

The establishment of *in vitro* screening methods for evaluating the susceptibility of sugarcane (*Saccharum* spp. hybrids) to the fungal disease, smut (causal agent: *Ustilago scitaminea* H. and P. Sydow) and the stalk borer, *Eldana saccharina* Walker (Lepidoptera: Pyralidae).

by

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ABSTRACT

The fungal disease smut (causal agent: *Ustilago scitaminea* H. & P. Sydow) and stalk borer *Eldana saccharina* Walker place major constraints on sugarcane agriculture in South Africa. The best approach for management is the introduction of resistant cultivars; however, conventional field-based screening for pest and disease resistance is a lengthy process. This study evaluated *in vitro* techniques combined with artificial inoculation of 12 week old *in vitro* plantlets and 8-10 week old embryogenic calli as rapid screening methods. Preliminary investigations were conducted on cultivars with known field ratings to smut and *E. saccharina*: NCo376, N26 and N39; and 5 'test' cultivars, whose identities were undisclosed until completion of experiments, were used to assess the accuracy of protocols. Infective *U. scitaminea* sporidia generated from teliospores, were used as inocula. Development of a callus protocol was unsuccessful due to sporidial and mycelial overgrowth, despite addition of a contact fungicide, Dithane M-45[®] (0.025 g/l) and a biocide/fungicide, PPM[™] (5 ml/l), to media. Plantlet inoculation by injection, 1 cm above the apical meristem, resulted in 12% and 20% of smut susceptible NCo376 plantlets producing smut whips after 5 weeks when inoculated with 1×10^6 and 1×10^9 sporidia/ml, respectively. Smut whip production in 5 of the 8 (63%) cultivars inoculated with the lower sporidial concentration correlated with their field resistance ratings. In addition, whips harvested from *in vitro* plantlets were a valuable source of aseptic teliospores for future research. Ongoing work involves inoculation of NCo376 calli with such teliospores and maintenance on medium with PPM[™] - emergence of whips from plantlets remains to be assessed. The *E. saccharina* screening protocol involved surface decontamination of eggs with 1% sodium hypochlorite (NaOCl) for 15 min. Feeding bioassays were conducted by placement of first instar larvae on *in vitro* plantlets and calli for 3 and 2 weeks, respectively. Larval mass, length and percentage infestation were recorded. Although greater larval size was expected in susceptible compared with resistant cultivars, the results did not support this. Significant differences in plantlet infestation were observed between susceptible (94-98%) and resistant (72-86%) lines. No significant differences were found in the callus feeding bioassay. However, a 24 h callus choice bioassay which investigated larval preference between callus genotypes compared with NCo376, showed significant differences and correctly discerned cultivar susceptibility according to field ratings.

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PREFACE

The experimental work described in this dissertation was conducted at the Biotechnology Department of the South African Sugarcane Research Institute (SASRI), Mount Edgecombe, Durban, from January 2009 to December 2010, under the supervision of Dr. S. J. Snyman (SASRI and UKZN-Westville) and Mr. C. Hunter (UKZN-Pietermaritzburg).

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

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LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
AFLP	amplified fragment length polymorphism
BAP	benzyl-amino purine
Bt	<i>Bacillus thuringiensis</i>
EC1	embryogenic callus liquid medium
EC2	embryogenic callus semi-solid medium
EC3	embryogenic callus germination medium
GM	genetically modified
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
KH ₂ PO ₄	monopotassium phosphate
LPD&VCCs	Local Pest Disease and Varieties Control Committees
MC1	meristem culture charcoal medium
MC2	meristem culture liquid medium
MC3	meristem culture semi-solid medium
MgSO ₄ .7H ₂ O	magnesium sulphate
MS	Murashige and Skoog basal salts and vitamins
NAA	α -naphthalene acetic acid
NaOCl	sodium hypochlorite
PM	plantlet maintenance medium

PPM TM	Preservative for Plant Tissue Culture Media
RFLP	restriction fragment length polymorphism
REML	Restricted Maximum Likelihood
RM	rooting medium
RSD	Ratoon Stunting Disease
SADC	Southern African Development Community
SASRI	South African Sugarcane Research Institute
SCMV	Sugarcane mosaic virus
SCYLV	Sugarcane yellow leaf virus
SCYP	Sugarcane yellows phytoplasma
SM	shoot multiplication medium
v/v	volume per volume
YLS	yellow leaf syndrome

1. INTRODUCTION

Sugarcane (family: Poaceae; genus: *Saccharum* L.) is an important agricultural crop in South Africa and is grown in the eastern region of the country. The South African sugar industry prides itself in being one of the leading producers of good quality, cost competitive sugar. This industry is a significant contributor to the country's economy, plays a key role in rural development and provides employment to an estimated 77 000 individuals (Anon, 2009/2010). Despite growing sugarcane in relatively adverse conditions further south than any other sugarcane producing country (O'Reilly, 1998), the South African sugar industry ranks as the 12th sugarcane producer worldwide (<http://www.southafrica.co.za>; <http://www.fao.org>). The direct annual income of this industry is an estimated R7 billion from the production of approximately 2.3 million tons of sugar per season (Anon, 2009/2010).

Approximately half of the sugar produced in South Africa is exported, primarily to the middle and far East. In addition, South Africa's sugar industry is a significant contributor to the development and rehabilitation of 9 neighbouring countries that belong to the Southern African Development Community (SADC) (<http://www.southafrica.co.za>). In order to continue being a major economic contributor in South Africa, sustainability is essential. Further, productivity needs to be enhanced to meet the ever-increasing demand for sugar and its by-products, in southern Africa and abroad. Several factors can affect sugarcane yield and one such cause for concern is the impact of pests and diseases. These are difficult to control and cause severe sugarcane yield losses (Bailey, 1979; Carnegie, 1983, Rott *et al.*, 2000).

According to Bailey (1995), sugarcane is vulnerable to diseases due to several characteristics of its agriculture in South Africa. Firstly, sugarcane is vegetatively propagated by planting setts and therefore, systemic pests and diseases may be spread at planting (Rott *et al.*, 2000; Anon, 2005). Secondly, sugarcane is a perennial monoculture crop which is harvested after 12-18 months, allowing systemic pathogens to propagate and increase from one ratoon to the next. Thirdly, poor practice with respect to crop rotation in South Africa and inadequate periods between replanting, after older crops are removed, enables the spread of pathogens (Bailey, 1995). Finally, sugarcane variety improvement is limited by environmental conditions and the lengthy process of crossing and selection which requires 12-15 years to complete (Butterfield and Thomas, 1996). New cultivars cannot be produced readily to offset the challenges of pests and diseases. In addition, the adoption

of new cultivars is a slow process due to the high cost of replanting and only 10% of a farm being replanted each year (Bailey, 1995; Parfitt, 2005).

Much research has been conducted into the management of pests and diseases by the South African Sugarcane Research Institute (SASRI) and although some strategies may alleviate the problem temporarily; pests and diseases persist, often reemerging under favourable conditions, causing significant yield loss (Carnegie, 1974; Flores and Osada, 1980; Bailey and B  chet, 1982; Bailey *et al.*, 1994; Bailey, 1995; Keeping 1995; Keeping, 2006). Due to the complexity of the sugar industry, it was necessary to establish the Local Pest Disease and Varieties Control Committees (LPD&VCCs) in 1982. These committees conduct regular pest and disease surveys and have regulations and guidelines to protect against serious threats to the industry (Thompson *et al.*, 1983; Anon, 2005).

One of the most important fungal diseases is sugarcane smut, caused by *Ustilago scitaminea* H. & P. Sydow. This disease was first detected in South Africa in 1877 and has since been observed worldwide (Bailey, 1979; Akalach, 1994; Riley *et al.*, 1999, Comstock, 2000). Sugarcane smut is characterized by the development of a smut whip from the apical region of infected sugarcane stalks. The smut whip is a modified shoot which contains black teliospores (Mohan Rao and Prakasam, 1956). Infected stalks tiller rapidly and appear grass-like compared with uninfected stalks (Sharma, 1956; Comstock, 2000). Yield losses associated with sugarcane smut may be as high as 75% in susceptible cultivars (Bailey, 1977; Rutherford *et al.*, 2003). Major outbreaks of smut in the past have cost the industry millions of rands (Bailey, 1979).

The major insect pest in South Africa is the stalk borer, *Eldana saccharina* Walker (Dick, 1945), a Lepidopteran (Pyralidae) moth indigenous to Africa. The larval stage of the pest's life cycle is damaging to sugarcane, as these bore into cane stalks to feed (Carnegie, 1974; Atkinson, 1980). Three major *E. saccharina* outbreaks have occurred in the past, however incidences continue to cause yield loss in South Africa. Annually, *E. saccharina* infestations cost the industry an estimated R50 million (Keeping, 1995).

Several control measures may be implemented to minimize potential sugarcane yield loss caused by pests and diseases but an integrated approach is often recommended. Good farming practices are

essential but do not guarantee eradication of infections. Chemical control is often expensive and has downstream negative effects on the environment; therefore it is not a method of choice (Webster *et al.*, 2005). Further, chemical control for stalk borers is ineffective due to the cryptic nature of the larvae. Biological control of *E. saccharina* has been investigated for several years but is difficult to implement in South Africa (Conlong, 1997a; Conlong, 1997b; Downing *et al.*, 2000). The large numbers of eggs produced by *E. saccharina* and their relatively short life-cycles (2-3 months) exacerbate infestations (Carnegie, 1974). The control of smut is difficult because fungal spores are often dispersed by wind across great distances and smut whips may release $1 \times 10^8 - 1 \times 10^9$ teliospores/day for approximately 3 months (Waller, 1969; Pedgley, 1982; Hoy and Grisham, 1988). The number of spores which are produced ensures a significant proportion will survive, infect and persist (Trione, 1980).

The planting of resistant cultivars is recommended as the best and most economical approach for controlling pests and diseases, having the least impact on the environment and increasing productivity without the need for other inputs, such as costly chemical applications or labour (Martin, 1973; O'Reilly, 1998). SASRI's Plant Breeding, Crossing and Selection Programme is tasked with the cross pollination of sugarcane cultivars with favourable characteristics and generating sugarcane interspecific hybrids with combinations of desirable traits (Butterfield and Thomas, 1996; Parfitt and Thomas, 2001). Conventional field-based screening for resistance to pests and diseases is a key component of the breeding programme prior to release of a commercial cultivar (O'Reilly, 1998; Anon, 2003a).

This programme is essential to the productivity of the South African sugar industry however output is poor as only 1 or 2 cultivars are released after 12-15 years of investigations. Screening for pest and disease resistance requires 3-5 years and is conducted after 5 years of selecting cultivars with other traits of interest, such as sucrose and fibre contents (Anon, 2003a). Unfortunately, a significant number of cultivars selected for favourable traits are discarded during screening for pest and disease resistance (Bailey and Béchet, 1982; Butterfield and Thomas, 1996). Therefore, a rapid technique to screen new cultivars for resistance to pests and diseases would be advantageous, particularly if it could be applied earlier in the selection process.

An important point to note is that sugarcane in a particular area may be vulnerable to many pathogens and insect pests. Breeding sugarcane that is resistant to multiple pests and diseases is

difficult due to the complex genome of sugarcane (Butterfield *et al.*, 2001; Butterfield, 2005). Kolobaev (1983) observed that a cultivar may exhibit resistance to one pathogen but is susceptible to several others. Following mapping of sugarcane molecular markers associated with smut and *E. saccharina*, Butterfield (2007) found a negative correlation between common markers, indicating that a cultivar resistant to *E. saccharina* is likely to be susceptible to smut and vice versa (Butterfield, 2007). Despite incorporating marker assisted breeding into the breeding programme at SASRI, thousands of new cultivars are produced and screening is time-consuming.

In vitro techniques could potentially be used for rapid screening and has been investigated in the present study. Many advances have been made in the field of *in vitro* plant tissue culture since the mid-1900s (Thorpe, 2007). Pioneer research in *in vitro* cell and tissue culture of sugarcane was published in 1969 and various methods have since been established (Barber and Nickell, 1969; Heinz and Mee, 1969; Barba *et al.*, 1977). The immature leaf roll of sugarcane is generally the explant used in sugarcane tissue culture (Barber and Nickell, 1969; Heinz and Mee, 1969; Barba *et al.*, 1977). Direct morphogenesis *in vitro* is achieved by meristem culture; or leaf roll disk culture which develops either via organogenesis into shoots or somatic embryogenesis before germinating into plantlets. Indirect morphogenesis is the production of undifferentiated callus cells which may be manipulated in culture by the application of exogenous growth regulators, via organogenesis or somatic embryo germination to produce sugarcane plantlets (Barber and Nickell, 1969; Snyman *et al.*, 1996; Snyman, 2001). Meristem culture has been shown to be beneficial in producing pathogen-free plants because the meristem contains embryogenic cells which do not contain pathogens (Rani and Raina, 2000; Fitch *et al.*, 2001; Ramgareeb *et al.*, 2010). In addition, cultured meristems are used for micropropagation as the resultant plantlets are genotypically and phenotypically stable (Lee, 1987; Hoy *et al.*, 2003).

1.1 Aim of this study

The aim of this study was to establish *in vitro* screening methods for evaluating the susceptibility of sugarcane cultivars to smut disease and *E. saccharina*. In order to accomplish this, protocol development for artificial inoculations of 2 types of plant material were investigated with first instar larvae of *E. saccharina* and different concentrations of *U. scitaminea* sporidial suspensions. The plant materials used were 12 week old plantlets derived from apical meristem culture and 8-10 week old embryogenic calli derived from immature leaf roll disk explants. The accuracy of methods

was assessed by evaluating several sugarcane cultivars (NCo376, N26 and N39) and 5 ‘test’ cultivars (A, B, C, D and E). NCo376 is susceptible to smut and *E. saccharina*, whilst N26 is susceptible to *E. saccharina*, resistant to smut; and N39 is resistant to *E. saccharina*, susceptible to smut. The identity of the ‘test’ cultivars and their associated field-resistance ratings for smut and *E. saccharina* were undisclosed until completion of the experiments. In addition, cultivar N27 and its genetically transformed counterpart (Bt40) which contained the *Bacillus thuringiensis* (Bt) toxin *cryIAb* gene (Snyman, pers. comm.¹) were evaluated for *E. saccharina* resistance.

For smut investigations, plantlet survival and whip production were recorded, and shoot germination was noted for callus experiments. Percentage infestation, larval mass and length were recorded for *E. saccharina* plantlet and callus investigations. In addition, a callus choice bioassay was investigated and callus genotype preference compared with NCo376 callus was noted for *E. saccharina*. The results obtained from the cultivars evaluated *in vitro* were compared with their field resistance ratings to smut and *E. saccharina* in order to determine the best *in vitro* screening methods.

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2. LITERATURE REVIEW

2.1 History of sugarcane breeding and the economic importance of the crop in South Africa

Sugarcane (*Saccharum* spp.) are large perennial grasses (Family: Poaceae) which are native to Asia and well suited to tropical and sub-tropical environments (Edgerton, 1955; Stevenson, 1965; Blume, 1985). It is a good agricultural crop for sugar production due to its favourable characteristics, such as high sucrose accumulation within the stalks and the ability to be vegetatively propagated by stalk cuttings also referred to as seedcane or setts (Earle, 1928; Blume, 1985; Soopramaniem, 2000). In addition, multiple harvests may be obtained from a single planting because sugarcane is a perennial, ratoon crop. Setts remain in the ground for several seasons because these regenerate after each harvest (Earle, 1928; Dodds, 1929; Soopramaniem, 2000).

There are 6 known *Saccharum* species, i.e. *S. spontaneum* L., *S. robustum* Brandes and Jeswiet ex Grassl; *S. officinarum* L., *S. barberi* Jeswiet, *S. sinense* Roxb. and *S. edule* Hassk. (Soopramaniem, 2000; Hodkinson *et al.*, 2002). However, the basis for modern commercial cultivars of sugarcane was provided by Dutch breeders in Java, who crossed *S. spontaneum* and *S. officinarum* producing interspecific hybrids. This cross produced the first hybrid noble cultivars (POJ 2725 and POJ 2878). These cultivars expressed desirable characteristics such as the good sucrose yields of *S. officinarum* and the robust traits of *S. spontaneum* (Earle, 1928; Price, 1965; Stevenson, 1965; Rao, 1968; Moore, 2005).

Sugarcane was first grown in South Africa in 1848 from cultivars derived from *S. officinarum* x *S. spontaneum* (O'Reilly, 1998). Following significant vulnerability to pests and diseases due to the narrow gene pool from the first cross, an Experiment Station was established at Mount Edgecombe in 1925 in order to expand the genetic base of commercial sugarcane by crossing with imported cultivars. In addition, new cultivars were screened for desirable traits and those cultivars which were well-suited to the local environment were released (Anon, 2003a; Moore, 2005).

Presently, the South African sugar industry incorporates agricultural practices with the manufacture of various sugars (its primary products) and by-products. By-products of sugarcane include animal feed, alcohol, furfural which is used for the purification of oils and as a nematicide, diacetyl and 2,3-pentanedione that are used as flavourants, dextran which has several medicinal uses and paper

from bagasse (Blume, 1985; Walter and Rodríguez-Kábana, 1992; Almazan *et al.*, 1998; O'Reilly, 1998; <http://www.illovo.co.za>).

The South African sugar industry produces 2.3 million tons of sugar per season, generating approximately R7 billion in annual direct income (Anon, 2009/2010). Sugarcane is farmed in the KwaZulu-Natal and Mpumalanga regions of the country (Fig. 1; Anon, 2003a). Currently, the industry is comprised of 38 200 registered sugarcane growers, employing approximately 77 000 individuals (Anon, 2009/2010).



Figure 1. Sugarcane growing areas, mills and SASRI Research Stations in South Africa, which are located along the east coast of the country (Anon, 2003a).

2.2 Impact of pests and diseases on the South African sugarcane industry

A major constraint to the productivity of the sugar industry is the impact of pests and diseases (Carnegie, 1974; Bailey, 1979; Carnegie and Smaill, 1980; Bailey *et al.*, 1994). A range of sugarcane insect pests exist and some, such as aphids, are vectors of microbial diseases (Rybicki and Pietersen, 1999). Important stalk borers include *E. saccharina* Walker and *Sesamia calamistis* Hampson (Smaill, 1978). Major sugarcane diseases in South Africa include bacterial diseases, such as Ratoon Stunting Disease (RSD) (*Leifsonia xyli* subsp. *xyli*) and leaf scald (*Xanthomonas albilineans*) (Roth and Thomson, 1970). Viral diseases of importance are mosaic caused by *Sugarcane mosaic virus* (SCMV) and yellow leaf syndrome (YLS) caused by *Sugarcane yellow leaf virus* (SCYLV) and sugarcane yellows phytoplasma (SCYP) (Goodman *et al.*, 1998; Lockhart and Cronje, 2000). Major fungal diseases are brown rust (*Puccinia melanocephala* H. & P. Sydow) and sugarcane smut (*U. scitaminea* H. & P. Sydow) (Bailey, 1979; Anon, 2003b). *Eldana saccharina* and sugarcane smut are considered to be the major pest and one of the most important diseases, respectively, in South Africa (King, 1956; Carnegie, 1974; Bailey, 1995; Anon, 2003a; Anon, 2005).

2.2.1 Sugarcane smut (*Ustilago scitaminea*)

2.2.1.1 History of sugarcane smut

The incidence of sugarcane smut is widespread internationally (Comstock, 2000). The initial occurrences of sugarcane smut across various countries are presented in Table 1. All sugar producing countries have at one time or another experienced smut infection and the most recent was Australia (Riley *et al.*, 1999). Smut was first detected in South Africa in 1877 at which time it severely affected 'China canes' (McMartin, 1945; King, 1956). A second outbreak occurred during 1945 and 1955, infecting cultivar Co301 (Bailey, 1979). A third smut outbreak was reported during the 1960s in the northern sugarcane producing region, affecting cultivar NCo310. During the 1980s, a fourth severe outbreak of smut occurred, in cultivar NCo376 (Bailey, 1995).

Table 1. Global observations of smut disease. A summary of the year in which sugarcane smut (*U. scitaminea*) was first observed in various sugarcane producing countries of the world.

Year	Country	Reference
1877	South Africa	McMartin, 1945
1940	Argentina	Briceño <i>et al.</i> , 2005
1946	Rhodesia (now Zimbabwe)	James, 1974
1963	Thailand	Juangbhanich and Wangnon, 1983
1971	Hawaii; Iran	Ershad and Bani-Abbassi, 1971; Byther and Steiner, 1974
1978	Cuba; Belize; Florida, USA; Venezuela	Flores and Osada, 1980; Pérez and Mauri, 1983; Briceño <i>et al.</i> , 2005; Comstock and Lentini, 2005
1981	Louisiana, USA	Koike <i>et al.</i> , 1981
1993	Morocco	Akalach, 1994
1998	Australia	Riley <i>et al.</i> , 1999

More recently during October 2007 and March 2008, severe smut infections were observed in 83% of farms inspected in Mpumalanga and seedcane from 70% of these farms had to be ploughed out (van den Berg *et al.*, 2008). Although smut infections had decreased slightly in 2009 and 2010 compared with 2008, significant incidences were observed in Mpumalanga (van den Berg *et al.*, 2009; Singels *et al.*, 2010). Smut remains a threat because teliospores are present and continues to infect susceptible cultivars during the selection process for new genotypes. Smut is a major disease in the industry due to the potential amount of associated sugarcane losses and difficulty in controlling severe outbreaks (Bailey, 1979; Anon, 2003b).

2.2.1.2 Characteristics of *U. scitaminea* and mode of infection

Sugarcane smut is characterized by the development of a sori-containing structure from the apical region or growing point of the sugarcane stalk (Fig. 2a), which is commonly referred to as a smut whip (Hoy *et al.*, 1991; Comstock, 2000). The whip is initially enclosed by the peridium, which is a thin membrane that disintegrates when mature, releasing black teliospores (Trione, 1980; Comstock, 2000). Teliospores make contact with sugarcane either by wind dispersal or its presence

in soil. Unintentional planting of infected cane is a third means by which smut is spread (Anon, 2003b).



Figure 2. Sugarcane infected with smut at SASRI, Mount Edgecombe, Durban. (a) Characteristic aerial smut whip from the apical region of the stalk. (b) Poor growth and excessive tillering of the smut-infected sugarcane stool.

Various morphological changes may occur in sugarcane infected by smut. Generally, infected sugarcane appears significantly thinner, tillers (produces secondary shoots) rapidly and appears grass-like when compared with uninfected sugarcane (Fig. 2b; Sharma, 1956; Anon, 2003b). Observations by Sharma (1956) revealed that sugarcane size was reduced, internodes were distorted and bud precursors were suppressed or longer and papery, whilst root primordia were either suppressed or fewer in number. The leaf sheaths were smaller, elongated and often failed to surround the node and the topmost leaves appeared split or absent. The morphology of floral structures was also affected, with a reduction in filament size or the absence of the stamen and an increased number of stigmas. In addition, atypical smut galls and paired apical whips were observed (Sharma, 1956).

The life cycle of *U. scitaminea* consists of a resting stage, vegetative stage and parasitic stage (Alexander and Ramakrishnan, 1977). According to Alfieri (1978), there are 2 distinct cycles of infection (Fig. 3). Primary infection occurs when chlamydospores (i.e. dormant teliospores) present

in soil or in seedcane cause infection of sugarcane stalks. Secondary infection occurs when chlamydospores infect sugarcane by wind, water, cultivation or equipment.

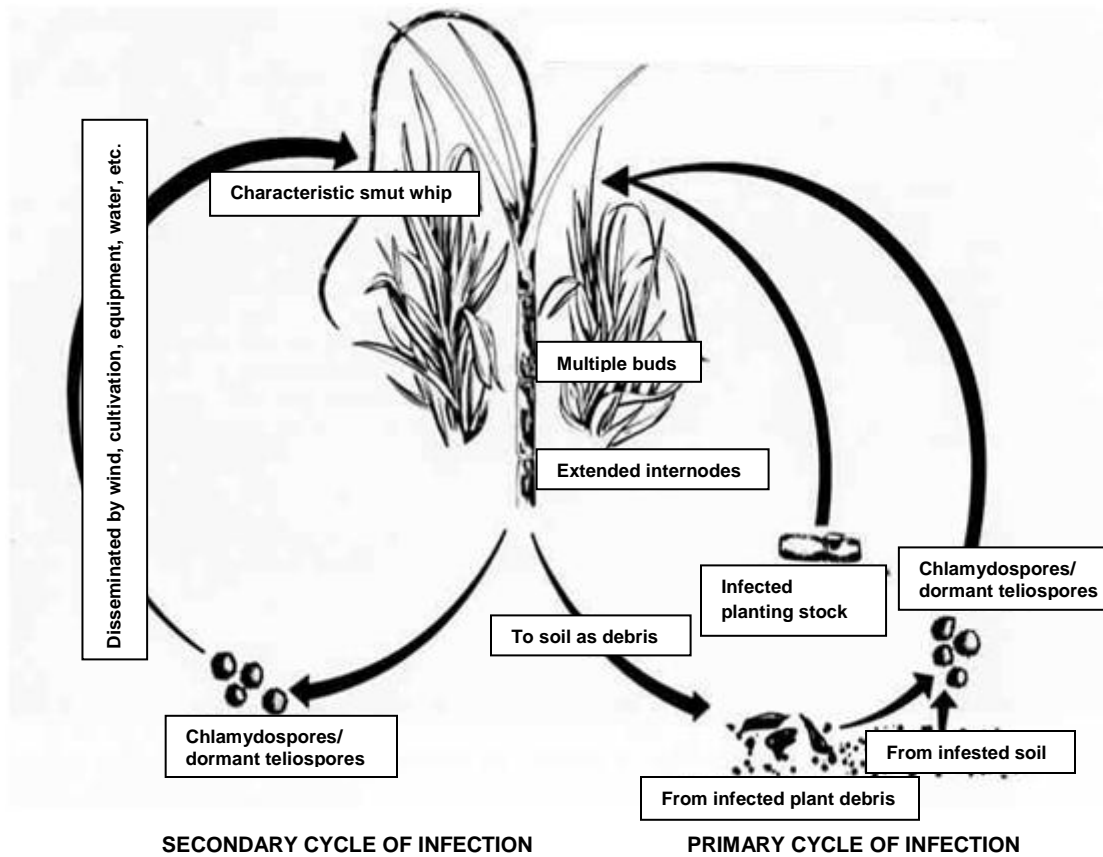


Figure 3. Life cycle of *Ustilago scitaminea* Sydow, the causal agent of sugarcane smut (adapted from Alfieri, 1978). This figure depicts 2 possible cycles of infection. Primary infection occurs by dormant teliospores in soil that may infect planting stock. Alternatively, during secondary infection dormant teliospores may be transmitted to sugarcane by wind or unsanitary farming practice. Both cycles result in the development of characteristic smut whips which contribute to a greater number of teliospores.

