SRA Research Project Final Report

Preparing the Australian sugar industry for exotic disease threats : final report 2009/033

SRA Project Code	**2009/033 (BSS331)**		
Project Title	**Preparing the Australian sugar industry for exotic disease threats**		
Key Focus Area in SRA Strategic Plan	3		
Research Organisation(s)	Sugar Research Australia		
Chief Investigator(s) **Authors**	Dr Rob Magarey, Principal Research Scientist Dr Rob Magarey, Dr Peter Samson, Dr Kath Braithwaite, Dr Nicole Thompson and Dr Nader Sallam		
Project Objectives	1. Develop and validate specific molecular diagnostic tests for Ramu stunt and downy mildew and define their distribution and variability in PNG 2. Develop efficient screening procedures for plant resistance to Ramu stunt, downy mildew and moth-borers, especially *Sesamia grisescens*; rapid screening procedures will be a key focus. 3. Determine pest and disease resistance ratings for 100-130 of the most important Australian commercial varieties, parents and promising clones. These will include recently released smut resistant varieties. 4. For moth-borers, investigate the occurrence of cross-resistance against the major borer species and develop an understanding of plant-insect relationships. 5. Refine incursion management plans for the targeted pests and diseases. 6. Incorporate resistance data into the BSES variety database (SPIDNet), provide wider access to data via QCANESelect and communicate results to the industry in each region.		
Milestone Number	Final report		
Milestone Due Date	1 May 2015	**Date submitted**	12 May 2015

Reason for delay (if relevant)	N/A
Milestone Title	Final Report
Success in achieving the objectives	☒ Completely Achieved ☐ Partially Achieved ☐ Not Achieved
SRA measures of success for Key Focus Area (from SRA Strategic Plan)	• Industry supported through effective pest, disease and weed diagnostic capabilities and awareness and training programs. • Development and adoption of SRA-developed packages for integrated management of key pests, diseases and weeds. • Weighted average disease ratings for varieties in each region. • Up-to-date dossiers to support contingency plans to minimize threats and impacts of key exotics. • Capability to provide entomology, pathology and weed expertise to meet the pest, disease and weed diagnostic and management needs of the industry.

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Quick summary

1. Previous field resistance trial data for downy mildew, Ramu stunt and *Sesamia grisescens* collected in PNG since the mid-1980s were accessed, analysed and information used as a foundation for project field resistance screening research.
2. Pot methods for moth borer resistance screening were reviewed, a visit to South Africa was made to view pot trial methodology and rapid test resistance screening research plans developed.
3. Field resistance screening trials for the moth borers (*Sesamia grisescens* and *Scirpophaga excerptalis*) and the diseases (downy mildew, (*Peronosclerospora* spp.) and Ramu stunt (tenuivirus)) showed promise in project research. Data collected has been analysed and resistance ratings for Australian commercial varieties assigned and loaded into SPIDNet.
4. A rapid resistance screen for the moth borers (manual infestation of test plants) and downy mildew (using oospores mixed into potting soil) show promise, but each method requires further refinement.
5. The causal agent of Ramu stunt was confirmed as a tenuivirus and a diagnostic assay validated. Suspect disease specimens can now be confidently assayed, should an Australian incursion occur. Some pathogen variation was noted around PNG; it is unknown if this will affect varietal resistance reactions.
6. Molecular tools for assaying for downy mildew were also developed; suspect disease specimens for DM can also now be confidently assayed. Significant DM pathogen variation in PNG was noted, with three taxa identified. One of these may be a new *Peronosclerospora* species.
7. The project has led to major steps forward in relation to knowledge of these pests and diseases. Our capacity to identify, manage and advise the Australian sugarcane industry with regard to PNG pests and diseases has improved greatly. The industry is now in a strong position to deal with the major pest and disease threats present in PNG.

Executive Summary

Papua New Guinea (PNG) is the centre of diversity for three *Saccharum* spp., including the domesticated *S. officinarum*, *S. edule* and the 'wild cane' *S. robustum*. *S. spontaneum* is also present in PNG but is considered to have been introduced several centuries ago. A closely related grass, *Miscanthus floridulus*, is also present at high altitudes (above 800 m). Pests and diseases of these grasses include moth borers (*Sesamia grisescens*, *Chilo terrenellus*, *Scirpophaga excerptalis*); this pest group includes some of the most important affecting sugarcane world-wide. Two major PNG diseases are downy mildew (DM, caused by *Peronosclerospora* spp.) and Ramu stunt (RS, caused by a tenuivirus). The two diseases pose a significant threat to the Australian sugarcane industry. DM was once present in Australia and caused very significant yield losses, but has since been eradicated. Ramu stunt was recognised for the first time in PNG in 1985, when it devastated the commercial estate at Ramu. Previous to the project, Ramu stunt was thought to be caused by a tenuivirus; this was confirmed in project research.

BSES scientists provided consultancy services to Ramu Sugar (Ramu Agri-Industries) from the mid-1980s; linked with this was the development of field-based resistance screening techniques. A total of 21 DM field trials were planted by Ramu

staff over 20 years and about 12 RS trials. SRDC funding provided in BSS331 enabled the collation and analysis of trial data and calculation of resistance ratings for potential standard varieties and other commercial varieties. Papers were published on the results (Kuniata et al, 2010a. Kuniata et al, 2010b).

Project funding enabled the export of the most important current Australian commercial varieties and these were included in moth borer, DM and RS trials planted in 2010, 2011, 2013 and 2014. Only a limited number could be sent through quarantine. Each trial included a set of 'standard' varieties that reflected the range in resistance of commercial varieties (resistant to susceptible). For the diseases, and some pests (especially *S. grisescens*), some varieties were known to be more susceptible than others. A knowledge of the resistance of Australian commercial varieties was considered essential to prepare for a possible Australian incursion.

Project research into field resistance screening for moth borers (*Sesamia grisescens*, *Scirpophaga excerptalis*) showed consistent varietal reaction amongst varieties between trials, but there was less consistency for resistance to *C. terrenellus*. The most 'resistant' and 'susceptible' varieties provided the most consistent and decisive reactions. There was a good correlation between stalk incidence and the extent of stalk colonisation with these borers. *S. grisescens* and *Rhabdoscelus obscurus* (weevil borer) incidence was positively correlated, while *S. grisescens* and *S. excerptalis* incidence was negatively correlated – the presence of some borers influences the incidence of others.

Downy mildew field-resistance screening also showed consistency between trials in the most resistant and susceptible varieties. Some variation in reaction of standard varieties in field trials was noted when compared to historical data (1985-2000 data). Pathogen variation may have contributed to these differences in reaction. Resistance ratings for the Australian commercial varieties for the PNG pests and diseases have been calculated.

Rapid pot resistance-screening tests for *S. grisescens* and downy mildew showed promise; overseas moth borer techniques adapted for *S. grisescens* pot trials were not successful, but a second technique showed promise and requires further testing. Downy mildew oospores were used to inoculate a pot soil into which single-eye test canes were planted; this is the first time oospores have been used in DM resistance screening. Promising results were obtained but further research is required to refine the technique.

Ramu stunt causal agent research clearly showed that a tenuivirus is the cause of Ramu stunt and the use of a molecular detection technique for the disease was validated. This means the test can now be used confidently in quarantine and for confirming disease identity, should an incursion occur. Some variation in the pathogen was noted in survey samples; the effect this may have on variety reaction is unknown – further research is required.

Downy mildew pathogen research led to the development of molecular tools to assay plant samples for the disease. Three known species of the causal agent (*Peronosclerospora*) have been described (*P. sacchari*, *P. miscanthi* and *P. philippinensis*); project research has also shown three different *Peronosclerospora*

taxa in PNG, but one of these is likely to be an undescribed species. Further morphological descriptions are needed along with parallel molecular characterisation.

Incursion management plans were updated with the latest research developments; resistance ratings were uploaded into SPIDNet and should an incursion occur, will be made available to industry via QCANESelect™ .

General background

Papua New Guinea (PNG) is the centre of diversity for three *Saccharum* spp., including the domesticated *S. officinarum* (chewing or noble canes), *S. edule* (pit pit – also domesticated and eaten as a cooked vegetable) and the 'wild cane' *S. robustum*. *S. spontaneum* (another more 'grass-like' wild cane) is also present but is considered to have been introduced to the island of PNG several centuries ago. The diversity amongst *S. spontaneum* individuals is limited compared to that present in the other three species. A closely related grass, *Miscanthus floridulus*, is also present at high altitudes (above 800 m). Each of these species hosts sugarcane pests and pathogens that attack commercial hybrid varieties around the world.

The Australian sugarcane industry is free of the serious moth borers that comprise some of the most serious pests of sugarcane (*Saccharum* hybrids) on a world-wide basis. However, Papua New Guinea (PNG), on our door-step, has several damaging species: the stalk borers *Sesamia grisescens* Warren (large pink stalk borer; Noctuidae) and *Chilo terrenellus* Pagenstecher (internode borer; Crambidae) and the top borer *Scirpophaga excerptalis* (Walker) (top shoot borer; Pyralidae). *S. grisescens* and *C. terrenellus* are restricted to the island of New Guinea whereas *S. excerptalis* is widespread through Asia. These borers damage commercial crops of sugarcane grown in the Ramu Valley in the north of PNG, and are also found in *Saccharum* spp. (*S. officinarum*, *S. edule*, *S. robustum* and *S. spontaneum*) growing in home gardens and in the wild.

Sesamia grisescens lays eggs behind the leaf sheaths. Young larvae of *S. grisescens* feed on the inner surfaces of leaf sheaths for several days and then bore into the stalk, where they feed gregariously in the upper internodes and usually damage the meristem, causing a 'dead heart'. Maturing larvae then migrate to nearby stalks, sometimes damaging the meristem (Young and Kuniata, 1992; Kuniata and Sweet, 1994; Kuniata, 1998). *C. terrenellus* and *S. excerptali*s both oviposit on the unfolded leaves. Larvae of *C. terrenellus* tunnel inside semi-mature and mature stalks (Kuniata et al., 2001). Newly hatched larvae of *S. excerptalis* enter the midrib of the top leaf on the stalk, emerge and tunnel to the meristem, with only one larva surviving in each stalk (Mukunthan, 1985). Meristem damage causes a dead heart and stops the stalk from growing, leading to side shooting and reduced stalk weight, while internode damage reduces stalk weight and sugar content and quality.



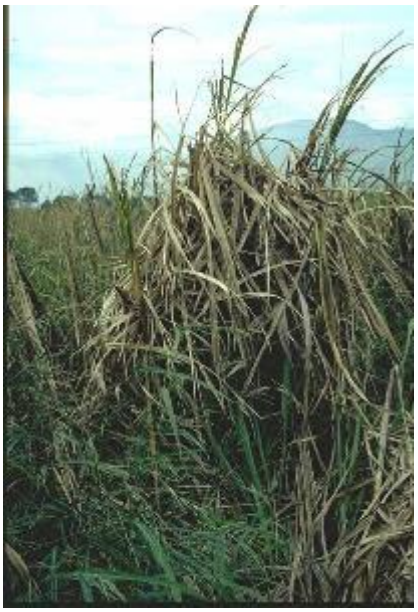


Figure 1: Symptoms of two different moth borers present in PNG: top – *Sesamia grisescens* (left - dead heart; middle - internal damage; right – dead crop); bottom - *Scirpophaga excerptalis* (left – shoot symptoms, middle – stalk damage, right – characteristic holes in affected leaves).

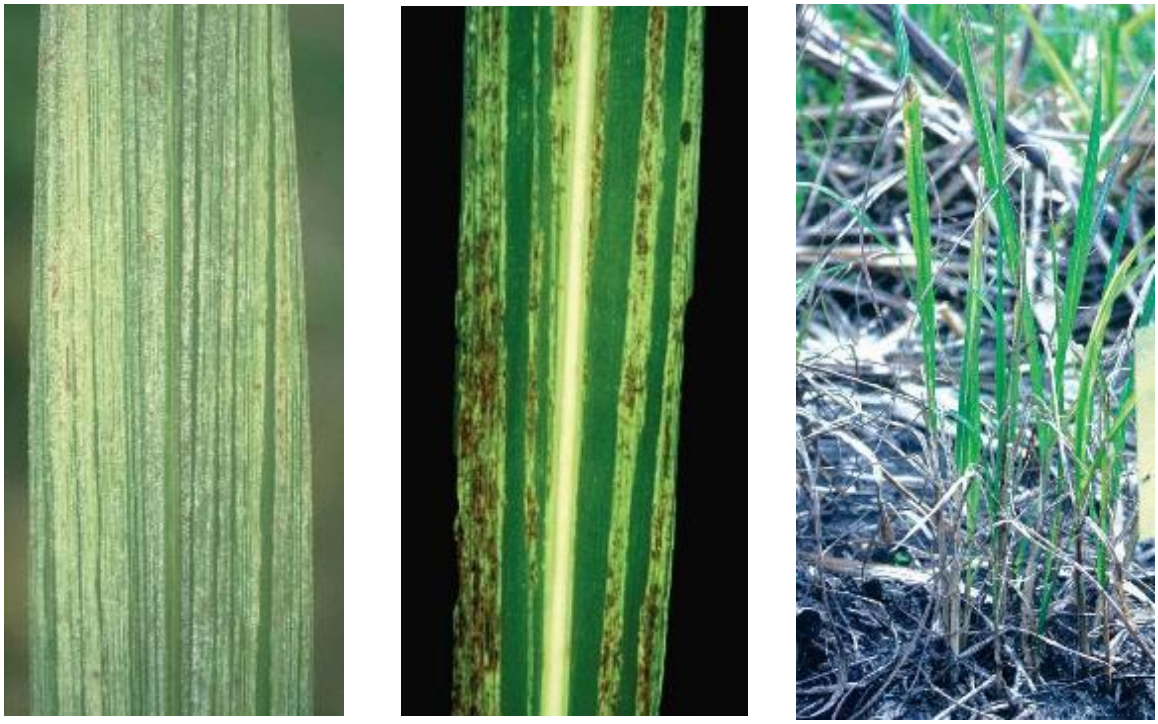


Figure 2: Symptoms of the two different diseases researched in BSS331 and present in PNG: top – Downy mildew (left – down production under an infected leaf; middle – well developed leaf streaks characteristic of DM; right – thin, poorly-grown stalks associated with systemic infection); bottom – Ramu stunt (left – stunted stool; middle – streaked mottled leaf symptom; right – loss of stools in a previously-affected crops).

Two major disease threats in PNG are downy mildew (DM, caused by *Peronosclerospora* spp.) and Ramu stunt (RS, caused by a tenuivirus). The two diseases pose a significant threat to the Australian sugarcane industry. DM is a systemic disease caused by an oomycete (fungus-like) pathogen. It invades sugarcane via bud and spindle-leaf infections, spreads through the growing plant, stunting the growth of susceptible varieties and leading to characteristic leaf streak symptoms. The delicate conidia (spores) are produced during warm humid nights, largely on the underside of leaves, in fluffy whitish-grey hyphal growth (hence the term 'down'); these spores are blown in the wind and spread to infect cane growing close-by. The spores last only a matter of hours before losing viability. Under cooler conditions, oospores are produced in infected leaves leading to leaf splitting (the vascular bundles within the leaves are forced apart by the developing spores). The role played by oospores in the disease epidemiology has not been studied extensively but is thought to be minor compared to the role of conidia. DM is favoured by warm humid conditions and spread occurs more rapidly in summer.

DM was introduced into Australia early in the history of sugarcane cultivation in this country and resulted in very significant yield losses in commercial crops. A focused eradication program, consisting of the cropping of resistant varieties, production of disease-free nursery cane, elimination of heavily-infested crops and rogueing of individual diseased stools eventually led to DM eradication in the late 1950s. DM is therefore capable of causing very significant yield losses under Australian conditions

Ramu stunt is a relatively recently-recognised disease, seen for the first time in PNG in 1985 when an epidemic devastated the commercial estate at Ramu. RS was first reported to be caused by a phytoplasma (Cronje et al., 1999). Evidence gathered at Ramu suggested the planthopper *Eumetopina flavipes* transmits the disease (Kuniata et al, 1994). RS is also a systemic pathogen and leads to very significant stunting of infected plants and premature death in highly susceptible varieties. Failed ratoon crops were common in the susceptible Ragnar in which the disease was first recognized at Ramu in 1985 (Suma and Jones, 2000; Kuniata et al., 2010b). The most obvious symptoms are severe stunting, a trashy stool appearance, varied leaf striping / mottling and stool death (Kuniata et al., 2010b). When the project began, there remained confusion as to the nature of the causal agent, whether it was caused by a phytoplasma or a virus.

From the mid-1980s, BSES scientists provided consultancy services to Ramu Sugar (Ramu Agri-Industries); linked with this was the development of field-based resistance screening techniques designed to gather information on the resistance of Australian commercial varieties to the two diseases. A total of 21 DM field trials were planted by Ramu staff over 20 years and about 12 RS trials. SRDC funding provided by project BSS331 enabled the collating of these data, their analysis and the application of resistance ratings to potential standard varieties and other commercial canes.

Exotic moth borers, DM and RS are important biosecurity risks to the Australian sugarcane industry. In order to be prepared for possible incursions, BSES Limited, in partnership with Ramu Agri-Industries, formulated a research proposal to further develop resistance screening techniques for Australian commenced varieties to each

of these PNG threats. Some Australian commercial cultivars and advanced clones were already being propagated in PNG while others were shipped to allow screening for resistance to the borers and diseases. *S. grisescens* was the main target in the borer trials, as it is judged the most damaging pest at Ramu A-I (Kuniata, 2000), but the other borer species were also recorded during sampling. The sugarcane weevil borer, *Rhabdoscelus obscurus* (Boisduval) (Coleoptera: Curculionidae), which occurs in both PNG and Australia, was also assessed in trials.

Project funding also facilitated the introduction to PNG (via quarantine) of the most recent commercial varieties released to the Australian sugarcane industry. This was essential for assessing the susceptibility of Australian commercial crops to both stem borers and PNG diseases. Initial introductions of these varieties was via propagation plots located at Leron Plains cattle ranch (around 80 km towards Lae along the Laue-Madang road). Propagation at this location, away from the commercial estate, enabled multiplication of the varieties in the absence of the stem borers and DM / RS.

References

Cronje CPR, Bailey RA, Jones P, Suma S (1999) The phytoplasma associated with Ramu stunt disease of sugarcane is closely related to the white leaf phytoplasma group. *Plant Disease* 83(6): 588

Kuniata LS (1998) Borer damage and estimation of losses caused by *Sesamia grisescens* (Lepidoptera: Noctuidae) in sugarcane in Papua New Guinea. *International Journal of Pest Management* 44: 93-98.

Kuniata LS (2000) Integrated management of sugarcane stemborers in Papua New Guinea. In 'Sugarcane pest management strategies in the new millennium.' (Eds PG Allsopp, W Suasa-ard) pp. 37-50. (International Society of Sugar Cane Technologists: Brisbane).

Kuniata LS, Young GR, Pais E, Jones P, Nagaraja H (1994). Preliminary observation on Eumetopina sp (Hemiptera: Delphacidae) a vector of Ramu stunt disease of sugarcane in Papua New Guinea. Jornal of the Australian Entomological Society 33: 185-186.

Kuniata LS, Chandler KJ, Korowi KT (2001) Management of sugarcane pests at Ramu, Papua New Guinea. *Proceedings of the International Society of Sugar Cane Technologists* 24: 382-388.

Kuniata LS, Magarey RC, Rauka GR, Suma S, Bull JI (2010) Screening for Ramu stunt resistance at Ramu Agri-Industries, Gusap, PNG 1986–2008. *Proceedings of the Australian Society of Sugar Cane Technologists* 32: 312-321.

Kuniata LS, Sweet CPM (1994) Management of *Sesamia grisescens* Walker (Lep.: Noctuidae), a sugar-cane borer in Papua New Guinea. *Crop Protection* 13: 488-493.

Mukunthan N. 1985. New aspects in the biology of sugarcane top borer, *Scirpophaga* (*Nivella* F.) *excerptalis* Wlk. Entomon 10: 235-238.

Suma S, Jones P (2000). Ramu stunt. In: A Guide to Sugarcane Diseases. Eds. P. Rott, R.A.

Bailey, J.C. Comstock, B.J. Croft, and A.S. Saumtally. CIRAD and ISSCT, pp 226-230.

Young GR, Kuniata LS (1992) Life history and biology of *Sesamia grisescens* Walker (Lepidoptera: Noctuidae), a sugarcane borer in Papua New Guinea. *Journal of the Australian Entomological Society* 31: 199-203.

Section 3: Background and Project Research

Chapter 1: Project reviews

Initial funding provided the opportunity to undertake three reviews of different aspects related to screening varieties for resistance to pests and diseases. Two related to the collation and analysis of PNG historical data on: i. *Sesamia grisescens* resistance, and ii. downy mildew and Ramu stunt resistance. The third involved a lap top study of methods used world-wide for moth borer resistance screening, plus a study tour to South Africa by a project staff member to view South African research into resistance screening methods for *Eldana sacchari*, a very serious sugarcane stem borer in Africa.

1.0 Review of previous resistance screening data for stem borers in PNG

We analysed the results of two variety trials planted in 2003 and 2007 with the aim of evaluating varietal responses to these borers, and particularly to *S. grisescens*. For two moth borers, *S. grisescens* and *S. excerptalis*, and for the weevil borer, damage and/or numbers differed significantly among clones in at least one trial. However, varietal differences were not detected for *C. terrenellus*. There was a negative correlation between damage from *C. terrenellus* and *S. grisescens* in one trial and it is possible that damage from the latter species may interfere with the activities of *C. terrenellus* or destroy evidence of its presence. Damage from *S. grisescens* and weevil borers was positively correlated, probably because weevil borers are known to be attracted to previously damaged cane. Cane yield was measured in one trial and was negatively correlated with damage from *S. grisescens* and weevil borers but not from *C. terrenellus* or *S. excerptalis*. This study confirms the importance of *S. grisescens* as a major pest of sugarcane and a key component of the borer complex in PNG, confirms the existence of resistance to this pest among sugarcane clones, and indicates that screening of Australian commercial varieties for resistance to *S. grisescens* in PNG is worthwhile. Apparent varietal differences in crop response to borers in PNG may be influenced by interactions among species.

The published paper is attached as Appendix 1.

2.0 Review of moth-borer resistance screening worldwide and report on visit to SASRI

Information on procedures for screening sugarcane varieties for resistance to moth borers and associated research was obtained by a search of literature and by a 2-week visit to SASRI (South African Sugarcane Research Institute).

Screening procedures are mainly of two types: in-field plots where plants are infested by naturally occurring populations of moths, sometimes encouraged by planting of susceptible host plants near the experimental plots and/or augmented by

the release of additional moth borers from laboratory culture in pots where plants are infested artificially, often using moth-borer eggs.

Each of these has advantages and disadvantages. Natural infestation of field plots allows the full range of resistance mechanisms – antixenosis, antibiosis and tolerance – to operate under commercial conditions, but results may be subject to considerable experimental variation due to variable environmental conditions and inconsistent numbers of borers. Artificial infestation of potted plants allows the experimenter to control environmental variables and apply a constant infestation pressure but some components of resistance (especially ovipositional antixenosis) may be missed. While artificial infestation has been used in numerous studies to elucidate resistance mechanisms, it seems to be currently used as a routine method of screening varieties for borer resistance only at SASRI against *Eldana saccharina*. Molecular markers and near-infrared spectroscopy are techniques that could aid with identification of potentially resistant varieties.

Data collected in resistance trials always includes a measure of borer damage, typically a count of bored internodes, and sometimes a measure of larval performance such as number or weight of borers or number of emergence holes indicating successful production of adults. A few studies have included plants from which borers are excluded, usually with insecticide, which allows crop tolerance to be measured, or have estimated tolerance by rating plant response according to indirect measures such as side-shooting or stalk breakage.

Systematic screening of varieties ideally includes a set of standard varieties covering the range of expected responses from susceptible to resistant. Most screening programs rate varieties as susceptible, resistant or intermediate, but the program at SASRI rates varieties on the 1-9 scale familiar to plant pathologists, with the results weighted according to experimental precision. Molecular markers for resistance have been identified that can help with choice of parents in breeding programs.

There is some evidence of ovipositional antixenosis, larval antixenosis and antibiosis and plant tolerance as mechanisms of genotypic sugarcane resistance against different species of moth borers. Of these, antixenosis or antibiosis acting against early-stage larvae seems the most common, preventing larval penetration of the stalk or delaying penetration so that small larvae are exposed to abiotic and biotic mortality factors. Near-infrared spectroscopy (NIR) has potential as a method for predicting resistance that is associated with stalk surface chemistry. Phenotypic resistance may be altered by plant nutrition; water stress or increased levels of nitrogen may increase susceptibility while increased silicon may promote resistance.

SASRI has adopted an annual program of screening varieties from stages 4 and 5 of their 5-stage breeding program in pots in a shadehouse for resistance to *E. saccharina*. This method ensures uniform infestation of plants, allows better control of environmental variables and requires less labour than similar field trials. However, yield trials in stages 4 and 5 are also sampled for *E. saccharina* damage but with fewer variables measured than in the shadehouse. Plants in the shadehouse are assessed for damage as bored internodes and larval performance as number of live larvae. Length of bored internodes and weight of larvae and pupae were measured when the program was begun several years ago but were strongly

correlated with the other two variables and so were dropped from the procedure. Six standard varieties are included in each screening trial and both standard and test varieties are rated on a 1-9 (resistant-susceptible) scale by a statistical method that weights the measured variables according to their variance and then calculates ratings according to the precision of the experiment; the latter calculation is fundamentally different from the method used by SRA plant pathologists.

The full review and report is attached as Appendix 2.

3.0 Review of historical DM and RS resistance screening data from PNG trials (Ramu)

With the provision of project funding for resistance screening research, all previous resistance trial data (as much as was available) from DM and RS field trials were accessed by project staff. The design of the previous DM field trials had included two rows of test canes planted between a single row of a systemically-infected susceptible variety. Test cane plots were 10 m long; trials consisted of two replicates of test canes planted in a randomised complete-block design.

RS stunt trials were similarly designed as per DM trials. Different standard varieties were employed to provide for a suitable scope of RS resistance. With RS, test cane infection relied on the spread of infected *E. flavipes* individuals from infected to healthy sugarcane stools.

Data from both DM and RS trials were collated and analysed using the current BSES resistance rating technique. The reaction of the standards was compared to their expected reaction, as assessed in previous trials and in commercial crops. Where significant variation from expected reaction was evident, resistance ratings were not applied to test canes. With each disease, more than sufficient numbers of standards had been included in the previous trials; data analyses enabled the best of these to be selected for inclusion in future trials (Kuniata et al, 2010a,b).

The results suggested that trial reliability with both diseases varied over the years. Generally low transmission levels led to unreliable resistance ratings (Kuniata et al, 2010a and b); low transmission was of uncertain cause but at times was related to low rainfall (personal observation). Increasing the level of disease transmission was seen as a priority. Australian commercial varieties proved more susceptible to DM than to RS; in fact, up to 50% of commercial varieties sourced from the Australian sugarcane industry were susceptible to DM, compared to much lower levels amongst varieties sourced from other breeding programs around the world (Kuniata et al, 2010a). In contrast only 25% of Australian varieties exhibited RS symptoms. In field trials, the most reliable disease resistance observations were sourced from the early first-ratoon crop; this meant resistance ratings could only be applied 18 months after a trial was planted. A priority for research was therefore to develop a rapid screening test for DM.

The results were published in the Proceedings of the Australian Society of Sugar Cane Technologists (ASSCT; Kuniata et al, 2010a. Kuniata et al, 2010b). Copies of these papers are included in Appendix 3.

References

Kuniata, L.S., Magarey, R.C., Rauka, G.R, Suma, S. and Bull, J.I. (2010a). Screening for downy mildew resistance at Ramu Agri-Industries, Gusap, PNG 1986-2008. Proc. Aust. Soc. Sugar Cane Technol., 32: 301-311.

Kuniata LS, Magarey RC, Rauka GR, Suma S, Bull JI (2010b) Screening for Ramu stunt resistance at Ramu Agri-Industries, Gusap, PNG 1986–2008. Proceedings of the Australian Society of Sugar Cane Technologists 32: 312-321.

Chapter 2: Field resistance screening trials – Stem borers

Objectives

- Develop efficient screening procedures for plant resistance to moth-borers, especially *Sesamia grisescens*; rapid screening procedures will be a key focus.
- Determine pest and disease resistance ratings for 100-130 of the most important Australian commercial varieties, parents and promising clones. These will include recently released smut resistant varieties.

Outputs and Achievement of Project objectives

Introduction

Five field trials to assess borer resistance were planted during the project. However, the first trial planted in 2009, SRT02-09 ('Sesamia Resistance Trial<Sequence number>-<Year of planting>'), was not significantly infested by borers and no useful data were collected. The remainder of the report refers to the four subsequent trials.

Methods

Four field trials to assess varietal resistance to borers were planted in each of 2010, 2011, 2013 and 2014. Each trial included 13 standard clones. Twelve of them were chosen to represent a range of levels of susceptibility to *S. grisescens* displayed in commercial fields and field trials at Ramu Agri-Industries, as judged by staff from Ramu A-I (note that 'Resistant' in this context refers to varieties that are the least liable to damage among the suite of varieties available at Ramu A-I rather than varieties that are truly resistant):

Resistant:	PN92-439, RQ117
Intermediate-Resistant:	PN92-31, PN92-339
Intermediate:	B72177, R570
Intermediate-Susceptible:	F177, Q127
Susceptible:	Cadmus, Q135, Q136, Q198[Φ.]

One more standard, Q219[Φ], was included in all trials after it showed high levels of damage from *S. excerptalis* in the trial planted in 2010. Details of the first three trials are given in Table 1. SRT01-14 was planted with 36 clones on 18/3/2014 but no destructive sampling was done during the project.

Table 1 Details of varietal resistance trials planted in Papua New Guinea.

Trial code	SRT01-10	SRT01-11	SRT01-13
No. of clones	32	33	32
Planting date	13/12/2010	28/11/2011	13/3/2013
Destructive sampling date, plant crop [a]	20/6/2011 (25)	5/6/2012 (24)	27/8/2013 (36)
Internodes/stalk (mean and clone range)	12.9 (10.8-15.4)	13.5 (9.9-16.2)	10.4 (8.7-12.7)
Harvesting date	22/10/2011	22/9/2012	?/11/2013
Destructive sampling date, first ratoon [a]	30/7/2012 (31)	30/7/2013 (32)	23/4/2014
Internodes/stalk (mean and clone range)	21.5 (18.0-25.8)	20.6 (15.8-25.4)	15.5 (13.0-17.5)

[a] Week of each year in parenthesis, for comparison with pheromone traps catches in Figs. 3-5

Each variety was planted in plots measuring 7.5 m or 8 m long by four rows wide at a 1.8 m row spacing, replicated four times in randomised complete-block designs. Clones believed to be susceptible to *S. grisescens*, Cadmus or Q136, were planted in a 'spreader' or 'infestation' row between each four-row column of trial plots and also around the outside of each trial to increase the local population of borers. Each plot was separated from the next plot within the column by a 1 m gap, and an empty row was left between each column of plots and the spreader row on either side. Trials were mainly intended to evaluate varietal response to *S. grisescens*, so cane tops infested with this borer were distributed around the outside of the trial when infested material was available.

The varieties in each trial were confirmed using DNA fingerprint analysis. After crop establishment, a leaf sample was taken from one stalk from the middle of each plot of one replicate in the trial. DNA was extracted and the fingerprint compared to the profiles held in the AGRF database. For Australian varieties, this was able to determine if the variety was correct or not. For PNG varieties this was able to show if the variety was consistent across all trials and propagations. A small number of discrepancies were noted during the trial program, presumably due to mis-labelling of varieties in PNG or errors during planting, and affected varieties were excluded from analyses.

Each plot row was planted by hand with 25 two-bud billets placed into pre-formed furrows. Metalaxyl was sprayed over the billets at 1 kg product/ha for control of downy mildew disease, fertiliser was applied, and billets were then covered with soil. Trial SRT01-13 was sprayed three times with lambda-cyhalothrin (Karate), in April, May and June 2013, in an attempt to limit stalk damage from *S. excerptalis* before the main flights of *S. grisescens*.

Sticky traps baited with a pheromone to lure moths of *S. grisescens* were used to monitor moth activity in the trials (Figure 1). During 2011, traps for *S. grisescens* were placed near trials planted in 2009 and 2010, with one trap at each trial until week 11 (18 March) and two thereafter. Similar trapping was done in subsequent years, with four traps per trial in 2012 and two per trial in 2013. Traps were inspected weekly and pheromone lures were replaced approximately monthly.

Figure 1: (a) Kaile Korowi inspecting a sticky trap baited with pheromone lure for *S. grisescens*, (b) *S. grisescens* moth in trap with pheromone-impregnated rubber lure visible at top.

Trials were destructively sampled for borers on the dates given in Table 1, at times when there was observed to be damage from *S. grisescens* as evidenced by dead tops. The table also gives a measurement of stalk development at the time of sampling, as internodes per stalk. In SRT01-13, numbers of internodes per stalk were low when sampled in the plant crop because of the short growing time. Ten stalks were randomly selected from each of the outside rows of each plot and any dead tops were classified as being the result of infestation by *S. grisescens* or *S. excerptalis*. Dead hearts from *S. excerptalis* occur higher in the stalk and are characterized by shortened internodes and stunted upper leaves (Fig. 2), compared with dead hearts caused by *S. grisescens*. Stalks were then sliced open longitudinally and total internodes, internodes damaged by each borer species, and numbers of larvae and pupae of each borer were counted. Tunnels of *C. terrenellus* could be distinguished from those of *S. grisescens* by their serrated margins and generally smaller diameter while tunnels of the weevil borer *R. obscurus* were packed with fibrous frass; damage from *S. excerptalis* was limited to a fine tunnel near the apical meristem. Each trial was harvested by cutting all stalks at ground level using a commercial cane harvester or by hand. Borer sampling was done once before the harvest, i.e., in the plant crop, and once in the first-ratoon crop.

Figure 2: Stalk with dead heart caused by *S. excerptalis*.

Moth borer egg batches were counted in some trials. Egg batches of *S. grisescens* can be felt as a swelling beneath the young leaf sheaths, while egg batches of *C. terrenellus* and *S. excerptalis* can be counted on the lamina. Dead tops of cane stalks caused by each of these species were also counted on some occasions. Egg batches and dead tops were counted in rows 1 and 4 of each 4-row plot for the whole plot length.

Plot totals (over 20 stalks) for numbers of internodes, damaged internodes (for *S. grisescens* and *C. terrenellus*) and infested stalks (for *S. excerptalis*), and numbers of each borer were analysed by generalised linear mixed models using GenStat 13.2. Numbers of damaged internodes were not analysed for *S. excerptalis* as larvae of this pest tunnel through only a few very young internodes; the primary damage is to the meristem which is almost invariably destroyed in infested stalks. Damage measurements as a proportion of total internodes or stalks were assumed to follow a binomial distribution with a logit link function. Borer counts were assumed to follow a Poisson distribution. The statistical probability of clonal effects was estimated by the Wald statistic with clone as the fixed term, replicate a random term, and the dispersion parameter either fixed or estimated, whichever gave the more conservative test. Relationships between damage measurements in each plot were analysed by correlation. Average damage to the standard clones from each borer species was compared among trials by simple correlation. Normalised values for the response of the standards in each trial were calculated from the mean and standard deviation (s) of transformed values (x) of the percentage damage from each borer after angular transformation (arsine √), using the formula $z = (x - mean)/standard\ deviation$ by the STANDARDIZE function in Excel 2010.

Crop yields were measured in some trials. Ten stalks were cut from each plot for fibre and juice analysis. In the plant and first ratoon crops of SRT01-10 and the first ratoon of SRT0111, the weight of cane in rows 2 and 3 was measured using a weighing bin and commercial harvester. In the plant crop of SRT01-11 and the first ratoon of SRT01-13, the weight of cane in rows 2 and 3 was measured by hand-cutting. Yields were converted to tonnes/ha according to the row spacing of 1.8 m. Quality measurements and yields were compared among treatments by 2-way analysis of variance. Clonal means at harvest were compared with previous damage measurements by simple correlation.

A final analysis was conducted over all trials to estimate susceptibility of all tested varieties to the four borers according to the criteria of proportion bored internodes for *S. grisescens*, *C. terrenellus* and weevil borer and proportion bored stalks for *S. excerptalis*. We fitted a linear mixed model to the data transformed as logits. Analyses for the 13 standard varieties were run over all the six data sets in the current trials (three trials, plant and first ratoon data) plus three data sets from previous trials conducted by Ramu Agri-Industries, while analyses for the test varieties plus standards were run over the current trials only. Means were separated by the least-significant difference test (P = 0.10).

Results

Flights of *Sesamia grisescens*

In 2011, there were peaks of moth flights during weeks 2-4 (14-28 January), 9-12 (4-25 March) and 19-20 (13-20 May) (Figure 3). The period between peaks corresponds with the generation time of *S. grisescens* of 7-11 weeks (Young and Kuniata, 1992).

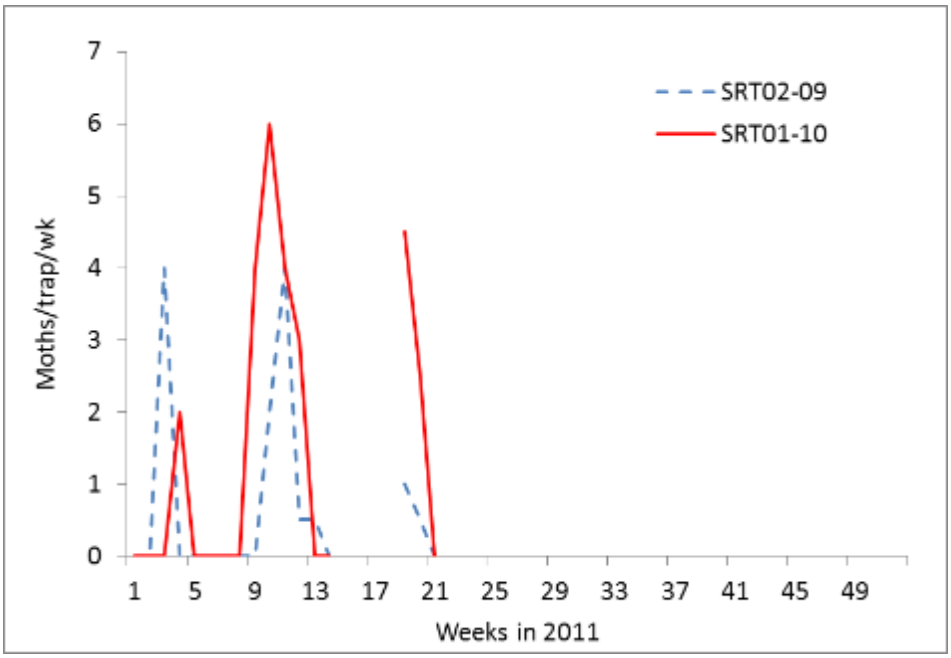


Figure 3: Weekly catches of *S. grisescens* moths in pheromone traps placed near trials SRT02-09 (first ratoon) and SRT01-10 (plant crop) during 2011.

Catches in 2012 and 2013 are given in Figures 4 and 5, respectively. Generations were much less clear in 2013 than in 2011.

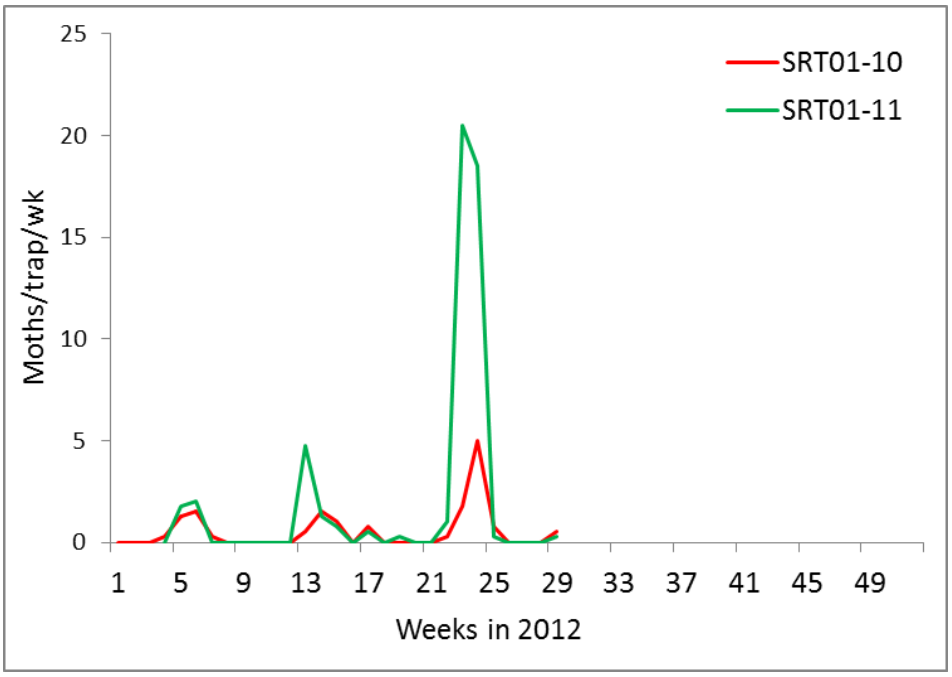


Figure 4: Weekly catches of *S. grisescens* moths in pheromone traps placed near trials SRT01-10 (first ratoon) and SRT01-11 (plant crop) during 2012.

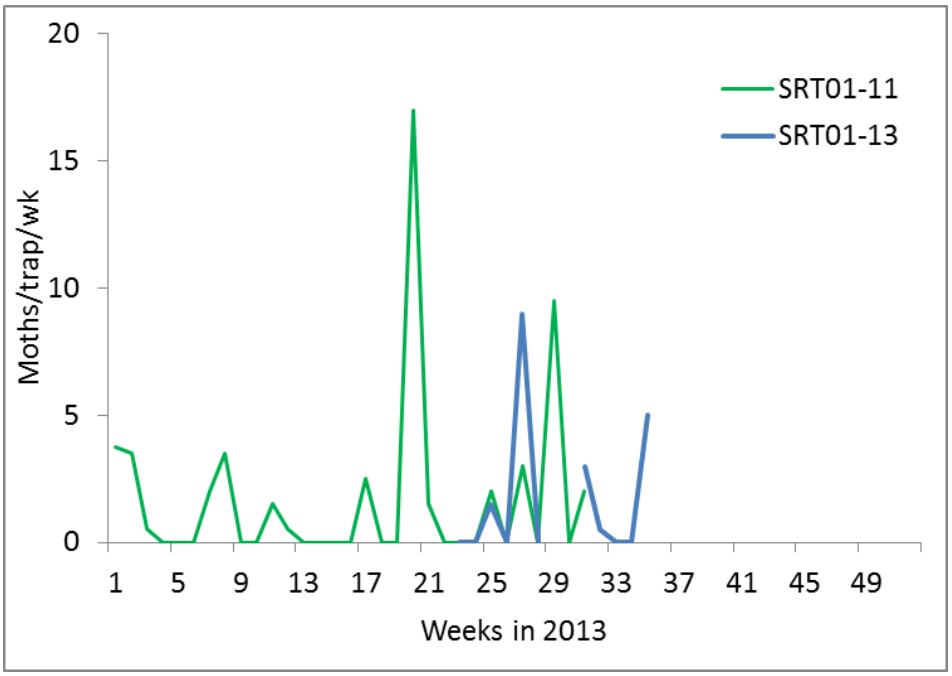


Figure 5: Weekly catches of *S. grisescens* moths in pheromone traps placed near trials SRT01-11 (first ratoon) and SRT01-13 (plant crop) during 2013.

