MAURITIUS RESEARCH COUNCIL

DETECTION OF THE PHYTOPLASMA ASSOCIATED WITH YELLOW LEAF SYNDROME OF SUGAR CANE

Final Report

April 2002

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MAURITIUS SUGAR INDUSTRY RESEARCH INSTITUTE

Title of project : Detection of the phytoplasma associated with yellow leaf syndrome of sugar cane

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GENERAL INTRODUCTION

In the 1960s a new symptom known as yellow wilt was observed in sugar cane in Tanzania. These symptoms were then found to be widespread in Central and East Africa. (Ricaud, 1968; Rogers, 1970). No pathogen was at that time associated with yellow wilt. Several years later yellow leaf syndrome (YLS) which has similar symptoms as yellow wilt of sugar cane was described in Hawaii (Schenck, 1990; Schenck and Hu, 1991). YLS is characterized by the yellowing of the midrib. The colour gradually extends to the leaf blade and is sometimes accompanied by a shortening of the upper internodes, producing a fan-like appearance. Thereafter YLS was reported in 34 countries (Lockhart and Cronjé, 2000). The syndrome was found to be transmitted by vegetative propagation (Schenck, 1990) suggesting that a pathogen was involved. Subsequently, a new luteovirus- *Sugarcane yellow leaf virus* (SCYLV)- was found to be associated with YLS in several countries. Furthermore Cronjé *et al*. (1996, 1998) during their search for the SCYLV, found electron microscopic evidence for phloem-inhabiting phytoplasma-like bodies.

Phytoplasmas, previously known as mycoplasma-like organisms (MLOs) were first discovered in the late 1960s by Doi *et al.* (1967) as wall-less microbes in electron micrographs of mulberry tissue affected by the "yellows" disease, mulberry dwarf. They were called "mycoplasma-like organisms" (MLOs) because of their morphological similarity to the wall-less mollicutes mycoplasmas, known to cause numerous disorders of human and animals. Recent evidence showing that MLOs are only distantly related to animal mycoplasma led to their designation as "phytoplasma" a name that reflects their primary plant hosts (Sears and Kirkpatrick, 1994). Phytoplasmas have been found to be associated with diseases of several hundred plant species (McCoy *et al*., 1989; Kollar *et al.*, 1990).

Cronje *et al*. (1998) presented evidence of phytoplasmas in sugar cane with symptoms of YLS based on PCR amplification of the 16S rDNA of the organism, RFLP analysis of the PCR products, scanning electron microscopy and transmission

studies. These authors further distinguished two groups of the phytoplasma (which they named *Sugarcane yellows phytoplasma* - SCYP I and SCYP II), present in both YLS symptomatic and asymptomatic field grown sugar cane. They also found poor association between the presence of SCYLV and YLS in field grown sugar cane varieties in eight African countries and 12 countries outside Africa.

In Thailand two important sugar cane diseases, sugar cane white leaf (SCWL) and sugar cane grassy shoot (SCGS), are caused by phytoplasma (Wongkaew *et al*., 1997). Analysis of the RFLP pattern and of the PCR product of the 16S rRNA and the sequence data of the spacer region between the 16S rRNA and the tRNA (Ile) showed that the SCWL and the SCGS diseases are caused by two different phytoplasmas. On the other hand sequences obtained from the intergenic spacer region between the 16S and the 23S rDNA genes of the ScYP I and ScYP II confirmed the identity of the phytoplasma as belonging to the Western X and SCWL phytoplasma groups (Cronje *et al.*, 1998).

In Mauritius no phytoplasma disease was recorded in sugar cane until recently. YLS was first observed in 1994 on variety CP 721210 imported from Florida, USA. Symptoms were also observed in local varieties a few months later. The presence of SCYLV was confirmed in 1996 and that of the phytoplasma in 1997.

This study was undertaken to investigate the distribution of the two pathogens associated with YLS, particularly the phytoplasma for which the incidence was unknown in Mauritius. The phytoplasma was detected by polymerase chain reaction (PCR) and the virus by reverse transcriptase PCR (RT-PCR). An attempt was made to relate symptoms to the presence of either or both pathogens. Strain variation in the phytoplasma was studied using PCR and RFLP analysis of the phytoplasma 16S rDNA and PCR products were cloned and sequenced using an ABI 310 DNA analyser. Tissue culture techniques were investigated to free diseased material from YLS.

MATERIALS AND METHODS

Plant material for the detection of SCYP

Leaf samples were collected from

1. imported sugar cane varieties grown in quarantine
2. a germplasm field collection available at Réduit and
3. commercial varieties grown islandwide.

DNA extraction and PCR

Genomic DNA was extracted from both symptomatic and asymptomatic leaves following the method of Harrison *et al*. (1994). Five grams of mature leaf tissue were cut into 2 mm strips and ground to fine powder in liquid nitrogen. The extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA pH 8.0, 100 mM Tris-HCl, 0.2% 2-mercaptoethanol) was pre-warmed to 65°C before grinding the leaf samples. Four milliliters per gram of leaf tissue were added to each sample and incubated at 65°C in a water bath for 60 min. The tubes were then cooled to room temperature and an equal volume of chloroform:isoamyl alcohol (24:1) was added, mixed well and centrifuged at 4000 *g* for 10 min. The upper layer was recovered and an equal volume of isopropanol and 5 ml of 5 M NaCl were added to each sample, mixed and incubated at –20°C for 2 h. Genomic DNA was spooled off, or precipitated at 3000 *g* for 10 min, air-dried and resuspended in sterile doubled distilled water.

DNA was also extracted from the stem of varieties M 695/69, CP 721210 and S 17 as follows: a middle node was selected and the outer rind was removed. The node was thinly diced and ground in a coffee grinder using liquid nitrogen. A separate blender was used for each sample. DNA was then extracted from the ground powder using the CTAB DNA extraction buffer.

DNA was amplified from the rRNA operons (Figure1) by a general amplification step followed by a nested amplification step. Three universal primer pairs were tested for the first round, P1/P7, SN910601/P6 and R16mR1/R16mF2. Primer pairs R16F2n and R16R2 were used for the nested round (Table 1).

Figure 1. Diagrammatic representation of a phytoplasma rRNA operon including the 16S and 23S rRNA genes and the intergenic spacer region. The direction and position of oligonucleotide primers used in PCR analysis are represented by arrows.

