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FINAL REPORT – SRDC PROJECTS:

BS187S – IMPLEMENTATION OF SENSITIVE PATHOGEN INDEXING METHODS IN SUGARCANE QUARANTINE

by

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SUMMARY

BSES provides a quarantine service primarily for sugarcane plant breeding, allowing safe intrastate and international exchange of germplasm. The aim of this project was to develop or obtain, and implement in quarantine, diagnostic tests for pathogens of key quarantine diseases of sugarcane. Important diseases include ratoon stunting disease, Fiji disease, sugarcane mosaic, leaf scald, maize and sugarcane streak, white leaf and grassy shoot.

ELISA testing is the industry benchmark for detection of ratoon stunting disease. This was compared with quantitative PCR for earlier and more sensitive detection. Samples were collected upon entry to quarantine and indexed using quantitative PCR. Following nine months growth, plants were screened by ELISA and the results compared. It was shown that this disease could not be reliably indexed upon entry and that samples should be collected after several months growth to increase the chance of detection.

A RT-PCR test was trialed and confirmed as suitable to index for Fiji disease. An internal control was developed to ensure confidence in the method during routine screening. Five Australian isolates and three overseas isolates were successfully detected using this test. RT-PCR was also the preferred method to test intrastate collections for sugarcane mosaic, after a comparison with ELISA. ELISA testing may be better for imported canes due to the variation between strains of mosaic. The internal control developed for Fiji disease indexing was also applied to this RT-PCR test. An extraction method for larger samples was developed to increase the likelihood of detection, when indexing for both mosaic and Fiji disease.

Several PCR tests and ELISA were compared for detection of leaf scald. PCR proved to be more sensitive, although the specificity of some tests was not adequate for quarantine. A PCR test reported to be highly specific was trialed to overcome false positives given with some tests. The new test gave improved specificity and will be trialed in a Bio-PCR system to obtain the greatest sensitivity possible.

PCR primers for the detection of maize and sugarcane streak viruses were designed from published sequences. These successfully amplified a specific fragment from plasmids containing segments of the genomes of these viruses. These primers were documented and the plasmid DNA retained as positive controls. An extraction protocol and PCR test for white leaf and grassy shoot phytoplasmas was obtained and documented. A test for the sugarcane smut fungus was also obtained. A test for sugarcane yellow leaf virus is available but was not implemented for routine use.

Sensitive indexing methods have been trialed and implemented for important quarantine pathogens of sugarcane. These methods will supplement visual indexing of glasshouse collections, upgrading quarantine to a state of best practice. Ongoing development of sensitive pathogen indexing methods will continue to maintain this level and guarantee safe exchange of sugarcane germplasm for the benefits of the Australian sugar industry.

1.0 BACKGROUND

BSES operates sugarcane quarantine for the Australian sugar industry, enabling germplasm to be moved with minimal risk of disease transfer both to and from other countries and between growing areas within Australia. This is principally to support BSES plant breeding, a program dependent on the exchange of germplasm, although services are available to other organisations. BSES quarantine facilities include containment glasshouses and laboratories at the David North Plant Research Centre, capable of processing approximately 60 imported clones and 150 interstation exchange clones each year. In addition, the facility holds a collection of Australian commercial varieties for export to other countries, in support of exchange agreements.

Pathogen indexing in BSES quarantine has relied almost exclusively in the past on visual detection of symptoms, which for some pathogens has very limited sensitivity. Recent progress in the field of molecular biology has facilitated the development of extremely sensitive, pathogen specific, diagnostic tests for numerous plant pathogens, including important quarantine pathogens of sugarcane. Tests are now available for ratoon stunting disease, Fiji disease, leaf scald, smut, sugarcane mosaic, gumming, maize streak, yellow leaf syndrome and white leaf/grassy shoot diseases. The regard of these tests as best practice justifies their implementation in quarantine for disease detection, although the practice of indexing is complicated by various issues.

Successful indexing of germplasm in quarantine involves implementing tests that give the best chance of detecting a pathogen. Consideration must be given to distribution of the pathogen within the plant, enabling a sampling and testing strategy to be developed for detection as soon as possible after entry to quarantine. Tests should prove to be reliable and cost effective, while retaining a high level of sensitivity and specificity for the target organism. Thirdly, test protocols must be feasible within quarantine operations and resources, allowing the application of this research for the benefit of the exchange program. Obtaining or developing, and implementing sensitive diagnostic tests for key pathogens of sugarcane will increase the security and confidence of disease detection in quarantine for safe exchange of germplasm.

