

SUGARCANE STREAK MOSAIC—RESEARCHING A RELATIVELY NEW DISEASE IN INDONESIA

By

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Abstract

SUGARCANE STREAK MOSAIC occurs in the south and south-east Asia regions and was first identified in Indonesia in 2005. Its relatively recent recognition means that much remains to be learnt about this viral disease. ACIAR-funded research has been investigating molecular and serological assays for the virus, yield losses, important modes of transmission, and an integrated disease management strategy to manage and minimise losses. So far our findings include: molecular variation in the virus around its geographic range; yield losses amount to approximately 20% in a susceptible variety; transmission may occur via application of infested sap to leaves; mechanical transmission (as on infested machinery surfaces) is unlikely to be significant. Surveys have shown the disease to be present in various parts of Indonesia (Java, Sumatera, Sulawesi and probably West Papua). Much remains to be learnt about its epidemiology.

Introduction

The Australian sugarcane industry recognises that knowledge and experience gained in research into exotic biosecurity threats provides a valuable defence for commercial sugarcane production in Australia. Major pest and disease threats are present in PNG, Indonesia, Thailand, Laos and Vietnam, and further afield around the world. When a new incursion occurs, it is imperative that a rapid, well-thought-out response is activated that clearly and quickly addresses the most important issues related to management of that pest or disease.

Sugarcane is grown on over 380 000 ha of land in Indonesia by over 140 000 farmers to produce around 30 Mt of harvestable product (Toharisman and Triantarti, 2014). Though previously centred on Java, the Indonesian sugarcane industry has expanded into Sulawesi, Sumatera, and West Papua. Farm sizes are small (average around 1 ha) and farming operations are almost exclusively undertaken by hand.

Sugarcane streak mosaic virus (SCSMV) is a pathogen only recognised in the late-1990s (Hall *et al.*, 1998). Some of the initial pathogen research centred on diagnostics and was undertaken in south Asia (Hema *et al.*, 1999; Hema *et al.*, 2003; Li *et al.*, 2011); this included work on pathogen characterisation and strain detection. The disease was first recognised in Indonesia in 2005 on the island of Java (Kristini *et al.*, 2006; Magarey *et al.*, 2012; Putra *et al.*, 2014). Putra *et al.* (2014) reported on some initial yield loss and transmission studies, but there remains much unknown about the virus and the disease it causes (Magarey *et al.*, 2012).

Several surveys have been conducted in Indonesian territory to determine the extent of its distribution and its proximity to Australia. In previous research (Magarey *et al.*, 2012), surveys of Javan cane fields showed that the disease was widespread across the island. Many of the 931 crops surveyed were infected by the disease.

Two recent surveys have been conducted, one in west Java and Sumatera (2016), the second in Sulawesi (2017). SCSMV was found in both regions. In Sumatera there appeared to be some relationship between incidence and the use of plant sources originating in Java. In Sulawesi, indicative SCSMV symptoms were identified in three out of the five regions surveyed; confirmation of the presence of SCSMV rests with upcoming research and specific specimen assays.

Important issues for cane farmers include:

- i. How can we be sure the symptoms we are looking at are SCSMV?;
- ii. How fast will it spread?;
- iii. What are the likely yield losses?;
- iv. Are our commercial varieties resistant?;
- v. How does it spread?; and
- vi. How do we best manage the disease?

Our current understanding of the nature of SCSMV and the results of research, are outlined in this paper.

Methods

Diagnostics

The aim was to develop two different types of assay: i. the most sensitive molecular test, to be used in quarantine and for assessing variation in the pathogen between regions and countries; and ii. a serological assay, that may provide a cheap but reliable test to be used within industry for assaying plant sources.

