Detection of Huanglongbing (Citrus Greening) Disease by Nucleic Acid Spot Hybridization

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Polymerase chain reaction (PCR) amplification with primers specific to the rDNA region successfully amplified the 1160-bp DNA fragment from a Huanglongbing (HLB)-infected sweet orange sample with mottling symptoms leaves, but not from healthy sweet orange plants. The PCR product of 1160-bp was used as probe labeled with biotin for detection of the HLB pathogen in the nucleic acid spot hybridization (NASH) test. It was found that the HLB pathogen could be detected up to 1:100 dilution in HLB-infected tissue. Total DNA extracted from HLB-infected tissue was diluted 2-fold as 900 ng in TE buffer and spotted on a nitrocellulose membrane. Strong signals were observed up to 225 ng of DNA dilution, whereas a moderate signal was recorded at 112 ng. No hybridization signal was observed in the healthy samples, while strong signals were observed in the positive control.

Key words: Huanglongbing, PCR Detection, Nucleic Acid Spot Hybridization, Non-Radioactive Probes

Introduction

Sweet orange (*Citrus sinensis* Osbeck) cv. *Sathgudi* is an important commercial cultivar in South India, and Andhra Pradesh (A. P.) occupies the first place in cultivation of *Sathgudi*. In India A. P. tops in terms of area followed by Maharashtra, Punjab and Karnataka. Citrus fruits are rich in vitamins and minerals and, namely used as fresh juice, for flavouring dishes of vegetables, fish, meat and salads. The citrus species is prone to 150 diseases and disorders caused by fungi, viruses, bacterial and phytoplasmal infections. Bud-transmissible diseases like Huanglongbing (HLB), tristeza, citrus yellow mosaic, exo-cortis and citrus ring spot cause huge loss in fruit production in all the citrus-growing areas.

The HLB disease is easily transmissible by graft and psyllid vectors from citrus to citrus. It can also be transmitted by dodder, *Cuscuta reflexa*. The bacterium-like organism is restricted to phloem sieve tube elements. This bacterium has a membranous cell wall of the Gram-negative type and belongs to the subdivision of the Proteobacteria. HLB is caused by *Candidatus Liberibacter africanums* in Africa, *L. asiatiums* in Asia (Garnier *et al.*, 2000) and *Ca. L. americanus* in Brazil (Teixeira *et al.*, 2005). Two psyllid vectors transmit the HLB disease. The Asian form of the HLB disease is spread rapidly by the Asian psyllid *Diaphorina citri*, while the African form of the HLB disease is transmitted by the African psyllid *Trioza erytreae*. It is widely accepted that both species of bacteria multiply in both of the psyllid vectors, but this has not been demonstrated with molecular evidence. However, Moll and Martin (1973) noticed marked increases in the number of HLB bacteria in *T. erytreae* vectors over 9 days and concluded that bacteria were multiplied in these vectors.

Since the bacterial nature of the HLB organism was established, Jagoueix *et al.* (1996) used universal primers for general amplification of prokaryotic 16S rDNA. Based on the sequence information, primers have been developed to amplify a 1160-bp region of ribosomal DNA for detection of the HLB disease by polymerase chain reaction (PCR). Ribosomal DNA primers have been widely used for detection of all forms

of HLB. These primers have been shown not to amplify 16S ribosomal sequences of other citrus pathogens (Jagoueix *et al.*, 1996).

The HLB bacterium infects citrus trees of almost all cultivars and causes substantial economic losses to the citrus industry by shortening the lifespan. It is an important epidemic disease and is difficult to control in India and several other Asian countries. Furthermore, the HLB disease has a long incubation period and many latently infected citrus plants occur in the field (Mc Clean, 1970; Ahlawat *et al.*, 1995). The development of diagnostics for virus and virus-like diseases of citrus and the production of virus-free planting material is an important strategy and is the first step for the management of bud-transmissible diseases (Gopal *et al.*, 2001). However, the detection of this fastidious bacterium is difficult because of its non-culturability, its low concentration and uneven distribution in its natural hosts (Su and Chang, 1974). The disease is diagnosed by biological indexing on indicator hosts, which is time-consuming, and symptom expressions depend on temperature. The disease, therefore, cannot be diagnosed easily by conventional procedures such as electron-microscopic examination of ultra-thin sections and bioassay on indicator plants. As an alternative, a rapid and reliable detection protocol by PCR was developed. However, high levels of polyphenols and tannins in citrus leaves generally interfere with obtaining good-quality DNA and thus affect the reliable detection of HLB organisms by PCR. Therefore a method was developed in which sodium sulfite is added to the extraction buffer EDTA during extraction of DNA from HLB-infected leaf midrib tissue (Gopal *et al.*, 2004). HLB isolates, namely sweet orange (EF 552698) and acid lime (EF 552699), are cloned, sequenced and deposited in the Genbank. Based on the sequence of the isolates a set of primers was designed and the amplified product was used for preparation of biotin-labeled non-radioactive probes for detection of the HLB disease. The results are reported in the present paper.