2.0 PROJECT OBJECTIVES

- Obtain and trial diagnostic tests for indexing germplasm for key systemic pathogens of sugarcane (ratoon stunting bacterium, Fiji disease virus, sugarcane mosaic virus, leaf scald bacterium, maize streak virus, SCYLV, and phytoplasmas).
- Develop sampling strategies for each pathogen and test.
- Establish and validate protocols for efficient and effective use of diagnostic tests in quarantine

Sensitive diagnostic tests have been obtained or designed and trialed for key quarantine pathogens of sugarcane. A number of sampling strategies has also been investigated. Protocols have been established and validated for routine indexing of glasshouse collections for ratoon stunting disease (RSD), Fiji disease, mosaic, leaf scald, streak and phytoplasma diseases, and these have been documented in the BSES Quarantine Indexing Manual. A test for the detection of the sugarcane smut fungus

was also obtained. Methods for detection of leaf scald and sugarcane gumming disease are continuing to be investigated.

3.0 METHODOLOGY, RESULTS and DISCUSSION

All technical data concerning the methods and results is contained within the BSES Quarantine Indexing Manual attached as Appendix 2 of this report. The following discussion includes general methods and their justification, including research outcomes for diagnostic tests for each disease and/or agent.

3.1 Ratoon stunting disease (RSD)

The EB-EIA (ELISA) test for *Leifsonia* (*Clavibacter) xyli* subsp. *xyli* is the industry benchmark for testing canes for the presence of the RSD bacterium, and has been implemented for routine use in quarantine. Initial testing using this method was performed on interstation exchange clones to be released in August 1997. Attempts were made to extract xylem fluid by the conventional means of positive pressure applied to the cut end of a billet. The cane, grown in the glasshouse, was thin and quite dry and insufficient fluid was obtained from most samples. Further samples were taken and fluid extracted by centrifuging. Stalk pieces were placed in 50ml Falcon tubes and centrifuged at 3000g for 5 minutes. In most cases, the required 0.5 ml was obtained, although a significant proportion of clones were unyielding. Attempts were made to supplement xylem exudate by pipetting water onto the billet end and centrifuging it through the stalk. The fluid obtained from many samples was dirty from debris falling off the stalk, and this inhibited the EB-EIA test.

In view of these difficulties and the work involved in sampling by centrifuging, a further attempt was made to obtain samples conventionally. This time, pots were heavily watered, samples were taken early the following morning, packed in plastic and refrigerated until sampling. Satisfactory samples were obtained. This was repeated twice and it was concluded this treatment was optimum for routine use. Interstation Exchange clones released in 1998 were successfully tested by this means. The method was documented in the Quarantine manual and is now routinely used for indexing of glasshouse collections for the presence of RSD.

The development of a diagnostic Taqman quantitative PCR assay in project UQ024 has provided a more sensitive alternative to ELISA testing. Samples were collected from stalk material of imported germplasm upon entry to quarantine in October and November 1999. These samples were indexed for RSD using the Taqman assay to determine if the bacterium could be detected upon entry to quarantine, allowing immediate measures to be taken for containment or eradication.

Samples were collected by crushing a one cm^2 piece of stalk tissue in a garlic crusher and collecting the juice. This was centrifuged at 13000g to concentrate cells and the pellet was twice washed with sterile distilled water and centrifuged. The final volume of resuspension for PCR testing was 50µL. The results of these tests indicated that one imported cane was positive for RSD. The canes were grown in quarantine until June 2000 and samples collected and tested by ELISA. The previously positive sample was also retested using the Taqman assay.

ELISA results revealed four imported canes to be positive for RSD, although the cane previously tested by Taqman was not positive. The variety that tested positive in initial screening was tested again using Taqman and was weakly positive. These results suggest that indexing upon entry to quarantine is not reliable for detection of RSD. Instead, indexing should continue to occur after the first year of growth. Indexing will remain by ELISA although development of the Taqman assay will continue for use in research and where extremely low level detection is desired.

