COMPARISON OF RSD ASSAYS FOR DIAGNOSIS AND SCREENING VARIETIES FOR RESISTANCE

By

BJ CROFT[1], J GREEN[2], KS BRAITHWAITE[3]
BSES [1]Woodford, [2]Mackay, [3]Indooroopilly
bcroft@bses.com.au

KEYWORDS: Ratoon Stunting Disease, *Leifsonia xyli* subsp. *xyli*, Tissue Blot Immunoassay, ELISA.

Abstract

RATOON STUNTING DISEASE (RSD), caused by the bacterium *Leifsonia xyli* subsp. *xyli*, has no specific external symptoms and internal symptoms are often unreliable. Immunoassays are widely used to diagnose the disease because they are sensitive, relatively inexpensive and are suitable for bulk screening. The most widely used immunoassays for RSD diagnosis are the enzyme-linked immunosorbent assay (ELISA) and the stalk tissue blot immunoassay. Phase-contrast microscopy is also used for diagnosis but one person can only process 100–200 samples in a day. These assay methods all use extracts from stalks of mature cane. Collecting stalks from mature crops of cane is heavy work and requires bulky stalk samples to be transported to the site where they are processed. RSD bacteria are known to occur in roots, stalks, leaf sheaths and leaves. Bacteria occur at low concentration in leaves. There have been two previous studies that have identified moderate to high levels of bacteria in leaf sheaths. Samples of leaf sheaths can be easily and quickly taken in the field and can be transported in small plastic bags. In this paper, we compare five different diagnostic methods for RSD; phase-contrast microscopy of extracts from stalks and leaf sheaths, ELISA on extracts from stalks and tissue blot immunoassay of stalks and leaf sheaths. Tissue blot of stalk tissue was the most sensitive method followed closely by ELISA. Tissue blot of leaf sheath tissue and phase-contrast microscopy had similar sensitivity but were less sensitive than tissue blot of stalk tissue and ELISA. The ELISA and tissue blot of leaf sheaths were compared for screening varieties for resistance to RSD. Twenty seven varieties were screened for resistance with both methods. The absorbance from the ELISA was highly correlated with the percent infected vascular bundles in the leaf sheath. This paper discusses the advantages and disadvantages of the various diagnostic assays.

Introduction

Diagnosis of ratoon stunting disease (RSD) has always played an integral role in the management of this disease. Steindl (1961) observed that the disease was associated with an orange-red discoloration of the vascular bundles at the nodes when stalks are sliced longitudinally.

Electron microscopy was initially used to identify the bacterium (Teakle *et al.*, 1973) and then phase contrast and dark field microscopic methods were developed (Gillaspie *et al.*, 1973). Microscopic methods use vascular extracts taken from mature stalks. Serological methods such as the stalk tissue blot enzyme-linked immunoassay (STB, Harrison and Davis, 1988) and the evaporative binding enzyme-linked immunoassay or ELISA (Croft *et al.*, 1994) were developed to enable processing of large numbers of samples.

RSD bacteria are found in xylem vessels in all parts of the plant but the highest concentrations are reported to be in the lower parts of the stalk (Bailey, 1977). Teakle *et al.* (1975) reported that diffusates from stalks, leaf sheaths and leaf mid-ribs could be used for diagnosis with an electron microscope. They found that the oldest leaf sheaths had the highest concentrations of bacteria. In Mauritius, the ratio of bacteria in stalks and leaf sheaths was reported to be 1.6:1 and in leaf sheaths, leaf mid-ribs and leaf blades the ratios were 25:5:1 (Anon., 1988).

This paper reports on experiments to compare phase contrast microscopy, ELISA and tissue blot of stalk and leaf sheath tissues for diagnosis of RSD. In addition, ELISA and tissue blot of leaf sheaths were compared for rating varieties for resistance to RSD.

Materials and methods

Plant samples and assays

All samples of RSD-infected and disease-free cane were collected from the BSES Woodford Experiment Station.

