UPDATE ON THE OCCURRENCE OF YELLOW LEAF SYNDROME IN THE SOUTH AFRICAN SUGAR INDUSTRY AND EVIDENCE FOR THE CAUSAL ORGANISM

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Abstract

In a survey of the South African and Swaziland sugarcane industries in 1997, symptoms of yellow leaf syndrome (YLS) were found in all areas and in a wide range of varieties. As in previous years, symptoms were present from late autumn to early summer. Symptoms were particularly common in the contrasting areas of the Midlands region of KwaZulu-Natal and the Mpumalanga lowveld, where the majority of stalks in whole fields often had symptoms. In the Midlands, widely grown variety N12, previously thought to have a degree of tolerance, invariably expressed conspicuous symptoms. In Mpumalanga, varieties N19 and N24 had more symptoms than other varieties. The usually wet conditions experienced over most of the industry in the winter months appear to have been conducive to the expression of YLS symptoms.

A total of 265 samples of field grown sugarcane were collected to determine the presence of the putative causal agents of YLS. The phytoplasma ScYP was detected by PCR in 162 of 163 samples with typical symptoms of YLS from all areas. The luteovirus ScYLV, detected by enzyme-linked immunoassay (ELISA), was found to be mainly restricted to certain genotypes undergoing selection in northern KwaZulu-Natal and Mpumalanga. The virus was not detected in commercial varieties in most of the sugar industry. This indicates that ScYLV is not a candidate casual agent for YLS as it commonly occurs in southern Africa. PCR and other evidence for the identity of the phytoplasma is presented.

Keywords: yellow leaf syndrome, phytoplasma, luteovirus

Introduction

Since its first appearance in late 1994, symptoms of yellow leaf syndrome (YLS) have become common in the South African sugar industry. Symptoms have appeared in all commercial varieties and in most parts of the industry. YLS symptoms most commonly appear in maturing cane in the cooler months of autumn to early summer (Bailey *et al.*, 1996). Some varieties appear more likely to develop symptoms than others. For example, variety CP66/1043 invariably develops highly conspicuous symptoms in the winter months, whereas in widely grown variety NCo376 symptoms are relatively mild.

A phenomenon not reported elsewhere is the temporary occurrence of symptoms in some situations in South Africa. It is now expected that symptoms that are often common in most commercial varieties in the cooler months will fade with the onset of better growing conditions in mid-summer, for example when relatively mature cane is 'stood over' to the next milling season (Bailey *et al.*, 1996).

It is now commonplace that YLS symptoms are most common and conspicuous when cane suffers from stress, for example when lacking in nutrients and when grown in very dry or over-wet soils. The sudden appearance of symptoms can also be elicited by borer damage. This sudden appearance of symptoms in many plants in a field and over large areas following stress or damage indicates that YLS is common in most parts of the industry in a latent form.

The causal agent of YLS is not known with certainty. Evidence for the association of a phytoplasma with symptoms of YLS in southern Africa has been presented previously (Cronjé *et al.*, 1997). Further evidence of this association is reported in this paper.

A luteovirus provisionally named sugarcane yellows luteovirus (ScYLV) has been reported to be associated with YLS by workers in the United States (Lockhart *et al.*, 1996). Despite numerous attempts, this virus was not found to occur in South Africa before mid-1997. A specific antiserum and the protocol for an enzyme linked immunoassay (ELISA) for ScYLV developed by BEL Lockhart (University of Minnesota) was made available in 1997 for testing in South Africa. This test was found to reliably detect ScYLV in some imported varieties at SASEX.

This paper reports the occurrence of YLS in the 1997-98 season and the results of a survey in which samples from all parts of the South African and Swaziland sugar industries were tested by a PCR test for the phytoplasma and also by the ELISA test for the luteovirus.

YLS in the 1997-98 season

A survey of all parts of the South African and Swaziland sugar industries was conducted from August to October 1997. Symptoms of YLS are most common and conspicuous at this time of year. As in previous years, symptoms were found in all parts of the two industries. One major difference from previous seasons was that YLS symptoms were found to be widespread and common in widely grown variety N12 in the Midlands region of KwaZulu-Natal. In this area symptoms appeared suddenly in late July and persisted for only a few weeks. Within this period the symptoms were very common over large areas, often appearing in the majority of stalks over

entire fields. This gave a predominantly yellow appearance to the cane in most fields in the region. Previous to this, symptoms had been rare in N12.

