DISTRIBUTION, ASSESSMENT AND LOSS CAUSED BY RATOON STUNTING DISEASE (*Leifsonia xyli* subsp. *xyli*) OF SUGAR CANE IN THE FIJI SUGAR INDUSTRY

A thesis presented in partial fulfillment of the requirements for the degree of Master of Sciences in Biology at the University of the South Pacific.

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DECLARATION

I declare that this thesis is my own work, except for those sections explicitly acknowledged, and that the main content of the thesis has not been previously submitted for a degree at any other university.

SAIMONE SABAKERA JOHNSON

CERTIFICATION

This is to certify that this thesis entitled "Distribution, Assessment and Loss caused by Ratoon stunting disease (*Leifsonia xyli* subsp *xyli*) of sugar cane in the Fiji Sugar Industry" submitted for the Master of Science in the subject of Biology to the University of the South Pacific, is a bona fide research work carried out by Mr. Saimone S. Johnson (S88048260) under our supervision and guidance. No part of the thesis has been submitted for any other degree.

The assistance and help received during the course of investigation has been duly acknowledged.

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ABSTRACT

New sugarcane varieties are developed and provided to the Fiji Sugar cane farmers by the Fiji Sugar Corporation through its Sugar Cane Research Centre at Lautoka. Currently, the average cane yield in Fiji is 45 – 70 tonnes/ ha. Due to the low cane yield in Fiji, research has been directed at increasing the cane yields. One of the causes of low cane yield is the ratoon stunting disease. The objective of this study was to determine the distribution, assessment and loss by ratoon stunting disease (***Leifsonia xyli*** subsp. ***xyli***) of sugar cane in the Fiji sugar industry.

A nationwide survey was conducted and positive samples were identified using two diagnostic technique, phase contrast microscopy and Evaporative-Binding immunoassay. A difference of 23% was observed with the results with EB-EIA being more sensitive. Positive samples were cultured in the laboratory. Cultured stocks were used in inoculating the cane plant for yield loss trial and resistance screening trial. The epidemiology of the ratoon stunting disease in the Fiji sugar industry showed that the disease is widespread in all the sugarcane sectors with varying amounts of infection averaging

29% and 6.6% based on evaporative-binding immunoassay and phase contrast microscopy respectively.

The difference in cane yield was seen with all the treatments in the cane yield loss experiment but the difference was not statistically significant. It was observed that the only notable effect of the disease was on the cane yield but very little effect on the sugar and percent of pure obtainable cane sugar (% pocs). The cane yield for the plant (first crop) and subsequent ratoon crops (third year, fourth year, etc.) differed with an average reduction of 37% but the average loss caused by RSD was 29%. The best duration of hot water treatment was identified as 2 hours at 50°C because it gave a higher average yield compared to the control (untreated) and 3 hours at 50°C.

In the screening of the fifteen commercial varieties; Vatu, Homer and Galoa varieties were susceptible to ratoon stunting disease based on reading of greater than 0.1nm on the ELISA reader while Kaba, Vomo, Yasawa, Naidiri, Ragnar, Ono, Waya, Mana, Spartan and LF82-2252 varieties were resistant to ratoon stunting disease with a reading of less than 0.05nm on ELISA reader. Only two varieties, Beqa and Aiwa were moderately susceptible to ratoon stunting disease. To control the disease

to manageable levels, an integrated approach including resistant varieties with hot water treated nurseries with best farming practices are recommended.

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CHAPTER 1

INTRODUCTION

Sugarcane belongs to the genus *Saccharum*, tribe Andropogoneae and family Poaceae and is characteristically tropical and subtropical being very abundant in the Old and New World (Daniels and Roach, 1987). Sugarcane is propagated vegetatively by cuttings (stem setts called seed cane); each sett containing one or more to raise the first crop called Plant. Subsequent crop(s) due to regeneration (tillering) are called Ratoon. The sugarcane plant needs a range of distinct climatic conditions to effectively produce the maximum cane sugar yield in its stalks. It is well understood that the sugarcane plant needs moist months with warm temperatures for germination and growth; long summer with interspersed rain days for continued growth and sugar production and dry, sunny and cool days to maximize sugar storage and access to good harvesting.

Sugarcane was grown in Fiji for chewing purposes when the early European discoverers and settlers arrived. Sugarcane's commercial growing and sugar manufacturing in Fiji was initiated in 1862 when Mr. David Whippy first produced sugar on the island of Wakaya. By 1883, it became Fiji's main export commodity

(Anon, 1993). The Colonial Sugar Refining Company (CSR), an Australian company stamped its mark in Fiji because of the resource and experience they brought with them and ran five sugar mills (Nausori, Rarawai, Labasa, Lautoka and Penang). These mills were bought by the Fiji Government in 1973 and renamed the Fiji Sugar Corporation Limited (FSC). Out of the five mills, only four are operational till now as Nausori mill was closed in 1959.

The sugar industry is comprised of 38 sugarcane sectors with an average of 600 to 700 farms per sector. Within each sector approximately fifteen to twenty gangs in about ten to fifteen locations are involved in the harvesting of the crop and supply sugarcane to the four existing sugar mills. Sugarcane is grown commercially on the drier sides of the islands with 2200mm rainfall annually (Anon, 2001). The Colonial Sugar Refinery Company (CSR) established the Agricultural Experiment Station in 1904 at Rarawai. It was moved to Lautoka in 1958 where it has been ever since under FSC control (Anon, 1993).

The history of ratoon stunting disease in Fiji is unknown but it is believed that it was introduced with sugarcane varieties when the sugar industry was set up in Fiji in the 1880s or it may

have been introduced through our imported germplasm. Germplasm collection prior to the 1940s were not restricted compared to nowadays because of the non establishment of plant quarantine at that time, which might have contributed to some of the sugarcane diseases that are now affecting sugarcane production in Fiji (Tamanikaiyaroi and Johnson, 1995). Ratoon stunting disease (RSD) was first recorded in Queensland, Australia during 1944 – 1945 on a new promising variety, Q28 when the variety produced extremely poor ratoon crops (Steindl, 1961). Ratoon stunting disease is a systemic disease, caused by a corynebacterium known as ***Leifsonia xyli*** subspecies ***xyli***, formerly known as ***Clavibacter xyli*** subspecies ***xyli*** (Davis *et al.*, 1984). Ratoon stunting disease was first identified in Fiji in 1953 at Nausori, Rarawai and Lautoka (Anon., 1953). The preferred control method in Fiji was using heat treatment of sugarcane sets before sowing (planting). Hot water treatment kills the bacteria present in sugarcane sets. Hot water treatment tanks were erected at Rarawai and Lautoka mills for establishing disease-free seedbeds of the major varieties with the intention of supplying sugarcane growers with disease-free material (Anon., 1957).

Research on ratoon stunting disease in Fiji was limited until the South African Sugar Experiment Station confirmed it in 1994

after sap samples from suspected RSD stalks were sent for analysis. Sap samples were identified positive (Tamanikaiyaroi and Johnson, 1995) for the bacterium ***Leifsonia xyli*** subspecies ***xyli***, using phase contrast microscopy and immunofluoroscence microscopy. The seriousness of ratoon stunting disease has never been studied in Fiji's situation before.

A plant is referred to as healthy or normal, when it is capable to carry out its physiological functions to the best of its genetic potential in favorable environment. Whenever the plants are attacked by pathogens or disturbed by environmental conditions, physiological functions are altered as compared to normal, and then the plants become stressed (Agrios, 1988). Ratoon stunting disease alters the physiological functions of sugarcane plant which reduces the yield. Sugar industries worldwide have been on the verge of collapse in the face of disease epidemics but in most cases recovered after substituting susceptible varieties with immune, resistant, or tolerant varieties (Walker, 1987).

Disease in sugarcane, as in any other plant, is the malfunction of host cells and tissues that results from their continuous irritation by a pathogenic agent or environmental factor. This leads to the development of symptoms, which may result in the

partial impairment, or death of the whole plant or its parts (Agrios, 1988).

Ratoon stunting disease (RSD) is one of the most devastating diseases of sugarcane in the world causing losses up to 30% per year (Hughes, 1974). It costs $10 million loss in the Australian sugar industry annually and if no control measures are practiced, losses could reach as high as $200 million (Croft *et al*, 1993). To-date, a survey and loss assessment has not been done to determine the effects of this disease in Fiji.

Historically the diagnosis of RSD by physical - visual symptoms was quite difficult because the internal and external symptoms were not the same in all sugarcane varieties. Macroscopic symptoms of the disease may be affected by varietal characteristics, climatic conditions, physiological status of the plant, and the presence of other diseases (Ricaud, 1974). With the improved diagnosis of ratoon stunting disease, accurate screening of ***Leifsonia xyli*** subspecies ***xyli*** is feasible (Matsuoka, 1980).

Ratoon stunting disease has always been managed by heat therapy of planting material, combined with well-organized

multiplication nurseries (Walker, 1987). Historically, varietal susceptibility to RSD has been one of the major incentives for setting up plant breeding programme. Numerous studies have been conducted to determine the feasibility of screening sugarcane varieties resistant to RSD.

The main aim of this study is to determine the distribution and loss by ratoon stunting disease caused by ***Leifsonia xyli*** subsp ***xyli*** in the Fiji sugar industry. This overall main objective is divided up into four specific objectives that are outlined below:

Specific Objective 1:

To conduct a survey in all sugarcane sectors in the Fiji sugar industry for the incidence of ratoon stunting disease (RSD).

Specific Objective 2:

To establish the presence of ***Leifsonia xyli*** subsp ***xyli*** in sugarcane in Fiji.

Objective 3:

To determine the loss caused by ratoon stunting disease.

Objective 4:

To develop a screening method for identifying resistant varieties to ratoon stunting disease for commercial growing in Fiji.

CHAPTER 2

LITERATURE REVIEW

2.1 Geographic Distribution

In 1961, Ratoon stunting disease (RSD) was limited to 20 countries (Steindl, 1961). In 1989, 48 countries recorded RSD (Ricaud *et al*., 1989) and by the year 2000, RSD had been reported from most of the 70 sugarcane-growing counties as shown in Table 1 (Rott *et al*., 2000).

2.2 History of Occurrence

The disease prior to 1949 was originally known as 'Q28 trouble' or 'Q28 disease' because of its association with the variety Q28 in Australia, but after 1949 it was called ratoon stunting disease because it mainly affects ratoon crops (Steindl, 1961). Steindl (1949) conducted experiments to determine the mode of disease transmission. It was found that RSD was transmissible and could be spread by artificial inoculation or on the blade of the cutter, planter or cane knife.

Table 1: Geographic Distribution of Ratoon Stunting Disease

Australia & Oceanic	**Asia**	**Africa**	**America / Caribbean's**
Australia	Bangladesh	Burkina Faso	Argentina
Fiji	China	Cameroon	Barbados
Hawaii	India	Congo	Belize
PNG	Indonesia	Cote d'Ivoire	Bolivia
	Japan	Egypt	Brazil
	Malaysia	Ethiopia	Colombia
	Myanmar	Kenya	Cuba
	Pakistan	Malagasy R	Dominican R
	Philippines	Malawi	El Salvador
	Sri Lanka	Mali	Guadeloupe
	Taiwan	Mauritius	Guyana
	Thailand	Mozambique	Martinique
		Nigeria	Reunion
		South Africa	St. Kitts & Nevis
		Sudan	Trinidad
		Swaziland	Jamaica
		Tanzania	Mexico
		Togo	Nicaragua
		Uganda	Panama
		Zambia	Peru
		Zimbabwe	Puerto Rico
			Uruguay
			USA
			Venezuela

(Source: C. Ricaud *et al.*, 1989 & P. Rott *et al.*, 2000)

The disease was first thought to be caused by a virus because of the difficulties in isolating the causal agent (Antoine, 1959, Steindl, 1961 and Bourne, 1965). Later it was realized that the causal agent of RSD was not a virus but a bacterium (Teakle *et al*., 1979). Using phase contrast and electron microscopy, they found that the size of the causal organism ranged from 0.15 – 0.3µm in width and 0.6 – 4.0µm in length (Teakle *et al*., 1979, Teakle, 1983 and Davis *et al*., 1984)

Alternate hosts of RSD are not common but some grasses were found to contain the bacterium after inoculation but without any external symptoms (Steindl, 1957). In Cuba and USA, sorghum and Sudan grass hybrid showed stunting (Wehlburg, 1956) and wilting (Benda, 1975) respectively. Gillaspie and Teakle (1989) recorded numerous grasses that were determined to be hosts after experimental inoculation, including *Zea mays, Sorghum* species, *Brachiara mutica, Brachiara miliiformis, Chloris gayana*, *Cynodon dactylon, Echinocola colonum, Imperata cylindrica, Panicum maximum*, *Pennisetum purpureum* and *Rhynchlelytrum repens*.

2.3 The Pathogen

2.3.1 The Pathogen name

Leifsonia xyli subspecies ***xyli*** (Davis *et al*., 1984)

2.3.2 Pathogen classification

Kingdom: MONORA; Organisms with genetic material (DNA) not organized into a nucleus (Prokaryote).

BACTERIA; have cell membrane and cell wall.

- Irregular, Gram – positive, non - sporing rods.

Genus: ***Leifsonia*** [formerly known as ***Clavibacter***]

Species: ***xyli***

Subspecies: ***xyli*** (Davis *et al*., 1984)

The change of name from ***Clavibacter*** to ***Leifsonia*** was described by Evtushenko *et al.* (2000) as a new actinomycete genus. ***Leifsonia poae*** was proposed as a new genus and species for a bacterium isolated from a nematode (***Subanguina radicicola***) on the grass ***Poa annua***. The authors

then found that their new bacterium was very closely related to some existing nematode - associated bacteria in ***Clavibacter***. Thus it was proposed to transfer ***Clavibacter xyli*** subsp ***xyli*** and subsp ***cynodontis*** to the new genus (***Leifsonia***) with the same subsp combinations (Evtushenko *et al*., 2000).

