FINAL REPORT 2014/049

SOLVING YCS

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ABSTRACT

Yellow canopy syndrome (YCS) is a sporadic condition presenting as golden-yellowing of the mid-canopy in sugarcane during the peak growing period of December to March. The key driver of YCS is growth rate and symptoms usually exhibit after rainfall. YCS can be induced or mitigated by altering sink strength and sugarcane can recover from a YCS event. Abiotic or biotic stress has a serious effect on the photosystems and the physiological fitness of the crop. There is a strong correlation between YCS expression, leaf sucrose and sink strength, independent of crop age. YCS symptomatic leaves always have high leaf sucrose and α-glucan content. Under experimental conditions the pyrethroid bifenthrin supresses insect stress, promotes increased sink growth and maintains low leaf sucrose and α-glucan levels. Induced senescence causes YCS plants to have a lower number of attached leaves. Yield loss precedes YCS expression and there is no correlation between YCS severity and cane yield or CCS. Lamina starch staining is a useful tool to assist in YCS identification. There is no strong evidence of genetic predisposition for YCS susceptibility. Industry-wide incidence and severity of YCS is too difficult to accurately assess due to its episodic nature, no single causal agent and the link to climate change and severe weather events. The data does not support the cause of YCS being a pathogen, specific insect or mite, soil borne agent, poor root health, nutrient deficiency, or heavy metal toxicity. YCS is a physiological disorder visualised as the terminal expression of metabolic perturbances caused by growth disruption.

EXECUTIVE SUMMARY

The occurrence of Yellow canopy syndrome in Gordonvale Nth Queensland in 2012 was concerning to the Australian Sugarcane Industry due to the unknown impact of this new sugarcane condition. The term YCS was coined when initial observations and analyses confirmed that leaf yellowing associated with the disorder was different to other types of yellowing in sugarcane. The research that unfolded from that point set about to accurately describe the syndrome, investigate what conditions are required for its development, identify how widespread it was, evaluate potential yield impact, explore management options and find the cause of YCS.

To address these issues the current project utilised the expertise of scientists and industry personnel in the areas of agronomy, plant physiology, pathology, metabolomics, chemistry, nutrition, plant stress, soil and root health, microscopy, anatomy, genetics, growth regulators, entomology, agronomic chemicals, farm management, remote sensing and yield modelling. The study presented here covers a research span of up to 7 years for some areas of focus between 2014-2020.

Comprehensive nutrient testing of soil and plant tissue show nutrient deficiencies, heavy metal toxicities, or compromised nutrient mobilization within the plant is not the cause of leaf yellowing in YCS affected plants. Crop age studies show that the onset of yellowing commences in the middle region of the mid-canopy during the peak growing period of December to March. This mostly affects source leaf +3 to +6, and initially, symptomatic leaves will have green leaves above and below them. Therefore, it is imperative that visual diagnosis is made early, before YCS affected leaves age and form a continuum with the lower senescent leaves. The episodic nature of YCS appears to move as a wave through the crop as prevalence of the condition comes and goes. However, symptom synchronicity is independent of crop age and therefore likely driven by external factors. Severity of

YCS usually increases after good rainfall following a dry or slow growth period and typically peaks in mid-February to late March. Symptoms subside as photoassimilation and growth slowdown during the shorter daylength and cooler months. Thus, growth rate is the key driver of YCS.

It is evident from the growth regulator, water stress and crop age experiments that manipulation of sink size or strength can induce or mitigate YCS. Therefore, incumbered sink growth is likely caused by any form of stress that impacts the crop. However, stress shield agrochemical treatments only offered temporary protection to the photosynthetic apparatus and were ineffective in increasing sink size or strength, merely delaying the onset of leaf yellowing. Any substantial limitation to sink size prior to rapid growth, will cause a reduction in mass flow between the source and non-photosynthetic sink tissue. Photosynthesis studies show that leaf +3 and +4 have the highest % C-fixation contribution of the canopy which explains why these two leaves are more susceptible to YCS. When photoassimilation rates exceeds the sink capacity, source leaf sucrose levels will rise, and to offset further accumulation carbon is repartitioned to the α-glucan pool. However, if sucrose accumulation continues and breaches the upper tolerable threshold, the outcome will be reduced stomatal conductance, increased internal heat, photooxidation, chloroplast destruction, cell death and leaf yellowing. Leaf yellowing is irreversible and terminal. Chlorophyll fluorescence studies show disruption to the photosystems well before the onset of yellowing and high levels of leaf sucrose and starch are evident at first light. This is indicative of disruption to the normal diurnal profile which is synonymous with YCS development. Hence, early morning starch staining of YCS affected lamina and midrib with iodine solution is a useful tool to assist in the identification of YCS.

Metabolite studies shown a strong correlation between leaf sucrose accumulation and YCS severity. The cause of leaf sucrose accumulation may be a physical blockage of the phloem. In support of this, microscopy and dye uptake studies show evidence of vascular occlusions and restricted sap movement likely caused by both abiotic and biotic agents. However, extensive microbial and molecular analyses have been unable to conclusively substantiate the existence of a pathogen in any tissue from YCS affected plants, including symptomatic plants derived from pathogen free tissue cultured clean source cane. There is also no association between YCS and soil biology. Furthermore, transmission studies show that seed cane, juice or leaf tissue is not the source of YCS. Some opportunistic endogenous microorganisms are shown to increase in abundance in YCS leaf tissue and are likely secondary in nature. It is also evident that the cause of elevated leaf sucrose may be due to reduced internode growth and sink strength which initiates a source sink imbalance slowing sucrose export from the leaf. Hence, the cause of reduced culm growth may be abiotic or biotic in origin.

Under experimental conditions, the broad-spectrum insecticide bifenthrin is highly effective in maintaining good internode growth and low levels of source leaf sucrose and α-glucans. Current entomological studies indicate that no specific insect is the cause of YCS, and that insect pressure aligned to weather conditions may impact directly on plant growth and sink strength. Therefore, chemical control of insects enables plant resources to be redirected from defence to growth.

Incidence and severity of YCS throughout the sugarcane industry is difficult to assess due to its episodic nature and the link to one or more growth limiting causal agents. The data from clonal assessment and variety trials do not support a genetic predisposition to YCS. Biomass studies show no correlation between YCS severity and cane yield (TCH) or commercial cane sugar (CCS). Leaf monitoring shows the average duration of a YCS event is 8 weeks. Calculations incorporating

reduced photosynthetic capacity as a % of the canopy of these impacted leaves equates to an approximate 2% yield loss. This is supported by APSIM modelling which suggests that YCS has limited, if any effect on yield. Therefore, it is the longevity of the YCS event and not the severity that will cause the greatest yield impact attributed directly to leaf yellowing. However, as YCS is driven by reduced growth, the main yield loss occurs prior to YCS development and expression. Thus, it is the intensity and scale of the growth stressor, be it biotic or abiotic, that is the cause of crop yield loss and not YCS per se.

Preventing the slowdown of crop growth by reducing abiotic and biotic stress on the crop prior to and during the period of high photoassimilation, will lower the risk of YCS development and expression. Therefore, whichever treatment or farming practice removes or reduces the most dominant stressor impacting crop growth, will be the best management option to prevent or mitigate YCS development.

Studies by Bonnett (2018) show that climate change has significantly impacted the northern Queensland sugarcane production over the past 70 years. As the effects of climate change and weather events become more severe, so too will the array of stressors that can reduce crop growth at particular intervals and increase the risk of YCS development. Therefore, we postulate that climate change and in particular extreme weather events, is the large-scale external change that triggered the occurrence of YCS in 2012.

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1. BACKGROUND

1.1. Yellow canopy syndrome (YCS)

When Yellow canopy syndrome was first noted in the Mulgrave Mill area in 2012 it was an unknown sugarcane anomaly of unknown geographical distribution, with an undetermined impact rating. In light of this, the Industry was keen to find the cause of YCS and to have it characterised so that any potential impact could be quantified. Once this was achieved a management program could be adopted to prevent or mitigate the condition. To achieve these goals a pilot project (2013/807) was established in 2013 to investigate the causative factors of YCS through establishment of monitoring sites in the Burdekin and Herbert regions, and to develop strategies to estimate yield impact.

Initial results from the pilot project showed that YCS could be induced through a shock water-stress treatment and that plant stress was a key factor in its development. Plant physiology studies revealed an accumulation of leaf starch and a possible compromised sugar transport system. There was also evidence of microbial entities in the leaf tissue that may be pathogenic in origin. The combined findings elucidated the complexity of YCS and the possible interaction with stress and other unknown entities.

With this knowledge in hand this research project builds off the progress made in 2013 to further characterise this syndrome and to solve YCS. In order to achieve this goal, the research used a multitude of technologies to unravel this complex conundrum.

1.1.1. YCS development and symptom expression

To understand how YCS develops and expresses a monitoring system was established to rate prevalence and severity. This methodology was deployed in all field and pot trials. Through this system a greater understanding was attained of where YCS symptoms start in the canopy, which leaves were first to be affected, and how long symptoms lasted. This understanding revealed that YCS is a mid-canopy condition that affects the main export source leaves. Characterising the physiology of this part of the canopy revealed that leaf +3 and +4 contribute the greatest % C-fixation to the canopy and were typically the first leaves to show YCS symptoms. The increased risk of high sucrose exporting leaves developing YCS concurred with findings of the pilot project study that showed YCS leaves had a compromised sugar transport system. This eventually led to the decision to focus on leaf +4 as the default leaf for YCS experimental focus and sampling within this project and across the YCS integrated research program.

1.1.2. Disruption to plant physiology

High leaf sucrose and starch (insoluble α-glucan) is apparent in all YCS symptomatic leaves. This finding led to the discovery of high levels of both metabolites in source leaves sampled at first light. Based on this information research conducted to understand the effects of high leaf sucrose showed a reduction in mid-canopy photosynthesis, stomatal conductance and C-fixation. Chlorophyll fluorescence studies revealed significant disruption to the PET chain well before the onset of leaf yellowing and the physiological fitness of the photosynthetic machinery.

High source leaf sucrose is either cause by an increase in synthesis creating an imbalance between supply and demand or impeded sucrose export. Reduced phloem transport may be caused by a physical blockage or reduced mass flow between the source and non-photosynthetic sink tissue. To

investigate this further, pathology, microscopy and dye uptake studies were employed to determine if a physical occlusion in the phloem was evident. Similarly, internode volume was evaluated as a proxy for sink strength in YCS symptomatic plants.

The disruption to carbohydrate metabolism in YCS asymptomatic and symptomatic leaves from the same stalk culminated in high levels of starch at first light. This understanding identified starch as a potential candidate for a diagnostic test. Iodine starch staining of the lamina and midrib was determined to be a useful tool to assist in the identification of YCS, and more targeted leaf sampling for research purposes.

1.1.3. Causes of YCS

Identifying the cause of sucrose accumulation in the leaf would be to reveal the cause of YCS. Therefore, addressing this research task involved systematically testing each of the following areas of interest through a series of experiments, field and pot trials and comprehensive sampling of all plant tissue types and surrounding soil:

- Plant stress – abiotic and biotic
- Nutrient deficiency and mobilisation
- Heavy metal toxicity
- Soil biology and root health
- Pathology and pests – viruses, bacteria, phytoplasmas, fungi, nematodes
- Entomology – insects and population studies
- Climate and weather events

1.1.4. Yield and variety susceptibility

To investigate the YCS impact on cane and sugar yield all trial treatments were monitored and scored for YCS prevalence and severity, and analysed for biomass and sugar content. Data was evaluated for varietal susceptibility to YCS and comparative analyses between treated and untreated plots. These agronomic trials were conducted in all sugarcane regions with the assistance of growers and sugar service providers. Broad scale APSIM modelling was also performed by Dr Geoff Inman-Bamber to assess yield losses to YCS in the Burdekin and Herbert regions.

The goal of the above strategies was to assess any correlation between YCS severity and tonnes cane/hectare (TCH) and commercial cane sugar (CCS), which would provide industry with an impact rating of this disorder.

1.1.5.YCS management

Characterisation of this disorder and understanding the cues or triggers that lead to its development sits at the centre of how to manage YCS. Identification of the agent/s that impact on growth and sucrose export from the source leaf will provide the means to develop management practices to prevent or mitigate YCS development.

2. PROJECT OBJECTIVES

The project objectives were to

- Establish whether YCS is caused by an abiotic and/or biotic factor
- Determine which environmental factors and mechanisms drive and or mitigate the development and expression of YCS
- Determine the extent of YCS distribution throughout the sugarcane growing regions
- Assess the impact of YCS on the sugarcane industry
- Explore management options to prevent or mitigate the development of YCS

3. OUTPUTS, OUTCOMES AND IMPLICATIONS

3.1. Outputs

3.1.1 The major outputs delivered by this project are:

- Development of a YCS monitoring, prevalence and severity scoring system
- Determining that the key driver of YCS is growth rate, irrespective of crop age
- Identifying that abiotic and/or biotic factors can inhibit growth rate and sink size, inducing a supply demand imbalance and high source leaf sucrose and starch accumulation during the peak growing season
- Confirming YCS is a mid-canopy syndrome mostly affecting leaf +3 to +6
- Establishing a link between crop stress, weather and YCS development
- Improved knowledge of baseline macro/micro-nutrient levels and nutrient mobility in sugarcane tissue
- A greater understanding of the association between leaf silicon content and crop stress
- Development of methodologies to investigate sugarcane root health
- Optimised molecular pathology protocols
- Increased understanding of sugarcane physiology, photosynthesis, anatomy and vascular transport
- Development of starch staining techniques for use in diagnostics
- Certified trained technician in the use of a scanning and transmission electron microscope (Nirosha Hewage Don)
- Development of microscopy and fluorescent dye uptake methodologies
- Contribution to a YCS management program
- Increased knowledge of sugarcane entomology
- Identification of the mealybug *Heliococcus summervillei* recorded for the first time in Qld on sugarcane
- Assessment of variety heritability and susceptibility to YCS
- Assessment of YCS impact to the sugarcane industry
- Publication from this study (Marquardt et al., 2016)

3.1.2 Adoption

The knowledge and methodologies developed in this project will be beneficial to sugarcane researchers, adoption officers and sugar service providers. This information will be useful to identify and manage YCS and to also assist in the advancement of sugarcane agronomy throughout the industry.

3.2. Outcomes and Implications

The project outputs will be of benefit to sugarcane researchers and industry personnel across the agronomy, physiology, chemistry, plant breeding, disease and pests, entomology, soil and plant nutrient and microscopy/anatomy sectors. The huge volume of research that was conducted to unravel the complexity of YCS has furthered industry understanding of how this crop behaves under a rapidly changing environment. Establishment of methodologies and baseline parameters specific to sugarcane in the areas of agronomy, physiology, nutrition, and entomology, in healthy plants across a large number of modern commercial varieties and clones, is an invaluable resource which can be utilised well into the future. This robust database should enable researchers to conduct quick comparative analyses whenever sugarcane anomalies arise.

The physiological changes attributed to YCS gives the industry a key insight to how this crop has responded to abiotic and biotic stressors that impact growth at key points in the cropping cycle. In particular, it allows for an assessment of the response by crops that are resource limited and those that are not. This will be hugely important for growers and all industry stakeholders as predicted climate change and extreme weather events unfold into the future. Understanding why and how the crop responds to adverse conditions was perhaps the most important outcome of this research project. This knowledge is invaluable to industry, as being able to predict the impact to the crop under certain environmental conditions is vital to the development of management plans to prevent or mitigate losses.

It should be noted that environmental changes also include changes to biotic agents that currently impact the crop and those that currently do not. It is evident from the data that under experimental conditions, the broad spectrum insecticide bifenthrin was highly effective in controlling insects that impacted crop growth. Entomological studies showed that the population dynamics of certain sugarcane insects are aligned with weather events and have a significant impact on plant growth under conducive conditions. While this is not surprising, it highlights one of many biotic entities that may transition from low impact to high impact under a changing climate. This is highlighted by the recent incursion of fall armyworm (Early et al., 2018). Therefore, when abiotic stressors such as water deficit combine with biotic stressors to influence crop growth, the impact to industry will be compounded. Fortunately, the data shows that in general, the impact to industry by YCS is minimal. However, as true yield impact precedes YCS symptom expression, assessment of the magnitude of impeded crop growth prior to YCS development would be more pertinent.

The condition known as YCS may only be a snapshot of how one crop has responded to a rapidly changing environment, but it does emphasize the challenges all crops face into the future. The research outputs of this project have given the industry a competitive advantage in preparedness to address and reduce the impact of future sugarcane anomalies. There are also valuable learnings to be made from this research by the wider agricultural community in Australia and internationally.

4. INDUSTRY COMMUNICATION AND ENGAGEMENT

4.1. Industry engagement during course of project

Communication of objectives and results throughout the project

4.1.1. Presentations to industry and scientific research community

- YCS Scientific Reference Panel reviews and workshops
- Grower updates and shed meetings throughout Qld 2014 - 2020
- Webinar May 6th, 2020 (Appendix 4)
- Productivity and sugar services groups throughout Qld
- Grower delegates and industry representatives
- Canegrowers organisation
- ASSCT conferences

4.1.2. Industry conference papers and posters

1) Braithwaite KS, Mills E, Olsen DJ (2017) A pathology-based investigation into the cause of Yellow Canopy Syndrome. Proceedings of the Australian Society of Sugar Cane Technologists 39: 99–106

2) Di Maggio L, Olsen DJ, Verrall R (2019) WinRHIZO™ software for evaluating effects of farming systems on sugarcane root systems. Proceedings Australian Society of Sugar Cane Technologists 41: 205-211

3) Joyce P, Don NH, Sousa M, Olsen D (2016) Starch accumulation in sugarcane in response to stress. Proceedings Australian Society of Sugar Cane Technologists 38: 20-28

4) Joyce P, Hewage Don N, Olsen DJ (2017) Physiological effects of YCS in sugarcane plants. Proceedings Australian Society of Sugar Cane Technologists 39: 222-225

5) Memory A, Olsen DJ (2017) The effect of defoliation on yellow canopy syndrome (YCS) severity in sugarcane. Proceedings of the Australian Society of Sugar Cane Technologists 39 (Poster)

6) Olsen DJ, Tippett O, Ostatek-Boczynski Z (2019) Plant-nutrient deficiency or heavy-metal toxicity as a cause of Yellow Canopy Syndrome. Proceedings of the Australian Society of Sugar Cane Technologists 41: 352–362

7) Olsen DJ, Ward AL (2019) Effect of neonicotinoid, pyrethroid and spirotetramat insecticides and a miticide on incidence and severity of Yellow canopy syndrome. Proceedings Australian Society of Sugar Cane Technologists 41: 359-366

8) Olsen DJ, Brownlee J (2017) Effect of plant growth regulators on expression of yellow canopy syndrome in sugarcane. Proceedings of the Australian Society of Sugar Cane Technologists 39: 107-113

9) Olsen DJ, Shafiei R, Botha FC (2016) the fast fluorescence kinetics: a sensitive tool for early detection of water stress in sugarcane. Proceedings of the Australian Society of Sugar Cane Technologists 38: 218-227

10) Olsen DJ, Magarey RC, Dibella L, Sefton M, Milla R, Sallam N, Sventek K, Calcino D (2015) Yellow canopy syndrome: A condition of unknown cause affecting sugarcane crops in Queensland. Proceedings of the Australian Society of Sugar Cane Technologists 37: 176-185

11) Ostatek-Boczynski ZA; Olsen DJ; Darain F; Schroeder BL (2016) Nutrient uptake mechanisms in YCS affected sugarcane. Proceedings Australian Society of Sugar Cane Technologists 38: 140 (Poster)

12) Ostatek-Boczynski ZA; Olsen DJ; Tippet O (2019) Nutrient uptake and accumulation in sugarcane affected by Yellow Canopy Syndrome. Proceedings Australian Society of Sugar Cane Technologists 41: 272 (Poster)

13) Pierre JS, Rae AL, Olsen DJ, Perroux JM (2018) Sugarcane root systems: developing a toolkit of methods to understand what's going on below ground. Proceedings of the Australian Society of Sugar Cane Technologists 40: 127-137

14) Tippett O, Olsen DJ, Ostatek-Boczynski ZA (2019) Is magnesium deficiency a causal agent of sugarcane Yellow Canopy Syndrome? Proceedings of the Australian Society of Sugar Cane Technologists 41: 221-229

4.1.3 Other

1) Communication through SRA newsletters, flyers, emails, videos, and website

4.2. Industry communication messages

Key communication points:

- YCS is a sugarcane condition that describes leaf yellowing of the mid-canopy mainly affecting leaf +3 to +6
- YCS symptomatic leaves have a golden-yellow colour
- The key driver of YCS is growth rate, not vice versa
- YCS symptomatic leaves always have elevated levels of leaf sucrose and starch at first light
- High leaf sucrose above an upper tolerable level initiates yellowing and accelerates senescence (aging)
- YCS plants always have fewer attached leaves due to premature induced senescence
- The cause of source leaf sucrose accumulation is the cause of YCS
- The research data does not support a single cause
- Abiotic or biotic factors can limit sucrose transport between the source and sink tissue causing high leaf sucrose accumulation
- High leaf sucrose accumulation initiates the onset of leaf yellowing
- YCS is not a disease

- YCS is always preceded by some form of stress that limits non-photosynthetic sink tissue (internode size)
- Environmental or biological stress or both can be the trigger for YCS
- To prevent or mitigate YCS development the crop growth must be prevented from slowing down during the peak growing period December – March.
- To manage YCS, every available resources should be used together with best practice farming to prevent or reduce the impact of the dominant plant stressor that is impacting growth that leads
- A crop can recover from a YCS event
- YCS usually expresses after good rain or irrigation in crops that have been previously stressed
- High photoassimilation, high leaf sucrose and high light intensity is required to initiate yellowing
- There is no commercial cane sugar (CCS) penalty caused by YCS
- There is no significant cane yield penalty caused by YCS
- Cane yield penalty (period of slow growth) precedes the development and expression of YCS; target this period to reduce yield loss
- There is no correlation between YCS severity and TCH or CCS
- It is highly likely that the occurrence of YCS in 2012 is a response to climate change and adverse weather conditions

5. METHODOLOGY

5.1 Pot and field trials

There were many pot and field trials conducted during the course of the project research 2014 - 2020. As results came to hand many of the trials were repeated and in the following year with variations to investigate new leads and hypotheses. To simplify these activities details of each trial is contained with Appendix 1. A summary of the pot and field trials, excluding those that were abandoned or inconclusive due to weather events or equipment failure, is presented in Table 1 and Table 2 respectively. Pot and field trials were all completed within approximately 12months from establishment and field trial ratooned for subsequent studies are noted in Table 2.

Table 1 Pot trials

Trial	Genotype	Location	Year
Water stress	KQ228$^{\Phi}$	Burdekin	2014
Soilless	KQ228$^{\Phi}$	Burdekin	2014
Variety Assessment	KQ228$^{\Phi}$, Q200$^{\Phi}$, MQ239$^{\Phi}$, Q232$^{\Phi}$, Q208$^{\Phi}$ & Q183$^{\Phi}$	Burdekin	2014
Hormone stress	KQ228$^{\Phi}$	Burdekin	2015

Tissue culture plant source water stress treatment	Q200$^{\phi}$, Q247	Mission Beach	2015
Pathology/stress	KQ228$^{\phi}$, Q200$^{\phi}$	Burdekin	2015
Pathology trial– Quarantine Q200$^{\phi}$ clean cane	Q200$^{\phi}$	Burdekin	2016
Plant source/pathology	KQ228$^{\phi}$, Q200$^{\phi}$	Burdekin	2017
Transmission	KQ228$^{\phi}$, Q200$^{\phi}$	Mission Beach	2015

Table 2 Field trials

Trial	**Genotype/Crop class**	**Location**	**Year**
Environmental stress	Q200$^{\phi}$ 5R	Ingham	2014
Confidor®	Q200$^{\phi}$ P	Stone River Ingham	2014
Insect/stress shield	Q200$^{\phi}$ P	Stone River Ingham	2015
Confidor® and water stress	KQ228$^{\phi}$ P	Burdekin	2014
Soil biology	Q200$^{\phi}$ P	Stone River Ingham	2015
Soil biology trial extension/ including soil fumigant	Q200$^{\phi}$ 1R	Stone River Ingham	2015
Crop age	KQ228$^{\phi}$ 3R	Burdekin	2014
Crop age – extension	KQ228$^{\phi}$ 4R	Burdekin	2015
Root studies	KQ228$^{\phi}$ 1R & 4R	Burdekin – Home Hill & Kalamia	2015
Clonal variation	20 clones P – see Chapter 'Variety assessment' results this report	Burdekin	2015
Clonal assessment (CAT)	60 families with their 15 parents P – see Chapter 'Variety assessment' results this report	Burdekin	2017
Develop clean cane source/Pathology screening	Q200$^{\phi}$ P	Burdekin	2015
Physiological studies	Q240$^{\phi}$ 1R	Burdekin	2016
Farm management (ameliorant)	KQ228$^{\phi}$ 4R	Burdekin	2015
Farm management (ameliorant)	Q232$^{\phi}$ 2R	Herbert	2015
Plant growth regulator	KQ228$^{\phi}$ P	Burdekin	2016

Magnesium	Q240$^{\phi}$ 1R	Ingham	2017
Bifenthrin and streptomycin	KQ228$^{\phi}$ 2R	Burdekin	2018
Regional variety trial (RVT)	16 varieties - see Chapter 'Variety assessment' results this report	Ingham	2017/18
Regional variety trial (RVT) bifenthrin treated	16 varieties - see Chapter 'Variety assessment' results this report	Ingham	2018-2020
Insecticide	KQ228$^{\phi}$ 1R, 2R	Burdekin	2017/19
Insecticide -targeted spray	KQ228$^{\phi}$ 3R	Burdekin	2019/20
Commercial insecticide strip treatment	Q200$^{\phi}$ P, KQ228$^{\phi}$ 2R, Q240$^{\phi}$ P, Q240$^{\phi}$ P	Ingham, Ayr, Mackay & Maryborough	2019

5.2 Clean plant source for trials

5.2.1 Apical meristem tissue culture

Six Q44^{A} sugarcane tops infected with Sugarcane mosaic virus (SCMV) were sourced from SRA Woodford. Tops were surface sterilized with 70% ethanol and the outer leaf whorls surrounding the meristem were removed with sterile forceps and scalpel. Using a dissecting stereo microscope, the very inner leaf whorls and tissue was carefully removed to expose the apical meristem (Cheong et al., 2012). The meristem was dissected and placed on MSB2 medium (Murashige and Skoog (MS) salts + 30g/L sucrose + 0.45mg/L BAP) on separate plates and grown under 16hrs light and 8hrs dark per day (Figure 1A). After approximately three weeks six independent plants regenerated (Figure 1B) which continued to multiply (Figure 1C) before being transferred to MS medium in the light for further growth, multiplication and establishment of a healthy root system (Figure 1D). Each plate contained approximately six clonal plants from the original apical meristem.

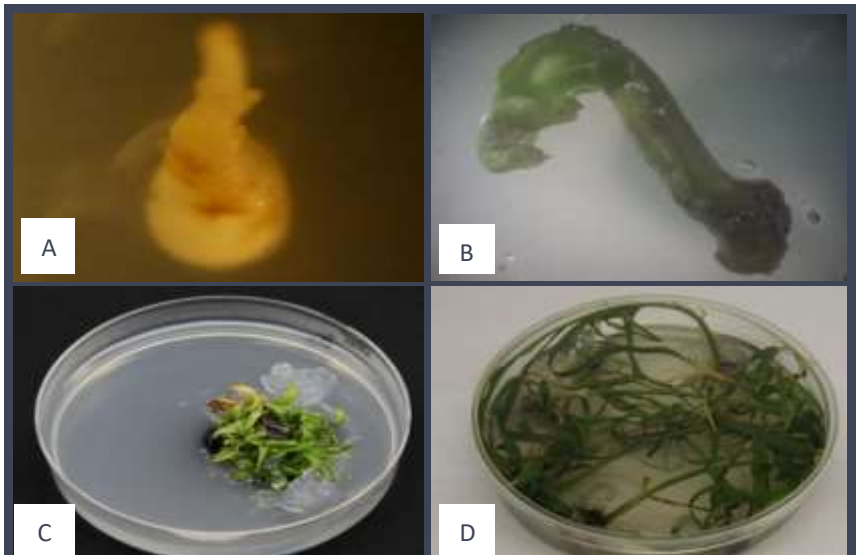


Figure 1 Apical meristem initiation (A) shoot regeneration 3 weeks after initiation (B) multiplication 6 weeks after initiation (C) clonal plants propagated 4 months after initiation (D)

5.2.2 RNA extraction and PCR

A random selection of ten leaves approximately 10mm long were harvested from plants in each tissue culture plate for SCMV screening. Leaves were placed in a 2mL grinding tube with 2 x 4mm plus 2 x 2.8 mm stainless steel balls and ground in a Geno/Grinder® @ 1500 strokes/min for 2 x 20 seconds. The ground material was removed, and RNA extracted using a Bioline Isolate II RNA Plant Kit BIO-52077. The quality of the RNA was checked by first producing cDNA using RT-PCR and then standard PCR with the following primers RubiscoRNAf 5' GGA TCC GGT GCA TGC AGG TG 3' RubiscoRNAr 5' GGG CTT GTA GGC GAT GAA CG 3'. One step Qiagen® RT-PCR kit together with the following SCMV primers **S400 551** 5'-ACA CAA GAG CAA CCA GAG AGG **S400 910** 5'-AGT CAA AGG CAT ACC GCG CTA was used to detect SCMV

5.3 Material sampling

For the purpose of this report material classified as Controls were any culms with mid canopy leaves that were asymptomatic for YCS. The amount of sample biomass and type of tissue collected for analysis varied between experiments and trials and was determined by the type analysis to be conducted. However, the following protocols were adhered to whenever possible.

5.3.1 Leaf, internode, sap, and roots

The first fully expanded leaf with a visible dewlap (FVD) attained the notation of Leaf +1 or Leaf 1 or L1 (Figure 2A) (Bonnett, 2013). Each leaf thereafter above or below L1 is in decreasing or increasing numerical order (Figure 2B). Mostly leaf sections were collected from the middle region of the leaf unless otherwise specified. Leaf punches were also collected from the same region on either side of the midrib depending on the research directive. For the majority of analyses samples were kept cool on wet ice or freezer blocks. Samples were snap frozen in liquid nitrogen or dry ice for RNA studies. For in-house metabolite studies leaf punches were placed in a 2 mL screw cap tube (Figure 2C) and snap frozen or dropped into a Thermos filled with boiling water for 15 mins and then transferred to a -20°C freezer. All tissue sampled for pathology analyses were taken from material that had the exterior surface first wiped with 70% methylated spirits or alcohol wipes. All instruments used for sampling were cleaned with 70% methylated spirits between samples.

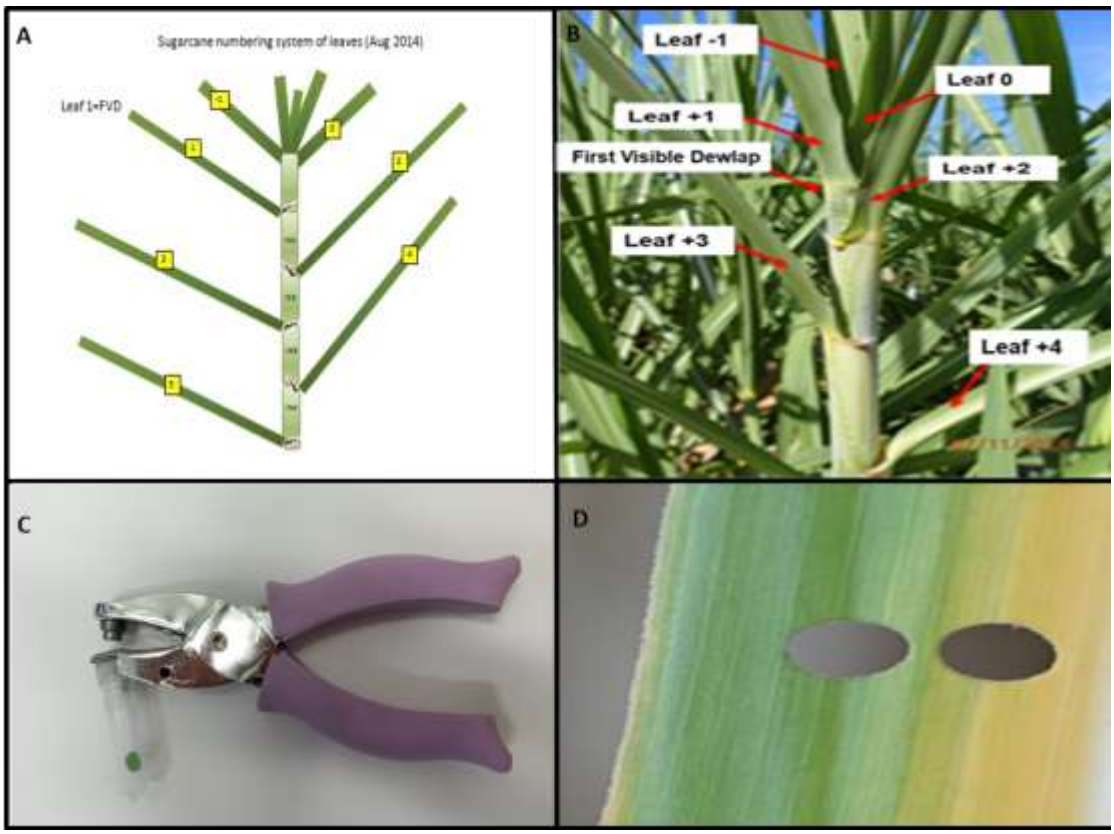


Figure 2 Schematic diagram of sugarcane leaf numbering system used during sampling (A) photograph indicating Leaf 1 in the leaf with the first visible dewlap from the top (B), modified Fiskars hole punch 6.35mm Ø and 2mL screw cap tube (C), example of leaf disk sample taken from early stage (ES) and late stage (LS) YCS lamina (also used for midrib) (D)

Internode samples were cut mostly on an angle on one end with secateurs and handled in the same way as leaf tissue unless otherwise stated. Compressed air was used to push sap from the internodes (in the direction from bottom to top) into collection tubes and stored at either -20°C or -80 °C. All equipment was sprayed with 70% methylated spirits or wiped with alcohol wipes between samples.

At each stool, all stalks were cut off at ground level and 8 soil cores (5cm x 30cm) were collected in a 30cm radius from the centre of the stool. Stalk height and biomass was measured. Soil cores were placed in a solution of 15% Sodium Bicarbonate and allow to soak overnight. Roots were then washed out, using a series of sieves to collect all root material. Root biomass was then measured. Alternately a soil core (200mm diameter x 200mm depth) was collected from within the row and adjacent to the stool. Soil cores were washed, passed through a series of sieves, and roots carefully removed. Roots were weighed before being placed in a clear Perspex tray and scanned at 800dpi with a flatbed scanner. Root analysis was then conducted on scanned images using WinRHIZO software (Regent, Quebec, Canada).

5.3.2 Nutrient investigation

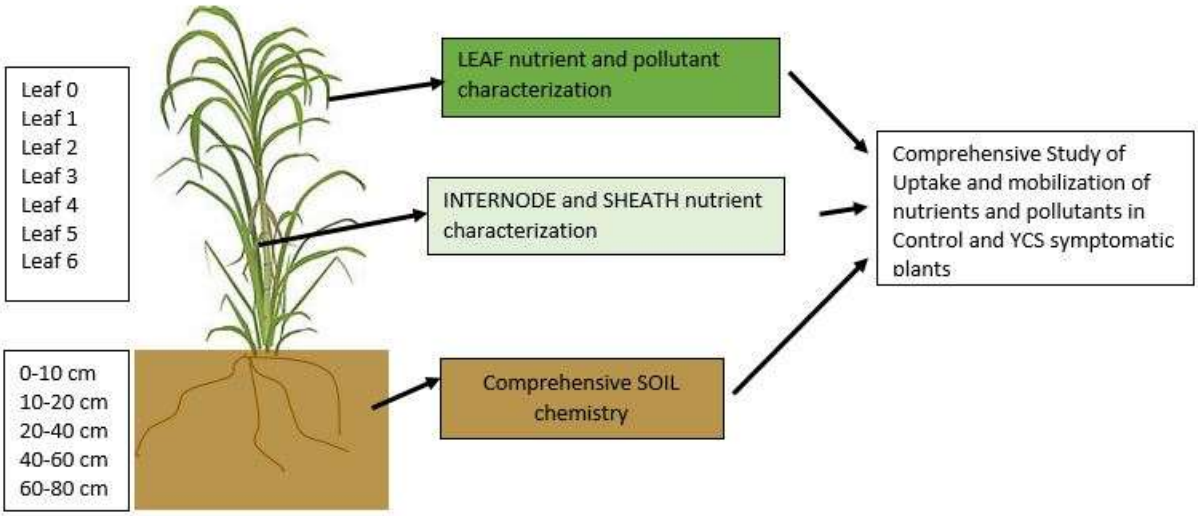
Twenty Leaf+1 (FVD) including lamina and sheath were collected for each genotype to make up one rep and three reps reps were collected for each site in the field. Leaves were folded in half (top to base) and a piece 100-200 mm length was cut out and the remainder discarded. The midrib was

stripped out and discarded. The material was washed it with deionised water and patted dry dry with paper towel. The material was then placed in a drying oven at 65°C for 3 days before processing for nutrient extraction. Internodes from the same plants were passed through a Dedini shredder before being oven dried at 65°C for 3 days. Roots from the same plant were collected and processed as described in section 5.3.1.

The sampling protocol included collecting samples of soil, internode, sheath and leaf. Soil and plant samples were collected at multiple locations from Control and YCS symptomatic sites at two selected districts: Burdekin and Herbert. The two regions were selected as they displayed YCS prevalence over the current and previous seasons and the regions represented contrasting soil types with Herbert predominantly with low pH soils and limited irrigation and from Burdekin with primarily neutral to alkaline soils and full irrigation as a standard agronomic practice.

The sampling protocol included two major varieties for the regions: Q208(ф) and KQ228(ф) (Figure 3).

Selecting appropriate soil and plant samples:



Selecting sampling regions and sites:

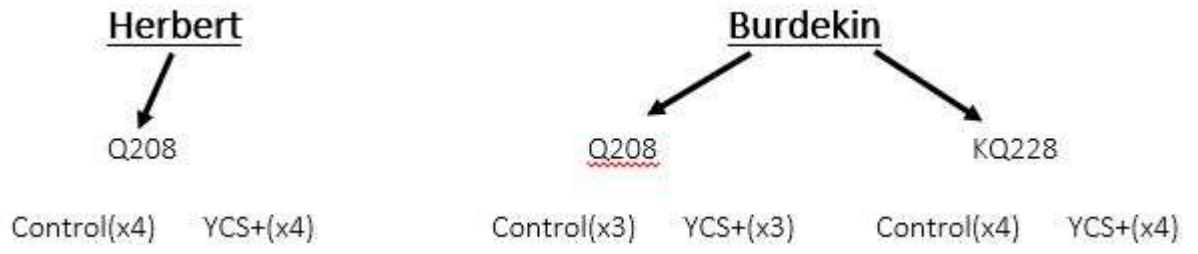


Figure 3 Soil and plant sampling and sugarcane regions

Three or four sites of Control and YCS symptomatic locations were chosen at each region for all varieties tested. At each sampling site: soil, internode, sheath and leaf samples were collected.

Soil sampling was conducted from 10 or 20 cm depth intervals from 0 to 80 cm; four cores were taken for each rep and subsequently the four sub-samples for each depth were combined and prepared for analysis.

In each location 30 or 40 soil samples were collected, in total 110 soil samples were collected and those were subjected to comprehensive chemical testing.

Selecting appropriate plant samples along the stalk:

Internode and the corresponding sheath and leaf samples were collected along the stalk. Collected samples included Internode 0 to Internode 6; sheath 0 to sheath 6 and leaf 0 to leaf 6; total samples per each rep included: 7 internode, 7 sheath and 7 leaf samples

In total 462 plant samples were collected:

- 126 plant samples from Q208 sampling at Burdekin (3 reps)
- 168 plant samples from KQ228 sampling at Burdekin (4 reps)
- 168 plant samples from Q208 sampling at Herbert (4 reps)

Analysis of all soil and plant samples are currently in progress, approximately 70% completed.

5.4 Yield

Biomass was determined by either hand or machine harvest. Hand harvest biomass is described in section 5.4.1 whereas machine harvest weights were measured with a weigh truck or determined by bin weights at the respective sugarcane mill. CCS was either determined in-house through NIR analysis or through receipt of mill data.

5.4.1 Biomass sampling to determine cane yield & CCS

Within the trial, 2 x 5m representative sections per plot (recommend: Rows 1 & 4) are selected and a stalk count is conducted in each. Thirty consecutive stalks from each plot were cut as close to the base as possible and all 60 stalks are weighed. Twenty randomly selected stalks (10 from each 5m section) were weighed as a whole. Ten of these stalks were measured from the base to the first visible dewlap (FVD) and then cut between the 5th and 6th node and the cabbage placed aside. All 20 stalks were stripped of all leaves and then stalks, and cabbage weighed separately to determine the proportion of millable stalk. From these stalks 3 were retained for wet dry weight and 6 were processed through SpectraCane™ 400 fully automated high-speed sugarcane analyser to determine CCS. Three stalks and 4 cabbage tops and leaves were mulched and a subsampled placed together with the mulched stalk for drying in an oven @60 degrees Celsius for approximately 7 days to establish a dry weight.

5.5 YCS Monitoring and scoring

The YCS rating system consisted of a severity scale from 0-4 (Table 3) and a prevalence rating based on the percentage of the crop showing that severity (Table 4). Ratings were conducted on different canopy portions: low (below leaf +5), mid (leaves 1-5), and upper canopy (above leaf +1).

Table 3 Prevalence rating key

Prevalence Rating	Description (% of plot affected)
0	0%
1	1-25%
2	26-50%
3	51-75%
4	76-100%

Table 4 YCS Severity rating key

Severity Rating	Description (degree of yellowing on leaves)
0	No YCS symptoms evident
1	Yellowing is present in approximately 25-50% of the leaf. It may be presented in a solid yellow form or as mottling either along the leaf edges, tips or on one side of the midrib only
2	Yellowing is present in approximately 50% of the leaf in either solid yellow or mottling form. Yellow colour exhibits a stronger orange hue than rating 1. Typically found on both leaf margins and leaf tip although symptoms can occur on one side of the midrib only
3	Yellowing is present in at least 75% of the leaf. Advanced yellowing across the entire leaf blade, with mottling now developed into solid colouring.

Weekly severity scoring was performed on Leaf+1 to L+7 in trials

The following formula was used to calculate a comparable rating between canopies:

Canopy YCS severity rating = sum of individual leaf ratings/the total leaf count between L+1 and L+7

5.6 Sample processing

5.6.1 Lyophilisation of samples

Metabolite sensitive material that was unable to be stored at -80°C was lyophilised in a CHRIST® Alpha 1-4 LSC Plus Freeze dryer. After lyophilisation of mid leaf samples, the midrib was removed and leaves passed through a small shredding mill. After shredding the required amount of material was transferred to 2 ml tubes containing a little sterile sand (3 x small spatula scoops) and 2 x 3mm and 2 x 2.38mm stainless steel balls and ground in a Geno/Grinder® for 10 min @1750 strokes/min. Powder from replicates was then equally combined and thoroughly mixed to make one stock

sample. Dry powder portions of 100 mg were sent to Metabolomics Australia University of Melbourne Victoria for metabolome analysis.

Lyophilisation was also used to determine water content by calculating the difference in sample mass before and after freeze drying. When a freeze dryer was unavailable a drying oven was substituted.

5.6.2 Extraction of carbohydrates from lyophilised material or a single fresh leaf disk

Finely ground lyophilised lamina (3.0 mg) or one whole fresh leaf punch was used for the extraction. Chlorophyll was extracted in 500 µL of 100% V/V acetone (precooled to -20°C), and vortexed thoroughly, left overnight at -20°C, vortexed and then centrifuged for 5min at 6000Xg at 4°C. Acetone solution containing chlorophyll was removed and kept aside for chlorophyll quantification. A further 500 µL (V/V) acetone (precooled to -20°C) was added to the pellet and chlorophyll re-extracted as above. Both supernatants were combined for chlorophyll determination.

The pellet was then left to air-dry before 200 µL deionised water was added and incubated at 70°C for 30 minutes followed by centrifugation at 16000Xg for 10 minutes. Supernatant was removed and retained, and the pellet re-extracted with water as before. The combined supernatant was filtered through a 0.45 µm PVDF filter and used to determine sucrose, glucose, fructose, and soluble alpha-glucan. Two small spatula scoops of sterile sand and 2 x 3mm and 2 x 2.38 mm stainless steel balls were added to the residual pellet and the sample ground in a Geno/Grinder® for 12 min @1750 strokes/min. Potassium hydroxide (20 µL of 4.2M) and 400 µL deionised water was added to the residual pellet and autoclaved for 2 hrs at 121° C (~210kPa), ground for a further 5 mins, then cooled, neutralised with acetic acid (70 µL of 1M) and centrifuged at 16,000xg for 10 minutes. Supernatant was removed and used for starch determination.

5.6.3 RNA extraction

Fresh leaf material was ground to a very fine powder under liquid nitrogen in a mortar and pestle. RNA was extracted from the laminar material using Qiagen RNeasy Plant Mini Kit (QIAGEN N.V., The Netherlands) according to the manufacturer's instructions with the addition of 2.5% (w/v) PVP-40, with the RNA eluted twice in the elution buffer. In brief, 600 µL of RLT buffer was added to tissue powder and centrifuged at 15,000 rpm for 5 minutes before passing through the QIAshredder column and eluting with 60µL RNase-free water passed through membrane twice. RNA quantity was checked with the NanoDrop for yields > 100 ng/µL and A_{260}/A_{230} > 1.0. RNA quality was checked with 1.5% TBE agarose gel containing 1 % SybrSafe to visualize 18s and 28s ribosomal RNA bands.

5.6.4 Extraction of metabolites for GC-MS (Untargeted) and LC-MS (Amino Acids and Untargeted Profiling)

Approximately 30 mg of homogenized leaf was added to a cryomill tube. Methanol (100%) (500 µL), and a quantitative internal standard containing 4% [(13C6-Sorbitol (0.5 mg/mL), 13C5-15N-Valine (0.5 mg/mL); 2-aminoanthracene (0.25 mg/mL) and pentafluorobenzoic acid (0.25 mg/mL)] was added. The sample was vortexed for 30 sec and was subsequently homogenized using a cryomill (Bertin Technologies) using program #2(6100-3 x 45 - 045) at -10°C. The sample mixture was then incubated at 30°C, and agitated at 850 rpm for 15 mins and then centrifuged at 13,000 rpm for 5 mins at 4°C. The supernatant containing methanol was then transferred into a new Eppendorf tube. Milli-Q Water (containing formic acid, 2%) (500 µL) was added to the remaining

pellet in the cryo- mill tube. The sample was vortexed for 30 sec and then centrifuged at 13,000 rpm for 5 mins at 4°C. The supernatant was then combined with the previous methanolic supernatant. A (50 µL) aliquot and a (5 µL) were transferred into glass inserts and dried in vacuo for subsequent TMS polar metabolite derivatisation. Extracted leaf tissue samples were placed in a snaplock bag with silica gel prior to derivatisation for GC-MS analysis. A 10 µL aliquot of the extract was transferred into an Eppendorf tube for subsequent amino acid metabolite derivatisation (LC-QQQ-MS) and a 50 µL aliquot was used for LC-QTOF-MS Profiling. Aliquots for LC-MS (Amino acid quantitation and untargeted profiling) were stored at -20°C prior to analysis.

5.6.5 Derivatisation of Polar metabolites

The dried samples were re-dissolved in 10 µL of 30 mg mL^{-1} methoxyamine hydrochloride in pyridine and derivatised at 37°C for 120 min with mixing at 500 rpm. The samples were then treated for 30 min with 20 µL *N*,*O*-*bis*-(trimethylsilyl)trifluoroacetamide (BSTFA) and 2.0 µL retention time standard mixture [0.029% (*v*/*v*) *n* dodecane, *n*-pentadecane, *n*-nonadecane, *n*-docosane, *n*-octacosane, *n*-dotriacontane, *n*-hexatriacontane dissolved in pyridine] with mixing at 500 rpm at 37°C. Each derivatised sample was allowed to rest for 60 min prior to injection.

5.6.6 Amino acids

Two different stock solutions were used, 1) Amino acids, containing a standard mix of 25 amino acids in water 0.1% formic acid and 2) Sulphur containing compounds: a 2.5 mM stock solution containing glutathione and s-adenosyl-homocysteine in water with 10 mM TCEP and 1 mM ascorbic acid. The solutions were mixed and diluted using volumetric glassware with water containing 10 mM TCEP and 1 mM ascorbic acid, 0.1% formic acid to produce the following series of combined standards: 0.1, 0.5, 1, 5, 10, 20, 50, 100 and 150 µM

5.6.7 Chemical elements

All samples were labelled and processed prior further chemical analysis. The midrib was removed from all fresh leaf samples, followed by drying at 65^{o}C until constant weight. The dry samples were ground to a particle size of less than 0.5 mm using a Culatti MFC micro-hammer mill (Kinematica AG, Lucerne, Switzerland). The traditional method of nitric-perchloric acid digestion was utilised for sample decomposition (Zasoski and Borau, 1997). This method was used for the extraction of nutrient elements (phosphorus, potassium, sulphur, calcium and magnesium) as well as trace elements (copper, zinc, iron and manganese).

The open vessel hot plate digestion procedure was conducted in duplicates to obtain two analytical replicates, using a 500 mg of each plant sample in a 10ml mixture of 4:1 concentrated nitric acid (69%, AR grade) and perchloric acid (70%, AR grade).

The Dumas dry combustion technique was performed in duplicates for the analysis of nitrogen (N) and carbon (C), using an Elementar Vario Max CN analyser. Sample sizes of plant material were restricted to 0.2-0.3 g, and results are reported as % dm.

Extraction of silicon (Si) was conducted using a modified method of Fox et al. (1969) consisting of dry ashing of 0.5 g prepared plant sample at 600°C for 4h, followed by NaOH fusion. This method is our reference method for the assessment of Si in plant tissue and has been correlated with well-

established XRF techniques (Ostatek-Boczynski and Haysom, 2003) (Haysom and Ostatek-Boczynski, 2006).

5.6.8 Starch staining

Leaf tissues were bleached to remove chlorophyll before staining. Leaf samples were boiled in 95% ethanol over a prolonged period (~1hour) and then rinsed with DI water. A small quantity of 2% Lugol's reagent was applied to the tissue for 30 seconds and any excess removed. Larger leaf sections were under 2% Lugol's for 15mins.

5.6.9 Protein extraction

Different machines to homogenise the tissues were tested to determine was best for sugarcane samples.

Four extraction methods and four buffers were tested. RIPA and Nonidet buffer, using a mortar and pestle gave the best profile a (FastPrep™ versus GenoGrinder™ versus Precelly™ versus mortar and pestle). All of the samples had detectable amounts of protein and loading large volumes of sample/buffer (10ul) did not affect how the gel ran; 14ul (maximum) is sufficient for loading

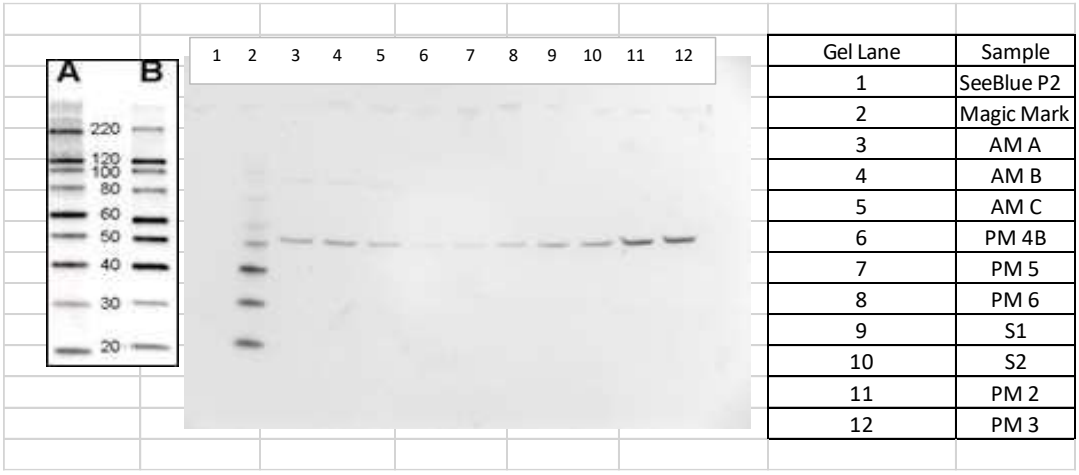
Samples extracted with RIPA or Nonidet buffer showed protein degradation with large amount of <3kdalton products (data not shown). Mortar and pestle extracted the most amount of protein followed by FastPrep™. Both the Precelly™ and Genogrinder™ methods produced less total protein. The extraction buffers (RIPA, Nonidet and TNT-4), were also assessed for amount and integrity of protein extracted (Table 5). Protein yields using three extraction buffers tested was low (<1mg/mL).

Table 5 Composition of extraction buffers for protein from sugarcane leaves

	TNT-4 Extraction Buffer	Nonidet-P40 (NP40) buffer	RIPA buffer (Radio Immuno Precipitation Assay buffer)	Tris buffer
Tris		50 mM	50mM	100 mM
Hepes (MW 238.3)	50mM			
NaCl (MW 58.4)	75mM	150 mM	150 mM	
KCl (MW 74.5)	75mM			
Detergent	0.05% (Tween-20)	1.0% NP-40 *	1.0% NP-40 *	
PEG (MW 3350)	1.00%			
Ethylene glycol	10.00%			
Thimerosal	0.01%			
Sodium deoxycholate			0.50%	
Sodium dodecyl sulphate			0.10%	
EDTA				10 mM
CaCl2				2 mM
BME				50 mM
pH	7.65	8.00	8.00	8.00
* NP40 or Triton X100				

To extract more protein, Tris buffer was used to successfully extract ~1mg/mL of soluble protein. Methods to transfer the proteins to the nylon membrane were optimised. Using the Tris buffer,

6.5ug of protein was loaded and transferred to the nylon membrane using the Iblot™. This step was optimised to enable visualisation of all protein molecular weights (data not shown). A Western detection of the antibody to the ADP glucose pyrophosphorylase was tested on the nylon membrane. Results showed that the transfer of proteins was uniform and the antibody was detected well and was of the expected size (Figure 4). Detection of the amylase antibody was also successful.



Gel Lane	Sample
1	SeeBlue P2
2	Magic Mark
3	AM A
4	AM B
5	AM C
6	PM 4B
7	PM 5
8	PM 6
9	S1
10	S2
11	PM 2
12	PM 3

Figure 4 Western blot probed with the ADP glucose pryophosphorylase antibody. Size of the band is ~50kDa which is the expected size

5.6.10 DNA extraction from insects

Insects were fragmented in a petri dish with a scalpel, then transferred to a 2ml screw-cap Eppendorf tube. A Qiagen DNeasy Blood and tissue kit Buffer was then used to extract DNA. Buffer ATL was added, and insect fragments were further ground with the small pestles. After the addition of Proteinase K and a ceramic bead (yellow ridged king), the tubes were shaken in the FastPrep 4.0m/s for 30 sec. followed by incubation at 56^{0}C for 1 hr. The protocol continues with several spins and addition of wash buffer and finally elution buffer. The DNA extracts were quantified using picogreen or the Qubit, and ranged between 15 to 100ng/uL. DNA was then stored at -20^{0}C until required.

5.7 Sample analyses

5.7.1 Quantification of carbohydrates

Sucrose content was determined using the standard enzymatic method (Bergmeyer and Bernt, 1974) with a spectrophotometer (BMG-Labtech, FLUOstar Omega) and 96-well UV-clear plate (Thermo Fisher, UV Microtiter).

Glucose composition was determined using Amplex® Red/glucose oxidase enzyme assay (Life Technologies) in a 96-well plate (Thermo Fisher, Microtiter) with a spectrophotometer (BMGLabtech, FLUOstar Omega).

Fructose content was determined using a BioVision Fructose Flurometric assay kit in a 96-well plate (Thermo Fisher, Microtiter) with a spectrophotometer (BMG-Labtech, FLUOstar Omega). A 1/10 dilution of the OxiRed probe and a running temperature of 37°C was optimal for this assay.

Starch and soluble α-glucan were digested in a sodium acetate buffer (100mM, pH 5.5) containing 10 U amyloglucosidase per reaction for 2h at 37°C. After cooling down to room temperature, glucose was measured in the resulting solution as described above (Bergmeyer and Bernt, 1974).

5.7.2 GC-MS analysis

Samples (1 µL) were injected in split less (lower and higher aliquots) into a GC-MS system comprised of a Gerstel 252 autosampler, a 7890A Agilent gas chromatograph and a 5975C Agilent quadrupole MS (Agilent, Santa Clara, USA). The MS was adjusted according to the manufacturer's recommendations using *tris*-(perfluorobutyl)-amine (CF43). The GC was performed on a 30 m VF-5MS column with 0.2 µm film thickness and a 10 m Integra guard column (J & W, Agilent). The injection temperature was set at 250°C, the MS transfer line at 280°C, the ion source adjusted to 250°C and the quadrupole at 150°C. Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1}. For the polar TMS metabolite analysis, the following temperature program was used; start at injection 70°C, a hold for 1 min, followed by a 7°C min^{-1} oven temperature, ramp to 325°C and a final 6 min heating at 325°C. For the polar metabolite analysis, the following temperature program was used; start at injection 70°C, a hold for 1 min, followed by a 7°C min^{-1} oven temperature, ramp to 325°C and a final 6 min heating at 325°C. Both chromatograms and mass spectra were evaluated using either the Agilent MassHunter Workstation Software, Quantitative Analysis, Version B.05.00/Build 5.0.291.0 for GC-MS. Mass spectra of eluting compounds were identified using the public domain mass spectra library of Max-Planck-Institute for Plant Physiology, Golm, Germany (http://csbdb.mpimp- golm.mpg.de/csbdb/dbma/msri.html) and the *in-house* Metabolomics Australia mass spectral library. All matching mass spectra were additionally verified by determination of the retention time by analysis of authentic standard substances. Resulting relative response ratios (area of analyte divided by area of internal standard, ^{13}C6-sorbitol) per sample FW (mg) for each analysed metabolite as previously described (Roessner et al. 2001). The data was also normalized in order to compare fold differences between groups. If a specific metabolite had multiple TMS derivatives, the metabolite with the greater detector response and improved peak shape within the dynamic range of the instrument was selected.

5.7.3 LC-QQQ-MS

LC-QQQ-MS - An Agilent 1200 LC-system coupled to an Agilent 6410 Electrospray Ionisation-Triple Quadrupole MS was used for quantification experiments. Injection volumes of 1 µL of samples or standards were used. Ions were monitored in the positive mode using a Dynamic Multiple Reaction Monitoring (DMRM) method optimized for each analyte. The source, collision energies and fragmentor voltages were optimized for each analyte by infusing a derivatised standard with LC eluent. The following source conditions were used: gas flow 10 L.min-1, nebulizer pressure 45 psi and capillary voltage 3800 V.

An Agilent Zorbax Eclipse XDB-C18 Rapid Resolution HT 2.1 x 50 mm, 1.8 µm column was used with a flow rate of 300 µL min^{-1}, maintained at 30°C, resulting in operating pressures below 400 bar with a 19 minute run time as previously described (Boughton et al., 2011). A gradient LC method (Table 6) was used with mobile phases comprised of (A) 0.1% formic acid in deionized water and (B) 0.1%

formic acid in acetonitrile. These conditions provided suitable chromatographic separation of modified amino acids. Co-elution was observed for some of the species, but this could be accounted for by using the mass-selective capabilities of the mass spectrometer using MRM (multiple reaction monitoring).

Table 6 Gradient LC Method for 6410-QQQ

	% B
0.00	1
2.00	1
9.00	15
14.0	30
14.1	1
19.0	1

5.7.4 Amino acid quantification

Derivatisation was done by using 10 µL aliquots of each standard or sample. These were added to 70 µL of borate buffer (200 mM, pH 8.8 at 25°C) containing 10 mM TCEP, 1 mM ascorbic acid and 50 µM 2-aminobutyric acid. The resulting solution was vortexed, then 20 µL of AQC reagent (200 mM dissolved in 100% ACN) was added and immediately vortexed. The samples were heated with shaking at 55°C for 10 minutes then centrifuged and transferred to HPLC vials containing inserts.

5.7.5 Photosynthesis

Measurements on all available green leaves starting from one above the youngest fully expanded leaf were taken throughout the day to encompass a range of vapour pressure deficit (VPD), radiation, light, and other environmental stress conditions. Following standard settings recommended for C_4 plants, leaf gas exchange measurements were made twice a day on control and YCS-symptomatic plants using two LiCOR 6400 instruments (Long et al., 1996). The stomatal conductance, leaf level photosynthesis, internal CO_2 (Ci) and intrinsic transpiration efficiency were also measured during the day.

During gas exchange measurements, the sample CO_2 concentration and airflow rate was maintained at 400 µmol$m^{-2}s^{-1}$ and 500 mol m^{-2} s^{-1}, respectively. The photosynthetically active radiation (PAR) was maintained at 1500 mmol m^{-2} s^{-1} with the internal red, blue, and green light sources. The intensity of blue light in the light source was 10%, while red was 80%. The standard CO_2 matching option was used after each set of measurements for greater accuracy.

5.7.6 Chlorophyll A fluorescence

Chlorophyll a O–J–I–P fluorescence transients (Strasser and Govindjee 1992) were recorded from leaves 1 to 6. Measurements were performed on the broadest midsection of the leaves, of a minimum of 10 plants for each group (with or without visual expression of YCS). Measurements were conducted with a PEA fluorescence meter (Hansatech Instruments Ltd., King's Lynn, Norfolk, PE 30 4NE, UK). The transients were induced by a red light (peak at 650 nm) of 3,200 µmol m^{-2} s^{-1} provided by the PEA instrument through an array of six light-emitting diodes (van Heerden 2014). The JIP-test (Strasser and R.J. 1995) was subsequently employed to analyse each recorded

transient. The following data from the original measurements were used: maximal fluorescence intensity (FM); fluorescence intensity at 50 µs (considered as F0); fluorescence intensity at 300 µs (F300 µs) required for calculation of the initial slope (M0) of the relative variable fluorescence (V) kinetics; the fluorescence intensity at 2 ms (the J step) denoted as FJ. VJ was calculated as (FJ - F0)/(FM - F0). The JIP-test (Strasser and R.J. 1995) was used to translate the original recorded data to biophysical parameters that quantify the stepwise energy flow through Photosystem II. A multi-parametric expression performance index (P_{ABS}), was also calculated (Strasser et al. 2000). The P_{ABS} considers the three main steps that regulate photosynthetic activity by a Photosystem II reaction centre (RC) complex, namely absorption of light energy (ABS), trapping of excitation energy (TR) and conversion of excitation energy to electron transport (ET). The formulae used to calculate each of these biophysical parameters from the original fluorescence measurements are as previously detailed (van Heerden et al. 2007b)

5.7.7 Chlorophyll content

A SPAD 502 Plus Chlorophyll Meter was used to measures leaf chlorophyll content in field. Ten measurements were made in the same position on the leaf and then averaged to obtain a more accurate representation of lamina chlorophyll abundance.

5.7.8 Nutrient

Chemical analyses (leaf, leaf sheath and internode)

Analytical determination of major nutrient elements: phosphorus (P), potassium (K), sulfur (S), calcium (Ca) & magnesium (Mg) as well as trace elements: copper (Cu), zinc (Zn), iron (Fe) and manganese (Mn) was conducted on an Agilent Technologies 725-ES simultaneous radial viewing ICP-OES (Inductively Coupled Plasma Optical Emission Spectroscopy).

Analysis of nitrogen (N) and carbon (C), was conducted using an Elementar Vario Max CN analyser. The results for all major nutrient elements were reported as % dry matter (dm) and trace elements in mg/kg dm.

The final analytical determination was conducted on an Agilent Technologies 725-ES simultaneous radial viewing ICP-OES instrument (Agilent Technologies Australia, Forest Hill, Victoria) using a Sturman-Masters double pass spray chamber and V groove nebulizer. Results were reported in % dm for major elements: Ca, Mg, P, K, S, Si and Na and in mg/kg dm for trace elements: Cu, Zn, Fe and Mn.

Additionally, as a standard procedure a Certified Reference Material (CRM) Citrus Leaves (NCS ZC73018) was used within all plant analytical methods, to ensure quality outputs.

Soil

Standard published soil analytical methods (Rayment and Lyons, 2011) were used for the estimation of soil chemical properties and assessment of plant available nutrients.

For the estimation of available silicon two methods were used: the BSES-P method (Method 9G2) as well as calcium chloride extraction method (BSES Soil Method 6) (Rayment and Lyons, 2011).

Both methods are used in conjunction across Australian Sugar Industry for the interpretation of plant available silicon in soil.

Heavy metals

Analysis was conducted at DSITI laboratory (Department of Science, Information Technology and Innovation, Science Division, 41 Boggo Road, Dutton Park, QLD 4102).

5.7.9 Residue analysis

Methodology

Crop samples (tops, billets and stalks) were taken from both untreated and treated plots, at various foliar applications and treatment timings, from 2.4 to 6.7 months after last application (MALA) of Actral® 250 EC at a single rate of 80 g a.i./ha of bifenthrin.
Expressed juice was extracted from the sugarcane stalks within two hours of collecting the stalk samples. Sugarcane tops, billets and expressed juice samples were frozen prior to dispatch to the SRA ChemLab facility in Indooroopilly for analysis.

The residues of bifenthrin were extracted from the plant matrices by solvent homogenisation. Column clean-up was carried out using C18 solid phase extraction. Quantification was possible using liquid chromatography with a single quadrupole mass detector (LC MS) and external standardisation.

The reported limit of quantitation (LOQ) for each sample portion in the method was set at the lowest fortification level and tested with acceptable recovery, precision and selectivity. These LOQs also meet the requirements set in APMVA, Agricultural and Veterinary Chemicals Code Instrument No. 4 (MRL Standard) 2012, in food commodities and animal feed commodities for bifenthrin with a MRL (maximum residue level) at or about the limit of analytical quantitation.

The LOQ for: sugarcane tops was set at 0.020 mg/kg; billets was set at 0.010 mg/kg and; expressed juice was set at 0.010 mg/L.

5.7.10 Transcriptome sequencing

Ribosomal RNA-depleted total RNA was checked for quality on the Agilent 2100 Bioanalyzer, then sequenced by LC Sciences (Tx,USA) on Illumina HiSeq 2000/2500. Samples were multiplexed 4 per lane, to give 8-14 times coverage. Between 69-101 million 2x100bp paired-end reads per sample were obtained for the FV9 Herbert samples. Reads were trimmed for quality (CLC Genomics Workbench v8.5) and mapped against the FV9 Herbert de novo contig set for differential expression analysis (CLC Genomics Workbench v8.5, assembled 63,708 de novo contigs with N50 of 1066).

5.7.11 Statistical analyses

Statistical analysis of the metabolite data was performed using MetaboAnalyst 3.0 http://Metabolanalyst.ca, (Xia et al., 2015). Fold change was calculated as the means ratios of each treatment compared with the asymptomatic tissues and T-tests with unequal variances were performed to compare data obtained between experimental groups. The false-positive rate associated with multiple comparisons was calculated using the false discovery rate (FDR) or Bonferroni-corrected *P* values were also calculated. All tests with significance of $P < 0.05$ were considered in the analyses (Xia et al., 2015).

Statistical analysis of in-house carbohydrates and yield data was through Statistix10

Statistical software package Statistix 10 was used to analyse carbohydrate data. An Analysis of Variance (ANOVA-completely randomised design) was used to compare starch, sucrose, glucose, and fructose means from mid-leaf extracts to identify if there are differences between sample groups. To identify which groups are significantly different a Tukey's HSD AllPairwise Comparisons Test was then applied to create confidence intervals for all pairwise differences (these are displayed as homogeneous groups A, B, AB, C etc). Different groups indicate that their means are significantly different from each another. A statistical check for normality was also performed using the Shapiro-Wilk test and where necessary the data was transformed (normalised) prior to performing the Tukey's HSD test.

Transcriptome

Differential expression of RNA analysis was determined using the CLC Genomics Workbench v8.5 software (CLCBio, Aarhus, Denmark). The results were subjected to EDGE statistical analysis, then filtered to give only those transcripts with an FDR-corrected p-value <= 0.1. These transcripts were then sent through the Blast2GO Pro software (https://www.blast2go.com) for Gene Ontology (GO) annotation.

6. RESULTS AND DISCUSSION

This study presents results conducted between 2014 and 2020 and builds upon the 2013/807 pilot project of 2013. This was the largest project undertaken within the integrated YCS Research Program and covered an extremely diverse range of investigative studies. Therefore, results are presented and discussed in a series of topical chapters, addressing the aetiology and management of YCS through research of a) the development and expression of YCS, b) associated changes to plant health, physiology, morphology, anatomy, growth rate and nutrition c) identification of episodic triggers, d) diagnostics, e) elimination or identification of the causal agent/s, f) genotypic variations, g) evaluation of farm management practices and options of prevention or mitigation, h) incidence, severity and potential yield impact i) and possible reasons for the emergence of YCS.

6.1. Pattern of YCS development, symptom progression, crop age and season

When YCS was first noted in 2012 there were many questions about which leaves were impacted, how symptoms progressed within the leaf canopy, whether the crop's physiological age influenced the prevalence and severity of the condition, and whether there was a YCS 'season'. Crop age field trials conducted in the Burdekin between 2014 and 2016 together with leaf monitoring and physiological measurements (see Appendix 1: 1.2.7 & 1.2.8 for details of trial planning and outline) were critical to answering these questions.

Research conducted by Scalia et al. (2020) gives a detailed account of the metabolic disruption within a leaf that causes the ensuing YCS development, golden-yellow expression and progression along a leaf and will not be revisited in this chapter.

6.1.1 Crop age trials

A crop age trial was established on 22/7/2014 on a grower's farm in Home Hill, Burdekin (Farm # 4888 Block # 9-1) and serves as a model of YCS development and expression. The aim was to assess whether the age of sugarcane plants affects YCS prevalence and/or severity. The hypothesis is that an external factor is triggering YCS symptoms on developing leaves and the age of the plant has no bearing on expression of symptoms. This trial field of KQ228(ϕ 3rd ratoon (3R) had been severely YCS-affected in the previous ratoon. Treatments consist of four different aged cane plots (plot size is 4 rows x 10m) in a randomized complete block design with 5 replicates coming into the period when YCS typically occurs (Nov - Apr). The age treatments were created by hand slashing the ratooning crop each month, so that four ages were created. Treatments are: Control-July 22nd harvested, September 4th slashed, October 3rd slashed, and November 4th slashed. Prior to commencement of the trial, a soil test was taken, and nutrients applied at Six Easy Steps rates (https://sugarresearch.com.au/growers-and-millers/nutrient-management/six-easy-steps-toolbox/). Weeds were chemically controlled and the trial was fully irrigated on a 7-day schedule.

6.1.1.1 YCS prevalence and severity

Representative samples were collected from each treatment on 25 February 2015 and the number of leaves in the upper, middle and lower canopy were recorded and categorised as either: leaves with YCS symptoms (1st YCS and 2nd YCS leaf number) the green leaves above (GA) and below (GB). The physiological age of plants was 3, 5, 7 & 9 months for November, October, September, and July treatments, respectively. Leaves were recorded from the apex down (Figure 5) where Leaf 3 is equivalent to the FVD = L+1. Table 7 shows there was a statistically significant difference between treatments for total number of leaves and dead leaves. However, the number of green leaves were mostly similar in all age treatments. Differences among treatments (age) for the leaf number (as counted from the apex) of first and second visible symptomatic YCS leaf were not statistically significant. The average leaf number of the 1st YCS leaf was L8 (L+5) in all 4 treatments. The 9th leaf (L+6) in all treatments showed yellowing with the exception of one leaf each in 2 replicates in the November slashed treatment (3 months old). These results suggested that the initial occurrence of visible yellowing was most likely during the development of L+5 and L+6.

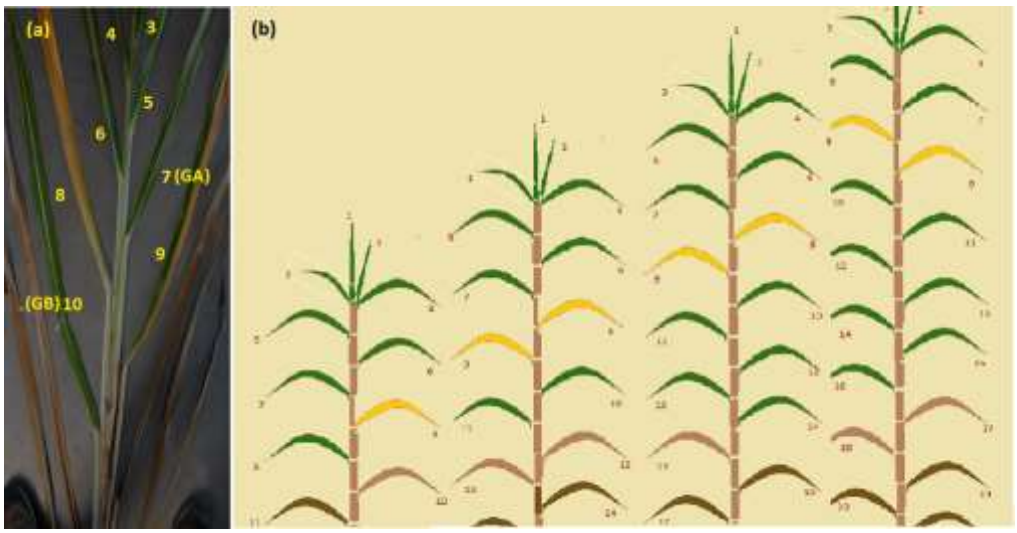


Figure 5 Stalk showing YCS symptoms (a); Leaf numbering in 4 age treatments (b) (from the visible spindle downwards) ***Note: Leaf 3 = FVD = L+1***

Table 7 Means of total number of leaves (total, green and senesced), and leaf number of 1st and 2nd YCS leaves in all the age treatments and their statistical significance.

	Treatments				
Attributes	**July**	**September**	**October**	**November**	**Lsd**
Total number of leaves	16.1	15.4	15.2	12.5	1.2**
Number of green leaves	10.8	10.9	11	10.2	ns
Number of senescent leaves	5.3	4.5	4.2	2.3	0.88**
1st YCS Leaf number (1st leaf)	8.6	8.3	8.6	8.3	ns
2nd YCS Leaf number (2nd leaf)	9.4	9.3	9.5	9.3	ns

Weekly YCS monitoring and scoring of YCS prevalence and severity (Table 3 & Table 4) as well as stalk height measurements (Figure 7) commenced in early November 2014 and concluded in May 2015. Prevalence is defined as the proportion of total stalks per plot showing YCS symptoms, whereas severity is the degree of yellowing exhibited, and plant height was measured from the base to the FVD. Prevalence and severity were rated for the crop canopy (above FVD), mid-canopy (leaves +1 to +5) and lower canopy (leaves below +5). Yellowing was first observed in the lower canopy in mid-November in all but the youngest aged crop (November treatment) (**Error! Reference source n ot found.**A). Symptoms arrived in the lower canopy of the younger crop approximately 4 weeks later in mid-December. Leaf yellowing spread rapidly through the stalk population and was observed in 75-100% of plants consistently in the lower canopy from early January right through until monitoring ceased in May with no difference between crop age during this time (Figure 6A). The prevalence of yellowing in the mid canopy, however, showed a different trend. First symptoms were recorded in mid-late December, 4+ weeks after they first arrived in the lower canopy (Figure 6B). Initially all crop ages were equally affected. Prevalence appeared to come and go like a wave in the mid canopy with peaks and troughs observed throughout the monitoring period. During the peak of YCS prevalence (early Feb), the older July plots had less incidence of YCS with 51-75% of stalks affected compared with 76-100% of stalks for the other 3 younger crops. By early May YCS was no longer evident in the mid canopy of any plot but was still present in the lower canopy (typical senescence). This incidence pattern separates YCS from other yellowing conditions and confirms that the mid canopy (L+1 to L+6) is the region affected by this condition.

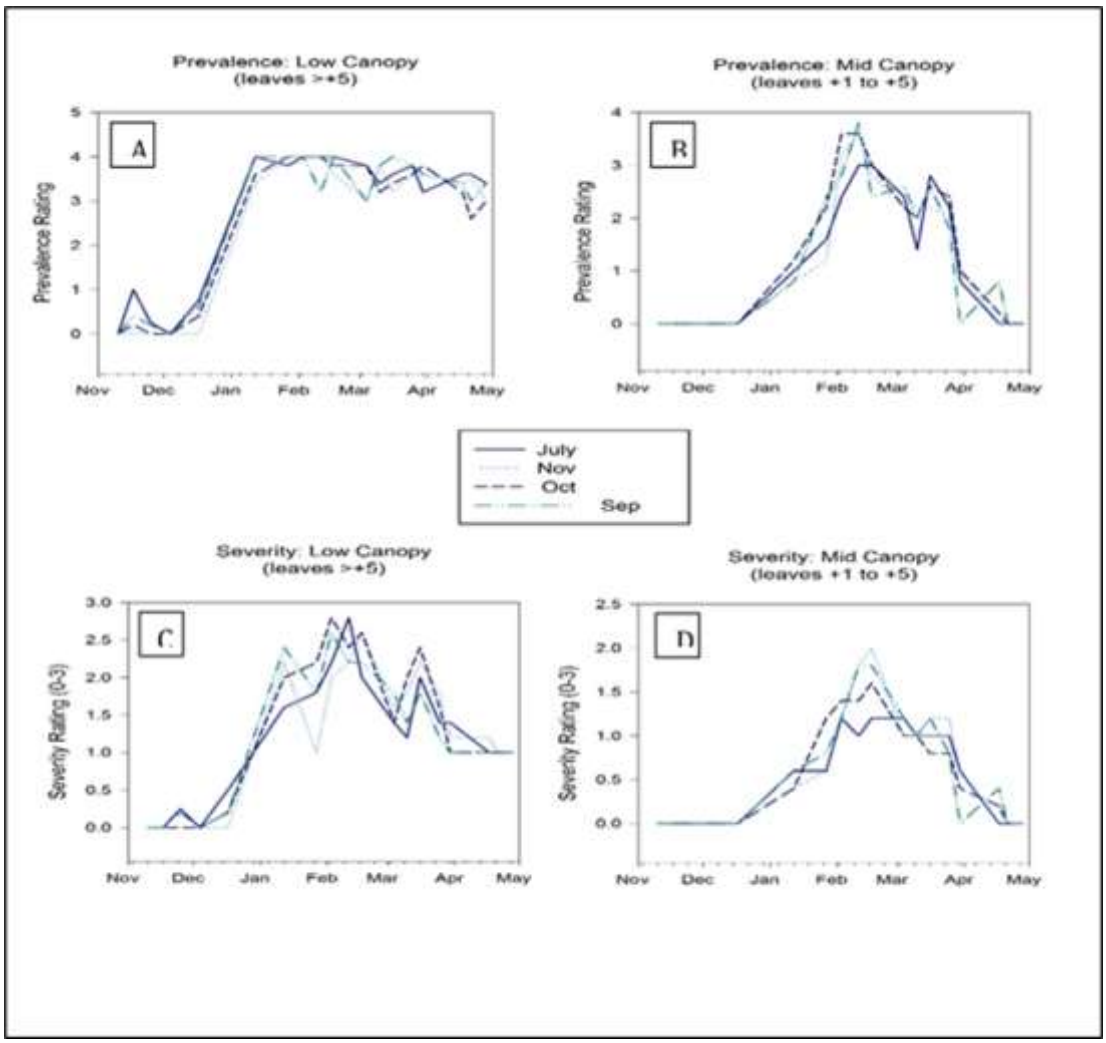


Figure 6 YCS prevalence and severity, measured weekly from November 2014 until May 2015. Lower canopy prevalence (A), Mid canopy prevalence (B), Lower canopy severity (C), and Mid canopy severity (D)

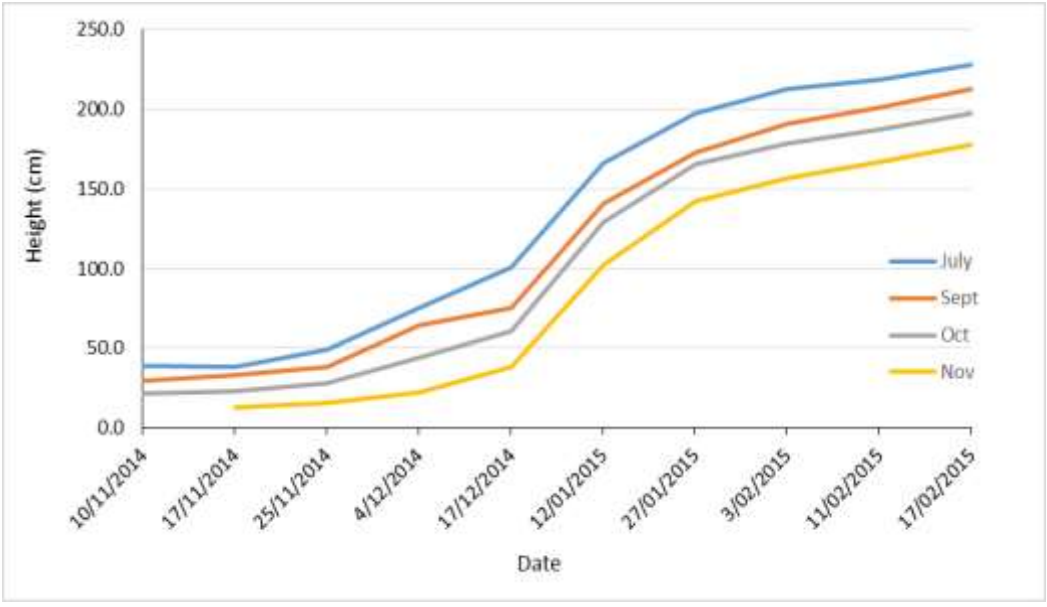


Figure 7 Stalk height at each monitoring date. For each treatment, ten stalks are measured per rep. Average height across reps for each treatment is presented. Height was measured from ground to the top visible dewlap.

Severity, or degree of yellowing, showed a similar trend in the lower canopy for all treatments. Yellowing rapidly progressed from mild to severe in late December, after which there were peaks and troughs of severity. By April, the symptoms had returned to mild and remained that way until the end of monitoring in May. Interestingly, although prevalence remains around 76-100% of stalks affected from Jan-April, the severity of the symptoms fluctuates from mild yellowing to severe (Figure 6C). In the mid-canopy we see a separation of severity by treatment during the YCS peak around 19th February with the older crop being less severely affected than the younger crops. There does not appear to be a growth penalty as the different aged crops maintain uniform height separation during this time (Figure 7).

The 2014 trial was ratooned in July for a 2015/16 trial to further explore the effect of age. The July harvest became the first age treatment with successive treatments created by slashing the ratooning crop in September and November 2015, and January 2016. Plots are 4 rows by 10 metres. There are five replicates with 6 monitored stalks per replicate. Plants first became YCS symptomatic in mid-January, with older plants (July and September) showing more severe symptoms than the younger November treatment (Figure 8). By late February, the January plants began to exhibit YCS symptoms. At this point the January plants were quite small, measuring around 30cm of stalk. January plants had been asymptomatic until this point, even though the larger plants around them were showing yellow YCS leaves. During March, the November and January treatments continued to express symptoms higher in their canopy and with greater severity than the July and September plants. Interestingly, during March, YCS severity plateaued in the July and September plants despite the sustained increase in severity in the two younger treatments. YCS peaked in early April and then began to decrease.

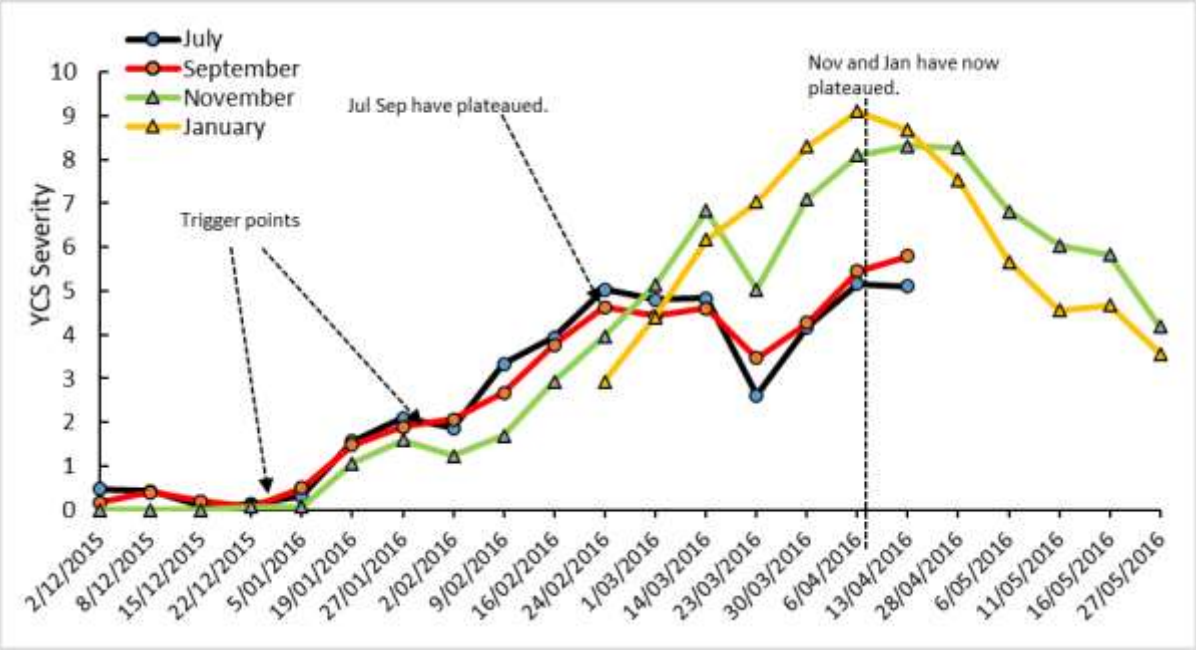


Figure 8 YCS severity score over time 2015/16. The score is calculated as the sum of the severity ratings for leaves +1 to +7. Each data point represents the average of 30 stalks. Means ± standard error.

6.1.1.2 Weather and growth

The initial incursion of YCS coincided with the first significant (>20mm) rainfall event of the season in late December after a very dry preceding period (14.8mm July-Nov). This also coincides with a period of increased average daily temperatures. Similarly, YCS increases following rainfall in early February, however this trend does not continue in March. Following early March rain, YCS severity increases only in the younger November and January plants while there was no corresponding increase for the older July and September plants. In fact, these plants plateau in their severity (Figure 9). We hypothesise that this is due to these plants transitioning from a period of rapid growth and canopy filling, to a more mature stage of their life cycle. July plants had, on average, 180cm of stalk on March 1st and were 150cm taller than the youngest January treatment (Figure 10). Therefore, within the YCS season (Jan-May), it is not the age of the plant per se, but rather the rate of growth that is the key driver for YCS development and severity.

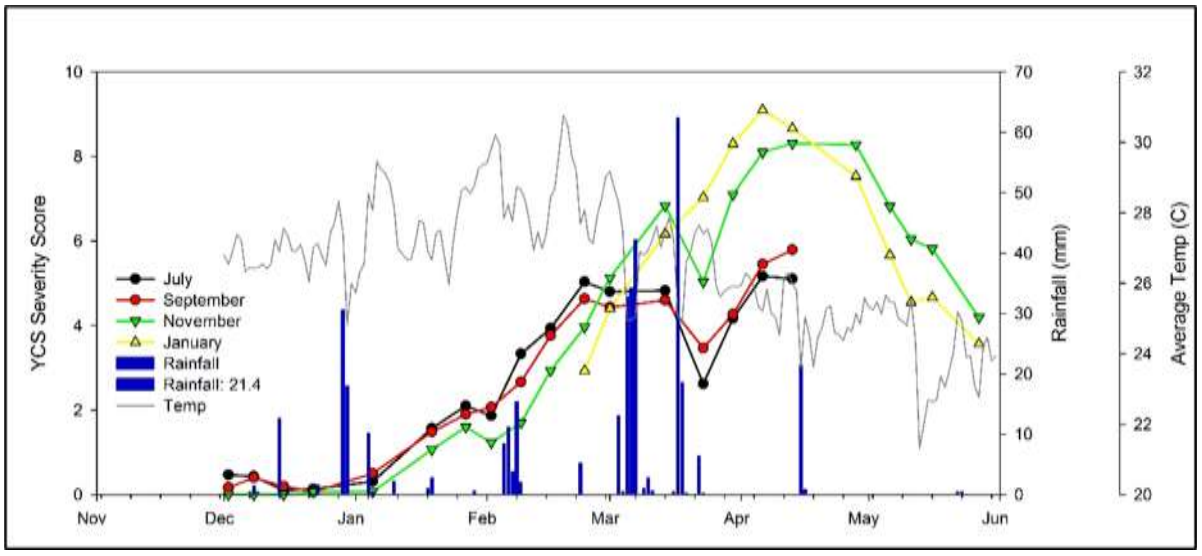


Figure 9 Rainfall, Average daily temperature, and YCS Severity over time

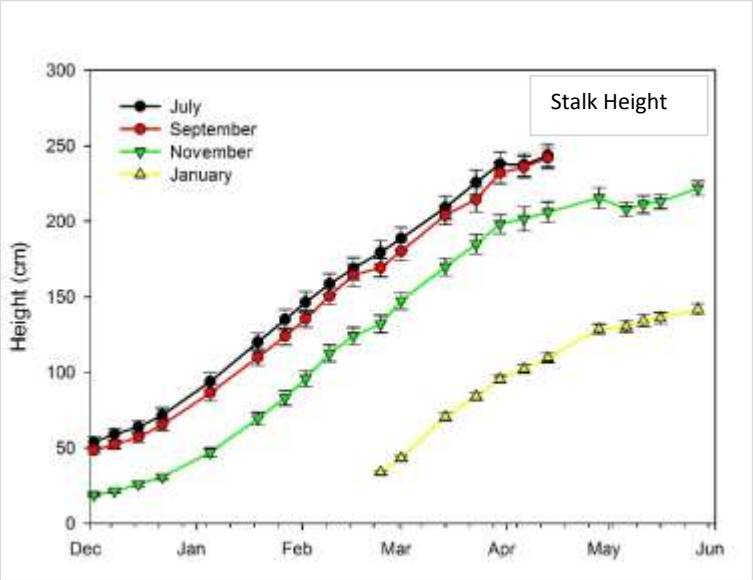


Figure 10 Average Stalk height (cm). Measured from ground to top visible dewlap. Each data point represents the average of 30 stalks. Means ± standard error

Although there was a good correlation between plant height and YCS for July, September and November treatments (r=0.81, r=0.91 and r=0.86 respectively), this was not the case for the January treatment (r-0.02) (Figure 11A). It seems that, inside the YCS season (Jan to May) even young and small plants can express YCS symptoms.

The number of leaves per stalk does not appear to be a particularly strong predictor of YCS severity. Coefficient of determination was, July R^2=0.59, September R^2=0.69, November R^2=0.35 January R^2=0.39. The January treatment showed strong YCS symptom expression at the 5-6 Leaf stage, while the other treatments did not (Figure 11B). This suggests that time of season is a stronger predictor of YCS and that as long as you are within the Dec-April period, YCS can occur regardless of number of leaves.

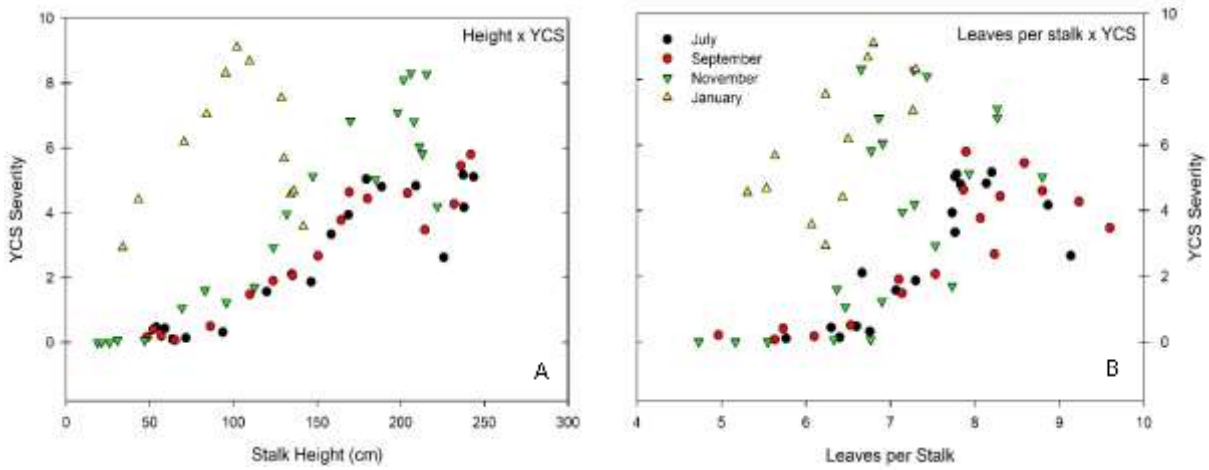


Figure 11 Correlation of YCS severity with stalk height (A) and number of leaves per stalk (B). Stalk height (cm) is measured from ground to top visible dewlap. The leaves per stalk count includes all leaves lower than and including leaf +1, excluding fully senescent leaves

As plants in all treatments are exposed to the same number of cumulative thermal units or degree days (°Cd) each will have a similar phyllochron (time taken between the appearance of successive leaves) irrespective of the treatment or age of plant (Robertson, 1998). During this peak growth period (Nov-April) Australian commercial varieties produce one new leaf approximately every 7-10 days depending on cumulative degree days (Inman-Bamber, 1994). Therefore, the number of leaves developed after any YCS event should be the same in all treatments. If symptoms are triggered at the same time regardless of crop age, this would then reveal whether a correlation exists between crop maturity and YCS development and expression. The influence of any critical environmental conditions during the initial developmental phases could then be investigated together with the first visible YCS symptoms in each treatment.

While the July, September and November treatments showed a good correlation between increasing degree days and YCS severity (r=0.81, 0.91 and 0.77 respectively), there was no such relationship for the January treatment (r=1.4) (Figure 12A). Even between July, September, and November treatments there was quite a difference between the magnitude of YCS severity at any given degree day, suggesting that some other unknown factor was influencing these results. To identify this unknown factor plant growth rates were calculated for the treatments (Figure 12B). When expressed this way, a clear linear trend becomes apparent with increasing rates of growth resulting in more severe YCS symptoms (Figure 12B). Analysis of thermal accumulation time (day degrees) and growth rates provides further evidence that time of year is a primary driver for YCS.

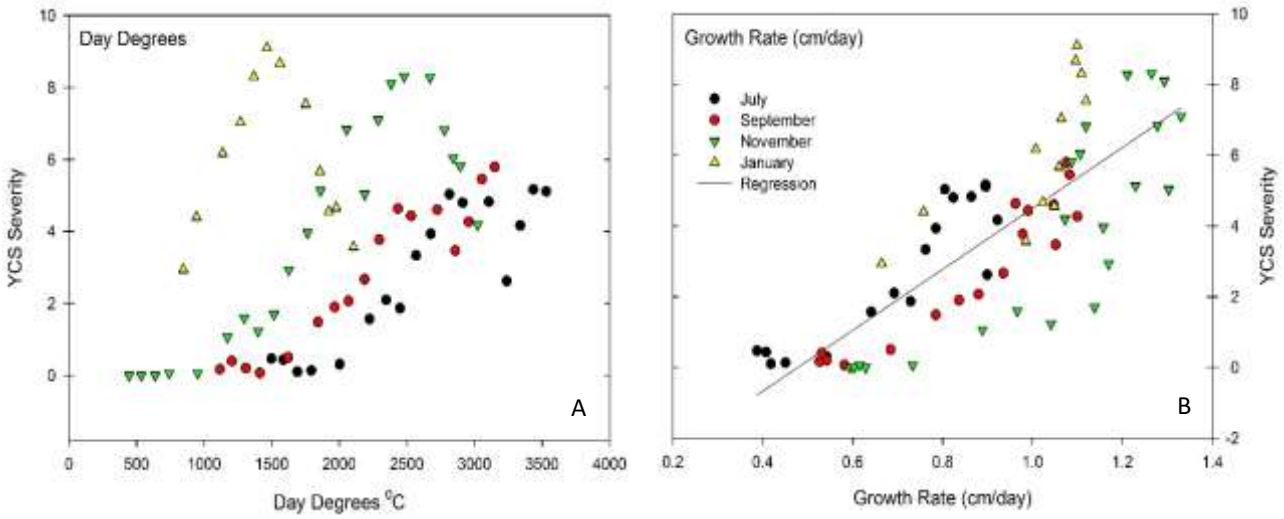


Figure 12 Correlation of YCS severity with thermal accumulation day degrees (A) and growth rate (B). Growth rate = stalk height (measured weekly) / days since ratooning. Regression line summarises all data points.

6.1.1.3 Discussion and conclusions

On completion of this two-year crop age study it can be surmised that YCS is a condition affecting the source leaves of the mid-canopy during the peak growing period of December to March. During this time of year Australian sugarcane varieties have a phyllochron of approximately 7-10 days, are

highly productive and have a rapid turnover of leaves. YCS yellowing tends to commence in the middle region of the mid-canopy, mostly between L+3 to L+6. The prevalence of yellowing below L+6 is akin to natural senescence and will not oscillate like a wave with peaks and troughs as it does in the mid-canopy. Therefore, when the condition first presents itself, there is a separation between YCS yellowing and senescent leaves, with green leaves below and above the impact zone. Monitoring shows that yellowing progresses up the canopy and does not exhibit above the FVD. As YCS affected leaves start to senesce, the band of green leaves between the mid and lower canopy will merge, producing a continuous zone of varying degrees of yellow-brown, yellow, and golden-yellow from bottom up. Therefore, visual diagnosis of YCS must be made early before this colour separation ceases to exist.

The prevalence of yellowing in the mid canopy, clearly showed a different trend to yellowing in the lower canopy. First symptoms were recorded in mid-late December, 4+ weeks after they first arrived in the lower canopy (Figure 6B). Initially all crop ages were equally affected. Prevalence appeared to come and go like a wave in the mid canopy with peaks and troughs observed throughout the monitoring period. During the peak of YCS prevalence (early Feb), the older July plots had less incidence of YCS with 51-75% of stalks affected compared with 76-100% of stalks for the other three younger crops. By early May YCS was no longer evident in the mid canopy of any plot but was still present in the lower canopy which correlates with natural senescence. This incidence pattern separates YCS from other yellowing conditions and confirms the mid canopy (L+1 to L+6) is the region affected by this condition. The synchronicity of YCS prevalence independent of the crop age indicates there is commonality to the external driver of the event.

Severity of YCS in the mid-canopy increased from late December and peaked by mid-February. This coincides with the time of accelerated growth rate due to high light intensity and temperature and a longer photoperiod. By April symptoms have returned to mild, in alignment with reduced growth rate and have all but disappeared by May. However, at this same time point symptoms are more severe in younger crops which are still actively growing and have not yet transitioned to a more mature status. There is a strong correlation between YCS severity, growth rate and time of year (cumulative thermal units). Evidently, higher rates of growth following good rainfall also correlate with outbreaks of yellowing across the treatments independent of age. The limitation of inconsistent rainfall (even under irrigated conditions) and other factors to maintain adequate uniform growth rates throughout the peak growing season, is the impetus for the waves of observed expression in the mid-canopy. Thus, it is rate of growth, not the physiological age of the crop per se that is the main driver of YCS.

6.2 Nutrients

Sugarcane is a crop with a rapid turnover of leaves and may exhibit many forms of leaf yellowing in its canopy at any one time. Leaf yellowing may be due to natural senescence, water stress and agrochemical phytotoxicity to name a few. Initially there was much conjecture that the cause of YCS may be a nutrient deficiency. To investigate this further, sampling of leaf, culm, roots, and soil was undertaken across the Herbert and Burdekin districts which have contrasting soil types. Primarily, samples were taken from fields with both YCS asymptomatic and symptomatic plants and analysed

for nutrient levels. As nutrient content is only one parameter associated with plant health, subsequent sampling and analyses also investigated nutrient uptake, mobility, and heavy metal toxicity.

Leaf Samples were also collected from a Confidor® trial conducted in Ingham 2014 (Appendix1: 1.2.2). Confidor® treatment induced a stay-green effect in this trial and this was used as the control to compare with YCS symptomatic plants in the untreated plots. Heavy metal analysis of these samples is also reported here.

6.2.1 Nutrient deficiencies

6.2.1.1 Macro and trace elements

The results for macro nutrients obtained for the diagnostic Leaf +1 from Q208 sampling at Burdekin and Herbert and KQ228 at Burdekin are presented in **Table 8**.

Table 8 Major nutrient elements in samples of Leaf +1 from green Control and YCS symptomatic plots.

	N	C	Ca	Mg	P	K	S	Si
% dm								
Critical Level	**1.80**	**N/A**	**0.20**	**0.08**	**0.19**	**1.11**	**0.13**	**0.70**
Burdekin Q208 (January 2015)								
Control (n=3)	1.45	45.1	0.221	0.217	0.237	1.70	0.195	1.14
SD	*0.04*	*0.1*	*0.017*	*0.018*	*0.014*	*0.13*	*0.016*	*0.12*
YCS+ (n=3)	1.48	44.1	0.216	0.186	0.252	2.05	0.246	1.40
SD	*0.05*	*0.10*	*0.024*	*0.016*	*0.004*	*0.07*	*0.016*	*0.11*
Burdekin KQ228 (March 2015)								
Control (n=4)	1.62	45.2	0.228	0.197	0.336	1.67	0.190	0.91
SD	*0.07*	*0.2*	*0.028*	*0.016*	*0.026*	*0.11*	*0.013*	*0.03*
YCS+ (n=4)	1.56	44.9	0.244	0.181	0.332	1.51	0.176	1.44
SD	*0.06*	*0.3*	*0.025*	*0.010*	*0.027*	*0.044*	*0.006*	*0.11*
Herbert Q208 (May 2015)								
Control (n=3)	1.77	46.5	0.283	0.140	0.185	1.45	0.155	0.90

SD	*0.08*	*0.1*	*0.015*	*0.011*	*0.004*	*0.041*	*0.008*	*0.11*
YCS+ (n=3)	1.74	44.4	0.390	0.153	0.219	1.34	0.151	1.69
SD	*0.13*	*0.3*	*0.062*	*0.018*	*0.013*	*0.068*	*0.013*	*0.29*

The values for majority of macro nutrient elements do not indicate clear deficiencies in diagnostic L+1. Notably, nitrogen values fall below the suggested critical level, however there is no indication that this occurs only in YCS symptomatic leaf samples. It is likely that the reduced nitrogen reading may be attributed to the time of sample collection as Burdekin Q208 sampling was conducted in January, KQ228 in March while Q208 sampling at Herbert was completed much later in May 2015.

The concentration of trace nutrients in control samples and YCS symptomatic are presented in Table 9.

Table 9 Trace nutrient elements in samples of Leaf +1 from Control (green) and YCS symptomatic plots.

	Cu	Zn	Fe	Mn	B
	mg kg^{-1} dm				
Critical Level	**2**	**10**	**50**	**15**	**1**
Burdekin Q208 (January 2015)					
Control (n=3)	4.29	15.1	47	51	3.1
SD	*0.23*	*1.1*	*3*	*7*	
YCS+ (n=3)	4.21	17.7	45	25	3.2
SD	*0.18*	*0.5*	*5*	*1*	
Burdekin KQ228 (March 2015)					
Control (n=4)	6.19	20.5	63	42	4.2
SD	*0.28*	*1.6*	*7*	*7*	
YCS+ (n=4)	5.83	18.4	45	40	3.4
SD	*0.19*	*1.0*	*1*	*6*	
Herbert Q208 (May 2015)					

Control (n=3)	4.57	19.1	59	81	2.6
SD	*0.13*	*0.2*	*7*	*11*	
YCS+ (n=3)	4.92	17.8	54	199	3.1
SD	*0.15*	*1.1*	*3*	*20*	

Table 8 and Table 9 show all macro and trace element nutrients to be at adequate concentration in both YCS asymptomatic and symptomatic plants.

This data confirms YCS is not caused by a major or trace element deficiency in the lamina.

6.2.2 Heavy metal content

Table 10 shows the heavy metals content of leaf samples collected in the Confidor® trial conducted in Ingham (see Appendix 1: 1.2.2).

Table 10 Heavy metals and trace elements (mg kg^{-1} dm) in samples from Control and YCS symptomatic plants.

	Cd	Co	Pb	Cr	Se	As
MDL	0.014	0.011	0.06	0.5	0.06	0.08
Leaf 0						
Control	0.017	0.098	0.28	0.9	<0.06	0.18
+YCS	<0.014	0.066	0.28	1.0	<0.06	<0.08
Leaf 1						
Control	<0.014	0.128	0.34	0.9	<0.06	0.11
+YCS	<0.014	0.086	0.26	1.1	<0.06	<0.08
Leaf 2						
Control	<0.014	0.134	0.32	1.3	0.06	0.12
+YCS	<0.014	0.101	0.35	1.8	0.06	0.08
Leaf 3						
Control	<0.014	0.162	0.38	1.1	<0.06	0.12
+YCS	0.017	0.118	0.41	1.5	0.08	<0.08
Leaf 4						
Control	<0.014	0.180	0.47	1.2	<0.06	0.13
+YCS	<0.014	0.115	0.44	2.0	0.06	0.08
Leaf 5						
Control	0.015	0.185	0.51	1.3	<0.06	0.14
+YCS	<0.014	0.130	0.53	1.7	0.08	0.11
Leaf 6						
Control	0.014	0.240	0.60	1.5	0.06	0.16
+YCS	<0.014	0.114	0.58	1.5	0.06	0.10

Comparison of YCS asymptomatic and symptomatic heavy metal content shows no marked differences in any leaf except for chromium (Cr) which trended higher in YCS symptomatic leaf tissue (Figure 13). However, the opposite is true for cobalt (Co) (Figure 14). Levels of mercury (Hg) and nickel (Ni) were at such low levels they fell below detection limits (DLs) of the analytical method used by the DSITIA Laboratory and are therefore deemed to be non-problematic.

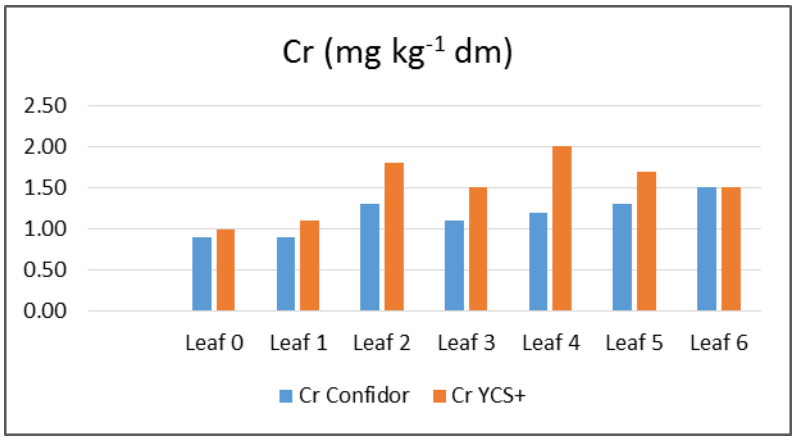


Figure 13 Q208$^{\phi}$ Chromium content in Confidor® trial

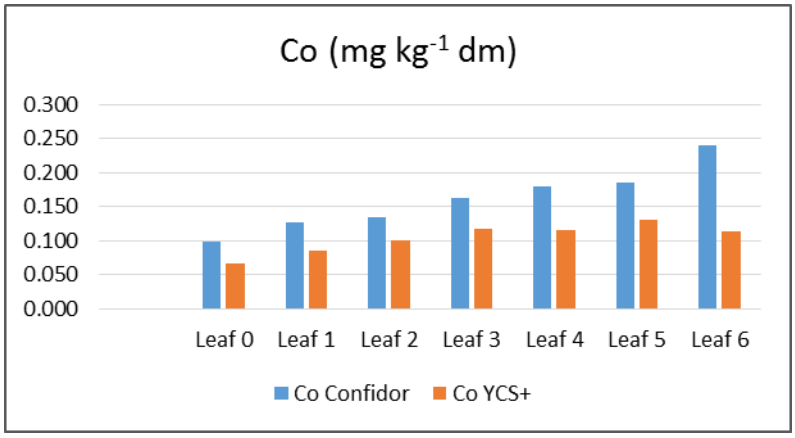


Figure 14 Q208^{A} Cobalt content in Confidor® trial

Heavy metal analysis was conducted by DSITI laboratory on selected soil and leaf samples. The results of heavy metal analysis for soil and plant samples from the Q208 sampling at Burdekin are presented in Table 11a and Table 11b, respectively. Results for mercury (Hg), nickel (Ni) and cobalt (Co) for all soil samples submitted for analysis are below detection limits (DL) for the analytical method used (DSITI Laboratory), Table 11a. Interestingly, concentration of majority of measured heavy metals are higher in soil samples from the Control plots when compared with the YCS symptomatic sites. All metals and trace elements are within typical soil range, as reported by Rayment and Barry (1993).

Table 11a Concentration of heavy metals and trace elements (mg kg^{-1}) in soil, Q208 Burdekin sampling.

	Pb	Cr	Se		As	Cd
MDL	10	3	0.1		0.1	0.05
Typical Soil range*	2-300	5-5000	0.01-1.2		0.1-40	0.01-2
			0-10 cm			
Control	25	16	1.01		2.44	0.05
YCS+	16	11	0.48		1.83	0.06
			10-20 cm			
Control	24	16	1.04		2.51	0.06
YCS+	14	11	0.42		1.81	<0.05
			40-60 cm			
Control	23	16	1.17		2.76	0.07
YCS+	18	12	0.40		2.11	0.09

Hg, Ni and Co below DL

*(Rayment et al., 1997)

There is no indication of heavy metal content being elevated in plant samples collected from the YCS symptomatic plots of Q208 in Burdekin. In contrary, slightly higher concentrations are found in majority of leaf samples obtained from the green Control plants, when compared with the YCS symptomatic. This finding further suggests that YCS symptoms are unlikely to be due to heavy metal toxicity.

Table 11b Concentration of heavy metals and trace elements (mg kg^{-1} dm) in leaf, Q208 Burdekin sampling.

	Ni	Co	Pb	Cr	Se	As
MDL	0.20	0.011	0.10	0.50	0.06	0.02
Leaf 0						
Control	0.66	0.059	0.27	0.85	<0.06	0.04
YCS+	0.55	0.024	0.11	0.82	0.12	0.02

Leaf 1						
Control	0.52	0.061	0.12	0.65	<0.06	0.05
YCS+	0.36	0.022	<0.10	0.44	0.14	0.02
Leaf 2						
Control	0.60	0.068	0.13	0.79	0.06	0.05
YCS+	0.53	0.024	0.11	0.62	0.16	0.03
Leaf 3						
Control	0.49	0.078	0.76	0.64	0.06	0.06
YCS+	0.46	0.027	<0.10	0.69	0.18	0.04
Leaf 4						
Control	0.51	0.068	0.11	0.68	0.08	0.07
YCS+	0.37	0.022	<0.10	0.52	0.15	0.03
Leaf 5						
Control	0.43	0.064	0.21	0.50	<0.06	0.07
YCS+	0.32	0.020	<0.10	0.38	0.14	0.03
Leaf 6						
Control	0.34	0.047	<0.10	0.35	<0.06	0.07
YCS+	0.33	0.016	<0.10	0.40	0.12	0.03

Hg and Cd below DL

Similar outcomes were found in soil and plant samples from all other sampling locations in terms of heavy metals. The combined data for all heavy metals and trace elements measured in diagnostic Leaf 1 are presented in Figure 15.

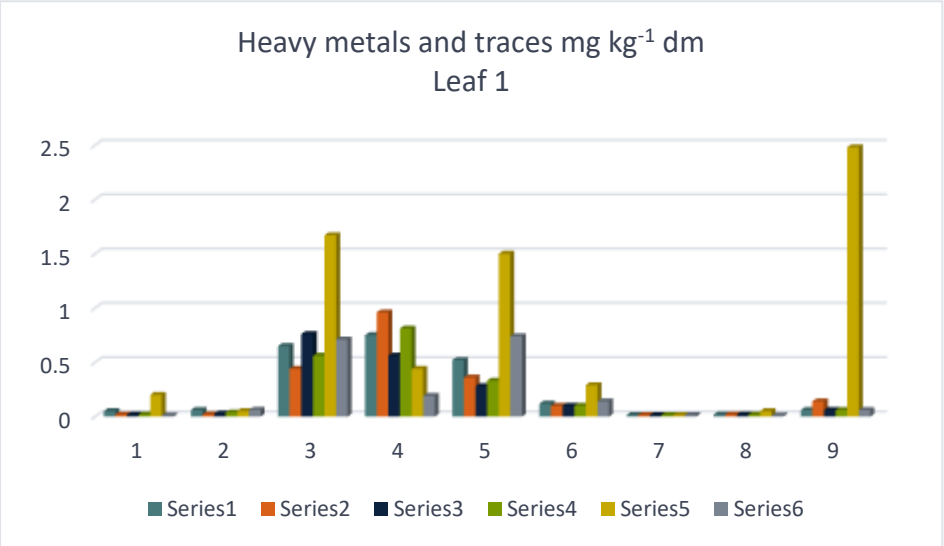


Figure 15 Concentration of heavy metals and traces in Leaf 1 of Control and YCS symptomatic samples from all sampling locations.

There are no clear trends suggesting elevated heavy metal values in Leaf 1 of the YCS symptomatic plants. In contrary, the values for a number of heavy metals are higher in Control green leaf samples when compared with the YCS symptomatic. This further emphasizes the view that it is rather unlikely for YCS to be a direct symptom of heavy metal toxicity.

Although numerous studies investigating the detrimental effect of heavy metals on plant growth have been published (Wyszkowski and Radziemska, 2010) (Prasad, 2004), no information is available on heavy metal toxicity levels for sugarcane. Changes in soil chemical properties in sugarcane soils, including heavy metals, have however been studied in the past, particularly in terms of recycling of industry by-products (Rayment et al., 1997). These published data provide a useful benchmark for the YCS heavy metal / trace element studies.

6.2.3 Nutrient mobility (Efficiency of nutrient uptake and nutrient balance)

This study investigated the soil-plant interactions and nutrient uptake study through internode, leaf sheath and finally accumulation in the leaf. The results of the uptake of plant mobile K and plant immobile Ca, from the Q208 sampling at Burdekin, are presented in Figure 16 and Figure 17 respectively.

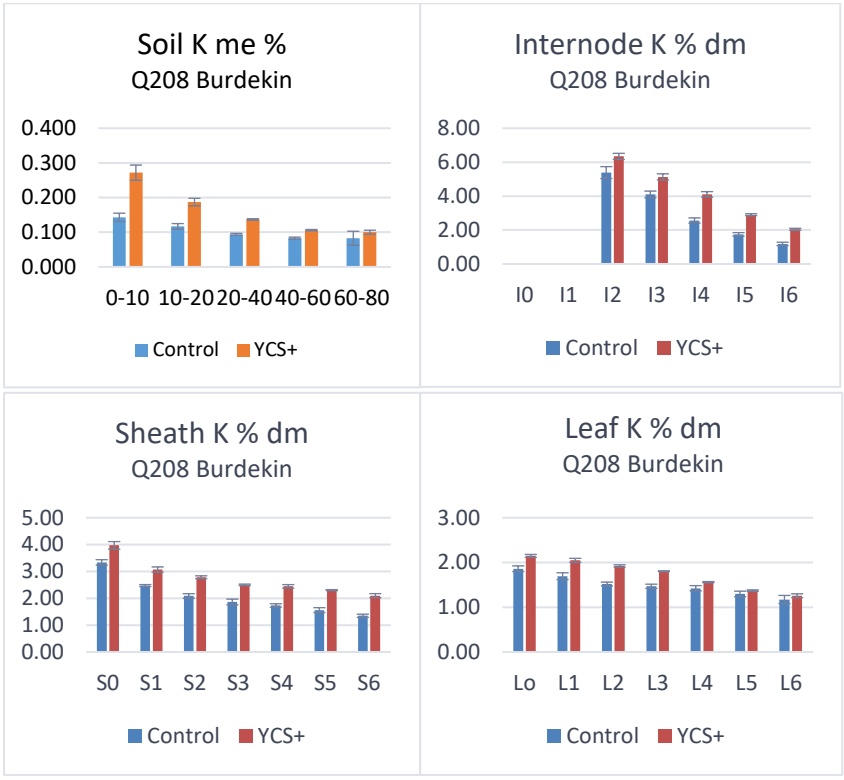


Figure 16 Concentration of potassium in soil and plant tissue. Q208 sampling January 2015, Burdekin. Soil X axis – depth in cm. Plant X axis – Internode/Sheath/Leaf numbers (L1=FVD, I1= internode under L1 sheath).

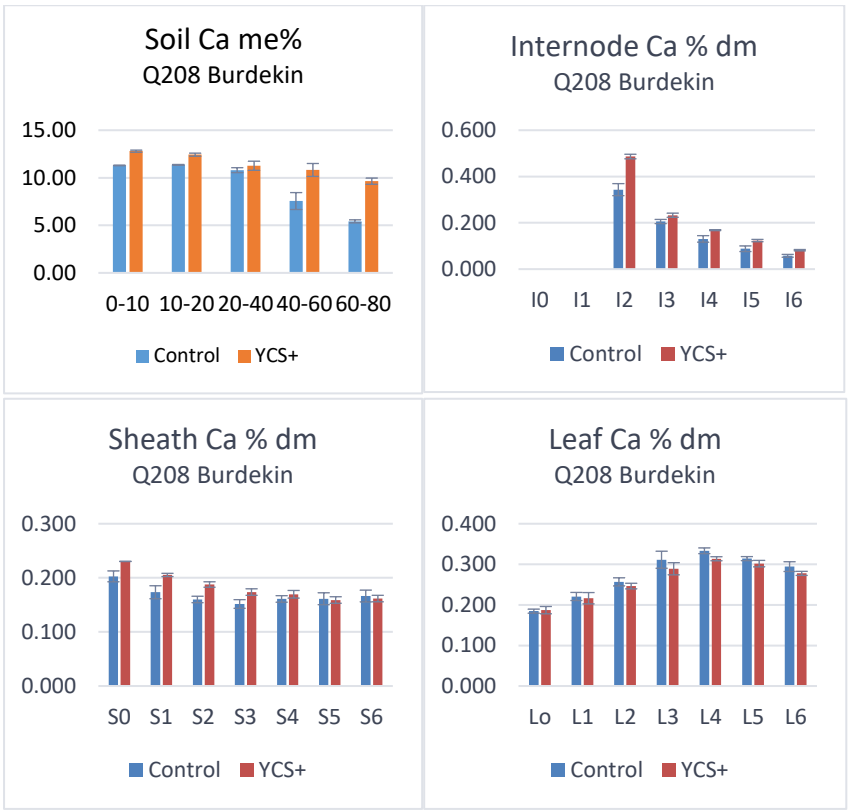


Figure 17 Concentration of calcium in soil and plant tisue. Q208 sampling January 2015, Burdekin. Soil X axis – depth in cm. Plant X axis – Internode/Sheath/Leaf numbers (L1=FVD, I1= internode under L1 sheath).

The results presented in Figure 16 and Figure 17 suggest the uptake trends of K (mobile) and Ca (immobile) are similar in green Control and YCS symptomatic plants, and are directly related to the supply in soil. There are no apparent unexpected changes in the measured concentration at any point of uptake along the stalk that would suggest blockages or disturbance of uptake. Potassium is characterized by high mobility in plants at all levels and as such is expected to be present at higher concentration in upper leaves. This is the case in our study. Higher accumulation of Ca is evident in older leaves however, as expected for plant immobile nutrients.

Similar trends of nutrient uptake from soil and distribution within the plant were found in all varieties, soil types and sampling locations included in this study (data not shown). This suggests that nutrient uptake mechanisms and mobility within the plant are not adversely affected by YCS.

An interesting trend was observed for silicon accumulation in leaf, with higher concentration found in YCS symptomatic plants in all leaf numbers, across all sampling sites and soil types, as presented in Figure 18.

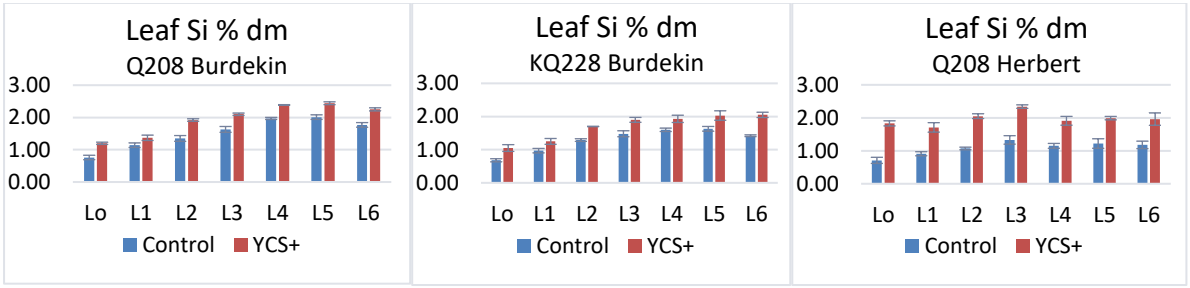


Figure 18 Concentration of Si in leaf samples collected from Control and YCS symptomatic plants for all sampling sites at Burdekin and Herbert. X axis – leaf numbers.

A significant difference was found in leaf Si concentration between control and YCS symptomatic sites (*p=000*). The elevated Si values in leaf samples from YCS symptomatic plants was independent of the supply from soil, with adequate concentration of available Si measured in all soil samples in this study, for all control and YCS symptomatic sites (Figure 19).

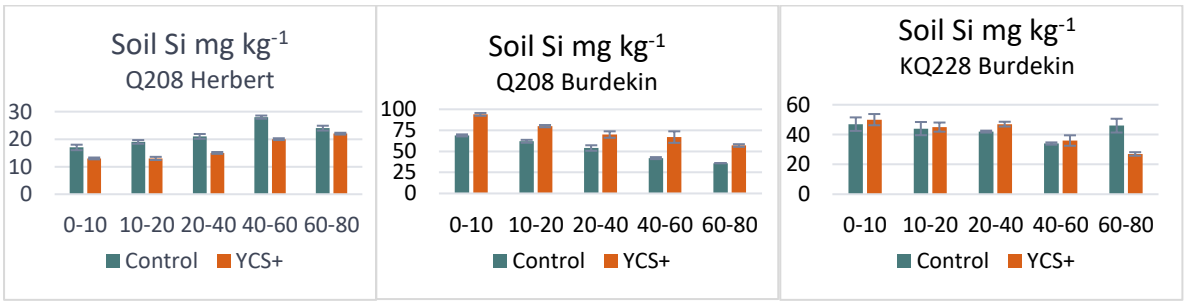


Figure 19 Concentration of available Si in soil samples collected from Control and YCS symptomatic plots, all sampling locations included. Presented results for $CaCl_2$ analytical method. X axis – soil depth in cm.

Further data analysis suggested that Mg concentration was decreased in leaf samples of YCS symptomatic plants when compared with green Control in majority of leaf samples analysed, this was evident for all sampling locations, varieties and soil types (data not shown). This finding prompted us to look further at relationships between nutrient elements in leaf samples, in particular Si and Mg. Based on the data obtained for all leaf samples included in this study, there was a significant difference found between the Si/Mg ratio in green Control and YCS symptomatic samples. This finding could potentially lead to the Si/Mg ratio to be considered as an indicator of YCS, however, further investigation is needed to find out if this phenomenon is unique to YCS or prevalent in other stresses such as drought or various known sugarcane diseases. Interestingly, recent studies have found that Si uptake by plants reduces stress and promotes growth.

Transmission electron microscopy (TEM) work concurs with chemical analyses that YCS leaf tissue has higher Si content than controls. Si aggregates are clearly visible in several TEM sections of YCS affected leaves. This includes small aggregates (blue circles) and also masses of Si –like particles within the cell walls (Figure 20).

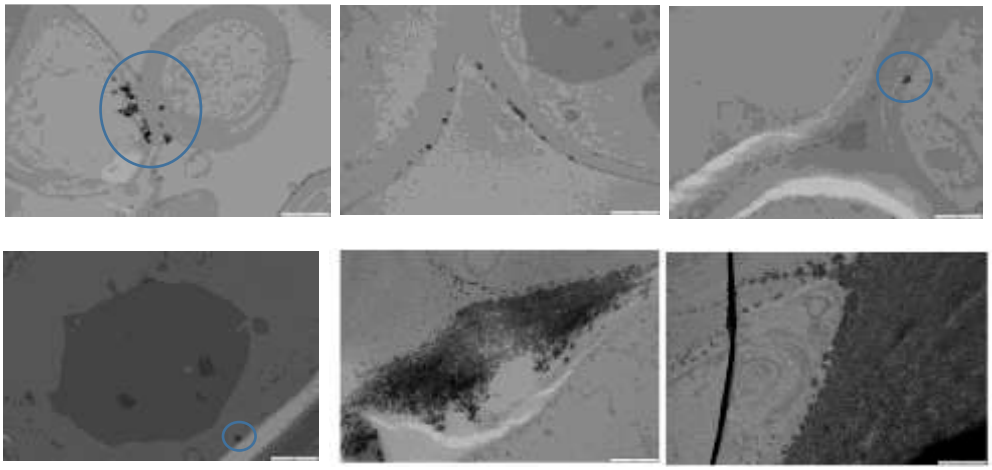


Figure 20 TEM images of leaf of YCS affected plants showing aggregates of Si in the cell

Silicon mediated regulation of genes has been linked to biochemical pathways involved in mitigating stress and promotion of plant growth recovery (Zhu et al., 2016; Manivannan and Ahn, 2017). Studies by Marquardt et al. (2017) found that major metabolic changes in YCS plants were associated with sucrose metabolism, carbon repartitioning, and an upregulation of the phenylpropanoid pathway in response to oxidative stress. This suggests that high leaf Si content in YCS plants is likely to be associated with an antioxidative or anti-stress response to mitigate the impact of YCS and assist in its recovery.

6.2.3 Magnesium studies

Although magnesium content was found to be lower in YCS symptomatic leaf tissue, levels were not below the critical threshold. However, Mg is a critical element required by sugarcane for active phloem loading and is also the central element of the chlorophyll molecule. Physiological studies show that YCS symptomatic plants have altered carbon partitioning in response to sucrose accumulation and reduced photosynthetic rates synonymous with a Mg deficiency (Marquardt et al., 2016). While molecular studies showed that sugar transporters and were functional (Marquardt et al., 2019), field trials and chemical analyses were continued to validate the data.

A field trial was established to investigate whether addition of magnesium effects the incidence and/or severity of YCS. The site was YCS affected at the time of trial establishment. Three treatments were evaluated; 1) foliar applied magnesium 50kg/ha rate, 2) soil applied magnesium 50kg/ha rate, and untreated control. There were three replicates of each. YCS monitoring as well as leaf, soil and diagnostic tests were conducted throughout the trial duration (see Appendix 1: 1.2.17). A "baseline" diagnostic measurement was conducted on February 19th before treatment application. Treatments were applied on February 25th, 2016 and a "follow up" diagnostics was conducted on March 31st 2016, approximately one month post treatment application.

YCS severity at the site naturally declined over time, treatment effect on YCS symptom expression proved not significant across all treatments during the observational period. It can be seen in that the soil applied magnesium had slight alleviating effects on symptom expression one month post treatment application (Figure 21). This effect, however, did not last or may have been influence by heavy rainfall experienced during the 1st and 3rd weeks in March, as can be seen in (Table 12). Foliar applied magnesium was found to exacerbate YCS symptom expression, however this was found to be non-significant.

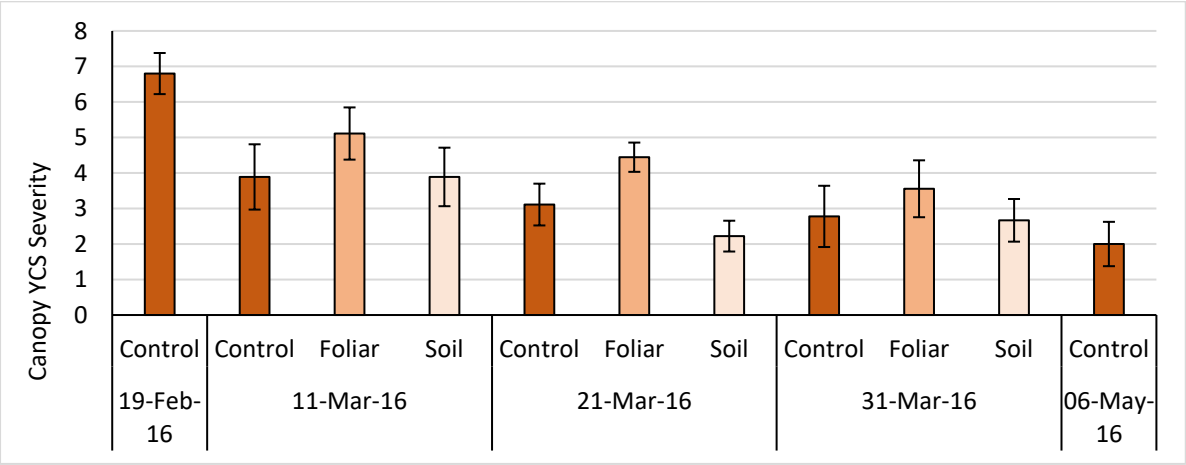


Figure 21 YCS canopy severity (sum of leaf severity ratings), variety KQ228$^{\Phi}$ with treatments of foliar applied magnesium (50kg/ha), soil applied magnesium (50kg/ha), and untreated control. Bars are the average of 9 stalks. Means ± standard error.

Table 12 Weather parameters during 2016- 2017 at Home Hill. GPS (19°39'21.3"S, 147°28'16.9"E).

Month	Temp	Humidity	Solar Radiation	Total Rainfall
	Avg (°C)	Avg (%)	Avg (MJ m^{-2})	(mm)
Jan	27.7	74.5	25.5	17.2
Feb	28.6	76.6	23.0	46.0
Mar	26.8	82.1	19.2	215.2
Apr	25.2	76.3	18.8	22.2
May	24.1	77.7	15.6	0.8

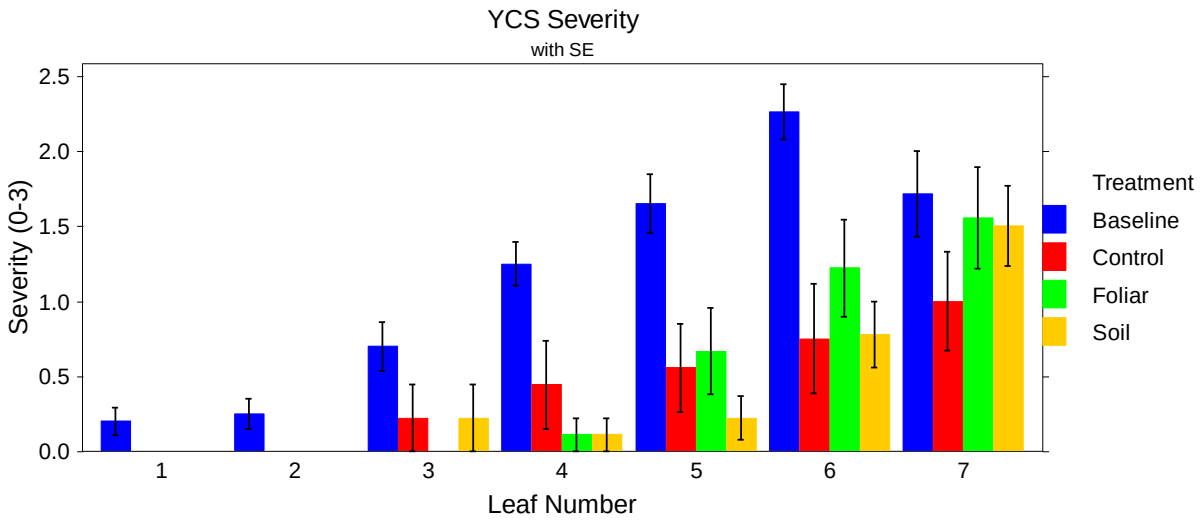


Figure 22 YCS severity in response to magnesium treatments. Plot shows severity ratings for leaves +1 to +7. Pre-treatment baseline levels are also shown for comparison. (n=9, means ± standard error).

In Figure 22 it can be seen that the yellow phenotype is the same across treatments with the exception of Leaf +5 having a slight treatment effect for soil applied magnesium. It is therefore concluded that there has been no improvement in phenotype attributable to the treatments.

From Figure 23(B) it is clear that baseline plants had a significantly higher YCS severity rating than the experimental treatments, both across the canopy and on diagnostic Leaf +4 Figure 23(C). Application of Mg did not reduce YCS severity relative to control Figure 23(B). The application of Mg did not result in any growth advantage (stalk height or rate of leaf elongation).

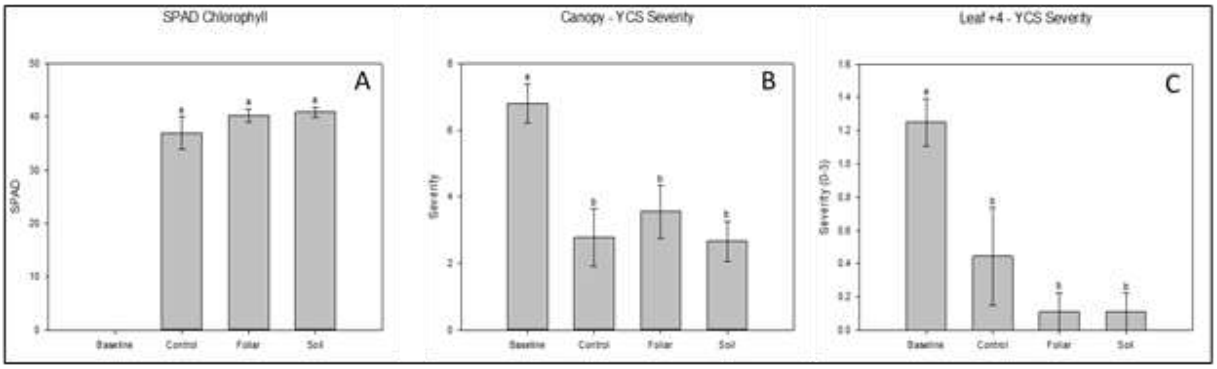


Figure 23 Agronomic measurements of YCS severity pre-treatment (baseline) and post treatment, SPAD chlorophyll content (A), YCS canopy severity (B), and Leaf +4 YCS severity (C) are shown. (n=9, means ± standard error)".

Leaf nutrient analysis was conducted on both Leaf +1 and Leaf +4. The experimental treatments differ from the baseline for a few elements, however do not differ significantly from each other (Figure 24(A) and (B)).

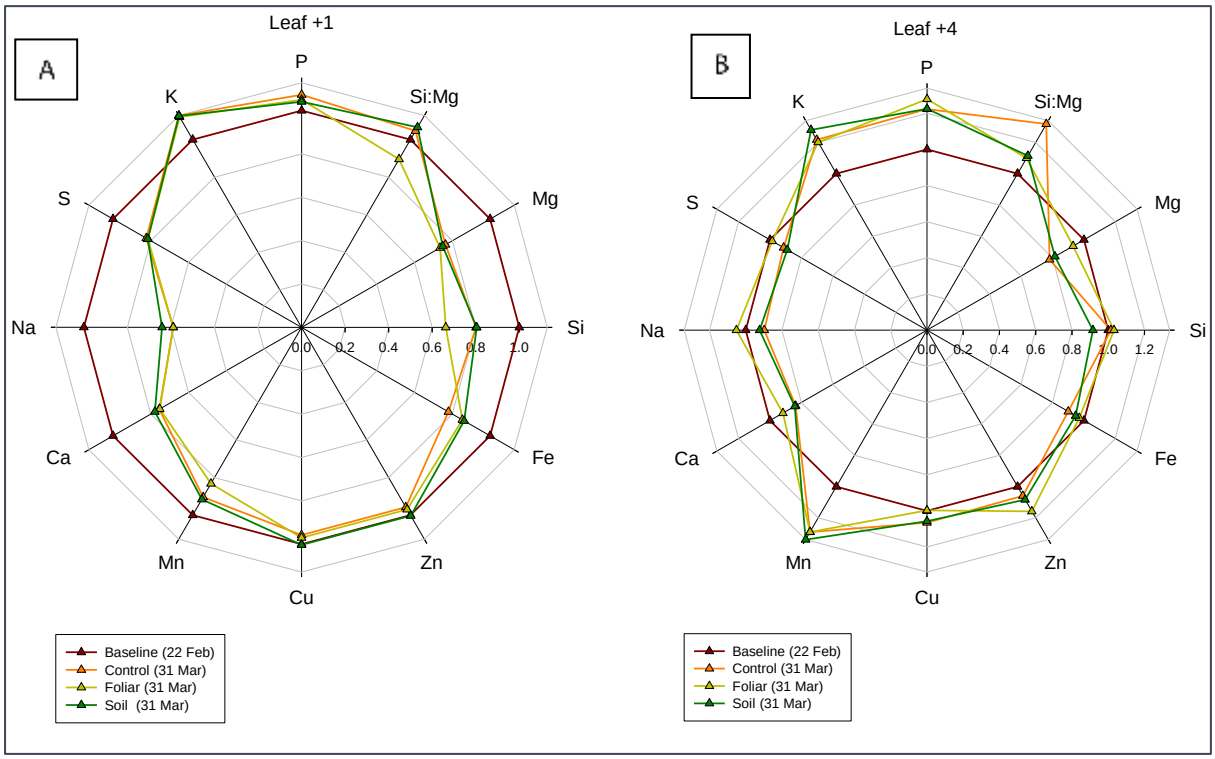


Figure 24 Leaf nutrient results. Leaf +1 (A) and leaf +4 (B). The radar plots show magnesium treatments relative to pre-treatment baseline values for 12 plant nutrient parameters. For each parameter the pre-treatment baseline has been normalised (x=1.0). Nutrient leaf analysis taken before and after treatment application for both Leaf +1 and Leaf +4.

The results of this trial show the application of Mg did not reduce incidence or severity of YCS symptoms relative to control. Foliar or soil-application of Mg did not result in higher Mg tissue

content than Control. There was no effect on plant growth, stalk height or leaf extension rates and no additional uptake of this element in response to the treatments.

Perhaps the significant lessening of YCS symptoms from February to March was not due to the treatments applied but some other unknown factor driving the phenotypic change. It is possible that the key driver during this experiment was the change in weather conditions. March was cooler, more humid, and significantly wetter than February.

In an attempt to gain further clarity around magnesium involvement in YCS development it was decided to conduct a comparative analysis experiment within another field trial. Application of magnesium sulphate (MgSO4) was included (foliar and soil) in this trial together with the addition of gypsum (CaSO4), potassium sulphate (K_2SO4), dolomite [$Mg(CO3)_2$], and compost to the soil. This trial was also investigating the effect of the insecticide bifenthrin to prevent YCS development and the possible involvement of insects as a causal agent.

Unfortunately, YCS symptoms were very low in the untreated controls with only 1-2 leaves affected at any time (Figure 25). Peak expression occurred in late January. At this time the gypsum, magnesium sulphate soil and compost treatments had the highest rates of YCS expression (though still low). The treatments that had the least YCS expression were the bifenthrin, magnesium sulphate foliar and potassium sulphate treatments. All treatments decreased in YCS expression in early February and continued to decline (Figure 25).

Although YCS expression was low in this trial it was still possible to evaluate the role of magnesium as a causal agent. Foliar applied Mg increased the leaf tissue Mg concentration significantly while, interestingly, the soil applied Mg at the same rate had a much lower impact which suggests foliar application is a more efficient method for plant uptake (Figure 26). However, the increased Mg did not have an effect on the incidence or severity of YCS as the Mg foliar treatment was no different to the Untreated Control with respect to the number of YCS leaves per stalk (Figure 25).

Other treatments resulted in lower tissue Mg than Control, particularly the K_2SO4, Dolomite and Gypsum. The levels of Mg seen in these treatments are similar to those measured in previous years in YCS symptomatic leaves. Given that these treatments did not show any increase in YCS expression than Controls (except for one week for the Gypsum) it can be concluded that lower (not below the critical threshold) Mg per se is an unlikely cause of YCS.

Furthermore, results from the Burdekin Insecticide trial corroborate these findings as despite regular magnesium sulphate foliar applications, which resulted in elevated magnesium tissue concentrations, there was no reduction in YCS incidence or severity (in fact the MgSO4 treatment was one of the most symptomatic). In addition, the bifenthrin treatments in this trial aligned with findings in the Burdekin Insecticide trial in that these treatments had the least YCS affected canopies.

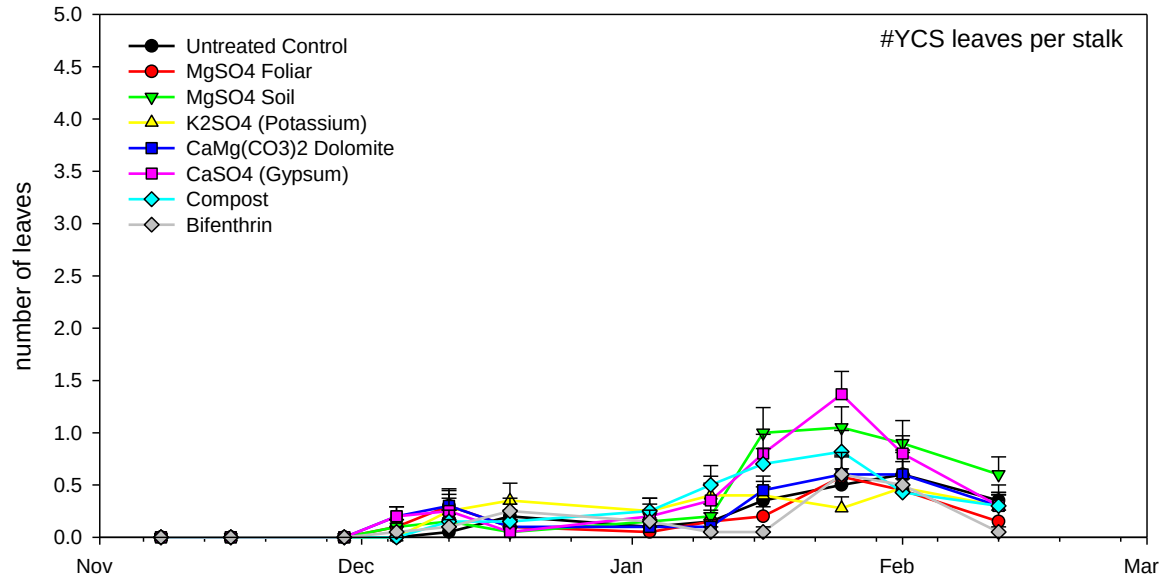


Figure 25 Average number of YCS leaves per stalk. Means of 20 monitored stalks ± standard error.

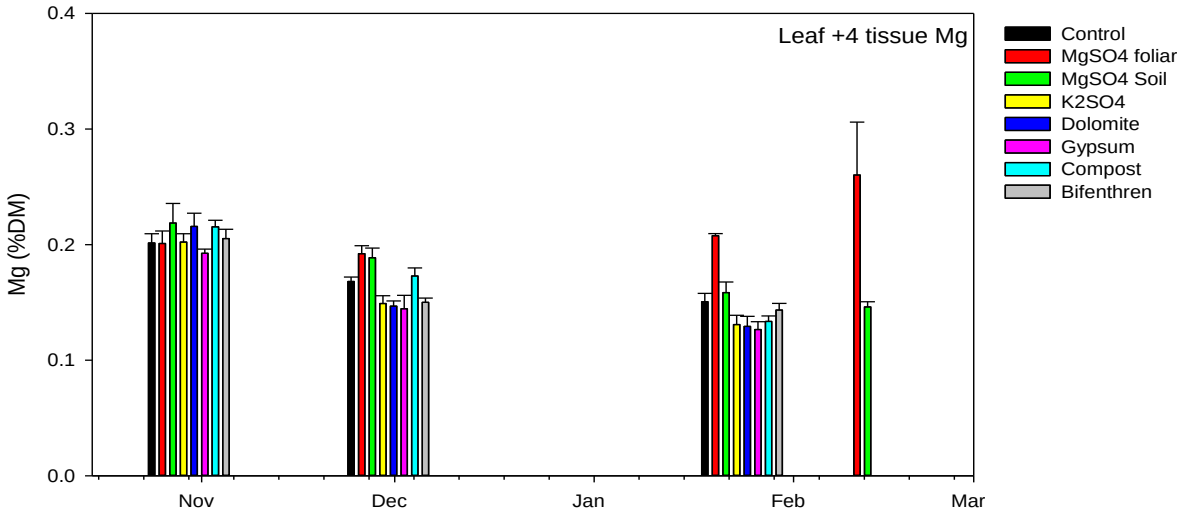


Figure 26 Leaf +4 tissue Mg. Means of 4 reps (20 individual leaves per rep) ± standard error.

6.2.4 Leaf magnesium and metabolite levels

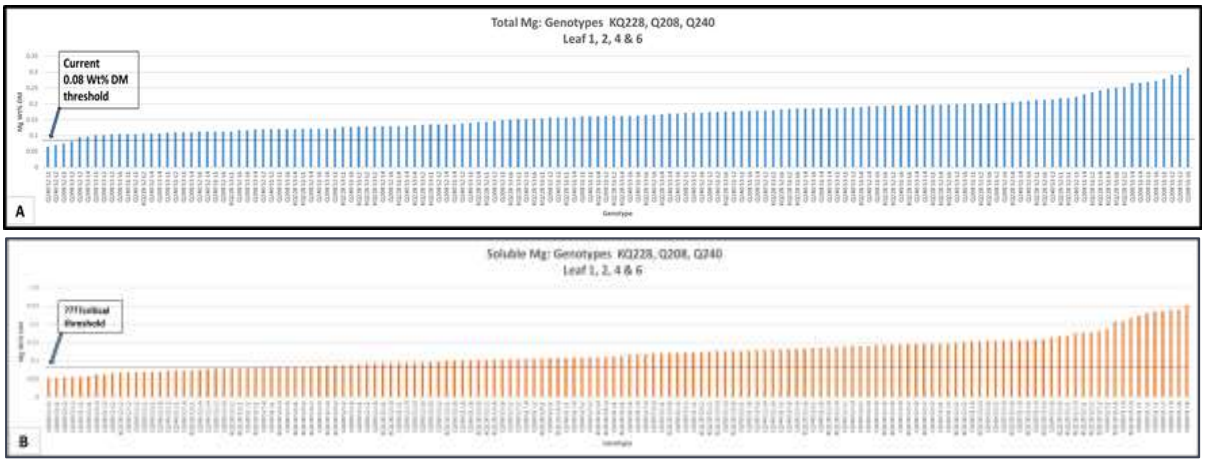
Leaf magnesium and carbohydrate accumulation

Magnesium (Mg) is a critical element that interacts with ATP to power the H^+-ATPase required to pump H^+ across the cell membrane of the companion cell to create a H^+ gradient. A sucrose H^+ symporter (SUT4) then combines with this gradient to transport sucrose from the apoplast across the companion cell membrane to concentrate in the phloem. This activity is the mechanism through which sugarcane actively loads the phloem. Deficient levels or inadequate supply of Mg also increases the plant's hypersensitivity to an extended photoperiod, which may culminate in a downregulation of the sucrose symporters. Therefore, a deficiency or inadequate supply of Mg coupled to a longer day length can result in impaired phloem loading and subsequent carbohydrate accumulation in the source tissue. Mg is also required by pyrophosphatases involved in long distance translocation of sugars and pyrophosphate hydrolysis. Hence, a magnesium deficiency may also affect movement of sucrose along the phloem from the source to sink tissue, inducing an imbalance that will result in further sugar accumulation in the source (Hermans et al., 2005)

Total and soluble Mg in sugarcane leaves (High yielding crops)

To gain a better understanding of the range across total and soluble Mg levels in sugarcane leaves, 144 samples comprised of Leaf +1, 2, 4 & 6 (4 reps) were collected from three genotypes (Q208$^{(l)}$, Q240$^{(l)}$ & KQ228$^{(l)}$) grown under best practice guidelines in three different sites in the Burdekin (crops @ >170t cane/ha).

Figure 27(A-C) shows there is clearly variation in Mg levels across the genotypes and between leaf numbers. Only two leaf +1 samples had total Mg levels below the 0.08 WT% DM minimum threshold currently accepted within the sugarcane industry. Figure 27(C) shows that leaf +1 total Mg levels are almost two fold higher than the accepted minimum and this also holds true for leaves +2, 4 and 6. These results would suggest that in well managed irrigated fields in the Burdekin there is no obvious Mg deficiency based on the current SRA recommendations



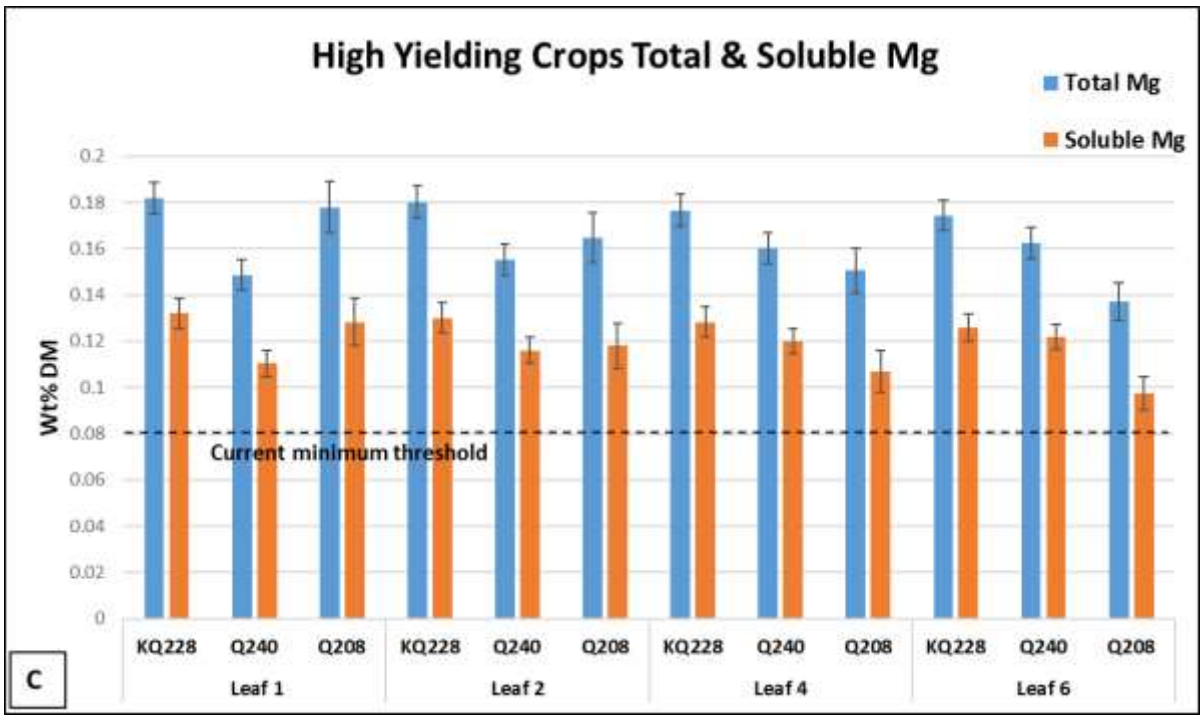


Figure 27 Total Mg (0.065-0.313 Wt% DM) A) soluble Mg (0.053-0.253) B) average total and soluble Mg in Leaf 1, 2, 4 & 6 across varieties Q208[A], Q240[A], KQ228[A] C)

Leaf sucrose and total α-glucans (High yielding crops)

Metabolite studies shows that sucrose and α-glucan levels are always elevated in YCS symptomatic leaves (Scalia et al., 2020). Therefore, establishing a sucrose and α-glucan baseline together with Mg levels in healthy leaves is crucial to understanding the involvement of Mg and YCS development.

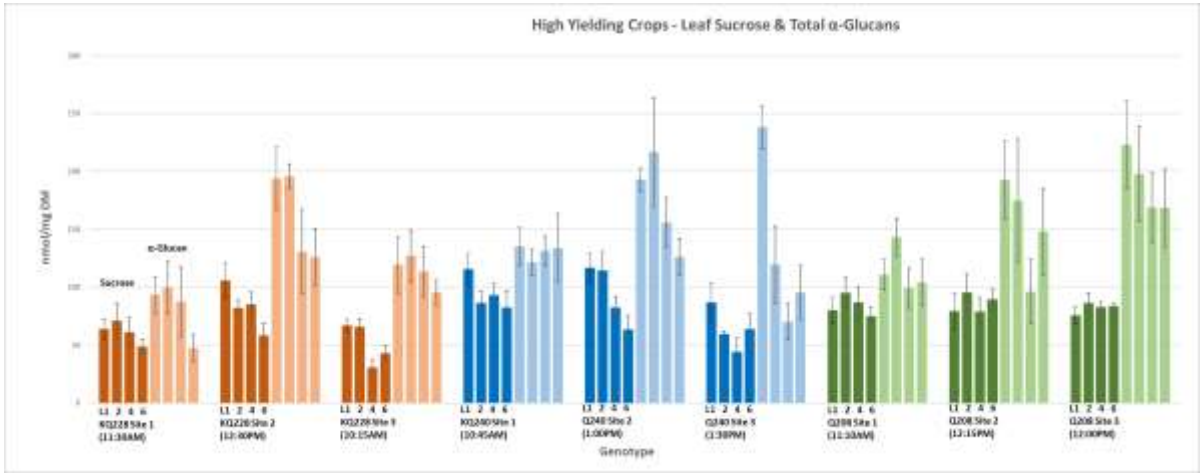


Figure 28 Sucrose and total α-glucan content (nmol/mg DM) in Leaf 1, 2, 4 & 6 across varieties Q208[A], Q240[A], KQ228[A]

Analysis of sucrose and α-glucan levels within leaf 1, 2, 4 & 6 across three sites and genotypes showed there was no accumulation of either metabolite (Figure 28) comparable to that previously measured in YCS leaves or above the 200 nmol/mg DM upper threshold (Scalia et al., 2020). Therefore, this was an excellent dataset to establish baseline parameters around sucrose, α-glucans and Mg.

Mg & sucrose/α-glucan relationship (High yielding crops)

Figure 29 (A & B) shows there is no correlation between total and soluble Mg levels with either sucrose or α-glucan under adequate Mg conditions. A more detailed analysis (data not shown) also showed no correlation between sucrose and total Mg for the four samples below the current Mg lower threshold highlighted in Figure 27A.

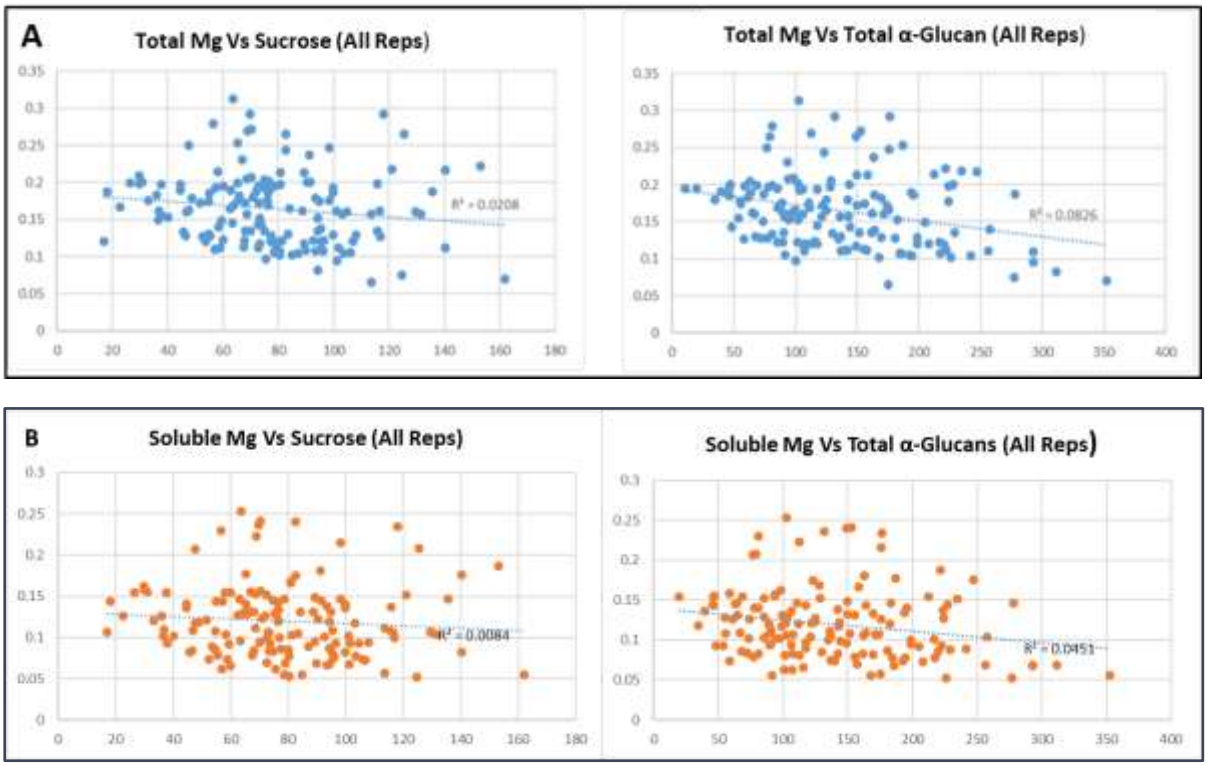


Figure 29 Correlation between leaf sucrose and Total α-glucan content with A) Total Mg and B) Soluble Mg for Leaf 1, 2, 4 & 6 across varieties Q208^{A}, Q240^{A}, KQ228^{A}

Mg related transcriptome expression

A comparative analysis of Mg related transcripts in leaf samples across three genotypes (Q200$^{\phi}$, KQ228$^{\phi}$ & Q208$^{\phi}$) and three field visits showed that the YCS expression did not match the transcriptome effects expected in a magnesium-deficient condition. While some Mg-related transcripts were differentially- similarly-annotated transcripts. For example, some transcripts were up-regulated while others with identical annotations were down-regulated. In addition, all of these transcripts have other functions aside from their Mg associations.

Overall, these results show that a magnesium-deficiency conferred response is not associated with YCS. Physiological and 'omics' studies show that YCS leaves always have elevated levels of sucrose (Marquardt et al., 2016; Scalia et al., 2020). Research by Scalia et al. (2020) indicates that a physical blockage of the phloem is not the cause of elevated leaf sucrose and reduced phloem flow. This concurs with nutrient mobilisation studies presented in section 6.2.3 of this report (Olsen et al., 2019). In response to rising leaf sucrose, feedback inhibition of photosynthesis causes an over-reduction of the photosynthetic electron transport (PET) chain and under-utilisation of trapped solar energy (Marquardt, 2019). The consequence of this is increased production of reactive oxygen species (ROS) and rapid photooxidation of the leaf tissue resulting in destruction of the chloroplasts

and yellowing (Schöttler and Tóth, 2014). Therefore, the lower Mg levels measured in YCS leaves may be due to remobilisation of resources out of the leaf as disruption to sucrose transport and photosynthesis increases during YCS development.

6.2.3 YCS Recovery Study

A field of Q240$^{\phi}$ sugarcane was identified in the Burdekin in February 2016, which had very clear YCS symptomatic cane and very green asymptomatic cane at opposing ends of the same block.
The aim was to test whether YCS symptomatic and asymptomatic cane were similar across a range of agronomic and physiological parameters, and to determine the cause of the phenotypic difference. In May, the YCS cane appeared to have spontaneously recovered. This presented an opportunity to test the same parameters to determine any significant differences or similarities between the previously symptomatic cane and asymptomatic cane.

The field trial of sugarcane variety Q240$^{\phi}$ 1R was on a commercial farm in the Burdekin and measurements were made on 20 stalks of YCS symptomatic and 20 stalks of YCS asymptomatic which were randomly selected from within the same block. In February samples were collected to evaluate the difference between symptomatic and asymptomatic cane. Later, in May, the same cane had appeared to fully recover and was all asymptomatic. This cane was then re-sampled to evaluate the apparent recovery. Leaf +1 and leaf +4 were analysed for nutrient content. Baseline samples were taken in early February and post-YCS samples were taken in late May. February and May were analysed separately. For each month analysis of variance (α=0.05) was determined using a split plot model with treatment the main plot factor and leaf number the sub-plot factor. LSD pairwise analysis was then conducted with significant difference shown by letter separations (a, ab, b, etc.). A comparison of nutrient concentrations of the symptomatic (Feb) and recovered (May) cane is now presented.

<u>Si and Mg</u>

Si was significantly higher in YCS leaf +4 compared to the asymptomatic cane and remained elevated despite having 'recovered' (Figure 30). Mg was found to be significantly lower in YCS leaf +4 and once the cane no longer demonstrated symptoms, the Mg returned to considerably higher levels equal to the control (Figure 31). The Si/Mg ratio was higher in YCS leaf +4 due to these differences and returned to normal levels once the cane had 'recovered' (Figure 32). Also note that Mg levels were lower in leaf +1 than in leaf +4, yet there was no yellowing of leaf +1 (Figure 31, left).

<u>P and K</u>

Levels of P were elevated in YCS leaf +1 during the YCS outbreak and once the cane returned to a healthy green, both YCS leaf +1 and leaf +4 had significantly higher concentrations of P compared to the control (Figure 33). Conversely, K showed significantly lower levels in YCS leaf +4 and by May had significantly higher levels of K (Figure 33).

<u>Zn and Fe</u>

Levels of Zn were equitable in both leaves across treatments in February and by May, Zn was significantly elevated in both YCS leaves (Figure 34). There was a similar finding for Fe in which levels

were consistent across treatments for both leaves in February and once recovery had appeared to take place, Fe levels were significantly lower for both YCS leaves compared to the control (Figure 36).

All other elements (S, Ca, Cu, Na,) showed no significant difference between symptomatic and recovered treatments (data not shown).

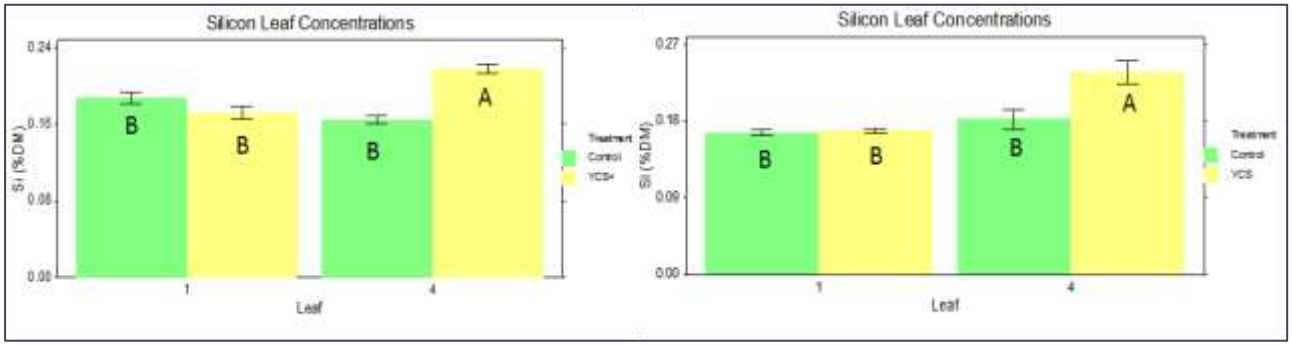


Figure 30 Silicon results for Burdekin Q240$^{\Phi}$ comparing leaves +1 and +4 mean values in Feb (left) and May (right). Si is significantly greater (p<0.05) in YCS leaf +4 in both instances.

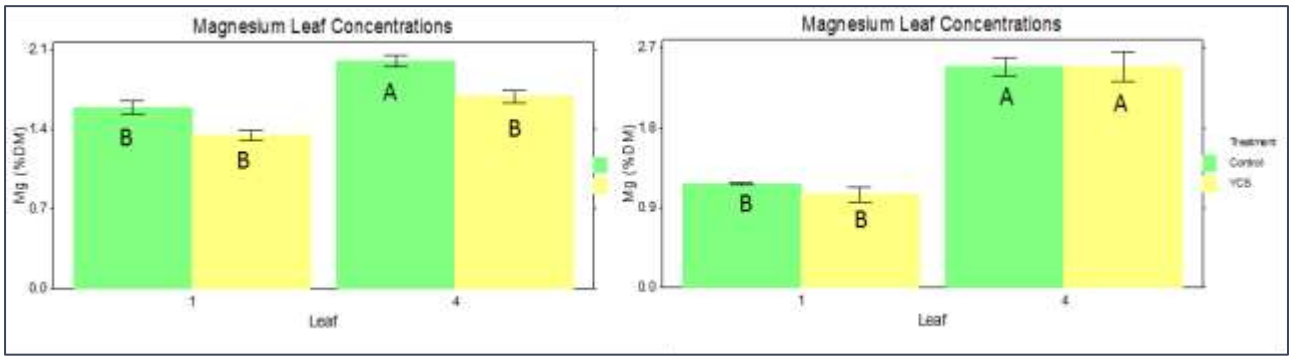


Figure 31 Magnesium results for Burdekin Q240$^{\Phi}$. In Feb (left) Mg is significantly lower (p<0.05) in YCS leaf +4 and in May (right) Mg levels are similar to the control.

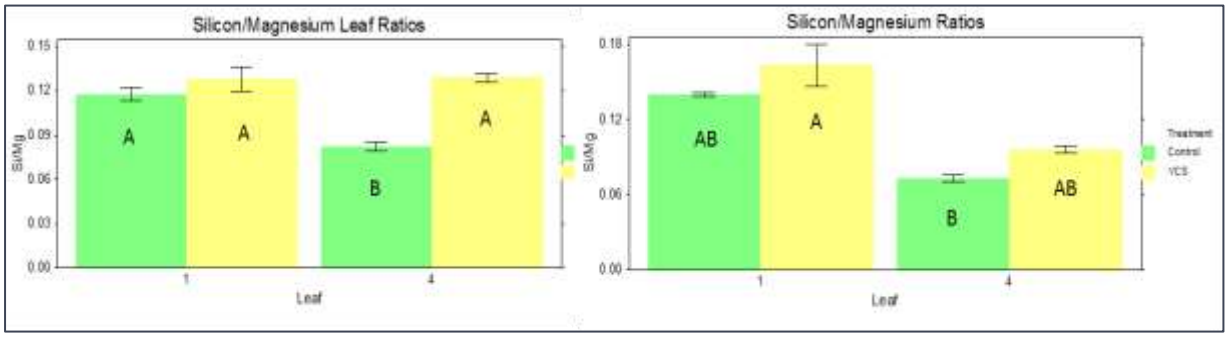


Figure 32 Silicon/Magnesium ratios for Burdekin Q240$^{\Phi}$. In Feb (left) Si/Mg is significantly higher (p<0.05) in YCS leaf +4 and in May (right) return to levels similar to the control.

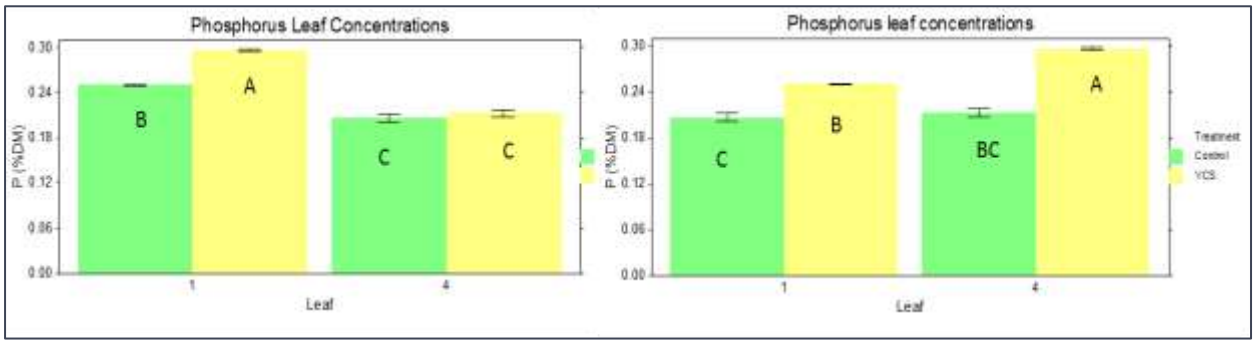


Figure 33 Phosphorus results for Burdekin Q240Φ. In Feb (left) YCS leaf +1 is significantly higher (p<0.05) and in May (right) both YCS leaf +1 and +4 are significantly higher than the controls.

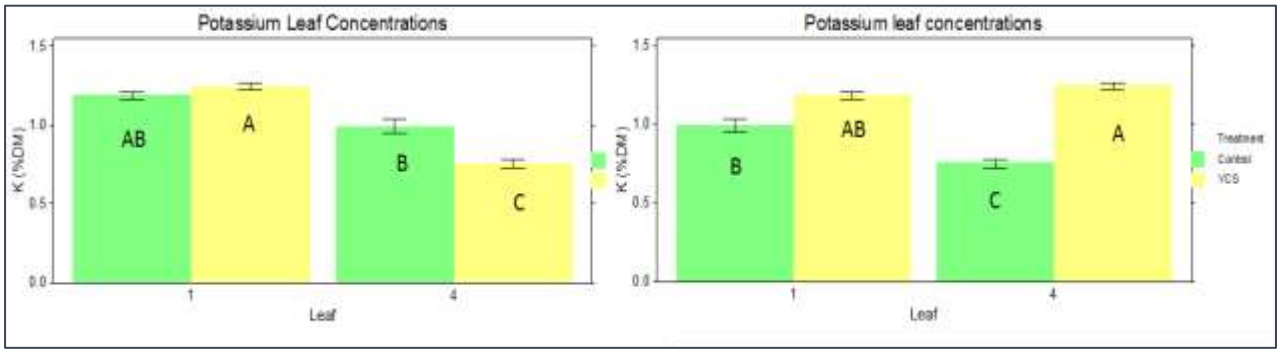


Figure 34 Potassium results for Burdekin Q240Φ. In Feb (left) K is significantly lower (p<0.05) in YCS leaf +4 and in May (right) YCS leaf +4 is significantly higher.

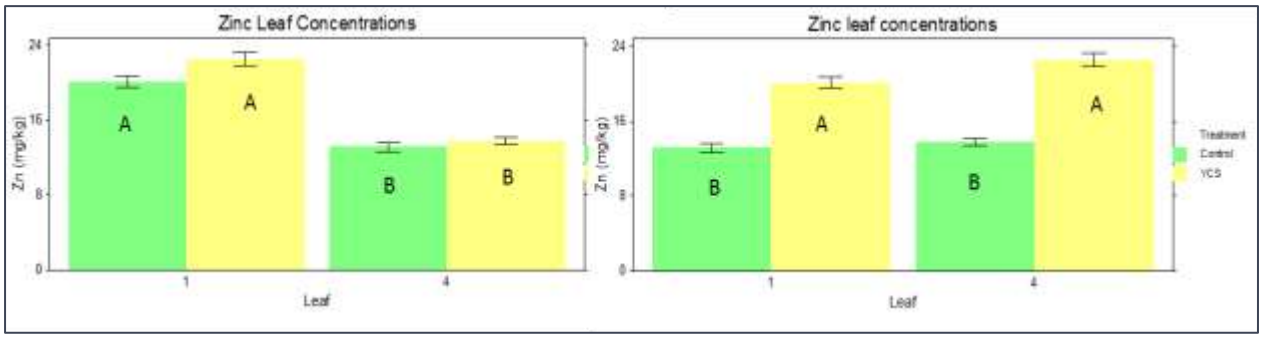


Figure 35 Zinc results for Burdekin Q240Φ. In Feb (left) Zn levels remain comparable between treatments and in May (right) both YCS leaf +1 and +4 are significantly higher (p<0.05) than the controls.

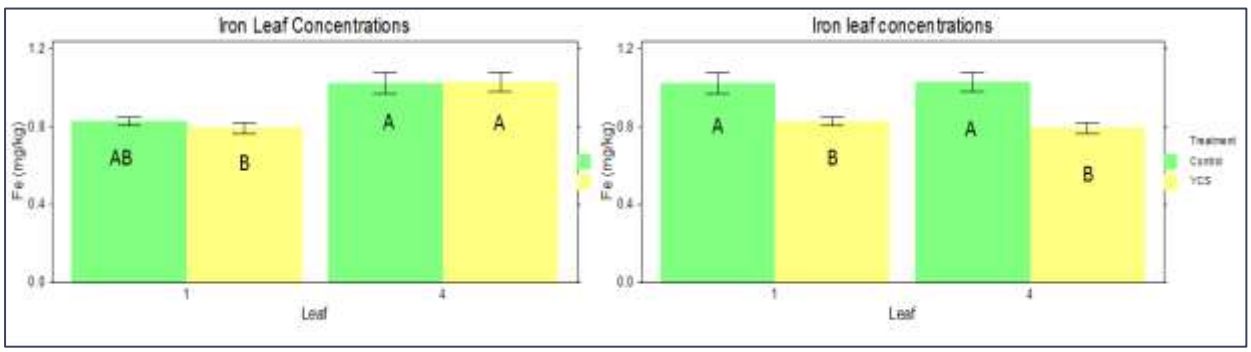


Figure 36 Iron results for Burdekin Q240Φ. In Feb (left) Fe levels remain comparable between treatments and in May (right) both YCS leaf +1 and +4 are significantly lower (p<0.05) than the controls.

These results concur with previous studies that have identified significant differences throughout the canopy in Mg of YCS symptomatic and non-symptomatic cane and the data presented here supports that discrepancy.
The Mg of leaf +4 during the YCS outbreak is significantly lower than the control and once the cane experienced spontaneous 'recovery' the Mg concentrations returned to higher levels in the equivalent leaf number, almost identical to that of the asymptomatic cane. The soil test results show that Mg levels in the soil remained the same throughout this growing period. Mg foliar sprays had not been applied at any stage to correct the deficiency, which indicates that there appears to be a disruption in Mg uptake either within the roots or within the plant during an outbreak of YCS. This could potentially be responsible for the symptomatic yellow leaves. This supports the hypotheses that YCS may cause low Mg or conversely that low Mg causes YCS.

Interestingly, Si demonstrated significantly higher concentrations in the YCS leaf +4 during the YCS outbreak and despite the cane returning to green later in the season, the Si remained high. This is not unexpected as Si uptake increases when a plant is under stress, assists with its recovery, and once accumulated is not mobile within the plant (Hernandez-Apaolaza, 2014; Manivannan and Ahn, 2017). Sugarcane is also a known Si-accumulator crop (Keeping, 2017). There were similar findings for P concentrations in which YCS leaf +1 was elevated during YCS expression and by May both leaf +1 and +4 were showing significantly higher levels despite having recovered. Additionally, K showed lower levels during the YCS event and conversely higher levels once the cane recovered. The micronutrients of Zn and Fe also showed inconsistencies in which there were no significant concentration variations to the asymptomatic cane in February and post-YCS had significantly higher and lower concentrations respectively.

The differing nutrient concentrations between leaf +1 and leaf +4 can be attributed to the mobility of particular nutrients. K is very mobile and as a consequence leaf +1 can have higher total concentrations as it is translocated from older leaves to developing leaves when required.
Fe is relatively immobile and can typically be seen in higher concentrations in the more mature leaves of a crop. Overall, leaf +4 seemed to demonstrate the most deviation in nutrient concentrations in YCS symptomatic cane when compared to the asymptomatic cane. This finding concurs with similar variations in metabolite disruption noted in this same leaf tissue (Scalia et al., 2020).

The findings of this nutrient study confirm that sugarcane can recover from a YCS event.

6.2.4 Discussion and conclusions

Comprehensive nutrient testing of soil and plant tissue confirms YCS is not caused by nutrient deficiencies, heavy metal toxicities, or compromised nutrient mobilization within the plant (Olsen et al., 2019). However, results did show elevated levels of Si and reduced and Mg content in all YCS samples across all leaves. After recovery from a YCS event leaf Mg levels returned to normal. This suggests the Si/Mg ratio anomaly is highly unlikely to be the cause of YCS, but rather a plant nutrient accumulation and mobilisation response prior to, during and post YCS expression. The levels of both elements either rise, remain static, or fall in response to external stressors, elevated leaf sucrose, over-reduction of the PET chain, photooxidation, chloroplast destruction or cell death.

6.3 Crop stress

The episodic nature of YCS at a regional and individual field level has been both intriguing and puzzling at the same time. Growers and industry service providers constantly reported outbreaks of canopy yellowing after good rainfall that was preceded by a very dry period during the peak growing season. The onset of yellowing was also noted to be very fast and often occurred within a few days to a week following rain. This was also evident in irrigated crops. Prior to these YCS events, rainfed fields under water-deficit conditions exhibited typical water stress characteristics of leaf rolling, a lighter yellow colouration of the lamina as well as natural senescence with browning from the tip and outer margins (Inman-Bamber, 2004; Inman-Bamber and Smith, 2005). However, in the counterpart irrigated fields, leaves appeared green and turgid prior to the YCS event. Growers reported that these crops appeared to have stopped growing prior to the rain, even though all the conditions for growth were available. This peculiarity is known as the reduced growth phenomenon (RGP). The main cause of RGP is thought to be a reduction in leaf nitrogen, reduced photosynthesis due to sugar mediated feedback downregulation of the photosystems, an increase in maintenance respiration, maturation of the culm, and possibly even tiller mortality and the timing of harvest (Park et al., 2005; van Heerden et al., 2010). While there are similarities between YCS and RGP our research indicates YCS plants do not have any leaf nutrient deficiencies (see section 6.2 of this report). Gene expression and metabolite studies by Scalia et al. (2020) show the status of the internodes to be a 'Feast' state which does not support an increase in maintenance respiration, which also concurs with culm sugar content data which consistently shows no CCS penalty.

One of the issues with working on YCS has been the transient and uncertain nature of symptom expression. Research conducted in the pilot project 2013/807 in 2013 suggested that YCS–like symptoms can be elicited in plants via a water stress and that this can be repeated when mature cane is present on those plants. The following studies presented in this chapter examine the potential link between YCS development and various stressors, including water deficit stress.

6.3.1 Water stress pot trial – Tissue Culture plant source - Mission Beach

Plants derived from tissue culture offer some of the 'cleanest' disease-free plant sources available in the sugarcane industry. If YCS is caused by an unknown pathogen, such plant sources may be YCS-free. Of relevance to the YCS research program is therefore whether a water stress treatment will elicit YCS-like symptoms in plants derived from tissue culture. Tissue culture plantlets of Q200 and Q247 (both varieties of which readily express YCS in affected districts) were sourced, transferred to small pots and quickly moved to a Mission Beach nursery, to avoid potential 'infection' by a YCS pathogen (if it exists) (see Appendix 1: 1.1.5). Plants were grown for nine months, until about 1.5 m of mature stalk material was present on shoots, and a water stress applied to half of the pots.

In early November 2015, the automatic irrigation on two replicates of the pot trial was switched off for two days. Separate water stresses were applied to Q200 and Q247. Some leaf symptoms of stress were noted, but these were not severe. Irrigation was then re-established and observations of the leaf canopy made.

Results

Within 24 hours, very significant leaf yellowing has occurred on those plants which were more exposed to the elements (on the edges of the trial). Symptoms appeared identical to those of YCS – full yellowing across the leaf blade was noted as well as the typical blotchy yellowing on the leaf lamina (Figure 37 & Figure 38).

Figure 37 Photos of the same plants after a water stress was applied to each (KQ228 –left; and Q200 – right).

Figure 38 Leaf yellowing that developed within 24 hours after a water stress (Q200 left); water-stressed vs non-water stressed (on right) in tissue culture plants at Mission Beach

The application of a water stress provided a trigger for YCS symptom development in the original pot trial conducted at SRA Tully using planting material sourced from either the Tully region or from YCS-affected crops in the Burdekin (see 6.8 of this report). It was not known whether perhaps an unknown YCS-associated pathogen was present in the Tully material; the water stress treatment may then have revealed that the lack of symptom development in Tully crops may have been because of the lack of a stress trigger in these crops.

For this reason, sourcing a potentially YCS-free plant source (tissue culture) provided an opportunity to investigate this further. The development of YCS-like symptoms in the tissue culture material answers some questions but poses others. If there is a pathogen involved in YCS, it may be that conventional tissue culture does not eradicate it. Some pathogens may survive the tissue culture process. To address this, meristem tissue culture was undertaken at SRA Brisbane to create another

potential YCS-free plant source. This tissue culture process has a greater certainty of eradicating pathogens (see section 6.8 of this report for details trials using this source material).

It is noteworthy that the water stress treatment followed by irrigation induced a rapid YCS response in plants on the perimeter of this pot trial. In the absence or detection of a known pathogen in the clean source tissue culture plants, the physiological response of these plants should first be considered to understand why YCS was able to be induced under these conditions. Plants that are 9 months old grown in small pots would be root bound and therefore have a much smaller root mass and sink size. This is often referred to as the pot effect and can be limiting depending on the aim of the investigation (Ogunkunle and Beckett, 1988). In this case a much smaller root mass would reduce sink strength and impact on the plants ability to maintain maximum growth rate. The crop age trials (see section 6.1.1 of this report) showed that the key driver of YCS was growth rate. Root bound plants would have similarities to field plants grown under soil compaction conditions which have a negative impact on growth of the other main sink tissue of the culm (Ogunkunle and Beckett, 1988; Smith et al., 2005). Both of these non-photosynthetic main sink tissues (roots and culm) are dependent on adequate supply of reduced carbon, water and nutrients (Rae et al., 2005; Wang et al., 2013). Therefore, the pot effect together with water restriction in this trial would be extremely sink limiting, making the plant highly susceptible to a supply demand imbalance if rapid growth was to suddenly occur. Manipulation of sink strength in the plant growth regulator trial (see section 6.6.1 of this report) reduced sucrose export from the source leaf, leading to increased sucrose accumulation in the leaf, reduced stomatal conductance and photosynthetic activity. This triggered photo-oxidation and leaf yellowing which is dependent on a sugar-mediated down-regulation of the photosystems and under-utilisation of trapped energy due to a lack of oxidised coenzyme (Ahmad, 2014; Schöttler and Tóth, 2014; Marquardt, 2019). This explains why plants on the edges of the trial that have the highest solar radiation interception are first to develop and express YCS symptoms.

6.3.2 Abiotic stress and abscisic acid (ABA)

6.3.2.1 Heat and water stress

Extreme climatic events can have a significant impact on crop health and production (Zhao and Li, 2015; Watson et al., 2017; Bonnett, 2018). It was hypothesised that periods of extreme heat could induce YCS symptoms in plants grown under water stress. A pot trial was established in the Burdekin to test this theory with 14 week old KQ228$^{(b)}$. The key objective was to investigate if there was a difference in response to individual and combined effects of water limitation and transient extreme heat stress.

Treatments involved two water limitations and heat bursts on 2 days at 11am-2pm by maintaining the pots at 42-44^{0}C. The maximum ambient temperature was 27-29^{0}C throughout the experiment.

1. Well-watered (80% field capacity) + no heat treatment
2. Well-watered (80% field capacity) + heat treatment
3. Water limiting (40% field capacity) + no heat treatment
4. Water limiting (40% field capacity) + heat treatment

<u>Results</u>

Table 13 Leaf response to different stress conditions

Treatment	Observations
Well-watered + no heat (no stress)	Normal leaf growth
Well-watered + heat	Pale green patches on leaf lamina (Figure 39A)
Water limiting + no heat	Tip drying mainly in younger leaves including the spindle, and leaf rolling (data not shown)
Water limiting + heat	Drastic yellowing in leaves 5-8 (Figure 39B)

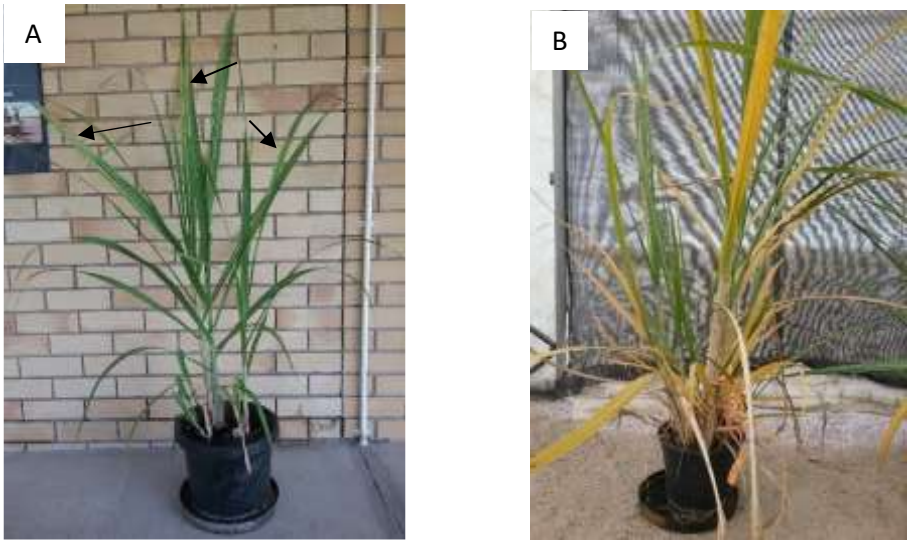


Figure 39 Leaf colour change in well-watered + heat (A) and water-limiting + heat (B) treatments 2 days after heat treatment

Observations were commenced 2 days after treatment (Table 13). There was a clear difference in leaf response to the individual and combined stress conditions (Table 13 & Figure 39). Pale green patches on heat stressed well-watered plants stayed pale until maturity, and plants in water-limiting conditions recovered completely after watering. However, yellowing leaves in plants under combined stress (high temperature and limited water) continued to senesce prematurely.

Summary

A combination of high heat and water stress was required to induce severe leaf yellowing and rapid senescence. However, the majority of the yellowing was in the lower canopy and was more akin to natural senescence than mid-canopy yellowing in YCS. YCS yellowing is also a more golden-yellow colour caused by high levels of zeaxanthin and anthocyanins resultant of changes to carbohydrate metabolism and chlorophyll destruction (Marquardt et al., 2016; Marquardt et al., 2017)

6.3.2.2 Exogenous Abscisic acid (ABA)

Role of ABA in a number of physiological processes such as development, responses to abiotic stresses and regulation of stomatal function has been well documented (Hauser et al., 2011; Khanna-Chopra, 2012; Saradadevi et al., 2017). Similarly, use of abiotic and biotic stresses and exogenous ABA to induce endogenous ABA levels in plants has also been shown in other crops (Aroca et al., 2008; Qin et al., 2011).
A pot experiment with KQ228$^{(\phi}$ was established to test whether exogenous ABA could induce early leaf senescence or YCS symptoms, and whether there is a leaf level or plant level difference in response to location of exogenous ABA application.

ABA treatments consisted of a concentration range from 4-10mM applied to the leaves and roots through soil drenching. During the experimentation, the most responsive leaf, as well as the most responsive leaf region to exogenous ABA were tested.

Results

Foliar application of ABA

A) Differential response of leaf age to exogenous ABA

Tips of first six fully expanded leaves in potted 14 week old KQ228^{A} plants were treated with either ABA (8mM) or water (control). Test plants had at least 8 leaves each, and all were >90 cm long. At the time of treatment, Leaf +7 and +8 were in the process of natural senescence.

Within 24 hours, leaves of ABA treated plants started yellowing from the tip to the base indicating the effect of exogenous ABA (Figure 40). The youngest fully expanded leaf leaf +1 showed the lowest visible response to external ABA, while leaf +5 had the most severe yellowing. All leaves in the control treatments were green (data not shown).

Figure 40 Within canopy variation to foliar application of ABA (leaf +1-6 from right to left)

B) Differential response of leaf regions to external ABA application

Leaf +2-4 were treated with 8mM external ABA or water (control) to the adaxial or abaxial tip, mid and basal leaf regions.

External ABA did not a show any negative impact on leaf growth. Adaxial applications always showed visible colour changes, mainly yellowing (Figure 41). Adaxial application to the tip regions showed induced tip yellowing which only continued up to ~1/3 of the leaf lamina. Application to basal regions induced significant yellowing and accelerated senescence in mature leaves. Adaxial application to the mid regions had not shown any visible changes in leaf growth or colour.