Primer	Sequence	Reference
P1	5'-AAGAGTTTGATCCTGGCTCAGGATT-3'	Deng and Hiruki (1991)
P7	5-CGTCCTTCATCGGCTCTT –3'	Smart *et al*. (1996)
SN910601	5'-GTTTGATCCTGGCTCAGGATT-3'	Namba *et al* (1993)
P6	5'-CGGTAGGGATACCTTGTTACGACTTA	Deng and Hiruki (1991)
R16mF2	5'-CATGCAAGTCGAACGGA-3'	Gundersen and Lee (1996)
R16mR1	5'-CTTAACCCCAATCATCGAC-3	Gundersen and Lee (1996)
R16F2n	5'-GAAACGACTGCTAAGACTGG-3'	Lee *et al* (1993)
R16R2	5'-TGACGGGCGGTGTGACAAACCCCG-3'	Lee *et al* (1993)

Table 1: Sequence of universal primers used in the amplification of the phytoplasma 16S rRNA operon.

A standard 25 µl reaction mixture consisted of the following: reaction buffer 1x (Boehringer Mannheim, BM), 200 µM dNTP (BM), forward and reverse primers (0.5 nM), 0.3 units *Taq* polymerase (BM) and total nucleic acids (100 ng). Alternatively, lyophilized PCR beads (Pharmacia Biotech) containing buffer, dNTPs and *Taq* polymerase were used for PCR reactions. One µl (or a dilution) of the first round amplicon was used as template for the nested round.

PCR conditions for the general amplification were as follows: an initial denaturation step at 94ºC for 3 min followed by 35 or 40 cycles of 94ºC for 50 s; 53ºC for 1.5 min and 72ºC for 1.5 min and a final extension step of 72ºC for 10 min. Nested amplification was carried out as follows: an initial denaturation step of 3 min at 94ºC, 35 cycles of: 94ºC for 50 s, 54.5ºC for 1min 30 s and 72ºC for 1 min 30 s and a final extension of 10 min at 72ºC.

Tubes with reaction mixture devoid of DNA templates were included as negative controls. PCR products were separated by electrophoresis on 1% agarose gel followed by staining in ethidium bromide. DNA bands was visualized using a UV transilluminator.

PCR conditions were optimized by changing the DNA concentrations, diluting the 1st round amplicon by varying the number of first round cycles and magnesium ion concentrations.

RFLP analysis of PCR products

The 16S rDNA amplified by PCR using the general primers R16mF2 and R 16mR1 followed by the nested primers R16F2n and R16 R2 were analyzed after restriction endonuclease digestion. Five microliters of each PCR product were digested separately with the restriction enzyme *Rsa*I. This enzyme was used because it is not a frequent cutter, and distinguishes between the different groups of phytoplasmas.

The following phytoplasmas groups (1-6) were also amplified in a single step PCR using primer pairs R16F2n and R16R2: European Aster Yellow (group 1), Faba Bean Phyllody (group 2), Peach Yellow Leaf Roll (group 3), Coconut Lethal Yellow (group 4), European Elm Yellow (group 5) and Tomato Big Bud (group 6). These were digested together with amplified PCR products of the phytoplasma from sugar cane.

The digested products were separated by electrophoresis on either 2% agarose or 6% polyacrylamide gels, stained with ethidium bromide and visualized under UV light. RFLP profiles of the sugar cane samples were compared with group specific phytoplasmas 1-6.

Transformation of *E. coli*

F2n/R2 amplified products from three samples showing different *Rsa*I RFLP profiles were selected as insert. PCR products were ligated into linearised pGEM-T Easy vector, then cloned into competent *E coli* cells. The following protocol was adopted: the nested PCR products were run on 1% low melting point agarose, stained with ethidium bromide and visualized under UV light. The 1250 bp band from each sample was carefully sliced from the gel avoiding contamination between samples. DNA was extracted from the gel using the Qiagen spin kit 50. Cleaned DNA was run against a molecular weight ladder for estimation of DNA concentration. The ligation reaction mixture (10 µl) was set up with the following; 2x T4 ligation buffer (5 µl), pGEM-T Easy Vector (50 ng), PCR insert (20 ng) and T4 DNA ligase (3 Weiss units). The reaction was allowed to proceed at 4ºC overnight for maximum ligation. High efficiency competent *E.coli* cells (JM 109) were used for cloning the ligated vector. The ligation mixture (2 µl) was transferred into three 1.5 ml microcentrifuge tubes and kept on ice. Frozen JM 109 cells were placed in an ice bath until just thawed then mixed by gently flicking the tube. The cells (50 µl) were transferred into tubes containing the ligation mixture and gently flicked to mix then returned on ice for 20 min. They were finally heat shocked for 45-50 s in a water bath at exactly 42ºC then placed again on ice for 2 min. The transformed cells were then mixed with 950 µl SOC medium (per litre: tryptone, 20 g; yeast-extract, 5 g; NaCl, 0.5 g; glucose, 3.6 g) and incubated for 1.5 h at 37ºC with shaking. Each transformation culture (100 µl) was then plated onto duplicate LB/ampicillin/IPTG/X-gal plates. The plates were incubated at 37ºC overnight (16 h) until the appearance of white/blue colonies.

Detection of insert from transformed colonies

PCR reaction mix (25 µl) with F2n/R2 primers were set up as described earlier. Individual colonies were scraped using a sterile tooth pick and transferred into the

PCR tubes and amplified. PCR products were run on 1% agarose. Positive PCR products were digested with *Rsa*I, separated on 2% agarose and their RFLP profile analyzed.

Plasmid extraction and sequencing

A single white colony tested positive for the insert was selected from each plate and inoculated into tubes containing LB (1.5 ml). The cells were grown overnight at 37ºC, then spun down. Plasmid was extracted from *E. coli* by the Qiaprep plasmid DNA extraction kit (Qiagen) and gel quantified. Sequencing PCR reaction mix (one tube per primer) were set up as follows; 8 µl Terminator ready reaction mix (Perkin Elmer), 200 ng plasmid, primer F2n or R2 (3.2 pmol) and sterile deionised water to make up to a total volume of 20 µl. The reagents were mixed and overlaid with one drop of mineral oil. Amplification was carried out using the following program: 94ºC, 2 min and 25 cycles of 94ºC for 30 s, 50ºC for 15 s and 60ºC for 3.5 min. The amplification products were precipitated in 2 µl sodium acetate (3 M) and 50 µl ethanol (95%). The precipitate was pelleted and washed with ethanol (70%). The pellet was dried in a fume cupboard, then heated at 85ºC for 1 min. The preparation was then used for sequencing on a ABI 310 DNA analyzer.

