FINAL REPORT 2013/357

Innovative approaches to identifying the cause of chlorotic streak and new management strategies

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ABSTRACT

Chlorotic streak is one of the major diseases in the Australian sugar industry. The disease occurs in areas of high rainfall and poorly drained fields and is a limitation to productivity, particularly during seasons that are wetter than average. The cause of the disease was unknown for 87 years before being identified *through* a combination of modern molecular techniques, particularly Next Generation Sequencing, and traditional pathology and microscopy. The causal agent is a novel biflagellated cercozoan and *Phytocercomonas venanatans* has been assigned as the tentative name. It is the first plant pathogenic cercozoan able to systemically infect higher plants and also the first to be successfully grown in axenic culture on common microbiological media.

The ability to culture the organism opens the way to developing innovative methods of control. Until now, there has been no reliable method to screen varieties for resistance to chlorotic streak due to the difficulty of setting up replicated screening trials with controlled infection. The project assessed both field-based and glasshouse-based screening. Using the cultured organism as inoculum was considered the most promising way of delivering a rapid resistance screening method. Other outcomes include improved diagnostic screening methods to assist Productivity Services staff, an understanding of epidemiology and a better understanding of the biology of the causal agent.

EXECUTIVE SUMMARY

Chlorotic streak (CS) of sugarcane has a worldwide distribution and is one of the most widespread diseases of the Australian sugar industry. The disease occurs in areas of high rainfall and poorly drained fields and is a limitation to productivity, particularly during seasons that are wetter than average. Infection reduces germination, ratooning, stalk number and weight, and is responsible for premature replanting and the loss of highly productive but susceptible cultivars. The disease can cause yield losses of up to 40 % of sugar yield in susceptible cultivars, costing the industry $8 - 10 M annually. Although the disease was first recognised 87 years ago, identification of the causal agent was unknown until 2016. The aim of this project was to trial a range of innovative molecular and traditional pathological techniques to identify the cause of chlorotic streak, study epidemiology and develop new management strategies.

We used a combination of modern molecular techniques, particularly Next Generation Sequencing (NGS), and traditional pathology and microscopy to identify the causal agent. Metagenomics NGS identified novel protistan ribosomal and nuclear genes (actin and polyubiquitin) in chlorotic streak-infected sugarcane. Phylogenetic analyses indicated that the causal agent belonged to the order Cercomonadida (Rhizaria, phylum Cercozoa) but did not match any known species and a new genus and species *Phytocercomonas venanatans* was assigned as the tentative name. Community profiling was used to show a consistent link between the cercozoan 18S ribosomal DNA and diseased sugarcane samples representing different plant tissues and collected from different locations.

A biflagellated organism with morphological features similar to the Cercomonads was isolated into pure axenic culture from internal stalk tissues of infected sugarcane. The organism was used to infect sugarcane plants, which subsequently developed symptoms characteristic of chlorotic streak. The organism was reisolated from infected plants, thus completing Koch's postulates and confirming that *P. venanatans* causes chlorotic streak.

The first-generation diagnostic test was improved by designing primers to target the ribosomal genes identified through NGS. This test works well on xylem sap and stalks, making it ideal to assist Productivity Services in screening clean seed plots. Potentially, CS and ratoon stunting disease (RSD) sampling and testing of seed plots could be combined, adding value to the existing SRA commercial diagnostic service. The impact of this service will be reduced losses through CS-free clean seed distributed to the industry. The test can also detect the organism in hydroponic water and soil.

Until now, there has been no reliable method to screen varieties for resistance to chlorotic streak due to the difficulty of setting up replicated screening trials with controlled infection. Reliable resistance ratings would assist growers to select the appropriate varieties to grow in disease-prone areas. The screening method would be incorporated into the core SRA Biosecurity and Plant Breeding programs. The impact will be reduced losses and replanting costs because CS resistant varieties will be targeted to high risk areas.

This project compared field and glasshouse based screening methods. Field-based screening attempts were made in Tully and NSW. The Tully trials were done at a site subjected to five flooding events over two years. The NSW inspections were made in a second-ratoon field subjected to four major floods and during the annual RSD surveys of seedbeds. For all field-based approaches, good results could be achieved if sufficient flooding occurred, however, selecting trial sites with sufficient flooding that does not compromise optimum growth conditions is challenging.

A gravel hydroponic transmission system had been developed in the 1960s and attempts were made to adapt this into a glasshouse-based screening system in Tully. Limited transmission occurred in a circulating hydroponic system, possibly due to rapid changes in solution pH and the associated plant nutritional effects. However, a non-circulating, non-aerated system provided better transmission and improved plant nutrition. The conditions needed to achieve significant disease levels in the test plants have now been established. The second glasshouse-based approach used axenic cultures of the pathogen to inoculate various parts of pot-grown sugarcane plants. A range of novel methods were trialled and methods based on combinations of root soaking with root damage were found to be most successful.

Epidemiological information was gained by disease monitoring in the field at Tully. Results showed that disease levels build up over time and that different lengths of inundation associated with flooding events may lead to uneven distribution of the disease in a commercial crop. Several novel methods of controlling the pathogen were attempted, and now that the causal agent has been identified, this work can be further expanded.

The outcomes from this project will contribute to increasing productivity by reducing losses from CS. If yield losses could be reduced from 10 % to 2 % through improved control strategies and methods to screen varieties for resistance, we estimate an increase in gross returns of around $8 M annually.

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1. BACKGROUND

1.1. Significance of the disease

Chlorotic streak (CS) disease is one of the most serious and widespread endemic diseases of the Australian sugar industry (Magarey, 2005, 2006). Despite its importance, research progress had been extremely slow due to its intractable nature. Chlorotic streak is spread through wet soil, and flood, drainage and irrigation water and infects through the root system, but the root/soil/water environment is difficult to study and control. The disease goes under the radar in dry years, but causes heavy losses in wet years. Symptoms are often transient, probably due to environmental conditions, including soil temperature. The industry has an incomplete set of resistance ratings due to the difficulty of achieving reliable replicated screening trials with controlled infection. Adding to the list of challenges is the astonishing fact that the causal agent remained unknown for 87 years. The lack of understanding of the causal agent and lack of a diagnostic assay seriously hindered successful disease management.

1.2. 1Previous research

In the late 1950s - 1960s, BSES pathologists led the world in CS research establishing fundamental disease parameters such as infection, incubation and dormancy periods, infection sources and alternative hosts, and achieved transmission through a hydroponic trough system (Sturgess, 1961). From 2000 to 2002, Rob Magarey led SRDC project BSS243 which carried out disease surveys, obtained preliminary field resistance and yield loss data, and highlighted the implications of recycling irrigation water in the Burdekin. Throughout this time, the causal agent was unknown although several organisms had been proposed, including fungi (Carpenter, 1940), insects (Abbott and Sass, 1945) and viruses (Sturgess, 1961).

In recent years BSES once again attempted to identify the causal agent, this time using molecular techniques. While testing a wide range of "universal" PCR (polymerase chain reaction) primers, a primer set was identified that generated a chlorotic streak-specific DNA fragment. The target gene (actin) was too conserved to assist in identifying the causal agent, but it indicated that it was a eukaryote and was developed into a first-generation diagnostic assay (Braithwaite and Croft, 2013).

Until project 2013/357 there was no reliable method for rating varieties for resistance to CS due to the difficulty of setting up replicated screening trials with controlled infection. A rapid chlorotic streak resistance screening test is essential so that growers can select appropriate varieties to grow in disease-prone areas. Past attempts to screen varieties for resistance to CS have used hydroponic trough systems and natural spread in the field (BSS243). A glasshouse-based method has the advantage that it is independent of weather conditions and can give relatively rapid results. Ratings for varieties will enable growers to avoid planting susceptible varieties in chlorotic streak-prone fields and will reduce losses from the disease.

Although hot water treatment, improved field drainage and resistant varieties are likely to be the mainstays of CS control, a better understanding of the biology of the causal agent may lead to new innovative methods of control. A greater understanding of pathogen dynamics in the field could lead to recommendations, advice and wider options for growers. Soil and water pathogen levels could be used to calculate risk thresholds to provide growers with advice.

Methods to inactivate the organism in recycled tail water would assist in the Burdekin district where it has been shown that irrigation water can transmit the disease and recycling tail water can add to the disease pressure (Magarey and Neilsen, 2002).

1.3. Aim of the project

The aim of this project was to trial a range of innovative molecular and traditional pathological techniques to identify the cause of chlorotic streak, study epidemiology and develop new management strategies for one of the most widespread serious diseases in our industry.

2. PROJECT OBJECTIVES

1) Identify the causal agent of chlorotic streak: through a range of molecular, microscopic and pathological techniques identify the organism(s) responsible for chlorotic streak and develop diagnostic assays.

2) Investigate disease epidemiology: improve our understanding of chlorotic streak spread and biology through disease surveys and transmission studies in the field and glasshouse.

3) Use the research findings to improve management strategies for chlorotic streak: develop innovative new management strategies based on an understanding of the causal agent.

4) Develop a reliable method to screen varieties for resistance: a temperature controlled hydroponic facility will be developed to screen varieties for resistance.

3. OUTPUTS, OUTCOMES AND IMPLICATIONS

3.1. Outputs

- Output #1: Causal agent of chlorotic streak determined (Category SCIENTIFIC KNOWLEDGE). The causal agent has been established as a novel biflagellated cercozoan tentatively named *Phytocercomonas venanatans*.
- Output #2: Diagnostic test (Category SOFT TECHNOLOGY). An improved PCR based diagnostic test has been developed to screen soil, plants and water for chlorotic streak.
- Output #3: Variety screening methods (Category SOFT TECHNOLOGY). Several glasshouse inoculation approaches have been trailed to screen sugarcane varieties for resistance to the disease.

Output #1 will be of interest to scientists and sugarcane pathologists around the world. One of the greatest sugarcane mysteries in the world has now been solved after 87 years. *P. venanatans* is the first plant pathogenic cercozoan able to systemically infect higher plants and also the first to be successfully grown in axenic culture on common microbiological media. Sunshine Sugar played an important role in this part of the project, through the supply of material from the Harwood region for Next Generation Sequencing.

Output #2 will be targeted to Productivity Services. Chlorotic streak has its greatest effect in the high rainfall wet tropics and poorly drained areas in other districts. NSW and Tully are two such regions, and the Sugar Service groups in those areas (Sunshine Sugar and Tully Cane Productivity Services Ltd) provided our greatest stakeholder support. Sap samples from the Tully clean seed plot were used for diagnostic test development. Further development of this output will occur in Project 2017010. The PCR-based diagnostic test will be extended beyond the research phase and used to assist productivity services to deliver clean seed to the industry. Delivery will be through integration with the existing SRA RSD diagnostic laboratory.

Output #3 will initially be targeted to the SRA breeding program. SRA pathologists and plant breeders have a close working relationship ensuring integration of any new variety screening test into the breeding program once fully operational. Sunshine Sugar assisted in this output by developing field-based resistance screening methods. Further development of a novel, rapid and reliable chlorotic streak variety resistance screening method will occur in Project 2017/010. Resistance ratings will be made available through the plant breeding database SPIDNet and the on-line decision support tool QCANESelectTM to provide better information to growers about newly released varieties.

All three outputs have already been communicated to the industry but Outputs #2 and #3 will be further promoted in Project 2017010 with the assistance of the SRA Adoption team. Regional grower meetings will be held in areas where CS is common to explain how growers can use the variety resistance ratings to minimise losses. Training of Productivity Service advisors in sample collection and interpretation of diagnostic results will be conducted.

3.2. Outcomes and Implications

This project has yielded a number of important breakthroughs on an intractable endemic problem. The outcomes that could arise from the research and that could be delivered to industry are:

- Reliable resistance ratings for commercial varieties: A novel screening method would be incorporated into the core SRA Biosecurity and Plant Breeding programs. Screening should target the accelerated and maximum propagation clones (about 25 per year), bringing CS into line with red rot, mosaic and nematode screening practices. The impact will be reduced losses and replanting costs because CS resistant varieties will be targeted to high risk areas.
- A diagnostic service for industry: Potentially, CS and RSD sampling and testing of seed plots could be combined adding value to the existing SRA commercial diagnostic service. The test would be targeted to sugar service groups and the impact of this service will be reduced losses through CS-free clean seed distributed to the industry.

The outcomes from this project will contribute to increasing productivity by reducing losses from CS. If yield losses could be reduced from 10 % to 2 % through improved control strategies and methods to screen varieties for resistance, we estimate an increase in gross returns of around $8 M annually.

The disease also has environmental implications because it discourages farmers from recycling irrigation tail water, due to fear of spreading the disease. Research planned for Project 2017010 will assess the potential for detecting CS in irrigation water. The studies will indicate the feasibility of providing risk assessments for CS in river and recycled irrigation water supplies in the future.

4. INDUSTRY COMMUNICATION AND ENGAGEMENT

4.1. Industry engagement during course of project

The key messages arising from the project are:

1) The cause of the disease has now been sorted out
    - Publications are in progress
    - The ability to grow the pathogen in culture opens up many new opportunities
2) We could finally obtain reliable ratings for our varieties
    - The method will be incorporated into the core SRA Biosecurity and Plant Breeding programs.

- It will target accelerated and maximum propagation clones (about 25 per year)
- This will bring CS into line with red rot, mosaic and nematode screening practices

3) A diagnostic test is available to productivity services
   - Best case: compatible with RSD-LSB
   - Even a test for soil and water
   - Worst case: a test for stalks (planting material)
4) The research opens up new ways of managing the disease due to:
   - Understanding of the biology of the organism
   - Understanding of pathogen dynamics in the field.

Communication through the SRA adoption team:

The key messages from this project were communicated to the industry through the CS and Biosecurity roadshows organised by Matt Reynolds, Adoption Officer – Biosecurity, in late 2016. Matt organised six events targeting productivity services staff and their board members. The events were run in Mackay, Proserpine, Ayr, Ingham, Tully and Meringa. The format focused on two components; the first, presented by Dr Kathy Braithwaite, summarised SRA's current work in chlorotic streak and the potential outcomes that future research could deliver, with the second, presented by Matt Reynolds, explored Biosecurity more broadly and aimed to identify opportunities participants saw within their regions. A further two presentations were organised by Matt and given at Ballina and Childers in 2017 without the biosecurity component.

The following events/publications were organised by the SRA communication team (particularly Brad Pfeffer):

- Northern industry information meeting April 2016
- Landline episode (ABC TV) 25 September 2016
- SRA media release 26 September 2016
- CaneConnection Spring 2016
- Chlorotic streak fact sheet ISI 7007

Table 1 summarises the considerable media coverage arising from the Landline episode and SRA media release, until 21 October 2016.

Table 1: Media coverage arising from the Landline episode and SRA media release, compiled by Brad Pfeffer

Date 2016	Media outlet	Audience size
25 September	ABC Landline	276000
25 September	ABC online	291000
27 September	ABC Country Hour	Unknown
27 September	ABC Rural Report (Townsville and Cairns)	Unknown
26 September	Cairns Post	16636
28 September	Seven News Cairns	21000
28 September	Seven News Mackay	13000
29 September	North Queensland Register	2827
1 October	Herbert River Express	2250
5 October	Whitsunday Guardian	1530
30 September	Burdekin Advocate	2000
30 September	Seven News Townsville	14000
1 October	Innisfail Advocate	3067
September	SRA CaneConnection	2600
10 October	Australian Canegrower magazine	2500
7 October	Northern Miner	2298
10 October	SRA e-newsletter	3000
29 September	Burdekin Canegrowers enews	Unknown
19 October	Mareeba Express	13,000
20 October	Port Douglas and Mossman Gazette	3773
	Total	**670,481**

We received positive feedback at all locations where the CS/ Biosecurity roadshows were held. Many Productivity Services were happy to prepare letters of support for future research on chlorotic streak, indicating that they were interested in supporting the adoption of project outputs. The comments from MAPS, BPS, SSP, HCPSL and TCPSL are provided below. Most Productivity Service groups saw the development of a rapid resistance screening method as the most important output, with one group more interested in the development of a diagnostic test.

Table 2: Industry interest in the adoption of project outputs resulting from the CS/ Biosecurity roadshows

Productivity Service	Correspondent and date of support letter	Main area of interest in adoption of project outputs as indicated in the support letter
MAPS	John Agnew 19/2/17	"A protocol for rapid screening of clones for disease reaction…will be of significant befit to the sugar industry because it will result in the release of more clones with disease resistance"
BPS	Rob Milla 17/1/17	"It would be of great benefit to the industry to have a rapid screening method for new varieties"
SSP	Peter Sutherland 30/1/17	"A rapid resistance screening method would prevent highly susceptible varieties such as Q238(ɬ) from being released'
HCPSL	Lawrence Di Bella 29/1/17	"A rapid resistance screening method would greatly assist in the selection of resistant varieties for our region"
TCPSL	Gerry Borgna 23/1/17	"A diagnostic test for our seed plots would help ensure delivery of clean seed"

Northern industry information meeting:

The northern meeting staged on Monday April 18th 2016 at the Brother's Leagues Club, Cairns, was devoted to CS project research. The title of the session was: *"New breakthrough on chlorotic streak"* presented by Rob Magarey.

Shed meetings:

A series of shed meetings for the Tully district was organised by the Tully Cane Productivity Service for early May 2016. CS was covered by Rob Magarey in the session on pathology research.

Industry training opportunities:

Introductory and advanced pathology courses were held at Woodford in March 2015 and February 2017. For both courses, current progress in CS research at SRA was presented in a seminar format and then workshop participants learnt the practical aspects of symptomology, diagnosis and control during the disease training sessions.

Laboratory tours and presentations:

Several Indooroopilly Biotech laboratory tours and informal presentations were made by Kathy Braithwaite and Chuong Ngo during the project. They discussed progress on identifying the causal agent and attempts to isolate and culture the organism. The visitors were:

- QDAF staff on 17 December 2015
- SRA board members on 27 January 2016
- Qld Government minister Leanne Donaldson 31 August 2016
- Pathologist and breeder from Argentina 29 November 2016

Conference presentations

- "Progress in understanding and managing chlorotic streak" by Kathy Braithwaite, Chuong Ngo, Mona Singh, Katherine Sventek, Robert Magarey and Barry Croft. Presented at the ISSCT XI Pathology and IX Entomology Workshops, Guayaquil, Ecuador September 2015.

- "Progress in understanding and managing chlorotic streak of sugarcane" by Kathy Braithwaite, Chuong Ngo, Barry Croft, Rob Magarey and Anthony Young. Presented at the 29th ISSCT Congress, Chiang Mai, Thailand, in December 2017. This paper won the award for the best paper in the biology section of the congress.
- "Insights into the epidemiology of chlorotic streak disease as determined by multiple field assessments" by Anthony Young and Mark Ensbey. Presented at the 37th ASSCT conference, Bundaberg 2015
- "Chlorotic streak transmission and crop dynamics research" by Rob Magarey, Katherine Sventek, Judi Bull and Kathy Braithwaite. Presented at the 38th ASSCT conference, Mackay 2016.
- "Chlorotic streak resistance screening in the Tully district" by Rob Magarey, Katherine Sventek, Judi Bull, Joanne Stringer and Ray Zamora. Presented at the 38th ASSCT conference, Mackay 2016.

4.2. Industry communication messages

The major forum for communicating the outcomes of this project to the industry was a CS/biosecurity roadshow held in late 2016 and early 2017. The dates and locations of these presentations is shown in **Table 3**.

Table 3: Dates and locations of the CS/ Biosecurity roadshows

Target District	Location	Date	Presenter
Mackay, Plane Creek	SRA Mackay	21/11/17	Kathy Braithwaite
Proserpine	Proserpine Canegrowers	22/11/16	Kathy Braithwaite
Burdekin	BPS	22/11/16	Kathy Braithwaite
Ingham	HCPSL	23/11/16	Kathy Braithwaite
Tully	SRA Tully	23/11/16	Kathy Braithwaite
Innisfail/Babinda Mossman	SRA Meringa	24/11/6	Kathy Braithwaite
NSW	Sunshine AG Advisor meeting, Sunshine Sugar Ballina Corporate Office	6/2/17	Kathy Braithwaite
Bundaberg, Isis, Maryborough	Southern Region Variety Approval Committee Meeting at Childers	9/2/17	Chuong Ngo

The six presentations given in 2016 were dedicated events for CS and biosecurity targeting productivity services staff and their board members. The participants were spread across the industry as shown in **Table 4**.

Table 4: Industry participation at the CS/ Biosecurity roadshows

Industry sector	Participants
Productivity Services	36
Growers	7
SRA staff	8
Canegrowers employees	2
Wilmar employees	2
DAF extension officer	1
Consultant	1
Tully sugar employee	1
Total	**57**

During the CS/ biosecurity roadshows held in late 2016, Matt Reynolds documented the feedback and questions arising from the presentations. The opportunity to ask questions identified a number of areas for further work: 1) understanding of the organism; 2) symptoms of CS, and 3) variety screening. This indicates that CS is considered an important disease in our industry, and that more research is required and that industry would welcome the adoption of project outputs.

1) *Understanding of the organism*

   Understanding where the organism exists outside of the sugarcane plant, in addition to aspects which would relate to the farm management of the disease was seen across all events.

2) *Symptoms of chlorotic streak*

   The potential for a plant to be infected with CS but not express disease symptoms, and the implications this could have on the management of the disease, was discussed at each location.

3) *Variety screening*

   Disease resistance data on CS was seen as being of great value, and brought up a number of questions regarding the process and how it would fit into the broader variety screening process.

The full list of questions is provided in Appendix 9.2.

The events held in 2017 were part of other events and no formal feedback was recorded. However, they were both well received, particularly by the NSW group. They immediately invited Kathy Braithwaite and Chuong Ngo to visit a nearby farm that had recently flooded and was now displaying chlorotic streak so that samples could be taken for diagnostic testing.

5. METHODOLOGY

5.1. Identification of the causal agent through molecular approaches

Previous research into identifying the causal agent of chlorotic streak (CS) identified an actin PCR primer set that generated a 750bp DNA fragment highly correlated with disease symptoms (Braithwaite and Croft, 2013).

Actin proteins are found only in eukaryotic cells and participate in many important cellular processes including muscle contraction, cell motility, cell division and organelle movement. Unfortunately, actin genes are highly conserved and Blast (Basic local alignment search tool) database searching could not determine the origin of the DNA fragment or any related organisms. Next Generation Sequencing (NGS) is a powerful tool that can produce an enormous volume of sequence data relatively cheaply. The NGS process begins with fragmentation of a DNA or complementary DNA (cDNA) template that is then sequenced in millions of parallel reactions. Sequence data can be aligned (mapping) to known reference sequences or reassembled without known sequences (de novo assembly).

5.1.1 NGS sample selection and sequencing

Target tissue selection. Sequencing an unknown organism with an unknown genome size, mixed with sugarcane's large genome poses some challenges. Determining the relative abundance, using quantitative PCR (qPCR), of CS actin to sugarcane actin in various tissues will assist with sample selection. Tissues with a higher CS ratio will be better candidates for sequencing, as they will contain a higher proportion of DNA sequences from the CS organism in the NGS data. Chlorotic streak actin was used to identify a homolog from sugarcane (86 % homology). Primers for qPCR were designed and verified for both CS and sugarcane actin (**Table 5**). The qCS Act R1a primer of the CS actin pair is positioned in an intron, which increases specificity. A second CS actin primer pair (qCS Act F3, qCS Act R3) was required to quantify CS RNA transcript levels.

Table 5: qPCR primers used for determining relative abundance of pathogen to host DNA.

Target gene	Primer	Nucleotide sequence (5' - 3')
CS actin (gDNA only)	qCS Act F1 qCS Act R1a	GAGCAAGCGTGGTATTCTCA GAAAAGCAAGGCAACAAACTC
CS actin	qCS Act F3 qCS Act R3	CGCAGAGCAAGCGTGGTATT CGTCCCAGTTCGTCACGAT
Sugarcane actin	qSo Act F2 qSo Act R2	GTTGCACCACCTGAGAGGA CATCTGTTGGAAAGTGCTGAG

All samples were collected from field-grown plants from the Woodford pathology farm, with the exception of the pot trial samples, which were glasshouse-grown. Stalk DNA was extracted using a Qiagen DNeasy Plant Mini Kit (used for subsequent DNA extractions on all tissues in this section), then used 25 ng of DNA per reaction. Unmodified xylem exudate was not prepared in any way and 5 µL was used per reaction. Xylem exudate DNA was first prepared by collecting xylem sap from several stalks, pooling then storing at -20°C.

DNA was extracted by first centrifuging 2 ml of exudate at 15,000 g for 1 min and then extracting DNA from the pellet. Quantifying DNA was not possible due to low concentrations and therefore 5 µL was used per reaction. Samples were also sourced from a fungicide pot trial at Woodford (Section 6.6). Symptomatic plants (all treatments excluding the short hot water treatment (HWT) control) were grouped as CS, while asymptomatic plants were grouped as HWT. Xylem exudate was collected from all stalks within the two groups and DNA extracted. Roots were sampled by germinating 40 (20 each of CS and HWT) single-eye setts taken from the lowest two nodes. Single-eye setts were placed in sterile damp vermiculite at 25°C. HWT setts produced roots within 4 days, while CS setts had no roots at the same time point. Roots were harvested after 14 days into liquid nitrogen and stored at -80°C.

Five µL and 25 ng of DNA extracted from exudate and roots were used per reaction, respectively. Relative pathogen abundance was also estimated from RNA. RNA was extracted from roots with a Qiagen RNeasy kit and cDNA synthesised using Promega DNase I, random primers and Promega ImpromII reverse transcriptase. Template cDNA was first screened for DNA contamination with PCR primers PFK5 F1 and PFK5 R1 (Zhu *et al.*, 2013, Section 5.4.2), ensuring only cDNA is measured. A total of 25 ng cDNA was used per qPCR reaction. Quantification was performed on a ViiA 7 real-time PCR system (Applied Biosystems) with SensiMix SYBR Low-ROX (Bioline, Alexandria, NSW, Australia). Primer concentrations were 200 nM, a 15 µl reaction volume and an annealing temperature of 60°C. Ct values were used to estimate relative abundance using the formula $2^{(\text{CSActin Ct - SoActin Ct})}$.

NGS sample collection and DNA extraction. Harwood, NSW was chosen as the location for sample collection due to having a two-year crop rotation and the prevalence of chlorotic streak; leading to an abundance of diseased material. All samples were from the variety Empire. Healthy samples were collected from field 3700 on farm 1198 (Fischer farm). The field had an elevated position and therefore would possibly contain less chlorotic streak compared to other farms. Four stools from 2 year old cane were selected and harvested (10 - 15 stalks) by Anthony Young from Sunshine Sugar early in the morning on 3rd March 2014. Closer inspection showed that stool 1 had symptoms on some stalks and therefore samples from each stool were pooled separately. Stalks were stripped of leaves, washed with Decon90 detergent and a scourer, rinsed under running tap water and then sprayed with 70 % alcohol. Xylem exudate was collected into 50 ml falcon tubes on ice and stored at -20°C until required (**Table 5**). A stalk from hot water treated (HWT) plant cane, from the Harwood approved seed plot, was also collected on the 4th March 2014 and was stored at 4°C.

Chlorotic streak affected samples were collected early morning on 4 March 2014 from field 1200 on farm 1101 (Young farm). Three sample types were collected: (1) stalks from 1-year cane, (2) stalks from 2-year cane and (3) suckers on 2-year cane (**Table 5**). Fifteen stalks were cut each from 2-year cane and suckers, both of which showed excellent symptoms. Short internodes were discarded from the 2-year cane, due to difficulty with collecting exudates. Thirty stalks of 1-year cane were collected and all stalks showed symptoms. Stalks were prepared and xylem exudate collected in the same manner as was performed for healthy samples.

In consultation with QFAB (http://www.qfab.org/) it was decided DNA sequencing (DNAseq) was the best approach. This was due to the higher pathogen to host ratio in DNA samples compared to RNA. Two samples were prepared for DNAseq: (1) exudate DNA from symptomatic chlorotic streak affected plants (CS); and (2) exudate DNA from asymptomatic healthy plants (H).

Due to lower than expected yields from DNA extractions, both CS and H samples were pooled from a number of samples. The CS sample was pooled from DNA extractions of CS S 1, CS S 2, CS 2y 1 and H 2y 4 (**Table 6**). The H sample was pooled from DNA extractions of H 2y 2, H 2y 3 and HWT plant cane.

Table 6: Xylem exudate samples collected for DNA extraction to be used in NGS analysis.

Xylem exudate sample	Sample name	Exudate volume collected	Symptoms present
Healthy, 2 year old, stool 1	H 2y 1	20 ml	yes
Healthy, 2 year old, stool 2	H 2y 2	13 ml	no
Healthy, 2 year old, stool 3	H 2y 3	17 ml	no
Healthy, 2 year old, stool 4	H 2y 4	35 ml	no
CS, 1 year old, tube 1	CS 1y 1	35 ml	yes
CS, 1 year old, tube 2	CS 1y 2	25 ml	yes
CS, suckers, tube 1	CS S 1	25 ml	yes
CS, suckers, tube 2	CS S 2	30 ml	yes
CS, 2 year old, tube 1	CS 2y 1	15 ml	yes

Xylem exudate DNA samples were screened by PCR using the actin F1 and R1 primers (Braithwaite and Croft, 2013) and sugarcane PFK5 F1 and R1. PFK5 primers were used as a PCR control (Section 5.4.2).

Sequencing. The Illumina HiSeq 2000 was chosen as the sequencing platform on recommendation from QFAB. One hundred bases were sequenced from both ends of 350 bp DNA fragments (paired end sequencing) and both CS and H samples were sequenced on 1 lane (samples are barcoded first). One lane generates approximately 15 to 20 Gb of sequence per sample. Two samples each approximately 5 µg DNA, at a concentration of 200 ng / µl were submitted on 15 April 2014 for library preparation and DNA sequencing to The Ramaciotti Centre for Genomics (http://www.ramaciotti.unsw.edu.au/). Libraries were produced using a TruSeq DNA PCR-Free Library Preparation Kit (Illumina, San Diego, CA, USA) that utilises Covaris shearing to produce a 350 bp average insert size. One hundred base pair paired-end sequencing was carried out on a HiSeq 2000 (Illumina, San Diego, CA, USA). Data from the DNAseq experiment was forwarded to QFAB for quality control of the raw DNA sequence reads and bioinformatics analysis.

5.1.2 Bioinformatics and gene cloning

Bioinformatics analyses were done using Geneious version 8 (Biomatters Ltd, New Zealand), unless specified.

QFAB bioinformatics. Sequencing data was forwarded to QFAB bioinformatics who performed quality control using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Initial analysis of NGS data involved the mapping of the short read sequences onto a QFAB curated database of pathogen and microbiome genome sequences. This was performed to quickly look for any organisms that are abundant in CS compared to H samples. Draft genome sequences from the human microbiome, bacterial genomes and viral genomes were collated along with potentially contaminating sequences from organellar genome databases, synthetic sequence databases and plasmid databases. The bowtie2 software (Langmead and Salzberg, 2012) was used to for mapping of reads against the sequence databases and the picard software (https://broadinstitute.github.io/picard/) was used to convert the resulting output into the binary and indexed bam format.

A second approach utilised the Kraken program (Wood and Salzberg, 2014) that looks for unique k-mer words produced from user-defined databases. Unique k-mers are generated using Jellyfish (Marcais and Kingsford, 2011) from DNA sequences of a collection of taxons within the user-defined database.

The unique k-mers were mapped to 95,000,000 joined reads (reads from a pair are joined with an N separating them i.e. read1:N:read2) each from the CS and H sequence data (**Figure 1**). Counts are made for k-mer matches to sequence reads and are assigned to a taxon. NCBI (National Center for Biotechnology Information) taxa results are differentiated at several levels including domain, kingdom, phylum, order, family, genus and species. Sequence databases of bacteria, algae, algae EST (expressed sequence tags), fungi, fungi EST and an SRA defined database (Appendix 9.3) were compiled from all publically available GenBank data for the groupings. The generated databases were used as inputs for Jellyfish and Kraken. Presence or absence and relative abundances of taxa in the CS and H samples were examined.

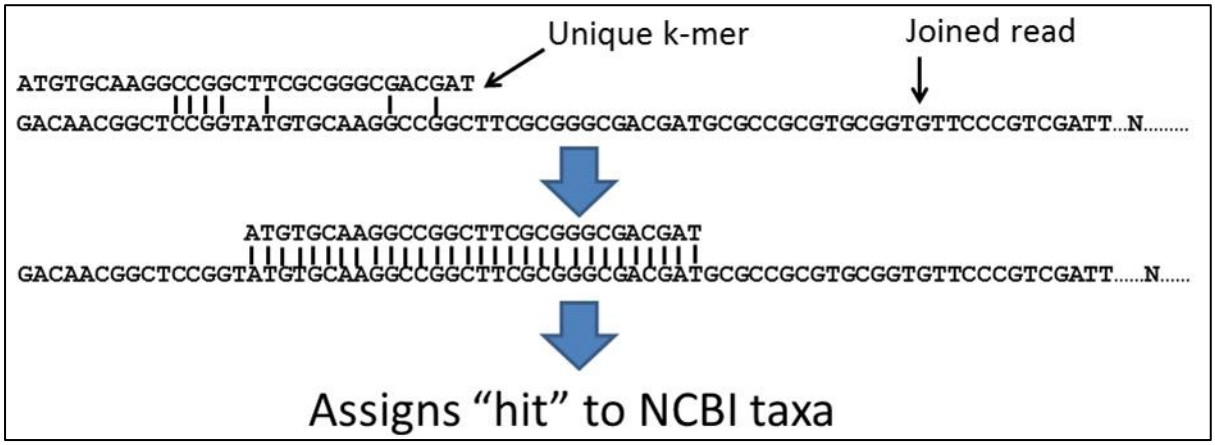


Figure 1: Generalised principle of sequence data analysis using Kraken.

Ribosomal DNA sequence. *Saccharum* spp. ribosomal sequences (GenBank accession KF184927, 18S position 8,033-6,228 and 28S position 5634-2,246) and *Cladochytrium* 18S and 28S sequences (KF711853 and KF711852) were used for mapping analyses. Mapping was performed using default parameters with a 20 % allowable mismatch and three iterations. Alignments were manually examined and novel reads were compared against GenBank sequences using BlastN. Contigs generated from novel reads were aligned back to *Saccharum* spp. ribosomal sequences. Primers were designed to sequence areas with low homology, PCR verified, Sanger-sequenced and then used as a reference for further mapping (0 % allowable mismatch) and sequence extension. This process was repeated until a single unambiguous sequence was achieved. Verification of the 10 kb ribosomal DNA sequence was performed by amplification using LongAmp Taq DNA Polymerase (New England Biolabs, Ipswich, MA, USA) on DNA extracted from cultures (extraction similar to xylem-exudates) and with the primers CS IGS F1: 5'-CGTAGAGGAGCAATGCATCA-3'; CS 28S R10: 5'-TGAAACGTTAGTGCCGAGAC-3'. Sequencing was performed using primers designed to cover the length of the sequence. The ribosomal 18S, 5.8S and 28S subunits were annotated by aligning the 18S small subunit to *Cercomonas plasmodialis* (GenBank accession AF411268) and the 5.8S and 28S subunits to *Plasmodiophora brassicae* (GenBank accession AB526843).

Actin gene sequence extension. Sequencing data from the CS sample was used to map to a 1,300 bp actin sequence (Braithwaite and Croft, 2013), with default parameters, a 5 % allowable mismatch rate and five iterations used to extend the sequence.

Consensus sequences were generated and mapping repeated until no further extension occurred. Several primers were designed to the new putative 5' and 3' regions of the sequence using the Genscript online primer tool (www.genscript.com).

Specificity was tested with PCR and Sanger sequencing on template DNA from diseased and healthy xylem-exudates. Amplification of actin was performed using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) with primers: CS Act F1: 5'- GGTGACTGACGTGGATTCTTT-3'; CS Act R6: 5'- GCTTCGTTAGTTGCTGATTCAA-3' on DNA extracted from cultures. An actin cDNA sequence was generated from infected root RNA extracted using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and cDNA synthesised using Improm II Reverse Transcription System (Promega, Madison, WI, USA). Actin cDNA was amplified with actin F1 and R1 primers (16) and sequenced. The cDNA sequence was used to annotate the intron area by aligning to the gDNA sequence.

Polyubiquitin gene sequence. Amplification of polyubiquitin was performed with Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA), DNA-template extracted from cultures
and the primers UBIQ1: 5'-GGCCATGCARATHTTYGTNAARAC-3' and IUB2:

5'-GATGCCYTCYTTRTCYTGDATYTT-3' (Archibald *et al.*, 2003). The UBIQ1 and IUB2 primer pair generates a ladder of ubiquitin gene products corresponding to repeats of polyubiquitin monomers. Fragments between 400-900 bp were cloned into pGEM-T Easy Vector Systems (Promega, Madison, WI, USA) and sequenced using M13 forward and reverse. The introns were annotated by comparing to a *Cercomonas edax* (GenBank accession AY099138) amino acid translation.

5.1.3 Phylogenetic tree construction

Phylogenetic tree construction was performed by David Bass from the Division of Genomics and Microbial Diversity, Department of Life Sciences, Natural History Museum, London, SW7 5BD, United Kingdom. A cercozoan reference sequence dataset was modified from Howe *et al.* (2011), including the recently described putative Cercomonad Kraken carinae (Dumack *et al.*, 2016). The chlorotic streak organism SSU rRNA gene contig (1,823 bp) was added to this and aligned using the E-ins-I algorithm within mafft version 7 (http://mafft.cbrc.jp/alignment/software/). The resulting alignment (121 sequences, 1781 positions analysed) was refined manually and analysed using Maximum Likelihood (ML) in RAxML BlackBox version 8 (http://sco.h-its.org/exelixis/web/software/raxml/) (Generalized time-reversible (GTR) model with CAT approximation (all parameters estimated from the data); bootstrap values mapped onto the tree with the highest likelihood value). A Bayesian consensus tree was constructed using MrBayes v 3.2.5 (http://nbisweden.github.io/MrBayes/). Two separate MC3 runs with randomly generated starting trees were carried out for 4 million generations each with one cold and three heated chains. The evolutionary model applied included a GTR substitution matrix, a four-category autocorrelated gamma correction and the covarion model. All parameters were estimated from the data. Trees were sampled every 1,000 generations. The first 1 M generations were discarded as burn-in (trees sampled before the likelihood plots reached stationarity) and a consensus tree was constructed from the remaining sample. The chlorotic streak 18S rDNA sequence that we generated is available from GenBank (accession KR704194).

5.1.4 Community profiling

Experiment 1: 16S, 18S and ITS amplicon sequencing. It was worthwhile investigating the possibility that other hidden organisms may be correlated with chlorotic streak symptoms. Community profiling is a technique based on the sequencing of PCR amplicons generated with degenerate primers on environmental samples.

The Australian Centre for Ecogenomics (ACE; www.ecogenomic.org) was used for this service, performing both library preparation and sequencing. Sequencing of bacterial 16S ribosomal RNA (rRNA), eukaryotic 18S rRNA and fungal internal transcribed spacer (ITS) amplicons was performed using the Illumina MiSeq platform, producing 250 bp paired reads.

The bacterial and archaea 16S rRNA primers used were 803F (5' – TTAGANACCCNNGTAGTC – 3', 4 primer mix) and 1392wR (5' – ACGGGCGGTGWGTRC – 3'). In *Escherichia coli*, it amplifies the 803-1392 region of the 16S gene. The eukaryotic 18S rRNA primers used were 563F (5' – GCCAGCAVCYGCGGTAAY – 3') and 1132R (5' – CCGTCAATTHCTTYAART – 3"). In *Saccharomyces cerevisiae* it amplifies the 563-1132 region of the 18S gene. The fungal ITS2 primers used were ITS3 (5' – GCATCGATGAAGAACGCAGC – 3') and ITS4 (5' – TCCTCCGCTTATTRATATGC – 3'). In *S. cerevisiae*, it amplifies the entire ITS, including the end of 5.8S and the start of 28S. A small number of samples (**Table 7**) were selected for sequencing and 3 ng / µL provided to ACE.

Table 7: Samples included in the initial community profiling analyses.

Sample	Sample description
Q241$^{\Phi}$	Xylem exudate pooled from infected Q241$^{\Phi}$ plants from Tully field.
Empire	Xylem exudate pooled from infected Empire plants from Harwood field.
RP CS	Xylem exudate pooled from infected RP193-67 plants from Woodford glasshouse.
RP H	Xylem exudate pooled from healthy RP193-67 plants from Woodford glasshouse.
RP F	Xylem exudate pooled from infected RP193-67 plants treated with various fungicides from Woodford glasshouse.
TC	Whole plant tissue from dying Empire plantlet from Indooroopilly tissue culture facility.
NEG	Negative control.

Only forward reads are processed from NGS and reads are discarded if they fail quality control, are single reads, have > 0.05 % abundance or have less than 60 % sequence identity to the data. The sum of read distributions are not 100 % due to discarded reads. Reads were assigned to an operational taxonomic unit (OTU) from the Greengenes database (http://greengenes.lbl.gov) for 16S, the SILVA database (http://www.arb-silva.de) for 18S and the UNITE database (https://unite.ut.ee) for ITS sequences.

