FINAL REPORT 2015/046

Securing Australia from PNG biosecurity threats

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ABSTRACT

Papua New Guinea (PNG) is the centre of diversity for several Saccharum species (*S. officinarum, S. edule, S robustum*) and supports indigenous pests and diseases of sugarcane. These threaten Australian commercial sugarcane production, but also directly affect commercial sugarcane yields at the only PNG sugar estate at Gusap, Morobe Province. Two major diseases (Ramu stunt, downy mildew) and a group of pests (moth borers) are recognised as the most important biosecurity threats. Australian scientists have been working in PNG for over 30 years to develop better management strategies for these organisms. A previous project (2009/033) progressed diagnostics and resistance screening research but further work was required. The current project investigated pathogen diversity and diagnostics for Ramu stunt / downy mildew, developed a rapid DM varietal resistance screening test, developed a rapid moth borer screening test (*Scirpophaga excerptalis*) and researched an artificial diet for *S. excerptalis*. This research will assist the development of improved detection and management of an incursion into Australia while improving productivity in PNG. Research identified possibly four *Peronosclerospora* taxa where only two were thought to be present previously, three of which may be new species. Pathogen variation was also identified in the tenuivirus causing Ramu stunt. Variation in both pathogens may lead to unexpected susceptibility in previously resistant varieties. The identified tenuivirus was shown to be the cause of Ramu stunt, while *Eumetopina flavipes* was confirmed as the virus vector. A rapid method for DM resistance screening was developed, as was a preliminary rapid test for resistance to *S. excerptalis*.

EXECUTIVE SUMMARY

Issue and objectives: Disease incursions have occurred on a semi-regular basis in the Australian sugarcane industry. Some of these have led to major constraints on sugarcane productivity and the loss of high-yielding germplasm. More recent examples include smut (*Sporisorium scitamineum*) which caused a significant disruption to the Australian commercial industry, principally through the loss of high-yielding varieties and important parental germplasm. Extreme losses were only prevented by prior knowledge of both the disease and pre-emptive resistance screening of Australian commercial varieties in Indonesia. Papua New Guinea (PNG) is the home to a number of biosecurity threats, including downy mildew (DM) (*Peronosclerospora* spp), Ramu stunt and moth borers – the latter are a major pest group present in many countries around the world. Previous research led to method development for varietal resistance screening for DM and Ramu stunt, but these required long-term trials. Some variation in both the DM and Ramu stunt pathogens had been detected; this extent of this variation had not been explored, nor the implications for disease diagnostics. The biology/ aetiology of Ramu stunt required further research to enable better disease management. Previous field trials have identified varietal resistance to moth borers but a rapid method for screening varieties for moth borer resistance was also needed, particularly a method that would isolate resistance to individual species rather than the complex of borers that infest field trials. Successful research on all these aspects would position the Australian industry well in dealing with any incursion of these PNG pathogens and pests.

R&D methodology: DM pathogen diversity was explored by collecting diseased material from the Ramu Estate and around PNG; leaves supporting 'down' were collected as well as leaves showing the leaf splitting symptoms. DM was also collected from several other *Saccharum* species to determine the diversity of the pathogen in closely related plant species. Molecular tools were used to examine the phylogeny of the isolates; traditional taxonomy was also employed. Ramu stunt-diseased plants were also collected during a disease survey; access to archived stunt specimens enabled further material to be examined for comparison purposes. Molecular tools were also used to determine pathogen variation. Concurrent research in the USA (including DNA sequencing) also informed Australian research outcomes. Transmission research aimed to confirm *Eumetopina flavipes* as the disease agent vector and that the identified tenuivirus causes the disease. This was progressed by collecting non-infested planthoppers, infecting them on diseased sugarcane, assaying the fed planthoppers using a molecular test, placing these on healthy tests plants and then assaying the test plants for the virus after a suitable latent period. A rapid DM resistance screen was developed which involved mixing leaf shred material (containing DM oospores) into disease-free soil and planting single-eye setts of test varieties into the infested soil. DM symptoms were monitored in the emerging cane plants. A rapid screen for moth borers incorporated the infestation of a single susceptible plant in the centre of a ring of un-infested individual plants of six varieties, and monitoring borer incidence in these plants over time. The focus in the moth borer research was on *Scirpophaga excerptalis* (top shoot borer).

Outputs include much improved knowledge of the pathogens causing the two diseases; significant pathogen variation was identified. At least three taxa of *Peronosclerospora* were recognised (possibly four) with 2-3 of these potentially being undescribed species. *P. sacchari*, the species most often associated with DM on a world basis, is one of the taxa present in PNG, but not *P. philippinensis*. Pathogen identity is critical when considering varietal resistance to DM. Further classification work is required with *Peronosclerospora* taxa. Significant variation was also identified in the Ramu stunt causal agent; an isolate from Alotau (eastern PNG) showed only 78 - 84 % homology with the pathogen from Ramu. Research clearly showed that Ramu stunt is caused by a tenuivirus and that *Eumetopina flavipes* is the insect vector. A rapid method for screening varieties

for DM resistance was developed based on the soil application of DM oospores obtained from leaf split material. A potential method for rapidly screening many sugarcane clones was thus identified.

A similar rapid test was developed for *Scirpophaga excerptalis* based on a glasshouse method with pest-free plants located adjacent to a single infested plant.

Outcomes / implications include the ability to confidently diagnose both DM and Ramu stunt, should an Australian incursion occur. Tools have been developed to identify DM pathogen taxa; this will benefit Australian scientists in deciding the implications of a DM incursion and assist with interpreting DM resistance rating variation in PNG. A rapid resistance test offers the possibility of screening Australian clones and varieties quickly (either at Ramu, or after an incursion in Australia) to generate essential resistance data for Australian commercial varieties. Molecular diagnostic tools for Ramu stunt provide similar benefit; a better understanding of the insect / pathogen biology will also benefit the industry in developing a suitable IDM strategy. A rapid resistance screening test will also be needed should a moth borer incursion occur; while resistance will not provide sufficient crop protection on its own, it will constitute an important part of any IPM strategy. Social and economic impacts of this project would be very large if an incursion occurred – via much improved management of these pests and diseases. Environmental impacts would arise from reduced use of chemicals (either pesticides or fungicides) through the better and more-timely application of resistance as a major pest or disease control strategy. Some aspects of the research will be immediately adopted, as for instance in quarantine diagnostics, not only within SRA but elsewhere in Government biosecurity activities. Personnel using the information will include SRA staff, industry Productivity Service staff plus biosecurity officers in Government departments around the country.

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1 BACKGROUND

1.1 Australian sugarcane biosecurity background

The Australian sugarcane industry has enormously benefited from the exclusion of major pests and diseases that affect overseas sugarcane production areas; we remain free from a significant number of major biological constraints. A particularly important exclusion has been stem borers; several damaging species are present in PNG, where they exert a very significant influence on commercial crop production. Major diseases (Ramu stunt / downy mildew - amongst others) are also present in PNG and of these downy mildew (DM) continues to adversely affect crop yields.

The 2006 sugarcane smut incursion in Eastern Australia reinforced the necessity to make adequate preparation for an incursion before it happens. Key preparations with smut included developing specific diagnostic tests, collecting varietal resistance information, formulating incursion contingency plans and identifying key management options. These preparations helped minimise commercial yield loss when smut entered the east coast Australian industry in 2006. For the same reasons it is extremely important that the industry prepares for possible incursions of major pests and diseases present in production areas very close to our shores (PNG and Indonesia). A failure to plan for these threats would leave the industry in a vulnerable position.

1.2 PNG biosecurity threats

Australian scientists recognised the value in becoming familiar with major exotic pests and diseases in the early 1980s. BSES established close links with the PNG industry; consecutive three-year 'Memoranda of Understanding' between BSES and PNG agencies formed the basis of the collaboration. Consultants in pests, diseases and breeding were provided to Ramu, leading to BSES/ SRA researchers becoming familiar with the most important biological constraints. The need to further collaborate with Ramu Agri-Industries was highlighted by the lack of resistance information on leading Australian smut-resistant commercial varieties; data were required on constraints such as *Sesamia grisescens* (stem borer), DM and Ramu stunt. There was no identified cause of Ramu stunt and no specific diagnostic assay and a lack of information on the variation associated with the DM mildew pathogen. Allowing this situation to continue would have led to an inability to adequately diagnose suspect infected samples during an incursion. Previous varietal resistance screening tests were unreliable and required refinement to enable Australian varieties to be properly assessed. SRDC funded a research project that aimed to fill these knowledge gaps (project 2009/033: *Preparing the Australian sugar industry for threats from exotic pests and diseases by identifying and using plant resistance mechanisms).* Project work led to the collation and analysis of previous resistance information and its publication: i DM (Kuniata *et al.*, 2010a), ii. Ramu stunt (Kuniata *et al.*, 2010b), and iii. stem borers (Korowi *et al.*, 2011). In project research, the Ramu stunt pathogen was found to be a tenuivirus (Braithwaite *et al.*, 2007)*;* more recently, a series of insect cages were used to successfully transmit the virus from diseased to healthy plants, confirming that the tenuivirus is the causal agent. A diagnostic RT-PCR test for Ramu stunt has been developed and validated (Braithwaite *et al.,* 2012); further work is needed to characterise the entire genome of the virus using next generation sequencing technologies (Adams *et al.,* 2013).

Significant variation was found in the DM pathogen (Magarey *et al*., 2012; Thompson *et al*., 2013). Research has suggested that two *Peronosclerospora* species (*P. sacchari*/ *P. miscanthi*) may be present in PNG. Their host range (sugarcane/ sorghum/ maize) has not been elucidated and this is important to define potential entry routes into Australia.

Further refinement of resistance screening tests has been made (Magarey *et al.*, 2012; Korowi *et al.*, 2013) as has the biology of the stem borer and the disease pathogens (Magarey *et al.*, 2012). Linkages were established between Australian (Nicole Thompson) and US researchers working on DM.

More work was required to understand: i. the specifics of the stunt and DM diagnostic tests (further work on pathogen characterisation / variation; where to sample plants; plant growth stage when the pathogen can be detected vs symptom development); ii. refinement of the DM rapid resistance test (work is needed to optimise inoculum levels, and to ensure rapid test data correlate with field test data) and iii. research on a rapid resistance screen for moth borers (*Scirpophaga excerptalis*)

2 PROJECT OBJECTIVES

2.1 Downy mildew

- Using molecular tools, clearly identify variation in the DM pathogen in commercial crops, garden canes and wild relatives in PNG enabling the specific taxa involved in DM causation to be scientifically described.
- Develop an accurate reliable DM diagnostic assay(s) that is specific to identified downy mildew variants.
- Validate a rapid resistance screening test utilising soil-applied oospores.
- Study host range of *P. miscanthi* and *P. sacchari* on two maize and two local sorghum varieties.

2.2 Ramu stunt

- Characterise genomic variations identified during disease surveys ensuring accuracy of the diagnostic test.
- Complete sequencing of the Ramu stunt genome using NGS technologies. As Ramu stunt appears to be a new Tenuivirus, the full genome sequence will allow it to be formally named and its taxonomic position to be determined.
- Define the biology of the host-vector-virus interaction and viral-host range in order for its taxonomic position to be determined.
- Define the biology of the host-vector-virus interaction and viral-host range in order to develop better management strategies and varietal resistance tests.

2.3 Other stem borers

- Develop methods for screening for resistance to *C. terrenellus* and *Scirpophaga excerptalis.*
- Develop an artificial diet for growth to maturity of larvae of *S. excerptalis* for use in IPM strategies.

3 OUTPUTS, OUTCOMES AND IMPLICATIONS

Outputs

3.1 Downy mildew

- Pathogen molecular research suggests there are most likely three undescribed *Peronosclerospora* species present in PNG. Significant pathogen variation explains why the resistance of some varieties has varied with time at Ramu, PNG. Detailed molecular information on this pathogen variation is now documented.
- Some information on the influence of pathogen variation x host susceptibility was obtained, with several *Saccharum* species and *Miscanthus* DM isolates and germplasm included in pathogenicity tests.
- The infectivity of DM oospores in a rapid DM resistance screen was confirmed and the technique offers promise for future resistance screening work.

3.2 Ramu stunt:

- Pathogen diversity in the Ramu stunt causal agent was investigated and significant differences identified related to location and host
- The tenuivirus associated with Ramu stunt was confirmed as the causal agent in transmission experiments, where the virus was identified in a disease-free test plant exposed to the vector (*Eumetopina flavipes*) with the virus detected in the vector after feeding on a diseased plant (before transmission to the healthy test plant).

3.3 Stem borers

- A shade-house pot experimental technique offers opportunity to screen for varietal resistance to *Scirpophaga excerptalis* (top shoot borer).
- Attempts to develop an artificial diet to rear *S. excerptalis* on a culture medium in the laboratory were unsuccessful.

Outcomes and Implications

Significant research is still required in the following areas: -

- *Peronosclerospora taxonomy*: having identified several distinct taxa of the DM genus, further research remains needed to characterise these taxa and to describe the new species.
  This will have implications for resistance testing as below, as well as being of significant scientific interest.
- *DM Resistance implications*: there will be a need to undertake further resistance x taxa research to determine not only how the different taxa affect DM expression amongst different sugarcane varieties/ clones, but also how the different taxa affect disease development in various *Saccharum* species. This will have implications in relation to how the disease(s) may spread around PNG/ closer to Australia and in the species that DM can be expected to infect.
- *Rapid resistance screening*: further experimentation using oospores as the inoculum source in resistance screening trials is needed. Preliminary data only have been obtained; there seems to be significant promise with the technique and further work will expedite implementation of the procedure. A rapid technique for screening for borer resistance also shows promise and could provide a foundation for further screening work with *S. excerptalis* (and other species).*Ramu*

stunt: there are similar implications arising from the Ramu stunt (tenuivirus) pathogen research. No screening of varieties for resistance to the different stunt pathogens has yet been undertaken and it is not known how the Alotau isolates relate to Ramu stunt development in different Saccharum species.

The target audience for the Ramu research includes Australian canegrowers (through a consideration of the resistance of Australian commercial varieties to DM and Ramu stunt, should incursions occur), Ramu Agri-Industries (in considering the resistance of commercial canes at Ramu); Australian Biosecurity staff in various Government agencies (in being able to assay for the *Peronosclerospora* / Ramu stunt taxa and to interpret the disease observations made in field surveys); Productivity Service staff (in understanding the background to potential disease risks to the Australian sugarcane industry); and SRA staff (disease background information and the application of disease assays in quarantine).

Adoption so far includes the update of contingency plans (making information and disease ratings available to the Australian sugarcane industry) and the application of improved disease assays in quarantine.

4 INDUSTRY COMMUNICATION AND ENGAGEMENT

4.1 Industry engagement during course of project

Key messages

- Pathogen variation in the causal agents of DM and Ramu stunt has been detected in PNG.
- New taxa (undescribed) of *Peronosclerospora* have been identified and these await description.
- The effect of pathogen variation on the resistance of Australian commercial varieties is yet to be determined, though variation in DM resistance with DM 'strain' has been observed in the past.
- Rapid resistance screening for both DM and top shoot borer (*Scirpophaga excerptalis*) looks promising.

Updates on the project have been provided at SRA industry meetings in collaboration with SRA Adoption staff. Such meetings have been held through the industry in the 2015-2017 period. In addition, papers have been delivered at annual conferences of the Australian Society of Sugar Cane Technologists (see the published papers listing). Information has also been supplied at SRA Pathology training events at Woodford during the project period.

4.2 Industry communication

Conference and workshop oral presentations:

Braithwaite KS, Magarey RC, Kuniata LS (2015). Ramu stunt: An important biosecurity threat to Australia. ISSCT XI Pathology and IX Entomology Workshop, September 2015, Guayaquil, Ecuador.

Braithwaite KS, Ngo C, Tom L, Kuniata LS (2017). Ramu stunt: an important viral disease from the home of sugarcane. ASSCT (American) conference, June 2017, New Orleans, Louisiana, USA

Braithwaite KS, Ngo C, Tom L, Kuniata LS (2017). Ramu stunt: an important biosecurity threat to the Australian sugarcane industry. SciPlant17 - *Science Protecting Plant Health* conference, September 2017, Brisbane

5 METHODOLOGY

5.1 Downy mildew

5.1.1 Defining the *Peronosclerospora* species present in PNG.

5.1.1.1 Survey

Leaves expressing DM symptoms were collected from a range of *Saccharum* species (including commercial sugarcane, *S. officinarum*, *S. robustum*) and related host species (including *Miscanthus*, corn and sorghum) during a survey of Ramu and surrounding areas in the Eastern Highlands/ Morobe Province in October-November 2016. Dr Roger Shivas and Dr Malcolm Ryley were participants on the survey and gave advice with regards to sample preparation and species identification. In addition, all samples from the survey were sent to the Queensland Herbarium for storage and analysis. Dr Yu Pei Tan has assisted greatly in this work, completing the Cox2 sequencing of the isolates.

Specimens collected during the survey were used in host transmission studies as well as for microscopic morphology studies and in molecular analyses and assay development work.

5.1.1.2 Microscopic analysis

As a guide to microscopic observation of oospores of the different DM taxa, data were sourced from Sivanesan and Waller, 1986, which outlines oospore size variation in known *Peronosclerospora* species infecting *Saccharum* spp. (Table 1)

Oospores

Light microscopy was used to examine oospores from leaf shred material collected during the project survey and in other project activities. All oospores measured ~50 µm, regardless of their collection location or host species. This suggests no microscopic evidence for the presence of *P. philippinensis* in PNG, as the oospores of this species are smaller. Morphological observations did not enable differentiation of other *Peronosclerospora* species since the oospores of many *Peronosclerospora* are around 50 µm.

Asexual structures (conidia/ conidiophores)

Examination of the conidiophores was also not conclusive, as these structures appeared to be typical for various *Peronosclerospora* species. It should be noted that only specimens with associated ‘down’ can be examined for conidia, so this limited morphological observations in many wild host species.

Table 1: Distinguishing features of *Peronosclerospora* species, as defined in Sivanesan and Waller, 1986.

Species	Distribution	Host	Symptom	Conidia	Oospores
P. sacchari	Australia*, Fiji, India, Indonesia, Japan, PNG, Philippines, Taiwan, Thailand	*Saccharum, Zea*	Leaf streaking and shredding	25-55 x 15-25 µm	50 µm with wall 3.5-5 µM thick
P. philippinensis	India, Philippines	*Saccharum, Euchlaena, Sorghum, Zea*	Leaf streaking	27-39 x 17-21 µm	15.5-22.5 x 2-4 µm
P. miscanthi	Fiji, Philippines, Taiwan, possibly PNG	*Saccharum, Miscanthus*	Leaf shredding	37.0-48.5 x 14-30 µm	32.5-56.5 µm diameter
P. spontanei	Thailand, Philippines	*Saccharum, Zea, Euchlaena, Miscanthus*	Leaf streaking	39-45 x 15-17 µm	Similar to *P. sacchari*

5.1.1.3 Sequence analysis

Specimens collected during the 2016 survey were submitted to the Queensland Plant Pathology Herbarium and sequenced by Yu Pei Tan, BQ (Table 5). Dr Tan used the Cox2-F and Cox2-RC4 primers as described in Choi *et al.* (2015) and she made the sequence data available for project analysis (prior to publication).

All DNA analyses were undertaken using the Geneious Version 11.0.2 (http:www.geneious.com, Kearse *et al.*, 2012). Alignment of the sequences with *Peronosclerospora* voucher specimens published in GenBank was undertaken using the ClustalX algorithm using default parameters.