Infestation levels

Infestation levels at destructive sampling of each trial are given in Table 2. Damage from each borer differed significantly among clones ($P < 0.05$) on all sampling occasions. Numbers of insects differed significantly among clones except for numbers of *S. grisescens* in the plant crop of SRT01-10, *S. excerptalis* in the first ratoon of SRT01-10, *R. obscurus* in the first ratoon of SRT01-11 and both *C. terrenellus* and *R. obscurus* in the plant crop of SRT01-13. Damage from *S. grisescens* was particularly heavy in the plant crop of SRT0111, with the most heavily infested clone, QN98-1947 (39.6% bored internodes), being almost destroyed (Figure 6). Damage from *S. excerptalis* was heavy in the plant crop of SRT01-13 despite spraying of lambda-cyhalothrin in the first half of 2013, although this may have had an effect earlier in the year before plots were sampled.

Extremely dry conditions at Ramu in 2014 together with a lack of irrigation meant that trial SRT01-14, which was planted in March 2014, was too small to be destructively sampled. However borer damage to the cane tops was evident late in 2014 so an assessment was made of damage by *S. grisescens* and *S. excerptalis*. Damage from *S. grisescens* was low (mean 1.6% dead tops, range 0.0-6.0%) and differences among varieties just failed to reach statistical significance ($P = 0.051$). Damage from *S. excerptalis* (mean 24.5% dead tops, range 10.9-40.8%) varied significantly among varieties ($P < 0.001$).

Table 2. Borer damage and numbers determined by destructive sampling of 20 stalks per plot in each trial

Data set	*S. grisescens*	*C. terrenellus*	*S. excerptalis*	*R. obscurus*
Damage (%); bored stalks for *S. excerptalis* and bored internodes for other species				
01-13 1R	11.2 (5.2–18.8)	7.9 (3.5–12.4)	30.3 (6.3–61.3)	1.0 (0.0–3.0)
01-13 P	6.6 (1.3–14.4)	10.9 (4.7–18.1)	30.9 (6.3–62.5)	1.9 (0.1–5.1)
01-11 1R	8.1 (2.2–16.5)	8.9 (6.1–12.8)	32.5 (8.8–51.3)	2.0 (0.3–5.8)
01-11 P	14.1 (2.9–39.6)	10.7 (4.6–18.0)	57.0 (17.5–92.5)	2.5 (0.1–6.3)
01-10 1R	6.8 (4.2–11.0)	9.8 (5.0–15.7)	14.5 (2.5–28.7)	5.5 (1.1–16.1)
01-10 P	8.8 (2.6–18.6)	8.5 (2.9–12.3)	33.1 (6.3–71.3)	3.4 (0.4–8.1)
Insects/20 stalks				
01-13 1R	6.6 (1.3-15.0)	3.4 (0.5-7.3)	5.1 (0.8-11.3)	0.8 (0.0-3.0)
01-13 P	2.7 (0.0-11.8)	6.7 (2.0-12.0)	5.5 (0.8-11.8)	0.7 (0.0-2.8)
01-11 1R	6.6 (1.0-40.0)	7.0 (3.3-13.8)	2.9 (0.0-7.3)	1.6 (0.0-4.8)
01-11 P	8.5 (0.0-32.8)	10.9 (4.5-21.5)	8.0 (1.3-14.5)	2.7 (0.0-7.3)
01-10 1R	4.1 (0.8-10.0)	8.9 (3.0-14.5)	1.4 (0.3-2.8)	5.3 (1.3-19.8)
01-10 P	26.2 (3.3-68.8)	6.3 (1.0-15.0)	6.1 (1.0-13.0)	4.0 (0.3-9.0)

Figure 6: Plot of QN98-1947 in the plant crop of SRT01-11, with extreme damage from *S. grisescens* when sampled in June 2012.

Damage and numbers of each borer

Numbers of each borer species and levels of their respective damage were significantly correlated ($P < 0.05$) in almost all destructive samples (Table 3). Correlations were particularly strong for *S. excerptalis* and *R. obscurus*. Numbers of *S. grisescens* were extremely variable in the first ratoon of SRT01-11 because sampling was conducted when some larvae were still in their gregarious habit early in development, and this probably contributed to the poor correlation in this data set.

Table 3: Correlations (*r*) between average measurements of damage (bored stalks for *S.excerptalis* and bored internodes for other species) and numbers of the causal borer species for clones in each trial; all correlations except for *S. grisescens* in SRT01-11 first ratoon are statistically significant at the 5% level

Data set	***S. grisescens***	***C. terrenellus***	***S. excerptalis***	***R. obscurus***
01-13 1R	0.69	0.73	0.97	0.87
01-13 P	0.78	0.66	0.98	0.83
01-11 1R	0.27	0.59	0.87	0.89
01-11 P	0.89	0.87	0.90	0.90
01-10 1R	0.60	0.55	0.78	0.92
01-10 P	0.64	0.69	0.99	0.94

Borer damage and number of internodes per stalk

Damage from *S. grisescens* tended to be negatively correlated with the average number of internodes present on stalks of each clone (Table 4). Trends were not clear or consistent for the other borers.

Table 4: Correlations (*r*) between average numbers of internodes on stalks of each clone and measurements of damage (bored stalks for *S. excerptalis* and bored internodes for other species); values marked with an asterisk are statistically significant at the 5% level

Data set	***S. grisescens***	***C. terrenellus***	***S. excerptalis***	***R. obscurus***
01-13 1R	-0.11	0.06	0.03	0.05
01-13 P	-0.25	0.07	0.36 *	-0.02
01-11 1R	-0.30	0.25	-0.21	-0.38 *
01-11 P	-0.30	0.45 *	-0.10	0.00
01-10 1R	-0.16	0.18	-0.36 *	-0.14
01-10 P	-0.35 *	0.08	0.03	-0.07

Internode damage and damaged stalks

The average proportion of internodes damaged by each borer on each clone was highly correlated with the proportion of stalks damaged (Table 5). The relatively poor correlation for *C. terrenellus* in the first ratoon of SRT01-11 corresponds to a small range of damage levels among clones in that trial (Table 2). For *S. grisescens*, the proportion of internodes damaged was also correlated with the average of the proportion of dead hearts for each variety, a characteristic that can be observed without destructive sampling, but the correlation was less strong.

Table 5: Correlations between the averages for each clone of the number of stalks damaged by each borer species and the proportion of damaged internodes

Data set	***S. grisescens*** **Dead hearts**	***S. grisescens***	***C. terrenellus***	***S. excerptalis***	***R. obscurus***
01-13 1R	0.82	0.96	0.94	0.96	0.97
01-13 P	0.84	0.95	0.89	0.96	0.97
01-11 1R	0.80	0.91	0.68	0.94	0.94
01-11 P	0.88	0.91	0.97	0.94	0.96
01-10 1R	0.78	0.91	0.83	0.98	0.94
01-10 P	0.73	0.95	0.90	0.94	0.96

Correlations among borers

Levels of damage were significantly correlated between species of borers in some trials (Table 6). Internode damage from *S. grisescens* tended to be negatively correlated with damage from *S. excerptalis*, though with a contrary result in SRT01-10 first ratoon, and dead hearts caused by the two species were negatively correlated in all but one trial. Damage from *S. grisescens* had no consistent correlation with damage from *C. terrenellus*. Damage from *C. terrenellus* and *S. excerptalis* was positively correlated in two trials but not in the other four; differences between trials could not be explained by overall levels of damage (Table 2). Damage from the weevil borer, *R. obscurus*, was positively and significantly correlated with damage from *S. grisescens* and positively but not significantly correlated with damage from *C. terrenellus*. Damage from *R. obscurus* was not correlated with damage from the top borer *S. excerptalis*.

Table 6: Correlations between measurements of borer damage (bored stalks for *Scirpophaga excerptalis* and bored internodes for other species except where indicated otherwise) to clones in each trial; *, P < 0.05

Trial	*S. gris./ C. terr.*	*S. gris./ S. exc.*	*S. gris.*[a]*/ S. exc.*	*C. terr. S. exc.*	*S. gris./ R. obs.*	*C. terr./ R. obs.*	*S. exc./ R. obs.*
01-13 1R	-0.20	-0.53 *	-0.65 *	0.50 *	0.28	0.28	0.09
01-13 P	0.53 *	-0.24	-0.30	-0.04	0.14	0.21	-0.06
01-11 1R	0.03	-0.14	-0.46 *	-0.04	0.64 *	0.23	-0.04
01-11 P	-0.32	-0.38 *	-0.57 *	0.43 *	0.45 *	0.15	0.03
01-10 1R	0.07	0.38 *	0.33	0.07	0.40 *	0.26	0.33
01-10 P	0.14	-0.11	-0.36 *	-0.08	0.36 *	0.22	0.17

[a] *S. grisescens* dead hearts

Correlations between numbers of each borer species in each trial mostly followed a similar pattern to correlations of their damage (Table 7 c.f. Table 6). In this analysis, numbers of the weevil borer, *R. obscurus,* were positively correlated with numbers of both of the stalk borers, *S. grisescens* and *C. terrenellus* (Table 7).

Table 7: Correlations between numbers of each borer species on clones in each trial; *, P < 0.05

Trial	*S. gris./ C. terr.*	*S. gris./ S. exc.*	*C. terr. S. exc.*	*S. gris./ R. obs.*	*C. terr./ R. obs.*	*S. exc./ R. obs.*
01-13 1R	-0.05	-0.34 *	0.28	0.39 *	0.42 *	-0.04
01-13 P	0.29	-0.36 *	0.03	0.01	0.40	-0.08
01-11 1R	0.30	-0.13	-0.25	0.29	0.29	-0.23
01-11 P	-0.31	-0.61 *	0.18	0.40 *	0.21	-0.21
01-10 1R	0.42 *	0.33	-0.04	0.32	0.42 *	0.34
01-10 P	0.16	-0.08	-0.18	0.02	0.27	0.09

Correlations among trials

Correlations of borer damage to the standard varieties among trials are given in Tables 8-11.

For *S. grisescens*, correlations among trial results were always positive, though not always statistically significant, for damage assessed as both proportion of dead hearts and proportion of bored internodes (P = 0.05) (Table 8). This comparison does not include the plant crop of SRT01-14 because of the low level of infestation and the different method of assessment.

Table 8: Correlations (*r*) between levels of *S. grisescens* damage to 13 standard clones among trials or crop years; *, P <0.05)

	01-13 1R	**01-13 P**	**01-11 1R**	**01-11 P**	**01-10 1R**
***S. grisescens* dead hearts**					
SRT01-13 P	0.71 *				
SRT01-11 1R	0.60 *	0.68 *			
SRT01-11 P	0.65 *	0.36	0.54		
SRT01-10 1R	0.55 *	0.67 *	0.79 *	0.61 *	
SRT01-10 P	0.33	0.71 *	0.63 *	0.24	0.45
***S. grisescens* internodes**					
SRT01-13 P	0.73 *				
SRT01-11 1R	0.63 *	0.46			
SRT01-11 P	0.66 *	0.25	0.78 *		
SRT01-10 1R	0.70 *	0.36	0.47	0.53	
SRT01-10 P	0.51	0.66 *	0.55 *	0.44	0.11

Damage from *C. terrenellus* to the 13 standard clones, as measured by the proportion of bored internodes, was poorly correlated among trials (Table 9). Only two correlations reached statistical significance at the 5% level.

Table 9: Correlations (*r*) between levels of *C. terrenellus* damage to 13 standard clones among trials or crop years, assessed as the proportion of bored internodes; *, $P < 0.05$

	01-13 1R	**01-13 P**	**01-11 1R**	**01-11 P**	**01-10 1R**
SRT01-13 P	0.11				
SRT01-11 1R	0.26	0.38			
SRT01-11 P	0.48	0.50	0.42		
SRT01-10 1R	0.04	0.29	0.71 *	0.32	
SRT01-10 P	0.14	0.70 *	0.11	0.45	0.30

Levels of *S. excerptalis* damage to the 13 standards mostly correlated well among trials (Table 10). *S. excerptalis* damage in SRT01-14 plant crop was judged by counting dead hearts rather than by destructive sampling. The crop was backward and variable when this assessment was done and, considering this, correlations with previous results are reasonable.

Table 10: Correlations (*r*) between levels of *S. excerptalis* damage to 13 standard clones among trials or crop years, assessed as the proportion of bored stalks; *, *P* < 0.05

	01-14 P	**01-13 1R**	**01-13 P**	**01-11 1R**	**01-11 P**	**01-10 1R**
SRT01-13 1R	0.49					
SRT01-13 P	0.86 *	0.77 *				
SRT01-11 1R	0.66 *	0.68 *	0.81 *			
SRT01-11 P	0.76 *	0.76 *	0.84 *	0.76 *		
SRT01-10 1R	0.48	0.59 *	0.51	0.40	0.47	
SRT01-10 P	0.77 *	0.64 *	0.78 *	0.67 *	0.89 *	0.60 *

For the weevil borer *R. obscurus*, correlations among trial results were always positive, though not always statistically significant (P = 0.05) (Table 11).

Table 11: Correlations (*r*) between levels of *R. obscurus* damage to 13 standard clones among trials or crop years, assessed as the proportion of bored internodes values; *, *P* <

0.05

	01-13 1R	**01-13 P**	**01-11 1R**	**01-11 P**	**01-10 1R**
SRT01-13 P	0.43				
SRT01-11 1R	0.46	0.30			
SRT01-11 P	0.40	0.40	0.44		
SRT01-10 1R	0.43	0.34	0.44	0.91 *	
SRT01-10 P	0.78 *	0.21	0.52	0.62 *	0.63 *

Standardised responses to *S. grisescens* showed a consistent difference in damage among 13 standard varieties (Figure 7). Three varieties selected in Papua New Guinea, PN92-339, PN92-439 and PN92-31, had the least damage, with other varieties exhibiting a range of responses increasing to the most susceptible variety, Q135.

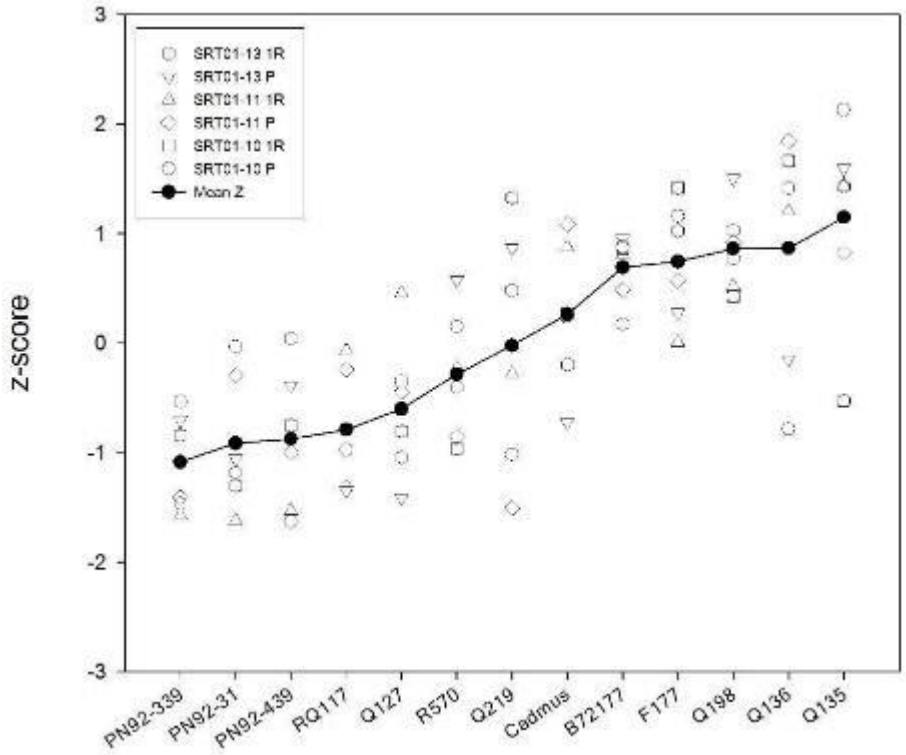


Figure 7: Standardised responses to *Sesamia grisescens* (proportion of bored internodes) of 13 standard varieties in six trial data sets, with varieties arranged in ascending order of mean z-scores.

For damage caused by *C. terrenellus*, the range of mean z-scores among the 13 standard varieties was similar to that recorded for *S. grisescens* (Figure 8 c.f. Figure 7) but there was considerable scatter of responses within varieties around the mean z-score for each.

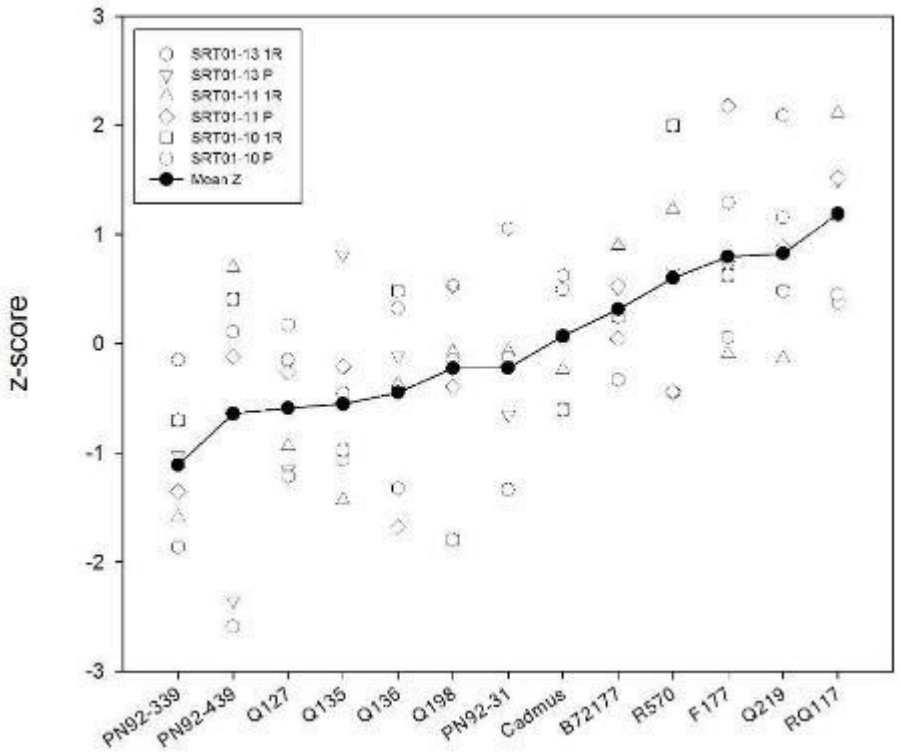


Figure 8: Standardised responses to *Chilo terrenellus* (proportion of bored internodes) of 13 standard varieties in six trial data sets, with varieties arranged in ascending order of mean z-scores.

For damage caused by *S. excerptalis* (Figure 9), the range of mean z-scores across varieties was greater than observed for *S. grisescens* and *C. terrenellus*, and with a tighter scatter around the means for each variety, indicating larger and more consistent responses than noted for the other two borers. The criterion of damage is also different: damaged stalks for *S. excerptalis* and damaged internodes for *S. grisescens* and *C. terrenellus*.

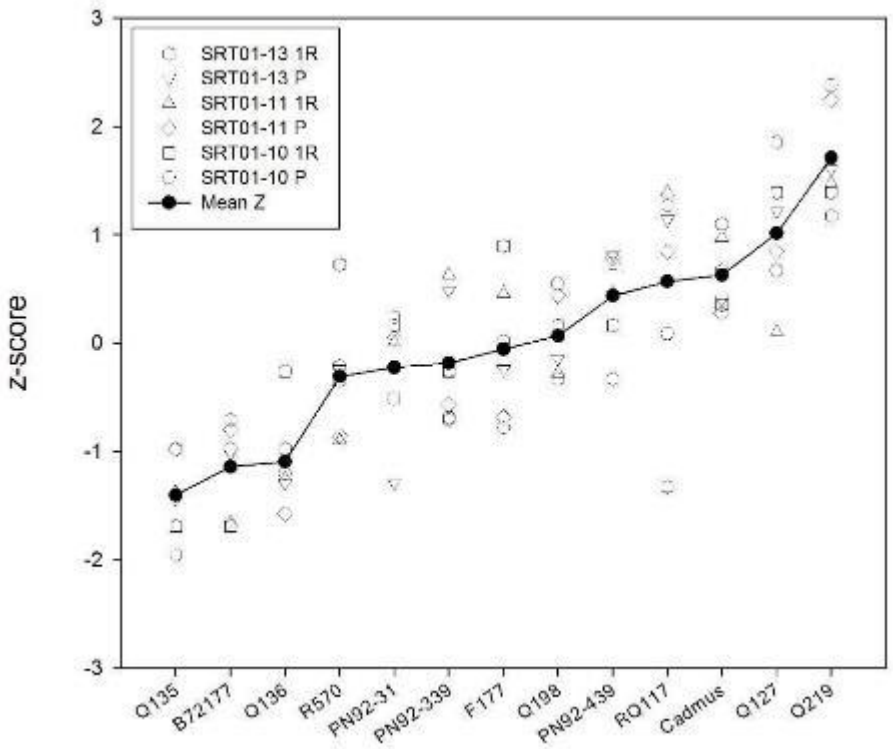


Figure 9: Standardised responses to *Scirpophaga excerptalis* (proportion of damaged stalks) of 13 standard varieties in six trial data sets, with varieties arranged in ascending order of mean z-scores.

For damage caused by *R. obscurus* (Figure 10), there was a considerable scatter of z-scores in each trial around mean z-scores for each standard variety. The relatively low level of damage from *R. obscurus* compared with damage from other borers (see Table 2) would have reduced the precision of damage estimates and contributed to the scatter in Figure 10, particularly for trial SRT01-13. The PNG-selected variety PN92-339 had a particularly low level of damage while Cadmus was the most susceptible.

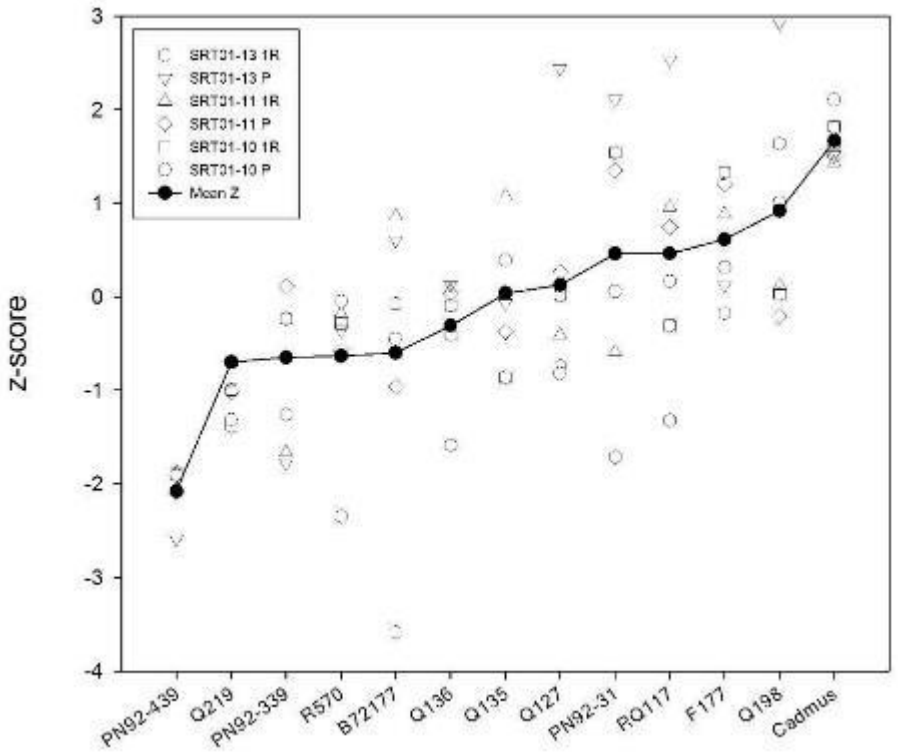


Figure 10: Standardised responses to *Rhabdoscelus obscurus* (proportion of bored internodes) of 13 standard varieties in six trial data sets, with varieties arranged in ascending order of mean z-scores.

Oviposition and borer damage

SRT01-10

In SRT01-10, *S. grisescens* egg batches were counted in the plant crop on 25 May 2011, i.e. week 21, soon after the third moth flight (see Figure 3). Numbers of stalks, egg batches and egg batches/100 stalks all differed significantly among clones (Table 12).

Table 12: Counts of stalks and *S. grisescens* egg batches in SRT01-10 in May 2011

Numbers/16 m	Mean	Clone range	$P_{variety}$
Stalks	263	168–387	<0.001
Egg batches	4.6	1.3–12.3	<0.001
Egg batches/100 stalks	1.8	0.6–3.6	0.004

Numbers of dead hearts caused by *S. grisescens* in SRT01-10 were counted on 7 June 2011, i.e. 2 weeks after the *S. grisescens* egg count. This should have allowed time for larvae hatching from eggs to kill the growing point on affected plants. We also counted dead hearts caused by the top borer *S. excerptalis*.

There were highly significant differences among clones for dead hearts caused by *S. grisescens* and *S. excerptalis* (Table 13). There was no correlation between numbers of stalks of each clone and the proportion of dead hearts caused by either pest, nor was there a correlation between the proportion of dead hearts caused by the two pests on the 32 clones ($P > 0.05$). There was a correlation between numbers of *S. grisescens* egg batches counted per stalk in May and proportion of stalks with dead hearts caused by *S. grisescens* in June ($r = 0.42$, $P = 0.017$, Figure 11).

Table 13: Counts of tops (dead hearts) killed by *S. grisescens* or *S. excerptalis* in SRT01-
10 in June 2011

% of stalks	Mean	Clone range	$P_{variety}$
S. grisescens 1° dead hearts	1.9	0.7–4.4	<0.001
S. grisescens 2° dead hearts	2.2	0.3–4.9	0.002
S. grisescens total dead hearts	4.1	1.0–7.8	<0.001
S. excerptalis dead hearts	15.7	3.2–41.7	<0.001

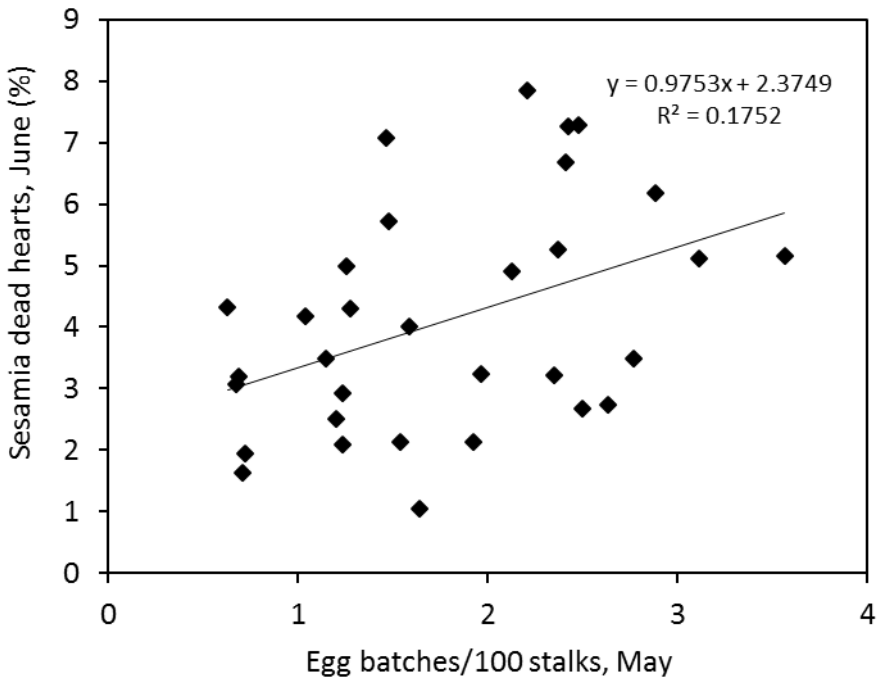


Figure 11: *S. grisescens* dead hearts on 7 June and egg batches on 25 May 2011 in SRT01-10, on 32 clones.

SRT01-11

In the plant crop of SRT01-11, moth borer egg batches were counted on the standard clones on 24 February 2012 (*S. grisescens*, *C. terrenellus* and *S. excerptalis*, 13 clones; week 8 of moth trapping in Fig. 4) and 21 April 2012 (*S. grisescens* only, 12

clones excluding Q219$^{\phi}$, week 16 of moth trapping in Figure 4). We decided to count only *S. grisescens* eggs in April so that searchers could concentrate on the stalks and leaf sheaths rather than also having to search the leaf blades where eggs of *C. terrenellus* and *S. excerptalis* are laid.

Numbers of *S. grisescens* eggs were very low in February but much higher in April. Numbers of stalks in February 2012 differed significantly among the 13 standard clones, but numbers of *S. grisescens* egg batches did not differ significantly on either occasion (Table 14). A different result had been obtained in the plant crop of SRT01-10 when egg batches were counted in May 2011 but in that trial we assessed all clones, not just the standards. We had decided to concentrate on the standards in SRT01-11 as this would give us an idea of the mechanism of resistance in a suite of clones of well-defined resistance ratings, while allowing more time to examine individual stalks. Numbers of *C. terrenellus* and *S. excerptalis* egg batches in February also did not differ significantly among clones (Table 14).

Table 14: Counts of stalks and moth borer egg batches in SRT01-11 in February and April 2012 on standard clones

Count per 2 rows	**Mean**	**Clone range**	$P_{variety}$
Stalks (Feb)	143	117-187	<0.001
S. grisescens			
Egg batches (Feb)	0.3	0.0-1.3	0.51
Egg batches/100 stalks	0.2	0.0-1.0	0.80
Egg batches (Apr)	10.2	3.5-20.3	0.06
Egg batches/100 stalks	7.2	2.5-11.1	0.12
C. terrenellus			
Egg batches (Feb)	1.83	0.5-3.0	0.11
Egg batches/100 stalks	1.3	0.3-2.3	0.08
S. excerptalis			
Egg batches (Feb)	4.6	0.8-8.3	0.76
Egg batches/100 stalks	3.5	0.4-6.3	0.96

Numbers of dead hearts in the plant crop of SRT01-11 were counted on 15 May 2012. There were highly significant differences among clones for dead hearts caused by *S. grisescens* and *S. excerptalis* (Table 15). Although there was no correlation between the proportion of dead hearts caused by the two pests on the 12 standard clones (r = -0.30, $P > 0.05$), there was a significant negative correlation when all 33 clones were considered ($r = -0.36$, $P = 0.037$). There was a correlation between numbers of *S. grisescens* egg batches counted per stalk in April and proportion of stalks with dead hearts caused by *S. grisescens* in May, on the 12 standard clones ($r = 0.65$, $P = 0.022$, Figure 12). Numbers of egg batches of *S. excerptalis* per stalk counted in February on 13 standard clones were similarly correlated with the proportion of dead hearts caused by this pest in May ($r = 0.66$, $P = 0.014$, Figure 13).

Table 15: Counts of tops (dead hearts) killed by *S. grisescens* or *S. excerptalis* in SRT01-

11 on 15 May 2012

% of stalks	Mean	Clone range	$P_{variety}$
S. grisescens	21.9	4.8-48.4	<0.001
S. excerptalis	26.0	7.6-49.9	<0.001

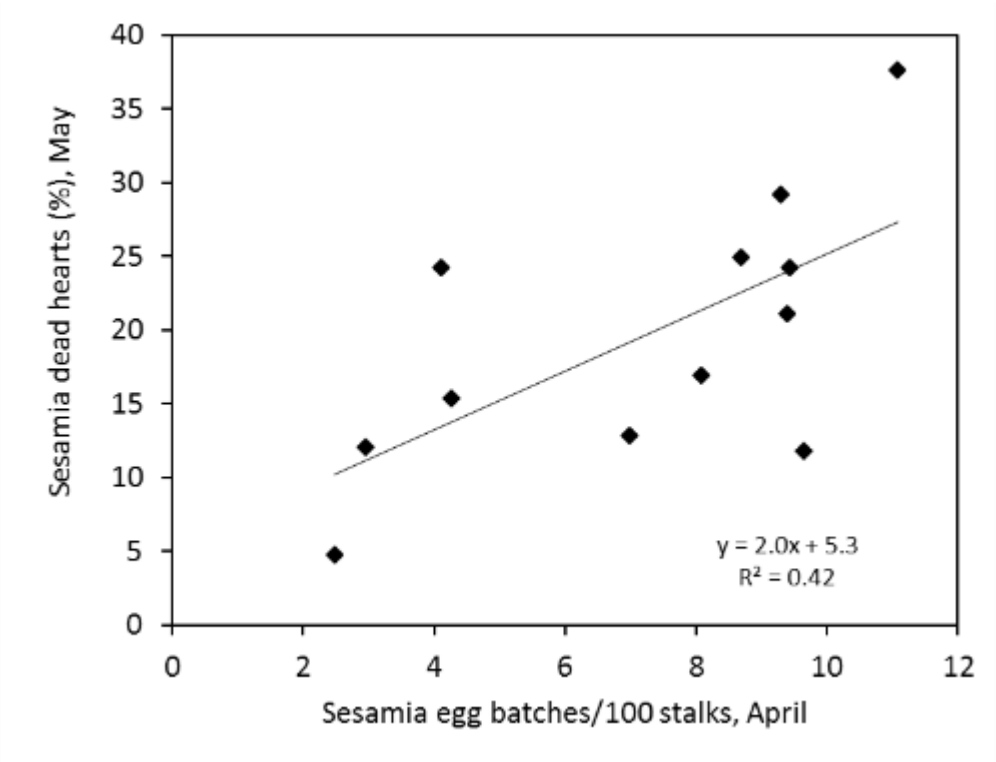


Figure 12: *S. grisescens* dead hearts on 15 May and egg batches on 21 April 2012 in SRT01-11, on 12 standard clones.

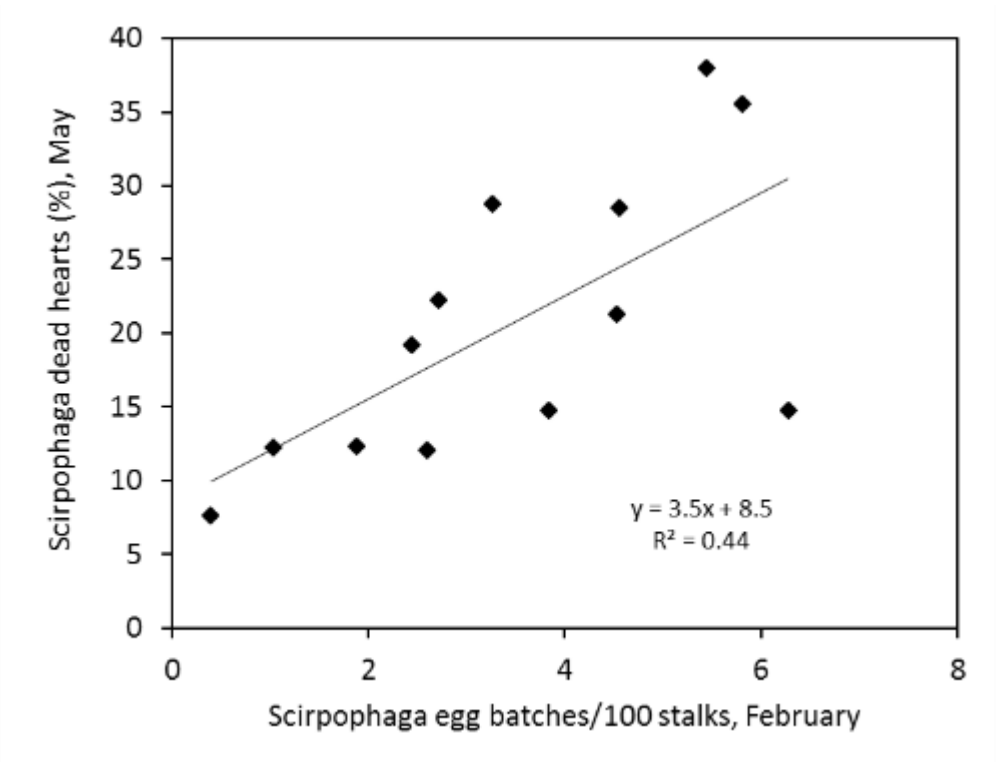


Figure 13: *S. excerptalis* dead hearts on 15 May and egg batches on 24 February 2012 in SRT01-11, on 13 standard clones.

In the first ratoon of SRT01-11, numbers of *S. grisescens* egg batches were counted on the standard varieties on 23 May 2013 (week 21 of moth trapping in Fig. 5, just after a large moth flight). Numbers of *S. grisescens* eggs differed significantly among varieties ($P < 0.001$), with fewest eggs on two varieties selected in PNG, PN92-31 and PN92-339. Unfortunately the total number of stalks of each variety was not counted.

For 13 standard varieties, *S. grisescens* damage as assessed as either dead hearts or bored internodes by destructive sampling in July-August 2013 was significantly correlated with numbers of egg batches counted on these varieties in May (Figure 14).

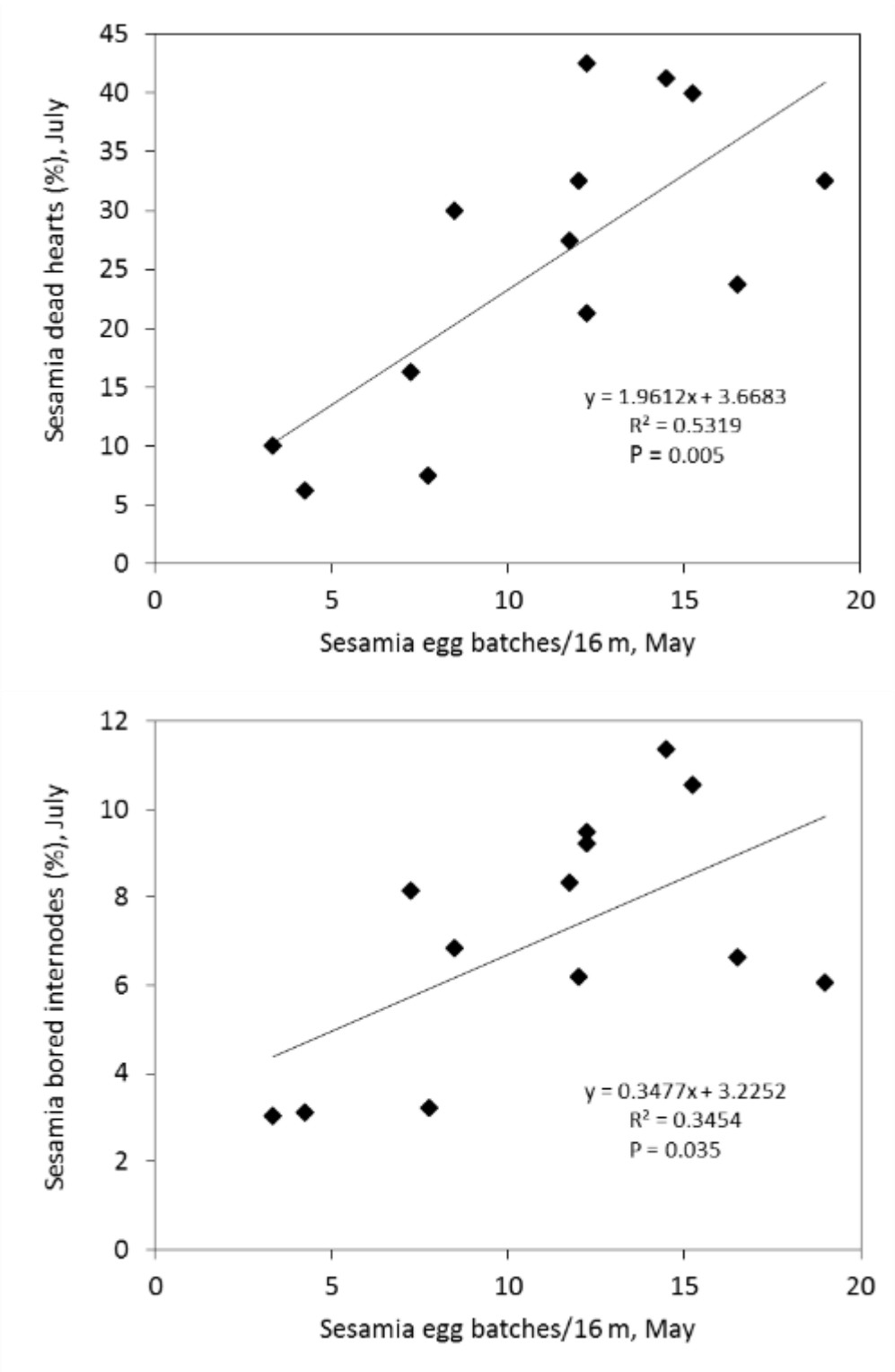


Figure 14: Relationships between damage from *S. grisescens* to 13 standard varieties, assessed as either dead hearts (top) or bored internodes (bottom) in July-August, and numbers of egg batches counted previously in May.

Crop yields

Mean sugar content (commercial cane sugar, CCS) differed significantly among clones in all harvested trials, while yield of cane and sugar differed significantly among clones in all harvested trials except SRT01-13 first ratoon. The plant crop of SRT01-13 was not commercially harvested but, instead, was cut back to allow ratooning. Juice analysis in the plant crop of SRT01-11 was not done because of the extremely poor quality of the cane after serious damage from moth borers and woolly aphids. Yields will obviously affected not just by borer damage but also by the intrinsic yield potential of the clones as well as extrinsic factors; e.g. heavy infection from downy mildew was noted in Q182$^{\Phi}$ which was the lowest yielding clone in SRT01-10.

Correlations between mean yield of each clone and prior measurements of borer damage are given in Table 16. Usually yield was negatively associated with prior damage from all borers, though correlations were mostly not statistically significant (P = 0.05). Negative correlations were mostly weaker for *C. terrenellus* than for the other two moth borers. The positive correlation between sugar content and damage from *S. excerptalis* in the first ratoon of SRT01-13 is surprising and unexplained, but cessation of stalk elongation caused by death of the apical meristem might encourage accumulation of sucrose in the stalk.

Table 16: Correlations (*r*) between crop yield parameters and measurements of borer damage recorded earlier in the year; values marked with an asterisk are statistically significant at the 5% level

Data set	Mean	*S. grisescens*	*C. terrenellus*	*S. excerptalis*	*R. obscurus*
Cane					
01-13 1R	41.2 t/ha	0.13	-0.11	-0.26	0.29
01-11 1R	47.0 t/ha	0.16	-0.03	-0.39 *	-0.20
01-11 P	12.1 t/ha	-0.36 *	-0.34 *	-0.30	-0.32
01-10 1R	71.5 t/ha	-0.10	0.05	0.03	-0.17
01-10 P	53.4 t/ha	-0.47 *	-0.23	-0.15	-0.29
CCS					
01-13 1R	12.0	-0.49 *	0.20	0.65 *	-0.06
01-11 1R	16.8	-0.09	-0.05	-0.29	0.11
01-10 1R	13.9	-0.20	-0.14	-0.20	-0.21
01-10 P	12.4	-0.47 *	-0.12	-0.15	-0.30
Sugar					
01-13 1R	4.9 t/ha	-0.11	-0.01	0.03	0.28
01-11 1R	7.9 t/ha	0.12	-0.05	-0.47 *	-0.18
01-10 1R	10.0 t/ha	-0.16	-0.04	-0.07	-0.19
01-10 P	6.7 t/ha	-0.52 *	-0.22	-0.19	-0.34

Susceptibility ratings for test clones

Analyses were first conducted for the 13 standard varieties across the six data sets generated in the current project plus three data sets from previous Ramu A-I trials.

There were significant differences in damage among the standards for all four borer species (Tables 17-20), with a continuum of responses across the range (see also Figs. 7-10 earlier, but note these show only results from the current trials and use a different transformation). Certain standard varieties were chosen from the suite of 13 to represent typical levels of susceptibility to each borer: susceptible, intermediate and resistant (S, I and R in Tables 17-20). The ratings of *S. grisescens* susceptibility for the chosen varieties are in close agreement with ratings provided by Ramu staff (see Materials and methods).