Material and Methods

Detection of HLB PCR

The bud sticks collected from sweet orange cv. *Sathgudi* at All India Co-ordinated Research Project on Citrus, Tirupati, India were grafted on 1-year-old seedlings. The HLB culture was maintained on different citrus species seedlings in an insect proof glasshouse. Available glasshouse isolates of the HLB disease were also used for detection of the HLB disease by PCR and nucleic acid spot hybridization (NASH) test using the 726-bp HLB DNA fragment cloned in the pRTZ5 vector.

DNA extraction

The total DNA was extracted from midribs of HLB-infected sweet orange cv. *Sathgudi* and from healthy plants. The samples were ground to a powder in liquid nitrogen. 1 ml of extraction buffer (0.1 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1 M KCl, 0.65% sodium sulfite) heated to 95 ºC was added to the ground tissue in an Eppendorf tube and incubated at 95 ºC for 10 min with occasional agitation. The homogenate was placed on ice for 2 min and centrifuged for 10 min at 12000 × *g*. The supernatant was treated with Rnase (100 μg/ml) and the DNA was precipitated with 0.6 vol. of ice-cold isopropanol. After centrifugation, sterile distilled water was added to the precipitate, and the mixture was heated briefly to 65 ºC to completely dissolve the DNA. DNA was re-precipitated with 2 vol. of ethanol and 0.1 vol. of sodium acetate (pH 5.2) at –20 ºC for 2 h. DNA was isolated by the sodium sulfite EDTA method used for detection of the HLB disease by PCR amplification as reported by Gopal *et al.* (2007).

PCR amplification

PCR was performed in 50 μl reaction mixture, using 1 μM of each primer, 200 μM each of dNTPs, 0.05 U/μl of Taq DNA polymerase, 1× PCR reaction buffer, 1.5 mM of $MgCl_2$ and 6 μl of DNA template. The amplification was performed in a thermal cycler (Corbett Research, Mortlake, Australia). PCR conditions used were as follows: 1 cycle of 95 ºC for 5 min, 40 cycles of 95 ºC for 1 min, 52 ºC for 1 min, 72 ºC for 1 min, and 1 cycle of 72 ºC for 10 min. The PCR product was analyzed by 1% agarose gel electrophoresis in 1X TAE (tri-acetate-EDTA) buffer containing ethidium bromide, and the gel was observed under a UV transilluminator and photographed.

Detection of HLB by NASH

Non-radioactive labeling of DNA

Non-radioactive labeling of DNA was done using the biotin labeling kit and detection system

kit (Fermentas). All necessary reagents and buffers were provided in the kit, and the manufacturer's protocol was followed as given below.

Probe preparation

The IMP-agarose block containing the respective DNA fragments was cut from the gel and purified using the gel extraction kit (Eppendorf). The probe was prepared in 50 μl reaction mixture, using 10 μl of DNA template (100 ng to 1 μg), 10 μl of decanucleotide in 5× reaction buffer and 24 μl of distilled water. The tube containing the reaction mixture was vortexed and spun down in a micro-centrifuge for 3–5 s, incubated in a boiling water bath for 5–10 min and cooled on ice. 5 μl of biotin labeling mix and 1 μl of Klenow fragment (5 U/μl) were added to the tube; the mixture was shaken and spun down in a microcentrifuge for 3–5 s. The reaction mixture was incubated for 1 h at 37 ºC, and the reaction was stopped by adding 1 μl of 0.5 M EDTA.

