EVALUATION OF SUGARCANE VARIETIES FOR RESISTANCE TO RATOON STUNTING DISEASE

by

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Submitted in fulfillment of
the academic requirements for the degree
of

MASTER OF SCIENCE

in the
School of Life and Environmental Sciences
University of Natal
Durban
September 2003

ABSTRACT

Ratoon stunting disease (RSD), caused by the bacterium *Leifsonia xyli* subsp *xyli*, is well established in most sugarcane growing regions of the world and is considered to cause more yield losses worldwide than any other sugarcane disease (Hughes, 1974). In South Africa, field trials have demonstrated that yield reductions under rainfed conditions can exceed 40% in highly susceptible varieties (Bailey and Bechet, 1986). When cane is grown under irrigation, yield losses are less noticeable but still significant in many varieties (Bailey and Bechet, 1995). It is estimated that RSD currently results in a one percent reduction in industrial production in South Africa and between 10 and 20% in other African countries where South African varieties are grown (Bailey and McFarlane, 1999; Rutherford *et al.,* 2003). For many years, the reaction of different sugarcane varieties to RSD has been based on large, replicated yield loss trials grown over a number of years under rainfed and irrigated conditions. Although these trials provide valuable information, they are time-consuming and require large areas of uniform land. They are therefore not suitable for incorporation into a routine disease screening programme in which large numbers of genotypes are assessed for their reactions to the important diseases occurring in the industry. As a result, the susceptibility of new commercial varieties to RSD is only known several years after release to the growers.

The main objective of this study was to establish a suitable method to reliably evaluate sugarcane genotypes for RSD resistance as part of the plant breeding and selection programme. Emphasis was placed on the use of the tissue blot immunoassay (TBIA) developed by Harrison and Davis (1988) and modified by Davis *et al* (1994), in relation to the more traditional methods of variety assessment, such as the rate of spread of RSD in the field at harvest and yield loss trials. Although the immunoassay protocol was not altered, slight modifications to the blotting procedure resulted in clearer blots that were easier to interpret. Internode position and the age of the cane were shown to have a marked effect on the extent of colonisation and ultimately the RSD resistance rating. A trial investigating the effect of the extent of colonisation on the rate of spread of RSD at harvest was conducted and showed that the relationship between spread and

colonisation was highly significant. This indicated that RSD spread more rapidly through varieties such as N14 and N22 that supported high populations of *L. xyli* subsp *xyli*. The control plots in the same trial provided useful information on the extent of colonisation in the twelve varieties planted. In another trial, the effect of RSD on the yield components of six commercially grown varieties was investigated and TBIA was also conducted to compare the two methods of variety assessment. The relationship between yield loss and the extent of colonisation was significant in both the plant and first ratoon crops. TBIA produced consistent results and the ranking of the six varieties was virtually identical, despite the different growing conditions during the two crop cycles. In an attempt to screen large numbers of genotypes under controlled glasshouse conditions, TBIA was also tested on RSD–infected sugarcane transplants (seedlings). The results of this trial were variable and could not be reliably used as a screening tool.

Based on the findings of this study, TBIA has now been adopted as a quicker and cheaper alternative to immunofluorescence microscopy for diagnosing RSD in sugarcane transplants. More importantly, TBIA has been accepted as a method of screening genotypes routinely for resistance to RSD and the first screening trial was planted in November 2002. It will now be possible to inform sugarcane growers of the RSD status of the new varieties as they are released, enabling them to make more informed decisions on how to manage each variety. This information will also be valuable when selecting parents in the crossing programme, with a long term view of improving the general resistance of commercially grown varieties to RSD. This should ultimately result in a substantial reduction in RSD levels in the industry.

PREFACE

The experimental work described in this dissertation was carried out at the South African Sugar Association Experiment Station Pathology Department from August 2000 to August 2003, under the supervision of Dr Barbara Huckett and Mr Roger Bailey.

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

Signed.....

SA McFarlane

ACKNOWLEDGEMENTS

Sincere thanks to:

Dr Barbara Huckett for her objective advice and encouragement, especially with the writing of this thesis.

Mr Roger Bailey, for his useful comments regarding this work and for sharing his vast knowledge and experience on sugarcane diseases and their importance.

The staff of the Pathology Department for their assistance in trial management, and data and sample collection.

Dave Dinkelmann for designing and constructing much of the equipment used for the TBIA.

Julie Richards for proof reading this manuscript.

My friends and colleagues, especially Sandy Snyman for her interest, constant encouragement and useful advice.

My parents, Ed and Lynn Tough, for their interest and support.

My husband and friend, Keith McFarlane for his patience and understanding .

For
Christopher John McFarlane –
the "very long worksheet" is finished!

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a.i.	active ingredient
ANOVA	analysis of variance
arcsin	arc sine
CV	coefficient of variation
cvb	colonised vascular bundles
EB-EIA	enzyme linked-immunosorbant assay
ERC	estimated recoverable crystal
IFM	immunofluorescence microscopy
ha	hectare
HWT	hot water treatment
LSD	least significant difference
L. xyli* subsp *xyli	*Leifsonia xyli* subsp *xyli*
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline-tween
PCM	phase contrast microscopy
RSD	ratoon stunting disease
SASEX	South African Sugar Association Experiment Station
SBS	single budded sett
se	standard error
t cane / ha	tons cane per hectare
t / ha	tons per hectare
TBIA	tissue blot immunoassay
TBS-T	Tris buffered saline-tween

GLOSSARY

Genotype: a new sugarcane hybrid in the variety development programme.

Nursery: fields designated for the production of healthy seedcane. These are usually located on growers' farms.

Plant crop: the first crop of sugarcane that develops after planting.

Sett: a vegetative propagule consisting of one or more nodes with buds and root primordia. These setts are often referred to as 'seed', 'seedcane' or 'seed material'.

Sugarcane transplants: sugarcane plantlets produced from single-budded setts.

Stool: a single sugarcane plant that develops as a cluster of sugarcane stalks arising from the germination of setts, or the regrowth after the crop has been harvested.

Ratoon: sugarcane that grows from the buds remaining in the stubble left in the ground after harvest. The old root system supports the regenerating crop until new roots develop.

Variety: a commercially grown sugarcane cultivar.

Volunteer: sugarcane that survives after the eradication of the previous crop. Volunteers often harbour systemic diseases such as ratoon stunting disease, smut and mosaic.

CHAPTER 1

INTRODUCTION

Sugarcane is a monocotyledon and a member of the family Gramineae, and is classified in the genus *Saccharum*. There are six *Saccharum* species but the commercial varieties cultivated today are complex hybrids involving two or more species, usually *S. officinarum* and *S. spontaneum* and to a lesser extent *S. sinense, S. robustum. S. edule and S. barberi* (Arcenaux, 1965).

The crop is grown commercially in many tropical and sub-tropical regions of the world in soils and climates displaying great variation (Barnes, 1974). Sugarcane provides about 65% of the sugar produced in the world. South Africa is one of the world's leading cost-competitive producers of high quality sugar, producing an average of 2,5 million tons of sucrose per season from approximately 23 million tons of cane (Anon, 2002).

The nature of the sugarcane plant and the manner in which it is grown often make it vulnerable to attack from a wide range of pathogens (Bechet *et al.*, 1992). It is vegetatively propagated and systemic diseases can therefore be spread in seedcane and from infected volunteers that survive from the previous crop. The crop is perennial, and in South Africa the ability of a variety to ratoon well is considered an important trait (Hunsigi, 1993). As a result, fields are only replanted every nine to ten years on average in South Africa, allowing systemic diseases to build up from crop to crop. The crop is largely grown as a monoculture in the coastal areas of KwaZulu-Natal and to a lesser extent in the KwaZulu-Natal Midlands and Mpumalanga, favouring epidemic development. This situation has improved to some extent over the past fifteen years with the availability of a wider range of varieties, most of which have some resistance to the important diseases occurring in the industry (Bailey *et al.*, 1994).

Only about 21% of the 420 000 hectares of land under sugarcane in South Africa is irrigated (Schmidt, 2001). A large proportion of the crop therefore frequently suffers

from moisture stress, increasing the risk of damage from certain diseases (Bechet *et al.*, 1992).

Sugarcane is affected by a large number of diseases, caused by fungi, bacteria, viruses and phytoplasmas. The most important of these in the South African sugar industry are ratoon stunting disease (RSD) (*Leifsonia xyli* subsp *xyli* Davis *et al.* 1984; Evtushenko *et al.* 2000), smut (*Ustilago scitiminea* H. & P. Sydow), mosaic (sugarcane mosaic virus), brown rust (*Puccinia melanocephala* H. & P. Sydow), red rot (*Glomerella tucumanensis* Spegazzini; von Arx & E. Muller), leaf scald (*Xanthomonas albilineans* Ashby 1929; Dowson, 1943) and streak (sugarcane streak virus) (Bailey and Bechet, 1982). The planting of resistant varieties is the most efficient and long-term method available to control important sugarcane diseases in most industries globally. In South Africa, genotypes are screened at the South African Sugar Association Experiment Station (SASEX) for those diseases that cause or are most likely to cause serious yield losses, and which cannot easily be controlled other than by satisfactory resistance. These include smut, mosaic, brown rust, red rot, leaf scald and streak. Disease screening is achieved by exposing the genotypes under test to the pathogen or pathogens of interest, either through inoculation (smut, leaf scald and red rot) or natural infection (smut, mosaic, brown rust and streak). For trials relying on natural infection, particularly in the case of mosaic and smut, planting dates are an important consideration in order to improve the chances of spread and infection.

No trials are currently conducted at SASEX to screen genotypes routinely for tolerance to RSD, a disease that is well established in most sugarcane growing regions of the world and is considered to cause more yield losses worldwide than any other sugarcane disease (Hughes, 1974). In South Africa, field trials have demonstrated that yield reductions under rainfed conditions can exceed 40% in highly susceptible varieties (Bailey and Bechet, 1986). When cane is grown under irrigation, yield losses are usually less, but are still significant in many varieties (Bailey and Bechet, 1995 and 1997). It is estimated that RSD currently results in a 1% reduction in industrial production in South Africa and between 10 and 20% in other African countries where South African varieties are grown (Bailey and McFarlane, 1999).

RSD incidence has been reduced in some areas to low levels using an integrated control programme (Comstock *et al.*, 1995; McFarlane *et al.*, 1999). This programme involves the planting of only healthy seedcane from heat-treated stock, the effective eradication of the previous (often RSD-infected) crop and ensuring the practice of adequate field hygiene. Despite this, the disease remains prevalent in many countries, including South Africa (Fig 1.1), where control programmes based on these measures have been in existence for many years (Bailey *et al.*, 1999, Croft *et al.*, 1994). The use of resistant genotypes may offer an alternative strategy for minimizing losses from RSD.

It was only recently that the first attempts were made to screen for varietal resistance to RSD in Canal Point, Florida (Davis *et al.,* 1994), although differences in varietal reactions to RSD have been recognised since 1950 (Steindl, 1950). This is mainly due to the fact that selection for disease resistance usually relies on the visual assessment of disease symptoms, and RSD has no easily recognisable symptoms.

Harrison and Davis (1988) introduced the tissue blot-enzyme immunoassay (TBIA) to detect and enumerate vascular bundles colonised by *L. xyli* subsp *xyli.* They showed that the TBIA could be used to measure both the incidence and severity of RSD, two parameters commonly regarded as being important in breeding for disease resistance in sugarcane. They found that results from the method correlated well with yield reductions caused by RSD in single stool plots. Davis *et al.* (1994) made some modifications to the equipment used for the TBIA that enabled the screening of large numbers of genotypes for RSD resistance.

Differences in varietal reactions to RSD in the South African sugar industry have already been demonstrated in trials investigating yield responses to RSD (Bailey and Bechet, 1986, 1995 and 1997), the rate of spread of RSD on cane knives (Bailey and Tough, 1992), and populations of *L. xyli* subsp *xyli* (McFarlane and Bailey, unpublished) in different varieties. However, these trials are time-consuming and require large areas of uniform land, and as a result the reactions of only a limited number of commercial varieties are known. A simple, efficient method to assess varietal reactions to RSD is therefore essential if routine screening of large numbers of genotypes is to be achieved.

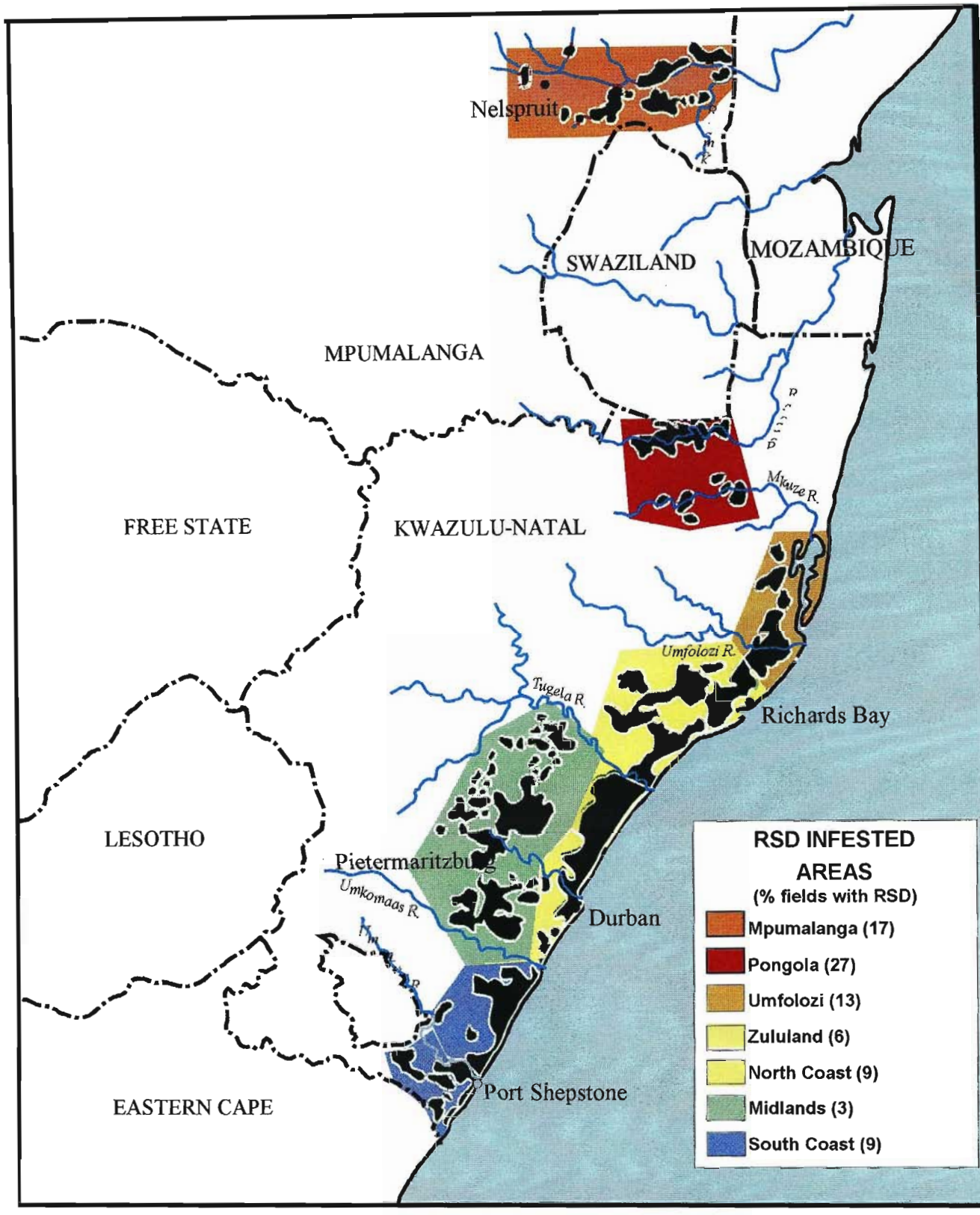


Figure 1.1 Occurrence of ratoon stunting disease in the South African sugar industry, 2002

(based on data obtained from the RSD diagnostic service operated at SASEX)

The main objective of this study was to determine the most efficient method to reliably rate the reactions of large numbers of sugarcane genotypes to infection by *L. xyli* subsp *xyli* as part of the variety development programme at SASEX. Emphasis was placed on the use of TBIA in relation to the more traditional methods of variety assessment, such as the rate of spread of RSD in the field and trials investigating the effect of RSD on yield. TBIA was also tested as a method of mass-screening RSD–infected sugarcane transplants under controlled glasshouse conditions.

CHAPTER 2

LITERATURE REVIEW

2.1 Ratoon stunting disease

2.1.1 Introduction

Ratoon stunting disease (RSD) of sugarcane, caused by the bacterium *Leifsonia xyli* subsp *xyli*, was first reported in Queensland during the 1944-45 season in the variety Q28 (Steindl, 1950). Subsequently it was soon identified as being well established in most sugarcane growing regions of the world and it is considered to be the disease causing more yield loss worldwide than any other sugarcane disease (Hughes, 1974; Davis and Bailey, 2000). Intolerant varieties have been shown to suffer yield losses as high as 50% when grown under unfavourable conditions (King *et al.,* 1965; Irey, 1986; Gillaspie and Teakle, 1989; Dean and Davis, 1990). In South Africa, yield reductions exceeding 40% have been recorded in susceptible varieties under rainfed conditions (Bailey and Bechet, 1986) (Table 2.1). When cane is grown under irrigation, yield losses are less noticeable but still significant in most varieties (Bailey and Bechet, 1995).

2.1.2 The incidence and effects of RSD in the South African sugar industry

The large-scale RSD diagnostic service that has operated in the South African sugar industry since 1977 has provided valuable information on the incidence of RSD in both commercial fields and intended seedcane sources. Initial surveys indicated that approximately 30% of fields in the southern regions of the industry were infected with RSD (Bailey and Fox, 1984). Intensive surveys conducted since 1997 indicated that the incidence has subsequently decreased substantially, and in 2000 it was estimated that about 13% of fields in this region were infested with RSD (Bailey *et al.*, 2000). The

levels of RSD within infested fields in the rainfed areas were low, with a mean of 1,3% stalks infected. The impact on production in these areas when growing conditions are favourable is therefore likely to be negligible.

Table 2.1 RSD-induced yield losses (tons cane per hectare) recorded in previous trials (plant to first ratoon)

Variety	Yield loss (%)				Overall rating
	Trial 1	Trial 2	Trial 3*	Trial 4*	
NCo376	-39	-22	-14	-19	Susceptible
N12	-17	-2			Intermediate-susceptible
N14	-38	-32	-15**	-13**	Highly susceptible
N17		-50	-8		Highly susceptible
N19		-11	-13	-8	Susceptible
N22		+2	+2		Intermediate
N23		-7		-11	Intermediate-susceptible
N24				-19	Susceptible
N25				-23	Highly susceptible

Trial 1 Bailey and Bechet, 1986
Trials 2 and 3 Bailey and Bechet, 1995
Trial 4 Bailey and Bechet, 1997
(* denotes irrigated trials; ** denotes underestimate since 'healthy' plots were infected)

This decrease in the incidence of RSD can be attributed to an increase in grower awareness of the disease due to the diagnostic service, active Local Pest, Disease and Variety Control Committees, and extension services. These services lead to an increase in the use of phytosanitary practices to control the disease (Refer 2.1.6).

Since the inception of large-scale diagnosis in 1977, the recorded incidence of RSD has been higher in the northern irrigated regions of KwaZulu Natal and in Mpumalanga where, until recently, levels exceeded 50% fields infected. Since then, progress has been made in these areas and recent surveys indicated that 29% of the fields were infested (unpublished). With a mean of 6,3% stalks infected, significant losses are still likely in a number of fields. In 1999, it was estimated that RSD caused losses of approximately 2% of annual production in the northern region of the South African sugar industry (Bailey and McFarlane, 1999). The overall loss due to RSD in the industry in

the 2000/01 season was estimated to be about R10 million or 1% of production (Rutherford *et al.*, 2003).

RSD surveys conducted in other African countries where production is based on South African varieties suggested that losses ranged from 10 to 20% (Bailey and McFarlane, 1999).

2.1.3 Causal organism

Although RSD had been recognised as an important disease of sugarcane for over 40 years (Steindl, 1950), it was only in the 1970s that Gillaspie *et al.* (1973) and Teakle *et al.* (1973) independently found a bacterium associated with the disease in Louisiana and Australia, respectively. These findings were confirmed in South Africa (Bailey, 1976), Taiwan (Chen *et al.*, 1975), Mauritius (Ricaud *et al.*, 1976), India (Rishi and Nath, 1978), Brazil (Gillaspie *et al.*, 1979) and Florida (Davis and Dean, 1984). Davis *et al.* (1980) reported the isolation of the RSD bacterium in axenic culture and showed it to be the causal agent. The bacterium was later identified as a corynebacterium and was taxonomically designated *Clavibacter xyli* subsp *xyli* (Davis *et al.*, 1984). Evtushenko *et al.* (2000) reclassified the bacterium, along with *C. xyli* subsp *cynodontis*, as *Leifsonia xyli* subsp *xyli* and *Leifsonia xyli* subsp *cynodontis*, respectively. This was based mainly on cell wall composition and the fact that a distinct phylogenetic branch was formed on analysis of the 16S rDNA.

2.1.4 Symptoms of RSD

2.1.4.1 Visual symptoms

RSD has no obvious external symptoms, and the only internal symptom, an orange-red to brown discolouration of the vascular bundles in the nodal regions of infected plants (Figure 2.1), is largely dependent on the variety involved and the conditions under which the crop is grown (Steindl, 1950).

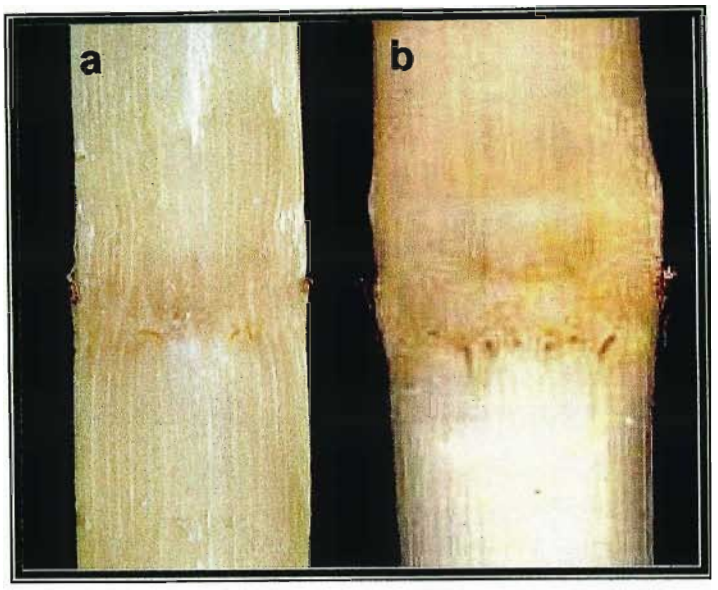


Figure 2.1 a) Healthy stalk b) Stalk showing internal symptoms of ratoon stunting disease

2.1.4.2 Effect on growth of sugarcane

The growth of RSD-infected plants may be stunted, but this is not a distinguishing characteristic because many other factors are well known to cause such stunting (Steindl, 1961). The stunting associated with RSD is often exacerbated by poor growing conditions, particularly drought stress, and usually worsens with each ratoon (Gillaspie and Teakle, 1989). Stunting in infected plants is often not uniform and fields infested with RSD often have a characteristic uneven appearance.

Transmission electron micrographs have shown that the bacterium is limited to the xylem vessels of the cane stalk (Worley and Gillaspie, 1975). Gillaspie *et al.* (1973) suggested that the physical plugging of the xylem vessels was the main cause of stunting and subsequent yield decline in plants infected with the disease. The increasing incidence of infection in fields during the harvesting process (refer 2.1.6.2) usually contributes to a decline in yield during successive ratoons (Roach, 1992a and b; Davis and Bailey, 2000). Infected setts may germinate slowly but overall germination is usually satisfactory (Steindl, 1950). Growth of infected plants is slower than that of healthy plants and the yield of the crop is ultimately adversely affected. The reduction in

yield is a result of shorter and thinner stalks rather than fewer stalks in infected stools and sucrose content is usually not adversely affected. It is only under extremely poor conditions for growth that reductions in stalk numbers may occur (Koike, 1974).

2.1.5 Methods of diagnosis

2.1.5.1 Microscopic diagnosis of RSD

A number of methods are now available to diagnose RSD. Phase contrast microscopy (PCM) is the most widely used microscopic method (Gillaspie *et al.*, 1973; Teakle *et al.*, 1973; Teakle, 1974). It has been used extensively in South Africa (Bailey and Fox, 1984; Bailey and Tough, 1991a) and Australia (Steindl, 1976; Egan, 1980) to diagnose RSD on a large scale. False positive results are rarely encountered with this method but false negatives are common when bacterial numbers are low.