Table 17: Standard varieties ranked in descending order of damage from *S. grisescens* (bored internodes); varieties followed by the same letter are not significantly different by lsd test (*P* = 0.10). Letters of S, I and R signify varieties chosen as susceptible, intermediate and resistant for later comparison with test varieties

Variety	Estimate	Standard Error	Back-transformed %	Letter Group
Q135 :S	-1.6449	0.1835	16.2	A
Q198[A] :S	-1.7915	0.1862	14.3	AB
Q136 :S	-1.8299	0.1856	13.8	AB
F177	-1.8627	0.1862	13.4	AB
B72177 :I	-2.018	0.1875	11.7	BC
Cadmus	-2.0218	0.187	11.7	BC
Q219[A]	-2.2221	0.2032	9.8	CD
R570 :I	-2.2414	0.1899	9.6	CD
Q127	-2.2934	0.1921	9.2	D
RQ117 :R	-2.4246	0.1922	8.1	DE
PN92-31 :R	-2.4976	0.1952	7.6	DE
PN92-439 :R	-2.6429	0.199	6.6	E
PN92-339 :R	-2.663	0.1976	6.5	E

Table 18: Standard varieties ranked in descending order of damage from *C.terrenellus* (bored internodes); varieties followed by the same letter are not significantly different by lsd test (*P* = 0.10). Letters of S, I and R signify varieties chosen as susceptible, intermediate and resistant for later comparison with test varieties

Variety	Estimate	Standard Error	Back-transformed %	Letter Group
F177 :S	-1.9273	0.1497	12.7	A
Q219[A] :S	-1.9423	0.1568	12.5	AB
RQ117 S	-1.9629	0.1474	12.3	AB

R570	-2.0831	0.1508	11.1	ABC
Q198[A]	-2.1516	0.1535	10.4	BCD
B72177 :I	-2.1581	0.152	10.4	BCD
Cadmus :I	-2.1818	0.1511	10.1	CD
PN92-31 :I	-2.2532	0.1533	9.5	CD
Q135	-2.2919	0.1544	9.2	CDE
PN92-439	-2.2973	0.1534	9.1	DE
Q136	-2.3139	0.1533	9.0	DE
Q127	-2.3376	0.1533	8.8	DE
PN92-339: R	-2.5067	0.1564	7.5	E

Table 19: Standard varieties ranked in descending order of damage from *S. excerptalis* (bored stalks); varieties followed by the same letter are not significantly different by lsd test (P = 0.10). Letters of S, I and R signify varieties chosen as susceptible, intermediate and resistant for later comparison with test varieties

Variety	**Estimate**	**Standard Error**	**Back-transformed %**	**Letter Group**
Q219[A] :S	-0.4697	0.4675	38.5	A
Q127 :S	-1.1006	0.4582	25.0	B
Cadmus	-1.2622	0.4594	22.1	BC
RQ117 :I	-1.2827	0.4618	21.7	BCD
PN92-439	-1.4123	0.4624	19.6	BCDE
Q198[A]	-1.6433	0.4628	16.2	CDEF
PN92-31	-1.6998	0.4640	15.4	DEF
F177	-1.8244	0.4621	13.9	EF
PN92-339	-1.8839	0.4634	13.2	FG
R570 :I	-1.9742	0.4635	12.2	FG
B72177 :R	-2.3350	0.4712	8.8	GH
Q136 :R	-2.5487	0.4735	7.3	HI
Q135 :R	-2.9237	0.4788	5.1	I

Table 20: Standard varieties ranked in descending order of damage from *R. obscurus* (bored internodes); varieties followed by the same letter are not significantly different by lsd test (P = 0.10). Letters of S, I and R signify varieties chosen as susceptible, intermediate and resistant for later comparison with test varieties

Variety	Estimate	Standard Error	Back-transformed %	Letter Group
Cadmus :S	-2.9844	0.2807	4.8	A
Q198	-3.3180	0.2858	3.5	B
Q135	-3.3332	0.2869	3.4	B
F177	-3.3671	0.2853	3.3	B
PN92-31	-3.4049	0.2895	3.2	B
RQ117 :I	-3.4707	0.2855	3.0	BC
Q127	-3.5830	0.2890	2.7	BC
Q136	-3.6155	0.2887	2.6	BCD
B72177 :I	-3.6725	0.2952	2.5	BCD
R570	-3.8039	0.2993	2.2	CD
PN92-339	-3.8253	0.2950	2.1	CD
Q219[A] :R	-4.0409	0.3238	1.7	DE
PN92-439 :R	-4.3196	0.3210	1.3	E

Cut-off levels of damage to define susceptible, intermediate and resistant categories of test canes were then calculated by, first, calculating mean levels of damage (logit-transformed) to each of the susceptible, intermediate and resistant standards for each borer, and then calculating the means of those means; these cut-offs are drawn as heavy lines in Table 21. For each borer, varieties above the first heavy line can be classed as susceptible, those below the second heavy line as resistant, and those between the two lines as having intermediate susceptibility.

Table 21: Varieties ranked in descending order of damage from four borer species. Letters of S, I and R signify standard varieties chosen as susceptible, intermediate and resistant, and heavy lines are drawn to delineate the susceptible, intermediate and resistant categories, for each species

Sesamia bored internodes		Chilo-bored internodes		Scirpophaga-bored stalks		Weevil bored internodes	
Variety	%	Variety	%	Variety	%	Variety	%
QN98-1947	25.2	Q151	16.4	Q209	43.6	QC02-275	7.9
QN99-770	20.0	QC02-275	15.4	Q235	42.2	Q234	6.5
QC01-6058	19.8	Q171	15.3	QC02-929	41.3	Q213	6.3
Q234	17.7	Q230	14.6	Q219^{A} :S	37.8	QN98-1947	6.2
Q182	17.7	QC98-2151	14.6	Q221	33.9	Q107	6.1
MQ239	16.9	Q243	13.8	Q190	32.5	QN99-770	5.8
QN97-2122	16.2	Q246	13.8	Q243	32.4	Q151	5.6
Q135 :S	16.1	Q241	13.7	QC98-2151	32.1	QN97-2122	5.0
QC02-1896	15.5	QC02-6656	13.7	Q182	31.9	Cadmus	4.8
Q213	15.2	Q220	13.5	MQ239	31.7	QC98-4103	4.7
Q208	15.0	Q234	13.1	Q107	31.4	Q230	4.7
Q151	14.8	QC02-1896	12.6	QC98-4103	28.9	Q177	4.4
Q124	14.7	Q177	12.6	Q215	27.6	QC02-6656	4.3
QN97-1972	14.4	F177 :S	12.5	KQ236	27.5	Q246	4.1
Q230	14.3	QC94-1771	12.4	QC02-6007	27.5	QN97-1972	4.1
Q198 :S	14.3	KQ228	12.4	QC02-6656	27.4	QC98-2151	4.0
Q246	14.3	Q219^{A} :S	12.3	QN97-2328	27.1	Q190	4.0
Q188	13.9	RQ117 :S	12.2	QN99-770	27.1	Q124	3.9
Q136 :S	13.7	MQ239	11.9	Q203	27.0	Q182	3.8
QC02-275	13.7	Q87	11.5	Q241	27.0	QC02-6007	3.7
Q231	13.6	QC98-4103	11.5	Q124	26.3	Q155	3.5
F177	13.6	Q231	11.3	KQ228	25.8	Q135	3.5
Q171	12.7	QC01-6058	11.3	QC01-6058	25.6	Q198	3.5
QC94-1771	12.5	KQ236	11.3	Q246	24.9	QC01-6058	3.5
Q209	12.1	Q183	11.2	Q127 :S	24.8	Q188	3.4

Q203	11.9	R570	11.2	QC94-1771	24.5	QN98-1064	3.3
Cadmus	11.7	QN97-2122	11.2	Q200	23.9	F177	3.3
B72177 :I	11.7	Q235	11.2	Q138	23.0	PN92-31	3.2
Q190	11.6	Q107	11.1	QN99-2265	22.9	Q220	3.2
QN98-1064	11.4	QS01-1078	10.9	Q231	22.9	Q183	3.1
QC98-4103	11.1	QN97-123	10.9	Q234	22.8	Q221	3.1
Q107	10.9	QN99-2265	10.9	Q183	22.6	RQ117	3.0
QC02-6007	10.9	Q113	10.8	RQ117: I	22.2	QC94-1771	3.0
Q177	10.8	Q242	10.8	Cadmus	21.9	Q215	2.9
Q221	10.1	Q155	10.8	Q151	21.7	QC02-929	2.9
KQ236	10.1	Q224	10.6	Q155	21.6	Q231	2.8
Q155	9.8	Q213	10.5	Q230	21.6	Q113	2.8
Q87	9.8	Q188	10.5	QS01-1078	21.5	Q243	2.8
Q215	9.8	Q138	10.5	Q213	21.4	Q127	2.8
QN98-175	9.6	B72177 :I	10.5	QN97-633	20.7	Q136	2.6
R570 :I	9.6	Q198	10.4	QN98-175	20.2	Q87	2.6
Q219	9.5	QC02-929	10.2	Q220	20.1	QC02-1896	2.5
Q113	9.5	Q221	10.2	PN92-439	19.9	B72177	2.5
Q242	9.4	QN98-1947	10.2	Q171	19.6	Q203	2.4
QN99-2265	9.3	QN98-1064	10.0	Q224	19.3	Q209	2.3
Q127	9.3	Cadmus :I	10.0	Q177	18.7	Q235	2.3
Q243	8.9	Q124	9.8	QN97-1972	17.7	R570	2.2
Q183	8.8	Q208	9.6	Q87	17.5	PN92-339	2.1
Q138	8.5	QC02-6007	9.5	QN97-2122	17.2	QN97-633	2.1
Q224	8.3	QN98-175	9.4	QN98-1064	16.6	Q138	2.1
Q220	8.3	QN97-2328	9.4	Q113	16.4	Q224	2.1
RQ117 :R	8.2	Q209	9.4	Q198	16.3	Q208	1.9
QC02-6656	8.1	QN99-770	9.3	PN92-31	15.6	QN97-2328	1.9
QN97-2328	7.9	PN92-31 :I	9.3	Q208	15.2	QN98-175	1.9
PN92-31 :R	7.7	Q135	9.2	QC02-275	14.7	MQ239	1.9
QC98-2151	7.7	Q136	9.2	Q188	14.6	Q200	1.8
KQ228	7.6	Q190	9.2	F177	14.1	Q219	1.8
QN97-633	7.3	QN97-633	9.0	PN92-339	13.3	Q241	1.5
QN97-123	7.3	PN92-439	8.9	QC02-1896	12.9	KQ228	1.5

Q241	6.7	Q127	8.8	Q242	12.2	QS01-1078	1.4
QC02-929	6.7	Q215	8.6	R570 :I	12.1	QN97-123	1.4
PN92-339 :R	6.5	QN97-1972	8.5	QN98-1947	11.0	PN92-439	1.3
PN92-439 :R	6.5	Q200	8.4	QN97-123	9.5	QN99-2265	1.2
QS01-1078	6.5	Q182	8.1	B72177 :R	8.7	Q171	1.2
Q200	6.3	PN92-339 :R	7.5	Q136 :R	7.6	Q242	0.9
Q235	6.1	Q203	7.2	Q135 :R	5.2	KQ236	0.8

Discussion

Damage levels differed among clones for all four borers in all trials. Kuniata (2000) has previously noted differences in susceptibility to *S. grisescens* among varieties. Varietal resistance has not been well-studied for *C. terrenellus* but has been documented for many other species of *Chilo* such as *C. auricilius* (Sharma *et al.*, 2007) and *C. sacchariphagus* (David and Joseph, 1984; Nibouche and Tibère, 2008). Varietal resistance to *S. excerptalis* has been identified in studies elsewhere (e.g. Chaudhary and Yadav, 1995). Varietal resistance to weevil borers has been measured previously in Australia (Robertson and Webster, 1995; Berding, 1996).

Numbers of each borer also differed among clones in most trials. Damage is an historic record of prior infestation whereas borer presence is transient and borer numbers can also be highly variable (especially for *S. grisescens* which is gregarious in early instars), so differences among clones are more likely to be detected by damage measurements. Damage and insect numbers of the respective borers at sampling were significantly correlated in most data sets, giving confidence that damage was being correctly ascribed to the causal borer species.

Damage from *S. grisescens* tended to be negatively correlated with the average number of internodes on stalks of each clone. This was also seen in two of three data sets from two previous trials at PNG (Korowi *et al.*, 2011) One explanation is that some of the infested stalks would have had dead hearts and so would have stopped elongating. However, a similar negative relationship might have been expected for *S. excerptalis*, which invariably causes dead hearts, but this was not consistently observed. Alternatively, clones with shorter stalks may be more susceptible to infestation by *S. grisescens*. More detailed observations over time would be needed to elucidate the relationship, if any, between stalk length of clones and likelihood of borer infestation.

The average proportion of internodes damaged by each borer on each clone was highly correlated with the proportion of stalks damaged. Categorisation of stalks as bored or unbored could provide a cost-effective method of rating clones. This is easy for *S. excerptalis* but not for the other species. For *S. grisescens*, *C. terrenellus* and *R. obscurus*, stalks would need to be sliced open to detect damage but there would be an option to cease examination once damage was detected and the stalk

could be categorized, with no need to count the damaged internodes. Alternatively, for *S. grisescens*, internode damage was correlated with the proportion of dead hearts and the latter could be used as an indicator of damage to each variety.

Levels of damage were correlated, positively or negatively, between species of borers in some trials. These correlations between species could reflect similarities or differences between resistance profiles of clones against different species, or could be due to direct or indirect interactions among species.
The weevil borer *R. obscurus* preferentially attacks cane that has already been damaged from other causes (Chang *et al.*, 1970) and stalks damaged by *S. grisescens* in PNG are known to be more prone to damage by *R. obscurus* (Kuniata and Sweet, 1994; Kuniata, 1998). Damage from *R. obscurus* was positively correlated with damage from *S. grisescens* and *C. terrenellus* in the present study (though not always with statistical significance), and also in previous resistance trials in PNG (Korowi *et al.*, 2011). Damage from *R. obscurus* was not correlated with damage from *S. excerptalis*, presumably because this borer only damages the meristem and top of the stalk and not the lower stalk which *R. obscurus* infests.

Internode damage from *S. grisescens* tended to be negatively correlated with damage from *S. excerptalis*, though with a contrary result in one data set, and dead hearts caused by the two species were negatively correlated in all but one trial. A similar negative correlation between damage from the two species was detected in an earlier trial in PNG (Korowi *et al.*, 2011). It is possible that stalks with previous damage from *S. excerptalis* are less attractive to moths of *S. grisescens* for oviposition because of the change in stalk architecture once the meristem has been killed. In an attempt to reduce damage from *S. excerptalis* early in crop growth, the plant crop of trial SRT01-13 was sprayed with lambdacyhalothrin during the first half of 2013 but this did little to reduce damage from *S. excerptalis* recorded in August. More residual insecticides could have been used but we did not want to interfere with later infestation by *S. grisescens*.

These correlations, if due to inter-species interactions, have the potential to confuse the apparent patterns of varietal susceptibility to particular borer species. However, infestations by multiple species are inevitable in field trials in PNG. Pot trials of plants artificially infested with borers would be one method to isolate varietal responses to individual borer species.

The lack of consistent positive correlations of damage among moth borers to the different clones, and the significant negative correlations in some cases, indicates that clones that appeared to be resistant to one borer species weren't necessarily resistant to the others. For example, PN92-439, which was relatively resistant to *S. grisescens* in the set of standard varieties, was quite heavily attacked by *S. excerptalis*. Q135, which was particularly susceptible to *S. grisescens* (see also Korowi *et al.*, 2011), was the least susceptible among the standard varieties to *S. excerptalis*.

There were strong positive correlations among trials for measured levels of damage to the standard varieties from *S. grisescens*, *S. excerptalis* and *R. obscurus*. Correlations were less consistently positive for *C. terrenellus*. Standardised plots of damage also showed that results were more variable among trials for *C. terrenellus*.

The strongest and most consistent difference among the standard varieties was measured for damage from *S. excerptalis*. The rank order of standardised plot of damage from *S. grisescens* was similar to the order proposed by staff of Ramu A-I (see Materials and methods), with four varieties selected in PNG, PN92-339, PN92-439, PN92-31 and RQ117, having the least damage in trials. Cadmus, which is believed to be the most susceptible variety and which suffered extreme damage during an outbreak at Ramu A-I in 1987 (Kuniata and Sweet, 1994), was not the most susceptible variety in our trials. However, it was the variety most damaged by the weevil borer *R. obscurus*, and the secondary attack by *R. obscurus* would have contributed to the losses from *S. grisescens* seen in commercial cultivation (see also Kuniata and Sweet, 1994).

Numbers of egg batches of *S. grisescens* differed significantly among clones in two of three trial/year combinations, where they were counted on either the full set of clones in the trial or the standards only, and there was a significant positive correlation between the number of egg batches and subsequent damage in all three. There was also a significant positive correlation between the number of egg batches of *S. excerptalis* and subsequent damage from that species in the one trial/year where data were available. This suggest that differences in numbers of eggs laid by moths on different clones (antixenosis) could be at least partly responsible for observed varietal differences in susceptibility to these two borers. Factors affecting susceptibility of sugarcane to damage from *S. grisescens* have not previously been studied. Kuniata (2000) noted that all varieties are attacked when young but, in older cane, varieties showing some resistance produced a constriction in the internode below the bored internode, preventing rot from extending down the stalk. This was not assessed in our trials (we attempted to assess in the first field trial, but the subjective nature of the assessment and the variation among the assessment team indicated that the results would be meaningless). Resistance of genotypes to *S. excerptalis* has previously been correlated with resistance to establishment of young larvae in the stalk (e.g. Chaudhary and Yadav, 1995; Mukunthan and Mohanasundaram, 1998), but not apparently with antixenosis. More detailed measurements would be needed to define mechanisms of resistance to the moth borers assessed in this project.

Crop yields differed significantly among varieties but, without borer-free controls, it is not possible to apportion this to differences in borer susceptibility. Usually yield was negatively associated with prior damage from all borers, with no particular species being the dominant factor.

Commercial Q-varieties exhibited a range of susceptibilities to each borer species, some being heavily attacked and some appearing relatively resistant. For most borers, resistance levels varied more or less continuously from the most susceptible to the most resistant variety, with few obvious steps. Therefore, delineating varieties as 'susceptible', 'intermediate' and 'resistant' is rather artificial. An attempt has been made to do that, but it is probably more useful to maintain the data set as a continuum of the original varietal responses. In the event of an incursion, results in this database could be combined with other known varietal performance traits to determine which varieties should be incorporated in an integrated pest management plan. In most cases, our results for the test canes come from only a single trial, though with data from both the plant crop and the first ratoon. It would be advisable to confirm

resistance levels of the better-performing varieties (in terms of both current commercial performance in Australia and resistance as determined in this project) in further trials in PNG, as well as testing new Australian varieties when a sufficiently large set becomes available.

Outputs / outcomes

- Field trials demonstrated significant differences among clones for damage from all borer species – three moth borers and the weevil borer *Rhabdoscelus obscurus*. Numbers of egg batches of *Sesamia grisescens* and *Scirpophaga excerptalis* were correlated with subsequent damage from each species, suggesting a role for antixenosis (ovipositional non-preference) as a resistance mechanism. No data were collected for *Chilo terrenellus*.
- Among the 13 standard clones included in four field trials, reasonably consistent responses were measured for *S. grisescens* and *S. excerptalis* but responses were more variable for C. *terrenellus*. The order of susceptibility varied among borer species.
- Ratings for susceptibility of test varieties to the different borers are now available to the industry.

References

Berding N (1996) Sugarcane weevil borer resistance: breeding strategy development using survey data. *Proceedings of the Australian Society of Sugar Cane Technologists* **18**, 9099.

Chang VCS, Tanimoto V, Ota AK (1970) Clonal resistance of sugarcane to the New Guinea sugarcane weevil (Coleoptera: Curculionidae) in Hawaii. *The Hawaiian Planters' Record* **58**, 103-118.

Chaudhary JP, Yadav SR (1995) Screening of promising sugarcane genotypes against top borer *Scirpophaga excerptalis* Walker (Lepidoptera: Pyralidae) under semi-natural conditions – II. *Indian Sugar* **45**, 173-182.

David H, Joseph KJ (1984) Mechanism of resistance in sugarcane against the internode borer,
Chilo sacchariphagus indicus (Kapur). *Proceedings of the Sugar Technologists' Association of India* **48**, 43-58.

Korowi KT, Samson PR, Kuniata LS (2011) Screening for borer resistance among sugarcane varieties in Papua New Guinea, 2003-2008. *Proceedings of the Australian Society of Sugar Cane Technologists* **33** (electronic format) 9 pp.

Kuniata LS (1998) Borer damage and estimation of losses caused by *Sesamia grisescens* (Lepidoptera: Noctuidae) in sugarcane in Papua New Guinea. *International Journal of Pest Management* **44**, 93-98.

Kuniata LS (2000) Integrated management of sugarcane stemborers in Papua New Guinea. In 'Sugarcane pest management strategies in the new millenium.' (Eds PG Allsopp, W Suasa-ard) pp. 37-50. (International Society of Sugar Cane Technologists: Brisbane).

Kuniata LS, Chandler KJ, Korowi KT (2001) Management of sugarcane pests at Ramu, Papua New Guinea. *Proceedings of the International Society of Sugar Cane Technologists* **24**, 382-388.

Kuniata LS, Sweet CPM (1994) Management of *Sesamia grisescens* Walker (Lep.: Noctuidae), a sugar-cane borer in Papua New Guinea. *Crop Protection* **13**, 488-493.

Mukunthan N. 1985. New aspects in the biology of sugarcane top borer, *Scirpophaga* (*Nivella* F.) *excerptalis* Wlk. Entomon 10, 235-238.

Mukunthan N and Mohanasundaram M. 1998. Failures of attack by the top borer, *Scirpophaga excerptalis* Wlk. in relation to resistance in sugarcane genotypes. *Insect Sci. Applic*. 18, 293-300.

Nibouche S, Tibère R (2008) Damage assessment for selection of resistance to the spotted stalk borer and genetic correlations for resistance and yield components in sugarcane. *Plant Breeding* **127**, 38-42.

Robertson LN, Webster DE (1995) Strategies for managing cane weevil borer. *Proceedings of the Australian Society of Sugar Cane Technologists* **17**, 83-87.

Sharma BL, Saroj J, Chaudhary OP (2007) Biochemical basis of host plant resistance in sugarcane varieties against stalk borer, *Chilo auricilius* Dudgeon. *Cooperative Sugar* **39**, 29-39.

Young GR, Kuniata LS (1992) Life history and biology of *Sesamia grisescens* Walker (Lepidoptera: Noctuidae), a sugarcane borer in Papua New Guinea. *Journal of the Australian Entomological Society* **31**, 199-203.

Chapter 3: Field resistance trials - Downy mildew

Objective

- Develop efficient screening procedures for plant resistance to downy mildew; a rapid screening procedures will be a key focus.

Outputs and Achievement of Project objectives

Introduction

Four field trials were planted in the project period, one in each of 2010, 2011, 2913 and 2014; in the latter trial, disease observations remain current. In each of these trials, test plots continued to be single-row, 10m long, with four replicates. Trial design was a randomized complete block. Two test rows were planted between single infection rows (infection rows were therefore planted every third row). Infection rows were planted 3-4 weeks before the test canes; the diseased material grew slower than the initially healthy test canes so an early planting provided for better diseased cane growth (less competition). Additionally, more advanced diseased material enabled test cane exposure to DM right from early shoot emergence. Details of the standard varieties planted and their resistance ratings are included in Table 1.

Table 1: The standard varieties included in DM field trials planted at Ramu, PNG during the project period.

Variety	Resistance rating
H58-5301	1.0
RQ117	2.0
Q121	3.0
S17	4.0
Q107	4.0
Cadmus	5.0
Q130	5.0
Q82	6.0
Q88	6.0
Q113	7.0
Q87	9.0

As per the stem borer field trials, variety identity was checked in each disease resistance screening trial using DNA fingerprint analysis. A leaf sample was taken from one stalk of each variety in one replicate of each trial. The DNA fingerprint was compared to the profile held in the AGRF database. Some discrepancies were noted and this was important (see later) in interpreting the reaction of several standard varieties. Trial details are outlined in Table 2

Table 2: Details of DM resistance trials planted at Gusap during the project period (20102014).

Trial year	2010	2011	2013	2014
No. of clones	17	17	17	17
No. standards	7	7	7	7
Replicates	4	4	4	4
Planting date	13/12/2010	23/12/2011	13/3/2013	6/5/2014
Inspection times	Monthly	Monthly	monthly	monthly

[a] Week of each year in parenthesis

Inspections of test cane plots generally occurred monthly in each of the trials. Assessments were based on + vs – infection in each stalk of the test canes in each plot; the derived value for comparison amongst varieties was therefore '% diseased stalks'. Large differences in infection levels were observed amongst both standards and test varieties.

Correlation with previous trial data

The usual method for assessing consistency with previous trial data was to correlate the level of the disease in the standard varieties with the level expected from historical data. Resistance ratings for the standards generated in the review paper (Kuniata et al, 2010a) were correlated with data from each of the project trials. In addition, correlations between the project trials was compared to correlations of each trial with the historical data. A poor correlation of disease in all the standards data, or an unusual level of disease in individual standards, was further investigated to determine the likely cause.

Application of resistance ratings

Stringer et al (2012) published a new method for the application of resistance ratings that uses all available plot data from all previous trials. Each set of new trial data is compared to the combined data from all previous trials and groups of varieties which differ significantly from each other are identified (Stringer et al, 2012). Successful trials identify groups of varieties that are 'resistant', 'intermediate' and 'susceptible' in reaction to the disease. These groupings are used to apply resistance ratings. This method was applied to project trial data; some analyses using this method were also applied to historical data from previous trials conducted at Ramu, PNG. These analyses will be reported as an addendum to the final report.

Results

Individual trial comparisons (2010-2013 series trials)

Data for the standards in individual trials were compared to the data for the standards in each of the other trials; these are illustrated in Figure 1.

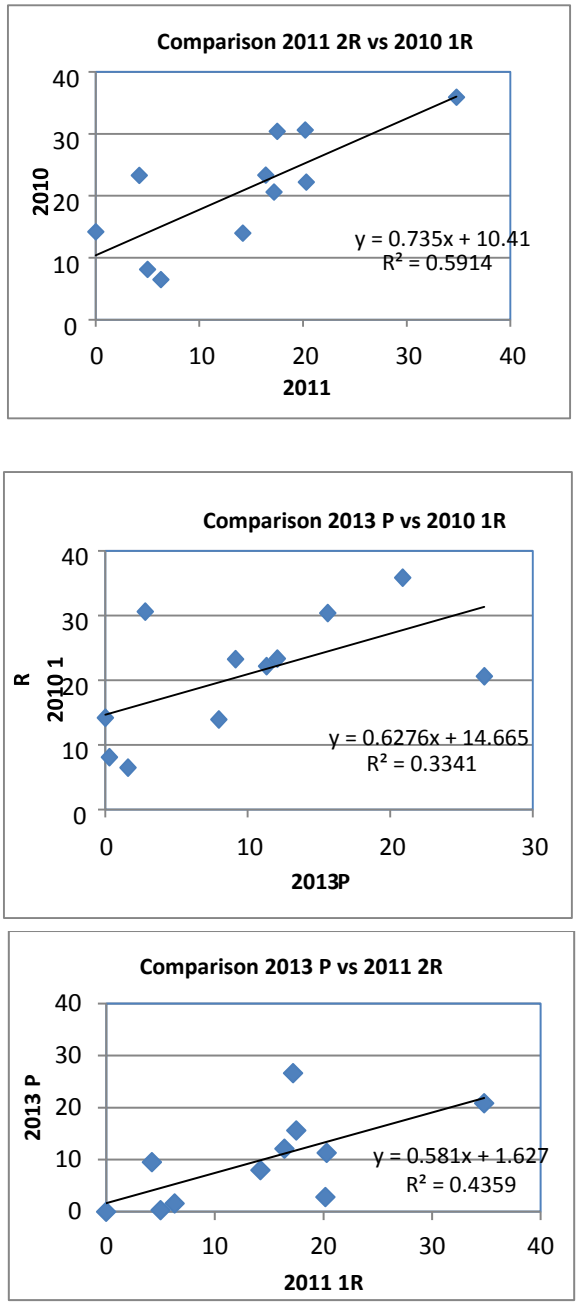
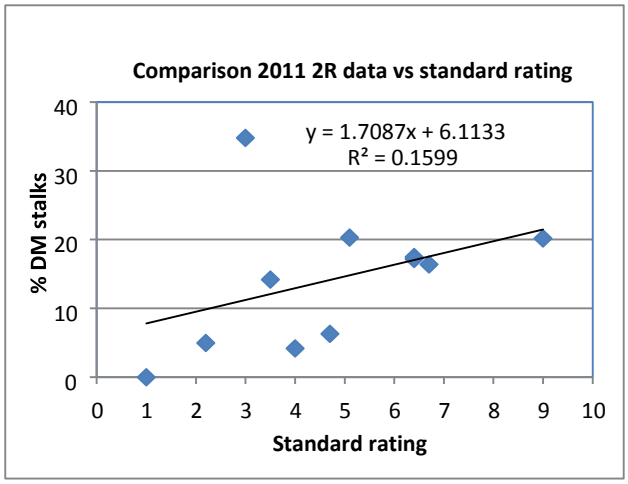
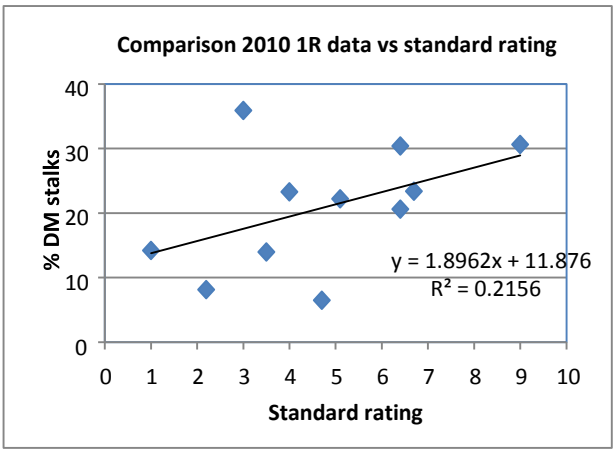


Figure 1: Comparison of the standards data in each trial compared with the data for the standards in each of the other project DM field trials.

Comparison with conventional standard ratings

The standards data for each trial were also compared to historical data for the same standards, based on field trial results gathered from the mid-1980s to the early 2000s (Figure 2). The coefficient of determination was lower in each case.





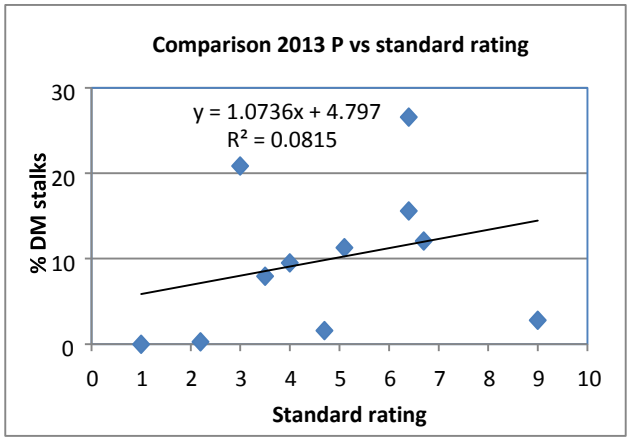
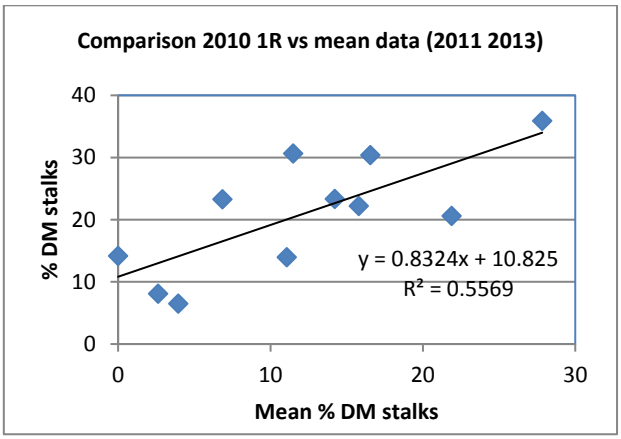
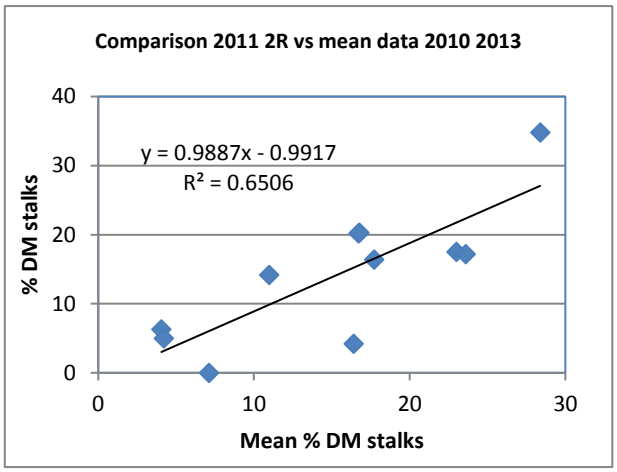


Figure 2: The relationship between standards data in each trial compared with the historical standards ratings calculated from earlier field trials at Ramu (mid-1980s to early 2000s).

Individual trial data vs mean standards data from two other 2010-2013 trials

Figure 3 illustrates the relationships between the standards reactions when individual trial data are compared to the mean data for the standards from the other two trials (relating each trial to the mean data for all three trials would lead to bias, related to the inclusion of data from the trial in question).





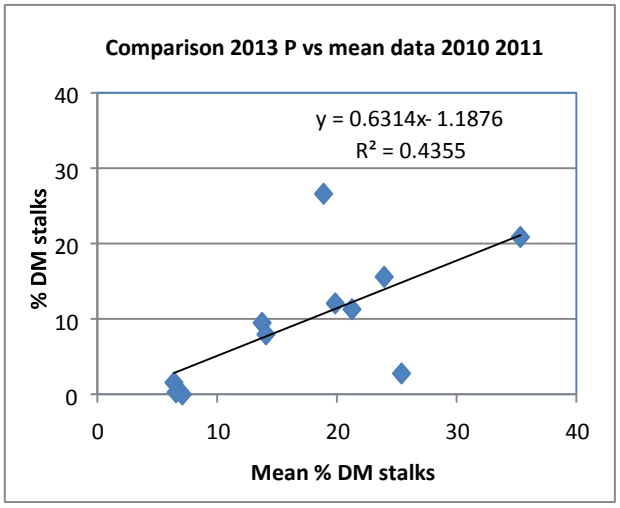


Figure 3: The relationship between standards data in each trial compared with the mean data for the other two trial plantings in 2009-2013 project research.

Summary data

A summary of the above figures is outlined in the following text and table.

When a comparison of the reaction of the standards in each trial was compared to the historical standards rating, the co-efficient of determination was low; however, this improved significantly if the reaction of the standards in each trial was compared to the mean data for the standards in the other two project research trials (Table 3).

Table 3: The coefficient of determination for comparison of the standards in each trial with either historical standard ratings, or new ratings derived from the mean figures for the two project trials (2010-2013 plantings).

Trial/Class		Standards (historical)	Standards (revised from 2 other trials)
2010	1R	0.22	0.56
2011	2R	0.16	0.65
2013	P	0.08	0.44

Revised standards ratings

Given the above results, new ratings for the standards were calculated based on the data for the 2010, 2011 and 2013-planted trials (Table 4).

Table 4: Summary data (% DM stalks) for the standards in each of the project trials along with revised standards ratings and the historical ratings for the same varieties.

Variety	2011	2013	2010	Mean	Old ratings	Revised ratings
RQ117	5.0	0.3	8.1	4.5	2.2	1.0
H58-5301	0	0	14.2	4.7	1.0	1.1
Cadmus	6.3	1.6	6.5	4.8	**4.7**	**1.1**
S17	14.2	8.0	14.0	12.0	3.5	3.3
Q107	4.2	9.5	23.3	12.3	4.0	3.4
Q113	16.4	12.1	23.4	17.3	6.7	4.9
Q87	20.2	2.8	30.6	17.9	**9.0**	**5.1**
Q130	20.3	11.3	22.2	17.9	5.1	5.1
Q82	17.5	15.6	30.4	21.2	6.4	6.1
Q88	17.2	26.6	20.6	21.5	6.4	6.2
Q121	34.8	20.9	35.9	30.5	**3.0**	**9.0**

Table 5: DM infection in all test canes in trials planted in 2010, 2011 and 2013.

2010		2011		2013	
Variety	% DM stalks	Variety	% DM stalks	Variety	% DM stalks
QN97-563	2.4	QN98-1453	0.0	H58-5301	0.0
Q230	3.3	KQ91-71304	7.1	MQ239	0.0
QC94-1771	6.3	QA95-1707	9.2	KQ91-71304	0.4
Cadmus	6.5	QN97-633	10.6	RQ117	1.0
QC93-745	6.9	QN99-2265	11.6	Q213	1.4
Q203	7.3	QN97-2353	11.6	QN97-387	2.6
Q117R	8.1	Q188	12.9	Q220	4.3
QC91-423	9.0	QN99-815	15.6	Cadmus	4.6
Q135	9.1	QN98-320	16.1	QN97-536	5.7

Q136	10.1	QC01-6446	16.2	QN97-470	9.1
QN95-939	10.5	QN97-542	17.8	KQ228	9.9
Q209	10.9	QC01-279	18.7	S17	10.5
QN97-542	12.7	QN99-1292	19.9	Q171	11.0
Q220	13.0	QN98-175	21.4	Q82	11.8
QN90-91	13.6	Q243	23.3	Q188	14.2
H58-5301	14.2	QC98-2551	24.0	Q209	14.6
B72177	16.2	QN97-23	25.8	Q87	15.5
Q231	17.5	QN92-1234	26.9	Q234	16.0
QN89-1574	18.4	QN97-563	28.0	Q235	16.0
Q198	18.8	Q242	29.1	Q242	16.0
QC92-991	21.1	QC02-106	31.2	Q243	16.0
QN97-633	21.4	Q221	36.4	Q88	16.5
Q224	21.9	QC93-896	45.4	Q107	19.1
Q130	22.2	KQ236	49.6	Q138	20.8
Q127	23.0	QC01-6058	52.6	Q219	21.0
Q107	23.3	QC02-156	55.0	Q130	21.4
Q113	23.4			QN98-320	23.0
Q190	23.7			QN92-1234	25.0
F177	23.7			Q113	26.1
QN97-1423	25.6			Q172	28.0
QN97-1423	26.0			Q121	33.6
Q183	26.4			KQ236	34.5
QS93-411	28.0			QN97-542	44.5
Q121	35.9			Q231	46.1
Q215	36.6			Q151	56.3
Q219	37.3			Q208	56.6
QS94-429	39.2			QN97-2024	56.8
QN88-185	41.0			QN98-184	58.3
Q182	41.3			QN98-1947	62.7
QS93-423	42.4			Q200	88.0
QS94-2392	43.8				
QA91-3469	45.1				
QN97-123	49.3				
QS93-980	58.3				

There were a number of Australian commercial varieties that exhibited relatively high levels of DM infection - for instance: Q200, Q231, KQ236, Q219 and Q182.

Discussion

On a previous visit to Ramu (October 2013), research staff were concerned at the quality of some of the data collected in DM field resistance screening trials; counts in individual plots varied in inspections undertaken at short intervals by Ramu staff. An apparent poor correlation of the standards data with historical ratings was potentially seen as an inspection problem. For this reason, other research staff visited

Ramu to speak to local staff and to assist in collecting a new data set for each project trial and to find out if other factors were contributing to the poor correlation analysis outcomes.

Inspections of the three project trials were undertaken quicker than anticipated, partly due to the methods adopted – writing plot results on flagging tape and tying this to the end stool in each plot; one person (in cases) counting both DM-infected and disease-free stalks in each plot. These methods provided an efficient alternative to normal Ramu practice and will be used in the future.

When data from the current and previous inspections were related to the historical data for the standards, poor coefficients of determination were apparent. In other words, the reaction of the standards in the current trials was unexpected when compared to the DM infection in standards in trials conducted in the period mid-1980s-early 2000s. However, the reaction of the standards in each project-planted trial correlated much better with mean data for all recently-planted trials (2010-2013); this raised the possibility of strain variation in the DM pathogen or mistaken variety identity. When the data were examined for individual varieties, the reactions particularly of Cadmus, Q121 and Q87 changed the most. There is a possibility that the pathogen has changed (new strain) leading to this result. This can only be confirmed by culture of *Peronosclerospora* on individual plants (it's an obligate pathogen) and inoculation of a set of standards with a number of pathogen 'isolates'; variation in reaction with isolate would show that pathogen variation had occurred. The suggestion of a change in the DM pathogen at Ramu is nothing new; in the early 1990s, the formerly resistant varieties Q107 and Cassius showed a much higher level of DM on the Estate, compared to on-going resistance screening trials conducted in a more isolated off-site trial site. Reference was made at that stage to DM strains 'A' and 'B'. Nicole Thompson has more recently identified molecular variation in isolates of *Peronosclerospora* obtained from around Ramu and other places in PNG; three possible taxa of the pathogen are believed to exist in PNG. More work is current sorting out these taxa and identifying if a new species of *Peronosclerospora* may be present on the Estate.

Data collected from the 2014-planted trial (outside this scope of this project) will add to the background data for the standards and will provide a better estimate of their current resistance on the Estate. Continually updated mean data for the standards should lead to better coefficients of determination in any future analysis of data for the standards – through better resistance estimates.

Confirmation of pathogen variation, if this is the cause of the differing results, may have important implications for resistance-screening research at Ramu: i. standard ratings may need to be updated regularly, ii. ratings for Australian varieties would become more uncertain, as the question would be – to which strain are the varieties resistant or susceptible?, and iii. could we rely on these ratings to gauge the resistance of Australian crops to DM, as which taxa is likely to gain entry to Australia?

Of interest is the reaction of some test canes to DM in the three trials (data still to be more fully analysed and reported). MQ239$^{\Phi}$ was very highly resistant to DM in the 2013-planted trials (0% disease), while Q200, Q231, KQ236, Q182 and several

others were highly susceptible. There remains quite a high level of susceptibility to DM in the Australian germplasm.

Outputs / outcomes

- There were consistent differences in disease incidence amongst the commercial varieties in the three field trials assessed during the project period.
- Differences in reaction, compared to historical data, with three standard varieties was noted; two of these varieties were probably incorrectly identified in Ramu nursery material and this could have contributed.
- Differences in relative disease infection levels may result from the influence of a different *Peronosclerospora* strain.
- Data obtained on Australian commercial varieties was analysed and uploaded to SPIDNet. There appears to be quite significant susceptibility to DM in Australian commercial varieties.

References

Kuniata, L.S., Magarey, R.C., Rauka, G.R, Suma, S. and Bull, J.I. (2010). Screening for Downy mildew resistance at Ramu Agri-Industries, Gusap, PNG 1986-2008. Proceedings of the Australian Society of Sugarcane Technologists. 32: 301-311

Stringer J, Croft B, Bhuiyan S, Deomano E, Magarey R, Cox M, Xu X. (2012). A new method of statistical analysis for sugarcane disease screening trials. Proceedings of the Australian Society of Sugarcane Technologists. 34: 10 pp

Chapter 4: Field resistance trials - Ramu stunt

Objective

- Develop efficient screening procedures for plant resistance to Ramu stunt.

Outputs and Achievement of Project objectives

Introduction

In previous RS field trials conducted at Ramu**,** low and variable levels of disease expression have hindered resistance screening of commercial varieties for RS resistance. Kuniata et al, 2010b) collated all the previous trial data (1986-early 2000s) and identified suitable standard varieties for use in future field resistance trials. In previous screening programs, infection rows of Ramu stunt-infected planting material had been planted between test cane rows, with transmission of the disease occurring via the *Eumetopina flavipes* planthopper (Kuniata et al., 2010b). A similar design was employed in BSS331 research.

Four RS field resistance screening trials were planted during the project period, in each of 2010, 2011, 2913 and 2014 (see Table 1). Disease observations in the latter are continuing into the first and later ratoon crops.

