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Sugarcane Pathogens: Detection and Elimination through *In Vitro* Culture

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ABSTRACT

Commercial sugarcane cultivation, which involves: (a) multiple ratoons in the field for several years, (b) propagation by means of vegetative stem cuttings, (c) large areas under sugarcane monoculture, (d) production in tropical and subtropical climates, can exacerbate pathogen infestation and the spread of diseases. This paper reviews important diseases in sugarcane, methodology for detection of pathogens and highlights several interventions for pathogen elimination such as hot water treatment of stem sections and *in vitro* culture using meristematic cells as the initiation material. These have ensured a multi-faceted approach for disease management and enabled the production of material without pathogens such as *Leifsonia xyli* subsp. *xyli*, *Xanthomonas albilineans*, sugarcane mosaic virus and sugarcane yellow leaf virus. Additionally, international germplasm exchange may benefit by incorporating aspects of *in vitro* culture at various stage of the process.

Keywords: meristem excision, molecular testing, viruses
Abbreviations: CIRAD, Centre de Cooperation Internationale en Recherche Agronomique pour le Developpement; **DAS-ELISA**, double antibody sandwich enzyme linked immunosorbant assay; **EB-EIA**, evaporative binding-enzyme immunoassay; **HWT**, hot water treatment; ***Lxx***, *Leifsonia xyli* subsp. *xyli*; **RT-PCR**, reverse transcription polymerase chain reaction; **RFLP**, random fragment length polymorphism; **SASRI**, South African Sugarcane Research Institute; **SCFDV**, *Sugarcane Fiji disease virus*; **SCMV**, *Sugarcane mosaic virus*; **SCSMV**, *Sugarcane streak mosaic virus*; **SCWL**, *Sugarcane white leaf phytoplasma*; **ScYLV**, *Sugarcane yellow leaf virus*; **SCYP**, *Sugarcane yellows phytoplasma*; **SrMV**, *Sorghum mosaic virus*; **TBIA**, tissue blot immunosorbant assay; **USDA**, United States Department of Agriculture

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INTRODUCTION

Sugarcane is a tropical crop and is vegetatively propagated on a commercial scale. This enables transmission of a suite of organisms with disease-causing potential; a topic that has been well studied and reviewed (Rott *et al*. 2000; Zhang *et al*. 2006). Consequently, a range of diagnostic techniques from visual examination to molecular tests are used to assist in pathogen detection and disease management. Pathogen elimination from stem sections (setts) using chemo- and/or thermotherapy is moderately successful for the control of some pathogenic agents (Benda and Ricaud 1978; Bailey 1983), but viruses have not been easy to eliminate. This has been a serious limitation in the international germplasm exchange programme. The development of specific *in vitro* culture techniques has been a valuable tool in removal of certain systemic pathogens and this review serves to highlight successful interventions. Two techniques that involve aspects of *in vitro* culture for pathogen control that have not been discussed here include: (a) mutagenesis: where cells undergo somaclonal variation either as a result of the *in vitro* process or exposure to mutagenic agents while in culture and subsequently become disease resistant (Larkin and Scowcroft 1983) and (b) genetic engineering: where foreign genes are inserted into sugarcane to confer resistance to pathogens such as *Sugarcane mosaic virus* (SCMV) (Ingelbrecht *et al*. 1999) and *Sugarcane yellow leaf virus* (ScYLV) (Gilbert *et al*. 2009; Zhu *et al.* 2011).

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Invited Mini-Review

OVERVIEW OF IMPORTANT DISEASES OF SUGARCANE

The nature of the sugarcane plant and the manner in which it is grown often make it vulnerable to a wide range of diseases caused by bacteria, fungi, viruses and phytoplasmas. While some widespread and damaging diseases such as brown rust (*Puccinia melanocephala* H. & P. Sydow) and orange rust (*P. kuehnii* E.J. Butler) are not systemic, most of the economically important diseases affecting sugarcane are and can be transmitted by planting infected seed material (Rott *et al*. 2000). For this reason, the production and distribution of healthy seedcane is an important component of integrated disease management strategies adopted by many sugarcane industries (Hoy and Flynn 2001; Fortmann *et al*. 2006; McGuire *et al*. 2009).