Scanning and transmission electron microscopy have been used successfully to examine the morphological characteristics of the bacterium (Kao and Damann, 1980; Davis *et al.*, 1984). Serologically specific electron microscopy (ISEM) was earlier found to be useful in diagnosing RSD when other reliable methods were not readily available (Damann *et al.*, 1977).

Greater sensitivity has been obtained by using fluorescent-antibody staining techniques to detect *L. xyli* subsp *xyli* by epifluorescence microscopy. Staining cell extracts on multiwell glass slides, immunofluorescence microscopy (IFM), was found to be at least ten times more sensitive than PCM (Harris and Gillaspie, 1978). Sensitivity was further improved by first staining and then concentrating the bacterium on membrane filters, a technique known as fluorescent-antibody direct count on filters (FADCF) (Davis and Dean, 1984). These techniques are however more expensive and time-consuming than PCM.

2.1.5.2 Immunoassays to diagnose RSD

Other serological assays have since been developed to improve the efficiency of RSD diagnosis to facilitate the processing of large numbers of samples. The enzyme linked-immunosorbant assay (ELISA) was tested for its suitability in the late 1970s but was found to be non-specific (Gillaspie and Harris, 1979). Harrison and Davis (1988) developed a tissue blot-enzyme immunoassay (TBIA) to enumerate colonised vascular bundles, a method that could also be used to diagnose RSD. This technique was found to be less sensitive but more efficient than IFM and FADCF. A dot blot-enzyme immunoassay was subsequently developed but a number of false positive results were recorded (Harrison and Davis, 1990). Leaman *et al.* (1992) developed and successfully introduced an enzyme linked-immunosorbant assay (EB-EIA) to replace PCM for the routine diagnosis of RSD in the Australian sugar industry. This method has since been adopted by the South African sugar industry to routinely diagnose RSD (McFarlane *et al.*, 1999).

2.1.5.3 DNA-based diagnostic methods

The polymerase chain reaction (PCR), the most sensitive method of diagnosis currently available, has recently been developed to detect *L. xyli* subsp *xyli* at a number of sugarcane research institutes. Pan *et al.* (1998), in the United States of America, were the first researchers to publish a PCR protocol using a 20-mer primer pair, Cxx2 forward (5' ACC CTG TGT TTT CAA CG) and Cxx1 reverse (5' CCG AAG TGA GCA GAT TGA CC) that successfully distinguished *L. xyli* subsp *xyli* from *L. xyli* subsp *cynodontis* and all other sugarcane pathogens tested. The primers, derived from the internal transcribed spacer region between the 16S and 23S rRNA genes of the bacterial rRNA operon, amplify a 438-base pair DNA product.

Shortly afterwards, Australian researchers (Fegan *et al.,* 1998), published a PCR protocol using a 19-mer primer pair, CxxITSf#5 forward (5' TCA ACG CAG AGA TTG TCC A) and CxxITSr#5 reverse (5' GTA CGG GCG GTA CCT TTT C). These were

selected from aligned sequences of *L. xyli* subsp *xyli* to amplify a 278-base pair DNA product specific to *L. xyli* subsp *xyli.*

The protocol developed by South African researchers amplifies a 237-base pair DNA product from both *L. xyli* subsp *xyli* and *L. xyli* subsp *cynodontis* with a 30-mer forward primer, T_8 (5' TTG TCC AGG CGC CGG ATC TGA GAC AGT ACT), paired with a 14-mer reverse primer, T_9 (5' TGC TCG CGT CCA CT) (van Antwerpen and Botha, 1999). Although the primers were unable to distinguish the two *L. xyli* subspecies, they did differentiate these from other sugarcane pathogens found in Africa.

The amplification reactions used with the different primer pairs varied in the number of cycles used as well as the times specified for the denaturing and annealing steps. The reaction mixtures also differed slightly for all three protocols.

These PCR methods are currently not used for routine RSD diagnosis but rather for quarantine and research purposes, where a high level of accuracy is required. In South Africa this is mainly due to cost factors and the risk of contamination between samples. The EB-EIA is sufficiently sensitive to routinely detect RSD infections in cane aged nine months or older.

2.1.6 Transmission and control of RSD

2.1.6.1 Planting material

RSD is a systemic disease and is therefore transmitted during routine commercial propagation by planting seedcane taken from RSD-infected plants. The use of RSD-free planting material from an established nursery is therefore important in the control of the disease (Steindl, 1961; Egan, 1980; Roach, 1987). Heat treatment of seedcane before planting is practised to eliminate bacteria before establishing seedcane nurseries to ensure the planting of relatively healthy commercial seedcane (Damann and Benda, 1983).

Hot water treatment at 50°C for 2 to 3 hours is the most commonly used method of heat treating seedcane to eliminate RSD (Steindl, 1961; Shukla *et al.*, 1974; Benda and Ricaud, 1978). Other methods include hot air (Steindl, 1961), moist air (Chu and Lee, 1968) and aerated steam (Mayeux *et al.,* 1979). All heat treatment methods can adversely affect germination, with some varieties, such as N12, N17 and N19 in South Africa, being more susceptible to injury than others (Anon, 2001). Hot water treatment is not always entirely successful in eliminating RSD, particularly in heavily infected seedcane, and repeated treatments are necessary to ensure RSD-free seedcane (Roach, 1987).

2.1.6.2 Harvesting operations

Steindl (1950) showed that healthy setts could be inoculated with sap from stunted stalks and become infected. He further demonstrated that when harvesting the cane crop, RSD could be transmitted when the sap from infected stalks contaminates the blades of harvesting equipment and is spread from plant to plant. These findings were confirmed by Bailey and Tough (1992) and Comstock *et al.* (1996) in experiments using cane knives at harvest. Mechanical harvesters have also been shown to spread the disease (Taylor *et al.*, 1988; Damann, 1992; Hoy *et al.*, 1999). The complete eradication of the previous crop is therefore essential, particularly if the crop was infected with RSD, as volunteers are an important source of inoculum. An adequate fallow period after crop eradication aids in the identification and elimination of volunteers before the subsequent crop is planted. Disinfection of harvesting implements, usually by means of chemical disinfectants such as quaternary ammonium compounds, ethanol or neutralized cresylic acid (James, 1996), is important in preventing healthy cane from becoming infected. Carbolic acid (10% vol/vol) is currently recommended in the South African sugar industry (King, 1956; Bailey and Tough, 1992). These disinfectants are applied either by spraying onto the cutting surfaces or by dipping or swabbing implements (Roach, 1987 and 1988; Taylor *et al.*, 1988; Gillaspie and Teakle, 1989; Bailey and Tough, 1992).

2.1.6.3 Survival of L. xyli subsp xyli in the soil

There is some evidence to suggest that RSD survives in the soil or plant debris after the eradication of a previously infected crop (Autrey *et al.*, 1991; Bailey and Tough, 1991b; Anon, 1992). Fallow periods of up to three months after all volunteers have been removed are recommended in South Africa to eliminate this source of infection. Some growers in Australia have found it particularly difficult to eradicate RSD from certain fields, despite all the necessary precautions being taken to prevent the disease. Dominiak (1996) therefore suggested that certain soils, particularly sandy soils, may be more prone to RSD.

2.1.7 Why is RSD still prevalent in most sugarcane industries?

An integrated control programme of heat-treating seedcane to ensure healthy stock, disinfecting farm implements and allowing for adequate fallow periods could effectively reduce the incidence of RSD in crops to a low level. Despite this, the disease remains prevalent in a number of countries where control programmes based on these measures have been in existence for many years (Chu and Lee, 1968; Damann *et al.*, 1984; Irey, 1985; Roach, 1987; Croft *et al.*, 1994 and 1995). Although the incidence of RSD has declined substantially in the South African sugar industry, RSD levels are still high in the northern most production areas of the industry (Bailey and Tough, 1991a, Bailey and McFarlane, 1999). This lack of control can be attributed to the reluctance of growers to heat treat cane regularly due to its effect on germination, escapes during heat treatment, inadequate farm hygiene and fallow periods, the etiology of the disease and the continued use of RSD-susceptible varieties (Roach, 1987). The problem is exacerbated by the fact that RSD produces no specific external symptoms and is therefore difficult to diagnose based on a visual assessment alone. However, the introduction of diagnostic services in some countries based on microscopic observations of xylem sap (Gillaspie *et al.*, 1976; Bailey and Fox, 1984; Davis and Dean, 1984; Roach and Jackson, 1990) and more recently using the EB-EIA (Croft *et al.*, 1994; McFarlane *et al.*, 1999) and TBIA (Harrison and Davis, 1988) have served to

increase the awareness of growers to the disease. The chances of detecting RSD in a relatively small sample of stalks (usually 20 to 50 per field) are small when the levels of the disease within fields are low (Bailey and Fox, 1984; Barry Croft, personal communication). This is particularly serious in nursery fields where, if RSD is present, levels tend to be low and may not be detected.

In South Africa, approximately 48 000 growers grow sugarcane on a small scale (Anon, 2003a) and attempts to control RSD by heat therapy and farm hygiene practices may have only limited success. This is particularly true when contractors harvest cane and move from one farm to another with no sterilisation of harvesting implements. In a number of countries, economic pressures are forcing sugarcane growers to increase the size of their farms and reduce labour and attention to farm hygiene is therefore often reduced (Roach and Jackson, 1992). Growers are also unwilling to leave fields fallow. The potential benefits and costs of screening for RSD resistance therefore deserve some consideration.

2.2 Screening for resistance to diseases

2.2.1 Introduction

Historically, the susceptibility of agricultural crops to diseases has been an important incentive in the introduction of plant breeding programmes (Skinner *et al.*, 1987). In most crops, this provides the primary method for continued and long-term increases in productivity. Consistent progress relies on an efficient selection programme to produce superior varieties.

New sugarcane genotypes are grown from seed that is produced by crossing one parent variety with another. A new population of progeny consisting of many thousands of genotypes is produced annually. Genotypes pass through several stages of selection, their numbers being reduced at each stage (Figure 2.2). It takes 11 to 15 years to produce a new variety in the South African sugar industry (Anon, 2003b).

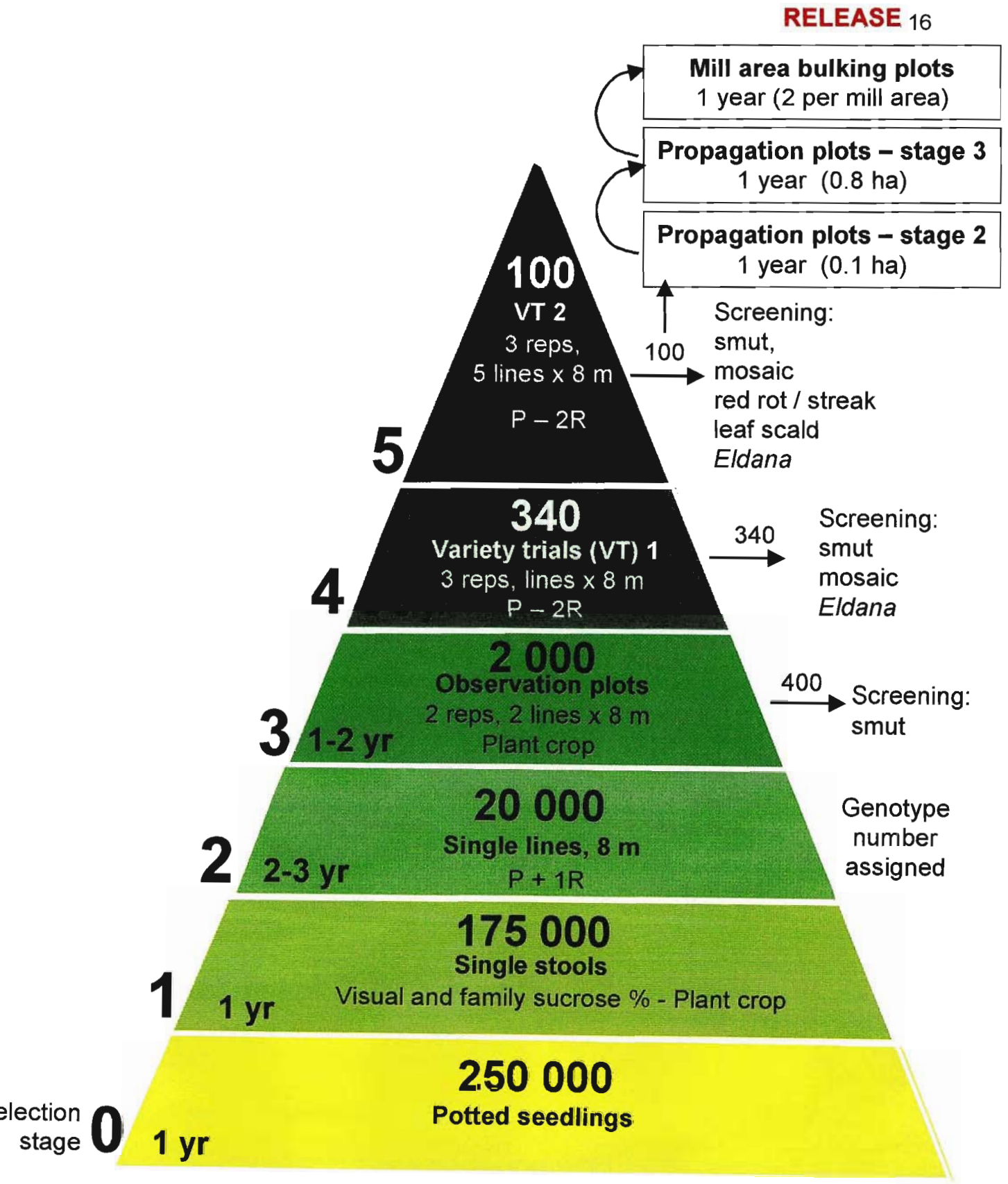


Figure 2.2 Diagrammatic representation of the selection programme at SASEX

2.2.1.1 Disease resistant varieties

Some sugarcane industries have all but been destroyed by disease epidemics but have recovered after the introduction of more resistant varieties (Edgerton, 1955). In South Africa, outbreaks of mosaic, streak and smut from 1910 to the early 1980s caused serious yield losses in susceptible varieties (Bailey, 1995). Today, the most useful long-term control of important sugarcane disease in most industries is based on selecting and releasing only those genotypes sufficiently resistant to current disease problems. For this strategy to work effectively the most economically important diseases need to be identified and sufficient selection pressure applied (Bailey and Bechet, 1982). The amount of effort expended to produce varieties resistant to a certain disease and the stages at which genotypes are assessed for their reactions to the various diseases are largely dependent on the importance of the disease under both favourable and poor growing conditions (Skinner *et al.*, 1987). The stage at which disease screening is introduced is also influenced by the problems associated with screening large numbers of genotypes satisfactorily.

2.2.2 Essential criteria for successful disease screening

The inclusion of known standards to act as controls and provide objectivity is essential in disease screening trials (Ricaud, 1981). Adequate replication is also essential for those diseases with variable symptom expression where an observed resistance rating can carry a large standard error (Skinner *et al.*, 1987). The screening techniques adopted need to be acceptably efficient so as not significantly delay the flow of new genotypes through the selection programme

2.2.3 Disease screening in the South African sugar industry

In the South African sugar industry, genotypes are screened for those diseases that cause or are most likely to cause serious yield losses, and which cannot easily be controlled other than by satisfactory resistance. These include smut (*Ustilago*

scitiminea), brown rust (*Puccinea melanocephala*), red rot (*Glomerella tucumanensis*), leaf scald (*Xanthomonas albilineans*), streak (sugarcane streak virus) and mosaic (sugarcane mosaic virus) (Bailey and Bechet, 1982).

Selection for disease resistance is applied at all stages of the sugarcane breeding programme in South Africa (Butterfield and Thomas, 1996). In the early stages of the selection programme, genotypes are exposed to the common diseases at the various selection sites in KwaZulu Natal. By the time genotypes reach the late stages of the programme, they have been exposed to a wide range of diseases under different environmental conditions. Those genotypes that are severely affected by a disease are discarded so some selection pressure is applied during the normal process of genotype selection. Adequate resistance to certain common and highly infectious diseases such as brown rust and gumming are obtained in this way. Special disease screening trials are necessary for those diseases where the natural levels of inocula are too low to provide reliable estimates of varietal resistance (Bailey and Bechet, 1982). The genotypes included in these trials are exposed to elevated levels of inocula either by artificial inoculation or by surrounding the plants with heavily infected, highly susceptible plants allowing for natural exposure to the pathogen. These trials are ratooned to obtain a better estimate of the effect of the disease over a period of time and over successive ratoons, which is particularly important with diseases such as smut that build up over time. The trials are conducted at a number of sites where conditions favour the development and spread of the specific diseases. Although artificial inoculation methods help eliminate susceptible genotypes more rapidly from the selection programme, it is essential that they are well correlated with the natural infection process and that the results are interpreted with this in mind (Skinner *et al.*, 1987).

2.2.4 Stability of resistance

Resistance to most sugarcane diseases has been largely successful and stable. Robinson (1976) suggested that this is due to the allopolyploid nature of sugarcane: oligogenes are unlikely to be expressed and the pathosystem depends on many genes

and is essentially quantitative in expression. Changes in the pathogen can however occur, resulting in a breakdown of this resistance. Evidence of such changes in disease status have been reported in Hawaii when smut attacked two previously resistant varieties (Ferreira and Comstock, 1989) and in Mauritius when *Xanthomonas campestris* pv *vasculorum,* the causal organism of gumming, caused severe damage to their industry (Anon, 1983). A breeding programme based on repeated combination and in which reasonable pressure is applied for resistance to diseases will build a stable, horizontal resistance system in sugarcane (Robinson, 1976).

2.2.5 The effect of disease screening on the breeding programme

A plant breeding and selection programme must be large enough to accommodate the number of criteria under selection. Skinner *et al.* (1987) discussed the theoretical economic costs of discarding parents and progeny for an additional character in breeding and selection programmes. They stated that progress in productivity in the breeding programme is adversely affected if too many characters are included in selection. They stressed that manipulation of characters by other means such as disease control programmes may be economically more feasible.

2.2.6 Screening for varietal resistance to RSD

Differences in varietal resistance to RSD have been recognised since 1950 when Steindl first identified the disease (Steindl, 1950). The use of resistant genotypes may offer an alternative strategy for minimizing losses from RSD (Roach, 1988, 1992a, b; Comstock *et al.,* 1997). Selection for disease resistance in most plant breeding and selection programmes is based on visual assessment of disease symptoms. Stunting is the only external symptom of RSD but is not visually apparent and is not characteristic of RSD alone. A number of procedures, largely based on the different diagnostic methods used (Refer 2.1.5), have been employed in an attempt to assess varietal reactions to RSD.

2.2.6.1 Yield trials

For many years, varietal reactions to RSD were based solely on large, replicated yield loss trials grown over a number of years and in different locations. For this reason the assessment of cultivar susceptibility has been difficult and time-consuming (Gillaspie and Teakle, 1989). As a result, the relative susceptibility of only a small number of varieties is known in South Africa (Bailey and Bechet, 1986 and 1995) and other countries (Koike *et al.*, 1982; Dean, 1983; Grisham, 1991). Single-stool plots (Dean, 1983) and microplots (Matsuoka, 1980) have also been used to screen varieties for RSD tolerance and results were comparable with those of larger trials. Alternative approaches to yield trials to screen sugarcane genotypes for their resistance to RSD have been investigated.

2.2.6.2 Anatomical features of different varieties

The vascular system in sugarcane stalks consists of discrete vascular bundles containing both xylem, which are inhabited by *L. xyli* subsp *xyli*, and phloem tissue (Julien *et al.*, 1989). The vascular bundles run longitudinally through the internodes until some branch into leaves, root primordia and buds at the nodes. Both greater branching of the vascular bundles (Teakle *et al.*, 1978) and greater resistance to hydraulic conductivity in non-infected nodal tissue by fewer xylem vessels passing through the stalk node without terminating (Teakle *et al.*, 1975) were correlated with lower yield loss due to RSD. This suggested that resistance was based on the inherent vascular structure in the nodal regions of the cane stalk whereby the spread of *L. xyli* subsp *xyli* within the xylem is limited in resistant genotypes, thus restricting its ability to colonise the plant.

Studies conducted in Brazil by Cruz (1983), Cruz and Tokeshi (1987) supported this theory. They demonstrated that the flow rate of staining solution and the number of functional xylem vessels are directly correlated with the severity of RSD and the susceptibility of the variety using a staining by transpiration method. With this method

the vacuum pump used in previous studies was replaced by the natural transpiration of the plant to stain those xylem vessels not impeded by *L. xyli* subsp *xyli* (Valarini and Tokeshi, 1981; Chagas and Tokeshi, 1994a, b; Giglioti *et al.*, 1997).

2.2.6.3 *Rate of spread of RSD*

Experiments have shown that the rate of spread on cane knives (Bailey and Tough, 1992) and mechanical harvesters (Damann, 1992) is dependent on the variety involved. Harrison *et al.* (1986) found that the concentration of *L. xyli* subsp *xyli* necessary to cause infection in resistant varieties is higher (approximately 10^8 cells / ml) than that required to infect more susceptible varieties (approximately 10^4 cells / ml). Davis *et al.* (1988a) counted approximately 10^8 *L. xyli* subsp *xyli* cells per ml of sap in more susceptible varieties while only about 10^6 cells / ml were counted in sap from more resistant varieties. Damann (1992) suggested that these were the two main reasons for the increased rate of spread of RSD in susceptible varieties.

2.2.6.4 *Population densities of L. xyli* subsp *xyli*

Many researchers have found that the relative densities of *L. xyli* subsp *xyli* in different genotypes were related to the resistance of those genotypes to damage from RSD. Population densities of *L. xyli* subsp *xyli* were assessed using a number of different methods. Gillaspie *et al.* (1976), Bailey (1977) and Roach (1990) used PCM to demonstrate that the highest populations of *L. xyli* subsp *xyli* were consistently associated with the most susceptible varieties. These findings were confirmed using the fluorescent antibody direct-count on filters (FADCF) technique (Davis, 1985) and EB-EIA (Croft *et al.*, 1994, Croft and Greet, 1997).

2.2.6.5 *Number of colonised vascular bundles*

Harrison and Davis (1988) used a modified immuno-blot assay, the tissue blot-enzyme immunoassay (TBIA), to detect and enumerate vascular bundles colonised by *L. xyli*

subsp *xyli*. In a later study they found that quantitative estimates of the number of colonised vascular bundles (cvb) obtained with TBIA were highly correlated with the relative densities of the pathogen population in adjacent tissue determined by counting the number of fluorescent antibody stained cells (Harrison and Davis, 1990). The method was found to be at least as sensitive as PCM and the dot blot immunoassay. They concluded that in a properly structured trial, TBIA could be used to measure both the incidence and severity of RSD. Seem (1984) defined incidence as the proportion or percentage of diseased entities within a sampling unit while severity was defined as the quantity of disease affecting entities within a sampling subunit. These two parameters are commonly regarded as being important in breeding for disease resistance in sugarcane. Either incidence or severity alone could be used to assess genotypes for RSD resistance, but used together they would offer greater discrimination amongst the genotypes. Screening for RSD resistance was incorporated into the United States Department of Agriculture – Agricultural Research Services (USDA-ARS) sugarcane varietal development programme at Canal Point, Florida in 1989 (Comstock *et al.*, 1995). Davis *et al.* (1994) made some important modifications to the apparatus used for the TBIA that permitted greater numbers of samples to be processed in a shorter period of time.

Comstock *et al.* (1996) compared the number of cvb using tissue blots with the rate of spread of RSD in different varieties. The study was conducted on fields of commercial cane and relied on infection through normal cultural practices. They found that the greatest spread of RSD occurred in varieties that had the highest number of cvb. They stated that a high level of resistance would be required to prevent the spread of RSD but low levels of the disease could be maintained in those varieties with some degree of resistance.

2.2.7 RSD screening programmes in different countries

RSD screening using TBIA is introduced from Stage II in the USDA cane varietal development programme at Canal Point, Florida (Comstock *et al.*, 1995). Approximately

1 000 genotypes are available for testing at this stage. This method of screening is also used in Louisiana, although the screening programme is smaller than that of Florida (Hoy and Flynn, 2001). Croft and Greet (1997) reported that the EB-EIA was a simple and efficient method for rating cultivars for resistance to RSD. The method is however, used only for advisory and research purposes and not in the plant breeding and selection programme in Australia (Barry Croft, personal communication). Genotypes in Brazil are evaluated by the staining and transpiration method (Chagas and Tokeshi, 1994a; Giglioti *et al.*, 1997). Although a number of other methods have been found to be useful in identifying susceptible varieties (Refer 2.2.6), they are time-consuming and not as suitable for routinely screening large numbers of genotypes as TBIA, particularly in countries where labour costs are high (Giglioti *et al.*, 1997).