***In situ* localisation of phytoplasma by DAPI**

Young cane tops were used as starting material for the *in situ* localization of phytoplasma by 4', 6-diamidino-2-phenylindole-2 HCl (DAPI). This technique involves six steps i.e fixation of the tissue, dehydration, infiltration, embedding, sectioning and staining. *Catharantus roseus* stem infected with Green Valley X phytoplasma was used as positive control.

The fixative, 4% paraformaldehyde in PBS (0.13 M NaCl, 0.007 M Na_2HPO_4 and 0.003 M NaH_2PO_4) was prepared under a fume hood and the pH adjusted to 7.0 with H_2SO_4. Fresh tissues were thinly cut into Petri dishes, then quickly transferred into Bijou bottles containing the fixative. The tissues were maintained in

paraformaldehyde at 4ºC for 1 week. To remove the fixative, the tissues were rinsed in 0.85% saline (8.5 g/l NaCl) then left in fresh saline (0.85%) for 30 min.

The following solutions (1:1 v/v) were successively used for the gradual dehydration of the tissues: 50% ethanol/0.85% saline, 70% ethanol/0.85% saline, 85% ethanol/0.85 saline, 95% ethanol/distilled water and absolute ethanol. Dehydration was carried in the cold room with 90 min incubation at each step. The tissues were finally maintained in absolute ethanol overnight at 4ºC. A last dehydration step with absolute ethanol was carried out the next day for 2 h at room temperature.

Infiltration was carried out in the fume hood at room temperature with an initial one hour 50% ethanol/50% Histoclear (Sigma) incubation step followed by three 1h 100% Histoclear incubations. Finally the tissues were incubated overnight in 50% Histoclear/50% molten Paraplast wax (Sigma) at 60ºC in an oven.

The 50/50 solution was replaced with molten wax (100%) in the oven and changed twice daily for 4 days. Once embedded, the tissues were placed in the centre of moulds previously coated with glycerol and freshly prepared molten wax was poured over. The samples were allowed to set in the wax at room temperature. Once hardened, the wax block was fixed on a microtome disc using some molten wax. The block was trimmed and mounted on the microtome. The tissues were then cut in 10 µm sections and the ribbon allowed to float on a water bath at 30ºC. This allows the ribbon to relax and spread out flat. The sections were then transferred to poly-1-lysine coated slides also pre-warmed at 30ºC. An overnight incubation at 50ºC was used to fix the tissues on the slides. The tissues were stained with DAPI and examined by florescence microscopy. Stained sections were photographed using 800 s films with exposure times varying from 5-30 min

Detection of SCYLV using RT-PCR

SCYLV was detected by RT-PCR using primers specific to the coat protein region of the virus. Total nucleic acids extracted from the youngest fully unrolled leaf.

Amplification was carried out using primer set YLS 462 and YLS 111 (US Sugar Corporation, USA). Sugar cane total nucleic acids samples and primer YLS 462 were boiled for 5 min, quenched on ice and reverse transcribed at 42^{o}C for 15 min. followed by denaturation at 99^{o}C for 5 min. Amplification was carried out as follows; 94^{o}C 1 min, 54^{o}C 1 min, 72^{o}C 20 min, 1 cycle; 94^{o}C 1 min, 54^{o}C 1 min, 72^{o}C 2 min, 40 cycles and a final elongation at 72^{o}C for 10 min. RT-PCR products were electrophoresed on 1.8% agarose gel, stained with ethidium bromide and photographed under UV light.

Elimination of SCYLV and SCYP by tissue culture

Two susceptible interspecific commercial varieties M 1658/78 and M 695/69 and nine noble cane (*Saccharum officinarum*) clones infected by SCYLV were used to optimize tissue culture conditions. Three explants were evaluated and consisted of young leaf rolls, axillary bud and meristem tip. Callus was produced from young leaf rolls, about 5 mm thick, cultured in the dark on callus induction medium (MS salts - Murashige & Skoog, 1962), Gamborg's B5 vitamins (Gamborg, 1968), 3.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 6.7 g/L glucose, 13.3 g/L sucrose and 1.8 g/L phytagel). Embryogenic callus was subcultured at monthly intervals and was regenerated on the same medium devoid of 2,4-D. To determine the minimum number of callus subcultures required for the elimination of the virus, plantlets were regenerated at each subculture. Axillary bud and apical meristem were cultured on modified MS medium.

Forty three varieties in quarantine found to be infected with either SCYLV or SCYP or with both pathogens were induced to produce callus *in vitro* from young leaf rolls. Nineteen varieties that successfully regenerated into plantlets were tested for the absence of SCYLV and SCYP by RT-PCR and PCR respectively. Rooted plantlets were transferred to the glasshouse and checked for both pathogens after three and twelve months.

RESULTS

Detection of SCYP by PCR

Of the 134 samples obtained from the various sources (quarantine, variety collection and commercial fields) and tested for the presence of the phytoplasma by PCR, 63 (48.8%) tested positive and produced a specific 1250 bp fragment of the phytoplasma 16S rDNA using the universal primers SN 910601/P6 combination and the nested primers R16F2 and R16R2 (Tables 2 and 3). The PCR products were amplified from symptomatic and asymptomatic varieties. Figure 2 shows an agarose gel electrophoresis of the PCR product of the 1250 bp fragment of the phytoplasma 16S rDNA. There was a positive significant correlation ($r = 0.36$) between the presence of the phytoplasma and the symptom where 47 symptomatic samples produced the 1250 bp PCR product. These results are in agreement with Cronje *et al*. (1998) in South Africa and Arocha *et al*. (1999) in Cuba where phytoplasma was highly correlated with YLS. On the other hand 16 asymptomatic samples tested positive for phytoplasma. Twenty-nine symptomatic varieties tested negative for phytoplasma of which 18 were positive for SCYLV (Tables 2 and 3) as revealed by RT-PCR. This demonstrates the difficulty of determining the identity of the YLS pathogen based on symptomatology alone.