Experiment 2: 16S, 18S and ITS amplicon sequencing with PCR blockers. Due to the over-representation of sugarcane reads in the initial community profiling data, the experiment was repeated using sugarcane specific PCR amplification blockers. PCR blockers bind to specific sequences in a sample and stop polymerases from amplifying that sequence. By reducing amplification of sugarcane sequences, this should increase amplification of less abundant sequences in a sample. We chose to use C3 based blockers on recommendation from ACE and due to cost.

Sugarcane mitochondrial and plastid sequences were blocked using ACE in-house blockers mit-C3 (5' - GGCAAGTGTTCTTCGGA – 3') and Plas-C3 (5' - GGCTCAACCCTGGACAG – 3') for the 16S bacterial primer set. Due to the position of these blockers, primers were different to the initial 16S community profiling experiment. Primers used were 341F (5' - CCTACGGGNGGCWGCAG – 3') and 806wR (5' – GACTACHVGGGTATCTAATCC – 3'). The 18S primer set used the SRA designed PCR blocker (5' – ACTCGACCCTTCTGCCGGCG – 3'), while the fungal ITS primer set used the SRA designed PCR blocker (5' - AAAGACACTCCCAACCCACCCGA -3'). Both 18S and ITS primer sets were identical to the initial community profiling experiment.

Experiment 3: 18S amplicon sequencing. Eukaryotic 18S community profiling was repeated to confirm associations discovered in community profiling experiment 1. Samples sequenced represented different sugarcane varieties from different locations (**Table 8**), samples from Koch's postulates experiments, cell cultures, and sorghum transfection (Section 6.2) and water from root exudates (Section 6.3).

Community profiling of cultures can also show whether there is a single or multiple organisms present.

ACE were again used to perform library preparation and sequencing using the Illumina MiSeq platform (250 bp paired reads). Primers are as per community profiling experiment 1. Two rounds of amplification were performed for samples and these were pooled. This was due to low DNA quantities extracted from small samples.

Table 8: Samples included in the third community profiling analyses.

Sample #	Sample name	Sample description
Xylem exudate		
CS 1	Q155 CS	Woodford field (originally from Rocky Point)
CS 2	Q155 H	Woodford field (originally from Rocky Point)
CS 3	Q241$^{\Phi}$ CS 1	Tully field (2014 trip)
CS 4	Q241$^{\Phi}$ H 4	Tully field (Kat sent down)
CS 5	Empire CS	Woodford field (originally from NSW)
CS 6	Empire H	Woodford field (originally from NSW)
CS 7	Q90 - CS from RP culture	Woodford GH. Koch's postulates plants - rep B
CS 8	Q90 - CS from Empire culture	Woodford GH. Koch's postulates plants - rep B
CS 9	Q90 - H from Q228 culture	Woodford GH. Koch's postulates plants - rep B
Cultures		
CS 10	Q90 - CS from RP culture	Woodford GH. Koch's postulates plants - rep B
CS 11	Q90 - CS from Empire culture	Woodford GH. Koch's postulates plants - rep B
CS 12	Q90 - H from Q228 culture	Woodford GH. Koch's postulates plants - rep B
CS 13	2T2 initial	Super culture from contamination experiment
CS 14	2T2 S8	1st sub into S8
CS 15	2T2 CM sub1	1st sub into corn meal
CS 16	2T2 CM sub2	2nd sub into corn meal
CS 17	2T2 PD sub1	1st sub into potato dextrose
CS 18	2T2 PD sub2	2nd sub into potato dextrose
Root water		
CS 19	Empire CS - Treatment A	Barry's root water experiment 19/6/15
CS 20	Empire CS - Treatment B	Barry's root water experiment 19/6/15
CS 21	Empire H - Treatment A	Barry's root water experiment 19/6/15
CS 22	Empire H - Treatment B	Barry's root water experiment 19/6/15
Sorghum		
CS 23	Sorghum root Tully	Tully GH troughs - good symptoms, PCR +
CS 24	Sorghum root Woodford	Woodford GH then field - poor symptoms, PCR +
CS 25	Sorghum root Harwood plant 2	Harwood field - no symptoms, PCR -
CS 26	H2O	Negative control

5.2. Pathology investigations and Koch's postulates

5.2.1 Isolation and Culturing of *P. venanatans*

Source material for the original *P. venanatans* isolations were the CS-infected sugarcane cultivars Empire, RP193-67 and Q238$^{\Phi}$, maintained in the field at the SRA Pathology Farm at Woodford. Individual stalks were only selected for pathogen isolations if their leaves displayed strong visual symptoms of the disease. Later *P. venanatans* isolations were made from experimentally inoculated plants.

Sugarcane stalk lengths were washed and scrubbed thoroughly, sprayed with ethanol, and under sterile conditions, re-cut and split longitudinally (**Figure 2A**).

Small pieces of internal stalk tissue were removed from the node regions (**Figure 2B**) and transferred initially to S8 broth media (Davis *et al.* 1980) in Nunc EasYFlask cell culture flasks (Thermo Fisher; **Figure 2C**). Cultures were incubated at 28°C in the dark without shaking. Culturing success was assessed by checking flask cultures under an Olympus CK2 inverse microscope (**Figure 2D**), counting cells on a hemocytometer under an Olympus BX50 light microscope (**Figure 2E**) and by PCR using the CS diagnostic primers (see Section 6.4).

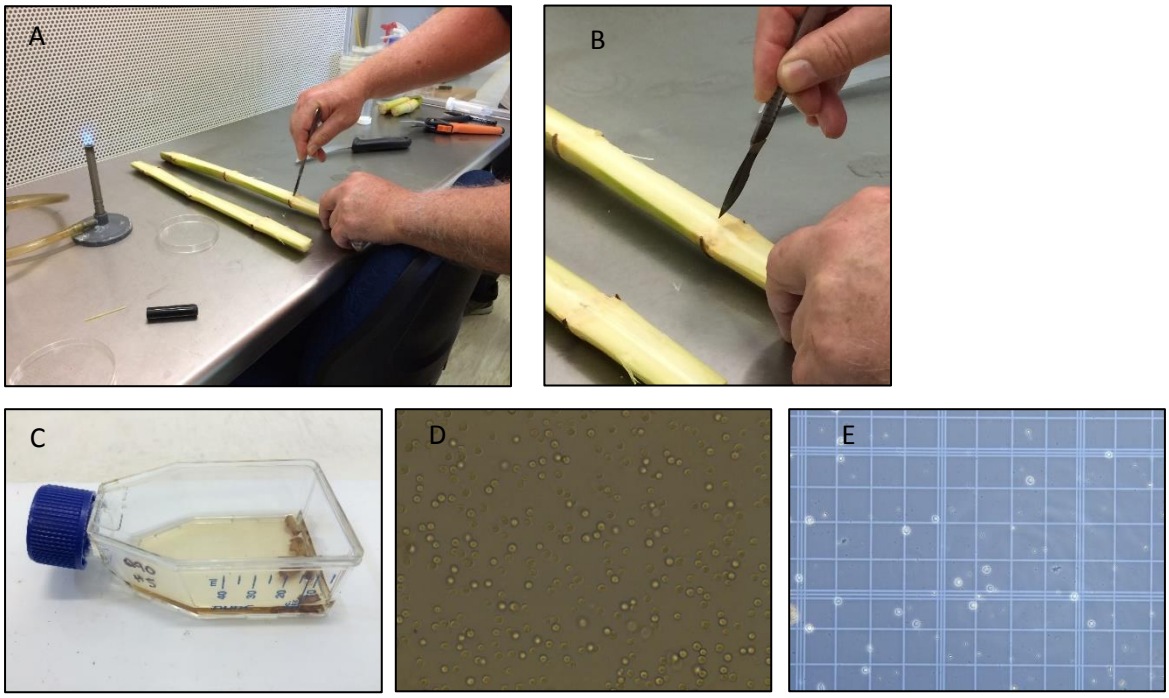


Figure 2: Steps in the method used to isolate *P. venanatans* and establish it in aseptic culture. A: washed stalks split longitudinally under aseptic conditions; B: small internal pieces of node tissue cut out; C: node tissue place in culture flask with liquid broth media; D: culture condition quickly checked by inverse microscopy; and E: cell counts made on a haemocytometer.

5.2.2 Establish growth curves for P. venanatans in culture

Growth curves were established for freshly initiated cultures and sub cultures. Cultures were initiated from CS-infected RP193-67 into S8 broth and cell counts were taken at various time points over 4 weeks. The best culture was subcultured onto two media, S8 broth and corn meal broth (40 g/l corn meal) six days after the original initiation with cell counts taken at various time points over three weeks.

A second experiment was performed to determine the optimal growth media. Sub culturing used the common pathological media, corn meal both and potato dextrose broth (200 g/l potato plus 20 g/l glucose) with or without supplemented peptone (4 g/l). A simple media of glucose (20 g/l) plus peptone (4 g/l) was also tested.

5.2.3 Inoculation experiments

To demonstrate Koch's postulates, cultures of *P. venanatans* were used to inoculate CS-free sugarcane plants by several methods. The cultivars Empire, RP193-67, Q183$^{\phi}$and Q90, sourced from the SRA Pathology Farm at Woodford, was prepared by exposing single-bud stalk cuttings to a hot water treatment of 50°C for 30 minutes (Bell, 1933).

Inoculation experiment 1. Root injection. Disease-free single-bud stalk cuttings of Q90 were planted into long flat-sided pots made from plastic conduit split in half lengthways. The open face was covered with clear, removable Perspex to allow access to the roots (Pearson *et al*., 1996). When 3 months old, 0.2 ml of one of three culture preparations were injected into the visible sett roots using a hypodermic syringe. The three culture preparations, all grown on S8 broth, comprised a fresh, actively motile 6-day old culture established from CS-infected Empire, an inactive (non-motile) 1-month old subculture originally established from CS-infected RP193-67, and a control cell-free culture derived from uninfected KQ228$^{\phi}$. For this initial trial, there was no attempt to quantify the

inoculum, as only the growth state of the cultures was being compared. There were two replicates per treatment. Plants were later transferred to 20 l pots and grown on the glasshouse floor.

Inoculation experiment 2. Root injection. Disease-free single-bud stalk cuttings of cultivars Empire and Q183$^{\phi}$ were germinated into 100 mm pots. When 2 months old, the root mass was removed from the original pot and the visible sett roots injected with 0.3 ml of a *P. venanatans* culture with a hypodermic syringe. Plants were immediately transplanted to larger 200 mm pots. The culture, established from CS-infected Q238$^{\phi}$ onto S8 broth, was 12-days old at the time of inoculation and actively motile with 790,000 cells/ml (based on haemocytometer cell counts). Control plants were injected with S8 media. There were three replicates per treatment.

Inoculation experiment 3. Stalk injection. Disease-free single-bud stalk cuttings of RP193-67, Empire and Q183$^{\phi}$ were germinated and grown for 5 months, by which time the plants had produced stalks approximately 20-30 mm in diameter. The plants were inoculated by injecting 0.1 ml of *P. venanatans* culture with a hypodermic syringe into each of three holes made with a fine needle in the root primordial region of the basal node of the stem. The culture was established from the severely infected Q183$^{\phi}$ plant inoculated in Experiment 2 through root injection. The culture was grown on S8 broth for 13 days and had 410,000 cells/ml. Control plants were injected with S8 medium. There were two replicates per treatment.

Inoculation experiment 4. Leaf whorl inoculations. Disease-free single-bud stalk cuttings of RP193-67 were germinated and grown for 3 months. One set of plants were inoculated by decapitating the shoot above the growing point with a pair of sharp secateurs and applying 0.2 ml of *P. venanatans* culture to the freshly cut surface of the leaf whorl. Another set was inoculated by injecting 0.1 ml of culture into each of three holes made with a syringe into the leaf whorl above the growing point of the shoot. Each set of plants had three replicates. The inoculum was a sub-culture of that used in Experiment 3 and grown for seven days on S8 broth to give 42,000 cells/ml. Control plants (two replicates) received no inoculation.

5.2.4 Transmission to other host plants

The initial attempts to transmit CS to sorghum were done prior to isolation *P. venanatans* in pure culture, replicating “natural” transmission.

Experiments were done at two locations using different approaches. At Woodford, pots of infected plants and test plants were arranged on glasshouse benches lined with geofabric to hold water. After a period of time to transmit the disease, the sorghum plants were planted in the field. At Tully, infected and test plants were grown in tubs of gravel. Symptomatic plants were sent to Indooroopilly for diagnostic PCR screening (see Section 6.4).

Corn and sorghum with the cultured organism. Seed of maize (*Zea mays* cultivar Terrific F1) and sorghum (*Sorghum* hybrid cultivar Cow Pow) were germinated and grown in commercial potting in 6-cell trays in the glasshouse. Seedlings were inoculated either by injecting 0.1 mL of *P. venanatans* culture into the leaf whorl above the growing point of the shoot, or by decapitating the shoot above the growing point and applying 1 drop of culture on the freshly cut stem surface. The culture was established from CS-infected Q238$^{\phi}$, and after its second subculture onto potato dextrose peptone broth, followed by seven days growth had 62,000 cells/mL. Control plants received no inoculation. Replicates ranged from six to eight for sorghum and five for corn.

5.3. Microscopy

5.3.1 Size determination

To determine the size of the organism prior to successful culture, xylem (sap) samples from CS-infected plants were successively filtered through Millipore filters in various combinations of 8 µm, 5 µm, 3 µm, 1.2 µm, 0.45 µm and 0.22 µm. DNA was extracted from the membranes with a MoBio PowerWater DNA extraction kit and the presence of the organism detected through diagnostic PCR screening (see Section 6.4).

5.3.2 General microscopy methods

Stalk and root tissue and fresh xylem exudate were examined by light microscopy on an Olympus BX50 DP controller software to capture images. Phase contrast microscopy used a Leica DM2000 LED microscope to view and capture images and video. Cells growing in liquid broth were visualized *in vivo* on a Nikon Eclipse E800 light microscope with images and measurements captured using the integrated LEICA™ camera and LuciaG software (Nikon). Differential interference contrast microscopy (Nomarski) was performed on a Leica DM6B.

Plant tissue were prepared in various ways:

1. Hand-sectioned tissues were viewed either unstained or stained with Toluidine Blue or Lacto-glycerol Blue. Tissues included roots, talks and buds.
2. Xylem sap collected by applying positive air pressure to stalk pieces
3. Root exudates obtained by placing roots in pure water at 28°C with no shaking for 2 hours up to 24 and 48 hours.
4. Node tissue from infected stalks were fixed with 10 % neutral buffered formaldehyde as well as formalin acetic alcohol and stained with haematoxylin and eosin.

5.3.3 *In situ* hybridization

The system chosen was random-primed DIG (digoxigenin) labelling of dsDNA using the Roche Applied Science system, with NBT/BCIP colour detection. The methods used were developed from Schwarzacher and Heslop-Harrison (2000), the DIG Application Manual for *in situ* Hybridisation supplied by Roche and the method supplied with the kit.

The dsDNA probes were derived from purified PCR products amplified from CS-infected stalks of Empire using the Actin, the 18S ribosomal gene and 28S ribosomal gene diagnostic primer sets. Labelling was done with the DIG High Prime DNA labelling and detection starter kit. While three probes were prepared, most work focused on the 18S probe as this was the most efficiently labelled.

5.4. Development of a diagnostic assay

5.4.1 Preparation of templates

Sugarcane DNA for PCR was generally prepared using the Qiagen DNeasy Plant mini kit, although the Qiagen DNeasy Plant maxi kit was used when larger amounts of starting material had to be processed, such as for stalks and leaf sheaths. Grinding of the tissue prior to DNA extraction generally used the Precellys bead-based system, but stalk material also required pre-grinding in a RobotCoupe homogenizer. A MoBio PowerSoil DNA extraction kit was used to extract DNA from roots. Xylem sap, expressed from stalks using positive pressure, was either subjected to full DNA extraction and purification with the Qiagen DNeasy Plant mini kit or a crude concentration step. DNA was extracted by centrifuging aliquots and resuspending the pellet in buffer AP1 supplied with the

kit, then continuing according to manufacturer's instructions. The crude template was prepared by centrifuging 100 µl aliquots for 5 minutes at 13,000 rpm, removing 90µL of supernatant and resuspending the pellet in the remaining liquid.

For cell cultures, 100 µl aliquots were centrifuged for 5 minutes at 13,000 rpm and the pellet resuspended in buffer AP1 supplied with the kit, then extracted according to manufacturer's instructions.

Soil and water PCR templates were extracted using the MoBio PowerSoil DNA extraction kit and MoBio PowerWater DNA extraction kit, respectively. The water was initially concentrated by filtering through a series of membranes, followed by extracting the DNA from the membrane. Millipore membranes (25 mm diameter) of 8, 1.2, 0.45 and 0.22 µm were used inside reusable screwed filter assemblies.

5.4.2 PCR primers for the routine detection of *P. venanatans*

The current routine gel-based PCR test for chlorotic streak is based on the ribosomal small subunit (SSU), and uses the PCR primers CS18SF1 and CS18SR2. An alternative set based on the ribosomal large subunit (LSU) is also available. An early generation test was based on actin primers supplied by Nicole Thompson (SRA) and Clint Magill (Texas A&M) and improved by designing a new reverse primer (Braithwaite and Croft, 2013). Routine diagnostic screening is always combined with an endogenous test to check for DNA quality and a range of primers were implemented depending on the various templates. All diagnostic primers used in the project are listed in **Table 9**.

PCR amplification for routine screening is based on Promega GoTaq Green master mix and performed on an Eppendorf Mastercycler proS or Applied Biosystems Veriti using a cycling program of 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 60°C for 1 minute and 72°C for 30 seconds, then a final cycle of 72°C for 2 minutes. PCR products were visualized by agarose gel electrophoresis with SybrSafe visualisation.

5.4.3 qPCR for the routine detection of *P. venanatans*

Quantitative PCR (qPCR) used primers targeting the ribosomal SSU for both the pathogen and host to quantify relative abundance of the pathogen (**Table 9**). qPCR was performed on a ViiA 7 Real-Time PCR system (Applied Biosystems) with SensiMix SYBR Low-ROX (Bioline, Alexandria, NSW Australia).

An annealing temperature of 60°C was used for all qPCR. Host to pathogen Ct values were compared to estimate a relative abundance.

Table 9: PCR Primers used throughout this study.

Test and target gene region	Primers	Nucleotide sequence (5' - 3')	Reference
First generation test for chlorotic streak: actin	Actin F1	GACAACGGCTCCGGTATGTGCAAG	Clint Magill (Texas A&M)
	Actin R1	GTCAGAATCTTCATCATGTAGTCCG	
Modified first generation test for chlorotic streak: actin	Actin R3	GCAAGGCAASAAACTCCTACC	Braithwaite and Croft (2013)
Current diagnostic test for *P. venanatans:* SSU	CS18SF1	GGGCGTTTATACGTCTGGTA	This study
	CS18SR2	CGGATGATCTGCAGTTGGTA	
Alternative diagnostic test for *P. venanatans:* LSU	CS28SF4	GTTGGCTGAGGTAGGAACCA	This study
	CS28SR2	ACCGTCCGGCTGTCTATATG	
Endogenous test 1 for sugarcane: sugarcane phosphofructokinase	ScPFK5F1	AGCCACATCAGATCAACAAG	Zhu *et al.* (2013)
	ScPFK5R1	TGAAGTTATACCCTGCCATT	
Endogenous test 2 for sugarcane: sugarcane actin-depolymerising factor	ADF-F	CTACTACTGTGGATTTGTACGCCATTA TAG	SRA in-house
	ADF-R	GGACCTTTTTTACACAGCAAGAAAC	
Endogenous test for cell cultures and environmental samples: Fungal SSU	NS7	GAGGCAATAACAGGTCTGTGATGC	White *et al*. (1990)
	NS8	TCCGCAGGTTCACCTACGGA	
qPCR pathogen combination: *P. venanatans* SSU	qCS18SF2	GGTTATCAGCCGAAGGAAGT	This study
	qCS18SR1	GTTGTATGCTCGATGCACCT	
qPCR sugarcane combination: sugarcane SSU	qSo18SF2	GGGGGCATTCGTATTTCATA	This study
	qSo18SR2	CCCCAACTTTCGTTCTTGAT	

5.5. Disease epidemiology

Two commercial crops in the Tully district were monitored for CS to learn more about disease transmission in the field. Sites were located in the Riversdale (Zamora) and Lower Syndicate (Harney) sub-districts.

5.5.1 Crop transmission dynamics

Zamora variety trial: Fully grown cane stools were inspected and mapped at the Zamora site in the Riversdale district, Tully. Though varietal differences were present (it was a variety trial), a comparison of the same (all) varieties in each replicate allowed for assessment of differences in disease distribution across the site. There was a definite slope across the block, with the northern end quite a lot higher than the southern (approximately 1 - 2 m) and significant differences in flood levels were observed during flood events. A single-row farming system was in operation with a trash blanket.

Harney: Crop growth was approximately waist height when the disease assessment was undertaken at this farm in the Syndicate area, Tully. The weather had been warm and dry and the assessment followed a very dry wet season (with wet season rainfall about half the average for the Tully area). The site is located in a flood-prone part of the district, though little spread in the last 12 months would have been expected because of the lack of flooding events. The crop was growing reasonably well and cultivated as part of a single-row farming system incorporating a trash blanket. Details of the inspected sites are outlined in **Table 10**.

Table 10: Field cropping sites inspected for chlorotic streak

Site	Zamora	Harney
Variety	Various	Q250$^{\Phi}$
Crop class	1R	2R
Area	0.16 ha	0.65 ha
Number of rows inspected	All plots	6 rows

5.5.2 Monitoring.

Where individual stools were assessed for CS, two assessors walked representative rows in the crops. Each individual stool was carefully observed for CS symptoms and GPS data for infected stools were recorded. A record was kept of stool numbers (both diseased and healthy), using a 'tele-counter'.

An opportunity arose to record individual disease stools at the Zamora variety trial; data were recorded on 10 April 2014, 20 October 2014 and 3 June 2015. The Harney cropping site was assessed at three different inspection times (10 December 2014; 10 May 2015; 9 November 2015).

5.5.3 Mapping.

Individual stool data were mapped using Mapinfo software. GPS data were uploaded and converted to 'Mapinfo' compatible files; stool data were manipulated using the software to determine the distribution of the disease within the crops.

5.6. New management strategies based on an understanding of the causal agent

5.6.1 Fungicide pot trial

A glasshouse pot trial was established at Woodford to test if three systemic fungicides could reduce the severity of chlorotic streak. The experiment also provided an opportunity to reduce or eliminate other organisms, particularly Oomycetes, to provide good quality experimental material for identification of chlorotic streak. The trial used the variety RP193-67 showing chlorotic streak symptoms. One-eye setts cut from symptomatic cane were dipped in water or fungicide and planted into untreated or fungicide-treated potting mix, three setts per pot, with four replicate pots (**Table 11**). In this experiment "control" plants are CS-infected while Treatment 2 plants grew from hot water treated setts, which should have rendered them disease-free.

Table 11: Details of the three fungicide treatments.

	Treatment	Active ingredient	Active against	Dip treatment	Soil treatment
1	Untreated control				
2	Hot water (50°C 30 min)		Standard CS treatment		
3	Fongarid	Furalaxyl 250 g/kg	Oomycetes	0.3g/L dip 10 min	0.08 g/4L potting mix
4	Intake Hiload Gold	Flutriafol 500g/L	Fungi	0.5 mL/L dip 10 min	0.4 mL/4L potting mix
5	Dynasty CST	Azoxystrobin 75 g/L Fludioxonil 12.5 g/L Metalaxyl 37.5 g/L	Fungi and Oomycetes	2 mL/L dip 10 min	0.53 mL/4L potting mix

5.6.2 Metronidazole pot trials

A glasshouse pot trial was established at Woodford to test if metronidazole, an antibiotic commonly used to treat protozoan infections in humans, is active against CS in plants and/or phytotoxic to plants.

The first trial used healthy and CS-infected symptomatic RP193-67, each cut from 12 stools, then recut into 18 setts per treatment, giving a total of 180 setts (90 CS and 90 healthy). Metronidazole (Sigma M3761-56) was prepared as 160ppm (0.64 g metronidazole + 4 L water) then diluted to 80, 40 and 20 ppm (**Table 12**). Setts were placed in the treatment solutions in plastic bags at room temperature overnight, then planted into 6-pack trays and placed in the germination chamber at 30°C for one week before being transferred to the glasshouse. The number of germinating shoots were counted over an 18 day period.

Table 12: Details of the first metronidazole trial.

Treatments	Code
Untreated CS infected control - soaked in water overnight	ControlCS
Untreated healthy control - HWT 30 min at 50°C then soaked in water overnight	ControlHWT
CS infected - soaked in 20 ppm metronidazole overnight	CS20ppm
CS infected - soaked in 40 ppm metronidazole overnight	CS40ppm
CS infected - soaked in 80 ppm metronidazole overnight	CS80ppm
CS infected - soaked in 160 ppm metronidazole overnight	CS160ppm
Healthy - HWT 30 min 50°C then soaked in 20 ppm metronidazole overnight	H20ppm
Healthy - HWT 30 min 50°C then soaked in 40 ppm metronidazole overnight	H40ppm
Healthy - HWT 30 min 50°C then soaked in 80 ppm metronidazole overnight	H80ppm
Healthy - HWT 30 min 50°C then soaked in 160 ppm metronidazole overnight	H160ppm

The experiment was repeated with a smaller number of treatments but testing a higher concentration of metronidazole. Only CS-infected RP193-67 was used in this experiment with 36 setts per treatment, totalling 108 CS infected one-eye setts. The cut setts were placed in plastic bags

with the treatments (**Table 13**) at room temperature for 24 hours, then planted in 6-pack trays and placed immediately into the glasshouse. After two weeks the number of germinating shoots were counted over a four-week period.

Table 13: Details of the second metronidazole trial.

Treatments	Code
Untreated CS infected control - soaked in water 24 hrs	ControlCS
CS infected - soaked in 200 ppm metronidazole	CS200ppm
CS infected - soaked in 400 ppm metronidazole 24 hrs	CS400ppm

5.6.3 The effect of metronidazole on the growth of P. *venanatans*

Potato dextrose +peptone (PDP) broth cultures of *P. venanatans* were established from CS-infected stalks of field-grown Q238[ф]. Cell counts were taken by counting on a haemocytometer (see Section 5.2) and the most actively growing culture at the time was subcultured into 10 mL PDP broths containing metronidazole at various concentrations ranging from 2.5ppm to 400ppm. Cell counts at day 0 were recorded and then taken again after 3 days growth at 28°C.

All treatments were done in triplicate. Three trials were necessary to establish the inhibitory range of metronidazole against *P. venanatans.*

5.7. NSW motherplot- and seedplot-based resistance screening methods

To gauge the susceptibilities of the varieties present under relatively uniform conditions, inspections were made of 24 varieties growing in a 2nd ratoon field that had previously served as a motherplot (MP). This field, planted in September 2011, was subject to four major floods (January 2012, January, February and March 2013), in addition to other waterlogging events. Random 20 m transects were measured out and the number of CS symptoms (i.e. individual leaves with one or more streaks) was recorded in a single row. These numbers were log10 transformed and then converted into a rating on a rounded scale of 1 - 9 by dividing throughout by the highest recorded incidence in a single variety and multiplying by 9.

During the annual RSD surveys of 2012, 2013 and 2014, seedbeds were given qualitative ratings for the amount of CS present (0-3) and the degree of crop unevenness (even, low, medium and high). Crop unevenness was included as CS will often only affect a proportion of stools in a field, and therefore can contribute to non-uniform growth. Variety and crop class (plant, replant, 1st ratoon, 1st replant ratoon etc.) were also recorded. Seedbed data were divided into the following categories: fallow plant (FP), replant (RP), ratoon from fallow plant (FR) and ratoons from replant (RR).

The average CS prevalence was calculated for the 20 varieties represented by 10 or more seedbeds. These were ranked and converted to a rounded ranking of 1 - 9 by dividing the CS prevalence for each variety with that of the highest, then multiplying by 9.

Varieties that had been in production for approximately 10 years or more were separated from varieties with less than 10 years of production to test if there is a general difference between crop uniformity and the susceptibility of ‘new’ and ‘old’ varieties grown at Harwood. This was done using the Wilcoxon Rank Sum Test with Statistix 10.

Data were sorted by conventional row spacings (1.50 m - 1.70 m single rows with or without controlled traffic) and dual row configurations (1.80 m - 1.83 m with controlled traffic). The relative amount of CS and crop unevenness was calculated for each system. Statistics were calculated using a log linear model test of independence using Statistix 10.

5.8. Tully field-based resistance screening methods

The Zamora site in the Riversdale district, Tully was used to develop a field-based screening method. The method relied on the natural transmission of CS in flood water and/ or root infection resulting from growth in infested soil. Standard varieties with a known field reaction were included (these were resistant, intermediate and susceptible) and were grown in replicated plots among a selection of commercial canes, thus facilitating a direct comparison of CS disease levels in test vs standard varieties. Disease incidence measurements were based on typical CS leaf symptom expression in individual stalks and stools over a two-year period. This enabled the application of resistance ratings to the test canes. No infection rows (consisting of CS-diseased planting material planted every third row, Magarey *et al.,* 2006) were included in the experiment. Infection relied totally on CS transmission from contaminated flood waters.

Disease-free planting material was sourced from the Tully Cane Productivity Services Approved seed plot at Merryburn Estate and also from the SRA Tully propagation site; standard varieties of known field reaction were specifically sourced from the SRA site. Many of the standards are older varieties that are not now grown commercially.

Trial essential details were as follows:

- number of commercial varieties tested: 17
- number of standards: 7
- replicates: 5
- plot size: 1 row x 9 metres
- trial design: randomised complete block

Flood events. The Zamora site is low-lying and located in an area regularly inundated by floodwaters. In the trial period, there were at least five flood events. Some of these lasted for 3 - 5 weeks.

There were a few events where water depth was as high, or higher, than the standing crop and this affected cane growth. Some stool death occurred in the lowest parts of the block and multiple side-shooting occurred on some stalks as a result of the flooding.

Trial harvest. The trial was harvested under commercial conditions on 6 August 2014. Harvest yields were not assessed as the principal aim was to assess disease levels and not yield loss. Maintaining disease-free plots for yield comparison would also have been impossible in the circumstances.

Monitoring. During the growth of the plant and first ratoon crops, varieties were inspected for CS. All shoots in all plots were inspected; disease incidence was recorded when typical leaf symptoms were observed. Records were based on a stalk or stool basis. Dates when inspections were undertaken are outlined in **Table 14**.

Table 14: Inspection dates and data recorded for the Zamora resistance screening trial planted in August 2013.

Data recorded	Inspection 1 (P) 10 April 2014	Inspection 2 (1R) 20 October 2014	Inspection 3 (1R) 3 June 2015
% stools diseased	+	+	+
% stalks diseased	+	-	+

5.9. Hydroponic-based resistance screening methods

5.9.1 Temperature-controlled root systems

Tully SRA has developed air-conditioned benches (Reghenzani, 1984) for Pachymetra root rot varietal resistance screening; root system temperature control is critical for Pachymetra root rot infection and symptom expression, and this is the case also for CS.

Initial testing of circulating hydroponic system. An air-conditioned bench was modified to suit a hydroponic growth system via the following modifications; a slope was added to the false floor to facilitate hydroponic solution drainage, and collection at the lower end. An NFT channel system that contained the hydroponic solution provided for the growth of test plant root systems. Sugarcane test plants were planted in the six NFT channels aligned across the bench (**Figure 3**). CS-infected plants were placed at the top of each NFT channel to provide an inoculum source for spread of the CS pathogen. Holes in the bench-top allowed for shoot growth of the 16 test plants in each of the six NFT channels, while maintaining the temperature-controlled air within the bench. Small plastic baskets were filled with inert clay balls ('pebbles', 1 - 2 cm diameter) and these supported the pre-germinated sugarcane test plants. Roots grew freely around the inert clay balls and into the hydroponic solution. At the lower end of the channels, hydroponic solution drained to a large storage container located within the air-conditioned bench. From there, the solution was pumped back to the top end of the channels to re-circulate past the sugarcane plants.

There was a maximum capacity of 96 plants in the bench (**Figure 3**). As CS is known to spread in drainage water, the system provided a suitable starting point to test CS transmission in a hydroponic system.

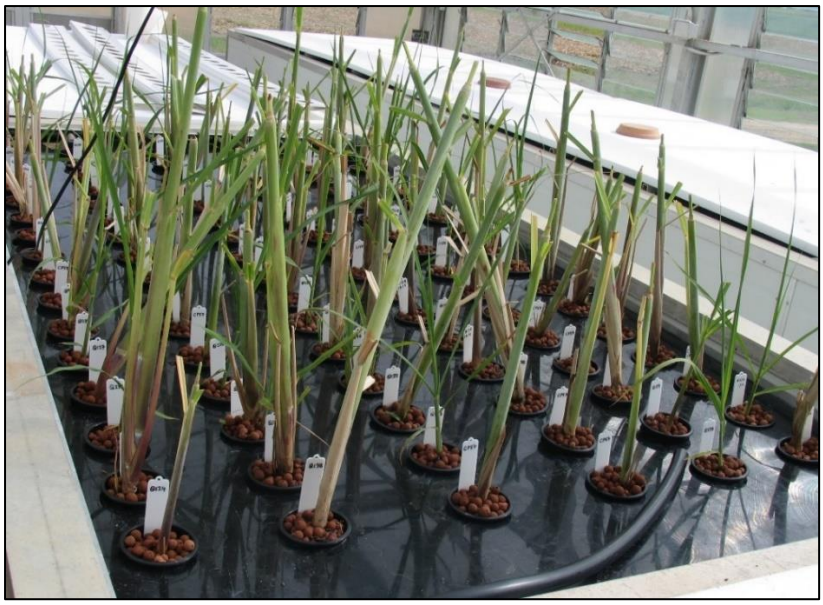


Figure 3: Set up of the test plants in the air-conditioned bench at Tully SRA.

The test varieties were randomly assigned within each NFT channel and were maintained in the bench for around four months. When mature stalk material was noted at the base of the developing shoots, the plants were ratooned; Egan (1965) had previously noted that higher rates of transmission occurred when plants were ratooned in the presence of the CS inoculum. Regular inspections were undertaken in both the 'plant' and 'ratoon' cane. The experiment was terminated after approximately 5 months.

Follow-up testing of circulating hydroponic system. The first experiment was repeated using similar methods but with regular adjustment of the solution pH using sodium hydroxide. An additional problem arose in this experiment with root infection by *Pythium arrhenomanes*. This pathogen has a zoosporic stage (motile spore) and hydroponic systems offer an ideal system for Pythium root rot spread. Previous soil research had identified the pathogen (Magarey and Croft, 1996); fungicides for disease management were also investigated (R.C. Magarey, unpublished data.). Furalaxyl was added to the hydroponic solution at 2 ppm, greatly reducing the effects of Pythium root rot.

5.9.2 Non-temperature controlled root systems

Still-gravel hydroponics. In order to multiply the CS inoculum and to provide CS-infected plants for glasshouse experiments, gravel-filled steel troughs, as used by Egan (1965), were established.

There was no management of root system temperature in this system. CS symptoms were evident in diseased material, there being very few problems with either plant nutrition, solution pH or Pythium root rot within these troughs. The need for large scale testing of varieties, and the bulky nature of the troughs, meant this system was generally unsuitable for large-scale varietal resistance screening. However, the success of this system may provide a useful model for a refined resistance screening procedure. Two 'gravel hydroponic' experiments were therefore established, using smaller plastic storage containers plus a modified inoculation system.

Egan (1965) showed that CS transmission can occur with just a two-hour dip of root systems in CS-infested hydroponic solution. He grew healthy test plants in disease-free solution until shoot roots had developed, dipped the root systems in infested solution for two hours, ratooned the plants and then established them in a gravel-filled steel trough. He changed the hydroponic solution every two weeks and monitored symptom development. Transmission was affected when several chemicals were introduced into the inoculum for the two-hour root inoculation: terramycin increased transmission while the general fungicide TMTD (Thiram) and the biocide PCNB reduced CS infection. His observations were used to design two 'gravel hydroponic' experiments.

Fifteen litre plastic storage containers were purchased and taps sealed into the base of each container (to allow for solution drainage). Washed 'blue metal' was obtained and used to two-thirds fill the containers. The susceptible varieties Pindar, Q238$^{\phi}$ and Empire were used in the experiment. Single-eye setts were germinated after a short hot water treatment (50°C, 30 min) to eliminate CS. Plants were initially grown in either perlite or vermiculite; plants were inoculated when mature stalk material had developed.

The following treatments were applied to the hydroponic solution, with test plant root systems dipped in the solutions for two hours:

1) *Healthy control*: roots dipped in water
2) *Disease control*: roots dipped in CS-infested hydroponic solution
3) *Terramycin*: roots dipped in CS-infested hydroponic solution with terramycin added at 0.02 % solution (w/v). 'Terramycin pinkeye powder', *oxytetracycline hydrochloride*, 20 mg/ g. Pfizer product.
4) *Thiram*: roots dipped in CS-infested hydroponic solution with Thiram added at 0.1 % (w/v) solution. TMTD, 500 g/ kg, Barmac product.

To prepare the treatment solutions, hydroponic solution was collected fresh from one of the CS diseased planting material troughs; plants growing in this trough were exhibiting excellent CS symptoms (leaf streaks) at the time. The solution was drained into a bucket and the treatment chemicals added within 20 min of collection.

The chemicals were weighed then mixed fresh with 5.0 L of water, and the roots submerged for two hours in the chemical solution. Water (control) and hydroponic solution (CS treatment) were prepared separately. Some root injury occurred at inoculation – this was found by Egan to increase CS infection (Egan, 1965). In this instance root injury was a natural consequence of washing away the potting medium from the roots at inoculation. Treatments were replicated four times.

Boxes containing the test plants were placed side-by-side in the glasshouse and care taken to avoid cross contamination.

The same experiment was repeated using identical methods; the first experiment was established on 7 July 2015, while the second was initiated on 30 July 2015. No diseased plants were placed with the test plants – infection relied solely on the two-hour inoculation. Plants were monitored closely over the next 12 weeks; plants were ratooned after 9 weeks. Soon after ratooning, symptoms began to develop, especially in the terramycin treatment and slightly later in the CS hydroponic solution treatment.

An attempt to analyse the results was made using a Generalised Linear Mixed Model assuming a Poisson distribution.

5.10. Woodford pot-based resistance screening methods

5.10.1 Plant material

Twelve varieties were selected from the small propagation plot at Woodford. An additional variety RP193-67, grown only at Woodford for screening trials, was included to provide experimental material for research. The clones were cut, subjected to a short hot water treatment at 50°C for 30 min and planted in May 2016. Germinated setts were transplanted to 100 mm pots, grown in the glasshouse on benches, and fertilised and sprayed for mites as required. Treatment 5 (see Section 5.10.3 below) required the soil pots to be placed inside a second pot containing sand in the bottom.

The trial required 30 plants of each variety (6 treatments by 5 reps). Plants were 10 weeks old at the time of inoculation (August 2016). All plants leaves trimmed on the day before inoculation.

5.10.2 Preparation of inoculum

Two weeks before the trial, *P. venanatans* was isolated from two stalks each of field-grown Q238$^{(\phi}$ and RP193-67, both excellent CS symptoms. After one week, contaminated and poorly growing cultures were eliminated and the six best were subcultured to six large (50 mL) flasks each containing 40 mL potato dextrose broth supplemented with yeast extract and peptone (PDYP). The subcultures were grown for a further week until the day of the inoculations. On the morning of the inoculations, all 36 flasks were combined to give approximately 1.4L of inoculum. Cell counts were determined and dilution factors calculated based on the volumes needed for each inoculation treatment (**Table 15**). Treatments 1, 2 and 4 needed smaller volumes of concentrated cells. Treatments 3 and 5 needed larger volumes of dilute cells. PDYP broth was used to make the dilutions, as we had previously determined that the *P. venanatans* is very sensitive to changes in media.

5.10.3 Inoculation methods

Five different methods of inoculation were trialled, while treatment 6 was the uninoculated control. A detailed description of each method is provided in **Table 15**. A simple code name was assigned to each method. **Figure 4** illustrates the five inoculation methods. During the inoculation proceedings, notes were made on the condition of the roots of each variety.

Table 15: Details of the inoculation methods and *P. venanatans* culture requirements to perform the Woodford glasshouse screening trial.