2.2.8 Reliability of data when assessing RSD susceptibility

As with all screening programmes, the reliability and repeatability of the data accumulated from one year to the next is essential. There have been reports that the accuracy of results of screening for RSD is dependent on the time of sampling. Gillaspie *et al.* (1976) found that there were seasonal differences in *L. xyli* subsp *xyli* populations and reported that screening for RSD resistance on the basis of bacterial populations was most successful in late September to October in Louisiana. Bailey (1977) and Davis and Dean (1984) observed bacteria by phase contrast microscopy in all parts of the plant, but at higher titres in mature stalk tissue late in the growing season. Research conducted in South Africa showed that *L. xyli* subsp *xyli* populations were low during the first six months of growth (unpublished). Levels reached their peak after nine months and tended to stabilise after this period. These findings were based more on absolute rather than relative bacterial concentrations in a limited range of varieties.

In their study to determine whether sugarcane clones could be reliably rated for RSD resistance using single stool plots in a range of situations, Roach and Jackson (1992) recorded a high variation in populations of *L. xyli* subsp *xyli* in single stalks sampled from one stool. This indicated that sampling of several stalks per stool would be

desirable to obtain reliable ratings for resistance. They found that the time of assessment of populations of *L. xyli* subsp *xyli* for resistance ratings was not critical, provided immature cane was avoided, as clones tended to retain their relative resistance ratings across a range of times and infection levels. Their research did however, suggest that variations in environmental conditions could make the absolute values of *L. xyli* subsp *xyli* populations unreliable as indicators of RSD resistance. Davis *et al.* (1988a) confirmed this but found that differences in relative population densities of *L. xyli* subsp *xyli* remained constant from plant to ratoon crops and between two sites. These studies suggest that screening procedures based on relative population densities of *L. xyli* subsp *xyli* may be useful due to the fact that there were apparently few limitations as far as time and location were concerned.

Davis *et al.* (1994) reported that some characteristics of the cane stalks such as excessive stalk splitting and pithy stalks with central cavities made it difficult to obtain tissue cores for TBIA in some genotypes. Previous studies have shown that although the number of vascular bundles is greater in the peripheral regions of the stalk sections, a lower percentage of these were colonised in infected stalks (Davis *et al.*, 1988b).

CHAPTER 3

The development of a laboratory-based technique to enumerate vascular bundles colonised by *Leifsonia xyli* susbp *xyli*

3.1 Introduction

The use of resistant varieties is the most practical and economically important method of disease control in sugarcane. However, although RSD is considered to be the most important disease of sugarcane worldwide (Gillaspie and Teakle, 1989; Davis and Bailey, 2000), screening for RSD resistance is only routinely practised in the breeding and selection programmes in the sugar industries of Florida and Louisiana (Comstock *et al.*, 1995).

In order to assess the reactions of different varieties to a disease, it must be possible to quantify the disease according to visual symptoms or laboratory assays that are capable of quickly assessing large numbers of genotypes. One of the reasons that genotypes have not, until recently, been routinely screened for RSD resistance is that the disease does not produce any easily recognisable external symptoms other than stunting, and internal symptoms are unreliable (Irvine, 1976; Gillaspie and Teakle, 1989). It is therefore not possible to accurately diagnose and rate varieties for their reaction to RSD according to symptoms observed in field trials.

Many of the techniques tested for RSD screening have proven to be useful in distinguishing varieties but are often not practical for screening large numbers of genotypes. For instance, yield loss trials that are run over a number of years require large, uniform tracts of land (Gillaspie and Teakle, 1989). Other methods investigating anatomical features (Teakle *et al.*, 1975 and 1978) and flow rates (Valarini and Tokeshi, 1981), population densities (Gillaspie *et al.*, 1976; Davis, 1985) and infectivity titrations (Harrison *et al.*, 1986) are labour-intensive. A tissue blot immunoassay (TBIA), developed by Harrison and Davis (1988) and modified by Davis *et al.* (1994) has proven to be useful in screening greater numbers of

genotypes by providing information on the incidence and severity of RSD in a single test.

This chapter describes the development of the TBIA for enumerating vascular bundles colonised by *L. xyli* subsp *xyli* as well as preliminary experiments to assess the extent of colonisation in some commercially grown South African varieties.

3.2 Materials and methods

3.2.1 Production of *Leifsonia xyli* subsp *xyli* polyclonal antiserum

3.2.1.1 Isolation of L. xyli subsp xyli

L. xyli subsp *xyli* was extracted from stalks of a highly susceptible variety, N14, according to the method of Dr Stevens Brumbley (personal communication). RSD-infected stalks were washed and surface-sterilised by immersing in 10% (v/v) sodium hypochlorite for ten minutes before spraying the stalk with 70% (v/v) ethanol and flaming. Internodes were cut from the basal portion of each stalk, where populations of *L. xyli* subsp xyli are known to be high (Bailey, 1977), using secateurs sterilised by flaming. Xylem sap was expressed with positive pressure using a Gelman Little Giant compressor. The sap was collected using a 1 ml micropipette and pipetted onto plates of MSCm agar (Davis, *et al.*, 1980) modified according to Dr Stevens Brumbley (personal communication) and streaked with a flamed loop before incubating the plates at 28°C for two to three weeks.

The MSCm agar consisted of the following: 1.7% (w/v) corn meal agar (Difco, Michigan), 0.4% (w/v) Bacto agar (Difco, Michigan), 0.8% (w/v) Bacto Soytone (Difco, Michigan), 30 ml bovine hemin chloride (Sigma type I) (0.1% solution in 0.05 M NaOH), 0.02% (w/v) $MgSO_4.7H_20$, 13 ml 0.1 M K_2HPO_4, 87 ml 0.1 M KH_2PO_4 in 800 ml polished water. The pH was adjusted to 7.5 with 1 M NaOH. The solution was autoclaved at 121°C for 20 minutes and allowed to cool in a waterbath to 50°C. The following solution was filter sterilised through a 0.2 µm filter and added to the agar: 0.5% (w/v) glucose, 0.05% (w/v) cysteine, (free base, Sigma, Missouri), 0.2% (w/v) bovine serum albumin fraction V (Sigma, Missouri) in 100 ml polished water. Nalidixic acid was added to the medium to give a final concentration of 30 µg/ml.

Colonies raised on the MSCm agar were 0.1 to 0.3 mm in diameter, circular with entire margins, convex and non-pigmented. These colonies were transferred to S8 broth and incubated for 2 weeks at 28°C with agitation at 180 rpm in a Labcon shaker incubator. The S8 broth consisted of the following: 0.8% (w/v) Bacto soytone (Difco, Michigan), 0.035% (w/v) K_2HPO_4, 0.11% (w/v) KH_2PO_4, 0.02% (w/v) $MgSO_4.7H_20$, 30 ml bovine hemin chloride (Sigma Type I) (0.1% solution in 0,05 M NaOH) in 900 ml polished water, pH ~6.6. The broth was autoclaved at 121°C for 20 minutes and allowed to cool before adding the following filter sterilised solution: 0.2% (w/v) glucose, 0.05% (w/v) cysteine, (free base, Sigma, Missouri) 0.2% (w/v) bovine serum albumin fraction V (Sigma, Missouri) in 100 ml polished water. Nalidixic acid was added to give final concentration of 30 µg/ml.

3.2.1.2 Preparation of L. xyli subsp xyli antigen

Whole cells of *L. xyli* subsp *xyli* were collected by centrifuging 50 ml aliquots of SC broth cultures at 9 700 rpm for 15 minutes in a Sorval centrifuge (du Pont Company, Wilmington, DE). The supernatant was discarded and the pellet was washed five times by re-suspending in 20 ml 0,1 M phosphate buffered saline (PBS) (0.1 M phosphate buffer, pH 7.4, 0.5 M NaCl) and centrifuging at 9 700 rpm for ten minutes. The pellet was re-suspended in 10 ml PBS and the concentration was adjusted to OD_{640} = 0.1 using a Beckman DU7500 diode array spectrophotometer (California). The resulting antigen preparation was divided into 1 ml aliquots in microfuge tubes, flash frozen in liquid nitrogen and stored at -20°C. New antigen preparations were prepared every two weeks.

3.2.1.3 Immunisation of rabbits

The immunogen was mixed to a thick emulsion with an equal volume of Freund's adjuvent (Sigma, Missouri) using two syringes connected through a leur fitting. New Zealand rabbits were injected with 0.5 ml of the inoculum. Booster injections were given at two weekly intervals. Blood samples were taken before each inoculation to test the titre of the antigen. A terminal bleed was conducted 52 days after the first inoculation.

3.2.1.4 Preparation of serum

The blood was allowed to clot for 60 minutes at 37°C and then refrigerated at 4°C overnight to contract. The serum was separated from the clot and the remaining insoluble material was removed by centrifuging at 9 200 rpm for ten minutes at 4°C. The serum was sterilised by filtering through a 0.2 µm filter (Micron Separations Inc, Massachusetts) and dispensed into 1 ml microfuge tubes. The serum was stored at -20°C.

3.2.2 Testing the specificity of the *L. xyli* subsp *xyli* antiserum

3.2.2.1 Sample preparation

Sap was extracted from 100 healthy and 100 RSD-infected stalks of N14 and 10 ml of each was transferred to separate sterile 15 ml centrifuge tubes (Corning Incorporated, New York). Approximately 100 mg fresh weight of *L. xyli* subsp *xyli* was transferred to a 1.5 ml microfuge tube and suspended in 1 ml of sterile deionised water. The samples were centrifuged at 9 200 rpm for ten minutes. The pellets were resuspended in 1 ml sample buffer that consisted of the following: 25% (v/v) stacking gel buffer (Harlow and Lane, 1988), 4% (w/v) sodium dodecyl sulphate, 20% (v/v) glycerol and 10% (v/v) 2-mercaptoethanol. The samples were held at 100°C for ten minutes before centrifuging at 10 000rpm for ten minutes to remove cell debris. The samples were stored at -20°C until required.

The samples (25 µg) were loaded into the wells of a 10% acrylamide gel with 5 µl sample buffer. A current of 100V was applied until the dye had collected at the bottom of the wells, and the current was then increased to 150V for approximately one hour.

3.2.2.2 Silver staining

The acrylamide gel was immediately placed in fixative (40% v/v methanol, 10% v/v acetic acid) for 30 minutes. This was replaced with two changes of fixative (10% v/v ethanol, 5% v/v acetic acid), each for 15 minutes. The gel was then incubated for five

minutes in an oxidizer (BioRad, California). This was followed by numerous five-minute wash steps with deionised water until all the yellow colour was removed from the gel. The gel was incubated in silver reagent (BioRad, California) for 20 minutes and then washed in deionised water for one minute. The gel was stained with three changes of developer (BioRad, California) (five minutes each) and the reaction was stopped with 5% (v/v) acetic acid.

3.2.2.3 *Western blotting*

The protein bands were transferred to nitrocellulose membrane (Micron Separations Inc, Massachusetts) by wet electrophoretic transfer (Hoefer Scientific Instruments, California) for one hour. The transfer was verified by washing twice with 2% Ponceau-S (3-hydroxy-4-{2-sulfo-4-(sulfo-phenylazo)phenylazo}-2,7-naphthalene disulfonic acid in 30% trichloracetic acid, 30% sulfosalicylic acid) for 10 minutes.

The blot was blocked by incubating in TBS-T (0.484% w/v Tris, 0.8% w/v NaCl, 2% v/v Tween 20, pH 7.8) and 3% (w/v) milk powder overnight at 4°C. The blot was then incubated for 90 minutes in the antiserum produced against *L. xyli* subsp *xyli* in the first rabbit (antiserum 1), diluted 1: 1000 in TBS-T and 0.3% (w/v) bovine serum albumin. This was followed by six five-minute washes in TBS-T. The blot was then incubated in goat anti-rabbit IgG whole molecule peroxidase conjugate (Sigma, Missouri), diluted 1: 1000 in TBS-T and 0.3% (w/v) bovine serum albumin. The blot was washed three times with TBS-T and 0.05% (w/v) SDS, followed by three washes in TBS-T. The blot was then incubated for one minute in the Enhanced Chemi-luminescence reagents (Amersham, Buckinghamshire) before being exposed to autoradiographic film (Hyperfilm-ECL) for five minutes.

The blot was stripped by washing twice with 0.29% (w/v) tri-sodium citrate, pH 3.2 for five minutes before washing with two changes of TBS-T for five minutes. The blot was then reprobed with the antibody produced in the second rabbit (antiserum 2) followed by the antibody (old antiserum) that had previously been used in all serological tests for *L. xyli* subsp *xyli.*

3.2.2.4 *Enzyme linked-immunosorbant assay*

The antibody was tested against a range of bacterial pathogens, including *Xanthomonas albilineans, Acidovorax avenae* subsp *avenae, Herbaspirillum rubrisubalbicans and X. axonopodis* pv *vasculorum* as well as other non-pathogenic bacteria found in sugarcane.

Flat-bottomed microtitre plates (Sterilin, Staffordshire), each well containing 200 µl of PBS with approximately 1 x 10^6 cultured bacterial cells or xylem extract containing non-pathogenic, symbiotic bacteria were processed according to the procedure of Croft *et al.* (1994) modified by McFarlane *et al.* (1999). The multiwell plates were centrifuged at 3 500 rpm for 20 minutes. The supernatant was removed from the wells using an Flexiwash plate washer (ASYS Hitech, Eugendorf, Austria), leaving approximately 10 µl of sap in each well. The plates were vortexed to resuspend the pellet and 200 µl of coating buffer (0.1 M potassium phosphate buffer, pH 7.0) was pipetted into each well. The plates were centrifuged at 3 500 rpm for ten minutes and the supernatant discarded. The wash procedure was repeated and once the supernatant had been removed for the third time, the plates were placed in a fan-forced incubator at 37°C overnight.

The plates were washed twice with PBS-T (0.1 M phosphate buffer, pH 7.4, 0.15 M NaCl, 0.01% (v/v) Tween-20) (five minutes each) and the wells were then blocked for 30 minutes with 200 µl of 5% (w/v) skim milk in PBS-T. After two washes with PBS-T, 100 µl of *L. xyli* subsp *xyli* antibody (1 mg/ml) diluted 1:1000 (v/v) in PBS-T was added to the wells and the plates were incubated for one hour at room temperature. The plates were washed twice with PBS-T before 100 µl goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma, Missouri) diluted 1:1 000 (v/v) in PBS-T was added to the wells and the plates were incubated for a further hour at room temperature. The plates were washed five times with PBS-T. Finally, 100 µl of substrate buffer (0.1% 4-nitrophenyl phosphate in 10% diethanolamine buffer, pH 9.8) was added and the absorbance at 405 nm was measured at the time of adding the substrate buffer and four hours later with a BioRad 450 Microplate reader (BioRad, California). Those samples with a reading greater than 0.15 after four hours were considered positive.

3.2.3 Preparation of immunoglobulin

A column of 8 to 10 ml bed volume of pre-equilibrated DEAE-cellulose (Whatman DE 52 pre-swollen cellulose anion exchanger) was prepared by washing with half-strength PBS until the pH of the cellulose was equivalent to that of the buffer. Two millilitres of the immunoglobulin preparation was pipetted on top of the cellulose. The immunoglobulin was washed through the column with half-strength PBS and the eluate was collected in approximately 1 ml fractions. The fractions were monitored at 280 nm and those containing the first protein peak eluted were combined. The OD_{280} of the combined fractions was measured and adjusted with half-strength PBS to approximately 1.4 (about 1 mg/ml). The immunoglobulin was filter-sterilized using a 0.2 µm filter, aseptically dispensed in 1 ml aliquots and stored at 4°C for up to six months.

3.2.4 Tissue blot immunoassay (TBIA)

3.2.4.1 Tissue blot procedure

Internodes were selected from the lower one third of the sampled stalks. Longitudinal cores were removed from each stalk piece using an 11 mm diameter cork borer mounted in a drill press (Figure 3.1a). A 10 mm cross section was excised from the core using a device constructed at SASEX under advice from Dr Jeff Hoy, Louisiana State University (Figure 3.1b).

The sections were blotted with tissue paper to remove excess moisture and placed onto the surface of nitrocellulose membrane (Micron Separations Inc, Massachusetts) within the wells of a filter-holding apparatus as described by Davis *et al.* (1994) (Figure 3.2). This apparatus was constructed at SASEX and consisted of two perspex plates, each with dimensions of 130 x 87 x 10 mm. The top plate had holes, 11 mm in diameter in a five by six array that formed 30 wells when the two plates were bolted together. A single membrane was stacked on top of two sheets of absorbant paper and at least five sheets of tissue paper within the apparatus. Each filter apparatus had the same dimensions as a standard 96-well microtitre plate.

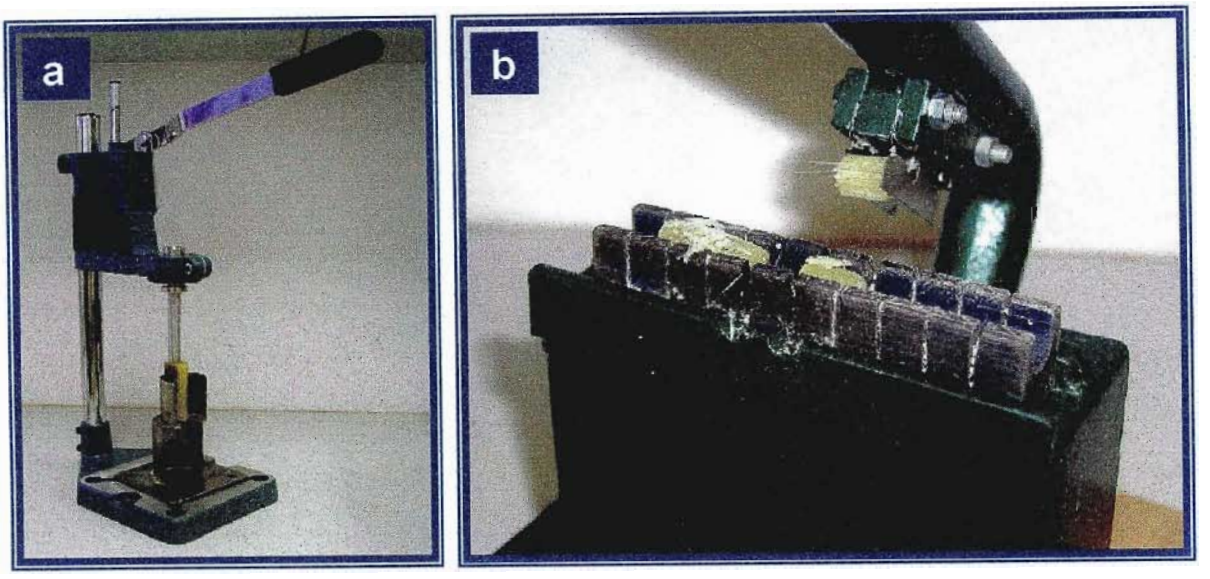


Figure 3.1 Apparatus used in the preparation of stalk sections for tissue blots. A tissue core was removed from a stalk piece using a cork borer (a) mounted in a drill press. A segment was excised from the tissue core using a cutting device (b) constructed at SASEX.

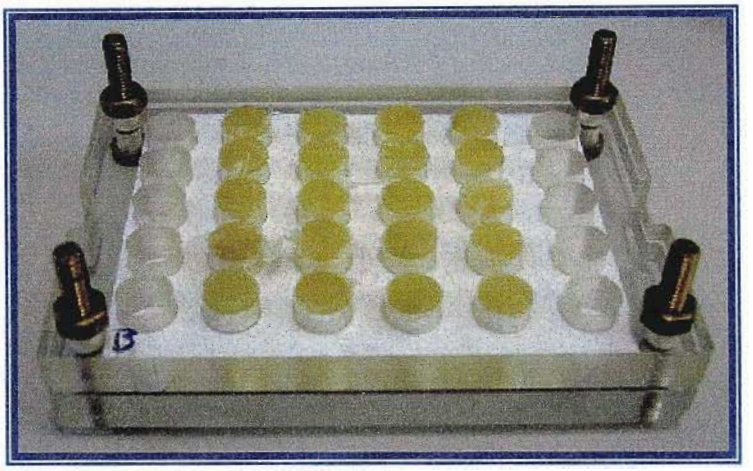


Figure 3.2 Filter holding apparatus. Tissue sections were placed in wells, resting on nitrocellulose membrane.

Two sets of apparatus were centrifuged at 3 000 rpm for ten minutes (Cenuturion, Scientific Limited, Essex) using a rotor attachment designed to carry multiwell trays. The membranes were then removed from the apparatus and dried at 80°C for one hour.

3.2.4.2 Immunoassay procedure

A modified indirect ELISA procedure was used to stain *L. xyli* subsp *xyli* cells deposited onto the membranes during the centrifugation process. Membranes were incubated for one hour at 28°C in *L. xyli* subsp *xyli* antiserum diluted 1:1 000 (v/v) in ELISA buffer (0.01 M PBS, pH 7.2, 2% (w/v) polyvinylpyrrolidone-10, 0.5% (w/v) bovine serum albumin (Sigma, Missouri), 0.2% (v/v) Tween-20, 0.02% (w/v) sodium azide). Sufficient volume of the diluted antiserum was added to the reaction vessel to totally immerse the membranes. After incubation, the membranes were washed with three changes (five minutes each) of ELISA washing solution (0.01 M PBS, pH 7.2, 0.2% v/v Tween-20, 0.02% w/v sodium azide). The membranes were then incubated in goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma, Missouri) diluted 1:1 000 (v/v) in ELISA buffer for a further hour at 28°C, followed by three washes as previously described. The membranes were incubated in substrate solution in the dark for 30 minutes at 28°C. The substrate solution was prepared by dissolving 0,03% (w/v) naphthol-AS-phosphate (sodium salt) in 0.5% (v/v) *N,N*-dimethyl-formamide and combining 1.25 ml of this solution with 250 ml 0,2 M Tris buffer, pH 9,1. 0.1% (w/v) Fast Blue BB and 0.1% (v/v) 0.1 M $MgCl_2$ were added immediately before use. Stained membranes were rinsed briefly in distilled water, immersed in 1% sodium hypochlorite for 30 minutes and again rinsed briefly in distilled water. The membranes were allowed to air dry before examining the impressions of the tissue sections on the membrane using a stereomicroscope (Zeiss, West Germany) at 40x magnification.

3.2.5 Analysis of data

The arcsin square root transform function in SigmaStat Version 2.0 was used to normalise the data (proportion of cvb). The data were then analysed using one way analysis of variance (ANOVA) when data were normally distributed. In some cases, the data were not normally distributed and ANOVA on ranks was used. SigmaPlot Version 5.0 was used to plot the data.

3.3 Results and discussion

Due to the success of the TBIA in screening varieties for resistance to RSD in Florida (Comstock *et al.*, 1995), preliminary experiments were conducted to test the application of this method in the South African sugar industry. Although the basic protocol required little modification, some adjustments were necessary to optimise the blots and standardise the sampling methods.

3.3.1 Isolation and culture of *L. xyli* subsp *xyli*

Colonies of *L. xyli* subsp *xyli* were evident on the MSCm agar plates after 19 days. All cultures were checked using phase contrast microscopy (PCM) to ensure that they were pure before sub-culturing into SC broth. Growth of *L. xyli* subsp *xyli* in broth was evident after 10 days.

3.3.2 Antiserum production

Fifty two days after inoculation, the polyclonal antisera from rabbits 1 and 2 had agglutination titres of 512 and 1 024 against approximately 1 x 10^9 *L. xyli* subsp *xyli* cells per milliliter, respectively.

3.3.3 Specificity of antisera using ECL hybridisation and EB-EIA

Silver staining indicated that protein bands were present in all samples, including the healthy sap (results not shown).

Single bands were detected with ECL hybridisation in the samples from the broth culture of *L. xyli* subsp *xyli* and RSD-infected xylem sap and not in the healthy sap when probed with both antisera 1 and 2 (Figure 3.3). According to the ECL results, both antisera 1 and 2 were more specific than the antibody that had previously been used in serological tests for *L. xyli* subsp *xyli.* At a dilution of 1:1 000, the reaction was stronger with antiserum 2 and this antiserum was tested further for specificity to *L. xyli* subsp *xyli* using EB-EIA.

