is left intact, contributing to resistance during the screening trials. However, in the present study, bypassing the rind by wounding it and inoculating larvae *via* the openings resulted in borer survival, damage and growth that did not entirely reflect the established resistance ratings of the cultivars in both lower and upper stalk regions. For example, $N17_{SR}$ exhibited moderate rind hardness, high fibre and nitrogen content in the bottom stalk parts but larvae from these internodes expressed high survival and growth and caused extensive stalk damage thereby indicating that resistance in $N17_{SR}$ may be largely due to stalk rind hardness. Conversely, larval survival and damage were low in the top internodes of $N33_R$, which expressed higher rind hardness, fibre and nitrogen content, thus suggesting other defences against the insect beyond the rind.

Observations by Bosque-Perez *et al.* (1997) in maize indicated that *E. saccharina* feeding in the stalk was limited by stalk hardness but different mechanisms may be involved in the ears where rind protection is unavailable. Santiago *et al.* (2003) observed that maize genotypes resistant to the borer *Sesamia nonagrioides* Lefèbvre (Lepidoptera: Noctuidae) had softer rinds than susceptible cultivars. This suggests that apart from stalk hardness, other mechanisms may contribute to resistance against stalk borers.

Gibson *et al.* (2009) attributed the inconsistent relationship between stalk hardness and survival of the borer *Diatraea grandiosella* Dyar (Lepidoptera: Crambidae) in different maize cultivars to plant chemistry, including the role of nitrogen in insect nutrition. In the present study, nitrogen content in the upper stalk parts of the semi-resistant and resistant cultivars $N12_{SR}$, $N41_{SR}$, $N29_{SR}$ and $N33_R$

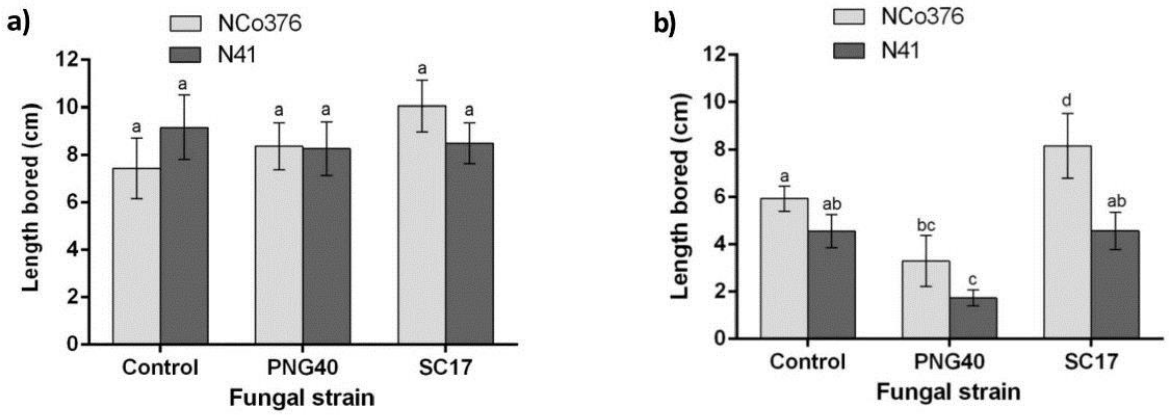

Fig. 9. The effect of PNG40 and SC17 on length bored by *Eldana saccharina* larvae in (**a**) top (P = 0. 298); and (**b**) bottom ($P \leq 0.001$) regions of $NCo376_S$ and $N41_{SR}$ stalks. Different letters above the bars indicate statistical significance ($P \leq 0.05$). Mean ± S.E., n = 6–10.

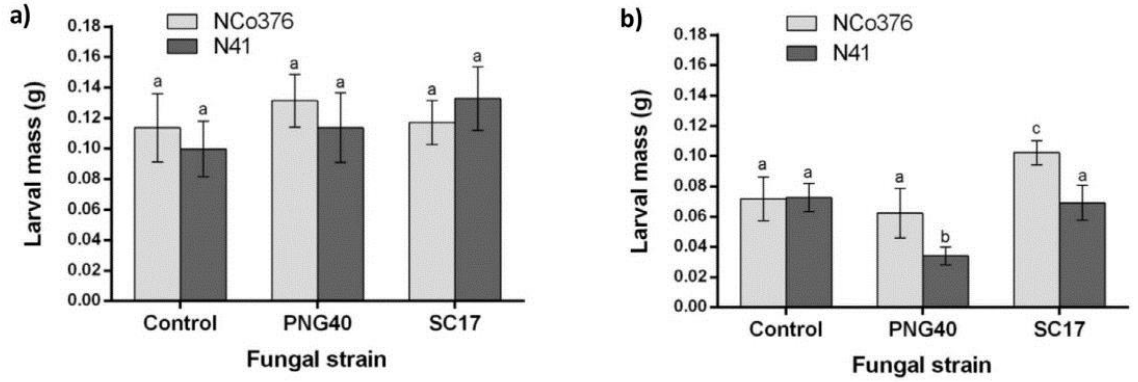

Fig. 10. Mass gained by larvae retrieved from (**a**) top (P = 0.089) and (**b**) bottom (P = 0.017) regions of $NCo376_S$ and $N41_{SR}$ stalks infected with PNG40 and SC17, three weeks after inoculation of infected plants with larvae. Different letters above the bars indicate statistical significance ($P \leq 0.05$). Mean ± S.E., n = 5–8.

was lower than in the susceptible $NCo376_S$ and $N11_{VS}$. The higher nitrogen levels, together with low fibre content, in the susceptible genotypes may render these cultivars more nutritious to *E. saccharina* than the resistant ones. Furthermore, nitrogen content in the top internodes of all cultivars, except for $N12_{SR}$, was higher than in the bottom stalk regions and fibre was lower in the former than in the latter. This is due to high nitrogen demand for protein synthesis in the young actively growing tissues of the upper stalk regions (Mattson 1980), whilst high fibre content in the bottom internodes is probably due to increased lignification of cell walls as the tissues mature (Gibson *et al.* 2009). This combination of low fibre and high nitrogen content may have led to the greater borer damage and growth observed in the susceptible cultivars, and upper stalk tissues, as this would have enhanced insect nutrition compared to the semi-resistant genotypes and bottom stalk parts, which exhibited high fibre and low nitrogen. However, in the resistant cultivar $N33_R$, damage was lower in the top internodes than in bottom ones, whilst larvae obtained from the top internodes of both resistant cultivars $N29_R$ and $N33_R$ displayed less mass gain than those from the bottom internodes, despite lower fibre and higher nitrogen in upper stalk tissues of these two cultivars.

Besides insect nutrition, nitrogen in plants is also utilized in the production of nitrogen-based secondary metabolites that act against insects (Mattson 1980). The anti-herbivory properties of nitrogen-based compounds such as benzoxazinoids (Falco 2001), vegetative storage proteins (Liu *et al.* 2005), cyanogenic glucosides (Ballhorn *et al.* 2006), chitinases, defensins, proteinase inhibitors, polyphenol and ascorbate oxidases (Rutherford 2014),

have been reported in different plants. High levels of dhurrin, a cyanogenic glucoside, can be present in young shoots of sorghum with accumulation in older plants being triggered by nitrogenous fertilizer application (Busk & Moller 2002). Benzoxazinoids are present in high concentrations in younger plant parts, including 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one (DIMBOA) and the derivative 2,4-dihydroxy-2H-1,4-benzoxazin-3-one (DIBOA), which act by inhibiting digestive proteinases (Rutherford 2014) and are suspected to be involved in resistance to aphids in sugarcane (Joshi & Viraktamath 2004). Polyphenol oxidases exhibit anti-nutritional activity against lepidopteran larvae (Thipyapong *et al.* 2007) and are highly active in meristematic tissues of sugarcane but activity declines with maturity (Bucheli *et al.* 1996). Nitrogen availability in sugarcane cultivars with the genetic potential to synthesize these anti-herbivory metabolites may enhance resistance to insects. However, nitrogen in tissues of cultivars in which the genetic capacity to produce these nitrogen-based secondary metabolites is absent may promote insect nutrition resulting in susceptibility of the plant. The genotypes $N29_R$ and $N33_R$ may, therefore, use available nitrogen, especially in the top internodes, for synthesis of compounds that act against insects. This may play a role in resistance in young shoots, reducing survival and *E. saccharina* populations in the field to the benefit of the mature crop. Biochemical activity against the insect may include other non-nitrogen anti-herbivory metabolites such as tannins, flavonoids and terpenes (Rutherford 2014).

Low infection levels by *F. pseudonygamai* SC17 and *F. sacchari* PNG40 were observed in the top internodes of $N41_{SR}$ and $NCo376_S$. Synthesis and accumulation of pathogenesis-related (PR) proteins and other antimicrobial metabolites in the nitrogen-rich upper stalk parts may have resulted in inferior infection levels compared to lower regions of the stem. As PR proteins, such as peroxidases, glucanases, defensins, thionins, trypsin and chymotrypsin inhibitors, are produced during pathogen attack (Rutherford 2014), their production after inoculation with the *Fusarium* strains may have been limited by nitrogen availability in lower stalk tissues resulting in greater infection than in the upper stem regions. Consequently, the impact of PNG40 and SC17 on *E. saccharina* survival, length bored and weight gain was not observed in the top internodes. However, infection by *E. saccharina*-promoting *F. pseudonygamai* SC17 and antagonistic *F. sacchari* PNG40 in the lower stalk influenced borer survival and damage in cultivars $NCo376_S$ and $N41_{SR}$ infected by the fungi.

Both varieties exhibited less borer damage when infected with PNG40 whilst $NCo376_S$ experienced more damage in SC17-colonized stalks. As a result, larvae from N41 that fed on tissue infected by PNG40 gained less whilst those from $NCo376_S$ that fed on SC17 gained more weight. These observations are supported by *in vitro* bioassays that showed the toxic and beneficial effects of these isolates on *E. saccharina* (McFarlane *et al.* 2009). Studies in maize also showed that stalks inoculated with *F. verticilloides* displayed increased damage by *E. saccharina* and other lepidopterans than in uninoculated controls (Schulthess *et al.* 2002). Ako *et al.* (2003) found higher oviposition rates and survival of larvae in maize stems inoculated with *F. verticilloides*. On the other hand, Logrieco *et al.* (1996) reported that 35 strains from different *Fusarium* species isolated from wheat, maize, barley and potatoes produced beauvericin, a well-known insecticidal compound. Furthermore, Dowd *et al.* (1989) demonstrated the toxic effect of trichothecenes, deoxynivalenol, dihydroxycalonectrin and 8-hydroxycalonectrin produced by *F. graminearum*, on growth of larvae of lepidopterans *Spodoptera frugiperda* (J.E. Smith) and *Heliothis zea* (Boddie). The observations from the present study, therefore, suggest that the impact of *E. saccharina* on a cultivar depends on whether it is infected by a *Fusarium* strain that is beneficial or antagonistic to the borer. This may potentially result in variations in resistance ratings of cultivars during screening or in the field. For instance, although cultivar $N41_{SR}$ is semi-resistant to *E. saccharina* whilst $NCo376_S$ is susceptible, infection of $N41_{SR}$ by a *Fusarium* strain toxic to the borer (*e.g.* PNG40) may result in a much higher resistance rating compared with uninfected or SC17-infected $NCo376_S$ plants. It may also be possible that a resistant cultivar colonized by a strain beneficial to the insect, *e.g.* $N41_{SR}$ colonized by SC17, may appear less resistant when compared to a susceptible one infected by an *E. saccharina*-antagonistic fungus.

While physical mechanisms are important in resistance to *E. saccharina* in sugarcane cultivars, evidence from this and other studies suggest that other strategies, for example nitrogen-based defence, may also play a role. Establishing the

presence of anti-herbivory metabolites in sugarcane cultivars, their relationship with resistance to *E. saccharina* and selection of genotypes expressing these compounds may be beneficial. Furthermore, the impact of *Fusarium* strains should be considered in the management of *E. saccharina*. Controlling infection by *Fusarium* strains that are beneficial to the pest may aid in reducing damage. In addition, it may be possible to employ antagonistic isolates such as *F. sacchari* PNG40 as biological control to form part of integrated pest management strategies as the method employed in this study to conduct *E. saccharina* resistance trials is less costly, less laborious and time-consuming than the traditional methods and may be of value to the South African sugarcane plant breeding programme.

ACKNOWLEDGEMENTS

We acknowledge and thank M. Keeping, N. Muthusamy and N. Sewpersad for their assistance, and the South African Sugarcane Research Institute, University of KwaZulu-Natal and National Research Foundation of South Africa (Grants 85573 and 85414) for funding.

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Accepted 25 June 2014

CHAPTER 4

In vitro generation of somaclonal variant plants of sugarcane for tolerance to *Fusarium sacchari*

Plant Cell Rep (2013) 32:249–262
DOI 10.1007/s00299-012-1359-0

ORIGINAL PAPER

In vitro generation of somaclonal variant plants of sugarcane for tolerance to *Fusarium sacchari*

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Received: 28 August 2012 / Revised: 5 October 2012 / Accepted: 9 October 2012 / Published online: 23 October 2012

Abstract
Key message **A combination of in vitro culture and mutagenesis using ethyl methanesulfonate (EMS) followed by culture filtrate-mediated selection produced variant sugarcane plants tolerant and resistant to *Fusarium sacchari*.**
Abstract *Eldana saccharina* is a destructive pest of the sugarcane crop in South Africa. *Fusarium sacchari* PNG40 (a fungal strain harmful to *E. saccharina*) has the potential to be an endophytic biological control agent of the stalk borer. However, the fungus causes Fusarium stalk rot in sugarcane. In the current study, sugarcane plants tolerant and resistant to *F. sacchari* PNG40 were produced by exposing embryogenic calli to the chemical mutagen ethyl methanesulfonate (EMS), followed by in vitro selection during somatic embryogenesis and plantlet regeneration on media containing *F. sacchari* culture filtrates (CF). The incorporation of 100 ppm CF in the culture media at the embryo maturation stage, at germination, or at both, resulted in callus necrosis and consequent reduced plantlet yield. Subsequent trimming of the roots of regenerated plants and their exposure to 1,500 ppm CF served as a further selection treatment. Plants produced from EMS-treated calli displayed improved root re-growth in the presence of CF pressure compared with those from non-treated calli. The tolerance of CF-selected plants was confirmed in greenhouse tests by inoculation with *F. sacchari* PNG40, re-isolation of *Fusarium* spp. from undamaged tissue of asymptomatic plants and establishment of the identity of fungal isolates as PNG40 using molecular analysis. The restriction of PNG40 presence to the inoculation lesion in some plants suggested their resistance to the fungus. Genotypes exhibiting symptomless endophytic colonization by PNG40 were identified and will be utilised for testing biological control strategies against *E. saccharina*.

Keywords Culture filtrate (CF) · *Eldana saccharina* · Ethyl methanesulfonate (EMS) · *Fusarium sacchari* · In vitro selection · Somatic embryogenesis

Introduction

Sugarcane (*Saccharum* spp. hybrids) is negatively affected by pests and diseases largely due to its vegetative propagation by stem sections (setts), perennial use of ratoons and monoculture in large adjacent fields, all of which result in the easy spread of microbial diseases and pests (Bailey 2004). The most important pest in the South African sugarcane industry is the stalk borer *Eldana saccharina* Walker (Lepidoptera: Pyralidae), the cause of extensive damage and economic losses since the 1970s (Mokhele et al. 2009). Among other control measures, the use of resistant varieties is the cornerstone of Integrated Pest Management strategies employed against it (Rutherford and Conlong 2010).

It takes 11–15 years to release a new improved cultivar of sugarcane through conventional breeding (Butterfield

Communicated by P. Lakshmanan.

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et al. 2001). Potential new commercial cultivars undergo a five-stage selection programme during which clonal material of selected genotypes is increased and clones are chosen for their sucrose content and resistance to pests and diseases (Parfitt 2005). Although clones combining high sucrose content and high sugar yield can be identified, they are often not released as they may lack resistance to one or more pests or diseases (Butterfield and Thomas 1996). Due to difficulties in conducting *E. saccharina*-inoculated screening trials for resistance, only those relatively few clones remaining in the later stages of the selection programme can be tested (Nuss 1991). Consequently, there is a considerable negative impact of pests and diseases on the selection programme, with 40 % of the best yielding clones at each stage being rejected due to susceptibilities (Butterfield and Thomas 1996).

In the case of *E. saccharina*, the combination of conventional breeding strategies with genetic engineering has been considered as a means to overcome the susceptibility of high sugar yielding clones to the insect (Meyer et al. 2000). However, the use of this technology in sugarcane has limitations, such as transgene silencing (Manners and Casu 2011), the cost and length of time required to prepare biosafety regulatory dossiers (Cheavegatti-Gianotto et al. 2011) and intellectual property restrictions.

