STARCH ACCUMULATION IN SUGARCANE IN RESPONSE TO STRESS

By

PRIYA JOYCE[1], NIROSHA HEWAGE DON[1],
MONICA SOUSA[1], DAVEY OLSEN[2]
[1]*Sugar Research Australia, Indooroopilly*
[2]*Sugar Research Australia, Brandon*
pjoyce@sugarresearch.com.au

KEYWORDS: Starch Accumulation, YCS, Senescence, Biotic, Stress.

Abstract

YELLOW CANOPY SYNDROME (YCS) is a new problem affecting the Australian sugar industry. It was first reported in 2012 and has increased in occurrence and spread from the initial reports in North Queensland to further south in Mackay last year. While the causal agent is still unknown, several physiological studies have been performed. This paper examines the accumulation of starch in sugarcane leaves. This was an initial observation in YCS affected leaf sections of tillers of KQ228$^{(\!)}$. Subsequently, a method for routine high throughput qualitative analysis of starch has been developed, enabling rapid assessment of this response. Follow up work has compared this phenomenon in affected stalks, both between leaves of different ages within a stalk, as well as within regions of the same leaf. The accumulation of starch in sugarcane plants in response to biotic stress and in naturally senescing leaves has been undertaken to understand this phenomenon better and will be described in this paper. Our results showed that unlike YCS affected leaves, starch did not accumulate in the leaves of diseased plants nor in senescing leaves collected from the field.

Introduction

Yellow canopy syndrome (YCS) was first observed in sugarcane at a farm north of Cairns, Queensland in 2012. It was hoped that this syndrome would be transient and not be seen again. However, since then, YCS has appeared further south, becoming widespread in the Herbert and Burdekin regions in 2013 and 2014 and in the Mackay district in 2015. Integrated research on establishing the cause and effects of YCS has been ongoing, and while its effects are well studied, the causal agent still remains unknown.

YCS generally occurs during the hot and humid summer months with peak symptom expression from late January to April. Typical symptoms show a rapid yellowing of the lower leaves, often more noticeable after a period of water stress (Figure 1). There appears to be no specific pattern of occurrence. Yellowing can vary from being localised on one half of the leaf lamina with the adjacent half remaining green, whole leaf becoming yellow, or can be expressed as yellow mottling in no specific pattern. However, unlike bright yellow midribs associated with sugarcane yellow leaf virus (SCYLV), the midrib remains white in YCS affected leaves.

Another difference is that YCS occurs during peak summer conditions, while SCYLV occurs during the winter months when the ambient daytime temperature is below 20 ^{o}C in Australia. The occurrence of YCS is independent of the sugarcane variety, crop class as well as soil type (Olsen *et al.,* 2015). To date, no YCS has been confirmed in the Plane Creek, Bundaberg, Isis, Rocky Point or New South Wales districts.

While investigating the physiological effects of YCS on affected leaves of KQ228$^{(\!)}$ tillers in the Burdekin, it was noted that leaves contained a hyper-accumulation of starch. Further studies on the nature of this accumulation in YCS plants from other regions and varieties has now been

conducted. In addition, we were curious to see whether this phenomenon was unique to YCS affected plants, or was a common response by sugarcane to yellowing and/or any biological induced stress. The effects of biotic-induced yellowing and natural senescence on starch accumulation are reported in this paper.

Fig. 1—Typical YCS symptoms in the field and a close-up of a typical leaf showing YCS symptoms.

Material and methods

Sample collection

YCS samples

Leaf samples were collected from YCS symptomatic and asymptomatic (referred to as healthy in this paper) stalks from the Herbert and Burdekin regions early in the morning between 7 am and 9 am in May 2014 as described below. Symptomatic stalks showed YCS yellowing on lower leaves.

Disease samples

Leaf samples were collected from the Woodford Pathology station from diseased and healthy plants from three different varieties showing strong symptoms of infection: variety Q124$^{\Phi}$ with Ratoon Stunting Disease (RSD) or Fiji Leaf Virus (FDV); variety Q44 with Leaf Scald Disease, and variety Q205$^{\Phi}$ with Sugarcane Mosaic Virus (SCMV) or Smut Disease. For the control samples, leaves were collected from un-inoculated healthy sugarcane of each variety.

Samples were collected in winter, early in the morning between 7 am and 9 am, in order to collect leaves with the lowest levels of starch accumulation (Du et al., 1998).