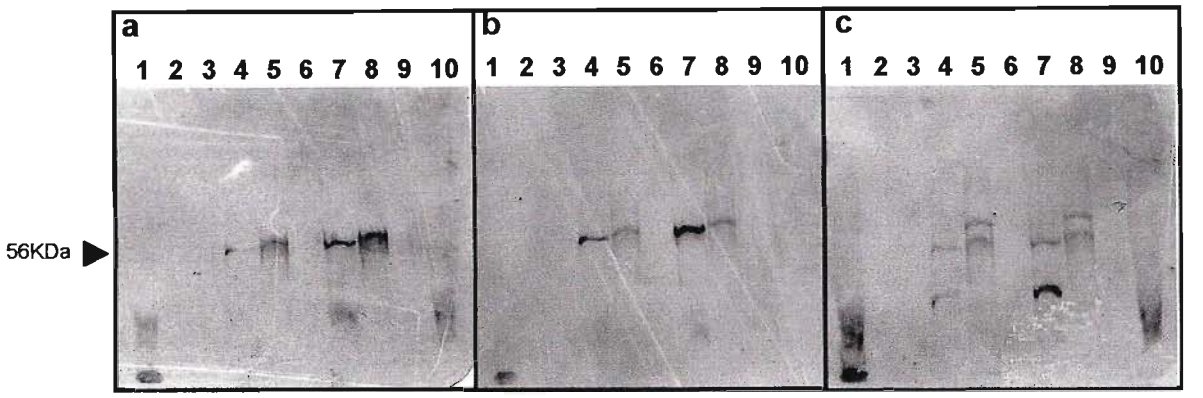


Figure 3.3 ECL hybridisation results: (a) antiserum 1, (b) antiserum 2 and (c) previously developed antiserum. Lane 1 Marker, 2 Blank, 3 healthy sap, 4 *L. xyli* subsp *xyli* broth culture. 5 *L. xyli* subsp *xyli*-infected sap, 6 healthy sap, 7 *L. xyli* subsp *xyli* broth culture, 8 *L. xyli* subsp *xyli*-infected sap, 9 blank, 10 marker.

When tested against xylem sap containing *L. xyli* subsp *xyli, Xanthomonas albilineans, Acidovorax avenae* subsp *avenae, Herbaspirillum rubrisubalbicans and X. axonopodis* pv *vasculorum* using EB-EIA, there was a significant difference in the absorbance readings obtained after four hours incubation (Figure 3.4). The antiserum produced strong reactions when incubated with *L. xyli* subsp *xyli* cells in RSD-infected xylem sap. It did not, however, react with the other bacterial pathogens isolated from sugarcane or to symbiotic bacteria observed in the numerous healthy stalks tested.

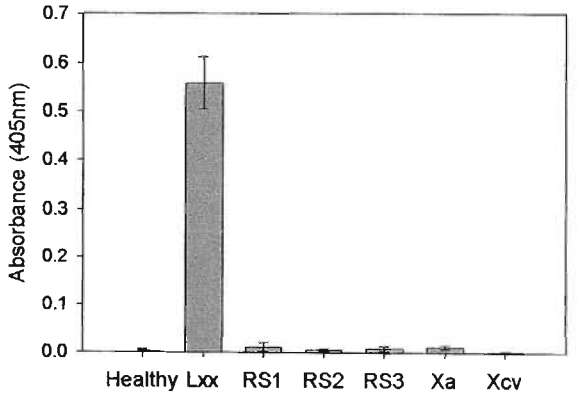


Figure 3.4 EB-EIA results for healthy xylem sap and xylem sap containing *L. xyli* subsp *xyli* (Lxx), *Acidovorax avenae* subsp *avenae* (RS1), *Herbaspirillum rubrisubalbicans* (RS2, RS3), *Xanthomonas albilineans* (Xa) and *X. axonopodis* pv *vasculorum* (Xcv). Data analysed using one way ANOVA. Bars represent se, n = 8)

The antiserum is now used for the routine diagnosis of RSD with the EB-EIA in the South African sugar industry and has been tested extensively against phase contrast microscopy in large-scale surveys throughout the industry (McFarlane *et al.*, 1999). Agreement between the two methods of diagnosis is 97%, indicating that the polyclonal antiserum has acceptable specificity to *L. xyli* subsp *xyli*.

3.3.4 Optimisation of the tissue blot immunoassay

Initial tissue blots were poor and certain modifications were required to reduce the number of blots that were unreadable due to the smearing and subesquent staining of excess xylem sap infected with *L. xyli* subsp *xyli*. Smearing was particularly evident in highly susceptible varieties such as N14 and N17 that are known to support high populations of *L. xyli* subsp *xyli* and frequently exhibited a high proportion of cvb.

3.3.4.1 Preparation of membrane

Although the original protocol did not prescribe pre-treating the nitrocellulose membrane (Davis *et al.*, 1994; Comstock *et al.*, 1995), this was investigated in an attempt to reduce the extent of smearing of the xylem sap.

Nitrocellulose membrane was therefore:

(i) used without pre-treatment or was pre-wet by soaking in distilled water (www.msifilters.com) or ELISA wash buffer. Tissue sections were then placed on the surface of the nitrocellulose membrane either five minutes after pre-wetting or after the membranes were allowed to air dry overnight;

(ii) rinsed in ELISA washing solution with gentle shaking for five minutes after centrifugation to remove unbound bacterial cells before baking; and

(iii) immersed in ELISA buffer containing 3% BSA or low fat milk powder for ten minutes with gentle shaking to block unreacted binding sites. The membranes were then rinsed in ELISA washing solution before the immunoassay was conducted.

Pre-wetting the membrane, blocking or rinsing the membrane after centrifugation did not improve the blots (Figure 3.5). If the membrane was wet when the tissue sections were placed on the surface, smearing was particularly severe and it was not possible to read the blots.

3.3.4.2 Preparation of tissue sections

Comstock *et al.* (1995) made no mention of the delay between sample collection and processing. When extracting sap for routine RSD diagnosis, samples are processed as soon as possible to ensure that sufficient sap is obtained for microscopic examination (Bailey and Fox, 1984) or the EB-EIA (McFarlane *et al.*, 1999). However for the TBIA, sufficient sap is only required to transport the bacterial cells from the xylem vessels and deposit them on the surface of the membrane. Excess sap may exacerbate the smearing on the membrane and hence interfere with the immunoassay.

Tissue cores were therefore removed from the stalk immediately after harvest. The same stalks were left uncovered overnight to reduce the amount of sap in the xylem vessels, and another tissue core was removed from the next internode. The samples were processed in the order they were collected in the field.

Once excised from the tissue core, tissue sections were either placed onto the membrane immediately, or they were first dried for five minutes in a fan-forced incubator at 37°C. The biggest influencing factor on the blots was found to be the length of time the stalk samples were stored before processing. By storing the stalks overnight to reduce the amount of xylem sap before removing the tissue cores, the smearing was markedly reduced, making blots easier to interpret. There was still sufficient sap to deposit any *L. xyli* subsp *xyli* cells present in the xylem onto the membrane surface during centrifugation.

There was little difference in the clarity of the blots when the tissue cores were removed after overnight storage or when the tissue sections were oven-dried at 37°C, except that the sections oxidised when dried in the oven and stained the circumference of the blots brown. The overnight storage of stalk samples was easier

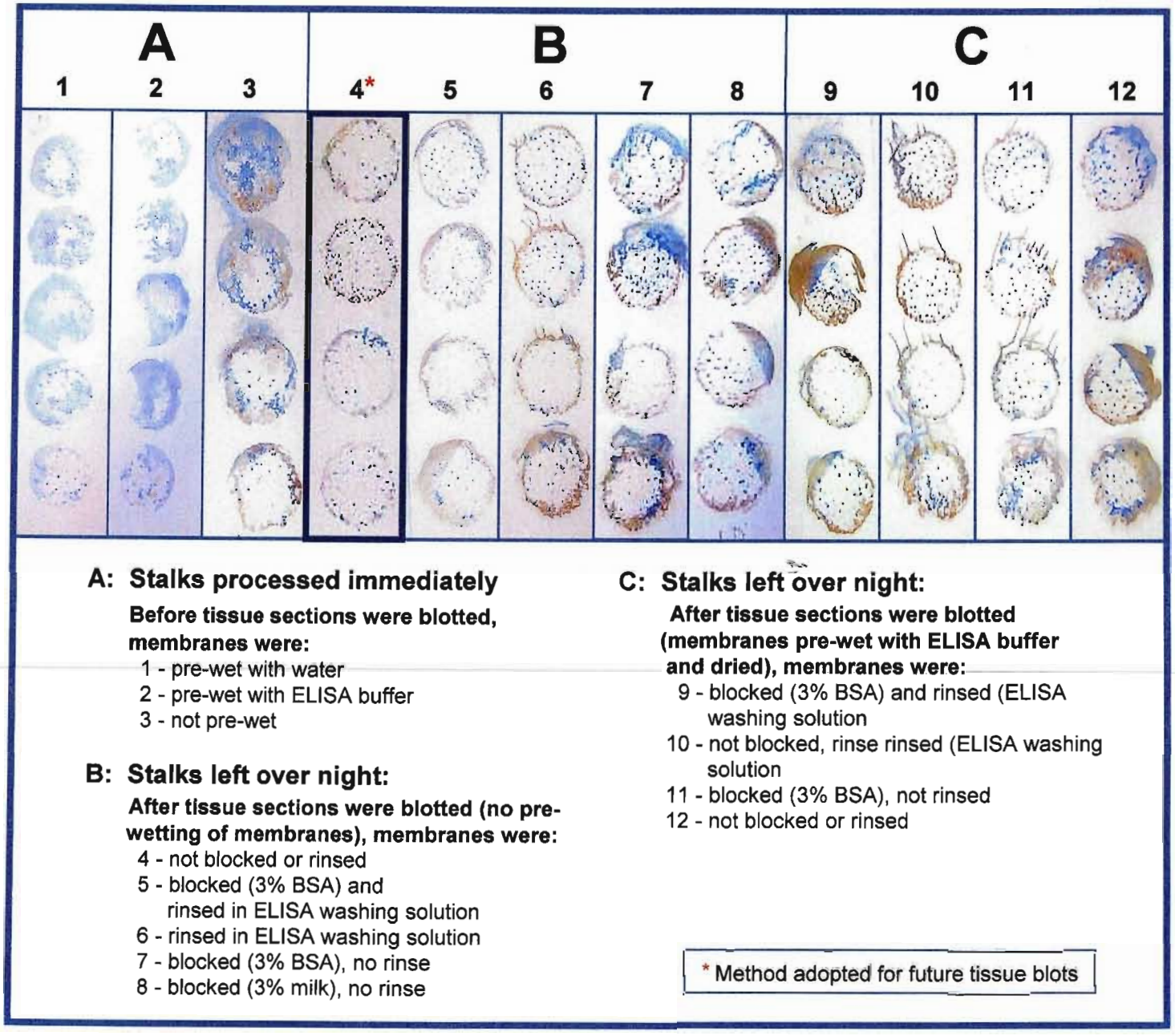


Figure 3.5 The effect of storing stalk samples after collection and pre-treating the nitrocellulose membrane before and after blotting on the quality of the tissue blots.

to manage than oven drying the tissue sections since it involved less sample handling. It reduced the risk of mixing samples because once removed from the stalks, the tissue sections were not individually labeled. Furthermore, overnight storage facilitated sample throughput, allowing stalk collection on one day and TBIA the following day. Storage for longer periods was not attempted because the results from the routine diagnostic service indicated that the likelihood of *L. xyli* subsp *xyli* detection was reduced after 24 hours (Bailey and Fox, 1984).

After these modifications, all the vascular bundles in the tissue sections made impressions on the membrane during centrifugation and could be counted to determine the total number of vascular bundles in each section (Figure 3.6).

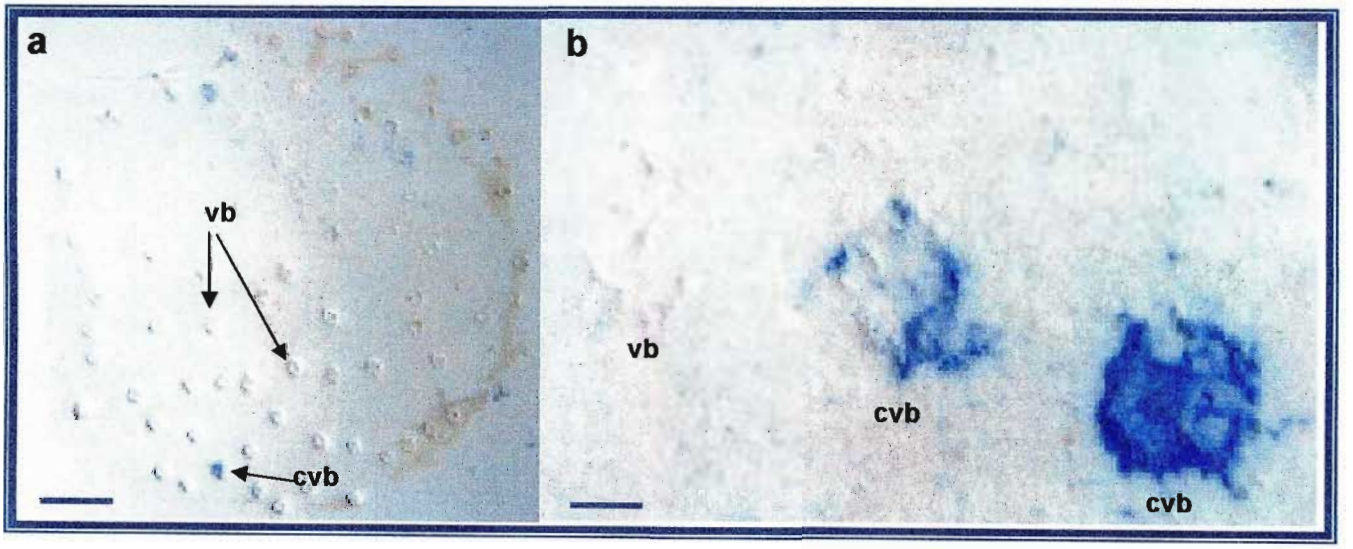


Figure 3.6 (a) Tissue blot showing impressions from vascular bundles (vb), most not colonised by *L. xyli* subsp *xyli*, bar =1.6 mm, 40x magnification. (b) Impressions of three vascular bundles, two colonised by *L. xyli* subsp *xyli* (cvb), bar = 0.25 mm, 63x magnification.

Those vascular bundles colonised by *L. xyli* subsp *xyli* were usually visible as discrete areas of blue stain on the membranes and could be counted and marked to determine the proportion of colonised vascular bundles (cvb) in each tissue section.

3.3.5 Effect of stalk age on the proportion of cvb

Since sugarcane produces tillers over a period of several months, all the stalks in one stool are at slightly different stages of development. Populations of *L. xyli* subsp

xyli increase as the age of cane increases (Gillaspie *et al.*, 1976; Bailey, 1977; Anon, 1991), and populations within a single stool are therefore likely to vary. This was investigated by cutting all stalks from three infected stools of N14, N16, N17 and NCo376 to ascertain the variability within stools and between varieties. The second lowest internode was cut from each stalk and the TBIA was performed.

Bailey (1977) and Davis *et al.* (1988b) reported that the rate of increase in populations of *L. xyli* subsp *xyli* differed according to variety, with the greatest increases occurring in the more susceptible varieties. The variation amongst stalks taken from the more susceptible varieties (NCo376, N14 and N17) was acceptable. The most mature stalks of all the varieties were consistently infected with RSD. However, the younger stalks of N16 were either not infected or the levels were too low to be detected, and the range in proportion of cvb amongst stalks was highest in this variety (Table 3.1).

Table 3.1 Variation in proportion of cvb within stools in four varieties

Variety	Mean cvb	Std deviation	Range
N14	0.79	0.09	0.29
N16	0.36	0.23	0.64
N17	0.84	0.08	0.23
NCo376	0.65	0.07	0.21

N16 is considered to have a similar RSD rating to N12 and would therefore be expected to support lower populations of bacteria than susceptible or highly susceptible varieties. The increase in the populations of *L. xyli* subsp *xyli* to detectable levels in younger stalks is also likely to be slower in these varieties. This investigation illustrated the importance of selecting the oldest stalk from each stool to be sampled.

3.3.6 Effect of internode position and variety on the proportion of cvb

The varieties grown in the South African sugar industry are known to react differently to RSD, with yield losses being more severe in certain varieties (Refer to Table 2.1). Different varieties have been shown to support different populations of *L. xyli* subsp

xyli (Gillaspie *et al.*, 1976; Bailey, 1977). These populations are also affected by the age of the tissue, with the highest numbers occurring in the oldest tissue at the base of the sugarcane stalks (Davis *et al.*, 1988a). Bailey (1977) found that, although populations of *L. xyli* subsp *xyli* declined from the oldest to the youngest tissue, this was not as marked in highly susceptible varieties where the bacterium could be detected in all internodes. To assess the effect of these factors on the proportion of cvb, the lowest four internodes were cut from each of five stalks of six varieties with a range of susceptibilities to RSD. This would be the first investigation of the differences in the extent of colonisation in a range of South African varieties. The varieties chosen were N22 (intermediate), N12 and N23 (intermediate-susceptible), NCo376 (susceptible), and N14 and N17 (highly susceptible) (Refer to Table 2.1).

3.3.6.1 Effect of internode position on the proportion of cvb

The mean proportion of cvb in all varieties in the first and second internodes was greater than that of the third and fourth internodes, although these differences were not significant (Figure 3.7).

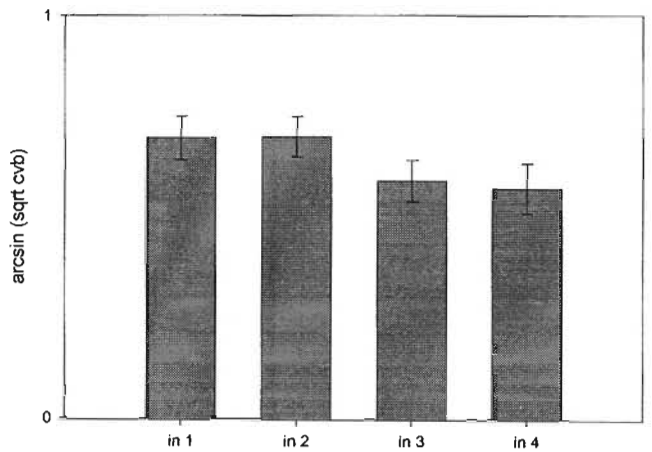


Figure 3.7 Effect of internode position on the mean proportion of cvb in six varieties. Data analysed using one way ANOVA. Bars represent se, n = 30.

There was little variability in the proportion of cvb from internodes one to four in varieties N12, N14 and N17 (Figure 3.8). A steady decline in the extent of colonisation was evident in NCo376.

Internodes three and four exhibited significantly fewer cvb than internode one ($P<0.05$). The cvb in internode two was also significantly higher than in internode three. There was also a significant difference in the proportion of cvb in internode three compared to the other internodes in N22.

The differences in populations from the basal region (internode one) to internode four in some varieties and the steady decrease in proportion of cvb from internodes one to four in NCo376, illustrated the importance of standardising internode selection. For the six varieties, the mean proportion of cvb in internode one was equivalent to internode two, and those in internodes three and four were similar. However, in N22 and NCo376 there was a significant difference between internodes two and three. It was also evident in this investigation that the degree of variability tended to be low in the second internode in most of the varieties tested.

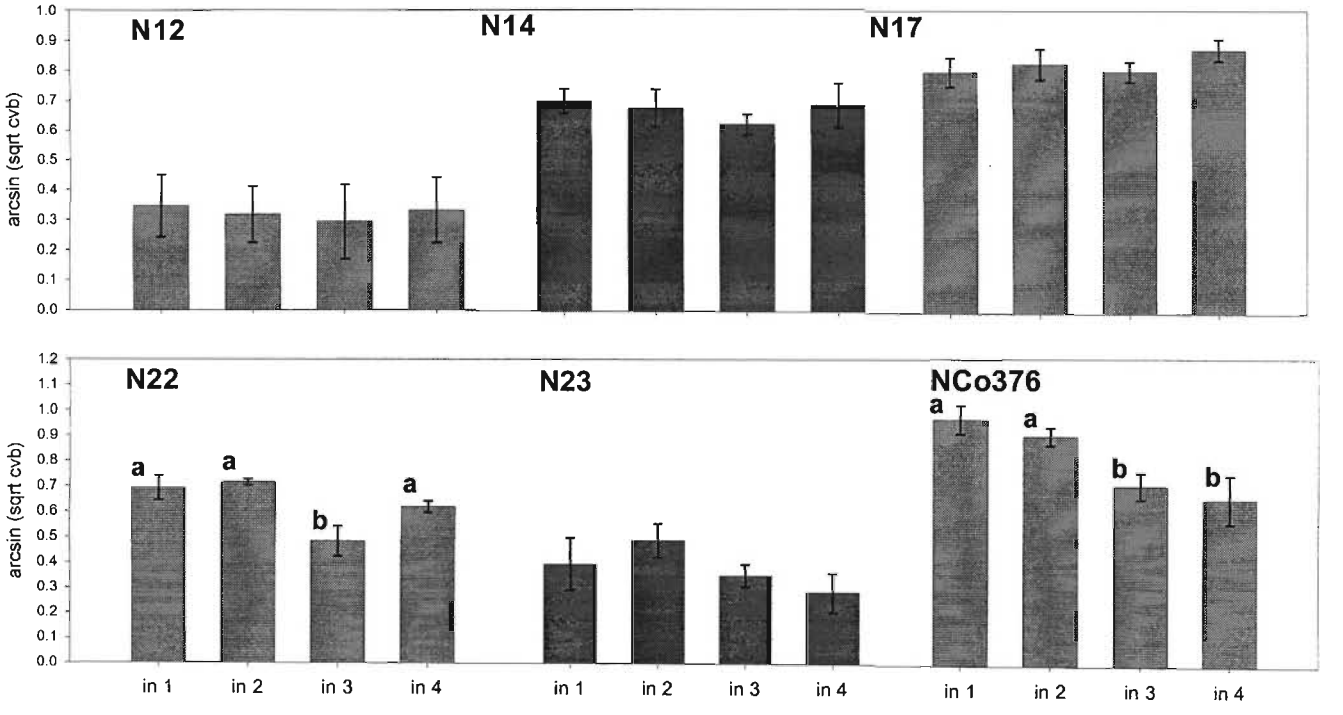


Figure 3.8 Effect of internode position on the proportion of cvb in six varieties. Data analysed using one way ANOVA. Different letters above columns denote significant differences ($P<0.05$). Bars represent se, n = 5.

Where possible, internode two was therefore selected from each stalk for the TBIA in subsequent experiments and field trials. Although populations of *L. xyli* subsp *xyli* in internode one were, in some instances, slightly higher than in internode two, this

internode was often damaged when stalks were plucked from the stools and could not be used for the TBIA.

3.3.6.2 *Effect of variety on the extent of colonisation*

Davis *et al.* (1994) showed that TBIA was effective in distinguishing varieties based on the incidence and severity of RSD infection. The data from the preliminary experiments reported in this chapter indicated that there were significant differences in the proportion of cvb in a number of the varieties tested (Figure 3.9).

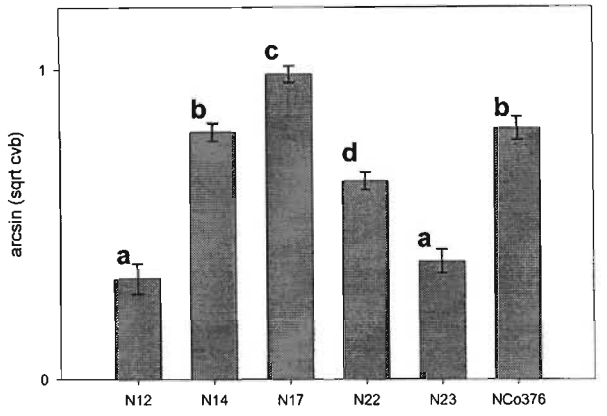


Figure 3.9 Mean proportion of cvb in six varieties. Data analysed using ANOVA on ranks. Different letters above columns denote significant differences (P<0.05). Bars represent se, n = 5.

Furthermore, the ranking of the varieties based on the extent of colonisation corresponded, in most instances, with that based on yield loss in previous trials (Refer 2.1.2), with N17, N14 and NCo376 being the most susceptible and N23 and N12 being more resistant. N22 was ranked as the most resistant variety in the yield loss trial but results from the TBIA indicated that it was more susceptible than N12 and N23. Such discrepancies will be investigated further in a combined trial using TBIA and yield data to assess the reactions of varieties to RSD.

3.4 Conclusions

Slight modifications to the sampling process resulted in improved blots, but the protocol for the immunoassay was not altered. It was clear from this investigation that precise sampling procedures are critical to the success of the TBIA and it was

necessary to standardise sampling methods in order to reduce variability. In subsequent investigations reported in this thesis, only the older stalks in the stool were sampled and the second lowest internode were selected for the TBIA after storing stalks overnight to reduce smearing.

With the TBIA, it was possible to distinguish a highly susceptible variety such as N17, from N23 and N12, two varieties known to show more resistance to RSD under most environmental conditions. Based on the information obtained in this chapter, further investigations into the use of the TBIA for RSD screening in the South African sugar industry were warranted.