3.2 Fiji disease

3.2.1 Selection of test

A RT-PCR test for detection of Fiji disease virus (FDV), the causative agent of Fiji disease, was developed by Smith *et al* (1992). This test was used to confirm the presence of FDV in crude nucleic acid extracts from diseased plants. Three primer pairs were compared and FDV7F/R was selected for further use following consistent results. Two RT-PCR kits were trialed, with the Boehringer Titan kit selected due to lower cost and better results. This kit was later replaced by the *C.therm* RT-PCR system (Roche) after a trial with this kit gave better results than the Titan kit. These investigations have established a testing protocol that was documented.

3.2.2 Sampling strategy

The next objective was to devise a sampling strategy which, using the same RT-PCR method, would detect FDV in billets (at the time of planting in quarantine), and in asymptomatic, infected plants. From diseased cane, tissue samples were taken from the pith, rind and eyes at the base and top of stalks. The same tissues were tested after 'spiking' with Fiji galls, which contain high FDV concentration. FDV was detected in eyes only when they had been spiked, but only weakly, indicating presence of inhibiting factors (possibly phenolics). FDV was routinely detected in rind and pith, indicating either to be suitable tissue for quarantine screening. Two clones with extensive galling at the Pathology Farm were tested to confirm the method for leaf tissue. FDV was detected in all samples from leaves with no galls. Some problems were experienced with weak, non-specific bands that co-migrate near the FDV band on agarose gels. Extensive efforts were put into development of a Southern blot test to verify PCR results. The FDV7 450bp fragment was cloned for use as the probe. No restriction sites were available for removal of primers from the probe, so the smaller internal fragment of FDV27 was used. In one experiment, this gave acceptable results, but the work was suspended. These spurious bands did not continue to be a significant problem.

The Interstation Exchange collections were indexed for FDV, after vector insects (*Perkinsiella saccharicida*) were detected in the glasshouse. The canes had been screened visually in the ratoon and were deemed disease-free, but possibly, the intruding insect(s) carried FDV. A series of experiments was run to develop a protocol for RT-PCR detection of FDV in insects. Extracts from whole insects trapped on diseased plants (at the Pathology Farm) gave positive results and those from Fiji-free areas gave negative results. The 139 clones in the collection were also

tested and FDV was not detected. This insect FDV test was documented for future use in new quarantine incursions, should they occur and for research.

Sampling for FDV detection was explored further following progress in milestones related to sugarcane mosaic virus (SCMV) detection (see below). Larger, pooled samples are collected from plant material (stalk material before planting or leaf material shortly after) and homogenised. These pooled samples were tested using RT-PCR and any positive results followed by testing of clones individually, thus reducing the initial test cost.

3.2.3 Development of internal control

Following advice from the review of 9 November 1998, the development of an internal control for the FDV RT-PCR test was investigated. Primers were designed based on the reported sequence of *Ribulose diphosphate carboxylase* (Rubisco) from sugarcane. These were combined in a duplex RT-PCR reaction with the FDV7F/R primers. The effectiveness of detecting FDV in infected samples was not altered by the addition of the Rubisco primers to the reaction. In the duplex reaction, amplification of the FDV product was preferential to amplification of the Rubisco product. This is advantageous as the FDV derived product is more important to detect than the control product in the case of a positive test. The Rubisco primers (designed to include a transcriptional splice site) amplified both DNA and RNA derived products, confirming success of both the reverse transcription and PCR stages of the test. The test was shown to work well from healthy and diseased samples using the total nucleic acid extraction (TNAE). Unfortunately, the rapid release prep routinely used in the FDV test does not yield endogenous sugarcane RNA of a quality sufficient for RT-PCR, so other extraction methods were tested. These included the Integrated Sciences *Fastprep* machine and *RNAgreen* extraction kit, the Qiagen *FastRNA* extraction kit and a variation of the rapid release prep using the TNAE reagents. The efforts to pursue this avenue were unsuccessful and an alternative was explored.

Nicole Thompson (SRDC scholar, University of Adelaide) had reported (Thompson and Randles, 1999) that tobacco mosaic virus (TMV) could be used as an internal positive control for RT-PCR. TMV extracts and specific primers were obtained from the University of Adelaide. Under the RT-PCR protocol used for the detection of FDV, a TMV-derived product was amplified. Using the FDV protocol with both FDV and TMV present, products from both viruses (450 bp and 400 bp) were amplified and clearly separated on an agarose gel. Under the same protocol, but with SCMV and TMV added, products of both viruses were amplified (360 bp and 400 bp). Using the same protocol to index for different viruses has benefits within the quarantine system and this was recorded in the quarantine manual. Appendix 2 (p17) shows a gel with these two duplexed tests. Attempts to test for both FDV and SCMV at once with the TMV control were unsuccessful. It is presumed the tests interfere with each other.