Table 1: Details of RS resistance trials planted at Gusap during the project period (20102014).

Trial year	2010	2011	2013	2014
No. of clones	32	24	24	24
No. standards	7	7	7	7
Replicates	3	3	4	4
Planting date	29-30/12/2010	30/11/2011	3/4/2013	7/5/2014
Inspection times	Monthly	Monthly	Monthly	Monthly

Methods

Each of the field trials were planted with both test canes and standard varieties (of known field reaction) in single-row 10 m plots / three replicates; a randomised complete-block design was employed. Infection of trial plots occurred via the planting of infection rows; in the 2013 trial, in an attempt to increase infectivity, dual rows were planted (roughly 30 cm apart); one row comprised the test canes and the other Ramu stunt infected planting material (PN97-54). The reasoning was to encourage the movement of the infectious planthopper (*Eumetopina flavipes*) from the diseased plants to the healthy test canes. The planthopper is known to move from leaf to leaf / plant to plant. Close canopy contact is likely to increase disease spread via the insect vector. Infection rows (except in the 2013 trial) were planted 3-4 weeks before the test canes to improve exposure of the test canes to RS infection.

All cane was planted by hand, with labelled bags used to transport the cane to the field plots (Figures 1 and 2). Details of the standard varieties planted and their resistance ratings are included in Table 2

Variety identity was also checked in each RS resistance screening trial using DNA fingerprint analysis. A leaf sample was taken from one stalk of each variety in one replicate of each trial. The DNA fingerprint was compared to the profile held in the AGRF database.

Table 2. The standard varieties included in RS field trials planted at Ramu, PNG during the project period.

Variety	Resistance rating
Cadmus	1.0
Q124	2.7
Q134	4.0
N7	5.7
Q125	5.8
BJ7013	6.1
Ragnar	9.0

Figure 1: Ramu field hands arranging the varieties, contained with bags, in order to speed planting of the field plots

Figure 2: The 2014-planted trial site after all plots were planted in June 2014 (?)

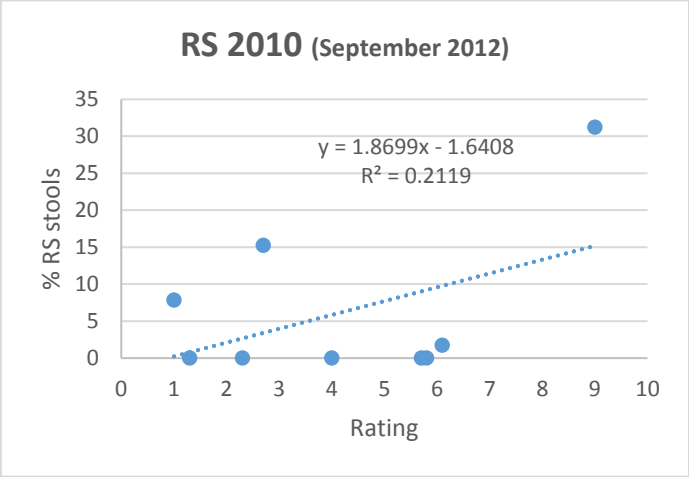
Inspections of test cane plots generally occurred at monthly intervals in each trial. Assessments were based on + vs – infection in each cane stool; the derived value for comparison amongst varieties was therefore '% diseased stools'.

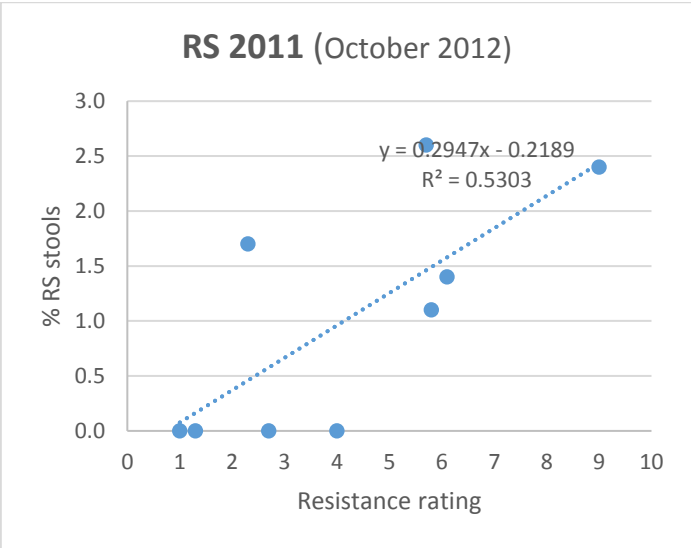
Statistical analyses: Data were entered into an Excel spreadsheet, mean data were calculated for total stools, total diseased stools and % Ramu stunt stools. Data for the standards were then examined more closely and a correlation analysis undertaken comparing the reaction of the standards with the historical 'standard ratings' for each variety. The coefficient of determination was calculated.

Resistance ratings for the standards generated in the review paper (Kuniata et al, 2010b) were correlated with data from each of the project trials. A poor correlation of disease in all the standards data, or an unusual level of disease in individual standards, was further investigated to determine the likely cause.

Results

A regression analysis was undertaken and the coefficient of determination calculated to illustrate how closely current trial data matched previous results (Figure 3).





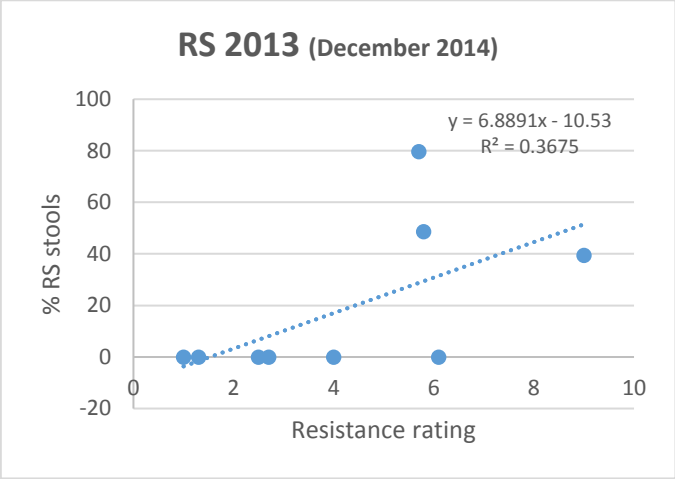


Figure 3: The relationship between standards data in the 2010, 2011 and 2013-planted trials compared with the historical standards ratings calculated from earlier field trials at Ramu (mid-1980s to early 2000s).

Table 3: Australian commercial varieties tested for resistance to Ramu stunt in field resistance trials at Ramu.

2010	**% RS stools**	**2011**	**% RS stools**	**2013**	**% RS stools**
QS94-2392	5.2	CO-6502	1.7	Q231	20.3
QS94-429	3.9	KQ236	0.0	KQ228	7.8
PN92-439	3.8	KQ91-7103	0.0	KQ236	0.0
PN92-339	3.6	Q243	0.0	MQ239	0.0
CO6502	0.0	QC01-279	0.0	Q127	0.0
Q125	0.0	QC01-6058	0.0	Q138	0.0
Q213	0.0	QC01-6446	0.0	Q188	0.0
Q215	0.0	QC02-106	0.0	Q200	0.0
Q220	0.0	QC02-156	0.0	Q208	0.0
Q231	0.0	QC02-929	0.0	Q213	0.0
QN97-1423	0.0	QN92-1234	0.0	Q219	0.0
QN97-542	0.0	QN97-2353	0.0	Q234	0.0
		QN98-320	0.0	Q235	0.0
		QN99-2265	0.0	Q242	0.0
		QN99-815	0.0	QC01-279	0.0
		QS01-152	0.0		

Infection on the RS trials was lower than anticipated and many canes showed no disease development. Higher disease levels were present in the 2013-planted trial. A couple of important canes have shown some disease – these include two significant commercial varieties: Q231 and KQ228.

Discussion

Previous research conducted in 1986-2000 showed that around 75% of Australian commercial varieties are resistant to Ramu stunt and show no symptom development when exposed to the disease. The review of these historical data conducted at the start of the project period (Kuniata et al., 2010b) concluded that less emphasis should therefore be placed on Ramu stunt research, especially in developing a rapid resistance screen test since most canes are resistant to the disease. The review also highlighted that the level of Ramu stunt in the previous trials tended to be low. We found the same thing in our trials, the percentage of varieties exhibiting disease symptoms was slightly lower than 25%, a result no doubt of the relatively low infection levels observed in these trials.

Attempts were made to increase disease levels in the stunt trials in project research. The vector *Eumetopina flavipes* (a planthopper (insect)), is known to move from leaf to leaf with limited broader dispersal, especially when population densities are low. For this reason, dual rows were employed in the 2013-planted trial, with one row of the dual rows comprising cane infected by Ramu stunt. Our results suggested that this strategy did lead to higher disease levels in the standard varieties, but the data were no more reliable, judging by the coefficient of determination. The coefficients of determination in each of the three trials (where significant data has been obtained) remained low compared to the same parameter calculated in Australian-based field trials. Dry weather during the project period, and a relatively dry location for 1 or 2 of the trials may have contributed to the low vector numbers. Future trials may benefit from locating the resistance trials in an area where supplementary irrigation could be applied, or in wetter parts of the Estate.

In Australia, Fiji leaf gall (also caused by an insect-vectored virus) resistance screening in undertaken by breeding the vector in cages surrounding diseased susceptible varieties, then transferring a known population of vectors to caged test plants. After a specific time period, these plants are then transferred to the field for symptom observation and disease assessments. This approach would not have been easily achieved within this project, but could be worth considering in the future. It would be time consuming and add extra costs to the procedure but may have improved trial outcomes. Such a method could be employed at Ramu, though it may be more labour intensive and require careful implementation.
Ramu stunt was observed in a few Australian commercial varieties. Of significance was the detection of stunt symptoms in Q231 and KQ228; it appears these varieties have a significant degree of susceptibility.

Outputs/outcomes

- Australian commercial varieties were screened for Ramu stunt in field trials at Ramu. Low levels of disease hindered the application of resistance ratings. Data were uploaded to SPIDNet.
- Some improvement in infection levels in the standard varieties occurred when dual rows were employed, with one row of test canes planted 60 cm from an infection row.
- Some disease was noted in KQ228 and Q231.

Reference

Kuniata, L.S., Magarey, R.C., Rauka, G.R, Suma, S. and Bull, J.I. (2010a). Screening for downy mildew resistance at Ramu Agri-Industries, Gusap, PNG 1986-2008. Proc. Aust. Soc. Sugar Cane Technol., 32: 301-311.

Kuniata LS, Magarey RC, Rauka GR, Suma S, Bull JI (2010b) Screening for Ramu stunt resistance at Ramu Agri-Industries, Gusap, PNG 1986–2008. Proceedings of the Australian Society of Sugar Cane Technologists 32: 312-321.

Chapter 5: Rapid resistance screen pot trials – Stem borers

Objective:

- Develop efficient screening procedures for plant resistance to moth-borers, especially *Sesamia grisescens*; rapid screening procedures will be a key focus.

Outputs and Achievement of Project objectives

Introduction: During the course of the project, five pot trials were conducted to test for varietal resistance to borers under controlled conditions. All trials took place in a shadehouse that was modelled on similar structures used in South Africa for screening varieties for resistance to *Eldana*. The completed shade house measures 19 m x 14 m and has a sloping roof 3.0 – 4.1 m in height. The roof is clear polycarbonate and the walls are white shadecloth (Figure 1). Later in 2014 ventilators were fitted and dark green stretches of shadecloth were used to cover sections of the roof to limit temperature escalation.

Figure 1: Shadehouse at Ramu: completed structure December 2010

Pot trial 1

Aim

Develop a method for rapidly screening sugarcane varieties for resistance to *Sesamia grisescens* by artificially infesting potted plants with eggs in a shadehouse. Specifically, this trial was intended to determine the number of eggs to place on each plant to discriminate between susceptible and resistant varieties.

Infestation procedure

Sesamia moths were kept in two cages with cut sugarcane tops supplied for oviposition. Egg batches were scraped from beneath the leaf sheaths and kept in the laboratory on moist filter paper. Sugarcane stalks were infested by lifting a young leaf sheath with a knife and placing a known number of black eggs – those almost ready to hatch – behind the sheath (Figure 2).

Main trial

Based on results from two preliminary experiments, the main trial used egg numbers of 20, 40 and 80 per pot to achieve good larval establishment. The experimental design was two varieties (Cadmus and PN92-439) and three egg densities, with each combination replicated eight times. Plants were grown in 35 L pots, in a potting medium consisting of creek topsoil: sand: chicken manure in the ratio 3:2:1. Five primary stalks were maintained in each pot (Figure 3). Pots were watered daily by hand and Urea (2 g) was added to each pot on 22 February 2011. Plants were infested from 24-28 May 2011 (about 22 weeks after transplanting). Black eggs were counted and inserted behind a leaf sheath on one stalk in each pot. The trail was harvested from 5-8 July 2011.

Figure 2: Oviposition cage used to collect *Sesamia* eggs (left), egg batches including some black eggs (top right) and inserting eggs behind a leaf sheath (bottom right)

Figure 3: Potted plants in the shadehouse in May 2011, when *Sesamia* eggs were added

Results

There was evidence of feeding by small *Sesamia* larvae, either on the leaf sheath or the internode immediately behind the sheath, on every stalk on which eggs were placed. However, larval establishment as assessed by recovery of at least one large larva or pupa from the experimental pots was poor (Table 1). Clearly there was no difference in establishment between infestation densities. The difference in establishment between varieties, 11 pots for Cadmus and 5 for PN92-439, was not statistically significant ($P = 0.12$ by Fisher's exact 2-tailed test for 2 x 2 tables).

Table 1: Number of pots from which *Sesamia* was recovered, out of eight for each variety/density combination, in the shadehouse trial

Variety	Eggs per pot	No. of pots where *Sesamia* recovered
Cadmus	20	3
	40	6
	80	2
PN92-439	20	2
	40	1
	80	2

The number of *Sesamia* recovered from pots differed significantly between varieties, with more found on Cadmus than on PN92-439 (Table 2). The proportion of the *Sesamia* population that was represented by larvae was very similar on both varieties: 0.64 and 0.60 on Cadmus and PN92-439, respectively. Mean weight of larvae and pupae was significantly greater on Cadmus – 0.48 g as against 0.45 g –

but this was based on very few pots (pots in which larvae established successfully). Significantly more larval exit holes were recorded on Cadmus than on PN92-439, and there was also a significant density effect and density*variety interaction.

Table 2: Numbers and average weight of *Sesamia* recovered from pots

Variety	Eggs per pot	No. of *Sesamia* larvae and pupae	Mean weight (g) [a]	Exit holes
Cadmus	20	1.9 ± 1.2	0.42 ± 0.00	0.5 ± 0.5
	40	3.6 ± 2.1	0.51 ± 0.06	1.1 ± 0.6
	80	5.9 ± 1.5	0.49 ± 0.01	4.1 ± 1.1
PN92-439	20	0.4 ± 0.3	0.38 ± 0.08	0.0 ± 0.0
	40	0.3 ± 0.2	0.59 ± 0.02	0.0 ± 0.0
	80	0.3 ± 0.3	0.35 ± na	0.3 ± 0.3
$P_{variety}$		<0.001	0.035	<0.001
$P_{density}$		0.25	0.56	0.005
$P_{interaction}$		0.21	na	0.016

[a] Including only pots where larvae established: see Table 2. Data were analysed by one-way ANOVA because of the many missing values, with separate analyses for variety and density

A total of 74 individual pupal weights were obtained for pupae collected from the potted plants or reared from larvae. Mean weights (± SE) were 0.42 ± 0.01 (n = 68) and 0.38 ± 0.04 g (n = 6) for Cadmus and PN92-439, respectively. There was no significant difference in mean weights (t = 0.77, df = 72, P = 0.44) when these data were analysed as independent observations.

More dead hearts were recorded on Cadmus than on PN92-439 and the number increased with increasing infestation density (Table 3). Both the proportion of bored internodes and length of borer tunnels were greater on Cadmus than on PN92-439.

Table 3: Damage recorded in pots

Variety	Eggs per pot	No. dead hearts (max. 5)	Proportion bored internodes (%)	Tunnel length (cm)
Cadmus	20	0.5 ± 0.3	4.1 ± 2.6	21 ± 13
	40	1.4 ± 0.7	5.1 ± 2.5	41 ± 23
	80	2.0 ± 0.6	10.6 ± 2.6	78 ± 19
PN92-439	20	0.1 ± 0.1	1.1 ± 0.7	6 ± 5
	40	0.3 ± 0.2	1.1 ± 0.4	6 ± 3
	80	0.9 ± 0.4	1.5 ± 0.5	14 ± 7
$P_{variety}$		0.016	0.001	0.002
$P_{density}$		0.039	0.14	0.06
$P_{interaction}$		0.60	0.22	0.19

Discussion

Sesamia establishment in the shadehouse was much lower than in the preliminary laboratory experiments even at the highest egg density of 80 per release stalk. We

suspect that plants were too large at the time of infestation. Plants were also heavily infested with woolly aphids and the internode borer *Chilo terrenellus*. There had also been a substantial infestation of the planthopper *Eumetopina flavipes* and associated ants earlier in plant growth. All these confounding factors would be minimised by infesting plants earlier in their growth. However, our results indicated poorer establishment of larvae and less damage on PN92-439 than on Cadmus, which is in agreement with their known resistance status. Larval growth as measured by larval or pupal weight was similar on both varieties, suggesting the resistance mechanism in PN92-439 may be reduced establishment of small larvae rather than poorer larval growth following stalk penetration. However, the poor larval establishment means that results must be interpreted cautiously.

Pot trial 2

Aim

The aim of this trial was to improve establishment of *Sesamia grisescens* larvae on potted plants. Specific questions being addressed in the second trial were:
What is best stage to infest plants to get good establishment on susceptible varieties?
How many eggs to place on each plant to discriminate between susceptible and resistant varieties, as affected by plant age?

First attempt

Methods were similar to the first trial but plants were infested at a younger age. Plants in the first trial had been infested 22 weeks after transplanting whereas two plant ages at infestation were included in the second: 12 and 18 weeks after transplanting. Four varieties were grown, two susceptible (Cadmus and Q135) and two resistant (PN92-439 and RQ117). Each combination of variety and plant age was infested with two egg densities, 20 and 50 per plant, replicated eight times. Plants were destructively sampled 37-38 days after transplanting (21-22 March 2012). All 128 pots were sampled, with the stalk on which eggs were released sampled first and the remaining four stalks sampled if larvae had established. Larvae had established in only five pots: Cadmus/young/50 eggs, Cadmus/old/20 eggs, Q135/young/50 eggs, PN92-439/old/50 eggs and RQ117/young/20 eggs. Initial feeding by first instars beneath the leaf sheath was detected in all but four of the 128 pots but the first instars were unable to penetrate the stalks. Various reasons were proposed for poor establishment: insufficient eggs, plants too wet or too dry, plants growing too fast, or predation by earwigs which were abundant. Eggs may have also been placed too high on the stalk while they would perhaps have a better chance of establishing where there is space between the sheath and the stalk.

Second attempt

The younger plants in the trial were still useful for a repeat of the experiment. The remaining four stalks in each pot were re-infested on 12-13 April (i.e. 20 weeks after transplanting) with either 50 or 100 eggs, with eggs placed under sheaths 5 or 6.
Plants were sampled on 16-18 May 2012, about 35 days after infestation. There was evidence of establishment in 25 of the 61 infested pots (Table 4). There was clearly no difference in establishment success between the two egg densities. There was a significant difference in establishment on the different varieties: 4/15, 13/15, 1/16 and 7/15 pots of Cadmus, Q135, PN92-439 and RQ117, respectively ($\chi^2 = 9.2$, $P = 0.027$). The difference was due to better results on variety Q135; establishment

success did not differ significantly among the other varieties (χ^2 = 3.9, P = 0.14). Good establishment on Q135 is gratifying as it is a known susceptible variety, but poor establishment on Cadmus is a concern.

Table 4: Number of pots with evidence of *S. grisescens* establishment, out of seven or eight for each variety/density combination, in the shadehouse trial

Variety	Eggs per pot	Dead hearts	Boring in stalk	Larvae in plants	Evidence of establishment
Cadmus	50	2	1	1	2
	100	1	2	1	2
Q135	50	6	6	6	6
	100	6	7	5	7
PN92-439	50	0	0	0	0
	100	1	1	0	1
RQ117	50	1	2	1	2
	100	1	5	1	5

The number of *S. grisescens* recovered from pots differed significantly between varieties (after transformation as log (x+1)), with more found on Q135 than on the other varieties (Table 5). There was no significant effect of egg density or density*variety interaction. The largest number of *S. grisescens* recovered from any single pot was 14 (Q135 with 100 eggs). There were more dead hearts, a greater proportion of bored internodes and a greater length of tunnels within stalks of Q135 than of the other varieties (Table 6). Density effects and variety*density interactions were not statistically significant for any of these measurements.

Table 5: Numbers of *S. grisescens* recovered from pots (mean ± SE)

Variety	**Eggs per pot**	**No. of *S. grisescens* larvae and pupae**
Cadmus	50	1.0 ± 1.0 b
	100	0.6 ± 0.6 b
Q135	50	3.5 ± 0.9 a
	100	4.4 ± 1.9 a
PN92-439	50	0.0 ± 0.0 b
	100	0.0 ± 0.0 b
RQ117	50	0.3 ± 0.3 b
	100	0.4 ± 0.4 b
$P_{variety}$		<0.001
$P_{density}$		0.95
$P_{interaction}$		1.00

Means followed by the same letter are not significantly different (P = 0.05 by lsd test)

Table 6: Damage recorded in pots (mean ± SE)

Variety	Eggs per pot	No. dead hearts (max. 5)	Proportion bored internodes (%)	Tunnel length (cm)
Cadmus	50	0.3 ± 0.2 b	1.5 ± 1.5 b	6.8 ± 6.8 b
	100	0.3 ± 0.3 b	1.8 ± 1.5 b	6.6 ± 6.6 b
Q135	50	1.4 ± 0.4 a	10.1 ± 2.7 a	53.4 ± 13.5 a
	100	2.0 ± 0.5 a	11.9 ± 3.1 a	65.3 ± 18.6 a
PN92-439	50	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b
	100	0.1 ± 0.1 b	0.2 ± 0.2 b	0.3 ± 0.3 b
RQ117	50	0.3 ± 0.3 b	2.0 ± 1.8 b	7.3 ± 7.0 b
	100	0.3 ± 0.3 b	3.7 ± 2.7 b	11.6 ± 10.1 b
$P_{variety}$		<0.001	<0.001	<0.001
$P_{density}$		0.29	0.46	0.54
$P_{interaction}$		0.54	0.96	0.93

Means followed by the same letter are not significantly different (P = 0.05 by lsd test)

Pot trial 3

Aim

The aim of this trial was to investigate factors that may influence establishment of *Sesamia* larvae and to ultimately develop a reliable infestation procedure. Larval establishment had been poor in the shadehouse which was at that stage covered with white cloth giving only 50% shading, while significantly higher establishment was achieved on cane tops placed in buckets in the laboratory. This pot trial investigated treatments that could either be applied to (a) individual stalks within pots or to (b) whole pots. The susceptible variety "Cadmus" was used in this trial.

(A) Test of infestation procedures on stalks within pots

Methods

This sub-trial investigated conditions that may influence the ability of larvae to establish: growing medium, fertiliser regime, watering regime, plant age and temperature, with cut tops (from both pot-grown plants and field-grown plants) included as a reference system where establishment has been successful previously (Table 9).

Table 7: Treatments in the third pot trial, sub-trial B

Tmt #	Treatment	Details
1	Standard	Standard growing conditions[1]
2	Dry	Begin to dry pots 2 weeks before infestation and continue after infestation (allow to wilt before rewatering)
3	Extra fertiliser	Add 2 g urea every 3 weeks after transplanting
4	Sand	Use coarse mixture, soil : sand : chicken manure at 1:4:1
5	Young	Plant and transplant 6 weeks later than others
6	Cool	Move pots to greenhouse (green shadecloth) before infesting
7	Cut-pots	Cut tops from potted stalks and place into water in whitehouse
8	Cut-field	Cut tops from field stalks and place into water in whitehouse

[1] Standard conditions as in sub-trial A, but with only six plants (and stalks) per pot and only one stalk infested in each pot. Treatments were replicated eight times.

All pot treatments except #5 were established as in sub-trial A, while single-eyes setts for treatment #5 were planted on 9 August and transplanted into pots on 18 September 2012. A first attempt at infestation was made from 11-15 November 2012, with 100 eggs placed behind one sheath in each pot. Eggs were mostly obtained from moths kept in cages inside the air-conditioned entomology laboratory, but eight pots were infested with field-collected eggs. The infestation stalks were cut and sliced open to assess establishment on 27 November.

Due to almost complete failure of larval establishment at the first attempt (see Results), infestation was repeated on one of the remaining stalks in each pot on 29 December 2012 to 5 January 2013, about 20 weeks after transplanting of the older plants. Eggs were inserted at an average sheath number of 4.5, counting from the top. Eggs were obtained from moths kept in cages under natural lighting and ambient conditions. Establishment was assessed from 14-17 January 2013.

Results

Eggs in treatments #2 and #3 were inserted higher and lower on the stalk, respectively, than those in all other treatments, which were all at about the same relative positions on the stalk (Table 8). This is in accord with the experimental design, wherein eggs in all treatments except #2 and #3 were to be inserted at the 'ideal sheath', the one that is used for oviposition in the field. Live larvae were found in the majority of stalks in all treatments except #1, and dead hearts caused by larval feeding were found in the majority of stalks in all treatments except #1 and #2 (Table 2). (There is no obvious reason why larval establishment should be so poor in treatment # 1, and this apparent anomaly in unexplained.)

There were significant differences among treatments for number of live larvae and tunnel length (Table 8). Treatments #1 and #2 performed poorly. Inserting eggs at one sheath lower than the 'ideal sheath', using the alternative insertion method, or sealing the sheath after insertion gave the best results. Using Tanglefoot on the stalks to exclude predators or frequent spraying of the sheaths with water was not advantageous.

Table 8: Results of sub-trial A: test of infestation procedures on stalks within pots; larval establishment was assessed 12-15 days after infestation with eggs

Tmt #	Treatment	Sheath infested (from top)	Stalks with live larvae	Stalks with dead hearts	No. of live larvae Mean ± SE	Tunnel length Mean ± SE
1	Ideal sheath	4.6	2	3	1 ± 1 d	1.0 ± 0.4 c
2	Ideal sheath-1	3.0	6	3	29 ± 10 c	2.3 ± 1.8 bc
3	Ideal sheath+1	5.1	8	6	56 ± 11 ab	19.1 ± 3.2 a
4	Alt. insertion	4.4	7	7	73 ± 6 a	23.6 ± 2.6 a
5	Sealed	4.6	8	8	61 ± 10 a	17.4 ± 3.0 a
6	Predator-free	4.1	5	6	21 ± 8 cd	6.9 ± 2.2 bc
7	Water spraying	4.0	8	7	32 ± 10 bc	8.4 ± 2.7 b
P					<0.001	<0.001

Means in columns followed by the same letter are not significantly different by Fisher's protected least-significant-difference test ($P = 0.05$)

(B) Test of growing conditions

Methods

This sub-trial investigated conditions that may influence the ability of larvae to establish: growing medium, fertiliser regime, watering regime, plant age and temperature, with cut tops (from both pot-grown plants and field-grown plants) included as a reference system where establishment has been successful previously (Table 9).

Table 9: Treatments in the third pot trial, sub-trial B

Tmt #	Treatment	Details
1	Standard	Standard growing conditions[1]
2	Dry	Begin to dry pots 2 weeks before infestation and continue after infestation (allow to wilt before rewatering)
3	Extra fertiliser	Add 2 g urea every 3 weeks after transplanting
4	Sand	Use coarse mixture, soil : sand : chicken manure at 1:4:1
5	Young	Plant and transplant 6 weeks later than others
6	Cool	Move pots to greenhouse (green shadecloth) before infesting
7	Cut-pots	Cut tops from potted stalks and place into water in whitehouse
8	Cut-field	Cut tops from field stalks and place into water in whitehouse

[1] Standard conditions as in sub-trial A, but with only six plants (and stalks) per pot and only one stalk infested in each pot. Treatments were replicated eight times.

All pot treatments except #5 were established as in sub-trial A, while single-eyes setts for treatment #5 were planted on 9 August and transplanted into pots on 18 September 2012. A first attempt at infestation was made from 11-15 November

2012, with 100 eggs placed behind one sheath in each pot. Eggs were mostly obtained from moths kept in cages inside the air-conditioned entomology laboratory, but eight pots were infested with field-collected eggs. The infestation stalks were cut and sliced open to assess establishment on 27 November.

Due to almost complete failure of larval establishment at the first attempt (see Results), infestation was repeated on one of the remaining stalks in each pot on 29 December 2012 to 5 January 2013, about 20 weeks after transplanting of the older plants. Eggs were inserted at an average sheath number of 4.5, counting from the top. Eggs were obtained from moths kept in cages under natural lighting and ambient conditions. Establishment was assessed from 14-17 January 2013.

Results

Results of the first infestation were assessed 12-16 days after eggs were placed on the plants. No eggs from the laboratory colony had hatched, whereas hatching was recorded on all eight of the plants receiving field-collected eggs, with successful tunnelling within the stalks in seven. At the time of the second infestation, young plants had fewer internodes than those planted earlier (Table 10), while pots kept dry or moved to cooler conditions had slightly fewer internodes than the standard pots. Stalks cut from the field had similar numbers of internodes to those grown in pots in the shadehouse.

Larvae established in most stalks with large numbers of dead hearts recorded (Table 10). Numbers of live larvae did not differ significantly among treatments. However there were significant differences in tunnel length. Tunnel length was quite large in stalks cut from plants and placed into buckets, and much less in the pots placed into the cooler shadehouse covered with green shadecloth.

Table 10: Results of sub-trial B: test of growing conditions; larval establishment was assessed 12-16 days after infestation with eggs

Tmt #	Treatment	No. of internodes[a]	Stalks with live larvae	Stalks with dead hearts	No. of live larvae Mean ± SE	Tunnel length Mean ± SE
1	Standard	11.4 a	8	8	60 ± 6	16.1 ± 2.1 b
2	Dry	10.8 b	8	7	53 ± 7	20.0 ± 3.1 ab
3	Extra fertiliser	11.8 a	8	5	49 ± 12	14.1 ± 3.1 bc
4	Sand	11.4 a	8	7	64 ± 8	15.6 ± 2.8 bc
5	Young	8.6 c	8	8	56 ± 9	18.0 ± 2.7 ab
6	Cool	10.8 b	5	4	31 ± 14	7.5 ± 4.1 c
7	Cut-pots	11.4 a	8	8	64 ± 5	26.0 ± 1.3 a
8	Cut-field	11.3 ab	7	8	43 ± 12	22.4 ± 4.7 ab
P		<0.001			0.15	0.003

[a] At time of infestation

Means in columns followed by the same letter are not significantly different by Fisher's protected least-significant-difference test ($P = 0.05$)

Discussion

Larval establishment was much better in this trial (both parts) than in previous pot experiments in the whitehouse, regardless of the treatment. Establishment in growing stalks in pots was almost as good as in cut stalks, contradicting what has been observed in previous experiments. The following positive observations can be made from the results:

- Larval establishment was reduced if eggs were placed behind sheaths too high on the stalk; what was judged as the 'ideal sheath' or one below was most suitable.
- The alternative insertion method (which involved gluing egg batches onto paper and placing behind the sheath) has promise as a convenient infestation method.

A number of treatments were found to offer no advantage to larval establishment under the trial conditions:

- Spraying sheaths with water
- Moving pots from the whitehouse to the cooler greenhouse
- Infesting at a younger age
- Applying extra fertiliser
- Using a different growing medium
- Excluding predators
- Drying of pots

However the following should be noted with respect to the last two points above:

- Predators (particularly earwigs) were not as numerous as in previous trials, so predation may still have played a role in poor establishment in those trials

- Due to problems with the watering system, it is likely that the standard pots were not as constantly wet as in the previous trial (see Pot trial II). Therefore, we have not shown that excess watering did not play a role in previous poor establishment.

In summary, the two parts of this trial have shown that it is possible to get good establishment of larvae of *S. grisescens* on potted plants in the 'whitehouse' that was built for the purpose. The reason(s) for poor establishment in previous trials were not elucidated.

Larval establishment was much better in this trial (both parts) than in previous pot experiments in the whitehouse, regardless of the treatment. Establishment in growing stalks in pots was almost as good as in cut stalks, contradicting what has been observed in previous experiments. The following positive observations can be made from the results:

Pot trial 4

Aim

The aim of this trial was to apply best experimental procedure to assess whether shadehouse resistance ratings agree with ratings obtained in the field. The varieties chosen for this trial are the 12 *Sesamia* standard varieties used in every field trial plus Q219[(b)], an additional standard that was added to field trials because of its extreme susceptibility to *Scirpophaga*. The experimental design for the trial is outlined below.

Experimental design:

- 13 varieties: B72177, Cadmus, PN92-31, PN92-339, PN92-439, F177, R570, RQ117, Q127, Q135, Q136, Q198, Q219
- 8 replicates x 100 eggs/pot
  = 104 pots and 10,400 eggs

Five single-eye setts of each variety were planted in each large pot and growth from each sett was pruned back to one stalk. Each pot was infested with 100 *Sesamia* eggs and replicated eight times. Pre-germinated setts were planted in the pots on 13/6/2013 and plants were then infested with eggs in late November.

Procedure

- Mixed potting medium – 3 topsoil (from creek bank): 2 sand: 1 chicken manure.
- Pots randomised - 13 varieties within eight rows; each row is a replicate. Plants not touching each other.
- Cut single-eye setts, pre-germinated in sterilised sand in seedling trays. Planted in trays on 1/5/2013.
- Five germinated setts were planted in each pot in shadehouse on 13/6/2013
- Trimmed leave tops to promote root development. Maintain five primary stalks in each pot.
- Pots fertilised with 5 g urea per pot.
- Infest experimental plants with black eggs
- Harvested. Cut release stalk, measured length of feeding under sheath and recorded any feeding on underlying sheaths 2 and 3 and stalk.

Data recorded:

- number of stalks per pot
- for each stalk
  - total length of stalk (minus tops)
  - number of internodes
  - number of bored internodes
  - length of bored internodes
  - length of red rot
- number of larvae and pupae per pot
- total weight of larvae and pupae per pot

Results

Table 11 shows pot trial 4 results. There was no significant difference between total dead heart, bored stalks, larval or pupal numbers in all varieties tested (tables 12).

However, F177 sustained the lowest proportion of bored internodes and shortest tunnel length (Tables 12 c & f), which is inconsistent with field observations. In addition, there was no apparent relationship between the normalised damage from *S. grisescens* to the standard varieties in field trials and that calculated for pot trial 4 (Figure 4). Although Q135 and Q198[Φ] were among the most heavily damaged varieties in both data sets, there were clear anomalies for some other varieties such as F177. Reasons for these inconsistencies are unknown. However, it should be noted that there was considerable variation among replicates in the pot trial, with little or no internode damage in many pots. It might be the case that an entire different pot trial design is required to test for varietal resistance to *S. grisescens*.

Table 11. Means generated from pot trial 4 for 13 varieties tested for resistance to *S. grisescens.*

Var	Dead heart	HT2TVD (cm)	Av nodes	Bored nodes	Prop. bored nodes	Tunnel length (mm)	No larvae	No pupa
B72177	0.500	213.875	17.375	2.050	0.120	199.250	1.125	1.100
Cadmus	0.300	192.125	20.075	1.550	0.081	99.250	0.875	0.750
F177	0.050	201.050	17.525	0.125	0.007	4.380	0.000	0.000
PN92-31	0.225	232.125	18.025	0.675	0.042	58.130	0.250	0.150
PN92-339	0.325	210.475	18.900	1.750	0.095	136.500	0.825	0.525
PN92-439	0.075	254.150	20.750	0.975	0.046	68.250	0.425	0.400
Q127	0.575	220.675	18.300	2.725	0.154	239.250	0.875	0.825
Q135	0.525	210.550	16.875	3.100	0.187	260.130	2.075	1.250
Q136	0.425	273.325	20.875	2.200	0.108	206.130	1.325	1.075
Q198	0.564	248.077	16.615	2.821	0.172	250.260	1.051	1.103
Q219	0.450	220.200	19.700	2.950	0.156	261.750	0.350	0.025
R570	0.550	204.900	16.600	2.350	0.147	186.380	0.975	0.675
RQ117	0.400	194.000	20.175	2.200	0.112	169.630	0.850	0.525

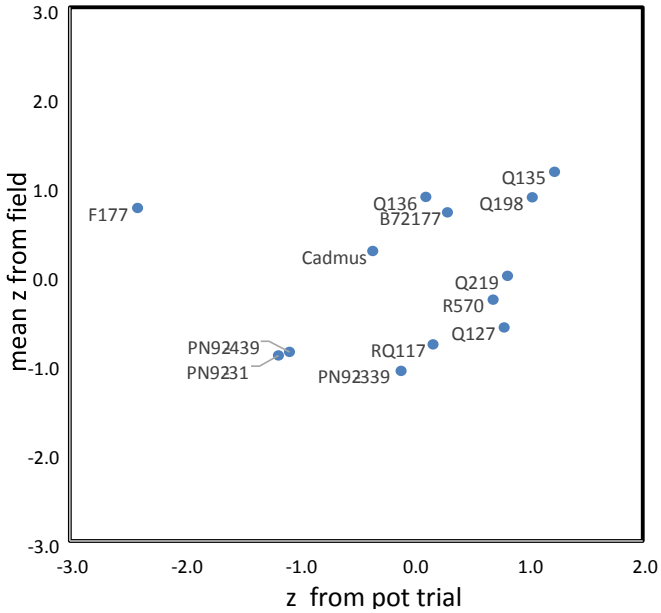


Figure 4. Relationship between the normalised damage from *S. grisescens* in the standard varieties in field trials and that calculated for pot trial 4

Tables 12: Ranking of 13 standard varieties based on their response to *Sesamia* infestation in a shadehouse pot trial.

A) Negative Binomial analysis of dead hearts summed
Effect=Variety Method=Tukey-Kramer(P<.05)

Obs	Variety	Estimate	Standard Error	Letter Group
1	Q127	1.0561	0.3028	A
2	Q198	1.0116	0.3061	A
3	R570	1.0116	0.3061	A
4	Q135	0.9651	0.3096	A
5	B72177	0.9163	0.3134	A
6	Q219	0.8109	0.3221	A
7	Q136	0.7538	0.3272	A
8	RQ117	0.6931	0.3327	A
9	PN92-339	0.4855	0.3537	A
10	Cadmus	0.4055	0.3627	A
11	PN92-31	0.1178	0.3992	A
12	PN92-439	-0.9808	0.6177	A
13	F177	-1.3863	0.7404	A

C) Logit Proportion of bored internodes
Effect=Variety Method=Tukey(P<.05)

Obs	Variety	Estimate	Standard Error	Letter Group
1	Q198	-1.8665	0.4713	A
2	Q135	-1.8701	0.4713	A
3	Q127	-1.9173	0.4713	A
4	Q219	-1.9891	0.4713	A
5	R570	-2.1286	0.4713	A
6	B72177	-2.4214	0.4713	A
7	RQ117	-2.6454	0.4713	AB
8	Q136	-2.8099	0.4713	AB
9	PN92-339	-2.8483	0.4713	AB
10	Cadmus	-3.2597	0.4713	AB
11	PN92-31	-3.7256	0.4713	AB
12	PN92-439	-3.9469	0.4713	AB
13	F177	-4.7433	0.4713	B

E) Poisson analysis of bored stalks summed
Effect=Variety Method=Tukey-Kramer(P<.05)

Obs	Variety	Estimate	Standard Error	Letter Group
1	R570	-0.01200	0.3540	A
2	Q127	-0.01200	0.3540	A
3	Q198	-0.01200	0.3540	A
4	Q135	-0.1455	0.3784	A
5	Q219	-0.1455	0.3784	A
6	Cadmus	-0.2997	0.4087	A
7	B72177	-0.2997	0.4087	A
8	Q136	-0.2997	0.4087	A
9	PN92-339	-0.2997	0.4087	A
10	RQ117	-0.2997	0.4087	A
11	PN92-439	-0.9928	0.5776	A
12	PN92-31	-0.9928	0.5776	A
13	F177	-2.0914	1.0002	A

B) Negative Binomial analysis of no. larvae summed
Effect=Variety Method=Tukey-Kramer(P<.05)

Obs	Variety	Estimate	Standard Error	Letter Group
1	Q135	2.2541	0.5424	A
2	Q198	2.1721	0.5824	A
3	B72177	1.6537	0.5611	A

Obs	Variety	Estimate	Standard Error	Letter Group
4	R570	1.5844	0.5741	A
5	Q136	1.5727	0.5629	A
6	Q127	1.5603	0.5724	A
7	PN92-339	1.5545	0.5984	A
8	Cadmus	1.4081	0.6132	A
9	RQ117	1.2213	0.5636	A
10	PN92-439	0.3496	0.6574	A
11	Q219	0.2584	0.6012	A
12	PN92-31	-0.08825	0.6466	A
13	F177	-13.8771	344.36	A

D) Negative Binomial analysis of no. pupae summed
Effect=Variety Method=Tukey-Kramer(P<.05)

Obs	Variety	Estimate	Standard Error	Letter Group
1	Q198	2.1963	0.6330	A
2	Q135	1.6800	0.5995	A
3	B72177	1.5448	0.6058	A
4	Q127	1.5442	0.6203	A
5	Q136	1.3775	0.6121	A
6	R570	1.3466	0.6310	A
7	Cadmus	1.1145	0.6656	A
8	PN92-339	1.0579	0.6417	A
9	RQ117	0.6716	0.6287	A
10	PN92-439	0.3016	0.6937	A
11	PN92-31	-0.5172	0.7570	A
12	Q219	-2.2777	1.1743	A
13	F177	-13.8496	339.20	A

F) Negative Binomial analysis of length tunnel sum
Effect=Variety Method=Tukey-Kramer(P<.05)

Obs	Variety	Estimate	Standard Error	Letter Group
1	Q198	7.3915	0.5748	A
2	Q219	7.1355	0.5506	A
3	Q127	7.1141	0.5562	A
4	Q135	7.0343	0.5588	A
5	B72177	7.0327	0.5682	A
6	Q136	6.8084	0.5639	A
7	R570	6.7974	0.5586	A
8	PN92-339	6.6969	0.5813	A
9	RQ117	6.4494	0.5777	A
10	Cadmus	6.2952	0.6065	A
11	PN92-439	5.4217	0.6409	AB
12	PN92-31	5.4027	0.6014	AB
13	F177	3.1633	0.6041	B

Pot trial 5

Aim

To develop a reliable technique to test for varietal resistance to *Chilo terrenellus* and *Scirpophaga excerptalis* under controlled conditions. Based on previous field results, a resistance pattern was evident for *Sesamia grisescens* and *Scirpophaga excerptalis* across all standard varieties tested. However, there was a large spread of standardized responses to infestation by *Chilo terrenellus* for each standard variety among all data sets, and a novel technique was required to test for varietal response to this species.

Method

Unlike *Sesamia*, fresh *Chilo* and *Scirpophaga* hatchlings readily disperse after hatching and settle on adjacent plants. We devised an infestation technique which takes advantage of this behaviour. This was done via placing one potted plant in the middle and surrounding it with four other potted plants of the same variety in the shadehouse facility (Figure 5). When plants were 6 months of age, two moth egg batches were placed on the underside of a young leaf on the central plant to serve as a source of infestation.

Figure 5: Pot trial 5 design showing potted plant arrangement in the shadehouse.