The pathogen is a small, xylem-inhabiting, coryneform bacterium that may be detected in xylem sap extracts (Davis and Bailey, 2000).

2.3.3 Pathogen morphology

a) Rod shaped bacterium normally measures about 0.25 – 0.5 µm by 1.0 – 4.0 µm but can be as long as 10 micrometer (Davis *et al*., 1984).
b) Bacteria are usually straight or slightly curved rods, but some cells are swollen at the tip or in the middle (Gillaspie and Teakle, 1989)
c) Mesosomes are often present and sometimes appear to be associated with septum formation (Teakle, 1974).
d) Unicellular, sometimes septate and occasionally in the form of branched filaments or micro – colonies consisting

of highly aggregated bacterial cells (Gillaspie and Teakle, 1989)

2.3.4 Pathogen Physiology

Leifsonia xyli subsp. ***xyli*** is aerobic, non-motile, gram positive, non-spore forming, non-acid fast, catalase positive and oxidase – negative bacterium (Davis *et al.*, 1980). The cardinal temperatures for this bacterium in cultures are approximately 15°C, 28°C and 31° C. The bacterium is sensitive to many antibiotics at 50µg/ml such as kanamycin, streptomycin, erythromycin chloramphenicol, tetracycline and penicillin, but not actidone (Teakle, 1983).

2.4 Pathogen Diagnosis

The diagnosis of RSD is relatively difficult because the internal and external symptoms are not the same in all varieties. The absence of definite symptoms of the disease made it difficult to diagnose. An orange-red discoloration of the vascular bundles at the node (Steindl, 1950) was observed during investigations. Although this symptom is also present in

healthy cane however this symptom was used for a survey in 1952 (Hughes and Steindl, 1956) that showed that the disease was widespread in all cane growing districts in Australia.

Ricaud (1974) reviewed the problems in the diagnosis of RSD and found that varietal characteristics, climatic conditions, and physiological status of the plant and the presence of other diseases may affect the macroscopic symptoms of the disease. Currently there are four major diagnostic techniques in use, which include infectivity assays, microscopy, immunological techniques and Nucleic acid-based methods.

2.4.1 Symptoms of the disease

External symptoms

a) Slow germinating and slow growth than that of a healthy crop (Steindl, 1950).
b) Contains fewer stalks that are short and thin with small tops (Gillaspie and Teakle, 1989).
c) An “up” and “down” (uneven) appearance in the sugarcane field (Hughes and Steindl, 1956).

d) Stunting and unthrifty growth associated with RSD are conspicuous only during a growing period with insufficient moisture (Gillaspie and Teakle, 1989).

Internal Symptoms

a) Nodes of very young cane have a general pink colour near the meristem (Hughes and Steindl, 1956).
b) Nodes of mature cane have a discoloration within individual vascular bundles that vary in colour from variety to variety (Gillaspie and Teakle, 1989).
c) A sliced disease stalk will show small reddish dots, commas and various straight or bent forms up to 3mm in length (Gillaspie and Teakle, 1989).
d) Discolored vascular bundles show that the xylem vessels are plugged with gummy substance (Hughes and Steindl, 1956).

2.4.2 Infectivity assays

There are three types of infectivity assays for ratoon stunting disease diagnosis: (i) Using juvenile symptoms in cane where single node cuttings are dipped in juice sample, planted and the stalk examined at the fourth and fifth node

for discoloration in the nodal area (Teakle, 1983). (ii) Using uprights of cane where after dipping node cuttings in juice sample are planted with one node under ground and the other node above the ground. The shoots from the cuttings are observed after 6 weeks for nodal pink discoloration (Teakle, 1983). (iii) Using mature symptoms after dipping in juice sample, they are planted and then observed after 2 – 6 months for nodal discoloration (Schexnayder, 1960).

2.4.3 Microscopy techniques

Microscopy has been used widely for the diagnosis of ratoon stunting disease. The most commonly used is phase contrast microscopy at a magnification of 1000 times. Bailey and Fox (1984) have used phase contrast microscopy for diagnosis since 1977. Amiet (1985) conducted field surveys using phase contrast microscopy in Australia. Fluorescence microscopy was also used in diagnosis as compared with phase contrast microscopy by Roach and Jackson (1990). They found that fluorescence microscopy might be useful at certain times of the year in providing an easy and rapid diagnosis of RSD. Roach (1990) outlined the method of preparing slides for phase contrast microscopy and found

that in general, it was preferable to sample more stalks from a plot to get the best estimate and by centrifuging sap from a number of stalks in one tube before observing under the microscope. Pathogen densities vary among varieties and are the greatest in the basal portion of mature stalks during these later parts of the growing season (Davis and Bailey, 2000).

2.4.4 Culture

Another possible diagnostic technique is based on the isolation of the causal organism on a culture medium (Gillaspie and Teakle, 1989). The fastidious nature of the pathogen requires nutritional additives in axenic culture as in the developed media, the MS (modified for sugarcane) agar medium (Davis *et al*, 1980). For isolation in culture, fresh sap extracts are placed on MS agar by the streak-plate or dilution-plate techniques (Davis and Dean, 1984).

2.4.5 Serological techniques

A serological assay known as the evaporative binding enzyme-linked immunoassay (EB-EIA) was developed by Croft

et al. (1994). This procedure generally involved the use of extracted cane sap from the cane stalk and dried on micro-plates with buffer and specific antiserum added. This in turn is read on the micro-plate reader taking the absorbance at 405 nm.

A more specific, sensitive and rapid method of diagnosis is known as the polymerase chain reaction (PCR) protocol. The technique generally requires some knowledge of the target DNA sequence, and relies on the binding of short oligonucleotides (primers) to known sequences on either side of the target region of pathogen DNA (Bridge, 2002). The utility of this technique in plant pathology depends on the application for which it is used. The two most common applications are for amplifying regions of DNA specific to individual pathogens for their subsequent identification (Seal *et al*., 1992), or for providing considerable quantities of selected DNA regions for subsequent analysis (White *et al*., 1990).

2.5 Epidemiology

2.5.1 Host

The anatomy of the sugarcane vascular bundles is such that in susceptible varieties, the number of xylem vessels passing through the node is larger than in resistant varieties (Teakle *et al.*, 1975). The pathogen densities in sap from different cultivars have been shown to be correlated directly with cultivar susceptibility; this indicates that cultivar resistance to RSD is also associated with reduced pathogen colonization of vascular tissue (Teakle, 1983). Pathogen densities vary among varieties, and are the greatest in the basal portion of mature stalks during the later part of the growing season (Davis and Bailey, 2000).

Some of the known susceptible, tolerant and resistant varieties of commercial cane in some of the countries are listed below

Table 2: Some of the Varieties with known Resistance to RSD

Country	***Susceptible varieties***	***Intermediate varieties***	***Resistant varieties***
Australia	Q113	Q120	Q135
	Q124	Q127	Q136
	Q142	Q132	Q50
	Q145	Q141	Q61
	Q158	Q147	Q95
	Q161	Q149	Q151
	Q162	Q157	Q152
	Q28		Q160
	Q94		Q164
	Q115		Q121
Australia	Q145		Q117
	Q153		
	Q96		
	Q138		
	Q110		
	Q146		
	Q144		
USA		H56-752	H60-6909

United States of America	CP51-21		CP70-1133
	CP53-1		CP29-116
	CP44-101		CP52-68
	CP70-321		CP63-588
	CP70-330		CP60-25
	CP72-1210		
South Africa	Nco376		
	N13		

2.5.2 Environmental Factors

The environment plays a major role in the development of diseases and frequently determines whether the RSD would occur. The environmental factors that most seriously affect the initiation and development of infectious plant disease are temperature, moisture, light, soil nutrients and soil pH (Agrios, 1988). Therefore, the favorable environmental conditions coupled with susceptible plant and infective pathogens are the three main factors that must be present for a disease to occur. Moisture is one of the environmental factors that contribute to the severity of ratoon stunting disease. Moisture either as drought or

water logging can increase yield loss due to the disease manifestation (Davis and Bailey, 2000). The stunting and "unthriftyness" associated with ratoon stunting disease are usually greater when there has been insufficient moisture during the growing period (Gillaspie and Teakle, 1989). In areas of the world where sugarcane is periodically subjected to water stress, yield loss caused by ratoon stunting disease may occur in susceptible varieties otherwise tolerant to the disease (Rossler, 1974).

Soil type as an environmental factor has been studied in the United States to estimate losses caused by ratoon stunting disease. It was found that losses caused by ratoon stunting disease in sandy and muck soils were significant (Dean *et al.*, 1989). However, losses were greater on sandy soils compared to weak clay soils (Dean and Davis, 1990).

Temperature has an effect only in cultured media. Davis *et al.*, (1980) found that after 2 weeks of aerobic incubation at 28° C, colonies appeared with a diameter of 0.1 – 0.3mm.

2.5.3 Effect of human cultural practices

The activities of humans have a direct or indirect effect on plant disease epidemics, some of them favoring and some reducing the frequency and the rate of epidemics. Continuous monoculture of sugarcane with large acreage combined with other factors such as fertilization increases the possibility and severity of epidemics (Agrios, 1988).

The ratoon stunting disease appears to spread primarily through the agency of human. In the case of ratoon stunting disease, mechanization of agricultural practices increased the transmission of diseases, especially as a result of indiscriminate contamination of farm equipment such as cutter-planters, or harvesters and cane cutting knives (Hughes and Steindl, 1955). They observed that transmission was evident when healthy and diseased canes were planted in yield trials. In one instance, the cutter-planter carried the infection of 60 consecutive plants. These were followed by additional diseased stalk plant interspersed with healthy plants as the cutting blade gradually became free of inoculum.

2.5.4. Yield loss due to ratoon stunting disease

Ratoon stunting disease is one of the most devastating diseases of sugarcane in the world causing losses up to 30% per year (Hughes, 1974). In evaluating Canal Point (CP) varieties, Dean and Davis (1990) found that losses in sugar per hectare caused by RSD averaged 5%. The loss to the Florida sugar industry was estimated at $27.2 million for the 74% of the area occupied in 1988 – 1989. Grisham (1991) found that losses increased with the number of years. A planting was cropped with losses averaging 14% in the first year crop and increased to 27% in the third year (second ratoon crop).

It was found that there was a difference in yield between plant cane and ratoon cane and also a difference between healthy and diseased cane (Steib, 1971). Plant crop yields were less affected by ratoon stunting disease than were the ratoons and there were indications that further reductions in cane yields could be expected in subsequent crops (Rossler, 1974). This is possibly due to the increase of RSD incidence in successive ratoon crops (Davis and Bailey, 2000). The inhibition of germination and growth, mortality

of shoots and suppression of growth caused by ratoon stunting disease is not always immediately apparent (Zummo, 1974). Dean *et al*. (1988) conducted four RSD yield loss trials on two different soil types and found significant loss in tons of sugar per acre. The disease has little effect on cane quality and stalk population, and losses in yield were almost entirely due to decreases in stalk mass (Bailey and Bechet, 1986).

Field experiments have shown that ratoon stunting disease can cause reductions in yield of 15 – 30% under good irrigated conditions and 20 – 40% under rainfall conditions in varieties that are widely grown in Africa (Bailey and McFarlane, 1999).

Correlations of bacterial population to yield loss were studied with more precise and sensitive techniques to enumerate bacterial cells of ***Leifsonia xyli*** subsp. ***xyli.*** Bacterial populations were found to develop at a faster rate and to a greater extent in susceptible cultivars and were correlated with yield reduction. The correlation coefficient calculated was r = 0.90 (Davis, 1985). Victoria *et al* (1995) found correlation between bacterial

concentration and parameters of response such as development index (DI). The development index was based on total volume of cane. Development indexes calculated using the product of volume and number of stalks / meter; tonnes cane / hectare and; tonnes sugar / hectare were highly significant in the range of 0.78 – 0.92.

2.6 RSD Management

The management of any disease is dependent on the knowledge of the symptoms, causes and mechanisms. The study of these allows the development of methods to combat plant diseases and, thus increases the quantity and improves the quality of plant products (Agrios, 1988).

The most commonly used control method for RSD is the physical method that includes hot water treatment of propagative parts (stem sets). The hot water treatment of sugarcane has been used commercially in almost all sugarcane-growing areas since the early 1950s. Although hot water treatment worked for some varieties in the reduction of RSD incidence, the use of heat therapy may cause the reduction in germinability. The use of

resistant clones was of secondary importance in ratoon stunting disease (Gillaspie and Teakle, 1989) during this time.

Varieties of sugarcane have been identified that are resistant to ratoon stunting disease. Wismer (1971) found that a Hawaiian variety, H60 – 6909 was highly resistant to RSD. The value of resistance in controlling plant diseases was recognized in the early 1900s (Agrios, 1988). The advances in the science of genetics and the advantages of growing a resistant variety, made the breeding of resistant varieties possible and desirable (Agrios, 1988). It has been suggested that the requirements for effective genetic controls of RSD are availability of resistant parent sources; availability of rapid and reliable screening procedures for ratoon stunting disease detection; and information on the inheritance of RSD resistance (Roach, 1992).

Sugarcane is allopolyploid in nature, therefore oligogenes are unlikely to be expressed and the pathosystem depends on many genes and is essentially quantitative in expression (Robinson, 1976). According to Walker (1987), a breeding program based on recurrent combination and in which reasonable pressure is exercised for resistance to diseases in the choice of the parents will build a stable, horizontal resistance system.

Breeding for resistance to the RSD pathogen has been suggested as a means of controlling the disease (Gillaspie and Teakle, 1989).

CHAPTER 3

MATERIALS AND METHODS

3.1: RSD Survey and Field Incidence

Objective: 1

To conduct a survey in all sugarcane sectors in the Fiji sugar industry for the incidence of ratoon stunting disease (RSD).

Materials:

i. Tools (includes knife, plant clippers, air pressure/vacuum pump and ice box).
ii. Ethanol (90%).
iii. 5ml sample tubes with lids (vials).
iv. Plant material (All sugarcane fields were randomly selected irrespective of variety).