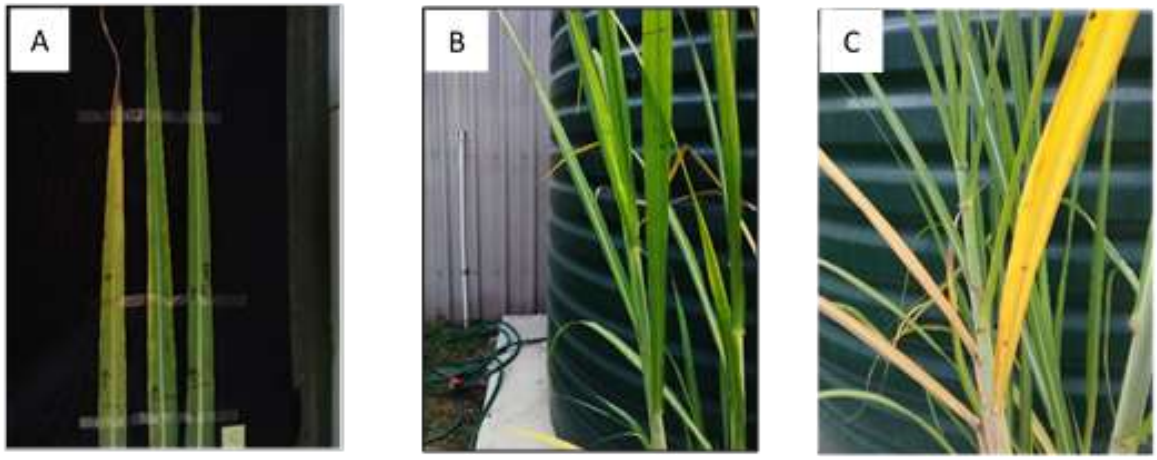


Figure 41 Differences in exogenous ABA induced yellowing in adaxial leaf regions; tip (left), mid (middle) and base (right)

Soil drenching with ABA

The potted plants received either a 5mM or 10mM ABA soil drenching. ABA 10mM triggered yellowing from the leaf tip to base within 2 days after treatment, mainly in lower leaves (Figure 42). Lower ABA concentration did not show any visible changes in the test plants.

Figure 42 Drastic leaf tip yellowing 2 days after soil drenching with ABA

Summary

Exogenous ABA (foliar and soil) induced leaf yellowing and accelerated leaf senescence in sugarcane, which always commenced from the tip and continued towards the basal region of the leaf. This is synonymous with the pattern of natural senescence from the oldest part of the leaf to the youngest; and from the oldest leaf to the youngest up the canopy. However, YCS symptoms tend to start where sucrose levels and solar radiation interception is highest (Scalia et al., 2020). A remarkable difference in visible colour change was observed with leaf age as well as location of application.

Crops grown in tropical conditions regularly experience multiple micro and macro climatic conditions within the canopy resulting, for instance, higher or varying canopy temperature (especially in the mid and lower canopy) than normal. In sugarcane, the leaf architecture, canopy shape and canopy cover may trap such micro-climatic conditions and make temporary physiological adjustments. Therefore, it is possible to have elevated ABA levels in sugarcane crops grown under abiotic stress conditions, which could trigger leaf yellowing and premature senescence as adjustments to adverse conditions. However, ABA induced senescence did not originate in the mid canopy as observed in YCS (see section 6.1 of this report).

6.3.3 Confidor® Trial – Stone River, Ingham

The trial was established on 7/9/2014 using asymptomatic plant source, variety Q200$^{\phi}$ sugar cane on a HCPSL rainfed managed site in Stone River (farm #0847A Block #2-1) (see Appendix 1: 1.2.2). The aim of the trial was to establish if Confidor® Guard (Bayer CropScience) reduces the prevalence and/or severity of YCS symptoms in field grown cane. The effect on soil-borne insects, root health, yield and sugar content were also studied as part of this trial. A randomized complete block design of four treatments with four replicates for a total of 16 plots were examined through the 2014/2015 growing season. Plots were planted five rows wide and 15 meters long with four rows of guard to

reduce edge effect. There were four treatments; Untreated Control, Confidor® 22mL/100m (label rate), Confidor® 44mL/100m (2 x label rate) and Confidor® 44mL/100m at planting + 22mL/100m at fill in (3 x label rate). Prior to commencement of the trial, a soil test was taken and nutrients applied at Six Easy Steps rates. Weeds were chemically controlled as per standard commercial practice. Monitoring of the trial commenced in early February was carried out weekly. YCS prevalence and severity was recorded as well as stalk height and soil moisture. A treatment effect became evident during the season and additional sampling was undertaken in the form of canegrub assessment, root biomass and final biomass determination. Samples were also sent to SGS laboratories, Brisbane for biochemical analysis.

YCS Monitoring

Standard protocol for YCS monitoring of prevalence and severity was adhered to throughout the trial (Table 3 & Table 4). YCS was first observed in the lower canopy in early February in the untreated control plots. Symptoms arrived in the lower canopy of the Confidor treated plots crop approximately 3-4 weeks later in early March (Figure 43A). YCS spread rapidly through the stalk population and was observed in 75-100% of plants consistently in the lower canopy around early May. All Confidor rates showed a similar trend for lower canopy prevalence that was quite different to the untreated plots. Untreated plots were clearly more affected from early onset right up until the YCS peak prevalence in late May (Figure 43A).

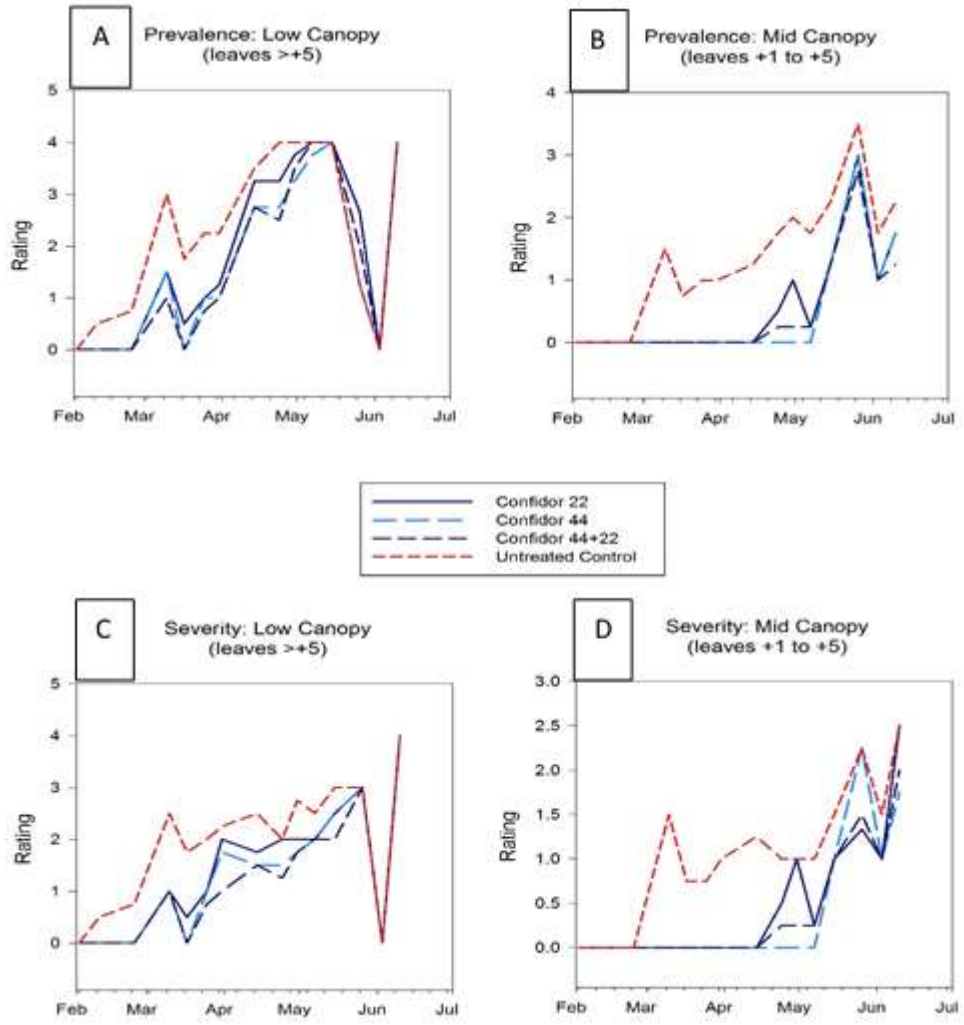


Figure 43 YCS prevalence and severity results, measured weekly from February 2014 until June 2015. Lower canopy prevalence (A), Mid canopy prevalence (B), Lower canopy severity (C), and Mid canopy severity (D)

YCS prevalence in the mid canopy showed a similar trend, although the delay in symptom onset in the treated plots was much longer with untreated plots showing YCS yellowing approximately 6-7 weeks before the treated plots (Figure 43B). Higher rate plots (Confidor 44 and Confidor 44 + 22) initially showed some greater delay in YCS onset, however by early May all of the treated plots followed a similar prevalence trend (Figure 43B). YCS was always more prevalent in the mid-canopy of untreated plots throughout the duration of the monitoring period (Figure 43B)).

Severity, or degree of yellowing, was always greater in the lower canopy of untreated plots throughout the peak season Feb-June (Figure 43C). Severity reached the maximum “severe” rating simultaneously in all plots in mid-May. There is some suggestion that higher Confidor rates resulted

in milder yellowing, particularly throughout April, however eventually all treated plots showed similar severity trends in their lower canopies (Figure 43C).

In the mid canopy, untreated plots showed greater degree of severity than treated plots across all time points. The high rate Confidor 44 delayed the onset of symptoms relative to the other treated plots by approximately 4 weeks. After this point, treatment rate did not make a difference.

Soil Moisture Results

This trial was rainfed only. Soil moisture was measured weekly at a range of depths (Figure 44). Overall, the surface profile was quite dry with adequate soil moisture only becoming apparent at depths ≥300mm during the course of the trial period. In general soil moisture increased from Feb-April before the whole profile underwent a gradual dry down from early May. This is a typical pattern for the Stone River region.

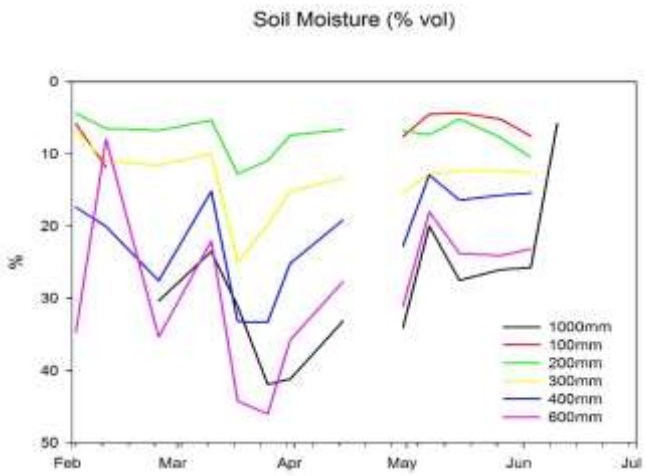


Figure 44 Soil moisture (%vol) at 100mm, 200mm, 300mm, 400mm, 600mm, and 1000mm depth over time. Data presented is an average of eight PR2/6 soil moisture probes (Delta-T Devices Ltd, Cambridge UK), installed 2 per treatment across the length of the trial.

Biomass

Final fresh weight biomass was measured for each of the monitored stalks (see section 5.4.1 this report). These measurements are presented below together with average YCS severity for each monitored stalk (Figure 45). Linear regression analysis, and Pearson's correlation test, was performed to determine the strength of the linear relationship (if any) between YCS and final biomass. There was no significant effect of confidor treatment on final stalk biomass, so treatment was not included as a predictor in the following regression analysis. YCS severity was not shown to be a significant predictor of final biomass ($p=0.417$) and YCS was not correlated with fresh weight biomass (Pearsons correlation $r= -0.013$) (Figure 45).

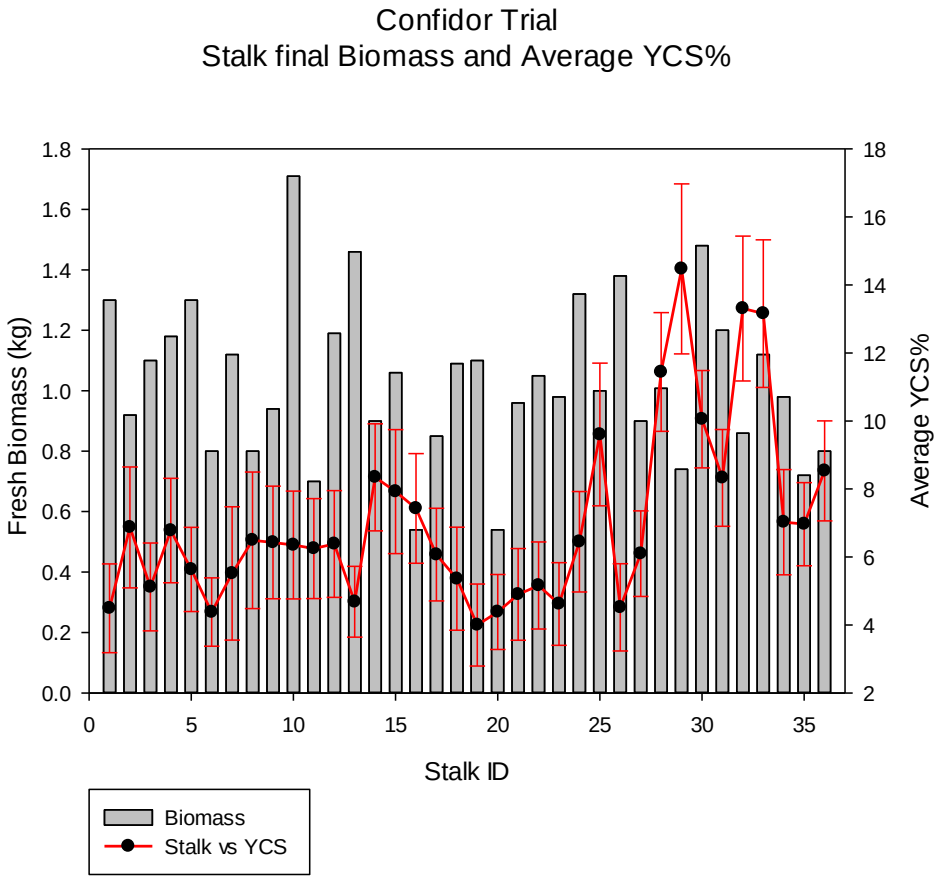


Figure 45 Stalk biomass and average YCS severity per stalk at the Herbert Confidor Trial. YCS severity has been averaged across 29 observations and is expressed as a % (number of YCS affected leaves per stalk / total leaves per stalk). Standard errors shown.

Biomass was not statistically different between treated and untreated plots, although the mean was lower in the untreated plots (Table 14). Plant heights were also very similar between treatments. There was some spread in the CCS means for plots, however no statistical difference was found. These results are consistent with findings of other YCS Confidor trials.

Table 14 Biomass results. ANOVA performed at 95% confidence. Difference between groups has been determined by a Tukey's HSD all-pairwise comparisons (p<0.05). Test (displayed as letter separations A, B, AB etc)

Treatment	Stalk Height (cm)	Stalks/10m	Total Biomass (t/ha fresh)	Millable Stalk (t/ha dry)	CCS
Confidor® 22	254.9	138.8	105.82	21.40	10.05
Confidor® 44	242.0	145.5	109.13	22.33	10.31
Confidor® 44+22	243.5	144.3	106.50	22.19	8.97
Untreated Control	245.2	133.0	93.87	18.89	9.31
p-value	0.512	0.545	0.321	0.187	0.086
CV	5.2	9.4	11.3	10.7	7.5

Compositional analysis

Leaf and culm samples from untreated control and Confidor® 44mL plots were sent to SGS for compositional analysis In June. Upper canopy leaves were taken from the zone above the FVD, mid-canopy leaves (leaves +1, 3 and 5) were cut at corresponding dewlap excluding sheath. Upper stalks, comprising internodes (0 to +5) and lower stalks (internodes 6+) were stripped of leaves and sheath. Table 15 shows there was no significant difference between Confidor treated and Untreated plots for the majority of biochemicals at the main factor level. The exception was glucose, which was decreased in Confidor treated plots. There were some differences at the sub-factor level, with upper canopy leaves (those above FVD) having lower levels of moisture, sucrose and fructose and significantly higher levels of crude fibre. Analysis of the interaction terms showed that there was no difference between Confidor treated and untreated plots in the upper canopy across the range of biochemicals for the combined upper and mid-canopy. However, metabolite levels in the mid-canopy YCS symptomatic leaves show higher sucrose, glucose, fructose and starch than asymptomatic treated leaves. Full metabolome analysis of the mid-canopy is presented in section 6.3.3 of this report.

Table 15 Leaf analysis results. Upper Canopy Leaves are all leaves above FVD, while Mid Canopy Leaves are leaves 1, 3 and 5. Analysis of variance conducted using a split-plot design with main factor (chemical treatment) and sub-factor (leaf location in the canopy). ANOVA performed at 95% confidence. Difference between groups has been determined by a Tukey's HSD all-pairwise comparisons (p<0.05) (displayed as letter separations A, B, AB etc) (compositional analysis conducted by SGS Food and Agriculture, Brisbane.)

Treatment	Starch %	Protein %	Dry Matter %	Crude Fibre %	Sucrose %	Glucose %	Fructose %
Confidor®	0.21^{A}	10.17^{A}	30.34^{A}	45.10^{A}	1.41^{A}	1.19^{B}	1.15^{A}
Untreated Control	0.32^{A}	8.66^{A}	31.99^{A}	41.45^{A}	2.77^{A}	1.42^{A}	1.31^{A}
p-value	0.122	0.068	0.131	0.059	0.136	0.017	0.054
Upper Canopy Leaves	0.24^{A}	9.61^{A}	30.43^{B}	44.62^{A}	1.46^{B}	1.25^{A}	1.16^{B}
Mid Canopy Leaves	0.29^{A}	9.22^{A}	31.89^{A}	41.93^{B}	2.72^{A}	1.36^{A}	1.30^{A}
p-value	0.267	0.138	0.003	0.001	0.002	0.087	0.039
Confidor® * Upper Canopy	0.20^{A}	10.19^{A}	29.92^{A}	46.42^{A}	1.34	1.19^{B}	1.14
Untreated Control * Upper Canopy	0.28^{A}	9.04^{A}	30.94^{A}	42.81^{AB}	1.78	1.31^{AB}	1.19
Confidor® * Mid Canopy	0.22^{A}	10.15^{A}	30.75^{A}	43.78^{B}	1.68	1.20^{B}	1.16
Untreated Control * Mid Canopy	0.36^{A}	8.29^{A}	33.04^{A}	40.09^{B}	3.76	1.52^{A}	1.44
p-value	0.561	0.177	0.164	0.958	0.052	0.126	0.088
CV	45.72	7.68	4.02	4.63	47.73	13.59	14.41

Results presented in Table 16 show that there was no significant difference between Confidor treated and Untreated culms for the majority of biochemicals in both the upper and lower sections. The exception was starch%, which was lower in Confidor treated plots. As expected, there were some differences between mature and immature culm sections, with upper stalk internodes having lower levels of dry matter and sucrose and significantly higher levels of protein, glucose and fructose. Analysis of the interaction terms showed that there was no difference between Confidor treated and untreated plots in the Upper Stalk internodes for sucrose and other biochemicals. This concurs with results showing no CCS penalty associated with YCCS. There were also no differences in the Lower Stalk internodes across these biochemical parameters.

Table 16 Stalk analysis results. Upper Stalk is comprised of internodes 0 to 5, while Lower Stalk is internodes 6 and lower. Analysis of variance conducted using a split-plot design with main factor (chemical treatment) and sub-factor (node location in the canopy). ANOVA performed at 95% confidence. Difference between groups has been determined by a Tukey's HSD all-pairwise comparisons (p<0.05) (displayed as letter separations A, B, AB etc)

Treatment	Starch %	Protein %	Dry Matter %	Crude Fibre %	Sucrose %	Glucose %	Fructose %	Maltose %
Confidor	0.20^{B}	4.87^{A}	21.26^{A}	21.68^{A}	17.93^{A}	2.97^{A}	12.92^{A}	1.05^{A}
Untreated Control	0.36^{A}	4.43^{A}	21.09^{A}	21.67^{A}	14.56^{A}	2.36^{A}	14.65^{A}	1.69^{A}
p-value	*0.006*	*0.156*	*0.756*	*0.985*	*0.082*	*0.444*	*0.119*	*0.134*
Upper stalk	0.32^{A}	5.59^{A}	16.97^{B}	22.24^{A}	4.74^{B}	3.96^{A}	17.00^{A}	1.57^{A}
Lower stalk	0.246^{A}	3.71^{B}	25.37^{A}	21.11^{A}	27.75^{A}	1.37^{B}	10.58^{B}	1.17^{A}
p-value	*0.131*	*0.000*	*0*	*0.064*	*0*	*0*	*0*	*0.232*
Confidor * Upper Stalk	0.25^{AB}	6.08^{A}	16.29^{B}	22.54^{A}	5.99^{B}	4.76^{A}	16.12^{A}	1.26^{A}
Untreated Control * Upper Stalk	0.40^{A}	5.11^{A}	17.65^{B}	21.94^{A}	3.50^{B}	3.16^{AB}	17.88^{A}	1.88^{A}
Confidor * Lower Stalk	0.159^{B}	3.66^{B}	26.22^{A}	20.81^{A}	29.87^{A}	1.18^{B}	9.72^{B}	0.84^{A}
Untreated Control * Lower Stalk	0.32^{AB}	3.75^{B}	24.51^{A}	21.40^{A}	25.62^{A}	1.56^{AB}	11.42^{B}	1.51^{A}
p-value	*0.819*	*0.027*	*0.013*	*0.318*	*0.517*	*0.026*	*0.978*	*0.929*
CV	56.7	13.480	7.61	7.24	23.31	44.27	17.97	65.88

Summary

This field was extremely water stressed for most of the trial with little moisture in the upper 300 mm soil profile (Figure 44). Observations of YCS prevalence and severity in the lower canopy (Figure 43) is therefore more likely a record of water stress senescence and should be interpreted with caution.

However, peak YCS expression in the mid-canopy during early March is characteristic of YCS. The leaf compositional analysis shows the Confidor treatment, although inducing a stay-green response in the mid and lower canopy overall, is not causing a significant change in sucrose content relative to the untreated control. While not statistically significant, there is reduced leaf accumulation of glucose, fructose, sucrose and starch in Confidor treated plants. However, YCS symptomatic leaves of the mid-canopy do show higher levels of sucrose and starch and lower levels of protein than asymptomatic leaves. This concurs with YCS studies showing a repartitioning of carbon to other metabolic pools as sucrose levels rise (Marquardt, 2019; Scalia et al., 2020). Reduced protein levels concur with reported decreases in PSII core protein D1, Calvin cycle CP12 and related carbon fixation proteins of the bundle sheath cells (Marquardt, 2019). Interestingly YCS asymptomatic and symptomatic leaves have a similar dry mass even though the latter has approximately 3-fold more sucrose content (Scalia et al., 2020). Cellular death and protein hydrolysis in YCS leaf tissue are the likely cause of the mass equilibrium.

Differences in culm biochemical composition between treated and untreated plots is not treatment or YCS specific, and can be explained by variations in ontogeny between the upper and lower internodes (Botha and McDonald, 2010). Data does not support a CCS penalty in YCS symptomatic stalks.

The reduction in visible yellowing in the lower and mid-canopy in the Confidor treatments does not correlate with increased biomass, nor is there any correlation between YCS severity and CCS. This suggests that the reduction in canopy yellowing by Confidor is a stress shield effect. This concurs with manufacture claims and studies that neonicotinoids increased heat and drought stress tolerance (Thielert, 2006; Ford et al., 2010; Geissler and Wessjohann, 2011).

6.3.4 Metabolite, transcript and chlorophyll fluorescence – Stone River Confidor® trial

Although visual yellowing is usually only evident in the lower leaves of the canopy (older than leaf +5) photosynthesis and stomatal conductance are reduced in YCS asymptomatic and symptomatic leaves (Marquardt et al., 2016). On a canopy basis photosynthesis is reduced by 14% and 36% in YCS symptomatic KQ228$^{\phi}$ and Q200$^{\phi}$ plants, respectively.

Sucrose levels increased significantly and reflects some of the earliest changes that is induced in the YCS symptomatic plants. In addition, there are disruptions on both electron acceptor and donor side of photosystem II (Marquardt et al., 2016). Some of these changes are characteristic of a degree of disruption to the protein structure associated with the electron transport chain. Based on the results we proposed that the first changes in metabolism in the YCS symptomatic plants is an increase in sucrose and that all the other changes are secondary effects modulated by this increased sugar levels.

To form a better understanding of the above we studied the metabolic and gene expression changes that accompany the expression of YCS in sugarcane (Botha et al., 2016). This information would be important to assist in developing management strategies as well as in the identification of potential causal factors.

In this study of the Confidor trial conducted in Stone River (farm #0847A Block #2-1) (see Appendix 1: 1.2.2) we analysed changes to the metabolome, transcriptome and chlorophyll *a* fluorescence. Samples of Leaf +2 and +4 were collected, processed and analysed as per standard protocols used throughout the YCS research program (see section 5.6 and 5.7 of this report). More than 200 metabolites were detected in the leaf samples and 84 of these could be identified. The results revealed intrinsic differences ($p<0.05$) between the metabolomes of the YCS symptomatic and asymptomatic plants. It was evident that significant metabolic changes occurred well before the development of leaf yellowing. The major metabolic changes were associated with sugar metabolism, the pentose phosphate cycle, and phenylpropanoid and α-ketoglutarate metabolism. The diurnal changes of sucrose concentrations (low in the morning and high at the end of the day) are absent in the YCS symptomatic plants even before symptom expression. Comparing the leaf transcriptomes of the symptomatic and asymptomatic plants show that a complex network of changes in gene expression underpins the observed changes in the metabolome (Marquardt et al., 2017).

Here we report on the metabolic changes in sugarcane variety Q200$^{\phi}$ in untreated controls and plants treated with three different concentrations off imidacloprid as Confidor®.

Results and discussion

The application of imidacloprid significantly altered the timing and pattern of yellowing in this crop. At time of sampling leaf +2 of the untreated control samples were just starting to yellow while leaf +2 of all the imidacloprid treatments was still green. Leaf +4 of the control was clearly YCS symptomatic with significant yellowing along the leaf blade while leaf +4 of the high imidacloprid treatment (double application) was still green.

To establish whether the imidacloprid treatments specifically influence chloroplast membrane functions the OJIP transients were recorded for leaf 2 and 4 across the treatments and analysed in detail. The original recorded OJIP fluorescence transients were normalised between the two fluorescence extremes O (*F*0) and P (*F*M) (Figure 46A & B). In these figures, each normalised transient represents the average of three individual transients.

Consistent with the observed phenotype the fluorescence kinetics between the leaf +2 samples are similar (Figure 46A). In contrast the shape of the transients recorded for leaf +4, of the different treatments were different between the control and imidacloprid treatments (Figure 46B). The imidacloprid effect was to decrease the variable fluorescence between 0.1 and 10mS (around the J phase of the kinetic transient).

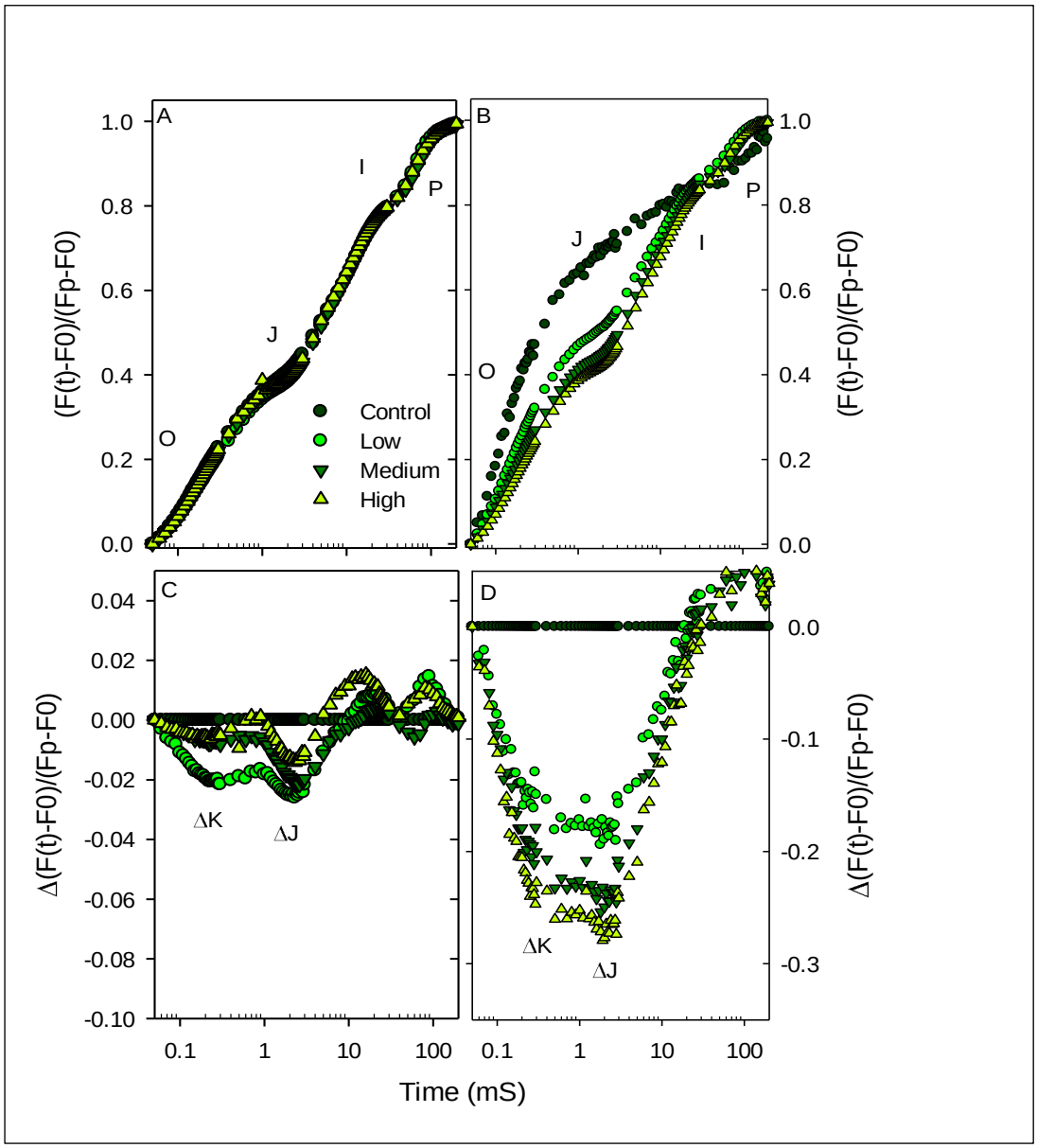


Figure 46 The polyphasic chlorophyll a fluorescence rise OJIP of YCS symptomatic Q200 leaf 2 A) and leaf 4 B). The OJIP fluorescence transients were (normalised between O (F_{50}µs) and P (F_{200}ms). Difference in variable fluorescence curves were constructed by subtraction of normalised (O–P) fluorescence values of the high imidacloprid treatment from the control and lower imidacloprid treatments (C,D). Confidor treatments 22cm^3 100m^{-1} (Low), 44cm^3 100m^{-1} (Medium) and 44cm^3 100m^{-1} followed by 22cm^3 100m^{-1} at fill in (High) (see text for details)

Difference in the variable fluorescence curves were constructed by subtraction of the normalised (O–P) values of high imidacloprid transients from the other transients (Figure 46C and D). The delta curves of the leaf two transients indicated that some differences in around PSII were already present between the control and Confidor® treatments. (Figure 46C). However, in the control leaf 4 samples a large increase in variable fluorescence was evident (Figure 46D), with two distinctive peaks in variable fluorescence at 300 µS (ΔK) and 2mS (ΔJ).

The J step (2 ms) represents the transition point between the light dependent or single turnover phase (O–J) and light independent or multiple turnover phase (J–I–P) involved in PSII photochemistry (Strasser and Strasser, 1995; Tsimilli-Michael and Strasser, 2013). Previously we have shown that in YCS symptomatic tissue fluorescence increases around PSII and that there are indications of a disruption of the water splitting activity, which provide electrons to PSII (Marquardt et al., 2016).

The decrease in fluorescence observed at ΔK and ΔJ in the Confidor® treatments relative to the YCS symptomatic tissues, suggest that the electron transport system continues to function normally, i.e. electrons flow to PSII. Alternatively, electrons are donated by non-photochemical reactions at the water splitting site and or that organic electron acceptors other the QA such as ascorbic acid accepts electrons from PSII.

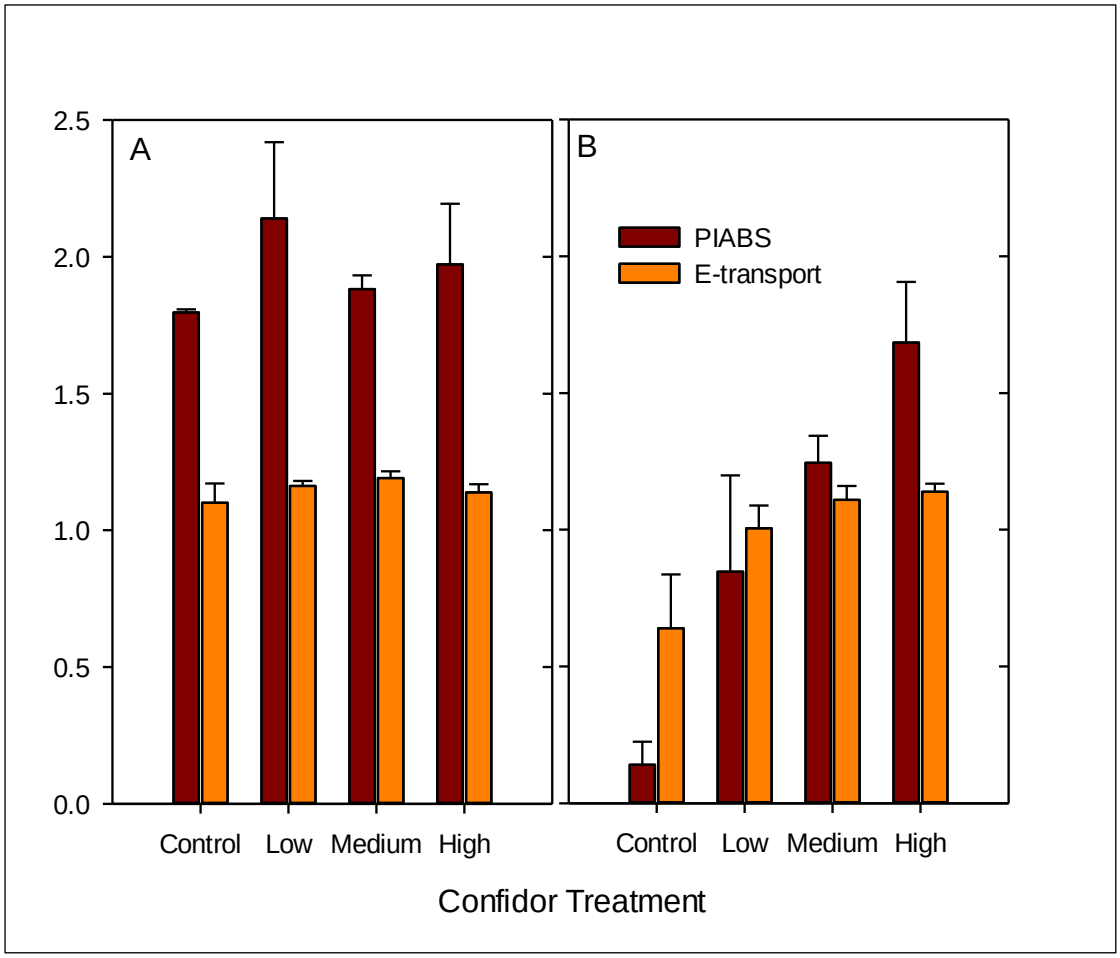


Figure 47 Performance index (PIABS) and electron transport efficiency (Ψo= Eto/Rc) recorded in leaf 2 A) and leaf 4 B) in YCS symptomatic sugarcane plants. The Confidor® treatments 22cm^3 100m^{-1} (Low), 44cm^3 100m^{-1} (Medium) and 44cm^3 100m^{-1} followed by 22cm^3 100m^{-1} at fill in (High) (see text for details)

Electron transport efficiency (Ψo) can be calculated from the OJIP transients (Ψo= Eto/Rc) (Strasser and Strasser, 1995; Strasser et al., 2000). In addition, the overall physiological fitness of the electron transport system (PI_{ABS}) can be determined (Kruger et al., 1997; Tsimilli-Michael and Strasser, 2013).

Despite the indications that changes in PSII starts to occur in leaf 2 and that there are differences between the control and imidacloprid treatments (Figure 46C) there are no significant changes in either electron transport efficiency or PI_{ABS} (Figure 47A). The overall physiological fitness of the electron transport system is more than 90% lower in the control leaf 4 samples than in the high imidacloprid treatment (Figure 47B). The data also illustrate how much more sensitive PIABS is than just calculating electron transfer efficiency (90% vs 15%) in leaf 4 of the high imidacloprid treatment. All the imidacloprid treatments significantly reduced the YCS impact on the electron transport system of leaf 4 (Figure 47B).

This protection or maintenance of electron transport efficiency by imidacloprid will ensure that photosynthesis is protected and also significantly reduces the risk of photooxidation and resultant leaf yellowing.

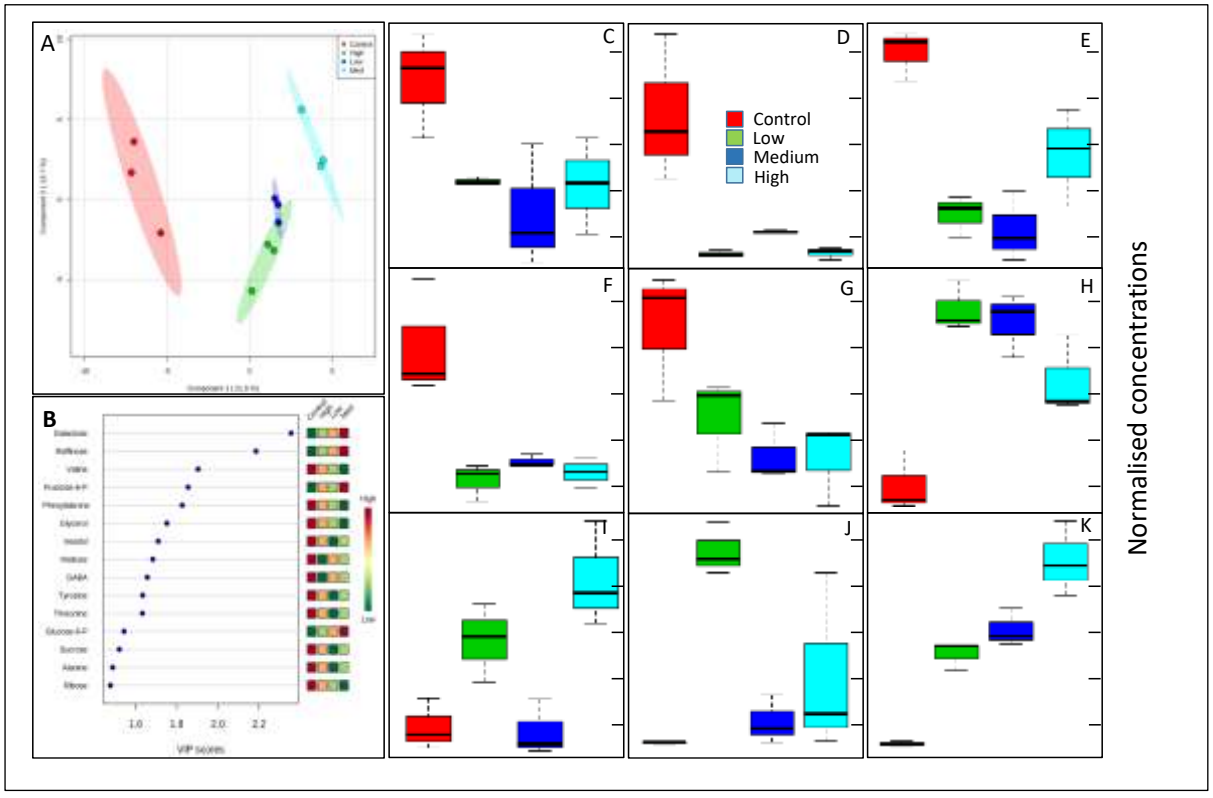


Figure 48 PLS-DA score plot (component 1 vs Component 2) for comparing metabolites from YCS symptomatic leaf 2 tissue with and without imidacloprid treatments A). The model was constructed from 84 identified metabolites generating a three-PLS-DA component model with R2 = 0.64 and Q2 = 0.25. Coloured ovals indicate 95% confidence regions. VIP scores with the corresponding heat map of statistically significant metabolites B). Green and red indicate decreased or increased metabolite levels. Relative abundance of sucrose C), maltose D), fructose E), GABA F), phenylalanine G), aconitate H), quinate I), raffinose J) and galactose K).

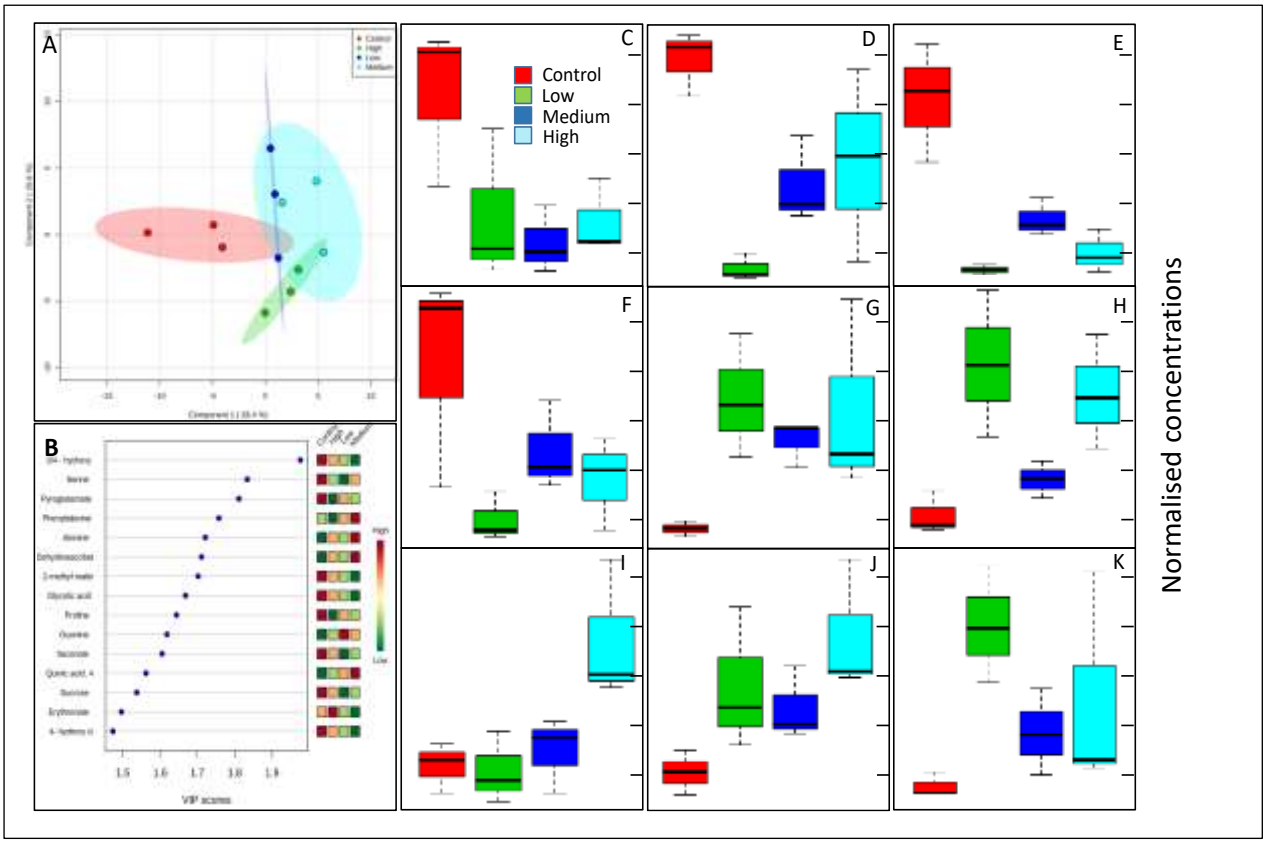


Figure 49 PLS-DA score plot (component 1 vs Component 2) for comparing metabolites from YCS symptomatic leaf 4 tissue with and without imidacloprid treatments A). The model was constructed from 84 identified metabolites generating a three-PLS-DA component model with R2 = 0.64 and Q2 = 0.25. Coloured ovals indicate 95% confidence regions. VIP scores with the corresponding heat map of statistically significant metabolites B). Green and red indicate decreased or increased metabolite levels. Relative abundance of sucrose C), maltose D), proline E), quinate F), raffinose G), ribitol H), phenylalanine I), dehydroascorbate J) and digalactosylglycerol K).

The PLS-DA performed on leaf +2 of the control and imidacloprid treatments revealed a clear differentiation between the samples (Figure 48A). The data showed a clear separation in the metabolites of the leaf material from the control and three treatments in PC1, which accounted for 21.5% of the total variation. There was not a clear separation based on the metabolites in PC2 which

accounted for 13.5% of the variance. Combined, these two components accounted for 45% of the variation between the samples. It is also noteworthy that the separation between the imidacloprid treatment is only evident in PC1, and not in accordance with the level of treatment.

Twenty-three metabolites showed significant changes (P <0.05) between the control and imidacloprid treatments; 15 metabolites with the highest VIP scores (Figure 48B) included 4 sugars, 2 sugar phosphates and 5 amino acids. Five of these metabolites (raffinose, GABA, phenylalanine, inositol and ribitol) are associated with stress metabolism. Some of these metabolites are down regulated by the imidacloprid (Figure 48 C, D, E, F) and are part of sucrose, starch and phenylpropanoid metabolism. Others are upregulated (Figure 48 G to K) and are components associated with stress protection and energy metabolism.

The PLS-DA analyses on leaf 4 did not result in a clear separation of the control and imidacloprid treatments (Figure 49A). This is despite the fact that PC1, accounted for 28.4% and PC2 for 21.6% of the total variation. Combined, these two components accounted for 50% of the variation between the samples.

Twenty metabolites showed significant changes (P 0.05) between the control and imidacloprid treatments. The 15 metabolites with the highest VIP scores (Figure 49B) only include the two sugars sucrose and maltose which are highest in the control (YCS symptomatic) samples (Figure 49C, D).

Heat maps of the twenty-five most important metabolites between the samples are presented in Figure 50A and B. In leaf 2 samples the control and imidacloprid treated are well separated with the 25 metabolites clustering into three groups (Figure 50A) according to the rate of imidacloprid application. The biggest cluster with 11 metabolites includes sugar, amino acids and stress induced components, which are all in high abundance. The imidacloprid treatments prevent this accumulation. In contrast, those metabolites associated with energy metabolism, antioxidant, and stress protectant properties are upregulated by the imidacloprid treatments.

The separation of the treatments is less evident in leaf +4 but the same trends and emphasis on different metabolic processes were present (Figure 50B).

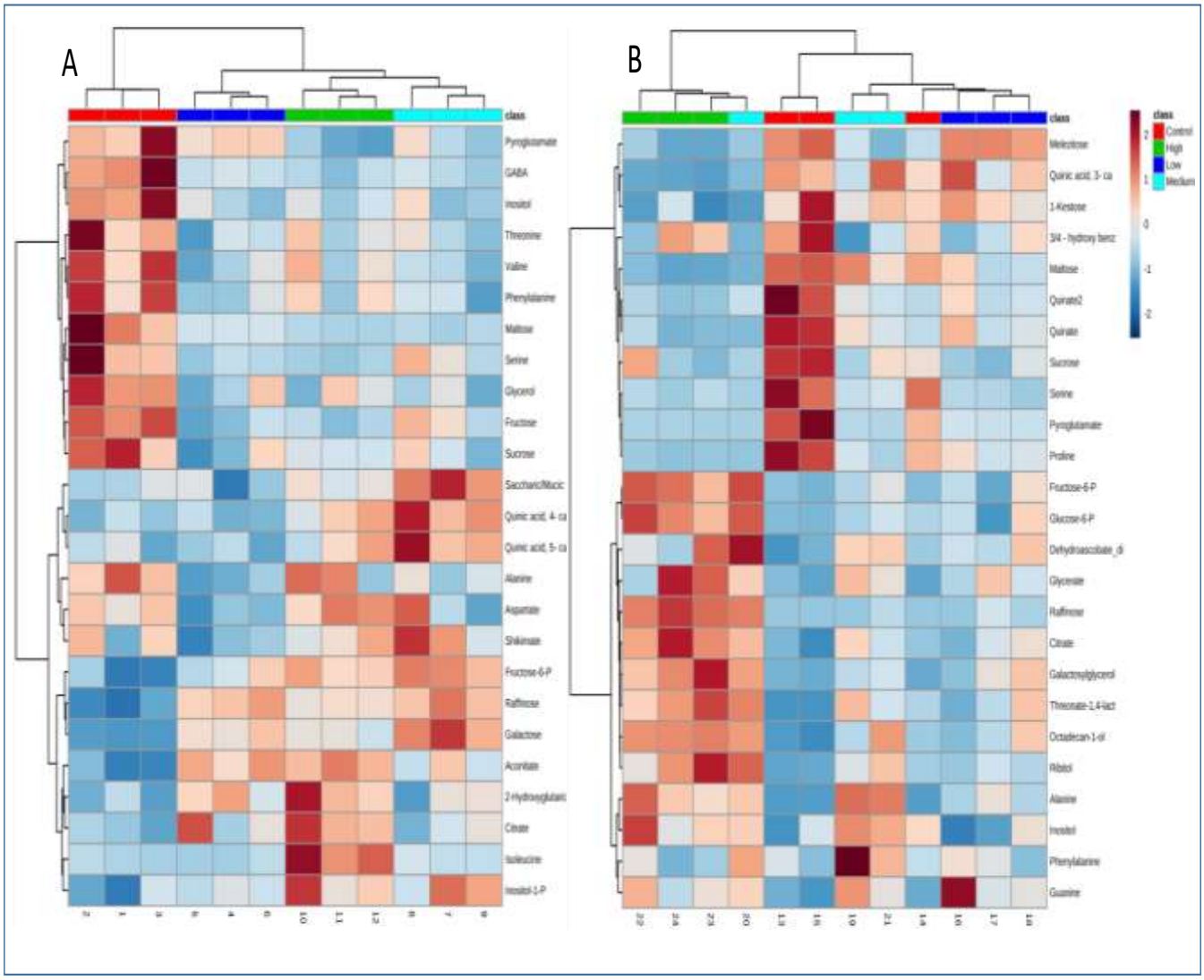
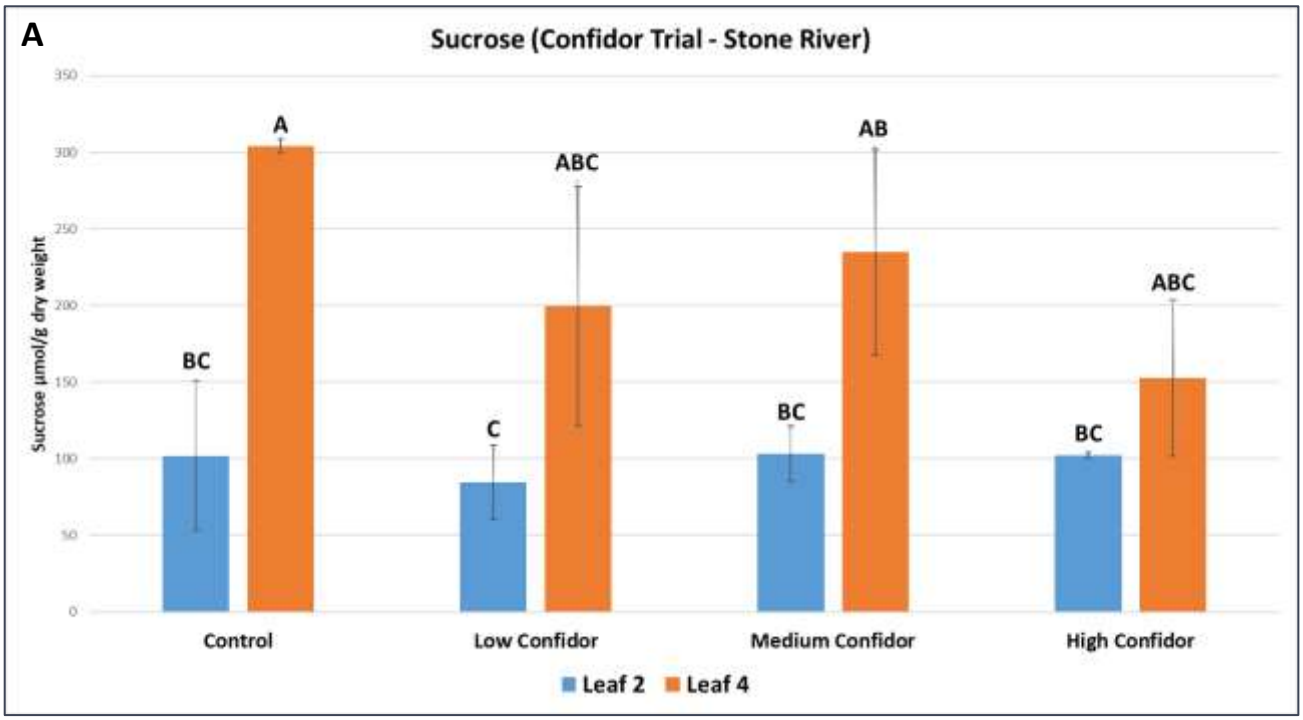
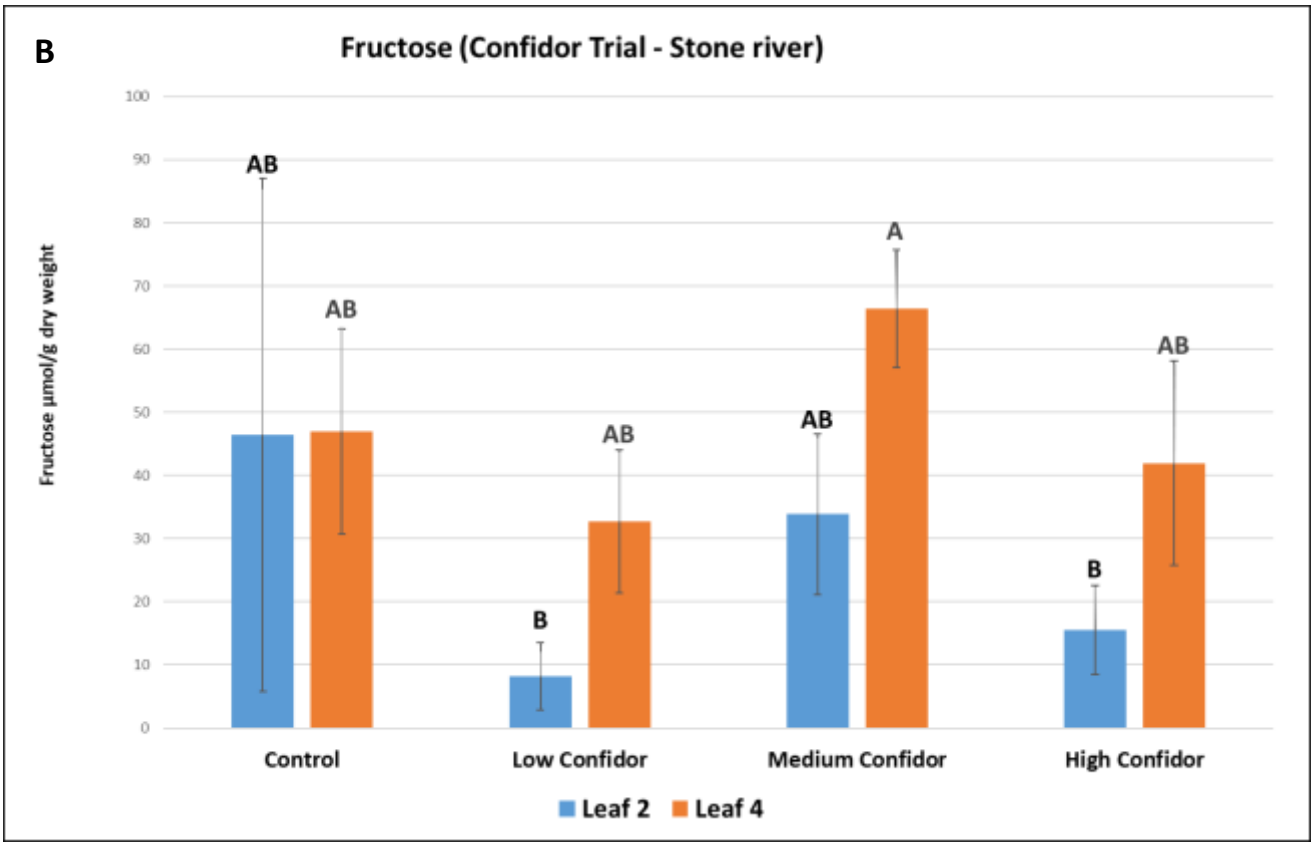


Figure 50 Metabolite heat maps Leaf 2 A) Leaf 4 B)

Sucrose analysis

In house sucrose assays were performed on the prepared lyophilised leaf material (see section 5.6 and 5.7 of this report)


A
Sucrose (Confidor Trial - Stone River)
Sucrose µmol/g dry weight
350
300
250
200
150
100
50
0
BC
A
C
ABC
BC
AB
BC
ABC
Control
Low Confidor
Medium Confidor
High Confidor
Leaf 2
Leaf 4


B
Fructose (Confidor Trial - Stone river)
Fructose µmol/g dry weight
100
90
80
70
60
50
40
30
20
10
0
AB
AB
B
AB
AB
A
B
AB
Control
Low Confidor
Medium Confidor
High Confidor
Leaf 2
Leaf 4

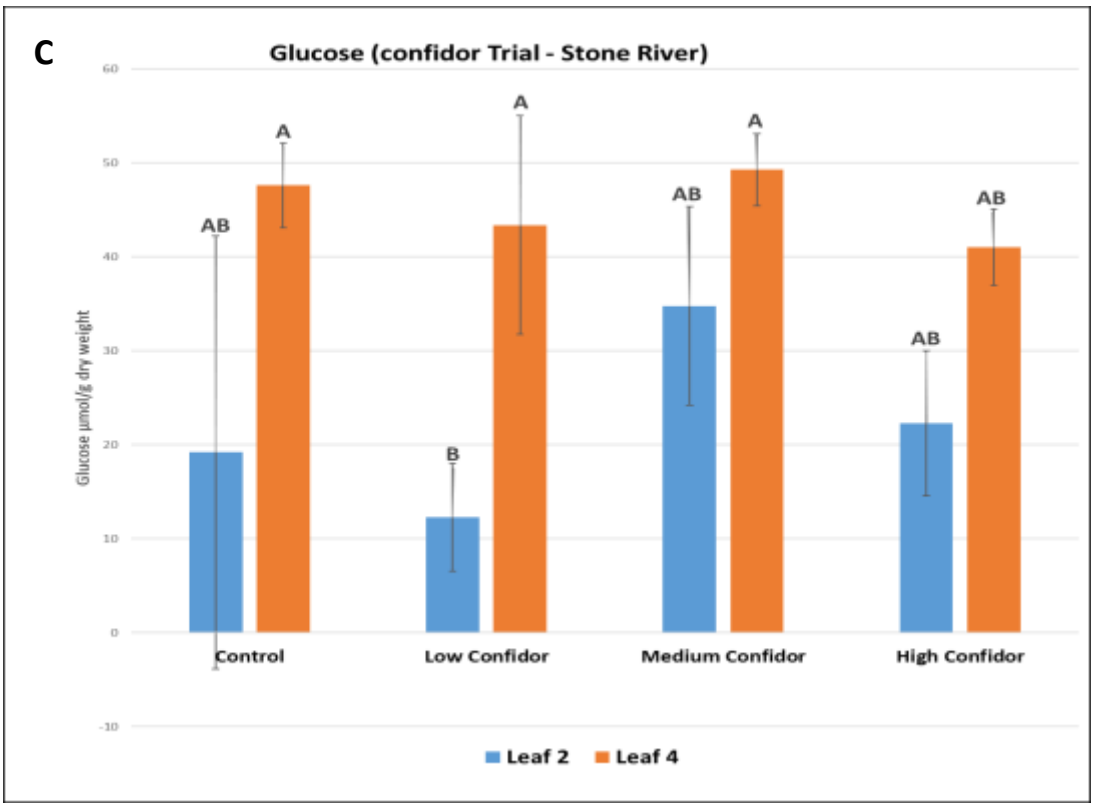


Figure 51 Confidor® rates (Low=22mL 100m^{-1}, Medium = 44mL 100m^{-1}, High = 66mL 100m^{-1}) of treatment and leaf sucrose A) fructose B) and glucose C)

Statistically there is no significant difference in mid-leaf sucrose, fructose and glucose levels between Confidor® treated and untreated controls for either leaf +2 or +4 (Figure 51 A-C). However, while not statistically significant, there is clearly reduced sucrose content in YCS symptomatic leaf +4 for all levels (22-66 mL 100m^{-1}) of Confidor® treatment (Figure 51A). In healthy plants as the photosynthetic rate increases during the day and sucrose accumulates, more carbon is partitioned to starch and stored. However, as daylength increases and light intensity diminishes sucrose levels drop off and starch levels closely follow during the dark period. This diurnal rhythm of sucrose and starch is well documented (Du et al., 2000). In plants affected by YCS the diurnal profile is disrupted resulting in high levels of leaf starch and sucrose in the early morning (Marquardt et al., 2016). Samples collected for this study were harvested at first light and results concur with these studies in leaf +4 of untreated Confidor® control plants exhibiting YCS symptoms. The levels of sucrose and starch in leaf +4 after the night period while statistically non-significant is trending lower in the medium to highest rate of Confidor® treated plants. These results would suggest that Confidor® confers some form of protection from leaf yellowing in rainfed Q200$^{(l)}$ sugarcane under very dry conditions.

Transcriptome analysis

The entire quality-trimmed transcript set was used to assemble de novo contigs in the CLC Genomics Workbench software (v8.5). This resulted in a set of 63,708 contigs with an N50 = 1066. These contigs were used to map the paired reads in the transcript set against, to generate an expression table. The differentially-expressed contigs were subjected to EDGE statistics, and the table filtered to give those contigs with an FDR-corrected p-value of less than 0.05.

Initially, the transcriptome analysis was run as a multigroup analysis experiment, where the expression values were compared between each treatment group in turn. While the Confidor® was applied at three different rates (low, medium and high), only the low Confidor® treatment yielded any statistically-significant results. (data not shown). This was unexpected, and closer inspection showed very high FDR-corrected p-values for the other group comparisons (up to 1.00). Possibly this was due to a lack of sequencing replicates, as due to cost, we pooled RNA samples prior to sequencing rather than sequencing each one separately.

For that reason, the analysis experiment was re-run as a two-group comparison of YCS untreated vs Confidor® treated, combining the Confidor®-treated samples as replicates. In order to have an equal number of treatments, the RNAseq data from YCS samples in FV3 and FV4 (both also Herbert Q200^{A} variety) were included in the untreated YCS group.

This analysis resulted in 572 contigs whose differential expression between the two groups were statistically-significant. Of the 572 contigs, 538 had lower transcript abundance after Confidor® treatment, and 34 had higher transcript abundance after Confidor® treatment. These 572 contigs were taken for further analysis using Blast2GO software.

A volcano plot of the expression table is shown in Figure 52, with the statistically-significant results shown in red.

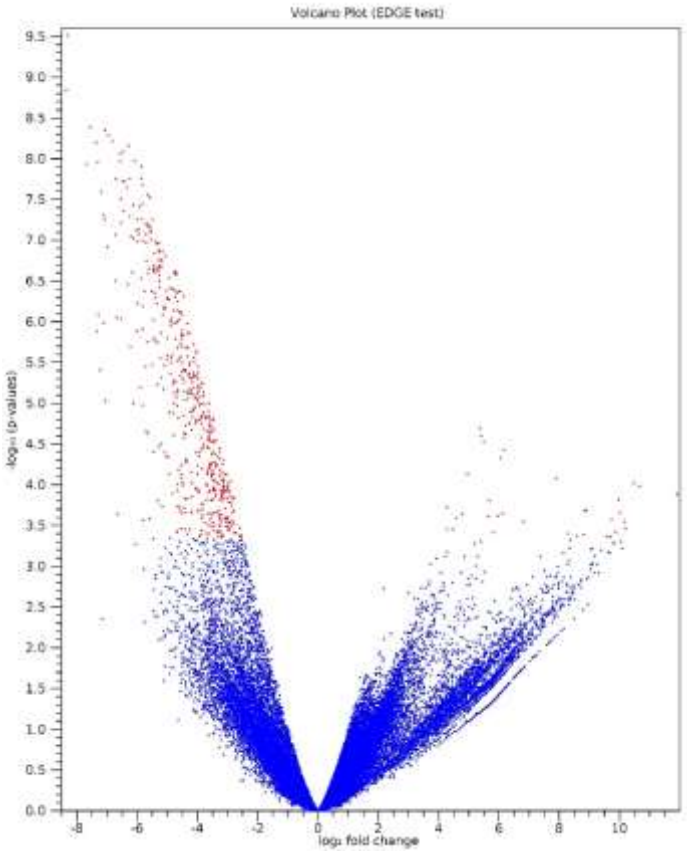


Figure 52 Volcano plot of differential expression for Untreated YCS vs Confidor® treated YCS plants. Red dots indicate the 572 statistically-significant results, with FDR-corrected p-value <= 0.05.

Higher transcript abundance after Confidor® treatment

34 of the contigs were up-regulated in the Confidor®-treated group, and were sent through the Blast2GO Pro software (BioBam, Spain) for identity and function analysis. 10 of these returned no hits on Blast. This is not surprising, given how little of the sugarcane genome is known and annotated. However, 24 of the contigs were identified, along with their role in the plant's metabolism (Figure 53).

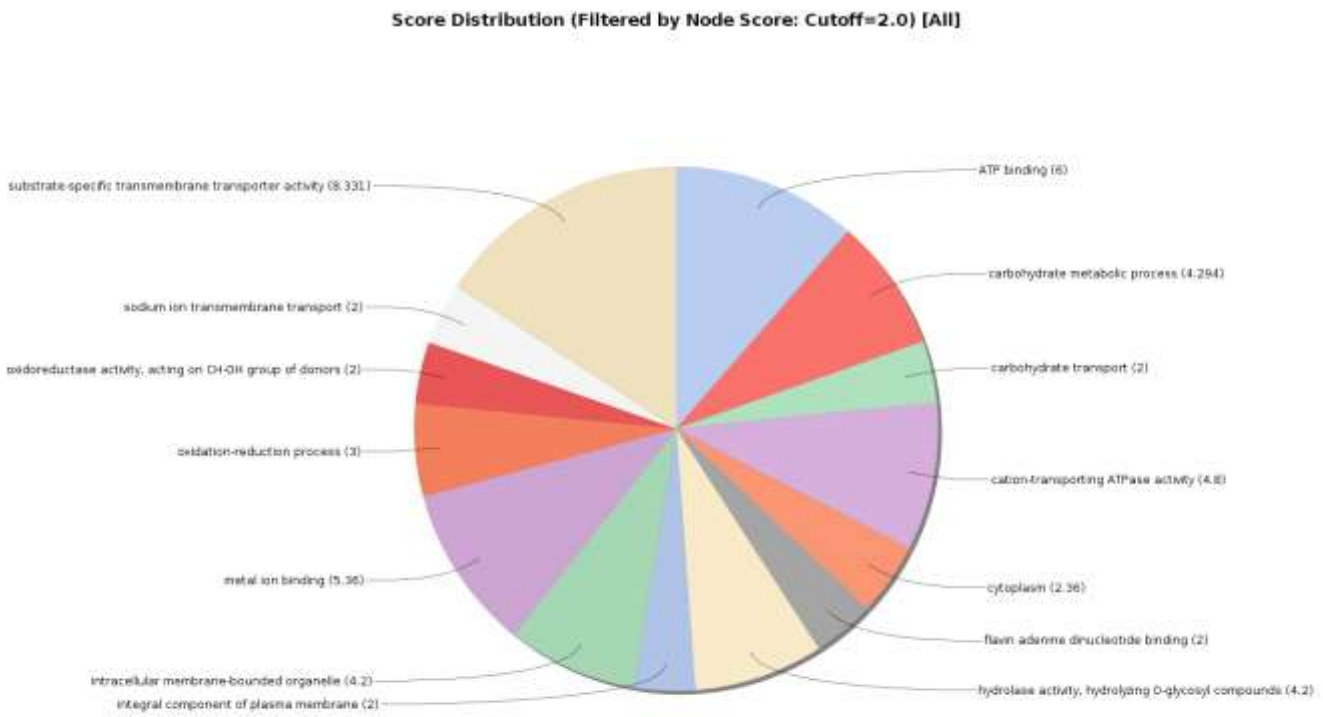


Figure 53 Gene ontology terms attributed to the contigs up-regulated in the Confidor® treated plants. The number in brackets following the term is the node score from the graph created in the software. A higher number indicates more sequences associated with that term.

Figure 53 shows a number of interesting gene categories have increased expression following Confidor® application. Of particular importance is the transmembrane transport and carbohydrate metabolism and transport. This supports the metabolite data shown in Figure 50A which clearly shows that Confidor® prevents the accumulation of carbohydrates in the leaf. Thus, upregulation of carbohydrate transport out of the cell will minimize disruption to cell metabolism and delay the onset of YCS symptoms.

The up-regulated contigs and their associated GO terms from level 4 of the graph were also plotted as a bar chart (Figure 54), with more specific functional categories.

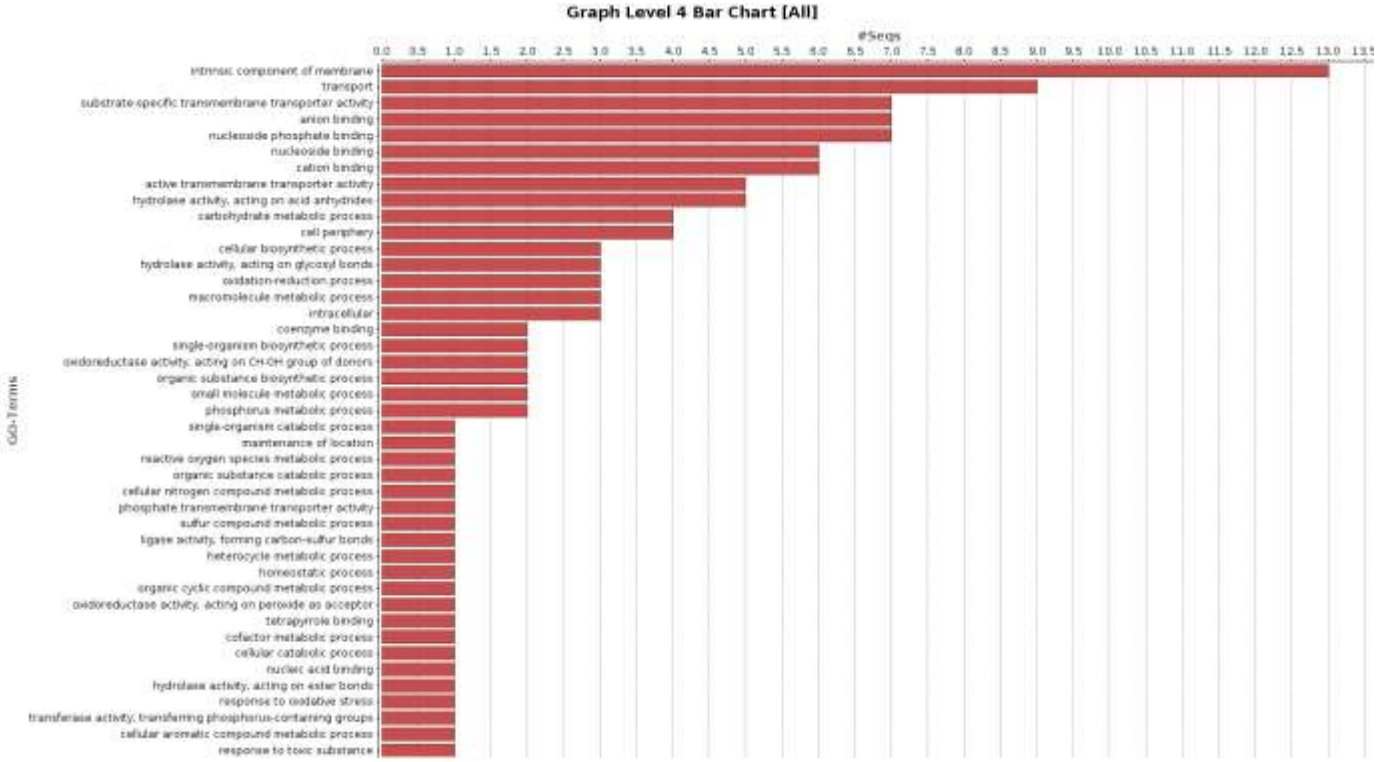


Figure 54 Gene ontology terms attributed to the contigs up-regulated in the Confidor®-treated plants, at Graph Level 4 for more specific category labels.

In addition to the transmembrane transport activity and carbohydrate metabolism, this figure shows the up-regulation of oxidoreductase activity in the Confidor®-treated plants, as well as the response to oxidative stress and response to toxic substance. This may be the plant's attempt to detoxify the Confidor® applied, and may not be related to the decrease in YCS symptoms.

Lower transcript abundance after Confidor® treatment

538 of the contigs were found in lower abundance in the Confidor®-treated plants. Of these, 229 contigs had no Blast hits at all. Two of these were highly significant in terms of fold change and FDR-corrected p-values (contigs _52058 and _17256). Adjusting the Blast settings and searching for these individually, they were both matched to sugarcane sequences but with no annotation or gene identity. As they had no matches to other annotated monocot species sequences, they were unlikely to be coding for important genes.

Of the 309 contigs identified, 172 were identified as retrotransposons or transposable elements, and the majority were classified as belonging in the LTR subclass, as either Ty1-copia-like or Ty3-gypsy-

like. Retrotransposons are highly abundant in plant genomes and can account for the majority of the genomic sequence (Santos et al., 2015). However, it is surprising to see so many of them have a lower transcript abundance after Confidor® treatment. It is possible that they play a role in gene regulation (Dhadi et al., 2015).

Looking in more detail at these elements, the GO id's are associated with zinc ion binding, nucleic acid binding and aspartic-type endopeptidase functions. Additionally, due to their mode of action, they are also associated with RNA and DNA binding, integration, replication and recombination functions. Together, these functions account for more than half of the score distribution results seen in Figure 55 and Figure 56.

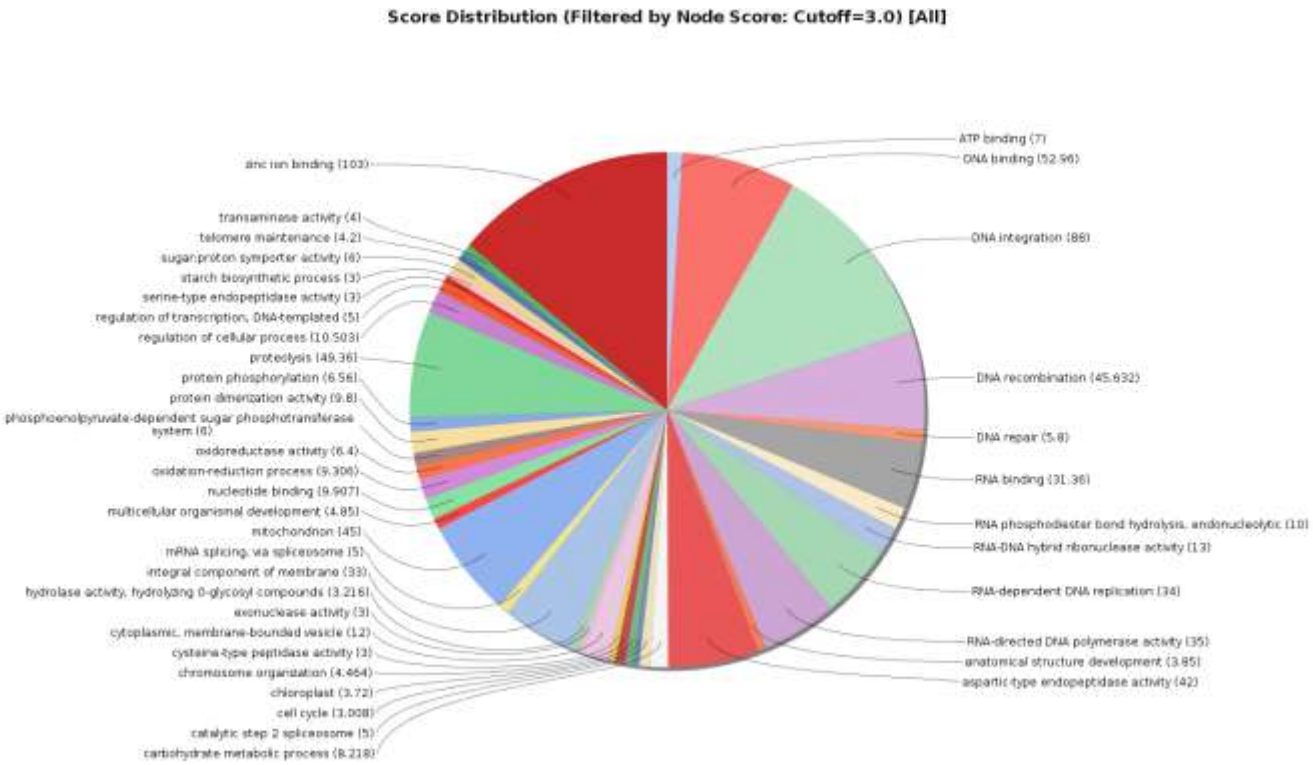


Figure 55 Gene ontology terms attributed to the contigs down-regulated in the Confidor®-treated plants. The number in brackets following the term is the node score from the graph created in the software. A higher number indicates more sequences associated with that term.

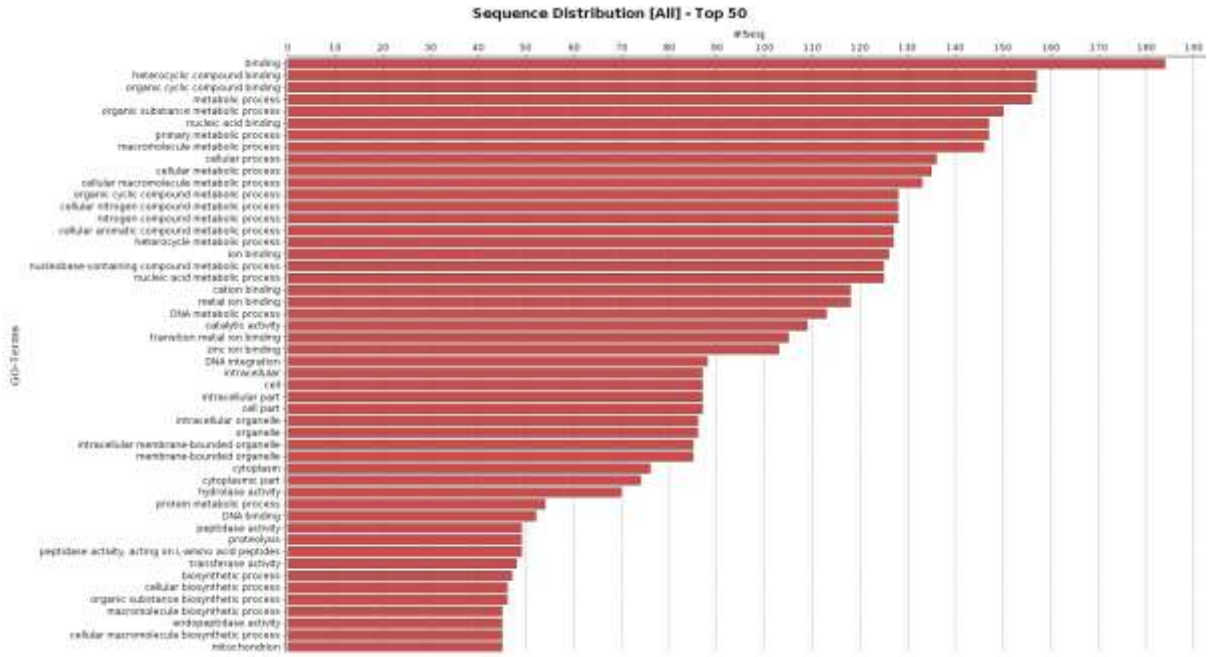


Figure 56 Gene ontology terms attributed to the Top 50 contigs down-regulated in the Confidor®-treated plants.

Summary

The data shows a clear separation in the metabolites of the leaf material from the control and three imidacloprid treatments. Twenty-three metabolites showed significant changes (P 0.05) between the control and imidacloprid treatments. Five of these metabolites are associated with stress metabolism. Some of these metabolites down regulated by the imidacloprid are part of sucrose, starch and phenylpropanoid metabolism. Others that are upregulated are components associated with stress protection and energy metabolism.

It is evident that Confidor alters plant metabolism and delays the onset of leaf yellowing and YCS. There is also evidence of electron transport system maintenance and minimised disruption to the photosystems. Chlorophyll fluorescence data also shows that physiological fitness of the photosynthetic apparatus is more robust in confidor treated pants than the untreated controls.

Neonicotinoid insecticides can provide a stress shield effect in plants under stress; mainly heat and drought stress in this trial (Ford et al., 2010; Geissler and Wessjohann, 2011; Stamm et al., 2014 and references therein). The physiological basis for this is not well understood. However, our transcriptome data offers some insight, identifying upregulation of genes associated with photosynthesis, carbohydrate and lipid metabolism, development of the cell wall and membrane organisation, and downregulation of genes associated with phytohormones and oxidative stress responses in imidacloprid treated plants. Application of the neonicotinoid imidacloprid is reported to slow down the development of YCS symptoms in sugarcane (see section 6.3.2, 6.3.4 and 6.13.2.1 of this report). However, with leaf +4 sucrose levels in asymptomatic high Confidor treated plants approaching the upper tolerance level, this protection is likely unsustainable and offers nothing more than a temporary stress shield.

6.3.5 Confidor and Water Stress Trial - Burdekin

A field trial 1R KQ228$^{\phi}$ was established at the SRA research farm at Brandon in September 2014 to explore YCS transmission, symptom expression under different irrigation regimes and Confidor (imidacloprid) applications. Variety KQ228$^{\phi}$ was planted (28/8/2013) from two sources: 1) Burdekin with YCS symptoms 2) Tully with no visible YCS symptoms. The two irrigation (flood) regimes consisted of a normal frequency (~ 10 days) and a reduced frequency where every second irrigation was skipped (~ 20 days). Three confidor treatments were established: 1) Control 2) 22 ml/100 m applied at planting 3) 22 ml/100 m applied at 'hill-up'. All plots also received Shirtan and Lorsban. Confidor was mixed with Lorsban when applied at planting. The trial was arranged as a split-split-plot with four replicates (see Appendix 1: 1.2.4).

YCS Monitoring

Standard protocol for YCS monitoring of prevalence and severity was adhered to throughout the trial (Table 3 & Table 4). Ratings were conducted on different canopy portions: low (below leaf +5), mid (leaves 1-5), and upper canopy (above leaf +1).

YCS developed in the lower canopy within this trial in late December-early January (Figure 57) This timing is typical of YCS. Development of symptoms in the mid canopy occurred later in the season. In the previous milestone there was an initial indication that YCS development in the mid-canopy was more advanced in the water stress treatments than in irrigated treatments. This trend continued with water stress treatments having the highest prevalence ratings in the mid-canopy at all sampling dates, except one. The confidor effect in this trial did not appear to be as large as the effect of water stress. However, within the irrigation treatment, plots treated with confidor only showed YCS symptoms in the mid-canopy on one occasion. Without confidor, irrigated plots showed YCS symptoms in the mid-canopy on all sampling dates from March.

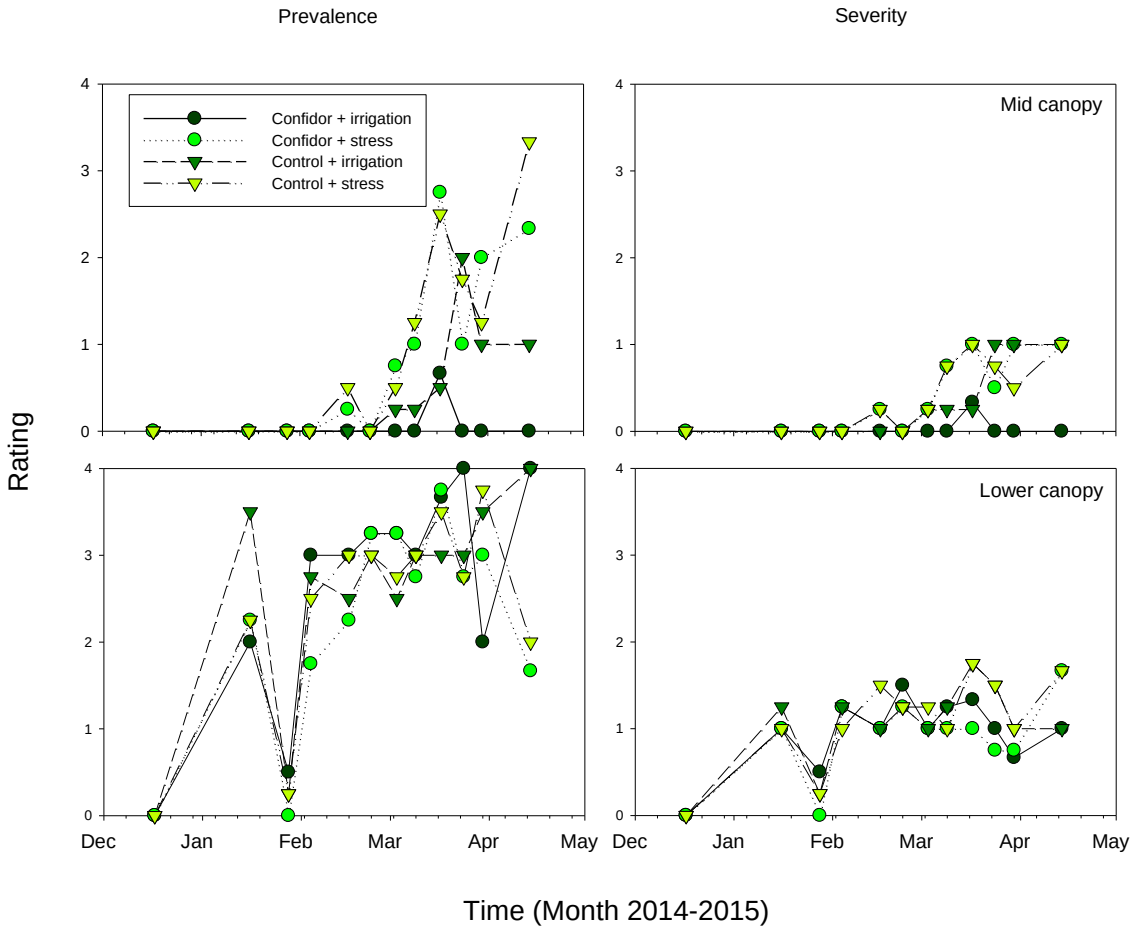


Figure 57 YCS prevalence and severity in the lower and mid canopy in the Burdekin

Soil Moisture

Initially (January-early February), no difference in soil moisture was evident between irrigated and water stress treatments (Figure 58). This was due to rainfall events in January (Figure 59). Differences in soil moisture were established with irrigation in February. Further irrigation maintained these effects. The occurrence of YCS in the mid-canopy coincided with the decline in soil moisture in the water stress treatment.

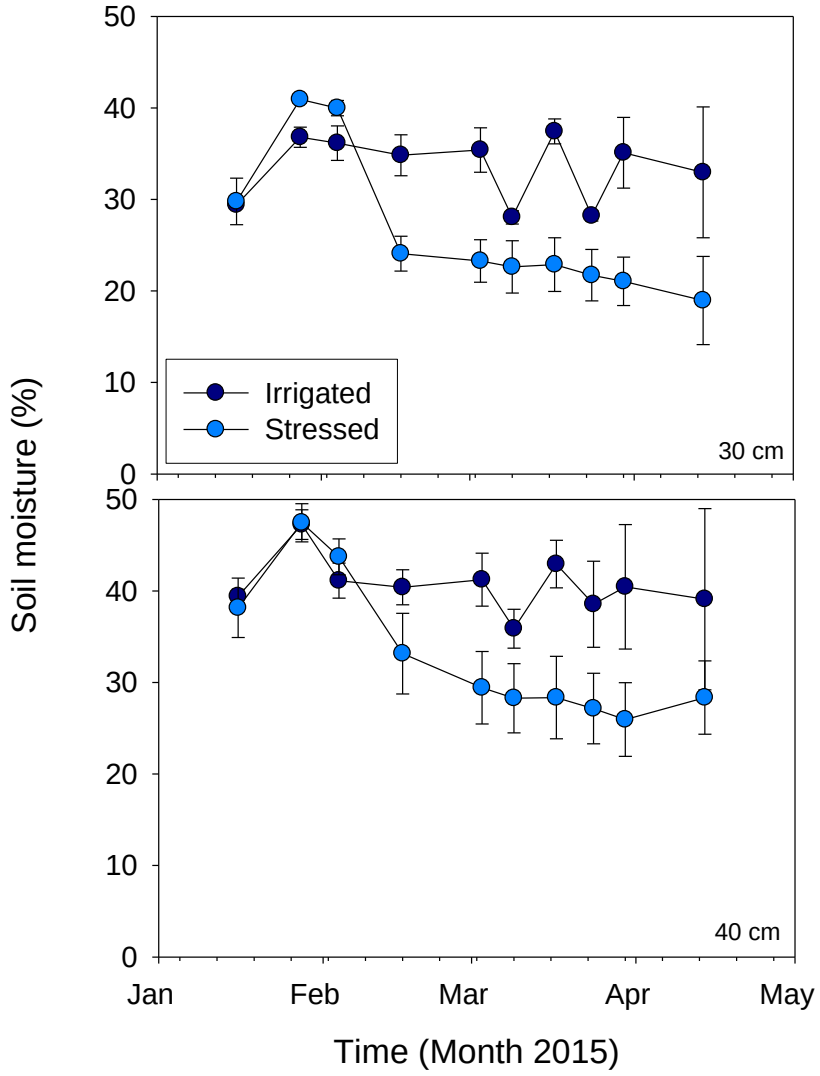


Figure 58 Soil moisture (%) at 30 and 40 cm in irrigated and water stress plots over time

Timing of YCS development in the lower canopy coincided with a rainfall event and high humidity in early January in the Burdekin region (Figure 59). However, there was no consistency in this relationship as a rainfall event in late January was initially followed by a period of low YCS symptoms. Monitoring of environmental conditions at a higher intensity (minutes) may be required to determine whether short periods of extreme conditions are linked to symptom development.

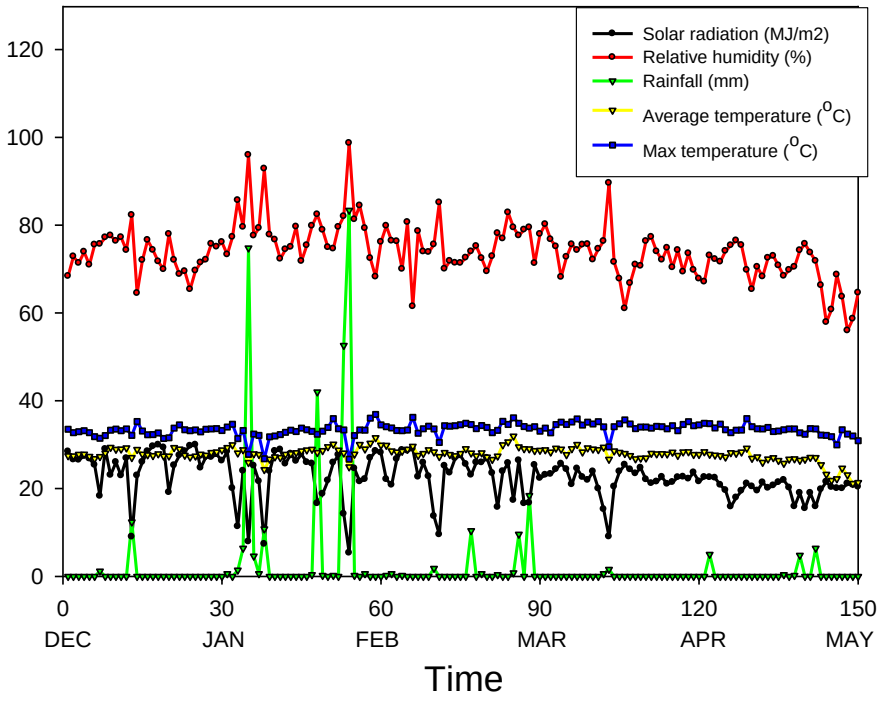


Figure 59 Environmental conditions at Ayr from December 2014 to May 2015

Biomass

Final biomass sampling was conducted on August 17th, 2015 (see section 5.4.1 of this report). Biomass results are presented in Table 17.

Table 17 Crop traits following irrigation (irrigated, water stressed) and chemical treatments (confidor, control). Analysis of variance conducted using a split-plot design with main factor (irrigation) and sub-factor (chemical treatment).

Treatment		Stalks/m2	Fresh biomass (t/ha)	Stalk % DM	TCH (assuming 85% millable stalk)	CCS	TSH
Irrigated		10.1	138.9	0.30	118.1	17.7	20.9
Water Stress		10.8	126.8	0.28	107.8	17.3	18.7
p value		*0.42*	*0.57*	*0.23*	*0.57*	*0.13*	*0.52*
Confidor		10.5	132.7	0.29	112.8	17.3	19.6
Control		10.4	132.9	0.29	113.0	17.7	20.0
p value		*0.78*	*0.98*	*0.86*	*0.98*	*0.50*	*0.82*
Irrigated	Confidor	10.1	139.7	0.30	118.8	17.8	21.2
Irrigated	Control	10.1	138.0	0.30	117.3	17.6	20.6
Water Stress	Confidor	10.9	125.7	0.28	106.9	16.8	18.1
Water Stress	Control	10.6	127.8	0.29	108.6	17.7	19.3
p value		*0.84*	*0.80*	*0.90*	*0.80*	*0.27*	*0.55*

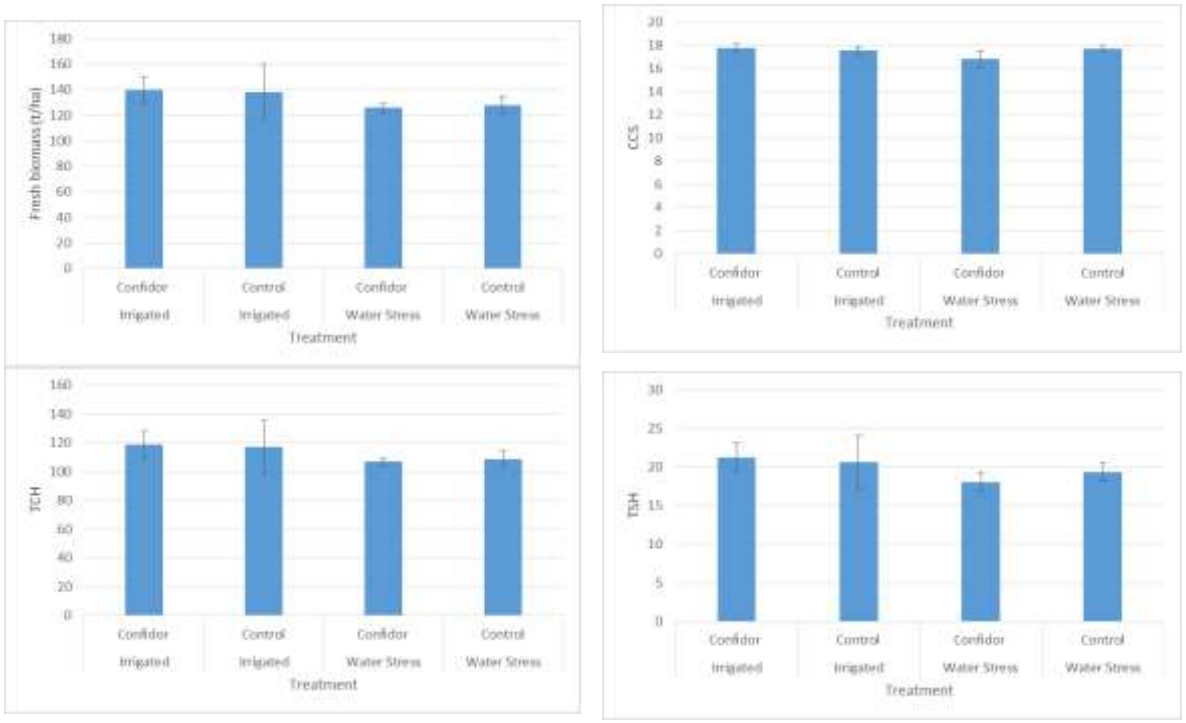


Figure 60 Total fresh biomass, cane yield, CCS and sugar yield for irrigation and chemical treatments in the Burdekin. Error bars ± standard error.

Even though irrigation, on average, resulted in an additional ~ 13 t/ha of sugarcane biomass, there were no statistically significant differences for any crop trait at final harvest (Figure 60).

Summary

Soil moisture readings show there was a significant moisture fluctuation in the top 30 and 40cm soil profile of irrigated plots in early March; possibly due to missed irrigation (Figure 58). A dry down in all treatments occurred up to the rainfall event at the end of February into early March (Figure 59) where the top 30cm soil moisture profile was held steady in non-irrigated treatments. This oscillation under high solar radiation and temperature (Figure 59) is the ideal condition for initiation of a source sink imbalance. YCS monitoring shows a rapid rise in prevalence and severity at this time point in all treatments including slight yellowing in the irrigated Confidor treatment (Figure 57). At this point in time imidacloprid is ineffective in preventing YCS development and expression in the mid-canopy. Biomass data shows no correlation between YCS severity and yield which has been a consistent observation across the research body of work.

This trial suggests a consistent soil moisture profile to maintain growth rate is more effective in mitigating YCS than imidacloprid treatment.

6.3.6 Metabolite and transcript analysis - Burdekin Confidor® trial

Here we report on metabolome and transcriptome changes associated with water stress and imidacloprid treatment in a field trial of 1R KQ228(ϕ) established at the SRA research farm at Brandon in September 2014.

Results and discussion

In the rain fed plants leaf +4 exhibited severe YCS symptoms and there is a major disruption of photosynthetic electron transport efficiency (Table 18). In contrast, both leaf 2 and 4 are green and the electron transport system is functional in the fully irrigated crop.

Application of imidacloprid only partially prevent the development of YCS symptoms in the rain fed crop. This is evident in both the degree of yellowing and electron transport efficiency.

Table 18 ***Leaf phenotype and photosynthetic electron transport efficiency PIabs.***

Treatment	**Phenotype**		**PIabs**	
	Leaf 2	**Leaf 4**	**Leaf 2**	**Leaf 4**
Rainfed	Green	Yellow	1.56	0.16
Irrigated	Green	Green	2.79	1.88
Rainfed + imidacloprid	Green	Green-yellow	2.25	0.36
Irrigated + imidacloprid	Green	Green	1.58	2.28

Twenty-two metabolites showed significant changes (P 0.05) between the rain fed and irrigated treatments (Figure 61B). These metabolites fall in similar metabolic pathways as previously reported in the Herbert Confidor trial (see section 6.3.3. of this report). Further analyses showed that leaf metabolism in the rain fed and irrigated cane separate into two clusters (Figure 61) and in the same fashion as the visible phenotype (Table 18). The most obvious change in metabolism between leaf +2 and +4 under rain fed conditions is the increase in sugars (Figure 61A & C). The increase in maltose would be consistent with a build-up of starch. The changes in metabolism between leaf +2 and +4 under rain fed conditions are absent under full irrigation.

Thirty-one metabolites showed significant changes (P 0.05) between the rain fed and irrigated treatments (Figure 62B). Metabolome analyses show that the metabolism of leaf +4 differ significantly from leaf +2 only under rain fed conditions. Imidacloprid can only partially circumvent the effect (Figure 62A & C). Noteworthy is that the imidacloprid treatment reduces accumulation of sucrose, fructose and maltose in leaf +4 of the rain fed cane.

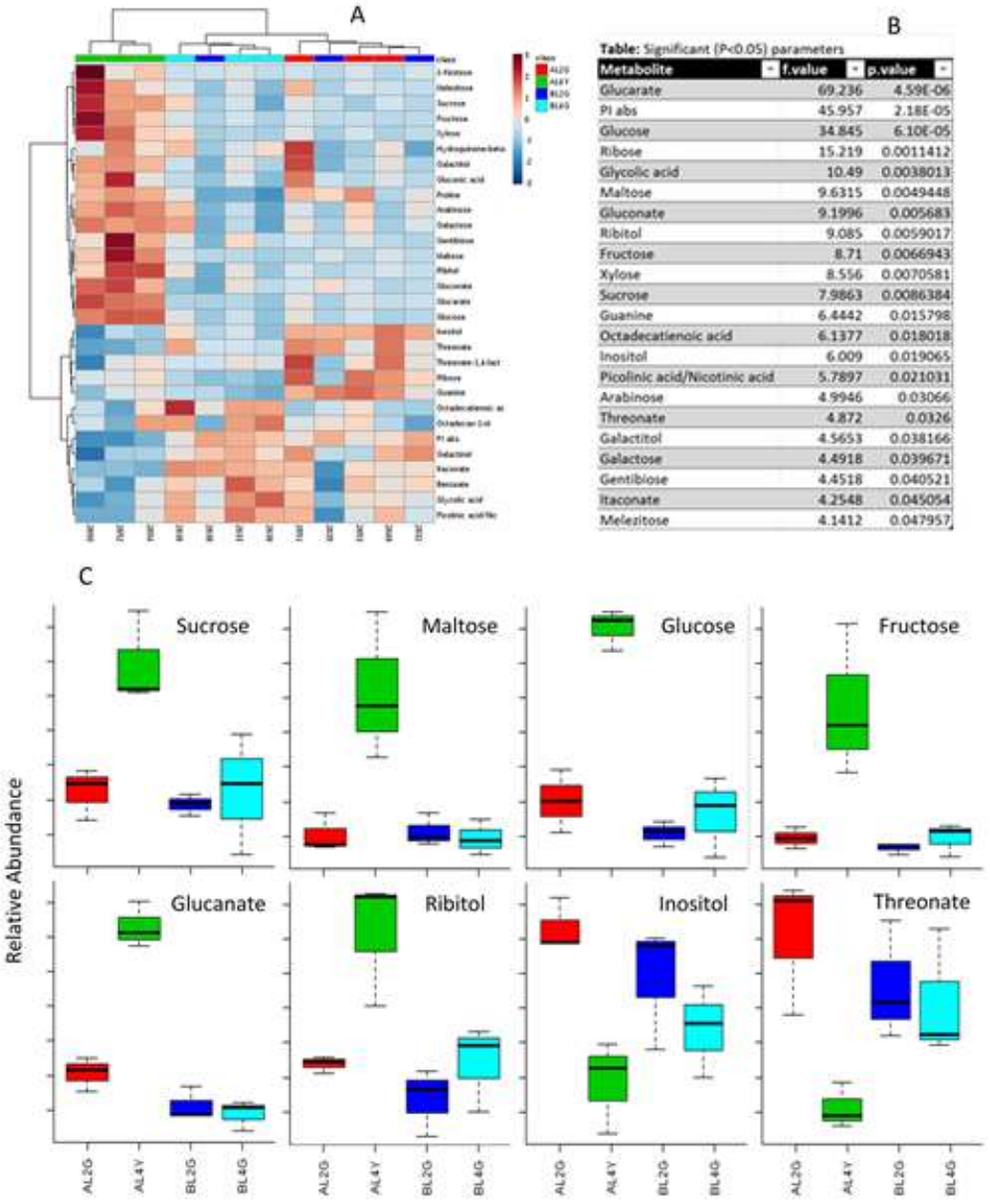


Figure 61 Metabolome data of leaf 2 and 4 of a YVS symptomatic KQ228$^{\Phi}$ crop under rainfed irrigated conditions. A metabolite heat map of the 30 most informative metabolites A), the 22 metabolites that digffer significantly (P<0.05) B), and changes in some metabolites previously reported to change during YCS expression C). AL2, AL4 (rainfed) and BL2 and BL4 (irrigated) leaf 2 and 4 material. The phenotype of the leaf (green = G) and (yellow =Y) is captured in the last letter.

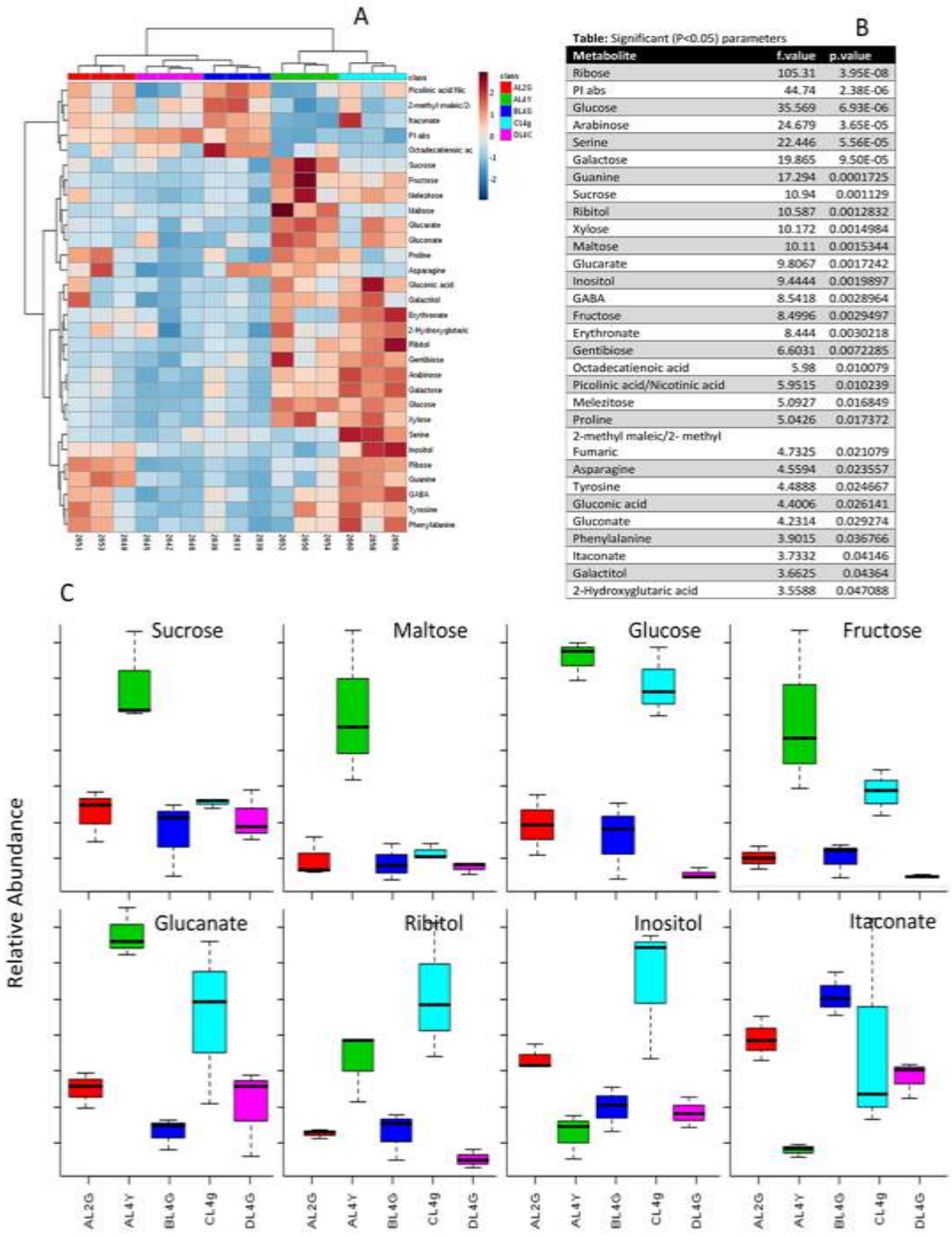


Figure 62 Metabolome data of leaf 2 and 4 of a YVS symptomatic KQ228$^{\Phi}$ crop under rainfed irrigated conditions, with and without a single application of imidacloprid at 22cm^3 100m^{-1}. A metabolite heat map of the 30 most informative metabolites A), the 22 metabolites that differ significantly (P<0.05) B), and changes in some metabolites previously reported to change during YCS expression C). AL2, AL4 (rainfed) and BL4 (irrigated) leaf 4, CL4 (rainfed) leaf 4 with imidacloprid and DL4 (irrigated) leaf 4 with imidacloprid. The phenotype of the leaf (green = G) and (yellow =Y) is captured in the last letter.

The RNAseq samples were de novo assembled into a reference contig set for use in the differential expression analysis, using CLC Genomics Workbench software (v8.5). This resulted in 38,456 contigs with an N50 of 1102.

The analysis was run as YCS-untreated versus Confidor-treated experiment. Mapping the samples back against this reference generated an expression table, which was then subjected to EDGE statistics. The table was filtered for FDR-corrected p-values less than 0.05.

This resulted in 85 transcripts that were statistically-significant in their differential expression. A volcano plot of the expression results is shown below in Figure 63, with the statistically-significant results shown in red.

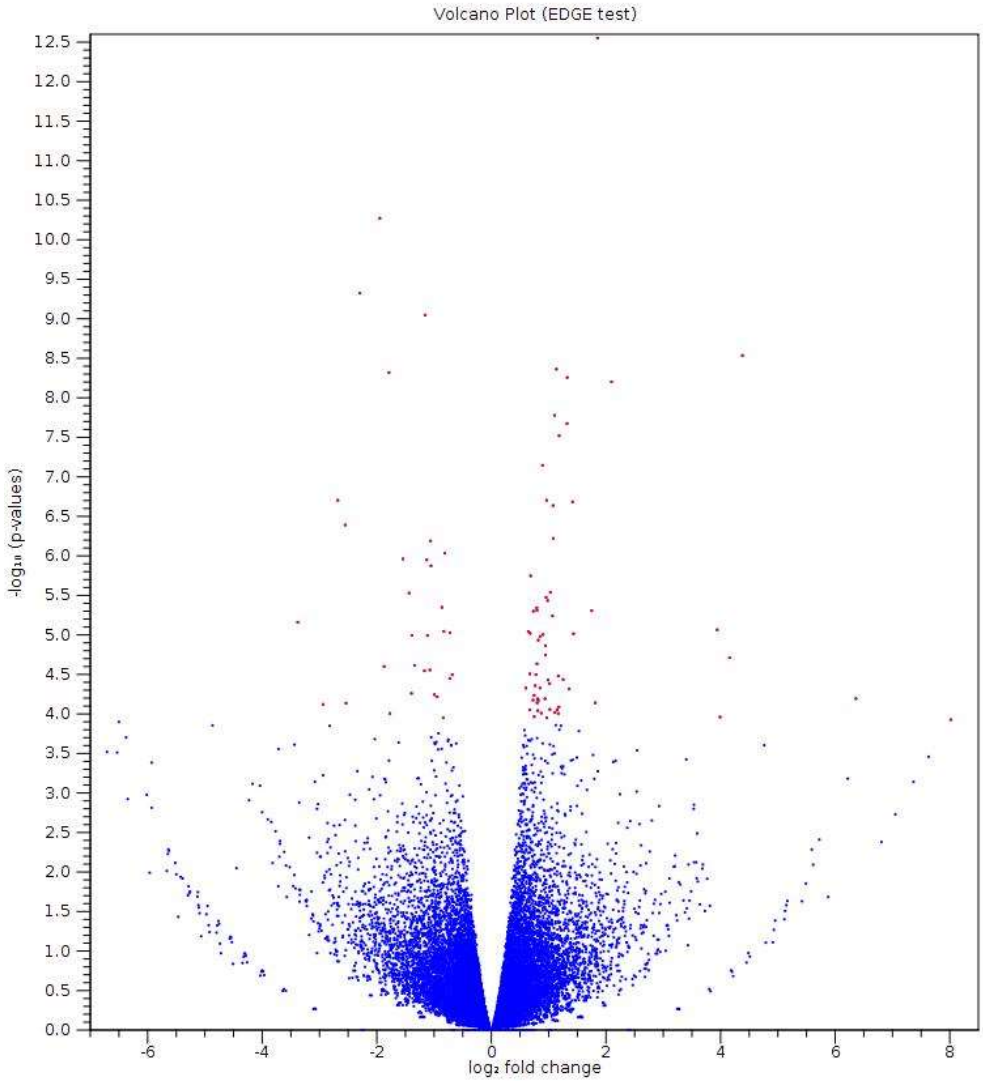


Figure 63 Volcano plot of differential expression for Untreated YCS vs Confidor® treated YCS plants. Red dots represent the 85 most statistically-significant results with FDR-corrected p-value ≤ 0.05

Of the 85 contigs, 32 had lower transcript abundance in the Confidor-treated plants and 53 had higher abundance in the Confidor-treated plants. These 85 contigs were investigated in more detail using Blast2GO Pro software (v1.9.3).

Given sugarcane's limited representation in the NCBI sequence database, it was not surprising to have 15 of the 85 return no blast hits at all, and another 2 of the 85 return 'unknown' as the result. Similarly, 14 of the 85 returned blast matches to merely predicted or hypothetical proteins of sorghum, maize and soybean.

From the 54 transcripts with proper blast results remaining, the two transcripts with the highest fold-change in expression (up-regulated in Confidor-treated plants) were matched by Blast2GO to cytochrome oxidase subunit partial (259-fold) and cytochrome c oxidase subunit I (82-fold) proteins. Cytochrome c oxidase is involved in the final step in the mitochondrial electron transfer chain, and regulates oxidative phosphorylation and ATP synthase activity (Li et al., 2006). Taken the disruption in the electron transport activity in the chloroplast, this could indicate that the upregulation of oxidative phosphorylation to supplement energy requirements might be linked to the stress shield effect of imidacloprid.

Combining all the significant results and looking at the metabolic functions enriched in the differentially-expressed transcripts in the Confidor-treated plants gives the following (Figure 64).

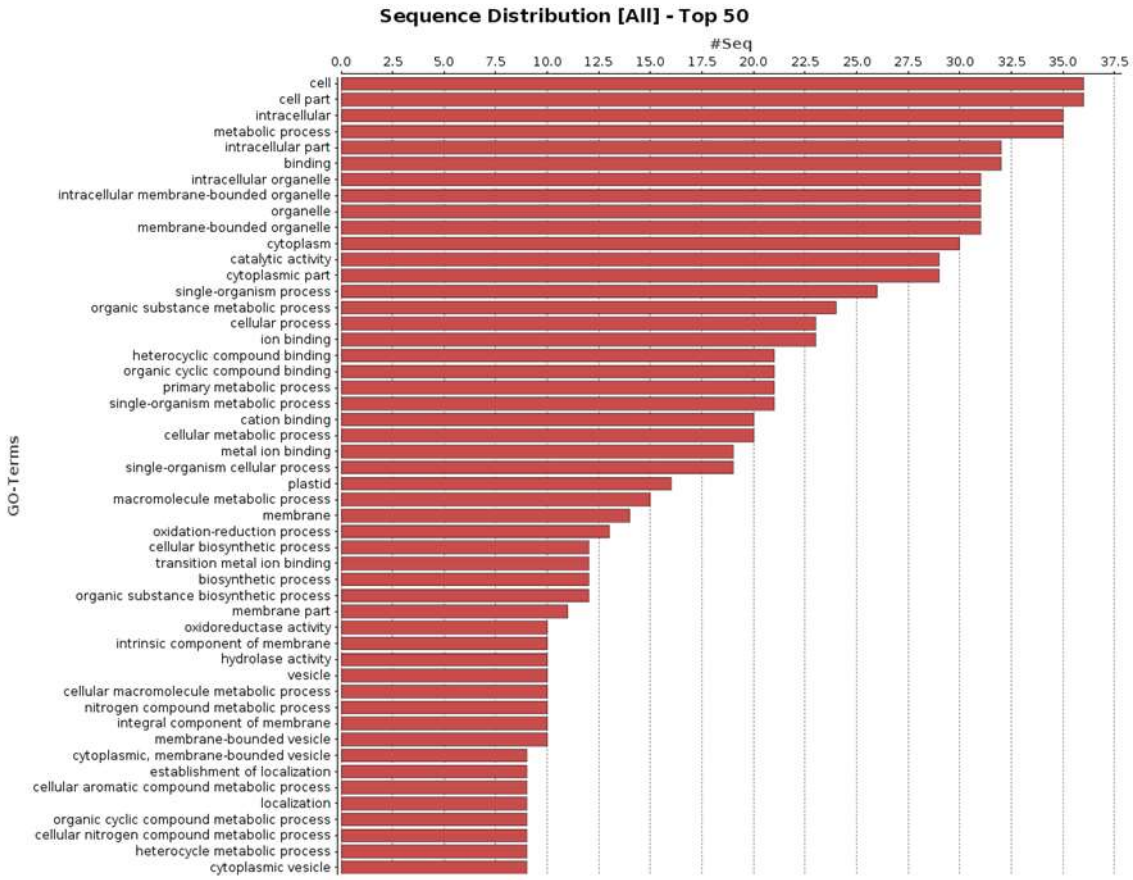


Figure 64 Gene ontology terms attributed to the top 50 contigs

Transcripts with lower abundance (down regulated in the Confidor-treated plants) include jacalin-related lectin, polyamine oxidase, disease resistance protein RPM1, retrotransposon Ty1-copia subclass, alcohol dehydrogenase, serine carboxypeptidase, NBS-LRR-like resistance, starch branching enzyme, and rubisco subunit binding-protein.
Transcripts with higher abundance in the Confidor-treated plants include chlorophyll a-b binding protein of LHCII, phytochrome B, lichenase-2 precursor, UDP-glycosyltransferase, probable -trehalose-phosphate synthase, cytochrome P450, trans resveratrol di-O-methyltransferase, protochlorophyllide reductase protein, trehalose-6-phosphate synthase, beta-amylase, pyruvate orthophosphate dikinase isoform 3, and glycerol-3-phosphate transporter 1.

Collectively this analysis suggests protection by imidacloprid of the intactness of critical metabolic functions in the chloroplast.

Summary

Chlorophyll fluorescence, metabolite and transcriptome data indicate that imidacloprid offers protection to the plant against canopy yellowing under drought conditions. Leaf +4 sucrose offer the best indication of YCS development with levels highest in water stressed YCS symptomatic plants. Irrigated non-treated plants show low leaf +4 sucrose accumulation and no YCS symptom expression. Therefore, while Confidor may offer a stress shield under drought conditions, irrigation is key to delay YCS development.

6.3.7Insecticide stress shield trial – Stone River, Ingham

This field trial of Q200$^{\phi}$ was established on 10/09/2015 on Farm #0053A Block #1-1, Stone River, Ingham (see Appendix 1: 1.2.3). The trial was testing a range of insecticide treatments to investigate whether 1) Confidor was eliciting a stress shield effect 2) other brands of imidacloprid would have a similar effect in mitigating canopy yellowing and 3) soil or air-borne insects are involved in causing YCS. Treatments involved were:

- Untreated Control
- Insecticide (Confidor) – low rate. Soil applied at plant
- Insecticide (Confidor) – label rate. Soil applied at plant
- Insecticide (Imidacloprid) - Confidor competitor. Soil applied at plant
- Insecticide (Thiamethoxam) [Neonicotinoid] [systemic] – Targets broad spectrum of sucking, soil and leaf-feeding pests. A neonicotinoide like imidacloprid and also claims a stress shield effect. Soil applied at plant
- Insecticide (Pyrethroid) [non-systemic] Broad spectrum insecticide. Foliar applied every 2 weeks.
- Miticide (Diafenthiuron) [non-systemic] – a miticide with pretty poor activity on most other pests. Foliar applied every 3 weeks.
- Insecticide (Spirotetramat) [systemic] – for the control of sucking pests. Long lasting residual with miticidal properties. Soil applied at plant

Results

YCS Severity

In general, all treatments followed the same trend. Symptom onset occurred in mid-late March with YCS peaking in mid-late May. The Control treatment was clearly more severe than many of the insecticide treated plots (Figure 65).

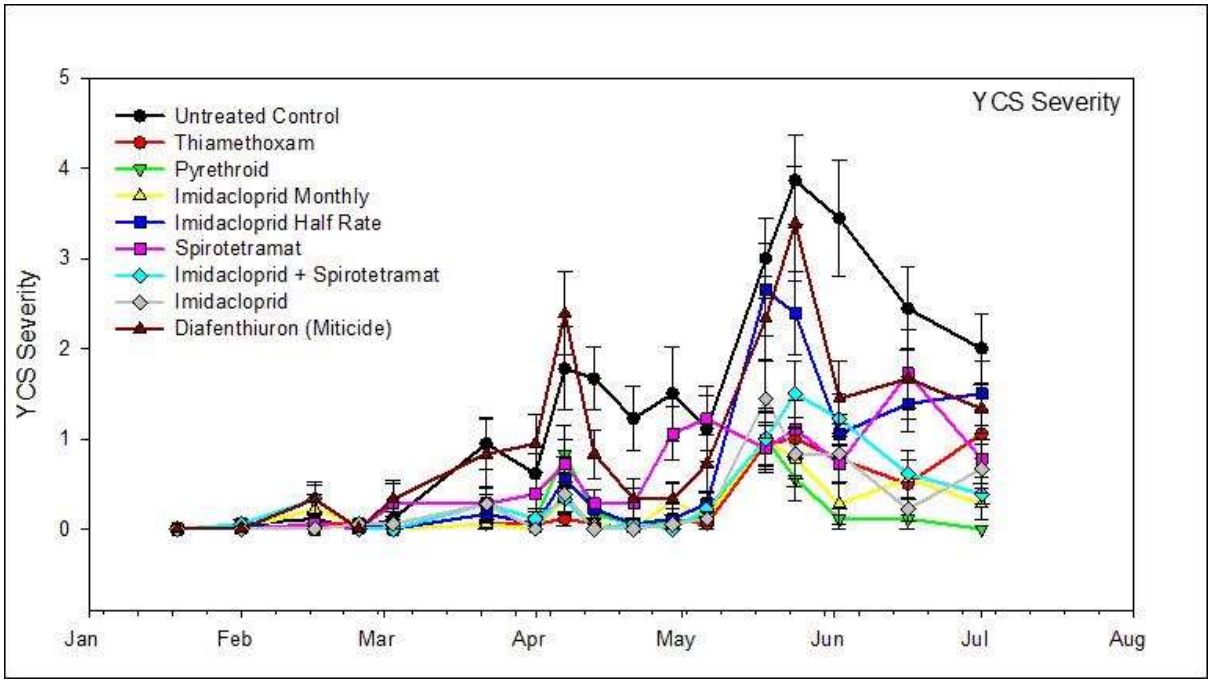


Figure 65 YCS severity score over time. The score is calculated as the sum of the severity ratings for leaves +1 to +7. Each data point represents the average of 18 stalks. Means ± standard error.

Neonicotinoid insecticides showed significantly less YCS than Control over the duration of the trial (Figure 66(A)). There was no difference between these neonicotinoids until mid-late May when the Imidacloprid Half Rate treatment showed greater YCS than the others. Even though the half rate was still present in leaf tissue at detectable levels (**Error! Reference source not found.**) we suspect that t he concentration was too low to provide any YCS benefit. We had speculated that the Imidacloprid reapplied monthly would provide a longer lasting stay-green response than the standard label application, however this was not the case (Figure 66(A)). The monthly applied Imidacloprid treatment did not show a greater reduction in YCS expression relative to the standard label rate.

This trial has shown that the stay-green phenotype first observed with Confidor brand Imidacloprid can also be elicited with a competitor brand (Nuprid) and a non-Imidacloprid product which is a member of the same Neonicotinoid family (Thiamethoxam).

The dedicated miticide treatment, for the most part, did not show reduced YCS expression relative to the Control (Figure 66(B)). The miticidal treatment did show a significant reduction in mites than the Control (and pyrethroid), (Figure 67), however YCS was not reduced. Although not a comprehensive

trial of miticides, we feel this is sufficient evidence to conclude that mites are not the primary causal agent of YCS. We were surprised by the response of the pyrethroid treatment, which showed reduced YCS relative to Control throughout the entire monitoring period (Figure 66 B).

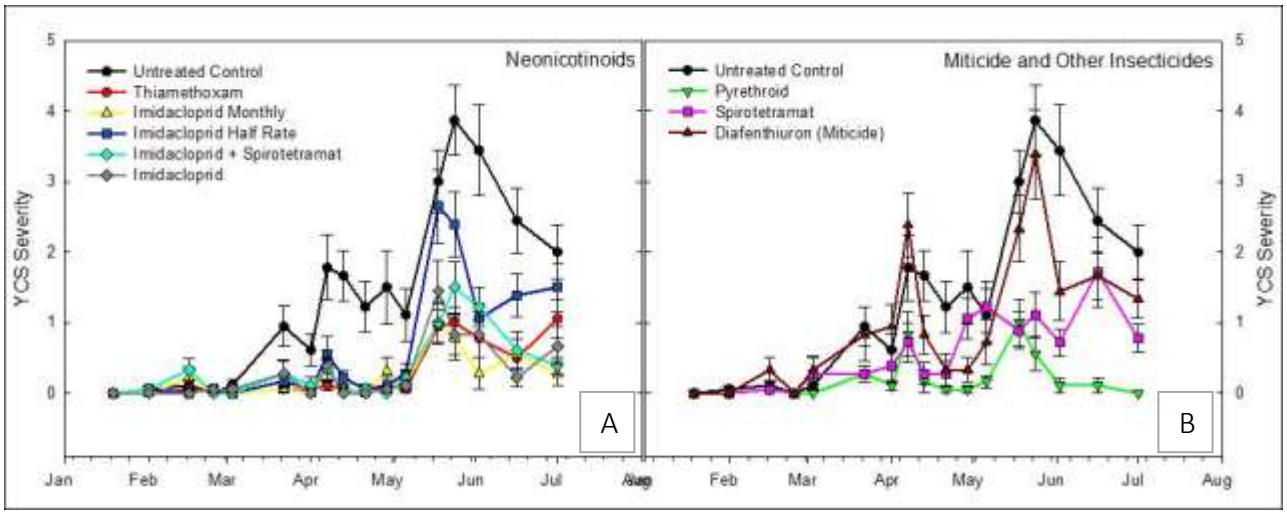


Figure 66 YCS severity score over time. Neonicotinoid treatments (A), and Other Treatments (B). The score is calculated as the sum of the severity ratings for leaves +1 to +7. Each data point represents the average of 18 stalks. Means ± standard error.

Chemical residue analysis

On 9-May-16 samples from leaf and stalk were collected from six of the treatments and sent to Bayer Crop Science (Melbourne) for chemical residue analysis. The treatments were; Imidacloprid applied monthly, Imidacloprid low rate, Imidacloprid (Confidor competitor), Spirotetramat, and Imidacloprid + Spirotetramat. Results are presented in Table 19.

Table 19 Insecticide residue analysis results.

Sample type	Treatment	Active Ingredient	Residue of A.I. detected (mg/kg)
Leaf	Control	Not applicable	N/A
	Confidor monthly	Imidacloprid	0.58
	Confidor low rate	Imidacloprid	0.02
	Nuprid	Imidacloprid	0.05
	Movento	Spirotetramat	<0.01
	Movento Energy	Imidacloprid / Spirotetramat	0.04 / <0.01
Stalk	Control	Not applicable	N/A
	Confidor monthly	Imidacloprid	0.19
	Confidor low rate	Imidacloprid	<0.01
	Nuprid	Imidacloprid	<0.01
	Movento	Spirotetramat	<0.01
	Movento Energy	Imidacloprid / Spirotetramat	0.01 / <0.01

Only the monthly applied Imidacloprid treatment was present in detectable levels in the stalk samples, whereas all Imidacloprid treatments were detected in leaf tissue (Table 19). In leaf tissue, the monthly applied Imidacloprid had a concentration eleven times that of the single-applied treatment. Interestingly, the low rate Imidacloprid (half the label rate) was still present at a detectable level. We

speculate that if there was an insect cause of YCS, in either the leaf or stalk, then the monthly applied treatment should stay-green. This was not the case (see Figure 66 A).

Mite sampling

Sampling was conducted on 10-May-16 to assess mite numbers as well as refine a sampling method for future studies on mites and YCS. Results showed a high number of mites in the bifenthrin (Astral 250) treatment which can be expected given the chemical is a pyrethroid (Figure 67). A higher number of mites also appears to occur in the control treatment which may have become more obvious with more stalks examined. However, with the current dataset there may not be enough difference to answer the question whether the chemical treatments were reducing the mite densities in the cane.

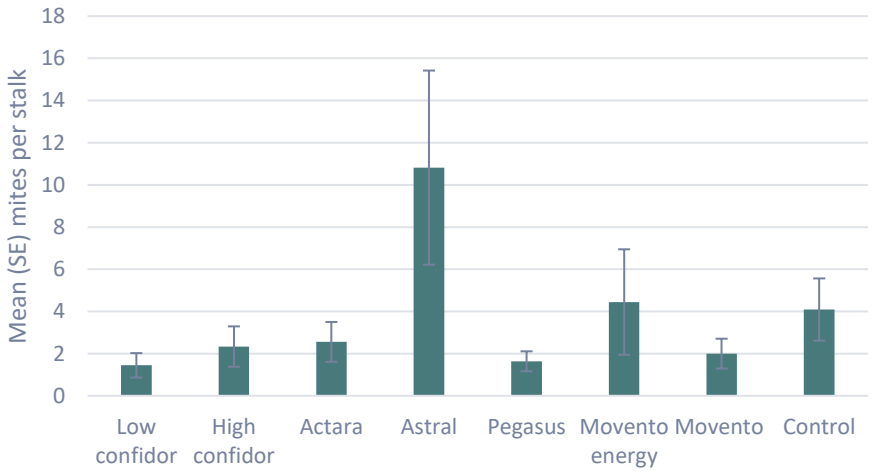


Figure 67 Mean (±SE) number of mites found per stalk in each treatment (n=9-11). Imidacloprid Half Rate (Low Confidor), Imidacloprid Monthly (High Confidor), Thiamethoxam (Actara), Pyrethroid (Astral), Diafenthiuron (Pegasus), Imidacloprid + Spirotetramat (Movento Energy), Spirotetramat (Movento) and Untreated Control (Control).

Canegrub assessment

On 5-May-16, two stools per plot were dug and assessed for canegrubs. Across the whole trial, only one canegrub was found. We conclude that canegrubs are not a contributing factor at this trial.

Biomass

Biomass was measured at 12 months. Results showed no difference in stalk population between neonicotinoid treatments and Control (Figure 68A). There were however significant differences in average stalk weight ($p<0.05$) and fresh weight biomass. Stalks were 26%, 23%, 18%, 16% and 15% heavier than Untreated Control for Imidacloprid Monthly, Thiomethoxam, Imidacloprid half rate, Imidacloprid label rate, and Imidacloprid + Spirotetramat respectively (Figure 68B). This resulted in significant differences in total biomass (t/ha). Only the Imidacloprid Half Rate did not have significantly greater tonnes/hectare than Control. Control had an average 79 t/ha compared with 108, 101, 99, and 91t/h for Thiamethoxam, Imidacloprid label rate, Imidacloprid Monthly, and Imidacloprid + Spirotetramat respectively (Figure 68C). Monthly re-application of Imidacloprid did

not result in a greater response than a single label application, however a half rate was clearly less effective. There was no difference in CCS between neonicotinoids treatments and Control (Figure 68 D).

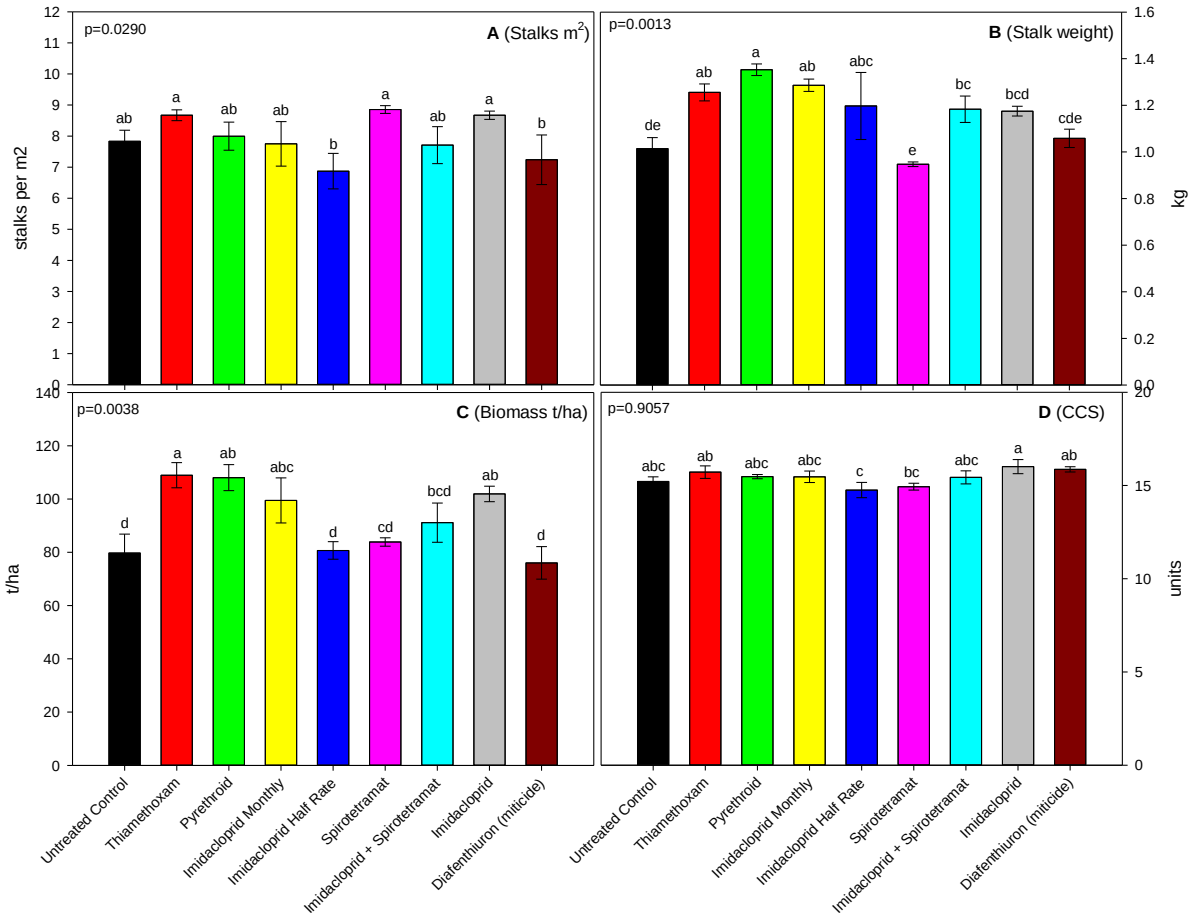


Figure 68 Biomass results. Stalks per m2 A), Average stalk weight B), Fresh weight Biomass C), and CCS D). Means ± standard error. Analysis of variance by completely randomised design (p<0.05) with LSD pairwise comparison shown by letter separations a, ab, b etc.

These results are quite telling, particularly given the significant improvement in symptom severity that the neonicotinoid treatments provided over Control (Figure 66 A). Also significant are the results for the Imidacloprid half rate treatment. This treatment was clearly the least effective at moderating YCS (Figure 66 A) and was the only neonicotinoid to show no difference to Control in final yield. The half rate was chosen on the advice of Bayer Crop Science who suggested that this low rate would still provide a stress shield response in the absence of a full insecticidal benefit. If this is the case, then the fact that this was the worst performing neonicotinoid suggests that the YCS and yield response was a result of insecticidal rather than stress shield benefit. However, this conclusion is contrary to that of other YCS imidacloprid trial outcomes.

Results showed no difference in stalk population between Diafenthiuron miticide, Spirotetramat, Pyrethroid and Untreated Control (Figure 68A). Average stalk weight was 33% (significant p<0.05)

higher than Control for the Pyrethroid treatment (Figure 68B). There was no difference between Miticide (Diafenthiuron), Spirotetramat and Control. Total biomass tonnes/hectare followed this trend. The Pyrethroid averaged 108 t/ha compared with 79t/ha for Control (Figure 68C) while there was no difference for the Miticide and Spirotetramat. There was no difference in CCS between any of these treatments and Control (Figure 68D).

The Pyrethroid (bifenthrin) was the most effective of all treatments, including neonicotinoids, at reducing YCS severity (Figure 66 A & B) and resulted in a significant ($p<0.05$) improvement in yield. The lack of response of the miticide, even though it was re-applied bi-weekly, suggests that mites are not a direct cause of YCS (Olsen and Ward, 2019). This result is contrary to recent entomological findings which includes translucent mites that may not have been previously observed.

Summary

Imidacloprid treatments and non-Imidacloprid product Thiamethoxam from the same neonicotinoid family, showed a significant treatment response in the field delaying the onset of canopy yellowing. Yellowing was also reduced by Confidor treatment in the nematicide trial without a reduction in nematode numbers, suggesting nematodes are not the cause of YCS.

The miticide treatment reduced mite numbers significantly yet failed to prevent YCS development and expression. Therefore, mites are unlikely to be the direct cause of YCS or indirectly responsible for increased stress on the plant in this trial.

The broad spectrum pyrethroid (Bifenthrin) was the most effective in reducing YCS severity. Interestingly, both the pyrethroid and the imidacloprid treatments did show a yield benefit in this trial compared to the untreated control. This appears to be an anomaly as previous imidacloprid trials failed to show a correlation between YCS severity and yield. This suggests that both bifenthrin and imidacloprid treatments are removing insect pressure in this field that was not evident in previous Confidor trials.

6.3.8 Discussion and conclusions

The crop age trials show that growth rate is the key driver of YCS. Growth regulator experiments indicate that manipulation of sink strength can induce YCS development and expression. This concurs with studies by Marquardt et al. (2019) that the source sink imbalance is not caused by an overexpression of the photosynthetic genes. Studies also show nutrient deficiencies are not associated with YCS and extensive microbial and molecular analyses have been unable to conclusively substantiate the existence of a pathogen in any tissue from YCS affected plants. Therefore, either abiotic or non-pathogenic biotic stress is the likely cause of growth rate impediment and sink limitation.

The water stress pot trial conducted in Mission Beach using clean cane tissue culture source material, is an important experiment as it showed YCS could be induced simply by turning water off for a few days and then on again. Water deficit was not long enough to cause water stress yellowing but sufficient to limit sink strength when combined with the 9-month growth perturbance of the pot

effect. When irrigation was returned photoassimilation and carbon export exceeded the sink capacity causing sucrose accumulation in the source leaf and initiation of YCS expression. The fact that yellowing of the lamina occurred so rapidly after irrigation, is indicative of a system already on the cusp of exceeding tolerable levels of leaf sucrose. This helps explain why growers report a flush of yellow that occurs almost overnight, when good rain follows a sink limiting dry period.

It is evident from the metabolite and YCS monitoring data that Confidor or imidacloprid treatments mostly provide a stress shield to the effects of heat and drought. However, this stay-green effect is temporary and does not offer long-term protection or prevent the development and expression of YCS. Biomass results show there is no correlation between YCS severity and cane yield. Similarly, the pyrethroid treatment bifenthrin also appears to mitigate YCS symptoms expression. Insecticide studies reported in section 6.9 of this report show that bifenthrin prevents the accumulation of source leaf sucrose and α-glucans (soluble and starch) under experimental conditions. Unimpeded export of reduced carbon from the source tissue was due to an increase in internode size or culm growth after bifenthrin application (Scalia et al., 2020). Therefore, in the absence of an identified pathogen vectored by a specific insect or group thereof, bifenthrin is possibly removing general insect pressure that would otherwise impact plant resources used for growth.

Crop stress has a serious effect on the photosynthetic light-dependent reactions which leads to an over-reduction of the electron transport chain, the production of reactive oxygen species (ROS), cellular damage and leaf yellowing (Gill and Tuteja, 2010). Chlorophyl fluorescence studies show well irrigated field have physiologically fit photosynthetic apparatus while water stress plants are significantly impacted. Evidently, imidacloprid treatment provides temporary protection of the photosystems under these conditions, delaying the onset of yellowing.

These combined data suggest that YCS is triggered by an abiotic or biotic stress event/s that limits growth rate prior to a period of high rates of carbon fixation and rapid growth.

6.4 Soil biology and root health

Soon after YCS was noted in 2012/13 reports from Industry service representatives stated that cane stools affected by YCS could be easily pulled from the soil. This implied that that YCS was caused by either a soil borne agent or poor root health. To test these hypotheses trials were established in 2014/15 and 2015/16 to investigate 1) any differences between roots of YCS plants and controls 2) whether application of soil treatments could manipulate root health and prevent or mitigate YCS development (see Appendix 1: 1.25 & 1.26). Yield decline research conducted between 1980-2000 showed that several soil-based treatments could have large effects on root health and size(Pankhurst et al., 2003; Garside and Bell, 2011, 2011).

6.4.1 Root Studies

Samples were collected from two field sites to determine if the YCS affected cane has smaller root systems than unaffected cane. Field sites were at two commercial farms in the Burdekin; site one at Home Hill (KQ228 1st ratoon) and site two at Kalamia (KQ228 4th Ratoon). The sites were chosen because they contained clear examples of YCS and green control cane within the same block (Figure

69). The sites were sampled on 5/2/15 and 19/2/15 respectively. At each site 3 x YCS symptomatic and 3 x YCS asymptomatic stools were selected. At each stool, all stalks were cut off at ground level and 8 soil cores (5cm x 30cm) were collected in a 30cm radius from the centre of the stool (Figure 70). Stalk height and biomass was measured. Soil cores were placed in a solution of 15% Sodium Bicarbonate and allow to soak overnight. Roots were then washed out, using a series of sieves to collect all root material. Root biomass was then measured.

Figure 69 YCS affected cane (left), and green control cane (right) at the Kalamia site.

Figure 70 Sampling protocol. Soil core holes surrounding the sugarcane stool which has had its stalks removed.

Table 20 and Table 21 show the above and below ground physiological measurements taken to assess the effects of YCS on root and culm growth.

Table 20 Home Hill (Site 1) findings. Statistical significance was tested using a 2 sample T-test at α=0.05

Treatment	Rep	Total Stalks	Average Stalk Height (cm)	Total Biomass (kg DM)	Average Biomass/ Stalk (kg DM)	Total Roots (g DM)	Roots / Soil Volume (g DM/cm3)	Roots/Stalk (g DM)	Roots/Total Biomass (g/kg DM)
Control	1	13.0	237.5	5.59	0.430	4.14	0.0018	0.318	0.740
Control	2	11.0	248.8	5.17	0.470	4.14	0.0018	0.376	0.800
Control	3	8.0	236.8	3.06	0.383	5.05	0.0021	0.631	1.648
	Mean	10.7*	241.0*	4.61*	0.428*	4.44*	0.0019*	0.442*	1.063*
YCS	1	9.0	215.8	2.14	0.238	2.43	0.0010	0.270	1.135
YCS	2	10.0	212.9	2.55	0.255	2.60	0.0011	0.260	1.018
YCS	3	7.0	221.7	2.22	0.317	2.30	0.0010	0.329	1.037
	Mean	8.7*	216.8**	2.30**	0.270**	2.44*	0.0010**	0.286*	1.064*

Table 21 Kalamia (Site 2) findings. Statistical significance was tested using a 2 sample T-test at α=0.05

Treatment	Rep	Total Stalks	Average Stalk Height (cm)	Total Biomass (kg DM)	Average Biomass/ Stalk (kg DM)	Total Roots (g DM)	Roots / Soil Volume (g DM/cm3)	Roots/Stalk (g DM)	Roots/Total Biomass (g/kg DM)
Control	1	8	208.8	2.00	0.250	3.66	0.0016	0.458	1.833
Control	2	8	201.7	2.27	0.284	2.61	0.0011	0.326	1.148
Control	3	14	208.6	4.39	0.313	4.39	0.0019	0.314	1.001
	Mean	10.0*	206.3*	2.88*	0.282*	3.55*	0.0015*	0.365*	1.327*
YCS	1	12	220.167	3.08	0.256	3.64	0.0015	0.303	1.183
YCS	2	16	197.385	4.91	0.307	4.13	0.0018	0.258	0.840
YCS	3	10	198.200	2.32	0.232	3.59	0.0015	0.359	1.549
	Mean	12.6*	205.2*	3.43*	0.265*	3.79*	0.0016*	0.307*	1.191*

Results at the Kalamia site indicated no statistical difference between YCS and Control stools (Table 21). The Home Hill site showed fewer roots in the YCS treatments and overall, significantly less roots per stalk (Table 20). There were significant differences in above ground biomass between the treatments which resulted in statistically similar root:shoot ratios overall (Table 21). The roots per stalk data is the most interesting, showing lower values for the YCS treatments at both sites, though not significant at Kalamia.

It should be noted that this was a preliminary study with a small sample size which makes it difficult to draw firm conclusions. Nonetheless, the study allowed protocols to be developed for use in larger field trials in which the application of soil treatments to manipulate root health was investigated.

6.4.2 Soil biology and root studies

Trial 1

To assess whether the control of some soil biological agents (soil fungi, insects, and nematodes) affects YCS prevalence and severity the following treatments were applied:

- Untreated Control:
- Systemic fungicide: Amystar 250 EC (600 mL / Ha)
- General fungicide: Mancozeb (600 ppm in soil)
- Insecticide: Confidor® (recommended dose for grub control)
- Nematicide: Nimitz (fluensulfone)
- Metham sodium (Tamifume) soil sterilization
- Irrigated Control: a treatment to alleviate water stress.

The experiment was established in September 2014 and planted with Q200 sourced from a plant source showing YCS symptoms. Plant growth and YCS symptoms were monitored to determine if any treatments led to reduced symptom expression. Soil sampling for nematode populations was undertaken mid-way through the growing season to determine whether soil-applied biocides had influenced pathogenic nematode populations (as indicators of root health). Soils were sampled to 25 cm depth along the row profile, using augers. Soils were kept cool and taken to the Tully soil assay laboratory for assay using a whitehead tray extraction technique. The trial was harvested by hand in October 2015.

Results

Assay of soils from four treatments that were likely to influence nematode populations showed that these treatments did reduce pathogenic nematodes numbers. Soil fumigation with Tamifume had the greatest effect, while Confidor® reduced numbers marginally (Table 22).

Table 22 Nematode populations as affected by treatments applied in the Macknade soil biology trial. Population are expressed as numbers per 200 mL soil.

Treatment	Root lesion	Root knot
Control	5289	216
Soil fumigant	590	255
Nematicide	1181	48
Confidor®	3616	608

Symptoms

YCS symptoms developed in all plots by the end of the trial period but were significantly delayed in the Confidor® treatment. Though soil fumigation had an obvious effect on soil nematodes (as indicators of root health), there was no effect on YCS symptom development (Figure 71B). The

irrigated treatment showed very little yellowing in the mid-canopy (Figure 71C) with Confidor® treatment devoid of YCS symptoms (Figure 71D)

Figure 71 YCS symptom development in treatments Control A) soil fumigant - Tamifume B) irrigated C) and imidacloprid – Confidor®. Soil biology trial Macknade farm Ingham.

Yield

Table 23 Harvest biomass yields. ANOVA performed at 95% confidence. Difference between groups has been determined by a Tukey's HSD all-pairwise comparisons (p<0.05).

Treatment	Fresh Biomass t/ha	Stalks per m²	Dry Biomass t/ha	Cane t/ha	CCS	Sugar t/ha	Fresh Tops and green leaf t/ha
Control	122^{AB}	10.7^{A}	34^{AB}	98^{A}	9.8^{A}	9.5^{AB}	17.4^{AB}
Amistar	123^{AB}	10.6^{A}	34^{AB}	95^{A}	11.2^{A}	10.6^{AB}	16.7^{AB}
Penncozeb	133^{AB}	10.3^{A}	38^{AB}	109^{A}	9.5^{A}	10.3^{AB}	15.8^{AB}
Confidor	138^{AB}	10.1^{A}	41^{A}	109^{A}	12.9^{A}	14.0^{A}	21.8^{A}
Nimitz	111^{B}	10.3^{A}	29^{B}	90^{A}	9.6^{A}	8.5^{B}	14.1^{B}
Metham	120^{AB}	9.7^{A}	34^{AB}	97^{A}	10.8^{A}	10.2^{AB}	16.4^{AB}
Irrigation	142^{A}	10.7^{A}	40^{A}	115^{A}	12.0^{A}	13.9^{A}	18.0^{AB}
	CV 9.8 p 0.024	CV 10.5 p 0.828	CV 11.3 p 0.006	CV 11.7 p 0.072	CV 16.4 p 0.094	CV 17.9 p 0.005	CV 15.3 p 0.023

Means in columns followed by the same letter are not significantly different. Tukey 0.05

Yield results show that treatment effects were largely non-significant, though Confidor® and irrigation both led to significant improvements in comparison with nematicide for sugar tonnes / ha (Table 23). While not statistically significant Irrigation, Confidor and Penncozeb all trended higher for cane yield (TCH). In terms of magnitude, irrigation and Confidor® led to approximately the same yields, with Confidor® best for decreasing symptom expression. This would suggest that Irrigation is the best non-chemical treatment to mitigate YCS and improve yields.

Trial 2

Treatments

- Soil Fumigant (Metham Sodium) – applied 2 weeks before planting
- Insecticide (Imidacloprid) – applied at plant
- Fungicide (Propiconazole) – foliar application once a month
- Nematicide (Fenamiphos) – applied at early tillering
- Fungicide (*Bacillus subtilis*) – applied at plant
- Fungicide (SpinFlo- Carbendazim)
- Untreated control

Results

YCS Severity

In general, all treatments followed the same trend. Symptom onset first occurred in mid-February, with increasing degree of severity from then until late June. It should be noted that by May/June it is was difficult to score YCS severity accurately due to the level of senescence in the crop. YCS peaked in mid-late May (Figure 72). Relative to the Untreated Control most treatments showed similar levels of YCS expression. The exception was the Imidacloprid treatment, which was consistently less severe than Untreated Control. Once again, the Cofidor® result concur with observations of the stress, insect and yield trials using this treatment (see sections 6.3, 6.9 & 6.13 of this report).

Of interest are the fungicidal treatments, which showed mixed results. The biological treatment (Bacillus subtilis) showed consistently more YCS than Untreated Control, while the Propiconazole was no different and Carbendazim treatment showed significantly reduced YCS at many time points (Figure 72). The Carbendazim was re-applied monthly as a foliar treatment, while the Propiconazole and Bacillus were applied as soil treatments at plant. Perhaps this explains the difference in symptom expression with Carbendazim treating the causal agent more effectively. However, given that the Imidacloprid treatment showed even less YCS severity than Carbendazim this is unlikely. The same reasoning also makes it unlikely that the Carbendazim was providing some protection against secondary fungal infection that other treatments were not.

There are some questions as to the efficacy of the Metham Sodium treatment and whether its level of activity had diminished. The effect of this treatment on YCS expression was mixed. A few time-points showed reduced severity relative to Untreated Control, while others showed no improvement (Figure 72). In fact, even early on in the season the Metham treatment was no better than the

Control. The fact that the Imidacloprid treatment showed far less symptom expression, suggests that a soil-borne agent is unlikely the cause of YCS.

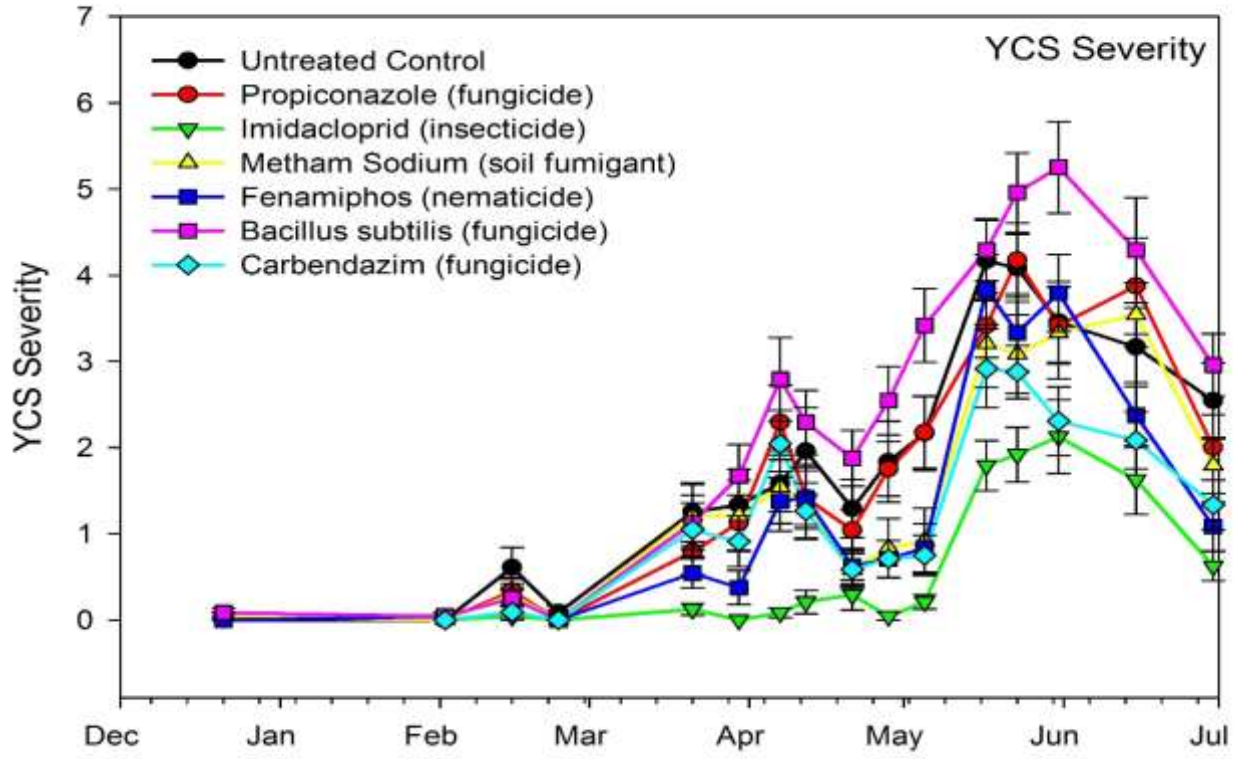


Figure 72 YCS severity score over time. The score is calculated as the sum of the severity ratings for leaves +1 to +7. Each data point represents the average of 24 stalks. Means ± standard error.

Root Assessment

Root sampling was undertaken on 25-Mar-16. One stool was randomly selected in each plot. All above-ground biomass was harvested, partitioned and weighed. A soil core (200mm diameter x 200mm depth) was collected from within the row and adjacent to the stool. Soil cores were washed, passed through a series of sieves, and roots carefully removed. Roots were weighed before being placed in a clear Perspex tray and scanned at 800dpi with a flatbed scanner. Root analysis was then conducted on scanned images using WinRHIZO software (Regent, Quebec, Canada). None of the treatments were statistically different ($p<0.05$) across a range of global and root class parameters (Table 24 and Table 25).

Table 24 Root and Biomass global parameters.

Treatment	Shoot Number	Average Shoot Wt (g)	Root Shoot Ratio (g)	Total Root Vol (cm^3)	Total Root Length (cm)	Average Diameter (mm)	Length per Soil Vol (cm/cm^3)
Pripiconazole (fungicide)	3.7	735.8	37.8	18.2	6671.1	2.4	1.0
Imidacloprid (insecticide)	4.7	792.1	51.1	16.3	5143.2	2.8	0.7
Untreated Control	5.0	637.3	25.0	24.0	6545.9	4.3	0.9
Metham Sodium (soil fumigant)	5.0	764.4	52.1	14.3	7042.6	3.1	1.0
Fenamiphos (nematicide)	5.3	610.8	54.9	10.1	3671.9	2.2	0.5
Bacillus subtilis (fungicide)	6.0	612.4	40.6	12.1	4707.5	3.1	0.7
Carbendazim (fungicide)	3.3	603.6	49.7	13.7	5814.7	1.8	0.8

no statistical difference between treatments for any of the parameters ($p<0.05$)

Table 25 Comparison of root classes. Primary root diameter: d >1.0mm, Secondary root diameter: 0.5 < d < 1.0mm, Tertiary root diameter: 0 < d < 0.5mm

Treatment	Primary Length (cm)	Secondary Length (cm)	Tertiary Length (cm)	Secondary / Primary Ratio (cm)	Tertiary / Primary Ratio (cm)
Pripiconazole (fungicide)	760.5	1225.9	4683.4	1.6	6.2
Imidacloprid (insecticide)	654.3	952.5	3535.5	1.4	5.2
Untreated Control	913.2	1290.0	4341.6	1.4	4.8
Metham Sodium (soil fumigant)	655.9	1061.7	5323.7	1.6	8.3
Fenamiphos (nematicide)	412.4	649.0	2609.0	1.7	6.9
Bacillus subtilis (fungicide)	533.7	833.4	3339.6	1.5	6.0
Carbendazim (fungicide)	624.0	978.7	4210.8	1.5	6.6

no statistical difference between treatments for any of the parameters ($p<0.05$)

Canegrub Sampling

Two stools per plot were dug and assessed for canegrubs. Across the whole trial, only 17 canegrubs were found. They were found in all treatments. We conclude that canegrubs are not a contributing factor at this trial.

Nematode Sampling

One soil sample was collected from every plot on 26-Apr-16 and sent to SRA Tully laboratory for analysis. As expected, the Fenamiphos treatment (nematicide) showed the fewest nematodes and lowest percentage of plant pathogenetic nematodes overall (Table 26). The Imidacloprid treatment had the greatest total number of nematodes and also the highest percentage of plant pathogenetic nematode species. Given the low rate of YCS symptom expression in the Imidacloprid plots, it is therefore unlikely that nematodes are a direct YCS causal agent. Also of note is the Metham Sodium treatment, which showed similar nematode counts to the Untreated Control. However, it is unclear whether this treatment had waned, or if it was less effective against nematodes.

Table 26 Summary of nematode results (n=4).

Treatment	Bacterivore / Fungivore	Bacterivore + Fungivore	Total Nematodes	% Pathgenogenic Nematodes
Propicinazole	1.8	990.3	2466.5	43.2
Imidacloprid	2.6	1132.8	3751.3	50.8
Untreated Control	0.8	865.3	2292.0	38.8
Metham Sodium	1.9	965.8	2351.5	43.3
Fenamiphos	1.4	628.8	1393.5	32.6
Bacillus subtilis	0.9	894.8	2242.5	39.5
Carbendazim	1.1	1212.8	3014.3	41.9

Biomass

Biomass was measured at 12 months. Results showed no difference in stalk population between any of the treatments and the Untreated Control (Figure 73A). There were some significant differences in average stalk weight between treatments. The Metham Sodium and Imidacloprid were 21% and 19% greater than Control respectively (Figure 73B). There was no difference between any of the other treatments and Control. Total biomass tonnes/hectare was statistically ($P<0.05$) lower in only the Propiconazole treatment (Figure 73C). The Untreated Control had an average yield of 77 t/ha compared with 90 and 87 for the Imidacloprid and Metham treatments, respectively. While this trend is higher it is not statistically significant at a 95% confidence interval. There was no difference in CCS between any of the treatments and Control (Figure 73D).

Imidacloprid showed a significant reduction in YCS severity over Control (Figure 72), however the Metham treatment was less so yet still resulted in an increased stalk weight. This suggests that severity and biomass are not tightly correlated which concurs with yield trial studies (see section 6.13 of this report).

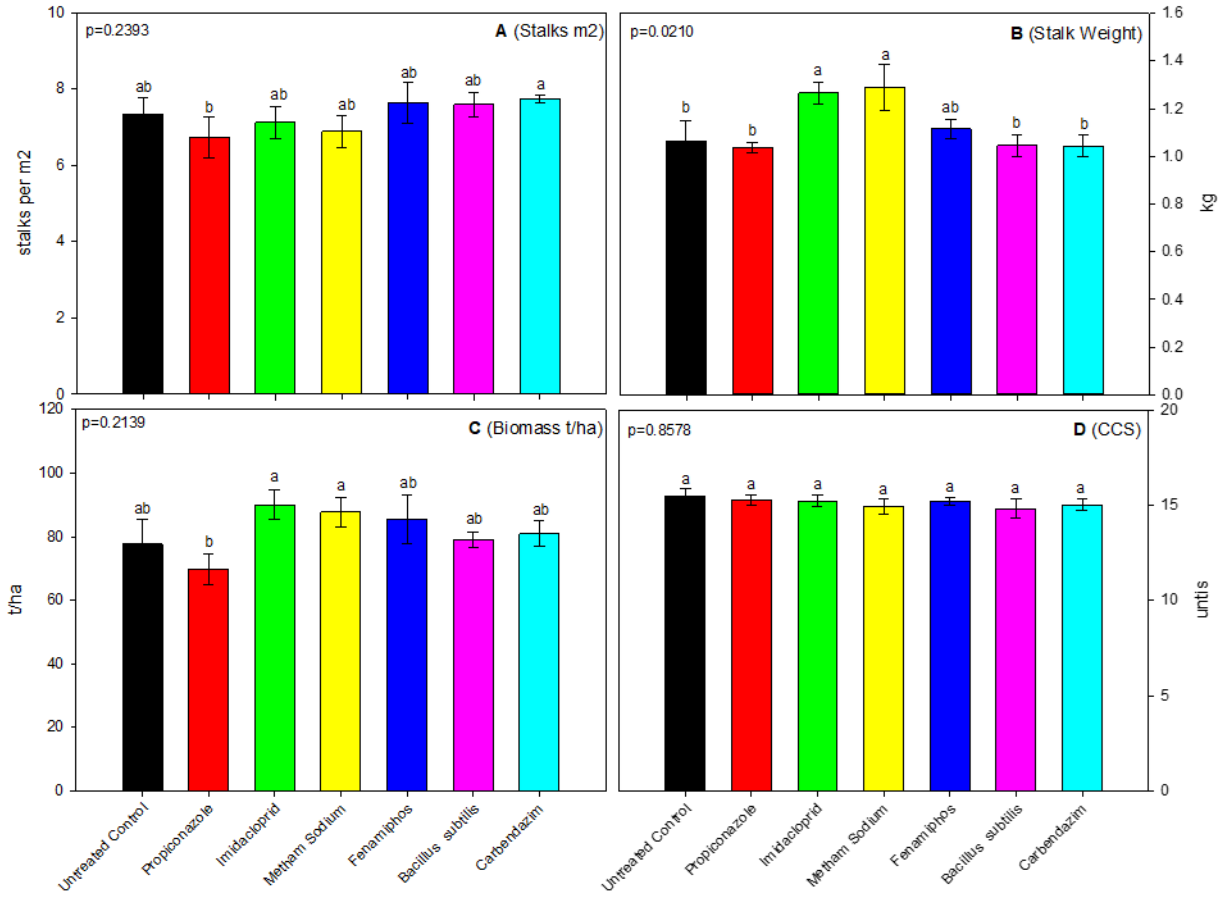


Figure 73 Biomass results. Stalks per m2 (A), Average stalk weight (B), Fresh weight Biomass (C), and CCS (D). Means ± standard error. Analysis of variance by completely randomised design (p<0.05) with LSD all pairwise comparison shown by letter separations a, ab, b etc.

These findings showed that fungicidal treatments were ineffective at improving yield relative to untreated control. The foliar applied Carbendazim (re-applied monthly) did show some reduction in symptom severity however there was no yield response. The soil applied Propaconazole was not effective at either reducing severity or improving yield. This suggests that a fungal pathogen is unlikely to be the cause of YCS. For the same reasons nematodes are also unlikely to be a causal agent (as shown by the Fenamiphos response). The Bacillus subtilis treatment was reported to provide a soil health benefit as well as some systemic fungicidal action. This treatment was also ineffective.

Imidacloprid once again is the best performing treatment. The mechanism for this is either insecticidal or stress relief (see section 6.3 of this report). The Metham sodium soil fumigant also had a positive response on stalk weight in this trial, but less so on YCS mitigation. This suggests that the yield response may have been in response to controlling an unknown soil organism rather than any YCS effect. The Metham treatment showed significantly ($p<0.05$) reduced total soil microorganisms and total fungi than the Control even 6+ months after initial application (data not shown).

6.4.3 Discussion and conclusions

These experiments set out to determine whether poor root health (per se) directly leads to YCS symptom development and the involvement of any soil borne agent. Treatments were applied that were known to affect root health; these included soil fumigation, fungicides, insecticides and nematicide treatments. Assessment of nematode populations during the growing season clearly showed they were affected by Nimitz (fluensulfone) and fumigant Metham sodium (Tamifume), and conversely, Confidor® had only a minor effect (root knot / root lesion nematodes). In contrast only the Confidor® treatment led to reduced symptom development closely followed by Irrigation, suggesting that root health (per se) is not the direct cause of YCS. Eventually, some symptoms did develop in the Confidor® treatment plots. The result in this experiment confirms observations made in other trials and in commercial crops where Confidor® has influenced YCS symptom development (see section 6.13.2.1 of this report). The fungicides were mostly ineffective, ruling out a fungal pathogen.

Above ground analyses showed that any chemical treatment that reduced YCS symptoms did not significantly improve yield. The best example of this was Imidacloprid which simply provided a stress shield stay-green effect without yield benefit. The Irrigation treatment was potentially the most effective as it reduced YCS symptoms and returned the highest yield. The collective results from the root health and soil biology studies indicate that YCS is not caused by a soil borne agent or poor root health. There is also no evidence that YCS causes poor root health. Studies by Rae and Pierre (2018) showed no difference in root system structure between healthy control and YCS symptomatic plants which concur with these results.

6.5 Plant physiology and YCS

Many hypotheses were put forward to explain the episodic nature of YCS observed within and between fields and regions throughout the peak growing season. These included pests and diseases, abiotic stress, farming practices, agrochemicals, soil and root health, nutrition and climate change. Even though the integrated YCS research program systematically set about to investigate each of these theories the research was interwoven by a common thread. This was to understand the changes to the plant's physiology which would help identify the cause of YCS development and expression. Very early in the program it was identified that YCS symptomatic plants accumulated high levels of sucrose and starch in the source leaves of the mid-canopy (Botha et al., 2015; Joyce et al., 2016; Marquardt et al., 2016). Further analysis confirmed there was a complete disruption to natural diurnal rhythm of these metabolites and extensive disruption to carbohydrate metabolism in general (Du et al., 2000; Marquardt et al., 2016; Marquardt et al., 2017). This result was the start of an in-depth analysis of the possible reasons for a disruption to sucrose translocation between the source and sink tissue. Here we report on the physiological studies conducted and perturbances to plant function associated with YCS.

6.5.1 Crop age trial

The experiment was established on a grower's farm at Home Hill, Burdekin (Farm # 4888 Block # 9-1). The cane was allowed to grow its ratoon by harvesting and slashing them in July (7months), September (5months), October (4 months) and November (3 months) 2014, respectively, and created the age differences as the main treatment within the block. (see section 6.1.1 & Appendix 1: 1.2.7 of this report). The aim of this study was to determine whether there was any pattern of YCS appearance in terms of leaf emergence in different age crops of KQ228$^{\phi}$. The hypothesis is that an external factor is triggering YCS symptoms on developing leaves and the age of the plant has no bearing on expression of symptoms. The number of leaves developed after the event should be the same in all treatments as the time taken between the appearance of successive leaves (phyllochron) expected to be the same for KQ228$^{\phi}$ (Robertson, 1998). This would suggest that the symptoms are triggered at the same time regardless the age of the crop. An evaluation of any critical environmental conditions that may influence the initial developmental phase of the first YCS symptomatic leaf in different age crops, would then be possible. The thermal time (^{0}Cd) can be estimated for each leaf development thereafter, as the sugarcane leaf development depends on temperature (Campbell et al., 1998).

Leaf counts and monitoring for prevalence and severity (Table 3 and Table 4) were recorded throughout the trial. Stalk samples were collected on 25 February 2015 when the youngest treatment was about 3 months old. Age at sampling of the other treatments were 4, 5 and 7 months. Representative YCS symptomatic stalks were identified and used for fresh and dry matter partitioning. The internodes attached to the selected leaves were also dissected and weighed separately. The total number of leaves, green leaves, YCS symptomatic leaves and dead leaves were recorded. Leaves were used for colour spectrum (RGB) analysis. The intensity of the green and yellow colour of the leaf lamina were carefully observed and leaves were grouped into five classes. They are green, green – blur (green to naked eye), yellow-green, yellow, and brown (dead). The RGB intensities were measured with the IDS camera and chlorophyll content at the time of measurement was recorded using SPAD and CL-02 Chlorophyll content meter, respectively.

Results

YCS began to be consistently observed from mid-December (Table 27) onwards in plants with >60cm of stalk (Figure 76). At this point, the November treatment had an average 38cm of stalk and did not exhibit symptoms until the next monitoring date 3 weeks later, when it then had 100cm of stalk (Figure 76). It appears time of year and plant height (age) are key factors influencing YCS expression. YCS was observed in the mid canopy of all treatments for the first time on 12 January. By mid-February, all plants were similarly affected by YCS regardless of age, however no YCS was recorded in the upper canopy of any treatments (Table 27).

Table 27 Monitoring data. Severity and prevalence ratings are shown for the lower canopy (leaf 6 and lower) and the mid canopy (leaf 1-5). Severity is rated in a scale of 0 to 4, corresponding to no symptoms, mild, moderate and severe symptoms respectfully. Prevalence (0-4) is rated according to the proportion of plants within the plot that are exhibiting symptoms. The scale (0-4) corresponds to no plants, 25%, 50%, 75% and 100% respectfully.

Canopy position	Canopy ratings	Treatment	Date										Mean
			10/11/2014	17/11/2014	25/11/2014	4/12/2014	17/12/2014	12/01/2015	27/01/2015	3/02/2015	11/02/2015	17/02/2015	
Low (leaves >5)	Severity (0-4)	July	0	0	0.25	0	0.5	1.6	1.8	2.2	2.8	2	1.115
		Sept	0	0	0.2	0	0.2	2.4	1.8	2.6	2.2	2.2	1.16
		Oct	0	0	0	0	0.2	2	2.2	2.8	2.4	2.6	1.22
		Nov	0	0	0	0	0	2.2	1	2	2.2	2.6	1
		Mean	0	0	0.11	0	0.23	2.05	1.7	2.4	2.4	2.35	1.12
	Prevelance (0-4)	July	0	0	0.25	0	0.75	4	3.8	4	4	4	2.08
		Sept	0	0	0.2	0	0.6	4	4	4	3.2	4	2
		Oct	0	0	0	0	0.4	3.6	4	4	4	3.8	1.98
		Nov	0	0	0	0	0	3.4	4	4	4	3.6	1.9
		Mean	0	0	0.11	0	0.44	3.75	3.95	4	3.8	3.85	1.99
Mid (leaves 1-5)	Severity (0-4)	July	0	0	0	0	0	0.6	0.6	1.2	1	1.2	0.46
		Sept	0	0	0	0	0	0.6	0.8	1.2	1.8	1.8	0.62
		Oct	0	0	0	0	0	0.4	1.2	1.4	1.4	1.6	0.6
		Nov	0	0	0	0	0	0.4	0.6	1.2	1.8	2	0.6
		Mean	0	0	0	0	0	0.5	0.8	1.25	1.5	1.65	0.57
	Prevelance (0-4)	July	0	0	0	0	0	1	1.6	2.4	3	3	1.1
		Sept	0	0	0	0	0	0.8	2.4	2.8	3.8	2.4	1.22
		Oct	0	0	0	0	0	1.2	2.2	3.6	3.6	3	1.36
		Nov	0	0	0	0	0	0.8	1.2	3.2	3.6	2.8	1.16
		Mean	0	0	0	0	0	0.95	1.85	3	3.5	2.8	1.21

Leaf and internode dry matter

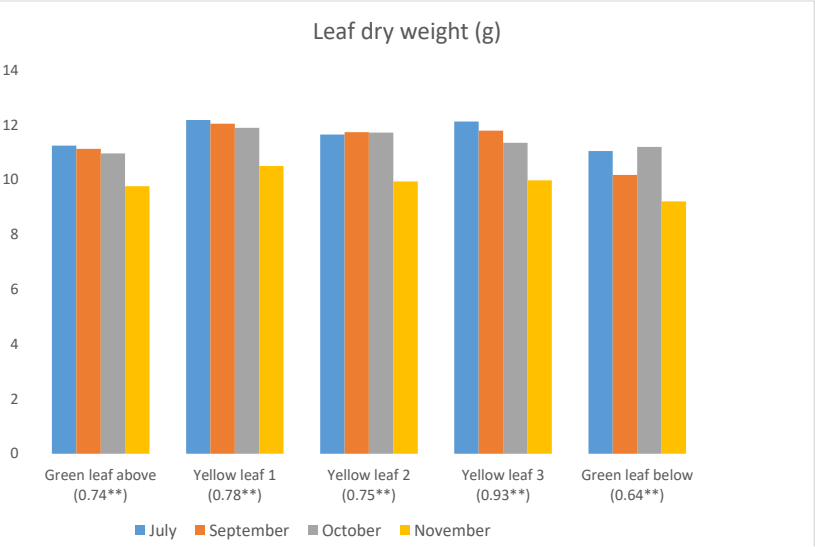


Figure 74 Dry weight of 5 leaf positions in the symptomatic stalk (green leaf above 1st YCS leaf, 1st YCS leaf, 2nd YCS leaf, 3rd YCS leaf and green leaf below the last YCS leaf) in 4 age treatments.

The leaf dry weight within each of the five leaf classes was significantly different between treatments (Figure 74). The November slashed treatment had the lowest leaf dry weight and it was significantly different from the other 3 age treatments. The October slashed treatment had the highest dry weight. The YCS leaf dry weight was not statistically different to that of the green leaves.

This concurs with studies by Scalia et al. (2020) which show that a YCS symptomatic leaf +4 has a similar dry mass to a green control leaf even though it contains approximately 3-fold more sucrose and α-glucans. Compositional analyses studies suggest this is due to reduced protein content in the YCS leaf (Table 15).

Internode dry weight

There was no significant difference between age treatments for the average dry weight of nodes attached to each leaf group (Figure 75). However, as expected the dry weight increased ($p<0.05$) with the maturity of the node in each stalk irrespective to the condition of the attached leaf. Among 4 treatments, the node attached to the last green leaf above the 1st YCS leaf had the lowest dry weight ($p<0.05$).

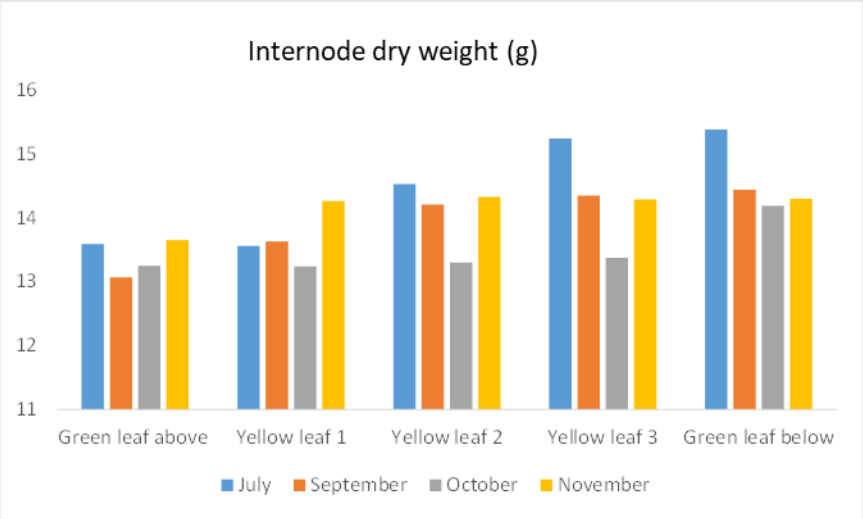


Figure 75 Dry weight of internodes attached to each 5 leaf positions in the symptomatic stalks (green leaf above 1st YCS leaf, 1st YCS leaf, 2nd YCS leaf, 3rd YCS leaf and green leaf below the last YCS leaf) in all 4 age treatments.

Internode length

Among age treatments, average internode lengths in 5 different leaf groups were not significantly different ($p>0.05$). However, the node length within each treatment (age group) showed an increasing trend as noted with the node dry weight.

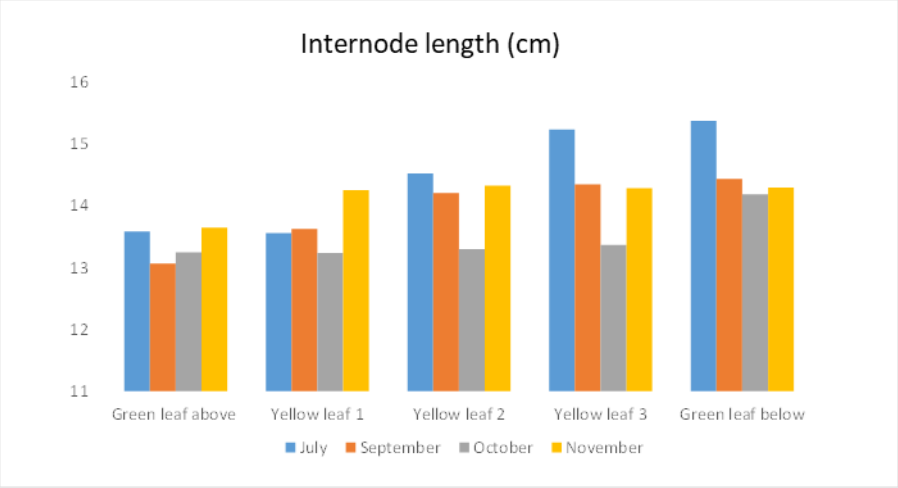


Figure 76 Length of nodes attached to each leaf 5 positions in the symptomatic stalks (green leaf above 1st YCS leaf, 1st YCS leaf, 2nd YCS leaf, 3rd YCS leaf and green leaf below the last YCS leaf) in all 4 age treatments.

RGB discrimination

The leaf samples collected from the age trial were analysed for red, green and blue (RGB) primary colour reflections. The leaves were grouped in to green, green – blur, yellow-green, yellow and brown (dead). The RGB intensities were measured with the IDS camera on each leaf (colour class) (Figure 77), and chlorophyll content at the time of measurements were taken using SPAD and Chlorophyll meter CL-02, respectively.

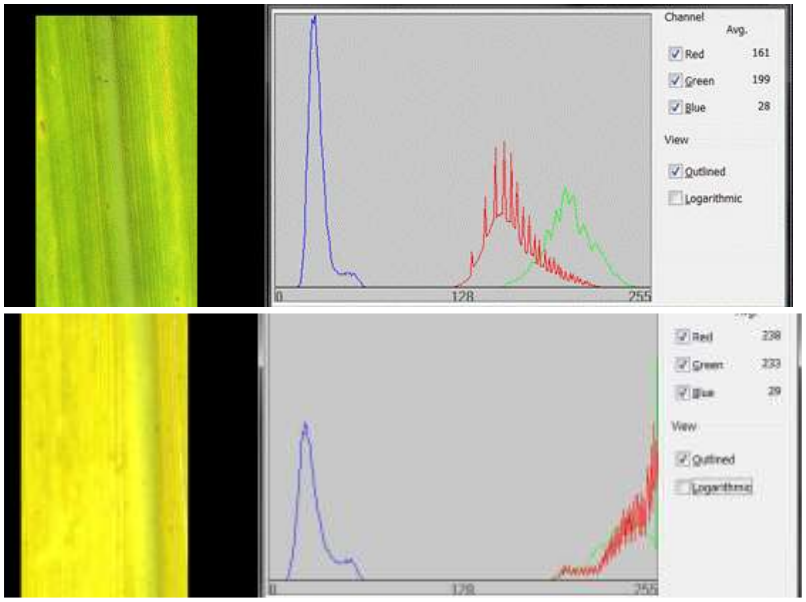


Figure 77 Primary colour (red, Green and Blue) intensities recorded for pre-YCS and YCS leaf samples from the IDS high resolution camera.

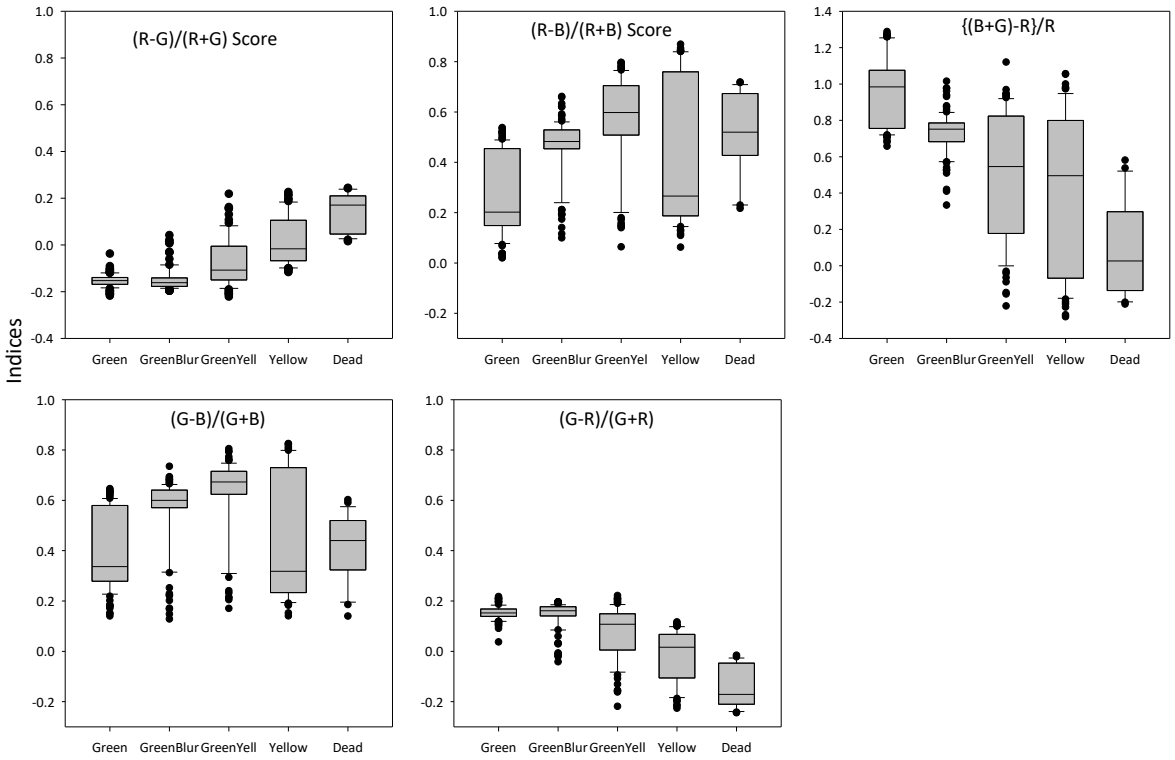


Figure 78 Boxplots demonstrating the relationship between leaf colour in green, YCS and dead leaves and several indices based on their visible RGB spectra.

Five different indices were developed for evaluation of leaf colours. Figure 78 shows the variation among different indices and the potential use as a tool in preliminary discrimination of green leaves from YCS leaves, especially during the early stages of yellowing. However, results show there would not be sufficient RGB discrimination between YCS leaves and the different stages of leaf senescence to use this as a tool for early detection of YCS yellowing.

Summary

Results show a synchronized occurrence of YCS symptoms irrespective of crop age, but this is influenced by the amount of formed cane or maturity of the culm. Altering the sink size during the peak photosynthetic period increases the risk of an imbalance between storage, and growth, development and maintenance respiration. There is no evidence that the symptomatic leaves or internodes attached to those leaves had any set back in biomass accumulation between the age treatments. This is to be expected as these leaves and internodes are of the same chronological age with similar YCS symptoms (see section 6.1.1 of this report). RGB discrimination is not a useful tool to detect early onset of YCS.

6.5.2 Physiology case study – Q240$^{\Phi}$ Burdekin

In early February 2016, a field of variety Q240^{A} 1R sugarcane was identified in the Burdekin which had very clear YCS symptomatic cane and very green asymptomatic cane at opposing ends. Recognising this as a unique opportunity, we conducted intensive sampling to determine the cause of the difference in symptom expression, and identify key agronomic and physiological YCS indicator parameters. During February, this site was also intensively sampled by the SRA project team (2015/016) and the Western Sydney University team (2014/082). At the time of sampling, the crop was 6 months old. Interestingly, by mid-May all cane at this site was asymptomatic. This presented a further opportunity to study whether the plants had indeed recovered or whether they were actually YCS affected yet not expressing symptoms.

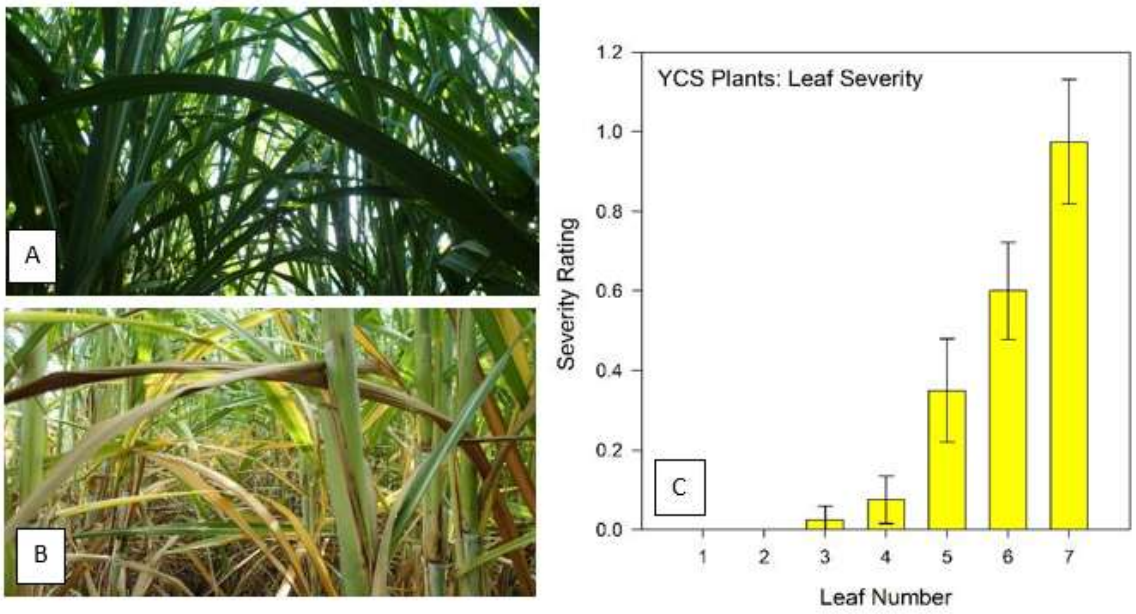


Figure 79 Q240^{A} Symptom expression, February 2016. Control A), YCS B) and Average Leaf Severity in the YCS plants C). Bars represent the average of 20 stalks. Means ± standard error.

YCS Severity

There was a strong contrast between asymptomatic Control and symptomatic YCS plants, with the Control plants showing no signs of yellowing anywhere in their canopy. Despite the fact that the YCS cane was located only 50 meters from the green controls, and within the same row, their canopy was strikingly yellow (Figure 79 A & B). Figure 80 shows a typical comparison between control and YCS, with strong yellowing evident on Leaf +7 and as high up as Leaf +3 (Figure 79 C). Farm inputs and management were identical for the two phenotypes.

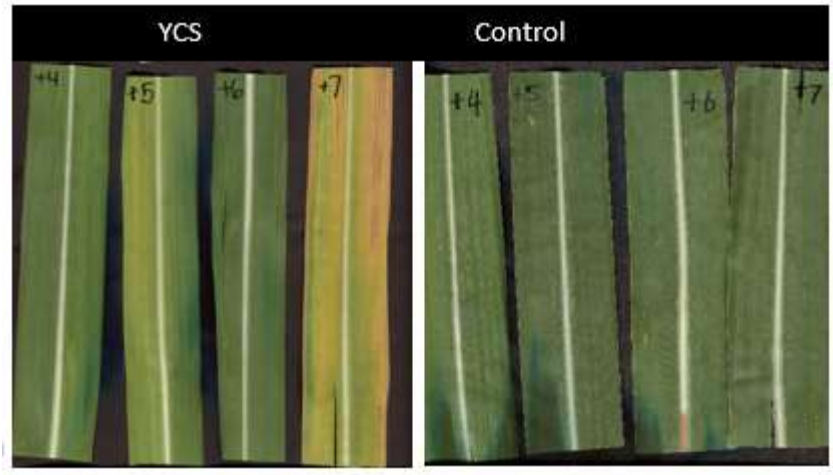


Figure 80 YCS leaf severity in YCS and Control. Images are of the middle 30cm of each leaf. Leaf numbers +4, +5, +6, and +7 are shown (left to right).

Growth, Starch and Sucrose

Despite having similar stalk heights (data not shown), the YCS cane had 2-3 fewer leaves per stalk. This was significant at the p=0.05 level (Figure 81). No other significant differences were measured. Both treatments showed similar leaf expansion rates (Figure 81(A)). The elevated sucrose and starch levels are typical of YCS affected leaves (Figure 81 C), even though our samples were taken from Leaf +4 which was primarily green at the time (Figure 79 C and Figure 80). More comprehensive measurement of metabolites was conducted by the SRA project team (2015016) (Marquardt et al., 2019; Scalia et al., 2020).