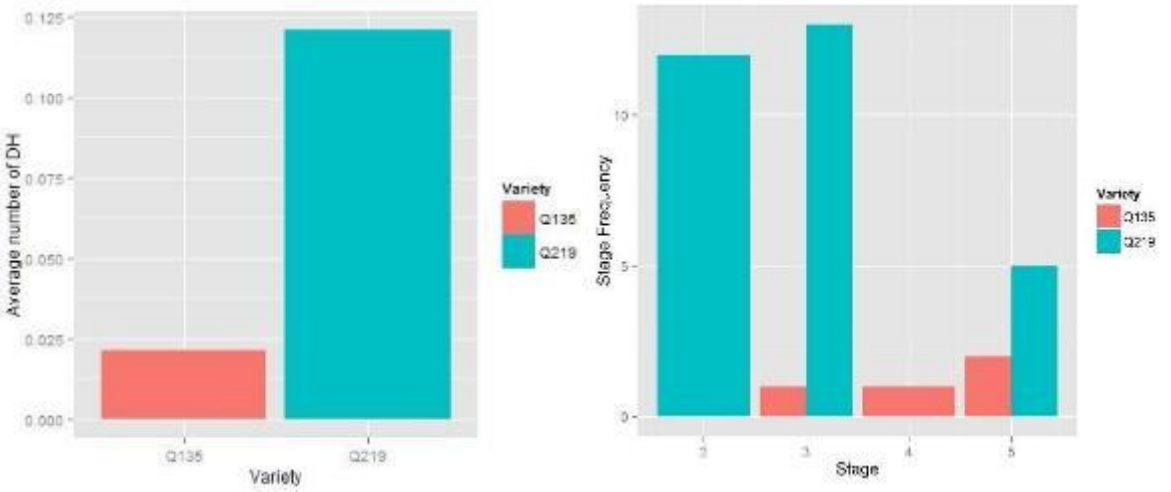
All potted plants were destructively sampled on 14, 15/1/2015 when plants were about 9 months old. We selected two varieties to be tested against *Chilo* (RQ117 and Q127) and two others to be tested against *Scirpophaga* (Q135 and Q219). These varieties were selected because they were on the opposite ends of the damage level spectrum by the corresponding borer according to field results.

Results

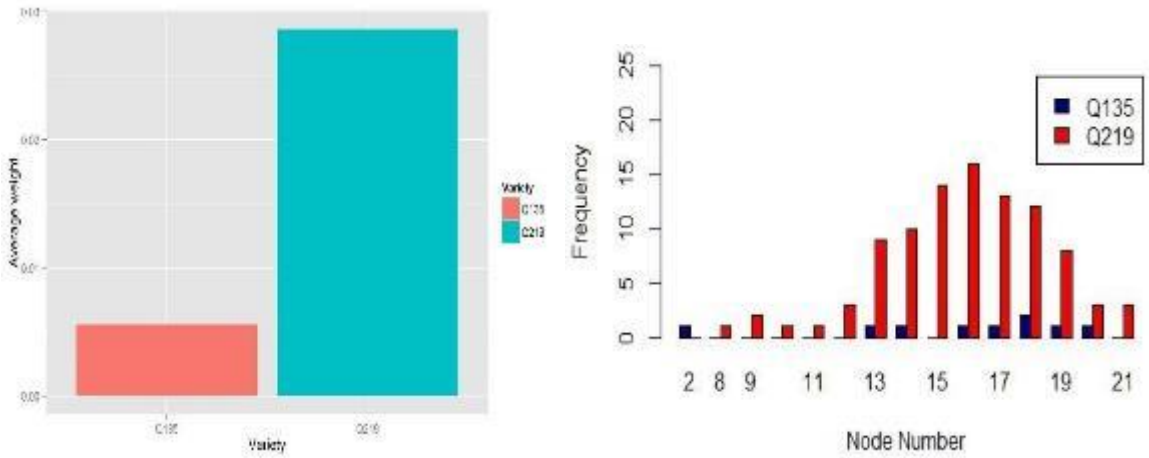
For *Scirpophaga*, Q219 showed a significantly higher percentage of dead heart symptoms, larval and pupal abundance, larval and pupal weight compared to Q135, which is consistent with field results (Figure 6).

For *Chilo*, RQ117 had more dead hearts compared to Q127, while there was no significant difference in the number of damaged internodes, entry holes, exit holes, tunnel length or frequency

of stages (P> 0.5). However, immature *Chilo* stages recovered from Q127 weighed more than those recovered from RQ117 (P< 0.5) (Figure 7).



b)



c)
d)

Figure 6. (a) Average dead heart/plant, (b) stage frequency, (c) average immature stage weight and (d) frequency of internodes damaged by *Scirpophaga excerptalis* for Q135 and Q219 in pot trial. DH = dead heart. Stage 2, 3, 4 and 5 are medium larva, large larva, pre pupa and pupa respectively.

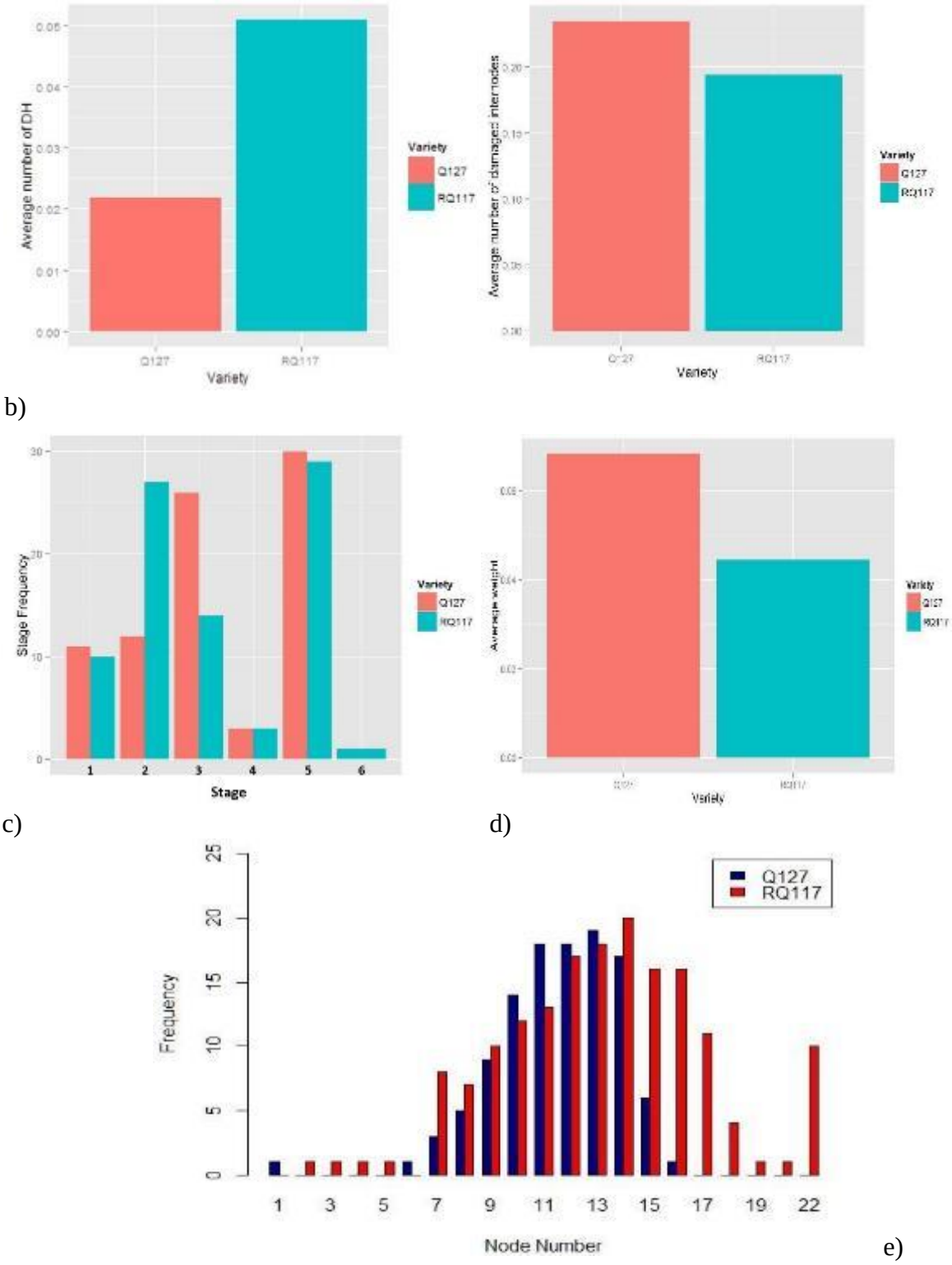


Figure 7. (a) Average dead heart/plant, (b) damaged internodes, (c) stage frequency, (d) average immature stage weight and (e) frequency of internodes damaged by *Chilo terrenellus* for Q127 and RQ117 in pot trial. DH = dead heart. Stage 1, 2, 3, 4, 5 and 6 are small larva, medium larva, large larva, pre pupa, pupa and exuvia respectively. Internode 22 is growing point.

Discussion

These results suggest that our infestation technique is a reliable method of testing for varietal resistance to *S. excerptalis* and *C. terrenellus*. The inconsistency showed by *Chilo* is typical of that species and might be an indication that selecting for varietal resistance to this species is difficult. We intend to improve this technique by placing a second set of pots to surround the original set. This will give *Chilo* hatchlings the chance to disperse further and settle on more plants, which will ultimately assist in detecting any potential resistance pattern among varieties.

Outputs / outcomes

- For *S. grisescens*, results from four pot trials conducted in the shadehouse facility didn't correlate with field trial results. Establishment of larvae after plants were artificially infested with eggs was inconsistent among trials and a satisfactory infestation technique was not identified.
- A novel infestation technique that took advantage of the dispersal behaviour of early larval stages of *S. excerptalis* and *C. terrenellus* was trialled with promising results. Early larval stages dispersed from a central potted plant and settled on surrounding plants.

Chapter 6: Rapid resistance screen pot trials - Downy mildew

Objective

- Develop efficient screening procedures for plant resistance to downy mildew
    - a rapid screening procedures will be a key focus.

Outputs and Achievement of Project objectives

Introduction

Problems with the DM field trials conducted at Ramu include the variable and uncontrolled environmental conditions experienced in the field, the rather lengthy trial period between planting and disease assessment and the costs of planting field trials (trial costs plus the lost opportunity costs associated with loss of a commercial crop harvest).

Peronosclerospora spp**.** (DM pathogen) produce two types of inoculum: i. conidia – short-lived, fragile spores that spread limited distances on warm humid nights to infect healthy cane via young spindle (leaf) and axial bud infection, and ii. oospores – robust spores produced under cooler conditions in infected leaf tissue. The role of oospores in disease epidemiology is not clear. Previous resistance screening trials have relied on conidia-infection under field conditions, where spore production in infection rows leads to spread of DM to test cane plots. However, the fragile nature of conidia makes spore collection and artificial inoculation difficult.

A series of experiments was therefore undertaken to investigate whether DM spores (conidia and oospores) could be used to infect test canes in short term pot trials. Two types of short term DM pot trials were experimented with in project research. The first aimed to utilize conidia by positioning DM-diseased plats above healthy test canes planted in pots in a greenhouse. The second utilised oospores, with high densities of oospores mixed into a pot soil, before single-eye setts of tests canes were planted into the infested soil. Some of this work has been published in a paper presented at the 36th (2014) conference of the Australian Society of Sugar Cane Technologists (Magarey et al, 2014).

Conidia-infection of young test plants

A shadehouse (whitehouse) was constructed for assessing varietal resistance to the moth borers (*S. grisescens* and other species) and the same structure was used for the first DM pot trials. An elevated platform was constructed (see Figure 1) to enable DM-infected plants to be maintained at a consistent height above healthy test canes. In these experiments, it was hoped that conidia falling from DM-infected cane would fall onto the healthy test cane varieties, leading to rapid and consistent infection of the latter material.

Figure 1: The set-up for the DM shadehouse transmission trial investigating infection via conidia.

Trial 1: DM-infested material of R570 was pre-germinated in soil contained in black plastic bags in early November 2010. Test canes (the DM standard varieties) were pre-germinated in August 2011; the following DM 'standard' varieties were included: Cadmus, RQ117, H585301, Q82, Q87, Q88, Q107, Q113, Q121, Q130, Q107 and S17. Eleven individual plants (replicates) of each variety were included in a completely randomised trial design.

Figure 2: Black plastic shroud used to increase relative humidity.

Approximately four weeks after germination, test canes were placed below the advanced DM 'infection plants' so that high levels of inoculum (produced on the under-side of infested leaves) would fall directly on the test plants. On 11 September 2011, the test canes were placed below the infected material, with black plastic 'curtains' were constructed to reduce the wind blowing across the pot trial and to attempt to elevate relative humidity amongst the test canes. The curtain extended from ground level to the height of the infection plants. (Figure 2). Monthly monitoring of DM infection continued until January 2012.

Results

Several factors made the trial operation more difficult than anticipated. It was difficult to gain access to the test plants (for inspections) under the platform supporting the infected plants and especially with the curtain material protecting the test canes. Secondly, temperatures in the whitehouse as high as 40^0 C were recorded during the trial period and these temperatures are in excess of the optimum temperatures for DM development. Thirdly, mite infections were extensive in the test plants, making identification of DM infection more difficult.

While some DM infection was recorded by Ramu staff, subsequent inspections by SRA scientists identified very little DM infection. For these reasons, the method was laid aside and the use of oospores as an infection source examined more closely.

Oospore-infested pot soils

Little previous research has been conducted into the use of oospores as an inoculum source for DM infection of sugarcane (Egan and James, 1989). However, one paper has been published from research conducted in Taiwan (Matsumoto et al., 1961). They found that test plants could indeed be infected with oospores, though not much detail was provided on inoculum levels. DM oospores cannot be cultured *in vitro* so any trial work requires the collection of shoots displaying leaf splitting symptoms in a susceptible variety. At Ramu, the variety Q136 appears to exhibit this symptom more frequently than other varieties. Project research on variation in the taxa of *Peronosclerospora* spp. present at Ramu has investigated the association of specific DM symptoms with pathogen variants (see later).

The use of oospores in DM resistance screening had never been attempted before. However, SRA staff are very familiar with the use of oospores in sugarcane resistance screening trials, with oospores used as inoculum with Pachymetra root rot.

Reliable resistance screening for Pachymetra root rot (caused by *Pachymetra chaunorhiza*) relies on the mixing of culture-derived oospores with potting mix and the planting of singe-eye setts of test canes in small pots filled with the inoculated potting mix (Croft and Magarey, 2000) Inoculum levels of around 50,000 spores / kg potting mix lead to excellent levels of disease in susceptible varieties. After 12 weeks, root systems are assessed for disease symptoms and test cane reactions compared with those in standard varieties. In this way, resistance ratings are applied to all test canes. The method is relatively quick, reliable and can be undertaken under controlled conditions (Croft et al, 1989).

Two 'rapid test' experiments were conducted at Ramu Agri-Industries Estate, Gusap in 2013. In both experiments, single-eye setts were planted in field soil infested with high levels of *P. sacchari* oospores; plants were grown in small black polythene bags for the duration of the experiments.

Experiment 1

Test plants

Planting material of Q221$^{\phi}$ and KQ236$^{\phi}$ was collected from Leron Plains (a largely DM-free site about 80 km from Gusap) in May 2013. Stalks were cut into single-eye setts and healthy buds selected for the experiment.

Inoculum

Oospores were extracted from field-grown shoots (Q136) showing typical DM 'leaf splitting' symptoms. Leaf splitting material was finely-cut using scissors and half (100 g fresh weight) blended in a food blender (10g at a time), while the other half was retained. A small amount of water was added with the leaves; the macerated tissue was washed through a flour sieve to remove large leaf pieces, and the filtrate rinsed in a 38um sieve to collect the oospores. Spores were suspended in 200 mL water and an estimate of oospore density made by counting spores in a known volume of solution under a microscope.

Figure 3: Washing DM oospores collected on a 38 um sieve.

Inoculated soils

Soil was collected from a non-sugarcane field, sieved and mixed to ensure uniform properties through-out; 300 g (moist weight) of additional soil was weighed into ziplock bags.

A comparison was made between oospores applied in solution and oospores applied in the finely-cut, leaf splitting material (oospores not extracted from the leaf material). The latter was added to give approximately the same number of oospores per pot as with the oospore suspension. To achieve this comparison, the 300 g soil contained in the ziplock bags was either inoculated with 10 mL of

oospore suspension or 10 g of finely-cut leaf split material. A wire stirrer was then used to mix the oospore suspension or the leaf split material with this soil.

The small polythene bags were three-quarter filled with soil and single-eye setts of two varieties (Q221$^{\phi}$ / KQ236$^{\phi}$) placed on the soil surface; the inoculated soil was then placed on top of the setts. Soil depth above the sett was approximately 2.5 cm. The method provided for immediate contact between infested soil and the bud. The following treatments were applied (Table 4)

Figure 4: Setts potted in black polythene bags with the oospores added in soil surrounding the setts (right) at Ramu in October 2013.

Table 1: DM oospore inoculation treatments (Experiment 1) applied in May 2013 at Gusap, PNG

Treatment	Variety	DM inoculation	Dose
1	Q221$^{\phi}$	None	-
2	KQ236$^{\phi}$	None	-
3	Q221$^{\phi}$	Oospores	3.35 million spores / kg
4	KQ236$^{\phi}$	Oospores	3.35 million spores / kg
5	Q221$^{\phi}$	Leaf split material	10 g leaf split / pot
6	KQ236$^{\phi}$	Leaf split material	10 g leaf split / pot

The experimental design included six treatments and five replicates. The disease status of test plants was assessed in late October 2013, with plants recorded as positive or negative for DM

Experiment 2

A second experiment was established in late October 2013. Fewer treatments were applied but many more replicates. As with trial 1, the oospore suspension was mixed through the soil in a bucket with a wire stirrer. The finely-cut leaf split material was added to 200 g soil and mixed by shaking in a sealed plastic container. The inoculated soil was then placed around the single-eye sett in the polythene bags, as per the first experiment. Treatments are detailed in Table 2.

Table 2: DM oospore inoculation treatments (Experiment 2) established in October 2013 at Gusap, PNG

Treatment	Variety	DM inoculation	Dose
1	Q221[(])	None	-
3	Q221[(])	Oospores	6.00 million spores / kg
5	Q221[(])	Leaf split material	2 g leaf split / pot

There were 25 replicates of each treatments with reps 1-8 incorporating one-eye setts cut from young stalks, reps 9-16 planted with the top buds from older stalks, and reps 17-25 planted with buds from the lower portions of older stalks (Figure 5).

Figure 5: Potting DM oospore-infested soil with single-eye setts of several experimental varieties in October 2013.

Results

Experiment 1

The results from the final assessment of plant status for each treatment suggested that significant disease has developed, particularly setts inoculated with finely-cut leaf split material (Table 3). With some DM infection evident, a follow up experiment was immediately instigated. Some plant death was also evident; it is not evident what caused this death.

Table 3: Results of DM oospore inoculations applied in May 2013 at Gusap, PNG

Treatment	Variety	DM inoculation	Results (% DM plants)
1	Q221$^{\phi}$	None	0
2	KQ236$^{\phi}$	None	0
3	Q221$^{\phi}$	Oospores	20
4	KQ236$^{\phi}$	Oospores	20
5	Q221$^{\phi}$	Leaf split material	60
6	KQ236$^{\phi}$	Leaf split material	60

Experiment 2

Data on test plant infection for each treatment is included in Table 4. This shows extensive infection of test plants by DM within four weeks of trial establishment in October 2013.

Table 4: DM infection of test canes exposed to DM oospores or leaf split material assessed five weeks after trial establishment

Treatment	Plants exhibiting DM symptoms	Total number of test plants	%
Control – no DM	0	25	0
Oospores	12	24	50
Leaf material	17	25	68

Discussion

A rapid test for DM requires only formation of above-ground symptoms; rapid discard of susceptible clones would be possible using these symptoms alone. Disease resistance screening and yield assessment activities in the plant breeding selection program are both costly exercises. Efficiencies gained in either activity ultimately may lead to better selection of promising clones and greater productivity in commercial varieties released to industry. Research for better resistance screening techniques for the early part of the selection program is therefore a priority.

Results from the use of oospores for DM screening are showing promise. We have shown that inoculation of soil with oospores may lead to rapid DM infection and symptom expression. If the technique leads to the discrimination of varietal resistance (resistant vs intermediate vs susceptible varieties – still to be tested), then the simplicity of the test, coupled with a short time frame and inoculum stability, makes early stage resistance screening a real possibility. Oospores are readily extracted from leaf material growing in the field and large numbers can be obtained.

Outputs / outcomes

- The suspension of plants infected with DM above young test cane plants did not successfully lead to significant DM infection in experiments conducted in this project. Factors that may have contributed were high temperatures in the shadehouse during the course of the trial.
- DM oospores mixed with pot soils into which single-eye sets of test canes were planted led to significant DM infection of test canes. The technique shows promise and should be further investigated to see if reliable resistance ratings can be obtained with this method.

References

Croft BJ (1989) A technique for screening sugarcane cultivars for resistance to Pachymetra root rot. *Plant Disease* **73**, 651-654.

Croft BJ, Magarey RC (2000) Pachymetra root rot. In 'A Guide to Sugarcane Diseases' (Eds P Rott, RA Bailey, JC Comstock, BJ Croft, AS Saumtally) pp. 126-130. (CIRAD-ISSCT: Montpellier, France).

Magarey RC, Bull JI, Atkin F, Dunne V, Pendrick R, Sventek K, Tom L (2014). Use of oospores as inoculum for early-stage resistance screening for downy mildew and Pachymetra root rot. *Proceedings of the Australian Society of Sugar Cane Technologists*, 36

Matsumoto T, Chen PC, Yang SM (1961). Downy mildew of sugarcane in Taiwan. III. Leaf splitting and infection by oospores. Rep. Taiwan Sugar Experiment Station 25: 95-110.

Chapter 7: Pathogen variability and diagnosis - Ramu stunt

Objectives

- Develop and validate specific molecular diagnostic tests for Ramu stunt and define its distribution and variability in PNG

Outputs and Achievement of Project objectives

Introduction

Ramu stunt was unknown until 1985-86 when poor yields, stool death and severe stunting became evident in commercial crops at Ramu Sugar (now Ramu Agri-Industries Limited, RAIL). The disease is characterised by stunting, failure to ratoon and various ill defined leaf symptoms which are believed to vary between varieties (Kuniata *et al.*, 2010). The vector is the island sugarcane planthopper *Eumetopina flavipes* (Kuniata *et al.* 1994). At the time of the epidemic, the causal agent was unknown but was suspected to be a virus or a phytoplasma.

As the disease has not yet been found in Australia, but the vector occurs in Torres Strait and Cape York, Ramu stunt is a major quarantine disease risk to the Australian sugar industry. Thus it was realised that the capability to monitor for Ramu stunt disease and prevent or manage potential incursions in Australia would be greatly facilitated by identification of the causal agent, understanding of the biology of the disease and the development of a robust diagnostic test. A diagnostic test for Ramu stunt would also allow safer movement of sugarcane germplasm.

Research carried out at BSES between 2004 and 2006 suggested that the causal agent was a virus (Braithwaite *et al.*, 2007). Cloning experiments identified a number of sequences with homology to known or proposed members of the viral genus *Tenuivirus*. A diagnostic RT-PCR test was developed based on a sequence with high homology to Tenuivirus RNA polymerase. Since then, various improvements have been made, with the target now the disease specific protein coding region. However, all such optimisations had been done on detached leaf pieces and the test had not been fully implemented in a field situation at Ramu. Factors such as test sensitivity and the optimal leaf and leaf position to sample needed to be determined. So a specific objective of 2009033 was to develop and validate specific molecular diagnostic tests for Ramu stunt and define its distribution and variability in PNG.

Methodology

When research into the causal agent of Ramu stunt commenced, access to good quality infected plant material was a limiting issue. Quarantine import conditions require that leaves brought into Australia under permit be irradiated, so fresh material was out of the question. It was found that using $CaCl_2$ as a drying agent was the preferred solution. Either 2 g fresh leaf could be stored in 50 mL tubes, or 20 leaf disks punched with a paper punch could be stored in 5 mL tubes. The samples collected from the field were stored in an esky during the day and were processed in the Ramu pathology lab later by chopping and placing into the $CaCl_2$tubes. For routine screening, samples consisted of punched leaf-disks stored in 5mL tubes. Sensitivity assays determined that Ramu stunt can be detected in as little as one positive in ten leaf disks (data not shown). On entry to Australia, the samples were surrendered to customs and irradiated according to the import permit conditions. At the SRA Indooroopilly laboratory, the RNA was extracted using a Qiagen RNeasy mini kit. The extractions were screened with an endogenous test based on Rubisco (RubiscoRNAf/ RubiscoRNAr) to check RNA quality, as per standard SRA quarantine operating practice. The RT-

PCR test for Ramu stunt (C1eF/C1fR) amplifies a band of 350bp from the open reading frame that codes for a protein known either as the disease specific protein (for Rice stripe virus) or the major non-capsid protein (for Maize stripe virus). It is located at the 5' end of Tenuivirus RNA4. As the project progressed, diagnostic primers were also designed for other isolates of Ramu stunt and Maize stripe virus. On some occasions, samples were also screened for sugarcane yellow leaf virus (SCYLV) using the primers developed by Girard *et al*. (2010). Amplified products were resolved on 2% agarose gels.

Eumetopina samples were collected on many occasions during the project and were stored in 90% alcohol. The same Ramu stunt diagnostic RT-PCR test for sugarcane was found to work extremely well for *Eumetopina*. Total nucleic acids were extracted using either the Rapid Release Prep from Thomson and Henry (1995) or the proteinase K method from Virtudazo *et al.* (2001). The endogenous test to check for template quality used the COII primers A-298 and B-tLys from Liu and Beckenbach (1992).

Variability amongst isolates was determined by sequencing a 1200 bp fragment of the Tenuivirus RNA4. RNA4 codes for the disease specific or major non-capsid protein at the 5' end, while the function of the 3' end is unknown. Using several internal primers, the 1.2kb of sequence was obtained from over 30 isolates of Ramu stunt. Initially the packages provided by VectorNTi, then later Geneious, were used to edit the sequences, carryout phylogenetic analyses and draw phylogenetic trees. Partial sequences from other genome regions have also been obtained.

While the RNA extracted from dried, irradiated leaves was adequate for diagnostic RTPCR, it not suitable for causal agent research. Fortunately RNA can be imported untreated, but this required performing RNA extractions on-site. The laboratory facilities at Ramu, while adequate for some pathology activities, are limited for molecular techniques. A Qiagen TissueRuptor grinder and disposable grinding probes were purchased for the project. Qiagen RNeasy Plant mini kits, purchased in Australia, were also taken to Ramu to perform the needed extractions. RNA was successfully extracted in the facilities available, although the length of time taken to bring the samples back into Australia was not ideal.

Viral mini-purifications were performed on the equivalent of 2 g fresh leaf that had been stored over $CaCl_2$ in 50 mL tubes, using methods developed by Lane; either by pelleting (http://plantpath.unl.edu/llane/text/overview.html) or use of a sucrose cushion (Lane, 1986). Viral proteins were then separated by polyacrylamide gel electrophoresis (PAGE) and detected by silver staining.

Results:

Validation of the diagnostic test

Validation of the plant crop

The Ramu stunt resistance screening trial (RST1/2010) planted in December 2010 was used as the source material for test validation. The trial includes standard varieties of known disease ratings (Kuniata *et al*., 2010). By taking leaf samples of the standard varieties, both with and without symptoms of Ramu stunt, and screening them with the diagnostic test, it allowed symptoms, disease rating and diagnostic result to be correlated.

Sampling of the plant crop was undertaken in August 2011. For each of the nine standards in three replicates, three randomly chosen stools with no apparent symptoms were sampled, and every stool showing any suspected symptom of Ramu stunt was also sampled. The symptoms were described and photographed. Of the 81 samples taken from stools showing no obvious symptoms of disease, one stool of the variety N7 tested positive for Ramu stunt (Table 1). For the plants that were showing symptoms, the diagnostic results show that all except one of these samples tested positive for Ramu stunt. As only the three most susceptible varieties (Q125, BJ7013 and Ragnar) were showing symptoms, the total number of samples taken was only 27. From the symptom descriptions and photographs taken in the field and laboratory, various symptom types could be identified. There appears to be a strong relationship between symptom type and diagnostic result (Table 2).

Table 1: Diagnostic screening results for the standards in plant crop.

Standard	**Mean Rating**	**No symptoms**		**Symptoms**	
		Number tested	**Diagnostic test result**	**Number tested**	**Diagnostic test result**
Cadmus	1.0	9	All negative	0	-
Q80	1.3	9	All negative	0	-
Co6502	2.5	9	All negative	0	-
Q124	2.7	9	All negative	0	-
Q134	4.0	9	All negative	0	-
N7	5.7	9	1 positive	0	-
Q125	5.8	9	All negative	4	All 4 positive
BJ7013	6.1	9	All negative	8	All 8 positive
Ragnar	9.0	9	All negative	15	1 negative
Total		**81**	**1 unexpected result**	**27**	**1 unexpected result**

Table 2: Occurrence of the different symptom types attributed to Ramu stunt amongst the 27 symptomatic samples. Note that some plants displayed more than one symptom type.

Symptom type	**Q125 4 stools**	**BJ7013 8 stools**	**Ragnar 15 stools**	**Diagnostic test result**
'Classic' leaf stripe	2	2	8	Always strong positive result
Stunting	1	1	8	Always strong positive result
Shoot death and yellowing	-	3	3	Always strong positive result
Faint streaks or flecks	1	3	2	Strong positive result: 1 Faint positive result:4 Negative result: 1

Although the disease levels in the trial were lower than expected, with only 27 symptomatic plants being observed amongst the standards in August 2011, validation of the diagnostic test was successful. For the plant crop, the symptoms observed by the field team correlated well with the disease rating of the standards and the diagnostic results, with only two out of 108 samples being incorrectly diagnosed. The results of the plant crop validation were presented at the ASSCT

conference in May 2012 in a paper titled "Ramu stunt: resistance screening and validation of the diagnostic test" by KS Braithwaite, R Kombukon, LS Kuniata, and RC Magarey.

Validation of the ratoon crop

Sampling of the ratoon crop was undertaken in March 2012. Unfortunately the trial had been sprayed a week before with the herbicide MSMA and the crop was showing severe herbicide damage. In addition, much of the crop was displaying strong symptoms of Ramu streak with small amounts of downy mildew. The most common leaf symptoms included brown mottling, chlorotic mottling and diffuse yellow or white stripes. Stool symptoms included stunting, grassiness and death. It was impossible to recognize the symptoms of Ramu stunt in the field with any confidence. Consequently a different sampling and screening strategy was used for the ratoon crop. For each of the nine standards, three randomly chosen stools with no apparent disease or disorder were sampled, not three per replicate. In addition, stools showing a range of leaf markings or stool symptoms that could represent either herbicide damage, Ramu streak or Ramu stunt were sampled and photographed. For the more resistant standards, nine more samples per variety were screened with the diagnostic test, giving 12 in total. For the more susceptible standards, at least nine samples were taken, so including the three symptom free samples, the total number of samples tested ranged from 12 to 22. Screening results are shown in Table 3.

Table 3: Diagnostic screening results for the standards in the ratoon crop.

Standard	**Mean Rating**	**No obvious symptoms**		**Various leaf and stool symptoms**	
		Number tested	**Diagnostic test result**	**Number tested**	**Diagnostic test result**
Cadmus	1.0	3	All negative	9	All negative
Q80	1.3	3	All negative	9	All negative
Co6502	2.5	3	All negative	9	All negative
Q124	2.7	3	All negative	9	All negative
Q134	4.0	3	All negative	9	All negative
N7	5.7	3	All negative	12	8 negative 4 RS positive
Q125	5.8	3	All negative	13	All negative
BJ7013	6.1	3	All negative	9	8 negative 1 RS positive
Ragnar	9.0	3	All negative	19	11 negative 8 RS positive
Total		**27**	**All negative**	**98**	**13 RS positive samples**

Because it was already known that there were low disease levels in the trial, the finding that only 13 out of 125 samples tested positive was not unexpected. At least the screening results show that only the most susceptible varieties test positive with the diagnostic test. Ragnar, as expected, had

the highest number of positive samples. Unfortunately, there is no possibility of correlating symptoms with diagnostic results for the ratoon crop. This shows why a diagnostic test for Ramu stunt is essential. Even in the best situations, the symptoms of Ramu stunt are variable and confusing and other cane disorders can mask the symptoms.

Ramu estate survey

The final phase of the Ramu stunt diagnostic test validation was to carry out a survey of commercial canes on the RAIL estate, particularly any showing unusual symptoms. This is basically a test of 'real world' samples and would show whether the test is useful for the Ramu pathology team.

Every year, the pathology group at Ramu carries out a disease survey. This involves assessing the levels of the major sugarcane diseases that exist so that appropriate management practices can be applied. Normally, the team surveys almost all ratoon fields in the estate by field, variety and crop class. They use a simple survey method where the surveyor assesses every 10th or 20th row of a field depending on the size of the field. The estate survey takes several weeks; in 2012, the survey occurred in November-December. The 2012 disease survey was an opportunity to combine visual diagnosis of Ramu stunt, with molecular testing.

A total of 54 samples were collected from across the whole estate (Table 4). Canes sampled were mostly commercial canes, with some nursery, propagation or experimental material, and some material from the Ramu stunt resistance screening trial RST1/2010. The plants targeted were those showing suspect Ramu stunt symptoms, such as yellowing, stunting, leaf stripes, etc, as decided by the Ramu pathology field inspection team. The vast majority of the commercial canes sampled were R570 showing various yellowing symptoms.

Twenty seven of the 54 plants (50%) tested positive for Ramu stunt. These are highlighted in red in Table 4. The vast majority of R570 that were diagnosed as being yellow (whether stunted, flecky, streaky etc) did not have Ramu stunt. By far the only symptom clearly associated with the diagnostic test is the stripe-type symptom noted as being 'classic'. Because there were so many yellow plants that did not test positive for Ramu stunt, the samples were then screened for SCYLV. Twenty two of the 54 plants (41%) tested positive for SCYLV but there is no obvious relationship between yellowing symptoms and test results.

Table 4: Symptom descriptions and screening results for the 54 samples taken during the estate survey. Varieties are arranged in order according to their Ramu stunt resistance rating. Samples testing positive for Ramu stunt are shown as (+).

Variety	Mean RS rating	Field	Ref#	Observations	RS result	SCYLV Result
RQ117	1.4	ES313	27	no obvious symptoms	-	+
		ES313	28	no obvious symptoms	-	+
R570	1.5	DS108	2	streaks on side shoots	+	-
		DS108	3	nothing obvious now	-	+
		DS108	4	faint flecks and a bit yellow	-	+
		DS108	5	excellent classic	+	-
		DS505 A	11	classic	+	-
		BS106	21	Yellow	-	-
		BS106	22	Ramu orange leaf?	-	+
		AN083	23	yellow	-	+
		AN083	24	very streaky and yellow	-	+
		AN083	25	yellow midribs	-	-
		AN083	26	stunted and yellow	-	-
		DN404	33	Streaky, yellow, DM and herbicide	-	-
		DN404	34	Streaky, yellow, DM and herbicide	-	-
		DN404	35	not obvious, also DM and herbicide	-	-
		AS109	36	Ramu orange leaf?	-	-
		AS109	37	yellow and flecks	-	-
		AS109	38	good symptoms in field, classic	+	+
		AS108	39	yellow and streaky	-	-
		ES205	45	yellow stripe	-	-
		ES205	46	faint yellow	-	+
		ES205	47	faint yellow	-	-
		ES205	48	faint yellow	-	-
B72-177	1.8	DS212	1	no leaf symptoms but plant stunted	-	+
PN92339	5.5	CS208	15	leaf stripes, classic	+	-
		CS208	16	leaf stripes, classic	+	-
		CS208	17	leaf stripes, flecky streaks, classic	+	-
		CS208	18	yellow	-	-
		CS208	19	yellow	-	-
		CS208	20	Yellow	-	-
		GN108 B	29	Streaky	-	-
		GN108 B	30	Streaky	+	-
		GN108 B	31	flecky and one streak	+	-
		GN108 B	32	flecky	+	-
BJ7013	6.1	DS505 A	12	streaky and flecky	+	+

		DS505 A	13	Classic	+	-
		DS505 A	14	very yellow and streaky, classic	+	+
PN97-54	7.0	DS504	6	strong stripes, classic	+	+
		DS504	7	very clear stripes classic	+	+
		DS504	8	classic stripe	+	+
		DS504	9	wispy yellow stripes	+	+
		DS504	10	classic stripe	+	+
		ES206	40	very strong classic	+	-
		ES206	41	normal classic	+	-
		ES206	42	normal classic	+	+
		ES206	43	Yellow	+	-
		ES206	44	very flecky and faint classic	+	+
Ragnar	9.0	CN107	50	one stripe	+	-
		CN107	51	Streaky	-	-
		CN107	52	slightly streaky	-	-
		CN107	53	streaky, classic	+	+
		CN107	54	streaks	+	+
		CN107	55	very faint streaks, classic	+	+

Unusual symptoms

There is a common belief that Ramu stunt is under control at RAIL and not causing losses to commercial production. However, in recent years there have been unconfirmed observations that:

- Disease incidence is increasing, including in commercial fields, and in varieties that have not shown it previously
- The symptoms are becoming more extreme

RAIL-bred canes were considered resistant if they were released from the breeding program with a PN designation. The situation is now such that RAIL trials use PN canes in the spreader rows because some appear to be more susceptible than Ragnar. During 2014, there was a high incidence of Ramu stunt in the commercial nursery with the plants showing a wide range of symptoms. One example was the Australian variety Q198 where six different stools were sampled showing three different symptom types (stunting, classic leaf stripe and yellow midribs). All tested positive with the diagnostic test. Infected plants now have to be rouged from the commercial nursery to minimize spread of the disease.

These observations suggest that there may have been a viral strain change. Seven isolates of Ramu stunt collected from commercial canes, mostly from 2006-2007, were sequenced prior to the start of this project. It was decided to sequence several more recent isolates showing interesting symptoms to see if sequence variation could indicate a strain change. Another eight more isolates have now been sequenced. Percent nucleotide identity across a 1.2kb fragment from RNA4 ranged from 99.3% to 100% identity, indicating only very minor sequence changes in this gene region (Appendix 1; see later Figure 4). Figure 1 shows examples of the symptom types thought to be more severe that the usual “classic”.

Table 5. Fifteen isolates of Ramu stunt collected from commercial canes on the RAIL estate sequenced for evidence of strain variation.

Collection Date	Variety	Ref #	Location	Crop type	Symptoms

Isolates sequenced prior to 2009033					
2001	Ragnar				
2006	N7				
2006	Q85N1802				
2007	BJ7013				
2007	PN93-18				
2007	Q125				
2007	Ragnar				Classic stripe (Fig. 1A)
Isolates with unusual symptoms					
21/11/12	PN97-54	40	ES206	Nursery 1R	Like maize stripe virus (Fig. 1B)
05/04/13	BJ7013	18	DS505a	RS 2011 trial	Extreme yellow stripes (Fig. 1C)
29/07/14	PN97-57	7	BN401	RAIL trial spreader row	extreme symptoms (Fig. 1D)
31/07/14	PN92-339	60	DS409	Commercial nursery	grassy
31/07/14	R570	63	EN102	Commercial field	very strong stripes
31/07/14	Q198	64	DS408	Commercial nursery	stunted
31/07/14	Q198	66	DS408	Commercial nursery	very strong classic
31/07/14	Q198	69	DS408	Commercial nursery	yellow midribs (Fig. 1E)

Figure 1. Examples of leaf symptoms seen in Ramu stunt-infected plants. A: Ragnar exhibiting the "classic" symptoms of pale and mid-green stipes with a rough edge; B: PN97-54 showing symptoms similar to Maize stripe virus; C: BJ7013 showing yellow stripes; D: PN97-57 showing mottle stripes; E: Q198 with yellow midribs.

Surveys and pathogenic variation

A specific objective was to validate the diagnostic test and define the distribution and variability of Ramu stunt in PNG. Ramu stunt symptoms are known to vary in commercial varieties and it would not be surprising if similar symptom variation occurred in garden and wild canes. There is only limited knowledge of how widespread the disease is in garden / wild canes and grasses. Prior to this project, the disease was thought to be widespread throughout PNG. However the true distribution could not be confirmed until a diagnostic test was developed. Because of the biosecurity risk to Australia posed by Ramu stunt, it is important to assess the prevalence of the disease in PNG and if alternate grass hosts exist. The risk of a disease incursion is greater if the pathogen is abundant, widespread, has a wide host range or a high capacity for pathogenic variation. A consequence of new races or strains appearing is that existing resistance ratings and diagnostic tests may become obsolete. These risks can only be determined by carrying out surveys. The surveys described here mostly targeted garden canes *Saccharum officinarum* (noble or chewing canes) and *Saccharum*

edule, and wild *Saccharum robustum. Miscanthus, Saccharum spontaneum* and grassy weeds were sampled occasionally.

Surveys

During the project period, several short and long surveys were carried out. Both the Ramu Valley and Kassam Pass are close to the RAIL estate and can be visited in an afternoon. Goroka and Madang can be reached by driving but need an overnight stay. Alotau requires flying and an overnight stay. The samples collected during the surveys showed various leaf stripes, flecks or chlorosis. The full details for all samples taken during the project are listed in Appendix 2. Brief descriptions of the surveys are given here:

Ramu Valley 2009

Two short visits to the Ramu Valley were carried out in 2009 (Appendix 2.1) with assistance from Iki Agoname, Wamba Batimane and Lastus Kuniata. Of the wild and garden canes and grasses, only three noble canes from a family garden plot, known as Block 48 near Kesewai, and a *Eumetopina* nymph from one of those canes, tested positive (Figure 2A).

Kassam Pass and Ramu Valley 2010

Two districts were surveyed in late August-September 2010 (Appendix 2.2). On 31 August, Kathy Braithwaite, Nicole Thompson, Rob Magarey and Lastus Kuniata travelled through the Kassam Pass to Yonki Dam in the Eastern Highlands Provence. This region is about 1500m above sea level. *S. robustum, Miscanthus* and Job's Tears (*Coix lacryma-jobi*) were collected from the road-side and *S. officinarum* from a traditional PNG garden.

Miscanthus is a suspected host for downy mildew and Job's Tears is an alternative host for Fiji disease virus. No sample tested positive for Ramu stunt.

On the following day, Kathy Braithwaite, Nicole Thompson and Wamba Batimane travelled north through the Ramu Valley as far as Walium Station. *S. officinarum* and maize were collected from traditional gardens, and one *S. robustum* sample from the road-side. Sample 13 (Figure 2B), a noble cane from the Sausi Evangelical Church, showed the "classic" symptom of leaf stripes and tested positive. Several noble canes from Block 48, a site tested previously, were again positive for Ramu stunt.

Kassam Pass, Goroka, Madang, Ramu Valley, Surinam Valley 2010

A longer survey over five days was carried out in June 2011 (Appendix 2.3). The Kassam Pass and Goroka regions were visited first by Kathy Braithwaite, Nicole Thompson, Rob Magarey, Peter Samson, Kaile Korowai and Baina. Then the Ramu Valley through to Madang was visited next by Kathy Braithwaite, Nicole Thompson, Peter Samson and Kaile Korowai. Finally, a short visit was made to the Surinam River Valley by Kathy Braithwaite, Nicole Thompson, Peter Samson, Kaile Korowai. Samples taken from road sides consisted of mostly *S. robustum, Miscanthus* and grassy weeds, while samples from traditional gardens were mostly *S. officinarum*, *S. edule* and grassy weeds. Only one sample had obvious Ramu stunt symptoms: sample 10, a noble cane from the Asasa Apostolic Church (Figure 2C) in the Ramu Valley, and it tested positive.

RAIL estate 2012

The Entomology Technician Wamba Batimane lives in a residential village on the RAIL estate. He very kindly volunteered to plant and maintain some Ramu stunt-infected noble canes in his garden. Four noble canes were sampled in March 2012 (Appendix 2.4) and three tested positive. W3 was the variety "Tinora" collected from Habina in Eastern highlands. It was very yellow and streaky, without the classic symptoms (Figure 2D). W4 was a red-stalked variety popular around estate. The main stalk was dead with stunted side shoots. The objective behind sampling these plants was to show if the Highland plant (W3) had a "highland" isolate, when compared to the local plant (W4), which was expected to have a "Ramu" isolate.