Eldana saccharina has been found to be associated with *Fusarium* species in maize (Schulthess et al. 2002) and sugarcane (McFarlane et al. 2009). In maize, endophytic colonisation by *F. verticilliodes* was correlated with higher *E. saccharina* infestation and damage compared with plants treated with fungicide, suggesting a beneficial relationship between the fungus and the insect (Schulthess et al. 2002). In sugarcane, *E. saccharina* damage is associated with infection by *Fusarium* spp. where larval borings facilitate access of the fungus to the inner stalk resulting in rot of tissue surrounding the insect borings (McFarlane et al. 2009). Usually, Fusarium stalk rot in sugarcane is minimal without such borer-inflicted wounds to the stalk rind as the fungus cannot overcome this barrier unaided. McFarlane et al. (2009) also showed that certain *Fusarium* isolates were beneficial to the stalk borer's survival and growth rate, whilst other isolates (e.g. *F. sacchari* isolate PNG40, which was isolated from a larval boring containing a moribund insect) were detrimental. Developing resistance or tolerance to these *Fusarium* strains in sugarcane can assist in controlling Fusarium stalk rot. In addition, the production of sugarcane genotypes exhibiting resistance to the *Fusarium* strains beneficial to *E. saccharina* may result in collateral increase in resistance to the stalk borer. Furthermore, selecting genotypes tolerant to strains antagonistic to *E. saccharina*, e.g. *F. sacchari* PNG40, may enable use of the fungus as an endophytic biological control agent against the stalk borer.

In vitro culture systems are known to be a potential source of variant plants from which those with desired traits may be selected. This approach has been widely applied in the improvement of a number of agricultural species (Van den Bulk 1991; Jain 2001; Lakshmanan et al. 2005; Snyman et al. 2011). In many such studies, physical (Ali et al. 2007; Sharma et al. 2010) or chemical (Imelda et al. 2000; Shah et al. 2009) mutagens, together with in vitro culture, have been used to increase mutation frequency to obtain disease-tolerant regenerated plants, e.g. bunchy top virus in banana (Imelda et al. 2000), *Fusarium* wilt in abaca (Purwati and Sudarsono 2007) and Black sigatoka and *Fusarium* wilt in banana (Jain 2010). In sugarcane, in vitro strategies that incorporate specific selection agents have been used to produce plants with improved tolerance to red rot disease (Ali et al. 2007), salt (Patade et al. 2008) and herbicide (Koch et al. 2012).

Purified toxins, or pathogen culture filtrates containing toxins, involved in pathogenesis, have been shown to be suitable selection agents for use in vitro (Daub 1986; Svabova and Lebeda 2005). For example, *Fusarium* produces fumonisin B_1, which triggers the salicyclic acid pathway and encourages necrotrophic colonisation (De la Torre-Hernandez et al. 2010). Reducing the plant's susceptibility to such toxins may lead to a decrease in damage by disease. This may be achieved by employing these compounds in selection of plants that permit fungal growth without toxin-inflicted damage (tolerance) or those that inhibit proliferation of the fungus (resistance) (Roy and Kirchner 2000). Studies in maize (Gengenbach et al. 1977), rice (Ling et al. 1985), barley, wheat (Wenzel and Foroughi-Wehr 1990) and sugarcane (Sengar et al. 2009) have confirmed a correlation between tolerance of plants to toxins, or culture filtrates, and that obtained to the pathogen. Nevertheless, it is important to expose such plants selected in vitro to the pathogen to confirm tolerance (Thakur et al. 2002; Sengar et al. 2009).

The objective of the present study was, therefore, to establish the feasibility of in vitro-induced mutagenesis, followed by fungal toxin-mediated screening, selection of somaclonal variant sugarcane cells and subsequent plantlet regeneration to achieve tolerance to *F. sacchari* PNG40. This is the first part of a larger study aimed towards employing *F. sacchari* PNG40 in control strategies against *E. saccharina*. Tolerance to *F. sacchari* will reduce Fusarium stalk rot, usually associated with *E. saccharina* damage, and also permit endophytic colonisation in sugarcane plants by PNG40. This will facilitate exploitation of the fungus as a biological control agent against the stalk borer.

Materials and methods

Fusarium sacchari culture and filtrate preparation

Fusarium isolate PNG40 was originally obtained from aborted *E. saccharina* borings in sugarcane stalks, identified as *Fusarium sacchari* and reported to be toxic to the stalk borer (McFarlane et al. 2009). To prepare cultures for storage, colonies were grown on potato-dextrose-agar (PDA) (Biolab, Wadeville, RSA) for 5 days, after which 5 × 5 mm mycelial squares were transferred to 15 % (v/v) glycerol (Merck, Wadeville, RSA) and stored at −80 °C. Starter cultures were prepared by placing a thawed mycelial square on PDA for 3 days. Thereafter, a mycelial square was cut from the leading edge of the *F. sacchari* PNG40 colony, transferred to 250 ml of potato-dextrose-broth (PDB) (Fluka, St Louis, USA) and agitated at 145 rpm at 28–30 °C. After 7 days, the culture was centrifuged at 12,000 rpm for 5 min. The supernatant was filtered through sterile muslin cloth to remove the mycelia and its dry mass (80 °C for 24 h) was determined. The filtrate was sequentially filtered through Whatman No. 1 filter paper, 0.45 and 0.2 µm filters (Millipore, Ireland). This culture filtrate (CF) was stored at 4 °C for a maximum of 24 h. The concentration of each batch of CF was expressed as fungal dry mass/volume of PDB used in the *Fusarium* liquid culture. To ensure batch-to-batch consistency, culture conditions with regards to duration, media and temperature were identical. The culture filtrate was diluted according to mycelial dry mass.

Indirect somatic embryogenesis and plantlet acclimation

The cultivar NCo376 was field grown at the South African Sugarcane Research Institute (SASRI), Mount Edgecombe, Durban. Leaf roll decontamination, explant preparation (30 × 2 mm-thick leaf discs/stalk) and culture conditions were as described by Snyman (2004), and all media (at pH 5.8) are listed in Table 1. Ten leaf discs/90 mm Petridish were initiated on 30 ml Embryo Initiation Medium (EIM) and incubated in the dark at 25–27 °C, sub-culturing on to fresh medium every 2 weeks. After 6–8 weeks, 0.2 g of embryogenic calli were transferred to 30 ml Embryo Maturation Medium (EMM) incubated in the dark at 25–27 °C for 3 weeks. After embryo maturation, the calli were transferred to 30 ml Embryo Germination Medium (EGM1) in 90 mm Petridishes and incubated in 16 h light (200 µm/m^2/s photon flux density)/8 h dark photoperiod, at 26–30 °C for 4–8 weeks. Individual rooted plantlets (>20 mm in height) were transferred to Sterivent® vessels (110 × 100 × 80 mm) (Duchfa, Belgium) with EGM1 (20 plants per vessel). For acclimation, plantlets (70–100 mm in height) were planted in polystyrene seedling trays (67 × 33 cm, 98 cells) containing a 1:1 peatmoss–vermiculite medium (v/v), supplemented with dolmitic lime (Calmasil®, Middleburg, RSA) (5 g/10 kg substrate). The trays were transferred to a poly-carbonate tunnel, watered for 5 min (600 ml/min) twice a day and fertilised every 2 weeks (NPK 5:1:5, Profert, Noordsberg, RSA) for 2 months.

Establishment of culture filtrate-selection treatments

To determine the effect of CF during embryo maturation, embryogenic calli were established for 6–8 weeks on EIM. The calli (0.2 g per replicate, n = 12–21) were placed on EMM containing CF and incubated in the dark at 25–27 °C, sub-culturing weekly. Different concentrations of CF (0, 20, 50 or 100 ppm) were used to determine a suitable concentration for screening and selection during embryo maturation. Callus necrosis (defined as at least 50 % of a callus piece being brown in appearance), fresh and dry mass (70 °C for 48 h) were recorded after 3 weeks. Embryo germination and plantlet growth were subsequently carried out on media without CF and, for each treatment, the total number of plants produced per 0.2 g callus was recorded over 12 weeks. For the CF effect on embryo germination, embryo initiation (6–8 weeks) and maturation (3 weeks) were both carried out on media without CF. Subsequently, embryogenic calli were transferred to EGM1 supplemented with 0, 20, 50 and 100 ppm CF, and maintained in the photoperiod growth room for 4–12 weeks, sub-culturing weekly. Callus necrosis and

Table 1 Media composition for embryo initiation (EIM), maturation (EMM), germination (EGM1) and plantlet establishment (EGM2) stages

Constituents (g/L)	Medium			
	EIM	EMM	EGM1	EGM2
MS salts and vitamins (Murashige and Skoog 1962)	Full-strength	Full-strength	Full-strength	Half-strength
Casein hydrolysate	0.5	0.5	0.5	–
Sucrose	20	20	20	5
2,4-Dichloro-phenoxyacetic acid	3	1	–	–
Agar–agar	8	8	8	8

Table 2 Treatments used to select ethyl methanesulfonate (EMS)-treated calli and controls tolerant to CF. Embryogenic calli (0.2 g) were exposed to EMS (32 mM) for 4 h. Culture filtrate was included at embryo maturation stage, at germination, or both

Treatment	EMS	Culture stages	
		CF (100 ppm)	
		Embryo maturation (3 weeks)	Embryo germination and plantlet establishment (4–8 weeks)[a]
1	−	−	−
2	+	−	−
3	−	+	−
4	+	+	−
5	−	−	+
6	+	−	+
7	−	+	+
8	+	+	+

[a] Until plants reached a height of 20 mm

number of plants per replicate were recorded after 4 and 12 weeks, respectively.

To investigate the effect of CF on established plantlets (70–100 mm in height), green leaves were pruned just above the ligule and roots were trimmed to <1 mm. The trimmed plantlets were transferred to Magenta® vessels (5 plants/vessel) containing EGM2 with 0, 750 or 1,500 ppm CF to establish an appropriate concentration for selecting plantlets tolerant to CF. After 3 weeks of incubation in the photoperiod growth room, root re-growth was determined by measuring root length.

Production of variant plants and CF-mediated selection

The EMS preparation and callus treatment were according to the methods of Koch et al. (2012). Embryogenic calli (0.2 g) were placed in 32 mM EMS solutions for 4 h, after which they were rinsed three times with liquid EMM; liquid EMM was used for the controls.

After EMS exposure, eight CF treatments (Table 2) were tested for selection. Calli were transferred to EMM for 3 weeks followed by EGM1 for 4–8 weeks. Culture filtrate at 100 ppm (the most toxic of the tested concentrations) was incorporated in either EMM or EGM1 and in both media (Table 2). Fresh and dry mass and number of plants per 0.2 g of callus were recorded after embryo maturation and germination, respectively; callus necrosis was recorded at each stage. Plantlets (20 mm in height) produced from each treatment were transferred to Sterivent® vessels until they were 70–100 mm tall. The roots and leaves of the plantlets were then trimmed before being transferred to Magenta® vessels containing 80 ml EGM2 supplemented with 1,500 ppm CF, the concentration deemed in preliminary studies to be suitable for selection of CF-tolerant plants. Root length was recorded after 3 weeks.

Ex vitro selection studies using *Fusarium sacchari*

After screening on medium containing 1,500 CF for 3 weeks, plantlets with roots that re-grew to at least 10 mm in length were used for ex vitro investigations. Such plants were acclimated in the glasshouse at 20/34 °C (night/day temperature), watered for 5 min (600 ml/min) twice a day and fertilised every fortnight with NPK (5:1:5). When they had 1–2 stem internodes they were transferred to pots (200 mm diameter; 170 mm height), placed in troughs and not watered for a week. They were then inoculated by stabbing the stem 2–3 cm above the soil surface with PNG40-colonised toothpicks (Gilbertson 1985). Controls were stabbed with either uncolonised or colonised toothpicks. Secondary infection was avoided by swabbing the stem with 70 % ethanol prior to stabbing and the protruding toothpick was cut and the wound wrapped with parafilm.

Seven to 8 weeks after toothpick stab inoculation, the stems of dead and live plants (some with necrotic, chlorotic and crinkled leaves) were surface sterilised by submerging them sequentially in 95 % (v/v) ethanol for 2 min, 10 % (v/v) sodium hypochlorite for 5 min and sterile water (twice) to rinse, after which they were dried in the laminar air flow cabinet for 5 min. Removal of surface contaminants was confirmed by pressing the leaves and stems on PDA. The leaves were cut across the blade and placed on Nash and Snyder (1962) medium. The stems were split into longitudinal sections and inoculation lesion severity for individual stems was visually rated as: 0, no lesions; 1, mild; 2, moderate; 3, severe. One of the two longitudinal sections was used for re-isolation of the fungus from the lesion and from the undamaged area above it, and the other section was used for staining. Fungal re-isolation from the inoculation lesion, and the undamaged area 20–30 mm above it, was carried out by cutting longitudinal stem sections and placing the segments on Nash and Snyder (1962) medium and incubating at 28–30 °C for 5 days. Isolates were visually compared with PNG40 and were subjected to molecular analysis by inter-simple sequence repeats (ISSRs). Stem tissue was stained with lactophenol cotton blue (Sigma, St. Louis, USA) and wet mounts prepared for compound light microscope detection of fungal colonisation, according to the method of De Mars and Boerner (1995).

Molecular analysis of isolated fungus

Fungal DNA extraction was conducted according to the PrepMan® Ultra Sample protocol (Applied Biosystems,

California, USA). The DNA concentration was determined using a spectrophotometer (NanoDrop Technologies, Delaware, USA) and adjusted to 200–250 ng/µl with elution buffer.

Regions between microsatellites were amplified using ISSR 1, ISSR 2, ISSR 4 and ISSR 8 primers (McFarlane et al. 2009) (Table 3). The efficacy of these primers to discriminate *Fusarium* species and isolates was tested by performing ISSR-PCR using six isolates belonging to *F. andiyazi, F. proliferatum* and *F. sacchari* (two isolates/species) obtained from the SASRI culture collection. PCR was carried out for each primer using a PCR kit (Kapa Biosystems, Massachusetts, USA) in a final volume of 19.5 µl composed of 13.42 µl PCR water (Ambion, Texas, USA), 2 µl Taq buffer with $MgCl_2$ (15 mM), 0.4 µl dNTPs (10 µM), 1.6 µl ISSR primer (10 µM), 0.5 U Taq polymerase and 2 µl DNA template. Cycling conditions for PCR amplification were 95 °C for 2 min, followed by 32 cycles of denaturation at 94 °C for 30 s, primer annealing (temperature in Table 3) for 30 s, extension at 72 °C for 30 s, and a single final extension at 72 °C for 5 min. PCR products were stained with GelRed™ nucleic acid stain and separated by gel electrophoresis with a 100 bp O'Gene™ Ruler DNA Ladder Mix (Fermentas, Maryland, USA) as the molecular weight marker. Banding patterns of the *Fusarium* isolates were visually analysed and polymorphic bands were noted. PCR was repeated three times with each primer. DNA extracted from re-isolated fungi from dead and live plants was subjected to ISSR-PCR using primers ISSR 1, ISSR 4 and ISSR 8, and the banding patterns were compared with those of PNG40 to confirm similarity between the inoculated *F. sacchari* PNG40 and the re-isolated fungus.

Statistical analyses

The data were analysed using Genstat statistical package 13th edition (VSN International, UK. 2010). The data were initially tested for normality using the Shapiro–Wilk test and for homogeneity using the Bartlett test. The Restricted maximum likelihood (REML) method was used to estimate random and fixed effects and significant differences amongst treatments were detected using the Holm-Sidak test.

Table 3 ISSR primer sequences and annealing temperatures used in discriminating *Fusarium* species and isolates

Primer	Sequence	Annealing temperature (°C)
ISSR 1	$CCCGCATCC(CA)_9$	57
ISSR 2	$CCCGGATCC(GA)_9$	55
ISSR 4	$(AG)_9G$	51
ISSR 8	$(CCA)_5RY$	45

R purine, *Y* pyrimidine

Results

Establishment of callus and in vitro plantlet screening conditions

Exposure of embryogenic calli to 0–100 ppm *F. sacchari* CF at either embryo maturation or germination stages resulted in callus necrosis, i.e. at least 50 % of a callus piece being brown in appearance, which intensified with increasing CF levels in the media (Fig. 1a). At the maturation stage, some necrotic calli, especially those exposed to 100 ppm CF, turned mucilaginous and developed root hairs after 3 weeks. Despite these results, no significant differences in fresh and dry mass were detected amongst the CF treatments during maturation (results not shown). When the embryogenic calli were subjected to CF stress only during embryo germination (Fig. 1a), necrosis set in during the first week of exposure and, by 4 weeks, nearly 100 % of calli on all tested CF treatments were necrotic.