Treatment and code	Description/details	Estimated volume inoculum/ pot	Total volume (mL)
1 Injection	Remove plant from pot and inject the shoot roots and largest roots with a syringe using 0.3 mL inoculum per plant.	0.3 mL at 100,000 cells/ mL	19.5
2 Decapitation	Decapitate plants with sharp secateurs just below 2nd dewlap ensuring the cut is horizontal. Immediately place 0.3 mL of inoculum on freshly cut surface, then cover shoot with alfoil.	0.3 mL at 100,000 cells/ mL	19.5
3 Pour roots (undamaged)	Remove plant from the pot and line the pot with gladwrap. Slip plant back into pot while pouring in 40 mL inoculum of down the side of the pot. incubate at 30°C for 2 hr.	40 mL at 22,000 cells/ mL	600
4 Knife	Cut 3 slots radially in outer area of soil with a knife and add 1 mL inoculum to each slot	3 mL at 100,000 cells/ mL	195
5 Soak roots (damaged)	Remove upper pot from lower pot and wash sand away from the roots. Trim the protruding roots to 2-3 cm. Sit roots and pot in a petri dish with 40 mL of inoculum and incubate at 30°C for 2hr. Replace upper pot back into lower pot.	40 mL at 22,000 cells/mL	600
6 Control	No treatment	0	0

Figure 4: Methods used to inoculate plants with *P. venanatans* during the Woodford pot based screeing trial. A: *P. venanatans* injected through the roots (method 1); B: decapitation of stalks then *P. venanatans* applied to the cut surface (method 2); C: root ball and *P. venanatans* incubated within gladwrap-lined pot (method 3); D: *P. venanatans* applied to knife cuts in the soil (method 4); E: clean trimmed roots emerging from upper pot (method 5); and F: plants with trimmed roots soaking in *P. venanatans* in incubator (method 5).

5.10.4 Rating of the trial

The first rating took place 10 weeks after inoculation (October 2016). Five leaves on each plant (top visible dewlap leaf (TVD), TVD-1, TVD-2, TVD+1 and TVD+2) were rated according to a 0 - 4 scale (**Table 16**). Examples of the symptoms assigned to each rating scale are shown in **Figure 5**. Disease severity was calculated as DS= ((sum severity scores for 4 leaves)/(number of leavesx4))*100.

By this stage the plants had become too big and overcrowded, so after rating, each was repotted into a 200 mm pot with individual drippers and established on the glasshouse floor in another chamber.

Plants received their final rating 10 weeks later (December 2016). Again, five leaves per plant were rated (TVD, TVD-1, TVD-2, TVD+1 and TVD+2), however, the 0 - 4 scale was modified so that wilting of the leaves was no longer assessed (**Table 16**).

The trial was conducted using a randomised complete block design in a glasshouse environment. After the first assessment, the trial layout in the glasshouse was modified because pot size was affecting plant development and the newly potted plants were moved to the floor of a separate glasshouse. The randomised complete block design was maintained, however, the layout of the blocks within the glasshouse was different. For this reason each rating was analysed separately and as a combined data set. Pairwise comparisons of inoculation method and variety effects were conducted using least square means and compact letter display. All plots and model results were produced using R (R Core team, 2016).

Table 16: Rating scale devised for the Woodford pot-based chlorotic streak screening trial.

Rating scale	
0	no symptoms
1	1 CS streak
2	2 CS streaks
3	>2 CS streaks
4	Many streaks and scorching/ wilting of leaves (October 2016)
4	Many streaks and scorching of leaves (December 2016)

Figure 5: Examples of the streaking symptoms assigned to each rating scale. From left to right is one streak (1), two streaks (2), more than 2 streaks (3) and many streaks and scorching (4).

6. RESULTS AND DISCUSSION

6.1. Identification of the causal agent through molecular approaches

6.1.1 NGS sample selection and sequencing

Target tissue selection. Quantitative PCR of samples from different tissue types showed that xylem exudate had the highest ratio of pathogen to host DNA (**Table 17**). Interestingly, the CS exudate DNA sample from the Woodford pot trial had twice the amount. Unmodified exudate also showed good relative pathogen abundance, however, it is unsuitable for NGS due to impurities.

Table 17: Relative abundance of host (SoActin) compared to pathogen DNA (CSActin). Ct refers to the average cycle number of two technical replicates, at which a similar amount to PCR product is present (i.e. a lower Ct means more starting DNA template was present). A lower fold difference equates to more pathogen DNA relative to host DNA.

Sample (Variety)	CSActin Ct	SoActin Ct	Fold difference (So/CS)
Stalk DNA (RP193-67)			
2-2 Top node	31.51	19.92	3,083
2-2 Top internode	31.58	20.49	2,178
2-2 Middle node	31.80	19.68	4,448
2-2 Bottom internode[1]	30.75	20.61	1,129
2-2 Bottom node	31.87	19.58	5,023
Unmodified xylem exudate (RP193-67)			
1-3 Top internode	29.90	25.93	16
2-1 Bottom internode	31.36	27.04	20
2-1 Top internode	31.31	25.33	63
2-2 Bottom internode[1]	32.12	27.04	34
3-1 Top internode	29.05	24.45	24
Xylem exudate DNA (Empire)			
CS affected 2 ml exudate	26.05	18.66	168
Healthy 2 ml exudate	undetectable	21.52	NA[2]
Woodford pot trial (RP193-67)			
CS exudate DNA	21.54	22.53	0.5[3]
HWT exudate DNA	undetectable	21.83	NA
CS sett root DNA	32.13	19.16	8,035
HWT sett root DNA	undetectable	19.05	NA
CS sett root cDNA	32.67	19.49	9,216
HWT sett root cDNA	undetectable	19.45	NA

[1] exudate taken from same tissue, [2] Not applicable, [3] Pathogen DNA concentration is 2 times higher than host DNA.

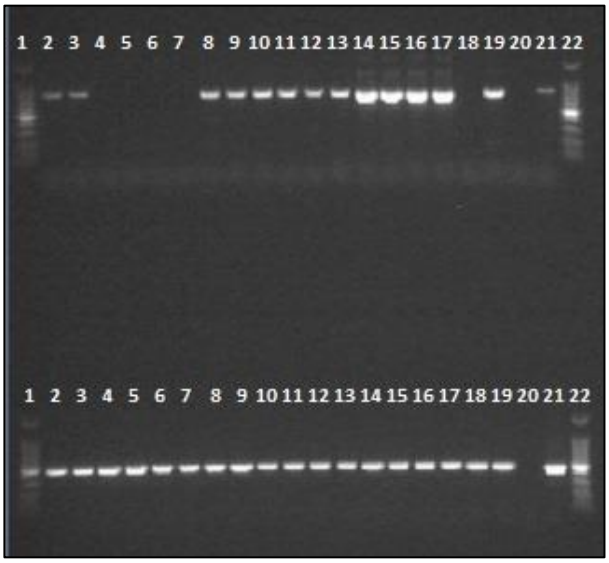
RNA sequencing was one of the initial methods considered to reduce the complexity of the sequence information. However, relative pathogen abundance was extremely low in root cDNA as well as in xylem exudate cDNA (**Table 17** and **Table 18**). Low levels of pathogen transcript in RNA led to us to take a DNAseq approach. Relative pathogen abundance was also low in stalk DNA. These results suggest targeting xylem exudate is probably the best approach for NGS.

NGS samples. Xylem samples from Harwood were first PCR screened then subjected to qPCR to estimate relative abundance. Initially, unmodified sap was used as template, however, only CS 2y 1 was positive (data not shown). The negative results may be due to the large volume of xylem exudate having a dilution effect.

This is supported by negative results for PFK5 in four of nine samples (data not shown). The PCR was repeated on DNA extractions from the xylem exudates.

There was sufficient DNA present in all samples as indicated by the positive PFK5 results (**Figure 6**). The H 2y 1 sample that showed leaf symptoms was positive, as was the H 2y 4 sample which did not

show visible symptoms (**Figure 6**). The remaining two healthy samples were negative. All chlorotic streak samples were positive, with suckers showing large amounts of PCR products (**Figure 6**).



Top row: actin F1 R1
Bottom row: PFK5 F1 R1

Lane	
1	100 bp ladder
2 - 3	H 2y 1
4 - 5	H 2y 2
6 - 7	H 2y 3
8 - 9	H 2y 4
10 - 11	CS 1y 1
12 -13	CS 1y 2
14 - 15	CS S 1
16 - 17	CS S 2
18 - 19	CS 2y 1
20	negative control
21	positive control
22	100 bp ladder

Figure 6: PCR screening of xylem exudate DNA from Empire. Samples were run as duplicate reactions.

Quantitative PCR was used to estimate relative abundance of pathogen and host DNA in samples collected for NGS (**Table 18**). Given the favourable relative abundance of the putative pathogen in xylem exudates, RNA extraction from xylem exudates followed by cDNA synthesis was trialled. Different actin primers were required for the cDNA (compared to DNA templates). Three of the four healthy samples show the presence of some pathogen DNA (**Table 18**) and demonstrates the sensitivity of qPCR compared to PCR. The Healthy stool 1 and 4 samples (H 2y 1 and H 2y 4) had comparable levels to CS affected 1 year old cane and the healthy stool 3 exudate sample had a minute amount present. Relative pathogen abundance was found to be very good for all CS exudate samples and especially good for exudate from suckers and two year old affected cane (**Table 18**). Although the H 2y 3 sample showed trace amounts of pathogen in the qPCR result, it was included due to lack of healthy exudates and low DNA yields from HWT plant cane. The low abundance of pathogen DNA in the H 2y 3 sample will be accounted for and will not affect our bioinformatics approach. Xylem samples from infected plants were chosen for the NGS sample based on relative abundances.

Table 18: Relative abundance of host (SoActin) compared to pathogen DNA (CS Actin). Ct refers to the cycle number (average of two technical replicates) at which a similar amount of PCR product is present for So Actin and CS Actin. The cDNA sample is an average of four replicates with two technical replicates each.

Sample (Variety)	CSActin Ct	SoActin Ct	Fold difference
Xylem exudate DNA (Empire)			
Healthy, 2 year old, stool 1	26.93	20.48	87
Healthy, 2 year old, stool 2	undetectable	19.61	NA [1]
Healthy, 2 year old, stool 3	32.94	20.44	5,791
Healthy, 2 year old, stool 4	25.28	20.15	35
CS, 1 year old, tube 1	25.55	20.79	27
CS, 1 year old, tube 2	26.57	20.56	65
CS, suckers, tube 1	23.37	20.47	7
CS, suckers, tube 2	22.93	20.99	4
CS, 2 year old, tube 1	23.27	19.91	10
Xylem exudate cDNA			
Healthy, 2 year old, stool 4	30.68	20.49	1,165

[1] Not applicable

Sequencing data. NGS of the chlorotic streak infected (CS) and healthy (H) xylem exudate DNA samples was successfully completed on the Illumina HiSeq 2000 platform. This was performed by The Ramaciotti Centre for Genomics (http://www.ramaciotti.unsw.edu.au/). Data was forwarded onto QFAB (http://www.qfab.org/) and was found to be of a good quality following examination with the FastQC program. It consisted of approximately 190,000,000 reads from each sample. Read pairs generated from the sequencing contain 100 bp of known sequence at both ends and are separated by 150 bp of unknown sequence (**Figure 7a**). The 350 bp fragment is a median within a 200 to 600 bp range. Mapping refers to the alignment of reads or read pairs to a reference sequence (**Figure 7b**). The paired end arrangement of reads can also be useful for finding unknown sequences through extending or extension mapping (**Figure 7b**).

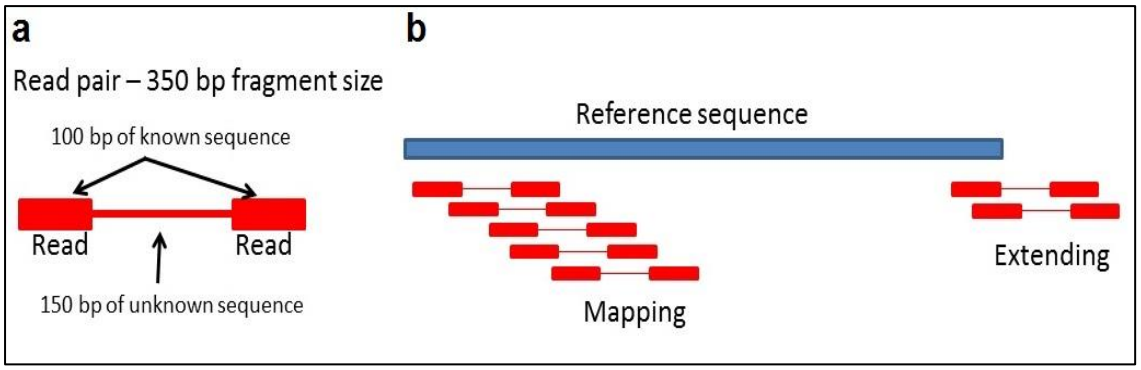


Figure 7: Graphical interpretation of terminology used in the NGS study. (a) Representation of a read pair. Thick bars represent forward and reverse sequence reads. Reads are separated by an area of known distance, but unknown sequence (thin bars). (b) Approaches for utilising the read pairs.

6.1.2 Bioinformatics and gene cloning

Characterisation of exogenous sequences. Note: This section has been taken from the QFAB report (Appendix 9.3) with minor revisions.

The initial strategy of QFAB involved the mapping of read pairs onto a collection of pathogen and microbiome genome sequences.

The complete and draft genome sequences from the human microbiome, bacterial genomes and viral genomes were collated along with potentially contaminating sequences from organellar genome databases, synthetic sequence databases and plasmid databases.

The bowtie2 software was used to perform mapping of reads against the genome sequence collection and the picard software was used to convert the resulting output into the binary and indexed bam file format. Distributions of expected and unexpected sequence mapping were investigated using proprietary QFAB methods that summarise the taxonomic distributions within the mapped data. The data did not show a remarkable bias in either of the datasets to exclusive or unique species but did identify a reasonable number of probable host sequence reads (data not shown). This mapping approach does not yield satisfactory results due to the insufficient breadth of the target sequence collection (the database is loaded with only complete and draft genomes and does not encompass the anticipated breadth of phytopathogens). This survey supports the assertion that the chlorotic streak organism is not likely to be a known bacteria or virus since no obvious difference in species were observed between the CS and H libraries. The bowtie software (and other mapping approaches) are expecting to map reads against complete genome or chromosome sequences and are not ideal tools for broader phylogenomic characterisation. To make use of the broadest known genomic resources would require a re-evaluation of the strategy.

Phylogenomics. Note: The following paragraph has been taken from the QFAB report (Appendix 9.3) with minor revisions.

To circumvent the limitations of the bowtie2 based approach a more comprehensive sequence scanning approach was adopted where the following logic was applied:

1) The NCBI database was searched to identify any DNA sequences (gene, genome or expressed sequence tags) that were annotated as being taxonomically a member of "bacteria", "fungi", "viruses", "algae" or a SRA defined group. The SRA group consisted on Stramenopiles (group containing oomycetes), Opisthokonta (group containing fungi) and Rhizaria (group containing *Plasmodiophora*). This was performed using proprietary software implemented for the purpose of filtering NCBI sequence collections for sequences of a given taxonomic assignment.
2) The sequence collections were structured using the Kraken software and Jellyfish was used to make a k-mer database of the DNA words that are observed in any of the sequence collections.
3) The Kraken software was used to identify the taxonomic nodes that could explain the kmers observed in each of the short read sequences prepared from the CS and H sequence libraries (figure 2.)
4) The Kraken mapping data was summarised to describe the numbers of DNA sequences that appear restricted to either the CS or H data. The data was further explored to identify the taxonomic levels where at least a 1.7-fold difference in the number of assigned sequences was observed.

The NGS sequence data from the CS and H libraries were compared using k-mer word counts to the available public sequence resources. This showed an enormous diversity in the number of taxonomic units present but failed to reveal a substantial number of sequences that could be unambiguously assigned to a potential pathogen in only the infected tissue.

A high stringency mapping of CS and H reads to our known actin gene resulted in 50 CS and 2 H (a read pair) reads mapping, meaning there is a small amount of pathogen DNA present in the H sample. This was expected as some xylem exudate with trace amounts of pathogen was included in the H sample.

From this result we should only look at relative amounts of hits, rather than presence or absence of hits.

The QFAB analysis did give a good ratio of CS to H hits for some chytrids, especially the *Cladochytrium* genus which showed 47.5 times more hits in CS compared to H. This approach was repeated by SRA concentrating on the Chytridiomycota phylum.

A stand out was the *Chytridium* genus with 32.7 more hits in CS compared to H. The Cercozoa phylum, which was identified in the “Mapping to reference genes” section in this report, was also examined using this approach. *Cercomonas* sp. ATCC 50367 (only one DNA sequence on GenBank), had 74.3 times more hits for CS compared to H. This approach is limited to available sequence data; as an example for less studied taxa there are only between one and ten DNA sequences available. This approach therefore, could not give a definitive answer when the pathogen is likely to be unknown to science. It may however, give good leads to explore through hits to similar organisms.

De novo assembly. Another approach to try and isolate DNA sequences from the chlorotic streak pathogen is by doing a de novo assembly. This technique does not utilise a reference sequence and generates contigs by aligning within the read pairs available. The CS NGS reads were filtered to help with the assembly. Filtering involves low stringency alignment of the reads from the CS data to completed genome sequences such as *Sorghum bicolor*, *Zea mays* and all chloroplasts and mitochondria. The remaining reads were assembled over a 2 week period at SRA. The largest 200

contigs generated (3.3 million in total) were blasted, however, all were found to be of sugarcane origin.

A second attempt at a de novo assembly was attempted, using more stringent mismatch allowances. Data was filtered using all available sugarcane sequences (*Saccharum* sp. hybrid cultivar, *Saccharum officinarum* BACS), *Saccharum spontaneum*, all bacteria, chloroplasts and mitochondria (from *Sorghum bicolor*, *Orzya sativa*, *Zea mays*), in addition to sorghum and maize genome data. The second attempt produced similar results to the first, therefore the de novo assembly approach was stopped. Lack of progress was largely due to the size of the sugarcane genome and the lack of publically available sequence data.

Mapping to reference genes. Ribosomal DNA (rDNA) is DNA sequence that codes for ribosomal RNA (rRNA). Eukaryote rDNA consists of tandem repeats, each containing an intergenic spacer (IGS; also known as a nontranscribed spacer NTS), external transcribed spacer (ETS), 18S small subunit (18S SSU), internal transcribed spacer 1 (ITS1), 5.8S, ITS2, and 28S large subunit (28S LSU). Most research on microorganisms centre on this area. Based on the QFAB analysis, *Cladochytrium* and *Saccharum* ribosomal sequences on GenBank were used as reference sequences. Reads from CS were mapped with 20 % allowable mismatches to these sequences to see if visual differences were present in the mapped alignments (the actin mapping work showed that it is sometimes possible to differentiate between host and pathogen reads simply by looking at the alignments). Following mapping, we visually found several 100 bp reads that were not host DNA and were possibly of pathogen origin (**Figure 8**). BlastN searches of the putative pathogen reads returned matches to the Cercozoa group.

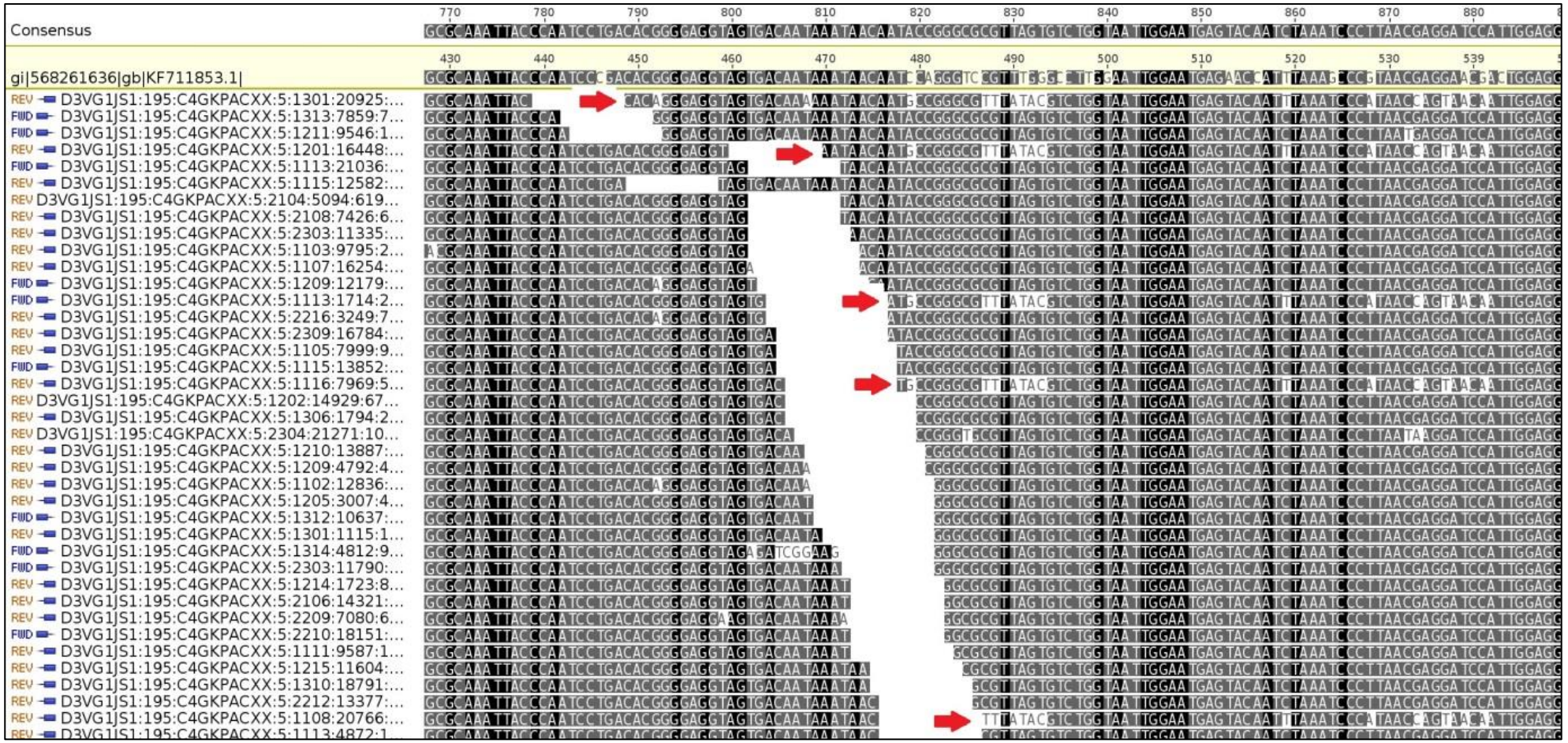


Figure 8: Unique groups of reads visually identified in mapping experiments. Arrows indicate similar 100 bp reads within alignments, possibly from the chlorotic streak pathogen. BlastN results show all other reads in the alignment are of sugarcane origin

Three non-continuous consensus fragments from the 18S ribosomal gene of the putative chlorotic streak pathogen were initially identified and aligned back to a sugarcane 18S gene. This alignment was used to find the position of the fragments and for selecting PCR primers that would limit amplification of host DNA. The alignments showed coverage of an area of approximately 600 bp. Two forward and two reverse primers were designed (in a similar manner to the actin primers) to span the unknown areas and PCR tested. PCR products were sequenced and a 565 bp sequence was determined. Similar sequences were found on GenBank using BlastN and tBlastN (**Table 19**)

Table 19: Selection of high ranking BlastN and tBlastN results from CS 18S (565 bp)

BlastN of 18S			
Taxon	**Coverage (%)**	**Homology (%)**	**Accession**
Paracercomonas sp. Panama107	98	89	FJ790735
Cercomonas sp. strain Panama61	98	89	AY884336
Eimeriidae environmental sample clone 1198	98	89	EF023757
Eimeriidae environmental sample clone 761	98	89	EF023421
Eimeriidae environmental sample clone 743	98	89	EF023404
tBlastN of 18S			
Taxon	**Coverage (%)**	**E Value**	**Accession**
Uncultured eukaryote	98	7e-85	AB572152
Uncultured cercozoan	98	6e-66	AM114800
Soil flagellate AND18	98	2e-66	AY965864
Paracercomonas sp. HFCC 918	98	6e-66	HM536163
Cercomonas metabolicus strain HFCC88	90	3e-68	DQ211597

A 1,140 bp consensus sequence was pieced together for the 28S gene. Four forward and four reverse primers were PCR tested and all primer combinations showed specificity for CS. Sanger sequencing produced a 1,005 bp fragment, which was blasted (**Table 20**).

Table 20: Selection of high ranking BlastN and tBlastN results from CS 28S (1,005 bp)

blastN of 28S			
Taxon	**Coverage (%)**	**Homology (%)**	**Accession**
Paracercomonas marina	97	89	DQ386164
Gymnophrys sp. ATCC 50923	97	88	FJ973379
Cercozoa sp. CC-2009d	97	87	GQ144690
Placocista sp. CC-Grouse Mountain	95	88	GQ144688
Protaspis grandis	97	87	GQ144689
tBlastN of 28S			
Taxon	**Coverage (%)**	**E Value**	**Accession**
Paracercomonas marina	95	8e-153	DQ386164
Cercomonas sp. C59	98	3e-151	DQ386165
Gymnophrys sp. ATCC 50923	96	7e-149	FJ973379
Cercozoa sp. CC-2009d	95	3e-147	GQ144690
Cryothecomonas sp. APCC MC5-1	97	3e-145	GQ144683

This process was repeated until a single unambiguous sequence was achieved. A 10 kb ribosomal DNA sequence was identified and verification performed by amplification of the entire length. The complete 18S SSU and 28S LSU sequences were annotated by aligning with other complete Cercozoan sequences.

Highly similar sequences, excluding uncultured and environmental sample sequences, on GenBank were found using BlastN. Similar to previous findings, the top five results for both 18S SSU and 28S LSU belong to Cercozoa (**Table 21**).

Table 21: Selection of high ranking BlastN results from CS 18S (1,823 bp) and 28S (3,569 bp)

BlastN of 18S SSU			
Taxon	**Coverage (%)**	**Homology (%)**	**Accession**
Eocercomonas sp. HFCC 908	99	88	HM536153
Cercomonas plasmodialis	99	88	AF411268
Cercomonas sp. HFCC 903	99	87	HM536148
Dimorpha-like sp. ATCC 50522	99	87	AF411283
Cercomonas sp. HFCC 904	99	87	HM536149
BlastN of 28S LSU			
Taxon	**Coverage (%)**	**Homology (%)**	**Accession**
Gymnophrys sp. ATCC 50923	83	86	FJ973379
Paracercomonas marina	76	87	DQ386164
Cercomonadida sp. C15_1A03	81	85	FJ032646
Protaspis grandis	79	86	GQ144689
Cercozoa sp. CC-2009d	78	85	GQ144690

We have uploaded the CS 18S sequence onto Genbank (accession number KR704194 - Uncultured Cercozoan clone CS SSU47 18S ribosomal RNA gene, partial sequence). The 10 kb sequence has also been deposited in GenBank (accession number MF034900)

Extending the actin sequence. The actin sequence was extended from 1,311 bp to 1,821 bp and was deposited into GenBank (accession number MF034898). BlastN of the full putative sequence shows less than 59 % of the actin sequence matches anything and at most there is only 88 % similarity between the sequences that match.

Organisms with most similar actin sequences at the DNA level are *Helicoverpa armigera* (insect), *Olpidium bornovanus* (a chytrid), *Physcomitrella patens* subsp. patens (moss) and rabbit. A Blast search using a translated nucleotide query on a translated nucleotide database (tBlastN) showed highest query coverage was 61 - 62 % with an E value of 0 to *Hordeum vulgare* subsp. vulgare (oats, accession AK369289), *Bigelowiella natans* (Rhizaria related to Cercozoa, accession AY251793) and *Dictyostelium purpureum* (Amoebozoa, accession XM_003292845).

Extending sequences using this technique is mainly limited by depth of sequencing coverage. Further extension of the actin gene, if required, may be possible as sometimes only one end of a read pair is mapped. The read at the other end can be manually found and used as the reference for further extension. The highly conserved nature of the actin gene throughout eukaryotes leads to matches with little taxonomic significance, however, it is interesting that the plant pathogenic chytrid, *Olpidium bornovanus* and a member of the kingdom Rhizaria, Bigelowiella natans were identified by this analysis.

Molecular signatures of Cercozoans. As a result of the similarity of the putative CS organism to Cercozoans, genetic characteristics that define the group were investigated. Cercozoa were found to contain a deletion in the 18S small subunit at the terminal loop of the V6 region (Bass *et al*., 2005), along with an insertion of one or two amino acids at the monomer-monomer junctions of the eukaryote polyubiquitin protein (Archibald *et al*., 2003; Bass *et al*., 2005).

Alignment of 18S SSUs from the putative CS organism, Animals, Plants, Fungi, Oomycetes and Cercozoa showed the 18S SSU from the putative CS organism has a deletion at the correct position (**Figure 9**).

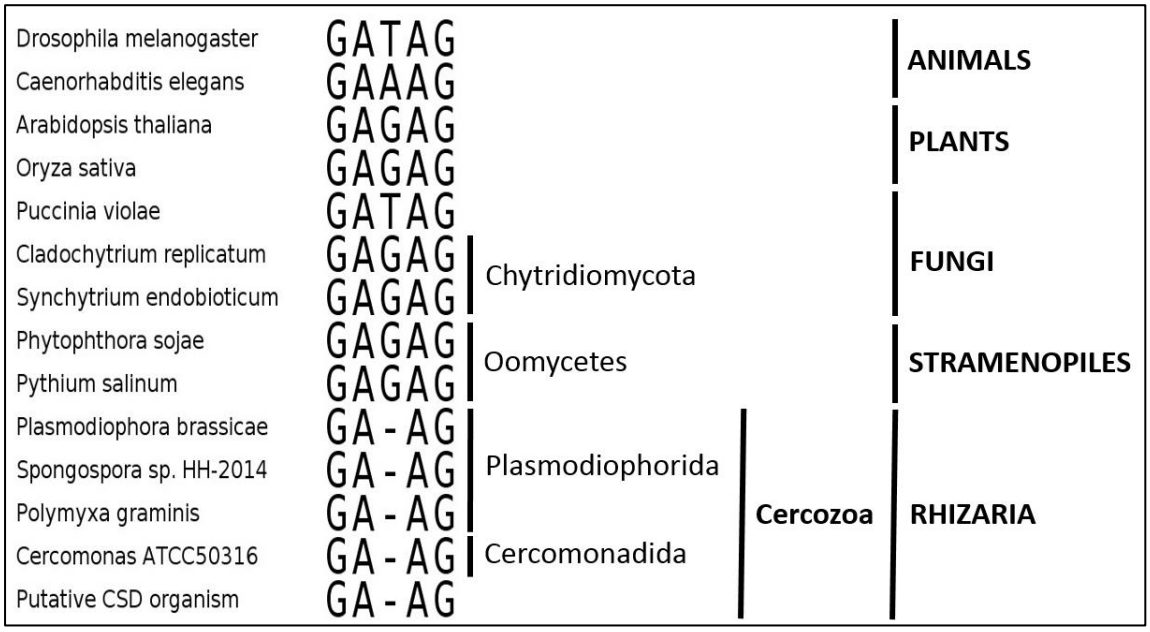


Figure 9: Alignment of the terminal loop of the V6 region of 18S SSU. All Cercozoa share the single nucleotide deletion

Most ubiquitin genes are found as repeats of similar monomers. Cercozoan polyubiquitin has either a one or two amino acid insertion between monomers, with the plant-pathogenic plasmodiophorids having one insertion.

Reads from the "diseased" NGS dataset were examined for the presence of any characteristic insertions in polyubiquitin.

Polyubiquitin sequences from seven *Cercomonas* sp. ATCC 50316 isolates were used as reference sequences for mapping with the CS NGS dataset. These were chosen because of the substantial sequence differences between the isolates. Due to the fact that the polyubiquitin sequence of the putative CS organism is unknown, this will theoretically assist with the mapping of the dataset to at least some reference genes. Reads that contained the characteristic amino acid insertions were found to map to two *Cercomonas* sp. ATCC 50316 sequences. Different to the plasmodiophorids, the putative CS organism contains a two amino acid insertion (**Figure 10A**), grouping them with the Cercozoa clades of Monadofilsoa and Proteomyxidea. Sugarcane polyubiquitin does not contain this insertion (**Figure 10B**). This analysis was repeating using the "healthy" NGS dataset and no insertions were found (data not shown). PCR was used to identify a CS polyubiquitin sequence and it has been deposited into GenBank (accession number MF034899).

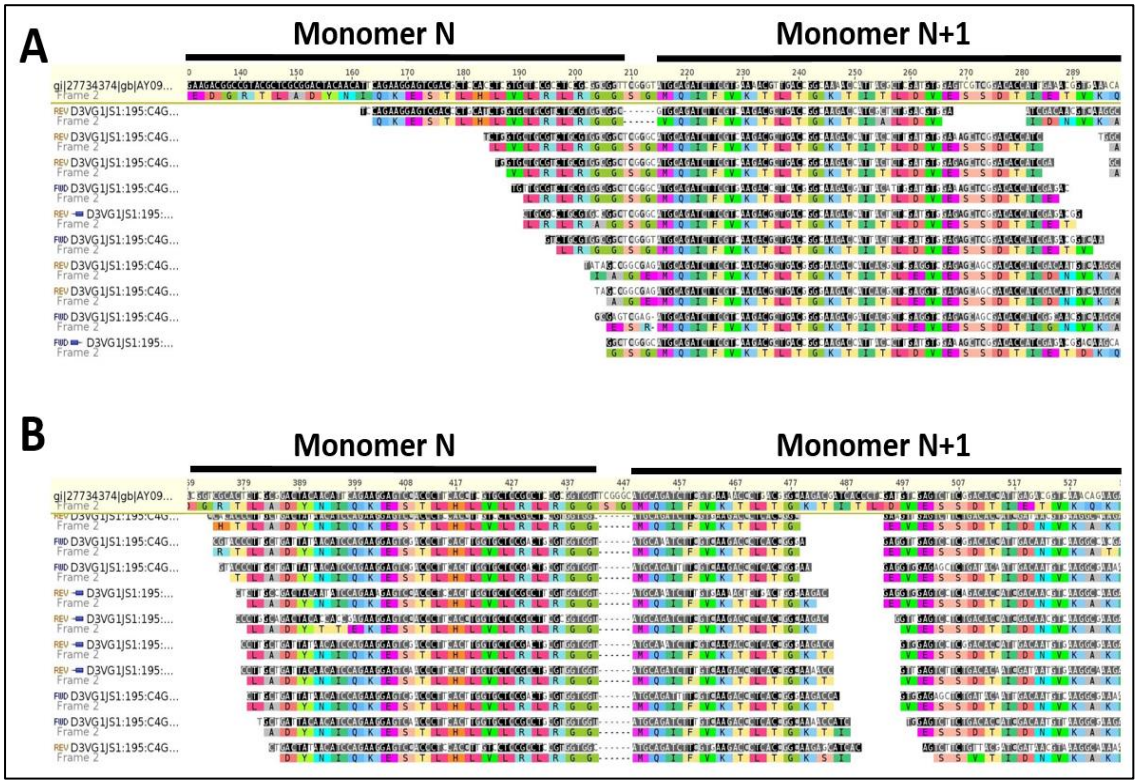


Figure 10: Polyubiquitin insertions found from mapping to a *Cercomonas sp*. ATCC 50316 isolate (uppermost sequence). (A) Characteristic "SG" insertions found in reads from the "diseased dataset". (B) Different area from the same mapping, showing host sequence lacking an insertion

6.1.3 Phylogenetics

Note: This section has been taken from correspondence with David Bass with minor revisions:

The identified Cercozoan associated with CS is a deep-branching Cercomonad, grouping near *Metabolomonas* and *Brevimastigomonas* (**Figure 11**). Cercomonads are usually free-living bacterivores but are also known to be influenced by plant associations (they include active and perhaps specialised rhizophere and phylosphere lineages). However, they are also opportunists that are able to group together to kill nematodes and probably have a very wide set of feeding options. However, your organism is a very distinct sequence type, as you can tell from the blast results.

Other relatives of free-living bacterivorous cercozoans in a related order - glissomonadida - have also specialised on feeding on algal cells and can destroy algal cultures in a few days. These are not closely related to your organism, but I mention it to illustrate that a plant/ parasitising-feeding strategy can evolve within otherwise largely bacterivorous groups. As the name implies *Brevimastigomonas anaerobica* grows in microaerophilic and anaerobic conditions, and there are apparently other anaerobic lineages in that part of the tree. This is perhaps relevant to the ability of your organism to develop a specialised lifestyle. Depending on its morphology and more thorough phylogenetic analyses possible names could be *Brevimastigamonas*/ *Metabolomonas phytophaga*, or perhaps a new genus is necessary. Some transcriptome data exist for *B. anaerobica* and I know people are working on genomics of at least one more strain, branching below *B. anaerobica* in this tree. However, I think these are all free-living taxa so comparable analyses of a related pathogen would be extremely interesting.

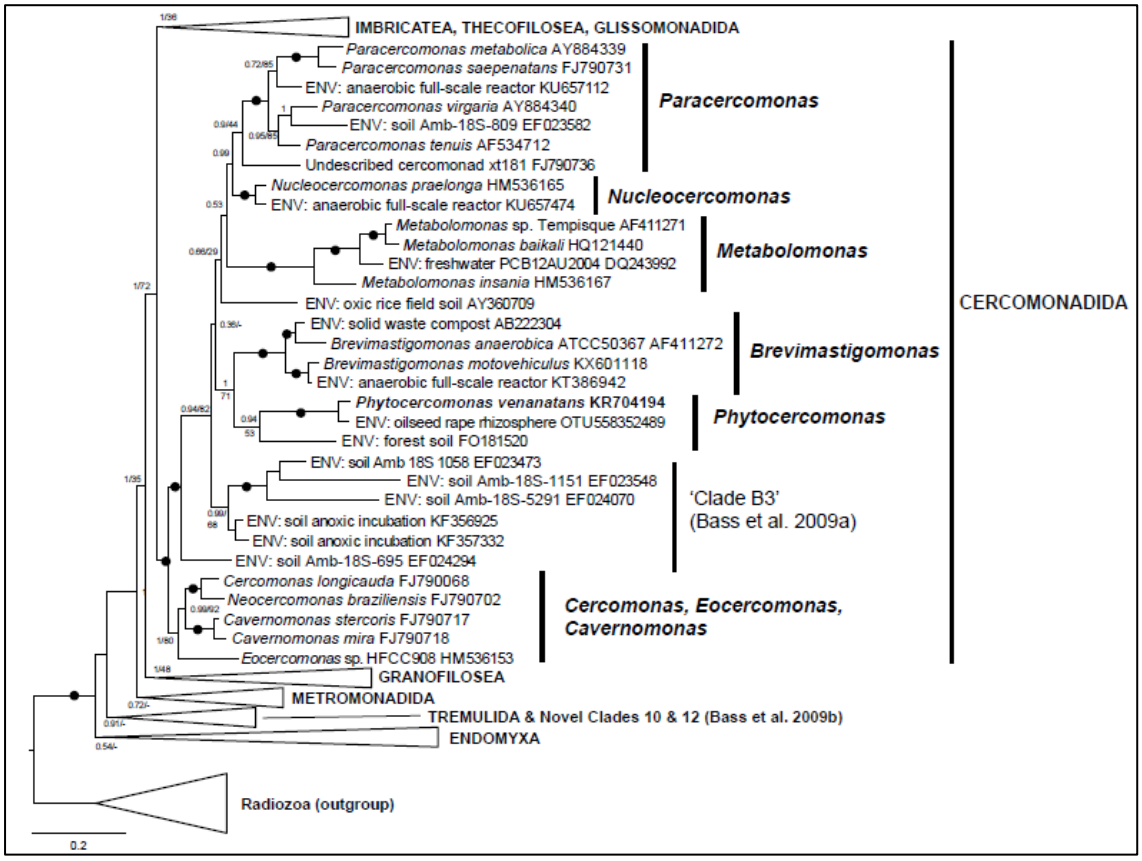


Figure 11: Bayesian 18S rDNA phylogeny including *Phytocercomonas venanatans* and 110 other Cercozoan sequences, rooted on Radiozoa. 1782 positions were included in the analysis. Non-Cercomonad clades are collapsed for clarity. Bayesian Posterior Probabilities/ Maximum Likelihood bootstrap values are indicated on nodes; black filled circles indicate values of ≥ 0.95 and 95 %, respectively

6.1.4 Community profiling

Experiment 1: 16S, 18S and ITS amplicon sequencing. Results for the three primer sets are presented as read distribution (relative abundance within the sample) following filtering of *Saccharum* and negative control sequences.

The 16S dataset examines if there are any bacterial species associated with diseased plants. Read counts ranged from 30,000 to 110,000. As expected, host chloroplast and mitochondrion sequences accounted for most of the reads.

Subtracting host and negative control reads gives a better indication of bacterial species present within the samples (**Figure 12**). Samples from the glasshouse have more diversity, possibly due to favourable environmental conditions for bacteria. The tissue culture sample contained no bacteria. All bacteria species are present in the RP H sample except for *Pseudomonas* sp. SRP3086, which is also absent in diseased Q241$^{\Phi}$ and Empire. There is no indication that any bacteria is associated with CS.