Both cycles produce the characteristic smut whip and infected sugarcane stalks develop multiple buds with longer internodes. The longevity of teliospores in soil was investigated by Hoy *et al.* (1993) and was found to decrease in the presence of moisture. In dry conditions, teliospores remained viable for 18 weeks (Hoy *et al.*, 1993). The parasitic phase of *U. scitaminea* is initiated by the germination of teliospores. Moisture is necessary for germination and each teliospore produces a promycelium, consisting of 3 septa, 4 cells and 4 sporidia (Alexander and Ramakrishnan, 1977).

Infection occurs when teliospores germinate at the bud or meristematic region of sugarcane and after successful fusion of opposite (+ and -) mating strains (Alexander and Ramakrishnan, 1977; Alexander and Ramakrishnan, 1980). A study conducted by Alexander and Ramakrishnan (1977) found that 3 types of fusion may occur after teliospore germination; namely, sporidial fusion, promycelial fusion or hyphal fusion, which result in the parasitic dikaryotic condition. Sporidial fusion occurs when sporidia produce short hyphae which fuse when opposite mating strains make contact. Promycelial fusion occurs when hyphae develop directly from either end of the promycelium, grow towards each other and fuse. Alternatively, promycelia from different teliospores may make contact and fuse without the development of a fusion hypha. Hyphal fusion occurs when one cell produces the fusion hypha which grows toward another cell in the same hypha or cell of neighbouring hypha to fuse. The nuclei migrate toward each other in the fused cell and this generates the dikaryotic infective hyphae (Alexander and Ramakrishnan, 1977).

When infective hyphae are produced within parenchymous tissue of immature sugarcane leaf rolls, growth of the leaf occurs simultaneously with the development of mature hyphae which leads to the formation of a smut whip (Alexander and Ramakrishnan, 1980; Hoy *et al.*, 1991). A study by Trione (1980) revealed that *U. scitaminea* cells grow rapidly in the whip compared with other plant tissues. In addition, the cytology and physiology of hyphae are transformed from a vegetative to reproductive state; thereby enabling the development of numerous teliospores.

2.2.1.3 Economic importance

Sugarcane smut has the potential to cause significant yield losses in severe outbreaks as was observed in South Africa during the 1970s. A survey was conducted to assess the incidence of smut during the 1977/78 and 1978/79 seasons in South Africa (Bailey, 1979). During that time cultivar NCo376, which was predominant in the northern growing region of South Africa, was significantly affected. In Komatipoort, 45% of fields showed greater than 5% smut-infected stools (growth shoots) during the 1978/79 season which increased from 13% of fields during the 1977/78 season. Malelane, Pongola and Monzi regions showed significant increases in smut infected stools during the 1978/79 season compared to previous years. Estimated losses during 1978/79 due to smut were 50 000 tons of sugarcane, with an associated sugar value of R1.4 million. At the time, sugarcane smut was reported as the most serious problem facing the South African sugar industry (Bailey,

1979). Without adequate control measures, a similar situation could arise again, therefore smut is still regarded as a threat to the productivity of the sugar industry.

2.2.1.4 Current methods of control

Several control measures may be implemented to reduce the risk of severe infection and consequent yield losses due to smut. These include the introduction of disease-free sugarcane at the seedcane stage and removal (roguing) of infected sugarcane in the field (Anon, 2003b). Entire fields may be ploughed out when levels within fields exceed a pre-determined threshold. Bailey (1979) assessed various hot water treatments of sugarcane setts in combination with fungicides and found the best treatment for the control of smut to be a 30 min hot water treatment at 50°C with 500 µg/ml triadimefon. Currently, the fungicide Bayleton® (active ingredient: triadimefon) may be used when sugarcane is heat treated (Benda and Ricaud, 1977; Anon, 2003b). However, a long-term approach is the introduction of resistant cultivars which has a low environmental impact and is cost-effective for the farmer. Therefore, the production and selection of new cultivars with good traits, including resistance to important diseases, are key objectives of SASRI's Plant Breeding, Crossing and Selection Programme.

2.2.1.5 Methods of conventional screening for smut resistant sugarcane cultivars

The numerical (0-9) rating scale for sugarcane diseases proposed by Hutchinson (1968) is still used when new cultivars are screened: 0-immune; 5-intermediate or average; 9-very highly susceptible. A broad numerical system was suggested by Ricaud (1981): 2-highly resistant; 5-intermediate, average; 8-highly susceptible. Generally, conventional screening for rating smut susceptibility in sugarcane involves artificial inoculation in the field with a concentrated smut teliospore inoculum; however, natural infection methods may be used as well (Byther and Steiner, 1974; Ferreira *et al.*, 1980; Ferreira and Comstock, 1989).

Immersion and wound-paste are 2 artificial inoculation methods generally used (Ladd *et al.*, 1974; Ferreira *et al.*, 1980; Ferreira and Comstock, 1989). The immersion method is widely used and involves submerging sugarcane setts in a teliospore suspension for varying time periods (Bailey and Béchet, 1982; McFarlane and McFarlane, 2002). Different countries have established particular concentrations (range 2.5×10^6 to 5×10^8 teliospores/ml) and exposure times (10 min to 12 hours)

of the inoculum (reviewed by Ferreira *et al.*, 1980). Sugarcane setts may be incubated in humid conditions (e.g. in plastic bags for 24 h or 0-4 days in a humidity tent) before being planted. The wound-paste method involves injuring the sugarcane stalk by 6 incisions made close to the bud. A paste of teliospores is then brushed over the wound (Ferreira *et al.*, 1980). A paste method has also been used, in which a concentrated suspension of teliospores is brushed over the bud on setts without wounding (Ladd *et al.*, 1974).

Natural infection involves planting rows of susceptible cultivars which are infected and produce smut whips containing numerous teliospores, amongst test cultivars (Bailey and B  chet, 1982; McFarlane and McFarlane, 2002). Thus, teliospores may naturally infect the test cultivars, similar to circumstances in the field (Ferreira *et al.*, 1980). Standing cane and setts were evaluated for smut susceptibility in Hawaii by Byther and Steiner (1974), in which 6 month old stalks were cut above the 8th node and the area between the 8th and 12th node was inoculated by various methods, such as spray, paste, wound-paste, and combinations of the above. These researchers found that the use of standing cane required less land and more replications were possible which resulted in a more reliable indication of susceptibility.

Conventional screening of sugarcane using an injection method was conducted by Burner *et al.* (1993), in which a teliospore inoculum was injected into the leaf whorl tissue above the apical meristem. These plants were obtained by *in vivo* direct morphogenesis and maintained in a greenhouse. This inoculation method was adapted from Ferreira and Comstock (1989) and was found to be useful for identifying smut resistant cultivars as this method accessed physiological resistance because the bud scales were bypassed (Burner *et al.*, 1993). Recently in Kenya, Co *et al.* (2008) investigated the inoculation of sugarcane seedlings using immersion (30 min soak), paste and wound-paste methods. The wound-paste method was found to be the best means of inoculation. However, conventional screening requires a large amount of smut teliospores which need to be harvested. A 6-step method for bulk harvesting of smut teliospores was proposed by Tokeshi (1980). Correct handling of teliospores is essential to maintain their viability.

Conventional screening is still widely used in the sugar industry for screening new cultivars from the breeding programme. However, it is inefficient due to land constraints and lengthy time required for screening. In addition, screening is carried out in the later stages of selection because the large

number of new cultivars produced cannot be screened in the initial stages (Parfitt and Thomas, 2001; Parfitt, 2005).

2.2.2 *Eldana saccharina*

2.2.2.1 History of *E. saccharina*

Eldana saccharina Walker (Lepidoptera: Pyralidae) is a stalk borer indigenous to Africa and has been found to attack Gramineae and Cyperaceae plants (Walker, 1865; Waiyaki, 1974; Atkinson, 1979; Nuss *et al.*, 1986). This Lepidopteran species was first described in Sierra Leone, western Africa in 1865 (Walker, 1865). Across central Africa, *E. saccharina* is identified as a pest of crops such as maize, millet and rice (Carnegie, 1974). *Eldana saccharina* was first recorded as a pest in South Africa in 1939 at the Umfolozi Flats, KwaZulu-Natal (Fig. 4), where it caused severe damage to the sugarcane cultivar, POJ 2725 and attacked 3 other cultivars in the area (Dick, 1945; Dick, 1950; Atkinson *et al.*, 1981).

The outbreak appeared to be contained within this region and lasted 13 years until 1953. Regular harvesting of susceptible cultivars and the introduction of the resistant sugarcane cultivar, Co 281, probably assisted in reducing infestation (Dick, 1950; Atkinson *et al.*, 1981). A second severe outbreak of *E. saccharina* occurred in 1970 at Hluhluwe which affected cultivar NCo376 (Carnegie, 1974; Atkinson *et al.*, 1981). This outbreak spread along the east coast of South Africa (Fig. 4), further north to Eastern Transvaal (Mpumalanga) (1973 and 1976) and far south to Port Shepstone (1980), significantly affecting sugarcane fields (Atkinson *et al.*, 1981). Recent surveys of *E. saccharina* in South Africa have found that populations are widely distributed outside of the sugarcane regions and may pose a threat to South Africa's maize agriculture as well (Assefa *et al.*, 2008).

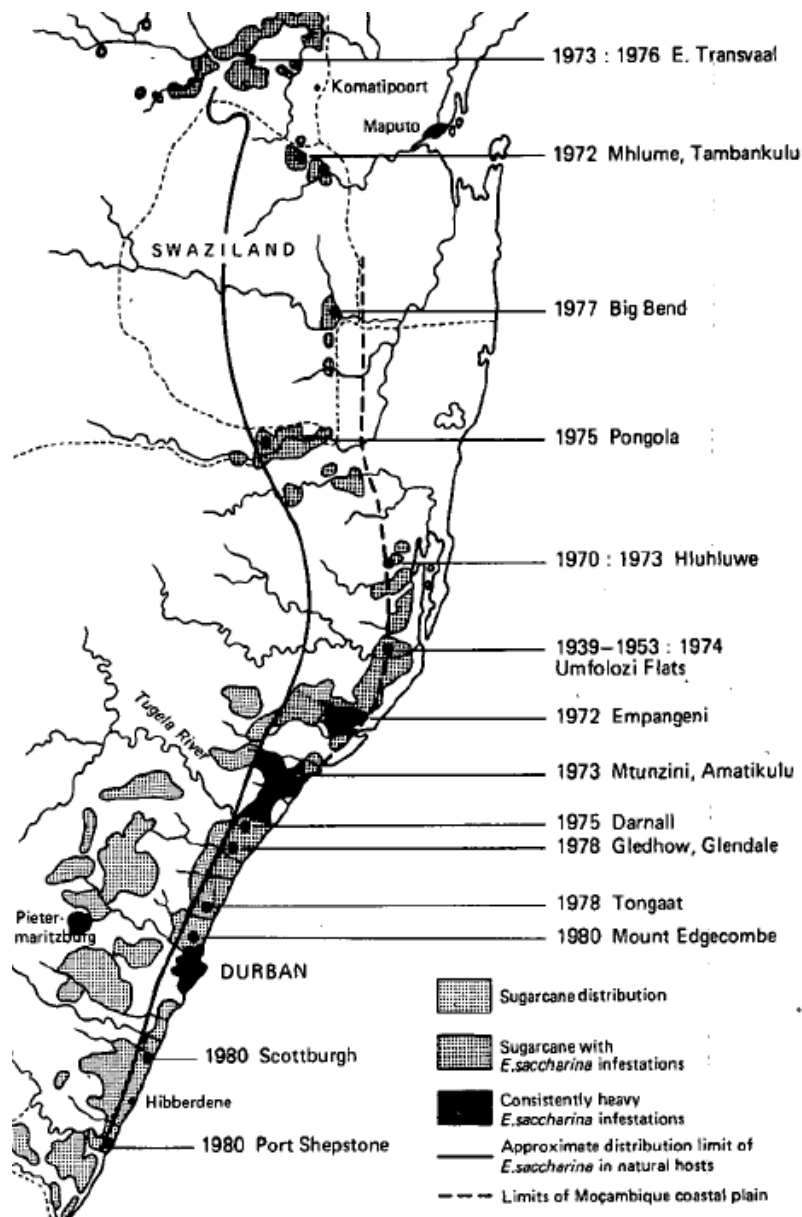


Figure 4. Outbreak history of *E. saccharina* in regions of southern Africa producing sugarcane (Atkinson *et al.*, 1981). These outbreaks were observed in South Africa and Swaziland during 1939-1980.

2.2.2.2 Characteristics of *E. saccharina* and mode of infection

Early studies of *E. saccharina* showed that adult moths emerge at night and ratios of males to females were equal, however males emerged 2-3 days before females (Dick, 1945). Moths are active only at night and mating usually occurs during the night of emergence. Females generally

begin oviposition the following night, regardless if mating has occurred. The number of eggs laid is greatest at the start of oviposition and gradually decreases. Each female is capable of laying 450-750 eggs during oviposition (Dick, 1945; Carnegie, 1974).

Egg clusters containing 20-200 eggs are laid in sheltered regions of the host plant, such as between dead leaves and the stalk or attached to dry trash (Fig. 5) (Dick, 1945; Atkinson, 1979). Eggs are oval, pale yellow in colour and are attached to the substrate by a reddish fluid secreted by the adult female (Dick, 1945; Waiyaki, 1974). The duration of the egg stage is influenced by temperature. The physiological time required to complete a life cycle stage is measured in heat units, which is degree days ($^{\circ}\text{C}\cdot\text{d}$) (Way, 1995; Horton *et al.*, 2002). First instar larvae range from 1.5-2 mm in length and hatch after 7-10 days (Dick, 1945; Dick, 1950). Initially larvae feed on dead leaves or organic matter (Dick, 1950; Carnegie, 1974).

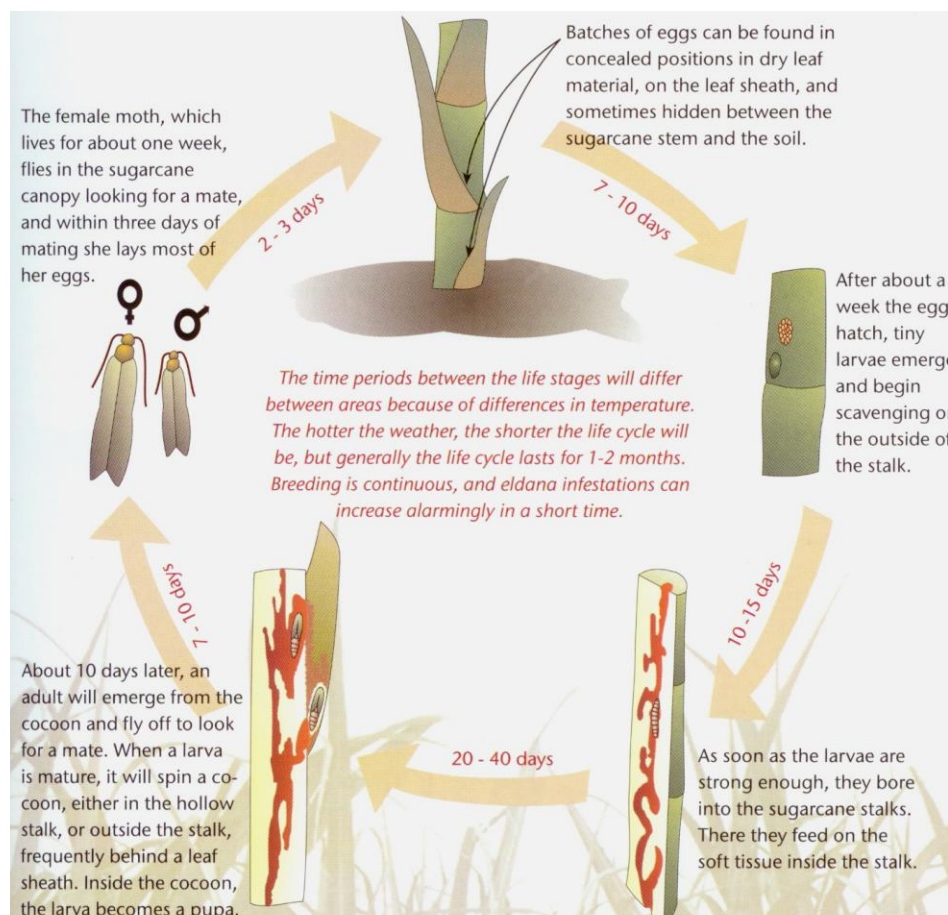


Figure 5. Life cycle of *E. saccharina* (Anon, 2005). The duration of a complete cycle is 46-78 days.

Larger larvae (approximately 10 mm) bore into sugarcane stalks to feed, generally at the node and bud or through cracks (Fig. 6a and b; Dick, 1945; Atkinson, 1979; Atkinson, 1980). During boring, larval excrement (frass) is pushed through the bore hole to the outside of the sugarcane stalk and observation of frass can assist in early detection of *E. saccharina* infestation (Carnegie, 1974).

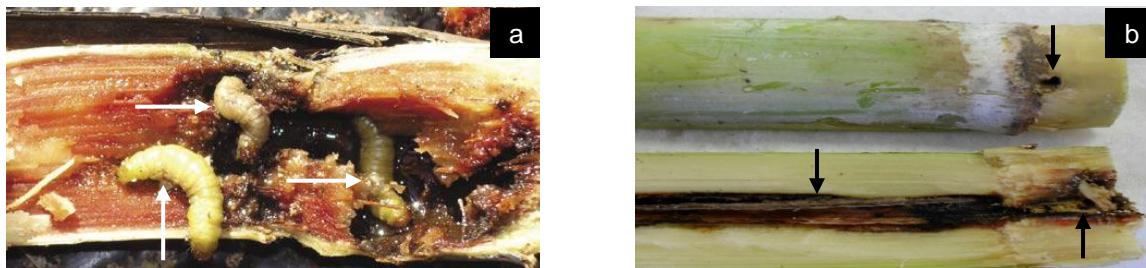


Figure 6. Longitudinal sections of split sugarcane stalks infected by *E. saccharina* larvae. (a) Severely infected sugarcane stalk (Anon, 2005). (b) Boring at the nodal region of the sugarcane stalk and damage within the infected stalk.

The larval stage lasts 20-40 days during which time they are destructive to their host plant. Mature larvae pupate by enclosing themselves in a cocoon (Carnegie, 1974). The pupal stage lasts 7-10 days, after which adult moths emerge and the cycle continues. The duration of each complete cycle is an estimated 2-3 months, however, it has been observed that various stages may simultaneously exist in a single sugarcane stalk or field (Dick, 1945; Carnegie, 1974).

2.2.2.3 Economic importance

Economic losses due to *E. saccharina* vary across affected areas and correlate with the severity of infestation (Carnegie and Smaill, 1980). In South Africa, mill surveys were conducted at 14 mills during the 1978/1979 season to assess the incidence of *E. saccharina* and yield loss (Smaill and Carnegie, 1979). This assessment revealed 0.1% loss of recoverable sugar associated with every 1% stalks damaged by *E. saccharina*. In an investigation conducted in the Ivory Coast during 1980-1981, 0.5% loss in weight of sugar produced by the mill for each 1% of internodes bored in the field was used as a basis for calculation of loss (Cochereau, 1982). An assessment in Swaziland by King (1989), found that 1% of internodes damaged by *E. saccharina* correlated with 1% loss in the yield of recoverable sucrose. In South Africa, an average infection rate of 1 larva/100 stalks during a

season has the potential to cause 0.5 ton sugarcane loss per hectare (Way *et al.*, 2003; Anon, 2005). This amounts to a considerable financial loss to sugarcane growers and the industry.

2.2.2.4 Current methods of control

Several factors may assist in controlling *E. saccharina* infestations and integration of strategies is often necessary to obtain adequate control. SASRI has released guidelines and recommendations for *E. saccharina* control in South Africa (Anon, 2005) and an Integrated Pest Management System (IPMS) has been suggested by Webster *et al.* (2005), which require early harvest of sugarcane at 12 months in order to avoid *E. saccharina* infestations because older cane is prone to attack (Way and Goebel, 2003). These recommendations include the introduction of good quality, healthy seedcane because the presence of *E. saccharina* eggs and larvae in seedcane, at the planting stage, leads to excessive sugarcane damage and yield losses (Anon, 2005). The nitrogen content of sugarcane needs to be regulated by monitoring the amount of fertilizer applied because the presence of excessive amounts of nitrogen support *E. saccharina* infestations (Anon, 2005). Pre-trashing which is the removal of dead leaves from stalks is recommended, particularly during August to October (Webster *et al.*, 2005).

Sugarcane experiencing moisture stress is prone to *E. saccharina* damage; therefore the water-holding capacity of soil needs to be investigated (Nuss *et al.*, 1986). High silicon concentrations within sugarcane can assist in protecting stalks from *E. saccharina* attack as plant material is strengthened by this mineral, such that larvae cannot easily penetrate the stalk (Keeping and Meyer, 2000; Kvedaras *et al.*, 2007). Adequate amounts of silicon should be included in soil before planting. Regular small scale surveys are conducted by the LPD&VCCs to enable early detection of possible *E. saccharina* infestations (Anon, 2005). Several factors are considered when selecting 'carry-over' fields after mill closure, such as cultivar resistance rating and potential for stress because mature sugarcane is prone to *E. saccharina* attack (Nuss *et al.*, 1986). Damage can be reduced in these 'carry-over' fields by the application of the insecticide, Fastac[®] (active ingredient: alpha-cypermethrin) (Leslie, 2003; Leslie *et al.*, 2006). However, Fastac[®] needs to be applied once every 2 weeks for 16 weeks and requires stringent guidelines and safety measures for labourers applying the chemical (Webster *et al.*, 2005). Therefore, the best approach to limit the impact of *E. saccharina* is the use of resistant cultivars (Anon, 2005).

2.2.2.5 Methods of conventional screening for *E. saccharina* resistant sugarcane cultivars

Sugarcane feeding bioassays, in shade-houses, is currently the main method used to assess the susceptibility of new sugarcane cultivars to *E. saccharina* at SASRI (Keeping, 1999). Desirable cultivars from the Breeding Programme are propagated; setts are planted in pots and maintained in shade-houses for approximately 8 months (Fig. 7) before being water-stressed for 1 month (Keeping, 2002). Fertilized *E. saccharina* eggs are obtained from the Insect Rearing Unit at SASRI and 200 eggs/pot are placed on concealed areas of the sugarcane stalks, similar to where female moths lay their eggs in nature (Keeping, 2002; Keeping, 2006). The temperature is closely recorded in order to monitor larval development by degree-days ($^{\circ}\text{C.d}$) and plants are maintained until 500°C.d is achieved (Way, 1995; Keeping, 2002). The accumulation of 500°C.d generally occurs over a period of 6 weeks (Keeping, pers. comm.²).



Figure 7. Conventional screening of sugarcane for evaluating *E. saccharina* susceptibility. Pot trials conducted in shade-houses at SASRI to determine the resistance ratings of new cultivars.

Sugarcane stalks are then cut longitudinally and the number of larvae are recorded and compared across standards with known resistance ratings. This occurs at 2 stages, primary screening which selects 3 possible resistant cultivars and secondary screening which selects 2 possible cultivars.

² Keeping, M. G. 2009. South African Sugarcane Research Institute (SASRI), Private Bag X02, Mt. Edgecombe, Durban, 4300, South Africa.

2.3 The Plant Breeding, Crossing and Selection Programme at SASRI

SASRI's Plant Breeding, Crossing and Selection Programme is tasked with cross pollinating sugarcane cultivars with good characteristics and generating sugarcane interspecific hybrids with combinations of desired traits (Parfitt and Thomas, 2001; Anon, 2003a). All countries producing sugarcane invest in breeding and selection of cultivars suited to that specific climate in order to maximize profit. Commercial sugarcane cultivars are polyploid hybrids with unequal chromosome contributions from the original wild type cross between *S. officinarum* and *S. spontaneum* (Butterfield *et al.*, 2001). In addition, several copies of each chromosome may be present, therefore breeding for desired traits is difficult because a gene cannot be fixed within specific genotypes (Butterfield *et al.*, 2001; Butterfield, 2005). Conventional screening for resistance to pests and diseases is a key component of the breeding programme prior to release of a commercial cultivar (O'Reilly, 1998; Anon, 2003a). This screening occurs at Stage 4 and 5 (Table 2) of the programme which may be after 5-8 years. Screening for smut resistance requires 3-5 years to complete; whilst screening for resistance to *E. saccharina* may take 2-3 years (Anon, 2003a).

Table 2. Details of the SASRI Selection Programme (Anon, 2003a).