Incorporation of CF in the embryo maturation media and subsequent embryo germination on EGM1 without CF resulted in decreased plant yield (Fig. 1b). Embryos from the control and 20 ppm treatments at maturation germinated normally. However, some non-necrotic calli only produced roots and those calli that were brown eventually turned black. No significant differences in plantlet yield were observed between the control and 20 ppm, and the 50 and 100 ppm CF treatments (Fig. 1b). However, the latter produced significantly fewer plants than the control ($P < 0.001$). Although there were no statistically significant differences with respect to plantlet yield amongst the tested CF concentrations (20, 50 and 100 ppm), there was a trend indicating an inhibiting effect of the CF (Fig. 1b). Although application of the CF stress at the embryo germination stage resulted in most calli being recorded as necrotic, the non-necrotic areas within each callus started greening during the first week on EGM1 + CF and plantlet yield was recorded by week 12 (Fig. 1b). Plant yield was significantly inhibited ($P < 0.001$) by increasing concentrations of CF with the least number of plants being produced at 100 ppm CF (Fig. 1b).

As expected, there was an inverse relationship between percentage callus necrosis and plantlet yield with the treatments recording the highest necrosis producing the lowest number of plants (Fig. 1). Also, callus exposure to CF at the embryo germination stage resulted in more severe necrosis than at the embryo maturation stage, with the highest percentage necrosis recorded at each stage being 95.5 ± 0.9 and 61.6 ± 3.9 %, respectively. Consequently,

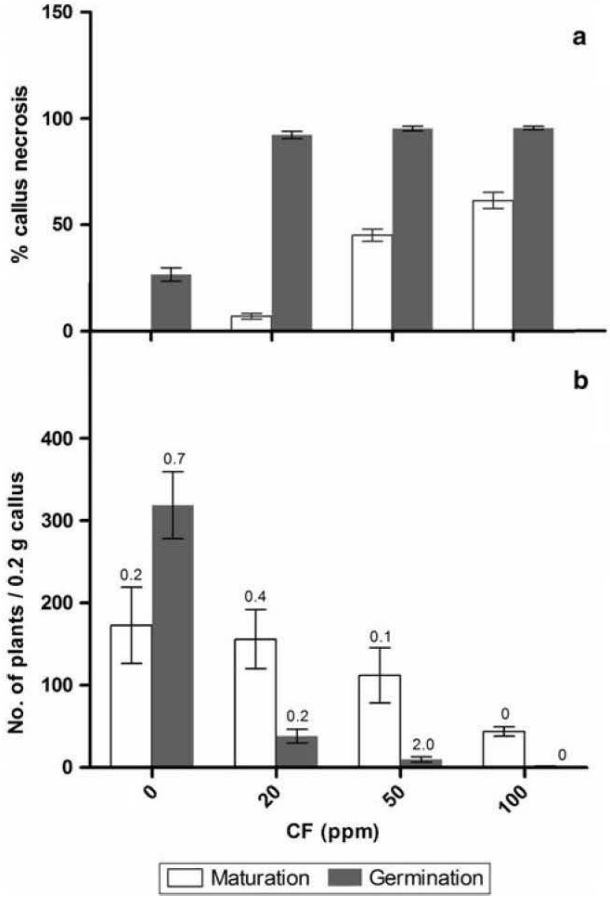

Fig. 1 The effect of culture filtrate concentration in either the embryo maturation or germination medium on **a** percentage callus necrosis, and **b** plantlet yield. The number above the *bar* indicates percentage abnormal plantlets. *Lines* above the *bars* represent standard error of the mean, $n = 12$–21

fewer plants were produced from the former than from the latter. The number of abnormal plants from each treatment after incorporation of CF during either maturation or germination was recorded and never exceeded 2 % (Fig. 1b).

Culture of plantlets with trimmed roots and leaves on EGM2 + CF resulted in stunted root growth and discolouration at the base of the stem after 3 weeks (Fig. 2a). Significant inhibition ($P < 0.001$) of root re-growth was observed in the 750 ppm and 1,500 ppm treatments with plant root lengths of 17.8 ± 1.7 and 8.5 ± 2 mm, respectively, compared with 39.4 ± 2.1 mm in the control plants (Fig. 2b). The 1,500-ppm CF was the most root re-growth inhibiting treatment after 3 weeks and was, therefore, adopted for selection of CF-tolerant plants. Since trimmed roots of 50 % of the plantlets cultured on medium containing 1,500 ppm CF re-grew to at least 10 mm in length, this value was set as the criterion for selection of CF-tolerant plants. The leaves re-grew to approximately the original length in all the treatments, although they were pale-green in the 750 and 1,500 ppm CF treatments compared with those of the control.

Selection of calli and plants putatively tolerant to *F. sacchari*

The effect of CF incorporated in either embryo maturation or embryo germination medium, and in both media on EMS-treated calli was investigated (Table 2). The EMS treatment did not result in callus necrosis, as no significant differences were recorded between the EMS-treated (treatments 4 and 8) and the non-treated calli (treatments 3 and 7) (Fig. 3a). Only calli cultured on EMM + CF (treatments 3, 4, 7 and 8) were found to be necrotic (Fig. 3a).

After embryo maturation, calli from treatments 1–4 were transferred to EGM1 − CF and those from treatments 5–8 to EGM1 + CF, until plantlets developed (maximum 12 weeks). As expected, after 4 weeks in germination medium, no callus necrosis was detected in calli from treatments 1 and 2 (no CF at both maturation and germination), and necrosis in calli from treatments 3 and 4 (CF stress at maturation only) remained at the end of the maturation stage (Fig. 3a). Also, as previously determined, necroses in calli from treatments 5 and 6 (CF stress at germination only) increased significantly ($P < 0.001$) during the germination stage. Further, they reached significantly higher values than those recorded when CF stress was imposed at maturation (treatments 3 and 4) (Fig. 3a). The calli from treatments 7 and 8 were exposed to CF during both maturation and germination stages. For treatment 7, the percentage callus necrosis increased significantly from the maturation to the germination stage, but no such difference was obtained for treatment 8. Again, there was no apparent effect of EMS on callus necrosis (treatments 3 vs. 4, 5 vs. 6, 7 vs. 8).

The results from all of the EMS and CF treatments, indicate that plantlet yield was not affected by EMS (treatments 1 and 2) but generally decreased with the severity of the CF-imposed stress (Fig. 3b). As in the previous investigation (Fig. 1b), the number of plants produced from calli exposed to CF during the germination stage (treatments 5, 6, 7 and 8) was significantly fewer ($P < 0.001$) than those from calli cultured on medium with CF during maturation (treatments 3 and 4). Despite treatments 7 and 8 having CF pressure in both stages, and treatments 5 and 6 in germination only, no significant differences in plantlet yield were observed (Fig. 3b). However, treatment 8 produced significantly more plants than treatment 6. The number of plants produced from the EMS + CF at maturation (treatment 4) was significantly higher ($P < 0.001$) than from the other EMS treatments (6 and 8). A relationship was again observed between number

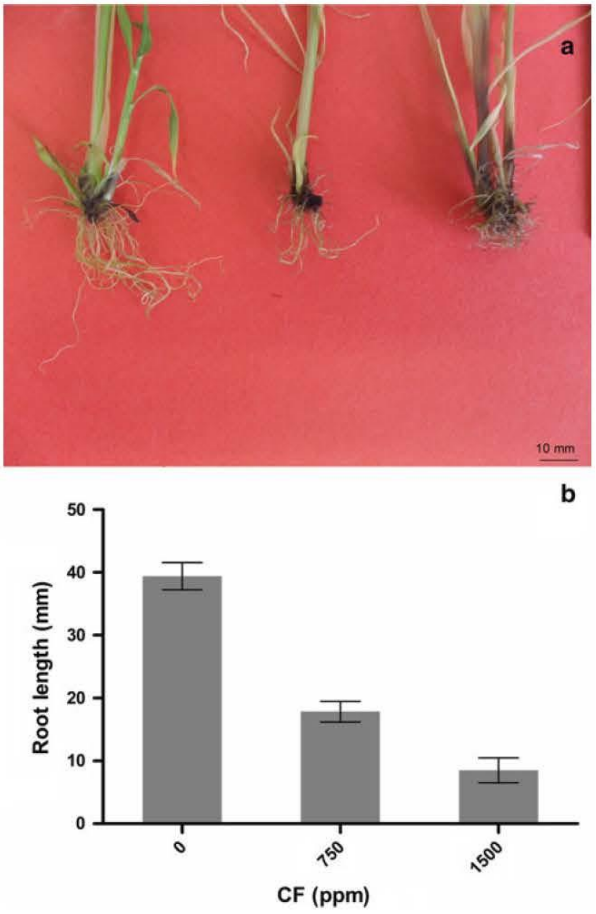

Fig. 2 The effect of *Fusarium sacchari* culture filtrate (CF) on **a** the visual appearance of roots, and **b** root re-growth from plantlets which had their roots and leaves trimmed before being placed on media with 0–1,500 ppm CF for 3 weeks. *Lines* above the *bars* represent standard error of the mean, $n = 10$

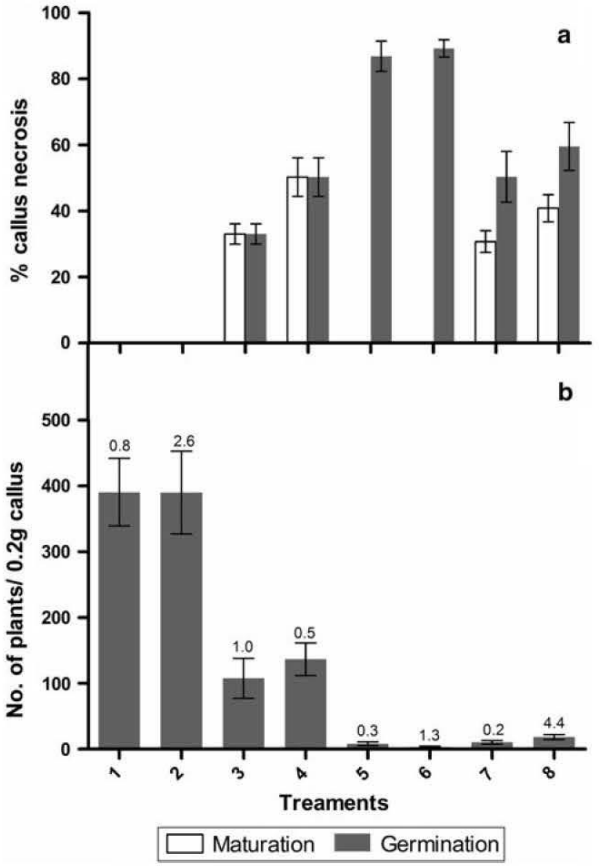

Fig. 3 The effect of culture filtrate on **a** percentage necrosis, and **b** plantlet yield of EMS-treated calli. The culture filtrate was incorporated at the embryo maturation stage or at germination or both stages. The *number* above the *bar* indicates percentage abnormal plants. *Lines* above the *bars* represent standard error of the mean, $n = 7–12$

of plants and percentage callus necrosis, with treatments that exhibited high percentage callus necrosis producing low plant yields (treatments 5–8). All treatments produced a relatively small percentage of abnormal plants, which included albino and chimeric individuals (Fig. 3b).

Since *Fusarium* CF inhibits root growth (Chen and Swart 2002; Khan et al. 2004), all of the plants that survived the manipulations described above (Fig. 3) were further screened for tolerance to CF based on root re-growth in the presence of 1,500 ppm CF for 3 weeks (Fig. 4). The root lengths for plants from most EMS treatments (treatments 2, 4 and 8) displayed wider inter-quartile ranges (distance between the 25th percentile and 75th percentile) than their corresponding controls [1 (+CF), 3 and 7, respectively] (Fig. 4). In addition, the EMS treatments 2, 6 and 8 had wider total ranges than their respective controls (1, 5 and 7) (Fig. 4). This reflects a greater variation in root length in the plants regenerated from the EMS-treated calli than in those from the non-EMS treatments. Furthermore, the EMS treatments resulted in more plants with at least 10 mm in root length than the non-EMS treatments (with the exception of treatment 6) (Fig. 4).

All plants with root length of 10 mm or more were inoculated with *F. sacchari* PNG40 by stabbing with *F. sacchari*-colonised toothpicks. Inoculated controls exhibited leaf crinkling, chlorosis and necrosis 3–4 weeks after stabbing the stems with *F. sacchari*-colonised toothpicks, and their shoot growing point dried and died after 7–8 weeks. Wilting leaves and dead shoot growing points were also observed in some plants from treatment 8 and one plant from treatment 3 became chlorotic and then necrotic. Longitudinal sections of stems from dead and live plants, from all treatments, revealed lesions of varying severity progressing from the stabbed area. Dead plants were observed only in the inoculated control and in treatment 8 (Table 4). Lesion severity rating for symptomless

plants ranged from 1 to 2, and from 2 to 3 in the symptomatic ones (Table 4).

Detection, re-isolation and confirmation of identity of *F. sacchari* PNG40 from putative-tolerant plants

Asymptomatic plants supporting endophytic colonisation were distinguished from the negative control plants by visualisation of hyphae (stained using lactophenol blue) in between plant cells above the inoculation lesion (results not shown). The presence of *Fusarium* was confirmed in stems of symptomatic, asymptomatic and dead plants, and symptomatic leaves by surface sterilising and placing transverse sections of these organs on selective Nash and Snyder (1962) medium. The test for microbial growth after pressing stems and leaves on PDA was negative for all samples, thus confirming the effectiveness of the surface sterilisation. *Fusarium*-like colonies grew from the lesions of the dead, symptomatic and asymptomatic plants and the symptomatic leaf sections. No growth was observed from the stem sections from non-inoculated plants. *Fusarium* could not be re-isolated from undamaged tissue above the lesion in 15 of the 24 asymptomatic plants from all treatments, but the fungus was retrieved from the other nine plants (Table 4). Visualisation of the fungus in undamaged tissue was also achieved in three asymptomatic plants. However, the retrieval of *Fusarium* from undamaged tissue of nine plants, including the three from which the fungus was visualised, asserted re-isolation as a better approach for determining endophytic colonisation than microscopic observation.

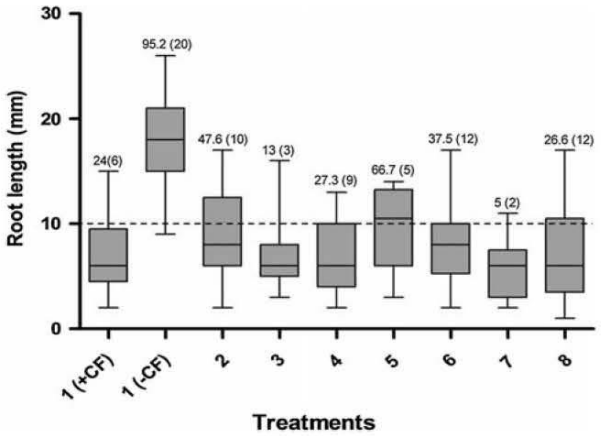

Fig. 4 The effect of EMS on root re-growth after 3 weeks in the presence of 1,500 ppm culture filtrate. The *boxes* represent the interquartile range and the lower, middle and upper limits of each *box* represent the 25th percentile, median and 75th percentile, respectively. The *lower and upper bars* represent lowest and highest values recorded, respectively. The *numbers* above the *bars* indicate the percentage number of plants (*outside the brackets*) and actual number of plants (*in brackets*), with a root length above the 10 mm threshold (*dashed line*), $n = 6$–45

Molecular analysis was carried out on DNA from the fungal isolates retrieved from the plants. The efficacy of primers ISSR 1, ISSR 2, ISSR 4 and ISSR 8 in distinguishing between different *Fusarium* isolates and species was tested by subjecting six isolates of *F. andiyazi*, *F. proliferatum* and *F. sacchari* (two isolates/species) to ISSR-PCR. The number of monomorphic, polymorphic bands and unique banding patterns obtained from the *Fusarium* isolates using each primer indicated that a combination of primers ISSR 1, ISSR 4 and ISSR 8 was able to separate each of the six different *Fusarium* isolates (results not shown). Using these primers, banding patterns generated from isolates retrieved from the lesions and the undamaged area above them were the same as for PNG40 (Table 4).