Ratoon stunt, a bacterial disease caused by *Leifsonia xyli* subsp *xyli* (*Lxx*; Davis *et al*. 1984), is one of the most widespread diseases internationally and can cause yield reductions of between 10 and 50% (Bailey and Bechet 1986, 1995; Comstock 2002). The disease does not have obvious external symptoms and can therefore be spread unwittingly from one area to another. Another widespread and economically important bacterial pathogen, leaf scald (*Xanthomonas albilineans* Dowson 1943), exhibits striking symptoms and can cause entire sugarcane stools to die but latent infections can result in the disease escaping detection (Ricaud and Ryan 1989). The fungal disease smut (*Sporisorium scitamineum* H. & P. Sydow) occurs in most sugarcane-growing countries and can result in yield reductions of 10 to 75% depending on the severity of the disease, climatic conditions and susceptibility of the variety being grown (Ferreira and Comstock 1989; Rutherford *et al*. 2003). Red rot (*Glomerella tucumanensis* [Spegazzini] von Arx & E. Muller) is another important systemic fungal disease that may result in substantial sucrose losses in subtropical sugarcane (Viswanathan and Samiyappan 2002). Mosaic (SCMV and *Sorghum mosaic virus* [SrMV], both potyviruses) is currently one of the most important viral diseases affecting sugarcane, with losses of between 7 and 50% being reported (Singh *et al*. 1997; Grisham 2000; Viswanathan and Balamuralikrishnan 2005). Studies conducted in South Africa showed that the yield of a susceptible variety could be reduced by 45% when SCMV incidence is high (Fox and Bailey 1987). In fields severely infected with yellow leaf (ScYLV, a luteovirus), losses are estimated to be between 5 and 16% in South African varieties (Rutherford *et al*. 2004), and up to 25% in susceptible varieties grown in other countries (Vega *et al*. 1997). While Fiji leaf gall (*Sugarcane Fiji disease virus* [SCFDV]) is not as widespread as some of the other diseases mentioned and has largely been controlled through varietal resistance, it is still considered one of the most important diseases of sugarcane (Smith 2000) and is regarded as a significant threat to industries that currently do not have SCFDV (Frison and Putter 1993). White leaf (Rishi and Chen 1989) and leaf yellows (Cronje *et al*. 1998) are caused by phytoplasmas and both have been observed to have a negative effect on yield. Ratoon crops have been abandoned in the past in Thailand and Taiwan when severely infected with white leaf, but the effects of the disease have been mitigated through varietal resistance (Rishi and Chen 1989).

TECHNIQUES USED FOR PATHOGEN INDEXING

Pathogen screening plays a vital role in limiting the spread of diseases in seedcane in commercial plantings and in international sugarcane germplasm exchange. Standard quarantine practices have been summarized by Frison and Putter (1993). Pathogens can remain latent and in low numbers in planting material, requiring diagnostic methods with high sensitivity, specificity and reliability for disease detection in sugarcane industries world-wide.

Prior to 2000, the main methods for diagnosing diseases were recognition of visual symptoms and isolation of pathogens such as *X. albilineans* on selective growth media, e.g. Davis *et al*. (1994). These methods remain integral to disease diagnosis in quarantine facilities. Both phase contrast (Gillaspie *et al*. 1973; Teakle *et al.* 1973; Bailey 1976) and immunofluorescence microscopy (Harris and Gillaspie 1978) are used for the detection of *Lxx*. The evaporative binding-enzyme immunoassay (EB-EIA) is currently used for the detection of *Lxx* on a large scale for commercial cane in Australia and South Africa (Croft *et al*. 1994; McFarlane *et al*. 1999). The enzyme-linked immunosorbent assay (ELISA) is used for detection of SCMV (Gauer *et al*. 2002) and ScYLV (Schenck *et al*. 1997; Scagliusi and Lockhart 2000). Other diagnostic tests developed for the detection of ScYLV include: serological tissue blot immunosorbent assay (TBIA; Schenck *et al*. 1997) and double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA; Scagliusi and Lockhart 2000; Chatenet *et al*. 2001).