5.1.2 Validate a rapid resistance screening test utilising soil-applied oospores

Previous research (Kuniata *et al.*, 2010a) has shown that Australian germplasm has a significant DM susceptibility; around 50 % of clones and varieties are susceptible. For Ramu Agri-Industries this means that a large portion of imported commercial varieties need to be discarded, prior to selection for high-yielding agronomic traits. Without a rapid resistance screening test, insufficient numbers of varieties are able to be screened to select those that are both resistant and high-yielding. Should there ever be a DM incursion into Australia, a rapid resistance screen would be needed to assess the vulnerability of the industry to DM-associated yield losses.

For these reasons a project objective was to develop a rapid resistance screen. Research in the preceding project (2009/033) had shown there was potential to use DM leaf shred material to infect single-eye setts, when the inoculum was mixed into pot soils (Magarey *et al.*, 2014). Previous overseas research had identified that *Peronosclerospora* oospores may on occasion lead to DM expression in inoculated cane (Lieu and Egan, 1989; Matsumoto, 1961; Matsumoto *et al.*, 1961).

The research reported here aimed to improve our knowledge of both the reliability of the test, as well as the ability to discriminate resistant from susceptible varieties.

Several experiments were therefore conducted over the course of the project, using DM leaf-shred material mixed into pot soils in short-term pot experiments. The data were analysed using a generalised linear mixed effect model with a binomial error distribution using restricted maximum likelihood (using ASReml-R).

The experiments conducted over the course of the project followed the same general pattern, as described below.

Peronosclerospora (DM) inoculum: consisted of leaf-shred material (Figure 1). *Peronosclerospora* infection, of some varieties in particular, leads to the production of oospores within the systemically-infected leaves. As the oospores develop (approximately 50 µm in diameter), they apply sideways pressure on the adjacent leaf material, which in turn causes the leaves to split longitudinally along the leaf venation. With abundant oospore production, the leaf blade becomes a tangled array of fibrous material.

This material was sought in commercial crops as inoculum for pot experiments. The variety most prone to leaf shredding at Gusap is Q136; leaf shredding is not seen in all varieties. Shredded leaves were collected, taken to the pathology laboratory, the mid-ribs removed and the leaf blades (what was left of them) cut into 5 mm, or less, lengths.

The fresh weight of this material was recorded.

Inoculation system: Forestry nursery soil (sandy loam) was obtained, weighed and calculated quantities of the leaf-shred material mixed into the nursery soil to create specific inoculum levels. Mixing was thorough in order to ensure an even inoculum distribution. Single-eye, disease-free setts of selected varieties were obtained and placed 3 - 5 cm below the soil surface, in close contact with the infested soil, to provide significant opportunity for the oospores to infect the sugarcane buds. Generally small polythene plastic 'pot' bags were used to contain the soil with the pots placed in the shade at ambient temperature immediately after potting. After 1 - 2 weeks the pots were shifted to a nursery bench to facilitate further cane growth. In one experiment, plants were planted into field plots for 2 - 3 months to provide for further disease expression.

Plant maintenance: No insecticides were applied during the course of these experiments; however, initial fertilising and regular watering were essential.

Disease assessment: DM incidence was the critical disease information recorded. This was assessed by examining leaves for the typical systemic leaf streaks that characterise DM. Multiple test plants were included in each replicate; statistical analysis was undertaken to assess treatment effects, clear differences between treatments were noted.

Experiment 1: DM infection using leaf shred material (January-March 2016)

Aim: to successfully use DM leaf shred material to generate diseased test plants.

With limited testing of leaf shred material as an inoculum source, the first experiment in the project focused on generating DM-diseased plants using this inoculum source.

Inoculum: During a visit made in September 2015, dry weather had made establishing a DM experiment impossible because of a lack of planting material. There was recognition however, that gathering and saving DM leaf-shred inoculum was essential if an experiment was to be established within 2 - 3 months. With this in mind, leaf-shred was collected from a commercial crop of Q136 by Ramu staff. The leaf material was cut into small pieces, dried and securely stored in the laboratory. With other Oomycete species, oospores provide a long-term survival structure and so the long-term viability of stored material was considered likely.

When the experiment was eventually conducted (January 2016), fresh leaf shred material was located and collected from a commercial Q136 crop, and this was used as a comparison to the older, stored material. Leaf shred was added to, and mixed in with, the pot soil.

Propagation material: In mid-January 2016, a search was made of the two main project propagation plots (established in the previous project) for potential test plant material; few mature stalks were found and a number of plots were empty – a result of poor stool survival associated with the 2015

drought. Some apparently suitable material was found in Estate block B North, where the 2014-planted Ramu stunt resistance screening trial was growing. The trial contained a number of Australian commercial varieties of known DM resistance. A known susceptible variety (Q221$^{(l)}$) was found in reasonable quantity and condition, with sufficient healthy stalks to cut the required quantities of single-eye setts for the experiment.

Treatments: the following treatments were established: -

1. Control – no DM inoculum
2. DM treatment 1 – stored leaf shred material
3. DM treatment 2 – fresh DM inoculum.

Replication: a completely randomised design was employed with 20 plants per treatment.

Experiment 2: DM dose-response experiment

Aim: to determine the optimum inoculum dose for resistance screening

The second experiment aimed to re-test the ability of leaf shred material to transmit DM, and also to examine inoculum dose as a potential influence on disease development.

Inoculum: during a visit in early May 2016, leaf split material was collected from Q136 in Estate Block A South. Three fertiliser bags of split leaves were collected by Ramu technicians. The material was cut into short pieces (aiming for 5 mm lengths). The leaf shred was stored overnight inside the pathology laboratory at ambient temperature.

Propagation material: Q221$^{(l)}$ from the 2013-planted Ramu stunt screening trial in B North was collected from rep 1 – no other propagation material of this variety was available at the time. All stalks were carefully inspected and disease-free material was cut for the experiment. A second inspection of shoots was made once the stalks had been removed from the trial site. Both the stalks and leaf split material were taken to the Agronomy office for processing. Stalk material was cut into single-eye setts on 3 May 2016 and placed in plastic buckets.

Soils: Two soils were used in the experiment: i. nursery soil – with a relatively high amount of organic matter in a loamy-silt soil, and ii. sandy soil – obtained directly from the Bora River.

Inoculation: The weight of the soil needed to fill the peat pots used in this experiment (8 cm x 8 cm x 8 cm deep) was calculated and sufficient batches of inoculum prepared to cater for each treatment. The weighed inoculum was mixed into a single batch of soil for each treatment. Mixing was achieved by placing a small amount of the soil for each treatment in a 15 L bucket and using a wire stirrer to mix the inoculum through the soil (roughly one quarter of the total soil).

The remaining soil was then added and the stirrer used to spread the soil/inoculum mixture through the bulk soil. The treatment soil was then transferred to a bench and spread/ mixed on plastic sheeting – hand mixing was achieved by heaping the soil into a cone shape and moving soil from one side of the cone to the other.

A small amount of un-infested soil was then added to the base of the peat pot, the sett (with no leaf sheath) placed bud-up, and the inoculated soil used to fill the peat pot. Soils were inoculated from the lowest to highest DM inoculum dose. Pots were then watered gently by hand to saturation.

The following amounts of each soil were added to individual pots: -

- Nursery soil (NS): 3.275 kg/ 15 pots (wet weight)
- Sandy soil (SS): 4.725 kg/ 15 pots (wet weight)

Treatments

1. Soil NS: No inoculum
2. Soil NS: DM leaf shred: 0.1g/ pot
3. Soil NS: DM 0.2g/ pot
4. Soil NS: DM 0.5g/ pot
5. Soil NS: DM 1.0g/ pot
6. Soil NS: DM 2.0g/ pot
7. Sand SS: No inoculum
8. Sand SS: DM 0.2g/ pot
9. Sand SS: DM 1.0g/ pot
10. Sand SS: DM 2.0g/ pot

***Variety*: Q221$^{\phi}$** ***Replicates*: 3** ***Plants / Rep*: 5**

DM rapid screening trial

Bench layout

Rep A	4	8	7
	1	9	6
	10	3	2
Rep B	5	10	5
	4	3	6
	7	9	8
Rep C	2	1	5
	3	1	9
	7	10	4
	6	8	2

Across the bench

Figure 1: Peat pots containing two different soils and a range of inocula comprising leaf split material, soon after trial establishment in late April 2016.

Figure 2: Close up view of the soils inoculated with the leaf split material.

Transplanting:

After four weeks, the test plants were planted into the field with the same trial design as per the placement on the benches. This enabled the plants to grow with minimal supervision and without the constraints of pot-bound root systems.

Experiment 3: *Identifying variation in disease expression associated with resistance*

Aim*: to determine if varietal resistance affects disease expression in this pathosystem*

Previous research suggested that *Peronosclerospora* (DM) oospores mixed with soil are infective. However, the conditions leading to optimum infection required more research – the following aspects in particular:

- *Symptom expression*: symptoms had been expressed transiently; a better method for obtaining disease expression was needed.
- *Relationship to field reaction*: ultimately the reaction of varieties in the pot screening technique needed to reproduce the reaction of varieties in commercial crops.

In this experiment the aim was to confirm again that leaf shred material leads to DM expression in susceptible varieties, but also to determine if varietal resistance influences disease incidence.

Propagation material: In a previous experiment, prior infection of some test plant material had confused the results. In this experiment, planting material was sourced from the isolated Leron quarantine plot, thus eliminating any risk of prior disease infection. Three varieties were used – two susceptible and one of intermediate resistance. These were sourced from the Leron plot on 21 December 2016; the cane was collected in stalk lengths and cut into single-eye setts at Gusap.

Inoculation: The healthy cane plus soil was first potted into small polythene bags to determine how much soil required infesting. After soil infestation with the required leaf split material, the small polythene bags were then one-third-filled with *Peronosclerospora* (DM) infested soil, and single-eye setts of each variety placed on top.

The bags were then filled with the remaining infested soil and placed alongside (but not immediately adjacent) to the un-infested plants/ pots (in a shaded position).

Inoculation dose:

- 13.2g/ pot, or
- 0.066g material/ g soil
- 6.6 g inoculum/ 100 g soil

A sample of the inoculum was taken and viewed under a microscope. Many oospores were attached to the split leaf pieces; the number released into the soil however is unknown. The density of the material in the soil was relatively high, so one would have expected buds and shoots to make reasonable contact with the soil-based oospores.

Treatments (10 replicates of each)

1.	MQ239$^{\phi}$	Resistant:	No DM
2.	MQ239$^{\phi}$		DM
3.	Q221$^{\phi}$	Susceptible:	No DM
4.	Q221$^{\phi}$		DM
5.	Q200$^{\phi}$	Susceptible	No DM
6.	Q200$^{\phi}$		DM

The plants were maintained on a concrete bench near the plant breeding shed. Pots were watered as required.

Disease recording: test plants were monitored weekly both for sett germination and also for DM symptoms. Inspections continued for approximately five weeks (until 31 January 2017) until little further disease expression occurred.

5.1.3 Host range studies

5.1.3.1 Conidia as inoculum

The host range of *Peronosclerospora* species causing sugarcane downy mildew (DM) is important in determining the potential threat posed by DM to the Australian industry. Typical DM symptoms are shown in Figure 3. There has been much work undertaken on assessing the DM resistance of Australian varieties, however little is known about the host range on related *Saccharum* species and wild grasses. An experiment was therefore established to determine if the DM pathogen causing leaf striping (classic symptoms) on sugarcane and corn would infect other species. This was repeated in the second year to determine if the leaf shredding (vs leaf striping) symptom occurs in other host species. In the original project plan, four host range experiments were designed: HR-1/HR-2 to assess conidia (asexual spore) inoculation and HR-3/HR-4 to assess oospore (sexual spore) inoculation.

Figure 3: Typical downy mildew symptoms on sugarcane. (L) Characteristic down on the leaf showing the classic symptom; (R) leaf shredding on Q136

Methods

HR-1 and HR-2 trials to assess conidia host range

Figure 4: Corn (L) and sugarcane (R) infected with downy mildew and chosen as sources of inoculum

These experiments investigated the host range of the DM taxa present in PNG. DM sources from both sugarcane and maize were used to inoculate several hosts (Figure 4).

Small greenhouses were purchased in Australia and shipped to Ramu (Figure 5). These provided shelter for the test plants and shielding from any external sources of *Peronosclerospora* inoculum.

The chambers were shaded to avoid excessive heat within the shade house. Plants were checked every day for water requirement and plant health.

Three chambers were allocated as:

- Chamber 1: Sugarcane conidia transmission trial (HR-1)
- Chamber 2: Maize conidia transmission trial (HR-2)
- Chamber 3: Healthy control plants – no inoculum present

Figure 5: Inoculation chambers at Ramu for containment of spores

Eight test species/varieties were selected based on availability and freedom from DM symptoms:

- Commercial sugarcane varieties
  - DM susceptible: Q121, Q200$^{\phi}$
  - DM resistant: Cadmus, MQ239$^{\phi}$
- Maize (*Zea maydis*)
- Pit pit (*Saccharum edule*)
- *Saccharum robustum*
- *Saccharum officinarum*

Test plants of *Miscanthus* were sourced from the Eastern Highlands, but did not survive transplantation, so this treatment was not included. *Saccharum spontaneum* was not able to be sourced due to drought/ fires which had destroyed much of the habitat where *S. spontaneum* is normally found.

The *Saccharum* species were cut into one-eye setts and hot-water treated at 52°C for 30 minutes to eliminate any DM infection. Corn was treated with hot air at 52°C for 24 hours to dry the kernels and reduce the chance of *Peronosclerospora* infection. Each was planted into potting mix in polyhouses, planting 2 one-eye setts/ pot with 3 reps each (except for *S. officinarum* – there was only enough planting material for 2 pots in Chamber 3).

The host species acting as the inoculum source was planted (untreated) into the appropriate polyhouse: corn seedlings were transplanted directly from the field; the infected R570 was cut into one-eye setts and propagated in polybags. Host plants were placed on the top shelves of the chamber, with two polybags containing 2 plants per chamber. There was no source of *Peronosclerospora* in the control chamber.

Plant germination was recorded and growth monitored. Leaf samples were collected at 5, 10 and 14 weeks after planting. At each time-point, observations were made for disease symptoms and a single leaf collected for PCR analysis. The leaf sample was cut into ~50 mm long pieces and dried over calcium chloride for import to Australia. Dead plants (either failed germination or other causes) were removed from the chamber to maximise the space for the living plants.

Sample import, DNA extraction and analysis was undertaken as per Magarey *et al.*, 2014 (SRA Project 2009/033 Final Report). In brief, the samples were dispatched to SRA Indooroopilly under import permit and irradiated at 50 kGray upon arrival, as per the import requirements.

DNA was extracted from the samples using a QIAGEN DNeasy Plant Extraction kit and the quality checked using spectrophotometry and an internal control PCR (Rubisco DNA and/or B-tubulin). In most cases the DNA was sufficient for analysis; any low quality samples were re-extracted. All samples were analysed using the Cox-1 PCR as developed previously. Analysis of the amplicons was done on a 2 % agarose gel.

All three time points were analysed for the presence of a Cox-1 amplicon, and observations were made for the presence of DM.

5.1.3.2 Oospores as inoculum.

The method developed by Magarey (this report) was adapted to test the host range of the *Peronosclerospora* taxa causing leaf shredding. As for the conidia HR trial, compromises were made due to the lack of available material. There was no sugarcane leaf shred material when the experiment was established, possibly resulting from a combination of unseasonal weather and the reduced amount of Q136 in commercial fields. Q136 is the only current commercial variety at Gusap that exhibits significant leaf shredding, but this variety is susceptible to smut. The latter disease was found for the first time at Gusap during the project period, and a smut management strategy is to terminate infested crops. The abundance of Q136 is therefore decreasing and reduced incidence of leaf splitting is a consequence.

A decision was made therefore to test the host range of leaf split inoculum sourced from *Miscanthus* and *S. robustum*. The treatments were expanded to allow testing of two different *S. robustum* inoculum sources.

Four chambers were established

1) Healthy (no inoculum) control
2) Inoculated with leaf shredding from *S. robustum* growing in the lowlands
3) Inoculated with leaf shredding from *S. robustum* growing in the highlands
4) Inoculated with leaf shredding from *Miscanthus* growing in the highlands.

Test plants represented the species below, with two replicates planted per chamber. The test plants were sourced and treated as per the conidia inoculum trial.

- *S. robustum*
- *S. officinarum*
- *S. edule*
- Maize
- *Saccharum* hybrid, variety Cadmus (resistant)
- *Saccharum* hybrid, variety Q87 (intermediate)
- *Saccharum* hybrid, variety R570 (intermediate)
- *Saccharum* hybrid, variety BJ6904 (susceptible).

One-eye setts of the test plants were placed in soil infested with leaf shred material contained within plastic polybags. The bags were 1/3 filled with potting soil, covered with 1 cm of shredded leaf material cut into 2 cm lengths, the one-eye setts were then placed on top and covered with a layer of shredded leaf material (Figure 6).

The pot was filled with potting mix and placed into the appropriate chamber. The control chamber was prepared prior to the other chambers to avoid any cross contamination. Individual pots were given sequential numbers for ease of analysis: samples 1 and 2 were uninoculated (chamber 1); samples 3 and 4 were inoculated with leaf shred from *S. robustum* from the lowlands (chamber 2); samples 5 and 6 were inoculated with leaf shred from *S. robustum* from the highlands (chamber 3); samples 7 and 8 were inoculated with leaf shred from *Miscanthus* (Chamber 4).

Figure 6: Setting up the experiment for inoculation with leaf shredding material. Higher inoculum doses were used in these experiments than in the rapid resistance screening tests to help ensure disease development

The new chambers were modified to reduce the temperature and humidity by adding mesh screening to a portion of the rear of the chamber, thus allowing airflow. Growth was monitored during the experiment.

Leaf sampling and symptom observations were made at 10, 17 and 24 weeks post inoculation, with PCR analysis for the presence of *Peronosclerospora* spp. undertaken on all samples. The PCR analysis was as described for conidia experiment.

5.2 Ramu stunt

5.2.1 Disease survey

Disease surveys assist in assessing the incidence and severity of a disease, which helps to gauge the potential for a pathogen incursion in Australia. Surveys may also identify new strains, highlighting the possible need to modify the diagnostic test. Surveys in PNG for Ramu stunt were undertaken during a previous related project (2009/033) and the ACIAR-funded project (CS1/1996/140). In all cases, garden canes, wild canes and weeds showing suspect symptoms of Ramu stunt were collected and screened with the diagnostic molecular test. When positive samples were identified, the entire 1.2kb RNA 6 genome fragment was sequenced for phylogenetic comparisons. In the current project, a one day survey in November 2016 was undertaken through the Ramu Valley.

The aims of this survey were to obtain more data on disease distribution and to find material that could be used in the genome sequencing aspect of the work.

A project specific survey took place on 3 November 2016, with Ramu staff (Leka Tom, Simeon Yamang and Wamba Batimane) assisting. The first sampling site was a crop of R570 on the Ramu estate known to be infected with Ramu stunt (infected seed cane had been utilised). Further sampling sites were in the Ramu Valley toward Madang. Leaf samples were collected and processed by storing in tubes over calcium chloride, multiple tubes were collected to provide sufficient material for genome sequencing. On arrival into Australia, samples were irradiated, as required by import permit conditions. RNA was extracted using a Qiagen RNeasy mini kit. All samples were screened with the standard RT-PCR diagnostic test for Ramu stunt (Braithwaite *et al.* 2012); this test uses the primers C1eF and C1fR, and an endogenous test for RNA quality, which in turn incorporates the sugarcane phosphofructokinase primers PFK5-F1 and PFK5-R1 (Zhu *et al.* 2013). Samples showing suspected symptoms of maize stripe virus (MSpV) were screened with the primers RotNCPaFWD and RotNCPcREV.