M1 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 M2

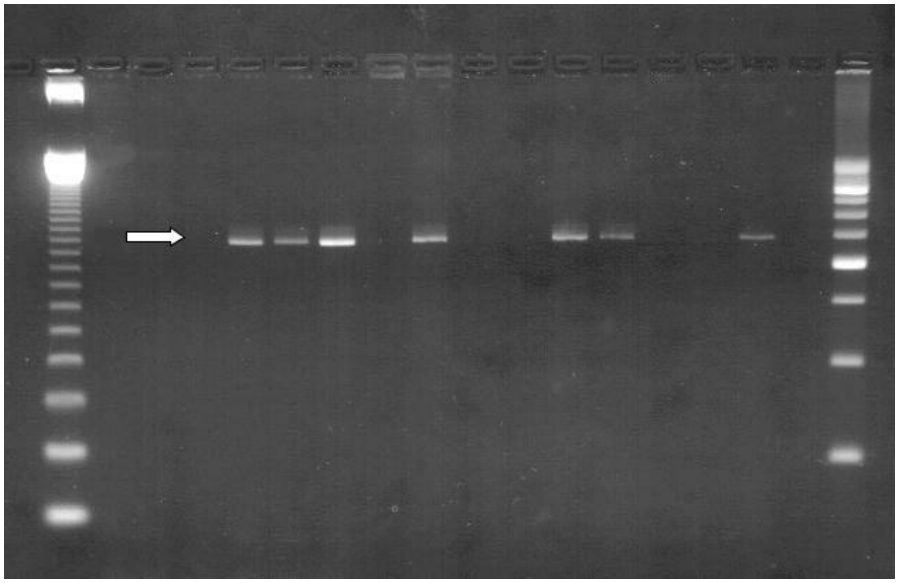

Figure 2:Polymerase chain reaction amplification of 16S rDNA sequence of SCYP using nested primers R16F2n and R16R2. A product of 1250 bp (arrow) in lanes 4-6, 8, 11-12 and 15 indicates presence of SCYP. M1 & M2: 123 bp and 250 bp ladder molecular weight markers.

Table 1. Detection of sugarcane yellow leaf virus and sugarcane yellows phytoplasma by RT-PCR and PCR in 134 samples derived from 113 varieties.

Sample No	*Variety	RT-PCR	PCR	Presence of symptoms	Sample No	*Variety	RT-PCR	PCR	Presence of symptoms
1	CC 8226	+	+	+	71	M 587/70	+	-	+
2	CC 8596	+	+	+	72	M 744/70	+	-	-
3	CC 8527	+	+	+	73	R 570	+	+	+
4	CC 8227	+	+	+	74	M 2597/79	-	-	+
5	CC 8215	+	-	+	75	M 554/79	+	-	+
6	CC 8592	-	+	-	76	M 2077/78	-	-	+
7	R 832089	-	-	-	77	M 2343/77	+	-	+
8	R 830288	+	+	-	78	M 1197/77	+	+	+
9	R 831592	+	+	+	79	M 1176/77	-	+	-
10	R 830395	+	-	-	80	M 937/77	+	-	+
11	R 840075	+	-	-	81	M 134/75	+	-	+
12	R 841289	+	+	+	82	M 1722/71	+	+	+
13	R 832065	+	+	+	83	M 1030/71	+	+	+
14	R 830547	+	+	+	84	M 1682/70	+	-	-
15	R 830680	-	-	-	85	SP 713501	+	-	-
16	R 840653	+	-	-	86	NCO 310	+	+	+
17	R 832276	+	+	+	87	M 1342/84	-	-	-
18	G 8537	+	-	-	88	M 1300/84	+	-	-
19	G 75368	+	-	-	89	M 5/83	+	+	+
20	G 8758	-	-	+	90	M 1412/32	+	+	+
21	G 8737	-	-	+	91	M 784/82	+	-	-
22	N 27	+	-	+	92	M 175/82	+	+	+
23	ROC 15	+	-	-	93	M 36/82	+	+	+
24	Q 136	-	-	-	94	M 33/82	+	+	+
25	Q 159	+	+	+	95	M 3/82	+	-	+
26	Q 154	-	-	+	96	M 1551/80	-	-	+
27	Q 135	-	+	+	97	RP 8068	-	+	+
28	Q 151	-	-	-	98	ROC 5	+	+	+
29	Q 138	-	-	+	99	ROC 2	+	+	+
30	Q 155	+	-	-	100	ROC 1	-	+	+
31	Q127	+	-	-	101	PB 739067	-	-	-
32	MEX 70485	-	-	-	102	R 575	+	+	+
33	SP 803280	+	-	-	103	Q 96	+	-	-
34	SP 80185	+	+	-	104	Q 72	+	+	+
35	ROC 14	+	+	+	105	CP 742005	+	+	+
36	ROC 13	+	+	-	106	RB 705051	+	-	+
37	MEX 801410	+	-	-	107	R570	+	-	+
38	MEX 68134	-	-	+	108	M 1658/78	+	+	+
39	MEX 79431	-	-	-	109	M 3035/66	-	+	-
40	MEX 73206	-	-	+	110	M 695/69	+	-	-
41	SP 803390	+	+	+	111	R570	+	-	+
42	SP 792233	+	+	+	112	M 1557/70	+	+	+
43	SP 801520	+	+	+	113	M 1551/80	+	-	-
44	S 17	+	+	+	114	M 1658/78	+	+	+
45	CO 527	+	-	+	115	M 52/78	-	+	-
46	CO 976	+	+	-	116	M 555/60	+	-	-
47	CO 1208	+	-	+	117	R570	+	-	+
48	CP 62258	-	-	+	118	R570	+	+	-
49	CP 65357	+	-	+	119	R570	+	+	-
50	CP 67412	+	+	+	120	M 1658/78	+	-	-
51	CP 70321	-	+	+	121	M 1557/70	+	-	-
52	CP 72355	-	-	-	122	M 1658/78	+	-	-
53	CP 72370	+	+	+	123	M 1658/78	+	+	-

