Progress in understanding and managing chlorotic streak of sugarcane

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Abstract Chlorotic streak is a major disease of sugarcane and has one of the highest incidences of any disease in Australia. It is a serious problem in flood-prone or waterlogged fields, where susceptible varieties can show losses of up to 40%. Management of the disease is hampered by the lack of knowledge about the causal agent which has remained unknown since the disease was first recognised in 1929. Australian scientists were actively involved in chlorotic streak research in the 1960s and they concluded that the disease was caused by a virus. Other workers suggested that it was caused by a chytrid fungus. Sugar Research Australia (SRA) recently resumed research and in 2012 identified a DNA sequence specific for the disease. This was a significant breakthrough, as it formed the basis of a diagnostic PCR test. Since then, increased research efforts have led to progress in a range of activities. The diagnostic test has been refined and can be used to screen field samples and monitor research experiments. We have been able to successfully detect the organism in sugarcane stalks, roots, soil and root exudates. Next Generation Sequencing was used to identify the causal agent, with phylogenetic analyses suggesting that it is a unique Cercozoa. Methods to culture the organism and transmit it experimentally to fulfil Koch’s Postulates are being developed. Resistance screening data is being generated from the field using various approaches and a rapid glasshouse system is being trialled as a better way to obtain variety resistance ratings. Our understanding of the causal agent may open up new management strategies that will reduce losses and decrease the cost of existing management programs.

Key words Chlorotic streak, diagnostic test, Next Generation Sequencing, hydroponics

INTRODUCTION

Chlorotic streak has a worldwide distribution and is one of the most serious and widespread diseases of the Australian sugar industry (Magarey 2005). The disease can cause sugar yield losses of up to 40% in susceptible varieties (Magarey and Nielson 2002). Despite its importance, research progress has been extremely slow due to its intractable nature. The disease is spread through wet soil, flood and drainage water and infects through the root system, but the root/soil/water environment is difficult to study and control. In the field, leaf symptoms are often transient, with the erratic behaviour believed to be due to environmental conditions, including soil temperature (Sturgess 1961).

Although first recognised around 1929, the causal agent is still unknown. A diverse range of organisms have been proposed as the causal agent, including a chytrid fungus (Carpenter 1940) and a leafhopper as vector (Abbott and Sass 1945). Later research clearly established the role of soil and water in disease transmission, rather than an insect (Antoine 1957). Transmission experiments carried out in the 1960s implicated a virus (Sturgess 1963), but Rogers et al. (2001) could find no evidence of a virus using modern virology techniques.

SRA recently resumed attempts 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 DNA fragment specific to chlorotic streak. This breakthrough led to increased research activities based in the laboratory, glasshouse and field. While there are still many questions to answer, we have made considerable progress. This paper reviews recent progress in identifying the causal agent, optimising the diagnostic test, developing a controlled transmission system, generating resistance ratings and understanding disease epidemiology.
One of the aims of our research program was to identify the causal agent using modern DNA sequencing techniques known as Next Generation Sequencing (NGS). We began with a working hypothesis that the causal agent belonged to one of three groups of plant pathogens that produce zoospores and transmit through water: the Cercozoa (Kingdom Rhizaria), Oomycetes (Kingdom Chromalveolata) or Chytrids (Kingdom Fungi).

By testing a wide range of ‘universal’ PCR primers, we identified a primer pair that generated a chlorotic streak-specific DNA fragment coding for actin, a conserved eukaryotic gene. Unfortunately, it was too conserved to allow us to identify the organism through the standard approach of sequencing followed by matching against GenBank using BlastN (Braithwaite and Croft 2013). However, the DNA fragment could form the basis of a diagnostic test which was further developed into a qPCR format (quantitative PCR). We screened a wide range of different chlorotic streak-infected plant tissues to quantify the relative concentration of pathogen actin DNA compared with sugarcane actin. We found that the relative proportion of pathogen DNA in some xylem extracts was more than 100 times greater than in stalk nodes and this guided us in the selection of sequencing template.