Interestingly, we once again see fewer leaves on YCS affected plants (Figure 81 A). This is a trend apparent in almost all of our trials and data sets. Given that we typically see no significant reduction in stalk height, or leaf expansion rate, we can assume that the reduction in leaf numbers is directly related to increased senescence rather than any decrease in leaf emergence.

Soil moisture and Irrigation

Soil moisture was measured beneath the stool (n=20) in the 0-20cm zone. Results show very similar levels of soil moisture at the time of sampling (%vol). Given the similarity of the soil types, and the fact that this site was regularly furrow irrigated (every 7-10 days) this is not surprising and suggests that a soil moisture deficit (or waterlogging) is not the cause of the yellow phenotype in this cane.

Leaf relative water content

Relative water content (%) was significantly higher in YCS than Control (Figure 81(B)). This is a trend we see in all of our data sets.

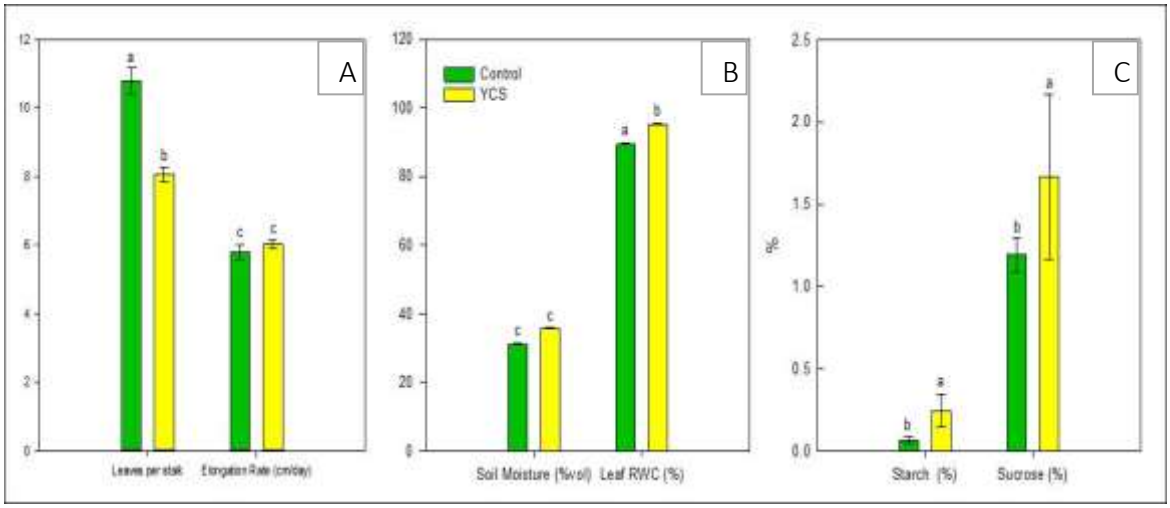


Figure 81 Average Leaves per Stalk and Leaf Elongation Rate A), Soil moisture and Leaf moisture B), and Leaf Starch and Sucrose content C). Bars are the average of 20 stalks. Means ± standard error. Statistical significance (p<0.05) indicated by letter separations.

Soil Test Results

A representative soil sample (0-20cm) was collected from within the row of both treatments and sent for laboratory analysis. The two soils were very similar across a range of soil physical and chemical parameters. Both were clay loam soils with relatively high sodium contents (ESP% of 11 and 13 for YCS and Control respectively). Overall, soil macro and micronutrients were in adequate supply and there were no measured chemical or physical parameters that differed significantly between the two soils (data not shown). We did not, however, test soil biology so we can make no conclusions regarding soil biotic agents at this site. Note that Western Sydney University did extensive metagenomics analyses on the soil and root/plant microbiome at this site (Hamonts et al., 2018). Interestingly, a discussion with the grower also revealed that the crop always grew better at the irrigated end than at the bottom end of the field, which he always put down to soil variation. Historically, this was well before YCS occurred in 2012.

Root Sampling

Root sampling was undertaken on 25-Mar-16. Three stools were randomly selected within YCS and Control sections of the field. All above-ground biomass was harvested, partitioned and weighed. A soil core (200mm diameter x 200mm depth) was collected from within the row and adjacent to the stool. Soil cores were washed, passed through a series of sieves, and roots carefully removed. Roots were weighed before being placed in a clear Perspex tray and scanned at 800dpi with a flatbed scanner. Root analysis was then conducted on scanned images using WinRHIZO software (Regent, Quebec, Canada).

Statistical significance was determined using a 2-Sample T-Test at a confidence of p=0.05. None of the 8 measured parameters were significantly different between the two treatments (p>0.05). However, the data shows a trend for the asymptomatic Control having smaller average root diameter, more fine root hairs, less primary and secondary roots, greater above-ground biomass, and greater total root length (Figure 82). These trends are interesting though not conclusive given

the small sample size. However, the larger above ground biomass suggests asymptomatic plants with finer root hairs and longer root system are better able to seek and absorb water and nutrients, and that these roots are healthier. Therefore, asymptomatic control plants would likely have higher sink strength or carbon sink capacity than the YCS symptomatic plants (Ogunkunle and Beckett, 1988; Smith et al., 2005). A stronger sink reduces the risk of a source sink imbalance (Bihmidine et al., 2013).

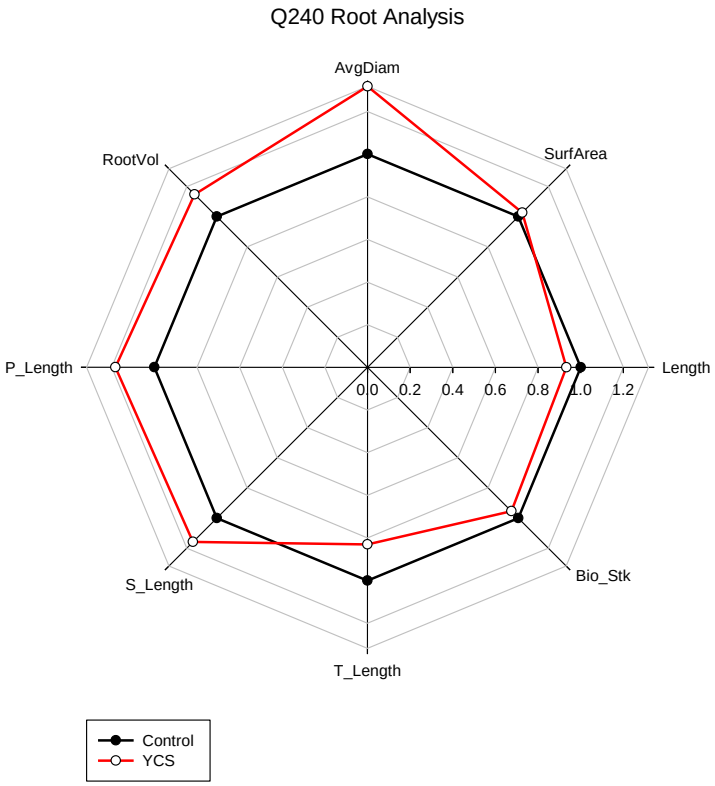


Figure 82 Root system results. The radar plot shows YCS relative to Control for 8 root parameters. For each parameter the Control has been normalised (x=1.0). Parameters; total root length (Length), biomass of above-ground cane (Bio_Stk), length of tertiary roots 0-0.5mm diameter (T_length), length of secondary roots 0.5-1.0mm diameter (S_Length), length of primary roots >1.0mm diameter (P_Length), total root volume (RootVol), average root diameter (AvgDiam), and total root surface area (SurfArea). n=3.

YCS Recovery

The site was revisited on 25-May-16, 3months after the first field visit in February. At this time cane which had previously been YCS symptomatic was largely asymptomatic. Previously asymptomatic Control was also asymptomatic. Visually the change in YCS expression was very clear and raised the question of whether the YCS cane had completely recovered, or whether it was still YCS impaired though asymptomatic. There was no doubt that the weather conditions had changed markedly since our initial Feb sampling. May was significantly cooler with less average solar radiation per day (Figure 83).

A complete set of diagnostics (**Error! Reference source not found.**) were re-run at in this May field v isit (Figure 84). Of particular note is the apparent recovery in number of leaves per stalk (Figure 84).

May YCS had 2-4 more leaves per stalk than they had in February although this was still significantly less than the Control treatment (Figure 84).

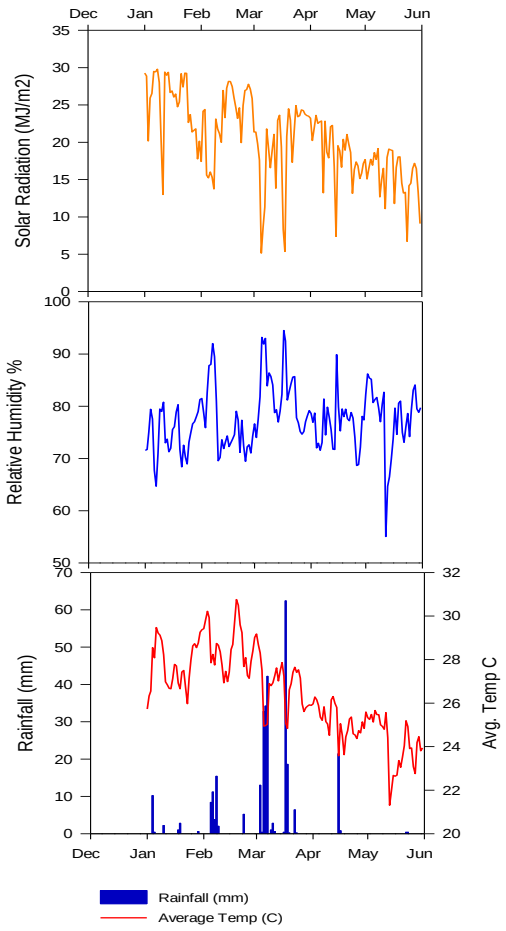


Figure 83 Weather parameters taken from Home Hill weather station.

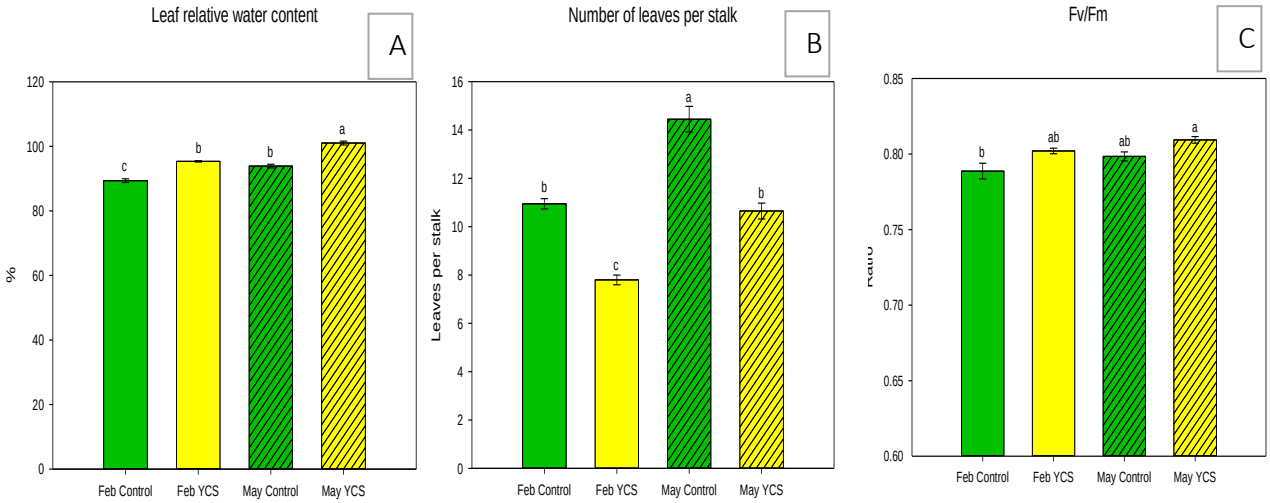


Figure 84 Comparison of YCS and Control cane during symptomatic (February) and asymptomatic (May) field visits. Leaf relative water content % (A), Average number of leaves per stalk (B), and Quantum yield of

photosystem II Fv/Fm (C). Bars represent the average of 20 stalks. Means ± standard error. Statistical significance (p<0.05) is shown by letter separations.

Biomass sampling

Biomass was measured on 27-Jul-16 when the crop was 11 month old. Sampling was conducted in the same area where all previous measurements had taken place. Two five-linear-meter sub-samples of cane row were cut from YCS and Control rows. Cane was partitioned, weighed and biomass determined for each of the components. Statistical significance was determined using a 2-Sample T-Test at a confidence of p=0.05.

With the exception of leaf biomass, results showed no significant difference (p>0.05) between YCS and Control across the range of measured parameters (Figure 85 A, B, C, D, E). YCS had significantly less (p<0.05) total leaf biomass (t/ha) than Control Figure 85 F). This is consistent with measurements of leaf numbers throughout the season, which showed Control plants to have an average of 2-3 more leaves per stalk than YCS affected plants (see Figure 84 B).

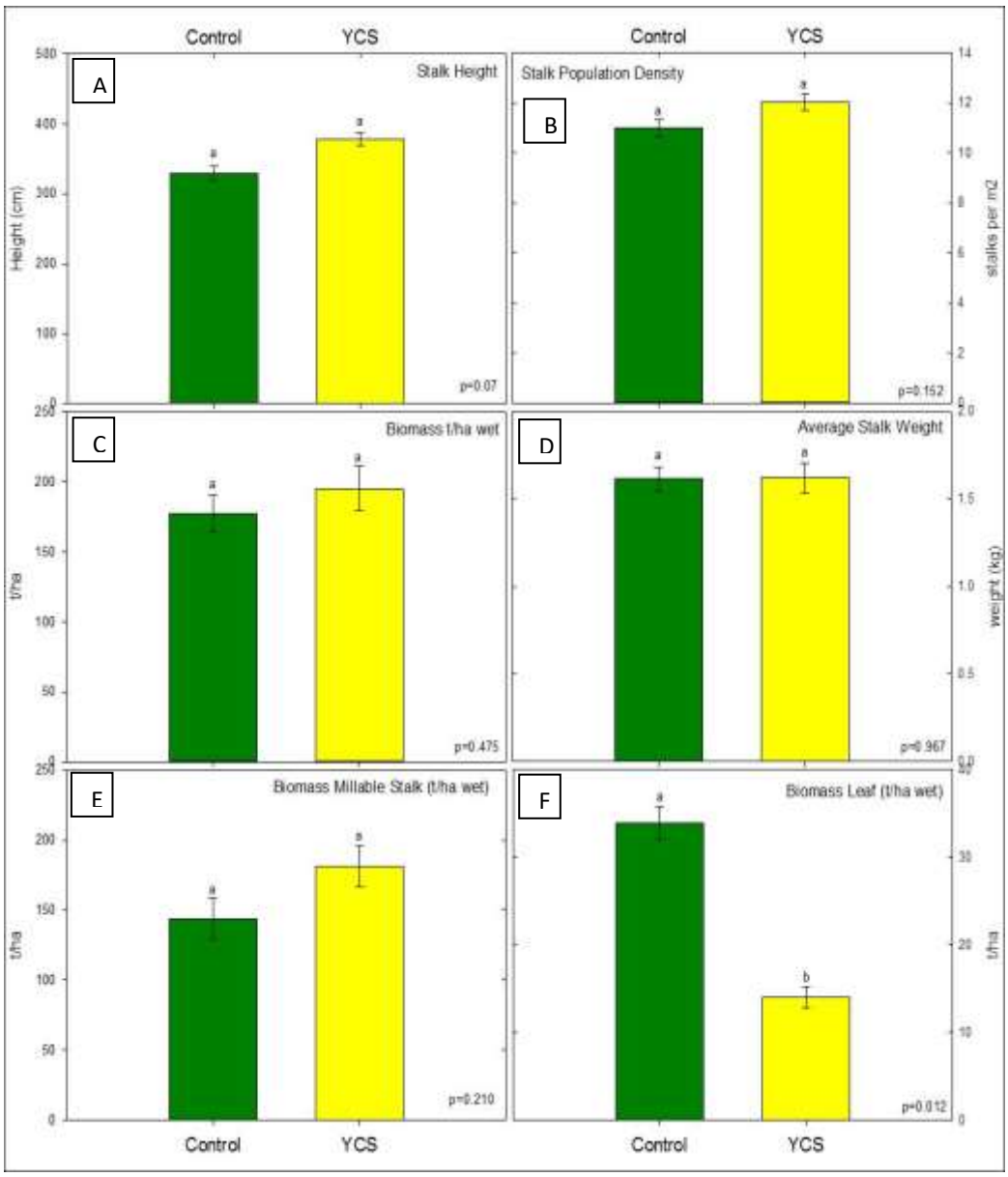


Figure 85 Q240A Biomass results. Panel shows average stalk height (A), stalk population density (B), biomass t/ha wet (C), Average stalk weight (D), Biomass of millable stalk t/ha wet (E), and Biomass of Leaf t/ha wet (F). Means ± standard error.

Internode Sampling

Sampling was conducted in late July, one week after biomass sampling. Twenty stalks were randomly chosen from Control and YCS affected cane. All stalks and internodes length and width were then measured from the top down where internode 1 is the internode attached to leaf +1. YCS cane showed a period of reduced internode length relative to Control in internodes 17-20. A shortening of internodes can be due to water and nutrient deficit stress or an impeded supply of reduced carbon from the source (Rae et al., 2014). Interestingly, internode growth recovered between internodes 21-24, being comparable to Control. Internode diameters were similar throughout.

To calculate when in the season this internode shortening took place, a timeline was imposed on the data. Assuming an average phyllochron of 10 days, approximate dates were assigned to each

internode. From this calculation working back from late July when internode +1 was formed, it becomes apparent that reduced internode growth occurred in late December through January. This is well before the onset of the yellow symptoms and peak period of YCS expression in February. Noteworthy is the rainfall event in early February (Figure 83) followed by high solar radiation at the time of YCS expression. This is synonymous with Industry reports of a flush of yellow moving through the crop after rainfall that has been preceded by a dry period or slower growth.

Summary

A distinct difference in visible golden-yellowing of the mid-canopy was observed within the same field. YCS symptomatic stalks had 2-3 fewer attached leaves than asymptomatic stalks, likely due to increased rate of senescence. Highest levels of sucrose and starch accumulation was evident in symptomatic mid-canopy leaves, with increased levels also recorded in asymptomatic leaves on the same culm. Mid-canopy leaves on YCS stalks always have higher levels of relative water content (RWC).

Soil moisture and composition was similar throughout the field. Roots of asymptomatic plants appeared healthier and supported a larger above ground biomass, indicative of increased sink capacity and strength. A balanced source sink maintains a strong sucrose gradient and limits sucrose accumulation in the export leaves. (Botha et al., 1996; Geiger et al., 1996; Bihmidine et al., 2013). This reduces the risk of YCS development and the onset of yellowing (Scalia et al., 2020).

A reduction in internode growth is evident prior to resumed internode growth and symptom expression after good rain and high solar radiation. A crop can recover from a YCS evident with no statistically significant biomass penalty.

6.5.3 Physiology and molecular studies

Physiological studies, molecular studies on DNA status performed was generated from samples collected over two visits to Mackay in Feb and March 2015. YCS samples were collected from the farm of Joe & Paul Schembri's (Q208$^{(l)}$- 2R) and the healthy plants from the Q208$^{(l)}$ plant crop at Mackay Area Productivity Services (MAPS) farm, at Victoria Plains Qld. Both crops were of a similar age.

All the leaves from leaf +1 = first visible dewlap (FVD) as well as the furled leaf (0) were collected and used for starch studies. The appearance of the leaves at harvest are shown (Figure 86).

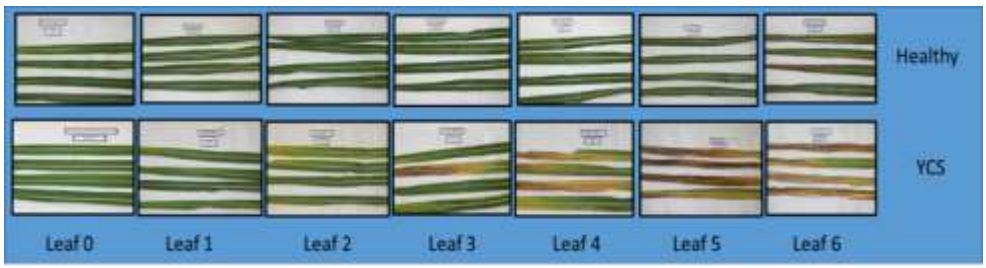


Figure 86 Starch accumulation in healthy and YCS affected plants. (A: Healthy leaves, B: YCS leaves.)

6.5.3.1 Electrical conductivity (EC) studies

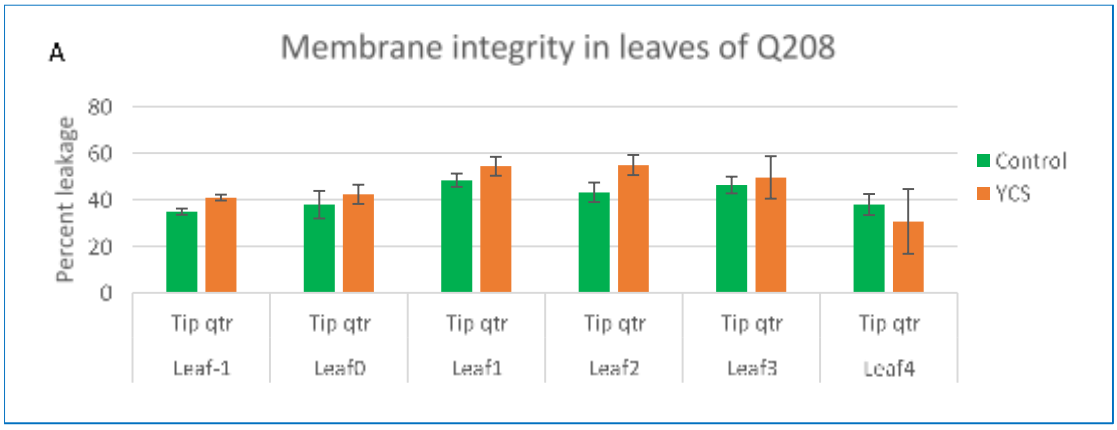
One of the earliest responses to stress is the loss of membrane integrity resulting in 'leaky' membranes. This work was performed to determine whether there is a perception of the stress signal; and whether it occurs before any strong visible symptoms appear; and whether there was any correlation between the observations and age of the cells.

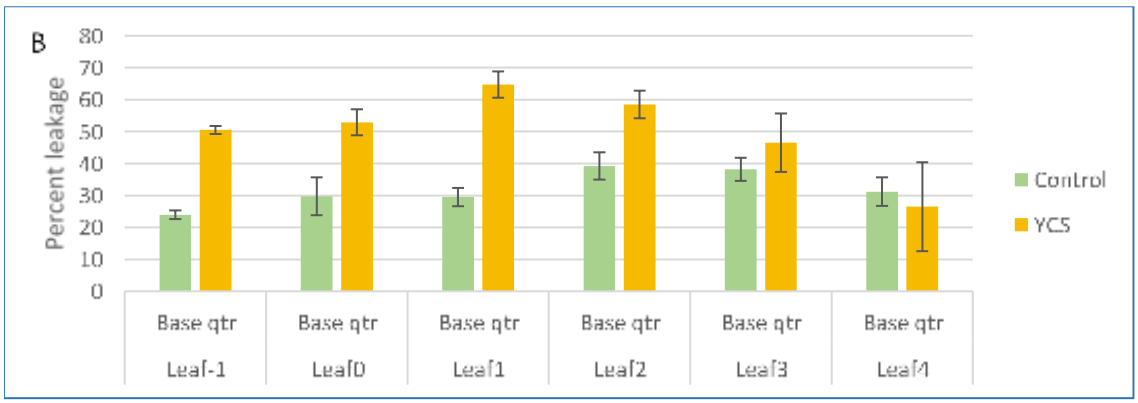
Protocol

There were three replicates and each replicate consisted of four stalks. The stalks were harvested in pairs from the same fields as above. Immediately after harvest, the leaf discs were punched straight into the Falcon tubes containing deionised (d.i.) water. Twenty leaf discs per replicate were taken from either the basal quarter or the tip quarter of each leaf. These were left in the tubes overnight. The next day, the discs were removed, adhering water removed quickly and then frozen at -80^{0}C. These samples were taken back to Brisbane on dry ice where the samples were thawed in a fresh d.i. water, and left for a further 12hours. The water from each tube was then measured for the electrical conductivity (EC), and leakage calculated as shown below.

Percent leakiness was expressed as =% [EC initial÷ (EC initial + EC Freeze thawed)]

Results show that in the tip quarter region, YCS and healthy leaves behaved similarly although the leakiness was higher in the YCS leaves (Figure 87 A). However, when the basal quarter region EC is compared, the YCS leaves are significantly leakier than the healthy leaves, releasing up to 65% of the cell ion content (Figure 87 B).





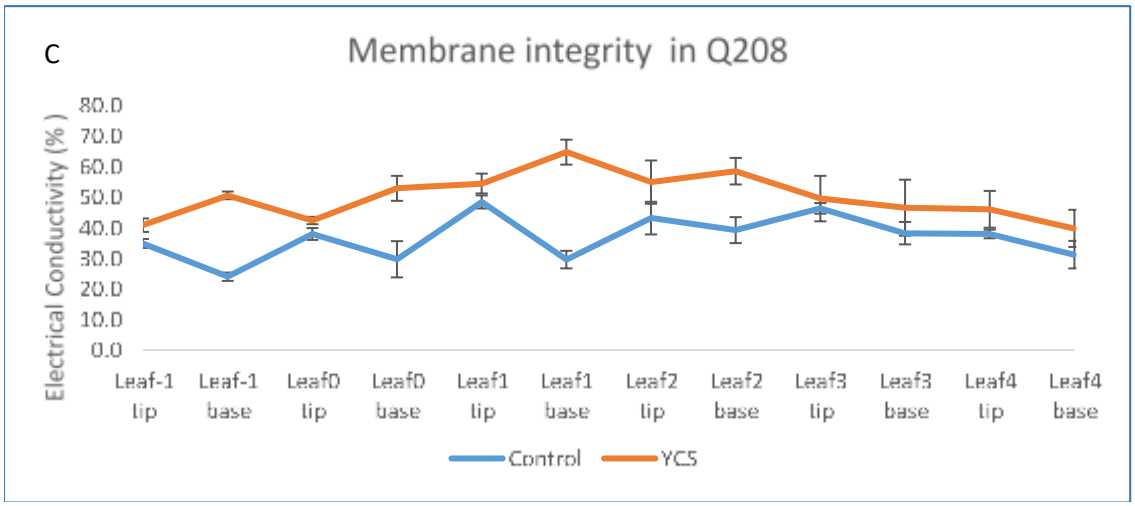


Figure 87 Electrical conductivity of the different regions of the sugarcane leaf in healthy and YCS leaves. The tip region of leaves A), Basal region of the leaves B), Comparison of EC between healthy and YCS plants in the leaf tip and basal region in all leaves C).

This result shows that the YCS plants have perceived the signal already in the furled leaf-1, showing an increase in membrane leakiness from 25% in healthy to 50% in the YCS leaves. This trend continued till leaf +2 after which the difference was less significant. When the data are plotted with all samples, YCS leaves up to leaf +2 clearly show an opposite trend to that in control plant leaves. From leaf +3 onwards, this trend changes and looks similar to that in control leaves (Figure 87 C).

If this difference in EC is consistent across varieties and unique to YCS plants, then this could serve as a potential diagnostic tool for YCS identification.

Relative water content was also measured in these leaves using leaf punches. The results showed that YCS affected leaves were not lower in their RWC and were comparable to that of asymptomatic green leaves (data not shown).

6.5.3.2 DNA laddering studies

Programmed cell death (PCD) is a genetically controlled event in the cell to induce its own destruction, in order to inhibit the organism's development, reproduction and senescence in the end

of the life cycle. PCD also plays an important role in the plant as a defence mechanism against biotic and abiotic stresses, and is a way of eliminating the infected or stress disturbed cells (Chen et al., 2014). PCD is characterized by a chromatin condensation, DNA fragmentation, cell shrinkage and cell apoptosis. In order to study if there was programmed cell death (PCD) in plants affected by YCS, we performed the following experiment and focussed on DNA fragmentation as an indication that PCD was occurring in YCS plants.

DNA was isolated using established protocols from the same Q208 plants collected at Mackay in the experiments described above. We first checked the DNA that was isolated in a pilot study to ensure that the method that we used produced non-sheared good quality DNA. We compared the DNA from control healthy leaf1 as well as YCS leaf1 and concluded that DNA isolated via centrifugation was as good as that which had been spooled up using a glass rod (Table 28). We also tested the ready –made DNA extraction kit (DNeasy) and obtained a similar yield and quality of the DNA, but favoured the CTAB extraction method due to slightly better DNA when the gel was run.

Table 28 DNA yield and quality from different extraction methods

Sample ID	ng/ul	260/280
Q208 Control Lf +1 (DNeasy)	247.89	1.85
Q208 YCS Lf +1 (DNeasy)	380.92	1.82
Q208 Control Lf +1 (CTAB - A)	508.34	1.86
Q208 YCS Lf +1 (CTAB - A)	805.46	1.89
Q208 Control Lf +1 (CTAB - B)	481.60	1.86
Q208 YCS Lf +1 (CTAB - B)	277.10	1.88

The DNA of all the leaves of Q208 YCS and control stalks were extracted using the CTAB method. We also included sections within the YCS leaf which were green or yellow, to see if there was any preferential laddering in these leaves. In addition, we obtained some Q208 leaves from the glasshouse which were naturally senescing and included these too.

The quality of DNA was checked with the Nanodrop (Table 28) and equal amounts of DNA (10µg) was loaded and run overnight on a 2% agarose gel at low voltage (40V), to enable good separation of DNA.

Table 29 DNA samples and yield from Q208 leaves of control and YCS plants

Sample ID	Yield (ug/g)
Q208 Control Lf 0	259
Q208 Control Lf +2	377

Q208 Control Lf +3	421
Q208 Control Lf +4	220
Q208 Control Lf +5	319
Q208 Control Lf +6	179
Q208 YCS green Lf 0	478
Q208 YCS green Lf+1	276
Q208 YCS green Lf +2	279
Q208 YCS green Lf +3	272
Q208 YCS green Lf +4	236
Q208 YCS green Lf +5	180

Q208 YCS Lf +2 yellow	110
Q208 YCS Lf +3 yellow	24*
Q208 YCS Lf +4 yellow	239
Q208 YCS Lf +5 yellow	169
Q208 YCS Lf +6 yellow	241
Q208 Control Lf +7 Senescent	133
Q208 Control Lf +8 Senescent	9
Q208 Control Lf +9 Senescent	104
Q208 Control Lf +11 Senescent	40

*= sample was lost

Results

Results showed that in the older leaves (+5 and +6), there was less DNA extractable, as was the case with yellow leaves too. The yield was the lowest in the oldest senescing leaf +11. No DNA pellet was obtained for senescing yellow leaf +8 (Table 29).

The DNA gel showed that there was no significant difference between the DNA of control and YCS green leaves. In the case of the yellow YCS leaves however, degradation of the DNA was clearly visible commencing at leaf +2 (Figure 88). The green YCS leaves showed DNA degradation from leaf +4 onwards. All the senescing leaves showed DNA degradation with leaves +9 and +11 showing highly degraded low molecular weight DNA.

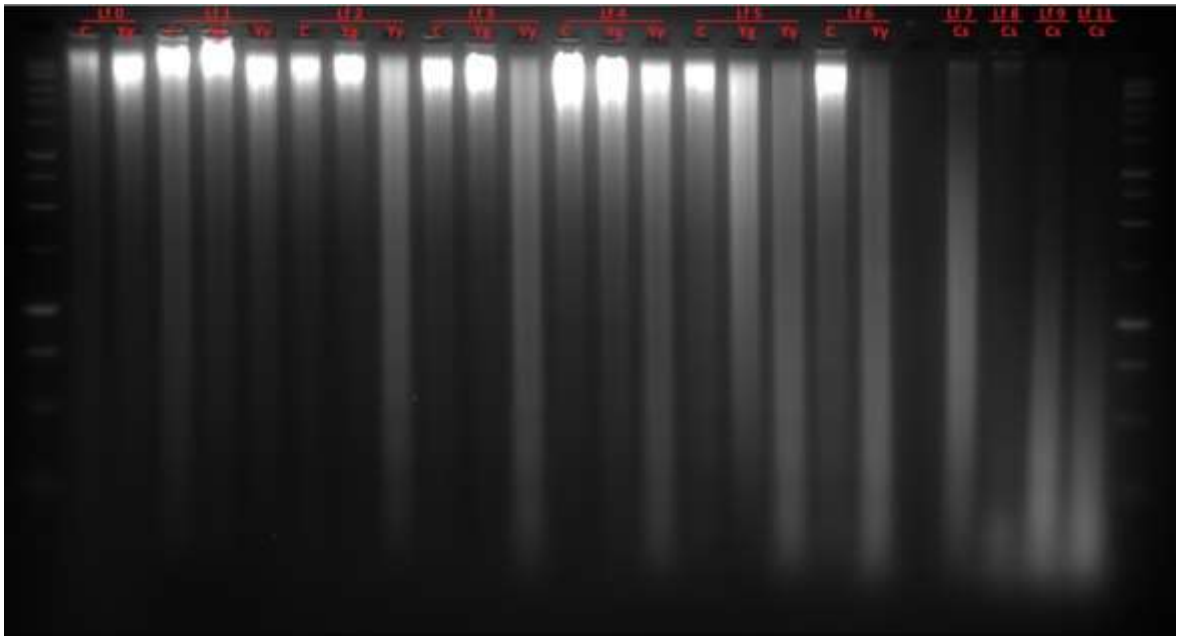


Figure 88 DNA gel showing the quality of DNA extracted from healthy, YCS affected green and yellow leaf sections as well as naturally senescing Q208$^{\Phi}$ leaves +7 to +11.

Summary

None of the DNA samples showed laddering. Thus, the likelihood of YCS and leaf yellowing being the result of PCD is unlikely.

6.5.3.3 Western blots for key enzymes in starch metabolism

Two key enzymes responsible for the metabolism of starch- ADP glucose pyro phosphorylase (synthesis of starch) and α-amylase (degradation of starch) have been studied.

Polyclonal antibodies to these enzymes were used to perform Western blots. Optimisation of methods for protein extraction, electrophoresis and Western blots were first performed before the samples could be analysed (see section 5.6.9 of this report).

Western blot for ADP glucose pyrophosphorylase (AGPase) which catalyses the reaction of glucose-1-phosphate with ATP to form ADP-glucose, which is the first step in the starch synthesis pathway. The YCS affected Q208$^{\Phi}$ samples used for the blot were collected from Mackay (24-25 Feb 2015), from Joe and Paul's Schembri's farm, while the asymptomatic healthy controls were from the Mackay Productivity Services demonstration plots (Table 30). Both groups of plants were of a similar age. Leaves were sampled early in the morning and placed directly into liquid nitrogen. Samples were stored at -80°C until ready for analysis.

The extracted proteins (10 µg) were run on PAGE-SDS gels, transferred to nylon membrane and probed with polyclonal antibodies against AGPase.

Table 30 Samples used for protein gel and Western blot analysis

Name	Variety	Type	Rep	Leaf no. (FVD = 1)
MC6	Q208	Healthy, asymptomatic	2	1
MC7	Q208	Healthy, asymptomatic	2	2
MC8	Q208	Healthy, asymptomatic	2	3
MC9	Q208	Healthy, asymptomatic	2	4
MC10	Q208	Healthy, asymptomatic	2	5
MY6	Q208	YCS symptomatic	2	1
MY7	Q208	YCS symptomatic	2	2
MY8	Q208	YCS symptomatic	2	3
MY9	Q208	YCS symptomatic	2	4
MY10	Q208	YCS symptomatic	2	5

Following transfer to the nylon membrane using the iBlot, the gel was stained with Coomassie blue to show that the loading was similar in all the wells (Figure 89).

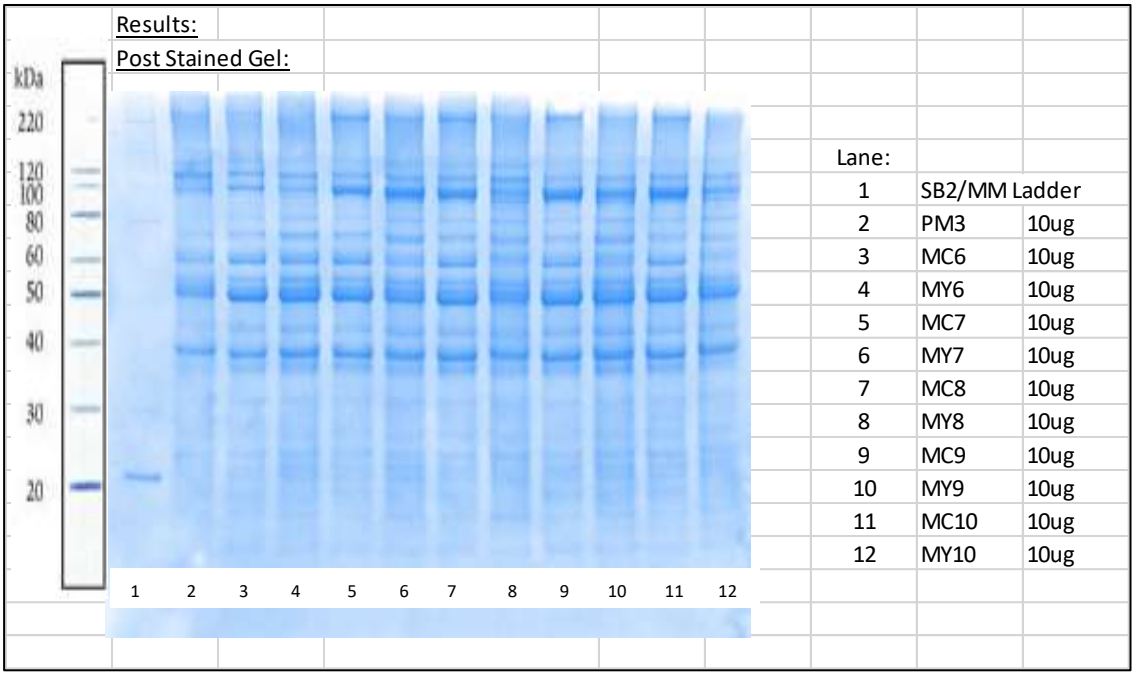
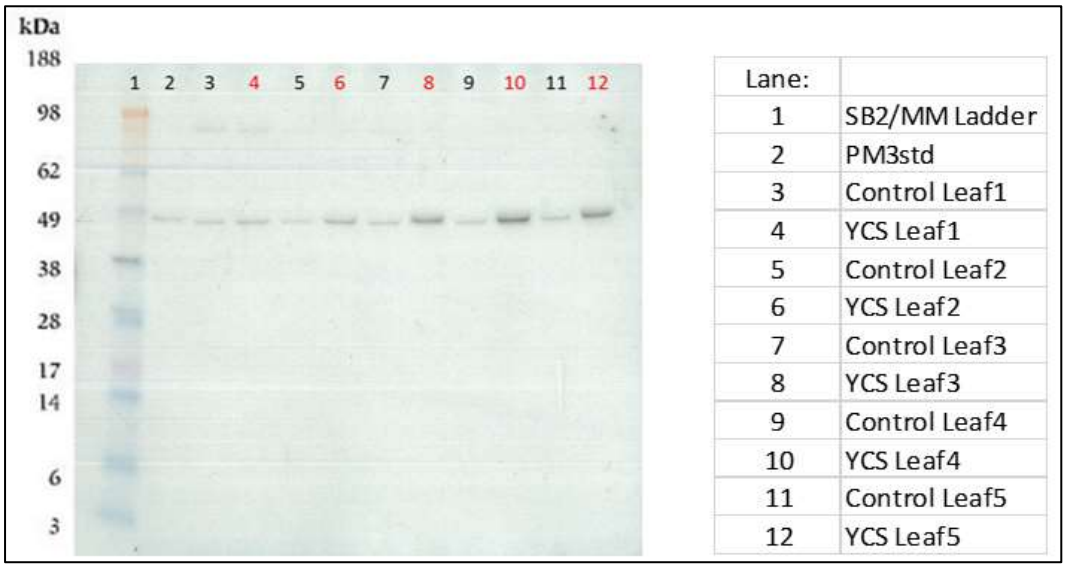


Figure 89 Coomassie stained protein gel to show loading was similar in all samples. Control sample PM3 extracted 2.11.16

A Western blot was performed as per the protocol recommended by the manufacturer of the antibody. Size of the protein was as expected ~50kDa. Results showed that in the YCS samples, there was more AGPase than in the asymptomatic healthy controls in all the leaves tested (Figure 90). This difference increased with the leaf age, becoming more pronounced from Leaf +2 onwards.



Lane:	
1	SB2/MM Ladder
2	PM3std
3	Control Leaf1
4	YCS Leaf1
5	Control Leaf2
6	YCS Leaf2
7	Control Leaf3
8	YCS Leaf3
9	Control Leaf4
10	YCS Leaf4
11	Control Leaf5
12	YCS Leaf5

Figure 90 Western blot showing the increased amount of the ADPglucose pyrophosphorylase in the YCS samples (shown in red numbers), detectable from leaf1 onwards

Our results showed that in the YCS samples, there was more AGPase than in the asymptomatic healthy controls in all the leaves tested. This difference increased with the leaf age, becoming more pronounced from Leaf+2 onwards (Figure 90).

Here, we describe the results we obtained for α-amylase on the same Q208$^{\phi}$ samples.

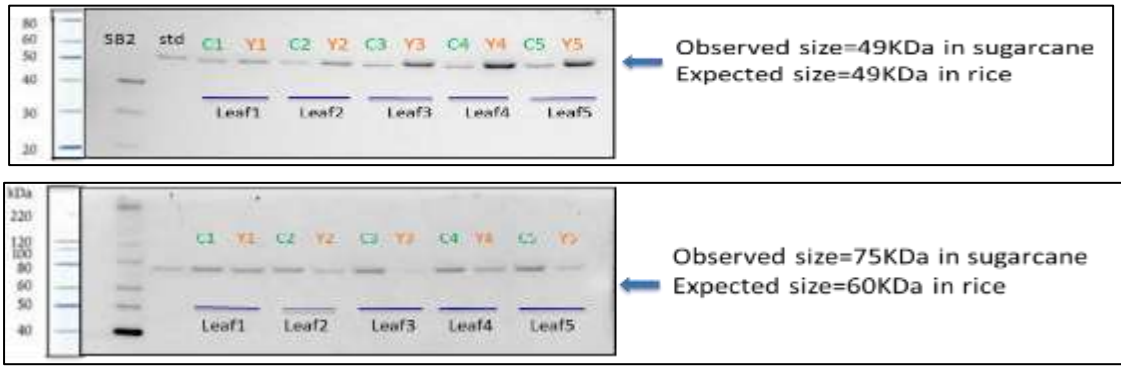


Figure 91 Western blot analysis of Q208^{A} leaf proteins for ADP glucose pryophosphorylase (top) and α-amylase (bottom) in control asymptomatic (C) and YCS (Y) affected plants

Our Western results showed that there is an increase in the levels of ADP glucose pyrophosphorylase in YCS affected leaves of Q208$^{\phi}$. Thus, an increase in synthesis accompanied by a decrease in breakdown would lead to an increased amount of starch in YCS leaves (Figure 91).

Antibodies to the following proteins (PR-1, peroxidase, PPO and dehydrins) were obtained and tested in Q208$^{\phi}$ to further characterise some of the biochemical changes, and provide more information on the nature of YCS (i.e. abiotic or biotic stress) (Figure 92). In addition, we have performed Western blots for other proteins, which are known to accumulate in response to abiotic/ biotic stress. These include antibodies specific for the pathogenesis-related protein (PR-1), polyphenol oxidase, peroxidase and dehydrin.

Pathogenesis Related Protein-1: Trend is increased levels of expression in YCS samples - leaf1 to 4. Leaf 5 seem to be lower intensity in the YCS sample.

PPO: Trend is increased intensity in YCS samples - Blurring together of bands, with no clear band being upregulated.

Dehydrin: Trend is decreased intensity of bands in the YCS samples.

Peroxidase: Trend is decreased intensity in YCS samples, especially in leaf +2 and leaf +3.

Thus, as the proteins, which are correlated with abiotic stress response (e.g., dehydrin and peroxidase) showed a trend to decrease in YCS samples; we can conclude that YCS is not abiotic in nature. Similarly, the PR-1 and PPO proteins showed increased band intensity, suggesting that YCS may be biotic in nature.

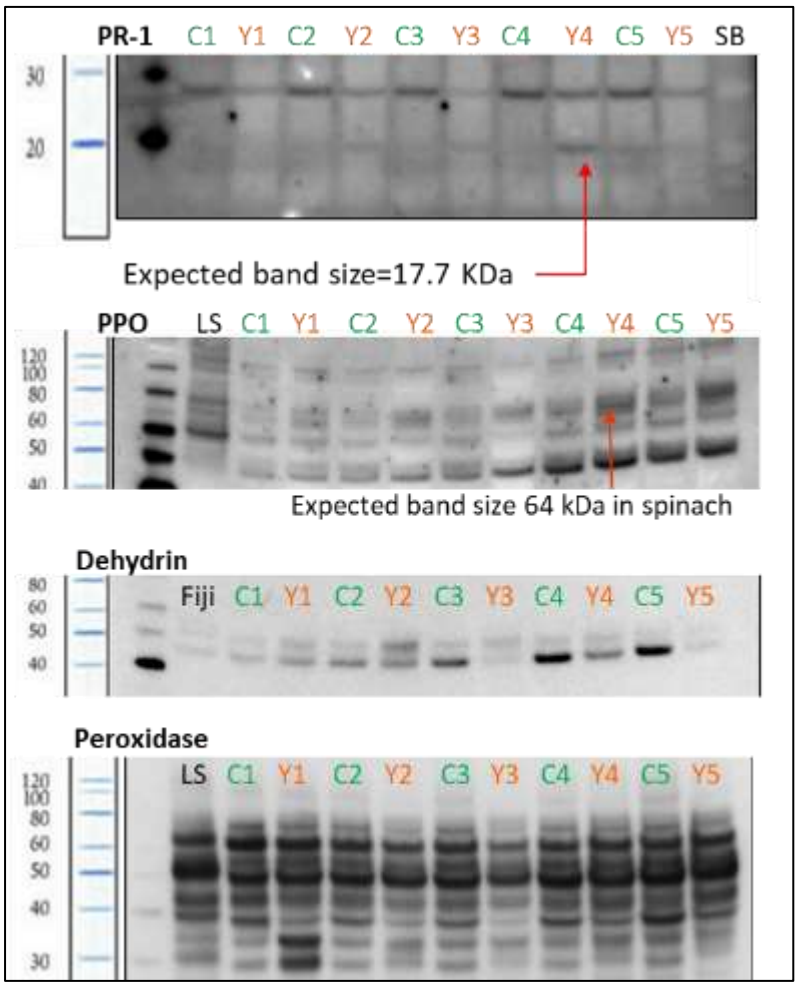


Figure 92 Q208$^{\Phi}$ Western blots probed with antibodies for PR-1, PPO, Dehydrin and Peroxidase proteins. SB=Sea Blue molecular marker; LS=leaf scald positive control; Fiji=Fiji disease positive control; C=asymptomatic control; Y=YCS symptomatic plant

Summary

In Q208$^{\phi}$, the accumulation of starch in older leaves correlated well with an increase in synthesis accompanied by a decrease in breakdown of starch, especially observed in leaf +2 and older. This result along with that of increased activity of PR-1 and PPO proteins, suggest the causal agent may be biotic in nature. However, the Western blots for the other proteins that we tested were not as clear, with trends suggesting an involvement of a biotic agent in the YCS plants. Further work using monoclonal antibodies may provide a clearer solution.

6.5.3.4 Starch

Preliminary research in pilot project 2013/087 indicated there was hyperaccumulation of starch in older leaves of YCS affected KQ228$^{\phi}$ plants. This research work was continued in this new project, with the goal to understand the reason for this starch accumulation with a view to elucidating the causal agent and develop a hypothesis on the mechanism involved.

Starch accumulation

This was first observed in the bundle sheath cells of leaves of young tillers of YCS cane. This was then verified in leaves of the mature cane, which was also found to accumulate starch (Figure 93 A, B).

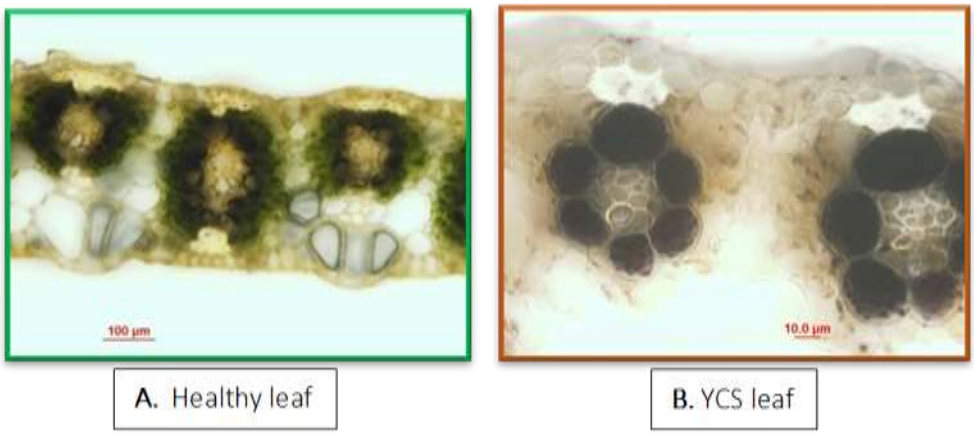


Figure 93 Leaf sections of healthy (A) and YCS infected (B) KQ228A plants stained with Lugol's reagent for presence of starch

Taking good hand sections is time consuming and requires fresh tissue, which is not practical when collecting samples from remote regions in north Queensland, requiring several days before samples can be processed at the Indooroopilly laboratory. Thus, a protocol to study starch accumulation was needed to be developed.

Leaf tissues have chlorophyll which masks colour development in response to a stain. To overcome this problem, it is essential to bleach or clarify the leaf tissues, prior to staining.

Two methods were tested:

1. Boiling in 95% ethanol over a prolonged period (~1hour)
2. Autoclaving in 95% ethanol (15min), followed by autoclaving in alkali (15min) and finally suspending the tissue in bleach (overnight).

Both methods worked well, but method 1 required constant attention; and a maximum of 24 samples (capacity of the heating block) could be processed at a given time.

Method 2 was simpler to perform and a large number of samples could be processed in the same run. In addition, tissue clarification was uniform. This method worked well for hand cut sections of leaf tissue too (Figure 94 A & B). We were also successful in sampling leaf punches using a paper punch, which made the sampling size uniform. In addition, frozen, as well as dry samples (oven dried or over $CaCl_2$) could be used with this method.

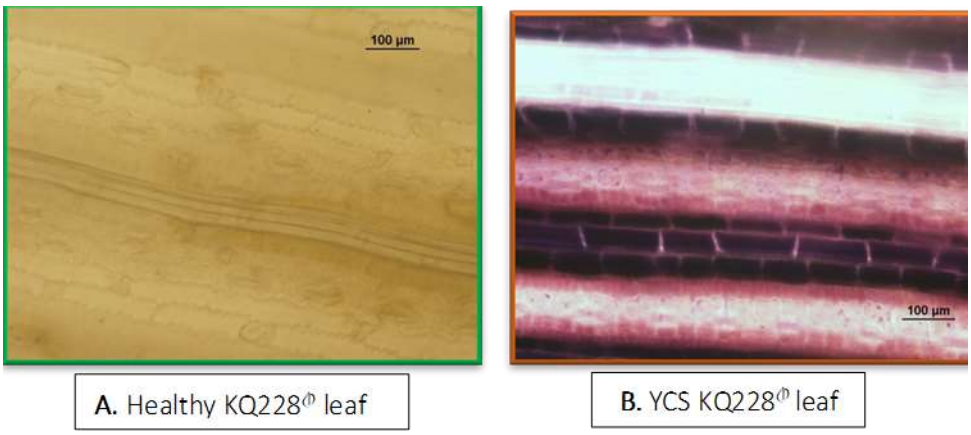


Figure 94 Clarified leaf sections of KQ228A stained with Lugol's reagent for the presence of starch (starch stains purple). Note the total lack of purple colour in the healthy samples (A) while the YCS samples show strong colouring due to the presence of starch in the cells

Using this method, we showed that punches of leaf +4 taken early in the morning <9am showed a clear difference between YCS and control asymptomatic plants and could be used to develop a diagnostic tool for YCS. The sheath did not show this clear distinction (see section 6.10.4 of this report).

Iodine staining appeared to show varietal staining (Figure 95). However, starch synthesis is tightly aligned with sucrose synthesis (Stitt and Quick, 1989). Studies show that as sucrose levels rise in the source leaf there will be carbon partitioning to starch through the triose phosphate transporter and will rise and fall with the diurnal rhythm (Du et al., 2000; Weise et al., 2011). In YCS leaves with impeded sucrose translocation there is downregulation of the triose phosphate transporter and which results in carbon retention in the chloroplast (Marquardt, 2019; Scalia et al., 2020). Therefore, the observed starch staining differences between varieties is likely due to leaf sucrose levels. Leaf quantification studies of both these metabolites are well investigated in YCS (Marquardt et al., 2019; Scalia et al., 2020).

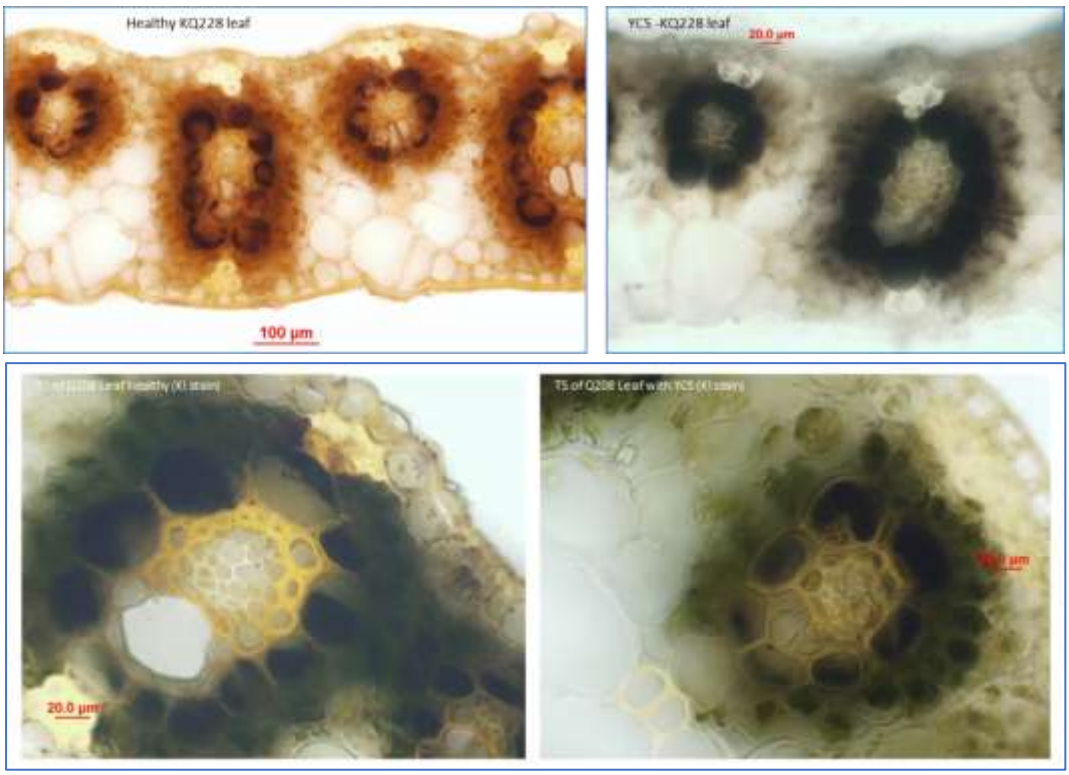


Figure 95 Leaf sections of healthy and YCS leaves of KQ228^{A} (top panel) and Q208^{A} (bottom panel) stained for starch

Symptom development

A holistic approach was adopted to study YCS symptom development in the whole plant including observations and analysis of the development of chlorosis and starch accumulation. Molecular studies of starch metabolism enzymes were discussed in section 6.5.3.3 of this report.

All data was generated from samples collected over two visits to Mackay in Feb and March 2015. YCS samples were collected from the farm of Joe & Paul Schembri's (Q208$^{\Phi}$ – 2nd ratoon) and the healthy plants from the Q208$^{\Phi}$ (Plant crop) at MAPS farm, at Victoria Plains. Both crops were of a similar age.

All the leaves from leaf +1 = first visible dewlap (FVD) as well as the furled leaf (0) were collected and used for starch studies. The appearance of the leaves at harvest are shown (Figure 96).

Figure 96 Q208$^{\phi}$ leaf 0 to +6 from healthy asymptomatic and YCS affected plants

Starch accumulation

Each leaf was sampled in four regions 10cms apart, starting from the tip end. Segments were labelled (A to D) from closest to the furthest from the tip respectively (Figure 97). The entire lamina (2cm) including the midrib region was taken, bleached and stained for starch using the protocol developed at SRA.

Results showed that starch accumulation in healthy asymptomatic leaves was minimal in the younger leaves with more accumulation occurring in the sections closest to the basal region of the leaf (section d) (Figure 97). In the YCS affected plants however, starch commenced to accumulate in the region closest to the tip (section a) in the youngest leaves (leaf 0); with progressively more regions showing accumulation in older leaves. In leaf 3 and older, starch accumulated in all the four sections (Figure 97).

Thus, in YCS affected Q208$^{\phi}$ plants, starch accumulation followed the age of the cells with older cells present in the tip region showing more starch accumulation than younger cells closer to the base of the leaf. Furthermore, starch accumulation in YCS leaves which were either green or chlorotic in the same leaf, behaved identical irrespective of leaf colour, and all accumulated starch (Figure 97).

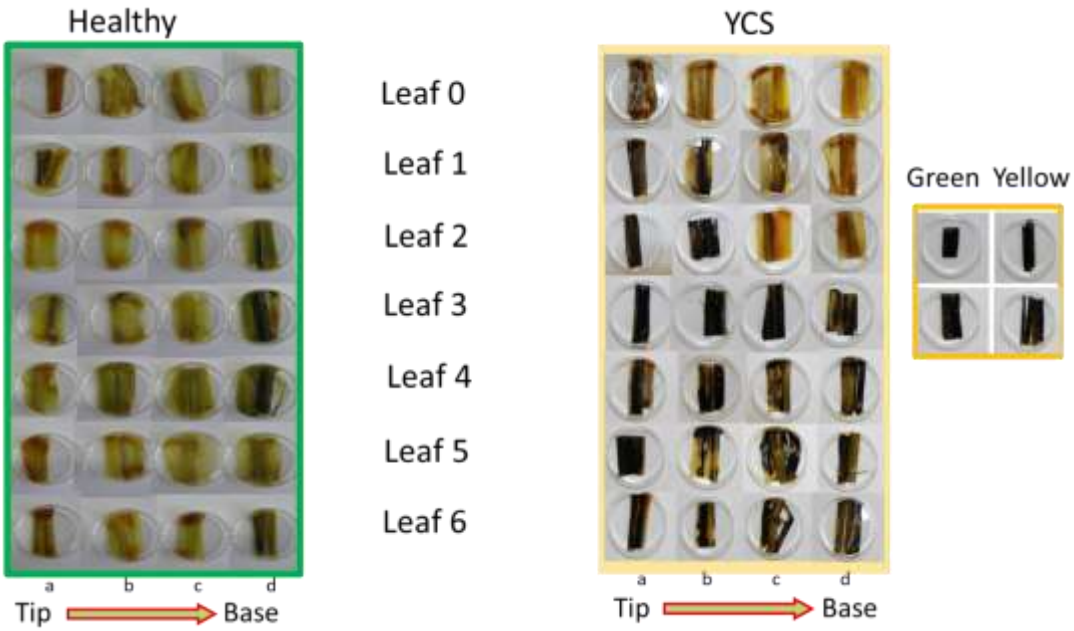


Figure 97 Starch accumulation in Q208$^{\phi}$ YCS symptomatic and asymptomatic leaves collected in Mackay

Starch levels in naturally senescing leaves were also investigated. Senescent leaves of field grown Q208$^{\phi}$ plants showed no starch accumulation, indicating that in normal senescence, the starch is mobilised out of senescing leaves.

Heat Stress and carbohydrate analysis

Scientists at the SRA Brandon station performed an experiment to test if YCS–like symptoms could be induced, following heat stress treatment on 3-month-old KQ228$^{\phi}$ sugarcane plants growing in pots (see section 6.3.2.1 of this report). Plants had approximately 6-8 leaves and were maintained outside (in an area near the tunnel houses in SRA Brandon), and no artificial light was given.

Only during the heat treatment (44^{0}C) were plants moved into the incubator. Treatment was commenced at 11am, and the duration of treatment was 3 hr/day (treatment period was 2 days). None of the leaves showed any yellowing prior to commencement of the heat stress treatment. Rapid yellowing of lower leaves started ~2 days after the treatment while the upper leaves remained green (Figure 98). This experiment was repeated at 42^{0}C and similar results were obtained.

Figure 98 KQ228$^{\Phi}$ potted plant appearance 2days after heat stress treatment

Starch accumulated in asymptomatic leaves 1 and 3 before any yellowing was evident. In symptomatic leaves +5 and +6, starch accumulated in sections taken closer to the base of the leaf but not in the ones near the tip of the leaves (Figure 99, Figure 100). Sucrose levels were highest across the entire leaf +1 and +3. In leaves +5 and +6, the sugars were more variable with a higher concentration of sugars closer to the base of the leaves than the tip (Figure 100).

Figure 99 Regions of the leaves of heat stress plant sampled (a to h) for starch (red) and sugars (blue)

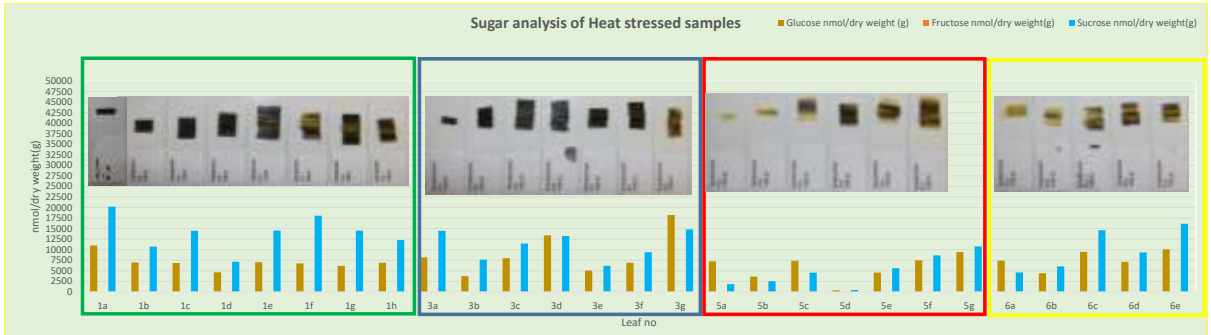


Figure 100 Sucrose and hexose profiles from tip to base (a-h) of heat stressed leaves (1, 3, 5 and 6). The starch test results of each of these samples is inserted above the graph

Starch in senescing leaves of Q208^{A}

Q208$^{\Phi}$ leaf samples were collected from Bundaberg Sugar Services filed in June 2015. Three stalks were selected, and the two oldest mature almost senescing leaves still attached to the stalks were

sampled (example 1A and 1B) (Figure 101 A). These leaves were placed on ice and brought back to Brisbane and analysed for starch. The aim was to study starch behaviour in naturally mature, almost senescing leaves in the field.

Figure 101 Method of sampling leaves from each of the three stalks, and within the leaf - top panels. Appearance of two consecutive mature (from top down), senescing leaves of Q208$^{\Phi}$ stalk 3, sampled for starch analysis - bottom panels

Starch analysis showed that in field grown mature cane, the mature older senescing leaves do not retain starch (Figure 102). Starch is translocated out of the leaves, with very little starch remaining at the early morning sampling period.

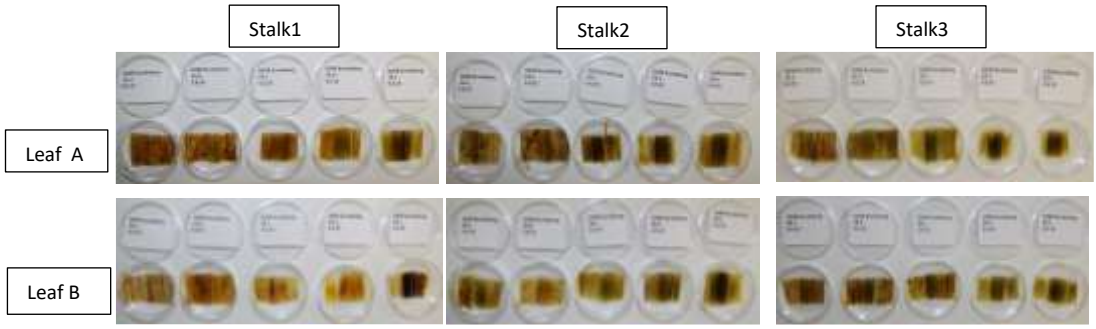


Figure 102 Starch assay in senescing leaves of mature stalks of field grown Q208$^{\Phi}$ at Bundaberg. Upper senescent leaf A) and lower senescent leaf B)

Whole Leaf, whole canopy staining

Iodine dye staining of a whole leaf of KQ228$^{\Phi}$ showed a strong correlation between starch load and YCS symptoms (Figure 103, Figure 104). We hypothesize that in YCS affected plants starch accumulates in the major exporting source leaves (leaf +3 to +6), and that upper mid-canopy (leaf +1 and +2) with reduced photosynthetic yield will mostly remain starch free. This concurs with studies by (Scalia et al., 2020).

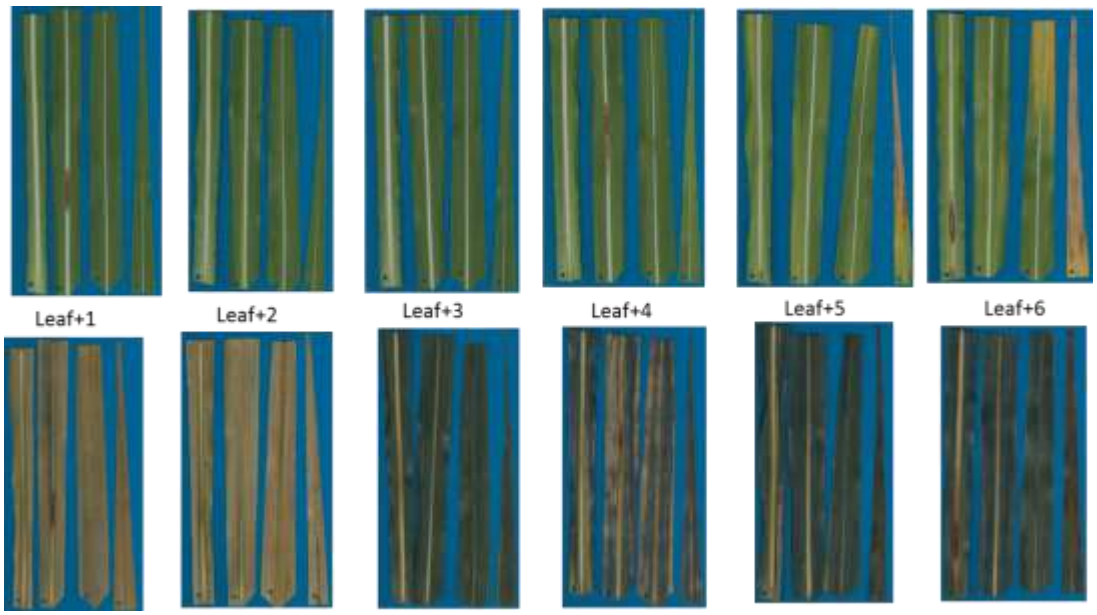


Figure 103 Whole Leaf starch iodine dye test on KQ228^{A} leaves taken from an YCS symptomatic block on 24th March 2016. Leaves +1 to +6 are shown. Top images show leaves before staining while bottom images show the same leaves after staining. Leaf >+3 showing YCS symptoms

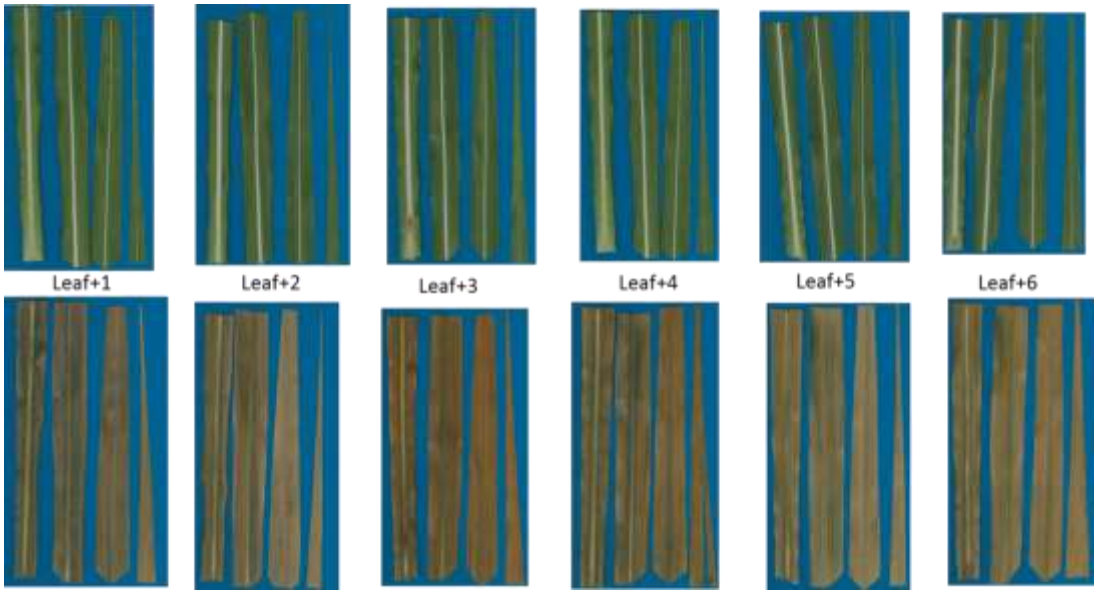


Figure 104 Whole Leaf starch iodine dye test on KQ228$^{\Phi}$ leaves taken from an YCS asymptomatic block on 24th March 2016. Leaves +1 to +6 are shown. Top images show leaves before staining while bottom images show the same leaves after staining. Leaves +1 to +6 showing no YCS symptoms

YCS Recovery

Dye tests showed starch accumulation when YCS symptoms are present and we questioned whether this was still the case when those plants had greened-up later in the season. To test this, we resampled previously symptomatic cane later in the season when it had appeared to be asymptomatic and fully recovered (Figure 105 A). Dye tests showed an absence of starch

accumulation in early morning consistent with asymptomatic leaves (Figure 105 B). From a starch perspective, it appeared our plants had recovered. This suggests, for the first time, that YCS may be a transitory condition.

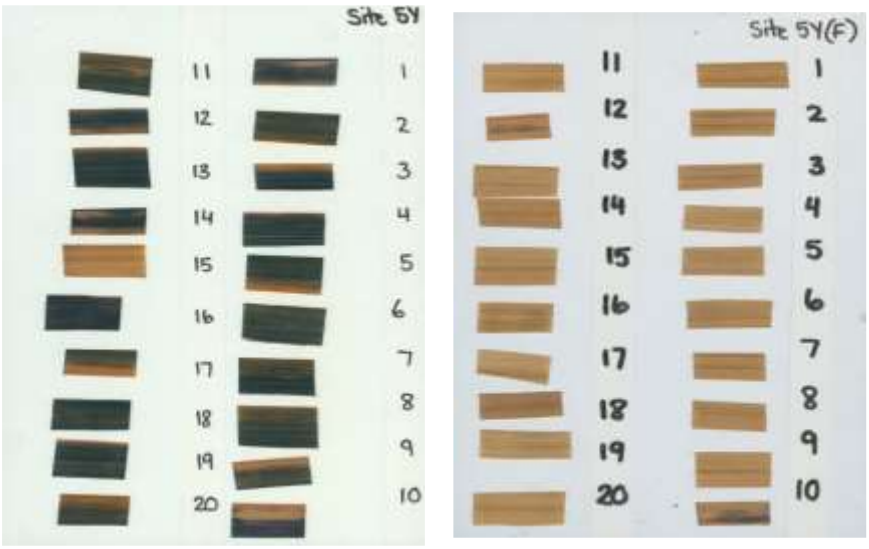


Figure 105 YCS Recovery. Starch dye stains of symptomatic (A) and asymptomatic (B) leaves. All leaves are Leaf +4 and were collected in early morning. Twenty individual plants are shown. The same plants were sampled on 19-Feb-16(A) and 6-May-16(B)

Summary

Starch accumulation is correlated with the age of the cells as well as age of the leaf in YCS plants. Older cells and older leaves in YCS affected plants also accumulate more starch than the younger leaves/cells. In contrast, heat stress induced more starch accumulation in younger leaves compared to the older leaves in pot trials. Unlike the retention of starch in older leaves, senescing leaves of field growing cane showed no accumulation of starch. This is most likely due to mobilisation of carbohydrates out of the leaf prior to death of the leaf. The results presented here indicate that the detection of pre-dawn starch in leaves of sugarcane could be a potential candidate for a diagnostic tool for YCS. This research has enabled the development of the starch mid-rib test for YCS (Scalia et al., 2020). Finally, the accumulation of starch in YCS symptomatic leaves is absent when the plant has recovered from YCS and leaves are green, which suggests that YCS may be a transitory condition developing in response to a trigger which may be a combination of biotic and/or abiotic plant stress.

6.5.4 Clonal variation physiology and YCS

This trial was established on 27 August 2015 in the Burdekin. After harvesting the 1R crop on 26 July 2015, the first irrigation, nutrient application and weed control practices were completed. The experiment was designed with water application treatment as the main treatment and 20 test clones as sub treatment. The water treatments were applied on 27 September and irrigation was

completely stopped for the drought treatment. Irrigated treatment received a rate of 22mm/100 mm while semi irrigated treatment received only 50% (see Appendix 1: 1.2.10).

6.5.4.1 Establish base level physiological functions in active green leaves of 20 clones

Weather data was recorded using an automatic weather station installed less than 200 meters from the trial site. Temperature, relative humidity, radiation, wind speed and rainfall were logged hourly (Figure 106).

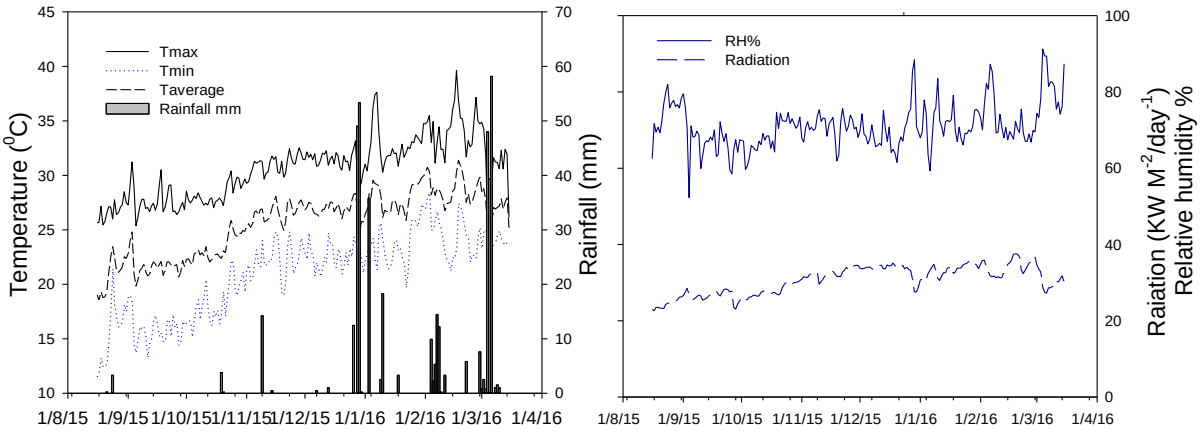


Figure 106 Weather data; temperature ^{0}C (maximum, minimum and average), rainfall (mm), relative humidity % and radiation during the period of August 2015 to March 2016

Soil moisture

Soil moisture variation during the crop cycle was characterized in the rainfed, half irrigated and irrigated treatments using enviro scans installed in each replicate (Figure 107). In the rainfed treatment, soil moisture was measured to the depth of 2m whereas in half irrigated and irrigated treatments up to 1.5 and 1 m depths, respectively (Figure 107).

In addition, soil water content in each plot of the rainfed treatment was measured using a Neutron moisture gauge through aluminium access tubes installed in the middle of each plot. Moisture content was measured at 20 cm intervals stating from 20 cm below the soil to a depth of 300 cm. Neutron scatter counts were converted to a count ratio based on a daily water count and a calibration equation was used to predict volumetric moisture content at each depth. Total soil water for each plot was calculated as the product of the measurement interval (20 cm) and the sum of individual volumetric moisture level at each measurement interval. The soil moisture variation during the drought period for each variety was estimated (Figure 108). There was no variation ($p>0.05$) among clones for water extraction during the observation period when most YCS was observed.

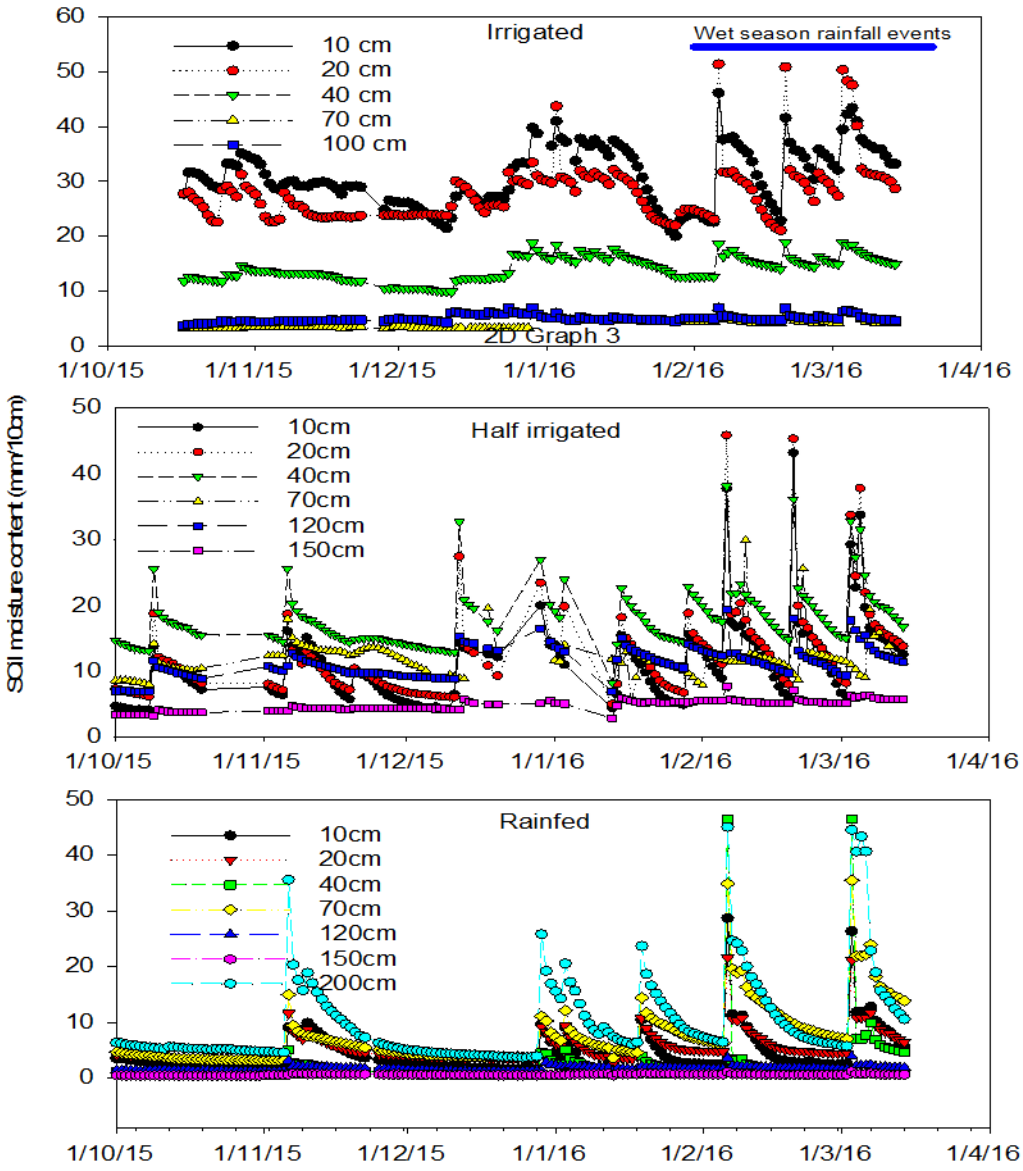


Figure 107 Soil moisture profiles (0 to 200cm depths) of 3 water treatments during the experimental period at Brandon.

Mid-trial sampling and measurements

Biomass harvest at 6 months stage in December 2015

The early biomass sampling was conducted on 17 December 2015 to estimate the biomass losses in clones in different water stress conditions imposed in the experiment. In total 16 shoots were sampled from the two guard rows and the total weight was recorded. A sub sample of 3 stalks were taken to estimate the total dry matter and the leaf area of each clone. There were no visible YCS-like symptoms at the time of biomass sampling. However, there was a significant variation ($P<0.05$) among 3 water treatment for biomass at 6 m stage. The average shoot dry weight of the irrigated, half irrigated and rainfed treatments were 96.61g, 88.3 and 73.1g, respectively. There was a 24% biomass reduction in the rainfed treatment when compared with irrigated treatment. There was no clone-by-water treatment interaction for the early biomass.

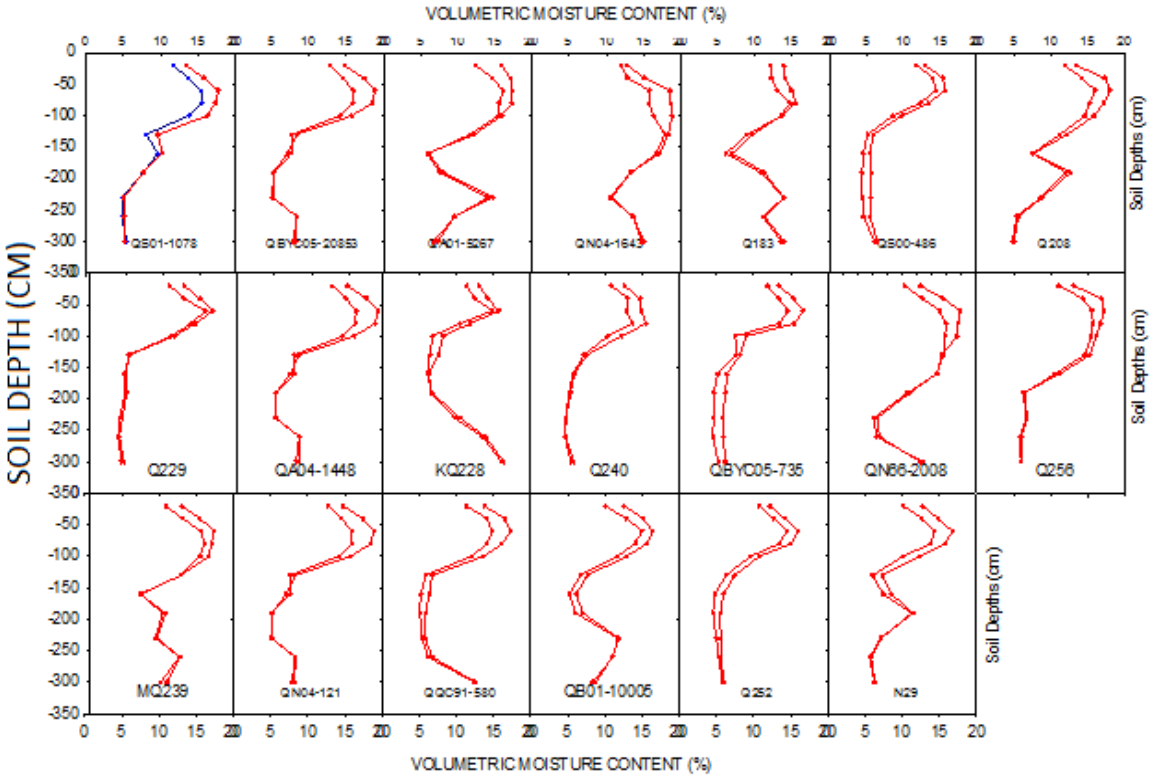


Figure 108 Soil moisture variation measured with neutron moisture meter (NMM) between 1 November 2015 (2nd line) and 2 December 2015 (first line) among 20 clones before the on-set of monsoonal rain in January 2016.

The average leaf area per shoot was measured in the sub samples. As observed for biomass, the treatment difference for leaf area was significant ($p<0.001$). There was a 23% leaf area reduction in rainfed treatment when compared to irrigated treatment. The clone variation was significant ($p<0.002$). However as seen in biomass, there was no clone–by-treatment interaction for leaf area.

The early biomass sampling after prolong drought period in the rainfed treatment in December 2015 and the root water depletion during the most stress period (Figure 108) clearly showed the severity of drought in the experiment.

Physiological observations

Leaf gas exchange was measured on the third fully expanded leaf (leaf +3) on tagged 2-3 plants in all three treatments and 3 replicates (Table 31). Measurements were taken in October 2015 when the moisture stress in rainfed treatment was mild to moderate. Photosynthesis (A), stomatal conductance (gs) and intercellular CO_2 concentration to ambient CO_2 concentration ratio (Ci/Ca) were measured using Licor 6400xt infra-red gas analyser and portable photosynthesis system. The Intrinsic transpiration efficiency (iTE) of the leaf samples were calculated as the ratio of A and gs. There was a significant difference ($p<0.05$) between 2 water treatments (while moderate stress in the rainfed treatment) for photosynthesis (21% difference) and conductance (31% difference). There was no clone–by-treatment interaction for photosynthesis. MQ 239 maintained highest

photosynthesis under rainfed while QA04-1448 had the lowest. There was no clone difference for gs and internal CO_2 (Ci) at this stage

Table 31 Gas exchange (leaf +3) observations of 20 clones in irrigated (non-stressed) and rainfed (mild stress) treatments.

Clones	Photosynthesis (mmolm-2 s-1)			Stomatal conductance ($molm^{-2}$ s^{-1})			Internal CO2 (Ci)($molm^{-2}$ s^{-1})		
	Irrigated	**Rainfed**	**Mean**	Irrigated	Rainfed	Mean	Irrigated	Rainfed	Mean
CT05-735	40.0	25.6	32.8	0.46	0.27	0.37	124.5	176.7	150.6
CT05-853	37.5	31.5	34.2	0.41	0.34	0.37	140.7	67.9	100.2
KQ228	37.4	32.0	34.4	0.52	0.34	0.42	138.2	137.3	137.7
MQ239	40.4	42.3	41.4	0.42	0.55	0.49	97.3	177.4	141.8
N29	35.2	27.3	31.7	0.37	0.30	0.34	81.4	196.7	132.7
Q183	27.7	27.6	27.6	0.26	0.26	0.26	138.0	108.0	121.3
Q208	38.6	26.1	31.6	0.48	0.27	0.36	139.3	160.8	151.2
Q229	40.8	32.0	35.9	0.62	0.34	0.46	136.3	170.2	155.1
Q240	39.2	25.7	31.7	0.43	0.24	0.32	127.0	154.4	142.2
Q252	33.9	26.8	29.9	0.34	0.27	0.30	148.3	159.0	154.2
Q256	39.0	25.3	31.4	0.38	0.26	0.31	130.5	177.6	156.7
QA01-5267	31.3	24.5	27.5	0.29	0.24	0.26	142.5	99.7	118.8
QA04-1448	38.8	21.0	28.9	0.61	0.19	0.38	182.8	153.9	166.8
QB01-5	37.4	27.3	31.8	0.36	0.25	0.30	128.7	165.4	149.1
QC91-580	36.8	32.7	34.3	0.44	0.30	0.36	149.9	119.2	131.5
QN04-121	35.0	32.0	33.1	0.46	0.27	0.34	135.5	95.3	109.9
QN04-1643	38.6	27.1	32.2	0.43	0.27	0.34	134.0	82.3	105.3
QN66-2008	33.6	27.3	30.1	0.34	0.28	0.31	117.8	180.8	152.8
QS00-486	34.4	24.8	29.1	0.32	0.24	0.28	136.7	168.0	154.1
QS01-1078	33.4	29.2	31.1	0.40	0.26	0.32	158.6	168.0	69.8
Grand Total	**36.5**	**28.5**	**32.1**	**0.42**	**0.29**	**0.34**	**133.6**	**135.9**	**134.9**
Lsd5%	Treatments		2.164			0.057			ns
	Clone		6.723			ns			ns
	CV%		12.66			ns			ns

Canopy Photosynthesis

Photosynthesis (A), stomatal conductance (gs) and internal CO_2 (Ci) were measured in December (5 months) in all the green leaves in the canopies of 20 clones to establish the benchmark leaf level photosynthesis in leaves 0 to +7 (Figure 109). Generally, in sugarcane, the average Ci/Ca ratio is higher in young leaves than the mature leaves. There was a significant variation among clones for A, gs and Ci/Ca ratio. The physiologically matured leaves (leaf + 3 and +4) showed the highest (28%) C fixation contribution to the canopy than other leaves. The upper mid-canopy leaves (leaf +1 and +2) had an average of 13% contribution, whereas the green leaves at the base of the canopy (leaf +7) had the lowest which was <10% of the total canopy photosynthesis. In general, the leaf canopy below the leaf +4 contributed approximately 15% of the plant total photosynthesis (Figure 110). This contribution could vary among test clones with their canopy size and age.

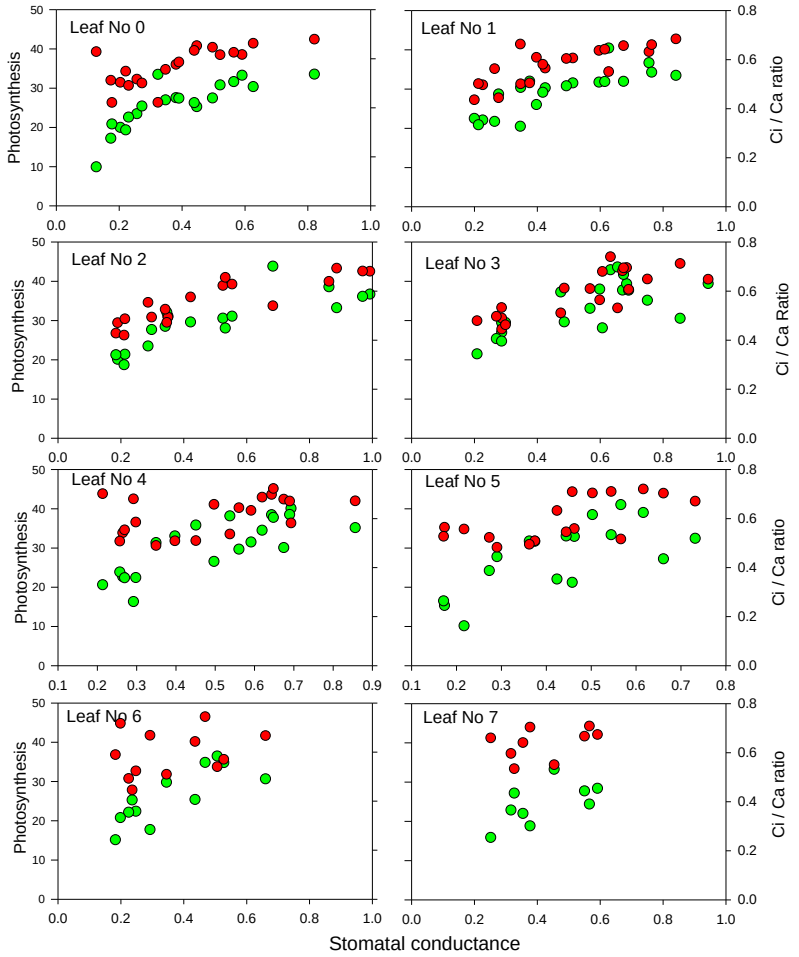


Figure 109 Stomatal conductance (H_20 mol m^{-2} s^{-1}) and its association between Photosynthesis (CO_2 mol m^{-2} s^{-1}) (green symbols), and the ratio of internal and external CO_2 (Ci/Ca ratio) (red symbols) as the reference physiological values for healthy (-ve YCS) leaves in 20 clones at 5 months stage.

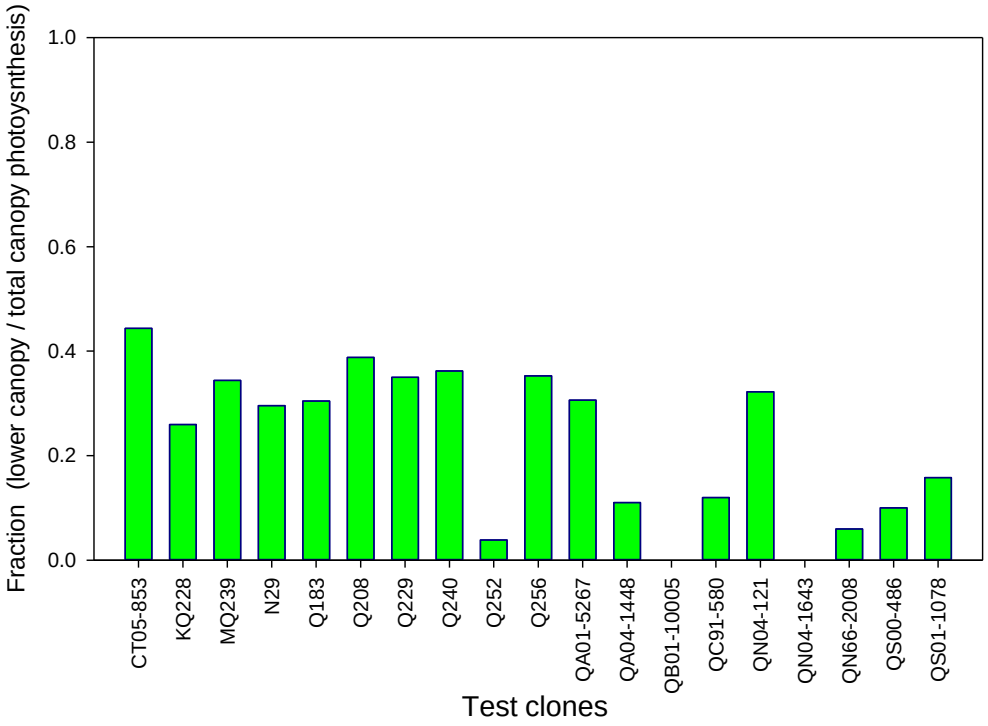


Figure 110 Variation in fractional contribution of photosynthesis of lower canopy below (leaf +4) to the total canopy at 5 months.

Inherent colour discrimination (NDVI) among clones

The canopy colour discrimination was estimated using Green Seeker in the irrigated and drought treatments at 5 month stage. The sensor emits brief bursts of red and infrared light and measures the amount of each type of light that is reflected back from the green canopy. The sensor displays the measured value of the normalized difference vegetation index (NDVI) reading (ranging from 0.00 to 0.99). The strength of the reading is a direct indicator of the greenness of the canopy where higher the reading, the greener the plant canopy. There was no full canopy formation at the time of measurements.

The results suggested a significant variation in NDVI index even with no visible YCS symptoms (Figure 111).

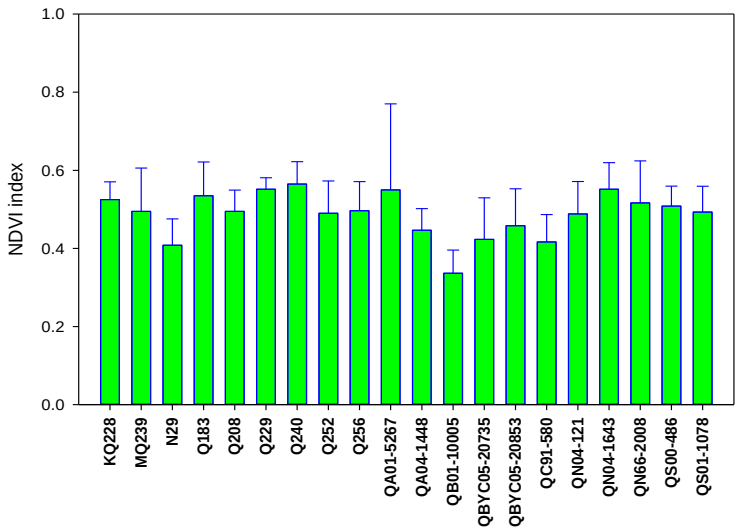


Figure 111 Clonal variation for NDVI index as measured at 5 month stage when the plants are in mild stress condition.

Treatment effects on YCS

The observations were made during the peak period of YCS in the experiment (January to April 2016). From each plot, three stalks with visible leaf yellowing either in top, middle or lower canopy (as defined) were selected from the two middle rows of the plots. The yellow leaf position and the number, and also the leaf position of the first senesced leaf were recorded (**Error! Reference source not found.**). The YCS incidence was expressed as the % of affected leaf in the canopy.

Estimation of leaf level productivity losses due to YCS

Photosynthesis (A) was measured in all green leaves (leaf 0-8) in the canopy well before the YCS occurrence in 20 clones (November) in the fully irrigated treatment (Table 32). The capacity of individual leaves for photosynthesis in the clean canopy was established when there was no reduction in leaf area due to YCS or yellowing.

Fractional photosynthetic capacity (Am) (Table 33) for each leaf was estimated as:

Am = (A/$\bar{A}$x),

where A is the measured photosynthesis on a leaf (1…8), $\bar{A}$x is the mean canopy photosynthesis (n=1,2,…8) of a clone. The average Am values of each leaf across 20 clones were considered as the normalized photosynthetic index of the particular leaf in the canopy of that clone (An).

This index is “0” for the YCS affected leaf/s at the time of observations of the canopy. The total photosynthetic capacity of the canopy was estimated as:

$[\sum_{n}^{1}(A_n)]$ where, n = total number of leaves present in the canopy at the time of observation

The YCS severity of affected canopy was expressed as a reduced photosynthetic capacity of the canopy with YCS affected leaves. These data were statistically analysed to test the treatment, clone and clone–by-treatment interactions (Table 34).

Table 32 The photosynthesis measured on the leaf canopy from the leaf above the top visible dewlap (0) to the last fully green leaf +8 in the well irrigated treatment

Clone	Leaf number (position)									Average
	0	1	2	3	4	5	6	7	8	
KQ228	23.48	28.74	31.08	36.99	22.68	24.21	22.40	19.52	11.71	24.54
MQ239	27.48	31.76	36.73	42.92	30.10	39.04	30.67	18.84	11.30	29.87
N29	33.27	31.96	36.14	37.74	34.52	33.37	15.16	24.39	14.64	29.02
Q183	20.86	22.52	21.40	25.38	23.87	27.76	22.15	15.35	9.21	20.94
Q208	22.61	30.40	21.28	35.85	29.50	31.71	34.80	22.01	13.21	26.82
Q229	31.66	31.56	30.58	40.11	37.71	32.95	36.49	22.88	13.73	30.85
Q240	30.39	33.48	36.57	39.36	38.47	38.51	34.86	27.75	16.65	32.90
Q252	29.94	21.75	21.37	37.27	27.60	24.05	18.78	16.20	9.72	22.96
Q256	25.28	20.51	28.08	35.15	35.19	32.47	17.73	28.41	17.05	26.65
QA01-5267	33.52	30.31	32.12	38.01	31.52	31.79	25.29	15.89	9.54	27.55
QA04-1448	22.24	20.90	27.70	33.08	24.75	21.12	20.01	15.32	9.19	21.59
QB01-10005	25.42	32.01	28.50	21.50	22.39	20.30	15.23	11.20	6.72	20.36
QC91-580	19.37	26.05	23.49	29.64	22.43	16.48	15.23	11.12	6.67	18.94
QN04-121	27.01	40.45	43.84	43.64	38.18	33.06	25.42	23.26	13.96	32.09
QN04-1643	29.20	32.93	34.10	36.27	28.01	22.83	20.12	16.30	9.78	25.50
QN66-2008	27.44	31.90	31.08	30.57	38.53	23.21	19.68	10.13	6.08	24.29
QS00-486	33.57	34.30	43.27	41.74	37.81	21.22	16.35	11.21	6.73	27.35
QS01-1078	26.29	30.82	33.26	28.11	26.55	27.20	23.21	20.10	12.06	25.29
QBYC05-20853	20.00	22.10	20.14	29.46	31.37	41.05	29.81	27.19	16.31	26.38
QBYC05-20735	24.79	32.59	37.27	43.27	31.58	36.08	30.41	21.00	16.30	30.37
Average	**26.69**	**29.35**	**30.90**	**35.30**	**30.64**	**28.92**	**23.69**	**18.90**	**11.53**	**26.21**

Table 33 The fractional photosynthetic capacity (Am) of each leaf in the canopy of 20 clones

Clone	Leaf number (position)									Total
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	
KQ228	0.89	1.09	1.18	1.40	0.86	0.92	0.85	0.74	0.44	8.37
MQ239	1.04	1.20	1.39	1.63	1.14	1.48	1.16	0.71	0.43	10.19
N29	1.26	1.21	1.37	1.43	1.31	1.26	0.57	0.92	0.55	9.90
Q183	0.79	0.85	0.81	0.96	0.90	1.05	0.84	0.58	0.35	7.15
Q208	0.86	1.15	0.81	1.36	1.12	1.20	1.32	0.83	0.50	9.15
Q229	1.20	1.20	1.16	1.52	1.43	1.25	1.38	0.87	0.52	10.52
Q240	1.15	1.27	1.39	1.49	1.46	1.46	1.32	1.05	0.63	11.22
Q252	1.13	0.82	1.81	1.41	1.05	0.91	0.71	0.61	0.37	8.83
Q256	0.96	0.78	1.06	1.33	1.33	1.23	0.67	1.08	0.65	9.09
QA01-5267	1.27	1.15	1.22	1.44	1.19	1.20	0.96	0.60	0.36	9.40
QA04-1448	0.84	0.79	1.05	1.25	0.94	0.80	0.76	0.58	0.35	7.37
QB01-10005	0.96	1.21	1.08	0.81	0.85	0.77	0.58	0.42	0.25	6.95
QC91-580	0.73	0.99	0.89	1.12	0.85	0.62	0.58	0.42	0.25	6.46
QN04-121	1.02	1.53	1.66	1.65	1.45	1.25	0.96	0.88	0.53	10.95
QN04-1643	1.11	1.25	1.29	1.37	1.06	0.87	0.76	0.62	0.37	8.70
QN66-2008	1.04	1.21	1.18	1.16	1.46	0.88	0.75	0.38	0.23	8.29
QS00-486	1.27	1.30	1.64	1.58	1.43	0.80	0.62	0.42	0.25	9.33
QS01-1078	1.00	1.17	1.26	1.07	1.01	1.03	0.88	0.76	0.46	8.63
QBYC05-20853	0.76	0.84	0.76	1.12	1.19	1.56	1.13	1.03	0.62	9.00
QBYC05-20735	0.94	1.24	1.41	1.64	1.20	1.37	1.15	0.80	0.62	10.36
Normalized (A_n)	**1.01**	**1.11**	**1.17**	**1.34**	**1.16**	**1.10**	**0.90**	**0.72**	**0.44**	**8.94**

Table 34 Analysis of variance for the differences in normalized photosynthesis index of canopies with varying degree of YCS among clones in 2 water treatments

Source of variation	DF	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	0.8189	0.4094	0.42	
Treat 1	3.1788	3.1788	3.30	0.11	
Residual	2	1.9276	0.9638	1.62	
Clone 19	192.1823	10.1149	16.99	<.001	
Treat x Clone	19	23.2880	1.2257	2.06	0.014
Residual	76	45.2541	0.5954		
Total 119	**266.6497**				

The differences among clones (Figure 112) and treatment-by-clone interactions ($p<0.05$) were significant. However, there was no difference ($p<0.05$) between irrigated and rainfed treatments for YCS occurrence (7.85 and 8.17).

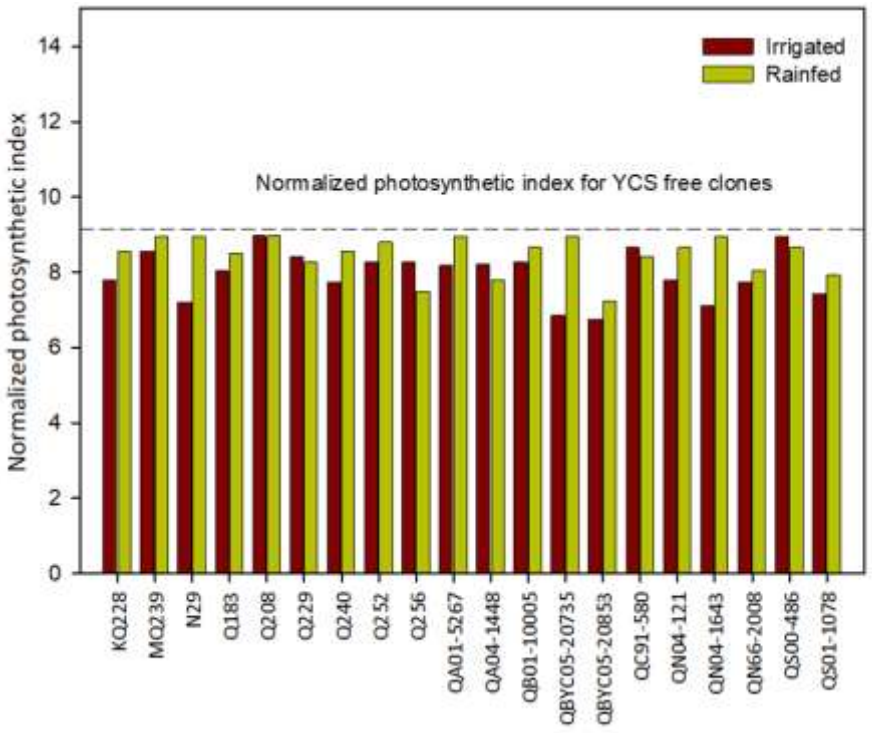


Figure 112 Variation between clones for normalized photosynthesis index under irrigated and rainfed conditions in the clone assessment trial.

YCS prevalence and the reduced photosynthetic capacity was highest in introgression BC1 line, QBYC05-20835 under irrigated and rainfed conditions. Generally, popular commercial clones and some advanced lines had more YCS symptoms on photosynthetic capacity under irrigated conditions than rainfed. Among them, KQ228, Q240A, N29, QN04-1643, QN04-121 had a significant drop in photosynthetic capacity under irrigated conditions. The introgression lines QBYC05-20735 maintained high photosynthetic capacity under rainfed but had a significant drop under irrigated conditions. Almost all the clones tested in this experiment had YCS and Q208$^{(l)}$ had the least effects on the photosynthetic capacity under both conditions.

6.5.4.2 Canopy level YCS occurrence and differences in starch accumulation patterns among clones in clonal evaluation trial

Leaf colour and starch accumulation in nine fully expanded leaves (leaf +1-9) including the senescing leaves and also in the young leaf (leaf 0) above the leaf with the first visible dewlap (FVD) in all clones were tested in all the replicates in the rain-fed and semi-irrigated treatments.

Leaf 0 to leaf +9 were collected and scored individually for YCS prevalence considering the pattern and total area of yellowing. Green leaves with no yellowing were scored as 0 and senesced leaves as 5. Leaves with 5-25% yellowing, 25-50% yellowing, leaves with yellow mottling or drying edges and >60% yellowing were given ratings of 1, 2, 3, and 4, respectively. Ratings for Leaf 0-3 (upper canopy), Leaf 4-6 (mid canopy) and Leaf 7-9 (lower canopy) were used to find out differences in yellowing at canopy level in all 20 clones under different water environments.

Presence of starch was tested using an Iodine test on leaf samples collected shortly before sunrise. Cross sections of 3-5 cm were taken from the mid regions of all the collected leaves and stored frozen until staining (see section 5.6.8 of this report for protocol). Presence of starch leaf sections with no starch was given a score of 0 whereas starch in only one half of the leaf blade (or 50% leaf blade) with or without mid-rib and whole leaf blade were given scores of 1 and 2, respectively.

Clone	Scores given for LLeaf colour and Starch in Leaf 0 - Leaf 9									
	L0	L1	L2	L3	L4	L5	L6	L7	L8	L9
KQ228	0	0	0	2	2	0	0	4	5	5
KQ228	0	0	0	1	1	0	2	2	2	0
MQ239	0	0	0	0	0	0	0	4	1	5
MQ239	0	0	0	0	1	0	0	2	0	1
N29	0	0	0	0	0	4	2	5		
N29	0	0	0	0	0	0	1	0		
Q183	0	0	0	0	0	1	0	2	3	5
Q183	0	0	0	0	0	2	0	2	1	1
Q208	0	0	0	0	0	0	0	5		
Q208	0	0	0	0	0	0	0	0		
Q229	0	0	0	0	0	5	0	4	5	
Q229	0	0	0	0	0	2	0	1	0	
Q240	0	0	0	0	0	0	1	4	4	5
Q240	0	0	0	1	0	0	1	2	2	0
Q252	0	0	0	0	0	1	2	4	5	
Q252	0	0	0	0	0	2	2	2	1	
Q256	0	0	0	0	0	1	0	3	4	5
Q256	0	0	0	0	0	1	1	1	1	0
QA01-5267	0	0	0	0	1	0	0	0	4	5
QA01-5267	0	0	0	0	2?	0	0	0	0	0
QA04-1448	0	0	0	0	1	5	5	5		
QA04-1448	0	0	0	0	-	2	2	0		
QB01-10005	0	0	0	0	0	0	2	4	5	
QB01-10005	0	2	0	1	2	2	2	1	0	
QBYC05-20735	0	0	0	0	0	0	2	5		
QBYC05-20735	0	0	0	0	0	0	1?	0		
QBYC05-20853	0	0	0	0	1	2	5			
QBYC05-20853	0	0	1	1	1	0	0			
QC91-580	0	0	0	0	0	0	2	0	5	
QC91-580	0	0	0	0	0	0	1	0	1	
QN04-121	0	0	0	0	0	0	0	0	1	1
QN04-121	0	0	0	0	0	0	0	0	2	1
QN04-1643	0	0	0	0	0	0	1	3		
QN04-1643	0	0	0	0	0	1	0	0		
QN66-2008	0	0	0	0	0	0	4	4	5	
QN66-2008	0	0	0	1	0	2	1	1	1	
QS00-486	0	0	0	0	4	4	4	5		
QS00-486	0	1	0	1	2	2	1	1		
QS01-1078	0	0	0	0	1	0	2	2	5	
QS01-1078	0	0	0	0	1	0	1	1	0	

Figure 113 Scores given for Yellowing and starch accumulation in individual leaves of all 20 clones in one replicate in the drought treatment in the clone assessment trial. Leaf colour Key and ratings; Green (0), 0-25% yellowing (1), 25-50% yellowing (2), yellowing or mottling (3), >60% yellowing (4), senescing leaves (5)

Results indicate a range of yellowing in mid and lower canopy levels in symptomatic clones, and a possible difference in severity in YCS prevalence in the 20 test clones (**Error! Reference source not found.**).

Results of Lugol's test indicates a range of starch accumulation patterns in the assayed clones (**Error! Reference source not found.**). As shown in Figure 113, in a few clones several green leaves and senescing or senesced leaves also had accumulated starch. However, there is a positive correlation between % canopy yellowing and the total rating for starch accumulation (R^2 =0.65).

The starch assay results together with the canopy yellowing patterns from all the replicates in different water treatments would help to conclude any clonal difference in YCS occurrence.

Summary

In this trial, irrigation significantly increased sugarcane biomass compared to rain fed irrespective of genotype. The NDVI index was used to establish the initial colour discrimination among test clones shows a natural variation between the clones of the irrigated treatment before any YCS symptom expression. The leaf level contributions of photosynthesis, stomatal conductance and internal CO_2 were measured for each green leaf (0 to +8) and the fractional photosynthesis capacity of canopy for each individual leaf was calculated. Leaf +3 and +4 showed the highest (~28%) C-fixation rate and leaves below leaf +4 contributing only 15% of the plants total photosynthesis. Starch staining intensity showed a correlation with YCS severity.

6.5.5 Microscopy

Microscopy is an excellent tool to investigate changes to organs, cells and organelles as well as visualising the location of certain metabolic deposits and the detection of microorganisms. Light and electron microscopy was used to compare YCS asymptomatic and symptomatic plant samples collected in field visits.

6.5.5.1 Light microscopy

Lamina

The following microscopy was investigating where starch was accumulating in YCS symptomatic leaves. Hand sections of leaves showing YCS symptoms were taken and compared to asymptomatic controls. Initial results in unstained lamina could not detect any obvious differences between the two samples (Figure 114). Sections of young KQ228$^{(\dagger)}$ tiller leaves, showed accumulation of starch grains in bundle sheath cells (Figure 115).

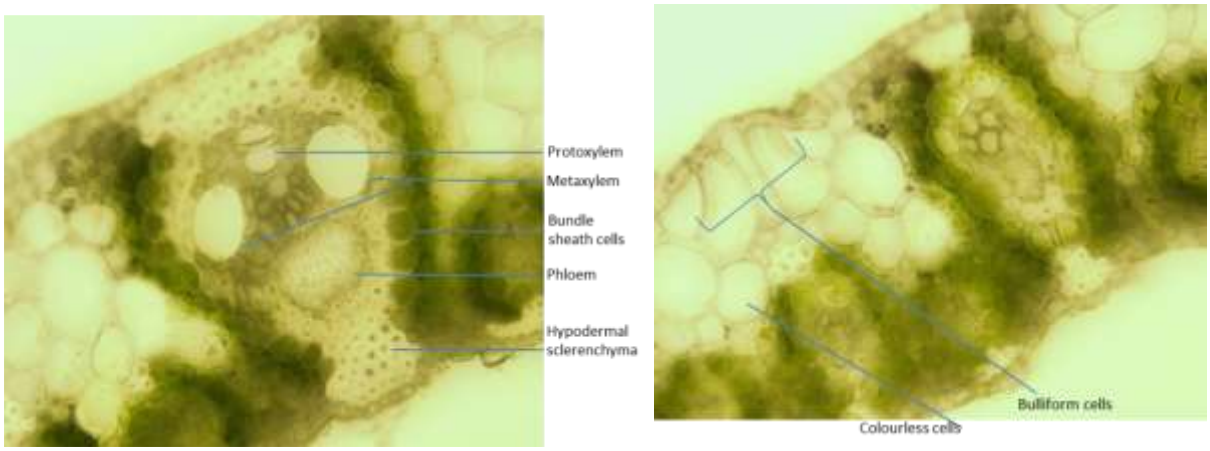


Figure 114 Unstained hand sections of lamina of KQ228^{A} showing the types and location of cells present

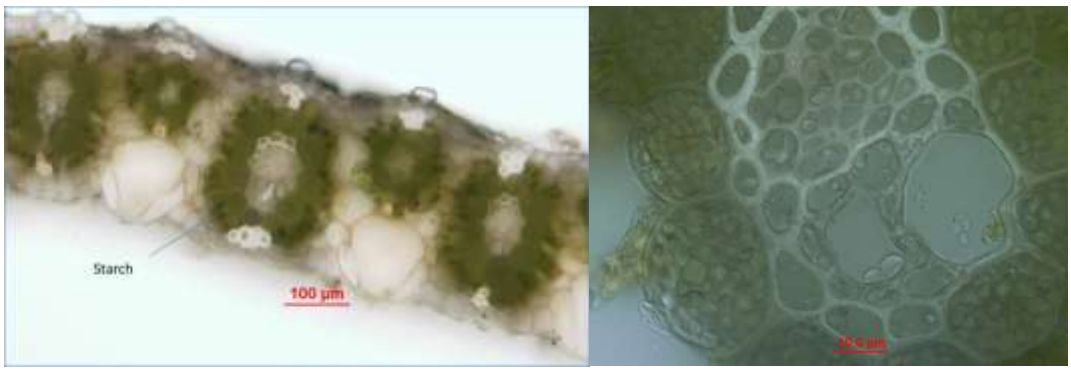


Figure 115 Unstained hand sections of lamina of a young tiller of KQ228$^{\Phi}$ showing starch grains in bundle sheath cells at low and high magnification

Sections of roots were also taken to firstly understand the anatomy and then look for differences in the asymptomatic controls with that of YCS symptomatic plants. Preliminary results showed that the sieve plates in the roots of YCS symptomatic plants looked abnormal (Figure 116, Figure 117). However, this result needs to be verified in many more samples to confirm whether this phenomenon is associated with YCS.

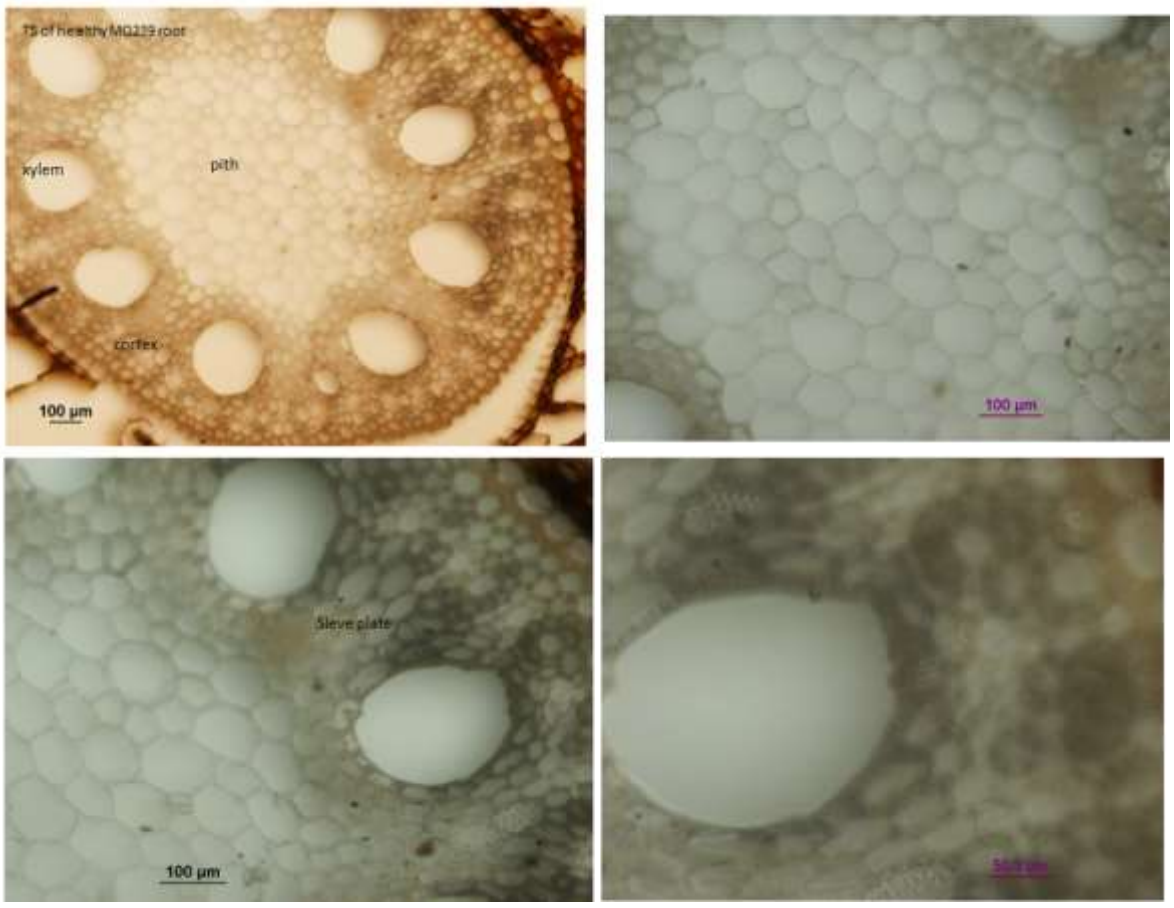


Figure 116 Transverse sections of MQ239^{A} roots from a asymptomatic control plant

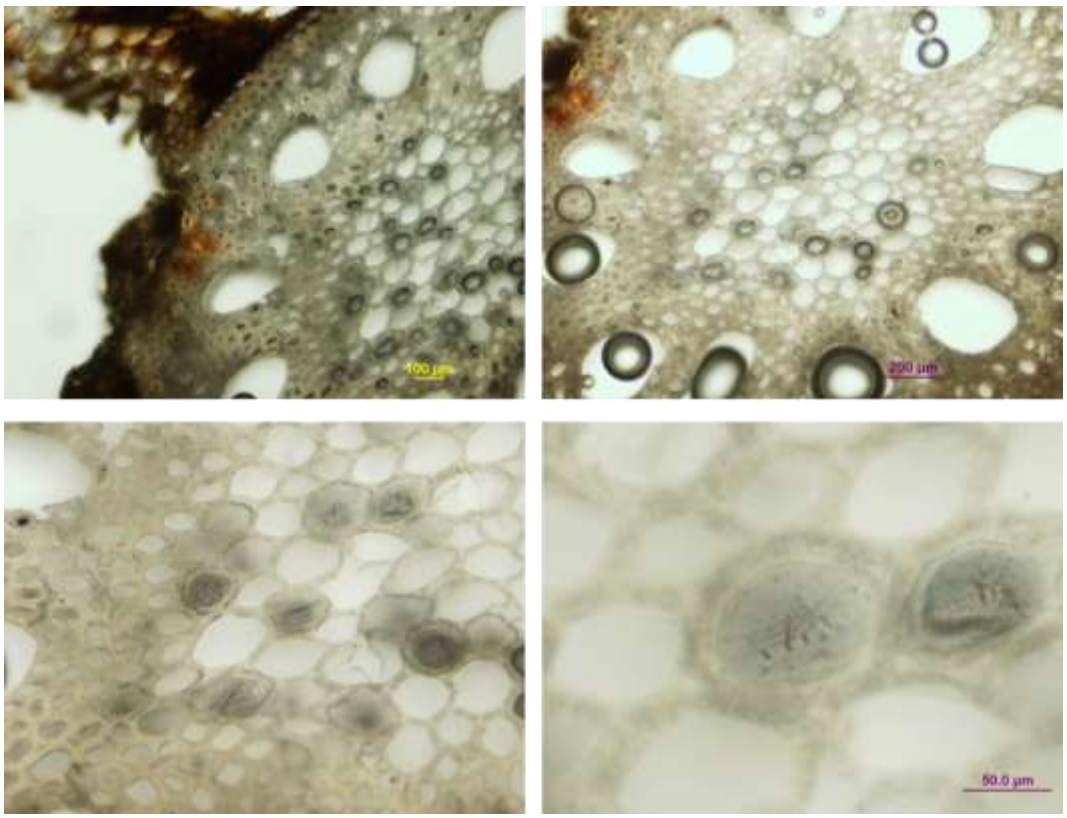


Figure 117 Transverse sections of MQ239$^{\Phi}$ roots from a YCS symptomatic plant

Lamina, midrib and sheath

Q240 tissue of healthy and YCS plants was sectioned by hand, stained with toluidine blue and then examined under a compound microscope. The lamina, midrib and leaf sheath were screened. There were no visible differences between healthy and YCS plants in any of the tissues examined (Figure 118).

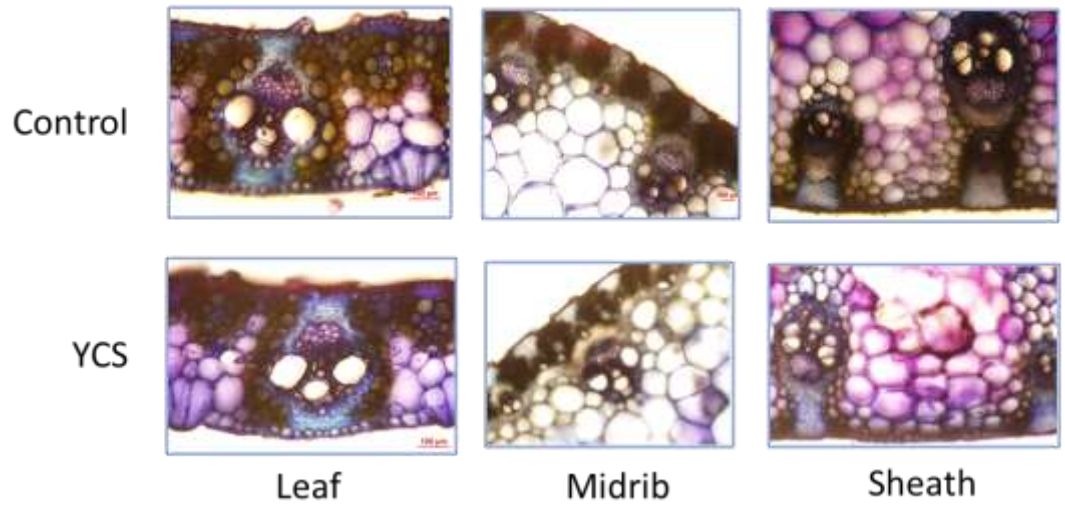


Figure 118 Transverse sections of healthy (A) and YCS (B) leaf, midrib and sheath of Q240 sugarcane

Optimised protocols were developed to preserve, dehydrate and microtome section samples collected from the Burdekin, Mackay and Herbert. Samples were collected from leaf, sheath, midribs, internodes and roots of YCS and asymptomatic (referred to as healthy hereafter) plants.

Internodes

The internode sections of YCS affected plants of KQ228$^{\phi}$ using the compound microscope are shown below (Figure 119 A-D). The figures show the close up of the parenchyma region (B), xylem (C) and phloem (D) regions of the vascular bundles in the internode.

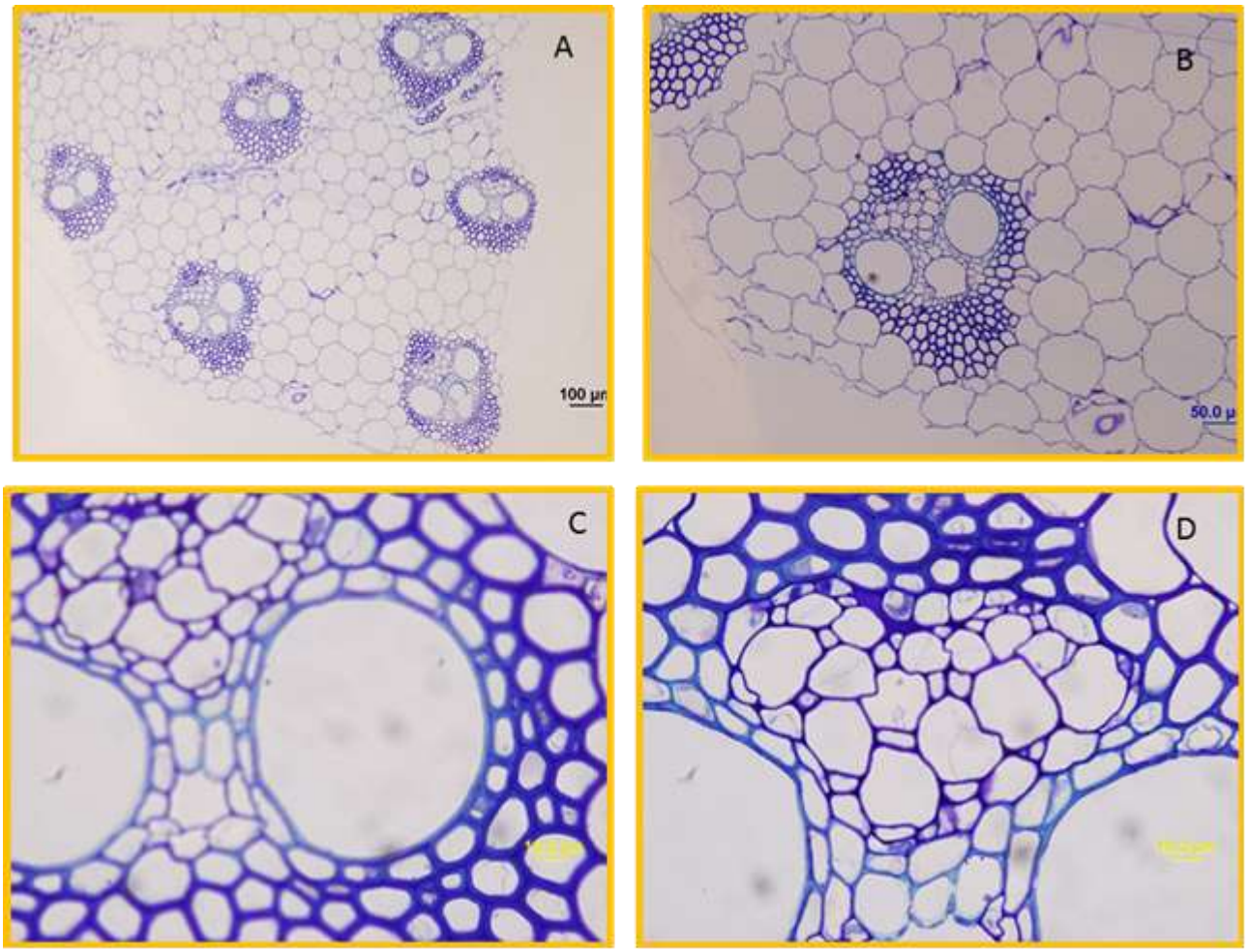


Figure 119 Internode sections of KQ228^{A} showing vascular bundles and parenchyma cells A and B) visualised under the compound microscope, close up of the xylem C) and close up of the phloem D).

As bright field microscopy was unable to reveal any anatomical differences between asymptomatic and YCS symptomatic tissues in any of the above-ground organs, a different approach to study the functionality of cells in these tissues was undertaken.

Fluorescent dyes

Based on the auto fluorescence emission spectrum conducted with the confocal microscope at UQ (data not shown), a green fluorescent dye carboxy fluorescein diacetate (5, 6-CFDA) (excitation 494 and emission at 521nm) and red Texas Red (TR) (excitation at 595 and emission at 615nm) were

selected to provide more information on the functionality of conducting tissues in sugarcane. These dyes were sourced and tested at Mackay SRA station. Stalks of Q208(ɸ) showing YCS symptoms were sampled from Paul and Joe Schembri's farm, while the asymptomatic controls were obtained from the Mackay Productivity clean plots farm.

Stalks were brought back to the station and the first leaf was separated from the stalk along with its subtending leaf sheath, and quickly placed in water upside down, with the leaf tip immersed in the water. The leaf was cut again to 1/3rd of its size and left overnight in the laboratory to take up the fluorescent dyes (Figure 120). Both dyes (Texas Red (TR) and Carboxy fluorescein diacetate (5, 6-CFDA)) were placed in the same tube, to enable simultaneous visualisation of both dyes in the same section. Leaves were sectioned and viewed under a stereo microscope using the green or red filter after 20 hours.

Figure 120 Set up for conducting overnight dye uptake studies in leaves in the lab

Sections of the leaf were closest 2cms in from the leaf sheath dewlap junction (distal) to the dye solution (Figure 121, Figure 122) and taken 2cms upstream of the cut end (proximal) (Figure 123, Figure 124). Results showed that the pattern of uptake of the two dyes was different especially in the proximal sections (Figure 123, Figure 124).

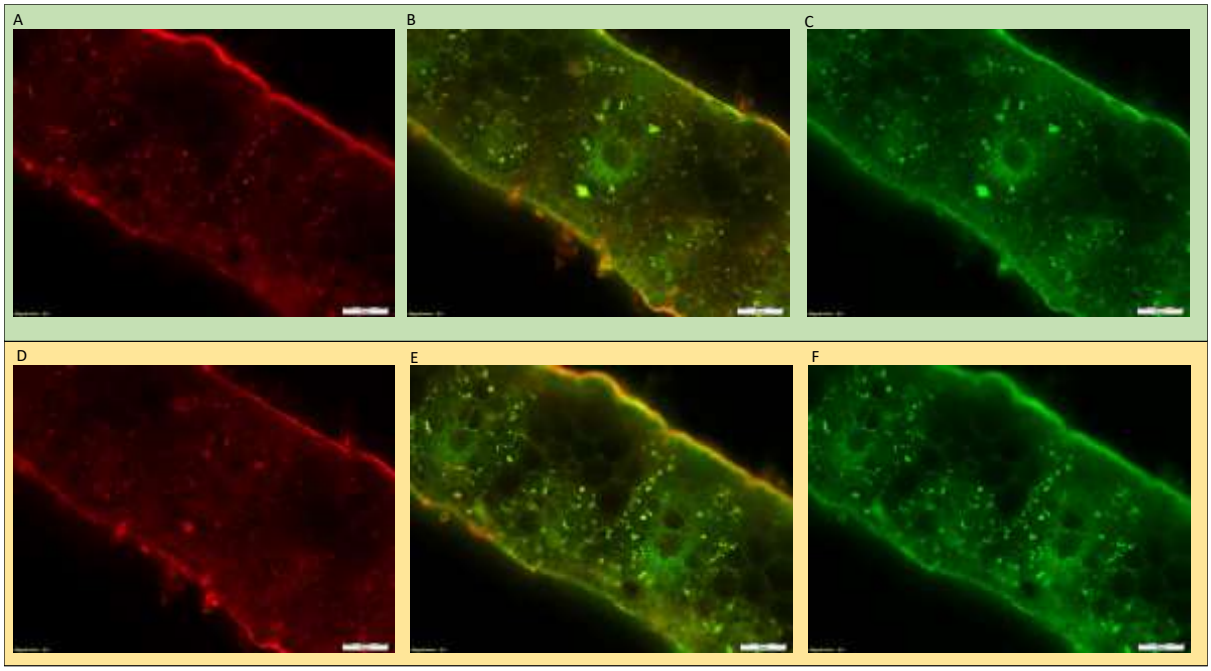


Figure 121 Dye uptake pattern in distal small vascular bundles of healthy (A-C) and YCS (D-F) leaves of Q208$^{\phi}$. The central panels are merged images of the red and green filters.

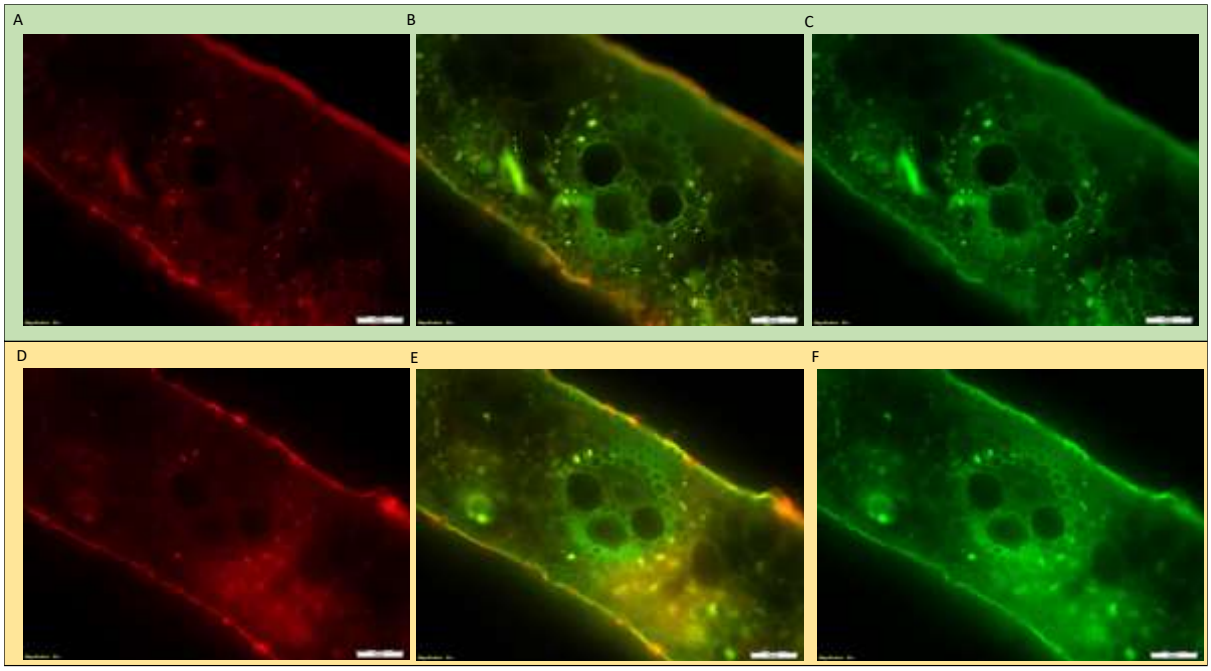


Figure 122 Dye uptake pattern in distal large vascular bundles of healthy (A-C) and YCS (D-F) leaves of Q208$^{\phi}$. The central panels are merged images of the red and green filters

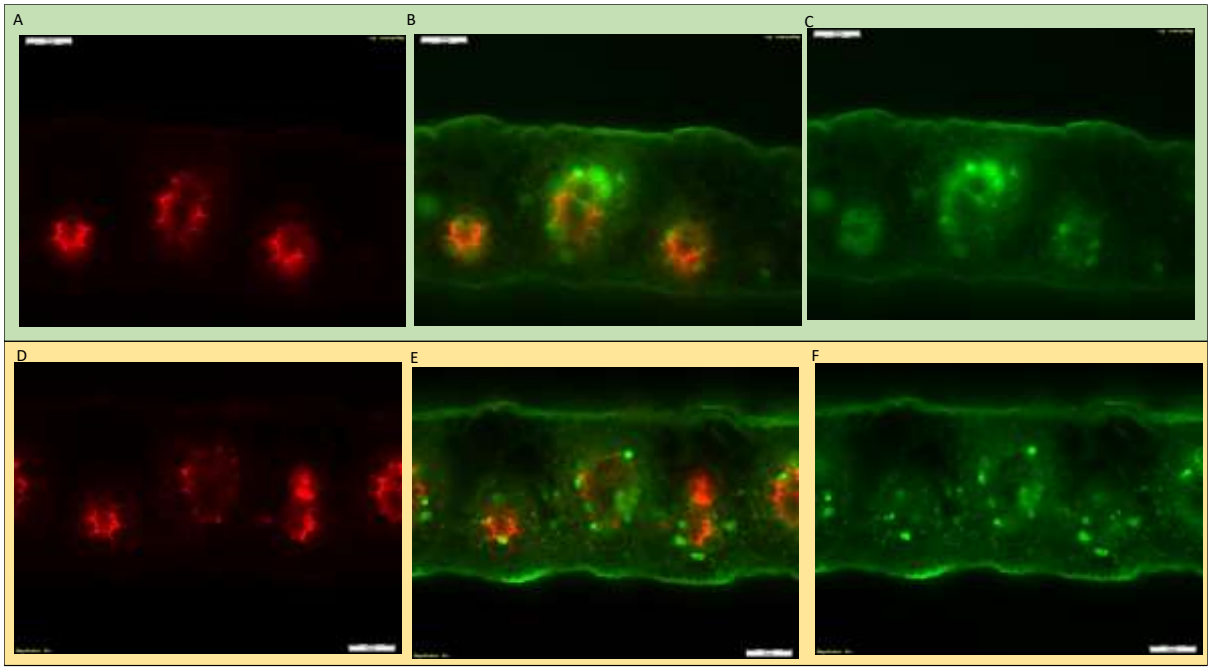


Figure 123 Dye uptake pattern in proximal small vascular bundles of healthy (A-C) and YCS (D-F) leaves of Q208$^{\Phi}$. The central panels are merged images of the red and green filters

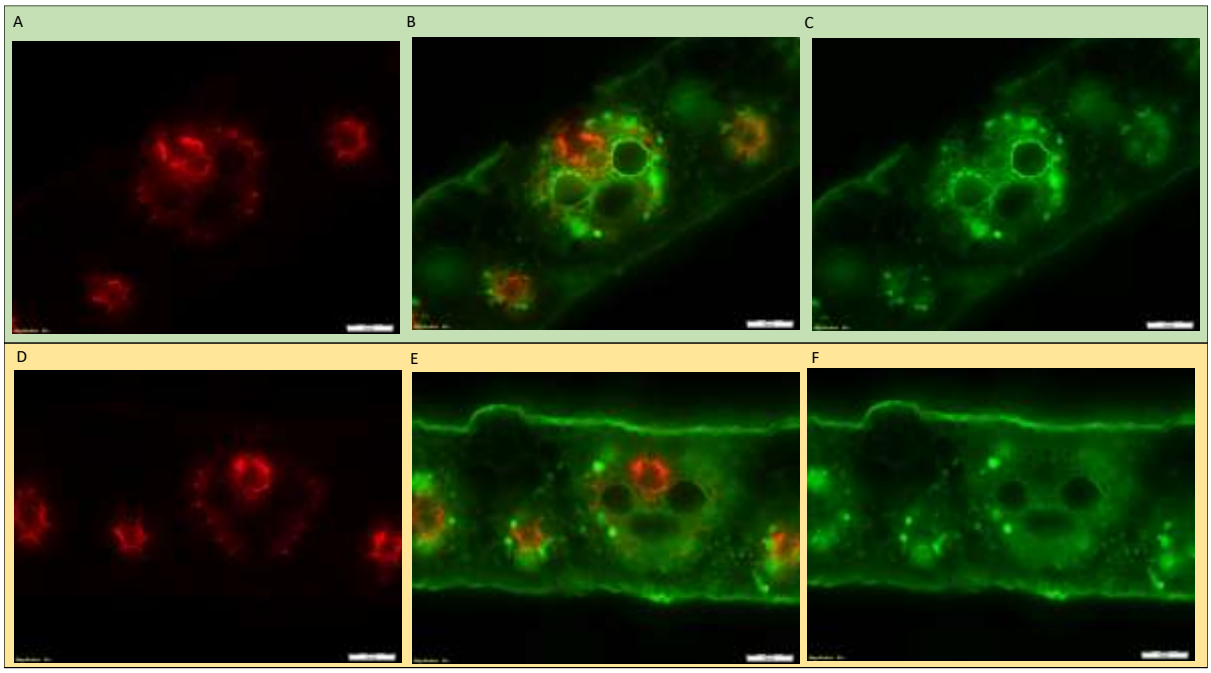


Figure 124 Dye uptake pattern in proximal large vascular bundles of healthy (A-C) and YCS (D-F) leaves of Q208$^{\Phi}$. The central panels are merged images of the red and green filters

The distal sections were not very informative as the tracer dyes were in insufficient quantity, to make any meaningful conclusions.

In the proximal sections, however, Texas red was predominantly in the vascular parenchyma in the small vascular bundles and in the large ones, in both the vascular parenchyma as well as the

protoxylem lacunae. No difference in the pattern between asymptomatic and YCS samples (Figure 123 A & D and Figure 124 A & D).

CFDA was present mainly in the bundle sheath cells of the smaller VBs (Figure 123 C & F) but occupied the entire cell volume in case of the asymptomatic leaf, while in the YCS leaf, the fluorescence was more subdues and not occupying the whole bundle sheath cells (Figure 123 F). In the large VBs, CFDA fluorescence was very intense in the xylem parenchyma cells of the asymptomatic leaf (Figure 124 C versus Figure 124F). In general, YCS affected leaves showed much less CFDA in their tissues, especially in the large vascular bundles.

Summary

The functionality of the large VBs is affected in YCS leaves and may be due to blockage in the phloem resulting in less tracer being transported and hence not detected. Another possibility is death of some of the xylem or phloem parenchyma cells leading to lack of CFDA expression. CFDA-SE is colourless when it enters cells by diffusion, and upon cleavage by intracellular esterase enzymes to form an amine-reactive fluorescent product, CFSE. This product produces a detectable fluorescence and covalently binds to intracellular lysine residues and other amine sources. In this study, this research was not examined any further.

Light microscopy with fluorescent tracer dyes

Dye uptake studies performed in the past year using several different dyes had been restricted to movement in the xylem mainly. In isolated leaves, we successfully introduced the fluorescent tracer 5,6 CFDA which we hoped would target the phloem. However, our results showed that this tracer was present in the vascular parenchyma surrounding the xylem vessels but was not present in the phloem. Two other fluorescent tracer dyes were sourced, 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS)-an apoplasmic tracer) and 8-acetoxypyrene-1,3,6-trisulfonic acid trisodium salt (symplasmic tracer) which have been reported to move in the phloem in sorghum (Milne *et al.* 2015). The studies reported here were performed to test feeding methods, optimise protocols, and verify whether these dyes behave in a similar manner in sugarcane. These dyes were used to develop protocols to study phloem functionality in YCS affected plants.

Protocol

Two types of experiments were undertaken- root to shoot (RS) and shoot to root (SR). Q208$^{\Phi}$ was used in the first experiment to test the RS system using the dye HPTS (expt 2). We then compared Q249$^{\Phi}$ stalks using both RS and SR systems and using the HPTS as well as the Evan's blue dye (expts 3 and 4) and examined the cells that the dyes targeted in the internodes and the vascular bundles (VB).

For RS experiments, the stalks were placed in the dye and allowed to take it up for 6 hours. For the SR protocol, reverse osmosis was used (Figure 125) and stalks removed after 6 hours. Sections of the internodes were taken and observed under the light microscope using fluorescence filters.

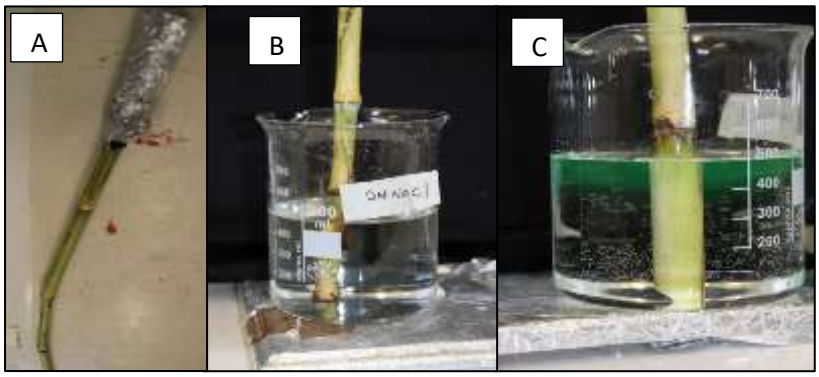


Figure 125 SR reverse osmosis experiment set up. Alfoil wrapped around Falcon tube containing light sensitive HPTS tracer dye (A). Base of stalk is placed in high osmotic solution to induce downward flow of the dye (B) and the dye is visible in the solution after 3hours (C).

Results showed that in the SR experiment, HPTS moved in the phloem in internodes +5 and older, while in the smaller internode +3 it was visible in the vascular parenchyma (Figure 126).

When the dye was fed to older stalk at internode +8, the fluorescence was not present in all the VB, but when present it was mainly in the phloem (Figure 127).

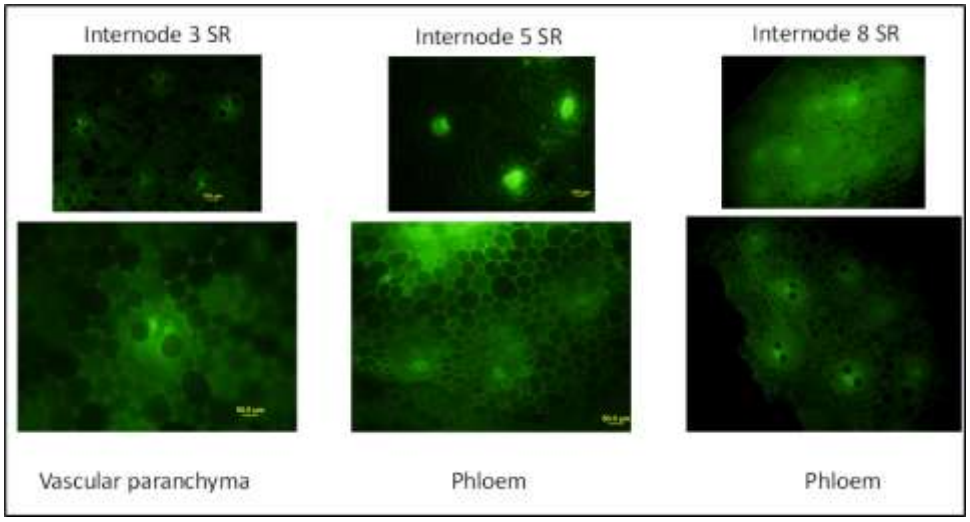


Figure 126 Location of fluorescent tracer dye HPTS (SR) in the cross section of internodes when fed to internode2

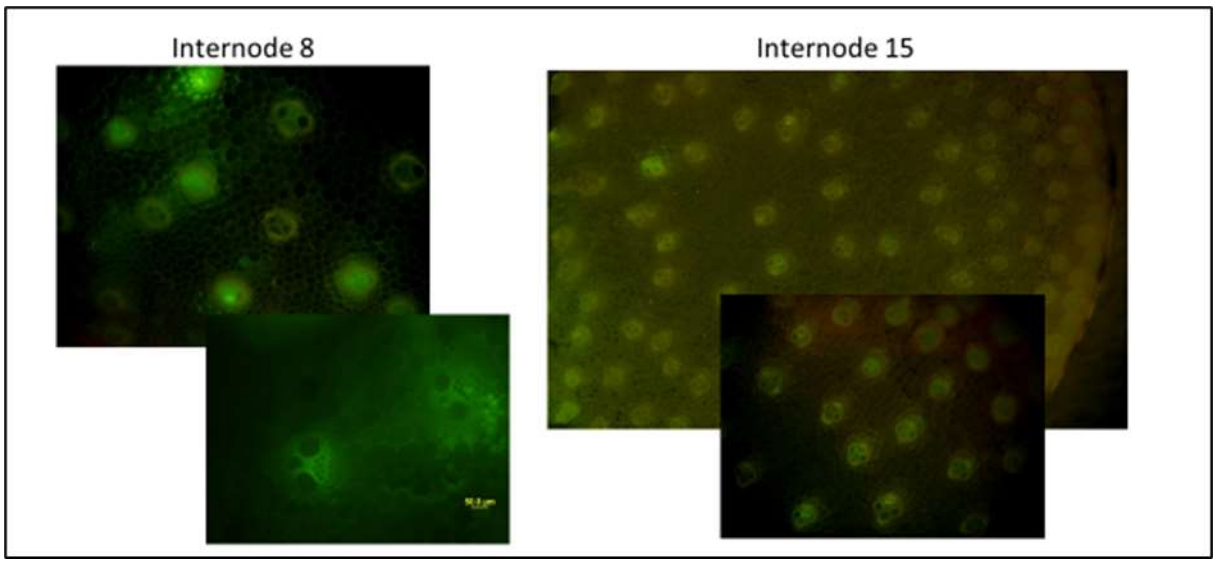


Figure 127 HPTS fed to older internode8 (SR) of Q249$^{\Phi}$, and visualised using WIBA filter

In SR experiments, HPTS is shown to be predominantly translocated through the phloem. Investigations using the DAPI filter show it is more sensitive to the presence of the dye which improves detection (Figure 128).

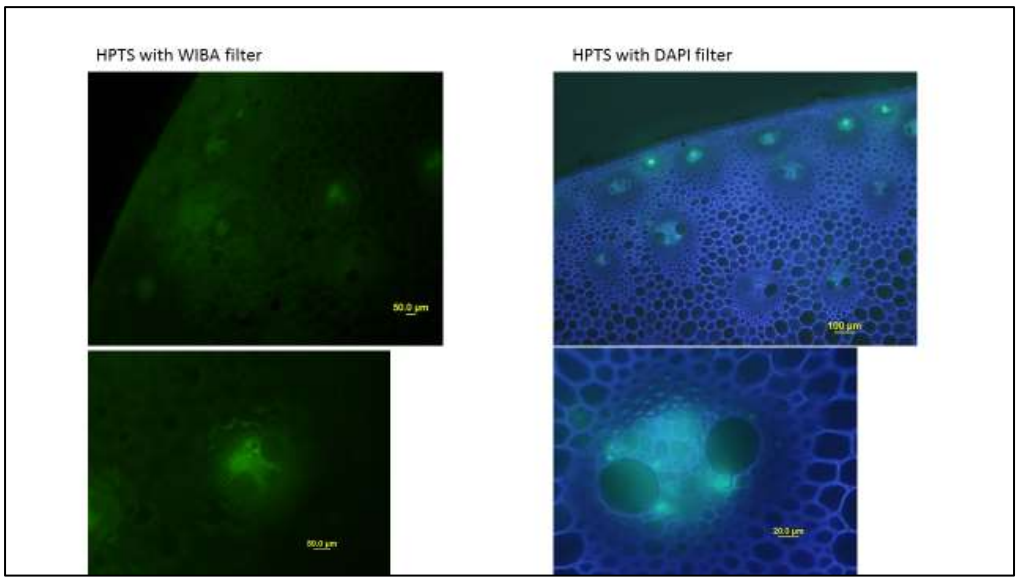


Figure 128 Fluorescence visualised using the WIBA filter versus the DAPI filter in stalks fed HPTS dye in Q249$^{\Phi}$

The fluorescent tracer dye *p*-Toluenesulfonic acid (PTSA) was used in the following study. A comparison performance of these two fluorescent dyes in stalks of Q249^{A} was conducted using the RS system (described above) that we had successfully tested using HPTS dye (Figure 129 A). (Figure 129 B) shows the three regions of the internode that we used to assess location and presence of the dye in the transverse sections.

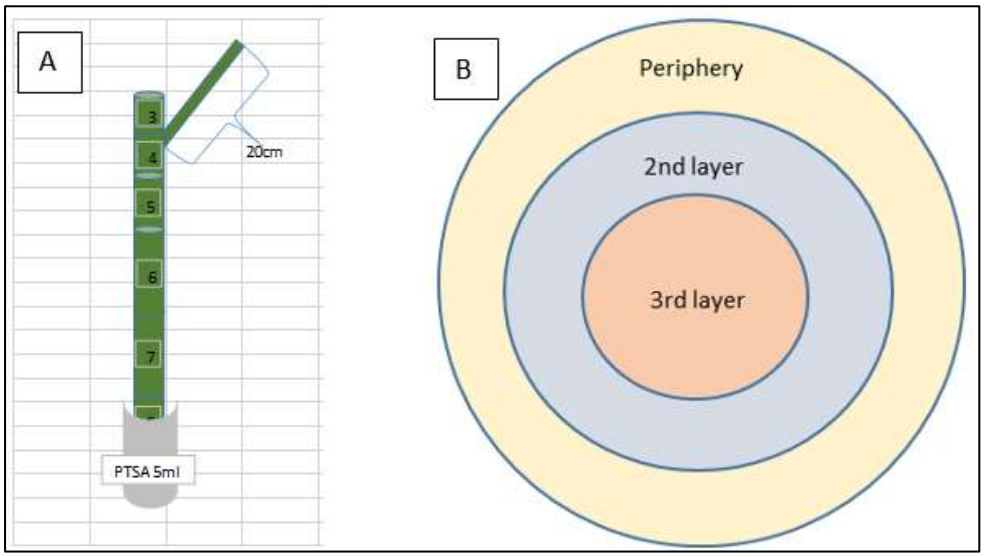


Figure 129 Cartoon of system used for RS using fluorescent dye and stalk cut at internode 8 with leaf 4 left attached (A). Diagrammatic representation of the transverse section of the internode to show the three regions analysed for presence of dyes (B)

Results showed that PTSA is more specific to movement in the phloem in the internodes (Figure 130) and appeared to be present in more of the VB than HPTS. Further results (Figure 131), show that PTSA is present in the phloem of all the VBs present across the internode, while HPTS is present in the phloem in the peripheral region, but in the vascular parenchyma in the central and core VBs.

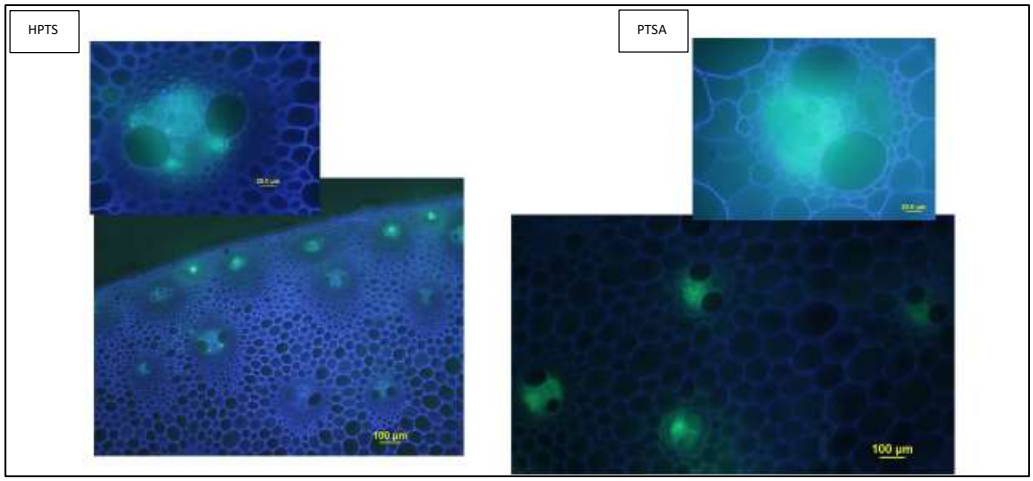


Figure 130 HPTS versus PTSA in internode 8 of Q249$^{\Phi}$. Both dyes were fed using the RS method. Fluorescence using the PTSA dye is stronger and present in more VBs than HPTS, which is predominantly present in the peripheral VB.

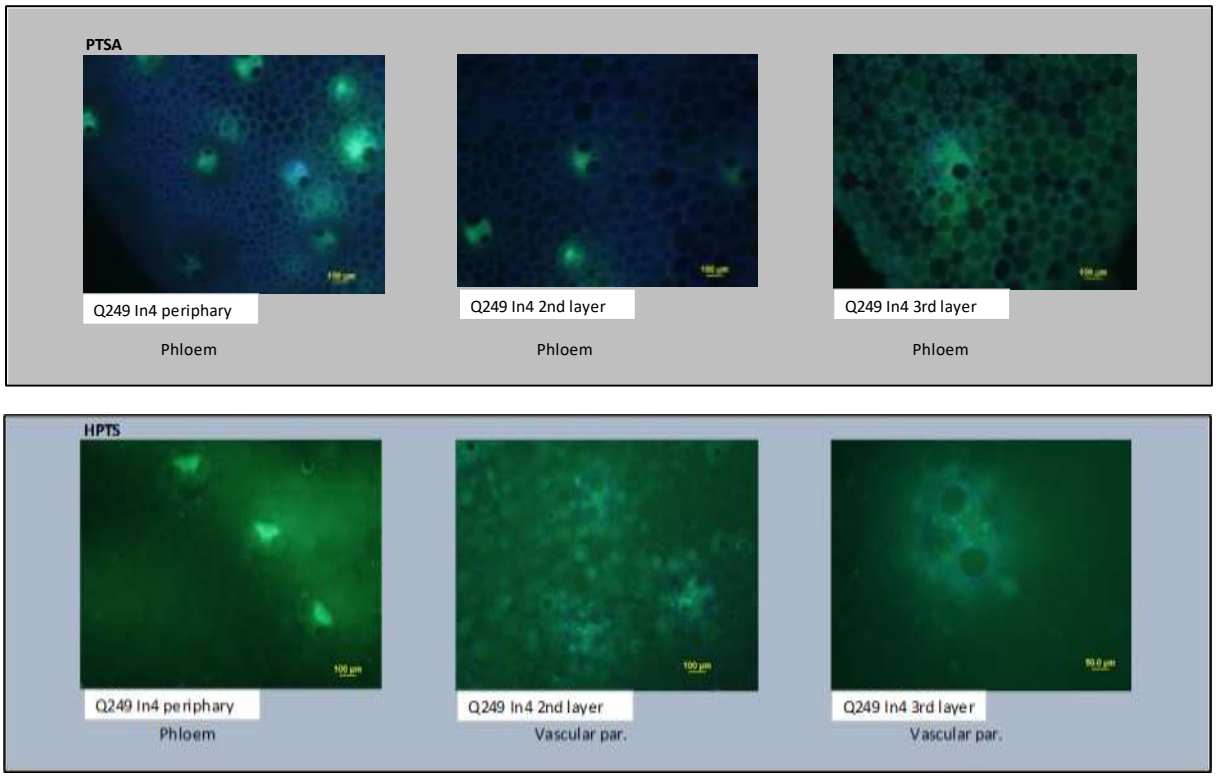


Figure 131 Free hand sections of internode 4 of RS experiment in Q249$^{\Phi}$. The two panels show fluorescence in VBs when either PTSA (A) or HPTS (B) was used

The location of the fluorescent dyes in the leaf +4 of the fed stalks were also examined (Figure 132). Results showed that in the leaf midribs, PTSA was detected in the phloem of the large VB, and in the vascular parenchyma and phloem of small and intermediate VBs; while HPTS fluoresced mainly in the vascular parenchyma of the VBs. In the lamina, there was more fluorescence in the HPTS dye-fed stalks, as it was present in both the xylem and phloem.

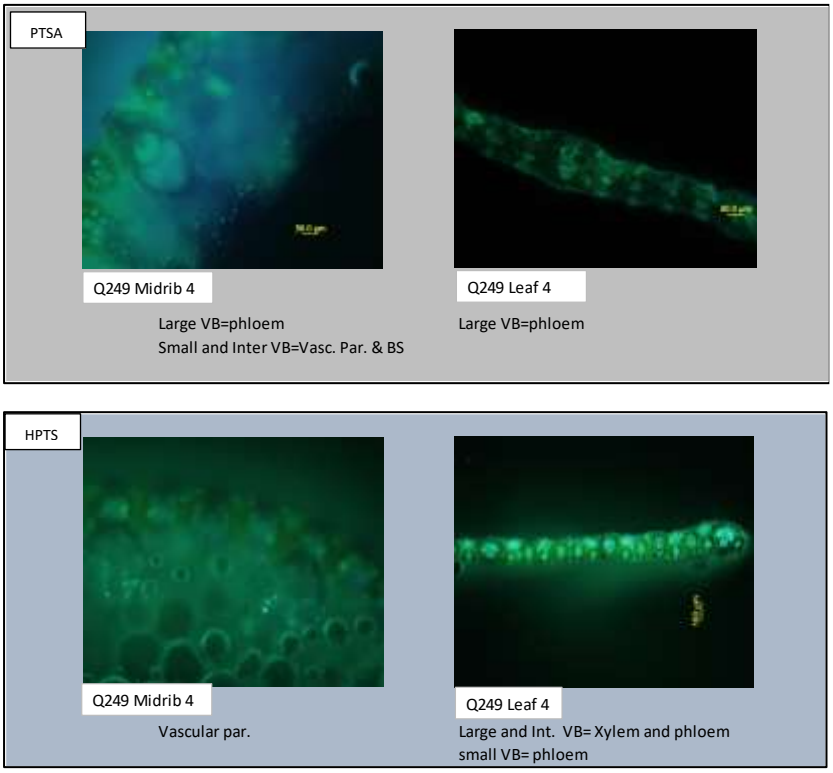


Figure 132 Free hand sections of leaf 4 of RS experiment in Q249$^{\Phi}$. The two panels show fluorescence when either PTSA (top panel) or HPTS (bottom panel) was used

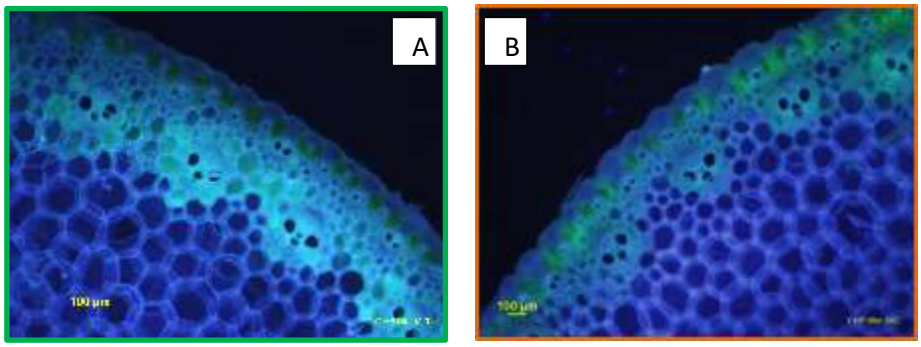
Summary

PTSA is phloem specific in stalks and large VB in leaves, while HPTS shows up in phloem or vascular parenchyma in stalk VBs, and no specific cell in the lamina. Equipped with the DAPI filter, two water soluble dyes which performed well in RS and SR experiments, the methodology was deployed to the field to investigate phloem transport in YCS plants. In 2019, this work was extended to using tracer dyes in the insecticidal trial at Brandon.

Tillers having up to 6 leaves were harvested and recut under water. A total of three YCS and three control tillers were harvested. The leaves were trimmed to ~20 cm and then stalks placed in dye for 2 hours (RS method). Fluorescent tracers, which were either apoplasmic (HPTS) or symplasmic (PTSA) in translocation within the plant were tested.

HPTS: Midrib of control tiller leaf 0 (Figure 133 A), showed fluorescence in vascular bundles (VB) and surrounding parenchyma cells, while in the YCS tiller, VBs showed reduced fluorescence while the surrounding parenchyma cells showed none (**Error! Reference source not found.**). In contrast, the s heath showed very high fluorescence in VB and surrounding cells in YCS tiller compared to the control sheath (Figure 133 C and Figure 133 D). This suggests a blockage of movement of the apoplastic tracer dye HPTS, from sheath to midribs in the YCS tillers.

Midrib and sheath



Sheath

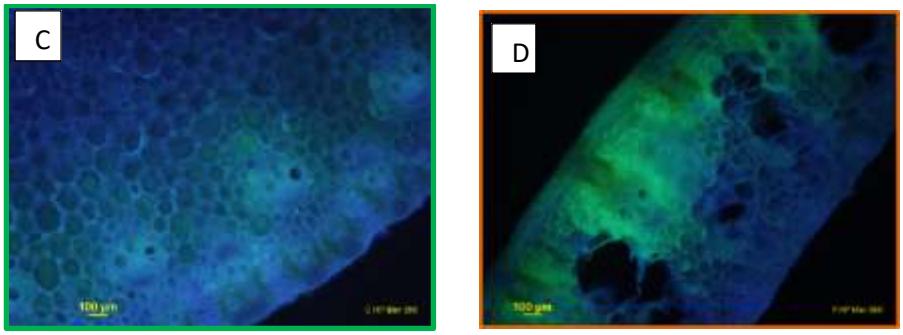
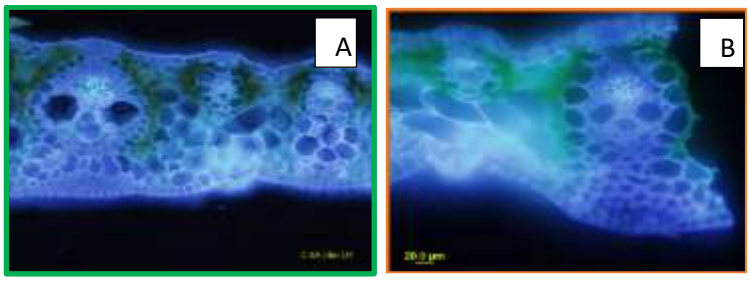


Figure 133 HPTS sections of midrib and leaf sheath of control (A & C) and YCS (B & D) tiller leaf. Note the hyper accumulation of dye in the sheath of YCS plants (D), concomitant with reduced presence of dye in the parenchyma cells of the midrib (B)

PTSA: Similar studies on uptake in leaf +1 of tillers of the symplasmically transported (i.e., via the phloem) tracer dye PTSA, showed similar occurrence and distribution of dye in the lamina (Figure 134 A & B). In the midribs, however, regions closest to the lamina (Figure 134 C & D) showed similar dye distribution in both control and YCS tillers, while in regions of the midrib furthest away from the lamina, very little dye was present in the VB and surrounding parenchyma cells (Figure 134 E & F). In the sheath, presence and intensity of dye is less in YCS tillers compared to control in the VB as well as surrounding cells (Figure 134 G & H). Additionally, there appears to be more dye deposited on the phloem cell wall, giving the appearance of reduced volume of the sieve tubes.

Lamina



Midrib

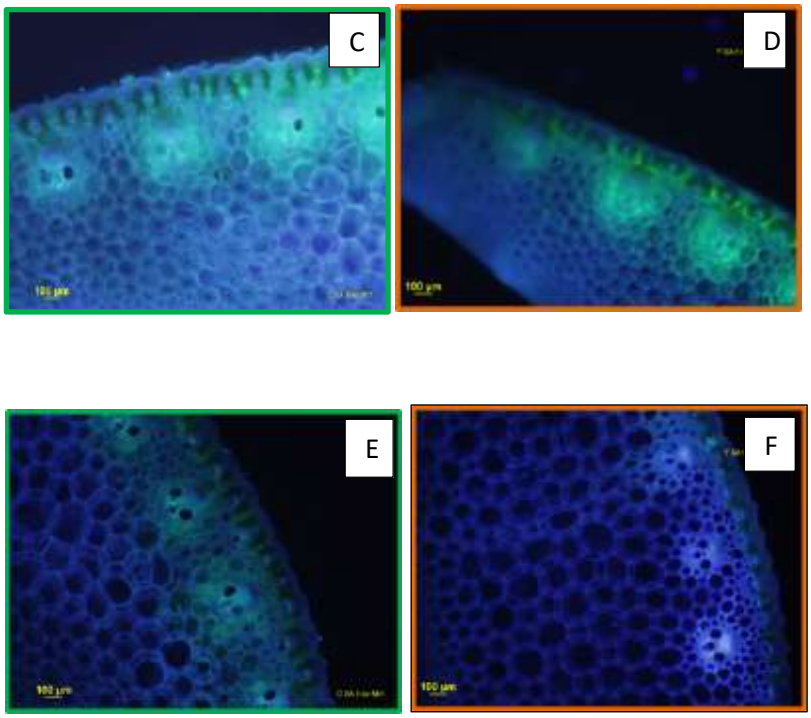


Figure 134 PTSA fed tiller (RS) All green borders are asymptomatic control plants and orange borders are YCS plants. Detection of fluorescence of tracer dye in lamina (A and B); midribs closer to the lamina (C and D) and furthest from lamina (E and F); and sheath (G and H). Note the absence of fluorescence in the midrib cells and vascular bundles furthest from lamina in YCS plants (F) while the dye is present in vascular bundles and surrounding parenchyma cells closer to the laminar (D)

Summary

These experiments using tracer dyes suggest that dye movement is compromised in tillers of YCS affected plants, especially in the midribs. Fluorescent tracer dyes were tested and verified and identified that the phloem transport from the sheath to midribs of leaves in YCS plants is reduced.

The VBs closer to the lamina are still able to function in dye delivery to the lamina and hence fluorescence is visible in the lamina sections. Further extensive investigation would be required to determine the exact location of any vascular blockages.

Leaves fed tracer dyes were sectioned in Indooroopilly lab and visualized under the compound microscope in 2019 as planned. Results showed that there was blockage in YCS leaves (Figure 135, Figure 136). The blockage was present in both dewlaps, midribs as well as leaf sheath VBs.

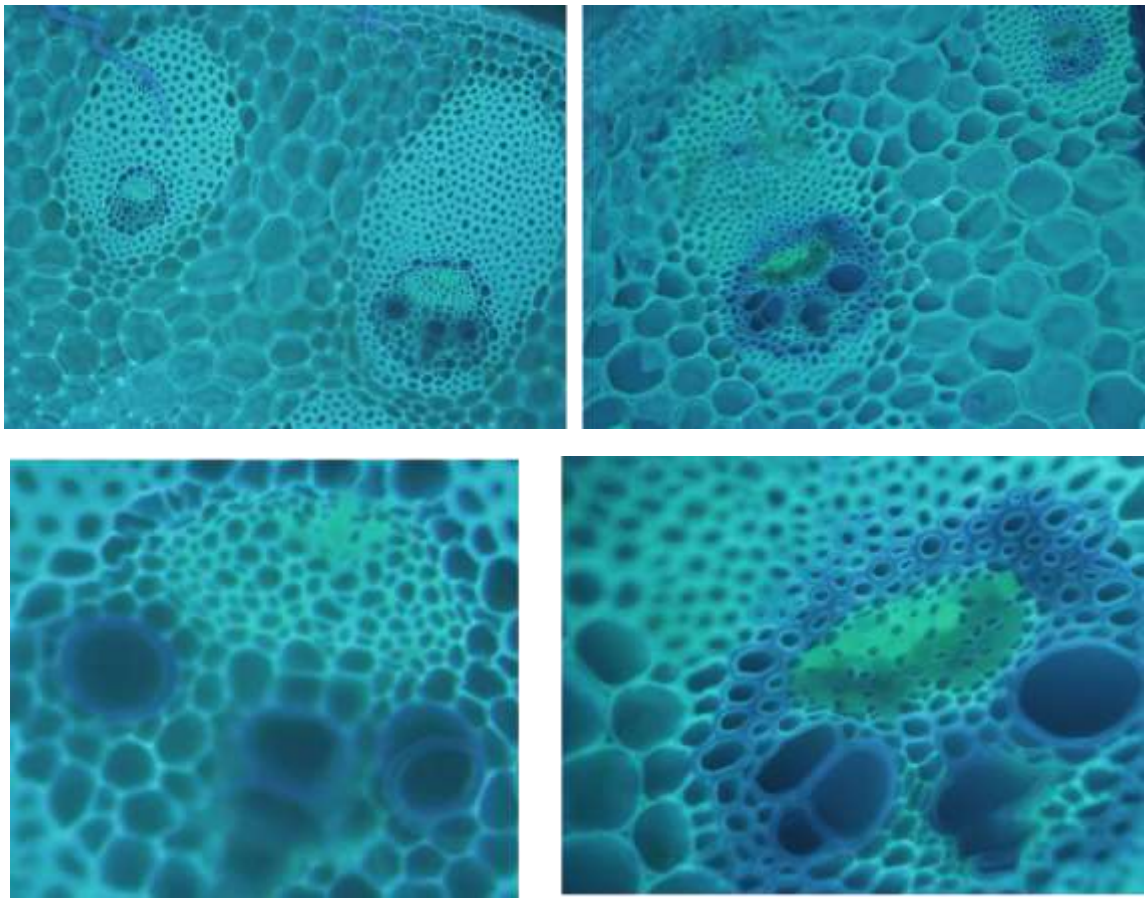


Figure 135 Transverse sections of dewlap2 of tracer (HPTS) fed leaf2. . Bifenthrin treated, asymptomatic control (LHS) and YCS (UTC) symptomatic (RHS). Note the blocked phloem of the YCS dewlap. Bottom panels are a close up of the VB region

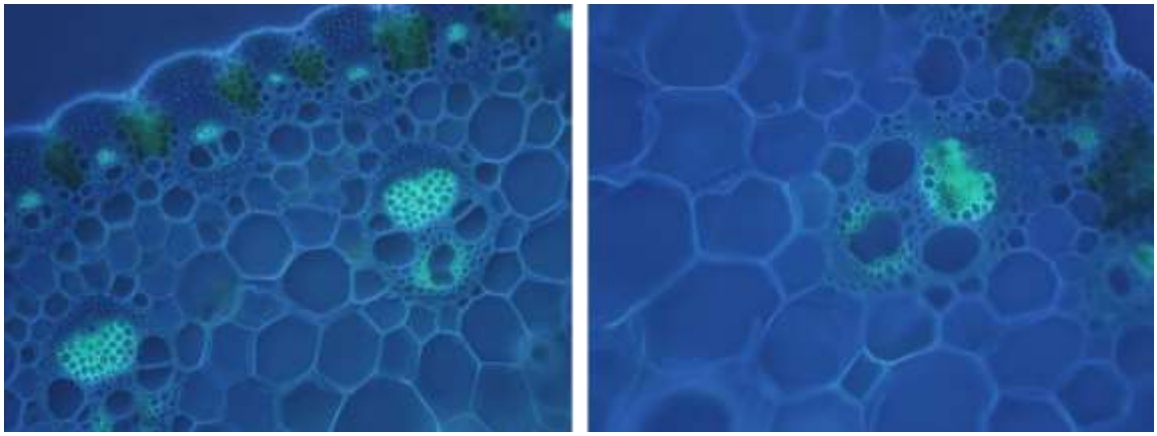


Figure 136 Transverse sections of midrib2 of tracer (HPTS) fed leaf2. Bifenthrin treated, asymptomatic control (LHS) and YCS (UTC) symptomatic (RHS). Note the blocked phloem of the YCS midrib vascular bundle

When internode sections were analysed, the blockage was visible albeit less obvious (Figure 137)

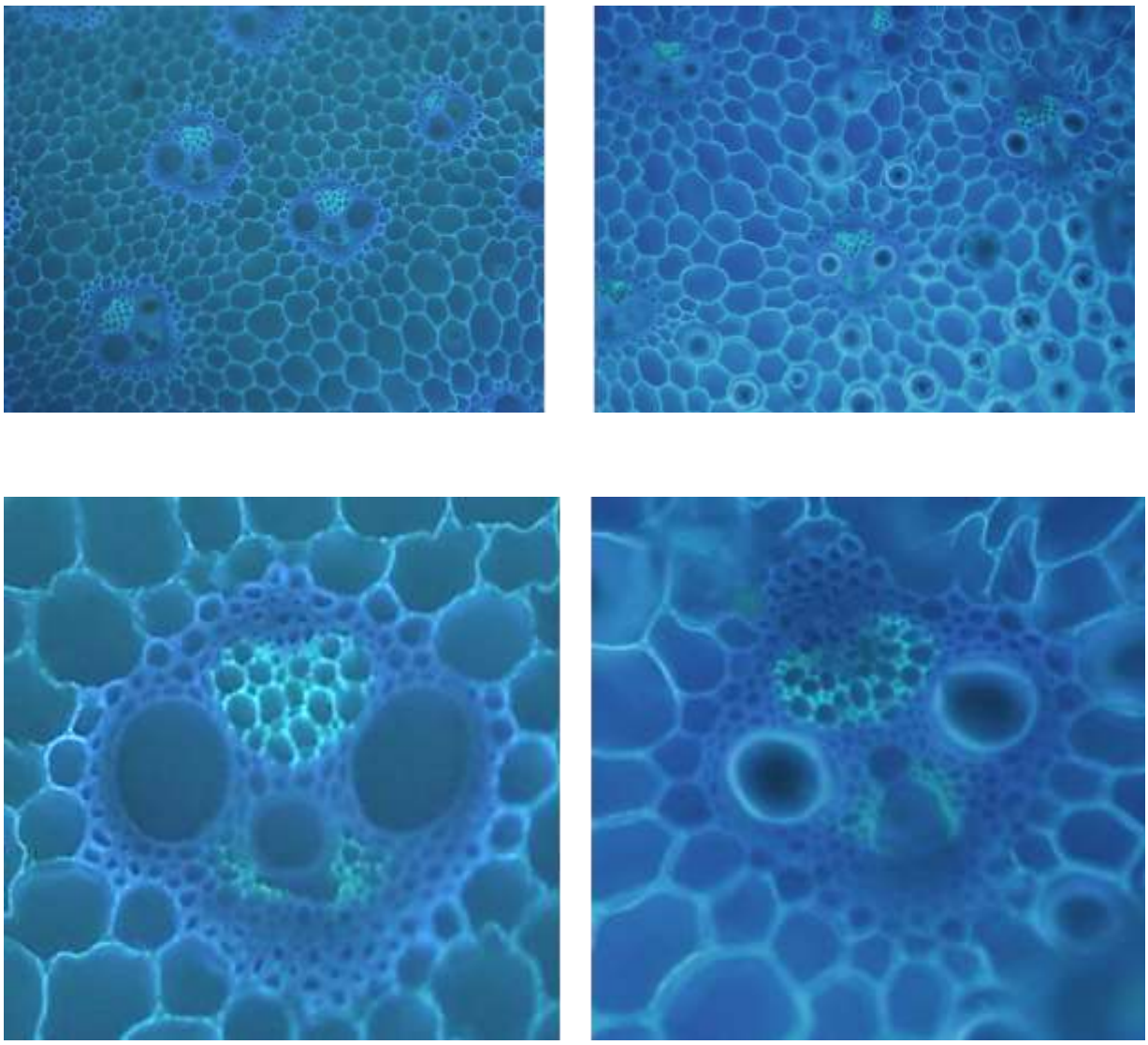


Figure 137 Internode 4 sections of Bifenthrin treated, asymptomatic control (LHS) and YCS (UTC) symptomatic (RHS) stalks after feeding of PTSA tracer dye. Bottom panel is zoomed in image of the same sections to show the subtle blockage occurring in some of the VBs in the UTC treatment

The microscopy results appear to show the presence of a physical blockage in vascular bundles of YCS plants.

Causal agents

The leaf and stalk were tested for the presence of phytoplasmas using Diene's stain. This stain is highly specific to phytoplasmas and would help us determine if YCS is due to this microorganism which resides in the phloem. The stain was prepared and tested on some FAA fixed and sectioned samples of KQ228$^{\phi}$ collected in 2014. In YCS leaves, while accumulation of the stain was visible in the phloem, this was not limited to the phloem region only. These sections showed dense staining of the bundle sheath, surrounding Kranz mesophyll as well as the sclerenchyma cells near the lower epidermis cells (Figure 138 B). The healthy sections did not show similar staining (Figure 138 A). The nature and cause of the mesophyll cell reaction is unknown.

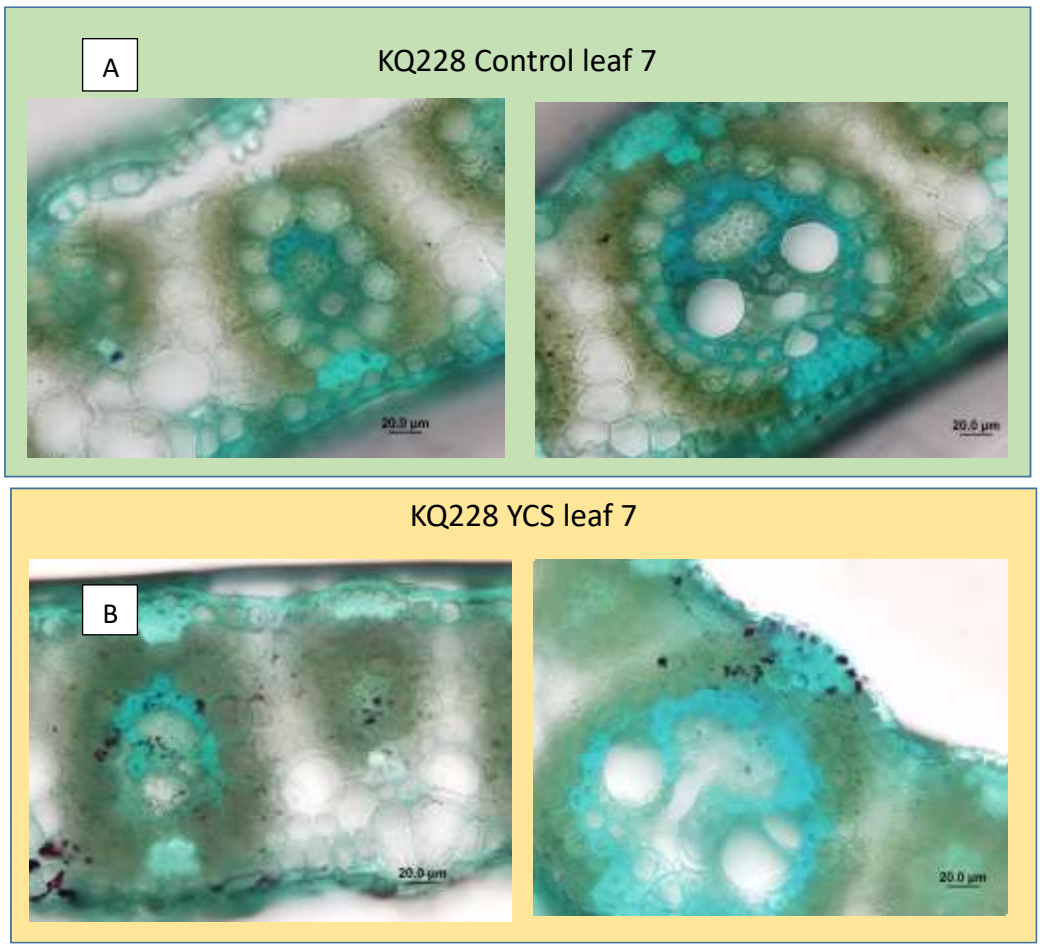


Figure 138 Diene's stain on FAA fixed leaf7 sections of KQ228$^{\Phi}$ control and YCS affected plants

Further leaf and stalk tissue collected from the Burdekin were used to repeat the test with Diene's stain as well as other stains. Staining also focussed on toluidine blue which had shown the presence of unknown particles especially in the older leaves of YCS affected plants (Fig. Figure 139 A & B).

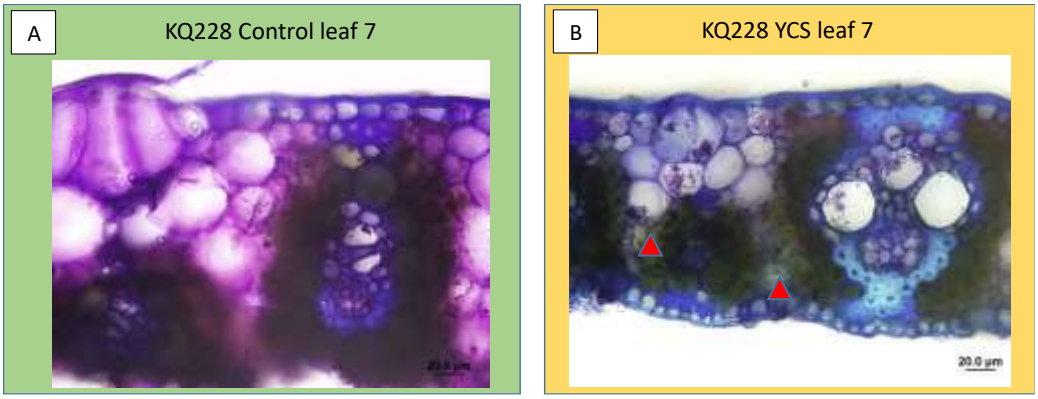


Figure 139 Toluidine blue stain on fresh leaf sections of KQ228$^{\Phi}$ control and YCS affected plants. Arrow heads point to the presence of numerous particles in the YCS samples

After consultation with UQ scientists, (microscopist Mr. Rick Webb and Prof. Daryl Joyce) regarding the nature of these particles, it was suggested that they might be phenolic compounds.

A suitable protocol for use of vanillin in sugarcane was then developed. Sections were stained with vanillin, to confirm the presence of the nature of these bodies. Results showed that both control and YCS leaves produced the characteristic bright pink colour soon after the addition of the vanillin stain. However, only YCS sections changed colour from pink to black after a 2hour incubation (Figure 140 A & B), confirming the presence of large amounts of phenolic compounds.