Molecular tests

It is important for development of any sensitive assay to start with an appropriate target pathogen and plant source. Twenty seven sugarcane samples displaying mosaic symptoms were therefore assayed at the University of Bogor for SCSMV and a second pathogen giving rise to mosaic symptoms – sugarcane mosaic virus (SCMV). Of the plant samples, seven were infected only by SCSMV using methods established previously (Putra *et al.*, 2014). The virus was inoculated into sorghum to provide for a comparison of the virus isolates, and assessment of any isolate variation, while setts were propagated to maintain the SCSMV source.

Previous work had seen the development of a RT-PCR test (Damayanti and Putra, 2011) and this was applied to known samples of both sugarcane mosaic virus (SCMV) and sugarcane streak mosaic virus (SCSMV) to determine the specificity of the test. SRA uses the CPf-AP3 primers in the RT-PCR (Damayanti and Putra, 2011) assay to confirm positives because it caters for detection of isolates with possible sequence variation. A Power SYBR Green RNA-to-CT 1-Step Kit (ThermoFisher) was used on an Applied Biosystems ViiA7 machine as it generally works well with viral assay of most samples, including dried plant samples and also insects.

An additional consideration is the method of transportation of leaf specimens to the laboratory for analysis. To this end, FTA cards (a simple card on which sample extracts can be dried and preserved) were tested for their use in sugarcane. To enable a side-by-side analysis, a leaf sample was split into two, with half used to prepare an extract that was preserved on FTA cards and half desiccated over calcium chloride. The RT-PCR assay was undertaken using specific primers after RNA preparation.

Quantitative RT-PCR (qRT-PCR) is a technique that enables a very sensitive quantitative assay of the presence of the virus in plant samples. Primers for this test were designed to detect the more conserved end of the SCSMV genome; 10 sets were designed and one primer has been optimised for all SCSMV isolates and tested on dried plant samples and also on insects. The test was also applied to extracts stored on FTA cards.

A potential rapid and specific field test (LAMP – Loop mediated isothermal amplification) was tested with a focus on design of the assay, testing of sample accuracy, sensitivity and

troubleshooting. Research used the program ‘LAMP Designer’ to design six sets of primers. The method is quite simple and involves an extraction, dilution, addition of enzyme and reaction components added with an incubation period. This potentially could be used in the field and our development so far has been on the use of a colour reaction (blue vs purple). We are also exploring the option of using a different detection method, based on recent publications.

Transmission

Mechanical – leaf application

With sugarcane mosaic virus (SCMV, not SCSMV) in Australia, infected leaf extracts are applied to the surface of test plant leaves via an infested scourer pad and this leads to effective mechanical transmission. With this inoculation method in mind, disease-free test plants were grown from disease-free plant sources. When test plants were at an appropriate size (6 weeks of age), diseased cane juice was extracted from both cane stalks and also cane leaves expressing SCSMV symptoms; extracts were maintained in PBS buffer. Abrasive pads dipped in the infected juice were used to inoculate the cane leaves (Figure 1). Juice was kept cool and used within one hour from juice extraction. The plants were grown for three months and regular observations made for SCSMV symptoms. Both positive and negative controls were included in the experiment.

Treatments included were:

- i. leaf extracts from healthy plants;
- ii. stalk extracts from healthy plants;
- iii. disease-free test plants (not inoculated);
- iv. leaf extracts from diseased plants;
- v. stalk extracts from diseased plants; and
- vi. SCSMV-infected plants (uninoculated).

Three varieties were also tested: the susceptible PS 864, an intermediate variety BL and the resistant PS 98-126 with five replications.



Fig. 1—Inoculating young leaves of test plants with SCSMV using the abrasive pad technique.

Mechanical – knife application

Previous work suggested the possibility of SCSMV transmission via knives contaminated with SCSMV-infested juice. With this in mind, infested juice was extracted from both cane stalks and leaves and applied to healthy stalks of cane by cutting them with a contaminated knife

(previously dipped in infested juice). The susceptible variety PS 864 was used in the experiment with treatments including disease-free control, diseased control (infected cane setts) and a third treatment being disease-free stalks cut with a knife dipped in infested juice.