Figure 2: Examples of Ramu stunt-infected noble canes seen during the surveys. A: healthy (left) and infected (right) stool at Block 48; B: sample 13 from Sausi; C: sample 10 from Asasa; D: sample W3 from Wamba's garden.

Alotau 2013

The final survey for the project was done in the Alotau region in April 2013 and involved Rob Magarey, Kathy Braithwaite, Nicole Thompson, Judi Bull and Andrew Greet and a driver supplied by NBPOL (Appendix 2.5). Most samples were taken from traditional gardens and were mostly *S. officinarum*. Three samples showing interesting fungal diseases of sugarcane were also collected for their scientific interest.

An extensive disease survey of PNG was carried out in 2001 as part of an ACIAR-funded project. During this survey, Rob Magarey found excellent symptoms of Ramu stunt in only a very small number of locations and one of these was Alotau (Table 6). However when these two samples were screened with the Ramu stunt diagnostic test, they did not test positive. Despite this, they showed the characteristic 35kDa protein associated with Ramu stunt-infected plants (Braithwaite *et al.*, 2007). This suggested that the virus in the Alotau plants may represent a different strain from that found in plants in the Ramu region to the north.

Alotau was chosen as the site of the final 2009033 survey, in the hope that more of these interesting Ramu stunt plants could be observed. Fortunately, one garden at Bitu Village had several stools showing either classic or unusual extreme symptoms (Figure 3). When these plants were screened with the diagnostic test, they did not test positive. As a result of attempts to sequence regions of the Ramu stunt Tenuivirus genome, a large range of PCR primers were available, in addition to the two used in the diagnostic test. Using various combinations of existing primers, and newly designed ones, the 1.2 kb region from each of the four 2013 Alotau viral isolates and the two 2001 Alotau isolates was eventually amplified and sequenced.

Figure 3: Strong striping and flecking symptoms seen in four noble canes infected with Ramu stunt at Bitu Village, Alotau. From left to right are samples 26, 27, 28 and 29.

Table 6: Two samples suspected to have Ramu stunt collected from the Alotau region in 2001 as part of an ACIAR funded project. Details are taken from the original sample database.

Sample	Suspected disease	Lat	Long	Species	Symptoms	Comments
220	Ramu stunt - suspect	10° 18.48	150° 27.22	officinarum	Excellent symptoms - classic stunt	Good symptoms - Garden on the edge of Alotau harbour - not very well cared for.
224	Ramu stunt - suspect	10° 17.70	150° 23.71	officinarum	Good symptoms	Bitu Village. Garden just off Airport - Alotau road.

Genetic variation between survey samples

Until the Alotau survey, the number non-commercial canes testing positive for Ramu stunt with the diagnostic test was 11, from four locations. All locations have been within, or close to, the Ramu Valley and all host plants have been noble canes from traditional gardens. A summary of all positive samples is shown in Table 7. *Eumetopina* collected from an infected noble cane also tested positive. No Ramu stunt has been detected in *S. robustum, S. edule, Miscanthus, S. spontaneum* or any of the grass weeds tested so far (blady grass, elephant grass, guinea grass or itch grass). At this stage it appears that Ramu stunt is not common or widespread away from the RAIL estate.

The 1.2 kb fragment spanning the target region has previously been sequenced from Ramu stunt-infected commercial canes on the RAIL estate. The corresponding region has now been sequenced from 11 Ramu stunt-infected noble canes (Table 7). The extent of sequence variation between viral sequences from commercial canes and from noble canes is presented in a phylogenetic tree (Figure 4). As mentioned in Results Section 1, there is minor sequence variation between commercial isolates on the RAIL estate. Isolates from the Ramu Valley are quite different, but not so different as to prevent the diagnostic primers from binding to their target region. The isolates collected from Wamba's garden were similar to each other despite the source of the cane and apparently intermediate between the estate and Valley. This suggests that they probably became infected at Ramu. The Alotau isolates were genetically very different to all other isolates to such an extent that the diagnostic test had to be modified for those isolates to be detected. Confirmation that they were infected with Ramu stunt came from the presence of the characteristic 35 kDa protein seen in viral purifications (see later; Figure 12). Alotau isolates shared only 80.6-82.0% sequence identity with the Ramu estate and Valley samples (Appendix 3). Only minor sequence variation existed amongst the 2001 and 2013 Alotau isolates. These results suggest that it would be valuable to conduct more

surveys in other parts of the country, particularly in the south, closer to Australia. The results from this work was presented as a poster at ASSCT titled: "The importance of disease surveys for understanding quarantine pathogens" by KS Braithwaite, LS Kuniata and RC Magarey.

Table 7: All survey samples collected since July 2009 which have tested positive for Ramu stunt.

Location	**Reference #**	**Collection date**	**1.2kb sequence in Fig.4**
Block 48, Kesewai, Ramu Valley	3	21/7/09	yes
	9	15/12/09	
	10	15/12/09	yes
	nymph	15/12/09	
	21	1/9/10	
	22	1/9/10	
	23	1/9/10	yes
Sausi Evangelical Church, Ramu Valley	13	1/9/10	yes
Asasa Apostolic Church, Ramu Valley	10	16/6/11	yes
Wamba's garden, Ramu estate	W2	13/3/12	
	W3	13/3/12	yes
	W4	13/3/12	yes
Bitu Village, Alotau	26	6/4/13	yes
	27	6/4/13	yes
	28	6/4/13	yes
	29	6/4/13	yes

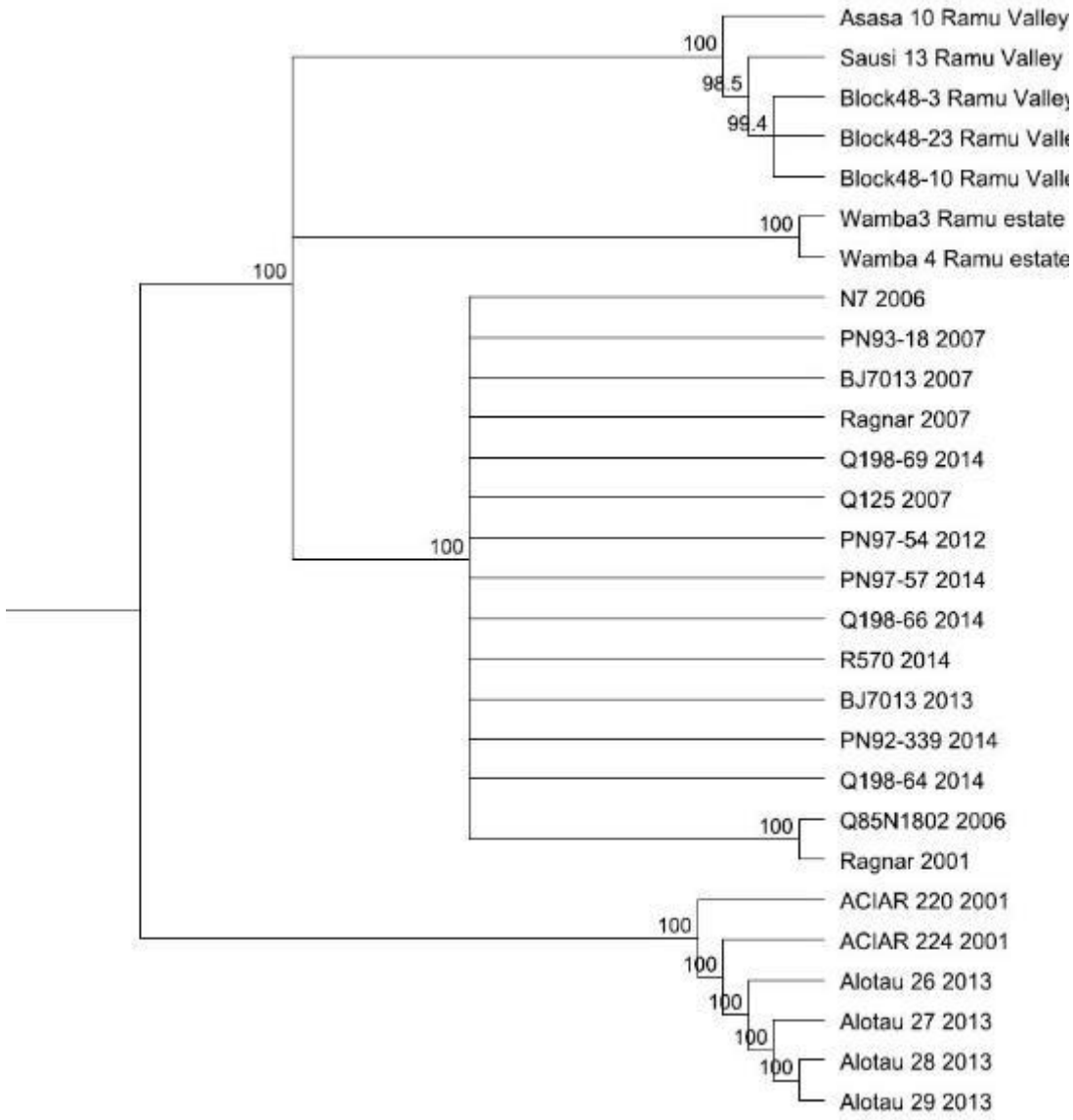


Figure 4: Phylogenetic tree showing the genetic relationship between 15 Ramu stunt isolates from commercial canes, 7 isolates from noble canes around the Ramu Valley and estate and 6 isolates from Alotau. A 1.2kb region of RNA4 was sequenced, aligned using ClustalW and the tree prepared using the UPGMA tree build model with 1000 bootstraps.

Other Tenuiviruses

During surveys in the Ramu Valley, the common weed itch grass (*Rottboellia cochinchinensis*), was regularly found to show leaf stripe symptoms extremely similar to those of Ramu stunt (Figure 5A) but consistently failed to give a positive diagnostic result with the Ramu stunt test. On further investigation it was found that the plants were infected with a different Tenuivirus, Maize stripe virus (MSV). A diagnostic set of primers has now been developed for this virus. MSV infecting maize has also been observed on the RAIL estate (Figure 5B), so it appears that the two Tenuiviruses can exist in close proximity.

Figure 5: Symptoms of Maize stripe virus in *Rottboellia* (A) and maize (B) seen in and around the Ramu estate.

Transmission experiments

Ramu stunt was recognized as a disease in 1985 and at that time, the causal agent was unknown. Research carried out by BSES and SRA identified a Tenuivirus as the potential cause of the disease but the process of rigorously demonstrating that a particular biological agent is the cause of a plant disease, known as Koch's Postulates, still had to be carried out. For Ramu stunt, this requires caged transmission trials, where the insect vector *Eumetopina flavipes* was used to transmit the virus from known infected plants to healthy test plants. Unfortunately Koch's Postulates in the strictest sense has not been carried out because a closed system was not used. Despite this, we have been able to experimentally transmit the virus and confirm the transmission by symptomology, RT-PCR, sequencing and protein analysis.

Experimental setup

The experimental system at Ramu was based on three cages (Figure 6). Two were covered in high quality white insect proof mesh. One of these had no openings and contained the control healthy plants (HH) which were to remain untouched throughout the course of the experiment. The second cage contained the healthy test plants (HD) and had openings to allow the introduction of the insects which were to transmit the virus. The plants were the variety Ragnar, sourced from the quarantine glasshouse in Indooroopilly. The third cage was covered in green shade cloth and contained known, infected stools of Ragnar (DD) sourced from a Ramu stunt resistance screening trial. *Eumetopina* were collected from the field and introduced to the green cage to feed on the infected cane and acquire the virus. The infected insects were collected and transferred to the test cage where they transmitted the disease.

Two previous attempts were made to transmit the disease. The first attempt in 2012, used feeding periods of only one week, which was found to be insufficient for virus acquisition. Another attempt was made in 2013 but due to very dry conditions, the *Eumetopina* could not establish in large numbers in the green cage.

The third, successful attempt was made between March and October 2014. For this attempt, the *Eumetopina* remained in the source cage for a minimum of 35 days, which was considered sufficient time for planthoppers to complete their life cycle. Also on this occasion, better care and attention was given to the plants by the Entomology group, particularly Baina. Despite the extra care, the trial still had problems. Firstly, *Eumetopina* numbers in the cages were hard to maintain because of predators; and secondly, mealybug infection was very high and their damage obscured the symptoms of Ramu stunt. However the disease was able to be successfully transmitted from infected to healthy plants and the two main symptoms of Ramu stunt infection (leaf steaks and

stunted stalks) were observed in the test plants. Various molecular tests confirmed that test canes were infected with the same virus as found in the source plants.

Figure 6: Three cage set-up used in the transmission trials. Cages from left to right are HH (healthy control); HD (test cage containing initially healthy cane destined to become infected) and DD (source cane known to be infected with Ramu stunt).

Experimental Progress

The time-line for the trial and summary of results is shown in Table 8. Several collections of virus-free *Eumetopina* were made and placed into the source cage for 7 to 62 days. At each time point, 40 *Eumetopina* were kept aside for screening with the RT-PCR test. At the time of transfer (Figure 7), 68% had acquired the virus. The test plants were then exposed to the *Eumetopina* for two months. At the July sampling stage (Figure 8), *Eumetopina* numbers in both the source and test cages were extremely low, mostly due to predation by other insects. However the test plants were showing midrib puncture marks, so the *Eumetopina* had been active and laying eggs for some of that time. Leaf symptoms were observed in three of the four stools, although no stunting was observed. Leaves from the symptomatic stools tested positive with the diagnostic test. Thus virus transmission has been experimentally demonstrated for Ramu stunt. The plants were ratooned and re-sampled three months later in October (Figure 9). In contrast to the July sampling, this time leaf symptoms were less clear (probably due to severe mealybug damage), but plant stunting was very obvious. Stools consisted of stunted and non-stunted stalks which were separated for sampling and testing. The diagnostic test showed a very strong relationship between stunting and a positive test result. Stool 3, which was uninfected in July, was still uninfected. The source plants, derived from infected setts planted in 2012, were completely dead, clearly showing the long term effects of Ramu stunt infection.

Table 8: Time line for the successful caged transmission experiment carried out in 2014.

	Source cage (DD)	Test cage (HD)	Control cage (HH)
Plant source	RS2010 trial CN107	Indooroopilly glasshouse	Indooroopilly glasshouse
Step 1 March 2014: Begin trial	Leaves show Ramu stunt symptoms. Stalks thin and stunted 8 Leaf samples tested: all positive	Leaves healthy and green. Stalks tall and not stunted in any way. 8 Leaf samples tested each: all negative	

Step 2 Collect insects	*Eumetopina* collected from ES104 on 5 occasions between 28th March and 22nd May 660 introduced to the cage. 40 kept aside for testing: all negative		
Step 3 May 2014: Transfer insects	Cage opened and *Eumetopina* collected. 40 kept aside for testing: 68% positive Plants look poorly due to mealybugs. Ramu stunt symptoms difficult to see. 6 Leaves samples tested: all positive	*Eumetopina* transferred from green cage to test cage	
Step 4 July 2014: Sample after two months	Cage opened and *Eumetopina* collected. 40 tested: 40% positive Plants very sickly. Sample mixed leaves: all positive	Cage opened and *Eumetopina* collected. 40 tested: 1 positive Sample from each of the 4 stools: Stools 1, 2 and 4 positive	Sample leaves from 5 stools: all negative
Step 5 October 2014: Ratoon samples	All plants dead. No samples taken.	Harvest every stalk from the 4 stools. Stalks separated into thin and stunted or tall (non-stunted). Stools 1, 2 and 4 positive	Sample leaves from 4 stools: all negative


Figure 7: Activities in May: Harvesting *Eumetopina* from the DD cage (left) and transferring to the HD cage (right).

Figure 8: Activities in July: Stunted and unhealthy condition of plants in the DD cage (left) and four harvested stools from the HD cage (right).

Figure 9: Activities in October: healthy green cane in the HH cage (left) and grassy, yellow appearance of canes in HD cage (right) with some tall stalks visible (arrow).

Details of the analyses carried out

At each of the major time points outlined in Table 8, a symptom description was recorded, leaves were sampled, wiped with alcohol wipes, photographed and stored in tubes of $CaCl_2$. Samples were either punched leaf-disks stored in 5mL tubes for RT-PCR or 2g pieces stored in 50mL tubes for viral mini-purifications. All analyses were done at the SRA Indooroopilly laboratory. A detailed description of the symptoms and the diagnostic screening results for July and October are shown in Table 9. All Ramu stunt RT-PCR positive samples are shown in red. There was an excellent relationship between symptoms and diagnostic result. Agarose gels showing the RT-PCR results for Ramu stunt are shown in Figures 10 and 11.

Viral purification, followed by polyacrylamide gel electrophoresis (PAGE) was performed on a subset of samples. Prior to this project, it was found that Ramu stunt-infected plants contain a large amount of a 35 kDa protein not found in uninfected plants (Braithwaite *et al*., 2007). This is believed to be the disease specific protein or major non-capsid protein and is very characteristic of Tenuivirus infections. Once again there was an excellent relationship between symptoms, diagnostic RT-PCR and viral protein result. Polyacrylamide gels showing protein patterns for the viral mini-preps are shown in Figure 12. The figure also shows several other interesting results which have been mentioned previously in this report. The Alotau survey samples were suspected to be infected by Ramu stunt even without a diagnostic test result because they showed the 35kDa protein. Figure 12 lane 22 shows sample 26 from Bitu Village. Similarly, *Rottboellia cochinchinensis* was suspected to be infected with Ramu stunt despite a negative diagnostic test result. However further

investigation revealed that it was a different Tenuivirus and the characteristic band shown in Figure 12 lane 25 is larger than that seen for Ramu stunt.

During this project, a 1.2 kb fragment representing part of the Tenuivirus RNA4 has been sequenced from over 30 Ramu stunt isolates, including two from Ragnar. To confirm the symptom observations, diagnostic screening results and viral protein analysis, six isolates, from both plants and *Eumetopina* in the trial, were sequenced and compared to previously sequenced Ragnar viral isolates (collected in 2001 and 2007). Sequence analysis showed that all isolates share 99.3-100% sequence identity over the 1.2 kb (Appendix 4), suggesting that the same viral isolate has been transmitted throughout the experiment. The minor sequence variation observed is not related to plant *vs* insect, source *vs* test plants or date of collection.

Table 9: Detailed symptom description and RT-PCR screening results for the July and October samples.

	Stool	**Ramu stunt symptoms and sample type**	**RT-PCR result**
July (2 month) sampling			**See Fig. 10 lane:**
Source cage (DD)		Leaves yellow, fungal and insect damage.	17-19: All positive
Test cage (HD)	1	4 leaves sampled; one has definite symptoms	10: positive
	2	4 leaves sampled; two have definite symptoms	11: positive
	3	4 leaves sampled; none have reliable symptoms. Probably *Eumetopina* or mealybug damage	12: negative
	4	4 leaves sampled; three have definite symptoms	13: positive
Control cage (HH)		5 stools, No RS symptoms	4-8: All negative
October Ratoon sampling			**See Fig. 11 lane:**
Source cage (DD)		All plants dead. No samples taken.	
Test cage (HD)	1	6 thin and stunted stalks: symptoms visible.	14-15: A and B positive
	2	8 thin stunted stalks: leaves very yellow. 1 tall stalk: outer leaves are dark green.	16-17: A and B positive 8: C negative
	3	4 thin stalks: leaf mealybug damage? Tall stalk 1: no obvious symptoms of RS Tall stalk 2: no obvious symptoms of RS.	18: A negative 9: B negative 10: C negative
	4	2 Medium stalks: 1 leaf has yellow stripes.	19: A negative 20: B positive 11: C negative

		8 very small stalks: leaf mealybug damage? Tall stalk 1: no obvious symptoms of RS Tall stalk 2: no obvious symptoms of RS	12: D negative
Control cage (HH)		4 stools. Leaves mostly green with a few mealybugs and brown leaf spots. No obvious symptoms of RS	4-7: All negative

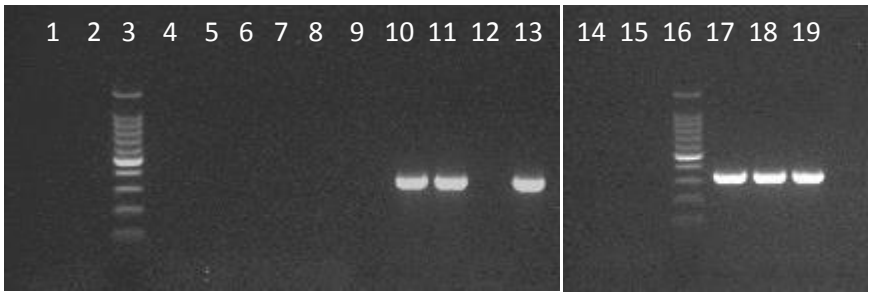


Figure 10: RT-PCR diagnostic screening results for July samples. Lanes 1 and 14 are no template controls; lanes 2 and 15 are healthy controls; lanes 3 and 16 are size markers; lane 9 is blank; lanes 4-8 are the 5 stools of HH which all tested negative; lanes 10-13 are the 4 stools of HD where stool 3 has tested negative; lanes 17-19 are 3 mixed samples from DD where all have tested positive.

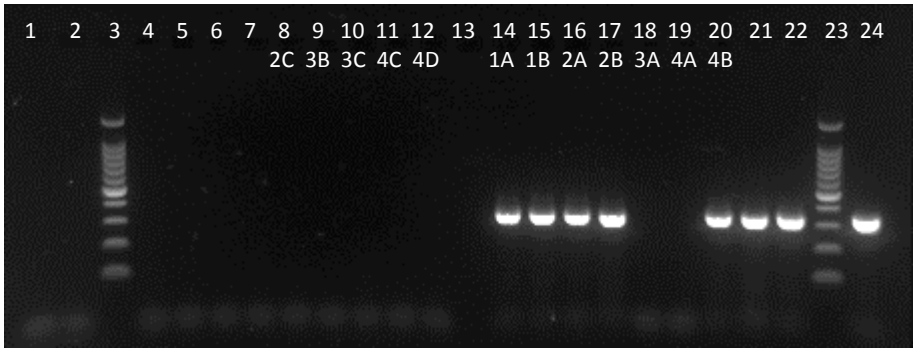


Figure 11: RT-PCR diagnostic screening results for October samples. Lane 1 is no template control; lane 2 is healthy control; lanes 3 and 23 are size markers; lane 13 is blank; lanes 4-7 are the 4 stools of HH which all tested negative; lanes 21, 22 and 24 are positive controls. HD stools are as follows:

- 14 and 15: HD1 samples A and B both positive
- 16, 17 and 8: DH2 samples A, B and C, respectively. Only A and B tested positive.
- 18, 9 and 10: DH3 samples A, B and C, respectively. None tested positive.
- 19, 20, 11, 12: DH4 samples A, B, C and D, respectively. Only B tested positive.

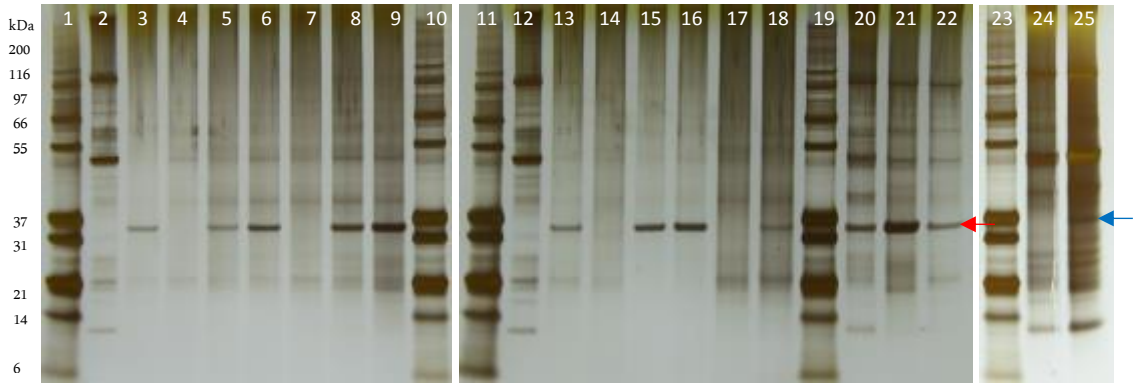


Figure 12. Silver stained, polyacrylaminde gels showing separation of viral proteins. Lanes 1, 10, 11, 19 and 23 are protein size markers. Approximate sizes in kDa are shown on the left hand side. Lane 2 and 12 are healthy Ragnar from the Indooroopilly glasshouse. Lanes 20-22 are Ramu stunt-infected positive controls as follows: Ragnar (20), PN97-54 (21) and noble cane 26 from Bitu, Alotau (22). All show the characteristic 35kDa band (red arrow). Lane 24 is healthy *Rottboellia* and lane 25 is Maize stripe virus infected *Rottboellia* which also shows a characteristic band (blue arrow). Transmission samples are as follows:

Lane 3 and 13: DD May, both showing the characteristic 35kDa band
Lane 4: HH July showing no band
Lanes 5-8: the 4 stools of HD July where stool 3 (lane 7) is lacking the 35kDa band
Lane 9: DD July showing the characteristic 35kDa band
Lane 14: HH October showing no band
Lanes 15-18: the 4 stools of HD October. Samples correspond to Table 9 and Fig. 11 where lane 15 is HD1 A and B; lane 16 is HD2 A and B; lane 17 is HD3A which lacks the 35kDa band; and lane 18 is HD4B.

Other aspects of host/virus biology

Distribution of the Ramu stunt virus within plants

Many of the basic aspects of the Ramu stunt – sugarcane host-pathogen system are still not well understood. Tracking the spread and distribution of the virus within the sugarcane plant would help to understand how the disease is spread through infected planting material, and the contribution of ratooning towards increasing the incidence of the disease.

Distribution within leaves

It is essential to identify the optimal tissue to sample for any diagnostic test. The distribution of the virus in leaves of symptomatic plants was studied using Ragnar from the spreader rows of the resistance screening trial RST1/2010. Four stools of Ragnar, each showing strong, clear leaf stripe symptoms of Ramu stunt on at least one stalk were selected. Individual stalks with and without symptoms were then identified and the youngest unfurled leaf (YUL), top visible dewlap (TVD) leaf and oldest green leaf (OGL) were sampled. Leaves were further sub-sampled into tip, middle and base. For stools that were not showing symptoms, no positive RT-PCR diagnostic results were obtained, regardless of the leaf sampled or the leaf sub-sample position (Table 10). For stalks with leaf symptoms, if the sampled leaf was showing symptoms, then a positive result was always obtained. If the leaf was not showing symptoms then a positive result could sometimes be obtained,

but not in all cases. Of the sub-sample positions (tip, middle and base), the most reliable diagnostic result was obtained from the region of the leaf showing the leaf stripe symptoms. Thus it appears that within the plant, high viral titre only occurs within leaves showing symptoms, particularly the specific area of the leaf with the stripe symptom. Positive diagnostic results could be obtained from non-symptomatic leaves from symptomatic stalks, but the results were not as reliable.

Table 10: Leaf position experiment. Stalks with and without symptoms of Ramu stunt were sampled and screened with the diagnostic test. A red cross indicates that a positive RT-PCR results was obtained.

	Oldest green leaf			**Top visible dewlap leaf**			**Youngest unfurled leaf**		
	Base	**Mid**	**Tip**	**Base**	**Mid**	**Tip**	**Base**	**Mid**	**Tip**
Stool 1 Leaf symptoms Diagnostic result	none -	none -	none -	none -	none -	none -	none -	none -	none -
Stool 2 Leaf symptoms Diagnostic result	none -	none -	none -	none -	none -	none -	none -	none -	none -
Stool 3 Leaf symptoms Diagnostic result	none -	none +	none -	none +	none -	none -	none +	yes +	yes +
Stool 4 Leaf symptoms Diagnostic result	none -	none -	none -	yes +	yes +	none +	none +	yes +	yes +

Distribution of the virus within young plants

The diagnostic test was used to screen a range of plant tissues and plants of various ages during two sampling periods in May and July 2014. The variety Ragnar was used and the plants were sourced from the SRA and RAIL Ramu stunt 2014 screening trials. Only Ragnar test cane from the trials was sampled, not spreader cane, as this way the plant must have acquired the virus via the insect vector *Eumetopina* within the course of the trial, and not from infected planting material.

Table 11 shows the early stages of virus acquisition in young plant cane and resultant spread throughout the plant. At 8 weeks after planting, no symptoms are visible and no virus can be detected in leaf tissue. By 12 weeks, the potential symptoms of Ramu stunt infection are just starting to become apparent and yet all plants parts test positive for the virus. Thus spread within the plant is very rapid and precedes the appearance of reliable symptoms. By 17 weeks, reliable symptoms can be seen on Ragnar and the sampling and screening confirm that all plant parts can harbor the virus.

Table 11: Distribution of the virus in young plant cane. A red cross indicates that a positive diagnostic test result was obtained.

Trial plant date	**Sampling date**	**Field site**	**Crop stage**	**Symptoms**	**Tissue tested**	**RT-PCR Results**
3/04/2014	28/05/2014	BN401 RAIL trial	plant crop 8 weeks	no definite symptoms yet	Leaf plant 1 Midrib plant 1 Leaf plant 2 Midrib plant 2	- - - -
7/05/2014	29/07/2014	BN401 SRA trial	plant crop 12 weeks	symptoms unreliable and not clear	Leaf 1 Midrib 1 Leaf 4 Midrib 4 Meristem Stalk and base Roots	+ + + + + + +
3/04/2014	29/07/2014	BN401 RAIL trial	plant crop 17 weeks	definite symptoms on leaf 4	Leaf 1 Midrib 1 Leaf 4 Midrib 4 Meristem Stalk and base Roots	+ + + + + + +

Distribution of the virus within young plants

Table 12 shows the distribution of the virus in mature cane plants. For this sampling, past screening trials were used. For the purposes of this experiment, leaf 1 is the first fully unfurled leaf and usually leaf 1, leaf 4 and leaf 7 (usually the oldest green leaf) were sampled. If symptoms were not present on any of these three leaves, it was noted in the table. Side-shoots (arising from buds on the standing stalks) and suckers (arising from the ground) with or without symptoms were sampled as indicated. Unfortunately no first-ratoon cane was available in May or July 2014 because harvest of the April 2013 trial had been delayed. The screening results are not as consistent as for young cane but confirm the results in Table 10. Generally symptoms are only seen on the younger leaves and only these test positive. No leaf-4 or leaf-7 have tested positive. Nevertheless, all plant parts, including leaves, midribs, stalk, stalk base and roots, whether part of the main stalks, or suckers or side-shoots, can harbor the virus.

Table 12: Distribution of the virus in mature cane. A red cross indicates that a positive diagnostic test result was obtained.

Details	**Symptoms**	**Tissue**	**RT-PCR results**
Year planted: April 2013 **Sampling date:** 27/05/2014 **Field site:** ES104, SRA trial **Crop stage:** Plant crop, 13 months	Symptoms visible on leaves 1 and 2	Leaf 1 Midrib 1 Leaf 4 Midrib 4 Leaf 7 Midrib 7 Meristem Stalk Stalk base Roots	- + - - - - + + + +
Year planted: April 2013 **Sampling date:** 29/07/2014 **Field site:** ES104, SRA trial **Crop stage:** Plant crop, 15 months	Top of main stalk lost to borers Symptoms visible on sideshoot leaves 2 and 3 Symptoms visible on sucker	Main stalk Main base Main roots	faint + - -
		Sideshoot leaf 1 Sideshoot midrib 1 Sideshoot leaf 4 Sideshoot midrib 4 Sideshoot meristem Sideshoot stalk	+ + - - + –

		Sucker leaf Sucker midrib Sucker base	- - +
Year planted: 2011 **Sampling date:** 27/05/2014 **Field site:** DS505a, SRA trial **Crop stage:** 2nd ratoon	Symptoms visible on leaves 1, 2 and 3 Sucker has 3 leaves and no symptoms	Leaf 1 Midrib 1 Leaf 4 Midrib 4 Leaf 7 Midrib 7 Meristem Stalk Stalk base Roots	+ + - - - - + + - -
		Sucker leaf Sucker meristem Sucker base	- faint + -
Year planted: 2010 **Sampling date:** 28/05/2014 **Field site:** CN107, SRA trial **Crop stage:** 3rd ratoon	Plant stunted and leaves yellow	Main stalk sprouting eyes Main stalk non-sprouting eyes Main stalk leaf Main stalk midribs	+ + + +
		Sideshoot leaf and midrib Sideshoot meristem Sideshoot base	+ + +
		Sucker leaf 1 Sucker midrib 1 Sucker meristem Sucker stalk Sucker base Sucker roots	+ + + + + +

Further characterisation of the Tenuivirus genome

Tenuiviruses have large genomes with four to six segments, the largest being 9 kb, and the smaller ones having an ambisense arrangement. In addition to the 1.2 kb of sequence obtained from RNA4 of the Ramu stunt genome, several other contigs over 1 kb have been obtained (Table 13). They all have homology to Tenuiviruses or the closely related group, Bunyaviruses. The longest match is to the viral RNA polymerase. This is normally about 9kb in other Tenuiviruses, so the full sequence is yet to be obtained. Several different sequences with some homology to a nucleocapsid (coat protein) have been obtained, so the exact sequence is yet to be determined. Only the longest sequence is listed in Table 13. It should be noted that the GeneBank matches are using the program BlastX, not the more common BlastN. There is almost no nucleotide homology to any organism (virus or otherwise) using BlastN. This indicates that the Ramu stunt virus is unique and not closely related to any other virus, although all evidence suggests that it is a Tenuivirus. As the Ramu stunt virus is very poorly characterised and its full relationship to other known Tenuiviruses is unknown, it is important to complete the sequencing. However, sequencing a genome of the size and complexity of Tenuiviruses by traditional methods is tedious but modern next generation sequencing technologies are now available to rapidly complete this.

Table 13: All Ramu stunt genome sequence contigs of over 1kb.

Size	Identity	Top Blast-X match	E value
1.2kb	Major non-capsid protein	Maize yellow stripe virus (proposed Tenuivirus)	E 10^{-6}
3.9kb	RNA polymerase	Rice stripe virus (Tenuivirus)	E 10^{-89}
1.4kb	Nucleocapsid	Sandfly fever Naples virus (Bunyavirus)	E 10^{-5}

Further confirmation that Ramu stunt is caused by a Tenuivirus has come from electron microscopy observations of viral preps. Two viral mini-preps (PN97-54 and Ragnar) were observed with the assistance of John Thomas and Kathy Crewe at the Ecosciences Precinct (Figure 13). Tenuiviruses are described as having particles of a filamentous, indeterminate length, 3-12 nm in diameter, and linear, circular, helical or supercoiled configuration. PN97-54 was sampled during the estate survey and showed very strong classic symptoms. Gel electrophoresis of this sample showed that it had a large amount of the 35kDa protein, relative to its total protein load (Figure 12 lane 21). This is possibly reflected in the extensive number of particles observed under EM. The Ragnar sample was collected in 2007 and is the reference isolate used in sequencing (for example Appendix 1 and 4). The age of the sample may explain the lower viral titre seen.

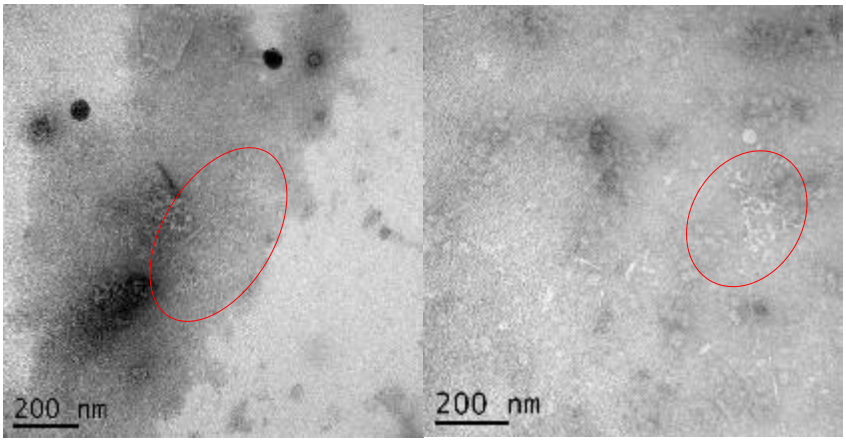


Figure 13: Tenuivirus particles observed under electron microscopy. A: PN97-54; B: Ragnar. The thin filamentous particles are indicated by the red circles.

Discussion

The validation of the Ramu stunt diagnostic test using the standards in the plant crop of the Ramu stunt resistance screening trial gave excellent correlations between the test results, the 'classic' leaf stripe symptom and Ramu stunt resistance ratings. It is clear that the leaf stripe symptom shown in Figure 1A is the definitive symptom for Ramu stunt, although plants showing other symptoms may still be infected. A diagnostic test is essential in these situations. This is similar to Fiji disease virus, where the only definitive symptom is the presence of galls, but plants may also display stunting, dead spindles and distorted tops.

Screening results from the estate survey found that the majority of plants thought to be infected because of leaf yellowing, particularly R570, were not. Plants which show yellowing only, without any other kind of symptom, are not infected with Ramu stunt. The reason why so many yellow plants were not positive for Ramu stunt could be because (1) they are affected by another disease, an insect or nutritional problem, or even herbicide damage or (2) perhaps Ramu stunt is yet to fully develop - the virus titre may increase as the season progresses. The yellowing is unlikely to be solely due to SCYLV. Although many plants did have SCYLV, there does not appear to be any relationship between yellowing symptoms and SCYLV test results.

Survey results show that Ramu stunt is not common or widespread away from the commercial crops on the estate. No Ramu stunt was detected in *S. robustum, S. edule*, or any of the grass weeds tested so far. This was despite most samples having a range of streak and fleck symptoms that *could* suggest Ramu stunt. For the noble canes that did test positive, classic leaf symptoms or stunting were always observed, although often only by a trained Ramu staff member. From the 32 sugarcane isolates sequenced during 2009033, it can be seen that there is sequence variation between commercial isolates on the RAIL estate and the noble isolates from the Ramu Valley, but not so much as to prevent the diagnostic primers from binding to their target region. In contrast, there is considerable variation between all northern isolates compared to the Alotau isolates, to such an extent that the diagnostic test had to be modified for those isolates to be detected. These results suggest that it would be valuable to conduct more surveys in other parts of the country, particularly in the south, closer to Australia. While the vast majority of 2009033 survey samples did not test positive for the specific disease being tested, the samples represent a valuable collection of sugarcane and grass germplasm from PNG. The samples are preserved over $CaCl_2$ at SRA Indooroopilly and could be used in the future for other biosecurity purposes.

We have been able to experimentally transmit the Ramu stunt virus from known infected plants to known healthy plants using the insect vector. In the strictest sense, Koch's Postulates cannot be said to have been completed because:

- The insects were collected from the field, not reared, and although free of Ramu stunt, they may have had other viruses
- The cage setup was not a closed system; weeds were present as well as other insects and insect predators
- The presence of so many mealybugs made leaf symptom recognition difficult

Despite this, we have been able confirm the transmission by symptomology, RT-PCR, sequencing and protein analysis. The control plants also suffered from weeds and insects and yet they did not test positive for Ramu stunt. However, these plants remained fairly free of mealybug damage. This is probably because the infestation of mealybugs in the test cage was due to accidental transfer from the source cage. It was hoped that further transmission experiments could be carried out during this project. Factors such as plant host range and vector life cycle stage are still not understood. However because the dry weather in 2013 delayed this trial, there was insufficient time to carry out further trials. If further transmissions are able to be carried out in future, efforts will be made to address some of the issues from this experiment.

This project has also been able to resolve some basic aspects of host/ virus biology, particularly relating to virus distribution in the plant. This is important to ensure that the optimal tissue is being sampled for the diagnostic test. A great deal of progress has been made in characterizing the virus but much remains to be done. Complete sequencing of the Ramu stunt genome would allow the virus to be formally named and its taxonomic position determined.

The Ramu stunt research results described here have clearly achieved the desired objective and associated output: *Specific disease-diagnostic tests that may be used in screening trials and the quarantine program and that can be applied in the event of a disease incursion.* The diagnostic test is available as an SRA quarantine diagnostic protocol and is used by the quarantine group. It has been used on numerous occasions to assist NAQS personnel to screen their PNG survey samples.

Outputs / outcomes

- The project confirmed that the causal agent for Ramu stunt is a tenuivirus.
- The Ramu stunt diagnostic test has been successfully validated using the standards from a resistance screening trial. There was a good relationship between symptoms, resistance rating and diagnostic test results. This test can now be confidently used to test plant samples for Ramu stunt.
- The distribution of the virus throughout the plant was determined, providing some basic information about the disease and ensuring that the optimal tissue is sampled for diagnostic screening.
- Disease surveys throughout PNG found that the disease is not common or widespread outside of the Ramu Valley.
- Some slightly different Ramu stunt viral isolates were detected in Alotau, suggesting there is some variation in the causal agent around PNG. The biological implications (whether this affects varietal resistance) is unknown at this stage.
- The causal agent of the disease, a previously unknown Tenuivirus, has been confirmed by carrying out caged insect transmission trials. This has also confirmed the identity of the causal agent.
- Fragments of the viral genome have been sequenced to further characterise the virus.

References

Braithwaite KB, Croft BJ and Magarey RC (2007). Progress in identifying the cause of Ramu stunt disease of sugarcane. Proceedings of the Australian Society of Sugar Cane Technologists, 29: 235-241

Girard JC, Fernandez E, Daugrois JH, Roques D, Roumagnac P and Rott P (2010). Genetic diversity of sugarcane yellow leaf virus in a sugarcane selection plot in Guadeloupe (FWI). Proceedings of the International Society of Sugar Cane Technologists, Vol 27, Veracruz, Mexico

Kuniata LS, Young GR, Pais E, Jones P, Nagaraja H (1994) Preliminary observations on *Eumetopina* sp. (Hemiptera: Delphacidae) as a vector of Ramu stunt disease of sugarcane in Papua New Guinea. *Journal of the Australian Entomological Society* 33: 185-186

Kuniata LS, Magarey RC, Rauka GR, Suma S, Bull JI (2010) Screening for Ramu stunt resistance at Ramu Agri-Industries, Gusap, PNG 1986–2008. Proceedings of the Australian Society of Sugar Cane Technologists 32: 312-321.

Lane LC (1986). Minipurification – a general purification procedure in: Propagation and purification of RNA plant viruses. Methods in Enzymology, 118: 687-696, Ch 48

Liu, H. & Beckenbach, A.T. (1992) Evolution of the mitochondrial cytochrome oxidase II gene among ten orders of insects. *Molecular Phylogenetics and Evolution* 1, 41–52.

Thomson D and Henry R (1995) Single-step protocol for preparation of plant tissue for analysis by PCR BioTechniques 19 (3): 394-397

Virtudazo EV, Nakamura H and Kakishima M (2001). Phylogenetic analysis of sugarcane rusts based on sequences of ITS, 5.8S rDNA and D1/D2 regions of LSU rDNA. J Gen Plant Pathol 67:28–36

Publications arising from this work

Braithwaite KS, Kombukon R, Kuniata LS and Magarey RC (2012). Ramu stunt: resistance screening and validation of the diagnostic test. Proceedings of the Australian Society of Sugar Cane Technologists, 34 (electronic format)

Braithwaite KS, Kuniata LS and Magarey RC (2014). The importance of disease surveys for understanding quarantine pathogens. Proceedings of the Australian Society of Sugar Cane Technologists, 36 (electronic format)

Appendices

Appendix 1. Genetic variation between 15 Ramu stunt isolates collected from commercial fields between 2001 and 2014.

Appendix 2. Details of samples collected from all surveys during 2009033

- Appendix 2.1 Survey samples collected from the Ramu Valley in July and December 2009
- Appendix 2.2 Survey samples collected from the Kassam Pass region and Ramu Valley in August/September 2010
- Appendix 2.3 Survey samples collected from Goroka, Ramu Valley, Madang and Surinam River regions in June 2011
- Appendix 2.4 Survey samples collected from a garden on the Ramu Estate in 2012
- Appendix 2.5 Survey samples collected from the Alotau region in 2013

Appendix 3. Genetic variation between 15 Ramu stunt isolates from commercial canes, 7 isolates from noble canes around the Ramu Valley and estate and 6 isolates from Alotau.