Survey samples testing positive for Ramu stunt were subjected to phylogenetic analyses. The entire 1.2kb of RNA 6 (based on the naming system of Mollov *et al.*, 2006) was sequenced and the data combined with sequence data from project 2009/033. Sequence editing, contig construction, multiple sequence alignments with Clustal W and tree construction were performed using programs supplied in the Geneious 8.1.8 package. The tree was generated using the Jukes-Cantor genetic distance model and UPGMA tree build method with 100 bootstraps.

5.2.2 Ramu stunt genome sequencing.

At the initiation of this project, there were no published Ramu stunt pathogen sequences; the only sequence information was that generated during the previous project (2009/033). The original intention of this project was therefore to sequence the genome of a Ragnar isolate. However, in September 2015, it was evident that Dr Dimitre Mollov, a molecular virologist from the National Germplasm Resources Laboratory (NGRL), USDA-ARS, Beltsville Maryland, USA, had almost completely sequenced the Ramu stunt viral genome. His viral source was also Ragnar, sent to Beltsville some years previously by Lastus Kuniata (Head of Research at Ramu Agri-Industries). The request for diseased Ragnar came from Clarissa Maroon-Lango, who had begun compiling a world collection of sugarcane diseases in the Plant Germplasm Quarantine Program (PGQP) high security glasshouse facility operated by the Animal and Plant Health Inspection Service (APHIS) at Beltsville, USA. The NGRL is currently using metagenomics to characterize Ramu stunt, sugarcane yellow leaf virus (SCYLV), sorghum and sugarcane mosaic viruses (SrMV/ SCMV) and is planning work on sugarcane mild mosaic virus (SCMMV).

A decision was made that SRA and USDA would collaborate to sequence more isolates of Ramu stunt. Dimitre was currently involved in the project "*Characterizing and detecting pathogens to ensure safe exchange of plant germplasm*" (Project Number: 8042-22000-302-00-D). In 2016 an informal collaborative arrangement was made to exchange Ramu stunt samples from PNG with the support of Leka Tom and Lastus Kuniata. It was decided that isolates would be sequenced by both SRA and NGRL. Dimitre was also interested in MSpV, another tenuivirus; MSpV is common on the estate and in the Ramu Valley, particularly in the weed 'itch grass' (*Rottboellia cochinchinensis*) which is found growing in association with sugarcane. Several samples were collected and sent to Beltsville.

RNA approach

The preferred approach for preparing RNA for sequencing at SRA was to extract RNA using a Qiagen kit in the Ramu pathology lab. It was intended to dispatch the RNA to Australia and then send it to a commercial sequencing provider. For sequencing at Beltsville, the preferred approach was to send living cane setts. The PGQP glasshouse, operated by APHIS at Beltsville, is able to accept diseased material.

However, neither approach was used. Extracting RNA at Ramu precludes the use of anything other than fresh leaves, which would exclude a preserved Alotau sample. Live material was sent to Beltsville but it arrived in very poor condition. This led to the use of imported leaf samples, stored over calcium chloride and irradiated on arrival into Australia. The material was then either analysed by SRA staff or resent onto Beltsville. The use of dried irradiated samples to prepare RNA for high throughput sequencing is not ideal, but adequate RNA quality was obtained.

Sample selection

Based on the phylogenetic groupings, it was decided that one isolate each from the commercial cane/Ramu estate cluster and the noble cane/Ramu Valley cluster would be sequenced by SRA and USDA. An additional isolate from the Alotau cluster was also sequenced by SRA. These are shown with arrows in Figure 20. Collection details are shown in Table 2. R570 (isolate 25) and Asas noble 1 (isolate 26) were collected during the survey. Several samples of MSpV in both maize and Rottboellia were also collected during the survey and sent to NGRL.

Table 2: Collection details for the three Ramu stunt virus isolates sequenced by SRA and two isolates sequenced by NGRL. Four isolates of Maize stripe virus (MSpV) were also sequenced by NGRL.

	Code	Host plant	Location	Year
SRA	PN97-54	Commercial	Block ES206, RAIL estate	2012
	Wamba-4	Noble	garden, RAIL estate	2012
	Alotau-26	Noble	garden, Bitu Village, Alotau	2013
NGRL	R570-25	Commercial	Block BS914, RAIL estate	2016
	Noble 1-26	Noble	garden, Asas Village, Ramu Valley	2016
MSpV	28	Maize	garden, Asas Village, Ramu Valley	2016
	31	*Rottboellia*	garden, Asas Village, Ramu Valley	2016
	6	*Rottboellia*	Creek near Block CN107 RAIL estate	2016
	8C	*Rottboellia*	Near Block ES104 RAIL estate	2016

SRA RNA sequencing

For the SRA samples, RNA was extracted using Qiagen RNeasy kits with on-column DNA digestion. The three leaf RNA samples (PN97-54, Wamba-4 and Alotau-26), each approximately 6 µg with a concentration of 160 ng µl was submitted to The Ramaciotti Centre for Genomics (http://www.ramaciotti.unsw.edu.au/) for library preparation and sequencing. We were advised that the RNA was degraded; not surprising since the samples were up to five years old, dried and irradiated, however, we proceeded anyway. Ribosomal RNA depletion was performed using an Illumina ScriptSeq leaf kit to reduce sequencing data produced from the abundant plant ribosomal sequences. Libraries were produced using an Illumina TruSeq Stranded Total RNA Library Prep Kit. 75 bp paired end sequencing with a median insert size of 210bp (100 bp – 450 bp range) was performed on an Illumina NextSeq500.

SRA Bioinformatics

Bioinformatics analysis was performed by Chuong Ngo. FastQC, part of the Geneious 8.1.8 package, was used to examine sequence quality and mapping. Quality was further examined by mapping both untrimmed and trimmed (bases with > 0.1 % chance of error) PN97-54 reads to the six GenBank Ramu Stunt virus RNA segments from Mollov *et al.* (2016, Table 3) using the "Medium Sensitivity/ Fast" default settings.

Generation of Ramu stunt contigs from PN97-54 and Wamba-4 was performed by mapping trimmed reads to the published Ramu stunt virus segments using a word length of 65 and allowable mismatches of 10 %. This allowed a good alignment of the reads to the published Ramu stunt virus segments. Consensus sequences generated from mapping PN97-54 and Wamba-4 sequence data were aligned back to Ramu stunt virus segments to check for accuracy.

Unfortunately Alotau-26 produced very few mapped reads. Untrimmed sequences were used to obtain maximum coverage for the analyses. Mapping parameters were adjusted until there was good mapping to one of the six Ramu stunt viral segments (Accession KR094119). A "good" mapping outcome was determined by aligning the generated consensus sequence from the mapping to the 1.2kb RNA 6 sequence (Alotau-26). These parameters (word length 18, allowable mismatch 25 %) were then used for mapping of the remaining five Ramu stunt genome segments. Further mapping was undertaken to improve the contig sequences by using the six generated Alotau-26 consensus sequences as references. Semi-complete Alotau-26 consensus sequences were aligned to the Ramu stunt virus segments. PCR verification and closing of gaps in the six Alotau-26 contigs is underway to generate complete sequences for the viral segments.

Mapping was also done using PN97-54 and Wamba-4 sequence data and the Alotau-26 sequence as the reference. This was to investigate if the Ramu stunt virus isolate from the Alotau region is present in plants that already contain the characterized Ramu stunt virus isolate of Mollov *et al.* (2016).

NGRL-USDA RNA sequencing

Two samples, R570-25 and noble 1-26 were received as dry and irradiated leaf material from SRA. Total RNA was extracted by a modified Qiagen protocol (using RNeasy plant extraction kit). Total RNA was sent to SeqMatic, Fremon,t CA for cDNA library construction and sequencing. Libraries were run on a NextSeq 500 illumina platform as 75bp single end reads. Analyses were undertaken using Geneious, CLC Workbench and SPAdes. The NGRL sugarcane virus detection workflow has three steps: 1. *De novo* assembly, largely just to "see what's there"; 2. Blast data against an in-house database containing one sequence of all known sugarcane viruses; and 3. Blast data against an in-house database containing all plant viruses in NCBI plus Arabidopsis.

5.2.3 Biology of the host-vector-virus interaction.

Two transmission trials were planned for this project. The first would determine which vector life stage (adults or nymphs) is responsible for transmitting the virus. The second trial would establish the host range of the virus. An isolate from the commercial cane 'Ragnar' would be transmitted to "wild' and "garden" sugarcanes. If transmission was successful, sequencing part of the viral genome would be undertaken to check that there had been no sequence change. As described below, the first trial had several issues and was not successful. So the testing of the vector life stage was repeated by combining it into the second trial.

Four new cages were constructed for transmission trials using Biomesh insect-proof mesh purchased in Bendigo, Victoria and shipped to Gusap in October 2015.

The four cages were to comprise:
the source cage, which would contain confirmed diseased Ragnar plants; two test cages, which would contain disease-free Ragnar plants and receive either adults or nymphs from the source cage, and finally a control cage, which would contain disease-free Ragnar plants and not be opened during the experiment. The cages were constructed in November - December 2015 on prepared ground (weeded and sprayed with herbicide).

In October 2015 Ramu stunt-infected Ragnar plants were sourced from the Ramu and SRA Ramu stunt screening trials at BN401, planted in 2014. Six stools were identified that displayed either stunting or leaf streaking and these were tagged and sampled. The leaf samples were processed and returned to SRA, Indooroopilly for diagnostic testing. Five infected, tagged stools were excavated from BN401 and transplanted to the source cage. By late January 2016, two diseased plants had become established in the diseased cage.

Trial 1

Disease-free Ragnar plants required for the test and control cages were supplied from the SRA quarantine glasshouse at Indooroopilly, on two occasions in early 2016. There were numerous problems involving import permit delays, delayed shipping and finally poor establishment due to a cricket infestation. Various measures were undertaken to control the crickets but none were successful.

An alternative experimental set up was required (Figure 7A). A total of 12 surviving plants, originally sourced from the Indooroopilly quarantine glasshouse, were repotted and the pots placed onto a concrete bench. This small number of plants only allowed for four control pots and eight test pots. Diagnostic assays were performed to ensure their disease-free status.

Due to the 2015 drought, a decision was made to source *Eumetopina flavipes* from garden canes in the Balus Hauslain (technician's village) where the plants were well-watered. Assays for Ramu stunt were carried out to ensure that the experiment started with Ramu stunt-free insects. 40 adults collected from village gardens were introduced into the diseased cage on 7th June 2016 and 60 adults on the 9th June. A subsample was kept aside for testing. The *E. flavipes* were left on the diseased plants for three weeks (until the week starting 27th June) to provide time to breed, lay eggs, for the eggs to hatch and develop into nymphs. The insects were harvested from the diseased cage in late June and sorted by eye into four size classes (adult, large nymphs, medium nymphs, small nymphs) and placed in two vials each for introduction to the test plants. A small subsample was kept aside for Ramu stunt assay.

The alternative transmission setup used white mesh sleeves placed over each test plant, instead of cages. The tops of the sleeves were tied to bamboo poles. Vials of insects were opened inside the

sleeve and then the base of the sleeve sealed around the pot with string and tape. The four size classes of insect were placed onto two replicate pots each. Four pots received no insects. The insects were allowed to leave the vials at will. The pots remained covered for three weeks to allow the insects to feed and transmit the virus. On the 19th July, the insects were killed with insect spray, the bags removed, all insects collected and the plants sampled. The plants remained on the bench for another two months to allow for further symptoms to appear, before sampling for the final time. During this period the plants were treated with Confidor to prevent vector breeding.

Figure 7: A. Transmission trial setups. A: alternative setup used for trial 1; B: cages constructed at the start of 2015/046 and used for trial 2.

Trial 2

The same two diseased Ragnar plants from BN401 established in the diseased cage in January 2016 were reused. They had been regularly screened to ensure their disease status, ratooned and were then replanted to provide three diseased stools.

Five plant sources representing three *Saccharum* species were collected from the Kainantu region (Eastern Highlands Province) on 20th July 2016 for use as test plant material in the trial, (Table 3). Stalk material was returned to the Ramu site, potted, and maintained on the concrete benches. By November the plants had germinated and were trimmed, repotted and sampled for stunt assay. In March 2017, plants were reorganized to ensure that there were exactly six plants in individual pots for each host species. Ragnar plants from the completed trial 1 were included for reuse in this trial. The 36 test plants were labelled and sampled again to ensure that they were virus-free at the start of the transmission period. Half of the top visible dewlap (TVD) leaf was sampled so as not to disturb the central growing point of the plant.

Table 3: Details of the test material to be used in the host range experiment.

Species	Common name	Location source
Saccharum officinarum	Red noble	National Agriculture Research Institute, Aiyura (NARI)
Saccharum officinarum	Yellow noble	
Saccharum edule	Red edule	
Saccharum edule	Yellow edule	Summer Institute of Linguistics, Ukarumpa
Saccharum robustum	Robustum	Roadside between Kainantu and SIL
Commercial sugarcane	Ragnar	Concrete benches from trial 1

In January, over 300 *E. flavipes* individuals were collected from village gardens and introduced to the diseased cage as the experiment commenced. Further smaller introductions were made in February. Testing of a subsample showed them to be virus-free.

The transmission treatments commenced in March 2017. The original cages constructed in 2016 were used in this trial (Figure 7B). One test cage each was assigned to “adults”, “nymphs” and “control” (no insects). The test cages had been moved away from the original cricket infestation site and the ground beneath treated with insecticide. Plastic was placed beneath the cages instead of weed mat. The 36 test pots were arranged so that two pots of each were placed randomly in the test and control cages.

All *E. flavipes* were harvested from the diseased cage on the 2nd March 2017 and sorted into adults and nymphs using a stereo microscope in the pathology laboratory. Subsamples of each were taken for later Ramu stunt assay.

The trial concluded in June 2017. For each of the 33 surviving test and control plants, all stalks in the stool were sampled and returned to the Ramu pathology lab. The number of stalks in the stool were counted, leaves were carefully observed for symptoms and each top visible dewlap leaf sampled for assay. Leaves from the diseased cage were also sampled for stunt assay.

Molecular assay

Leaf samples were collected and processed as described previously. At SRA, Indooroopilly, leaves were screened with the standard diagnostic assay for Ramu stunt (Braithwaite *et al.* 2012) and the endogenous test for RNA quality.

E. flavipes individuals were collected and stored in 90 % ethanol. A modified proteinase K method was used to extract total nucleic acids (Virtudazo *et al*., 2001). Ramu stunt assays utilised the same RT-PCR test as for plants. The endogenous test was a COI PCR using the barcoding primers LCO1490 and HCO2198 from Folmer *et al*. (1994).

Test plants testing positive for Ramu stunt (indicating transmission), were further characterised by sequencing the entire 1.2kb of RNA 6. Test plant sequence data were compared to source plant, *E, flavipes* and field control sequence data to show that the same isolate had been transmitted. Sequence editing, contig construction and multiple sequence alignments were as described previously.

5.3 Stem borers

5.3.1 Develop methods for screening for resistance to *Chilo terrenellus* and *Scirpophaga excerptalis*

Two shade house trials were conducted in the Ramu white-house with similar methods used in both. The aims were to: 1. assess the tolerance/resistance capacity of six varieties to the sugarcane top borer *Scirpophaga excerptalis* Walker (Lepidoptera: Crambidae), and 2 confirm that the method, which relies on larval dispersal to adjacent plants, is a reliable resistance assessment technique. Undertaking two similar experiments provided a measure of the reliability of the technique.

The trials consisted of 16 replicates (clusters) with each cluster consisting of a central pot surrounded by six peripheral pots. This was unlike the trial design used in project 2009/033 to test for resistance to *Sesamia grisescens*, where each pot was separated from its neighbours and individually infested with eggs. This time one variety (RQ117) was planted in a central pot and six peripheral pots were planted with test varieties (three of these were consistent between the trials – RQ117, Q135 and Q219$^{\phi}$). All peripheral pots were distributed randomly around the central pot (Figure 8 and Figure 9). The number of stalks in each pot was maintained at five. Stalks were infested

by stapling two top borer egg batches onto the underside of two leaves chosen randomly in the central pot (previous trials confirmed the active dispersal of neonate larvae to adjacent plants). Number of dead hearts, tunnel length (cm) and weights (g) of the recovered immature stages were recorded for all infested stalks in all replicates.

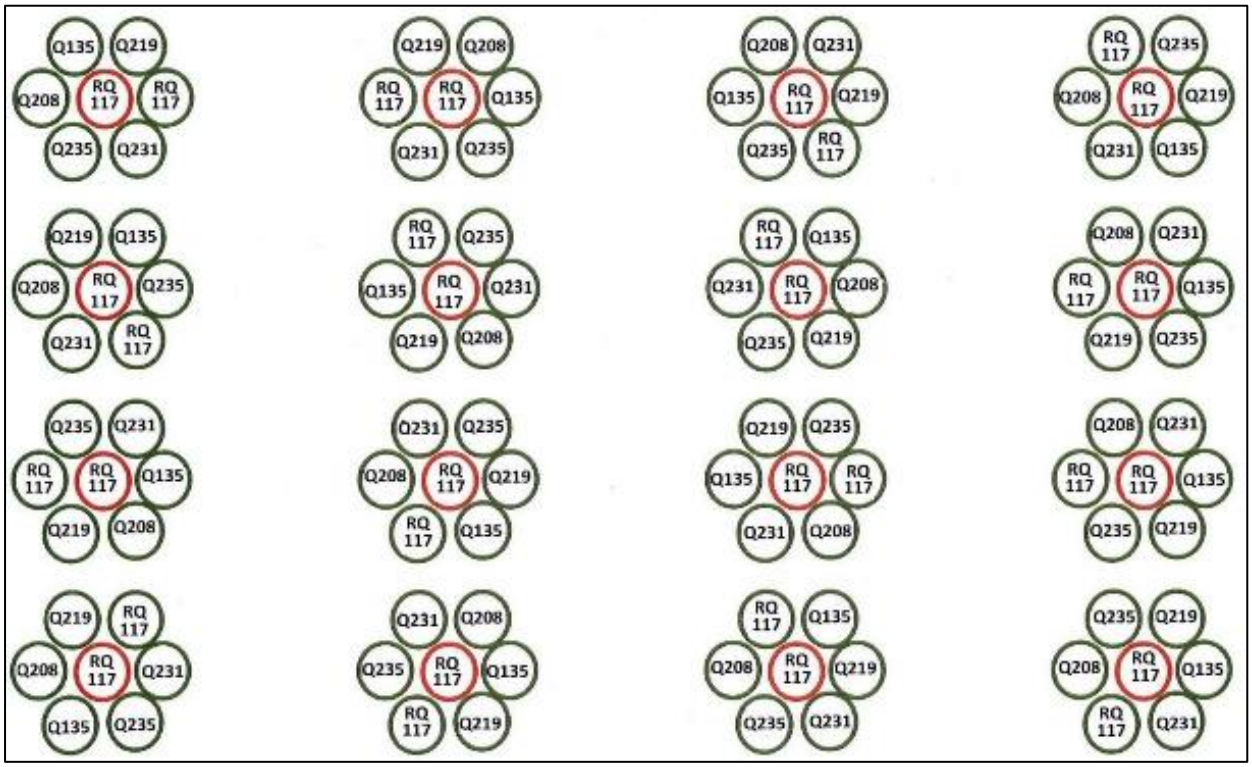

Figure 8: Lay out of the *Scirpophaga* pot trial (Sc1-16) in the white house at Ramu

Figure 9: *Scirpophaga* pot trial (Sc1-16) in the white house at Ramu; photos taken in June 2016

Two experiments were established during 2016-2017.