The RSD resistance screening experiment was planted at the BSES Woodford Experiment Station. One-eye setts were inoculated with RSD-infected juice of Q110 extracted with a small mill. The juice was filtered through 4 layers of fine nylon material and was applied to the setts of the test varieties as they were cut with a Felco 19 spraying secateur in July 2010.

The setts were germinated in peat pots and transplanted to the field in September 2010. There were three replicates of 27 varieties planted in a randomised complete block design with eight plants in each plot (Table 4). Eight varieties of known resistance to RSD were included in the experiment.

Stalk vascular extracts for ELISA and phase contrast microscopy were collected by applying positive air pressure to stalk pieces. The stalk pieces were taken from towards the base of the stalks. The extracts were collected in microcentrifuge tubes and frozen at –20 °C until required. In some experiments, an additional extract was collected for phase contrast microscopy by centrifuging sections of leaf sheaths.

Three to four leaf sheath sections were placed in a 1.5 mL microcentrifuge tube and centrifuged at 5000 rpm for 5 min. The plant tissue was removed and the vascular extract was stored at –20 °C until required.

The ELISA procedure has been described by Croft (2002). Absorbance of the colour reaction in the ELISA was read on a Biorad microplate reader at 405 nm. Samples with an absorbance reading of >0.05 were considered positive.

Phase contrast microscopy (PCM) was performed on an 8 µL aliquot of the vascular extracts examined at 1000 magnification. Ten fields of view were examined and the concentration of RSD bacteria was scored on a 0–5 scale where: 0 = no bacteria, 1 = <1, 2 = 1, 3 = 2–10, 4 = 10–20 and 5 = 20–40 bacterial cells/field of view.

Samples for STB were taken from a sound internode at the base of the stalk. An 8 mm longitudinal core was taken from an internode, one end of the core was cut transversely with a safety razor and the fresh cut was blotted by pressing it firmly onto a 0.45 µm pore size nitrocellulose membrane.

In one experiment, the whole stalk was assayed by removing the rind from the stalk section, cutting the section into quarters and blotting each quarter onto a membrane. The membranes were dried at 70 °C for 1 hr and stored until they were processed. The enzyme-linked immunoassay procedure for the STB has been described by Harrison and Davis (1988).The total number of

vascular bundle imprints and the number with the positive blue coloration were counted under a dissecting microscope. The percent positive vascular bundles was calculated.

Samples for the leaf sheath tissue blot enzyme-linked immunoassay (LSTB) were taken from the oldest green leaf sheath except where noted. A 12 × 100 mm strip of leaf sheath was taken from the centre of the base of the leaf sheath.

The basal end of the strip was cut with sharp secateurs and the cut end was blotted by firmly pressing it on a nitrocellulose membrane. In some experiments, the last 10 mm of the strip of the leaf sheath above the cut end was squeezed between a plastic pinch-type hose clamp (Nunc) before it was blotted onto the membrane.

The LSTB membranes were processed by the same procedure as the STB and the same method was used to assess the results.

Two experiments were conducted to investigate whether sampling different leaf sheaths on the same stalk would improve diagnosis in moderately resistant and resistant varieties. In the first experiment, three leaf sheaths starting with the oldest green leaf sheath were sampled from two stalks of four varieties and processed with the LSTB.

Samples from the same stalk were processed with the STB. Extracts from stalks and leaf sheaths were also examined by phase contrast microscopy and ELISA. A stalk from a disease-free plot of Q110 was included as a control.

The second experiment was designed to test if the distribution of the infected vascular bundles within the stalk was related to the infected vascular bundles in the leaf sheaths.

Samples were taken from six stalks and the stalk and leaf sheaths were blotted so that the alignment on the blot was exactly the same as occurred on the stalk before the samples were taken (Figure 3).

A cross section from the base of the stalk was blotted onto the membrane by carefully removing the rind, cutting the stalk into quarters and blotting each quarter onto the membrane. Each leaf sheath was divided down the centre in line with the corresponding bud on the stalk and each half of the leaf sheath was blotted onto the membrane so that it was in the same relative position to the stalk blot.

