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Rapid Detection of Sugarcane Phytoplasma by PCR Amplification using Specific Primers

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ABSTRACT. *A part of conserved sequence of 16SrRNA gene of sugarcane phytoplasma in Sri Lanka (SP-SL) was analyzed to design polymerase chain reaction (PCR) primers for the detection of SP-SL in plant tissues. Newly developed sugarcane phytoplasma specific eighteen mer primers SPP1/SPP2 were used to amplify 321 bp long rDNA sequence of SP-SL and the DNA fragment was visualized on agarose gel for the determination of presence of SP-SL in the substrate. Phytoplasmas associated with several symptomatic plants, both graminaceous and non-graminaceous, found in the SP-SL affected sugarcane fields were differentiated from SP-SL with the use of universal primer pair P1/ P2 followed by amplifying with the new specific primers. The amplification of SP-SL DNA was possible even at very low concentrations of template DNA such as about 0.04 ng in 20 µl of reaction mixture. This direct PCR method using new SPP1/ SPP2 primers is very specific, sensitive and rapid in the detection of SP-SL in plants.*

INTRODUCTION

The sugarcane phytoplasma disease first reported to have appeared in Kantale, Sri Lanka in 1972 and named "grassy shoot disease" (Jayathilake, 1973) has caused severe crop losses in sugarcane plantations from time to time and has become the most serious sugarcane disease in Sri Lanka. The most common foliar symptoms are narrowing and partially or almost totally chlorotic leaf lamina. Stunting, excessive tillering and formation of side shoots from the bottom to the top of the stalks are other diagnostic symptoms of this disease. Severely infected younger plants that appear as yellowish or whitish rosettes of grass may die eventually. An important characteristic feature of the symptomatic plants is on and off masking of symptoms hiding the pathogen inside.

The association of a phytoplasma with this disease was first established in 1997 (Jones *et al*., 1997) and a few samples of sugarcane phytoplasma collected from Sevanagala and Uda Walawe areas were found similar to sugarcane white leaf phytoplasma (Kumarasinghe and Jones, 2001) which has been recorded to be associated with sugarcane white leaf disease (SWLD) in Thailand, Taiwan and Japan (Chen, 1973; Nakashima *et al*., 1994). Another disease in sugarcane caused by phytoplasma having similar symptoms to SWLD is named sugarcane grassy shoot disease (SGSD) which has been reported from India, Bangladesh, Malaysia, Nepal and Pakistan (Chona *et al*., 1960; Kabir *et al*., 1988; Rishi and Chen, 1989). Even though, the two diseases can

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hardly be differentiated only on the disease of disease symptoms, they have been differentiated through the sequence analysis of the causal phytoplasmas in other countries (Wongkaew *et al*., 1997).

No phytoplasma has been cultured *in vitro* and detection of phytoplasma in plants has been left to on microscopy and symptom expression. During the 1980s, polyclonal antisera and monoclonal antibodies were produced against several phytoplasmas (then mycoplasma-like organisms) to detect phytoplasmas in plants (Sarindu and Clark, 1993; Vera and Milne, 1994; Viswanathan, 1997). However, the major limitations of this technique were with the purification and the specificity of phytoplasmas and lack of sensitivity (Jones *et al*., 1997). Advances in molecular diagnosis based on DNA hybridization, amplification and sequencing have recently been developed for the detection and classification of phytoplasma isolates (Bertaccini *et al*., 1990; Klingkong and Seemuller, 1993). At present, PCR (Polymerase Chain Reaction) assays using oligonucleotide primers based on sequences derived from the phytoplasmal 16SrRNA gene provide one of the most sensitive, reliable, rapid and accepted methods for detecting phytoplasmas in infected plants (Ahrens and Seemuller, 1992; Wongkaew *et al*., 1997; Marcone *et al*., 1997).

Apart from the diagnosis of the disease in a few samples sent to laboratories overseas, no method has so far been established to detect sugarcane phytoplasma in Sri Lanka (SP-SL). Use of available methods for detection of phytoplasma associated with SWLD and SGSD in the detection of SP-SL would be difficult due to practical implications and lack of technical know-how in Sri Lanka. Detection of SP-SL in plant tissues for the differentiation of healthy and affected plants is a basic requirement for studies and management of the disease especially due to the masking nature of its symptoms. Identification of possible alternative hosts for the pathogen would also be of practical importance in the development of strategies for disease management.