Setts were grown in small pots and placed within an insect-proof net. Regular inspections were conducted examining the developing plants for symptoms of SCSMV; this occurred over a 12 week period. SCSMV incidence was then summarised for all treatments. Each treatment was replicated 5 times.

Epidemiology

The spatial pattern of a disease may provide clues as to the mode of disease transmission; data also suggest how fast the disease spreads. With this in mind, a disease-free plant source of the susceptible PS864 and a diseased plant source of the same variety were identified. A suitable field was selected at Kediri in central eastern Java and both diseased and healthy cane planted to assess spread from diseased to healthy cane.

The plot dimensions were approximately 60 m x 100 rows; the area was planted in July 2015. Each stool in the crop has been inspected on a monthly basis since then with GPS records of disease incidence on a stool-by-stool basis.

A second crop near Pasuruan (east Java) has also been regularly monitored; this cane had been planted by the time the project started. Monitoring aims to quantify spread within a more heavily-diseased crop. Again monthly monitoring using GPS equipment is providing data on the speed and pattern of spread.

Yield loss

Assessment of yield losses caused by SCSMV is being undertaken by relating disease incidence in plots with yield parameters (stalk populations, stalk weights, sugar content). Varying disease levels have been created by planting mixtures of diseased and healthy SCSMV-infected setts. A randomised complete block design with five replicates was employed. Plots consisted of four rows, 12 m in length, of the susceptible PS 864.

All plots were surrounded guard cane of the more resistant variety DL to limit disease spread (4 m sections at the ends of plots; two guard rows). Disease levels included the following: 0, 5, 10, 15, 20, 25, 30, 50, 75 and 100% diseased plants.

Monthly disease observations were undertaken to quantify SCSMV spread both within and between plots. Yield in the harvested plant crop (Figure 2) was assessed via the following parameters: stalk height, stalk diameter, stalk weight, stalk number, and stool populations. Sugar content was not quantified in the plant crop.



Fig. 2—Harvesting the yield loss experiment at Kediri at the end of the plant crop.

Results

Diagnostic assay development

The RT-PCR tests developed by Damayanti and Putra (2011) clearly distinguished between the two viruses (Figure 3). This provided confidence in the RT-PCR-based tests to distinguish the two viruses.

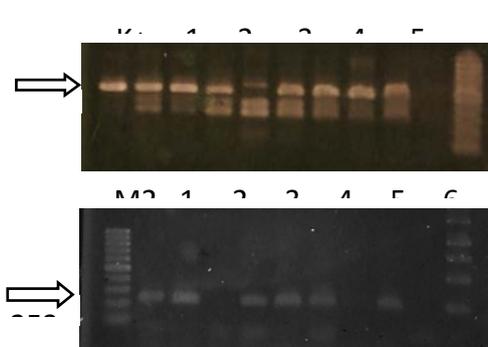


Fig. 3—Differences in the bands associated with PCR detection of SCSMV and SCMV (SCSMV above, SCMV below).

The results of tests comparing dried leaf specimens versus FTA cards (with dried leaf extracts) showed that the FTA cards detected SCSMV in fewer samples compared with the dried leaves. This is likely to be because the size of the sample is much smaller for the FTA cards. In future experiments these will also be analysed in duplicate. The ease of handling, importing and analysis makes this method of transportation and preservation very convenient.

RT-PCR assays have worked well on all samples, including dried tissue and extracts dried on the surface of FTA cards. FTA cards, coupled with RT-PCR, were used to store and assay samples from the 2016 survey of Sumatera and West Java.

Quantitative RT-PCR (qRT-PCR) primers were designed to detect the more conserved 3' end of the SCSMV genome. FTA cards have proved a little problematic; the RT-PCR is done with the filter paper in the tube and this may interfere with the setup of the qRT-PCR when attempting large scale/robotic systems.