The older leaf sheaths were blotted closest to the stalk blot. Two of the six samples were taken from disease-free plots to act as negative controls.

Statistical analysis

An analysis of variance of the results from the RSD resistance experiment was conducted on the absorbance from the ELISA and the logit-transformed percent infected vascular bundles in the LSTB using SAS Mixed model procedure (SAS Institute, USA). Pair-wise comparisons of means were conducted using the Tukey-Kramer method ($P<0.05$).

Results and discussion

Diagnostic assay methods

The vascular bundles in the leaf sheath (Figure 1) occur at regular intervals between parenchyma cells and air spaces. Densely packed thick walled sclerenchyma cells, immediately above and below the vascular bundles, give the leaf sheath a rigid framework.

The LSTB was able to detect RSD infection in initial trials. Squeezing vascular bundle contents out of the leaf sheath increased the area of blue stain in positive samples, making it easier to identify positive samples (Figure 2).

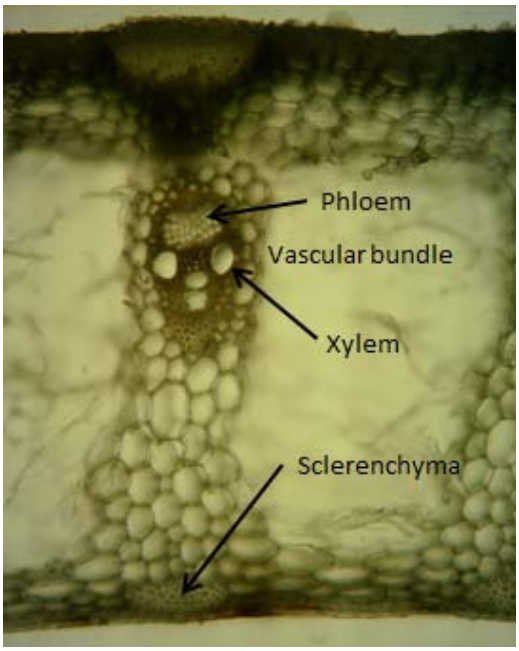

Fig. 1—Cross section through a sugarcane leaf sheath.

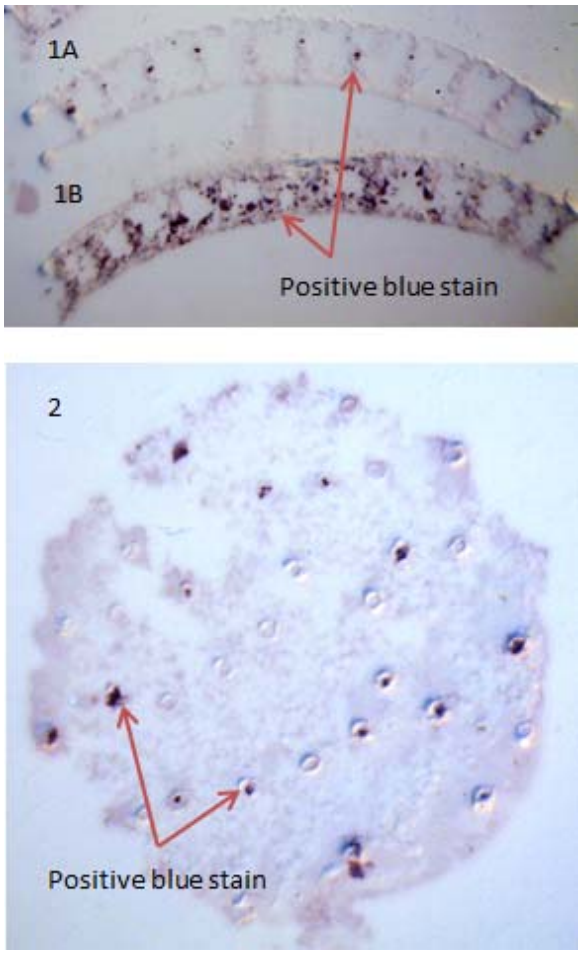

Fig. 2—Typical positive blue stain in tissue blots of leaf sheath (1) and stalk (2). The same leaf sheath was printed onto the membrane with (1B) and without squeezing (1A) to force vascular fluid out of the tissue. Both leaf sheath and stalk samples were from the same stalk of RSD-infected Q170$^{(b)}$.