Fig. 1. Top: Read distribution of the 18S dataset. The yellow bars show that *Saccharum* 18S ribosomal sequences account for most of the reads. The sample codes are as follows: Q241: xylem sap from infected Queensland field-grown Q241; Empire: xylem sap from infected NSW field-grown Empire; RP CS: xylem sap from infected glasshouse-grown RP193-67; RP H: xylem sap from healthy glasshouse-grown RP193-67; RP F: xylem sap from infected, fungicide-treated glasshouse-grown RP193-67; TC: whole tissue from Empire plantlet established in tissue culture; NEG: negative (no DNA) control.

Bottom: Read distribution of the 18S dataset following filtering of *Saccharum* and negative control sequences. Sample codes are as above.
Chlorotic streak-infected and uninfected (healthy) xylem sap DNA samples were sequenced on the Illumina HiSeq 2000 platform. Bioinformatic analyses combined with PCR verification identified the entire ribosomal DNA coding region of an organism present in the infected xylem sample. Based on BlastN searches of GenBank, the small and large subunits (SSU or 18S and LSU or 28S, respectively) of the ribosomal sequence indicated that the causal organism is most likely a Cercozoa, but is distinct from any known organism. 18S ribosomal DNA phylogenies show that the causal organism is unique and considerable characterization is still required to complete its taxonomic identification.

While the exact identification of the causal agent is yet to be made, we have been able to show a link between the pathogen 18S ribosomal DNA and the disease by using community profiling, a technique based on sequencing PCR amplicons generated with degenerate primers from environmental samples. The 18S was used to show if there are any eukaryote species associated with diseased plants. Results are presented as read distribution (relative abundance within the sample, Fig. 1 top) and read distribution following filtering of *Saccharum* and negative control sequences (Fig. 1 bottom). From a range of samples representing different tissues and locations, the majority of DNA was from sugarcane. However, if subtracted from the analysis, DNA of lower abundance becomes more obvious and Cercozoa DNA is consistently represented in every diseased sample.

**DIAGNOSTIC TEST**

Our initial diagnostic test was based on the actin gene (Braithwaite and Croft 2013) but diagnostic PCR primers are now available for the ribosomal SSU and LSU. The latter two were developed from the NGS results. All three genes show equally good specificity for the chlorotic streak organism, but the repetitive ribosomal genes give more intense signals (Fig. 2). The ribosomal SSU primer set has become the standard diagnostic combination for routine screening for chlorotic streak. We combine the specific test with one of two endogenous tests. PCR primers for phosphofructokinase are used for sugarcane samples and universal fungal SSU primers are used for water and soil testing.

![Fig. 2. Comparison between three diagnostic PCR combinations (actin, ribosomal SSU and LSU). H indicates uninfected (healthy) samples; CSD indicates chlorotic streak disease-infected samples. Sample codes are H1: RP193-67 xylem; H2: Empire roots; H3: Q1700 pith; C1: Q1700 pith; C2: Empire stalk; C3: Empire stalk; C4: Q2410 stalk (not positive in this example); C5: Q2080 stalk; C6: Empire roots.](image)

We have screened a wide range of plant tissues to determine the optimal sampling strategy. We have obtained our most reliable screening results when ground stalk is use to prepare the DNA template for PCR amplification. Unfortunately, specialized grinding equipment is needed to prepare the stalk material. A much easier approach has been to use xylem sap, expressed from the stalks using the same procedure as that used for routine ratoon stunting disease (RSD) testing in the Australian industry. While straight sap is not always as sensitive as stalk DNA, a DNA extraction is not required and sensitivity can be improved with a brief centrifuge. This approach has the potential to provide combined RSD and chlorotic streak testing. Leaves, while easy to sample, were found to give variable diagnostic results, suggesting that the pathogen
is not reliably present in the leaves even when symptoms are present. Roots were more reliable than leaves, but the distribution of the organism within the roots appears to be uneven.

We have attempted to use our diagnostic test on water and soil samples in the industry with mixed results. This is believed to be due to sample size limitations and needs further optimisation. We have obtained limited positive test results from field soil when specialised soil DNA extraction kits are used. However, the kit’s requirement to use only 20 mg of soil as template is a problem because, as with roots, the distribution of the organism is uneven. So far we have not been able to obtain a positive result from field drainage water and again, the dilution factor and sample size could be the limitation. We have however, been able to obtain positive results from water in experimental systems, as will be explained below.

