DETECTION AND CHARACTERISATION OF SUGARCANE YELLOWS PHYTOPLASMA

By

S.M. ALJANABI, Y. PARMESSUR, P. JONES, S. SAUMTALLY and A. DOOKUN
Mauritius Sugar Industry Research Institute, Réduit, Mauritius
Tropical Virus Unit, Plant Pathology Department, IACR Rothamsted, Harpenden, Herts, AL5 2SQ, England

Abstract

A study was carried out to assess the distribution and association of sugarcane yellows phytoplasma—ScYP in sugarcane affected by yellow leaf syndrome (YLS) in Mauritius. The polymerase chain reaction was used to detect and characterise the phytoplasma. Samples were collected from clones undergoing quarantine, in a variety collection plot, in mature commercial fields and from 6 month old commercial fields. A 1.25 kb DNA fragment encoding for the phytoplasma 16S rRNA was consistently amplified by nested-PCR. Of the 204 samples with and without symptoms derived from 166 varieties, 95 (57%) tested positive for ScYP by PCR. Restriction fragment length polymorphism analysis of the phytoplasma 16s rDNA amplified product indicated that 3 phytoplasma groups are present in sugarcane in Mauritius. The results indicated that detection of YLS based on symptoms only is not reliable, since many asymptomatic varieties were positive for the phytoplasma early in the growing season.

Introduction

Yellow leaf syndrome (YLS) of sugarcane was first described in 1989 in Hawaii (Schenk, 1990) and is characterised 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-leaf appearance. Symptoms also include accumulation of sucrose in the midrib (Lockhart and Cronjé, 2000). YLS is prevalent in adult canes, and stress conditions such as cold and excess moisture tend to favour symptom development. No cause was found at that time. Following the occurrence of YLS in Hawaii, several countries recorded the syndrome and it has since been observed in 34 countries (Lockhart and Cronjé, 2000). A number of factors such as abiotic stresses and insect damage can induce similar effects as YLS, rendering identification based on symptoms difficult. YLS has been found to be transmitted by vegetative propagation of cane setts (Schenk and Hu, 1991) suggesting that a pathogen is involved. Subsequently, a luteovirus, named sugarcane yellow leaf virus (SCYLV), was detected by Lockhart et al. (1996) and presence of the virus was also confirmed by Vega et al. (1997). YLS was first observed in Mauritius in 1994 on variety CP72-1210. Symptoms were subsequently observed in several other varieties, and the presence of SCYLV was confirmed in 1996 (Saumtally and Moutia, 1997).

Investigations carried out by Cronjé et al. (1996, 1998) in South Africa showed that a phytoplasma was associated with YLS symptoms. Detection was based on PCR amplification of the 16s rDNA of the organism as well as by electron microscopy. The phytoplasma has also been found in symptomless plants. It has provisionally been named sugarcane yellows phytoplasma and the strains divided into subgroups I and II (ScYP I and ScYP II) based on RFLP profile (Cronjé and Bailey, 1999).

This study was undertaken to investigate the distribution of YLS in Mauritius and to characterise the phytoplasma that has been associated in previous studies with the disease. Varieties undergoing quarantine were also screened for the pathogens to identify infected clones.

Materials and methods

Plant material

One hundred and thirty four leaf samples derived from 116 varieties were collected from mature and flowering cane in 1999. The varieties were grown either in a closed quarantine glasshouse, a variety collection plot, or in commercial fields island-wide. Seventy additional samples from 50 commercial varieties were collected from 6 month old symptomless cane plants. Material taken from each stool was visually assessed by three scorers for the presence (yellow midrib and yellowing of the lamina) or absence of symptoms (green leaves).

Total nucleic acids extraction

Total nucleic acids were extracted following the method of Harrison et al. (1994). A phytoplasma enrichment method (Harrison et al., 1994) was also used to extract DNA from sugarcane.

Polymerase chain reaction (PCR) for diagnosis of phytoplasma

Total genomic DNA was subjected to PCR assay using universal primers designed to amplify a specific sequence within the 16s rDNA region of phytoplasmas. Several primer sets were tested but the

KEYWORDS: Sugarcane Yellows Phytoplasma, PCR, Quarantine, Yellow Leaf Syndrome.
nested approach described by Gunderson and Lee (1996), with different first round primers and thermal cycling profiles, was adopted as it produced consistent results. Primers are listed in Table 1. The PCR was performed in a total volume of 25 μL, as described by Cronje et al. (1998). The PCR products were electrophoresed on 1.8% agarose gels, stained with ethidium bromide and photographed under UV light.