The method is not currently under further development, though it seems to be quite robust when separating the RT from the qPCR reaction. LAMP assay research showed that recognition of the colour reaction of the test was problematic under field conditions. In addition, Indonesian-collected samples failed to react in some tests; this was thought to be because of significant viral sequence variation. In order to eliminate detection variation by colour recognition, a system that is detected by optical instruments is being tested.

Various facets of molecular research have suggested there is significant variation in the viral genome and further studies will aim to sequence a more conserved part of the genome, leading to a reliable assay that is not dependent on virus origin. Research with SCSMV samples sourced from Myanmar and Sri Lanka confirmed that the outermost primer set is not optimised for the Indonesian samples, and this may explain why the initial LAMP test failed.

A comparison of isolates showed that those from Pasuruan exhibited a homology of between 97.4–98.8%, while there was slightly lower homology in isolates obtained from West Papua (95.7–96.6%). Whether the sequence difference is biologically significant remains unknown. Further work will centre on sequencing parts of the genome, and will be undertaken using a range of samples collected from around Indonesia. Sequence variation in the coat protein region was found in samples from countries other than Indonesia; this is an important consideration in the development of a serological assay. This variation is being taken into account for the production of antisera based on the coat protein region.

Transmission

Mechanical transmission – leaf application:

Very good transmission was obtained when infested juice, from either diseased stalks or leaves, was applied to healthy leaves using a scourer. Infection rates were above 90% (Figure 4). There was also significant differences between the three varieties, with the susceptible PS 864 exhibiting much more disease than the intermediate BL and resistant variety PS 98-126. Infested juice extracted from cane stalks appeared to lead to higher levels of disease compared to leaf extracts from diseased cane. No disease was seen in the disease-free controls and 100% disease was observed in the ‘disease’ controls.

Mechanical transmission – knife application:

In contrast very little transmission occurred when a knife was used to apply infested cane juice to the cut ends of setts. Around 6% diseased plants were associated with knife transmission; further research is needed to be sure this level of disease incidence reflects actual knife transmission. The current results suggest that such transmission is unlikely to be significant in the field context.

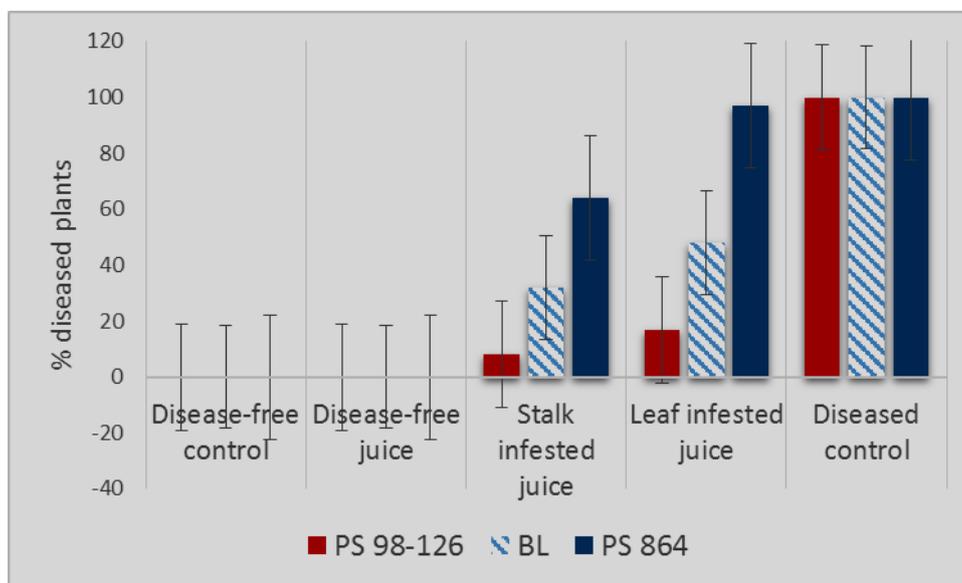


Fig. 4—SCSMV incidence in test plants of three varieties inoculated using extracts from either SCSMV-infected leaves or stalks. The varieties varied in disease resistance: PS 864 (susceptible), BL (intermediate) and PS 98-126 (resistant). Standard errors for each treatment are shown.