**BIOLOGY OF THE ORGANISM**

Our ultimate goal is to isolate the organism in pure culture and then complete Koch’s Postulates, but we are still in the early stages of developing the experimental systems to do this. This work has been greatly assisted by the diagnostic test, which allows us to monitor the success of experiments.

Our starting hypothesis for the cause of chlorotic streak was that the organism must have zoospores or some other motile structures, at least for part of its life cycle to explain transmission through water and infection through the roots. Using PCR and microscopy, we have attempted to produce these structures under controlled conditions and to characterize them. The production of PCR-positive water is easy to obtain, simply by placing roots in pure water at 28°C with no shaking for 2 h. To eliminate the chance that the positive test results have come from infected roots and not motile organisms, we used a series of filters, including 25 µm and 10 µm stainless steel filters, to remove small root pieces, followed by Millipore filters ranging from 8 µm to 0.22 µm. From this we have determined that the organism is about 8 µm to 10 µm in size. While chlorotic streak root exudates are easy to generate, it is surprisingly difficult to identify the exact organism involved. A large number of motile organisms and bacteria can be generated from both chlorotic streak -infected and healthy roots.

We have attempted to set up experimental transmission systems to research various aspects of the pathogen’s biology. In the glasshouse, pots of infected plants and test plants were arranged on benches lined with geofabric to hold water and transmit the disease (Fig. 3 left). Alternatively, infected and test plants were grown in tubs of gravel (Fig. 3 centre). Both of these methods were able to successfully transmit chlorotic streak from sugarcane to sorghum (Fig. 3 right), based on symptoms. Various plant parts from the sorghum were then screened with the diagnostic test, but only the roots and stalk base tested positive. When sorghum root pieces were placed in water for 24 h as described above, exudates tested positive.

We are attempting to establish laboratory-based transmission systems. Ideally we will establish the organism in pure culture, although Cercozoa can be a challenging group to culture. The plant pathogenic Cercozoa are usually obligate pathogens and unable to be cultured. Others, such as marine Cercozoa, can be grown on unusual media using bacteria as the food source. We are also attempting to establish sterile cultures of chlorotic streak-infected plants for experimental
material. This would be an ideal way to test the effect of fungicides and other control agents *in vitro*. However, this has not been successful so far as the infected plants struggle to remain alive in tissue culture.

**RESISTANCE SCREENING**

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. A rapid chlorotic streak resistance screening test is essential so that farmers can select appropriate varieties to grow in disease-prone areas. Two approaches for assessing differences in varietal reaction have been tested. One is based on natural spread from infected plants in the field, while the second is based on a glasshouse hydroponic system.

Field-based screening programs are underway in both North Queensland and New South Wales. The Queensland screening site at Tully was subjected to at least five flooding events over a 2-year period (Magarey et al. 2016a). Infection levels were over 60% in the most susceptible variety and several current Australian commercial varieties were found to be very susceptible, including Q241\(\text{a}\) and Q251\(\text{a}\). Experiments were rated for ‘% stools diseased’ and ‘% stalks diseased’. Rating stools is faster and crop inspections are significantly easier, especially in mature crops where symptom expression is often higher.

The New South Wales based field screening compared two approaches (Young et al. 2013; Young and Ensbey 2015). Firstly, inspections were made in a second-ratoon field that had previously served as a motherplot and had been subjected to four major floods. In this situation, varieties were grown under relatively uniform conditions. Ratings for 24 varieties were derived from the number of symptomatic leaves counted over a 20 m transect. For the second method, qualitative ratings were given during the annual RSD surveys of seedbeds. This method proved valuable in determining how canes performed in the field and provided data on the role of crop class, weather and different farming practices on chlorotic streak incidence.

Selecting resistance trial sites is a balance between optimum flooding events versus optimum growth conditions. Significant resources can be wasted in planting of field trials where disease development is limited. 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. A hydroponic system similar to that developed by Egan (1965) has the potential to provide a rapid screening system independent of the weather. The setup could also be used to study the biology of the organism and test different control strategies.