TE extraction

The leaf material of HLB-infected and healthy citrus species was ground separately in TE buffer (1:1 w/v) and pressed through muslin cloth. 200 μl of the supernatant were collected, and the DNA was denatured by adding 0.5 M NaOH for 10 min. 25 μl of 3 M sodium acetate (pH 5) were added and dilutions of 1:1, 1:25, 1:50 and 1:100 in TE buffer were made.

Blotting

A piece of membrane was cut out, drew a lattice of 1 cm squares was drawn with a soft lead pencil and one edge to indicate the orientation was cut off. The membrane was soaked in 20X SSC for 30 min and placed on a sheet of Whatman 3 mm filter paper. 5 μl of each sample were pipetted carefully in each square of the membrane and the spots t allowed o dry at room temperature; the membrane was baked in a hybridization oven (HS II, Amerex Instruments Inc., Lafayette, CA, USA) at 80 ºC for 2 h.

Pre-hybridization

The membrane was kept in a hybridization bottle and pre-hybridization solution [6X SSC, 5X Debheart's reagent (0.1% (w/v) BSA, 0.1% (w/v) Ficoll, 0.1% (w/v) PVP), 0.5% (w/v) SDS and 50 μg/ml denatured herring sperm DNA] was added at the rate of 0.2 ml/cm^2. Then the bubbles were removed and the membrane incubated at 42 ºC for 4 h in a hybridization oven with gentle rotation.

Hybridization

The biotin-labeled DNA probe was denatured in boiling water for 5 min, added to pre-hybridization solution (25–100 ng/ml) and incubated at 42 ºC over night in a hybridization oven with gentle rotation. Thereafter the hybridization solution was discarded and the membrane washed twice with 2X SSC + 0.1% SDS for 10 min at room temperature with gentle rotation. Final washes were given twice with 0.1X SSC + 0.1% SDS for 20 min at 65 ºC with gentle rotation. Later the washed membrane was dried on filter paper and the detection procedure was performed using the detection kit.

Detection procedure

The membrane was transferred to the plastic tray containing 30 ml of the blocking/washing buffer mixture and incubated at room temperature for 5 min. Then the blocking/washing solution was discarded and 30 ml of the blocking solution were added. The mixture was incubated at room temperature for 30 min. Later the blocking solution was discarded, 20 ml of diluted streptavidin-AP conjugate were added and the mixture was incubated at room temperature for 30 min. Again the solution was discarded, 60 ml of blocking/washing buffer were added two times and the mixture is incubated at room temperature for 15 min. Later, the blocking/washing solution was discarded and 20 ml of detection buffer added; the mixture is incubated at room temperature for 10 min. The detection solution was discarded, 20 ml of substrate solution were added and incubated at room temperature in the dark overnight. The substrate solution was discarded, 30 ml double distilled water added and the membrane rinsed for a few seconds. Then the membrane was air-dried and stored at 4 ºC.

Results and Discussion

Isolation of good-quality DNA was done by adding sodium sulfite in Tris-EDTA at a content of 0.65% to the existing protocol and standardized, which appeared to be promising for the detection of the HLB disease by PCR. DNA was extracted from leaf midribs of sweet orange hav-