Leaf samples were numbered as follows: leaf +1 was designated as the first fully expanded leaf from top to bottom of the sugarcane stalk corresponding to the first visible dewlap (FVD). Leaves below the FVD (older leaves) were numbered upwards in succession, and leaves above the FVD were numbered (0, –1, –2) until the spindle leaf (Figure 2, A and B).

Leaf samples were collected from three sugarcane plants of the same condition and variety, kept separate and treated as replicates. Ten leaf punches were collected directly into 2 mL screw cap eppendorf tubes, from leaves of healthy and symptomatic plants avoiding the midrib, starting from the youngest furled leaf to the lowest mature leaf, using a paper punch (Figure 2, C).

Tubes were stored on dry ice during collection and transported back to the SRA Indooroopilly laboratories for starch analysis. In some experiments, instead of leaf punches, 1 cm leaf sections across the leaf blade (from margin to midrib to margin) after discarding ~30 cm of the narrow and dying tip end were tested.

This sampling method was modified to study starch accumulation trends within the leaf. In addition, 1 cm pieces of the leaf sheath subtending the leaf was also collected and analysed for starch as above.

Leaves with SCYLV were sourced from foreign clones present in the glasshouse at Indooroopilly. In addition, leaf material of an unknown disease showing symptoms similar to YCS, called Golden Leaf Syndrome, and was brought back via quarantine from Papua New Guinea (PNG). The leaf samples from PNG were brought back in Falcon tubes dried over $CaCl_2$ (as per quarantine regulations). Leaf punches were taken from this dried material and starch analysed using the method developed for fresh leaf samples. Details of diseases studied are shown in Table 1.

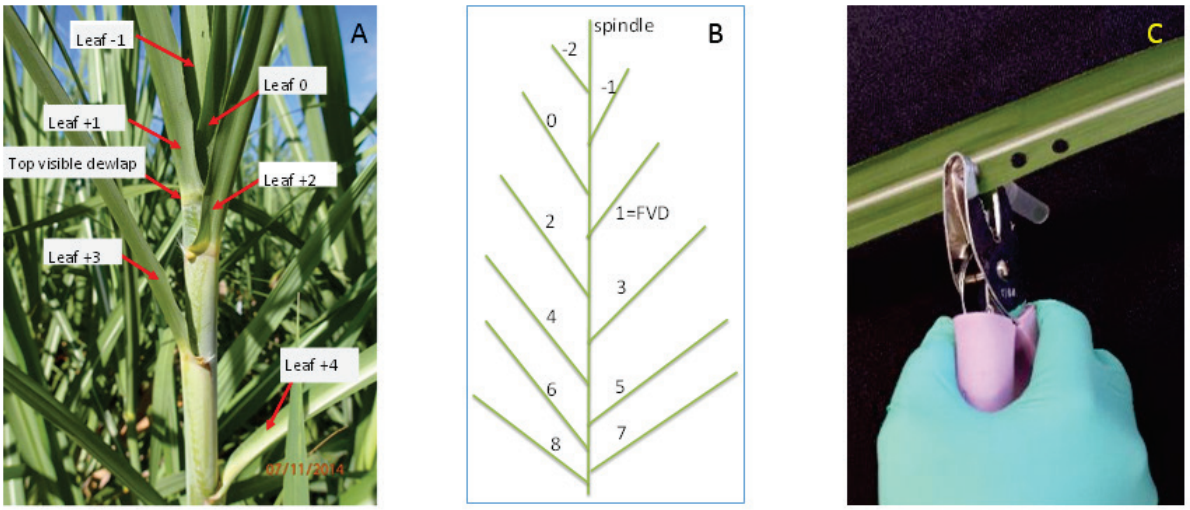

Fig. 2—(A) and (B) leaf numbering of leaves on the stalk, where the first visible dewlap (FVD) is leaf +1. (C) leaf punches taken from leaves in the field.

Table 1—Sugarcane diseases tested for starch accumulation in leaves.