5.3.1.1 **Experiment SC1-16**: *Scirpophaga* trial Sc1-16 was established on 30 March 2016. Standard varieties were Q117, Q135 and Q219$^{\Phi}$ with the additional test canes being Q208$^{\Phi}$, Q231$^{\Phi}$ and Q235$^{\Phi}$. Plants in all central pots (RQ117) were infested between16 - 22 August 2016. Plants were given an extra dose of phosphorous and iron fertilizers when significant signs of chlorosis were noticed in several pots. The symptoms disappeared following fertilizer application and the trial was destructively sampled on 10 October 2016, when plants were about 6 months old.

5.3.1.2 **Experiment SC2-16:** *Scirpophaga* pot trial Sc2-18 was established on 24th October 2016, the central pots (RQ117) in each group of plants were infested on 21st March 2017 and individual stalks / plants destructively sampled on the 6th June 2017. The standard varieties (Q117, Q135 and Q219$^{\Phi}$) were again included with Q241$^{\Phi}$, Q243$^{\Phi}$ and Q246$^{\Phi}$ as the test canes.

Statistical analyses

There were three assessments of interest: 1. dead heart: a bernoulli (0, 1) measurement that indicates whether the growing point is dead (1) or alive (0); 2. number of bored internodes; and 3. tunnel length (cm), measuring the distance from the borer's point of entry to where it finally pupated/exited the stalk as an adult moth. A longer tunnel indicates greater damage.

The analysis was undertaken in four parts (one each for dead heart and number of bored internodes and two for tunnel length).

The first analysis, based on a generalised linear mixed model (GLMM), was fitted to the dead heart bernoulli (0, 1) data with the logit function as the canonical link for this model. Varieties were fitted as a fixed effect, to test for differences between the six varieties. The random terms included blocks, pots within blocks and one indexed the observational units of stalks within pots. To fit these data to the GLMM we assumed the responses from the individual stalks to be independently and identically distributed. Because of this assumption, the scale parameter was fixed to be 1. This model was fitted using the ASReml function in the ASReml-R package (Butler, 2009) in R (R Core Team, 2016).

The second analysis involved the bored internode variable. If the data did not follow the usual Poisson distribution, partly due to an over-abundance of zeros, the non-zero data were firstly analysed using a linear mixed model. If the model assumptions were not met, a second analysis was undertaken using a generalized linear model with a quasi-Poisson distribution. This distribution attempts to describe the variance in the data that cannot be explained by a Poisson distribution. This model was fitted using the glm function in R (R Core Team, 2016), therefore, replicate and pot within replicate were fitted as fixed terms in the model. Also, for completeness, the data were examined using Fisher's exact test of independence, to determine whether there was a relationship between the number of bored internodes and variety (ignoring replicate and pot within replicate). The last two analyses relied on linear mixed models using ASReml-R (Butler, 2009). These were used to examine the relationship between tunnel length and variety and then between tunnel length and the number of bored internodes. The first model is: tunnel length mean + variety + rep + rep:pot. The second model is: tunnel length mean + bored internodes + variety + bored internodes:variety + rep + rep:pot

The model assumptions are that the residuals are normally distributed, they have a constant variance and are independent; the factor level variances are equal for the treatments (tested using the Brown-Forsythe test). To determine whether the random term in the model is statistically significant, a log-likelihood ratio test was used. To test H0 : The reduced model is true; H1 : The current model is true. To test the null hypothesis, that an arbitrary group of k coefficients from the

model is set equal to zero (e.g. no relationship with the response), two nested models were fitted: 1. reduced model which omits the k predictors in question, and 2. current model which includes them.

A multiple comparison test was used to determine which of the treatment means were different; a Tukey's multiple comparison test was applied (family significance level of 5%). The data contained results for the central pot and these were removed for this analysis. The same method was used to analyse both experiments.

5.3.2 Develop an artificial diet for growth to maturity of larvae of *S. excerptalis* for use in IPM strategies

Diet components used for the breeding of *Eldana saccharina* Walker in South Africa were obtained in Australia and taken to RAIL, PNG on 29/3/2016 (Table 4). Preliminary tests demonstrated the inability of *Scirpophaga* larvae to develop on that diet, nor were they able to develop on the diet used at RAIL to breed the Ramu borer *Sesamia grisescens* Warren. Therefore various components from the *Eldana* and *Sesamia* diets were trialled in combination.

Table 4: Dietary components of the Eldana diet

Ingredient	**(gram or ml)**
Dried crushed cane	200.0g
Ground chickpea	100.0g
Yeast extract	3.0g
Casein	17.14g
Sodium propionate	9.14g
Ascorbic acid	3.34g
Calcium lactate	1.14g
Tri-sodium citrate	2.29g
Sodium chloride	0.57g
Citric acid	2.29g
Nipagin	2.00g
Dithane M45	0.17g
Streptomycin	1.50 ml
Denol (70 %)	35.0ml
Agar	4.00g
Water (for agar)	1000.0ml
Water (balance)	0.00 ml

6 RESULTS AND DISCUSSION

6.1 Downy mildew

6.1.1 Defining the *Peronosclerospora* species present in PNG.

6.1.1.1 Survey

Details of the specimens collected during the project survey are outlined in Table 5.

Table 5: Specimens collected during survey of Ramu plantation (samples BRIP65983-BRIP65995 and BRIP66004-66005); Eastern Highlands (BRIP65996-66003) and Ramu valley to Madang Province (BRIP66006-66011). The SRA accession and BRIP codes are shown.

BRIP	Host	ColCollectionDate	SRA Accession	LocPreciseLocation
65983	Sugarcane	1/11/2016	A1621-01A	FN 306
65984	Sugarcane	1/11/2016	A1621-02A	FN 305
65985	Sugarcane	1/11/2016	A1621-03A	BN 101
65986	Sugarcane	1/11/2016	A1621-04A	BN 401
65987	Sugarcane	1/11/2016	A1621-05A	Ramu office prop plot
65988	Sugarcane	1/11/2016	A1621-06A	Ramu office
65989	Maize	1/11/2016	A1621-07A	E South Corn plot
65990	Sorghum	1/11/2016	A1621-08A	GN
65991	Sugarcane	1/11/2016	A1621-09A	BN 101
65992	Sugarcane	1/11/2016	A1621-10A	BN 401
65993	Maize	1/11/2016	A1621-11A	E South Corn plot
65994	Maize	1/11/2016	A1621-12A	E South Corn plot
65995	Sugarcane	1/11/2016	A1621-13A	BN 401
65996	Coix sp.	2/11/2016	A1621-14A	O'ekara
65997	Maize	2/11/2016	A1621-15A	O'ekara
65998	Sugarcane	2/11/2016	A1621 16A	O'ekara
65999	Maize	2/11/2016	A1621-17A	O'ekara
66000	Maize	2/11/2016	A1621-18A	O'ekara
66001	Robustum	2/11/2016	A1621-19A	Norekora
66002	Miscanthus	2/11/2016	A1621 20A	Between Yonki and Kainantu
66003	Miscanthus	2/11/2016	A1621-20C	Between Yonki and Kainantu
66004	Sugarcane	3/11/2016	A1621-21A	BS 104
66005	Sugarcane	3/11/2016	A1621-22A	Asas
66006	Wild sorghum growing within LS robustum	3/11/2016	A1621-23A	River site from 2009
66007	Robustum	3/11/2016	A1621-24A	Walium bridge
66008	Maize	3/11/2016	A1621-25A	Popoeta
66009	Robustum	3/11/2016	A1621-26A	Digicell tower on road towards Madang
66010	Robustum	3/11/2016	A1621-27A	River site from 2009
66011	Robustum	3/11/2016	A1621-28A	Walium bridge

DM was observed in hybrid sugarcane, *S. officinarum, S robustum* and *Miscanthus* spp.

6.1.1.1.1 Microscopic analysis

Oospores

Light microscopy was used to examine oospores from leaf shredding survey samples and other project-related specimens. All oospores measured ~50 µm, regardless of their collection location or host species (Figure 10). This suggests that there is no microscopic evidence for the presence of *P. philippinensis* in PNG as oospores of this species are smaller. Morphological observations did not enable differentiation of other *Peronosclerospora* species since oospores of many others are around 50 µm.

Asexual structures (conidia/ conidiophores)

Examination of the conidiophores was also not conclusive, as these structures appeared to be typical of those for various *Peronosclerospora* species (Figure 11). It should be noted that only specimens that produce down can be examined for conidia, so this limited morphological observations in many wild host species.

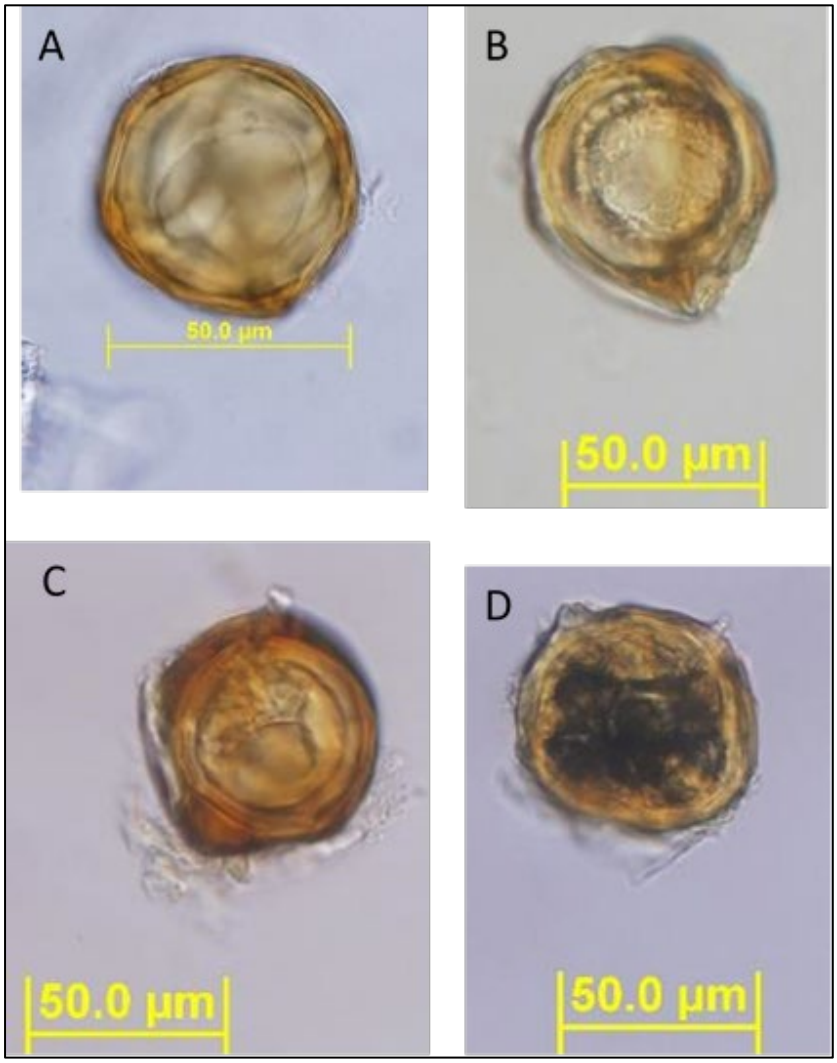

Figure 10: Oospores collected during project. A: from Q136, RAIL estate; B: from *Miscanthus*, Eastern highlands; C: from *S. robustum*, Eastern highlands; D: from *S. robustum*, Madang Province/Lowlands

Figure 11: Conidiophores and conidia collected from R570 on Ramu estate

Discussions with Dr Marco Thines suggest that oospores are generally only produced on the primary/natural host, i.e. it is host-species specific (M Thines, pers comm). The non-primary host reaction is the formation of asexual conidiophores and conidia. This lends weight to the theory of multiple species being present in PNG, as the leaf shredding was observed on a wide range of hosts, except for corn.

Further discussion with Dr Shivas, Dr Ryley and Dr Marco Thines suggested that the *Peronosclerospora* that infect graminicolus plants in PNG are not well described, and that molecular DNA sequence analysis is most likely the best way of determining the species relationships in PNG.

6.1.1.1.2 Sequence analysis

Molecular assay of the various DM specimens suggested an alignment into two distinct groups

The DNA sequence relationship was further analysed using a phylogenetic tree (Neighbour Joining, 1000 bootstraps), and is shown in Figure 12. For this analysis, *Sclerophthora macrospora* was added as an out group, but did not resolve, indicating that a high amount of sequence diversity is present in the analysis.

The two major groups identified in the alignment are clearly visible. Unfortunately, the discrimination between the published voucher specimens of the *Peronosclerospora* is not clear, with no resolution between the species in this case. This was unexpected, and further suggests that the variation in PNG is as great as the variation observed between characterised specimens. Additionally, the pairwise identity is close to 100 % for a range of samples, including *P. sorghi*, *P. miscanthi* and *P. sacchari* (data not shown).

When other factors, such as host and location, are taken into account there are few 'logical' groupings. There are two groups that did resolve which could indicate new species: one clade that infects *Miscanthus* in the highlands (and a maize that was sourced from the highlands); and one that infects *S. robustum* from the lowlands area. These are the only two clades that showed any consistency with host and location. Interestingly, *P. miscanthi* from the database does not fall within this group, so it is likely that the leaf shredding on *Miscanthus* in PNG is a distinct species.

The greater conclusion, however, is that the Cox2 gene sequence is not able to resolve species differences in the grass-infecting *Peronosclerospora* in PNG, and that the sequence may need to extend to the Cox1 and Cox 1-2 spacer region.

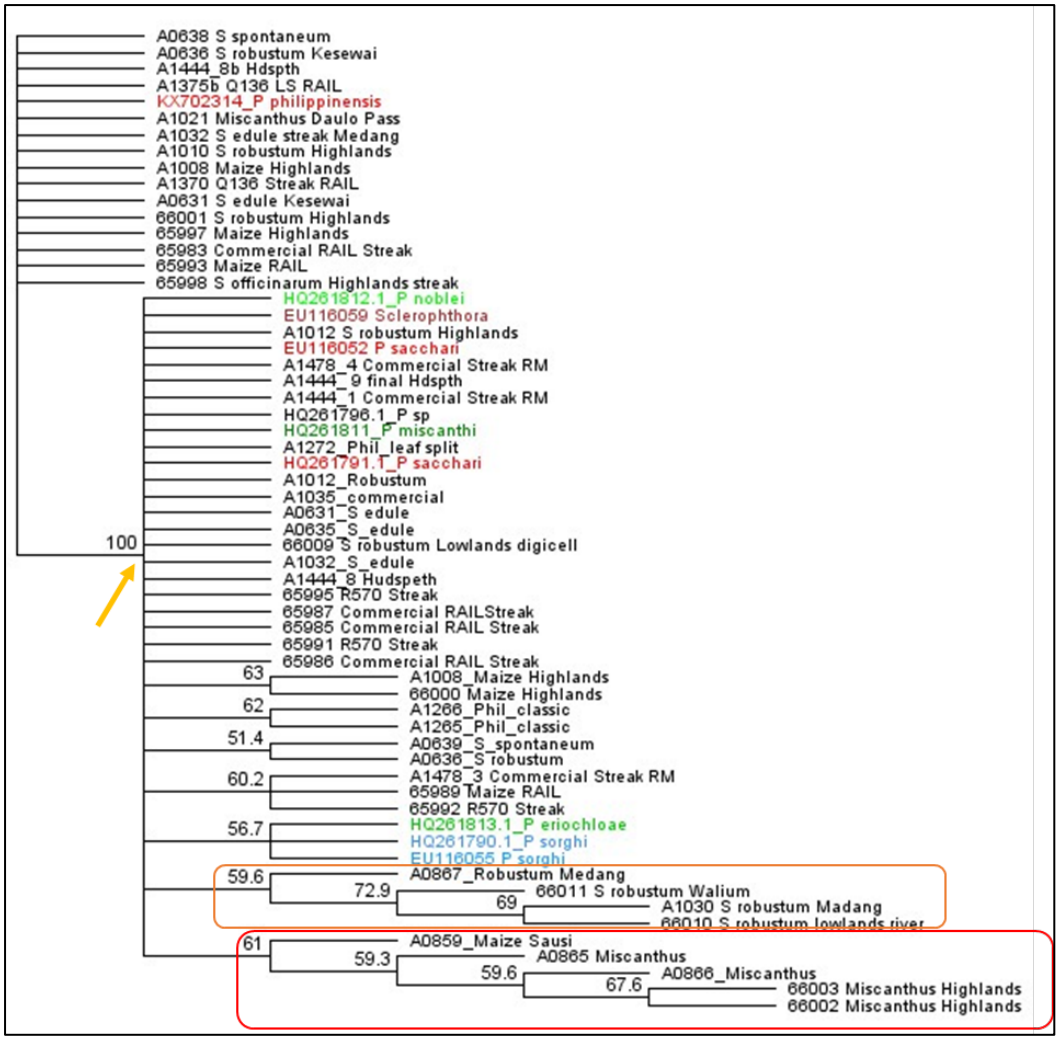

Figure 12: Cox2 sequence analysis of specimens from PNG. Coloured samples are vouchered specimens from GenBank. The tree has been populated with samples collected during the project. BRIP accessions are all 6NNNNN numbers. The arrow indicates the node separating the two major clades. The two circled clades are supported by physical differences

A comparison of Cox2 sequences from specimens collected through this project, and the previous one, clearly show that there are two main groups (100 % support in the tree above), as well as some further differentiation within one of the clades (50 - 68 % supported). However, there is not 100 % sequence agreement with any deposited specimen in Genbank, indicating that the specimens are different.

Cox1 analysis

The Cox1 primers developed during project 2009/033 are in the Cox-1 and Cox1-2 spacer region, and are able to pick up the more subtle differences of the grass-infecting *Peronosclerospora*, as illustrated in Figure 13, and conclusively found in project 2009/033 research. *Sclerophthora macrospora* is an effective outgroup in the analysis of this region.