During the 1990's molecular techniques were introduced to diagnostic laboratories for the detection of sugarcane pathogens. Molecular tests are able to detect low titres of microorganisms making them ideal for pathogen screening. Polymerase chain reaction (PCR) is used for the detection of *Lxx* (Pan *et al*. 1998; van Antwerpen and Botha 1999; Fegan *et al*. 2002), *Maize streak virus* (MSV; Rybicki and Hughes 1990), *X. albilineans* (Pan *et al*. 1997, 1999), *S. scitamineum* (Albert and Schenck 1996) and phytoplasmas such as those causing leaf yellows (SCYP), grassy shoot and white leaf (Deng and Hiruki 1991). Generic primers are available for the detection of *Luteoviridae* (Chomic *et al*. 2010) and *Potyviridae* (Hall *et al*. 1998; Chen and Adams 2001; Zheng *et al*. 2010) in sugarcane.

To detect the presence of SCMV, a reverse transcription-PCR (RT-PCR) was developed (Smith and Van De Velde 1994). RT-PCR-RFLP (RFLP; random fragment length polymorphism) methods have been published for SCMV strain discrimination and detection of SrMV (Yang and Mirkov 1997). Subsequent refinement of this technique improved specificity and facilitated distinction of SCMV strains A, B, D and E, as well as strains from Africa not yet described (Alegria *et al*. 2003), but could not detect strains from China (Fernandez *et al*. 2006). Primers for *potyviruses* of *Poaceae*, developed by Marie-Jeanne *et al*. (2000), detected all strains of SCMV and SrMV but were unable to distinguish between these two viruses in a concurrent infection (Fernandez *et al*. 2006). Sequencing is currently the only reliable method to assess SCMV and SrMV genetic diversity (Perera *et al*. 2009). Amplification and sequencing a specific fragment from diseased sugarcane leaf samples confirmed the existence of several strains of *Sugarcane streak mosaic virus* (SCSMV) in Asia (Fernández *et al*. 2006). At the South African Sugarcane Research Institute (SASRI) and Centre de Cooperation Internationale en Recherche Agronomique pour le Developpement (CIRAD) quarantine facilities, a one-step RT-PCR is routinely used for detection of SCMV as well as for other members of the potyviruses such as SrMV (van Antwerpen *et al*. 2005; Fernández *et al*. 2006).

Luteoviroids are usually present in lower concentrations than other viruses in plants and are restricted to phloem tissue which is more difficult to disrupt during sample preparation (Chomic *et al*. 2010). ScYLV can be detected using RT-PCR (Comstock *et al*. 1998) and quantitative RT-PCR (qRT-PCR) (Zhu *et al*. 2010). RT-PCR can detect the virus in earlier stages of infection and is routinely used in quarantine facilities such as United States Department of Agriculture (USDA) (Xie *et al*. 2009), BSES Ltd (B Croft pers. Comm.), SASRI (van Antwerpen *et al*. 2005) and CIRAD (Girard *et al*. 2006). Although the TBIA is 10^3 to10^4 fold less sensitive than RT-PCR (Zhu *et al*. 2010), it is a faster and cheaper method to detect ScYLV and can be used for rapid screening of large numbers of samples (Schenck *et al*. 1997). A multiplex RT-PCR test for the simultaneous detection of four sugarcane viruses (SCMV, SrMV, SCSMV and ScYLV) was developed by Xie *et al*. (2009).

Unknown pathogens causing symptoms in quarantined

Table 1 A review of *in vitro* explants used for multiplication of pathogen-free cells and treatments used for the elimination of sugarcane pathogens.