Disease (Biotic stress)	Causal agent	Collected from	Variety
Ratoon stunting disease (RSD)	Bacterium	SRA Woodford	Q124$^{\phi}$
Leaf scald	Bacterium	SRA Woodford	Q44
Smut	Fungus	SRA Woodford	Q205$^{\phi}$
Fiji leaf gall	Virus	SRA Woodford	Q124$^{\phi}$
Sugarcane mosaic (SCMV)	Virus	SRA Woodford	Q205$^{\phi}$
Sugarcane yellow leaf virus (ScYLV)	Virus	SRA foreign clones	From S. America
Yellow leaf syndrome (YLS)	Unknown	Burdekin productivity board	QS02-1032
Golden leaf syndrome (GLS)	Unknown	PNG	PN92-339 and B72-177

Senescing samples

Leaves from Q208$^{\phi}$ plants growing in the Bundaberg productivity board fields were collected in June 2015. The aim was to study starch behaviour in mature almost-senescing leaves in the field under natural conditions. Three stalks were selected and the two oldest mature almost senescing leaves still attached to the stalks were sampled (Figure 3). Whole leaves were collected, placed on ice and brought back to SRA Brisbane laboratories for starch analysis. In this experiment, leaf pieces from margin to margin were stained for starch as this provided a more thorough analysis of the starch status of the leaf.

Fig. 3—Cartoon showing the method of sampling the sections taken for the starch assay. One cm leaf sections were cut 10 cm apart, commencing after the narrow and often dead tip region was discarded (~30 cm), and assayed for starch.

Starch assay

A high throughput qualitative assay was developed after testing different methods. These included treatment with acetone, bleach, 80% lactic acid or 100% ethanol. Various methods for the ethanol treatment were also tested including microwaving, boiling water bath and autoclaving. Of the various combinations tested, the most consistent method which we have adopted and used for all our assays was the following:

One mL of 95% ethanol was added to each eppendorf tube containing the leaf punches. Tubes were then autoclaved on liquid cycle for 20 min at 121°C. After the cycle was completed, the ethanol was discarded and 1 mL of sodium hydroxide (1%) was added to the tubes and they were re-autoclaved as before. After the end of the cycle, the remaining solution of sodium hydroxide was discarded. Next, 1 mL of ten-fold diluted commercial bleach was added to the tubes and samples were left overnight.

After these steps the leaf punches were discoloured, the commercial bleach solution was then discarded and MilliQ water was added to remove the remaining bleach. The leaf punches of each sample were placed on glass slides and 100 µL iodine/ potassium iodide solution (1% Lugol's stain) was added to it. When starch was present, the characteristic dark colour was observed in the leaves. A visual colour index was developed and samples given a rating for the colour developed in response to the stain (Figure 4).

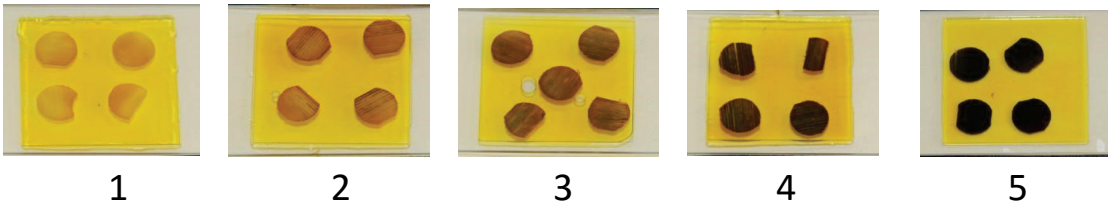

Fig. 4—Visual Rating index adopted for starch staining of leaf discs, showing a gradation from no starch (1), very light (2), medium (3), heavy (4) and very heavy (5) staining for starch.

The visual scores were the average of ratings of leaf punches of each leaf of the three replicate stalks tested for each disease. The ratings were rounded to the closest whole number. Generally, all four punches showed very similar colour in the starch test.

Results

YCS samples

Samples of leaves collected from the trip to the Herbert showed that starch accumulated in all leaves from leaf +2 and older (Figure 5). It also showed that while samples from healthy plants collected in the morning showed no starch present, by the afternoon (PM samples) some starch accumulation was visible in these plants. Leaf sheath however, did not show any difference between healthy and YCS plants irrespective of the sampling time with the exception of the oldest leaf 5 (Figure 5).

Disease leaf samples

Leaf punches from a range of sugarcane varieties with various diseases (bacteria (leaf scald and RSD), fungi (smut) and viruses (SCMV and Fiji)) were collected from the SRA disease testing station at Woodford (Table 2) and tested for presence of starch using the method described above. All the samples were rated using the visual rating index for the presence of starch.