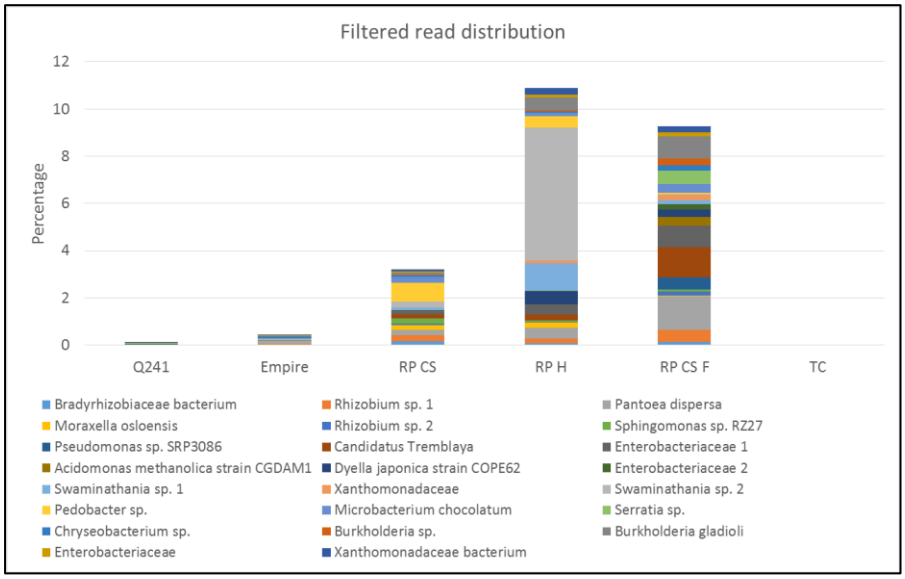


Figure 12: Read distribution of the 16S dataset following filtering of *Saccharum* and negative control sequences

The 18S dataset examines if there are any eukaryote species associated with diseased plants. Read counts ranged from 60,000 to 180,000. Like the 16S dataset, host ribosomal sequence accounted for most of the reads. Following the subtraction of host and negative control reads, very few OTUs remain (**Figure 13**). All samples from diseased plants have the CS associated Cercozoan present. This provides more support for the hypothesis that CS is caused by a Cercozoan pathogen.

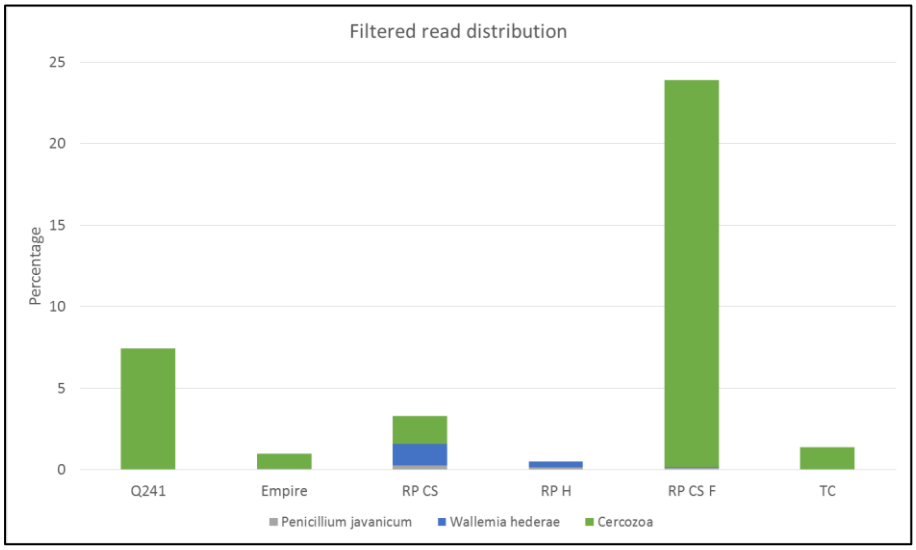


Figure 13: Read distribution of the 18S dataset following filtering of *Saccharum* and negative control sequences

The ITS primers are meant to be specific for the kingdom Fungi, however, this was not found to be the case. Future community profiling experiments will require optimisation to limit amplification of host DNA. There was a single read belonging to a Cercozoa within the 75,000 reads from the RP H sample.

Apart from this, Cercozoa are again present in all samples from diseased plants. Interestingly, two Cercozoan sequences were identified in this dataset. The second Cercozoan sequence differs in 9 bases at the 5' end of the 250 bp amplicon corresponding to the 5.8s ribosomal area. It shares homology with a *Saccharum* sequence at the same area.

This may be an artefact of PCR or NGS, as this sequence had no identical reads when compared to our earlier NGS DNAseq data using mapping. Like the 16S and 18S datasets, host sequence again accounted for most of the reads. All fungal species are present in the RP H sample and low abundance species in this sample are also low or absent in samples from diseased plants (**Figure 14**). This suggests that fungi are not the likely cause of CS.

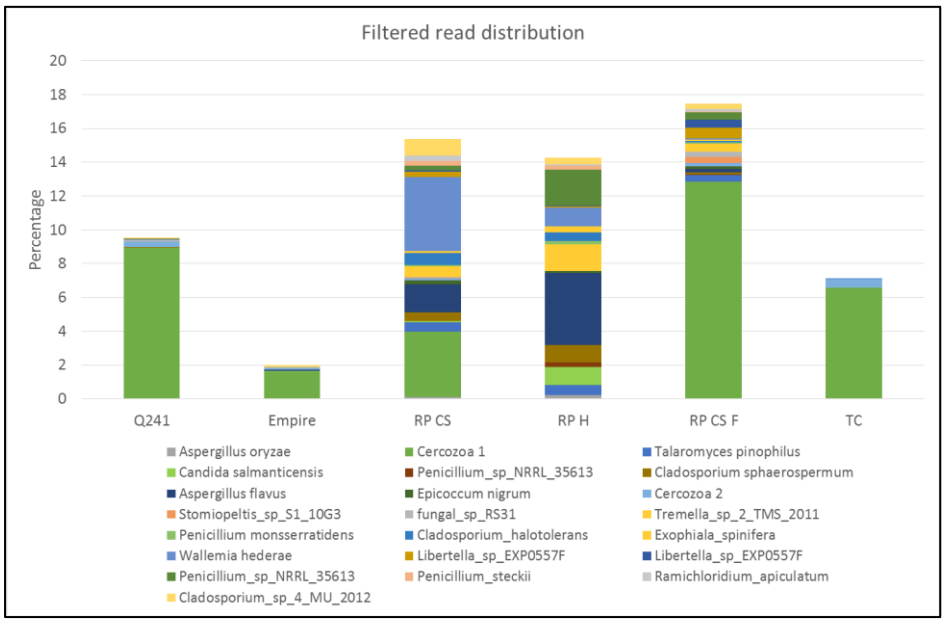


Figure 14: Read distribution of the ITS dataset following filtering of *Saccharum* and negative control sequences

Experiment 2: 16S, 18S and ITS amplicon sequencing with PCR blockers. The PCR blocking experiment didn't worked as expected. There was a reduction in overall reads and some samples did not amplify at all. The reduction of intended sequences was also not successful. We also found the OTU profiles to be different from the first experiment. Due to these issues, results from this experiment cannot be used with confidence. Optimising PCR reaction conditions can improve results when using PCR blocking for future experiments.

Experiment 3: 18S amplicon sequencing. Community profiling was repeated using only the 18S primer set on a wider range of samples. This was done to further confirm the association found in section 6.1.4 Experiment 1.

Xylem exudates from field plants. Xylem exudates from field grown Q155, Q241$^{\Phi}$ and Empire were examined for their eukaryotic profiles. Following the filtering of *Saccharum* spp. sequences, all three cultivars showed that only Cercozoa were present in infected plants (**Figure 15**, **Figure 16**). Healthy plants had no Cercozoa present.

The Q241$^{\Phi}$ healthy sample did have contamination present. Xylem exudates from healthy Q241$^{\Phi}$ plants were collected in Tully at a different time to the infected sample and were sent down to Brisbane. Contamination probably occurred during the collection step.

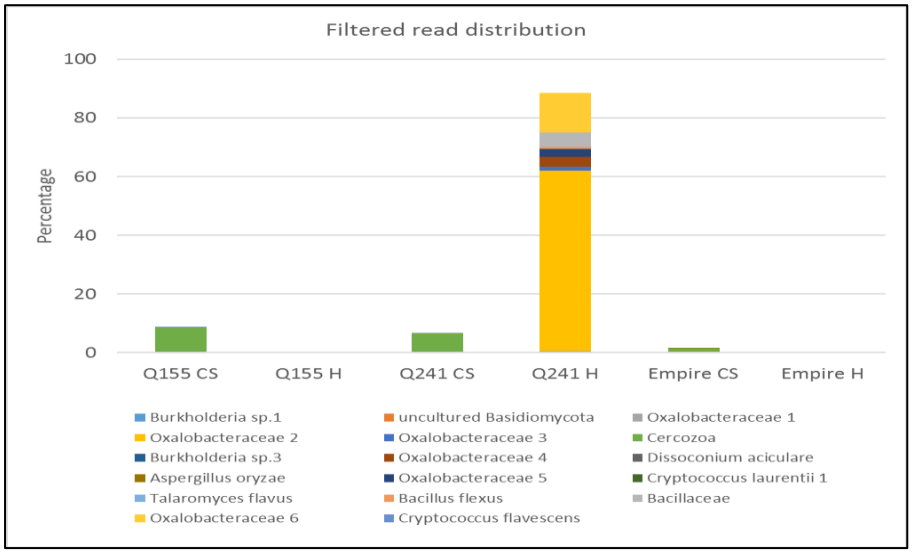


Figure 15: Read distribution of the xylem exudate 18S dataset from field plants following filtering of *Saccharum* and negative control sequences

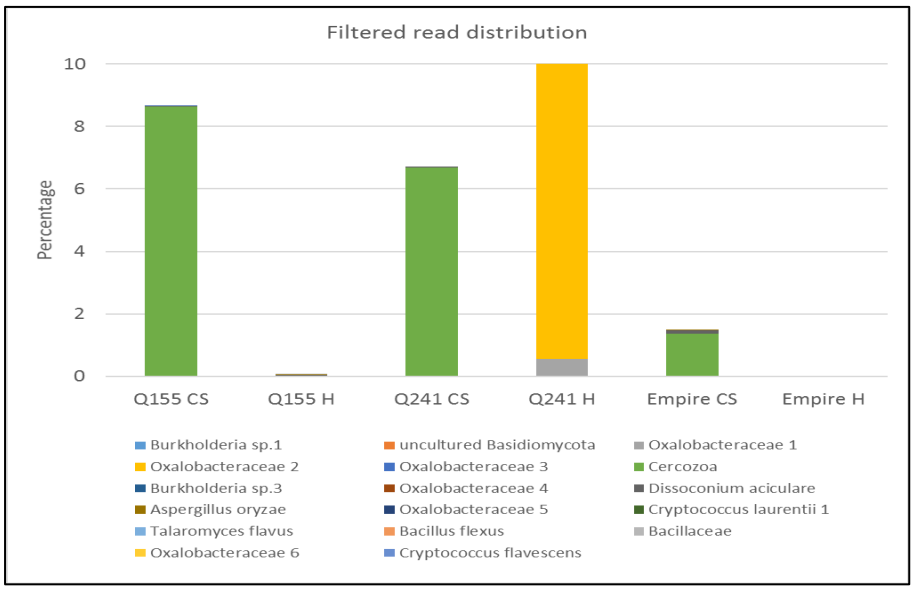


Figure 16: Read distribution of the xylem exudate 18S dataset from field plants following filtering of *Saccharum* and negative control sequences. Axis has been scaled to better show abundance of Cercozoa

Cultures. Cell cultures were examined to determine whether they were pure and if there was any diversity within the cells (**Figure 17**). Culture subcultures were also examined to determine if *Saccharum* spp. sequences could be reduced in serial subcultures. Interestingly very little *Saccharum* spp. sequences were present in the data. This is in contrast to results from the Koch's postulates samples (see below). There was also very few contaminating sequences from the maize in the cornmeal media and potato in the potato extract media. This is useful information for any future genome sequencing work.

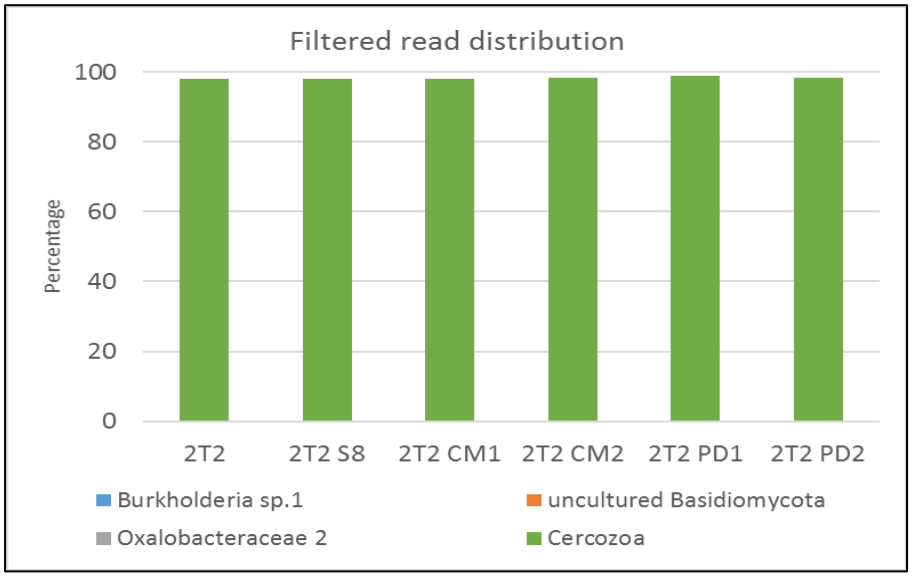


Figure 17: Read distribution of the 18S dataset from cultures following filtering of *Saccharum* and negative control sequences

Root water. Examination of water from root soaks was performed to further confirm the association of Cercozoa with infection. Detection of a Cecozoa in the water was unsuccessful for all samples (data not shown). This may be due to the low number of cells that actually leave the roots.

Sorghum transfection. Although symptoms were present in the Tully and Woodford material, there were very few Cercozoan reads found in the data (**Figure 18**). This may be due to extremely low levels present in sorghum, or that the organism was no longer present in the plants at the time of sampling.

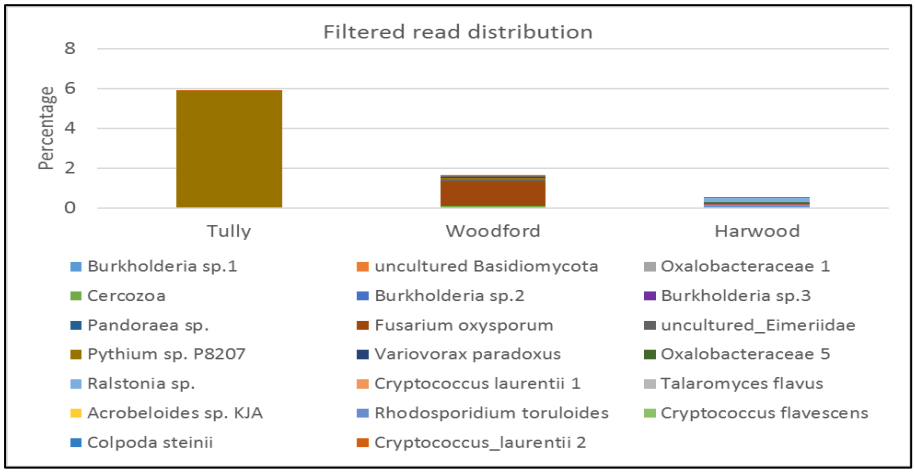


Figure 18: Read distribution of the 18S dataset from Sorghum following filtering of *Saccharum* and negative control sequences

Koch's postulates. Only inoculation using active cells (from Empire) resulted in infection of Q90 (**Figure 19**). Interestingly there was an abnormal abundance of Cercozoan reads for a xylem exudate sample. Community profiling of cultures from the same plants show a lower than expected abundance of the Cercozoan (**Figure 20**). This was probably an error with mixing up similarly labelled samples.

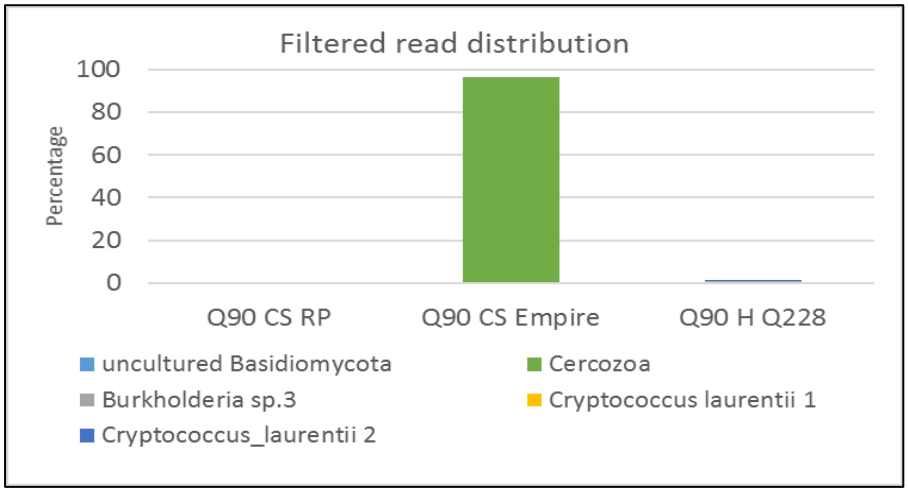


Figure 19: Read distribution of the xylem exudate 18S dataset from Koch's postulate Q90 plants. *Saccharum* and negative control sequences have been filtered

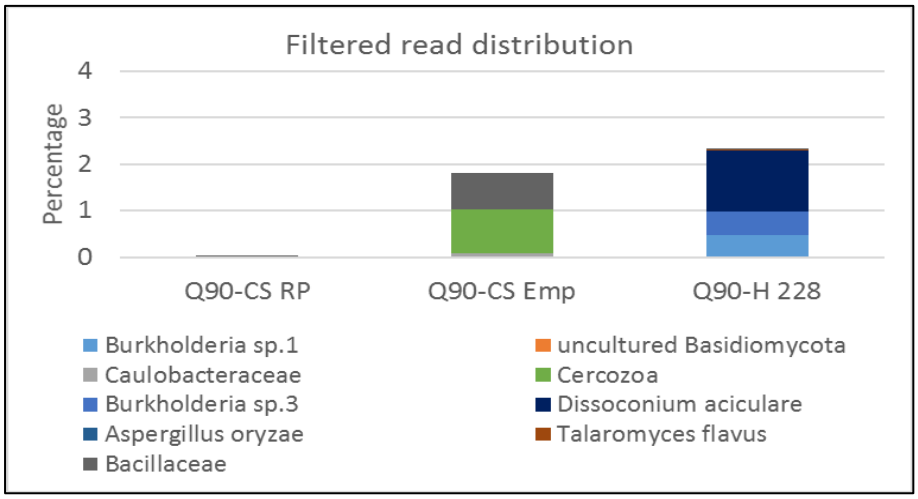


Figure 20: Read distribution of the culture 18S dataset from Koch's postulate Q90 plants. *Saccharum* and negative control sequences have been filtered

6.1.5 Discussion

This study demonstrates the use of new molecular techniques to uncover the elusive and probable causal organism of chlorotic streak of sugarcane. This is the first known pathogenic association between a Cercomonad and a higher plant. The use of NGS to uncover concealed pathogens in both plants (Candresse *et al.,* 2014) and animals (Hartikainen *et al.,* 2014b) is now becoming common-place. This approach was tried because considerable work had been done over the years to identify the causal agent, without success.

The existing chlorotic streak diagnostic actin sequence (Braithwaite and Croft, 2013) was especially useful in assisting with selection of pathogen-enriched and pathogen-limited samples for NGS, allowing for a high chance of successful identification of novel DNA sequences. Quantitative differences between CS and H datasets using the Kraken program suggested several possible candidates, however, this approach was limited due to the novelty of the organism and therefore the lack of any publicly available DNA sequences. Mapping of the diseased dataset to *Saccharum* spp. ribosomal sequences revealed a small number (< 1 %) of novel ribosomal reads that were of Cercozoan origin. Primers used to create larger ribosomal contigs showed specificity similar to the diagnostic actin sequence.

Initially the short length of the novel sequences and the lack of closely related sequences on GenBank limited the identification of *P. venanatans* as a Cercozoan. Complete ribosomal small and large subunits were assembled revealing similarity to sequences from Cercomonadida. These sequences were identical to ribosomal sequences amplified from *P. venanatans* isolated in culture.

Further evidence of this affiliation, was the presence of a deletion in the 18S small subunit and two amino acid insertions between the polyubiquitin monomers. Rhizaria are unique in having either one or two amino acid insertions between the polyubiquitin monomers, with only Cercozoa containing the deletion in the 18S ribosomal sequence (Bass *et al.,* 2005; Burki *et al.,* 2010). *P. venanatans* has a serine-glycine insertion that further differentiates them from the pathogenic Endomyxa group that contain only one polyubiquitin insertion. An intron is also present in polyubiquitin and similar introns are present in several other Cercomonads (Archibald *et al.,* 2003).

Amplicon sequencing (or community profiling) is used to study often unculturable, microbial communities (Lindahl *et al.,* 2013; Hartikainen *et al.,* 2014a; Bass *et al.,* 2015). This technique was used to determine whether other organisms may also be associated with chlorotic streak. Results indicate a definitive association between the disease and *P. venanatans* with no associations found for bacteria or fungi, suggesting *P. venanatans* is the likely cause.

The eukaryotic supergroup Rhizaria comprises Cercozoa, Endomyxa, and Retaria (Foraminifera and Radiozoa) and formal recognition of this supergroup was based on molecular phylogenetics, mainly of the 18S SSU DNA (Burki and Keeling, 2014). Rhizaria are one of the most poorly understood supergroups, mainly due to the fact that many are hard to culture and there are no known rhizarian parasites of humans. The best known pathogenic rhizarians belong to Endomyxa, of which Plasmodiophorida (one of two orders within class Phytomyxea) infect plants and oomycetes (Neuhauser *et al.,* 2014), and members of class Ascetosporea (haplosporidians, paramyxids, paradinids, and mikrocytids) that infect commercially important invertebrates. Plasmodiophorids contain organisms such as *Plasmodiophora brassicae*, the causal agent of club root in brassicas, *Spongospora subterranea* which causes powdery scab of potato and *Polymyxa graminis* an obligate parasite of plant roots, while only weakly pathogenic itself, it is responsible for transmission of important plant viruses. Haplosporidians (including the genera *Haplosporidium*, *Minchinia*, *Urosporidium* and *Bonamia*) and Paramyxida (containing *Marteilia*, *Paramarteilia*, *Marteilioides*, *Eomarteilia*, and *Paramyxa*) infect bivalves and crustaceans in both marine and freshwater habitats (Hartikainen *et al.,* 2014a, b; Ward *et al.*, 2016). They can devastate aquaculture and some are listed in international legislation concerning the movement of live animals for aquaculture (Stentiford *et al.,* 2013). ,, rhizarian parasites and eukaryvores are not restricted to Endomyxa. Many Cercozoa (=Filosa) are flagellates or amoebae, gliding or swimming heterotrophs, and are abundant in marine, soil, and freshwater habitats (Bass and Cavalier-Smith 2004), although the range of cell morphologies is broad and include two lineages that have become algae by enslaving photosynthetic microbes. Cercozoan parasites include *Pseudopirsonia* and *Cryothecomonas*, parasites of diatoms, the termite gut-endosymbiont *Cholamonas*, and doubtless many more not yet characterised, but none are yet known from the order Cercomonadida, to which *P. venanatans* belongs.

Cercomonads are particularly abundant in soil and freshwater habitats, where they are important bacterivores, but are also known to predate other microbial eukaryotes and larger organisms, e.g. nematodes (Bjørnlund and Rønn, 2008). The diversity of Cercomonads has been rapidly expanding in recent years (Bass *et al.,* 2009a, Brabender *et al.,* 2012; Dumack *et al.,* 2016; Gawryluk *et al.,* 2016).

The creation of *Phytocercomonas* brings the number of genera to eight; prior to Bass *et al.* (2009b) there was only one.

Genomic and transcriptomic analyses of *Brevimastigomonas* (sister to *Phytocercomonas*) have recently shed light on the earliest stages of mitochondrial adaptation to low oxygen conditions (Gawryluk *et al.,* 2016), however, *Phytocercomonas* grows very well in aerobic culture conditions, like most other known Cercomonads.

The erection of the new genus *Phytocercomonas* is supported by the phylogenetic distance from its closest relative *Brevimastigomonas*, differences in morphology between the two genera and the presence of a related OTU associated with the rhizosphere of oilseed rape (**Figure 11**). Morphologically, *P. venanatans* differs from *B. anaerobica* in having a larger cell size, longer anterior flagellum, rarely present pseudopodia and it is also non-metabolic (Bass *et al.,* 2009b; Brabender *et al.*, 2012). A full morphological description is provided in Section 6.3. Microscopic examination of diseased plant tissues identified *P. venanatans* cells in low counts in xylem exudates, suggesting it is living within xylem vessels. We could not determine if the organism is confined to the xylem or whether it is present in other cells within plants (see Section 6.3).

6.2. Pathology investigations and Koch's postulates

6.2.1 Isolation and Culturing of *P. venanatans*

P. venanatans has proved to be relatively simple to culture using the methods presented in Section 5.2. We successfully established cultures from three sugarcane cultivars, RP197-63, Q238$^{\Phi}$ and Empire, originally sourced from different locations across the Australian sugar industry. Attempts to culture the organism on solid agar media were unsuccessful (data not shown). Because the organism will only grow in broth cultures the contamination rate is fairly high, with yeast the most common contaminant, usually becoming apparent within about two days.

6.2.2 Establish growth curves for *P. venanatans* in culture

Two stalks of CS-infected RP193-67 were used to initiate replicate cultures of *P. venanatans* on S8 broth media. **Figure 21** shows that one culture (from stalk 1, replicate flask 2) was found to have extremely high cell counts, relative to the others. Motile cells increased in number for six to 13 days after the culture was initiated and then started to reduce in number. This coincided with increasing numbers of non-motile cells without flagella.

The high cell count culture (from stalk 1, flask 2) was subcultured onto two media (S8 broth and corn meal broth) six days after the original initiation. **Figure 22** shows that for most subcultures, motile cells increased in number for about seven days and then then started to reduce in number. The variation in cell number between individual subcultures was much less than that seen in initiated cultures.

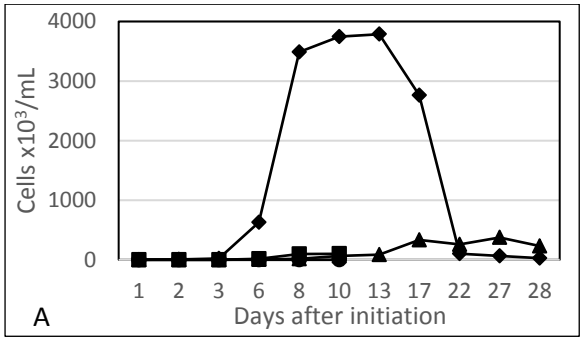


Figure 21: Variation in growth curves for four independent *P. venanatans* cultures established from CS-infected cultivar RP193-67 and grown on S8 media over a four week period. Stalk 1-1 (▲); stalk 1-2 (♦); stalk 2-1 (■); stalk 2-2 (●)

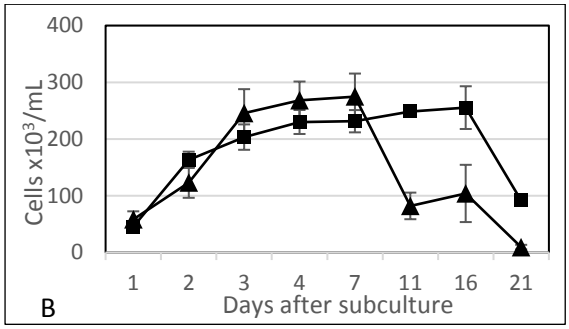


Figure 22: Growth curves for subcultures derived from stalk 1-2 grown on S8 (▲) and corn meal broth (■) over a three week period. Data points represent average ± SE of three replicate cultures

For both the initial isolation and subculture, we determined that between 7 and 14 days was the period of peak growth. Cultures initiated from RP193-67 onto S8 broth were subcultured onto a range of growth media. The optimal media was potato dextrose broth supplemented with peptone (**Figure 23**). Even potato dextrose alone without peptone encouraged higher growth rates than corn meal with peptone. The supplemented potato dextrose broth was so successful that it allowed those cultures to be successively subcultured five times, which could not be achieved on any other media (data not shown).

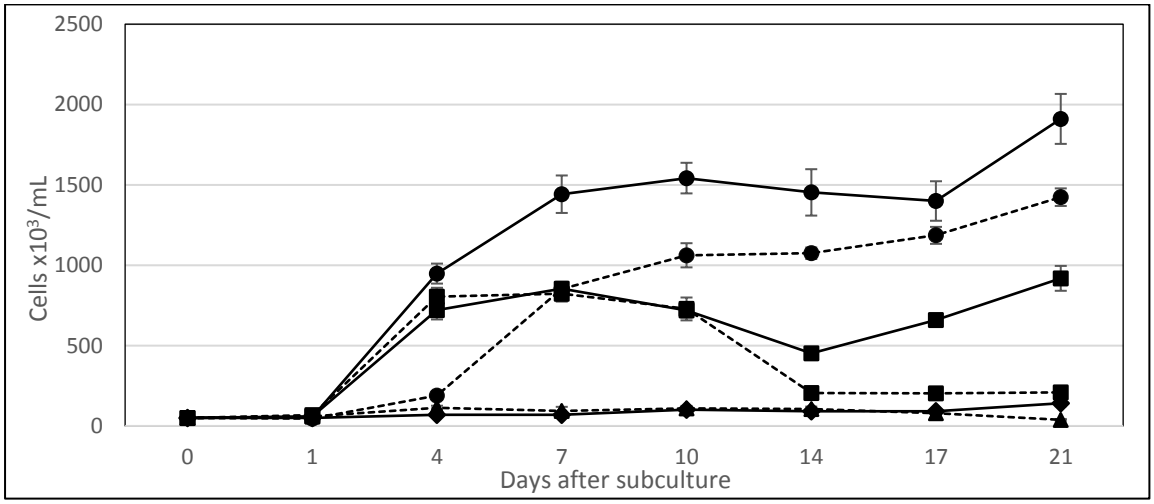


Figure 23: Growth curves for *P. venanatans* subcultures grown over a three week period on four broth media: S8 (▲); glucose plus peptone (♦); corn meal (■); potato dextrose (●). Solid lines represent media supplemented with peptone and dashed lines represent media without added peptone. Data points represent average ± SE of six replicate cultures

6.2.3 Inoculation experiments

The ultimate confirmation that *P. venanatans* is the causal agent of chlorotic streak is to complete Koch's Postulates. Using actively motile cultures of the pathogen and three different inoculation techniques we were able to inoculate healthy sugarcane plants that subsequently developed the characteristic symptoms of chlorotic streak. Infection was confirmed by PCR screening of plant tissues and by re-isolation of the pathogen into aseptic culture with PCR and microscopic confirmation.

Experiment 1. Root inoculations. We achieved successful inoculation of sugarcane by injecting roots with cultures of *P. venanatans* (**Figure 24A**). A motile cell culture produced chlorotic streak symptoms 6 weeks after inoculation (**Table 22**).

Both replicate plants showed typical symptoms of CS, namely leaf streaks with necrosis and scalding on the leaf tips (**Figure 24B**). No symptoms were visible on plants injected with the older non-motile culture or the control culture.

We successfully re-isolated *P. venanatans* from the symptomatic plants inoculated with the active culture. The two replicates were sampled for *P. venanatans* isolations 13 weeks and 16 weeks after inoculation, respectively. At the 16 week stage there was a clear difference between the heights of the symptomatic plant injected with the motile culture compared to the plants injected with the inactive or control cultures (**Figure 24C**). For each re-isolation, 10 S8 broth cultures were established using tissue pieces removed from inside the stalks. Stalk material was also sampled for DNA extractions. The presence of *P. venanatans* was assessed by microscopy and PCR screening of cultures and stalk pieces (**Table 22**). *P. venanatans* was not detected in the stalks of plants injected with the inactive or control cultures, nor could successful isolations be made from those plants.

Table 22: Presence of *P. venanatans* in Q90 test plants after injecting roots with three cultures, a 6-day old motile culture, a 28-day old non-motile culture and the control culture containing no cells. New isolations were established from the inoculated material and the presence of *P. venanatans* confirmed by PCR and microscopy

***P. venanatans* culture**		**Q90 test plants**		***P. venanatans* cultures established from test plants**	
Status	**Replicate**	**CS symptoms**	**PCR confirmation of *P. venanatans* in stalks**	**Microscopic confirmation of *P. venanatans***	**PCR confirmation of *P. venanatans***
Motile	A	Streaks and scalding	10/10	10/10	10/10
	B	Streaks and scalding	nt[a]	10/10	10/10
Non-motile	A	No symptoms	0/10	0/10	0/10
	B	No symptoms	nt	0/10	0/10
control	A	No symptoms	0/10	0/10	0/10
	B	No symptoms	nt	0/10	0/10

[a] nt: not tested

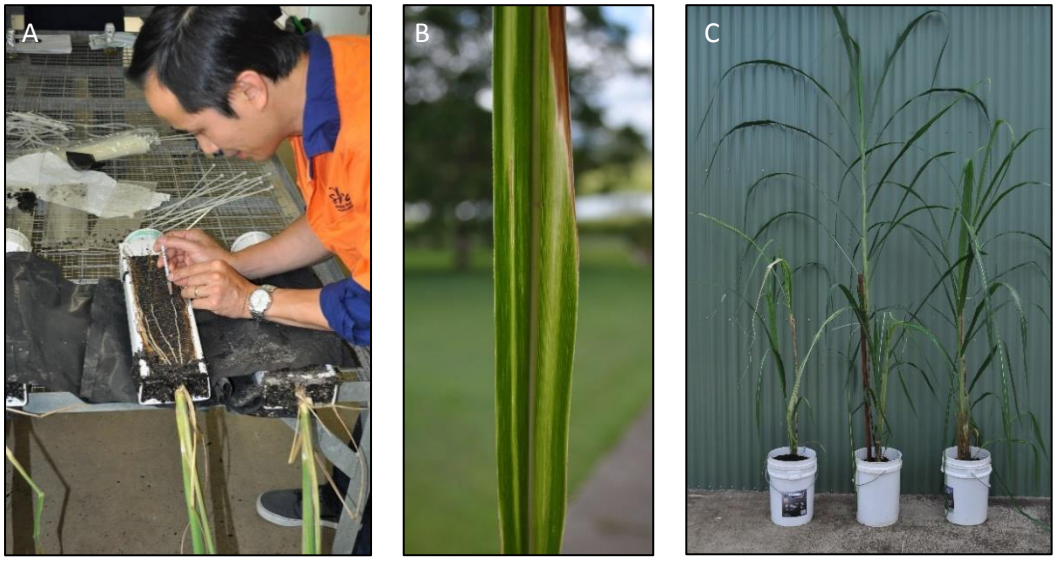


Figure 24: Q90 plants inoculated with *P. venanatans*. A: injecting roots in plastic conduit pots; B: symptoms of leaf streaks with necrosis and scalding on the leaf tips seen in Q90 injected with the active culture; C: variation in the height seen between three Q90 plants injected with the active culture (left), control culture (centre) and inactive culture (right)

Experiment 2. Root inoculations. Root injections were repeated, this time using a known concentration of active cells and different sugarcane cultivars (**Figure 25A**) Chlorotic streak symptoms were first observed 5 weeks after inoculation and by 10 weeks, one Empire plant had severe symptoms (necrosis and scalding of leaf tips in addition to streaks; **Figure 25B**) and was starting to die (**Table 23**). Symptoms were also seen in Q183$^{\phi}$, with one considered severe (**Figure 25C**). No symptoms were seen on control plants. Xylem exudate, collected from 5-cm-long stalk pieces by low speed centrifugation, was subjected to PCR screening to confirm the disease status of the inoculated plants (**Table 23**).

Only the two plants with severe symptoms tested PCR-positive and these were used to establish new cultures of *P. venanatans.*

Table 23: Transmission results for sugarcane plants injected with *P. venanatans* into the roots or stalks

Inoculum	Test Cultivar	CS symptoms on test plants	PCR confirmation of test plants	Re-isolation of *P. venanatans* from test plants
S8 control	Empire	0/3	0/3	nt[a]
S8 control	Q183$^{\phi}$	0/3	0/3	nt
P. venanatans	Empire	1/3	1/3	1/1[b]
P. venanatans	Q183$^{\phi}$	2/3	1/3	1/1[b]

[a] nt: not tested

[b] Isolation was only attempted from the plants with strong CS symptoms.

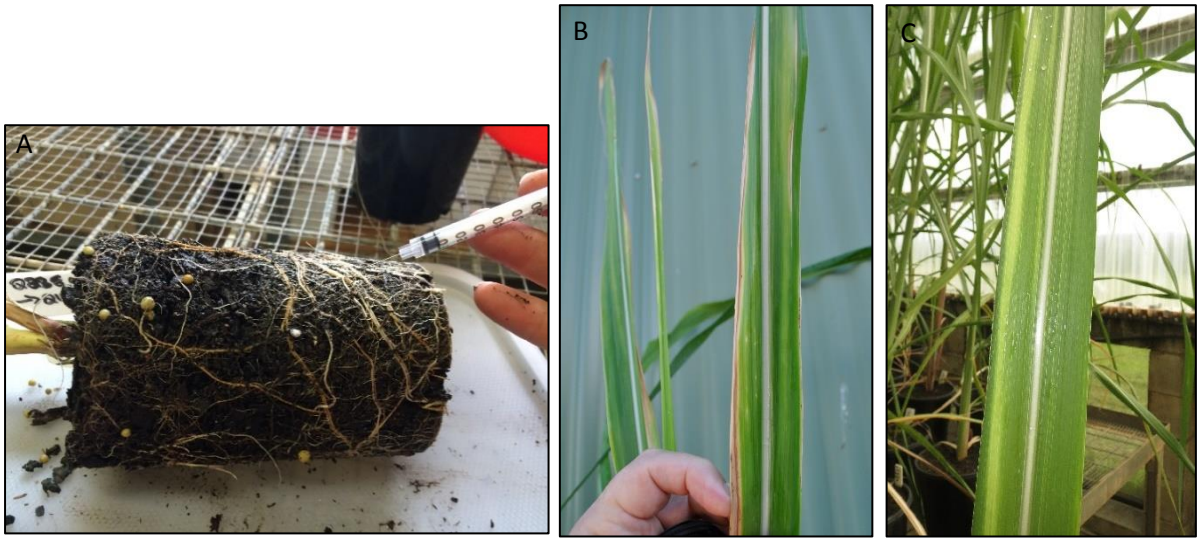


Figure 25: Method used to inject roots of Empire and Q183$^{\Phi}$ (A). B: severe symptoms of necrosis and scalding of leaf tips seen in one Empire plant 10 weeks after inoculation; C: typical symptoms of leaf streak seen in one Q183$^{\Phi}$ plant five weeks after inoculation

Experiment 3. Injection into the stalk. We achieved successful inoculation of sugarcane by injecting stalks with *P. venanatans* (**Figure 26A**, **B**). Chlorotic streak symptoms were first observed in the cultivar Empire 7 weeks after inoculation. By 9 weeks, symptoms were observed on all *P. venanatans* inoculated plants of all cultivars and no symptoms were observed on control plants (**Table 24**). The inoculated plants showed typical leaf streaks, and in some cases necrosis and scalding on the leaf tips (**Figure 26C**). *P. venanatans* was re-isolated from all inoculated plants but not from the control plants.

Xylem exudate and stalk pieces were sampled for PCR screening to confirm the disease status of the inoculated plants (**Table 24**). All plants with symptoms tested PCR-positive and cultures of *P. venanatans* were established from the stalks.

Table 24: Transmission results for sugarcane plants injected with *P. venanatans* into the roots or stalks

Inoculum	Test Cultivar	CS symptoms on test plants	PCR confirmation of test plants	Re-isolation of *P. venanatans* from test plants
S8 control	Empire	0/2	0/2	0/2
S8 control	Q183$^{\Phi}$	0/2	0/2	0/2
S8 control	RP193-67	0/2	0/2	0/2
P. venanatans	Empire	2/2	2/2	2/2
P. venanatans	Q183$^{\Phi}$	2/2	2/2	2/2
P. venanatans	RP193-67	2/2	2/2	2/2

[a] nt: not tested

[b] Isolation was only attempted from the plants with strong CS symptoms.