54	CP 79318	+	-	+	124	M 3035/66	+	-	-
55	CP 681026	-	-	+	125	M 52/78	-	-	-
56	CP 681067	-	+	-	126	M 695/69	+	+	-
57	CO 975	+	-	-	127	R570	+	-	-
58	CP 701527	-	+	+	128	R570	-	-	-
59	CP 721210	+	+	+	129	R573	-	-	-
60	F 149	+	+	+	130	1658/78	+	-	-
61	F 166	+	+	+	131	M 96/82	+	-	-
62	F 170	+	-	-	132	M 695/69	+	-	-
63	F 172	+	-	-	133	R570	+	+	-
64	H 328560	+	-	+	134	M 695/69	+	+	-
65	H 605657	+	+	+					
66	H 699092	-	+	+					
67	M 13/56	+	+	-					
68	M 555/60	+	+	+					
69	M 574/62	+	+	+					
70	M 527/68	+	-	+					

*Source of material: samples 1-43 (quarantine)
44-106 (variety collection plot)
107-134 (commercial fields island wide)
RT-PCR detects sugarcane yellow leaf virus and PCR detects sugarcane yellows phytoplasma
+ = detection positive; - = not detected

Samples	Total	Sy + V + Ph +	Sy + V + Ph –	Sy + V - Ph +	Sy + V - Ph -	Sy – V + Ph +	Sy – V + Ph –	Sy – V – Ph +	Sy – V – Ph –
Quarantine	**43**	**14**	**2**	**1**	**6**	**3**	**10**	**1**	**6**
Variety collection plot	**63**	**24**	**13**	**5**	**5**	**2**	**9**	**2**	**3**
Commercial fields	**28**	**3**	**3**	**0**	**0**	**6**	**11**	**2**	**3**
Total	**134**	**41**	**18**	**6**	**11**	**11**	**30**	**5**	**12**

Table 2. Summary results of survey for yellow leaf syndrome. Samples were tested for sugarcane yellows phytoplasma (SCYP) and sugarcane yellow leaf virus (SCYLV). Sy+: sugarcane leaves with YLS symptoms; Sy-: sugarcane leaves without symptoms; V + or -: sugarcane yellow leaf virus detected or failed to be detected by RT-PCR, Ph + or -: sugarcane yellows phytoplasma detected or failed to be detected by PCR.

Restriction digestion of the PCR product with *Rsa*I

Digestion with *Rsa*I showed RFLP profiles typical of phytoplasma (Figure 3). Samples A, B and G showed four bands and their profiles appeared to be a combination of groups 3 and 4 or group 4 and 6 phytoplasmas. Sample C appears to be a group 3 phytoplasma, sample D; group 1 and samples E and F; group 4. These results suggest the presence of at least four strains of SCYP in sugar cane.

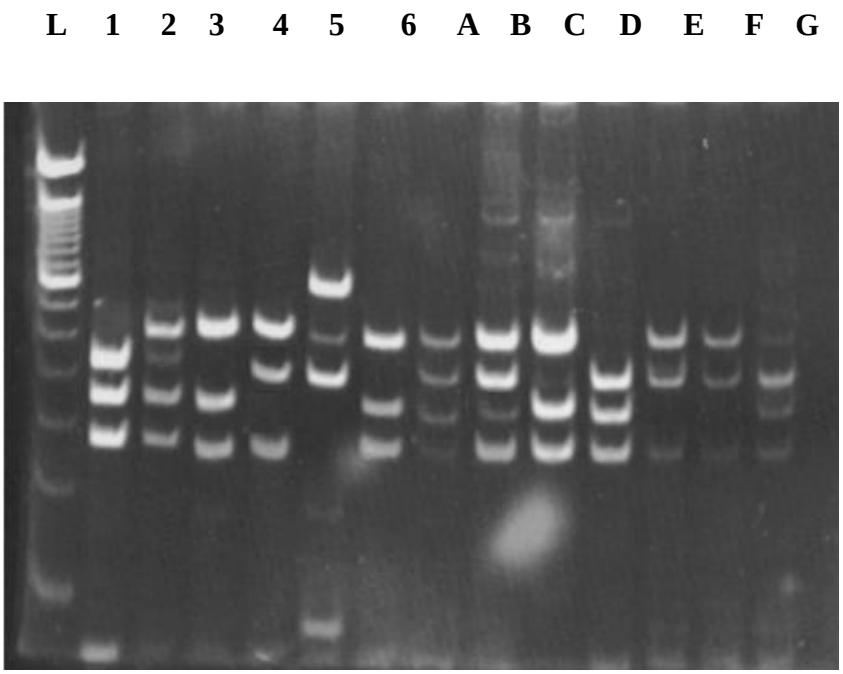

Figure 3: RFLP profiles of PCR products amplified with primers F2n/R2 following digestion with Rsa I and separation on 6% polyacrylamide. Lane 1-6; groups 1-6 of phytoplasma, lane A-G; sugarcane samples and lane L; 100 bp ladder.

PCR products from three samples showing differing *Rsa*I restriction profiles were selected for sequencing; sample B (mixed group 3 and 4 or 4 and 6), sample C (group 3) and sample D (group 1). PCR products were cleaned using the Qiagen spin 50. Quality and quantity was estimated by electrophoresis on 1% agarose and a 1:1 molar ratio was used for ligation with pGem vector II.

Transformed *E. coli* cells were spread plated on LB containing Ampicillin/ Xgal/IPTG. An overnight incubation was sufficient for appearance of the colonies (Figure 4). Three types of colonies were present (1) white colonies representing transformed cells containing the insert, (2) blue colonies, cells containing the vector without the insert (3) pale blue colonies whereby vector contains an insert but LacZ is only partially disrupted. . More than 90% of cells were transformed with the cloned vector.