F. sacchari PNG40-tolerant and -resistant plants

The inoculation with *F. sacchari* PNG40 confirmed the tolerance or resistance of CF-selected plants as follows: (1) Six of the seven plants from treatment 1 inoculated with PNG40-colonised toothpicks (positive controls) displayed symptoms, lesion severity ratings (LSR) of 2–3 and three of them died after 8 weeks. A single control was symptomless and showed an LSR of 2. Stabbing with sterile toothpicks had no adverse effects on the plants (negative controls) (Table 4). (2) Twenty-three out of the 29 inoculated plants from all the other treatments were symptomless and exhibited a LSR of 1–2, 8 weeks after toothpick stab inoculation (Table 4). Re-isolation of *Fusarium* from undamaged tissue and ISSR analyses of the isolates from eight of these plants indicated that they permitted potential endophytic colonisation by *F. sacchari* PNG40 and were, therefore, tolerant to the fungus (Table 4). The single asymptomatic inoculated control was also considered tolerant. In the other 15 asymptomatic plants, the fungus was retrieved from the inoculation lesions only. These plants were classified as resistant to the fungus as they appeared to limit PNG40 growth and proliferation. (3) Six plants were regarded as susceptible as one plant showed symptoms only (treatment 3), another exhibited an LSR of 3 (treatment 5) and the other four displayed symptoms, a LSR of 3 and died (treatment 8) (Table 4).

Discussion

Callus and plantlet response to fungal culture filtrate

Fungi produce toxic metabolites that are involved in plant pathogenesis (Yoder 1980). In vitro plant cultures of

susceptible and tolerant genotypes vary in their response to these compounds, thus making such phytotoxins useful in in vitro selection strategies for disease tolerance (Binarova et al. 1990). In this regard, establishment of an appropriate phytotoxin concentration that negatively affects cells, tissues, organs and whole plants increases the probability of obtaining tolerant lines (Daub 1986). *Fusarium* spp. culture filtrates and purified toxins have been used widely in callus (Toyoda et al. 1984; Hidalgo et al. 1998; Thakur et al. 2002), root growth (Baker et al. 1981; Chen and Swart 2002; Khan et al. 2004) and leaf necrosis (Hidalgo et al. 1998) tests to select tolerant genotypes. Similarly, in the present study, inclusion of different concentrations of *F. sacchari* PNG40 CF in the culture media resulted in deleterious effects on both embryogenic callus and plantlets. The fungal filtrate inflicted dose-dependent callus necrosis (Fig. 1a) and consequent decrease in plant yield (Fig. 1b), as well as inhibition of root re-growth in regenerants (Fig. 2). Furthermore, necrosis was found to be a better indicator of the effect of CF on calli during embryo maturation than callus mass as no significant differences in fresh and dry mass were obtained amongst the CF treatments. However, the effect of the CF was more severe when incorporated at the embryo germination stage than at maturation, as evidenced by the higher levels of necrosis at the former than the latter (Fig. 1a). Further, there were no significant differences in necrosis at the germination stage after inclusion of CF in both maturation and germination media and when it was added in the germination medium only (Fig. 3a). This is significant as no reports on

Table 4 A summary of disease response and tissue colonisation by *Fusarium sacchari* PNG40 in plants (treatments 1–8) 2 months after toothpick stab inoculation with the fungus. Plants selected from the root re-growth test were inoculated and re-isolation was carried out from the lesion and area above it to confirm the presence of the fungus in the plant tissues. Inter simple sequence repeats (ISSR)-PCR analyses were used to confirm identity of isolates as PNG40

Treatment	Plant no.	External symptoms[a]	Severity of lesion[b]	Dead (D) or alive (A)	Re-isolation on NS agar from lesion[c]	ISSR comparison of isolate with PNG40[d]	Re-isolation on NS agar from undamaged area above lesion[e]	ISSR comparison of isolate with PNG40[f]	Resistant (R), tolerant (T) or susceptible (S)
T1 (sterile toothpick)	1a	0	0	A	N	nd	N	nd	ni
	1b	0	0	A	N	nd	N	nd	ni
	1c	0	0	A	N	nd	N	nd	ni
T1 (inoculated with PNG40)	1d	1,2,3,4,5	3	D	Y	+	nd	nd	S
	1e	2,3,4,5	3	D	Y	+	nd	nd	S
	1f	2,3	2	A	Y	+	N	nd	S
	1g	0	2	A	Y	+	Y	+	T
	1h	3,4	3	A	Y	+	N	nd	S
	1i	3,4	3	A	Y	+	N	nd	S
	1j	3,4,5	3	D	Y	+	nd	nd	S
T2	2a	0	1	A	Y	+	Y	+	T
	2b	0	2	A	Y	+	Y	+	T
	2c	0	2	A	Y	+	N	nd	R
	2d	0	2	A	Y	+	N	nd	R
T3	3c	0	1	A	Y	+	Y	+	T
	3g	0	2	A	Y	+	Y	+	T
	3j	2,3	2	A	Y	+	N	nd	S
T4	4a	0	1	A	Y	+	N	nd	R
	4h	0	1	A	Y	+	Y	+	T
	4l	0	2	A	Y	+	N	nd	R
T5	5a	0	2	A	Y	+	N	nd	R
	5b	0	2	A	Y	+	N	nd	R
	5c	0	3	A	Y	+	N	nd	S
	5e	0	2	A	Y	+	N	nd	R
	5f	0	1	A	Y	+	N	nd	R
T6	6b	0	2	A	Y	+	Y	+	T
	6d	0	1	A	Y	+	N	nd	R

Table 4 continued

Treatment	Plant no.	External symptoms[a]	Severity of lesion[b]	Dead (D) or alive (A)	Re-isolation on NS agar from lesion[c]	ISSR comparison of isolate with PNG40[d]	Re-isolation on NS agar from undamaged area above lesion[e]	ISSR comparison of isolate with PNG40[f]	Resistant (R), tolerant (T) or susceptible (S)
T7	7a	0	1	A	Y	+	Y	+	T
	7b	0	1	A	Y	+	N	nd	R
T8	8a	0	2	A	Y	+	N	nd	R
	8f	4,5	3	D	Y	+	nd	nd	S
	8g	0	1	A	Y	+	Y	+	T
	8i	4,5	3	D	Y	+	nd	nd	S
	8j	0	2	A	Y	+	N	nd	R
	8k	0	2	A	Y	+	N	nd	R
	8n	0	1	A	Y	+	N	nd	R
	8r	4,5	3	D	Y	+	nd	nd	S
	8s	0	1	A	Y	+	N	nd	R
	8t	4,5	3	D	Y	+	nd	nd	S

ni not inoculated

[a] External symptoms: 0, no symptoms; 1, leaves crinkling; 2, leaves chlorotic; 3, leaves necrotic; 4, dead growing point; 5, wilting

[b] Lesion severity rating: 0, no lesions; 1, mild; 2, moderate; 3, severe

[c] Y, fungus re-isolated; N, fungus not re-isolated

[d] Isolate retrieved from lesion: +, isolate similar to PNG40; −, isolate not similar to PNG40; nd, not determined (no fungus retrieved)

[e] Y, fungus re-isolated; N, fungus not re-isolated; nd, not determined (no undamaged tissue)

[f] Isolate retrieved from undamaged area above lesion: +, isolate similar to PNG40; −, isolate not similar to PNG40; nd, not determined (no fungus retrieved)

incorporation of CF at the germination stage of embryogenesis were found in the literature. In most studies, for example in alfalfa (Binarova et al. 1990), gladiolus (Remotti et al. 1997), carnation (Thakur et al. 2002), and rose geranium (Saxena et al. 2008), CF was supplied during embryogenic callus induction and surviving calli were then transferred to germination medium without CF. The observed response of CF during embryo germination may be due to light-dependant toxin activity (Asai et al. 2000) or differential effects of the CF on the various biochemical and physiological processes occurring at embryo germination or maturation stages. Nevertheless, a few necrotic calli produced plants suggesting that a callus piece could consist of both tolerant and susceptible cells. However, inadequate exposure of susceptible embryos to the CF may also have resulted in production of plants, i.e. 'escapes'. In a study to select tomato plants tolerant to *Fusarium* CF, Toyoda et al. (1984) pointed out that it is critical to ensure infiltration of the CF to deeper callus cell layers to avoid escapes. In that study, adequate exposure to *F. oxysporum* CF was achieved by submerging tomato calli placed on semi-solid medium incorporated with CF, and in liquid medium also containing the CF (liquid-on-agar method). In another approach, Jin et al. (1996) incorporated *F. solani* CF in soya bean embryogenic suspension cultures to select CF-tolerant embryos. Incorporation of CF in liquid media during culture using methods such as suspension or temporary immersion cultures, together with use of smaller callus pieces can, therefore, ensure adequate exposure of callus cells to the CF. However, work in our laboratory (unpublished) has shown that sugarcane calli do not survive such immersion treatments.

In the current study, 'escapes' that may have resulted as a consequence of inadequate exposure of cells to CF stress at the embryo maturation and germination stages were discarded by assessing root re-growth in regenerated plants on CF-containing medium (Fig. 4). In addition, as tolerance expressed by single cells may vary from that of the whole plant (Van den Bulk 1991), this test was employed to screen the putative tolerant lines at the whole plant level. The inhibition of root growth by *Fusarium* CF reported in other studies (Chen and Swart 2002; Khan et al. 2004) and that observed in the current one using *F. sacchari* CF, proved that assessing root re-growth is a suitable approach for this purpose. Hence, the observed responses of calli and regenerated plants to the CF treatment indicated its suitability as an in vitro selecting strategy for screening plants tolerant to this toxin at various stages of morphogenesis. In this regard, the established CF concentration for selection during embryogenesis was 100 and 1,500 ppm for root re-growth.

Production of EMS-induced variants and in vitro and ex vitro selection for tolerance to *F. sacchari*

The ability of EMS to induce mutations in plants is well documented (Jabeen and Mirza 2002; Lee et al. 2003; Hoffmann et al. 2004). Mutagenesis can enhance in vitro-induced variation, thereby improving the probability of selecting plants with desired attributes. Although in vivo mutagenesis using EMS has been undertaken in a number of plant species (Khairwal et al. 1984; Jabeen and Mirza 2002; Sharma et al. 2010), the use of in vitro cultures allows for the treatment of single cells, thus preventing formation of chimeras, and screening and selection under controlled conditions. In this regard, there are several reports on EMS treatment of calli, followed by appropriate screening protocols, for production of plants tolerant to herbicides (Jander et al. 2003; Koch et al. 2012), salt (Luan et al. 2007) and diseases (Imelda et al. 2000; Purwati and Sudarsono 2007; Matsumoto et al. 2010). An attribute of EMS that makes it a popular mutagen is its ability to induce high point mutation frequencies without causing lethal abnormalities to the chromosomes (Waugh et al. 2006). In the present study, there were no significant differences in callus mass and necrosis (Fig. 3a) between EMS-treated and non-treated calli. As stress in calli due to EMS treatment, i.e. growth inhibition (Svetleva and Crino, 2005) and necrosis (Koch et al. 2012) has been reported to be directly related to the mutagen dose, these observations indicated the suitability of the employed mutagenic treatment. However, whilst it is important to minimise negative effects during a mutagenic treatment, it is also critical to attain high mutation frequencies, i.e. variation. Sadat and Hoveize (2012) reported that treatment of sugarcane calli with 32 mM EMS for 4 h induced variation whilst causing minimal negative effects. Similarly, in the current study, although the EMS treatment did not induce negative effects in calli, variability was achieved as evidenced by plants with greater contrast in root re-growth in the presence of CF pressure than the controls (Fig. 4). This observation suggested that treatment of calli with EMS improves mutation frequency compared with in vitro culture without the mutagen.

Fusarium sacchari CF-tolerant plants were obtained from the variants generated from the EMS treatment by selection of treated embryogenic calli and subsequently regenerated plants with the desired mutation(s) using CF at the established concentrations. More plants (2–5 times) with improved root re-growth were obtained from EMS treatments (Fig. 4, treatments 2, 4 and 8) than their respective controls (Fig. 4, treatments 1(-CF), 3 and 7), suggesting that the mutagenic treatment resulted in enhanced ability to overcome CF-induced root re-growth inhibition, i.e. increasing the production of CF-tolerant plants. It is possible that the generation of a mutation(s) that deters the action of root-growth inhibiting compounds produced by *Fusarium* spp., e.g. fusarubin, javanicin, anhydrofusarubin (Baker et al. 1981) and the peptide Nep1 (Bae et al. 2006), may have led to this result. Challenging the plants exhibiting improved root re-growth with *F. sacchari* PNG40 verified their tolerance or resistance to the fungus (Table 4). This ability of EMS to induce tolerance to *Fusarium* spp. has been described in other studies (Purwati and Sudarsono 2007; Shah et al. 2009; Matsumoto et al. 2010). Sharma et al. (2010) reported that in vivo EMS treatment of seeds was more effective in producing genotypes tolerant to *Fusarium* wilt in garden pea than selection of in vitro culture-induced variants using CF. However, a combination of in vitro mutagenesis using EMS and selection via CF pressure allows for better penetration and uniform exposure of callus cells to both the mutagen and the CF, thereby improving chances of selecting the desired mutation(s). In addition, as observed in the present study, in vitro mutagenesis and selection allow for screening at different stages of morphogenesis, i.e. embryo maturation, germination and plantlet establishment. The results indicated that screening at the embryo germination stage is more stringent than at maturation. Further, escapes can be discarded and putative-tolerant lines selected from the surviving regenerants by assessing root re-growth in whole plants exposed to CF pressure.

Based on studies in which tolerance to CF displayed by calli was uncorrelated to that exhibited to the toxin-producing pathogen (Vardi et al. 1986; Rowe and Stortz-Lintz 1993), there is a possibility of plants achieving tolerance to other compounds present in the CF and not the putative toxin (Van den Bulk 1991). Inoculation of CF-selected plants with the toxin-producing fungus is, therefore, necessary to confirm tolerance. Afolabi (2008) reported that toothpick stab inoculation of maize with *Fusarium* spp. induced lesions in the stem and severity was associated with the level of tolerance of the genotypes. In the current study, after toothpick stab inoculation with PNG40, most of the CF-selected plants exhibited no disease symptoms, whilst those from the controls (not exposed to CF or EMS) (treatment 1) were symptomatic or dead and displayed lesion severity ratings of 2–3 (Table 4). Re-isolation of the fungus from dead, symptomatic and asymptomatic plants and confirmation of the identity of the retrieved fungus as PNG40 using molecular analysis (Table 4), supported the tolerance or resistance of the symptomless plants to *F. sacchari* PNG40. Nine of the twenty-four asymptomatic plants were regarded tolerant as they allowed PNG40 growth without causing damage to the occupied tissue (endophytic colonisation), presumably because they were unaffected by toxins produced by the fungus. Studies in

maize indicate that symptomless endophytic colonisation of young plants by *Fusarium* spp. resulting from artificial inoculation of seeds or seedlings persists up to their maturity (Munkvold and Carlton 1997; Schulthess et al. 2002). Similarly, endophytic colonisation of young plants by *F. sacchari* was determined in the current study and it is also possible that the fungus' occupation of plant tissue may perpetuate over long periods. However, a 10-month investigation has been initiated to confirm such long-term colonisation in these plants. The other 15 symptomless plants appeared resistant to *F. sacchari* as they limited the presence of PNG40 to the inoculation lesion, possibly due to biochemical or physiological mechanisms that inhibited fungal growth and proliferation in the plant tissues (Roy and Kirchner 2000).

These observations support a correlation between tolerance to the CF in vitro and that displayed to the organism (*F. sacchari*), thereby confirming the usefulness of CF as an in vitro selection agent. Such a validation has been reported in several crop species (Gengenbach et al. 1977; Ling et al. 1985; Wenzel and Foroughi-Wehr 1990; Sengar et al. 2009). *Fusarium* spp. produce phtyotoxins, such as fumonisin B_1, moniliformin (Van Asch et al. 1992) and fusaric acid (Diniz and Oliviera 2009), known to elicit disease development. As illustrated in the present study, it can be expected that developing tolerance to such compounds (purified or in culture filtrate) may also lead to tolerance or resistance to *Fusarium* spp. However, *Fusarium* CF are also known to contain mutagenic compounds, e.g. Fusarin C (Lu and Jeffrey 1993). Successive exposures to the CF during screening may, therefore, lead to undesirable mutations which result in plants exhibiting susceptibilities to biotic and abiotic factors (Matsumoto et al. 2010). This may explain the high mortality observed in treatment 8 in the present study.

The reported findings, therefore, indicate that plants both tolerant and resistant to *F. sacchari* can be produced by treating calli with 32 mM EMS for 4 h, exposing them to 100 ppm CF at the embryo germination stage and assessing root re-growth in regenerated plantlets in the presence of 1,500 ppm CF. Whilst phenotypic analysis of these EMS-induced variants and tests to establish long-term presence of endophytic *F. sacchari* in tolerant plants are necessary in ongoing work, the present study indicated the feasibility of the described approach in obtaining tolerance to the fungus. This protocol has value in developing tolerance and resistance to *F. sacchari* in commercially important cultivars and possibly even to other sugarcane pathogens. Further, genotypes expressing these traits may be used as parents to provide sources of tolerance and resistance genes in sexual crosses for development of new commercial cultivars. The tolerance to *F. sacchari* PNG40 and associated endophytic colonisation by the fungus obtained using the approach described in this study, are essential prerequisites towards the exploitation of PNG40 in biological control strategies against the *E. saccharina*. Future investigations will employ these tolerant genotypes to test the effect of the fungus on larval survival and growth of the stalk borer.