CHAPTER 4

The relationship between extent of colonisation of sugarcane by *Leifsonia xyli* subsp *xyli* and the rate of spread of ratoon stunting disease

4.1 Introduction

Ratoon stunting disease (RSD) was first recognised as a mechanically transmissible disease by Steindl in 1950, before Gillaspie *et al.*, (1973) and Teakle *et al.*, (1973) discovered that the disease was caused by a bacterium. Early studies showed that extensive spread of the disease was possible, not only by planting infected seedcane, but also by harvesting implements (Hughes and Steindl, 1955; Steib *et al.*, 1957). This form of spread was shown to occur when sap from infected stools was transferred on the surfaces of the equipment to the cut surfaces of healthy stools. More recently, studies confirmed this method of spread during manual (Gillaspie and Teakle, 1989; Bailey and Tough, 1992) and mechanical (Taylor *et al.*, 1988) harvesting operations.

The relative densities of *L. xyli* subsp *xyli* have been found to vary in different varieties and can be predictive of the resistance of the varieties to RSD (Bailey, 1977; Davis *et al.*, 1988a and b; Harrison and Davis, 1988). The rate of spread of RSD appears to depend on the populations of *L. xyli* subsp *xyli* supported by the different varieties (Damann, 1992; McFarlane, unpublished; Hoy *et al.*, 1999) and can therefore also give an indication of resistance to the disease. Investigating a number of varieties differing in their susceptibility to RSD, Comstock *et al.* (1996) related the rate of spread to the mean number of colonised vascular bundles using the tissue blot immunoassay (TBIA). These investigations showed that RSD spread more rapidly in the more susceptible varieties exhibiting a higher number of colonised vascular bundles (cvb).

This chapter describes a field trial investigating the influence of the extent of colonisation by *L. xyli* subsp *xyli* on the rate of spread of RSD in twelve varieties grown

commercially in the South African sugar industry. The trial served to test the hypothesis that, by growing varieties that support lower populations of *L. xyli* subsp *xyli*, the rate of spread of RSD would be limited and as a result, would benefit the growers.

4.2 Materials and methods

4.2.1 Establishment of field trial

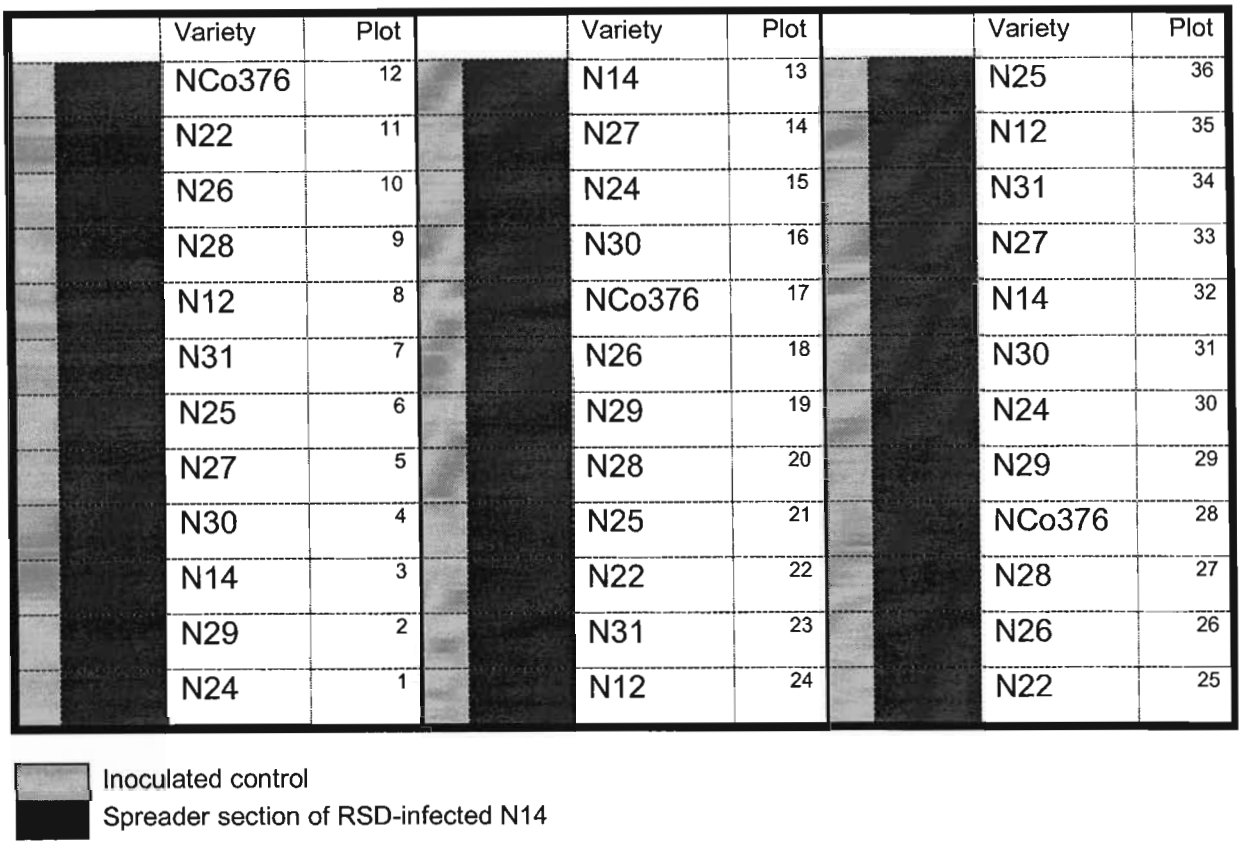
The trial was conducted under rainfed conditions on a uniform, deep sandy clay loam soil at Mount Edgecombe in KwaZulu-Natal (29° 42'S and 31° 02'E).

4.2.1.1 Choice of varieties

Twelve released varieties were included in the trial. Six of these had previously been included in yield loss trials and as a result, could be rated for RSD resistance (Refer to Table 2.1). N22 (intermediate), N12 (intermediate-susceptible), NCo376 (susceptible) and N14 (highly susceptible) were used as standards. Infected control plants of each variety were included to compare the ranking of the eight varieties obtained in the yield loss trials to the extent of colonisation by *L. xyli* subsp *xyli.* The controls also provided information on the reaction of the four varieties that did not have an RSD rating.

4.2.1.2 Layout of trial

The trial consisted of three replications of twelve rows in a randomised complete block design (Figure 4.1). Plots consisted of single rows, each 17 m long, representing the different varieties included in the trial, at an interrow spacing of 1,2 m. Each row was planted with one inoculated transplant of the variety under test to serve as the control, four transplants of infected N14 to serve as RSD spreader plots within the row, followed by 24 healthy transplants of the variety under test, planted 0,5 m apart.



Variety	Plot	Variety	Plot	Variety	Plot
NCo376	12	N14	13	N25	36
N22	11	N27	14	N12	35
N26	10	N24	15	N31	34
N28	9	N30	16	N27	33
N12	8	NCo376	17	N14	32
N31	7	N26	18	N30	31
N25	6	N29	19	N24	30
N27	5	N28	20	N29	29
N30	4	N25	21	NCo376	28
N14	3	N22	22	N28	27
N29	2	N31	23	N26	26
N24	1	N12	24	N22	25

Figure 4.1 Layout of trial investigating the rate of spread of RSD and the extent of colonisation of the vascular bundles.

4.2.1.3 Preparation of planting material

Seedcane of the twelve varieties was cut into single-budded setts (SBS), hot water treated (HWT) for 2 hours at 50°C and planted in seedling trays to produce transplants for the trial. Control plants and the plants to be used as the spreader plots at the beginning of each row, were produced by dipping SBS into *L. xyli* subsp *xyli*-infected cane juice expressed from stalks of the highly susceptible variety N14, for 30 minutes before planting.

Transplants were germinated in the dark at 30°C at a relative humidity of between 80 and 95%. Once germinated, the trays were transferred to outside benches where they were irrigated three times daily for 10 minutes. The plants were fertilised weekly with a

hydroponic fertiliser (3:1:3 (38), Gromor®) at a rate of 2.5 g in 5 litres per tray. After eight weeks, the transplants were ready to be transferred to the field. Extra SBS were planted and only the most vigorous transplants were selected for planting in the trial.

4.2.1.4 Growth and harvesting of trial

The trial was planted in October 1998 and all the transplants survived the transfer to the field.

The trial was harvested manually and at each harvest, three cane cutters were assigned to the trial, with each cutter being responsible for harvesting one replication. At harvest, each row was systematically cut, starting from the infected control stool, moving through the spreader section of N14 and continuing through the uninoculated plants to the end of the row. The knives were disinfected at the end of each row by dipping in 10% (v/v) Jeyes Fluid (a.i. carbolic acid). The trial was harvested annually, and was carried through to the second ratoon.

4.2.2 Sampling and laboratory analysis

All control, spreader and uninoculated stools were tested for RSD using immunofluorescence microscopy at 13 months (November 1999), before the first harvest. The first and second ratoon crops were sampled before harvest at 11 months (October 2000) and 12 months (October 2001) respectively (Figure 4.2).

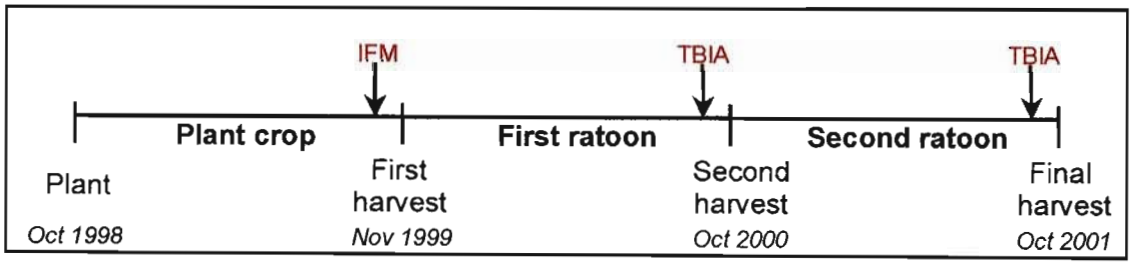


Figure 4.2 Diagrammatic representation of sampling and harvesting operations in the trial, 1998 to 2001.

The oldest stalk was plucked from each uninoculated stool and labelled according to the row number and the position of the stalk along the row. One stalk was also taken from the infected control stool in each row. The spreader sections of N14 were sampled after the first harvest to assess the degree of colonisation but were not sampled in the second ratoon crop.

4.2.2.1 Assessment of RSD infection

Diagnosis of RSD and the proportion of colonised vascular bundles (cvb) were determined using TBIA on the single stalk samples according to the procedure described in Chapter 3.

The TBIA was used as a quantitative measure of **RSD incidence** based on whether stalks were infected with *L. xyli* subsp *xyli* or not. This was used to estimate the **rate of spread of RSD** along the row, with each stalk sample representing 0,5m of the row.

The TBIA was also used as a qualitative test to determine the proportion of cvb, describing the **severity of infection** within those stalks that were infected.

4.2.3 Statistical analysis

The raw data were transformed and statistically analysed as described in Chapter 3. Bar charts and graphs were generated using Excel and SigmaPlot Version 5,0.

4.3 Results and discussion

In this chapter, the rate of spread of RSD and the proportion of cvb were investigated as methods of assessing varietal reactions to the disease. Differences in the reactions of South African varieties to RSD are known to occur (Bailey and Bechet, 1986,1995 and 1997) and one factor that is thought to play an important role in resistance is the relative populations of *L. xyli* subsp *xyli* in infected stalks (Davis *et al.*, 1988a). This in turn is

reported to influence the rate of spread and increase of RSD in different varieties during harvesting operations (Damann, 1992; Comstock *et al.*, 1996; Hoy *et al.*, 1999).

4.3.1 RSD status in the plant crop

4.3.1.1 RSD in the inoculated control plots

RSD was detected in all varieties, and populations of *L. xyli* subsp *xyli* were high in most of the inoculated control plots, apart from N26 and N27 (Table 4.1). This suggested that all varieties included in the trial had some degree of susceptibility to RSD.

Table 4.1 Mean rating of *L. xyli* subsp *xyli* populations in the plant crop, as determined by immunofluorescence microscopy

Variety	*L. xyli* subsp *xyli* rating		
	Control	Spreader	Uninoculated
N12	4,0	4,0	0
N14	4,0	4,0	0
N22	4,0	4,0	0
N24	4,0	4,0	0
N25	4,0	4,0	0
N26	3,6	4,0	0
N27	2,3	4,0	0
N28	4,0	4,0	0
N29	4,0	4,0	0
N30	4,0	4,0	0
N31	4,0	4,0	0
NCo376	4,0	4,0	0

Rating (per field of view):

0	No bacteria
1	1-3 bacteria
2	3-10 bacteria
3	11-15 bacteria
4	>25 bacteria

The fact that two of the three stools of N26 had high levels of RSD indicated that the variety is susceptible, but population development in the one stool was limited. Although every attempt was made to expose all SBS to similar levels of inoculum before planting,

some SBS may have been exposed to lower levels, if they were not completely immersed in the infected juice.

One inoculated control plot of N27 was not infected with *L. xyli* subsp *xyli* and the other two stools had lower populations of the bacterium. This suggests that the variety may have some resistance to infection.

4.3.1.2 RSD in the inoculated spreader plots

Populations of *L. xyli* subsp *xyli* were high in all stalks taken from the spreader sections of N14. The inoculum pressure at the beginning of each row should therefore have been similar and would be unlikely to affect the rate and extent of infection along each row.

4.3.1.3 RSD in the uninoculated rows

No RSD was detected in the uninoculated stools in the plant crop (Table 4.1). It could therefore be assumed that any stools after the spreader sections testing positive for RSD in the first and second ratoon crops, became infected via the N14 spreader sections during the harvesting operations.

4.3.2 RSD status in the inoculated stools in the first and second ratoon crops

TBIA was used to measure RSD incidence and severity in the trial after the first and second harvests (i.e. in the first and second ratoon crops) in the control and spreader plots.

4.3.2.1 Populations of L. xyli subsp *xyli in spreader plots of N14*

There was no significant difference ($P<0,05$) in the proportion of cvb in the spreader sections of N14 used as a means of inoculating each variety (Figure 4.3). This supported the severity ratings given in the plant crop when checked using immunofluorescence microscopy (Table 4.1).

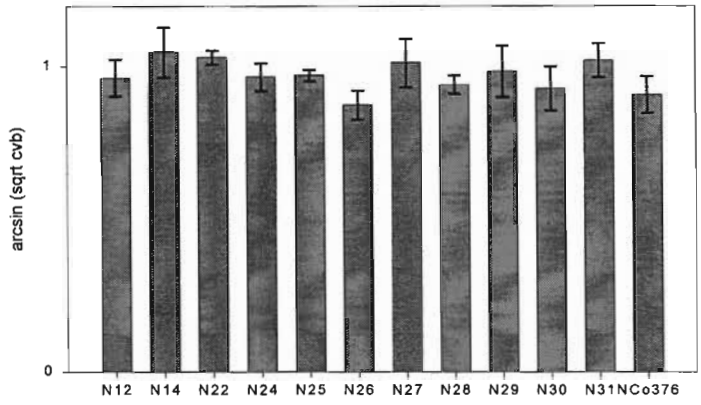


Figure 4.3 Mean proportion of cvb in spreader sections of N14 at the beginning of the rows of each variety shown on the x axis. Bars represent se, n = 6.

4.3.2.2 Populations of L. xyli subsp xyli in inoculated controls

The mean proportion of cvb and the RSD incidence in the controls differed between the varieties in the trial, in some cases significantly (P<0,05) (Figure 4.4). Three of the four standards reacted as expected, but N22 exhibited a high degree of colonisation (similar to N14) and all inoculated stools in this variety were infected with RSD. The standard

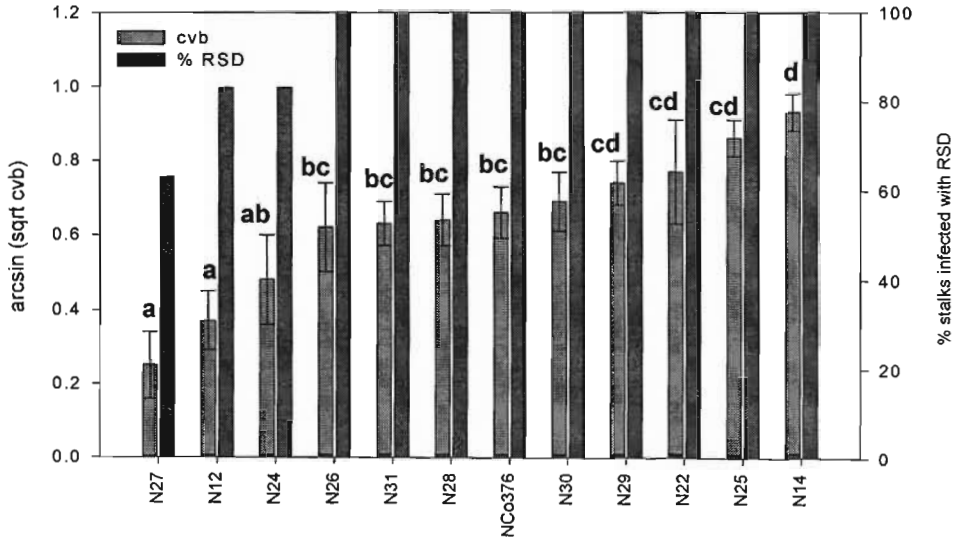


Figure 4.4 Mean proportion of cvb and RSD incidence in inoculated control plots (first and second ratoon crops). Data analysed using ANOVA on Ranks. Different letters denote significant differences (P<0.05). Bars represent se, n = 6.

error was highest in N22, giving an indication of the variability in the extent of colonisation in this variety.

RSD was not detected in some of the inoculated control plots of N27, N12 and N24. In N27, one inoculated stool remained RSD-free through to the second ratoon. With the exception of N12, the extent of colonisation was significantly lower in N27 than the other varieties.

Although *L. xyli* subsp *xyli* was detected in all control plots of N12 and N24 in the plant crop, one stool of N12 did not show infection in the first ratoon, but again showed infection in the second ratoon. No RSD was detected in one stool of N24 in the second ratoon, suggesting that the negative results in these two varieties were due to sampling error and should therefore be viewed as false negatives. In Chapter 3, no RSD was detected in the younger stalks of N16, a variety that is considered to be intermediate in its reaction to RSD. N12 is similar in its reaction to RSD, and it is possible that had younger stalks been inadvertently selected from the control stools, populations of *L. xyli* subsp *xyli* may have been below detectable levels. This may explain the negative result in one of the inoculated control plots of N12 in the first ratoon.

N24 is considered to be susceptible to RSD based on yield loss results. All stalks would therefore be expected to support detectable levels of *L. xyli subsp xyli* since the cane was over nine months old and was in its second ratoon (Anon, 1991). Bailey and Fox (1984) found that it was essential to examine all stalks for external damage if sufficient sap were to be extracted for routine RSD diagnosis. Had the stalk selected for the TBIA been dry due to, for instance, extensive stalk borer damage or cracking, insufficient xylem sap would have been present within the stalk to deposit the bacteria onto the membrane during centrifugation. This may provide one explanation for the negative result in the second ratoon.

The false negative results in the N12 and N24 control plots would have affected the mean proportion of cvb. The mean proportion of cvb in N24 in the first and second

ratoon increased from 0,25 to 0,3 if the levels in only the five infected stalks were considered. This was then similar to N31, N28, N26, NCo376 and N30. If the same principle was applied to the other two controls in which all stools did not test positive for RSD, the mean proportion of cvb in N27 increased from 0,09 to 0,14 and in N12 from 0,16 to 0,19. This would not affect the relative ranking of the two varieties as intermediate and intermediate-susceptible respectively.

4.3.3 RSD status of stools planted after the spreader plots in the first and second ratoon crops

This trial confirmed that the rate of spread and increase of RSD differs from one variety to another. Comstock *et al.* (1996) suggested that for little or no spread to occur, an average of less than one cvb for a particular variety was required. Although the proportion of cvb rather than absolute numbers was used in this study, no variety grown commercially in South Africa has shown such low levels of colonisation. In this trial, all the varieties were found to have some degree of susceptibility to the disease, with RSD spreading from infected to healthy stools in all the varieties tested. This was most evident after the second harvest, with the mean RSD incidence and severity increasing significantly in all varieties from the first to the second ratoon crop (Figure 4.5)

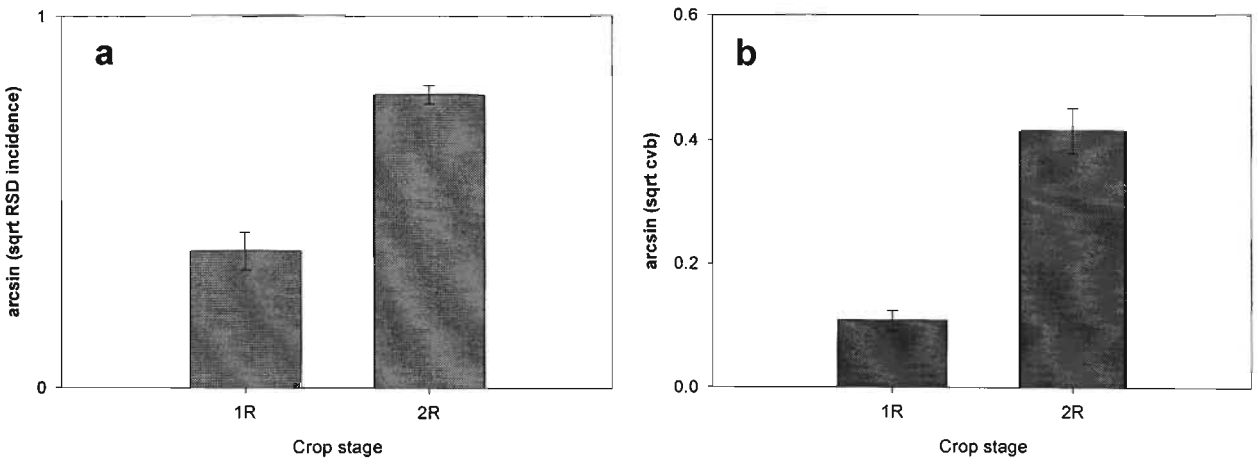


Figure 4.5 Differences in (a) RSD incidence and (b) proportion of cvb from the first to second ratoon crop. Data analysed using ANOVA on Ranks. Bars represent se, n = 36 and 288 respectively.

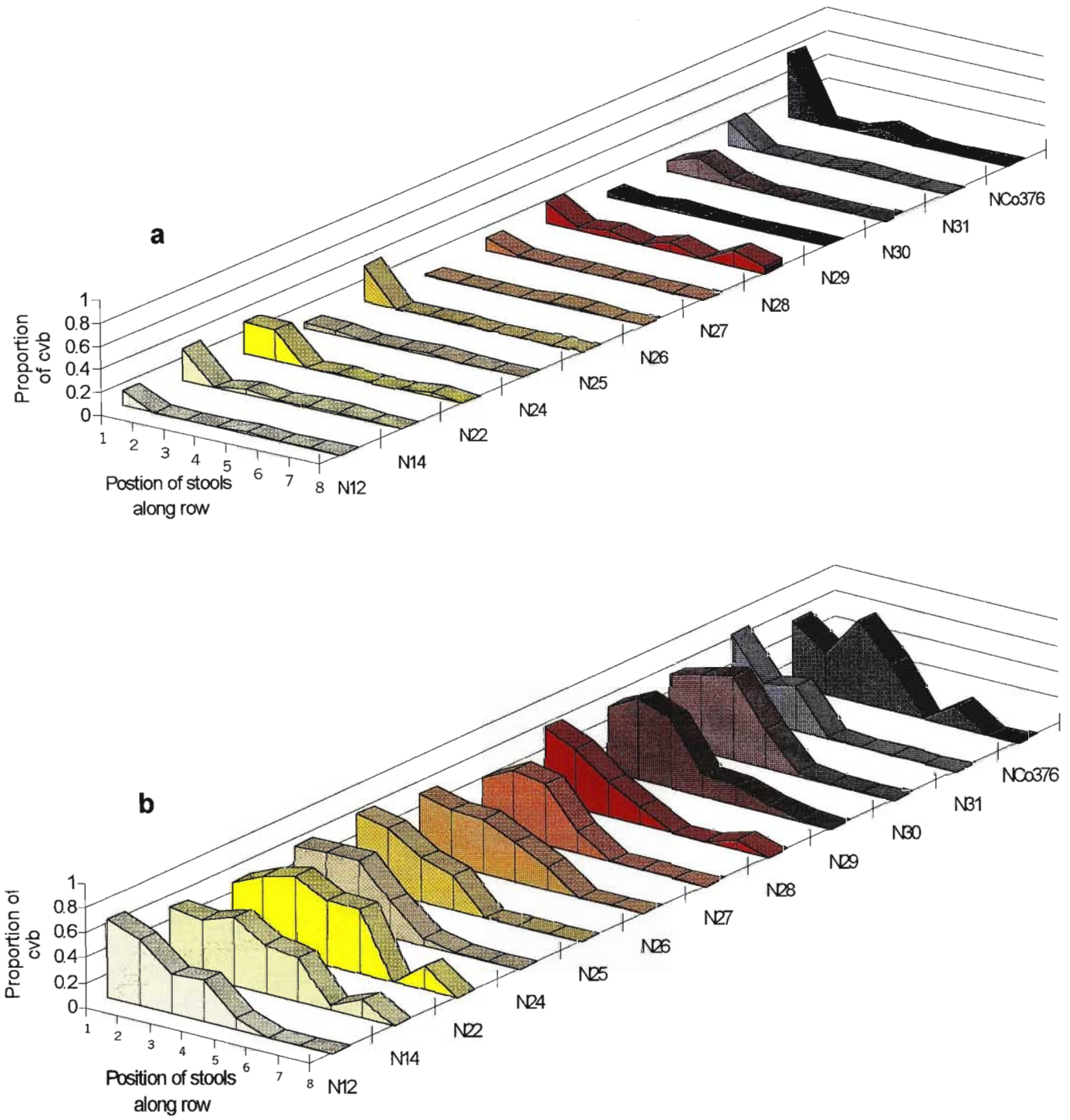


Figure 4.6 Mean rate of spread of RSD and extent of colonisation along the rows of each variety in (a) the first and (b) second ratoon crops.