Selection Stage	No. of clones per site/total	Trial design	Year	No. reps	No. crops	Select Rate %	Selection Criteria
Seedlings NURSERY	50 000 x 5 250 000	Potted seedlings	1	Family	1	70	Visual assessment and freedom from diseases
Stage 1 SINGLE STOOLS	35 000 x 5 175 000	Replication of families	2		1	11	Mean sucrose per family
Stage 2 SINGLE LINES	4 000 x 5 20 000	8m row	3		2	10	Sucrose yield – plant and ratoon Family performance from cross summary
Stage 3 OBSERVATION TRIAL	400 x 5 2000	2 x 8m row	5	2	1	15	Plant cane sucrose yield At Pongola – rating from smut inoculation trial
Stage 4 PRIMARY VARIETY TRIAL	60 x 5 300	5 x 8m row	7	3	3	30	Plant cane sucrose yield (reselect in (reselect in 2 nd ratoon) Smut/mosaic/eldana screening trial
Stage 5 SECONDARY VARIETY TRIAL	20 x 5 100	5 x 8m row, 4 off-station trials per region	11	3	3		Compilation of all yield and pest/disease results Yield stability or specific adaptability
BULKING UP	1-2	Further variety trials					Grower acceptance Agronomy - interactions with environment and management

This programme is essential to the productivity of the South African sugar industry but is a lengthy process because only 1 or 2 cultivars are released after approximately 12 years of investigations (Fig. 8). An estimated 40-50% of cultivars, selected for good sucrose content and yield traits, are discarded at this stage of screening (Bailey and B  chet, 1982; Butterfield and Thomas, 1996). Therefore a rapid technique for screening pest and disease resistance would be advantageous, particularly if it could be applied earlier in the screening process.

Sugarcane grown in a particular area may be vulnerable to several pathogens and insect pests. The selection of a good cultivar which exhibits resistance to multiple diseases and insect pests is difficult as it has been observed that a cultivar resistant to a single pathogen may be susceptible to another (Kolobaev, 1983). Butterfield (2007) mapped molecular markers (RFLP and AFLP) associated with smut and *E. saccharina*. Common markers were found to be genotypically and phenotypically negatively correlated, meaning that a cultivar resistant to *E. saccharina* is likely to be susceptible to smut and vice versa (Butterfield, 2007). At SASRI, molecular marker assisted breeding has been conducted since 2002 in order to generate cultivars with improved resistance to smut and *E. saccharina* (Butterfield, 2005). However, the thousands of new cultivars produced by marker assisted breeding need to be screened and conventional field-based screening is time-consuming.

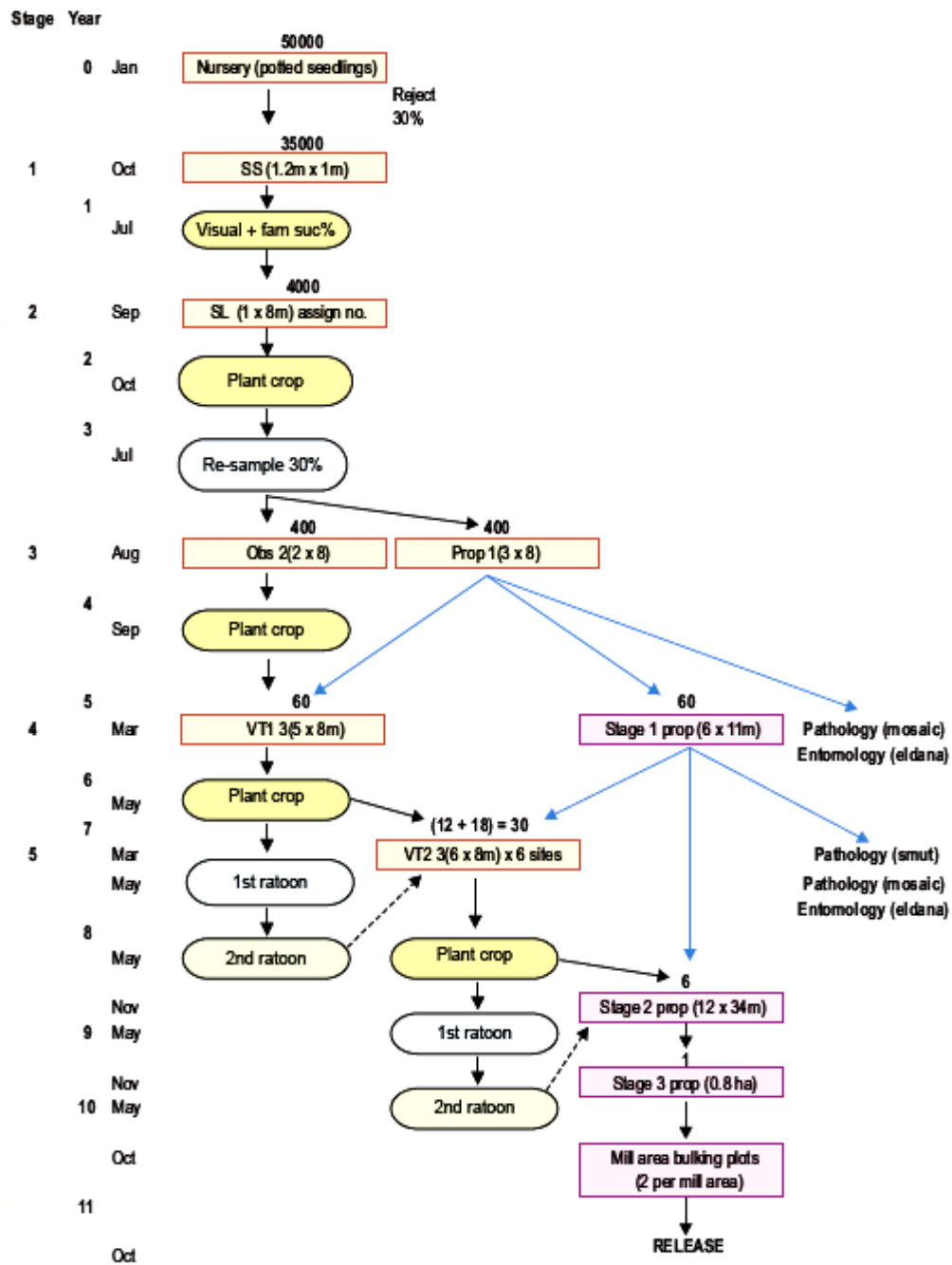


Figure 8. A summary of the Plant Breeding selection process at the Empangeni Research Station. The diagram indicates when conventional screening for pest and disease resistance is conducted (Anon, 2003a).

2.4 *In vitro* culture of sugarcane

In vitro technology, enhanced by totipotency of plant cells, has allowed various manipulations to plant cells through 2 developmental routes, i.e. organogenesis and embryogenesis (Street and Withers, 1974). Organs, tissues and cells have been successfully used *in vitro* to obtain plants with characteristics of interest; whilst, a greater yield of desired plants is achievable through micropropagation (Maribona *et al.*, 1983; Lee, 1987). Thus, selection for improved traits and mass production (micropropagation) of certain plant species, as well as plant products is possible. *In vivo* and *in vitro* (direct and indirect) routes of morphogenesis have been established for sugarcane and are summarized in Fig. 9.

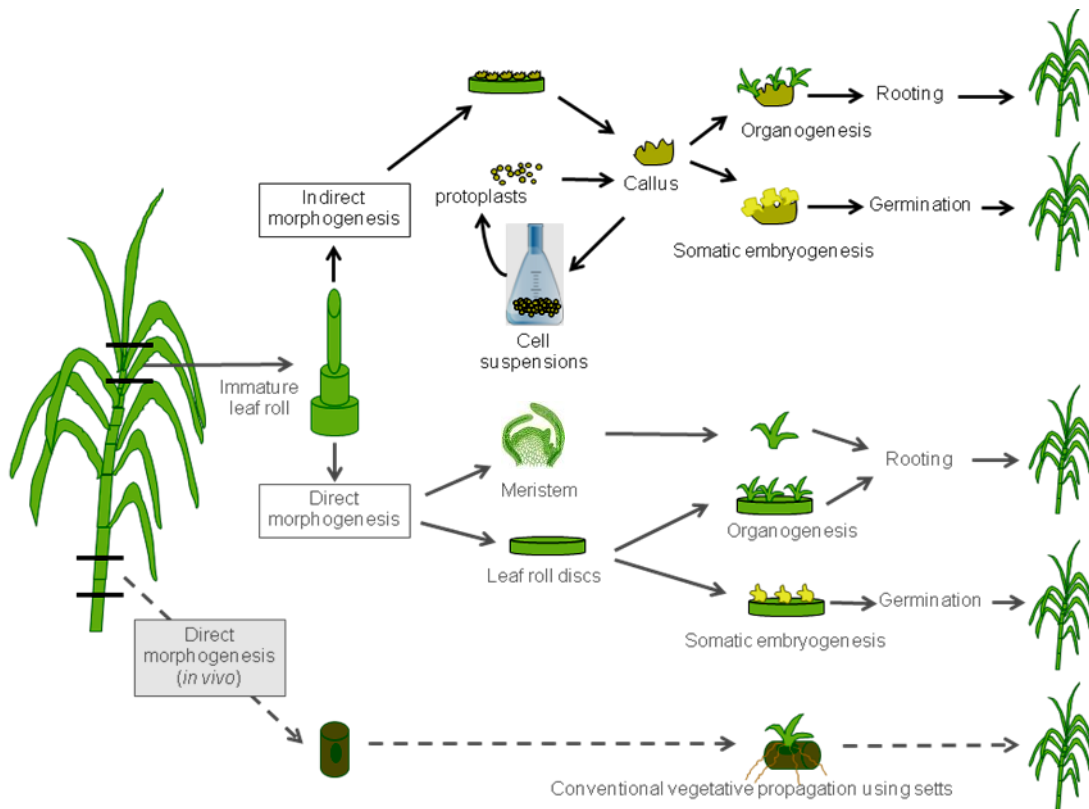


Figure 9. Diagrammatic representation of the different routes by which sugarcane plants can be regenerated (Koch, 2010; modified from Snyman, 2001).

Conventional vegetative propagation of sugarcane, which is widely used commercially, occurs *in vivo* by planting sugarcane sets, containing the bud and root primordia (Soopramanien, 2000). For *in vitro* sugarcane culture, the immature leaf roll is the plant material harvested because this region

contains meristematic tissue (Chen *et al.*, 1988; Snyman *et al.*, 1996). Growth and development of explants are controlled by exogenous plant growth regulators that are added to culture media. An interaction between adequate amounts of cytokinin (e.g. BAP and kinetin) and auxin (e.g. IBA and NAA) is necessary to induce a specific route of morphogenesis (Fakhrai and Fakhrai, 1990).

Direct morphogenesis of sugarcane *in vitro* may occur by the culture of the apical meristem, which develops into shoots in the presence of a cytokinin followed by rooting when exposed to an auxin (Lee, 1987; Grisham and Bourg, 1989; Ramgareeb *et al.*, 2010). Alternatively, immature leaf roll disks may be induced to directly produce shoots (organogenesis) in culture which may be rooted, producing plantlets. In addition, plantlets may be generated from the germination of somatic embryos obtained from leaf roll disk culture (Irvine and Benda, 1985; Snyman *et al.*, 2000).

Indirect morphogenesis occurs by the production of undifferentiated callus cells which may be manipulated in culture by plant growth regulators to produce plantlets via organogenesis or somatic embryogenesis (Barber and Nickell, 1969; Liu and Chen, 1974; Barba *et al.*, 1977; Liu *et al.*, 1980). In addition, sugarcane cell suspensions may be produced from callus from which protoplasts can be isolated.

Optimisation of *in vitro* methods for sugarcane micropropagation has been researched across all countries producing sugarcane (Lorenzo *et al.*, 1998; Visessuwan *et al.*, 1999; Lorenzo *et al.*, 2001; Ali *et al.*, 2007; Singh *et al.*, 2008). In South Africa, nutrient concentrations and combinations, as well as different types of media (i.e. semi-solid or liquid) have been investigated (Sweby *et al.*, 1994; Nkwanyana *et al.*, 2010). For the purposes of this study, 2 routes of morphogenesis were exploited, i.e. direct morphogenesis by means of meristem culture and indirect morphogenesis by somatic embryogenesis of immature leaf roll disks.

2.4.1 Direct morphogenesis by meristem culture

Meristematic regions in plants contain embryogenic cells which are undifferentiated and have the ability for multiple divisions and differentiation into specific plant cells that develop into shoots and roots (Binding and Krumbiegel-Schroeren, 1984; Kane, 2000; Starr and Taggart, 2001). According to Kane (2000), meristem culture is the culture of the apical dome alone, which is free of pathogens. Culture of shoot and root meristems offer a rapid method for plant propagation whilst maintaining

genetic stability (Lee, 1987; Cheema and Hussain, 2004). This is possible because meristem tissue is organized in comparison to undifferentiated callus and this factor is important to maintain plant traits of interest (Kane, 2000). The use of meristem culture in sugarcane provides a means to generate plants free of bacterial and viral infections because these cells are seldom infected by pathogens (Rani and Raina, 2000; Chatenet *et al.*, 2001; Fitch *et al.*, 2001; Cha-um *et al.*, 2006; Ramgareeb *et al.*, 2010). In addition, meristem culture offers similar advantages to other tissue culture methods because selected plants can be maintained and propagated aseptically, in a relatively short time period, whilst maintaining genetic stability (Davies, 1981; Lee, 1987; Grisham and Bourg, 1989).

2.4.2 Somatic embryogenesis

Somatic (non-gamete) embryos may develop directly from immature sugarcane leaf roll disks or indirectly through callus (Fig. 9). The development of plantlets from somatic cells, through a route similar to natural embryology, is termed somatic embryogenesis (Fig. 10; Street, 1973; Zimmerman, 1993). A single embryogenic cell exhibits bipolarity which results in the development of root and shoot primordia, similar to zygotic embryogenesis (Street and Withers, 1974).

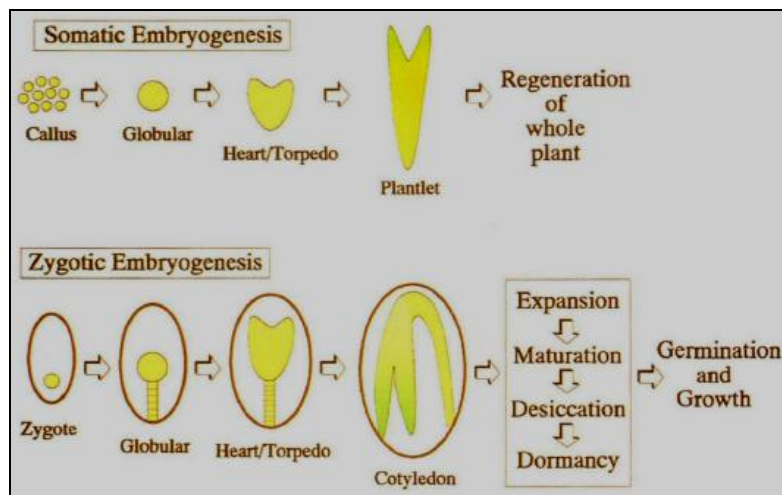


Figure 10. A comparison of somatic and zygotic embryogenesis (Zimmerman, 1993).

Embryogenic callus is induced to develop from sugarcane explants when maintained in the dark on MS (Murashige and Skoog, 1962) medium supplemented with the auxin, 2,4-dichlorophenoxyacetic

acid (2,4-D). Various concentrations of this auxin have been investigated for sugarcane since 1969. A 2,4-D concentration range of 1 - 5 mg/l is required to initiate and maintain callus production, and 3 mg/l of 2,4-D is widely used for somatic embryogenesis in sugarcane (Barber and Nickell, 1969; Nader *et al.*, 1978; Sweby *et al.*, 1994).

Germination of somatic embryos and plant production in sugarcane was first demonstrated by Ho and Vasil (1983) and this approach allowed for rapid plant propagation. Histological studies by Guiderdoni and Demarly (1988) found that 2 distinct and organized types of calli may be induced from sugarcane, i.e. nodular callus and friable callus. Nodular calli gave rise to somatic embryos, whilst friable calli resembled root meristems. Somatic embryogenesis has been used for the micropropagation of sugarcane plants (Lee, 1987; Snyman *et al.*, 2008), induction of mutations by exploiting somaclonal variation (Koch, 2010) and genetic engineering for improved traits (Bower and Birch, 1992; Bower *et al.*, 1996; Enríquez *et al.*, 2001).

2.5 Applications of *in vitro* tissue culture for the screening of sugarcane cultivars and inducing resistance to pests and diseases

The use of *in vitro* technology for the production of somaclonal variants that are resistant to microbial pathogens and insect pests has been widely reported in the literature for sugarcane (summarized in Table 3). Although these studies employed *in vitro* techniques to derive plant material; the screening processes were modifications of conventional field-based methods (Bravo *et al.*, 1989). In addition, a considerable number of *in vitro* sugarcane studies have focused on the production of pathogen-free plantlets, induced resistance by culture techniques or genetic modifications (GM) (Table 3).

Fereol (1984) investigated *in vitro* inoculation of sugarcane with pure cultures of *U. scitaminea* and found positive infection. The pathogen penetrated the plant tissue and smut whips developed in plantlets. *In vitro* screening of sugarcane for smut susceptibility in South Africa was reported by Singh (2004). Four sugarcane cultivars were evaluated for smut resistance and that study was successful at inducing smut infection *in vitro*. The results obtained by Singh (2004) correlated with the field susceptibility ratings of the cultivars investigated. The author suggested that the method would be advantageous for pre-screening large numbers of new cultivars for smut resistance (Singh, 2004).

Table 3. Applications of *in vitro* sugarcane tissue culture techniques for eradication of/protection against pests and diseases.

Somaclonal variation/induced resistance		
<i>In vitro</i> route	Pathogen/Disease/Pest	Reference
Somatic embryogenesis	Fiji disease	Krishnamurthi and Tlaskal, 1974
	'eye spot' disease	Maribona <i>et al.</i> , 1986
	<i>Diatraea saccharalis</i> F. (borer)	White and Irvine, 1987
	Rust	Bravo <i>et al.</i> , 1989
	SCYLV, SCYP	Parmessur <i>et al.</i> , 2002
	Red rot	Singh <i>et al.</i> , 2008
Pathogen-free sugarcane		
<i>In vitro</i> route	Pathogen/Disease	Reference
Direct organogenesis (Meristem culture)	Fiji disease	Wagih <i>et al.</i> , 1995
	SCYLV	Fitch <i>et al.</i> , 2001; Chatenet <i>et al.</i> , 2001
	SCMV, SCYLV	Ramgareeb <i>et al.</i> , 2010
	Bacteria & Fungi	Cha-um <i>et al.</i> , 2006
Transgenic sugarcane		
Introduced gene	Pest	Reference
<i>cryIA(b)</i> for <i>Bt</i> endotoxin	borer (<i>Diatraea saccharalis</i>)	Arencibia <i>et al.</i> , 1997
Synthetic gene for aprotinin	borer (<i>Scirpophaga excerptalis</i>)	Christy <i>et al.</i> , 2009
Potato proteinase inhibitor II	canegrub (<i>Antitrogus consanguineus</i>)	Nutt <i>et al.</i> , 2001

An *in vitro* insect feeding bioassay was conducted in 2002 to determine resistance of transgenic rice plants to *Chilo suppressalis* Walker, which is a Lepidopteran similar to *E. saccharina* (Marfa *et al.*, 2002). The rice plants were transformed with *cryIB* and maize proteinase inhibitor (*mpi*) genes and were inoculated with *C. suppressalis* larvae hatched from surface sterilized eggs. That study was successful because larvae allowed to feed on rice plants transformed with Bt *cryIB*, died within days of infestation compared with larvae fed with untransformed or *mpi* transformed rice plants (Marfa *et al.*, 2002). The above-mentioned screening techniques for evaluating plant resistance to smut and a Lepidopteran insect demonstrate the potential of an *in vitro* approach and were the basis for this study.

3. MATERIALS AND METHODS

3.1 Plant material and *in vitro* culture

3.1.1 Collection and preparation of sugarcane

Sugarcane cultivars used in this study (NCo376, N26, N39, N27, Bt40 and ‘test’ cultivars: A, B, C, D and E) were harvested from SASRI, Mt Edgecombe, KZN. Secateurs were used to cut the top half of cane stalks and remove large leaves. Processing of cane stalks was conducted in the laboratory and explants were placed in culture within 6 h of harvesting. Immature stalk sections were inverted and wiped with absolute ethanol [100% (v/v)] (Fig. 11a). Individual leaf roll whorls were unraveled by hand. Stalks were wiped with ethanol after each layer was removed until the innermost node was visible. Stalk tips were inverted and immersed in absolute ethanol until excision (Snyman *et al.*, 1996). Further processing and excision was conducted under aseptic conditions within a laminar air-flow cabinet.

3.1.2 Apical meristem culture

Young leaf roll whorls were removed using a scalpel [Swann-Morton[®] (Sheffield, England), carbon steel surgical blade, no. 10A] and forceps. The explant material, containing the uppermost node and meristem, was trimmed to 2 cm. This was immersed in liquid MC2 medium (Table 4) to minimize the production of phenolic compounds. Meristems were excised under a dissecting microscope (Carl Zeiss, 16x mag). Meristematic tissue was sequentially removed from the basal explant until the meristem (size range: 1-5 mm) was exposed (Fig. 11b). This was excised and embedded in semi-solid meristem-initiation medium (MC1; Table 4). Three meristems were placed on each Petri dish, sealed with parafilm and incubated in the dark at $28 \pm 1^\circ\text{C}$ for 1 week (Fig. 11c). The developing meristems were sub-cultured to a new region on the medium every 2 days to avoid the negative influence of phenolic compounds secreted by the explant.

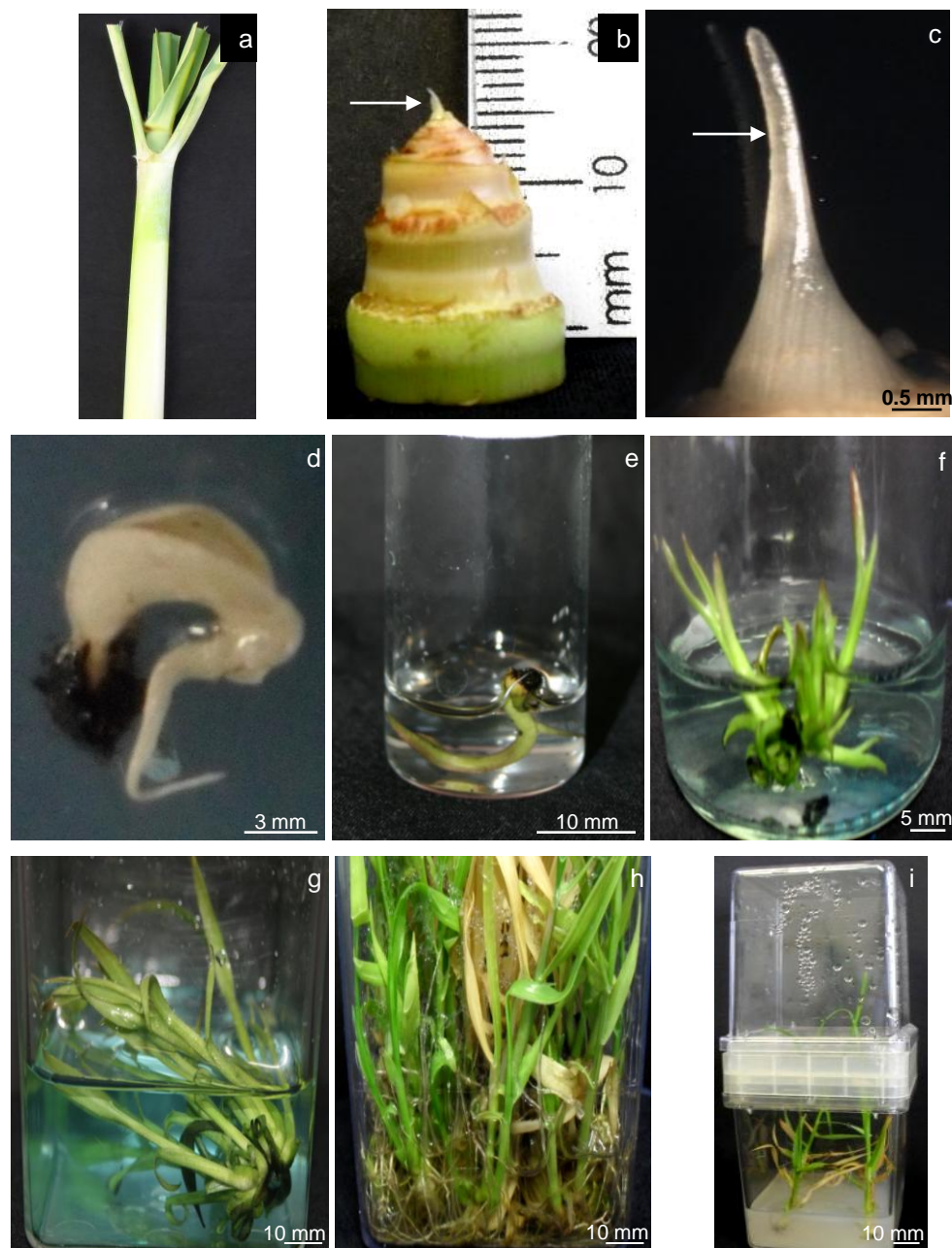


Figure 11. Apical meristem culture and propagation of plantlets. (a) Immature section of NCo376 sugarcane stalk, length = 35 cm. (b) Macroscopic image of exposed apical meristem. (c) Microscopic image of the apical meristem on semi-solid MC1 medium. (d) Developing meristem on semi-solid MC3 medium after 2 weeks. (e) Shoot elongation in liquid MC2 medium after 4 weeks (45 ml culture tube). (f) Shoot development in liquid SM medium after 6 weeks. (g) Further multiplication of shoots in a Magenta[®] vessel (Sigma-Aldrich (Pty) Ltd, Johannesburg, SA) containing SM medium, after 8 weeks in culture. (h) Rooted plantlets in liquid RM medium after 12 weeks. (i) Plantlets maintained on semi-solid PM medium.