Five RSD diagnostic methods were used to test 72 samples from 19 varieties. The LSTB was tested with and without squeezing the leaf sheaths. The samples included 15 taken from putatively disease-free sources and 57 from sources inoculated with RSD. No RSD was detected in any of the samples from the disease-free sources by any method (Table 1).

The STB detected the highest number of positive samples from the RSD-inoculated cane, followed closely by the ELISA. The ELISA failed to detect RSD in two samples that were positive with the STB, but ELISA and PCM on the leaf sheath detected RSD in another sample that the STB failed to detect. Previous comparisons have shown that the STB is more sensitive than the ELISA (Hoy *et al.*, 1999).

PCM on stalk extracts has been the standard method used in Australia for microscopic diagnosis. This method was less sensitive than the STB and ELISA, failing to detect five positives that were identified with the other methods.

The LSTB with and without squeezing was less sensitive than the STB and ELISA, failing to detect bacteria in eight and seven samples respectively that were positive with the other methods. Squeezing the leaf sheath to force vascular contents onto the cut surface slightly improved the sensitivity of the LSTB and made it easier to observe positive reactions in the assay.

All three samples of the intermediate-resistant variety, Q183(ᵎ) from the RSD source were positive with the STB and ELISA but were negative with the LSTB and PCM on leaf sheath extracts. Most of the other samples that the LSTB failed to correctly diagnose were from varieties rated intermediate or resistant to RSD and with low numbers of infected vascular bundles in the STB and low absorbance readings in the ELISA.

PCM on leaf sheath extracts was the least sensitive method. However, the number of bacterial cells per field of view was estimated to be seven times higher in leaf sheath extracts compared to stalk extracts for those samples that were positive by both methods (data not shown). Extremely high numbers of bacterial cells were observed in the leaf sheath extracts from some samples.

Table 1—Summary of number of positive and negative reactions in 57 inoculated and 15 disease-free samples tested for RSD with five methods. The LSTB method was tested with and without first squeezing the leaf sheath.

Method	Source			
	RSD-inoculated		RSD-free	
	Pos.	Neg.	Pos.	Neg.
STB	43	14	0	15
ELISA	42	15	0	15
PCM stalk	38	19	0	15
LSTB squeeze	36	21	0	15
LSTB direct	35	22	0	15
PCM leaf sheath[1]	33	23	0	15

[1]No extract was available from one leaf sheath sample

In the experiment to compare detection of RSD in different leaf sheaths on the same stalk, the eight stalks tested were rated positive with ELISA, PCM and STB (Table 2). The STB had only 3% infected vascular bundles in samples 5, 6 and 7. The LSTB on samples from the same stalk gave variable results with the oldest and second oldest leaf sheaths detecting 6 of the 8 positive samples and the third oldest leaf sheath detecting only 4 of the 8 positive samples.

LSTB of the oldest leaf sheath failed to detect disease in the two samples of Q183(ᵎ) which had low numbers of infected vascular bundles in the STB. The LSTB failed to detect disease in this

variety in the previous experiment. One of the samples of Q183$^{\phi}$ did produce a positive result for the second oldest leaf sheath. Detailed observations were made on the leaf sheaths in this experiment. Some leaf sheaths were only partly green with, in some cases, up to 50% of the leaf sheath brown and desiccated.

In some samples, parts of the leaf sheath had internal reddening from fungal infection. There was no clear relationship between the health of the leaf sheath and the reaction in the LSTB.

Table 2—Comparison of ELISA, PCM, STB and LSTB performed on multiple samples from nine stalks. All TB results are the percent infected vascular bundles.