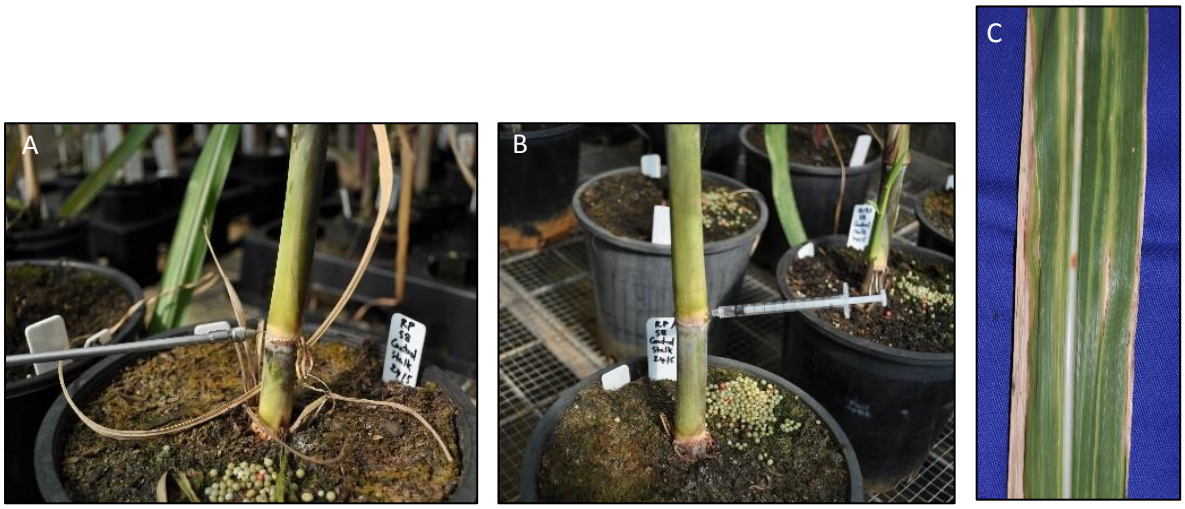


Figure 26: Inoculation of plants by injecting stems. A: making a hole in the root primordial region of the basal node of the stem with a fine needle; B: injecting the culture; C: symptoms of necrosis and scalding seen in inoculated Empire

Experiment 4. Leaf whorl inoculation. In the final trial we achieved infection by inoculating the top of the stalk, specifically the leaf-whorl region above the meristem (**Table 25**). By applying the inoculum to the cut surface of the top of the stalk (**Figure 27A**), severe symptoms were produced on the newly produced leaves 5 weeks after inoculation. The young leaves had many chlorotic streaks, scorching of the leaf tips and wilting of the young spindle leaves (**Figure 27B**). In contrast, the plants that were inoculated by injecting into the leaf whorl showed transitory streak symptoms on the expanding leaf with injection holes only 17 days after inoculation (**Figure 27C**). However, only one of the plants went on to develop symptoms in newly produced leaves and this took 12 weeks to develop (**Table 25**).

We saw no symptoms on control plants. We re-isolated *P. venanatans* from the stalks of all inoculated plants showing symptoms, but not from the control plants.

Table 25: Transmission results for RP67-193 plants inoculated with *P. venanatans* into the leaf whorl

Inoculation method	CS symptoms on test plants	Re-isolation of *P. venanatans* from test plants
Control (no inoculation)	0/2	0/2
Apply to cut surface of leaf whorl	3/3	3/3
Injection into leaf whorl	1/3	1/3[a]

[a] Re-isolation of *P. venanatans* was only attempted from the plant with CS symptoms.

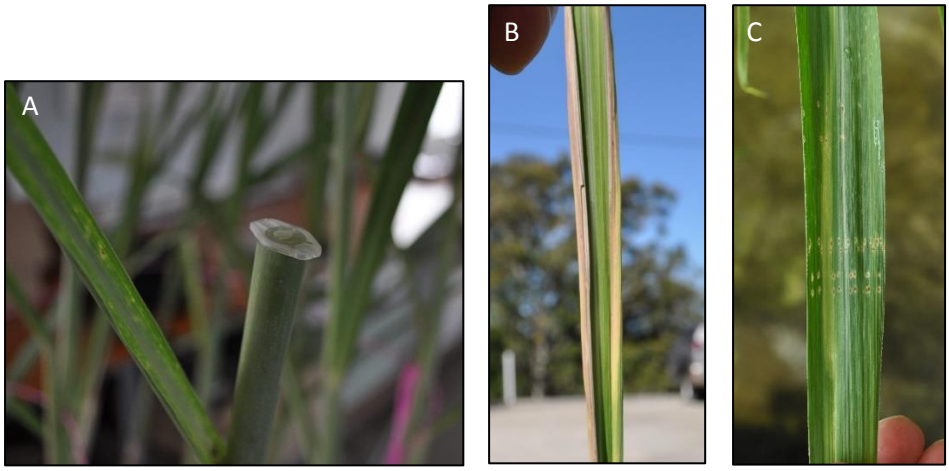


Figure 27: Inoculation of plants through the leaf whorl region. A: applying *P. venanatans* culture to the freshly cut surface of the leaf whorl; B: severe symptoms of streaks and scorching of the leaf tips in the young spindle leaves after inoculating through decapitation; C: milder leaf streaking and injection holes seen in the unfurled leaf after injecting the leaf whorl

6.2.4 Transmission to other host plants

We were interested in transmitting CS to sorghum and corn for several reasons: firstly, to demonstrate that we could transmit the disease experimentally under controlled conditions, confirming the work of Egan (1965); secondly, sorghum would make a good sequencing candidate for future NGS work aimed at sequencing the genome of *P. venanatans* as its genome is much smaller than that of sugarcane and has been completely sequenced; and thirdly seedlings of corn and sorghum are easy and quick to raise and could be developed into a rapid bioassay system to test aspects of
P. venanatans's biology.

"Natural" transmission to sorghum. Potential symptoms of CS were observed in one plant inoculated at Woodford through geofabric (**Figure 28A**, **B**). Potential symptoms of CS were observed on two plants in the gravel hydroponic setup at Tully (**Figure 28C**, **D**). Various plant parts from the three symptomatic plants were screened with the diagnostic test (data not shown). All three plants were shown to be infected with CS, but only the roots or the base of the stalk tested positive. When sorghum root pieces were placed in water for 24 hours as described above, three exudate samples tested positive.

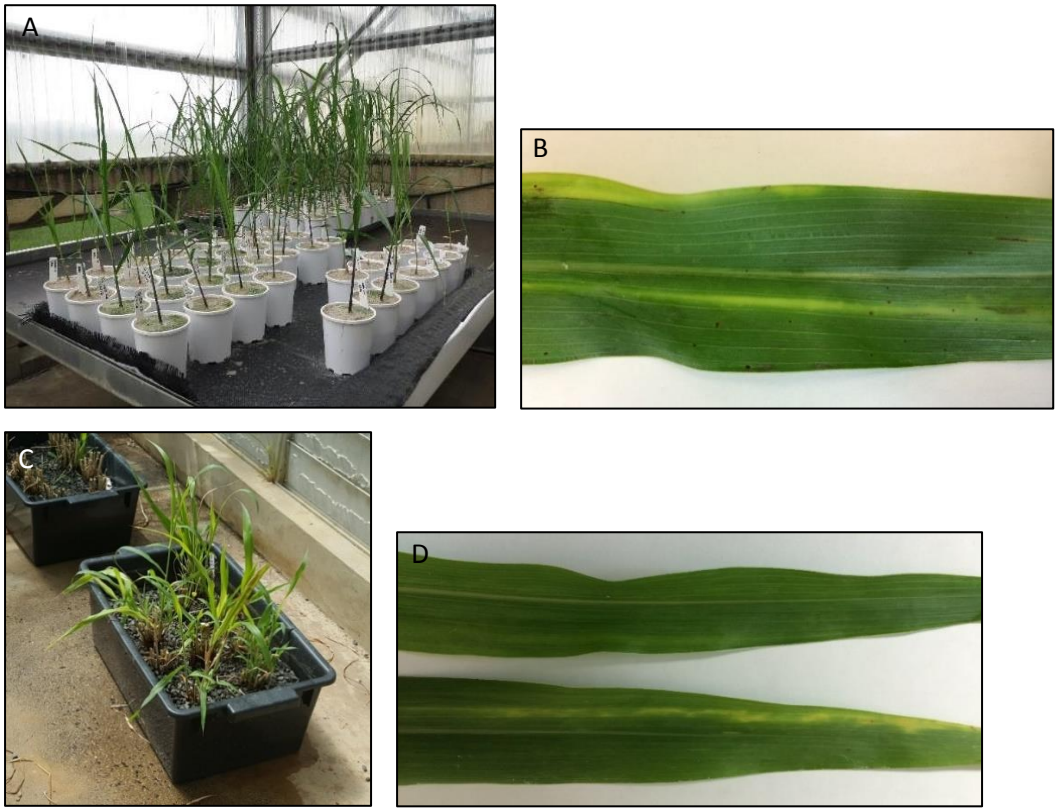


Figure 28: Experimental transmission set-up for sorghum. A: Pots on geofabric on the glasshouse bench at Woodford; B: symptoms seen on Woodford sorghum; C: plants in tubs of gravel at Tully; D: symptoms seen on 2nd Tully sorghum plant

Leaf whorl inoculation of corn and sorghum. Maize (*Zea mays* cultivar Terrific F1) and sorghum (*Sorghum* hybrid cultivar Cow Pow) seedlings were inoculated either by injecting into the leaf whorl, or by decapitating the shoot. Both corn and sorghum displayed symptoms on at least one test plant after inoculation through decapitation. (**Table 26**). Symptoms of extensive wilting and necrosis of leaves, scorching leaf tips, mottled streaks observed on sorghum are shown in **Figure 29**.

Table 26: Transmission results for plants inoculated with *P. venanatans* into the leaf whorl

Test host	Inoculation method	CS symptoms on test plants
Corn Terrific F1	Control (no inoculation)	0/5
	Apply to cut surface of leaf whorl	2/5
	Inject into leaf whorl	0/5
Sorghum Cow Pow	Control (no inoculation)	0/6
	Apply to cut surface of leaf whorl	4/6
	Inject into leaf whorl	1/8

Figure 29: Severe symptoms of streaks and scorching of sorghum leaves after inoculating through decapitation above the leaf whorl

6.2.5 Discusssion

The ability to successfully grow *P. venanatans* in axenic broth culture is a unique feature for this organism compared with other plant pathogenic cercozoans. Well-known plant pathogenic cercozoans include *Plasmodiophora brassicae* (clubroot in brassicas), *Spongospora subterranea* (powdery scab of potato) and *Polymyxa graminis* and *Polymyxa betae*, vectors of several plant viruses (Neuhauser *et al.*, 2010). They belong to the Plasmodiophorids (class Phytomyxea) and this group of Cercozoa are obligate biotrophic pathogens unable to be cultured. As discussed in Section 6.1, the Phytomyxea belong to the subphylum Endomyxa, while the chlorotic streak pathogen belongs in the subphylum Filosa, a group not usually known for containing pathogens and only distantly related to the other plant pathogenic Cercozoa.

P. venanatans has proved to be relatively simple to culture. The initial attempts to isolate and culture the pathogen were done on S8 broth media (Davis *et al.*, 1980). This is a highly complex medium designed to support growth of the fastidious RSD bacterium (*Leifsonia xyli* subsp. *xyli*) that lives within the xylem of sugarcane plants. Because the chlorotic streak causing pathogen appears to live in the same environment, we theorised that this medium could also support growth of the pathogen. However, we found that the cultures could only be successfully subcultured once on S8, and when subcultured again, showed a large drop in the rate of multiplication, while a third successive subculture from the original stalk isolation was generally not successful. Subsequently, we found that the organism will grow successfully on a range of common plant-extract broth media including corn meal and potato dextrose. Potato dextrose agar supplemented with peptone supported the highest growth rates during several subcultures, providing the cells are subcultured during the peak growth phase of 7 - 14 days. Cells have been observed to divide and some cells briefly display four flagella (Section 6.3).

Many free-living heterotrophic Cercozoans must be grown in xenic cultures containing living bacteria (for example, Schuster and Pollak, 1978). A plant source such as cereal leaf extracts (Cerophyll), wheat grains or rice starch are common Protist media inclusions (see https://www.ccap.ac.uk and https://www.atcc.org for recipes) but we have been able to achieve axenic culture on plant-based microbiological media alone. That we could not culture the organism on solid agar media may explain why earlier researchers were unable to successfully isolate the causal agent using traditional pathological isolation techniques. Initiation of cultures is relatively easy providing the variable distribution along the stalk is allowed for by taking samples from multiple sections of stalk.

The contamination rate due to yeast is fairly high, but again, taking samples from multiple sections of stalk overcomes this.

Addition of antibiotics to control contaminants has not been investigated but we would expect this to be successful. The ability to reliably produce large amounts of the pathogen will greatly assist sequencing of the genome of the organism, production of inoculum for cultivar screening experiments and studies on interaction of the organism with the plant.

We have been able to take the cultured cells of the pathogen and infect sugarcane which subsequently gives rise to the characteristic symptoms of chlorotic streak and then re-isolate for the second time, thus demonstrating Koch's postulates. Infection has been achieved using cultures derived from several sugarcane cultivars and through inoculation of roots, stalks and leaf whorls. Successful infection could be replicated, even with slight procedural modifications, showing that the infection methods are simple, adaptable and reliable. The technique of injection to transmit the disease to healthy plants is not new. Sturgess (1961) reported that limited transmission could be achieved when the roots of healthy plants were inoculated directly by means of a fine hypodermic needle or by immersion of the intact root system for 7 days with diluted diseased root extracts. We have also demonstrated transmission to corn and sorghum, confirming earlier work by Egan (1965) that the disease could be transmitted to a range of grasses.

By inoculating roots, stalks and leaf whorls, we have demonstrated that the pathogen can move from the roots throughout the whole stalk, from the base of stalk throughout existing higher nodes and move from the leaf whorls down into the stalk, confirming that the Cercozoan is systemic in sugarcane. In some experiments the symptoms obtained were severe, probably due to the use of inoculum concentrations much higher than what would occur in nature. To date, we have not attempted to optimize the inoculum concentration and this important parameter remains to be determined. For all three tissue types, symptoms were generated within a matter of weeks to months.

6.3. Microscopy

Numerous attempts were made during the first years of the project to visualize any organisms that could be the causal agent. However, we suffered from the same problems that other researchers had experienced over the last 87 years (Carpenter, 1940; Abbott and Sass, 1945), namely that without knowing what to look for, it was extremely difficult to identify any obvious unusual organisms in sugarcane tissues.

By successive filtration of sap samples, combined with DNA extractions and diagnostic PCR screening, we determined that on each occasion only the 8µm filter reliably tested positive by PCR. This led us to conclude that the CS organism is 8 - 10 µm which is much larger than originally proposed by Sturgess (1961) of less than 0.5 µm. From then on, microscopic observations focussed on any structures that were about 8 - 10 µm. Since a surprising array of appropriately sized structures were observed in all tissue types, it was still difficult to be confident that they were the correct structure. Also a large number of motile organisms and bacteria could be generated from healthy sugarcane material.

6.3.1 Observations on cells in pure culture

Once the pathogen was established in axenic liquid culture (see Section 6.2) microscopic observations were much easier (**Figure 30**). An organism, consistent with the morphology of the Cercomonadida, was observed, and as we had suspected, was approximately 10µM in size. The cells are easily visible with simple light microscopy, although we often used phase contrast to clearly see the flagella.

Occasionally differential interference contrast microscopy (Nomarski) was used. The organism appears to multiply through division, and at times briefly displays four flagella. Old cells become non motile and lose their flagella, becoming darker in colour.

We initially thought that these structures were cysts, but we have no evidence at this stage that the organism produces a long-term survival structure as part of its life cycle.

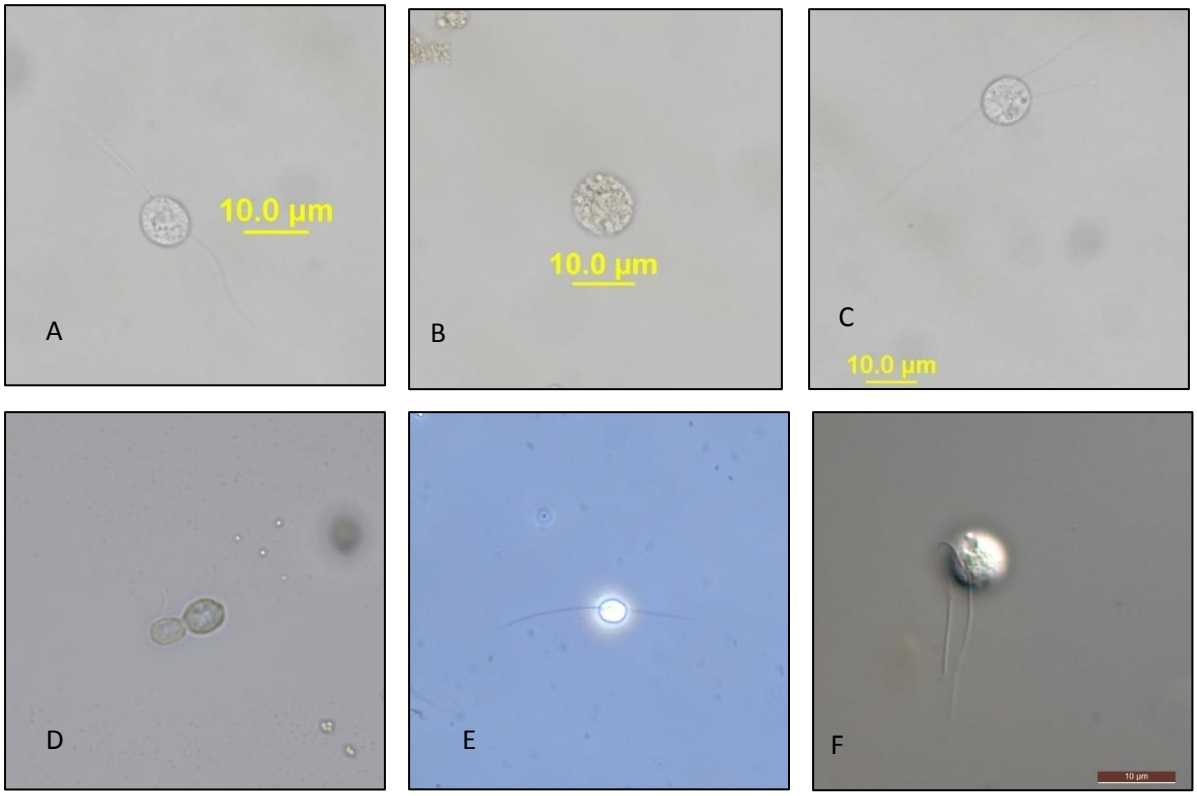


Figure 30: Various microscopic views of *P. venanatans*. A: typical view of a motile cell; B: old non-motile cell; C: cell with four flagella; D: dividing cell; E view under phase contrast microscopy; F: view under Nomarski microscopy

6.3.2 *In situ* hybridization

In situ hybridization was developed in the hope that specific structures of the pathogen could be identified in plant tissues. Based on the equipment available at Indooroopilly, it was decided that colourmetric *in situ* hybridisation (CISH) would be performed.

Three types of hybridisation template were trialed: root exudate (water from which CS-infected roots had been allowed to soak); xylem sap expressed from the stalks; and roots, either hand-cut transverse sections or viewed whole longitudinally. In all cases, PCR screening was carried out first to ensure that the material was positive for CS. Tissues were fixed with paraformaldehyde. As to be expected, some optimisation was required with the major problem being high non-specific background. Early progress was limited by the fact that we were not sure of the actual structure of the causal agent, making it difficult to decide if the correct organism had been labelled. However, once the organism was established in pure culture, the technique could be optimized on cultured cells.

By taking fresh samples of the cultured organism, fixing for 3 hours with paraformaldehyde and then applying to microscope slides, we could achieve excellent hybridisation with labelled ribosomal probes.

The cultures tested so far have been initiated from CS-infected RP193-67 and Empire (**Figure 31**). While fixation often distorts the shape of the cells, they are clearly recognizable by the intense staining. The flagella are often visible.

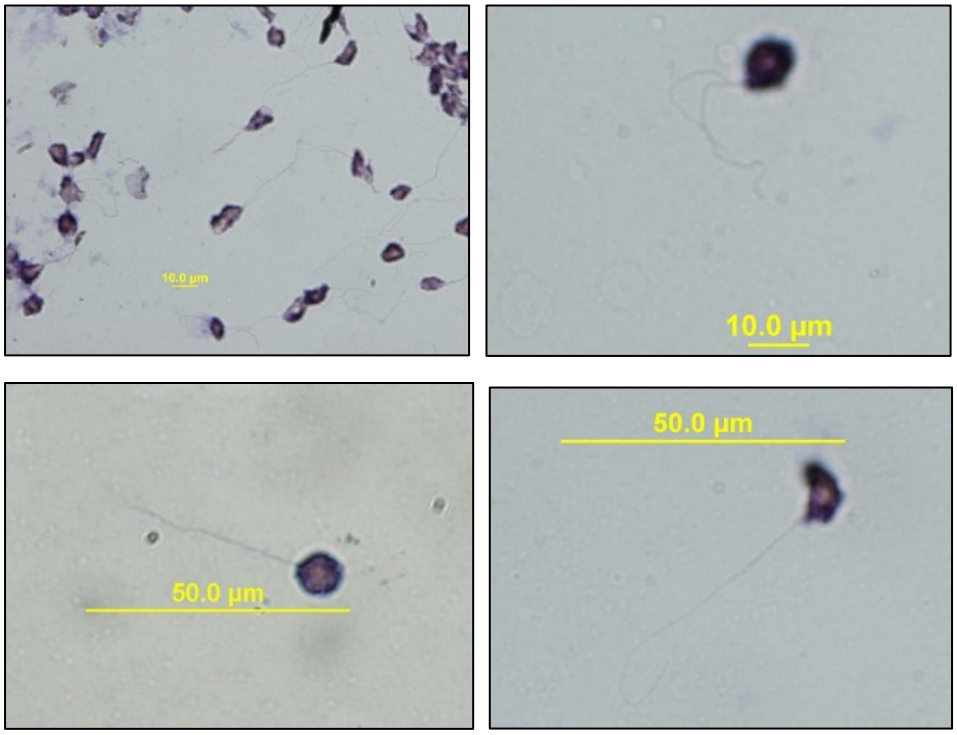


Figure 31: Various hybridized cultured cells of *P. venanatans* isolated from either CS-infected Empire or RP193-67 and probed with DIG-labelled ribosomal probes. The cell body is approximately 10 µm in diameter and the flagella can be up to 50 µm long

The optimized technique was then applied to water from which CS-infected roots had been allowed to soak, xylem sap expressed from the stalks, and roots, either hand-cut transverse sections or viewed whole longitudinally. The organism has now been successfully detected in both root exudates and xylem (**Figure 32**) although only limited numbers of cells are observed in these substrates. Once we became familiar with observing the cells, we realized that typical cells could be observed directly in the xylem, providing it was fresh and not frozen. This explains some of our earlier failed attempts when material was frozen before handling.

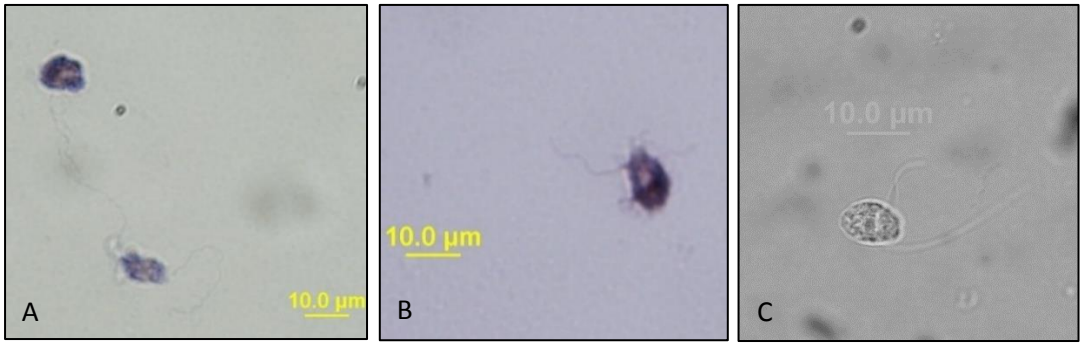


Figure 32: *In situ* hybridisation results for CS-infected RP193-67. A: 24-hour root water exudate probed with DIG-labelled 28S; B: xylem probed with DIG-labelled 18S; C: a view of untreated fresh xylem

However, the situation for infected roots is still not resolved. Purple hybridisation products were able to be observed in CS-infected roots cells (**Figure 33**) but the staining does not seem to indicate a recognizable organism or biological structure.

More research is needed to be able to observe *P. venanatans* within plant cells. At this stage we have not confidently observed the organism in any sectioned plant tissue.

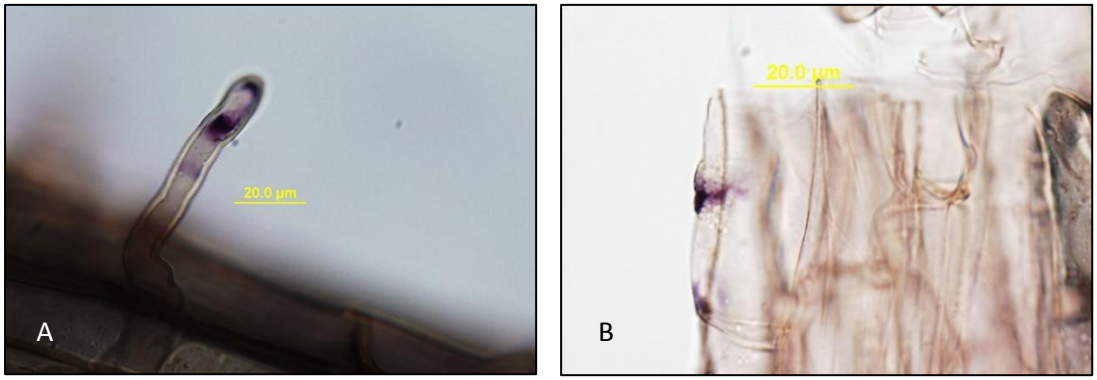


Figure 33: *In situ* hybridisation results for CS-infected RP193-67 roots probed with DIG-labelled ribosomal probes. A: hybridisation in a root tip; B: hybridisation at the root surface

6.3.3 Discussion

Researchers had been trying for many years to observe the causal agent of CS in sugarcane using both light and electron microscopy. From our experience, there was no shortage of possible structures; a wide range were observed, but similar structures can be observed in healthy tissues. A specific visualization technique was clearly needed. However, even *in situ* hybridisation did not produce clear results. We had established through PCR that the organism exists in large amounts in the xylem and this has now been confirmed through light microscopy and *in situ* hybridisation. We hypothesize that this is how the organism spreads within the plant. We have no evidence of the pathogen in any sugarcane cellular tissue and this may be because it does not multiply to any great extent in cells.
We also do not know how the organism infects naturally though the roots, how it causes disease, how cells released back into the soil and how the organism survives for extended periods outside of the plant.

We have established that the pathogen has the morphology, size, and movement consistent of Cercomonadida. A formal description of its morphology has been provided by David Bass (Division of Genomics and Microbial Diversity, Department of Life Sciences, Natural History Museum, London, UK):

> *Diagnosis: Cells round to ovoid to drop-shaped, 6-15 µm long, ventrally flattened. Two flagella, subapically inserted, directed anteriorly (AF) and posteriorly (PF). Flagella extend +/- equally beyond cell in both directions, typically by 1.5x to 3x cell length. PF attached to ventral surface of cell, sometimes in a groove. Both flagella may be (partially) in contact with the substrate when gliding, with most contact, attachment, and motor force provided by PF. Both flagella free and extended when swimming. Gliding movement very variable in speed, from small localized movements to fast (up to 40 µm/s observed) directed gliding, with anterior-posterior rocking/jiggling. Cells frequently not in contact with the substrate, even not in directed movement. Pre-division cells with two anterior and two posterior flagella frequently seen. Swimming motion fast and direct, with flagella extended in front of and behind cell, directing movement by a helical/flickering motion. Cells inflexible in shape; non-metabolic; small pseudopodia only very rarely observed. One to four contractile vacuoles. Nucleus in a central and anterior position within cell, with prominent nucleolus. No cysts observed, but cells may be stationary in apparent*

resting mode for extended periods. Non-marine; grows in organically enriched freshwater culture media.

6.4. Development of a diagnostic assay for *P. venanatans*

6.4.1 Primer design and choice of optimal tissue to sample

The first generation diagnostic test used actin primers supplied by Nicole Thompson (SRA) and designed by Clint Magill (Texas A & M). These primers were unsuitable for detection in roots due to non-specific amplification (Braithwaite and Croft, 2013) and were improved by designing a new reverse primer which targets the actin intron, giving the combination (Actin, F1-R3). Once the full ribosomal sequence was obtained by Next Generation Sequencing (see Section 6.1), new diagnostic primers targeting the ribosomal small subunit (SSU, F1-R2) and ribosomal large subunit (LSU, F4-R2) could be developed. All three genes have been shown to have equally good specificity for the CS organism, but the repetitive ribosomal genes give more intense signals (**Figure 34**). The ribosomal small subunit primer set has become the standard diagnostic combination for routine screening for chlorotic streak.

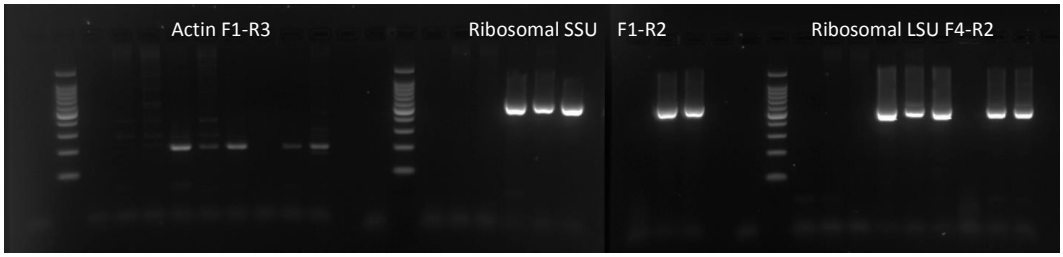


Figure 34: Comparison between three CS-specific diagnostic PCR combinations. The primers amplifying actin, the ribosomal small subunit and the ribosomal large subunit and show similar specificity in that no apparently healthy samples are detected and five out six potentially CS -infected samples could be detected, however, intensity is much higher for the ribosomal genes. Samples are H1: RP193-67 H xylem; H2: Empire H roots; H3: Q170 H pith; CS1: Q170 CS pith; CS2: Empire CS stalk; CS3: Empire CS stalk; CS4: Q241 CS stalk (not positive in this example); CS5: Q208 CS Stalk; CS6: Empire CS roots

Routine screening is always combined with an endogenous test to check for DNA quality (**Table 9**). Sugarcane tissue DNA samples were initially checked with primers targeting phosphofructokinase. These primers give excellent results for stalks, leaves, roots and leaf sheaths, but give unreliable and variable results for xylem sap. Surprisingly, the universal fungal SSU primers gave better results for xylem sap. Later in the project the ADF primers were introduced and found to give excellent endogenous amplification from xylem. The universal fungal SSU primers are now only used in endogenous tests for environmental samples (water and soil) and for *P. venanatans* cell-cultures.

From work reported in Braithwaite and Croft (2013) and work carried out in the early phase of this project, we believed that stalks and xylem were the optimal tissue for detection with the actin primer-based PCR test. We trialed midrib and leaf sheath fluids as target tissue, and while these tissues are easy to sample in the field and the fluids easy to obtain, endogenous test results showed that the DNA was not able to be amplified, probably due to PCR inhibitors such as phenolics. Several leaf experiments were conducted with variable results (see Section 6.4.5). The most consistent diagnostic results were seen with stalks, probably because a large sample (1g) was used. However, the time and labour necessary to prepare the stalks for DNA extraction is considerable. The next best tissue was xylem, which has the advantage of requiring no DNA extractions and sampling is done in the same way as for RSD. As the project progressed the diagnostic test switched to ribosomal SSU-based primers and qPCR became available. Despite these improvements, the optimal samples were still stalks and xylem as will be shown below.

6.4.2 Abundance and distribution of *P. venanatans* within sugarcane stalks

To determine the distribution of the pathogen within infected sugarcane stalks, six mature stalks of field grown RP193-67, all showing symptoms of the disease, were split longitudinally and pieces of internal tissue were removed from the nodal region, with five to seven nodes sampled per stalk, depending on the stalk length. Nodes were numbered from the top down, and one internode was also sampled. DNA extractions were performed on three independent pieces of tissue per node. A single extraction was used for a gel-based diagnostic screen to ensure that the stalks were infected, while the three replicate DNA extractions (technical replicates) were used for qPCR.

Figure 35 shows that there was considerable variability among stalks, among nodes from the same stalk and even among the samples from within the same node. There was no apparent trend in abundance of *P. venanatans* along the length of the stalk. The gel-based PCR results are shown at the left in each panel of **Figure 35** and it can be seen that standard PCR gave comparable results to qPCR, with discrepancies (such as stalk 2, node 7) due to reliance on a single sample for PCR, compared to the average of three for qPCR.

The experiment was repeated with one field grown stalk of RP193-67. Nodes and internodes were numbered from the top down as before. The stalk was split longitudinally, then split again into quarters, as shown in **Figure 36**. Samples were taken from just below the rind (outer pith, A) and from the middle of the pith (inner pith, B). For each sample point, two 'A' sections were pooled, and two 'B' sections were pooled, for each node or internode. No gel-based PCR was performed in this experiment.

The results presented in **Figure 37** show that once again there was no apparent trend in abundance of *P. venanatans* along the length of the stalk. There was also no apparent difference between nodes and internodes from the same the same region of the stalk. However, the outer pith (just inside the rind) contained considerably more pathogen than in the centre of the stalk. This region is also where the majority of the vascular bundles are in sugarcane stalks, and supports the idea raised in section 6.3, namely that the pathogen is predominantly within the xylem, rather than intercellular. The result also supports the finding that stalks were considered a reliable diagnostic tissue because large (1g) samples were taken.

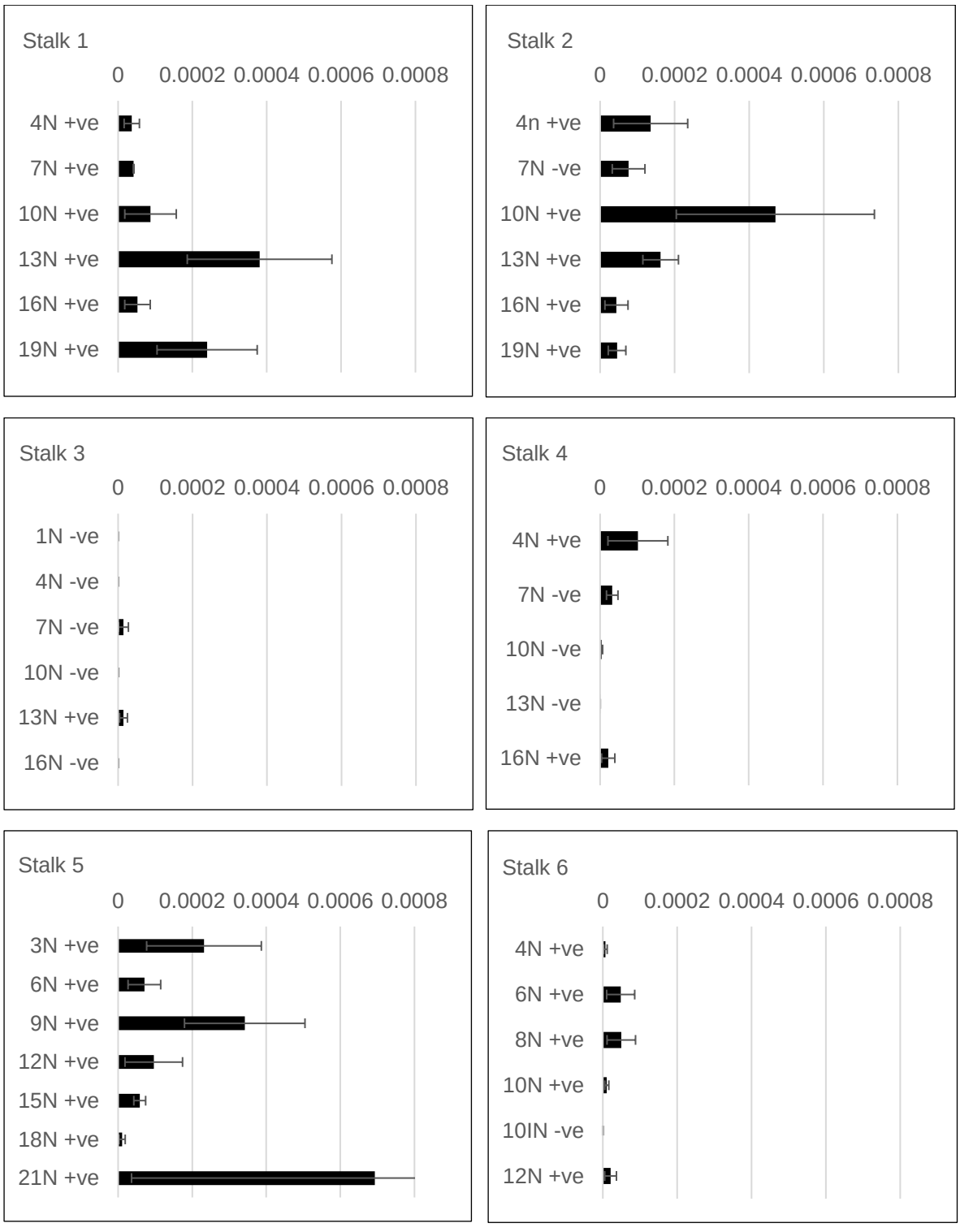


Figure 35: Relative abundance of *P. venanatans* along the length of six CS-infected stalks of RP193-67 assessed by qPCR with primers targeting the ribosomal SSU of both sugarcane and pathogen. Bars represent average ± SE of three replicate node pieces. Codes at the left of each figure indicate the node (N) or internode (IN) number and the gel-based PCR result (either positive or negative) for each individual node piece

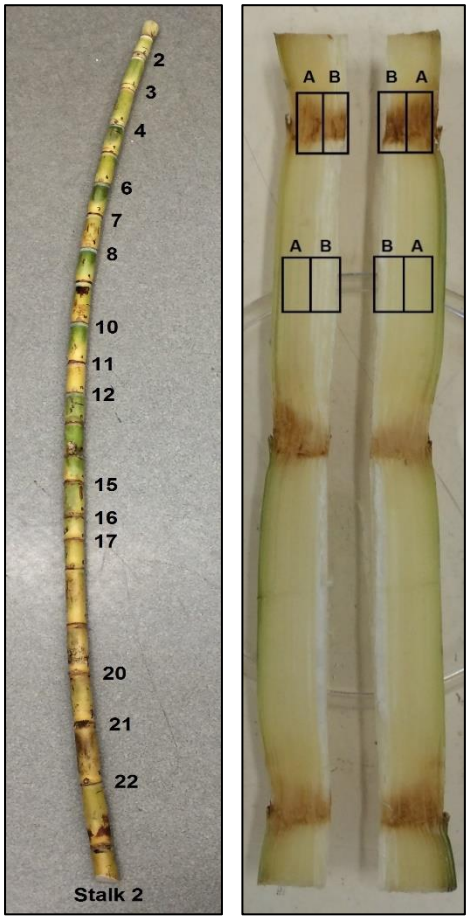


Figure 36: Sampling strategy to compare relative abundance of *P. venanatans* along the length of the stalk and within the stalk. A: numbering of nodes from the top down; B: stalks split longitudinally and sampled from just inside the rind (outer pith, A) and from the middle of the pith (inner pith, B). The duplicate 'A' samples were pooled, as were the duplicate 'B' samples

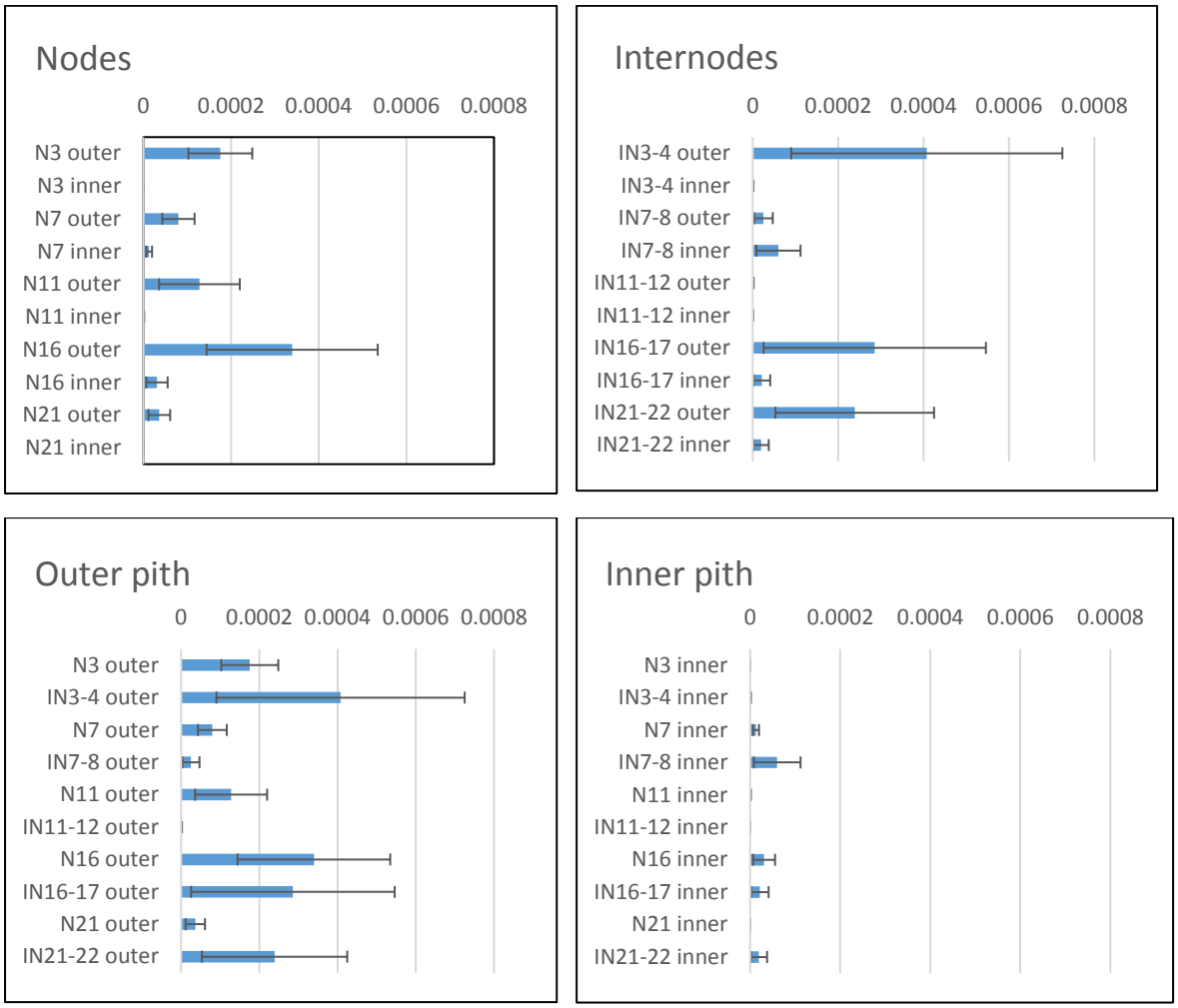


Figure 37: Relative abundance of *P. venanatans* along the length of one CS-infected RP193-67 stalk assessed by qPCR with primers targeting the ribosomal SSU of both sugarcane and pathogen. Bars represent average ± SE of two replicate node (N) or internode (IN) pieces. The pieces were taken from either just inside the rind (outer pith) and from the middle of the pith (inner pith)

6.4.3 Effect of uneven distribution in the stalk on the detection of *P. venanatans* in xylem sap

The variable distribution of *P. venanatans* within the stalk has consequences for using xylem as the diagnostic target tissue. To confirm this, the variety RP193-67 was sampled from the field at Woodford. Four stalks from each of six stools were sampled, giving 24 stalks in total. Leaf symptoms on each stalk were recorded. Three stalks showed no symptoms, while eight stalks showed mild symptoms and 13 showed strong symptoms. Five two-eye setts were sampled along the length of the stalk from top to base and the xylem was extracted and subjected to PCR diagnostic testing.