Table 4. Media compositions used for plantlet generation from meristem culture. Liquid and semi-solid media based on MS* were used.

Meristem Culture Media						
Constituent	MC1	MC2	MC3	SM	RM	PM
MS*	4.42	4.42	4.42	4.42	4.42	4.42
BAP (mg/l)	0.1	0.1	0.1	0.1	-	-
kinetin (mg/l)	0.015	0.015	0.015	0.015	-	-
IBA (mg/l)	-	-	-	-	1.0	-
methylene blue	1.0	1.0	1.0	1.0	-	-
sucrose (g/l)	20	20	20	20	30	5
activated charcoal (g/l)	3.5	-	-	-	-	-
agar (g/l)	8	-	8	-	-	8
pH**	4.5	4.5	4.5	5.3	5.8	5.8

* MS basal salts and vitamins (Murashige and Skoog, 1962); Highveld Biological (Pty) Ltd., Johannesburg, SA.

**pH of media was adjusted using either 1M KOH or 1M HCl solutions before being autoclaved at 121°C for 20 min.

MC1- Meristem Culture Charcoal Medium;
 MC2- Meristem Culture Liquid Medium;
 MC3- Meristem Culture Semi-solid Medium;
 SM - Shoot Multiplication Medium;
 RM - Rooting Medium;
 PM - Plantlet Maintenance Medium.

After 8 days, viable meristems (size range: 10-15 mm) with emerging shoots were transferred onto MC3 medium (Table 4) and maintained at $26 \pm 1^\circ\text{C}$ with a 16 h light/8 h dark photoperiod in a controlled growth room (Fig. 11d). Illumination was provided by Phillips Master TL-D florescent lighting with an intensity of $35 \mu\text{mol.m}^{-2}.\text{s}^{-1}$. Sub-culturing was performed every 2 days until the meristem grew to 1 cm. It was then transferred to 3 ml of MC2 medium in 45 ml glass culture tubes (Fig. 11e).

After 2 weeks, shoots (± 2 cm) were transferred to larger glass culture bottles (100 ml) containing 20 ml of SM (Table 4) for shoot multiplication (Fig. 11f). Resulting shoot clusters were separated and transferred to fresh SM. Sub-culturing was carried out fortnightly for 6-8 weeks (Fig. 11g). When the shoots were ± 5 cm, they were separated and placed in liquid RM (Table 4) for root induction (Fig. 11h). Rooted plantlets were maintained on semi-solid PM (Table 4) and used in subsequent investigations (Fig. 11i).

3.1.3 Somatic embryogenic callus production

Immature portions of sugarcane stalks for embryogenic callus production were processed according to Snyman (2004). Thick outer leaf whorls were removed until the diameter of leaf roll was ≤ 1 cm (Fig. 12a). The soft immature leaf roll was placed in a Petri dish and cut into 2-3 mm transverse sections whilst partly immersed in liquid EC1 medium (Table 5), using a scalpel (E-MED Surgical blade, no. 10).

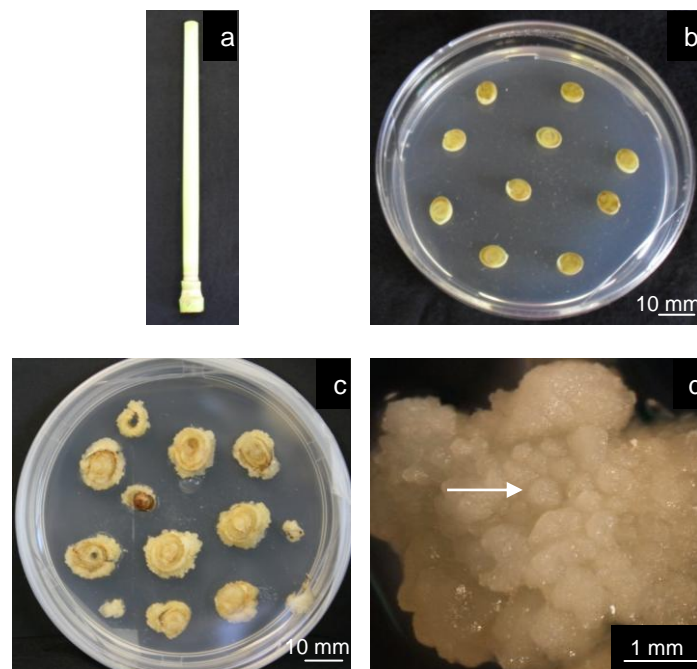


Figure 12. The production of somatic embryogenic callus from immature leaf roll culture. (a) Section of sugarcane stalk after processing in the lab, showing immature leaf roll (NCo376; 25 cm). (b) Transverse sections (2-3 mm) of the leaf roll on semi-solid EC2. (c) Development of callus after 4 weeks in culture. (d) Microscopic image of embryogenic callus after 8 weeks in culture.

Ten leaf roll disks were placed on each Petri dish (Fig. 12b), with the adaxial surface in contact with EC2 medium (Table 5). Each plate was sealed with parafilm and maintained in the dark at $28 \pm 1^\circ\text{C}$. Explants were transferred to fresh EC2 medium fortnightly. After 8 weeks, embryogenic calli were ready for use in subsequent investigations. Medium devoid of 2,4-D, EC3 (Table 5), was used for embryo germination.

Table 5. Composition of media used for the production of embryogenic calli from immature sugarcane leaf roll disks.

Somatic Embryogenesis Media			
Constituent (g/l)	EC1	EC2	EC3
MS	4.42	4.42	4.42
casein hydrolysate	0.5	0.5	0.5
sucrose	20	20	20
2,4-D (mg/l)	3	3	-
agar	-	8	8

pH of media were adjusted to 5.8, using 1M KOH or 1M HCl solutions, before being autoclaved at 121°C for 20 min.

3.2 *Ustilago scitaminea* inoculum preparation and *in vitro* inoculation

3.2.1 Collection and maintenance of *U. scitaminea* teliospores

Smut whips were collected from smut-infected sugarcane plants grown at SASRI (Fig. 13a). Teliospores were scraped off whips (Fig. 13b) and maintained overnight at 37°C to dry. Dried teliospores were stored in an air-tight container, containing activated silica-crystals and kept at ambient laboratory temperature ($21\text{-}25^\circ\text{C}$).

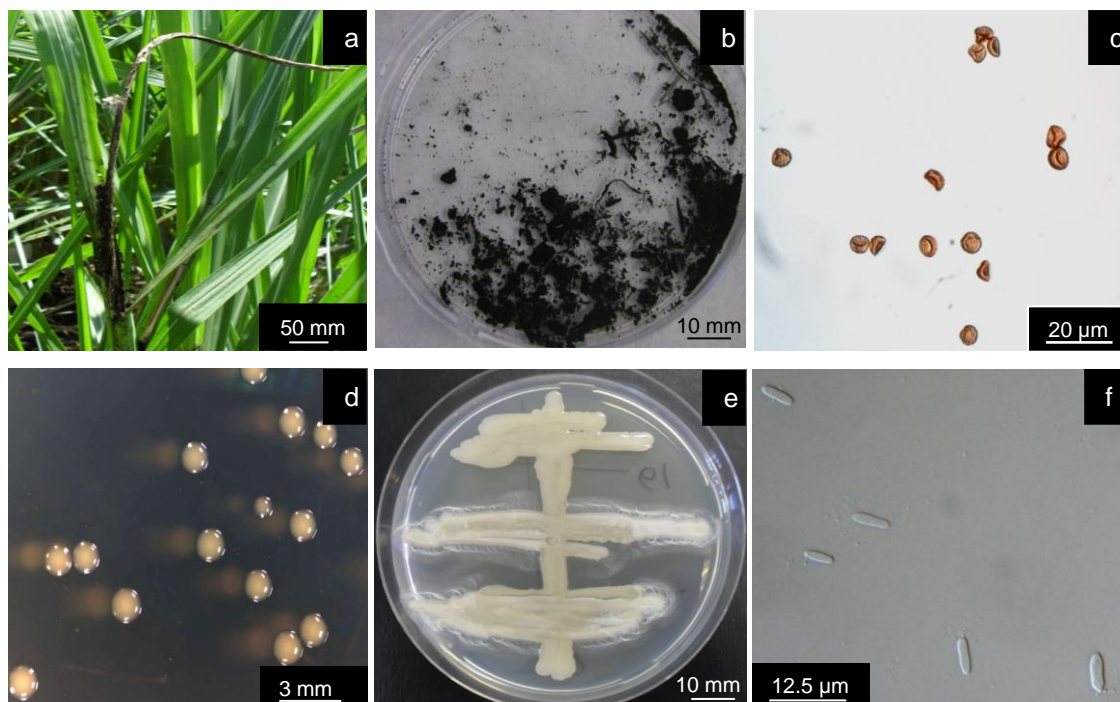


Figure 13. Steps in the preparation of *U. scitaminea* inocula. (a) Mature smut whip on infected sugarcane. (b) Smut teliospores scraped off from mature smut whip. (c) Microscopic image of *U. scitaminea* teliospores. (d) Sporidial colonies grown on PDA. (e) Cross-streaked sporidia on PDA which produced mycelia from the interaction of opposite mating strains. (g) Single mating-type sporidia.

3.2.2 Surface decontamination of teliospores and isolation of individual mating-type sporidial colonies

Teliospores were added to 1 ml of streptomycin sulphate solution [0.5 g/l (dissolved in sterile deionised water); Sigma-Aldrich (Steinheim, Germany)]. After 10 min, this solution was centrifuged for 2 min at 2000 x g (Tomy capsulefuge, PMC-060). The supernatant was discarded and spores were washed 3x in the streptomycin sulphate solution, after which, serial dilutions (10^{-1} – 10^{-4} sporidia/ml) were made in sterile deionised water. Each serial dilution (100 µl) was used to inoculate Potato Dextrose Agar [PDA; Merck (Wadeville, Gauteng, SA)] (Juangbhanich and Wangnon, 1983). This medium was made as per the manufacturer's instructions, pH 5.6 ± 0.2 . Sterile glass beads were placed on the agar surface and gently swirled to evenly spread the teliospore solution (Fig. 13c), after which the beads were removed. PDA plates streaked with either smut teliospores or sporidia were maintained at ambient laboratory conditions (21-25°C).

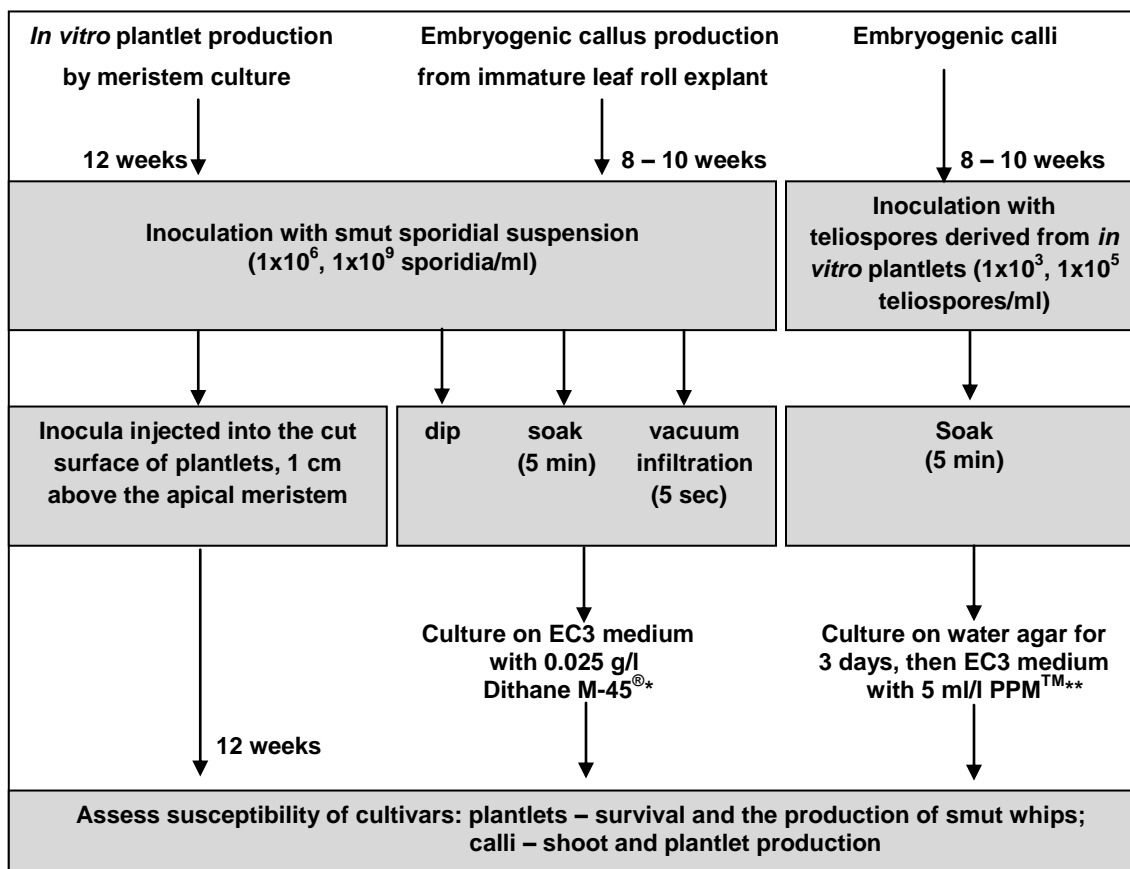
Teliospore development was checked after 18 h using a dissecting microscope (Leica MZ12₅; x40 mag). When germinating sporidia were observed, the surface of the medium was rinsed with 1 ml streptomycin solution and 100 µl of this liquid, containing sporidia, was added to 900 µl of the streptomycin solution. Serial dilutions were made and 100 µl of each dilution was used to streak PDA. After 24 h, discrete colonies were microscopically visible (Fig. 13d). A sterile metal loop was used to transfer cells from individual sporidial colonies onto fresh PDA for 3 days to allow growth.

3.2.3 Characterization of + and – sporidia

In order to determine mating compatibility between sporidial colonies, cells from individual colonies were cross-streaked on PDA (Saxena and Singh, 1966; Fereol, 1984; Albert, 1996). Cells from a single colony were streaked vertically on a Petri dish, whilst cells from 3 other colonies were streaked horizontally, separate from one another and labeled accordingly (Fig. 13e). These were maintained at ambient laboratory conditions (21-25°C) until mycelia, characterized by white ‘fuzz’, was observed (Fig. 13e). Successfully mated colonies were labeled as either + or – (Singh, 2004).

3.2.4 Preparation of sporidial suspensions and *in vitro* experimental approach

Individual colonies of opposite mating strains were grown on PDA for 5 days. A sterile metal loop was used to transfer sporidia into a plastic conical tube (10 ml), containing 5 ml sterile deionised water, which was vigorously shaken. A hemocytometer (Fuchs-Rosenthal Bright Line) was used to determine the concentration of these stock suspensions and subsequent dilutions (1×10^9 , 1×10^6 and 1×10^3 sporidia/ml) of single mating-type sporidia were made (Fig. 13f). Suspensions of opposite mating strains were combined (1:1 ratio) and inocula were used within 6 h (Singh, 2004). Susceptibility of sugarcane cultivars to *U. scitaminea* infection was assessed with 2 types of plant material, namely 12 week old *in vitro* plantlets and 8-10 week old embryogenic calli. A summary of the refined experimental approach is presented below (Fig. 14). Plantlet survival, the number of regenerated plantlets from embryogenic calli and the production of smut whips were assessed.



* Dithane M-45[®] – contact fungicide (Rohm & Haas, Philadelphia, USA) [active ingredient – 800g/kg Mancozeb: a coordination product of zinc ion and manganese ethylene bisdithiocarbamate (80%)]
 ** PPM[™] – Preservative for Plant Tissue Culture Media; Plant Cell Technology – Washington, USA [active ingredients: 5-Chloro-2-methyl-3(2H)-isothiazolone (0.1350%) and 2-methyl-3(2H)-isothiazolone 0.0412%]
 (www.ppm4plant-tc.com)

Figure 14. Experimental design for evaluating cultivar susceptibility to smut disease. A comparison was made between 2 types of plant material; *in vitro* plantlets and embryogenic calli. For plantlet investigations, cultivar susceptibility was determined by assessing smut whip production from plantlets and plantlet survival, 12 weeks post-inoculation. Shoot and plantlet production were recorded for calli investigations.

3.2.5 Plantlet inoculation by injection

Plantlets obtained 12 weeks after meristem culture were cut 1 cm above the apical meristem. A Hamilton syringe (10 μ l; Anatech Instruments (Pty) Ltd., Durban, SA) was used to inject 0.5 μ l sporidial suspension into this cut surface at a depth of 2 mm (Singh, 2004; Fig. 15). In the initial

experiment, inoculated NCo376 plantlets were maintained on semi-solid PM medium, however, this experiment was unsuccessful in sustaining plantlet growth or producing *in vitro* smut whips due to sporidial overgrowth in the medium. In a second approach sterile soil, which was autoclaved 3x, was used to sustain plantlets. This consisted of a 1:1 ratio of peat moss to vermiculite, supplemented with full strength MS (basal salts and vitamins) and 20 g/l sucrose. Although, *in vitro* smut whips were produced in the latter approach, contamination by various fungi was observed on the soil and plantlets, which affected plantlet survival.

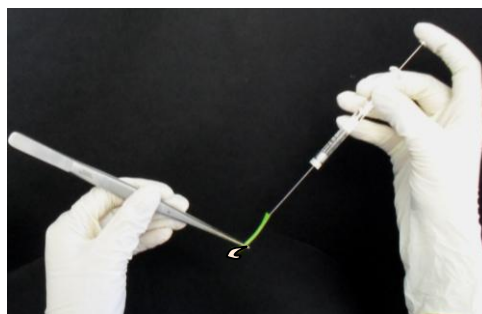


Figure 15. Plantlet inoculation with *U. scitaminea* sporidial suspension. Plantlets were cut 1 cm above the apical meristem and 0.5 μ l of the inoculum was injected into the leaf whorl.

The first approach was then refined by inclusion of the contact fungicide, Dithane M-45[®] (active ingredient: 800 g/kg mancozeb; Rohm and Haas, Philadelphia, USA) in PM medium. A range of fungicide concentrations (0-0.5 g/l) was investigated to determine the lowest concentration that eliminated sporidial growth. Inoculated plantlets were maintained at a 16 h light/8 h dark photoperiod in a controlled growth room with a temperature of $26 \pm 1^{\circ}\text{C}$. Plantlets were transferred to fresh medium every 4 weeks for a period of 12 weeks.

3.2.6 Embryogenic callus inoculation by immersion

Embryogenic calli obtained 8-10 weeks after leaf roll disk culture were used for assessing smut infections. Initially, immersion methods used to inoculate calli were a dip, 5 min soak and 5 sec vacuum infiltration in teliospore suspensions; however, sporidial overgrowth and sensitivity of calli to Dithane M-45[®] was a problem. Refinement of the protocol by changes to inoculum concentrations and composition of media were necessary. NCo376 calli (0.5 g/replicate) were used and a summary of all experiments is presented in Table 6.

Table 6. Experiments conducted during refinement of a protocol for callus inoculation with *U. scitaminea*.

	Inocula	Method of inoculation	Medium	Problem
Exp 1 (n = 3)	1x10 ⁶ and 1x10 ⁹ sporidia/ml	dip, 5 min soak, 5 sec vacuum infiltration	EC3	sporidial overgrowth on callus and in medium; no shoot germination
Exp 2 (n = 3-4)	1x10 ⁶ and 1x10 ⁹ sporidia/ml	dip, 5 min soak, 5sec vacuum infiltration	EC3 with 0.025 g/l Dithane M-45 [®] ; maintained for 2, 4 or 6 days then transferred to EC3	mycelial overgrowth on callus; no shoot germination
Exp 3 (n = 5)	1x10 ⁶ and 1x10 ⁹ sporidia/ml	5 min soak	EC3 with 5ml/l PPM [™]	mycelial overgrowth on callus; no shoot germination
Exp 4 (n = 5)	1x10 ³ , 1x10 ⁴ and 1x10 ⁵ sporidia/ml	5 min soak	EC3 with 5ml/l PPM [™]	sporidial & mycelial overgrowth on callus; no shoot germination
Exp 5 (n = 10)	1x10 ³ , 1x10 ⁵ teliospores/ml*	5 min soak	Water agar for 3 days, then transferred onto EC3 with 5 ml/l PPM [™] ; sub-cultured fortnightly	[to be assessed]

* Note: In Experiment 5, sterile teliospores obtained from the whips produced on *in vitro* plantlets were used as inocula.

3.3 *Eldana saccharina* inoculum preparation and *in vitro* inoculation

3.3.1 Surface decontamination of *E. saccharina* eggs

Five day old fertilized *E. saccharina* eggs laid on tissue paper (Fig. 16a), were obtained from the Insect Rearing Unit at SASRI, Mt. Edgecombe. The tissue paper was cut around egg clusters and eggs were immersed in sodium hypochlorite (NaOCl) solutions [0 (sterile deionised water), 0.5% and 1.0% v/v] for different time periods (5, 10, 15 or 20 min). Gentle swirling assisted detachment of eggs from the paper. After treatment, eggs were transferred to sterile filter paper, placed in a metal strainer over a beaker (Fig. 16b), and subsequently rinsed 3x with sterile deionised water. The liquid was allowed to drain into the beaker for 10 min.

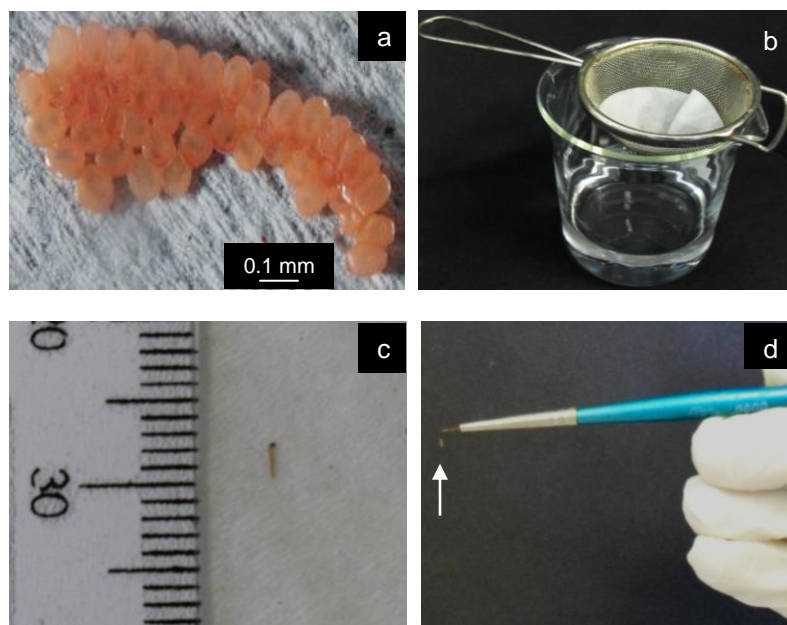


Figure 16. Preparation of *E. saccharina* inoculum. (a) Fertilized *E. saccharina* egg clusters laid on tissue paper, 5 days after oviposition. (b) Eggs were placed on filter paper and rinsed 3x with sterile deionised water after exposure to NaOCl. (c) First instar larva (± 2 mm). Image was captured within 12 h of larval emergence. (d) Method of larval transfer: a sterile fine-tip paint brush was used to gently transfer larvae onto plantlets or calli. Arrow shows larva suspended from the brush tip.

Ten eggs from each treatment were transferred to 523 medium [comprised of 10 g/l sucrose, 8 g/l casein hydrolysate, 4 g/l yeast extract, 2 g/l KH_2PO_4 , 0.15 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 8 g/l agar (Viss *et al.*, 1991)]; with 3 replicates per treatment. Plates were maintained at ambient laboratory

temperature (21–25°C). Microbial growth on the medium was monitored for 2 weeks. In order to assess larval emergence, eggs exposed to the same treatments described above were placed on sterile filter paper and enclosed in Petri dishes until larvae emerged. The treatment which yielded the best larval emergence and elimination of microbes was used to decontaminate eggs in later experiments.

3.3.2 Incubation of eggs and emergence of larvae

In subsequent investigations, surface decontamination of eggs involved a 15 min exposure to 1.0 % NaOCl, followed by a 3x rinse with sterile deionised water. Filter paper containing eggs were removed from the metal strainer and laid on a Petri dish for 5 min. Eggs were removed from the filter paper and placed in sterile Eppendorfs. First instar larvae emerged after 2-3 days (Fig. 16c).

3.3.3 *In vitro* experimental approach

A summary of the experimental approach used to assess the susceptibility of *in vitro* sugarcane plantlets and embryogenic calli to *E. saccharina* infestation is presented in Fig. 17.