It was in this context that a PCR assay for the detection of SP-SL was attempted as a pioneer experiment towards the studies on SP-SL in order to acquire knowledge for the management of this devastating sugarcane disease in the country.

MATERIALS AND METHODS

The experiments were conducted in the laboratories at the College of Natural Resources and Environment, South China Agricultural University (SCAU), Guangzhou, P.R. China and at the Sugarcane Research Institute (SRI), Uda Walawe, Sri Lanka.

Plant material

Sugarcane shoots severely affected with SP-SL were collected from sugarcane growing areas of Sri Lanka as the source for target phytoplasma. Samples were also collected from naturally infected plants where SP-SL symptoms are masked. To secure healthy sugarcane samples, another set of plants had been raised from apical meristem tissues of apparently healthy sugarcane selected from a field, which had no history of SP-SL and planted on sterilized soil in insect proof cages. Leaf samples of each of three weeds with SP-SL related symptoms namely, *Brachiaria* sp. (Gramineae), *Calyptocarpus viatis* (Compositae) and *Borreria hispida* (Rubiaceae) found close to SP-

SL affected sugarcane plants were collected with non-symptomatic plants of the three types. Samples of rice plants affected with yellow dwarf disease caused by phytoplasma and non- symptomatic rice plants provided by the Laboratory of Plant Virology, SCAU were also included in the experiment. All the samples were stored below –20 ºC immediately after collection until they were used for the extraction of DNA.

DNA extr action

Total DNA was extracted following a slightly modified method of Nakashima *et al*. (1991).

A sample of 0.3 g of plant tissues cut into small pieces was flash frozen in liquid nitrogen and ground into powder in a mortar. Before the powder begins to thaw, warm 900 µl of extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl at pH 8.0 and 0.2 % 2-mercaptoethanol) was added and the slurry was transferred into a 2.0 ml centrifuge tube. The slurry was then incubated at 65º C in a water bath for 30-40 minutes. After adding and mixing of 450µl of chloroform: isoamyl alcohol (24:1), the homogenate was centrifuged at 13000 g for 5 minutes. The supernatant was pipetted out to a new centrifuge tube and equal volume of chloroform: isoamyl alcohol (24:1) was added. After shaking several times, the mixture was centrifuged as previously. The supernatant was transferred into a new centrifuge tube and 1/10 volume of 3 M sodium acetate (at pH 5.2) was added in it, followed by the addition of double the volume of cold purified absolute ethanol for precipitation of DNA. The mixture was then kept at –20º C for 2-3 hrs or overnight. The tube was centrifuged at 13000 g for 15 minutes and the supernatant was discarded. The pellet was washed with 70% and 95% ethanol respectively and the air-dried DNA was suspended in 50-100 µl of TE buffer (10mM Tris, 1mM EDTA) depending on the size of the pellet. DNA concentration was estimated with a spectrophotometer (UV 1600-UV visible, SHIMADZU) whenever, it was necessary.

DNA amplification and visualization

The PCR amplifications were conducted using two oligonucleotide primers whose sequences matched the 16SrRNA gene of the phytoplasmas. The first set of primers consisted of primer P_1, 5′–ACGAAAGCGTGGGGAGCAAA-3′ and P_2, 5′–GAAGTCGAGTTGCAGACTTC-3′ (produced by BIOASIA, Shanghai, China) designed by Ahrens and Seemuller (1992). The second set of primers designed from this study included primer SPP_1, 5′–ATTAAAGTGCCCATCATG-3′ and SPP_2, 5′–GTACTAAGTGTCGGGATT-3′ (produced by BIOASIA, Shanghai, China). After a series of experiments on optimization of reaction mixture and thermal cycle, PCR amplification was performed in a 20 µl reaction mixture containing 2 µl genomic DNA (appro. 20 ng/µl) of plants, 1.6 µl dNTP mixture (each at 2.5 mM), 0.7 µl of each primer P_1 and P_2 (10 µM), 0.1 µl Taq DNA polymerase (5U/ µl, Takara Biotechnology, Dalian, China) and 2 µl 10x PCR reaction buffer containing Mg^{+2} (Takara Biotechnology, Dalian, China). Thirty-five PCR cycles were operated in a Progene (Techne Cambridge Ltd, Cambridge, UK) thermocycler for the amplification of target DNA with the primers P_1/P_2. Each cycle consisted of 1minute (5 minutes for the first cycle) denaturation at 94 º C, 25 second annealing at 60º C and 75 second (10 minutes for the last cycle) extension at 72º C. For the new set of primers SPP_1/SPP_2, thirty six PCR cycles were performed with altered annealing temperature of 52º C for 45 seconds.