Acknowledgments Many thanks to the National Research Foundation (NRF)-South Africa and the South African Sugarcane Research Institute (SASRI) for funding to carry out this study.

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CHAPTER 5

Potential of *Fusarium sacchari*-tolerant mutants in controlling *Eldana saccharina* and borer-associated Fusarium stem rot in sugarcane

Eur J Plant Pathol
DOI 10.1007/s10658-014-0582-7

Potential of *Fusarium sacchari*-tolerant mutants in controlling *Eldana saccharina* and borer-associated Fusarium stem rot in sugarcane

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Accepted: 14 December 2014

Abstract Some *Fusarium* spp. such as *F. sacchari* PNG40, found in association with the African sugarcane stalk borer *Eldana saccharina*, have potential in biological control against the insect. However, *Fusarium* can cause stem rot in sugarcane thereby constraining its application. The present study tested the usefulness of *F. sacchari* PNG40 tolerant sugarcane mutants in the control of Fusarium stem rot and *E. saccharina* using endophytic *F. sacchari* PNG40. In vitro mutagenesis and selection of embryogenic calli (cultivar N41) were employed to produce *F. sacchari*-tolerant mutants. Stalks of 8 months-old N41 mutants and previously-produced NCo376 mutants were inoculated with PNG40 and then with *E. saccharina* 2nd instar larvae after 3 weeks. Length bored was 36–82 % less in inoculated stalks (1.00–4.67 cm) of the NCo376 and N41 mutants and their respective parents than their uninoculated controls (3.83–8.67 cm). Mass gain was significantly less in larvae retrieved from incubated stalks (0.028–0.045 g) of MutA and MutE of NCo376, and Mut1 and Mut27 of N41 than their controls (0.054–0.072 g). The NCo376 and N41 mutants displayed significantly less percent stalk area discoloured (Fusarium stem rot) (10.6–22.0 %) than their respective parents (N41 - 28.9 % and NCo376 - 30.2 %). Re-isolation of PNG40 from undamaged tissue indicated endophytic colonisation. MutA of NCo376 and Mut1 and Mut23 of N41 were identified for future field studies aimed at curbing Fusarium stem rot and developing endophytic *Fusarium*-mediated biological control against *E. saccharina*, as part of an integrated pest management approach.

Keywords Biological control · *Eldana saccharina* · *Fusarium sacchari* · Mutants · Sugarcane · Tolerance

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Introduction

The stalk borer *Eldana saccharina* Walker (Lepidoptera: Pyralidae) is a major constraint to sugarcane production in South Africa. Significant losses are incurred in the sugar industry through reduction in valuable biomass by borer feeding on stalk tissue, and poor juice quality and decreased sucrose content caused by opportunistic fungal infection of bored tissue (Way and Goebel 2003). The SA sugar industry loses an estimated total of US$ 82 000 000 due to direct *E. saccharina* damage and indirect losses due to early harvesting of the crop at 12 months to minimise borer damage, instead of the more economically viable age of 15–18 months (Keeping et al. 2014). To control this pest, integrated management approaches employ cultural, chemical and biological strategies (Webster et al. 2005). Amongst these, the use of resistant cultivars is considered the best

Published online: 05 January 2015

approach. However, the polyploid and aneuploid nature of the sugarcane genome complicates the development of genetically-improved genotypes via conventional breeding, a lengthy and resource-intensive process (Ming et al. 2006). Further, *E. saccharina* resistance in sugarcane is largely dependent on physical mechanisms such as stalk rind hardness and fibre content (Keeping and Rutherford 2004). Hard stalk rinds provide barriers that deny larvae access to the inner stalk tissue whilst high fibre is assumed to reduce the digestibility of tissue by the insect. However, whilst these characteristics may contribute to borer resistance, they are undesirable due to their negative relationships with sucrose content and sucrose recovery in the mill (Singh et al. 2013). Therefore, development of control strategies to complement existing management approaches for *E. saccharina* is essential.

Resistance to *E. saccharina* in sugarcane is inversely related to resistance against the biotrophic pathogen *Sporisorium scitamineum* (Syd.) Piepenbr & Oberw., causing sugarcane smut. Both the insect (Leslie 1993) and fungus (Croft and Braithwaite 2006) prefer to enter sugarcane stalks via the nodal buds. It has been suggested that this negative correlation could be due to negative cross-talk between salicylate (SA) and jasmonate/ethylene (JA/ET) defence signalling systems in which elevated levels of SA in the buds confer resistance to *S. scitamineum*, whilst inhibiting JA/ET-mediated defence against *E. saccharina* (Heinze et al. 2001; Rutherford 2014). Damage by *E. saccharina* in sugarcane is associated with opportunistic infection by necrotrophic *Fusarium* species of the section Liseola which cause a reddish-brown discolouration of internal stalk tissues (Fusarium stem rot). Compatible biotrophic pathogens such as *Sporisorium scitamineum* induce primarily JA/ET responses, whilst compatible necrotrophic pathogens such as *Fusarium* induce SA-dependent cell death responses (van Loon et al. 2006). For example, some *Fusarium* species of the section Liseola produce gibberellins which are antagonistic to the JA pathway and could serve to increase susceptibility to the fungus (Traw and Bergelson 2003). Liseola section fusaria produce toxins which contribute to necrotrophic colonisation by activating the salicylate pathway (SA) and inducing oxidative stress and cell death (De la Torre-Hernández et al. 2010; Sánchez-Rangel et al. 2012). Certain *Fusarium* isolates from sugarcane are known to be beneficial to *E. saccharina* (McFarlane et al. 2009). This could be partly due to suppression of JA induced defence responses favouring both the fungus and *E. saccharina*.

Nevertheless, the potential of *Fusarium* spp. in biological control of crop insect pests is well established. For example, pathogenicity of *Fusarium* spp. against insects has been reported in the sugar beet root maggot *Tetanops myopaeformis* (Roder) (Majumdar et al. 2008), chilli thrips *Scirtothrips dorsalis* Hood, chilli broad mite *Polyphagotarsonemus latus* (Banks) (Mikunthan and Manjunatha 2008), wheat stem sawfly *Cephus cinctus* Norton (Wenda-Piesik et al. 2009), rice weevil *Sitophilus oryzae* L. (Batta 2012) and mealworm *Tenebrio molitor* L (Guo et al. 2014). In sugarcane, *Fusarium oxysporum* was shown to be harmful to *Chilo auricilius* Dudgeon, *C. infuscatellus* Snellen and *Sesamia inferens* Walker in bioassays (Varma and Tandan 1996). However, the utility of most of the entomopathogenic *Fusarium* spp. in insect control is limited by their pathogenicity to plants. Some such *E. saccharina*-associated *Fusarium* spp. that were isolated from stalks exhibiting limited borer damage, were found to have harmful effects on larval growth and survival in addition to causing stem rot, e.g. *F. sacchari* PNG40 (McFarlane et al. 2009). Although Fusarium stem rot in sugarcane is a caveat to pursuing fungus-mediated insect control, the development of *Fusarium* tolerant sugarcane genotypes may aid in combating *E. saccharina* damage and associated Fusarium stem rot, particularly if a symptomless endophytic relationship between *F. sacchari* PNG40 and sugarcane could be established.

Endophytic colonisation of a host plant can occur when pathogen virulence and host defence mechanisms are in balance, such that neither is negatively impacted by the association (Schultz et al. 1999). Navarro-Meléndez and Heil (2014) showed that symptomless endophytism was associated with increased JA signalling and repressed SA signalling suggesting that defence against insects and necrotrophic pathogens would be heightened. Indeed, most fusaria have an initial hemibiotrophic phase where early colonisation relies on a living host (biotrophic), before eventually transitioning to killing and consuming host cells (necrotrophic) (Ma et al. 2013). Increased JA signalling and repressed SA signalling could serve to maintain the hemibiotrophic symptomless state.

Induction of mutations that enhance defence mechanisms against *F. sacchari*, or that limit its necrotizing ability (e.g. resistance to the toxin fumonisin B1) could

lead to establishment of tolerance to the fungus through such a balance (Mahlanza et al. 2013). This might alleviate the phytopathogenicity of the pathogen in plant – fungus – insect interactions and limit disease associated with entomopathogenic activity of the fungus. It is hypothesised that *Fusarium*-tolerant mutants endophytically colonised by PNG40 may circumvent Fusarium stem rot and permit fungal insecticidal activity against *E. saccharina*, thereby enabling the application of PNG40 in biological control strategies against the borer in sugarcane.

We previously reported on the use of in vitro mutagenesis in sugarcane to gain tolerance to *F. sacchari* PNG40 in the NCo376 sugarcane cultivar (Mahlanza et al. 2013). The present study aimed to test the potential utility of *F. sacchari* PNG40-tolerant mutants of NCo376 and of newly produced mutants of N41 to control both *E. saccharina* and borer damage-associated Fusarium stem rot. The NCo376 cultivar is susceptible to *Fusarium* and *E. saccharina* whilst the more commercially important N41 exhibits intermediate resistance to *E. saccharina* and *Fusarium*. As in vitro mutation induction is a random process that may affect non-target traits, the impact of mutagenesis on stalk rind hardness, fibre and nitrogen content, sugarcane characteristics involved in *E. saccharina* resistance mechanisms, were also investigated.

Material and methods

Mutagenesis of calli and selection of N41 *Fusarium sacchari*-tolerant plants

Fusarium sacchari PNG40 tolerant plants of the cultivar N41 were produced according to the protocol of Mahlanza et al. (2013). Briefly, 8 weeks-old embryogenic calli were exposed to a solution of the mutagen ethyl methanesulphonate (EMS) (32 mM) for 4 h and then transferred to embryo maturation [MS salts and vitamins (4.4 g/l); 2.4D (1 mg/l); casein hydrolysate (0.5 g/l); sucrose (20 g/l) and agar (8 g/l)] and germination (maturation without 2.4D) media (Snyman 2004), containing *F. sacchari* culture filtrate (CF) (100 ppm), for 3 and 4 weeks, respectively. Thereafter, the roots of the surviving regenerants were trimmed and the shoots were transferred to rooting medium (germination medium with half-strength MS) incorporated with 1500 ppm CF. Plants that exhibited a root length above a threshold of 10 mm, established in preliminary studies, were acclimated in the glasshouse and inoculated with *F. sacchari* PNG40 by inserting fungus-colonised toothpicks into the stem of the plants (Gilbertson 1985). Symptoms of *Fusarium* infection, lesion severity (rating 0–3) and fungal infection were determined after 2 months. Symptomless plants with low lesion severity (rating 1–2) from which the fungus was re-isolated from both the lesion and undamaged tissue above the lesion were regarded as tolerant (Table 1). Intersimple sequence repeat (ISSR) analyses were conducted to confirm the identity of the isolates as PNG40 according to the protocol of (McFarlane et al. 2009).

Molecular analysis of regenerants

Genomic DNA was extracted from leaf material of the tolerant plants using the protocol of Dellaporta et al. (1983). Twenty four random amplified polymorphic DNA (RAPD) primers (Table 2) were used to detect genetic differences between the parent N41 cultivar and the tolerant plants. For each line, a polymerase chain reaction (PCR) was conducted in a 25 μl mix containing 100 ng genomic DNA template, 2.5 μl 10×buffer, $MgCl_2$ (2.5 μM), dNTPs (200 μm), primer (0.25 μM) and Taq polymerase (1.25 units). The PCR cycling condition were as follows: an initial denaturation at 92 °C for 4 min 30 s then 40 cycles of denaturation at 92 °C for 1 min, primer annealing at 35 °C for 1 min and extension at 72 °C for 2 min, and a final extension for 15 min at 72 °C. The PCR products were separated on a 2 % agarose gel containing 80 μl/l ethidium bromide and visualised using a UV transilluminator. For each primer, the reaction was repeated at least twice and for those that generated polymorphisms, the reaction was conducted a third time. Only reproducible bands were considered during scoring.

Fusarium sacchari and *Eldana saccharina* inoculation

The N41 tolerant mutants and the previously obtained NCo376 mutants (Mahlanza et al. 2013), that supported endophytic colonisation (i.e. from which PNG40 was re-isolated from undamaged tissue) were used. In this report, the NCo376 mutants 2a, 4 h, 3c 7a and 8 g from Mahlanza et al. (2013) are referred to as MutA-E, respectively. The NCo376 and N41 mutants were grown in riversand in 20 l buckets placed in troughs containing water and fertilised (NPK – 5:1:5) monthly for 8 months

Table 1 *F. sacchari*-tolerant mutants of N41 produced by in vitro mutagenesis. After inoculation with PNG40, lines without symptoms and from which PNG40 was re-isolated from undamaged tissue were regarded tolerant. Five lines (Mut5, 8, 16, 23, 27) were selected for further studies

Line	Symptoms[a]						Re-isolation	
	Wilt	Dead growing point	Leaf crinkling	Leaf necrosis	Leaf chlorosis	Lesion[b] rating	Lesion	Undamaged tissue
C1	–	–	–	–	–	0	nd[c]	nd
C2	–	–	–	–	–	0	nd	nd
C3	–	–	–	–	–	0	nd	nd
C4	√	√	–	√	√	3	√	√
C5	–	–	–	–	–	2	√	–
C6	–	–	–	√	–	2	√	√
C7	√	√	–	–	–	3	√	–
C8	–	–	–	–	–	2	√	√
Mut1	–	–	–	–	–	2	√	–
Mut2	–	–	√	–	–	2	√	–
Mut3	–	–	–	–	–	3	√	√
Mut4	–	–	–	–	–	2	√	–
Mut5*	–	–	–	–	–	1	√	√
Mut6	–	–	–	–	–	1	√	–
Mut7	–	–	–	–	–	1	√	–
Mut8*	–	–	–	–	–	1	√	√
Mut9	–	–	–	–	–	2	√	–
Mut10	–	–	–	–	–	3	√	–
Mut11	–	–	–	–	–	2	√	–
Mut12	√	√	–	–	–	3	√	√
Mut13	–	–	–	–	–	3	√	–
Mut14	–	–	–	–	–	2	√	√
Mut15	–	–	√	–	–	3	√	–
Mut16*	–	–	–	–	–	1	√	√
Mut17	–	–	–	–	–	2	√	√
Mut18	–	–	–	–	–	2	√	–
Mut19	–	–	–	–	–	2	√	√
Mut20	–	–	√	–	–	2	√	–
Mut21	–	–	–	–	–	2	√	√
Mut22	–	–	–	–	–	2	√	√
Mut23*	–	–	–	–	–	1	√	√
Mut24	–	–	–	–	–	2	√	–
Mut25	–	–	–	–	–	3	√	–
Mut26	–	–	–	–	–	1	√	–
Mut27*	–	–	–	–	–	1	√	√

[a] Symptoms : √–present;– =absent

[b] Lesion severity rating: 0=no lesion - 3=severe

[c] nd=not determined. These were negative control plants that were not inoculated

*Tolerant lines selected for further studies

Table 2 Random amplified polymorphic primers used in molecular analyses and the bands generated from DNA of the mutant and parent lines of N41

Primer name	Primer sequence	Total bands produced	Monomorphic bands	Polymorphic bands	Approximate range of band size (bp)
262	CGCCCCCAGT	8	7	1	460–1310
B-10	CTGCTGGGAC	9	9	0	340–1250
K19T	AGTTCAGGC	10	10	0	620–1300
OPA-1	CAGGCCCTTC	9	9	0	710–1350
OPA-04	AATCGGGCTG	8	8	0	610–1450
OPA-05	AGGGGTCTTG	9	9	0	320–1200
OPA-07	GAAACGGGTG	14	12	2	450–1350
OPA-08	GTGACGTAGG	10	10	0	480–1360
OPA-10	GTGATCGCAG	12	12	0	600–1300
OPA-11	CAATCGCCGT	8	8	0	540–1350
OPA-17	GACCGCTTGT	6	6	0	740–1350
OPB-11	GTAGACCCGT	5	5	0	640–1300
OPC-1	TTCGAGCCAG	12	12	0	440–1380
OPM-01	GTTGGTGGCT	7	7	0	680–1220
UBC-65	AGGGGCGGGA	12	12	0	470–1380
OPW-6	AGGCCCGATG	8	8	0	400–1300
OPA-19	CAAACGTCGG	14	13	1	640–1360
OPS-04	CACCCCCTTG	12	12	0	520–1200
OPG-06	GTGCCTAACC	7	7	0	600–1390
OPA-09	GGGTAACGCC	8	6	2	550–1260
OPA-17	GACCGCTTGT	8	8	0	520–1300
OPB-02	TGATCCCTGG	7	4	3	640–1320
OPAB-08	GTTACGGACC	12	12	0	580–1280
OPAB-09	GGGCGACTAC	12	12	0	420–1400
Total		227	218	9	

in a glasshouse (20–34 °C). For each genotype, tillers were marcotted and inoculated with *F. sacchari* PNG40 (Mahlanza et al. 2014). Briefly, the stalks were cut, the cut ends were immersed in a weak acid solution [H_2SO_3 (1.8 μM), H_3PO_4 (0.77 μM), H_2 SO_4 (0.38 μM) and HNO_3 (0.60 μM)] and 3rd–5th nodes from the cut end were covered in soil in metal canisters (6 l). After 2 weeks, roots had developed in the nodes covered in soil, the acid solution was discarded and the plants were watered and fertilised (NPK – 5:1:5). After 4 weeks, a wound was made in the stalk in the 2nd node from the soil surface and a *F. sacchari* PNG40 conidial suspension (10^5 conidia/ml) was introduced into the wound. For each mutant genotype and the NCo376 and N41 parents, inoculated and uninoculated treatments were established with five plants per treatment arranged in a complete randomised block design. After 3 weeks, all plants were inoculated with *E. saccharina* using a modified protocol of Kvedaras and Keeping (2007). Preweighed second instar larvae were placed into modified Eppendorf tubes and these were attached to the stalk over the wounds made during *Fusarium* inoculation using adhesive gum. The bases of the tubes were cut to allow movement of the larvae into the stalk and the lids were adapted to allow ventilation in the tubes. The plants were harvested after 3 weeks and stalks segments were surface sterilised and cut longitudinally under aseptic conditions to expose the borings, larvae and discoloured and undamaged tissue. The length of stalk tissue bored and mass gained by the larvae were recorded. Images of the stalk tissue were used to determine the area discoloured due to *Fusarium* infection. Re-isolation of *F. sacchari*-PNG40 was conducted from discoloured tissue to confirm PNG40 as the cause of discolouration.