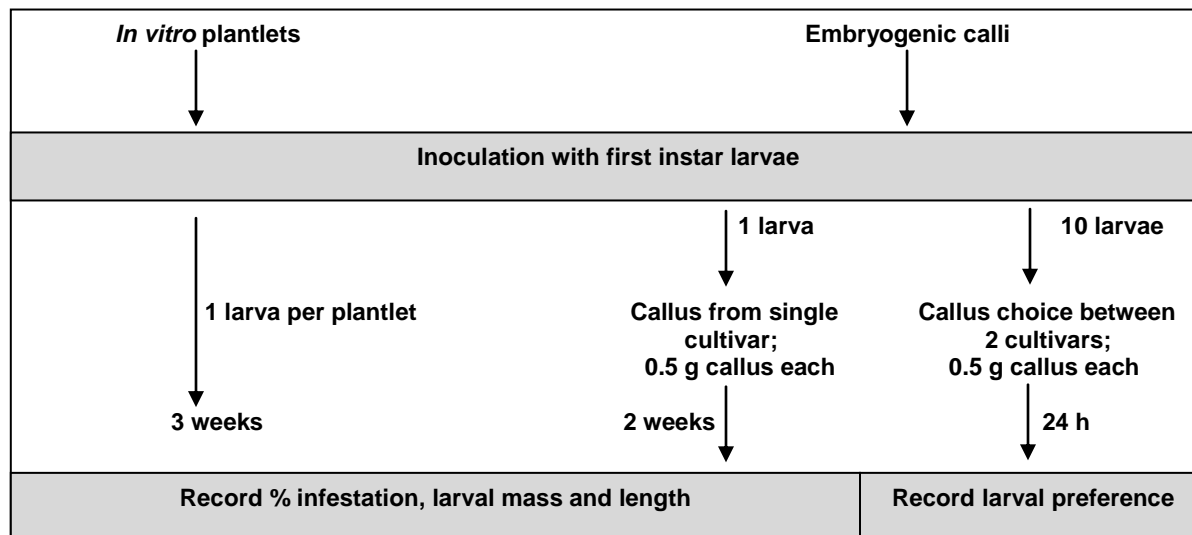


Figure 17. Experimental design for evaluating the susceptibility of sugarcane cultivars to *E. saccharina* infestation. *In vitro* plantlets and embryogenic calli were inoculated with first instar larvae. Susceptibility was assessed by comparing percentage infestation, larval mass and length after larval feeding on *in vitro* plantlets and calli. Larval preference by callus choice assessment was determined by exposing 10 larvae to calli derived from NCo376 and the other cultivars evaluated (N26, N39, N27, Bt40 and test cultivars – A, B, C, D and E) for 24 h, and counting the number of larvae per callus genotype.

3.3.4 Plantlet inoculation

A sterile fine paint brush [Winsor Newton™ (London, UK), Cotman Series 0000] was used to transfer first instar larvae (Fig. 16d) on 12 week old *in vitro* plantlets (height: 10-12 cm). Inoculated plantlets were maintained on semi-solid PM medium (Table 4), at growth room conditions (section 3.2.5). Visual assessment for frass (positive diagnosis for infestation) was conducted and the number of plants infested was recorded weekly. Larvae were removed from plantlets, 3 weeks after inoculation. Larvae were placed in individual Eppendorfs and in a freezer for 6 h before larval length and mass were recorded.

3.3.5 Callus bioassay

Initially for the callus feeding bioassay, embryogenic calli (0.5 g) were placed on filter paper, dampened with sterile deionised water, over EC2 medium and 5 larvae were placed on the calli. Plates were sealed with parafilm and masking tape and maintained in the dark. Larval mass and length were recorded after 2 weeks (n = 10). However, larval recovery from this experiment was poor. A second approach was then carried out by placing a single larva on 0.5 g calli in universal bottles, containing 10 ml EC3 medium, which were maintained in the dark.

In order to investigate larval preference, a callus choice bioassay was conducted. Calli from NCo376 and each of the other cultivars evaluated were placed opposite each other (approx. 70 mm apart) on dampened filter paper, and 10 larvae placed in the middle of the Petri dish. Plates were sealed and maintained in the dark for 24 h. Larval preference was determined by counting the number of larvae per callus genotype.

3.4 Assessment of *in vitro* screening methods

Methods were applied to N26 (susceptible to *E. saccharina*; resistant to smut), N39 (susceptible to smut; resistant to *E. saccharina*), and 5 'test' cultivars (A, B, C, D and E) in order to assess the accuracy of the protocols. The identity and associated field-resistance ratings of the 'test' cultivars were undisclosed until completion of the experiments. In addition, screening methods developed for *E. saccharina* were applied to N27 (susceptible to *E. saccharina*) and Bt40, the latter of which is a genetically transformed N27, with the *Bacillus thuringiensis* (Bt) toxin *cry1Ab* gene which has been shown to kill insects when ingested (Gill *et al.*, 1992).

3.5 Data analysis

The statistical program, Genstat version 12, was used to analyze data in this study. The data were initially tested for normality and homogeneity using the Kolmogorov-Smirnov and Bartlett's tests ($P < 0.05$), respectively. In addition, SAS version 9.2 (2009), was used to analyze percentage infestation data obtained from *E. saccharina* investigations. Details of specific analyses are presented in the appropriate sections. Microsoft[®] (2007) Excel was used to generate graphs, calculate means and Standard Error (SE).

3.6 Photography and microscopy

Macroscopic images were captured with a Samsung (ES-15) 10.2 megapixel digital camera. A Nikon DS-Fi1 camera attached to either a Nikon SMZ1500 or Nikon Eclipse 80i microscope were used to capture light microscopic images. Stereo images were captured by a Nikon DXM 1200C camera and Nikon AZ100 compound microscope. A Leo 1450 Scanning Electron Microscope (SEM) was used to capture high magnification images of smut whips and teliospores.

4. RESULTS

4.1 *Ustilago scitaminea*

4.1.1 Preliminary investigations and establishment of protocols for *in vitro* smut screening

4.1.1.1 Assessment of inoculum concentrations and the control of sporidial overgrowth by the contact fungicide, Dithane M-45[®]

An initial investigation was conducted to determine an appropriate sporidial suspension concentration range for inoculation. Opposite mating strains of *U. scitaminea* sporidia were combined in a 1:1 ratio (Singh, 2004) and 50 μ l of 3 concentrations (1×10^3 , 1×10^6 and 1×10^9 sporidia/ml) were streaked on PDA. Plates were observed for 2 weeks for mycelial development characterized as a white ‘fuzz’ phenotype. There was a significant difference in mating success amongst the 3 concentrations of sporidial suspensions (results not shown, data were logit transformed and analyzed using a Generalized linear mixed model analysis, $P < 0.05$, $df = 2$, F statistic = 265.57, $n = 10$). Sporidial concentrations of 1×10^6 and 1×10^9 sporidia/ml were adequate to allow opposite mating strains to fuse. These concentrations were used to inoculate plant material in subsequent investigations.

In preliminary smut investigations, sporidial overgrowth was observed in media maintaining inoculated calli (EC3) and plantlets (PM) after a few days, resulting in necrosis of all plant material. In order to establish a means to control the sporidial overgrowth, a contact fungicide, Dithane M-45[®], was incorporated into EC3 medium. An effective concentration of the fungicide was determined by streaking 50 μ l of 1×10^9 sporidia/ml on EC3 medium incorporating 0 (control), 0.0025, 0.005, 0.01, 0.025 and 0.05 g/l Dithane M-45[®]. There was a significant difference in sporidial growth after 2 weeks (data were logit transformed and analyzed by a Generalized linear mixed model analysis, $P < 0.05$, $df = 5$, F statistic = 717.98, $n = 10$) (Fig. 18). The lowest concentration of Dithane M-45[®] which successfully inhibited the growth of sporidia was 0.025 g/l. The effect of this fungicide concentration on the development of plantlets and calli was further investigated.

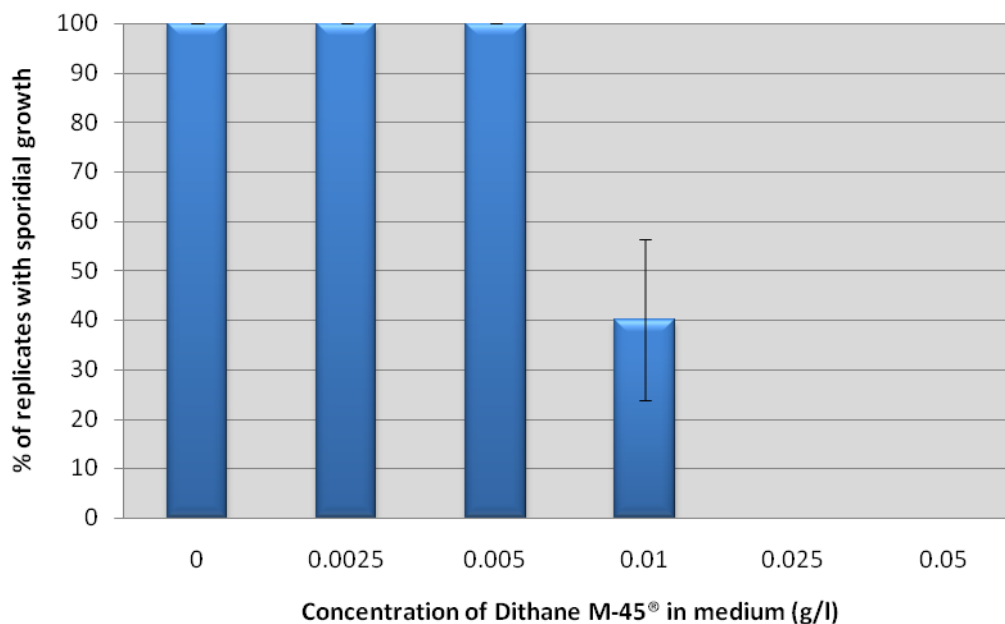


Figure 18. The effect of increasing Dithane M-45® concentration on *U. scitaminea* sporidial development. Data were logit transformed and subjected to a Generalized linear mixed model analysis. Sporidial growth on EC3 medium, after 2 weeks, was significantly different, $P < 0.05$, mean \pm SE, df = 5, F statistic = 717.98, n = 10.

4.1.1.2 Protocol development for plantlet inoculation

The method of inoculation described by Singh (2004) was used in the current study. Stalks of *in vitro* plantlets were cut transversely in order to inject the sporidial suspension using a Hamilton® syringe. It was necessary for this inoculation to be as close to the meristem as possible because smut whips develop from the growing point of sugarcane stalks. A preliminary investigation was conducted with NCo376 plantlets which were cut to 3 lengths, i.e. 1, 2 and 3 cm and injected with 0.5 μ l of sterile deionised water. These were maintained on semi-solid PM medium for 3 weeks in order to determine the effect of plantlet length and inoculation by injection on plantlet survival. Each Magenta® vessel contained 4 plantlets and 3 replicates were used per treatment. The percentage plantlet survival from each treatment is presented in Fig. 19. There was no significant difference in plantlet survival (Generalized linear mixed model analysis, $P > 0.05$, df = 2, F statistic = 0.41, n = 3).

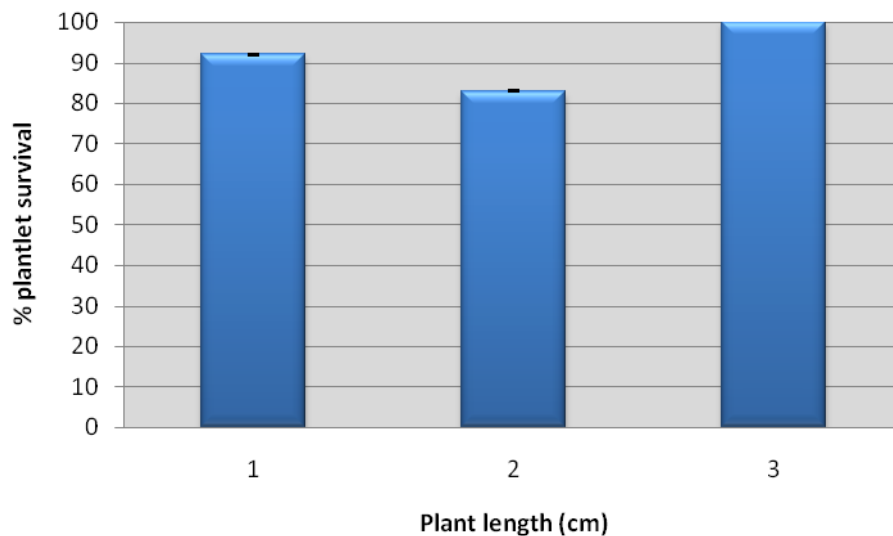


Figure 19. The effect of plantlet length (cut to 1, 2 and 3 cm) and inoculation with 0.5 μ l sterile deionised water on the survival of NCo376 plantlets after 3 weeks in culture. Data were logit transformed and analyzed with a Generalized linear mixed model analysis, $P > 0.05$, $df = 2$, F statistic = 0.41, mean \pm SE, $n = 3$.

Subsequently, *in vitro* NCo376 plantlets were cut to 1 cm and injected with 0.5 μ l of 1×10^6 and 1×10^9 sporidia/ml. In the initial experiment, inoculated plantlets were maintained on PM medium in a 16 h light/8 h dark photoperiod. As mentioned in section 4.1.1.1, *U. scitaminea* sporidia developed rapidly in PM medium maintaining the plantlets which resulted in 100% mortality of NCo376 plantlets. A second approach involved plantlet maintenance on a 1:1 ratio of sterile peat moss and vermiculite supplemented with MS nutrients and 20 g/l sucrose. Although smut whips developed from inoculated plantlets after 10 weeks in culture, the soil was contaminated by fungi other than *U. scitaminea*. Despite the application of 0.025 g/l Dithane M-45[®] to the soil, the fungi continued to develop on plantlets.

The former approach was then refined by the addition of 0.025 g/l Dithane M-45[®] to PM medium. Plantlets were cut to 3 lengths, injected with sterile deionised water and maintained in the same conditions as above for 3 weeks. No significant difference was observed in plantlet survival (binomial data, logistic regression analysis, $\chi^2 p > 0.05$, $df = 2$, mean deviance = 2.21 for $n = 25$) (Fig. 20). Therefore for subsequent *U. scitaminea* investigations, plantlets were cut to 1 cm and maintained on PM with 0.025 g/l Dithane M-45[®].

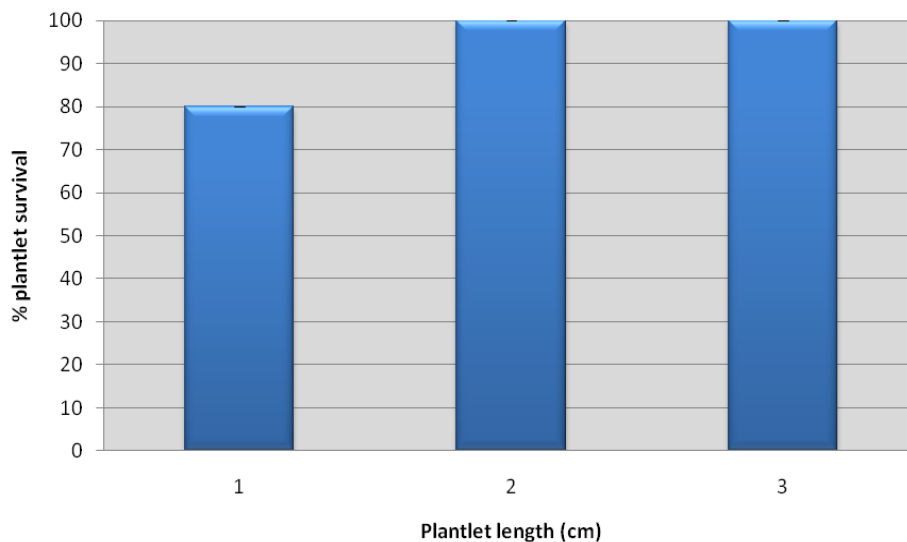


Figure 20. The effect of 0.025 g/l Dithane M-45[®] on the survival of plantlets cut to 3 lengths (1, 2 and 3 cm) and injected with 0.5 µl of sterile deionised water. A logistic regression analysis revealed no significant difference in plantlet survival, chi pr > 0.05, df = 2, mean deviance = 2.21, mean ± SE, n = 25.

4.1.1.3 Protocol development for callus investigations

In order to determine the effect of Dithane M-45[®] on callus development, 0.5 g per replicate of NCo376 callus was cultured on EC3 medium incorporating 0.025 g/l Dithane M-45[®]. Cultures were maintained in a 16 h light/8 h dark photoperiod and sub-cultured fortnightly for 6 weeks. A visual comparison of calli from treatment and control after 6 weeks is presented in Fig. 21. There was a significant difference in shoot production between the treatment and control [data subjected to Mann-Whitney U (Wilcoxon rank-sum) test, $P < 0.05$, $U = 0.0$, $n = 10$]. NCo376 calli were extremely sensitive to 0.025 g/l Dithane M-45[®] and all embryos failed to germinate.

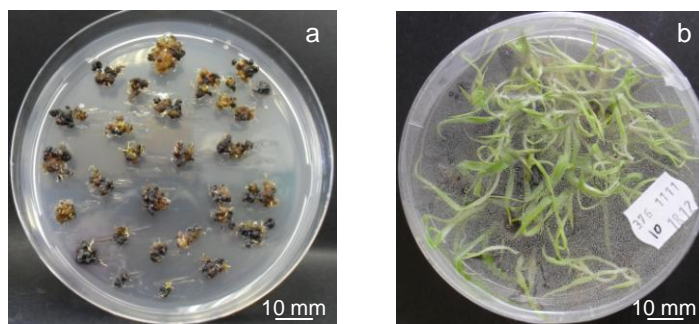


Figure 21. A visual comparison of NCo376 embryogenic calli maintained on semi-solid EC3 medium (a) including 0.025 g/l Dithane M-45[®] and (b) devoid of Dithane M-45[®]. Embryos failed to germinate and were necrotic when exposed to Dithane M-45[®] for 6 weeks whilst embryos from the control successfully germinated and produced shoots. Data were analyzed using a Mann-Whitney U (Wilcoxon rank-sum) test, $P < 0.05$, $U = 0.0$, $n = 10$.

Several experiments were conducted in order to refine a protocol for callus inoculation with *U. scitaminea* sporidial suspensions. As mentioned previously (section 4.1.1.1), 100% necrosis of inoculated calli was observed when cultured on EC3 medium due to sporidial overgrowth in the medium. In light of the fact that calli are sensitive to sustained exposure to Dithane M-45[®] (Fig. 21), a second experiment investigated temporary exposure of inoculated calli to Dithane M-45[®]. Calli inoculated by a dip, 5 min soak and 5 sec vacuum infiltration with 2 inoculum concentrations (1×10^6 and 1×10^9 sporidia/ml), and sterile deionised water as a control, were maintained on EC3 medium including 0.025 g/l Dithane M-45[®] for 2, 4, and 6 days, followed by transfer to fresh EC3 medium devoid of Dithane M-45[®]. However, sporidial and mycelial overgrowth was observed within a week of inoculation, in all instances (Fig. 22).



Figure 22. NCo376 callus (0.5 g) inoculated with 1×10^9 sporidia/ml and cultured on EC3 medium incorporating 0.025 g/l Dithane M-45[®] for 6 days. Image shows overgrowth of sporidia and white mycelia on necrotic calli and the surrounding medium.

In a further attempt to control sporidial overgrowth, the broad spectrum tissue culture biocide/fungicide, PPM™, was included in EC3 medium. A third experiment involved the culture of inoculated calli, as described above, on EC3 medium including 5 ml/l PPM™. The inclusion of PPM™ was effective in preventing the development of sporidia and mycelia on the medium. However, mycelial overgrowth was observed on the surface of calli and caused necrosis whilst the control calli, inoculated with sterile water were uncontaminated and later produced shoots. More mycelia were observed when calli were inoculated with 1×10^9 compared with 1×10^6 sporidia/ml (Fig. 23).

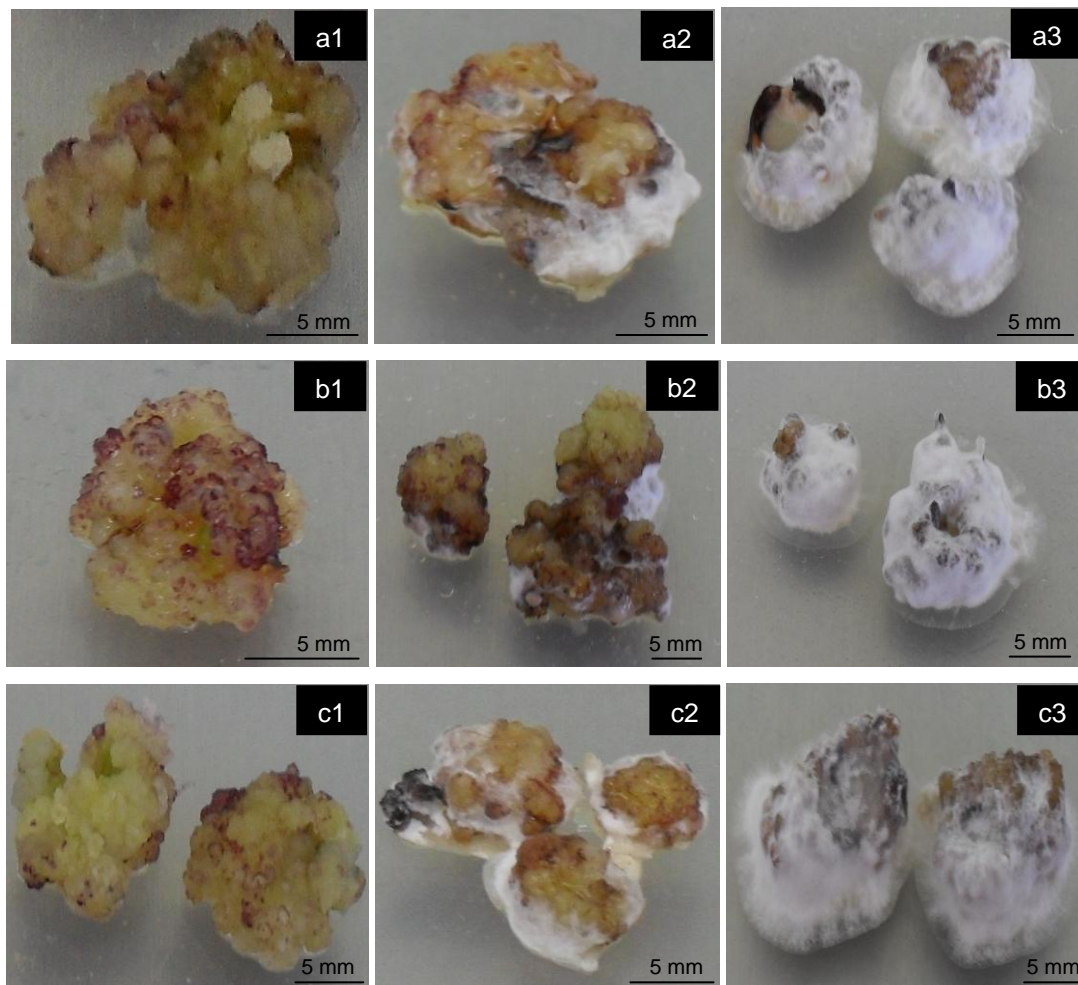


Figure 23. A visual comparison of inoculated NCo376 embryogenic calli maintained on EC3 medium incorporating 5 ml/l PPM™. Images were captured 1 week after inoculation with *U. scitaminea* sporidial suspensions by the dip (a1 - a3), 5 min soak (b1 - b3) and 5 sec vacuum infiltration (c1 - c3) methods. a1, b1 and c1 represents controls in which calli were inoculated with sterile deionised water. a2, b2 and c2 show calli inoculated with 1×10^6 sporidia/ml and a3, b3 and c3 show calli inoculated with 1×10^9 sporidia/ml. All calli inoculated with sporidial suspensions were necrotic whilst control calli successfully produced shoots.

The protocol described above was further refined by inoculation of calli with lower concentrations of *U. scitaminea* sporidial suspensions, i.e. 1×10^3 , 1×10^4 and 1×10^5 sporidia/ml using only a 5 min soak treatment. Inoculated calli were cultured on EC3 medium with 5 ml/l PPM™. Sporidial overgrowth was observed on the surface of calli inoculated 1×10^3 and 1×10^4 sporidia/ml; whilst mycelia and sporidia developed on the surface of calli inoculated with 1×10^5 sporidia/ml. Therefore, the lower 2 sporidial concentrations did not facilitate successful mating. This experiment was unsuccessful as all calli were necrotic after 1 week and failed to produce shoots.

In summary, the outcome of preliminary plantlet investigations for protocol development revealed that plantlets could be cut to 1 cm and inoculated by injection with 1×10^6 and 1×10^9 sporidia/ml. Maintenance of cultures in semi-solid PM medium incorporating 0.025 g/l Dithane M-45® was adequate to prevent plantlet death caused by sporidial overgrowth in the medium. However, protocol development for callus investigations was challenging. The dip, 5 min soak and 5 sec vacuum infiltration treatments did not affect calli survival or the production of shoots; however, calli were necrotic when exposed to the inoculum and were sensitive to 0.025 g/l Dithane M-45®. The inclusion of 5 ml/l PPM™ in EC3 medium was adequate for the prevention of sporidial overgrowth in the medium; however sporidial and mycelia overgrowth on the surface of calli resulted in necrosis. An additional investigation for embryogenic callus inoculation with *U. scitaminea* was conducted later in this study and is described in section 4.1.2.2.

4.1.2 *In vitro* screening of cultivars for *U. scitaminea* susceptibility

4.1.2.1 Plantlet inoculation

Following inoculation with 0.5 µl of 1×10^6 and 1×10^9 sporidia/ml suspensions by injection into the cut surface of plantlets, the innermost leaf continued to grow whilst the outer leaves, around the 1 cm area, browned (Fig. 24a, b and c). White mycelia were observed at the cut surface of some plantlets within a week of inoculation. Sporidia were observed on the surface of the medium around the base of plantlets in some cultures, 1 week after inoculation. However, sporidia did not penetrate the medium as was previously observed when inoculated plantlets were maintained on PM medium devoid of Dithane M-45®. Sporidial overgrowth was observed in all cultures 6 weeks post-inoculation.