4.3.3.1 *Rate of spread (RSD incidence) and severity of RSD along the row*

RSD spread from the N14 spreader sections into the rows of all varieties after one harvesting operation. This spread was quite extensive in some varieties, but the proportion of cvb was generally low. The decrease in incidence and severity of RSD from the spreader sections to the end of the row indicated that the spread of the disease occurred in the direction of cutting in all varieties. Since RSD did not spread past the eighth stool in all rows after two harvests, only the RSD incidence and severity up to this point were assessed. Figure 4.6 illustrates the mean proportion of cvb at each position along the rows of the different varieties in the three replications.

When the trial was sampled after the second harvest, the disease had not spread much further, but the proportion of cvb had increased substantially in most varieties. Had the third ratoon of the trial been sampled, one would have expected the spread to be far more extensive, 'fuelled' by the higher populations of *L. xyli* subsp *xyli* that had developed in the infected stools along each row in the second ratoon.

A number of varieties showed intermittent infections along the rows, particularly in the first ratoon crop after only one harvesting operation. This suggested that some stools escaped infection even when the subsequent stool became infected. This has been reported by other researchers (Dr Jeff Hoy, Dr Michael Grisham, personal communication) and may relate to the manner in which that particular stool was harvested or sampled. In one trial conducted in Australia using mechanical harvesters, Taylor *et al.,* (1988) reported that no spread of RSD was detected when the stools were cut below ground level. This has never been shown when cane is cut manually. However, there were significant differences in RSD incidence and severity between replications in both the first and second ratoon crops (Figure 4.7), each cut by a different cane cutter. The RSD incidence in replication one was significantly different to replication two in the first ratoon and to replication three in the second ratoon crop. There were differences in the proportion of cvb, particularly in replication one in the first ratoon and replication three in the second ratoon crop, but these differences were not

significant. This suggests that the manner in which the cane was harvested may have influenced the rate of spread of the disease.

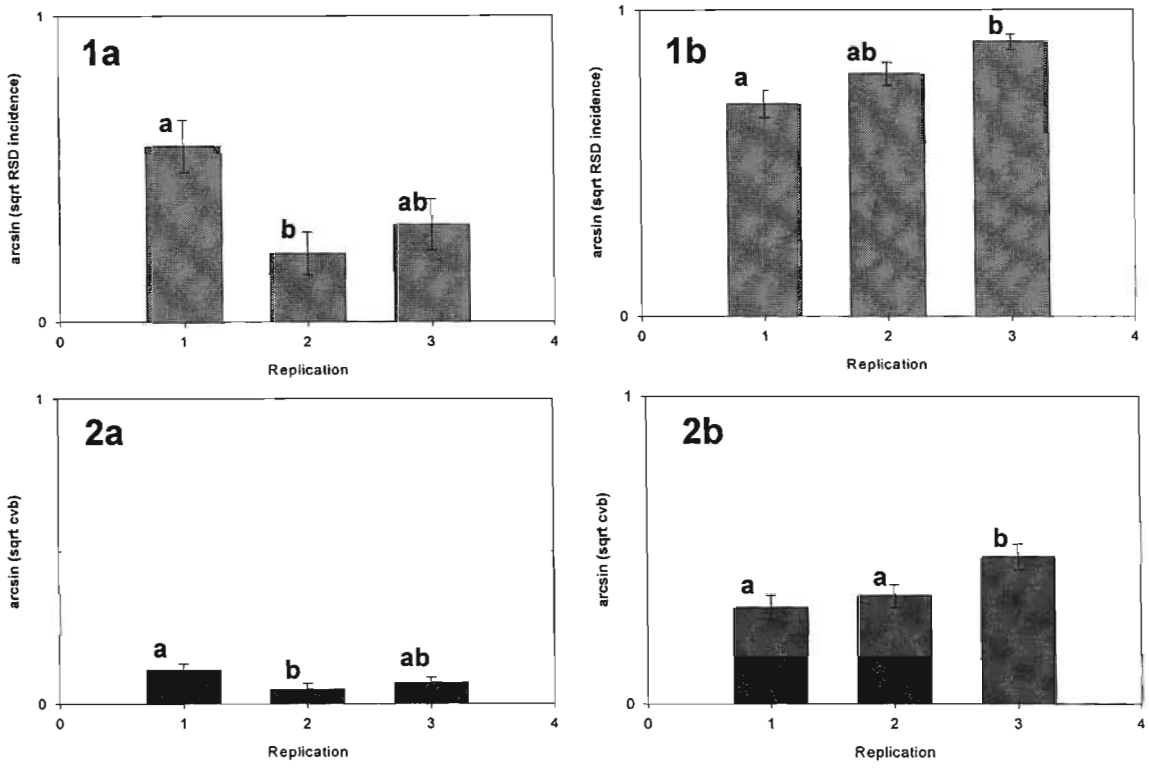


Figure 4.7 Differences in RSD incidence in (1a) first ratoon and (1b) second ratoon and proportion of cvb in (2a) first ratoon and (2b) second ratoon) in different replications. Data analysed using ANOVA on Ranks. Different letters above columns denote significant differences (P<0.05). Bars represent se, n = 96 and 12 respectively.

The spread of RSD was most extensive in N14 (highly susceptible standard) and N28 in the first ratoon, followed by N24, N22 (intermediate standard) and N26. The extent of colonisation was greatest in N28 after one harvest and this increased quite substantially in the variety after the second harvest, particularly in the first two stools, but RSD did not spread further along the row. The proportion of cvb in the first two stools along the rows of N14 and N22 were higher than N28, but the overall colonisation in these two varieties was lower. The extent of colonisation was low in N24 and N26.

By the second ratoon, the increase in severity in both N14 and N22 at most positions along the row was more substantial than in N28, with N22 having the highest overall

proportion of cvb in all the varieties tested. This would indicate that N14 and N22 were the most susceptible of the varieties tested. As discussed previously, N14 is highly susceptible and intolerant to RSD but N22 does not suffer significant yield loss when infected with the disease, and is considered to be intermediate in its reaction to RSD (Bailey and Bechet, 1995, 1997). These results do however, confirm that N22 appears to be a variety that supports high populations of *L. xyli* subsp *xyli.*

The proportion of cvb in the first stool after the spreader section was highest in the susceptible standard NCo376. RSD was detected in only two stools of this variety after one harvest but the disease spread rapidly along the row and the extent of colonisation was high in the second ratoon crop. This was not as high as in N14 and N22, but from this trial, NCo376 could be considered susceptible to the disease. This is in agreement with previous findings based on yield loss and the results obtained from the inoculated control plots in this trial.

RSD spread and severity were low in the intermediate-susceptible standard, N12, after one harvest. The extent of colonisation was particularly high in the first two stools after two harvests, but decreased steadily along the row. The variety would be considered susceptible based on the data from this trial, a finding not supported by data from previous trials. Other varieties that had similar patterns of spread along the row in the second ratoon were N29, N24, N26, and N30 and would therefore be rated susceptible in this trial.

Although the proportion of cvb in N25 was relatively high in comparison with many of the other varieties in the first ratoon, the spread along the rows was lowest with only the first stool after the spreader section testing positive. The extent of colonisation in the second ratoon was high in the first two stools after the spreader section, but decreased steadily along the row, with only four stools testing positive. This slow rate of spread after two harvests was only evident in two other varieties, N27 and N31. Both these varieties had a low proportion of cvb after one harvest in the two stools that tested positive. The proportion of cvb along the row was generally lower in N27 and N31 in the second ratoon. Based on this information, N27 and N31 would be considered the most

resistant of the varieties under test, with N25 slightly more susceptible. Although recent evidence indicates that N27 does have some resistance to RSD (McFarlane, 2002), N25 has been found to be almost as susceptible to RSD as N14 (Bailey and Bechet, 1995). This low rating was also not supported by the results from the inoculated controls. The reaction of N31 is not known, but the results from the inoculated control plots indicated that this variety was susceptible to the disease.

In summary, the differences in the rate of spread and severity between the varieties after two harvests were not substantial, making the grouping of varieties difficult. All the varieties tested would be considered susceptible to highly susceptible to the disease. The spread was least extensive in N27, N31 and N25 and severity was lowest in N27 and N31. Spread and severity were greatest in N14 and N22, suggesting that these two varieties are highly susceptible to the disease. The other varieties fell between these two groups. Presenting the information obtained from the trial in this manner did not adequately distinguish the varieties. Certain problems in the layout and sampling of the trial were identified that may have influenced the findings presented here. Infected stools surviving from the previous crop (volunteers) provide an important source of *L. xyli* subsp *xyli* inoculum in the newly planted crop. Using a highly susceptible variety such as N14 in the spreader plots, which supports large populations of *L. xyli* subsp *xyli,* can provide useful information on this form of spread through all varieties. Perhaps, however, a better test of the varieties would however have been to use the inoculated controls as spreader plots, thus representing the situation when infected seedcane is planted. In varieties such as N27 where the initial proportion of cvb was low, it can be assumed that spread would have been significantly slower and this may have separated the varieties better.

A 12 metre row of uninoculated plants was found to be too long in this trial and rows with fewer stools, spaced further apart to facilitate sampling would be advantageous. By the second ratoon, individual stools were not easily identified and pegs at sampling points along each row would reduce sampling errors. Additional replications would be

beneficial in reducing variability and increasing the possibility of detecting significant differences between the varieties under test.

4.3.3.2 Relationship between RSD incidence and severity

The relationship between RSD incidence and proportion of cvb is shown in figure 4.8. In this figure, the relative positions of the infected stools along the rows of each variety have been ignored. Rather, the mean RSD incidence in the three replications was plotted against the mean proportion of cvb in each variety in the first and second ratoon crops. The best correlation was obtained with a straight line (y = 143,24x + 15,995). The relationship was highly significant (P=0,001) at 23 degrees of freedom (r = 0,92) suggesting that an increase in the extent of colonisation could be expected to facilitate the spread of the disease.

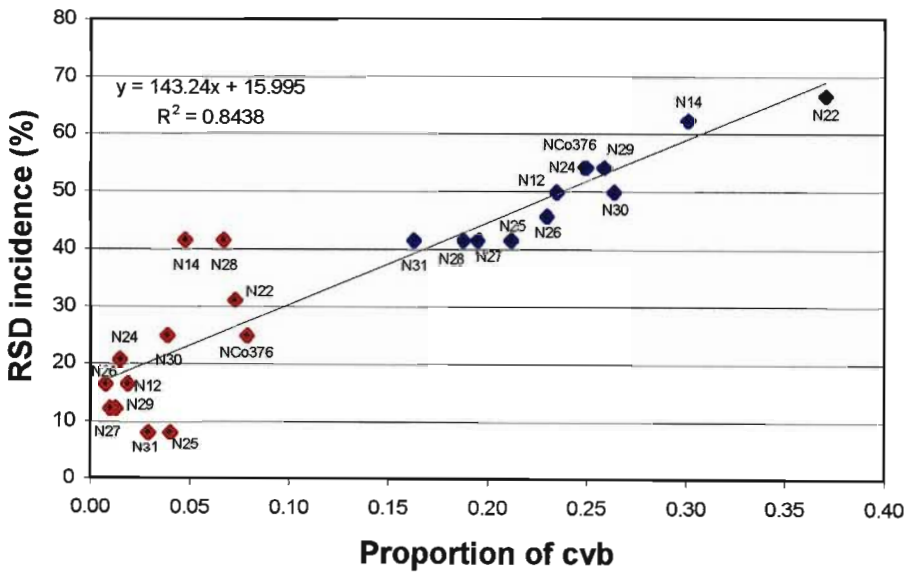


Figure 4.8 Relationship between RSD incidence (%) and proportion of cvb after one (♦) and two (♦) harvests.

After the first harvest, RSD incidence was lowest in N31 and N25 (8,3% stalks infected) and highest in N14 and N28 (41,7% stalks infected). The severity of RSD, based on the proportion of cvb in all eight stools sampled, was lowest in N26 (0,02 cvb). NCo376 had

proportion of cvb in all eight stools sampled, was lowest in N26 (0,02 cvb). NCo376 had the highest proportion of cvb (0,08) but RSD incidence (25% stalks infected) was close to the first ratoon average.

After the second harvest, the extent of colonisation by *L. xyli* subsp *xyli* was lowest in N31, N28 and N27. RSD was detected in 41,7% of the stalks tested in these varieties. The highest proportion of cvb and RSD incidence was recorded in N22. The greatest increase in RSD incidence and severity from the first to the second ratoon occurred in N29 and N22 respectively. N28 showed the smallest increase in these two parameters, with RSD incidence remaining the same and the proportion of cvb increasing from 0,07 to 0,19.

RSD incidence and severity along the row and in the inoculated control plots indicated that N14 and N22 were the most susceptible of the varieties tested. N14 is known to be highly susceptible to RSD, but results from previous yield loss trials (Bailey and Bechet, 1995 and 1997) suggested that N22 suffered very little yield loss when infected and has therefore been rated intermediate in its reaction to the disease. However, a recent study of the spread of RSD in commercial fields in Umfolozi has indicated that the disease can spread rapidly through this variety (unpublished). In one field, *L. xyli* subsp *xyli* was detected in 13% of the stalks tested in a 160-stalk sample in the plant crop. This increased to 30% in the first ratoon crop and 77,5% after the second harvest. This, along with the fact that the proportion of cvb was high in this trial and in previous experiments (refer to Chapter 3), suggests that this variety supports high populations of *L. xyli* subsp *xyli*, which results in the rapid spread of the disease, but that the variety is tolerant to infection. This should be seen as one limitation of the TBIA technique, in that it will not be possible to distinguish tolerant varieties that are able to support high populations of *L. xyli* subsp *xyli* from those that are susceptible and suffer significant yield losses when infected with RSD. However such tolerant varieties pose a threat to other susceptible and highly susceptible varieties and where possible, should be avoided. In a study conducted by Hoy *et al.* (1999), CP79-318, a variety that does not suffer yield loss when infected with RSD, fell within the group of varieties exhibiting the

greatest spread. They stated that the levels of inoculum in the spreader plots could influence the rate of spread. In this trial, all spreader sections of N14 were found to have high populations of *L. xyli* subsp *xyli* and there were no significant differences from one spreader section to another. This is therefore unlikely to have influenced the severity and rate of spread of RSD in N22. They also stated that these factors could be influenced by environmental conditions. Although this could play a role when comparing results from trials at different locations, it is unlikely to have had an effect in this trial where all varieties were exposed to the same conditions and the soil was uniform.

4.4 Conclusions

The data from this trial were difficult to interpret and some modifications to the design of future trials investigating RSD spread may facilitate the grouping of varieties according their resistance to RSD.

Based on the data from the inoculated control plots, the varieties could be ranked for RSD susceptibility as follows: N27 (intermediate); N12 (intermediate-susceptible); N24, N31, N28, N26, NCo376, N30 (susceptible) and N29, N22, N25 and N14 (highly susceptible). Most of these rankings, apart from N22, are in broad agreement with previous findings (Bailey and Bechet, 1995, 1997).

Based on the incidence and severity of RSD at specific points along the row, all varieties were rated susceptible or highly susceptible. Better discrimination was obtained by plotting the overall incidence against severity, particularly in the second ratoon. The findings did not however support the results of the inoculated control plots. Certain modifications to the design of future trials may improve the overall results, making such trials more meaningful.

N22 appears to support high populations of *L. xyli* subsp xyli but is reported to suffer very little yield loss when infected. TBIA was unable to distinguish this variety from N14.

Although this should be considered a limitation, it is important to identify those varieties that support high populations of *L. xyli* subsp *xyli*.

The fact that the correlation between the proportion of cvb and the mean RSD incidence was highly significant suggests that greater spread occurred in those varieties that were more extensively colonised by *L. xyli* subsp *xyli*. This would support the hypothesis that by growing varieties that supported low populations of *L. xyli* subsp *xyli*, the rate of spread at harvest could be reduced.

CHAPTER 5

The effect of RSD on the yield of six sugarcane varieties and relationship to extent of colonisation by *Leifsonia xyli* subsp *xyli*

5.1 Introduction

The reaction of commercially grown South African varieties to ratoon stunting disease (RSD), caused by the bacterium *Leifsonia xyli* subsp *xyli*, was based in the past, on large, replicated yield loss trials grown over a number of years under rainfed and irrigated conditions (Bailey and Bechet, 1986, 1995 and 1997). These trials have provided valuable information on the effect of *L. xyli* subsp *xyli* on the different varieties, and have shown that, even under good growing conditions, the disease can cause substantial reductions in yield. However, these trials are time-consuming and require large areas of uniform land. Environmental conditions that affect growth and yield (Gillaspie and Teakle, 1989) may vary from year to year. They are therefore not suitable for application in a routine disease screening programme where it is necessary to assess large numbers of varieties. As a result, the susceptibility of new varieties to RSD is currently not known at the time of release to the growers in the South African sugar industry.

Alternative approaches to yield loss trials to screen sugarcane genotypes for their resistance to RSD have been investigated, but TBIA has been found to be the most efficient and simple method currently available (Davis *et al.*, 1994; Comstock *et al.*, 1995). Using this method, it is possible to routinely screen up to approximately 1 000 genotypes each season (Comstock *et al.*, 2001)

The trial described in this chapter had two main objectives. The first was to use the effect of *L. xyli* subsp *xyli* on yield as a means of assessing the susceptibility of four commercially grown varieties. The second objective was to examine the relationship

between yield loss and the extent of *L. xyli* subsp *xyli* colonisation of these four varieties and the two standards included in the trial.

5.2 Materials and methods

5.2.1 Establishment and management of trial

The trial was conducted under rainfed conditions on a uniform, deep sandy loam soil at Mount Edgecombe, KwaZulu-Natal. The plant crop received 1 045 mm of rain and the first ratoon, 1 300 mm.

5.2.1.1 Choice of varieties

Four commercially grown varieties, N16, N27, N29 and N35, with unknown reactions to RSD were included in the trial as well as two standard varieties, N12 (intermediate-susceptible) and N14 (highly susceptible).

5.2.1.2 Layout and planting of trial

The trial was planted in October 2000. A split plot randomised complete block design with six replications was used, with varieties as whole plots and diseased or healthy cane as sub-plots. Each sub-plot consisted of four rows, 7 m long with a row spacing of 1,2 m. The healthy plots were planted first to ensure that the bundles of healthy and infected seedcane were not mixed. The trial was bordered on all sides by healthy NCo376 to reduce border effects.

The trial was fertilised with 60kg nitrogen and 60 kg of potassium per hectare at the time of planting and top dressed after harvest. The herbicides Sencor ® Dow AgroSciences (a.i. metribuzin) (3 l/ha) and Diuron® Bayer (a.i. substituted urea) (2 l/ha) were applied in combination in the plant and first ratoon crops.

5.2.1.3 Preparation of seedcane

Seedcane for the trial was obtained from the plant crop of specially grown propagation plots. The healthy plots were established with healthy seedcane that was hot water treated at 50°C for two hours. The seedcane for the RSD-infected propagation plots was cut into three-budded setts and the freshly cut ends were immediately dipped in juice from crushed stalks of severely infected N14. The inoculated stalks were covered with a plastic sheet and left for 24 hours before planting.

Before planting the trial, the source material from the propagation plots was tested for *L. xyli* subsp *xyli* using phase contrast microscopy and EB-EIA. All the healthy plots were free from *L. xyli* subsp *xyli* but levels in the diseased plots were relatively low and the seedcane was reinoculated before planting using the same method as described previously.

5.2.1.4 Harvesting of trial

The trial was burnt before each harvest. The plant crop was harvested in October 2001 at the age of 12,5 months and the first ratoon in September 2002, 11,5 months later. At harvest, precautions were taken to prevent the spread of RSD to the healthy plots. Cane cutters were assigned to harvesting either the healthy or the RSD-infected blocks. Cane knives were disinfected in 10% (v/v) Jeyes Fluid (a.i. carbolic acid) after harvesting each row of healthy cane. Knives were not disinfected when harvesting the RSD-infected blocks to facilitate the spread of the disease to any stools that were not infected in the plant crop.

5.2.2 Sampling and laboratory analysis

5.2.2.1 RSD diagnosis using EB-EIA

One week before harvesting the plant crop in October 2001, twelve stalks were collected

from each sub-plot. The healthy sub-plots were sampled before the RSD-infected sub-plots to avoid cross contamination during sample preparation in the laboratory.

The lowest undamaged internode was cut from each stalk. The one end of the stalk piece was placed in a specially constructed moulded resin adaptor (Croft and Witherspoon, 1982), and the xylem sap expressed using low-pressure compressed air (Richardson, 1978). The sap from each stalk was pipetted directly into a well of a standard 96 well flat-bottomed microtitre plate (Sterilin, Staffordshire) so that twelve wells represented the stalk samples from one sub-plot. The microtitre plates were processed according to the EB-EIA procedure described in Chapter 3, section 3.2.2.4.

5.2.2.2 Assessment of RSD incidence and severity using TBIA

A tissue core was taken from the second internode of all stalks sampled from the diseased sub-plots and used for the TBIA as described in Chapter 3, section 3.2.4. The impressions of all vascular bundles were visible on the developed membranes, with those colonised by *L. xyli* subsp *xyli* appearing as distinct blue dots. The number of infected stalks and the proportion of colonised vascular bundles (cvb) per stalk were determined in each RSD-infected plot.

TBIA was used as the sole method of RSD diagnosis in the first ratoon crop.

5.2.3 Assessments of yield and cane quality

Stalk counts were carried out the day before each harvest. At harvest, each row was weighed separately and yield assessments were based on all four rows in each sub-plot. Three stalks were randomly selected from each row to provide a 12-stalk sample per sub-plot for mill analysis. The height (measured from ground level to the top visible dewlap) and diameter of the internode one metre above ground level of each of these stalks were measured.

Yield was expressed as tons cane and tons estimated recoverable crystal (ERC) per hectare.

$$ERC = \mathbf{a}*S - \mathbf{b}*N - \mathbf{c}*F$$

Where S = sucrose % cane
N = non-sucrose % cane
F = fibre % cane

The coefficients are based on the milling statistics of the previous three seasons.

a accounts for losses in filtercake and undetermined means
b accounts for loss in molasses (related to non-sucrose content)
c accounts for loss in bagasse (depends on level of fibre in cane)

ERC was also expressed as a percent of the cane yield (ERC % cane) and was used as a measure of cane quality.

5.2.4 Analysis of data

Trial data were analysed using two way analysis of variance (ANOVA) in SigmaStat 2.0. TBIA results were transformed using the arcsin square root transform function in SigmaStat 2.0. Graphs and bar charts were generated with Excel and SigmaPlot 5.0.

5.3 Results and discussion

5.3.1 RSD incidence in the plant and first ratoon crops

Between 86 and 100% of the stalks tested in the diseased plots were infected with *L. xyli* subsp *xyli* in the plant crop. The lowest incidence of RSD was detected in plots of N27 and N35 (Table 5.1). By the end of the first ratoon crop, all stalks sampled from the diseased plots were infected with RSD.

Despite the precautions taken when planting the trial, some *L. xyli* subsp *xyli* was detected in the plots planted with seedcane from the healthy propagation plots. Populations of *L. xyli* subsp *xyli* may have been present in these plots at levels below the threshold of EB-EIA and phase contrast microscopy. However, since the levels were considerably lower in the healthy plots, it was still possible to make comparisons and estimate yield losses due to RSD infection.

Table 5.1 Percent stalks with *L. xyli* subsp *xyli* in plots planted with seedcane from healthy and RSD-infected propagation plots (plant crop and first ratoon).