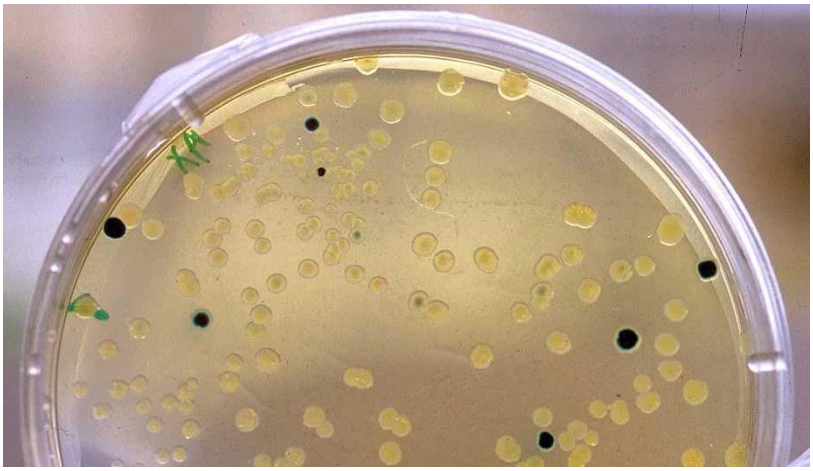

Figure 4: E. coli transformed with pGEM Vector on LB plates containing Ampicillin/ Xgal/IPTG.

Seventeen white colonies were randomly selected from the three plates and the presence of the insert was tested by PCR using F2n/R2 primers. All colonies were found to contain the 1250 bp insert.

One colony previously tested positive for the insert was selected from each plate and the plasmid extracted using a Qiagen plasmid extraction kit. Estimation of plasmid quantity was carried out on a gel using a molecular weight marker. Plasmids from each sample were amplified using a sequencing amplification program. PCR products were precipitated, then used for sequencing.

To check for the integrity of the insert, 9 amplicons (three transformed colonies per sample) were digested with *Rsa*I and their profiles verified with groups 1-6 phytoplasmas (Figure 5). Restriction digest of PCR products from colonies C were identical to insert. Prior to cloning, sample B showed a mixed 4 bands pattern (groups 3 and 4). After cloning, RFLP digest from 3 colonies (B1-B3) showed the existence of two types of 3-banding pattern. Fragment from colony B1 appeared as a group 3 and fragment from colonies B2 and B3 appeared as a group 4 phytoplasma. These results may suggest the presence of two operons within the same phytoplasma. These are amplified independently by the same primers and show a combined RFLP pattern upon digestion. Through cloning, each fragment was transferred to a different *E. coli* cell, each showing different restriction digests. The existence of two strains of phytoplasma, each belonging to different groups, within the same plant is also a likely explanation.

Sample D showed a three- banding pattern typical of group 1 prior to cloning. RFLP from two colonies tested (D2 and D3) maintained this pattern. However colony D1 from the same plate revealed the existence of two new fragments of size 170 bp and 260 bp with the disappearance of a main upper band (430 bp). A point mutation within the upper band may have led to the formation of a new *Rsa*I restriction site leading to the disappearance of the upper band and the appearance of two additional bands of smaller size. Further characterization of these inserts by sequencing needs to be carried out for a better understanding.

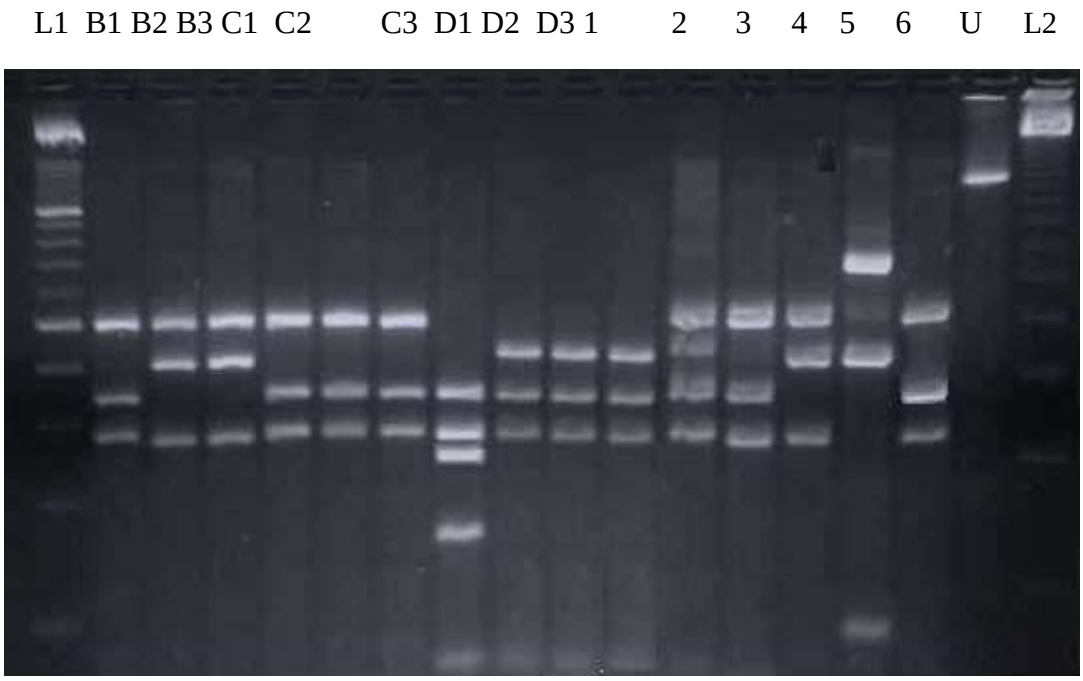

Figure 5: RFLP pattern of transformed colonies digested with RsaI showing the existence of two operons from sample B (lanes 2-4) and sample D (lanes 8-10). Lane 1 (L1); 100 bp ladder, sample C; lane 5-7, lanes 11-16; groups 1-6 phytoplasmas, lane 17 undigested sample and lane 18 (L2); 123 bp ladder.

DNA Extraction

In order to determine the most suitable tissue to be used for the SCYP detection, DNA was extracted from the stem and from the leaves of twenty plants (varieties M 695/69, CP 721210 and S17) and amplified using primers R16mF2/R16mR1 followed by F2n/R2. Eight plants were found positive for the phytoplasma when DNA extracted from the stem was used for amplification. Instead, only one plant showed the presence of phytoplasma when DNA extracted from the leaves was used for amplification. A better detection of the phytoplasmas from the stem probably suggests that the phytoplasmas are more likely to be in higher titres in the stem. Alternatively, the absence of inhibitors especially polyphenols from DNA extracted from the stem may also account for better PCR amplifications. However, the use of DNA from the stem for the detection of phytoplasmas is a destructive method.