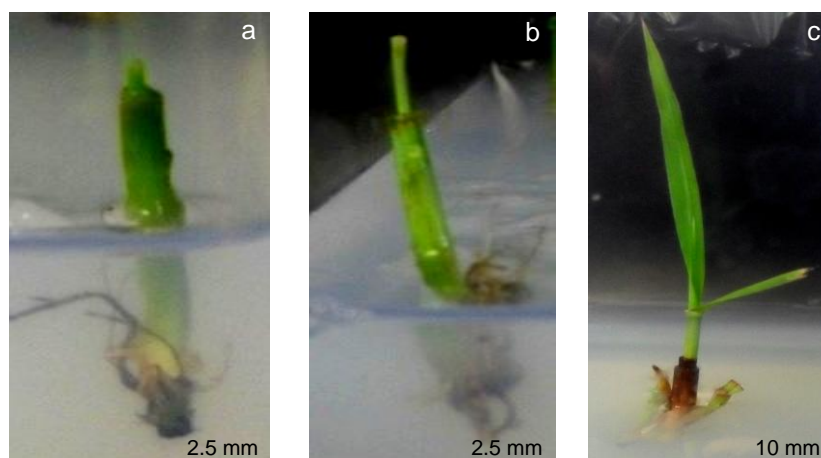


Figure 24. Development of *in vitro* NCo376 plantlets after inoculation with *U. scitaminea* sporidial suspension. (a) Emergence of the innermost leaf, 2 days after cutting and inoculation. (b) Browning of the cut area and emergence of the new leaf, 5 days after inoculation. (c) Sturdy plantlet, 2 weeks after inoculation.

At 12 weeks post-inoculation, plantlet survival and whip production was recorded (Table 7). Survival was poor in most cultivars. The greatest percentage survival was observed when ‘test’ cultivar E was inoculated with 1×10^6 sporidia/ml; whilst all inoculated N39 plantlets died (regardless of sporidial concentration).

Table 7. A comparison of plantlet survival and whip production across cultivars, 12 weeks post-inoculation. Plantlet survival and whip production are compared between inoculum concentrations and total plantlet survival is compared across cultivars. Dissimilar alphabet characters denote a statistical significance (data subjected to a Generalized linear mixed model analysis, $P < 0.05$, $n = 50$).

Cultivar	% Plantlet survival		% Total plantlet survival*	% Plantlets producing whips	
	inoculum concentration			inoculum concentration	
	(sporidia/ml)			(sporidia/ml)	
	1×10^6	1×10^9		1×10^6	1×10^9
NCo376	60	16	38 ^{cd}	12	20
N26	16	20	18 ^{bc}	0	4
N39	0	0	0 ^a	0	0
A	4	24	14 ^b	0	8
B	56	52	54 ^d	16	4
C	16	16	16 ^{bc}	0	0
D	8	20	14 ^b	0	0
E	84	36	60 ^d	12	0

* Mean plantlet survival observed with both inoculum concentrations

A comparison of percentage plantlet survival between inoculum concentrations revealed that plantlet survival increased for N26 and 'test' cultivars A and D when inoculated with 1×10^9 sporidia/ml compared with 1×10^6 sporidia/ml; whilst plantlet survival were equal at both concentrations for 'test' cultivar C. All other cultivars showed a decrease in survival when inoculated with the higher inoculum concentration.

Statistical analysis of plantlet survival data revealed no significant difference in the mean survival between plantlets inoculated with 1×10^6 and 1×10^9 sporidia/ml (Generalized linear mixed model analysis, $P > 0.05$, $df = 1$, F statistic = 3.79, $n = 25$). However, the percentage of total plantlet survival was significantly different (Generalized linear mixed model analysis, $P < 0.05$, $df = 7$, F statistic = 7.91, $n = 50$) (Table 7). Statistical differences were observed between N39; 'test' cultivars A and D; and 'test' cultivars B and E. The greatest total plantlet survival was observed in the latter 2 'test' cultivars, 54% and 60%, respectively.

Smut whip production observed in 5 of the 8 (63%) cultivars inoculated with 1×10^6 sporidia/ml, correlated with the cultivars' field resistance ratings. However, N26 (field resistant) produced a smut whip *in vitro* when inoculated with the higher inoculum concentration (1×10^9 sporidia/ml) (Table 7). NCo376 and 'test' cultivar B produced whips when inoculated with both concentrations of sporidial suspensions. NCo376 produced a whip earlier than the other cultivars, after 5 weeks in culture. N26 and 'test' cultivar A plantlets only produced smut whips when inoculated with 1×10^9 sporidia/ml, whilst 'test' cultivar E only produced whips when inoculated with 1×10^6 sporidia/ml. The highest percentage of smut whips was produced by NCo376 plantlets inoculated with 1×10^9 sporidia/ml. However, no significant difference was found in the production of smut whips across cultivars (binomial data analyzed by a Generalized linear mixed model analysis, $P > 0.05$, $df = 7$, F statistic = 1.66, $n = 50$).

In order to microscopically examine the structures of an *in vitro* smut whip and verify *U. scitaminea* teliospores, a whip was removed from an NCo376 inoculated plantlet (Fig. 25a-f). The *in vitro* derived whip closely resembled smut whips which develop in field-infected sugarcane, albeit proportionally smaller in size (Fig. 25a). The whip epidermis was silver, papery thin, and had burst at several regions (Fig. 25b and c). Within the whip was a dense compaction of dark brown teliospores (Fig. 25c) and the whip appeared folded (Fig. 25d).

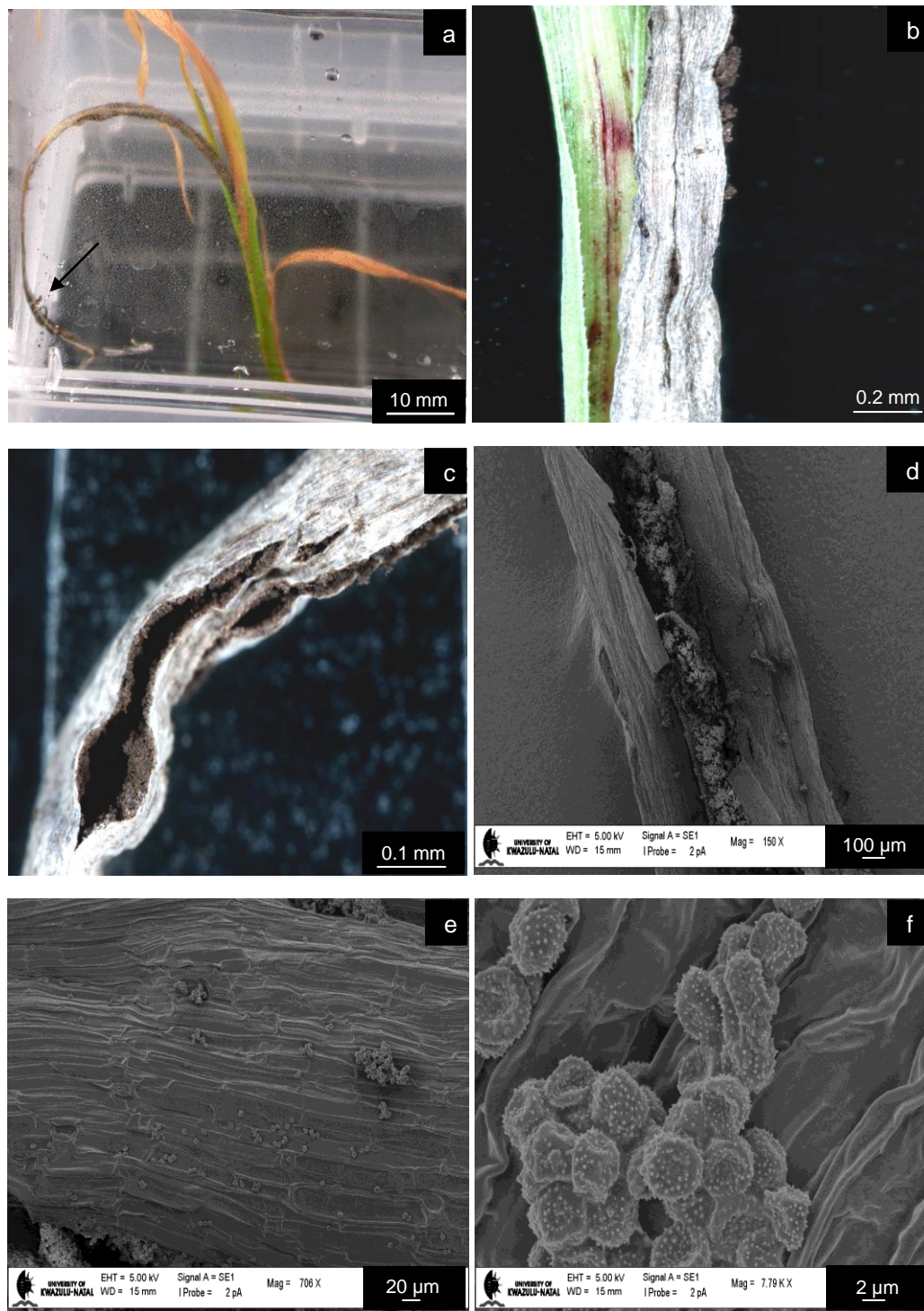


Figure 25. Microscopic examinations of an *in vitro* smut whip (a-f). (a) An *in vitro* smut whip which developed on a NCo376 plantlet. (b) Lower surface of whip shows silvery outer layer. (c) Burst whip showing dark brown teliospores within whip. (d) SEM image of folded whip and teliospores within. (e) Interior surface of whip which consists of structures resembling the shape of typical plant cells with teliospores dispersed on its surface. (f) Higher magnification of previous image shows a cluster of *U. scitaminea* teliospores.

Upon closer examination by SEM, the shape of typical plant cells were observed in the inner surface of the whip (Fig. 25e). Further examination revealed distinct clusters of *U. scitaminea* teliospores attached to the inner surface of the whip (Fig. 25f).

In order to detect the presence of fungal mycelia in plant tissue of infected *in vitro* NCo376 plantlets, the leaf tissue was stained with Lactophenol blue and viewed with a light microscope. Fungal mycelia were observed in a longitudinal section of leaf tissue from the upper region of the infected plantlet (Fig. 26). In addition, stained stem tissue from inoculated plantlets which did not produce whips revealed that fungal mycelia were present in the tissue.

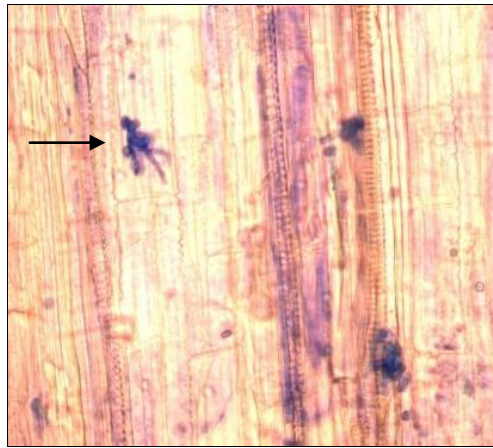


Figure 26. Longitudinal section of infected NCo376 leaf tissue showing fungal mycelia stained with Lactophenol blue (x200).

A visual comparison was made between teliospore suspensions obtained from smut whips produced in *in vitro* plantlets and field infected sugarcane (Fig. 27a and b). As expected, teliospore suspensions from the field contained foreign debris and possibly contaminating fungi, whilst *in vitro* derived teliospores were uncontaminated.

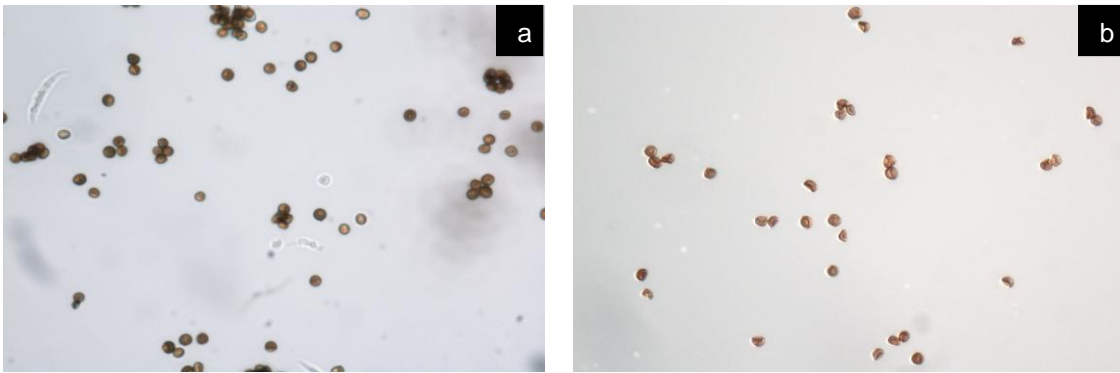


Figure 27. A visual comparison of teliospore suspensions from (a) a smut whip from field infected sugarcane and (b) an *in vitro* smut whip (x400).

In order to determine viability of the teliospores which developed *in vitro*, a concentration of 1×10^3 teliospores/ml was cultured on PDA medium and water agar, and maintained for 5 days at ambient temperature (21-25°C) (Fig. 28). Germinating teliospores produced numerous sporidial colonies when cultured on the nutrient-rich PDA. Teliospores cultured on water agar did not produce sporidial colonies. Instead, the characteristic white ‘fuzz’ phenotype was directly initiated.

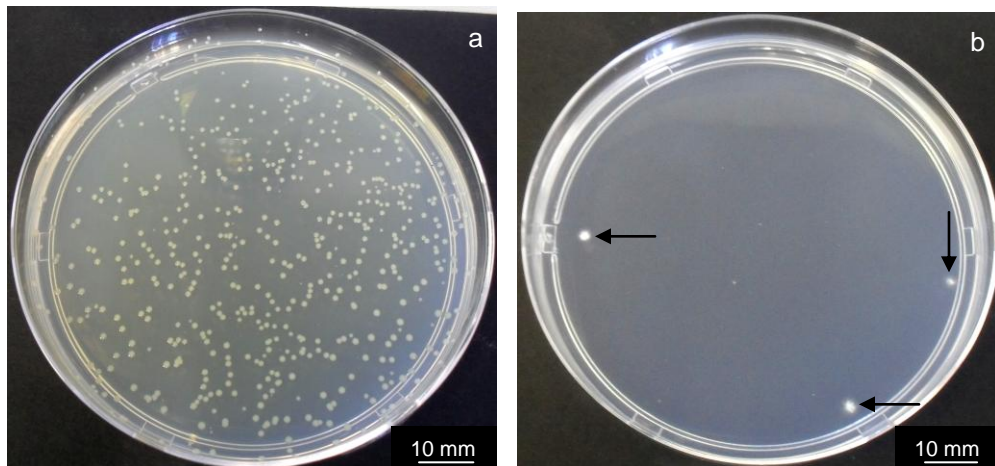


Figure 28. Germination of sterile teliospores (1×10^3 sporidia/ml) harvested from an *in vitro* NCo376 plantlet on (a) PDA and (b) water agar. Teliospores cultured on PDA produced numerous individual mating-type sporidial colonies whilst teliospores cultured on water agar produced white mycelia after 5 days.

4.1.2.2 Callus inoculation

As previously mentioned (section 4.1.1.1), all preliminary callus experiments resulted in sporidial and mycelial overgrowth on calli. Therefore in a last attempt to develop a successful protocol, sterile teliospores harvested from *in vitro* smut whips were used as inocula. NCo376 calli were soaked for 5 min in sterile teliospore suspensions of 2 concentrations, i.e. 1×10^3 and 1×10^5 teliospores/ml. Inoculated calli were cultured on water agar for 3 days, to lessen the likelihood of sporidial production from germinating teliospores, before being transferred to EC3 medium incorporating 5 ml/l PPMTM. Although sporidial overgrowth and calli necrosis were observed in 40% of replicates ($n = 10$) (Fig. 29a), shoots successfully developed from calli inoculated with *U. scitaminea* teliospore suspensions (Fig. 29b, c and d).

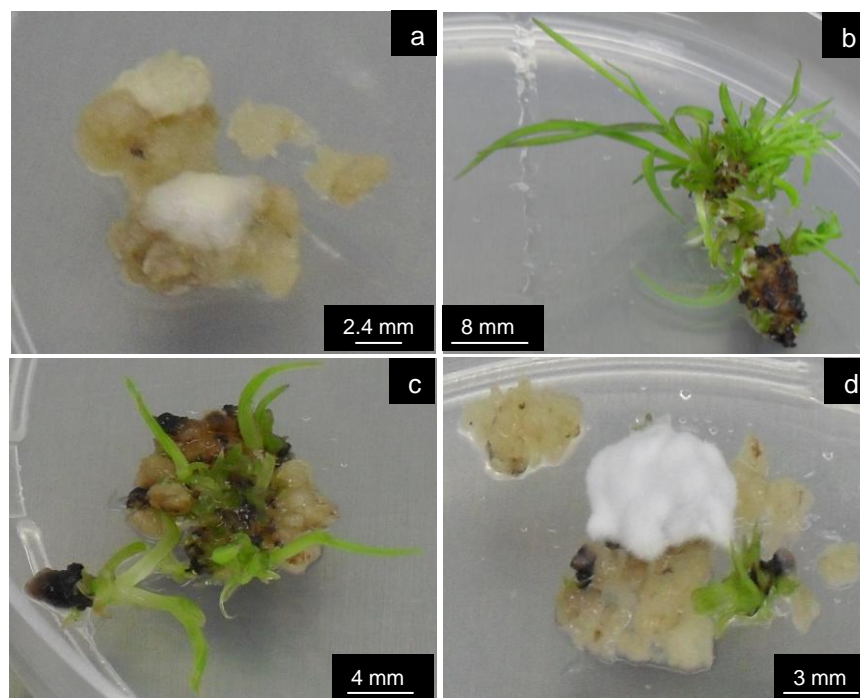


Figure 29. NCo376 germinating embryos inoculated by a 5 min soak with sterile teliospores harvested from *in vitro* plantlets. Images were captured 2 weeks post-inoculation. (a) Necrotic callus with sporidial overgrowth on the surface. (b) Sturdy shoot development observed from the control calli. (c) Shoots produced from callus inoculated with 1×10^3 teliospores/ml; no sporidial or mycelial overgrowth was observed on the callus surface. (d) Callus inoculated with 1×10^5 teliospores/ml; *U. scitaminea* mycelia developed on the surface and some green shoots were observed.

At time of writing, the shoots observed in Fig. 29 (b, c and d) had developed into sturdy plantlets and had been in culture for 8 weeks post-inoculation. A visual inspection of plantlets revealed that the control plantlets were larger than those exposed to *U. scitaminea* teliospores and distorted leaves were observed when calli were exposed to 1×10^3 and 1×10^5 teliospores/ml (Fig. 30). The number of shoots produced from the inoculated calli were significantly different (ANOVA analysis, $P < 0.05$, $df = 2$, F statistic = 3.88, $n = 10$) and there was a difference in plantlet survival between the treatments after 8 weeks (ANOVA analysis, $P < 0.05$, $df = 2$, F statistic = 6.06, $n = 10$) (Table 8). These plantlets will remain in culture for an additional 4 weeks to observe if smut whips develop; thereafter, microscopic examinations will be conducted to detect fungal mycelia within the stem and leaf tissues.



Figure 30. Plantlets produced from calli inoculated with sterile teliospores, (a) and (b) represent plantlets produced from calli inoculated with 1×10^3 and 1×10^5 teliospores/ml, respectively. Abnormal leaves are shown by arrows.

Table 8. A comparison of shoot production (4 weeks) and plantlet survival (8 weeks) of NCo376 calli inoculated with 2 concentrations of sterile teliospores. Dissimilar alphabet characters denote a statistical significance (data analyzed by an ANOVA analysis, $P < 0.05$, $df = 2$, mean \pm SE, $n = 10$).

Teliospore concentration (teliospores/ml)	Mean no. of shoots	Mean no. of plantlets
1×10^3	9 ± 2.75^a	7 ± 2.11^a
1×10^5	11 ± 3.36^{ab}	7 ± 2.13^a
0 (control)	22 ± 4.49^b	17 ± 4.33^b

4.2 *Eldana saccharina*

4.2.1 Establishment of a surface decontamination protocol for *E. saccharina* eggs and monitoring its effect on larval emergence

The 2 concentrations of NaOCl investigated for surface decontamination of *E. saccharina* eggs were 0.5% and 1.0% v/v, with a control treatment using sterile deionised water. Ten fertilized eggs were exposed to each concentration for 5, 10, 15 or 20 min. Eggs were maintained on 523 microbial medium (Viss *et al.*, 1991) and growth of microbial colonies on this medium was observed for 2 weeks. Microbial contamination was observed from the control treatment for each of the exposure periods tested but was successfully eliminated when eggs were exposed to 1% NaOCl for 15 and 20 min, or 0.5% NaOCl for 20 min (Fig. 31). When comparing the interaction of NaOCl concentration and time, there was a significant difference in microbial contamination between treatments (data were logit transformed and analyzed using a Generalized linear mixed model analysis. $P < 0.05$, $df = 6$, F statistic = 18.27, $n = 10$).

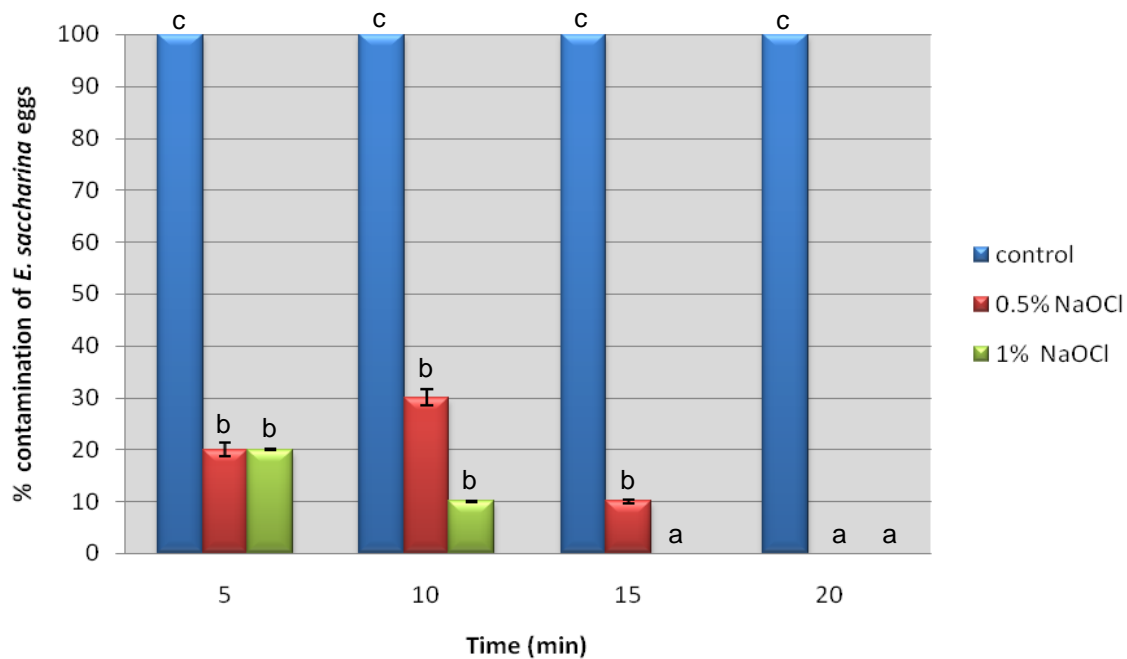


Figure 31. The percentage of *E. saccharina* eggs with microbial contamination after exposure to 2 concentrations of NaOCl at 4 time periods. Dissimilar alphabet characters denote a statistical significance (logit transformed data were analyzed by a Generalized linear mixed model analysis, $P < 0.05$, $df = 6$, F statistic = 18.27, mean \pm SE, $n = 10$).

In order to determine the effect of the decontamination treatments on larval emergence, 10 eggs were exposed to the same treatments as described above but placed on dampened filter paper for 2-3 days. Statistically, there was an interaction between NaOCl concentration and time, and larval emergence was significantly different between treatments (ANOVA analysis, $P < 0.05$, $df = 6$, F statistic = 2.92, $n = 10$). The best larval emergence was recorded for eggs exposed to 1% NaOCl for 15 min (Fig. 32).

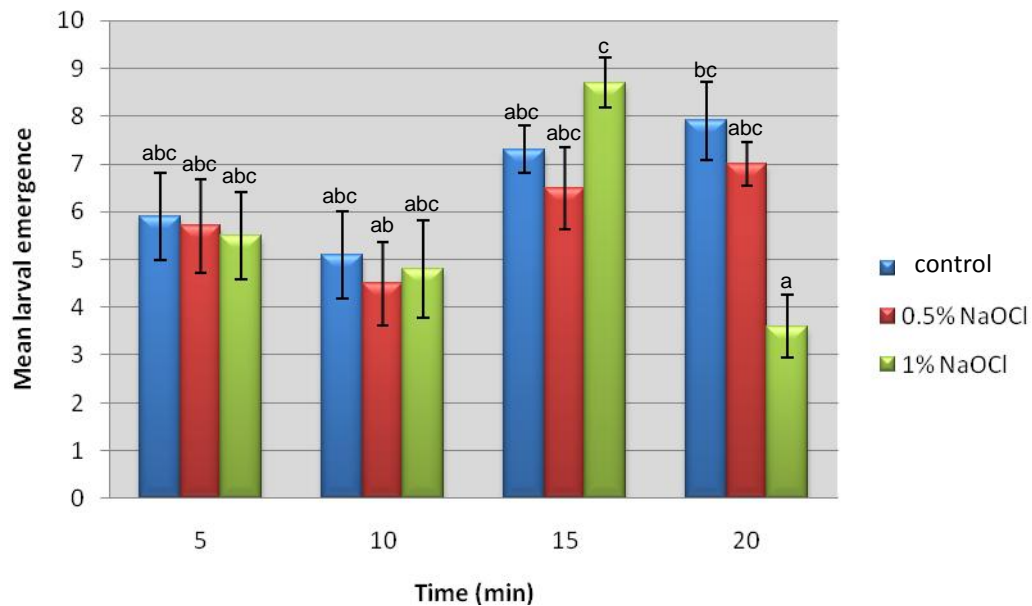


Figure 32. The effect of egg surface decontamination by treatment with 2 concentrations of NaOCl at 4 time periods on the mean larval emergence of *E. saccharina*. Dissimilar alphabet characters denote a statistical significance (data were analyzed using an ANOVA, $P < 0.05$, $df = 6$, F statistic = 2.92, mean \pm SE, $n = 10$).