To test the possibility of the presence of more than one strain of phytoplasma in one variety, the PCR products were cloned into pGEM-T Easy vector (Promega). Several positive colonies were used for PCR with the nested primers R16F2n and R16R2.

### RFLP analysis of PCR products

The 16S rDNA products of positive samples amplified by PCR using the nested primers R16F2n and R16R2 were analysed by restriction endonuclease digestion. In addition, the following six phytoplasma groups were amplified in a single step PCR using primer pairs R16F2n and R16R2: European aster yellow (gp 1), Faba bean phyllody (gp 2), Peach yellow leaf roll (gp 3), Coconut lethal yellow (gp 4), European elm yellow (gp 5) and Tomato big bud (gp 6). These amplicons were digested along with amplified PCR products from the sugarcane samples and the cloned second round PCR products from positive colonies using the 4-cutter enzymes Mse I (Tru 91), Rsa I, Sau 3A and Taq I. The products were separated by electrophoresis in 3% agarose (2% agarose + 1% Methaphore™ agarose, FMA Bioproducts, ME, USA). RFLP profiles of samples were compared with profiles from group specific phytoplasmas.

### Results

#### Detection of phytoplasma by PCR

Out of 134 mature and flowering stools sampled, 76 showed typical YLS symptoms and, out of these, 47 were positive for the ScYP. For the symptomless stools tested, 29 were positive for the phytoplasma. For the younger 6 month-old stools, where no symptoms were observed, 32 samples were identified positive for ScYP by PCR. Altogether, 95 out of the 204 samples gave the expected 1.25 kbp fragment of the phytoplasma 16S rDNA using the nested primers R16F2 and R16R2 (Figure 1). Forty-seven (62%) of the 76 flowering samples with typical YLS symptoms and 16 (28%) of 58 symptomless flowering cane were positive for ScYP (Table 2). There was a positive significant correlation (r = 0.36) between the presence of the phytoplasma and YLS symptoms in the samples tested. Nineteen out of 43 foreign clones

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>PCR</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>General (F)</td>
<td>5'-AAGAGTTTGATCTTGCTGCTAGGATT-3'</td>
<td>Deng and Hiruki, 1991</td>
</tr>
<tr>
<td>P7</td>
<td>General (R)</td>
<td>5'-CACTGCGAGCCGACCA-3'</td>
<td>Smart et al., 1996</td>
</tr>
<tr>
<td>SN910601</td>
<td>General (F)</td>
<td>5'-GTTGTACCTGGCGTGCAAGGATT-3'</td>
<td>Namba et al., 1993</td>
</tr>
<tr>
<td>P6</td>
<td>General (R)</td>
<td>5'-GATGAGGAATACCTGGTTGCTACGACTTA-3'</td>
<td>Deng and Hiruki, 1991</td>
</tr>
<tr>
<td>R16mF2</td>
<td>General (F)</td>
<td>5'-CGATTCAAGTGCGACCA-3'</td>
<td>Gunderson and Lee, 1996</td>
</tr>
<tr>
<td>R16mR1</td>
<td>General (R)</td>
<td>5'-CTTACAACCACTATGAC-3'</td>
<td>Gunderson and Lee, 1996</td>
</tr>
<tr>
<td>R16F2n</td>
<td>Nested (F)</td>
<td>5'-GAAAGCTGCTAGACTGCG-3'</td>
<td>Lee et al., 1993</td>
</tr>
<tr>
<td>R16R2</td>
<td>Nested (F)</td>
<td>5'-TGCGCCGCGGTTGCTGCAAACCCCG-3'</td>
<td>Lee et al., 1993</td>
</tr>
</tbody>
</table>
Table 2—A summary of results for the survey carried out on 134 samples at flowering stage and 70 six month old symptomless samples.

<table>
<thead>
<tr>
<th>Plant Stage</th>
<th>Number of samples</th>
<th>PCR + Sym</th>
<th>PCR + Sym</th>
<th>PCR - Sym</th>
<th>PCR - Sym</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flowering cane</td>
<td>134</td>
<td>47</td>
<td>16</td>
<td>29</td>
<td>42</td>
</tr>
<tr>
<td>Six months</td>
<td>70</td>
<td>32</td>
<td>32</td>
<td>38</td>
<td>38</td>
</tr>
<tr>
<td>Total</td>
<td>204</td>
<td>47</td>
<td>48</td>
<td>29</td>
<td>80</td>
</tr>
</tbody>
</table>

introduced in quarantine were infected with ScYP. Forty-two symptomless flowering samples were tested negative for phytoplasma (Table 2). Out of 70 young symptomless varieties, 32 (46%) tested positive for the phytoplasma while 38 (54%) sample were free of the pathogen.