The abrasive pad method proved to be very effective in transmitting SCSMV in each of the varieties and disease incidence was consistent with acknowledged field resistance ratings of the varieties.

Epidemiology

Monitoring of the Kediri plot has shown a small increase in disease incidence, with diseased stool scattered across the field. Infection levels are at approximately 10% after the plant and first ratoon crops. This crop will be further monitored in order to ascertain any patterns in disease spread.

The field at Pasuruan has higher disease levels (approximately 20% infected stools) and the SCSMV spatial pattern four months after ratooning is shown in Figure 5. To identify if there is any significant spatial pattern (potentially suggesting either vector or in-field mechanical transmission), the data are being analysed using appropriate software.

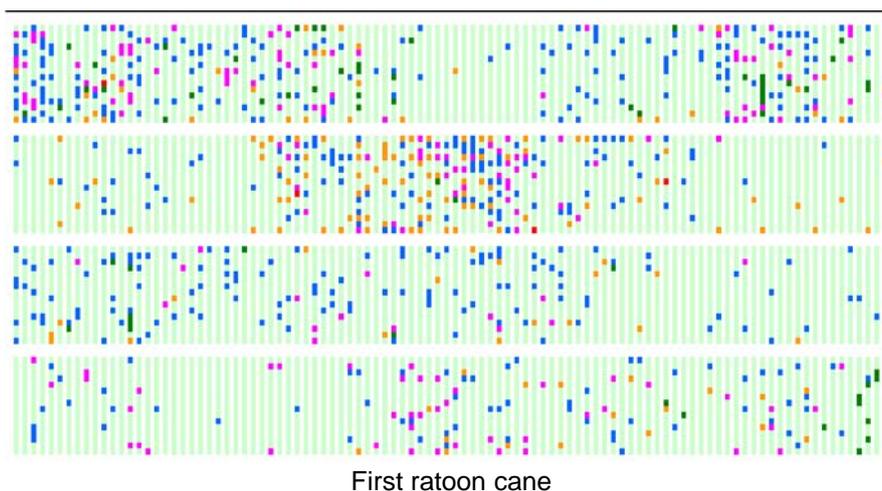


Fig. 5—Spatial pattern of SCSMV in nursery cane at Pasuruan in the early first ratoon (four months). Each colour represents the additional SCSMV incidence observed at each monthly inspection time.

Yield loss

Disease levels remained fairly constant within plots over the plant and first ratoon crops; this suggests little spread between plots (Figure 6). Harvest and monitoring data suggest that yield reductions occurred, principally at the highest disease levels; average loss of cane yield (0 vs 100% diseased stalks) was around 22% (Figure 7).

Symptom expression in the different plots took some time to reach the indicated levels (5–6 months after planting) (Figure 6).