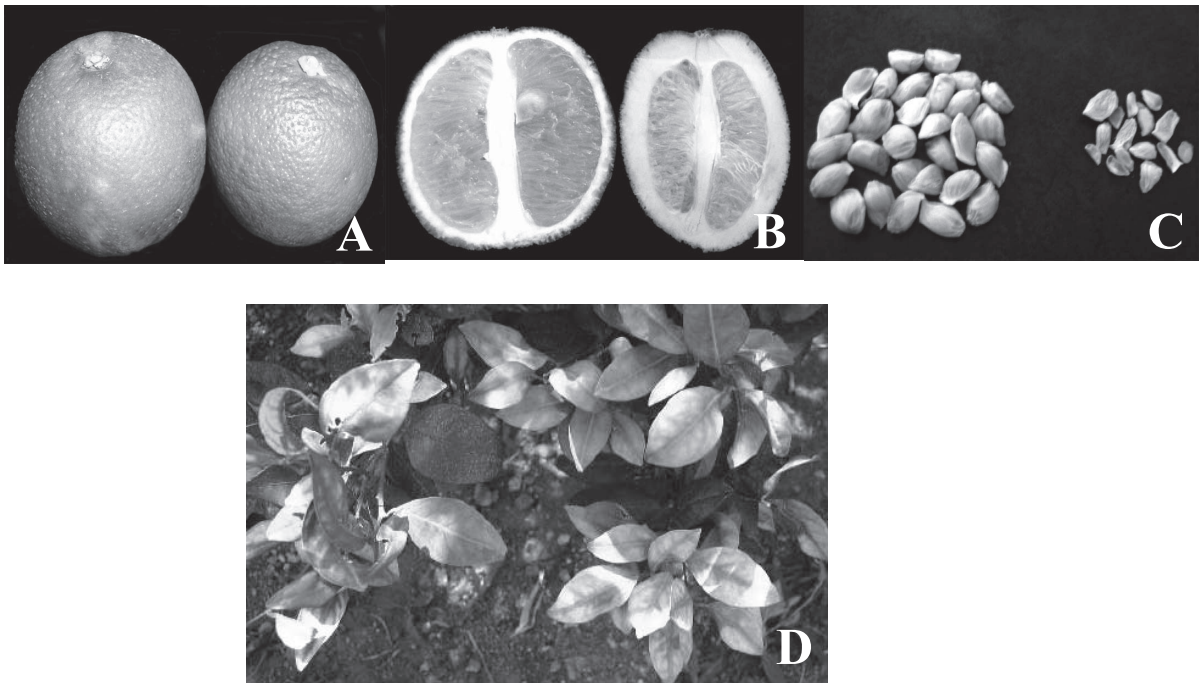

Fig. 1. (A) Healthy and HLB-infected sweet orange cv. *Sathgudi* fruits. (B) Longitudinal section of fruit showing lobsided growth. (C) Abortive seeds. (D) Mottling symptoms on *Sathgudi* sweet orange.

ing mottling symptoms (Fig. 1) and subjected to PCR for the detection of the HLB disease.

PCR amplification with primers specific to the rDNA region successfully amplified a 1160-bp DNA fragment from a HLB-infected sample, but not from healthy sweet orange plants which indicated the specificity of the primers (Fig. 2).

The PCR product of the HLB-infected sample was used for probe preparation. Different dilutions of infected tissue used in the NASH test showed that the HLB pathogen was detected in 1:1, 1:25; 1:50; 1:100 dilution (Fig. 3). 900 ng of total DNA were diluted 2-fold as 900 ng 450 ng, 225 ng, 112 ng, 56 ng in TE buffer and spotted onto a nitrocellulose membrane giving strong signals in 900 ng, 450 ng and 225 ng and a moderate signal in 112 ng dilution, whereas a low signal was observed in 56 ng. No hybridization signal was observed in the healthy sample used as negative control, while strong signals were observed in the positive control. It is clear that the developed biotin-labeled DNA probe may be used in the budwood certification programme successfully for the detection of HLB disease pathogens.

For diagnosis of the HLB disease identification of the best tissue source for isolation of quality DNA is an important aspect as the bacterium resides in the phloem tissue. The extraction of HLB DNA was carried out by a method wherein sodium sulfite was added in Tris-EDTA, which yielded improved HLB DNA (Gopal *et al.*, 2007). The presence of high levels of polyphenols and tannins in citrus leaves interferes with obtaining good-quality DNA, and thus affects the reliable detection of pathogens by PCR. The addition of sodium sulfite to Tris-EDTA (extraction buffer) could inhibit the effect of phenols and tannins on DNA, hence good-quality DNA was obtained, which in turn improved the amplification of DNA in PCR. In order to prevent interference of the polyphenolics in nucleic acid extraction the addition of sodium sulfite to the extraction buffer has reduced the degradation of DNA and improved the yields, quality and stability of DNA (Byrne *et al.*, 2001). Sodium sulfite-extracted DNA survived at various temperatures much longer than that extracted without addition of sodium sulfite (Baranwal *et al.*, 2003). Jagoueix *et al.* (1996), Nakashima *et al.* (1996) and Krishna Reddy (2000) used the

