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MAINTAINING ACCESS TO FOREIGN GERMPLASM BY DEVELOPING METHODS TO DETECT UNIDENTIFIED VIRUSES IN SUGARCANE IN QUARANTINE

by

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1.0 SUMMARY

Four types of generic tests for detecting unidentified viruses in plants were trialed for their suitability for sugarcane. The tests were: double stranded RNA (dsRNA) analysis, sap inoculation of indicator plants, sap examination by electron microscopy and polymerisation chain reaction (PCR) amplification using 'group-specific' primers.

- dsRNA extraction and purification was optimised for leaves infected with Fiji disease virus (FDV). The 10 dsRNA segments can now be used as molecular weight markers for comparison to unknown samples. The test is time consuming and complex and will not be used for routine screening, but will be used when required.

- Sap inoculation onto maize, wheat, barley and sorghum was trialed in the glasshouse. Maize and sorghum gave good responses to mosaic viruses and sorghum was chosen as the most suitable host for routine screening. This test has been implemented in quarantine.

- Sap examination under the electron microscope (EM) was tested on a limited number of occasions. It is slow, only suitable for high titre viruses, and requires an experienced operator. The test will not be used for routine screening, but will be used when required.

- PCR using 'group-specific' or degenerate primers was used with limited success for luteoviruses and very good success for potyviruses. A 'group-specific' enzyme linked immunosorbent assay (ELISA) kit is also available for potyviruses. Screening for potyviruses has been implemented in quarantine.

Detailed protocols for the detection of unidentified viruses using these four tests have been prepared and will be included in the BSES quarantine manual.

A new disease, yellow leaf syndrome (YLS), thought to be caused by an unidentified virus appeared in quarantine during the project. A number of diagnostic techniques including dsRNA analysis, western blotting, leaf dips and immunosorbent electron microscopy (ISEM), and PCR with 'group-specific' primers were trialed in an attempt to identify the causal agent which was suspected to be a luteovirus. YLS research is now carried out in a separate project.

2.0 BACKGROUND

Improved sugarcane varieties are the key to the continuing profitability and sustainability of the Australian sugar industry. Importing foreign cane for use in breeding programs is fundamental to the genetic improvement of Australian sugarcane. Access to foreign germplasm depends on the continued operation of a high security quarantine service. BSES and collaborators have already developed sensitive and specific tests for known viral pathogens of sugarcane including sugarcane bacilliform virus (SCBV), sugarcane mosaic virus (SCMV) and FDV. However as sugarcane is a vegetatively propagated

grass, it can also contain agronomically important, unidentified and sometimes symptomless viruses. Such viruses are a concern to AQIS and the Australian sugar industry. The development and implementation of tests for viruses in sugarcane will minimise the risk of importing exotic viral diseases. Only general tests are able to detect an unidentified virus.

The four major general tests for detecting viruses are:

1. Analysis for the presence of dsRNA: Plants do not normally contain dsRNA but many viruses have single stranded RNA (ssRNA) genomes, and a few have dsRNA genomes. The ssRNA viruses often produce dsRNA while replicating. Therefore detection of dsRNA is a strong indication that the plant is infected with an RNA virus. The technique is not suitable for viruses with DNA genomes.

2. Sap inoculation of indicator plants: Bioassays of mechanically transmitted viruses are usually highly sensitive and easy to perform. The test is not suitable for non-mechanically transmitted viruses.

3. Sap examination by electron microscopy: Examination of plant sap with the electron microscope allows direct viewing of viral particles. Mixed infections of viruses can also be detected by this method. The technique is most suitable for rod shaped viruses present in high titre. Isometric viruses and those present in low titre are difficult to detect.

4. PCR amplification using ‘group-specific’ primers: PCR technology can be used to detect new members of characterised viral groups. The PCR primers, often degenerate, are designed to be complementary to conserved genomic sequences shared by all known members of a virus group, and are used to detect new related members. Non-specific amplification is often a problem with degenerate primers and the list of viral groups for which primers are available is limited.

The aim of this project was to develop and implement general diagnostic tests for unidentified viruses in sugarcane germplasm held in quarantine. The availability of these general tests has provided an opportunity to significantly improve the security of sugarcane quarantine.

3.0 OBJECTIVES

- Determine the efficacy and suitability of generic tests for detection of unidentified sugarcane viruses.
- Evaluate these tests for the detection of suspected viruses in sugarcane.
- Develop protocols for routine screening of sugarcane germplasm for unidentified viruses.
- Implement the tests as part of standard quarantine operating procedure, and recommend diseases for which more specific tests should be developed.