Methods:

Sugarcane stalk samples were taken from the randomly selected farms in a random survey of 15 farms per sector. From each of these farms, sugarcane stalks of nine months and older were sampled from the headlands (Figure 3.1). The samples were collected from the farms selected irrespective of the sugarcane

varieties grown (Survey form in Appendix 1). Twenty stalks of cane were sampled per farm by selecting the healthiest looking cane stalk from a poor looking cane stool at the headlands or the corners of the field.

Figure 3.1: Sampling of cane from the Headlands

The stalks were cut into one node pieces with one end cut straight and the other at 45 degrees so that the straight cut end is fitted properly onto the pump tube and the slanted end of the cane is aimed at the sample tube. The cane sap was extracted by using a pressure pump to exude the sap into a sample bottle. The sample bottle was then placed in an ice box. Once all the samples per sector were collected, the ice box

containing the samples were taken back to the lab and analyzed for the presence or absence of the bacterium using two diagnostic technique, Phase Contrast Microscopy (PCM) and Evaporative Binding-Enzyme-linked Immuno Assay (EB-EIA).

3.2 RSD Establishment through Culture

Objective: 2

To establish the presence of ***Leifsonia xyli*** subsp ***xyli*** (Davis *et al*., 1984) in sugarcane in Fiji.

Materials:

i. Tools (includes knife, plant clippers, air pressure/vacuum pump, sample tubes (vials), ice box, centrifuge machine, micro plate reader, beaker, magnetic stirrer and pipettes).

ii. Chemicals [70% ethanol, 90% ethanol and components of the M-SC media (corn meal agar, soytone, Bovine hemin chloride magnesium sulphate –hydrous, di-potassium hydrogen phosphate, potassium di-hydrogen phosphate deionised water, glucose cysteine-free base and bovine serum albumin-fraction V).

iii. Plant material (All sugarcane farms randomly selected irrespective of variety).

Methods

Two diagnostic techniques were used to establish the presence of ***Leifsonia xyli*** subsp ***xyli*** (Davis *et al*., 1984). These diagnoses were done by Phase contrast microscopy (PCM) and an enzyme-linked immunoassay technique known as evaporative binding enzyme-linked immunoassay (EB-EIA) was used to diagnose for this disease. Both these diagnostic techniques were carried out at the Sugarcane Research Center at Lautoka. The Phase Contrast Microscopy (PCM) technique includes the use of a Phase contrast microscope. A drop of the sugarcane sap is put on a microscope slide with a cover slip and observed under oil immersion at the 100x magnification. The presence of the bacterium is positive when the bacterium presence was observed with its characteristic Y clubbed shape. The evaporative – binding enzyme linked immunoassay technique generally involves the use of extracted cane sap from the cane stalk and dried on micro-plates with buffer and specific antiserum added. This in turn is read on the micro-plate reader taking the absorbance at 405 nm at time zero (immediately after adding substrate solution) and at one

hour after incubation of substrate. The ELISA procedure is given in Appendix 5.

Once the site with positive identification was determined, the bacterium was cultured on a selected media, M-SC (modified sugarcane) agar as per Objective 1. The lower three or four internodes of each clean stalk are surface sterilized by submersion in 70% ethanol for 1-2 minutes, then dipped in 90% ethanol and flamed (Davis and Dean, 1984). The sterilized piece (1 square centimeter) was then placed on M-SC media and kept at 27°C in an incubator. The cultures were constantly observed and were sub cultured when bacterial growth was confirmed.

3.3. Effect of RSD on Sugar Cane Yield

Objective: 3

To determine the loss caused by ratoon stunting disease.

Materials:

i. Tools for cultivation (tractor with plough, harrow and drill attachments), cane knife and hoe.

ii. Plant material - sugar cane (Mana Variety of 8 – 9 months old).

Methods:

A seedbed of diseased cane was established at the Ratoon stunting disease nursery at Nausori Highland, Nadi (Figure 3.2). They were planted in 5-pint polythene pots. The prepared seedbed was used for planting RSD infected sugarcane sets to use as seed material for planting a trial to determine the loss caused by RSD. Mana was used in this trial because it is the main variety (80-90%) planted in Fiji and was the variety most affected with RSD. Once the seed cane was ready for planting at 9 months old, they were then harvested and planted as disease-loss trial at the Lautoka Sugar Cane Research Centre.

Figure 3.2: Maintenance of RSD – infected cane plants

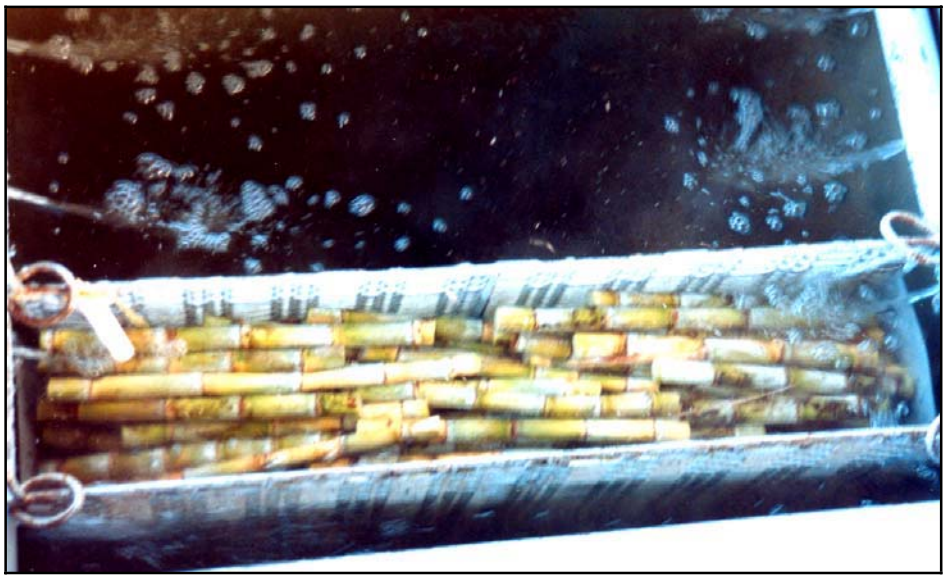


Figure 3.3: Sugarcane planting material undergoing Hot Water Treatment (HWT)

The experimental design used was a random complete block design. The three treatments were diseased cane (control with no heat treatment), diseased cane - hot water treated cane for 2 hours and diseased cane - hot water treated cane for 3 hours as shown in Figure 3.3. Once the plants in the pot were about one and half months old, they were transferred in the field with a plot size of 10m X 1.37m X 2 rows or an area of 27.4m^2 (Figure 3.4).

At harvest time, each plot of all the rows were harvested for the plant crop and the ratoon crop and manually weighed. This was used to determine the yield per unit area. Cane samples consisting of nine stalk samples were taken at random from each plot for cane juice analysis. The pol, brix and fibre values were used to determine the %Pure Obtainable Cane Sugar (%pocs) as described by Powell (1955) as shown in Appendix 3. The product of cane yield per unit area and %POCS gave total tonnes sugar per hectare. The sugarcane yield in tonnes per hectare (TC/HA) was analyzed and compared in two treatments. All data were analysed by standard ANOVA procedures using an Analytical Software, the *STATISTIX® 8* statistical package.

Figure 3.4: Trial design (RCBD) for RSD Yield –loss trial

B (plot 1)	C (plot 6)	C (plot 7)	B (plot 12)
A (plot 2)	B (plot 5)	A (plot 8)	C (plot 11)
C (plot 3)	A (plot 4)	B (plot 9)	A (plot 10)
Rep 1	Rep 2	Rep 3	Rep 4

Key:

A = diseased cane untreated with HWT (0 hrs)

B = diseased cane treated with HWT (2 hrs)

C = diseased cane treated with HWT (3 hrs)

3.4: Screening for RSD Resistance

Objective: 4

To develop a screening method in identifying varieties resistant to ratoon stunting disease for commercial growing in Fiji.

Materials:

i. Tools for cultivation (tractor with plough, harrow and drill attachments) and hoe.

ii. Tools (includes knife, plant clippers, air pressure/vacuum pump, sample tubes, ice box, centrifuge machine, micro plate reader, beaker, magnetic stirrer and pipettes).

iii. Chemicals [70% ethanol, 90% ethanol and components of the M-SC media (corn meal agar, soytone, Bovine hemin chloride magnesium sulphate –hydrous, di-potassium hydrogen phosphate, potassium di-hydrogen phosphate deionised water, glucose cysteine-free base and bovine serum albumin-fractionV).

iv. Plant material:

Sixteen cane varieties comprising of Mana, Kaba, Waya, Vomo, Yasawa, Ragnar, Ono, Mali, Beqa, Aiwa, Spartan, Homer, Galoa, Naidiri (LF82-2122) and LF82-2522.

Methods:

Cane varieties with resistance, susceptible and intermediate resistances were identified using the evaporative binding enzyme-linked immunoassay technique of diagnosis (Croft *et al.*,

1994) at the Sugarcane Research Center, Lautoka. A promising variety (in development) and fifteen commercial varieties were inoculated with RSD infected sap and planted in the field. The analysis of the infection was carried out by evaporative binding – enzyme linked immunoassay or EB – EIA (Croft *et al.*, 1994). Inoculation was done in stools after harvesting the standing crop of eight months old. About four to five drops of the RSD infected sap (0.2 – 0.3ml) was smeared on the cut end of the stalk and then covered with aluminum foil. Assessments using EB-EIA was done 12 months after inoculation.

The EB–EIA diagnosis involved the extraction of cane sap using a 12V vacuum pump from 5 cane stalks in each replication from each of the variety and stored in a freezer. The diagnosis took two days to complete as described in an Instruction manual (Croft *et al.*, 1993), which is summarized below. The samples were defrosted, 0.2ml of each sample and a pair of negative and positive standards put into the 96-microplate well with 88 wells for test samples and 4 wells for the standards. The other 4 wells were left empty as shown in Figure 3.5.

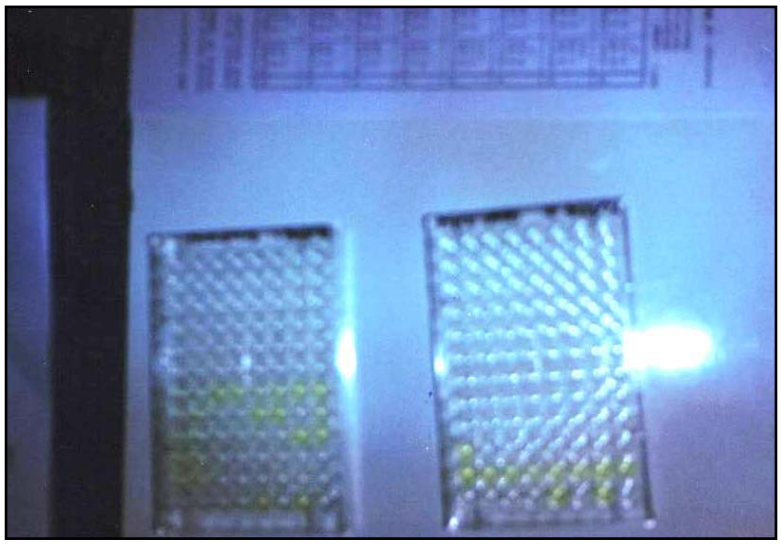


Figure 3.5: An example of a 96-miroplate well in the lab as used in EB-EIA

The plates were then centrifuged at 3000rpm for 20 minutes. Coating buffer was added to the wells and centrifuged again at 3000rpm twice consecutively for 10 minutes each. The coating buffer was removed and kept in an incubator overnight at 37° C. The following day, the plate was blocked with 5% skim milk in Phosphate – buffered saline (PBS) – Tween for 30 minutes and then washed with PBS – Tween. Ratoon stunting disease – specific antiserum is added to each well and incubated at room temperature for 1 hour and then washed with PBS – Tween. The second antibody, goat anti – rabbit alkaline phosphatase conjugate, is added to each well and incubated for 1 hour at

room temperature and then washed with 5 washes of PBS – Tween. A substrate solution of 1.0% 4 – nitro phenyl phosphate in 10% diethanolamine buffer (pH 9.8) was added and the absorbance at 405nm is measured at time 0, 30 and 60 minutes with a micro plate reader.

CHAPTER 4

RESULTS

4.1: RSD Survey and Field Incidence

Ratoon stunting disease (***Leifsonia xyli*** subsp ***xyli***) was confirmed using phase contrast microscopy as shown in Figure 4.1 and EB-EIA (Evaporative binding - Enzyme linked immuno assay) showing different levels of infection as shown in Table 3.

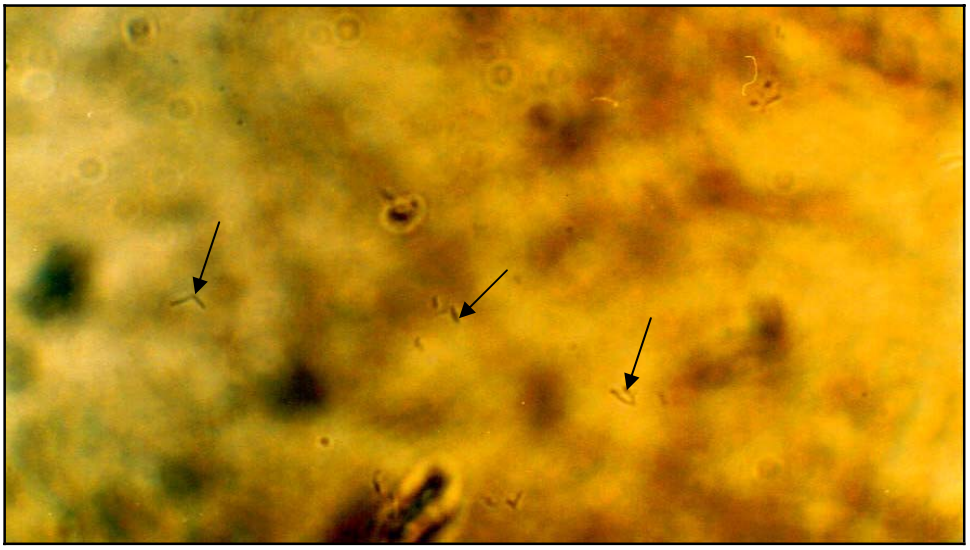


Figure 4.1: Photograph of *Leifsonia xyli* subsp *xyli* (with arrows) from the field using PCM

The presence of ***Leifsonia xyli*** subsp ***xyli*** was found in seven sectors using phase contrast microscopy compared to EB-EIA where

all the thirty eight sectors in the Fiji sugar industry showed the presence of ***Leifsonia xyli*** subsp ***xyli***.