Detection of the SCYLV by RT-PCR

RT-PCR amplification of the SCYLV RNA showed that 100 samples (68.7%) were infected with the virus (Tables 2 & 3). The expected fragment size of 352 bp (Figure 6) was amplified from symptomatic as well as asymptomatic varieties with primers YLS 462 and YLS 111. Of the 100 SCYLV infected samples, 58 expressed typical YLS symptoms while 42 were asymptomatic. There was a non-significant correlation ($r = 0.09$) between the presence of the SCYLV and the symptoms. Despite the high number of SCYLV-positive varieties the correlation coefficient was non-significant. This is probably due to the high sensitivity of the RT-PCR assay, which can detect low titer of the virus that is not sufficient enough to cause visible symptoms.

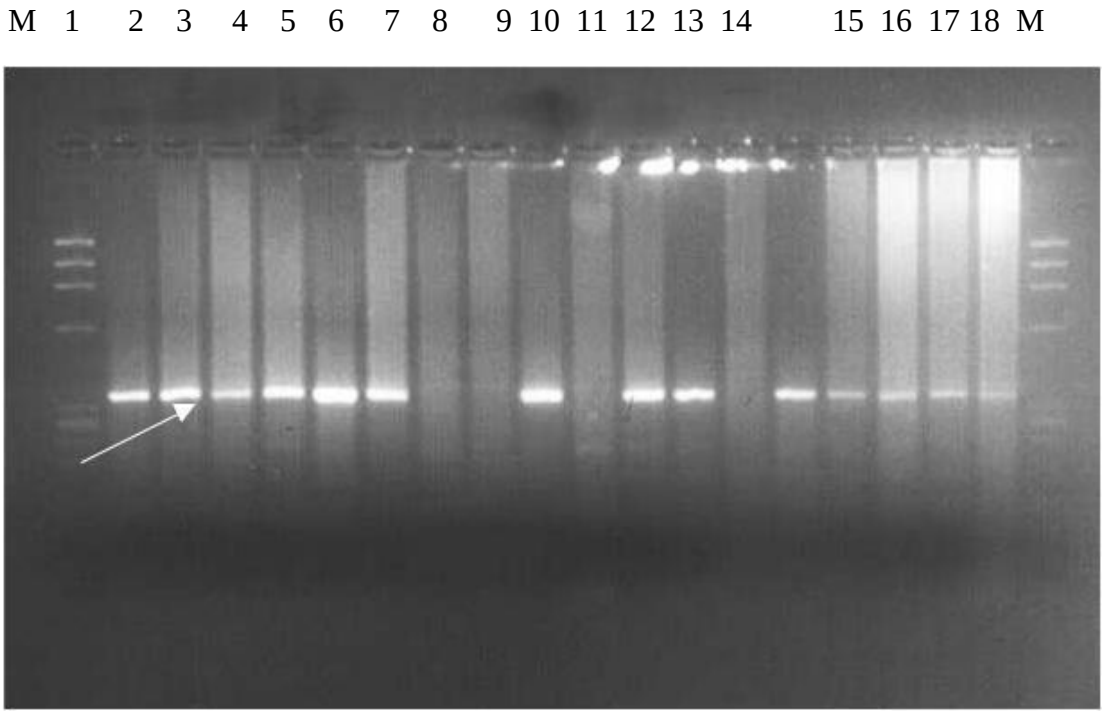

Figure 6: Detection of SCYLV in 18 sugar cane samples (lanes 1-18). M: 100 bp ladder. Positive detection of SCYLV is indicated by a band of 352 bp (arrow).

Elimination of SCYP and SCYLV by tissue culture

Out of 43 varieties of sugar cane from quarantine, 28 varieties were found infected with SCYLV and 19 varieties with SCYP. Among these, 17 were co-infected with both pathogens. All varieties were induced to form callus *in vitro*. Some failed to form embryogenic callus and could not be regenerated. After two subcultures of one month each, nineteen varieties were regenerated into plantlets and tested for SCYLV and SCYP. No virus or phytoplasma could be detected in any *in vitro* plantlet regenerated through callus culture. Plantlets from these 19 varieties were transferred to the glasshouse and all tested for the two organisms. No pathogen could be detected from the plants three and twelve months after transfer to the glasshouse indicating their elimination.

A likely explanation for the elimination of both pathogens is their localisation within the phloem. In sugar cane, somatic embryos arise mainly from non-vascular tissues (Guiderdoni and Demarli, 1988). Lack of connection between the somatic embryos and the phloem limits movement of the virus or the phytoplasma. Therefore, plantlets regenerated from callus are free from both pathogens. Secondly, a high concentration of auxin in tissue culture medium may also inhibit viral or phytoplasma replication. Prolonged exposure may lead to their death and eventual elimination from newly formed plantlets.

In this experiment, it has been possible to eliminate both the virus and the phytoplasma from infected plants by the culture of callus derived from leaf rolls. This opens new avenues as the ‘detect and destroy’ strategy so far adopted for quarantine material need not be applied. Infected material with SCYLV and ScYP could be cultured *in vitro* for their elimination and released for evaluation by breeders. This technique requires little skill and plantlets free from both organisms can be produced within 4-5 months. However callus culture of sugar cane should be treated cautiously due to possible somaclonal variation.

In situ localisation of phytoplasma from sugar cane

No phytoplasma was detected from sugar cane using the DAPI technique. In fact sugar cane tissues appeared to have an inherent ability to fluoresce. Changing exposure times did not help in the distinction between sugar cane tissues and nucleic acids following staining with DAPI. Extensive lignification of the vascular bundles may account for such extreme fluorescence. Alternatively the youngest part of the stem (which appears less lignified) must be used for sectioning and localization of phytoplasma.

CONCLUSION

This study has enabled an assessment of the distribution of yellow leaf syndrome in Mauritius. The disease was found to be widespread in several commercial cultivars and in a high proportion of clones introduced from various countries. YLS probably spread from one country to another as the disease was unknown until recently. Although this investigation was focused on the phytoplasma associated with the disease, the associated virus had also to be taken into account to understand the disease, particularly with respect to symptomatology.