4.0 OUTCOMES

- Tests for unidentified viruses have been implemented into routine quarantine screening.

- Detailed protocols for the detection of unidentified viruses have been prepared and will be included in the BSES quarantine manual.

- A supply of seed of indicator hosts for known and unidentified sugarcane viruses has been collected and stored.

- Methods were optimised for dsRNA extraction and analysis from sugarcane and detection of potyviruses by either PCR or ELISA.

- Yellow leaf syndrome, a new disease of sugarcane caused by an unidentified virus, was detected in BSES quarantine in 1994. Preliminary research into the causal agent of this disease formed the basis of a new research project.

5.0 RESEARCH METHODOLOGY, RESULTS AND DISCUSSION

5.1 Yellow leaf syndrome

Symptoms similar to those reported for YLS were observed in canes held in the BSES quarantine facility at Indooroopilly and in the field in 1994. A luteovirus was considered the most likely pathogen based on the yellow symptom and results from overseas. Because of the potential risk to the Australian sugar industry from YLS, this new disease was used as a model for application of diagnostic tests to determine the identity of the unidentified virus. A number of diagnostic techniques were tested for luteovirus detection and identification. These included dsRNA analysis, western blotting, leaf dips and ISEM, and PCR with 'group-specific' luteovirus primers.

dsRNA extractions and a number of virus purification procedures were attempted initially. The optimal purification method was based on that used for tomato yellow top luteovirus (TYTV) (Thomas, 1984). A large range of antisera raised to different luteoviruses, such as barley yellow dwarf virus (subgroups RPV, MAV and PAV), beet western yellows virus, potato leaf roll virus and TYTV, was used for western blotting. The PAV and TYTV antisera consistently recognised proteins from YLS samples, however the bands detected on the western blots did not correspond to the expected size of the luteovirus coat protein, suggesting that a new, previously unidentified luteovirus may be involved.

Luteoviruses generally occur in low viral titre and sap examination under the EM was found to lack sensitivity. However using an antisera to trap particles, known as immunosorbent electron microscopy (ISEM), was more successful. Small spherical particles were observed under EM when the TYTV or barley yellow dwarf virus RPV antisera was used to trap particles from YLS samples. This observation is consistent with the fact that luteoviruses have small isometric particles 25 - 30 nm in diameter.

Group-specific PCR primers for luteoviruses (Robertson et al, 1991) were synthesised and assessed for detection of luteovirus-specific RNA in YLS infected cane. Only limited success was achieved. A 530 bp fragment was amplified using the primers and the published reverse transcriptase PCR (RT-PCR) protocol, however non-specific products were also synthesised. This result is further evidence that YLS is caused by a luteovirus.

Most of the initial research in BS116S was on YLS. Because this disease was considered a serious quarantine risk, a recommendation was made for increased staff and resources. A separate research proposal to identify the causal agent and develop a specific test was submitted to the CRC-TPP for funding and this project commenced in October, 1995.

5.2 dsRNA analysis

Sugarcane infected with FDV was used to optimise the extraction and detection of dsRNA. FDV has a genome of 10 dsRNA segments and once purified they can be used as molecular weight markers for analysing unknown samples. The technique was then trialed on sugarcane from the museum collection at the Eight Mile Plains Pathology Farm, sorghum infected with Johnson grass mosaic virus (JGMV), a noble cane from the international glasshouse showing unusual symptoms (OE14) and an orchid from north Queensland believed to be infected with a reovirus. It was found that large amounts of leaf material were required to do the extraction (10 - 30 g) and that in some cases ssRNA and DNA complicated the detection. Digesting with RNase can indicate if ssRNA is involved. dsRNA analysis was not found to be suitable for routine screening because of the time and amount of leaf material required. However it will be used for quarantine germplasm showing unusual symptoms.

5.3 Sap transmission

A collection of seeds from indicator hosts was obtained and bulked up. The collection includes:

(a) the three indicator hosts used by AQIS for clonally propagated grasses: *Zea mays* cv Supagold, *Triticum aestivum* cv Hartog and *Hordeum vulgare* cv Tallon;

(b) the indicator host used by USDA for sugarcane: *Sorghum bicolor* cv Rio;

(c) indicator hosts of known sugarcane viral diseases: *Nicotiana benthamiana* and *Chenopodium amaranticolor* for peanut clump virus and *Nicotiana occidentalis*, a possible host for SCBV. *Sorghum bicolor* cv Rio is also an indicator host for sugarcane mosaic virus and a possible host for sugarcane mild mosaic virus (SCMMV).