After amplification, each sample was added with 4 µl of loading buffer (50% glycerol, 5 mM EDTA, 0.25% v/v bromophenol blue) and the mixture (8 µl/well) was electrophoresed on 1.0% agarose gel containing 0.5 µg/ml ethidium bromide at 60V for 40 minutes in 0.5 x TBE (Tris, Boric acid, EDTA). The gels were scanned under an UV transilluminator to analyze the bands.

Cloning and sequencing of target DNA

Samples of severely SP-SL affected leaf tissues were used to extract the target DNA for cloning and sequencing. A 557 bp sequence of the 16SrRNA gene of the SP-SL was amplified in PCR primed by universal phytoplasma primers P_1/P_2 and purified using PCR purification kit (Qiagen Co., Hilden, Germany) as per instructions of the manufacturer. The purified target DNA was ligated to the vector, pUC 18-T using the instructions of the supplier (BIOASIA, Shanghai, China) and transformed into *Escherichia coli*, strain DH10B using $CaCl_2$ mediated heat shock method. The cloned target DNA was sent to BIOASIA, Shanghai, China, where the sequencing was performed with an ABI prism automated sequencer.

Primer designing

The new set of primers SPP_1/SPP_2 was designed to differentiate sugarcane phytoplasma from other phytoplasmas, based on the sequences producing significant alignments to the target sequence (557 bp) amplified by the universal phytoplasma primers P_1/ P_2, resulted by BLAST (Basic Logical Alignment Search Tool) provided by the National Center for Biotechnology Information (NCBI), USA (http://www.ncbi.org). The amplified target sequence of the 16SrRNA gene of SP-SL was expected to be 321 bp in the PCR with the use of SPP_1/SPP_2.

Detection procedure

Several experiments were conducted to evaluate the suitability of the use of new specific primers SPP_1/SPP_2 in the detection of SP-SL. At least three PCR observations were performed for each DNA sample, which relates a particular treatment. Sterile distilled water was used instead of template DNA as the control.

Most suitable dilution for optimum PCR amplification was determined by evaluating template DNA (appro. 200 ng/µl) diluted at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. Six DNA samples extracted from top-most leaf sheaths of severely SP-SL affected sugarcane clumps and one sample from healthy sugarcane clumps were used for DNA extraction. Each dilution was used in PCR primed by SPP/SPP_2 for the detection of SP-SL.

Most suitable tissues of sugarcane plant for DNA extraction and subsequent PCR with the primers SPP_1/SPP_2 were determined by evaluating samples from top of the stalks, top-most leaf sheaths, middle portions of leaf blades and apices of the leaf blades of severely SP-SL affected shoots. Each type was represented by three different

samples.

To investigate the specificity of the new specific primers SPP_1/SPP_2 in the detection of SP-SL over the universal phytoplasma primers P_1/P_2, DNA extracted from sugarcane infected with SP-SL, symptom-masked sugarcane, rice infected with yellow dwarf phytoplasma (YDP), symptomatic weeds namely *Brachairia sp*., *Calyptocarpus viatis* and *Borreria hispida* was tested for PCR using both sets of primers. DNA extracted from healthy sugarcane, non-symptomatic rice and other weeds was also used as treatments. Three samples from each category were used in the experiment.

RESULTS AND DISCUSSION

Design of primers

The sequence of 557 bp long amplified product of the part of 16SrDNA of SP-SL with the primers P_1/P_2 showed 100% sequence similarity with the corresponding sequence of the sugarcane Mollicutes (GenBank accession No. X76432). The new primers SPP_1/SPP_2 designed to differentiate the target sequence of sugarcane Mollicutes (phytoplasma) from the closest allies of SP-SL (Table 1) amplified a 321 bp long sequence depending on their places of mismatches along the DNA sequences. The accuracy of the primer design was checked by the use of rice yellow dwarf phytoplasma and phytoplasma associated with white leaf symptoms of *Brachiaria sp*. The latter was reported closely related to Bermuda grass white leaf phytoplasma in Thailand (Wongkaew *et al*., 1997) and Italy (Marcone, *et al*., 1997) in the present study.