Using phase contrast microscopy it was recorded that out of all the four sugar mills in Fiji, Rarawai mill had the highest average incidence (8.4%) while Labasa mill had the lowest average incidence (2.0%). The total average for all the sugar mills in Fiji using PCM was 6.6% as shown in Table 3.

The results of the survey using EB-EIA diagnosis showed that the Penang mill had the highest average (33.5%) incidence while the Lautoka mill had the lowest average (26.3%) incidence of ratoon stunting disease. Overall the average ratoon stunting disease incidence for all the four sugar mill districts using EB-EIA was 29.6% as shown in Table 3.

The highest incidence of ratoon stunting disease for the Lautoka mill area (Table 3) was recorded in Saweni and Nawaicoba sectors with 47% while the lowest incidence was recorded in Malolo sector with 7% as shown in Figure 4.2. The crop from which the samples were taken were 3.6% (plant), 40.5% (ratoon) and 55.9% (other ratoons) as in (Appendix 2).

Table 3: Incidence of Ratoon Stunting Disease in the Fiji Sugar Industry

Lautoka mill			Rarawai mill			Labasa mill			Penang mill		
Sector	% incidence		Sector	% incidence		Sector	% incidence		Sector	% incidence	
	E	P		E	P		E	P		E	P
Drasa	33	0	Yaladro	27	0	Waiqele	40	0	Ellington 1	47	0
Lovu	20	0	Drumasi	53	0	Wailevu	13	13	Ellington 2	33	0
Lautoka	27	0	Tagitagi	7	0	Vunimoli	67	0	Malau	27	33
Saweni	47	8	Naloto	33	0	Labasa	13	0	Nanuku	27	0
Natova	27	0	Varavu	33	67	Bucaisau	53	7			
Legalega	27	0	Veisaru	33	0	Wainikoro	47	0			
Meigunyah	13	17	Rarawai	7	0	Daku	20	0			
Qeleloa	27	0	Koronubu	47	0	Natua	27	0			
Yako	20	0	Mota	27	0	Solove	7	0			
Malolo	7	0	Varoko	20	17	Bulivou	13	0			
Nawaicoba	47	13									
Lomawai	40	0									
Cuvu	33	0									
Olosara	27	75									
Average	26.3	7.5		28.7	8.4		30.0	2.0		33.5	8.3
% Total Average incidence (EB-EIA)											**29.6**
% Total Average incidence (PCM)											**6.6**

Note: ***E = Evaporative-binding enzyme linked immunoassay (EB-EIA) diagnosis***
P = Phase contrast microscopy (PCM) diagnosis

The highest incidence of ratoon stunting disease for the Rarawai mill area (Table 3) was recorded in Drumasi sector with 53% while the lowest incidence was recorded in Tagitagi and Rarawai sectors with 7% as shown in Table 3 and Figure 4.3. The crop from which the samples were taken were 0% (plant), 59.3% (ratoon) and 40.7% (other ratoons) as in (Appendix 2).

The highest incidence of ratoon stunting disease for the Labasa mill area (Table 3) was recorded in Vunimoli sector with 67% while the lowest incidence was recorded in Solove sector with 7% as shown in Figure 4.4. The crop from which the samples were taken was 15.3% (plant), 43.4% (ratoon) and 41.3% (other ratoons) as in (Appendix 2).

The highest incidence of ratoon stunting disease for the Penang mill area (Table 3) was recorded in Ellington 1 sector with 47% while the lowest incidence was recorded in Malau and Nanuku sectors with 27% as shown in Figure 4.5. The crop from which the samples were taken was 0% (plant), 71.7% (ratoon) and 28.3% (other ratoons) as in (Appendix 2).

Figure 4.2: RSD Incidence (EB-EIA) in Lautoka mill area

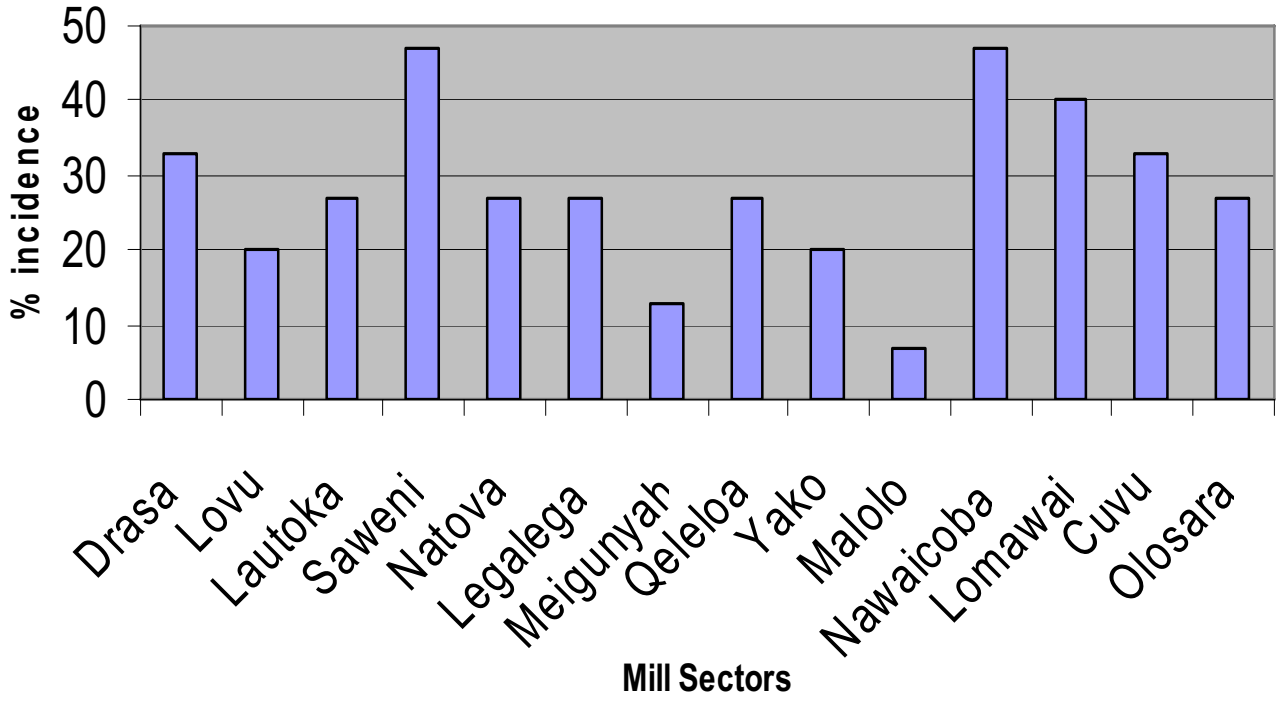
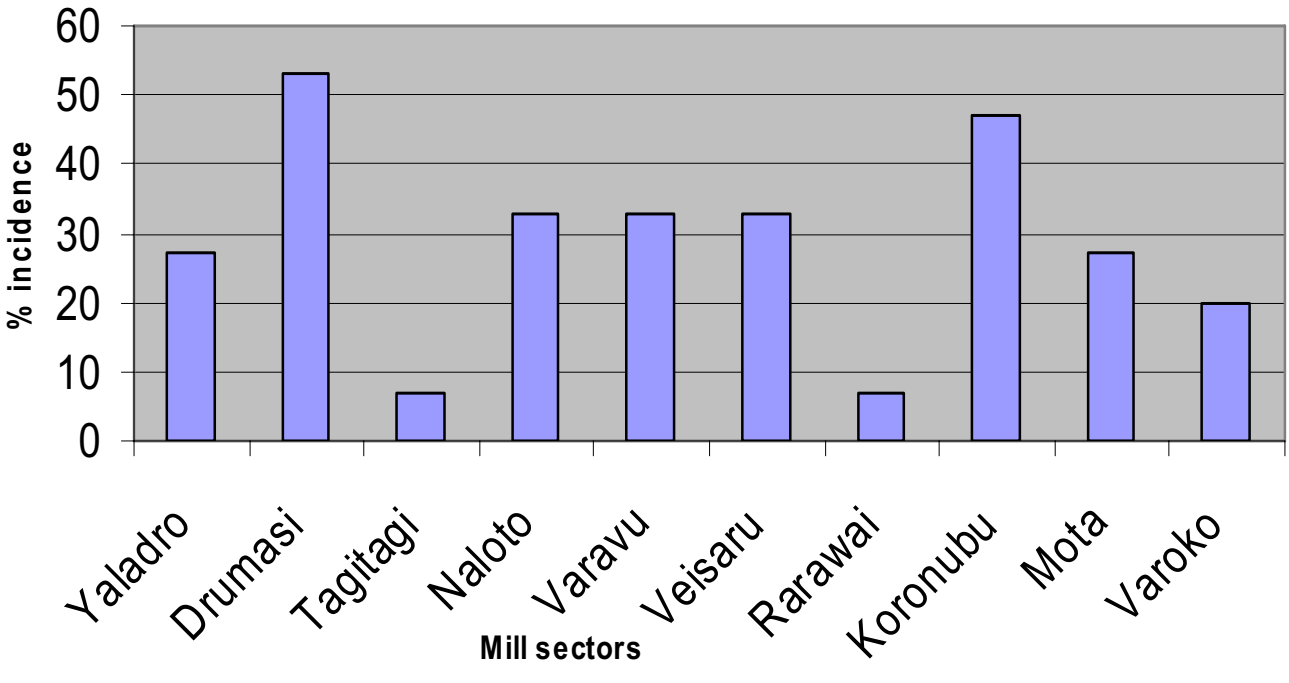


Figure 4.3: RSD Incidence (EB-EIA) in Rarawai mill area



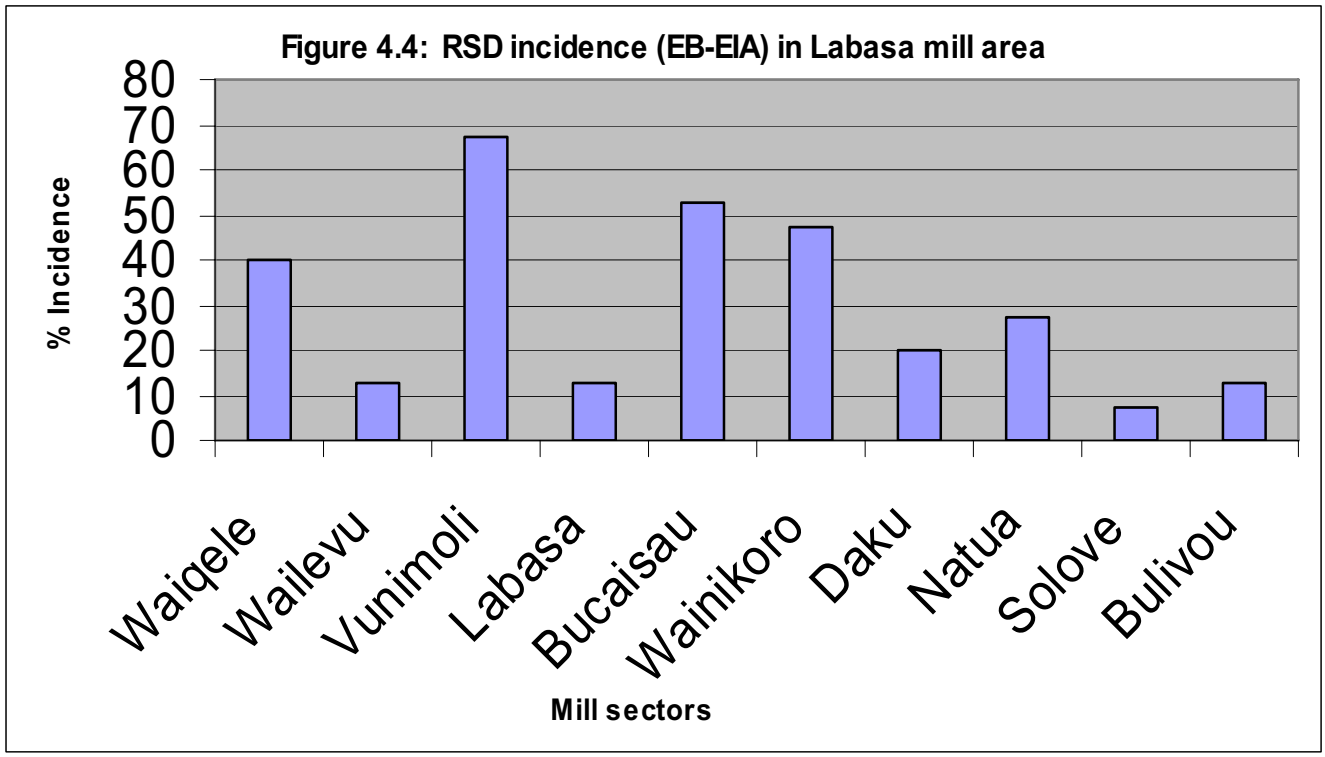


Figure 4.4: RSD incidence (EB-EIA) in Labasa mill area
% Incidence
80
70
60
50
40
30
20
10
0
Waiqele
Wailevu
Vunimoli
Labasa
Bucaisau
Wainikoro
Daku
Natua
Solove
Bulivou
Mill sectors