RFLP analysis of PCR products

RFLP analysis of the 1.25 kbp fragment of the 16S rDNA, amplified with primers P6/SN910601 for the first round amplification and R16F2n/R16R2 for the nested PCR from positive plants, with 4 restriction enzymes, identified one group related to Western X phytoplasma (data not shown). Nested PCR products generated by R16mF2/R16mR1 for the first round and R16F2n/R16R2 for the second round amplification digested with 4 restriction enzymes identified 3 distinct groups and 1 subgroup (Figure 2). The RFLP pattern produced from the second round amplification of the 16S rDNA from both plants and controls was consistent and reproducible. The three identified groups belong to group 1, 3, and 4 according to the classification of Lee et al. (1998). Group 1 belongs to European aster yellow I-B, group 3 to Western X III-A or peach yellow leaf roll III-A, and group 4 to coconut lethal yellow V-A. The subgroup shared similarity with group 1, which is related to tomato big bud I-A. RFLP analysis of the cloned PCR products indicated the presence of more than one phytoplasma in one variety (Figure 2). Two restriction enzymes, Rsa I and Mse I, produced a well-defined pattern with a larger number of bands than Taq I and Sau 3A, and they were used to analyse PCR products from all positive plants.

Fig. 2—RFLP analysis of PCR products generated with nested primers combination (R16F2n and R16R2) of the phytoplasma 16S rDNA sequence amplified from positive plants (lanes 1–9) and from control groups g1–g6 (lanes 10–15) using Rsa I, Mse I, Sau 3A and Taq I restriction enzymes. Roman numbers on top of solid bars representing phytoplasma groups. Lane 16 uncut PCR product of the 16S rDNA sequence. M1, 100 bp molecular weight marker (Boehringer Mannheim), M2, 123 bp ladder, (Sigma).
Discussion

Sugarcane yellow leaf syndrome was found to be widespread in several commercial sugarcane varieties in Mauritius and in 30 of 43 clones undergoing quarantine (Table 2). Cronjé and Bailey (1999) suggested a closer association of the phytoplasma in sugarcane varieties grown in South Africa. In a previous study, ScYP was found in varieties tested in Mauritius by reverse transcriptase-PCR (RT-PCR) but there was no significant correlation with YLS (Aljanabi et al., 2001).

In 42 flowering cane samples, neither phytoplasma nor symptoms were detected. This suggests that plants were either tolerant or resistant to phytoplasma infection. The lack of symptoms might be related to the low titre of phytoplasma that could not cause significant or detectable damage to the chloroplast. Also, the sensitivity of the nested PCR is high enough to detect very low population of phytoplasma. Unfavourable environmental conditions for phytoplasma growth and proliferation could be another factor contributing to the absence of symptoms, regardless of the presence of the pathogen in plant tissue.

The results indicated that primers used for the general amplification of the phytoplasma, P6 and SN910610, can amplify one group of phytoplasma (data not shown). Using these two primers in combination with R16F2n and R16R2 amplified ScYP I, which is related to Western-X phytoplasma as described by Cronjé and Bailey (1999). Also, we noticed that similar band size amplified from different groups in the second round might co-migrate on agarose gels. This was confirmed when we cloned several PCR products of 1.25 kb amplified with R16F2n and R16R2 primers. Testing some of these clones revealed two different RFLP patterns indicating, in some cases, that two phytoplasmas belonging to different groups were present in the same plant (Figure 2).

The amplification of the phytoplasma 16S rDNA from asymptomatic samples was not unexpected as substantial latent infection can occur, which complicated early diagnosis based on symptoms. The best option would be to test sugarcane varieties, regardless of symptoms, as they are not completely reliable as to the causal agent involved. Our findings that some symptomatic plants failed to show the presence of either pathogen confirm the unreliability of diagnosis based solely on symptomology as our methods of detection (nested PCR) are the most sensitive available.

The effect of the disease on yield has not been accurately estimated, but figures of 20% loss of recoverable sugar has been reported (Lockhart and Cronjé, 2000). Infected plant material can be freed from the virus (Delage et al., 1999) and the phytoplasma (MSIRI, unpublished) by tissue culture.