Results showed that in the variety Q205$^{\Phi}$, the healthy plants had high starch present in all the leaves. In contrast, smut and SCMV affected plants showed much lower starch ratings in all the leaves. In Q124$^{\Phi}$ and Q44 plants, the trend was similar with leaves of healthy plants showing starch ratings higher than or equivalent to those affected by Fiji or RSD in Q124$^{\Phi}$; and leaf scald in Q44 (Table 2).

In none of these diseases studied was the starch rating higher in affected leaves than in healthy un-inoculated controls.

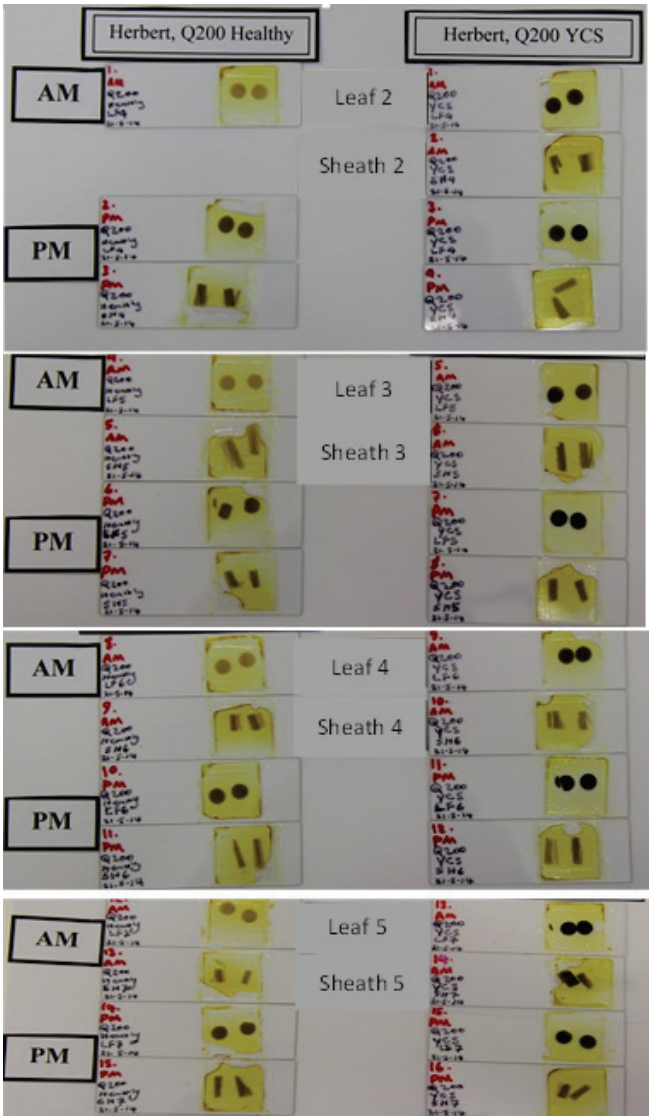

Fig. 5—Starch accumulation in YCS leaf punches stained with Lugol's reagent in Q200$^{\phi}$ plants.

To evaluate whether the presence of yellow colour correlated with the accumulation of starch, leaf samples of healthy and SCYLV infected plants growing in the Quarantine glasshouse at Indooroopilly were tested for presence of starch (Table 3).

Table 2—Visual ratings for presence of starch in leaf punches (Figure 4) from the youngest to oldest leaves of healthy and diseased sugarcane plants.

Variety	Q205$^{\phi}$			Q124$^{\phi}$			Q44	
Leaf #	Healthy	Smut	SCMV	Healthy	Fiji	RSD	Healthy	Leaf Scald
–3	5		1		5	2	2	1
–2	5		2	4	5	3	2	1
–1	4	1	1	5	5	5	1	1
0	4	1	1	5	3	5	2	1
1	3	1	1	4	1	4	2	1
2	4	1	1	3	2	2	1	1
3	5	1	2	4	4	3		1
4		1	2	4	4	2		
5		2	1	3	3			

Table 3—Correlation between SCYLV, leaf colour and presence of starch in sugarcane clones present in the quarantine glasshouse.