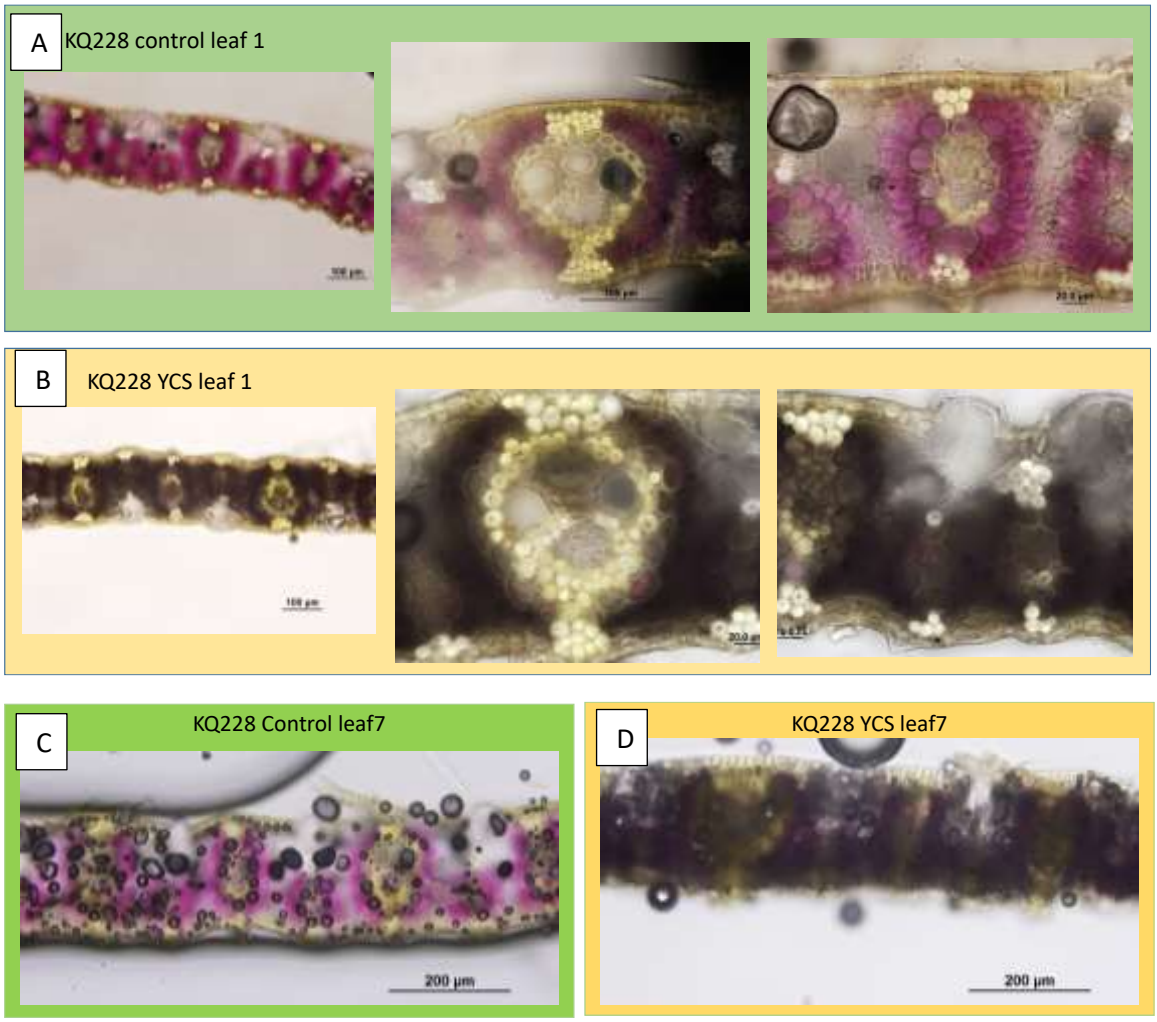


Figure 140 Vanillin stain on fresh leaf sections of KQ228$^{\phi}$ control (A and C) and YCS (B and D) affected plants. Stained leaf sections showed that YCS leaves contained a lot more phenolics producing a dark stain after 2 hours, while the controls remained pink

More recently, we have optimised the use of aniline blue as a fluorescent stain to detect callose accumulation (Koh et al., 2012). Using this method, we have revisited samples collected from the insecticide trial at Brandon, SRA. Hand sections of bifenthrin treated asymptomatic plants were compared with untreated controls (UTC) showing YCS symptoms. Microscope results showed that the callose and blockage was more prominent in the midribs of the UTC leaf +4 tested compared to that in the lamina vascular bundles (Figure 141, Figure 142). However, both lamina and midribs in the UTC showed that the phloem sieve tubes are not as clear as that in the bifenthrin treated

samples, which could be partly responsible for the reduced transport of water and metabolites in the plant.

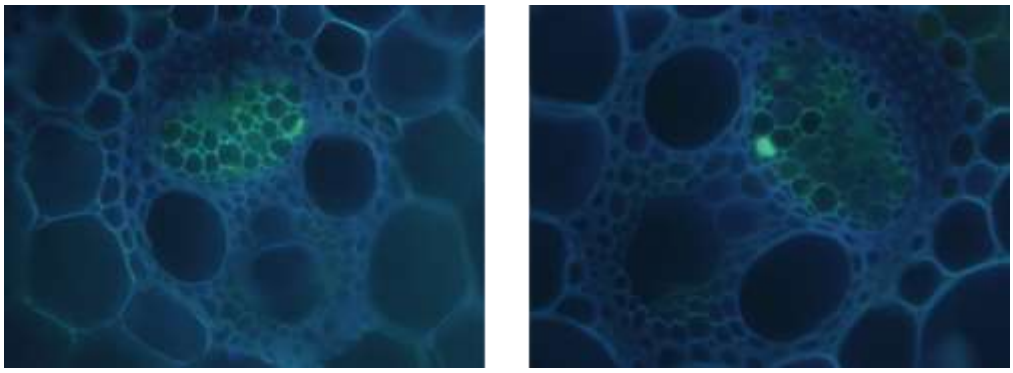


Figure 141 Aniline blue viewed as a fluorescent dye in free-hand sections of Bifenthrin asymptomatic leaf4 (LHS) and UTC YCS symptomatic leaf4

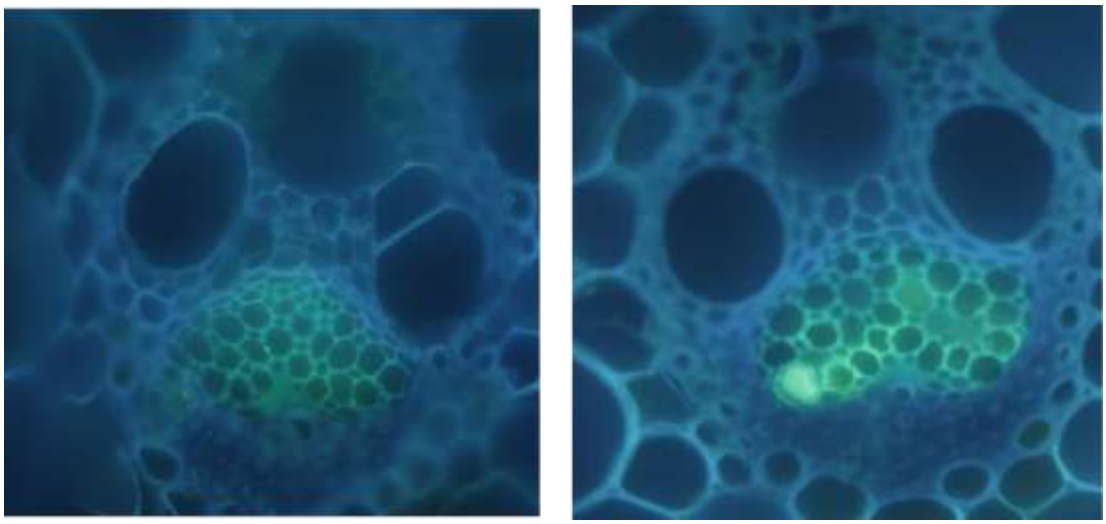


Figure 142 Aniline blue viewed as a fluorescent dye in sections of Bifenthrin asymptomatic midrib of leaf4 (LHS) and UTC YCS symptomatic midrib of leaf4.

Summary

Fluorescent and tracer dyes show there is some callose blockage of vascular tissue in YCS symptomatic leaf tissue which may be site section specific or more extensive. A larger number of sample sections would need to be processed and view to draw any firm conclusions. Furthermore, quantitative fluorescence spectroscopy studies of large sections of lamina, midrib and sheath showed no correlation between callose quantity and YCS symptomatic plants (Scalia et al., 2020). This suggests that higher levels of callose may be site specific rather than extensively located throughout the vasculature. No pathogenic organisms were confirmed in association with YCS plants in any of the stained sections.

6.5.5.2 Electron microscopy (EM)

Scanning electron microscope (SEM) research

Presented below are the photographs (Figure 143) and SEM images (Figure 144, Figure 145, Figure 146) of healthy and YCS leaves of KQ228$^{\phi}$ collected during the first trip to Burdekin in January. No obvious differences were immediately visible.

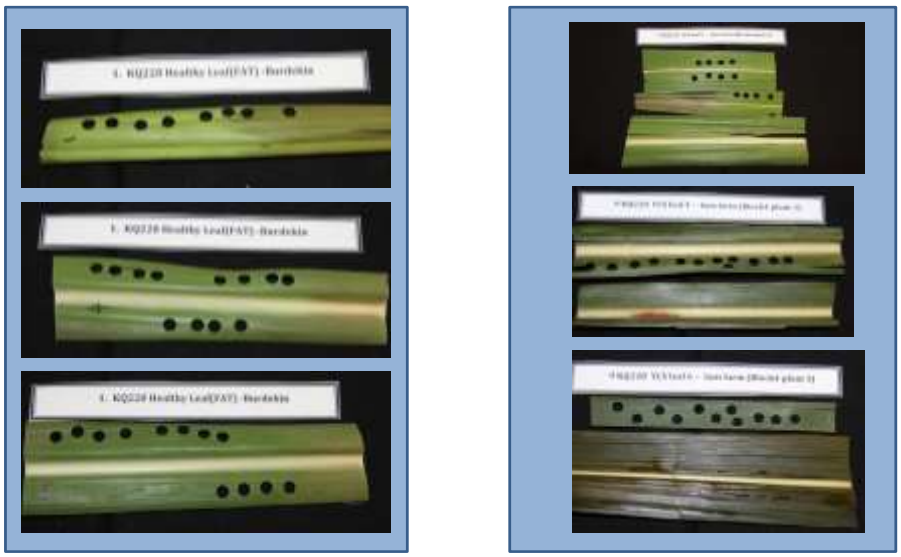


Figure 143 Sampling method of KQ228$^{\phi}$ leaves for SEM analysis

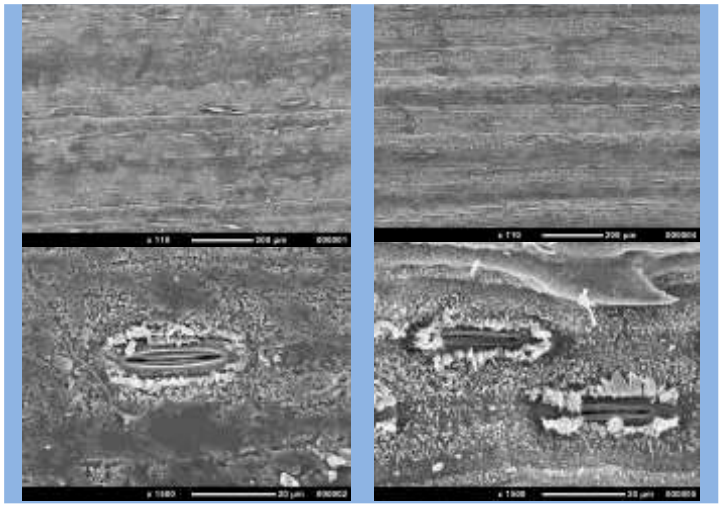


Figure 144 SEM images of the first not fully unfurled leaf-1 (FVD=leaf 1) of healthy and YCS leaves

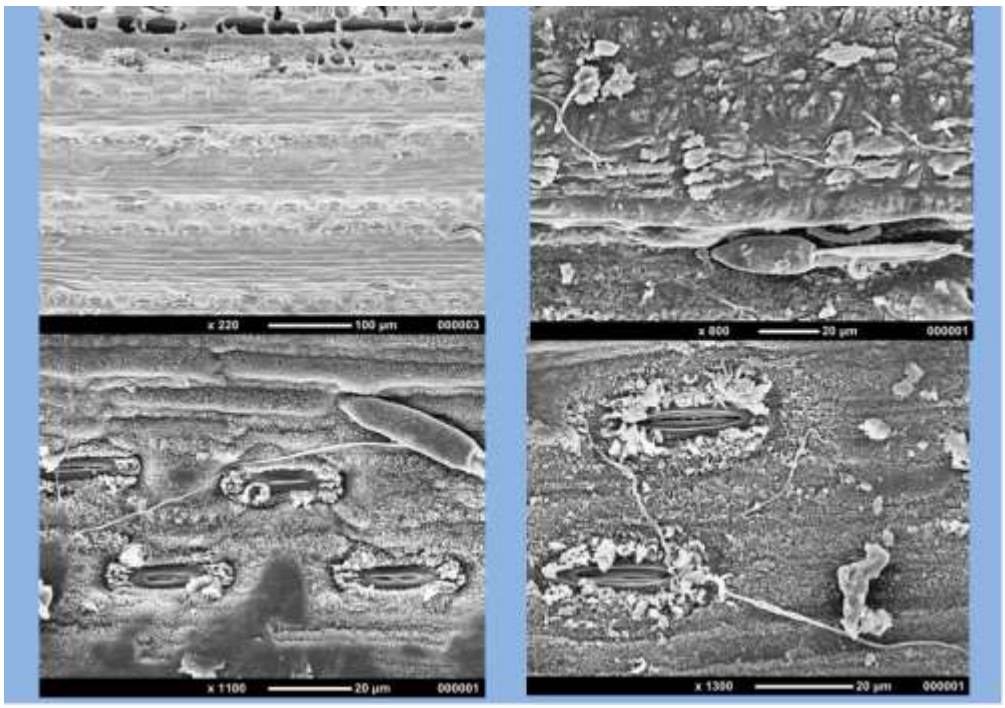


Figure 145 SEM images of the leaf 2 of healthy and YCS leaves

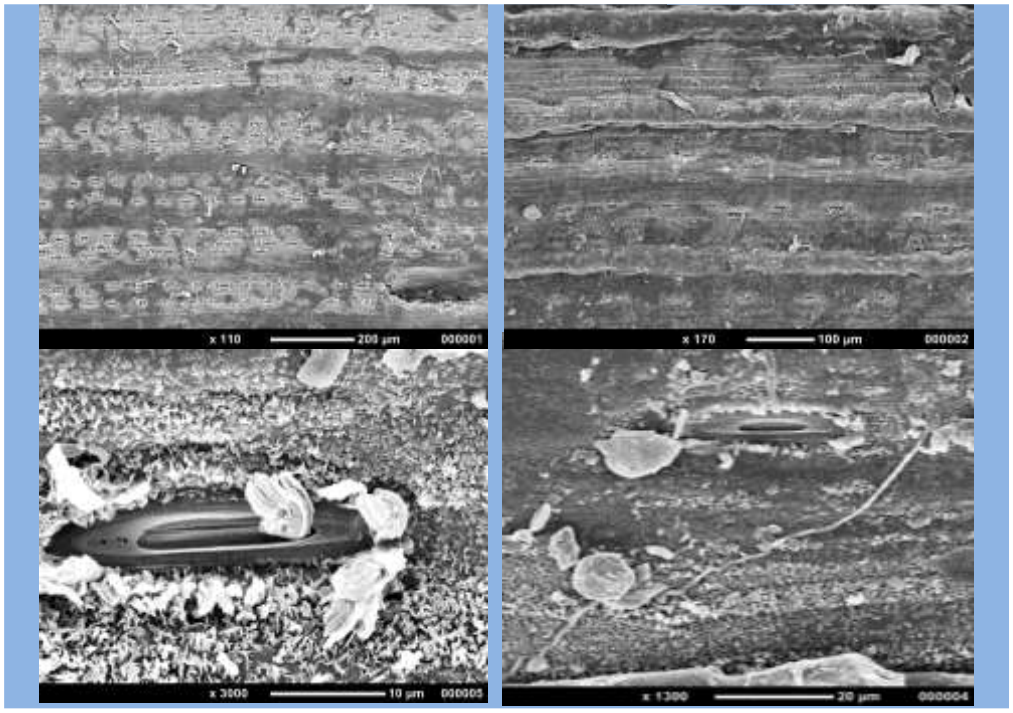


Figure 146 SEM images of leaf 4 of healthy and YCS leaves

Phytoplasmas

A rapid screening method using SEM on glutaraldehyde preserved tissues was developed and used to investigate various tissue (Table 35). Phytoplasma-like organisms were observed (Figure 148, Figure 149, Figure 150) in many of these samples as described by (Lebsky and Poghosyan, 2014). (Figure 147). However, it is worth noting that the appearance of starch granules and phytoplasmas are very alike. Table 35 shows a summary of results.

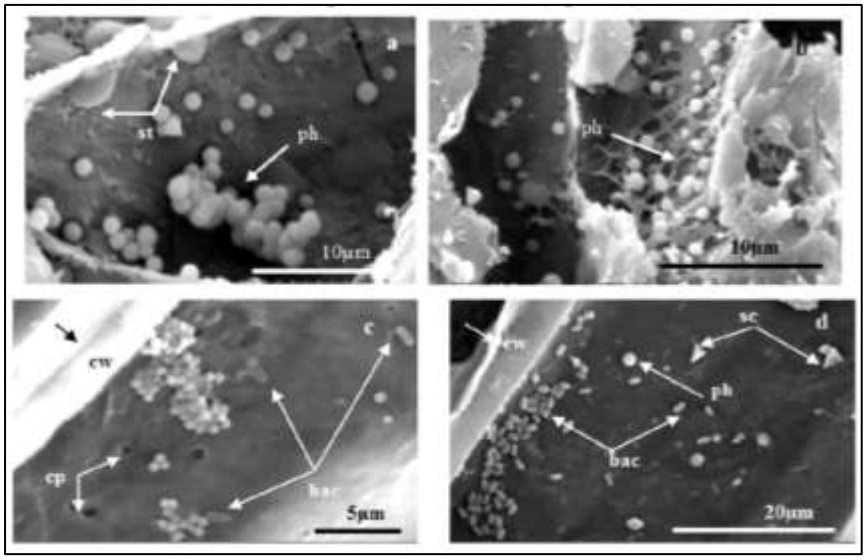


Figure 147 SEM images of phytoplasmas (ph) and bacteria (bac) in phloem tissues of field-grown (a and d) and indexed tomato plants (b and c). Starch grains (st), plasmadesmatal pores (cp), cell wall (cw) and salt crystals (sc) (Lebsky and Poghosyan, 2014)

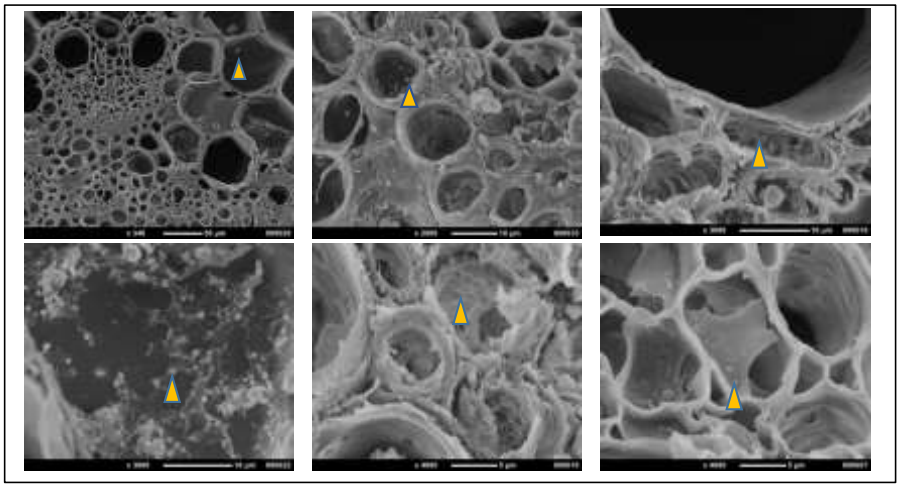


Figure 148 SEM images of midrib of Q208[A], showing presence of globular organisms in the vascular bundles in phloem sieve tubes as well as phloem parenchyma cells (arrowhead)

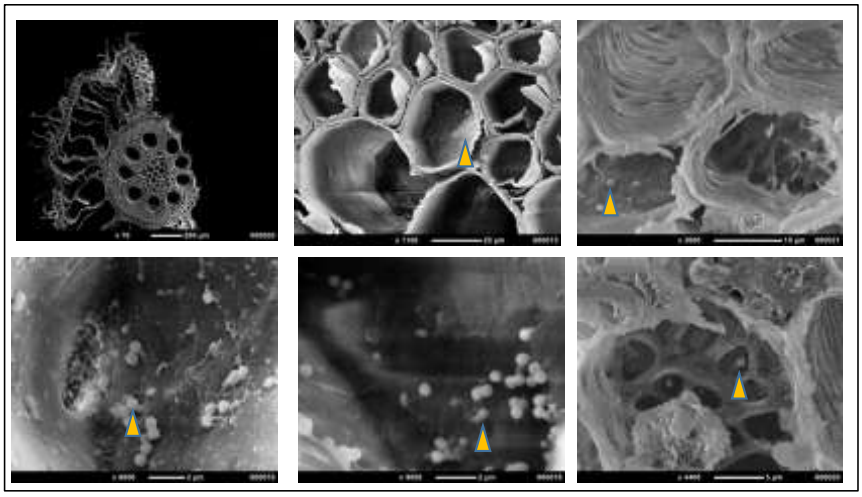


Figure 149 SEM of KQ228$^{\Phi}$ roots showing presence of phytoplasma-like organisms in the phloem sieve plates (arrowhead)

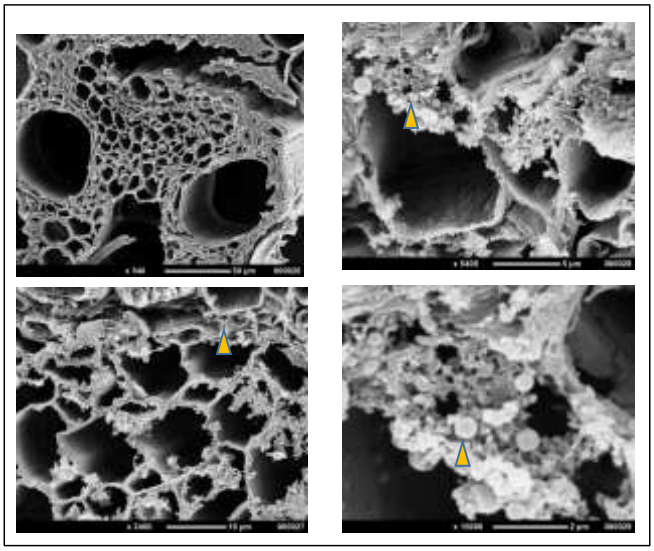


Figure 150 SEM images of midrib of YCS leaf1 in Q240$^{\Phi}$ showing presence of phytoplasma-like bodies (arrowhead) in phloem cells of the vascular bundle

Table 35 Plant samples collected and processed for SEM

Variety	Collection Date	Date processed	Collection place	Control/YCS	Processed Organ	SEM observation date	Presence of Phytoplasma like structures
Q208	18.04.2017	08.05.2017	Marybrough	Control	Leaf3	x	
					Midrib3	15.05.2017	Y/NO ?
					Sheath3	25.05.2017	No
Q208	18.04.2017	08.05.2017	Marybrough	YCS	Leaf3	22.8.17 /21.9.17	yellow Lf3 :No
					Midrib3	15.05.2017	Yes
					Sheath3	x	
KQ228		25.05.2017	from Burdekin	Control	Xylem sap	27.05.2017	No
			by Gerard	YCS	Xylem sap	27.05.2017	Yes
KQ228	3.3.2016	07.06.2017	Burdekin	Control	Roots	24.07.17	No
				YCS	Roots	07.06.17/9.07.17	Yes
Q208	3.3.2016	07.06.2017	Burdekin	Control	Roots	09.06.17	No
				YCS	Roots	07.06.17/09.06.17	No
Q240	3.3.2016	07.06.2017	Burdekin	Control	Roots	24.07.17	No
				YCS	Roots	15.08.17	
Q200	13.6.2017	16.06.2017	Burdekin	YCS	Xylem sap		No
Q208	13.6.2017	16.06.2017	Burdekin	YCS	Xylem sap		No
Q240	13.6.2017	16.06.2017	Burdekin	YCS	Xylem sap	01.08.2017	No
KQ228	13.6.2017	16.06.2017	Burdekin	YCS	Xylem sap		No
Q208		30.06.2017	from HT	control(TC)	Leaf	x	
				control(TC)	Roots	25.07.17	No
Q208		30.06.2017	from Marybrough TC	YCS	Roots	10.07.17 /13.10.17	Yes
Q240			from HT	Control(TC)	Roots	x	
Q240		30.06.2017	from Marybrough TC	YCS	Roots	13.07.17	
KQ228	13.6.2017	30.06.2017	Burdekin	YCS	In1,meristem	10.7.17/18.7.17	Yes
Q240	13.6.2017	30.06.2017	Burdekin	YCS	In1, meristem	10.7.17/18.7.17	Yes
Q200	13.6.2017	30.06.2017	Burdekin	YCS	In1, meristem		
Q200	03.07.2017	17.07.2017	from NSW	Controls	In1		
Q208	03.07.2017	17.07.2017	from NSW	Controls	In1		
Q240	03.07.2017	17.07.2017	from NSW	Controls	In1	17.07.2017	
KQ228	03.07.2017	17.07.2017	from NSW	Controls	In1	17.07.2017	
Q200	03.07.2017	27.07.2017	from NSW	Controls	In1, whorls		No
Q208	03.07.2017	27.07.2017	from NSW	Controls	In1, whorls	01.08.17	No
Q240	03.07.2017	27.07.2017	from NSW	Controls	In1, whorls	01.08.17	No
KQ228	03.07.2017	27.07.2017	from NSW	Controls	In1, whorls	01.08.17	No
KQ228	31.08.2017	05.09.2017	Tully	Untreated	Internodes and nodes	08.09.2017	Yes
				Streptomycin treated		08.09.2017	
KQ228	31.08.2017	14.09.2017	Tully	Untreated	Internodes and buds		
				Streptomycin treated			
Q240	23.02.2016	06.10.2017	Burdekin	control	Mr1,4,6	10.10.2017 and	
				YCS		16.10.2017	
Q208	12.10.2017	23.10.2017	Quarantine glass	control	Lf1,Mr1	24.10.2017	
			house Indo:				
Q208	23.01.2015	23.10.2017	Mackay	control	Lf1,Mr1	24.10.2017	
				YCS			
KQ 228	14.06.2016	23.10.2017	Burdekin	Control	Roots	24.10.2017	
				YCS			Yes

Further analysis of samples did not show unequivocal presence of micro-organisms in YCS samples. Additionally, these images were sent to world experts in UK (Dr. Phil Jones, Rothamsted Research) and Italy (Prof. Assunta Bertaccini, University of Bologna). Both experts were reserved and not convinced these images were specifically of phytoplasma. SRA pathology research also support this conclusion.

Transmission Electron Microscopy (TEM)

TEM at UQ was used to analyse sections of leaf, midribs and sheath of YCS and non-symptomatic plants taken from a cross-section of field visits. The original protocol took approximately two weeks before samples could be assessed which was extremely time consuming. A new method developed in consultation with the microscopists at UQ enabled a turnaround time in two days (Figure 151). This new protocol retains both shape and structure and is amenable to toluidine blue staining. In addition, further tracking of regions of interest using this method is feasible as thicker sections are

first taken for observation with the light microscope, then followed by TEM of interesting samples warranting closer examination.

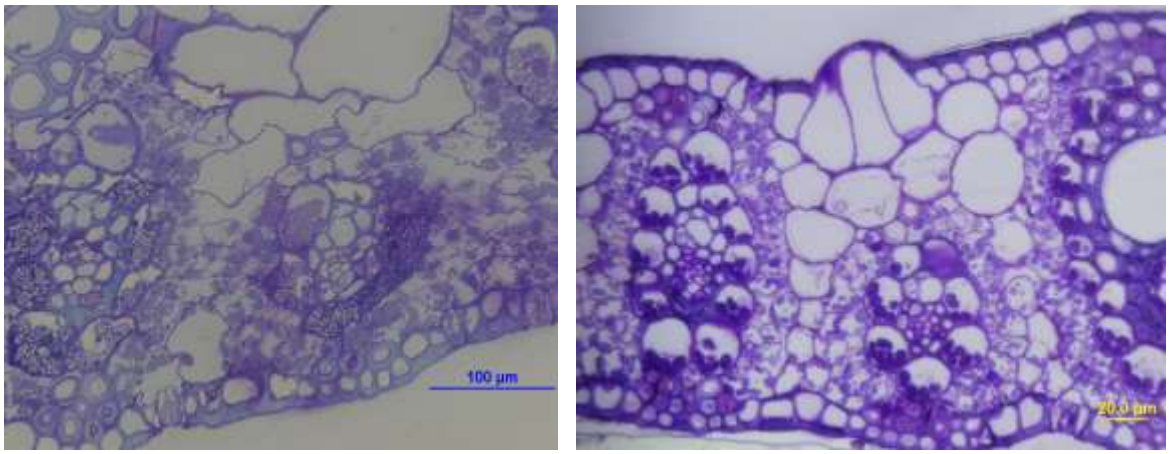
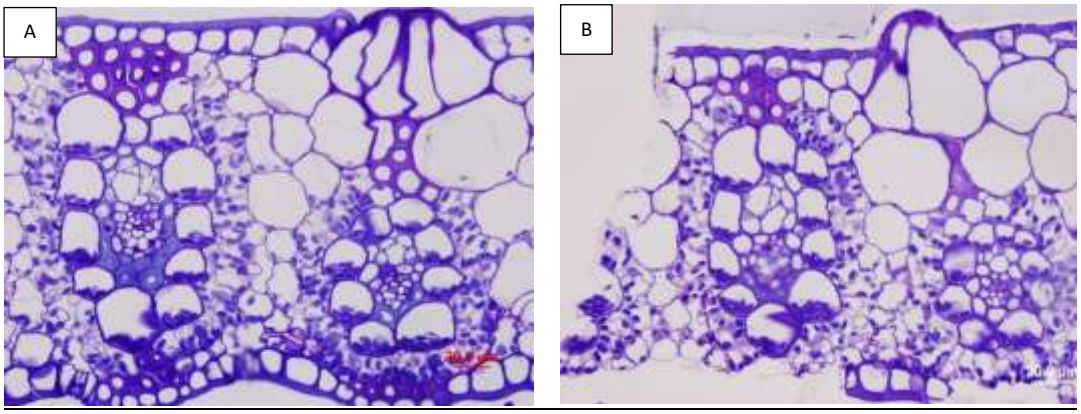


Figure 151 KQ228$^{\phi}$ leaves processed with the old protocol (left) or new one (right)

Using this new protocol, systematic comparisons of healthy and YCS affected tissues was conducted. Sections of leaf, midribs and sheath (Figure 152) for two varieties have been taken using the new protocol and critically examined with the light microscope.

Healthy KQ228$^{\phi}$ leaf section YCS KQ228$^{\phi}$ leaf section



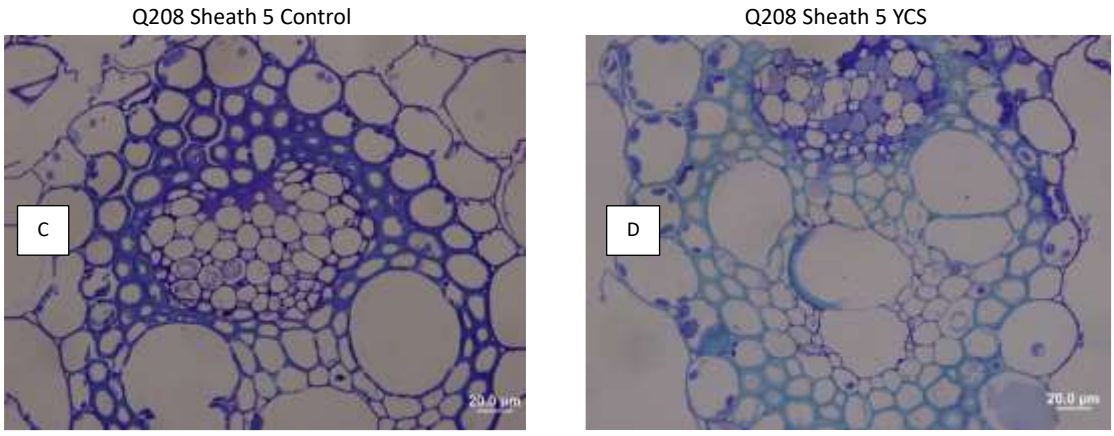


Figure 152 Light microscope sections of leaf (A and B) and sheath of control and YCS affected plants (C and D)

Using the new protocol, several hours were spent scrutinising the grids under TEM, and only occasionally did we see any micro-organisms in KQ228$^{\phi}$ (Figure 153, Figure 154, Figure 155).

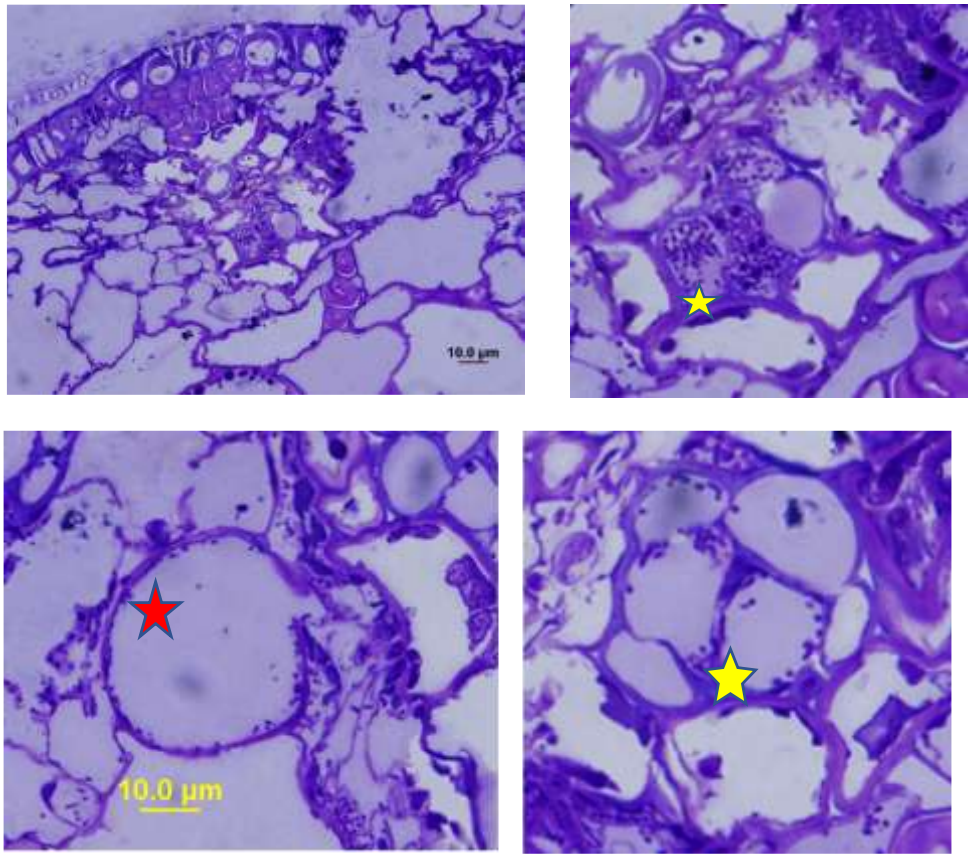


Figure 153 TEM image of YCS affected leaf 5 showing presence of bacteria in phloem ( ) and mesophyll cells surrounding the VB ( ). Also note the general appearance of cells showing collapsed cell walls.

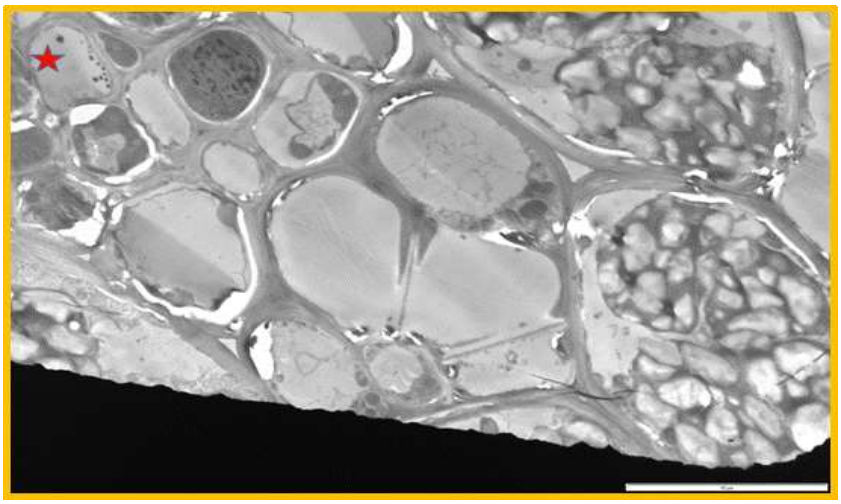


Figure 154 Close up of lamina of YCS affected plant showing presence of starch in the bundle sheath cells and also dark dense bodies in the phloem cell (★)

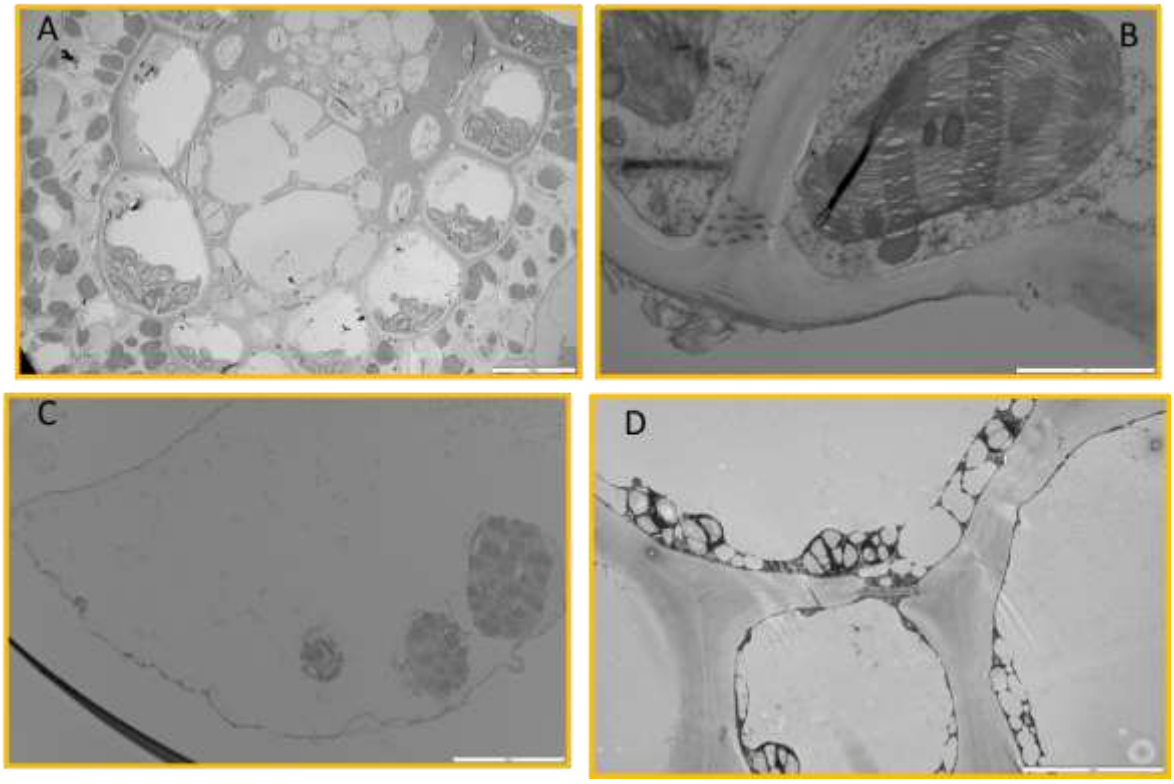


Figure 155 TEM of a Q240$^{\phi}$ YCS plant. A- Leaf 6 showing starch in the bundle sheath cells, B- Presence of starch in the mesophyll cells C. Degrading chloroplast in the sheath of YCS plant D. Starch accumulation in the dewlap of the YCS leaf

The TEM work has detected micro-organisms in some of the samples, but as many of the prepared samples have not yet been analysed under the TEM, it is difficult to conclude whether these micro-organisms are involved in YCS. For firm conclusions to be made using this technology the precise

target cells /tissue would need to be identified and many more sections would be required analysing organs and organelles in different varieties.

Roots

Root samples were collected from the field in the Burdekin in February 2016 and brought back to Brisbane on ice. They were preserved in glutaraldehyde, as per the standard protocol, and stored at 4^0C until ready to process further. Samples were then prepared for TEM using the developed method, and thick (~1000µm) for light microscopy and thin (~200 µm) for TEM sections taken with the microtome. These sections were then compared under the compound microscope.

Results shown below (Figure 156) show that the size and integrity of the cells in both healthy and YCS roots look similar (A and B). However, closer examination of the xylem vessels showed that there was severe occlusion in the YCS plants compared to the health controls (C and D). In addition, examination of the phloem sieve plate also indicated a similar occlusion (E and F). Other regions of the roots did not show any significant differences under the light microscope (G and H).

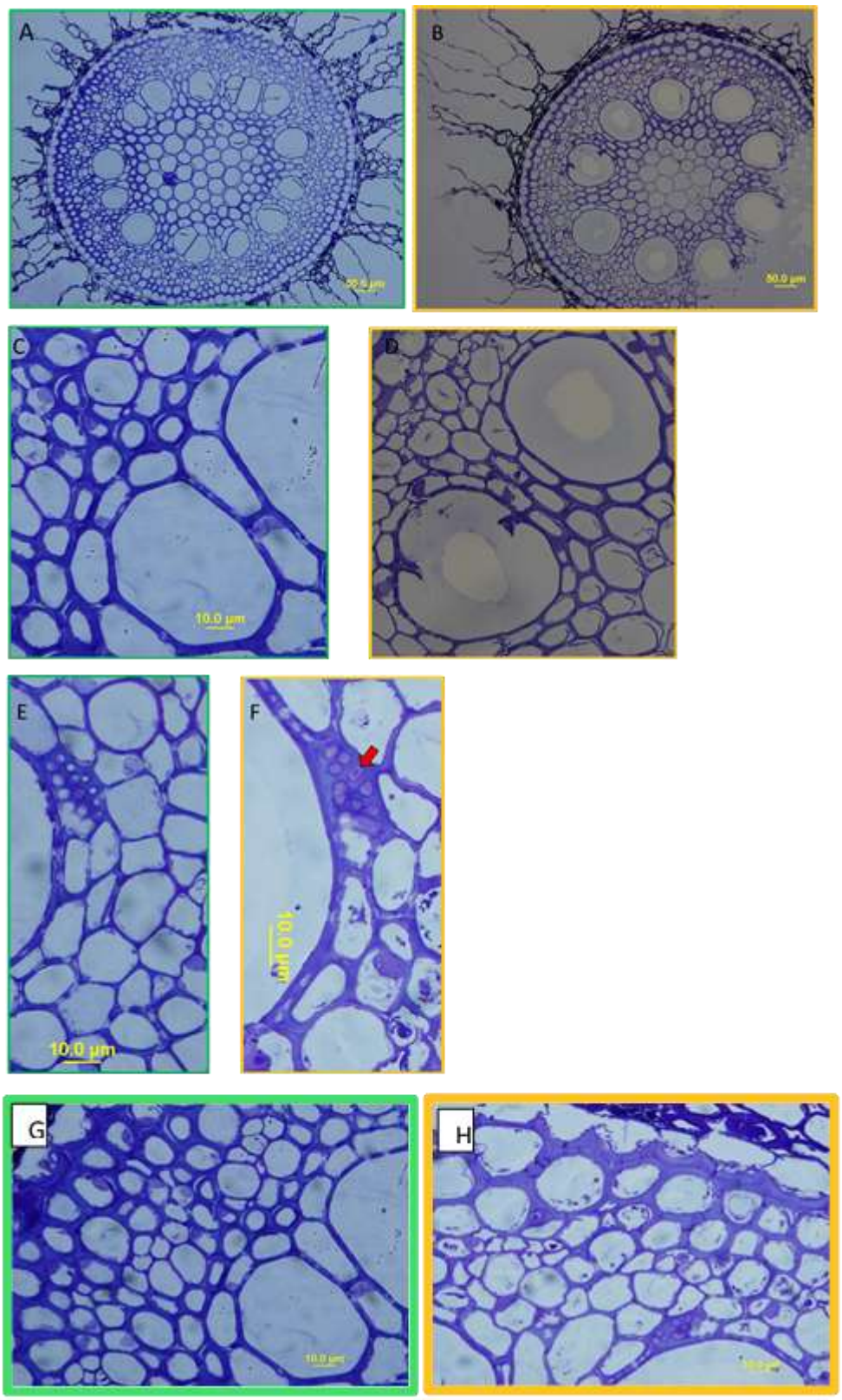


Figure 156 Transverse sections of roots of healthy (green border A,C,E and G) and YCS plants (orange border B,D,F and H) of KQ228$^{\Phi}$. Note the occlusion present in the xylem vessels in YCS affected roots (B and D). Arrowhead in F) showing occlusion /blockage in the sieve pores

Previous samples collected and preserved in glutaraldehyde were processed and viewed with TEM. This study focussed on KQ228$^{(\phi}$ as this variety showed the most severe symptoms to YCS in the Burdekin. Leaf +1 and leaf +4 of healthy and YCS stalks were fixed, dehydrated, embedded, stained and sectioned for EM. Each of these steps had to be optimised for sugarcane. This enabled identification of the different cell types, organelles and orientation of the sections. The bundle sheath cells showed starch accumulation in the leaves of healthy controls which concurred with starch assays performed previously (Figure 157, Figure 158). However, to visualise minute changes in structure or anatomy, thinner sections required the use of a diamond-edge knife. Comparisons of cells of healthy and YCS leaf sections at this magnification using bright field microscopy, did not show any obvious differences in the appearance of YCS affected cells. However, one initial grid of leaf sections of KQ228 leaf +4 did show the possible presence of viruses (Figure 159 B-D). This concurs with studies by Dr. Kathy Braithwaite that shows an abundance of viral particles present in sugarcane plants (see section 6.8.1.3 of this report).

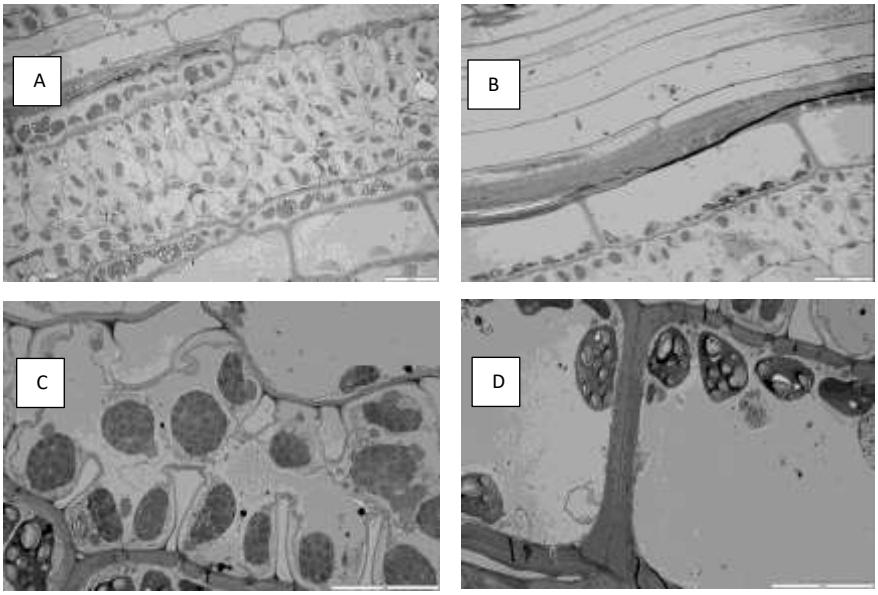


Figure 157 Leaf sections of the youngest healthy leaf +1 showing the components of the leaf. The starch granules in the bundle sheath cells are clearly visible in figure 9D

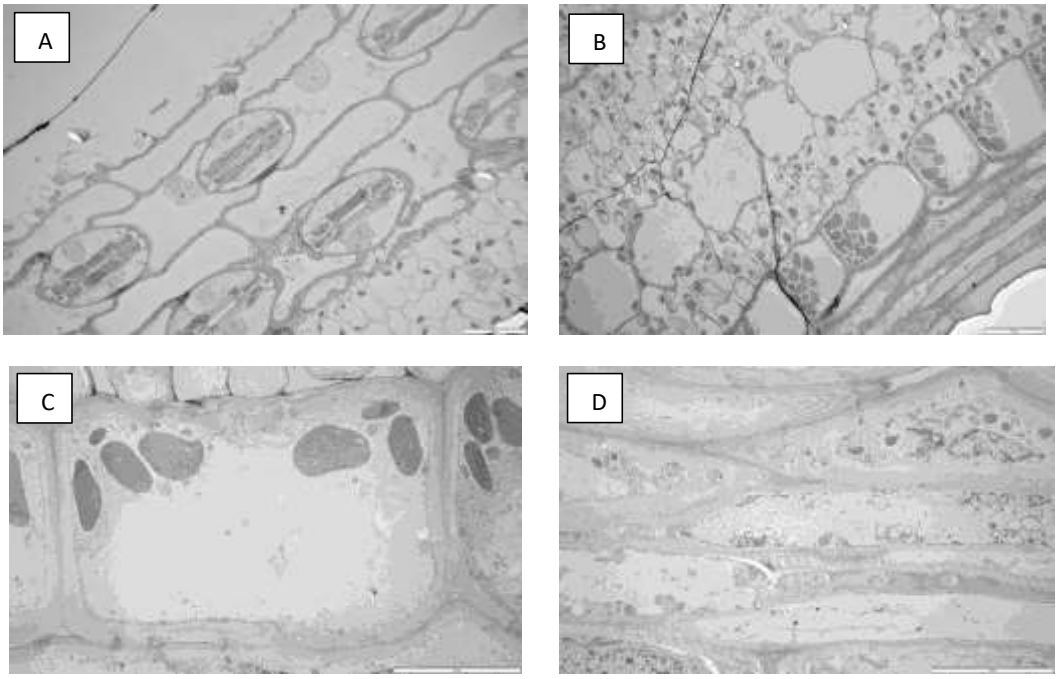


Figure 158 Leaf sections of the youngest YCS leaf +1 showing the components of the leaf. Less starch is present in the bundle sheath cells in this leaf (C). Figure 10D shows the LS of the phloem

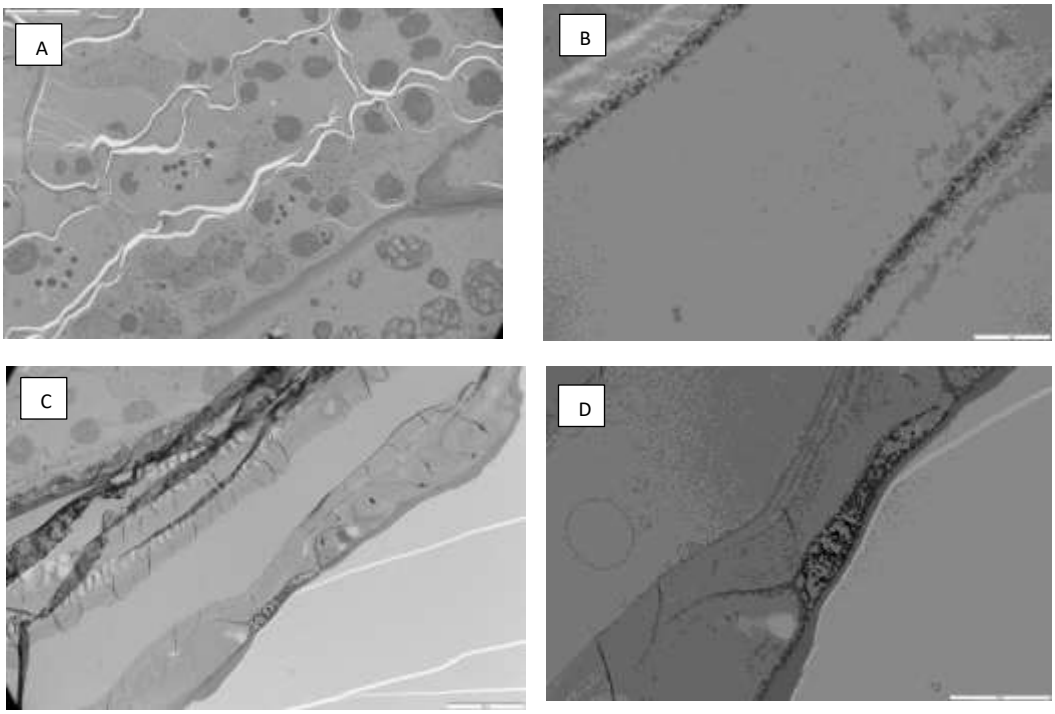


Figure 159 Initial images of phloem of leaf 4 of KQ228 showing YCS symptoms (FVD= leaf 3). Starch granules are visible in the bundle sheath cells A). Electron dense particles are visible in B), and in the phloem C) and magnified in D).

In December 2017, all microscopy worked ceased under the recommendation of the SRP. Table 36 shows the various samples collected between 2015-2017 and the level of completion.

Table 36 Samples collected from the field for processing for electron microscope work. A number of samples are at various stages of the processing protocol as shown in the table.

	Fifth sample collection for TEM (YCS FV07 26.02.2015)								
Variety	Tube no	Leaf no	dehydration completed?	Processing method (old/new)	Microtomy done?	1000nm sections (Y/N)	Number of samples	Light microscopy done? (Y/N)	Number of samples
Q208Y	44	green Lf6							
Q208Y	45	Yellow Lf6							
Q208Y	46	Green Lf5 distal							
Q208Y	47	Green leaf 5 proximal							
Q208H	48	Leaf 4							
Q208H	49	Lf6							
Q208H	50	Lf5							
Q208Y	51	Lf4							
Q208Y	53	Lf3							
Q208Y	54	Lf 3 MR							
Q208Y	55	Lf2							
Q208Y	56	Lf2 MR							
Q208Y	57	Lf0							
Q208Y	58	Lf0 MR							
Q208H	59	Lf3							
Q208H	60	Lf3 MR							
Q208H	61	Lf2							
Q208H	62	Lf2 MR							
Q208H	63	Lf0							
Q208H	64	Lf0 MR							
		being processed							

23-02-16 collection

Variety	Sample no	Leaf no	type of section	GA/GA+C	dehydration completed? (Y/N)	Processing method (old/new)	Microtomy done?	1000nm sections (Y/N)	Number of samples
Q240Y1	1	Lf1	Lf	GA	Y	New	Y	Y	
Q240Y1	2	Lf1	Mr	GA					
Q240Y1	3	Lf1	Sh	GA					
Q240Y1	4	Lf1	Dw	GA	Y	New	Y	Y	
Q240Y1	5	Lf1	Lf	GA+C	Y	New	Y	Y	
Q240Y1	6	Lf1	Mr	GA+C					
Q240Y1	7	Lf1	Sh	GA+C					
Q240Y1	8	Lf1	Dw	GA+C					
Q240Y1	9	Lf4	Lf	GA	Y	New	Y	Y	
Q240Y1	10	Lf4	Mr	GA					
Q240Y1	11	Lf4	Sh	GA	Y	New	Y	Y	
Q240Y1	12	Lf4	Dw	GA	Y	New	Y	Y	
Q240Y1	13	Lf4	Lf	GA+C					
Q240Y1	14	Lf4	Mr	GA+C					
Q240Y1	15	Lf4	Sh	GA+C					
Q240Y1	16	Lf4	Dw	GA+C					
Q240Y1	17	Lf6	Lf	GA	being processed				
Q240Y1	18	Lf6	Mr	GA					
Q240Y1	19	Lf6	Sh	GA					
Q240Y1	20	Lf6	Dw	GA					
Q240Y1	21	Lf6	Lf	GA+C					
Q240Y1	22	Lf6	Mr	GA+C					
Q240Y1	23	Lf6	Sh	GA+C					
Q240Y1	24	Lf6	Dw	GA+C					
Q240H1	25	Lf1	Lf	GA	Y	New	Y	Y	
Q240H1	26	Lf1	Mr	GA					
Q240H1	27	Lf1	Sh	GA					
Q240H1	28	Lf1	Dw	GA	Y	New	Y	Y	
Q240H1	29	Lf1	Lf	GA+C	Y	New	Y	Y	
Q240H1	30	Lf1	Mr	GA+C					
Q240H1	31	Lf1	Sh	GA+C					
Q240H1	32	Lf1	Dw	GA+C					
Q240H1	33	Lf4	Lf	GA	Y	New	Y	Y	
Q240H1	34	Lf4	Mr	GA					
Q240H1	35	Lf4	Sh	GA	Y	New	Y	Y	
Q240H1	36	Lf4	Dw	GA	Y	New	Y	Y	
Q240H1	37	Lf4	Lf	GA+C					
Q240H1	38	Lf4	Mr	GA+C					
Q240H1	39	Lf4	Sh	GA+C					
Q240H1	40	Lf4	Dw	GA+C					
Q240H1	1	Lf6	Lf	GA	being processed				
Q240H1	42	Lf6	Mr	GA					
Q240H1	43	Lf6	Sh	GA					
Q240H1	44	Lf6	Dw	GA					
Q240H1	45	Lf6	Lf	GA+C					
Q240H1	46	Lf6	Mr	GA+C					
Q240H1	47	Lf6	Sh	GA+C					
Q240H1	48	Lf6	Dw	GA+C					
								EM analysis soon	

Variety	Tube no	23/01/2015		Sample	dehydration completed? (Y/N)	Processing method (old/new)	Microtomy done?	1000nm sections (Y/N)	Number of samples	Light microscopy done? (Y/N)	Number of samples	100nm sections (Y/N)	TEM (Y/N)
Q200 Y-1	1	v	Lf 1	Lf									
Q200 Y-1	2	v	Lf 1	Mr									
Q200 Y-1	3	v	Lf 1	Sh									
Q200 Y-1	4	v	Lf 1	IN									
Q200 Y-1	5	v	Lf3	Lf 3									
Q200 Y-1	6	v	Lf3	Mr									
Q200 Y-1	7	v	Lf3	Sh									
Q200 Y-1	8	v	Lf3	IN									
Q200 Y-1	9	v	Lf5	Lf 5									
Q200 Y-1	10	v	Lf5	Mr									
Q200 Y-1	11	v	Lf5	Sh									
Q200 Y-1	12	v	Lf5	IN									
Q200 H-1	31	v	Lf 1	Lf									
Q200 H-1	32	v	Lf 1	Mr									
Q200 H-1	33	v	Lf 1	Sh									
Q200 H-1	34	v	Lf 1	IN									
Q200 H-1	35	v	Lf3	Lf									
Q200 H-1	36	v	Lf3	Mr									
Q200 H-1	37	v	Lf3	Sh									
Q200 H-1	38	v	Lf3	IN									
Q200 H-1	39	v	Lf5	Lf									
Q200 H-1	40	v	Lf5	Mr									
Q200 H-1	41	v	Lf5	Sh									
Q200 H-1	42	v	Lf5	IN									
KQ228 Y-1	76	v	Lf 1	Lf 1	Y	new	Y	Y	4	Y	4	Y	Y
KQ228 Y-1	77	v	Lf 1	Mr									
KQ228 Y-1	78	v	Lf 1	Sh									
KQ228 Y-1	79	v	Lf 1	IN									
KQ228 Y-1	80	v	Lf5	Lf 5	Y	new	Y	Y	2	Y	1	N	N
KQ228 Y-1	81	v	Lf5	Mr									
KQ228 Y-1	82	v	Lf5	Sh									
KQ228 Y-1	83	v	Lf5	IN									
KQ228 Y-1	84	v	Lf6	Lf 6	Y	new	Y	Y					
KQ228 Y-1	85	v	Lf6	Mr									
KQ228 Y-1	86	v	Lf6	Sh									
KQ228 Y-1	87	v	Lf6	IN									
KQ228 H-2	107	v	Lf 1	Lf 1									
KQ228 H-2	108	v	Lf 1	Mr									
KQ228 H-2	109	v	Lf 1	Sh									
KQ228 H-2	110	v	Lf 1	IN									
KQ228 H-2	111	v	Lf5	Lf 5									
KQ228 H-2	112	v	Lf5	Mr									
KQ228 H-2	113	v	Lf5	Sh									
KQ228 H-2	114	v	Lf5	IN									
KQ228 H-2	115	v	Lf6	Lf 6									
KQ228 H-2	116	v	Lf6	Mr									
KQ228 H-2	117	v	Lf6	Sh									
KQ228 H-2	118	v	Lf6	IN									
Q208 Y-1	122	v	Lf 1	Lf 1									
Q208 Y-1	123	v	Lf 1	Mr									
Q208 Y-1	124	v	Lf 1	Sh									
Q208 Y-1	125	v	Lf 1	IN									
Q208 Y-1	126	v	Lf3	Lf 3									
Q208 Y-1	127	v	Lf3	Mr									
Q208 Y-1	128	v	Lf3	Sh									
Q208 Y-1	129	v	Lf3	IN									
Q208 Y-1	130	v	Lf5	Lf 5									
Q208 Y-1	131	v	Lf5	Mr									
Q208 Y-1	132	v	Lf5	Sh									
Q208 Y-1	133	v	Lf5	IN									
Q208 H-1	153	v	Lf 1	Lf 1									
Q208 H-1	154	v	Lf 1	Mr									
Q208 H-1	155	v	Lf 1	Sh									
Q208 H-1	156	v	Lf 1	IN									
Q208 H-1	157	v	Lf3	Lf 3									
Q208 H-1	158	v	Lf3	Mr									
Q208 H-1	159	v	Lf3	Sh									
Q208 H-1	160	v	Lf3	IN									
Q208 H-1	161	v	Lf5	Lf 5									
Q208 H-1	162	v	Lf5	Mr									
Q208 H-1	163	v	Lf5	Sh									
Q208 H-1	164	v	Lf 5	IN									

TEM analysis of YCS affected plants of KQ228$^{\Phi}$ showed the occurrence of bacteria/phytoplasma like organisms, present in both phloem cells as well as in mesophyll cells (Figure 160**Error! Reference source not found.**). While these dense bodies resemble microorganisms, failure of consistent presence in all YCS affected samples may indicate these to simply be opportunistic organisms rather than the causal agents. Also, the sourcing of a healthy control is a high priority prerequisite to enable us to make this conclusion.

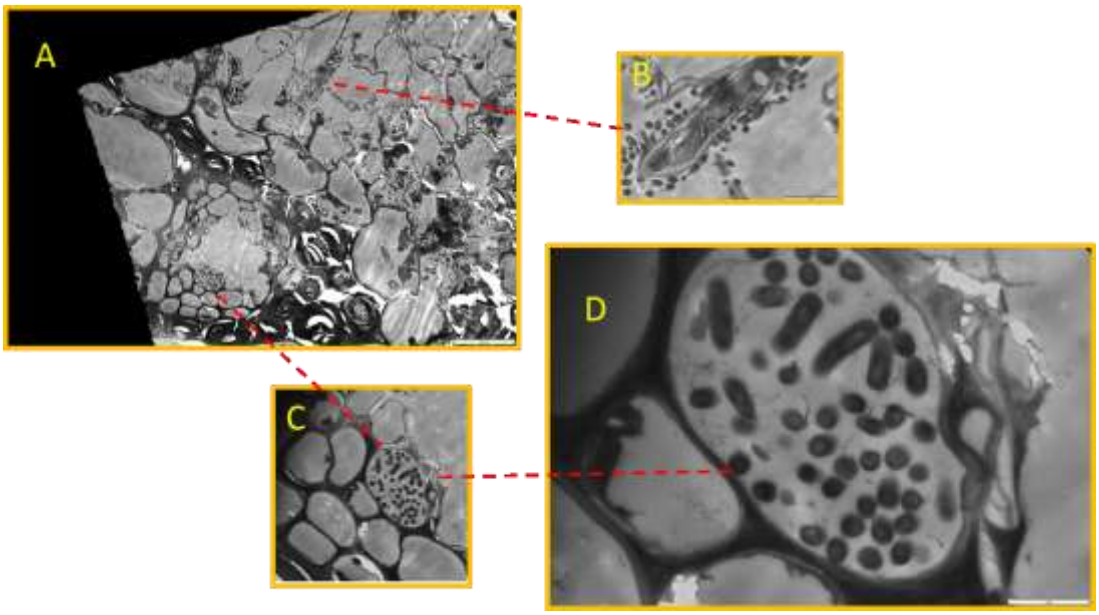


Figure 160 Images of the lamina of KQ228$^{\Phi}$ YCS affected plant showing presence of micro-organisms resembling bacteria/phytoplasmas present in phloem and mesophyll cells

Summary

SEM and TEM show the presence of microorganisms in some YCS symptomatic samples. These organisms have characteristics of phytoplasma, bacteria and viruses. TEM also shows occlusions in root xylem and phloem tissue. However, to be confident these organisms are YCS causal agents and not opportunistic endogenous microbes or secondary infections, they would need to be confirmed in all YCS samples and satisfy Koch's postulates. The pathology research presented in this report would suggest these organisms are secondary in nature (see section 6.8).

6.5.6 Dye uptake – water and metabolite transport

These studies were commenced to develop a method to study translocation of metabolites in sugarcane stalks and leaves. The hypothesis is that the yellowing of leaves in YCS plants is because the conducting tissues are blocked, thus inhibiting the movement of assimilates and nutrients within the plant.

Protocol development

Stalks of healthy KQ228(ϕ) sugarcane plants were obtained from SRA Woodford Pathology Station and brought back to Indooroopilly. These stalks were then recut under water and placed in a beaker containing either the 4% red or blue food dye (Figure 161 A). The dye Evan's blue was also tested as this is the only dye taken up by dead cells i.e., it cannot penetrate intact live cells. They were left for 24 hours in the solution and then assessed for the presence of dye. Stalks were cut above and below each node to assess the extent of dye uptake and whether the dye was present in all internodes. Leaf hand sections were also taken either close to the base i.e near the stalk (labelled proximal to dye) or closer to the leaf tip (labelled distal to dye).

Results showed that either colour dye was effective to study uptake in sugarcane (Figure 161 B). Both dyes were uniformly taken up into the leaves and could be easily visualised in the intact cabbage leaf whorls along the vascular bundles (Figure 161 C). The red dye however was less intense than the blue dye.

Sections of the stalks were taken and assessed for presence and distribution of the dye using the stereo microscope (Figure 162). Both blue and red dyes showed a similar pattern of distribution which was also similar in both internode 3 and internode 6. The dye seemed contained in the vascular bundles, without spreading into the adjoining parenchyma cells.

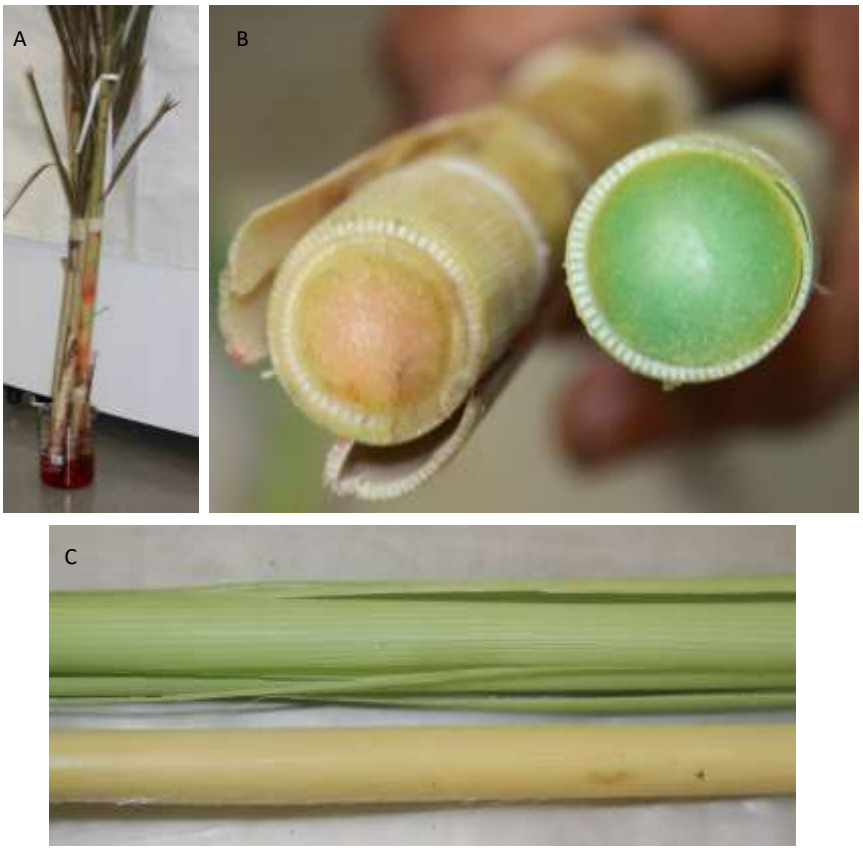


Figure 161 Laboratory setup for studying dye uptake by sugarcane stalks A). Stalks cut above the node to show uptake of both red and blue food dye B) and dyes taken up are visible in the vascular bundles of the leaves C).

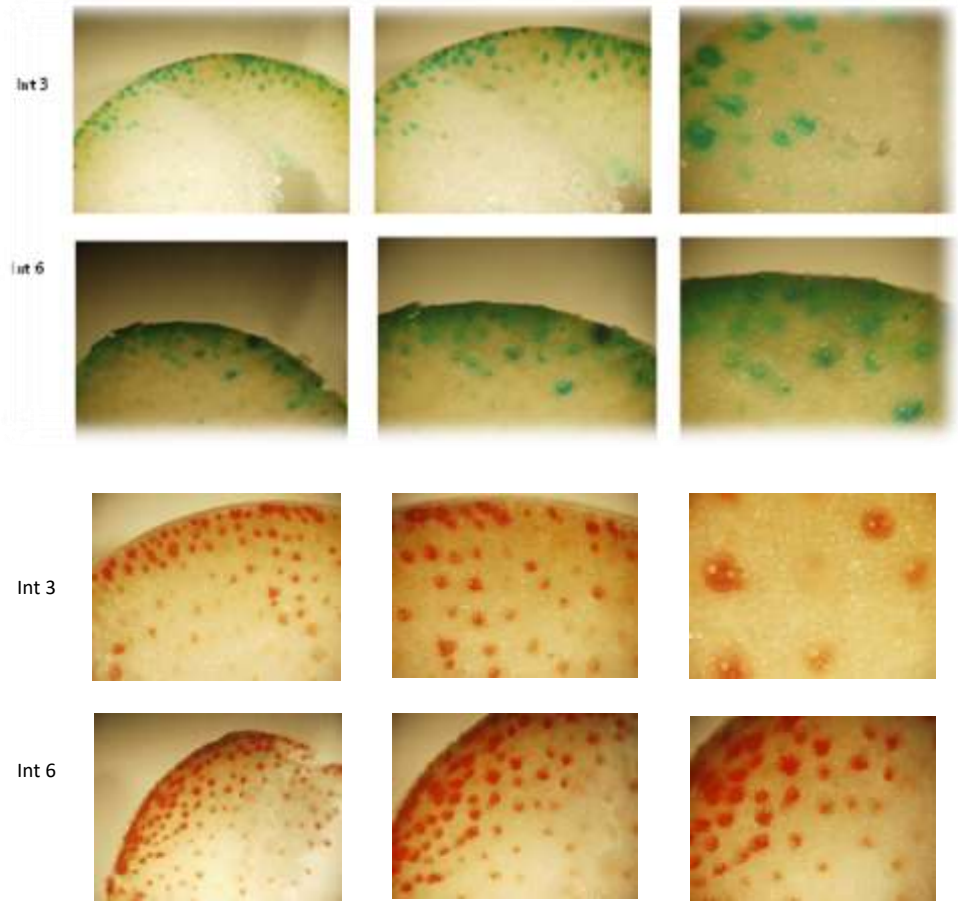
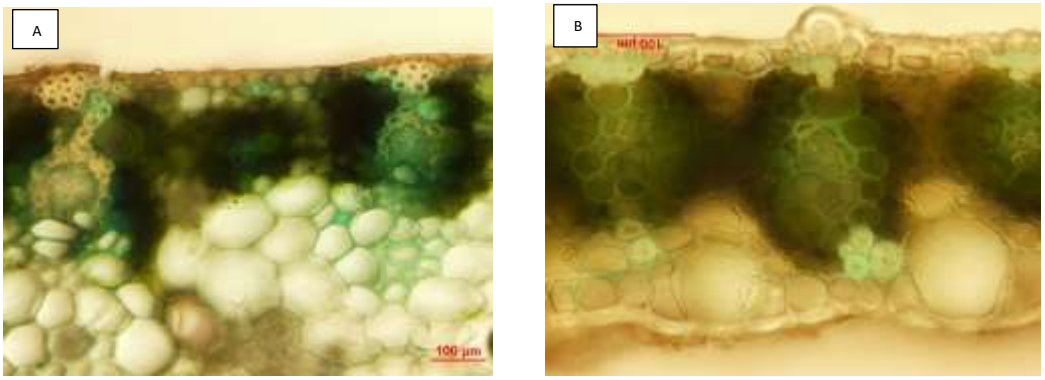


Figure 162 Visualisation of the dyes in internodes 3 and 6 using the stereo microscope at low, medium and high magnification

Leaf sections showed presence of dye in the vascular bundles in proximal (Figure 163 A & B) and distal (Figure 163 C & D) sections using blue and red food dyes. Comparing proximal to distal sections it is evident that the dye in the proximal region has infiltrated the apoplastic region of the cells surrounding the vascular bundles. In the distal sections the spread of the dye into the apoplast of surrounding cells is much less (Figure 163 B & D). The sections also show that there is less dye present when the red dye was used as compared to the blue dye.



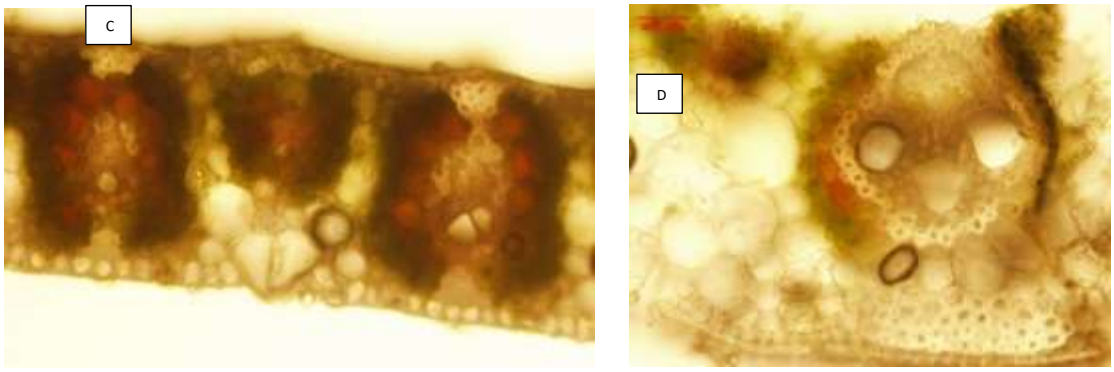


Figure 163 Leaf sections showing presence of dye in the apoplastic region of cells surrounding the vascular conducting tissues. A and C are of the region proximal to the dye while B and D are distal to the dye.

Uptake of Evan's blue compared to the blue food dye showed that the former is contained and only visible in the xylem conducting tissues while the food dye spreads in to the apoplast of adjoining cells (Figure 164).

Figure 164 Uptake pattern of blue food dye (left) versus Evan's blue (right) by KQ228 healthy stalks over a 24-hour period

1. Uptake studies in healthy and YCS symptomatic stalks of Q240$^{\Phi}$

Having successfully developed a protocol to study uptake of dyes in sugarcane stalks, the uptake of different dyes was tested in Q240 stalks (healthy and YCS) that were sent to Brisbane from the Burdekin. For this section of the report, the asymptomatic stalks will be referred to as 'healthy'.

The stalks were cut under the dye solution and left to take up the dye for 24 hours. Four different dyes were tested:

1. Red food dye (16%)
2. Blue food dye (4%)
3. Red and Evan's blue dyes mixed (8% +0.25% respectively)
4. Evan's blue dye at 0.5% (wt/vol)

Internodes were cut out and observed under the stereo microscope as described above.

Results

Uptake of all dyes occurred in the 24-hour period, and could be clearly seen in the leaves and sheath of healthy and YCS stalks (Figure 165 A-C).

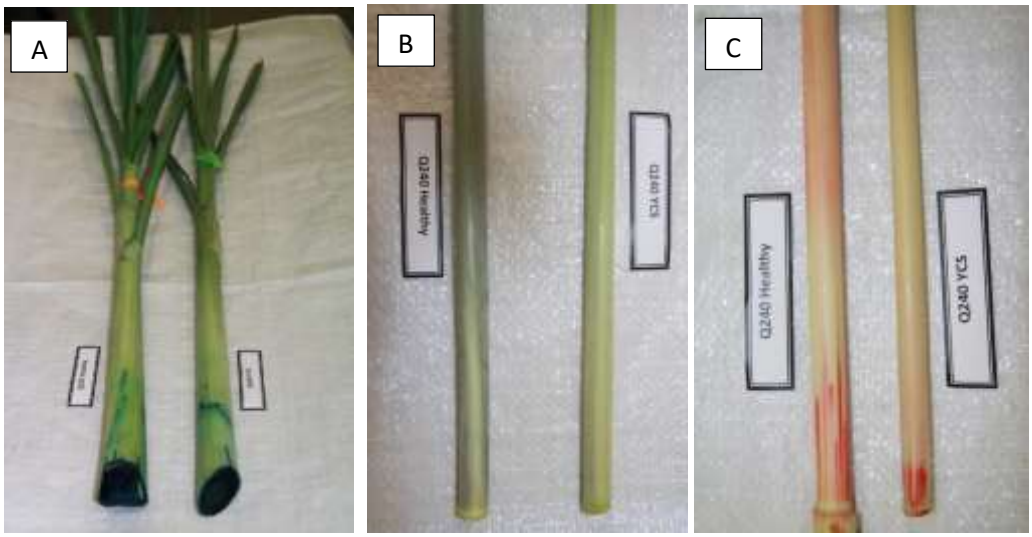


Figure 165 Uptake of various dyes by stalks of healthy and YCS affected Q240 plants.

Stereo microscopy was used to view staining of the vascular tissue within healthy and YCS stalks. Evan's blue was uniformly localised in the vascular bundles over the entire internode of healthy plants (Figure 166 A), while YCS stalks showed reduced dye colour (Figure 166 B). Also, the dye pattern especially in internode +3 was patchy in the YCS stalks with colour diffusing into adjacent cells. This could be due to loss of membrane integrity resulting in leakiness, and dye diffusing into the cell.

In contrast, the food dyes showed more patchiness in internodes +1 and +2 when compared to YCS stalks (Figure 167). A possible reason for this could be that the uptake was a lot faster in healthy stalks, resulting in the dye diffusing into the apoplastic area.

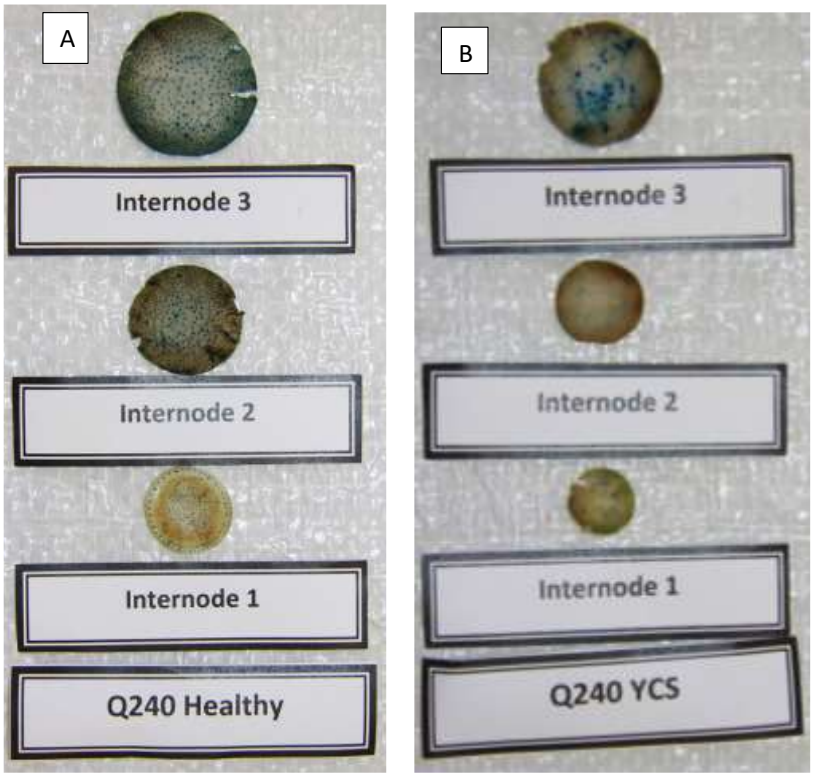


Figure 166 Uptake of Evan's blue dye by healthy and YCS stalks. Internodes were cut after 24 hours of uptake and examined under the stereo microscope

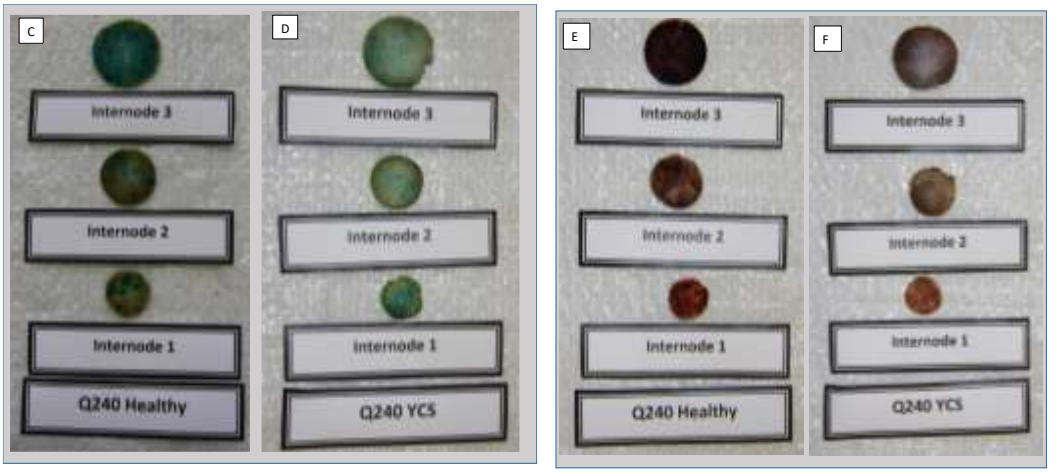


Figure 167 Uptake of blue (C-D) and red (E -F) food dye by healthy and YCS stalks. Internodes were cut after 24 hours of uptake and examined under the stereo microscope

From these experiments a suitable protocol for dye uptake studies in sugarcane was developed, using food dyes as well as Evan's blue. Internode +3 or older internodes is recommended for future studies, as the younger internodes are too variable and inconsistent to uptake. Differences in dye uptake were observed between healthy and YCS affected stalks of Q240. Results using Evan's blue

suggest that in YCS stalks, there is a breakdown of membrane integrity, resulting in the dye diffusing into living cells.

2. Dye uptake in isolated leaves

Evans blue dye was introduced into stalks or leaves of Q208$^{\Phi}$ healthy and YCS stalks. The dye was placed in vials and leaves placed upside down and allowed to take up the dye overnight in the laboratory at SRA Mackay Station (Figure 168). Leaves of different ages (+1 to +6) were placed in the dye to assess for blockage in the vascular bundles.

Similarly, for uptake via the transpiration stream, the stalks were cut at different internodes under water and then left in the dye overnight. The leaf and stalks were brought back to Brisbane on ice and sections taken and examined under the microscope.

Figure 168 Leaves of Q208$^{\Phi}$ standing in Evans blue dye

Results for the dye uptake via the leaves, showed that in some of the YCS leaves, the vascular bundles in the midribs, showed absence of dye colour. This was observed in leaf +1 (Figure 169 D) and leaf +4 (Figure 169 E), but not leaf +6 (Figure 169 F) of YCS leaves, while the healthy controls did not show this (Figure 169 A, B and C).

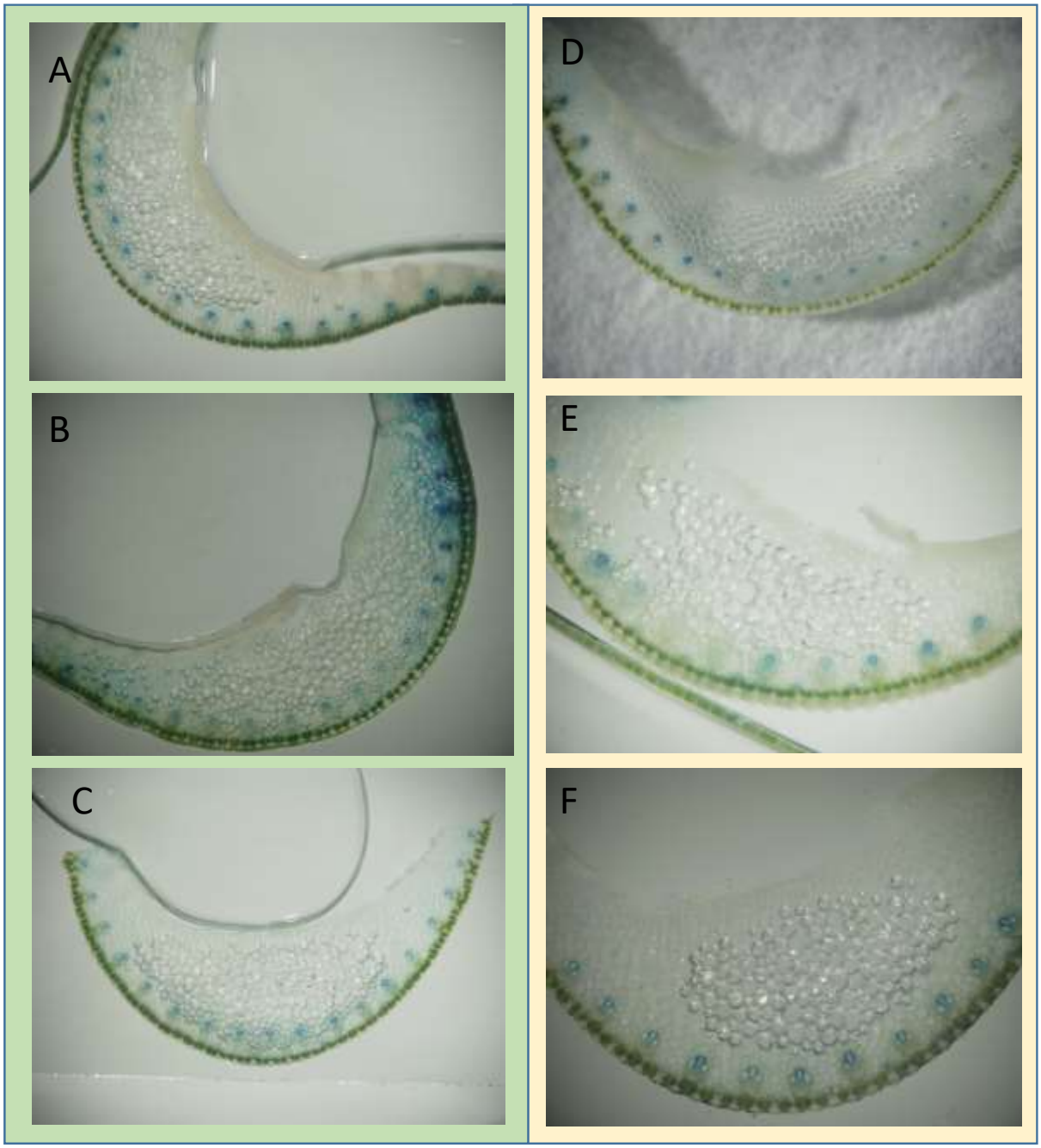


Figure 169 Dye uptake in the leaf number 1, 4 and 6. (Control: A, B, C, YCS: D, E, F)

When the uptake via the stalks was examined, the internodes of YCS plants showed reduced dye in the vascular bundles of internode +3 (Figure 170).

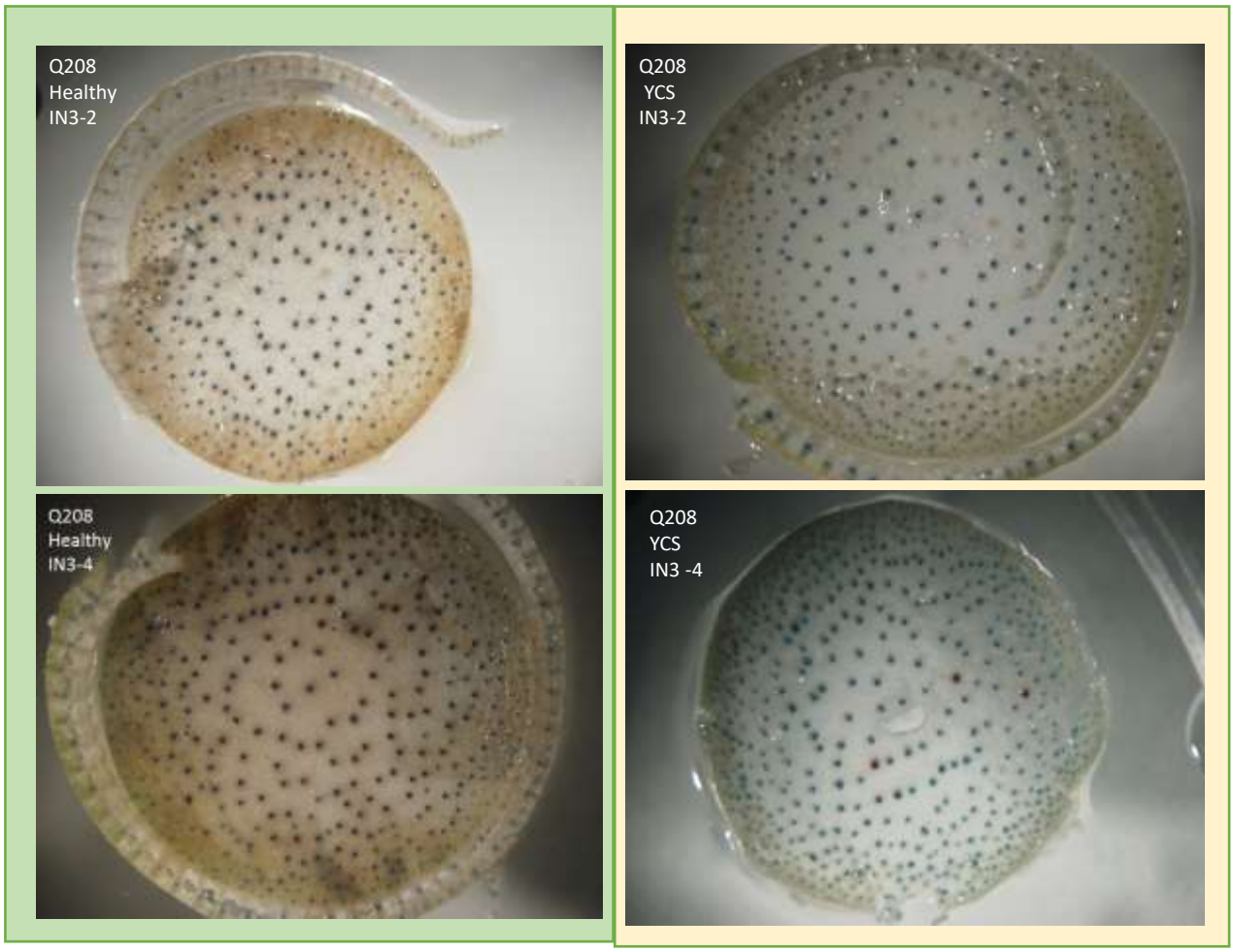


Figure 170 Dye uptake in internodes of healthy and YCS affected stalks of Q208$^{\Phi}$

This work shows that the vascular bundles of midribs and internodes of YCS plants may be compromised.

Dye uptake by 9-month old KQ228^{A} stalks taken from the field

Field grown stalks of both asymptomatic and YCS affected plants, which were 9 months old were harvested and brought back to the station for dye uptake studies. All stalks were placed in a bucket of clean water until ready to place in the dye. Stalks were trimmed to the 12th internode and then placed in one of the four dyes studied (Table 37).

Table 37 Dyes, concentration used, initial volume and volume taken up by the stalks

Dye	Conc.	Vol prep	Volume taken up by 2 stalks after 20 hours
Evans blue	0.5%	200mL	90mL
Methylene blue	1%	200mL	90mL
Sulphorhodamine G	4%	100mL	35mL
Tetrazolium salt	1%	100mL	75mL

At the end of 20 hours, the stalks were harvested and cut at each internode, photographed, packaged, and sent on ice bricks by overnight transport to the Brisbane labs for further studies. The data (Table 37) showed that the dye Sulphorhodamine G was taken up the least. This dye accumulates in the sumps.

The stalks were cut just above the node and assessed for presence of dye. The central 1cm core was taken using a cork borer and assessed under a dissecting microscope. The total number of VB which were white or coloured, showing presence of dye were counted, and expressed as a percent of the total number of VB. The intensity of colour was also recorded.

YCS affected stalks showed a higher percent of white VB in all the internodes below and including internode 4 when compared to asymptomatic ones (Table 38). Methylene blue showed a similar trend (Table 39).

Table 38 Presence of Evans Blue dye in vascular bundles at each of the internodes of asymptomatic control and YCS stalks of KQ228[A]

Evans blue											
Asymptomatic KQ228						KQ228 YCS					
Internode no.	No. of VB	% White	% Pale blue	% Mid-blue	% Dark blue	Internode no.	No. of VB	% White	% Pale blue	% Mid-blue	% Dark blue
3	100	93	1	2	4						
4	64	8	14	47	31	4	100	65	0	5	30
5	60	3	17	55	25	5	80	60	0	21	19
6	52	4	19	38	38	6	52	42	6	33	19
7	48	4	10	52	33	7	48	17	21	33	29
8	48	0	4	21	75	8	48	21	17	21	42
9	44	0	14	41	45	9	48	17	21	21	42
10	44	0	0	9	91	10	48	21	17	21	42
11	40	0	5	20	75	11	48	31	17	10	42

Table 39 Presence of Methylene blue dye in vascular bundles at each of the internodes asymptomatic control and YCS stalks of KQ228[A]

Methylene blue											
Asymptomatic KQ228						KQ228 YCS					
Internode no.	No. of VB	% White	% Pale blue	% Mid-blue	% Dark blue	Internode no.	No. of VB	% White	% Pale blue	% Mid-blue	% Dark blue
3	100	20	20	30	30	3	120	42	25	17	17
4	80	0	13	25	63	4	100	25	30	20	25
5	44	0	0	9	91	5	80	13	25	31	31
6	44	2	7	23	68	6	56	18	7	36	39
7	52	10	10	19	62	7	48	13	13	38	38
8	48	0	10	27	63	8	48	8	13	50	29
9	48	0	17	42	42	9	44	11	14	30	45
10	52	8	10	48	35	10	40	10	13	15	63
11	52	12	8	52	29	11	40	25	13	13	50

Table 40 Presence of Sulphorhodamine G dye in vascular bundles at each of the internodes of asymptomatic control and YCS stalks of KQ228[A]

Sulphorhodamine G									
Asymptomatic KQ228					KQ228 YCS				
Internode no.	No. of VB	% White	% Pale red	% Dark red	Internode no.	No. of VB	% White	% Pale red	% Dark red
1	124	8	92	0	1	124	6	85	8
2	124	8	80	4	2	124	4	80	16
3	56	18	73	9	3	56	14	50	36
4	56	9	73	18	4	48	17	63	21
5	40	38	43	20	5	48	21	58	21
6	36	14	69	17	6	48	10	58	31
7	36	22	56	22	7	44	11	55	34
8	36	17	36	42	8	40	15	60	25
9	36	28	56	17	9	40	25	50	25
10	36	22	56	22	10	40	25	50	25

Table 41 Presence of Tetrazolium dye in vascular bundles at each of internodes of asymptomatic control and YCS stalks of KQ228[A]

Tetrazolium salt							
Asymptomatic KQ228				KQ228 YCS			
Internode no.	No. of VB	% white	% pale red	Internode no.	No. of VB	% white	% pale red
1	64	66	34				
2	120	17	83	2	100	80	20
3	120	4	96	3	100	30	70
4	60	83	17	4	100	60	40
5	60	83	17	5	96	90	10
6	48	83	17	6	86	91	9
7	40	80	20	7	40	88	13
8	40	63	38	8	40	75	25
9	36	78	22	9	40	63	38
10	32	56	44	10	40	63	38
11	32	56	44	11	40	50	50

The results for the two remaining dyes- Sulphorhodamine G and tetrazolium were different (Table 40 and Table 41). The trend of presence of dyes were similar in both YCS and asymptomatic stalks.

The former dye is used as a tracer for apoplastic movement of solutions and accumulating in sumps, while tetrazolium dye is an indicator of respiration.

These results suggest that in YCS affected mature stalks of KQ228^{A}, there is a reduced uptake of dyes which move through the VBs and could be due to blocked VBs or reduced transpiration or both. However, neither the movement and accumulation of Sulphorhodamine G in the sumps nor respiration in the internodes of these stalks were affected. It is worth noting that intact YCS plants in the field show reduced transpiration due to stomatal closure initiated by high leaf sucrose (see section 6.5.4.1 & 6.5.4.2 of this report) (Scalia et al., 2020)

Intact plant uptake experiments

Methylene blue dye uptake experiments were conducted on intact potted KQ228$^{\phi}$ plants in March and June 2016. One pot of healthy asymptomatic and one of YCS symptomatic plants were used for this study. Soil was removed and the stools were cut in half and each half placed in a separate pot. Thus, there were two pots of YCS affected plants, and two of healthy plants. Two different treatments were given to each of the YCS and healthy pots:

1. Root to shoot (RS) (= uptake): Addition of 1% methylene blue to the rooting medium to one symptomatic and one asymptomatic pot (~6litres per pot) (Figure 171A).

2. Shoot to root (SR) (= reverse flow): Addition of 6litres of 1M sucrose to the rooting medium to one symptomatic and one asymptomatic pot (~6litres per pot). The stalks of these pots were trimmed so that a 50mL Falcon tube with its end cut-off could fit snugly over the cut stalks (see photos). There were 6 stalks in the YCS stool and only 2 stalks in the control pot. The Falcon tubes were filled with 1% methylene blue. (Figure 171 B).

The pots were left outside the shed and by the next day, leaves had turned blue in the treatment (2). As the tubes were almost empty, they were refilled once with 50mL of tap water. The plants were all harvested after 10 days, packaged and sent via overnight transport to Brisbane with ice bricks. In Brisbane, stalks were cut into one-eye setts and photos were taken.

Figure 171 Treatment 1 (RS) stalk and root system in buckets filled with dye A). Treatment 2 (SR) falcon tubes with methylene blue, attached to the stalks, in buckets containing 1M sucrose solution B).

Results:

RS treatment-

Each of the two stalks in the control healthy pot showed good uptake of blue dye (Figure 172 top panel) clearly visible up to internode 7. In the YCS affected stalks, there is less uptake of dye, with almost no dye visible above internode 9. In one of the YCS stalks1, the uptake is very patchy and almost no uptake after internode11, while in stalk2 blue colour is visible up to internode 9.

Figure 172 Dye uptake by stalks of KQ228$^{\Phi}$. Top panel shows two of the stalks placed from youngest to oldest internode (left to right); lower panel shows dye uptake in YCS affected stalks

Roots of both healthy and YCS plants showed similar blue colour (Figure 173) (which is expected, as they were bathing in the dye for ten days).

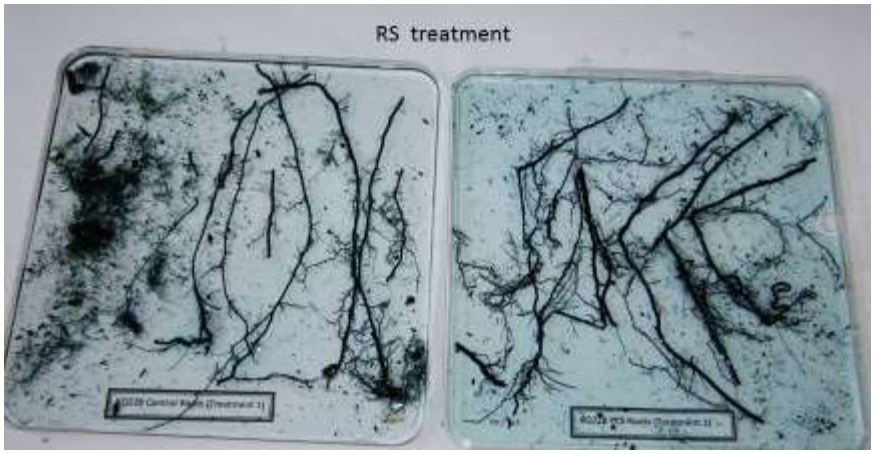


Figure 173 Colour in roots of healthy controls (left) and YCS affected (right) plants

SR treatment-

Dye movement from the top to the base of the stalks was examined. (Figure 174 top two panels) show the presence of blue dye in all the internodes to the base in both stalks. In YCS affected stalks, presence of dye is reduced and is present closer to the outer edge of the stalks (Figure 174 two lower panels & Figure 175).

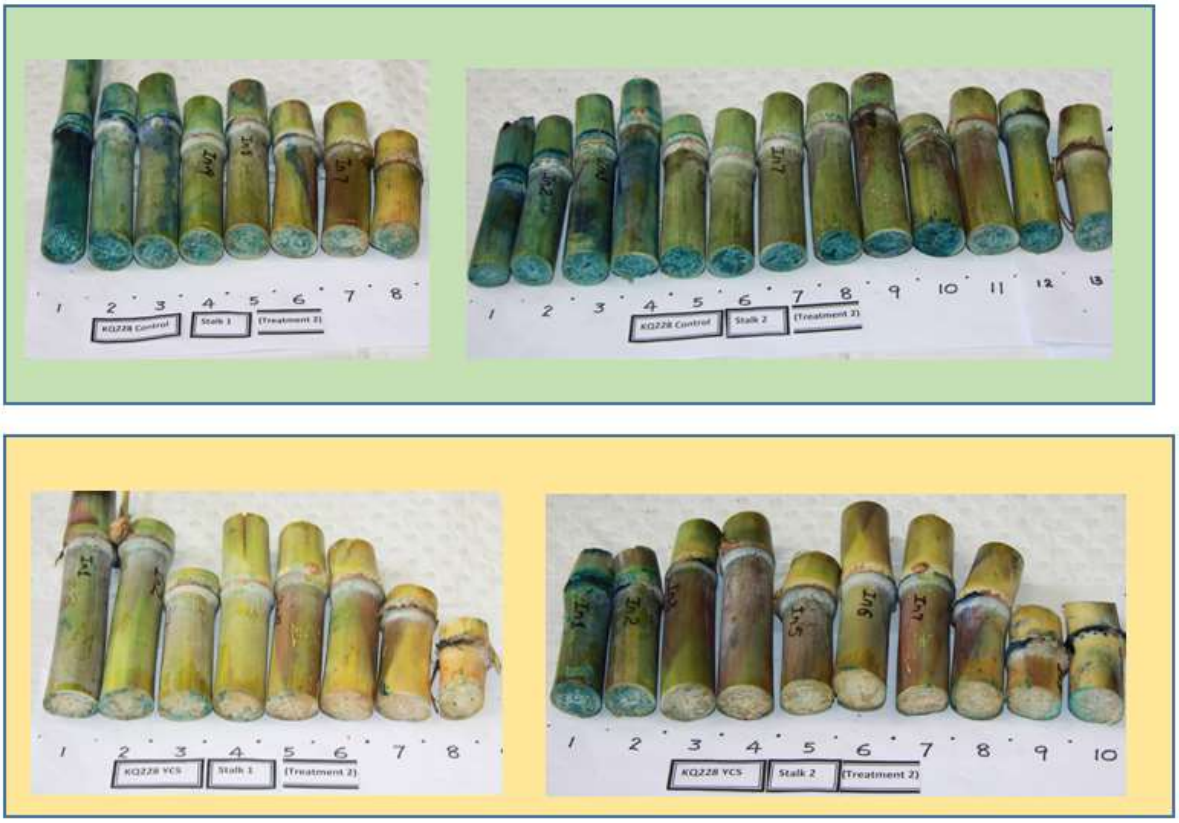


Figure 174 Reverse flow experiments in the healthy (top panel) and YCS (bottom panel) affected stalks of sugarcane placed in a 1M solution of sucrose, after 10 days of application of methylene blue dye to the internodes 8 - 13 nodes above the soil

Figure 175 Close up of internode 7 in two healthy (LHS) and YCS affected stalks (RHS) of KQ228$^{\Phi}$ roots

Despite not showing much colour in the stalks, the roots of YCS plants showed strong blue colour, much more so than that in the controls (Figure 176). This could indicate that either the movement out of the roots was severely inhibited in YCS plants, causing it to accumulate within the roots; or that in the control roots the dye was rapidly oxidised to the colourless product. Low concentrations of methylene blue are used as an indicator of respiration. As the amount of dye present in the rooting medium was not measured, this observation would require further investigation to be able to draw conclusions.

Figure 176 Roots of healthy and YCS affected plants after 10 days of methylene blue dye treatment to shoots of potted plants

Whole plants with roots experiments

Twelve whole stalks of KQ228(ⴱ) (six of each- 1R for YCS and P crop for controls) were dug out including the roots at 9am. Whole stools were dug up with the roots and brought back on the trailer to SRA. As much soil was removed by tickling the root zones, prior to placing the stools on the trailer. Upon arrival at SRA the roots were washed in running water to further remove as much of the soil as possible and rehydrate the roots. Supermarket sucrose was used for the osmotic agent.

Treatment 1:
Three reps for both treatments were included. Each stool was placed in a tall 20L bucket with no drainage hole. Stools were trimmed and stalks removed, so that only four were left in each stool. All cabbage including Leaf +1, 2, 3 and 4 were left on the stalks to help in uptake of dye. 1% Methylene blue solution made up in water was added to cover all the pots (12L/pot =120g/pot). Thus for 6 pots, we needed 720g of methylene blue. All stalks were placed in 10L of dye as in the intact plant system (Figure 180). From each of the six stools, one stalk was harvested after 1, 3 and 6 days. On the 6th day harvest, in addition to the stalk, some of the roots were also harvested and packed in ziplock bags. The fourth remaining stalk was used for sap flow experiments, with sap flow monitored for 5 days. Photos were taken and stalks were cut into smaller pieces, wrapped in clear cling plastic film and then transported overnight to the SRA Brisbane laboratory for analysis.

Figure 177 Methylene blue uptake (RS) by healthy (LHS) and YCS (RHS) stalks placed in the dye for 6 days. Internodes from 1 to the oldest internode are presented sequentially

Results of presence of dye in this RS experiment, showed a similar trend to that observed in the potted plants (Figure 177), suggesting a reduced movement of solutes in the vascular bundles of YCS plants.

Treatment 2:
Intact stools were placed in a bucket with 10L of a 2M sucrose solution. As in treatment 1, each stool was trimmed to have 4 stalks. The top of each stalk was cut off near internode 5 or 6 and a PVC pipe was placed over it. The pipe was sealed with Consolidated Alloys Butyl rubber sealant (Bunnings part # 10266) at the junction of the PVC tube and the stalk and covered with Nescofilm to prevent leakage (Figure 179). In these tubes, 50mL of 1% methylene blue was added; and then topped up with another 50mL that afternoon and the next day. In general, the YCS plants were not using much of the dye and hence were not being topped up much.

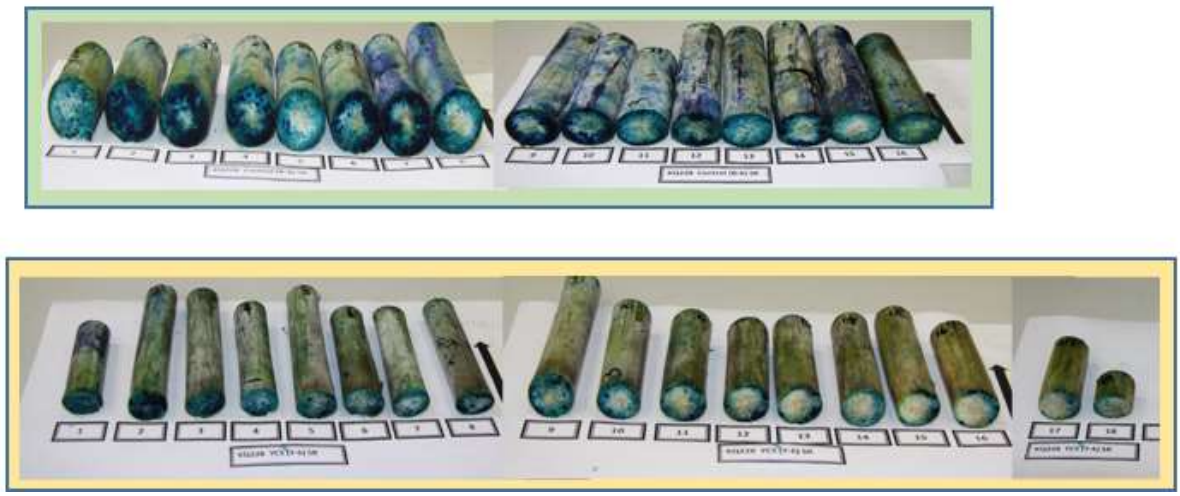


Figure 178 Dye uptake (SR) of methylene blue in stalks of healthy (top panel) and YCS (bottom panel) sugarcane plants after 6 days of application of the dye

In this experiment, the differences between healthy and YCS stalks was present, with the lower internodes showing less dye in YCS stalks compared to healthy controls; like the pot experiment (Figure 178). This suggests that the movement of metabolites down the stalks is also impeded by YCS.

Figure 179 Topping up the dye in the SR stalk experiment (LHS) and close-up of the system used (RHS).

Figure 180 Presence of blue dye in the bucket in the SR experiment tested with a piece of paper towel. Control bucket on the left and YCS on the right

Sap flow experiment

A set three healthy asymptomatic controls and three YCS affected stalks brought to the shade house were fitted with sap flow meters to assess movement of sap within the intact stalks. These were one of four stalks of the stool which remained from the dye uptake experiments (Figure 181).

Figure 181 Sap flow meter being attached to a cane stalk in the shade house

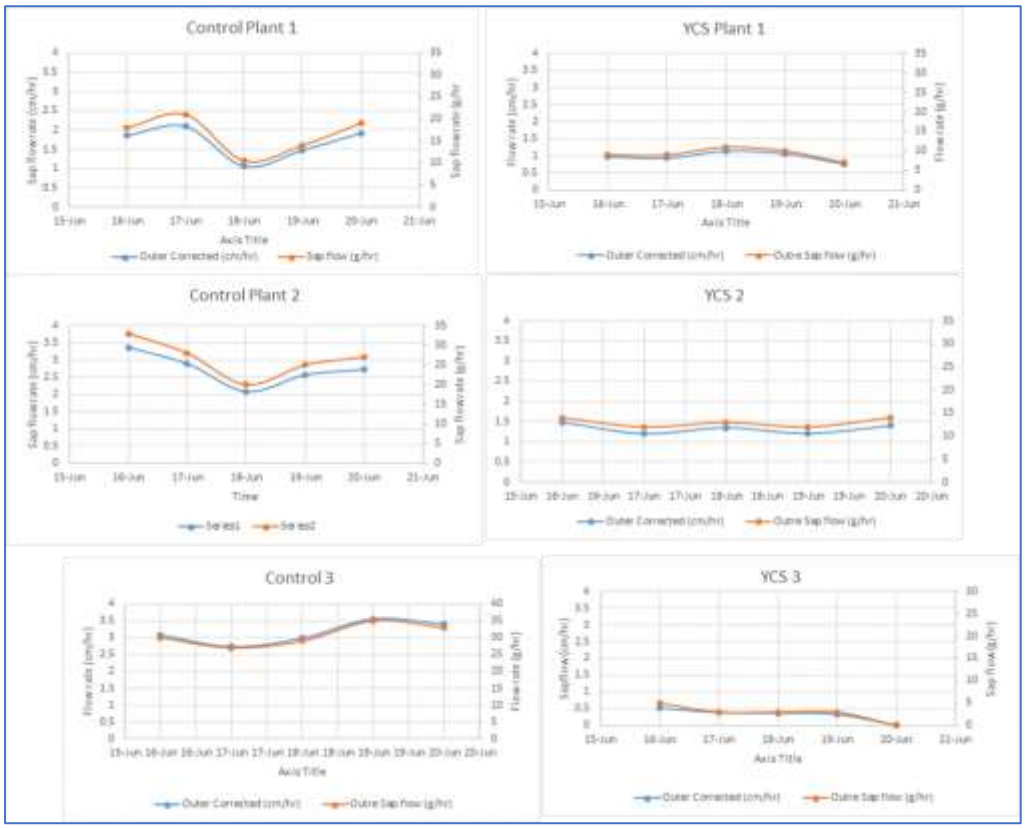


Figure 182 Graphs showing sap flow in control healthy stalks and YCS ones. The sap flow was monitored over five days

Results showed that the YCS stalks had a lower flow of sap compared to the controls. All three replicates showed a 50% reduction in flow (from 2.5 to 3.5 in controls, down to 0.5 to 1.5 in YCS samples) (Figure 182).

While this work does show that the YCS stalks have reduced sap flow, the readings on all samples are around 10 to 15% of that in the field. This is not unexpected as whole mature stools have been uprooted and although kept in water, are bound to have experienced shock and stress from the conditions. This next experiment was performed using younger 7-month-old, field grown cane plants, to overcome some of the problems experienced here with mature cane stalks.

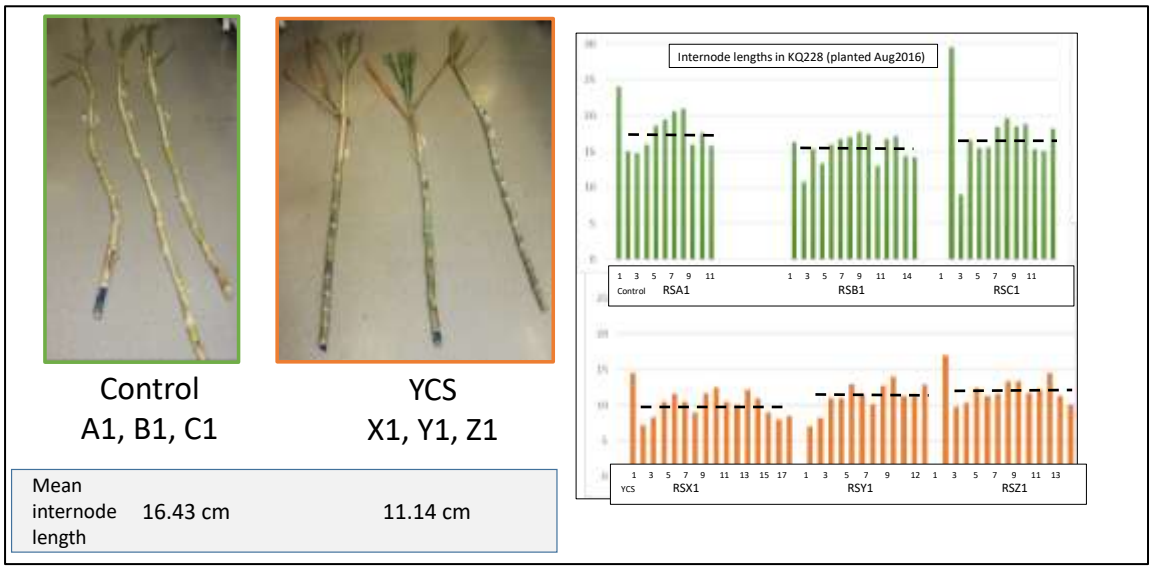


Figure 183 Appearance of 7month old KQ228$^{\Phi}$ stalks (asymptomatic controls versus YCS). Internode lengths of each internode are shown on the right-hand side

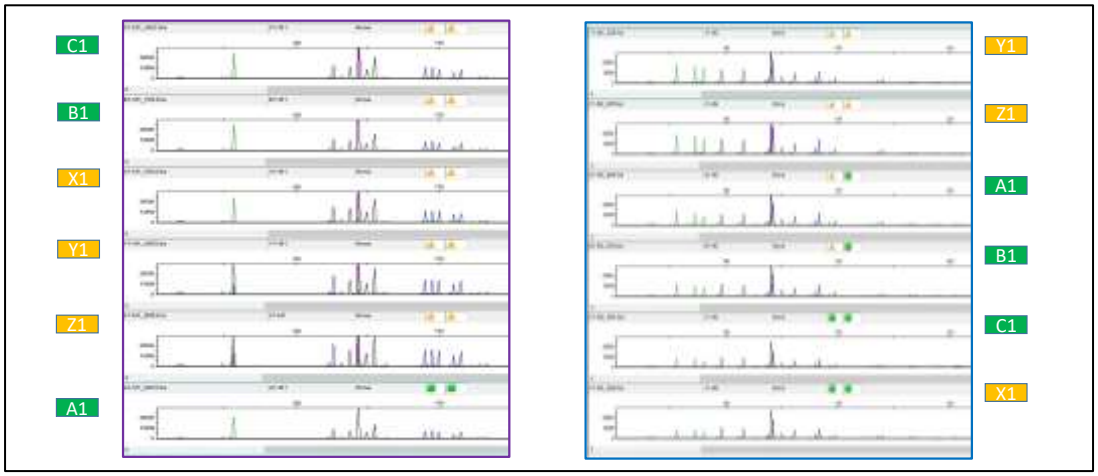


Figure 184 SSR results on all six stalks collected from the Burdekin. Both SSR primers results confirmed that all stalks are of the same KQ228$^{\Phi}$ variety

Optimised conditions for uptake studies were completed of which the following should be adhered to for both RS and SR experiments:

- the age should be 6-8 month old best for field grown plants
- Dye uptake period should be between 1~2 days
- leaves should be trimmed to a similar size
- selected stalks should be of similar length.

Dye uptake experiments in isolated organs as well as in intact plants (pot and field-grown sugarcane) have shown consistently that there is a reduced translocation of dyes both via the transpiration stream (xylem) as well as in the phloem as evidenced in the reverse osmosis experiments (SR) within the YCS affected plants. This hypothesis is further strengthened in Sap-flow analysis which has also

shown a ~ 50% reduction in the flow rate in YCS stalks (from 35 down to <15 g/hour). The repeated dye uptake work was repeated in intact plants to confirm previous results. Our studies were performed on 7-month old KQ228$^{\Phi}$ plants growing in the SRA station at Burdekin. Our results compared well with that obtained in similar dye uptake experiments in 2016, with potted plants as well as mature cane (12-month old). YCS stalks showed reduced movement of dye in both root to shoot (RS) as well as shoot to root (SR) experiments (data not shown). We observed vastly different phenotypic characteristics in the YCS and control plants (Figure 183). However, DNA fingerprinting confirmed that all six stalks were KQ228$^{\Phi}$ using SSRs (Figure 184).

Dye uptake in glasshouse turgid versus wilted Q208^{A} and Q240^{A} stalks

This experiment was performed to study the uptake of dyes by water stressed stalks under glasshouse conditions. Two dyes (Methylene Blue and red food colouring) were tested, four stalks for each dye- two varieties and two treatments (turgid and stressed). Each stalk was placed in 50 mL of 1 % Methylene Blue dye or 50 % Pillar Box red food colouring and left for 18 hours. The internodes were then cut and photos taken (Figure 185). For Q208$^{\Phi}$: stalks from young spindle to Internode 8 with 4 leaves plus leaf 0 and leaf -1 in both dyes.

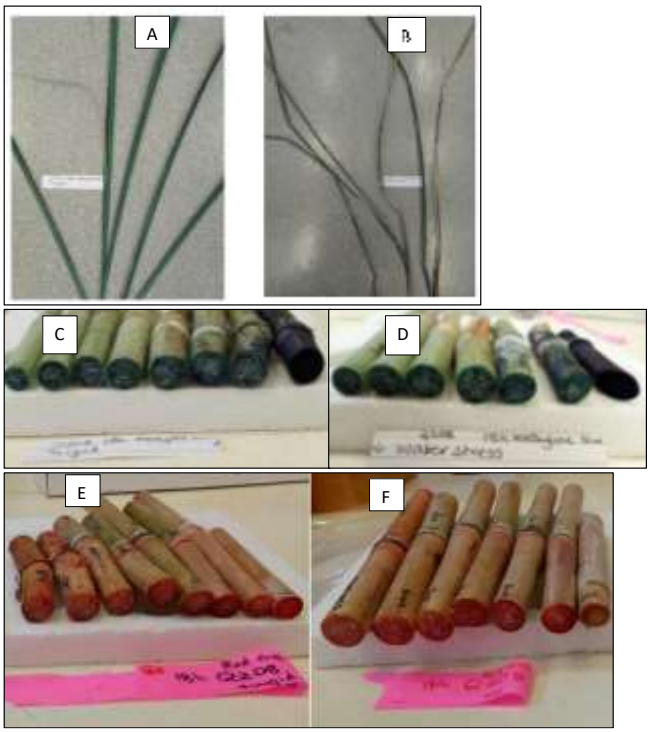


Figure 185 Appearance of leaves on turgid and water stressed Q208$^{\Phi}$cane stalks after uptake of blue dye (A and B). Internodes showing similar amount and pattern of blue dye (C, D) and red (E, F) in the two internodes of both treatments. Internode sections are arranged from oldest (left) to youngest (right) in the photos.

Stalks of Q240$^{\Phi}$ also showed similar pattern of uptake of dyes as was the case in Q208$^{\Phi}$ stalks, except that there was less dye taken up in the water stressed stalks (data not shown). Thus, the pattern of

dye uptake in turgid and water stressed stalks was similar, but uptake was slower in stressed ones in both varieties studied.

Summary

It is evident from dye uptake experiments that there is membrane breakdown in YCS plants. Movement of dyes in both directions is compromised in YCS affected plants. This is consistent with physiological studies that show source leaf sucrose accumulation is a result of reduced phloem transport between the source and non-photosynthetic sink tissue. High sucrose accumulation evidently moves into the apoplastic space (Scalia et al., 2020) and transpiration stream where ABA mediated signalling induces an increase in guard cell hexokinase expression which hastens stomal closure (Kelly et al., 2013). Hence, a reduction in sink strength due to inhibited culm growth or partial phloem blockage, may initiate sucrose accumulation resulting in compromised vascular transport observed in dye uptake and sap flow experiments. Noteworthy is the reduced internode length (~25%) observed in YCS symptomatic stalks compared to asymptomatic controls (Figure 183).

6.5.7 Discussion and conclusions

The crop age trial investigated if an external factor is triggering YCS symptoms in developing leaves and whether the age of the plant has any bearing on symptom expression. This was an extremely important experiment as it not only investigated any correlation between YCS and crop age, but it also shows what happens when you alter the sink size before the peak photosynthetic period from December to March. The occurrence of YCS symptoms was in unison across the treatments irrespective of age, but dependent on the level of ontogeny. However, unlike the other treatments the November slashed plants did not show YCS symptoms in mid-December when they were physiologically only 6 weeks old. At that time point these plants had approximately 38cm of cane and about 6 internodes based on a phyllochron of 150°Cd and leaf base temperature (Tbase) of 10°C (Inman-Bamber, 1994; Campbell et al., 1998; Sinclair, 2004; Bonnet, 2013). Internode elongation does not cease until about internode +8 to +10, approximately 380°Cd (Tbase = 18°C) after the formation of the FVD, and expansion is mostly arrested by internode +12 to +14 (Inman-Bamber, 1994; Lingle, 1997, 1999). However, this may vary as internode elongation is driven by available water, temperature, carbon supply and sink metabolism, and heavily influenced by both abiotic and biotic stress (Inman-Bamber, 2013) (Rae et al., 2006). Therefore, the November treatment source leaves would be feeding elongating and expanding internodes very close to a very large root sink that had established itself in the ratoon crop before slashing. It should be noted, it was long thought that the root system dies and is replaced after harvest but studies shows that the die-back can be as low as 17% and that the old root system to be functional at depths of 2.0m, and able to contribute to crop growth for approximately 4 months (Wood and Wood, 1967; Glover, 1968; Ball-Coelho et al., 1992). This of course is affected by abiotic and biotic stress, availability of water, soil type and cultivar (Smith et al., 2005).

In the November treatment, supply is unlikely to exceed the sink capacity or demand due to the close proximity of source leaves to the large root system which ensures maintenance of a strong sugar gradient (Botha et al., 1996; Geiger et al., 1996; Bihmidine et al., 2013). However, in early January when this treatment had amassed a further 60cm of cane it became YCS symptomatic. This

is likely driven by reduced culm growth rate causing a more immediate source response than the roots due to the close proximity of a larger number of immature internodes beneath the affected source leaves. This concurs with studies by Scalia et al. (2020) that shows sucrose accumulation in the mid-canopy source leaves of YCS affected crops is primarily influenced by culm growth limitations. If conditions return where either 1) export of reduced carbon meets the demands of sink metabolism or 2) the production of photosynthate is reduced during the cooler lower energy capture months, a balanced source sink will ensue, and the crop will grow out of the YCS event. This was evident in the crop age trial.

The occurrence of distinct YCS symptom expression in mid-canopy leaves in only one part of a field allowed for a detailed investigation of the physiology of this syndrome in the Burdekin case study. YCS symptomatic and asymptomatic leaves on the same stalk have sucrose and starch content higher than asymptomatic control counterparts. This concurs with results attained across the YCS program (Joyce et al., 2016; Marquardt et al., 2016; Marquardt et al., 2017; Scalia et al., 2020). Note, this field was also extensively studied at a metabolome, transcriptome, proteome and microbiome level (Hamonts et al., 2018; Marquardt, 2019). No measurable difference in soil type and moisture content was apparent in this field, yet root system scans suggest asymptomatic control plants are healthier and able to support a larger above ground biomass. The reduced above ground sink size is a possible cause of supply exceeding demand and leaf sucrose accumulation in symptomatic plants. Interestingly, the grower confirmed that historically he has noted a variation in growth between the top and bottom end of this field well before the occurrence of YCS in 2012.

Analysis of internode size (data not shown) of YCS symptomatic stalks revealed a slowdown in growth, or a reduction in sink strength, in the month prior to good rainfall and the onset of yellowing in February. After this time point internode growth was comparable to the that of the asymptomatic control. This suggests that even though the crop was irrigated, the dryer period prior to expression may have been sufficient to reduced culm growth under very high temperatures and solar radiation (Figure 83) and trigger YCS in plants with a compromised root system. Reduced growth when a crop has all necessary requirements is akin to a condition known as the reduced growth phenomenon (RGP) (Park et al., 2005; van Heerden et al., 2010). However, unlike RGP affected crops this field did recover and there was no statistically significant biomass penalty attributed to YCS.

The clonal variation trial studies provided a better understanding of baseline physiology across 20 clones. Interestingly, significant variation in the NDVI index existed between the clones in YCS asymptomatic plants, yet varietal assessment studies could not confirm that some genotypes were more YCS susceptible than others. This suggests that there is no predisposed advantage of delayed YCS development in plants that have a higher abundance of chlorophyll or leaf greenness. This is perhaps not surprising as disruption to the PET chain, that leads to the destruction of chloroplasts and the onset of yellowing in YCS affected plants, is in response to the inefficient use of trapped energy for continued CO_2 fixation under high cellular sucrose concentrations (Marquardt et al., 2016; Marquardt et al., 2017). To offset the carbon load carbon repartitioning to insoluble starch is evident from leaf iodine staining but evidently inadequate to prevent rising sucrose levels. Therefore, reduction of the light trapping apparatus through destruction of chloroplasts and early induction of leaf senescence are obvious mechanisms to ratify this dilemma. A lower number of attached leaves

on YCS plants is evident in this trial and consistently so in canopy monitoring since the start of the research project.

In general, photosynthesis (A), stomatal conductance (gs) and internal CO_2 (Ci) studies show that as a % C-fixation contribution of the canopy (leaf 0 to +7), leaf +3 and +4 were highest (~28% each), leaf +1 and +2 (~ 13% each) and leaves below leaf +4 contributing only 15%. This helps explain why leaf +3 and +4 are potentially more susceptible to YCS development than other mid-canopy leaves. Any impedance of sucrose transport from these highly productive source leaves would therefore increase the risk of unhealthy levels of sucrose accumulation. Metabolite studies by (Scalia et al., 2020) show that when leaf sucrose levels exceed an upper tolerable threshold of approximately 200µmol/g DM photooxidation is triggered and irreversible leaf yellowing occurs (Marquardt, 2019). Canopy photosynthesis studies of YCS plants show a reduction in C-fixation of approximately 36% (Marquardt et al., 2016). For this exercise, assume yellowing first occurs in leaf +4 and as the plant ages and new leaves are produced, YCS will appear to move up the canopy. However, the symptoms actually remain in the mid-canopy with leaf +3 above it next becoming symptomatic. As time proceeds leaf +3 will become leaf+4 and leaf +4 becomes leaf +5 and so on. Eventually, the senescing YCS symptomatic leaves will merge with the naturally occurring senescent leaves below. This will continue until the plant recovers from YCS. Leaf monitoring across trials show that a YCS event may last for 4 – 12 weeks in which an average of 8 leaves will be affected. Using these observations and C-fixation rates as a guide, it is possible to predict potential yield loss directly to YCS yellowing. Based on cumulative °Cd over a 12 months cropping cycle a total canopy of approximately 40 leaves will be produced. Of these approximately 10-30% of the leaves may be affected by YCS. Yield loss from yellowing can be calculated as follows:

C-fixation % reduction (YCS) = -36%

Leaf +4 C-fixation rate contribution = +28%

C-fixation loss due to YCS (0.36x28) = -10%

% of total leaves affected (8/40) = 20%

Yield loss (0.1 x 20) = 2%

Using this methodology, it is easy to appreciate that the longer the YCS event lasts the greater the magnitude of yield penalty. More importantly however, is an understanding that the main yield loss precedes YCS expression and is not caused by YCS. It should be noted that YCS development is driven by reduced growth rate, which in turn causes leaf sucrose retention and the onset of yellowing, not vice versa. Therefore, it is the severity and scale of the growth stressor, be it biotic or abiotic, that is the cause of yield loss which has been incorrectly assigned by Industry to YCS.

Membrane leakiness increased in YCS leaves which concurs with transcriptome studies that showed an upregulation of membrane degradation and oxidative stress in YCS plants (Scalia et al., 2020). YCS is not associated with programmed cell death. The detection of pre-dawn leaf starch is a useful tool to help identify YCS and whether a plant has recovered from YCS. Leaf metabolite levels show that plants can recover from a YCS event.

Microscopy and fluorescent dye investigations show callose occlusions in YCS vascular tissue which may be responsible for impeded phloem and xylem transport. It should be noted that high levels of source leaf sucrose can enter the apoplastic space and transpiration stream to initiate stomatal closure and reduced transpiration. Reduced sink growth and strength or a physical phloem blockage may be the cause of high leaf sucrose. The extent of reduced sap flow between the source and non-photosynthetic sink tissue due to callose or other physical impediments cannot be determined from microscopy without further extensive tissue sectioning. Stained sections viewed under light microscopy did not reveal the presence of any pathogenic organisms. However, SEM and TEM did show the presence of microbes characteristic of phytoplasma, bacteria and viruses. TEM detected occlusions in xylem and phloem, but no firm conclusions can be made as to the pathogenicity or opportunistic nature of these microorganisms or their link to YCS.

6.6 Growth rate and sink strength

The crop age trials identified that crop age and growth rate interacted with YCS severity (see section 6.1 of this report). These factors together were stronger predictors of yield loss than YCS severity alone. The outcome of the trials showed that growth rate, and not physiological age, was the key driver of YCS. To investigate the link between growth rate and YCS further it was hypothesised that YCS could be induced by manipulating sink strength. A field trial was established at the SRA Burdekin Station to test this hypothesis through the use of plant growth regulators (see Appendix1: 1.2.16).

6.6.1 Plant Growth Regulator Trial

Treatments were first applied on 9 November 2016, and re-applied monthly thereafter. All treatments, with the exception of Paclobutrazol, were foliar applied. Paclobutrazol was soil applied.

1. Paclobutrazol (50uM): GA inhibitor
2. Gibberelic Acid (300uM)
3. 6-Benzylaminopurine (250uM): Cytokinin
4. Aviglycine (Retain) (1/2 label rate): ethylene inhibitor
5. Ethyphon (Promote 900) (1/3 label rate): ethylene
6. Trinexapac-Ethyl (Moddus) (1/2 label rate): GA inhibitor
7. Shade (50% shade cloth)
8. Untreated control

Monitoring

All plots were monitored weekly from 7 November 2016 to 21 March 2017. Leaves +1 to +7 were visually rated for YCS severity according to the standard rating key (Table 4). Stalk height (cm) was also recorded at the time of rating, measured from the base of the stalk to the first visible dewlap (FVD). New leaves were numbered as they emerged so that a record of lifetime leaf production was kept.

Results

YCS first appeared at this site in mid-November 2016 and was followed by another spike in symptoms in mid-December 2016 (Figure 186). Strongest symptom expression was seen from February to March 2017.

GA and GA Inhibitors

Compared to Untreated Control, Gibberellic Acid (GA) showed lower rates of YCS throughout the trial period (Figure 186 left), however this was not statistically significant (Figure 187). The GA-inhibitors Paclobutrazol (PAC) and Trinexapac-Ethyl (TXE) showed mixed results. The PAC was similar to Control while the TXE showed significantly more YCS overall (Figure 187). TXE showed lower or similar rates of YCS up until February. At this point the TXE plants became significantly more YCS affected than Control (Figure 186 left). This trend was similar for PAC, however the increased YCS was not sustained into March as was the case for TXE. Throughout February and March TXE had, on average, 10 - 15 % more of its canopy leaves YCS symptomatic than Control.

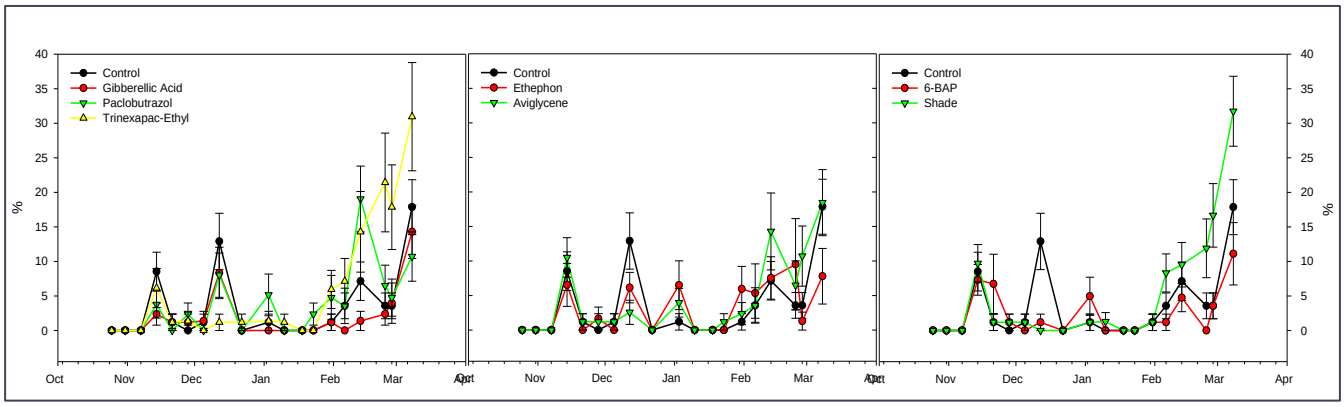


Figure 186 Proportion (%) of canopy leaves (+1 to +7), which are YCS symptomatic. Left panel shows Gibberellic Acid (GA) and GA-inhibitor treatments Paclobutrazol and Trinexapac-Ethyl. Middle panel shows Ethylene (Ethephon) and ethylene-inhibitor (Aviglycene). Right panel shows Cytokinin (6-Benzylaminopurine) and Shade treatments. Data points are means of 12 stalks ± standard error

Ethylene and Ethylene Inhibitor

The Ethylene (Ethephon) treated plants looked quite different in the field. They were generally shorter with smaller leaves and a much-reduced upper canopy size. Yellowing in these plants were more akin to natural senescence with a lighter yellow and browning appearance. Visually, the ethylene inhibitor Aviglycine (AVG) plants appeared similar to Control. There was no real difference in YCS incidence between the Ethephon, AVG and Control. They all followed a very similar record of peaks and troughs in YCS incidence (Figure 186 middle). Furthermore, there was no statistical difference in YCS incidence between Ethephon, AVG and Untreated Control but it should be noted that Ethephon yellowing was different to YCS (Figure 187). This finding showed that altering the natural senescence-related physiology of the plant did not pre-dispose the plants to more or less YCS than untreated controls. This suggests that potential senescence-related causal agents, such as premature programmed cell death, are an unlikely cause of YCS.

BAP and Shade

The BAP treatment generally showed less YCS throughout the trial and appeared visually greener than the Control (Figure 186 right), however this was not statistically significant (Figure 187). Interestingly, the shade treatment showed very little yellowing up until February. However, YCS monitoring after this point it showed YCS incidence increased rapidly and was greater than Control (Figure 186 right). This was later deemed to be an error as the plants were much taller and with fewer tillers than the control due to light seeking. Due to this it was very difficult to score these plants looking up through naturally senescing leaves. When stalks were harvested for internode volume measurements in March, this was confirmed. Therefore, YCS severity rating for the Shade treatment after February should be disregarded.

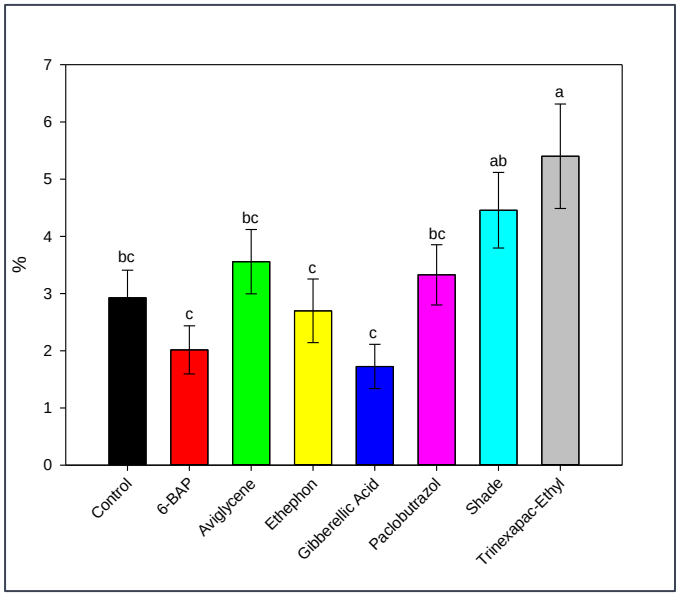


Figure 187 Proportion (%) of canopy leaves (+1 to +7), which are YCS symptomatic. Means of 12 stalks ± standard error. Analysis of variance (p<0.05) by repeated measures design with LSD all pairwise analysis shown by letter separations (a, ab, b, etc.)

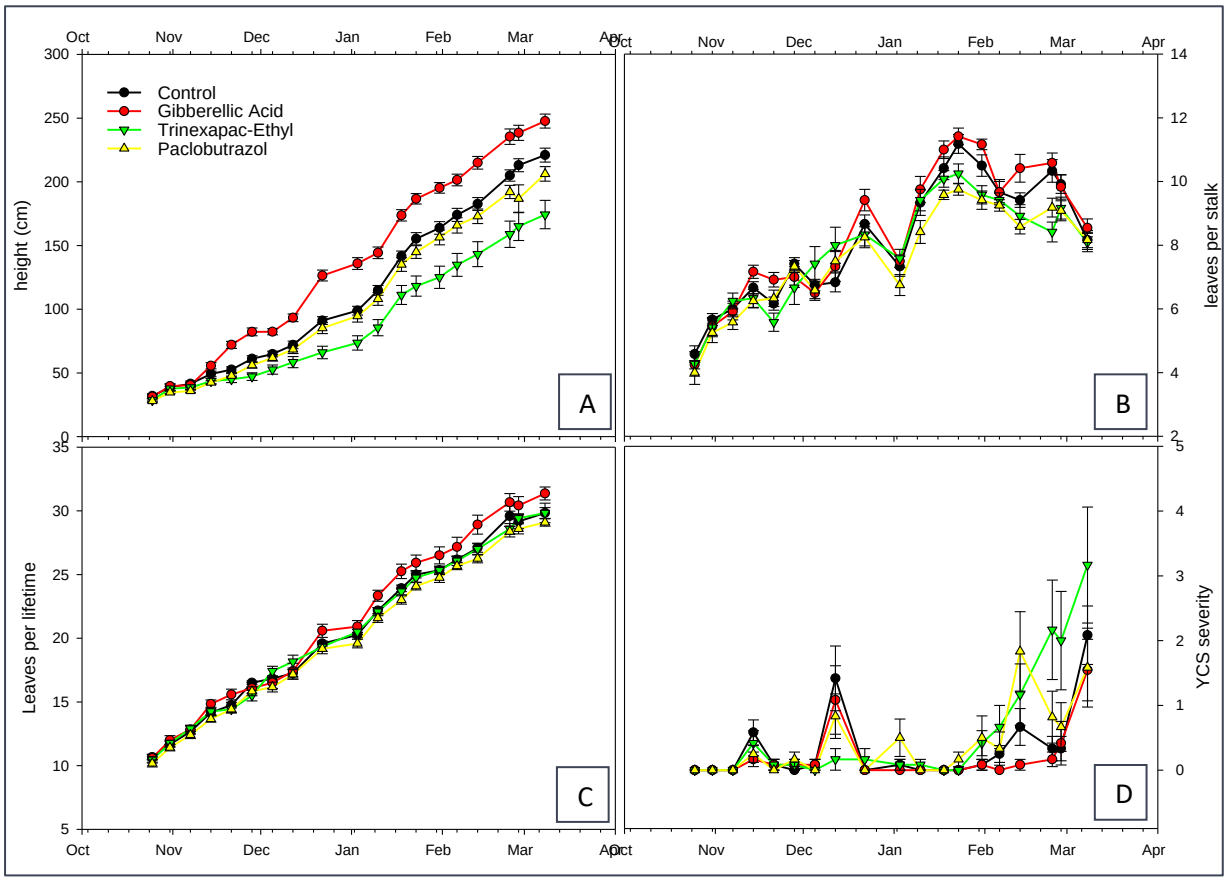


Figure 188 Plant growth and YCS comparison of Control, Gibberellic Acid, and GA-inhibitor treatments. Panel A- Stalk height (from ground to TVD), Panel B- Number of leaves per stalk, Panel C- total number of leaves produced from germination to the end of the trial period, Panel D- YCS severity (sum of severity scores for all leaves +1 to +7). Means of 12 stalks ± standard error.

Comparing GA treatments

The height of GA and TXE treated plants was significantly different from Control (Figure 188A), with GA approximately 30 cm taller throughout the trial period. TXE plants showed an opposite trend. They were approximately 50 cm shorter than Control and 80 cm shorter than GA plants. This was quite a striking difference in the field. The PAC plants were generally smaller than Control, however not significantly so. Given the strong treatment response of the TXE, the PAC treatment was less effective than we expected. We speculate that the method of application, or perhaps rate, was not ideal. PAC was the only soil-applied treatment in this trial.

From planting through to early January, the 4 treatments showed similar numbers of leaves per stalk (Figure 188B). After this point, however, there are some differences. Both of the GA inhibitors tend towards fewer leaves than the Control and GA treatments. They had, on average, 2 fewer leaves during the period coinciding with increased YCS incidence (late January onwards). This concurs with leaf counts recorded throughout the project in YCS symptomatic cane.

GA plants produced 2 extra leaves over the trial period (31 leaves compared with 29 for Control) (Figure 188C). This suggests that the gibberellic acid has altered the phyllochron within these plants due to the increased growth rate (Rai et al., 2017). There was no difference in the total number of leaves produced for TXE and PAC plants, even though their stalk height (in the case of TXE) was significantly different.

6.6.2 Discussion and conclusions

The use of plant growth regulators to alter growth rate and incidence and severity of YCS has been a major research finding. GA inhibitor treatment Trinexapac-Ethyl (Moddus) induced the most severe YCS symptoms, with lowest symptom expression visible in the GA treatment. Metabolite studies of leaf tissue collected from this field in March 2017 show that Leaf +4 sucrose content to be above the upper tolerable threshold of 200µmol/g DM in TXE and YCS symptomatic leaves (Scalia et al., 2020). All other treatments had sucrose levels ≤100 µmol/g DM. The upper tolerable threshold is a guide to the physiological fitness of the plant and a good indicator of YCS susceptibility. If leaf sucrose levels can be maintained below this threshold leaves will remain green and healthy in appearance. At the time of March sampling only the TXE and YCS symptomatic plants had YCS symptomatic leaf +4.

Prior to YCS expression, carbon is repartitioned to other pools and tissue in an attempt to lessen the carbon load and prevent sucrose levels transitioning from a healthful to harmful state (Marquardt et al., 2017; Marquardt et al., 2019; Scalia et al., 2020). Studies by Marquardt et al. (2017); Marquardt et al. (2019) showed altered carbon partitioning in both asymptomatic and symptomatic leaf tissue. Carbon is mostly repartitioned to the soluble α-glucan and starch (insoluble α-glucan) pools with high levels of accumulation measured in the lamina, midrib and sheath (Scalia et al., 2020). High levels of sucrose are also measured in the midrib and sheath. If sucrose levels continue to rise after these pools are saturated, the upper tolerance level will be exceeded and the irreversible onset of yellowing occurs (Marquardt, 2019).

Sink strength studies showed that TXE, ethylene and YCS symptomatic plants had an internode 4 volume approximately 60 % smaller than the GA, BAP, Shade and healthy Control plants (Scalia et al., 2020). This reduced physical size of internode 4 will eventually limits its capacity to fully accommodate all carbon exported from the source leaves above it during this peak growth time of the year. A diminished sink strength will reduce the call for carbon creating a weak sugar gradient between the source and sink, reducing sucrose translocation (Botha et al., 1996; Geiger et al., 1996; Bihmidine et al., 2013). Reduced phloem flow will cause sucrose levels to rise in the leaf and trigger YCS development and symptom expression under high light interception.

Transcriptome studies show that sugar transporters involved in phloem loading are functional and internodes are in a 'feast' state or not carbon starved (Bihmidine et al., 2013; Marquardt, 2019; Scalia et al., 2020). In support of this, sucrose concentration studies between the lamina and internode do not support any major vascular occlusion. Therefore, this collective data from the growth regulator trial shows that YCS is a physiological disorder that can be induced or mitigated by altering sink strength. Metabolite studies of high yielding crops (>170 t/ha) over a full crop cycle, showed maintenance of a source sink balance where supply did not exceed growth demands and little to no YCS was evident (Scalia et al., 2020) (Botha et al., 1996; Bihmidine et al., 2013).

6.7 Farm management

During the course of this research project many 'solutions' were proposed by growers, and others with vested interests for financial reward, as effective in preventing or mitigating YCS development and expression. Replicated field trials were established in the Herbert and Burdekin districts to evaluate a range of YCS treatment and management practices. These trials were developed in collaboration with Herbert Cane Productivity Services Limited (HCPSL) and Burdekin Productivity Services (BPS) with treatments chosen specifically to address questions asked by growers in those districts (see Appendix 1: 1.2.14 & 1.2.15).

6.7.1 Burdekin

YCS severity ratings were conducted at two time points: 3-Mar-16 and 23-Mar-16 during the peak of the YCS season. There was no statistical difference (p=0.5811) between the two dates in terms of YCS severity (data not shown). There was however a treatment effect. Although no treatment was significantly less severe than Control, gypsum, UV shield (Raynox), and Silica treatments were significantly more YCS affected than Control (Figure 189). These results suggest that amendments which increase soil nutrient stores, such as mill mud, six easy steps (6ES) high rate, compost, and Seasol have little effect on YCS severity and therefore cannot be recommended as management options.

Biomass was measured on 23-Feb-16 when the cane was 6 months old. Results show that none of the treatments had significantly greater yield than the Control (Figure 190). The tissue culture plants had a reduced biomass, but this can be explained by the slow establishment of tissue culture compared to the rapid growth of the ratoon crop. Interestingly, the treatments that showed increased YCS severity relative to Control, have no yield penalty. These findings suggest that none of the treatments could confidently be used to prevent the incidence of YCS or to reduce severity with any meaningful improvement on yield.

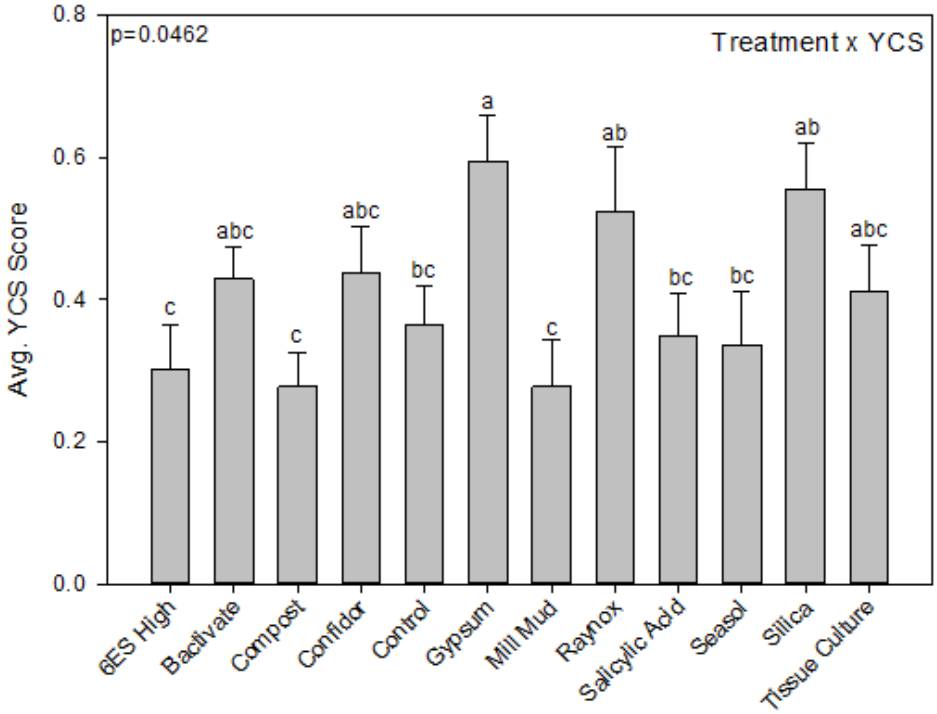


Figure 189 Average YCS severity of all canopy leaves (leaf +1 to +7). Means of two field visits are shown (3-Mar-16 and 23-Mar-16). Each bar represents the mean of 18 stalks ± standard error. Analysis of variance by repeated measures design (p<0.05) with LSD all pairwise comparison shown by letter separations a, ab, b etc.

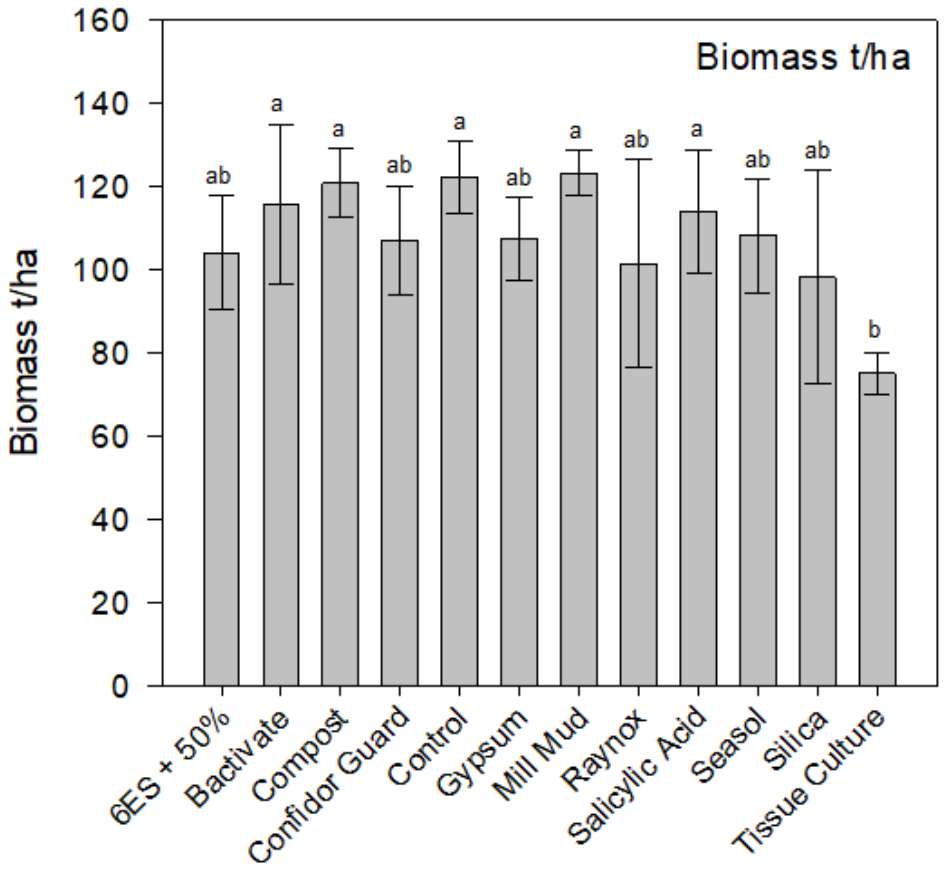


Figure 190 6 Month Biomass. Whole stalk fresh weight. Treatment mean ± standard error. Analysis of variance by randomised complete block design (p<0.05) with LSD all pairwise comparison shown by letter separations a, ab, b etc.

6.7.2 Herbert

Results of YCS severity ratings showed a significant treatment effect for Nuprid (Imidacloprid) only relative to Control (p<0.05) (Figure 191). There was some variability in the degree of yellowing with the silica (cement) treatment showing most symptoms, however all other treatments were similar to Control. This suggests that, with the exception of Nuprid, none of these treatments were effective at mitigating YCS incidence or severity. The Nuprid effect is consistent with findings in other field trials.

Across the 5 months of YCS monitoring a peak of YCS expression was noted in late December to early January, followed by another peak in late February (Figure 192). This is typical of the wave-like pattern seen in previous years.

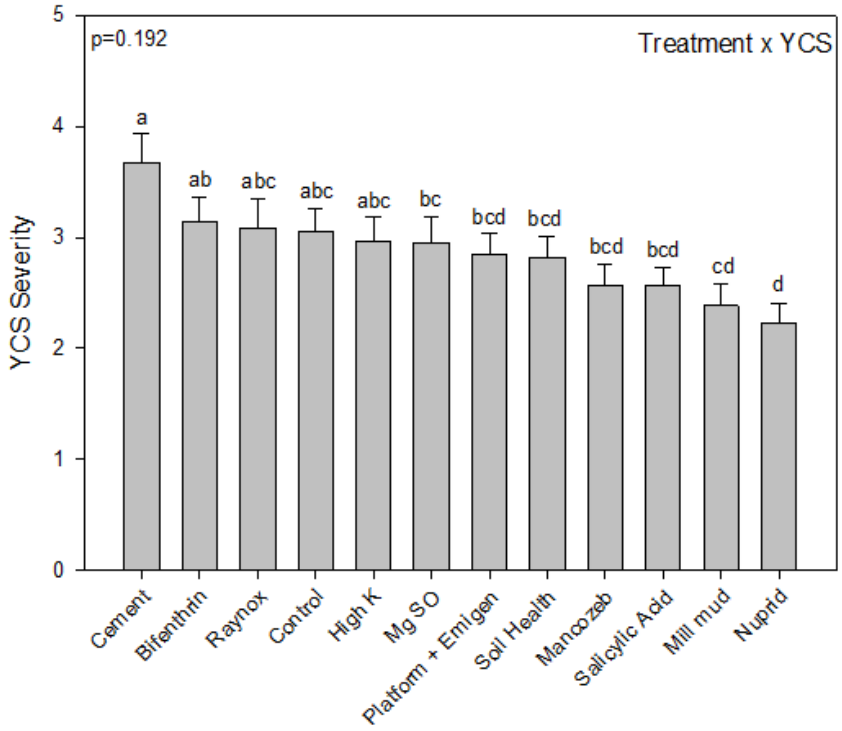


Figure 191 Average YCS severity of all canopy leaves by treatment. Bars are means of 17 field visits ± standard error. Analysis of variance by repeated measures design (p<0.05) with LSD all pairwise comparison shown by letter separations a, ab, b etc.

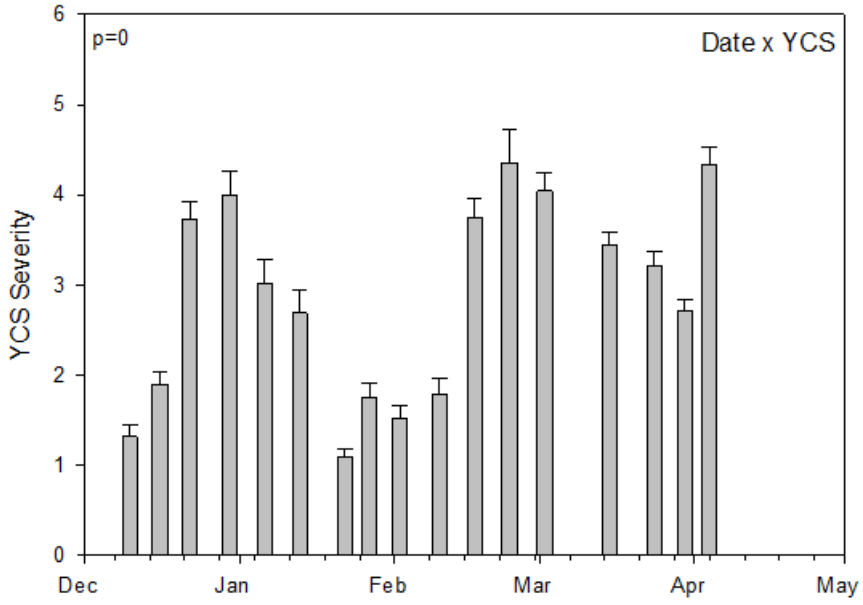


Figure 192 Average YCS severity by date. Bars represent means for all treatments combined ± standard error. Analysis of variance by repeated measures design (p<0.05) with LSD all pairwise comparison shown by letter separations a, ab, b etc.

Biomass and CCS were measured at 12 months. Results showed some significant variability in final yield between treatments, however only the Mill mud treatment had a greater yield than the Control (Figure 193). As there was no improvement in YCS severity for mill mud, this is most likely due to improved crop establishment arising from the high moisture content of the mill mud. Interestingly, the only treatment to show a positive effect on YCS severity (Nuprid) did not result in a statistically significant greater final yield. Once again, we see that the correlation between YCS severity and final yield is not strong. Results of CCS showed a similar trend, with variability between treatments, with only 2 treatments showing a significantly higher final CCS than Control; Mancozeb and Raynox (Figure 194). Neither of these treatments had any effect on YCS severity so their effect on CCS must be due to some other unknown factor.

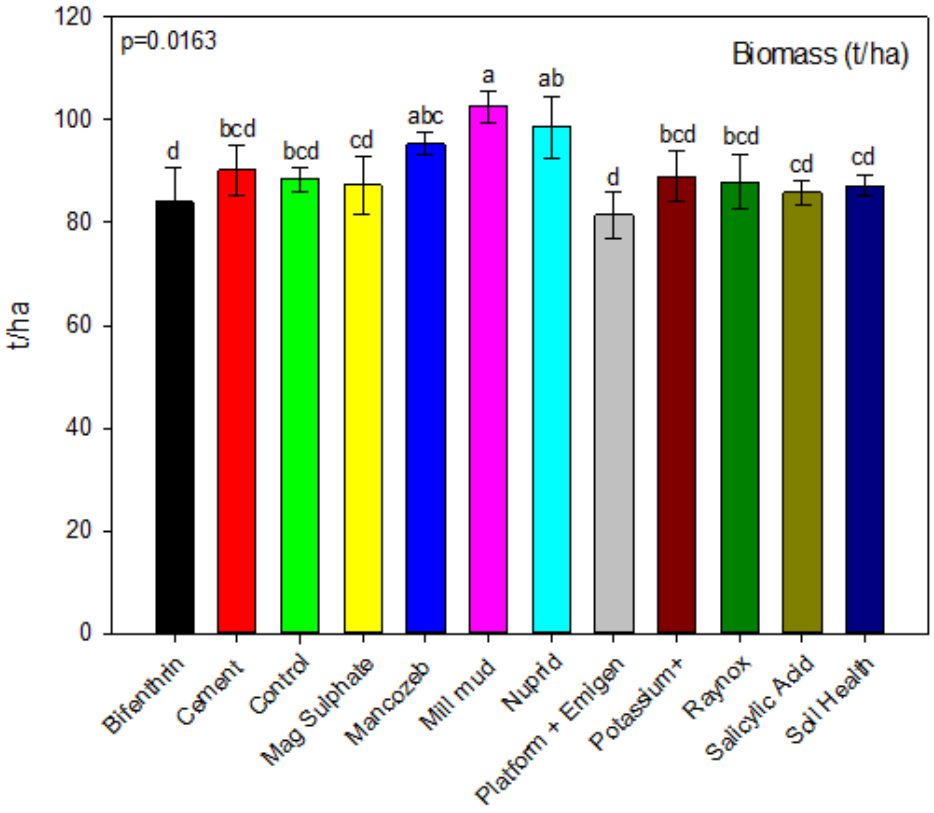


Figure 193 12-Month Biomass. Whole stalk fresh weight. Treatment mean ± standard error. Analysis of variance by randomised complete block design (p<0.05) with LSD all pairwise comparison shown by letter separations a, ab, b etc.

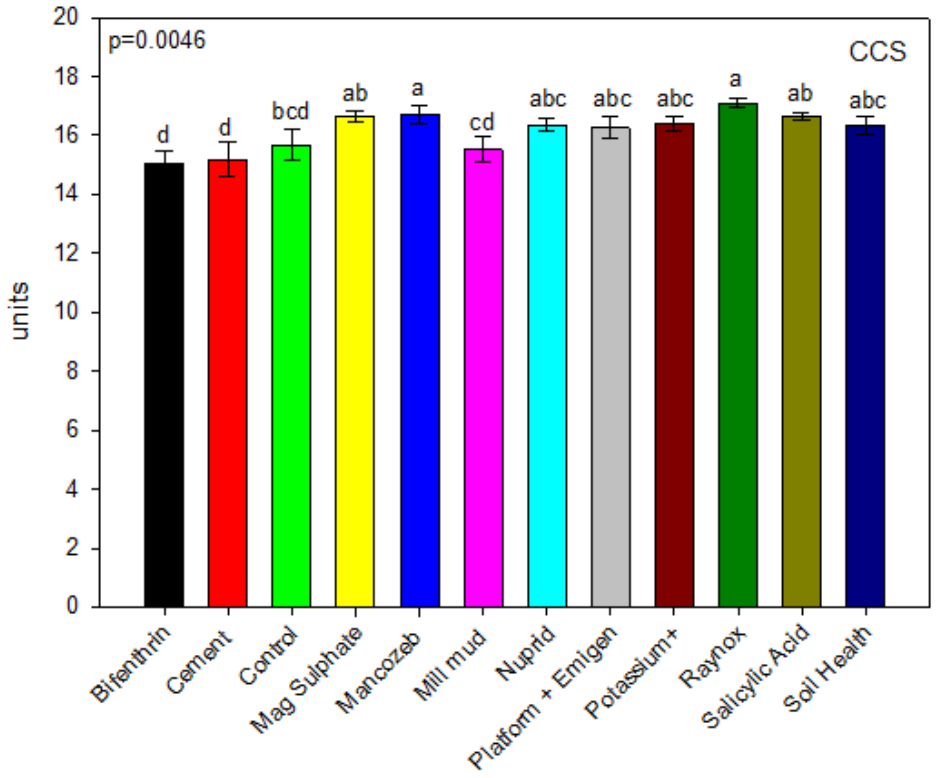


Figure 194 12-Month CCS. Treatment mean ± standard error. Analysis of variance by randomised complete block design (p<0.05) with LSD all pairwise comparison shown by letter separations a, ab, b etc.

6.7.3 Discussion and conclusions

YCS symptoms are reduced by some of the treatments while others appeared worse. While the imidacloprid treatment (Nuprid) was successful in reducing YCS symptom severity it failed to equate to a yield or CCS benefit. This concurs with results from the Confidor® trials (section 6.3 of this report) which show that imidacloprid acts as a stress shield, inducing a stay-green effect. No treatment showed a strong correlation with reduced YCS severity as well as improved cane yield and CCS. This further supports the argument that the duration of a YCS event is more likely to effect cane yield than YCS severity alone. The crop age trials showed that growth rate is the key driver of YCS (see section 6.1 of this report). When a well-timed application of growth supplement was added, YCS symptoms were either prevented or reduced (See section 6.6 of this report). It is tempting to argue that a similar result may be achieved by critical timepoint application of an ameliorant like mill mud that showed a trend for increased cane yield and reduced YCS severity. Overall, the outcome of these farm input management trials suggest it is unlikely YCS can be prevented or mitigated simply through the application of potential ameliorants.

In conclusion, we found that none of the 12 applied treatments could be confidently recommended to industry as a management option for YCS.

6.8 Pathology

The disruption to carbohydrate metabolism and photosynthesis prior to and during the onset of YCS symptoms is well described (Botha et al., 2016; Marquardt, 2019; Scalia et al., 2020). It well known and documented in the world literature that yellowing of the lamina in the *Poaceae* is caused by high carbohydrate accumulation (Tollenaar and Daynard, 1982; Krapp and Stitt, 1995; Jensen, 1996; Russin et al., 1996; Rajcan and Tollenaar, 1999; Graham and Martin, 2000; Braun et al., 2006). YCS is a condition that effects the main source leaves of the mid canopy and metabolite studies show that carbon is repartitioned to other metabolic pools such as soluble α-glucan and starch to offset further sucrose accumulation (Marquardt et al., 2017; Marquardt et al., 2019; Scalia et al., 2020). However, if leaf sucrose accumulation continues to rise above an upper tolerable threshold, the destruction of chloroplasts is triggered and the onset of irreversible yellowing ensues (Scalia et al., 2020). Leaf sucrose accumulation can be caused by 1) an increase sucrose synthesis which exceeds export rates, 2) inefficient phloem loading, 3) reduced sink strength (physical and/or metabolic) or 4) a physical blockage of the vascular system.

The yellowing symptoms of YCS are similar those caused by luteoviruses. Sugarcane is already known to be affected by one well characterised luteovirus, sugarcane yellow leaf virus (SCYLV), but it is possible that others exist. SCYLV is sometimes associated with a condition known as yellow leaf syndrome, where the midribs turn yellow, but the virus can also be asymptomatic. Ripening cane, dry, cool weather and sugarcane yellows phytoplasma (SCYP) also cause yellow leaf syndrome (Rutherford, 2004). Phytoplasmas, phloem-limited prokaryotes, are associated with yellowing. Several other phytoplasmas known to affect sugarcane are white leaf, grassy shoot and green grassy shoot.

This section of the report investigates whether a pathogen is responsible for causing high sucrose accumulation in the source leaves of sugarcane with YCS.

6.8.1 Molecular pathology - in-depth analysis of samples collected between 2013 to 2015.

Unless stated otherwise, sample collection was focussed on field-grown material rather than pot grown, and included a range of tissue type throughout the plant.

6.8.1.1 Tests carried out for yellowing diseases

An initial investigation of samples from the Burdekin, Herbert and Tully areas collected as part of the pilot project 2013/807 (Table 42) and 2014/049 (

Table 43), were screened with 11 specific and generic PCR or RT-PCR tests for pathogens.

Table 42 Samples collected in 2013 that have been tested in detail.

Date	Location	Variety	Causes of yellowing					Fungal-like organisms					Bacteria	Viruses
Healthy			1 Luteovir	2 SCYLV	3 SCWL	4 Phyto	5 Phyto	6 IGS	7 Chytrid	8 Oomy	9 Pythium	10 CS	11 Bacteria	12 Viral preps
2/08/13	Tully	Q200	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks
2/08/13	Tully	Q208	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks
28/08/13	Patane, Burdekin	Q183	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks
Yellow Canopy Syndrome														
12/07/13	Lenzo's Pappins Rd	Q200	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks
12/07/13	Mathew's Pappins Rd	Q200	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks
12/07/13	Pombell Rd, Herbert	Q208	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks
28/08/13	Patane, Burdekin	Q183	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks	stalks

Table 43 Samples collected 28-29 January 2014 that have been tested in detail.

Location	Variety and code	Causes of yellowing					Fungal-like organisms					Bacteria
Healthy		1 Luteovir	2 SCYLV	3 SCWL	4 Phyto	5 Phyto	6 IGS	7 Chytrid	8 Oomy	9 Pythium	10 CS	11 Bacteria
Burdekin station FAT	KQ228 (H1)	Leaves	Leaves	Leaves stalks	Leaves stalks	Stalks	Stalks roots	Stalks roots	Stalks roots	Stalks roots	Stalks roots	Stalks
Burdekin station BS trial	KQ228 (H2)	Leaves	Leaves	Leaves stalks	Leaves stalks	Stalks	Stalks roots	Stalks roots	Stalks roots	Stalks roots	Stalks roots	Stalks
Ian Shepherdson, Burdekin block 4	KQ228 (H3)	Leaves	Leaves	Leaves stalks	Leaves stalks	Stalks	Stalks roots	Stalks roots	Stalks roots	Stalks roots	Stalks roots	Stalks
Hobbs, Herbert	MQ239 (H4)	Leaves	Leaves	Leaves stalks	Leaves stalks	Stalks	Stalks roots	Stalks roots	Stalks roots	Stalks roots	Stalks roots	Stalks
Yellow Canopy Syndrome												
Mario Privitera, Burdekin	KQ228 (Y5)	Leaves	Leaves	Leaves stalks	Leaves stalks	Stalks	Stalks roots	Stalks roots	Stalks roots	Stalks roots	Stalks roots	Stalks
Ian Shepherdson, Burdekin block 4	KQ228 (Y6)	Leaves	Leaves	Leaves stalks	Leaves stalks	Stalks	Stalks roots	Stalks roots	Stalks roots	Stalks roots	Stalks roots	Stalks
Ian Shepherdson, Burdekin block 9	KQ228 (Y7)	Leaves	Leaves	Leaves stalks	Leaves stalks	Stalks	Stalks roots	Stalks roots	Stalks roots	Stalks roots	Stalks roots	Stalks

Location	Variety and code	Causes of yellowing					Fungal-like organisms					Bacteria
Chiesa, Herbert	MQ239 (Y8)	Leaves	Leaves	Leaves stalks	Leaves stalks	Stalks	Stalks roots	Stalks roots	Stalks roots	Stalks roots	Stalks roots	Stalks

1. **Generic luteovirus RT-PCR test:** nine degenerate primers designed by Chomič et al. (2010) are used in five combinations to detect most luteoviruses. One combination detects SCYLV and all SCYLV positive controls performed as expected. No samples from the target lists tested positive.
2. **Specific test for SCYLV**: a one-step RT-PCR test developed by CIRAD to detect all strains Girard et al. (2010) has been used on every YCS sample collected (not just those listed in Table 42 and Table 43). No sample has tested positive.

Specific and generic PCR tests for phytoplasmas. A first round PCR test using the universal primers P1/P7 (expected size 1830bp; Schneider et al. (1995)) is carried out. This is followed by a series of nested PCR combinations, all with an expected size of approximately 1600bp:

3. Specific for sugarcane white leaf: SCWL/SGS (Tran-Nguyen et al., 2000) Generic: R16F2n/M23SR (Padovan et al., 1995)
4. Generic: R16F2n/R16R2 (Gundersen and Lee, 1996)

Three samples (healthy and YCS Q183- Patane stalks and YCS KQ228-Y6 leaves) produced bands but they were not the expected size of known phytoplasmas. This suggests that the PCR products were probably non-specific PCR products produced by other prokaryotes.

Generic tests for fungi and fungal-like organisms

DNA extracted from the samples in Table 42 and Table 43 were screened with the following PCR tests:

5. **A generic test for fungi** targets the ribosomal intergenic spacer (IGS) with the primers LR12R and 5SRNA as described by James et al. (2001)
6. **A generic test for Chytrid fungi** uses the primers ITS5 and ITS-Chy as described by Nikolcheva and Bärlocher (2004)
7. **A generic test for Oomycetes** uses the primers ITS5 and ITS-Oom as described by Nikolcheva and Bärlocher (2004)
8. **A generic test for the Oomycete *Pythium*** uses the COI primers FM35 and FM58 designed by Martin (2000)
9. **Chlorotic streak**: the diagnostic test developed by Braithwaite and Croft (2013) was used to screen all samples in Table 42 and Table 43. No samples tested positive.

Note that the well-known universal fungal primer combinations ITS1 or ITS5 with ITS4 (White et al., 1990) were not used. Previous experience has found that these primers generate too many non-specific products, including the ITS region of sugarcane.

The IGS and Chytrid-ITS primer combinations produced strong PCR products from many stalk and root samples, both healthy and YCS. Generally, these strong products were unique to each sample. Rarely was the same sized product amplified from more than one YCS sample and absent in healthy samples. This was only observed on two occasions. The YCS stalk samples: Q200-Lenzo and Q200-Mathew generated similar sized IGS products that were not present in the healthy samples. Similarly, the YCS stalk samples: Q200-Mathew and Q208-Pombell generated similar sized Chytrid-ITS products. The PCR products were sequenced and compared to Genbank sequences using BlastN. The top BlastN scores did not match obvious pathogens of sugarcane. The IGS product matched *Armillaria cepistipes,* a common wood-rotting basidiomycete fungus and the Chytrid product matched *Adineta vaga*, a rotifer.

The Oomycete-ITS combination did not amplify products from stalks but did produce many PCR products from roots, including both healthy and YCS samples.

The *Pythium* COI combination did not amplify products from stalks but did produce faint PCR products in several root samples. Only the YCS sample MQ239-Y8 listed in Table 43 produced a strong product that was not found in the matching healthy sample MQ239-H4.

Bacteria

10. **Bacteria**: the generic universal test developed by Weisburg et al. (1991) using the primers fD1-rP1 was used to screen all samples in Table 42 and Table 43, followed by digestion of the PCR products with EcoRI. Several stalk samples were shown to contain bacteria: healthy and YCS Q183- Patane, which agrees with the phytoplasmas results, healthy KQ228-H1 and YCS KQ228-Y5 and Y6. The results do not appear to be correlated with YCS.

Viruses

11. **Viral preps:** simple viral preparations were performed for all stalk samples in Table 42 and the extractions were analysed by polyacrylamide gel electrophoresis (PAGE) followed by silver staining. Two samples, Q208-Pombell and Q183-Patane displayed proteins unique to the YCS viral preps that were not present in healthy preps. These two samples plus Q200-Mathews were all found to have isometric viral particles when viewed under the electron microscope.

This in-depth analysis of healthy and YCS symptomatic samples collected in 2013 and 2014 from field-grown material, represents stalks, leaves and roots and three regions. Generic and specific tests for viruses, fungi, fungal-like organisms, phytoplasmas and bacteria were carried out, although not on all tissues from all samples.

Only three tests produced no bands in any sample: the luteovirus, SCYLV and chlorotic streak tests. For all DNA and RNA extractions, an endogenous test (Phosphofructokinase for DNA and Rubisco for RNA) is performed to check the quality of the nucleic acid, so a lack of product with these tests is not due to the quality of the extraction.

The three phytoplasma tests produced non-specific PCR products in the one healthy and two YCS samples. The absence of expected sized products suggests that phytoplasmas are not associated with YCS.

The generic fungal and bacterial tests produced complex results, with PCR products amplified from many of the samples, both healthy and YCS. It is to be expected that a wide range of organisms would be detected in field grown sugarcane, and this was certainly the case. To further complicate the interpretation of the results, YCS is known to occur in waves and so samples considered "healthy" may not be healthy, but simply not expressing YCS at the time of sampling. If YCS is associated with a biotic factor, that organism may still be present in the samples not expressing symptoms of YCS. Even so, no PCR product was produced consistently across all YCS samples.

To investigate the isometric viral particles observed through electron microscopy (EM), several YCS samples were prepared as mini-viral preps and subjected to polyacrylamide gel electrophoresis (PAGE) and electron microscope observations:

- Using PAGE, two samples showed proteins unique to the YCS samples that were not in healthy (green) samples. These were stalk preps from Q183 and Q208.
- Eight samples have been examined under the EM (3 leaf preps, 3 stalk preps, 1 root prep and 1 leaf sheath prep). All three stalk preps had low numbers of isometric viral particles ranging from about 20-30nm.

6.8.1.2 Inclusion body staining

This technique combines light microscopy and specific stains to look for the presence of viral inclusion bodies. Many plant viruses induce distinctive intracellular inclusions. The inclusion may be the actual viral particle itself, or they may be the products of the viral genome, and in some cases, modified cell constituents. Their detection can provide a rapid and relatively inexpensive method for determining viral infection. It was hoped that this technique could be used to supplement electron microscopy, currently being done at the EcoSciences Precinct in Brisbane.

Two stain combinations are commonly used. The O-G stain is a combination of calcomine orange and "Luxol" brilliant green and detects inclusions containing protein. The second stain is Azure A and detects inclusions containing nucleic acids. The methods being trialled predominantly focus on staining epidermal strips (Christie and Edwardson, 1977, 1986). As YCS is suspected to involve alterations in starch accumulation and sugar transport, it is more appropriate to look for viruses within vascular bundles that could be blocking phloem transport. Consequently, the methods had to be adapted for transverse leaf sections and stalk vascular tissues.

Stained structures resembling inclusion bodies have been detected in some O-G stained YCS samples (Figure 15). One of these was Q183 stalk tissue, one of the three stalk samples where viral particles were observed under EM. The other sample shown in Figure 195 is a transverse section through a vascular bundle in a B72-177 leaf.

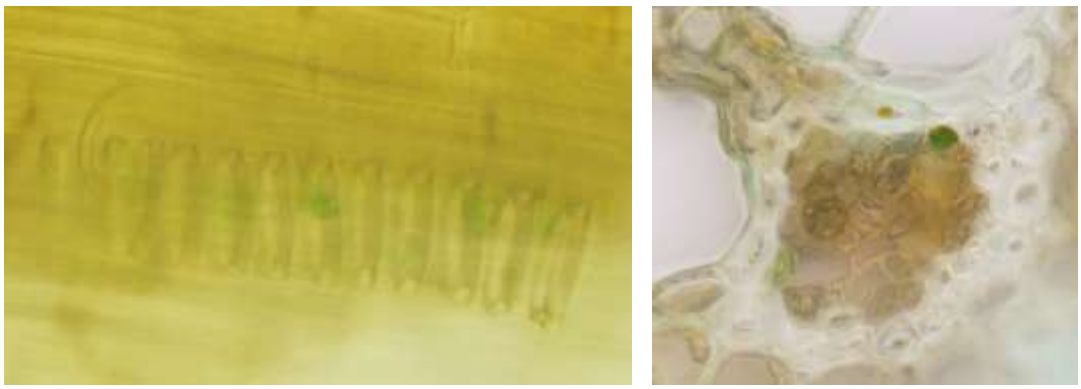


Figure 195 O-G staining of YCS samples. Green inclusion bodies observed in stalk vascular tissue in Q183 A) and in a phloem cell of a B72-177 leaf B).

Other YCS-affected leaf samples collected in Mackay have been transversely sectioned and stained. There was no overwhelming evidence for the presence of viral inclusion bodies, except for limited OG staining in the vascular tissue of Q249 from block 20-1 (Figure 196). This cane was severely affected by YCS.

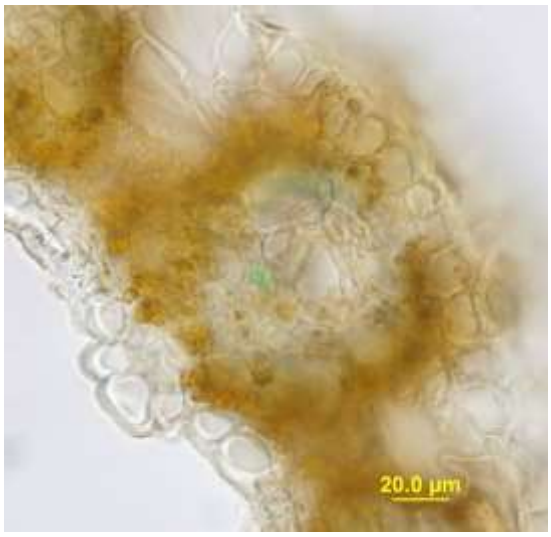


Figure 196 Green inclusion in the vascular tissue of a Q249 leaf after staining with the OG combination.

Stained structures resembling inclusion bodies were detected in two O-G stained YCS samples and one GLS sample (image not shown).

6.8.1.3 Viruses

Generic Luteovirus primers, viral minipreps and double stranded RNA (dsRNA)

Since isometric particles were observed under the EM, the search for isometric viruses known to block the phloem and interfere with sugar transport is a logical starting point. Luteoviruses fill these criteria. Luteovirids represent three genera within the family *Luteoviridae*. They have isometric particles of 25-30nm, are transmitted by aphids, and induce yellowing symptoms in their host. They

cause significant yield loss around the world in economically important crops such as beet, cereals, potatoes and legumes. They are present in low concentration and restricted to the phloem. A very large number of luteovirus sequences are publically available allowing the development of generic RT-PCR primers for their detection. Initially the primer sets of Chromic *et al*. (2010) were trialed. Chromic *et al.* (2010) describes nine degenerate primers which when used in five combinations are able to detect two of the three *Luteoviridae* genera (*Luteovirus* and *Polerovirus*, but not *Enamovirus* or some unassigned viruses). One of the five combinations is specifically designed to detect sugarcane yellow leaf virus (SCYLV). As known SCYLV-infected leaf samples are available at SRA, these samples were able to act as positive controls in the RT-PCR experiments. Initially RNA was extracted from 16 leaf and stalk samples showing symptoms of YCS or known to be infected with SCYLV and screened with the five primer combinations. Only the SCYLV-infected samples tested positive with the correct primer combination.

In February 2015, a sampling trip was undertaken in the Mackay area. Samples collected are shown in Table 2. RNA was extracted from leaf disks punched from the 10 Mackay samples and screened with the nine degenerate primers in five combinations. The 6th PCR is an endogenous check for RNA quality based on the Phosphofructokinase gene. All samples gave a good endogenous results and the SCYLV positive controls tested as expected. No Mackay sample tested positive with any of the Luteovirus combinations.

The viruses listed in Table 44 do not appear to be known viruses of sugarcane. It is possible, however, that different strains are involved which our current diagnostic tests are unable to detect.

Table 44 YCS and healthy samples collected from Mackay in February 2015.

Collected	Variety	condition	Farm	District	Crop
Feb 2015	Q200	H	MAPS Victoria Plains ASP		
	Q208	H	MAPS Victoria Plains ASP		
	Q240	H	MAPS Victoria Plains ASP		
	Q249	H	MAPS Victoria Plains ASP		
	Q200	YCS	MAPS Victoria Plains ASP		
	Q200	YCS	Paul and Joe Schembri #1154A	13-4	5R
	Q208	YCS	Paul and Joe Schembri #1154A	3-1	2R
	Q240	YCS	Paul and Joe Schembri #1154A	10-3	Plant
	Q240	YCS	Kevin Zahb #4012A	6-1	Plant
	Q249	YCS severe	Paul and Joe Schembri #1154A	20-1	Plant

Viral minipreps

Viral prep and poly acrylamide gel electrophoresis (PAGE) techniques were used to screen samples from the Burdekin, Ramu Agri-Industries Limited, PNG and the Herbert in May 2014. The Ramu samples were displaying a condition known as golden leaf syndrome (GLS). This syndrome, first observed in 2009, has symptoms very similar to those of yellow canopy syndrome. Viral preps (40), representing either leaf and/or stalk material were extracted from 22 plant samples (Table 45). The electrophoresis, followed by silver staining, is performed to identify proteins unique to YCS samples, not present in healthy of the same variety, which may indicate the presence of a virus.

Many of the leaf samples analysed showed unique YCS proteins. An example is shown in Figure 197. Three YCS-affected KQ228 leaves collected from Burdekin farms displayed several strong protein bands in the 21.5 kDa size range that were absent from healthy KQ228 leaves. Other samples, including MQ239 from the Herbert and Q183 from the Burdekin, show faint bands in the YCS, that do not appear to be present in healthy samples.

Table 45 2014 samples processed using the viral mini-prep method.

Collected	Variety	condition	Farm	District	Material
Jan 2014	KQ228	H	Burdekin station FAT	Burdekin	Stalks, leaves
	KQ228	H	Burdekin station BS YCS trial	Burdekin	Stalks, leaves
	KQ228	H	Ian Shepherdson block 4	Burdekin	Stalks, leaves
	KQ228	YCS	Ian Shepherdson block 4	Burdekin	Stalks, leaves
	KQ228	YCS	Ian Shepherdson block 9	Burdekin	Stalks, leaves
	KQ228	YCS	Mario Privatera	Burdekin	Stalks, leaves
	MQ239	H	Hobbs	Herbert	Stalks, leaves
	MQ239	YCS	Chiesa	Herbert	Stalks, leaves
Mar 2014	PN92-339	H	Ramu Estate	PNG	leaves
	PN92-339	GLS	Ramu Estate	PNG	leaves
	B72-177	H	Ramu Estate	PNG	leaves
	B72-177	GLS	Ramu Estate	PNG	leaves
May 2014	Q183	H	Patane	Burdekin	Stalks, leaves
	Q183	YCS	Patane	Burdekin	Stalks, leaves
	Q208	H	ASP Stone River Confidor trial	Herbert	Stalks, leaves
	Q208	YCS	ASP Stone River Confidor trial	Herbert	Stalks, leaves
	Q200	H	Hobbs, Abergowrie	Herbert	stalks
	Q200	YCS	Girgenti	Herbert	stalks
	PN92-339	H	Ramu Estate	PNG	leaves

	PN92-339	GLS	Ramu Estate	PNG	leaves
	B72-177	H	Ramu Estate	PNG	leaves
	B72-177	GLS	Ramu Estate	PNG	leaves

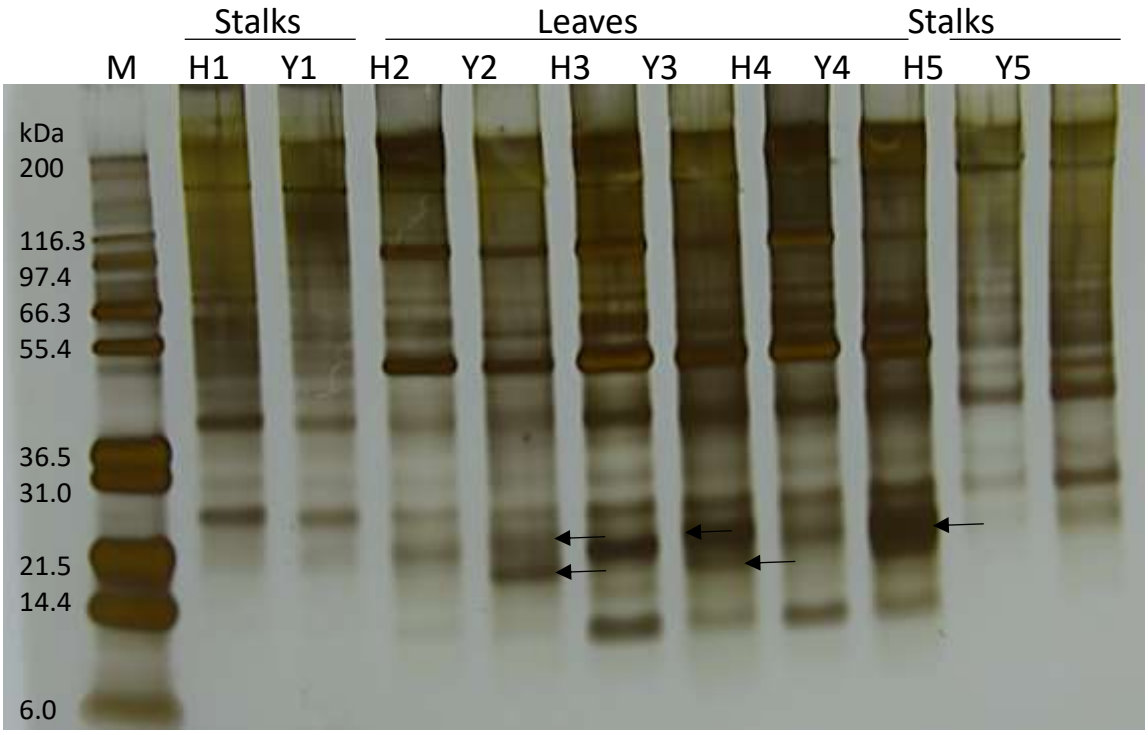


Figure 197 Silver stained poly acrylamide gel showing proteins extracted during virus mini-purifications. The arrows point to various proteins of about 21.5kDa that are in the YCS KQ228 leaves but not in the healthy KQ228 leaves. Lanes from left to right are:

M: Marker, H1: KQ228 stalk, Burdekin station, Y1: KQ228 stalk, Ian Shepherdson block 9, H2: KQ228 leaf, Burdekin station, Y2: KQ228 leaf, Mario Privatera, H3: KQ228 leaf, Burdekin station, Y3: KQ228 leaf, Ian Shepherdson block 4, H4: KQ228 leaf, Ian Shepherdson block 4, Y4: KQ228 leaf, Ian Shepherdson block 9, H5: Q208 stalk, Herbert Confidor trial, Y5: Q208 stalk, Herbert Confidor trial.

Further sample processing shows asymptomatic *vs* YCS leaf viral prep proteins separated on a polyacrylamide gel (Figure 198). The effects of YCS on the leaf protein complement are clearly seen (three double arrows). However, these protein differences did not correspond with viral particle observations when observed by electron microscopy. In contrast, the dried GLS-infected leaves of B72-177 showed a 41kDa band, not present in the asymptomatic (data not shown). This sample was later found to contain several viral particles. Samples were examined by electron microscopy at the EcoSciences Precinct, Brisbane with the assistance of Dr. John Thomas and Dr. Kathy Crew (Table 46).