Endophytic colonisation and spread of the fungus across internodes was determined by re-isolation from undamaged tissue from within the inoculated internode and the one immediately above. Sections of the damaged and undamaged tissue were excised aseptically and placed on Nash and Snyder (1962) agar, a semi selective medium for *Fusarium*. Percent sections colonised were recorded after 5 days of incubation at 30 °C. ISSR analyses were conducted to confirm the identity of the isolates.

Stalk rind hardness, fibre and nitrogen analyses

To determine the effect of mutagenesis on factors that may influence resistance of *E. saccharina* viz. stalk rind hardness, fibre and nitrogen analyses were conducted as described by Mahlanza et al. (2014) on stalks of the mutant lines and the parent cultivars. Internodes 5–7 for each plant were cut and their rind hardness was measured using a durometer (RexGauge Company, Glenview, IL). The stalk sections were shredded and fibre content (g/100 g dry mass) was measured using a fourier transform near-infrared (FT-NIR) spectrometer (Matrix F, Bruker, Bryanston, SA). Fine stalk material obtained after passing the shreds through a 1 mm sieve was used for analysis of nitrogen content (g N/100 g dry mass) using a Leco combustion analyser (TruSpec CN, Leco Corporation, St. Joseph, MI).

Data analyses

Data were analysed for normality using the Shapiro-Wilk test. Stalk rind hardness, fibre, nitrogen contents and area discoloured were analysed using analysis of variance (ANOVA) and Sidak tests. The grand mean stalk rind hardness, fibre, nitrogen contents of NCo376 lines were compared to those of the N41 ones using Student t tests. The stalk length bored and larval mass were analysed using the restricted maximum likelihood (REML) and Sidak tests. All analyses were conducted using Genstat statistical package (14th edition, VSN International, Hemel Hempstead, UK) and differences were statistically significant at $P \leq 0.05$.

Results

Genetic variation in regenerated N41 mutants

Twelve N41 *F. sacchari*-tolerant plants were produced by in vitro mutagenesis and selection using CF (Table 1). These plants showed no symptoms, lesion severity was low (rating 1–2) and PNG40 was re-isolated from the lesions and undamaged tissue above it. The genetic variability generated in these *F. sacchari* PNG40-tolerant variants in comparison with the parent lines was assessed by molecular analysis using RAPD primers. This generated a total of 227 reproducible bands from 24 primers, an average of 9.5 bands per primer, with the band sizes ranging from approximately 340–1450 bp (Table 2). Out of these bands, 218 were monomorphic whilst nine polymorphic ones were detected in seven mutant lines from the five primers 262, OPA07, OPA-19, OPA-09 and OPB-02, representing a 3.69 % rate of polymorphism (Table 2). Amongst the nine polymorphic bands found in the mutants, eight were absent and one had a different intensity from banding patterns generated from the parent DNA (Fig. 1a-d). The least number of band was produced from primer OPB11 (five bands) and the highest from OPA-07 and OPA-19 (14 bands) (Table 2). The highest number of polymorphic bands produced per primer was three from primer OPB-02.

Fusarium sacchari activity against *Eldana saccharina* in *Fusarium*-tolerant mutants

To establish the effect of PNG40 on *E. saccharina* in the *F. sacchari*-tolerant plants, stalks of five mutant and parent lines of each NCo376 and N41 cultivar were inoculated with the fungus and then with larvae. The stalk length bored in NCo376 mutant plants inoculated with PNG40 (1.17–4.67 cm) was approximately 36–82 % less than their uninoculated controls (6.67–8.67 cm) (Table 3). However, these differences were only statistically significant in MutA in which the inoculated plants displayed 1.17±0.44 cm and 6.67± 2.91 cm in the controls (P=0.045). Similarly, the inoculated N41 mutants (1.00–2.50 cm) exhibited 48–71 % less damage than their uninoculated controls (3.83–8.17 cm). Statistically significant differences were detected between inoculated stalks and

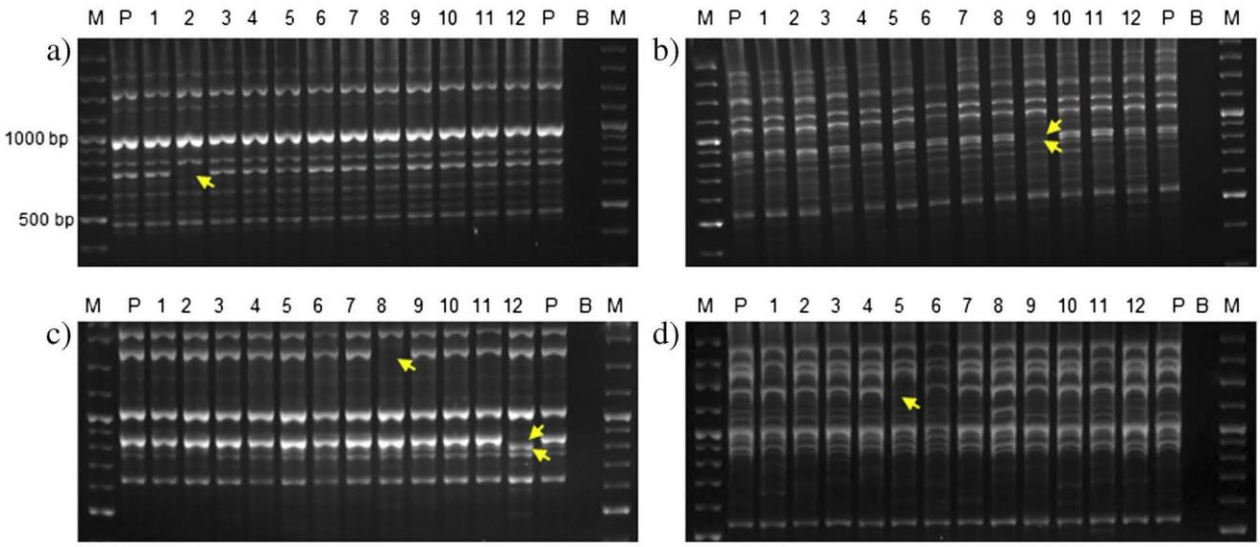

Fig. 1 Banding patterns generated from RAPD analyses of the N41 parent and tolerant mutants using primers **a**) 262; **b**) OPA-07; **c**) OPB-02; and **d**) OPA-19. Lanes *M*=marker (100 bp) *P*=parent, *B*=blank (water) and 1–12=mutants. Arrows indicate polymorphic bands

controls of the N41 mutants Mut5, Mut8, Mut23 and Mut27 ($P<0.001$) (Table 4). The length bored in stalks of parent cultivarsNCo376 and N41 inoculated with PNG40 also recorded significantly less damage than their uninoculated controls (Tables 3 and 4). However, no differences were detected between PNG40-infected stalks of the mutants and those of their respective parents (Tables 3 and 4). More damage was observed in stalks of the parent NCo376 (inoculated - 2.83±1.17 cm; control - 9.00±1.00 cm) (Table 2) than those of N41 (inoculated - 1.00±0.50 cm; control - 5.67±2.33 cm) (Table 4).

The mass gained by larvae retrieved from inoculated stalks was significantly less than those from the uninoculated controls of NCo376 mutants MutA and MutE ($P=0.040$) (Table 3), and Mut5, Mut27 and parent of N41 ($P=0.036$) (Table 4). The percent stalk area discoloured was recorded as a measure of Fusarium stalk rot. Stalks of NCo376 mutants MutA, MutD and MutE inoculated with PNG40 displayed significantly less area discoloured than those of their parent

Table 3 Comparisons of *Eldana saccharina* damage, larval mass gain and stalk tissue area discoloured due to *Fusarium* infection, amongst stalks inoculated with PNG40 and uninoculated controls of NCo376 mutants and parent. Stalks were inoculated with the fungus and, 3 weeks later, with 2nd instar larvae

Line	Length bored (cm)*		Δ Larval mass (g)*		Area discoloured (%)**	
	Uninoculated	Inoculated	Uninoculated	Inoculated	Uninoculated	Inoculated
NCo376	9.00 ± 1.00^{a}	2.83 ± 1.17^{de}	0.052 ± 0.021^{abc}	0.027 ± 0.002^{a}	6.14 ± 2.02^{a}	30.24 ± 3.81^{d}
MutA	6.67 ± 2.91^{abcd}	1.17 ± 0.44^{e}	0.066 ± 0.012^{bc}	0.029 ± 0.010^{a}	4.04 ± 1.75^{a}	10.61 ± 1.59^{b}
MutB	6.67 ± 0.67^{bc}	3.67 ± 1.96^{bde}	0.045 ± 0.010^{ab}	0.034 ± 0.018^{ab}	7.56 ± 2.08^{ab}	18.42 ± 7.63^{bcd}
MutC	7.33 ± 2.19^{abc}	4.67 ± 1.76^{bcd}	0.082 ± 0.010^{c}	0.065 ± 0.005^{c}	3.63 ± 1.24^{a}	21.97 ± 4.37^{cd}
MutD	8.67 ± 4.18^{abcd}	4.50 ± 1.32^{bd}	0.083 ± 0.019^{c}	0.054 ± 0.024^{abc}	5.54 ± 2.51^{a}	16.68 ± 4.83^{c}
MutE	7.00 ± 0.58^{c}	3.50 ± 1.26^{cd}	0.070 ± 0.005^{c}	0.045 ± 0.006^{ab}	5.17 ± 2.24^{a}	13.28 ± 2.97^{bc}

Different superscript letters indicate significant differences ($P\leq0.05$)

*REML and Sidak tests; Mean±SE, $n=3$–5

** ANOVA and Sidak tests Mean±SE, $n=5$

Table 4 Comparisons of *Eldana saccharina* damage, larval mass gain and stalk tissue area discoloured due to *Fusarium* infection, amongst stalks inoculated with PNG40 and uninoculated controls of N41 mutants and parent. Stalks were inoculated with the fungus and, 3 weeks later, with 2nd instar larvae

Line	Length bored (cm)*		Δ Larval mass (g)*		Area discoloured (%)**	
	Uninoculated	Inoculated	Uninoculated	Inoculated	Uninoculated	Inoculated
N41	5.67 ± 2.33^{ad}	1.00 ± 0.50^{c}	0.042 ± 0.011^{ab}	0.021 ± 0.005^{c}	5.84 ± 1.74^{a}	28.88 ± 3.85^{c}
Mut5	7.00 ± 1.53^{d}	2.00 ± 1.00^{bc}	0.054 ± 0.008^{bd}	0.028 ± 0.010^{ac}	5.88 ± 1.87^{a}	13.24 ± 5.54^{ab}
Mut8	6.33 ± 2.73^{ad}	2.50 ± 0.29^{b}	0.061 ± 0.019^{bd}	0.057 ± 0.009^{bd}	4.82 ± 4.69^{a}	21.30 ± 5.39^{bc}
Mut16	4.50 ± 2.02^{bd}	2.17 ± 1.42^{abc}	0.036 ± 0.014^{abc}	0.023 ± 0.007^{c}	4.63 ± 2.69^{a}	16.79 ± 5.52^{b}
Mut23	3.83 ± 1.69^{bd}	1.00 ± 0.29^{c}	0.042 ± 0.016^{abc}	0.036 ± 0.005^{abc}	2.93 ± 1.07^{a}	15.08 ± 4.97^{b}
Mut27	8.17 ± 2.74^{d}	2.50 ± 1.04^{abc}	0.072 ± 0.009^{d}	0.038 ± 0.012^{abc}	3.33 ± 1.11^{a}	17.36 ± 2.16^{b}

Different superscript letters indicate significant differences ($P\leq0.05$)

*REML and Sidak tests; Mean±SE; n=3–5

** ANOVA and Sidak tests Mean±SE; n=5

($P<0.001$) (Table 4). Also, PNG40-infected N41 mutants Mut5, Mut16, Mut23 and Mut27 exhibited significantly less discolouration than their parent cultivar ($P<0.001$) (Table 4). Stalks of N41 mutant Mut5 inoculated with PNG40 displayed the least area discoloured amongst N41 mutants and the parent, and this damage was not significantly different from its uninoculated control (Table 4).

Endophytic colonisation of stalk tissue sections

To establish the presence of PNG40 in discoloured stalk tissue, re-isolation of the fungus from stalk tissue sections on a *Fusarium* semi-selective medium was conducted. Further, re-isolation was carried out from sections of undamaged tissue from within the inoculated internode and the one immediately above to determine symptomless endophytic colonisation and spread of the fungus across internodes. The fungus was re-isolated from all discoloured sections from NCo376 and N41 mutants and parents (Table 5). In the undamaged tissue within the inoculated internodes of NCo376 mutants and parent, 50–80 % stalk sections were colonised by PNG40 with mutants MutD and MutE displaying the lowest and highest colonisation, respectively. Amongst those of N41, percent undamaged sections colonised ranged between 25 and 100 % with mutant Mut16 exhibiting the lowest levels of colonisation whilst the highest was recorded in Mut8 (Table 5). A comparison of the mean percent colonised sections from undamaged tissue in the inoculated internodes of the NCo376 and N41 lines did not indicate statistically significant differences ($P=0.953$) (Table 5). From the undamaged tissue sections from the internode above the inoculated one, PNG40 was retrieved in 0–50 % of the sections from NCo376 mutants and parent whilst 25–75 % of those from N41 were colonised by the fungus. Unlike in the inoculated internode, mutant MutD recorded the least percent sections colonised and MutE displayed the highest amongst NCo376 mutants and parent. Amongst those of N41, mutant Mut16 displayed the lowest and Mut8 and Mut23 the highest percent sections colonised. Mutants MutA and MutC of NCo376 and Mut5, Mut8 and Mut23 of N41 exhibited higher percent sections colonised from undamaged tissue from both the inoculated internode and the one above, than their respective parent cultivars (Table 5). In these internodes, statistically significant differences were detected between the mean percent section colonised in NCo376 and N41 lines. PNG40 was not recovered from tissue from the uninoculated controls.

To establish the impact of the EMS treatment on traits that contributed to *E. saccharina* resistance, stalk rind hardness, fibre and nitrogen contents were analysed in the mutants and compared with the parent cultivars. No significant difference in these parameters were recorded between mutants of both NCo376 and N41 and their respective parents (Table 6). Further, a comparison of the mean rind hardness ($P=0.506$) and fibre content ($P=$

Table 5 Re-isolation of *F. sacchari* PNG40 from NCo376 and N41 mutants. Re-isolation was carried out from inoculated internodes and the one above 6 weeks after inoculation with the fungus

Cultivar	Line	Stalk sections colonised (%)		
		Discoloured tissue	Undamaged tissue (not discoloured)	
			Inoculated internode	Internode above
NCo376	NCo376	100	66.7	20
	MutA	100	75	50
	MutB	100	66.7	40
	MutC	100	75	33.3
	MutD	100	80	0
	MutE	100	50	25
Mean*		100	68.9±4.34[a]	28.05±7.10[A]
N41	N41	100	66	60
	Mut5	100	83.3	66.7
	Mut8	100	100	75
	Mut16	100	25	25
	Mut23	100	75	75
	Mut27	100	60	40
Mean*		100	68.2±10.36[a]	56.95±8.29[B]

* Different superscript letters indicate significant differences ($P \leq 0.05$), Students t-test; Mean±SE

0.693) of NCo376 and N41 lines did not reveal any significant differences. However, the mean nitrogen content in N41 lines was significantly lower than that of the NCo376 ones ($P<0.001$).