Explant	Pathogen(s) eliminated	Reference
1. Immature leaf roll	*Lxx*, ScYLV	Snyman *et al*. 2007
	ScYLV, SCYP	Parmessur *et al*. 2002
2. Meristem excision	ScYLV	Chatenet *et al*. 2001; Fitch *et al*. 2001
	SCMV	Visessuwan *et al*. 1988, 1991, 1999
	SCMV, SCSMV	Mishra *et al*. 2010
3. Thermotherapy of setts, followed by meristem excision	*Lxx*, *X. albilineans*, SCMV	Victoria *et al*. 1999
	ScYLV, SCMV	Ramgareeb *et al*. 2010
	Lxx, ScYLV	Guevara and Ovalle 2005
4. Chemotherapy of excised meristems	SCMV	Balamuralikrishnan *et al*. 2002
	SCWL	Wongkaew and Fletcher 2004

Lxx – Leifsonia xyli subsp. *xyli,* SCMV: *Sugarcane mosaic virus*, SCSMV: *Sugarcane streak mosaic virus*, SCWL: *Sugarcane white leaf phytoplasma,* ScYLV: *Sugarcane yellow leaf virus*, SCYP*: Sugarcane yellows phytoplasma*

material can be sequenced and compared to other sequences on the NCBI BLASTn database to establish the identity of the causal agent (van Antwerpen and Botha 1999). In the interests of biosecurity, van Antwerpen and Rutherford (2008) identified emerging new viruses that could potentially infect sugarcane in the future and designed or obtained generic primers for most viral families to allow screening of material in the SASRI quarantine glasshouse.

SUMMARY OF METHODOLOGY TO ELIMINATE PATHOGENS FROM SUGARCANE

Pre-treatment of setts

Hot water treatments have been used since the 1920's to eliminate pathogens from plant material and heat regimes of between 34 and 52°C are commonly used to produce healthy plants (reviewed by Wang *et al*. 2007). The standard treatment for sugarcane setts is a cold soak in running water for 48 h to remove *X. albilineans*, followed by a hot water treatment of 50°C for 2-3 h to eliminate *Lxx* (Frison and Putter 1993; Dookun *et al*. 1996; Guevara and Ovalle 2005). Dipping in a broad-spectrum fungicide and insecticide is recommended (Frison and Putter 1993). Following sett treatment, thermotherapy is recommended during germination (2-3 weeks growth at 41°C after planting in trays; Guevara and Ovalle 2005).

Surface decontamination of sugarcane setts using detergent and/or ethanol, followed by germination of buds *in vitro* has been attempted for the removal of bacterial contaminants and to eliminate SCFDV (Wagih *et al*. 1995). However, only 28% of the germinated plants were virus free, suggesting that in addition to hot water treatments of setts and thermotherapy during germination of shoots, alternative methods and smaller explants (e.g. excised meristems) should be considered for propagation of virus-free cells.

In vitro techniques

Early work on plant meristems (Grout 1990) showed that the shoot and root tip meristematic cells of plants were free from virus for the following reasons: (a) meristematic cells have high metabolic activity and viruses were unable to replicate in all of these cells; (b) viruses spread rapidly through the plant by means of the vascular system and since the meristematic region does not have vascular tissue the viruses infect cells by osmosis spreading via the plasmodesmata. This is a slow process and viruses are therefore unable to infect dividing cells; (c) meristematic cells have a higher auxin concentration than cells from other regions of the plant and a high level of endogenous plant auxin is believed to inhibit virus replication.

Although no chemical or physical treatment is known to eradicate viruses from infected sugarcane plants, virus-free material can be propagated by: (a) meristem excision followed by either callus development and shoot multiplication (Leu 1978; Visessuwan *et al*. 1988, 1999; Fitch *et al*. 2001) or direct shoot organogenesis (Chatenet *et al*. 2001; Ramgareeb *et al*. 2010); (b) immature leaf roll culture followed by indirect somatic embryogenesis (Leu 1978; Dean 1982; Visessuwan *et al*. 1991, 1999; Parmessur *et al*. 2002), direct organogenesis (Irvine and Benda 1987) or direct somatic embryogenesis (Snyman *et al*. 2007) (**Table 1**). Most of these *in vitro* interventions resulted in pathogen-free plants in 80 to 100% of cases, emphasizing the need to verify the status of material derived from the process.