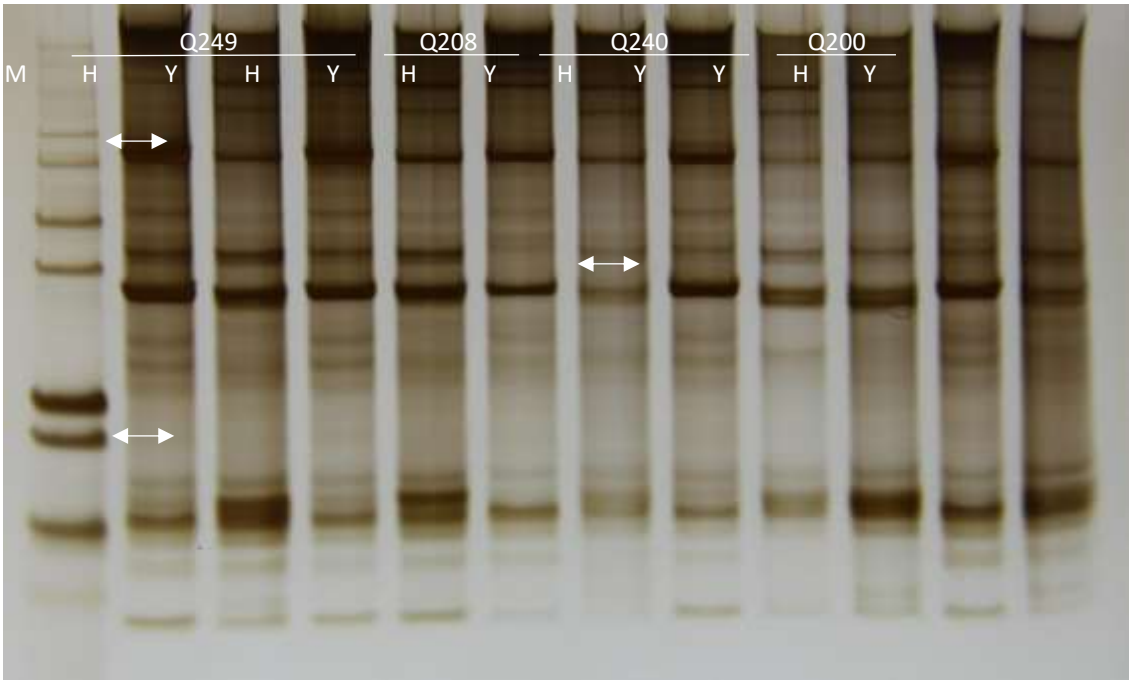


Figure 198 Leaf proteins extracted during the viral miniprep procedure from asymptomatic and YCS canes from Mackay. "H" indicates asymptomatic (green) leaves. Double arrows indicate protein differences between asymptomatic and YCS leaves.

Table 46 Viral minipreps examined by electron microscopy.

[1] The number of confirmed isometric, bacilliform and rod shaped particles observed

[2] Indicates that viral-like structures could not be confirmed

Date	Material	Condition	Location	Viruses?	Isom[1]	Bacil[1]	Rod[1]
Jul-13	Q208 stalks	YCS	Pombell Rd	yes	3	1	2
Jul-13	Q200 stalks	YCS	Pappins Rd	no			
Aug-13	Q183 stalks	YCS	Patane, Burdekin	yes	3		
Aug-13	Q183 roots	YCS	Patane, Burdekin	no			
Aug-13	KQ228 leaves	YCS	Burdekin	no			
Aug-13	KQ228 leaf sheath	YCS	Burdekin	yes			1
Jan-14	KQ228 leaves	YCS	Privatera, Burdekin	no			
Jan-14	KQ228 leaves	YCS	Shepardson, Burdekin	?[2]			
Jan-14	MQ239 leaves	YCS	Chiesa, Herbert	no			
May-14	Q183 leaves	YCS	Patane, Burdekin	Yes	4	3	3
May-14	Q183 leaves	Asympt	Patane, Burdekin	Yes	3	5	1
May-14	B72-177 leaves	GLS	Ramu, PNG	Yes	4		4
Feb-15	Q249 leaves	YCS	Schembri, Mackay	?			
Feb-15	Q208 leaves	YCS	Schembri, Mackay	?			
Mar-15	Q231 leaves	YCS	Hesp, Cairns	?			
Mar-15	Q200 leaves	YCS	Rhegenzhani, Cairns	?			

Some viral observations could not be confirmed by Dr. Thomas because they were confused with phytoferritin, flagella or other matter. Regardless, it can be seen that many samples with YCS were viral-free. Of the samples with viral particles, one was asymptomatic and the other had golden leaf syndrome (GLS). A range of particle types were observed, including isometric particles, bacilliform particles and various rod-shaped particles, as shown in Figure 199. The lack of particles in some YCS samples and the wide range of particle types observed in others, suggest that viral infection could not be the cause of YCS. However, it is possible that viruses contribute to the stress which triggers YCS. It is also possible that stressed YCS plants are more susceptible to viral infections.

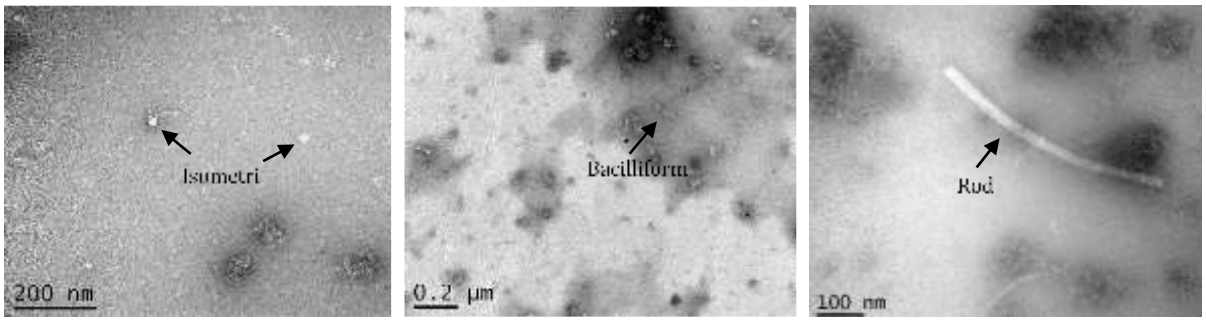


Figure 199 Three examples of the range of particles observed by electron microscopy.

Double Stranded RNA (dsRNA)

A non-specific approach to detecting viruses is double stranded RNA (dsRNA) analysis. The iNtRON Biotechnology Double Viral dsRNA mini kit was used to process samples. Sugarcane leaves infected with Fiji disease virus (FDV) and sugarcane mosaic virus (SCMV) were used as controls. FDV has a dsRNA genome of 10 fragments and the kit was able to extract these successfully, with 8 of the 10 routinely visualised on agarose or acrylamide gels. SCMV has a ssRNA genome and appeared only as a faint smear on gels. None of the YCS samples processed, including those from Table 2 known to have viral particles, gave more than faint smears. As this kit uses only a maximum of 300mg of leaf tissue, the starting material may be too low for the detection of ssRNA viruses which only have dsRNA as their replicative form.

Viral detection based on particle shape and size

As sugarcane is vegetatively propagated it is not surprising that viruses would accumulate in field grown plants. Some of the particle types observed could correspond to known viruses of sugarcane, for example:

- sugarcane yellow leaf virus (SCYLV): isometric, 24-29 nm diameter
- sugarcane bacilliform virus (SCBV): bacilliform, 60-900nm (ave 130nm) x 30nm
- sugarcane mosaic virus (SCMV): flexuous rods, 680-900nm x 11-13nm
- peanut clump virus (PCV): rods, 190nm and 245nm x 21nm.

As a starting point, attempts were made to confirm the identity of some observed viruses using the suite of diagnostic primers available through the SRA quarantine pathology group (Thompson et al., 2012). Most YCS-infected canes received at Indooroopilly have been screened with an RT-PCR diagnostic test for SCYLV. No Australian YCS cane sample has tested positive so far, although the GLS cane, B72-177 from Ramu, PNG, did test positive. Also, most samples collected since 2013 have been screened with a generic luteovirus test. Again, no Australian YCS cane sample has tested positive, although B72-177 from Ramu did. Data does not support that luteoviruses are associated with YCS. Also, while SCYLV is 24-29nm in diameter, the particles seen in Q183 from Patane were approximately 35nm.

A smaller range of samples focusing on those showing rod shaped particles, have been screened with RT-PCR diagnostic tests for PCV and SCMV. PCV causes red leaf mottle of sugarcane in addition to causing disease in peanuts, groundnuts, sorghum and maize. It is transmitted in soil by the fungal-like organism *Polymyxa graminis*. The virus is a member of the Pecluvirus family and shows considerable strain variation with more than 5 serotypes in existence. A broad-spectrum RT-PCR test was supplied by CIRAD (unpublished). No sample gave a PCR product of the expected size.

Mosaic on sugarcane can be caused by members of the potyvirus family including sugarcane mosaic virus (SCMV) and the closely related sorghum mosaic virus (SrMV). Many strains of SCMV have been reported worldwide, but in Australia, only SCMV-A is found in the southern districts of Queensland. Strain A is considered to be mild and is controlled in Australia by quarantine and the use of resistant varieties. A specific RT-PCR test for SCMV-A and a general potyvirus RT-PCR test able to amplify strains of SCMV, sorghum mosaic virus (SrMV) but not sugarcane streak mosaic virus (SCSMV) are routinely used at SRA. Both mosaic tests were trialed with no sample giving a PCR product of the expected size.

6.8.1.4 Pathology summary (2013-2015)

This section includes a summary of all pathology findings since 2013, including pilot project 2013/807 and screening results for phytoplasmas and bacteria.

Sample collections

Since 2013, 46 individual plant samples have been taken for pathology analysis and most are stored at -70°C. The 46 samples came from the Burdekin, Herbert, Mackay and Mulgrave and comprise mostly leaves and stalks. There is also a small collection of Golden Leaf Syndrome (GLS) samples collected from Ramu estate, PNG in 2014.

Viral mini preps

Over 70 viral mini preps have extracted, mostly from leaves and stalks, using a modified method from Les Lane (http://lclane.net/text/minipurprotocol.html). They were all separated by polyacrylamide gel electrophoresis, to check for quality and also for any obvious protein changes

which could indicate the presence of viral coat proteins. There were clear differences between green and YCS leaves, due to chlorophyll breakdown. There were no obvious differences between stalk proteins from green and YCS plants and no obvious proteins suggesting viral coat proteins.

Twenty-two viral preps have been viewed by electron microscopy with the assistance of Drs John Thomas and Kathy Crew at the EcoSciences Precinct at Boggo Road, Brisbane (Table 47). The 22 preps were mostly extracted from leaves and some stalks and represent 16 individual plants. As it can be difficult to confidently distinguish viral particles from cellular debris and other contaminating structures such as phytoferritin, flagella and bacteriophages, Dr Thomas was only able to confirm the presence of viral particles in 7 plants (Table 48). These represent a range of particle types. Isometric particles were generally either about 20nm in diameter, an unusually small size for plant viruses but characteristic for nanoviruses; and 35nm, possibly representing cryptic viruses. The bacilliform shaped particles were most likely sugarcane bacilliform virus, and while this virus is common in noble canes, it occurs less often in commercial canes. It is associated with symptoms of leaf flecking. Various rod-shaped particles were observed screened by RT-PCR for peanut clump virus (PCV), sugarcane mosaic virus (SCMV) and generic potyviruses, with all samples testing negative.

Table 47 All samples where viral preps were observed by electron microscopy.

Collection date	Variety	Condition	Location	Tissue	Viruses?
July 2013	Q208[A]	YCS	Pombell Rd, Herbert	stalks	**Yes**
July 2013	Q200[A]	YCS	Mathew's Pappins Rd, Herbert	stalks	possible
August 2013	KQ228[A]	YCS	Burdekin	Leaves leaf sheath	No **yes**
August 2013	Q183[A]	Asympt	Patane, Burdekin	Stalks Leaves roots	**Yes** **Yes** **yes**
August 2013	Q183[A]	YCS	Patane, Burdekin	Stalks roots	Possible no
January 2014	KQ228[A]	YCS	Mario Privatera, Burdekin	leaves	no
January 2014	KQ228[A]	YCS	Ian Shepardson, Burdekin	leaves	possible
January 2014	MQ239	YCS	Chiesa, Herbert	leaves	no
May 2014	Q183[A]	Asympt	Patane, Burdekin	Stalks leaves	**Yes** **yes**
May 2014	Q183[A]	YCS	Patane, Burdekin	Leaves stalks	**Yes** **yes**
February 2015	Q249[A]	YCS	Schembri, Mackay	leaves	no
February 2015	Q208[A]	YCS	Schembri, Mackay	leaves	no
March 2015	Q231[A]	YCS	Hesp, Cairns	leaves	possible
March 2015	Q200[A]	YCS	Rhegenzhani, Cairns	leaves	**yes**

December 2009	Q136	GLS	Ramu, PNG	leaves	no
May 2014	B72-177	GLS	Ramu, PNG	leaves	**yes**
Total=16 plants					

Table 48 Summary of viral particle types observed in 22 samples representing 16 plants by electron microscopy.

Observations	Isometric particles	Bacilliform particles	Rod shaped particles	Totals
Definite particles	4	4	7	7
Possible particles	3	-	1	4
No particles seen	-	-	-	5

Is YCS associated with a luteovirus?

Luteoviruses are a logical hypothesis for the cause of YCS because they are located in the phloem and chiefly cause symptoms of yellowing. Sugarcane yellow leaf virus (SCYLV) has a weak association with yellow leaf syndrome (YLS), where the midribs turn yellow but the blades remain green. Seventy four samples (representing 50 individual plants), mostly comprising YCS and asymptomatic samples, but also a small number showing YLS and GLS symptoms, have been screened for SCYLV using the test of Girard et al. (2010) which detects all nine known strains. No Australian cane was positive for SCYLV, but several samples from PNG were. From previous screening, it is known that approximately 50% of commercial canes on the Ramu Estate have SCYLV.

Thirty-six samples comprising YCS, asymptomatic, YLS and GLS symptoms have been screened for luteoviruses using the generic series of primers from Chomič et al. (2010). Once again, no Australian cane was positive for luteoviruses, but the PNG samples positive for SCYLV could be detected with the SCYLV primer combination.

Is YCS associated with a phytoplasma?

Phytoplasmas are also a logical hypothesis for the cause of YCS because they are located in the phloem and can cause symptoms of yellowing. The work described here commenced in July 2015 when a decision was made to focus on possible causes of phloem blockage and to sample tissues other than leaf blades, such as midribs, leaf sheaths and dewlaps. Several phytoplasmas are known to occur in sugarcane: sugarcane white leaf, grassy shoot and green grassy shoot and PCR primers are available for these. There is also a very large, published collection of generic primers for phytoplasmas, many used in a nested PCR format.

Approximately 110 DNA extractions representing 40 individual plants were screened initially with the well-known first round combination P1/P7 (Schneider et al., 1995), followed by three nested combinations. SCWLf/SGSr detects phytoplasmas in the sugarcane white leaf family (Tran-Nguyen *et al.,* 2000), while R16F2n/M23Sr (Padovan et al., 1995) and R16F2n/R16R2 (Gundersen and Lee, 1996) are generic combinations. P1/P7 followed by R16F2n/M23Sr was found to be the best combination for sugarcane and two plant sources consistently tested positive. These were Q183^{A} from the Patane farm, Burdekin and Q200^{A} from the Reghenzani farm, Mulgrave. The PCR products were sequenced and their identity determined by BlastN matching in GenBank. However, it was discovered that neither PCR product was derived from a phytoplasma. The Q183$^{\phi}$ amplicon matched *Bacillius megaterium,* an endophyte, and the Q200$^{\phi}$ amplicon matched *Lactococcus lactis,* a common plant bacterium.

The 110 DNA extractions representing 40 individual plants were screened with the generic bacterial combination fD1/rP1 (Weisburg *et al.,* 1991). While several samples produced PCR products, they were later shown to be various species of *Pantoea* spp, a group that includes rhizobacteria and endophytes.

Insect vectors and phytoplasmas

Following the review in December 2017, the recommendations of the Scientific Reference Panel (SRP) was that all dye uptake and microscopy related work should cease, and our team should focus on the molecular aspects for determination of the causal agent of YCS, specifically for the presence of phytoplasmas. This work was the predominant focus in the project 2016064 and readers are requested to please access this final report for detailed methodology and results of all plant-based analysis for the presence of phytoplasma. This work has been reported in detail in the final report of project 2016/064 and will not be repeated in this report on the SRA eLibrary. Results showed that plant PCR analysis of over 1000 DNA samples taken from Harvey Bay region to north Queensland, as well as from the insecticide trial at the Brandon SRA station was unable to conclusively detect any phytoplasmas, despite testing 15 different variables.

During a visit to the Burdekin in March in 2018, it was noted that plant hoppers were abundant on the cane leaves of both insecticide treated and untreated plants (Figure 200).

Figure 200 Panthopper observed in the SRA Burdekin insecticde trial

As all plant phytoplasmas are vectored by phloem feeding insects attention was directed to insects associated with YCS (see section 6.9 of this report) (Tran-Nguyen et al., 2000; Arocha et al., 2005). Over 300 plant hoppers were collected from the Burdekin trial and DNA was extracted (Qiagen DNeasy Blood and Tissue kit) and analysed for the presence of phytoplasma.

PCRs were performed on the insect DNA using the P1P7 universal primers followed by nested PCR reactions (Table 49).

Table 49 Universal and nested PCR reaction setup

P1/P7 Universal PCR Planthopper xtn i1 to i9 set up and run on the Eppendorf thermocycler.

SRA P1/P7	X1	X5	Eppendorf			
2 x GoTaq Green	10	50	95 C	2 min	x1	
MQ	8.2	41	95 C	30 sec	x35	40%ramp
P1	0.4	2	53 C	1 min		40%ramp
P7	0.4	2	72 C	2 min		40%ramp
	19	19ul/rxn	72 C	10 min	x1	
Template	1	1/rxn				
Total	50					

Bordeaux cycling parameters used with adjusted ramping - based on most successful recent plant PCR's.
5ul template used in each reaction.

Nested R16F2n/R16R2 with Planthopper i1 to i9 set up and run on the Eppendorf thermocycler:

Nested R16 PCR	X1	X5	Eppendorf			
2 x GoTaq Green	10	50	94 C	2 min	x1	
MQ	8.2	41	94 C	1 min	x35	40%ramp
R16F2n	0.4	2	55 C	2 min		40%ramp
R16R2	0.4	2	72 C	3 min		40%ramp
	19	19ul/rxn	72 C	10 min	x1	
P1/P7 PCR Template	1	1/rxn				
Total	50					

Bordeaux cycling parameters used with adjusted ramping. 4hr program.
1ul template from P1/P7 PCR's used in each reaction.
5ul of P1P7; 1ul in nested

DNA was isolated from 324 insects using the protocol described above. The DNA from three insects were pooled and considered as one sample, to ensure sufficient DNA was extracted. Thus, we performed 108 DNA extractions to isolate DNA from all of the insects that had been collected. PCRs with different combinations of PCR conditions as well as other variables (listed below) for amplification of phytoplasma genes as well as nested PCR products as listed below were performed.

Different variables and conditions as listed below were tested to improve the detection of phytoplasma.

Primers tested

1) Direct PCR using P1/P7 primer pair, followed by nested PCR using the following primer pairs:

a) AG1/AG2 (new primers designed by Andrew Geering -UQ)

b) Fu3/u5

c) XH (forward and reverse)

2) Direct PCR with R16F2n and R16R2 with

a) Fu3/u5

b) M1/M2

PCR Conditions:

1) Comparing PCR machines

2) Template titration -now aiming for 100ng/reaction

3) Ramping vs. No ramping

4) PCR cycles altered for Fu3u5 primers updated from original to the R16 Bordeaux conditions.

5) Pooling batches of 10 extractions in the one PCR reaction (33 hoppers equivalent) to improve phytoplasma detection.

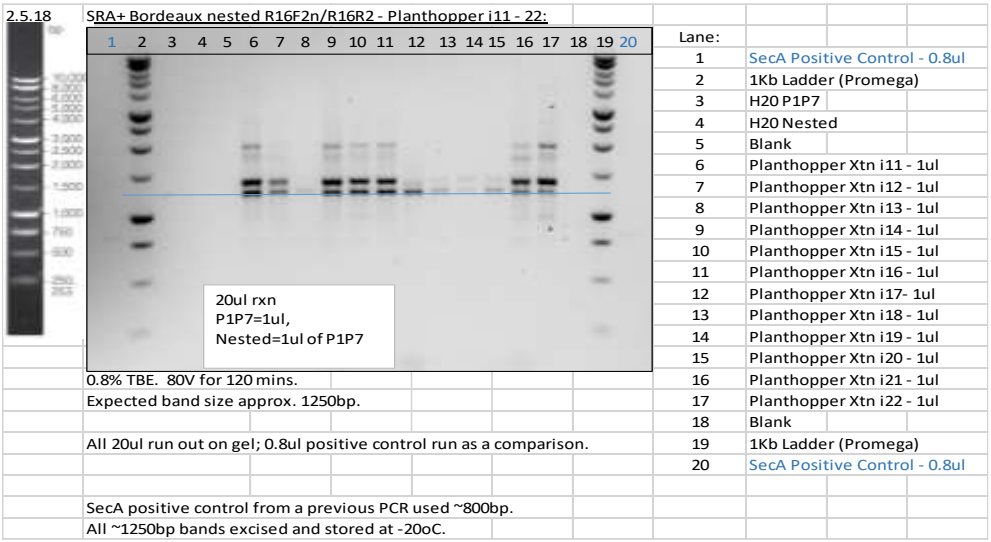


Figure 201 PCR gel of insect DNA showing the amplification of bands of the correct size

PCR Results:

Bands produced of the expected size (Figure 201) were excised, gene cleaned using the Promega PCR clean –up kit and then sent to AGRF for Sanger sequencing.

Sequencing results at AGRF:

Of the 44 sequences that were submitted to AGRF covering 33 extractions, the most abundant microorganism sequences present was that of bacterium: Nesterenkonia, Bacillus megaterium, and Pantoea. However, one sample showed a very strong band (Figure 202). This band was sequenced as

being that of Goosegrass SGS. As the positive control we were using was Sorghum green grassy shoot which is in the same group, we were concerned, that this result may have been due to accidental contamination, and not the phytoplasma causing YCS.

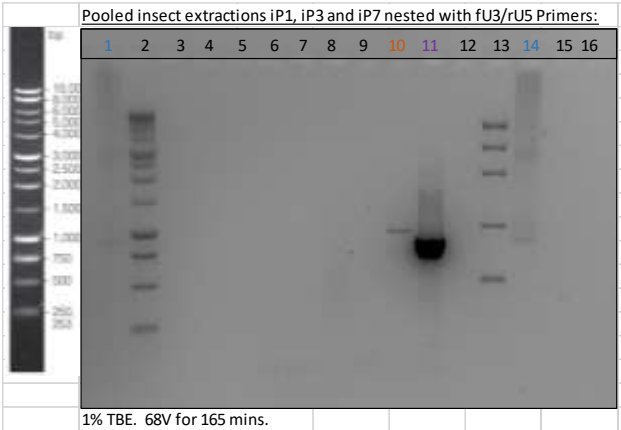


Figure 202 PCR gel of insect DNA amplified with fU3/U5. Only sample11 showed a strong band of the correct size

We were keen to perform further analysis to explore the possibility that phytoplasma specific sequences were present but masked by the large abundance of other bacterium also present in the samples. In order to perform this in-depth sequencing, we isolated the bands and sent them to the Australian Centre for Ecogenomics (ACE) at UQ.

The in-depth sequencing reads of PCR bands of the correct size from four insect DNA samples were analysed:

A3233_S20_paired_contig_3

1) Bacillus megaterium strain S30 16S ribosomal RNA gene, partial sequence

Sequence ID: MF594061.1 Length: 1404 Number of Matches: 1

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
2316 bits(1254)	0.0	1257/1258(99%)	1/1258(0%)	Plus/Plus

A3234_S21_paired_contig_1

2) Nesterenkonia sp. strain APA H6-5 16S ribosomal RNA gene, partial sequence

Sequence ID: MG279104.1 Length: 1432 Number of Matches: 2

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
2135 bits (1156)	0.0	1266/1319(96%)	7/1319(0%)	Plus/Plus

Alignment statistics for match #2

S268 bits(145)	3e-67	157/163(96%)	0/163(0%)	Plus/Minus

A3235_S22_paired_contig_1

3) Nesterenkonia sp. AC84 16S rRNA gene, isolate AC84

Sequence ID: AJ717365.1 Length: 1500 Number of Matches: 1

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
2412 bits (1306)	0.0	1439/1504(96%)	5/1504(0%)	Plus/Minus

A3236_S23_paired_contig_1

4) Nesterenkonia sp. GY074 16S ribosomal RNA gene, partial sequence

Sequence ID: KT751085.1 Length: 1491 Number of Matches: 2

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
2226 bits (1205)	0.0	1324/1382(96%)	6/1382(0%)	Plus/Minus

All samples showed an abundance of *Nesterenkonia* bacteria present in the submitted planthopper DNA tested. None of the insect samples showed phytoplasma sequences, which indicates they were not vectoring this micro-organism.

A small sample set of linear bugs and planthoppers that were of high abundance on YCS symptomatic cane in the Burdekin (Figure 203) were collected in March 2017. The insects were stored in ethanol and processed for DNA extraction using two methods and while both amplified the COI gene, the intensity was greater in the kit extracted samples for both the COI and 16S rRNA genes (Figure 204). DNA processing and Phytoplasma screening was conducted at the SRA biotechnology laboratory in Indooroopilly, Brisbane.

Figure 203 Photos of Linear bug (left) and plant hopper (right) collected at the Burdekin

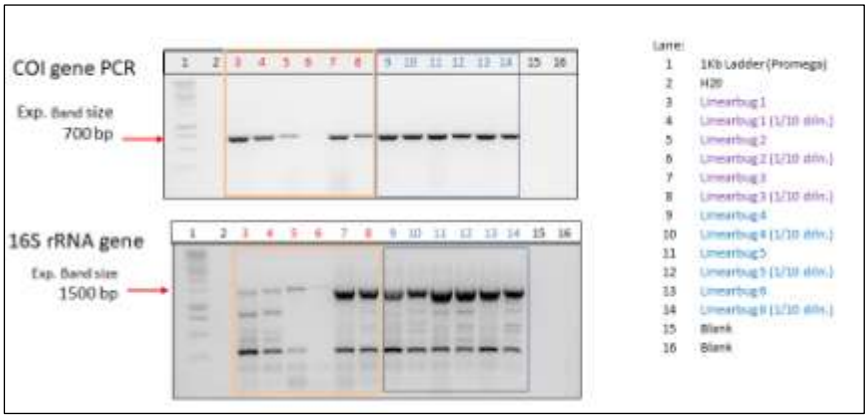


Figure 204 PCR amplification of COI and16S rRNA genes from three linear bugs using either the laboratory-made kit (numbers in red) or purchased Qiagen DNeasy Blood and tissue kit (numbers in blue)

Universal phytoplasma primers: P1/P7 for PCR1 and 16F2n/R16R2 for the nested PCR2 were used against insect and YCS affected sugarcane samples. PCR amplification was optimised using a combination of SRA (PCR1 with P1/P7) and Bordeaux (Nested PCR2 with R16F2n/R16R2) methods. This approach produced bands with one of the five plant hoppers tested (Figure 205). Plant hopper #5 produced seven bands of which five were closest to the target size. These bands were cut out and sent for sequencing which confirmed the amplicons were bacterial in nature (KY515296.1 Nesterenkonia lacusekhoensis strain EMLA3 16S ribosomal), and not from phytoplasma.

Further plant hopper samples were collected from the Herbert region and processed using Qiagen DNeasy Blood and tissue kit and the optimised nested PCR method. Once again no phytoplasmas were detected.

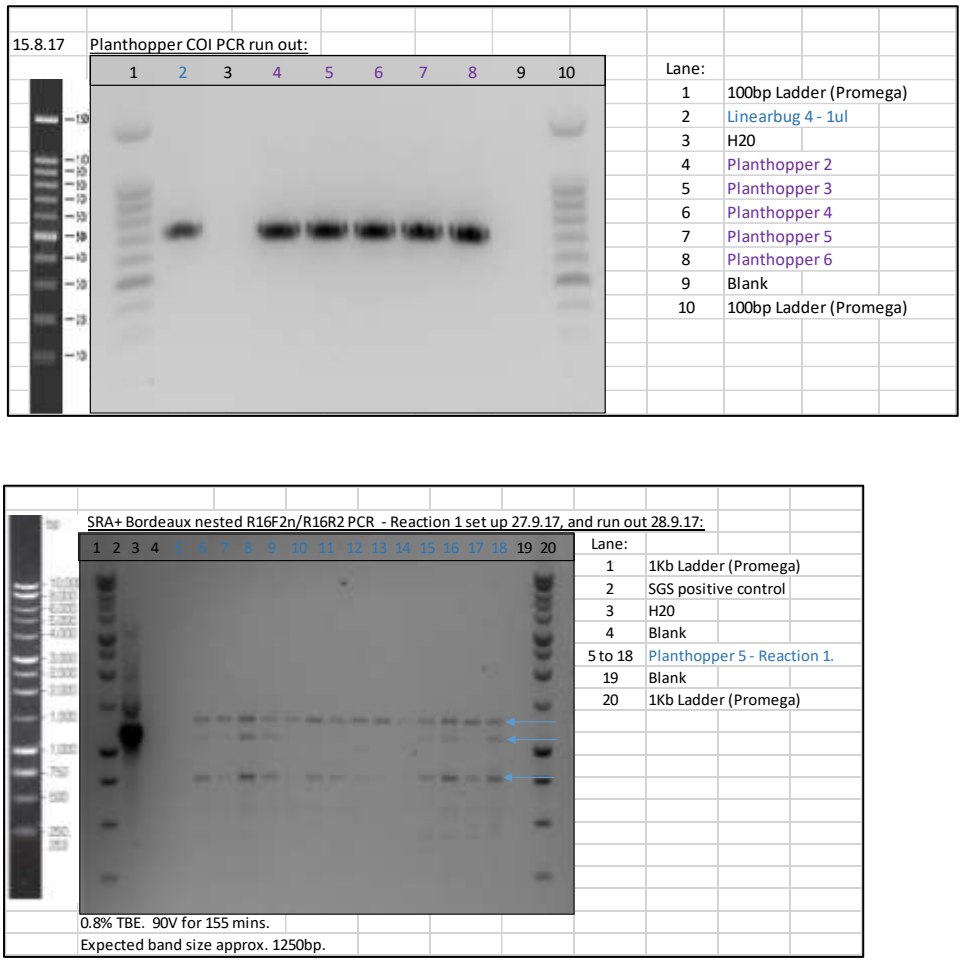


Figure 205 PCR gel for the COI of five plant hoppers collected from Burdekin (top panel) and nested PCR of plant hopper5 using phytoplasma specific primers (bottom panel)

White and pink mealybugs

White and pink mealybugs were collected from Meringa Nth Qld and processed and analysed in the SRA Indooroopilly laboratory. White mealybug has been associated with pasture dieback. While pasture dieback symptoms are completely different to YCS, they were analysed to see if they may be associated with vascular blockage or acting as a potential vector of a phloem blocking organism.

The main objectives were to

1) Isolate DNA, perform PCR and sequence the 16sR gene PCR products to see if we could identify any phytoplasma in either insect DNA
2) Compare bacteria present in the two insects
3) Take microscope images of infected leaves and see what damage the white mealy bug caused to the plant cells.

Results show that there were no phytoplasma in either mealybug DNA and the bacteria present were of a different species. Using the P1-P7 primers for direct PCR and then using 2uL of this direct product as template for the nested PCR. 16srF2n /R2n primers for the nested PCR gave the expected size bands.

White mealy bug sequenced PCR product using 16nr_F1

HM449982.1 Candidatus Tremblaya phenacola clone NH19 16S ribosom...	702	0.0
HQ819338.1 Uncultured organism clone ELU0180-T56-S-NIPCRAMgANa_0...	660	0.0
KT869366.1 Advenella kashmirensis strain Kav2 16S ribosomal RNA ...	577	1e-160
FJ592527.1 Uncultured bacterium clone H06_SB4A 16S ribosomal RNA...	577	1e-160

Pink mealy bug sequenced PCR product using 16nr_F1

AB374415.1 Candidatus Tremblaya princeps gene for 16S rRNA, part...	1528	0.0
M68890.2 Unidentified bacterial endosymbiont natural-host Planoc...	1495	0.0
AF322017.1 Planococcus citri beta-proteobacterial endosymbiont 1...	1483	0.0

White mealy bug nymphs were present on the abaxial leaf surface and were <0.5mm long **(**Figure 206). When the leaves were examined for presence of callose using fluorescent aniline blue stain, some vascular bundles showed increased presence of callose. Vascular bundles also showed blockage of the phloem cells (Figure 207).

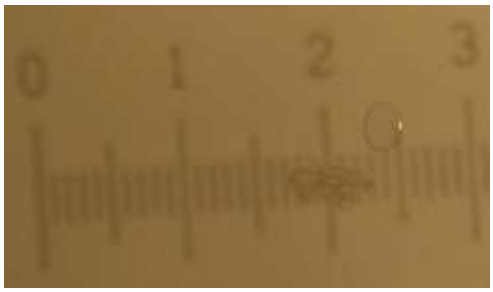


Figure 206 White mealy bug nymph

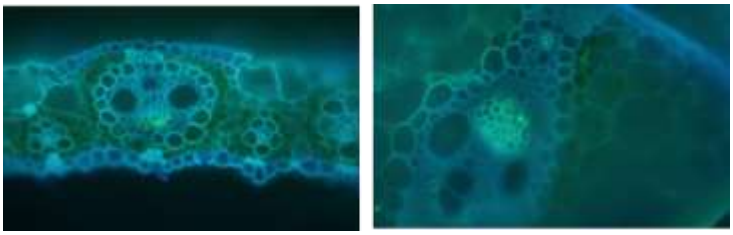


Figure 207 Transverse sections of leaves infested with white mealy bugs stained for callose using aniline blue fluorescence. On the left is from leaf -2 and on the right is a VB of the sheath of leaf -1

In conclusion, this study shows an association between white mealy bugs and the presence of callose and partial blockage in the vascular bundles of the leaves in the upper canopy. This is not surprising as callose deposition is a typical plant response to insect attack (Will and van Bel, 2006; Julius et al., 2018; Varsani et al., 2019). Nonetheless, callose deposition will cause some disruption of metabolite transport in these leaves. However, it should be noted that YCS affects the mid-canopy leaves and quantitative fluorescence spectroscopy analysis of leaf +3 and leaf +4 lamina, midrib and sheath show no significant difference between YCS asymptomatic and symptomatic plants (Scalia et al.,

2020). Studies by Scalia et al. (2020) also show no strong correlation between either leaf sucrose or callose synthase gene expression and callose levels.

In summary, no amplification of phytoplasma specific sequences have been detected in YCS asymptomatic or symptomatic plants and insect tissue despite testing many variables and PCR conditions. This result is contrary to the findings in YCS project 2016/064 (Geering et al., 2020). However, results presented here do concur with SRA phytoplasma studies conducted within 2016/064 by Dr Priya Joyce (Geering et al., 2020).

Fungi and fungal-like organisms

23 stalks and root DNA extractions representing 15 individual plants were screened with the following PCR tests:

- A generic test for fungi targeting the ribosomal intergenic spacer (IGS; LR12R and 5SRNA), as described by James et al. (2001)
- A generic test for Chytrid fungi targeting the ribosomal internal transcribed spacer (ITS; ITS5 and ITS-Chy), as described by Nikolcheva and Bärlocher (2004)
- A generic test for Oomycetes based on the ITS primers ITS5 and ITS-Oom, as described by Nikolcheva and Bärlocher (2004)
- A generic test for the Oomycete *Pythium* based on cytochrome oxidase I (COI) using primers FM35 and FM58, designed by (Martin, 2000).

The IGS and Chytrid-ITS primer combinations produced strong PCR products from many stalk and root samples, both YCS and asymptomatic, but rarely was the same sized product amplified from more than one YCS sample and absent in asymptomatic samples. This was only observed on two occasions and the PCR products matched *Armillaria cepistipes,* a common wood-rotting basidiomycete fungus and *Adineta vaga*, a rotifer. The Oomycete-ITS combination did not amplify products from stalks but did produce many PCR products from roots, including both asymptomatic and YCS samples. The *Pythium* COI combination did not amplify products from stalks but did produce faint PCR products in several root samples.

Light microscopy - inclusion body staining

Two stain combinations were trialed: The O-G stain is a combination of calcomine orange and “Luxol” brilliant green and detects inclusions containing protein. The second stain is Azure A and detects inclusions containing nucleic acids. Stained structures resembling inclusion bodies were detected in two O-G stained YCS samples and one GLS sample.

Fungal hyphae staining

Leaves and stalks were either sectioned transversely or viewed longitudinally and stained with the general fungal stain lacto-glycerol blue. As expected, occasional fungal structures were observed, but there was no evidence of extensive hyphal colonisation, spores, sporangia or other reproductive structures in YCS leaves.

Detailed study from one farm

While it is difficult to estimate yield losses caused by YCS, it is clear that some farms are more severely affected than others. It is possible that YCS on these farms is associated with stresses brought on by plant pathogens. One crop that has been extensively studied in this project is Q183[ф] from the Patane farm in the Burdekin. It was severely affected in 2013 but less so in 2014. Both YCS and "green" (asymptomatic) cane was sampled in both 2013 and 2014 and further subsampled into leaves, stalks, dewlaps and midribs when possible. This farm provides an opportunity to assess the pathogen load in such badly affected canes, and to look at the distribution of systemic microorganisms to ensure that the optimal tissue is being sampled.

Table 50 presents results for phytoplasma PCR screening and electron microscopy. Although the PCR products generated by the primer combination P1/P7 followed by R16F2n/M23Sr were later shown to be bacterial and not phytoplasma in origin, they were detected in every tissue sampled from both asymptomatic and YCS cane and from both 2013 and 2014. Viral particles were observed in almost every tissue viewed under the EM. The relative number of particles seen is shown in Table 50. SCBV was the most common virus and was seen in almost every tissue viewed. Rods and isometric particles were also observed. As sugarcane is vegetatively propagated, it is not surprising that viruses would accumulate in field grown plants. However, the number of viruses observed in this crop, could explain its poor growth performance or be secondary in nature in response to a crop under extreme stress.

Table 50 Distribution of potential pathogens in Q183[A] tissues, from both green (asymptomatic) and YCS-affected cane. nt: not tested; viral particles observed ranged from 0 (-), 1-5 (+), 6-10 (+++), >10 (++++).

	Phytoplasma PCR products (R16F2n/M23Sr)		**Viral particles observed under EM**	
	Asymptomatic	YCS	Asymptomatic	YCS
Leaves 2013	nt	nt	+	nt
Stalk 2013	✓	✓	++++	+
Roots 2013	nt	nt	+	-
Leaves 2014	✓	✓	+++	+++
Stalk 2014	✓	✓	+++	+++
Midrib 2014	✓	✓	nt	nt

Leaf sheath 2014	✓	✓	nt	nt
Dewlap 2014	✓	✓	nt	nt

Possible involvement of retrovirus in YCS

Badnaviruses are double-stranded DNA pararetroviruses and some have been shown to integrate into the host genome, termed endogenous pararetroviruses (EPRVs). The best studied EPRV is banana streak virus which integrates into the host banana genome and in certain cultivars is activated by stress, giving rise to episomal infections. The abiotic stresses that trigger activation include micropropagation by *in vitro* culture processes, temperature differences and water stress (Côte et al., 2010). Other proposed EPRVs include those occurring in taro, fig and yam (Yang et al., 2003; Laney et al., 2012) (Umber et al., 2014).

Australian commercial sugarcane is known to be occasionally infected with sugarcane bacilliform virus (SCBV), although the virus is more common in noble sugarcanes (Braithwaite et al., 1995). The symptoms are freckles, striate flecks and mottling. It is vectored by the pink sugarcane mealybug *Saccharicoccus sacchari*. The genome of the closely related banana streak virus is known to integrate into the host banana genome and in certain cultivars, stress can activate the virus, giving rise to episomal infections. The abiotic stresses that trigger activation include micropropagation by *in vitro* culture processes, temperature differences and water stress (Côte et al., 2010). Of the 16 individual plants examined by electron microscopy, only four plants had bacilliform particles. Three out of these four came from the same variety on one farm: Q183[A] Patane, Burdekin; the other was Q208[A], Pombell Road, Herbert. On that basis it is difficult to conclude that a bacilliform-shaped episomal virus is the cause of YCS. Several other observations suggest that SCBV could not be the cause of YCS:

- The symptoms of SCBV infection in sugarcane are freckles and striate flecks, although symptoms can be absent
- SCBV has been present in Australia for many years, probably since the introduction of noble canes
- SCBV is present worldwide, including countries that do not have YCS.

So far there are no published reports of SCBV integrating into the genome of sugarcane. However, it is appreciated that integration could still be occurring, but has not yet been detected, or another endogenous pararetrovirus or other retrovirus could affect sugarcane.

Summary

The data show that both YCS and asymptomatic cane can contain viruses and bacteria. Screening also shows the presence of fungi in many samples (Braithwaite et al., 2017). No phytoplasmas in plants or insects were detectable and no consistent identifiable organism has been found associated with YCS across the sample set.

6.8.2 Transmission pot trial

An initial YCS 'transmission' trial was planted on 30 April 2013 at Tully Sugar Experiment Station, soon after the widespread incidence of YCS was recognised in the Burdekin and Herbert regions. The object of this experiment was to see if either juice from symptomatic cane, or leaf material exhibiting symptoms, could lead to YCS-like symptoms in plants derived from asymptomatic crops. Treatments included sett inoculation using juice extracted from cane showing severe YCS symptoms as well as the addition of macerated YCS-affected leaves to potting mix with planting material from non-affected crops. Plant sources included symptomatic cane from the Burdekin region as well as cane from non-symptomatic crops located in Tully.

Results

Initially all plants, either grown from YCS-affected cane or from crops not showing YCS, grew well and did not exhibit any YCS symptoms. This included all treatments where transmission of YCS was attempted. Later it was found that water stress induced YCS-like symptoms in planting material derived from both YCS-symptomatic and YCS –asymptomatic crops. Sett inoculation with juice from YCS cane showed no more symptoms than setts grown from YCS-free crops. No evidence for YCS transmission was obtained from the trial results. The major finding was that water stress could induce YCS-symptom development.

A difficulty with YCS transmission research was determining the difference between cane that is asymptomatic and cane that is YCS-free. At the time of this experiment the causal agent / mechanism was unknown, and with no definitive YCS assay. This highlighted the issue of not knowing whether the control was YCS free, particularly when conducting transmission trials. Even cane sourced from regions where no, or few, YCS symptoms have been seen has exhibited extensive YCS-like symptoms when a water stress has been applied followed by watering (this was confirmed and reported in sections 6.3.1 and 6.8.5 of this report).

Woodford Transmission Trial

The aim of the yellow canopy transmission field trial at Woodford was to determine whether yellow canopy syndrome can be transmitted in seed cane. The trial included $Q200^{A}$, $KQ228^{A}$ and $Q247^{A}$ sourced from yellow canopy affected plots in the Herbert and Burdekin and non-affected plots at Bundaberg, Mackay and NSW. Some yellowing was observed on all plants on the oldest leaves during the trial but there was no yellowing on younger mid-canopy leaves in any variety. $KQ228^{A}$ showed more yellowing on older leaves than $Q200^{A}$ and $Q247^{A}$. There was no difference in yellowing observed in plants grown from cane sourced from yellow canopy affected sites and yellow canopy-free sites

Summary

It is evident from these experiments that YCS is unlikely to be caused by the transmission of an agent through the seed cane. YCS can be induced through water stress followed by irrigation.

6.8.3 Develop a clean cane source

Perhaps the greatest challenge of the pathology research was having certainty that control plants were YCS free. To assist in this dilemma, SRA's biosecurity department made available a widely grown commercial variety that was part of the SRA Quarantine Export collection, housed in a glasshouse at Indooroopilly. This stock of variety Q200$^{\phi}$ was originally sourced from Meringa in October 2002, long before YCS appeared in the industry. It was disease tested in 2003 and maintained in the Export Collection glasshouse with continued disease testing every five years to ensure it remains free of known sugarcane diseases.

As there was only one plant in the quarantine glasshouse, initial experiments made use of a small number of 1-eyed setts (see section 6.8.4 of this report). To attain sufficient numbers of plants for larger trials, the use of tissue culture propagation technology was employed. Stock was established from leaf whorl propagation as described by Lakshmanan et al. (2004). While a large number of plants were able to be generated through this technique, they are not guaranteed to be virus free. However, random samples were selected for pathogen screening and no known sugarcane viral pathogens were detected (data not shown).

To improve the confidence in attaining viral free stock, an apical meristem tissue culture methodology (Cheong et al., 2012) was developed to generate virus free cane from a source plant +ve for Sugarcane mosaic virus (SCMV) (see section 5.2.1 of this report). This technique was approximately 85% successful (Figure 208). Based on this success sugarcane tops were sourced for three widely grown genotypes Q200$^{\phi}$, KQ228$^{\phi}$ and Q208$^{\phi}$ from SRA Brandon propagation plots. Apical meristem initiation was used to generate and propagate thousands of clean cane stock (PCR screened) for use as healthy reliable controls in field and pot experiments.

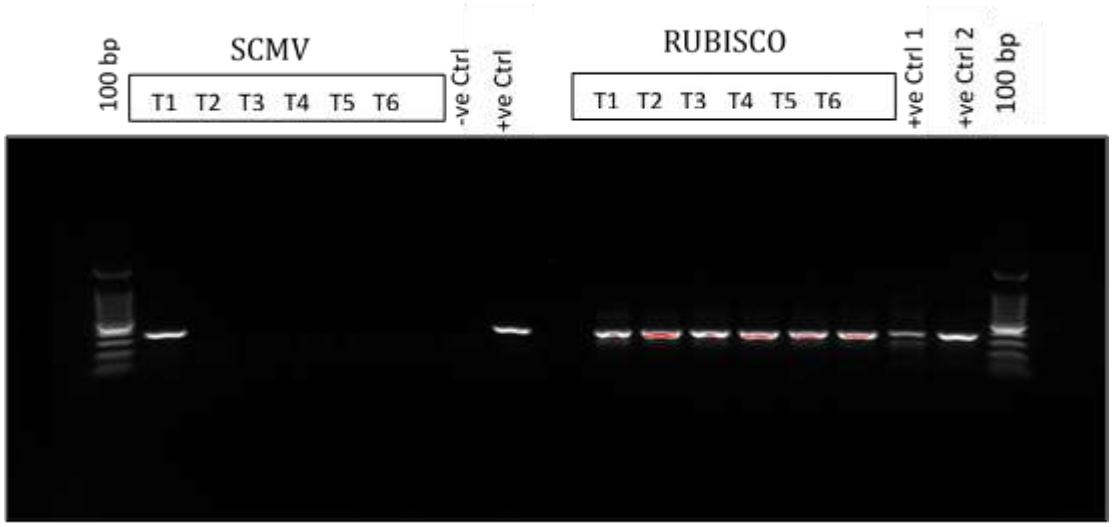


Figure 208 Gel image SCMV screened apical meristem tissue cultured plants

6.8.4 Pathogen isolation and culturing

The material for the isolation studies was sourced from the pathology pot trial established at Ian Shepherdson's farm in the Burdekin in spring 2015. This trial was planted with the "clean cane source" of Q200ф sourced from Meringa in October 2002, long before YCS appeared in the industry, and maintained in the SRA Quarantine Export glasshouse at Indooroopilly. It was initially disease tested in 2003 and then subjected to continued disease testing every five years to ensure that it remained free of known sugarcane diseases. 1-eye setts from the Indooroopilly glasshouse were sent to the Ian Shepherdson's farm and planted into pots buried in the ground along with other varieties including KQ228ф. Cane in the pathology trial began to display YCS symptoms in March 2016 and was sampled and sent to the Biotech lab at Indooroopilly in April. A further sampling was made in May when the cane was in a YCS-asymptomatic (green) phase. Q200ф remaining in the quarantine glasshouse was used as the YCS-free (healthy) control material.

The plant sources taken from Ian Shepherdson's farm and from the glasshouse are listed in (Table 51).

Table 51 Plant material used for direct PCR and pathological isolations

Variety	Trial Rep	Stalk	Trial Conditions	Date received	Condition
Burdekin pathology trial					
Q200ф	Rep 3	S1	Stressed soil	6/04/2016	symptomatic
Q200ф	Rep 2	S2	Stressed soil	6/04/2016	symptomatic
Q200ф	Rep 1	S3	Stressed soil	6/04/2016	symptomatic
Q200ф	Rep 4	S4	Stressed soil	6/04/2016	symptomatic
KQ228	Rep 4	S1	Irrigated soil	6/04/2016	symptomatic
KQ228	Rep 4	S2	Stressed soil	6/04/2016	symptomatic
KQ228	Rep 3	S3	Stressed soil	6/04/2016	symptomatic
KQ228	Rep 3	S4	Irrigated soil	6/04/2016	symptomatic
Q200ф	Rep 3	S1A and B	Irrigated soil	1/06/2016	asymptomatic
Q200ф	Rep 4	S2A and B	Irrigated soil	1/06/2016	asymptomatic
Q200ф	Rep 3	S3A and B	Stressed soil	1/06/2016	asymptomatic
Q200ф	Rep 4	S4A and B	Stressed soil	1/06/2016	asymptomatic
Healthy Control Plants					
Q200ф	Indooroopilly	S1	-	16/04/2016	YCS-free
Q200ф	Indooroopilly	S1	-	29/07/2016	YCS-free

Q200[◊]	Indooroopilly	S2	-	29/07/2016	YCS-free

The material was processed into leaves, stalks and/or xylem sap. For the symptomatic material sent in April, the leaves were subsampled into Leaf +4, regardless of symptoms (because this is the standard sampling leaf for YCS) and one yellow leaf, usually either Leaf +5 or Leaf +6. Xylem sap was blown from the stalk using the method used for RSD sampling. The material was then used in two ways:

a. DNA was extracted directly from the three sugarcane tissues (leaves, stalks and sap), followed by PCR;

b. Fungal and bacterial organisms were isolated from leaves and stalks. Then DNA was extracted from the microorganisms, followed by PCR.

For microbiological isolations from leaves supplied in April, leaf pieces were initially washed, then surface sterilized by soaking in 90% ethanol, followed by 1% bleach, followed by three washes in sterile water. Leaf pieces were plated out on agar plates and grown at 28°C.

Three types of microbiological isolations were made from stalks: pieces in liquid broth, pieces on agar plates, and water exudates on agar plates. In all cases, the stalk lengths were prepared by scrubbing clean then spraying with 90% ethanol, briefly flamed, then split longitudinally. For the material supplied in April, small pieces of internal tissue were removed and placed in liquid broth. For material supplied in June, either small pieces of internal tissue were removed and plated on agar plates to isolate fungi, or left to sit in sterile water for 30 minutes and the exudate spread on agar plates to isolate bacteria.

All culture types were grown at 28°C. Standard microbiological media was used throughout: potato dextrose agar, corn meal and MSC for fungal plates, S8 broth for liquid cultures and LB agar for bacterial plates.

After one to two weeks of growth, any colonies that were clearly growing out of the tissue and not accidental contaminants on the plates were subcultured so that all cultures were free of the original sugarcane tissue. DNA was extracted from the subcultured colonies. Note that no effort was made to subculture by preparing single spore or single colony cultures; it was assumed that any cultures shown to be pathogens of interest would be subject to further detailed microbiological and pathological analyses. Fungal or bacterial material was scraped off the plates and DNA extracted.

DNA extractions were performed using a Qiagen DNeasy Plant mini kit. Generic PCR tests were performed as follows:

1. Potential phytoplasmas were detected with the first round combination P1/P7 (Schneider et al., 1995), followed by the nested combinations R16F2n/M23Sr (Padovan et al., 1995) and R16F2n/R16R2 (Gundersen and Lee, 1996).
2. Bacteria were detected with the generic combination fD1/rP1 (Weisburg et al., 1991).
3. Fungi were detected with ribosomal primer combinations from White et al. (1990): ITS5 and ITS4 which amplifies the ribosomal internal transcribed spacer (ITS); and NS7 and NS8 which amplifies the ribosomal small subunit (SSU).

Only strong PCR products proceeded to gel-purification and sequencing. Sequence identities were determined by BLAST matching.

Results

Direct PCR

The leaf, stalk and sap samples used in direct PCR are listed in (**Table 52**). Very few tissues generated PCR positive products, and of those, only five YCS samples and one control sample produced strong enough gel bands to be used in sequencing. The sequencing results are presented in (**Table 53**).

Table 52 Processed plant samples used in direct PCR. Variety, stalk and trial replicate details come from Table 51. For leaves, G refers to Leaf 4 and Y refers to either Leaf +5 or +6. The PCR test that generated a product for sequencing is shown in bold.

Source code (Var, stalk, rep)	Sample type	Phytoplasma PCR	Bacterial PCR	PCR product sequenced?
Q200 S1 R3	Leaf 4 (G)	positive	negative	No – too faint
	Leaf 6 (Y)	**positive**	negative	**yes**
	Stalk	negative	negative	
	Sap	negative	negative	
Q200 S2 R2	Leaf 4 (G)	negative	negative	
	Leaf 6 (Y)	negative	negative	
	Stalk	negative	negative	
Q200 S3 R1	Leaf 4 (G)	negative	negative	
	Leaf 5 (Y)	positive	negative	No – too faint
	Stalk	**positive**	negative	**yes**
Q200 S4 R4	Leaf 4 (G)	positive	negative	No – too faint
	Leaf 5 (Y)	negative	negative	
	Stalk	negative	negative	
KQ228 S1 R4	Leaf 4 (G)	negative	negative	
	Leaf 6 (Y)	negative	positive	No – too faint
	Stalk	negative	positive	No – too faint
	Sap	negative	positive	No – too faint
KQ228 S2 R4	Leaf 4 (G)	negative	negative	
	Leaf 5 (Y)	positive	**positive**	**yes**
	Stalk	positive	negative	No – too faint
KQ228 S3 R3	Leaf 4 (G)	negative	negative	
	Leaf 6 (Y)	negative	positive	No – too faint
	Stalk	negative	negative	
KQ228 S4 R3	Leaf 4 (G)	negative	negative	
	Leaf 5 (Y)	negative	negative	
	Stalk	negative	negative	
Q200 S1 R3	Stalk A	Not done	positive	No – too faint
	Stalk B	Not done	positive	No – too faint

Source code (Var, stalk, rep)	Sample type	Phytoplasma PCR	Bacterial PCR	PCR product sequenced?
	Sap B	Not done	positive	No – too faint
Q200 S2 R4	Stalk A	Not done	negative	
	Stalk B	Not done	negative	
	Sap B	Not done	**positive**	**yes**
Q200 S3 R3	Stalk A	Not done	negative	
	Stalk B	Not done	negative	
	Sap B	Not done	positive	No – too faint
Q200 S4 R4	Stalk A	Not done	negative	
	Stalk B	Not done	negative	
	Sap B	Not done	**positive**	**yes**
Q200 S1 CONT	Leaf 4	negative	negative	
	Leaf 5	negative	negative	
	Stalk	negative	negative	
	Sap	negative	**positive**	**Yes**

Table 53 Sequencing results generated from sugarcane by direct PCR.

Sample	PCR test	Highest Blast N matches and interesting comments
Q200 S1 R3 Leaf 6	R16F2n/R16R2	*Bacillus megaterium*: endophyte, some fix N
Q200 S3 R1 stalk	R16F2n/R16R2	*Bacillus megaterium*
KQ228 S2 R4 Leaf 5	fD1/rP1	*Pantoea dispersa*: Inhabits plants, soil and water
Q200 S2 R4 sap B	fD1/rP1	*Pantoea dispersa*
Q200 S4 R4 sap B	fD1/rP1	*Pantoea dispersa*
Q200 S1 control sap	fD1/rP1	*Brevibacterium linens*: Non-pathogenic. Found in soil, on human skin and some cheeses

PCR from microbiological isolations

Only a limited number of fungi were isolated compared to the number of bacterial colonies obtained. As many bacterial colonies appeared to be very similar, only representative types based on colony morphology and colour were selected for DNA extraction and PCR. The bacterial and fungal colonies identified through PCR are shown in (Table 54).

Table 54 Details of bacterial and fungal isolations used in PCR. Variety, stalk and trial replicate details come from Table 51. For leaves, G refers to Leaf 4 and Y refers to either Leaf +5 or +6. (-) indicates that no fungal or bacterial colony was obtained. The sequencing code relates to Table 55.

Source code (Var, stalk, rep)	Sample type	Bacterial or fungal colonies obtained	Sequencing code if PCR product sequenced
Q200$^{\phi}$ S1 R3	Leaf 4 (G)	2 bacteria	no
	Leaf 6 (Y)	1 fungus, 1 bacteria	F1, B3
	Stalk	-	
Q200$^{\phi}$ S2 R2	Leaf 4 (G)	-	
	Leaf 6 (Y)	-	
	Stalk	1 fungus	F3
Q200$^{\phi}$ S3 R1	Leaf 4 (G)	2 bacteria: A and B	Only B= B5
	Leaf 5 (Y)	-	
	Stalk	-	
Q200$^{\phi}$ S4 R4	Leaf 4 (G)	1 fungus, 1 bacteria	F2, B6
	Leaf 5 (Y)	-	
	Stalk	-	
KQ228$^{\phi}$ S1 R4	Leaf 4 (G)	-	
	Leaf 6 (Y)	-	
	Stalk	-	
KQ228$^{\phi}$ S2 R4	Leaf 4 (G)	1 fungus	F4
	Leaf 5 (Y)	-	
	Stalk	-	
KQ228$^{\phi}$ S3 R3	Leaf 4 (G)	1 fungus, 1 bacteria	F5, B7
	Leaf 6 (Y)	1 bacteria	no
	Stalk	-	
KQ228$^{\phi}$ S4 R3	Leaf 4 (G)	1 fungus, 2 bacteria: A and B	F6, only A= B9
	Leaf 5 (Y)	-	
	Stalk	-	
Q200$^{\phi}$ S1 R3	Stalk A	4 bacteria	PD-1, PD-2, LB-3, LB-6
Q200$^{\phi}$ S2 R4	Stalk A	2 bacteria	Only PD-4
Q200$^{\phi}$ S3 R3	Stalk A	1 bacteria	no
Q200$^{\phi}$ S4 R4	Stalk A	1 bacteria	PD-5
Q200$^{\phi}$ S1 Cont	Stalk A	6 bacteria	PD1, CM1-3, LB1-2
Q200$^{\phi}$ S2 Cont	Leaf 3	-	
	Leaf 4	-	
	stalk	-	

Table 55 Sequencing results generated from microbiological isolations. The sequencing code relates to Table 54.

Code	Sample	PCR test	Highest Blast N matches and interesting comments
Fungi isolated from the April samples			
F1	Q200$^{\phi}$ S1 R3	ITS and SSU	*Cochliobolus* species: plant pathogens; *C. lunatus* and *C. stenospilus* cause brown stripes in sugarcane
F2	Q200$^{\phi}$ S4 R4	ITS and SSU	*Alternaria* species including *A. brassicicola:* plant pathogens that cause dark Leaf spots
F3	Q200$^{\phi}$ S2 R2	ITS SSU	*Exophiala spinifera*: common environmental fungus in soil, plants, decaying wood and water Top matches *Candida* sp. or other Saccharomycetales
F4	KQ228$^{\phi}$ S2 R4	ITS and SSU	*Cladosporium* species: common environmental moulds found in plants, soil and air. *C. cladosporioides* is a plant pathogen that infects already necrotic plants
F5	KQ228$^{\phi}$ S3 R3	ITS and SSU	*Phoma* species: common soil fungi and plant pathogens causing heart rot and dry rot
F6	KQ228$^{\phi}$ S4 R3	ITS only	Xylariales including *Podosodaria*: found on plants, dung and in insect nests. Many are pathogens.
Bacteria isolated from the April samples			
B3	Q200$^{\phi}$ S1 R3	fD1/rP1	*Kineococcus* species including *K. radiotolerans*: ubiquitous environmental bacteria
B5, B9	Q200$^{\phi}$ S3 R1 KQ228$^{\phi}$ S4 R3	fD1/rP1	*Curtobacterium oceanosedimentum* and C. citreum: common on plants, *C. citreum* is an endophyte in rice
B6, B7	Q200$^{\phi}$ S4 R4 KQ228$^{\phi}$ S3 R3	fD1/rP1	*Herbaspirillum* species: *H. seropedicae* is an endophyte of rice and sugarcane, *H. rubrisualbicans* causes mottled stripe disease in sugarcane
Bacteria isolated from the June samples			
PD-1	Q200$^{\phi}$ S1 R3A	fD1/rP1	*Bacillus* species, including *B. subtilis* and *B. amyloliquefaciens*: soil bacteria
PD-2	Q200$^{\phi}$ S1 R3A	fD1/rP1	*Pseudomonas* species: environment bacteria and also opportunistic pathogens in humans
LB-3 PD-5	Q200$^{\phi}$ S1 R3A Q200$^{\phi}$ S4 R4A	fD1/rP1	*Curtobacterium oceanosedimentum* and C. citreum: common on plants
LB-6 PD-4	Q200$^{\phi}$ S1 R3A Q200$^{\phi}$ S2 R4A	fD1/rP1	*Pantoea* species, including *P. dispersa*: inhabit plants, soil and water
Bacteria isolated from control samples			

PD1	Q200$^{\phi}$ S1 Cont	fD1/rP1	*Chitinophaga pinensis* - a non-pathogenic soil bacterium
CM1	Q200$^{\phi}$ S1 Cont	fD1/rP1	*Pseudomonas hibiscicola* and *Stenotrophomonas maltophilia:* found in water, soil, plants
CM2	Q200$^{\phi}$ S1 Cont	fD1/rP1	*Pantoea* species, including *P. dispersa*: inhabit plants, soil and water
CM3	Q200$^{\phi}$ S1 Cont	fD1/rP1	*Stenotrophomonas panacihumi*: soil bacteria
LB1	Q200$^{\phi}$ S1 Cont	fD1/rP1	*Pseudomonas monteilii*: soil bacteria. *Pseudomonas plecoglossicida*: - endophyte, in waste water, soil,
LB2	Q200$^{\phi}$ S1 Cont	fD1/rP1	*Haererehalobacter* species: Halophilic bacteria found in and marine environments

Summary

A wide range of organisms have been detected in the YCS-affected Q200$^{\phi}$ and KQ228$^{\phi}$ in the pathology pot trial. However, most of them were endophytes or common soil organisms.

Phytoplasmas: in alignment with results from other phytoplasma screening presented in this report, the phytoplasma primers amplified very few PCR products from YCS sugarcane, and when they do, sequencing and BLAST searching reveals them to be bacteria, not phytoplasmas. Similar results were obtained this time.

Bacteria: A wide range of bacteria were identified either through direct PCR or through doing isolations followed by PCR. Many proved to be common and were obtained from both varieties at both sampling time points, from a range of tissues and through different culturing techniques. The common species include *P. dispersa, B. megaterium* and *Curtobacterium* sp. Both *B. megaterium* and *P. dispersa* detected here concur with results presented in section 6.8.1.4 of this report. Most bacteria were endophytes or common environmental microorganisms.

Fungi: Far fewer fungi were cultured than bacteria. Most of the groups of fungi detected contain species that can be pathogenic to plants, but generally they do no produce symptoms compatible with YCS.

The control material: The Q200$^{\phi}$ grown in the glasshouse was also subjected to direct PCR or isolations, followed by PCR, and one stalk was found to contain several bacterial species. *Pantoea* sp. has now been detected in both glasshouse and field material.

Summary

A supply of Q200$^{\phi}$, isolated from the industry for 13 years, was planted into the field in a known YCS hotspot. Within one crop cycle (spring to autumn), it displayed symptoms of YCS. Direct PCR and microbiological isolations followed by PCR detected no obvious sign of a pathogen associated with YCS.

6.8.5 Pathology/Stress Trial – Burdekin (2015)

The pathology/stress trial was established on 1/10/2015 on field #4888A Block 4-1 at Home Hill, Burdekin (see Appendix 1: 1.1.6). Of note, this field has been YCS symptomatic for the two years previous. The objective was to compare different plant sources to evaluate their YCS incidence and severity relative to the surrounding commercial cane which acted as a control in this trial. Control cane was KQ228$^{\phi}$ 1st R. The site was fully irrigated by furrow. Pots were dug into the rows amongst the control cane and were watered via dripper irrigation.

There were three plant source treatments and two soil treatments arranged in a factorial design.

1. Control: ratoon cane KQ228 1stR
2. Burdekin: cane grown from one-eye-setts sourced from Ratoon cane parent (plant cane)
3. Mission Beach: KQ228 cane grown from one-eye-setts sourced from tissue culture raised away from industry
4. Quarantine: Q200 cane grown from one-eye-setts sourced from SRA quarantine glasshouse. Material in quarantine since 2003

Soil treatments were, field soil, a non-soil pearlite/vermiculite mix. An additional two treatments were added; these were systemic fungicide, and the addition of a shaded cane treatment. The surrounding ratoon crop is the control. Treatments were monitored weekly for YCS severity as per standard protocol (Table 4).

Results and discussion

A summary of 2016 YCS incidence at this trial is presented in Figure 3 below. YCS symptoms usually first appear in early February and this trial was no exception. The severity rating of control plants in the surrounding field show how a wave of yellow leaf expression moved through the canopy during February and March (Figure 209). Interestingly the clean source quarantine Q200^{A} plants generated from 1-eye setts consistently scored the highest YCS rating during this time. Also noteworthy is the lower YCS severity rating of the surrounding field cane (KQ228^{A} Burdekin) than all other treatments (Figure 209). This suggests that a pathogen cannot be the cause of YCS as both of the clean source canes 1) quarantine (Q200^{A}) and 2) KQ228^{A} sourced from stock grown in a non-sugarcane area in Mission Beach, had more severe YCS symptoms than the control. In support of this there was no difference between the soil and non-soil treatments (Figure 210) which excludes any soil borne pathogen in this trial. Thus, the data does not support an endogenous, environmental or vectored pathogen as the cause of YCS.

The fungicide treatment failed to prevent YCS development which also excludes a fungal pathogen as the causal agent. Shade treatment delayed the onset of YCS by one month, with increased YCS severity from the start of March. This is likely the result of reduced solar radiation interception and lower leaf sucrose accumulation, which concurs with data from a similar treatment in the growth regulator trial (see section 6.6.1 of this report).

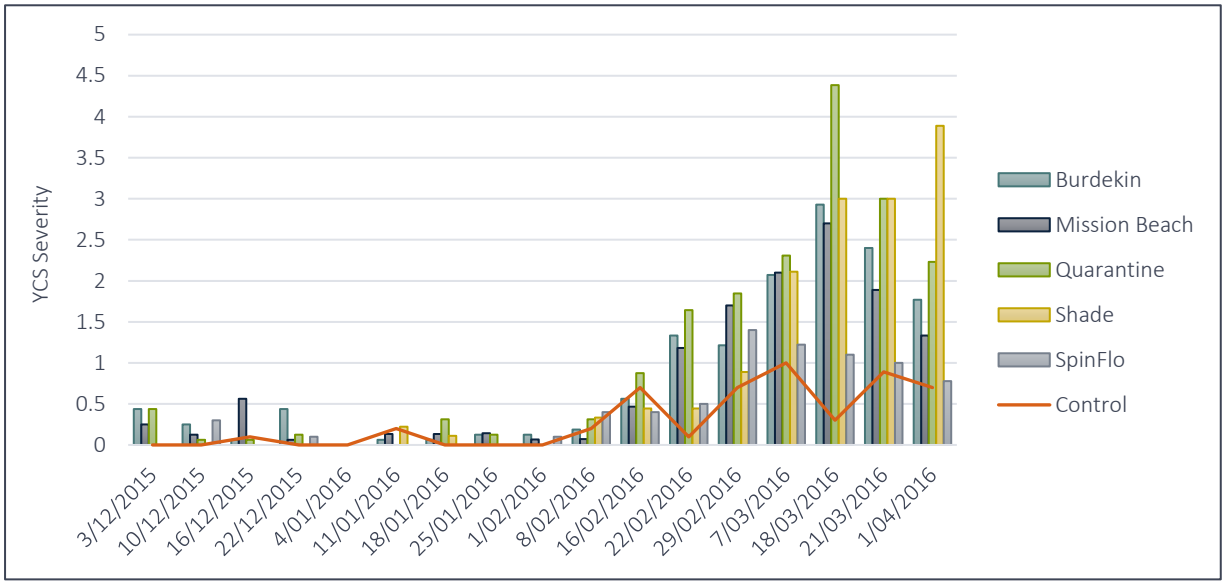


Figure 209 YCS severity score over time. The score is calculated as the sum of the severity ratings for leaves +1 to +7. Each data point represents the average of 8 stalks. The SpinFlo treatment is Carbendazim systemic fungicide. To date there has been no difference between soil and non-soil treatments (data not shown).

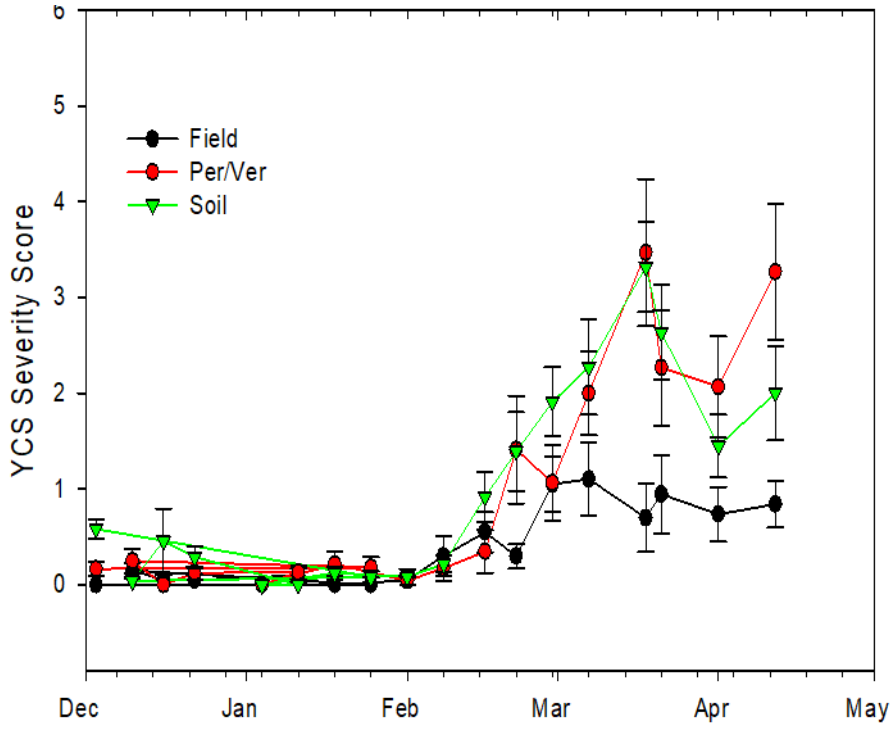


Figure 210 YCS severity score averaged across soil and soilless treatments

The difference in YCS severity between field grown KQ228$^{(\flat}$ control and all other pot grown treatments is striking. The greatest YCS severity differential commences in mid-late February at the peak of the growing season. This increase in YCS severity across the pot trial treatments is likely due

sink limitation caused by the pot effect. A similar scenario was observed in the Mission Beach water stress pot trial (see section 6.3.1 of this report). While the field of KQ228$^{\phi}$ in this trial did develop YCS, symptoms were less severe. This suggests that the roots and culm of the field grown plants were not as sink limited as the pot confined treatments. Therefore, the magnitude of the source sink imbalance and the subsequent YCS symptoms were diminished.

Conclusion

Clean source canes did not remain YCS free and had higher YCS severity than the surrounding field grown cane. No difference in YCS severity could be discerned between soil and non-soil grown treatments. This suggests that a pathogen is not the cause of YCS. Sink strength limitation attributed to the pot effect is the likely cause of increased YCS symptoms in all pot grown treatments in this trial.

6.8.6 Discussion and conclusions

An extensive microbiological analysis was carried out on material growing commercially and in field and pot trials. Sampling represented all tissue types for most varieties grown widely across all regions of the industry. Wide-ranging investigation was conducted on a clean cane source of Q200$^{\phi}$, originally derived from the Indooroopilly quarantine glasshouse. Clean sugarcane material generated through apical meristem tissue culture developed YCS in the field and tested negative for pathogens. Soil samples were also analysed for potential pathogens associated with pathology pot and field trials. Transmission trials show that YCS is not transmitted through seed cane, juice or leaf tissue. Organisms investigated included bacteria, viruses, fungi, protozoa and phytoplasmas. The methods adopted to screen plant and insect material for pathogens included direct PCR and culturing of bacteria and fungi, followed by PCR and sequencing. Tissue staining techniques together with light and electron microscopy were used to detect and visualise organisms in various plant tissue. A very wide range of organisms were detected but very few were pathogenic. Those that were potentially pathogenic, do no produce symptoms compatible with YCS.

Evidently no biotic agent is consistently present during YCS development and expression. This suggests that the cause of high sucrose accumulation in the source leaves of the mid-canopy is not due to a pathogen disrupting phloem loading or transport. However, it is possible that high numbers of microbes observed in crops with severe YCS symptoms could indicate a biotic agent is acting as a stressor. In this scenario the biotic agent may be disrupting growth rate and triggering the onset of YCS. Alternatively, high populations may simply be an opportunistic secondary response to a weakened defence system and a plentiful food supply caused by some other form of stress. Nonetheless, this data does not support the hypothesis that a pathogen is the cause of YCS.

6.9 Insects, insecticides and YCS

It was noted in 2015 during the insecticide stress shield and soil biology trials (see section 6.3 & 6.4 of this report) that neonicotinoid and pyrethroid treatments were effective in reducing YCS symptoms. However, further studies revealed the neonicotinoid (imidacloprid: Confidor®) simply offered a stay-green effect and was ineffective in providing sustained prevention of YCS development and expression. However, the pyrethroid (Bifenthrin) treatment was effective in reducing YCS symptoms as well as limiting leaf sucrose and α-glucan accumulation under experimental conditions. Other insecticides appeared to be of little benefit in mitigating YCS. Regardless of the efficacy of the treatment to manage YCS, much was learned about the types of insects and mites that inhabit sugarcane and their potential involvement in triggering or causing YCS.

The following is an account of entomological research conducted to ascertain the efficacy of the insecticide treatments to mitigate or manage YCS and whether a specific insect, or group thereof, is responsible for YCS development. Similarly, the research also encompassed an evaluation of acaricides and mite populations. Agronomic performance, plant physiology and insect population studies are also reported here.

6.9.1 Insect exclusion tents

In 2017 twelve insect exclusion tents were custom manufactured to investigate the involvement of insects in YCS development (Figure 211) (see Appendix 1: 1.2.22). These tents were constructed of a fine mesh on all sides including the bottom, with a single sealed zipper entrance on one side only. Large 35L pots of potting medium were planted with different types of seed cane source and established with drip irrigation in the tents. Depending on the hypothesis being tested, insects and/or plant material of interest were either excluded or introduced to the tents. Symptom monitoring was conducted from outside the tents and sampling and measurements at appropriate times as to not compromise the integrity of the tents. A summary of objectives and activities is presented in Table 56.

Figure 211 Insect exclusion experiment. Twelve insect tents on site at the SRA Burdekin station.

Table 56 Exclusion tent objectives and activities

Hypothesis	Potting medium	Seed cane source	Treatments	Sampling/Measurements
Plant leaf hoppers (*Perkinsiella saccharicida)* cause YCS	High quality potting mix	KQ228 one-eye setts from Tully clean seed plots soaked in streptomycin prior to germination	1. Insect tent (addition of 200+ *P . saccharicida*)	Prior to introduction of insects
Plant leaf hoppers (*Perkinsiella saccharicida)* vector a phytoplasma			2. Exclusion Control (minus all insects)	Starch/sucrose test
			3. Outside Control (minus tent).	Whole stalk sample for phytoplasma testing
Insects cause YCS	Pasteurised high quality pot	Heat treated KQ228 one-eye setts from Tully clean seed plots soaked in streptomycin prior to germination	1. Many different insects introduced at staggered times between November-February	After introduction of insects
Insects vector a phytoplasma			2. Bifenthrin treated	▪ store a subsample of insects at -80 (or ethanol) for phytoplasma testing
			3. Non-sprayed	▪ Insect observations – are they still alive? Is there a predominant species?
			4. Sugarcane stalks introduced from field	After onset of YCS symptoms
				▪ Starch test to confirm symptoms
				Sample of insects inside the tent for phytoplasma testing
				Whole stalks for phytoplasma testing
				Laboratory, molecular, TEM analysis
				▪ Stalk and insects for phytoplasma testing

Results

Unfortunately, the exclusion tent experiments were plagued with issues throughout the course of investigation. This is testament to the high degree of difficulty involved to successfully conduct these types of experiments in a field setting. Severe weather events and exclusion material breaches by weeds and insects through the bottom barrier of the tents compromised the exclusive integrity of the structures. It was also realised upon identification of insects within tents that the mesh aperture was inadequate to prevent cross-contamination of very small insects between tents, including the insect free controls. Due to this, no results are presented in this report.

Summary

A higher integrity fabric with smaller aperture pores would be necessary to successfully conduct this type of experiment in a field setting. However, this will not reduce the risk from adverse weather events.

No conclusions can be confidently deduced from any of the exclusion tent experiments.

6.9.2 Insecticide trial 1 – Burdekin (2017-2018)

The following insecticide trial of KQ228$^{(\flat}$ 1R was established on station at SRA Burdekin (Farm #6007 Block #3-1) in September 2017 (see Appendix 1: 1.2.21). The objectives of this trial were to further investigate 1) the efficacy of the pyrethroid bifenthrin to reduce YCS incidence and/or severity, 2) the optimal time of insecticide application to mitigate YCS and 3) what insects are being controlled by bifenthrin. Treatments consisted of an Untreated control (UTC), weekly application November-March (Continuous) and once per week for the duration of each of the months of November,

December, January, February and March. Insect capture, monitoring, identification and population data analyses, YCS monitoring (Table 4) together with a suite of physiological and leaf metabolite levels were conducted before, during and after YCS symptom expression. Magnesium treatment in this trial was part of the nutrient studies (see section 6.2.3 of this report) and was also monitored in this study.

Results

6.9.2.1 YCS monitoring and physiology

Monitoring of the treatments and YCS severity scoring took place on a weekly basis from late October (Figure 212). Low incidence of YCS was recorded across all treatments up until a significant rainfall event that occurred in late January (Figure 213). This coincided with the first significant rainfall event of the season (45mm). Following this event all unsprayed treatments began to express increasing rates of YCS expression (February, Magnesium sulphate, March and Untreated control). Conversely, all treatments that had been sprayed with Bifenthrin did not express significant YCS symptoms (November, December, January and Continuous).

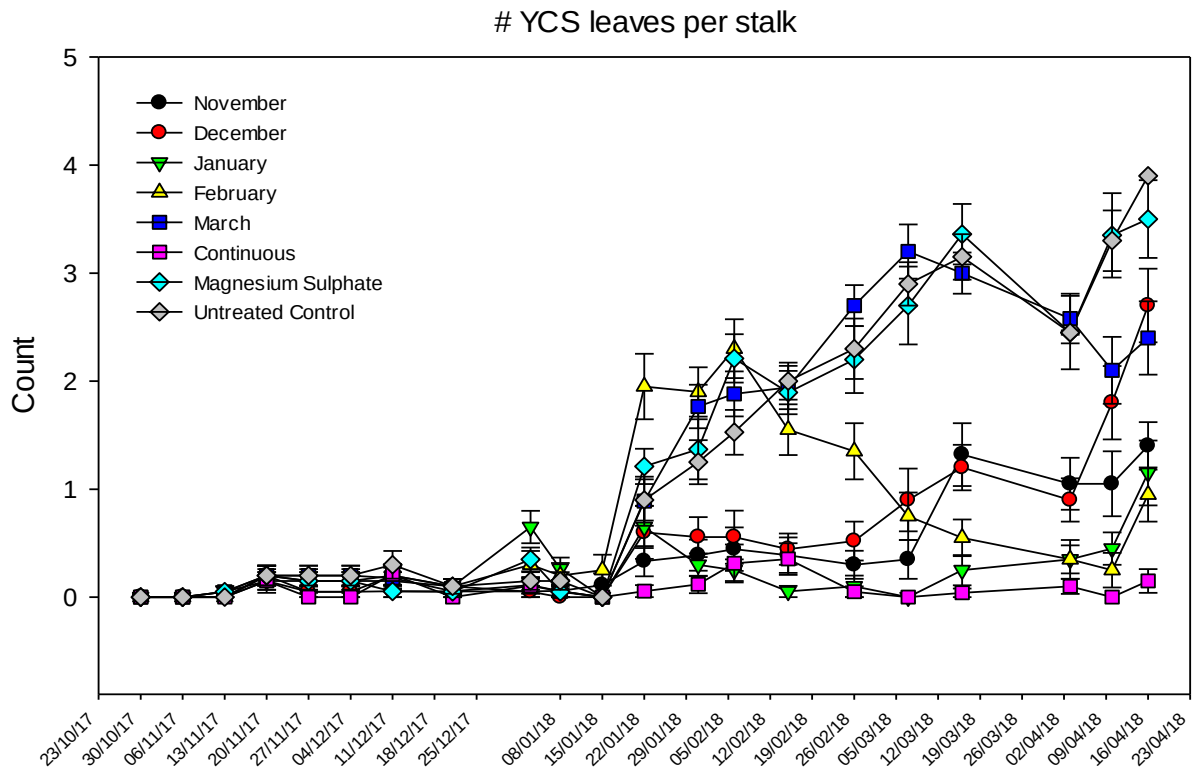


Figure 212 Average number of YCS leaves per stalk of the top 7 (+1 to +7) leaves of the canopy monitored weekly across treatments.

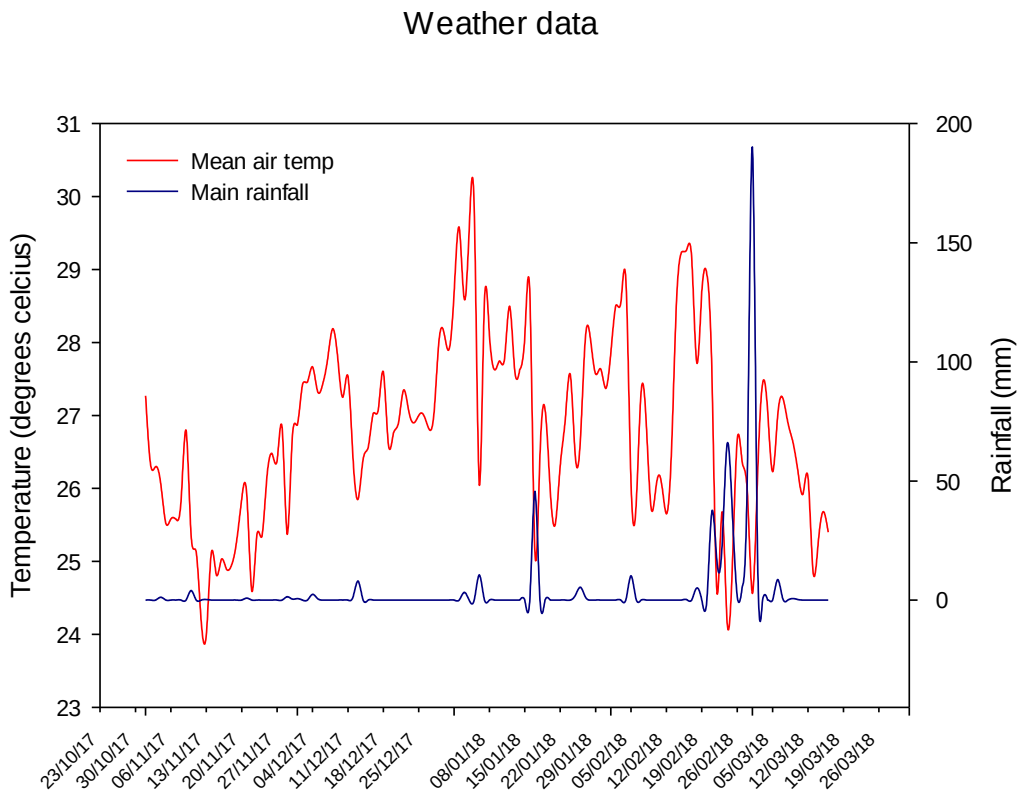


Figure 213 Rainfall (blue) and temperature (red) data recorded from the Brandon on-site weather station.

In early February clear differences could be seen between the Untreated Control and Continuous plots (Figure 214). The Continuously sprayed treatment remained green and asymptomatic throughout the monitoring period while the Untreated Control showed high levels of YCS symptom expression. Importantly, this is the first time a treatment had been able to maintain green leaves in a YCS effected field. This had important implications for future trial work, namely, the ability to effectively evaluate treatment effects and it also created the possibility of determining any yield and/or sugar impact with greater confidence. There also appears to be a trigger event around the middle of January which lead to a sharp rise in symptom expression for all unsprayed treatments (Figure 212). This occurred at the same time as the first good rainfall (45mm) of the season (Figure 213).

Figure 214 YCS response to insecticide treatments. Untreated Control plot (left) and Continuous plot (right). Photos taken 8-Feb-18.

As for timing, it was surprising that cane treated with Bifenthrin back in November and December remained relatively green right through March. All leaves initially sprayed would have been replaced by new leaves in the canopy by March. Therefore, if insects were the cause of YCS, then YCS should have returned to the new untreated leaves in the November and December plots. This suggests that if insects are involved, they may be much less mobile than anticipated and that populations were decimated during the weekly spraying in respective months. Despite the decreased YCS expression the November treatment was not significantly different to the UTC final yields. However, December had higher yield statistically than the UTC (Figure 225). This suggests timing of sprays may play a pivotal role in reducing yield penalties.

An interesting finding was the February treatment, in which prior to being sprayed had begun to express significant YCS symptoms, however after two spray treatments these symptoms subsided and by mid-February was expressing the same level of YCS as the sprayed treatments (November, December, January and Continuous). It seems the YCS was halted post-spray (Figure 212). The March treatment did not however follow this trend. After insecticide applications it showed a slight recovery but not to the degree seen in the February treatment. We speculate that as bifenthrin is a non-systemic insecticide it may have been less effective at this time due to the much higher rainfall experienced in March (Figure 213).

Bifenthrin residue analysis

A permit was required for use of bifenthrin in the research trials as this pyrethroid is not registered for above ground use in sugarcane. This residue work aimed to reveal how long bifenthrin remained on or in the plant after application, and to collect evidence to assist in a possible future APVMA application to register this product for industry use.

Sugarcane samples were collected from mature sugarcane in this trial. Bifenthrin residues were determined in sugarcane tops, billet and expressed juice by LC DAD MS. This work was performed at the SRA Indooroopilly chemistry laboratory (see section 5.7.9 of this report).

Reportable residue of bifenthrin were found in tops samples from the continuous treatment at 2.4 months after last application, and in billets samples from various treatments at 2.5 to 4.6 months

after last application. No other quantifiable residues of bifenthrin were found in the analysed expressed juice samples.

Sucrose and starch

(Note: in this section of the report all references to quantitative starch = total α-glucan (soluble α-glucan + starch)

Leaf punches (leaves +2, +3, +4) were taken on 22-Dec-18 when all plots were asymptomatic. The punches were assayed for starch (total α-glucan) and sucrose (Bergmeyer and Bernt, 1974). These represent a pre-YCS baseline. If sucrose levels rise above an approximate upper tolerable threshold of 200 µmol/g DM, leaf yellowing will occur (Scalia et al., 2020). Leaf +4 sucrose levels are well below this upper threshold in all treated and untreated plot except for December leaf +4 (Figure 215). Based on December leaf +2 and +3 sucrose and leaf +4 starch (total α-glucan) levels, leaf +4 sucrose is likely experimental error (Figure 215).

All plots were re-sampled on 6-Feb-18 when some plots were clearly asymptomatic while others were strongly YCS symptomatic. Leaf punches were taken from leaves +2, +3, and +4. Results show significantly higher concentrations of both starch (total α-glucan) and sucrose in all plots which had not received an insecticidal treatment at the time of sampling (February, March, Mag Sulphate, and Untreated Control) (**Error! Reference source not found.**). All of these plots were symptomatic at the t ime of sampling (Figure 212). Starch (total α-glucan) and sucrose levels for asymptomatic plots (November, December, January, and Continuous) were very similar to those of the baseline 22nd December cane. These findings suggest that the asymptomatic cane was in fact YCS free as opposed to YCS affected but not showing symptoms.

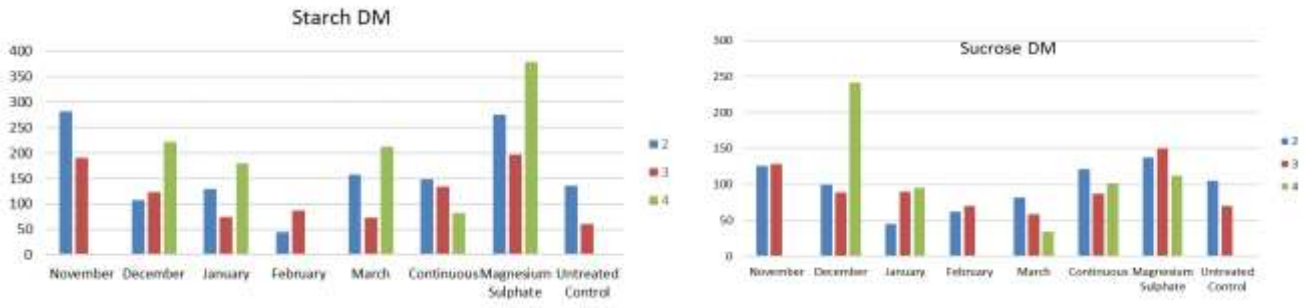


Figure 215 Baseline results for starch (total α-glucan) (left) and sucrose (right). Leaves +2, +3, and +4 are shown. Sampling was undertaken on 22-Dec-18 and all treatments were YCS asymptomatic at the time. Bars represent the mean of 12 leaves ± standard error.

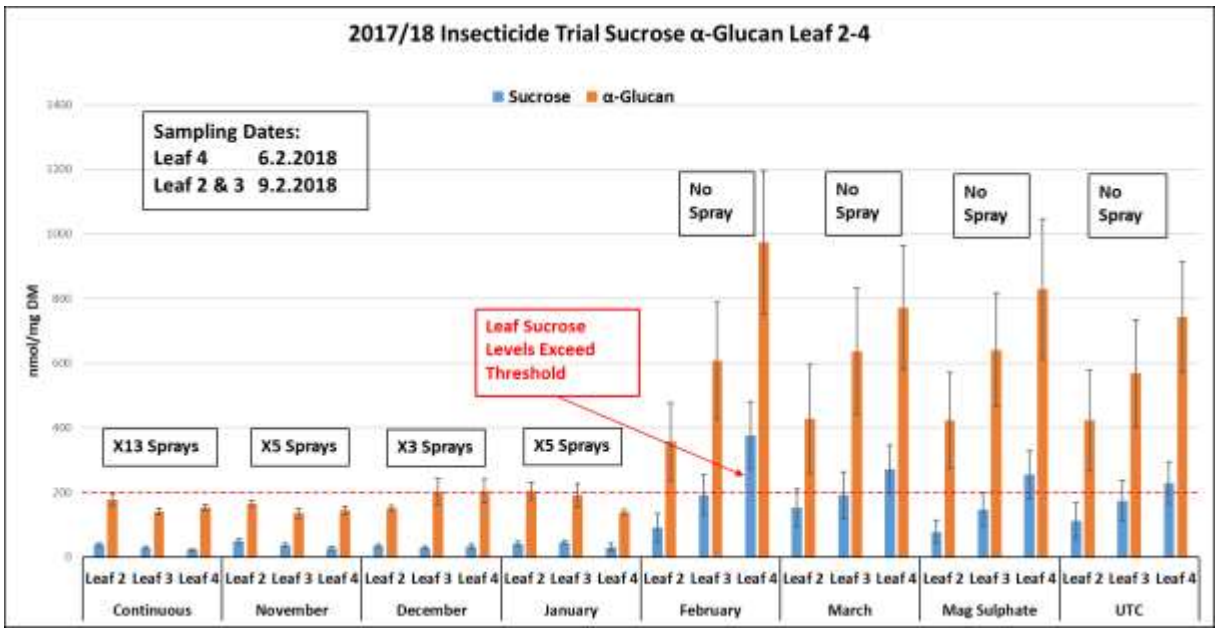


Figure 216 Source leaf sucrose and total α-glucan accumulation. Sucrose exceeds upper threshold in February (note: α-glucan units nmol glucose equivalent/mg DM) (Scalia et al., 2020)

Chlorophyll fluorescence

Leaf chlorophyll content was measured on 6-Feb-18 using a SPAD 502Plus Chlorophyll Meter (Spectrum Technologies INC, Aurora, Illinois). All 8 treatments and 4 reps were sampled. In each plot three stalks were randomly chosen, and measurements were taken on leaves +2, +3, and +4 from the midpoint of the leaf. The data fell into two distinct groups; cane which has been sprayed with bifenthrin and cane which was not. When the fluorescence values of the healthy green Continuous treatment are subtracted from the other treatments (to create delta curves), the magnitude of the difference can be easily seen together with the location of the disruption in the electron transport chain (Figure 217). It is clear that the electron transport chain is compromised around the J-step. This indicates a reduced capacity in the photochemical phase of photosystem II (PSII) together with a reduction in plastoquinone quenching post-PSII. This has arisen from the deactivation of light harvesting complexes and the resultant increase in photo-quenching stress on the remaining active antennae complexes (data not shown).

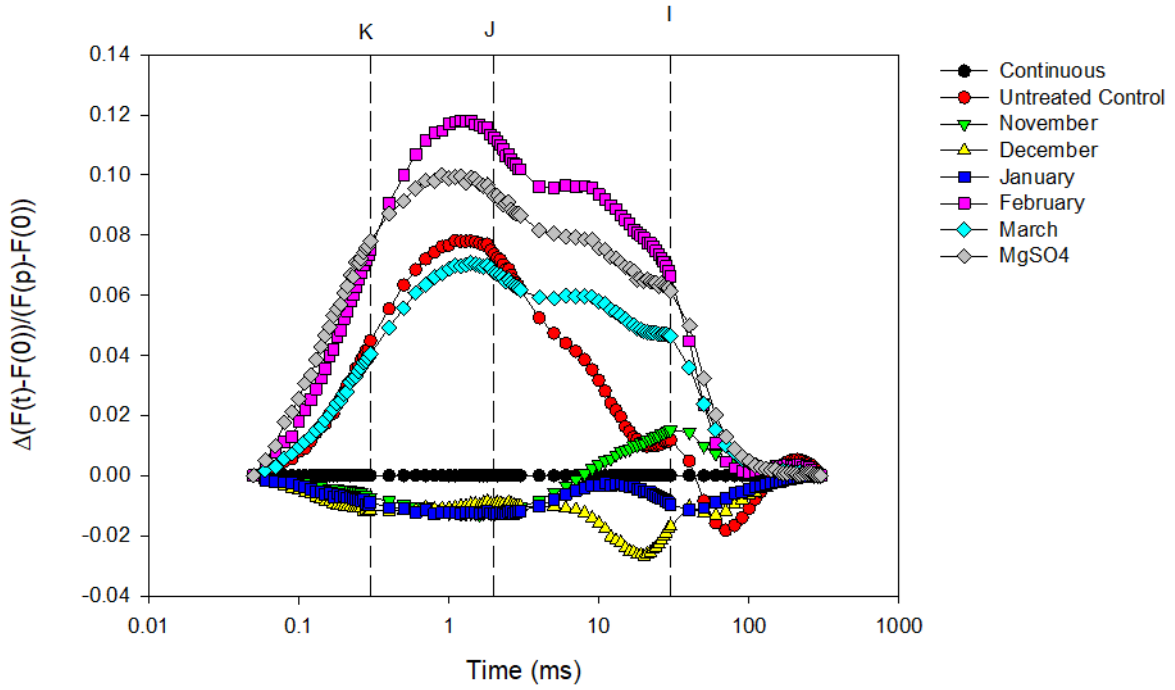


Figure 217 Leaf +4 delta curves of chlorophyll fluorescence. The Continuous treatment (black) has been used the healthy standard for which to compare all other treatments. Time points corresponding to OJIP steps are shown by vertical dashed lines

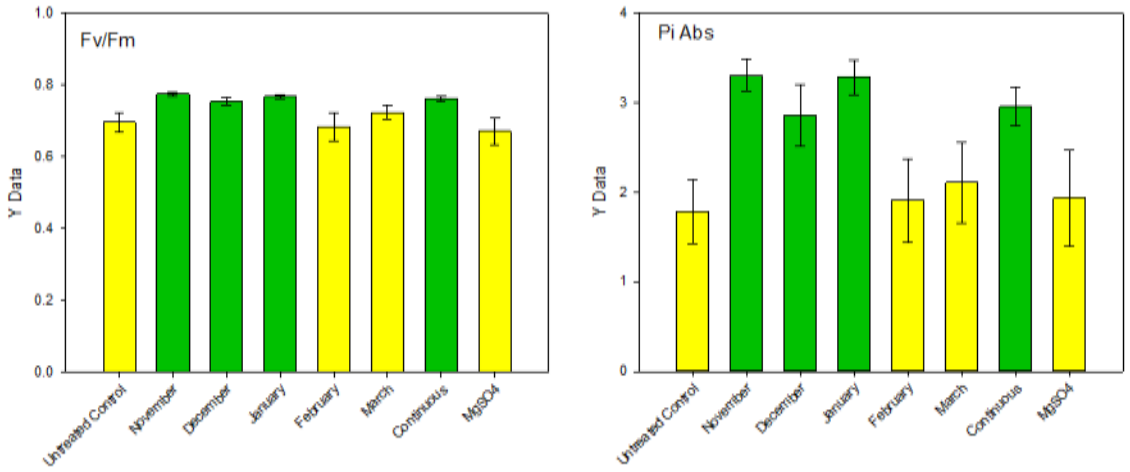


Figure 218 Fv/Fm (left) describing the maximum potential quantum efficiency of PSII, and PiAbs (right) a general performance index of the phyotosynthetic electron transport chain. Data for leaf +4. Green bars represent asymptomatic treatments at the time of measurement, while yellow bars are YCS symptomatic. Data are means of 12 leaves ± standard error.

Further indication that a stress-induced disruption of photosynthesis can be seen by comparing Fv/Fm values for the various treatments (Figure 218 left). Once again, the treatments separate according to whether they have received an insecticidal spray treatment. Overall fitness of the electron transport chain is reduced in YCS affected (non-sprayed) treatments as indicated by PiAbs (Figure 218 right).

Internode volume (sink size)

The internode volumes were measured in June just prior to harvest to determine if treatments had any effect on sink size and yield. A similar methodology to that used in the Burdekin physiological case study (see section 6.5.2 of this report) was adopted to investigate any correlation between treatments and culm growth. To approximately calculate when a new leaf and internode were formed a phyllochron of 150°Cd and leaf base temperature (Tbase) of 8°C were used (Inman-Bamber, 1994; Campbell et al., 1998; Sinclair, 2004; Bonnet, 2013). A Tbase of 18°C (below which internode growth stops) was applied for internodes. From this a timeline was aligned with the internode measurements to analyse when changes to culm growth occurred and when internode elongation stopped. This was then cross-referenced with treatment applications to determine if any correlation existed between timing of treatment and YCS symptom severity in comparison to the untreated control. This data is presented in Table 57.

Table 57 Treatments and time of application, Cumulative °Cd and internode volume (Leaf Tbase = 8°C). Internodes are in numeric order from top to bottom (Scalia et al., 2020)

Date	Internode Cummulative °Cd		Internode #	Internode Volume cm^3 Continuous	Continuous		Internode Volume cm^3 UTC	UTC		Internode Volume cm^3 November	Nov		Internode Volume cm^3 December	Dec		Internode Volume cm^3 Januarys	Jan		Internode Volume cm^3 February	Feb		Internode Volume cm^3 March	March		Internode Volume cm^3 $MgSO_4$
6/05/2018	20.99		1	23.48			13.09			14.70			13.18			22.78			19.46			13.88			13.82
28/04/2018	58.09		2	27.23			16.16			18.61			17.69			23.49			25.08			17.34			16.83
19/04/2018	108.19		3	31.96			12.80			24.43			21.60			26.52			22.82			20.00			14.88
10/04/2018	169.27		4	37.18			14.67			27.64			21.63			26.57			26.11			20.10			15.41
2/04/2018	232.58	Final Bifenthrin Spray	5	37.51	Spray		14.08			21.86			22.72			31.94			27.07			21.75			16.91
25/03/2018	297.01		6	37.82	Spray		16.20			26.07			25.05			34.70			27.82			25.28	No Spray 100mL		19.88
17/03/2018	366.76	Internode Elongation Stops	7	40.04	Spray		16.86			26.26			26.96			37.14			28.68			25.04	Spray		19.85
9/03/2018	428.89		8	40.45	Spray		18.54			29.40			29.22			39.24			29.46			20.27	Spray		20.10
2/03/2018	490.92		9	38.91	Spray		18.57			33.08			31.35			39.11			28.26			19.96	Spray		22.07
22/02/2018	549.72		10	36.30	Spray		19.50			34.45			34.77			39.29			30.69	Spray		20.72			25.59
15/02/2018	624.31		11	38.17	Spray		20.41			33.87			36.49			42.29			31.54	Spray		20.60			24.53
8/02/2018	683.80	Internode Expansion Stops	12	37.16	Spray		21.98			35.28			36.32			40.69			30.67	Spray		22.26			23.93
1/02/2018	750.71		13	37.40	Spray		22.37			35.55			34.85			39.32			29.01	Spray		21.73			28.25
25/01/2018	816.52	YCS Appears	14	38.61	Spray		22.33			36.89			33.80			37.09	Spray		28.68			21.66			28.83
17/01/2018	883.49		15	38.57	Spray		21.83			34.71			33.12			35.51	Spray		25.46			22.88			26.40
10/01/2018	953.51		16	36.27	Spray		21.81			31.67			29.67			33.36	Spray		23.49			22.85			25.05
3/01/2018	1025.81		17	35.49	Spray		23.30			33.09			28.58			32.82	Spray		22.40			23.33			25.06
27/12/2017	1095.98		18	34.28	No Spray		24.24			32.09			27.66	No Spray		31.08			21.91			23.57			25.16
20/12/2017	1158.96		19	33.91	Spray		23.91			30.13			26.45	Spray		31.44			20.74			22.43			26.19
13/12/2017	1220.08		20	31.71	Spray		24.10			28.14			24.43	Spray		29.66			19.30			21.20			23.00
6/12/2017	1286.47		21	28.82	Spray		22.52			27.62			25.39	Spray		28.67			19.32			22.58			19.81
29/11/2017	1349.29		22	27.77	Spray		26.30			25.20	Spray		25.59			25.01			18.73			22.03			20.70
21/11/2017	1412.36		23	28.41	Spray		31.24			23.29	Spray		24.52			31.91			12.62			16.67			24.81
13/11/2017	1468.28		24	27.67	Spray		29.07			21.93	Spray								11.50						
5/11/2017	1528.23		25	26.07	Spray					23.14	Spray														
30/10/2017	1593.18	Bifentrin Treatment Starts	26	27.01	Spray					23.05	Spray														
20/10/2017	1644.27		27	27.08						19.62															

YCS monitoring (Figure 212) and leaf metabolite data (Figure 215) show that the first signs of YCS symptoms occurred in late January 2018. This aligns with internode +14 (Table 15). At that point in time the two internodes beneath the symptomatic source leaf are internodes +15 and +16 which have a high demand for carbon from that leaf (Botha and McDonald, 2010). A quick comparison of those two internode volumes shows that untreated plants had a much smaller sink capacity than treated. Also of note is the increase in internode volume in each treatment after bifenthrin sprays

and the correlation with YCS severity. This data and metabolite results has been well analysed and documented in the 2015/016 Final Report (Scalia et al., 2020).

It was previously noted that the monthly only bifenthrin treatments offered protection well after new leaves had been formed despite no further insecticide applications. A possible explanation for this may well be associated with the long-term residual effect bifenthrin had in this trial. Interestingly, after the last spray application pyrethroid residue was found on the cane tops and culm at 2.4 and 4.6 months, respectively. Figure 219 shows three treatments from the 2017/18 field trial and the possible control of flying and crawling insects when considering time of Bifenthrin application and the residual effect. The continuous treatment is the only treatment to have both the culm and cabbage sprayed every week up to the April 10th, 2018 (a total of 21 sprays). All other treatments had either 3, 4 or 5 sprays on both the culm and cabbage (Table 57). There is also a substantial increase in the internode volume after the spray for all treatments other than Continuous treatment which implies that flying insects may play a lesser role in YCS development (Table 57).

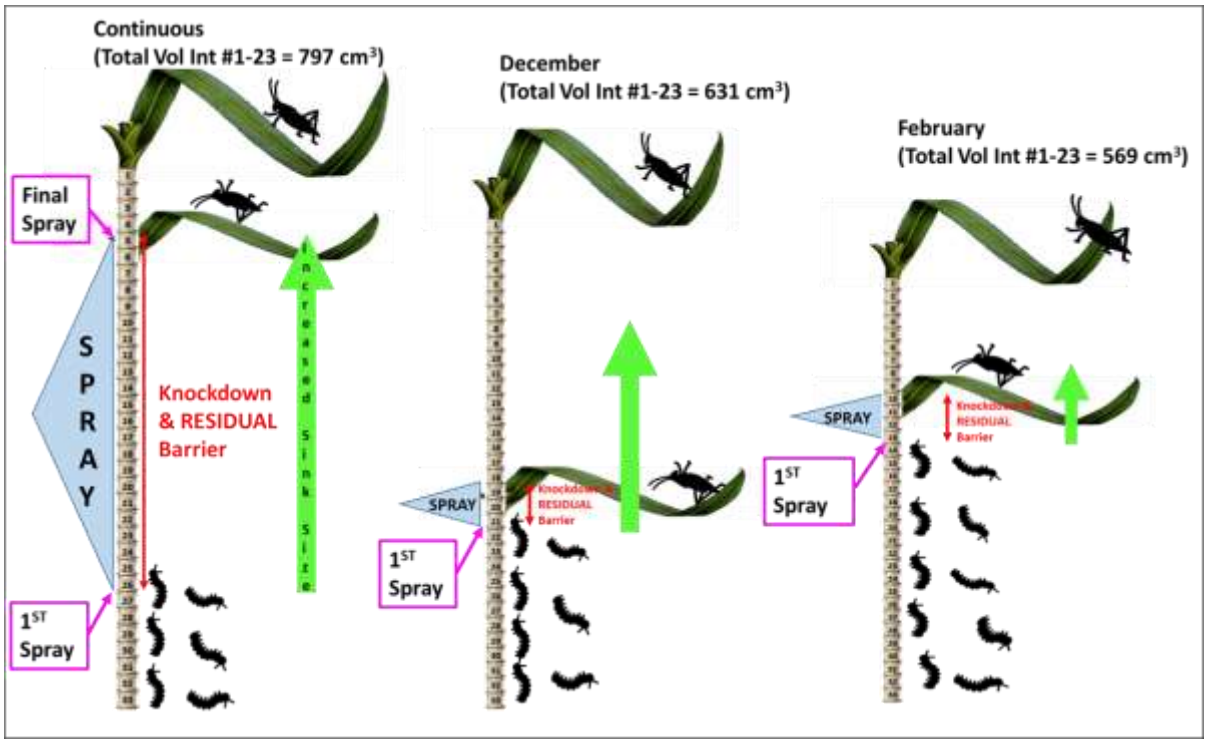


Figure 219 **Possible implications of Bifenthrin residual effect on crawling and flying insects and the impact on sink size and YCS [(Scalia et al., 2020) image modified]**

6.9.2.2 Entomology

A primary objective of this trial was to capture weekly data on the population dynamics of sugarcane planthopper species *Perkinsiella saccharicida* through various trapping methods. Traps were scored for *Perkinsiella* while the remaining captured insects were stored for future reference. Although we are only scoring for *Perkinsiella,* we have the ability to revisit this collection if other insects become of interest in the future.

In general, the sticky traps, pan traps, and knockdown canvas were largely ineffective at capturing planthoppers and gave us no concrete data on what bifenthrin was actually controlling. The light trap and tanglefoot sticky traps did yield some results and these are discussed below.

Light trap

A light trap was placed at a location >100m from the trial site and activated weekly for a 12-hour overnight period. This gave us a weekly indication of insects present in the vicinity of the trial (Figure 220). The traps were then scored for Perkinsiella while the remaining captured insects were stored for future reference. Main findings were:

- 4 major peaks in abundance – a similar trend as seen in other planthopper studies.
- Note that pathogen presence may not coincide with these peaks.
- Highest peak coincides with a significant rain event in late January and an incremental increase in average air temp.
- We observed the onset of significant YCS expression at this time also
- Temp increase could mean faster development, increase in population size and in turn higher feeding activity

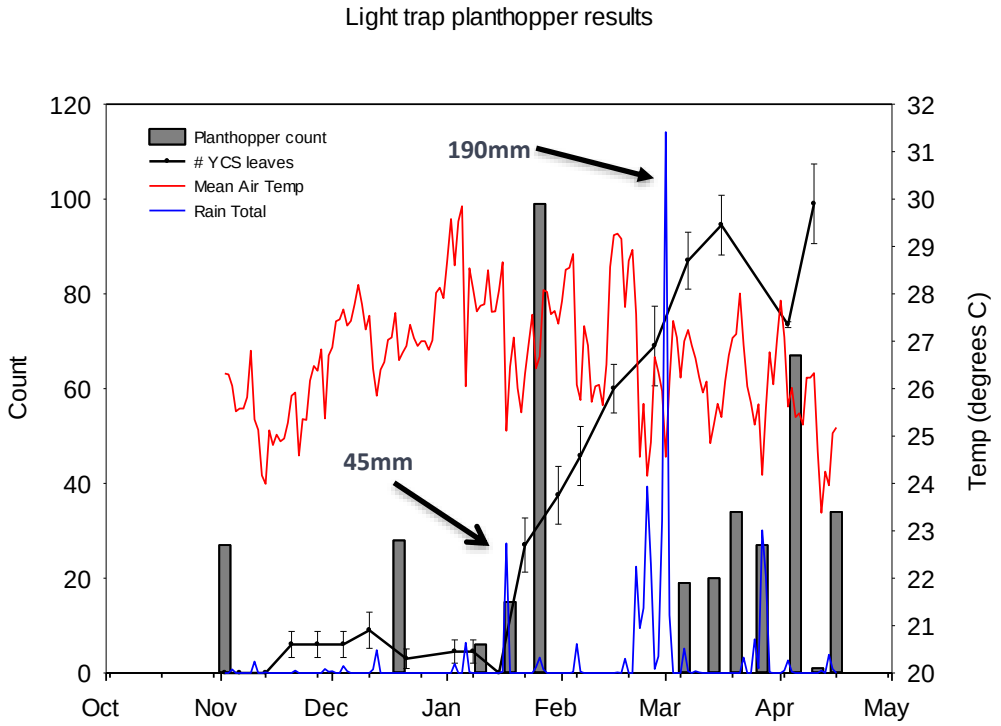


Figure 220 Population counts of Perkinsiella saccharicida collected weekly from a light trap placed >100m from the Insecticide trial site at the SRA Burdekin Station from October 2017 to May 2018. YCS monitoring data and local weather station data has been overlaid.

Tanglefoot stalk traps

Tanglefoot is a sticky, non-drying compound that is designed to trap insects. This substance was painted on electrical tape that was then wrapped around the cane stalk at fist visible dewlap level where the planthoppers have been observed to be most active. On 19-Apr-18 these traps were placed on 5 randomly selected stalks from each of the 4 reps for each treatment for a total of 20

stalks per treatment. This method aimed to capture planthoppers as they moved up and down the stalk and proved to be much more effective than trying to capture them on the wing using yellow sticky traps. The data is presented in Table 58. Although the Continuously sprayed treatment recorded the fewest planthoppers, there were no statistically significant treatment effects.

This data has shown that there are significant populations of *Perkinsiella* present in the field trial, even from the earliest point in the trial (Nov). Within the March treatment, an average of 6.75 (±1.5) planthoppers were trapped per 5 stalks which equals 1.4/stalk. Given there are approximately 500 stalks per treatment plot (4 rows by 10m), this would theoretically equate to 675 planthoppers per plot over the course of a week! This suggests that planthopper numbers were quite high in the plots. This trapping method proved far more successful when compared to the sticky traps. For example, 2 planthoppers were captured in 16 sticky traps in the March treatment compared with 7 from only 5 Tanglefoot traps over the same time period.

Table 58 Tanglefoot mean insect count for Perkinsiella placed in the field on 8 April 2018 for one week. A total planthopper count of the 5 stalks for each rep was scored. The mean and standard errors were then calculated for the 4 reps for each treatment.

Treatment	Mean total count (5 stalks/rep)	(± Std error)
March	6.75	1.5
Continuous	3	0.7
February	5	1.8
MgSO4	4.75	1.7
November	5.75	1.4
January	7.75	2.3
Control	5	2.1
December	7	1.1

6.9.2.3 Final Yield

A pre-harvest biomass was conducted in August in order to mitigate the margin of error associated with machine harvesting and to capture accurate yield data. Stalk biomass partitioning, stalk heights, stalk counts, and subplot biomass data was collected and the results are shown below.

The stalk biomass partitioning results as shown in Figure 221 was calculated from the average weights of 20 randomly selected stalks from each treatment. The millable stalk weights were measured from the ground to the top of the stalk between internode 5 and 6 with the remaining tops classified as leaf and cabbage. Despite significant differences between the treatments of whole stalk weights, it appears the overall proportion of biomass attributed to the leaf and cabbage is fairly consistent across all treatments. There appears to be quite variable results across individual millable stalk weights which significantly impacts final yield.

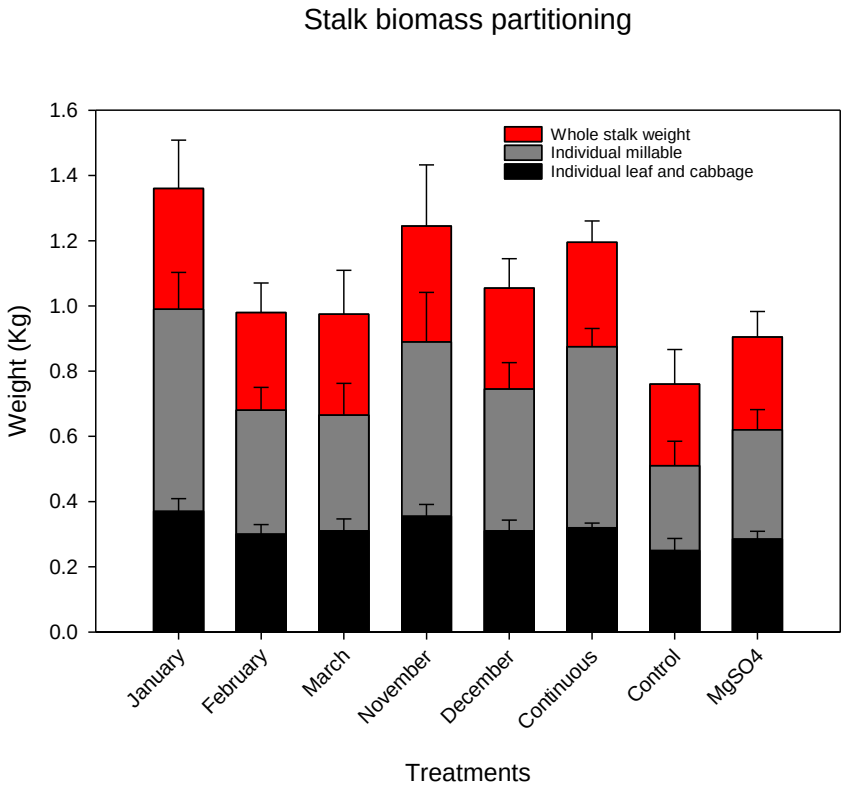


Figure 221 Mean weights (±SE) of individual canes harvested in the Burdekin, August 2018. Each treatment represents an average of 20 stalks. Whole stalk is the millable stalk plus the leaf and cabbage. Millable stalk is measured from the ground to the top between internode 5 and 6 with the remaining biomass classified as leaf and cabbage.

Stalk height measurements were made from the ground to the top visible dewlap on 20 randomly selected stalks from each treatment and the results are shown in Figure 222. The treatments that were statistically taller included Continuous, January and November treatments. The shortest cane was found in the UTC while the remaining treatments fell in the middle.

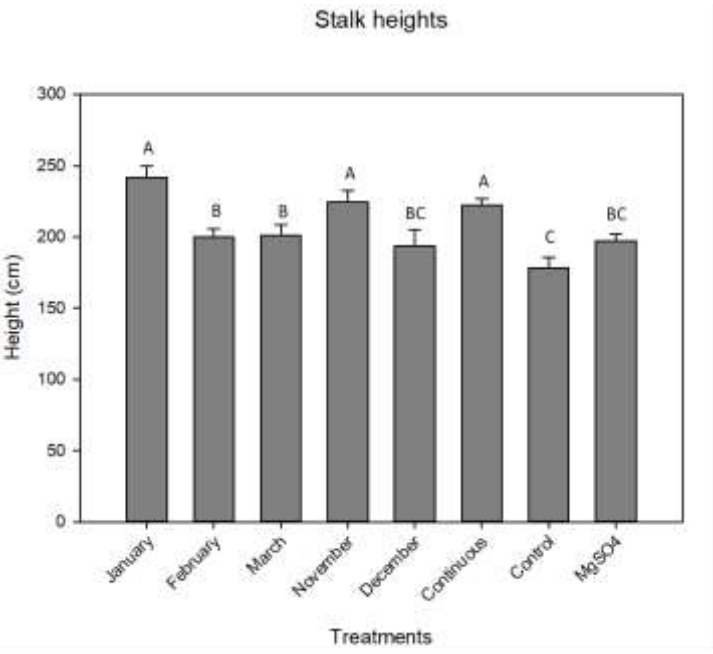


Figure 222 Mean stalk heights (±SE) of individual canes harvested in the Burdekin, August 2018. Each treatment represents an average of 20 stalks. The stalks were measured from the ground to the top visible dewlap. Significant treatment differences (p≤0.1) are separated by letters (LSD).

Two 5 x 1.52m sections were marked out in each rep and total stalk counts were made. The results are presented in Figure 223. A similar trend is observed in which the Continuous and January treatments yielded the highest stalk counts compared to the remaining treatments. The Continuous and January treatments were 23.7% and 23.8% higher than the UTC respectively.

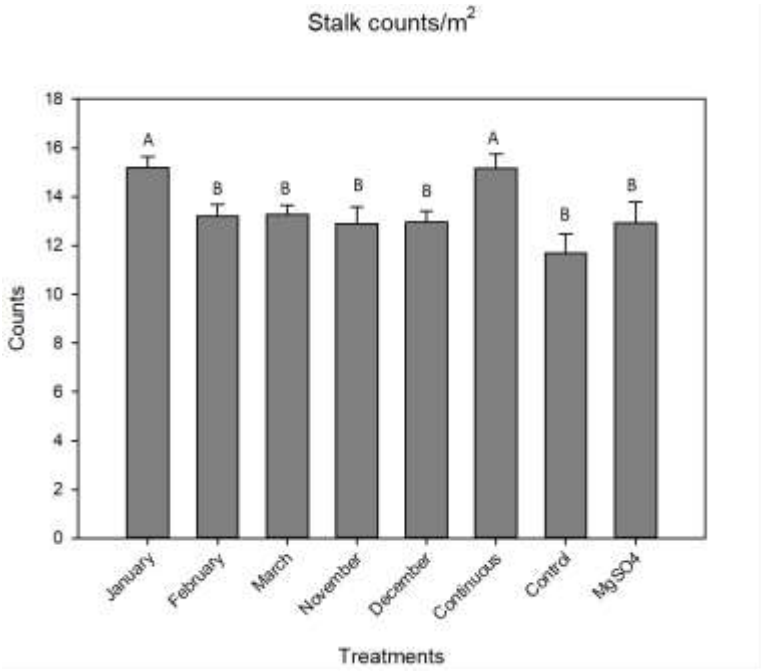


Figure 223 Mean stalk counts (±SE) of 1 m^2 of cane in the Burdekin, September 2018. Each treatment represents an average of 8 counts. Significant treatment differences (p≤0.1) are separated by letters (LSD).

Two 5 x 1.52m sections of cane were marked out in each replicate and harvested to calculate a subplot biomass as shown in Figure 224. These results were then converted to tonnes/ha as per industry standard (Figure 225). Both the Continuous and January treatments had the highest biomass which was statistically higher than the UTC, March and MgSO4 treatments. The Continuous and January treatments were 41.2% and 42.7% higher in biomass than the UTC respectively. This translates into a potential crop yield of >100t/ha for healthy cane versus just over 60t/ha for YCS affected cane.

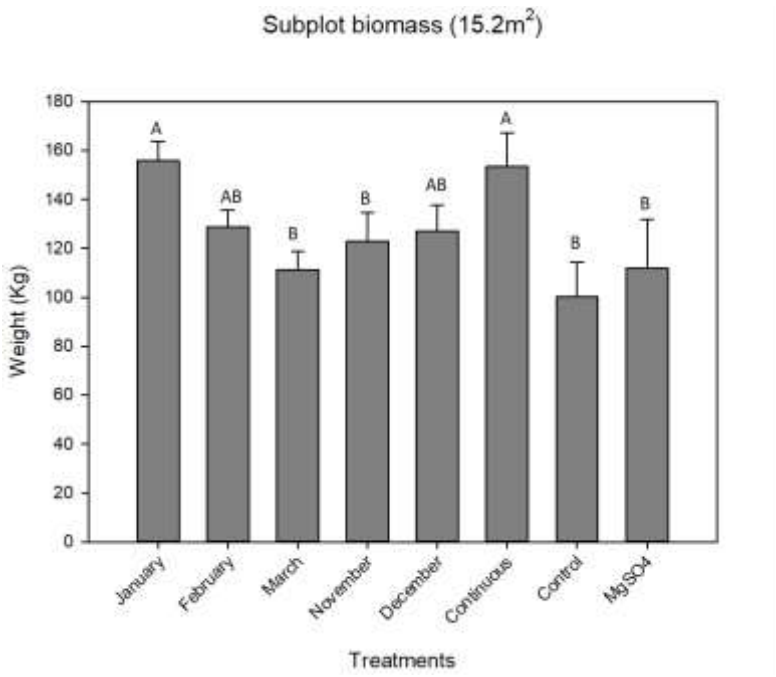


Figure 224 Mean stalk weights (±SE) of a harvested 15.2m^2 subplot of cane in the Burdekin in September, 2018. Each treatment represents an average of 4 biomass weights. The whole stalks were included in the weights. Significant treatment differences (p≤0.1) are separated by letters (LSD).

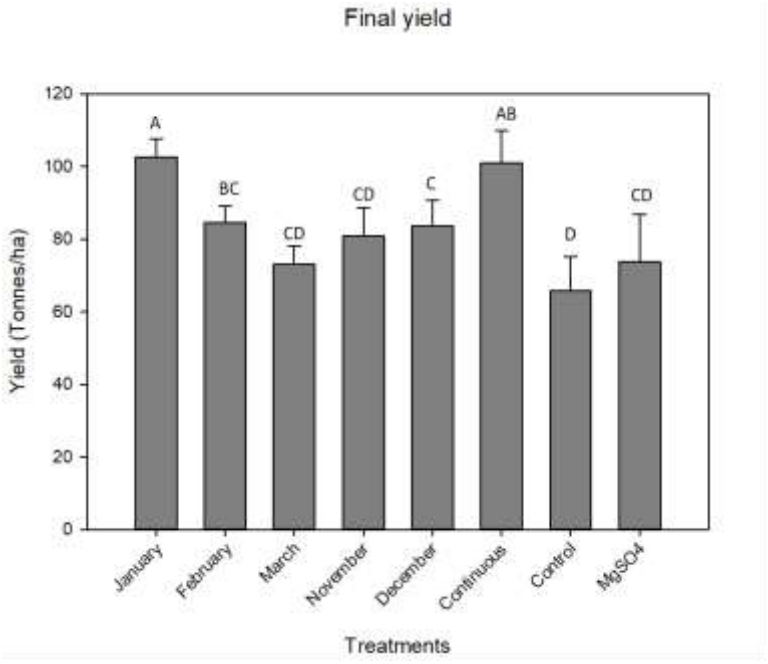


Figure 225 Mean yield (±SE) harvested in the Burdekin in September, 2018. Each treatment represents an average of 4 subplot weights. The whole stalks were included in the weights. Significant treatment differences (p≤0.1) are separated by letters (LSD).

The block was burnt and machine harvested on the 20th of September and the results are presented in Figure 226. The findings closely resemble that of the subplot biomass data with the January and Continuous treatments yielding the highest tonnes per hectare. Control and Magnesium sulphate treatments had the lowest yields.

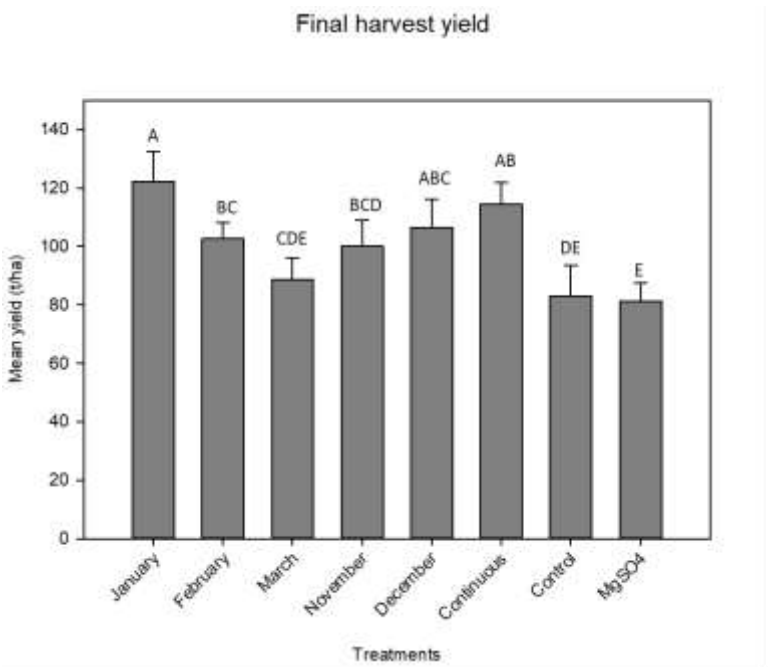


Figure 226 Mean yield (±SE) machine harvested in the Burdekin in September, 2018. Each treatment represents an average of 4 replicates. Significant treatment differences (p≤0.1) are separated by letters (LSD).

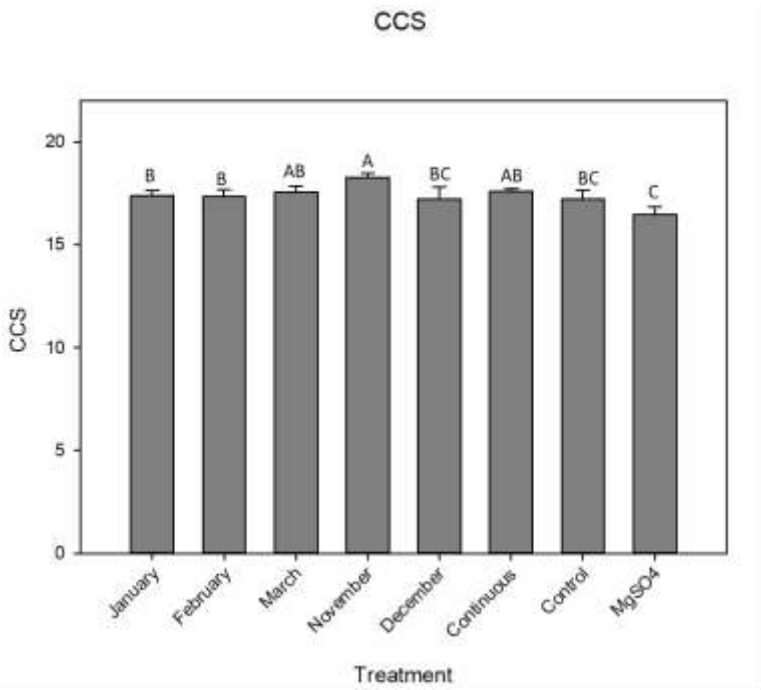


Figure 227 Mean CCS (±SE) harvested in September, 2018. Each treatment represents an average of 6 individual stalks. Significant treatment differences (p≤0.1) are separated by letters (LSD).

Commercial cane sugar (CCS) results shown in Figure 227 are quite consistent across treatments which suggests that the economic loss attributable to YCS is associated with compromised yield and not a decline in CCS.

The trial results have provided answers to many of the research questions that were posed early on. It was found that bifenthrin does reduce the incidence and severity of YCS which supports earlier trial findings. This is reflected by the Continuous treatment in which the cane stayed green throughout the duration of the trial despite other treatments presenting with severe YCS within very close proximity. This enabled for the first time relatively accurate reporting regarding the yield penalty associated with YCS within the parameters of this trial. The yield loss was above 40% which is a very significant economic penalty. However, it should be pointed out that this yield variation is largely due to stalk numbers (Figure 223). The bifenthrin weekly treatment had approximately 35% more stalks/m^2 than the untreated control. The reason for the stalk death in the UTC is unexplained and no details about when these stalks died is available. As no previous trial has shown bifenthrin to prevent stalk death it is difficult to draw firm conclusions about cane yield from this result. Stalk death occurs after canopy closure (Inman-Bamber, 1994) or due to above and below ground pests and disease (Allsopp, 2010; Vargas et al., 2015). As control plants were the slowest growing it is unlikely that rapid canopy closure could account for this level of loss. As bifenthrin is a non-systemic and was foliar applied it would also be unlikely to be controlling root feeding pests and there was also no evidence of any significant stem pest or disease that could account for this level of stalk death. Therefore, it is difficult to draw firm conclusions about the yield benefit attributed to the bifenthrin weekly treatment. (*Note: monthly stalk counts were conducted in the 2018/19 insecticide trial and results are presented in section 6.9.3.3 of this report.)*

It was found that planthoppers are effectively controlled by bifenthrin and the timing of sprays is crucial. The January treatment performed significantly and consistently better in terms, YCS incidence and severity when compared to the UTC. The yield data results were statistically comparable to the Continuous treatment, which suggests this may be an appropriate time to apply sprays to mitigate the impacts of YCS. As insect populations are primarily driven by weather and food availability (Johnson et al., 2016; Li et al., 2017), life cycle and population studies of sugarcane insects would be required to determine the appropriate time to apply treatments. This could be very difficult to determine given the weather extremes during the sugarcane peak growing period in Queensland.

Summary

The onset of leaf yellowing due to YCS occurred in late January to early February after the first significant rainfall event. Bifenthrin weekly was the most effective treatment to prevent YCS expression under experimental conditions. Bifenthrin treatments of one-month duration are effective in mitigating YCS development. This may be explained by reportable levels of the insecticide active on sugarcane tops and culm up to 2.4 and 4.6 months respectively, after the last application; bifenthrin was not detectable in juice. If insects are involved in causing YCS then this result suggests they are not very mobile. Monthly treatments remained YCS free up to 3-months after their last spray, after which 12 new leaves would have formed. No YCS development in these leaves suggests flying insects are not involved. After bifenthrin treatment internode size increases and YCS severity decreases. Leaf sucrose above the upper tolerable threshold coincides with YCS symptom expression and is well correlated to internode growth and YCS severity. Chlorophyll fluorescence measurements showed a clear disruption in the electron transport chain function in plants which were unsprayed (symptomatic) compared to bifenthrin sprayed (asymptomatic) plants.

Further evidence that asymptomatic plants were actually YCS-free. Insect light traps show four major peaks in planthopper populations which are affected by climatic conditions. Bifenthrin treated cane had a higher biomass than untreated controls but no CCS penalty was recorded in YCS symptomatic cane.

6.9.3 Insecticide trial 2 – Burdekin (2018-2019)

The following insecticide trial of KQ228[A] 2R was established on station at SRA Burdekin (Farm #6007 Block #3-1) in September 2018 (see Appendix 1: 1.2.23). This trial investigated the efficacy of pyrethroid insecticides bifenthrin and permethrin in preventing YCS development/expression and the effect on yield and CCS.

The following treatments were applied by knapsack during the months of November to April. Weekly application of two different pyrethroid treatments (bifenthrin and permethrin) were conducted, as well as sprays at 1500- and 2000-degree days on 18 December 2018 and 15 January 2019. In addition, permethrin which is currently registered for sugarcane use, was evaluated to assess its efficacy in decreasing the incidence and severity of YCS as a potential management option. Streptomycin was an addition to this trial as part of the pathology research (see section 6.8 of this report).

Results

6.9.3.1 YCS monitoring and physiology

Monitoring of the treatments and YCS severity scoring took place on a weekly basis from early November (Figure 228). Low incidence of YCS was observed all throughout January across all treatments. Symptoms then began to rapidly increase in mid-February in all treatments except the bifenthrin weekly and the bifenthrin 1500- and 2000-degree days treatments. The bifenthrin weekly observations builds upon previous trial results (see section 6.9.2 in this report) in which the cane remained green throughout the season. The bifenthrin 1500-2000 treatment also consolidates previous findings in that the January and February treatments, in which bifenthrin was sprayed throughout the month, was effective in reducing the incidence and severity of YCS for approximately 8 weeks. Conversely, we observed the permethrin weekly and permethrin 1500 & 2000 treatments demonstrate high levels of YCS expression similar to that of the UTC. Streptomycin 3 weekly treatment showed intermediate levels of YCS. The streptomycin at peak treatment had high levels of YCS expression and the peak spray application elicited little to no response. The bifenthrin at peak had high levels of YCS expression prior to the spray and there was a slight response in which symptoms decreased initially but returned to previously similar levels.

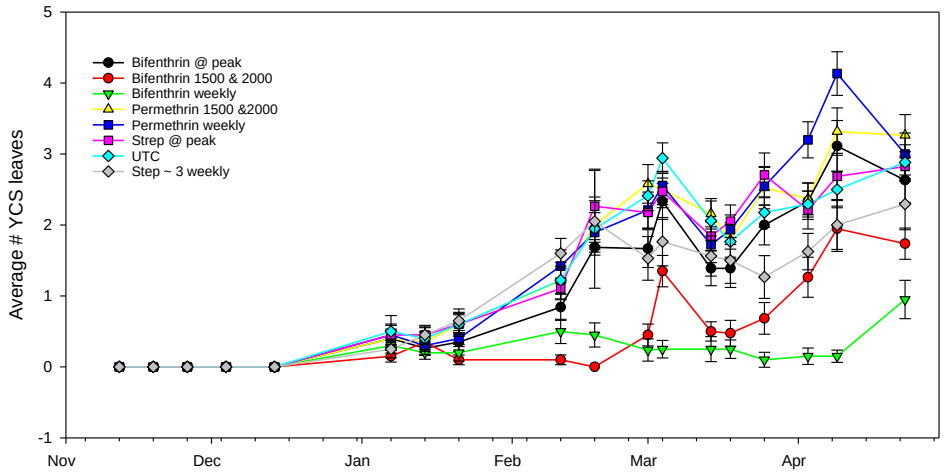


Figure 228 Average number of YCS leaves per stalk of the top 7 leaves of the canopy monitored weekly across treatments. Means of 20 stalks ±SE 2018-2019.

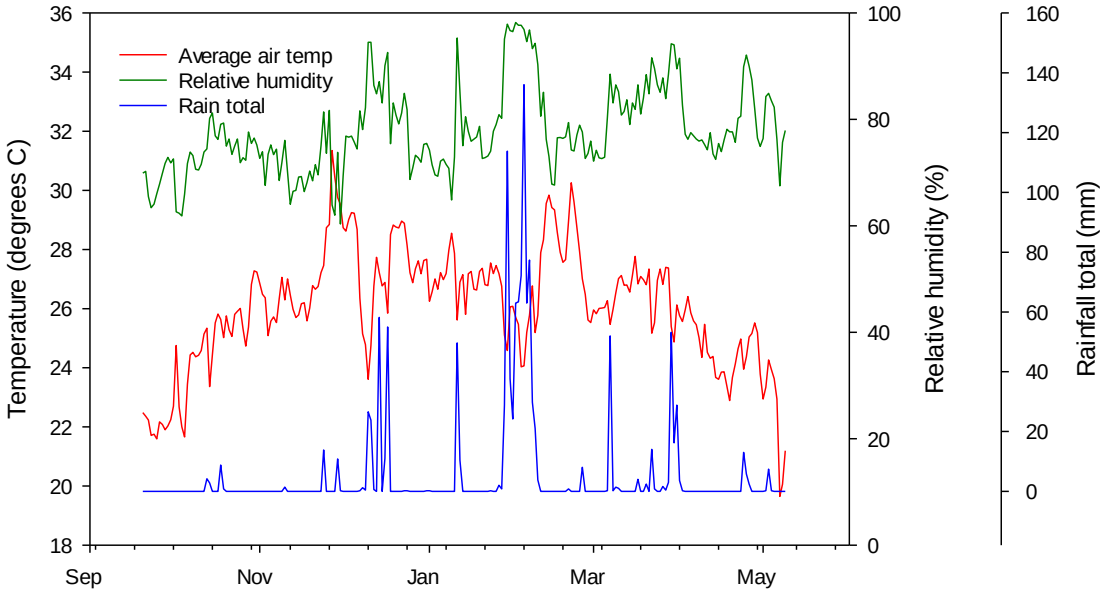


Figure 229 Climatic data from the 2019 Ratooned Insecticide Trial growing season.

The 2019 season was climatically challenging (Figure 229) with early December heatwaves followed by storms and flooding events in late January and early February. The weather data shows that 806 mm fell at the Brandon station in January and February alone and a total of 1238 mm from September 20, 2018 to the end of April. The trial was waterlogged for a minimum period of between 4 – 6 days which caused the cane stress and was expressed through the senescence of the mature leaves. The unprecedented rainfall not only impacted the spray schedule but could have diminished the residual effect of the insecticide sprays. These factors coupled with the flooding stress event may have played a role in the reduced effectiveness of certain treatments.

The rainfall events may go some way in explaining why the bifenthrin weekly treatment did exhibit yellowing in the lower to mid canopy. Having said this, the findings are still compelling in that continuously applied bifenthrin was the most effective treatment for two consecutive years. There

was minimal YCS expression throughout the season and arguably some yellowing events may be attributable to water stress.

Interestingly, the bifenthrin 1500-2000 treatment also consolidated the findings of the 2018/19 trial in which targeted sprays were shown to have a lasting effect of up to 8 weeks after the last spray was applied. The two sprays were applied on December 18 and January 15 prior to the flooding event and remained effective until early March. This supports conducting further research into timing of targeted spray applications for optimizing potential YCS management strategies.

The bifenthrin at peak treatment elicited a minimal response however just after the spray was applied there was another significant rainfall event which is likely to have impacted the result.

Permethrin appeared to be an ineffective treatment at both preventing onset and reducing incidence and severity of YCS. Permethrin weekly treatment exhibited higher YCS expression than the UTC and the permethrin 1500-2000 treatment had similar YCS symptoms to the UTC.

Streptomycin 3 weekly treatment had an intermediate level of YCS expression which was less than that of the UTC but not as effective as bifenthrin weekly. Streptomycin at peak showed minimal response post-spray application which may have been impacted by the rainfall event that followed. YCS expression in mid-March went down across all treatments which we suggest may be due to monitoring error.

Leaf metabolite, sink size (internode) and YCS expression analysis 2019

Leaf +4 punches were collected weekly and assayed for sucrose and total α-glucan. At the start of the trial, sucrose levels remain below the upper tolerable threshold of 200 µmol/g DM in all treatments (Figure 230). However, levels steadily begin to rise in January and eventually exceed healthy levels in February in all treatments except the bifenthrin treated plants (Figure 231). This concurs with results from the 2017-2018 insecticide trial (see section 6.9.2.1) where the onset of YCS (Figure 228) coincides with significant rainfall events (Figure 229), and correlates with leaf accumulation of sucrose and α-glucan exceeding upper tolerance levels. It is noteworthy that bifenthrin weekly and 1500- and 2000 °Cd treatments maintain very low levels of leaf sucrose and minimal YCS expression throughout the trial period (Figure 230, Figure 231).

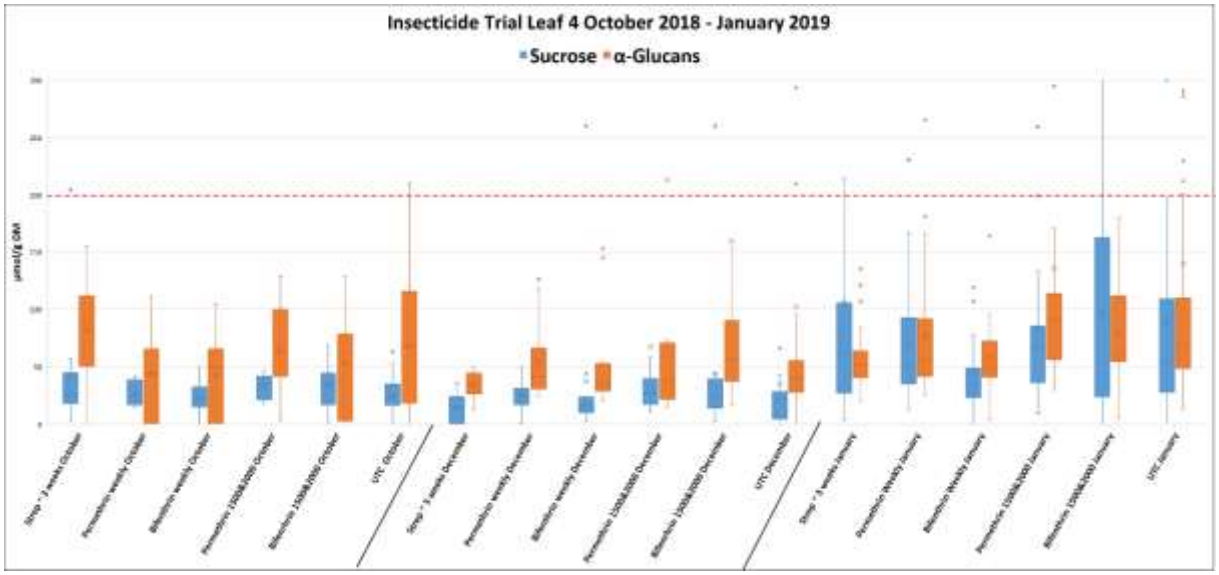


Figure 230 Insecticide trial leaf +4 sucrose and α-glucan content below 200µmol/g DM upper threshold October 2018-January 2019

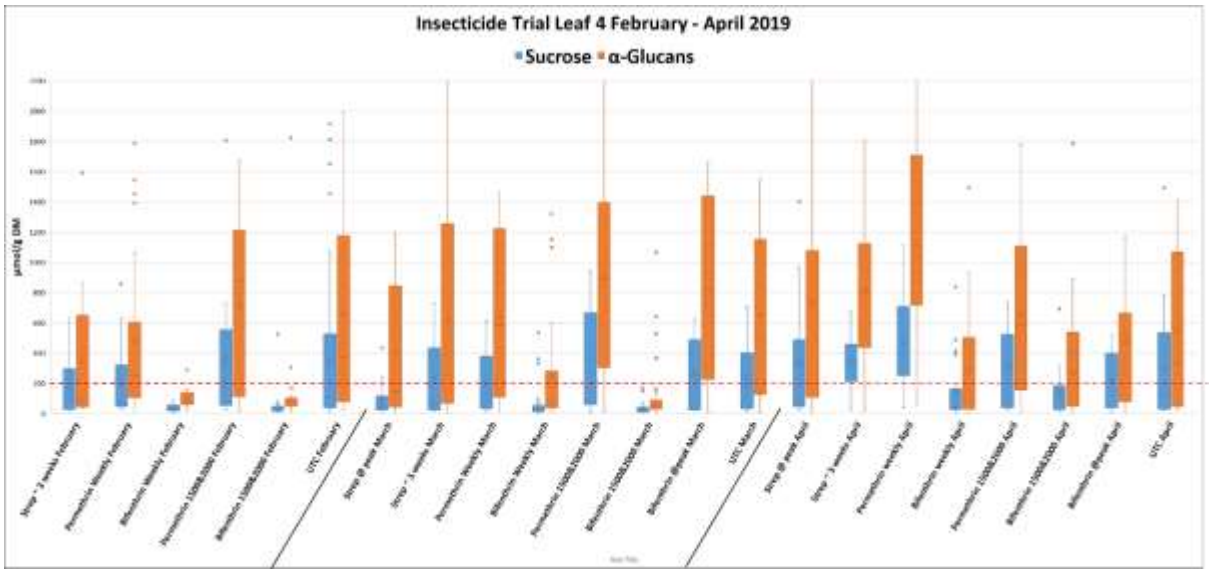


Figure 231 Insecticide trial leaf +4 sucrose and α-glucan content. Upper tolerance threshold 200µmol/g DM (Feb-April 2019)

An evaluation of treatment effect on sink size was conducted through measurements of internodes in August, prior to the September machine harvest. A phyllochron of 150°Cd and leaf base temperature (Tbase) of 10°C and internode (Tbase) of 18°C (Inman-Bamber, 1994; Campbell et al., 1998; Sinclair, 2004; Bonnet, 2013) were used to construct a timeline of treatment application and associated internode growth (Table 59). An event of note is the extreme rainfall and flooding event during late January to February, that impacted the trial for the second consecutive year

Table 59 Insecticide trial (2018-2019) treatments and time of application, cumulative °Cd and internode volume (leaf Tbase = 10°C)

						Bifenthrin @ Peak		Bifenthrin 1500 & 2000		Bifenthrin Weekly		Permethrin 1500 & 2000		Permethrin Weekly		Strep @ Peak		Strep ~ 3 weeks		UTC		
Date	Leaf Cumulative °Cd	Internode Cumulative °Cd	Activity/Event	Rainfall (mm)	Internode # From Top Down	Av Vol cm^3		Av Vol cm^3		Av Vol cm^3		Av Vol cm^3		Av Vol cm^3		Av Vol cm^3		Av Vol cm3		Av Vol cm^3		Date
18/08/2019	39.1	7.3			1	2.313134486		2.18		2.40		2.39		2.89		2.50		2.09		2.78		18/08/2019
5/08/2019	162.2	32.2			2	7.540745871		8.10		7.84		6.70		6.72		7.58		6.75		7.70		5/08/2019
21/07/2019	304.5	54.8			3	12.06980971		13.99		14.40		10.43		9.51		11.23		12.12		12.30		21/07/2019
4/07/2019	454.0	84.2			4	13.8236775		16.96		19.48		12.23		10.88		13.42		14.41		15.27		4/07/2019
17/06/2019	602.1	103.6			5	15.23174702		18.63		21.67		13.49		11.96		14.92		15.60		16.90		17/06/2019
3/06/2019	743.8	139.3			6	16.15072849		20.06		23.54		15.79		13.97		16.89		16.90		17.39		3/06/2019
21/05/2019	901.5	193.0		0	7	17.3608845		21.77		27.46		17.99		15.96		17.69		19.10		18.95		21/05/2019
10/05/2019	1044.8	248.3		0.8	8	19.10487487		22.73		24.00		19.81		18.28		20.62		20.20		20.72		10/05/2019
29/04/2019	1190.5	306.0		2.2	9	22.02919763		25.59		26.19		23.49		22.18		24.97		23.31		24.17		29/04/2019
20/04/2019	1326.6	370.1	Internode Elongation Stops	15.2	10	25.56057175		31.06		33.05		27.65		26.02		28.65		27.88		29.70		20/04/2019
10/04/2019	1467.5	431.0	Final Spray	0	11	41.05369489		37.21		38.92	Spray	31.90		31.25	Spray	31.41		32.00		32.66		10/04/2019
1/04/2019	1608.6	500.1		3	12	33.4331182		40.43		43.76	Spray	35.56		32.65	Spray	32.98		34.45		36.67		1/04/2019
23/03/2019	1760.9	580.4		58	13	35.69546134		40.19		45.80	Spray	37.00		32.71	Spray	33.73		35.07		37.48		23/03/2019
15/03/2019	1900.0	655.5		18.4	14	37.607081		40.11		45.97	No Spray	37.68		33.87	No Spray	35.03		35.12		37.65		15/03/2019
7/03/2019	2036.4	727.9		88	15	38.7214081	Spray	40.27		45.33	Spray	38.87		35.82	Spray	36.80	Spray	35.75		38.14		7/03/2019
26/02/2019	2182.2	801.7	YCS Appears max. dry mass)	4.4	16	40.43533092		42.20		57.73	Spray	42.46		38.13	Spray	39.44		35.23		41.30		26/02/2019
18/02/2019	2330.9	886.4		24.8	17	45.04399588		43.49		50.78	Spray	42.11		42.25	Spray	44.26		38.52		43.38		18/02/2019
11/02/2019	2467.5	967.0		0	18	46.49976843		46.00		55.60	Spray	44.81		47.29	Spray	44.58		42.10		48.71		11/02/2019
3/02/2019	2600.0	1035.5	FLOODS	241.4	19	45.6221969		48.54		57.02	No Spray	48.55		45.56	No Spray	43.65		44.44		50.33		3/02/2019
25/01/2019	2742.6	1106.1	FLOODS	305.4	20	49.89109637		45.66		54.26	No Spray	48.07		45.29	No Spray	44.75		44.24		48.04		25/01/2019
17/01/2019	2877.0	1176.5		0.8	21	49.99866116		42.97		56.51	Spray	49.85		43.43	Spray	44.36		45.12		46.00		17/01/2019
9/01/2019	3013.4	1248.9	2000°Cd Spray	74.6	22	48.50361909		43.43	Spray	51.52	Spray	48.98	Spray	40.14	Spray	47.65		43.21		52.53		9/01/2019
1/01/2019	3149.6	1321.1		0	23	46.79020082		44.20		47.27	Spray	49.56		41.19	Spray	50.96		44.29		50.16		1/01/2019
24/12/2018	3287.4	1394.9		0	24	51.15822612		46.53		53.28	No Spray	49.41		48.28	No Spray	52.62		43.89		49.66		24/12/2018
16/12/2018	3435.6	1479.1	1500°Cd Spray	63	25	56.8064861		50.92	Spray	59.87	Spray	51.63	Spray	44.95	Spray	56.13		51.99		51.68		16/12/2018
8/12/2018	3566.2	1545.7		77.4	26	65.89546008		59.38		59.04	Spray	48.04		48.23	Spray	55.64		57.33		57.88		8/12/2018
1/12/2018	3714.5	1618.0		18.2	27	56.97238475		60.70		53.22	No Spray	48.84		48.34	No Spray	54.30		54.28		69.92		1/12/2018
23/11/2018	3859.9	1719.4		50	28	60.02049887		67.66		64.04	Spray	39.10		36.40	Spray	55.66		51.16		46.75		23/11/2018
15/11/2018	3992.2	1787.7		0.6	29	70.26836848		45.79		60.13	Spray	46.16				44.18		56.92		31.64		15/11/2018
6/11/2018	4137.5	1861.0	First Spray	3.2	30					96.65	Spray	62.13						59.73				6/11/2018
28/10/2018	4287.0	1938.5		0.2	31							60.32										28/10/2018

Using the time of YCS appearance in late February as a reference to investigate any visible impact to internode growth prior to the event, casts focus on internode +17 and +18 (Table 59). Figure 235 shows that bifenthrin weekly has a significantly larger sink volume in this zone of the culm than any other treatment. Sink size in this region correlates with both leaf metabolite levels and YCS severity scores for all treatments except bifenthrin 1500-2000 treatment which has a smaller internode +17-18 sink size than the bifenthrin weekly. However, this appears to be a slightly delayed response as it aligns with monitoring which shows an increase in YCS severity for bifenthrin 1500-2000 just after this timepoint and a drop back down again 3 weeks later. Furthermore, sink size above the YCS zone to the point where internode elongation stops (internode +16 to +10) shows that bifenthrin weekly and 1500-2000 treatments have the largest sink and lowest YCS severity rating of all treatments (Figure 233, Figure 228). As there is no literature in support of bifenthrin acting as a plant growth stimulant, results suggest bifenthrin is controlling insects that have a significant effect on culm growth at specific times. Pathology data does not support a phloem blocking pathogen vectored by insects which implies impeded growth may be a direct response to insects themselves. This could be due to reallocation of plant resources from growth to defence when there is either 1) high insect attack after population increases following favourable weather conditions or 2) high populations of opportunistic phloem dwelling endogenous microbes during periods of high insect stress (for review Huot et al., 2014). One such microorganism that consistently amplified in PCR and confirmed through sequencing is *Curtobacterium* spp. (see section 6.8 of this report) (Geering et al., 2020). This group of bacteria are usually non-pathogenic endophytic organisms, but are noted to occasionally cause leaf yellowing in other crops; albeit with different symptoms to YCS (Silva Jr et al., 2012; Osdaghi et al., 2020). Evidently, sink limitation at specific points of ontogeny is visible. However, when the entire culm is considered the variation between treatments is minimal (Figure 234).

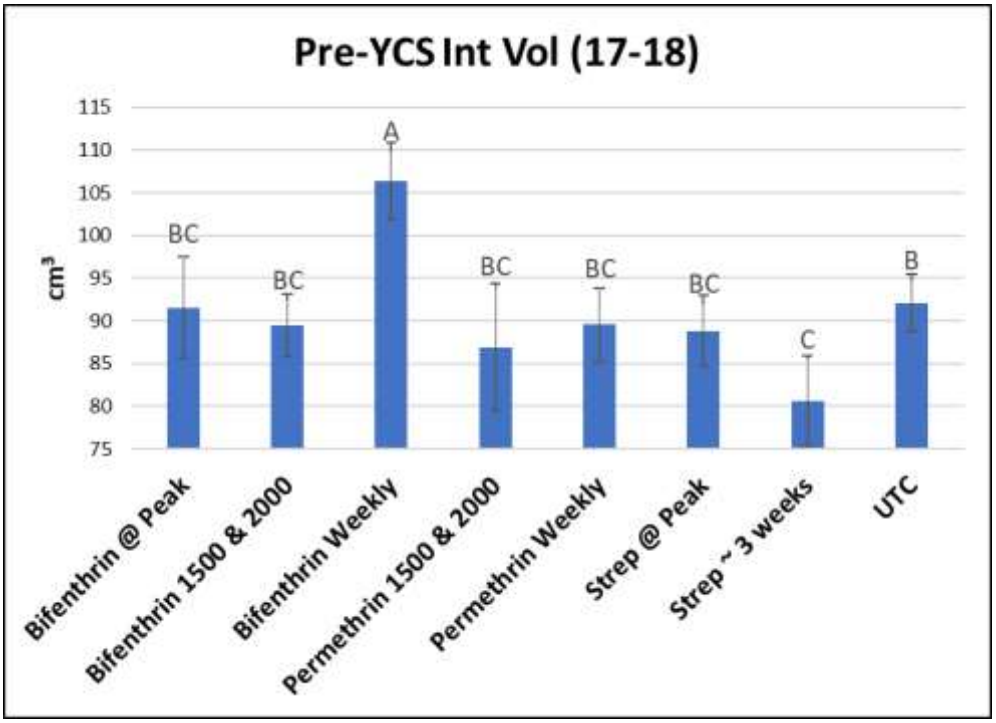


Figure 232 Insecticide trial pre-YCS expression, total internode volume (+17 to +18). LSD all-pairwise comparisons (p<0.05).

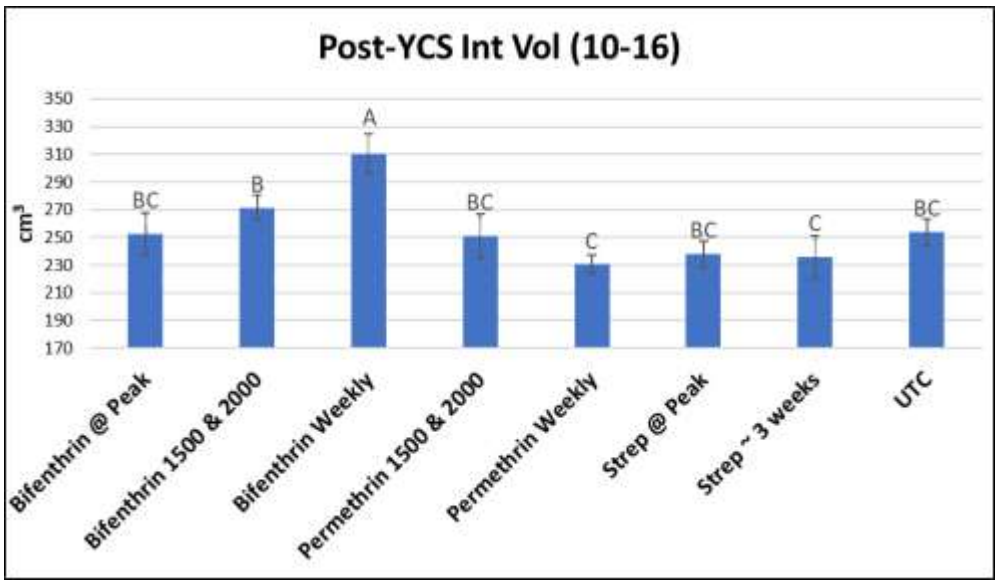


Figure 233 Insecticide trial post-YCS expression, total internode volume (+10 to +16). all-pairwise comparisons (p<0.05).

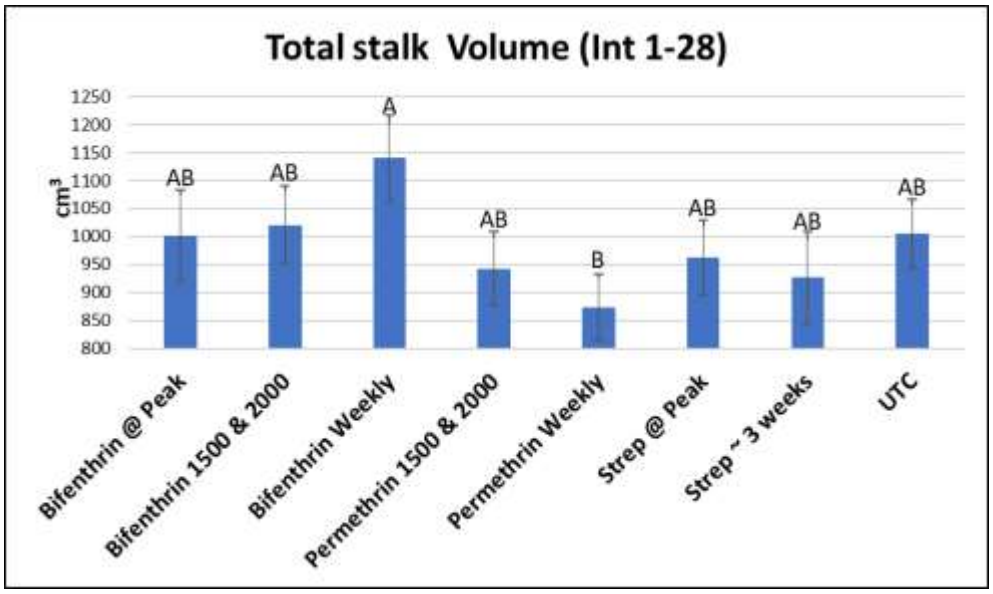


Figure 234 Insecticide trial total culm internode volume (+1 to +28). Tukey HSD all-pairwise comparisons (p<0.05).

6.9.3.2 Entomology

To determine if there was a link between YCS expression and insect abundance insects were monitored during the ratooned insecticide field trial using a variety of trapping techniques (see Appendix 1: 1.2.2.4). All Insect samples collected during the insecticide trial were dispatched to SRA Meringa and examined microscopically for the presence of insects.

Results and Discussion

Bifenthrin and Permethrin Treatments

As the bifenthrin weekly and bifenthrin 1500-2000 treatments had the most effect on suppression of insect populations and YCS expression, whereas in contrast the weekly permethrin was shown to be one of the least effective treatments, a summary of provisional results for these selected groups, based on analysis of yellow sticky trap counts only, are reported here.

Thrips

At least four species of thrips were detected in cane and pooled data of all species is presented (Figure 235, Figure 236, Figure 237). In the untreated control there is a bimodal peak in thrip population abundance. The largest peak in abundance occurs in mid-November followed by a smaller peak in early January around the period of early YCS expression. Thrip populations naturally decline from mid-Feb to mid-April and are at their lowest when peak YCS expression occurs.

In terms of insecticide control (as assessed using yellow sticky traps to monitor abundance) the weekly treatments of both permethrin and bifenthrin reduced thrip populations up to mid-February (Figure 235, Figure 236). In contrast the bifenthrin 1500-2000 treatment had no apparent impact on thrip populations (Figure 237). Thrips were clearly the most abundant pest (as assessed by sticky trap numbers). However, the suppression of thrips by both weekly treatments, combined with the fact that thrip populations peak in mid-November (with a smaller secondary peak in early January) and YCS expression is evident in permethrin treated cane from January-April, make it unlikely that thrips are involved in YCS symptom expression. In addition, in the field trials carried out in FNQ thrip populations (data not shown) far exceed those in this Burdekin insecticide trial, yet no clear YCS expression was observed at all four field sites.

Mites

At least four species of mites were detected in cane and pooled data of all species is presented (Figure 238, Figure 239, Figure 240). Other pest species although present are not included on the graphs due to very low abundance, these include linear bugs, planthoppers and aphids. Mite populations, in the untreated control, showed bimodal distribution one peaking in early January just prior to the commencement of YCS expression and a second peak in March-April coinciding with a further rise in YCS expression.

The bifenthrin weekly treatment reduced populations of mites (Figure 238) from December through to mid-April. The 1500-2000 bifenthrin treatment was also effective against mites (Figure 240) although YCS expression was not as markedly reduced as with the weekly treatment. The weekly permethrin treatment was also effective in reducing mite populations and both peaks where markedly reduced (Figure 239). However, YCS expression was not different between the weekly permethrin and the untreated control.

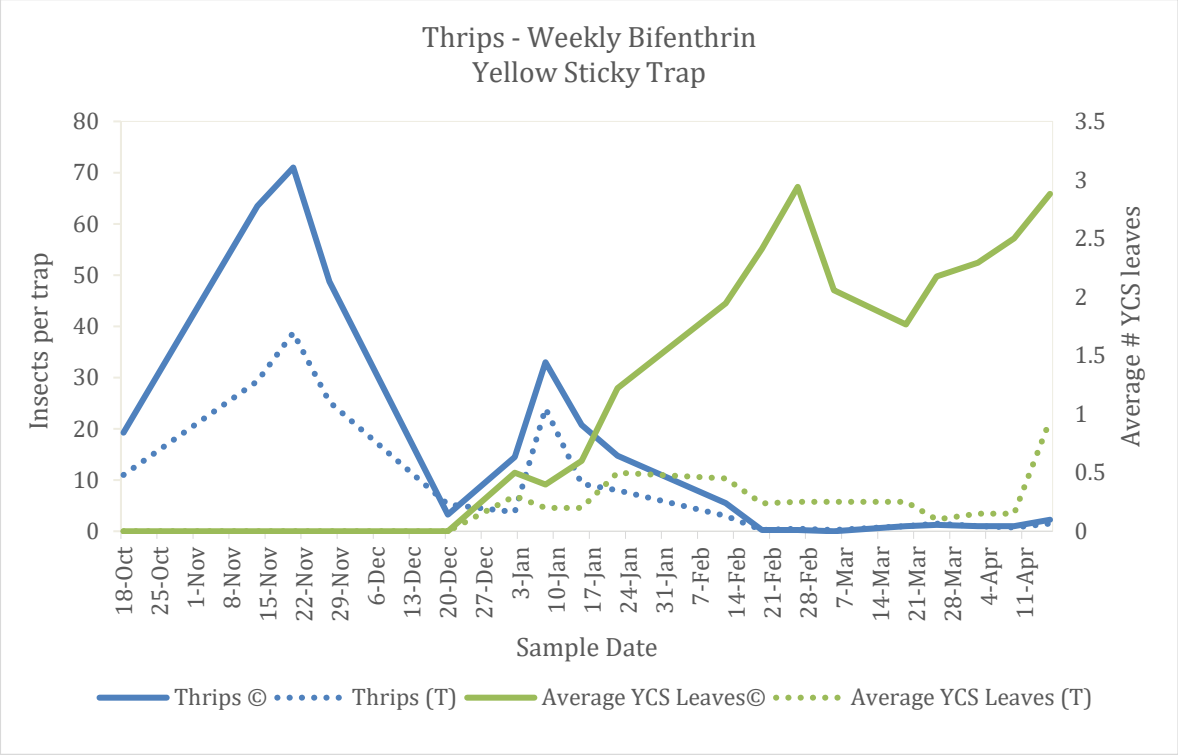


Figure 235 Mean thrip abundance per yellow sticky trap in sugarcane canopy of ratoon cane, in relation to weekly bifenthrin application and YCS expression. Based on four canopy sticky trap per treatment ©=control no spray, (T) = weekly bifenthrin spray treatment.

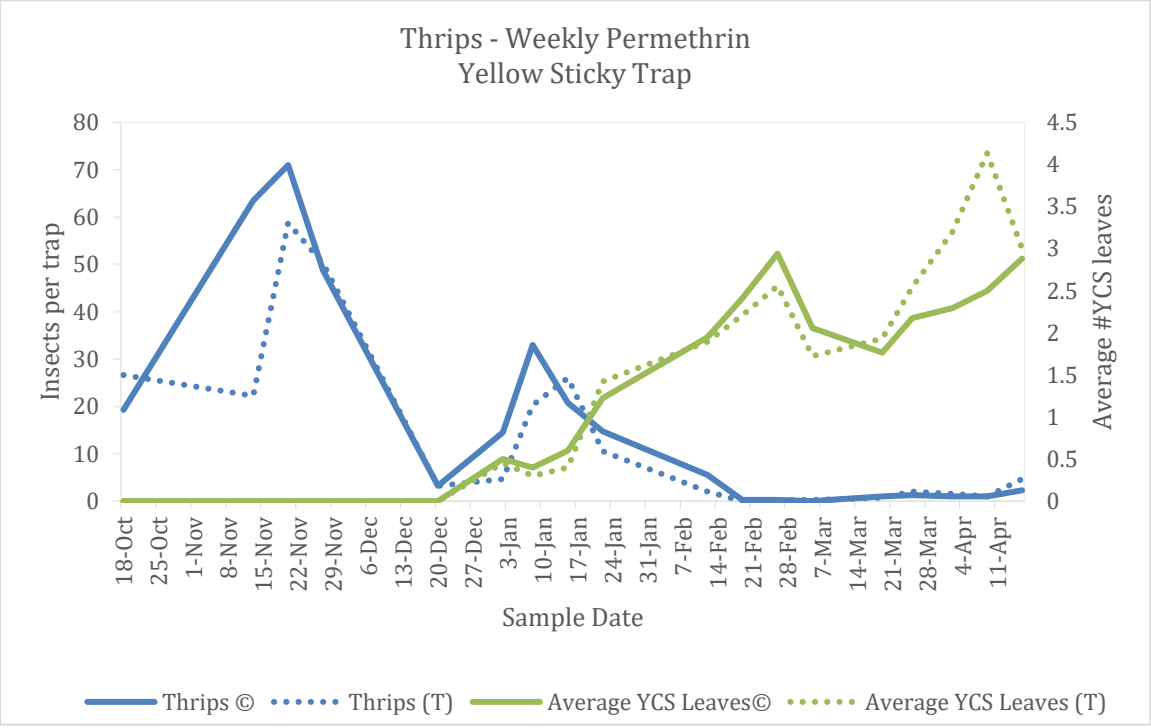


Figure 236 Mean thrip abundance per yellow sticky trap in sugarcane canopy of ratoon cane, in relation to weekly permethrin application and YCS expression. Based on four canopy sticky trap per treatment ©=control no spray, (T) = weekly permethrin spray treatment.

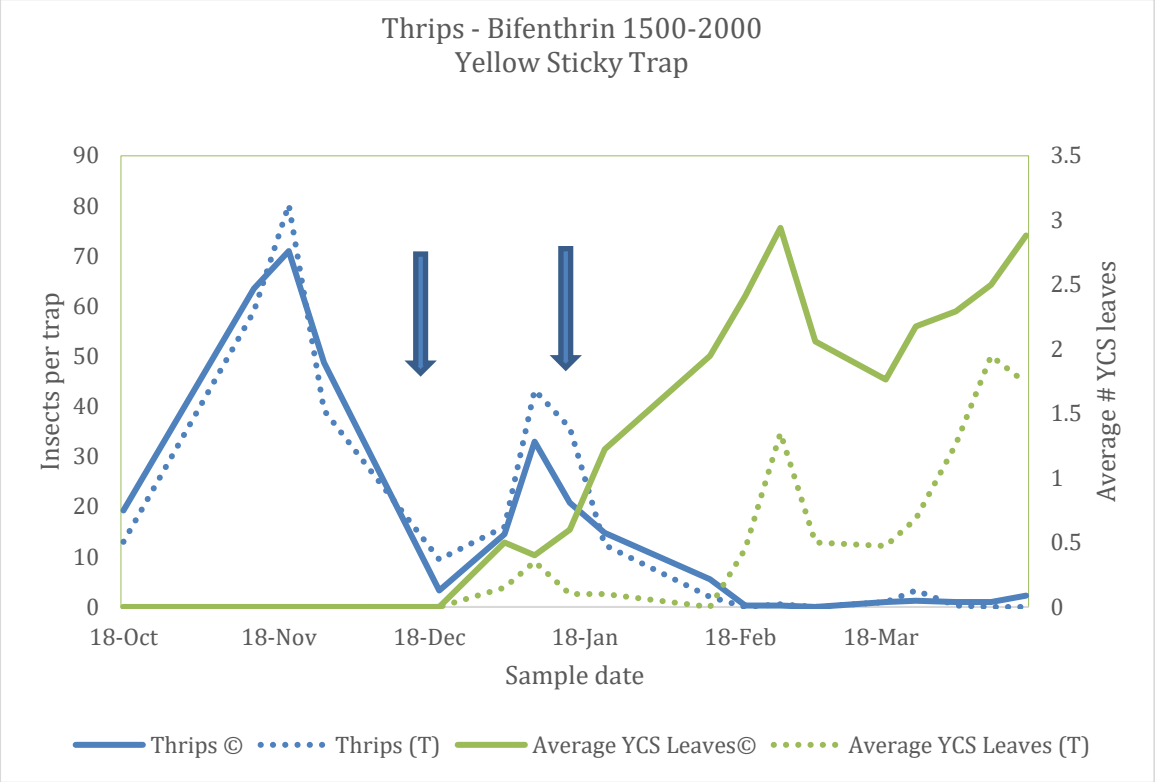


Figure 237 Mean thrip abundance per yellow sticky trap in sugarcane canopy of ratoon cane, in relation to bifenthrin 1500-2000 application and YCS expression. Based on four canopy sticky traps per treatment ©=control no spray, (T) = bifenthrin 1500-2000 spray treatment. Arrows indicate spray application date.

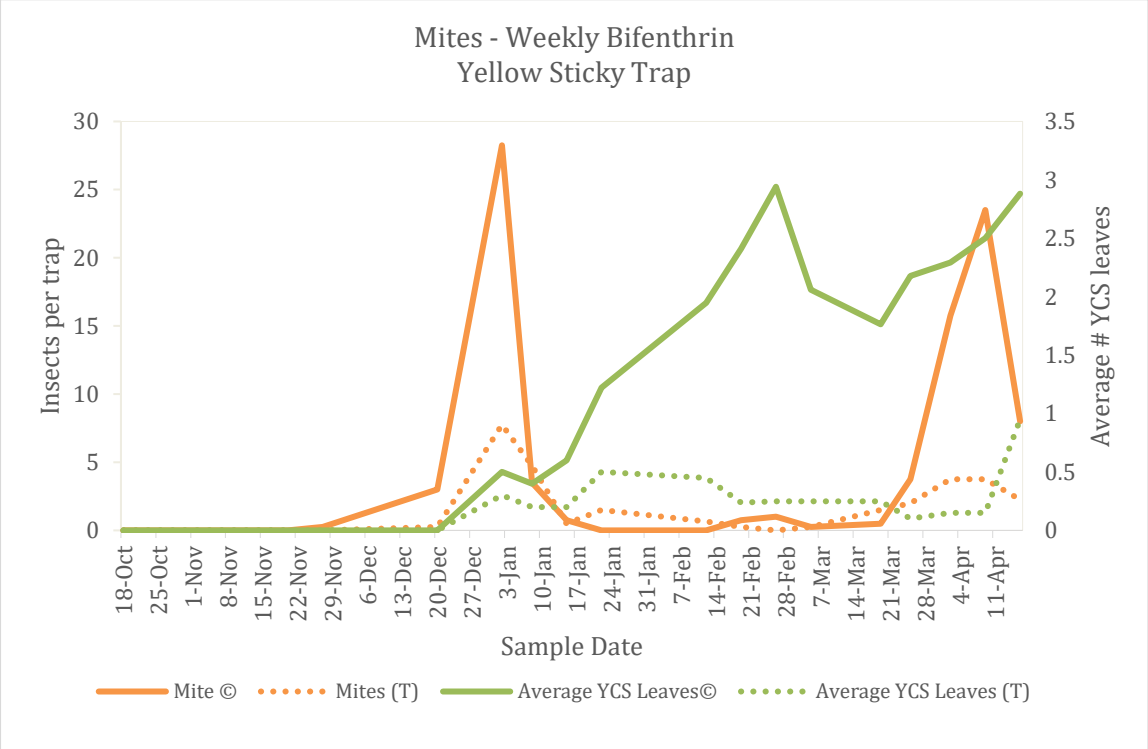


Figure 238 Mean mite abundance per yellow sticky trap in sugarcane canopy of ratoon cane, in relation to weekly bifenthrin application and YCS expression. Based on four canopy sticky traps per treatment ©=control no spray, (T) = weekly bifenthrin spray treatment.

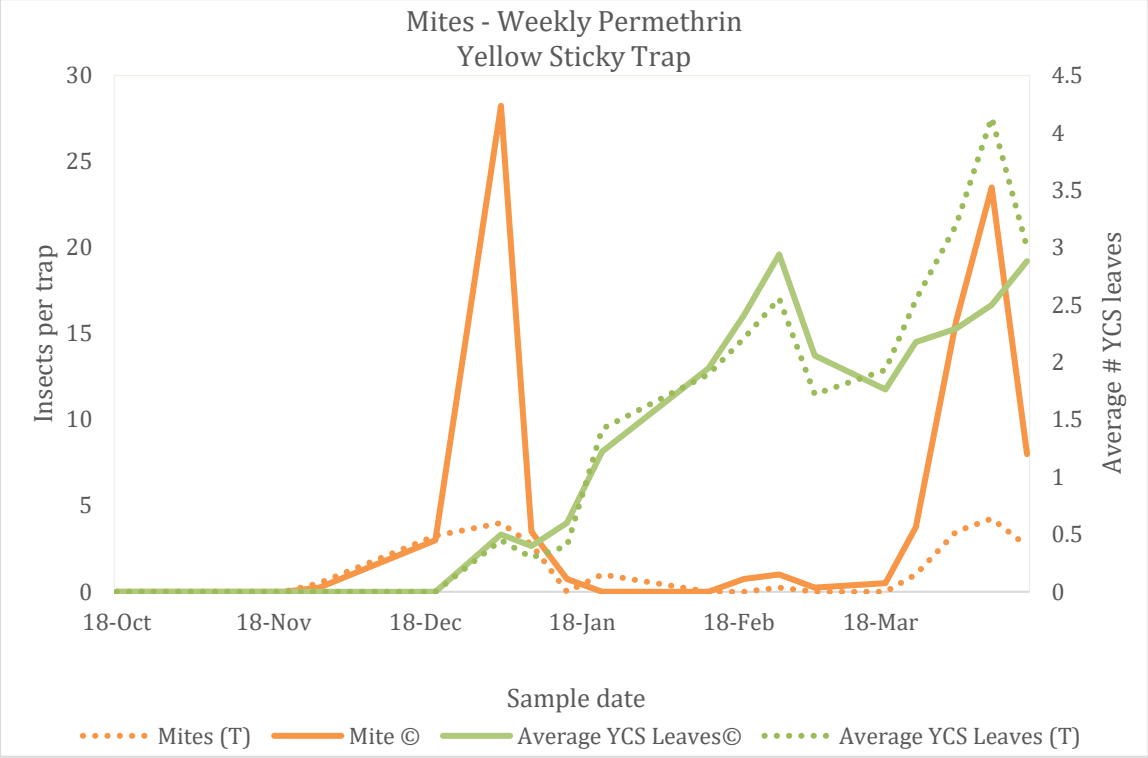


Figure 239 Mean mite abundance per yellow sticky trap in sugarcane canopy of ratoon cane, in relation to weekly permethrin application and YCS expression. Based on four canopy sticky traps per treatment ©=control no spray, (T) = weekly permethrin spray treatment.

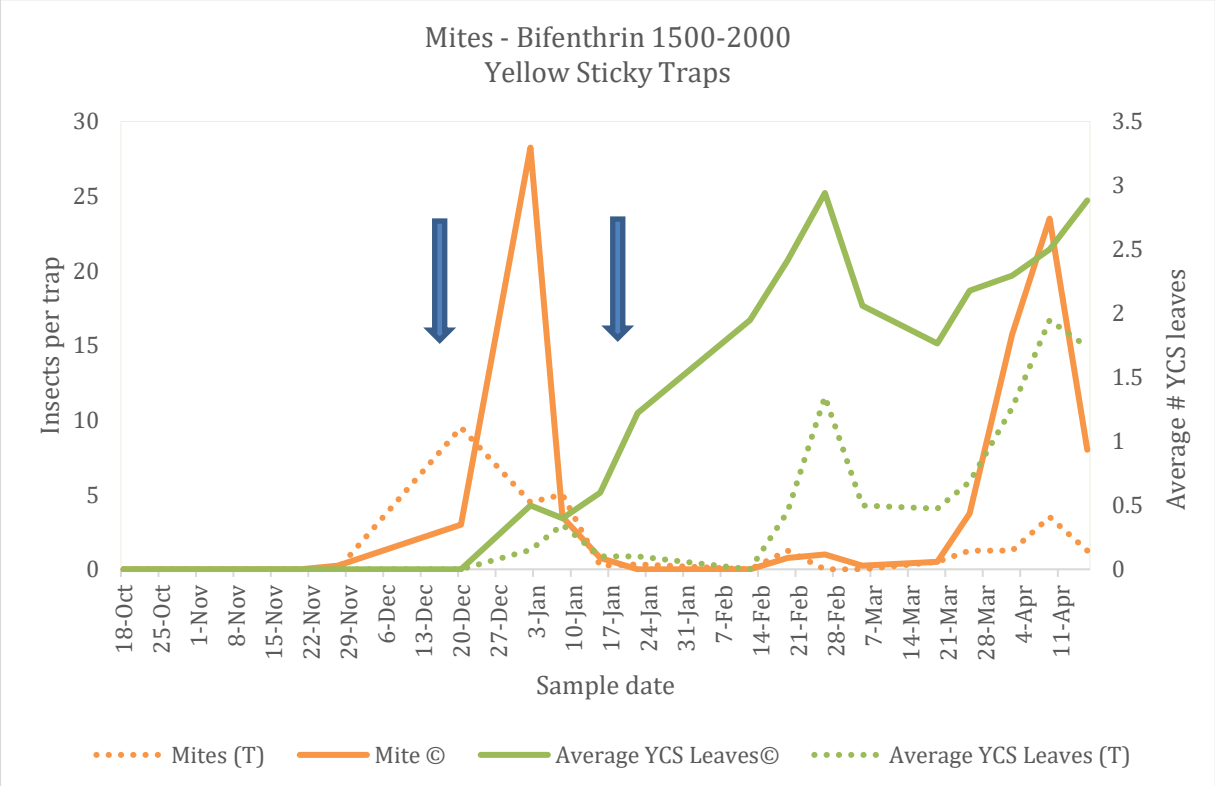


Figure 240 Mean mite abundance per yellow sticky trap in sugarcane canopy of ratoon cane, in relation to bifenthrin 1500-2000 application and YCS expression. Based on four canopy sticky traps per treatment ©=control no spray, (T) = bifenthrin 1500-2000 spray treatment. Arrows indicate spray application date.

Mealybugs

At least two species of mealybug were detected in cane and pooled data of all species is presented (Figure 241, Figure 242, Figure 243). Mealybug populations peaked between December-January just prior to peak YCS expression followed by a smaller peak in March (Figure 241). The weekly bifenthrin treatment reduced populations of mealybugs during the December-January peak but not the March peak (Figure 241).

In contrast to weekly bifenthrin, the weekly permethrin treatment was less effective against mealybugs in January and a second peak of mealybug was evident in March (Figure 242). This also coincided with a large peak in YCS expression from permethrin treated cane. The 1500-2000 bifenthrin treatment was not effective against mealybugs (Figure 243) even though YCS expression was lower in the bifenthrin 1500-2000 treatment.

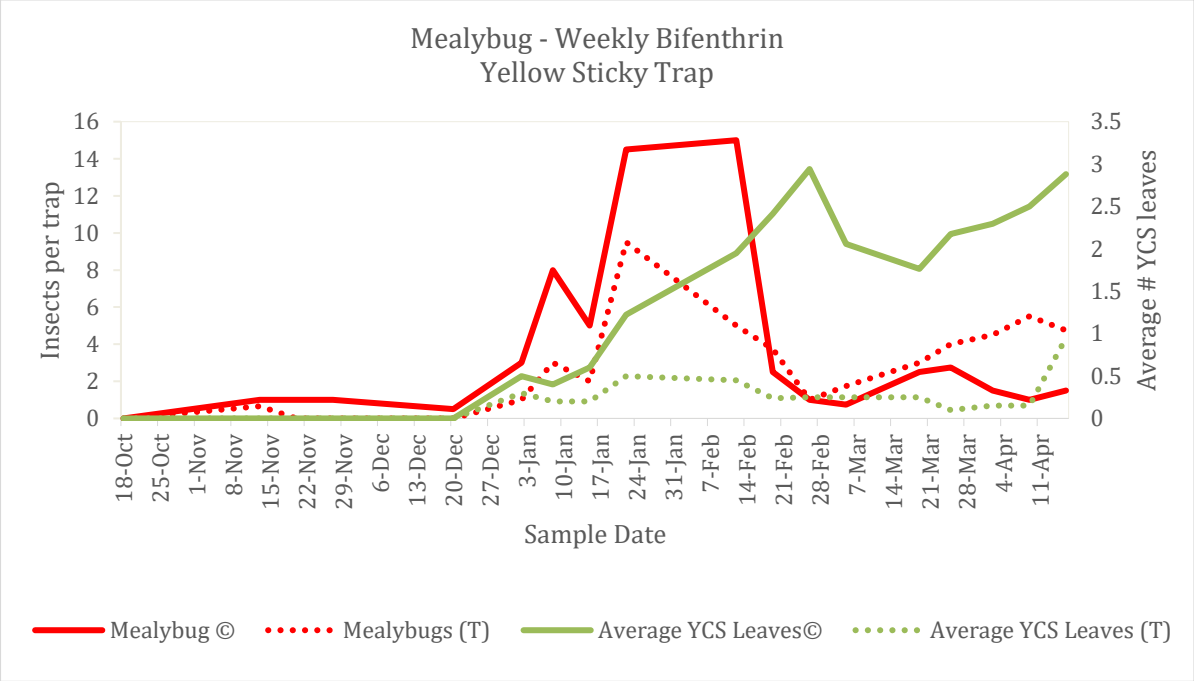


Figure 241 Mean mealybug abundance per yellow sticky trap in sugarcane canopy of ratoon cane, in relation to weekly bifenthrin application and YCS expression. Based on four canopy sticky trap per treatment ©=control no spray, (T) = weekly bifenthrin spray treatment.

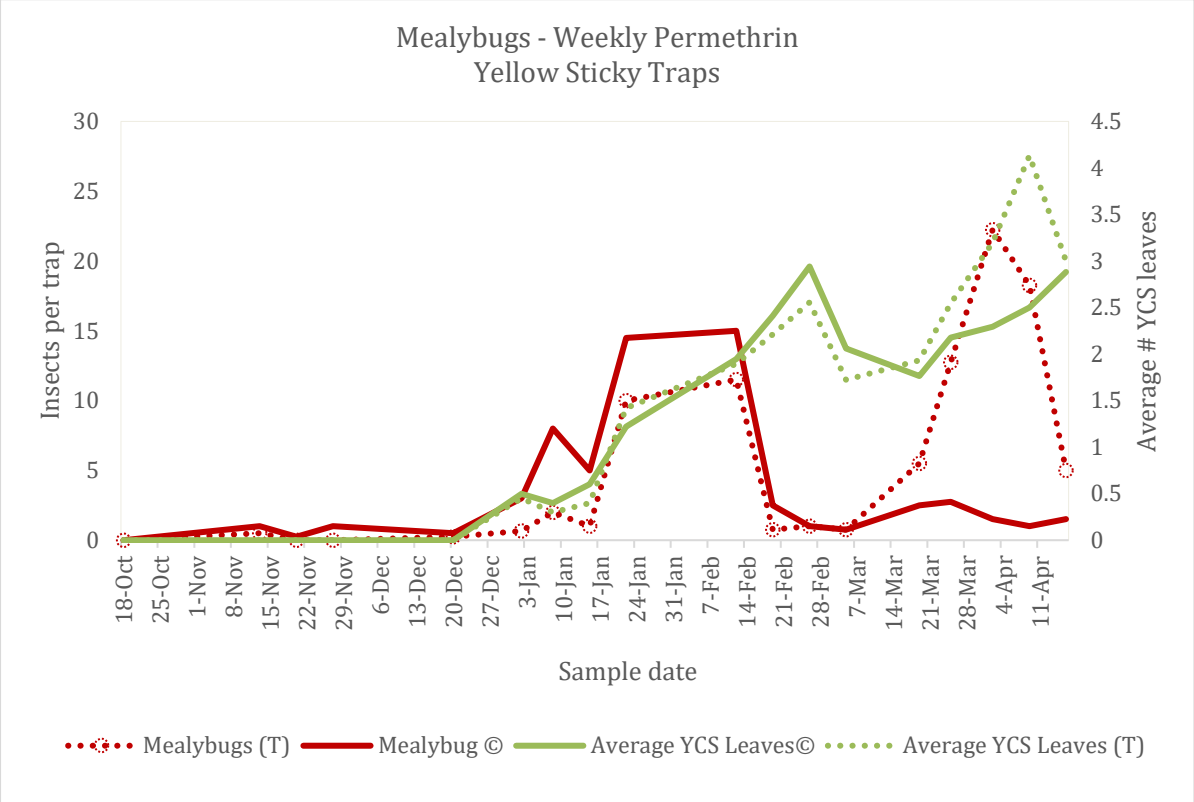


Figure 242 Mean mealybug abundance per yellow sticky trap in sugarcane canopy of ratoon cane, in relation to weekly permethrin application and YCS expression. Based on four canopy sticky traps per treatment ©=control no spray, (T) = weekly permethrin spray treatment.

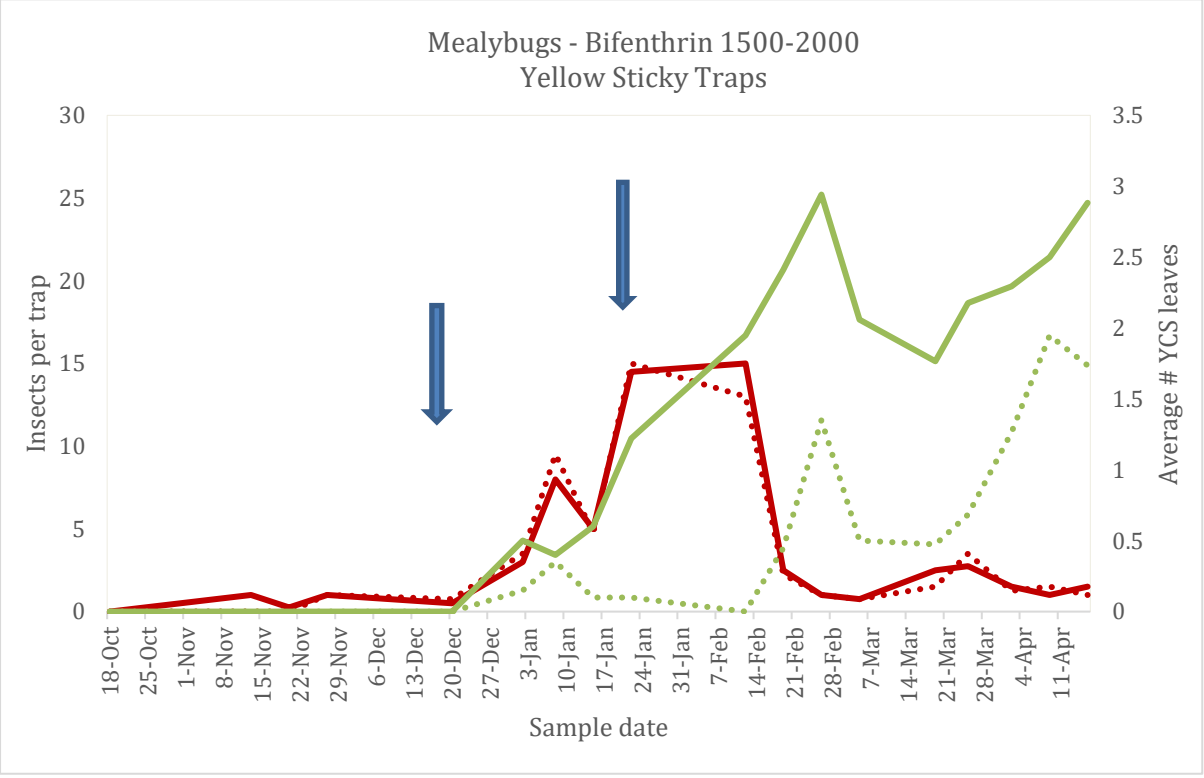


Figure 243 Mean mealybug abundance per yellow sticky trap, in relation to relation to bifenthrin 1500-2000 application and YCS expression in sugarcane canopy of ratoon cane. Based on four canopy sticky traps per

treatment ©=control no spray, (T) = weekly bifenthrin 1500-2000 spray treatment. Arrows indicate application date.