Table 6 Sugarcane stalk rind hardness, fibre and nitrogen content of 8 months-old NCo376 and N41 mutants. Internodes 5–7 of the stalks were used in the analyses

Cultivar	Line	Rind harness*	Fibre (%)*	Nitrogen (%)*
NCo376	NCo376	28.50±0.60	9.96±0.76	1.12±0.24
	MutA	29.63±1.29	10.41±1.33	1.13±0.64
	MutB	27.47±0.83	8.33±1.23	0.92±0.05
	MutC	30.07±0.91	9.22±1.50	0.83±0.22
	MutD	26.13±0.79	10.93±0.82	1.02±0.21
	MutE	27.50±1.25	9.01±0.33	1.10±0.06
Grand mean**		28.22±0.60[a]	9.64±0.39[a]	1.02±0.05[a]
N41	N41	30.00±0.60	10.54±1.68	0.57±0.12
	Mut1	28.17±2.32	10.01±0.84	0.84±0.11
	Mut8	26.90±0.92	7.29±2.52	0.67±0.09
	Mut16	28.87±0.70	8.47±0.53	0.69±0.19
	Mut23	28.43±1.45	12.14±1.33	0.69±0.12
	Mut27	30.17±1.30	11.47±2.30	0.72±0.29
Grand mean**		28.76±0.50[a]	9.99±0.75[a]	0.70±0.04[b]

Different superscript letters indicate significant differences ($P \leq 0.05$)

*No significant differences detected within amongst lines of each cultivar; ANOVA and Sidak tests; Mean±SE, $n=5$

**Mean rind hardness, fibre and nitrogen contents of the NCo376 and N41 lines compared with Student's t test ($P \leq 0.05$)

Discussion

The phytotoxicity of some entomopathogenic *Fusarium* spp. constrains their use in biological control of insect pests. For example, in a greenhouse experiment, Wenda-Piesik et al. (2009) inoculated wheat plants with five different *Fusarium* spp. and demonstrated mycosis of stem sawfly larvae by these fungi. However, those strains cause Fusarium crown rot and Fusarium head blight in wheat thereby precluding their utility in control of the insect. However, the present study describes an approach to modify a plant – pathogen interaction into a plant – endophyte association which benefits plant health through symptomless fungal colonisation and fungus-mediated defence against insects. Mutation induction in sugarcane cells by in vitro mutagenesis using EMS and screening using CF and the fungus conferred tolerance to *F. sacchari* PNG40 and improved endophytic colonisation by the fungus in some selected mutant lines.

Genetically modifying the microorganism may also amend a plant – pathogen interaction to achieve endophytic colonisation by the microorganism. For example, pathogenicity genes in the plant pathogens *Ustilago maydis* (Bolker et al. 1995), *Alternaria alternata* (Akamatsu et al. 1997) and *Colletotrichum magna* (Redman et al. 1999) were disrupted using transformation techniques to produce non-pathogenic strains. Freeman and Rodriguez (1993) exposed *C. magna*, a pathogen of cucurbit plants, to UV irradiation and obtained non-pathogenic mutants that endophytically colonised watermelon and cucumber seedlings. However, genetically altering the host plant can also modify the plant – pathogen association into a mutualistic plant – fungus relationship. In the present study, the *F. sacchari* tolerant mutants displayed less Fusarium stem rot than their respective parent cultivars and re-isolation of PNG40 from undamaged tissue confirmed the ability of the mutants to support endophytic colonisation by the fungus. It is likely that plant genotype can influence endophytic colonisation. For example, Bailey et al. (2005) assessed fungal endophyte diversity in different genotypes of cottonwoods (*Populus* spp.) and established that plant genetic variation and phytochemistry, e.g. tannin concentration, have an impact on endophytes that colonise the plant. In the present study, higher levels of endophytic colonisation were observed in the N41 cultivar than in NCo376. This is corroborated by indications from previous studies that N41 is amenable to endophytic colonisation by *Fusarium* spp. that eventually cause Pokkah boeng in sugarcane (Govender et al. 2010). However, observations in the present study indicate that the symptomless colonisation was enhanced in some mutants of N41. Further, wide variations in colonisation were recorded amongst mutants of each cultivar. This, therefore, indicates that genetic improvements can be undertaken to develop plant genotypes amenable to endophytic colonisation, which can then be utilised in endophytic biological control strategies. It shows that, from a population of mutants, it is possible to select plant genotypes that have developed mechanisms to counter fungal pathogenicity to achieve a host – microorganism equilibrium where endophytism occurs and in which the plant still benefits from fungal insecticidal activity.

The genetic differences observed between the mutants and parent cultivars detected with RAPDs are the result of a random mutagenesis approach in which non-target traits could have been altered. Analyses of stalk traits known to be involved in *E. saccharina* resistance, i.e. rind hardness, fibre and nitrogen content, were not different from those recorded in the parent cultivars. The retrieval of the fungus from undamaged tissue in the internode above the inoculated one also indicated the ability of the fungus to spread across internodes. This will be important in extending PNG40 insecticidal activity against *E. saccharina* throughout the entire stalk.

The strategy of manipulating plant – endophyte relationships in insect control is well recognised (Azevedo et al. 2000). Symptomless colonisation of the tolerant NCo376 and N41 mutant plants by PNG40 limited stem rot whilst the fungus negatively affected *E. saccharina*. The inoculation of stalks with PNG40 reduced damage by the borer in the NCo376 and N41 mutants and their parent cultivars. The fungus also caused less weight gain in larvae from some of the mutants and the N41 parent cultivar than those from their uninoculated controls. It is well documented that *Fusarium* spp. produce a range of toxins and some of these have insecticidal properties which include trichothecenes, zearalenone (Teetor-Barsch and Roberts 1983), beauvericin and fusaproliferin (Logrieco et al. 1996). Guo et al. (2014) reported the negative effects of *F. culmorum* on larvae of the mealworm *Tenebrio molitor* L in in vitro assays and detected insecticidal enniatins and beauvericin in extracts from the fungus. Furthermore, liquid and gas chromatography-linked mass spectrometry analyses of extracts from sugarcane stalk tissue infected with

Fusarium spp. showed that PNG40 and *F. pseudonygamai* ZN12, another *E. saccharina*-antagonistic strain, produced beauvericin whilst the *E. saccharina*-beneficial strain *F. pseudonygamai* SC17 did not produce the toxin (McFarlane unpublished data). In the current study, production of these insecticidal metabolites by PNG40 may have negatively affected *E. saccharina* fitness and led to reduced stalk damage. It is, therefore, possible that inoculation of sugarcane cuttings or seedlings with PNG40 may provide stalks with protection against the borer. Whilst, burning of stalks at harvest and extensive processing of cane juice, which includes heating, may eliminate mycotoxins that may be harmful to animal and human health, it is possible that reduced stalk rot in *Fusarium*-tolerant mutants will result in lower levels of fungal mycotoxins, e.g. fumonisins, possibly due to the ability to detoxify these compounds developed in the mutant genotypes. However, further studies need to be conducted to investigate any association between reduced stalk rot and *Fusarium* mycotoxin levels in the mutants.

As the N41 cultivar is known to be more resistant to *E. saccharina* than NCo376 (Keeping and Rutherford 2004), it was not surprising to find that the stalks of the former exhibited less borer damage and larval mass gain than the latter. Also, out of the five mutants from each cultivar, stalks of four N41 (Mut8, Mut16, Mut23, Mut27) mutants inoculated with PNG40 displayed significantly less stalk length bored than their controls compared with only one mutant of NCo376 (MutA). It is proposed that this was a combination of the insecticidal activity of PNG40 and the innate *E. saccharina* resistance mechanisms of each cultivar. The lower levels of nitrogen in the N41 than NCo376 lines may contribute to resistance as the former offers low nutrition to the borer. In addition, endophytic colonisation of plant tissue by *Fusarium* spp. may trigger elevation of endogenous levels of jasmonic acid, thus enhancing anti-herbivory response in the host (Navarro-Meléndez and Heil 2014). It is, therefore, possible that endophytic colonisation of NCo376 and N41 stalks by PNG40 may have activated host anti-herbivory metabolic pathways and augmented plant defences against *E. saccharina*. As the mutagenic treatment did not result in significant changes in rind hardness, fibre and nitrogen contents in the mutants compared with their parents, it is likely that the contribution of these traits to *E. saccharina* resistance was unaffected in the mutants.

MutA of NCo376, and Mut5 and Mut23 of N41 were identified for use in future studies because: 1) *E. saccharina* damage and larval masses were less in the inoculated stalks of these lines than their uninoculated controls; and 2) they displayed significantly less stalk tissue discolouration due to *Fusarium* infection than their respective parent cultivars; they also showed high levels of symptomless colonisation in the inoculated internode and that above it. Future work will focus on validating tolerance to *Fusarium* and efficacy of inoculated PNG40 against *E. saccharina* under field conditions. This will lead to application of these plant – fungus – insect associations as part of much needed integrated control approach for *E. saccharina*.

Acknowledgments We acknowledge and thank Nelson Muthusamy for the technical assistance, Nikki Sewpersad for advice on statistical analyses, Malcolm Keeping advice on *E. saccharina* inoculation, and the South African Sugarcane Research Institute, University of KwaZulu-Natal and National Research Foundation of South Africa (Grants 85573 and 85414) for funding.

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Chapter 6

Overview discussion and future prospects

6 Overview discussion and future prospects

Eldana saccharina is the single most important production constraint in the SA sugar industry and is extending to new areas previously thought to have unfavourable conditions for borer survival and development (Conlong, 2001; Assefa *et al.,* 2008; Kleynhans *et al.,* 2014). Larvae enter sugarcane stalks via nodes or cracks and feed on inner tissue (Dick, 1945; Leslie, 1993), thereby providing opportunistic *Fusarium* spp., which cannot gain entry into the stalk unaided, access to inflict Fusarium stem rot (Bourne, 1961; McFarlane *et al*., 2009). Consequently, revenue is lost due to the reductions in biomass and sucrose (Baker, 2014). An integrated pest management (IPM) approach has been implemented in the SA sugar industry to combat the pest (Conlong and Rutherford, 2009). These strategies involve early harvest, destruction of infested crops (Webster et al., 2005), application of insecticides, e.g. α-cypermethrin (Leslie, 2006), and use of resistant cultivars (Keeping, 2006). Resistance to the borer is the best measure to control *E. saccharina* (Keeping and Rutherford, 2004). However, additional strategies, such as biological control, and improvements to current approaches, are necessary for sustained management of the pest.

Understanding the underlying mechanisms for *E. saccharina* resistance is important for improved screening and selection of borer-resistant genotypes (Keeping and Rutherford, 2004). Seven sugarcane cultivars, with varying *E. saccharina* resistance ratings based on pot trial data (Keeping, 2006), were used in the current study to test the impact of stalk rind hardness, fibre and nitrogen content on resistance to the borer (Chapter 3). Susceptible cultivars generally exhibited low rind hardness and fibre content and high nitrogen content whilst resistant genotypes displayed hard rinds, high fibre and low nitrogen content (Mahlanza *et al.,* in press; Chapter 3). However, by-passing the rind at inoculation with *E. saccharina* larvae showed that these stalk characteristics may contribute to borer survival, damage and growth to variable extents in the different cultivars. The stalk rind is a physical barrier that impedes larval entry into the stalk whilst plant tissue high in fibre has low nutritional quality for borers (Kvedaras *et al.*, 2007). Nitrogen is a major component of numerous plant metabolites involved in antiherbivory defences (Mattson, 1980; Mithofer and Boland, 2012; Furstenberg-Hagg *et al.*, 2013; Rutherford, 2014) and the amount of free nitrogen in plant tissues is a major determinant to the nutritional value of the plant to the insect (Awmack and Leather 2002; Throop and Lerdau, 2004). Hence, the net effect of rind hardness, fibre and nitrogen on *E. saccharina* establishes the quality of a genotype as a host for borer thereby determining resistance to the insect. This proposal is supported by the findings that borer resistant sugarcane genotypes displayed hard rinds, high fibre and low nitrogen, stalk characteristics that constitute a poor quality host (Mahlanza *et al.,* in press; Chapter 3). However, hard rinds

and high fibre are undesirable as they complicate cane cutting and sucrose recovery (Singh *et al.*, 2013a). Screening for resistance to the sugarcane borer *D. saccharalis* in the USA (White *et al.,* 2006) and for *E. saccharina* resistance in South Africa (Zhou, 2013), result in inadvertent selection of sugarcane genotypes with high fibre resulting in the release of low sugar yielding cultivars. Developing genotypes with alternative forms of resistance will negate this relationship between stalk borers resistance and low sugar yields thereby allowing selection of the low fibre-high sucrose genotypes that are usually discarded due to borer susceptibility during the breeding programme.

Plant nitrogen content is major factor in determining host quality for insects (Throop and Lerdau, 2004). Unlike plants which use carbohydrates, animals use proteins as structural building-blocks and are less efficient nitrogen users, excreting significant amounts of the element in their waste (Mattson, 1980). Hence, nitrogen is a limiting factor for herbivores which need to source nitrogen to meet their physiological demands and compensate for their low nitrogen-use efficiency. In plants, the allocation of assimilated nitrogen towards plant defences or other physiological processes, e.g. growth, ascertains host quality (Cronin and Hay, 1996; Throop and Lerdau, 2004). For instance, nitrogen-based defence allelochemicals such as alkaloids, terpenoids and cyanogenic glucosides produced by plants antagonise herbivores (Mithofer and Boland, 2012), whilst soluble amino acids and enzymes such as ribulose bisphosphate carboxylase, which are easy for insects to extract and digest, enhance herbivore nutrition (Bernays and Chapman, 1994). Further, nitrate accumulation in plant tissues may cause toxicity to insects (Mattson, 1980). This allocation of plant nitrogen can vary amongst genotypes (De Jong and Van Der Meijden, 2000). Hence, the variable nitrogen-use efficiencies amongst sugarcane genotypes (Robinson *et al.,* 2007; Weigel *et al.,* 2010) may impact host quality. For instance, in the current study, larvae recovered from the low fibre, nitrogen-rich immature internodes of the tested cultivars gained more weight with the upper stalk parts exhibiting greater damage, than the mature sections which recorded low nitrogen. However, larvae retrieved from the immature parts of resistant cultivars N33 and N29 gained less mass, with N33 exhibiting less damage, than the mature stalk parts. Hence, it may be hypothesised that certain genotypes, such as N29 and N33, have the genetic potential to utilise supplied nitrogen in growth processes and metabolism of nitrogen-based anti-herbivory compounds and less nitrogen towards insect nutrition, thus resulting in a poor quality host for the herbivore which leads to resistance. It was also observed that comparisons of *E. saccharina* damage, growth and survival in immature internodes amongst the tested cultivars did not correspond to those recorded in mature ones (Chapter 3). This indicates that genotypes may display borer resistance at different ages depending on the impact of morphological and physiological changes which occur during maturity on host quality.

Genotypes that express resistance at the immature stage may be attractive as they will negatively affect borer populations which will benefit the mature crop. Also, Fusarium stem rot caused by infection by SC17 and PNG40 was less in the immature internodes compared with the mature ones. As nitrogen is essential to synthesis of antimicrobial metabolites in the plant (Rutherford, 2014), the lower levels of infection by SC17 and PNG40 recorded in the immature internodes compared with the mature ones (Chapter 3), possibly indicate high antimicrobial activity in the nitrogen-rich young tissues. Also, higher infection levels by borer-beneficial fusaria in mature internodes than immature parts, as recorded in current study, may contribute to higher *E. saccharina* damage observed in lower stalk parts than the upper ones (Mazodze *et al.,* 2003).