Sample number	Variety	ELISA Absorb.	PCM Stalk	STB	LSTB LS1[2]	LSTB LS2	LSTB LS3
1	Q110[1]	–0.01	0	0	0	0	0
2	Q110	0.35	3	34	10	50	0
3	Q110	0.16	2	21	8	15	31
4	Q138	0.53	4	22	50	10	18
5	Q138	0.16	2	3	22	20	0
6	Q183$^{\phi}$	0.27	3	3	0	11	0
7	Q183$^{\phi}$	0.14	2	3	0	0	0
8	KQ228$^{\phi}$	0.34	2	27	27	0	23
9	KQ228$^{\phi}$	0.35	3	13	42	27	33
	Average		3	16	20	17	13

[1] Sample from RSD-free plot
[2] LS1 = the oldest green leaf sheath, LS2 = second oldest and LS3 = third oldest

The leaves and leaf sheaths on sugarcane are arranged alternately at each node with the centre of the leaf sheath corresponding with the bud at the node. The RSD-infected vascular bundles within the stalk are not always evenly distributed across the stalk, especially in lightly infected stalks, where only one or two vascular bundles may be infected.

Because the connection of the leaf sheaths alternate on each side of the stalk, it was thought that the leaf sheath on the same side of the stalk as an infected vascular bundle in the stalk may be more likely to also be infected. The distribution of RSD infected vascular bundles were compared in four RSD infected stalks and four leaves on these stalks.

The samples from Q110, Q138 and Q242$^{\phi}$ gave a high percentage of infected vascular bundles in all stalk sections and in nearly all leaf sheath sections (Table 3). For these varieties, it appears that the samples could be taken for the LSTB from any position on any of the older leaf sheaths. One half of the youngest leaf sheath in Q110 had no infected vascular bundles.

Again, Q183$^{\phi}$ had a low percentage of infected vascular bundles in most sections of the stalk and only two of the leaf sheath blots had infected vascular bundles. The ELISA and PCM were both positive for the sample of Q183$^{\phi}$.

The number of infected vascular bundles in the stalk of Q183$^{\phi}$ relative to the infected vascular bundles in the leaf sheaths is illustrated in Figure 4.

The quarter of the stalk with the highest number of infected vascular bundles aligned with the oldest leaf sheath section with the highest number of vascular bundles. This suggests that there is a relationship between distribution of infected vascular bundles in the stalk and in corresponding leaf sheaths, but many more samples would be required to confirm this relationship.

It may be possible to increase the chances of a correct diagnosis with the LSTB by sampling at least two alternate leaf sheaths on a stalk.

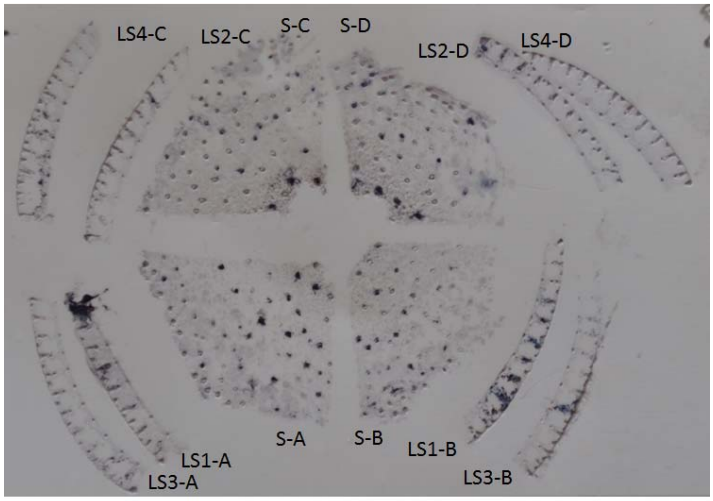

Fig. 3—Tissue blot of cross sections of a stalk and four leaf sheaths of Q138. The stalk sections were labelled S-A, S-B, S-C and S-D .The four leaf sheaths were blotted on the same side of the stalk as they occurred *in situ*. The oldest green leaf sheath was labelled LS1 and next oldest is LS2 and so on. Each leaf sheath was cut in half down the centre and the left and right half were labelled A and B for leaf sheaths 1 and 3 and C and D for leaf sheaths 2 and 4 so that the letter corresponded with the stalk piece with the same letter. A positive TB result is shown by the dark blue stain in the vascular bundles.