The rationale for using an endogenous control is that it acts as an extraction control as well as a PCR control. TMV was added to the extraction mix and was amplified in PCR. This dual control function was then achieved.

The review panel warned that TMV would have the propensity to contaminate the laboratory. We will manage this risk by using blank controls and our standard hygiene approaches. We are not aware of any other risks arising from potential TMV contamination.

3.2.4 Validity of RT-PCR test for FDV for use in quarantine

Sequencing of the FDV genome has been limited to effectively one isolate, and no conclusions can be drawn about the genetic variability of the virus. Therefore, we cannot assure this primer set will detect all isolates of the virus. To assess the effectiveness of the primers, we obtained samples of FDV-infected leaves from five Australian mill areas and three foreign countries. The test detected FDV in each sample. This permits substantial confidence in use of the test in quarantine.

A further validation of the FDV test is to determine if the virus can be detected before development of symptoms within the plant. This was tested in a set of 10 varieties planted at the Pathology Farm under infection pressure from vector insects reared on Fiji diseased plants. At regular intervals, leaf samples were collected randomly from five out of nine stools per plot and tested by PCR. At the same time, the plants were inspected for the presence of Fiji disease symptoms. Plants failed to show symptoms of Fiji disease until well after final samples were collected. Of the 50 plots (with 8-10 plants per plot), only five plants, from separate plots, expressed disease symptoms. These samples tested negative for Fiji disease. It is likely that the random sampling may not have allowed a sample from the positive plants to be collected. This work will be repeated in a future glasshouse trial to confirm the efficacy of the test for quarantine indexing.

3.3 Sugarcane mosaic

3.3.1 Selection of test

SCMV indexing in the past included visual inspection for symptoms and inoculation of indicator hosts. Visual inspection has low sensitivity, relying on the appearance and detection of disease symptoms. Indicator host inoculation is more sensitive, but has substantial time and glasshouse requirements. Sensitive tests now available include ELISA and RT-PCR techniques, and research included both of these.

Work initially concentrated on ELISA. A commercial kit for detection of potyviruses, manufactured by AGDIA Inc, was tested. SCMV was detected beyond a 3000x dilution of ground leaf tissue of diseased plants. Johnson grass mosaic virus was also detected. The kit uses a monoclonal antibody specific to a highly conserved amino acid sequence within the coat protein, and detects nearly all of the potyviruses on which it has been tested. The test is suitable for use in quarantine, although it would be necessary to verify its detection of all known strains of mosaic viruses infecting sugarcane in the world

Early work on RT-PCR concentrated on degenerate primers designed by Langeveld *et al* (1991) for detection of a broad range of potyviruses. These primers had detected SCMV with the First Strand cDNA Synthesis kit, but did not work with the Titan RT-PCR System, on which our laboratory had standardised for sugarcane yellow leaf

virus (SCYLV) and FDV. For cost savings and the opportunity to develop a multiplexed test with FDV and SCYLV, we worked with the Titan kit and the degenerate primers. It was identified that the Titan kit was intended as a high fidelity RT-PCR kit, and therefore included a proof-reading enzyme which breaks any non-specific linkages. Degenerate primers work by substituting inosine at highly variable sites, as inosine binds to adenosine, cytidine, guanosine and thymidine. Linkages formed by inosine were being broken by the proof-reading enzyme. To overcome this, we sought to obtain the components of the Titan kit, less the proof-reading enzyme. The manufacturer would not cooperate, so by researching literature referred to by the manufacturer, we attempted to deduce the kit components. The buffers and enzymes trialed successfully detected SCMV. In one attempt at a multiplexed reaction, both FDV and SCMV were detected.

The project review of November 1998 cautioned against this multiplex testing. The *C.therm* RT-PCR kit was trialed at this time and proved to give better results for FDV detection than Titan. This kit was also tested for detection of mosaic and results concurred. Following development of an internal control it was decided that the *C.therm* kit would be used for indexing both viruses, with the TMV control applied to both tests (see Appendix two, p17).