Sample ID	SCYLV	Leaf colour	Starch rating
Q247$^{\Phi}$ Healthy	–	Green	5
	–	Yellow	2
M2372/95 Healthy	–	Green	5
	–	Yellow	2
M1799/94 SCYLV	+	Green	3
	+	Yellow	5
CC98-577 SCYLV	+	Green	3
	+	Yellow	2

Results showed that in healthy plants, yellow leaves contained less starch than green ones, while in SCYLV affected plants, there was no correlation between the occurrence of green or yellow leaf colour and presence of starch in the leaves.

Furthermore, $CaCl_2$ preserved leaf samples of plants surveyed in PNG for viral diseases (Ramu stunt virus and/or SCYLV) showed no correlation between presence of virus (one or both), yellow colour of leaf and accumulation of starch (data not shown).

Natural senescence in the field

Occurrence of starch in naturally senescing leaves of Q208$^{\Phi}$ plants was also evaluated, to further elucidate the fate of starch in plants growing under field conditions (Figures 3 and 6). Very little starch accumulated in all senescing leaves, irrespective of distance from the base. Many of the leaves showed brown rust which appeared as brown specks in the starch assay (Figure 6).

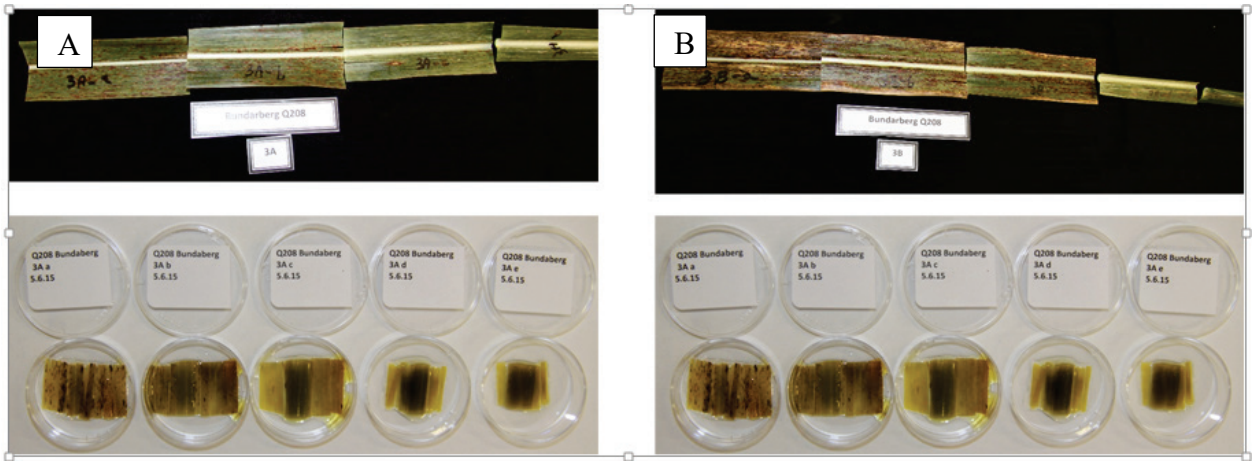

Fig. 6—Presence of starch in the two consecutive senescing Q208$^{\Phi}$ leaves. Appearance of leaf pieces after staining for starch, from the tip sections to the base region of the leaf in leaf A and B.

Golden leaf syndrome

Visually, YCS symptoms appear very similar to those of golden leaf syndrome (GLS), a condition affecting sugarcane in PNG. To ascertain if they were in fact the same condition leaf samples of GLS affected plants were collected and brought back to the Brisbane laboratory by Dr Kathy Braithwaite.

These leaves were tested for starch accumulation using the developed protocol. GLS affected leaves showed very high amounts of starch (Figure 7) in all leaves tested, while the healthy control leaves showed no starch accumulation. The symptomatic leaf 4 showed starch accumulation despite being green.