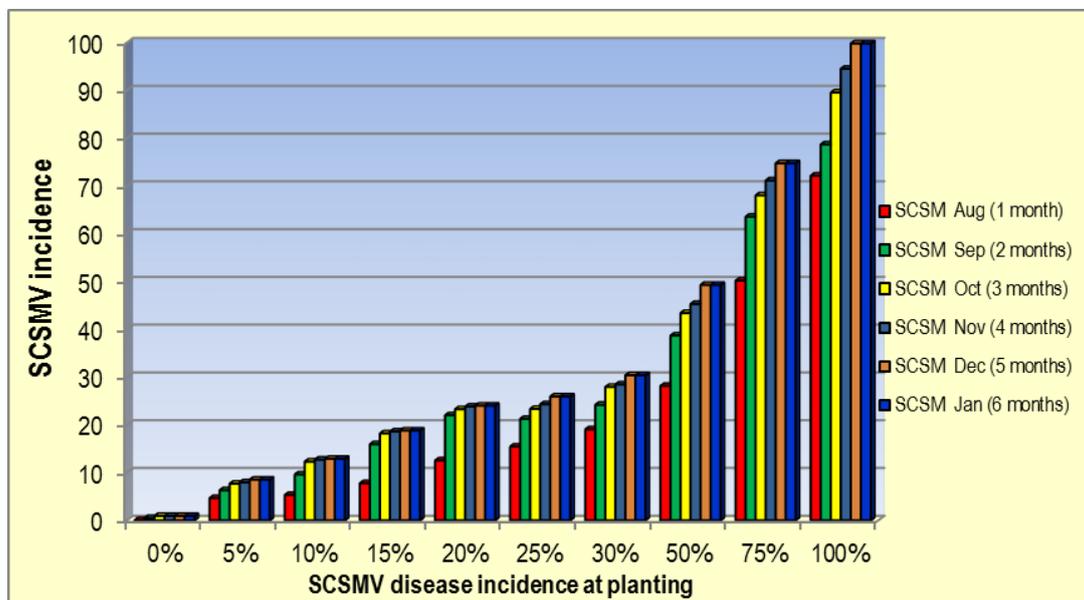


Fig. 6—Disease expression in plots in the Kediri yield loss experiment in the plant crop.

An examination of both stalk population and stalk height data revealed little difference between treatments but there may have been an effect on stalk diameter (data not presented). There appeared to be a consistent negative effect of SCSMV on yield, with a more rapid increase in yield losses at lower levels of the disease.

At higher disease incidence, losses appeared to plateau (Figure 7) at around 20%. A cubic relationship was fitted to the data (highest coefficient of variation).

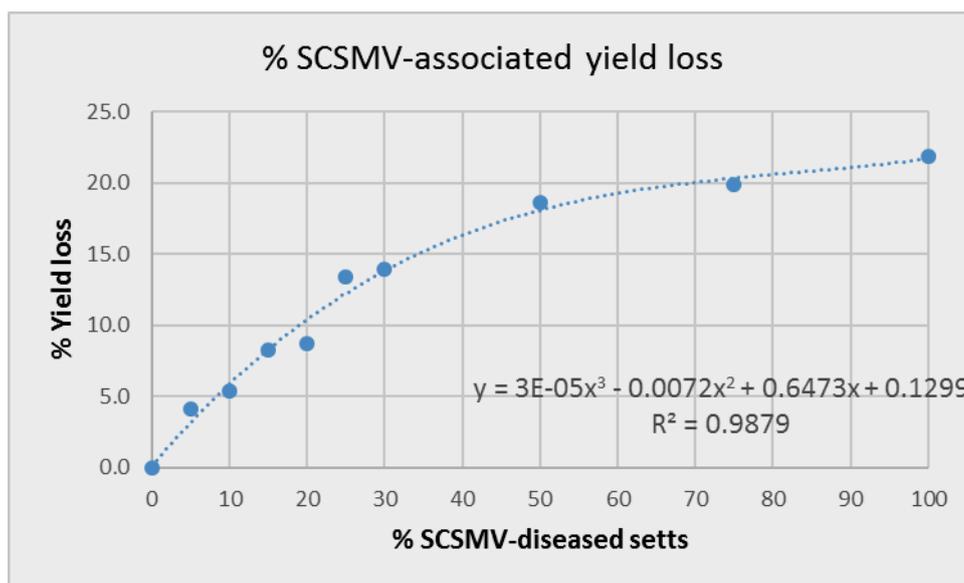


Fig. 7—Calculated yield as affected by SCSMV in plots of the Kediri yield loss experiment.

Discussion

With a recently recognised disease, many basic aspects of pathogen infection, transmission, losses and management will require basic research. So it is with SCSMV. Research is obtaining information on a number of critical disease and management issues. Additional research is still required but the magnitude of the losses associated with high disease incidence confirms the importance of the virus to sugarcane production in Indonesia, and its potential influence on Australian productivity.