3.3.2 Sampling Strategy

Our strategy has been to develop indexing protocols that use stalk tissue. It is desirable to index clones immediately upon entry to quarantine, enabling rapid containment and replacement. Clones enter quarantine as billets, so leaf tissue is not available for some weeks. Previously (using FDV), we showed that rind and pith tissue were suitable for PCR, whereas PCR was inhibited in eyes.

To investigate whether pith tissue could be used in preference to leaves for indexing SCMV, tests were conducted using degenerate potyvirus primers. RNA was prepared using the Rapid Release method, in which tissue was ground in microfuge tubes with micropestles then extracted in chloroform/isoamyl alcohol. From diseased stalks of ten varieties, samples of pith from the centre and outside of the stalk were taken from lower (mature) and upper (immature) stalk tissue. Even when tests of leaf tissue were positive, tests of stalk tissue were usually negative and sometimes weakly positive. Two questions were raised, firstly that the sensitivity of the degenerate primers was inadequate, and secondly that the sampling method was sometimes not obtaining virus.

Further tests were conducted with specific primers, S400-910/551, developed by Smith and Van de Velde (1994). These should have a lower detection endpoint than the degenerate primers, and indeed proved much more efficient. Samples from diseased stalks of 10 varieties were tested. Positive tests were less frequent from pith in the upper stalk than the lower stalk, and from the stalk than the leaves. Negative tests also occurred from diseased leaves in some varieties. While this study was not exhaustive, it indicated that the natural distribution of SCMV in the plant is low and uneven. We extracted from full transverse sections of stalks, using mortar and pestle, and this appeared more reliable than the small scale method, but it is unsuitable for indexing numerous samples as it is liable to contamination, and also impractical. The problem may have related to the size of samples, or the efficiency of extractions.

Several variations were made to sampling and small scale extraction procedures, but inconsistent results continued to occur.

A tissue homogeniser (UltraTurrax) was investigated and found very suitable. It rapidly and efficiently homogenises large quantities of leaf or stalk tissue from mature plant samples. Nucleic acid extractions based on the current chloroform/isoamyl alcohol protocol, but scaled up to work with ~1 gram of plant material (as opposed to ~50mg previously), were performed on symptomatic tissue with SCMV and FDV. Both of these viruses were detected in RT-PCR tests, including the TMV RT-PCR control where extractions were spiked with a crude TMV preparation. Alternative extraction methods and the Integrated Sciences Fastprep system trialed previously were much more laborious, far less effective and could not be used for large samples. Samples are pooled from several clones, and individuals are only tested when a positive test is made. This extraction procedure allows the use of TMV as a control for both extraction and RT-PCR steps of indexing.

The sampling protocol and RT-PCR tests, including the TMV internal control, were documented in the Quarantine Indexing Manual. FDV and SCMV can be tested for from the same extraction if desired. It is important to note that several strains of SCMV exist, but only strain A is in Australia. The documented primers will detect some strains, but not every strain that may enter if present in international sources. The ELISA test is also documented in the indexing manual for routine use in screening imported varieties. Leaf samples will be taken after four months growth in the glasshouse and screened by RT-PCR (Australian varieties) or ELISA (foreign varieties).

3.4 Leaf scald

3.4.1 Selection of test and sampling strategy

The basic method for indexing for the leaf scald bacterium, *Xanthomonas albilineans* (*Xa*), is to isolate on Wilbrink's medium and identify by taxonomic methods. Identification is simplified by using a specific ELISA test, for which we hold an antiserum. Samples were obtained by crushing pith with a garlic crusher and plating out the juice. *Xa* was cultured on modified Wilbrink's medium from diseased stalks obtained from the BSES pathology farm. The Indirect ELISA test was then applied successfully on colonies. To avoid the need to culture at the busy time of quarantine introduction, it is desirable to store the samples before culturing. Infected billets and juice samples were frozen at –20 ºC and –70 ºC for different periods. Successful isolations were repeatedly made after thawing. These methods were documented in the BSES Quarantine Manual for routine indexing. However, this approach is not ideal, because it is time-consuming, labour intensive and requires well-grown stalks. A much more sensitive test is also desirable.