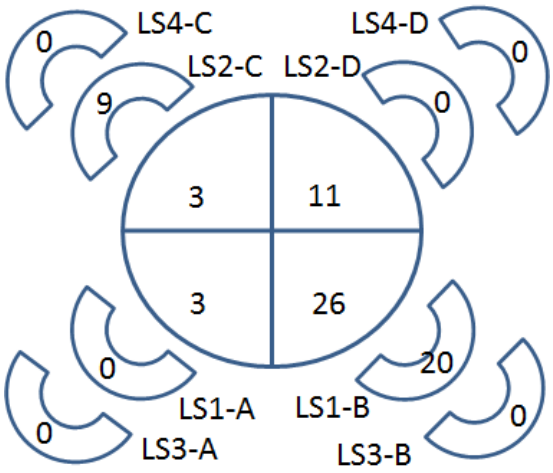

Fig. 4—Diagrammatic representation of the distribution of RSD-infected vascular bundles in one stalk of Q183$^{\phi}$. The percent infected vascular bundles for each sub-sample are shown by the figures. The stalk is represented by the circle cut in quarters and the leaf sheaths by the semicircular sections. The arrangement of the leaf sheath samples is explained in Figure 3.

Varietal resistance to RSD

Comparison of yield loss between disease-free and infected plots has been used to rate varieties for resistance or tolerance to RSD (Gillaspie and Teakle, 1989). It is expensive to conduct yield trials, especially when plots of both disease-free and RSD-infected cane have to be included for each variety.

The number of bacteria in vascular extracts taken from different varieties is correlated with yield loss of the varieties (Koike *et al.*, 1982). Harrison and Davis (1988) showed that the number

of infected vascular bundles in the STB was strongly correlated with the number of bacteria in vascular extracts and that both these measurements were correlated with the resistance of the varieties to RSD.

Table 3—Comparison of ELISA, PCM, STB and LSTB performed on multiple samples from six stalks. The code for the position of the samples from stalks and leaf sheaths is shown in Figure 3. All TB results are the percent infected vascular bundles.

Assay method/ sample	RSD-Free		RSD-inoculated			
	Q110	Q138	Q110	Q138	Q183$^{\phi}$	Q242$^{\phi}$
ELISA absorb.	0.01	–0.01	0.13	0.17	0.03	0.68
PCM stalk	0	0	3	2	1	4
Tissue blots						
S-A	0	0	62	65	3	36
S-B	0	0	51	74	26	48
S-C	0	0	45	33	3	17
S-D	0	0	36	39	11	39
LS1-A	0	0	70	33	0	60
LS1-B	0	0	50	70	20	73
LS2-C	0	0	27	11	9	70
LS2-D	0	0	45	33	0	100
LS3-A	0	0	20	20	0	100
LS3-B	0	0	36	30	0	100
LS4-C	0	0	54	50	0	82
LS4-D	0	0	0	9	0	100

Varietal resistance to RSD is correlated with the absorbance in the ELISA of vascular extracts taken from different varieties (Croft, 2002), the percent infected vascular bundles in the STB (Comstock *et al.*, 1996) and bacterial concentration in leaf mid-rib tissue measured with quantitative polymerase chain reaction (PCR) (Grisham *et al.*, 2007). The rate of spread of RSD by harvesters is slower in varieties that have lower bacterial concentrations and fewer infected vascular bundles (Comstock *et al.*, 1996; Hoy and Grisham, 2006).