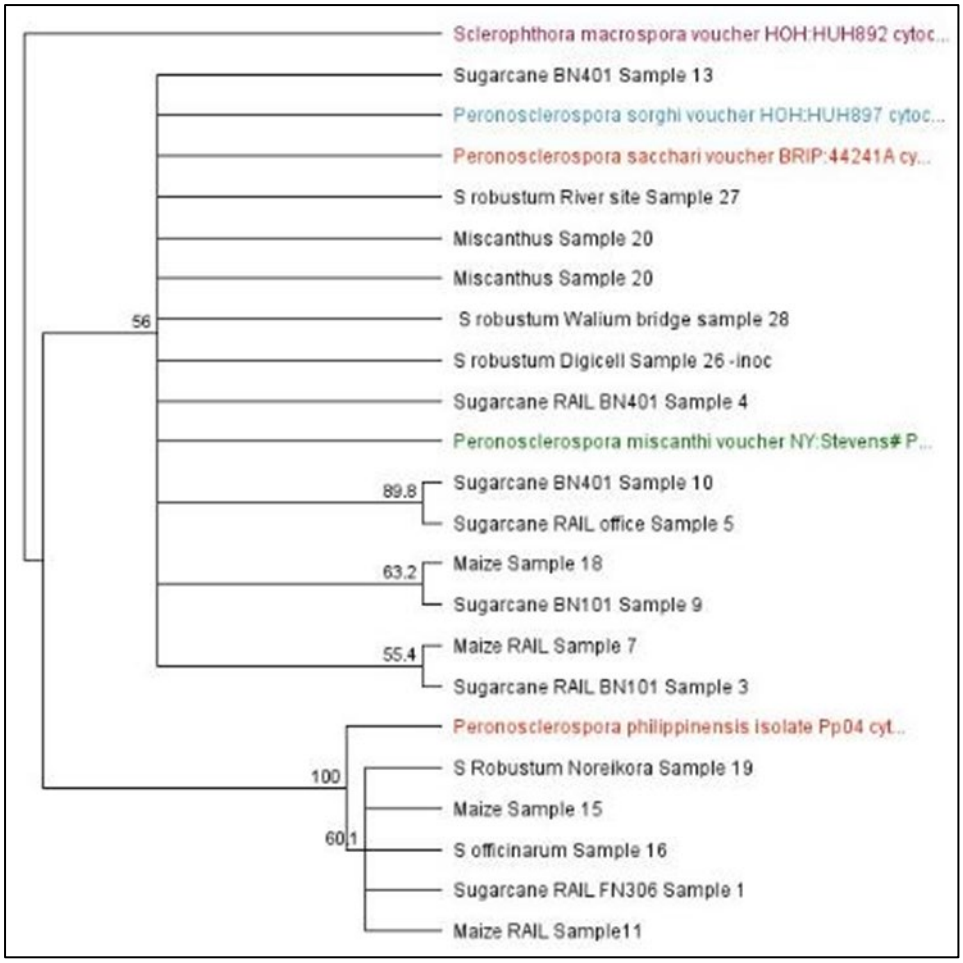

Figure 13: Cox1 analysis using protocol developed in previous studies. The coloured specimens are vouchered specimens from GenBank. The host and location of all specimens is on the figure

The Cox1 phylogenetic tree shows some similarities to the Cox2 analysis: There are two major clades, one of which is more closely related to *P. philippinensis* and one that may have further differentiation within. No specimens collected were identical to vouchered sequences, so this is further support that the specimens in PNG are unique.

There are definitely two species of *Peronosclerospora* causing DM in PNG: one that groups with *P. philippinensis* and one other large clade (most likely species complex). No evidence for *P. philippinensis* has been found using traditional microscopic taxonomy, and there is not 100 % sequence identity to the *P. philippinensis* voucher specimen. It is therefore likely that *P. philippinensis* is not in PNG, but a closely related species is.

Within the large species complex, there appear to be a further three species: one infecting *Miscanthus* (a new species, genetically different from the *P. miscanthi* already described); one infecting *S. robustum* in the lower Madang and Gusap Province; and then another undescribed species complex that can infect a wide range of hosts.

On commercial crops at Gusap, there appear to be at least two species: one causing leaf shredding in Q136 and one (or more) causing down symptoms. DNA sequence analysis strongly suggests that these are distinct species. Q136 displays leaf shredding symptoms, which suggests indicates that the DM infecting this variety is native to the commercial sugarcane variety Q136. It would be interesting to investigate the parentage of this variety and compare it to the other varieties in PNG - to determine if there is an ancestral variety unique to Q136. The main variety at Gusap is R570, which does not exhibit leaf shredding. These two varieties are from different breeding programs (Australia

and Reunion) so it is likely that they are genetically distant. There are currently a large number of Australian varieties in PNG, so it would be worthwhile to look for leaf shredding in those varieties that are more closely related to Q136.

Corn is a very effective crop for distributing a wide range of DMs. It is able to act as a reservoir for DM and, as the disease is seed-borne, it allows for wide dispersal of the pathogen. Corn should therefore be treated with fungicide to stop DM spread. It is possible that DM may 'jump' between related grasses, so new variants are likely to be continually evolving.

6.1.2 Validate a rapid resistance screening test utilising soil-applied oospores

Experiment 1:

Test plants were monitored over an extensive time period, but no DM was observed in any test plants. The reason for this remains unknown.

Experiment 2:

Four inspections were undertaken during the period, starting from the time of germination on the seedling bench to growth of the test plants in the field (last week of June 2016; Table 6). Records were kept of the status of each test plant in terms of viability and DM expression.

Table 6: Numbers of living plants and the DM status of test plants at four inspection times in the dose response x soil type pot experiment that utilised leaf-split material

Summary	27 May 2016		7 June 2016		17 June 2016		25 June 2016	
Treatment	DM	Total plants	DM	Total plants	DM	Total plants	DM	Total plants
1	1	12	2	13	4	13	4	13
2	1	13	2	12	5	12	5	12
3	0	14	2	12	4	12	5	12
4	3	13	3	12	5	12	7	12
5	2	13	2	12	5	11	7	11
6	1	11	2	12	3	10	5	10
7	0	12	2	13	2	11	4	11
8	3	12	1	13	2	11	4	11
9	0	12	0	13	1	11	2	11
10	5	14	0	13	0	12	1	12
	16	**126**	**16**	**125**	**31**	**115**	**44**	**115**

Some DM infection was noted but there appeared to be no relationship to applied treatments.

Experiment 3:

A pleasing aspect of this experiment was the absence of DM in any of the uninoculated plants, while there was good symptom expression in plants where soils had been inoculated with leaf shred material. Substantial disease development occurred after 5-6 weeks growth of the test plants (Table 7).

Table 7: Germination and DM incidence in leaf split-inoculated plants in experiment 3.

DM – leaf split treatments		*Assessment*	*Control*		*Assessment*
Pot Label	**Germination Date**	**DM positive (yes/no)**	**Pot Label**	**Germination Date**	**DM positive (yes/no)**
MQ239$^{\phi}$					
2A	02/01/2017	Yes	1A	26/12/2016	No
2B	01/01/2017	Yes	1B	26/12/2016	No
2C	01/01/2017	No	1C	29/12/2016	No
2D			1D		
2E	02/01/2017	No	1E	26/12/2016	No
2F			1F	02/01/2017	No
2G			1G	01/01/2017	No
2H	02/01/2017	Yes	1H	29/12/2016 (died)	No
2I	02/01/2017	No	1I		
2J	28/12/2016	Yes	1J	28/12/2016	No
	DM infection	**4 / 7 (57.1 %)**			**0 / 8**
Q221$^{\phi}$					
4A	02/01/2017	Yes	3A	14/01/2017	No
4B			3B	27/12/2016	No
4C	02/01/2017	No	3C	14/01/2017	No
4D	29/12/2016	No	3D	01/01/2017	No
4E	08/01/2017	Yes	3E	03/01/2017	No
4F	08/01/2017	Yes	3F	03/01/2017	No
4G	08/01/2017	Yes	3G	27/12/2016	No
4H	08/01/2017	Yes	3H	30/12/2016	No
4I	28/12/2016	Yes	3I	28/12/2016	No
4J	14/01/2017	Yes	3J	29/12/2016	No
	DM infection	**7 / 9 (77.8 %)**			**0 / 10**
Q200$^{\phi}$					
6A	02/01/2017	Yes	5A	02/01/2017	No
6B	30/12/2016	No	5B	01/01/2017	No
6C	30/12/2016	Yes	5C	26/12/2016	No
6D	01/01/2017	Yes	5D	26/12/2016	No
6E	09/01/2017	No	5E	27/12/2016	No
6F			5F	27/12/2016	No
6G	01/01/2017	Yes	5G	03/01/2017	No
6H	29/12/2016	No	5H	27/12/2016	No
6I	28/12/2016	No	5I	28/12/2016	No
6J		Yes	5J	03/01/2017	No
	DM infection	**5 / 9 (55.6 %)**			**0 / 10**

Note: Those plants failing to germinate are without dates

.

This data is summarized and illustrated in Figure 14

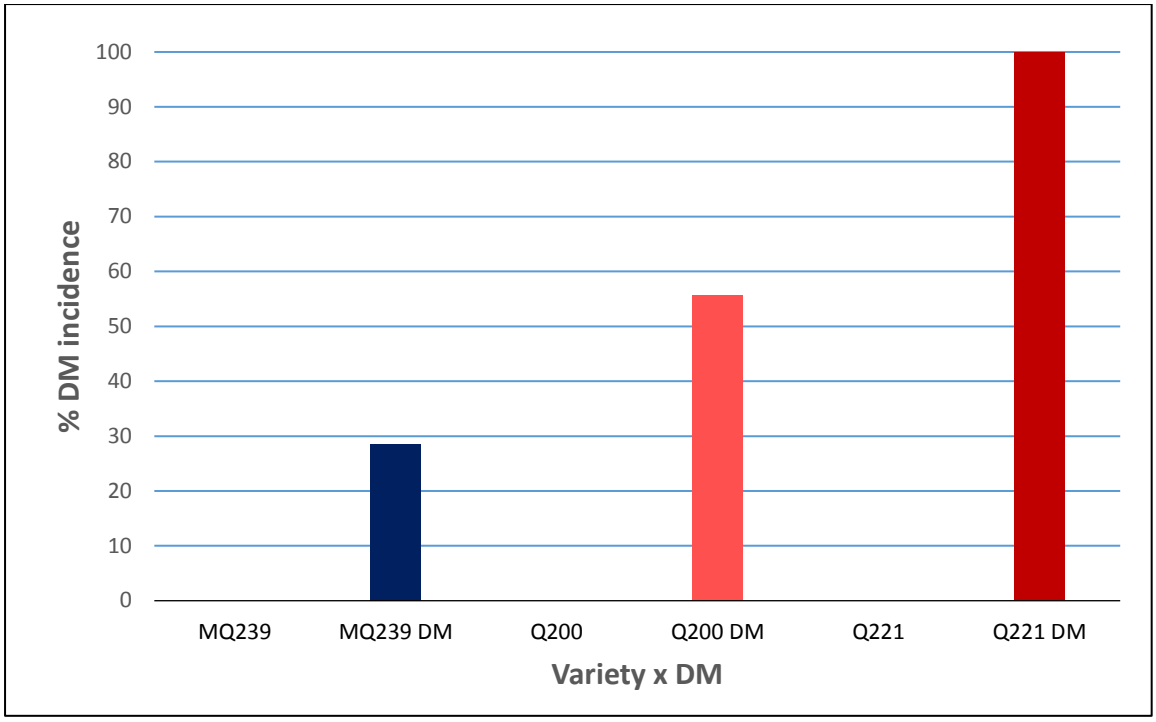

Figure 14: DM incidence in the leaf shred inoculation experiment (3) showing differences in incidence amongst the varieties of differing resistance.

In the first experiment, the failure of any treatment to give rise to DM expression was a cause for concern. The lack of disease in the stored leaf shred inoculated material was perhaps understandable, since there are no experimental data relating *Peronosclerospora* oospore viability with storage time. Oospores generally are long-lasting; oospores of the soil borne pathogen *Pachymetra chaunorhiza* are thought to remain viable in soil for over five years (Magarey *et al.*, 1994). The lack of infectivity with the freshly-collected leaf shred material is unexplained.

In the second experiment, the lack of suitable disease-free planting material confused the results. Even though planting material showing no symptoms was selected, the close proximity of this material with diseased cane seems to have led to latent infection in the stalks used in the experiment. Downy mildew predominantly spreads within crop through the abundant conidia (asexual spores) produced in association with the typical down symptom caused by the disease. These spores do not travel far (maximum about 400 m) and also lose viability quickly (in just 1 - 2 hours in daylight). Conidia land on the bud scales of the standing crop; they infect through the buds without accompanying symptoms. When the buds germinate, the pathogen becomes active in the plant and this may give rise to the disease. This appears to have been the case in experiment 2. The level of disease in the uninoculated treatments indicates the extent of disease spread through the propagation source, though several attempts were made to discard any suspect material (both at the field stalk selection stage and also when stalks were being cut into single-eye setts).

In experiment three, the disease-free nature of the planting material sourced from Leron was of paramount importance to the outcome of the experiment. The disease-free (no inoculation) checks showed no disease whatsoever, confirming the disease-free status of the planting material.
The Leron plots are set amongst beef-grazing paddocks with no commercial or wild canes close by. Ramu Agri-Industries in association with BSES/ SRA have in the past located their variety import quarantine plots at this site; this experiment supports the original decision to base a quarantine station at Leron Plains.

Pleasingly, the inoculated treatments showed abundant disease development, with the most susceptible variety (Q221$^{\phi}$) exhibiting 100 % infection.

This is important for any resistance screening technique - that susceptible varieties show high levels of disease. The variety Q200$^{\phi}$ is also susceptible, but not as susceptible as Q221$^{\phi}$ (from personal observations). Just over 50 % DM incidence was recorded in this cane. MQ239$^{\phi}$ is more resistant than either of the other two varieties; <30 % disease incidence was recorded in this variety. These results suggest the technique is promising; disease expression follows expectations, judging from other data and field observations. Statistical analyses however suggested a non-significant treatment effect (P=0.05) of either varieties or DM treatment. Trial design should be carefully re-evaluation in future experimentation.

6.1.3 Host range studies

6.1.3.1 Conidia as inoculum

Table 8 below shows the results for PCR test plant assay from the conidia inoculum experiment. Unfortunately, there was a lot of mite damage in the chambers, so symptoms of DM were not clear.

Table 8: Results of the conidia transmission trial. Each variety/ species is shown in triplicate for each of the three time points. Yellow results show positive results; blank spaces indicate plant death.

Chamber 1 : R570 DM Inoculum				
		1 month	2 month	3 month
Clone	**Pot No**	**Cox-1**	**Cox-1**	**Cox-1**
Cadmus	1	Negative	Negative	Negative
Cadmus	2	Positive	Negative	Negative
Cadmus	3	Positive	Negative	Negative
Edule	1	Positive		
Edule	2	Negative		
Edule	3	no sample		
MQ239	1	Positive	Positive	Positive (faint)
MQ239	2	Negative	Positive	Negative
MQ239	3	Positive	Positive	Negative
Officinarum	1	no sample		
Officinarum	2	Positive	Negative	Negative
Officinarum	3	Positive	Negative	
Q121	1	Positive	Negative	Negative
Q121	2	Negative	Negative	Negative
Q121	3	Negative	Negative	Negative
Q200	1	Negative	Negative	Negative
Q200	2	Negative	Negative	Negative
Q200	3	Negative	Negative	Negative
Robustum	1	Positive		
Robustum	2	Negative		
Robustum	3	Negative		

Chamber 2 : DM corn inoculum				
		PCR	PCR	PCR
Clone	**Pot No**	**Cox-1**	**Cox-1**	**Cox-1**
Cadmus	4	Positive	Negative	Negative
Cadmus	5	Positive	Negative	Positive
Cadmus	6	Positive	Negative	Negative

Chamber 2 : DM corn inoculum				
		PCR	PCR	PCR
Clone	Pot No	Cox-1	Cox-1	Cox-1
Edule	4	Positive		
Edule	5	dead		
Edule	6	Positive		
MQ239	4	Positive	Negative	Negative
MQ239	5	Negative	Positive	Negative
MQ239	6	Negative	Negative	Negative
Officinarum	4	Positive	Positive	Positive
Officinarum	5	dead		
Officinarum	6	Negative	Positive	Negative
Q121	4	Positive	Positive	Positive
Q121	5	Positive	Negative	Positive
Q121	6	Positive	Positive	Positive
Q200	4	Positive	Negative	Negative
Q200	5	Negative	Negative	Negative
Q200	6	Negative	Negative	Negative
Robustum	4	Positive	Positive	Positive (faint)
Robustum	5	Negative	Negative	Positive (faint)
Robustum	6	Positive		
Corn H	4	Positive		
Corn H	5	Positive		
Corn D		Positive	Positive	Positive

Chamber 3: no inoculum				
		PCR	PCR	PCR
Clone	Pot No	Cox-1	Cox-1	Cox-1
Cadmus	7	Negative	Positive	Negative
Cadmus	8	Negative	Negative	Negative
Cadmus	9	Negative	Negative	Negative
Edule	7	Negative	Negative	Negative
Edule	8	Negative	Negative	Negative
Edule	9	Positive		
MQ239	7	Negative	Negative	Negative
MQ239	8	Negative	Negative	Negative
MQ239	9	Negative	Negative	Negative
Officinarum	7	Negative	Positive	Negative
Officinarum	8	dead		
		dead		
Q121	7	Positive	Positive	Negative
Q121	8	Negative	Negative	Negative
Q121	9	Negative	Positive	Negative
Q200	7	Negative	Negative	Negative
Q200	8	Negative	Positive	Negative
Q200	9	Positive	Negative	Positive (faint)
Robustum	7	Negative	Negative	
Robustum	8	Negative		
Robustum	9	Negative	Negative	
Corn H	7	Negative		
Corn H	8	Positive		
Corn H	9	Positive		

Chamber 3: no inoculum				
		PCR	PCR	PCR
Clone	Pot No	Cox-1	Cox-1	Cox-1
Corn H		Positive		

Sugarcane as an inoculum source

Cox-1 PCR analysis of all time points did show some size variation of the pathogen, most notably in time point 2 (see MQ239$^{\phi}$ 1 and MQ239$^{\phi}$ 2 in Figure 15). As the experiment progressed, there were fewer positive samples, especially when analysing the samples from Chamber 1 (R570 inoculum). As can be seen in Figure 15, at the final time point there were no DM-diseased plants detected by PCR in Chamber 1 (R570 inoculation). This is in contrast to the final time point of Chamber 2, where corn was the host plant supplying the *Peronosclerospora* inoculum (see below Figure16).