In order to determine the best decontamination protocol for *E. saccharina* eggs, elimination of microbial contaminants (Fig. 31) and the best larval emergence (Fig. 32) were considered. A treatment of 15 min exposure to 1% NaOCl was used in further experiments.

4.2.2 *In vitro* screening of sugarcane for *E. saccharina* susceptibility

4.2.2.1 Plantlet feeding bioassay

In this investigation, 1 larva was placed on a single *in vitro* plantlet and maintained at a 16 h light/8 h dark photoperiod for 3 weeks. The number of plantlets in which frass was observed was recorded weekly. Larval mass and length were recorded after 3 weeks. Frass was observed within a week of inoculation and infested plantlets were dead after 3 weeks (Fig. 33).

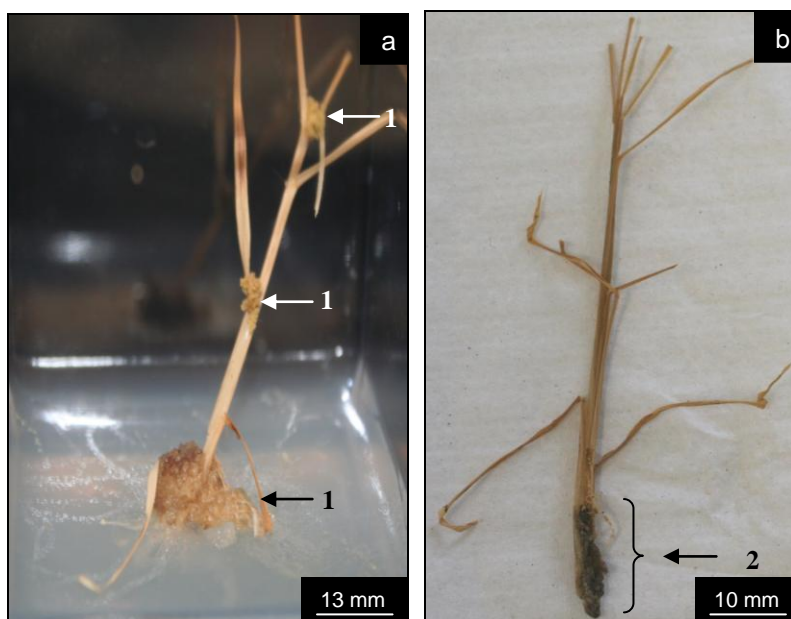


Figure 33. Visual assessment of NCo376 plantlet damage, 3 weeks after larval inoculation. (a) Areas of frass observed on dead plantlet in culture as shown by arrow. (b) Infested plantlet with larva within the base of the dead stem. 1 – frass; 2 – larva within stalk.

There was a significant difference in plantlet feeding across cultivars (categorical data, GENMOD analysis, $\chi^2 p < 0.05$, $df = 9$, $n = 35-50$). The greatest percentage of infestation was observed in NCo376 plantlets, closely followed by ‘test’ cultivar C (98% and 96%, respectively); 72% of ‘test’ cultivar D plantlets were infested which was the lowest value recorded (Fig. 34). The percentage infestation for Bt40 and N27 were 80% and 89%, respectively. The alphabetic characters assigned to NCo376 (susceptible) and N39 (resistant) were ‘a’ and ‘b’, respectively. Therefore all cultivars assigned ‘a’ would be expected to be susceptible and cultivars assigned ‘b’ would be expected to be resistant; whilst those assigned ‘ab’ are probably intermediate. Statistical analysis of data revealed

no significant difference in the rate of infestation across cultivars, from week 1 to week 3 (results not shown, regression analysis, $P > 0.05$, $R^2 = 74.5$, $n = 35-50$).

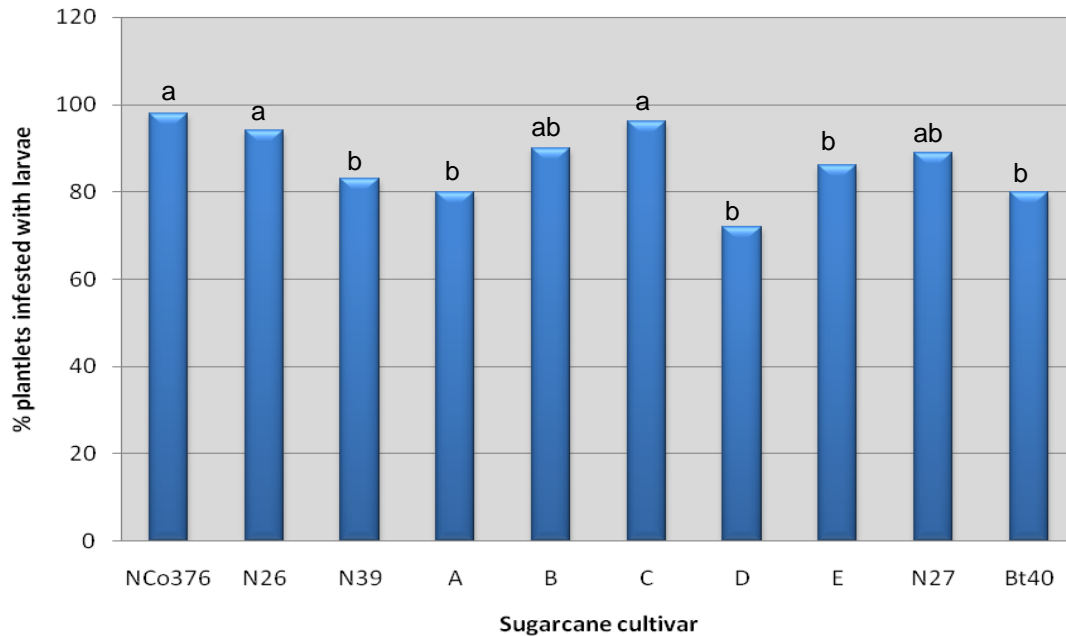


Figure 34. A comparison of percentage plantlets infested by *E. saccharina* larvae across cultivars, 3 weeks post-inoculation. Plantlet infestation was significantly different between cultivars. Data were analyzed using a GENMOD analysis, $\chi^2 p < 0.05$, $df = 9$, $n = 35-50$. ($n = 50$ for all cultivars except N27 for which $n = 35$).

The mass and length of larvae recovered from inoculated plantlets after 3 weeks were recorded in order to evaluate susceptibility across the cultivars investigated. The size of larvae varied within a cultivar and across the cultivars evaluated (Fig. 35 and Table 9).



Figure 35. Variation in the size of larvae harvested from *in vitro* NCo376 plantlets, 3 weeks post-inoculation.

Table 9. Plantlet feeding bioassay. A comparison of plantlet infestation by larvae across cultivars investigated. The number of larvae recovered from cultivars, mean larval mass and length after 3 weeks are shown. Dissimilar alphabet characters denote a statistical significance (REML analysis, $P < 0.05$, $df = 9$, $\text{mean} \pm \text{SE}$, $n = 50$). * note: $\text{mean} \pm \text{SE}$, $n = 35$.

Cultivar	Total no. of larvae recovered	Mean larval mass (mg)	Mean larval length (mm)
NCo376	49	20.20 ± 1.81^{bc}	10.31 ± 0.33^{bc}
N26	44	11.49 ± 0.59^{ab}	7.36 ± 0.17^a
N39	39	17.61 ± 1.37^{ab}	9.26 ± 0.25^{bc}
A	40	18.69 ± 2.12^b	9.64 ± 0.35^{bc}
B	43	14.97 ± 1.92^a	7.55 ± 0.36^a
C	46	18.97 ± 2.25^{ab}	9.21 ± 0.45^b
D	34	17.65 ± 1.62^b	9.20 ± 0.30^b
E	42	18.54 ± 1.89^{bc}	9.60 ± 0.28^{bc}
N27*	31	32.58 ± 3.58^c	10.82 ± 0.49^c
Bt40	36	13.97 ± 0.71^{ab}	7.91 ± 0.32^{ab}

The data for larval mass and length were analyzed using a Restricted Maximum Likelihood (REML) variance component analysis. The mass of larvae harvested from inoculated cultivars was significantly different ($P < 0.05$, $df = 9$, F statistic = 6.87 for $n = 35-50$). The greatest larval mass (32.58 ± 3.58 mg) was recorded for N27 (field susceptible to *E. saccharina*). However, the lowest larval mass (11.49 ± 0.59 mg) was recorded for N26 which is also susceptible to *E. saccharina*. A significant difference was observed in larval length across the cultivars investigated ($P < 0.05$, $df = 9$, F statistic = 11.24, $n = 35-50$). The shortest larval lengths were recorded for N26 and ‘test’ cultivar B; whilst the longest lengths were observed for N27 and NCo376 (the latter 2 are field susceptible to *E. saccharina*).

4.2.2.2 Callus feeding bioassay

This investigation was conducted to evaluate the use of callus for *in vitro* screening of sugarcane cultivars for susceptibility to *E. saccharina*. A single larva was placed on 0.5 g of callus per replicate and maintained in the dark for 2 weeks (Fig. 36a). Evidence of larval feeding on calli was observed by the production of frass (Fig. 36b).

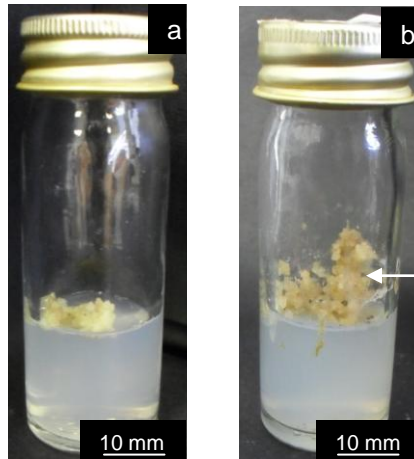


Figure 36. NCo376 callus (0.5 g) on EC3 medium in universal glass bottles. (a) Before larval inoculation and (b) 2 weeks after inoculation showing frass.

The percentage of calli replicates infested per cultivar, as observed by frass and growth of larvae, is represented in Fig. 37. Overall, the cultivars showing the highest and lowest percentage larval infestation of calli were N27 and ‘test’ cultivar E, respectively. The percentage infestation of NCo376 equaled that of N26 at 80%, which was greater than that of N39 at 70%. With regards to the ‘test’ cultivars, B had the highest percentage infestation of 75%, whilst E had the lowest of 50%. A and C = 65% and D = 70%. Infestation of Bt40 was much lower than its untransformed counterpart N27, at 55% and 80%, respectively. However, there were no significant differences in larval feeding between cultivars investigated (GENMOD analysis, $P > 0.05$, $df = 9$, $n = 20$).

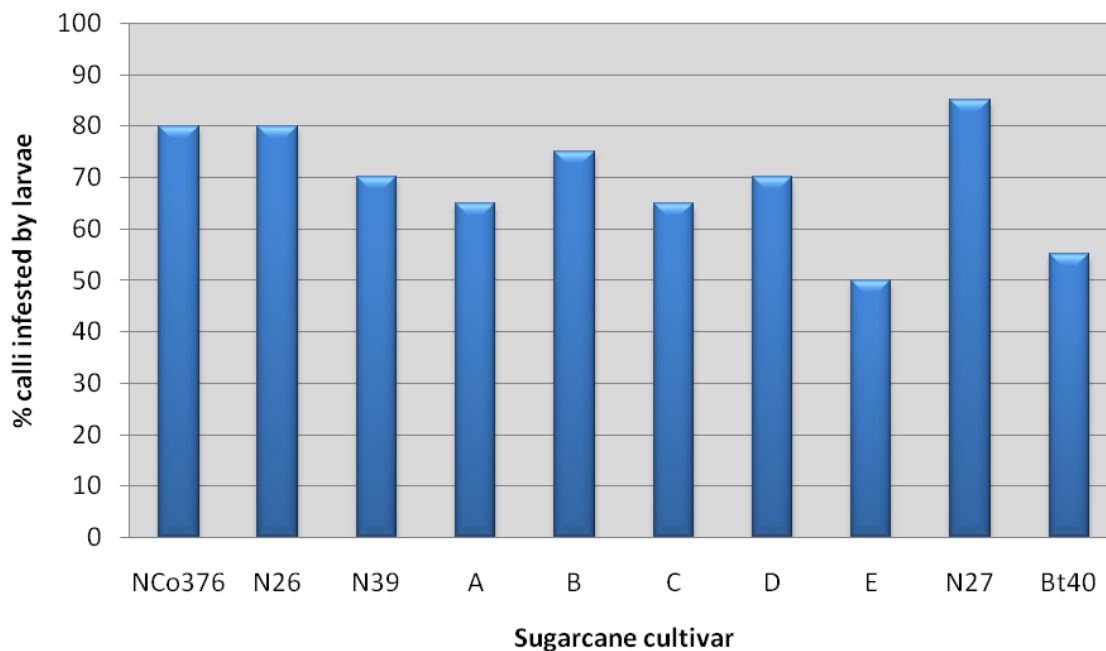


Figure 37. A comparison of percentage calli infested by larvae amongst the cultivars investigated. There was no significant difference in calli infestation across cultivars. Data were analyzed with GENMOD, $P > 0.05$, $df = 9$, $n = 20$.

The size of larvae recovered from inoculated calli was recorded. A significant difference in larval length was observed after 2 weeks (REML variance components analysis, $P < 0.05$, $df = 9$, F statistic = 4.4, $n = 20$) (Table 10). However, the only difference was found in larvae that fed on NCo376 calli which were longer compared with the other cultivars. Therefore, these results did not correlate with field ratings. No significant difference was observed in larval mass (REML variance components analysis, $P > 0.05$, $df = 9$, F statistic = 1.85, $n = 20$) (Table 10).

Table 10. Callus feeding bioassay. A comparison of calli infestation by larvae across the cultivars investigated. The total number of larvae recovered from the different calli genotypes after 2 weeks, as well as mean larval mass and length are presented. Dissimilar alphabet characters denote a statistical significance (REML analysis, $P < 0.05$, $df = 9$, mean \pm SE, $n = 20$).

Cultivar	Total no. of larvae recovered	Mean larval mass (mg)	Mean larval length (mm)
NCo376	16	43.52 \pm 2.38	14.34 \pm 0.45 ^b
N26	16	41.93 \pm 1.45	11.08 \pm 0.29 ^a
N39	14	41.51 \pm 1.49	10.49 \pm 0.28 ^a
A	13	42.46 \pm 4.25	10.90 \pm 0.56 ^a
B	15	38.73 \pm 4.52	10.61 \pm 0.76 ^a
C	13	40.92 \pm 6.20	10.27 \pm 0.99 ^a
D	14	39.86 \pm 1.70	11.35 \pm 0.27 ^a
E	10	42.77 \pm 2.71	11.44 \pm 0.51 ^a
N27	17	32.04 \pm 4.44	10.88 \pm 0.63 ^a
Bt40	11	28.03 \pm 4.00	9.96 \pm 0.96 ^a

4.2.2.3 Callus choice bioassay

Larval preference between embryogenic calli produced from different sugarcane genotypes was investigated in this experiment. Calli (0.5 g) from NCo376 (control), N26, N27, N39, Bt40 and ‘test’ cultivars A, B, C, D and E were compared with NCo376 calli in choice experiments. Ten larvae were used per replicate and results were recorded after 24 h. Although most larvae made a choice between NCo376 and other cultivar evaluated, some larvae did not. A visual representation of these results is shown in Fig. 38. A significant difference in the percentage of larvae which did not make a choice was observed (ANOVA analysis, $P < 0.05$, $df = 9$, F statistic = 3.27, $n = 20$). The largest percentage of larvae which did not make a choice was 37%, observed for N39, which was significantly different to ‘test’ cultivars E and D (the lowest values), and the rest of the cultivars (Fig. 38). No difference was observed between N27 and its transgenic counterpart Bt40.

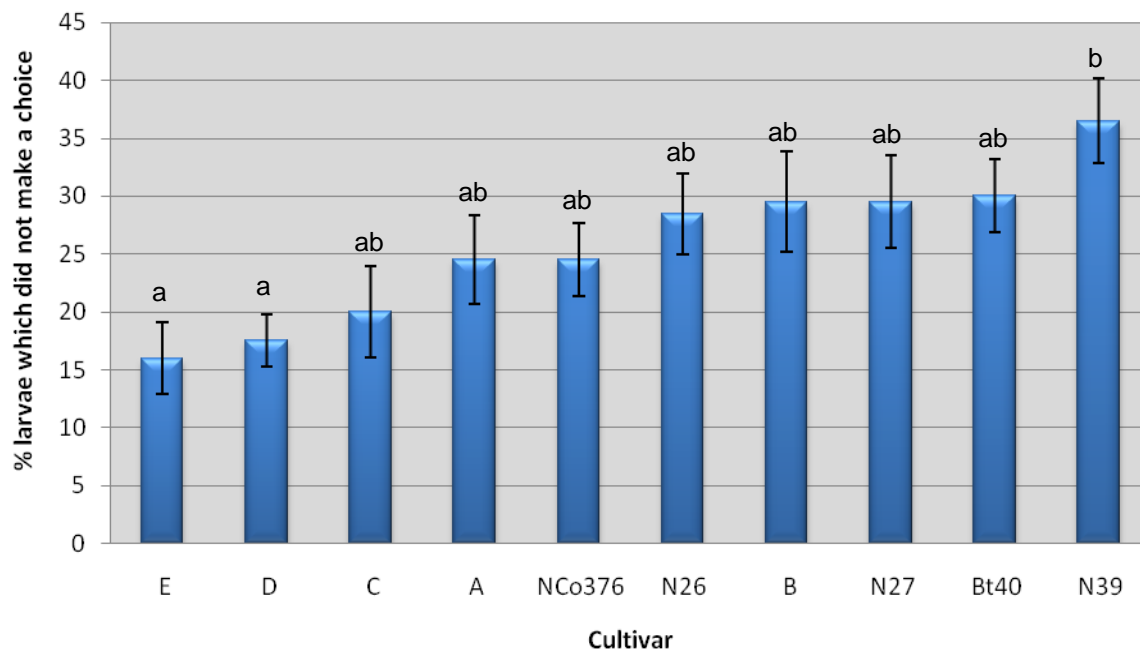


Figure 38. The percentage of larvae which did not infest calli when given a choice between NCo376 and the other cultivars evaluated, including control NCo376. Dissimilar alphabet characters denote a statistical significance (data subjected to an ANOVA analysis, $P < 0.05$, $df = 9$, F statistic = 3.27, mean \pm SE, $n = 20$).

Data collected from the callus choice bioassay were transformed into a ratio of the number of larvae that preferred callus from the cultivars investigated (A, B, C, D and E), N26, N27, N39, Bt40 and control NCo376, compared with NCo376 callus. A graphical representation of the ratios is shown in Fig. 39. For example, a 50:50 (cultivar: NCo376) ratio of larvae = 1; a 75:25 (cultivar: NCo376) ratio of larvae = +3; a 25:75 (cultivar: NCo376) ratio of larvae = -3. Therefore, the closer the bar is to 1 the less the difference in larval preference between NCo376 and the other cultivar evaluated. There was a significant difference in larval preference across the callus genotypes investigated (ANOVA analysis, $P < 0.05$, $df = 9$, F statistic = 7.04, $n = 20$). The results show that larval preference compared with NCo376 calli was greatest for N27 (field susceptible to *E. saccharina*) and ‘test’ cultivar C. The least larval preference was observed in Bt40 and N39 (field resistant to *E. saccharina*).

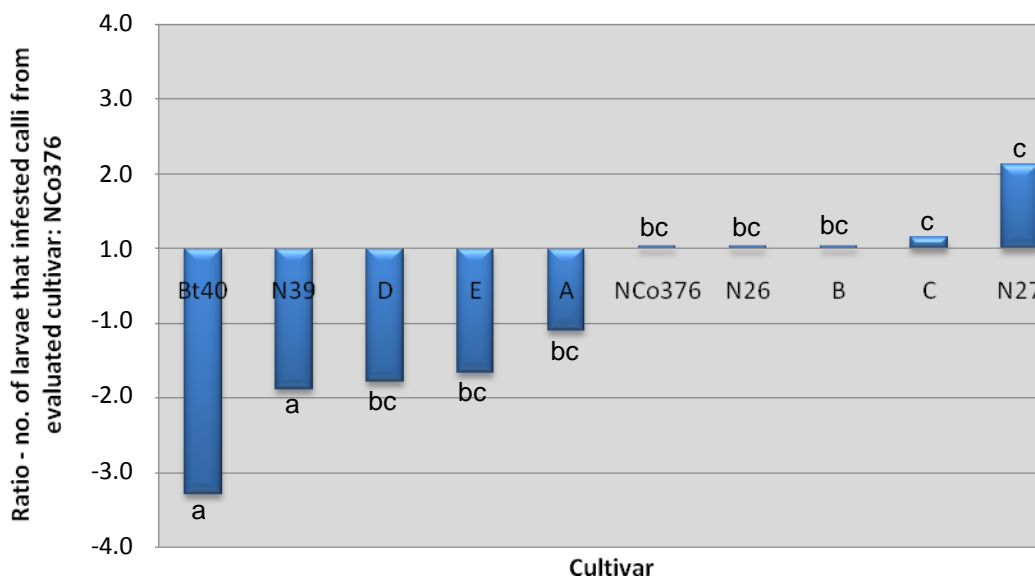


Figure 39. The ratio of percentage larvae which preferred calli derived from the ‘test’ cultivars (A, B, C, D and E), N26, N27, N39, Bt40 and control NCo376 cultivars compared with NCo376 calli. The positive and negative bars represent preference and lack of preference, respectively, to calli from that cultivar compared with NCo376. Dissimilar alphabet characters denote a statistical significance (ANOVA analysis, $P < 0.05$, $df = 9$, F statistic = 7.04, $n = 20$).

4.3 Identity of ‘test’ cultivars

The identities of the 5 ‘test’ cultivars were revealed upon completion of investigations (Table 11; Appendix 1). The field-based ratings for smut and *E. saccharina* have been included so that the accuracy of the *in vitro* methods to predict cultivar performance in the field could be evaluated.

Table 11. The identity of the 5 ‘test’ cultivars evaluated in this study and a comparison of their smut and *E. saccharina* field ratings. Field ratings were obtained from SASRI Variety Information Sheets (<http://www.sugar.org.za>)

‘Test’ cultivar	Identity	Smut field rating	<i>E. saccharina</i> field rating
A	N21	Resistant	Resistant
B	N22	Highly resistant	Intermediate-susceptible
C	N27	Resistant	Susceptible
D	N39	Susceptible	Resistant
E	N41	Intermediate	Intermediate-resistant

5. DISCUSSION

In this study, 2 types of plant material were explored for the establishment of *in vitro* screening methods for evaluating the susceptibility of sugarcane cultivars to smut (caused by *U. scitaminea*) and the stalk borer *E. saccharina*. The approach for smut screening was adapted from Singh (2004) and involved the use of *in vitro* sugarcane plantlets which were transversely cut to 1 cm and inoculated with *U. scitaminea* sporidial suspensions by injection. The development of a characteristic smut whip from inoculated plantlets was noted as a diagnostic tool for positive infection. The results of Singh's (2004) study correlated with the field smut ratings of the 4 cultivars evaluated.

In vitro screening of sugarcane for susceptibility to *E. saccharina* has not been previously investigated; however, a study by Marfa *et al.* (2002) used an *in vitro* insect-feeding bioassay to screen transgenic rice (*Oryza sativa* L.) plants for resistance to the striped stem borer, *Chilo suppressalis* and found the method to be rapid and easy to detect the resistance of plants transformed with the Bt *cry1B* gene to *C. suppressalis*. In addition to the use of *in vitro* plantlets, the current study explored the use of embryogenic calli for screening sugarcane susceptibility to smut and *E. saccharina*.

5.1 Towards the development of *in vitro* screening methods for smut and *E. saccharina*

Ustilago scitaminea

Singh (2004) investigated a range of *U. scitaminea* inocula concentrations (1×10^1 – 1×10^6 sporidia/ml) and observed infection at concentrations greater than 1×10^5 sporidia/ml. In the current study, the probability of successful mating between opposite mating types of sporidia was investigated using 3 concentrations of sporidial suspensions (1×10^3 , 1×10^6 and 1×10^9 sporidia/ml) and the latter 2 were adequate to allow mating which was essential for positive infection (section 4.1.1.1).

The use of sporidial inocula rather than *U. scitaminea* teliospores, which are widely used during conventional screening, was necessary because teliospores harvested from field-infected sugarcane contain other microbial contaminants which may confound results. In order to ensure elimination of bacterial contaminants, teliospores were washed with a 0.5 g/l streptomycin solution and cultured

on PDA. This medium provides a favourable environment for teliospore germination and results in production of a promycelium composed of 3 or 4 cells. Each cell has the ability to form a sporidium and under such conditions sporidia may either germinate to form hyphae or further multiply by budding-off more sporidia, similar to yeast cell multiplication (Antoine, 1961; Alexander and Ramakrishnan, 1977; Izadi and Moosawi-Jorf, 2007). Although the concentration of sporidial suspensions was determined before inoculation, the favourable conditions presented *in vitro* by nutrient-rich media most likely promoted excessive budding of sporidia leading to overgrowth in PM and EC3 media. Sporidial overgrowth around the base of plantlets was also observed in all plantlets in the study by Singh (2004). According to that author, the sporidial streak did not affect whip production from plantlets.

Singh (2004) transversely cut plantlets to 1 cm before the sporidial suspension was inoculated by injection. In the current study, the effect of that method on plantlet survival was investigated by cutting plantlets to 3 lengths (1, 2 and 3 cm) and inoculating with sterile water (Fig. 19). No significant difference in plant survival was observed after 3 weeks. In subsequent investigations, plantlets were cut to 1 cm before inoculation because the inoculation was required to be as close to the meristem as possible.