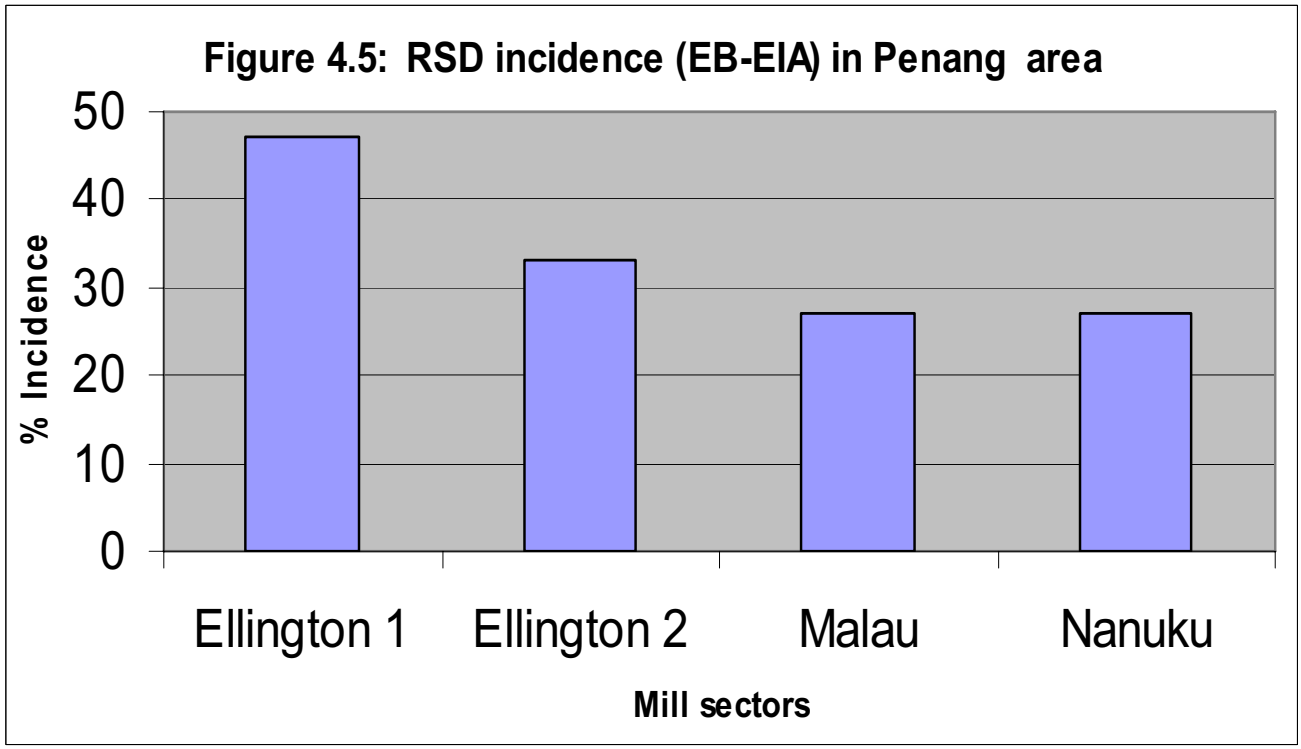


Figure 4.5: RSD incidence (EB-EIA) in Penang area
% Incidence
50
40
30
20
10
0
Ellington 1
Ellington 2
Malau
Nanuku
Mill sectors

4.2: RSD Establishment through Culture

The culture of the bacterium, ***Leifsonia xyli*** subsp ***xyli*** was carried out in-vitro with yellow and orange coloured colonies. Those samples that had high infestation level were selected to be cultured as shown in Figure 4.6. The cultures were sub cultured and pure cultures were obtained and used in the resistance screening experiment. It was observed that in the first few cultures there were some contamination by other bacteria, especially pseudomonas so we had to subculture only areas in the culture that showed pure colonies of ***L. xyli*** subsp. ***xyli*** which were confirmed by streaking a sample on to a microscope slide and viewing it under the phase contrast microscope as shown in Figure 4.7.

Figure 4.6: **Photograph of a culture of *Leifsonia xyli* subsp *xyli* in the lab**

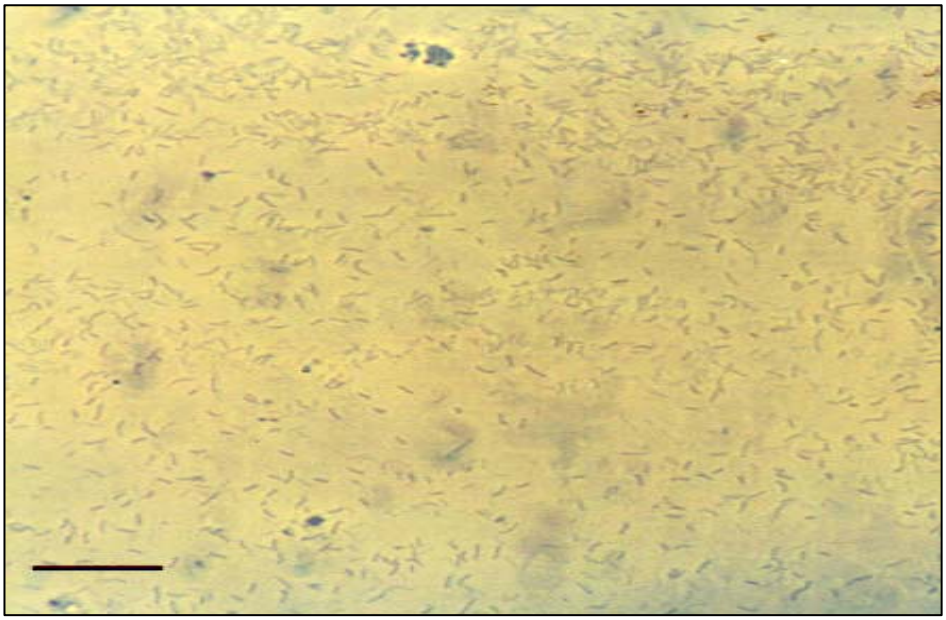


Figure 4.7: **Photograph of *Leifsonia xyli* subsp *xyli* in culture using phase contrast microscopy (Bar represents 1 µm) at 1,000 X magnifications.**

4.3: Effect of RSD on Sugar Cane Yield

Hot water treatment used in this study did not affect % pocs, cane and showed no significant difference in the sugar yield between the three treatments for variety Mana in the plant crop as shown in Table 4. However, cane treated at 50°C for 3 hours produced higher cane and sugar yield compared to other treatments investigated in the study.

The untreated plot had the lowest cane yield ($tcha^{-1}$) and sucrose content ($tsha^{-1}$). The total rain days for the year were 142 days with a total rainfall of 2044.2mm. The high yield for the plant crop was expected as a good amount of rainfall was experienced during the growing period from planting to the sixth month (Table 4).

Table 4: Sugarcane yield (plant crop) of ratoon stunting disease infected cane with three different duration of hot water treatment from a RCBD experiment with four reps.

Location	Plant date/ Date harvest	Variety & Crop	Treatment (Hot water treatment at 50°C for different amount of time - hours)	Cane Yield ($tcha^{-1}$)	POCS (%)	Sugar Yield ($tsha^{-1}$)
				P	P	P
Lautoka *142 raindays and 2044.2mm*	Jun/2001 Jul/2002	Mana Plant	A. Cane untreated	119	10.43	12.3
			B. Cane treated in hot water at 50°C for 2 hours	134	10.93	14.6
			C. Cane treated in hot water at 50°C for 3 hours	149	10.90	16.1
			LSD 5%	NS	NS	NS
			CV%	14.35	4.52	15.47

$tcha^{-1}$ **tonnes cane per hectare**

$tsha^{-1}$ **tonnes sugar per hectare**

P **plant crop**

NS **not significant**

CV **coefficient of variation**

Table 5 shows that cane treated in hot water did not significantly increase cane and sugar yield in the first ratoon crop. However, cane treated at 50° C for 2 hours produced higher cane and sugar yield compared to cane treated at 50° C for 3 hours as observed in plant crop.

The total rain days for the year were 144 days with a total rainfall of 1704.4mm. Overall cane yield declined in first ratoon crop as expected due to the lower amount of rainfall and the rains not coming during the active growing months of the ratoon crop. The effect of ratoon stunting disease infection was now visible as the crop reduced in its yield.

Table 5: Sugarcane yield (first ratoon crop) of ratoon stunting disease infected cane with three different duration of hot water treatment from a RCBD experiment with four reps.

Location	Plant date/ Date harvest	Variety & Crop	Treatment (Hot water treatment at 50°C for different amount of time - hours)	Cane Yield ($tcha^{-1}$)	POCS (%)	Sugar Yield ($tsha^{-1}$)
				R	R	R
Lautoka *144 raindays and 1704.4mm*	Jun/2001 Oct/2004	Mana 1st ratoon	A. Cane untreated	98	12.88	12.6
			B. Cane treated in hot water at 50°C for 2 hours	133	12.73	16.9
			C. Cane treated in hot water at 50°C for 3 hours	124	12.45	15.4
			LSD 5%	NS	NS	NS
			CV%	8.40	4.40	11.23

$tcha^{-1}$ **tonnes cane per hectare**

$tsha^{-1}$ **tonnes sugar per hectare**

R **ratoon crop**

NS **not significant**

CV **coefficient of variation**

There was a sharp decline in cane yield compared to plant and first ratoon crop as shown in Table 6. The total average reduction in cane yield from plant to ratoon was 36.6%. However, cane treated at 50° C for 2 hours produced higher cane and sugar than untreated and cane treated at 50° C for 3 hours.

The total rain days for the year were 109 days with a total rainfall of 11459.3mm. The significant decline shown was attributed to dry conditions, which affected vegetative growth during active growth period from May to November and the effect of ratoon stunting disease and its spread from infected plots. The average difference of cane yield ($tcha^{-1}$) between the plant and the two ratoon crops was 28.7% thus establishing that the average loss caused by ratoon stunting disease in this trial was 28.7%.

Table 6: Sugarcane yield (other ratoon crop) of ratoon stunting disease infected cane with three different duration of hot water treatment from a RCBD experiment with four reps.

Location	Plant date/ Date harvest	Variety & Crop	Treatment (Hot water treatment at 50°C for different amount of time - hours)	Cane Yield ($tcha^{-1}$)	POCS (%)	Sugar Yield ($tsha^{-1}$)
				S	S	S
Lautoka *109 raindays and 1459.3mm*	Jun/2001 Jul/2005	Mana Other ratoon	A. Cane untreated	47	12.93	6.1
			B. Cane treated in hot water at 50°C for 2 hours	58	12.83	7.5
			C. Cane treated in hot water at 50°C for 3 hours	49	12.30	6.1
			LSD 5%	NS	NS	NS
			CV%	31.37	4.82	31.72

$tcha^{-1}$ **tonnes cane per hectare**

$tsha^{-1}$ **tonnes sugar per hectare**

S **other ratoon crop**

NS **not significant**

CV **coefficient of variation**

4.4: Screening for RSD Resistance

Randomly collected sugarcane sap from all the clones in different sectors were analyzed for the presence of ***Leifsonia xyli*** subsp. ***xyli*** (Appendix 6). All the commercial varieties screened for the presence of ***Leifsonia xyli*** subsp. ***xyli*** had varying results as shown in Table 7.

Out of the 15 varieties screened, three varieties namely Vatu, Homer and Galoa had higher readings (X > 0.1nm) on the microplate reader by reading absorbance at 405nm; two varieties, Aiwa and Beqa had moderate readings (0.1nm < X > 0.5nm) on the microplate reader by reading absorbance at 405nm; while ten varieties, Kaba, Vomo, LF82-2252, Yasawa, Naidiri, Ragnar, Ono, Waya, Mana and Spartan had low readings (X < 0.05nm) on the microplate reader by reading absorbance at 405nm.

Table 7: ELISA Readings of the sugarcane varieties screened for the presence of *Leifsonia xyli* subsp. *xyli*.

Variety	T E S T O N E					T E S T T W O					TOTAL
	R 1	R 2	R 3	Tot	Average	R 1	R 2	R 3	Tot	Average	Average
A iwa	-0.15	-0.07	-0.02	-0.25		-0.17	0.508	0.03	0.37		
	-0.08	-0.02	0.105	0.01		-0.09	0.307	0.046	0.268		
Average	-0.23	-0.09	0.08	-0.24	-0.0785	-0.25	0.815	0.076	0.638	0.21267	0.0670667
	R 1	R 2	R 3	Tot	Average	R 1	R 2	R 3	Tot	Average	
K aba	-0.3	-0.24	-0.12	-0.66		-0.36	-0.22	0.043	-0.53		
	-0.13	-0.1	-0.05	-0.27		-0.2	-0.12	0.052	-0.26		
Average	-0.43	-0.33	-0.17	-0.94	-0.312	-0.55	-0.33	0.095	-0.79	-0.264	-0.288
	R 1	R 2	R 3	Tot	Average	R 1	R 2	R 3	Tot	Average	
Vomo	0.23	-0.26	-0.26	-0.29		-0.24	0.36	0.111	0.231		
	0.112	-0.11	-0.11	-0.1		-0.12	0.192	0.183	0.256		
Average	0.342	-0.37	-0.37	-0.39	-0.1313	-0.36	0.552	0.294	0.487	0.16233	0.0155
	R 1	R 2	R 3	Tot	Average	R 1	R 2	R 3	Tot	Average	
V atu	0.164	-0.26	-0.04	-0.14		0.593	0.367	0.327	1.287		
	0.082	-0.11	-0.03	-0.05		0.274	0.144	0.162	0.58		
Average	0.246	-0.36	-0.07	-0.19	-0.0627	0.867	0.511	0.489	1.867	0.62233	0.2798333
	R 1	R 2	R 3	Tot	Average	R 1	R 2	R 3	Tot	Average	
LF82-2252	-0.08	-0.24	-0.26	-0.58		0.328	-0.01	-0.29	0.03		
	-0.03	-0.1	-0.11	-0.24		0.171	-0.02	-0.15	– 0		
Average	-0.11	-0.35	-0.37	-0.82	-0.2737	0.499	-0.03	-0.45	0.026	0.00867	-0.1325
	R 1	R 2	R 3	Tot	Average	R 1	R 2	R 3	Tot	Average	
Yasawa	-0.28	-0.08	-0.24	-0.6		-0.18	-0.27	-0.37	-0.81		
	-0.12	-0.04	-0.1	-0.26		-0.09	-0.3	-0.21	-0.6		
Average	-0.4	-0.12	-0.33	-0.85	-0.284	-0.27	-0.57	-0.58	-1.41	-0.4713	-0.377667
	R 1	R 2	R 3	Tot	Average	R 1	R 2	R 3	Tot	Average	
Naidiri	-0.13	0.187	0	0.056		-0.17	-0.23	0	-0.4		
	-0.05	0.089	0	0.036		-0.09	-0.11	0	-0.2		
Average	-0.18	0.276	0	0.092	0.03067	-0.26	-0.34	0	-0.6	– 0 . 2	-0.084667
	R 1	R 2	R 3	Tot	Average	R 1	R 2	R 3	Tot	Average	
Ragnar	0.408	-0.13	-0.24	0.043		-0.35	0.301	-0.42	-0.47		
	0.21	-0.05	-0.1	0.058		-0.2	0.17	-0.23	-0.27		
Average	0.618	-0.18	-0.34	0.101	0.03367	-0.55	0.471	-0.66	-0.74	-0.2457	-0.106
	R 1	R 2	R 3	Tot	Average	R 1	R 2	R 3	Tot	Average	
Homer	0.934	0.863	0	1.797		-0.06	-0.28	0.475	0.138		
	1.017	0.249	0	1.266		-0.01	-0.13	0.221	0.074		
Average	1.951	1.112	0	3.063	1.021	-0.07	-0.41	0.696	0.212	0.07067	0.5458333
	R 1	R 2	R 3	Tot	Average	R 1	R 2	R 3	Tot	Average	
O n o	-0.31	-0.27	0	-0.57		-0.38	-0.19	0.249	-0.31		
	-0.13	-0.11	0	-0.24		-0.21	-0.1	0.2	-0.11		