Leafhoppers

At least six species of leafhoppers were detected in cane and pooled data of all species is presented (Figure 244, Figure 245, Figure 246). Leafhoppers were present in the canopy throughout the season with a variety of abundance peaks occurring. Overall leafhopper populations appeared low in sticky traps. Due to the low counts, it is difficult to determine treatment effects. However, it does appear that weekly bifenthrin maybe having a strong effect in suppressing populations (Figure 244) from December-February and March-April periods. The permethrin weekly and 1500-2000 bifenthrin treatments appear to have either minimal or no suppressive effect respectively (Figure 245, Figure 246). Overall, there does not appear to be any clear relationships with leafhopper abundance and YCS expression.

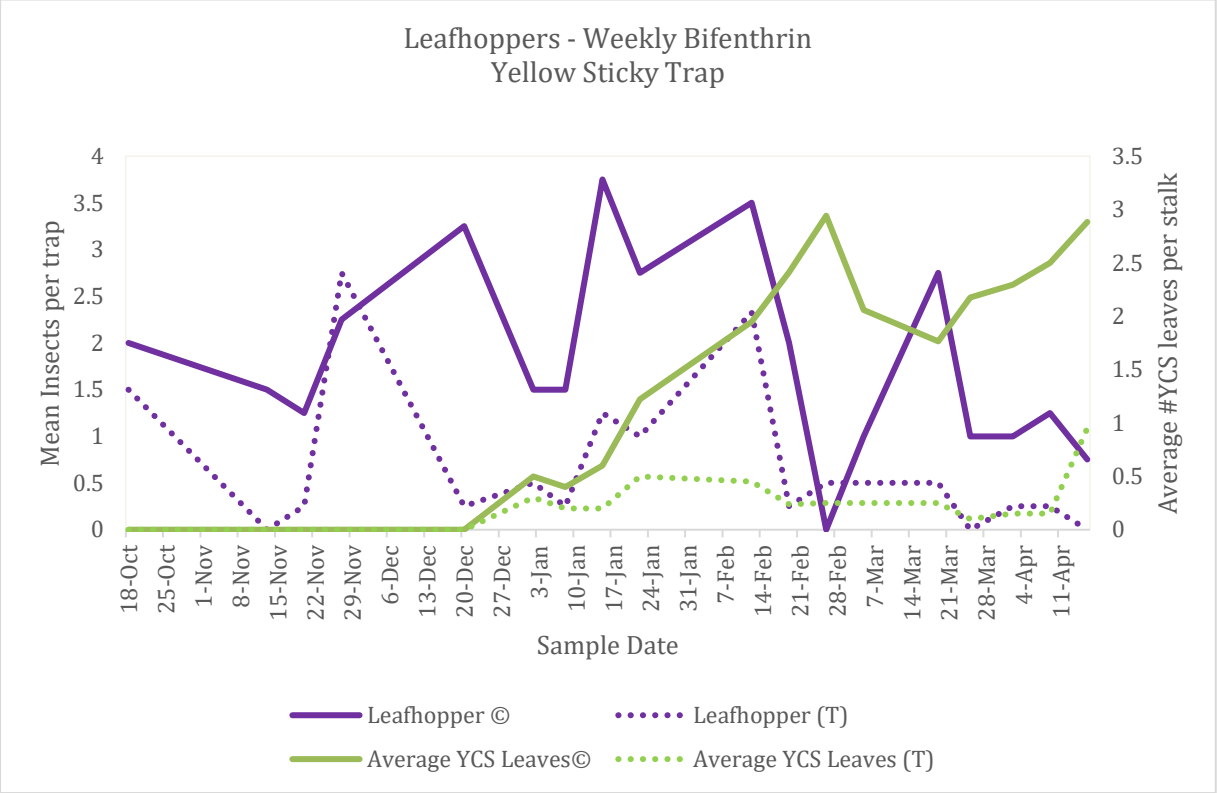


Figure 244 Mean leafhopper abundance per yellow sticky trap in sugarcane canopy of ratoon cane, in relation to weekly bifenthrin application and YCS expression. Based on four canopy sticky trap per treatment ©=control no spray, (T) = weekly bifenthrin spray treatment.

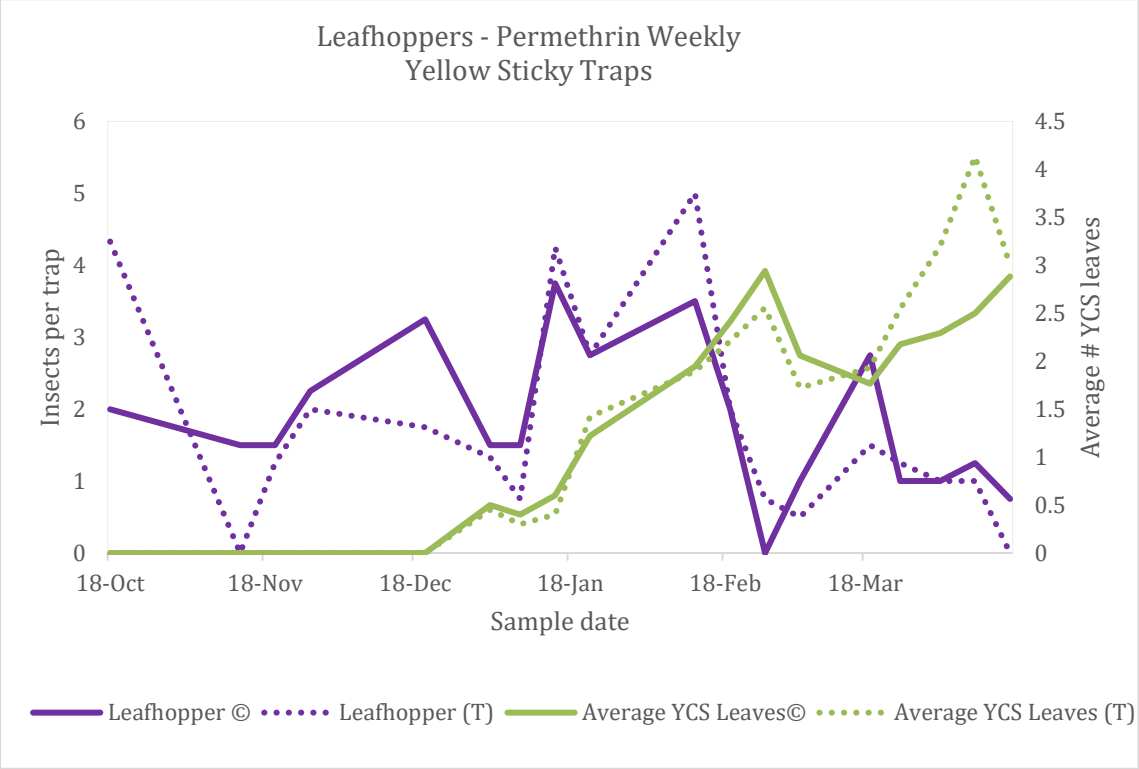


Figure 245 Mean leafhopper abundance per yellow sticky trap in sugarcane canopy of ratoon cane, in relation to weekly permethrin application and YCS expression. Based on four canopy sticky traps per treatment ©=control no spray, (T) = weekly permethrin spray treatment.

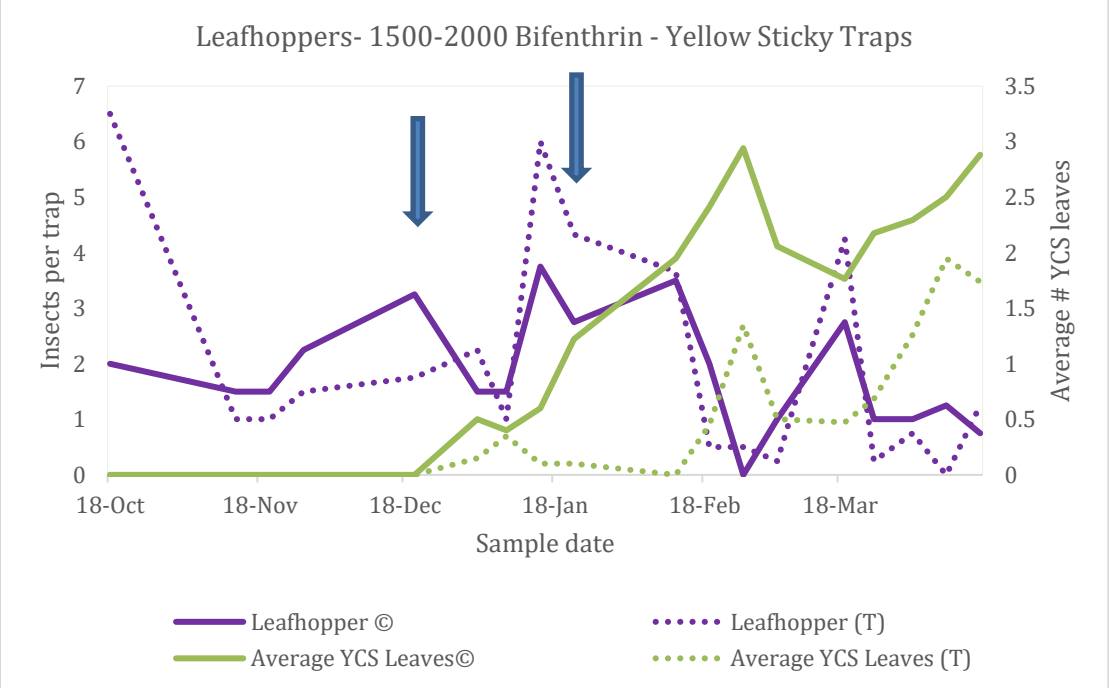


Figure 246 Mean leafhopper abundance per yellow sticky trap, in relation to relation to bifenthrin 1500-2000 application and YCS expression in sugarcane canopy of ratoon cane. Based on four canopy sticky traps per treatment ©=control no spray, (T) = weekly bifenthrin 1500-2000 spray treatment. Arrows indicate application date.

6.9.3.3 Final yield

A review of yield variation noted in 2017-18

Stalk counts

It was found in the 2017-18 insecticide trial that green, bifenthrin weekly treated cane yielded 42.7% higher biomass than YCS symptomatic cane (see section 6.9.2.3 of this report). However, it was noted that bifenthrin treated cane had approximately 35% more stalks/m^2 than the untreated control. This implied that a higher rate of stalk death was taking place in YCS affected plots. As the reason for stalk death was unknown, monthly stalk counts were conducted to investigate this further during this trial. It should be noted that this trial is conducted in the same field and is a ratoon crop. The position of treatment plots is based on a randomised block design and this changes each year to avoid positional effects. The data shows there is an initial increase in stalk numbers followed by a sharp decrease at which point the stalk numbers appear to plateau (Figure 247). This is a typical pattern of stalk growth and tiller death following canopy closure and interception of photosynthetically active radiation (PAR) below 70% (Inman-Bamber, 1994). No difference in stalk numbers was evident between treatments in this trial (Figure 247). This suggests that the variation in stalk numbers noted in the 2017/18 trial cannot be ascribed to bifenthrin treatment or YCS and therefore the yield penalty attributed to YCS is likely minimal.

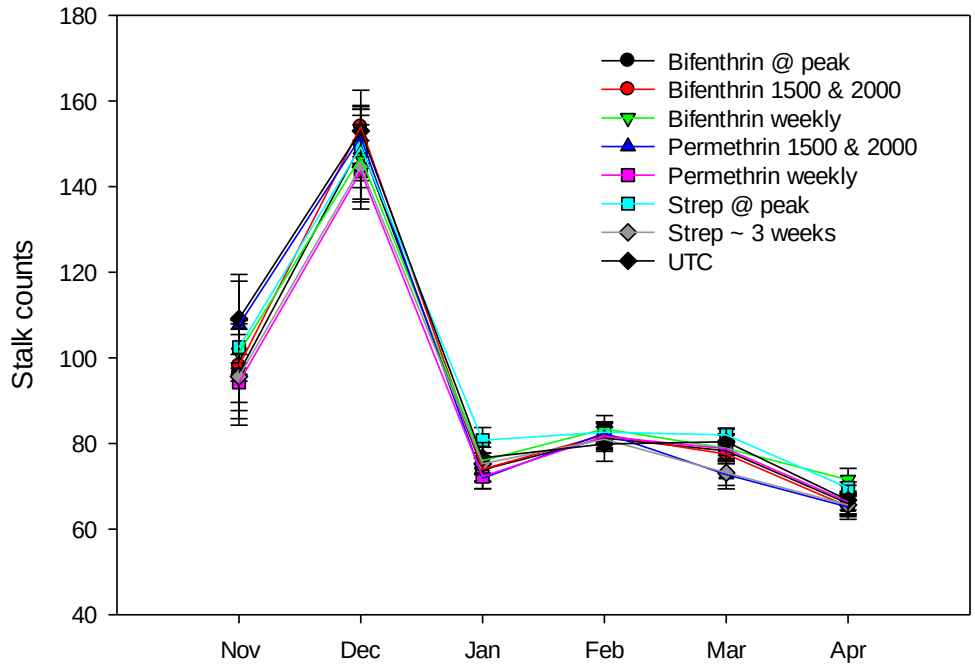


Figure 247 Number of stalks in each treatment across the season. Two 5m linear lengths of cane were counted per rep per treatment (±SE).

Final yield 2018-2019

The crop was 11 and 12 months of age at the time of hand and machine harvest, respectively. Sub-plot stalk counts, and biomass sampling (hand harvest) was conducted on August 20th prior to the machine harvest September 26th, 2019. Timing of this year's harvest is consistent with that conducted in 2018 to allow for comparative analyses. In the hand harvest, 20 randomly selected stalks from each treatment were cut between internodes 5 and 6 and stripped before being weighed to determine the millable stalk (sink) mass. The remaining tops and trash were weighed and classified as leaf and cabbage (source). Commercial cane sugar (CCS), fibre and water content were measured through SpectraCane™ analysis from 24 stalks collected randomly for each treatment.

Figure 248 shows no significant difference in the ratio between source and sink mass across the treatments. Research from project 2015016 has shown that leaf sucrose accumulation past a toxic upper threshold is due to a source sink imbalance which culminates in chloroplast breakdown and symptom expression. However, trial results show that despite the development of YCS in this field there is no severe disruption to the overall proportion of source to sink tissue mass between the treatments when the entire growing season is considered. This suggests that the period of imbalance over the life of the crop must be minimal and should be reflected in the measurable final yield across treatments. This concurs with a detailed sink size analysis of internode growth either side of the point of YCS expression in the stalk when the whole stalk is considered (Figure 233) (see section 6.9.3.1 of this report). In retrospect one could argue that an extended period of YCS severity and expression beyond that visualised in this 2018/19 season is necessary for source sink mass imbalance to be of significance.

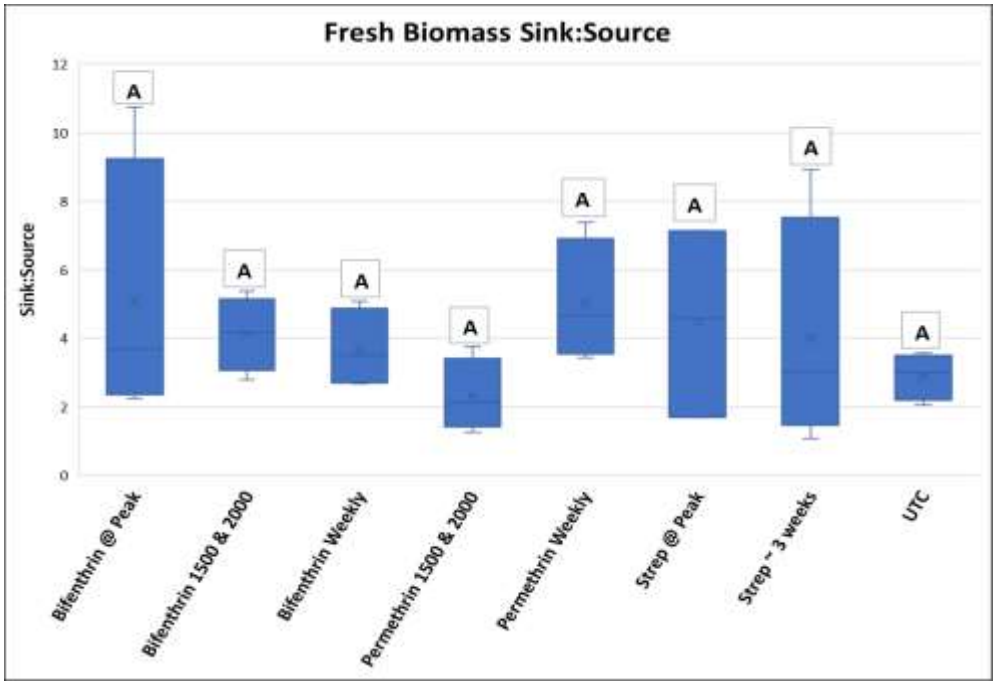


Figure 248 Ratio of mean mass (±SE) of individual canes harvested in August 2019. Each treatment represents an average of 20 stalks. Sink is classified as the millable stalk and source is the equivalent of the leaf and cabbage combined. Tukey HSD all-pairwise comparisons (p<0.05).

Stalk height measurements were made from the base of the culm to the top visible dewlap on 20 randomly selected culms from each treatment. In contrast to last year's trial, where the untreated control (UTC) was significantly shorter that the Bifenthrin continuous treatment, no measurable difference between treatments was observed this year (Figure 249).

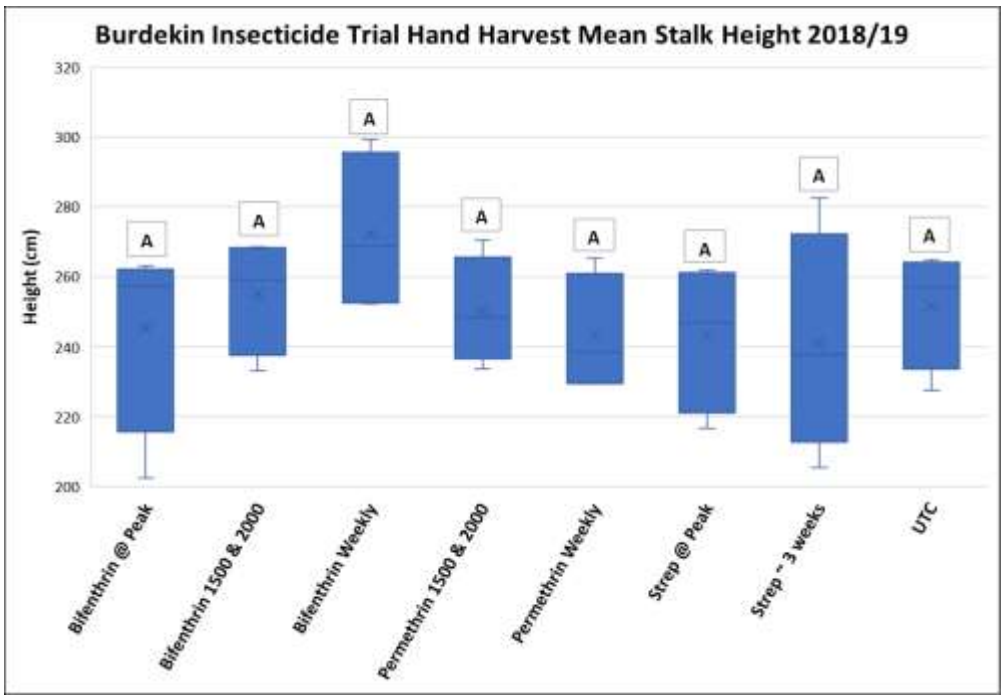


Figure 249 Mean stalk heights (±SE) of individual canes harvested in August 2019. Each treatment represents an average of 20 stalks. The stalks were measured from the base to the first visible dewlap (FVD). Tukey HSD all-pairwise comparisons (p<0.05).

Culm counts were made within eight 5 x 1.52m sections of each treatment (Figure 250). In contrast to last year, there was no measurable difference between Bifenthrin weekly and UTC. Interestingly the only treatment of significant stalk count difference to Bifenthrin weekly was the Streptomycin ~ 3 weeks. It is worth noting that the Streptomycin treatment was intended to increase from 3 weekly to fortnightly applications but, when attempted, high levels of phytotoxicity were observed (Geering et al., 2020). Even though treatment was returned to the original 3 weekly schedule, it is possible that some stalks may have died back after this period of unintentionally induced stress, which could account for the count difference.

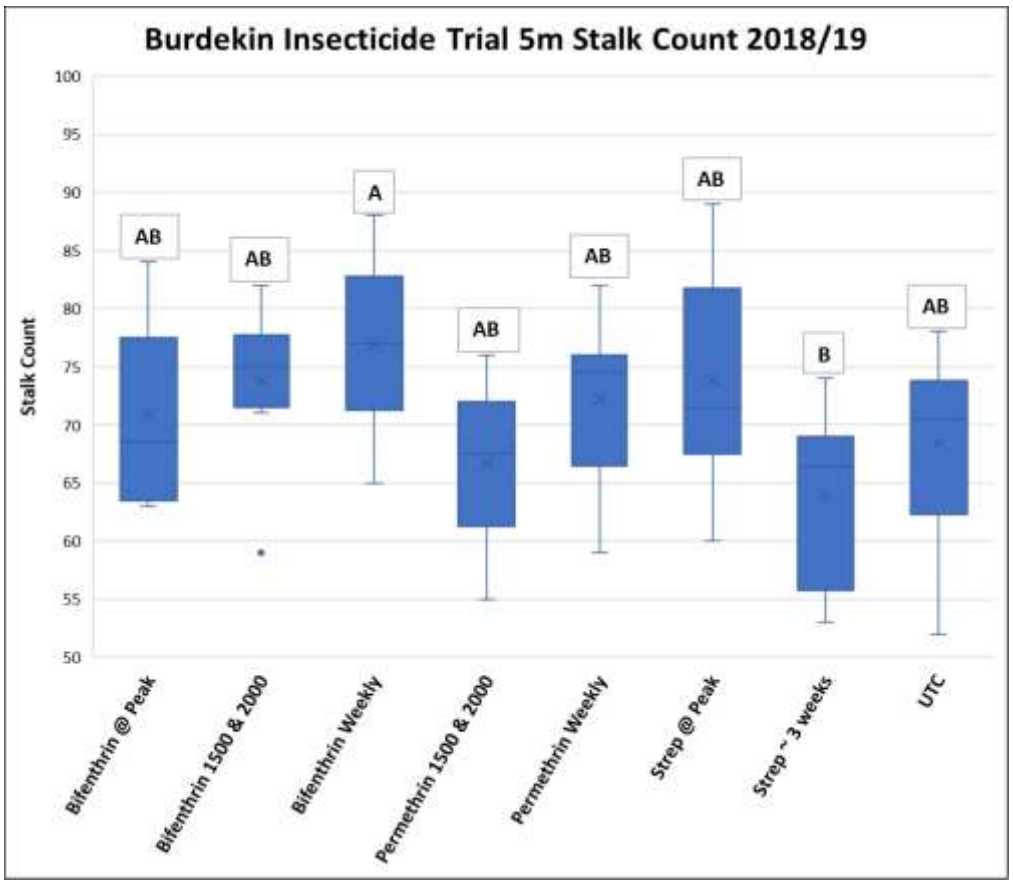


Figure 250 Mean stalk counts (±SE) 5 x 1.52m of cane in August 2019. Each treatment represents an average of 8 counts. Tukey HSD all-pairwise comparisons (p<0.05).

Yield represented by tonnes of cane per hectare (TCH) was calculated for each treatment using the mean sublot stalk counts and millable stalk biomass (Figure 251). Statistically there is no difference between the treatments and the UTC which contrasts with last year. Interestingly there is the same relationship pattern exhibited between treatments for yield and stalk count. The same is also true for the only significant variation exhibited between Bifenthrin weekly and Streptomycin ~ 3 weeks. This suggests that yield is highly dependent on stalk numbers.

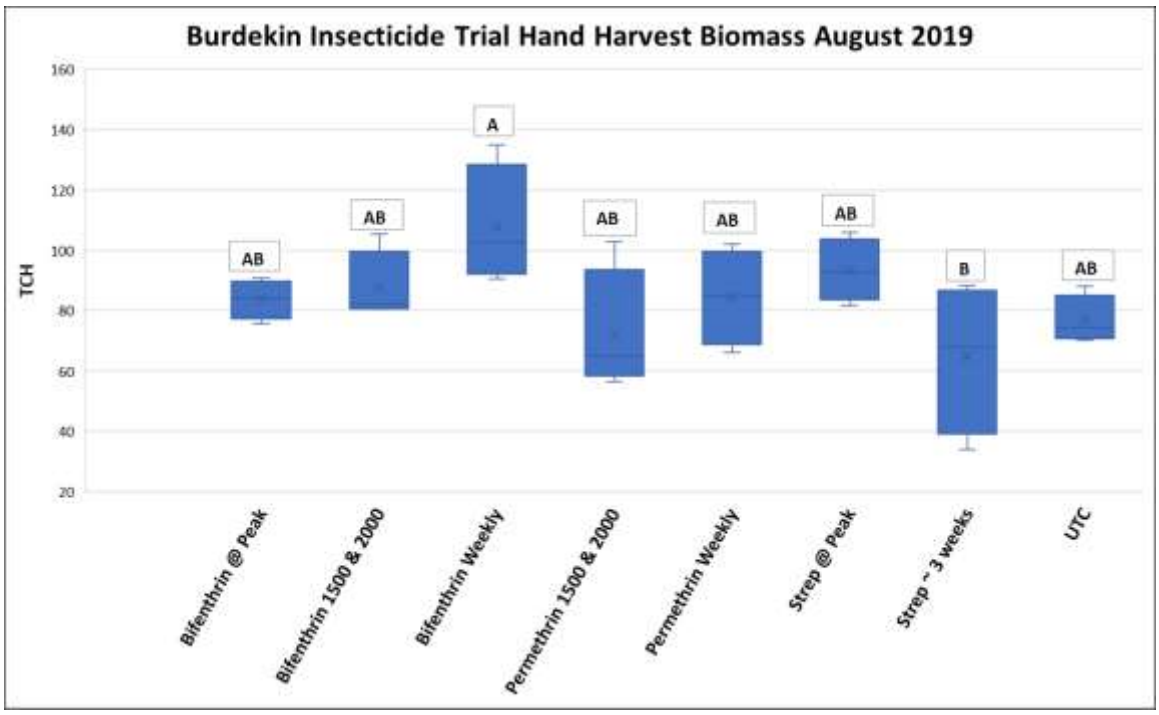


Figure 251 Mean stalk mass (±SE) of a hand harvested 15.2m^2 subplot of cane in August 2019. Each treatment represents an average of 4 biomass weights. Tukey HSD all-pairwise comparisons (p<0.05).

The field trial was burnt on September 25th and machine harvested September 26th, 2019. A haul out tractor with tipper weigh bin was used to record cane weights for each plot (Figure 252). All cane was then dumped on farm (SRA Brandon) for dry down and future destruction by burning. While the final yield (TCH) for each treatment from the machine harvest differs to that of the subplot hand harvest the yield pattern is similar. There is no significant difference in yield between the treatments and the UTC, with the only statistically significant variation recorded between Streptomycin~3 weeks and the UTC (Figure 253).

Figure 252 Burdekin insecticide trial machine harvest and weigh bin September 26th, 2019.

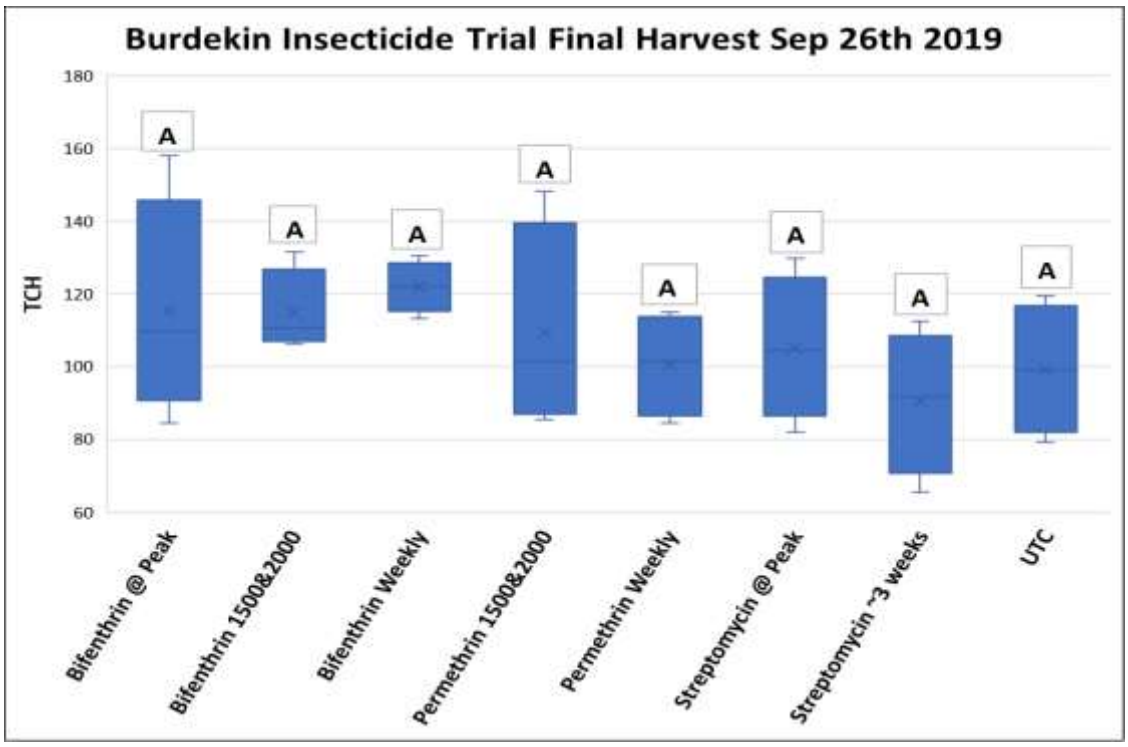


Figure 253 Mean yield (±SE) machine harvested in September 2019. Each treatment represents an average of 4 replicates. Tukey HSD all-pairwise comparisons (p<0.05).

Comparative analysis indicates there is only a 6% mean cane yield difference between the 2018 & 2019 continuous Bifenthrin treatments (115 &122 t/ha respectively). However, there is a 19% mean cane yield variance for the UTC from the same trials (83 & 99 t/ha respectively). These results suggest that the majority of the variation is due to lower stalk numbers and that the level of YCS severity and the duration of the season in 2017/18 was far worse than 2018/19. In support of this statement, YCS expression (Figure 254) correlates well with leaf sucrose and α-glucan content (Figure 255) surpassing the toxic upper threshold or 200 nmol/mg DM very late in the season (February 25th, 2019) and lasting approximately 6 weeks. In the 2017/18 YCS season leaf sucrose and α-glucan accumulation exceeded the upper threshold earlier in mid-January, lasting approximately 12 weeks (Figure 256).

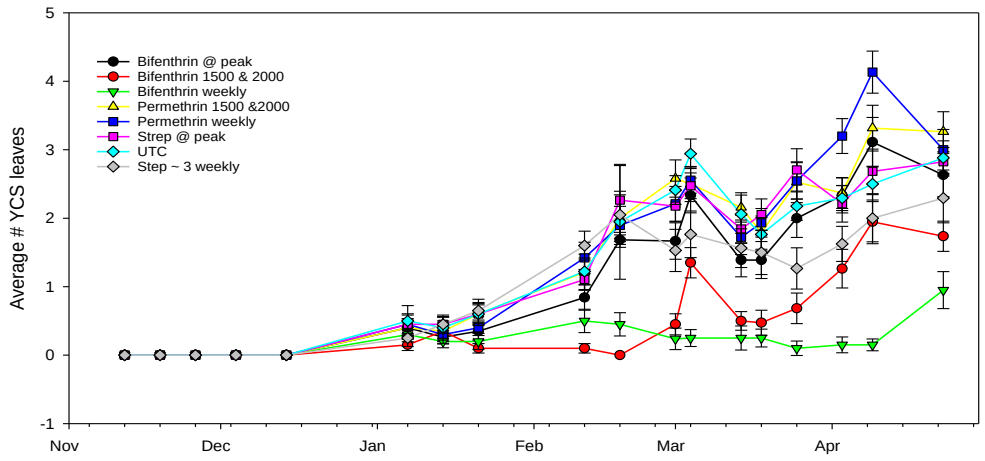


Figure 254 2018/19 average number of YCS leaves per stalk (of top 7 leaves of canopy) monitored weekly across treatments. Means of 20 stalks ±SE.

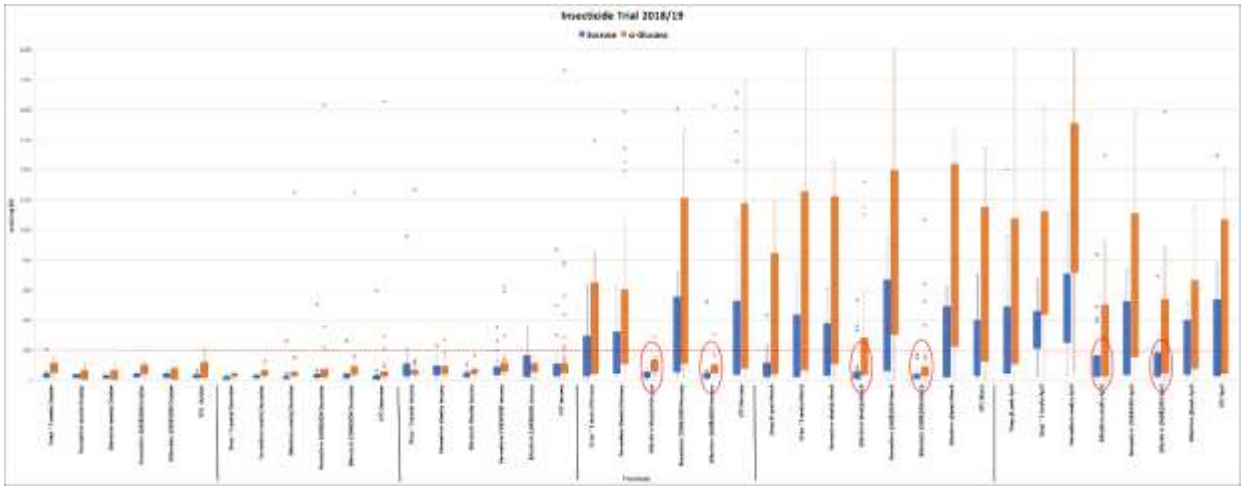


Figure 255 Leaf 4 sucrose and α-glucan content exceed the 200nmol/mg DM upper threshold 25th February 2019

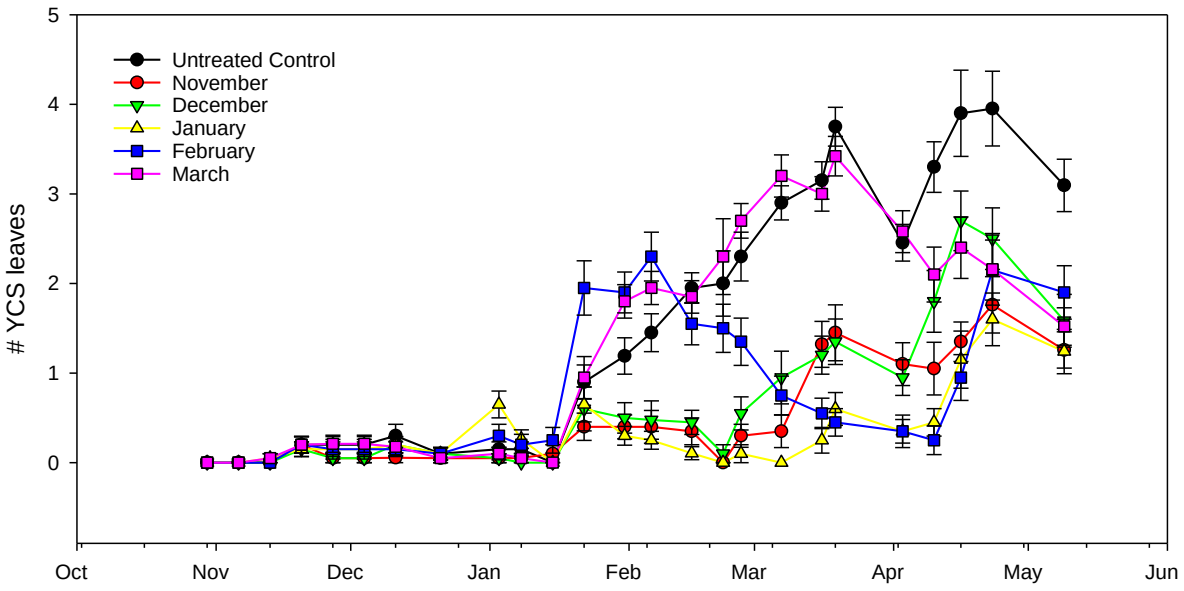


Figure 256 2017/18 average number of YCS leaves per stalk (of top 7 leaves of canopy) monitored weekly across treatments. Means of 20 stalks ±SE.

When YCS leaf severity rating is analysed in conjunction with cane yield there is no evidence of any correlation (Figure 257). As yield is a measure of carbon assimilation and export rates, the first step would be to i) assess photosynthetic rates ii) ascertain the level of feedback regulation and iii) determine the level of carbon export disruption from the source tissue and assign this to the YCS severity rating. This YCS severity rating can then be used together with the period of YCS duration and the proportion of the overall canopy affected to calculate the impact of YCS on yield. However, if available the best estimate would be obtained via the following four parameters i) YCS severity and ii) length of YCS duration iii) internode size beneath impacted leaves iv) the internode volume proportion of the total culm impacted (see conclusion in section 6.9 of this report). Given that the

2017/18 YCS duration was twice that of 2018/19 it would be reasonable to assume the yield impact would also be greater.

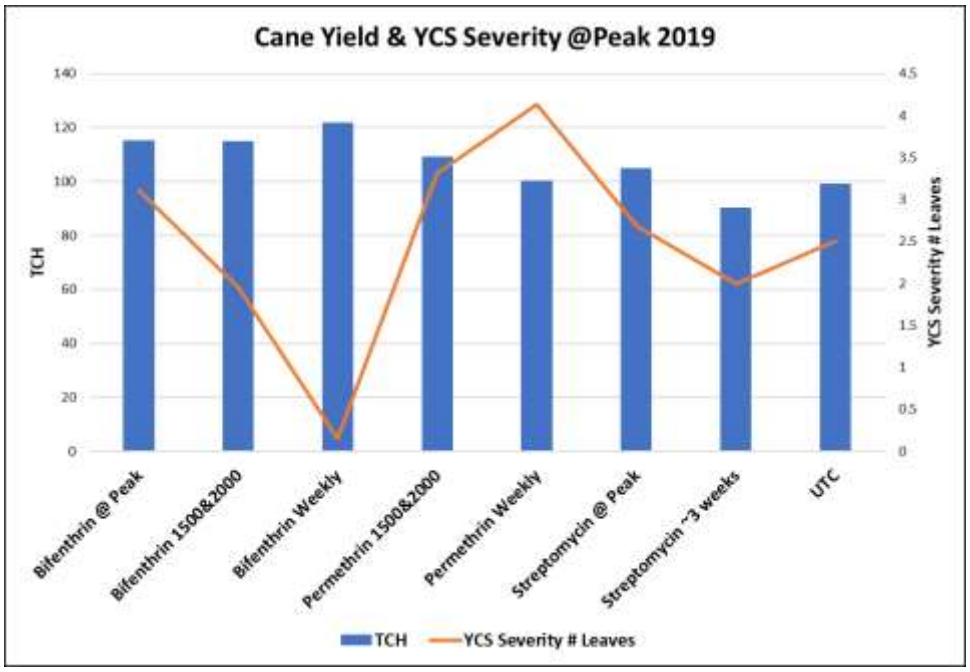


Figure 257 Burdekin 2019 insecticide trial yield and YCS severity rating

Figure 258 shows there is no Commercial Cane Sugar (CCS) penalty associated with YCS. This is consistent with the 2017/18 trial. It is also worth noting that none of the treatments (chemical or time of application) over the past two years have had any effect on CCS. Figure 259 shows YCS has no significant impact on sugar yield (tonnes of sugar per hectare - TSH)

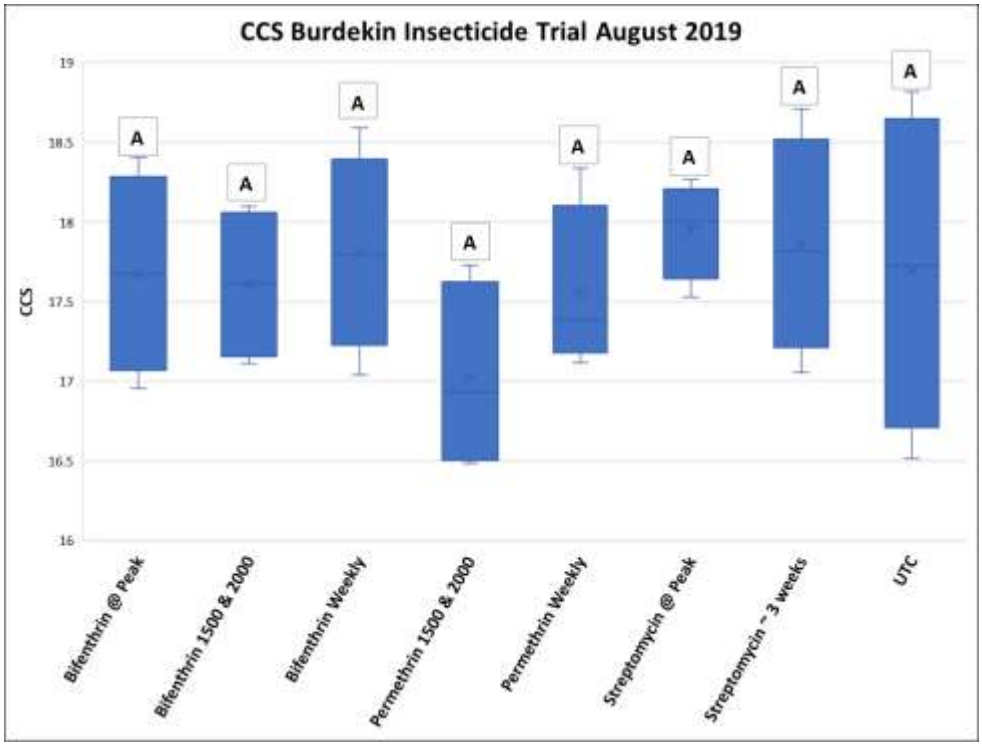


Figure 258 Mean CCS (±SE) harvested in August 2019. Each treatment represents an average of 24 individual stalks. Tukey HSD all-pairwise comparisons (p<0.05).

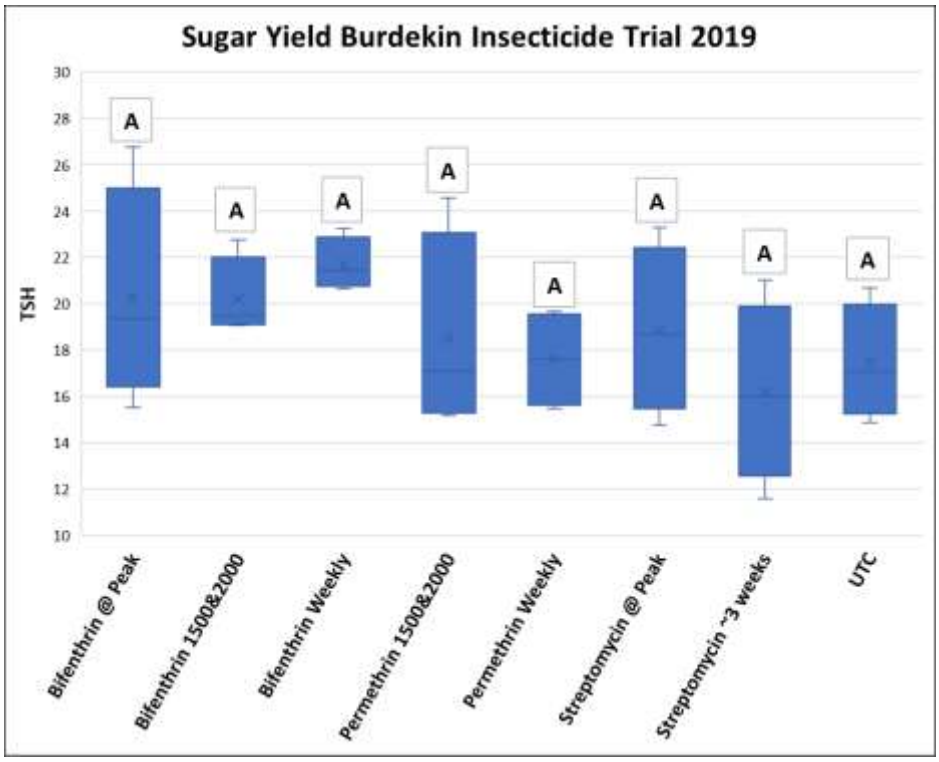


Figure 259 Mean TSH (±SE) harvested in September 2019. Tukey HSD all-pairwise comparisons (p<0.05).

Summary of Findings

- Bifenthrin weekly was the most effective treatment for the second consecutive season.
- Bifenthrin 1500 & 2000 reduced the incidence and severity of YCS for 8 weeks post application.
- Bifenthrin at peak elicited a minimal response that we speculate may be due to a rainfall event that occurred shortly after application.
- Permethrin was ineffective at preventing onset and reducing the incidence and severity of YCS.
- These findings were impacted by unprecedented seasonal rainfall events which not only interrupted the spray schedule but also may have reduced the residual effect of these non-systemic insecticides.
- Further research into timing of targeted sprays for potential management options is supported.

Summary

The suppression of insects through weekly application of Bifenthrin or at two time points (1500 & 2000 °Cd) slowed YCS symptom development by preventing the accumulation of sucrose and α-

glucans in the leaf. Bifenthrin weekly treatment was superior to the alternate pyrethroid permethrin and was the most effective treatment for the second consecutive season in preventing YCS development. While not as effective as the weekly treatment bifenthrin sprays at 1500 and 2000 °Cd reduced the incidence and severity of YCS for 8 weeks post application. Peak sprays, regardless of treatment type was ineffective in preventing YCS development or reducing severity. This indicates that the cause of YCS occurs prior to the onset of yellowing which cannot be reversed in the leaf. This concurs with studies by Scalia et al. (2020) that show once leaf sucrose tolerance levels are breached, irreversible leaf yellowing is triggered. YCS expression was noted after significant rainfall and concurs with data reported in this project and the integrated YCS program. A strong correlation exists between leaf +4 sucrose content and YCS symptom expression. Bifenthrin weekly treatment promoted better culm growth and a larger sink size than all other treatments. This correlated with the lowest YCS symptom expression but did not equate to a cane or sugar yield benefit. There was no correlation between YCS severity and cane yield or CCS which concurs with previous trial data reported here. The yield benefit attributed to bifenthrin in the 2017/18 insecticide trial was not confirmed in this trial. It was concluded that stalk numbers have a significant influence on cane yield which were not influenced by bifenthrin treatment.

Preliminary entomological studies suggest that mites and thrips are not the cause of YCS as they are effectively controlled by both types of pyrethroids in this trial, yet permethrin treatment does not reduce YCS symptoms. Bifenthrin weekly treatment was only effective in controlling mealybug populations at all times throughout the YCS season and permethrin was even less effective. The bifenthrin 1500-2000 sprays were not effective in controlling mealybugs and leafhoppers even though this treatment had a positive effect on supressing YCS symptoms.

6.9.4 Insecticide trial 3 – Burdekin (2019-2020)

The 2018/19 field trial in the Burdekin showed that weekly bifenthrin application was the most effective treatment in supressing YCS symptom development by preventing the accumulation of sucrose and α-glucans in the leaf. Interestingly, applications of Bifenthrin at two time points 1500 & 2000 °Cd was also effective in suppressing symptoms. The insecticide permethrin and bifenthrin peak applications were ineffective in preventing sucrose and α-glucan accumulation, but firm conclusions cannot be drawn from a single small-scale trial so were revisited in this trial.

The results of the Streptomycin treatments were ineffective in suppressing YCS development in either of the two previous trials. These treatments were replaced by targeted sprays to the upper and lower zones of the plant to investigate the effect on insect populations specific to these regions and/or to their mode of movement (crawling/flying).

The main objective of this trial was to determine if YCS can be managed by a particular insecticide active, and whether targeted and well-timed sprays would reveal if a specific insect/s is the cause of YCS or whether insect pressure simply acts as a YCS trigger. The impact on yield and CCS was also evaluated.

Research outcomes from the following insecticide trial of KQ228$^{\phi}$ 3R was established on station at SRA Burdekin (Farm #6007 Block #3-1) in September 2019 (see Appendix 1: 1.2.24). Treatments involved weekly applications of pyrethroids bifenthrin and permethrin, sprays of both insecticides at

1500 and 2000 °Cd and targeted sprays of bifenthrin to the base and apical meristem region and at time of peak YCS symptoms.

Results

6.9.4.1 YCS monitoring and physiology

YCS Monitoring severity scoring of treatments took place on a weekly basis from November 2019 to April 2020. There was very little YCS symptoms throughout the duration of the trail. The first signs of visual yellowing occurred in late February to early March with YCS severity never rating higher than 1.5 leaves for any treatment (Figure 260). This coincided with significant rain after a very dry period (Figure 264).

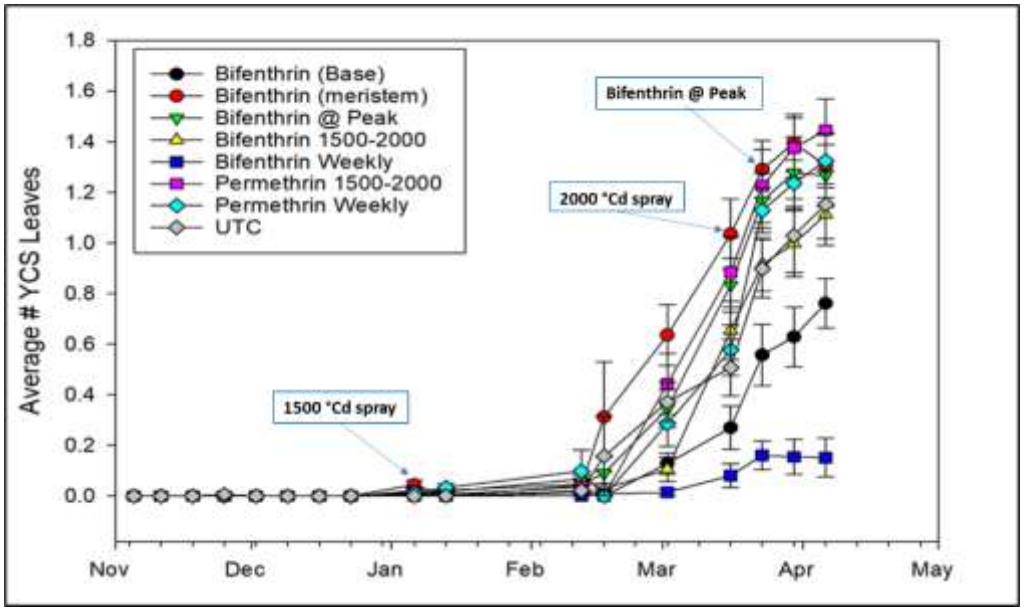


Figure 260 Average number of YCS leaves per stalk of the top 7 leaves (+1 to +7) of the canopy monitored weekly across treatments 2019-2020.

Leaf metabolites, sink size (internode) and YCS expression analysis 2020

Leaf +4 punches were collected monthly and assayed for sucrose and total α-glucan content. As discussed previously, leaf sucrose levels must be maintained below an approximate upper threshold of 200µmol/g DM to prevent the destruction of chloroplasts and the onset of chlorosis (Figure 261 A-D). Permethrin is the least effective treatment in preventing leaf sucrose accumulation; even when applied weekly (Figure 261 D). This concurs with studies conducted in the 2018/19 insecticide trial (see section 6.9.3.1 of this report). However, is should be noted that the Bifenthrin at Peak treatment was not applied until March 23rd, 2020 one week after leaf samples were collected in that month. Therefore, Bifenthrin at Peak is comparable to the untreated control (UTC).

The Bifenthrin weekly has a different Leaf 4 sucrose mean to all other treatments except Bifenthrin 1500 and 2000 °Cd (Figure 261 D). While there is a trend for Bifenthrin treatment at 1500 and 2000 °Cd to keep leaf sucrose well under the upper tolerable threshold, its mean is not statistically

different to the other bifenthrin treatments and UTC (Figure 261 D). There is also no real difference in leaf +4 metabolite accumulation between the basal and meristem bifenthrin treatments and the UTC (Figure 261 D).

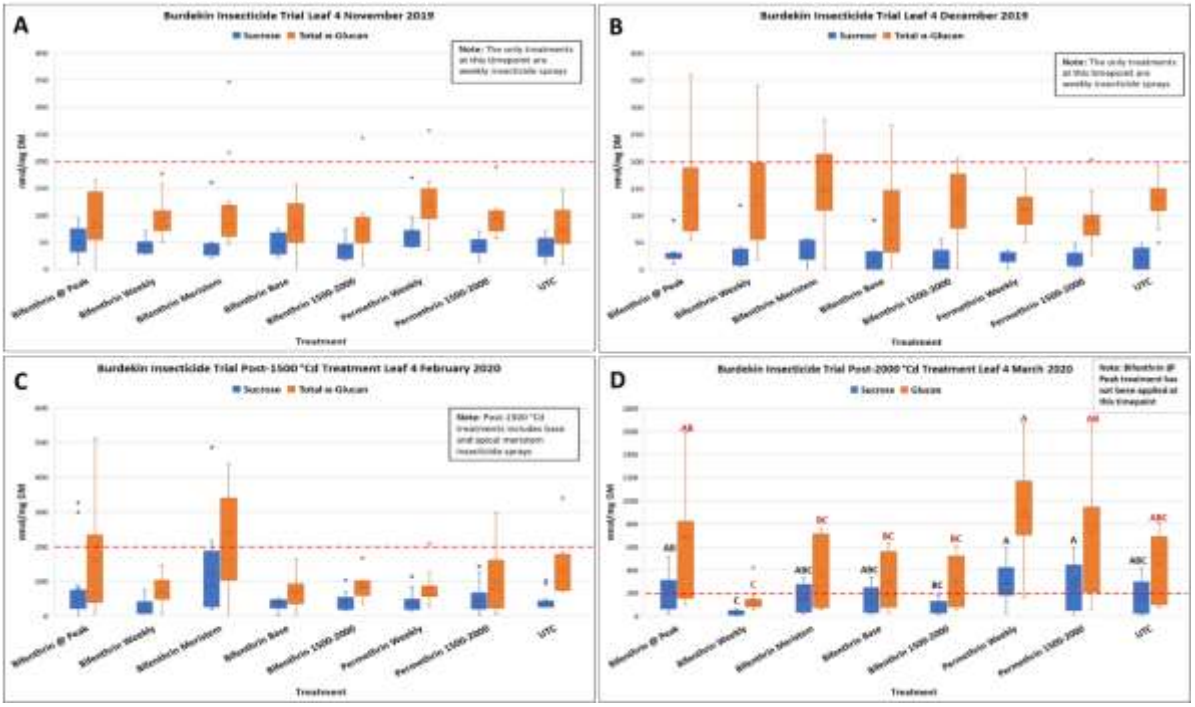


Figure 261 Leaf +4 KQ228[A] sucrose and total α-glucan content per treatment in November A), December B), February C) and March. Tukey HSD all-pairwise comparisons (p<0.05). Consider each metabolite separately. (note: no samples collected in January due to extreme wet weather event)

Differences in total glucan levels follow a similar trend to sucrose between treatments. This data suggests that applications of Bifenthrin to the whole plant at 1500 and 2000 °Cd (ie. removal of both crawling and flying insect pressure) is slightly more effective in reducing leaf sucrose accumulation than targeted sprays to either the base or meristem regions of the plant at these same time points. Our physiology case study and growth regulator trial study show that sink size has a significant effect on source leaf sucrose levels. Based on this, internode volumes were measured in April to see if there was any notable treatment effect on sink size. Removal of insect pressure at 1500 and 2000 °Cd is not as effective as weekly Bifenthrin sprays which induces a significantly ($p<0.05$) larger sink size (internode +1-13 volume) (Figure 262 B).

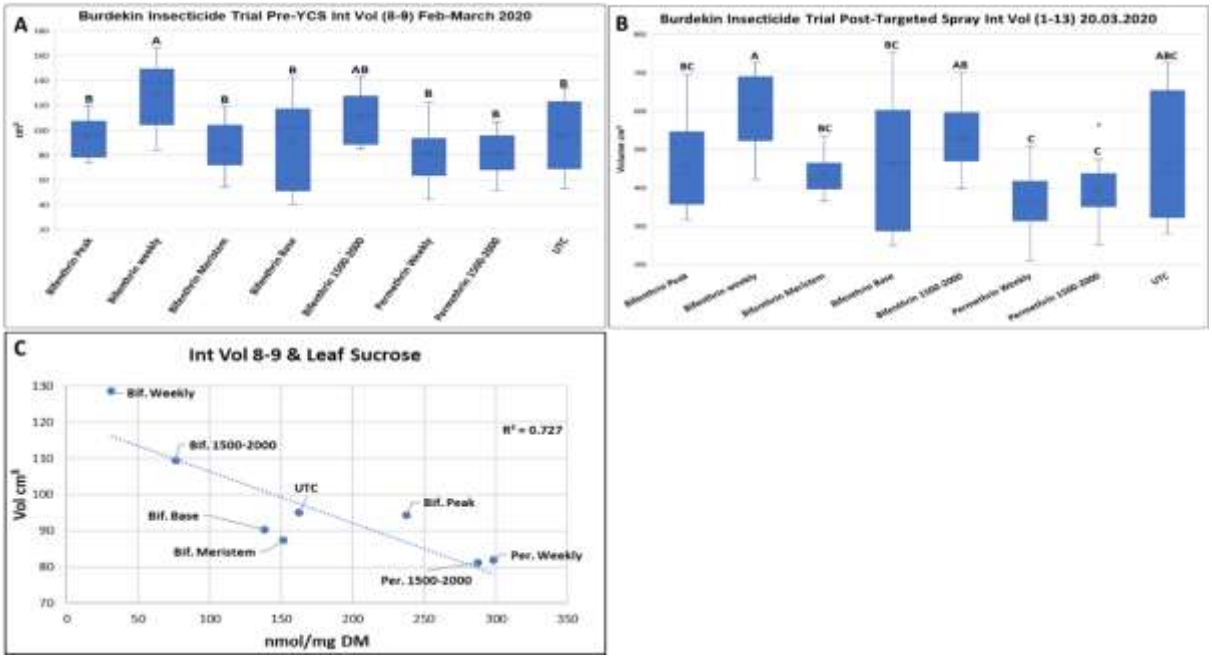


Figure 262 Sink size total internode volume +8 to +9 A), internode +1 to +13 B) and sink size to leaf sucrose correlation C). Tukey HSD all-pairwise comparisons (p<0.05).

Using culm volume (Int 1-13) as a proxy for physical sink/strength shows that bifenthrin weekly is the most effective in enhancing plant growth or vigour (Figure 262 B). While not statistically significant, a similar trend is true for bifenthrin 1500-2000 °Cd treatment. Analysing internode sizes in the immediate area below where YCS symptoms were first noted in late February to early March (Table 60) shows that internode volumes 8 & 9 (Figure 262 A) have a positive correlation (0.727) between sink size and leaf sucrose accumulation (Figure 262 C). In contrast to the bifenthrin treatments, permethrin reduces plant vigour (sink strength), inducing a source sink imbalance and leaf sucrose accumulation above the tolerable upper threshold (Figure 261 D). However, targeted bifenthrin treatments to the base and meristem were comparable to the UTC (Figure 261 D, Figure 262 A & B).

Visual YCS severity scoring (score of 0-3 as a factor of the top 7 mid canopy leaves) groups the permethrin treatments with the bifenthrin meristem treatment (applied at 1500 & 2000 °Cd) and bifenthrin @ peak (applied on March 23rd, 2020) with worst symptom expression than the UTC (Figure 260). Inclusion of bifenthrin meristem treatment in this category does not correlate with mean leaf +4 sucrose levels which remain under the upper tolerance threshold (Figure 261 D). This suggests the yellowing observed and rated as YCS in this treatment may be incorrect. Metabolite levels are in fact a mirror image to those of the UTC (Figure 261 D). Sink size (Int 8 & 9) at the commencement of YCS expression in late February to early March as well as plant vigour (Int 1-13) also do not support this YCS severity score (Figure 262 A-C).

Table 60 & Figure 262A show that when YCS expression appeared on March 2nd, 2020 the combined Int 8-9 sink volume directly beneath the YCS symptomatic leaf area was 18-57% larger in Bifenthrin weekly treated plants than the other treatments. This data supports the growth-defence trade-off phenomenon (Huot et al., 2014) observed where the plant's signalling molecules direct energy away

from internode growth to the JA pathway in defence of insect attack (Sehr et al., 2010; Agusti et al., 2011; Yang et al., 2012; Lin et al., 2016; Patil et al., 2019).

Table 60 Insecticide trial (2019-2020) treatments and time of application, cumulative °Cd and internode volume (leaf Tbase = 11°C)

						Bifenthrin @ Peak		Bifenthrin Weekly		Bifenthrin Meristem		Bifenthrin Base		Bifenthrin 1500 & 2000		Permethrin Weekly		Permethrin 1500 & 2000		UTC
Date	Leaf Cummulative °Cd	Internode Cumulative °Cd	Activity/Event	Rainfall (mm)	Internode # From Top Down	Av Volume cm³		Av Volume cm³	Continuous	Av Volume cm³		Av Volume cm³		Av Volume cm³		Av Volume cm³	Continuous	Av Volume cm³		Av Volume cm³
17/04/2020	64.9	36.9		0	1	5.93		7.73		8.49		7.93		6.41		6.49		6.40		7.93
10/04/2020	169.2	92.2		0.8	2	14.82		23.61		13.58		16.22		16.92		10.42		11.95		15.03
31/03/2020	315.7	168.7		2.4	3	19.98		32.52		20.56		22.90		27.30		16.26		17.50		21.77
22/03/2020	452.3	242.3	Final Spray (Bifenthrin @ Peak)	0	4	24.55	Spray	37.47	Spray	23.99		25.90		29.88		18.23	Spray	20.14		26.29
12/03/2020	599.2	319.2	Actual 2000°Cd Spray (whole plant and targetted sprays)	31	5	32.13		43.92	Spray	27.09	Spray	30.10	Spray	34.16	Spray	22.80	Spray	23.94	Spray	31.51
4/03/2020	737.9	401.9	Internode Elongation Stops	36	6	37.92		50.10	No Spray	34.47		36.39		40.44		27.81	No Spray	30.44		35.72
25/02/2020	875.8	483.8	YCS Appears	148.4	7	44.54		61.38	No Spray	41.92		44.05		50.45		35.97	No Spray	36.01		40.95
17/02/2020	1016.1	568.1		1.6	8	47.99		64.28	Spray	43.48		44.27		55.45		41.07	Spray	40.82		46.33
10/02/2020	1156.1	659.1		0	9	46.33		64.41	Spray	43.84		46.02		54.04		40.81	Spray	40.35		48.78
2/02/2020	1295.3	742.3			10	44.60		62.00	No Spray	42.19		42.19		55.04		40.62	No Spray	39.06		53.00
24/01/2020	1442.4	826.4	Extreme Rainfall Event	512.6	11	45.18		57.67	No Spray	42.52		44.70		50.85		38.65	No Spray	37.96		51.24
16/01/2020	1588.5	916.5			12	48.86		51.96	No Spray	47.00		48.90		53.74		37.43	Spray	41.23		48.93
8/01/2020	1728.5	1000.5	Actual 1500°Cd Spray (whole plant and targetted sprays)	0	13	50.56		51.71	Spray	48.02	Spray	49.84	Spray	56.85	Spray	39.17	Spray	44.35	Spray	51.17
31/12/2019	1863.7	1079.7		38.6	14	46.14		55.13	No Spray	45.44		46.29		55.02		45.58	No Spray	44.77		49.02
23/12/2019	1997.5	1157.5		0	15	38.27		51.15	Spray	41.42		35.43		48.86		44.67	Spray	36.00		46.29
15/12/2019	2135.5	1239.5		0	16	32.31		58.48	Spray	28.15		32.50		38.44		36.23	Spray	19.84		21.35
7/12/2019	2271.7	1319.7		0	17	26.30		46.83	Spray	29.74		32.18		48.78		39.95	Spray	25.71		31.18
29/11/2019	2405.5	1397.5		0	18	33.39		27.21	Spray	28.79		26.48		40.43		27.05	Spray	17.25		
20/11/2019	2542.4	1471.4		0	19	20.91				37.05		27.31		28.46		23.00	Spray	13.37		
11/11/2019	2677.1	1543.1		0	20					33.35		29.52				22.64	Spray	8.10		
1/11/2019	2823.3	1619.3	First Spray (Bifenthrin & Permethrin weekly)	1.8	21					30.40										
22/10/2019	2958.6	1684.6		2.8	22					30.41										
12/10/2019	3103.9	1759.9		0	23					29.71										
1/10/2019	3242.1	1821.1		0	24					24.63										

Figure 263 shows the pattern of internode growth for each treatment and the time points where growth was rapid or impeded. The cause of plant stress and the impact on growth may be biotic or abiotic. The impact of rainfall and temperature on plant growth, timing of insecticides, insect (biotic) pressure and YCS expression is also worthy of consideration (Table 60, Figure 263 & Figure 264). The extraordinary rainfall event on January 26-29th and follow up in early February 2020 was almost equivalent to the annual Burdekin rainfall. As noted many times in experimental trials and commercial fields, YCS occurred after significant rainfall following a very dry period; this trial was no exception. Therefore, any treatment which was ineffective in maintaining adequate sink growth prior to this period of rapid growth, would be at greater risk of supply exceeding demand and YCS development. Total internode volume (1-13) for the period after the 1500 °Cd treatment on Jan 11th, 2020 shows the largest sink is grown when bifenthrin is applied weekly (Figure 262 B). The removal of insects and mites (sees section 6.9.4.2 of this report) by the broad-spectrum insecticide bifenthrin together with long residue life, ensures resources are not directed away from growth. In contrast, the ineffective permethrin treatment has the smallest sink. Hence by the time of March 16th sampling, the source sink balance is in effect proportional to the efficacy of the insecticide treatments. It is evident a correlation exists between leaf sucrose and total glucan levels, sink size and insecticide efficacy.

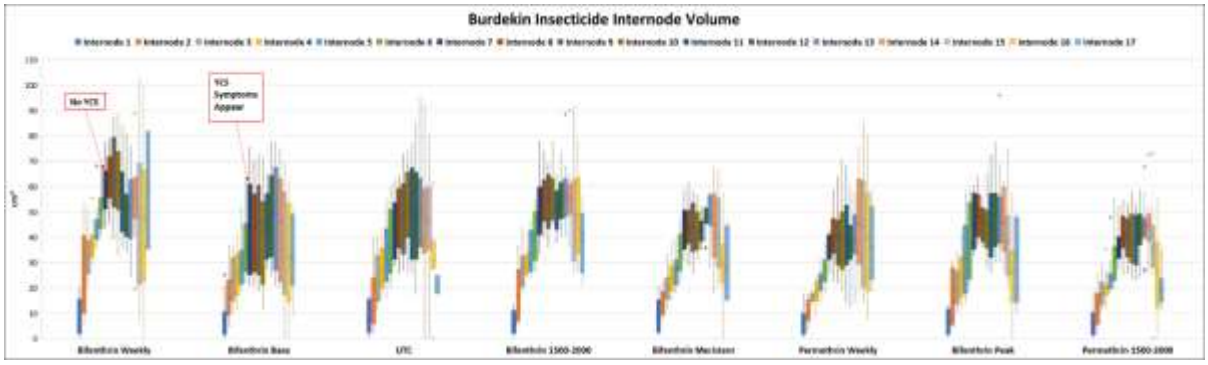


Figure 263 Internode volume +1 to +17 (top down) and treatment (December 2019 – April 2020)

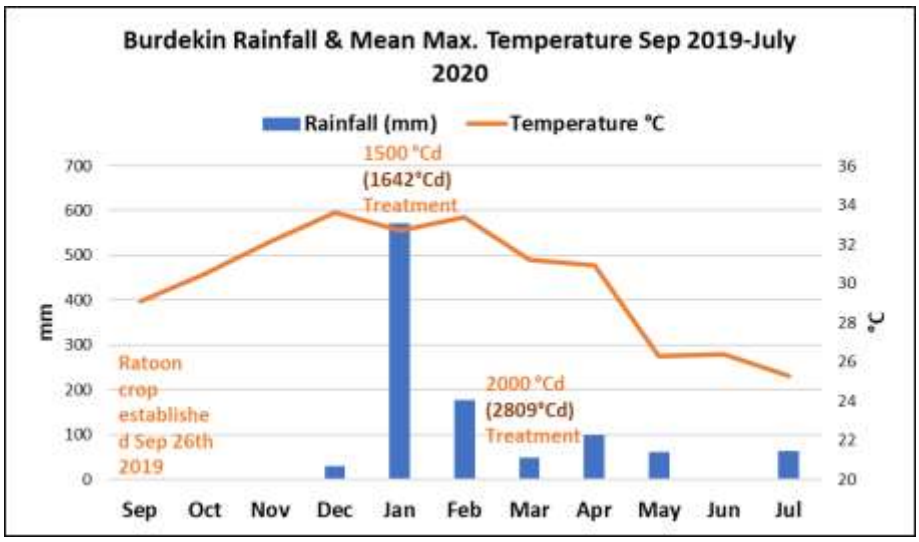


Figure 264 Rainfall and mean max temperature during the trial September 2019 – July 2020 and time of 1500 & 2000 °C day (actual) applications

6.9.4.2 Entomology

In the Ratooned Insecticide Field Trial (second ratoon) conducted in the Burdekin in 2018-2019 a number of treatments were shown to have minimal effect on both YCS expression and insect or mite populations. They included two streptomycin treatments. Consequently, the streptomycin @ peak YCS expression and streptomycin @ every 3 weeks treatments were therefore not included in the Ratooned Insecticide Field Trial (third ratoon) for 2019-2020. They were replaced with two new treatments bifenthrin applied to the base of the cane stalk (Bifenthrin@base) and bifenthrin applied to the cane meristem (Bifenthrin@meristem). These treatments were chosen to determine if insects derived from either the meristem tip or near the stem base were responsible for YCS development. In addition to the basal and meristem applications, three bifenthrin treatments (as used in the 2018-2019 trial) were applied – weekly, @peak and @1500 and 2000 day degrees (°Cd). Two permethrin treatments were also repeated; permethrin weekly and permethrin@1500 and 2000 °Cd.

All methodologies (field layout and treatment application rates) are described in Appendix 1: 1.2.21, 1.2.23 & 1.2.24. All insect samples collected during the insecticide trial were dispatched to SRA Meringa and examined using a binocular light microscope and the presence of insect and mite groups quantified.

Treatment applications were dependent on weather conditions and any gaps in treatment application (Table 61) were mainly due to rainfall activities restricting access to the sites. The three main rain events during Jan-March, as well as restricting weekly treatment applications, are also likely to have impacted pest populations. Although several insect and arachnid orders were collected and quantified only those with potential significance to YCS expression are reported.

Overall YCS expression at the site (Figure 260) was much lower and delayed in timing than in the 2018-2019 season (Figure 228). This lower expression could possibly be due to seasonal weather

conditions (most notably high rainfall events). Weather conditions almost certainly affected insect abundance during the season. As in the previous season, the bifenthrin weekly treatment appears to reduce YCS expression markedly and in addition bifenthrin @base is also somewhat effective.

Table 61 Treatment application timing for the Burdekin 2019-2020 insecticide field trial.

Treatment	Dates applied	Missing application (reason)
Bifenthrin (x 2) 1500 & 2000 °Cd	11 Jan & 18 Mar	-
Permethrin (x 2) 1500 & 2000 °Cd	11 & 18 Mar	-
Bifenthrin @base (x 2) 1500-2000 °Cd	11 Jan & 18 Mar	-
Bifenthrin @peak YCS symptoms	23 Mar	-
Bifenthrin @apical meristem (x 2) 1500-2000 °Cd	11 Jan & 18 Mar	-
Bifenthrin Weekly	5 Nov to 23 Mar	30 Dec - 5 Jan (Xmas break) 20 Jan - 10 Feb (2 x large rain events 150 mm & 200 mm) 24 Feb - 9 Mar (1 x large rain event 150 mm)
Permethrin Weekly	5 Nov to 23 Mar	30 Dec - 5 Jan (Xmas break) 20 Jan - 10 Feb (2 x large rain events 150 mm & 200 mm) 24 Feb - 9 Mar (large rain event 150 mm)

Results

Although a range of different sampling methods were used in this trial only data from sticky traps is presented as these samples were more indicative of overall arthropod diversity and relative abundance. Selected insect/mite groups are discussed as follows:

1. Mealybugs

Canopy - Yellow Sticky Traps

As with the previous trial (in 2018-2019) using canopy sticky traps two mealybug species were detected and the predominant species was the sugarcane mealybug *Saccharicoccus sacchari* with smaller numbers of *Helioccocus summervillei*. The smaller numbers of this are likely due to the fact that they are located on the underside of leaves and are largely flightless so less likely to be moved by wind onto the sticky traps. Therefore, their actual abundance in the crop could be higher than the sticky trap assessment. The general population abundance of mealybugs appears to follow a similar trend to YCS leaf expression (Figure 260) with a single large peak but peaking earlier in mid-March.

When analysing canopy sticky traps in contrast with the previous season, where abundance peaked in late January-mid February, in 2020 mealybugs peaked in late February-mid March (Figure 265). The mean abundance was 23 mealybugs per trap in the UTC, whereas in 2018-2019 at the same site mealybug abundance was slightly lower 14 mealybugs pear UTC trap. This change in the timing of mealybug peaks is most likely due to the heavy rainfall events from 20 January to 10 February as mealybug populations only started to gradually increase from 11 February onwards. Most treatments had minimal effect on mealybugs except where applications were applied at 2000 °Cd on

18th March where there is a noticeable drop in mealybug abundance for all but two treatments (bifenthrin @1500-2000 °Cd and Bifenthrin@ base). Of all the treatments applied Permethrin @ 1500 & 2000 °Cd and Bifenthrin @ peak were the least effective. The bifenthrin weekly treatment was the most effective up until mid-March and the lack of application between 24 February to 9 March and a large rain event of 150 mm may be have reduced its efficacy. In the previous 2018-2019 trial bifenthrin also reduced mealybug abundance. Permethrin weekly also reduced mealybug populations but only until early March. More mealybugs were present in the sugarcane canopy than in the field borders (data not presented). Overall, the predominant seasonal difference in mealybugs was the shift in peak abundance and slightly lower abundance in 2019-2020 most likely due to heavy rainfall events.

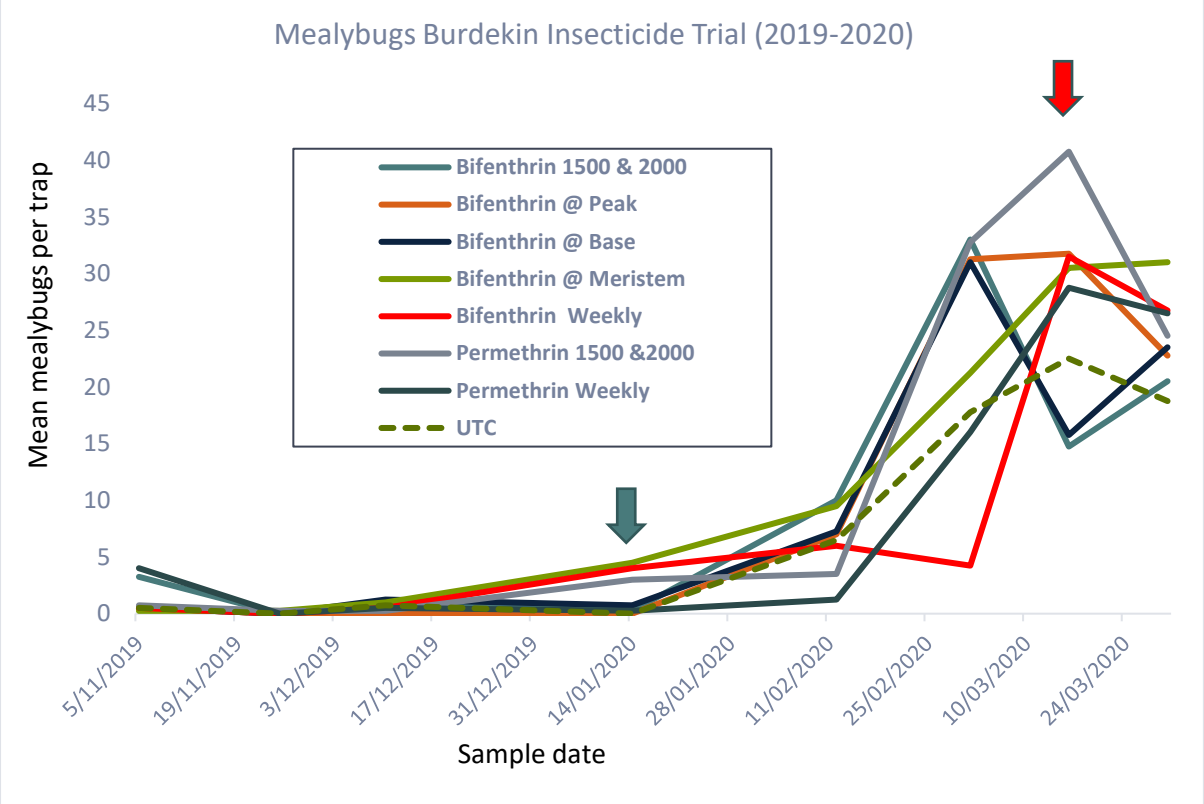


Figure 265 Mean mealybug abundance per yellow sticky trap in sugarcane canopy of third ratoon sugarcane in relation to insecticide application. Based on the mean of four canopy sticky traps per treatment and control (UTC). The blue and red arrow respectively represent the 1500 day degrees (°Cd) and 2000 °Cd application timings.

Stem - Yellow Sticky Traps

As with the previous trial (in 2018-2019) using mid-stem sticky traps one mealybug species was detected the sugarcane mealybug *Saccharicoccus sacchari*. However, in 2019-2020 traps were located in two positions on the stem to determine relative peaks of abundance and treatment efficacy in different parts of the cane stem. Mealybugs are known to spend part of their life-cycle below ground and part above ground. Sticky tape traps were located either near the cane stem base or mid-stem. Samples were collected monthly from mid-November to mid-February only.

From the mid-stem traps, it is apparent that mealybugs were most abundant in mid-February (Figure 266) and three treatments were relatively ineffective against this mealybug species; bifenthrin @meristem; bifenthrin @base and permethrin @1500-2000 °Cd. In contrast the most effective treatments were permethrin weekly and bifenthrin weekly (as also observed from canopy sticky trap data).

From the basal stem traps abundance overall was lower (around 50%; Figure 267) and this may reflect that by mid-November (when traps were first used) some mealybugs may already have moved higher up the cane stem to establish colonies near the internodes. Basal traps again confirmed that mealybugs were most abundant in mid-February and two treatments were relatively ineffective against this mealybug species: bifenthrin @meristem and more surprisingly Bifenthrin @base. In contrast the most effective treatments were permethrin weekly and bifenthrin weekly.

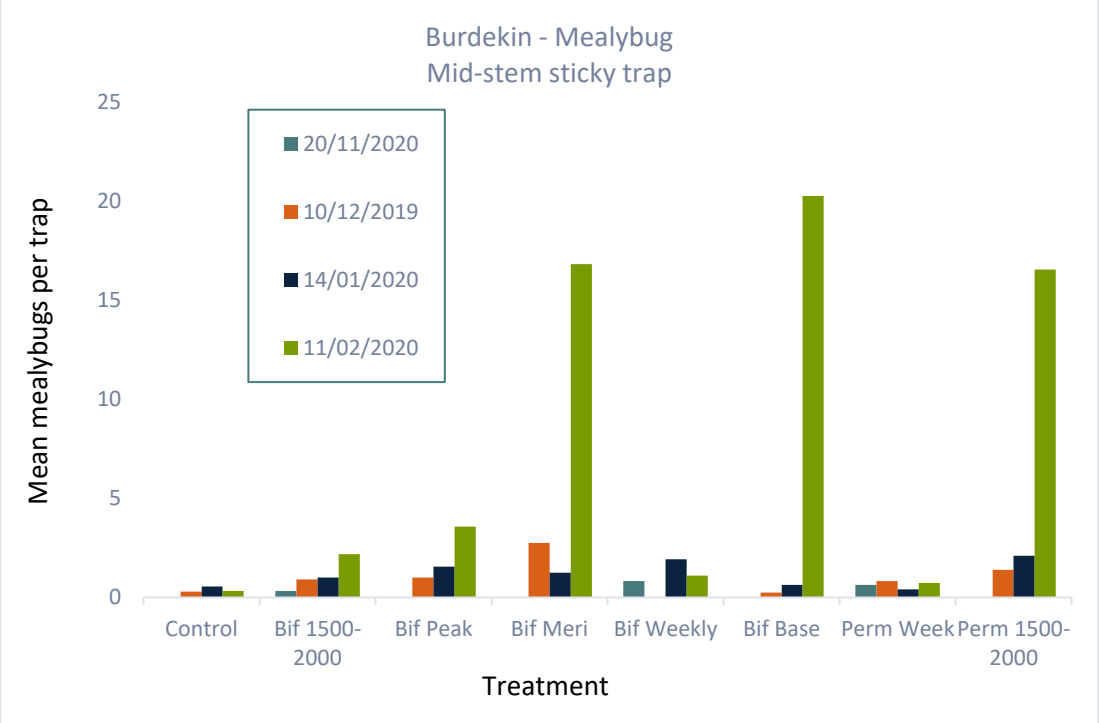


Figure 266 Mean mealybug abundance per yellow sticky mid-stem trap of third ratoon sugarcane in relation to insecticide application

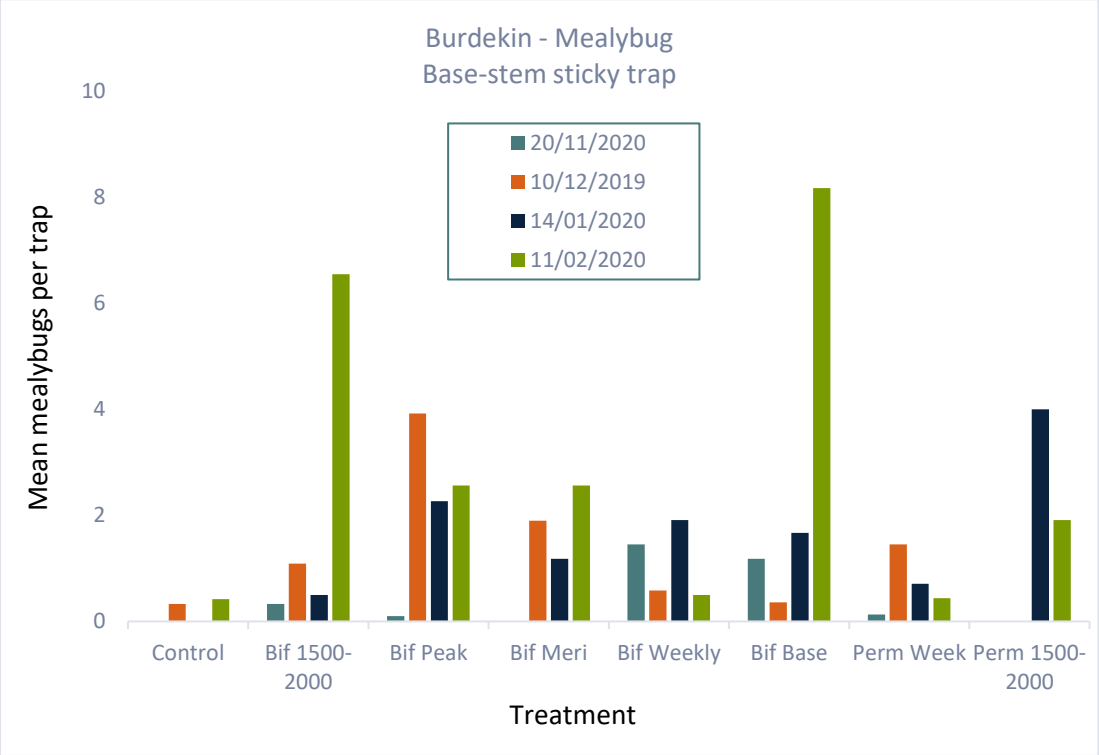


Figure 267 Mean mealybug abundance per yellow sticky base stem trap of third ratoon sugarcane in relation to insecticide application. Based on the mean of twelve sticky stem traps per treatment and control (UTC).

2. Mites

Canopy - Yellow Sticky Traps

As with the previous trial (in 2018-2019) several mite species were detected. However, in contrast with the 2018-2019 season, where two mite peaks of similar abundance were detected (one in late December (26 mites) and one in late March (25 mites), in the control there was only a single large peak in relative abundance in late March (137 mites in UTC) and a small peak in early January (Figure 268).

The general population abundance of mites appears to follow a similar trend to YCS leaf expression (Figure 260) with a single large peak but peaking earlier in mid-March.

In early-January the main mite present was a pest spider mite *Oligonychus* species which peaked at a maximum of four mites per trap only. The later March peak corresponded with presence of a beetle mite (Oribatida) which is not likely to be a pest species. Three treatments, Permethrin at 1500-2000 °Cd; Bifenthrin @ peak and Bifenthrin @ meristem were the least effective treatments. Of all the treatments applied, bifenthrin @ base were effective and the most effective was bifenthrin weekly. There is a noticeable contrast in efficacy between bifenthrin @ meristem and bifenthrin @ base which is indicative of the fact that beetle mites are often derived from the soil. In the previous 2018-2019 trial bifenthrin weekly was also the most effective treatment for mites, despite reports that bifenthrin is not always an effective pesticide for mites.

Three treatments had minimal effect on mites in early March but where applications were applied at 2000 °Cd on 18th March where there is a noticeable drop in mite abundance except for one treatment (Bifenthrin @2000 °Cd).

More mites were present in the sugarcane canopy than in the field borders (data not presented). The predominant seasonal difference in mite species was the very low abundance of *Oligonychus* species in January and the higher abundance of *Oribatida* mites in March. Rainfall events are likely to have reduced mite population abundance in January-February.

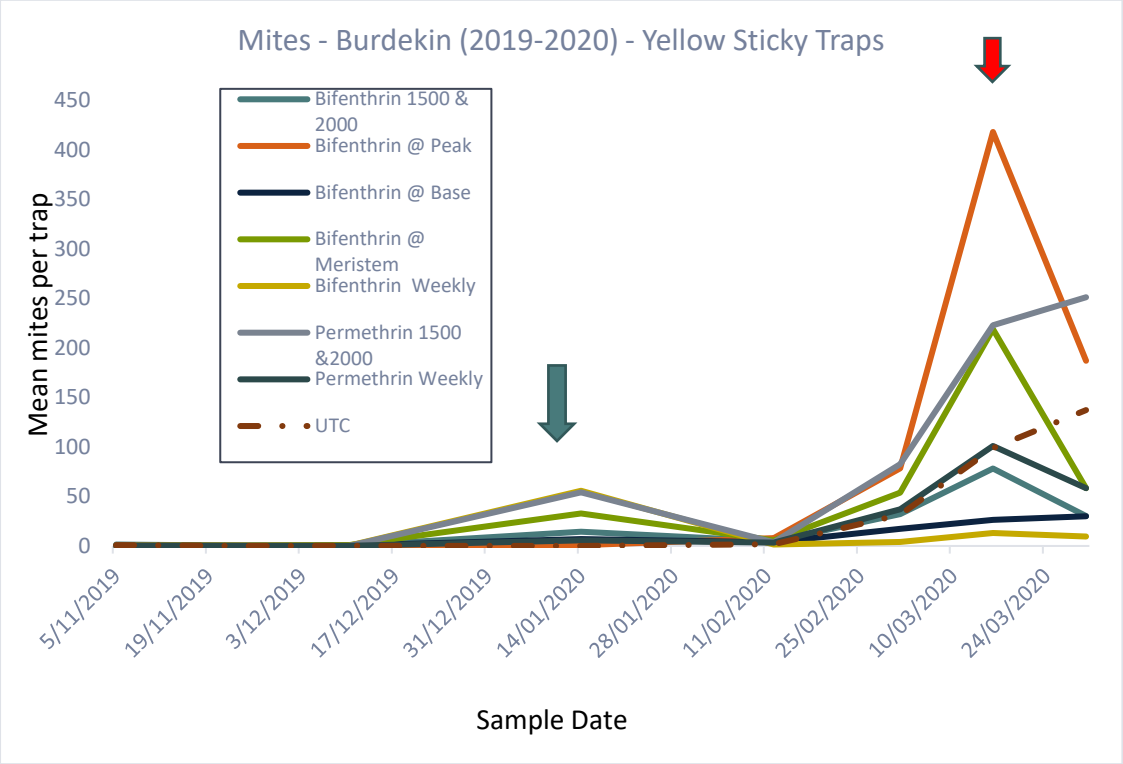


Figure 268 Mean mite abundance per yellow sticky trap in canopy of third ratoon sugarcane in relation to insecticide application. Based on the mean of four canopy sticky traps per treatment and control (UTC). The blue and red arrow respectively represent the 1500 day degree (°Cd) and 2000 °Cd application timings.

Stem - Yellow Sticky Traps

As with the previous trial (in 2018-2019) using canopy sticky traps few mite species were detected on sugarcane stems from traps set in the mid-stem position only. In 2019-2020 traps were located in two positions on the stem to determine relative peaks of abundance and treatment efficacy in different parts of the cane stem. Sticky tape traps were located either near the cane stem base or mid-stem. Samples were collected monthly from mid-November to mid-February only.

From the mid-stem traps (Figure 269) it is clear that mites were most abundant in both mid-January and mid-February (also observed in canopy sticky trap data). Two treatments were relatively ineffective against this mealybug species: bifenthrin@1500-1500 °Cd and permethrin@1500-2000 °Cd. In contrast the most effective treatments were permethrin weekly, bifenthrin weekly, bifenthrin

@peak and bifenthrin @base. The efficacy of bifenthrin @ base is indicative that mites are likely to moving up from the soil. Similar mite numbers were detected in the basal traps compared to the mid stem traps and the overall low abundance indicates that the mites detected do not prefer the stem and are more likely to be found on the foliage (as indicated earlier using canopy stem traps).

Basal stem traps again confirmed that mites were most abundant in both mid-January and mid-February (Figure 270) and four treatments were relatively ineffective against this mealybug species; bifenthrin @meristem; bifenthrin @1500-2000 °Cd and permethrin @1500-2000 °Cd. In contrast the most effective treatments were permethrin weekly; bifenthrin weekly and bifenthrin @base. However, because numbers were low on stem traps more reliable data is obtainable from the yellow sticky traps placed near the canopy.

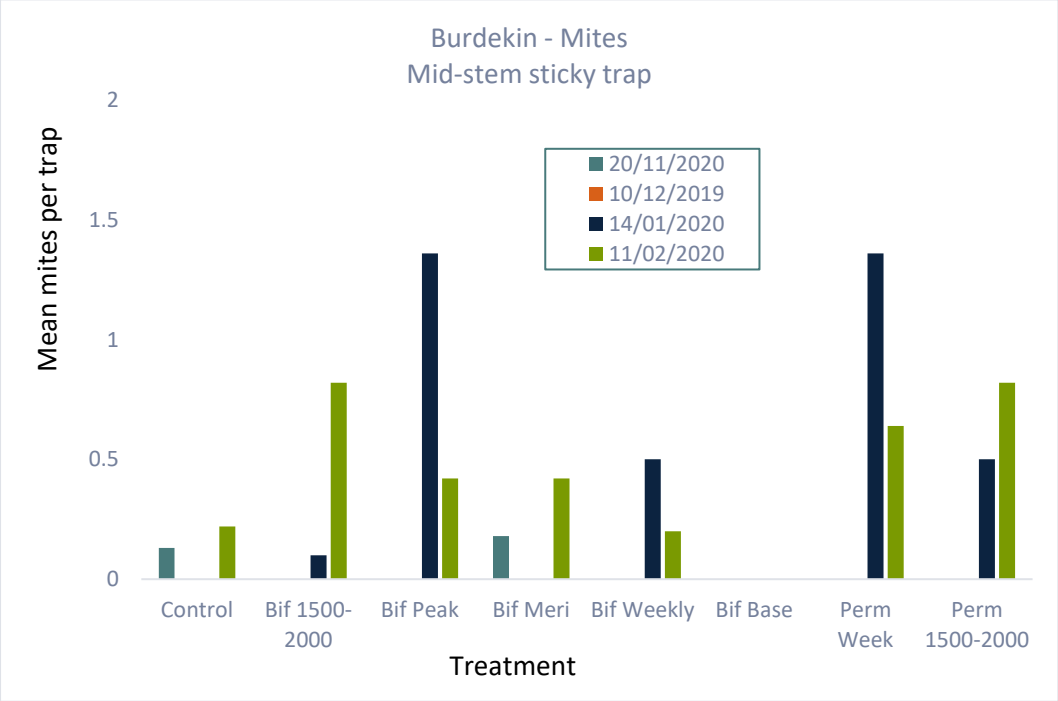


Figure 269 Mean mite abundance per yellow sticky mid-stem trap of third ratoon sugarcane in relation to insecticide application. Based on the mean of 12 sticky stem traps per treatment and control (UTC).

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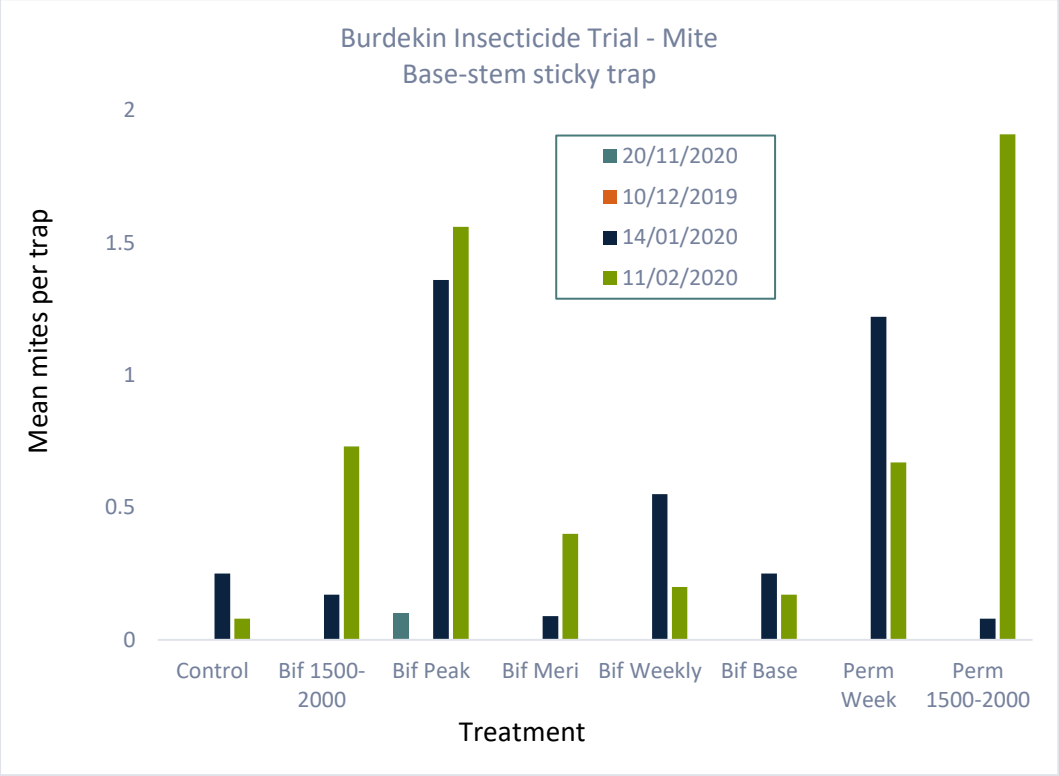

Figure 270 Mean mealybug abundance per yellow sticky base-stem trap of third ratoon sugarcane in relation to insecticide application. Based on the mean of 12 sticky stem traps per treatment and control (UTC).

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3. Thrips

Canopy - Yellow Sticky Traps

As also observed in the previous trial (in 2018-2019) multiple thrip species were detected in 2019-2020. In the 2018-2019 trial two peaks occurred with an early large peak in mid-November (70 thrips) and a smaller one in mid-January (25 thrips). In the 2019-2020 trial the peak abundance differed, with the larger peak being in in mid-January and the smaller peak in mid-November (Figure 271). In mid-January for all treatments, thrip abundance decreased and treatments applied in March (e.g., any @2000 °Cd or @peak treatments) would have had no effect as population abundance was extremely low due to natural decline.

More thrips were present in the field borders than in the sugarcane canopy and this has been observed at other sites as many of the thrip species appear to prefer grassy headlands. Rainfall events may also have reduced thrip population abundance in January, although generally thrip populations are low after January, regardless of weather conditions.

Thrip peak abundance occurred in late December and mid - January. As YCS expression peaked in March-April (Figure 260) it is unlikely that this insect is involved with YCS expression.

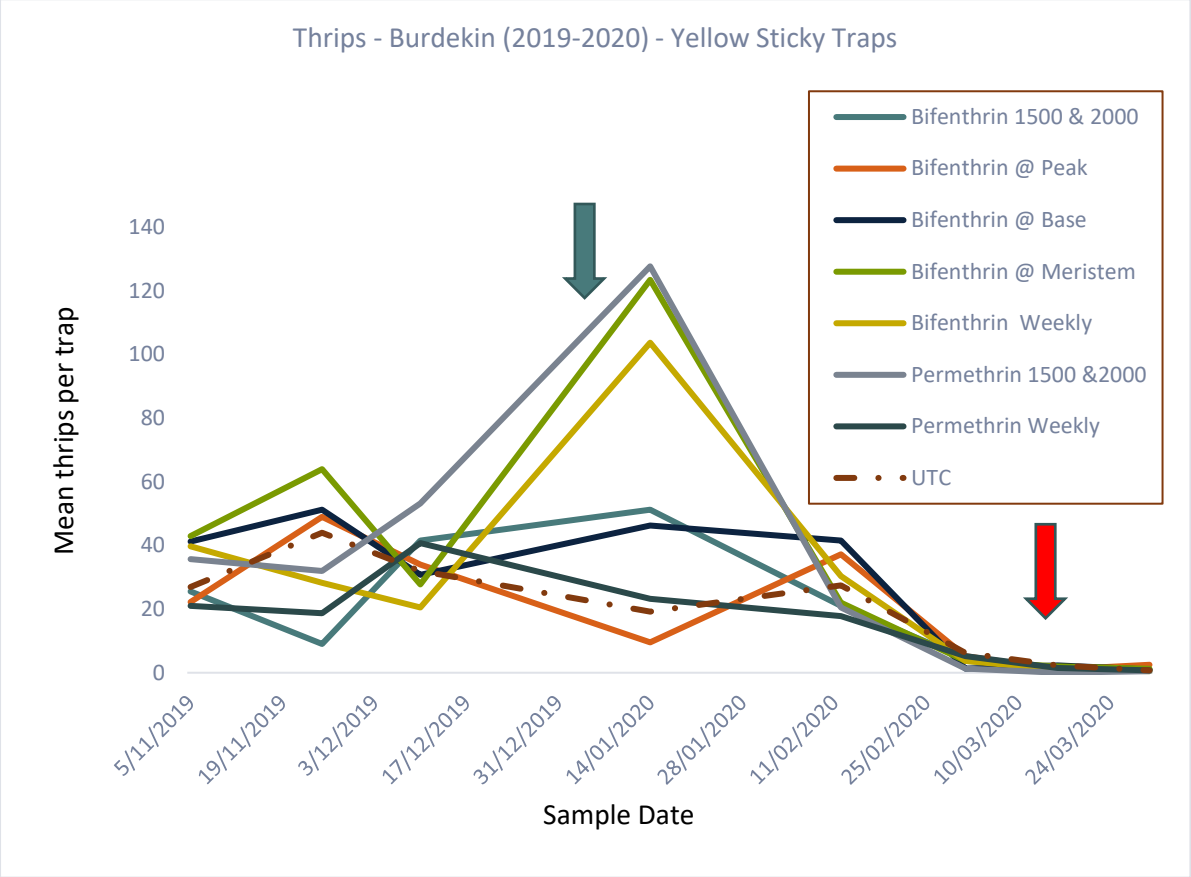


Figure 271 Mean thrip abundance per yellow sticky trap in sugarcane canopy of third ratoon sugarcane in relation to insecticide application. Based on the mean of four canopy sticky traps per treatment and control (UTC). The blue and red arrow respectively represent the 1500 day degree (°Cd) and 2000 °Cd application timings.

4. Leafhoppers

Canopy - Yellow Sticky Traps

As with the previous trial (in 2018-2019) using canopy sticky traps multiple leafhopper species were detected in 2019-2020 and one of the predominant species was *Myrmecophryne formiceticola* a root-feeding leafhopper. In the previous season (2018-2019) multiple peaks of abundance occurred in mid-December, late January and mid-March and abundance was relatively low (<3 per trap in UTC). In contrast in 2019-2020, there were two abundance peaks only (Figure 272) and abundance was higher one in late November (9 per trap in UTC) and one in mid-February (36 per trap in UTC). The weekly bifenthrin treatment appeared the most effective until mid-December and after that there was a resurgence in leafhopper abundance until a sharp decline post mid-January. Most treatments applied @ 1500 °Cd resulted in a sharp decline in leafhopper populations. The least effective treatments were bifenthrin @peak and bifenthrin @base. The most effective treatments overall were permethrin weekly and bifenthrin @1500-2000 °Cd. More leafhoppers were present in the sugarcane canopy than in the field borders (data not presented). Overall, the predominant seasonal difference in leafhoppers was a larger peak in February and higher abundance in 2019-2020.

Leafhopper peak abundance occurred between late December to late February, whilst YCS expression peaked in March-April (Figure 260) it is unlikely that this insect group is involved with YCS expression, unless a root-feeding leafhopper has migrated onto the root system and is therefore being under-represented in above ground trap catches.

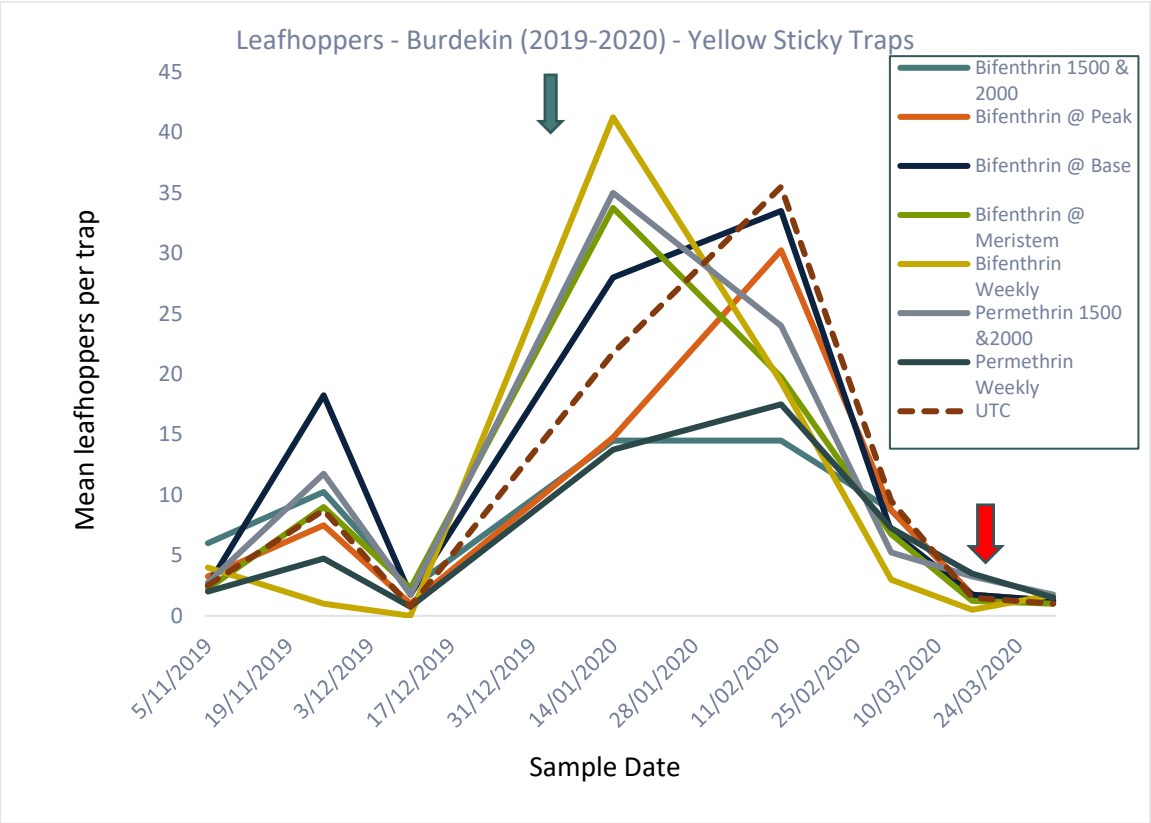


Figure 272 Mean leafhopper abundance per yellow sticky trap in sugarcane canopy of third ratoon sugarcane in relation to insecticide application. Based on the mean of four canopy sticky traps per treatment and control (UTC). The blue and red arrow respectively represent the 1500 day degree (°Cd) and 2000 °Cd application timings.

5. Linear bugs and planthoppers

Linear bugs and two planthopper species (*Perkensiella saccharicida* and *Lophops saccharicida)* were predominantly detected in yellow sticky traps during March but as they were usually <3 per trap the data is not presented.

6.9.4.3 Final yield

Machine harvest occurred on October 7th, 2020. Bifenthrin base treatment yield, although not significant (p=0.05), has a has a higher mean TCH than all other treatments (Figure 273 A). This does not correlate with individual stalk sampling and total culm volume measured on April 20th, 2020

which showed that bifenthrin weekly had the highest culm volume. This suggests that the bifenthrin base treatment had a larger number of stalks or tillers per plot than bifenthrin weekly. While the weekly spray also received application to the base of the plant, a more targeted and prolonged spray to this region may be responsible for maintaining stalk numbers. The field was ratooned on September 26th, 2019 and would have approximately 14 weeks of growth before the first application of bifenthrin to the bottom 40cm of the stool on January 11th, 2020. By this timepoint in an irrigated field in the Burdekin, the canopy should have already closed (Inman-Bamber, 1994). Stalk death occurs up to and after this point in time as smaller tillers compete for light (Inman-Bamber, 1994; Dias et al., 2019). Therefore, for the treatment to prevent stalk death it would have to remove a large population of soil borne crawling insects, thus allowing plant resource to be directed away from defence to growth. This would allow uniform stalk growth to reach the light at a similar time. Interestingly, Figure 263 shows uniform internode growth above internode +14 which is the point in time of the bifenthrin application (1500 °Cd). However, this is also true of the UTC and untreated Bifenthrin@Peak but without stalk numbers it is difficult to draw any firm conclusions. The weekly bifenthrin treatment with the largest individual culm volume (Figure 262 B) had spray treatment applied to the entire plant (including the base), yet has a smaller mean yield (TCH) than the base treatment (Figure 273 A). This suggests that the application of bifenthrin to the meristem and mid-region has possibly been detrimental to maintenance of the number of stalks per stool.

There is no significant variation in CCS between treatments and therefore sugar yield (TSH) is directly proportional to cane yield (TCH) (Figure 273 C & D).

Figures 6 A-C show there is no correlation between YCS severity and yield or CCS. This is consistent with previous research findings.

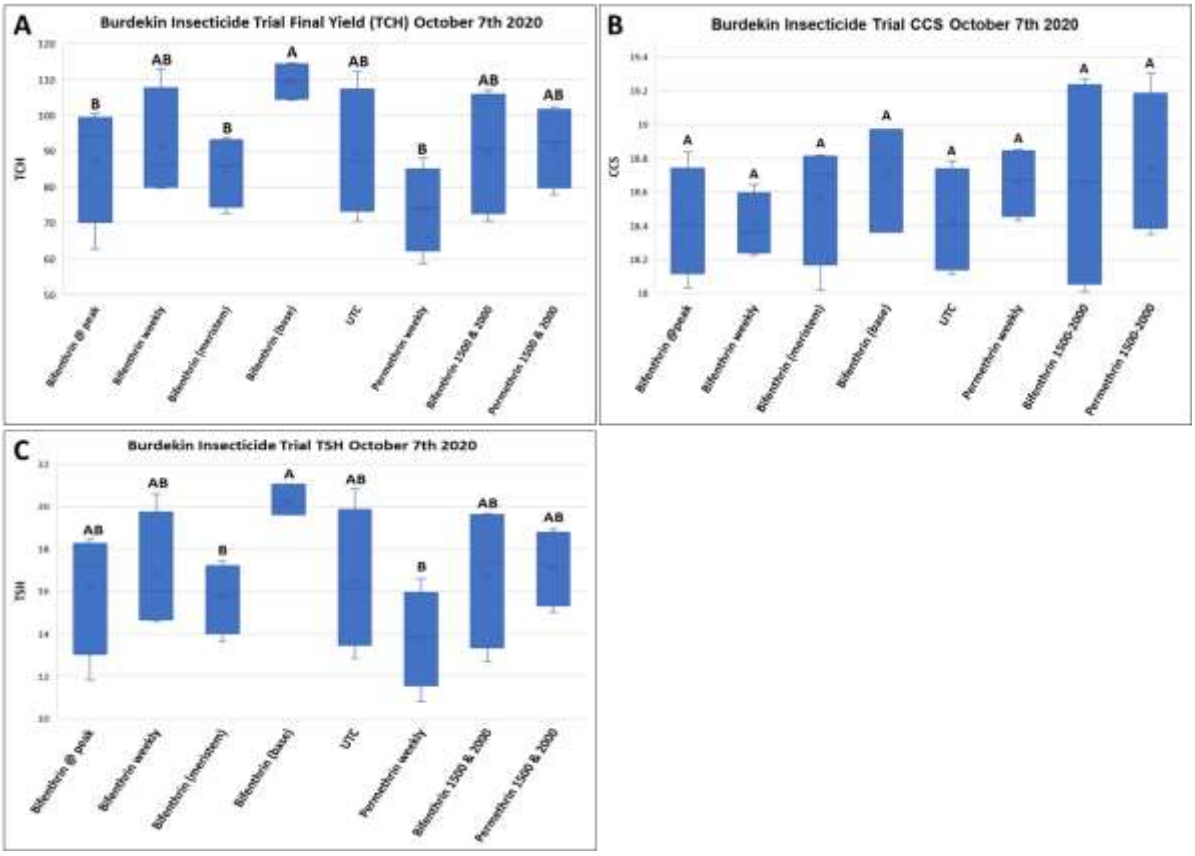


Figure 273 Burdekin insecticide trial tonnes cane per hectare (TCH) A), commercial cane sugar (CCS) B) and tonnes of sugar per hectare (TSH) C). Tukey HSD all-pairwise comparisons (p<0.05).

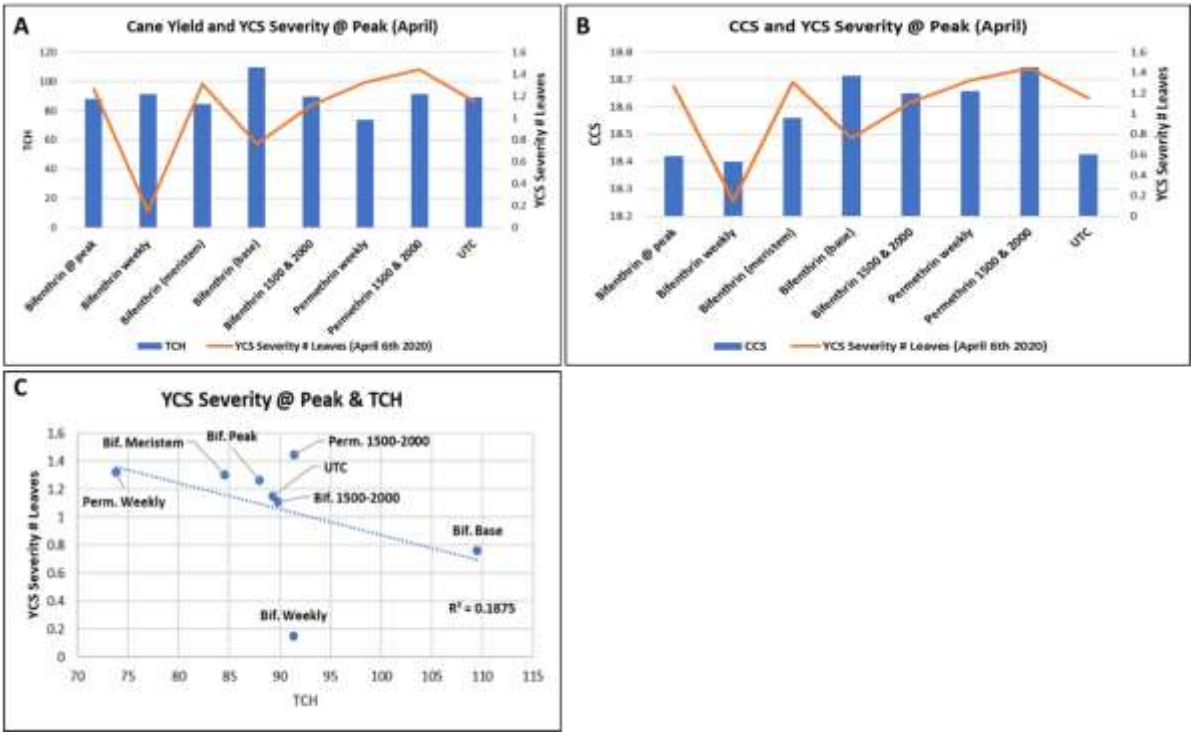


Figure 6 Burdekin insecticide trial YCS severity and yield TCH A), CCS B) and regression analysis C)

Summary

There was very little YCS development in the field trial across all treatments. Once again, the onset of yellowing due to YCS was evident after the first significant rainfall event following a very dry period. Application of bifenthrin on a weekly basis was the most effective of all treatments in improving internode growth or sink size, maintaining lower levels of leaf sucrose and α-glucans and preventing YCS development under experimental conditions. In contrast to this, permethrin was the least effective treatment to reduce YCS severity. Targeted sprays to the base and meristem region and at two well-timed points in the season seemed ineffective in maintaining healthy levels of leaf sucrose and glucan levels. There was no correlation between YCS severity and cane yield or CCS.

As YCS expression was lower than previous seasons, and rainfall is likely to have impact arthropod population abundance, it is difficult to form any definitive conclusion as to whether insect or mites are involved at this stage. However, some insects such as thrips are clearly not likely to be involved as they peak in abundance much too early to cause YCS expression. Other groups warrant further investigation including mites, mealybugs and leafhoppers as their peaks occur closer to those of YCS peak expression and they are generally impacted by weekly bifenthrin applications.

6.9.5 Insecticide variety trial (RVT) - Herbert (2018-2019)

In 2017 Herbert Cane Productivity Services Limited (HCPSL) established a sugarcane variety trial on a commercial farm in Ingham (Reinaudo 0127A). This trial is referred to as the Reinaudo variety trial (RVT) (see Appendix 1: 1.2.19). Commercial and non-commercial (experimental) varieties were utilised in this assessment. Two adjacent fields were established two months apart with one field planted on June 28th (early plant) and the other on 30th August (late plant) 2017. Access to these fields was gained for YCS research. The efficacy of the insecticide bifenthrin in preventing YCS development and symptom expression and the effect, if any, on yield and CCS was investigated in a comparative varietal assessment. The early plant field was used as the untreated control and the late plant field was treated weekly with bifenthrin using an inter-row boom spray (see Appendix 1: 1.2.20). Leaf +4 punches were taken monthly and assayed for sucrose and α-glucan content.

Commercial varieties assessed were SRA3[A], Q250[A], Q200[A], Q232[A], Q240[A], Q242[A], KQ228[A], Q208[A] and experimental varieties consisted of QN05-237, QN05-1380, QN07-496, QS06-7991, QN08-2274, QA05-2486, QC05-1281, QN08-488. During the course of this study variety QA05-2486 was released for commercial production in 2019 and is now classified as WSRA24[A].

Results

6.9.5.1 YCS monitoring and physiology

There was very little difference in YCS symptoms between bifenthrin weekly and untreated controls. The highest expression of YCS occurred in variety Q250[ф] in early April with an average of 1.8 (data not shown) affected leaves across the monitored stalks which is considered very low. There was unprecedented rainfall in the Herbert region from late January causing severe flooding which may also have impacted results. Leaf +4 sucrose and glucan levels concur with monitoring results with no unhealthy levels of accumulation noted between September 2018 and January 2019 (data not shown). However, in February a small number of commercial varieties showed increased levels of both metabolites. Of these varieties only Q250[ф] had leaf sucrose levels exceeding the upper tolerable threshold (Figure 274). However, by April leaf sucrose levels had subsided to a healthy status in Q250[ф] but risen above the tolerable threshold in Q232[ф]. Mean sucrose levels of all experimental varieties remained low throughout the trial (Figure 275). Interestingly, when the mean sucrose and glucan levels are considered for each of the commercial and experimental genotypes cohorts there is a similar pattern within each varietal group. However, it is clear that the bifenthrin treatment has maintained lower levels of both metabolites in both the cohorts. There was very little difference in YCS symptoms between bifenthrin weekly and untreated controls. The highest expression of YCS occurred in variety Q250[ф] in early April with an average of 1.8 (data not shown) affected leaves across the monitored stalks which is considered very low. There was unprecedented rainfall in the Herbert region from late January causing severe flooding which may also have impacted results. Leaf +4 sucrose and glucan levels concur with monitoring results with no unhealthy levels of accumulation noted between September 2018 and January 2019 (data not shown). However, in February a small number of commercial varieties showed increased levels of both metabolites. Of these varieties only Q250[ф] had leaf sucrose levels exceeding the upper tolerable threshold (Figure 274). However, by April leaf sucrose levels had subsided to a healthy status in Q250[ф] but risen above the tolerable threshold in Q232[ф]. Mean sucrose levels of all experimental varieties remained low throughout the trial (Figure 275). Interestingly, when the mean sucrose and

glucan levels are considered for each of the commercial and experimental genotypes cohorts there is a similar pattern within each varietal group (Figure 276). It is noteworthy that the bifenthrin treatment has maintained lower levels of both metabolites in both cohorts. However, leaf sucrose levels are well below the tolerable upper threshold. Experimental variety leaf +4 sucrose was the only metabolite that was not significantly different between treated and untreated.

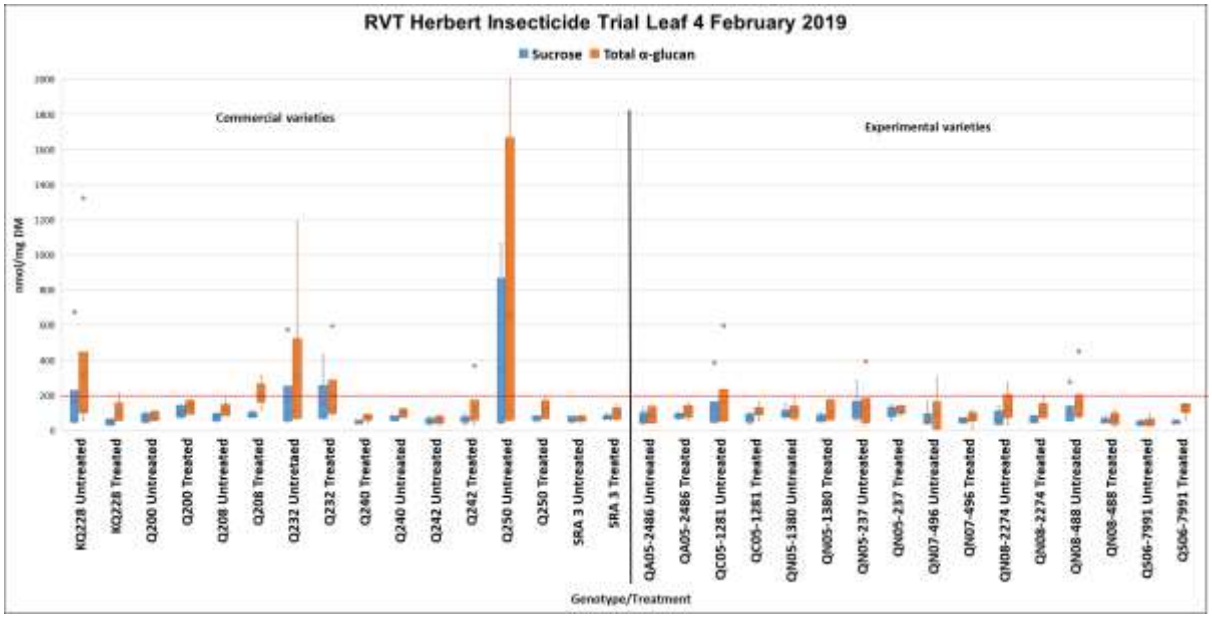


Figure 274 Insecticide variety trial commercial and experimental genotypes bifenthrin treated and untreated leaf +4 sucrose and total α-glucan content February 2019

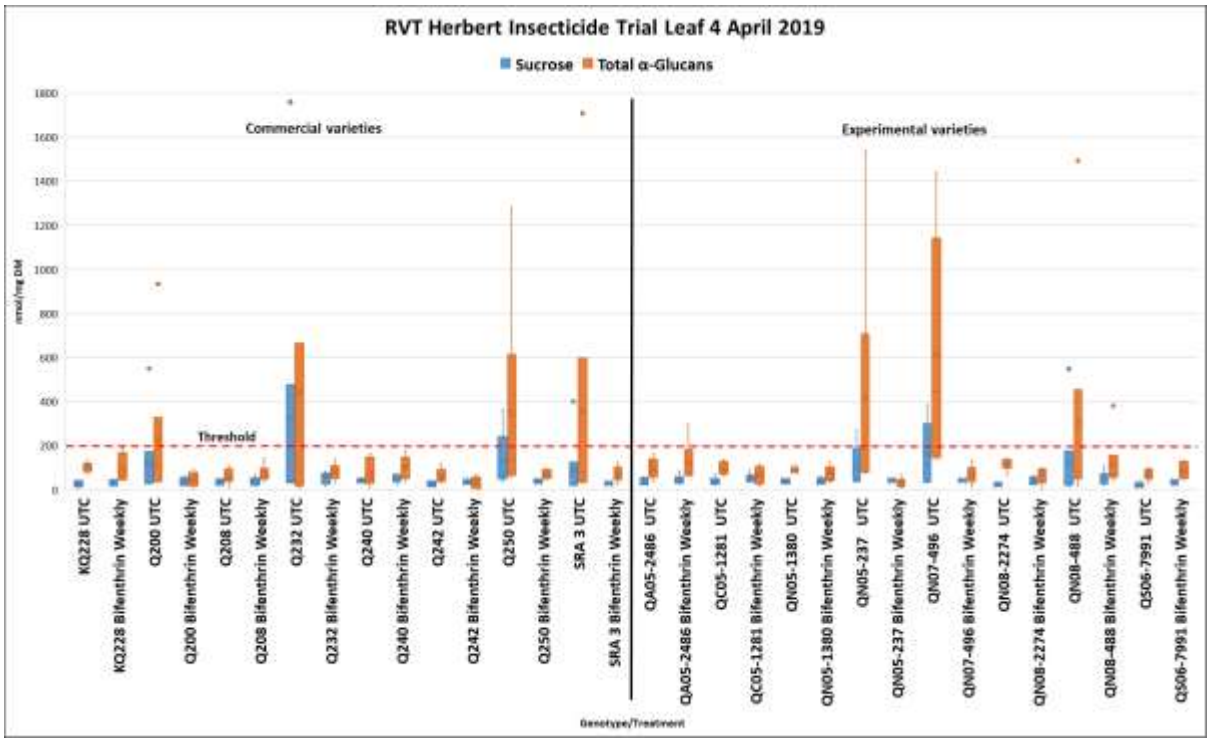


Figure 275 Insecticide variety trial commercial and experimental genotypes bifenthrin treated and untreated leaf +4 sucrose and total α-glucan content April 2019

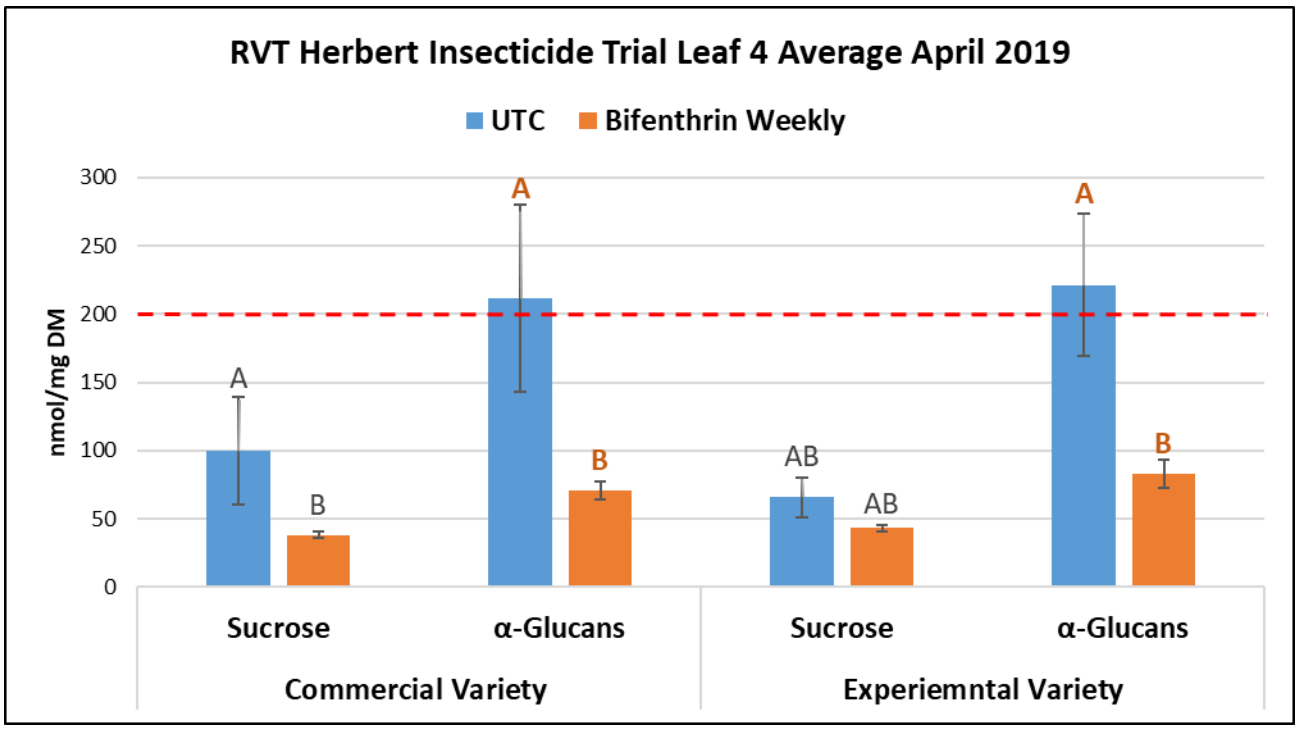


Figure 276 Insecticide variety trial bifenthrin treated and untreated leaf +4 mean sucrose and total α-glucan content of grouped commercial and experimental varieties April 2019. Analysis of variance by completely randomised design (p<0.05) with LSD all pairwise comparison shown by letter separations A, B, AB (compare each metabolite separately).

6.9.5.2 Final Yield

Yield results in the untreated control field show a range across both the commercial and experimental varieties to be approximately 60-120 TCH (Figure 277). All varieties showed some signs of leaf yellowing during the months of February to April but analysis between YCS severity scoring and cane yield shows there is no correlation (Figure 278).

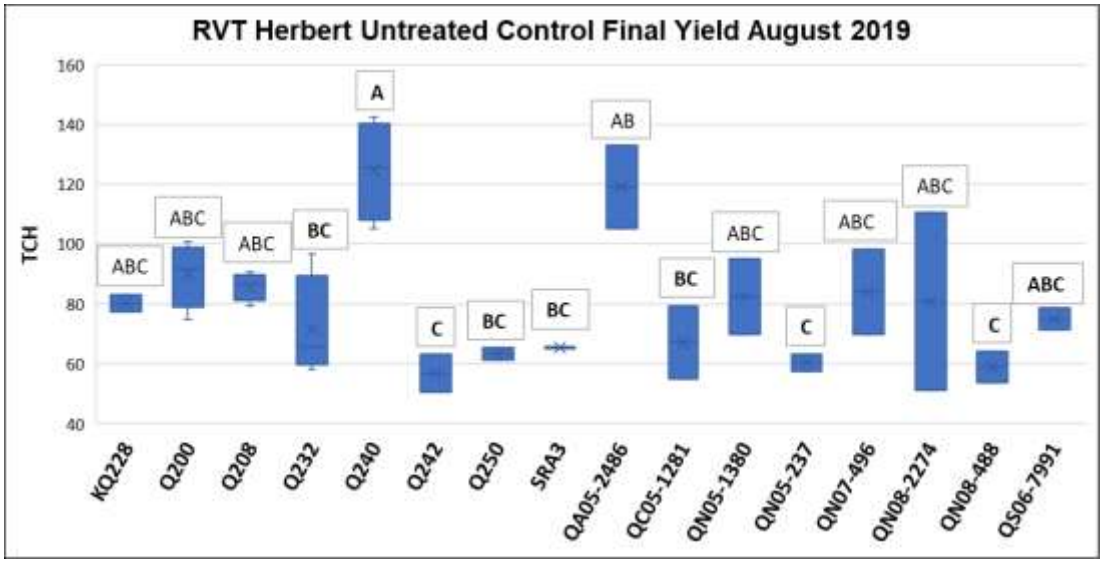


Figure 277 Mean culm mass (±SE) of a hand harvested 18.2m^2 subplot of cane in July 2019. LSD All-Pairwise Comparisons (p<0.1).

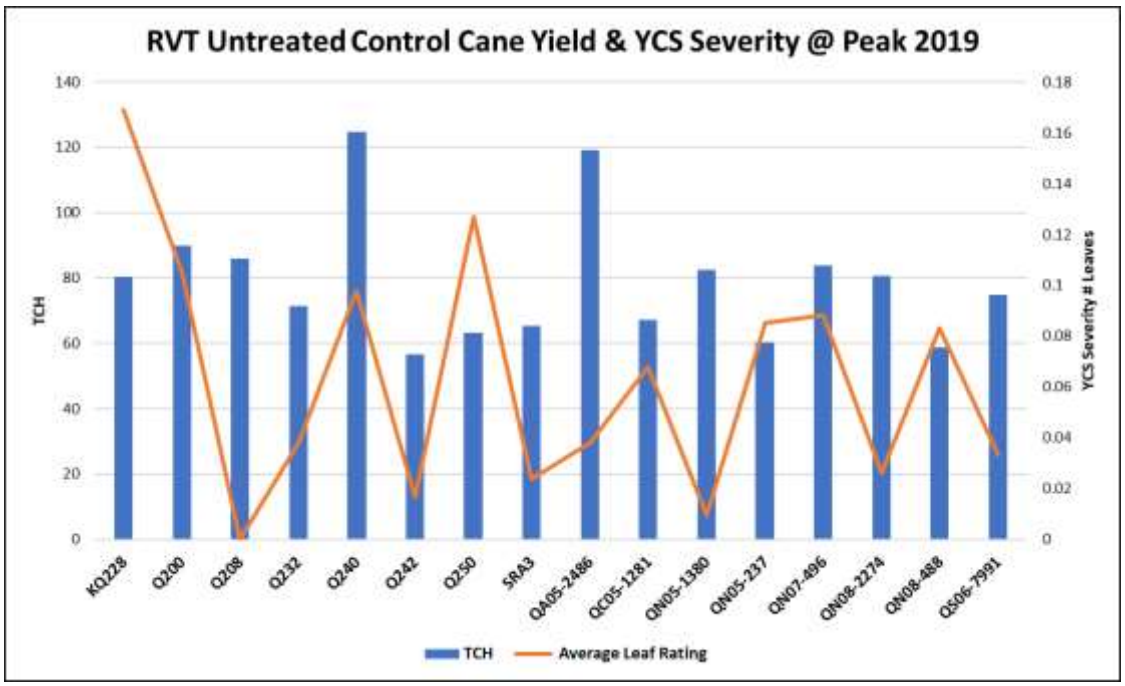


Figure 278 RVT 2019 insecticide untreated control yield and YCS severity rating

Weekly application of bifenthrin was effective in preventing unhealthy levels of leaf sucrose and α-glucan accumulation. Interestingly, there was a 2-fold variation between the lowest and highest cane yield (50-100 TCH) across the Bifenthrin treated field (Figure 279) which is the same ratio in the untreated control (60-120 TCH). No correlation exists between yield and YCS severity scoring in the insecticide treated field (Figure 280).

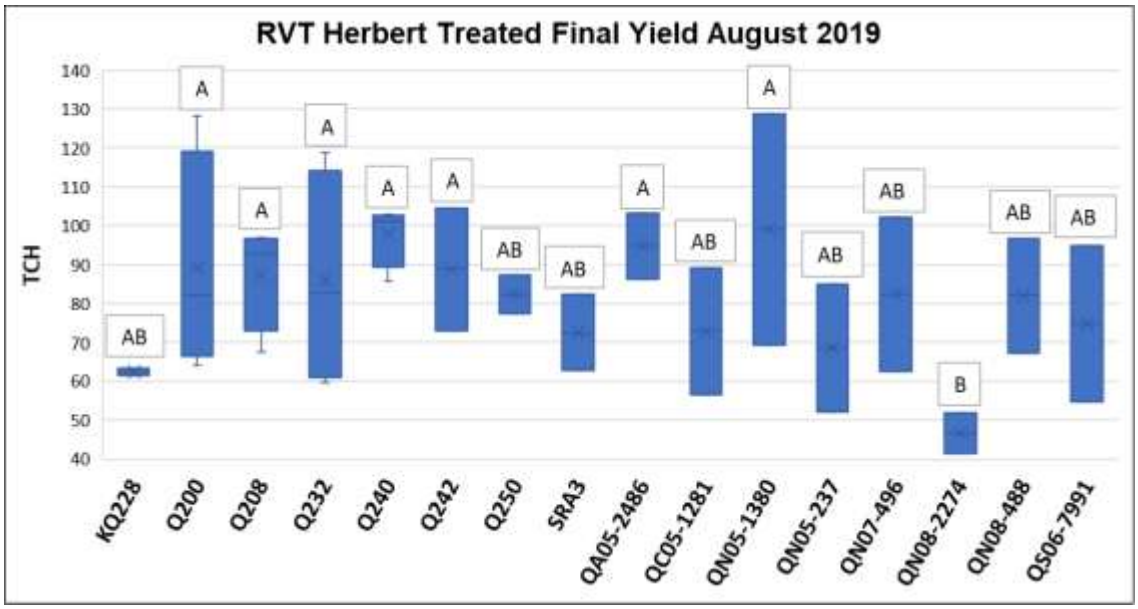


Figure 279 Mean stalk mass (±SE) of a hand harvested 18.2m^2 subplot of cane in July 2019. LSD All-Pairwise Comparisons (p<0.1).

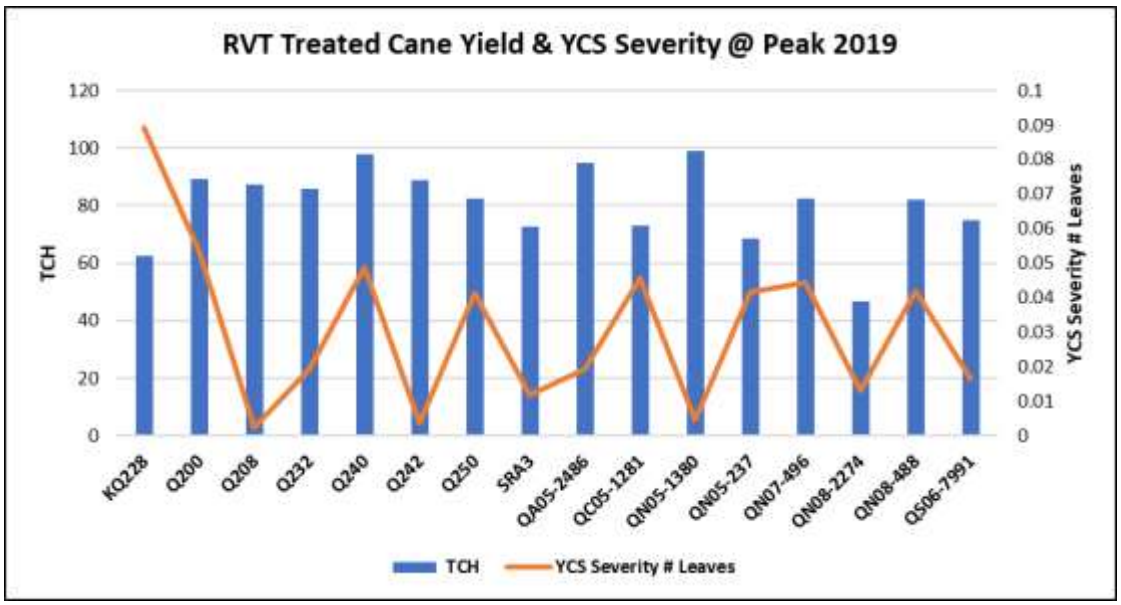


Figure 280 RVT 2019 insecticide treated yield and YCS severity rating

Figure 281-Figure 283 show variation in yield (TCH & TSH) and CCS across the 16 varieties in the RVT trial between bifenthrin treated and untreated controls. It should be noted that as there was close to no visible symptom expression, it is unlikely that the yield variation is due to YCS.

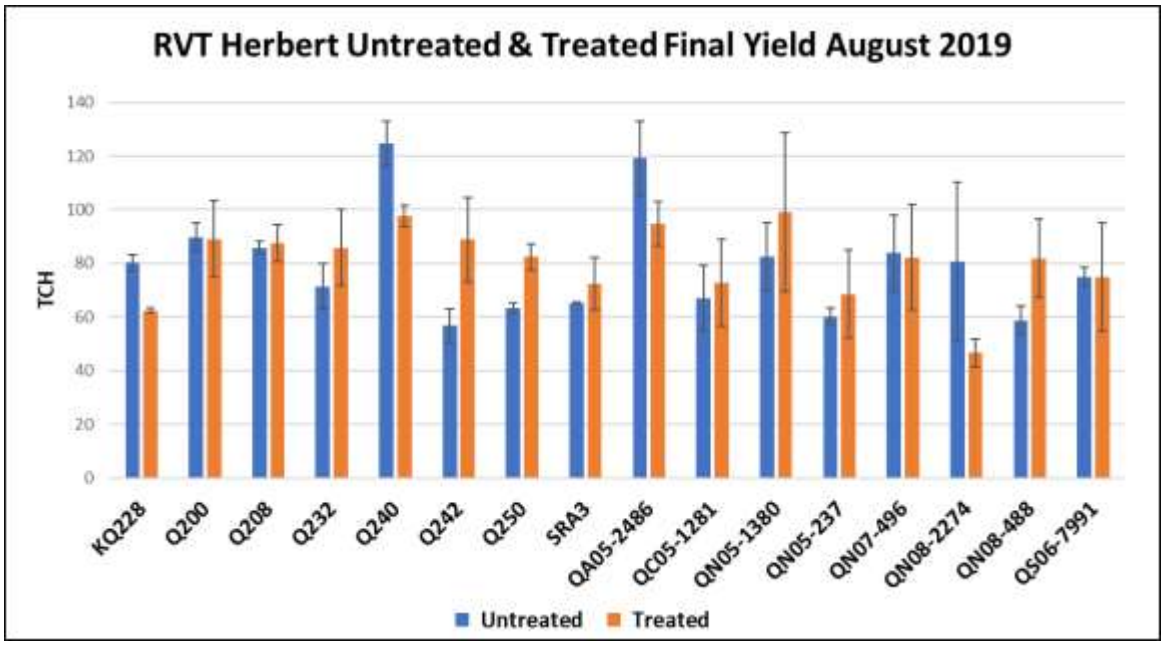


Figure 281 RVT 2019 Bifenthrin Treated and Untreated Control yield (TCH)

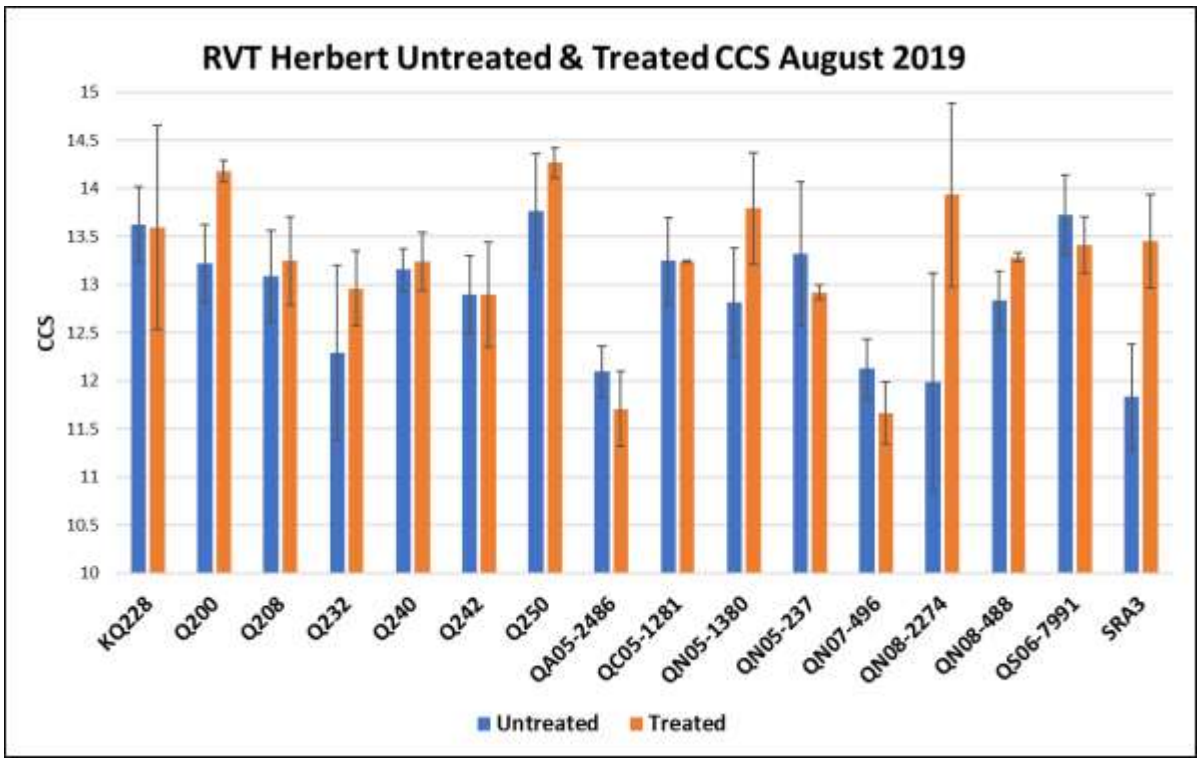


Figure 282 RVT 2019 Bifenthrin Treated and Untreated Control CCS.

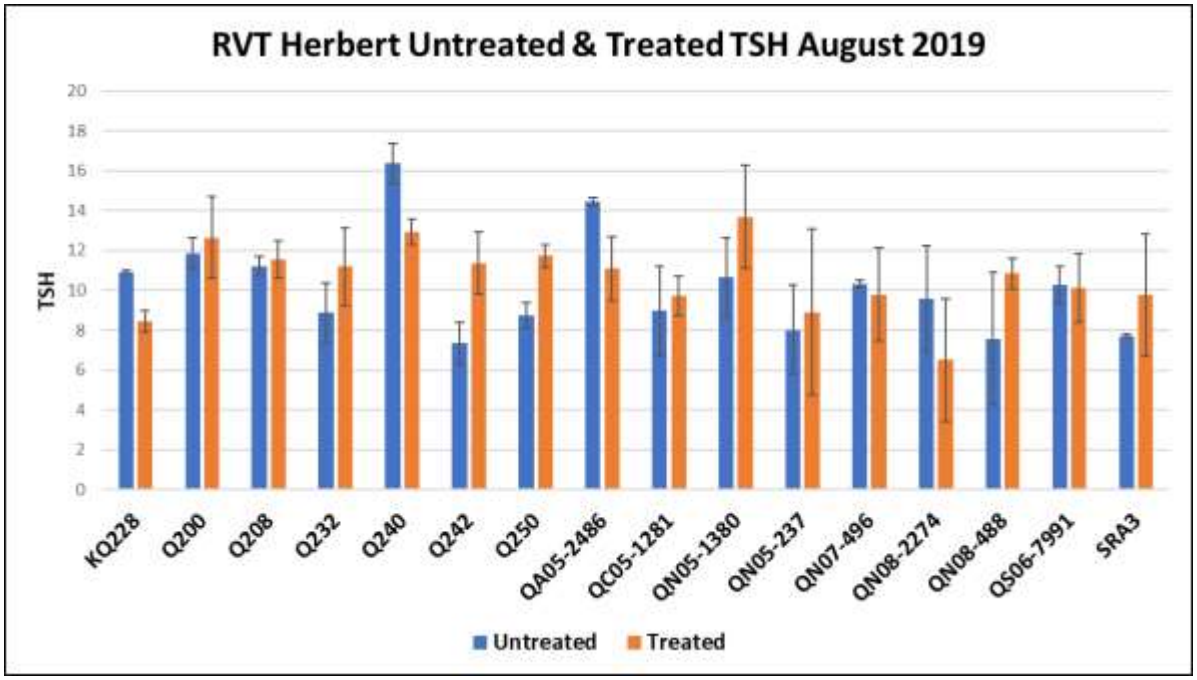


Figure 283 RVT 2019 Bifenthrin Treated and Untreated Control tonnes sugar per hectare (TSH).

Summary

Leaf +4 metabolite levels remained low in both the commercial and experimental varieties with only Q250ϕ and Q232ϕ accumulating unhealthy levels of sucrose but not for any sustained period of time. It could be argued that these two varieties are at higher risk of developing YCS, but given that each episode was short-lived, and no yield penalty was reported, firm conclusions cannot be made. In fact, harvest results indicate no difference in yield (TCH, TSH) or CCS between the insecticide treated plots and the untreated control. Commercial varieties have a more uniform CCS than the near commercial varieties while there is little difference in yield TCH & TSH. YCS severity rating through visual assessment of leaf yellowing, does not correlate with cane yield.

6.9.6 Insecticide variety trial (RVT) - Herbert (2019-2020)

The 2018/19 RVT was repeated in the 2R crop of 2019/20. Once again the efficacy of bifenthrin weekly treatment to mitigate YCS and any potential impact on cane and sugar yield was investigated. This is the third year for the RVT trial which is comprised of 9 commercial (variety QA05-2486 was released in 2019 and is now classified as WSRA24ϕ) and 7 experimental varieties. All treatments, monitoring and sampling were kept the same as in 2018/19 (see Appendix 1: 1.2.20).

Results

6.9.6.1 YCS monitoring and physiology

YCS monitoring between October 2019 and March 2020 did not report any YCS development in either treatment. Metabolite analyses of leaf +4 leaf punches collected monthly, concur with monitoring observations for each varietal cohort (Figure 284A-F). Leaf +4 mean sucrose levels remained below the upper tolerable threshold through the 2019/2020 season. There was a trend for maintenance of lower leaf +4 sucrose content in the bifenthrin treatment than the untreated control.

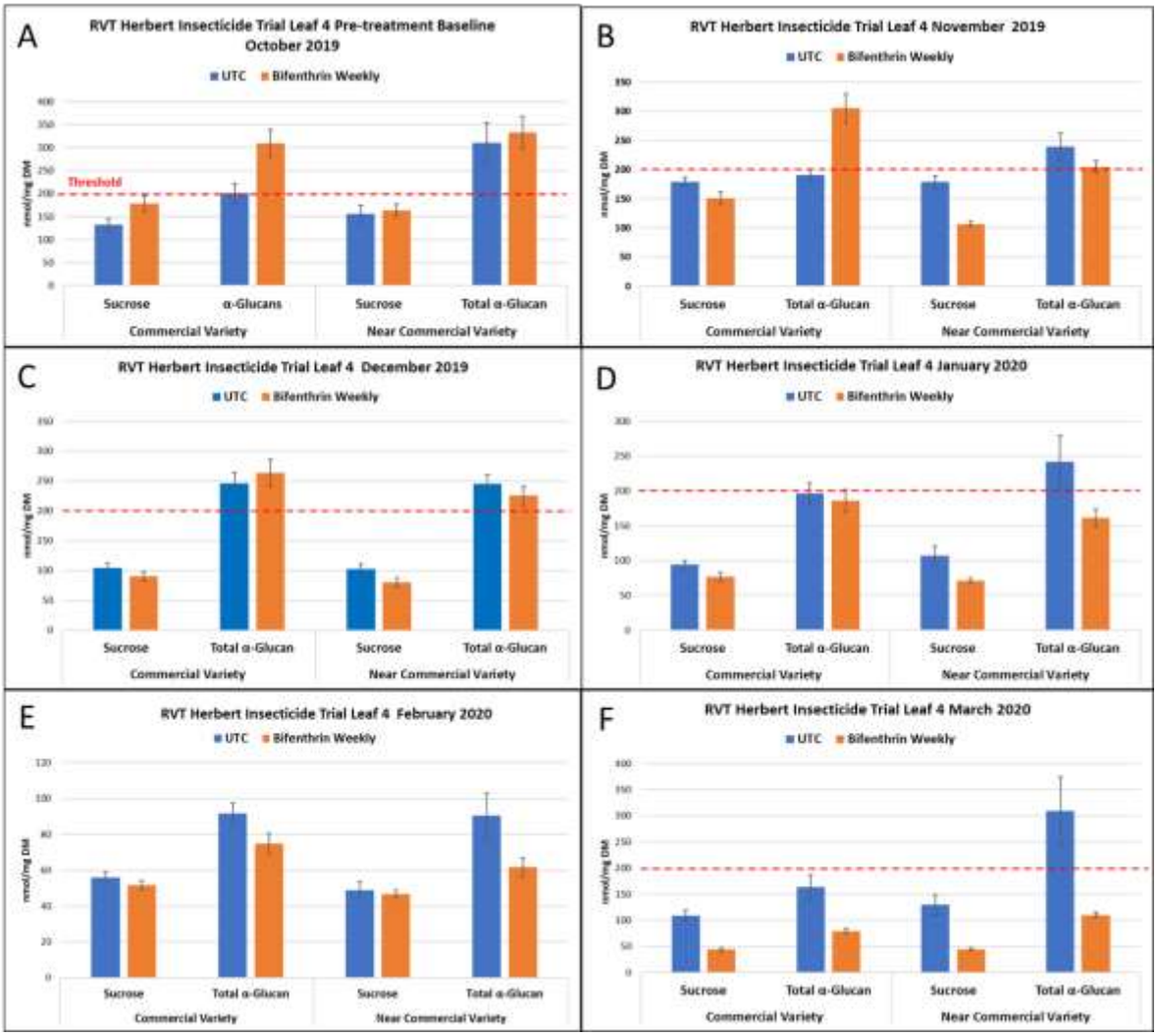


Figure 284 Insecticide variety trial bifenthrin treated and untreated leaf +4 mean sucrose and total α-glucan content of grouped commercial and experimental (near commercial) varieties October 2019 to March 2020 (A-E). (note: October samples had not received any bifenthrin treatment – baseline only)

A detailed review of leaf +4 sucrose content recorded in February and March, which is typically when highest YCS severity is recorded, shows no accumulation breaching the upper tolerance threshold for any variety within either the commercial or experimental groups (Figure 285 A & B). This result is very similar to the that of the 2018/19 trial (see section 6.9.5 of this report).

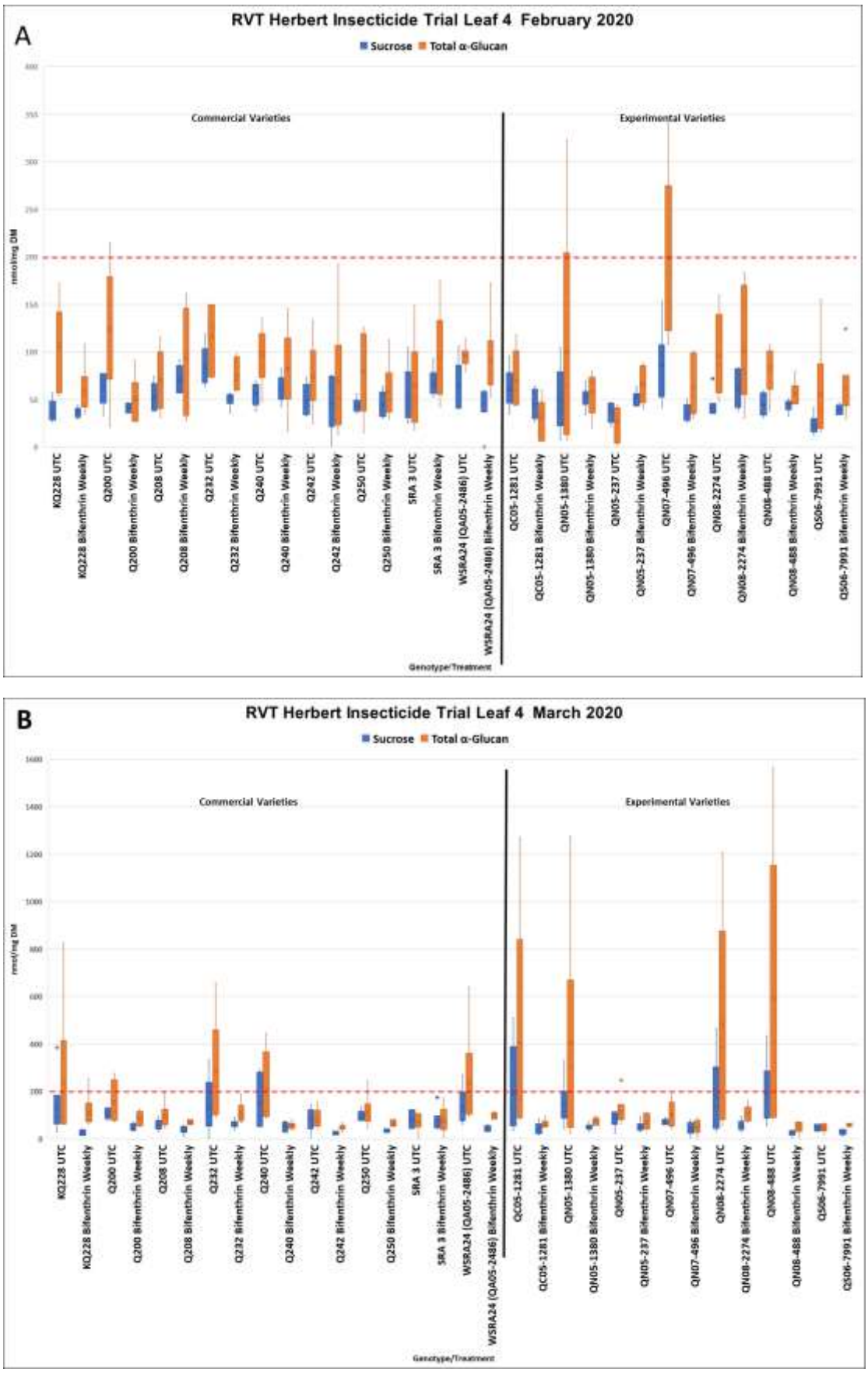


Figure 285 Insecticide variety trial commercial and experimental genotypes bifenthrin treated and untreated leaf +4 sucrose and total α-glucan content February A) March B) 2020

The RVT site is totally reliant on rainfall for water input and during the very dry period from August to December 2019 (Figure 286) there is notable partitioning of carbon to total α-glucans (Figure 287 A-D). This is a typical response of plants under stress (Utrillas and Alegre, 1997; Gupta and Kaur, 2005). These plants had little to no culm growth during the 4 months to December 2019 and plants were extremely water stressed and in very poor health. However, after good rainfall received in December and January leaf sucrose and glucan levels began to fall (Figure 287 C & D). This scenario is similar to that reported in the crop age trial where the November treatment was slashed back and did not develop YCS symptoms until the culm had attained approximately 100cm of cane (see section 6.5.7). The close proximity of a large root sink and drawing down of the glucan pool for growth after the water stress period, is likely responsible for reduced levels of sucrose and glucans in the source leaf. Continued rainfall through February and March (Figure 286) would have prevented a slowdown in internode growth producing a large culm sink. Under these conditions the risk of supply exceeding demand would be minimal, hence no source leaf sucrose accumulation or YCS expression reported in the field (Figure 285 A & B).

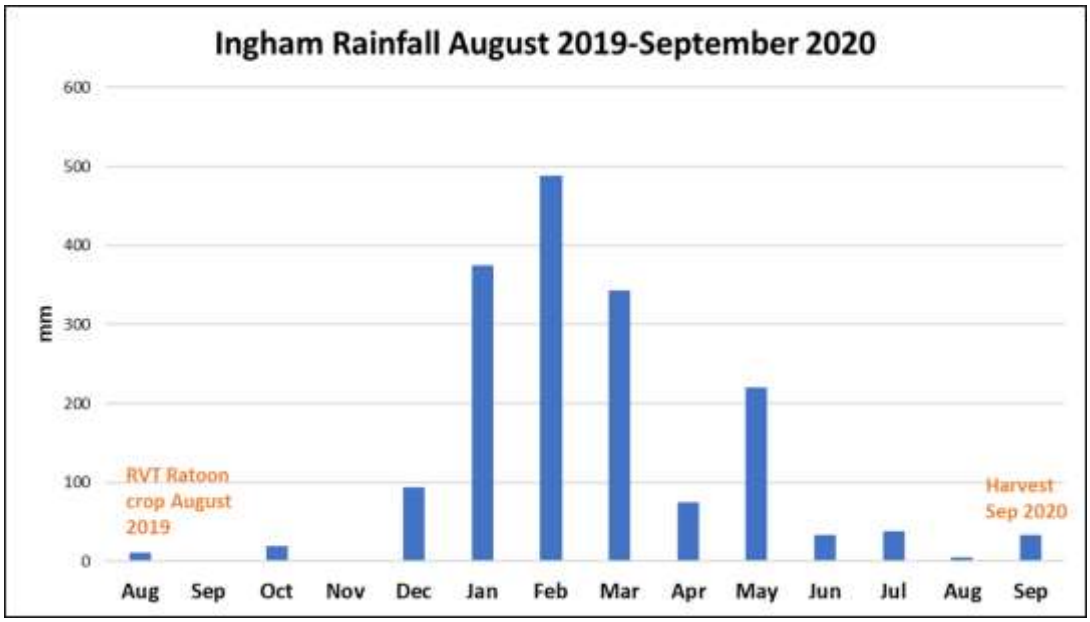
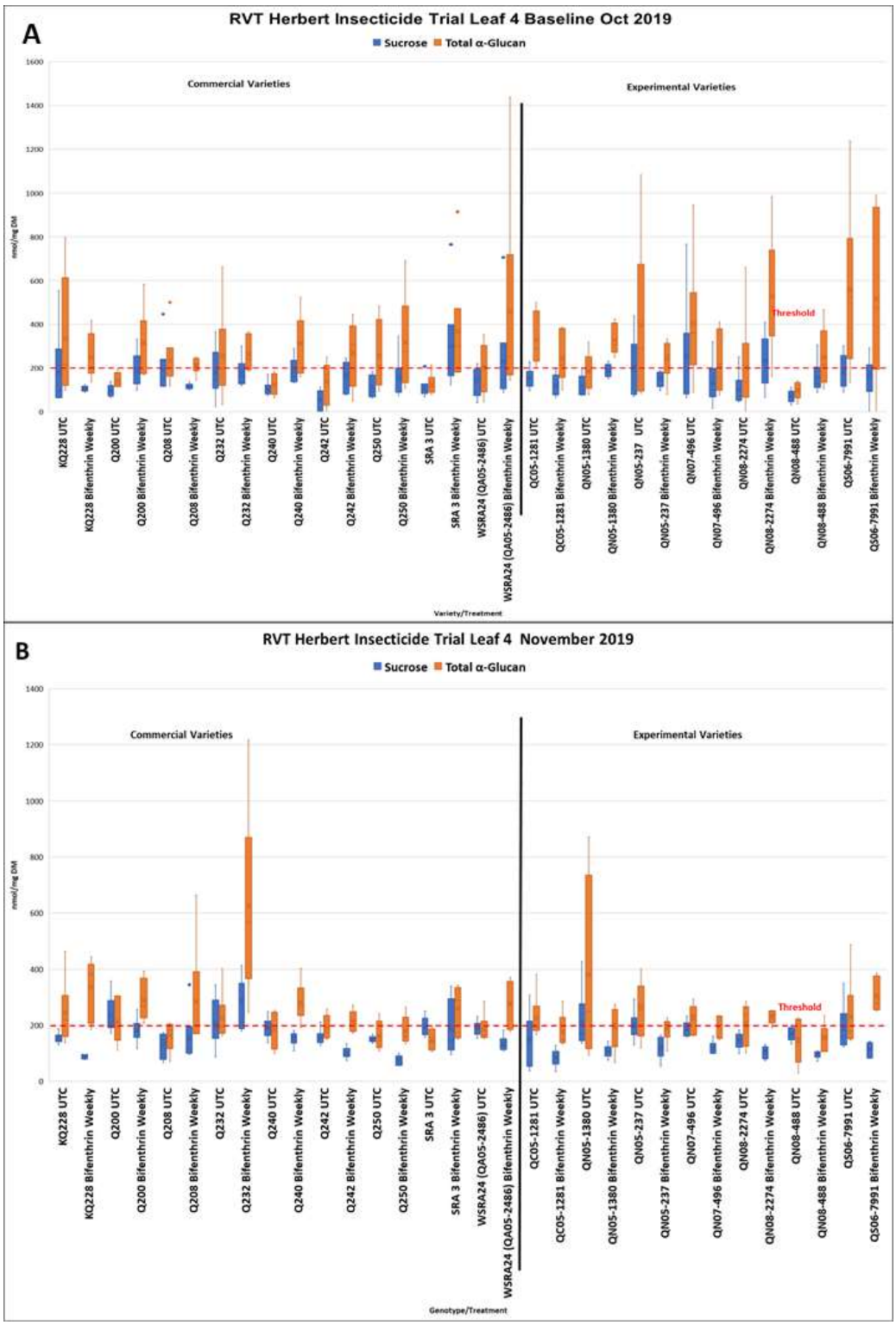


Figure 286 Ingham rainfall August 2019 to September 2020


A
RVT Herbert Insecticide Trial Leaf 4 Baseline Oct 2019
Sucrose
Total α-Glucan
Commercial Varieties
Experimental Varieties
nmol/mg DM
Threshold
KQ228 UTC
KQ228 Bifenthrin Weekly
Q200 UTC
Q200 Bifenthrin Weekly
Q208 UTC
Q208 Bifenthrin Weekly
Q232 UTC
Q232 Bifenthrin Weekly
Q240 UTC
Q240 Bifenthrin Weekly
Q242 UTC
Q242 Bifenthrin Weekly
Q250 UTC
Q250 Bifenthrin Weekly
SRA 3 UTC
SRA 3 Bifenthrin Weekly
WSRA24 (QA05-2486) UTC
WSRA24 (QA05-2486) Bifenthrin Weekly
QC05-1281 UTC
QC05-1281 Bifenthrin Weekly
QN05-1380 UTC
QN05-1380 Bifenthrin Weekly
QN05-237 UTC
QN05-237 Bifenthrin Weekly
QN07-496 UTC
QN07-496 Bifenthrin Weekly
QN08-2274 UTC
QN08-2274 Bifenthrin Weekly
QN08-488 UTC
QN08-488 Bifenthrin Weekly
QS06-7991 UTC
QS06-7991 Bifenthrin Weekly
Variety/Treatment
B
RVT Herbert Insecticide Trial Leaf 4 November 2019
Sucrose
Total α-Glucan
Commercial Varieties
Experimental Varieties
nmol/mg DM
Threshold
Genotype/Treatment

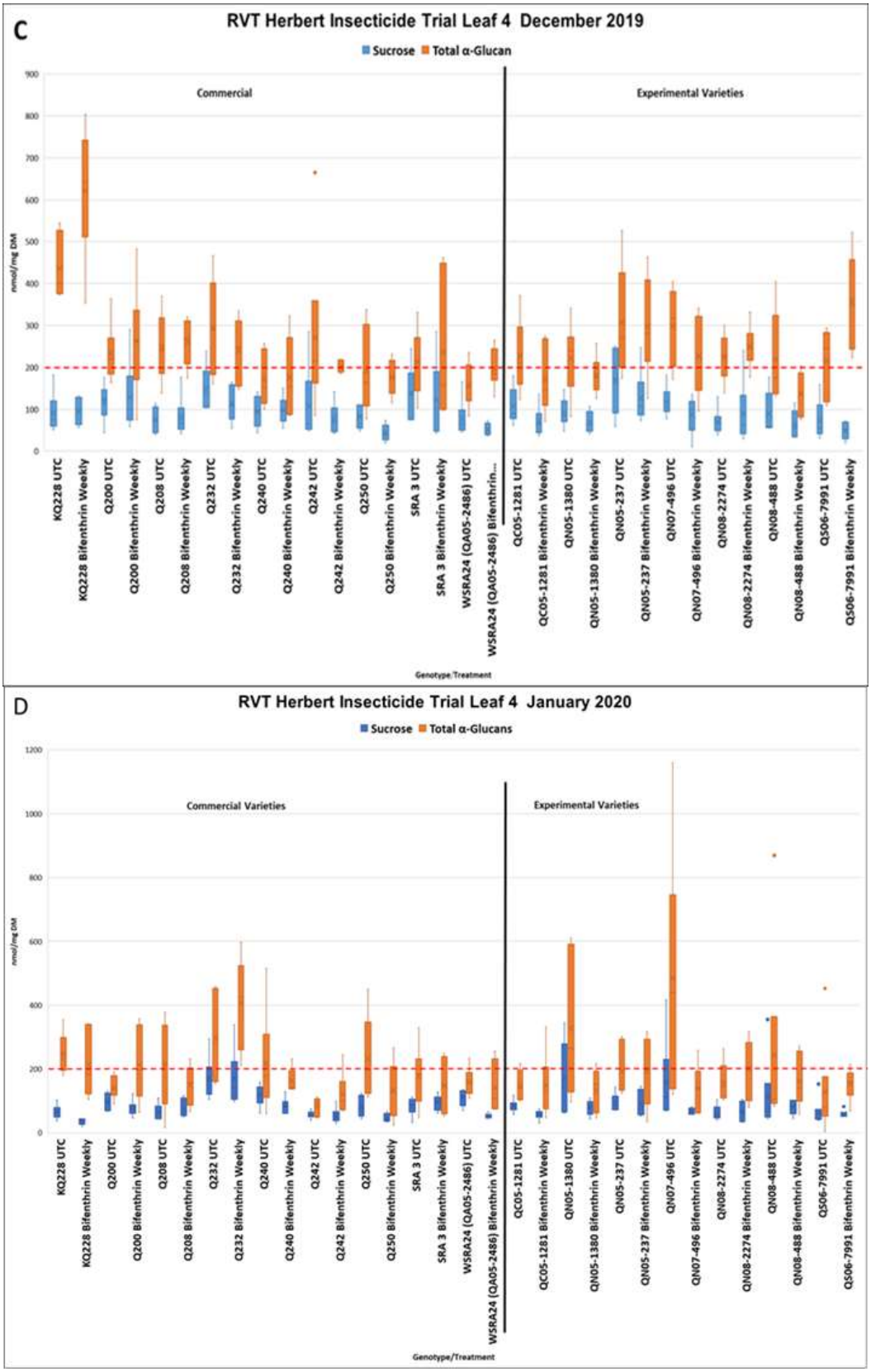


Figure 287 Insecticide variety trial commercial and experimental genotypes bifenthrin treated and untreated leaf +4 sucrose and total α-glucan content October A) November B) December C) and January D) 2020

6.9.6.1 Entomology

In the Ingham RVT Variety trial eight commercially available sugarcane varieties were monitored using yellow sticky traps on a weekly basis. Bifenthrin was also applied on a weekly basis until mid-February 2020 to treated plots. To examine general insecticide (rather than varietal trends data has been pooled for all varieties.

1. Mites

A variety of mites were detected in sticky traps, but abundance was higher (maximum of 35 per UTC trap) than in the Ingham insecticide strip trial (maximum of 8 per UTC trap). In contrast to the Ingham insecticide strip trials two, rather than three, major abundance peaks were observed with the largest peak in late January and a smaller peak in mid-March in the untreated controls. The bifenthrin treatments effectively reduced mite populations compared to the untreated control in both peak periods (Figure 288).

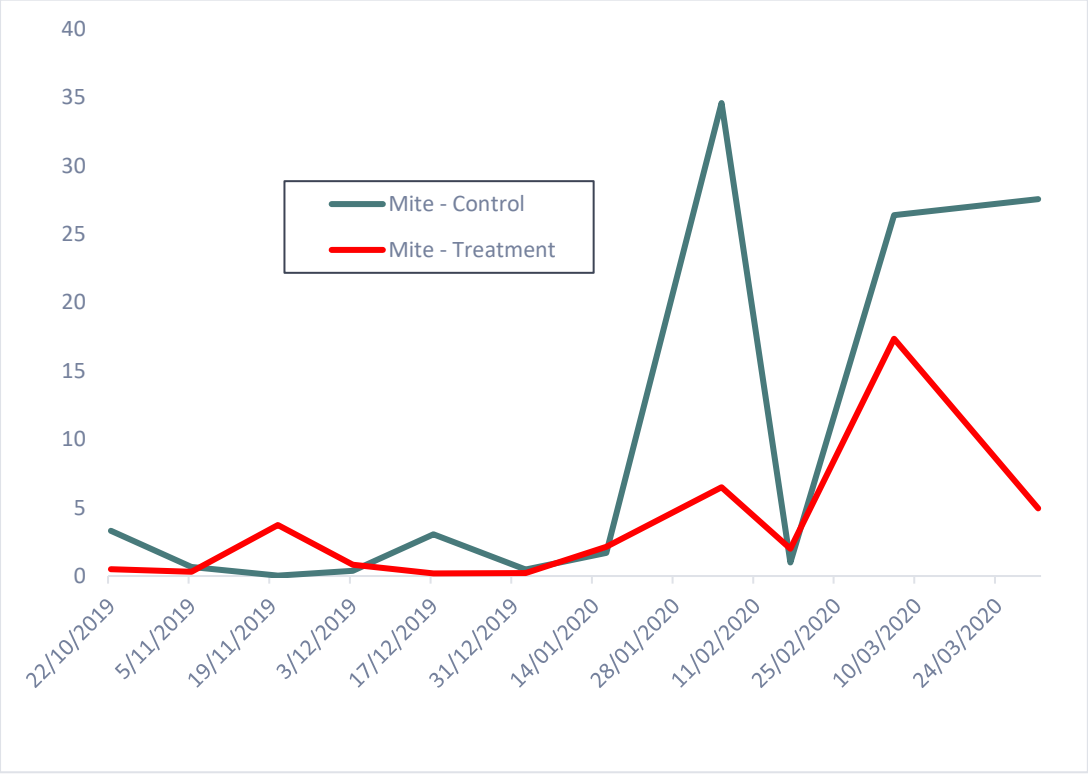


Figure 288 Mean mite abundance per yellow sticky trap in sugarcane canopy of plant cane in relation to insecticide application. Based on the mean of 16 canopy sticky traps per treatment and control (UTC). The blue and red arrow respectively represent the 1500 °Cd and 2000 °Cd application timings.

2. Mealybugs

The predominant mealybug at this field site was *Saccharicoccus sacchari* and population abundance was relatively low (Figure 289) in the control plots (especially compared to the Ingham insecticide strip trial. The last bifenthrin application was on 19th February and mealybug populations appeared to resurge in March in the bifenthrin-treated cane varieties. Th maximal peak is likely to have been late March, but as trapping was discontinued in mid-March this is a speculative assumption. In contrast to the insecticide strip trial data the bifenthrin applications in this variety trial appeared to have little impact on mealybugs. However, as data was pooled from eight sugarcane varieties further analysis needs to be conducted to determine if any varieties are more tolerant to mealybugs. The rainfall event of 250 mm between 26th – 31st January could also have reduced the treatment efficacy and delayed emergence of mealybugs from the root system.

Figure 289 Mean mealybug abundance per yellow sticky trap in sugarcane canopy of plant cane in relation to insecticide application. Based on the mean of 16 canopy sticky traps per treatment and control (UTC). The blue and red arrow respectively represent the 1500 °Cd and 2000 °Cd application timings.

3. Leafhoppers

There was likely a major peak in leafhopper abundance in early October, but sampling only commenced in late October (Figure 290). Leafhopper populations were relatively stable from Dec-March but at low levels (around 2 per trap). Leafhopper populations were supressed throughout the season by bifenthrin applications.

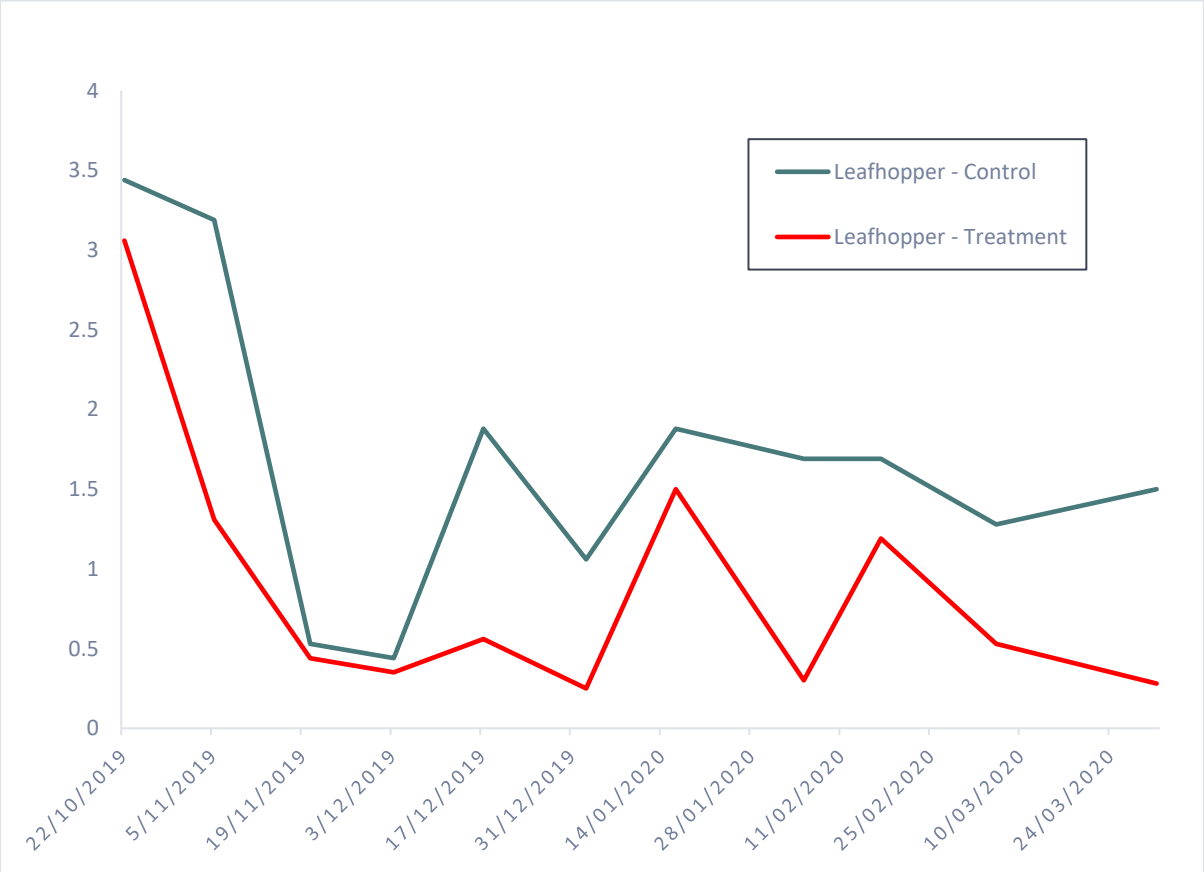


Figure 290 Mean leafhopper abundance per yellow sticky trap in sugarcane canopy of plant cane in relation to insecticide application. Based on the mean of 16 canopy sticky traps per treatment and control (UTC). The blue and red arrow respectively represent the 1500 day degree (°Cd) and 2000 °Cd application timings.

4. Thrips

A variety of thrips were detected in sticky traps and abundance was relatively high (Maximum of 710 in UTC Trap). A population peak is likely to have occurred in early November and populations gradually decline to negligible levels by late January (Figure 291). Populations in the cane treated with bifenthrin were reduced from November through to the end of December.

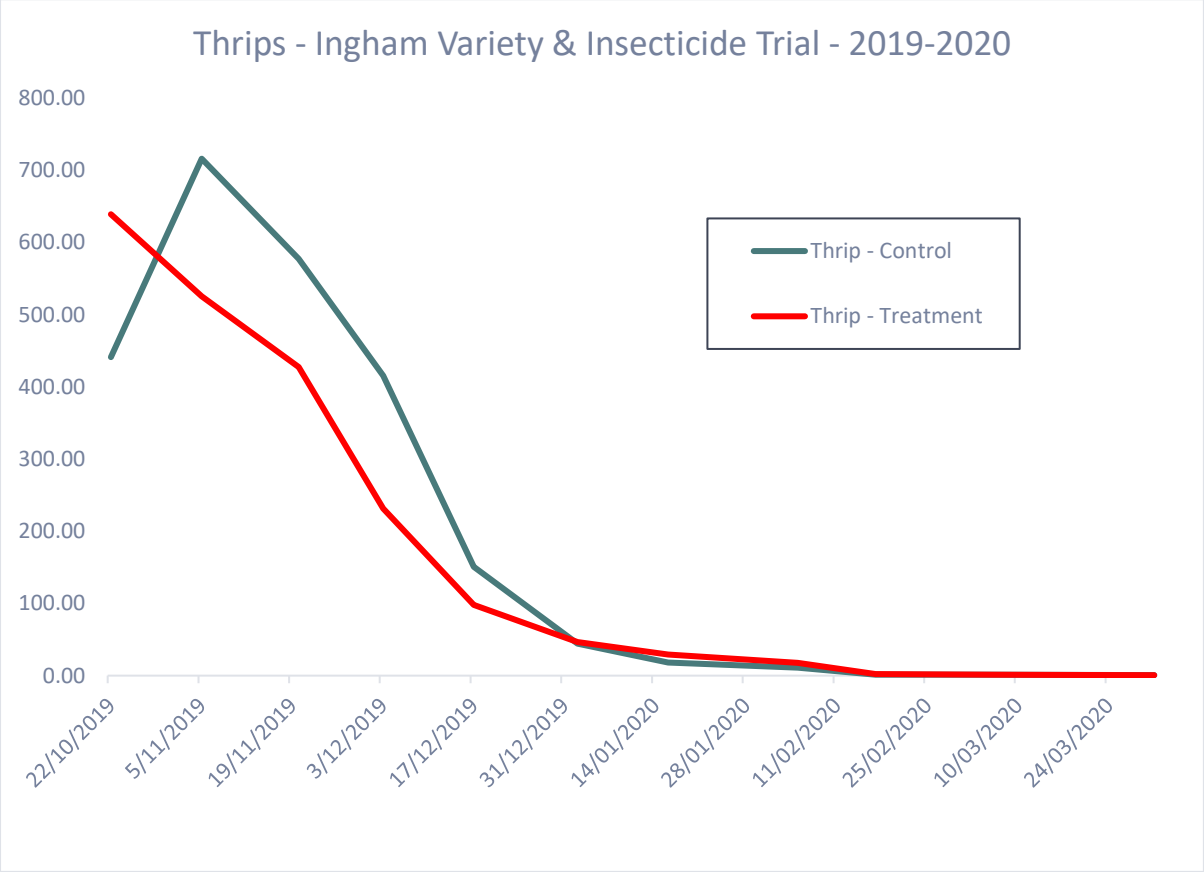


Figure 291 Mean thrip abundance per yellow sticky trap in sugarcane canopy of plant cane in relation to insecticide application. Based on the mean of 16 canopy sticky traps per treatment and control (UTC). The blue and red arrow respectively represent the 1500 °Cd and 2000 °Cd application timings.

6.9.6.1 Final yield

Commercial varieties (KQ228$^{\Phi}$, Q200$^{\Phi}$, Q242$^{\Phi}$ and Q250$^{\Phi}$) treated with bifenthrin weekly have a higher mean TCH yield than UTC; four varieties (Q208$^{\Phi}$, Q232$^{\Phi}$, Q240$^{\Phi}$ and SRA3$^{\Phi}$) show no yield difference between treated and UTC and WSRA24 has a smaller yield than the treated counterpart. In the experimental varieties two varieties show no yield difference between treatments, four have increased yield when treated, and one variety has higher yield in the UTC (Figure 292). Many of the untreated plots had rat damage (

Table 62) and no estimate of yield loss has been accounted for in these plots.

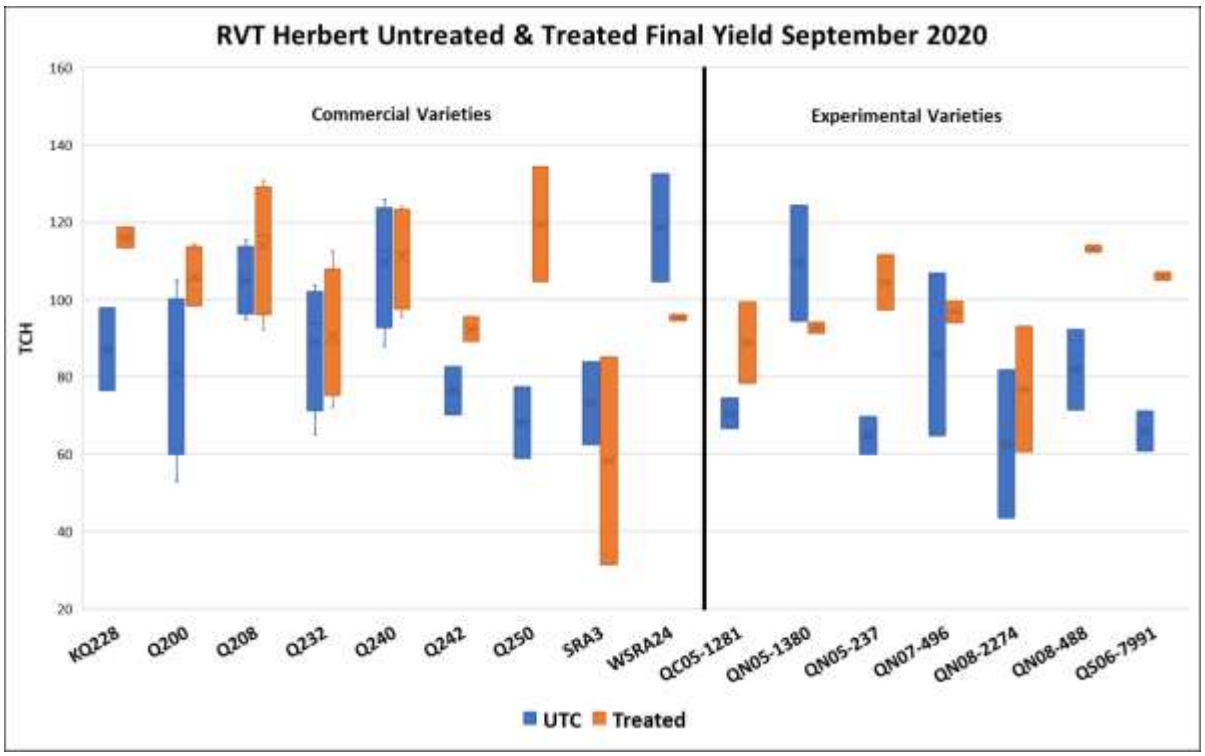


Figure 292 RVT commercial and experimental varieties cane yield tonnes cane/hectare (TCH) bifenthrin weekly treated and untreated controls

Table 62 Rat damage and smut in the following treatments and plots

Variety	Treated	UTC
Q200		Rat Damage 10%
Q208		Rat Damage 50%
Q240		Rat Damage 30%
Q250		Rat Damage 30%
QC05-1281		Rat Damage 80%
QN08-2274		Rat Damage 60%
QN08-488		Rat Damage 50%
QS06-7991		Rat Damage 60%
SRA3	SRA 3 Smut affected in treated	2m Gap Smut 4

While the UTC leaf +4 sucrose content does not exceed the upper tolerable threshold, it is significantly higher than the bifenthrin weekly treated plants (Figure 284 F). This suggests the bifenthrin treated plants have grown a larger sink and are better able to accommodate sucrose exported from the source leaves. The mean yield (TCH) across the commercial and near commercial varieties shows a similar trend, with bifenthrin treated plants having a higher biomass than the UTC (Figure 293). This suggests that leaf +4 sucrose content is a good measure of plant health or plant vigour.

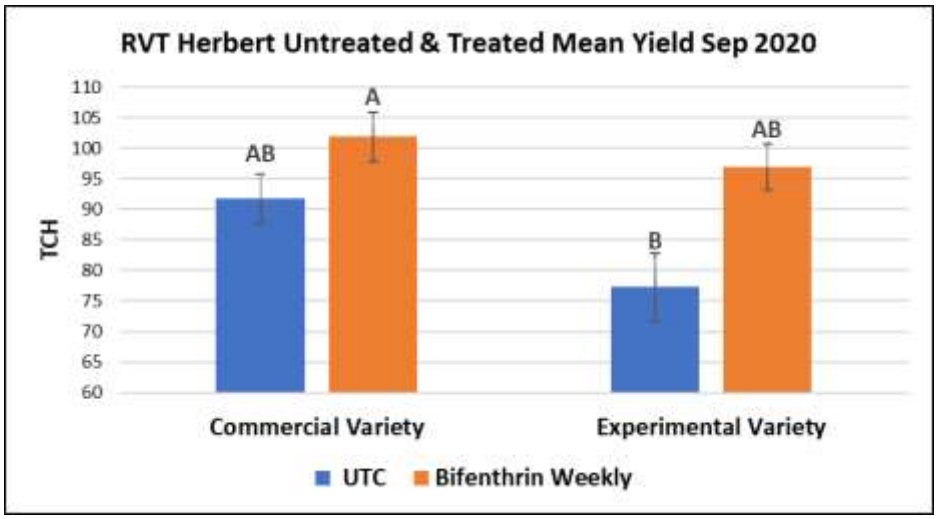


Figure 293 RVT mean commercial and experimental varieties cane yield tonnes cane/hectare (TCH) for each cohort, bifenthrin weekly treated and untreated controls

The only commercial varieties of the nine investigated in the RVT trial to show any variation in specific internode volumes (p,0.05) between treatments are Q200 (Int 9 & 10) and SRA 3 (Int 5) (Figure 294 A & B). This data concurs with leaf metabolite data measured between October and March (Figure 284 A-F) and the lack of any visible YCS symptoms throughout the season. It also suggests the crop maintained a source sink balance during the peak growth period from December to March which correlates with consistent rainfall, favourable weather conditions and good growth during this period.