Variety	% stalks infected			
	Plant crop		First ratoon	
	Healthy	Infected	Healthy	Infected
$N12^{IS}$	0	99	4	100
$N14^{HS}$	7	100	12	100
N16	0	99	2	100
N27	1	88	2	100
N29	4	100	10	100
N35	1	86	3	100

Standards: $N12^{IS}$ = intermediate-susceptible, $N14^{HS}$ = highly susceptible

5.3.2 Effect of RSD on germination and certain yield components

5.3.2.1 Germination

Steindl (1961) reported that RSD-infected setts often germinated slowly and erratically. Differences in germination between healthy and diseased plots have been noted in more recent trials conducted in the South African sugar industry (Bailey and Bechet, 1986).

Good germination was recorded in most varieties and plots in this trial, with the exception of N12. Overall, significantly more germinating shoots and tillers were recorded in the healthy plots ($P<0,05$) (Table 5.2). Differences were, however, not significant within the six varieties (Figure 5.1).

Germination was particularly poor in two adjacent plots of N12. Gaps in these plots were appropriately filled with diseased or healthy seedlings, but at harvest stalk counts were low in both plots, particularly in the RSD-infected plot. This extended into the first ratoon crop and the data from these plots were therefore replaced with the means for the five replications of diseased and healthy N12.

Table 5.2 Effect of RSD on mean germination (plant crop), stalk populations and stalk characteristics (plant and first ratoon) of six varieties grown under rainfed conditions.

	Plant crop				First ratoon		
		Stalk			Stalk		
	Germination	Pop^{n} (1 000s/ha)	Height (cm)	Diam (cm)	Pop^{n} (1 000s/ha)	Height (cm)	Diam (cm)
Healthy	191	119	118	2.3	151	205	2.3
Diseased	182*	110**	112**	2.3	147	195**	2.3
SED	4.2	2.7	1.5	0.1	2.2	2.7	0.1
LSD 0.05	8.6	5.4	3.2	0.4	4.6	5.5	0.4
LSD 0.01	11.5	7.3	4.2	0.5	6.1	7.4	0.5
CV%	9.5	9.8	5.7	7.5	6.3	5.7	7.2

Difference between healthy and RSD-infected plots significant (*) at P<0,05 or (**) at P<0,01 using two way ANOVA

There was a substantial, but not significant difference in the germination of the remaining healthy and diseased plots of N12, with the healthy plots having a higher population after three months. This variety is known to germinate slowly and in many cases, poorly (Anon, 2002). Dipping the ends of the N12 setts in RSD-infected juice may have encouraged the growth of other micro-organisms (Zummo, 1974) that, with *L. xyli* subsp *xyli,* may have had more opportunity to colonise the setts and delay germination. Previous trials with N12 showed little difference in germination (Bailey and Bechet, 1986 and 1995), but the seedcane for the diseased plots was planted directly from the propagation plots and was not dipped in RSD-infected juice before planting.

Reduced germination was also recorded in diseased plots of N27 and N35, but this was not reflected in the stalk populations before harvest.

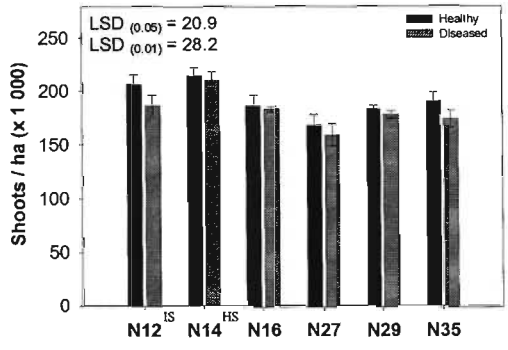


Figure 5.1 The effect of RSD on the germination of six varieties (plant crop). Data analysed using two way ANOVA. Bars represent se, n = 12. Standards: N12^{IS} = intermediate-susceptible, N14^{HS} = highly susceptible.

5.3.2.2 Stalk populations

There was an 8% difference in the mean stalk populations between the healthy and diseased plots before harvest in the plant crop, with the healthy plots having significantly more stalks than the diseased (P<0,01). Below average, erratic rainfall was recorded during the plant crop of the trial, which is likely to have influenced germination and stalk populations, particularly in the diseased plots. Growing conditions were excellent the following season and record crops were harvested in most parts of the industry (Anon, 2003a). Although there was a difference in the stalk populations before harvest in the first ratoon, this was not significant. This would support previous findings that RSD is less likely to have an effect on stalk populations when rainfall is adequate (Steindl, 1961; Bailey and Bechet, 1986).

When comparing the stalk populations in the healthy and diseased plots of the six varieties grown in the trial, N12 was the only variety that had a significantly lower population in the diseased plots (Figure 5.2). This was evident in both the plant and first ratoon crops and was likely to have been influenced by the poor germination of the infected plots. The stalk population in the healthy plots of N12 in the plant crop was the highest of the varieties included in the trial. N27 has a tendency to produce a low

population in the plant crop and this was evident in this trial. There was little difference between the populations in healthy and diseased plots of this variety.

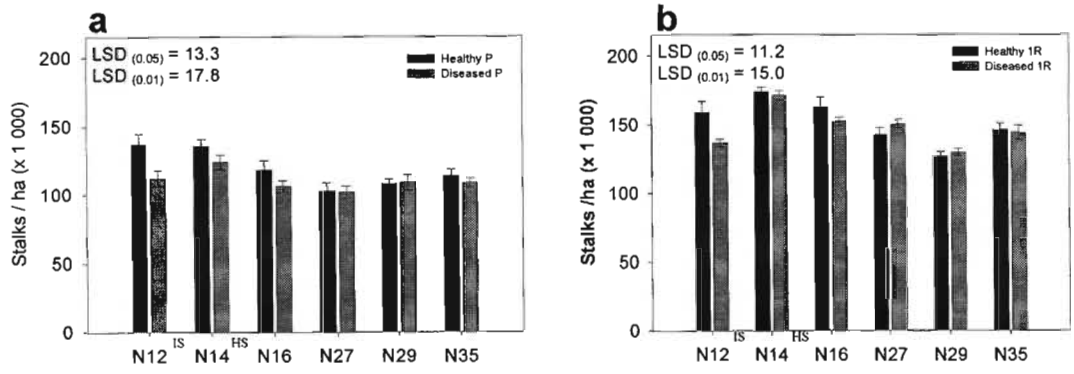


Figure 5.2 The effect of RSD on the stalk populations of six varieties [Plant (a) and first ratoon (b)]. Data analysed using two way ANOVA. Bars represent se, n = 12. Standards: $N12^{IS}$ = intermediate-susceptible, $N14^{HS}$ = highly susceptible.

5.3.2.3 Stalk characteristics

In many varieties, *L. xyli* subsp *xyli*, usually causes sugarcane stalks to become shorter and thinner, ultimately resulting in a reduction in yield (Koike, 1974; Bailey and Bechet, 1986). In most cases, these differences become more substantial from one crop to the next (Gillaspie and Teakle, 1989). In this trial, there was a significant difference ($P<0,01$) in the average stalk length between healthy and diseased plots in both the plant and first ratoon crops (Table 5.2).

Differences in stalk length were significant ($P<0,05$) in N16 and N29 in the plant crop, and in N12 and N29 ($P<0,05$) in the first ratoon (Figure 5.3). There was no difference in the mean stalk diameter between healthy and diseased plots in both the plant and first ratoon crops and the differences within varieties were not significant.

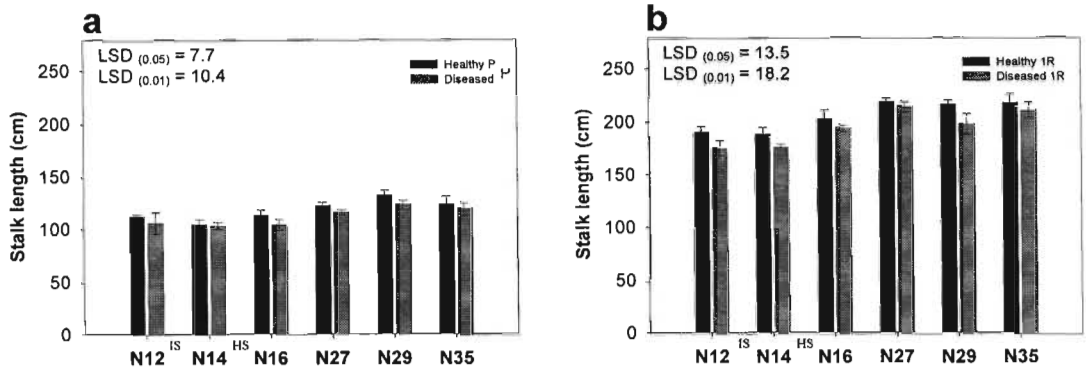


Figure 5.3 The effect of RSD on stalk length in six varieties [plant (a) and first ratoon (b)]. Data analysed using two way ANOVA. Bars represent se, n = 12. Standards: $N12^{IS}$ = intermediate-susceptible, $N14^{HS}$ = highly susceptible.

5.3.3 Effect of RSD on the cane and sucrose yield

The effect of RSD is known to increase with each ratoon (Gillaspie and Teakle, 1989) and this was clearly demonstrated in this trial. Bailey and Bechet (1995) reported that RSD could cause substantial yield losses, even under favourable growing conditions. The fact that yield differences in the first ratoon crop of this trial were significant (P<0,01) even though rainfall was adequate again emphasised the importance of RSD in all growing conditions.

The overall cane yield in the healthy plots of the plant crop was satisfactory with a mean of 75 t/ha after 12,5 months (Table 5.3). This was significantly different (P<0,01) to the cane yield in the diseased plots. This in turn resulted in a significant reduction in the yields of ERC in the RSD-infected plots (P<0,01). When expressed as a percentage of the cane yield (ERC % cane), there was no significant difference between the healthy and infected plots, indicating that RSD did not have an effect on the quality of the cane.

Growth in the first ratoon was good and an average yield of 122,7 tons cane/ha was obtained in the healthy plots after 11,5 months. The 12% reduction in cane yield in the RSD-infected plots was significant at the 1% level, as was the difference in ERC (t/ha). ERC % cane was higher in the diseased plots, but not significantly so.

Table 5.3 Mean cane and ERC yield (t/ha) and ERC % in healthy and RSD-infected plots of six varieties grown under rainfed conditions (plant crop 12,5 months, first ratoon 11,5 months).

	Plant crop			First ratoon		
	Cane yield t/ha	ERC t/ha	ERC %	Cane yield t/ha	ERC t/ha	ERC %
Healthy	75	8,7	11,6	123	16,9	13,8
Diseased	68**	7,7**	11,4	108**	15,2**	14,0
% loss	-9	-12	-2	-12	-11	+1
LSD 0,05	4	0,5	0,4	6	0,8	0,4
LSD 0,01	6	0,7	0,5	8	1,1	0,5
CV%	12	13	7	11	11	6

Difference between healthy and RSD-infected plots significant (*) at P<0,05 or (**) at P<0,01 using two way ANOVA

Growth in the first ratoon was good and an average yield of 122,7 tons cane/ha was obtained in the healthy plots after 11,5 months. The 12% reduction in cane yield in the RSD-infected plots was significant at the 1% level, as was the difference in ERC (t/ha). ERC % cane was higher in the diseased plots, but not significantly so.

5.3.3.1 Effect of RSD on cane yield of six varieties

RSD caused a reduction in cane yield (tons / ha) in five of the six varieties in the plant crop and all varieties in the first ratoon (Table 5.2). The extent of the reduction in yield differed among the varieties (Figure 5.4). The controls, N12 and N14, reacted as expected in the plant crop, but yield loss in N12 was as high as that of N14 in the ratoon crop.

Yield losses were significant at the 5% level in N29 (-15%) and N14 (-15 %) in the plant crop, suggesting that N29 is susceptible to RSD. The reduction in yield in the plant crop of N29 seemed to be associated with the significant decrease in stalk height and slightly thinner stalks, rather than a decrease in stalk population, as was the case with N14. Although yield losses were substantially higher in N16 (-13%) and N12 (-11%) than in

N35 (-4%), these losses were not significant. RSD did not reduce cane yield in N27 in the plant crop.

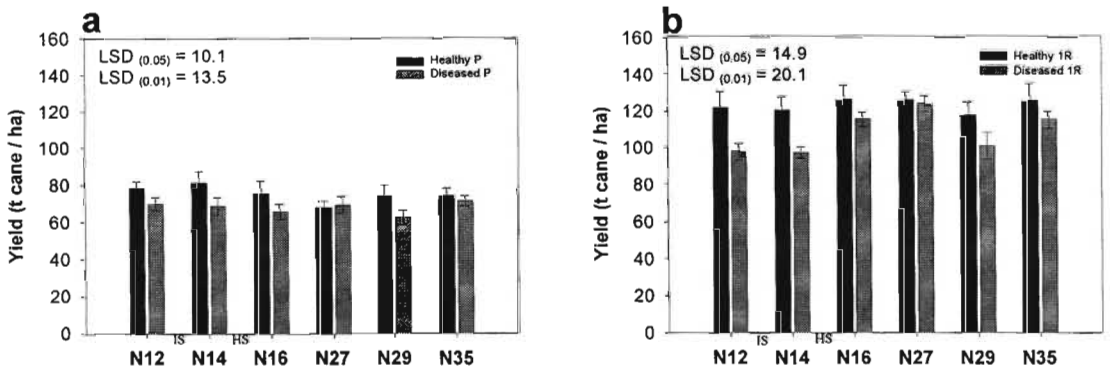


Figure 5.4 The effect of RSD on the cane yield (t cane/ha) of six varieties [plant (a) and first ratoon (b)]. Data analysed using two way ANOVA. Bars represent se, n = 12. Standards: $N12^{IS}$ = intermediate-susceptible, $N14^{HS}$ = highly susceptible.

In the first ratoon, losses were significant at the 1% level in N12 (-19%) and N14 (-19%) and at the 5% level in N29 (-14%). The losses recorded in N12 were unusually high for a variety that is considered to be intermediate-susceptible to RSD (Refer to Table 2.1). These losses were most likely due to the significant reduction ($P<0,01$) in the stalk population in the diseased plots, and to a lesser extent, the significant decrease in stalk height ($P<0,05$). N12 has been included in a number of yield loss trials, and in most cases has been considered intermediate-susceptible in its reaction to RSD based on the mean percent yield loss over two to three ratoons. However, the variety has shown considerable variation in yield loss from one ratoon to the next. In one trial grown under rainfed conditions, N12 showed losses of 28%, making it the third most susceptible variety of the eight varieties included in the trial (Bailey and Bechet, 1986). This was mainly due to reductions in stalk populations, possibly due to the low rainfall in the later stages of the plant crop and early stages of the first ratoon.

Losses were also high in N16 (-9%) and N35 (-8%), but these were not significant. Yields of N27 were suppressed slightly by RSD, but the results from the two crops indicated that the variety has some resistance to the disease.

5.3.3.2 *Estimated recoverable crystal*

The effects of RSD on ERC yield were similar to those on cane yield since RSD did not have a significant effect on cane quality in most varieties when infected with *L. xyli* subsp *xyli* (Table 5.2, Figure 5.5).

N12 lost substantially more ERC yield (tons / ha) due to RSD in the first ratoon crop when compared to the plant crop, and there was a slight reduction in ERC yield in N27. Losses in N14 and N16 were proportionally less in the first ratoon and those in N29 and N35 were comparable in both crops.

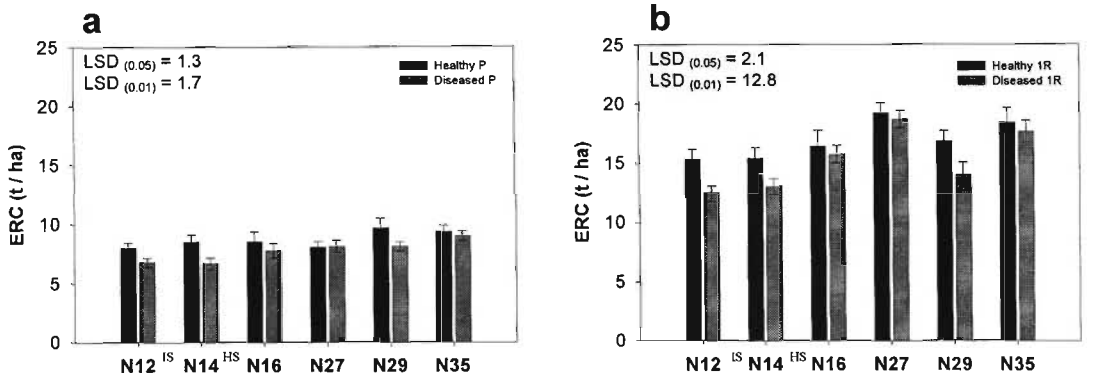


Figure 5.5 The effect of RSD on the ERC yield (t/ha) of six varieties [plant (a) and first ratoon (b)]. Data analysed using two way ANOVA. Bars represent se, n = 12. Standards: N12^{IS} = intermediate-susceptible, N14^{HS} = highly susceptible.

5.3.3.3 *Cane quality*

Gillaspie and Teakle (1989) and Rossler (1974) reported significant improvements in cane quality when RSD-infected sugarcane suffers a shortage of water, but many trials have not shown this. Although slight improvements in cane quality as a function of ERC % cane were evident in some RSD-infected plots in this trial, these improvements were only consistent from the plant to first ratoon crop of N16 (Figure 5.6). None of these improvements were, however, significant. There was a marked decrease in cane quality in the plant crop of N14, but this was reversed in the first ratoon.

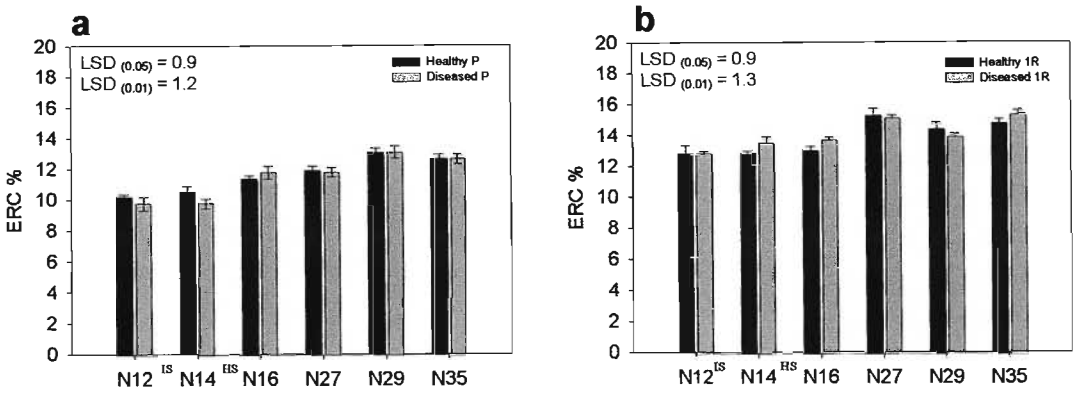


Figure 5.6 The effect of RSD on the ERC % of six varieties [plant (a) and first ratoon (b)]. Data analysed using two way ANOVA. Bars represent se, n = 12. Standards: $N12^{IS}$ = intermediate-susceptible, $N14^{HS}$ = highly susceptible.

5.3.4 Assessment of RSD severity using TBIA

In the plant crop, the mean proportion of cvb in N27 and N35 was significantly lower than the other varieties (P<0,05), while N14 had the highest degree of colonisation (Figures 5.7 and 5.8). There was no significant difference between N29, N12 and N16.

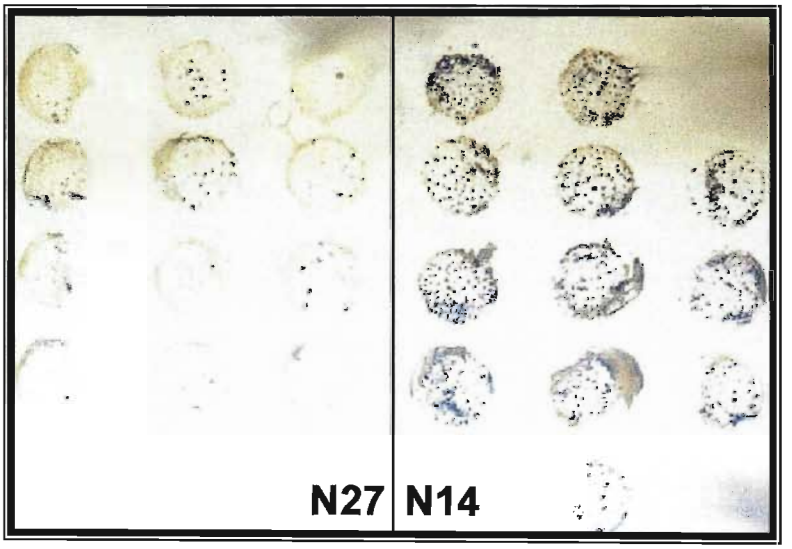


Figure 5.7 TBIA results showing tissue cores of varieties N27 and N14 with vascular bundles colonised by *L. xyli* subsp *xyli* stained blue.

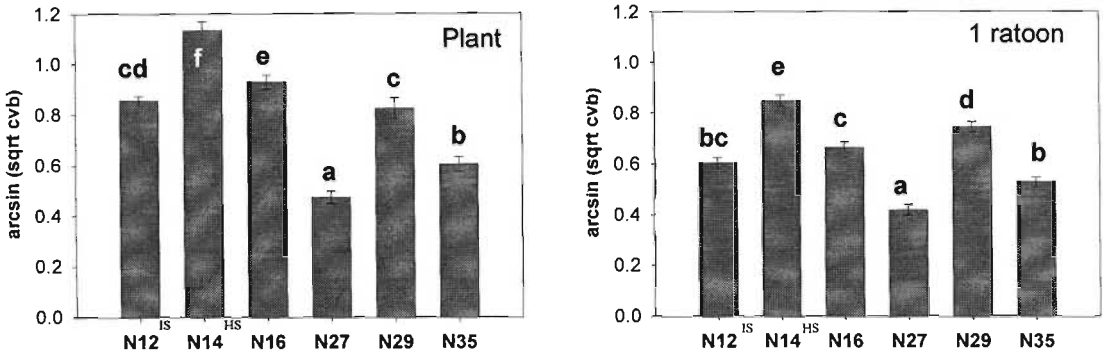


Figure 5.8 Extent of colonisation in six varieties (plant and first ratoon). Data analysed using two way ANOVA. Different letters above columns denote significant differences (P<0.05). Bars represent se, n = 72. Standards: ^{IS} = intermediate-susceptible, ^{HS} = highly susceptible.

In the first ratoon, although the overall colonisation was slightly lower than the plant crop, the ranking of varieties was almost identical. This would indicate that the method has some consistency from one year to the next, even though conditions varied greatly during the two crops. Other researchers have also reported that the absolute values of populations of *L. xyli* subsp *xyli* varied according to environmental conditions but that the relative population densities remained reasonably constant (Davis et al., 1988a; Roach and Jackson, 1992). The extent of colonisation was still lowest in N27, supporting the assumption that this variety has some resistance to RSD. N35 had significantly more cvb than N27, but the extent of colonisation in this variety was low compared to the other varieties included in the trial. N14 again had the highest proportion of cvb, significantly more than the other varieties. The difference in the proportion of cvb between N12 and N16 was not significant. N29 was the one variety that showed proportionally more cvb in the ratoon crop than the plant crop when compared to the other varieties.

5.3.5 Relationship between yield loss and the extent of colonisation

A good correlation was achieved between the mean percent yield loss and the proportion of cvb in the six varieties tested (Figure 5.9). This correlation was significant

at 5 degrees of freedom in the plant crop (P = 0,01, r = 0,91) and in the first ratoon (P=0,05, r = 0,076).

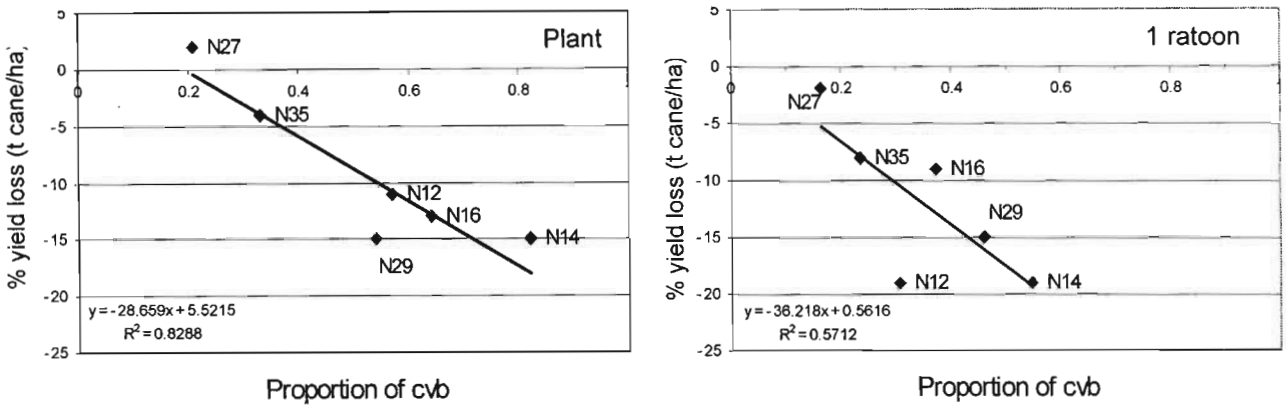


Figure 5.9 Relationship between yield loss (t cane / ha) and the proportion of cvb (plant and first ratoon).