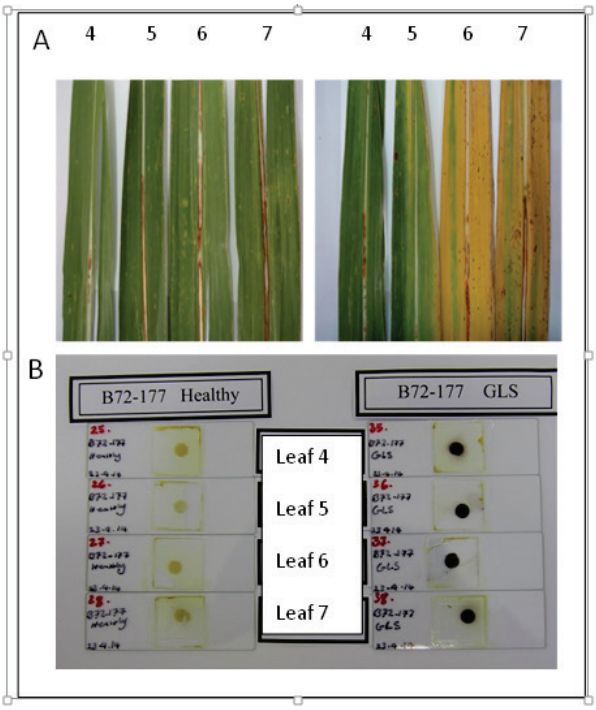

Fig. 7—Leaves of healthy and GLS affected plants of variety B72-177 collected from PNG showing typical golden symptoms and tested in Australia for the presence of starch.

Discussion

Since observing starch accumulation in YCS affected leaves, we have been queried if this response was specific to YCS or was a general response to biotic or abiotic stress.

There has been much speculation whether the causal agent of YCS is abiotic or biotic in nature. While this is still unknown, the response of sugarcane plant to YCS has been studied and more understanding has been gained. YCS seems to affect all varieties, albeit to differing severity, and has been reported in differing soil types and various growing regions, with Mackay region being severely affected in 2015 (Olsen *et al.,* 2015). One distinct characteristic observed in YCS affected leaves is the accumulation of starch.

Du *et al.* (1998) studied the effect of mild and severe water stress on leaf metabolism of sugarcane and concluded that levels of starch in stressed plants was reduced, while sugars were unaffected. These authors concluded that a combination of reduced stomatal conductance and decreased starch synthesis leads to an overall reduction in starch levels in sugarcane plants exposed to water stress.

It has been shown in many crops that biotic stress especially by organisms which reside in the conducting tissues (phloem or xylem), block translocation of assimilates, thus leading to accumulation of non-structural carbohydrates in the leaves (Tesci *et al.,* 1996; McElrone *et al.,* 2003).

In healthy sugarcane plants, during the day, carbon assimilation peaks around 4–5 h after sunrise when the rate of sugars produced is higher than the export of these sugars out of the leaves. This excess sugar is then converted to starch and stored in the chloroplasts of the bundle sheath cells of the leaf. At night, this stored starch is broken down and translocated out of the leaves to the sink tissues, so that by the end of the night period, there is very little starch remaining in the leaves (Lehrer *et al.,* 2007). However, when the leaves are infected with a virus or bacterium which resides in the conducting tissues, the export of the assimilated carbon is hampered, resulting in a build-up of carbohydrates and starch still present after the end of the night period around dawn.

In sugarcane, similar observations have been reported for SCYLV (Yan *et al.,* 2009), where there is a four-fold increase in accumulation of starch in leaves of infected sugarcane.

However, molecular analysis revealed no differences in transcript levels of sucrose phosphate synthase, sucrose transporters or callose synthase between the SCYLV-susceptible and -resistant cultivars and between SCYLV-infected and SCYLV-free sugarcane line (ElSayed *et al.,* 2013). This lead the authors to conclude that the reduction of assimilate export is neither due to lower sucrose transporter levels nor to a physical block of sieve tubes.

There has been some suggestion that the YCS causal agent maybe a bacterium akin to RSD. The accumulation of bacterial polysaccharides, production of gels, gums, and tyloses by the host in response to infection, and/or accumulation of bacterial cell masses that physically clog the elements can result in reduced water uptake and stress-related responses. Reductions in shoot hydraulic conductivity can be caused by embolism (Tyree and Sperry, 1989) or by a reduction in the vessel diameter (Lovisolo and Schubert, 1998).

Both, a physical blockage of the conducting tissues (unpublished data), as well as a reduction in the stomatal conductance and photosynthesis (Basnayake pers. comm.) has been observed in YCS affected plants. *Xanthomonas albineansis* (leaf scald) has been reported to reduce starch in the bundle sheath chloroplasts in infected sugarcane (Legaz *et al.,* 2011) which concurs with our results. The effects of RSD on carbohydrates in the leaves suggest a possible accumulation of starch in the stalk of RSD infected sugarcane (Steindl, 1950); however this author also claimed that it was not sufficiently constant to be of much diagnostic value.