Success rates for eradication of virus vary and may be influenced by the size of the meristem, the susceptibility of the cultivar and the consequent viral load. For sugarcane virus elimination, a suitable meristem size likely to be free of virus is in the range of 0.2 – 1.5 mm in length (Chatenet *et al*. 2001; Fitch *et al*. 2001; Ramgareeb *et al*. 2010). Although it is difficult to successfully culture explants of a size less than 0.5 mm in length (i.e. meristem dome only), excision and subsequent *in vitro* establishment and recovery of plants of meristems sized 0.2 (Visessuwan *et al*. 1998, 1991, 1999) and 0.3 mm (Chatenet *et al*. 2001; Fitch *et al.* 2001) has been accomplished. To use explants larger than 0.5 mm and ensure virus eradication, meristem culture was linked with heat therapy (Victoria *et al*. 1999; reviewed by Zhang *et al*. 2006; Ramgareeb *et al*. 2010). The exposure of the parent material to high temperatures (30-40°C) is thought to inhibit viral RNA synthesis and inactivate the virus. Ramgareeb *et al*. (2010) demonstrated that ScYLV and SCMV were only removed from meristems in size categories of > 0.5 mm and = 2 mm but not for larger explants (> 2 but < 10 mm).

Other reported methods of virus inactivation include chemotherapy using the antiviral agent ribavirin for removal of SCMV (Balamuralikrishnan *et al*. 2002) and a combination of antibiotics and meristem culture for eliminating sugarcane white leaf phytoplasma (SCWL) (Wongkaew and Fletcher 2004).

An alternative explant to meristems, namely leaf discs excised from immature leaf whorls from diseased plants followed by direct somatic embryogenesis or direct organogenesis, was assessed for disease elimination (Irvine and Benda 1987; Snyman *et al*. 2007). It was shown that clean plantlets from cultivars N14 and N32 could be regenerated from explants infected with *Lxx* and ScYLV, respectively (Snyman *et al*. 2007). However, inconsistent results were obtained for SCMV, where 92% of the plants were found to be free of the virus in N32 and only 25% success was reported for a more susceptible variety, NCo376 (Snyman *et al*. 2007). Similarly, SCMV was eliminated from an average of 77% of the acclimated plants tested in the Irvine and Benda (1987) study. The approach using the leaf roll as an explant source and the *in vitro* technique of direct somatic embryogenesis is suitable for the elimination of some pathogens (i.e. *Lxx* and ScYLV) but limited in the case of SCMV – especially when viral titre is high.

Although little field trial data is available to compare the effect of disease-free *in vitro* plants on yield, two studies recorded improved yields when *in vitro*-derived material was compared with hot water treated setts and untreated