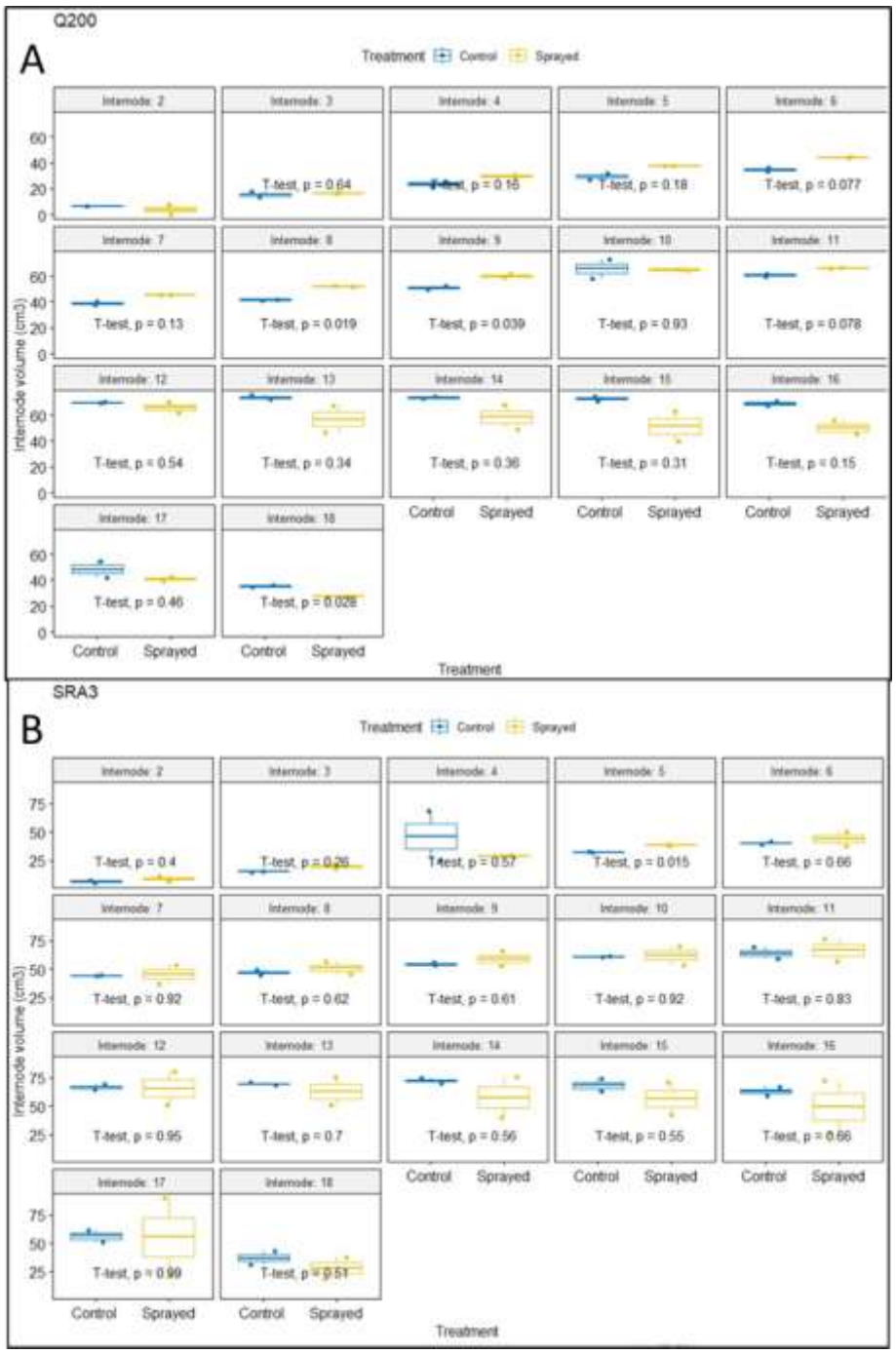


Figure 294 RVT internode volume +2 to +18 variety Q200$^{\Phi}$ A) and SRA3$^{\Phi}$ B)

The variety trial is typical of variation seen in TCH, CCS and TSH between genotypes (Figure 507 A-C).

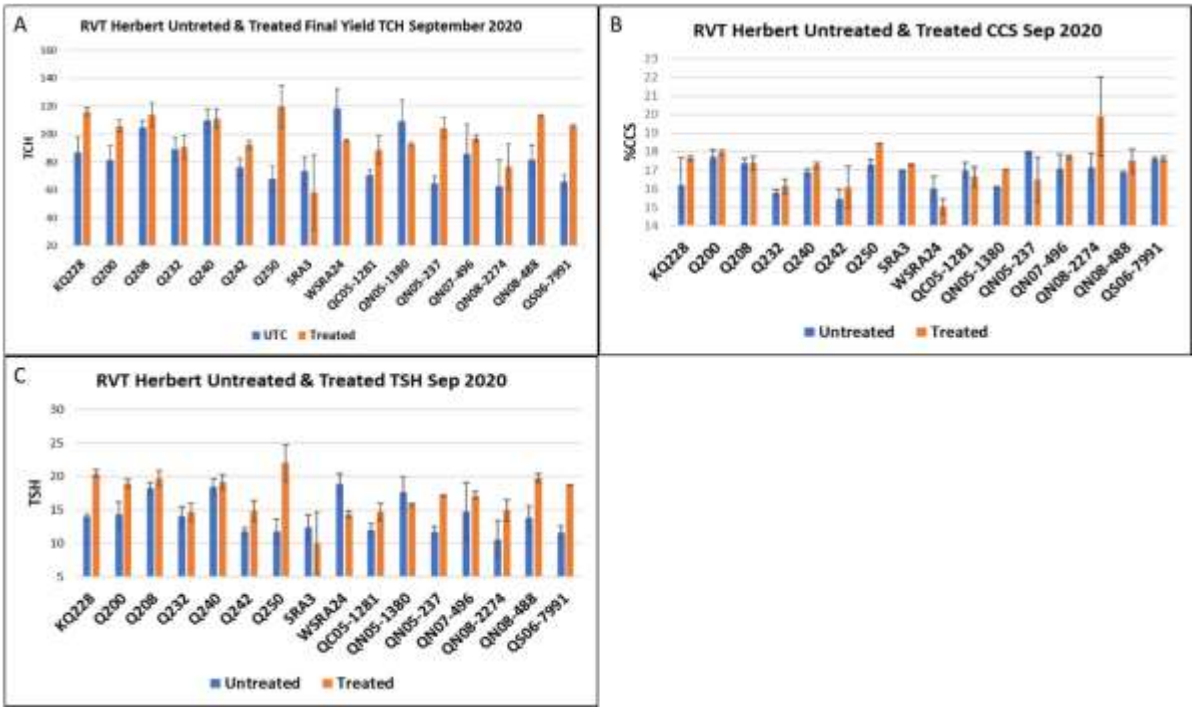


Figure 295 RVT mean commercial and experimental varieties yield, cane tonnes cane/hectare A), commercial cane sugar B) and tonnes of sugar/hectare C) for each cohort, bifenthrin weekly treated and untreated controls

Summary

No metabolic or visible evidence of YCS reported in the 2019-2020 RVT insecticide trial. Changes to leaf sucrose and total α-glucan levels did fluctuate throughout the season, however sucrose accumulation failed to breach the upper tolerance threshold in each varietal cohort. As the crop transitioned out of a severe water-stressed period, lack of mature cane above a sizeable ratoon root sink failed to induce a supply demand imbalance. It is evident from internode analyses that steady supply of rain throughout the peak growing season maintained adequate growth rate and a large culm sink was produced keeping the crop YCS free. However, there was no correlation between bifenthrin treatment and TCH, CCS and TSH in either varietal group. No conclusions can be drawn on the efficacy of bifenthrin to mitigate YCS or whether certain genotypes are more or less susceptible to developing YCS. However, it is evident that bifenthrin weekly treatment prevents leaf sucrose accumulation.

Mites, leafhopper and thrip populations were all supressed by regular bifenthrin applications. However, bifenthrin appeared to cause a resurgence in mealybug populations. It is unclear why this was observed it may be a function of weather conditions at the site or possibly an insecticide resistant mealybug population. Interestingly in the Burdekin insecticide strip trial bifenthrin did not effectively control mealybugs either (see section 6.9.7.2 of this report). As no obvious YCS symptoms were seen at this field site, and arthropod populations were likely impacted by rainfall events, no firm conclusions can be drawn as to the influence of insects or mites on YCS expression.

6.9.7 Commercial insecticide strip trials

Insecticide trials conducted in Burdekin and Herbert since 2017 show that the insecticide bifenthrin supresses YCS development and symptom expression. Analysis of samples from treated plants shows sucrose levels do not accumulate above the upper tolerable threshold of 200µmol/g DM in the source leaves of the mid-canopy. In these trials application of the insecticide has been applied with a knapsack spray under experimental conditions in a small four row, four rep, 10m plot trial. Application of the chemical in this way completely saturates the canopy and stalk of the plant and is most likely dissimilar to the spray penetration and contact achieved by commercial spray boom application. To test whether comparable results could be obtained under commercial conditions and on a larger scale was the objective of the grower strip trials. This would then determine if this mode of application would be a suitable option to mitigate YCS development should research identify an effective non-systemic registered agrochemical product. In addition, insects were monitored and identified to investigate their involvement in YCS development.

Fields that develop YCS each year were identified in each of the regions and growers were approached to participate in the trial. Four sites were selected including Ingham, Ayr, and Mackay but unfortunately the fourth site in Maryborough had to be abandoned as bifenthrin could not be applied due to continued wet weather. However' insect studies in this field have been included for review.

Two applications of bifenthrin were applied with a commercial boom spray at 1500 and 2000 °Cd. Insect and leaf punch samples were collected prior to and post treatments for analysis (see Appendix 1: 1.2.25).

The APVMA issued Permit 87959 (Appendix 2) to SRA which allowed for all cane treated with Bifenthrin to be milled.

6.9.7.1 Ingham site

The Q200$^{\phi}$ plant crop was established in July 2019 and was harvested in October 2020. Application of bifenthrin at 1500 and 2000 °Cd occurred in December and January, respectively.

Results

This site was totally rainfed and endured a hot and very dry start to the cropping cycle, with little to no rain between July and December 2019 (Figure 296). Baseline leaf +4 metabolite data shows elevated sucrose and glucan levels in samples collected prior to the 1500 °Cd bifenthrin treatment and prior to the first decent rain since the crop was established (Figure 297 A). This agrees with results from the water stressed field in the Ingham variety trial (RVT) (see section 6.9.6 of this report). Similarly, as water availability was abundant no further interruption to growth occurred and no YCS symptoms were reported. Crop age studies conducted in the Burdekin in 2014 concluded that growth rate and not crop age per se is the key driver of YCS. This correlates with leaf +4 sucrose and glucan levels which continue to decrease in February and March 2020, indicative of an adequate sucrose concentration gradient between the source and sink tissue (Figure 297 B & C). Maintenance of this gradient requires a strong call for carbon from the non-photosynthetic sink tissue which is dependent on good growth for storage and consumption (Bihmidine et al., 2013). At no point does

leaf sucrose content exceed the upper tolerable threshold to trigger the onset of yellowing (Figure Figure 297 A-C). This concurs with the absence of any YCS symptoms in this trial.

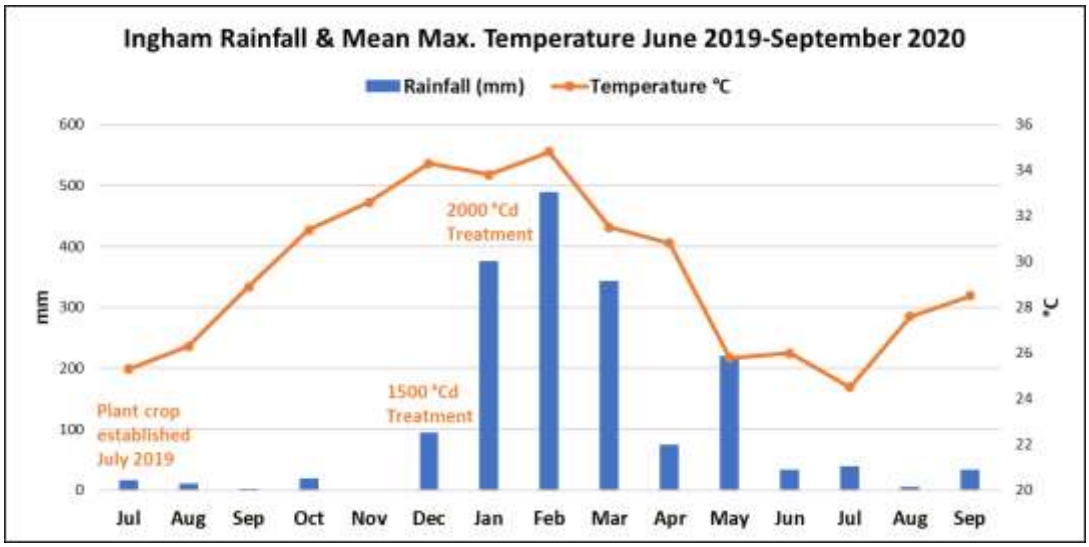


Figure 296 Ingham commercial insecticide strip trial rainfall (mm) and mean maximum air temperature (°C) 2019-2020

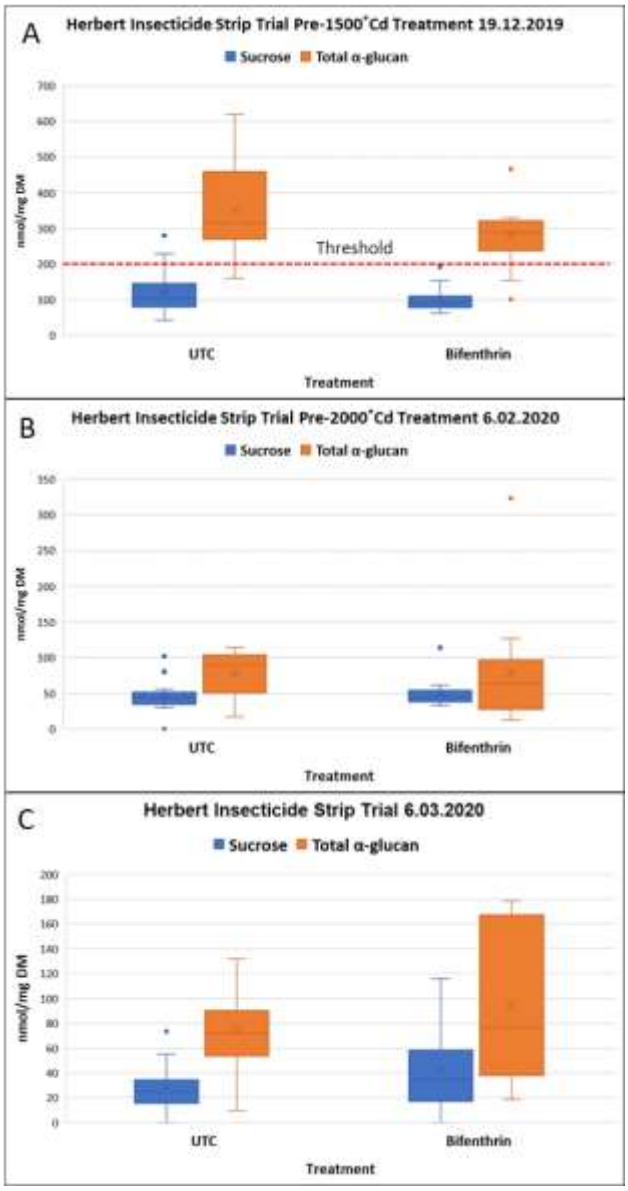


Figure 297 Commercial insecticide strip trial leaf +4 sucrose and total α-glucan content, December A), February B) and March C)

Entomology

Weather data from the trial indicated high rainfall events in January, February and March (Figure 296) with almost 500 mm in February and a 250mm rain event over 5 days in January, which may have impacted on both some populations and insecticide efficacy and any residual activity.

1. Mealybugs

The predominant mealybug at this field site was *Saccharicoccus sacchari*. No *Heliococcus summervillei* was detected in traps. A single peak in mealybug population was observed in early March (Figure 298) with 125 mealybugs per trap (UTC). In contrast populations in the cane treated with bifenthrin @1500 °Cd and 2000 °Cd were reduced by 50% (treatment at peak = 64 per trap). Mealybug populations had declined markedly by late March. Based on the population abundance in the control only the Bifenthrin @2000 °Cd application is likely to have impacted mealybug population abundance as populations were very low in December. The rain event of 250 mm

between 26th and 31st January could also have reduced the treatment efficacy and delayed emergence of mealybugs from the root system.

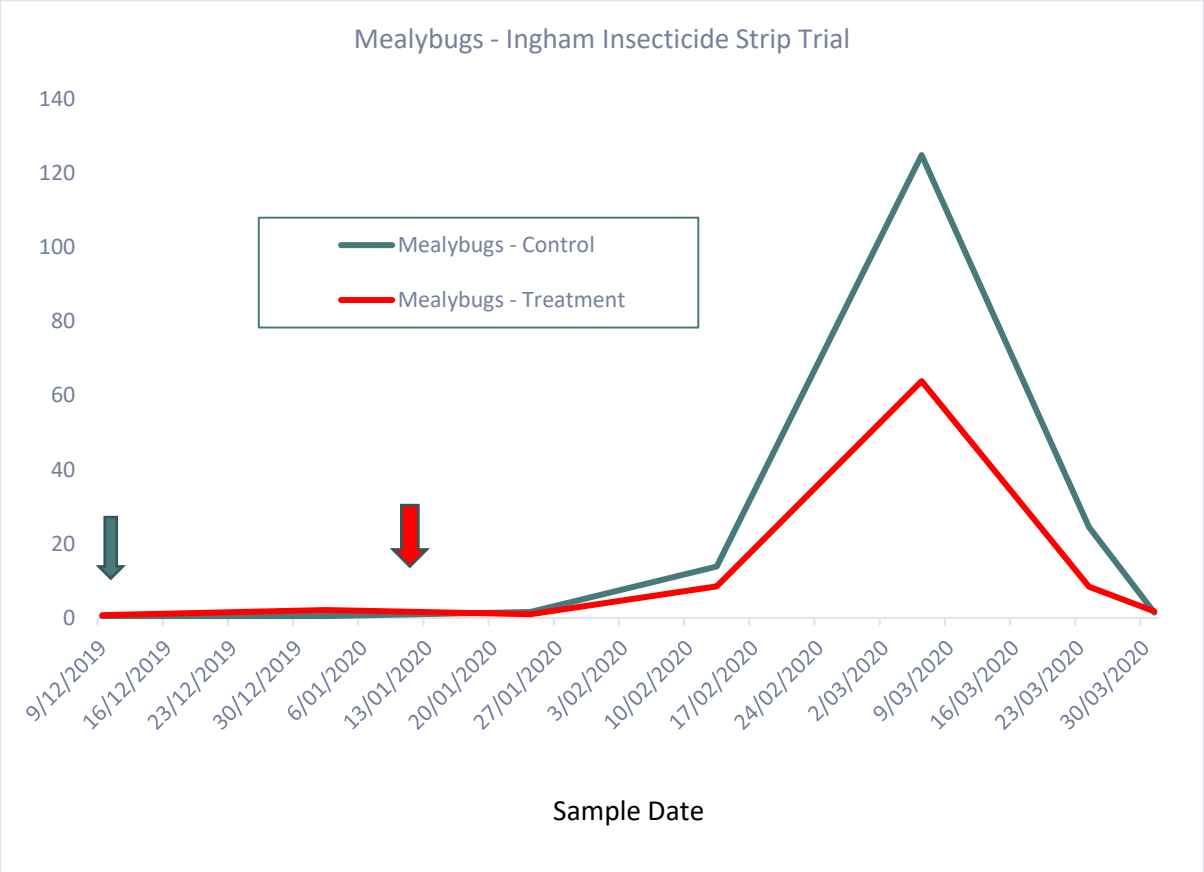


Figure 298 Mean mealybug abundance per yellow sticky trap in sugarcane canopy of plant sugarcane in relation to insecticide application. Based on the mean of 12 canopy sticky traps per treatment and control (UTC). The blue and red arrow respectively represent the 1500 °Cd and 2000 °Cd application timings.

2. Leafhoppers

A variety of leafhoppers were detected in sticky traps, but abundance was relatively low (2-3 leafhoppers per trap maximum) and no leafhopper was predominant. Two population peaks were evident (Figure 299) one in early January and one in early March. Populations in the cane treated with bifenthrin @1500 and 2000°Cd were reduced by around 50% (Treatment at peak = 64 per trap). Leafhopper populations had declined markedly by late March. Based on the bimodal population abundance both bifenthrin @1500 °Cd and 2000 °Cd applications are likely to have impacted leafhopper population abundance. The rain event of 250 mm between 26th and 31st January could also have reduced the @2000 °Cd treatment efficacy and caused a natural population decline.

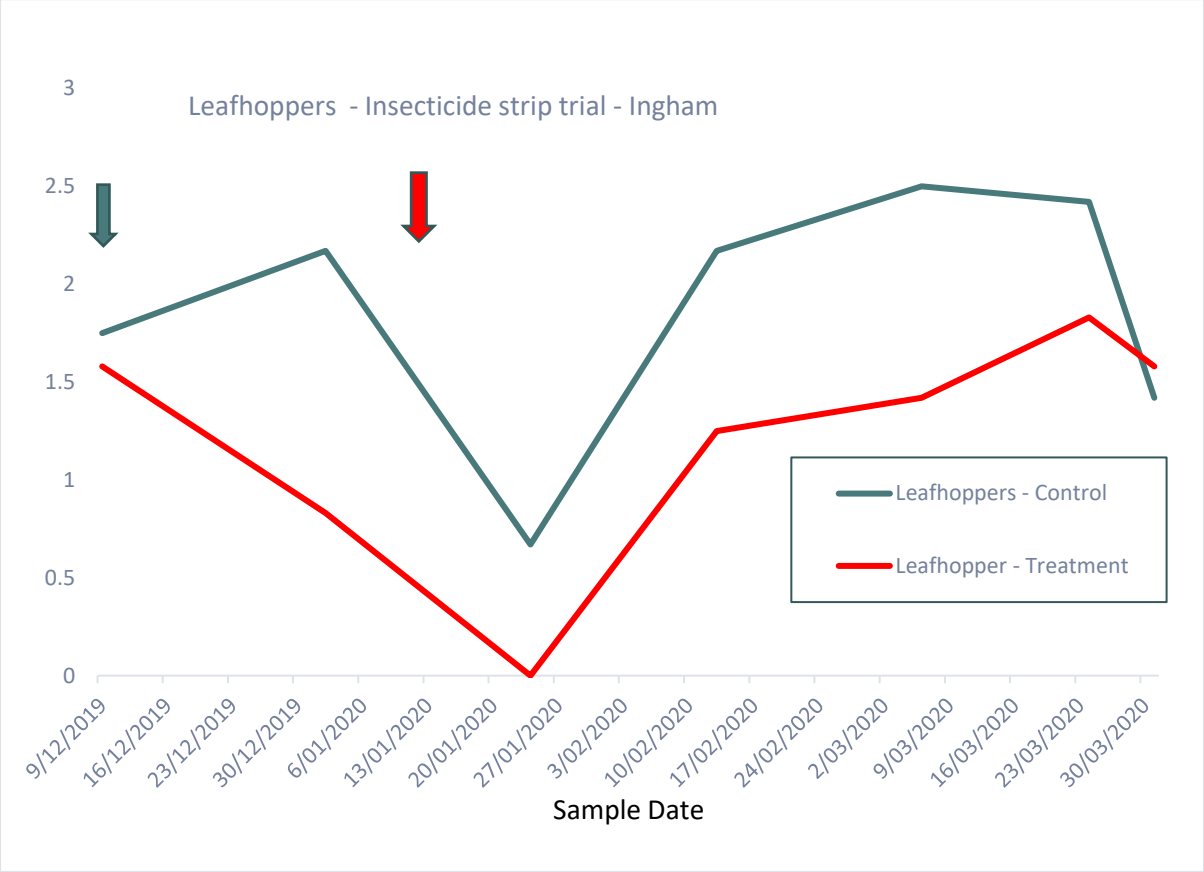


Figure 299 Mean leafhopper abundance per yellow sticky trap in sugarcane canopy of plant sugarcane in relation to insecticide application. ***Based on the mean of 12 canopy sticky traps per treatment and control (UTC). The blue and red arrow respectively represent the 1500 day degree (°Cd) and 2000 °Cd application timings.***

3. Mites

A variety of mites were detected in sticky traps, but abundance was relatively low (8 mites per trap maximum) and no mite species was predominant. Three population peaks were evident (Figure 300) in late December, mid-February and mid-March. Populations in the cane treated with bifenthrin @1500 and 2000°Cd were not reduced until March. Mite populations had declined markedly by late March. Based on the bimodal population abundance both bifenthrin @1500°Cd and 2000°Cd were likely to have impacted mite population abundance reducing it by around 50% in December and March. The rain event of 250 mm between 26th and 31st January could also have reduced overall treatment efficacy. Rainfall events in January, February and March may also have caused declines in peak abundance of mites.

Figure 300 Mean mite abundance per yellow sticky trap in sugarcane canopy of plant sugarcane in relation to insecticide application. ***Based on the mean of 12 canopy sticky traps per treatment and control (UTC). The blue and red arrow respectively represent the 1500 day degree (°Cd) and 2000 °Cd application timings.***

4. Thrips

A variety of thrips were detected in sticky traps and abundance was relatively high (340 thrips per trap maximum) and no thrip species was predominant. A population peak is likely to have occurred before early December and populations gradually decline to negligible levels by late January (Figure 301). Populations in the cane treated with bifenthrin @ 1500 and 2000 °Cd were reduced in January markedly. The bifenthrin applied at @2000 °Cd would have had minimal impact as thrip populations were in a natural decline phase.

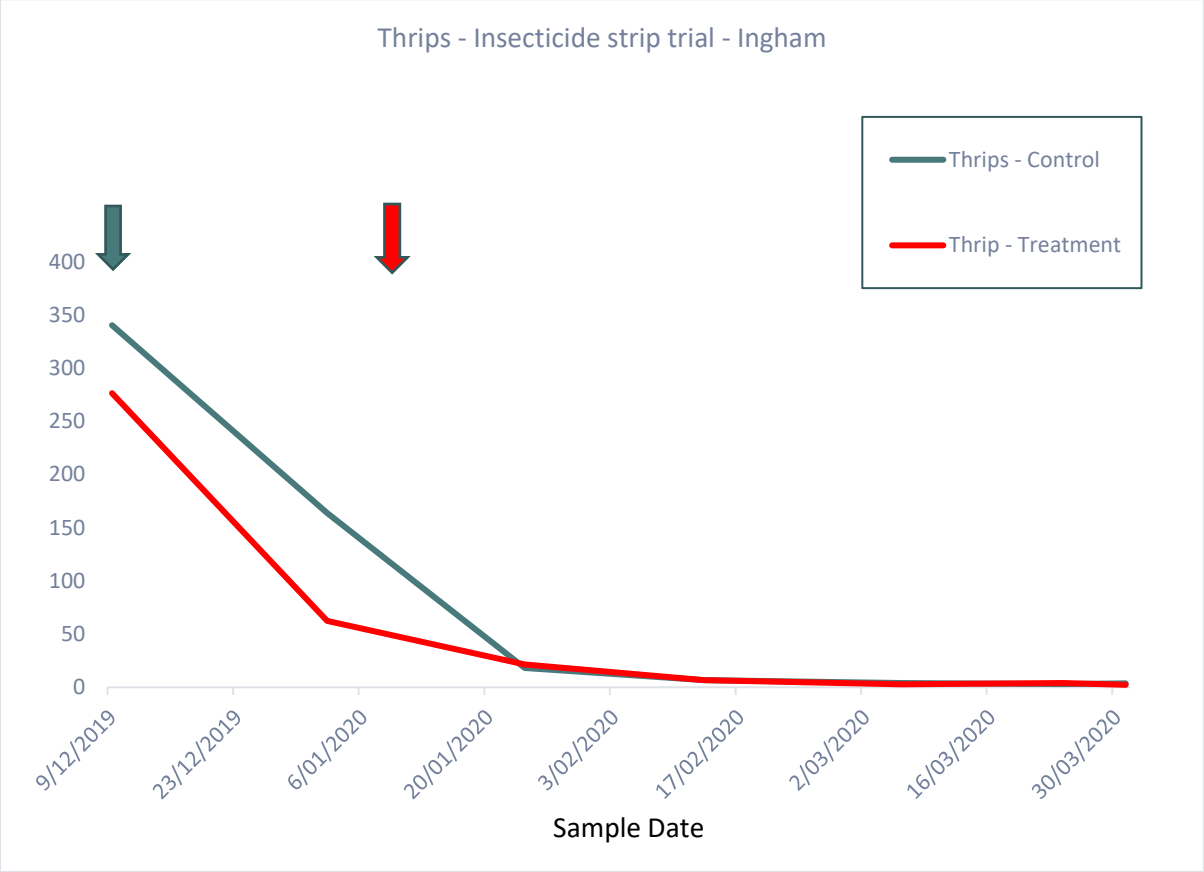


Figure 301 Mean thrip abundance per yellow sticky trap in sugarcane canopy of plant cane in relation to insecticide application. Based on the mean of 12 canopy sticky traps per treatment and control (UTC). The blue and red arrow respectively represent the 1500 day degree (°Cd) and 2000 °Cd application timings.

Final yield

There was no statistical difference in final cane yield (TCH) and CCS between bifenthrin treated and untreated controls under commercial conditions (Figure 302 & Figure 303).

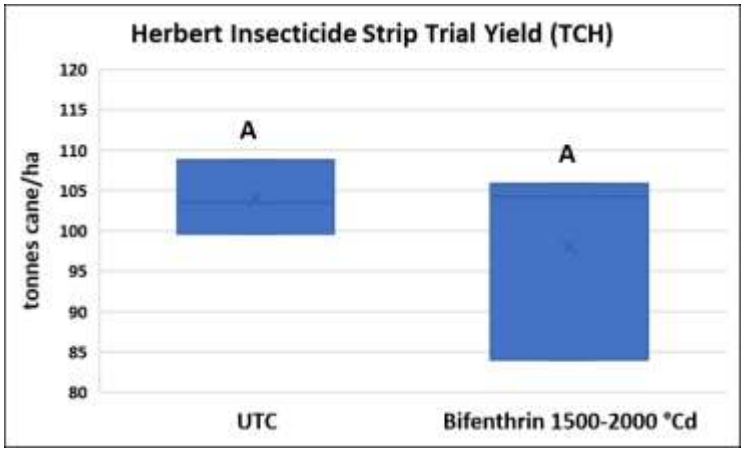


Figure 302 Commercial strip trial Q200^{A} tonnes cane/hectare (TCH), bifenthrin treated and untreated control. Tukey HSD All-Pairwise Comparisons (p<0.05)

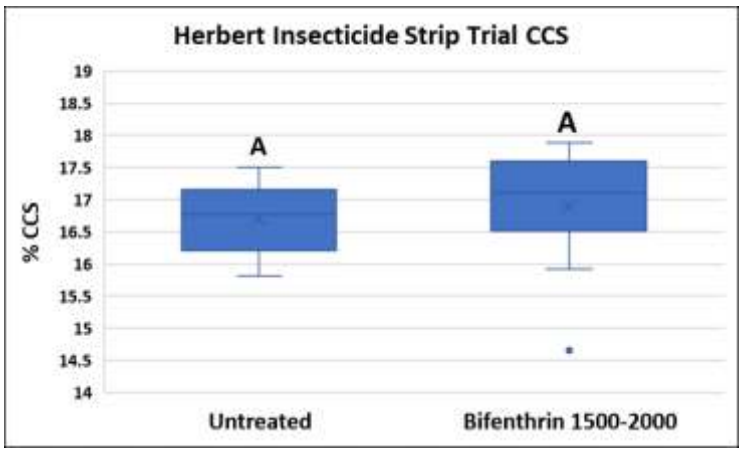


Figure 303 Commercial strip trial Q200$^{\Phi}$ commercial cane sugar (CCS), bifenthrin treated and untreated control. Tukey HSD All-Pairwise Comparisons (p<0.05)

Summary

YCS symptoms were absent in this field and this is confirmed by leaf metabolite data. There was only 50mm of rainfall over the 5-month period from July to November 2019 inclusive. As this is a rainfed field, plants had very little growth or culm development after the July harvest up to the first rainfall of 29mm on the 11-12th December 2019. The rain induced growth in mid-December would have been insufficient to cause a source sink imbalance due to the lack of cane under the new source leaves. Therefore, export of sucrose would be mainly directed to the root sink due its close proximity and energy requirements. The 65mm of rain that fell on December 31st would have been sufficient to grow a sizeable culm sink to accommodate sucrose export from the source leaves after the rainfall of 85mm on Jan 11th 2020. After this period throughout the peak photosynthetic months to April there was sufficient rain and growth to maintain a balanced source sink relationship despite any insect pressure and hence no YCS development. This is confirmed by the metabolite and yield data and concurs with previous studies that the timing of growth and the rate of growth are the key drivers of YCS.

Bifenthrin applications supressed populations of mites, leafhoppers, mealybugs and thrips. Rainfall events in January, February and March could also have supressed some insect and mite population build up. As no obvious YCS symptoms were seen at this field site and arthropod populations were likely impacted by rainfall events conclusions cannot be drawn as to the influence of insects or mites on YCS expression.

No difference in mean yield (TCH) and CCS is evident between treated and untreated control which confers with metabolite and monitoring data. As no YCS symptoms developed in the field trial no conclusions can be drawn on the efficacy of bifenthrin treatment under commercial conditions to mitigate YCS.

6.9.7.2 Ayr site

The KQ228$^{\Phi}$ 3R crop was established in July 2019 and was harvested in August 2020. Application of bifenthrin at 1500 and 2000 °Cd occurred in December and January, respectively.

Results

The Burdekin strip trial was a furrow irrigated field on an average 10-day cycle. Baseline leaf +4 metabolite data shows low levels of sucrose and glucan prior to the 1500 °Cd bifenthrin treatment (Figure 304 A). This is in contrast to much higher metabolite levels observed in the water stressed Herbert strip trial (Figure 297 A). Leaf sucrose levels reduced by approximately 50% over the following month prior to the 2000 °Cd treatment; glucan content remaining steady (Figure 304 B). The reduction in sucrose accumulation correlates with the good rainfall (~40mm) and high temperature in December (Figure 305). Even though this is an irrigated field, the input of extra moisture from rain for the first time in 5 months, together with high temperature has increased sink strength. These are perfect growth conditions for C4 plants to assimilate carbon and to grow rapidly (Botha, 2007; Moore and Botha, 2014). Internode analysis in April shows no significant difference in culm growth between the bifenthrin treatment and UTC (Figure 306). No YCS was reported in this field trial.

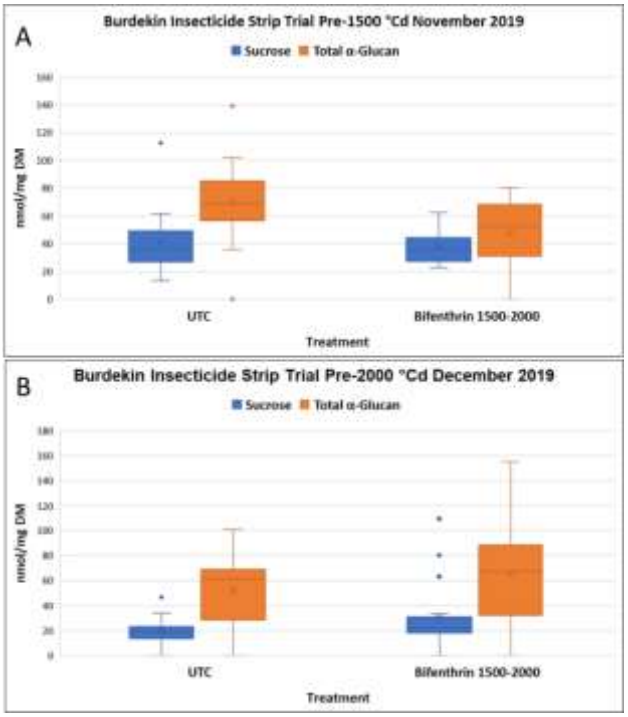


Figure 304 Commercial insecticide strip trial leaf +4 sucrose and total α-glucan content, November A) and December B)

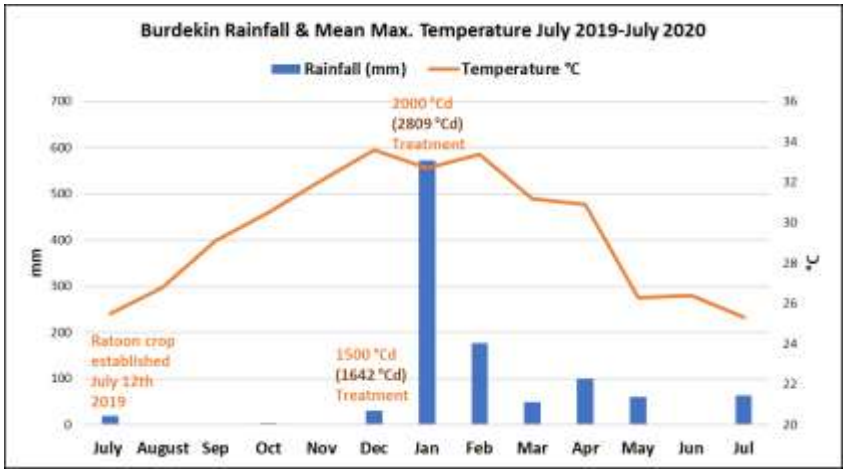


Figure 305 Burdekin commercial insecticide strip trial rainfall (mm) and mean maximum air temperature (°C) 2019-2020

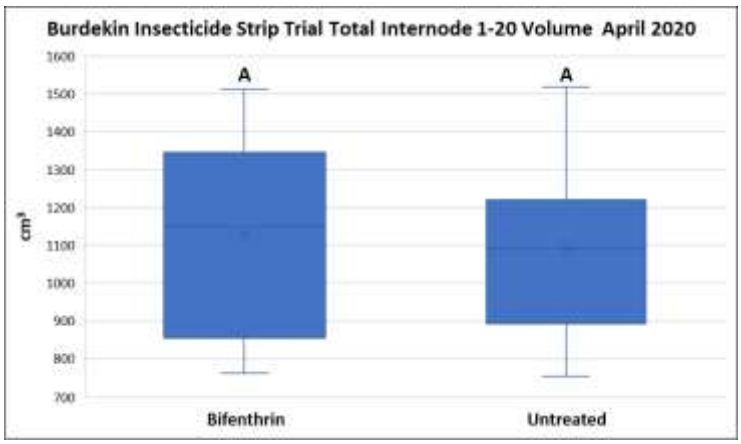


Figure 306 Burdekin commercial insecticide strip trial, total internode volume (Int +1 to +20) of Bifenthrin and untreated control from 4 stalks per plot (20 stalks total). Tukey HSD All-Pairwise Comparisons (p<0.05).

Entomology

Weather data from the trial indicated high rainfall events in January (Figure 305), with over 500 mm, which may have impacted some populations and insecticide efficacy.

1. Mealybugs

The predominant mealybug at this field site was *Saccharicoccus sacchari*. No *Heliococcus summervillei* was detected in traps. A single peak in mealybug population was observed (Figure 307) in late December with over 60 mealybugs per trap. The bifenthrin @1500 and 2000°Cd treatments only appeared to marginally reduce mealybug populations. Mealybug populations had naturally declined markedly by mid-February and this may have been influenced by the high rainfall events in January (Figure 305).

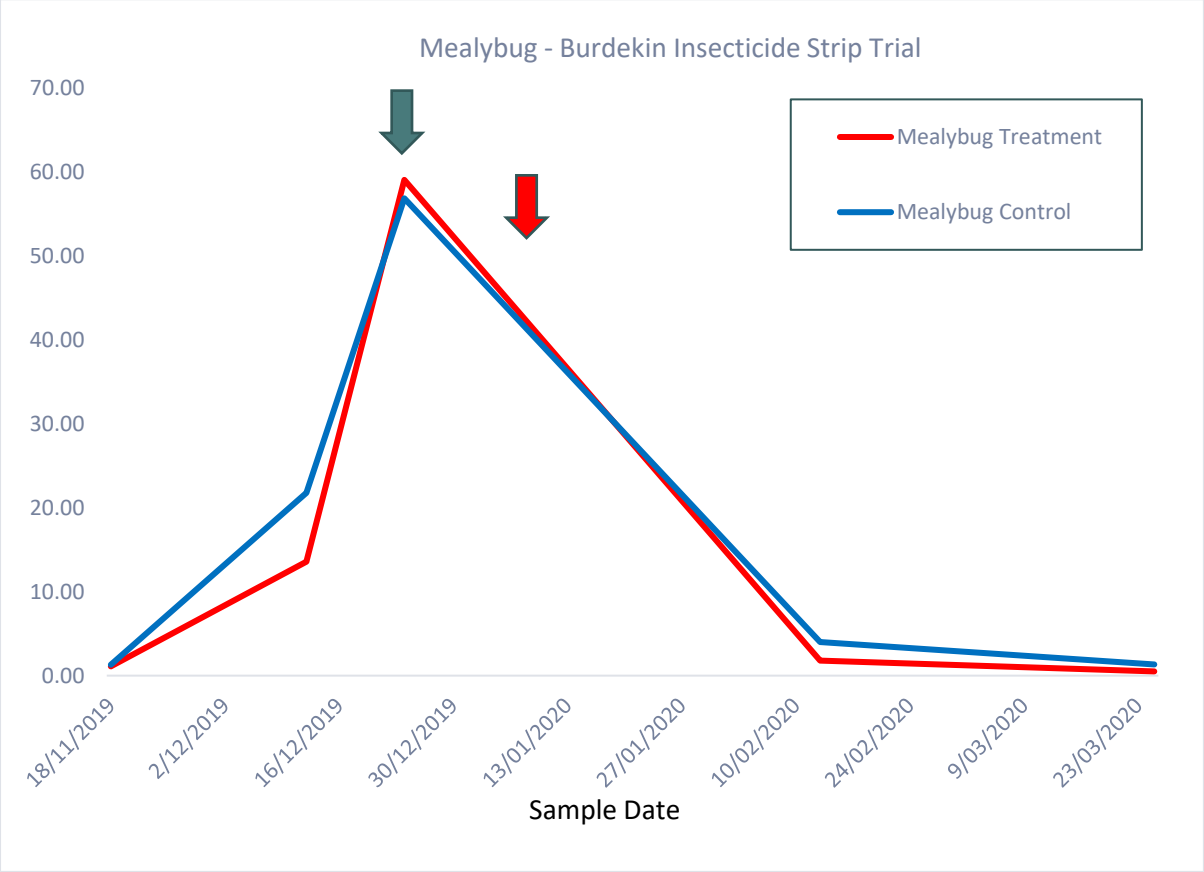


Figure 307 Mean mealybug abundance per yellow sticky trap in sugarcane canopy of plant sugarcane in relation to insecticide application. Based on the mean of 10 canopy sticky traps per treatment and control (UTC). The blue and red arrow respectively represent the 1500 °Cd and 2000 °Cd application timings.

2. Mites

Although populations were relatively low (maximum of 8 per trap recorded) several mite species were detected in traps. The peak of abundance was likely to be late March-April but as traps were only monitored till late-March the specific date of peak abundance is uncertain. The bifenthrin @ 1500 and 2000°Cd treatments effectively reduced mite populations compared to the untreated control (Figure 308). Mite populations started to increase markedly in the untreated control plots from mid-February but did not increase in the bifenthrin treated plots. Mite populations are likely to have been supressed in January and this may have been influenced the high rainfall events (Figure 305).

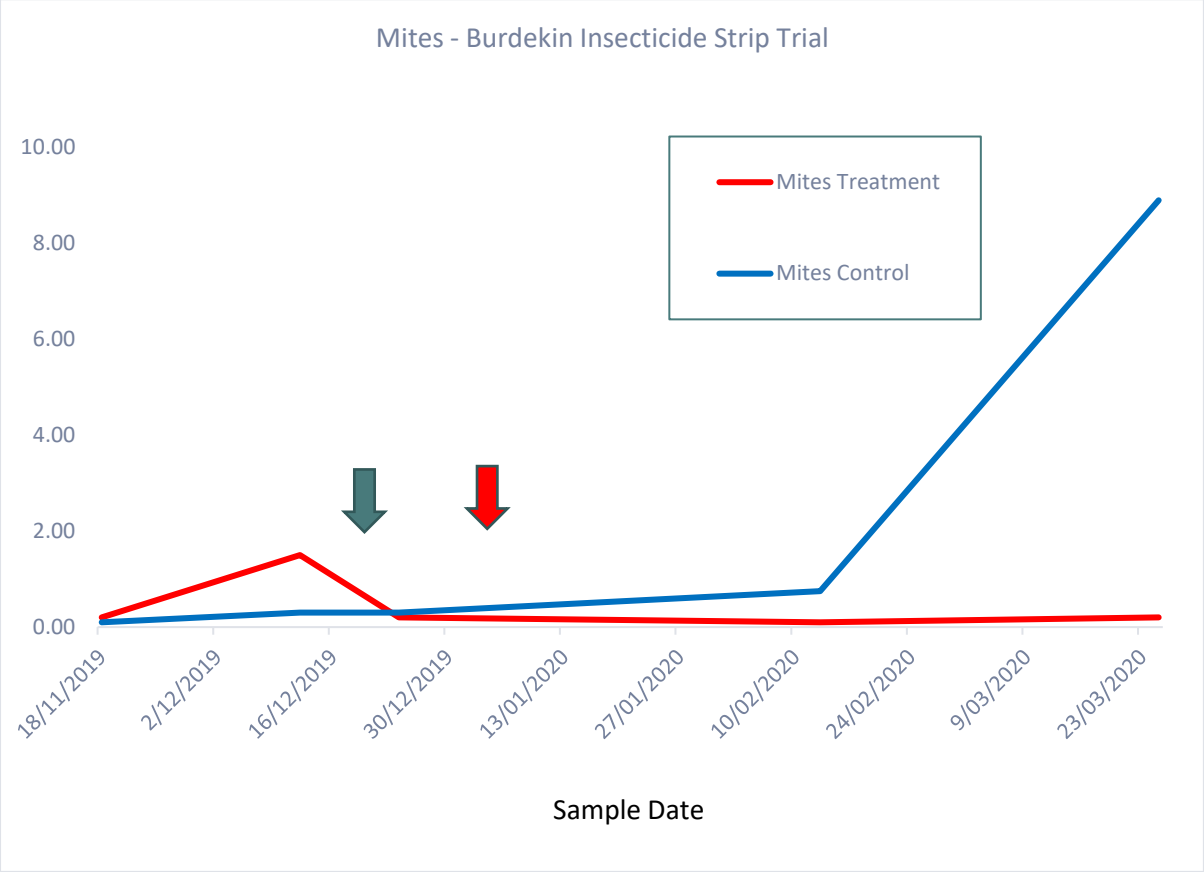


Figure 308 Mean mite abundance per yellow sticky trap in sugarcane canopy of plant sugarcane in relation to insecticide application. Based on the mean of 10 canopy sticky traps per treatment and control (UTC). The blue and red arrow respectively represent the 1500 °Cd and 2000 °Cd application timings

3. Thrips

Although thrip populations were relatively low (maximum of 30 per UTC trap) several thrip species were detected in traps. The peak of abundance was likely to be before mid-November when sampling commenced but there was a second peak observed in late December. The bifenthrin @1500 and 2000 °Cd treatments effectively reduced thrip populations especially after mid-December compared to the untreated control (Figure 309). Thrip populations started to naturally decline in the untreated control plots from late December and this could also have been influenced by high rainfall in January.

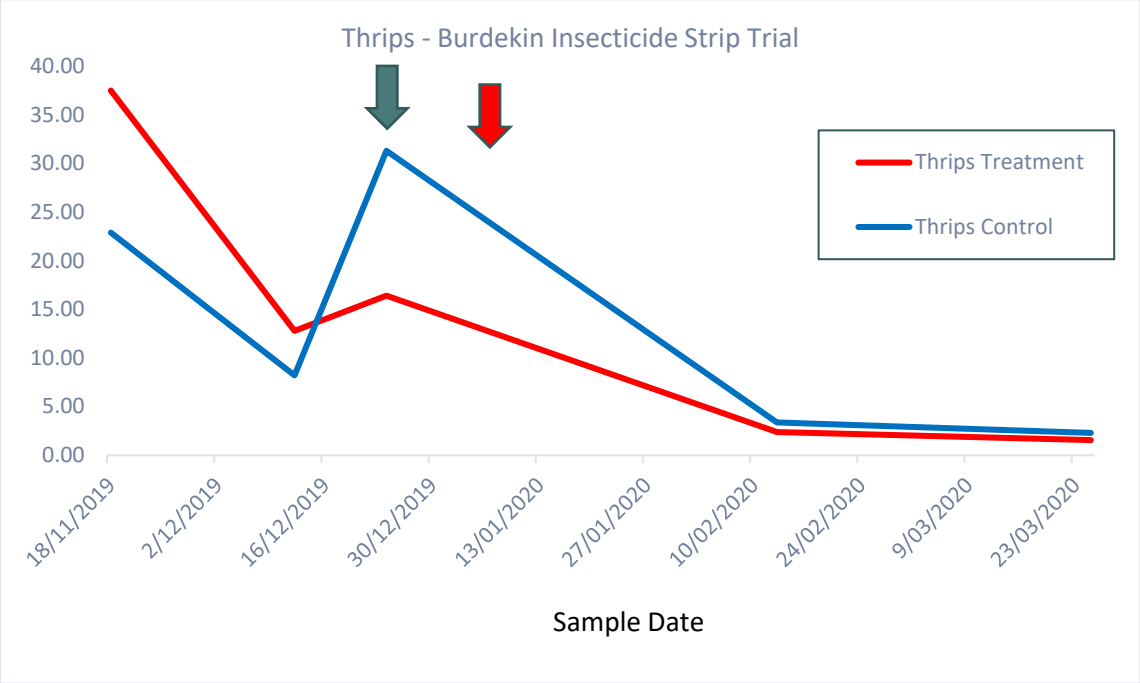


Figure 309 Mean thrip abundance per yellow sticky trap in sugarcane canopy of plant sugarcane in relation to insecticide application. Based on the mean of 10 canopy sticky traps per treatment and control (UTC) and eight field border traps. The blue and red arrow respectively represent the 1500 °Cd and 2000 °Cd application timings

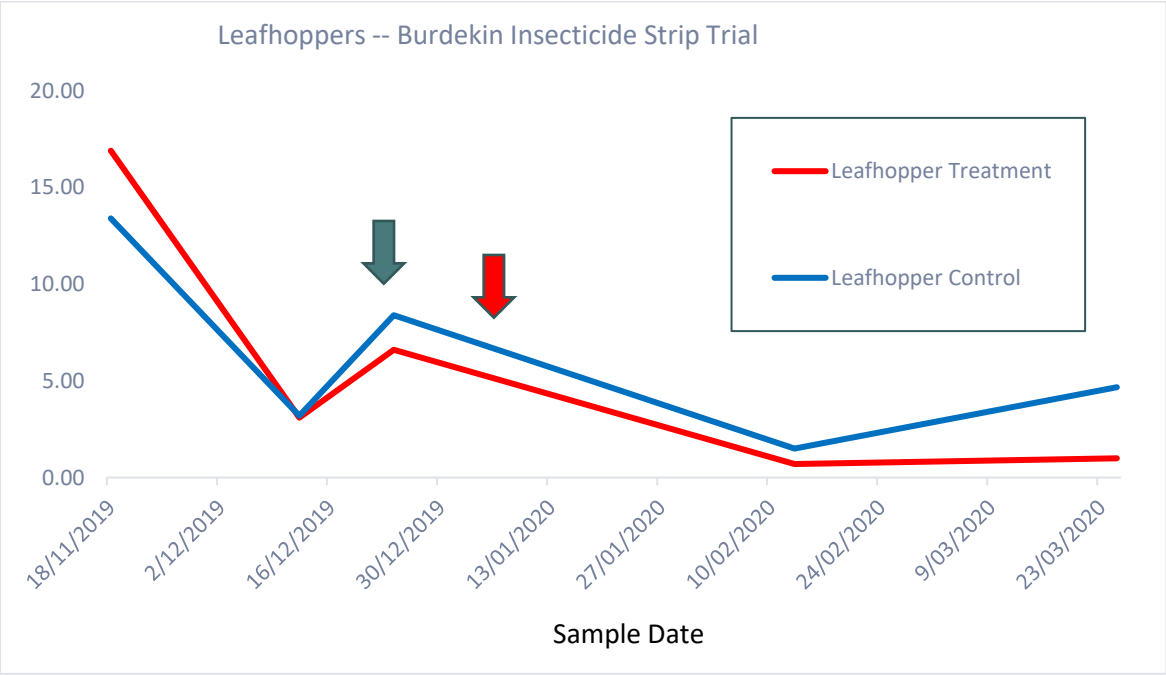


Figure 310 Mean leafhopper abundance per yellow sticky trap in sugarcane canopy of plant sugarcane in relation to insecticide application. Based on the mean of ten canopy sticky traps per treatment and control (UTC). The blue and red arrow respectively represent the 1500 °Cd and 2000 °Cd application timings

4. Leafhoppers

Leafhopper populations are likely to have peaked earlier than mid-November and exceeded 10 insects pre trap which indicates high population level (Figure 310). However, populations then declined by around 50% in untreated blocks, especially in January, likely as a consequence of high rainfall events. Consequently, the Bifenthrin applications @1500 and @2000 °Cd only marginally supressed leafhopper populations in January but this suppression extended into February and March.

Final yield

There was no statistical difference in mean cane yield tonnes cane/treatment and CCS bifenthrin treated and untreated controls under commercial conditions (Figure 311 A & B).

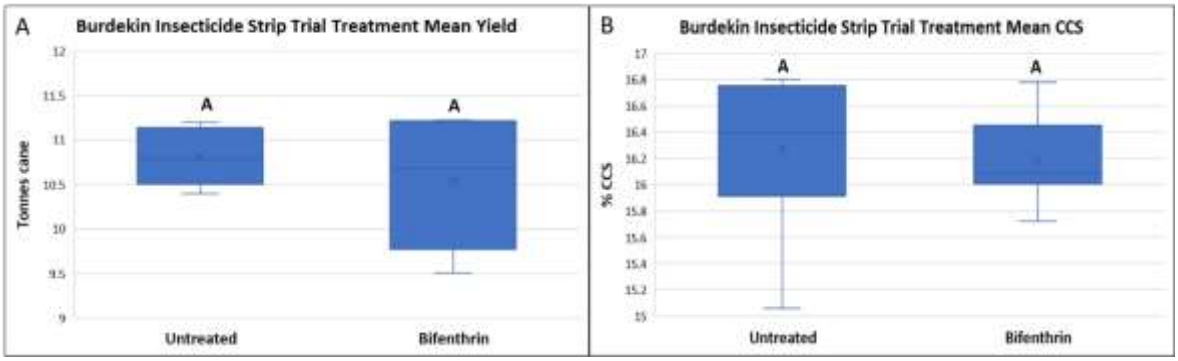


Figure 311 Burdekin commercial insecticide strip trial mean strip yield treated and untreated control A) and CCS B). Tukey HSD All-Pairwise Comparisons (p<0.05). Note: mill data unavailable, cane yield determined by weigh truck and CCS by SRA SpectraCane.

Summary

Leaf +4 sucrose levels remained well below the upper tolerable threshold throughout the duration of trial monitoring. Total α-glucan levels also remained low which is indicative of a healthy turnover of polysaccharides in sync with the diurnal rhythm (Du et al., 2000; Watt et al., 2005; Weise et al., 2011). In plants affected by YCS the diurnal profile of the source leaf is disrupted and causes a change to carbon partitioning and an increase in α-glucans to offset the rising carbon load (Marquardt et al., 2017; Scalia et al., 2020). It is evident there is no such disruption in this crop which supports the lack of YCS expression in this field throughout the trial. Metabolite and internode studies support a maintenance of good growth rate in both the treated and untreated strips. This is supported by yield data that shows no significant difference to cane yield or CCS between treatments.

Bifenthrin applications especially the application applied @ 1500 °Cd prior to high rainfall in January supressed populations of mites, leafhoppers and thrips but not mealybugs. Mealybugs are

particularly susceptible to rainfall, often washing off the canopy and stem and migrate down to the root system. It is likely mealybug populations did not recover from waterlogged soils in January. At other sites and in previous seasons mealybug populations usually peak in February to March. As no obvious YCS symptoms were seen at this field site and arthropod populations were likely impacted by rainfall events, conclusions cannot be drawn as to the influence of insects or mites on YCS expression.

In the absence of YCS symptoms throughout the duration of the field trial, no conclusions can be drawn on the efficacy of bifenthrin treatment under commercial conditions to mitigate YCS.

6.9.7.3 Mackay site

The Q240◊ 1R was established in June 2019 and was harvested in July 2020. Application of bifenthrin at 1500 and 2000 °Cd occurred in December and February, respectively. This field is mostly rainfed and only received one irrigation on December 13th, 2019 after the extremely dry period preceding it.

Results

Pre-1500 °Cd bifenthrin treatment leaf sampling was conducted on December 12th, 2019 to establish baseline sucrose and α-glucans levels. At the time of sampling, it was thought there was some evidence of YCS in the mid-canopy, but it was difficult to call due to the amount of leaf yellowing due to water stress in the field. Leaf+4 sucrose content shows levels just exceeding the tolerable upper threshold of approximately 200 µmol/g DM (Figure 312 A). Therefore, it is likely YCS was starting to exhibit in the field. It is worth noting that prior to the sample point in December, this rainfed only field had received a mere 15 mm of rain since the 9th July, comprised of insignificant daily precipitation of only 1 or 2 mm. The following day after sampling and application of bifenthrin, this field was irrigated due to the extremely dry conditions. Fortuitously, this field received 190mm of rain between December 15-31, 2019 and a further 292mm prior to next sampling and 2000 °Cd bifenthrin treatment on February 4th, 2020 (Figure 313). Leaf +4 sampling on February 3rd shows sucrose and glucan levels have fallen below 100 µmol/g DM in both treatments (Figure 312 B). This suggests that the high rainfall events in December and January have initiated good growth to establish a strong sink and sugar gradient between the culm and source leaves of the mid-canopy. Continued rain in February and March saw a further 588mm across the Mackay district. March 31st sampling showed an additional decline in leaf +4 metabolites in both treatments (Figure 312 C). This is further evidence of a large healthy and strong sink which has established due to continued supply of water. As metabolite levels are similar in both the treated and untreated cane this result cannot be attributed to the bifenthrin treatment.

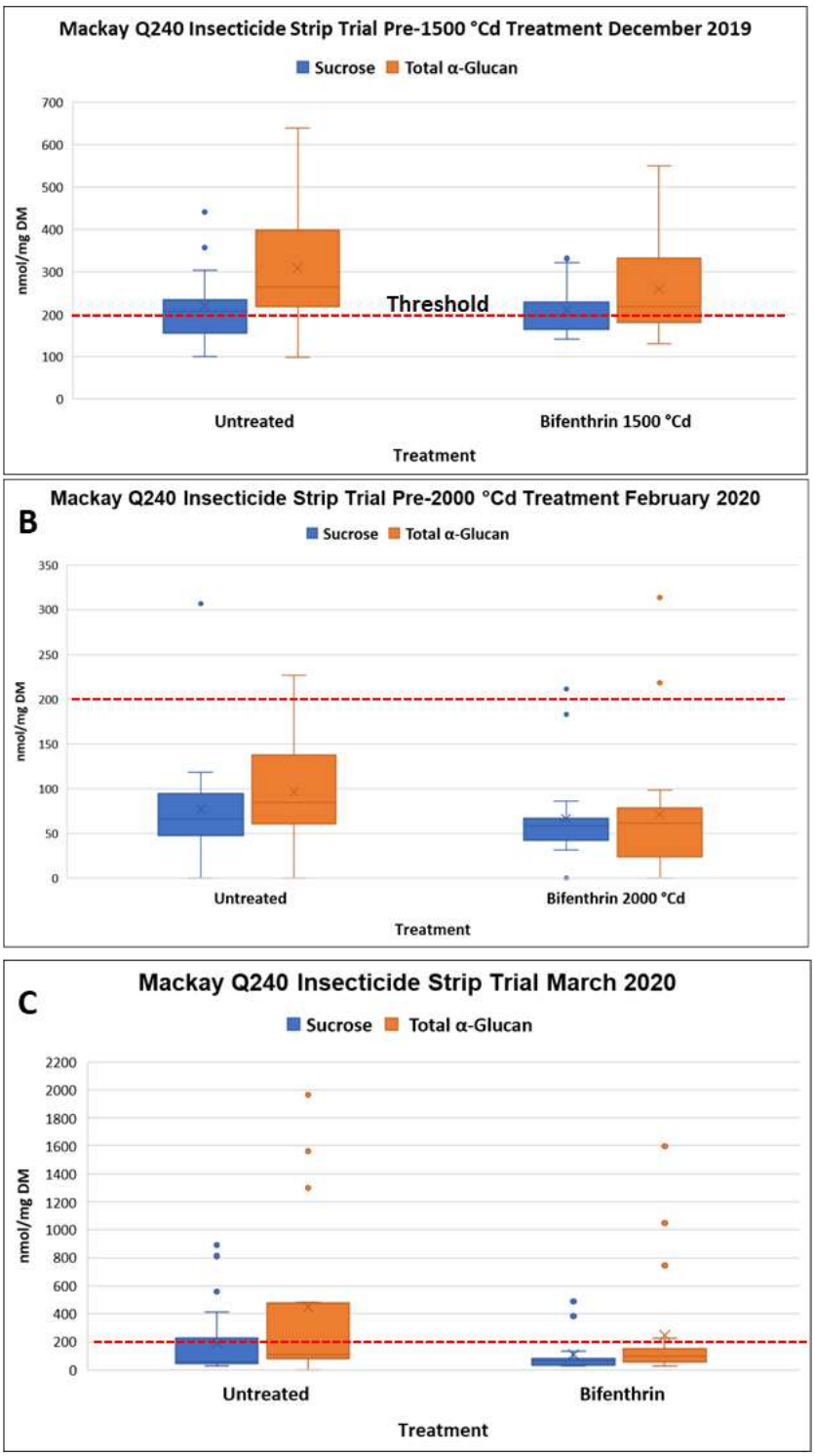


Figure 312 Commercial insecticide strip trial leaf +4 sucrose and total α-glucan content, December A) February B) and March C)

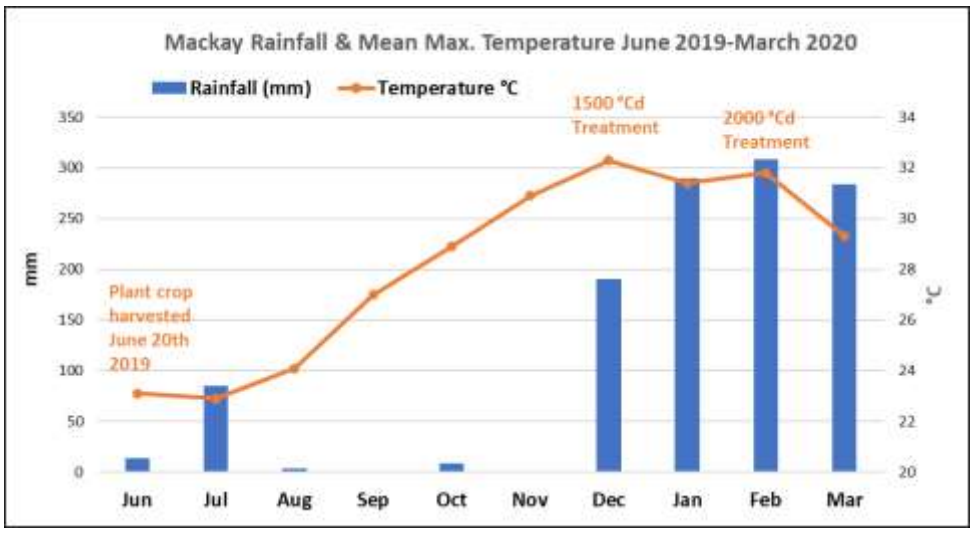


Figure 313 Mackay commercial insecticide strip trial rainfall (mm) and mean maximum air temperature (°C) 2019-2020

Entomology

Weather data from the trial indicated some high rainfall events but only on single days in December, January, February, and March which are unlikely to have impacted some populations or insecticide efficacy.

1. Mealybugs

The predominant mealybug at this field site was *Saccharicoccus sacchari*. Even though populations were relatively low, multiple fluctuating peaks of abundance and decline were observed (Figure 314) which is typical for mealybug populations. However, the bifenthrin @1500 and 2000 °Cd treatments appeared to have had no effect on mealybug populations. The reasons for this apparent lack of efficacy are unclear. Mealybug populations appeared to resurge in March and the maximal peak is likely to have been late March, but as trapping was discontinued in mid-March this is a speculative assumption.

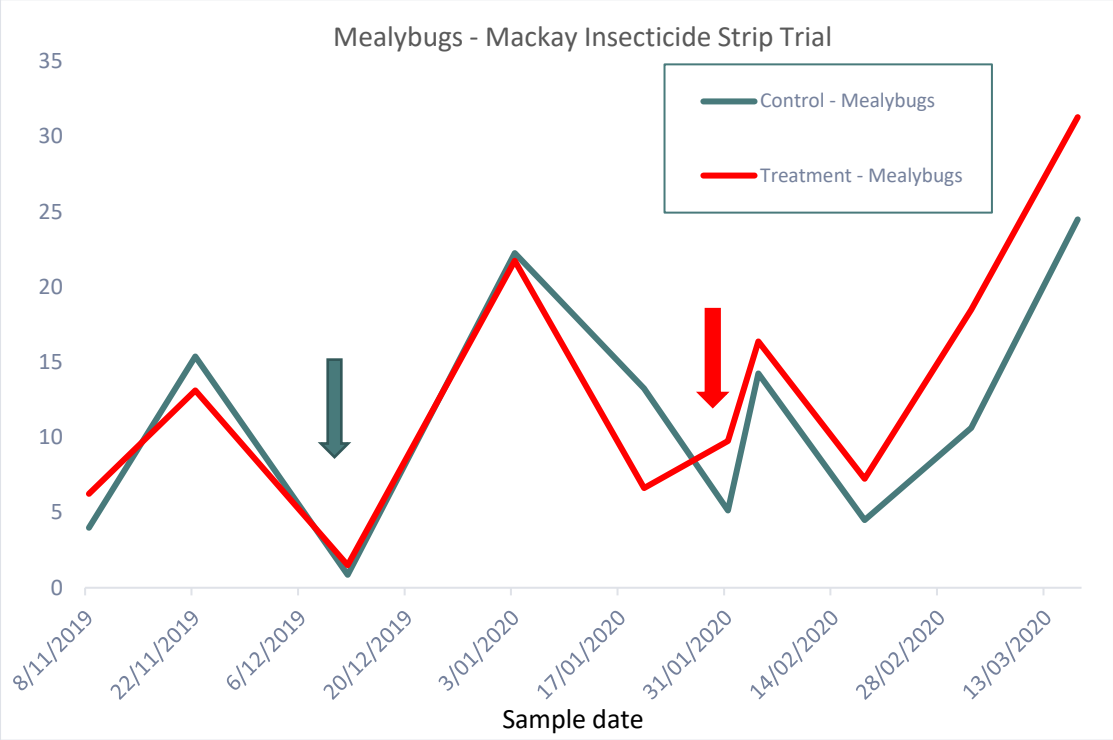


Figure 314 Mean mealybug abundance per yellow sticky trap in sugarcane canopy of plant sugarcane in relation to insecticide application. Based on the mean of eight sticky traps per treatment and control (UTC). The blue and red arrow respectively represent the 1500 °Cd and 2000 °Cd application timings.

2. Mites

Mite populations were relatively high (maximum of 51 per UTC trap) and several mite species were detected in traps. A bimodal distribution was observed with the largest peak in early January and a smaller peak in mid-February in the untreated controls. Both the bifenthrin @1500 and 2000 °Cd treatments effectively reduced mite populations compared to the untreated control (Figure 315).

3. Thrips

Thrip populations were multiple species and relatively high (maximum of 84 per UTC trap). A unimodal distribution was observed with the peak in late November. Both the bifenthrin @1500 and 2000 °Cd treatments had no impact on thrip populations compared to the untreated control (Figure 316).

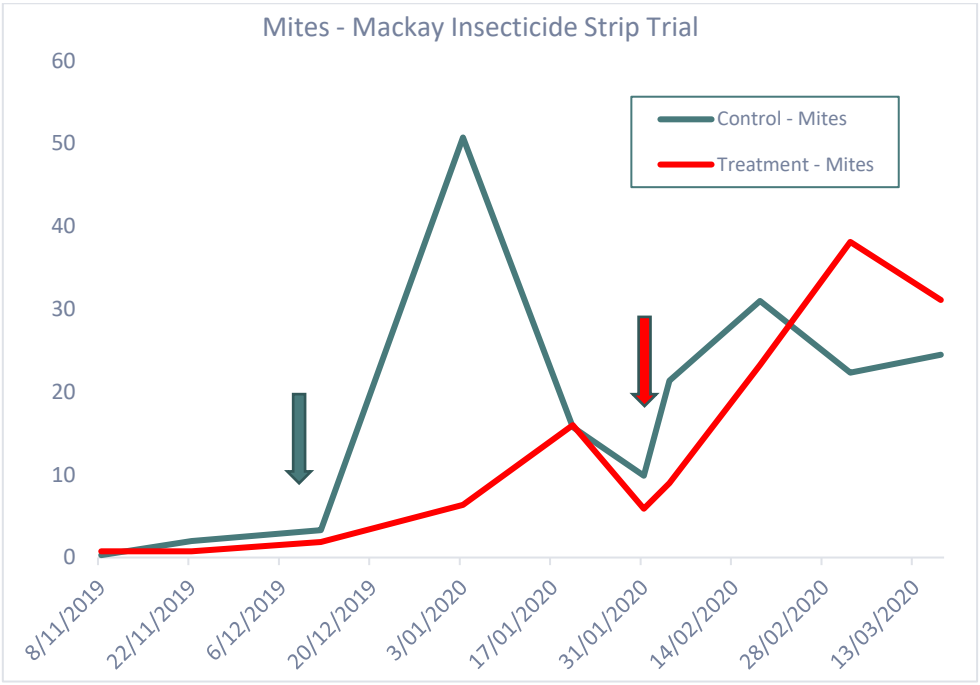


Figure 315 Mean mite abundance per yellow sticky trap in sugarcane canopy of plant cane in relation to insecticide application. Based on the mean of eight sticky traps per treatment and control (UTC). The blue and red arrow respectively represent the 1500 °Cd and 2000 °Cd application timings.

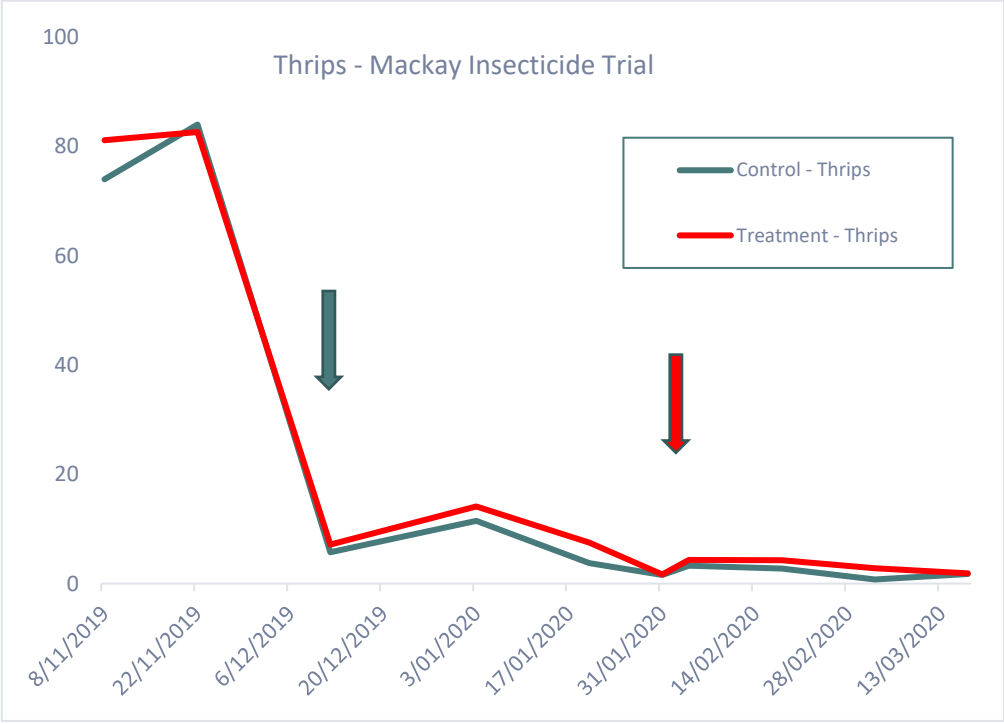


Figure 316 Mean thrip abundance per yellow sticky trap in sugarcane canopy of plant cane in relation to insecticide application. Based on the mean of eight sticky traps per treatment and control (UTC). The blue and red arrow respectively represent the 1500 °Cd and 2000 °Cd application timings.

4. Leafhoppers

Leafhopper populations were multiple species and relatively low (maximum of 6 per UTC trap). A unimodal distribution was observed with the peak in early February. The bifenthrin @1500 °Cd treatments had no minimal impact on leafhopper populations compared to the untreated control but the 2000 °Cd did have a small impact reducing populations in February and March (Figure 317).

5. Coleoptera

Coleoptera populations consisting mainly of ladybeetles were relatively high (maximum of 21 per UTC trap) and several species were detected in traps. A bimodal distribution was observed with the largest peak likely to be in late March and an early peak in January. Both the bifenthrin @1500 and 2000 °Cd treatments effectively reduced Coleoptera populations compared to the untreated control (Figure 318). This could be potentially problematic if this treatment were to be used it would affect the natural predator-pest complex in the sugarcane canopy. Ladybeetles are known to feed on small nymphs of Hemipteran insects such as mealybugs and aphids.

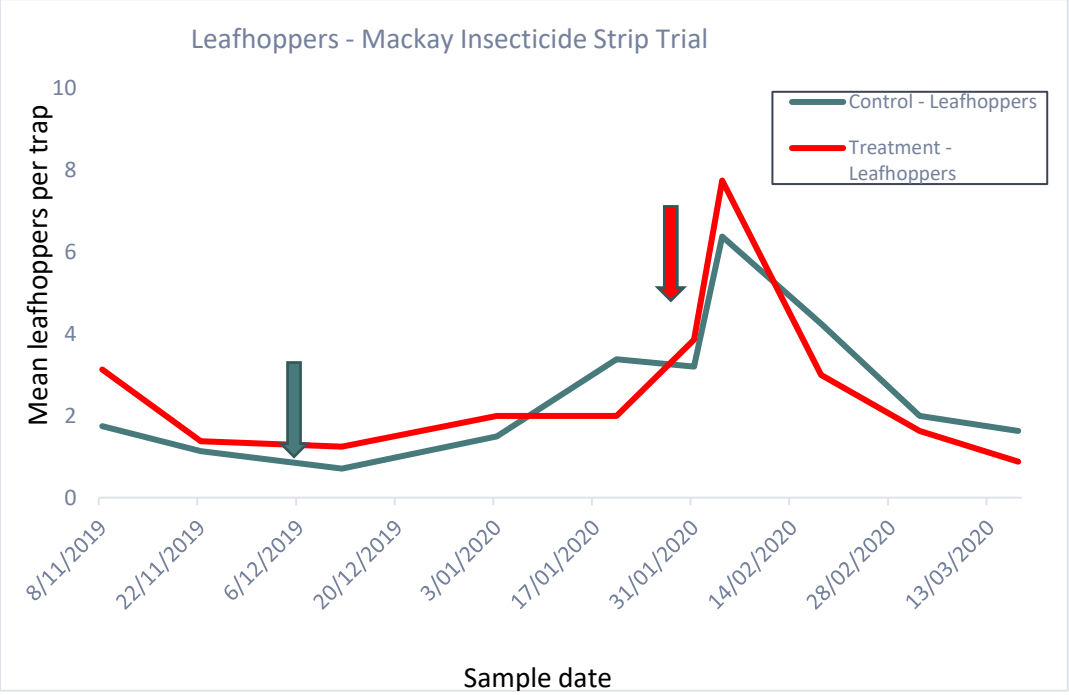


Figure 317 Mean leafhopper abundance per yellow sticky trap in sugarcane canopy of plant cane in relation to insecticide application. Based on the mean of eight sticky traps per treatment and control (UTC). The blue and red arrow respectively represent the 1500 °Cd and 2000 °Cd application timings.

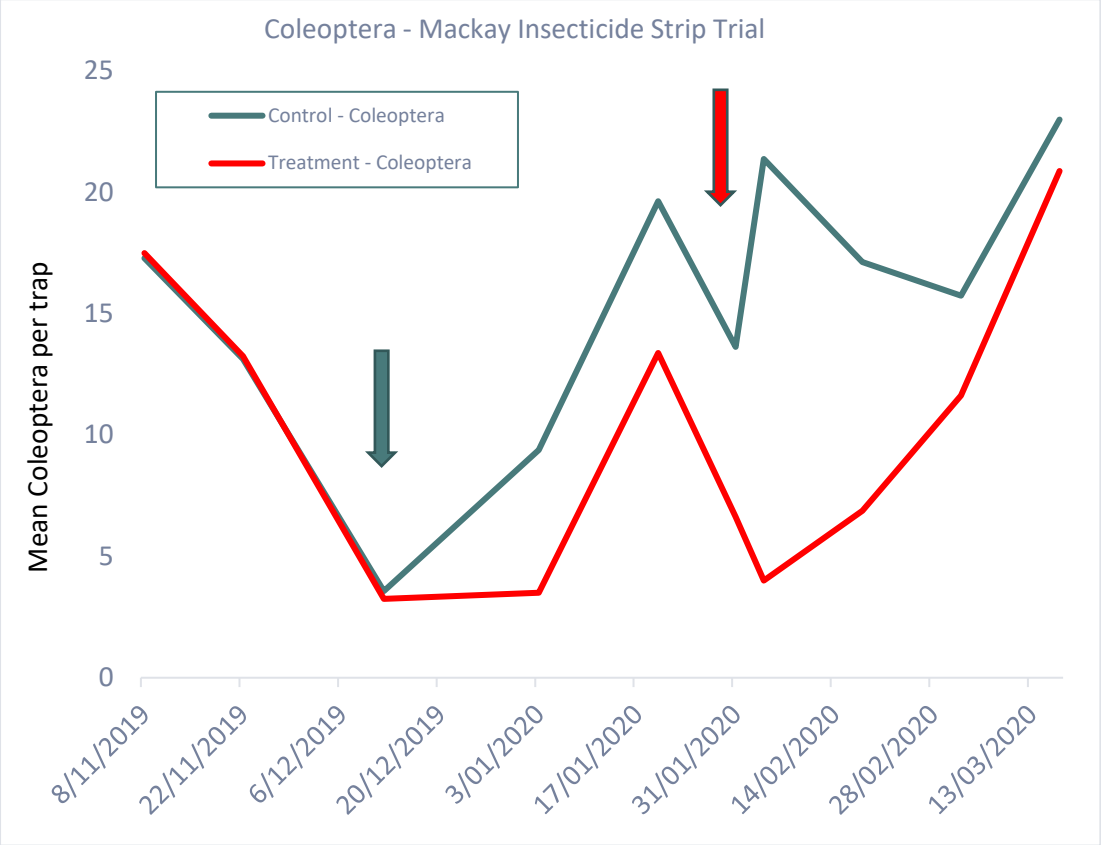


Figure 318 Mean Coleopteran abundance per yellow sticky trap in sugarcane canopy of plant cane in relation to insecticide application. Based on the mean of eight sticky traps per treatment and control (UTC). The blue and red arrow respectively represent the 1500 °Cd and 2000 °Cd application timings.

Final yield

Due to the wet conditions during harvest only mill data is available. There was no difference in mean cane yield tonnes cane/treatment and CCS bifenthrin treated and untreated controls under commercial conditions (Figure 319 A & B).

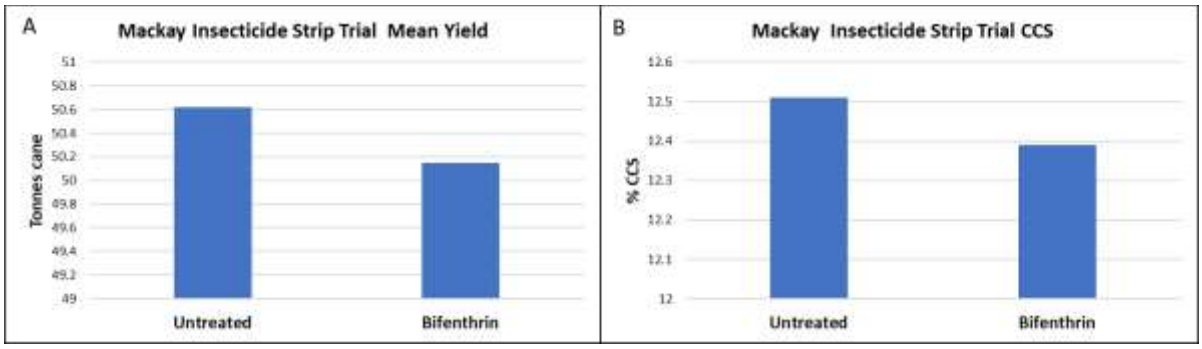


Figure 319 Mackay commercial insecticide strip trial mean yield treated and untreated control A) and CCS B). Note: cane yield and CCS data supplied by Mackay Sugar Ltd.

Summary

Leaf +4 levels of sucrose and total α-glucan were elevated prior to the first application of bifenthrin. However, after significant rainfall in the following four months levels of both metabolites fell to that of a healthy diurnal profile. There was no significant difference in leaf metabolites and yields between treatments.

Bifenthrin applications supressed mites and leafhoppers but not mealybugs and thrips. By the time of the first application @1500 °Cd (early December) thrips had naturally declined to minimal levels. Mealybugs had multiple population peaks which indicates that as rainfall events were sporadic (only one day high rainfall events in January, February and March) this was influencing population migration from below to above-ground. Mealybugs are particularly susceptible to rainfall often washing off the canopy and stem and migrate down to the root system. It is likely mealybug populations did recover from these one-off events but bifenthrin was unable to supress the resurgent populations. Coleoptera (predominantly predatory beetles) were severely impacted by bifenthrin and as they predate on small nymphs of mealybugs. This may also have influenced mealybug population abundance. However, only minimal YCS symptoms were seen at this field site and arthropod populations may have been impacted marginally by rainfall events. Therefore, conclusions cannot be drawn as to the influence of insects or mites on YCS expression.

As the trial site was devoid of YCS symptoms in both the treated and untreated cane, no conclusions can be made about the efficacy of bifenthrin application under commercial conditions to manage YCS.

6.9.7.4 Maryborough site

At the Maryborough field site, the trial was originally established to examine the impact of insecticide on YCS. However due to extreme waterlogged soil no insecticide applications could be applied. Consequently, only trap data from untreated control plots and border traps is presented.

1. Mealybugs

Mealybug populations were extremely low (Figure 320) this may have been due to the rainfall events impacting population build up. Interestingly at this site more mealybugs were detected in the field borders than on the plant cane throughout the sampling period. This is in contrast to all other field sites in this report.

2. Mites

Mite populations were relatively low at this site (Figure 321). This low abundance may have been due to the rainfall events impacting population build up. However, my early March a single peak in abundance was observed (18 mites per trap in UTC). At this site very few mites were detected in the field borders throughout the sampling period.

3. Leafhoppers

Leafhopper populations were relatively low at this site until February (Figure 322) and then there was a gradual build up to >80 leafhoppers per trap in the control. This low abundance initially may have been due to the rainfall events impacting population build up. At this site very few leafhoppers were detected in the field borders (<7 per trap) throughout the sampling period.

4. Thrips

Thrip populations were relatively low at this site reaching a maximum peak of 60 (Figure 323). This low abundance may have been due to the rainfall events impacting population build up. By mid-January thrip populations had started to decline in the crop but remained more stable in the field borders.

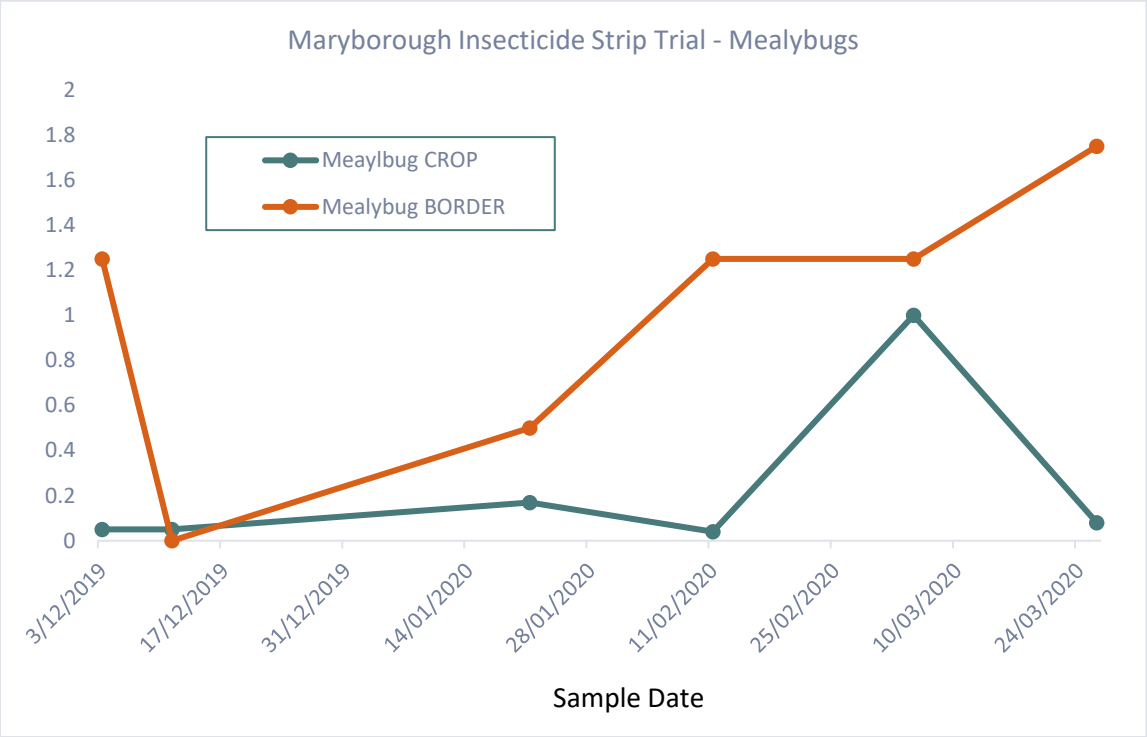


Figure 320 Mean mealybug abundance per yellow sticky trap in sugarcane canopy of plant cane in relation to insecticide application. Based on the mean of 20 canopy sticky traps in untreated control (CROP) and four field border (BORDER) traps.

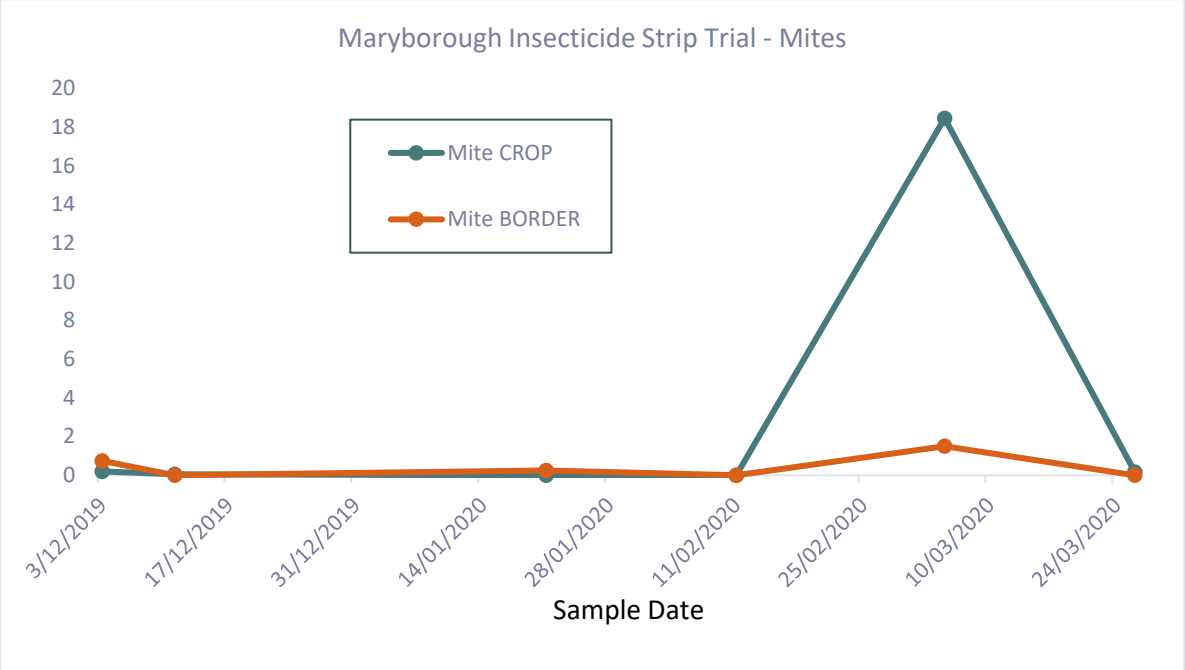


Figure 321 Mean mite abundance per yellow sticky trap in sugarcane canopy of plant cane in relation to insecticide application. Based on the mean of 20 canopy sticky traps in untreated control (CROP) and four field border (BORDER) traps.

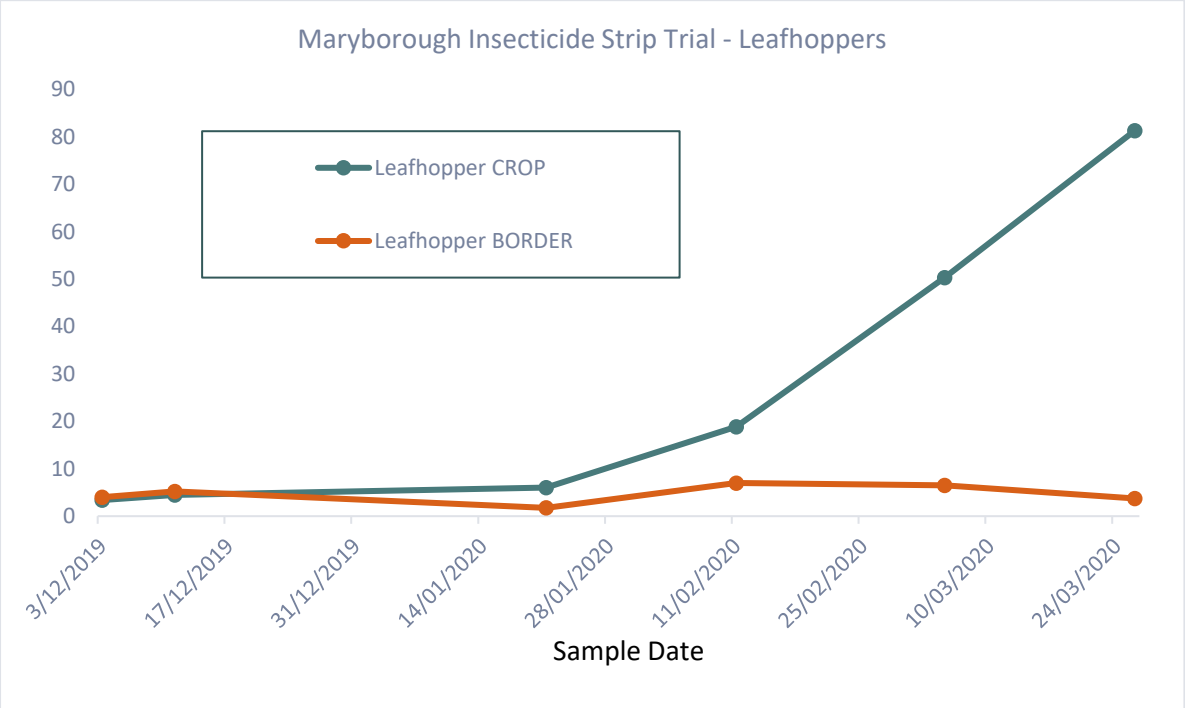


Figure 322 Mean leafhopper abundance per yellow sticky trap in sugarcane canopy of plant cane in relation to insecticide application. Based on the mean of 20 canopy sticky traps in untreated control (CROP) and four field border (BORDER) traps.

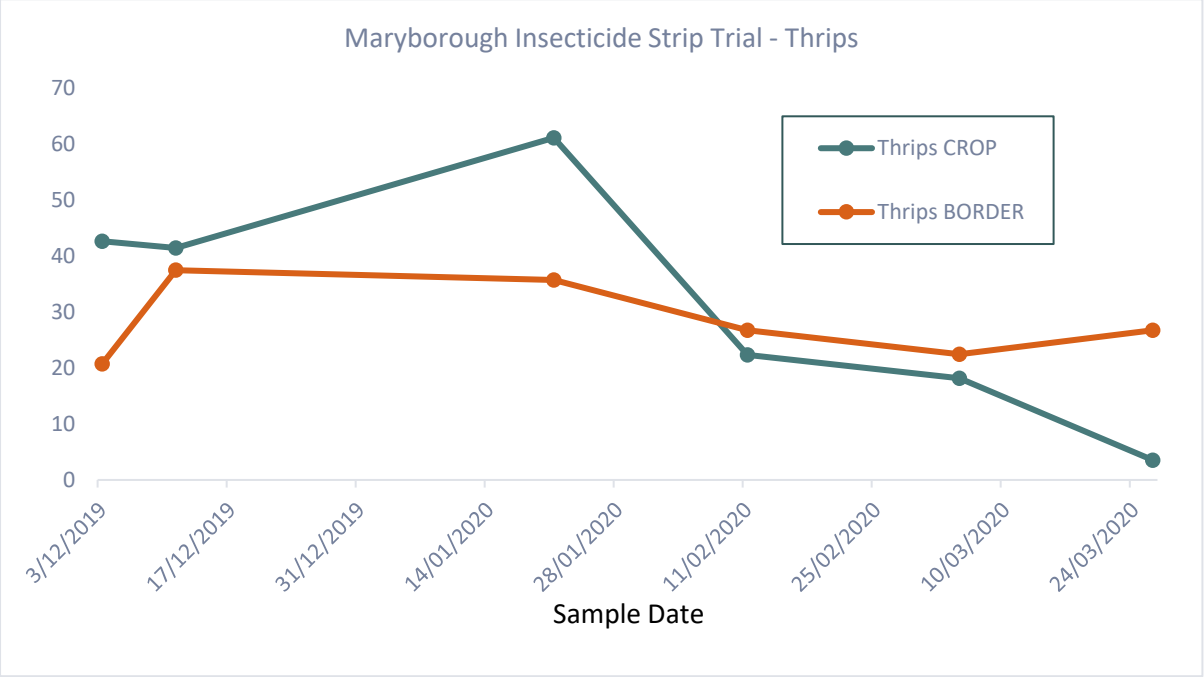


Figure 323 Mean thrip abundance per yellow sticky trap in sugarcane canopy of plant cane in relation to insecticide application. Based on the mean of 20 canopy sticky traps in untreated control (CROP) and four field border (BORDER) traps.

Summary

Bifenthrin could not be applied at this field site and only data on arthropod populations has been presented. No YCS was seen at all at the site and heavy rainfall would have likely cause waterlogged soils and impact on arthropod populations. This is very evident for mealybugs which are both root and stem/leaf feeders which had <1 mealybug per trap throughout the monitoring period. Other foliar feeders like mites and leafhoppers did start to resurge in February and March as rainfall events reduced in intensity. As YCS symptoms were not seen at this field site and arthropod populations were likely to have been severely impacted by rainfall events and waterlogged soils, conclusions cannot be drawn as to the influence of insects or mites on YCS expression.

6.9.8 Entomology summation

Insect and mite identification

Identification

Identification of insects and mites in this study has proved a challenge. Because most insect pests of sugarcane are regarded as only minor pests, with the exception of cane grubs and soldier flies, most SRA literature has no records of many of the species monitored. Studies on the diversity of insects and mites in sugarcane canopies and field borders are extremely limited in wider scientific literature. This has meant that there were no available simple methods of identification for most of the species caught in traps at the multiple field site locations. Literally thousands of insects and mites and well over one hundred different species were collected during this study. Identification relies on

specialist taxonomists for identification. Some species could be identified using taxonomic keys whilst others required specialist skills including dissection of adult male genitalia. As there was a large diversity of species only selected species were therefore identified to species level, although all have been retained for future taxonomic studies. A summary of some of the main species identified is shown in Table 64.

Seasonal Population Dynamics

The population dynamics of insects and mites can only really be effectively studied at multiple sites over multiple consecutive seasons. This was not always possible in this study. Multi season studies are important as firstly YCS expression appears to differ in intensity and location. Weather conditions especially large rainfall events are likely not only to influence YCS expression levels but also insect and mite population dynamics. As shown in Table 63 abundance peaks and relative abundance can vary markedly between sites in different regions. It was also clear that peak abundance varied between seasons. Compared with 2018/2019 trial data it is apparent that for the 2019/2020 season had lower insect abundance. This also coincides with lower YCS expression in 2019-2020.

Table 63 Summary of selected insect and mite abundance on untreated sugarcane at eight field sites in the 2019-2020 season. Showing key insect group with the timing of peak abundance and maximum number per trap.

Site & trial	Mealybug	Mites	Leafhopper	Thrips	Planthoppers
Mackay - Insecticide	15 (4 peaks) *Mid Nov* *Early Jan* *Early Feb* Mid MAR	51 (2 peaks) Early JAN *Mid Feb*	8 (1 peak) Late JAN	84 (1 peak) Late NOV	7 (1 peak) Mid FEB
Maryborough	1 (1 peak) Mid MAR	18 (1 peak) Early MAR	81 (1 peak) Late MAR	60 (1 peak) Mid JAN	4(1 peak) Early MAR
Rinella - Burdekin	56 (1 peak) Late DEC	8 (1 peak) Late Dec	17 (2 peaks) Mid NOV *Late Dec*	38 (1 peak) Mid NOV	15 (1 peak) Late MAR
CGF - Ingham	125 (1 peak) Early MAR	9 (3 peaks) *Early Jan* Mid FEB *Late Mar*	3 (2 peaks) Early Mar *Late DEC*	350 (1 peak) Early DEC	<1 (1 peak) Late JAN
RVT - Ingham	8 (1 peak) Late DEC	35 (2 peaks) Late JAN *Late Mar*	3 (1 peak) Late OCT	716 (1 peak) Early NOV	<1(1 peak) Early NOV
Burdekin Station - Insecticide	23 (1 peak) Mid MAR	137 (1 peak) Late MAR	36(1 peak) Mid FEB	44 (1 peak) Late NOV	3 (1 peak) Late MAR
Mirriwinni – FNQ*	2 (1 peak) Mid MAR	6 (1 peak) Late DEC	38 (3 peaks) *Late Jan* Early FEB *Early Mar*	125 (1 peak) Mid NOV	1(1 peak) Mid FEB
Mount Sophia – FNQ*	5 (2 peaks) Late NOV *Mid Feb*	6 (1 peak) Early JAN	6 (3 peaks) Mid NOV *Late Dec*	184 (1 peak) Mid NOV	<1(1 peak) Late MAR

			Mid Feb		

*sites monitored in FNQ but data not presented as no YCS expression.

Future studies – Arthropods and YCS

No definitive association between any particular arthropod group and YCS expression could be determined from the studies conducted to-date. However, that does not mean that arthropods are not involved in YCS expression. It is recommended to continue to examine the potential association between arthropods and YCS – one major reason being that clearly selective use of pesticides (Confidor and Bifenthrin), seem to reduce YCS symptom expression therefore implies that some insects or mites may be involved.

What our research has shown is that some arthropod groups are unlikely to be involved in YCS expression for a number of reasons (i) their early peaks in abundance which are well before YCS expression, (ii) there relatively late peak abundance after YCS expression has occurred or (iii) their low relative abundance (although sampling methodology could bias this assumption).

Based on the studies conducted so far the arthropod groups not recommended for further study therefore include:

1) **Thysanoptera (Thrips).** At least five species of were observed in sticky traps and were the most abundant group detected. Thrips are extremely difficult to identify. Thrips are very delicate insects and removal from sticky traps without damaging morphological features required for identification is extremely difficult. However, one species could be identified to species level and this species was a known minor pest of sugarcane. Thrips peak in abundance was (i) always much earlier than YCS expression (generally at least 2 months) and (ii) in sites with very high abundance, especially FNQ (2018-2019 season), YCS expression was not observed at all. It is however recommended to identify all species collected.

2) **Fulgoroidea (Planthoppers) and Lophoidea.** Although at least three species of planthopper were observed the most abundant species was the sugarcane planthopper *Perkinsiella saccharicida.* Perkensiella peaked in abundance much later than YCS expression (March-April) and was in low abundance even in sites with YCS expression. *Lophops saccharicida* was also detected at some sites. This species is known to feed on sugarcane and was detected in low abundance however it would be worth examining in future studies as it is quite possible that its abundance was underestimated using the sampling techniques used. It is however recommended to identify all species collected.

3) **Aphids.** Aphids were very low in abundance at all sites (usually <1 insect pre trap) despite the fact that yellow sticky traps are the main method of sampling for this insect group. Due to their relatively low abundance, they were quantified but not identified to species level and are highly unlikely to be associated with YCS.

4) **Heteroptera (True bugs and Linear bugs).** Although Linear bugs and other true bugs were observed and caught in sticky traps their abundance was relatively low and even when abundant this was generally in March – April. Linear bugs were studied earlier in this project and even though they cause some crop yellowing this is different to YCS yellowing.

However, there are some groups which do warrant further study as follows:

(i) **Cicadellidae (Leafhoppers)**. Although leafhoppers are not reported as pests of sugarcane in Australia multiple species (6-10) were detected in yellow sticky traps albeit in low abundance. However, it is likely that leafhoppers were present in higher abundance and a different type of sampling (such as pan water traps) may be required to get a more representative picture of their abundance. The Cicadellidae group of leafhoppers is known for its ability to vector phytoplasmas. Within this study several different species (some awaiting identification) were present including at least two genera which are known as phytoplasma vectors. These leafhoppers were not tested for phytoplasma and future studies could examine this aspect. Another relatively abundant species detected is known as a root feeder on sugarcane so further study of this species would require a root sampling method to be developed.

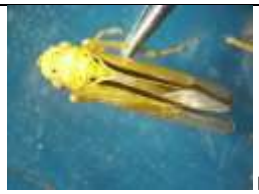
(ii) **Pseudococcidae and Coccidae (Mealybugs and scale insects)**. Mealybugs and scale insects can sometimes be misidentified by non-entomologists and both groups are phloem feeders. Mealybugs can also be vectors of viruses and phytoplasmas. In this study two species of mealybug were confirmed. One species, *Saccharicoccus sacchari* is common to most sugarcane fields in Australia but is only regarded as a minor pest. It usually congregates around the stalk nodes protected by the leaf sheath. This was the species detected in relatively high numbers on sticky stem traps and in yellow sticky traps. If this mealybug were to be involved with YCS this is only likely if (i) it is in very high abundance (ii) sugarcane varieties are more susceptible to attack (possibly due to higher sucrose contact in phloem) or (ii) they are vectoring an as yet undetected pathogen. A second species detected was *Heliococcus summervillei* the pasture mealybug which is associated with Pasture Dieback disease. This species was caught in relatively low abundance in yellow sticky traps but because it was observed on the underside of leaves expressing YCS yellowing and in higher abundance than in traps. This species has not been reported on sugarcane in Australia before but has been reported on cane in India and is found on Paspalum in Australia. Scales were detected in sticky traps but in very low abundance (insufficient for identification purposes) and are therefore not shown in any of the chapters. Monitoring of scale insects is usually only effective by visual observations of leaves and stalks which was not conducted in this study. There is a species of sugarcane scale insect called *Aulacaspis* know to be a minor pest of sugarcane in Australia.

(iii) **Acarina (mites)** In this study at least five species of mites were detected some of which are predatory, and others known minor pests although none have yet been identified to the species level. One genera of mite known to impact cane as a minor pest only was *Oligonychos* species. This spider mite species is usually found on the leaf surface and feeds on phloem sap. It is very small and transparent to the naked eye. Unfortunately, it can only be identified to species level through male morphology and the insects collected in sticky traps were predominantly female. The predatory mite species at most sites appeared later in the season in March and is likely to predating on small insects such as smaller mites, mealybug and planthopper nymphs. Yellow sticky traps may not be the best method for collecting mites and could underestimate abundance as some mites are very small and transparent.

(iv) **Other groups.** In our studies we have monitored multiple insect and mite groups and have specifically focused on those groups that are sap feeders. Even though in some instances (such as pasture mealybug and some leafhoppers) we may have underestimated abundance it is unlikely that there are other above ground insect or mite pests which we have missed during sampling. Even with above ground sampling we have detected insects which spend part of their life cycle on the roots and above ground (e.g. root-feeding leafhoppers and mealybugs). It would certainly be worth further exploring these two insects which alternate between above- and below ground habitats. The only other group of insects which could be underrepresented are those that feed exclusively on the roots such as earth pearls. Although these have not known to be associated with crop yellowing they are certainly sap feeders and feed on sugarcane and grass roots and are regarded as a minor pest in some regions of QLD. Further studies could examine these insect pests.

Table 64 Examples of key selected insect and mite with confirmed identifications on sugarcane in field studies conducted 2018-2020.

Insect Group	**Common name**	**Hosts**	**Vector**	**Image & Identifier**
Mealybugs				
Heliococcus summervillei	Pasture mealybug	Paspalum (Australia), Sugarcane (India) – causes pasture dieback?	Possible vector	 M. Schultz
Saccharicoccus sacchari	Pink sugarcane mealybug	Sugarcane	UNK	 M. Schultz
Thrips				
Anaphothrips sudanensis		Grasses (Poaceae), sugarcane	UNK	 L. Derby
Mites				
Gaeolaelaps species?		Predator	NO	J. Beard
Oligonychus species?		Sugarcane	UNK	 J. Beard
Oribatida	Beetle mites	Generally soil feeders some are predators	NO	 J. Beard
Leafhoppers				
Myrmecophryne formicetecola		Root feeder on sugarcane and grasses	UNK	 M. Fletcher
Cicadulina bimaculata		Multiple incl. sugarcane	Known phytoplasma vector	 B. Loecker

Scaphoideus foshoi		Multiple	Known phytoplasma vector	B.Loecker
Conoguinula coeruleopennis		Grasses and sugarcane	UNK	 K. Powell
Exitianus plebius?		Grasses and sugarcane	Possible phytoplasma vector	 B. Loecker
Planthopper				
Lophops saccharicida		Sugarcane	Possible phytoplasma vector	 B Loecker
Perkensiella saccharicida		Sugarcane	Known virus vector	 K Powell
Coleoptera				
Phyllotocus sp.	Nectar Scarab beetle		NO	 J. Bartlett
Monolepta australis	Red shouldered leaf beetle	soybeans, sugarcane & cotton	NO	 J. Bartlett
Halmus chalybeus	Steel blue ladybeetle	Feed on Feed on aphids, scales and small insects.	NO	 J. Bartlett
Coelophora inaequalis	Variable ladybeetle	Feed on aphids, scales and small insects.	NO	 J. Bartlett

Hispellinus multispinosus	Spiny leaf beetle	Grasses	NO	 J. Bartlett

6.9.9 Discussion and conclusions

Unfortunately, the exclusion tents were unable to exclude or contain very small insects and were structurally problematic under adverse weather conditions. Therefore, from these experiments it was not possible to ascertain if certain insects were involved in YCS development, either directly, or by vectoring a pathogenic agent. This type of experiment is notoriously difficult to execute under field conditions.

The onset of mid-canopy yellowing following good rainfall was a common element within the Burdekin insecticide field trials. In 2018 YCS symptoms appeared in last week of January, while in 2019 and 2020 expression was first noted in the last week of February. Preceding each of these events substantial precipitation was received followed by high temperature and solar radiation. This concurs with studies by Scalia et al. (2020). In 2018 symptoms progressed and peaked in mid-March and early April in 2019 and 2020. In all three trials bifenthrin weekly was the most effective treatment in maintaining leaf +4 sucrose content below the upper tolerance level of approximately 200 µmol/g DM. All leaf punch samples for metabolite assays were collected prior to 8AM and low levels of total α-glucans reported in plants treated weekly with bifenthrin are reflective of a healthy diurnal profile. Although efficacy was lower than the weekly treatment, bifenthrin treatment at 1500 and 2000 °Cd was effective in reducing YCS symptoms, and one-month only treatments were shown to mitigate YCS for up to 3-months under experimental conditions. It is highly likely that this long-term protection is attributable to reportable levels of bifenthrin residue found on sugarcane tops and culm up to 2.4 and 4.6 months after application, respectively. It also suggests that insects controlled by bifenthrin are unlikely to be highly mobile. Evidently, the ineffectiveness of bifenthrin treatment at peak YCS to reverse or halt YCS, confirms that the cause of YCS precedes the onset of leaf yellowing which cannot be reversed. Targeted bifenthrin treatment at 1500 and 2000 °Cd time points to the culm base were somewhat effective, while sprays to the meristem region of the stalk was ineffective in preventing or reducing YCS symptom expression. However, while not statistically significant, the bifenthrin base treatment produce the highest cane yield. This suggests the higher involvement of a crawling insect or arachnid. Streptomycin and the alternative pyrethroid permethrin were the least effective treatments.

Bifenthrin treatment promotes an increase in internode growth, producing a larger culm sink. This suggests that insects are either directly involved or vectoring an agent that leads to reduced culm growth. As YCS pathology studies (see section 6.8 of this report) have been unable to identify a phloem blocking pathogen it is likely that insects are impacting growth directly. High insect pressure which is primarily driven by favourable weather conditions and food availability, may cause a substantial diversion of plant resources from growth to defence. This may also lead to a population increase of opportunistic non-pathogenic endophytic organisms that could slow phloem transport. One such organism with the potential to do so, and which consistently appeared in PCR screening

and sequencing, is *Curtobacterium spp.* (see section 6.8 of this report) (Singh et al., 2018). Crop age studies (see section 6.5.1 of this report) revealed that growth rate is the key driver of YCS, and this concurs with increased internode growth in response to bifenthrin treatment. Any change to sink strength in close proximity to the source leaves of the mid-canopy that are usually affected by YCS, will either prevent or induce YCS development. Disruption to the source sink balance where supply exceeds demand will present as sucrose accumulation in the source leaf. Chlorophyll fluorescence studies show a clear disruption to the PET chain well before the onset of yellowing and is tightly correlated with leaf sucrose levels. Bifenthrin treatment prevented high sucrose accumulation in the source leaf and disruption to the photosystems. Leaf +4 sucrose content is therefore an excellent measure of source leaf health. Levels of this metabolite exceeding 200nmol/mg DM is considered toxic to the leaf, triggering a disruption to the photosystems I & II, degradation of the chloroplast, cell death and leaf yellowing. Leaf sucrose is well aligned with internode growth, the onset of yellowing and YCS severity. High levels of soluble and insoluble α-glucans are a result of carbon repartitioning in the leaf to draw down the carbon load. This altered carbon partitioning in the leaf is an attempt to offset damage to the photosystems caused by sugar mediated downregulation of the photosynthetic apparatus and underutilisation of trapped solar energy (Marquardt et al., 2016; Marquardt et al., 2017).

A lack of symptom expression in the Herbert varietal trial for two consecutive years does not allow for any firm conclusions to be drawn on varietal susceptibility to YCS. Leaf +4 sucrose and glucan levels were consistently low in both the bifenthrin treated and untreated controls. Therefore, no conclusions can be made about the efficacy of bifenthrin to prevent YCS development or the impact of insects in these trials.

Little to no YCS was recorded in any of the three commercial strip trials. Leaf +4 sucrose and glucan content confirm the lack of YCS expression in both the bifenthrin treated and untreated controls. Therefore, the efficacy of bifenthrin treatment to manage YCS under commercial conditions cannot be concluded from any of the three site trials.

It is evident that the pyrethroid bifenthrin was the most effective insecticide and treatment to maintain low levels of source leaf sucrose and total α-glucan across all of the trials. However, the prevention of YCS development and expression did not equate to a significant yield benefit in either TCH or CCS. Furthermore, there was no correlation between YCS severity and yield.

Depending on weather events and time of year there is a correlation between certain insect and mite population spikes prior to the first major rainfall event after which YCS development is triggered. This suggests that insect pressure in general is directly impacting on plant growth and altering sink strength prior to the period of rapid growth and photoassimilation after the rain event. Obviously, different species and groups of insects or mites will be more or less abundant depending on various factors such as weather events, food availability, waterlogging, temperature and predation. It is evident from the data that these influences on insect and mite populations dynamics vary from year to year. This will therefore influence when this type of biotic pressure will have the greatest impact on plant growth. If the period prior to the rain event was also very dry the combined impact of abiotic and biotic stress on growth rate would exacerbate the source sink imbalance and heighten the onset of YCS yellowing and severity. However, in the absence of a vectored pathogen, the bifenthrin suppression of large insect populations driven by favourable conditions, would enable a diversion of resources from plant defence to growth. Similarly, a larger internode sink can be

grown after good rainfall following a dry period of impeded growth. In either scenario, or a combination of both, improved culm growth in close proximity to the mid-canopy source leaves enables improved phloem transport between the source and sink. If adequate supply of reduced carbon meets the storage and metabolic demands of the sink, and leaf sucrose levels are maintained below the upper tolerance threshold, YCS development and expression will be prevented. Evidently a constant supply of good rainfall and growing conditions can counter any potential impact by insects on growth as seen in the RVT and commercial strip trials. This was confirmed by metabolite and internode analyses.

The key message here is that preventing the slowdown of crop growth by removing either abiotic or biotic stress on the crop during the period of high photoassimilation and high solar radiation, will reduce the risk of YCS development and expression. Therefore, whichever treatment or farm management practice removes or reduces the most dominant stressor impacting crop growth during the peak growing season, will be how the development of YCS can be prevented or managed.

6.10 Diagnostics

Leaf yellowing in sugarcane is a common condition as leaves are impacted by heat, water stress, pests and diseases, nutrient deficiencies, pathogens, agrochemical phytotoxicity and natural senescence. Thus, without a unique diagnostic, identification of YCS is difficult. Metabolic analyses show that sucrose and starch content is elevated in both asymptomatic and symptomatic leaves on the same culm (Marquardt et al., 2016; Scalia et al., 2020). When leaf samples taken at first light were analysed, high levels of sucrose and starch content indicated a complete disruption to the normal diurnal oscillation of these two metabolites (Marquardt et al., 2016; Marquardt et al., 2017). High leaf carbohydrate accumulation is known to cause leaf yellowing in the *Poaceae* (Fontaniella et al., 2003) suggesting that either sucrose or starch may be useful in developing a YCS diagnostic when coupled to unique YCS characteristics. Other parameters investigated as potential diagnostic indicators are silica/ magnesium, leaf fluorescence, chlorophyll and water content.

Leaf +4 has shown to demonstrate significant differences between YCS and asymptomatic green control plants over the range of diagnostic indicators. Therefore, sampling to mid-section of this leaf was the standard used for these analyses.

Evaluating Diagnostic Indicators

Sampling was conducted at six field sites in the Burdekin. Three sites were YCS symptomatic and three were green asymptomatic. A summary of site characteristics is presented in Table 65.

Table 65 Summary of site characteristics for the six diagnostic evaluation sites. Means for 20 stalks per site ± standard error.

Site #	ID	Variety	Class	Sample Date	Crop age (days)	Stalk Height (cm)	Leaves per Stalk	% Symptomatic Leaves
1	Control	Q240	1R	09-Feb-16	212	148.8	11.0	0.0
2	Control	KQ228	Plant	26-Feb-16	205	188.1	12.2	0.0
3	Control	KQ228	Plant	18-Mar-16	229	215.9	11.1	0.0
					215.3 (±1.3)	184.3 (±5.2)	11.4 (±0.22)	0.0
4	YCS	Q240	1R	09-Feb-16	212	234.2	7.8	22.4
5	YCS	KQ228	4R	19-Feb-16	179	154.2	6.9	58.5
6	YCS	KQ228	4R	23-Feb-16	222	219.5	7.7	33.9
					204.3 (±2.4)	202.6 (±4.4)	7.4 (±0.14)	38.2 (±2.9)

Sites were chosen which were strongly symptomatic and asymptomatic and represented both plant and ratoon crops. Asymptomatic sites remained YCS-free for the entire season.

6.10.1 Water Content

YCS affected cane showed significantly greater relative water content (RWC) % than Control cane (Figure 324). YCS averaged 94.8% compared with 90.9% for Control. This is an unusual result which may have been influenced by Leaf +4 being YCS asymptomatic in both fields at the time of sampling. Cane at all sites appeared fully turgid. It is possible that the higher RWC of YCS Leaf +4 is due to the increased sucrose contents of these leaves which reduces the water holding volume of the cells. Comparison with water content studies in a similar field shows that YCS symptomatic Leaf +4 has approximately 65% water content while controls have approximately 75% (Figure 325). High leaf sucrose content causes a stomatal conductance penalty of approximately 42% and as high as 58% in YCS leaves (Marquardt et al., 2016). The reduction in transpiration is initiated by high concentration of sucrose in the apoplastic space of which some enters the transpiration stream. This in turn causes abscisic acid (ABA) mediated hexokinase increases in the guard cells which triggers stomatal closure (Kelly et al., 2013). Leaf sucrose levels fluctuate with the diurnal rhythm and amount of disruption to phloem transport or sink demand (Black et al., 1995; Du et al., 2000; Koch, 2004; Morey et al., 2019). Therefore, RWC or water content variability would make this indicator unsuitable for a YCS diagnostic tool.

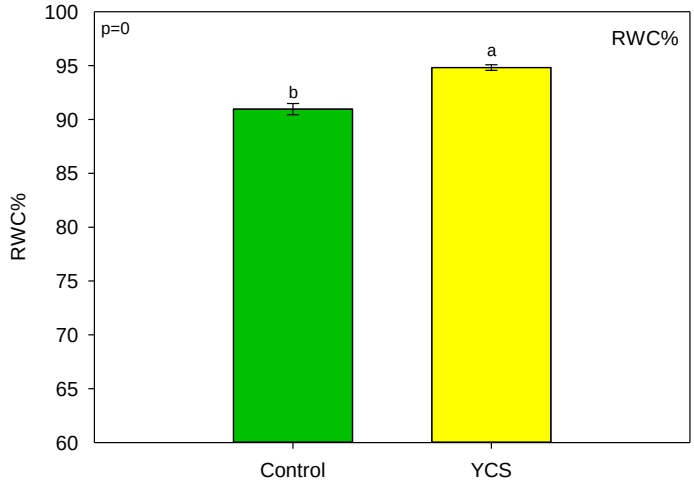


Figure 324 Relative water content (%) of leaf +4. Means for 60 leaves (20 leaves per site x 3 sites) ± standard error. Analysis of variance by two sample T-test (p<0.05) with LSD pairwise comparison shown by letter separations a, ab, b etc.

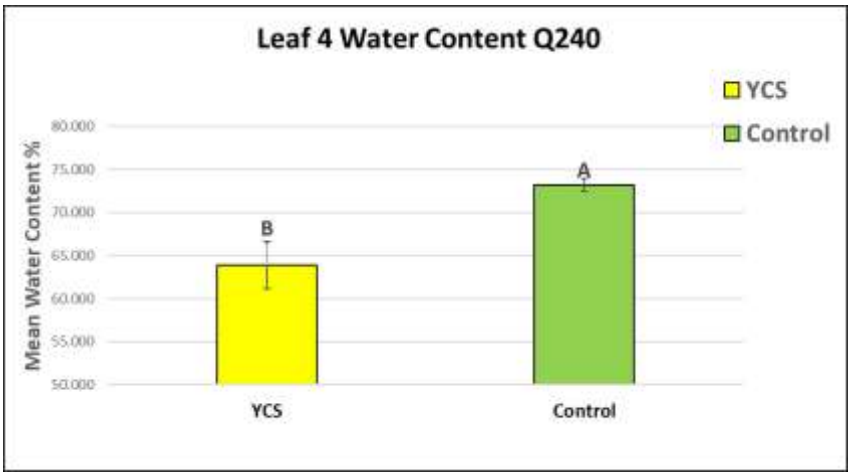


Figure 325 Water content Leaf +4 Means for 20 leaves ± standard error. Analysis of variance by two sample T-test (p<0.05) with LSD pairwise comparison shown by letter separations A, B

6.10.2 Chlorophyll Fluorescence

Chlorophyll fluorescence is known to be the most sensitive tool to measure the efficiency of photosynthesis and, consequently, can be used as an indicator of plant general vitality (Maxwell and Johnson, 2000). Fluorescence can give insights into the ability of a plant to tolerate environmental stresses and also the extent to which those stresses have damaged the photosynthetic apparatus (Maxwell and Johnson, 2000).

The kinetics of chlorophyll fluorescence after dark adaptation can provide valuable additional information on the organisation, efficiency, and linkage of electron transport (Strasser et al., 2004). The polyphasic rise in chlorophyll-a (Chla) fluorescence (OJIP) can be used to investigate the

behaviour of PSII with functional parameters calculated by the JIP-test (Strasser and Strasser, 1995). Analysis of the fast fluorescence rise according to the JIP-test allows establishment of structural and functional parameters, providing a quantification of the system's behaviour (Strasser and R.J., 1995; Kruger et al., 1997). Additionally, the OJIP fluorescence curve analysis can be used to monitor the effect of various biotic and abiotic stresses affecting the structure and function of the photosynthetic apparatus (Strasser *et al.,* 2004).

Under intense actinic light, a sequence of Chla fluorescence energy states is evident as a series of steps (OJIP). The O-J steps represent the electron acceptor of PSII from the ground state or minimal (O=F0) to the J peak (J=Fj) which occurs at 2 ms after the light exposure. The O-J step has been described as corresponding to the photochemical phase (Schreiber and Neubauer, 1987; Eullaffroy et al., 2009) Whereas, J-I phase corresponds to plastoquinone (PQ) quenching which (FI) takes place between 20-30 ms post illumination. The I-P (Fp=Fm) peak is the result of maximum concentration of fluorescence yield resulting from oxidation of plastocyanin (PC) as well as photo-oxidation of P700+ in and about PSI (Strasser et al., 2000; Schansker et al., 2003; Oukarroum et al., 2009; Tóth et al., 2011)

Chlorophyll fluorescence was measured on Leaf +4 at all sites. Results show the average fluorescent curve for YCS leaves falls below that of Control leaves (Figure 326 A). This indicates that there are fewer reaction centers participating in electron capture and transfer. When the data is normalized and the Control values subtracted from the YCS over time, the location of the photosynthetic disruption becomes apparent (Figure 326 B and C). YCS leaves show disruption in electron transport from photosystem II to photosystem I (shown be the peak around the J and I step - Figure 326 C). This is interesting given that these leaves were generally asymptomatic at all sites – YCS symptoms were seen in lower leaves at YCS sites.

A closer look at some of the OJIP parameters, which describe photochemical structural and functional organization as well as various fluxes through the electron transport chain, provides clearer picture of how these findings can be used as a potential diagnostic tool. Of the 50+ OJIP parameters measured and calculated, the 10 most highly significant are shown in Figure 327. These parameters describe a photosynthetic apparatus which has fewer functioning reaction centers (Fv and REo/RC), reduced electron transfer from Qa into the transport chain (Sm and N), and reduced efficiencies of electron transfer from Qa to Qb (psiEo, phiEo and phiRo). All of which is captured in the performance index and driving force parameters (Pi Total and DF Total respectively). These parameters may be the most applicable chlorophyll fluorescence based diagnostic. PI Total represents a performance index for energy conservation from photons absorbed by PSII antenna until the reduction of PSI acceptors while DF Total represents the total driving force until PSI acceptors. Both of these parameters are highly significant.

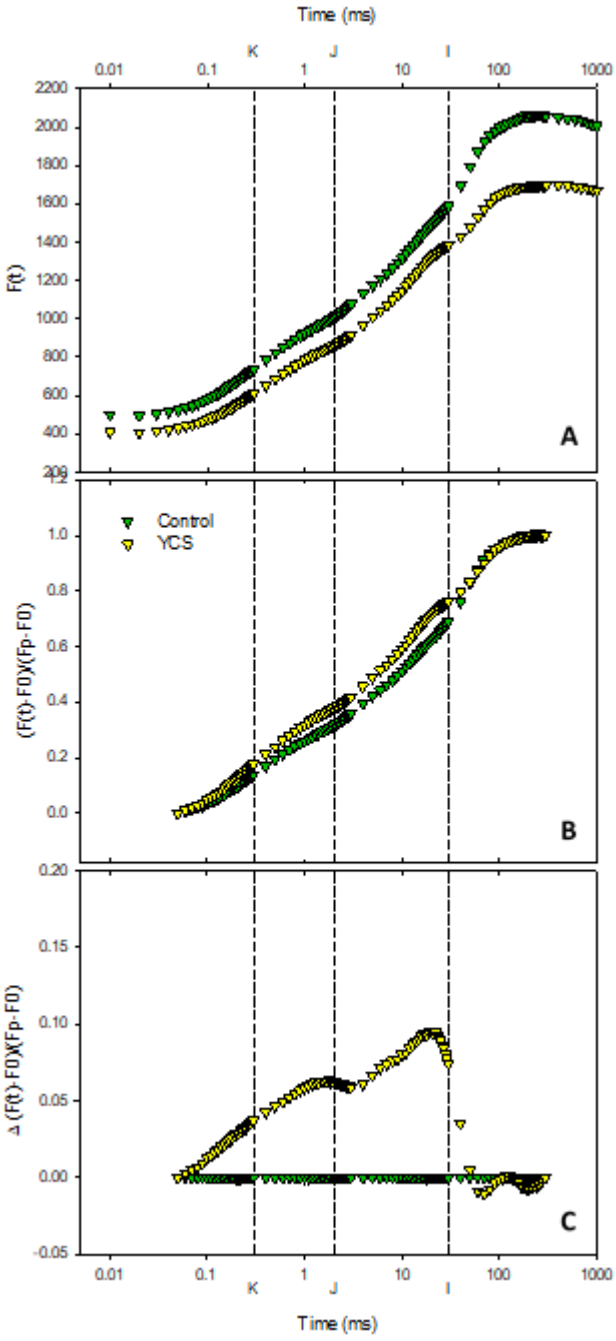


Figure 326 Leaf +4 fluorescence transients for YCS symptomatic (yellow) and asymptomatic (green). Non-normalised fluorescence transients (A), transients normalised between F0 and Fm (B), and delta Δ fluorescence shown after subtraction of green Control (C). Time points corresponding to OJIP steps are shown by the vertical dashed lines. Each data point is the mean of 60 samples.

It is evident from the data that there are signs of electron uncoupling and disruption of electron flow between photosystem II (PSII)and photosystem I (PSI) even in asymptomatic leaf +4 of a YCS plant. This will eventually lead to a significant decline in photosynthetic efficiency which can be evaluated through PI abs (Strasser et al., 2000). This concurs with studies by Marquardt et al. (2016) that show reduced photosynthetic activity by up to 36% in two varieties affected by YCS. However, a reduction in electron transport efficiency and increased fluorescence emission has also been recorded in

several studies of water stressed sugarcane plants (Silva et al., 2008; Graça et al., 2010; Silva et al., 2013). Therefore, use of chlorophyll fluorescence to discern between YCS and water stress as an effective indicator of YCS is inconducive.

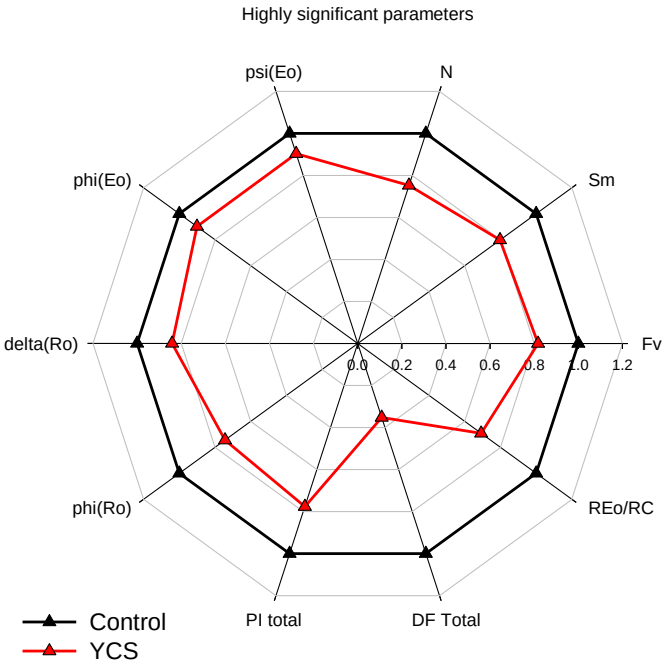


Figure 327 Radar plot comparing fluorescence parameters for Control and YCS-affected leaves. Values for the YCS treatment have been normalised to their corresponding Control so that the Control has values of 1 for all parameters and the stress treatment is expressed as a proportion of the control. All parameters were statistically significant at the p<0.001 level (n=60)

6.10.3 Silica, Magnesium and the Si:Mg ratio

Results from the six fields in the Burdekin diagnostic study showed that Silica and Magnesium levels were elevated in YCS leaves, however there was no difference in Si/Mg between YCS and Control (p=0.93) (Table 66). This result is contrary to results obtained in previous investigations (see section 6.2 of this report).

Table 66 Silica, Magnesium and Silica:Magnesium ratio for diagnostics sites. Means ± standard error. Statistical significance shown (t-test p<0.05). There were 3 reps at each site consisting of 20 x leaf +4 samples per rep.

Treatment/Site		Si %dm	Mg %dm	Si/Mg
Control				
	Site 1	2.00	0.16	12.21
	Site 2	1.60	0.21	7.54
	Site 3	1.88	0.18	10.21
		1.83 (±0.06)	0.19 (±0.007)	9.98 (±0.70)
YCS				
	Site 4	1.69	0.22	7.77
	Site 5	2.93	0.24	12.25
	Site 6	2.82	0.28	10.22
		2.48 (±0.20)*	0.24 (±0.008)*	10.07 (±0.66)^{ns}
	p-value	*0.006*	*0.001*	*0.93*

In addition to Si, Mg sampling as part of the diagnostics validation, sampling has also been conducted to determine if these Si/Mg trends are unique to YCS or if they also occur in sugarcane affected by other conditions, such as diseases and abiotic stresses. Samples of diseased cane have been collected from SRA Woodford and subjected to analysis. Collected samples included nutrient diagnostic Leaf +1 and YCS diagnostic Leaf +4, obtained from healthy and diseased plants. The collected material included samples from varieties: NCO-310 (Fiji disease), Q200^{A} (RSD), Q205^{A} (Smut) and Q44 (Mosaic). Additionally, samples from the water stress trial in the Burdekin were re-examined and analysed for Si in addition to the previously conducted tests. These samples presented a good opportunity to evaluate plant response to abiotic stress. All plant samples collected from Burdekin and Woodford were subjected to comprehensive chemical testing. Full nutrient analysis, including Si, were conducted on those, to determine if the increased Si:Mg ratio observed in YCS symptomatic plants is prevalent in other biotic and abiotic stresses.

Biotic Stress

The diagnostic leaf nutrient data (macro elements) obtained for the Leaf +1 samples collected from healthy and diseased plants from SRA Woodford are presented in Table 67.

Table 67 Nutrient data for diagnostic Leaf +1, samples collected from healthy and diseased plants from SRA Woodford, March 2016.

Variety/Disease	Ca	Mg	P	K	S	Si
	% dm					
CV	0.20	0.08	0.19	1.10	0.13	0.55
Q200[A] Healthy	0.37	0.31	0.22	1.10	0.18	1.57
Q200[A] RSD	0.33	0.32	0.19	0.84	0.19	1.86
Q44 Healthy	0.41	0.32	0.19	0.81	0.16	1.00
Q44 Mosaic	0.27	0.25	0.22	1.33	0.21	0.94
NCO-310 Healthy	0.34	0.21	0.18	0.86	0.16	0.94
NCO-310 Fiji	0.66	0.34	0.19	0.80	0.21	2.14
Q205[A] Healthy	0.52	0.32	0.17	0.74	0.19	1.29
Q205[A] Smut	0.82	0.36	0.18	0.82	0.25	1.58

The Leaf +4 results for Si and Mg, obtained for the leaf samples collected from healthy plants and diseased cane from SRA Woodford, are presented in Table 68.

Table 68 Mg and Si data for YCS diagnostic Leaf +4, Woodford samples from healthy and diseased plants. Average values from 3 field replicates.

Variety/Disease	Mg	Si	Si:Mg
	% dm		
Q200[A] Healthy	0.32	1.82	5.7
Q200[A] RSD	0.34	2.27	6.7
Q44 Healthy	0.34	1.65	4.8
Q44 Mosaic	0.30	1.11	3.8
NCO-310 Healthy	0.30	1.28	4.6
NCO-310 Fiji	0.49	3.19	6.5
Q205[A] Healthy	0.40	2.27	5.7
Q205[A] Smut	0.36	2.04	5.7

Abiotic stress

Field samples of Q208[Φ] and KQ228[Φ] were collected in October 2014 from the water stress trial in the Burdekin. As the cane was still very young, only Leaf +0 to Leaf +4 were available for collection.
The results for uptake trends of selected nutrients, specifically for Mg and Si, in Control and Drought affected plants of Q208[Φ] and KQ228[Φ] are presented in Figure 328 and Figure 329.

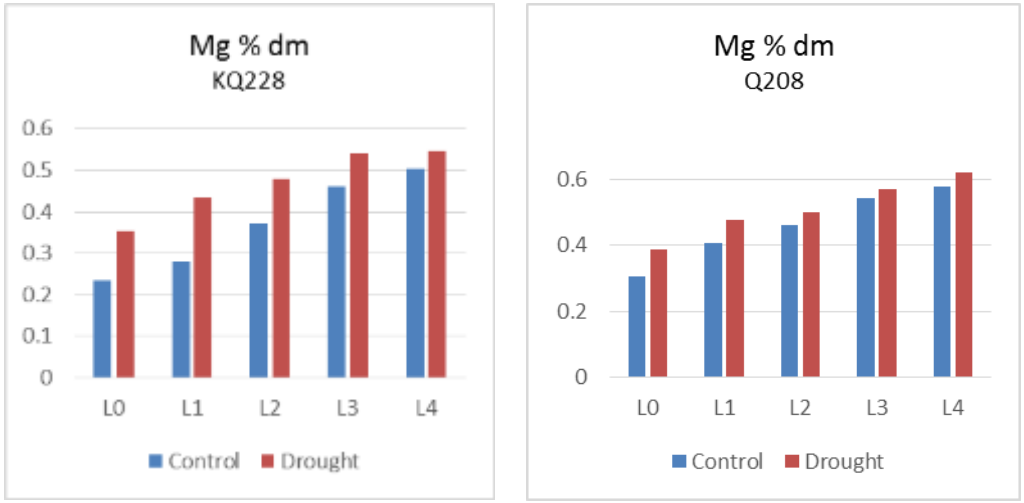


Figure 328 Uptake of Mg in Control and Drought affected young cane, varieties KQ228Φ and Q208Φ.

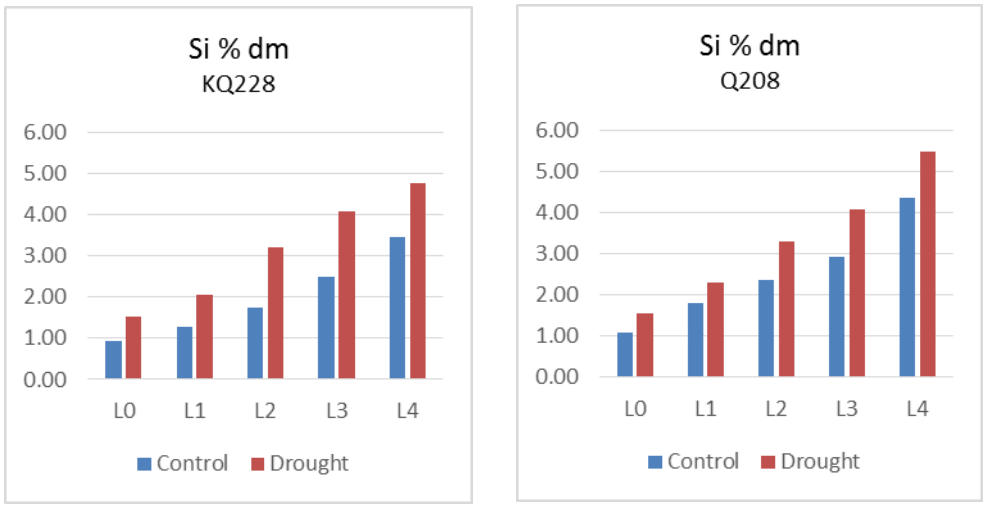


Figure 329 Uptake of Si in Control and Drought affected young cane, varieties KQ228Φ and Q208Φ.

The results presented in (Figure 328)and (Figure 329) show that both Mg and Si were taken up at a higher concentrations in drought affected cane. This was evident for all leaf numbers. These results confer with studies that show Si is heavily involved in mediated regulation of genes involved in mitigated abiotic stress; particularly ABA-dependent pathways and water stress (Hernandez-Apaolaza, 2014; Manivannan and Ahn, 2017). As the leaf samples were very young, Mg results suggest senescence was not advanced enough for mobilization out of the leaf. Table 69 shows high leaf Si:Mg ratio is also present in water stressed plants and not specific to YCS.

Table 69 Ratio Si:Mg in nutrient diagnostic Leaf +1 and YCS diagnostic Leaf +4 of KQ228Φ and Q208Φ collected from control and drought affected young cane.

Leaf Number	KQ228		Q208	
	Si:Mg			
	Control	Drought	Control	Drought
Leaf +1	4.6	4.8	4.4	4.8
Leaf +4	6.8	8.7	7.0	9.4

It is evident from these results that high leaf Si accumulation is not unique to YCS with RSD, Fiji disease and water stressed plants all showing increased accumulation (Table 68 & Figure 330). This confers with studies showing increased uptake of Si in stressed plants to aid in recovery (Hernandez-Apaolaza, 2014; Manivannan and Ahn, 2017). Similarly, Si:Mg ratios also show significant differences between healthy and diseased plants as found between YCS asymptomatic and symptomatic plants. Thus, the Si:Mg ratio anomaly observed in YCS is inconsistent and unsuitable for use as diagnostic.

6.10.4 Iodine Starch Test

Lugol's reagent stains starch a blue-violet colour when iodine attaches to its amylose component to form a amylose-iodine complex (Smith, 2007; Geigenberger, 2011). This potentially makes it a useful detection method for visualisation of insoluble α-glucan easy in different sugarcane leaf tissue. A quick and easy protocol was developed for clarifying leaf tissue for staining and better visualisation of where starch was accumulating in the leaf (see section 5.6.8 of this report). Leaf punch samples collected from the Herbert shows staining of lamina punches is able to discern between YCS symptomatic and controls when sampled early in the morning (Figure 331 A-C).

A similar principle was applied for samples collected from the six Burdekin diagnostic fields. Twenty Leaf +4 samples were collected at each site. Samples were taken before 9am at all sites. For each leaf a 2cm x 2cm section of lamina was cut from the mid-point of the leaf. Samples were soaked in 90% ethanol for a minimum of 7 days to bleach all chlorophyll. Samples were then soaked in Lugols iodine dye for 15 minutes before being rinsed and arranged on a flatbed scanner. Results are presented in Figure 332.

YCS symptomatic sites showed accumulation of starch as dark staining, while the asymptomatic Control sites were starch free (Figure 332). Within each YCS site there was some variability with some leaves appearing starch free, however there were no Control leaves which produced a positive stain. Screening of water stress, natural senescence, and a range of sugarcane disease samples showed no evidence of starch accumulation in the lamina (Joyce et al., 2016). Further testing relating to other stressor agents and diseases would be necessary for inconclusive evidence that this simple test could be used as a YCS diagnostic.

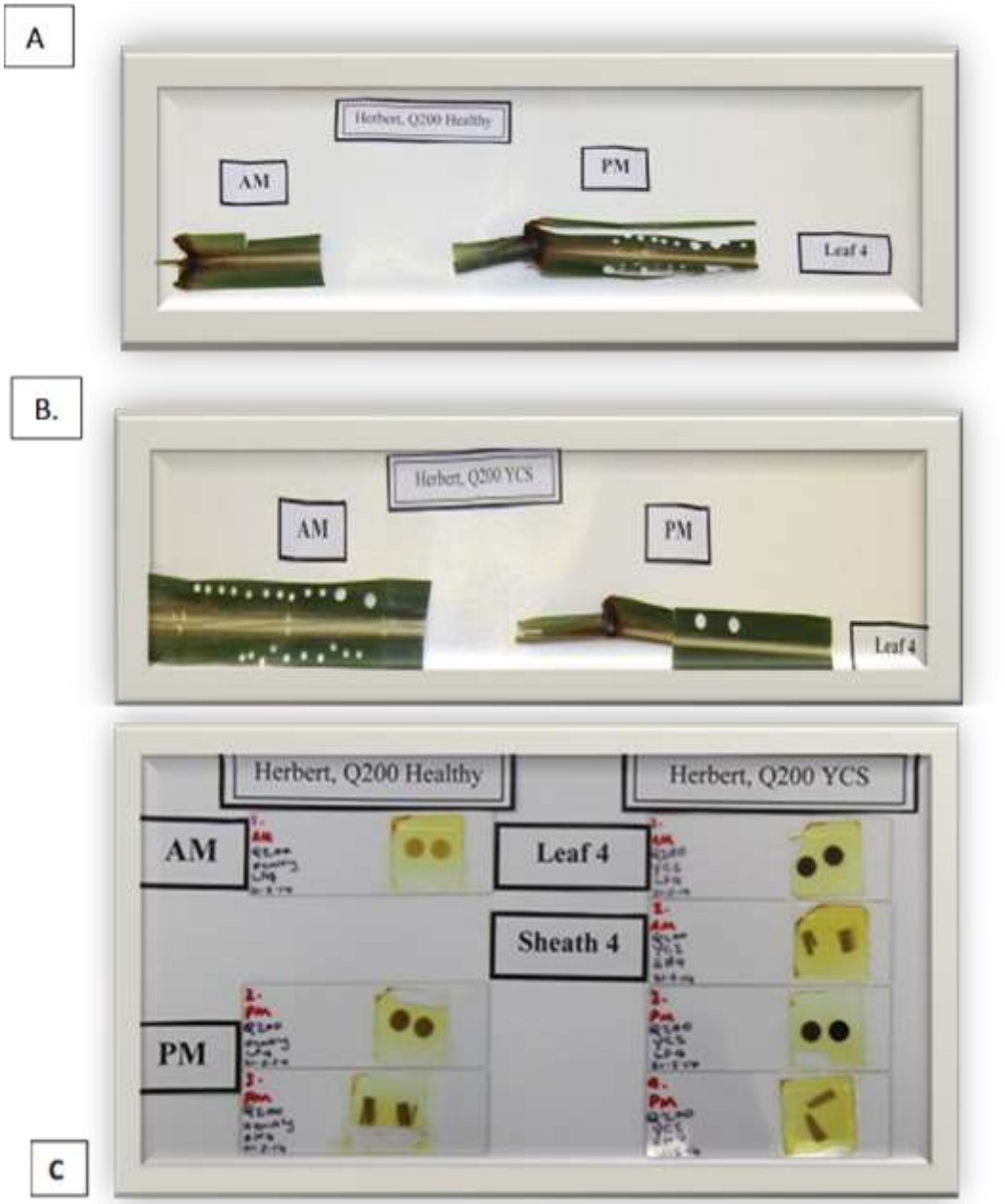


Figure 331 Q200[A] leaf+4 samples of asymptomatic control A) and YCS B) stalks. Leaf punches from A.M. and P.M. samples stained for the presence of starch C). YCS plants showed starch accumulation from leaf 4 irrespective of A.M. or P.M., while healthy asymptomatic plants showed starch accumulation in P.M. samples only.

Figure 332 Leaf +4 iodine starch stains. Sites 1-6 are shown. For each site 20 individual leaves were stained (1-20). Sites 1, 2, and 3 (top panels) were asymptomatic Controls while 4, 5, and 6 (bottom panels) were YCS symptomatic sites.

Further testing involved green, yellow, and brown leaf discs which were soaked in acetone for 48 hours in a 2mL screw capped eppendorf tube at room temperature. Following this treatment, some of the green discs were treated with 10% bleach to clear the tissue further. All discs were then treated with Lugol's reagent and assessed for presence of starch by the colour developed.

The use of acetone alone was as effective as those discs treated with acetone and bleach (Figure 332). Green leaf discs had the most starch, with reduced and no starch in yellow and amount of starch brown ones respectively (Figure 333). The midrib also showed presence of starch on the abaxial region (Figure 334).

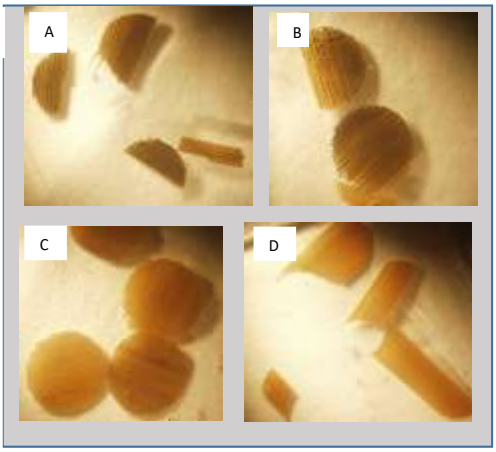


Figure 333 Leaf pieces stained for starch with Lugol's reagent. Treated with acetone only; A) Treated with acetone and then bleach B) Yellow leaf treated with acetone C) and brown leaf treated with acetone D)

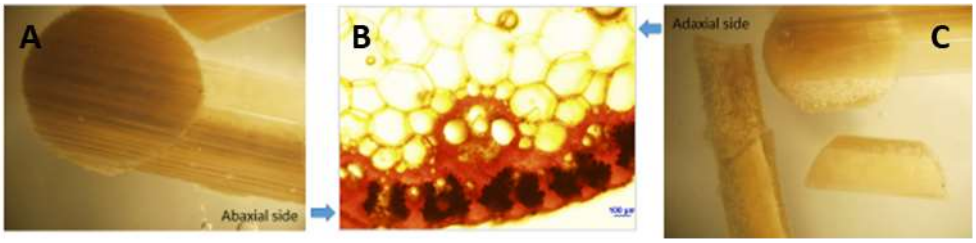


Figure 334 Presence of starch in acetone treated midribs of green leaves. Starch is only present in the abaxial region A) compared to the adaxial region C) as this is where the vascular bundles are B)

Use of Carborundum to abrade the leaf and then add Lugol's reagent was another method investigated. A 2cm green leaf piece (same leaf sample as used in method1) was placed between carborundum paper and abraded well. This was then eluted into a zip lock bag using 1mL of water and shaken well. To this bag, 2 drops of 5% Lugol's reagent was added and colour development observed.

Results showed that there was very little starch present in the pieces of leaf and may have been due to insufficient access of the reagent to the tissue (Figure 335). In addition, results were not uniform which is a potential problem with this subjective abrading method. This method was therefore unsuitable for a quick and easy starch staining method.

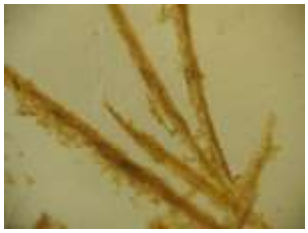


Figure 335 Starch test on abraded leaf pieces using Carborundum

Starch assays are not easy to do under field conditions and as glucose is the monosaccharide component of starch attention was drawn to this this metabolite as a possible diagnostic candidate. Methods to measure glucose was tested using Accuvin test strips. The method involved applying a drop of the sample to the test strip and waiting for the colour change. Preliminary results looked encouraging as the YCS samples showed a darker colour than the healthy controls (Figure 336). However, further testing showed the sensitivity of the strips to be insufficient to discern between YCS asymptomatic and symptomatic plants.

Figure 336 Accuvin strips showing development of dark purple colour in response to presence of glucose in YCS leaf sample

6.10.5 Discussion and conclusions

Th investigation of a potential YCS diagnostic has been a difficult and arduous task. Of all the diagnostic indicators tested, the lamina starch iodine stain has shown the best potential. Studies by Scalia et al. (2020) used a quick and easy in-field staining technique of the midrib which proved to be approximately 90% accurate. However, this was only achieved when used in association with a series of flash cards that highlighted key YCS characteristics such as leaf colour and canopy position. Results of the midrib test were validated through metabolite analyses. A parallel study investigating a novel biomarker identified through transcriptome studies proved unsuccessful (Scalia et al., 2020). As YCS is a growth inhibited physiological disorder without a single causal agent, it is unlikely that it will be amenable to a diagnostic test.

6.11 Variety assessment

As the incidence of YCS increased after 2013/14, reports from Industry suggested that some varieties were more susceptible, or had a higher risk of developing YCS than others. Other observations indicated that all varieties were equally affected by YCS. To investigate these claims, studies were conducted of crops grown within commercial operations, SRA and Industry breeding program assessment trials and experimental trials (see Appendix 1: 1.2.10 & 1.2.11)

6.11.1 Clonal assessment trial

Clonal assessment trials (CAT) are used by SRA's plant breeding program to assess various traits of new clones. Productivity and disease resistance performance are evaluated before data is made available to Regional Variety Committees for consideration. Through this process genotypes are

selected for a final assessment trial (FAT) before recommendations are made for propagation and release as a commercial clone.

6.11.2 Variability in genotype response to YCS

A second ratoon CAT comprised of twenty sugarcane genotypes in Brandon Qld was used for this study Experimental plot size was 4 rows x 10 m with a row spacing of 1.52 m (see Appendix 1: 1.2.10). Clone performance and YCS susceptibility was evaluated under three water levels; rainfed (RF) half irrigated (HF; 50% of normal irrigation frequency) and irrigated (IR; approximately 0.7ML/ha). The experiment was designed in a split block design with three replications. The rainfed treatment received no regular irrigation except the supplementary irrigation provided to establish the ratoon crop in July 2015. During January-February 2016, three supplementary irrigations were done to ease the severe water stress experienced by the crop. Throughout the growing season, the fully-irrigated treatment received adequate water (10ML) and the semi-irrigated treatment received only 50% of this. The variation among the 20 clones for YCS severity was significant. The number of green leaves in different canopies (clone variation) vary from 7-9. In most clones YCS appears on 4th leaf and subsequently the 5, 6 and 7th. The YCS on 7th leaf is difficult to distinguish from natural leaf senescence as symptoms of YCS and senescence were mostly similar. The leaf area of the top canopy was higher in irrigated treatment than the rainfed in most clones. Therefore, exposure of leaf No 4 and 5 to high radiation was relatively smaller in most clones under irrigated conditions than the rainfed treatment. Contrary to shade effects (delay) on YCS, the clones in irrigated treatment shown early YCS (2-3 days) than rainfed where there was less canopy shading on 4th and 5th leaves at the same age.

The pattern of YCS severity among test clone was consistent across water treatments indicating less interactions of clones with moist environments for YCS. However, these results are mostly applicable when YCS appears in the late stage of growth.

6.11.2.1 Heritability of YCS.

The experimental data provided the opportunity to estimate the genetic components of YCS and the heritability to be able to understand the genetics of the syndrome. The broad sense heritability of YCS severity based on starch rating and visual observation were estimated. The broad sense heritability explained the fraction of variation among clones due to repeatability of the clone genetic effects across replications and water treatments. The clone effects were considered as random and treatment and replicates effects were fixed for the genetics variance component analysis.

An analysis of variance (ANOVA) was initially performed for each of the treatment to examine heterogeneity of error and genetic variances for YCS. The following statistical model was used to partition the genetic variance (Cockerham, 1963).

$$Y_{ij} = \mu + g_i + b_j + (gb)_{ij}$$

where Y_{ij} = observed YCS of the *i*th genotype in *j*th block (average of two samples per plot); μ = mean of all observations; g_i = effect of the *i*th genotype; i = 1 to 20 (number of clones); b_j = effect of *j*th block; $(gb)_{ij}$ = interaction effect between the *i*th genotype and the *j*th block (referred to as experimental error).

Genetic and error variances were estimated and broad sense heritability (h^2_b) on the basis of genotype means (YCS) was estimated for each of the treatments as described by Fehr (1987) : $h^2_b = \frac{\sigma^2_g}{\sigma^2_g + \frac{\sigma^2_e}{n_r}}$.

where σ^2_g and σ^2_e are genetic and error variances and n_r is the number of replications.

Analyses were also conducted across water treatments for starch and visual observations and the variance components of genetic and genotype x treatment interaction were estimated. Genotypes (or clones) were considered as random effects while the water treatments were treated as fixed effects for the variance component analysis within the following model (Cockerham, 1963).

$Y_{ijk} = \mu + t_j + b_{kj} + g_i + (gt)_{ij} + (gb)_{ijk}$

where, Y_{ijk} = observed YCS of the *i*th genotype in the *j*th water treatment in the *k*th block (mean); μ = mean of all YCS observations; t_j = the effect of *j*th water treatments; *j*=1, 2; b_{kj} = effect of *j*th treatment within *k*th block; k=1, 2 or 3 (error 1); g_i = effect of the *i*th genotype; i = 1 to 20; $(gt)_{ij}$ = interaction effect between the *i*th genotype and the *j*th water treatment; and $(gb)_{ijk}$ = interaction effect between the *i*th genotype and the k^{th} *block* within the *j*th treatment (error 2). The genetic (σ^2_g) and phenotypic (σ^2_p) variance components were estimated from the combined analysis, and heritability of YCS was estimated on the experiment mean. The broad sense heritability for YCS was estimated using the following formula: $h^2_b = \frac{\sigma^2_g}{\sigma^2_g + \frac{\sigma^2_{gt}}{n_t} + \frac{\sigma^2_e}{n_t n_k}}$

where, σ^2_g, σ^2_{gt}, σ^2_e are genetic (G), G×water treatment (GxE) interaction and error variance components, respectively, and n_t and n_k are the numbers of observations and blocks, respectively.

Genetic correlations between YCS measurements and TCH were estimated from Kempthorne (1989): $\gamma_g = \frac{Cov_{g(xy)}}{\sqrt{\sigma^2_{gx}}\sqrt{\sigma^2_{gy}}}$

where, $Cov_{g(xy)}$ is the genetic covariance of the product of two measurements (YCS and TCS) and σ^2_{gx} and σ^2_{gy} are the genetic variances of the two traits. Phenotypic variances and co-variances were used to estimate the phenotypic correlation using the same statistical procedure.

6.11.2.2 Genetic variation

Genetic variance for YCS varied across water treatments, as did error variance. Genetic and error variances were not related to mean levels of YCS, and generally no significant associations were found between genetic variance, error variance, and most weather variables (e.g. daily temperature, vapour pressure deficits).

Analyses of variance across treatments were conducted for measurements collected at peak YCS period. These showed that genetic variance was significant in all cases and for most cases 3-fold greater than genotype x treatment interaction variance, indicating generally relative consistency in genotype resistance to YCS across different water availability conditions.

6.11.2.3 Genetic correlation between environments

Genetic correlations between YCS indices across all water treatment were generally positively correlated (0.56). The correlation matrix for genotype performance between 2 treatments and 3 replications (i.e.treatment x replicates) of YCS index was analysed using principal component analysis. The first component described approximately 43% of the total variance within the data, which represents a high proportion of non-error variance, considering that the average broad sense heritability for the 2 water treatments was approximately 0.5. The relatively high proportion of variance accounted for by the first component and positive loading of 2 water treatments is indicative of a generally consistent pattern of ranking of genotypes across many environments (predictive), and is consistent with the results from analyses of variance across treatments and the consistently positive genetic correlations between.

6.11.2.4 Genetic correlations between YCS index and cane yield

Genetic correlations between YCS, for each water treatment and cane yield of the clones were estimated. The average cane yield used for these correlations was calculated with the data from the 20 clones 2 water. Generally, low but positive genetic correlations were observed, but these varied from 0.19 (drought) to 0.24 (irrigated). This result indicates an overall positive association between and average cane yield among the genotypes, but with varying strength of the relationship.

The effects of weather or crop conditions (e.g. maximum and average temperature, evaporation, vapour pressure, level of crop water stress) at the time of YCS measurement on estimates of genetic correlations between YCS and TCH (replicate x treatment combination, 6 pairs of TCS and YCS data) were investigated. The maximum canopy temperature during the time of each measurement was negatively correlated (r = -0.23) with the magnitude of these genetic correlation values, while mean temperature and the vapour pressure had lower but still statistically significant negative correlations of -0.25, -0.18, respectively. Indicators of general water stress such as the water supply to demand ratio were not associated with magnitude of the genetic correlation between YCS and cane yield. However, these observations need to be further investigated in different zones with different conditions.

6.11.3 Variability in genotype response to YCS

Genetic variation for YCS in Clonal Assessment Trial; CAT2016 subset of population

The preliminary investigation on genetic variation among twenty advanced clones suggested that there is a considerable broad sense heritability for YCS. This study warranted further investigation on genetic control of YCS in a breeding population. In order to understand the genetic control of YCS, a subset of genetic population (15 parents and their offspring (60)) were selected from the 2016 clonal assessment trial (CAT) and planted in a replicated trial. During the crop growth period, YCS, agronomic and physiological observations were collected from these populations and parents. The objectives of this study were to investigate the genetic variation among and within families for YCS, and estimate the narrow sense heritability (NSH) using parent's offspring regression method and establish genetic background of YCS in some breeding populations.

Observations

Physiological differences among test clones:

The physiological observations were collected during a 3-6 month growth stage (October 2016 to January 2017 period). Gas exchange measurements with a portable photosynthesis analyser (LICR6400XT) were collected to determine genetic variation for rate of C fixation (A), stomatal conductance (gs) and the intrinsic transpiration efficiency (MS9) among progenies and parents. The intrinsic transpiration efficiency was calculated using the internal and ambient CO_2 concertation as described by (Farquhar and Richards, 1984)

Intrinsic Transpiration Efficiency (iTE) = $$TE_i = \frac{A}{g_s} = 1 - \frac{C_i}{C_a}$$

Whereas, Ci and Ca represent the internal and ambient CO_2, respectively. The iTE is depending on stomatal conductance (gs) and photosynthesis (A) as well.

Visual observation on YCS

A trial within the clonal assessment trial (CAT 2016/ 2017) was conducted to investigate the narrow sense heritability of YCS in sugarcane. Genetic material, experiment design and the procedure for sampling were presented in the MS 8. CAT trials designed with 6m single row plots of about 2000 genetic identity. Each clone typically producing 60 - 90 shoots per plot. The physiological observations were taken during the 3 - 6 months stage of the 60 progenies grown in 2 replications. YCS visual observations were made during the peak period of YCS in February 2017 when the crop was about seven months old. Three independent observers were employed to take visual observations on YCS incidence in each plot. Five stalks were picked randomly for rating by each observer. Three levels of ratings were given based on the intensity of YCS. Level 1 was given for a clean stalk without any YCS leaves, level 2 for stalks with 1 or 2 YCS leaves at the lower canopy (>7 - 9 leaves) and level 3 for stalks with YCS on either 4, 5 and 6.

Altogether 15 stalks were sampled by 3 observers for the survey and estimated the average rating of YCS for each clone. Sixty clones in 2 replications were analysed in a randomized block design for the YCS rating based on average of 15 stalks per clone per replicate.

Diagnostic starch test

The starch diagnostic test was conducted for the 60 clones and 15 parents in 2 replications. The 4th and 5th leaves were collected early in the morning from the most matured stalk and sampled 1/3 of

the base of the leaf lamina. Leaf samples were sealed in zip lock bags and stored in a cool container during sample collection. These samples were taken to the lab and kept in a deep freezer until use them for starch test. The standard dye stain test was conducted after removing all chlorophyll from the leaf as described earlier in previous milestones. The YCS intensity score was given based on the intensity of the stain on leaf lamina, midrib and the leaf margin (Table 70)

Statistical method

The rates can be made more nearly normal and at the same time the variance can be made relatively independent of the mean by transferred to square roots. As the counts are under 10 the most appropriate transformation is:

$$\text{Yi} = \sqrt{(Y + 0.5)}$$

The narrow sense and broad sense heritability were estimated using the methods described by Falconer and Mackay (1996).

Table 70 Leaf starch intensity and the score given for each clone based on the starch stain test.

LEAF STARCH	Score
No starch	0
20% all over	1
one side	2
one side + MR	3
both sides	4
both sides + MR - edges	5
both sides + MR + edges	6

Results

Physiological differences among Progenies and parents.

The variation among parents and progenies for photosynthesis, stomatal conductance, internal CO_2 and transpiration efficiency were significant (P<0.05). Clones KQ07 - 5410, KQ07 - 5107 and Q232$^{\phi}$ had significantly higher photosynthesis and stomatal conductance than Q208$^{\phi}$ and Q238$^{\phi}$. The average performance of parents and their offspring for these physiological characters are similar (Table 71).

These parental clones are among the mostly used promising parents in the hybridization program in SRA. The popular commercial clone Q208$^{\phi}$ showed less prevalence of yellow canopy syndrome and had more moderate photosynthesis and transpiration efficiency than Q232$^{\phi}$.

Table 71 Physiological observations; photosynthesis, stomatal conductance, internal CO_2 and intrinsic transpiration efficiency among parents and their families in the CAT population.

Progenies	Photosynthesis mmolm^{-2} s^{-1}	Conductance molm^{-2} s^{-1}	Internal CO_2 mmolm^{-2} s^{-1}	Intrinsic Transpiration (iTE)
KQ07-5107*N29	34.52	0.35	148.07	0.63
KQ07-5107*Q231	37.92	0.38	140.11	0.65
KQ07-5107*Q235	39.10	0.39	126.30	0.68
KQ07-5410*N29	39.50	0.36	140.47	0.65
KQ07-5410*Q235	41.74	0.48	145.70	0.64

Q208*KQ07-5107	39.17	0.33	130.57	0.67
Q208*QC91-580	40.42	0.71	175.14	0.56
Q232*KQ09-1547	45.46	0.56	145.89	0.64
Q232*Q252	40.16	0.56	175.19	0.56
Q238*Q232	37.85	0.38	159.73	0.60
Q252*N29	38.39	0.48	166.24	0.58
QC03-6239*Q235	41.39	0.48	146.09	0.63
QN80-3425*Q247	40.24	0.45	140.24	0.65
QS06-8140*N29	38.82	0.44	162.54	0.59
Average	**40.01**	**0.46**	**149.88**	**0.63**
Lsd 5 %	**6.56**	**0.16**	**21.32**	**0.08**
Parents				
KQ07-5107	44.89	0.59	143.35	0.64
KQ07-5410	45.16	0.51	135.48	0.66
KQ09-1547	43.05	0.57	161.76	0.60
N29	37.45	0.38	119.41	0.70
Q208	36.28	0.39	149.13	0.63
Q231	37.26	0.46	163.84	0.59
Q232	45.70	0.51	129.56	0.68
Q235	39.31	0.41	143.19	0.64
Q238	35.01	0.36	140.84	0.65
Q247	41.40	0.45	150.44	0.62
Q252	40.87	0.47	153.63	0.62
QC03-6239	41.21	0.42	125.31	0.69
QC91-580	38.54	0.45	143.38	0.64
QN80-3425	42.82	0.47	135.08	0.66
QS06-8140	37.00	0.38	149.74	0.63
Average	**40.39**	**0.45**	**142.94**	**0.64**
Lsd 5 %	**6.56**	**0.16**	**21.32**	**0.08**

Visual observation on YCS

Though the YCS prevalence during the first observation in March 2017 was relatively low, the variation among clones was highly significant (P<0.001) in the CAT population (Table 72). The variation among parents is significant (, P=0.004). Highest incidence was recorded on QS06-8140 (rating 1.95) while Q208$^{\phi}$ did not show any symptoms at this stage (rating 1). KQ09-1547 (1.55) and Q238$^{\phi}$ (1.45) had significantly higher YCS prevalence than Q208$^{\phi}$.

The physiological observation suggested that KQ09-1547 maintained relatively higher photosynthesis and conductance than Q208$^{\phi}$, nevertheless Q238$^{\phi}$ had a very similar capacity to Q208$^{\phi}$. Similarly, KQ07-5410 had low YCS prevalence (1.1) than KQ09-1574 (1.55) (P=0.004) even though the rate of photosynthesis is comparable.

Table 72 The variation among parents and offspring of the CAT2016 population for YCS prevalence. (1= no incidence 2 = at least 1 in lower canopy below leaf No 5, and 3 = at least 1 leaf on top canopy)

Cross	YCS prevalence Rating			
	Family	Mid parent	Female	Male
KQ07-5107 x N29	1.38	1.25	1.25	1.25

KQ07-5107 x Q231	1.28	1.20	1.25	1.15
KQ07-5107 x Q235	1.28	1.23	1.25	1.20
KQ07-5410 x N29	1.13	1.18	1.10	1.25
KQ07-5410 x Q235	1.23	1.15	1.10	1.20
Q208 x KQ07-5107	1.35	1.13	1.00	1.25
Q208 x QC91-580	1.18	1.10	1.00	1.20
Q232 x KQ09-1547	1.46	1.38	1.20	1.55
Q232 x Q252	1.08	1.13	1.20	1.05
Q238 x Q232	1.40	1.33	1.45	1.20
Q252 x N29	1.63	1.15	1.05	1.25
QC03-6239 x Q235	1.53	1.15	1.10	1.20
QN80-3425 x Q247	1.26	1.15	1.25	1.05
QS06-8140 x N29	1.69	1.60	1.95	1.25
Average	**1.35**	**1.22**	**1.23**	**1.22**
Lsd 5 %	0.41			

The variance components were estimated for genotypes and error variances in the genetic population and the broad sense heritability was estimated (0.59) (Table 73). The heritability estimate was comparable with the broad sense heritability estimated from the 20 clones in the previous experiment. A second set of observation has been made in April 2017 for further investigation on broad sense heritability for YCS in the same trial.

Table 73 Variance components estimated from the analysis of variance among 60 progenies.

Source	Variance component	Standard error of the estimates
Genotype	0.0594	0.0198
Residual	0.0806	0.01484
Heritability	**0.595**	

Parent offspring regression and the narrow-sense heritability (NSH)

We investigated the narrow sense heritability (Falconer and Mackay, 1996)(in the test CAT 2016 population. NSH is the proportion of total phenotypic variation due to additive effects of genes, which occurs in one of three ways: by mean trait values, variance components and parent-offspring regression. We adapted the parent- offspring regression to estimate the NSH in this experiment.

The progeny mean (1.35) was relatively higher than the mid parents (1.22) rating in the test CAT population.

The mid parent values were estimated by accounting the average performance of parents in each cross tested under same condition in the same trial. The means of 14 progenies were regressed against the mid parent values as shown in Figure 337.

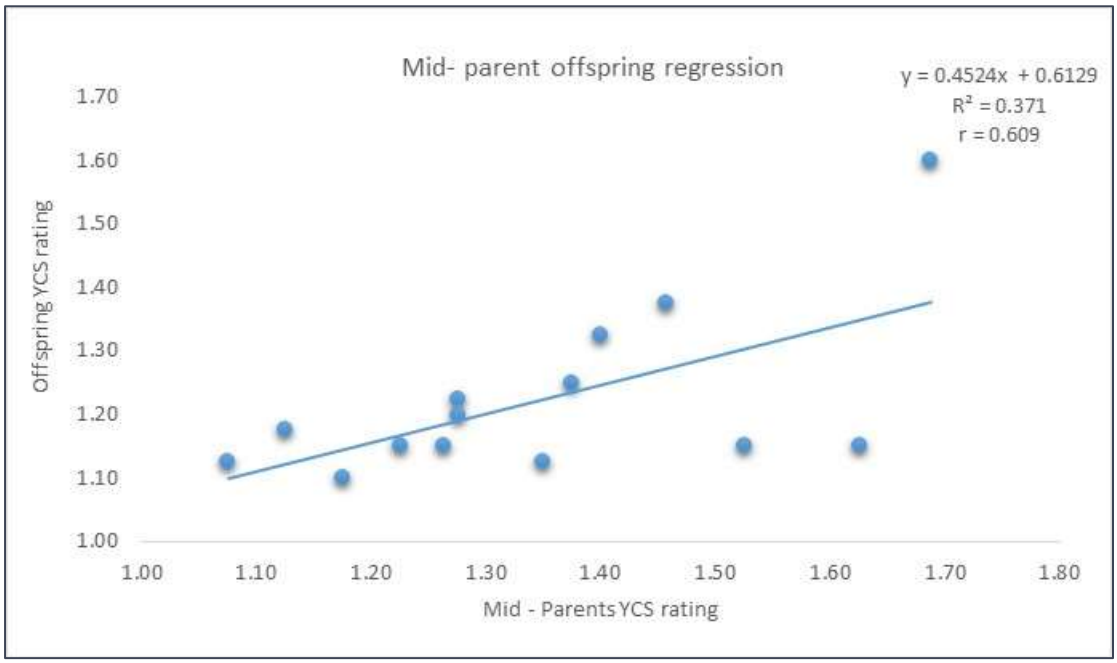


Figure 337 Parent offspring regression (NSH) estimate for the YCS prevalence score in the CAT 2016 population.

The narrow sense heritability estimate was 0.61 which is relatively closer to broad sense heritability (0.59). The moderate NSH suggests that the additive effects has some influence (60 %) on the YCS prevalence in the test CAT population. This observation is based on the YCS score in March 2017. However, continuous observation in ratoon crop would be important to understand the genetic contribution for YCS.

Association between YCS score and the physiological parameters

The phenotypic correlation between progeny mean and mid parents YCS score and the progeny mean gas exchange measurements were investigated. The correlation between observed progeny mean and mid parent values of the YCS score represents the narrow sense heritability of YCS (0.601) (Table 74).

Table 74 Correlation matrix between physiological traits (gas exchange and intrinsic transpiration efficiency) and YCS rating $P_{0.05}$ = 0.49 when n=14

Observations	*A*	*gs*	*Ci*	*TE*	*P-YCS*
Photosynthesis (A)					
Stomatal conductance (gs)	0.559*				
Internal CO2 (Ci)	-0.022	0.665*			
Intrinsic transpiration efficiency (iTE)	0.052	-0.650*	-0.997*		
Progeny mean YCS Score (P-YCS)	-0.053	-0.133	0.079	-0.105	
Mid Parent YCS Score (MP-YCS)	-0.004	-0.161	0.076	-0.070	0.601*r

However, there was no association between physiological traits and YCS based on progeny mean or mid parent values of YCS. However, the narrow sense heritability (0.601) is significant and it is similar to the broad sense heritability prediction.

Conclusions

The subset of a genetic population (CAT2016) provided an opportunity to investigate the inheritance of YCS in sugarcane. Although significant differences among progenies were observed, there is no distinct association between the YCS prevalence and the underlined physiological traits.

The visual rating based on the prevalence of YCS on the leaf canopy indicated that YCS can be inherited from their respective parents (NSH =0.61). However, investigation on the ratoon 1 crop (during the YCS season end of December 2017) could further confirm the inheritance of YCS. The starch test would confirm the visual ranking of YCS and provide valuable data for the genetic analysis.

6.11.4 Discussion and conclusions

The initial clonal assessment trial of 20 clones showed that YCS can occur similarly in drought stress and well irrigated conditions. While there are differences in visual symptoms among clones for YCS appearance and severity, there was no evidence of YCS free clones in the experiment. Even though starch accumulation correlates well with YCS symptom expression, it is at best a measure of plant health or stress. The presence of high leaf starch could be used for early detection of YCS-like symptoms but not to rate a genotypes susceptibility to YCS. Partitioning of carbon to starch and soluble α-glucan pools is in response to rising levels of leaf sucrose, and is initiated as a carbon offset mechanism. Metabolite studies by Scalia et al. (2020) showed that thousands of samples analysed from commercial varieties and experimental genotypes have a leaf sucrose tolerance level of approximately 200µmol/g DM. All varieties that are YCS asymptomatic have sucrose levels below this threshold. When sampled during the morning, YCS asymptomatic plants with high vigour have leaf sucrose levels of 0-100µmol/g DM (Scalia et al., 2020).

There is a no water stress effect for the severity or initiation of YCS in sugarcane clones. This is not surprising as water stress events precedes YCS development and expression which is triggered by rapid growth after rainfall (see section 6.3 of this report). Earlier genetic studies within this trial suggested that there is a moderate broad sense heritability for YCS. The moderate genetic correlation for YCS index between water treatments suggested some genetic control of the syndrome. However, there was no significant phenotypic correlation between TCH and YCS, and the genetic association between TCH and the YCS is relatively small.

Irrigated clones proved to have a larger upper canopy area than clones that were rainfed. Subsequently the larger surface area shaded the mid-canopy zone where YCS develops and expresses. It was noted during YCS scoring that these clones showed lower incidence and severity of YCS, and a delayed YCS response. However, in irrigated clones where there was less shading of the mid canopy leaf + 4 and Leaf +5, YCS expressed 2-3 days earlier than rainfed clones where there was less canopy shading of 4^{th} and 5^{th} leaves at the same age. This key finding concurs with results from other trials that show a period of high solar radiation interception, rapid growth and high photoassimilate production is required for a source sink imbalance to occur. Taking this into consideration, it is tempting to argue that high early sugar clones would be at higher risk of developing YCS. However, sink strength magnitude, photosynthetic rate and radiation use efficiency are instrumental to whether leaf sucrose exceeds upper tolerance levels, and these may all be

heavily influenced by climatic conditions. Therefore, it is not surprising that these physiological analyses involving trait characterisation under various environmental conditions, have proven less than successful in ratifying a genetic pre-disposition for YCS susceptibility.

6.12 YCS regional surveys

YCS distribution across districts during 2014/2015

After consultation with HCPSL, BPS and the SRA PEC Unit, a process for monitoring the incidence of YCS across districts has was established in 2014/15. The PEC officers were responsible for investigating any unusual reports of YCS. The SRA PEC Unit worked closely with the relevant productivity services to monitor and report on the distribution and severity of YCS. Productivity services reported to PEC officers bi-weekly, offering an assessment of areas within their districts which are impacted as well as an indication of the severity.

SRA PEC officers from all regions reported on YCS incidence and severity in their districts: Burdekin, Herbert, Mackay-Plane Creek, Mossman-Tablelands- Mulgrave, Tully-South Johnstone-Innisfail-Babinda, and Bundaberg South. The PEC officers sought input from their respective productivity boards for their reports. These reports were a source of current information about the prevalence and severity of YCS across the industry. Further input from the respective Productivity Services was also used to compile the reports of YCS distribution across districts during the 2014/15 crop year.

A summary of key observations from each reason is presented in Table 75.

Table 75 YCS observations by industry in the Burdekin, Wet tropics, Herbert and Central regions 2014/15

Region	YCS observations 2014/15
Burdekin	• Symptoms noted Jan – March, gone by end of June • Symptoms are below FVD • YCS symptoms do not seem to follow and consistent pattern • KQ228^{A}, Q247^{A} and Q238^{A} appeared to have the highest level of visible symptoms of YCS, while Q208^{A} seemed least affected • No link to soil health or specific farming practices • Wave effect of symptoms increasing, and decreasing was observed • Worst symptoms in mid-Feb to early March • Rubbery stalks seen in worst cases • Difficult to estimate yield loss
Wet tropics	• Moderate to severe YCS from late Feb • No YCS in Innisfail 2014/15 • YCS first appeared in Mossman in early December • Mossman and Mulgrave – more severe in late February • Mild YCS symptoms were recorded in Tully from September through to early December, however it is not clear if these were merely water stress symptoms brought on by the lower than normal rainfall for that time period • No consecutive YCS expression in the same field from year to year
Central	• YCS confirmed in Mackay late 2013 • 2014/15 confirmation of YCS in Proserpine

	• All varieties affected • Waves of yellow due to stress from dry & wet • CCS penalty – however CCS is most likely due to poor ripening i.e. the crop was still actively growing • Lodging worse – poor roots • Difficult to estimate yield loss • Crops that were affected last year are ratooning well
Herbert	• Waves of expression • All varieties affected • Rubbery stalks • Wet and dry stress – waves of yellow • Link between Pachymetra and YCS – crops with high Pachymetra are prone to YCS development • Yield loss hard to estimate

Although observations made in each of the regions were made independently, there is a common thread throughout the comments. The inconsistency of any YCS pattern or repeat event in the ratoon crop, all varieties affected, dry stress and wet initiation of yellowing, episodic waves of expression, symptoms most severe in mid to late February and difficulty estimating any potential yield loss. While these observations were made in 2014/15, they are still pertinent at the time of writing this report and is testament to the accuracy of the observations early in the research program.

6.12.1 Survey and monitoring error

YCS surveys were also conducted over a two-week period in late May 2014. A district-wide YCS survey was conducted in the Burdekin and Herbert. Project technicians travelled all major roads in both districts and rated every block along the roadside. A total of 886 and 892 blocks were assessed in the Burdekin and Herbert respectively and rated for severity.

Unfortunately, the extensive data set generated from the regional surveys and monitoring across the districts will not be presented here as it was fundamentally flawed, and no conclusions can be drawn. This is due to the manner in which the surveys were conducted, time of year, and the way YCS severity was scored. This comment is not implying that the work was poorly done; it simply reflects that as more knowledge came to hand about how YCS develops and expresses, parameters measured within the surveys became obsolete. It is now known that YCS is a mid-canopy condition any scoring above L+1 and below L+6 would be a misdiagnosis. Furthermore, YCS is notoriously difficult to identify if it is not observed at the start of symptom expression (Scalia et al., 2020). Therefore, it is impossible to make an accurate call on YCS from a vehicle in a drive past monitoring survey, let alone comment on its level of severity without careful closeup scrutiny of the leaf and canopy (see section 6.1 of this report). YCS also has a distinct golden-yellow colour which can be difficult to discern when merged with other yellowing leaves on the same culm of adjacent to it

(Scalia et al., 2020). YCS symptoms tend to start in December and peak in February, so any surveillance outside of this period is likely to be confused with other forms of leaf yellowing in the crop.

6.12.2 Discussion and conclusions

To attain an accurate assessment on YCS incidence and severity across all sugarcane regions would be very difficult to achieve for five main reasons 1) lack of a unique diagnostic test 2) the many other forms of yellowing in the sugarcane canopy at any one time that look similar to the untrained eye 3) detection of symptoms very early in an event before leaf yellowing merges with the older senescent leaves below 4) unpredictability due to the possible link to more than one causal agent/s and 5) the episodic nature of the condition. It is worth noting that remote sensing and GIS has been trialled with no success as YCS is a mid-canopy condition which renders this type of surveillance impotent (Robson, 2014).

6.13 Cane yield and CCS

Assessing any potential cane yield and CCS penalty associated with YCS has been a goal from the commencement of the project but difficult to achieve for many reasons. Unfortunately, the episodic nature of YCS made it difficult to predict where to conduct trials. Thus, the attainment of robust data to assess cane and sugar yields was not always possible if YCS symptoms failed to develop or were very mild. Nonetheless a large number of trials were conducted and the impact of YCS on yields were assessed. Appendix 1 contains details of all pot and field yield trials and a generic methodology to determine biomass and CCS is presented in section 5.4.1 of this report.

The following results, analyses and reports are a cross sectional representation of the research conducted between 2014 and 2020 to address the issue of YCS impact on cane and sugar yields.

6.13.1 Herbert and Burdekin intensive monitoring sites

Assessment of 2014/2015 YCS impacts on yield and CCS in progress. Including gathering of mill data, correlation with known YCS blocks and maintenance of database. (Davey Olsen)

Burdekin and Herbert Monitoring Sites

In December 2014, Herbert Cane Productivity Services (HCPSL) and Burdekin Productivity Services (BPS) began bi-weekly monitoring at YCS sites across their respective districts. BPS monitored 50 blocks while HCPSL monitored 30 blocks. Initial focus was on KQ228 in the Burdekin and Q200 in the Herbert. In January, the decision was made to include a number of other blocks which had become severely affected. These additional blocks represent many other varieties and ratoon classes. These blocks are rated for YCS prevalence and severity (Table 3 & Table 4). Additionally, a range of background block parameters such as yield and CCS history, plant and harvest dates etc. are being added to this data through mining of mill and productivity board data. This group of 80 sugarcane

blocks were used evaluate the impact of YCS on yield and sugar at the conclusion of the 2015 harvest.

Additional notes on methodology

SRA worked with HCPSL to determine long term farm yield and CCS averages for all of these monitored blocks. Five and ten year averages were calculated to form the foundation for impact assessment. For each block, 2015 yields and CCS was compared to their long-term farm averages and a % gain or loss calculated. This will allow block performance to be compared across varieties, classes and districts. Blocks were then grouped according to their relative % loss or gain against long term average and compared with their YCS rating history and background block histories to determine the drivers behind yield/CCS loss due to YCS.

Determining the yield, sugar, and economic losses attributable to YCS is of primary concern to industry. In previous years, estimates have been imprecise. This year we will estimate losses and economic impact with greater rigour by applying statistical and crop modelling techniques to carefully compiled data sets. Dr Geoff Bamber assisted in this analysis.

Monitoring Database

In December 2014, Herbert Cane Productivity Services (HCPSL) and Burdekin Productivity Services (BPS) began bi-weekly monitoring at YCS sites across their respective districts. BPS monitored 55 blocks while HCPSL monitored 35 blocks. Initial focus was on KQ228^{A} in the Burdekin and Q200^{A} in the Herbert. Some of these blocks have shown YCS symptoms, while others have not. In January the decision was made to include a number of other blocks which had become severely affected. These additional blocks represent many other varieties and ratoon classes. These blocks are rated for YCS prevalence and severity (see Table 3 & Table 4 for the rating keys).
In addition to YCS prevalence and severity data, the database contains data on production history, mill averages, weather, soil types, and a wide range of farm inputs and management practices This group of 80+ sugarcane blocks will be used evaluate the impact of YCS on yield and sugar at the conclusion of the 2015 harvest.

Statistical analysis of survey data (Herbert and Burdekin)

YCS monitoring was conducted weekly during which YCS prevalence and severity were assessed. Prevalence is defined as the proportion of total stalks per plot showing YCS symptoms, whereas severity is the degree of yellowing exhibited. Prevalence and severity were rated for the crop canopy (above TVD), mid-canopy (leaves +1 to +5) and lower canopy (leaves below +5). Scores for prevalence and severity were assigned according to the rating key's presented in Table 3 and Table 4. The yield variation among 98 farms due to unknown factors (biotic and abiotic) were considerably high in the survey data collected in Burdekin and Herbert during last 3 years. The simple correlation estimates between YCS occurrence and the respective yield difference in symptomatic and asymptomatic crops/years may be inaccurate because of the heterogeneity of unknown variations.

To minimize these variations, we observed the pattern of yield variation among farms using principal component analysis (PCA). PCA developed a single score (PC 1) representing the yield data (different years and ratoons) without changing the direction of variation. At this stage, PCA was conducted with the survey data collected from the 39 farms during last 3 years. The PC 1 (generally explain most of the yield variation) can be used as a vector for yield (TCH) across farms for further correlation analysis with YCS observations at different times of the year. The biplot graphically displays the two-way (farm-crop years) data and allows visualization of the interrelationship among farms and crop years. The biplot determines whether the target surveyed farms are homogeneous or should be divided into different groups as they are grouping into clusters.

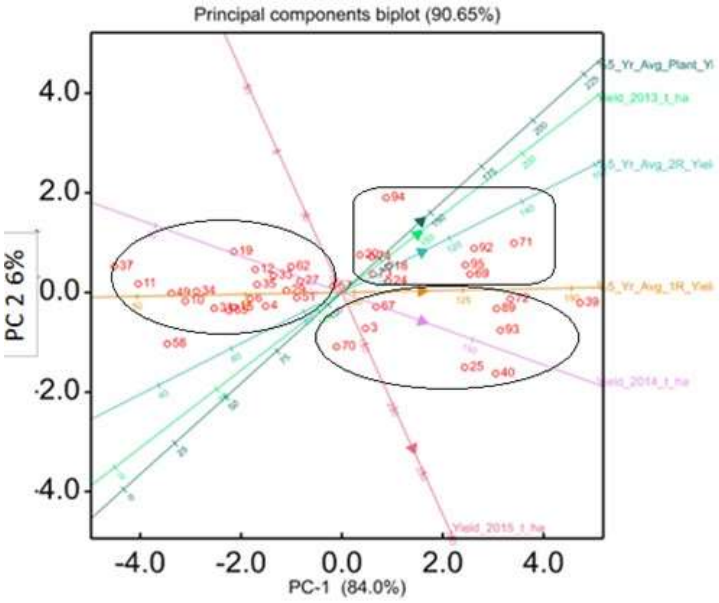


Figure 338 The loading of yield variables (lines) and score of 39 monitoring sites for the first two PCs in the PCA analysis. The numbers denote the farms and fonts denote the yield variables.

The PC1 and PC2 contributed 91% of the total variation in yield across farms whereas PC1 solely contributed 84% (Figure 338). The PC1 score showed a strong correlation with 2015 yield data (R^2=0.89, p<0.001). Hence, we used the PC1 as the vector for TCH to investigate the correlation between yield and YCS.

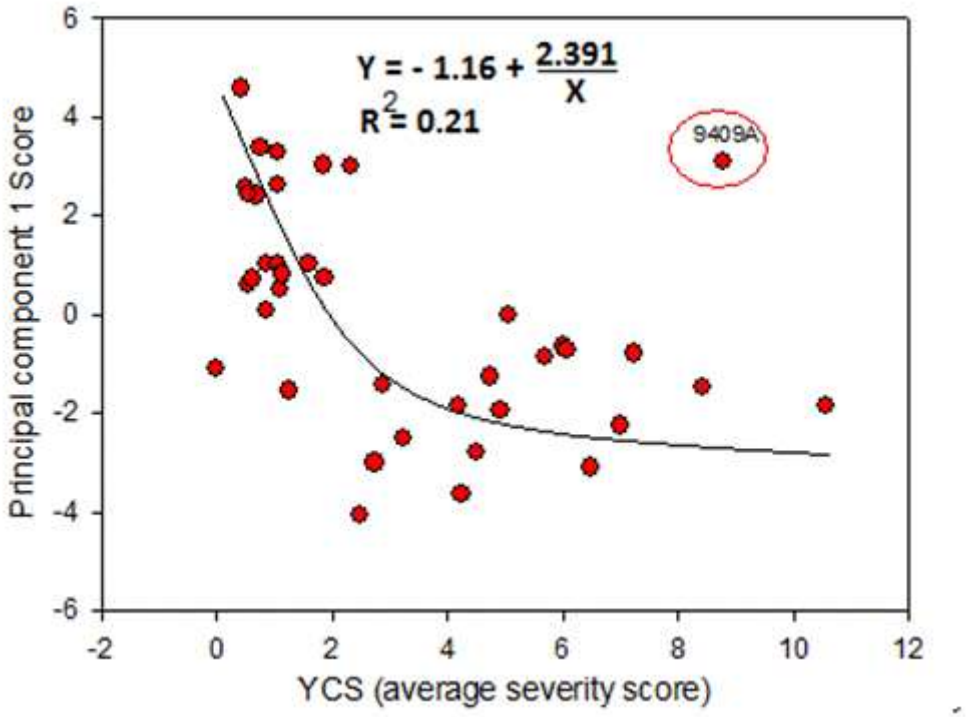


Figure 339 The association between YCS severity (average) and PC I (average yield response) for the 39 farms in Burdekin and Herbert region.

The coefficient of determination between YCS intensity and PC 1 (represents average yield trend) was 0.21 ($p<0.05$) (Figure 339). This suggests that the average YCS severity score could explain 21% of the variation in average yield response in these farms. However, these results were based only on 39 farms where the complete yield data was available.

Industry Averages - trends

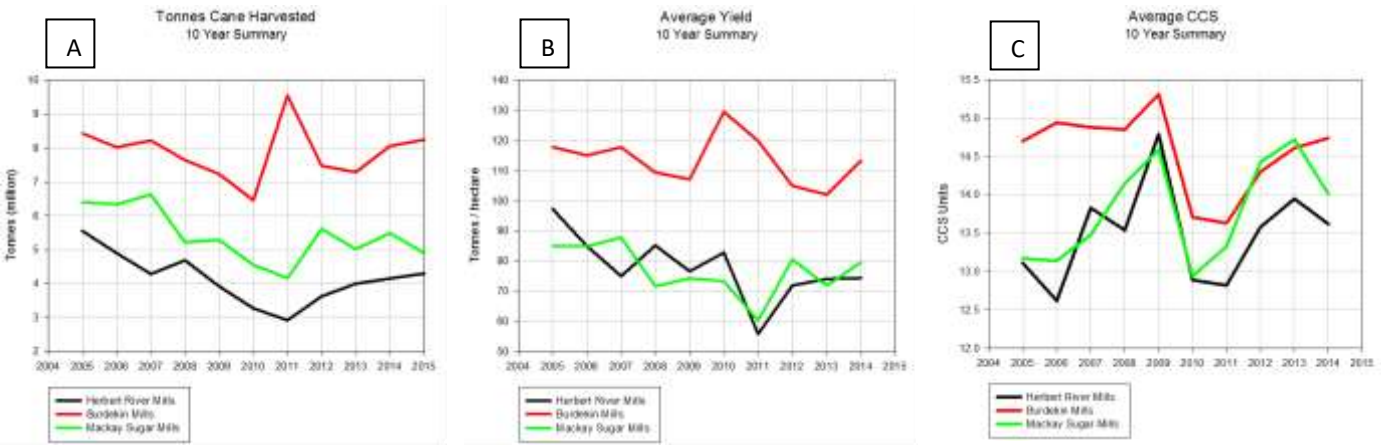


Figure 340 10 Year Mill averages for the Burdekin, Herbert and Mackay region. Tonnes harvested A), Average yield B) and Average CCS C). YCS was first noticed in 2012.