Pan *et al*. (1997) published a PCR test using primers specific for *Xa*. This method was trialed upon cell suspensions of three-day-old broth cultures and found to be highly sensitive (about 100 fold greater than ELISA). One drawback of this protocol was the presence of non-target fragments, possibly amplified from other unidentified bacteria commonly found in sugarcane. Pan *et al.* (1999) later reported the development of a second set of highly specific PCR primers for the detection of *Xa*.

These primers were trialed and confirmed to be more specific than the previous primers. A sampling strategy and PCR protocol were established and used to detect the bacterium in washed sap samples obtained from stalks of diseased plants, from the BSES pathology farm.

A more exhaustive trial was established to evaluate the sampling strategy and to confirm the integrity of this test for indexing in quarantine. A set of stalks was taken from a plot that recently had been inoculated with *Xa*, but was not symptomatic. Juice was taken from small sections of billets from the top, middle, and bottom internodes. These juice samples were tested by PCR; some were positive and others negative. All billets were then planted. These were grown until leaf scald should have developed if infection is present. The presence or absence of infection would show whether any false negatives occurred in the PCR indexing.

Plants were grown at the pathology farm until pencil line symptoms characteristic of leaf scald disease had developed in a number of plants. Juice samples were collected and tested by PCR. The results did not correlate with the initial findings and doubt was cast upon the testing method. After consulting BSES pathologist Barry Croft, it was evident that the sporadic nature of the leaf scald bacterium had compromised the trial and the results were not indicative of the integrity of the testing methods.

3.4.2 Test implemented

Using the Pan *et al.* (1999) PCR primers in the protocol reported for Milestone 5, 66 clones imported from seven countries during 1999 were tested for leaf scald. Stalk pieces were sampled from each clone prior to hot water treatment and planting, and stored at –70°C. Washed juice from these samples was subjected to PCR. All canes from five countries were negative, but bands indicative of leaf scald were found in canes from two countries.

To verify this putative positive result, juice from these samples was cultured on Modified Wilbrink's medium. Colonies similar to *Xa* were screened by PCR and amplified fragments were cloned and sequenced. Some clones had 95 % sequence similarity to the sequence of *Xa* reported by Pan. Others were more similar to the reported sequence of an unidentified saprophyte of sugarcane (Pan *et al* 1999). The cultures with putative positive PCR tests were then screened with ELISA using an antiserum to *Xa*, and results were negative. These results indicated that Pan's test is not diagnostic for leaf scald and another set of primers were sought.

Wang *et al* (1999) published primers for detection of leaf scald in PCR assays and compared several detection methods. These primers were tested and proved more specific than Pan's primers. No product was amplified from the washed sap samples collected from the import canes. The Wang paper also compared the sensitivity of ELISA, direct immunoassay, PCR and Bio-PCR. It was reported that Bio-PCR was at least 100 fold more sensitive than standard PCR for detection *Xa*. The Bio-PCR technique was tested using our documented modified Wilbrinks medium and the Wang PCR primers. It was shown that the Bio-PCR test was up to 1000-fold more sensitive, detecting approximately 1.2 X 10^2 CFU mL^{-1} in a pure culture dilution series. Further work will involve testing from juice samples, but a more selective medium is to be trialed for this. The standard PCR test using Wang's primers is

documented in the indexing manual and will be used until reagents for the highly selective media are obtained.

3.5 Yellow leaf syndrome

Symptoms of YLS have been observed widely in Australian sugarcane, requiring review of its previous high quarantine status. A virus and a phytoplasma are reported to be associated with this syndrome and one or both may be the causal agents. Presence of an assigned member of the luteoviridae (sugarcane yellow leaf virus, SCYLV) has been confirmed in samples from different regions throughout the Australian sugar industry, so probably there is little benefit in screening for it in quarantine. Reports from South Africa that the phytoplasma has been detected in Australian samples remain unverified, so the phytoplasma remains quarantinable. An SRDC project (NTU001) studying phytoplasmas associated with YLS has not yet delivered a test that is suitable for implementation in the BSES quarantine. This syndrome will not be indexed until the aetiology of YLS has been clarified and it has been established as a significant quarantine disease.

3.6 Streak

Plasmid DNA containing sequences of maize streak virus (MSV) for use as positive controls for PCR were provided by Assoc. Professor Ed Rybicki (University of Cape Town, South Africa). PCR primers reported by Rybicki & Hughes (1990) were in stock at BSES and when used in a PCR under conditions reported in this publication, amplified the reported 254 bp fragment. This protocol was added to the indexing manual, with the plasmid DNA stored as a positive control.