Table 1. Phytoplasmas closely related to SP-SL in relation to the sequence amplified in PCR with the primers P_1/P_2.

Phytoplasma	GenBank accession no.	Alignment %	No. of mismatches	Length of sequence (bp)
Sorghum grassy shoot	AF509325	99	5	557
Bermuda grass white leaf	AF248961	98	8	557
Cynodon white leaf	AF509321	98	9	558
Rice yellow dwarf	D12581	98	11	556
Phytoplasma (Strain gall)	Y15865	96	18	557
Mollicutes (*Catharanthus roseus*)	X76429	96	17	555
Stylosan. little leaf phytoplasma	AJ289192	96	22	559
Ashyellows phytoplasma	AF105316	95	23	559

Optimization of PCR with different primers

Owing to the low level of amplification with the primers P_1/P_2, possible mispriming and misincorporation of nucleotides; the primer concentrations, template

DNA concentrations and annealing temperatures were changed and other requirements were adjusted accordingly for accurate and effective amplification. With the increased concentrations of the primers, transient annealing resulted in unreliable bands and decreased concentrations gave faint bands. Slight changes in the concentrations of primers SPP_1/SPP_2 had no effect on the amplification of template DNA.

Altered concentration of template DNA has influenced PCR when the primers SPP_1/SPP_2 were used (Table 2). The detection of SP-SL from affected samples used for the experiment was 88.9% when the template DNA suspension was diluted at 10^{-1} (appro. 20 ng/ µl). Undiluted template DNA and dilutions at 10^{-2}, 10^{-3} and 10^{-4} also gave positive results of 55.5%, 72.2%, 50% and 22.2% respectively. The dilution of 10^{-5} gave no positive result in any sample. Perhaps, substances like phenolic compounds abundant in sugarcane tissues can have an inhibitory effect on amplification with undiluted template DNA samples as cited by Jones and Sutton (1997). Physical disturbances of different types of molecules in the reaction mixture cannot be ignored in explaining this low PCR amplification. Further dilutions of the template DNA may have caused subsequent decrease of amplification after 10^{-1}.

Table 2. Percentage[1] PCR detection of SP-SL primed by SPP_1/SPP_2 using different dilutions of template DNA extracted from the tissues of leaf sheaths of SP-SL affected and healthy sugarcane.

Tissues of leaf sheaths	Dilution level of template DNA					
	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
SP-SL affected	55.5	88.9	72.2	0	22.2	0
Healthy	0	0	0	0	0	0

[1] Based on eighteen PCR observations.

The yield of extracted DNA appeared to be qualitatively and/or quantitatively different with the use of different types of plant tissues even of the same clump. Highest SP-SL detection percentage is seen on the tissues of leaf sheaths (Table 3). This may be due to the inclusion of relatively higher number of vascular bundles in the sample at the DNA extraction thus providing higher amount of phytoplasma DNA. Detecting SP-SL on non-symptomatic plant tissues shows the usefulness of the detecting method. The overall low detection of SP-SL on non-symptomatic tissues may be due to the low level of pathogen density in the tissues. Further, a comparatively higher concentration of chlorophyll and phenolic compounds (Jones and Sutton, 1997) may have accounted for low detection of the disease on non-symptomatic tissues.

Table 3. Percentage[1] PCR detection of SP-SL primed by SPP_1/SPP_2 using template DNA extracted from different tissues of SP-SL affected sugarcane.

Plant portion for DNA extraction	Non-symptomatic tissues	SP-SL symptomatic tissues
Top of stalk	44.4	66.7
Leaf sheath	77.8	88.8
Middle of leaf	55.5	77.8
Apex of leaf	44.4	66.7

[1] Based on nine PCR observations.

Annealing temperature for the two sets of primers was crucial for accurate and effective amplification in PCR. Increased annealing temperature (60ºC) was effective for the primers P_1/P_2, in avoiding mispriming or misincorporation of nucleotides. For the primers SPP_1/SPP_2, a relatively low temperature (52ºC) was used to increase the efficiency with other optimized conditions.