In contrast to more lethal diseases, SCSMV doesn't drastically affect the yield of individual plants nor is it terminal; however, mean data from the Indonesian yield loss experiment suggests crop losses of up to 20% with much of this occurring at relatively low disease levels (30% diseased stools). These results are consistent with those reported by Putra *et al.* (2014). Observation of affected, individual crops may not immediately raise dire concerns; however, a feature of SCSMV is that many crops where it occurs are 100% diseased.

These crops may not show dramatic growth limitations in individual stools or patches within the crop but the overall yield losses experienced match those where more dramatic losses occur in just sections or parts of a crop. The result is that overall losses may be just as high. An unsuspecting farmer may think that the weather during the season did not favour crop growth, when a disease may be the real cause of the lack of crop vigour. Personal observations by the authors suggest many sugarcane crops through SE Asia are widely diseased, suffering significant SCSMV-associated losses, which are economically crippling on a whole-of-industry basis. SCSMV is an important economic issue for sugarcane production in Indonesia, especially in Java.

Transmission studies suggest that abrading leaves using a scourer dipped in infested cane stalk juice is able to lead to high disease incidence. Scourer transmission has been used successfully in resistance screening trials for sugarcane mosaic virus (SCMV); the same methodology appears to offer a good method for screening canes for SCSMV. Cane knives contaminated with SCSMV-infested juice have not led to significant disease transmission when healthy planting material is cut with infested knives – though low levels of transmission were detected. This is in contrast to results reported by Putra *et al.* (2014) who reported significant knife transmission. If SCSMV was easily transmitted on cane knives, management would be very difficult indeed. Further research is needed on mechanical transmission. Epidemiology research is expected to highlight potential modes of transmission under field conditions through analysis of spatial pattern data. Certain spatial patterns are characteristic of mechanical and vector-transmitted diseases.

Diagnostics research is progressing well, with several molecular methods able to reliably detect the causal agent. Research has shown that there is molecular variation in the pathogen, both between continents (India, Pakistan, Myanmar vs Indonesia) and also within Indonesia.

This is consistent with the report of some strain variation in the virus (Li *et al.*, 2011). This is important since any diagnostic test must be able to firstly, identify the pathogen and secondly, identify the specific unique identifiers of the 'strain' involved.

Our research is seeking to find out how significant such variation is, both in terms of detection but ultimately in terms of biological outcomes—whether detected variation affects pathogenicity. The latter may ultimately fall outside the scope of this proje

Research into molecular techniques is also addressing detection technologies such as LAMP, a potential rapid, in-field test, and also the use of FTA cards, a means of transporting extracts in a stable format for assay at a later date. Such technology would be very useful for surveys where extract stability is essential to obtain reliable assay results.

Serological assays are being developed, ultimately with the view to providing industry with a cheap and reliable assay. The variation in the viral coat protein may influence the serology of the test developed.

There are two main methods of developing antisera, one using a host animal (rabbit) while the other does not. Technology now allows sequence information to be given to a commercial technology firm who can create an antiserum without a live animal host. SRA are working with this firm to determine the best approach. Serological tests will be compared with molecular assays in terms of sensitivity, specificity and cost when these assays have been fully developed.

A third SCSMV survey, of the Indonesian Archipelago, will occur in 2018 and will shed more light on the incidence and severity of SCSMV in territories close to Australia. This survey will be critical for Australian interests since Indonesian territory involved close-by the Australian continent.

Research conducted in this and previous projects has thrown new light on the biosecurity threat posed by SCSMV. Much remains to be researched, including methods of transmission, potential insect vectors, and the resistance of Australian commercial varieties.

Strong links with our Indonesian counterparts are being maintained, thus providing a strong basis for on-going research, development and collaboration. Ultimately, an array of biosecurity measures related to SCSMV will be built into contingency plans to guide the Australian industry in response to a SCSMV incursion, should this occur.

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