In addition to the MSV plasmid, two clones containing sugarcane streak virus (SSV), a geminivirus infecting sugarcane in Africa and some surrounding regions, were also provided. The clones are derived from two different geographical isolates of SSV (from Mauritius and Natal). The primers failed to amplify reproducibly even after optimisation of the PCR conditions. This may be due to the primer design, and new primers based on SSV sequences reported in Genbank were designed and tested. These primers successfully amplified a specific product from both SSV isolates, as well as the MSV sample. This test was optimised and documented in place of the MSV protocol above.

It was recently reported by Bigarre *et al* (1999) that several species of SSV are found in sugarcane. Alignment of the sequence data available indicates the primers we have developed will detect each of these. This test will be used to index imported clones from countries where MSV or SSV have been reported.

3.7 Phytoplasma diseases

A PCR test for the white leaf and grassy shoot phytoplasmas was obtained from the laboratory of Karen Gibb at NTU (progress of SRDC project NTU001). Phytoplasma DNA extraction methods and PCR primers (Tran-Nguyen *et al*, 2000) were obtained and trialed upon samples provided by Lucy Tran-Nguyen during a visit to BSES. These samples included a white leaf phytoplasma in couch grass and grassy shoot phytoplasma in *Whitechloa cymbiformis*. These samples are representative of the

phytoplasmas causing white leaf and grassy shoot diseases in sugarcane. The extraction method and PCR test were trialed and confirmed to successfully detect the presence of phytoplasmas. The test was also able to distinguish between the two different phytoplasmas, allowing the specific phytoplasma present to be determined. The protocol was documented for screening clones imported from risk countries, with the test samples held as positive controls.

3.8 Smut

An incursion of smut in the Ord River Irrigation Area during the project period prompted the development of a diagnostic test for the sugarcane smut fungus, *Ustilago scitaminea*. Purified smut DNA and a plasmid with the smut *b*E gene were imported for positive controls. Primers were obtained using the sequences published by Albert and Schenck (1996). The plasmid was cloned, and the test was successfully run on the positive controls. The test was documented and controls stored in case they are needed for future use.

4.0 Impact

This project has provided the Australian sugar industry with a battery of highly sensitive indexing protocols for use in sugarcane quarantine. Implementing a range of diagnostic tests allows earlier detection and containment, improved sensitivity over visual indexing and potentially a reduced quarantine period. Although it is likely that quarantine services elsewhere are adopting tests similar to those reported here, implementation into practice requires a significant investment in time and resources. This project has fulfilled these requirements, and will increase the security of germplasm exchange both intrastate and internationally. Future research into diagnostics should continue to focus on important and newly arising quarantine threats, to maintain the current level of best practice.

5.0 Technical summary

The development of an internal control for RT-PCR testing for FDV and SCMV is an important achievement. This should be reported for the benefit of others involved in applying tests for these or other RNA pathogens. PCR primers for detection of sugarcane and maize streak viruses have also been developed by BSES.

6.0 Recommendations

The diagnostic tests obtained or developed during this project are documented for use as indexing tools in quarantine (Appendix 2). Several of these tests are currently used routinely for screening collections for known pathogens. Tests that are not used routinely should be employed when germplasm from risk areas is imported. Research should continue to identify methods for detection of pathogens that may become significant quarantine threats. Thus recommendations are:

Interstation exchange canes

- All canes indexed for RSD by ELISA after 10-12 months growth.
- RT-PCR test for FDV implemented for routine screening all canes from Isis south during first year.
- Visual indexing for SCMV and leaf scald supported by testing if required.

Imported canes

- All canes indexed for RSD by ELISA after 10-12 months growth
- All canes indexed for mosaic by ELISA after 4 months growth.
- All cane indexed for Leaf scald by PCR (or Bio-PCR when available) after 10-12 months growth.
- Canes from Fiji disease, streak or phytoplasma risk areas tested by PCR after 4 months growth.

Further work

- Bio-PCR experiments completed for leaf scald detection.
- Tests for other known quarantine threats, including gumming disease and Ramu stunt to be investigated.
- Tests for new quarantine threats obtained and trialed as necessary.

7.0 Publications

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APPENDIX 1 APPS Paper
APPENDIX 2 BSES Q indexing manual