Symptoms were also common in the geographically distant and bioclimatically distinctly different region of the Mpumalanga lowveld. In this area were common in all the main varieties, N14, N19 and N24.

A feature throughout the South African sugar industry was that symptoms were often most common and conspicuous in the wetter parts of fields. It seems likely that the widespread, first appearance of symptoms in the Midlands region during 1997 was due to there being more rainfall than usual over the winter months, so that conditions were wetter than usual, as well as being cool.

Symptoms in all commercial varieties in all areas faded after the cooler months. Towards early summer, the only varieties in which symptoms persisted were some apparently highly susceptible varieties undergoing selection in trials and some foreign varieties.

Materials and methods

Survey and sampling

All extension areas of the sugar industry were visited during August to October 1997, when symptoms of YLS were most common. Leaves with and without symptoms of YLS were collected from cane of different varieties and ages and from different growing conditions. Leaves were collected from positions 3-5 on both symptomatic and asymptomatic stalks, counting leaf 1 as the uppermost fully unfurled leaf. One leaf was collected per stalk and each sample consisted of a total of four to five leaves from different stalks. The leaves were cut transversely into 1mm strips, including the midrib, and frozen at –85°C for several hours to overnight. The frozen samples were ground to a fine powder in a coffee grinder, using dry ice as a coolant and to facilitate grinding. The ground samples were divided into two equal amounts and processed further for ScYLV and ScYP detection.

ScYLV detection

Samples of 2 g of ground leaf sample were suspended in 100 mM phosphate buffer (pH 6,0), containing 1% Na_2SO_3 and 0,05% Tween-20. The samples were resuspended by stirring thoroughly, and the resultant slurry filtered through cheesecloth. The samples were then transferred to microtitre plates coated with ScYLV antibodies. Samples were repeated twice or more per plate, and a known positive control was added to each test.

ELISA

The ELISA protocol and antibodies used were supplied by BEL Lockhart (University of Minnesota, USA). The protocol was used unchanged. The results were read on a Bio-Rad 550 plate reader 4 and 22 hours after adding the susbstrate. Readings two times higher than the background reading of healthy leaf samples on the plate were deemed positive for ScYLV.

ScYP detection

DNA Extraction

For the detection of ScYP, 10 g of powdered leaf was resuspended in 50 ml of extraction buffer. This buffer contained 100 mM K_2HPO_4, 31 mM KH_2PO_4, 0,3 M sucrose, 0,15% bovine serum albumin (fraction V), 2% polivinylpyrrolidone, 10 mM EDTA and 30 mM ascorbic acid, pH 7,4). The samples were stirred thoroughly to resuspend and then filtered through cheesecloth. The filtrate was clarified by centrifugation (3 000 x g, 5 min), and the supernatant transferred to clean tubes, which were then centrifuged at 20 000 x g for 35 min. Nucleic acids were extracted from these pellets by the procedure of Dellaporta *et al.* (1983), and finally resuspended in TE buffer (10 mM Tris-HCl, pH 8,0, 1 mM EDTA) containing 50 mg/ml RNAse and incubated at 37°C for one hour. DNA solutions where stored at –20°C until use.

PCR

Several universal primer pairs designed for the amplification of the 16S-rDNA region of phytoplasmas were tested. The combinations finally selected were the Asian Forward and Asian Reverse (SN910601, SN910502, Namba *et al.*, 1993) primers for general 16S-rDNA amplification, and LD16-1 (Harrison *et al.*, 1994) in combination with Asian Reverse for specific amplification of Western X phytoplasma. All samples were tested as standardised 25 µl reactions containing 2,5 µl 10x buffer (Geneamp PCR buffer, 100 mM Tris-HCl, pH 8,3, 500 mM Kcl, 15 mM $MgCl_2$, Perkin-Elmer South Africa (Pty) Ltd, Johannesburg), 0,2 µl of a 20 mM dNTP solution (Boehriger Mannheim, Johannesburg), 0,5 µl of a 10 mM solution of each primer and 0,125 µl Tag polymerase (Amplitaq Gold, 5 U/µl, Perkin-Elmer South Africa (Pty) Ltd, Johannesburg). Sterile double distilled water was added to this solution to a final volume of 24 µl. One µl of template DNA was added to the mix. The cycling protocol used with the Namba primers was as follows: 95°C for 12 min (1 cycle); 94°C for 30 sec, 54-58°C for 1 min, 72°C for 1 min 30 sec, 35 cycles; 72°C for 10 min, 1 cycle. PCR products were electrophoresed on 1% agarose gels containing ethidium bromide. Gels were viewed on a UV transilluminator.