Average	-0.44	-0.38	0	-0.81	-0.2707	-0.59	-0.29	0.449	-0.42	-0.1413	***-0.206***
	R 1	**R 2**	**R 3**	**Tot**	***Average***	**R 1**	**R 2**	**R 3**	**Tot**	***Average***	
Waya	0.118	-0.04	-0.11	-0.03		0.031	-0.22	0.288	0.099		
	0.042	0.003	-0.04	0.003		0.017	-0.1	0.14	0.058		
Average	0.16	-0.04	-0.15	-0.03	-0.0087	0.048	-0.32	0.428	0.157	0.05233	***0.0218333***
	R 1	**R 2**	**R 3**	**Tot**	***Average***	**R 1**	**R 2**	**R 3**	**Tot**	***Average***	
Beqa	-0.26	-0.23	0.466	-0.02		-0.09	0.259	0.312	0.478		
	-0.43	-0.09	0.141	-0.38		0.083	0.152	0.154	0.389		
Average	-0.69	-0.32	0.607	-0.4	-0.1337	-0.01	0.411	0.466	0.867	0.289	***0.0776667***
	R 1	**R 2**	**R 3**	**Tot**	***Average***	**R 1**	**R 2**	**R 3**	**Tot**	***Average***	
Mana	-0.26	-0.29	0	-0.55		-0.35	0.383	0	0.031		
	-0.11	-0.13	0	-0.23		-0.2	0.228	0	0.033		
Average	-0.37	-0.42	0	-0.78	-0.26	-0.55	0.611	0	0.064	0.02133	***-0.119333***
	R 1	**R 2**	**R 3**	**Tot**	***Average***	**R 1**	**R 2**	**R 3**	**Tot**	***Average***	
Spartan	-0.3	0.431	0.1	0.227		-0.12	-0.15	-0.31	-0.58		
	-0.13	0.201	-0.03	0.042		-0.12	-0.25	-0.16	-0.53		
Average	-0.43	0.632	0.07	0.269	0.08967	-0.24	-0.4	-0.47	-1.11	-0.3703	***-0.140333***
	R 1	**R 2**	**R 3**	**Tot**	***Average***	**R 1**	**R 2**	**R 3**	**Tot**	***Average***	
Galoa	0.032	-0.07	0.663	0.626		-0.27	-0.25	0.644	0.126		
	0.014	-0.04	0.276	0.249		-0.14	-0.12	0.093	-0.17		
Average	0.046	-0.11	0.939	0.875	0.29167	-0.41	-0.37	0.737	-0.04	-0.0147	***0.1385***

High reading (X > 0.1nm)

Moderate reading (0.1nm < X > 0.05nm)

Low reading (X < 0.05nm)

CHAPTER 5

DISCUSSIONS

Ratoon stunting disease (RSD) was easily masked in Fiji because of the weather conditions with an average rainfall of 1112.6mm (93 years long term mean) for all the four sugar mills (Anon, 2003). The factors that made diagnosis difficult were the lack of knowledge of the disease in its physical symptoms and the lack of diagnostic kits. These kits were only available in Fiji after other sugarcane countries such as Australia and South Africa developed diagnostic techniques such as enzyme-linked immunoassay (ELISA). Symptoms of weakness in sugar cane plant growth due to the infection of RSD were usually confused with poor nutrition and lack of water (drought conditions). The use of diagnostic techniques such as ELISA showed the presence of RSD and its extent in the sugarcane fields was also used as a means of screening all of the varieties grown in Fiji for resistance to RSD.

5.1: RSD survey and Field Incidence

The results indicate that all the thirty eight sugarcane growing sectors in Fiji are infected with RSD. This confirms the initial findings of the presence of ratoon stunting disease in

Fiji (Tamanikaiyaroi and Johnson, 1995). The pathogen is a small, xylem-inhabiting, coryneform bacterium that may be detected in xylem sap extracts (Davis and Bailey, 2000). Sampling from the headlands or corner of the field is an assumption that as the disease is transmitted by harvesters or cane knives, the first area to be affected is the headlands or corners of the field. Similar sampling technique was employed by Croft *et al*. (1995) for ease of sampling and to increase the chances of detection since disease is more likely to occur on the corners of a field if the cane has been infected by contaminated harvesters or planters. With the same number of samples taken from each sugar cane sector, it was possible to predict the percentage infection for the whole sugar industry for both the diagnostic technique.

The different diagnostic technique used showed some difference with the EB-EIA compared to the PCM technique with the EB-EIA having a higher percentage infection compared to PCM. Phase contrast microscopy has been used extensively for RSD diagnosis in other sugar cane growing countries worldwide (Amiet, 1985; Davis and Dean, 1984; Gillaspie *et al*., 1976; Roach, 1990; Roach and Jackson, 1990) which is a simple technique but gives too many false negatives when bacterial concentrations are low

(Davis and Dean, 1984). A difference of 23% was noted between the two techniques with EB-EIA giving a higher infection of ***Leifsonia xyli*** subsp. ***xyli*** with an average percent infection of 29.6% compared to PCM with an average percent infection of 6.6%. Evaporative Binding – Enzyme linked Immuno Assay results gave the more reliable data of the percentage infection of sugar cane by ***Leifsonia xyli*** subsp. ***xyli***. Evaporative Binding – Enzyme linked Immuno Assay is suitable for large scale RSD diagnosis and is relatively inexpensive when handling large number of samples (Croft *et al.,* 1994) and less time consuming compared to PCM. Phase contrast microscopy is too time consuming and labour intensive for screening large numbers of samples under Australian conditions (Leaman *et al*., 1992). This technique (EB-EIA) provides significant improvement in accuracy of diagnosis over PCM method and can be used in epidemiology studies of RSD where intensive sampling is required to detect low numbers of infected stools (Croft *et al*., 1994).

The infection and spread of ***Leifsonia xyli*** subsp ***xyli*** in all the sectors can be attributed to the planting of infected seed material and to some extent, the harvesting tool such as the cane knife and most recently the sugar cane mechanical harvester. The spread of ratoon stunting disease by both modes

has been studied extensively since the 1950s (Hughes and Steindl, 1955; Steib *et al.*, 1957; Bailey and Tough, 1992). The use of mechanical harvesters would be increasing in Fiji, especially in Lautoka mill area and this would contribute to the easy spread of the disease compared to manual harvesting because of the vast difference in rate of harvest per day. Disease incidence could possibly increase in all the sugar cane sectors if no control procedures are put in place as the RSD is widespread in all the sugar cane sectors.

The highest percent infection (67%) was found in Vunimoli sector followed by Drumasi sector with 53% and Nawaicoba and Ellington sectors with 47%. The lowest percent infection was found in Malolo, Tagitagi, Rarawai and Solove sectors with 7%. The explanation for such a difference can be attributed to the different crops (plant or ratoon), the samples were taken from. The sectors with high percent infection had samples taken from multiple ratoons i.e. crops with more than 3-4 ratoons while the sectors with low percentage had samples from either the plant crop or crops with less than 3 ratoons, which usually has low infection. Ratoon fields would have substantially higher disease incidence than plants (Croft *et al.*, 1995).

It is common knowledge that practicing monoculture will bring changes to the habitat such as experienced by the sugar industry in Fiji. Continuous monoculture of sugarcane with large acreage combined with other factors such as fertilization increases the possibility and severity of epidemics (Agrios, 1988). It is expected that the disease will keep on spreading because of the harvesting system used in Fiji where a group of cane cutters harvest sugar cane on a farm to farm basis, therefore spreading the disease by the infected cane harvesting knife. The retention of multiple (more than three) ratoons where the bacterial density will tend to increase as the ratoons are retained, which also becomes a source of the disease. Cane knives or chopper-harvester blades mainly transmit RSD, and the rate of spread during harvest can be rapid during the crops cycle i.e. the plant crop followed by a few ratoons (Bailey and Tough, 1992).

Crops with more than three ratoons (Other ratoons) in 2001 and 2002 covered an area of 58% and 72.8% respectively of the total sugar cane crop in Fiji (Anon, 2002). The large proportion of area under other ratoons increases the chance of RSD spreading to newer ratooning areas if not kept in check. The effects of RSD are markedly accentuated in the ratoon crop because the

diseased cane are slower in growth as compared to healthy crops, particularly in dry weather when the sugarcane stubble may remain dormant for weeks or even months (Hughes, 1974). Farmers who practice long and short fallow may have some advantage in that the RSD causal organism can be reduced but the presence of "volunteers" in the ploughed field can harbour RSD. The pathogen can also remain infectious for up to several months in dead or moribund plant debris and in the soil itself (Bailey and McFarlane, 1999).

5.2: RSD Establishment through Culture

The presence of the ratoon stunting disease causal agent, ***Leifsonia xyli*** subsp ***xyli*** was confirmed by culturing the bacteria in-vitro. The infected sap, once cultured had to be isolated and pure cultures done because of contamination by other bacteria such as pseudomonas and some fungal contamination. The colonies gave a yellow and orange colour which is consistent with studies previously done. Most of the colonies that developed from the ground gall suspension plated on corynebacterial agar (CB) were yellow, orange yellow or orange (Evtushenko *et al.*, 2000). The MS (modified sugarcane) agar medium developed by Davis *et al.* (1980), has been the medium used in Queensland and now done in Fiji. This medium is

proved efficient based on causal organism characters such as colonies of 0.1 – 1.3 mm in diameter and circular in shape with entire margins, convex and non – pigmented shape (Davis *et al*., 1980). Figure 4.6 and 4.7 show the culture and the view of the bacteria at 1,000 times magnification with the distinctive characters in the yellow and orange colonies.

These cultures were positively confirmed for the presence of the bacterium using evaporative binding immuno assay. The cultured bacteria were used in experiment 3 and 4 for inoculation purposes. Using the phase contrast microscope, the bacteria was seen to be a small, non-motile, rod shaped bacterium as recorded previously by Gillaspie *et al*. (1974) and Teakle (1974). The physical symptoms as recorded by Gillaspie and Teakle (1989) were difficult to diagnose. Fiji sugarcane varieties did not show any physical symptoms as described by Gillaspie and Teakle (1989) as discolouration within individual vascular bundles and small reddish dots, commas and various straight or bent forms up to 3mm in length.

5.3: Effect of RSD on Sugar Cane Yield

The difference in cane yield was seen with all the treatments in this experiment but it was not statistically significant to draw any concrete conclusions. This may be due to the variety, used in the study because preliminary experiments using Mana cultivar for heat treatment showed that it was more tolerant to heat treatment compared to other commercial varieties such as Kaba and Aiwa. Losses in resistant clones may not be significant, whereas losses in the highly susceptible clones, in both yield of cane and sugar per unit area, may approach 50% (Gillaspie and Teakle, 1989). It was seen that the only notable effect of the disease was on the cane yield but very little effect on the sugar and percent pocs. The disease has little effect on cane quality and stalk population, and losses in yield were almost entirely due to decreases in stalk mass (Bailey and Bechet, 1986).

Generally, the use of only one cane variety may be biased in drawing such conclusions but it is important for the Fiji sugar industry because farmers prefer growing the variety Mana, which accounts for eighty percent of all varieties planted (Anon, 2002). It is also clear that the cane yield for the plant and other ratoon crops was different. Similar results for other ratoons

were recorded by Rossler (1974). The plant crop yields were less affected by ratoon stunting disease than were the ratoons and there were indications that further reductions in cane yields could be expected in subsequent ratoon crops. The disease seems to be more prominent in the ratoon crop compared to the plant crop. Yield losses were greater in the stubble crop (ratoon) than in the plant – cane crop (Koike, 1980). The decrease in yield in other ratoon was more obvious as shown in Table 6, which could also be due to the disease spreading to other plots during harvesting as studied by Bailey and Tough (1992). Some varieties may die as a result of infection by the bacterium, however, RSD does not have any consistent effects on cane juice quality (James, 2005). The present average yield of 45 – 70 tonnes/ ha (Anon., 2003) in the Fiji sugar industry is similar to those shown in Table 6, but on the lower range of the average. It is likely that the declining cane yield in the Fiji sugar industry may be partly due to the infection by ratoon stunting disease. Stalk populations may be affected when the disease is severe and RSD-infected ratoon crops grow more slowly and the yield losses are larger (James, 2005).