Response of phytoplasma DNA for different primers

Two sets of primers P_1/P_2 and SPP_1/SPP_2 were able to generate easily-detectable bands in gel profiles at 557 bp and 321 bp levels respectively with the use of SP-SL DNA as the template. Plate 1 and 2 show the ability of SPP_1/SPP_2 to differentiate phytoplasma pathogens in other plants both graminaceous and non-graminaceous from SP-SL. Only DNA samples of sugarcane plants affected by SP-SL have given positive results with the specific primers SPP_1/SPP_2. The DNA of Rice affected by YDP, symptomatic *Brachiaria sp*. and symptomatic *C. viatis* gave positive results in addition to affected sugarcane when primed by P_1/P_2. On a few occasions, however, template DNA of rice affected by YDP generated very faint bands at 321 bp level with the primers SPP_1/SPP_2. Wongkaew *et al*. (1997) demonstrated that the SWLD phytoplasma and phytoplasma that infects *Brachiaria sp* in Thailand were closely related by RFLP and sequencing. The differentiation of SP-SL from the phytoplsma which affects *Brachiaria sp* support the finding of Wongkaew *et al* and it depicts the high level of specificity of the new set of primers used in this study. Therefore, the possibility of SP-SL being harbored in those weeds as alternative hosts can be ruled out.The finding is valuable in formulating management practices for the disease.

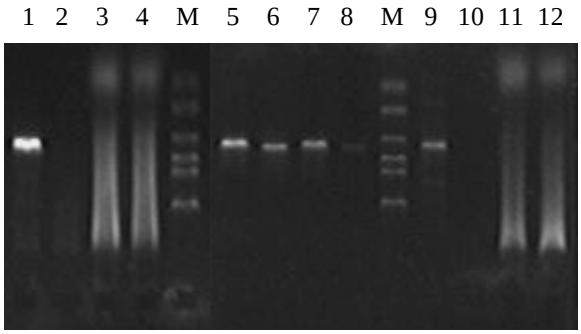

Plate 1. Amplification of template DNA extracted from sugarcane, rice and a few weeds with the primers P_1/P_2. M- DNA marker DL 2000 (fragment sizes- 2000, 1000, 750, 500, 250 and 100 bp), 1- sugarcane (infected with SP-SL), 2- sugarcane (healthy), 3- *C. viatis* (non-symptomatic), 4- *Brachiaria sp.* (non-symptomatic), 5- rice (infected with YDP), 6- *Brachiaria sp*. (symptomatic), 7- *C. viatis* (symptomatic), 8- *B. hispida* (symptomatic), 9- sugarcane (SP-SL symptom- masked), 10- water control, 11- rice (non-symptomatic), 12- *B. hispida* (non-symptomatic).

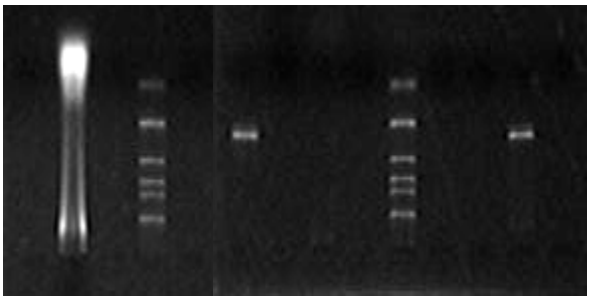

Plate 2. Amplification of template DNA extracted from sugarcane, rice and a few weeds with the primers SPP_1/SPP_2. M- DNA marker DL 2000 (fragment sizes- 2000, 1000, 750, 500, 250 and 100 bp), 1- sugarcane (healthy), 2- rice (infected with YDP), 3- *C. viatis* (non-symptomatic), 4- *Brachiaria sp.* (non-symptomatic), 5- sugarcane (infected with SP-SL), 6- rice (non-symptomatic), 7- *Brachiaria sp*. (symptomatic), 8- *B. hispida* (symptomatic), 9- *C. viatis* (symptomatic), 10- water control, 11- sugarcane (SP-SL symptom- masked), 12- *B. hispida* (non-symptomatic).

(Compare lane 5,6 and 7 of plate 1 with 2, 7 and 9).

CONCLUSIONS

The newly developed PCR assay is sensitive, reliable and accurate enough for the detection of SP-SL in plants, if DNA extractions and subsequent PCR conditions are

properly combined. The method would undoubtedly be helpful in obtaining etiological and epidemiological information about the disease and thus, disease management in the sugarcane industry of the country will be facilitated.

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