IMPROVEMENT OF QUARANTINE PROCEDURES FOR THE DETECTION OF SUGARCANE PHYTOPLASMAS

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Abstract

The importation of sugarcane varieties from other countries is necessary for the breeding of new varieties suited to the diverse environmental conditions in South Africa. To prevent the introduction and spread of new diseases, it is crucial that any imported pathogens are detected before the cane is released from quarantine. Although not present in South Africa, Grassy Shoot (GS) and Sugarcane White Leaf (SCWL) are economically significant phytoplasmal diseases that cause yield loss in sugarcane in a number of countries. Despite phytoplasmas being important pathogens that have been implicated in more than 300 plant diseases worldwide, these organisms have as yet not been cultured *in vitro*. The most reliable and rapid method of detection has been the use of nested polymerase chain reaction directed to conserved sequences in the 16S rRNA gene, where DNA isolation follows a phytoplasmal enrichment procedure. The process is both expensive and time consuming. This study investigated the use of FTA^{TM} paper (Whatman BioScience) for DNA extraction, and the use of a radioactive probe in conjunction with a tissue blot procedure to detect phytoplasmas in sugarcane leaf samples exhibiting symptoms of GS and SCWL, and in Bermuda grass leaf samples showing symptoms of Bermuda Grass White Leaf disease. The FTA^{TM} paper method was effective in extracting the DNA from which specific identifications could be made. As well as being more rapid, this method effected a two-thirds reduction in cost compared with the enrichment method. The radioactive probe was less useful because both mitochondrial and phytoplasmal DNA were detected.

Keywords: sugarcane, sugarcane diseases, Sugarcane White Leaf, SCWL, Bermuda Grass White Leaf, BGWL, Grassy Shoot, GS, DNA extraction, quarantine

Introduction

Although phytoplasmas have been associated with diseases in several hundred plant species, none have thus far been cultured *in vitro*. Past differentiation and classification of phytoplasmas relied primarily on biological properties such as specificity of plant and insect hosts, and symptoms exhibited in affected plants. The less laborious molecular-based analyses introduced in the past decade have proved more accurate and reliable for identification of phytoplasmas (Lee *et al*., 1998). Polymerase chain reaction (PCR) has been developed as a very useful diagnostic tool for phytoplasmal diseases (Namba *et al*., 1993).

Two economically important sugarcane diseases caused by phytoplasmas are Sugar Cane White Leaf (SCWL) and Grassy Shoot (GS). SCWL has been observed in Japan, Pakistan, Sri Lanka, Taiwan and Thailand, and has been the cause of serious yield losses (Chen and Kusalwong, 2000). The geographical distribution of GS include countries such as Bangladesh, India, Iran, Malaysia, Myanmar, Nepal, Pakistan, Sri Lanka, Sudan and Thailand. GS has been the cause of significant yield losses for many years in India (Viswanathan, 2000).

The quick and accurate identification of these pathogens in any imported variety is crucial to the prevention of these diseases being introduced to South Africa.

Methods and Materials

Plant samples

Sugarcane leaf samples exhibiting symptoms of SCWL and GS were collectedfor this study from Siyabalanduwa in Sri Lanka. The effectiveness of the methods investigated was also tested on Bermuda grass leaf samples showing symptoms of Bermuda Grass White Leaf (BGWL), samples of which were collected from areas surrounding Komatipoort in Mpumalanga Province, and Durban harbour and Pongola in the KwaZulu-Natal Province of South Africa. Leaf samples were stored at –80°C until required.

DNA extraction

The DNA enrichment method, which is a modified CTAB method (Harrison *et al.*, 1994), was used to extract DNA from all samples of sugarcane and Bermuda grass. The FTA^{TM} paper method (Whatman BioScience) was used as an additional extraction method for all samples. The FTA^{TM} paper method entailed capturing the DNA on a paper matrix, after which the DNA was washed three times with FTA Purification Solution^{TM} (Whatman BioScience) and twice with 1x TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and allowed to dry for one hour. Small discs of paper were used immediately thereafter for PCR.

PCR

A nested PCR was used to amplify phytoplasma DNA. The first round primers used were Asian F 5’ GTTTGATCCTGGCTCAGGATT 3’ (Namba *et al.*, 1993) and DH6R 5’ TGGTAGGGATACCTTGTTACGACTTA 3’ (Deng and Hiruki, 1991). For the nested reaction, the primers used were PLONEST F 5’GAAACGACTGCTAAGACTGG3’ and PLONEST R 5’TGACGGGCGGTGTGTACAAACCCCG3’ (Gundersen and Lee, 1996). Both universal primer pairs are based on the sequence of 16s rRNA of phytoplasmas. The PCR reaction was performed according to Berry *et al.* (2002). The nested PCR products were resolved on 1% agarose gels containing ethidium bromide and visualised using a UV transilluminator.