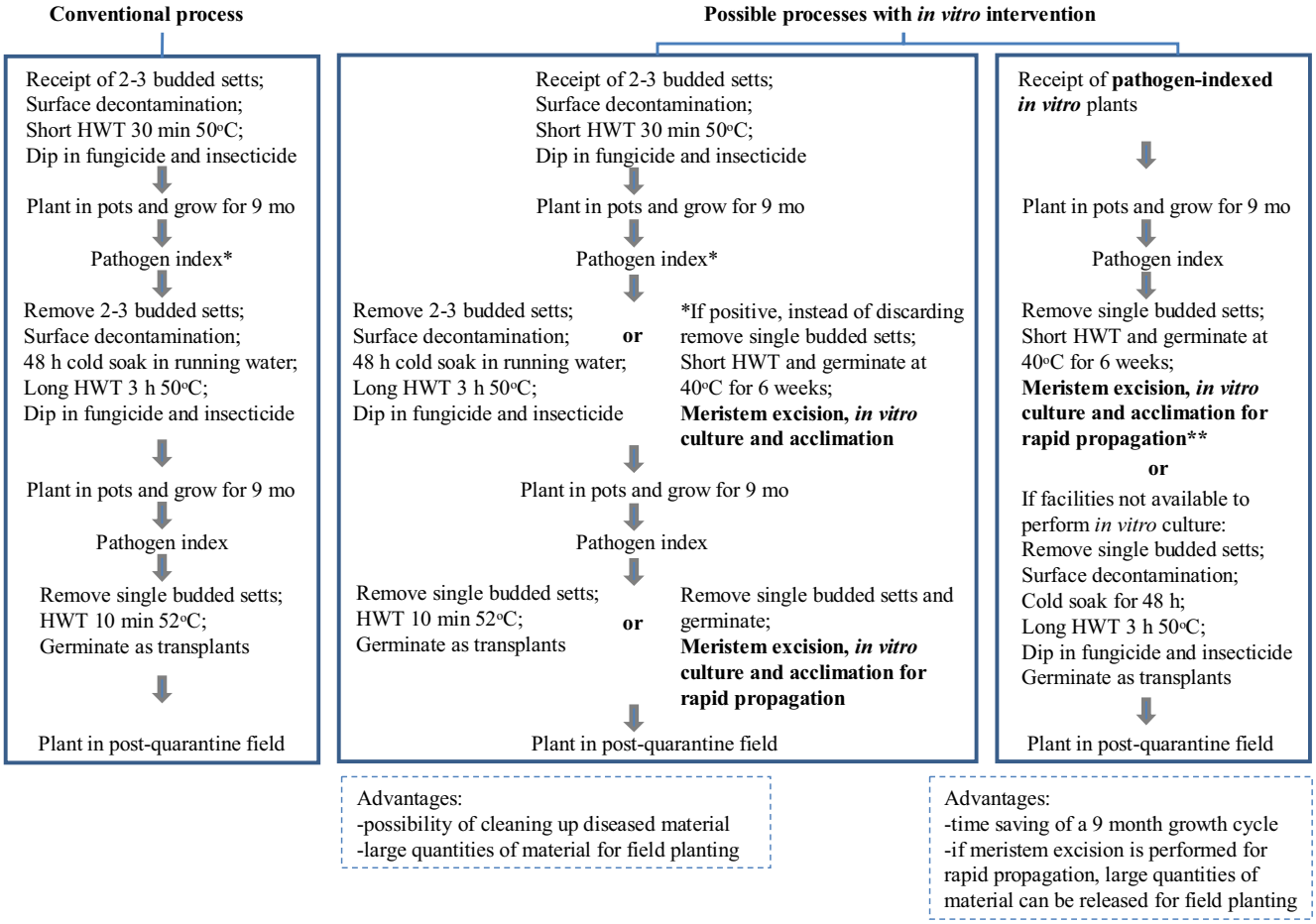

Fig. 1 A summary of the steps involved in processing sugarcane germplasm to facilitate international transfer. In addition to the conventional process (on left), two modified approaches have been proposed which incorporate meristem excision followed by *in vitro* culture for pathogen elimination and rapid propagation of clean material. *Discard if positive for a pathogenic organism; ** *In vitro* material can be released from quarantine after one growth cycle if pathogen indexing is negative (Frison and Putter 1993; Bailey *et al*. 2000). However, some quarantine units (e.g. SASRI and USDA; van Antwerpen pers. comm.) elect to go through the conventional two cycles of vegetative propagation prior to release to open quarantine fields.

material in both Columbia (Victoria *et al*. 1999) and Florida, USA (Flynn *et al*. 2005).