No symptom-free stalk tested positive for *P. venanatans*. Only one stalk with poor symptoms tested positive and 11 of the 13 stalks showing strong symptoms tested positive but none of them were consistently positive along the entire length of the stalk. The diagnostic screening results for the 12 stalks testing positive are shown in **Table 27**. The results confirm the unequal distribution although generally, the region between the middle and top of the stalk had the highest number of positive samples. As xylem samples are a good compromise between the reliability of the result and ease of sampling, approaches such as sampling from several node pieces, then centrifuging the xylem to concentrate it, or taking replicate samples, would overcome the patchy distribution along the stalk.

Table 27: Effect of stalk position on diagnostic screening results using untreated xylem. Only the 12 stalks testing positive out of the 24 are shown. Samples positive for *P. venanatans* are shown as (+) and negative are shown as (-)

Stool	1	2				3	4	5			6		Total per position
Stalk	1-3	2-1	2-2	2-3	2-4	3-1	4-2	5-1	5-2	5-3	6-1	6-3	
Top	-	-	+	-	-	-	-	-	+	-	+	+	4
Top-middle	+	+	+	+	+	+	+	+	+	+	-	+	11
Middle	+	-	+	+	-	-	-	+	+	+	+	+	8
Middle-Base	+	+	+	-	-	-	-	-	+	-	+	+	6
Base	-	+	-	-	-	-	-	+	-	+	+	-	4
Total per stalk	3	3	4	2	1	1	1	3	4	3	4	4	

6.4.4 Potential for screening RSD sap samples for chlorotic streak

As proposed in Braithwaite and Croft (2013), we felt there was the potential to combine RSD and chlorotic streak disease testing using the existing RSD testing infrastructure. Large numbers of sap samples are already collected by Productivity Service and other extension staff and sent to the SRA Indooroopilly lab where they are screened for RSD using an ELISA test. Left-over material is then disposed of. However, using this material for CS testing would eliminate the need for field staff to take and send additional samples and also avoids the need for DNA extractions from stalks. While there is the potential to use multiplex PCR to detect both pathogens in the future, at this stage they are detected with very different tests: protein-based for RSD and DNA-based for chlorotic streak, and so multiplexing was not attempted.

A trial was carried out in 2014 to ensure that samples collected for RSD were suitable for CS testing using PCR. This trial was needed at the time because sap samples routinely had the preservative Microshield 5 (Johnson and Johnson; active ingredient Chlorhexidine gluconate) added to prevent bacterial contamination. It quickly became apparent that Microshield 5, while having no effect on the protein-based ELISA test, is inhibitory to DNA-based PCR tests. Fortunately the RSD ELISA lab eventually stopped using Microshield 5, but our first attempt to implement a diagnostic test for the industry by hampered by the presence of Microshield 5.

The field samples to be screened were collected by Graham Cripps of the Tully Cane Productivity Services (TCPSL). These were collected and sent to the RSD lab during June - July 2014. Graham agreed to intentionally sample some stalks with symptoms of CS which was recorded on the sample grid sheets. An unfortunate complication was that some samples contained the usual Microshield 5, while some contained Chlorhexidine surgical scrub 4 % (Orion Labs Australia) which was even more inhibitory than Microshield 5. Despite this, most samples could be purified sufficiently, based on the endogenous SSU PCR results (data not shown). CS screening results for 86 RSD samples are shown in **Table 28**. There were 45 samples with symptoms of CS either in the sampled stalk or somewhere in the block, and 11 of these tested positive. From that group it appeared that Q200, Q241 and Q251 were the varieties in the Tully region most susceptible to CS. So a further 21 samples representing those varieties were randomly chosen, however, none tested positive for CS.

The last batch of samples (box 38) came from low lying areas described by Graham as where inundation would be experienced in the wet season. None tested positive.

Table 28: Tully RSD samples screened for chlorotic streak using standard gel-based PCR

Box	Farm	Variety and crop	Reason for testing	# tested	#CS positive
17	Ugana 9099	Q241$^{\phi}$ P	Symptoms	5	0
19	Musumeci 9048	Q251$^{\phi}$ 1R	Symptoms	3	0
20	DiMauro 4001	Q200$^{\phi}$ P	Symptoms	8	4
21	Nicotra 9291	Q241$^{\phi}$ 1R	Symptoms	4	0
21	Strathalma 5573	Q241$^{\phi}$ 1R	Symptoms	5	1
23	Davis 9849	Q241$^{\phi}$ RP	Symptoms	6	3
23	Vipiana 9486	Q251$^{\phi}$ P	Symptoms	6	0
34	Johnston 3914	Q241$^{\phi}$ 1R	Symptoms	5	3
38	Moran 9611	Q251$^{\phi}$ 1R	Symptoms	3	0
		Total		**45**	**11**
17	Musumeci 9267	Q241$^{\phi}$ P	Susc variety	5	0
17	Ugana 9099	Q241$^{\phi}$ P	Susc variety	5	0
21	Strathalma 5573	Q200$^{\phi}$ P	Susc variety	3	0
34	Johnston 3914	Q251$^{\phi}$ P	Susc variety	8	0
			Total	**21**	**0**
38	Moran 9611	Q208$^{\phi}$ 1R	Low lying	5	0
38	Moran 9611	Q208$^{\phi}$ 2R	Low lying	6	0
38	Moran 9611	Q238$^{\phi}$ P	Low lying	2	0
38	Moran 9611	Q214 P	Low lying	2	0
38	Moran 9611	Q250$^{\phi}$ P	Low lying	5	0
			Total	**20**	**0**

Screening was attempted again in 2016 but unfortunately both NSW and Tully suffered from very dry conditions during the year and chlorotic streak was not widely observed at either site.

Numerous inspections of the Harwood trial plots were made by Anthony Young and Mark Ensbey up until the end of July. However, as conditions were not conducive to symptom expression, no samples were taken for PCR screening and inspections ceased. Only QS97-2282 showed some streaking, but it was in the lowest, most water-logged part of the plot.

Jake McLagan, TCPSL Productivity Officer, agreed to sample the 1st ratoon crop at the Merryburn seed plot while doing his 3rd round of disease inspections. That block was chosen because it was the lowest block at the seed-plot and CSD had been observed previously. However, no visual symptoms of CSD where detected when sampling. 88 xylem (sap) samples representing nine varieties: Q200$^{\phi}$, Q208$^{\phi}$, Q219$^{\phi}$, Q231$^{\phi}$, Q232$^{\phi}$, Q240$^{\phi}$, Q241$^{\phi}$ Q250$^{\phi}$ and Q251$^{\phi}$ were provided. Microshield 5 was not added to the samples. None of the Tully samples tested positive for CS.

6.4.5 Detection of *P. venanatans* within sugarcane leaves

From work reported in Braithwaite and Croft (2013) and work carried out in the early phase of this project, we believed that *P. venanatans* does not accumulate to high level in leaves. To confirm this, we screened material that had been experimentally inoculated with *P. venanatans.*

The CS-specific primer combination of CS18SF1-CS18SR2 and the endogenous combination of ScPFK5F1-ScPFK5R1 were used. Table 6.4.3 shows the screening results for leaves 1, 4, 6 and 8 (with leaf 4 being the top visible dewlap leaf) from an RP193-67 plant injected through the stalk with a culture of *P. venanatans.* The leaves were subsampled into white streak areas, dead areas within the streak, green "healthy" areas and the leaf sheath. No leaf sample tested positive with the CS diagnostic test.

Leaf screening was repeated by taking only top visible dewlap leaves from plants inoculated by injecting *P. venanatans* into the stems of RP193-67, Q183$^{\phi}$ and Empire as part of Experiment 3 described in Section 6.2. Leaves from replicate plants were subsampled into white streak areas, green "healthy" areas and the midrib. Once again, no leaf sample tested positive with the CS diagnostic test (**Table 29**). Note that the leaf samples were not subjected to qPCR screening because the results did not look promising enough to warrant further testing. The results suggest that either *P. venanatans* is not present in the leaf, and symptoms are the result of a transportable toxin, or perhaps only present in young leaves or in the early stage of streak formation.

Table 29: Diagnostic PCR screening results for leaves collected from plants inoculated through the stalk with *P. venanatans*

A. RP193-67 injected through the stalk	**CS result**	**Endogenous result**
L1-White leaf	negative	positive
L1-Green leaf	negative	positive
L4- Leaf sheath	negative	positive
L4-White leaf	negative	positive
L4-Dead leaf	negative	positive
L4-Green leaf	negative	positive
L6- Leaf sheath	negative	positive
L6-Dead leaf	negative	positive
L6-Green leaf	negative	positive
L6-White leaf	negative	positive
L8- Leaf sheath	negative	positive
L8-Green leaf	negative	positive
L8-Dead leaf	negative	positive
L8-White leaf	negative	positive
B. Three varieties injected through the stalk		
RP193-67 TVD1		
White leaf	negative	positive
Green leaf	negative	positive

midrib	negative	positive
RP193-67 TVD2		
White leaf	negative	positive
Green leaf	negative	positive
midrib	negative	positive
Q183 TVD1		
White leaf	negative	positive
Green leaf	negative	positive
Midrib	negative	positive
B. Three varieties injected through the stalk		
Q183 TVD2		
White leaf	negative	positive
Green leaf	negative	positive
B. Three varieties injected through the stalk		
Midrib	negative	positive
Empire TVD1		
White leaf	negative	positive
Green leaf	negative	positive
Midrib	negative	positive
Empire TVD2		
White leaf	negative	positive
Green leaf	negative	positive
Midrib	negative	positive

6.4.6 Detection of *P. venanatans* within leaf sheaths

Recently, there has been interest from the industry in implementing the leaf sheath biopsy method (LSB; Young *et al*., 2014) for the detection of RSD. If the industry decides to proceed with the LSB, then a multiplex procedure for RSD and CS would be desirable. To assess whether *P. venanatans* could be detected in leaf sheaths, we screened material that had been experimentally inoculated with *P. venanatans***.** The varieties RP193-67, Q183$^{(b}$ and Empire were inoculated by injecting *P. venanatans* into the stalks as part of Experiment 3 in Section 6.2. Pieces of leaf sheath (not punches) were sampled and placed in zip lock bags with deionised water. As it was unknown if *P. venanatans* was tolerant of cold temperatures, half the samples were kept at room temperature overnight, with the other half was stored at 4°C overnight, as per the recommended LSB method of Young *et al.* (2014). Pieces of leaf were sampled and treated in the same way. DNA was extracted from the tissue pieces and the water was concentrated through brief centrifugation, similar to the method used for screening sap.

Samples were screened by with the CS-specific primer combination of CS18SF1-CS18SR2 and the endogenous combination ADP by gel-based PCR and then by qPCR. By this stage of the project, the

ADP primers had been implemented as the endogenous test as they were found to be superior for xylem, compared to PFK. Samples were also screened with the qPCR sugarcane SSU combination (qSo18SF2 and qSo18SR2) and a positive result was obtained in all cases.

This primer combination can be considered an endogenous test in this situation. Some samples did not have a matching water sample and for those that did, the sample was used up during the standard PCR, so not available for qPCR.

The results presented in **Table 30** confirm the results presented above in that leaves are not a reliable substrate for *P. venanatans* detection.

Results from the limited water testing that was done, also do not suggest that the water, left to soak at either 4°C or at room temperature overnight, is a promising substrate. However, leaf sheaths do appear to be a potentially promising sampling source for diagnostics at this stage.

Table 30: Diagnostic standard PCR and qPCR screening results for leaves and leaf sheaths collected from plants inoculated through the stalk with *P. venanatans.* qPCR was performed in triplicate and the positive (+) or negative (-) results for each replicate are shown. Standard PCR was not replicated

	qPCR for tissue	Standard PCR for tissue		Standard PCR for water	
CS samples	**CS result**	**CS result**	**ADF result**	**CS result**	**ADF result**
Leaf sheath + water (4°C ON)					
RP193-67 CS1 LS	+++	positive	positive	negative	positive
RP193-67 CS2 LS	+++	positive	positive	negative	positive
Q183 CS1 LS	+++	positive	positive	negative	positive
Q183 CS2 LS	+++	positive	positive	negative	positive
Empire CS1 LS	+++	positive	positive	negative	positive
Empire CS2 LS	+++	positive	positive	negative	positive
Leaf sheath + water (RT ON)					
RP193-67 CS1 LS	+++	positive	positive	nt	nr
RP193-67 CS2 LS	+++	positive	positive	negative	+ve
Q183 CS1 LS	+--	positive	positive	nt	nr
Q183 CS2 LS	-+-	negative	positive	negative	+ve
Empire CS1 LS	+++	positive	positive	nt	nr
Empire CS2 LS	+++	positive	positive	negative	+ve
Leaf + water (4°C ON)					
RP193-67 CS1 Leaf	---	negative	positive	negative	+ve
RP193-67 CS2 Leaf	---	negative	positive	negative	+ve
Q183 CS1 Leaf	--+	negative	positive	negative	+ve
Q183 CS2 Leaf	+++	positive	positive	negative	+ve
Empire CS1 Leaf	---	negative	positive	negative	+ve

	qPCR for tissue	Standard PCR for tissue		Standard PCR for water	
CS samples	**CS result**	**CS result**	**ADF result**	**CS result**	**ADF result**
Empire CS2 Leaf	---	negative	positive	negative	+ve
Leaf + water (RT ON)					
RP193-67 CS1 Leaf	---	negative	positive	nt	nr
RP193-67 CS2 Leaf	+++	negative	positive	negative	positive
Q183 CS1 Leaf	---	negative	positive	nt	nr
Q183 CS2 Leaf	+++	positive	positive	negative	positive
Empire CS1 Leaf	-+-	negative	positive	nt	nr
Empire CS2 Leaf	---	negative	positive	negative	positive
Healthy samples					
Leaf sheath + water (4°C ON)					
RP193-67 H1 LS	---	negative	positive	nt	nr
RP193-67 H2 LS	---	negative	positive	negative	positive
Q183 H1 LS	---	negative	positive	nt	nr
Q183 H2 LS	---	negative	positive	negative	positive
Empire H1 LS	---	negative	positive	nt	nr
Empire H2 LS	---	negative	positive	negative	positive

nt: not tested

nr: no result

In the next sampling trial, leaf sheath punches were taken according to the method described in Young *et al.* (2014). This trial involved taking leaf sheath punches from field-grown CS-free (healthy) Q200$^{(b)}$ and CS-infected Q238$^{(b)}$ from the Woodford Pathology farm. The "healthy" and CS punches were combined in zip-lock bags in ratios that always totalled 50. Water was added to cover the punches and the bags were left overnight at either 4°C or room temperature. The bags were then frozen until the time of diagnostic testing. DNA was extracted from 1g of a representative subsample of punches using the Qiagen Maxi kit and water was processed by pelleting 1.5 mL of liquid and resuspending the pellet in 50µL of water. Both water and punches were tested for *P. venanatans* using standard and qPCR. Samples were also screened with the ADP endogenous combination for gel-based PCR and the sugarcane SSU combination for qPCR, and positive results were obtained in all cases, even for the water samples (data not shown).

The results presented in **Table 31** show that no sample tested positive by standard PCR and only one sample gave a detectable qPCR reading on one of the three replicates. At this stage it appears that the leaf sheath biopsy approach is not a suitable detection method for *P. venanatans* in field grown plants, although further testing of different varieties, different leaf sheaths along the stalk or at different times of the year is needed to confirm this.

Table 31: Diagnostic standard PCR and qPCR screening results for combinations of healthy and CS-infected leaf sheaths collected from field grown plants. qPCR was performed in triplicate and positive (+) or negative (-) results for each replicate are shown. Standard PCR was not replicated

# of CS punches	# of Healthy punches	Overnight soak	qPCR result for punches	qPCR result for water	Standard PCR for punches	Standard PCR for water
0	**50**	4°C	---	---	negative	negative
10	**40**	4°C	---	---	negative	negative
20	**30**	4°C	---	---	negative	negative
30	**20**	4°C	---	---	negative	negative
40	**10**	4°C	-+-	---	negative	negative
50	**0**	4°C			negative	negative
0	**50**	RT	---	---	negative	negative
10	**40**	RT	---	---	negative	negative
20	**30**	RT	---	---	negative	negative
30	**20**	RT	---	---	negative	negative
40	**10**	RT	---	---	negative	negative
50	**0**	RT	---	---	negative	negative

6.4.7 Detection of *P. venanatans* in soil

The ability to detect chlorotic streak in the soil could provide the industry with a diagnostic service similar to that used for *Pachymetra* and nematodes. A MoBio PowerSoil DNA extraction kit was used to extract the DNA, but it is important to note that the kit uses only 250 mg of soil which is possibly below the CS detection level. The endogenous fungal SSU test was included and found to give positive results in all cases, showing that there was no issue with the quality of the soil DNA extracted.

Field soil samples were only taken on limited occasions throughout the project from NSW, Woodford and Tully. Initially no positive results were obtained (**Table 32**) but by switching the diagnostic primers from actin to ribosomal SSU, we obtained more sensitive results.

Table 32: Diagnostic screening results for soil samples collected during the project

Variety	Location	Initial results: Actin F1-R3	Improved results: 18S F1-R2
Empire CS	Woodford field	-	-
Q155 CS	Woodford field	-	-
Empire CS	Young Farm 1101, NSW	-	-
Empire H	Harwood mill Mother plot, NSW	-	+ve
BN88-3347 CS	Newrybar Swamp Road, NSW	-	+ve
Q241$^{\phi}$ CS	Strathalma farm, Tully 5573-13A	-	+ve
Q241$^{\phi}$ CS	Mac Farms, Tully 9726-52	-	-
Q208$^{\phi}$ CS	Mac Farms, Tully 9726-52	-	-
Q241$^{\phi}$ CS	Mac Farms, Tully 9726-52	-	-

6.4.8 Detection of *P. venanatans* in water

To assess whether we could detect *P. venanatans* in water, we sampled field and hydroponic water from Tully. The water samples were filtered and then a MoBio PowerWater DNA extraction kit was used to extract DNA. We had previously shown that the combination of filtering, DNA extraction and PCR could be used to detect *P. venanatans* in root water exudates. We soaked roots from CS-infected plants for various periods of time (see Section 6.3) and the organism could be detected after incubation periods of only two hours. However, environmental samples are more challenging due to the dilution factors involved. The volume that the kit is able to process is limited by how much particulate matter blocks the filters. Prefiltering can help, but unless large volumes can be tested, we suspected that field water may be below the CS detection level. The endogenous test (fungal SSU) worked in all cases so there was no issue with the quality of the DNA on the filters.

Water samples were collected on three occasions during the project. The initial attempts were made when detection was still based on the actin primers. As we became more familiar with the technique, we found we had to increase the sample volume being filtered and use some type of prefiltering, either glass fibre disks or filter paper, to remove particulate matter. Previous work has shown that this normally removes the majority of fungal matter. The flow-through was then trapped on a small pore (0.22µm or 1.2 µm) Millipore membrane and the DNA was extracted from the membranes. Initially no positive results were obtained (**Table 33**) but with use of the ribosomal SSU diagnostic primers, we were able to obtain a positive result for hydroponic water. Further optimization of both water and based detection *P. venanatans* is required.

Table 33: Diagnostic screening results for water samples collected during the project

	Source of water	Volume and filtering details	PCR test details and results
	Attempt 1: Collected March 2014		
1	Crude trough containing gravel and stationary water in a glasshouse(symptoms visible on some plants)	Initial attempt used 20 mL then repeated with 100 ml → 8 µm → 0.22 µm	PCR test: Actin Results: no sample tested positive
2	Crude trough containing gravel and stationary water outside the glasshouse		
3	Circulating hydroponics system with in a glasshouse		
4	Zamora farm, Tully district		
	Attempt 2: Collected August 2014		
1	Crude trough containing gravel and stationary water in a glasshouse	100 mL → glass fibre disks → 8 µm → 0.22 µm	PCR test: Actin Results: no sample tested positive
2	Crude trough containing gravel and stationary water outside the glasshouse		
3	Circulating hydroponics system with in a glasshouse		
4	Drainage canal water below block 52 (Q241[Φ] with symptoms) from MAC Farms (# 9726)		
	Attempt 3: Collected November 2014		
1	Trough #1 inside the glasshouse	150 mL → Whatman #2 filter paper → 1.2 µm	PCR test: CS18S Result: *sample 6 tested positive
2	Trough #1 inside the glasshouse		
3	Trough #1 inside the glasshouse		
4	Trough #2 outside the glasshouse		
5	Trough #3 outside the glasshouse		
6*	Trough #1 inside the glasshouse		

6.4.9 Discussion

A first-generation diagnostic test was developed by Braithwaite and Croft (2013) and used to screen different sugarcane tissues for the chlorotic streak pathogen. Once the full ribosomal sequence was obtained (Section 6.1), new diagnostic primers targeting the ribosomal small subunit rather than the actin gene were designed, offering increased sensitivity. The test has also been converted to qPCR format to determine the amount of pathogen in sugarcane tissues. In this study we found that end-point PCR gave comparable results to qPCR with only a slight reduction in sensitivity and is suitable for diagnostic screening if only presence/ absence of the pathogen is needed.

Xylem exudate, or stalk vascular extract, and stalk tissue were shown to be reliable sources of the pathogen, offering the potential for a diagnostic test for clean planting material for sugarcane industries. As planting material free of both RSD and CS is the goal of clean seed plots, it makes sense to test the same material for both diseases, saving time and money.

There has been interest from the industry in implementing a leaf sheath biopsy method (Young *et al.,* 2014) for RSD but at this stage is not clear that *P. venanatans* could be detected in the same way. CS could still be multiplexed with RSD, either in the sampling and testing phase (sap screened by PCR for both pathogens) or only in the sampling phase (RSD screened by ELISA and CS by PCR). Either way, there is a saving in time for sugar service staff.

Variation in the distribution of the pathogen along the stalks was shown to be high, even between single nodes. We have observed flagellated cells of *P. venanatans* in xylem exudates, suggesting that it moves within xylem vessels (Section 6.3), but we have not yet determined if the organism is confined to the xylem or also present in other cells within infected plants.

It is yet to be determined how the pathogen moves through the stalk but the uneven distribution revealed by PCR is supported by field observations that not all buds from diseased stalks produce diseased plants (Egan, 1989).

There is also potential to deliver a soil- and water-based assay for chlorotic streak. Soil assays are essential for successful management of poor soil health caused by root diseases. For example, SRA Tully has provided a commercial nematode and Pachymetra soil assay service for the Queensland sugar industry since 1997 (Magarey *et al*., 2004). DNA-based diagnostics for sugarcane pathogens are currently being developed at SARDI (http://www.sardi.sa.gov.au/diagnostic_services). The SARDI facility has the capacity to process up to 160 x 500g soil samples per day using an in-house DNA extraction method, robotic pipetting stations and high-throughput real-time PCR (Ophel-Keller *et al.,* 2008). Their best known packages are PreDicta B and PreDicta Pt. Farmers access the services via agronomists accredited by SARDI to interpret the results and provide advice on management options. This is achieved because DNA levels are related to disease thresholds, which in turn can provide disease risk assessments.

DNA tests for *Pachymetra* and nematodes are currently under development at SARDI and chlorotic steak is an ideal candidate to add to the suite of tests. A great deal of research was done several years ago to link *Pachymetra* soil spore numbers to root rot severity (Magarey, 1989) and a similar approach could be investigated for CS. A greater understanding of pathogen dynamics in the field could lead to recommendations, advice and wider options for growers. Soil and water pathogen levels could be used to calculate risk thresholds to provide growers with advice. Methods to inactivate the organism in recycled tail water would assist in the Burdekin district where it has been shown that irrigation water can transmit the disease and recycling tail water can add to the disease pressure (Magarey and Neilsen, 2002).

6.5. Disease epidemiology

Field monitoring of commercial crops has been expedited by the development of GPS technology; this enables disease incidence to be recorded on a stool-by-stool basis. GIS systems enable mapping of this information and provide an easily visualised understanding of the disease spatial pattern. Further information on the incidence and spread of the disease may provide a better understanding of factors that may be manipulated for improved CS management.

6.5.1 Disease levels

Zamora resistance trial. This field trial was planted in 2013 in the Riversdale area of the Tully district. A progressive increase in disease was evident over the crop monitoring period (2013 - 2015) (**Figure 38**). This corresponds with a number of significant flood events over the period, particularly in the 2014 calendar year. Replicate one is at the higher end of the cropping site, while replicate five is at the lower (higher flood-prone) area (**Figure 38**).

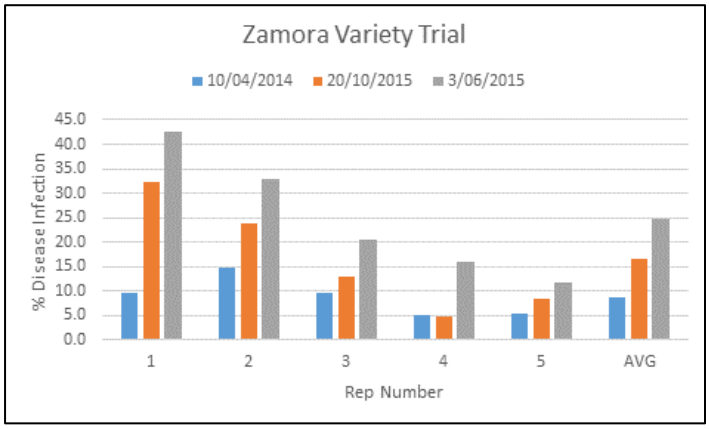


Figure 38: Disease levels present in each of the replicates (average over all varieties) in the variety trial at Ray Zamora's farm, Riversdale (data are % diseased stools')

Harney crop site. Disease levels were recorded for the Harney site, Syndicate in December 2014, May 2015 and December 2015 (**Figure 39**).

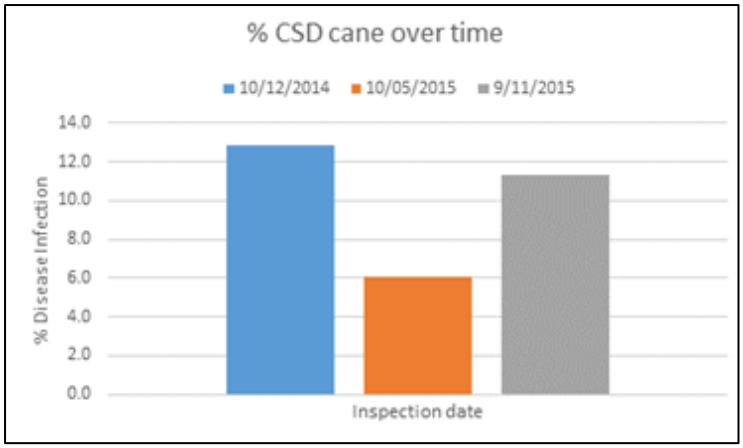


Figure 39: The percent diseased stools in Q241$^{\Phi}$ at the Harney field crop site, Syndicate, at three different inspection times (10 December 2014; 10 May 2015; 9 November 2015)

6.5.2 Crop site maps

The incidence of CS within the monitoring sites is illustrated in **Figure 40** and **Figure 41**. These show clearly the level of CS that developed at each site at the time of the inspections.

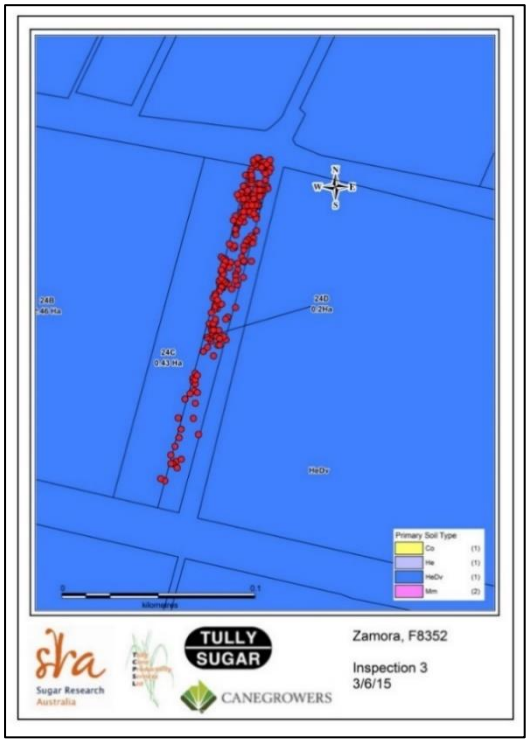


Figure 40: The spatial pattern of CS diseased stools at the Zamora variety trial site, Riversdale in June 2015. Note the difference in disease incidence between the higher (northern end) and lower parts of the site.

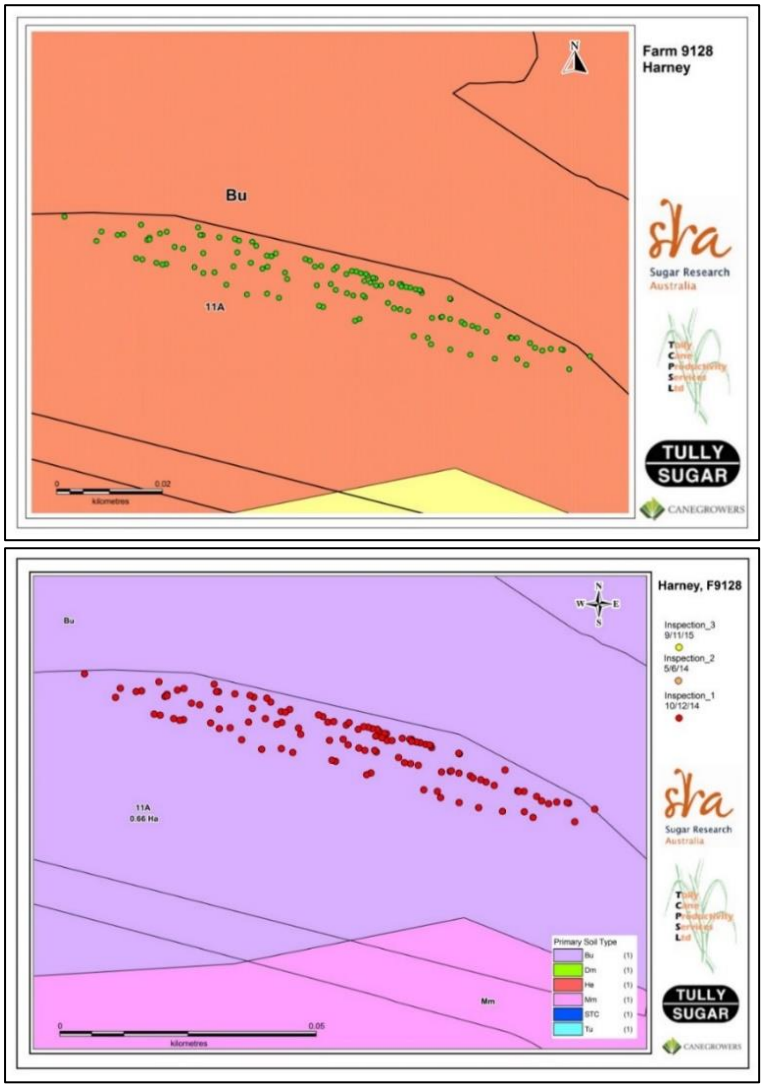


Figure 41: The CS spatial pattern at Harney in November 2015 (above) vs the spatial pattern at the same site in December 2014 (below). There was very little difference in extent and spread of the disease at each inspection (12 month period)

6.5.3 Discussion

The Zamora crop site showed a definite trend in disease incidence with varying height of the block; the lower ends of the site had a lower incidence of the disease that was independent of the total stool population (even though flood events had affected some stool survival). The reason for this variation in incidence could be related to the ecology of the causal agent; further causal agent research may lead to a better understanding of the reasons for the difference in spatial pattern. Differences in elevation at this site will have affected the length of time soils were waterlogged and this could have affected the causal agent.

Observations at the Harney cropping site showed little difference in disease levels over the 12-month period. This is not surprising since there were few flooding events during this time; rainfall was considerably lower, with wet season rainfall about half the normal average. The even disease distribution across the site probably parallels the lack of slope and drainage lines in this field; there was little influence of drainage on the disease at this site.

6.6. New management strategies based on an understanding of the causal agent

6.6.1 Fungicide pot trial

A glasshouse pot trial was established to test if three systemic fungicides could reduce the severity of chlorotic streak in the variety RP193-67. The number of shoots per pot was recorded after 14, 21 and 28 days (**Figure 42A**). The number of streaks per plant and plant height were recorded after 28 days (**Figure 42B** and **C**). The results shows that no fungicide treatment improved the growth of plants compared to the infected control plants. Only the height of the hot water treated plants was significantly different to all other treatments. The number of streaks on the leaves was not significantly different in the fungicide treated plants compared to the control infected plants. The hot water treated plants had no leaf streaks. None of the biocides/ fungicides appear to significantly reduce the leaf symptoms or stunting caused by CS.

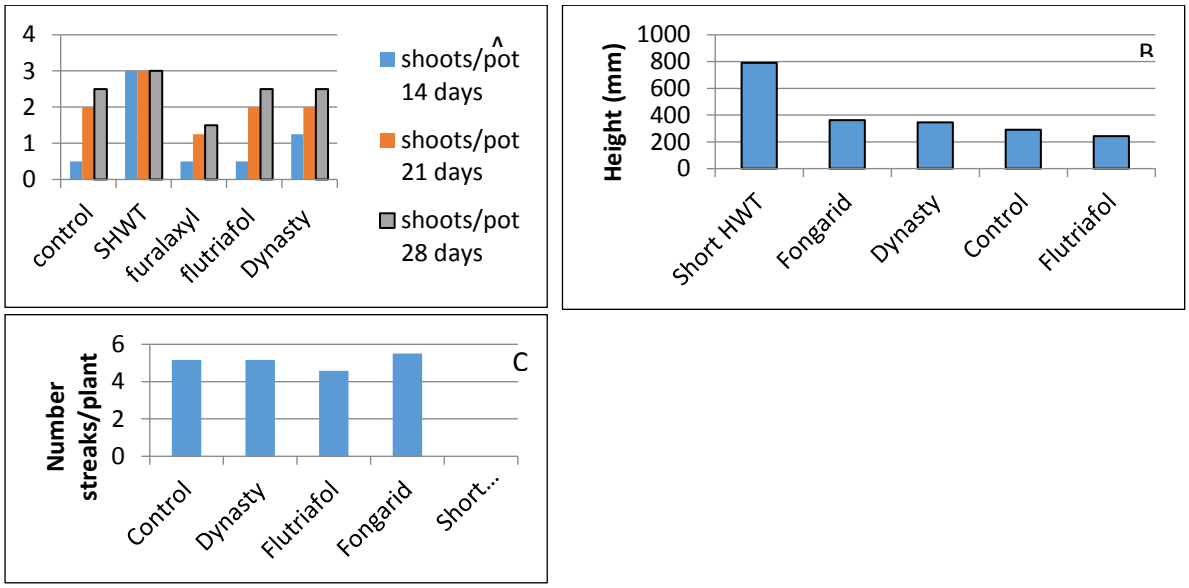


Figure 42: Effect of fungicides on growth of CS-infected plants. A shows the number of shoots per pot; B shows the average plant height after 3 months growth, and C shows the average number of streaks per plant.
The hot water treated plants had no symptoms of chlorotic streak

6.6.2 Metronidazole pot trials

Two glasshouse pot trials were established to test if metronidazole, an antibiotic commonly used to treat protozoan infections in humans, could reduce the severity of chlorotic streak in the variety RP193-67. The initial trial used concentrations of 20 to 160ppm and the number of germinating shoots per pot was recorded between one to two weeks (**Figure 43**). There was no significant effect in the number of shoots produced by the germinating setts from any treatment. Surprisingly, there was also no difference between the number of shoots germinating from infected and healthy shoots. While this experiment did not show any beneficial effects of metronidazole in controlling chlorotic streak, it at least showed that it was not phytotoxic to sugarcane.

The experiment was repeated with a smaller number of treatments but with higher concentrations of metronidazole and over a longer period of time. In this trial metronidazole was able to improve the germination of diseased setts (**Figure 44**).