Fungicides have been incorporated into tissue culture media by various researchers for the control or elimination of fungal contamination (Shields *et al.*, 1984; Zimowska, 2006). Non systemic or contact fungicides (e.g. Dithane M-45[®]) are effective in preventing fungal contamination on the surface of plants. Zimowska (2006) investigated the effect of 10 fungicides, including Dithane M-45[®], on the growth and development of *Seimatosporium hypericinum* (Ces.) Sutton *in vitro*. This approach was similar to the current study because a range of fungicide concentrations (1, 10 and 100 µg a.i./cm³) were incorporated into PDA medium onto which *S. hypericinum* culture was streaked. A concentration of 100 µg a.i./cm³ of the active ingredient, Mancozeb, was effective in inhibiting fungal growth in that study. The current study investigated the effect of Dithane-M45[®] incorporated into EC3 medium on the development of *U. scitaminea* sporidia and the lowest concentration which eliminated sporidial growth was 0.025 g/l (Fig. 18).

Contact fungicides are not generally absorbed by the plant. However, a study by Aggarwal *et al.* (2005) found that the growth of sunflower plants was adversely affected when exposed to Dithane M-45[®]. However, exposure to 0.025 g/l Dithane-M45[®] did not affect *in vitro* sugarcane plantlet

survival in this study (Fig. 20). The roots of *in vitro* plantlets were in contact with the medium containing Dithane M-45[®]. Sugarcane roots are typically made up of 3 layers of tissue, i.e. an epidermal layer of thickened sclerenchyma cells, a cortex of thin parenchyma cells and endodermis that surrounds the vascular tissue (Soopramanien, 2000). The outer tissue layer probably prevented the uptake of the fungicide into the plantlet therefore exposure to Dithane M-45[®] did not affect plantlet survival.

However, calli were found to be extremely sensitive to this fungicide (Fig. 21). Undifferentiated callus cells lack the physical barriers present in plantlets and Nadar *et al.* (1978) found that sugarcane embryogenic calli were thin-walled and cytoplasmically rich. Therefore, it is likely that the active ingredients of Dithane M-45[®] entered the cells and were toxic. In addition, the inclusion of this fungicide in EC3 medium did not prevent *U. scitaminea* overgrowth (Fig. 22). Sporidial and mycelial overgrowth were observed on the surface of NCo376 calli. This may have occurred because calli were inoculated by immersion in the sporidial suspensions; however, only the lower surface of calli were in contact with the medium incorporating 0.025 g/l Dithane M-45[®]. Considering the yeast-like budding of sporidia, this overgrowth on calli may have significantly increased the concentration of the pathogen, therefore the concentration of Dithane M-45[®], previously shown to eliminate sporidial growth in EC3 medium, was ineffective in this instance.

The broad spectrum biocide/fungicide for plant tissue culture, PPM[™], was then investigated for callus inoculation with *U. scitaminea*. Compton and Koch (2001) assessed the effect of several concentrations of PPM[™] on the organogenesis of 3 plant species and found that a concentration of up to 5 ml/l did not adversely affect callus production or somatic embryo germination. This finding was confirmed in the present study because 5ml/l PPM[™] did not affect shoot production from sugarcane callus (Figs. 23 and 29). In addition, this concentration was effective in preventing *U. scitaminea* overgrowth on EC3 medium; however, as mentioned previously, sporidial and mycelial overgrowth was observed on the surface of calli. Therefore, the use of *U. scitaminea* sporidia as inocula for calli investigations was not supported in this study.

In summary, an optimum protocol for plantlet inoculation with *U. scitaminea* involved injection of 1×10^6 and 1×10^9 sporidia/ml into the cut surface of 1 cm *in vitro* plantlets and maintenance in semi-solid PM medium with 0.025 g/l Dithane M-45[®] at a 16 h light/8 h dark photoperiod. The

outcome of callus protocol development was the incorporation of 5 ml/l PPM™ into semi-solid EC3 medium which successfully prevented sporidial and mycelial growth in the medium.

Eldana saccharina

An *in vitro* screening method for evaluating sugarcane susceptibility to *E. saccharina* had not been investigated before this study. Therefore, the current approach was adapted from Marfa *et al.* (2002). These researchers conducted an *in vitro* feeding bioassay to evaluate resistance of transgenic rice plants transformed with an insect resistance gene (*cry*) to larvae of the striped stem borer, *Chilo suppressalis*. The plantlets generated in that study were aseptic and therefore it was necessary for the larvae to be free of microbial contaminants as well. This was achieved by 2 surface sterilization treatments of *C. suppressalis* eggs with 0.8% NaOCl for 10 min and 5% formol solution for 5 min, followed by a 3x rinse with sterile water. The study found that larval emergence improved when eggs were treated with NaOCl compared with formal (Marfa *et al.*, 2002).

Many laboratory insect-rearing studies have used NaOCl to surface sterilize insect eggs. Trudel *et al.* (1995) treated *Dioryctria abietivorella* (Lepidoptera: Pyralidae) eggs with 5% NaOCl for 2-3 min in order to assess laboratory rearing techniques. Rahman *et al.* (1998) investigated growth parameters of *Spilosoma obliqua* (Lepidoptera: Arctiidae) and used a decontamination treatment of 1% NaOCl for 5 min. Here, 2 NaOCl concentrations (0.5% and 1% v/v) and a range of exposure times (5, 10, 15 and 20 min) were investigated to establish an optimum treatment for microbial elimination and larval emergence of *E. saccharina* (Figs. 31 and 32).

In vitro bioassays have shown promise as a rapid screening technique for evaluating insect resistance (Marfa *et al.*, 2002; Sharma *et al.*, 2005). Sharma *et al.* (2005) investigated 4 host plants' resistance to the noctuid, *Helicoverpa armigera* (Hüner) by using an *in vitro* detached leaf assay. These researchers concluded that this technique could be used to evaluate germplasm, isolate breeding materials, and map populations for resistance to *H. armigera* under uniform conditions in a short time and with minimal cost.

In this study, plantlet and callus feeding bioassays were conducted by the placement of an *E. saccharina* larva on a single plantlet or 0.5 g callus. The standard 16 h light/8 h dark photoperiod was used to maintain plantlets and calli were maintained in the dark. The duration of the feeding bioassay conducted by Marfa *et al.* (2002) ranged from 8 to 17 days; however in this study, the

duration of the bioassay was 3 weeks (21 days). The sugarcane plantlets used were 10-12 cm compared with 3-4 cm rice plantlets investigated by Marfa *et al.* (2002). Therefore, more food material was available to the insect. In addition, the duration of the larval stage of *E. saccharina* lasts 20-40 days and this is influenced by temperature (Carnegie, 1974; Way, 1995; Horton *et al.*, 2002). A duration of 3 weeks for the bioassay was adequate for larvae to feed before pupating.

Callus feeding and choice bioassays have been used by several researchers to determine insect resistance of host plants (Williams *et al.*, 1987a; Williams *et al.*, 1987b; Davis *et al.*, 1989). These researchers compared larval mass between resistant and susceptible host plants. Williams *et al.* (1987a) investigated maize (*Zea mays* L.) susceptibility to 3 corn borers and found positive correlations between larval mass and maize susceptibility after 7 days. Likewise, choice experiments were accurate as fewer larvae chose to feed on callus from resistant maize compared with susceptible varieties.

In the current study, the mass of callus investigated was small in comparison with plantlets evaluated; therefore the duration of the callus feeding bioassay was reduced to 2 weeks. Croughan and Quisenberry (1989) who evaluated the use of callus culture as a screening technique for determining fall armyworm (*Spodoptera frugiperda* J. E. Smith) resistance in 4 varieties of bermudagrass, found that field resistance ratings did not correlate with differences in larval mass on callus. Therefore, these researchers concluded that the use of callus culture for screening did not offer any advantages over conventional leaf screening. Therefore, in addition to a callus feeding investigation, a callus choice bioassay was conducted in order to evaluate larval preference to different cultivars compared with NCo376 (Fig. 39).

In summary, the protocol developed here involved surface decontamination of *E. saccharina* eggs by treatment with 1% NaOCl for 15 minutes followed by a 3x rinse in sterile deionised water. Inoculation for plantlet and callus feeding bioassays was conducted by placement of a single first instar larva on the plantlet (10-12 cm) or on 0.5 g embryogenic callus. In addition, a callus choice bioassay was conducted which compared larval preference between different callus genotypes and NCo376.

5.2 Are *in vitro* screening methods suitable for evaluating the susceptibility of sugarcane to smut (*U. scitaminea*) and *E. saccharina*?

In vitro tissue culture of sugarcane has been used to improve sugarcane cultivars through somaclonal variation and induced mutations (Table 3). However, resultant plants were conventionally screened to evaluate resistance to pests and diseases (Krishnamurthi and Tlaskal, 1974; Maribona *et al.*, 1986; Bravo *et al.*, 1989). This study focused entirely on *in vitro* screening of cultivars and investigated its potential as an accurate and rapid technique compared with conventional field screening.

Smut

Positive smut infection in *in vitro* plantlets was achieved by artificial inoculation with 1×10^6 and 1×10^9 sporidia/ml (Table 7). The disease progressed from within plant tissue towards the modification of the apical meristem into a characteristic smut whip. Successful smut infection of *in vitro* plantlets was also shown by Fereol (1984) and Singh (2004). Singh (2004) compared results obtained *in vitro* with the field susceptibility ratings of the cultivars and found a positive correlation. However, this was not observed here.

As expected, smut whips developed in NCo376 plantlets because this cultivar is susceptible to smut in the field (Fig. 25 a-f). However, cultivar N26 which is resistant to smut in the field produced a whip *in vitro* when inoculated with 1×10^9 sporidia/ml (Table 7). Although the development of smut whips from inoculated N39 plantlets was expected as this is a susceptible cultivar, all plantlets died within 6 weeks. Therefore, the survival of *in vitro* inoculated plantlets may be an indicator of resistance. ‘Test’ cultivars A, B, and E produced smut whips *in vitro*; however, the identities of A and B were N21 and N22, which are resistant and highly-resistant to smut, respectively (Table 11). ‘Test’ cultivar E was N41, whose field rating is intermediate to smut. The only ‘test’ cultivar susceptible to smut was D (N39) however no whips were produced *in vitro* (Table 7 and 11).

Two types of resistance mechanisms to smut have been reported for sugarcane, namely, mechanical and physiological resistance (Dean, 1982; Burner *et al.*, 1993). Mechanical resistance to smut is associated with bud morphology at the nodes of sugarcane setts because this is the primary site of infection in the field. Correlation of bud scale compaction and resistance to smut has been reported as a first line of defense (Waller, 1970; Alexander and Ramakrishnan, 1980). Waller (1970) found

that inoculation below bud scales and morphological changes to buds during germination resulted in increased infection. Early research involved inoculation of sugarcane setts by immersion in concentrated teliospore suspensions followed by planting in the field. A different approach was investigated by Ferreira and Comstock (1981), who inoculated sugarcane seedlings by injection with 1×10^3 teliospores/ml. The smut infection observed in that study correlated with results from immersion techniques. This injection technique was adapted by Singh (2004) however sporidia was used instead of teliospores.

The observation that field resistant cultivars developed whips in the current study may be attributed to the method of inoculation employed which by-passed mechanical barriers of sugarcane to the pathogen. The *in vitro* plantlets used were 3 months old and had not developed sufficiently to produce nodes or lateral buds. In addition, the inoculum was injected into the plantlet directly above the apical meristem. Therefore, cultivars resistant to smut in the field but which produced whips *in vitro* are probably influenced by bud resistance in the field.

However, some researchers found no correlation between bud characteristics and susceptibility to smut infection (James, 1969; Rampersad and Braithewaite, 1985). Therefore, other factors may influence cultivar susceptibility to smut. This may be attributed to physiological resistance influenced by the biochemistry of plant cells. James (1973) investigated the germination of smut teliospores on the internode surface of 40 cultivars and concluded that resistance to smut may be influenced by a chemical rather than a mechanical mechanism. Lloyd and Pillay (1980) isolated highly active, low molecular weight compounds (flavonoid glycosides) from bud scales of sugarcane and found that the concentration of these compounds correlated with resistance to smut. Therefore, these authors postulated that these compounds were inhibitors of spore germination and could be used as a chemical assay for detecting smut resistance (Lloyd and Naidoo, 1983).

In addition, histopathological investigations of stalk and meristem colonization by *U. scitaminea* showed that pre-infection resistance associated with the bud scale was independent of post-infection resistance (Lloyd and Pillay, 1980). Other studies confirm the assumption of Lloyd and Pillay (1980) in that glycoproteins, phenolics and polyamines are enhanced in sugarcane in response to *U. scitaminea* influences smut infection (Legaz *et al.*, 1998; Piñon *et al.*, 1999; Fontaniella *et al.*, 2002; Millanes *et al.*, 2005; Blanch *et al.*, 2007; de Armas *et al.*, 2007). In addition, specific genes activated by a cascade of sugarcane defense mechanisms to *U. scitaminea* were investigated by

Thokoane and Rutherford (2001). A putative chitin receptor kinase, a Pto ser/thr protein kinase interactor, and an active gypsy type LTR retro-transposon were expressed by the resistant cultivar, N52/219, in response to *U. scitaminea* (Thokoane and Rutherford, 2001).

Recently, it has been shown that different compounds from smut fungi can elicit physiological changes in sugarcane (Santiago *et al.*, 2008; Santiago *et al.*, 2010). Santiago *et al.* (2008) found that medium molecular mass compounds such as negatively charged proteins, peptides and glycopeptides from fungal extracts increased the accumulation of free phenolics in sugarcane leaves. The resistant cultivar (Mayarí 5514) accumulated hydroxycinnamic acids whilst hydroxybenzoic acids were enhanced in the susceptible cultivar (Barbados 42231). In addition, peroxidase production was enhanced in the resistant cultivar. This enzyme has been shown to activate important mechanisms of sugarcane resistance to fungal pathogens by using free phenolics as substrates (Lebeda *et al.*, 2001; Santiago *et al.*, 2008). Elicitors isolated from smut teliospores have been shown to increase the thickness of sclerenchyma and xylem cell walls by enhancing lignin content (Santiago *et al.*, 2010). This has been postulated as a possible mechanical defense response to the entry of the fungus.

As mentioned previously, embryogenic calli were also explored for the development of a rapid *in vitro* screening method to evaluate cultivar susceptibility to smut. As overgrowth of sporidia and mycelia was a persistent problem, inoculation of calli with sterile teliospores derived from *in vitro* inoculated plantlets was conducted as a final attempt to develop a protocol in this study. In a similar approach, Héctor *et al.* (1992) investigated the effect of *U. scitaminea* culture filtrates on the development of sugarcane callus. Calli derived from a highly resistant (POJ 2878) and highly susceptible (B 42231) cultivars were cultured on *in vitro* plantlet regeneration medium incorporating 60% culture filtrate of *U. scitaminea*. In addition, the effect of indole-3-acetic acid (IAA) in the medium, with and without the culture filtrate was investigated. Regeneration of the resistant cultivar was stable in all treatments and significantly different to the susceptible cultivar. Those authors speculated that *U. scitaminea* affects the optimal hormonal balance required for tissue development.

In this study, shoots were successfully produced when NCo376 calli were inoculated with sterile teliospores (Fig. 30 and Table 8). The leaves produced from calli exposed to *U. scitaminea*, were visually distorted compared with control plantlets (Fig. 30). At present, it is unclear if the

inoculation was successful and plantlets will remain in culture for 12 weeks post-inoculation to observe whip production, after which, microscopic examinations will be conducted on the stem and leaf tissues to detect the presence of fungal mycelia. N26, N39 and the 'test' cultivars in this study were not evaluated by this method due to time constraints.

Eldana saccharina

The current study revealed that *E. saccharina* larvae consume sugarcane plantlets and calli *in vitro*. This was observed by the production of frass in plant material and larval growth (Figs. 33, 35 and 36). Comparisons of larval mass and length were conducted across the cultivars evaluated similar to the study by Marfa *et al.* (2002). Infestation recorded from the plantlet feeding bioassay was statistically different (GENMOD analysis, $P < 0.05$, $n = 35-50$) and the trend was similar to the known field ratings of the cultivars, as percentage infestation of resistant cultivars were lower compared with intermediate and susceptible cultivars (Fig. 34). As expected, a lower infestation was observed with Bt40 compared with N27 and test cultivar C (N27). Bt40 contains the *cryIAb* gene which has been shown to kill Lepidopteran larvae when ingested (Gill *et al.*, 1992; Vojtech *et al.*, 2005). Larval mass and length across cultivars did not exactly correlate to the known field resistance ratings for the cultivars; however some similarities were observed (Table 9).

Two terms are widely used in the literature when discussing plant–insect interactions. Antixenosis, proposed by Kogan and Ortman (1978) is used to describe non-preference and antibiosis describes an antagonistic association of organisms in which one produces compounds which are harmful to another (Lawrence, 2000). Mathes and Charpentier (1969) (cited by Meagher *et al.*, 1996), classified components associated with stalkborer resistance. These were (1) unattractiveness of plants to adults for oviposition (antixenosis), (2) plant characters unfavourable for larval establishment in the plant (antixenosis and antibiosis), (3) plant characters that inhibit or retard larval development (antibiosis) and (4) plant tolerance. At SASRI, Mabulu and Keeping (1999) conducted glasshouse tests to investigate ovipositional antixenosis of sugarcane cultivars to *E. saccharina* and found it to be a negligible resistance mechanism. Therefore, plant characters which influence larval establishment and development are essential for cultivar resistance.

Field investigations that assessed *E. saccharina* larval establishment was conducted at SASRI and stalk penetration after 4 days was lower in a resistant cultivar (N8) compared with 2 susceptible cultivars (Leslie, 1993). An important finding was that larvae recovered from N8 were found on

dead leaf sheaths rather than buds or cracks as was observed in susceptible cultivars. Therefore, larval antixenosis could be an important resistance mechanism. Antibiosis may be an early resistance mechanism affecting larval establishment in sugarcane as a field study by Mabulu (2002) found that larval recovery after 7 days was lower in a resistant cultivar (N21) compared with a susceptible cultivar (N11).

Larval antixenosis and antibiosis may be influenced by different plant characters. Many researchers have attributed stalk borer resistance to rind hardness and fibre content (Martin *et al.*, 1975; White *et al.*, 2006). However, the release of *E. saccharina* resistant cultivars (N39 and N41) with high sucrose and moderate fibre content from SASRI suggests that stalk toughness is of minor importance in the resistance of these cultivars (Keeping and Rutherford, 2004). This was confirmed in the current study as the *in vitro* plantlets were young and had not developed a hard rind in comparison with field sugarcane. However, the trend of plantlet infestation *in vitro* across cultivars correlated to field cultivar resistance (Fig. 34). Keeping and Rutherford (2004) suggested that borer resistance is influenced by plant chemistry and physiological reactions to damage.

Rutherford *et al.* (1993) investigated sugarcane phytochemical correlations with resistance to *E. saccharina* and found that resistance could be associated with bud scale chlorogenate and flavonoid composition. In addition, surface wax composition may influence larval antixenosis as a high alcohol to aldehyde ratio was linked to *E. saccharina* resistance (Rutherford and van Staden, 1996). Therefore, the chemical composition of the sugarcane plant surface is important to resistance and this is supported by results observed for plantlet infestation in the current study (Fig. 34).

Falco and Silva-Filho (2001) summarized several aspects of the complex interaction between plants and insects as well as some sugarcane genes expressed in response to insect attack. In sugarcane polyphenol oxidase (PPO) is a defense-related protein which is activated by the octadecanoid pathway. Sugarcane has expressed sequence tags (ESTs) that encode a Ca^{2+} dependant protein kinase, which is a mitogen-activated protein (MAP) kinase, and a putative phospholipase which are involved in the activation of this pathway (Falco and Silva-Filho, 2001). Protein investigations with maize have found that a unique 33-kD cysteine proteinase is accumulated in response to insect feeding by fall armyworm (*S. frugiperda*) in resistant varieties and may be linked to plant resistance (Pechan *et al.*, 2000; Pechan *et al.*, 2002). This proteinase disrupts the peritrophic matrix of caterpillars that surrounds the food bolus and affects its digestive processes therefore an increase in

33-kD cysteine proteinase by host plants when attacked by an insect represents a resistance mechanism to feeding (Pechan *et al.*, 2002).

Statistical analysis of data recorded from the callus feeding bioassay revealed no significant difference in percentage infestation and larval mass (Fig. 37). Although a significant difference was observed for larval length (ANOVA analysis, $P < 0.05$, $n = 20$), the only difference was found in NCo376, therefore the results did not discern cultivar susceptibility (Table 10). This bioassay was a 'no choice' assay therefore the callus presented to larvae was the only source of food and larvae were probably forced to consume it. This may be the reason for no correlation between results and cultivar susceptibility to *E. saccharina*. Although, the plantlet feeding bioassay did not offer a choice to larvae, that assay was probably influenced by other aspects of plantlet resistance, as mentioned above, whilst the callus, comprised of exposed cells, did not have additional protective mechanisms.

In addition, the 33-kD cysteine proteinase expressed in maize plants has been found in maize callus and has been associated with larval growth inhibition of fall armyworm (Jiang *et al.*, 1995). Therefore, biochemical compounds which are up-regulated during insect feeding in plants may be expressed in calli as well. The data recorded in the callus choice bioassay were the number of larvae that did not make a choice and larval preference between callus genotypes (Fig. 38 and 39). Although there was a significant difference in the percentage of larvae which did not make a choice amongst cultivars (ANOVA analysis, $P < 0.05$, $n = 20$), the results did not correlate with field cultivar susceptibility to *E. saccharina* (Fig. 38). However, larval preference to different callus genotypes compared with NCo376 callus was statistically different and correctly reflected cultivar susceptibility (Fig. 39). Therefore the callus choice bioassay is the most promising screening method for *E. saccharina*.

5.3 Concluding remarks and future work

In vitro tissue culture offers many advantages in terms of controlled conditions, time and space-saving; however, one shortcoming encountered in this study was that the favourable conditions may exacerbate growth of microbial pathogens being investigated, as was observed with sporidial and mycelial overgrowth. The optimum protocol for plantlet inoculation with *U. scitaminea* sporidia investigated in this study was 0.5 μ l of 1×10^6 and 1×10^9 sporidia/ml by injection above the apical

mersitem of *in vitro* plantlets cut to 1 cm. Inoculated plantlets were maintained in semi-solid PM medium with 0.025 g/l of the contact fungicide, Dithane-M45[®]. However, this protocol was unsuccessful as a screening method for evaluating smut susceptibility. Further investigations to optimize this protocol are necessary. Although the results for smut screening in the present study did not correlate with the field resistance ratings of the cultivars investigated, a positive outcome is the production of sterile teliospores from inoculated plantlets. There is potential for teliospores derived through *in vitro* plantlet inoculations to be used in future investigations which eliminate the need to decontaminate and process teliospores collected from the field. Therefore, the use of sporidia may not be necessary for future *in vitro* investigations.

The establishment of a successful protocol for callus inoculation with *U. scitaminea* was challenging due to sporidial and mycelial overgrowth and necrosis of calli. In addition, the use 0.025 g/l Dithane-M45[®] to control *U. scitaminea* overgrowth was unsuccessful due to calli sensitivity. Another outcome of this study was that inclusion of 5 ml/l PPM[™] in the regeneration medium (EC3) eliminated sporidial overgrowth. As a last resort, teliospores harvested from *in vitro* plantlets were used to inoculate NCo376 calli. Shoots were produced from calli when maintained on EC3 medium with 5 ml/l PPM[™] and distortion of some leaves was observed. Future work will entail evaluating these plantlets, 12 weeks post-inoculation by observation of whips and microscopy to detect fungal mycelia within the plant tissue. Should infection be validated in these plantlets, it would be worth assessing other cultivars.

The optimum protocol for *E. saccharina* screening involved surface decontamination of eggs with 1% NaOCl for 15 min. Microbial contamination of the medium maintaining plant material was negligible. This study found that larvae consumed plantlets and calli *in vitro*. For plantlet feeding investigations, the trend of percentage infestation was similar to the field resistance ratings of cultivars. However, infestation was not significantly different for callus feeding. In addition, larval mass and length obtained using both types of plant material did not discern cultivar susceptibility to *E. saccharina*.

The callus choice bioassay which evaluated larval preference of different callus genotypes compared with NCo376 callus, correctly discerned cultivar susceptibility to *E. saccharina* (Fig. 39). Further assessment of this method and future studies to optimize this protocol would be advantageous because this method required the least time compared with the feeding bioassays. The

production of embryogenic callus required 8-10 weeks and the duration of the screening process was 24 h.

In conclusion, plant resistance to pests and diseases is a multi-factorial process. The aim of this study was to develop screening methods for one pest and one pathogen; however in nature, sugarcane is affected by several pests and diseases. An important point to note is that this study has focused on screening physiological resistance of sugarcane to *U. scitaminea* and *E. saccharina* because the plantlets were young and were unlikely to have fully developed mechanical resistance mechanisms compared with field sugarcane. In addition, calli lacked any physical barrier to infection and insect feeding. Screening methods that target physiological resistance are advantageous over methods used to screen mechanical resistance because in the event that a mechanical barrier should fail in nature, inherent resistance mechanisms could prevent severe disease or pest outbreaks. Therefore, improvement of the methods investigated in this study and further research into developing *in vitro* screening methods for multiple pests and diseases should be explored.

6. REFERENCES

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Appendix 1



29 November 2010

To whom it may concern

Verification of the identity of 'test' cultivars

The following sugarcane cultivars were evaluated in this study:

<u>'Test' cultivar</u>	<u>Identity</u>
A	N21
B	N22
C	N27
D	N39
E	N41

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