Conclusions

We demonstrated that the detection of YLS based solely on symptoms is totally unreliable because phytoplasma was detected in symptomless plants. YLS probably spread from one country to another as the disease was unknown until recently and due to the wrong assumption that symptomless plants are free of pathogens and that heat treatment of cuttings before planting can eliminate a number of pathogens. However, this is not the case at least for the phytoplasma. RFLP analysis detected three major phytoplasma groups (1, 3 and 4) and one subgroup related to group one in Mauritius (Lee et al., 1998). Cloning of the second round PCR proved to be useful in resolving the presence of more than one phytoplasma strain in one plant.

Acknowledgments

The authors would like to thank Drs Jean Claude Autrey (Director) and Claude Soopramanien (Deputy Director–Biology) for the helpful criticisms of the manuscript.

REFERENCES


DETECTION ET CARACTERISATION DU PHYTOPLASME AGENT CAUSAL DE LA MALADIE DE LA FEUILLE JAUNE DE LA CANNE A SUCRE

S.M. ALJANABI, Y. PARMESSUR, P. JONES1, S. SAUMTALLY et A. DOOKUN
Mauritius Sugar Industry Research Institute, Réduit, Mauritius
1Tropical Virus Unit, Plant Pathology Department, IACR-Rothamsted, Harpenden, Herts, AL5 25Q, England

Résumé

Une étude fut entreprise à Maurice pour déterminer la distribution et l'association du phytoplasme, agent causal de la maladie de la feuille jaune de la canne à sucre aussi connue comme le syndrome de la feuille jaune. La technique d’amplification en chaîne par polymerase (ACP) fut utilisée pour détecter et caractériser le phytoplasme. Des échantillons furent pris des clones en serre de quarantaine, en collection variétale, dans des champs commerciaux âgés de six mois et en pleine maturité. Un fragment d’ADN de 1.25 kb encodant pour le 16S ARNr du phytoplasme fut amplifié de manière consistante par la technique ACP. Des 204 échantillons avec ou sans symptômes, pris de 166 variétés, 95 (57%) furent trouvés positifs pour le phytoplasme par la technique ACP. L'analyse du fragment 16S ARNr du phytoplasme par la méthode dite « Polymorphisme de longueur des fragments de restriction (PLFR) » révèle que trois groupes de phytoplasme étaient présents dans la canne à Maurice. Les résultats indiquèrent aussi que la détection de la maladie dite syndrome de la feuille jaune ne pouvait se faire d’une façon fiable sur la base des symptômes seulement car un grand nombre de variétés sans symptômes s’avèrerent positives par rapport à l’agent pathogène tôt pendant le cycle de croissance.

Mots clés: phytoplasme du jaunissement de la feuille de la canne à sucre, ACP, quarantaine, syndrome de la feuille jaune.
DETECCIÓN Y CARACTERIZACIÓN DEL FITOPLASMA DEL AMARILLAMIENTO DE LA CAÑA DE AZÚCAR

S.M. ALJANABI, Y. PARMESSUR, P. JONES, S. SAUMTALLY y A. DOOKUN
Mauritius Sugar Industry Research Institute, Réduit, Mauritius
1Tropical Virus Unit, Plant Pathology Department, IACR Rothamsted, Harpenden, Herts, AL5 2QJ, England

Resumen
Se realizó un estudio para evaluar la distribución y asociación del fitoplasma del amarillamiento de la caña de azúcar-ScYP en caña de azúcar afectada por el síndrome de la hoja amarilla (YLS) en Mauritius. Se empleó la reacción en cadena de la polimerasa (PCR) para detectar y caracterizar el fitoplasma. Las muestras se colectaron en clones que se encontraban en la cuarentena, en parcelas de la colección de variedades, en campos comerciales próximos a cosecha y en campos comerciales de 6 meses de edad. Un fragmento de 1.25 kb ADN codificado para el fitoplasma 16S rARN se amplificó de manera consistente por medio del PCR anidado. De un total de 204 muestras con y sin síntomas obtenidos en 166 variedades, 95 (57%) resultaron positivas para ScYP por medio de PCR. El análisis de polimorfismo del fragmento de restricción del fitoplasma 16S rADN indicó que 3 grupos de fitoplasmas se encuentran presentes en caña de azúcar de Mauritius. Los resultados mostraron que la detección del YLS basado en tan solo los síntomas no es efectiva puesto muchas variedades asintomáticas resultaron positivas para el fitoplasma en los estados iniciales de crecimiento.

Palabras claves: fitoplasma del amarillamiento de la caña de azúcar, PCR, cuarentena, síndrome de la hoja amarilla.