Appendix 4. Genetic variation between Ragnar and *Eumetopina* isolates collected from the transmission trial, compared to field grown Ragnar.

Chapter 8: Pathogen variability and diagnosis - Downy mildew

Objective:

- Develop and validate specific molecular diagnostic tests for downy mildew and define its distribution and variability in PNG

Outputs and Achievement of Project objectives

Introduction

Sugarcane downy mildew (DM) is currently one of the most serious diseases of sugarcane at the Ramu plantation in Papua New Guinea. It is rated as one of the highest exotic disease risks for the Australian sugar industry. It can be caused by several Oomycete (fungal) species in the genus *Peronosclerospora* including *P. sacchari*, *P. spontanea*, *P. miscanthi* and *P. philippinensis*. SDM caused by *P. sacchari* was eradicated from Australia in 1957 by intervention and the use of resistant varieties. Recently, however, there has been an increase in the use of susceptible varieties in Australia, with an estimated 60% of current varieties being intermediate or susceptible to SDM (prior to this project). Before this project, there was no molecular diagnostic assay for these pathogens in Australia, and the pathogen species are difficult to distinguish by traditional taxonomy.

Some of the work in this final report has been published in ASSCT papers (Magarey et al., 2012; Thompson et al., 2013) and presented at ISSCT plant pathology workshop (2012).

Downy mildew in sugarcane

Saccharum infecting *Peronosclerospora* can spread through both asexual and sexual phases. These produce different leaf symptoms on the plant: the ‘classic’ leaf streaking with down produced on the underside of the leaf; and leaf shredding/splitting symptoms (Figure 1).

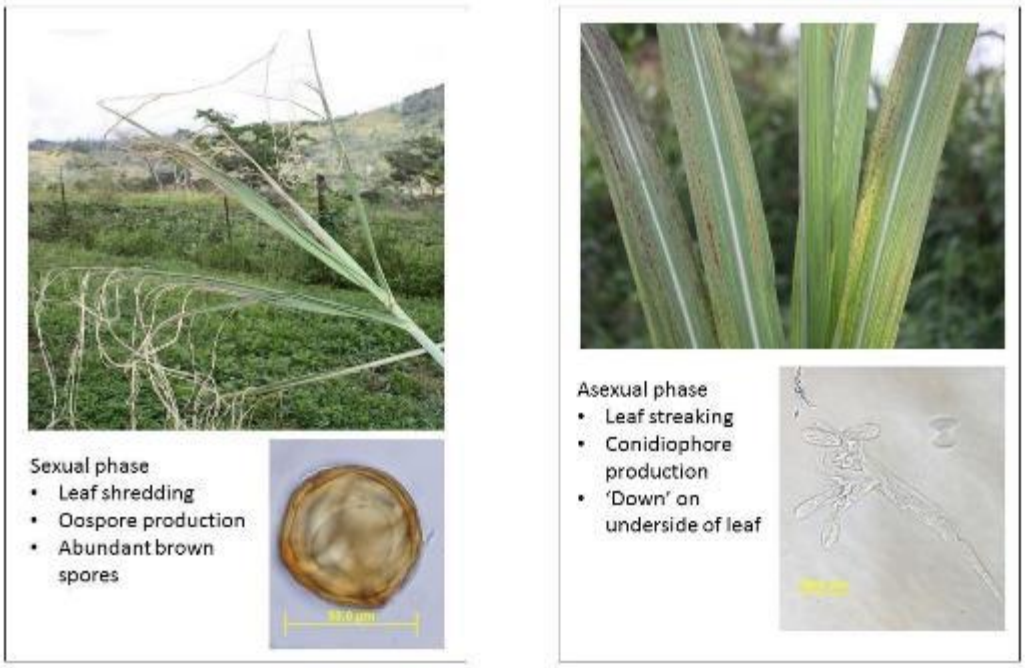


Figure 1: Typical leaf symptoms of downy mildew for both the sexual and asexual phases of *Peronosclerospora* species.

In PNG there are symptoms of leaf shredding in *Saccharum* and *Miscanthus* species: the latter had been observed by RAIL staff in the Eastern Highlands not far from the Ramu estate. It was unknown if this was caused by the same organism as the leaf shredding observed on commercial varieties on the estate. Leaf shredding was also widely observed on *Saccharum robustum* throughout the Ramu valley, and the relatedness to that found on commercial varieties unknown. Leaf streaking has been observed on maize and various *Saccharum* species (Figure 2).

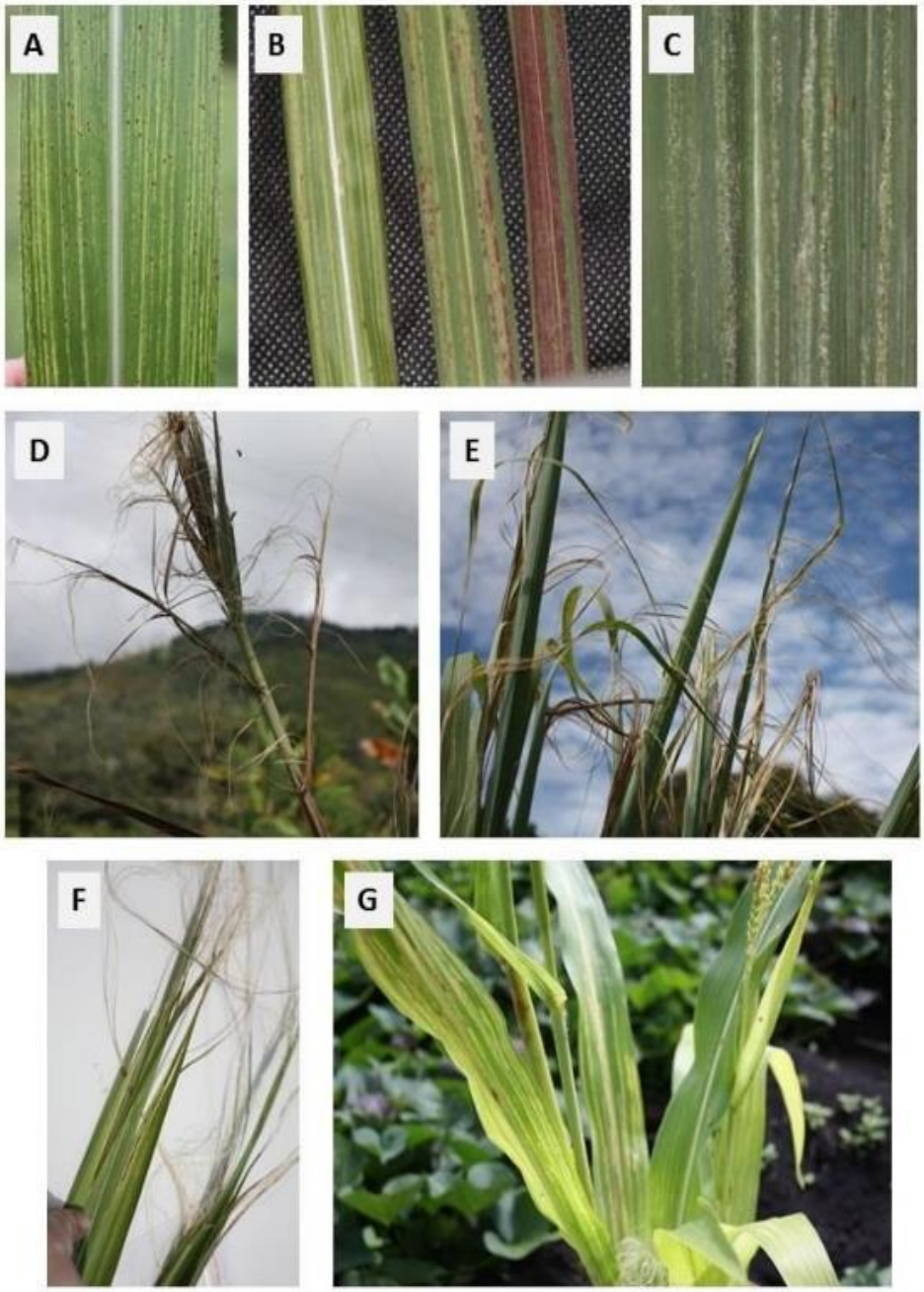


Figure 2: Leaf symptoms of downy mildew and leaf shredding in Papua New Guinea. A) Leaf streaking on commercial sugarcane; B) Reddened leaf streak symptoms on *S. edule*; c) Close-up of down formation along the leaf streaks on commercial sugarcane; D) Leaf shredding symptoms on *Miscanthus*; E) Leaf shredding symptoms on *S. robustum*; F) Leaf shredding on commercial cane; G) Downy mildew leaf symptoms on corn (*Zea mays*) (Thompson et al., 2013).

Microscopic analysis

The main distinguishing features of the *Peronosclerospora* from scientific descriptions are compiled in Table 1. For *Saccharum* infecting DM, the size of the oospores is the most easily distinguished feature. The literature describes *P. philippinensis* as having oospores of ~35um while *P. sacchari* and *P. miscanthi* have oospores of around 50um. Unfortunately, most of the conidia and conidia structures overlap in their size and observable features.

Table 1: Defining morphological features of *Peronosclerospora*: information compiled from CABI species descriptions of the *Peronosclerospora* (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*

Previous work

Prior observations by RAIL staff had observed a suspected ‘strain change’ of DM on the estate when a resistant variety became susceptible to the disease. Whether this was caused by the introduction of a different *Peronosclerospora* or the mutation of an existing strain is unknown.

A SRDC Travel and Learning Project (BSS322) was completed in 2009 that allowed collaboration with leading world experts on the molecular detection of *Peronosclerospora* species infecting sugarcane and maize. Dr Clint Magill (Texas A&M University, TX) and Dr Doug Luster (USDA-ARS, Ft Detrick, MD) have sequenced part of the genomes of isolates of *P. sacchari*, *P. philippinensis*, *P. sorghi* and *P. maydis*, and made this unpublished data available for the development of diagnostic primers.

Objectives

Develop and validate specific molecular tests and define the distribution and variability in PNG.

This section of the final report has been split into three main parts: development of diagnostic test; surveys; DM variability. Each section includes methods and results with the final discussion combined at the end.

Methods

Specimens

Samples were collected over the lifetime of the project. The samples collected include specimens from the sites and hosts with various leaf symptoms as described (Appendix 1). All specimens were collected and dried over calcium chloride then imported into Australia under import permits, irradiated at 25 kGray or 50 kGray, released from Quarantine and then stored at SRA Indooroopilly. All specimens have a unique identifier (A*) which was allocated on arrival in Australia and is used as the primary identifier throughout this section. A selection of samples will be deposited in the Queensland Plant Pathology Herbarium after consultation with the curator.

Microscopic analysis

Analysis of the oospores from DM was done by macerating the shredding leaf in lactoglycerol and observing under a light microscope. Morphological features that can distinguish the *Peronosclerospora* species are shown in Table 1.

Conidiophores and conidia were extremely difficult to observe because of the timing of the sporulation and the lack of a proper microscopic facility at RAIL. The down which is a matt of conidiophores and conidia are produced only during the night, and conidia are generally shed early in the morning. The conidiophores dry out rapidly and lose their diagnostic structures by early-mid morning, unless the humidity remains high. Therefore, only a limited amount of slide preparation was done. Slides were prepared by gently washing the down from the underside of the leaf using lactoglycerol, and placing under a coverslip for observation. The microscope facility at RAIL did not have the necessary objectives (20X and 40X) for observation, so slides were imported to Australia without knowing the quality of the slides. A few conidiophores were observed (Figure 1b), but this was insufficient for diagnosis and scientific description.

DNA extraction

DNA was extracted from the specimens by one of three methods, depending on the downstream application.

QIAGEN preparation

This method was used when sequencing of the samples was required. The extraction was done using a QIAGEN DNEasy Plant Mini Kit as per manufacturers instructions, excluding the use of

betamercaptoethanol. Sample grinding was done using either a FastPrep, Beadmill or QIAGEN Tissue Ruptor in the RLT buffer as per manufacturers instructions. DNA concentration was determined by Nanodrop spectrophotometer.

TPS extraction for rapid analysis

This method was used for analysis of a large number of samples, such as for the surveys. The quality of these extractions is poorer than that of the QIAGEN kits, however this method was suitable for the survey samples because of its reduced cost and reduced chance of contamination. Extractions were done using a modified method (Thomson and Dietzgen, 1995): the tissue was homogenised using a FastPrep 24 machine with TPS buffer prior to incubation at 100°C for 10 minutes. The optimal dilution of the extract was then determined by using ITS1-ITS4 or B-tubulin PCR prior to the Cox1 PCR. Specimens of interest were then subsequently re-extracted using the QIAGEN kit for sequencing analysis

Spore trap tape analysis

DNA was extracted from a section of each tape, using the method developed by Kathy Braithwaite for analysis of sugarcane smut spores (Magarey et al., 2008). The extract was diluted to determine the optimal dilution for detection, and PCR was done on a dilution series using ITS primers to detect any fungi and the Cox1 primers were used to detecting and determining differences in DM.

Analysis of specimens

PCR protocol

All specimens were subjected to PCR for analysis

1. **Determination of amplifilability of the specimen (positive control)**
    - Done using ITS1 and ITS4 (White et al., 1990) and/or Beta-tubulin primers (B-tubF1/ B-tubR1 from Dr. Clint Magill)
    - This was important if the quality of the DNA was questionable, such as for the TPS and spore trap tape extracts.
2. **Determine if *Peronosclerospora* is present**
    - Beta-tubulin primers (B-tubF1 / B-tubR1)
    - This step was sometimes eliminated if step 1 was successful and the specimen had known DM symptoms.
3. **Determine the variant of *Peronosclerospora***
    - Cox1 primers (Cox1F2/Cox1R1)
    - This was done on all specimens.

The PCRs were all done using Promega GoTaqGreen (containing 1.5mM $MgCl_2$) as per manufacturer's instructions including 1uM each primer. The ITS primers were used as described (White et al., 1990). Thermal cycling for step 2 and 3 was a 95°C denaturation for 2 minutes followed by 40 cycles of [95°C for 30 sec, 50°C for 30 sec, 72°C 45 sec (B-tubulin)] or 40 cycles of [95°C for 30 sec, 62°C for 45 sec, 72°C 45 sec (Cox1)] with a final extension of 72°C for 5 minutes. The thermal cycling was done on an Eppendorf ProS machine.

A subset of specimens were subjected to PCR using Cox2 primers as described (Hudspeth, Nadler, and Hudspeth, 2000) and analysed by DNA sequencing.

Gel-based analysis

PCR products were analysed on a 2% agarose gel with Sybr safe for visualisation under blue light. A 100bp DNA ladder (Promega) was included on each gel, as well as three positive control amplicons for comparison: one short (~200bp), one mid (~250bp) and one long (~300bp). This allowed rapid identification of the Cox1 variants.

Sequence analysis

Bands were excised from the agarose gel and cloned into the pGEM-Teasy vector (Promega) as per manufacturer instructions, and transformed into Alpha-Select Gold competent cells (Bioline) using standard molecular biology procedures. Plasmid DNA was extracted using FastPlasmid Mini Kit (5Prime) and the DNA sequence was determined using Sanger sequencing at AGRF (Brisbane) in both directions for each clone. All sequence analysis was done in Vector NTI (Invitrogen, until 2013) and then from 2013 Geneious Version 7.1.5 http://www.geneious.com (Kearse et al., 2012) was used. The forward and reverse reads were used to create a contig which was then edited manually if required. The ends were trimmed so that the start and end were the sequences of the primers used in the amplification. A BlastN search of GenBank database was done to ensure that the amplicon was highly similar to *P. sorghi* and/or other oomycetes (i.e. to check that the amplification was specific).

Phylogenetic analysis

Analysis of the Cox1 sequence required the use of multiple analysis programs because of significant gaps in the sequence: the primers were designed to amplify over a deletion, so the difference in the gap size and location is significant for the phylogenetic analysis. Most multiple sequence alignment programs and phylogenetic analyses have an inbuilt assumption that a gap in the sequence indicates an error in the sequence data. This leads to either the data being deleted from the analysis or the gap being coded as a 5th character nucleotide. Neither of these assumptions are good for including large gaps because it assumes that the gap is unrelated to the relatedness of the sequences: i.e. it is not a significant deletion. This problem is recognised by those working in the field of phylogenetics (Nagy et al., 2012; Simmons and Ochoterena, 2000) but is often overlooked by some researchers.

There are several programs available for analysing data with gaps (Borchsenius, 2009; Nagy et al., 2012; Young and Healy, 2003), and the method used in this report was chosen because of the flexibility in the analysis and the availability of free software. The first step is to use a program that allows multiple sequence alignment to use the gaps for alignment and not ‘stretch’ the sequence out. For this purpose MAFFT Alignment (Katoh et al., 2002) in Geneious v 7.1.5 (Kearse et al., 2012) with the option of E-INS-i with a scoring matrix of 200 PAM/k=2 and a gap open penalty of 1.52. The multiple sequence alignment was then edited in Geneious to trim the ends, so that no end-gaps were introduced into the analysis.

The alignment was exported as a FASTA (text) file, edited in text editing software (e.g. NotePad) then the gaps coded using FastGap v1.2 (Borchsenius, 2009). The data from FastGap was edited to a Nexus file for input into the standalone MrBayes phylogenetic analysis software (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Two data partitions were created: the DNA and a binary input using restriction for the coded gaps. Phylogenetic trees were constructed using standard Markov chain Monte Carlo ‘mcmc’ settings (Geyer, 1991; Hastings, 1970; Metropolis et

al., 1953) in MrBayes and allowed to converge until the average standard deviation of split frequencies was less than 0.01. The data was exported to FigTree (Rambaut, 2006-2014) for visualisation.

Analysis of the Cox2 amplicons was a simplified process completed within Geneious v 7.1.4, using many of the same settings as for Cox1 so that the analyses could be compared. MAFFT was used for alignment using an iterative method, and phylogenetic trees were calculated using the MrBayes Geneious plugin with default settings and 1000000 iterations. A consensus tree was constructed and visualised using Consensus Tree.

Other phylogenies were produced in Geneious v 7.1.4 and MEGA 5 (Tamura et al., 2011) with specifications as shown in figure legends.

Surveys

A summary map of survey locations in PNG is shown in Figure 3. This shows the location of the RAIL estate in Gusap (Morobe Province), Goroka (eastern Highlands Province), Madang (Madang Province) and Alotau (Milne Bay Province).

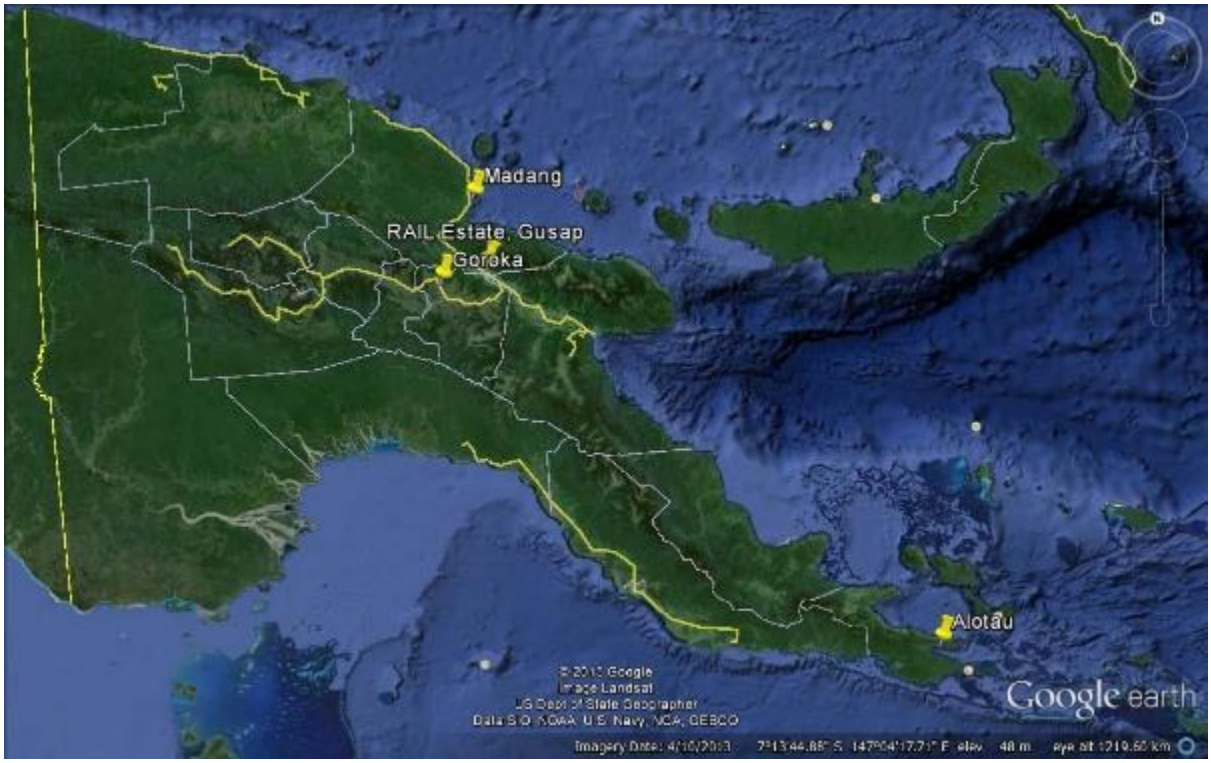


Figure 3: Map of PNG showing areas of survey. The yellow markers show the places included on the survey. The yellow lines are major roads, and the blue lines are Provincial borders.

RAIL estate

A survey of the RAIL estate was done on 20-21 November 2012 by Nicole Thompson, Kathy Braithwaite and RAIL pathology staff. This was carried out during the annual RAIL estate pathology survey as described in the Ramu Stunt portion of this report. Forty-one specimens of DM were taken from around the estate from the fields as shown in Figure 4. The symptoms were noted as leaf streaking or leaf shredding and Cox1 PCR was done to determine the variation on the estate. Specimens were labelled A1333-A1379 as in Appendix 1. Other specimens taken at different times from the estate have been included in the analysis (Appendix 1).

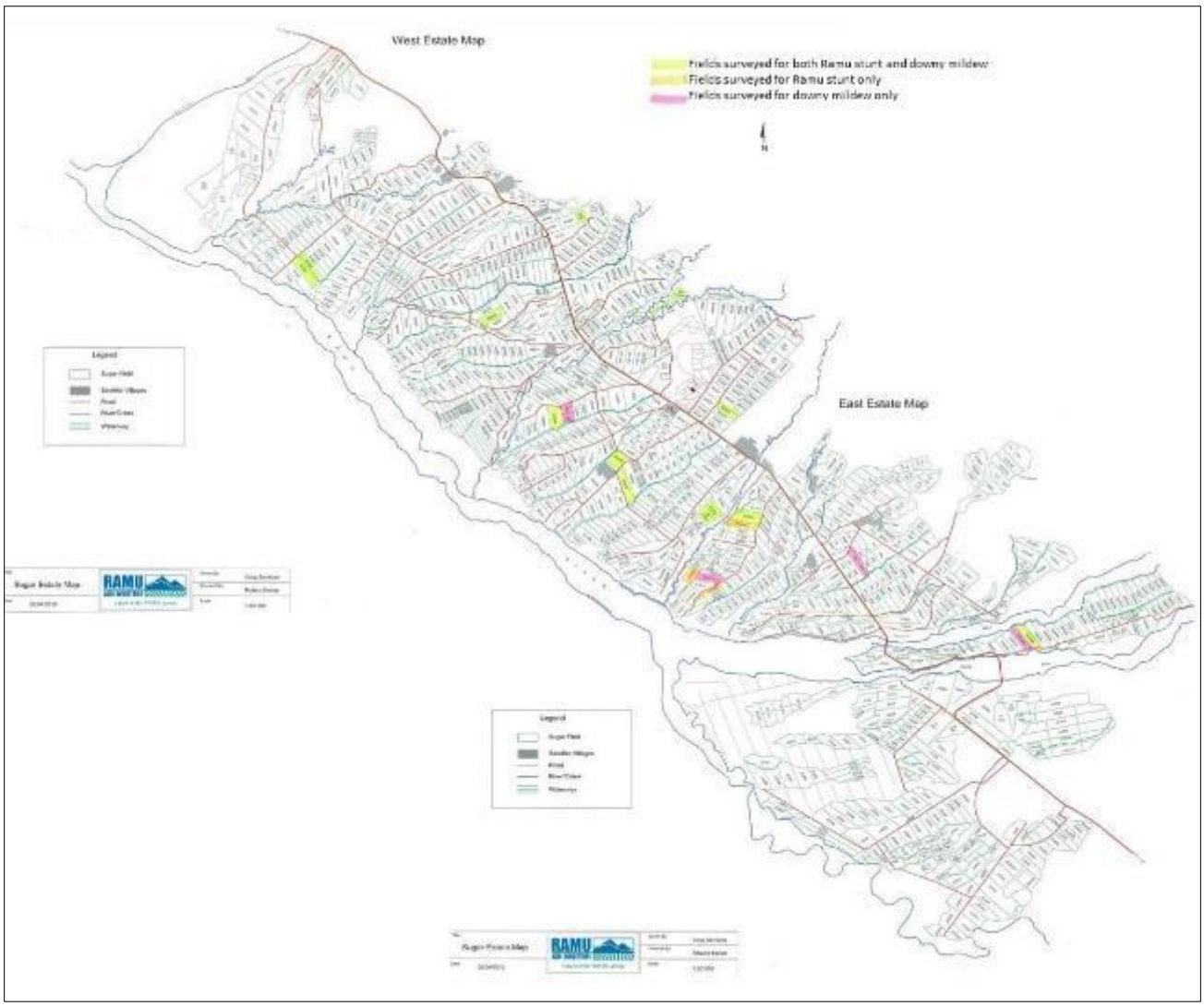


Figure 4: The RAIL estate map showing fields from which samples were collected, as in the legend.

The Ramu River borders the western side of the estate. The dark red line running from the north west to south east is the sealed road, leading towards Madang in the north and Lae in the south. Fields on the northern side of the road are designated as "N" and fields on the southern side designated as "S". The blocks are allocated from A through J from the northern most block to the southernmost blocks, with the road partitioning N from S. Therefore names such as AS303

represents Block A, south of the road, field 303. Blocks in the extreme southern portion (below the river junction) are exclusively oil palm, so no samples were taken from these blocks. Blocks in the extreme northern portion are free-holder owned, so no samples were taken from these blocks.

Eastern Highlands, Morobe and Madang Province

A survey was done of the Eastern Highlands, Morobe and Madang Provinces which followed the major roads in the area (Figure 5). The main part of the survey was done over four days June 14-17, 2011 and varied in elevation from 30m to 2474m. Some additional samples were collected from the Eastern Highlands and Ramu Valley on other visits to add to the specimen collection (Appendix 1). Details of the surveys, including staff that attended, can be found in the Ramu Stunt section.

Figure 5: Downy mildew sample collection points from 2009-2011 of the Ramu Valley (Morobe Province), Eastern Highlands and Madang Province.

Alotau province

A survey of this area was done on April 7-8, 2013 to establish if DM was present in this part of Milne Bay Province. The primary aim of this survey was for Ramu Stunt (see section on Ramu Stunt surveys) as a previous ACIAR-funded survey had found RS symptoms in this region. In contrast, only one possible report of DM-like symptoms had been reported from this area before. During this survey, no DM was found, although numerous other diseases were observed and specimens collected (Figure 6, Appendix 1).

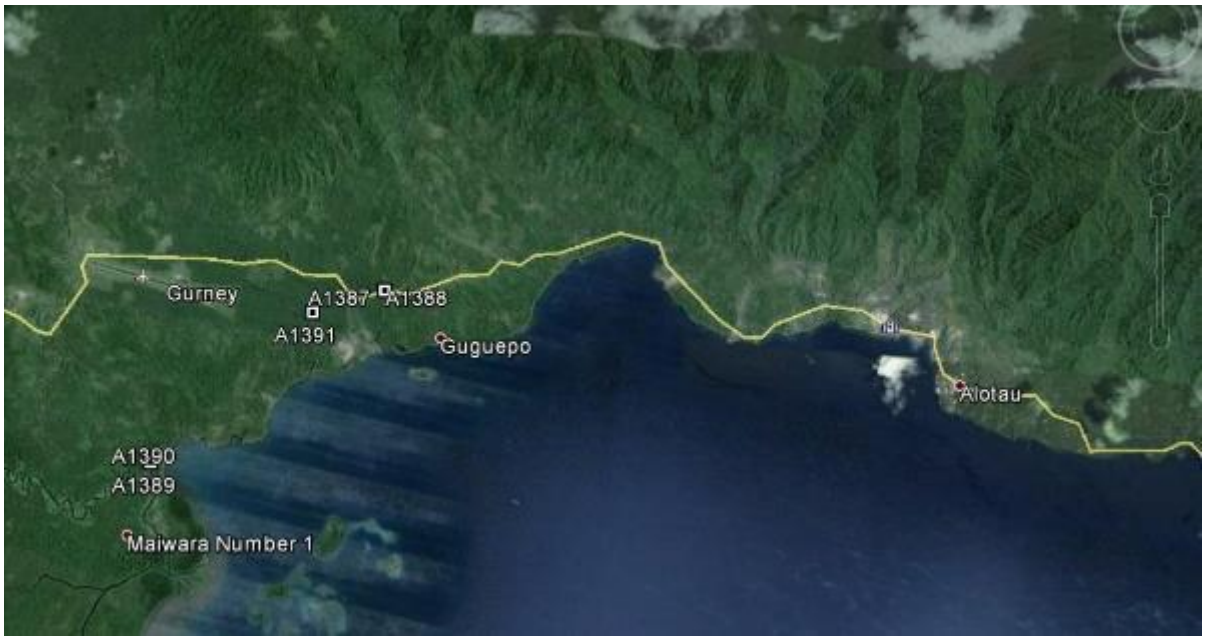


Figure 6: Specimen collection points from the survey of Alotau, Milne Bay Province. The yellow marker shows the town centre of Alotau and sample collection points are annotated in white.

Results

Microscopic analysis

Oospores were easily observed from all leaf shredding samples. All oospores observed throughout this project were brown, had a smooth thick wall and a size of between 50um and 55um. An example is shown in Figure 7.

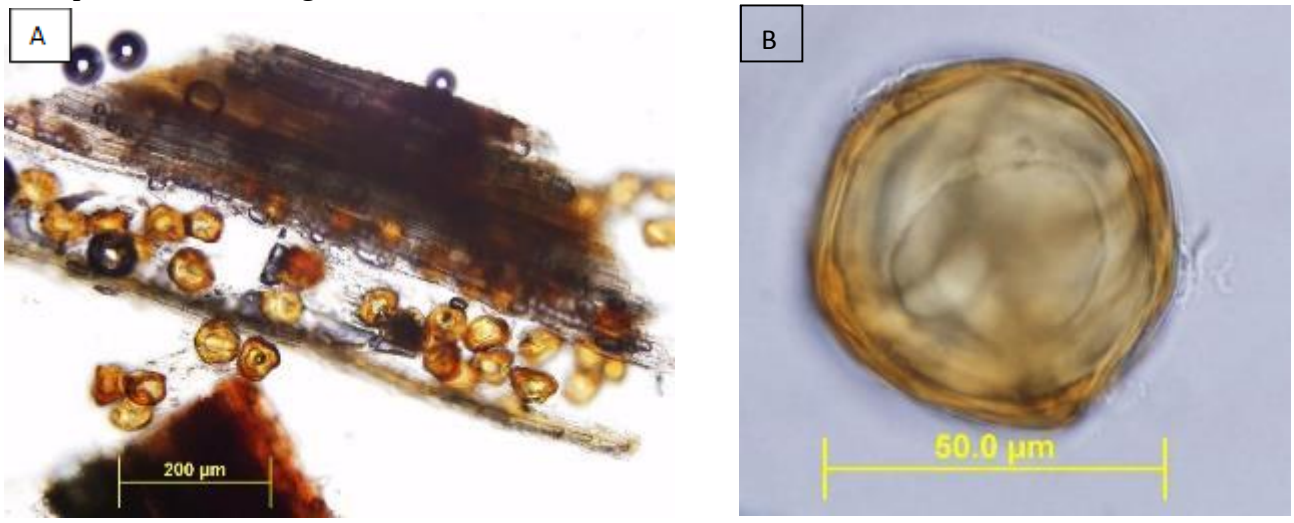


Figure 7

A: Leaf shredding sample showing abundance of brown oospores in S. robustum (A1010);
B: Oospore approximately 50um in width from Q136 leaf shredding sample (A0646)

Conidia and conidiophores were rarely observed due to the inability to obtain specimens at the time of sporulation and the lack of good microscopic facilities at RAIL. Of the slides made, there was not enough structures observed to make taxonomic conclusions. An example of a conidiophore structure observed from a specimen on the RAIL estate is shown in Figure 1.

Development of diagnostic test

Primer design

Several sets of primers that could potentially show differences between *Peronosclerospora* species were designed (the B-actin primers were a gift from Dr Clint Magill, Texas A&M University; Table 2). It was unknown if the primers would amplify sugarcane infecting *Peronosclerospora*, as only 2 DNA sequences were available at the time.

Table 2: PCR primers and their estimates of amplification size

Species	**Cox1**	**EF-1a**	**Btubulin**	**ITS-1**	**B-actin**
*P sacchari**	185/295	470?	?	100	?
*P philippinensis**	?	420?	?	100	?
P maydis	?	410?	400?	100	~600
P sorghi	317	?	400?	100	~600

* indicates only one sequence was available for each

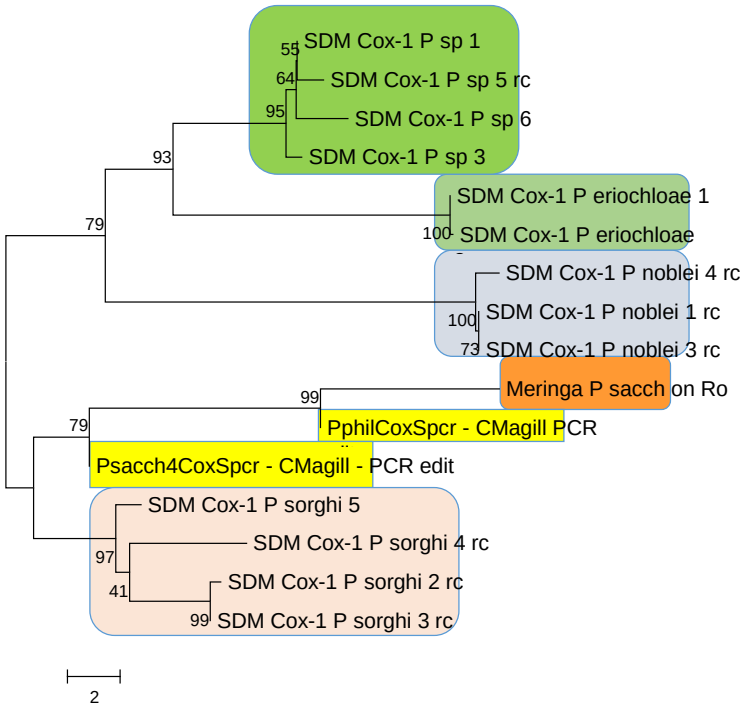
Primers tested on herbarium specimen Peronosclerospora

A "Proof of concept" was required before deciding which primer series to use for analysis of DM in PNG. To do this several samples were acquired from the Qld Plant Pathology Herbarium collection and analysed with all sets of primers. The results are summarized in Table 3.

Table 3: Results from amplification of primer sets for each species from the herbarium.

		Primer sets for analysis				
Species	**BRIP**	**Cox1**	**EF-1a**	**B-tub**	**ITS**	**B-actin**
P. eriochloae	13692	Different sizes for each species. *P. trifolium* (dicot infecting) did not amplify	Complex banding patters	One band. Amplifies all *Peronosclerospora*	Also amplified non-*P.* species	Complex banding pattern
P. sacchari	2104					
P. sorghi	44937					
P. sp ?	50933					
P. noblei	39880					
P. trifoliorum	48264					

The Cox1 amplicons showed the most promise for distinguishing between *Peronosclerospora* species, so sequence and phylogenetic analysis was done (Figure 8).



Neighbour joining, 1000 bootstraps (Mega 5)

Figure 8: Neighbour joining tree displaying differences between Cox1 amplicon sequences. Groups show the different species of *Peronosclerospora*.

These results show that the Cox1 amplicon is able to distinguish between species of *Peronosclerospora* from the herbarium collection. It should be noted that the 3 sugarcane-infecting species that were available at the time do not fall in the same clade. This is likely due to the limited material and information that was available but could also be indicative of variation in the sugarcane infecting *Peronosclerospora*.

Validation of diagnostic test

Sample analysis

Validation of the molecular diagnostic test was done on DM infected *Saccharum* collected from Papua New Guinea. Samples were collected from the RAIL sugarcane estate in Gusap Downs and from roadside locations in the Ramu Valley adjacent to the estate. Samples were from plants exhibiting both leaf streaking and leaf shredding on various host plants including commercial sugarcane, *Saccharum edule* (pit-pit), *S. spontaneum* and *S. robustum* (wild canes). DNA was extracted and Cox1 PCR done and the amplicons analysed by electrophoresis (Figure 9).

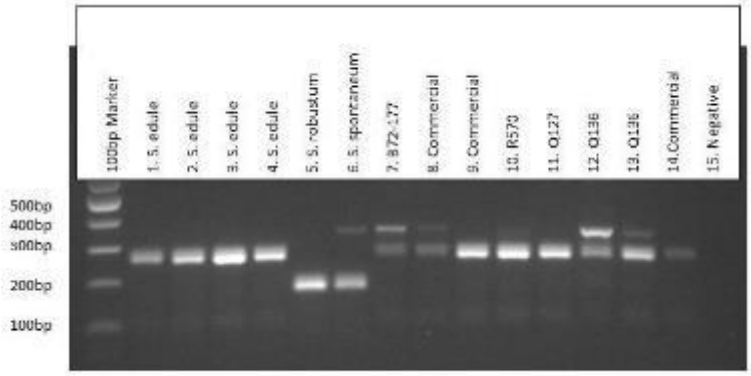


Figure 9: Agarose gel showing size differences of Cox1 amplicon from samples collected in Ramu Valley. NB: the uppermost band seen in some samples (e.g. 6, 7, 12) has been since eliminated from the PCR through redesign of the primers.

The bands were excised, sequence determined and a BlastN search of Genbank showed the greatest similarity to *Peronosclerospora sorghi* and then other oomycetes.

Two findings can be taken from this analysis:

1) The Cox1 primers are able to amplify DM infecting sugarcane in PNG; and

2) The Cox1 primers are able to show differences in the *Peronosclerospora* in PNG.

Spore trap analysis

Proof of concept

A validation experiment was designed to determine whether DM spores can be collected and detected on spore trap tapes in a similar way to the detection of sugarcane smut (Magarey *et al*., 2008). If successful, the use of spore traps could be added to the incursion management plan for DM.

The 'DM platform' inside the shadehouse at RAIL, PNG was used as an inoculum source during a DM conidiophore pot-transmission experiment. Spore tapes were placed at increasing distances from the platform (from 0m to the maximum of 14m) within the shadehouse, and left exposed for 1 week. The tapes were collected and sent to Indooroopilly for DNA extraction and Cox1 PCR analysis.

Although not all samples were amplified (Figure 10), both the long and short variant sizes were present on the tapes during this preliminary study, so an analysis of field samples was also completed to determine if detection was possible under field conditions. The reason for the amplification failure was likely to be the condition of the tapes: they were placed on the ground and therefore a lot of dirt, insects, water and other contaminants were present. In a spore trap machine the exposure of the tape is more controlled, so we would expect better results.

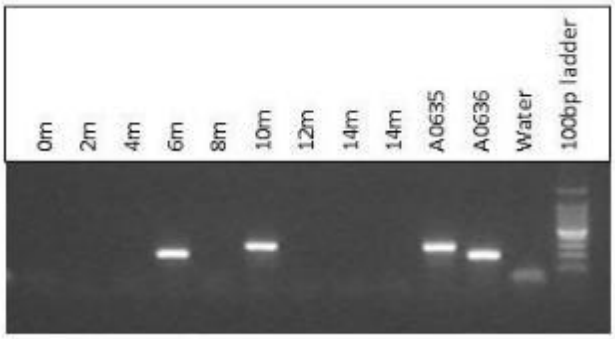


Figure 10: Cox1 amplification of specimens from shadehouse spore trapping. The distance from the cage is shown in metres for each lane; samples A0635 and A0636 were used as controls for the different amplicon sizes.

Field collected spore trap tape analysis

Sugarcane smut has not been found in Papua New Guinea, so as part of their incursion management plan RAIL has begun to use spore traps to capture spores from around the estate. A series of traps were run in May to July of 2012 and tapes representing 50 sites on the estate were sent to SRA Indooroopilly for analysis. The DNA was extracted from the spore trap tapes and diluted 1:10 in water before PCR using the Cox1 primers (Figure 11).

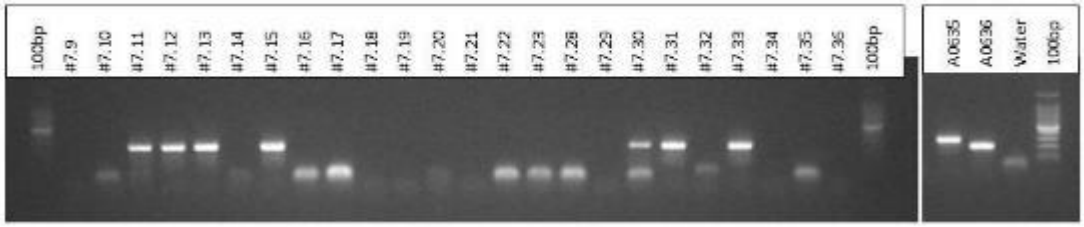


Figure 11: Cox1 PCR analysis of a subset of the spore trap tapes (Spore trap #7; tape analysis numbers 9 to 36). The size standards A0635 and A0636 were run on the next lane of the agarose gel and are included here for comparison. The Cox1 band for the spore trap tapes is ~300bp; the <100bp band seen at the bottom of some lanes is excess primer due to no amplification.

Of the 50 sites tested, there were 16 sites (32%) which had detectable DM spores for the time periods that the tapes were running.

There was no Cox1 size variation in the field sites: all spore traps detected Cox1 amplicon of about 300bp, indicative of leaf streaking.

Leaf shredding was expected to be detected, but no short amplicons were detected.

Most of the positive sites were on the southern side of the estate, which is traditionally the wetter side of the estate. This is consistent with the characteristics of the disease and field observation.

The notable exception was a site adjacent to the DM resistance screening field trial, which was located on the northern side of the estate (B North).

Distribution and variation of DM in PNG

On the Ramu estate

DM was found throughout the Ramu estate. The majority of the samples collected were of leaf streaking (i.e. down on the underside of the leaf) with only a few specimens of leaf shredding being noted from Q136 in FN303. Q136 is a variety that readily shows leaf shredding and FN was also known to show shredding in previous years. As this was an older ratoon, this is not surprising.

This confirms the findings from the spore trapping which showed a high level of disease on the southern side estate, with the implication of only leaf streaking being present because of the amplicon size.

When analysing the Cox1 variants on the estate, longer and shorter variants were detected which were consistent with leaf streaking and leaf shredding, respectively. Therefore there are two variants on the estate.

In other surveyed areas of PNG

DM symptoms have been found in the Eastern Highlands, Morobe and Madang Provinces. The symptoms include a leaf shredding (*Miscanthus*, *S. robustum*, *S. spontaneum*, and commercial cane) and leaf streaking (*S. officinarum*, *S. robustum*, commercial cane, *S. edule* and maize). There are three size variants of Cox1 PCR determined from gel analysis of the survey samples: two of which occur on commercial varieties and one which seems limited to the highlands. There is some crossover of the Cox1 variants and the host, especially when it comes to samples from the highlands.