CHALLENGES FOR INTERNATIONAL EXCHANGE OF GERMPLASM

Pathogen diversity

While there appears to be little genetic variation in the global populations of *Lxx* (Young *et al*. 2006), studies have shown diversity in the populations of other important systemic diseases. The threat of new pathotypes of these diseases is significant when importing sugarcane from other countries as there may be low levels of inherent resistance in the germplasm of commercial varieties being grown in the recipient industry. *S. scitamineum* populations in the Americas and Africa show little variation (Braithwaite *et al*. 2004; Raboin *et al*. 2006) indicating that varietal resistance is a viable and stable management option in these regions. However, Asian populations formed a genetically distinct group and the diversity within this group was relatively high, which may pose a risk to other sugarcane industries (Braithwaite *et al*. 2004; Raboin *et al*. 2006). The SCMV complex consists of four distinct Potyviruses and includes many strains of the pathogen (Shukla *et al*. 1992; Yang and Mirkov 1997; Perera *et al*. 2009; Viswanathan *et al*. 2009). Three distinct pathotypes of ScYLV showing varying degrees of virulence have been identified in worldwide collections (Abu Ahmad *et al*. 2007).

In addition to pathogen diversity, another disease-related risk is unknown or previously uncharacterized diseases entering a sugar industry. The use of sensitive molecular methods to detect these threats especially when foreign varieties are imported through quarantine has reduced but not eliminated this risk in South Africa (van Antwerpen and Rutherford 2008).

Transport of *in vitro* material

The safest and preferred method for international germplasm exchange is via *in vitro* cultures (Frison and Putter 1993). In order to facilitate this, neither antibiotics nor charcoal should be added to the growth medium as temporary depression of viral loads or masking of pathogens is undesirable (Frison and Putter 1993). However, certain problems may be encountered when *in vitro* material is transported overseas: (a) sterile containers can be opened by uninformed customs officials, causing contamination and ultimately destruction of the consignment; (b) exposure to extreme temperatures in flight if placement in a particular compartment of the aircraft is not specified; (c) the cost of producing *in vitro* material is higher than that of setts; (d) not all quarantine facilities have the necessary expertise to send and /or receive *in vitro* cultures; (e) if material is shipped in semi-solid medium, proper handling en-route is necessary.

POTENTIAL APPROACHES FOR PRODUCTION OF DISEASE-FREE MATERIAL

Fig. 1 provides a summary of the procedures for processing sugarcane germplasm when received by a quarantine facility. In addition to the conventional procedure for handling imported material (Frison and Putter 1993), two slightly

modified procedures have been shown to demonstrate the advantages of meristem excision and *in vitro* culture for pathogen elimination, rapid propagation of clean material and reducing the length of time required for quarantine screening by one growth cycle if pathogen-indexed *in vitro* material is supplied (as suggested by Frison and Putter 1993; Bailey *et al*. 2000; Snyman *et al*. 2011).

CONCLUSIONS

Varietal resistance is key to limiting the spread and impact of most of the important diseases affecting sugarcane, with emphasis currently being placed on breeding for smut, mosaic and brown rust resistance. Other management strategies for mosaic, smut and ratoon stunt include planting healthy seedcane and ensuring that fields are fallowed for a minimum of three months to enable the identification and removal of volunteers from the previous crop that may have been infected with these diseases. Sugarcane that is infected with *Lxx* can be cured over a number of years with a series of hot water treatments (50°C for 2 h) (Benda and Ricaud 1978) and a combination of thermotherapy and meristem tip culture is also reported to effectively eliminate the pathogen (Victoria *et al*. 1999). Smut can be eliminated from seedcane by hot water treating at 50°C for a minimum of 30 min in a fungicide such triadimefon (Bailey 1983). In contrast, once infected with mosaic, ScYLV or SCYP, sugarcane cannot be cured using hot water treatment alone, but meristem excision followed by *in vitro* culture can ensure propagation of virus-free cells provided that subsequent molecular pathogen diagnosis is conducted. In addition to producing virus-free plants, meristem culture also provides the following advantages of *in vitro* clonal propagation (Lee 1987), an internationally accepted method of plant production for export and import with respect to quarantine regulations (Frison and Putter 1993) and the cryopreservation of the apical meristem for long term storage of important commercial varieties (Taylor and Dukic 1993).

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