In the plant crop, N27 had the lowest proportion of cvb and did not lose any yield. N35 also exhibited low levels of colonisation and yield loss. The relationship between yield loss and colonisation was also significant in N12, N16 and N14. N29 was the one variety that suffered more yield loss than the proportion of cvb indicated. The ranking of N27, N35 and N14 did not change from the plant to the first ratoon. In the first ratoon, N12 was considered to be an outlier, losing more yield than expected for an intermediate-susceptible variety. However, the TBIA results supported the assumption that N12 is intermediate-susceptible in its reaction to RSD.

Based on yield loss recorded in this trial, N27 could be considered intermediate-resistant to RSD since the variety lost very little yield and did not support high numbers of *L. xyli* subsp *xyli*.. This variety has numerous other valuable characteristics such as high sucrose, smut and mosaic resistance, and general hardiness (Anon, 2002) and is likely to become increasingly popular in the South African sugar industry. With its apparent lower populations of *L. xyli* subsp *xyli,* and tolerance to infection, the variety may have an impact on the levels and effects of RSD in many of the sugarcane growing areas of South Africa. N35 could be considered intermediate and is another variety that looks

promising in this regard. The trial confirmed the previous assumption that N16 is intermediate-susceptible in its reaction to RSD. N29 was found to be susceptible to RSD based on yield loss and extent of colonisation.

5.4 Conclusions

RSD caused substantial yield losses in five of the six varieties included in this trial. N27 appeared to have some resistance to RSD, losing little yield in both crops.

The TBIA produced consistent results in the plant and first ratoon; the ranking of the varieties was virtually identical in both crops, despite the different growing conditions.

The results from the TBIA, measuring the extent of colonisation by *L. xyli* subsp *xyli,* generally supported the yield loss findings and the relationship between yield loss and the proportion of cvb in the six varieties tested was significant in both the plant and first ratoon crops. According to the TBIA and yield loss data, the varieties could be broadly ranked as follows: N27 (intermediate-resistant), N35 (intermediate), N12 and N16 (intermediate-susceptible), N29 (susceptible) and N14 (highly susceptible).

CHAPTER 6

Tissue blots as a means of detecting and enumerating vascular bundles colonised by *Leifsonia xyli* subsp *xyli* in sugarcane transplants

6.1 Introduction

Sugarcane transplants are produced in the South African sugar industry as an alternative means of propagation (Anon, 1988; Anon, 1990). They are produced by cutting stalks into single-budded setts (SBS), approximately 25 mm long. These are hot water treated, at 52°C for 40 minutes to control ratoon stunting disease (RSD) (Goodall *et al.,* 1998), before planting into 72- or 98- celled seedling trays. A primary shoot and a band of roots emerge from each SBS to produce a sugarcane transplant that can be transferred to the field after six to eight weeks in the nursery.

The production of sugarcane transplants is carefully managed and the resulting plants are usually disease free. They are therefore used primarily by growers for the establishment of farm nurseries in the South African sugar industry (Tucker, 1992). One advantage of transplants is that they can be used to rapidly bulk up varieties when seedcane is in short supply, particularly within the first few years after the release of a new variety.

Transplants or seedlings have been used to screen for resistance to leaf scald (Lopes *et al.*, 2001) and rust (Autrey *et al.*, 1996) in other sugar industries. They are useful in that they require less space and can be carefully managed under glasshouse or nursery conditions.

The objectives of this glasshouse trial were to ascertain whether RSD could be detected in the vascular bundles of transplants grown from SBS inoculated with *L. xyli* subsp *xyli*

using TBIA and whether the assay could be used to assess the extent of colonisation in RSD-infected transplants.

6.2 Materials and methods

6.2.1 Establishment and management of trial

6.2.1.1 Choice of varieties

The twelve varieties chosen for this trial were the same as those included in the field trial investigating the spread of RSD in different varieties (Chapter 4). Comparisons can therefore be made between the extent of colonisation observed in the inoculated control plots in the field trial in Chapter 4 and that of the inoculated transplants in this glasshouse trial. N22 (intermediate), N12 (intermediate-susceptible), NCo376 (susceptible) and N14 (highly susceptible) were again used as standards.

6.2.1.2 Layout of trial

Each 98-celled seedling tray used in the trial was divided into four sections. Each section, representing one treatment plot, was planted with 15 SBS of one variety. The treatment plots were randomised and the experiment was replicated four times.

6.2.1.3 Preparation of planting material

Seedcane for the experiment was obtained from a propagation plot established with healthy seedcane that was treated for two hours at 50°C before planting. Seedcane was collected from the propagation plot, nine months after planting. The stalks were cut into SBS using a twin-bladed circular saw.

SBS of each variety were dipped for 10 minutes in *L. xyli* subsp *xyli*-infected cane juice expressed from stalks of the highly susceptible variety, N14. The inoculated SBS were

sealed in plastic 'Ziplock' bags and left overnight, before planting into seedling trays the following day.

Transplants were germinated in the dark at 30°C at a relative humidity of between 80 and 95%. Once germinated, the trays were transferred to the glasshouse. The transplants were maintained at 25 to 28°C and were watered twice daily. The transplants were fertilised weekly with a hydroponic fertiliser (3:1:3 (38), Gromor®). The leaves of the transplants were trimmed every second week to encourage the development of more vigorous primary shoots.

6.2.2 Testing for RSD using TBIA

Transplants were harvested ten weeks after planting. The entire transplant, including the root ball, was removed from the tray and the primary shoot was removed as close to the SBS as possible. The shoot was washed and dried thoroughly and the base of the shoot was trimmed with a scalpel blade. The freshly cut base was pressed firmly onto the surface of nitrocellulose membrane and held for 20 seconds. Once all the transplants from one treatment plot had been blotted, the membrane was baked at 80°C for one hour. The immunoassay was conducted according to the procedure described in Chapter 3.

The membranes were allowed to air dry before examining the impressions of the tissue sections on the membrane using a stereomicroscope (Zeiss, West Germany) at 40x magnification.

6.2.3 Analysis of data

The data were analysed using one way ANOVA on Ranks and the Mann-Whitney Rank Sum Test in SigmaStat 2.0. Bar charts were generated using SigmaPlot 5.0.

6.3 Results and discussion

6.3.1 Germination

The germination of some of the varieties was poor and as a result, only the first ten transplants in each tray were collected for the TBIA.

6.3.2 TBIA for detecting *L. xyli* subsp *xyli* in sugarcane transplants

The TBIA was effective in diagnosing RSD in transplants. The vascular bundles of the sugarcane transplants made impressions on the nitrocellulose membrane (Figure 6.1a). Those colonised by *L. xyli* subsp *xyli* stained blue (Figure 6.1b).

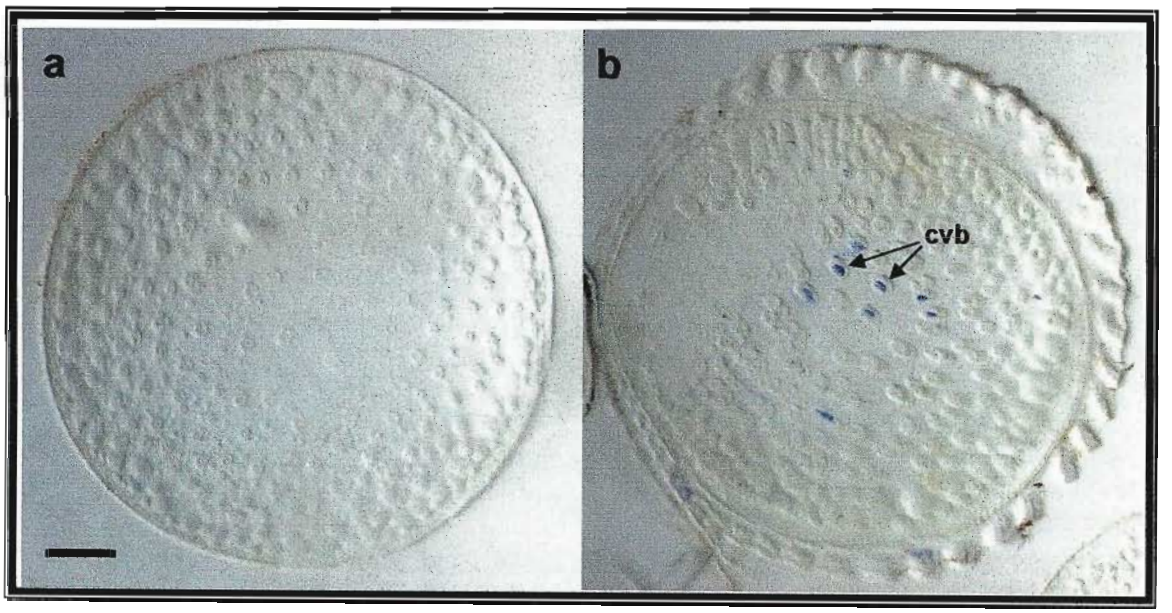


Figure 6.1 TBIA on (a) healthy and (b) RSD-infected sugarcane transplants. Vascular bundles colonised by *L. xyli* subsp *xyli* (cvb), stained blue, bar = 1 mm, 40x magnification.

6.3.3 RSD incidence in the different varieties

RSD incidence was lowest in N27, with 56% of the transplants testing positive for *L. xyli* subsp *xyli* (Figure 6.2). N12, N14, N25 and N30 were the only other varieties to have

less than 80% of the plants infected. In previous trials, N27 also showed slightly more resistance to infection than the other varieties (Chapters 4 and 5). However, N14 is a variety that normally contracts RSD extremely quickly (Roger Bailey, personal communication), so this lower level of infection was unexpected. N28, N31 and N26 had the highest levels of RSD with more than 95% of the transplants becoming infected.

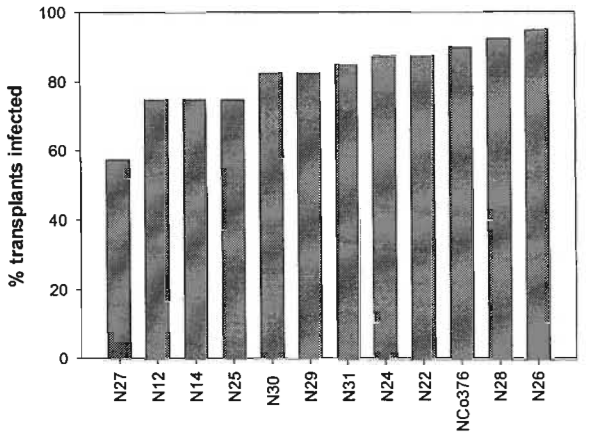


Figure 6.2 Percent transplants infected with RSD after inoculation with *L. xyli* subsp *xyli*, n = 40.

6.3.4 Extent of colonisation in transplants of different varieties

Due to the large number of vascular bundles in the transplants only those colonised by *L. xyli* subsp *xyli* and consequently stained blue, were counted. This therefore gave an indication of the number, rather than the proportion of cvb in each transplant. Since more than 1 000 genotypes are tested using TBIA in the United States each year, only the number of cvb per stalk section is taken into account (Comstock *et al.*, 1995). They have found that the results obtained can be used to satisfactorily distinguish susceptible from resistant varieties.

The results from this glasshouse trial were extremely variable from one replication to another when comparing the number of cvb in the different varieties. The mean cvb of all the replications for each variety are shown in Figure 6.3.

The controls did not react as expected, with N14 falling into the group with the lowest degree of colonisation and N12 being amongst the most severely infected. NCo376 was similar to N14, while N22 had the second highest degree of colonisation. This has been observed previously (Chapters 3 and 4) and again indicates that N22 supports high numbers of *L. xyli* subsp *xyli* despite being relatively tolerant to yield loss when infected.

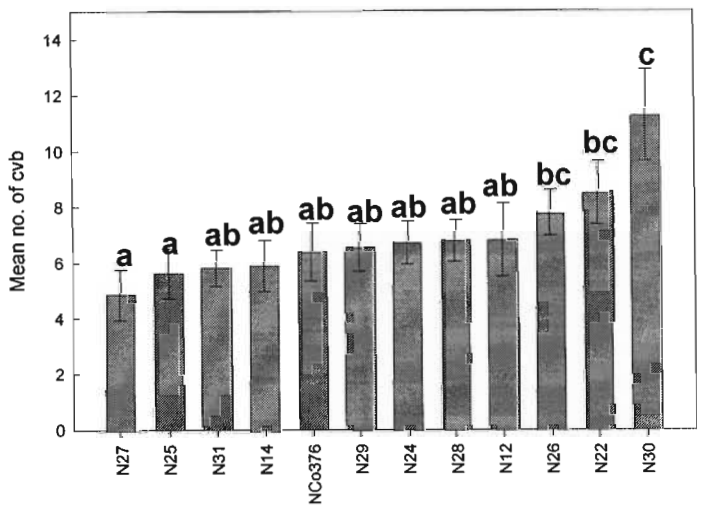


Figure 6.3 Mean number of cvb in sugarcane transplants inoculated with *L. xyli* subsp *xyli*. Data analysed using Rank Sum Test. Different letters above columns denote significant differences (P<0.05). Bars represent se, n = 40.

N27 had the lowest number of cvb of all the varieties, mainly due to the higher number of healthy transplants. If only the infected transplants were considered, colonisation in N27 was relatively high. The highest number of cvb recorded in this variety was 25, one less than that recorded in N14. The highest number of cvb recorded in a transplant was 46 in N30. This variety had the highest degree of colonisation overall.

At ten weeks, the transplants were possibly too young to discriminate between the different varieties based on the degree of colonisation. Although it is likely that other mechanisms are involved in resistance to RSD, one that seems feasible is the degree of branching in the nodal region of cane stalks (Teakle *et al.*, 1975 and 1978). In this trial, the primary shoot was cut as close to the SBS as possible, and in most cases no nodes were present when the assay was done. Once *L. xyli subsp xyli* has effectively colonised

the SBS, the transplant may have no effective defense mechanism until the nodes begin to develop.

6.4 Conclusions

TBIA was successful in diagnosing RSD in sugarcane transplants inoculated with *L. xyli* subsp *xyli.* Using this method, it was possible to quantify the cvb in the different varieties. The results were, however, variable and could not be reliably used to discriminate varieties based on the extent of colonisation.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

TBIA was successfully developed and applied for enumerating vascular bundles colonised by *L. xyli* subsp *xyli*, the cause of RSD in sugarcane. The extent of colonisation could in turn, be used a measure of varietal resistance to RSD, with those varieties showing high levels of colonisation usually falling into the susceptible or highly susceptible groups (Table 7.1). Both yield loss (Refer 5.3.3.1) and the extent of colonisation (Refer 5.3.5) have been shown to vary from year to year. Yield loss trials are particularly prone to variation from trial to trial (Refer Table 2.1) due to climatic conditions, germination and 'healthy' plots becoming infected with RSD. For this reason, rankings cannot be assigned to varieties according to specific ranges of yield loss. It is therefore essential to compare the varieties under test to known standards when ranking varieties for RSD resistance.

Although the method described by Davis *et al.* (1994) was not altered substantially, some modifications to the blotting procedure ensured that the blots obtained, particularly those from varieties supporting high populations of *L. xyli* subsp *xyli*, were clearer and easier to interpret. Storing the stalk samples overnight before processing had the greatest influence on the clarity of the blots (Refer 3.3.4.2). This resulted in a reduction in xylem sap and ultimately a reduction in smearing during the centrifugation process. Critical to the success and repeatability of the assay were stalk maturity and selection (Refer 3.3.5), as well as internode position (Refer 3.3.6). This was particularly important when testing those varieties supporting lower populations of *L. xyli* subsp *xyli*.

When TBIA was used in conjunction with the rate of spread of RSD along rows of different varieties, the results were inconclusive. All varieties were found to be susceptible or highly susceptible. However, the correlation between the mean RSD

Table 7.1 RSD ratings based on the extent of colonisation (TBIA), yield loss and RSD incidence.

Variety	Rating for RSD resistance								Overall rating
	Chapter 2	Chapter 3		Chapter 4		Chapter 5		Chapter 6	
	Yield loss (2.1.2)	TBIA (3.3.5)	TBIA (3.3.6.2)	TBIA (4.3.2.2)	Incidence & severity (4.3.3.2)	TBIA (5.3.5)	Yield loss (5.3.5)	TBIA (6.3.4)	
NCo376	S	S	S	S	S	-	-	IS	IS
N12	IS	-	I	IS	IS	IS	HS	IS	IS
N14	HS	HS	S	HS	HS	HS	HS	IS	HS
N16	-	IS	-	-	-	IS	IS	-	IS
N17	HS	HS	HS	-	-	-	-	-	HS
N22	I	-	IS	HS	HS	-	-	S	S (Tolerant)
N23	IS	-	IS	-	-	-	-	-	IS
N24	S	-	-	S	S	-	-	IS	S
N25	HS	-	-	HS	IS	-	-	I	S-HS
N26	-	-	-	S	IS	-	-	S	S
N27	-	-	-	I	I	IR	IR	I	IR-I
N28	-	-	-	S	I	-	-	IS	IS
N29	-	-	-	HS	S	S	S	IS	S
N30	-	-	-	S	S	-	-	HS	S
N31	-	-	-	S	I	-	-	-	IS
N35	-	-	-	-	-	I	I	-	I

Key:
IR Intermediate-resistant
I Intermediate
IS Intermediate-susceptible
S Susceptible
HS Highly susceptible

incidence and severity in the plant and first ratoon crops was highly significant, indicating that the disease spread more rapidly through those varieties with higher degrees of colonization (Refer 4.3.3.2). Some modifications to the design of future trials investigating RSD spread would be required to provide better discrimination between the varieties. These would include using the inoculated controls of the varieties under test as the spreader plots rather than a highly susceptible variety that supports large populations of *L. xyli* subsp *xyli*, and better spacing of the uninoculated plants in shorter rows (Refer 4.3.3.1). The inoculated control plots did however provide useful information on the extent of colonisation in the twelve varieties included in the trial. Variety N27 exhibited the lowest degree of colonisation and was rated intermediate while the highly susceptible standard, N14, had the highest proportion of cvb. The intermediate standard, N22, was similar to N14. This confirmed results from the preliminary experiments described in Chapter 3, in which N22 exhibited a high degree of colonisation, and suggests that the extent of colonisation may not be indicative of resistance in those varieties with some tolerance to yield loss. Such varieties may support high populations of *L. xyli* subsp *xyli* but suffer lower yield losses than susceptible varieties. This can be seen as one of the limitations of assessing varieties for their reaction to RSD based on the extent of colonisation by *L. xyli* subsp *xyli* alone. However, these varieties will provide an significant source of inoculum and pose a threat to susceptible varieties, and should therefore be avoided. Trials investigating yield loss due to RSD will still be necessary to provide information on the most widely grown varieties. This information is seen as an incentive to growers to continue with an integrated programme to control RSD on their farms. Estimates can be made on the effect of RSD in the South African sugar industry and will also serve to identify those varieties that are tolerant to infection.

The trial comparing yield loss and the extent of colonisation was seen as the ultimate test of TBIA as a screening tool for RSD resistance. Of the six varieties included in the trial, N27 suffered the lowest yield loss (2% in th first ratoon crop) (Refer 5.3.3.1) and exhibited the lowest degree of colonisation with approximately 20% of the vascular bundles being colonised in both the plant and first ratoon crops (Refer 5.3.5). Yield loss

and colonisation was also limited in N35. On the other hand, N14 suffered significant yield losses and the extent of colonisation by *L. xyli* subsp *xyli* was high with more than 50% of the vascular bundles being colonised. N12 was considered an outlier in the first ratoon because yield losses of 19% were recorded and were as severe as N14, although TBIA supported previous reports that the variety is intermediate-susceptible in its reaction to RSD with 30% of the vascular bundles being colonisd. The correlation between yield loss and the extent of colonisation in both the plant and first ratoon crops was significant at the 1 % level (Refer 5.3.5). Based on this finding along with the results from the other trials and experiments, TBIA was accepted as a method for assessing varieties for their reaction to RSD.

The first RSD screening trial was planted at Mount Edgecombe in November 2002. Forty-eight released varieties and promising genotypes were included in the trial and will be assessed in August 2003. It is necessary to assess the costs involved in screening for RSD resistance both for an overall economic assessment as well as in deciding at what stage to introduce RSD screening in the selection program if the methods developed and tested prove to be successful.

SASEX is reluctant to discard genotypes based on RSD susceptibility alone. The varieties that are released in the South African sugar industry must have some resistance to those important diseases that have highly efficient modes of dispersal such as smut, mosaic and rust. Although not always completely effective as practiced by growers, there are a number of options other than variety resistance for RSD control. It is therefore likely that RSD screening trials will only become part of the late stage of the routine disease-screening programme at SASEX. Such trials will not result in genotypes being rejected solely on their RSD rating, unless extremely intolerant, but the ratings will be considered along with productivity, performance and other disease data.

Not only will the TBIA be beneficial in providing valuable information to growers on the likely susceptibility of varieties to RSD as they are released, the method can also be used as a useful diagnostic tool. It is now used to diagnose RSD in sugarcane

transplants (6.3.2) and may provide a cheaper alternative to immunofluorescence microscopy when diagnosing RSD in mature cane.

Although many growers appear to follow all the recommendations for RSD control, the disease persists in their fields. This may be due to the fact that heat treatment is not always totally effective in eliminating RSD from infected seedcane or that an intended seed source has a low number of infected stalks that are not selected when sampling for RSD diagnosis. Perhaps the most common problem is the incomplete eradication of the previous crop that may have been infected with RSD. The disease spreads from this infected regrowth to the newly planted, healthy crop during the first harvest and continues to spread with subsequent harvests. There are therefore a number of areas where rating genotypes for RSD resistance would be beneficial in the control of the disease.

The reactions of new genotypes to RSD are currently only known several years after their release to growers, since this information is obtained from lengthy yield loss evaluation trials. It would be useful to advise growers on the status of each new variety before it is released for commercial propagation. This would enable growers to take special precautions when managing productive but RSD-susceptible genotypes. These precautions would include effective knife cleaning when harvesting these varieties and more frequent hot water treatment (HWT) of seedcane. Comstock *et al.* (1996) suggested that the spread of RSD could be controlled using genetic resistance and that the effort and cost of a HWT programme could be reserved for more susceptible genotypes. In the South African sugar industry, HWT of all varieties would still be encouraged, regardless of their RSD rating, mainly because a minority of new varieties bred from the current gene pool are evidently usefully resistant. However, in small-scale grower areas where HWT facilities are often lacking, varieties that show some resistance to infection would be useful in limiting the spread of the disease in seedcane. The benefits for both large and small-scale growers are likely to lie more in improved yields and counter-acting the spread of RSD on cane knives and mechanical harvesters. Although the trial reported on in Chapter 4 in this study did not provide

conclusive results on the effect of *L. xyli* subsp *xyli* populations on the rate of spread of RSD, Comstock (1996) reported that this depends largely on the susceptibility of the variety to the disease. It was stated that near-zero cvb was required to prevent or limit in-field spread of RSD, particularly if the recommended methods of RSD control such as HWT and field sanitation were not strictly adhered to. With the increasing use of mechanical harvesters in the South African sugar industry, and the number of contractors harvesting cane grown by small-scale growers, such varieties would be invaluable in reducing the extent of field to field and farm to farm spread of RSD.

N27 was found to have some resistance to RSD based on the fact that the variety suffered little yield loss and showed low levels of colonisation by *L. xyli* subsp *xyli.* Although heritability was not investigated in this study, the fact that N52/219, a variety with some resistance to RSD, is a parent of N27 supports the finding that RSD resistance is a heritable trait (Miller *et al.,* 1995 and 1996). The information provided from RSD screening trials could therefore be used to select or discard parents based on their reaction to RSD, with a long term view of improving the general resistance of commercial varieties.

With the success of the RSD screening programme and the increased use of RSD resistant parents, a substantial improvement in the RSD status of fields planted with new, resistant varieties is expected in the Florida region, both in percent stalks infected and average cvb per sample (Comstock *et al.*, 1995). With the likely inclusion of RSD screening trials in the routine disease screening programme at SASEX, a similar outcome is possible in the South African sugar industry, as well as those industries throughout Africa where South African varieties are grown.

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