RFLP

Aliquots of 5 µl of each amplimer were cut with restriction enzymes *RsaI* and *HaeIII* (Boehringer Mannheim), using the appropriate incubation buffers. Samples were incubated at 37°C for 2 h, and then electrophoresed through 1,5% agarose gels, and the bands visualised with a transilluminator as before.

Sequence analyses

Some of the PCR products were exised from the gel and the DNA recovered with a Quiagen extraction kit using the recommended protocol. The recovered DNA was then prepared for sequencing on a ABI-Prism 310 automated sequencer, using the protocol supplied by the manufacturer.

The sequences were compared with those of other phytoplasmas found in the GENBANK database using the BLASTN programme (Altshul *et al*, 1990).

Results

ScYLV detection

ScYLV was detected by ELISA in a number of samples by the ScYLV antibodies, and the OD values of positive samples were usually substantially higher than background reactions after 22 hours incubation. The virus was present in 19,7% of all samples tested (Table 1). These samples included a few new local genotypes undergoing selection, certain imported varieties in second stage quarantine, and several commercial varieties (Table 2).

Commercial varieties N28 and N30 were always infected by ScYLV, irrespective of where the samples originated in the industry. Varieties N24, NCo376, N19 and N14 were occasionally found infected with ScYLV at some locations in northern KwaZulu-Natal and Mpumalanga but not in the southern part of the industry. In 11 of 18 commercial varieties with YLS symptoms, including N12, ScYLV was detected.

Table 1.Occurrence of sugarcane yellows phytop[lasma (ScYP) and sugarcane yellow leaf virus (ScYLV) in leaf samples from field grown sugarcane with and without symptoms of YLS (numbers of samples, excludes experimental material).

Area	Samples with symptoms					Samples without symptoms				
	Total	ScYP+ ScYLV+	ScYP only	ScYLV only	Neither	Total	ScYP+ ScYLV+	ScYP only	ScYLV only	Neither
South Coast	11	0	11 (3F)	0	0	12	0	9 (4F)	0	3
Midlands	19	0	19	0	0	17	0	9 (3F)	0	8
North Coast	36	1*	35 (15F)	0	0	8	2	2 (1F)	0	4
Zululand	21	0	21 (10F)	0	0	2	0	1 (1F)	0	1
Northern	51	23 (8F)	27 (9F)	0	1	54	19 (9F)	25 (12F)	4	6
Swaziland	25	2	23 (7F)	0	0	9	1 (?)	6 (6F)	0	2
Totals	163	26 (8F)	136 (44F)	0	1	102	22 (9F)	52 (27F)	4	24

(*F) = Faint PCR product (positive RFLP)
(?) = Uncertain ELISA positive

ScYP detection

PCR

PCR products of the expected size (1 360 bp) for ScYP were amplified from samples collected throughout the industry. It was observed that in those samples where ScYLV was also present, the PCR products were often faint and in some cases could only be seen after a second PCR using a semi-nested primer combination. In most cases only ScYP was amplified using the Namba *et al.* (1993) primer pair, but some of the other bacteria found in cane were also occasionally amplified. The second primer combination of LD16-1 (Harrison *et al.*, 1994) with Asian reverse (Namba *et al.*, 1993) consistently yielded products that were only associated with the 16S-rDNA genome of ScYP.

The phytoplasma was detected in all varieties tested, and could be linked to symptom expression in all cases (Table 2).