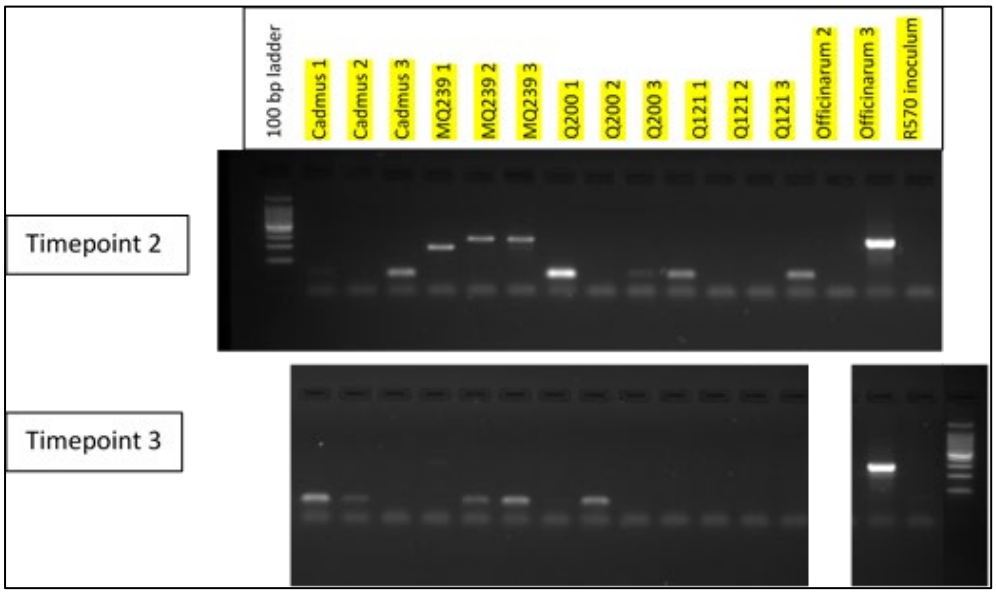

Figure 15: Agarose gel showing Cox1 amplification of selected samples inoculated from DM-infected R570. The gels have been adjusted to show the lanes and results for each replicate over time

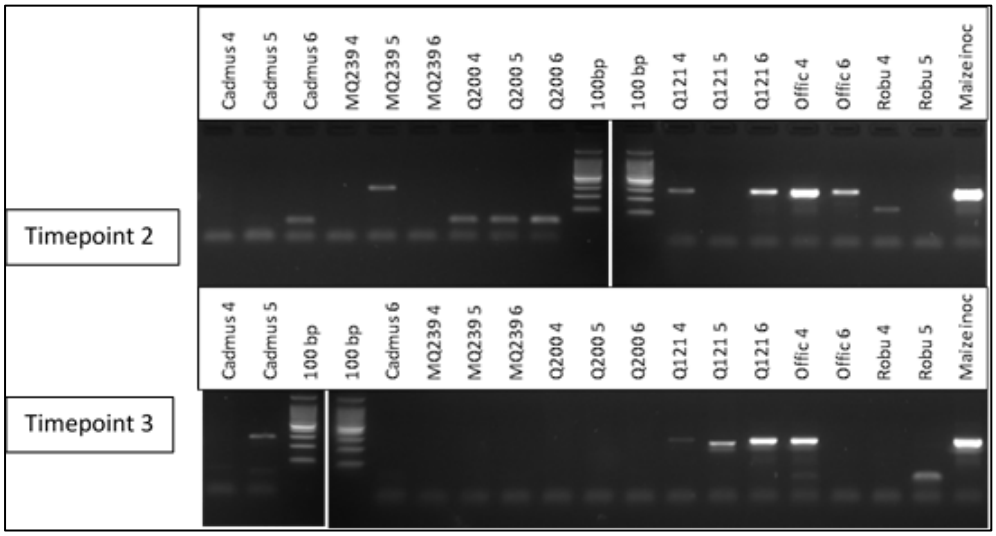

Figure 16: Agarose gel showing Cox1 amplification of selected samples inoculated from DM-infected corn. The gels have been labelled and show the lanes and results for each replicate over time

Corn as an inoculum source

Many more plants tested positive for *Peronosclerospora* in the chamber where corn was the inoculum source (at all time points). *S. officinarum* and Q121, both susceptible to DM, tested PCR-positive at the final time point. In addition, only *S. officinarum* had clear downy mildew symptoms (Figure 17). A slight size difference was observed for one of the Q121 replicates, but this was not further analysed.

The uninoculated control tested positive by PCR at the first time point, which unfortunately suggests test plant contamination; this most likely originated from the 'healthy' test corn, thus contaminating the chamber. Once corn *Peronosclerospora* infection was suspected, the corn was removed from both test chambers as well as the control chamber. It is possible however that this compromised the experiment. In any repeat experiment, the test corn should be sourced from Australia (or another reputable DM-free source).

There was a high death rate of plants in all chambers, indicating that growth conditions were not ideal for sugarcane growth. The temperature and humidity in the chambers were excessive on some days, leading to higher levels of other diseases, rotting of the plants and plant death. In subsequent experiments (including the oospore inoculation experiment) the chambers were modified to allow greater air flow. Such experiments may need to be undertaken inside more sophisticated isolation chambers to eliminate contamination.

Figure 17: *S. officinarum* from Timepoint 3 of conidia host range experiment, inoculated from DM-infected corn: (L) plant showing DM symptoms (R) leaf showing down on underside

A general trend was observed during the experiment: at the first time point there were many more positive results than in the later ones (Table 9). It is possible that the PCR assay detected spores that had landed on the leaf but which subsequently were not able to establish infection. This finding suggests the varieties should be maintained longer than 14 weeks before assay. This was also taken into account in the oospore inoculation experiment.

Chamber 2 (corn as the inoculum) exhibited a higher level of disease suggesting that corn is a better host for spore production. Higher DM levels could have arisen because of test plant inoculation at an earlier growth stage and/or a higher spore load associated with the diseased corn. The corn rapidly died, most likely due to adverse chamber environmental conditions, so infection must have occurred very early (as the test plants were germinating). In contrast, plants in Chamber 1 was infected by planting *Peronosclerospora*-infected R570: spores would only have been produced after the plants had germinated/ grown to a relatively large size. The R570 survived much longer in the chamber, and in theory a more consistent spore load would have been created. However, the temperature and humidity was not conducive to spore formation. It was concluded that corn is a more effective host for *Peronosclerospora*-inoculation, especially early in its growth.

Rapid trials using conidia as an inoculum would best be done in a chamber with diseased corn interspersed amongst the test plants, thus facilitating higher infection. A large chamber with temperature regulation would be needed, as this is less likely to overheat as with the small chambers. The early PCR DM detection on leaves needs to be confirmed in a longer term study.

An interesting finding was the PCR-positive *Saccharum robustum* at the first time point. *S. robustum* is known to become infected by *Peronosclerospora* species, but rarely (if ever) shows the characteristic 'down' symptoms. In this experiment, the *S. robustum* had no DM symptoms and the plants died during, or near the end of the experiment - so it was uncertain if the DM symptoms would have been leaf shredding, down or neither.

S officinarum was sourced from gardens in the housing area at Ramu, indicating that garden cane potentially harbours DM. This has implications for small farms/garden, as corn and *S. officinarum* are usually both grown in gardens, with ready access to cross infection. In commercial fields, this is managed via fungicide treatment of corn seed, but this is not practised in gardens.

6.1.3.2 Oospores as inoculum.

An example of the outcome of oospore infection of test plants is illustrated Figure 18; the result summary is illustrated in Table 9.

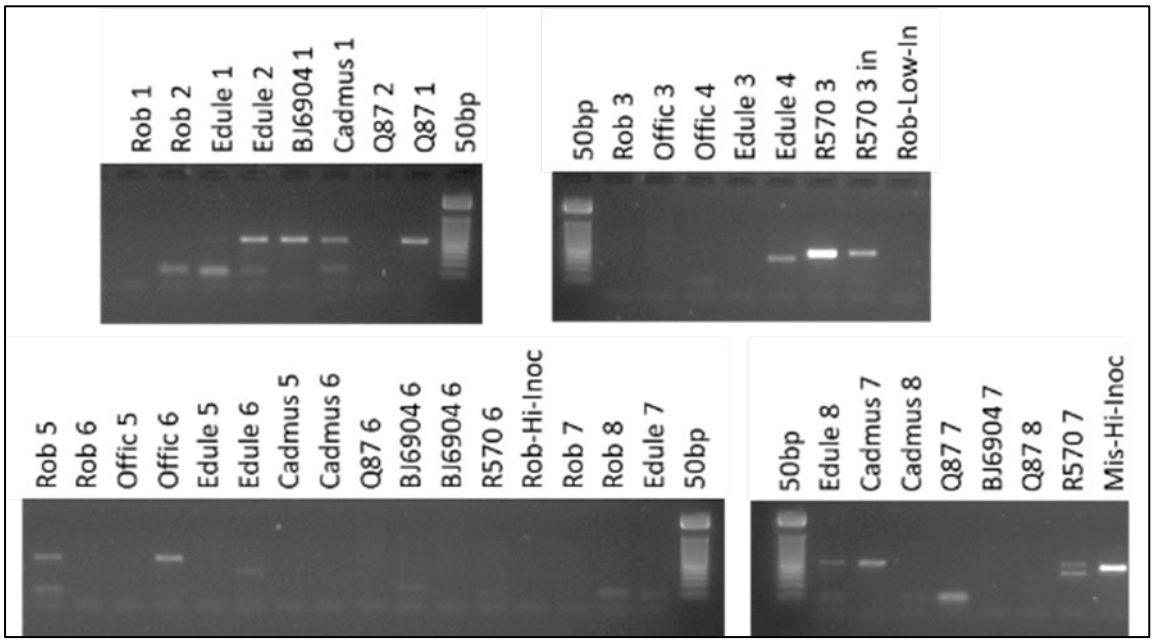

Figure 18: Agarose gel showing the final timepoint for the HR analysis. The table below shows the designation of the samples

Table 9: Host range PCR results for oospore inoculation. Each chamber is indicated as a group, with samples in duplicate per chamber. Cox1 PCR results are shown for all three time points, with positive samples highlighted in yellow. If the band size was not typical, this is indicated in the table text.

		10 weeks	17 weeks	24 weeks
CHAMBER 1: UNINOCULATED	Robustum 1	Negative	Negative	Negative
	Robustum 2	Positive	Negative	Negative
	Edule 1	Positive	Negative	Faint Positive
	Edule 2	Negative	Negative	Positive
	Cadmus 1	Negative	Positive	Positive
	Q87 1	Negative	Positive	Negative
	Q87 2	Negative	Negative	Positive
	BJ6904 1	Negative	Negative	Positive
	BJ6904 2	Negative		
CHAMBER 2: LOWLANDLEAF SPLITTING ROBUSTUM	Robustum 3	Negative	Negative	Negative
	Officinarum 3	Negative	Negative	Negative
	Officinarum 4	Positive	Negative	Negative
	Edule 3	Negative	Negative	Negative
	Edule 4	Positive	Negative	Positive (smaller)
	Maize 4	Positive		
	Cadmus 3	Negative	Positive	
	BJ6904 3	Negative	Negative	
	R570 3	Positive	Positive	Positive
CHAMBER 3: HIGHLANDS LEAF SPLITTING ROBUSTUM	Robustum 5	Negative	Negative	Positive
	Robustum 6	Negative	Negative	Negative
	Officinarum 5	Negative	Negative	Negative
	Officinarum 6	Negative	Positive	Positive
	Edule 5	Negative	Negative	Negative
	Edule 6	Positive	Negative	Positive (smaller)
	Cadmus 5	Negative	Negative	Negative
	Cadmus 6	Negative	Negative	Negative
	Q87 6	Positive	Negative	Negative
	BJ6904 6	Negative	Negative	Negative
	R570 6	Negative	Negative	Negative
CHAMBER 4: HIGHLANDS MISCANTHUS INOCULUM	Robustum 7	Negative	Negative	Negative
	Robustum 8	Negative	Negative	Negative
	Edule 7	Negative	Negative	Negative
	Edule 8	Negative	Positive	Positive
	Cadmus 7	Negative	Negative	Positive
	Cadmus 8	Negative	Negative	Negative
	Q87 7	Negative	Negative	Negative
	Q87 8	Negative	Negative	Negative
	BJ6904 7	Negative	Negative	Negative
	R570 7	Negative	Negative	2 bands

Visual experimental observations were more certain in this case, as there was little mite damage; the results are shown in Table 10. There was considerable insect damage at the second time point, caused by crickets. The planthopper *Eumetopina flavipes* was also observed, as well as some minor plant diseases such as pokkah boeng.

Table 10: Observations made in oospore host range trial

Chamber	Variety	Number	January 11th	March 1st	April 18th
			Observations	Observations	Observations
Healthy controls	Cadmus	1	No symptoms	1 plant, no DM	1 plant, no DM
	BJ6904	1	No SDM, brown spot	1 plant, no DM	1 plant, rust? No DM
	Robustum	1	No SDM	2 plants, no DM	2 plants, no DM
	Robustum	2	No SDM, some pale leaves	2 plants, no DM	2 plants, no DM
	Q87	1	No SDM	1 plant, no DM, insect damage	2 plants, no DM
	Q87	2	No SDM	2 plants, no DM, insect damage	2 plants, no DM
	Edule	1	No SDM	2 plants, no DM, insect damage	2 plants, no DM
	Edule	2	No SDM	2 plants, no DM, insect damage	2 plants, no DM
Leaf shredding inoculum from *S. robustum* (Madang)	Edule	3	No SDM	1 plant, no DM	1 plant, no DM
	Edule	4	No SDM	1 plant, no DM, one leaf dead at tip	1 plant, stooling, pale stripes, no DM
	Officinarum	4	No SDM, paler leaves	1 plant, pale leaves, no DM, Eumetopina	1 plant, insect damage, no DM
	Officinarum	3	No SDM	1 plant, pale leaves, no DM	1 plant, some paleness, no DM
	Robustum	3	No SDM, pale leaves, 2 brown spot lesions	1 plant, leaf tip dead on 2 leaves, no DM	1 plant, insect damage, no DM
	Cadmus	3	No SDM	1 plant, no DM, dying on tip	1 plant, no DM
	BJ6904	3	No SDM, Pale leaves	1 plant, no DM, dying on tip	1 plant, insect damage, no DM
	R570	3	No SDM, pale leaves	1 plant, DM	1 plant, stooling, leaf splitting with DM
	Maize	4	Possible DM*	dead	
Leaf shredding inoculum from *S robustum* from Eastern Highlands	BJ6904	6	No SDM, pale stripes	1 plant, pale leaves, no DM	1 plant, insect damage, no DM
	Q87	6	No SDM, pale leaf	1 plant, no DM	1 plant, no DM
	R570	6	No SDM, some pale margins, brown spot	1 plant, no DM, but leaf edge dying?	1 plant, pale, pokkah boeng?, no DM
	Cadmus	5	No SDM	2 plants, dead, only a little green leaf	
	Cadmus	6	No SDM, some pale striping	1 plant, some pale stripes	1 plant, stooling, no DM
	Robustum	5	One plant possible SDM but no sporulation, pale near base of leaf	2 plants, no DM, dead leaf tips	2 plants, no DM
	Robustum	6	No SDM, pale striping on leaves	2 plants, no DM	2 plants, no DM
	Edule	5	No SDM	dead heart, 1 plant, pale	2 plants (1 dead), no

Chamber	Variety	Number	January 11th	March 1st	April 18th
			Observations	Observations	Observations
				leaves, no DM	DM
	Edule	6	One small lesion, possibly SDM approx. 1cm long**	1 plant, no DM	1 plant, no DM
	Officinarum	5	No SDM	1 plant, pale, no DM	1 dying plant, yellow spot, no DM
	Officinarum	6	Pale stripe, no SDM	1 plant, pale, leaf has pale streaks	1 dying plant, no DM
Leaf shredding inoculum from Miscanthus from Eastern Highlands	R570	7	No SDM, brown spot and another spot, some mid-rib clearing/whiteness ?	1 plant, pale leaf symptoms (Pokkah boeng?)	1 plant, stooling, pale spots? Sclerophthora-like?
	BJ6904	7	No SDM, same sort of mid-rib clearing/whiteness	2 plants, something DM-like on older leaves, but severe other leaf symptoms (Pokkah boeng?)	2 plants, no DM, rust?
	Robustum	7	No SDM, some pale stripes on leaves	2 plants, pale stripes, no DM	2 plants, no DM, rust?
	Robustum	8	No SDM, same pale striping as #7	2 plants, pale leaf symptoms (Pokka boeng?)	2 plants, no DM, rust?
	Edule	7	No SDM, some pale striping, some brown spot	2 plants nearly dead, pale, no DM	1 plant, no DM
	Edule	8	No SDM, pale leaf	1 plant, no DM	1 plant, no DM
	Cadmus	7	Possible SDM	1 plant, pale leaf symptoms, no DM	1 plant, no DM
	Cadmus	8	Possible SDM	2 plants, no DM, pale leaf	2 plants, no DM

A comparison of DM symptom expression vs PCR test results showed an imperfect match, with more PCR-positive plants than those exhibiting DM symptoms. Only one plant with clear DM symptoms was observed: R570 inoculated with *Peronosclerospora* from the lowlands. The symptoms included characteristic leaf striping, some leaf shredding but also death of the meristem/ excessive tillering, an atypical symptom of DM (Figure 19:). The interior portion of the meristem was excised and tested by PCR for the presence of *Peronosclerospora*; positive results were obtained. No conidia were observed.

Figure 19: Downy mildew symptoms on R570 inoculated with oospores from *S. robustum* (lowlands specimen). The red arrow shows the leaf stripe on the R570. Excessive tillering, leaf shredding and internal meristem damage are also shown.

Plants in the uninoculated chamber showed numerous PCR-positive results, indicating a high level of contamination. The chambers were modified in this experiment to increase airflow to avoid overheating. Unfortunately, this appears to have allowed contamination of the 'disease-free' chamber; transmission conclusions are therefore inconclusive. The contamination appeared to vary from time point to time point, which could indicate the fluctuation of contaminating conidia in the air.

Interestingly, unlike the conidia experiment, multiple banding patterns were observed (Figure18 page 39, R570); this indicates a dual infection, something which has been observed on the estate before.
This could indicate contamination from a number of sources on the estate.

Positive results were recorded in all chambers, but even given the extended experimental timeframe (from 12 to 24 weeks), only one plant exhibited symptoms – this was in R570 inoculated with leaf shred from *S. robustum* collected from the lowlands area. This plant showed a clear DM-like stripe on the leaf, some potential leaf shredding, a dead meristem and subsequent tillering. Some of these are not confined to, or characteristic of, DM so a secondary infection may have taken place, or this symptom was caused by excessive oospore inoculum with R570.

Leaf shred was the only inoculum source leading to consistent DM symptoms through the course of the experiment. This suggests that the *S. robustum* leaf shredding is able to infect sugarcane and cause symptoms, however the PCR result was not conclusive because the inoculum specimen was unable to be analysed (most likely due to degraded DNA). The band sizes were significantly different from that of PCR-positive samples from the same chamber, but it is unclear whether this is contamination or a real result. DNA sequencing of these isolates may provide further information, and these have been submitted to the Queensland Herbarium for further analysis. The plant was destroyed at the end of the experiment, as we did not want to potentially cause a DM outbreak in commercial crops by a potentially new strain of the pathogen.

R570 is the major variety at Ramu, and if infection occurred as a result of a strain change, it may be highly-infective. A sample from the infected plant was dried, irradiated and specimens preserved for future analysis.

General conclusion from the host range studies

Common garden crops (corn, *S. officinarum* and *S. edule*) are susceptible to *Peronosclerospora* infection via conidia; however this conclusion is based on PCR results, and not visual symptoms, so more research is required to verify this. These crops (especially corn) could act as a disease reservoir: the impact on commercial crops is probably minor in PNG because of the high DM incidence anyway, but in the case of a disease incursion in Australia, disease surveys should include *Saccharum*, corn, and related grasses and the presence of these species considered in eradication and management strategies.

Peronosclerospora species causing leaf splitting in *S. robustum* could infect sugarcane, if inoculum levels are sufficiently high. Inoculum levels of *S. robustum* are controlled by the natural burning of the roadsides, but any large reservoirs of leaf splitting should be taken note of, if they are near to commercial canes.

6.2 Ramu stunt

6.2.1 Disease survey

Collection details for the survey material are shown in Table 11. Only three sugarcane samples tested positive for Ramu stunt. This included commercially grown R570 on the estate and two noble canes from a garden in Asas village. The entire 1.2kb of RNA 6 was sequenced from these three isolates and the data were combined into the tree shown in Figure 20. The isolates group into clusters based on sampling location and host and are consistent with previous results. Figure 20 shows two main clusters; within the top cluster are sub-clusters representing isolates from noble canes in gardens in the Ramu Valley; isolates from commercial canes on the Ramu estate; and isolates from noble canes growing in home gardens on the estate. The second major cluster contains the isolates from Alotau in Milne Bay Province. All isolates collected from Alotau, both in 2013 and during the ACIAR survey of 2002, are genetically different from isolates collected around the Ramu estate and the Ramu Valley.

Several samples of maize and the weed *Rottboellia cochinchinensis* suspected of being infected with MSpV were collected on the survey and later confirmed to be infected using a diagnostic test developed in project 2009/033.