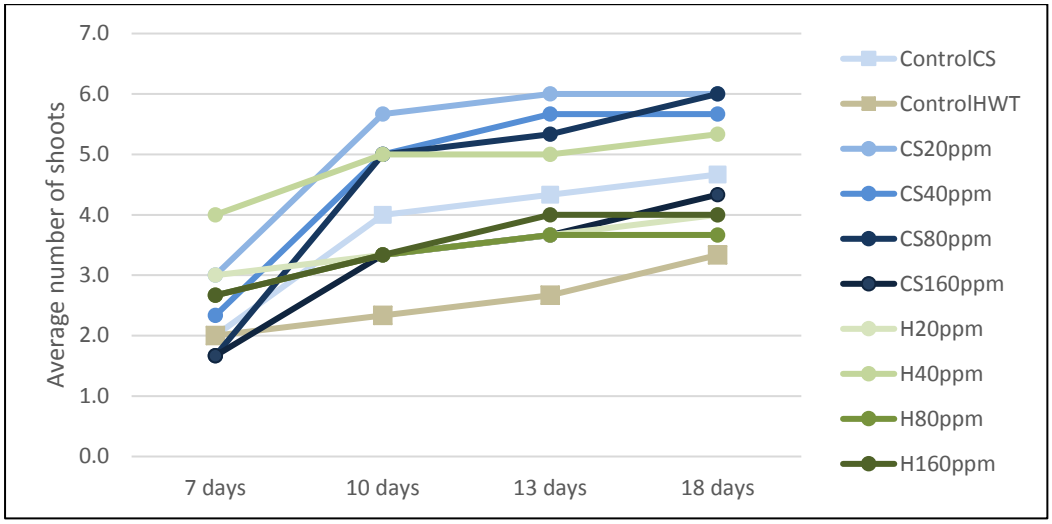


Figure 43: Effect of metronidazole on the germination of both healthy and CS-infected sugarcane setts. Data points represent the average ± SE of three replicate pots

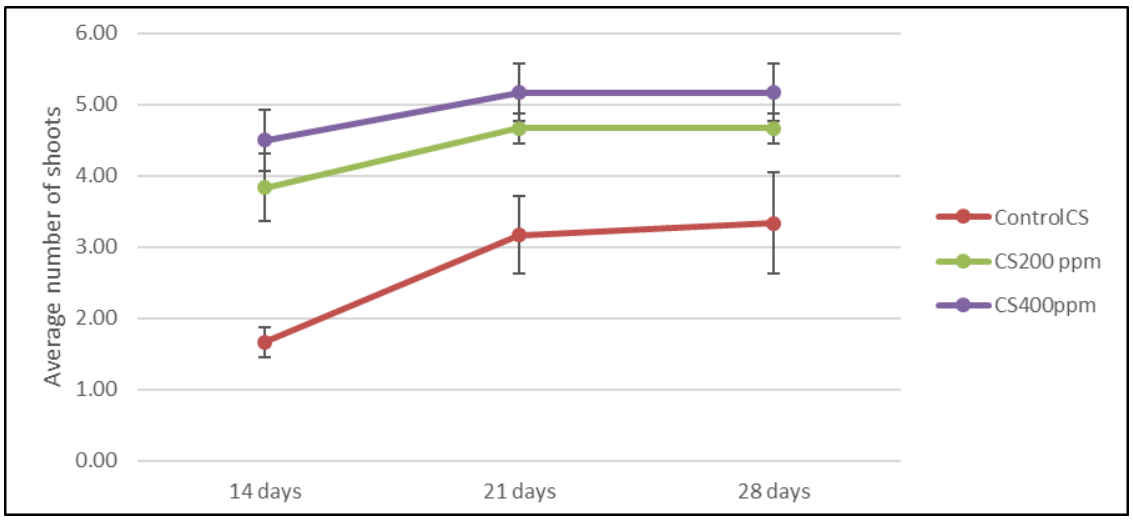


Figure 44: Effect of metronidazole on the germination of CS-infected sugarcane setts. Data points represent the average ± SE of six replicate pots

6.6.3 The effect of metronidazole on the growth of P. *venanatans*

Cultures of *P. venanatans* initiated from CS-infected Q238$^{\phi}$ were established in potato dextrose +peptone (PDP) broth and subcultured into 10 mL PDP broths containing metronidazole at various concentrations. Three trials were necessary to establish the optimal concentration of metronidazole to inhibit the growth of *P. venanatans.* In all cases *P. venanatans* was exposed to metronidazole for three days. Trial 1 (**Figure 45A**) started with the concentrations found to be suitable in the glasshouse based pot trial (see **Figure 44**), and flasks were seeded with 116,000 cells at day 0. All concentrations of metronidazole were very inhibitory, while the *P. venanatans* cells in the treatment with no added metronidazole multiplied 37 times in three days. Trial 2 (**Figure 45B**) used lower concentrations of metronidazole and flasks were seeded with 130,000 cells at day 0. Again, all treatments of metronidazole were very inhibitory, while the control treatment multiplied 23 times in three days. In the third trial, counts were taken every day to track how quickly the inhibitory effects of metronidazole took to inhibit growth of *P. venanatans.* **Figure 45C** shows that the effect is very rapid. The flasks were seeded with 137,300 cells, with the control multiplying 13 times over three days.

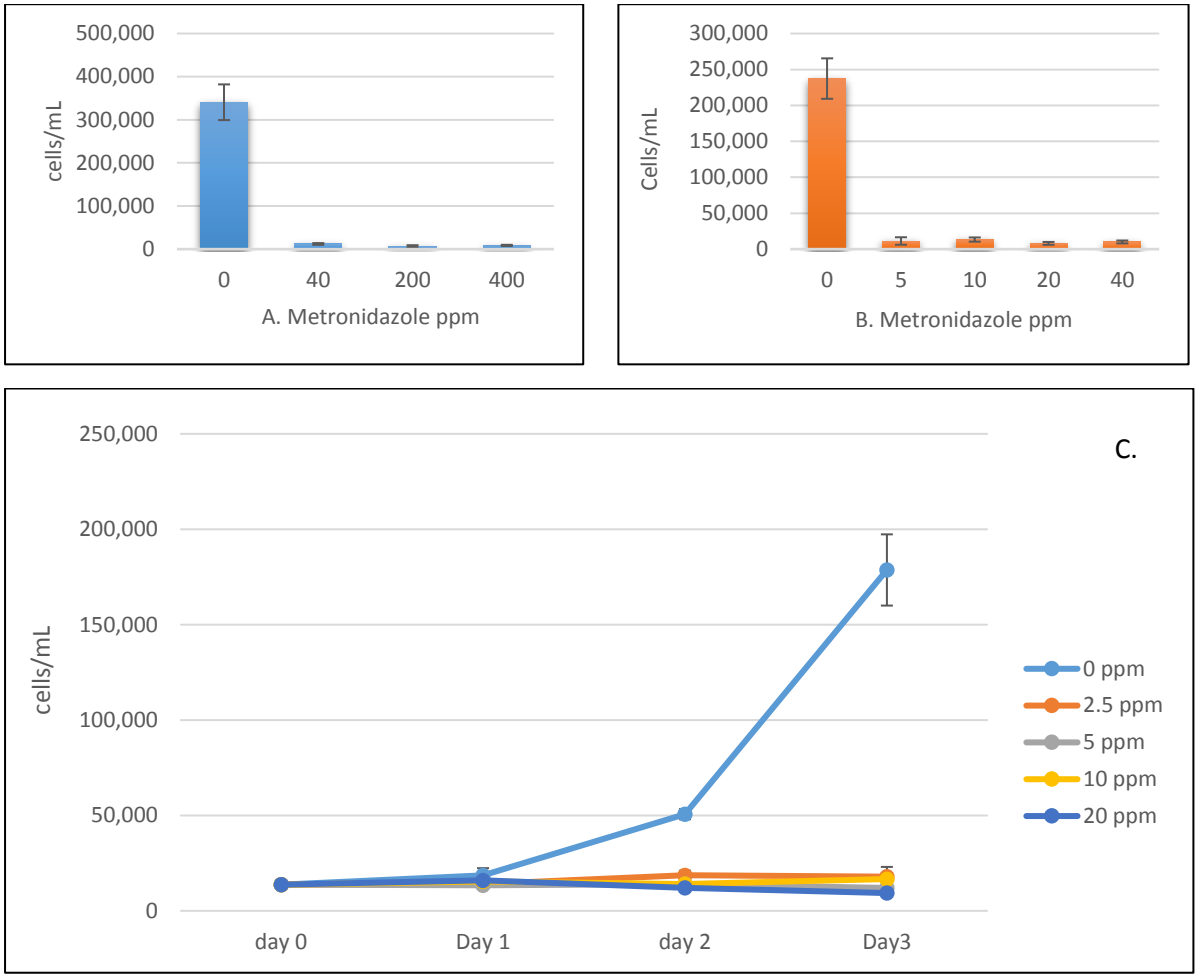


Figure 45: Effect of metronidazole on the germination of *P. venanatans* in culture. A, B and C present the result for three trials, each grown for three days. Bars and data points represent the average ± SE of three replicate flasks

6.6.4 Discussion

For chlorotic streak, the current management advice to growers is to use disease free planting material and improve drainage of low-lying cane fields. These recommendations have been in place for many years without knowing the causal agent.

It has been known since the 1930s that a simple hot water treatment could eliminate CS (Bell, 1933). A hot water treatment of 50C for 30 minutes is now a standard industry practice to eliminate the disease from planting material. Seed cane should be planted on the least flood prone and best drained section of the farm to reduce infection. Highly susceptible varieties should not be planted in fields prone to the disease. The use of resistant varieties is the preferable method of control and once we developed methods for inoculating plants with the cultured pathogen we realized that we could deliver better project outputs by concentrating on developing a rapid resistance screening method (see Section 6.9).

Although hot water treatment, improved field drainage and resistant varieties are likely to be the mainstays of CS control, a better understanding of the biology of the causal agent may lead to new innovative methods of control based on approaches used for other closely related organisms. A good starting point are the well-known plant pathogenic Cercozoans, such as *Plasmodiophora brassicae* (clubroot in brassicas), that belong to the Plasmodiophorids (class Phytomyxea). While *P. venanatans* belongs to a separate subphylum, it still shares many functional features as a result of its soil- and water-borne nature.

Many different strategies for disease management of *P. brassicae* have been researched and applied over the years. These have been reviewed by Porth *et al.* (2011). Resistant varieties are the most effective means of control, although resistance can be unstable. Raising soil pH to 7.1 - 7.2 with lime to create unfavourable conditions is well documented. High pH has been suggested to disrupt zoospore release, although it is unclear as to the mechanism of action and disease can still occur in alkaline soils. Crop rotation is another frequently recommended cultural control method, however, infested fields need to be rotated out of Brassica-family crops for a minimum of 5 - 7 years. This strategy is of limited benefit because the pathogen can infect weed hosts and resting spores can remain dormant in a field for up to 20 years. Improving drainage to reduce soil moisture can significantly reduce disease pressure, as *P. brassicae* thrives in saturated soil conditions. Disinfesting equipment and farm hygiene are also key measures to reduce spread of the disease. Recent control methods such as non-ionic surfactants, calcium cyanamide fertiliser and bait crops have also been trialled with some success.

Fungicides have also been used, with many giving inconsistent results or others that need to be used at high rates making them prohibitively expensive. No single fungicide has emerged as an effective and practical management tool. Pentachloronitrobenzene (PCNB) and Fluazinam are currently the recommended fungicides for control of clubroot and are most effective when applied to the root zone (Porth *et al*., 2011; Donald *et al*., 2008). PCNB has shown inconsistent results and may cause phytotoxicity in high concentrations, but can provide effective control in combination with liming. The application of boron has also been used to manage clubroot for more than 60 years (Donald *et al*., 2008; Porth *et al*., 2011). Fumigation is useful for controlling small outbreaks, but expensive for large areas (Donald *et al*., 2008).

The effectiveness of fungicides for controlling chlorotic streak was determined in the 1960s through hydroponic experiments (reviewed by Egan, 1989). The fungicides TMTD (thiram) and PCNB, and the sterilant benzalkonium chloride inhibited transmission, whereas terramycin increased the transmission rate. Other antibiotics and fungicides had no effect.

In addition to the chemicals used to control plant pathogenic Cercozoans, there are also novel control agents for human protozoan parasites. For example, the therapeutic drugs metronidazole, chloroquine and quinacrine control human protozoan infections including *Giardia* sp., *Plasmodium* sp. and *Trichomonas* sp. Metronidazole also controls some anaerobic bacterial pathogens including *Clostridium difficile* and *Helicobacter pylori*, (Jjemba, 2002; Samuelson, 1999). The results presented here show that *P. venanatans* is very sensitive to metronidazole when applied to the organism growing in liquid broth cultures, but less so when applied to plants in pots. Thus designing a method of delivery for plants in the field could be challenging. An alternative is to use the chemicals in more strategic ways. For example smut control of seed cane can be achieved by adding fungicides to the hot water treatment (Comstock, 2000; Bhuiyan *et al.* 2014). A method to inactivate *P. venanatans* in recycled tail water would assist in the Burdekin district where it has been shown that irrigation water can transmit the disease and recycling tail water can add to the disease pressure (Magarey and Neilsen, 2002). Now that techniques are available to isolate and quantify the organism experimentally, we can design further glasshouse experiments to test a wider range of control chemicals.

6.7. NSW motherplot- and seedplot-based resistance screening methods

CS had a significantly deleterious impact on NSW productivity during recent *La Niña*-defined years (as defined by the Bureau of Meteorology, 2012). Following an unprecedented three consecutive January floods (2011–2013), there have been perfect conditions for the disease. Following the 2011 and 2012 floods, Harwood field staff implemented a program to monitor CS to better gauge its

impact and help improve its management. The most urgent objective was to assess the susceptibility of the varieties available for propagation. Field-based screening methods in NSW had previously been attempted by Young *et al.* (2013).

To gauge the susceptibilities of NSW varieties under relatively uniform conditions, inspections were made in a 2nd ratoon field that had previously served as a mother plot. CS ratings were generated for 24 varieties (**Table 34**). New varieties had an average rating of 5.5, while older varieties had an average rating of 6.6. Q203 was the most susceptible variety in the ex-MP and during seedbed inspections.

Of these 24 varieties, field incidence ratings were generated for 20 that were represented by 10 or more seedbeds. The results of the field observations were broadly consistent with ratings generated from the ex-MP. However, some varieties, particularly Q183$^{\phi}$ and Q244$^{\phi}$, exhibited high symptoms in the ex-MP but low symptoms in the field.

Table 34: CS ratings based on incidence in previously inundated ASPs and MPs. Ratings for 24 varieties were generated by counting the number of leaves with streak symptoms in 20 m rows of a 2nd ratoon mother plot (MP). Field ratings were generated using average CS incidence data for varieties with 10 or more seedbeds inspected

Variety	Old (O) or New (N)	CS symp. /20 m	Log10 (symp.)	2nd ratoon MP rating	Seedbeds inspected	Field rating
Q203[Φ]	O	72	1.86	9	65	9
Q183[Φ]	N	62	1.79	9	21	2
Q244[Φ]	N	49	1.69	8	17	5
75C-326	O	49	1.69	8	16	7
Empire	O	48	1.68	8	99	8
SP79-2313	O	32	1.51	7	22	6
Q200[Φ]	O	29	1.46	7	5	N/A
Q234[Φ]	N	25	1.40	7	57	8
BN83-3120	O	22	1.34	7	195	6
KQ228[Φ]	N	22	1.34	7	4	N/A
Rogan	O	18	1.26	6	14	5
Q255	N	17	1.23	6	1	N/A
Arris	O	16	1.20	6	69	6
Q210[Φ]	O	15	1.18	6	14	5
Q235[Φ]	N	14	1.15	6	16	8
Q240[Φ]	N	13	1.11	5	43	3
Q242[Φ]	N	13	1.11	5	20	3
Q213[Φ]	O	13	1.11	5	1	N/A
Q193[Φ]	O	11	1.04	5	12	5
Q155	O	8	0.90	4	17	5
Q232[Φ]	N	6	0.78	4	184	4
Q243[Φ]	N	5	0.70	3	25	7
BN81-1394	O	3	0.48	2	5	N/A
Q208[Φ]	N	1	0.00	1	153	3

6.7.1 CS prevalence between 'old' and 'new' varieties

Of varieties with 10 or more seedbeds inspected, 535 seedbeds could be classified as 'old' varieties, while 536 could be classified as 'new'. Varieties that were classified as old were 75C-326, Arris, BN83-3120, Empire, Q155, Q167, Q193, Q203, Q210, Q212 (Rogan) and SP79-2313. Varieties that were classified as new were Q183, Q208, Q232, Q234, Q235, Q240, Q242, Q243 and Q244. Newer varieties had greater crop uniformity ($p<0.05$) and less streak ($p<0.05$). Average CS ratings were 5.5 and 4.9 for newer varieties compared with 6.6 and 6.2 for older varieties for ex-MP and field ratings

respectively. There was a relatively high correlation (0.90) between both ratings systems for older varieties (those in propagation for ten years or more), versus a low correlation (0.09) for newer varieties. Based on field incidence and ex-MP assessments, there was a higher amount of CS and crop unevenness in older varieties than new.

6.7.2 Seedbed inspections

A total of 1,106 fields were assessed, comprising 32 varieties. The number of seedbeds inspected for 2012 - 2014 were 245, 688 and 173 respectively. Inspections occurred between July and September. There was a statistically significant difference in CS incidence and crop unevenness in relation to the year seedbeds were inspected. The decline in CS incidence across the 3 years may have been associated with decreasing rainfall recorded at the Harwood Mill weather station in the preceding 12 months (June to May) (**Figure 46**).

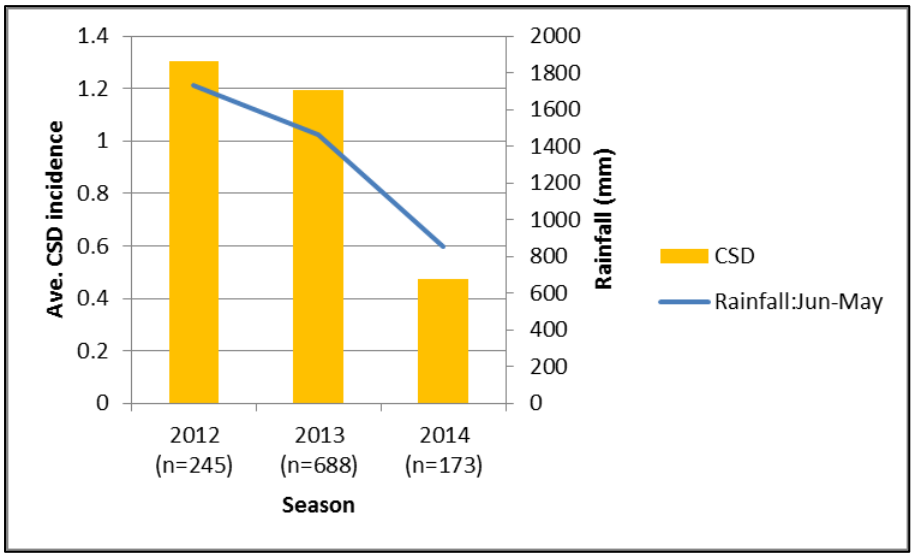


Figure 46: The relationship between the average incidence of CS in infected seedbeds and rainfall in the preceding 12 months (previous June until May)

CS prevalence and crop uniformity were both related to variety ($p<0.05$) for the 12 varieties with 20 or more seedbeds inspected. CS prevalence was not related to crop class (**Figure 47**). However, crop unevenness was related to crop class, with the least uniform being ratoon from replant (**Figure 47**). A comparison among crop classes of crop uniformity and 2014 yields indicated that crop uniformity may be a useful proxy for crop health (**Figure 48**).

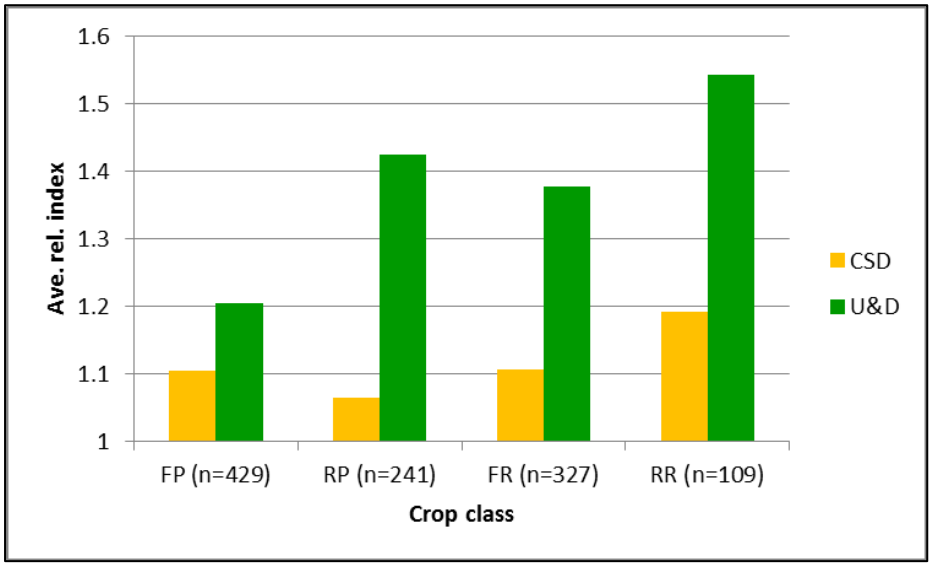


Figure 47: CS incidence and crop unevenness for different seedbed classes: FP=fallow plant, RP=replant, FR=ratoon from fallow plant and RR=ratoon from replant

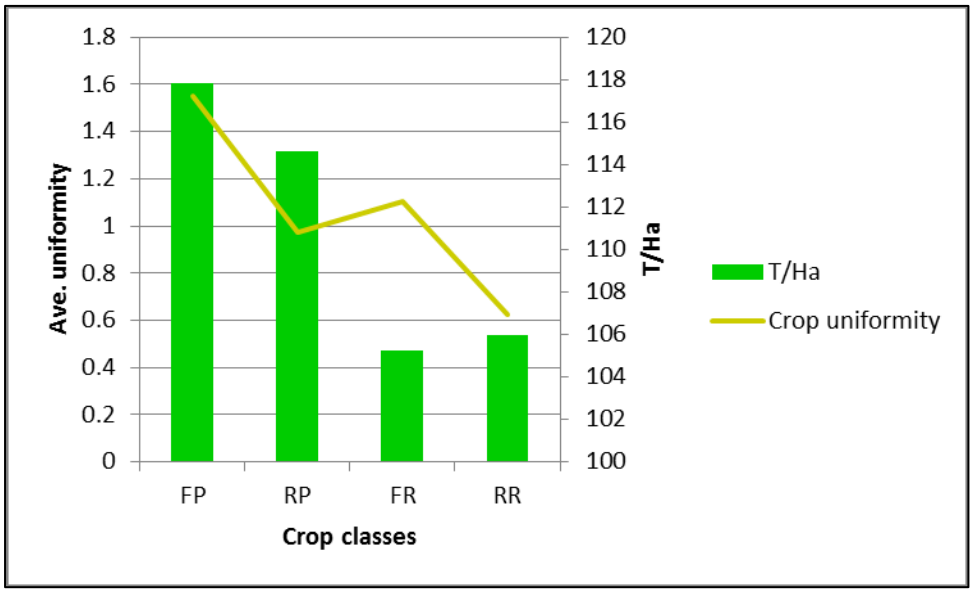


Figure 48: 2014 season crop performance and 2012 - 2014 crop uniformity (calculated by U=3-(U&D)2, where U is crop uniformity and U&D is the 'up-and-down' index of crop unevenness

6.7.3 Effect of row configuration on CS and crop uniformity

There was a statistically significant ($p<0.05$) lower amount of CS in dual row configurations relative to conventional row spacings; however, there was no difference in crop uniformity (**Figure 49**). Dual row growers were 15 % more likely to test newer varieties than conventional growers (**Figure 50**), but the reduced amount of streak under dual row configurations appears to be independent of variety as there was a reduction in the amount of streak present in both older and newer varieties in dual row crops (**Figure 51**).

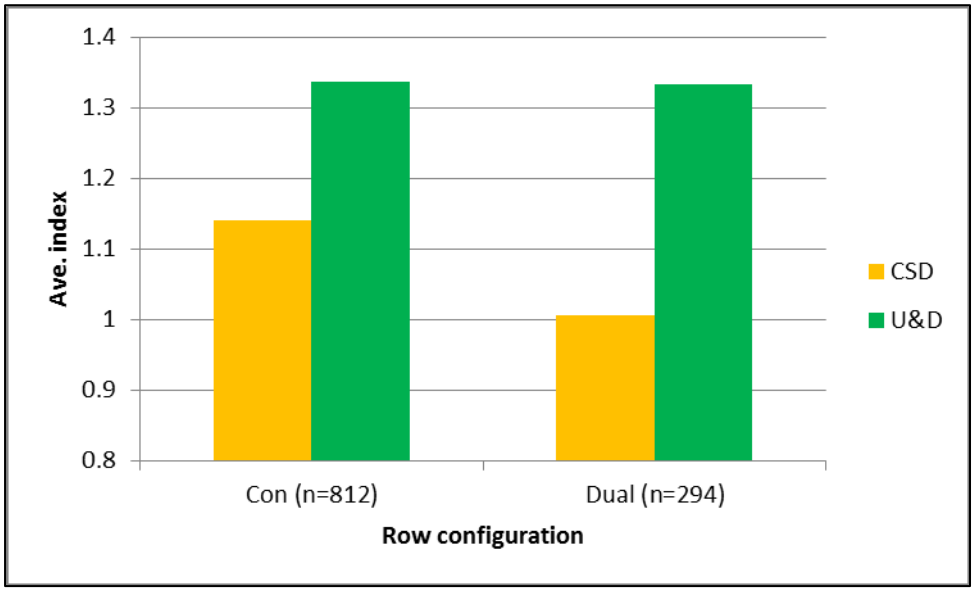


Figure 49: Average incidence of CS and crop unevenness in seedbeds under either conventional or dual row configuration

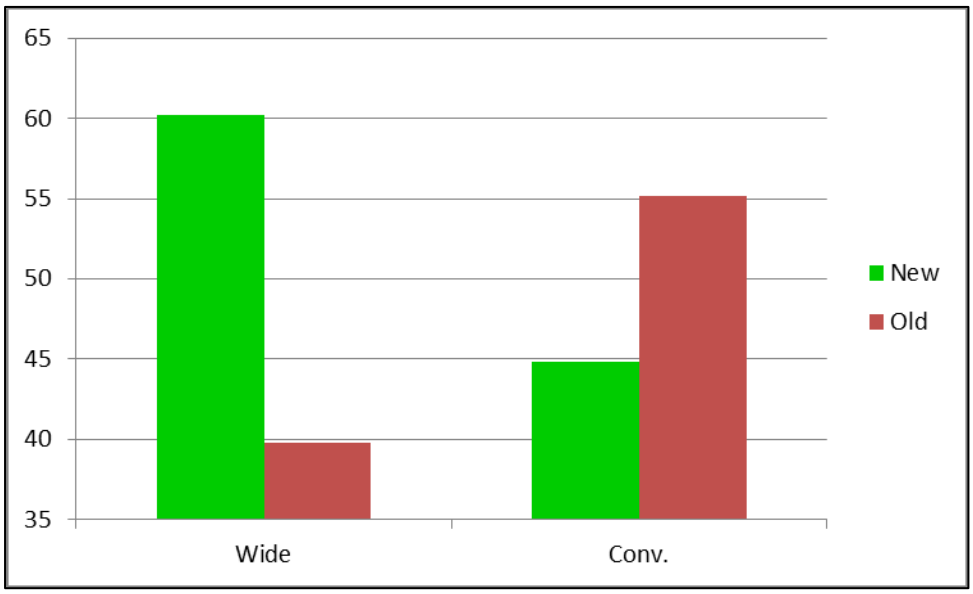


Figure 50: Percent of either new or old varieties tested for growers under either dual-row or conventional propagation

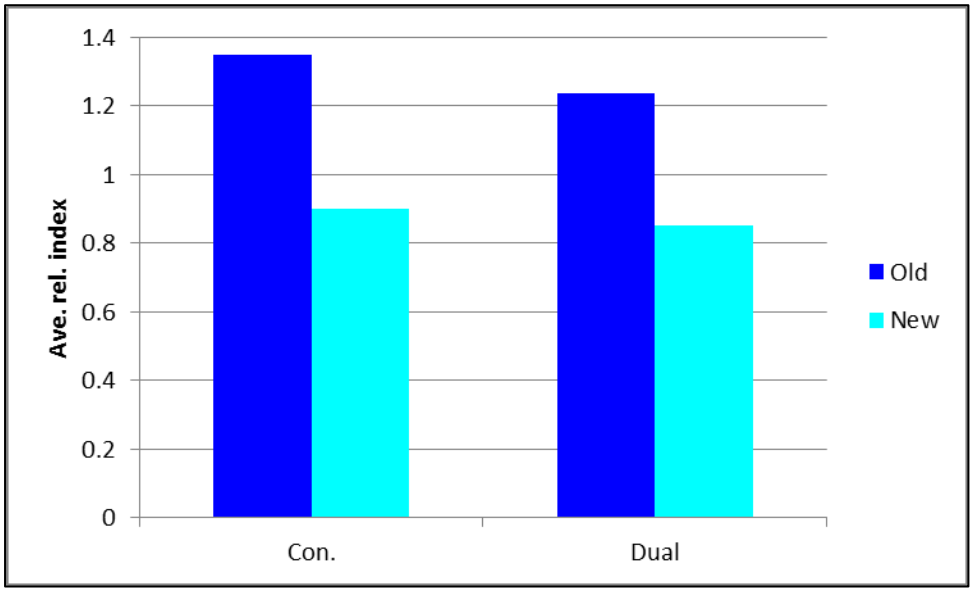


Figure 51: Relative incidence of CS for new and old varieties under conventional or dual row configuration

6.7.4 Discussion

The CS susceptibility ratings generated in this study (**Table 34**) have helped NSW cane farmers select the right variety for low-lying areas subject to inundation. The first method, whereby the number of symptomatic leaves was counted over a 20 m transect, provided a uniform series of ratings for 24 varieties because they were all in a single field which had been inundated at least four times and consequently all varieties had a presumed equal opportunity for infection. Furthermore, as the crop was 2nd ratoon ex-mother plot, all of the canes could be assumed to have been free of the disease prior to planting in September 2011 as they had undergone hot water treatment (Bell, 1933).

The second method proved valuable in determining how canes performed in the field and facilitated investigations into the role of crop class, weather and different farming practices in relation to CS.

While there may be a bias towards minimising the incidence of CS as only prospective seedbeds were targeted, with over 1,100 fields surveyed, and particularly for varieties represented by a large number of fields sampled, the relative incidences are indicative of CS performance in the field. This approach facilitated analysis of CS incidence over three years, revealing a statistically significant lower amount of CS during the unusually dry 2014 season.

On average, newer varieties had marginally lower ratings than older varieties using both methods. In some cases, like Q183 and Q244, the field incidence was far less than what may have been expected based on the ex-MP ratings. However, it is possible that, being released within the past 5 years, there has been less time for the disease to build up in vegetative planting stocks. Likewise, being new varieties, growers may have taken extra care with them, situating seedbeds on higher, better-drained soils, less prone to inundation. In the case of Q243$^{\phi}$, its relative resistance in the ex-MP was overshadowed by a poor performance in the field, contributing another factor to its abandonment. While the reason for the poor confluence of ratings systems for newer varieties relative to older varieties is unclear, it may be suggested that, being 'closer' to hot water treatment which destroys the infection (Bell, 1933), there has been a relatively lower amount of vegetative transmission in newer varieties in the field.

There was no significant relationship between crop class and CS incidence. This is not surprising given the major mode of transmission is free water, and all potential classes of planting material were equally subject to flooding events. However, there was a significant relationship between crop

class and the degree of unevenness of the crop, with fallow planted seedbeds exhibiting the highest crop uniformity and ratoon seedbeds from replant showing the highest crop unevenness. This indicates that non-CS factors have a high influence on crop uniformity. When 2014 yield data were plotted against the observed uniformity of the different crop classes, it appeared that uniform crops tend to out-yield uneven crops.

There was a statistically significant higher incidence of CS in conventional (1.5 m - 1.7 m single rows with or without controlled traffic) versus dual rows (1.8 m + with controlled traffic). Given the lower observed incidence in newer versus older varieties, it was considered possible that the lower observed streak may be due to more rapid variety adoption for dual row growers. It was found that, over the three-year period, dual row growers were 15 % more likely to test newer varieties than conventional growers. However, when CS incidence in newer and older varieties was compared for both dual row and conventional farming systems, it was clear that there was less CS in dual row configurations, regardless of the categorisation of older or newer varieties.

An intuitive explanation for the reduced CS in dual row configurations is improved drainage associated with defined compaction zones. Following inundation, this would tend to reduce the time required for water to get off the field, and so reduce, in relative terms, the opportunity for infection. While Egan (1966) demonstrated that relatively short exposure periods of an hour can transmit CS, it may be expected that any shortening of the exposure time would result in reduced incidence. Likewise, many dual row growers employ raised beds, which may also reduce transmission associated with inundation. Another associated factor may be that dual row growers are more likely to purchase ASP cane than conventional growers, meaning that planting stocks would have relatively reduced vegetative transmission of the disease. Similarly, as it may be expected that dual row growers proportionally represent more progressive growers, general drainage features may be in superior order than under many conventional systems.

This study facilitated relatively passive accumulation of CS epidemiological information in conjunction with annual RSD seedbed surveys. It demonstrated a clear reduction in CS incidence during the dry 2014 relative to preceding years, and reduced incidence under dual row configurations. This method also facilitated generation of field-based variety susceptibility ratings, which is particularly valuable for regions with a diverse mixture of varieties. It is likely that future management strategies will revolve around resistant varieties and deployment of resistant parents in breeding programs. This method of field rating may assist field staff in making variety recommendations and help growers select the best possible planting material to improve productivity.

Probably the most significant finding was the disparity between the MP and field ratings for varieties, which was seemingly based on whether they were ‘old’ (R=0.90) or ‘new’ (R=0.09) varieties. While there is limited statistical rigour with such a small sample size, it suggests that there is a much greater amount of vertical transmission of the agent than perhaps previously thought.

6.8. Tully field-based resistance screening methods

A field resistance trial was conducted on the Riversdale farm in the Tully area that incorporated the latest smut-resistant commercial varieties.

6.8.1 Disease levels in Zamora trial

Significant CS infection was noted in plots of the variety resistance trial at Zamora’s farm. More disease was particularly noted in the varieties rated intermediate and susceptible to CS. ‘*Percent diseased stalk*’ populations were far lower than the *‘Percent diseased stools*’ (**Figure 52**), as one stalk

showing symptoms in a stool was sufficient for the whole stool to be classified as diseased (June 2015 data). The mean data across all varieties and all plots in the first ratoon crop (maximum disease incidence) were 4.7 *% diseased stalks* and 27.8 *% diseased stools*.

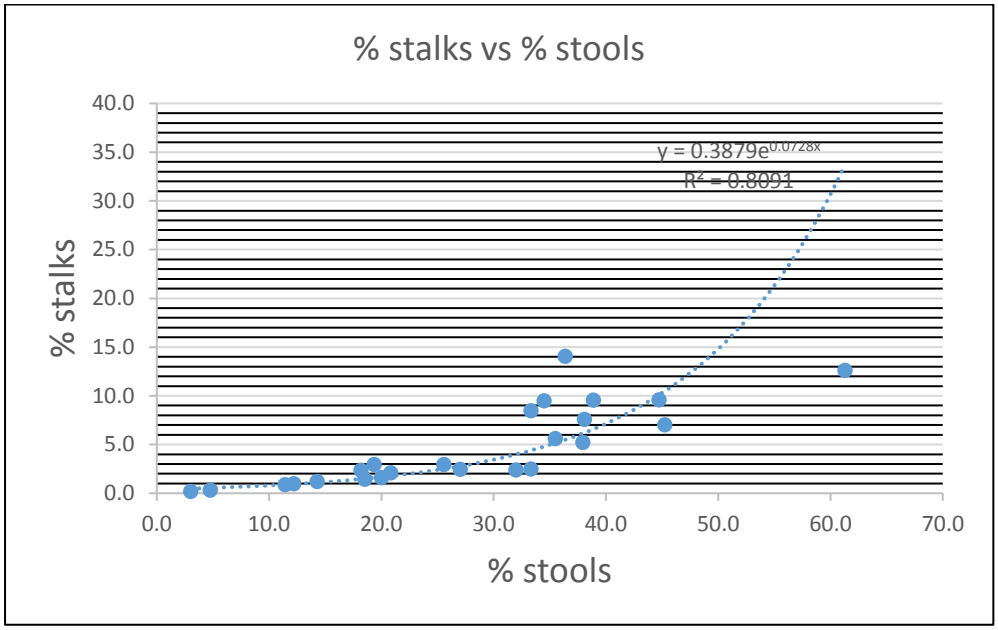


Figure 52: The relationship between '% diseased stalks' and '% diseased stools' in first ratoon data (recorded in June 2015) from the Zamora site

Data for each individual variety are listed in **Table 35**.

Table 35: CS-infected stalk and stool information for all varieties planted on the farm of Ray Zamora, Riversdale

	% stalks			% stools	
Clone	**April 2014**	**June 2015**	**Clone**	**April 2014**	**June 2015**
CP57	9.4	9.5	CP57	25.6	34.5
KQ228$^{\phi}$	2.2	1.2	KQ228$^{\phi}$	10.4	14.3
Pindar	2.8	7.6	Pindar	7.1	38.1
Q124	1.3	0.3	Q124	6.5	4.8
Q127	3.4	2.3	Q127	15.1	18.2
Q138	2.9	9.6	Q138	11.3	44.7
Q159	4.7	2.1	Q159	9.3	20.8
Q183$^{\phi}$	9.2	7.0	Q183$^{\phi}$	28.3	45.2
Q200$^{\phi}$	0.2	3.0	Q200$^{\phi}$	1.8	25.6
Q208$^{\phi}$	2.7	0.2	Q208$^{\phi}$	7.7	3.0
Q219$^{\phi}$	1.8	8.5	Q219$^{\phi}$	6.7	33.3
Q231$^{\phi}$	2.4	2.5	Q231$^{\phi}$	14.9	27.0
Q232$^{\phi}$	1.9	0.9	Q232$^{\phi}$	7.0	11.4
Q237$^{\phi}$	1.3	2.9	Q237$^{\phi}$	3.2	19.4
Q238$^{\phi}$	1.7	14.0	Q238$^{\phi}$	6.8	36.4
Q240$^{\phi}$	1.7	1.6	Q240$^{\phi}$	10.0	20.0
Q241$^{\phi}$	5.9	12.6	Q241$^{\phi}$	23.3	61.3
Q250$^{\phi}$	2.3	5.6	Q250$^{\phi}$	13.6	35.5
Q251$^{\phi}$	0.5	9.5	Q251$^{\phi}$	2.1	38.9
Q96	2.5	1.4	Q96	10.2	18.5
QN02-893	1.8	2.4	QN02-893	7.0	32.0
QN04-121	0.9	2.5	QN04-121	4.3	33.3
QN04-218	1.3	1.0	QN04-218	7.1	12.2
QN04-914	0.0	5.2	QN04-914	0.0	37.9
Mean	2.7	4.7	Mean	10.0	27.8

Very preliminary ratings (data from more than one experiment is needed to confirm the resistance of varieties) for each variety, using data from June 2015, are provided in **Table 36**.

Table 36: CS resistance ratings for all varieties planted on the farm of Ray Zamora, Riversdale

Clone	% stools	Rating
Q241[Φ]	61.3	S
Q183[Φ]	45.2	S
Q138[X]	44.7	I-S
Q251[Φ]	38.9	I-S
Pindar	38.1	I-S
QN04-914	37.9	I
Q238[Φ]	36.4	I
Q250[Φ]	35.5	I
CP57	34.5	I
Q219[Φ]	33.3	I
QN04-121	33.3	I
QN02-893	32.0	I
Q231[Φ]	27.0	I
Q200[Φ]	25.6	I
Q159	20.8	I
Q240[Φ]	20.0	R
Q237[Φ]	19.4	R
Q96	18.5	R
Q127	18.2	R
KQ228[Φ]	14.3	R
QN04-218	12.2	R
Q232[Φ]	11.4	R
Q124	4.8	R
Q208[Φ]	3.0	R

[X] Standard varieties are in bold text

6.8.2 Discussion

Resistance screening research has been conducted in Queensland for a number of years but there has been no consistent screening over time, unlike with the other major diseases. The first report of resistance screening was made in the mid-1930s, with at least three trials planted in the Babinda area (Bell, 1935, 1938, 1939, 1940; Magarey *et al.,* 2006). Test varieties were planted at a flood-prone site with CS transmission occurring via floodwaters. Early varieties screened included Q2, Q4, Q12, Q16, Badila and other current commercial varieties of the day. Industry notes suggest that further resistance trials were planted in the area but no record of trial details or results can be found.

More recently, Magarey *et al.* (2006) established field resistance screening trials in both the Tully and Burdekin regions, examining the resistance of the current commercial varieties and the reliability of the technique. Test varieties were planted between infection rows (every third row) and the most useful CS assessments occurred in the young first ratoon crops. Higher disease levels, and more reliable data, were obtained in the Tully (vs. Burdekin) trials – no doubt a result of the higher rainfall environment in Tully and the wetter soil conditions.

During this project it was found that the Zamora trial site provided excellent conditions for CS infection with the very significant flooding experienced during the 2013 - 2015 period. With the disease transmitted in floodwater, disease levels peaked as high as 60 % diseased stools (Q241$^{\phi}$) in the mature first ratoon crop – providing good resistance discrimination between varieties. Some current commercial varieties appeared to be resistant, including Q208$^{\phi}$, Q232$^{\phi}$ and KQ228$^{\phi}$. Known susceptible varieties, such as Q238$^{\phi}$ and Pindar exhibited disease levels in the higher range; further data are needed to confirm the ratings for some test canes. Disease incidence on a stalk basis was much lower, since a single stalk may give rise to an infected stool. The relationship between 'percent infected stalks' and 'percent infected stools' illustrates that with just a slight increase in 'percent infected stalks', there is a rapid increase in 'percent infected stools'. At lower CS disease levels, recording data on a stools basis is therefore more sensitive to increases in disease levels associated with susceptible varieties.