Different hydroponic systems were trialled in the glasshouse in Tully (Magarey et al. 2016b). Initially, it was found that very little transmission occurred when diseased plants and healthy test plants were grown in a circulating hydroponic system (Fig. 4 left). The reason for this remains unclear, though rapid changes in solution pH, with associated plant nutritional effects, may have contributed. A non-circulating, non-aerated hydroponic system (Fig. 4 right) provided better transmission and plant nutrition appeared to be improved. Adding the antibiotic terramycin increased transmission and symptom expression, as found by Egan (1965). A 2-hour inoculation period, where roots were dipped in infected hydroponic solution containing the antibiotic, was sufficient to lead to significant disease levels in the test plants.

**Fig. 4.** Glasshouse hydroponic systems tested for chlorotic streak transmission. Left: circulating hydroponic system on air-conditioned benches; right: the ‘still’ (non-circulating) gravel-hydroponic system.
EPIDEMIOLOGY

Field-based monitoring of disease epidemiology has been assisted by the development of GPS technology as it enables disease incidence to be recorded on a stool-by-stool basis. Geographical Information Systems (GIS) enable mapping of this information and provide an easily visualised understanding of the disease spatial pattern. Information on the incidence and spread of the disease may provide a better understanding of factors that may be manipulated for improved chlorotic streak management.

Two commercial crops in the Tully district have been monitored for chlorotic streak. Both farms were planted with hot-water-treated seed-cane and then monitored over the year. One site showed a definite relationship between disease incidence and site elevation of the block. Unexpectedly, the lower ends of the site, where longer, deeper flooding occurred, exhibited less disease (Fig. 5). The length of time that soils were waterlogged could have affected the survival of the causal agent, or could have triggered a plant-based response to the floodwaters. The second site showed little difference in disease levels over the 12-month period but suffered few flooding events and rainfall was considerably lower. 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.

![Image of Tully trial site map](image)

**Fig. 5.** An example of field-based disease monitoring obtained through GPS technology. The figure shows the spatial pattern of diseased stools at a Tully trial site in June 2015. Note the difference in disease incidence between the higher (northern end) and lower parts of the site.

DISCUSSION AND CONCLUSIONS

Chlorotic streak is one of the most intractable diseases of sugarcane in the world. A great deal of research has been undertaken over the last 85 years (Egan 1989; Magarey and Egan 2000) and, although much is known about various aspects of the disease, some key issues had remained - including the identity of the causal agent, a diagnostic assay for
screening planting material and a rapid resistance screening test. By tackling the disease with combined laboratory-, glasshouse- and field-based approaches, we have been able to greatly progress our understanding of the disease and attempt to address those outstanding issues. We hope to be able to formally identify the pathogen, establish it in pure culture and complete Koch's Postulates in the near future.

The evidence suggests that the causal agent belongs to the Cercozoa, a phylum in the poorly understood Rhizaria supergroup (Burki and Keeling 2014). They are abundant in soil and freshwater and saltwater habitats where they can be parasites of plants, invertebrate animals and other protists or free-living, feeding on bacteria, fungi, algae, other protozoa or even microscopic animals (Bass and Cavalier-Smith 2009). The best known plant pathogenic Cercozoa belong to the Plasmodiophorids (class Phytomyxea) and include Plasmodiophora brassicae, (clubroot in brassicas), Spongospora subterranea (powdery scab of potato) and Polymyxa graminis, the vector of several plant viruses (Neuhauser et al. 2010).

It has been known for many years that the chlorotic streak disease can be eradicated from infected planting material by hot-water treatment at 50°C for 30 min (Wilbrink 1932). The current management advice to growers is to improve drainage, use disease-free planting material and resistant varieties, and avoid ratooning under wet conditions. Although these strategies are likely to remain the mainstays of chlorotic streak control, a better understanding of the biology of the causal agent may lead to new innovative methods of control. For example, a range of disease management strategies are available for the plant pathogenic Plasmodiophorids. While the chlorotic streak pathogen and the Plasmodiophorids do not appear to be closely related, they both share many functional features as a result of their soil- and water-borne nature. The various transmission systems that we are attempting to establish have the potential to provide a rapid screening system and an experimental system to help understand the biology of the causal agent and the processes of pathogen infection and colonisation.

ACKNOWLEDGEMENTS

We thank SRA technicians Mona Singh, Katherine Sventek and Judi Bull for their hard work, and Rick Beattie (NSW Sugar), Jake McLagan and Graham Cripps (TCPSSL) and Andre Drenth (University of Queensland) for providing ideas, help and access to material. We acknowledge funding by SRA and the Queensland Department of Agriculture and Fisheries.