Table 11: Collection details and screening results for survey samples collected on the Ramu estate and Ramu Valley.

Code	Host	Location and GPS	Symptom description	Result
25-27	R570	BS914 (5° 58' 19" 145° 49' 55")	excellent symptoms	Positive for Ramu stunt
28-30	Noble 1	Asas (5° 43' 37" 145° 33' 44")	strong flecking	Positive for Ramu stunt
31-33	Noble 2	Asas (5° 43' 37" 145° 33' 44")	excellent RS and flecking	Positive for Ramu stunt
34-36	Maize	Asas (5° 43' 37" 145° 33' 44")	excellent MSpV	Positive for MSpV
37	Noble 3	Asas (5° 43' 37" 145° 33' 44")	faint yellow stripes	Negative for both
38	Noble 4	Asas	faint narrow yellow stripes	Negative for both

Code	Host	Location and GPS	Symptom description	Result
		(5° 43' 37" 145° 33' 44")		
39	*Rottboellia*	Asas (5° 43' 37" 145° 33' 44")	excellent MSpV	Positive for MSpV
40	Noble 5	Asas (5° 43' 37" 145° 33' 44")	Ramu streak?	Negative for both
41	Maize	Popoeta (5° 33' 53" 145° 25' 44")	excellent MSpV	Positive for MSpV
42	Noble 6	Popoeta (5° 33' 53" 145° 25' 44")	Ramu streak?	Negative for both
43	*Rottboellia*	Wallium Bridge (5° 33' 52" 145° 25' 44")	excellent MSpV	Positive for MSpV

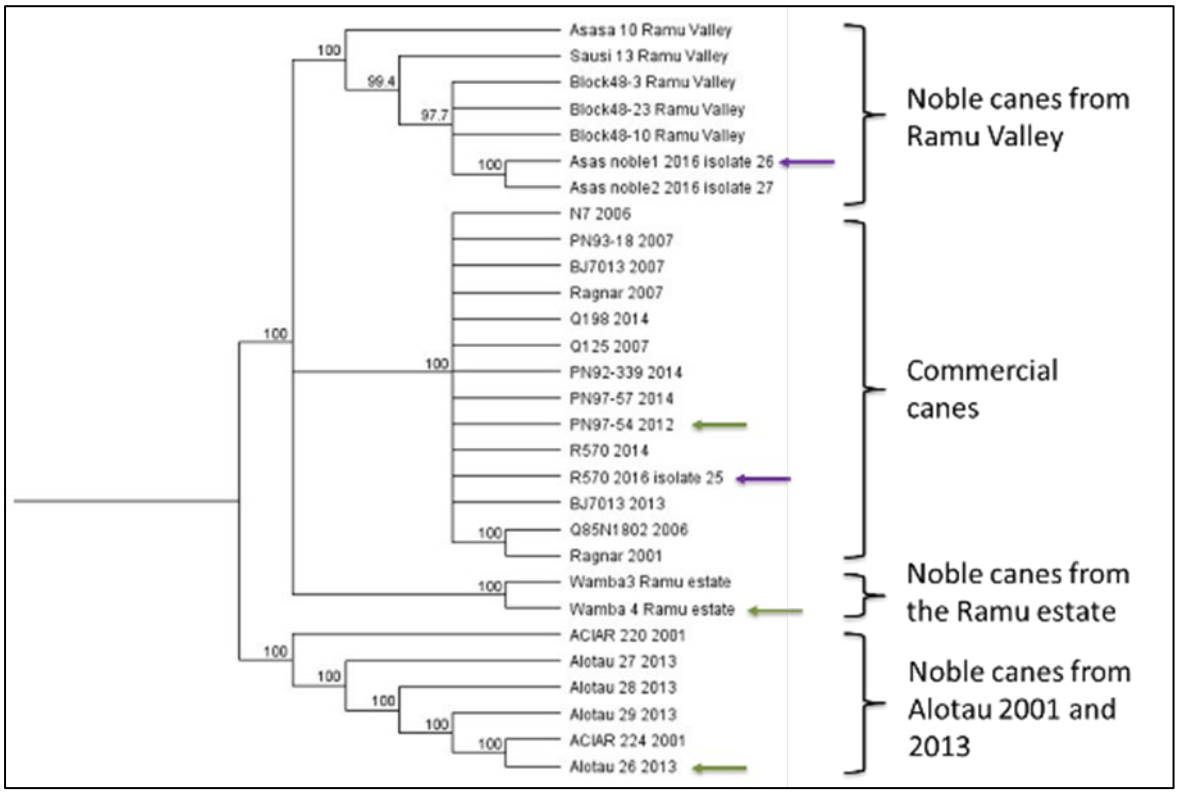

Figure 20: Phylogenetic analysis of 29 isolates of Ramu stunt based on 1.2kb of sequence data representing RNA 6. The nucleotide alignment consensus tree was generated using the Juke-Cantor genetic distance model and UPGMA with 1000 bootstraps. The three new sequences generated in this project are Asas noble 1 and 2 2016 (isolate 26 and 27) and R570 2016 (isolate 25). Arrows are explained in the methods section (page 19)

Three samples were identified as infected with Ramu stunt during the survey. The R570 finding shows that constant vigilance is required by Ramu staff to manage the disease on the estate, seeing that R570 is the most widely-grown commercial variety. Poor nursery practices allowed Ramu stunt-infected cane to be planted into a commercial field in this instance.

During the survey, a single garden at Asas village, Ramu Valley, was found to have a surprising array of viral diseases. Growing within a small area were noble canes with Ramu stunt, while maize and *Rottboellia* were infected with maize stripe virus. The MSpV specimens were sampled to provide material for Dimitre Mollov. MSpV was found at other locations, but no other Ramu stunt specimens were found during the survey.

There was no need to modify the existing diagnostic assay for the survey specimens, although this has been necessary in the past. Samples with excellent stunt symptoms were collected during a previous survey in the Alotau region, but these did not test positive with the stunt assay. Phylogenetic analyses confirmed that Alotau isolates are diverse (See Figure 20); the sequence alignment data were used to design a new series of primers, RSNCPaF and RSNCPcR. These primers are available, and will be important, if a stunt incursion occurs in Australia.

The survey data generated in projects 2015/046, 2009/033 and the ACIAR-funded CS1/1996/140 support several observations about Ramu stunt:

- Based on RNA 6 sequence data, the phylogenetic grouping of isolates is strongly linked to location and host
- The disease is present outside the Ramu estate, but is not common
- The disease has been found so far only in noble canes in gardens and commercial canes on the estate
- No Ramu stunt has been detected so far in *S. robustum, S. edule*, blady grass, elephant grass, guinea grass or itch grass.

6.2.2 Ramu stunt genome sequencing.

SRA sequencing results

Approximately 148 million reads were obtained from PN97-54 and Wamba-4, and 115 million reads from Alotau-26. FastQC, was used to examine sequence quality and showed that using RNA from irradiated leaf tissue can be successful. Further quality checks were undertaken by comparing trimmed and untrimmed reads from PN97-54, to check if sufficient numbers of reads could be mapped if using only high quality sequence. Table 12 shows that a sufficient number of reads did map, following trimming of sequence data, so both trimmed and untrimmed data can be used for mapping analyses.

Table 12: Ramu stunt virus RNA segments determined by Mollov *et al.* (2016) and mapping read data for PN97-54.

RNA fragment and GenBank accession	Size (bp)	Possible function of ORF	Untrimmed reads mapped	Trimmed reads mapped
RNA 1 KR094115	8,902	RNA dependant RNA polymerase	11,303,276	3,686,325
RNA 2 KR094116	1,675	Nucleoprotein	6,431,915	2,049,618
RNA 3 KR698381	1,583	Hypothetical protein	11,117,728	3,823,227
RNA 4 KR094117	1,574	Hypothetical protein	1,581,021	520,482
RNA 5 KR094118	1,377	Nucleocapsid	2,258,372	762,877
RNA 6 KR094119	1,201	Hypothetical protein	4,681,338	1,460,491

Consensus sequences corresponding to the six Ramu stunt virus segments were generated for all three samples sequenced by SRA. Small gaps are present in four of the six Alotau-26 consensus sequences generated in-silico and closing of these gaps is currently being attempted.

Reads belonging to the Ramu stunt virus isolate from Alotau were found in PN97-54 and Wamba-4 sequence data. The abundance of these reads was low (70 in PN97-54 and 12 in Wamba-4) in comparison to the number of reads from the published viral isolate in these two samples.

This suggests that both viral isolates can be present in the same plant, but at very different titres.

NGRL-USDA Ramu stunt and MSpV sequencing results

The sample from R570 (isolate 25) produced 30,744,044 raw data reads. Only 29 reads were removed after trimming and QC. The sample from Asas noble 1 (isolate 26) produced 26,180,603 raw data reads with 28 reads discarded after trimming. Sequence reads from R570-25 and noble 1-26 were assembled into contigs using CLC Genomic Workbench and generated 37,610 and 37,911 contigs respectively. For both samples, all six genomic RNAs of Ramu stunt virus could be identified amongst the contigs. Number of reads and average coverage (nucleotides per each position of the Ramu stunt genome) are shown in Table 13.

In addition to the expected Ramu stunt sequences, reads with low homology to umbraviruses were identified in both samples. This shows the value of screening the raw data with both the in-house sugarcane virus database and the larger plant virus database. The umbraviruses sequences were then checked against the SRA samples and a sequence in Wamba-4 was found to be identical to the sequence found in R570 (isolate 25). SCYLV was also detected in the Asas noble-1 (isolate 26). MSpV reads were detected in all *Rottboellia* and maize samples sent to Beltsville, and the maize sample also had reads matching SCYLV.

Table 13: Mapping read data for the NGRL-USDA Ramu stunt samples.

	R570 (isolate 25)		Asas noble 1 (isolate 26)	
RNA fragment and GenBank accession	**Reads mapped**	**Average coverage**	**Reads mapped**	**Average coverage**
RNA 1 KR094115	1,202,365	10,237	1,458,110	12,358
RNA 2 KR094116	1,060,045	47,843	980,220	43,987
RNA 3 KR698381	1,433,417	68,040	768,244	35,676
RNA 4 KR094117	551,101	26,536	911,600	43,787
RNA 5 KR094118	287,476	15,825	550,141	30,104
RNA 6 KR094119	1,703,638	107,521	1,350,041	84,896

Combined analysis

The generated segments for all five isolates were compared to the GenBank Ramu stunt virus segments (Table 14.) The consensus sequences from commercial cane isolates (PN97-54 and R570) were most similar to the published sequences from the Ragnar isolate. Consensus sequences from noble cane isolates had slightly lower similarity, while the segments generated from the Alotau-26 data showed least similarity.

Table 14: Similarity between the GenBank Ramu Stunt virus segments and consensus sequences generated from five more isolates. Data are presented as % nucleotide identity.

RNA fragment and GenBank accession	R570-25 consensus	PN97-54 consensus	Wamba-4 consensus	Noble 1-26 consensus	Alotau-26 consensus
RNA 1 KR094115	99.6	99.9	99.2	93.6	81.4
RNA 2 KR094116	99.4	99.6	99.3	96.0	85.9
RNA 3 KR698381	99.6	99.8	98.5	96.3	82.8
RNA 4 KR094117	99.6	99.7	98.4	95.2	82.1
RNA 5 KR094118	99.7	98.1	98.8	94.1	79.3
RNA 6 KR094119	99.7	99.8	98.2	95.6	81.6

During project 2009/033, the causal agent of Ramu stunt was shown to be a tenuivirus (Braithwaite *et al.,* 2007). Several viral genome fragments of over 1kb had been assembled from shorter cloned contigs.

Unfortunately there was no nucleotide homology between the contigs and known tenuiviruses using the program BlastN, although the program BlastX gave presumptive identifications for three of the contigs. This shows that without a reference sequence and without sufficient homology to known genes or proteins, it is extremely difficult to assemble the genome of a newly discovered, segmented virus.

Tenuiviruses have large genomes with four to six segments, the largest being around 9kb, and the smaller ones having an ambisense arrangement (Falk and Tsai, 1998). For rice stripe virus (RSV), the type member of the species, the predicted functions for some of the encoded proteins are known. RNA 1 has a single open reading frame (ORF) encoding the RNA-dependent RNA polymerase; RNA 2 encodes a putative polyprotein with similarity to phlebovirus membrane glycoproteins (pc2); RNA 3 encodes the nucleocapsid (N) protein (pc3) and RNA 4 encodes the major non-capsid protein, NCP (p4). The functions of the products of the other ORFs have not yet been definitively determined. Some isolates of RSV have five RNAs and rice grassy stunt virus (RGSV) has six.

The full sequence of the Ramu stunt viral genome from the cultivar Ragnar has been published by Mollov *et al.,* 2016, who provisionally named it Ramu stunt virus (RmSV). Six contigs associated with plant viruses were assembled and assigned as RNA 1 through RNA 6 based on length (Table 3). Once again, all matching had to be done using BlastX, not BlastN. RNA 1, at almost 9kb, codes for the RNA polymerase. Of the remaining five segments, only two have an ambisense arrangement. None of the RNA segments 2 to 6 were over 2kb, unlike most other tenuivirus segments. It was concluded by Mollov *et al.* (2016) that fragment lengths were complete because all six RNAs showed the complementary 5' and 3' terminal 10-nucleotide sequences typical of all tenuiviruses (Falk and Tsai, 1998). Mollov *et al.* (2016) also concluded that the six RNAs obtained from the RNA Seq experiments comprise the entire Ramu stunt viral genome, because no other plant virus-related contigs were identified. Thus it appears that the genome of the Ramu stunt virus is smaller (16kb) than the genomes of most well characterized tenuiviruses (usually 18 - 19kb).

Mollov *et al.* (2016) attempted to place the Ramu stunt virus in a phylogenetic context. Blast analyses showed that the RNA polymerase protein had the highest identity of 38 % to RSV, followed by RGSV. Phylogenetic analysis of the nucleoprotein amino acid sequence (from RNA 2) indicated that, while most closely related to tenuiviruses, it was more intermediate between tenuiviruses and phleboviruses but distinct from tospoviruses, the only plant virus genus in the family *Bunyaviridae*. The four hypothetical proteins coded for by RNAs 3, 4 and 6, had highest homology to three hypothetical proteins identified by Mahmoud *et al.* (2007) from maize yellow stripe virus (MYSV), a proposed tenuivirus. Mahmoud *et al.* (2007) demonstrated the presence of five genome segments ranging from 9.5kb for RNA 1, to 2.4kb, 2.1kb, 1.6kb and 1.6kb, for RNAs 2 to 5, respectively. However, apart from RNA 5, all other segments were only partially sequenced. At the time of publication, MYSV had no nucleotide or peptide sequence similarities between the five sequenced segments and any virus sequences, including tenuiviruses, available in the databases. It now appears that MYSV and the Ramu stunt virus share some sequence similarity.

With the publication of Mollov *et al.* (2016), we were able to revisit the early sequence data from project 2009/033. Remarkably, all six segments obtained by Mollov *et al.* (2016) were already assembled in 2009/033, some as early as 2009 (Table 15). Four of the six are full length (RNAs 2, 3, 5 and 6) and two are partial (RNAs 1 and 4). It was established early in this project that the 1.2kb contig with the code name of "MYSVseg3" had highest homology to MYSV segment 3 with GenBank

protein accession number CAI94656 (Mahmoud *et al.,* 2007). This contig, now identified as RNA 6, was used to design the diagnostic test (Braithwaite *et al.* 2012). There are still several contigs over 1kb that can't be matched, but they may not even be viral sequences.

Table 15: Ramu stunt virus contigs assembled in 2009033 and their relationship to the six segments obtained by Mollov *et al.* (2016)

2009033 contigs: Code name and size (bp)	Matching fragment from Mollov *et al.* (2016)	Match (determined from BlastN): Identities and Expect (E) value
RNApol 3,850bp	RNA 1 (KR094115) 8,902bp	3832/3850 = 99 % identity, E=0
KB40 1,675bp	RNA 2 (KR094116) 1,675bp	1667/1675 = 99 % identity, E=0
MYSVseg4-2 1,583bp	RNA 3 (KR698381) 1,583bp	1580/1583 = 99 % identity, E=0
KB444 943bp	RNA 4 (KR094117) 1,574bp	923/925 = 99 % identity, E=0
KB124 1,377bp	RNA 5 (KR094118) 1,377bp	1377/1377 = 100 % identity, E=0
MYSVseg3 1,201bp	RNA 6 (KR094119) 1,201bp	1200/1201 = 99 % identity, E=0

6.2.3 Biology of the host-vector interaction

Trial 1

This trial required the *Eumetopina flavipes* recovered from the diseased source cage to be separated into adults and nymphs. However, when the insects were harvested from the diseased cage, only very low numbers could be recovered, including only 11 adults. They were sorted into size classes and placed on the test pots; after three weeks (to allow the insects to feed and transmit the virus) the bags were removed, all insects collected and the plants sampled. No plant tested positive but one of the recovered *E. flavipes* did. The experiment was terminated in November 2016. At this stage there were only two surviving control plants and eight test plants. Again, no plant tested positive for Ramu stunt and no reliable symptoms were visible. Detailed screening results for this trial are presented in Table 16.

Table 16: Ramu stunt diagnostic screening results for the first transmission trial.

Date	Source of material	Sampling details and activities	Number tested	RS test result
Diseased Ragnar				
Oct 2015	BN401	Disease Ragnar transplanted from the field	6	5 +ve
12 May 2016	Diseased cage	Two stools of diseased Ragnar at time of ratooning	2	Both +ve
29 Jun 2016	Diseased cage	Two stools of diseased Ragnar at time when *Eumetopina* harvested	2	Both +ve
1 Nov 2016	Diseased cage	Two stools of diseased Ragnar at completion of experiment	2	Both +ve
Test and control Ragnar				
Dec 2015	Indooroopilly quarantine glasshouse	Ragnar (healthy)	1	-ve
29 Jun 2016	Bench out front of office	8 test and 4 control plants just before receiving *Eumetopina* from diseased	12	All -ve

Date	Source of material	Sampling details and activities	Number tested	RS test result
		cage		
19 Jul 2016	Bench out front of office	8 test and 4 control plants after *Eumetopina* feeding for 3 weeks	12	All -ve
1 Nov 2016	Bench out front of office	8 test and 2 control plants at completion of experiment	10	All -ve
Eumetopina flavipes				
12 May 2016	Balus village gardens	Host plants for *Eumetopina*	3	All -ve
12 May 2016	Balus village gardens	Preliminary test of *Eumetopina*	90	All -ve
7-9 Jun 2016	Balus village gardens	100 adult *Eumetopina* collected and introduced to diseased cage	13	All -ve
29 Jun 2016	Diseased cage	Harvest *Eumetopina* and introduce to test plants	5	All -ve
19 Jul 2016	Test pots	Recover all remaining *Eumetopina*	9	1 nymph +ve Details below:
		Nymph from Adult-2B pot		+ve

The screening results show that: (i) all source plants in the disease cage tested positive for Ramu stunt throughout the experiment; (ii) All *E. flavipes* collected from village gardens tested negative prior to introduction to the diseased cage, and (iii) all "healthy" test plants tested negative prior to introduction of the *E. flavipes.* Thus, all source material tested as expected throughout the experiment. However, at the end of the experiment, no plants tested positive for stunt and no reliable symptoms were visible, so viral transmission was not successful.

Trial 2

Prior to commencing this experiment, Ramu staff had made some efforts to deal with the cricket problem, so it was decided to use the four cages as originally planned. As the first trial failed due to low numbers of *E. flavipes*, it was decided to repeat the first trial by combining adult *vs* nymph assay with host range testing.