Both SCYLV and SCYP were diagnosed in symptomatic as well as asymptomatic plants. Therefore the detection of YLS based solely on symptoms is unreliable. In some of the plants that showed symptoms, neither SCYP nor SCYLV could be diagnosed suggesting either that symptoms similar to YLS could be misdiagnosed. In such cases, the yellowing could be due to abiotic factors such as poor soil fertility, soil compaction or soil inhabiting fungi.

Sugar cane yellow leaf virus was found to be well spread in commercial cultivars in Mauritius. However no positive correlation was found between YLS symptoms and the presence of the virus. On the other hand a positive correlation was observed between YLS symptoms and SCYP.

The phytoplasma could not be visualized by fluorescent staining. However, restriction fragment length polymorphism analysis has demonstrated that three major phytoplasma groups and one subgroup are present in Mauritius. This diversity is important and further studies are anticipated to determine their distribution as well as their reaction on different cultivars of sugar cane.

The elimination of both SCYP and SCYLV by tissue culture techniques is an important step in obtaining disease-free plants. Infected material could be freed from the pathogens and clean seed developed by micropropagation. Although it has been shown that the virus can be eliminated by tissue culture, it is the first time that it has been demonstrated that the phytoplasma can also be expelled in this manner.

REFERENCES

Arocha, Y., Gonzalez L., and Peralta E. L. 1999. First report of virus and phytoplasma pathogens associated with yellow leaf syndrome of sugar cane in Cuba. Plant Dis. 83:1177.

Cronjé C.P.R., Wesley-Smith J. and Maartens P. 1996. Yellow leaf syndrome of sugar cane: the search for a causal agent. Microscopic Soc. Southern Afr. 26: 45.

Cronjé C.P.R., Tymon A. M., Jones P. and Bailey R.A. 1998. Association of a phytoplasma with a yellow leaf syndrome in sugar cane in Africa. Ann. Appl. Biol. 133: !77-186.

Deng, S. and Hiruki, C. 1991. Amplification of 16S rRNA genes from culturable and nonculturable *Mollicutes*. Journal of Microbiological Methods 14:53-61.

Doi, Y., Teranaka, M., Yora, K., and Asuyama. H. 1967. Mycoplasma or P.L.T group-like microorganisms found in the phloem element of plants infected with mulberry dwarf, potato witches'-broom, aster yellows or paulownia witches'-broom. Ann. Phytopathol. Soc. Jpn. 33:259-266.

Gamborg O.L., Miller R.A. and Ojima K. 1968. Nutrient requirement of suspension cultures of soybean root cells. Experimental Cell Research 50: 151-158.

Guideroni E. and Demarli Y. 1988. Histology of somatic embryogenesis in cultured leaf segments of sugarcane plantlets. Plant Cell, Tissue and Organ Culture 14: 71-88

Gunderson, D. E. and Lee I-M. 1996. Ultrasensitive detection of phytoplasma by nested-PCR assays using two universal primer pairs. Phytopath. Medit. Vol 35:144-151.

Harrison N.A. Richardson P.A. Jones P. Tymon A.M. Edengreen S.J. and Mpunami A.A. 1994. Comparative investigation of MLOs associated with Caribbean and African coconut lethal decline disease by DNA hybridisation and PCR assays. Plant Disease 78: 507-511.

Kollar A., Seemullar E., Bonnet F., Saillard C., and Bové J.M. 1990. Isolation of the DNA of various plant pathogenic mycoplasma-like organisms associated with various plant diseases. J. Phytopathology 127: 177-186.

Lee I. M., Hammond R.W., Davis R.E., and Gundersen G.E. 1993. Universal amplification and analysis of pathogen 16s rDNA for classification and identification of mycoplasma like organism. Phytopathology 83: 834-832.

Lockhart B.E. and Cronjé, C.P.R. 2000. Yellow leaf syndrome. pp. 291-296. In: A Guide to Sugarcane Diseases. P.Rott, R.A. Bailey, J.C. Comstock, B.J. Croft and A.S. Saumtally (editors). CIRAD/ISSCT. 399 pp.

McCoy, R. E., Caudwell, A., Chang, C. J., Chen, T. A., Chiykowski, L. N., Cousin, M. T., et al., 1989. Plant diseases associated with mycoplasma-like organisms. Pages 545-560 in: The Mycoplasma. Vol. 5. R. F. Whitecomb and J. G. Tully, eds. Academic Press, New York.

Murashige T. and Skoog F, 1962. A revised medium for the rapid growth and bioassays with tobacco tissue cultures. Physiological Plant 15: 473-497

Namba, S., Kato S., Iwanami, S., Oyaizu, H., Shiozawa, H. and Tsuchizaki, T. 1993. Detection and differentiation of plant pathogenic mycoplasma-like organisms using polymerase chain reaction. Phytopathology, Vol 83:786-791.

Ricaud C. 1968. Yellow wilt of sugar cane in Eastern Africa. Sugar cane Pathologists' Newsletter 1: 45-49.

Rogers P.F. 1970. Yellow wilt of sugar cane in Eastern Africa. Sugar cane Pathologists' Newsletter 4: 53-54.

Schenk S. 1990. Yellow leaf syndrome- a new disease of sugar cane. Ann. Rep. Exp. Sta. Hawaiian Sugar Planters Association Exp. Sta. 1990: 38.

Schenk S. and Hu J.S. 1991. Cause of yellow leaf syndrome. Ann. Rep. Exp. Sta. Hawaiian Sugar Planters Association. 1991: 26.

Sears B. B., and Kirkpatrick B. C, 1994. Unveiling of evolutionary relationships of plant-pathogeneic mycoplasma like organisms. Am. Soc. Microbiol. News. 60: 307-312.

Smart C.D., Schneider B., Blomquist C.,., Guerra J., Harrison N. A., Ahrens U., Lorenz K. H., Seemuller E., and Kirkpatrick B. C 1996 : Phytoplasma specific PCR primers based on sequences of the 16S-23S rRNA spacer region. Applied and Environmental Microbiology 62 No 8 2988-2993

Wongkaew, P., Hanboonsong Y., Sirithorn P., Choosai C., Boonkrong S., Tinnangwattana T., Kitchareonpanya R., and Damak S. 1997. Differentiation of phytoplasmas associated with sugar cane and graminaceous weed white leaf disease and sugar cane grassy shoot disease by RFLP and sequencing. Theor Appl Genet. 95: 660-663.