RFLP

Restriction fragment length polymorphism analyses of the products generated by PCR indicated that ScYP is closely related to the Western X group of phytoplasmas, and is distinctly different from other phytoplasmas previously found in sugarcane, namely sugarcane white leaf (SCWL) and grassy shoot disease (GSD) (Figure 1).

Sequence analyses

Sequence analyses of the PCR products generated with the various primer combinations indicated a 98%+ homology of ScYP with the Western X group of phytoplasmas and lower homologies with other groups of phytoplasmas. There was a 89% homology of ScYP with the SCWL and GSD group, and ScYP is clearly a different phytoplasma to these.

Discussion

ScYLV

The virus was found in symptomatic as well as asymptomatic cane leaves of several varieities. However, in many of the virus positive cases there was no close association between presence of virus and YLS symptoms. Serological tests failed to detect ScYLV in most cane samples with YLS symptoms in southern and central KwaZulu-Natal, although YLS symptoms were common throughout these areas.

It was observed that where ScYLV was readily detected, the symptoms of YLS were often mild or even absent when compared with varieties grown in close proximity where only the phytoplasma could be detected (see N28 compared with N14 in Table 2).

Data collected so far indicate that ScYLV has only recently been introduced into South Africa and it is possible that it

passed undetected through quarantine. It seems possible that varieties imported recently from the USA, Mauritius and Zimbabwe were the source of this introduction.

ScYP

The phytoplasma was detected in 162/163 symptomatic samples and in 74/102 asymptomatic samples, which indicates there is substantial latent infection of sugarcane by the phytoplasma. We found that the extraction of DNA from ScYLV infected samples and the subsequent PCR of such samples often failed to detect the phytoplasma in the first round of PCR, and a nested PCR approach had to be used for amplifying products. These observations indicate a degree of competition between the pathogens.

Table 2. Summary of the occurrence of ScYP and ScYLV in commercial varieties in the South African sugar industry.

Variety	Total samples	Samples with YLS symptoms	ScYLV	ScYP
CP66/1043	4	4	0	4
N12	51	29	0	32
N14	18	14	1	16
N16	14	11	0	13
N17	2	1	0	1
N19	24	13	4	18
N21	4	2	0	3
N22	9	6	0	9
N23	2	2	0	2
N24	13	11	7	12
N25	4	1	0	3
N26	3	1	1	2
N27	5	3	0	4
N28	10	2	10	3
N29	3	1	0	2
N30	3	0	3	1
N31	1	1	0	1
NCo376	40	23	5	34

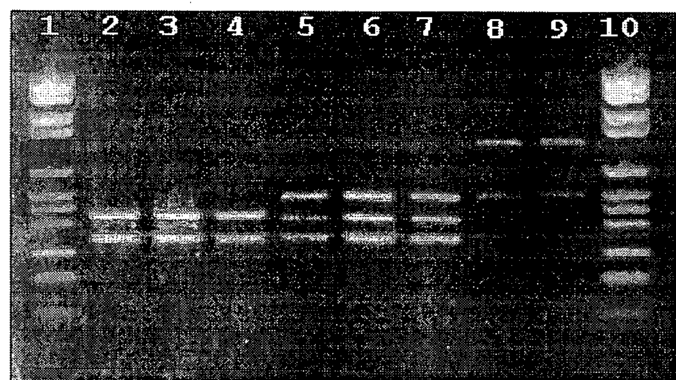

Figure 1. RFLP analyses of products generated with the Namba *et al.* (1993) primers from DNA samples of several phytoplasmas using *Rsa* 1 as cutting enzyme. Lanes 1, 10: molecular weight marker VI (Boehringer Mannheim); 2: stolbur; 3: chlorante; 4: clover phyllody; 5: sugarcane variety N27 with YLS; 6: Western X; 7: green valley X disease; 8: sugarcane white leaf disease; 9: sugarcane grassy shoot disease.

Conclusions

Our results suggest that the phytoplasma ScYP is the primary pathogen associated with YLS throughout the South African and Swaziland sugar industries. The virus ScYLV occurs in some varieties, but has a limited distribution and has not been found in the southern part of the industry. Much remains to be learned about the etiological aspects of YLS, but it is possible that there is an interaction between the ScYLV and ScYP.

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