A greater effort was made in this trial to increase the starting number of *E. flavipes*. In January 2017, over 300 *E. flavipes* were collected and introduced to the diseased cage. Further smaller collections were made in February 2017. However, only 200 *E. flavipes* individuals could be recovered from the diseased cage in March 2017 for introduction to the test cages. This indicates that there was considerable mortality or predation in the cages, probably by geckos and earwigs. The 200 *E. flavipes* were sorted into adults (80) and nymphs (120) using a stereo microscope in the pathology lab. Subsamples of each taken for Ramu stunt assay showed 60 % of the adult subsamples and 80 % of the nymph subsamples tested positive. So although low in number for this experiment, the *E. flavipes* had a high viral acquisition rate.

The experiment was terminated in June 2017. No *E. flavipes* could be recovered from either the adult or nymph cages. Three plants died during the experiment: a control Ragnar and both red *S. edule* plants in the nymph cage. Detailed screening results for this trial are presented in Table 17.

Table 17: Ramu stunt diagnostic screening results for the second transmission trial.

Date	Source of material	Sampling details	Number tested	RS test result
Diseased Ragnar				
Oct 2015	BN401	Disease Ragnar transplanted from the field	6	5 +ve
1 Nov 2016	Diseased cage	Two stools of diseased Ragnar ratooned	2	Both +ve
Diseased Ragnar				
2 Mar 2017	Diseased cage	Three stools sampled when insects collected	3	All +ve
29 Jun 2017	Diseased cage	Completion of experiment	mixed leaves	All +ve
Healthy test plants				
19 Jul 2016	Kainantu region	5 host species collected	5	All -ve
1 Nov 2016	Bench out front of office	5 - 6 pots of 5 host species	Mixed leaves	All -ve
1 Nov 2016	Bench out front of office	10 pots Ragnar recovered from trial 1	10	All -ve
1 Mar 2017	Bench out front of office	36 test pots prepared, sampled and placed in test cages	TVD sampled	All -ve
Eumetopina flavipes				
12 Jan 2017	Balhus village gardens	*Eumetopina* introduced into diseased cage	40	All -ve
11 Feb 2017	Balhus village gardens	Second introduction of *Eumetopina* into test cage	35	All -ve
2 Mar 2017	Diseased cage	Adults harvested from diseased cage and transferred to “Adult” test cage	10	3 of 7 females 3 of 3 males +ve
2 Mar 2017	Diseased cage	Nymphs harvested from diseased cage and transferred to “Nymph” test cage	15	12 of 15 nymphs +ve
Test plants				
29 Jun 2017	Control cage	11 control pots at completion of experiment	11	All -ve
29 Jun 2017	“Adult” cage	12 test pots at completion of experiment	12	All -ve
29 Jun 2017	“nymph” cage	10 test pots at completion of experiment.	10	2 +ve Details below:
		• red noble (N2-16) • yellow noble (N1-21)		+ve +ve

Once again, the screening results show that all source material tested as expected: (i) all plants in the disease cage were Ramu stunt-positive throughout; (ii) All *E. flavipes* collected from village gardens originally tested negative, and (iii) all “healthy” test plants originally tested negative. This time however two test plants at the end of the experiment tested positive: a red noble and a yellow noble (both *S. officinarum*), both from the nymph cage. Five plants from the nymph cage were thought to have suspect symptoms, including the two that tested positive. However, symptom recognition was unreliable because the plants were generally yellow. This was most likely resulted from plants being root-bound and nutrient deficient.

In order to demonstrate successful transmission of Ramu stunt from Ragnar to the *S. officinarum* test plants, the 1.2kb RNA 6 fragment from selected samples was sequenced and compared to reference isolates. Table 18 shows the nucleotide percent identity between nine viral isolates. Five isolates were from this experiment, while four sequences were obtained from previous samples and are used as reference sequences.

The current isolates were obtained from: Ragnar from the diseased cage at the start of the trial (November 2016); a *E. flavipes* nymph at the time of transfer from the diseased cage to test cage (March 2017); the red and yellow noble canes from the nymph cage that tested positive (June 2017); and Ragnar from the diseased cage at the completion of the trial (June 2017). The reference isolates were all from Ragnar and include: a field isolate from the same field as the original source for both trials; Ragnar samples collected earlier (2001 and 2007); and the published sequence from Mollov *et al.* (2016) with GenBank accession KR094119. The results show that sequence identity over the nine isolates ranges from 99.6 % to 100 %. This represents up to five bases varying over the 1.2kb.

The variation is similar, regardless whether the isolate was an older field isolate or from the recent trial. Thus this experiment has demonstrated that a commercial isolate can be successfully transmitted to garden canes.

Table 18: Percent identity (% of bases /residues which are identical) over the 1.2kb RNA 6 fragment from nine Ramu stunt isolates. Five of the isolates are from transmission trial 2 (in blue), while four isolates are included for reference purposes.

	Nymph Mar17	Red noble Jun17	Yellow noble Jun17	Ragnar Jun17	Ragnar BN401	Ragnar 2007	Ragnar 2001	KR094119.1
Ragnar Nov16	99.6	100	99.6	100	99.6	99.7	99.8	99.7
Nymph Mar17		99.6	100	99.6	100	99.6	99.8	99.6
Red noble Jun17			99.6	100	99.6	99.7	99.8	99.7
Yellow noble Jun17				99.6	100	99.6	99.8	99.6
Ragnar Jun17					99.6	99.7	99.8	99.7
Ragnar BN401 Field 2016						99.6	99.8	99.6
Ragnar 2007							99.8	99.8
Ragnar 2001								99.8

Although it has been known since the 1990s that *Eumetopina flavipes* is the vector of Ramu stunt (Kuniata *et al.*, 1994), very little is known about the way in which it transmits the virus. However, an understanding of the virus-vector-plant relationship is essential for the control and management of any viral disease. It has been noted that Ramu stunt trials conducted at Ramu exhibited lower disease levels over time (Kuniata *et al.,* 2010b). This is a concern. Several options were proposed to increase disease incidence and to improve trial reliability:

i. Increase the proportion of diseased infection material within the trial, which may add to the inoculum pressure.

ii. Increase the populations of the vector, *Eumetopina flavipes*. Additional population studies within resistance screening trials, and knowledge of the population dynamics of the vector on the Estate, may identify when and where the vector populations peak.

iii. A third option could be to propagate the test varieties as single-eye setts in a shade house and to add infective planthoppers to mesh cages housing individual test canes. This would allow quantification of the inoculum pressure and guarantee a specific disease pressure.

The first option was addressed in the later stages of project 2009/033, where spreader rows of infected Ragnar were planted in every second row of the trials, while in another trial dual rows were planted.

The aim of the first transmission trial in this project was to confirm which vector life stage (adults or nymphs) are responsible for acquiring and transmitting the virus. Kuniata *et al.* (1994) reported that symptom expression took 9 - 12 weeks when *E. flavipes* nymphs were introduced to cages, but 18 - 20 weeks for other treatments. There are three sequential phases to transmission of tenuiviruses by Delphacid planthoppers: acquisition, incubation and inoculation. According to Falk and Tsai (1998), tenuiviruses can be acquired from infected plants through feeding times of 15 minutes to four hours. The latent period can last from four to 31 days, during which time the vector is not able to transmit the virus, and then the vector can transmit to a new host plant through feeding, in some cases for only 30 seconds, but more usually for a few minutes to hours.

The timing of our experiment was based on the estimated lifespan of *Eumetopina flavipes* of about 30 - 35 days (L. Kuniata, Pers. Comm.), and the theoretical acquisition, incubation and inoculation times for planthoppers and tenuiviruses. It was decided that the adult *E. flavipes* would be exposed to the virus in the diseased cage for three weeks, to provide time to breed, lay eggs, for egg hatching and development into nymphs, but not adults. Thus only one generation would be present but all stages of the life cycle could have potentially acquired the virus.

The trial was initially delayed by a very unusual and unprecedented problem with crickets. An alternative set up was required and fine mesh sleeves used for FDV screening in Australia was used instead of cages. This setup was simple to implement at Ramu and operated without any obvious operational problems.

Unfortunately the number of insects recovered from the diseased cage to be used in the transmission experiment was extremely low. For this experiment the insects were not allowed to establish a breeding colony to build up numbers, and there was obviously a high death rate. This meant that very low numbers were introduced to each of the test plants — about five per plant and only nine were recovered at the end. Egg puncture marks were observed on one test plant suggesting that the pot-mesh sleeve setup was suitable for *E. flavipes* survival and could be developed for additional transmission work.

The only successful transmission trial conducted in the previous Ramu project (2009/033) was the 2014 trial where over 660 insects were introduced to the diseased cage. It is becoming clear that transmission is difficult if vector populations are low. So in the final transmission trial conducted in this experiment, efforts were made to increase the starting number of *E. flavipes* in the diseased cage. Although the trial started with over 300 *E. flavipes* in January, only 200 were recovered in March, and by June, none survived. Successful transmission only occurred when nymphs were used as the vector. However, low vector numbers and rates of infection make it difficult to conclude that nymphs are the preferred vector life stage. The lack of transmission in other cages and plants suggests that *E. flavipes populations* are not establishing in the cages and not moving from plant to plant. Transmission is relying only on the introduced insects to vector the disease.

Future transmission trials will require more effort to maintain vector populations possibly through the control of vector predators.

Although the transmission rate was low, two noble canes were able to be infected with an isolate of Ramu stunt from commercial cane. Our data show that there is a natural genetic separation of isolates from noble canes and commercial canes. So while commercial cane isolates can infect noble canes, it does not appear to happen often. This may be due to limited vector movement from the estate to the Ramu valley, or there may be other host–isolate factors not yet understood.

Ramu staff are looking to raise infective planthoppers in cages to improve the resistance screening trials. However further work to understand the virus-vector-plant relationship is clearly needed and better facilities for transmission.

6.3 Stem borers

6.3.1 Develop methods for screening for resistance to *Chilo terrenellus* and *Scirpophaga excerptalis*

6.3.1.1 Experiment Sc1-16

Results for each of the recorded parameters associated with borer infestation are outlined below.

Dead heart

The analysis of variance (ANOVA) suggested there was a significant variety effect on dead heart (p= 0.003). The log-likelihood ratio test suggested a significant variance associated with replicate (p<0.001), and a significant variance associated with pot within replicate (p< 0.001).

The predicted values for variety are in Table 19.

Table 19: The effect of variety on the incidence of dead heart caused by *Scirpophaga excerptalis* in Sc1-16. Varieties followed by the same letter are not significantly different (P=0.05).

Variety	Predicted value	Back-transformed value	Standard error	Statistical groups
RQ117	-0.90	0.29	0.440	a
Q219$^{\phi}$	-1.64	0.16	0.473	ab
Q235$^{\phi}$	-1.98	0.12	0.506	ab
Q231$^{\phi}$	-2.55	0.07	0.559	ab
Q208$^{\phi}$	-3.47	0.03	0.712	b
Q135	-4.62	0.01	1.101	b

Varieties Q135 and Q208$^{\phi}$ had significantly fewer dead hearts than RQ117 (5% family significance level).

Bored Internodes per bored stalk

The variances of the variety data are the same (as the Brown-Forsythe Test, p= 0.251) and the Shapiro-Wilk test of normality showed that the residuals were normally distributed (Shapiro-Wilk p=0.072). The ANOVA suggested there was a significant variety effect on bored internodes (p<0.001). The predicted values for variety are in Table 20. Varieties followed by the same letter are not significantly different (P=0.05).

Table 20: The effect of variety on the incidence of bored internodes caused by *Scirpophaga excerptalis* in Sc1-16. Different letters in the statistical group column suggest those varieties where significant differences in bored internodes were recorded.

Variety	Predicted value	Standard error	Statistical groups
Q235$^{\phi}$	1.9	0.1	a
Q208$^{\phi}$	2.2	0.2	ab
Q219$^{\phi}$	2.2	0.1	ab
Q231$^{\phi}$	2.4	0.1	b
RQ117	2.4	0.1	b
Q135	2.5	0.3	ab

Q235$^{\phi}$ had significantly fewer bored internodes than Q231$^{\phi}$ and RQ117 (5% family significance level).

Tunnel length x Variety

The Shapiro-Wilk normality test confirmed that the population was normally distributed (Shapiro-Wilk p= 0.051). The ANOVA suggested a significant variety effect on tunnel length (p< 0.001) (Table 21).

Table 21: The effect of variety on tunnel length caused by *Scirpophaga excerptalis* in Sc1-16. Different letters in the statistical group column suggest those varieties where significant differences in tunnel length were recorded.

Variety	Predicted value	Standard error	Statistical groups
RQ117	24.5	0.798	a
Q219$^{\phi}$	26.10	0.987	b
Q231$^{\phi}$	26.95	1.404	ab
Q208$^{\phi}$	27.46	2.039	ab
Q135	27.72	3.513	ab
Q235$^{\phi}$	32.18	1.144	b

RQ117 and Q219$^{\phi}$ had significantly shorter tunnel lengths compared to Q235$^{\phi}$ (5% family significance level).

Tunnel length x Bored Internodes

The ANOVA suggested no significant effect of bored internodes on tunnel length (p=0.434). The full statistical analysis is reported in Appendix 2.

6.3.1.2 Experiment Sc2-16

Dead heart

The analysis of variance (ANOVA) suggested there was a significant variety effect on dead heart (p = 0.001). The log-likelihood ratio test suggested a significant variance associated with replicate (p<0.001), but no significant variance associated with pot within replicate (p= 0.162) (Table 22).

Table 22: The effect of variety on the incidence of dead heart caused by *Scirpophaga excerptalis* in Sc2-16. Different letters in the statistical group column suggest those varieties where significant differences in dead heart incidence were recorded.

Variety	Predicted value	Back-transformed value	Standard error	Statistical groups
Q219$^{\phi}$	-0.21	0.81	0.305	b
Q243$^{\phi}$	-0.26	0.77	0.306	b
RQ117	-0.83	0.44	0.318	b
Q246$^{\phi}$	-0.92	0.40	0.323	b
Q241$^{\phi}$	-1.04	0.35	0.329	b
Q135	-2.99	0.05	0.554	a

Q135 had significantly fewer dead hearts than all the other varieties (5% family significance level).

Bored Internodes

The variances of the variety data are the same (as the Brown-Forsythe Test, p= 0.099) and the Shapiro-Wilk test of normality showed that the residuals were not normally distributed (Shapiro-Wilk p=0). The ANOVA suggested no significant effect of variety on bored internodes.

Several other statistical tests (Fisher's Exact Test / Generalised Linear Mixed Model with a Poisson family distribution) also confirmed a lack of effect of variety.

Tunnel length x Variety

The Shapiro-Wilk normality test showed that the residuals are not normally distributed (Shapiro-Wilk p= 0.004). The ANOVA suggested a significant variety effect on tunnel length (p=0.03) (Table 23).

Table 23: The effect of variety on tunnel length caused by *Scirpophaga excerptalis* in Sc2-16. Different letters in the statistical group column suggest those varieties where significant differences in tunnel length were recorded.

Variety	Predicted value	Standard error	Statistical groups
Q135	44.50	12.471	a
RQ117	47.31	5.394	a
Q243$^{\phi}$	55.01	4.698	ab
Q246$^{\phi}$	58.58	5.561	ab
Q219$^{\phi}$	68.27	4.665	b
Q241$^{\phi}$	68.89	5.835	ab

RQ117 had significantly shorter tunnel lengths compared to Q219$^{\phi}$ (5% family significance level).

Tunnel length x Bored Internodes

The Shapiro-Wilk test suggested the residuals were normally distributed (Shapiro-Wilk value p=0.553). The interaction of variety and the number of bored internodes was tested and found not to be significant (p>0.05) (Table 24).

Table 24: The predicted values for the number of bored internodes is outlined for Sc2-16 below. Different letters in the statistical group column suggest significant differences.

Bored internodes	Predicted value	Standard error	Ranking
1	12.92	14.404	ab
6	143.28	14.404	e
2	33.55	5.840	a
3	54.00	2.823	bd
4	66.32	3.358	c
5	88.54	11.760	cd

ANOVA suggested there was a significant effect of bored internodes on tunnel length ($p<0.001$), but no significant effect of bored internodes on tunnel length ($p=0.434$). The full statistical analysis is reported in Appendix 3.

In one of these experiments (Trial Sc1-16) sampling revealed a lack of infestation in several replicates, and with five clusters not showing any visible sign of infestation. This was unexpected since active dispersal and establishment of neonate *S. excerptalis* larvae has been demonstrated in previous trials. Lastus Kuniata suggested that a combined infestation by plant hoppers and scale insects (which results in the excretion of honey dew) hampered larval establishment.

Overall, there was a noticeable effect of variety on dead heart symptoms and tunnel length with Q135 being the least affected and RQ117 and Q219$^{\phi}$ being the most affected varieties; this was consistent between Sc1-16 and Sc2-16 suggesting consistency in this measure between experiments. This is consistent with results obtained in field trials during our previous project (2009/033).

In Sc1-16, there was a significant effect of varieties on bored internodes, but this effect was absent in Sc2-16. As one of the main effects of *Scirpophaga excerptalis* is on the viability of the apical meristem (top shoot health), it appears that this short term pot experiment may identify resistance at least in part and would be a method worth exploring further with a larger number of varieties.

Based on the experiments reported here, the larval dispersal technique offers an alternative resistance screening method to longer term field experiments. However, strict measures are needed to ensure the viability of the eggs used to infest plants, and that the potted plants are free of any arthropod infestations that might hinder borer establishment.

Lack of *Chilo terrenellus* infestation at Ramu, and the lack of significant identifiable resistance in varieties on the Estate, meant that a *C. terrenellus* screening trial was not undertaken; two *S. excerptalis* experiments were conducted instead.

6.3.2 Develop an artificial diet for growth to maturity of larvae of *S. excerptalis* for use in IPM strategies

No young larvae completed their development on any diet combination. On the other hand, a percentage of older larvae did pupate and emerge as moths in a particular diet combination (2016-01) but only when introduced to that diet in their late larval instars. A detailed report prepared by RAIL is attached to this report (Appendix 4).

It is not clear why *Scirpophaga* larvae in particular fail to develop on artificial diets. It might be the case that the diet substrate (i.e. textural property) is unconducive to the survival of younger instars, which mainly develop in the soft tissues of the growing point. A softer 'semi-solid' or 'colloid' diet formulation might therefore be required. The use of Carrageenan as an alternative gelling agent to Agar may improve the diet's texture. It also might be the case that larval nutritional requirements

change as they develop into later instars. *Scirpophaga* larvae move down towards the growing point as they develop into later instars. Hence, segments/portions of different diets could be vertically layered in a long vial to emulate the borer's natural microhabitat, and therefore the different instars could obtain their required nutrition as they make their way down the vial.

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10 APPENDIX

Appendix 1 Metadata Disclosure

Table 25 Metadata Disclosure 1

Data	Ramu stunt, downy mildew, Scirpophaga, moth borers, taxonomy, rapid resistance screen, causal agent
Stored Location	SRA
Access	Available on request
Contact	Dr Rob Magarey

Appendix 2 Scirpophaga trial Sc1-16 analysis

Appendix 2
Scirpophaga trial Sc

Appendix 3 Scirpophaga trial Sc2-16 analysis

Appendix 3
Scirpophaga Pot tria

Appendix 4 Scirpophaga Ramu artificial diet report

Appendix 4
Scirpophaga Ramu a