REFERENCES

**Résumé.** La strie chlorotique est une maladie majeure de la canne à sucre qui a l’une des incidences les plus élevées de toutes les maladies en Australie. C’est un problème sérieux dans les champs inondables ou gorgés d’eau où les variétés sensibles peuvent subir des pertes atteignant 40%. La gestion de cette maladie est générée par le manque de connaissances sur l’agent causal qui est resté inconnu depuis que la maladie a été reconnue pour la première fois en 1929. Des scientifiques australiens furent activement impliqués dans les recherches sur la strie chlorotique dans les années 1960, et ils conclurent que la maladie était causée par un virus. D’autres ont suggéré qu’elle soit causée par un champignon chytride. Sugar Research Australia (SRA) a récemment repris les recherches et en 2012 a identifié une séquence d’ADN spécifique de la maladie. Cette découverte a été une percée significative car elle a constitué les bases d’un test de diagnostic PCR. Depuis lors, des efforts accrus de recherche ont permis des progrès dans toutes sortes d’activités. Le test de diagnostic a été affiné et il peut être utilisé pour cibler des échantillons provenant des champs et superviser des expériences. Nous avons pu détecter avec succès l’organisme dans des tiges et des racines de canne à sucre, dans le sol et dans des exsudats racinaires. Le séquençage de nouvelle génération a été utilisé pour identifier l’agent causal, des analyses phylogénétiques suggérant qu’il s’agisse d’un Cercozoa particulier. Des méthodes pour cultiver l’organisme et le transmettre expérimentalement afin de satisfaire au Postulat de Koch sont en cours de mise au point. Des tests au champ faisant appel à différentes approches génèrent des données de criblages pour la résistance, et un dispositif en serre rapide est en cours d’essai pour obtenir un meilleur moyen de noter la résistance variétale. Notre connaissance de l’agent causal pourrait ouvrir la voie à de nouvelles stratégies de gestion qui réduiraient les pertes et diminueraient le coût des programmes de gestion existants.

**Mots-clés:** Strie chlorotique, test de diagnostic, séquençage de nouvelle génération, culture hydroponique

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**Progreso en la comprensión y manejo de la raya clorótica de la caña de azúcar**

**Resumen.** La raya clorótica es una enfermedad importante de la caña de azúcar y tiene una de las mayores incidencias que otras enfermedades en Australia. Es un problema grave en lotes propensos a inundaciones o anegados, donde las variedades susceptibles pueden mostrar pérdidas de hasta el 40%. El manejo de la enfermedad se dificulta por la falta de conocimiento sobre el agente causal, que ha permanecido desconocido desde que la enfermedad fue reconocida por primera vez en 1929. Los científicos australianos estuvieron involucrados activamente en la investigación de la raya clorótica durante la década de 1960 y llegaron a la conclusión que era causada por un virus. Otros trabajos sugirieron que era causada por un hongo quitridio. El Sugar Research Australia (SRA) reanudó recientemente investigaciones y en el 2012 identificó una secuencia de ADN específica para la enfermedad. Este fue un avance significativo, ya que era la base para una prueba de diagnóstico por PCR. Desde entonces, el aumento de los esfuerzos de investigación ha permitido avanzar en una serie de actividades. La prueba de diagnóstico se ha refinado y se puede utilizar para evaluar muestras de campo y monitorear los experimentos de investigación. Hemos sido capaces de detectar con éxito el organismo en los tallos y raíces de caña de azúcar, el suelo y exudados de las raíces. La secuenciación de nueva generación se utilizó para identificar el agente causal, sugiriendo los análisis filogenéticos que se trata de un Cercozoa único. Se están desarrollando métodos para cultivar el organismo y transmitirlo de forma experimental para cumplir con los postulados de Koch. Se están generando datos de resistencia en campo utilizando diversos enfoques y un sistema rápido en invernadero, que se encuentra en fase de prueba, como una mejor manera de obtener las clasificaciones de resistencia en variedades. Nuestra comprensión del agente causal puede abrir nuevas estrategias que reduzcan las pérdidas y disminuyan el costo de los programas de manejo existentes.

**Palabras clave:** Raya clorótica, prueba de diagnóstico, secuenciación de nueva generación, hidroponía