Summary

- Industry figures show a positive trend for tonnes harvested and average yield since 2011 (Figure 340 A & B)
- CCS shows mixed results (Figure 340 C). There are a range of
- Climate is the primary productivity driver, and conditions have been good the last few years. Growers will suggest that productivity in recent years should have been much greater given these good conditions.
- The long-term averages do not show any significant decrease that we could attribute to YCS. These district averages may hide the impact to individual growers.
- We speculate that the variability in YCS impact, at the grower scale, is due to differences in severity, and perhaps timing of the condition. Our database analysis may provide some answers.

6.13.2 Chemical induced control

6.13.2.1 Confidor® Trials

2014 (Stone River, Ingham)

A field trial was conducted to determine if Confidor® (Imidacloprid) reduces the prevalence or severity of YCS symptoms in sugarcane. The trial was established at HCPSL approved seed plot site in Stone River, Herbert district. Four treatments were imposed: Confidor® (22mL/100m), Confidor® (44mL/100m), Confidor® (44mL/100m+22mL/100m at fill in), and an Untreated Control. Plots were 4 rows by 10m, with 4 replicates. Variety was Q208 plant cane. The trial was planted on 15 July, 2013 and was monitored through to final harvest at 11 months. Final biomass was measured on 17 June, 2014 (see Appendix 1: 1.2.2)

Final fresh weight biomass was measured for each of the monitored stalks. These measurements are presented below (Figure 341) together with average YCS severity for each monitored stalk. Linear regression analysis, and Pearson's correlation test, was performed to determine the strength of the linear relationship (if any) between YCS and final biomass. Statistix software was used (version 10.0). There was no significant effect of treatment on final stalk biomass, so treatment was not included as a predictor in the final regression analysis

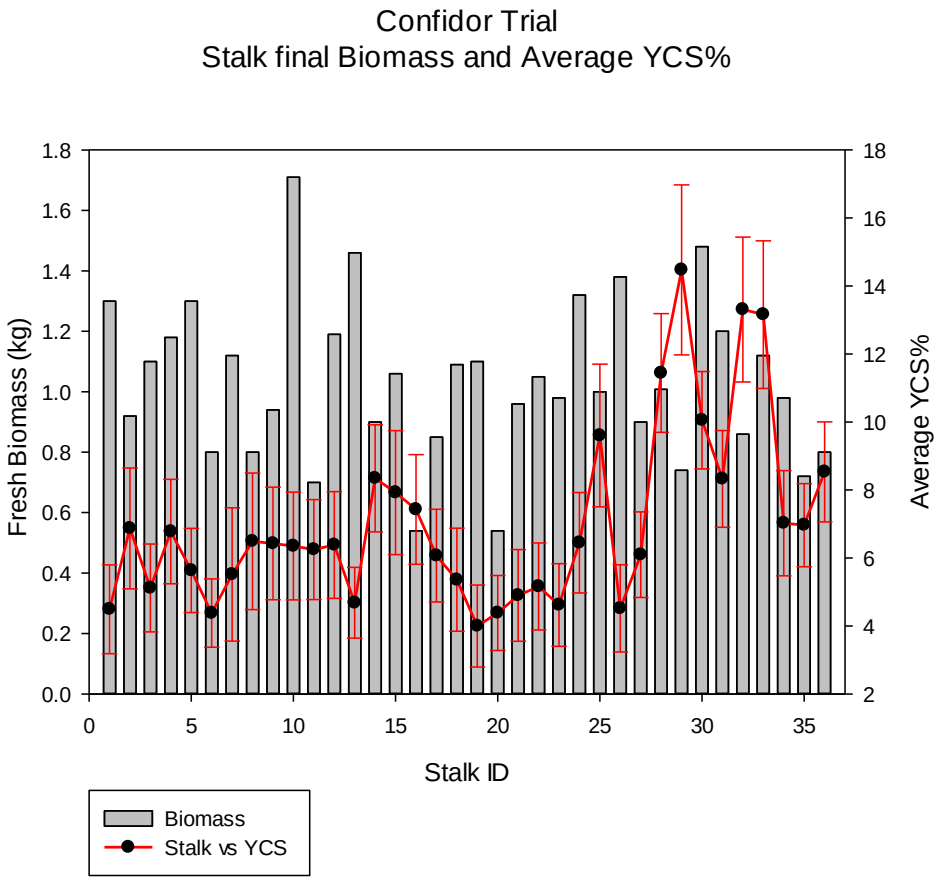


Figure 341 Stalk biomass and average YCS severity per stalk at the Herbert Confidor Trial. YCS severity has been averaged across 29 observations and is expressed as a % (number of YCS affected leaves per stalk / total leaves per stalk). Standard errors shown.

Summary

YCS severity was not shown to be a significant predictor of final biomass (p=0.417). At this site there was YCS was not correlated with fresh weight biomass (Pearsons correlation r= -0.013) (Figure 341)

2015 (Stone River, Ingham)

The purpose of this experiment is to evaluate whether Confidor® reduces the prevalence and/or severity of YCS symptoms, whether soil-borne insects or root health are contributing factors, and whether yield and sugar are impacted as a result (see Appendix 1: 1.2.2).

Biomass and CCS

Biomass sampling was undertaken on June 17th, 2015. All 4 treatments and 4 reps were measured. Analysis of variance of means was undertaken using Statistix10 at a 95% confidence level. Data was analysed as a randomized complete block design.

Biomass was not statistically different between treated and untreated plots, although the mean was lower in the untreated plots (Table 76). Plant heights were also very similar between treatments. There was some spread in the CCS means for plots, however no statistical difference was found.

Table 76 Biomass results. ANOVA performed at 95% confidence. Difference between groups has been determined by a Tukey's HSD all-pairwise comparisons (p<0.05).

Treatment	Stalk Height (cm)	Stalks/10m	Total Biomass (t/ha fresh)	Millable Stalk (t/ha dry)	CCS
Confidor® 22	254.9	138.8	105.82	21.40	10.05
Confidor® 44	242.0	145.5	109.13	22.33	10.31
Confidor® 44+22	243.5	144.3	106.50	22.19	8.97
Untreated Control	245.2	133.0	93.87	18.89	9.31
p-value	0.512	0.545	0.321	0.187	0.086
CV	5.2	9.4	11.3	10.7	7.5

Summary

These results are consistent with findings of other YCS Confidor® trials (see sections 6.3.2 & 6.3.4 of this report) where a treatment induced a stay-green effect, or reduced YCS severity, does not equate to a significant cane yield or CCS benefit.

6.13.3 Water stress physiology & YCS

Water stress, photosynthesis and YCS observations

In this section, physiological reasoning for differences in carbon assimilation between water stress and irrigated treatments in the clonal evaluation trial in Brandon, and the correlation between cane yield (TCH) and YCS (visual grading and starch diagnostic test) are discussed.

Photosynthesis impairment due to water stress and YCS

There was a significant genetic variation among clones for both photosynthesis (A) and stomatal conductance (g_s) (see section 6.11 in this report). There was a strong correlation between stomatal conductance and photosynthesis (r^2 =0.9) in all 20 clones (Figure 342). The relationship between A and g_s was mostly linear with a higher slope under water stress conditions (Figure 342). The limitation in stomatal conductance has resulted in low A in drought affected clones.

During the early stages (5-6 months) of water stress (August – December 2015), the average rate of photosynthesis was approx. 28.5 mmol m^{-2} s^{-1} in the drought treatment, which was only 75% of the full potential (37.5 mmol m^{-2} s^{-1}) of irrigated conditions (Figure 342). This reduction in photosynthesis

under water stress was mainly caused by the variation in stomatal limitation among clones (Basnayake *et al.* 2015). When water was limited, a rapid stomatal response (g_s) was observed resulting in a rapid decline in photosynthesis. Potential genetic variation for photosynthesis among the test clones was observed under irrigated conditions where there was no stomatal limitation.

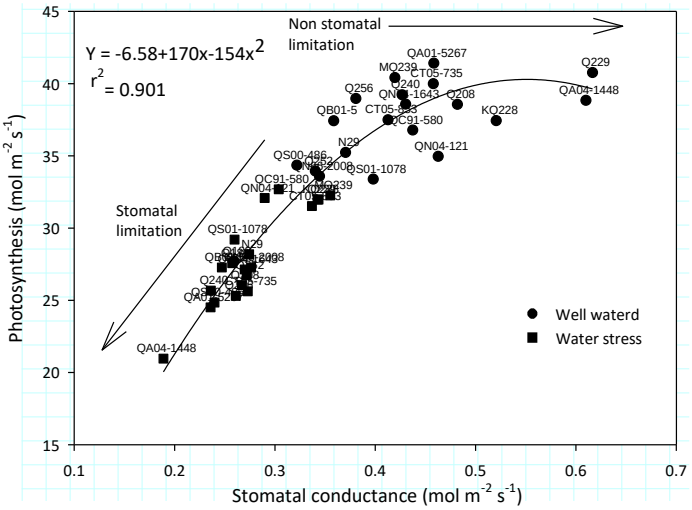


Figure 342 Relationship between Photosynthesis and stomatal conductance in all 20 clones in ratoon 2 crop under well-watered and water stress conditions.

A high photosynthesis clone, QA04-1448, has shown a severe stomatal limitation under water stress and hence had the lowest level of photosynthesis. However, most of the clones in the drought treatment have recovered well during the wet season, thus the reduction in TCH (20%) under water stress was not proportionate to the reduction in photosynthesis at the early stages of growth. YCS yellowing was observed in almost all the varieties regardless of the water availability. However, there was a significant yield difference (TCH) between treatments. The difference in YCS occurrence and TCH within each treatment was significant.

As reported earlier (Marquardt et al., 2016), the leaf with YCS symptoms had photosynthesis impairment mainly during the YCS peak period. In our study, first YCS observation was recorded in the irrigated treatment. During a 12-month period, stalks have produced an average of 32±2 leaves/stalk in the irrigated treatment whereas only 28±2 in the water stress treatment. The number of symptomatic leaves varied with the period of observations. The photosynthesis impairment caused by water stress was much higher than that of YCS effects on mature leaves in the canopy.

Comparison of Leaf colour (visual) and starch scores for leaves + 3 to +9

At the peak of YCS season, leaf samples (Leaf + 3 to +9) from YCS symptomatic stalks were collected from the ratoon 2 crop of the field trial in Brandon. Samples were collected early in the morning from all 3 replicates of irrigated and water stress treatments. Leaves were individually scored based on the relative greenness and YCS prevalence.

Table 77 Comparison of visual grading and diagnostic starch test scores of leaves + 3 to +9 in all 20 clones in the ratoon 2 crop in Brandon.

	Leaf greenness	Starch diagnostic test

Clones	Water stress	Irrigated	Average	Water stress	Irrigated	Average
CT05-735	9.3	11.0	10.2	12.0	8.0	10.0
CT05-853	14.7	11.0	12.8	8.3	8.8	8.6
KQ228	9.7	9.5	9.6	7.0	3.5	5.3
MQ239	5.3	8.5	6.9	2.3	2.0	2.2
N29	10.3	7.5	8.9	6.7	1.5	4.1
Q183	10.3	10.0	10.2	6.3	5.0	5.7
Q208	2.3	4.0	3.2	0.0	1.5	0.8
Q229	11.7	12.8	12.2	10.0	11.5	10.8
Q240	11.7	4.0	7.8	3.0	1.5	2.3
Q252	12.0	10.0	11.0	8.0	3.0	5.5
Q256	6.3	3.0	4.7	3.3	1.0	2.2
QA01-5267	6.0	4.0	5.0	1.0	3.0	2.0
QA04-1448	10.0	10.5	10.3	8.3	6.5	7.4
QB01-5	9.0	12.0	10.5	7.7	6.5	7.1
QC91-580	5.0	3.0	4.0	1.7	1.5	1.6
QN04-121	4.3	4.5	4.4	1.3	1.5	1.4
QN04-1643	8.0	10.5	9.3	3.7	9.0	6.3
QN66-2008	10.0	10.0	10.0	3.3	9.5	6.4
QS00-486	9.7	4.0	6.8	4.7	0.0	2.3
QS01-1078	8.7	7.5	8.1	6.0	7.5	6.8
Mean	**8.7**	**7.9**	**8.3**	**5.2**	**4.6**	**4.9**
Lsd 5% (clone)			**3.2**			**1.5**
Treatment difference not significant						

Green leaves with no yellowing were scored as 0 and senesced leaves as 6. Leaves with 5-25% yellowing, 25-50% yellowing, green leaves with yellow or dead edges of 2-5mm, 25% yellowing with drying edges and >60% yellowing had scores of 1, 2, 3, 4 and 5, respectively. For starch scores, the standard ratings were given to the iodine stained leaves. The total ratings of + 3 to +9 leaves were statistically analysed to test the variation among clones, treatments and clone x treatment interactions. The difference among clones was highly significant ($P<0.001$), whereas the treatment and treatment-by-clone interactions for both visual and starch tests were non-significant (Table 77).

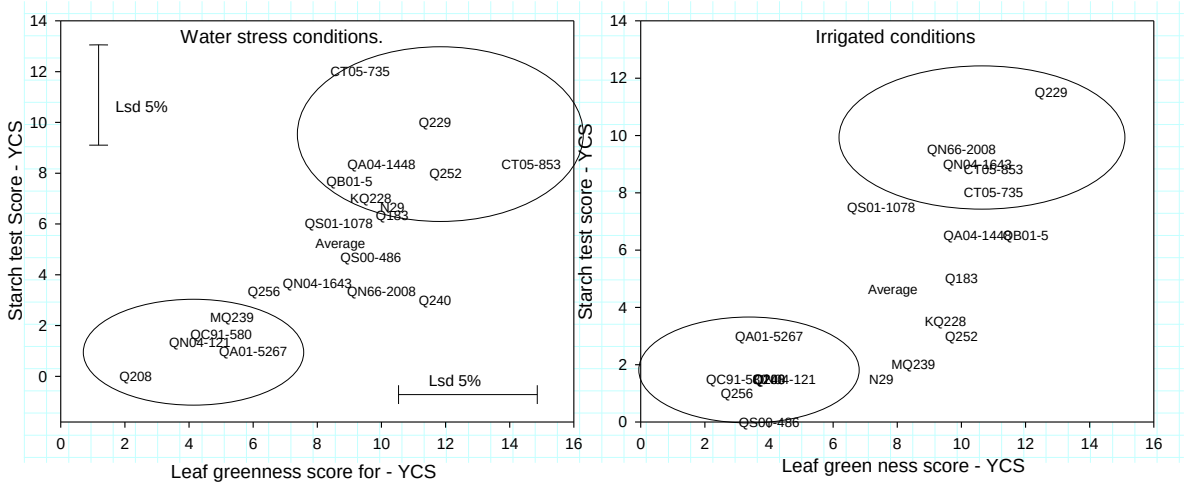


Figure 343 Comparison of scores for Leaf greenness, and presence and amount of starch in leaves +3 to +9 in 20 clones sampled from the irrigated and drought treatments in the field trial.

The relationship between to the 2 scoring methods was used to identify clones with similar response (Figure 343). In all 20 clones, a degree of yellowing was evident mainly in lower leaves, however, the individual starch scores have not followed the same pattern. Using the scores for greenness and presence and amount of starch, clones with high, moderate and low YCS incidence were identified.

The differences in ranking of clones for YCS under irrigated and water stress conditions were investigated. Among all 20 test clones, Q229$^{(\phi)}$, CT05-853 and CT05-735 had the highest rating while Q208$^{(\phi)}$, QC91-580, QN04-121 and QA01-5267 had the lowest under both water treatments. Clones Q252$^{(\phi)}$, KQ228$^{(\phi)}$, Q183$^{(\phi)}$ were in the high YCS group under water stress and moderate under irrigation (Figure 343). However, the clone x YCS interaction for the 2 scoring methods was not significant (P>0.05).

Clone responses to different water stress conditions and relationship with cane yield (TCH) and sugar (CCS)

At 12 months, the ratoon 2 crop was machine harvested and TCH, CCS, TDM and TSH were measured Table 78). At harvest, eight mature stalks from the two middle rows of each plot were sampled at random for detailed measurements, and total plot weights were measured using an electronic weigh bin. Stem of each mature stalk was separated from the shoot top at the point of the youngest visible node, the fresh and dead leaves in the eight stalks were removed and weighed, and a sub-sample was used to determine the fresh and dry weight ratio. The ratio of millable stalk weight to Leaf weight of 8-stalk sample was estimated for each sample. The fresh and dry weights of samples were used to estimate total dry matter (TDM) of each clone.

To obtain juice for analysis, four stalks from each 8 stalk sample were finely shredded using a cutter-grinder, 500 g of shredded material was transferred into a steel cylinder and juice was extracted using a Carver Press at 15.7 MPa for 60s. The extracted juice sample was used to measure Brix and pol % in juice, and the commercial cane sugar (CCS) was estimated. After extracting juice, the remaining cane residue was weighed and oven-dried to determine the fibre content (%) of cane. The Spectra cane was used simultaneously for juice and fibre analysis. The total Leaf dry matter per hectare was estimated using the Leaf to millable stalk weight ratio of the sub sample collected from each plot at harvest. The cane yield and sugar data are presented in Table 78.

Table 78 Mean values of tonnes cane per ha (TCH), commercial cane sugar (CCS), tonnes sugar per ha (TSH), total dry matter (TDM) and Fibre % in well irrigated and drought treatments. Summary statistics are given for the combined analysis of two water treatments and 20 clones. ns = not statistically significant.

Clones	Water stress					Irrigated				
	TCH	CCS	TSH	TDM	Fibre %	TCH	CCS	TSH	TDM	Fibre %
CT05-735	86.9	11.7	10.6	27.8	11.2	110.9	11.8	13.8	36.3	11.8
CT05-853	79.6	7.7	6.4	26.0	14.7	91.7	6.6	6.3	29.6	15.0
KQ228	96.6	12.4	12.4	30.8	11.3	114.1	14.7	17.4	37.7	11.9
MQ239	109.7	11.7	13.4	37.6	13.9	108.4	11.1	12.6	39.1	15.3
N29	78.3	9.9	8.2	25.2	12.4	92.3	11.0	10.6	29.4	11.4
Q183	59.5	11.9	7.5	18.8	11.1	102.8	13.4	14.3	34.2	11.7
Q208	88.2	11.5	10.6	27.9	12.1	105.6	9.8	10.9	34.0	12.2
Q229	85.8	12.4	11.2	26.8	11.5	128.5	10.5	14.0	40.7	10.6
Q240	94.8	13.0	12.8	30.3	12.0	125.0	13.2	17.2	41.3	12.1
Q252	65.2	13.1	8.8	21.3	11.1	104.4	13.3	14.5	33.5	11.4
Q256	107.9	12.1	13.5	34.8	11.2	120.1	12.3	15.5	38.2	11.2
QA01-5267	65.3	12.2	8.4	21.5	11.5	106.3	14.2	15.7	35.5	10.8
QA04-1448	85.4	10.4	9.3	25.3	10.0	112.4	10.4	12.2	33.5	10.2
QB01-5	21.8	6.8	2.0	6.3	10.9	56.2	3.9	2.2	18.4	17.9
QC91-580	47.5	10.9	5.5	14.6	11.9	96.0	9.9	9.9	30.0	11.4
QN04-121	54.7	10.8	6.1	16.5	10.9	107.6	9.9	11.2	33.4	10.9
QN04-1643	64.6	11.8	7.9	20.3	10.8	93.9	12.7	12.4	30.2	10.4
QN66-2008	51.2	9.5	5.1	15.9	11.4	72.2	10.0	7.4	23.4	12.3
QS00-486	88.2	13.6	12.6	28.8	10.9	103.2	12.7	13.7	33.1	11.0
QS01-1078	63.4	11.3	7.5	21.1	12.6	85.2	11.7	10.5	29.0	12.9
Grand Total	74.7	11.2	9.0	23.9	11.7	101.8	11.2	12.1	33.0	12.1
Mean	74.7	11.2	9.0	23.9	11.7	101.8	11.2	12.1	33.0	12.1
Lsd 5%										
Clones	20.38	1.64	2.85	6.61	0.91	20.38	1.64	2.85	6.61	0.91
Treatment (T)	22.60	ns	3.09.	7.30	ns	22.60	ns	3.09.	7.30	ns
Clone x T	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

In general, the clones with high yields performed best under both non-stressed and stressed conditions (Table 78). The average cane yield, TSH and TDM were lower in the water stress treatment than in the corresponding irrigated treatment, with the yield reduction of about 24% (Table 78) compared to the irrigated treatment. A similar trend was evident for TSH and TDM. This suggests that the water deficit in the water stress treatment in the 2R crop had a proportionately greater impact on yield per cumulative stress-days (around 80 days). The key difference between these two treatments was the

duration, and thus the severity of the water stress, with a longer duration of stress earlier in the growth cycle in the water stress treatment, while in the irrigated treatment only few stress-days during maturing prior to harvest. However, the 20 clones in the water stress treatment recovered remarkably during the wet season in January 2016 resulting only 24% yield reduction.

The relationship of YCS with cane yield and CCS

The relationship between YCS observations (starch ratings) with TCH and CCS of the 20 clones were estimated for irrigated and water stress environments. There was no association between YCS and TCH or TDM (data not shown) at harvest. Though there was a negative trend, the correlation between YCS severity and CCS under the two water treatments was poor (P>0.05) (Figure 344). According to this results, clones such as Q229(ↀ) and CT05-735 had produced higher TCH under both water treatments regardless of high YCS prevalence at 7-9 months stage. KQ228(ↀ) had moderate YCS rating among all but maintained relatively higher TCH and CCS under both water conditions.

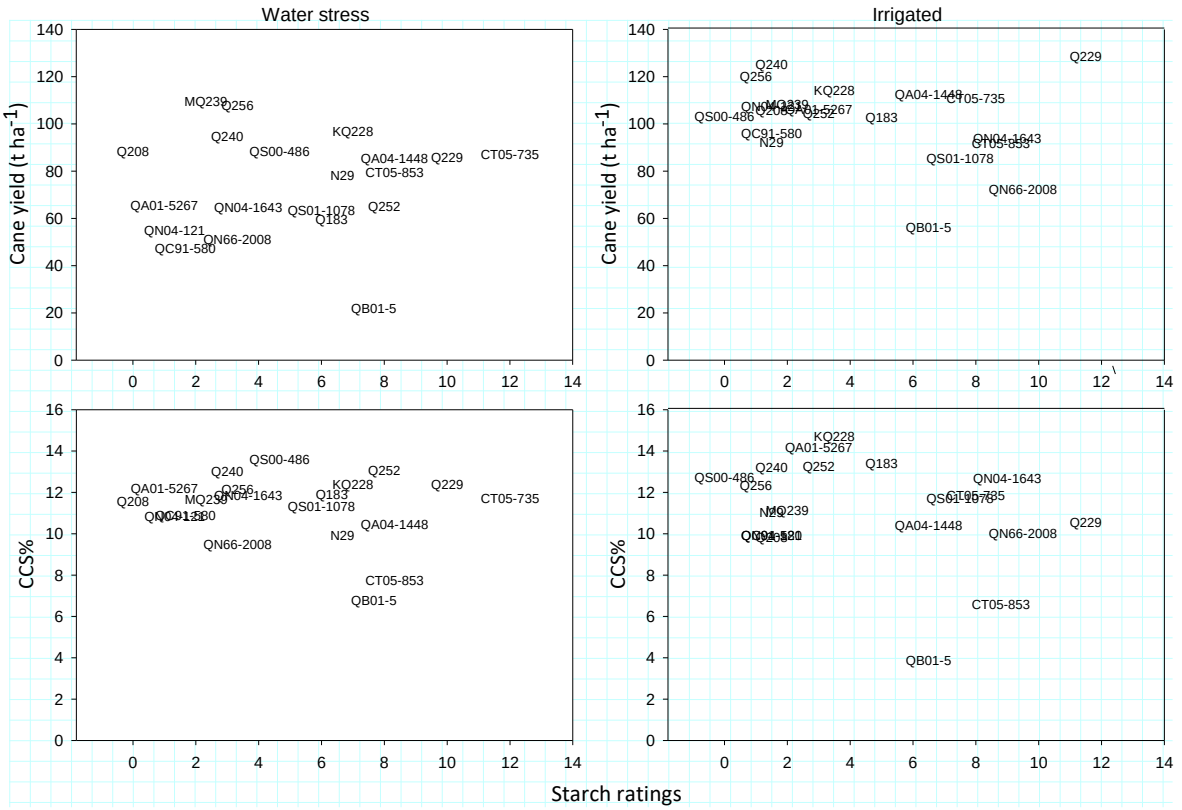


Figure 344 The relationship between YCS incidence (using starch test scores) with TCH and CCS in 20 clones under water stress and irrigated conditions.

Generally, YCS appears during summer months (between late December to following March) where the crop growth is at the peak stage. The severity of YCS varies among clones and it affects the effective leaf area of respective clones. Therefore, to understand the consequences of YCS on leaf area, the leaf area index (LAI) was estimated after the YCS period and compared with YCS ratings and the final TCH (Figure 345).

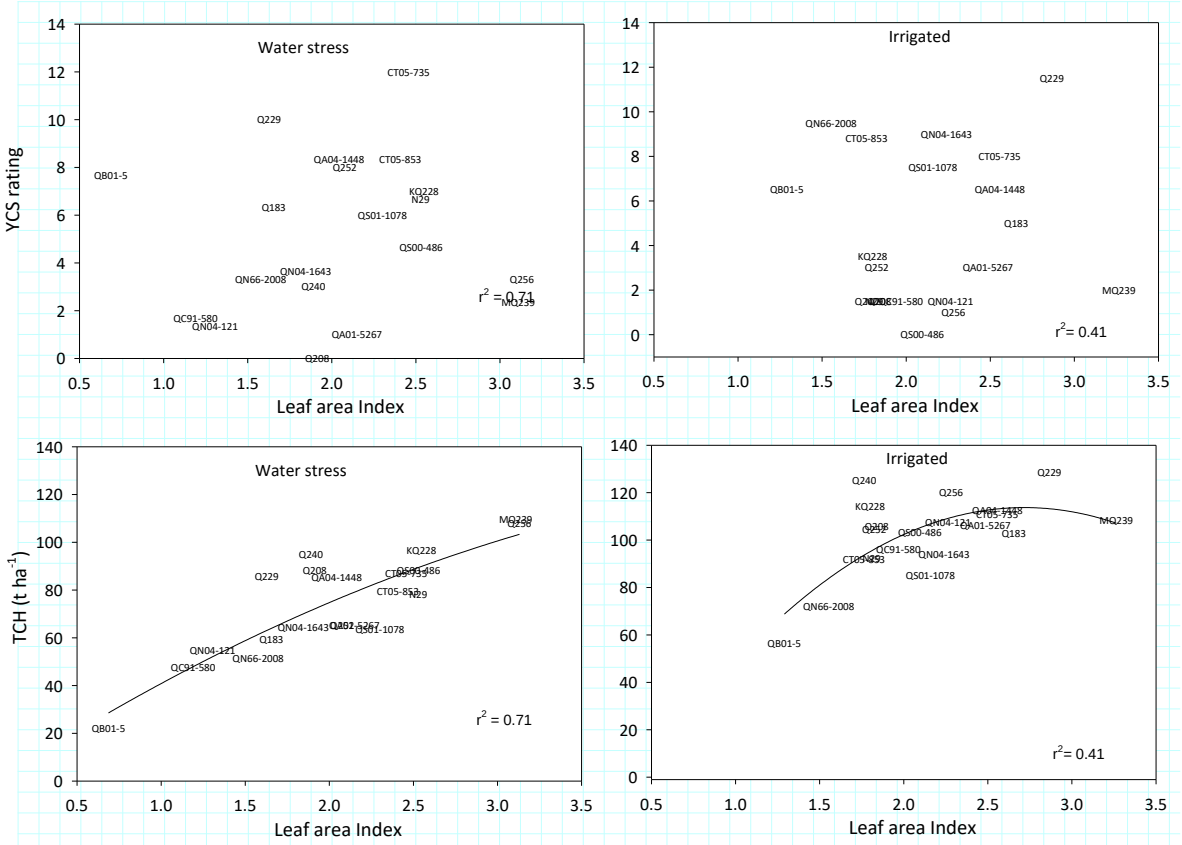


Figure 345 The relationship between Leaf area index after YCS period with the YCS ratings and TCH in 20 clones under water stress and irrigated conditions.

The results clearly showed that there is no correlation between YCS and leaf area index (Figure 345: upper panel). However, there were significant correlations between leaf area index and TCH under both water stress and irrigated conditions. Those clones with high leaf area index after the YCS episode continued higher photosynthesis and produced higher biomass. This association was stronger under irrigated conditions, because most of the leaf area development occurred during or after the wet season. The rapid growth occurred under wet season rainfall must have compensated the leaf area reduction due to YCS.

Summary

The yield reduction under drought was mainly caused by reduction in photosynthesis due to stomatal limitation. The YCS was not observed during this phase in both irrigated and drought treatments. However, there was a significant biomass variation between water stress and irrigated treatments at 6 months stage. Most of the test clones had higher YCS ratings during the peak YCS period, however, there was no correlation between YCS and TCH at harvest. There was a positive correlation between leaf area index after YCS period and TCH, and this correlation was stronger under water stress conditions. The clones with high leaf area index were able to maintain high radiation interception and hence high radiation use efficiency for high biomass production.

6.13.4 Is there an association between paddock cane yield and YCS score?

6.13.4.1 Herbert report - Geoff Inman-Bamber 18.11.2016 (Inman-Bamber et al., 2016)

Background

It was shown that the APSIM-Sugar model accounted for 53% (49% after cross validation) of the year to year variation in the mean sugar yield in the Herbert (2010-2015). Yields for the 'YCS years' (>2011) were excluded in order estimate what yields should have been without YCS. From the analysis, it was concluded that yields for 2012, 2013 and 2014 were very close to the yields expected from the modelling but the yield for 2015 was 12 ± 7% lower than expected, possibly because of the YCS phenomenon.

In this report we consider yields and YCS scores of individual paddocks being monitored in the Herbert. The approach is similar to the one for estimating average yields for the whole region but in this case, we have some information about the planting date and we know the ratoon date for each paddock. We can associate a weather station with the locality of the paddock and we can use the 10-year average yield as a guide to what soil parameters to use in the model, for each paddock.

Soil properties have a major influence on yields in rainfed areas such as the Herbert. Without accurate details about soil hydrology it would be difficult to simulate crop growth and yield accurately even in well controlled experiments. Yields of commercial paddocks differ from those of experimental plots because of many factors not represented in the APSIM model. Nevertheless, this model offers the best way of accounting for the impact of climate crop yield.

Methods

Planting dates

Planting dates were recorded as 'early', 'late season' and 'replant'. In the model we have to assign a unique date for planting and after advice from Mr Lawrence di Bella it was decided to 'plant' early- and late-season crops on 10 April and 7 September respectively. Crops recorded as 'replant' were assumed to be planted on 27 October. The year of planting was recorded and taken into account but most of the monitored crops were planted in 2012. If the planting date was not known, it was assumed to be a mid-season planting on 30 May.

Climate

Daily climate records were obtained (with thanks) from the Long Paddock website, operated by the Science Delivery Division of the Department of Science, Information Technology and Innovation (DSITI) of the Queensland government. The 'Patched point' database was accessed in order to associate a specific climate recording station to the district in which each paddock was located (Table 79). Daily rainfall, radiation, maximum and minimum temperature were required for the simulation of the crop in each paddock.

Table 79 Districts and their associated climate station

District	Climate station
Ingham Line	Bambaroo
Central	Ingham

Lower Herbert	Bambaroo
Wet Belt	Longpocket
Victoria	Victoria
Macknade	Victoria

Soils

Soil type information for the monitored paddocks was not provided. Instead, 10-year mean cane yields were used to select the depth of the soil and hence the water holding capacity (PAWC) for each paddock (Table 80). Other physical and chemical properties were obtained from the APSIM soil library (Macknade 923 P).

Table 80 Allocation of soil depth and PAWC to each paddock based on its 10-year mean cane yield.

10-year mean cane yield	Soil depth (mm)	PAWC (mm)
<=70 t/ha	600	72
>70 and <87 t/ha	1200	144
>= 87 t/ha	1800	216

Crop physiology

APSIM-Sugar can simulate accurately the development and yield of crops growing in well controlled experiments and new features have been added recently to improve the simulation of these experiments(Inman-Bamber et al., 2016). These features include the effect of water stress on transpiration efficiency (TE), the reduction in photosynthesis with crop development and the respiration of sucrose. It is hoped that the latter two features will help in accounting for the difference in simulated yield and the yield obtained from sugarcane grown on a commercial scale. There is uncertainty about the exact coefficients for simulating these processes, so a small range of coefficients were included in the settings giving rise to 12 ensembles for estimating the yield of each paddock. These are the same ensembles used for the simulation of historical yields of the Herbert region.

Table 81 Physiological factors and levels used in the new APSIM sugarcane module (v 7.7). (Codes used in Table 81 are in parenthesis).

Factor	Level 1	Level 2	Level 3
Growth slow-down between leaf #10 and #24	0% (sd1)	20% (sd2)	40% (sd3)
Respiration of sucrose	None (re1)	As a function of temperature (re2)	
Transpiration efficiency response to water stress	None (te1)	Responsive (te2)	

YCS observations

Paddocks in which YCS was observed, were monitored repeatedly for development of the syndrome. The canopy was separated into three components for these observations and severity (S) and prevalence (P) were rated separately for each component. The component (*i*) above the TVD leaf (leaf 1) was considered the most important as far as impact on yield is concerned; leaves 2 to 5, comprising the second component (*j*), were considered to be less impactful and the lower leaves (>5) were in the least important component (*k*). The overall YCS score (Y) was derived as: $3S_iP_i + 2S_jP_j + S_kP_k$, giving appropriate weightings to the different components of the canopy.

Various expressions of the YCS score were analysed for impact on yield. These included,

1) The average of all scores for the paddock
2) The total score for the paddock
3) The maximum score
4) The integral of the YSC score curve with respect to time
    a. From the earliest to the latest observation for each paddock
    b. While simulated leaf area index (LAI) was less than 3 (as for young crops or during severe stress periods)
    c. While the crop was < 150 days old

Results

Graphical analysis

There were 34 paddocks for which YCS scores were available for crops harvested in 2015 (Figure 346). Yield records were available for 2013 and 2014 but YCS monitoring started in 2015 and not before. Yields for 2015 for 30 of the 34 monitored paddocks were provided.

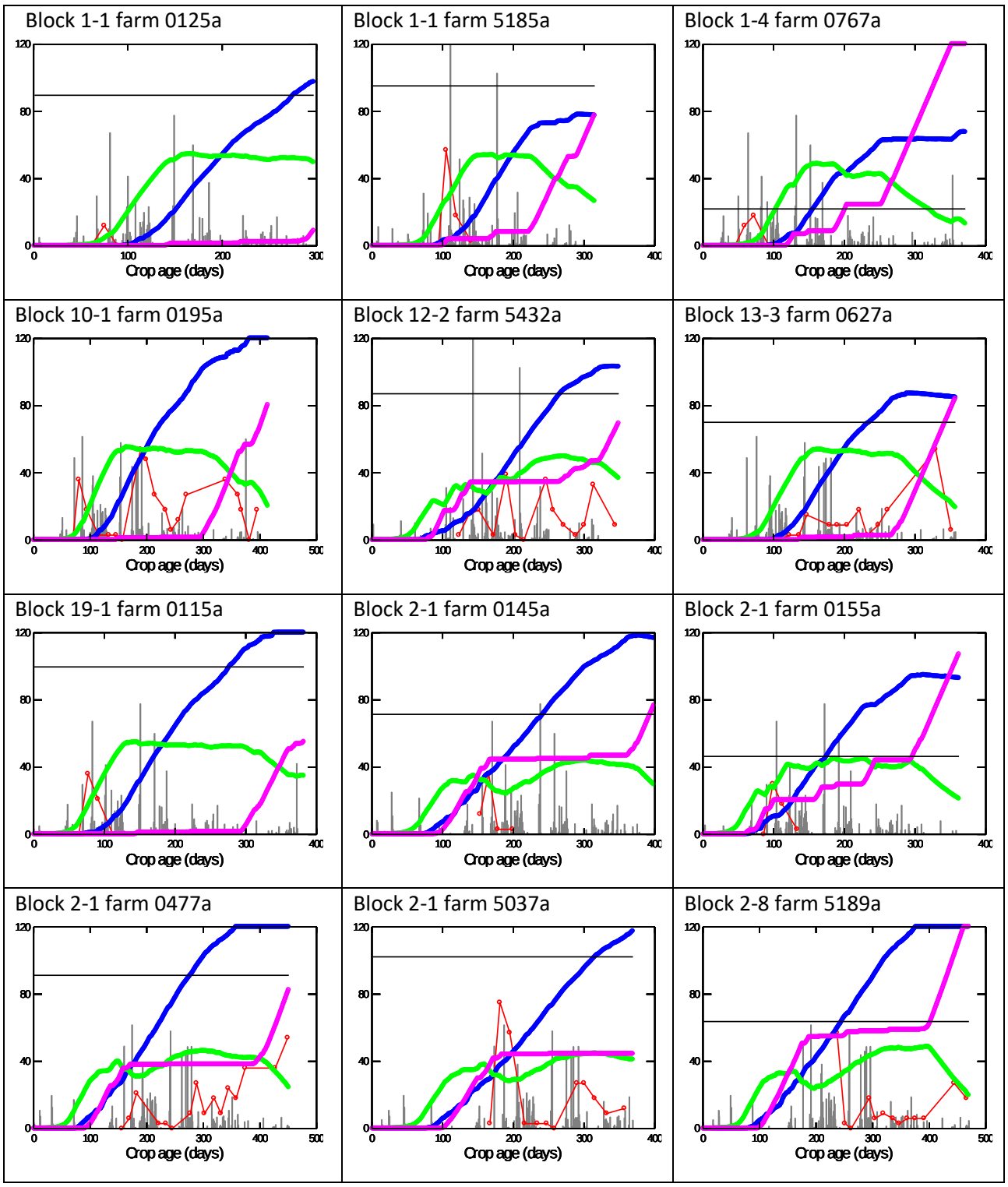
Several questions can be put to the data in Figure 346 where results of the simulations are presented together with the YCS score and the recorded cane yield. The simulations provide some idea of crop development in terms of leaf area index (LAI) and the degree of water stress endured by the crop, in terms of cumulative stress-days. YCS scores in Figure 346 have been scaled (x3) for clarity.

1) Does the overall YCS score explain the difference between simulated and observed yield.
    a. Block 1-4, farm 0767a produced a very poor yield. The simulated yield was also low but at least twice the observed yield. YCS was all but absent in this paddock and probably not responsible for the poor yield. Severe water stress was a likely cause.
    b. Block 3-2, farm 0505a had three waves of YCS, increasing in magnitude over time and the observed yield was similar to the simulated yield. YCS appeared therefore to have little impact on yield.
    c. Block 3-8, farm 0618a had no YCS and the observed yield was greater than the simulated yield which is a result we might have expected.

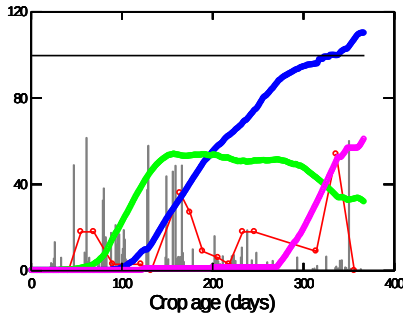
   d. Block 4-1 farm 5333a and Block 7-1 farm 0520a had very high YCS scores initially. Block 4-1 yielded about half the simulated yield and block 7-1 about 20% less. YCS could have limited yield here.

2) Is there a particular wave or peak in YCS that is associated with the difference between observed and simulated yield?
   a. Block 1-1, farm 0125a had a small YCS wave when the crop was small and Block 4-1, farm 5430a had a large wave at this stage. Observed and simulated yields were similar in both cases.
   b. Block 2-1, farm 5037a and Block 2-8, farm 5189a both had large YCS waves during the period of rapid cane growth. Observed cane yield was similar to simulated yield for farm 5037 and about half the simulated yield for farm 5189. The evidence here is conflicting.
   c. Block 3-2, farm 0505a, Block 13-3, farm 0627a and Block 7-7, farm 0211a all had a large YCS waves close to harvesting. Farm 505 yielded about the same as the simulation, and farms 627 and 211, a little less than the simulation. Block 2-1, farm 0477a ended up with a large YCS score and the observed yield was much lower than the simulated yield. The evidence here is again conflicting.
3) Is the yield difference between observed and simulated yields associated with the number of YCS waves?
   a. Block 1-1, farm 0125a and Block 1-4, farm 0767a each had one small YCS wave. The observed yield was similar to simulated yield for the one paddock and less than half the simulated yield for the other; conflicting evidence once more.
   b. Block 3-2, farm 0505a, Block 3-3, farm 5144a and Block 4-1, farm 5430a each had four YCS waves and their yields were similar to those simulated.
4) Is the duration and severity (area under the YCS curve) of YCS associated with the difference in between observed and simulated yield? Confidence in the answer to this question depends on the regularity of the YCS observation. From the records, it appears that some paddocks were visited at regular intervals and others not so.
   a. This is not true for Block 1-4, farm 0767a.
   b. Block 7-2, farm 0533a, Block 7-7, farm 0211a and Block 9-2, farm 0143a all had high YCS scores over a long time and their recorded yields were well below their simulated yields. However other paddocks (Block 5-1, farm 0463a, Block 5-4, farm 5026a) with shorter and less severe YCS 'infestations' yielded even less compared to the yield expected from the simulations.
5) Does the YCS score follow any particular rainfall or crop stress pattern
   a. Block 10-1, farm 0195a had two large YCS waves, one during a period or frequent rain and minimal crop stress and the other during a period of minimal rain and increasing crop stress. Block 13-3, farm 0627a developed severe YCS during a period of water stress and Block 1-1, farm 5185a during a period when stress minimal.

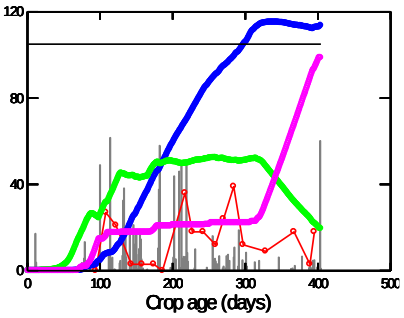
The evidence from Figure 346 does not lead to a clear conclusion about the impact of YCS on yield or on the impact of rainfall on the expression of YCS. Nevertheless, it is worth doing some regression analysis to determine if some associations have been overlooked.


Block 1-1 farm 0125a
Block 1-1 farm 5185a
Block 1-4 farm 0767a
Block 10-1 farm 0195a
Block 12-2 farm 5432a
Block 13-3 farm 0627a
Block 19-1 farm 0115a
Block 2-1 farm 0145a
Block 2-1 farm 0155a
Block 2-1 farm 0477a
Block 2-1 farm 5037a
Block 2-8 farm 5189a
Crop age (days)

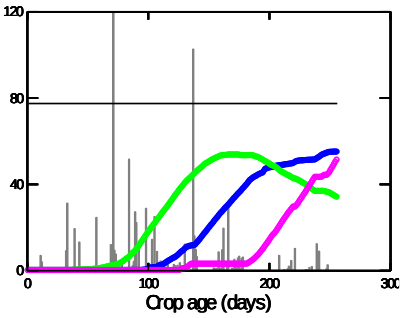
Block 3-2 farm 0505a



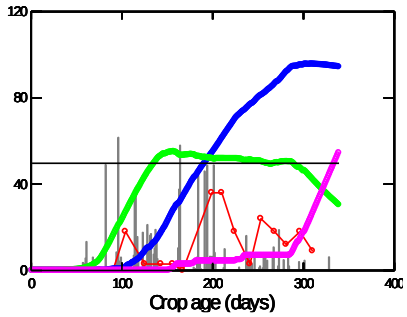
Block 3-3 farm 5144a



Block 3-8 farm 0618a



Block 4-1 farm 0250a



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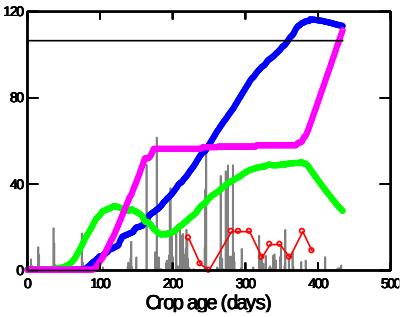

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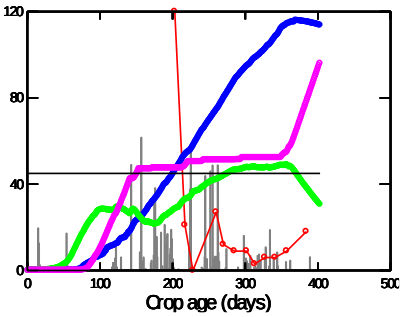

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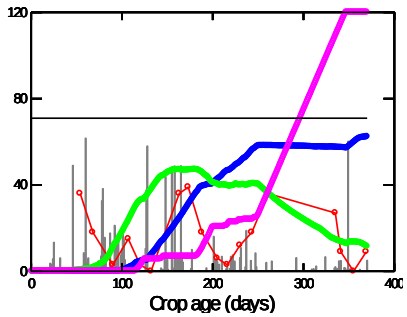

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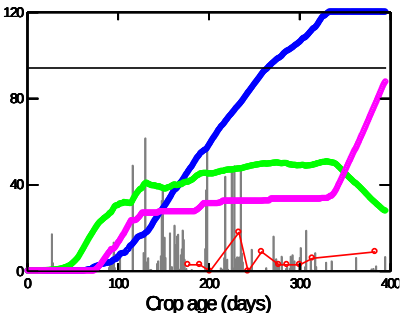
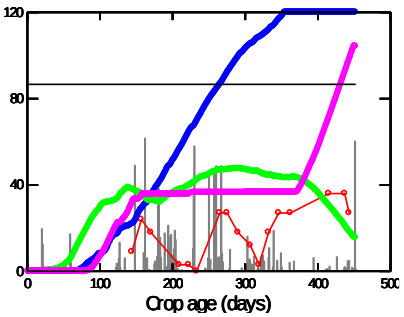


Block 48-2 farm 0455a



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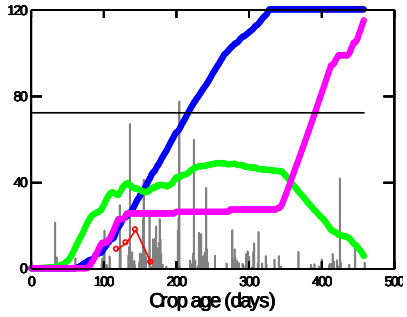
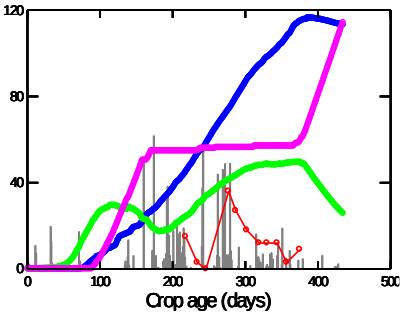
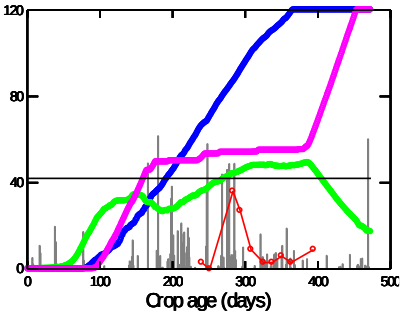


Block 5-1 farm 5080a



Block 5-4 farm 5026a



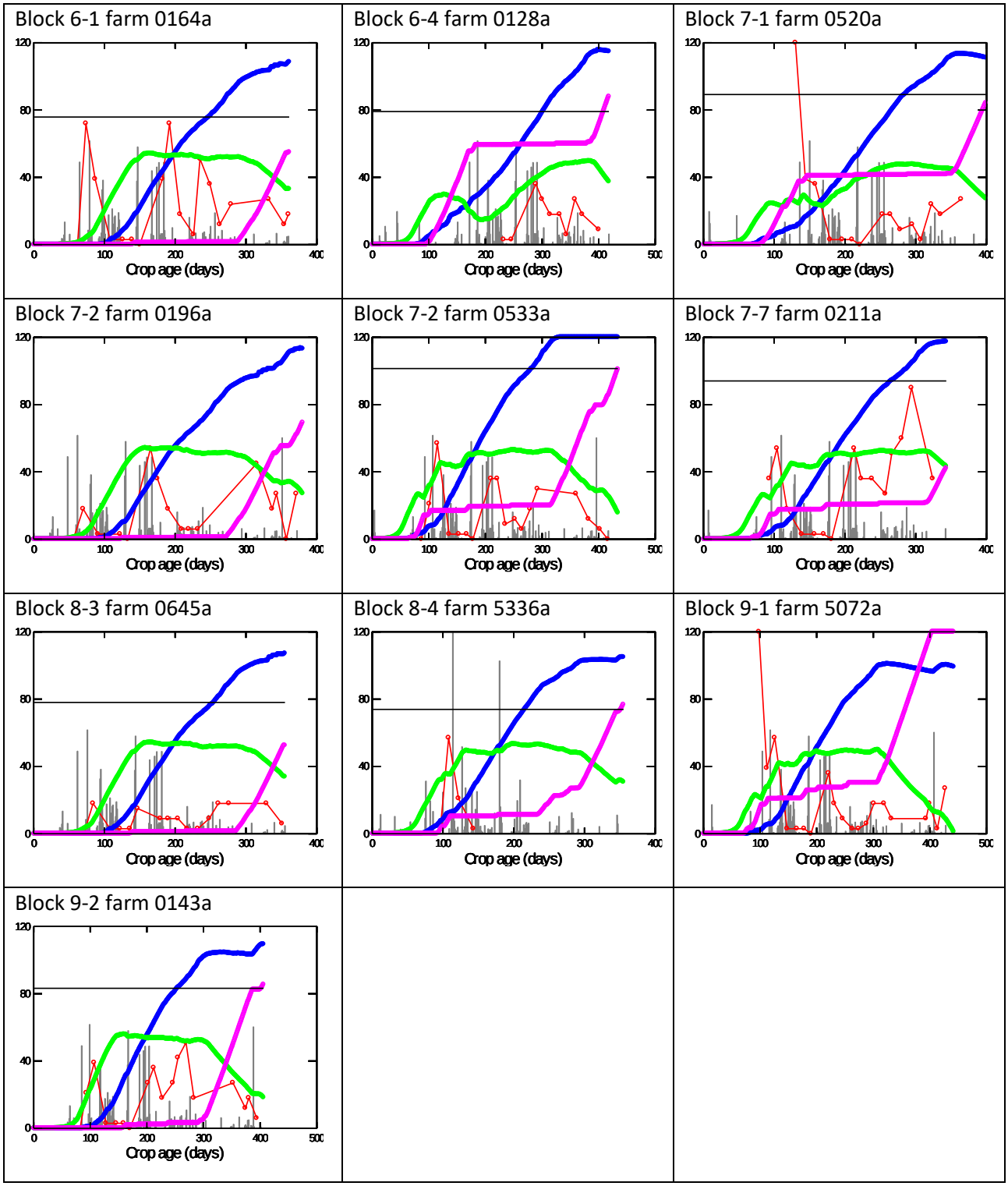


Figure 346 YCS scores x 3 (red line), LAI (green), simulated cane yield blue), water stress days (magenta), rainfall in (grey bars), recorded paddock yield (black horizontal line) for 34 monitored paddocks in the Herbert, harvested in 2015. Values greater than 120 were limited to 120 to fit on the graph

Scatter plot and regression analysis

Farm 10-year average yield accounted for 28% of the variation in paddock cane yield in 2015 (Figure 347) and the best of the physiological settings accounted for only 25% of this variation. When the soil settings were dissociated from the 10-year farm average yield, none of the simulations were significantly related to cane yields recorded in 2015. The 10-year average yields therefore had an

overriding influence on the simulations when they were used to define soil parameters as in Table 81. The simulations were useful for mapping YCS scores to crop development as in Figure 346 but with the 10- year mean farm averages available and accounting for more variation than the model, it was decided to use these data to remove as much variation from the 2015 yield data as possible, in order consider the impact of YCS on yield.

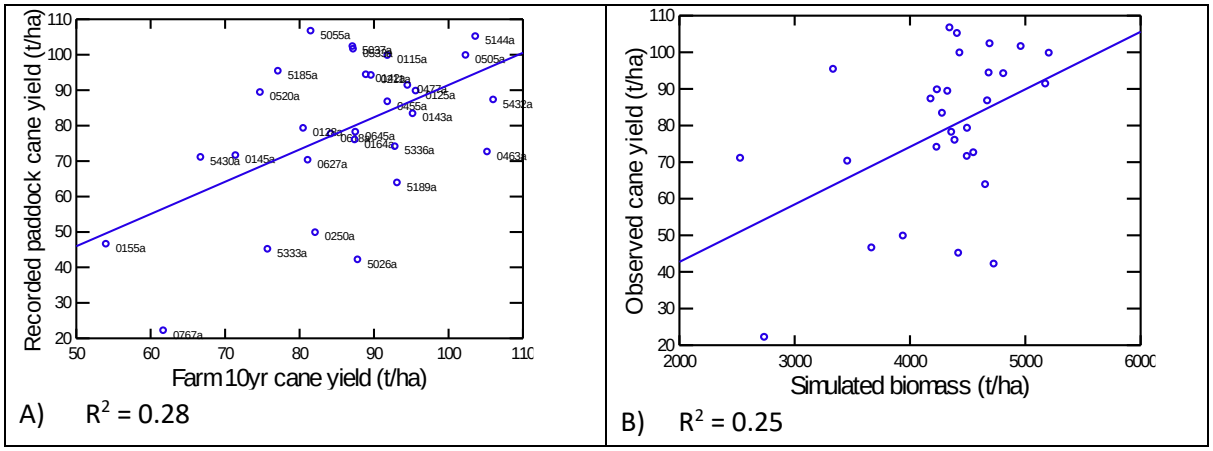
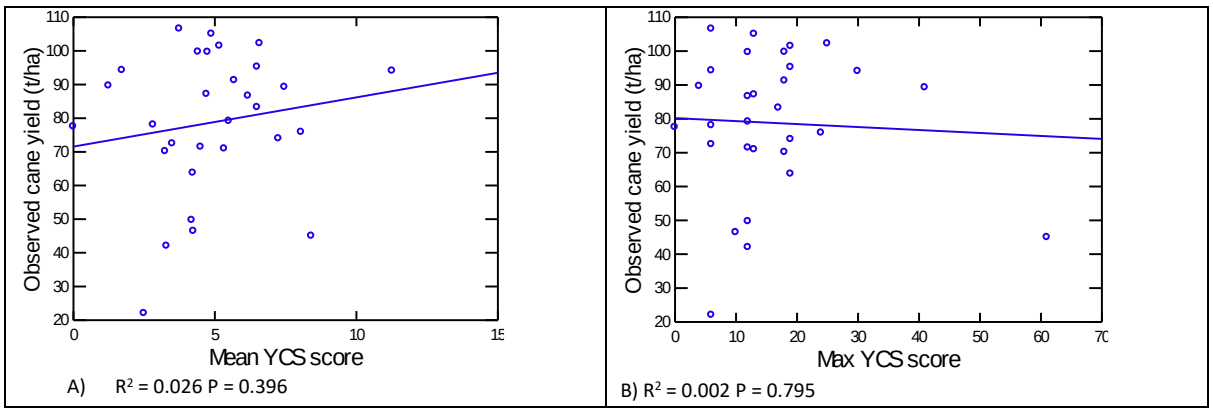


Figure 347 Recorded paddock cane yield and 10-year mean farm yield A), and biomass yield simulated with the best model setting B). Paddock numbers are given in A.

The various expressions of YCS alone, were not directly related to cane yield, although some approached significance in a positive direction which is not what we would expect (Figure 348). Different expressions of the YCS ratings were tried in regression models with 10-year mean farm yield as the primary independent variable. None of these expressions of YCS were significant (Table 82).



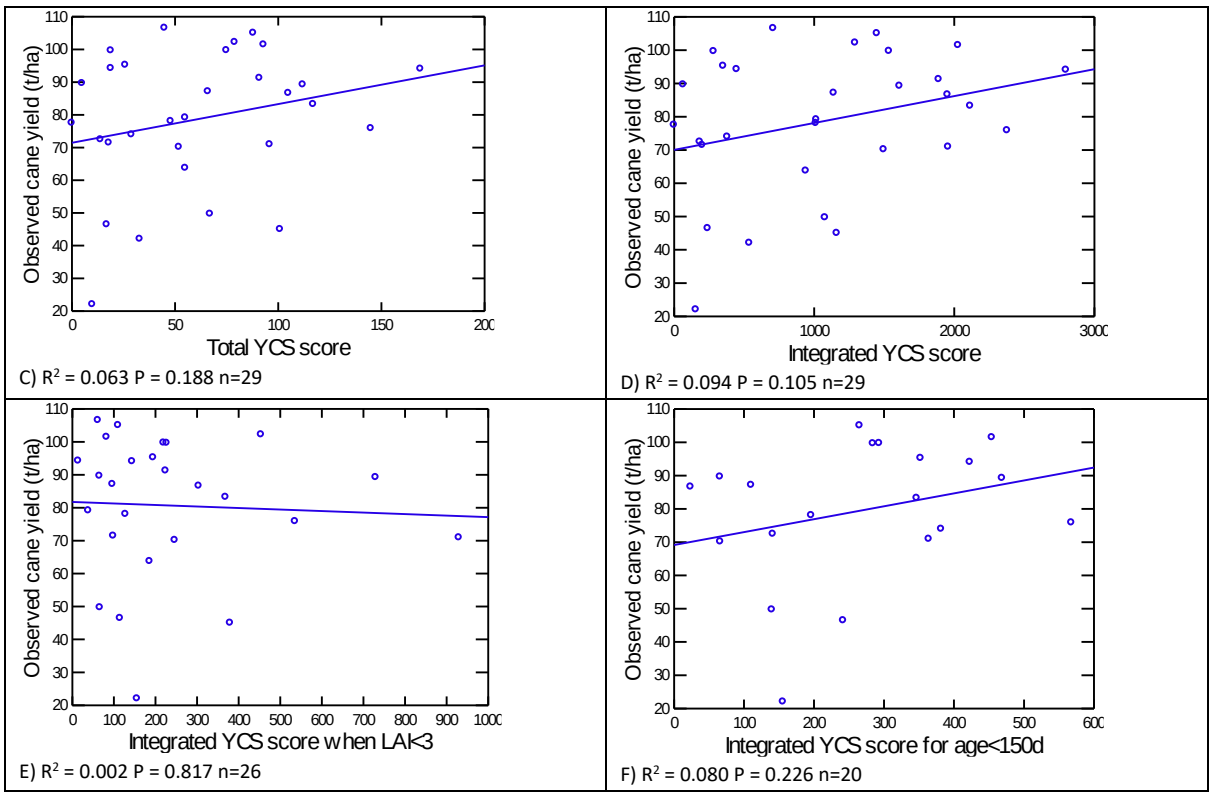


Figure 348 Maximum YCS score and crop age at maximum score A). Paddock cane yield and average YCS score B)

Table 82 Statistics for the regression of observed cane yield on 10-year mean farm yield and various expressions of the YCS score.

YCS expression	General regression statistics					Farm 10 year mean yield		YCS	
	n	R2	SEy	Const-ant	ANOVA P	slope	P	slope	P
None	30	0.285	18	0.544	0.002				
Total YCS score	30	0.340	18	-0.604	0.006	0.865	0.004	0.080	0.312
Integral, all obs	30	0.325	18	0.248	0.005	0.843	0.005	0.006	0.213
Integral, LAI<3	30	0.327	18	-12.749	0.005	1.014	0.001	0.021	0.201
Integral, age<150	30	0.329	18	-4.727	0.005	0.919	0.002	0.025	0.195
Mean YCS score	30	0.311	18	-6.766	0.007	0.910	0.002	1.473	0.321

Max YCS score	30	0.285	18	0.165	0.011	0.911	0.003	0.015	0.959

Summary

The impact of YCS on cane yield for 2015, of 30 paddocks in the Herbert was assessed by considering a number of derivations (expressions) of the YCS scores in relation to recorded yields. This was done by considering the timing and severity of YCS for each paddock both graphically and by regression analysis. The association between the various expressions of YCS and yield was then assessed after accounting for 'normal' variation in yield due to climatic, management and soil factors, using multiple regression analysis. The APSIM Sugar module and the 10-year mean farm yield were used to help explain 'normal' variation. Yields for 2015 were not related significantly to any expression of YCS either before normal variation was removed or after. Normal variation was best accounted for by the 10-year farm mean yield but 72% of this variation was still left unexplained. The model accounted for 25% of the yield variation (75% unexplained) only after using the 10-year farm mean yield to select soil parameters for the simulations. YCS did not account significantly for any of the remaining variation.

Conclusions and suggestions

YCS as recorded had no measurable effect on cane yield as recorded. That said, a large amount of variation in yield was not explained. YCS scores were done regularly for some paddocks and not for others. The regularity of scoring may have been due to the presence or absence of YCS. For this type of analysis, it would be best to record YCS at regular intervals regardless of its presence or absence. Most of the derivatives of the YCS recordings depended on this regularity, the area (integral) under the YCS curve in particular. It is also concerning that so little variation in paddock yields could be accounted for with the modelling. Detailed soil data is usually needed for such modelling and this was not available in this case. The impact of YCS on yield may best be studied in well controlled experiments rather than in the monitoring of farm paddocks. YCS cannot be 'applied' as a treatment in a standard field plot experiment but these experiments can offer opportunities to correct some of the deficiencies of the monitoring type of study. Soils can be described in more detail and YCS can be monitored more regularly for example.

The work reported here did not consider the impact of climate on the expression of YCS symptoms, apart from some observations made on the data in Figure 346. There were no obvious climatic conditions leading to YCS but a more comprehensive consideration of the data may be worthwhile.

6.13.4.2 Burdekin report - Geoff Inman-Bamber March 2017 (Inman-Bamber et al., 2016)

The impact of YCS on cane yield for 2015, of 42 paddocks in the Burdekin was assessed by considering a number of derivations (expressions) of the YCS scores in relation to recorded yields. This was done by considering the timing and severity of YCS for each paddock both graphically and by regression analysis. The association between the various expressions of YCS and yield was then assessed after accounting for 'normal' variation in yield due to climatic, management and soil

factors, using multiple regression analysis. Yields for 2015 were not related significantly to any expression of YCS either before normal variation was removed or after. Normal variation was best accounted for by the 10-year farm mean yield but 72 % of this variation was still left unexplained.

This report follows a similar one on the results of a similar study in the Herbert. Conclusions were similar and the wording in some parts, is identical. Here we consider yields and YCS scores of 54 individual paddocks that were monitored in the Burdekin during 2015. Yield and other management records were also available for 2013 and 2014. Yields vary from year to year and paddock to paddock for many reasons including the climate, soil, type and standard of management as well pest and disease. The climate experienced by the crop also depends on when it is planted and harvested and the ratoon stage of the crop. In order to measure the impact of any disease or phenomena, such as YCS on yield, it is necessary to remove, as much as possible, the influences on yield of all the other factors involved. The APSIM Sugar model represents the growth and yield of sugarcane very well when the climate, soil and management details impinging on the crop are well described. These details usually come from well controlled small plot experiments where the model can account for 80 to 90 % of the variation in yield (Inman-Bamber et al., 2016). For this study, we have some, but limited details for the factors that could affect yields of the commercial paddocks that were monitored. It is nevertheless appropriate to attempt the removal of the effects on yield by these factors with the use of this model, before trying to account for the effects of YCS on yield. Records of the average yield over 10 years for whole farm, were also available for many of the monitored paddocks. These average yields could be expected to reflect the type of management and the soils that could be partly responsible for the yield of each paddock. For the Herbert, 10-year farm mean cane yields were used to select the depth of the soil and hence the plant available water-holding capacity (PAWC) for each paddock. In this Burdekin study the 10-year mean farm yields were used independently and the appropriate PAWC was selected statistically as explained below.

Methods

The APSIM model requires details about the planting and harvest dates, the climate, the soil and irrigation and fertiliser management in order to complete a simulation of a given crop.

Planting dates

Planting dates were recorded as ‘early’, ‘late season’ and ‘replant’. In the model we need to assign a unique date for planting. It was decided to ‘plant’ early and late-season crops on 10 April and 7 September respectively. Crops recorded as ‘replant’ were assumed to be planted on 27 October. The year of planting was recorded and taken into account but most of the monitored crops were planted in 2012. If the planting date was not known, it was assumed to be a mid-season planting on 30 May.

Climate

Daily climate records were obtained (with thanks) from the Long Paddock website, operated by the Science Delivery Division of the Department of Science, Information Technology and Innovation (DSITI) of the Queensland government. The ‘Patched point’ database was accessed in order to associate a specific climate recording station to the district in which each paddock was located (Table 83).

Daily rainfall, radiation, maximum and minimum temperature were required for the simulation of the crop in each paddock.

Table 83 Districts and their associated climate station.

District code	Climate station
INK	Inkerman
PIO	Shirbourn
KAL	Kalamia
Clare	Clare
INV	GiruPO
AERO	Ayr_DPI

Soils

Details required to represent water and nutrient supply properties of soils for simulations by APSIM, were not available for the monitored paddocks in the Herbert or the Burdekin. Soil depth was a major factor in determining simulated yield for the Herbert as would be expected. For the Burdekin, the effect of soil depth on yield is expected to be reduced by the availability of full irrigation in that region.

A medium clay (Mulgrave No819, Table 83) was selected from the library of soils available with the APSIM software. Three depth levels were used in the simulations and the depth combinations that resulted in the best correlation between simulated and observed yields, were accepted as representative of all the paddocks in the study.

Table 84 Hydraulic properties of a Mulgrave medium clay, including the lower and upper limits of available water, saturated water content (Sat), bulk density (BD), runoff (RO) and drainage coefficients.

Depth interval	Lower	Upper	Sat	BD	RO weighting	Drainage
(mm)	(mm/mm)	(mm/mm)	(mm/mm)	g/cc	(0-1)	(0-1)
0. - 150.	0.230	0.330	0.450	1.440	0.762	0.300
150. - 300.	0.260	0.360	0.460	1.420	0.190	0.300
300. - 500.	0.310	0.400	0.480	1.377	0.048	0.300
500. - 700.	0.310	0.390	0.470	1.404	0.000	0.150
700. - 900.	0.300	0.380	0.450	1.440	0.000	0.150
900. - 1100.	0.300	0.370	0.450	1.450	0.000	0.150
1100. - 1300.	0.310	0.370	0.460	1.430	0.000	0.150
1300. - 1700.	0.280	0.340	0.420	1.536	0.000	0.150
1700. - 2000.	0.240	0.300	0.370	1.669	0.000	0.150

Irrigation

Irrigation cycle times for high (November to March) and low (April to October) demand periods, were recorded for each paddock (Table 85) and these were used to simulate irrigation, assuming that the soil profile would be filled with each irrigation event. Cycle time probably depended on soil type and probably varied over time but details on general practice were available rather than practice, specific to each paddock on the farm or each year.

Table 85 Irrigation intervals (days) used for each paddock in the simulations.

Farm	Block	High	Low	Farm	Block	High	Low
1927C	1-02	14	28	0247A	4-01	7	7
0259A	3-01	9	28	0089A	12-02	7	14
1062A	3-01	7	14	6218B	5-01	8	30
0271A	3-1	7	14	6022A	22-01	7	18
6707A	12-3	14	21	6533A	5-01	7	10
6031A	2-1	10	30	9140A	4-01	8	21
9083A	21-01	7	15	0361A	1-05	7	14
2452A	8-01	10	10	0267B	7-06	7	21
6650A	3-02	10	20	0246A	2-02	7	15
6649A	3-01	7	18	9424A	9-01	6	9
6189A	3-02	10	29	6234A	2-03	7	15
2524A	5-01	14	20	5755A	66-01	7	10
0095A	16-03	7	28	6988A	3-03	8	16
9044A	2-01	21	21	4271A	6-01	9	14
8099A	5-01	7	19	9065A	3-02	5	7
0429A	3-01	7	14	9046A	8-02	9	15
4247B	1-04	7	14	9205A	39-01	8	16
7028A	22-01	7	20	0023A	2-02	10	14
9017B	7-01	7	20	4888A	5-01	7	14
9018A	3-01	8	19	1986A	1-02	14	14
0220A	45-02	8	19	5231A	4-07	12	19
0674A	74-01	8	19	9409A	4-03	8	10
0321A	1-01	7	14	9409A	4-04	8	10
9054A	4-02	7	20	9006A	3-05	8	16
7114B	22-01	8	20	9091A	6-01	7	14
1273A	1-01	7	10	9091A	14-01	7	14
0256A	2-02	10	21	1345A	2-01	6	6

Crop Physiology

APSIM-Sugar can simulate accurately the development and yield of crops growing in well controlled experiments and new features have been added recently to improve the simulation of these experiments (Inman-Bamber et al., 2016). These and other features were used in factorial combination along with three soil depths (32 combinations) in attempt to capture the soil and physiological conditions of the crops that were monitored for this study on YCS (Table 86).

Table 86 Soil and physiological factors and levels used in the new APSIM sugarcane module (v 7.7). Factor/ level codes are in parenthesis

Factor	Level 1	Level 2	Level 3
Soil depth / PAWC (mm)	2000/148 (rd1)	1300/106 (rd2)	90/80 (rd3)
Growth slow-down with crop development	None (sd1)	30% between leaves #24 and 50 (sd2)	10% between leaves #10 and 25 and 30% between leaves #24 and 50 (sd3)
Lodging	None (lg1)	Lodging as in Inman-Bamber et al (2004). (lg2)	
Root vigour linked to above ground vigour linked to biomass	No (vg1)	Yes (vg2)	

YCS observations

Paddocks in which YCS was observed, were monitored repeatedly for development of the syndrome. The canopy was separated into three components for these observations and severity (S) and prevalence (P) were rated separately for each component. The component (*i*) above the TVD leaf (leaf 1) was considered the most important as far as impact on yield is concerned; leaves 2 to 5, comprising the second component (*j*), were considered to be less impactful and the lower leaves (>5) were in the least important component (*k*). The overall YCS score (Y) was derived as: $3S_iP_i + 2S_jP_j + S_kP_k$, giving appropriate weightings to the different components of the canopy.

Various expressions of the YCS score were analysed for impact on yield. These included,

5) The average of all scores for the paddock
6) The total score for the paddock
7) The maximum score
8) The integral of the YSC score curve with respect to time

Results

Removal of background variation (noise) in observed yields

Only three (rd3_sd2_vg1_lg1, rd2_sd2_vg1_lg1, and rd3_sd3_vg1_lg1) of the 36 soil x physiology settings of the APSIM model produced biomass yields that were significantly correlated with recorded paddock yields over three years (2013 to 2015). Statistics for the regression of recorded cane yield on the mean simulated biomass of these three settings were: $R^2 = 0.036$, $p = 0.04$, $n=118$.

While correlations with these settings were statistically significant, they removed very little of the background variation (<4 %). None of the 36 settings could account significantly for variation in cane yield recorded for 2015 when YCS was monitored.

10-year mean farm cane yield (for plant, 1st and 2nd ratoon crops combined) accounted significantly for 18.3 % of the variation in paddock cane yield recorded over three years (n=84) and 18.1 % of the variation for 2015 (n=34). Cane yield of individual paddocks monitored in 2015, was not correlated significantly with the 10-year mean farm yield, when the crop class (plant crops or ratoons) for the mean farm yield was the same as for the paddock concerned.

Thus, the best method for removing background variation in yield was the 10-year mean farm cane yield for plant, 1st and 2nd ratoon crops.

Graphical analysis

There were 42 paddocks for which YCS scores were available for crops harvested in 2015 (Fig 1). Yield records were available for 41 of these paddocks. YCS scores were considerably lower in the Burdekin than in the Herbert (Figure 349).

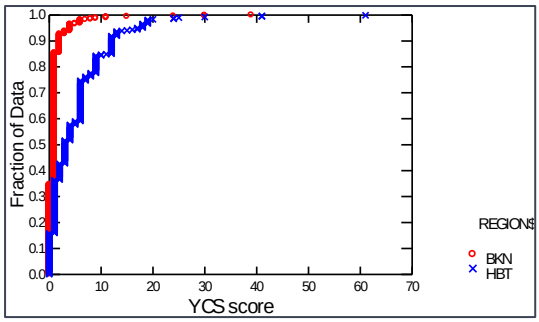


Figure 349 Cumulative frequency distributions of YCS scores in the Herbert and Burdekin

As for the Herbert, several questions can be put to the data in (Figure 349) where results of the simulations are presented together with the YCS score and the recorded cane yield, where available. The simulations provide some idea of crop development in terms of leaf area index (LAI). YCS scores in (Figure 349) have been scaled (x5) for clarity. Simulated yield in this case was regarded as the benchmark or yield potential.

6) Does the overall YCS score explain the difference between simulated and observed yield.

   a. Farm 9409a, blocks 4-03 and 4-04 experienced the highest YCS incidence of all 42 paddocks surveyed. The yield for block 4-04 was well below the simulated yield (potential yield) but the yield for block 4-03 was equivalent with the potential yield and was one of the highest yields recorded in the study. Other paddocks produced yields that were well below simulated yields but this could not be attributed to YCS, which scored low (Farm 0089a Block 12-02, Farm 0256a Block 2-02, and Farm 0674a Block 74-01).

7) Is there a particular wave or peak in YCS that is associated with the difference between observed and simulated yield?

   a. YCS peaked during periods of rapid growth, well after canopy closure for Farm 9409a, blocks 4-03 and 4-04, where yields were high and low respectively, compared to simulated yields.

b. Smaller waves occurred in other paddocks; late in the crop cycle for Farm 9054a Block 4-02 and Farm 9083a Block 21-01, and early and late for Farm 1273a Block 1-01. Cane yields for the first two paddocks were only slightly lower than simulated yields, and the yield for Farm 1273a, the second highest recorded in the study, was well above the simulated yield.

8) Is the yield difference between observed and simulated yields associated with the number of YCS waves?

   a. Farm 9409a, blocks 4-03 and 4-04 experienced only one large YCS peak, with an apparent effect on yield in one case but not the other. Other paddocks had two small peaks without an apparent effect on yield.

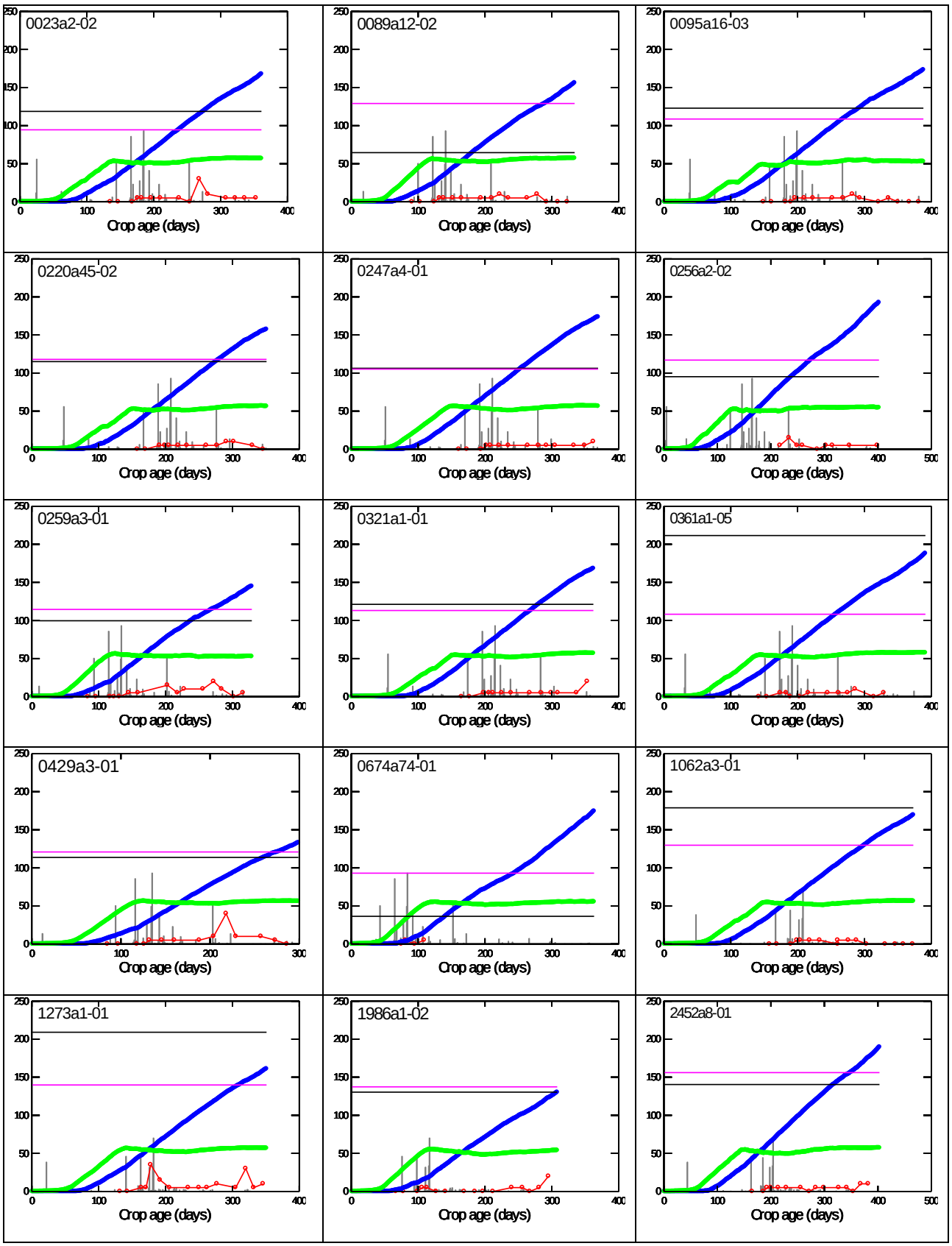
9) Is the duration and severity (area under the YCS curve) of YCS associated with the difference in between observed and simulated yield?

   a. YCS was present for more than more than 50 days in the case of Farm 9409a, blocks 4-03 and 4-04 where scores were high and in the case of Farm 9083a Block 21-01 and Farm 6649a Block 3-01 where the scores were much lower. There were no consistent impacts apparent on yield.

10) Does the YCS score follow any particular rainfall or crop stress pattern

   a. YCS symptoms occurred soon after rain events in case of Farm 0023a Block 2-02, 29 Farm 6988a Block 3-03, Farm 6022a Block 22-01, 10 and Farm 0429a Block 3-01. These types of patterns may give the impression that YCS follows rain events but there were other cases where YCS occurred long after rain. The two large peaks in YCS recorded for Farm 9409a, blocks 4-03 and 4-04, occurred about 80 days after a large rainfall of about 200 mm. Small YCS peaks occurred at about the same time in Farm 9205a Block 39-01 and Farm 9140a Block 4-01.

The evidence from (Figure 349) does not lead to a clear conclusion about the impact of YCS on yield or on the impact of rainfall on the expression of YCS. Nevertheless, it is worth doing some regression analysis to determine if some associations have been overlooked.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1273a1-01
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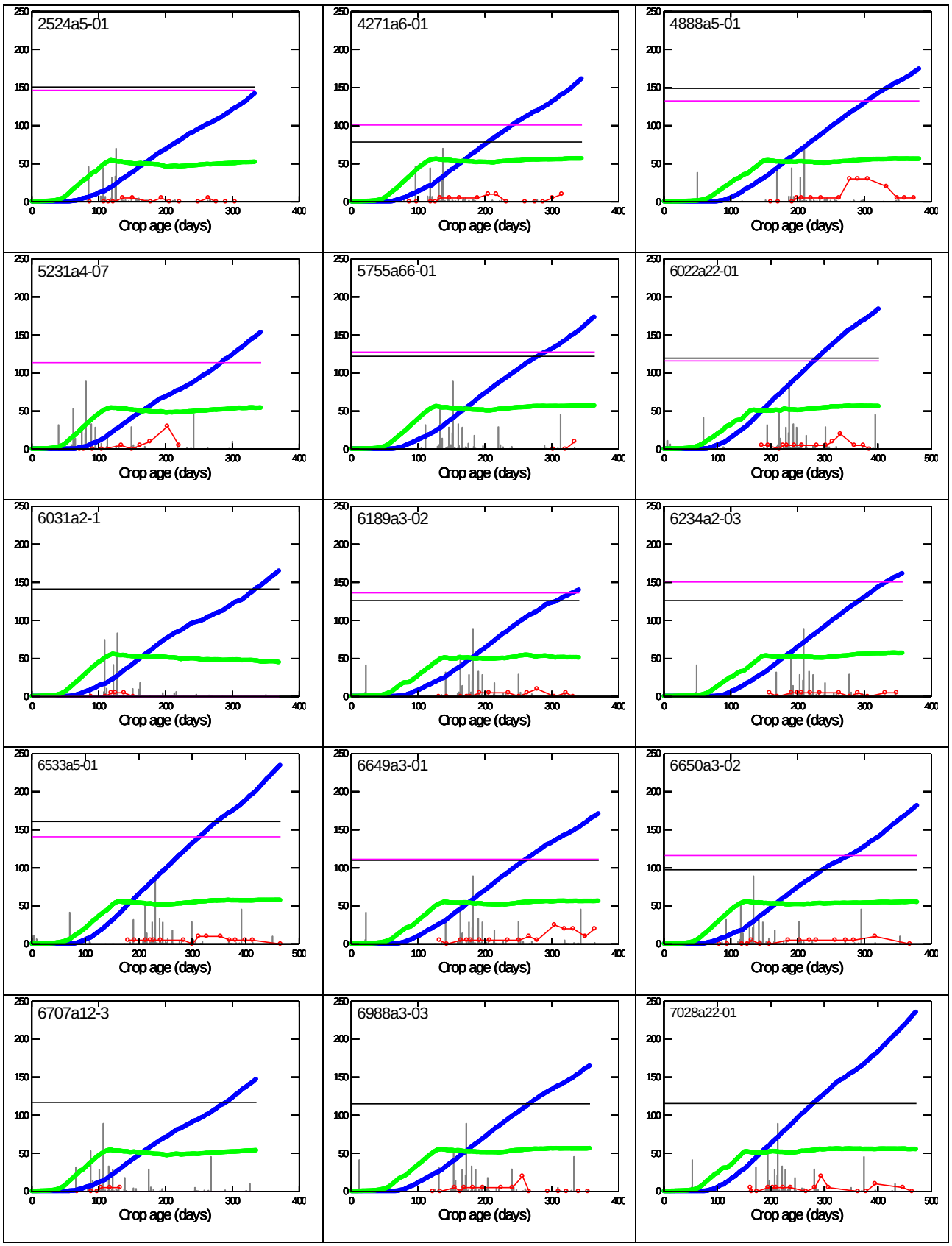
Crop age (days)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6988a3-03
7028a22-01
Crop age (days)

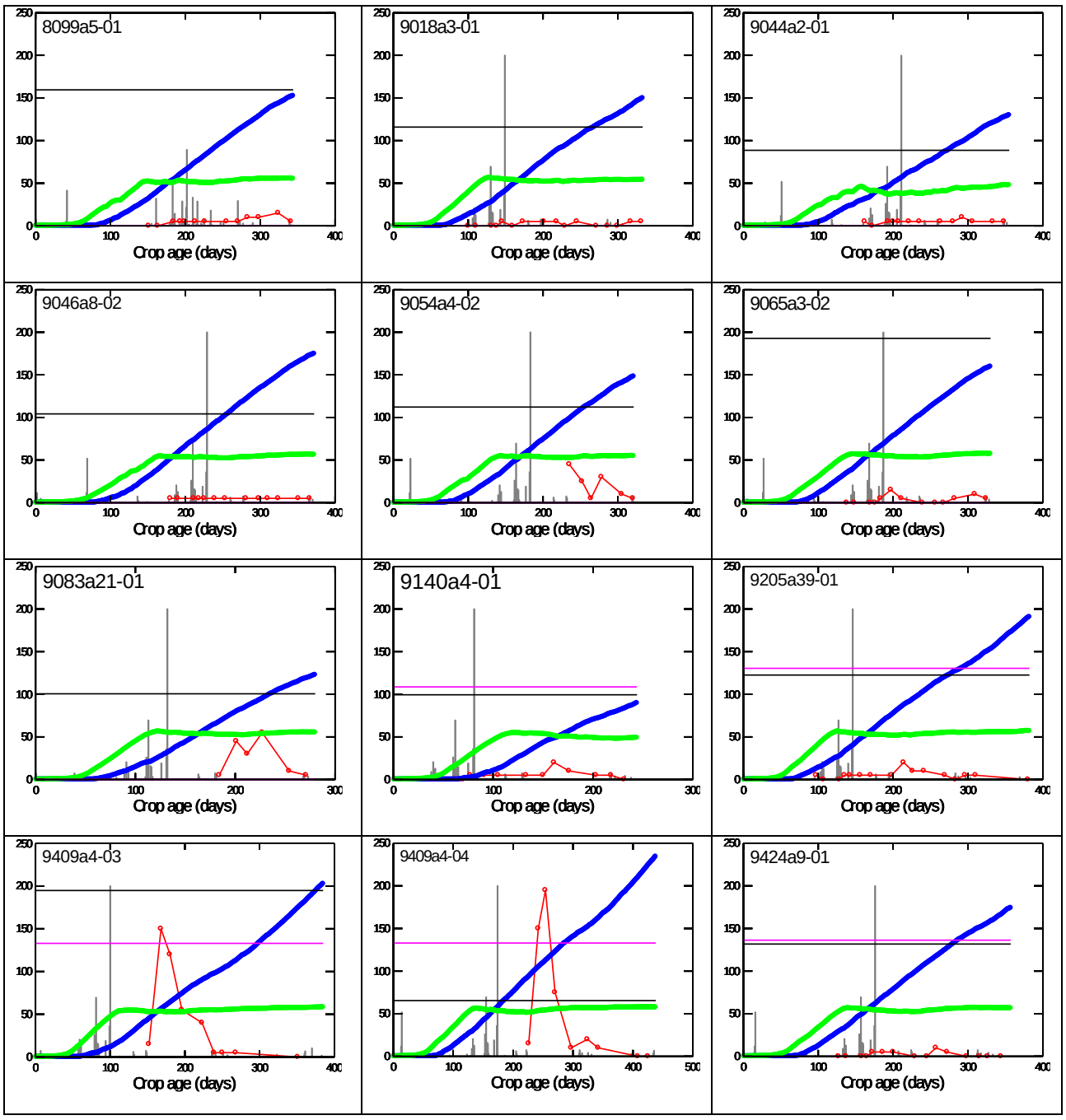


Figure 350 YCS scores x 5 (red line and symbols), LAI x 10 (green), simulated cane yield (blue), rainfall in (grey bars), recorded paddock yield (black horizontal line), 10-year mean farm yield (magenta horizontal line) for 42 monitored paddocks in the Burdekin, harvested in 2015. Farm and block numbers are shown.

Scatter plot and regression analysis

None of the various individual expressions of YCS were directly related to cane yield (Figure 351). Different expressions of the YCS ratings were tried in regression models with 10-year mean farm yield as the primary independent variable. None of these expressions of YCS were significant (Table 87).

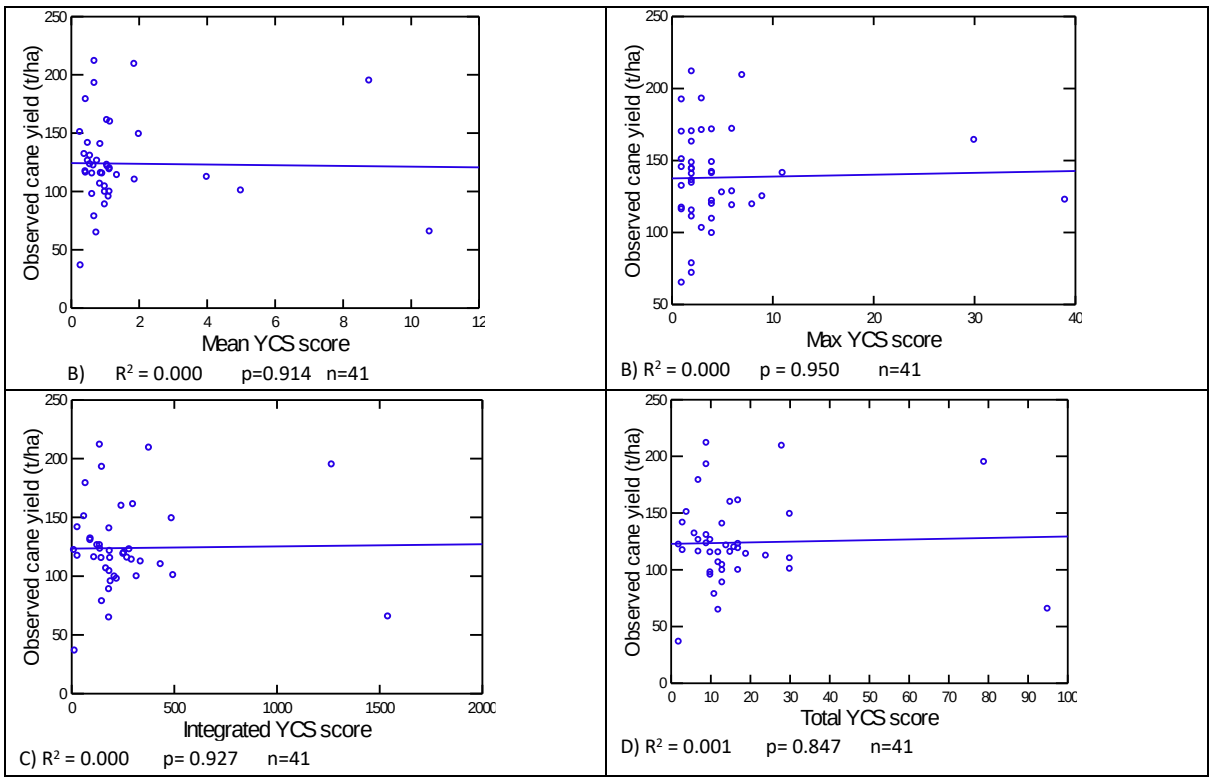


Figure 351 Maximum YCS score and crop age at maximum score A). Paddock cane yield and average YCS score B).

Table 87 Statistics for the regression of observed cane yield on 10-year mean farm yield and various expressions of the YCS score.

YCS expression	General regression statistics					Farm 10 year mean yield		YCS	
	n	R2	SEy	Constant	ANOVA P	slope	P	slope	P
None	34	0.181	35	-1.398	0.012	1.029	0.012		
Total YCS score	30	0.177	37	-4.243	0.072	1.037	0.025	0.008	0.981
Integral, all obs	30	0.178	37	-4.474	0.071	1.045	0.023	-0.002	0.922
Mean YCS score	30	0.178	38	-4.896	0.070	1.050	0.023	-0.551	0.860
Max YCS score	30	0.179	37	-4.862	0.069	1.052	0.022	-0.212	0.805

5.1.3 Conclusions and Suggestions

YCS as recorded had no measurable effect on cane yield as recorded. That said, a large amount of variation in yield was not explained. It is also concerning that so little variation in paddock yields could be accounted for with the modelling. Detailed soil data is usually needed for such modelling and this was not available in this case. The impact of YCS on yield may best be studied in well controlled experiments rather than in the monitoring of farm paddocks. YCS cannot be 'applied' as a treatment in a standard field plot experiment but these experiments can offer opportunities to correct some of the deficiencies of the monitoring type of study. Soils can be described in more detail and YCS can be monitored more regularly for example.

The work reported here did not consider the impact of climate on the expression of YCS symptoms, apart from some observations made on the data in (Figure 350). There were no obvious climatic conditions leading to YCS but a more comprehensive consideration of these and other data may be worthwhile.

6.13.5 APSIM modelling

6.13.5.1 Simulation of average annual yield (2013 to 2015) of monitored paddocks in the Herbert using best ensembles

The best APSIM model settings explained 53% (49% after cross validation) of the year to year variation in the mean sugar yield in the Herbert. Yields for 2010 to 2015 were lower than the yields expected from the model. This could be partly due to the presence of YCS which became obvious after 2012, however cyclonic weather and excessive rainfall in 2011 may have contributed to these low yields as well. Cumulative rainfall over three years preceding the crop that was crushed, explained an additional 9% of the annual variation in yield. When this was taken into account, yields obtained in 2012, 2013 and 2014 were very close to the yields expected from the modelling. However, yields obtained in 2015 were still more than one standard deviation lower than the expectation from the combined APSIM-cumulative rainfall model. Mean sugar yield for the 20 monitored cane paddocks trended down from 2013 to 2015 while yield expectations from the modelling trended upwards.

First approximation of YCS impact on yields in the Herbert

A yield loss estimate of 12 ± 7% in the Herbert for 2015 can be deduced from the data in (Figure 355) of the attached report. To put this into perspective, this represents an approximate loss to the Herbert of $10.8 million for the 2015 season based on the following calculations:

Assuming a conservative 50% of the Herbert was YCS affected
4.5M tonnes harvested x 50% = 2.25M
Assumed cane price of $40/t
Potential cane value of YCS crop = 2.25 x $40 = $90M
YCS impact = 12% x $90M = $10.8M

APSIM Modelling of YCS Yield Loss – Report

6.13.5.2 Is YCS affecting Herbert region yields? Geoff Inman-Bamber, May 2016

Summary

The best APSIM model settings explained 53% (49% after cross validation) of the year to year variation in the mean sugar yield in the Herbert. Yields for 2010 to 2015 were lower than the yields expected from the model. This could be partly due to the presence of YCS which became obvious after 2012, however cyclonic weather and excessive rainfall in 2011 may have contributed to these low yields as well. Cumulative rainfall over three years preceding the crop that was crushed, explained an additional 9% of the annual variation in yield. When this was taken into account, yields obtained in 2012, 2013 and 2014 were very close to the yields expected from the modelling. However, yields obtained in 2015 were still more than one standard deviation lower than the expectation from the combined APSIM-cumulative rainfall model. Mean sugar yield for the 20 monitored cane paddocks trended down from 2013 to 2015 while yield expectations from the modelling trended upwards. When YCS scores become available it may be possible to assess if YCS is responsible for lower than expected yields.

Background

The background to this approach for estimating the effect of climate, soils and management on mean annual yield is found in Everingham et al. (2007)and Inman-Bamber (2007).

Essentially we use 1000's of sensible settings for the APSIM model to simulate biomass yields and then we correlate these with yields from the catchment supplying a given terminal. We then select the best 10 to 30 models and obtain their average biomass estimates. We use these to derive a regression equation from which we get an estimate for the yield for the next crush year. Cross validation with a 'leave-one-out' technique can be used to check that the correlations are not spurious.

We use biomass estimates because often a yield forecast is required before cane stalk growth or sucrose accumulation has begun. However, in this case we are not trying to forecast yields but to understand what factors other than climate are affecting yields of a given catchment identified by the name of the shipping terminal. What follows is an initial attempt to assess the reduction in yield in the past three years caused by the advent of YCS. This is work in progress and the report constitutes a progress report on the work agreed on for analysing data from paddocks being monitored.

One difficulty with the Herbert region is that crop has access to water from water tables for unknown periods of time after rainfall (Rudd and Chardon, 1977). In a dry region of the Herbert, we measured water at 40 cm below the soil surface after 76 mm rain was recorded. After about 2 months the water table had receded to 1.5 m but was probably still accessible to sugarcane roots

(Inman-Bamber et al., 1999). Few Herbert growers irrigate but in these simulations, we used different levels of irrigation to represent the water table. The concern here is that we may be masking some long-term effects of low rainfall on the water table. Water tables could be viewed as store of water like a dam with a certain catchment size and storage capacity. The 'dam' option in APSIM was tried in order to simulate the water table more realistically but initial attempts did not help. Radiation during crop development has a strong influence of crop yields (r^2 ~ 0.2 to 0.4) in the Herbert (not as much as in the Tully region) so rainfall, which is often in excess, and cloud cover are negative factors for the region but low rainfall could also be impacting yields negatively particularly in the southern Herbert region.

A new version of the APSIM Sugarcane model (v 7.7) was used in this study. This version has some important new features dealing with plant water relations, photosynthesis and respiration.
Data for 2013, 2014 and 2015 were excluded in the development of the model because we suspect that YCS had reduced these yields and we needed a way of estimating yields without the influence of disease. Data for 2000 and 2001, when orange rust was prevalent, were excluded for the same reason.

Sugar yields for blocks being monitored for YCS by the productivity board

Sugar yields for 20 sugarcane blocks for 2013, 2014 and 2015 range from less than 4 t/ha to nearly 17 t/ha (Table 88).

Table 88 Sugar yield (t/ha) for 20 blocks being monitored for YCS in the Herbert.

Paddock	2013	2014	2015
3-8	9.1	10.5	9.0
1-4	3.1	7.2	3.5
12-2	14.7	14.0	9.3
3-5	10.6	11.8	12.1
1-1	6.5	9.6	11.2
4-1	14.9	11.3	8.9
6-1	17.1	10.4	8.7
8-4	13.5	13.3	8.6
2-1	13.1	11.3	11.8
7-2	14.7	12.9	12.3
7-7	10.7	10.1	11.6
9-2	12.9	10.2	12.2
5-1	18.3	9.2	11.0
8-3	13.3	15.0	12.9
48-2	16.8	13.5	13.6
13-3	5.3	10.5	11.7
3-2	17.0	11.8	15.4
7-1	11.9	10.7	12.5
3-3	13.2	13.3	13.0
19-1	11.6	13.1	15.3
Mean	12.4	11.5	11.2

Model settings

Climate, management and physiological settings were applied in a factorial arrangement as an attempt to cover the range of growing conditions in the Herbert. A total of 1620 settings were presented to the APSIM model derived from factorial combinations of the factors in Table 89.

Table 89 Climate, management and physiological factors which were varied in the new APSIM sugarcane module (v 7.7). (Codes used in Table 89 are in parenthesis).

Factor	Level 1	Level 2	Level 3
Climate station	Bambaroo (ba)	Victoria mill (vi)	Lucinda (lu)
Plant available water capacity (PAWC) of the soil	72 mm	144 mm	216 mm
Ratoon date	15-June	15-Aug	15-Oct
Growth slow-down between leaf #10 and #24	No (no)	20%	40%
Respiration of sucrose	No (no)	As a function of temperature (yes)	
Transpiration efficiency response to water stress	No (no)	Responsive (yes)	
Irrigate at deficit as a fraction of PAWC	No irrigation (dry)	0.2	0.4 (0.6 and 0.8 as well)

Best models (settings)

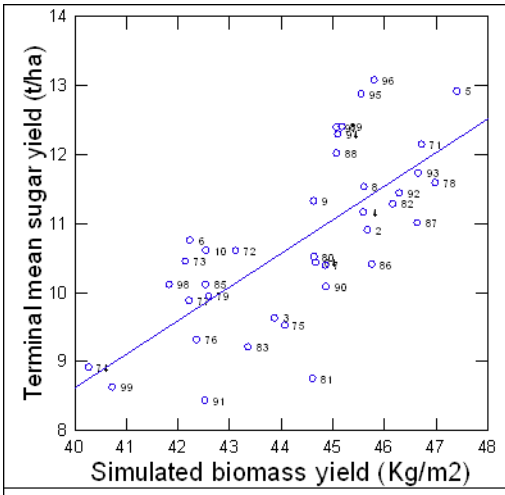
Of the 1620 settings tested, 18 explained more than 35% of the variation in the annual sugar yield of the Herbert region (Table 90). The best settings came from a wide range of settings presented to the model thus representing a wide range of soils in terms of PAWC, accessions to water tables, growing cycles in terms of ratoon dates and other physiological settings. The Bambaroo and Victoria climates both featured strongly but the Lucinda climate did not appear to be particularly influential. Other climate stations are available for the region and could be tested in future. The 12 best model settings for the Herbert region in the study by Inman-Bamber (2007) accounted for only 27% to 32% of the variation in the annual mean yields (1977 to 1999). It appears that the new APSIM sugar module and more experience in yield forecasting have helped to improve the hindcasting (and forecasting) of yields.

Table 90 Regression statistics for the best models (with adjusted r2>0.35) for the Herbert region, mean sugar yield (t/ha) versus simulated biomass yield for years 1971 to 2010, excluding 2000 and 2001 when orange rust was prevalent and 2013, 2014, and 2015 when YCS was prevalent (n=38).

PAWC	Date	Slow down	TE	Resp	Irrig	Climate	R^2	SE	Cons-tant	Slope	P
216	aug	no	No	No	0.4	ba	0.368	1.002	-6.331	0.253	0
216	aug	20%	No	No	0.4	ba	0.361	1.007	-4.932	0.289	0
216	oct	40%	No	No	dry	vi	0.365	1.004	0.225	0.297	0
216	oct	40%	Yes	No	dry	vi	0.405	0.972	-1.36	0.338	0
216	oct	40%	Yes	Yes	dry	vi	0.37	1.001	-1.398	0.372	0
216	oct	20%	No	No	0.2	vi	0.365	1.005	-6.163	0.317	0
216	oct	20%	Yes	No	0.2	vi	0.35	1.016	-4.893	0.292	0
144	aug	no	No	No	0.4	ba	0.352	1.014	-6.134	0.253	0
144	aug	20%	No	No	0.2	ba	0.359	1.009	-6.543	0.337	0
144	aug	20%	No	No	0.4	ba	0.366	1.004	-5.7	0.306	0
144	aug	20%	Yes	No	0.2	ba	0.432	0.95	-8.422	0.368	0
144	jun	40%	Yes	Yes	0.2	vi	0.353	1.014	-3.734	0.447	0
72	aug	40%	No	Yes	0.2	vi	0.407	0.971	-7.282	0.598	0
72	aug	40%	Yes	Yes	0.2	vi	0.412	0.967	-7.37	0.587	0
72	aug	20%	Yes	No	0.2	ba	0.377	0.995	-6.199	0.349	0
72	jun	40%	No	No	0.2	ba	0.379	0.994	-4.785	0.433	0
72	jun	40%	No	Yes	0.2	ba	0.362	1.007	-6.354	0.544	0
72	jun	40%	Yes	Yes	0.2	ba	0.365	1.005	-6.149	0.523	0

Best combined model

Mean biomass yield of all 18 models explained 53% of the variation in the annual sugar yield in the Herbert region (Figure 352). The standard error for each annual sugar yield estimate was 0.9 t/ha. The mean biomass yield of all 18 models thus provided a better account of historic yields than any of the 18 settings (models) used in isolation (Table 90).



Dependent Variable	TSPH
N	40
Multiple R	0.734
Squared Multiple R	0.539
Adjusted Squared Multiple R	0.526
Standard Error of Estimate	0.907
Cross validated R^2	0.493

Regression Coefficients B = $(X'X)^{-1}X'Y$

Effect	Coefficient	Standard Error	Std. Coefficient	Tolerance	t	p-Value
CONSTANT	-12.531	3.480	0.000	.	-3.601	0.001
BIOMASS	0.523	0.078	0.734	1.000	6.660	0.000

Figure 352 Scatter plot and regression statistics for the Herbert region mean sugar yield (t/ha) versus mean simulated biomass of the best 18 models, for years 1971 to 2010, excluding 2000 and 2001 when orange rust was prevalent and 2013 to 2015 when YCS was symptomatic (n=38). Crush year is shown in the scatter plot.

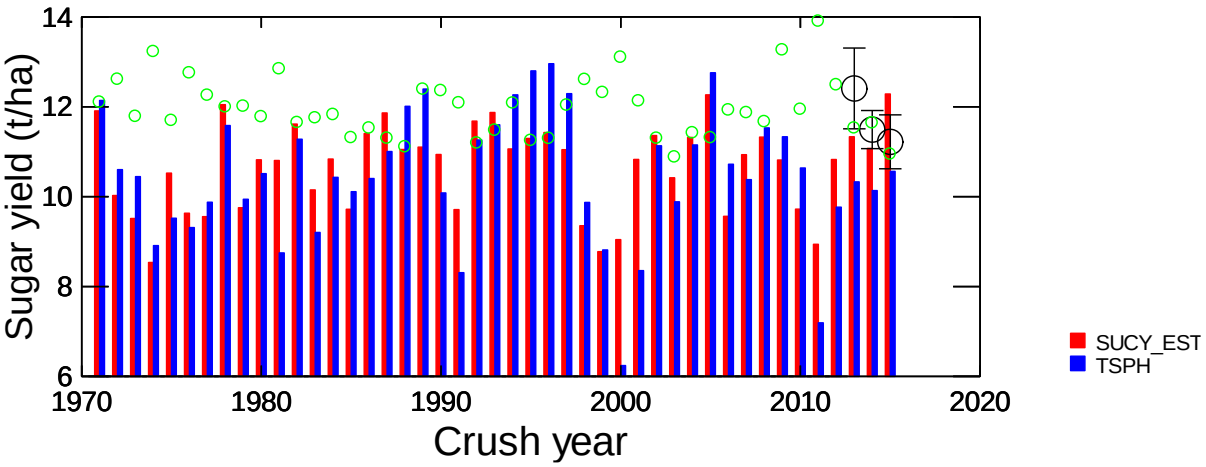


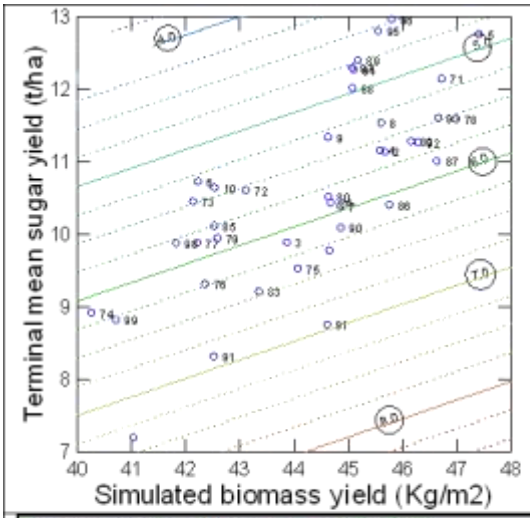
Figure 353 Simulated yield estimate for the 1971 to 2015 period (red bars) and actual yield for Herbert region (Lucinda terminal) for the period 1971 to 2015 (blue bars) and actual yields from paddocks monitored for YCS in 2013 to 2015 (black symbols and error bars)

Best combined model plus recent rainfall

It is conceivable that rainfall in years preceding the crop developing for a given crush year, could affect that crop in positive or negative ways. High rainfall could lead to rising water tables which could help the crop through dry periods. However excessive rainfall could also have long term negative effects on the root system growing in an anaerobic environment, and through the release

of ethylene, as well as additional compaction around the stool from harvesting machinery and through disruption to the replanting schedule. Hindcasting of past yields was improved considerably by including in the linear regression, the total rainfall over the three growing seasons before the season that produced the crop for a given crush. For example, total rainfall for the seasons 2000/2001, 2001/2002 and 2002/2003 had a significant effect on the crop developing in 2003/2004 for the 2004 crushing season (Figure 355).

High rainfall in 2010/2011 reduced the yield estimate for 2013 and 2014 compared to an estimate based only on simulated biomass (Figure 355).



Dependent Variable	TSPH
N	37
Multiple R	0.802
Squared Multiple R	0.643
Adjusted Squared Multiple R	0.622
Standard Error of Estimate	0.829
Dependent Variable	TSPH
Cross validated R^2	0.593

Regression Coefficients $B = (X'X)^{-1}X'Y$

Effect	Coefficient	Standard Error	Std. Coefficient	Tolerance	t	p-Value
CONSTANT	-8.650	3.669	0.000	.	-2.357	0.024
BIOMASS	0.479	0.078	0.653	0.933	6.153	0.000
LAG_RAIN	-0.344	0.112	-0.326	0.933	-3.076	0.004

Figure 354 Scatter plot and regression statistics for Lucinda terminal (Herbert region) mean sugar yield (t/ha) versus mean simulated biomass of the best 18 models, for years 1974 to 2010, excluding 2000 and 2001 when orange rust was prevalent (n=38). Total rainfall (m) over 3 years before each crush year was included in the linear regression. Rainfall contours are shown in the scatter plot with the total rainfall amount encircled.

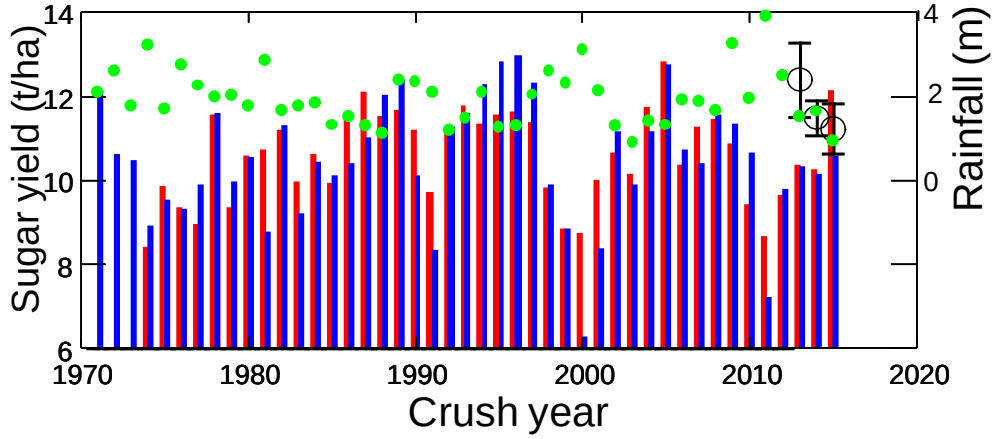


Figure 355 Simulated yield estimate for the 1974 to 2015 period (red bars) and actual yield for Herbert region (Lucinda terminal) for the period 1971 to 2010 (blue bars) and actual yields from paddocks monitored for YCS in 2013 to 2015 (black symbols and error bars). Mean in-crop rainfall (m) for each year is shown as green symbols.

Future work

1. Improve yield hindcasting using the Dam module in APSIM
2. Simulate crops in the monitored paddocks (20 for the Herbert) one at a time
3. Determine the statistical significance of YCS on the yield of these paddocks
4. Repeat all this work for the Burdekin

Conclusions

The best APSIM model settings explained 53% (49% after cross validation) of the year to year variation in the mean sugar yield in the Herbert. Yields for 2010 to 2015 were lower than the yields expected from the model, by more than one standard deviation. This could be partly due to the presence of YCS which became obvious after 2012 however cyclonic weather and excessive rainfall in 2011 may have contributed to these low yields as well. Cumulative rainfall over three years preceding the crop that was crushed, explained an additional 9% of the annual variation in yield. When this was taken into account, yields obtained in 2012, 2013 and 2014 were very close to the yields expected from the APSIM model plus the influence of rainfall over the preceding 3 years. However, yields obtained in 2015 were still more than one standard deviation lower than the expectation from the combined APSIM-cumulative rainfall model. The influence of excessive rain in 2011 was absent in the models prediction for 2015.

Mean sugar yield for the 20 monitored cane paddocks in 2013, 2014 and 2015, was substantially higher than the mean yield of sugar shipped out of the Herbert region. The yield of the monitored paddocks was also greater than the model's estimate for 2013 and 2014 but not for 2015. YCS data was not provided for these paddocks and it will be interesting to see if YCS was much worse for 2015 than for 2013 and 2014.

6.13.5.3 Is YCS affecting Burdekin region yields noticeably? Geoff Inman-Bamber, March 2017

The background to this approach for estimating the effect of climate, soils and management on mean annual yield, was explained in a similar report on Herbert region yields and is based on publications by Everingham et al. (2007)and Inman-Bamber (2007). Essentially we use 100's of sensible settings for the APSIM model to simulate biomass yields and then we correlate these with mean yields from the catchment supplying sugar to a given terminal. We then select the best 10 to 30 models and obtain their average biomass estimates. We use these to derive a regression equation from which we get an estimate for the yield for the next crush year. Cross validation with a 'leave-one-out' technique is used to check that the correlations are not spurious. We use biomass estimates because a yield forecast is often required before cane stalk growth or sucrose accumulation has begun. However in this case we are not trying to forecast yields but to understand what factors other than climate are affecting yields of a given catchment, identified by the name of the shipping terminal. A new version of the APSIM Sugarcane model (v 7.7) was used in this study (Inman-Bamber et al., 2016). This version has some important new features dealing with plant water relations, photosynthesis and respiration. Yield data for 2013 to 2016 were excluded in the development of the model because we suspect that YCS had reduced these yields and we needed a way of estimating yields without the influence of disease.
Data for 2000 and 2001, when orange rust was prevalent, were excluded for the same reason.

Model settings

Climate, management and physiological settings were applied in a factorial arrangement as an attempt to cover the range of growing conditions in the Burdekin. A total of 972 settings derived from factorial combinations of the factors in Table 91, were presented to the APSIM model.

Table 91 Climate, management and physiological factors which were varied in the new APSIM sugarcane module (v 7.7).

Factor	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6
Climate station	Ayr DPI	Clare	Giru PO	Inker-man	Kala-mia	Shirb-ourne
Plant available water capacity (PAWC) of the soil	140 mm	106 mm	80 mm			
Ratoon date	mid-June	mid-August	mid-October			
Growth slow-down between leaf #10 and #24	None	Low	High			
Lodging	Off	On				
Irrigation cycle	7 days	10 days	14 days			

Best models (settings)

Of the 972 settings tested, 13 explained more than 27 % of the variation in the annual sugar yield for the Burdekin region (Table 92). The best settings came from ratoons starting August and a wide range other settings presented to the model thus representing a wide range of soils in terms of PAWC, irrigation cycles and physiological attributes. Mean simulated biomass for the ensemble of 13 settings accounted for 39 % of the variation in the annual sugar yield of the Burdekin region (Figure 356 A). The seven best model settings for the Burdekin region in the study by Inman-Bamber

(2007)accounted for 39 to 49 % of the variation in the annual mean yields (1977 to 1999). The ensemble in
Table 92 accounted for 59 % of the yield variation in those years. The new APSIM sugar module and more experience in yield forecasting have helped to improve the hindcasting (and forecasting) of yields for the Herbert and Burdekin. The yields for these regions have proved more difficult to forecast than other for regions (Inman-Bamber, 2007). The mean biomass yield of all 13 model settings provided a better account of historic yields than any of the settings used in isolation (Table 92). Mean sugar yield for a given year was affected significantly by rainfall received during the crop preceding the one that produced the yield. The effect was negative (Figure 356 B) and could be ascribed to the effect of wet weather harvesting on the subsequent crop and harvest delays resulting in a shorter growing season than normal.

Table 92 Regression statistics for annual mean sugar yield (t/ha) of the Burdekin, versus biomass yield simulated with the best models (with adjusted r^2>0.27) for years 1971 to 2012. Years excluded were 000 and 2001 when orange rust was prevalent and 2013, 2014, and 2015 when YCS was prevalent (n=40).

PA-WC	Date	Slow-down	Lodg-ing	Climate	Irriga-tion	R^2	SE	Const-ant	Slope	P
80	August	High	On	Inkerman	14 days	0.381	1.01	9.59	0.16	<0.001
106	August	High	On	Inkerman	14 days	0.368	1.02	9.86	0.16	<0.001
140	August	High	On	Inkerman	14 days	0.361	1.03	9.93	0.15	<0.001
80	August	High	On	Inkerman	10 days	0.345	1.04	10.36	0.14	<0.001
106	August	High	On	Inkerman	10 days	0.334	1.05	10.51	0.14	<0.001
140	August	High	On	Inkerman	10 days	0.332	1.05	10.54	0.14	<0.001
106	August	High	On	Kalamia	14 days	0.304	1.07	11.53	0.12	<0.001
80	August	High	On	Kalamia	14 days	0.304	1.08	11.52	0.12	<0.001
140	August	High	On	Kalamia	14 days	0.298	1.08	11.59	0.12	<0.001
80	August	Low	On	Inkerman	14 days	0.293	1.08	9.97	0.13	<0.001
80	August	None	Off	Ayr DPI	7 days	0.291	1.09	2.66	0.21	<0.001
80	August	High	On	Inkerman	7 days	0.284	1.09	11.44	0.12	<0.001
106	August	High	On	Inkerman	7 days	0.280	1.09	11.45	0.12	<0.001
Average biomass for all models excluding years 2000, 2001, 2013-2016						0.390	1.01	8.84	0.170	<0.001
Rainfall (m) for preceding season for all years (1972-2016)						0.128	1.27	18.04	-0.914	0.016

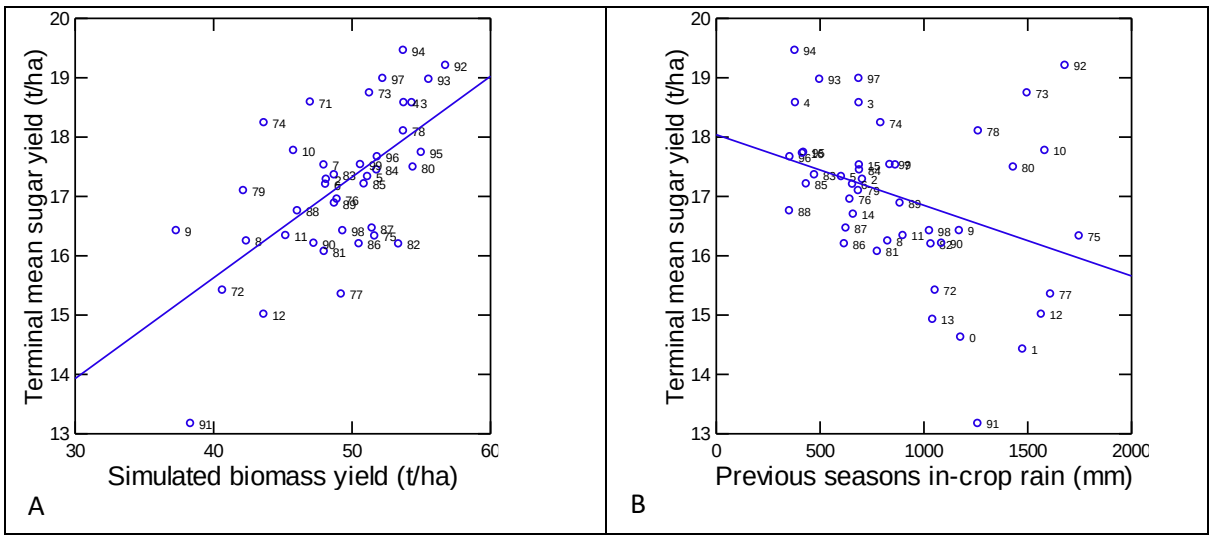


Figure 356 Scatter plots for Burdekin region annual mean sugar yield versus; A) mean simulated biomass of the best 13 models, for years 1971 to 2010, excluding 2000 and 2001 when orange rust was prevalent and 2013 to 2016 when YCS was symptomatic (n=40); and B) in-crop rainfall for the preceding season (antecedent rainfall) for all years, 1972 to 2016 (n=45). Each data symbol is labelled with the crush

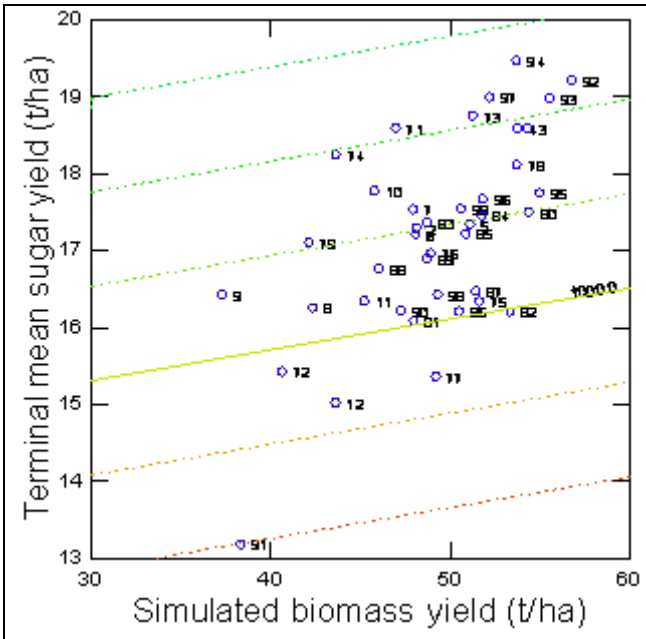
Best combined model plus preceding season's rainfall

Accounting for variation in Burdekin annual sugar yield, increased from 39 to 46 % when antecedent rainfall was added to the regression equation (Figure 357). The standard error of the estimate decreased from 1.01 to 0.96 t/ha with this addition.

The variance inflation factor (VIF) was low, indicating that antecedent rainfall and biomass simulated with current rainfall, were acting separately, as one would expect. When cross validated, the combined model accounted for 32 % of the variation in annual sugar yield.

Sugar yields in 1991, 2000, 2001 and 2013 were substantially lower than expected from the model (Figure 358). Cyclonic weather was probably responsible for the low yield in 1991 and orange rust for the low yields in 2000 and 2001. Floods in 2010 prevented the harvesting of a large proportion of the crop which then had to be carried over to the next year. This resulted in a major disruption of the planting and harvesting operation for the next season and may have also resulted in higher than expected yields for 2010 (Figure 358). Older ratoons with relatively low yields tend to be harvested later in the season and these may have been excluded from the 2010 crush. The disruption to the harvesting and planting schedules could be responsible for the lower than expected yield in 2013. The average yield for the monitored paddocks in 2013 was nearly 19 t/ha and was only slightly lower in 2014 and 2015. These paddocks were probably selected for good management as well as for their history of YCS. The mean yield for these paddocks was higher than the district average. We don't know if the yields could have been higher without YCS from these data.

Average regional yields for 2014, 2015 and 2016 were well within the error of yield estimate for each of those years. There was therefore no detectable effect of YCS on yield for those years. The effect of orange rust on yield was apparent for the years 2000 and 2001 and YCS does not appear to be limiting yield to the same extent as orange rust. Effects by disease on yields that are less than the error of the sugar yield estimate (about 1 t/ha or 7 %) would go undetected.



Dependent Variable	TSPH
N	39
Multiple R	0.675
Squared Multiple R	0.456
Adjusted Squared Multiple R	0.426
Cross validated R^2	0.322
Standard Error of Estimate	0.960

Regression Coefficients B = (X'X)-1X'Y

Effect	Coefficient	Standard Error	Std. Coefficient	VIF	t	p-Value
CONSTANT	9.483	1.732	0.000	.	5.474	0.000
LAG_RAIN	-0.579	0.385	-0.187	1.026	-1.503	0.142
BIOMASS	0.166	0.033	0.620	1.026	4.983	0.000

Figure 357 Scatter plot and regression statistics for Burdekin region mean sugar yield (t/ha) versus mean simulated biomass of the best 13 models, for years 1972 to 2012, excluding 2000 and 2001 when orange rust was prevalent (n=39). Total in-crop rainfall (mm) for the season before each crush year was included in the linear regression. Rainfall contours are shown in the scatter plot.

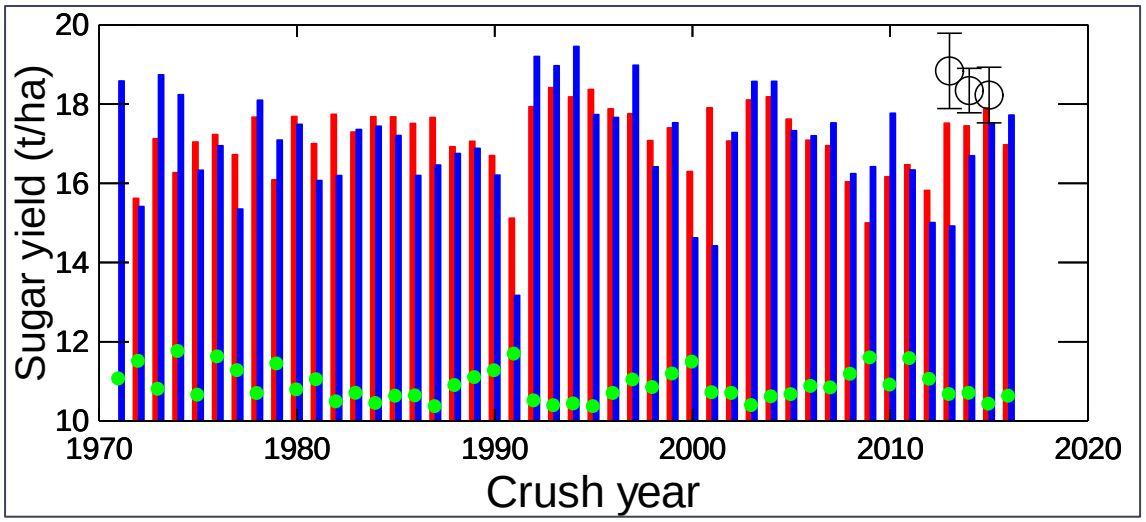


Figure 358 Simulated yield estimate for the 1974 to 2015 period (red bars) and actual yield for Herbert region (Lucinda terminal) for the period 1971 to 2010 (blue bars) and actual yields from paddocks monitored for YCS in 2013 to 2015 (black symbols and error bars). Mean in-crop rainfall (m) for each year is shown as green symbols.

Conclusions

The best APSIM model settings together with rainfall for the preceding season, explained 46 % (32 % after cross validation) of the year to year variation in the mean sugar yield in the Burdekin between 1971 and 2012. This leaves a lot of unexplained variation. Some of the 'unexplained' variation when using the model, could be explained by flooding and disease, which the model could not account for.

The standard error of the model was about 1 t sugar/ha. Simulated (estimated) yields and actual yields differed by less than 1 t/ha for the years 2014, 2015 and 2015 when YCS was prevalent. Low yields in 2012 and 2013 were probably due to the disruption of the planting harvest schedules following heavy rains and flooding in 2010/ 2011. It appears that YCS had limited effect (<7 %) if any on the sugar yield in the Burdekin region.

6.13.6 Discussion and conclusions

It is evident from the results presented in this chapter that assessing the impact of YCS on cane and sugar yield is a complex issue. However, it is noteworthy that whatever the analytical method used, a lack of correlation between YCS and cane/sugar yield penalty is universal. Interestingly the data indicates there is a trend, though not statistically significant, that increased YCS severity reduces cane yield but has no effect on CCS. It is tempting to argue that it is not YCS severity but the longevity of a YCS event that would potentially have the greatest impact on cane yield. Physiological studies (see section 6.5 of this report) show that at worst a YCS leaf is photosynthetically impacted by 40% at peak severity. Similarly, these studies show YCS usually affects 4-12 leaves during an episode so the impact on a total canopy production of approximately 40 leaves/year is in the vicinity of 2%. Obviously if more leaves were impacted the potential loss would be greater. The collective data over the course of this research project also supports this conclusion with the exception of the 2017/18 Burdekin insecticide trial that showed an approximate 30% cane yield increase under bifenthrin treatment. Further investigation into this anomaly revealed the yield differential to be a result of reduced stalk numbers in the untreated YCS plots. The cause of this stalk reduction is unknown but cannot be attributed to YCS as this condition does not cause plant death. YCS is a temporary affliction which the crop eventually grows out of in the cooler shorter photoperiod months from April/May (see section 6.2.3 of this report). Slightly higher cane yields recorded in other insecticide trials is likely attributed to the growth-defence trade-off (Huot et al., 2014) as bifenthrin is a non-selective insecticide removing most feeding insects, including beneficials.

Contrary to anecdotal evidence that YCS causes a CCS penalty, not a single sample from any experiment or trial is in support of this claim. Research by Scalia et al. (2020) shows the internodes adjacent to the YCS symptomatic leaves to be in a 'feast' state i.e. sugar content comparable or higher than asymptomatic cane.

6.14 The emergence of YCS in 2012

It is evident from the data presented in this report and research conducted by Scalia et al. (2020) that YCS is not a disease. No pathogenic organism could be consistently linked to the development

and expression of YCS in any of the thousands of samples of tissue types screened. Therefore, YCS is best described as a physiological disorder which may be induced by one or more stressor.

Our collective research shows that the key driver of the condition is growth rate which may be influenced by one or more abiotic or biotic agent. If there is substantial reduction in culm growth during the peak growing period from December to March, the sugarcane crop is more susceptible to developing YCS when rapid growth resumes. During this time of year conditions are usually perfect for growth of this C_4 plant which prefers high temperatures and long daylengths (Sage and Kubien, 2007; Ghannoum, 2009). Given access to a regular supply of water and nutrients it is possible for crops to exceed 200 tonnes cane per hectare (Strickland, 2016). This makes sugarcane one the highest biomass crops grown for either food or bioenergy (Moore and Botha, 2014). For sugarcane to attain high biomass, there must be a supply of photoassimilate from the source leaf that meets the growth demand of the non-photosynthetic sink tissue (McCormick et al., 2009; van Heerden et al., 2010). If supply exceeds demand, and in the absence of a phloem occlusion caused by or in response to a pathogen, there must be a deficiency in sink strength. Under these conditions sucrose export from the source will slow and accumulate in the leaf lamina, midrib and sheath (Marquardt et al., 2019; Scalia et al., 2020). The repartitioning of reduced carbon and sugar-mediated feedback inhibition of photosynthesis to decrease the carbon load in the source, is unsustainable and sucrose levels eventually breach the upper tolerable threshold of the leaf. At this point the level of metabolic and PET disruption, underutilisation of captured energy, and ROS production triggers a cascade of events that causes photooxidation, destruction of the chloroplasts and yellowing (Marquardt et al., 2016; Marquardt et al., 2017; Scalia et al., 2020).

To address the issue of reduced growth, investigation into soil health, nutrition, insects and farm management practices were undertaken in this study. None were forthcoming with evidence of any significant deterioration, deficiency or agricultural changes that correlated with the noted occurrence of YCS in 2012. This suggests that whatever triggered the source sink imbalance and expression of the condition in 2012 was not and incursion of an exotic agent, or widespread adoption of radical farming methods. It does however infer that a subtle large-scale external change has created or exacerbated existing stressors such as water and insects, causing a supply-growth imbalance that manifests in YCS. Perhaps the first clue that points toward the possible trigger is that YCS exhibits when good rainfall and growth occurs after a significant dry period. In this scenario smaller internode size caused by the dry period diminishes sink strength. This limitation instigates an imbalance due to reduced demand for fixed carbon which is now in ready supply following the rain and hot sunny days. The second clue is about season or time of year. During December to March the incidence of solar radiation is at its highest (Figure 359) and both high leaf sucrose and excess captured energy are required to cause leaf yellowing (Scalia et al., 2020); this is why YCS presents itself at this time. YCS symptoms are all but gone by the cooler, shorter daylength months. The third clue is that until 2015 there was no confirmed case of YCS in Tully Nth Qld, even though YCS was evident in districts to the north and south of the town. It is worth noting that Tully is the wettest town in Australia with an average rainfall of 4000mm and usually has an average of 180 rain days per year. In 2013 and 2014 Tully averaged 3,840 mm of rain and 184 rain days. However, when YCS was confirmed in Tully in 2015, ten fewer rain days were experienced with a much lower total of 2,850mm of rain for the year (BOM, 2020). During 2013 and 2014 average growth was maintained with an average yield of 80 t cane/ha, increasing by 20% to 100 t cane/ha in 2015. Under a greater

number of higher solar radiation days, increased photoassimilation increased the risk of a source sink imbalance and YCS was triggered.

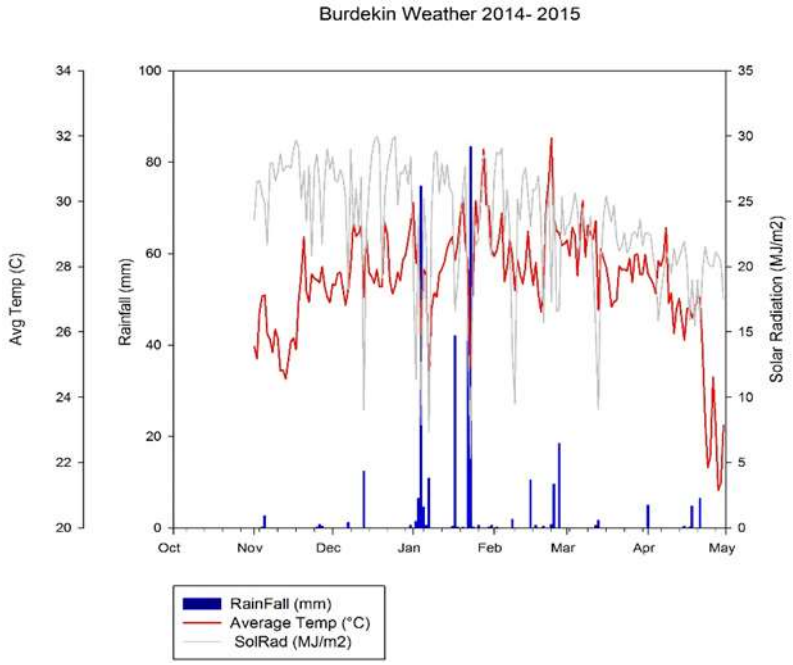


Figure 359 Burdekin weather 2014 – 2015 showing average daily rainfall, temperature and solar radiation.

Data obtained from the Australian Bureau of Meteorology shows that Queensland is now approximately 1.5°C warmer than in 1910 (Figure 360) (BOM, 2019). This phenomenon is not unique to Qld, but to all of Australia (Figure 361). It is worth noting that YCS was first reported in the summer/autumn of 2012/13 which correlates with this start of a peak temperature period that has continued to be consistently high (2013-2019) (Figure 360). Summers during 1999-2019 are now on average 1 month longer, and winters 3 weeks shorter than in they were between 1950-1969. When the historical annual mean temperature change since 1910 is considered, it is striking that the greatest temperature increase on the east coast of Australia is in the zone between Ingham and Mackay (Figure 362). The Ingham to Mackay belt covers the Herbert, Burdekin and Central regions. This area produces approximately 70% of Australia's sugarcane and correlates with the regions reporting highest incidences of YCS. Interestingly, the temperature increase in this this zone is comparable to the same temperature change experienced in the dead centre of Australia (Figure 362). Noteworthy is that approximately one third (0.5°C) of this 110 year temperature increase has occurred in the last 7 years and commenced when YCS was first reported in 2012/13 summer/autumn (Figure 360). This correlation between a period of extreme temperature change and the occurrence of YCS is compelling. Climate studies of the Mulgrave shire where YCS was first noted in 2012/13 show that over the past 70 years the average maximum and minimum temperatures have increased by over 1°C (Bonnett, 2018). However, consideration of this one variable alone is insufficient to draw any firm conclusions. Therefore a more in-depth analysis at a seasonal level incorporating rainfall and yield is presented in Figure 363 A-F.

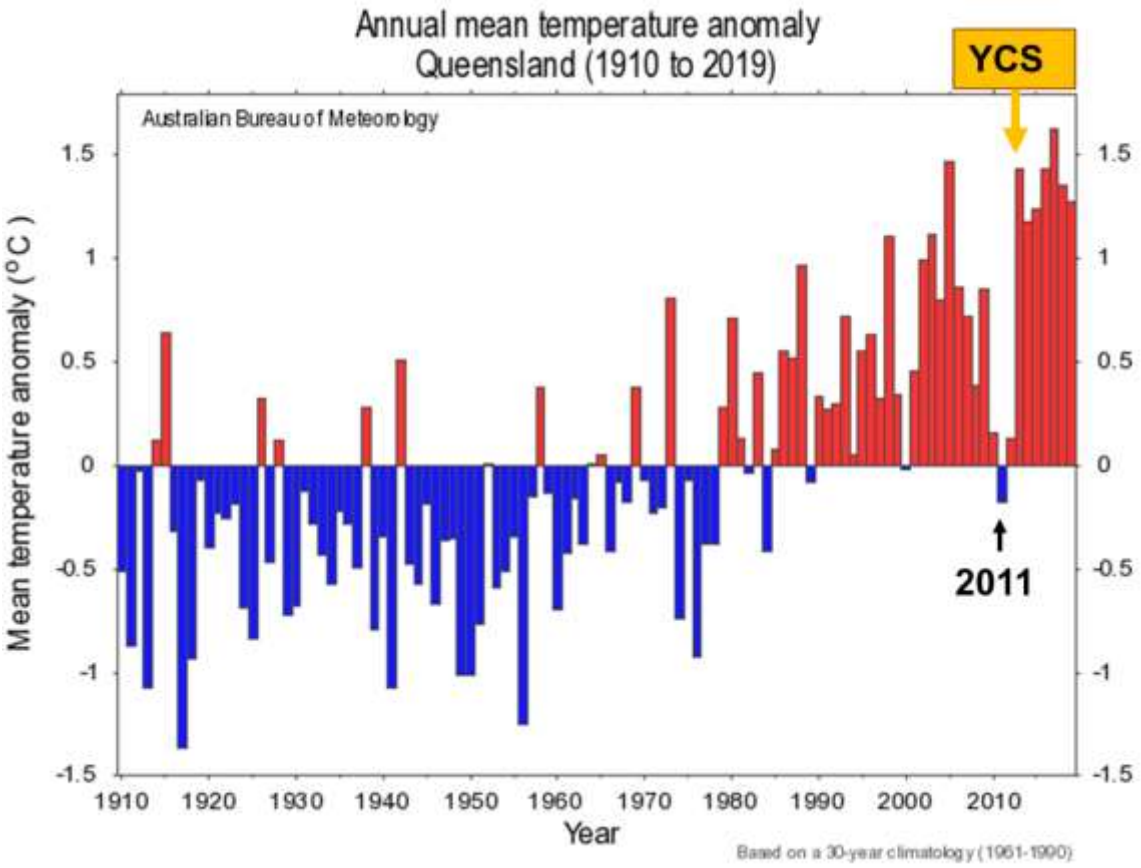


Figure 360 Queensland climate variability and change, annual mean temperature 1910 to 2019

(http://www.bom.gov.au/climate/change/index.shtml#tabs=Tracker&tracker=timeseries&tQ=graph%3Dtmean%26area%3Dqld%26season%3D0112%26ave_yr%3D0)

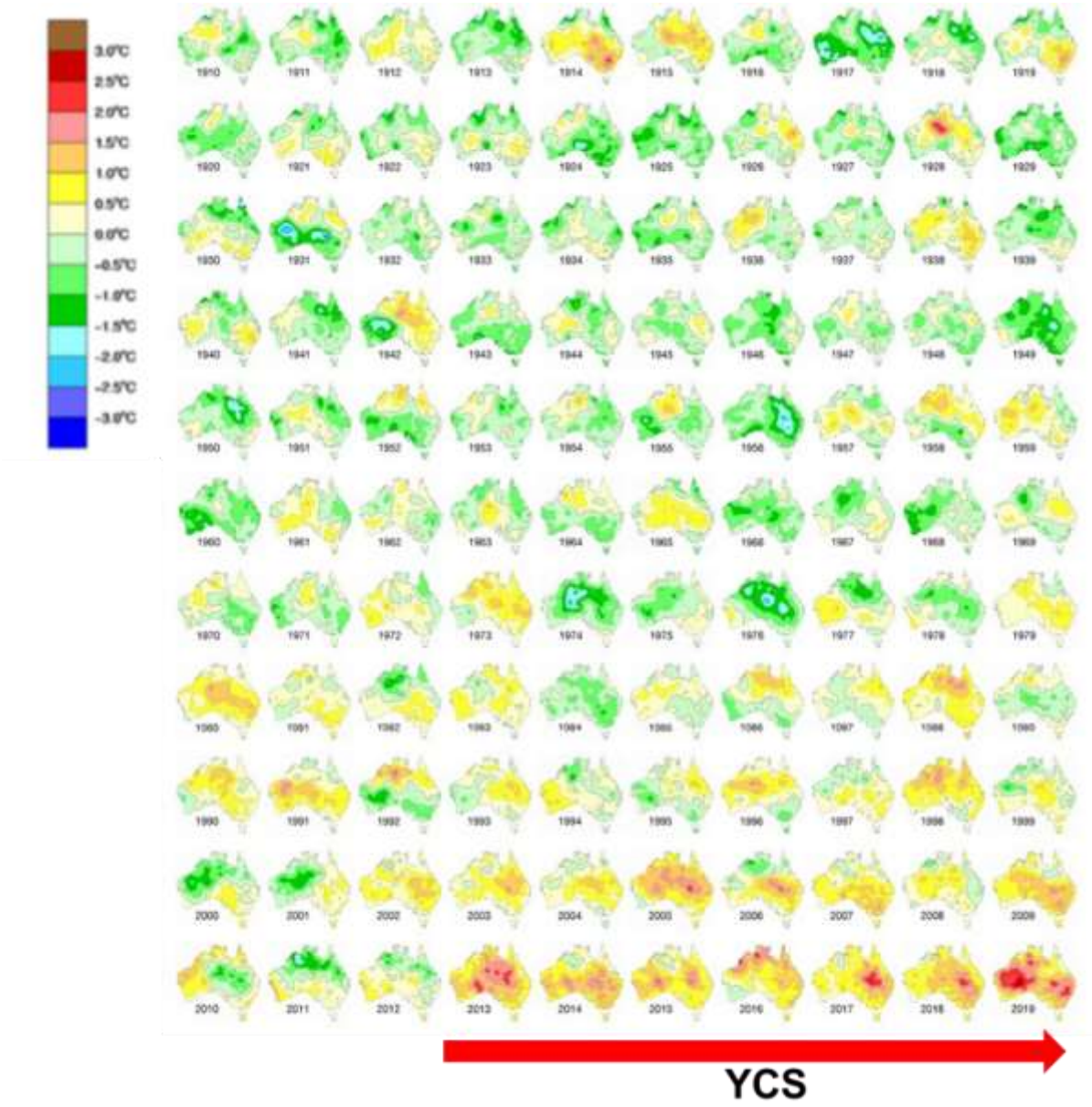


Figure 361 110 years of Australian temperature, 12 monthly mean temperature anomaly °C
http://www.bom.gov.au/climate/history/temperature/

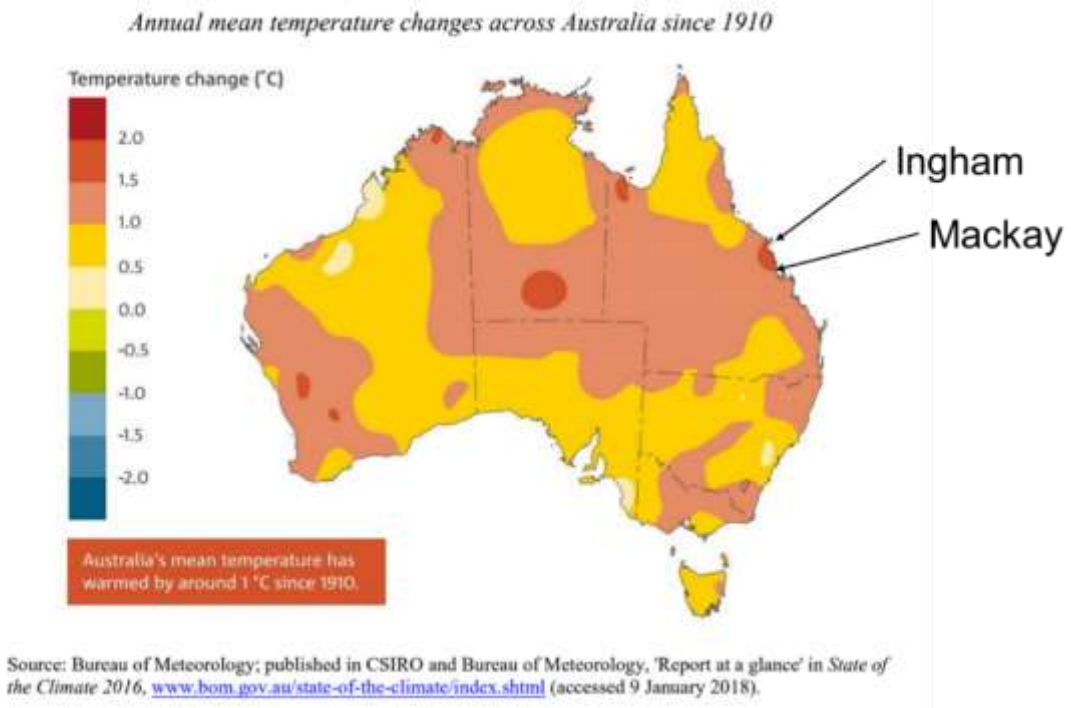


Figure 362 Annual mean temperature since 1910 shows a significant increase of approximately 1.5°C in the sugarcane belt between Ingham and Mackay covering three major districts of the Herbert, Burdekin and Central

In the context of growth-demand disruption and the triggering of YCS through a source sink imbalance during the peak growing season, it is more pertinent to consider seasonal rather than annual data for rainfall and weather. A representative timeline of rainfall, annual yield, first confirmed occurrence of YCS and severe weather events for each main sugarcane district is presented in

Figure 363 A-J. In the following analysis the impact of climate and weather on plant growth rate will be considered to determine if any correlation exists with the timing or occurrence of YCS. YCS was first reported near Gordonvale in the summer of 2012/13 and rainfall data shows that the spring and summer months leading up to YCS expression were very dry. This dry spring and summer period were the driest period in the last decade for this district (

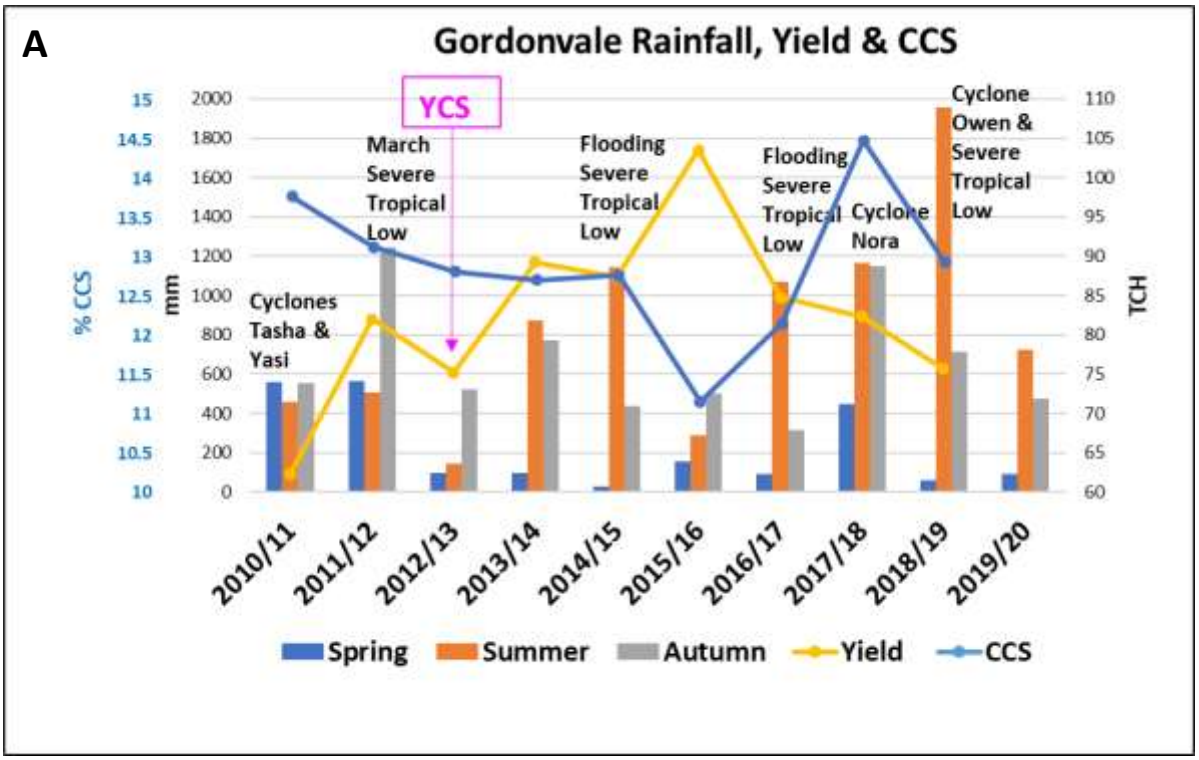
Figure 363 A). This reduced rainfall event would have significantly impacted culm growth and reduced sink strength, increasing the risk of a supply demand imbalance after the first decent rain that followed. YCS was reported in Gordonvale after good rainfall towards the end of summer into early autumn. This scenario can be investigated in each of districts represented in

Figure 363 A-J to deduce if any correlation exists with first reports of YCS. Using this methodology, it is evident that prediction of YCS outbreaks can be made by simply looking for periods of significant dry preceding wet weather events. However, as water availability is the only parameter impacting plant growth this model is limited. Consideration must also be given to other agents that may also inhibit plant growth such as nutrient availability, insect and other pest pressure, soil compaction and other farming methods, weed pressure and agrochemical toxicity to name a few. However, when considering the data presented in

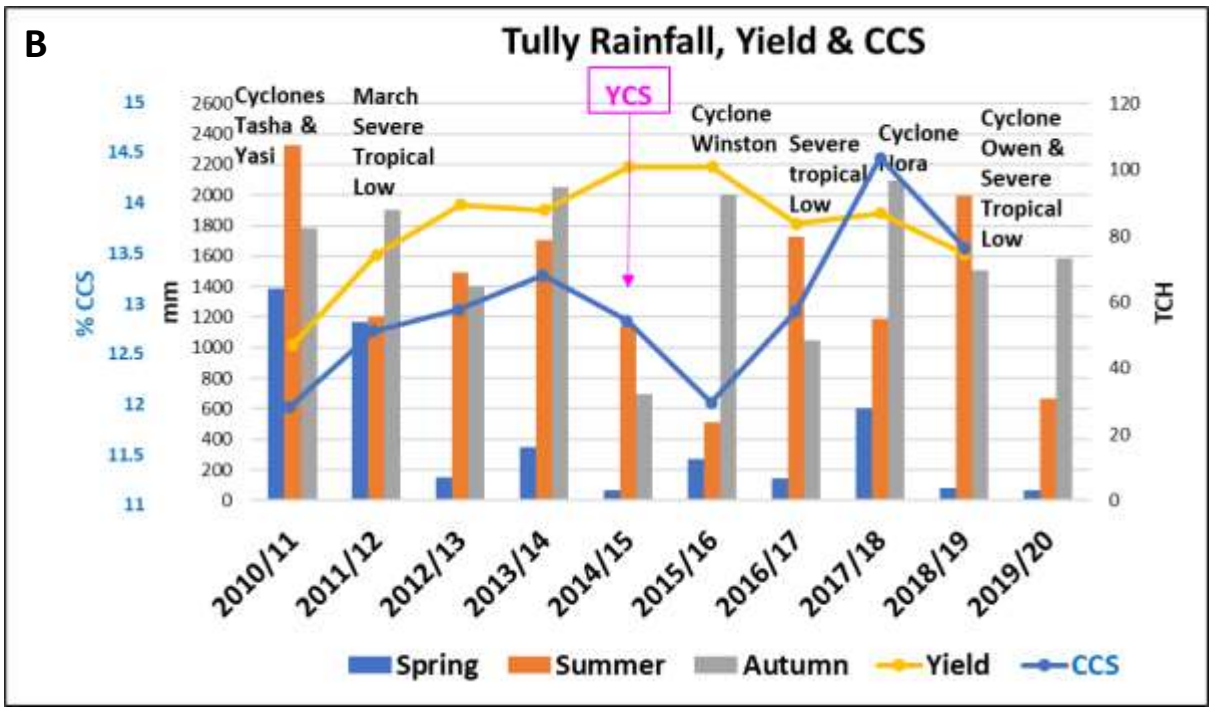
Figure 363 A-J the methodology is mostly sound. It was mentioned earlier in the report that YCS was not confirmed in Tully until 2015 despite YCS widely reported to the north and south.

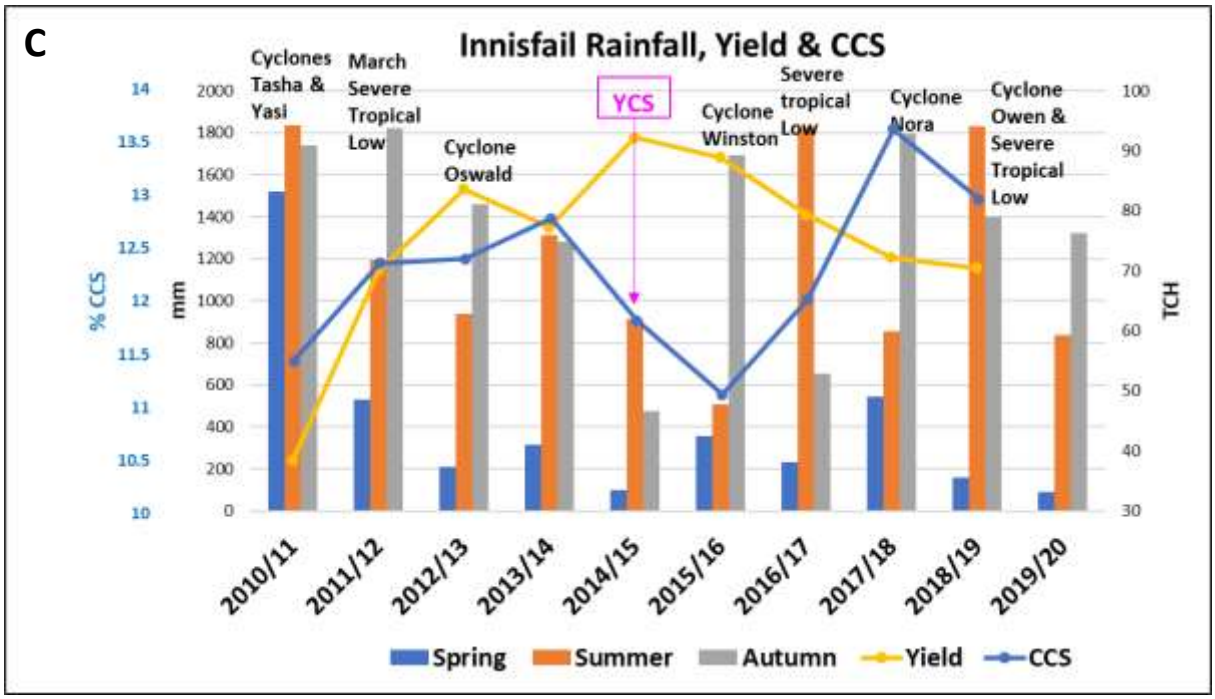
Figure 363B shows that 2015 was Tully's driest year in the last decade, with a particularly unusual dry spring leading into a wet but drier than usual summer. This scenario sets the perfect combination of elements for a source sink imbalance. It is noteworthy that even though YCS was confirmed for the first time in 2015, the highest annual yield in the past decade was achieved through increased photoassimilation due to higher solar radiation inception (fewer rainy days) and less water logging. Without this increase in carbon fixation, supply would be unlikely to exceed the growth demands of the crop to induce YCS development and expression. It is therefore not surprising that 2015 and 2016 seasons saw high incidences of YCS with confirmation of the condition as far south as Maryborough and Bundaberg. Both of these years saw high cane yields for the Industry throughout the state, indicative of increased plant growth and more consistent rainfall across the seasons. Of course, increased growth increases the risk of oversupply if the sink has been limited by stressors such as increased insect pressure under favourable weather conditions. This helps explain why our research has failed to find any correlation between YCS and cane yield penalty. Interestingly CCS tends to be a mirror image of cane yield (

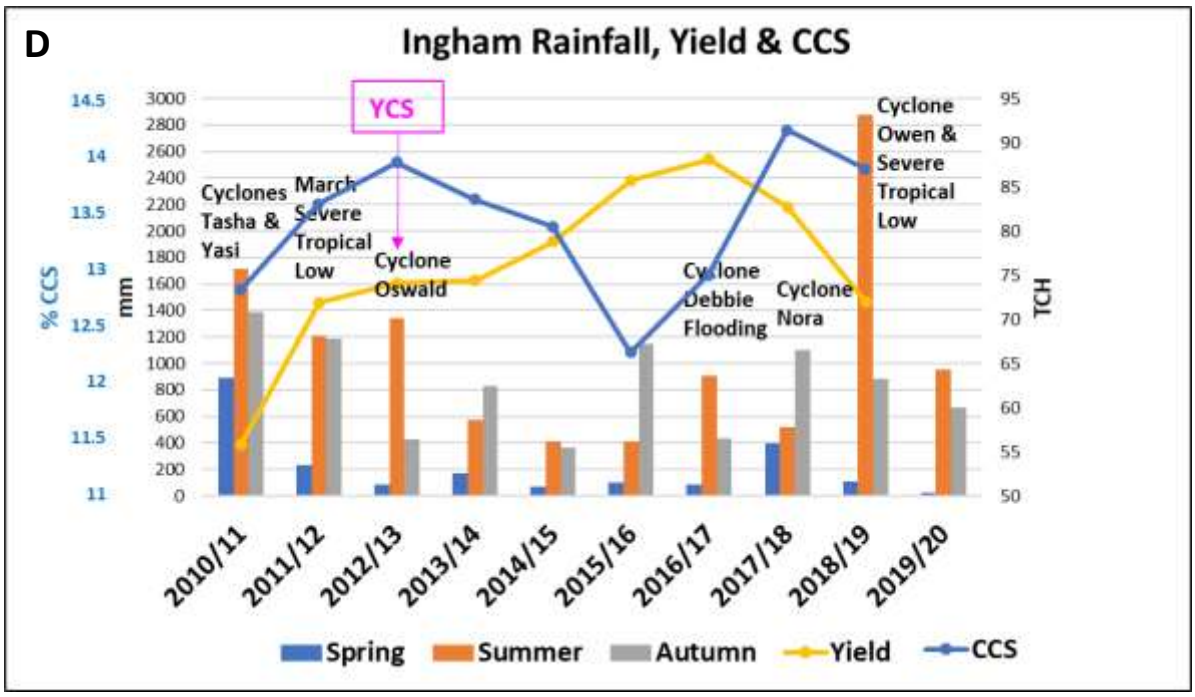
Figure 363 A-J). This does not suggest YCS has a negative impact on CCS, but rather there is a preference for partitioning of carbon to biomass during periods of high growth than to plant maturation and sucrose storage. Optimal seasonal conditions conducive for continued growth rather than drying down and ripening, is the mechanism behind low CCS and high biomass. Also of note is the effect of severe weather events. Noteworthy is the low yields and zero incidence of YCS during 2010/11 which saw extensive flooding from spring to autumn, more rain days, severe cyclones, and lower temperatures throughout Qld (Figure 360).

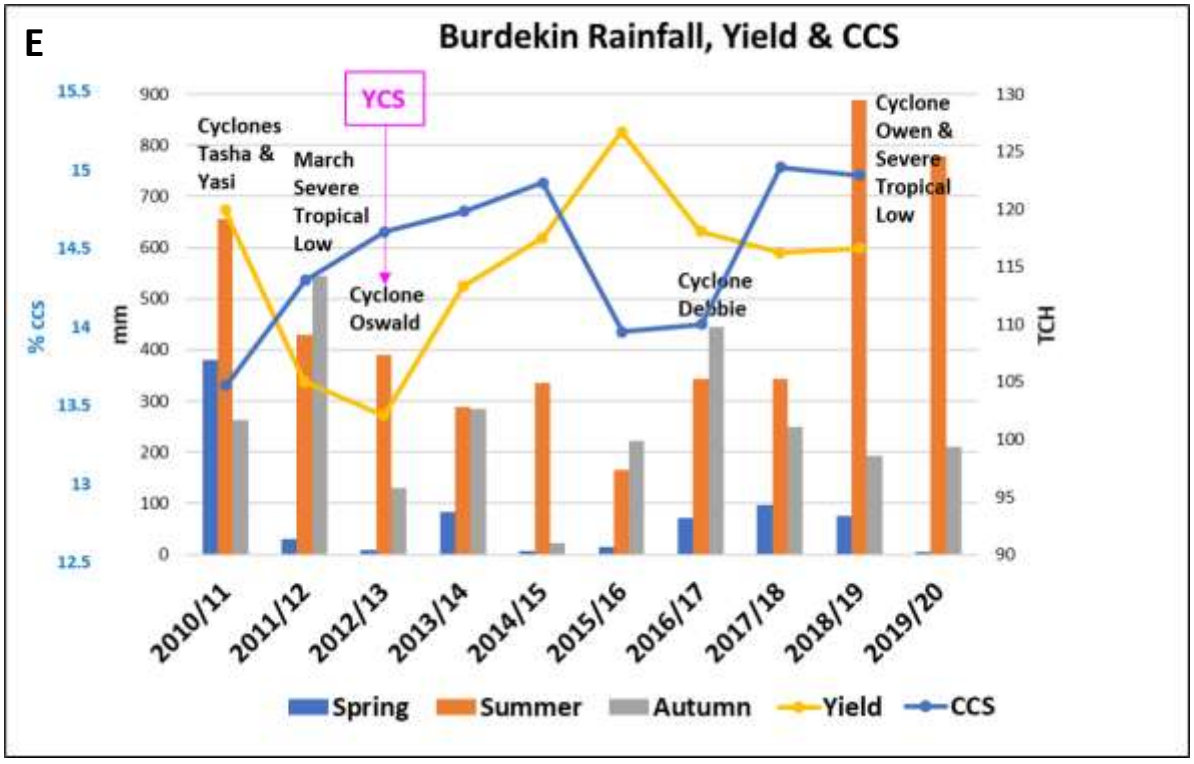


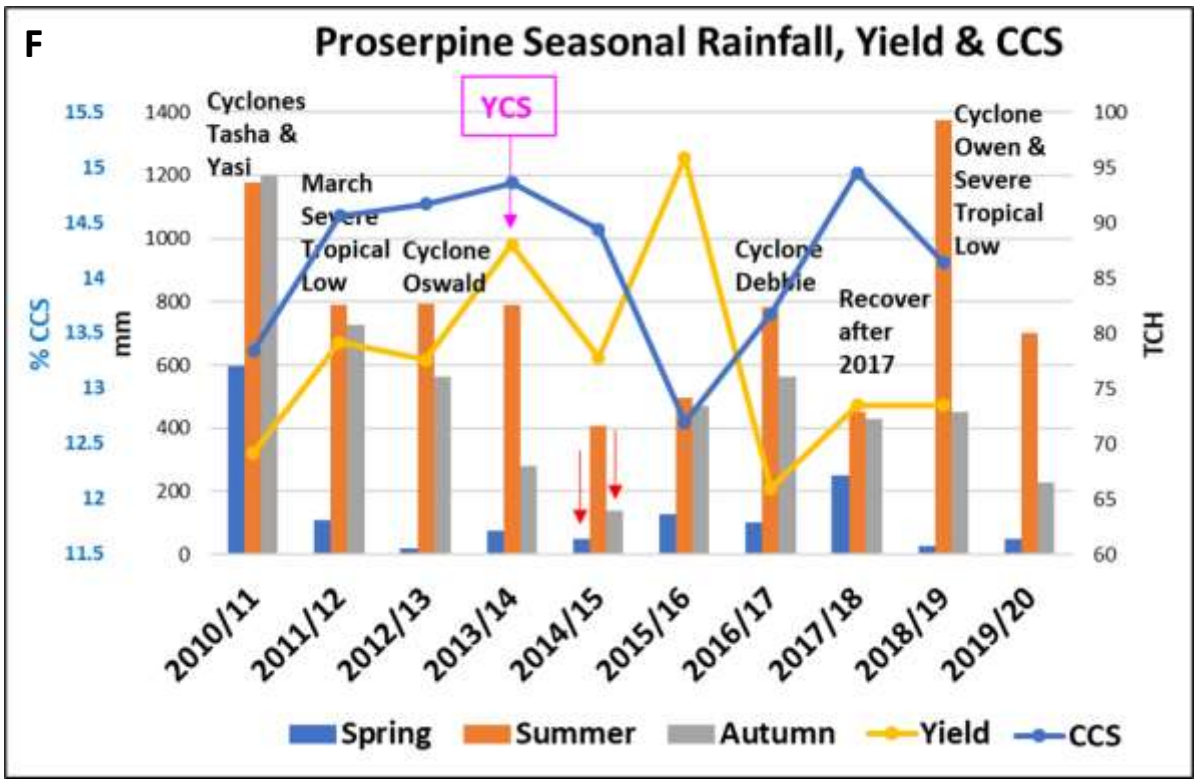
www.Canecalcs.com-

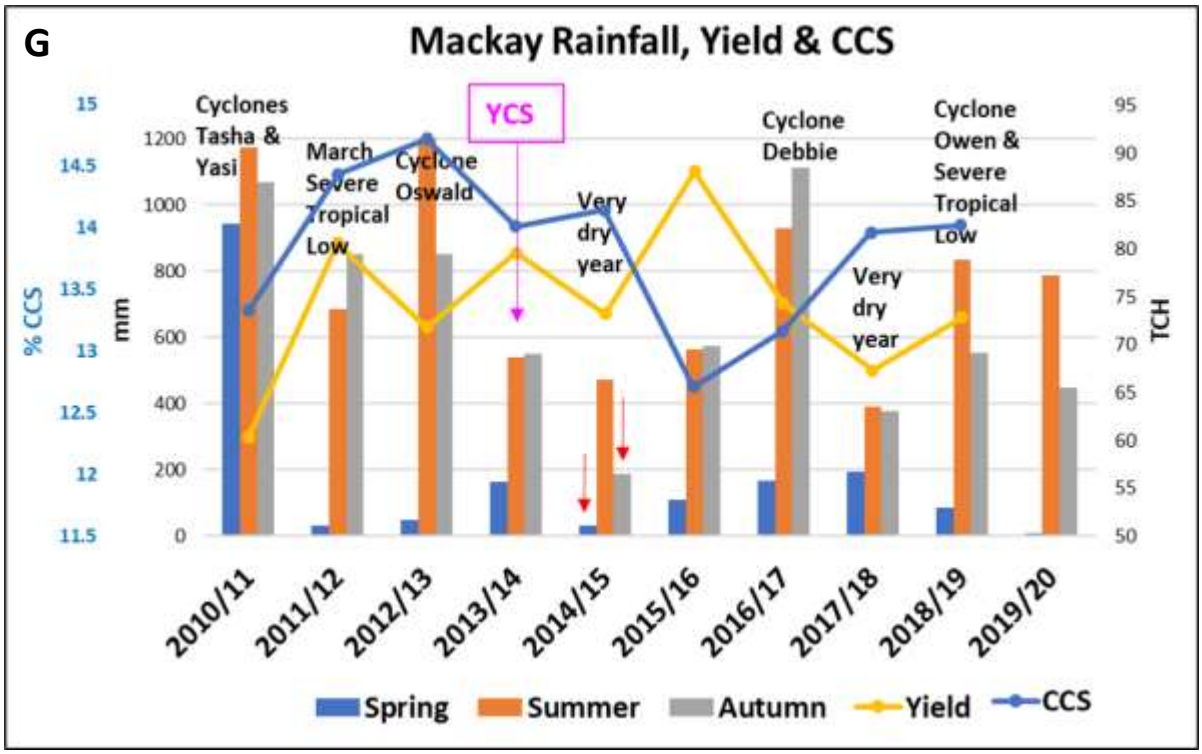

B
Tully Rainfall, Yield & CCS
Cyclones Tasha & Yasi
March Severe Tropical Low
YCS
Cyclone Winston
Severe tropical Low
Cyclone Nora
Cyclone Owen & Severe Tropical Low
% CCS
mm
TCH
2010/11
2011/12
2012/13
2013/14
2014/15
2015/16
2016/17
2017/18
2018/19
2019/20
Spring
Summer
Autumn
Yield
CCS


C
Innisfail Rainfall, Yield & CCS
Cyclones Tasha & Yasi
March Severe Tropical Low
Cyclone Oswald
YCS
Cyclone Winston
Severe tropical Low
Cyclone Nora
Cyclone Owen & Severe Tropical Low
% CCS
mm
TCH
2010/11
2011/12
2012/13
2013/14
2014/15
2015/16
2016/17
2017/18
2018/19
2019/20
Spring
Summer
Autumn
Yield
CCS

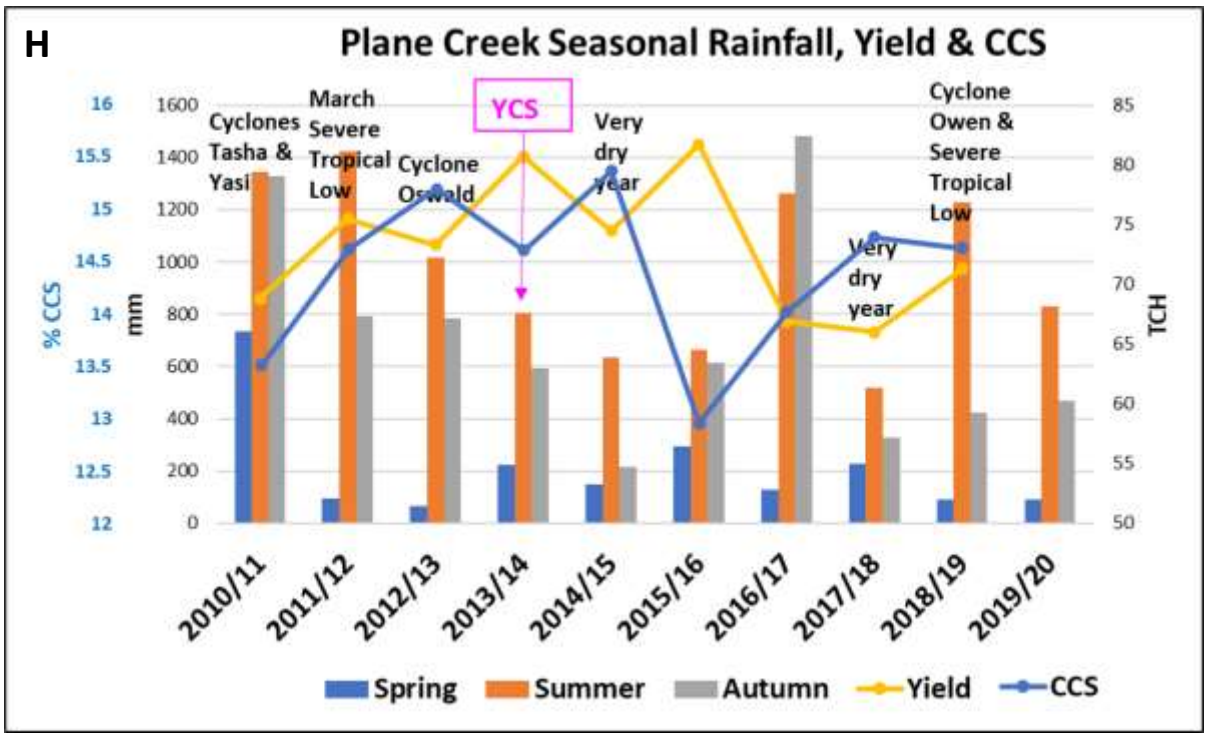

D
Ingham Rainfall, Yield & CCS
YCS
Cyclones Tasha & Yasi
March Severe Tropical Low
Cyclone Oswald
Cyclone Debbie Flooding
Cyclone Nora
Cyclone Owen & Severe Tropical Low
% CCS
mm
TCH
2010/11
2011/12
2012/13
2013/14
2014/15
2015/16
2016/17
2017/18
2018/19
2019/20
Spring
Summer
Autumn
Yield
CCS


E
Burdekin Rainfall, Yield & CCS
YCS
Cyclones Tasha & Yasi
March Severe Tropical Low
Cyclone Oswald
Cyclone Debbie
Cyclone Owen & Severe Tropical Low
% ccs
mm
TCH
2010/11
2011/12
2012/13
2013/14
2014/15
2015/16
2016/17
2017/18
2018/19
2019/20
Spring
Summer
Autumn
Yield
CCS

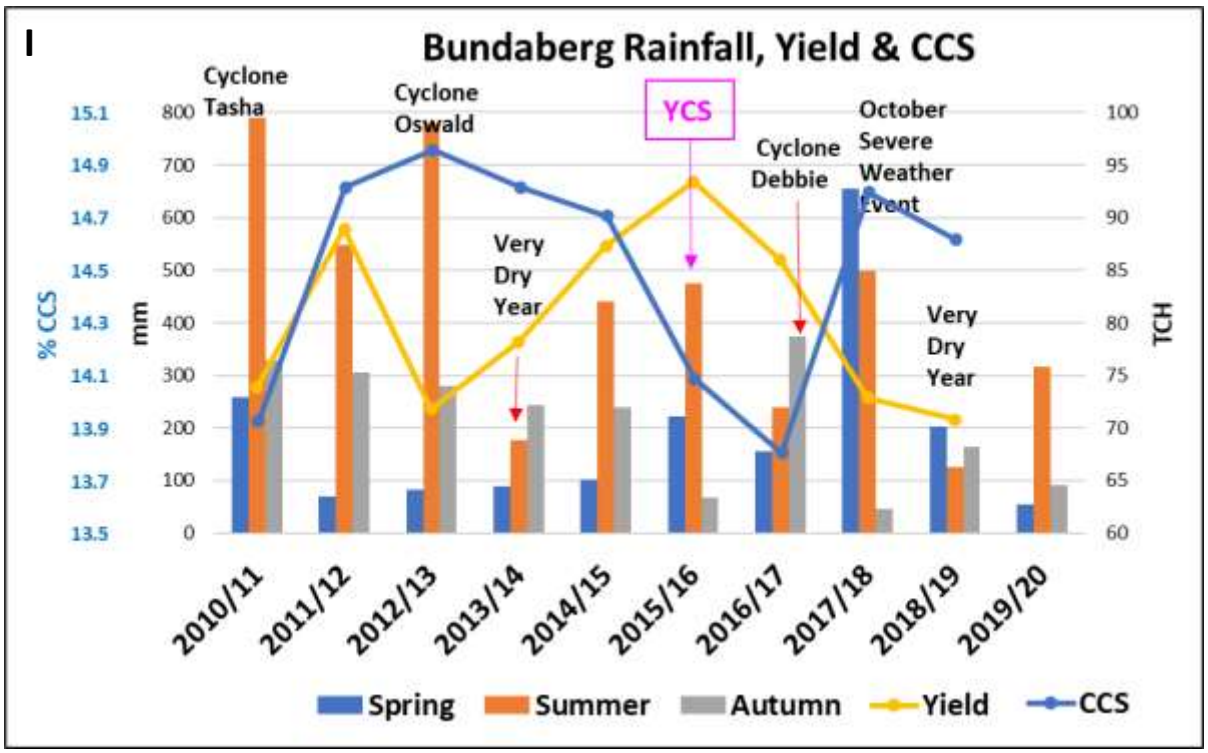

F
Proserpine Seasonal Rainfall, Yield & CCS
Cyclones Tasha & Yasi
YCS
March Severe Tropical Low
Cyclone Oswald
Cyclone Debbie
Recover after 2017
Cyclone Owen & Severe Tropical Low
% CCS
mm
TCH
2010/11
2011/12
2012/13
2013/14
2014/15
2015/16
2016/17
2017/18
2018/19
2019/20
Spring
Summer
Autumn
Yield
CCS


G
Mackay Rainfall, Yield & CCS
Cyclones Tasha & Yasi
YCS
March Severe Tropical Low
Cyclone Oswald
Very dry year
Cyclone Debbie
Very dry year
Cyclone Owen & Severe Tropical Low
% CCS
mm
TCH
2010/11
2011/12
2012/13
2013/14
2014/15
2015/16
2016/17
2017/18
2018/19
2019/20
Spring
Summer
Autumn
Yield
CCS

H


Plane Creek Seasonal Rainfall, Yield & CCS
% CCS
mm
TCH
Cyclones Tasha & Yasi
March Severe Tropical Low
Cyclone Oswald
YCS
Very dry year
Very dry year
Cyclone Owen & Severe Tropical Low
2010/11
2011/12
2012/13
2013/14
2014/15
2015/16
2016/17
2017/18
2018/19
2019/20
Spring
Summer
Autumn
Yield
CCS

I


Bundaberg Rainfall, Yield & CCS
% CCS
mm
TCH
Cyclone Tasha
Cyclone Oswald
YCS
Cyclone Debbie
October Severe Weather Event
Very Dry Year
Very Dry Year
2010/11
2011/12
2012/13
2013/14
2014/15
2015/16
2016/17
2017/18
2018/19
2019/20
Spring
Summer
Autumn
Yield
CCS

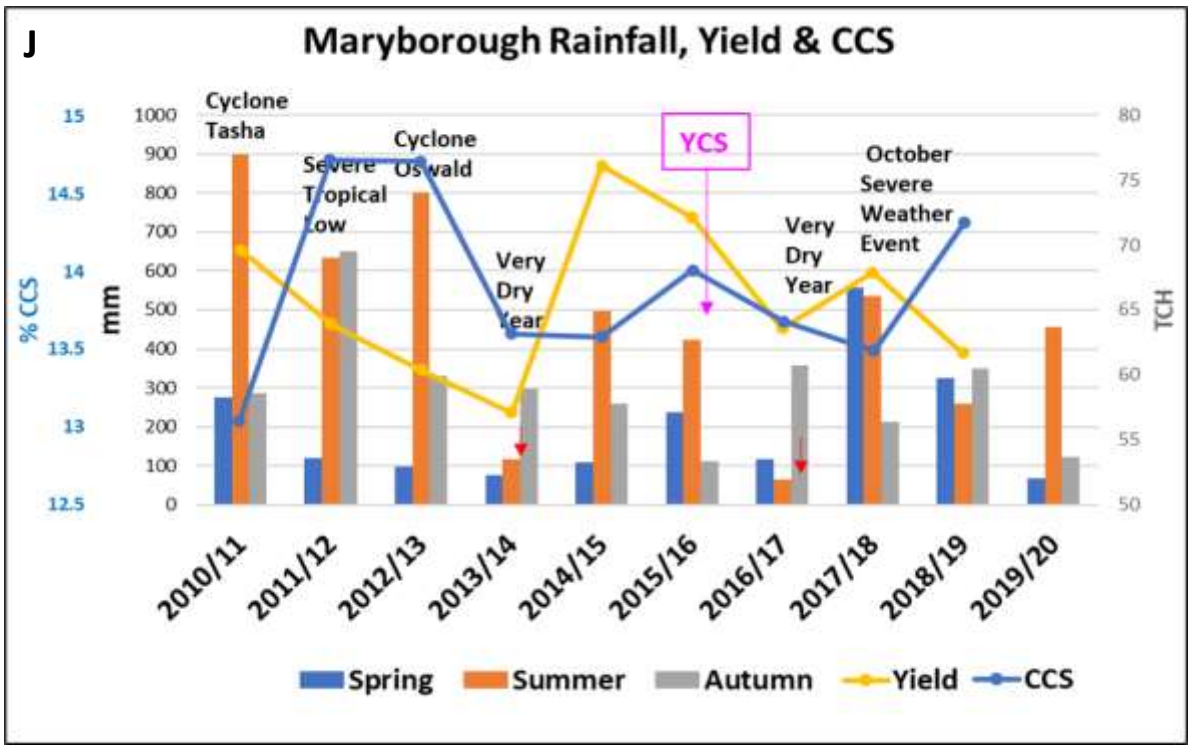


Figure 363 Seasonal (spring, summer, autumn) rainfall, cane yield (TCH), CCS, chronological timing of severe weather events 2010 spring/summer-2020 summer/autumn, time of first confirmed YCS occurrence, Gordonvale (A), Tully (B), Innisfail (C), Ingham (D), Burdekin (E), Proserpine (F), Mackay (G), Plane Creek (H), Bundaberg (I) and Maryborough (J)

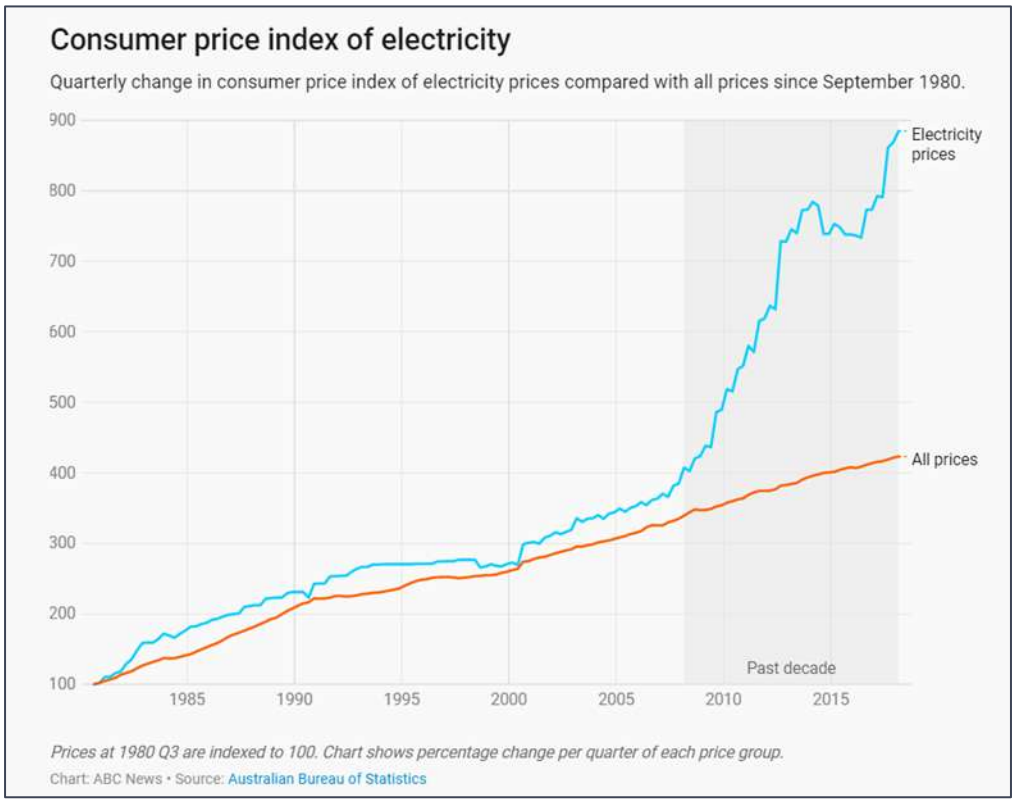


Figure 364 Electricity costs have increased by 120 % in the past decade (Australian Bureau of Statistics)

6.14.1 Discussion and conclusions

The mean temperature anomaly between 1910-2019 as identified by the Australian BOM shows a significant and consistent increase to mean temperature since 2012/13 (BOM, 2019). This rise in temperature of approximately 0.5°C has increased the incidence of severe weather, rainfall variability and stress on crops throughout Australia (Zhao and Li, 2015; Watson et al., 2017). Studies by Bonnett (2018) show that climate change has significantly impacted the northern Queensland sugarcane production over the past 70 years. A significant increase in temperature aligns with the main sugarcane growing area between Ingham and Mackay on the east coast of Qld where YCS was first reported. While C_4 plants are well adapted to high temperatures, access to adequate water supply is required to maintain growth requirements and avoid water stress (Ghannoum, 2009). Growers with crops under irrigation would need to increase the frequency of watering cycles to maintain a supply growth balance to mitigate YCS. Rising costs of water and electricity for pumping, has added further economic pressure to the challenge of preventing water stress and reduced growth (Figure 364). As the effects of climate change and weather events become more severe, so too will the array of stressors that can reduce crop growth and increase the risk of YCS development. Therefore, we postulate that climate change and in particular extreme weather events, is the large-scale external change that triggered the occurrence of YCS in 2012.

7. CONCLUSIONS

This extensive seven year study has utilised almost every area of plant research and agronomy to unravel the complexity of YCS, to better understand its development, symptoms, distribution, impact to industry, cause and management. From the moment YCS was noted in Nth Qld during the 2012 -2013 summer, it secured its place in the history books as one of the most puzzling sugarcane anomalies to be encountered by the Australian industry.

This research has confirmed early reports that symptoms appeared to come and go within a season, not return the following year, affect one section of a field and not another, have symptomatic and asymptomatic stalks in the same stool, and recover from an event. Within this study there were five key experiments and observations that helped progress the research forward to characterise YCS and identify the cause:

1) The crop age experiment identified growth rate was the key driver of YCS, independent of crop age. Monitoring of the condition provided evidence of symptom development initiating in the mid-canopy with green leaves above and below. This explained the wave like observations reported by growers and industry representatives, and the progression of leaf yellowing up the canopy over an average period of 8 weeks before recovery. Prevalence of the yellowing was synchronised independent of the crop age, indicating there was a common link to an external driver of the event. It was also noted that artificially producing treatments of different age in the trial altered sink size which affected YCS development and expression.

2) The water stress experiment was able to induce a rapid YCS response in plants with the highest exposure to solar radiation after irrigation was re-introduced. This scenario created a very strong source sink imbalance and concurs with reports of YCS expression moving like a wave through the crop after good rain following a period of limited water or poor growth.

3) The growth regulator trial showed how manipulation of sink strength could induce, prevent or mitigate YCS. Understanding the effect of the GA inhibitor on internode volume and sink strength confirmed the link between culm growth and YCS development.

4) The broad spectrum insecticide bifenthrin treatment promoted internode growth and maintained low levels of leaf sucrose and starch. Weekly treatment and control of insect pressure maintained YCS free plants. This confirmed the link between redirected resources to growth rather than defence, known as the 'growth-defence trade off' (Huot et al., 2014). Entomological studies have not identified any one or more specific insects species or groups that is responsible for causing YCS at the time of producing this report.

5) In the pathology/stress pot trial, disease free tested tissue culture and quarantine clean source cane did not remain YCS free and had higher YCS severity than the surrounding field grown cane. Included in the pot treatments was seed cane sourced from the surrounding field and it too had more severe YCS symptoms than the surrounding cane. This experiment confirmed two things a) YCS is not a disease (later confirmed by extensive pathology screening) and b) limiting sink size in treatments grown in pots exacerbated the source sink imbalance, increasing the severity of YCS symptoms.

These collective activities confirm the link between sink limited growth and YCS development and rule out the involvement of a sink limiting pathogen. Furthermore, the sporadic nature of YCS did not fit the typical epidemiology of a disease and this was confirmed through pathological studies. However, there is evidence of physical occlusions in the vasculature of YCS symptomatic plants and increased abundance of non-pathogenic endogenous microorganisms. These events are likely opportunistic and secondary in nature but may contribute to restricting phloem transport or limiting sink growth.

One other key observation at the start of the project was the absence of YCS in Tully. Tully is noted for being the wettest area in Australia, recording an average 180 rain days of lower solar radiation. Under these conditions it would be difficult for photoassimilation rates to exceed the sink capacity, explaining why the crop remained symptomless. However, YCS was confirmed in Tully in 2015 when rainfall reduced by 30% and yield increased by 20%. This concurs with final biomass trials that show no correlation between YCS and cane yield or CCS. However, it is evident that YCS prevalence and severity correlates with high yielding seasons in each of the regions. This is not surprising given that yield penalty precedes YCS development, and the magnitude of YCS severity is highest during periods of rapid growth following such limitations. Hence, YCS is caused by growth limitations and not vice versa. This concurs with trial biomass data and modelling which show the direct impact of YCS on yield is minimal. Any significant yield losses should not be ascribed to YCS expression but to the pre-YCS hindered growth period. Therefore, YCS management should centre around identification of the dominant stressor impacting growth and implementation of appropriate measures to counter or mitigate its influence.

Climate change data from the BOM align with the occurrence of YCS in the summer of 2012/13. It is likely that climate change and more frequent severe weather events are the drivers of YCS development and the impetus for the current distribution. YCS has now been recorded and confirmed in all sugarcane regions as far south as Maryborough. It is worth noting that YCS has not moved down the Qld coast, but rather its expression is a behavioural response to environmental changes impacting its physiology. Therefore, YCS is the visual terminal expression of metabolic perturbances caused by growth disruption in sugarcane plants under abiotic or biotic stress.

8. RECOMMENDATIONS FOR FURTHER RD&A

The impacts of climate change are real, and preparedness is the key to reducing detrimental impacts to crops in the future. In unchartered territory, the best course of action is to invest in modelling to predict impending environmental changes. This would then require further investment in areas of 1) targeted trait development to build tolerance and resistance into the germplasm to counter the identified risks, and 2) management practices to mitigate any genetic deficiencies.

9. PUBLICATIONS

(Marquardt et al., 2016)

Industry publications – see section 4.1.2

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12. APPENDIX

12.1. Appendix 1 Field and pot trials

Appendix 1 Pot Trial Outline and Me

12.2. Appendix 2 APVMA permit

PER87959.pdf

12.3. Appendix 3 DNA sequences

Appendix 3 DNA sequencing.docx

12.4. Appendix 4 Presentations

Note: Throughout the course of this project many presentations were deliver. As very few of the original research team are available for input only a few presentations are included here. However the ASSCT conference papers are a good representations of the main topics presented in this project

Industry webinar https://www.youtube.com/watch?v=SDe4L00cBLI&t=7s

ASSCT Nutrient uptake affected by \

12.5. Appendix 5 METADATA DISCLOSURE

Table 93 Metadata disclosure 1

Data	All data, scripts, images, analyses, files, reports and presentations associated with the project
Stored Location	Sugar Research Australia "J:\2014049YCS\"
Access	Access is restricted
Contact	Gerard Scalia (gscalia@sugarresearch.com.au) or SRA's IT Department/Steve Comerford (scomerford@sugarresearch.com.au) for access