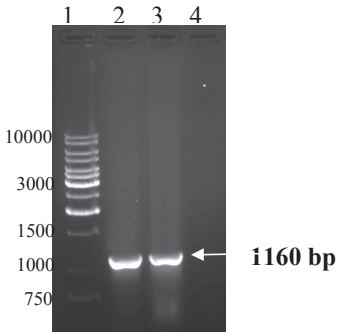

Fig. 2. Detection of HLB by PCR in sweet orange cv. *Sathgudi* with mottling symptoms using HLB-specific primers. Lane 1, marker (1-kb DNA ladder); lanes 2 and 3, sweet orange with mottling symptoms; lane 4, healthy.

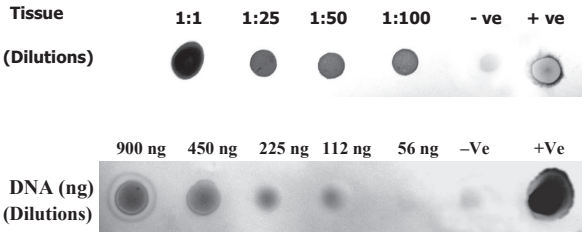

Fig. 3. Detection of HLB bacterium by nucleic acid spot hybridization using HLB-infected tissue and DNA extracted from HLB-infected sweet orange cv. *Sathgudi* (–ve, negative control; +ve, positive control).

PCR method for amplifying the 16S rDNA fragment of the HLB pathogen by using HLB-specific primers. Jagoueix *et al.* (1996) extracted DNA from both healthy and HLB-infected citrus leaves and subjected it to PCR amplification by using primers defined on the sequence of the 16S rDNA. Amplified DNA of the expected size 1160 bp was observed not only with citrus infected by *Liberibacter asiatius* from China, India, Indonesia, Nepal, Philippines and Taiwan but also with *L. africanus* from South Africa and Zimbabwe. Subandiyah *et al.* (2000) conducted agarose gel electrophoresis of DNAs amplified with primers of OI1 and OI2c and produced an amplified fragment of about 1,200 bp from the cv. Orlando tangelo which had been inoculated with one of the isolates OK 901, KINI, OKS7, OKS13 and OKS14.

Detection of plant pathogens by PCR in crude extracts is known to be difficult because of the presence of PCR inhibitors. To avoid this, capture of pathogens by antibodies (Jansen *et al.*, 1990) prior to amplification has been largely used. However, in case of uncultured *Liberibacter* of the HLB disease, this method cannot be applied, as many serotypes of the bacterium have been shown to occur and none of the monoclonal antibodies that have been produced so far is able to react with all *Liberibacter* serotypes (Garnier *et al.*, 1991; Gao *et al.*, 1993). This is why a procedure was developed to eliminate the PCR inhibitors in the plant extracts, a protocol based on differential centrifugation, use of antioxidants and dilution of the extract, which gave amplification of HLB DNA consistently enough for reliable diagnosis; similar results were also reported previously by Jagoueix *et al.* (1996) and Nakashima *et al.* (1996).

For effective and accurate detection of HLB, a very sensitive assay based on PCR using a pair of primers chosen from the sequence of the cloned HLB-specific DNA fragment has been developed (Jagoueix *et al.*, 1996; Nakashima *et al.*, 1996). The present study also showed that PCR-based diagnosis by using primers specific to rDNA and 16S–23S spacer regions, which were used in screening of budded plants for detection of HLB, is very rapid and specific. PCR amplification with primers specific to the rDNA region successfully amplified the 1160-bp DNA fragment from a HLB-infected sample, but not from healthy sweet orange plants, indicating the specificity of the primers. In the present investigation the total DNA was made into 2-fold dilution, and signals were detected up to a lower content of 112 ng. These results revealed the efficiency of probe to detect the presence of HLB at a low content of 112 ng. Diagnosis of HLB by PCR and NASH was found reliable, essential for screening of HLB-disease-free plants and to establish HLB-disease-free citrus nursery. These tests are used extensively for HLB diagnosis in bud-banks of sweet orange cv. *Sathgudi* at Citrus Research Station, Andhra Pradesh Horticultural University, Tirupati (A. P.), India.

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