Variation analysis

The variability of DM was assessed by Cox1 PCR (Figure 12) and DNA sequencing followed by phylogenetic analysis as described. Most specimens collected in this project have been DNA sequenced, but for brevity only a subset of each size variant is included in the final analysis. Many samples had identical sequence data, especially when collected from the same location, host and time.

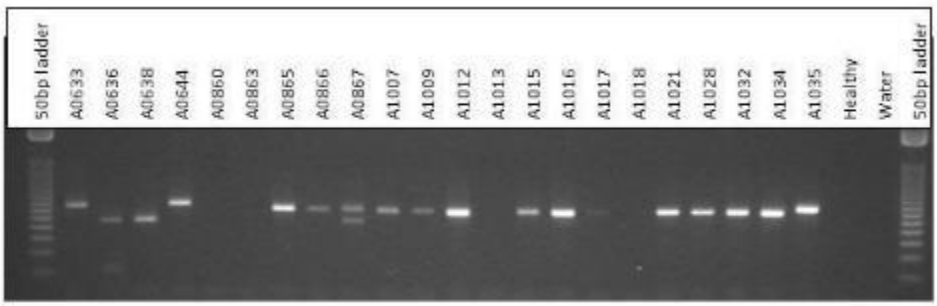


Figure 12: Cox1 amplification of a selection of samples collected from PNG. The marker is a 50bp ladder (Promega). The three size variants can be seen (e.g. A0633, A0636 and A1035). Samples that failed were subsequently re-analysed.

Further analysis of selected samples was done using Cox2 PCR with published primers. Only a select number of samples were analysed this way. Cox2 analysis has been used previously to classify Peronosclerospora, including *P. sacchari*, *P. philippinensis* and *P. miscanthi* (Hudspeth, Nadler, and Hudspeth, 2000; Thines et al., 2008) and it was hoped that this could add clarity to the Cox1 analysis.

Cox1 DNA sequence alignment

28 representative samples were chosen for analysis by sequencing of the Cox1 amplicon. The Cox1 primers are at the conserved ends of the alignment and designed to go across the gap(s) as shown in the Figure 13. These samples were chosen to demonstrate the differences between known *Peronosclerospora* species (either from amplicon sequencing (Cox1) or DNA sequence (nt)) and samples collected from within PNG for both the host species, symptom and the location (labelled with A*). Alignments were done as described and are shown in Figure 13.

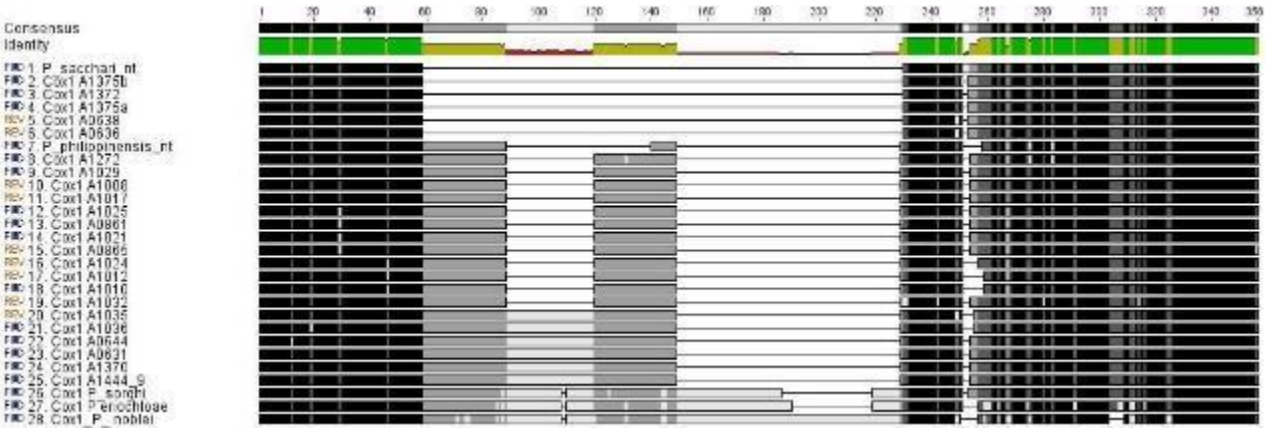


Figure 13: MAFFT alignment of 28 samples of Cox1 DM amplicon. Black blocks represent areas of high sequence similarity, dark grey for less similarity and the light grey are areas with little sequence similarity between the 28 samples. A consensus is shown in green-yellow-red above and shows the areas of greatest similarity in a graphical representation. Gaps in the sequence are illustrated by a dash.

Cox1 Phylogenetic analysis

The MAFFT alignment was used to construct a phylogenetic tree in MrBayes that included coding of the gaps in FastGap as described. The tree was displayed in FigTree and annotations added.

The figure shows that the phylogenetic analysis can separate the amplicon sizes. The species from the preliminary tree are interspersed within the analysed tree (Figure 14).

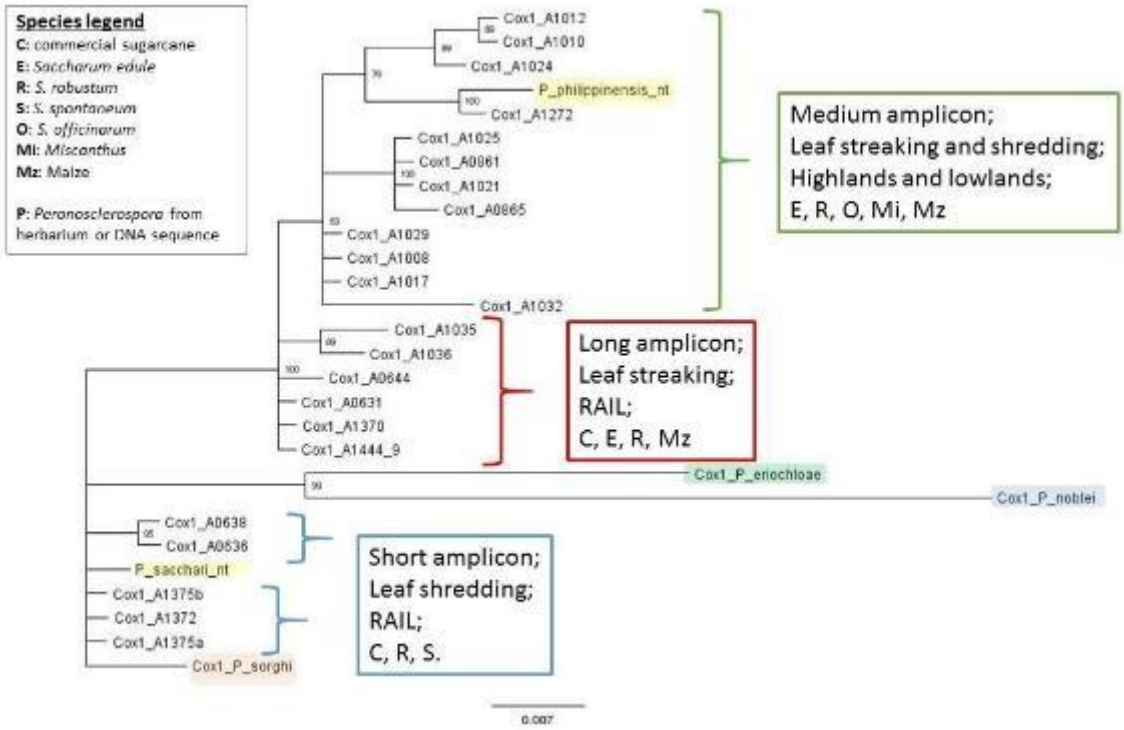


Figure 14: Phylogenetic representation of the relatedness of the Cox1 amplicons. The node strength is shown as a percentage and the genetic distance estimate is shown in the scale bar.

Cox2 DNA sequence alignment

The Cox2 region has been identified as a potential tool for analysis of the *Peronosclerospora* (Hudspeth, Nadler, and Hudspeth, 2000) and has been used several times for classification (Telle et al., 2011; Thines et al., 2008; Thines and Kummer, 2013). Thirty five sequences were selected for analysis and these were subject to analysis as described (Figure 15). Not all collected samples were analysed because of the large number of specimens; instead a selection was made that included a large number of published sequences to determine the difference of the PNG samples to the published *Peronosclerospora* species. If this proved to add to the phylogenetic separation of the *Peronosclerospora* in PNG, then Cox2 sequence analysis could be extended to all specimens.

Table 4: Specimens from Genbank included in Cox2 analysis

Genbank accession	Description
HQ261811 *	*Peronosclerospora miscanthi* voucher NY:Stevens# Philippine Fungi, Island of Luzon, No. 811 cytochrome c oxidase subunit 2 (cox2) gene, partial cds; mitochondrial
HQ261791 *	*Peronosclerospora sacchari* voucher BRIP:44241a cytochrome c oxidase subunit 2 (cox2) gene, partial cds; mitochondrial
HQ261796 *	*Peronosclerospora sp.* ST-2011 voucher BRIP:46735 cytochrome c oxidase subunit 2 (cox2) gene, partial cds; mitochondrial
HQ261790 *	*Peronosclerospora sorghi* strain 2.ps001 cytochrome c oxidase subunit 2 (cox2) gene, partial cds; mitochondrial

HQ261813 *	*Peronosclerospora eriochloae* cytochrome c oxidase subunit 2 (cox2) gene, partial cds; mitochondrial
HQ261812 *	*Peronosclerospora noblei* cytochrome c oxidase subunit 2 (cox2) gene, partial cds; mitochondrial
AY286224 †	*Peronosclerospora sorghi* cytochrome oxidase subunit II (COX2) gene, partial cds; mitochondrial gene for mitochondrial product

* (Telle et al., 2011); † (Hudspeth, Nadler, and Hudspeth, 2000)

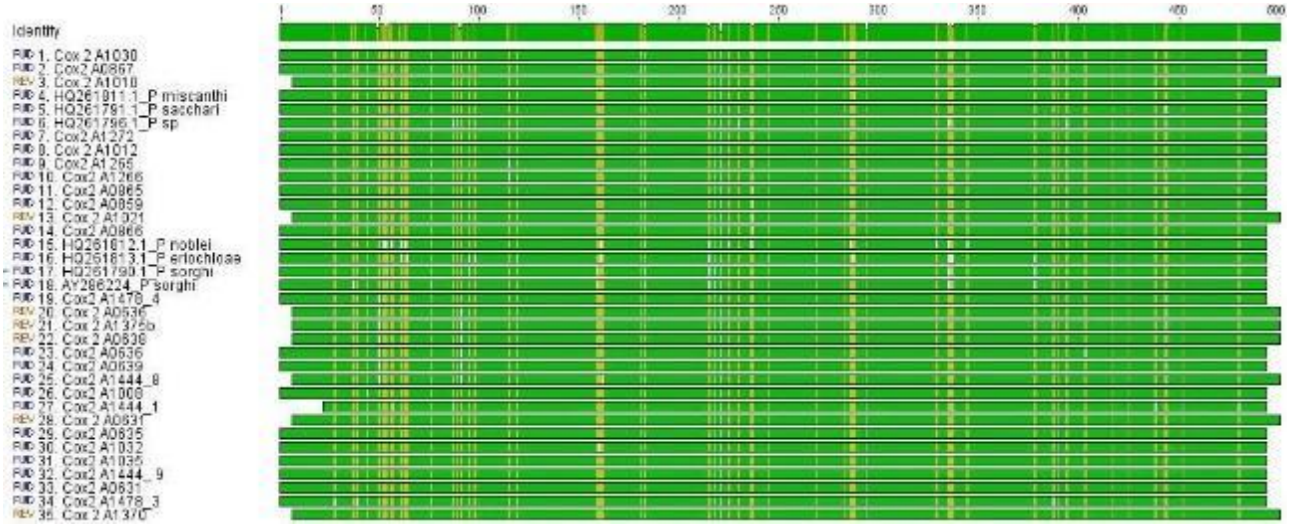


Figure 15: MAFFT alignment of Cox2 amplicons from selected specimens and sequences from Genbank (see Table 4). The green indicates a high level of sequence similarity, yellow less similar and white/pale blue indicates regions of sequence difference.

Cox2 phylogenetic tree analysis

The MAFFT alignment was used to create a phylogenetic tree in Geneious using the MrBayes plugin and Consensus Tree as described (Figure 16). This simplified process of phylogenetic tree analysis was possible because the sequence did not have large regions of gaps (Figure 15).

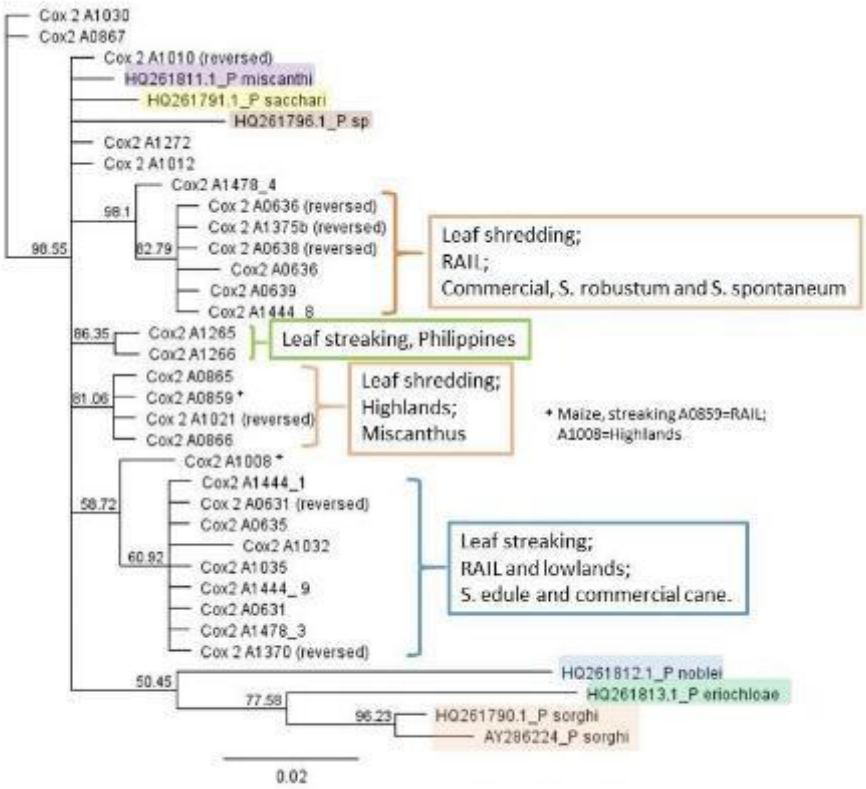


Figure 16: Phylogenetic representation of the relatedness of the Cox2 amplicons. The node strength is shown as a percentage and the genetic distance estimate is shown in the scale bar. Sequences extracted from GenBank are shown with their accessions and species.

As can be seen by this analysis, the Cox2 primers were less successful in separating the published sequences from each other and of separating the PNG specimens into meaningful groups. There are some loose groups for leaf streaking and leaf shredding but there are some outliers which do not fit in the analysis: these are samples that have not been highlighted or grouped in Figure 16.

Discussion

Diagnostic assay

The diagnostic analysis of sugarcane DM should be done as follows:

1. Collect leaf sample showing symptom, or spore trap tape
2. Extract DNA from symptomatic leaf tissue or spore trap tape
3. PCR analysis in 3 steps:
    a. ITS PCR (any combination of general ITS primers) to determine presence of amplifiable fungal DNA (very important for spore trap analysis);
    b. B-tubulin PCR to determine if *Peronosclerospora* is present;
    c. Cox1 to determine differences between *Peronosclerospora*.

Spore trapping

The equipment and methods developed for analysis of sugarcane smut spore traps can be used to analyse for sugarcane DM in the case of an incursion. Once published, this will make a valuable addition to the incursion management plan for sugarcane DM for surveillance, epidemiology, etc.

The spore traps can pick up all Cox1 variant sizes, however a high proportion of the Cox1 long amplicon was observed from RAIL, probably because of the high proportions of classic leaf streaking (down) observed on the estate. The conidiophores from the down are considered to not travel very far which can lead to the conclusion that: 1) there is a high proportion of DM on the Ramu estate or 2: that the spores can travel further than initially thought. This should be clarified by further study including a distance matrix, etc, however this will be difficult to construct in a place with endemic and widespread DM.

Peronosclerospora variation in PNG

P. philippinensis has not been found in Papua New Guinea during this project: no oospores smaller than 50um have been observed. It should be noted that in some reports, *P. philippinensis* is said not to produce oospores so further analysis of conidia and conidiophore structure should be done in conjunction with sequencing analysis to fully determine freedom from *P. philippinensis*.

The variation of *Peronosclerospora* in PNG is greater than expected. This can be shown through the inadequacy of current published taxonomic Cox2 DNA methods for distinguishing samples in PNG. Preliminary sequence analysis with the B-tubulin and ITS1-ITS4 primers was done, but there was not enough variation present to be meaningful (data not shown). We therefore conclude that the Cox1 primers are the best molecular method for detecting variation of *Peronosclerospora* in PNG.

There are three size variants of Cox1 amplicon. The Cox1 gene is a single copy gene in the mitochondria of *Peronosclerospora* and therefore should not demonstrate any differences within a species. This was supported by the preliminary taxonomic findings during the validation of the PCR, where the different *Peronosclerospora* species showed different amplicon sizes. In PNG there are clearly three size differences: one of which clusters with the unpublished *P. sacchari* sequence; one of which clusters close to (but not identical with) the unpublished *P. philippinensis* sequence; and one which is of a unique size (Figure 13). When looking at the phylogenetic representation in Figure 14 it is obvious that there is a lot of variation in PNG: this is illustrated by the fact that a defined species such as *P. sorghi* clusters closer to *P. sacchari* than to most specimens from PNG. The only specimens that remained as outgroups in all analyses are *P. noblei* and *P. eriochloae* while *P. sorghi*, *P. philippinensis* and *P. sacchari* are within the variation exhibited by the specimens from PNG. The specimen that is closest to *P. philippinensis* in Figure 14 is a specimen from the Philippines that showed leaf shredding on commercial sugarcane. Upon microscopic observation, the oospores were of a size with *P. sacchari* (50 um) so this is most likely to be correctly identified as a *P. sacchari* isolate from the Philippines. NB: In the Philippines it is regarded that *P. sacchari* causes the leaf shredding and that *P. philippinensis* causes the leaf streaking (R.T. Lazuran, Philsurin, personal communication). This is in contrast to traditional taxonomy that the leaf shredding and leaf streaking are caused by the same organism. The same organism should have the

same size Cox1 amplicon because this is a relatively conserved mitochondrial gene and size variation is associated with species: clearly further work on these organisms is required.

The Cox1 taxonomic study suggests that *Saccharum* and *Miscanthus* infecting *Peronosclerospora* from PNG have not been very well scientifically defined. For both Cox1 and Cox2, the variation present in the specimens collected from PNG was greater than the variation between species, and this includes known herbarium specimens and published DNA sequences. The species key was unable to distinguish the species based on the limited microscopic analysis and the overlap of most morphological features (i.e. the oospore size).

From analysis of the specimens collected we conclude:

- The 'Cox1 long amplicon' is most likely to represent a new species or taxa of *Peronosclerospora*, based on the host range and leaf symptom. This could have the 'Cox1 medium amplicon' within the clade, as they are more closely related to each other than to the 'Cox1 short amplicon'. This variant is found on the RAIL estate.
- The 'Cox1 medium amplicon' could represent a new taxa that encompasses the established *P. miscanthi* (when infecting *Miscanthus*), and some *P. sacchari* when infecting *Saccharum. P. philippinensis* could fall in this group, but we are yet to analyse a reliable herbarium specimen. This is not found on the RAIL estate, but could be a subgroup of the Cox1 long amplicon.
- The 'Cox1 short amplicon' is most likely to represent *P. sacchari*, based on the host range and oospore size. This has the main symptom of leaf shredding and is found on various host species on the RAIL estate.

This area of *Peronosclerospora* taxonomy needs to be re-analysed again from first principles.

Host range conclusions

Maize can act as a host for various *Peronosclerospora*

The maize sample from the highlands grouped with the lowlands samples, and vice versa in both the Cox1 and Cox2 analysis. It is common that maize is transported around PNG and is known that DM of maize is seed transmitted. When asked, the owners of this maize in the highlands said that they had got their seed from the lowlands and vice versa. This suggests that plants other than sugarcane could be implicated in the spread of DM throughout PNG. This could be another route for entry of DM to the RAIL estate and/or Australia.

Saccharum species can host more than one variant

Saccharum species such as *S. edule*, *S. officinarum* and *S. robustum* are widespread in PNG. These appear to be able to host any local *Peronosclerospora* strain. This is a concern because commercial sugarcane is a hybrid of *S. officinarum* and *S. spontaneum/S. robustum*. The mechanisms of resistance to DM are unknown but presumed to be genetic. Potentially, commercial cane could be susceptible to all Cox1 size variants which could lead to resistance breaking as has been observed previously on the RAIL estate. A host range study could be done to further investigate this.

The DM research results described here have achieved the desired objective and associated output: *Specific disease-diagnostic tests that may be used in screening trials and the quarantine program and that can be applied in the event of a disease incursion.* The diagnostic test is available as an SRA quarantine diagnostic protocol and will be converted into a national diagnostic protocol once published. It has been used to assist NAQS and other research personnel to screen specimens collected from various locations overseas. The use of spore traps has also been investigates that these can be used in addition to traditional sampling in the event of a disease incursion.

Outputs / outcomes

- Diagnostic PCR tests were developed for the DM pathogens present in PNG
    - A generic test (using the *Peronosclerospora* beta-tubulin gene)
    - A taxa-specific test (using the *Peronosclerospora* Cox1 gene)
- The taxa-specific test detects three different taxa, only two are believed to be described species
    - One similar to *P. sacchari*
    - One similar to *P. miscanthi*
    - A third likely to be an unclassified *Peronosclerospora* species
- The taxa close to *P. sacchari* and the unclassified *Peronosclerospora* are found in commercial cane and closely related *Saccharum* species. Variation in resistance ratings in individual varieties is therefore probable / likely in screening trials conducted at Ramu Agri-industries.
- Spore trapping can be used with the diagnostic test for detection of downy mildew
- Downy mildew is found readily in the Eastern Highlands, Morobe and Madang Provinces of PNG

References

Borchsenius, F. (2009). FastGap 1.2. Department of Biosciences, Aarhus University, Denmark. Published online at http://aubot.dk/FastGap_home.htm

FastGap 1.2. Denmark: Department of Biosciences, Aarhus University

Geyer, C. J. (1991). Computing Science and Statistics: Proceedings of the 23rd Symposium on the Interface, Fairfax Station.

Hastings, W. K. (1970). Monte-Carlo sampling methods using Markov chains and their applications. *Biometrika* **57**(1)**,** 97-109.

Hudspeth, D. S. S., Nadler, S. A., and Hudspeth, M. E. S. (2000). A COX2 molecular phylogeny of the peronosporomycetes. *Mycologia* **92**(4)**,** 674-684.

Huelsenbeck, J. P., and Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**(8)**,** 754-755.

Katoh, K., Misawa, K., Kuma, K., and Miyata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**(14)**,** 30593066.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., and Drummond, A. (2012). Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **28**(12)**,** 1647-1649.

Magarey, R. C., Braithwaite, K. S., Bade, G., Croft, B. J., and Lonie, K. J. (2008). Spore trap detection of sugarcane smut: 12 months experience and outcomes. *Proceedings of the Australian Society of Sugar Cane Technologists* **30,** 72-86.

Magarey, R. C., Kuniata, L. S., Samson, P. R., Korowi, K. T., Braithwaite, K. S., Thompson, N. P., Kombukon, R., and Bull, J. I. (2012). Preparations to enhance Australia's biosecurity: Part 2 - Resistance screening and pathogen research at Ramu Agri-Industries, PNG. *Proceedings of the 34th Conference of the Australian Society of Sugar Cane Technologists held at Cairns, Queensland, Australia, 1-4 May 2012***,** 22-Ag 22.

Metropolis, N., Rosenbluth, A. W., Rosenbluth, M. N., Teller, A. H., and Teller, E. (1953). Equations of state calculations by fast computing mechanisms. *Journal of Chemical Physics* **21,** 1087-1091.

Nagy, L. G., Kocsube, S., Csanadi, Z., Kovacs, G. M., Petkovits, T., Vagvoelgyi, C., and Papp, T. (2012). Re-Mind the Gap! Insertion - Deletion Data Reveal Neglected Phylogenetic Potential of the Nuclear Ribosomal Internal Transcribed Spacer (ITS) of Fungi. *Plos One* **7**(11).

FigTree v 1.4.2 1.4.2. Edinburgh: Institute of Evolutionary Biology, University of Edinburgh

Ronquist, F., and Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**(12)**,** 1572-1574.

Simmons, M. P., and Ochoterena, H. (2000). Gaps as characters in sequence-based phylogenetic analyses. *Systematic Biology* **49**(2)**,** 369-381.

Sivanesan, A., and Waller, J. M. (1986). Sugarcane Diseases. *In* "Phytopathological Paper (United Kingdom), Commonwealth Mycological Institute", Vol. 27, pp. 88, Kew (United Kingdom).

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony method. *Molecular Biology and Evolution* **28,** 2731-2739.

Telle, S., Shivas, R. G., Ryley, M. J., and Thines, M. (2011). Molecular phylogenetic analysis of Peronosclerospora (Oomycetes) reveals cryptic species and genetically distinct species parasitic to maize. *European Journal of Plant Pathology* **130**(4)**,** 521-528.

Thines, M., Goeker, M., Telle, S., Ryley, M., Mathur, K., Narayana, Y. D., Spring, O., and Thakur, R. P. (2008). Phylogenetic relationships of graminicolous downy mildews based on cox2 sequence data. *Mycological Research* **112,** 345-351.

Thines, M., and Kummer, V. (2013). Diversity and species boundaries in floricolous downy mildews. *Mycological Progress* **12**(2)**,** 321-329.

Thompson, N., Kuniata, L. S., Kombukon, R., and Magarey, R. C. (2013). Detection and variability of the causal agent of sugarcane downy mildew. *In* "ASSCT", Vol. 35, pp. Ag23. ASSCT, Townsville, Australia.

Thomson, D., and Dietzgen, R. G. (1995). Detection of DNA and RNA plant viruses by PCR and RT-PCR using a rapid virus release protocol without tissue homogenization. *Journal of Virological Methods* **54**(2-3)**,** 85-95.

White, T. J., Bruns, T., Lee, S., and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* "PCR Protocols: a guide to methods and applications" (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, Eds.), pp. 315-322. Academic Press, New York.

Young, N. D., and Healy, J. (2003). GapCoder automates the use of indel characters in phylogenetic analysis. *Bmc Bioinformatics* **4**.

General Discussion

Develop and validate specific molecular diagnostic tests for Ramu stunt and downy mildew and define their distribution and variability in PNG

Project research very successfully developed diagnostic techniques for both Ramu stunt and downy mildew. In fact the Ramu stunt work confirmed for the first time the nature of the stunt causal agent and showed conclusively that the Island Planthopper, *Eumetopina flavipes*, is the virus vector. These assays will be very important if an incursion of either disease occurs in Australia. As Ramu stunt symptoms are variable and difficult to diagnose by symptoms alone, this development will be very important for Australia's sugarcane biosecurity. Surveys around PNG suggest there is some variability ion the stunt pathogen. For the first time we now have data on stunt pathogen (tenuivirus) variability. Further molecular research coupled with additional surveys are needed to identify both the distribution and variation in Ramu stunt around PNG. Our work also examined where the virus is located within diseased sugarcane plants and this knowledge is imperative for selecting plant parts to sample when diagnosing the disease.

DM pathogen research also provided very interesting and relevant information. Different species of *Peronosclerospora* have been described and three species were thought to induce DM symptoms in sugarcane (*P. sacchari*, *P. miscanthi* and *P. philippinensis*); only the first two were considered present in PNG. Our research for the first time used molecular techniques to study in detail the pathogens causing DM in PNG. Significant variation in the pathogen was found. The traditional view of the pathogen in PNG has viewed *P. sacchari* as the causal agent, while *P. philippinensis* is considered the causal agent in the Philippines. Our work suggests that *P. philippinensis* is not present in PNG and that a third undescribed species may be affecting sugarcane. Previous resistance screening work undertaken at Ramu has noted that the resistance of some varieties appears to have changed over time; a second race of the pathogen was suggested in the early 1990s (Kuniata et al., 2010a). The presence of another *Peronosclerospora* taxa, as indicated in our work, would be consistent with this observation. The molecular assays developed will be critical for identifying the causal agent should a DM incursion occur in the Australian sugarcane industry. Tools have been developed that will separate *P. sacchari* from *P. philippinensis* and *P. miscanthi*, and the third postulated taxa.

Develop efficient screening procedures for plant resistance to Ramu stunt, downy mildew and moth-borers, especially Sesamia grisescens; rapid screening procedures will be a key focus.

Detailed studies of methods for moth borer resistance screening in commercial sugarcane varieties were conducted during the project. The field trials were successful in identifying that consistent differences in the reaction of varieties could be obtained in four trials planted in different years for three of the four moth borers (*Sesamia grisescens*, *Scirpophaga excerptalis* and *Rhabdoscelus obscurus*), while data for *C. terrenellus* were more variable. At Ramu, *S. grisescens* and *Scirpophaga excerptalis* are the two most important species; the difference in reaction amongst varieties suggests that varietal resistance could be an important management tool for these pests. This is consistent with commercial crop experience at Ramu, where a major commercial variety in the late 1980s (Cadmus) was susceptible to *S. grisescens* and was replaced by more resistant varieties in later years. Research suggested that the percentage of stalks infested was related to the extent of stalk injury caused by the moth borers. For *S. excerptalis* external symptoms of stalk infestation are unique and more easily identified than for the other species. Stalk colonisation for *S. grisescens*, *C. terrenellus* and *R. obscurus* requires more careful examination and visualisation of internal stalk symptoms may be needed to differentiate the species. Resistance screening techniques

for these species when they occur together will therefore require a greater investment of resources. We can now proceed to screen varieties for resistance to PNG moth borers in order to obtain resistance ratings for Australian commercial canes. The required data for a number of Australian varieties exported to Ramu have been collected in this project. Further analysis of these data will be undertaken shortly (as indicated previously) and ratings will be applied to our commercial varieties.

Rapid screening techniques for screening for *S. grisescens* and *Chilo terrenellus* were tested in project research. Some test plant colonisation occurred; methods used in South Arica to screen for Eldana saccharina resistance were unsuccessful. However, a newly devised method showed promise in the final experiment conducted.

The review of the previous disease resistance screening trials conducted at Ramu pre-project suggested that DM was the higher priority disease, since a much greater proportion of the commercial variety germplasm is susceptible to this disease. Higher priority was therefore given to DM in both field trials and in the development of a rapid resistance screen. In four field trials, consistent differences amongst varieties were noted in relation to disease incidence. When the reaction of the standard varieties was related to historical data for these standards (mean data gathered over a 20 year period), there was a higher correlation between data from the three project trials analysed as compared to the historical data. Two issues affecting these correlations were i. possible mistaken variety identity (shown via our molecular fingerprinting tools) and ii. possible pathogen variation. The latter is consistent with the DM pathogen variation studies and illustrates the possible consequences such pathogen variation can have on the resistance of varieties under field conditions. In the early 1990s, two previously commercially resistant varieties at Ramu (Cassius, Q107) became susceptible, even though data from DM screening trials conducted off-site continued to suggest they were resistant.

As found in previous Ramu stunt trials low disease levels in resistance trials hindered the application of resistance ratings. We attempted to address this by planting our infection rows as a dual row alongside a test row. This did seem to improve the level of disease incidence but the correlation of the standard varieties with their expected relative disease incidence did not improve greatly. The way forward for Ramu stunt resistance screening needs to be further considered. With Fiji leaf gall in Australia, tests plants are colonised under controlled conditions utilising infested populations of the vector (with individual plant cages). This could be worthwhile exploring in relation to Ramu stunt, though this would require more significant technical input and well-controlled environmental conditions for the screening work.

Rapid resistance screening research for DM examined both the use of conidia and oospores; conidia are fragile, short-lived spores and project research trialled suspending infected plants (producing conidia) above young test plants. This was not successful, perhaps because of the environmental conditions prevailing in the shadehouse. A second method utilised robust oospores as the inoculum source and methods similar to those used for Pachymetra root rot resistance screening. The method offers several advantages; i. more uniform application of inoculum, ii. potential storage of inoculum for long periods, iii. rapid screening, and ease of screening large numbers of varieties. Little research on the use of DM oospores as inoculum has occurred before; research conducted in this project suggested the method may be successful. The disease was transmitted using these spores but insufficient data was obtained to validate the technique – further research is needed to refine the technique. Such research will include: i. refining the level of inoculum, ii. ensuring the resistance ratings calculated are consistent with field reaction, and iii. defining the optimum time for disease inspections in test plants.

Determine pest and disease resistance ratings for 100-130 of the most important Australian commercial varieties, parents and promising clones. These will include recently released smut resistant varieties.

The most important Australian commercial varieties were exported to Ramu both before and during the project period. Also included were promising clones in the latter stages of the Australian selection program; such varieties are also used as parents – whether or not they reach commercial status. The most recent commercial varieties exported were smut resistant, and all current Australian commercial canes have some level of resistance. The actual number of varieties screened was less than 100; there was a limit to how many varieties could be sent to Ramu and it was decided that having two resistance ratings on the most important commercial varieties was more important (for reliability reasons) than having one rating on a larger number of less important varieties / clones. Further analysis of the resistance trial data will be undertaken very shortly and resistance ratings for all canes screened will be extended to the Australian sugarcane industry.

For moth-borers, investigate the occurrence of cross-resistance against the major borer species and develop an understanding of plant-insect relationships.

The direct examination of the cross resistance of varieties between borers was not directly tested in our research. However, data was obtained on the incidence of the moth borers under conditions of natural infestation. There appeared to be some relationships between the incidence of the borer species, with *Rhabdoscelus obscurus* positively correlated with *Sesamia grisescens* incidence, and *S. grisescens* negatively correlated with the incidence of *C. terrenellus*. There were some postulated mechanisms for such relationships, but there were no direct experimental data that addressed the issue.

Refine incursion management plans for the targeted pests and diseases.

Incursion management plans have been updated as a result of the outcomes of project research. These modifications include details related to the diagnostic tests for Ramu stunt and downy mildew, the resistance of Australian commercial varieties and aspects related to disease distribution and management.

Incorporate resistance data into the BSES variety database (SPIDNet), provide wider access to data via QCANESelect and communicate results to the industry in each region.

Resistance data will be uploaded to QCANESelect, once further statistical analyses have been completed and the ratings calculated.

Identify new knowledge / processes, practices, products, technology, and capacity building

The project has generated very significant new information, particularly in relation to the causal agents of Ramu stunt and downy mildew. The nature of the causal agent of Ramu stunt was confirmed and its distribution in infected plants determined. We now have confidence in assaying suspected infected plats and crops. The variation in *Peronosclerospora* species in PNG was also assessed and identified on a molecular level; this is the first such study undertaken in PNG. A new potential taxa of *Peronosclerospora* has been identified; further work will be needed to fully delimit this variation and potentially to classify the new taxa. The use of oospores as an inoculum source for resistance screening is a first in the sugarcane world and new information on methods for this technique were developed during the project period. Techniques for screening varieties / clones for the moth borers in PNG (except *C. terrenellus*) were also developed and new techniques were

developed. These will be useful for both Ramu and the Australian sugarcane industries. Spore trap research, used successfully to detect sugarcane smut in the Australian sugarcane industry, was shown to be able to detect conidia of *Peronosclerospor*a spp. This is new information on a world basis. Its usefulness is more limited than the application for smut, since the symptoms of DM are relatively more obvious and easily diagnosed. However, it could be a useful technique under specific circumstances. Very significant capacity building occurred during the course of the project, ranging from scientist's and technician's exposure to the pests and diseases in PNG, ability to undertake molecular techniques associated with pathogen assays, networking with PNG staff and general field experience. This places SRA staff in a very favourable position to deal with the major PNG pests and diseases, should an Australian incursion occur.

Intellectual Property (IP) and confidentiality

Intellectual property (IP) was generated during the course of these studies (molecular primers, disease specimens, pathogen sequences etc) but these are of no commercial value.

Industry communication and adoption of outputs

Industry communication occurred via the following routes: -

1. PEC Officer involvement: a PEC officer visited the Ramu site with the project research team and learnt first-hand about the pests and diseases at Gusap and the research being undertaken
2. Extension of information to industry:

- **ASSCT papers**:
  - Kuniata, L.S., Magarey, R.C., Rauka, G.R, Suma, S. and Bull, J.I.(2010). Screening for Ramu stunt resistance at Ramu Agri-Industries, Gusap, PNG 1986-2008. Proc. Aust. Soc. Sugar Cane Technol., 32: 312-321.

  - Kuniata, L.S., Magarey, R.C., Rauka, G.R, Suma, S. and Bull, J.I. 2010. Screening for downy mildew resistance at Ramu Agri-Industries, Gusap, PNG 1986-2008. Proc. Aust. Soc. Sugar Cane Technol., 32: 301-311.

  - Korowi KT, Kuniata LS, Samson PR (2011). Screening for borer resistance among sugarcane varieties in Papua New Guinea. Proceedings of the Australian Society of Sugar Cane Technologists, 32.

  - Braithwaite KS, Kombukon R, Kuniata LS and Magarey RC (2012). Ramu stunt: resistance screening and validation of the diagnostic test. Proceedings of the Australian Society of Sugar Cane Technologists, 34

  - Magarey RC, Kuniata LS, Samson PR, Korowi KT, Braithwaite KS, Thompson N, Kombukon R, Bull JI (2012). Preparations to enhance Australia's biosecurity: Part 2 – resistance screening and pathogen research at Ramu Agri-Industries, PNG. Proceedings of the Australian Society of Sugar Cane Technologists, 34

  - Thompson N, Kuniata LS, Kombukon R, Magarey RC. 2013. Detection and variability of the causal agent of sugarcane downy mildew. Proceedings of the Australian Society of Sugar Cane Technologists, 35.

- Korowi KT, Samson PR (2013). Screening for borer resistance among sugarcane clones in Papua New Guinea, 2010-2012. Proceedings of the Australian Society of Sugarcane Technologists. 35.

- ASSCT posters:

  - Braithwaite KS, Kuniata LS and Magarey RC (2014). The importance of disease surveys for understanding quarantine pathogens. Proceedings of the Australian Society of Sugar Cane Technologists, 36 (electronic format)

- Australasian Plant Pathology Society conference

  - Thompson N, Croft BJ. 2009. *Sugarcane downy mildew: development of molecular diagnostics*. Presented at Australasian Plant Pathology Society, Newcastle, NSW

  - Kathryn Braithwaite, Barry Croft and Robert Magarey (2011) Ramu stunt, a quarantine risk to the Australian sugarcane industry. ACPP-APPS Darwin, April 2011, p96.

- **Industry diseases training courses**
  - Woodford Pathology Training Courses:
    - BSES Limited "Sugarcane diseases workshop" 18-19 November 2009 "Exotic Diseases"
    - BSES Limited Sugarcane diseases workshop 18-19 October 2011 "Exotic Diseases"
    - BSES Limited "Sugarcane Diseases Advanced Workshop" 5-6 March 2013 "Exotic disease threats & preparedness"
    - BSES Limited "Sugarcane Diseases Introductory Workshop" 7-8 March 2013 "Exotic disease threats & preparedness"
    - BSES Limited "Extension Refresher Course" December 2010 "Downy mildew" "Ramu stunt" and "Other exotic threats"
  - Extension Disease Refresher Course, Cairns December 2010
  - SRDC Expo:
    - Session within the expo on project research
      - Various locations and project staff presenters in Queensland and New South Wales industry locations
      -
- BSES / SRA fact sheets
    - On the various PNG pests and diseases

- Thompson N, Sallam N, Braithwaite KS, Samson PR, Magarey R, Croft, B, Goebel, R. 2010. *BSES biosecurity: safeguarding the sweetest industry*. Presented at Global Biosecurity: Safeguarding agriculture and the environment, Brisbane, QLD

- International Society of Sugarcane Technologists Pathology Workshop (oral presentation)
  - Thompson N, Magarey R, Kuniata L, Croft B. (2012) *Development of molecular diagnostics for sugarcane downy mildew.* Presented at 10th ISSCT Pathology Workshop, Nanning, China.

Environmental impact

The development of resistance ratings for the moth borers may provide a positive environmental impact in the future, with the greater exploitation of varietal resistance to *Sesamia grisescens*. A decreased reliance on insecticides could be possible, leading to reduced release of pesticides into the environment. This may require more directed breeding and resistance screening to be undertaken by Ramu Agri-Industries scientists and staff. There were no other environmental impacts arising from this study.

Recommendations and future industry needs

The following areas arising from the project require further research: -

- Further identification of the different *Peronosclerospora* taxa present in and around Ramu cane fields and PNG and implications for resistance ratings and diagnostic tests
- Oospore inoculum levels, and incubation conditions, required to obtain reliable and repeatable DM infection in short term, rapid test pot trials at Ramu.
- The Ramu stunt virus is very poorly characterised and its relationship to other known Tenuiviruses is unknown; this requires further clarification..
- The biology of the host-vector-virus interaction, viral host range and extent of genetic variation is not well understood.
- Refined methodologies to screen for varietal tolerance to infestation by *Scirpophaga excerptalis* and *Chilo terrenellus*.

Future industry needs will include the following:

- The ongoing need to continue to screen Australian commercial varieties to the pests and diseases present in PNG. To fulfil this need, the Australian sugarcane industry will need to maintain contact with, and involvement in, the PNG sugarcane industry.

Publications arising from the project

1. Kuniata, L.S., Magarey, R.C., Rauka, G.R, Suma, S. and Bull, J.I. 2010. Screening for Ramu stunt resistance at Ramu Agri-Industries, Gusap, PNG 1986-2008. *Proceedings of the Australian Society of Sugar Cane Technologists*, 32: 312-321.

2. Kuniata, L.S., Magarey, R.C., Rauka, G.R, Suma, S. and Bull, J.I. 2010. Screening for downy mildew resistance at Ramu Agri-Industries, Gusap, PNG 1986-2008. *Proceedings of the Australian Society of Sugar Cane Technologists*, 32: 301-311.

3. Korowi KT, Kuniata LS, Samson PR. 2011. Screening for borer resistance among sugarcane varieties in Papua New Guinea. *Proceedings of the Australian Society of Sugar Cane Technologists*, 32. (electronic format)

4. Braithwaite KS, Kombukon R, Kuniata LS and Magarey RC. 2012. Ramu stunt: resistance screening and validation of the diagnostic test. *Proceedings of the Australian Society of Sugar Cane Technologists*, 34. (electronic format)

5. Magarey RC, Kuniata LS, Samson PR, Korowi KT, Braithwaite KS, Thompson N, Kombukon R, Bull JI. 2012. Preparations to enhance Australia's biosecurity: Part 2 – resistance screening and pathogen research at Ramu Agri-Industries, PNG. *Proceedings of the Australian Society of Sugar Cane Technologists,* 34. (electronic format)

6. Magarey RC, Bull JI, Atkin F, Dunne V, Pendrick R, Sventek K, Tom L 2014. Use of oospores as inoculum for early-stage resistance screening for downy mildew and Pachymetra root rot. *Proceedings of the Australian Society of Sugar Cane Technologists*, 36 (electronic format)

7. Thompson N, Kuniata LS, Kombukon R, Magarey RC. 2013. Detection and variability of the causal agent of sugarcane downy mildew. *Proceedings of the Australian Society of Sugar Cane Technologists*, 35. (electronic format)

8. Korowi KT, Samson PR. 2013. Screening for borer resistance among sugarcane clones in Papua New Guinea, 2010-2012. *Proceedings of the Australian Society of Sugar Cane Technologists*, 35. (electronic format)

ASSCT poster

- Braithwaite KS, Kuniata LS and Magarey RC. 2014. The importance of disease surveys for understanding quarantine pathogens. *Proceedings of the Australian Society of Sugar Cane Technologists*, 36 (electronic format)