To understand this phenomenon better in diseases of sugarcane caused by different types of micro-organisms, this work was performed as described in this paper. In our experiments, we were unable to show an increased accumulation of starch in response to infection in sugarcane inoculated with any of the known causal agents. In fact, in many of these diseased leaves that we tested, the starch content was reduced, compared to that in healthy plants. Also the two diseases which infect the conducting tissues (RSD and Leaf Scald), did not show enhanced starch accumulation in any of the leaves when compared to the healthy control plants.

The healthy controls were sampled a second time to verify the results of the previous collection, and were found to be almost identical to the first samples collected. None of the known diseases present in the Pathology station showed hyper accumulation of starch, suggesting that this phenomenon in YCS plants is unique to this disorder.

Interestingly, starch did not accumulate in naturally yellowing senescing leaves, which were almost depleted of their starch. This is what one would expect as plants will translocate out all nutrients and metabolites before the leaf is completely dead.

Future work will look at the location and distribution of the accumulated starch within different organs, it's correlation with the development of yellow colour and also study occurrence in different varieties and varying severity during the next YCS season. This will test the hypothesis that starch accumulation precedes yellow colour development and the use of starch accumulation as a potential rapid screening method to confirm the occurrence of YCS.

Acknowledgements

We wish to thank the Sugar Research Australia funding body for funding this project and Mr Barry Croft (SRA, Woodford), Dr Kathy Braithwaite and Ms Elizabeth Wilson (SRA, Indooroopilly) for providing diseased sugarcane leaf samples; Mr Lawrence DiBella of the Herbert Cane Productivity Service Limited (HCPSL), Mr Peter Hanson (SRA Bundaberg) for collection of field grown senescing leaves and Ms Rosa Schafiei (SRA, Indooroopilly) for help with the starch assays.

REFERENCES

Du Y, Nose A, Wasano K, Uchida Y (1998) Responses to water stress of enzyme activities and metabolite levels in relation to sucrose and starch synthesis, the Calvin cycle and the C4 pathway in sugarcane (*Saccharum* sp.) leaves. *Australian Journal of Plant Physiology* **25**, 253–260.

ElSayed AI, Weig AR, Sariyeva G, Hummel E, Yan S-L, Bertolini A, Komor E (2013) Assimilate export inhibition in Sugarcane yellow leaf virus-infected sugarcane is not due to less transcripts for sucrose transporters and sucrose-phosphate synthase or to callose deposition in sieve plates. *Physiological and Molecular Plant Pathology* **81,** 64–73

Legaz M, Blanch M, Piñón D, Santiago R, Fontaniella B, Blanco Y, Solas M-T, Vicente C (2011) Sugarcane glycoproteins may act as signals for the production of xanthan in the plant-associated bacterium *Xanthomonas albilineans. Plant Signaling & Behavior* **6***,* 1132–1139

Lehrer AT, Moore PH, Komor E (2007) Impact of sugarcane yellow leaf virus (ScYLV) on the carbohydrate status of sugarcane: comparison of virus-free plants with symptomatic and asymptomatic virus-infected plants. *Physiological and Molecular Plant Pathology* **70,** 180–188

Lovisolo C, Schubert A (1998) Effects of water stress on vessel size and xylem hydraulic conductivity in Vitis vinifera L. *Journal of Experimental Botany* **49,** 693–700.

McElrone AJ, Sherald JL, Forseth IN (2003) Interactive effects of water stress and xylem-limited bacterial infection on the water relations of a host vine. *Journal of Experimental Botany* **54,** 419–430.

Olsen D, 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.

Steindl DRL (1950) Ratoon stunting disease. *Proceedings of the International Society of Sugar Cane Technologists* **7,** 457–465.

Tecsi LI, Smith AM, Maule AJ, Leegood RC (1996) A spatial analysis of physiological changes associated with infection of cotyledons of marrow plants with cucumber mosaic virus. *Plant Physiology* **11,** 975–985.

Tyree MT, Sperry JS (1989) Vulnerability of xylem to cavitation and embolism. *Annual Review of Plant Physiology and Plant Molecular Biology* **40,** 19–38

Yan S, Lehrer A, Hajirezaei M, Springer A, Komor E (2009) Modulation of carbohydrate metabolism and chloroplast structure in sugarcane leaves which were infected by Sugarcane Yellow Leaf Virus (SCYLV). *Physiological and Molecular Plant Pathology* **73,** 78–87.