Restriction fragment length polymorphism (RFLP) analysis

Each PCR product was digested with restriction endonucleases *Rsa* I, Hpa II, *Taq* I, *Tru9* I and *Alu* I. Products were electrophoresed on a 1.5% agarose gel containing ethidium bromide and visualised using a UV transilluminator.

Sequencing

Amplified products obtained were recovered from the gels using a QIAquick gel extraction kit (Qiagen Ltd). The DNA fragments were prepared for direct sequencing, with the ABI 310 Genetic Analyser (Applied Biosystems), using a DNA Sequencing Kit (Applied Biosystems). Utilising the BLAST search protocol (www.ncbi.nlm.nih.gov/blast), resulting sequences were compared with the International Genbank database.

Tissue blot DNA hybridisation

The method was performed according to Pan *et al.* (1998). The product that was obtained from amplifying with phytoplasmal primers was used as a probe and labelled with P^{32}.

Results and Discussion

The DNA preparations via the enrichment method and the FTA™ paper method were used in PCR reactions to determine the presence of phytoplasmas. The samples with symptoms of SCWL, GS and BGWL all showed positive results for both extraction methods, indicating the presence of phytoplasmas. As seen in Figure 1, positive samples showed a product of ~1200 bp to be present.

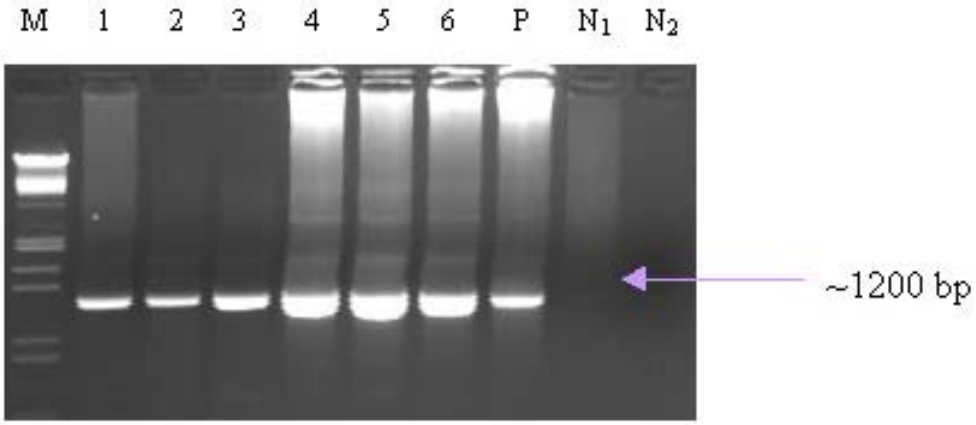

Figure 1. Nested PCR products, from DNA isolated via the FTA™ paper method (lanes 1-3) and the enrichment method (lanes 4-6), were resolved on a 1% gel. Samples tested: 1 - SCWL, 2 - GS, 3 - BGWL. P represents a positive control, while N_1 and N_2 both represent negative controls from the first and second round PCR, respectively.

Using the FTA™ paper method, samples can be processed more rapidly, with a two-thirds reduction in cost when compared with the DNA enrichment method. Sequencing (results not shown) and RFLP analysis (Figure 2) confirmed phytoplasma infection of samples with SCWL, GS and BGWL symptoms, although no clear distinction could be made between SCWL and GS sequences.

According to RFLP patterns, specifically the banding patterns generated by the enzyme *Hpa II*, when compared with results from a study done by Wongkaew *et al*. (1997), it was clear that samples showing SCWL and GS symptoms were all infected with SCWL phytoplasma. No cases of BGWL have previously been reported in South Africa.

Detection of phytoplasma infection using the tissue blot DNA hybridisation method with radioactive labelled probe was unsuccessful due to the detection of background bacterial and mitochondrial DNA. If a more specific probe could be designed to detect phytoplasmal DNA, this method would prove extremely useful and cost effective.

The FTA™ isolation method is now routinely used in the pathology laboratory of the South African Sugar Association Experiment Station for the detection of phytoplasmas in imported varieties.