The technique of sap inoculation was trialed in a glasshouse at the pathology farm, Eight Mile Plains, using sap from sugarcane infected with SCMV inoculated onto Supagold, Hartog, Tallon and Rio. Both Supagold and Rio gave clear results (easily seen mosaic symptoms), however Rio produced the symptoms in less time. In addition, large amounts of seed of Rio can be produced easily under glasshouse conditions. For these reasons, Rio will be used as the major indicator host for routine screening. This test was implemented in quarantine in 1997. Sap extracts from 43 noble canes from Hawaii were inoculated

onto Rio. The test was done in the international quarantine glasshouse and sap from JGMV infected sorghum was used as a positive control (JGMV should not infect sugarcane). The positive control plants were grown in an insect proof cage.

5.4 Sap examination

Initial sap examinations were made using the electron microscope at QUT but in 1996 we negotiated access to a transmission electron microscope in the Plant Protection Unit, QDPI, Indooroopilly, which is closer to BSES. Material screened by EM included sugarcane infected with known viruses (SCBV and SCMV), cane showing symptoms of YLS, and cane showing unusual symptoms (OE14). It requires experience to become familiar with the use of the microscope and to gain a feel for discriminating viral particles from plant organelles. Sap examination is also slow and limited to viruses in high titre. This test will only be used for quarantine germplasm showing unusual symptoms.

5.5 'Group-specific' PCR

Degenerate or 'group-specific' PCR primers for potyviruses, (Langeveld et al, 1991), luteoviruses (Robertson et al, 1991) and maize streak virus (Rybicki and Hughes, 1990) were synthesised from published literature. The luteovirus 'group-specific' primers were used with limited success for detecting YLS. The potyvirus specific RT-PCR primers were used more extensively and this test has been implemented into routine quarantine screening. The degenerate RT-PCR primers allow detection of SCMV in leaves with mosaic symptoms and symptomless leaves of plants known to be infected, but not in leaves of 'healthy' plants. The test could also detect the other closely related potyviruses, JGMV, maize dwarf mosaic virus and sorghum mosaic virus.

A similar approach is the use of 'group-specific' antisera in an ELISA test. A commercial antisera for the detection of potyviruses was trialed and compared to the PCR test. The test could detect SCMV in sugarcane but could not detect the closely related JGMV in Johnson grass or sorghum. This was due to the limitation of the monoclonal antisera included in the kit. The ELISA test is easier to perform than the RT-PCR test, but there is a risk that the commercial antisera may not detect all unidentified viruses.

The PCR test for sugarcane streak was not trialed because we had no test material, but recent negotiations with Prof Rybicki (University of Cape Town) to obtain a positive control and an antisera have been successful. Other 'group-specific' primers are available including primers for detection of phytoplasmas.

5.6 Discussion

In the first year of this project, two serious problems occurred in the BSES quarantine system. The first was the appearance of red/yellow flecks on noble canes introduced from India. All of these canes were subsequently destroyed. The second was the appearance of yellow midribs, characteristic of YLS, in commercial canes, mostly from Canal Point. Both were initially thought to be due to viruses. This demonstrates the need for protocols and contingency plans able to deal with new unidentified pathogen problems in quarantine. YLS was used as a model system for the application of diagnostic tests to

identity unidentified viruses. The experience gained from performing virus isolations and purifications, ‘group-specific’ primer PCR, EM, and dsRNA isolations, contributed towards developing generic tests for unidentified sugarcane viruses.

The aim of this project was to develop protocols, methods and experience to detect unidentified viruses and these protocols are an outcome of the project. To prevent losing this knowledge, a manual was prepared detailing the methods used to carry out each test trialed in this project. The manual also includes other tests for known viruses and other pathogens of sugarcane recently developed and implemented at BSES. This manual will be inserted into the BSES quarantine manual where it can be referred to by the quarantine pathologist or research assistant at any time. Not all of the generic tests will be used in routine screening, but they may be needed in specific situations, for example, if a new quarantinable disease arises. The methods will need to be constantly updated as new tests are developed or obtained from overseas, existing tests are improved due to new technologies and new sugarcane diseases appear.