In the experiment comparing 27 varieties for resistance to RSD, there were significant differences between the varieties in the absorbance from the ELISA and the percent infected vascular bundles in the LSTB (Table 4). Although the ranking of varieties, based on the ELISA and LSTB, was in general agreement with the rating of the standard varieties, there were no significant differences between the standard varieties in the ELISA (Tukey-Kramer, $P<0.05$). The most susceptible standard, Q110, was significantly different to the resistant standards Q117, Q200$^{\phi}$ and Q208$^{\phi}$ in the LSTB test. There were more significant differences between varieties with the LSTB than the ELISA. There was a highly significant correlation between the ELISA absorbance and the percent positive vascular bundles from the LSTB for all 27 varieties was 0.73 (Pearson's correlation $P<0.0001$). This suggests that both methods would give similar ratings for varieties. The ELISA and LSTB showed that there is a wide range of susceptibility to RSD in the modern Australian commercial varieties. The important commercial varieties, Q200$^{\phi}$ and Q208$^{\phi}$, had low scores for both the ELISA and the LSTB. The highest ELISA absorbance and percent infected vascular bundles were found in the variety Q242$^{\phi}$.

The ELISA sampling for this experiment required 324 stalks of cane to be cut, transported to the shed and then the vascular fluid was extracted. Cutting, carrying, transporting and extracting from this many stalks is a physically demanding and time consuming exercise. The 12 × 100 mm leaf sheath sections for the LSTB can easily be cut in the field and transported to the laboratory in a small plastic bag. Cutting and blotting the samples in the laboratory is easy and quick. Processing

the LSTB membranes is less technically demanding, uses fewer consumables and uses less expensive laboratory equipment than the ELISA. The LSTB can give similar RSD resistance ratings for the varieties with less cost.

Table 4—Absorbance from the ELISA and the percent positive vascular bundles for the LSTB of 27 varieties inoculated with RSD, ranked in descending order of ELISA absorbance. The standard varieties are rated susceptible (S), intermediate (I) or resistant (R) based on previous experiments. Each figure is the mean of four samples taken from each of three replicates.

Variety	Standard rating	ELISA absorb.	LSTB % positive
Q242$^{\phi}$		0.98 a[1]	52 a
Q235$^{\phi}$		0.51 b	25 abcd
KQ228$^{\phi}$		0.33 bc	38 abc
Q238$^{\phi}$		0.30 bc	20 abcdef
Q171$^{\phi}$		0.26 bc	50 ab
Q110	S	0.24 bc	22 abcde
Q138	S	0.23 bc	11 cdefghi
Q124	S	0.19 bc	20 cdefghi
Q241$^{\phi}$		0.19 bc	12 defghij
Q237$^{\phi}$		0.18 bc	18 abcdef
Q226$^{\phi}$		0.17 bc	3 ghij
Q90		0.15 bc	33 abcd
Q183$^{\phi}$	I	0.14 bc	7 efghij
Q243$^{\phi}$		0.14 bc	4 fghij
Q212$^{\phi}$		0.13 c	4 fghij
Q210$^{\phi}$		0.12 c	27 abcdefg
Q117	R	0.12 c	1 ij
Q177$^{\phi}$	I	0.11 c	14 bcdefgh
Q231$^{\phi}$		0.10 c	1 ij
Q240$^{\phi}$		0.09 c	7 defghij
Q200$^{\phi}$	R	0.09 c	2 hij
Q208$^{\phi}$	R	0.09 c	2 hij
Q232$^{\phi}$		0.08 c	4 fghij
Empire		0.06 c	2 hij
MQ239$^{\phi}$		0.06 c	1 hij
KQ236$^{\phi}$		0.06 c	1 ij
Q230$^{\phi}$		0.05 c	0 j
Mean		0.19	14

[1]Values within a column followed by same letter are not significantly different using the Tukey-Kramer test (P<0.05). The significant differences were calculated on the logit-transformed data for the LSTB.

Conclusions

This paper has shown that LSTB can diagnose RSD but it is not as sensitive as STB and ELISA. Sampling more than one leaf sheath from a stalk might increase the sensitivity of diagnosis with the LSTB. Sampling the oldest two green leaves gave the best diagnosis.

The LSTB can be used to rate varieties for resistance to RSD. The LSTB will give similar ratings to the ELISA and will be considerably cheaper than the ELISA method.