The use of hot water treatment in assessing the loss in yield is relevant because hot water treatment (HWT) has been known to

control ratoon stunting disease. In this instance, the Mana seed cane was infected with RSD prior to hot water treatment and observing the treated plots with the control. As clearly shown in Tables 5 and 6, the cane and sugar yield for the HWT at 50°C for 2 hours was higher as compared to the control and HWT at 50°C for 3 hours. The optimum temperature for hot water treatment has been found to be at 50°C for 2 to 3 hours (Steindl, 1961). Cane treated at 50°C hot water for 3 hours gave higher cane and sugar yield in the plant crop compared to control while cane treated at 50°C in hot water for 2 hours produced higher cane and sugar yield than untreated (controlled) and cane treated at 50°C in hot water for 3 hours in the ratoon crop. The difference between sugarcane treated for 3 hours compared to 2 hours may be due to the prolonged heat on the cane which may have killed some of the eye setts. Therefore the best hot water treatment was identified as 2 hours at 50°C. In previous experiments (Gawander unpublished), hot water treatment did not show any significant effect on the yield of three commercial varieties; Mana, Mali and Ragnar. The results may be due to the absence of the ratoon stunting disease bacterium (***Leifsonia xyli*** subsp ***xyli***) in the seed cane as the objective of this experiment (Gawander unpublished) was to observe the effect of heat treatment and not RSD on the three varieties.

This disease requires an integrated approach to manage it to economical level. In principle, RSD can be controlled by heat therapy but in practice it has not worked well as expected because this method requires great care to avoid re-infection (Dean and Davis, 1990). Hot water treatment does not totally eliminate the infection but merely diminishes it to a level to enable the grower to establish a nursery with a very low level of infection (James, 2005). Roach *et al.* (1992) recommended that to control RSD, there was a need to increase grower awareness of the disease; screen potential new cultivars for RSD susceptibility; minimise planting of RSD infected cane using heat treatment for RSD-free plant sources; and increase effectiveness of farm hygiene measures.

Moisture is one of the environmental factors that show the effect of ratoon stunting disease in sugar cane fields. The amount of rainfall determines the moisture content in the soil and when RSD – infected field is exposed to less moisture, the susceptible sugar cane varieties show the physical symptoms of stunted growth. Moisture either as drought or water logging can increase yield loss due to the disease manifestation (Davis and Bailey, 2000). During the second (2002) and third ratoon (2003), the weather conditions were bit more dry than usual as

seen in Table 6 where all the treatments had an average reduction of 36.6% loss in cane yield ($tcha^{-1}$). As the growth effects of RSD tend to be the greatest when crops are stressed, losses are greatest when the precipitation is erratic under rain fed conditions or when irrigation management is poor (James, 2005). The high concentration of the bacteria in the vascular bundle in susceptible varieties is the cause of the blockage that ultimately leads to the stunted stature of the sugar cane plant. It is easier for farmers to mistakenly diagnose the stunted growth of the cane crop because of soil infertility and moisture stress because of the lack of knowledge of ratoon stunting disease among farmers.

The effect of RSD on cane quality [Pure Obtainable Cane Sugar] was insignificant but the loss caused by RSD to the cane yield was 28.7% which falls under the range found by Hughes (1974) making RSD as one of the most devastating disease of sugarcane in the world causing losses up to 30% per year. The disease has little effect on cane quality and stalk population, and losses in yield were almost entirely due to decreases in stalk mass (Bailey and Bechet, 1986). Studies done in Florida had similar results with losses in cane tonnage attributed to RSD were apparently due to reduction in stalk height and diameter and not

due to reduction in the total number of stalks (Irey, 1986). As shown in tables 4, 5 and 6 the % pocs increased from the plant to subsequent ratoon crops. Cane quality may be slightly increased by the presence of RSD when the crop receives adequate water but may change when subjected to moisture stress (Rossler, 1974) as shown in tables 5 and 6. The actual degree of loss depends partly on the tolerance of a particular variety to the disease and partly on the environment, notably the moisture available since the RSD bacterium causes plugging of the vascular bundles (Hughes, 1974).

5.4: Screening for RSD Resistance

The fifteen varieties screened for the presence of ***Leifsonia xyli*** subsp ***xyli*** had different responses to the bacterium. Their different response can be explained on the basis of their susceptibility to the ratoon stunting disease causal agent, ***Leifsonia xyli*** subsp ***xyli***. The resistance of the varieties screened using EB-EIA is based on the relativity of the bacterial population present in the cane sap. Genetic variability for resistance to ***Leifsonia xyli*** subsp ***xyli*** exists and the first practical step in utilizing genetic variation in RSD resistance for control of the disease is to screen existing

commercial cultivars for resistance (Roach, 1992b). The absorbance from EB-EIA was found to be highly correlated with bacterial population and bacterial population is related to resistance (Croft *et al*,. 1994).

In the present investigation, Vatu, Homer and Galoa varieties were susceptible to ***Leifsonia xyli*** subsp ***xyli*** with a reading of greater than 0.1nm difference on the ELISA reader while Kaba, Vomo, Yasawa, Naidiri, Ragnar, Ono, Waya, Mana, Spartan and LF82-2252 varieties are resistant to ratoon stunting disease with a reading of less than 0.05nm difference on the ELISA reader. Only two varieties, Beqa and Aiwa had moderate readings that can be classified as moderately susceptible to ratoon stunting disease. There are large differences between varieties in RSD susceptibility / tolerance but highly resistant varieties are rare (James, 2005). Studies carried out in Florida on screening sugar cane for RSD resistance using an ELISA technique known as Tissue blot – enzyme linked immuno assay found that RSD reactions on a continuous scale with the lowest colonized vascular bundle (cvb) in ***Leifsonia xyli*** subsp ***xyli*** inoculated plants being the most resistant plant (Comstock *et al*., 2001).

The ratings in the two tests varied for each variety as shown in Table 7 but the average was used to determine the final rating. The repeatability of the resistance ratings to RSD varied in trials conducted in Florida but this could be higher in the later stages of the program (Comstock *et al*, 2001). A sugarcane clone's RSD resistance was shown by Dean *et al*. (1988) to be inversely related to ***Leifsonia xyli*** subsp ***xyli*** population levels, while there was a positive correlation between bacterial population levels and the numbers of colonized vascular bundles. ELISA techniques such as EB-EIA appears to offer a simple method of rating resistance to RSD since absorbance from EB-EIA was found to be highly correlated with bacterial population and bacterial population in infected varieties is related to resistance (Croft *et al*., 1994). The resistance shown by the sugar cane varieties indicates that the selection of parents either intentional or unintentional during the breeding and selection stages contributed to the high number of resistant varieties now planted in sugar cane fields. The use of resistance appears logical since the spread of RSD has been reported to be slower in resistant cultivars than in susceptible cultivars in both commercial plantings and experimental trials (Damman, 1992; Comstock *et al*., 1996). Croft et al. (1994) found that EB-EIA is suitable for large scale RSD diagnosis

since it is relatively inexpensive and provides a simple method of rating varieties for resistance to RSD.

The breeding program can utilize the results of the screening of the commercial varieties together with other characters such as agronomic and other data on disease in the decision to breed and release new varieties after screening the hybrid progeny. All sugarcane breeding programs contain at least some element of recurrent breeding and selection, with superior selected clones being returned as parents to the breeding pool (Roach, 1992b). Breeding for resistance is a long term and expensive exercise but with the selection of the right parents during the breeding program, the benefits of releasing a RSD – resistant variety will be more beneficial to the sugar industry. Use of RSD – resistant clones as parents should increase the frequency of RSD resistance in the progeny, even if selection pressure in the routine breeding and selection program is absent (Miller *et al*., 1996). The control of RSD through breeding would be sustainable over the long run and there is a clear danger that without selection pressure in the breeding programs, current resistance levels may not be maintained (Dean and Davis, 1990). Total reliance on resistance is not necessary but it could complement the use of heat therapy and phytosanitation to control RSD

(Comstock *et al.*, 2001). Integrated methods of control including RSD resistant varieties that are heat treated and planted in nurseries for farmers would ensure the control of ratoon stunting disease in Fiji.

CHAPTER 6

CONCLUSIONS

The epidemiology of the ratoon stunting disease in the Fiji sugar industry showed that the disease is widespread in all the sugarcane sectors with varying amounts of infection. The incidence of RSD in Fiji is 27% with all the thirty eight sugar cane sectors infected with different levels of infection. The cultures were not only a good source of inoculum, but as a confirmation of the presence of ***Leifsonia xyli*** subsp. ***xyli*** The presence of ratoon stunting disease could be a contributory factor to yield decline of sugar in Fiji.

The continued presence of RSD in undetected field will always be a source of inoculum as manual harvesting done by cane cutting gangs and also the increased use of mechanical harvesters now available in Fiji. The spread of RSD from farm to farm will continue to increase until phytosanitary awareness programs are implemented. This includes the cleaning of cane knives used from field to field; the breeding for RSD resistance; and also the use of heat treated seed material as a recognized source of

certified clean seed to be used by farmers. This will help to reduce the ratoon stunting disease levels in Fiji.

As seen from the yield loss experiment, the other ratoons crop figures were similar to the average yield currently observed in the Fiji sugar industry of 45 - 70tonnes /ha. The plant figures showed that the disease was not so widespread in the plots to show any significant difference between treated and untreated plots. After the plant crop, the figures start to change (decline) and the effect of the presence of ratoon stunting disease coupled with dry conditions was observed with an average reduction of 36.6%. The average loss due to RSD in this trial was 28.7% in cane yield but cane quality (POCS) remained somewhat constant. The decline in the yield was attributed to the effect of the ratoon stunting disease as it is systemic and the further the ratoon crop is maintained and cultivated, the higher the disease will manifest itself.

The resistance of the variety Mana and the other varieties (Kaba, Vomo, Yasawa, Naidiri, Ragnar, Ono, Waya, Mana, Spartan and LF82-2252) reaffirms that although a variety is resistant in the plant stage, the ratoon stunting disease can increase its population to reduce the yield of the crop from plant crop to

other ratoons. Mana which covers almost 80% of the variety planted in Fiji is resistant but with the presence of inoculums via the use of infected knife or harvester, the disease can easily spread and show its effect in the ratoon crops. Breeding for RSD resistance by selecting RSD resistant parents will ensure that RSD resistant progenies will be released to growers, therefore reducing the effect of RSD to acceptable levels. Continued management method should include both breeding for resistance and hot water treatment as means of reducing disease to minimal levels. The control of RSD could be achieved by planting RSD – resistant varieties that have undergone hot water treatment and planted in clean seed nurseries.

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Appendices

Appendix 1: RSD SURVEY FORM

A. FIELD

Sample Number:_____ Sample Collector:_____
Sampling date:_____ Sector:_____
Location:_____ Farm Number:_____
Farmer's Name_____ Cane Variety_____
Age:_____ Ratoon category_____
Topography:_____ Soil Type:_____
Area of field (Ha):_____ Date of analysis:_____

B. LABORATORY DIAGNOSIS

Total volume of sap collected (ml)_____

Stalk no.	Date diagnosed	PCM	Date diagnosed	ELISA
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				

Appendix: 2 RSD sugar cane sample survey

LAUTOKA MILL

CROP / SECTOR	PLANT (P)	RATOON (R)	OTHER RATOON (S)
Drasa	2	4	9
Lovu	1	2	12
Lautoka	0	7	8
Saweni	0	12	3
Natova	1	7	7
Legalega	0	11	4
Meigunyah	1	7	7
Qeleloa	0	5	10
Malolo	0	2	13
Yako	2	3	10
Nawaicoba	0	5	10
Lomawai	0	7	8
Cuvu	0	6	9
Olosara	2	5	8
TOTAL	**7**	**79**	**109**
SAMPLE PERCENTAGE (%)	**3.6**	**40.5**	**55.9**

RARAWAI MILL

CROP / SECTOR	PLANT (P)	RATOON (R)	OTHER RATOON (S)
Varoko	0	4	11
Mota	0	6	9
Koronubu	0	9	6
Rarawai	0	10	5
Veisaru	0	12	3
Varavu	0	4	11
Naloto	0	7	8
Tagitagi	0	8	7
Drumasi	0	15	0
Yaladro	0	14	1
TOTAL	**0**	**89**	**61**
SAMPLE PERCENTAGE (%)	**0**	**59.3**	**40.7**

PENANG MILL

CROP / SECTOR	PLANT (P)	RATOON (R)	OTHER RATOON (S)
Nanuku	0	12	3
Malau	0	9	6
Ellington 1	0	11	4
Ellington 2	0	11	4
TOTAL	**0**	**43**	**17**
SAMPLE PERCENTAGE (%)	**0**	**71.7**	**28.3**

LABASA MILL

CROP / SECTOR	PLANT (P)	RATOON (R)	OTHER RATOON (S)
Waiqele	1	6	8
Wailevu	3	4	8
Vunimoli	4	6	5
Labasa	3	7	5
Bucaisau	1	7	7
Wainikoro	4	7	4
Daku	0	13	2
Natua	1	2	12
Solove	2	9	4
Bulivou	4	4	7
TOTAL	**23**	**65**	**62**
SAMPLE PERCENTAGE (%)	**15.3**	**43.4**	**41.3**

Appendix 3: % POCS Calculations from Polarimeter Recordings

a. % Cane Sugar in juice = $\frac{\text{(Pol reading) x 26.00}}{\text{99.718 x App sp gravity } 20/20^0\text{C}}$

where – 26.00 g is the normal weight when the polarimeter used is fitted with the international scale.

- 99.718 x app. sp gravity $20/20^0$C is equal to the weight in grams of 100 ml solution.

* Apparent specific gravity $20/20^0$C is obtained from table 16 In "Cane Sugar Handbook" (Meade and Chen, 1977).