The mean 'percent stalk population' over all varieties was around 4 %; in previous trials conducted on an SRA Tully site (Magarey *et al.*, 2006), there were much higher 'percent infected stalk' populations. The presence of diseased infection rows (CS-infected planting material planted every third row in the trial) (Magarey *et al.*, 2006) may have increased the level of inoculum in these previous trials. Even so, good discrimination between varieties of differing resistance was obtained in the experiment reported here. With adequate flooding, infection rows are not needed.

It would appear that *'percent diseased stools*' is the most suitable parameter to record in resistance trials. The appearance of CS within a stool will establish a point of infection for the crop in future years – both in the infection of other stalks within the stool, and infection of other stools within the crop. Varieties generally considered to be CS-susceptible also exhibit high levels of stool infection. In infected fields of highly susceptible varieties, CS infection is easily noticed as whole stools tend to exhibit reduced growth and obvious CS disease symptoms. This is not the case in varieties possessing slightly higher levels of resistance. As such, the exponential-type relationship between 'percent diseased stalks' and 'percent diseased stools' makes biological sense. In field inspections undertaken in a range of field trials over a 15 - 20 year period (Magarey *et al*., 2006), only in the most highly susceptible varieties do most shoots in a stool exhibit CS symptoms. Often in these same stools growth is noticeably reduced and yield losses severe. When the biology of the causal agent is better understood, and the processes of infection and colonisation of a stool described in better detail, the reason for the high stalk incidence and yield losses in susceptible varieties will be better explained.

While leading to relatively high disease levels, the flooding negatively affected cane growth in the lower parts of the experiment. There was a significant loss of stool in some varieties, while in others there was a high level of side-shooting on stalks. Generally, growth in the plant crop was worse than the first ratoon due to the timing and extent of flooding in 2013 - 2014. Selecting trial sites is a balance between optimum flooding events vs optimum growth conditions – and the two are often contrary to each other. This is where the development of a reliable glasshouse resistance screen (Magarey *et al.,* 2016) would be a huge advantage, not only in a reduction in the time-frame for applying resistance ratings, but also in reducing the pressure on selecting field sites that exactly fit the requirements for disease screening. In NSW it was found that a drier than normal wet season led

to very little infection in some field experiments (**Figure 46**); significant resources can be put at risk in the planting of field trials where disease development is very limited.

An interesting observation in this experiment (at this site) was the relationship between disease incidence and site elevation (Section 6.5). Lower ends of the site, where longer, deeper flooding occurred, exhibited less CS. This may be related to the survival of the causal agent, but equally could be a plant-based response to the floodwaters. Not only is flooding important for CS transmission (per se), but specific conditions related to flooding may also be important. This needs further investigation in field studies. Knowledge of the causal agent would be of assistance in this regard. Farmers intending to crop Q241$^{\Phi}$, Q183$^{\Phi}$ and Q251$^{\Phi}$ should be aware of the potential effect of CS on these varieties. With susceptibility comes a propensity for very significant yield losses.

6.9. Hydroponic-based resistance screening methods

Research undertaken in the late 1950s-early 1960s clearly elucidated the most important mechanisms associated with disease transmission. Hydroponic growth systems successfully led to CS transmission to disease-free test plants (Bird *et al*., 1958, Egan, 1989).

Egan (1965) developed a glasshouse-based screening technique based on a gravel hydroponic system which included no circulation of the hydroponic solution to assess the resistance of various *Saccharum* species and some commercial varieties. He found significant differences in resistance among varieties and *Saccharum* spp. His work was curtailed by the outbreak of Fiji leaf gall in the Bundaberg area.

Once again CS transmission was attempted using several hydroponic systems to identify the best potential resistance screening technique. One system includes root system temperature control, as this was shown previously to be critical for CS symptom expression (Sturgess, 1962).

6.9.1 Temperature controlled root systems

Initial testing of circulating hydroponic system. Very little disease transmission was noted in the 'plant' crop and many plants failed to ratoon. Various leaf patterns/ chlorosis were associated with poor plant nutrition. Investigation revealed that the pH of the hydroponic solution dropped markedly with time, falling to 3.5 or lower.

Follow up testing of circulating hydroponic system. In the follow up experiment, limited CS transmission was noted while nutritional problems lingered (rapid lowering of the solution pH). The reason for the pH reduction is not known.

6.9.2 Non-temperature control of root systems

Still-gravel hydroponic experiments. Plant growth was much better with this system with no nutrient deficiency symptoms (**Figure 53**). Terramycin doubled the CS transmission rate in test plants with over 50 % plants showing symptoms after dipping in CS-infested inoculum (**Table 37**). This compares to approximately 25 % disease in the 'no chemical' inoculum. There was no infection in the TMTD and
un-inoculated control treatments. There appeared to be a variety effect with Empire exhibiting more disease than either Pindar or Q238$^{\Phi}$. The statistical analysis using a Generalised Linear Mixed Model failed as the model would not converge due to there being too many zeros.

Figure 53: The still-gravel hydroponics system in the Tully SRA glasshouse

Table 37: The effect of the biocide terramycin, the fungicide TMTD and varieties on CS transmission in a gravel hydroponic trial at Tully (data refer to '% plants diseased')

	Inspection date		
Variety	**14 October 2015**	**30 October 2015**	**9 November 2015**
Empire	0	31.3	68.8
Pindar	0	12.5	25.0
Q238[◊]	0	6.7	20.0
Inoculum treatment	14 October 2015	30 October 2015	9 November 2015
Control (CS-free)	0	0	0
CS-inoculation	0	12.5	25
CS+terramycin	0	21.7	52.2
CS+TMTD	0	0	0

6.9.3 Discussion

A great deal of research into CS has been undertaken over the last 85 years (Egan, 1989; Magarey and Egan, 2000). Although much is now known about various aspects of the disease, some key issues remained until 2013357 — including the identity of the causal agent, a suitable assay for screening planting material, and a rapid CS resistance screening test. The latter is essential for providing resistance data to plant breeders, and to farmers in general, so that the best choices can be made with regard to breeding strategies and variety selection for commercial crops. With the disease leading to losses as large as 60 %, farmers will want to minimise losses by planting varieties of appropriate resistance in areas prone to high levels of the disease.

The work described in this section focused on rapid transmission experiments. Previous work (Bird *et al.*, 1958; Sturgess, 1964, Egan, 1965) showed that CS transmission may occur via various forms of hydroponic systems. Such transmission is consistent with CS spread in floodwaters.

In our work, very little transmission occurred when diseased plants were located with healthy test plants in a circulating hydroponic system. The reason for this remains unclear, though rapid changes in solution pH, with associated plant nutritional effects, may well have contributed.

A non-circulating, non-aerated hydroponic system provided a better transmission environment. Plant nutrition appeared to be improved, and coupled with a two-hour inoculation of root systems, CS transmission was much better. Adding the antibiotic terramycin increased the CS transmission/ symptom expression, as found by Egan (1965). We don't have data on hydroponic solution biology to determine why more CS was observed with the terramycin treatment, but it is possible that biological interactions, and perhaps competition between organisms in the infested hydroponic solution, are affected by the chemical. Our intention is to incorporate terramycin in a 2-hour infection treatment for plants that are then placed within an air-conditioned bench hydroponic system. In this modified system, the hydroponic solution will not be circulated — to simulate conditions in our gravel system.

The results from our work offer hope of a potential rapid resistance test and, if further work can demonstrate reliability, a system for rapid screening may not be far away.

6.10. Woodford pot-based resistance screening methods

The first replicated resistance screening trial using cultured *P. venanatans* was performed in the glasshouse at Woodford in 2016.

6.10.1 Statistical considerations

Initial Trial design. The trial was conducted using a randomised complete block design in a glasshouse environment. Plants were assessed for a specified set of visible symptoms twice through the course of the experiment. A disease severity score was calculated for each assessment.

Modifications. After the first assessment, the trial layout in the glasshouse was modified because of concerns pot size was affecting plant development and newly potted plants were moved to a separate glasshouse. The randomised complete block design was maintained, however, the layout of the blocks within the glasshouse was different to address said concerns. For this reason primary results were calculated for each rating separately. Results from the combined data set should be interpreted with the above considerations in mind.

Residuals. The distributions of model residuals were visually assessed using q-q plots and histograms. Residual distributions were relatively symmetrical and consisted of sharp peaks and long tails. No distribution tested was found to be a better fit than a normal distribution, hence the normal distribution was chosen.

Model selection. Fixed effect and random effect linear models including inoculation method, variety, the interaction between inoculation and variety and a block effect were compared. No advantage was found using the random effect model so the simpler fixed effect model was selected for use for rating 1 and rating 2 results. A combined fixed effects model including the above terms and a rating effect was also run. Due to trial design and location changes between ratings mentioned above, neither multi-environment or repeated measures random effects models were deemed appropriate. Pairwise comparisons of inoculation method and variety effects were conducted using least square means and compact letter display.

6.10.2 Disease severity scores

When the disease severity distribution is plotted (**Figure 54A**), it can be seen that the majority of plants were free of symptoms, giving a disease severity of 0. When uninfected plants were removed

from the analysis and disease severity distribution replotted (**Figure 54B**), the results were skewed towards 100 %. This was due to the decapitation treatment (**Figure 55**). The rating scale was modified between the first and second rating because it became apparent that wilting (a symptom included in score 4) was observed in too many plants inoculated through decapitation (treatment 2). Thus decapitating the stalk, regardless of inoculation with *P. venanatans,* was found to be too damaging to the plants. Ideally a control consisting of decapitation with no *P. venanatans* should have been included but this was not envisioned at the start of the trial.

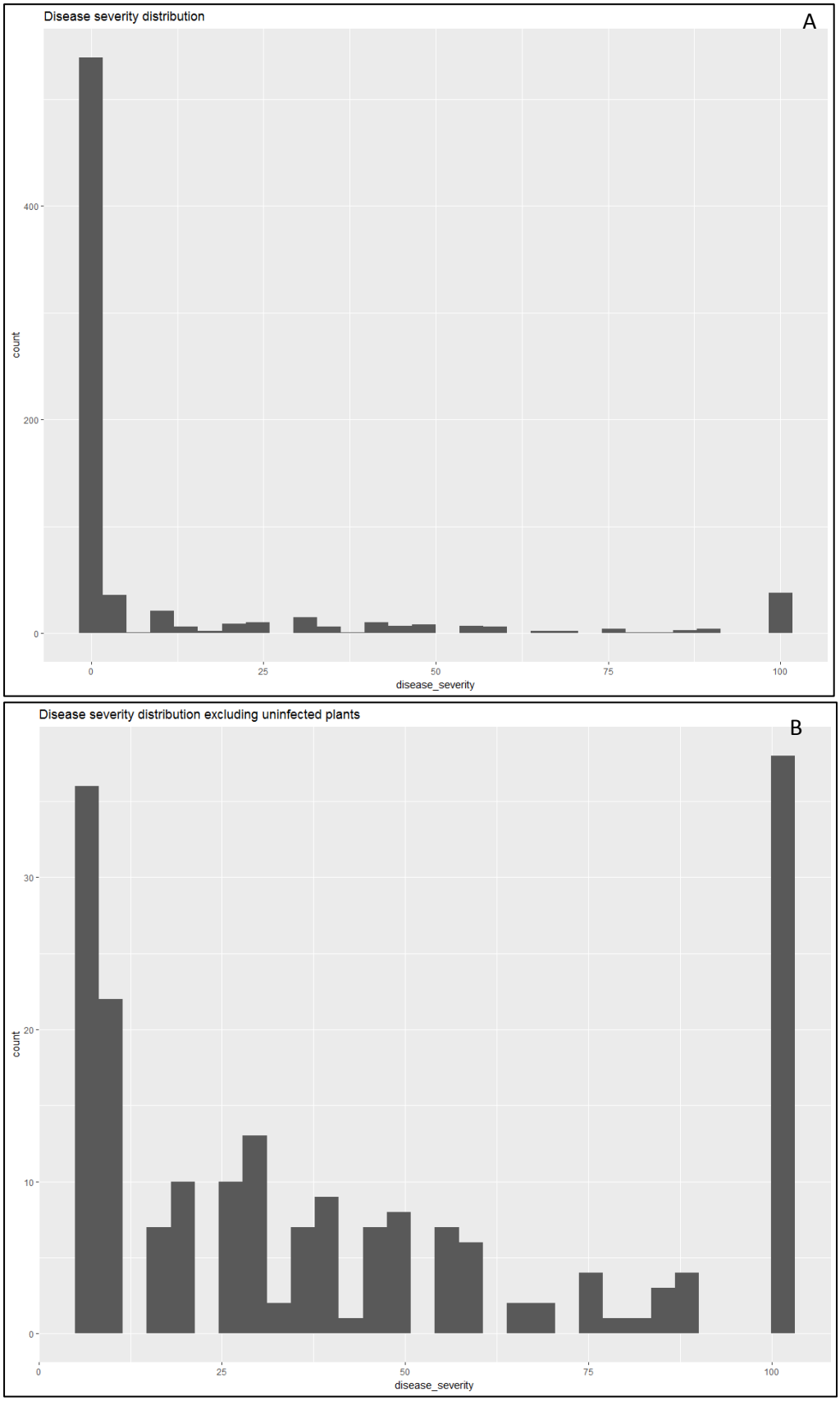


Figure 54: Distribution of combined disease severity scores from the Woodford pot screening trial. A displays data from all plants; B displays the data excluding the uninfected plants

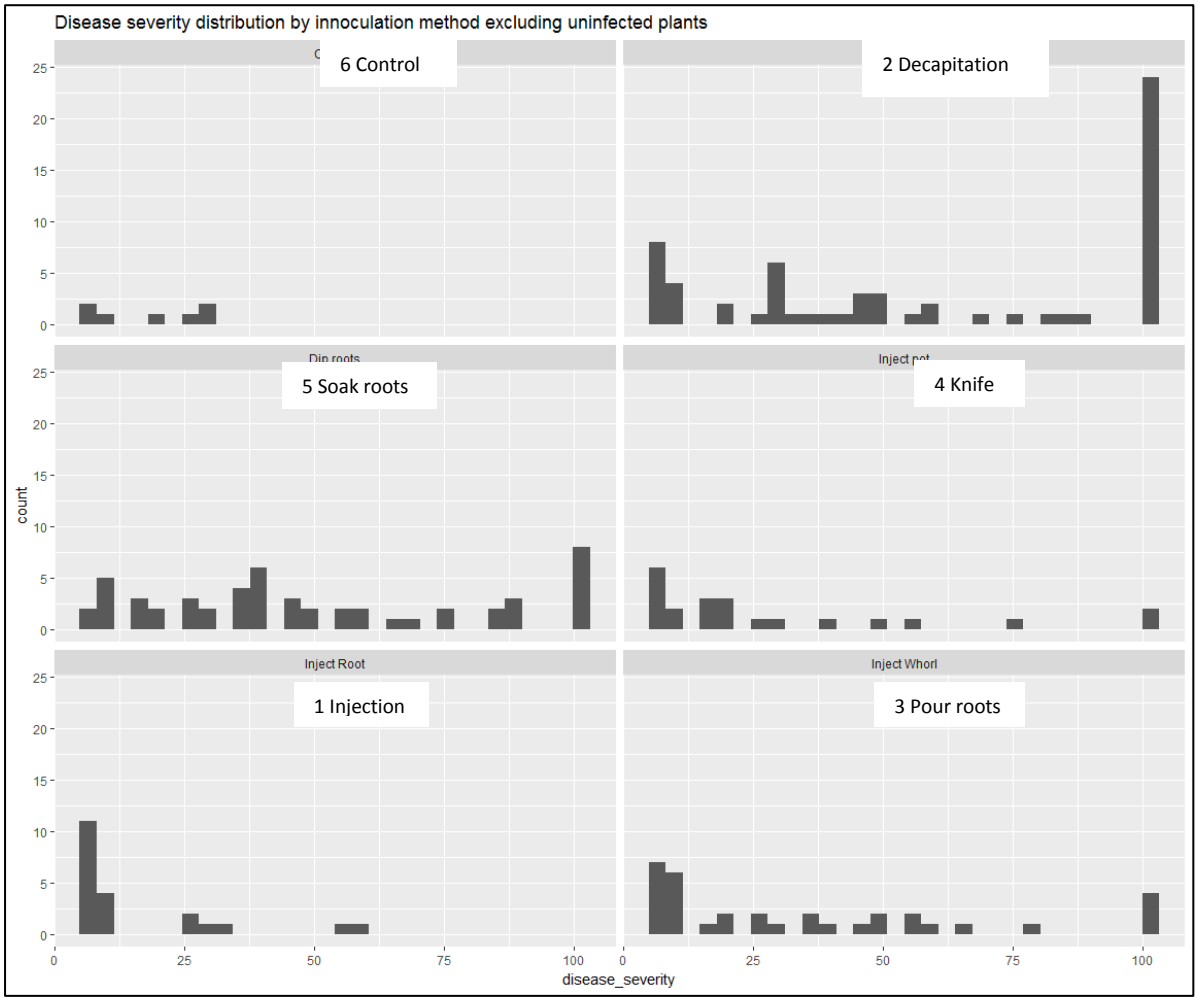


Figure 55: Distribution of combined disease severity scores for each treatment in the Woodford pot screening trial. The data excludes uninfected plants

6.10.3 Effect of treatment and variety

Analysis of Variance for treatments (**Table 38**) shows that only treatment 5 (Soak roots, damaged) and treatment 2 (Decapitation) gave significant results, however, as mentioned above, treatment 2 is not considered reliable because of the severity of the decapitation technique. Analysis of Variance for varieties (**Table 39**) shows that there are statistically significant differences between the resistance reactions of the varieties. A comparison between previously obtained ratings (extracted from SPIDNet) and results obtained in this trial are shown in **Figure 56**. SPIDNet ratings for the 13 varieties are presented graphically in the upper part of the figure. Note that there is no rating for Q231$^{\phi}$ and RP193-67. In the lower part of the figure are the disease severity scores for the same 13 varieties. It can be seen that some varieties showed very different reactions compared to the results in SPIDNet, with KQ228$^{\phi}$ and Pindar standing out. During the inoculations, notes were made on the condition of the roots of each variety. Pindar and Q200$^{\phi}$ were noted as having poor root growth 10 weeks after germination, possibly explaining why disease severity scores for Pindar were lower than expected. A larger trial with more replication may be able to show a variety interaction that relates to resistance mechanisms. Direct injection of the pathogen may overcome resistance mechanisms that operate under “natural’ infection in the field. SPIDNet ratings may also be inaccurate due to the interaction of other pathogens. Both the hydroponic methods and pot inoculation methods have the advantage of testing the effect of only one single pathogen.

Table 38: Analysis of Variance for six treatments in the Woodford pot screening trial. Statistical groupings are for combined and individual Rating 1 (October 2016) and Rating 2 (December 2016). Pairwise comparisons of inoculation method were conducted using least square means and compact letter display. Only the least square means and standard error for combined results are shown

Treatment	LS mean for combined	SE for combined	Group for combined ratings	Group for rating 1 only	Group for rating 2 only
6 Control	1.02	1.90	1	1	1
1 injection	2.49	1.87	12	1	1
4 Knife	5.34	1.92	12	1	1
3 Pour roots	9.36	1.95	2	1	1
5 Soak roots	20.77	1.88	3	2	2
2 Decapitation	34.58	2.07	4	3	2

Confidence level used: 0.95

P value adjustment: tukey method for comparing a family of 6 estimates

Significance level used: alpha = 0.05

Table 39: Analysis of Variance for 13 varieties in the Woodford pot screening trial. Statistical groupings are for combined and individual Rating 1 (October 2016) and Rating 2 (December 2016) results. Pairwise comparisons of variety effects were conducted using least square means and compact letter display. Only the least square means and standard error for combined results are shown

Variety	LS mean for combined	SE for combined	Group for combined ratings	Group for rating 1 only	Group for rating 2 only
KQ228$^{\Phi}$	1.00	2.73	1	1	1
Q124	4.33	2.73	12	1	123
Q200$^{\Phi}$	6.413	2.79	123	1	12
Q231$^{\Phi}$	7.56	2.79	123	12	123
Pindar	6.48	3.40	1234	1	123
Q232$^{\Phi}$	7.94	2.73	1234	1	123
RP193-67	10.33	2.73	12345	1	123
Q208$^{\Phi}$	16.50	2.73	2345	12	23
Q240$^{\Phi}$	16.71	2.79	2345	12	123
Empire	18.51	3.18	345	2	123
Q238$^{\Phi}$	20.84	2.79	45	12	123
Q138	21.33	2.76	5	12	123
Q183$^{\Phi}$	21.41	2.796	5	12	3

Confidence level used: 0.95

P value adjustment: tukey method for comparing a family of 13 estimates

Significance level used: alpha = 0.05

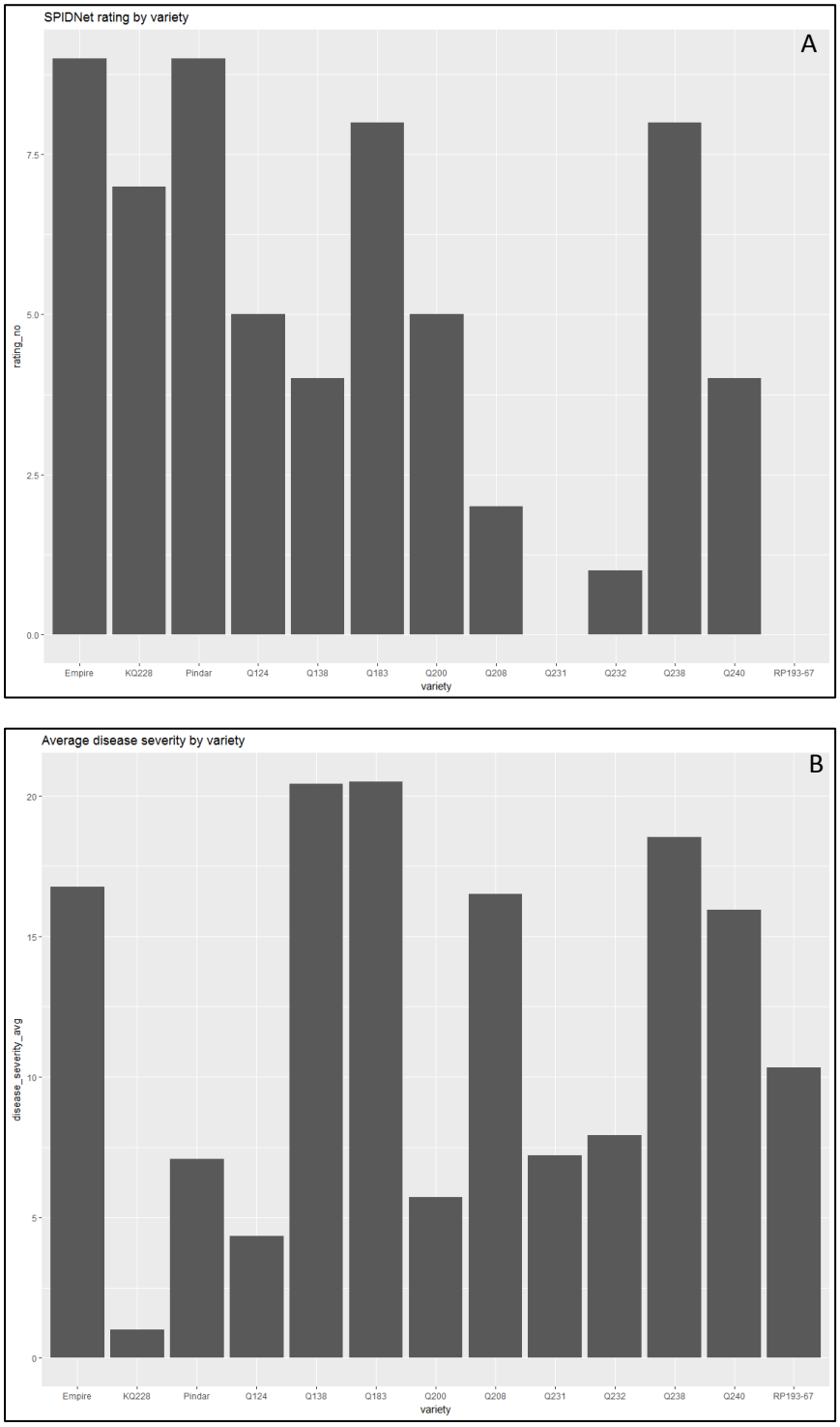


Figure 56: Comparison between SPIDNet ratings for 13 varieties (A) and the disease severity scores obtained in this trial (B)

6.10.4 Resources to run the trial

A number of factors need to be considered before concluding if the use of *P. venanatans* to inoculate large screening trials is achievable. These factors include the labour, time and resources to run the trial in the glasshouse (**Table 40**). The fastest and easiest methods were treatment 2 (decapitation) and treatment 4 (knife), however, treatment 2 gave unreliable results and treatment 4 gave a low level of disease severity. Treatment 5 (soak roots) was considered the most reliable method based on disease severity but needed double potting.

We think that a combination between treatment 3 (pour roots) which is based on the use of plastic to line the pots prior to a 2 hour incubation, and treatment 5, which includes root damage (in this case by trimming the roots with scissors), offers the best compromise. These are essentially the same methods using the same concentration of inoculum and a 2 hour root soak. The addition of a step where the roots are intentionally damaged distinguishes treatment 5 from treatment 3. Egan (1965) found that root damage increased CS infection in the hydroponic setup.

Table 40: Comparison between time, labour and inoculum volume needed to carry out each of the inoculation treatments

Treatment	Pre-preparation	Amount of inoculum	Labour (people)	Time to inoculate
1 Injection	Simple	20 mL	2	full day
2 Decapitation	Simple	20 mL	1	½ day
3 Pour roots (undamaged)	Simple	3.5 L	2	½ day
4 Knife	Simple	195 mL	1	½ day
5 Soak roots (damaged)	required double potting with the inclusion of sand in the lower pot	3.5 L	2	½ day
6 Control	Simple	0	-	-

6.10.5 Discussion

A major objective of this project was to establish reliable methods to screen varieties for resistance to chlorotic streak. When the project was first proposed, we considered the glasshouse hydroponic setup to be the most promising way of overcoming the limitations of doing screening field trials, where selecting trial sites that will receive optimum flooding can be challenging. Although we were able to successfully achieve disease transmission via the hydroponic setup, the ability to grow the pathogen in pure culture and use it to inoculate plants opens up new ways to improve screening methods further.

As part of the work to complete Koch's postulates, we demonstrated that by injecting roots or stalks with cultured cells, we could obtain symptoms within a few months in the glasshouse. This suggested that a rapid resistance screening method could be developed that, unlike all other methods tried previously, would be based on a known and controlled level of inoculum. Our Koch's Postulates experiments commenced in late 2015, running over the summer and autumn, and we found that injecting cell cultures into roots or stalks could lead to symptoms in susceptible varieties within 5 - 6 weeks. In contrast, our attempts to develop a screening trial occurred during the winter at Woodford and symptom expression took longer. Despite cooler temperatures, we could produce enough symptoms to calculate disease severity scores within 10 - 20 weeks after inoculation.

This work describes a preliminary trial and at this stage we are not in a position to use the data to calculate accurate resistance ratings. While the techniques look promising, optimization of the methods remain a high priority, specifically for:

1. Mass production of inoculum: We need to determine the optimal number of cells and age of cells to achieve high levels of disease in the trials. Factors to be considered will be culture medium, incubation temperature, culture source and of course inoculum concentration.

2. Method of inoculation: Several different methods have already been trialed, but further novel inoculation methods will be tested and earlier methods that appeared promising will be improved.
3. Rating method: Different factors to consider include trial length, severity vs incidence assessments, the need for ratooning, the extent of replication needed, the selection of appropriate standards and the data analysis required to develop resistance ratings.

Once the optimal conditions are established, they must be adaptable for routine screening programs providing a balance between time, labour, glasshouse space, consumables and cell culture requirements. It is also essential that glasshouse obtained ratings correlate with the natural reaction of varieties in the field.

6.11. General discussion

Although first recognised 87 years ago and in spite of significant research investment, identification of the causal agent of chlorotic streak had remained elusive. As part of project 2013/357 we developed methods for the isolation, culturing and inoculation of a novel pathogen of sugarcane, *Phytocercomonas venanatans* and we demonstrated that this organism is the causal agent of the disease. This is a major breakthrough in the understanding and management of a long-standing disease problem for sugar industries around the world. The project has achieved all of its objectives and the outputs have been communicated to industry.

Compelling circumstantial evidence that a Cercozoan was involved in the disease was obtained through Metagenomics Next Generation Sequencing, which identified DNA associated with infected sugarcane but absent in healthy sugarcane. A biflagellated Cercozoan was subsequently observed in liquid broth cultures established from infected sugarcane stalks. The DNA sequences and the morphology of the organism did not match any known species and a new genus and species, *Phytocercomonas venanatans,* has been proposed. Community profiling supports that *Phytocercomonas venanatans* is probably the cause of chlorotic streak. This is the first reported member from Cercomonadida showing a pathogenic association with higher plants.

We then confirmed that *P. venanatans* it is the cause of the disease by completing Koch's postulates. We were able to take the cultured cells of the pathogen and infect sugarcane using three different inoculation techniques. The sugarcane subsequently gave rise to the characteristic symptoms of chlorotic streak. We then reisolated for the second time, thus demonstrating Koch's postulates. We were able to link plant symptoms and plant diagnostic PCR screening with the isolation and reisolation of *P. venanatans*, microscopy and PCR confirmation of the cultures, to strengthen the association between *P. venanatans* and the disease. Infection has been achieved using cultures derived from several sugarcane cultivars and through inoculation of roots, stalks and leaf whorls.

While a first-generation diagnostic test was available before 2012/357, we have it improved the test by designing primers to target the ribosomal genes identified through NGS. This test works well on xylem sap and stalks, making it ideal to assist Productivity Services in screening clean seed plots. Potentially, CS and RSD sampling and testing of seed plots could be combined adding value to the existing SRA commercial diagnostic service. The test can also detect the organism in hydroponic water and soil.

Where chlorotic streak is a serious problem, the use of resistance cultivars, hot-water treatment of seed cane and improving field drainage are the recommended disease management options.

Now that the causal agent is known, we can better target control strategies based on an understanding of the biology of the causal agent. We have been able to demonstrate that *P.*

venanatans is extremely sensitive to anti-protozoan antibiotics, whereas it is relatively insensitive to most fungicides excluding TMTD (thiram) and PCNB. While there is potential for novel chemical solutions to chlorotic streak, our finding that we could experimentally inoculate plants with cultured cells and obtain symptoms within a few months led us to concentrate our efforts towards developing a rapid resistance screening method in the final year of the project.

Until now, there has been no reliable method to screen cultivars for resistance to chlorotic streak due to the difficulty of setting up replicated screening trials in the field where flooding events and inoculum levels can't be controlled. During the project there were two attempts to obtain resistance ratings from the field. One attempt, based in Tully, was done at a site subjected to at least five flooding events over a 2-year period. The other field-based attempt in NSW combined mother plot and seed-plot inspections with RSD surveys. Although field screening can give good results if sufficient flooding occurs, selecting resistance trial sites is a balance between optimum flooding events versus optimum growth conditions. Significant resources can be wasted in planting field trials where disease development is limited during dry years. The presence of other diseases, either soil-borne or even RSD can confuse the reactions. A glasshouse screening trial would be an advantage, reducing the time taken to obtain ratings, allowing standardisation of inoculum and infection conditions and reducing the need to find suitable field sites.

From our Koch's postulates work, we could generate symptoms within a matter of weeks to months. This offers the potential to develop a glasshouse-based screening method to obtain disease resistance ratings for sugarcane cultivars. This could be done either by manually infecting plants in pots as was done at Woodford, or through mass inoculations in a glasshouse-based hydroponic system. Methodologies for both types of screening trials were successfully developed in this project.

The methods established in this project that screened a range of varieties: NSW field screening, Tully field screening and Woodford glasshouse screening (Sections 6.7, 6.8 and 6.10) can be compared with the SPIDNet rating (**Table 41**). Only three of the standards used in the Tully field trial were included in the Woodford trial: Pindar (9), Q124 (5) and Q138 (4). All three gave disease severity scores that differed to some degree from what was expected: Pindar (I), Q124 (R) and Q138 (S).

It is clear that chlorotic streak screening has suffered from the lack of a reliable method to assess varieties for CS resistance. It should be kept in mind that single ratings from an individual resistance screening trial, using any method (even with other diseases), does not always provide an accurate assessment of cultivar resistance. For this reason, clones and cultivars need to be tested on multiple occasions to ascertain a more reliable guide on likely CS resistance under commercial conditions. There are some similarities but also many differences between ratings generated via the different methods in **Table 41**; further data for many more varieties, on a replicated basis (both within and between trials) is now needed to assess which resistance screening method provides the best results. This baseline data will provide the basis for ongoing resistance screening/ interpretation of the collected data.

Table 41: Comparison between the SRA SPIDNet and NSW field results, Tully field results and Woodford glasshouse trial. Only varieties that are common between at least two trial sites are included

Variety	SPIDNet Rating	Harwood Section 6.7[1]	Tully Section 6.8[2]	Woodford Section 6.10[3]
Empire	9	8	-	IS
Pindar*	9	-	IS	I
Q238ϕ	8	-	I	IS
Q183ϕ	8	2	S	S
KQ228ϕ	7	-	R	R
Q124*	5	-	R	R
Q200ϕ	5	-	I	IR
Q138*	4	-	IS	S
Q240ϕ	4	3	R	I
Q208ϕ	2	3	R	I
Q232ϕ	1	4	R	I
Q231ϕ	-	-	I	IR
RP193-67	-	-	-	I

*Standard varieties used in the Tully trial

1. Values are field ratings from **Table 35**
2. Values are ratings based on % stool June 2015 from **Table 36**
3. Values are based on the combined data group from **Table 39**

This project has been able to confirm and extend some of the ground-breaking research findings of Brian Egan and Owen Sturgess, the BSES pioneers of chlorotic streak research in the 1960s. These include:

- That not all setts planted from a diseased stool will develop symptoms has been confirmed by qPCR, which highlighted the uneven distribution within the stalk
- That the disease is readily controlled through a short hot water treatment is due to the causal agent being a simple single celled organism with a cell wall.

Our transmission work in both pots and hydroponics has confirmed that:

- A gravel hydroponic trough setup can achieve transmission
- The addition of terramycin to the hydroponic water increases transmission efficiency
- Damaging the roots increases transmission efficiency
- The disease can be transmitted to other monocots
- Disease transmission can be achieved by injecting roots with extracts using a hypodermic syringe
- Generally fungicides do not control the disease
- Only a two hour incubation period is needed to achieve transmission.

Interestingly, one aspect that could not be confirmed relates to the size of the organism. Sturgess used a variety of filtering, centrifuging and precipitation techniques in an attempt to isolate the organism and found that filtrates with a particle size of less than 0.5 µm were infectious, and this was the basis for his claim that chlorotic streak was caused by a plant virus. Unfortunately the experimental details are very brief and the filters are described only as a graded series of filter pads, the final one having an initial retention of all particles less than 0.5 µm. There is no mention of brand

or chemical composition of the filters. We have clearly shown through light microscopy and in situ hybridisation that the organism is about 8 - 10 µm. We have no explanation at this stage for the earlier results, other than perhaps there are other life stages that we have not yet identified.

While there has been tremendous progress in the last four years, there are still many gaps in our knowledge. Using the techniques we developed in this project to visualize, isolate, grow and quantify *P. venanatans* experimentally, we can begin to fill some gaps:

- Resistance ratings for current commercial varieties using controlled inoculation under standard conditions are required.
- The biology of the pathogen and its life cycle is completely unknown. We also have very limited information about how the pathogen infects sugarcane: how it enters the plant through the roots, how it spreads in the stalk and causes disease, how cells are released back into the soil and how the organism survives for extended periods outside of the plant.
- Can better, novel management options be developed as we increase our understanding of the causal agent?

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10. APPENDICES

10.1. Appendix 1 - METADATA DISCLOSURE

Table 9.42 Metadata disclosure 1

Data	• **Next Generation Sequencing data from xylem exudate DNA** • **Community profiling – amplicon sequencing data**
Stored Location	Bioinformatics analyses were done using Geneious version 8 (Biomatters Ltd, New Zealand) and are stored on the SRA server. Raw data for DNAseq and amplicon sequencing are also stored on the SRA server.
Access	Key sequences are publically available on GenBank (accession numbers are shown): • 18S rDNA (KR704194) • Entire ribosomal DNA region (MF034900). • Polyubiquitin (MF034899) • Actin (MF034898)
Contact	Dr Chuong Ngo, SRA

10.2. Appendix 2 - Northern Queensland Chlorotic streak/ biosecurity roadshow feedback

Table 9.2 Questions received around chlorotic streak recorded by Matt Reynolds

Understanding of the organism
Where is it in nature, Is it in other grasses or crops soils
What is CS doing to cause the disease expression
Where is it outside of sugarcane/ where does it come from / its movement within the broader sugarcane system (recycled water??)
What is CS actually costing the industry
How far is it moving/is the high water moving the CS into the plant from the soil
Understanding of the CS organism
Q238$^{\Phi}$/ use of recycled put water, is it a risk/ could you test the recycled waters/does dilution of recycled water with fresh resolve the CS issue
At what level of dilution/threshold/levels associated with disease expression
How's the protozoan entering the plant from the soil/ where's CS existing within the plant
Where are they multiplying
Symptoms of chlorotic streak
What about expressing the symptoms but not affecting yield loss / vice versa
Symptom expression and yield loss are they related
Is no expression of CS causing greater expression of other diseases/pests as a result of a compromised system (Is Cs making the plant vulnerable to other organisms?)
Disease without symptoms
Is harvesting transmitting the CS, harvesting in wet conditions, juice transmission during harvesting / extractor fans blowing fresh juice onto exposed cane
Trial idea - Can we run a harvester over CS and clean cane and see if the harvester transmits the disease (RSD type experiment)
Transmission through the SAP / same experiments in the SAP (Kathy's)
Can you have affected plants not expressing the symptoms/ are there other symptoms associated with CS associated with particular times of the year
No expression of symptoms and the presence of the disease within the plant
Does NIR offer any opportunity to CS detection
Variety screening
Is the visual rating system relevant

How do you control CS at a farm level/managing it out of the farm
How would it fit into the broader screening
A test could remove the significance of CS into the future/through our understanding of variety resistance
The relationship between trial resistance and in the field resistance and how growers interpret the information
most likely control method going forward compared to how other protozoan are managed
Don’t we currently have resistance ratings for CS
Trial idea - Could do rapid selection through Robs gravel technique. Pick up seedling trays, chop roots and dunk for 2 hrs and then they will naturally screen
Really want information before varieties are released
Very valuable to be able to screen for RSD and CS at the same time through LSB

10.3. Appendix 3 - Organisms included in the SRA defined database

Table 9.3 Organisms included in the SRA defined database. The presence of a genome sequence, number of DNA and EST sequences have are indicated

Group	Species	Genome	Nucleotide	EST
Rhizaria				
Phytomyxea	*Plasmodiophora brassicae*		328	324
Phytomyxea	*Spongospora subterranea*		157	11
Phytomyxea	*Phagomyxa*		5	
Phytomyxea	*Polymyxa*	y	2,974	541
Phytomyxea	*Sorosphaera*		8	
Phytomyxea	*Woronina*		2	
Chlorarachniophyte	*Bigelowiella natans*	y	608	3,472
Foraminifer	*Reticulomyxa filosa*	y		
Stramenopiles				
Oomycete	*Hyaloperonospora arabidopsidis*	y	2,291	
Oomycete	*Phytophthora parasitica*	y	44,235	17,466
Oomycete	*Phytophthora kernoviae*	y	2,669	
Oomycete	*Phytophthora lateralis*	y	8,229	
Oomycete	*Phytophthora infestans*	y	32,982	165,133
Oomycete	*Phytophthora alni*	y	71,956	
Oomycete	*Phytophthora capsici*	y	4,509	58,277
Oomycete	*Phytophthora ramorum*	y	56,702	1
Oomycete	*Phytophthora sojae*	y	1,687	40,897
Oomycete	*Phytophthora rubi*	y	80	
Oomycete	*Phytophthora fragariae*	y	152	
Oomycete	*Phytophthora pinifolia*	y	13,815	
Oomycete	*Phytophthora cryptogea*	y	10,676	
Oomycete	*Phytophthora cambivora*	y	37,313	
Oomycete	*Pythium ultimum*	y	6,053	100,391
Eustigmatophytes	*Nannochloropsis gaditana*	y	47,987	
Eustigmatophytes	*Nannochloropsis oceanica*	y	628	
Blastocystis	*Blastocystis hominis*	y	589	47,234
Amoebozoa				
Mycetozoa	*Dictyostelium purpureum*	y	14,108	23,786
Archamoebae	*Entamoeba histolytica*	y		

	Entamoeba nuttalli	y		
	Entamoeba invadens	y		
	Entamoeba dispar	y		
Fungi				
Chytrids	*Batrachochytrium dendrobatidis*	y	9,434	
Chytrids	*Homoloaphlyctis polyrhiza*	y	346	
Chytrids	*Spizellomyces punctatus*	y	26	3,991
Chytrids	*Allomyces macrogynus*	y	134	5,082