Further testing of the LSTB for RSD diagnosis should be conducted because of the ease of sampling with this method. The problems with collecting, transporting and processing large numbers of stalks for the STB, ELISA and PCM methods limits the number of samples that can be handled. Increasing the number of samples collected from a field greatly increases the probability of detecting low-level infections and the LSTB may allow many more samples to be collected.

The LSTB is also suitable for situations where it is important to conserve stalks for propagation such as in quarantine glasshouses. Leaf sheaths should be investigated as the source of samples for PCR diagnostic methods. The increased sensitivity of PCR may increase the detection of bacteria in leaf sheath extracts.

Acknowledgements

We thank Andrew Greet, Sam Azzopardi, Micah Croft and Carrie Croft for their technical assistance.

REFERENCES

Anon. (1988) Annual report of the Mauritius Sugar Industry Research Institute, p 36.

Bailey RA (1977) The systemic distribution and relative occurrence of bacteria in sugarcane varieties affected by ratoon stunting disease. *Proceedings of South African Sugar Technologists' Association* **51**, 55–56.

Comstock JC, Shine JM Jr, Davis MJ, Dean JL (1996) Relationship between resistance to *Clavibacter xyli* subsp. *xyli* colonisation in sugarcane and spread of ratoon stunting disease in the field. *Plant Disease* **80,** 704–708.

Croft BJ (2002) A method for rating sugarcane cultivars for resistance to ratoon stunting disease based on an enzyme-linked immunoassay. *Australasian Plant Pathology* **31**, 63–66.

Croft BJ, Greet AD, Leaman TM, Teakle DS (1994) RSD diagnosis and varietal resistance screening in sugarcane using the EB-EIA technique. *Proceedings of the Australian Society of Sugar Cane Technologists* **16**, 143–151.

Gillaspie AG Jr, Davis RE, Worley JF (1973) Diagnosis of ratoon stunting disease based on the presence of a specific microorganism. *Plant Disease Reporter* **57**, 987–990.

Gillaspie AG Jr, Teakle DS (1989) Ratoon stunting disease. In 'Diseases of sugarcane-major diseases' (Eds C Ricaud, BT Egan, AG Gillaspie Jr, CG Hughes) pp. 59–80. (Elsevier: Amsterdam).

Grisham MP, Pan Y-B, Richard EP Jr (2007) Early detection of *Leifsonia xyli* subsp. *xyli* in sugarcane leaves by real-time polymerase chain reaction. *Plant Disease* **91**, 430–434.

Harrison NA, Davis MJ (1988) Colonisation of vascular tissues by *Clavibacter xyli* subsp. *xyli* in stalks of sugarcane cultivars differing in susceptibility to ratoon stunting disease. *Phytopathology* **78**, 722–727.

Hoy JW, Grisham MP (2006) Effects of harvester type, inoculum source, and cultivar on spread of ratoon stunting disease. *Sugar Cane International* **24**, 10–14.

Hoy JW, Grisham MP, Damann KE (1999) Spread and increase of ratoon stunting disease of sugarcane and comparison of disease detection methods. *Plant Disease* **83**, 1170–1175.

Koike H, Gillaspie AG Jr, Benda GTA (1982) Cane yield response to ratoon stunting disease. *International Sugar Journal* **84**, 131–133.

Steindl DRL (1961) Ratoon stunting disease. In 'Sugar cane diseases of the World Vol. 1 Major diseases' (Eds JP Martin, EV Abbott, CG Hughes) pp. 433–459. (Elsevier: Amsterdam).

Teakle DS, Smith PM, Steindl DRL (1973) Association of a small coryneform bacterium with the ratoon stunting disease of sugar-cane. *Australian Journal of Agricultural Research* **24**, 869–874.

Teakle DS, Smith PM, Hayward AC (1975) Diagnosis of RSD by electron microscopy of sugar-cane tissue diffusates. *Proceedings of the Queensland Society of Sugar Cane Technologists* **42**, 115–116.