This relationship between genotype and the contribution of nitrogen to host plant quality may offer insights into conflicting reports on the role of nitrogen fertilisers in *E. saccharina* damage (Meyer and Keeping, 2005; Rhodes *et al.,* 2013). Increased sugarcane susceptibility to the borer during water stress, especially after fertiliser application (Keeping *et al.,* 2012), may be a consequence of enhanced host quality arising from insect nutrition profiting from nitrogen unutilised due to retarded growth and compromised biochemical defences. A proposal is to establish the concentration of the major forms of available nitrogen, e.g. amino acids, enzymes and nitrate in *E. saccharina*-susceptible and -resistant genotypes. Further, metabolic profiles of *E. saccharina*-resistant and -susceptible genotypes can be elucidated through chromatography and spectrometry techniques to detect anti-herbivory metabolites. For example, Brennan *et al*. (1992) used gas chromatography to compare the metabolomes of blackcurrant genotypes resistant and susceptible to the gall mite (*Cecidophyopsis ribis* Westw.) and established a correlation between resistance and mono- and sesquiterpenes, compounds known to have anti-nutritional activity against insects (Asakawa *et al.,* 1980; Perry *et al.,* 2008). Also, using nuclear magnetic resonance spectroscopy, Leiss et al. (2009) established that ragwort genotypes resistant to western flower thrips (*Frankliniella occidentalis* Pergande) produced higher levels of alkaloids and flavonoids than the susceptible ones. Establishing the underlying genetics of those induced physiological defences may yield molecular markers for borer resistance which may be used in selection of resistant genotypes. For instance, combined metabolomics and gene expression studies by Liu et al. (2009) in rice genotypes resistant and susceptible to the brown planthopper (*Nilaparvata lugens* Stal) identified secondary metabolites and genes associated with resistance to the insect. Also, Brennan *et al*. (2009) developed a PCR-based marker that was associated with resistance to gall mite in blackcurrant. Once *E. saccharina* resistance markers are established and used for early screening, only lines containing the markers can then progress to *E. saccharina* resistance trials.

Previous studies in sugarcane indicated that *Fusarium* spp. may be beneficial or antagonistic to *E. saccharina in vitro* (McFarlane *et al.,* 2009; Govender *et al.,* 2010). The present study demonstrated that such *Fusarium* strains influence borer damage, growth and survival *in vivo* (Chapters 3). *Eldana saccharina* susceptible cultivars NCo376 and resistant N41 displayed less insect damage when colonised by the borer antagonistic strain PNG40 than the controls (uninoculated stalks), whilst NCo376 stalks infected by the beneficial strain SC17 exhibited more damage (Chapter 3). This corroborated the proposal that *Fusarium* strains impact *E. saccharina* resistance negatively or positively, depending on the strain (borer-beneficial or -antagonistic) colonising the stalk. The pathogenicity of *Fusarium* spp. to insect pests has been reported in many plant species (Majumdar *et al.*, 2008; Mikunthan and Manjunatha 2008; Wenda-Piesik *et al.*, 2009; Batta 2012; Guo *et al.*, 2014), with the beneficial effect of *Fusarium* spp. on *E. saccharina* damage and fecundity being reported in maize (Schulthess *et al.*, 2002; Ako *et al.*, 2003). As *Fusarium* spp. are ubiquitous, the implications of findings from those studies, and the current one, should be considered in *E. saccharina* management strategies. It is possible that susceptible genotypes may exhibit increased resistance if colonised by an *E. saccharina*-antagonistic *Fusarium* strain whilst a borer-resistant genotype may appear more susceptible if infected by a strain beneficial to the insect. Hence, measures to eliminate *Fusarium* spp., e.g. fungicide treatments and use of *Fusarium* resistant cultivars, should be part of *E. saccharina* control approaches. Infection of plants by *Fusarium* spp. should be controlled during *E. saccharina* screening trials to avoid susceptible genotypes infected by borer-antagonistic strains being selected as resistant. Fungicide treatments may also be applied in *E. saccharina* resistance screening pot trials to eliminate *Fusarium* spp. for a more judicious assessment of genotype resistance.

The present study demonstrated the negative *in vivo* effect of *F. sacchari* PNG40 against *E. saccharina*, thus establishing the potential of the fungus in controlling the insect (Chapters 3 and 5). The fungus caused a reduction in length bored in stalks of NCo376 and N41 and their mutants (Chapter 5). Most studies report toxicity of *Fusarium* spp. against insects in *in vitro* bioassays (Varma and Tandan, 1996; Ganassi *et al.,* 2000; Majumdar *et al.,* 2008; McFarlane *et al.,* 2009; Batta, 2012; Guo *et al.,* 2014). However, the present investigation describes the negative impact of *F. sacchari* on *E. saccharina in vivo* and the consequent reduction in insect damage in sugarcane stalks. This is a major step towards implementing *Fusarium*-mediated insect control, as it proves the ability of the fungus to prevent *E. saccharina* damage in sugarcane stalks. This harmful effect of PNG40 on the borer may have been due to elevation of JA levels in tissues as a result of colonisation by endophytic *F. sacchari* (Navarro-Meléndez and Heil, 2014), acting in concert with possible production of the prominent insecticidal toxins beauvericin (Gupta *et al.,* 1991) fusaproliferin (Logrieco *et al.,* 1996) and enniatins (Guo *et al.,*

2014) by the fungus. The accumulation of JA and its intermediates activates expression of defence genes responsible for synthesis of proteinase inhibitors, anti-nutritional compounds, repair proteins and signalling molecules which amplify the defence response in the plant (Leon *et al.,* 2001). Whilst some entomopathogenic *Fusarium* strains that have potential in biological control are endophytic, some cause disease in plants (Majumdar et al., 2008; McFarlane *et al.,* 2009; Wenda-Piesik *et al.,* 2009). The latter is the case with *F. sacchari* PNG40, which despite its toxicity to *E. saccharina,* causes stem rot in sugarcane, subsequently hindering its utility in biological control of *E. saccharina*. However, in the current study, the production of *Fusarium*-tolerant genotypes was employed to overcome this impediment (Chapter 5). Unlike resistance which inhibits fungal growth, tolerance permits symptomless endophytic colonisation of plant tissue (Roy and Kirchner, 2000). This is an attractive remedial strategy for plant-entomopathogen-insect interactions in which the fungus is also a phytopathogen as endophytic colonisation alleviates disease and maintains the fungus in the plant to act against the insect.

The *Fusarium*-tolerant mutants produced in this study exhibited less Fusarium stem rot and showed endophytic colonisation in the inoculated internode and the one above it (Chapter 5). This supports the hypothesis that endophytism is the net effect of a balanced antagonism between plant resistance mechanisms and fungal pathogenicity (Schultz *et al.,* 1999). Hence, enhancing plant defences or diminishing pathogen virulence factors in a plant-pathogen relationship may achieve equilibrium in this antagonism, thereby circumventing disease. In some studies (Freeman and Rodriguez, 1993; Bolker *et al.,* 1995; Akamatsu *et al.,* 1997; Redman *et al.,* 1999), the fungal pathogen was genetically altered to weaken pathogenicity, thus achieving the endophytic equilibrium. In contrast, the present study illustrated an approach in which the plant is genetically altered via mutagenesis to achieve endophytism, i.e. disease tolerance. The mutagenic treatment employed in this study may have elicited random mutation events which enhanced plant defences against *F. sacchari* possibly through stimulation of constitutive expression of pathogenesis related genes (Duggal *et al.,* 2000; Zhang *et al.,* 2003), disruption of suppressor genes inhibiting resistance ones (Kwon *et al.,* 2004), and inactivation of fungal effector targets (Berestetskiy, 2008). This approach presents an appealing alternative to genetically altering the fungal pathogen as this may also protect the plant against new pathogenic strains. *Fusarium* spp. have also been shown to exhibit *in vitro* toxicity to *Chilo* and *Sesamia* spp. (Varma and Tandan, 1996), stem borers that pose a threat to production of the crop (Way *et al.,* 2012; Nikpay *et al.,* 2014) and may also offer defence against these pests.

It has previously been inferred, from observations of variable *E. saccharina* resistance in N41 stalks from the field, that the cultivar was readily colonised by different endophytic *Fusarium*

strains (Rutherford, pers. comm.). Observations in the current study corroborated this proposal as N41 and its mutants experienced higher levels of endophytic colonisation than NCo376 and its mutants. Furthermore, some N41 mutants expressed higher levels of colonisation than their parent (Chapter 5). This supported the hypothesis that endophytic colonisation may be genotype-dependant and breeding and selection of genotypes amenable to endophytic colonisation may be possible (Bailey *et al.,* 2005; Rutherford, 2014). In addition, identification of more *E. saccharina*-antagonistic *Fusarium* isolates, and assessing the receptiveness of different cultivars to endophytic colonisation to these strains through inoculation studies, may reveal cultivar-isolate relationships that are most effective for biological control purposes. As Fusarium stem rot results in sucrose loss and consequent lower sugar yields (Way and Goebel, 2003), it will be beneficial to test the impact of endophytic colonisation on sucrose content in the *Fusarium*-tolerant mutants. Indeed the mutant genotypes produced in this study may constitute genetic resources to introgress tolerance to *Fusarium* into commercial sugarcane varieties. Differential gene expression in the tolerant mutants and parent cultivars can be assessed using suppression subtractive hybridization (Legay *et al.,* 2011) or RNA seq (Ramskold *et al.,* 2012) to establish genes involved in defence against *Fusarium*. These genes may be used as markers for *Fusarium* tolerance. Future studies to assess Fusarium stem rot, endophytism and anti-herbivory activity of the fungus in the tolerant mutants under field conditions are necessary in order to advance towards implementation of biological control against the insect using endophytic *Fusarium*.

This study illustrated a novel technique for assessing borer resistance that may complement or even replace the *E. saccharina* resistance screening method currently used in the South African sugarcane breeding programme (Mahlanza *et al.,* 2014). The current practice entails growing plants in 25 L pots for 7-8 months in a shade house and inoculating with eggs at the base of the stalk (Keeping, 2006). This process is a laborious, time and space consuming exercise, resulting in restriction of the number of clones that can be screened. However, the method developed in the present study may offer improvements to the current one. Stalks from the field were marcotted in the glasshouse thereby producing plants that are ready for *E. saccharina* inoculation within 5 weeks compared to 7-8 months using the current method. The 6 L metal cylindrical canisters used offer an efficient use of limited space and labour compared with 25 L pots used in the standard bioassays. Inoculation of plants with larvae versus eggs lessens the time between inoculation and larval penetration of the stalk thus curtailing predation of eggs by ants and exposure to other mortality factors which affect hatching efficiency. Whilst rind hardness may still contribute to borer resistance, by-passing the rind at inoculation may place greater weight on inducible physiological mechanisms in assessing

resistance than the current method. Rind-based resistance can then provide auxiliary defence in the selected genotypes during cultivation in the field.

The *Fusarium*-tolerant mutants were produced via *in vitro* mutagenesis using ethyl methanesulphonate (EMS) and selection with fungal culture filtrates (CF) incorporated into culture media followed by inoculation of regenerated plants with the fungus (Mahlanza *et al.,* 2013; Chapter 4). This illustrated the effectiveness of this strategy in development of disease-resistant sugarcane genotypes, and possibly of tolerance to other biotic and abiotic stresses. The approach avoids some complications created by the complex sugarcane genome and problematic reproductive biology relied on by conventional breeding practices. These include poor pollen viability, seed sterility, unsynchronised flowering and polyploidy that results in crosses producing large numbers of clones which are highly variable in a range of characteristics and require a lengthy selection procedure (James, 2004). Whilst the role of conventional breeding practices is central in sugarcane improvement, *in vitro* mutation breeding can play a complementary part. Breeding thrives on creation of variation in plant populations from which desired traits can be selected (Acquaah, 2007). Variation generated by natural mutations and in segregating populations during conventional breeding approaches, is low and limits crop genetic improvement (Acquaah, 2007). However, higher somaclonal variation and induced mutation frequencies occurring in *in vitro* plant cultures present an alternative source of variation (Patade *et al.,* 2008; Rutherford *et al.,* 2014). As demonstrated in the current study, somaclonal variation, enhanced via induced mutagenesis using EMS, can generate variation from which desired traits may be selected. This may also yield traits that are not available in the gene pool (Van Harten, 1998). In the current study, plants from EMS treatments displayed greater variation in root length than those from non-EMS treatments (Mahlanza *et al.,* 2013; Chapter 4). In addition, polymorphisms were detected in *Fusarium*-tolerant mutants using RAPD markers indicating the ability of EMS to induce mutations in sugarcane cells (Chapter 5). Single base pair changes in genes may modify or disrupt their function resulting in expression of a desired trait (Kwon *et al.,* 2004). For example, more plants with improved root length from the EMS treatments than the non-treated ones were obtained in the present investigation, thus indicating that exposure of sugarcane cells to EMS possibly induced mutations which enhanced defence against *Fusarium* toxins (Mahlanza *et al.,* 2013; Chapter 4).

In vitro selection of large numbers of lines using appropriate selection agents can be employed under controlled screening conditions and limited space and time (Van den Bulk, 1991; Clemente and Cadenas, 2012). Plants selected *in vitro* using *Fusarium* CF exhibited tolerance or resistance when inoculated with the fungus in the glasshouse (Mahlanza *et al.,* 2013; Chapter 4; Chapter 5), thus showing the suitability of CF as a selection agent as they contain

fungal toxins involved in pathogenesis (Daub, 1986; Van den Bulk 1991; Chandra *et al.,* 2010). *Fusarium* spp. are known to produce phytotoxins such as fusaric acid (Bacon et al., 1996), trichothecenes (Desjardins and Hohn, 1997), moniliformin (Marasas *et al.,* 2000) and fumonisins (Munkvold and Desjardins, 1997; Nishiuchi, 2013). In the present investigation, toxicity of PNG40 CF was displayed by callus necrosis, plantlet yield decline and inhibition of root growth (Mahlanza *et al.,* 2013; Chapter 4). Re-isolation of the fungus was conducted to confirm Koch's postulates (Parry, 1990), i.e. confirming that the observed symptoms were caused by the inoculated fungal strain. Whilst most studies use morphological features to confirm the identity of the retrieved isolates (Swart *et al.,* 1999; Chen and Swart, 2002; Tahmatsidou *et al.,* 2006), the current investigation used ISSR markers, which widely used to detected variation amongst *Fusarium* strains (Mishra *et al.,* 2006; Gurjar *et al.,* 2009; McFarlane, *et al.,* 2009; Baysal et al., 2010; Dinolfo et al., 2010; Vitale et al., 2011), for a more accurate approach.

Indeed variation generated by *in vitro* mutagenesis and stringent screening and selection strategies may be harnessed to develop sugarcane genotypes expressing tolerance to herbicides, salinity, drought, heat and diseases (Rutherford *et al.,* 2014). Although targeted mutagenesis techniques such as zinc finger nucleases, transcription factor-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPRs) are emerging as tools for more precise mutation induction, knowledge of the genetic mechanisms responsible for the desired trait is a prerequisite to their utility (Gaj *et al.,* 2013; Chen and Gao, 2014; Fichtner *et al.*, 2014). However, as illustrated in this study, random mutagenesis offers the flexibility of mutating genes without prior knowledge of the genetic mechanisms involved. Nevertheless, disruption of important traits by non-target mutations is of concern in random mutagenesis (Van Harten, 1998). Exposing a large number of cells to the mutagen enhances chances of obtaining a desired mutation event that is accompanied with minimal lethal effects. The exposure of embryogenic callus cells to a mutagen as conducted in the current investigation is, therefore, advantageous over treating seeds or tissue explants as more regeneratable somatic cells are treated. As commercialisation of transgenic sugarcane continues to be hampered by technological, regulatory and marketing challenges (Burnquist, 2006; Meyer and Snyman, 2013; Birch, 2014), the present study demonstrated the utility of *in vitro* mutagenesis and selection approaches as successful tools for sugarcane genetic improvement.

In conclusion, the findings from the present study showed that, high rind hardness and fibre and low nitrogen content are associated with resistance to *E. saccharina*. The extent to which each of these stalk characteristics contribute to host quality unfavourable to *E. saccharina* varies between different genotypes. The association between lower nitrogen content and

resistance to the borer gave some insight into possible roles of this element in resistance and potential strategies to improve *E. saccharina* resistance screening for selection of high sucrose yielding *E. saccharina*-resistant genotypes. The beneficial and antagonistic effects of *Fusarium* spp. on *E. saccharina* damage was also demonstrated *in vivo*, thus indicating the possible influence of these fungi on borer damage and underlining the importance of controlling *Fusarium* spp. in *E. saccharina* management strategies. A protocol was established for production of *Fusarium*-tolerant sugarcane mutants using *in vitro* mutagenesis via exposure of embryogenic calli to EMS and selection with fungal culture filtrate at the embryo maturation, germination and plantlet stages. This provided evidence for the applicability of this approach in sugarcane genetic improvement. Lastly, the toxicity of *F. sacchari* PNG40 to *E. saccharina* and reduced Fusarium stem rot was demonstrated in *Fusarium*-tolerant mutants of NCo376 and N41. This presented a strategy to modify plant-pathogen interactions into mutually beneficial plant-endophyte associations for disease management and biological control purposes. MutA of NCo376, and Mut5 and Mut23 of N41 were selected (Chapter 5) for further studies which will include field experiments and molecular characterisation of the observed tolerance to *Fusarium*. This will motivate for the utilisation of *Fusarium*-sugarcane endophytic interactions in integrated management approaches for *E. saccharina*.

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