* "Pol reading" is the reading obtained from polarimeter.

b. % Cane sugar in cane = % cane sugar in juice x $\frac{\text{100-(\%Fibre+5)}}{100}$

c. % Soluble solids in cane = Brix of juice x $\frac{\text{100-(\%Fibre+3)}}{100}$

d. % Impurities in cane = (% Soluble solids in cane) – (% Cane sugar in cane)

e. % POCS = % Cane sugar in cane – ½ (% Impurities in cane)

Appendix 4: Statistical Analysis for Yield Loss Experiment

Statistix 8.0 LC0102P8, 8/30/2005, 4:57:10 PM

Randomized Complete Block AOV Table for tcha

Source	DF	SS	MS	F	P
block	3	4742.67	1580.89		
trt	2	1801.50	900.75	2.44	0.1680
Error	6	2217.83	369.64		
Total	11	8762.00			

Grand Mean 134.00 CV 14.35

Tukey's 1 Degree of Freedom Test for Nonadditivity

Source	DF	SS	MS	F	P
Nonadditivity	1	125.03	125.033	0.30	0.6082
Remainder	5	2092.80	418.560		

Relative Efficiency, RCB 1.77

Means of tcha for trt

trt	Mean
0	119.25
2	133.50
3	149.25

Observations per Mean 4
Standard Error of a Mean 9.6130
Std Error (Diff of 2 Means) 13.595

Statistix 8.0 LC0102P8-POCS, 8/30/2005, 4:58:18 PM

Randomized Complete Block AOV Table for POCS

Source	DF	SS	MS	F	P
block	3	10.4167	3.47222		
trt	2	0.6350	0.31750	1.34	0.3296
Error	6	1.4183	0.23639		
Total	11	12.4700			

Grand Mean 10.750 CV 4.52

Tukey's 1 Degree of Freedom Test for Nonadditivity

Source	DF	SS	MS	F	P
Nonadditivity	1	0.24892	0.24892	1.06	0.3495
Remainder	5	1.16941	0.23388		

Relative Efficiency, RCB 4.42

Means of POCS for trt

trt	Mean
0	10.425
2	10.925
3	10.900

Observations per Mean 4
Standard Error of a Mean 0.2431
Std Error (Diff of 2 Means) 0.3438

Statistix 8.0 LC0102P8-sucrose, 8/30/2005, 4:59:46 PM

Randomized Complete Block AOV Table for tsha

Source	DF	SS	MS	F	P
block	3	38.9167	12.9722		
trt	2	28.1667	14.0833	2.83	0.1361
Error	6	29.8333	4.9722		
Total	11	96.9167			

Grand Mean 14.417 CV 15.47

Tukey's 1 Degree of Freedom Test for Nonadditivity

Source	DF	SS	MS	F	P
Nonadditivity	1	3.9926	3.99255	0.77	0.4197
Remainder	5	25.8408	5.16816		

Relative Efficiency, RCB 1.34

Means of tsha for trt

trt	Mean
0	12.500
2	14.500
3	16.250

Observations per Mean 4
Standard Error of a Mean 1.1149
Std Error (Diff of 2 Means) 1.5767

Appendix 5: ELISA Diagnosis Procedure

This procedure is done in two days to complete. After the cane sap is defrosted, the samples are ready for diagnosis. This procedure is adapted from Croft, B.J., Teakle, D.S. and Leaman, T.M., 1993. Serological diagnostic tests for ratoon stunting disease. In **Sugarcane Instruction Manual, BSES publication**

DAY ONE

1) The samples are defrosted and each vial sap is noted.
2) The vials are shaken on the shaker and suspended matter allowed to settle for a minute.
3) With a pipette, 0.2ml of each sample is added to the micro plate beginning at column 2, leaving column 1 for the standards. After filling each well, the pipette tips are soaked into a detergent solution. Column 1 well E1 and G1 are used for positive controls while column 1 well F1 and H1 are used for negative controls.
4) The micro plate(s) are then stacked in each centrifuge plate holders and are centered. It is important that the holders have the same weight to avoid spillage. The centrifuge machine is set at 3000rpm for 20 minutes and the machine started.
5) The liquid from the centrifuged plates is removed using the immuno washer starting at column 2 to column 12. The immuno washer tips are rinsed in distilled water, and then liquid from row 1 is removed. Wash immuno washer again in distilled water and process next plate to avoid any of the positive standards in

row 1 accidentally carrying over to the subsequent rows.

6) Add 0.2ml coating buffer to each row using a pipette.
7) Re-centrifuge plates as above for 10 minutes at 3000rpm.
8) Remove liquid from plate with immuno washer as in step 5 above.
9) Add 0.2ml coating buffer as in step 6 above and re-centrifuge plates as in step 7 for 10 minutes at 3000rpm.
10) Remove liquid from plate with immuno washer as in step 5 and dry in incubator over night at 37°C.

DAY 2

11) Fill the wells with PBS-Tween and soak for 5 minutes
12) Weigh out 1g of skim milk and mix with 20ml PBS-Tween. Stir immediately and rapidly with a stirring rod.
13) Remove PBS-Tween from the wells using immuno washer and repeat step 11.
14) Remove PBS-Tween from the wells and refill with skim milk solution. Stand the plate at room temperature for 30 minutes. Take care not to scratch the slides of the wells with the immuno washer from hereon.
15) Weigh out 1g of skim milk for Antiserum dilution with 40ml PBS-Tween and 1 needle drop of Antiserum A in 15ml solution and 1 drop of Antiserum B in 15ml solution per plate.

16) Remove skim milk solution after 30 minutes and refill wells with PBS-Tween and allow it to soak for 5minutes.
17) Add a drop of Antiserum A into skim milk solution and stir on a magnetic stirrer.
18) Remove PBS-Tween from the wells and add 0.1ml of Antiserum A solution into the wells and allow it to stand for an hour.
19) Add a drop of Antiserum B into skim milk solution and stir using magnetic stirrer. Remove substrate tablets from freezer and allow it to reach room temperature before using in step 23.
20) Remove Antiserum A solution using immuno washer and refill with PBS-Tween and allow it to stand for 5 minutes.
21) Remove PBS-Tween solution from the wells and refill with 0.1ml of Antiserum B solution and allow it to stand for an hour.
22) Remove PBS-Tween solution from the wells and refill with the same solution and allow it to soak for 5 minutes. Repeat this step for four consecutive times.
23) Dissolve 5mg substrate tablets in 10ml distilled water to make the substrate buffer solution.
24) Remove PBS-Tween from the wells and add 0.1ml of substrate buffer. Make sure there are no bubbles and the bottom of the plate wiped clean
25) Place the micro plate in the micro plate reader and read at time = 0, time = 30 minutes and time = 60 minutes

26) After retrieving all the wanted data, subtract values of time 30 minutes from time 60 minutes to attain the differential absorbance reading.

SOLUTIONS REQUIRED FOR EB-EIA PROCEDURES

i. Phosphate-buffered saline (PBS) stock solution

Phosphate-buffered saline (PBS) is a reagent that stimulates the conditions found in blood serum. Antiserum is able to behave in PBS much as it would in blood serum, with little or no less in activity or specificity.

To make 500ml of a 10x stock PBS solution:

42.37g Sodium chloride (NaCl)

0.525g Potassium di-hydrogen phosphate (KH_2PO_4) – anhydrous

3.0g di-potassium hydrogen phosphate (K_2HPO_4) – anhydrous

1.0g Sodium Azide (NaN_3)

1. Dissolve the above ingredients in 500ml deionised water.
2. Adjust pH to 7.2 using HCL and or NaOH. Store on the shelf until needed.
3. Dilute 1/10 in deionised water before use.

ii. PBS-Tween stock solution

PBS-Tween is a buffered detergent solution used to wash away excess antiserum from the wells.

To make 500ml PBS-Tween:

500ml	1xPBS
0.25ml	TWEEN-20

1. Add 0.25ml TWEEN-20 to 500ml 1x PBS and mix.

iii. Coating buffer

Coating buffer is used to assist the adhesion of the blocking solution to the wells of the ELISA plate in EB-EIA tests.

To make 500ml coating buffer

0.8g	Sodium carbonate (Na2CO3)
1.47g	Sodium bicarbonate (NaHCO3)
0.2g	Sodium azide (NaN3)
500ml	Deionised water

1. Adjust pH to 9.8 with 10M HCl. Store in refrigerator.

Appendix 6: ELISA DIAGNOSIS FOR RSD RESISTANCE SCREENING

Date of diagnosis: 14/02/02
All reps

Well	Variety	Read # 1	Read # 2	Read # 3
E1	Standard	0.047	0.368	0.692
F1	Standard	0.045	0.254	0.535
G1	Standard	0.009	0.127	0.292
H1	Standard	-0.001	-0.022	-0.012
A2	LF82-2252R1	0.045	0.202	0.373
B2	Naidiri	-0.033	-0.11	-0.202
C2	Beqa	-0.021	-0.062	-0.114
D2	Ono	-0.031	-0.197	-0.407
E2	Galoa	-0.054	-0.182	-0.325
F2	Aiwa	-0.039	-0.122	-0.207
G2	Yasawa	-0.016	-0.097	-0.191
H2	Ragnar	-0.022	-0.166	-0.37
A3	Vatu	0.042	0.361	0.635
B3	Kaba	-0.046	-0.204	-0.402
C3	Homer	-0.022	-0.068	-0.082
D3	Mana	-0.02	-0.177	-0.372
E3	Waya	0.01	0.024	0.041
F3	Vomo	-0.03	-0.151	-0.27
G3	Spartan	-0.02	-0.13	-0.253
H3	Mali R1	*	*	*
A4	Beqa R2	0.01	0.117	0.269
B4	Ono	-0.055	-0.14	-0.241
C4	Vomo	0.039	0.207	0.399
D4	Homer	0.053	-0.091	-0.224
E4	Mali	-0.016	-0.071	-0.129
F4	Mana	0.038	0.193	0.421
G4	Naidiri	-0.02	-0.135	-0.247
H4	Aiwa	0.033	0.234	0.541
A5	Vatu	0.008	0.231	0.375
B5	Kaba	-0.037	-0.138	-0.254
C5	Ragnar	0.038	0.169	0.339
D5	Yasawa	0.04	-0.07	-0.229

E5	LF82-2252	0.022	0.039	0.017
F5	Spartan	-0.004	-0.084	-0.158
G5	Galoa	0.013	-0.114	-0.234
H5	Waya R2	-0.002	-0.123	-0.222
A6	Vatu R3	0.024	0.189	0.351
B6	Homer	0.032	0.286	0.507
C6	Waya	0.034	0.182	0.322
D6	Spartan	-0.038	-0.186	-0.346
E6	Mana	0.023	-0.038	-0.057
F6	Kaba	0.032	0.065	0.075
G6	Vomo	0.004	0.063	0.115
H6	Mali	0.059	0.222	0.405
A7	Galoa	0.217	0.768	0.861
B7	Yasawa	-0.057	-0.22	-0.427
C7	Aiwa	-0.022	-0.038	0.008
D7	Ragnar	-0.069	-0.261	-0.493
E7	LF82-2252	-0.039	-0.179	-0.332
F7	Ono	0.063	0.112	0.312
G7	Beqa R3	0.005	0.163	0.317

ELISA DIAGNOSIS FOR RSD RESISTANCE SCREENING

Date of diagnosis: 25/01/02
All reps

Well	Variety	Read # 1	Read # 2	Read # 3
E1	Standard	0.008	0.2	0.407
F1	Standard	0.146	0.001	-0.112
G1	Standard	-0.017	0.003	0.086
H1	Standard	0.006	0.175	0.336
A2	LF82-2252	0.12	-0.027	-0.136
B2	SpartanR3	0.01	0.05	0.11
C2	Vatu	-0.005	-0.019	-0.049
D2	Waya	0	-0.067	-0.109
E2	Kaba	-0.022	-0.099	-0.145
F2	Ragnar	-0.016	-0.151	-0.252
G2	Mali	-0.003	0.32	0.577
H2	Galoa	0.077	0.464	0.74
A3	Aiwa	-0.026	-0.167	-0.272
B3	Vomo	-0.02	-0.069	-0.283
C3	Homer	-0.006	-0.069	-0.112

D3	Beqa	-0.002	0.323	0.464
E3	Mana	-0.001	0.006	0.008
F3	Yasawa	-0.021	-0.158	-0.256
G3	Ono R3	-0.021	-0.094	-0.138
H3	Naidiri	*	*	*
A4	Waya R2	-0.013	-0.054	-0.051
B4	Vatu	-0.022	-0.17	-0.277
C4	Galoa	-0.016	-0.044	-0.085
D4	Homer	0.007	0.621	0.87
E4	Kaba	-0.014	-0.154	-0.251
F4	LF82-2252	-0.02	-0.16	-0.263
G4	Spartan	0.009	0.239	0.44
H4	Naidiri	-0.011	0.087	0.176
A5	Mana	-0.028	-0.193	-0.318
B5	Ono	-0.02	-0.18	-0.288
C5	Ragnar	-0.01	-0.088	-0.139
D5	Aiwa	-0.005	-0.058	-0.075
E5	Yasawa	-0.004	-0.043	-0.082
F5	Vomo	-0.001	-0.155	-0.262
G5	Beqa	-0.044	-0.181	-0.271
H5	Mali R2	-0.019	-0.156	-0.251
A6	Aiwa R1	-0.022	-0.095	-0.173
B6	Kaba	-0.015	-0.188	-0.318
C6	Vomo	0.001	0.119	0.231
D6	Vatu	0.024	0.106	0.188
E6	LF82-2252	-0.004	-0.056	-0.085
F6	Yasawa	-0.018	-0.182	-0.301
G6	Naidiri	-0.022	-0.1	-0.153
H6	Ragnar	-0.001	0.197	0.407
A7	Homer	0.102	1.017	1.036
B7	Ono	-0.026	-0.2	-0.331
C7	Waya	-0.007	0.069	0.111
D7	Beqa	-0.012	-0.163	-0.27
E7	Mana	-0.027	-0.182	-0.287
F7	Spartan	-0.026	-0.201	-0.33
G7	Galoa R1	-0.02	-0.002	0.012