In addition to optimising the tests for sugarcane tissues, factors such as cost, speed, reliability and amount of material required, have to be considered before the test can be implemented into routine quarantine screening. dsRNA extraction and analysis is complex and time consuming. Sap transmission is simple and inexpensive, but labour intensive and requires a large area within the quarantine glasshouse. Sap examination is slow, lacks sensitivity and requires an experienced operator. Current rate for the use of the QDPI microscope is $60 per hour. ELISA is simple and easy to perform and many samples can be done at once. An ELISA plate reader was not available at BSES at the start of the project but funds allocated to the purchase of a PCR machine were used instead to buy a plate reader. RT-PCR is more complex than ELISA and degenerate primers can lead to unreliable results. However once optimised, PCR tests are very sensitive, can be multiplexed, and total nucleic acid extractions can be used for a number of PCR tests.

Currently, detection of potyviruses by PCR or ELISA and sap inoculation onto sorghum cv. Rio have been implemented into routine screening. dsRNA analysis, sap examination and other PCR tests will be used if unusual symptoms are observed in quarantine, if results from the other tests are positive and need further analysis, or in specific situations (eg the test for streak will be used if germplasm is imported from South Africa). A number of tests for known pathogens have recently been implemented in quarantine at BSES. Germplasm can now be screened for SCBV (ISEM), SCMV (RT-PCR), FDV (RT-PCR), YLS (RT-PCR), ratoon stunting disease (ELISA) and leaf scald (isolation techniques). Most of these methods have been included in the quarantine manual (see appendix 9.2), and more tests will be included when they are implemented. In the near future, tests for phytoplasmas and smut could be implemented.

5.7 Recommendations

The initial focus in this project was YLS because it was perceived as a serious quarantine problem caused by an uncharacterised virus. It soon became apparent that there was insufficient staff input and resources for YLS. It was recommended that a separate project

be developed for the identification of the causal agent of YLS and development of a specific test. This project was funded by the CRC-TPP and began in 1995.
Methods, experience and the equipment necessary to carry out diagnostic virology need to be maintained in a quarantine laboratory so that if a quarantine problem arises, it can be dealt with. A manual describing the methods needed to carry out each test has been prepared for this reason. BSES now has access to an electron microscope and there is considerable virology expertise at both QUT and QDPI.

The second objective of this project was to evaluate the generic tests for the detection of suspected viruses in sugarcane. Considerable progress was made on YLS, suspected to be caused by a virus. A number of other diseases are also suspected to be caused by viruses, including striate mosaic, chlorotic streak and Ramu stunt. Research carried out at the Waite Campus, University of Adelaide suggests that the causal agent of striate mosaic may be a carlavirus. This conclusion has come from isolating, cloning and sequencing dsRNA. The causal agents of other diseases such as chlorotic streak may also be identified by this approach.

6.0 PUBLICATIONS

Smith, G R, Fraser, T A, Braithwaite, K S and Harding, R M. (1995) RT-PCR amplification of RNA from sugarcane with yellow leaf syndrome using luteovirus group-specific primers. Proceedings of the 10th Biennial Australasian Plant Pathology Society Conference, p84.

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Robertson, N L, French, R and Gray, S M. (1991) Use of group-specific primers and the polymerase chain reaction for the detection and identification of luteoviruses. J. Gen. Virol. 72: 1473-1477.

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Thomas, J E. (1984) Characterisation of an Australian isolate of tomato yellow top virus. Ann. appl. Biol. 104: 79-86.

8.0 ACKNOWLEDGMENTS

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9.0 APPENDICES

9.1 Abstract

Smith, G R, Fraser, T A, Braithwaite, K S and Harding, R M. (1995) RT-PCR amplification of RNA from sugarcane with yellow leaf syndrome using luteovirus group-specific primers. 10th Biennial Australasian Plant Pathology Society Conference, Lincoln, New Zealand, p84.

9.2 Extract from BSES Quarantine Manual

Hekmeijer, S. (1998) Diagnostic procedures for the identification of sugarcane diseases.

This manual was initially prepared in 1997. The draft included with this final report is the second draft updated in February 1988. In addition to the methods developed in BS116S, it includes diagnostic tests developed or implemented as part of BSES project 3118 (Quality assurance and improved diagnostics in sugarcane quarantine) between July 1996 - June 1997 and SRDC project BS187S (Implementation of sensitive pathogen indexing methods in sugarcane quarantine) between July 1997 - February 1998. The test for sugarcane yellow leaf virus, although implemented, has yet to be included in the manual. Other tests for sugarcane pathogens will be included in the future.