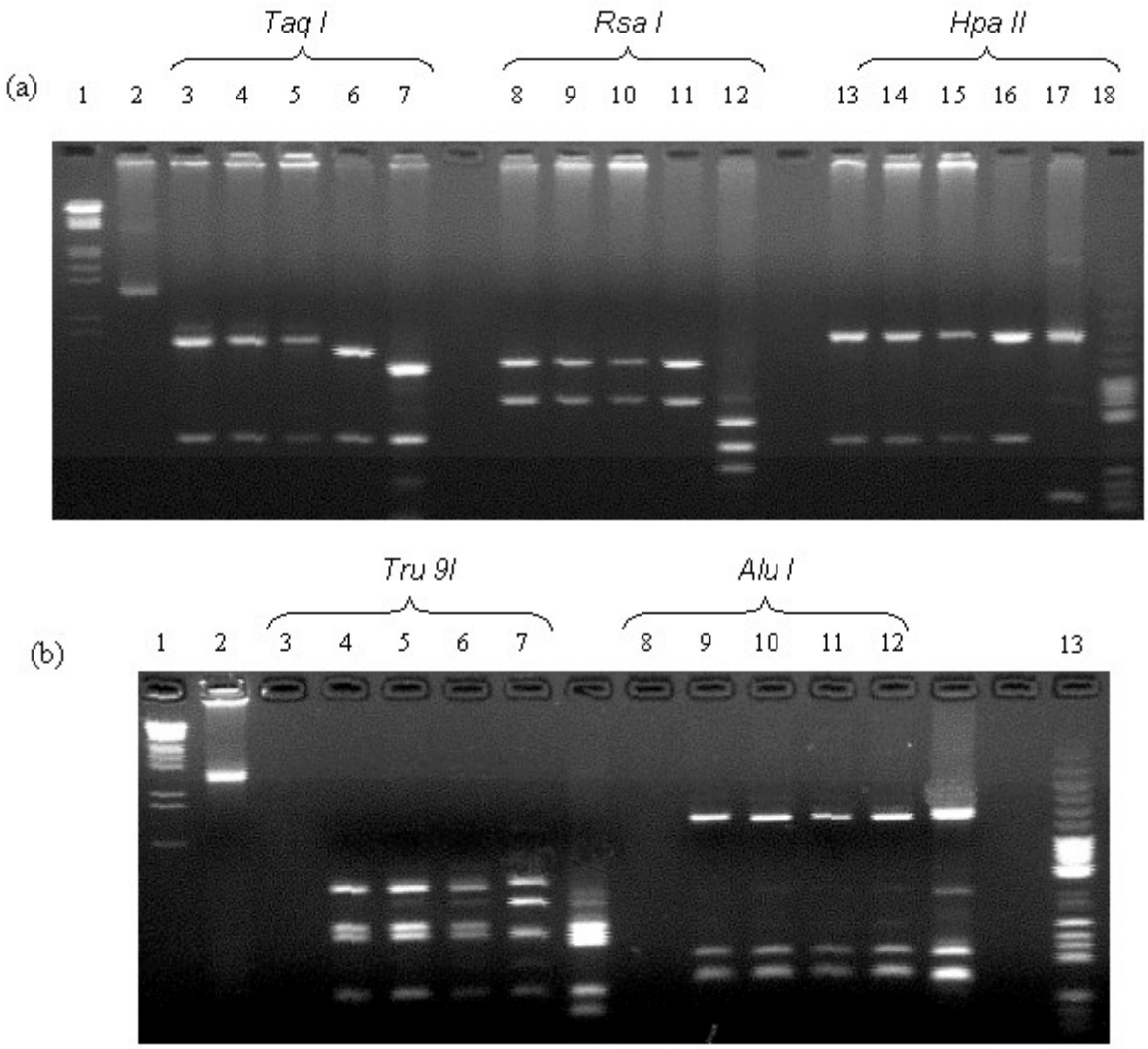

Figure 2. RFLP products were resolved on a 1.5% agarose gel. Samples used in (a) and/or (b): 3, 8 and 13 - SCWL sample 1; 4, 9 and 14 - GS; 5, 10 and 15 - SCWL sample 2; 6, 11 and 16 - BGWL; 7, 12 and 17 - Peach Yellow leaf roll (PYLR). Molecular weight marker 3 (lanes 1a and 1b) and molecular weight marker 5 (lanes 18a and 13b) as well as an undigested control (lanes 2a and 2b) were used as reference lanes. No differences in restriction patterns could be seen for the SCWL and GS samples. BGWL could be distinguished with the use of *Tru 9I* and *Taq I*.

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