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Detection of *candidatus liberibacter* asiaticus associated with citrus greening (huanglongbing) of mandarin by template preparation

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ABSTRACT

The polymerase Chain Reaction (PCR) diagnosis is more reliable and sensitive diagnostic tool for greening bacterium than other conventional approaches like Electron microscopy, DNA-DNA hybridization and immunofluorescence (IF) for detection of citrus greening. During experiment, it was observed that sodium sulphite method of DNA isolation provided higher yield and better quality DNA than other methods. Primer C (450 bp) was more efficient in amplifying the DNA of greening bacterium even at a very low concentration of 0.1 pg. To confirm the reliability of PCR, the greening bacterium was also detected in graft-inoculated plants, which showed typical greening bacterium was also detected in graft-inoculated plants, which showed typical greening symptoms. Results showed amplification of 450 bp in PCR suggesting sampling in March is more suitable for PCR detection of greening bacterium.

1. Introduction

Citrus belongs to family Rutaceae and has approximately 150 genera and 1600 species, which are found in tropical, temperate regions of the world. Globally citrus is grown in 114 countries. Out of these, 53 countries grow citrus commercially. On production basis, Brazil tops the list amongst citrus producing countries with 19.9 million tonnes followed by USA with 14.1 million tonnes, China with 12.1 million tonnes, Mexico with 6.4 million tonnes, Spain with 5.7 million tonnes and India 4.4 million tonnes at 6^{th} position.

Citrus is the third important fruit crop of India after banana and mango with the acerage of 5.63 lakh ha and production of 56.8 lakh tons and average productivity of 10.1 t/ha. The most important commercial citrus cultivars in India are, the mandarin (*Citrus reticulata* Blanco), followed by sweet orange (Citrus *Sinensis osbeck*) and acid lime (*Citrus aurantifolia* Swingle) sharing 65, 25 and 10 per cent, respectively, of all citrus fruits produced in the country. The state of Maharashtra is considered as the top most citrus growing state in terms of area (2.38 lakh ha) and second in terms of production (14.4 lakh tones) and 10^{th} in terms of productivity (6.0 t/ha) according to Indian Horticulture Database, 2005.

In India, the major pathogens of economic importance in citrus are citrus tristeza virus (CTV), citrus yellow mosaic badna virus (CYMV), indian citrus ring spot virus (ICRSV), Viroids diseases like citrus exocortis viroid and a fastidious prokaryote causing citrus greening disease. Citrus greening disease is an important disease of citrus which greatly affects the production of citrus fruits in several parts of India (Ahlawat, 1997). In India, the greening disease was first identified by L.R. Fraser in 1965 and its wide spread occurrence was confirmed by Varma *et al.* (1993), Ahlawat and Pant (2003).

Confirmation that a citrus tree is affected by greening has up to now relied on the electron microscopical identification of the bacterium, but due to erratic distribution of the pathogen in sieve tubes, it was cumbersome process to cut the right tissue having greening bacterium. However, several indirect approaches such as biological indexing, immunofluorescence tests with monoclonal antibodies and DNA-DNA hybridization with radioprobes have been used for greening diagnosis (Ahlawat and Pant, 2003). Biological indexing is a time consuming procedure and temperature dependent. It requires a well-equipped glass houses and long term maintenance of indicator hosts. The use of monoclonal antibodies for field diagnosis has proven unsatisfactory (Korsten *et al.*, 1993). Detection by DNA probes, though an accurate method for detection, but requires handling of radioactive elements and is being discouraged now a day. Moreover, these are not practically feasible methods for handling a large sampling unit. The recent study by Hocquellet *et al.* (2000) and Ahlawat *et al.* (2003) have shown that gene amplification of β operon ribosomal protein is a sensitive and promising technique for detection and differentiation of greening bacterium. However more work is required for standardization of detection of greening organism Polymerase Chain Reaction (PCR) and its application on large scale.

2. Materials and methods

2.1. Confirmation of infection in trees

The three trees showing typical symptoms of greening were identified and labeled. The symptomatic leaves from individual tree were taken as source material for extraction of DNA and confirmation of infection by PCR as described by Ahlawat *et al*., (2003).

2.2. Graft transmission

The scions from these six trees were grafted on mandarin sweet orange in the glasshouse. The observation for symptoms development in grafted plants was taken periodically and finally grafted plants were tested in PCR.

2.3. DNA extraction from citrus tissue

The total DNA was isolated from midrib and petiole of symptomatic leaves of infected field trees. Three methods of DNA extraction viz. DN-easy Plant Mini kit (QIAGEN, Germany), sodium sulphite (Baranwal *et al.*, 2003), Nucleic Acid Technique were employed.

2.4. DNA extraction protocol by commercial kit method (The protocol of QIAGEN was followed)

150 tissues from leaves of greening infected midrib were ground in liquid nitrogen in sterilized pestle and mortar. The tissue powder was transferred into 1 ml eppendorf tube. 400 µl of buffer AP1 and 4 µl of RNAse-A stock solution (100 mg/ml) to a maximum of 100 mg of ground plant tissue was added and vortexed vigorously. The mixture was incubated for 10 minute of 65^0C and was thoroughly mixed 2-3 times during incubation by inverting the tube. 130 µl of buffer AP2 was added to the lysate, mixed and incubated for 5 minute on ice. The tube was centrifuged for 5 minutes at 12000 rpm. The lysate was applied to the QIA shredder spin column (Lilac) sitting in 2 ml collection tube and centrifuged for 2 minutes at 12000 rpm. The flow through fraction was transferred to new tube without distributing the cell debris pellet. 1.5 ml volume of buffer AP3 was added to the cleared lysate and mixed by pipetting. 650 µl of mixture from above tube was transferred to DNase mini spin column sitting in 2 ml collection tube. It was centrifuged for 1 min at 6000 x g and flow through was discarded. The above step was repeated with remaining sample and flow though was discarded. The DNase column was placed in new 2 ml collection tube and 500 µl buffers AW was added to the DNeasy column and centrifuge for 1 min at 6000 x g. 500 µl of buffer AW was added to the DNeasy column and centrifuged for 2 min at maximum speed to dry the membrane. DNeasy column was transferred to 1.5 ml micro centrifuge tube and 50 µl of preheated 65^0C buffer AE was directly added into the DNeasy membrane and it was incubated for 5 min at room temperature and centrifuge for 1 min at 6000 rpm. The above step was repeated.

2.5. DNA extraction by sodium sulphite method (Baranwal *et al.*, 2003)

150 midrib of leaves were ground in liquid nitrogen. 10 ml extraction buffer was prepared (Appendix). 10 ml extraction buffer was heated to 65^0C before adding to powdered tissue. The powdered tissue was taken into eppendorf. 1 ml hot extraction buffer was added. The eppendorf containing powdered tissue in extraction buffer was kept at 95^0C heating block for 10 min. Regular vortexing after every 2 min was done. Kept on ice for 2 min. The tube was centrifuged at 12000 rpm for 5 min. Approximately 800 µl of supernatant was taken and transferred to new tube containing 5 µl of RNase. Incubated at 37^0C for 20 min. 480 µl of isopropanol was added to it and mixed by gentle rocking. The tube was centrifuged for 5 min at 12000 rpm. To the pellet, 30 µl of sterile distilled water was added. To dissolve, DNA pellet with distilled water was heated briefly at 50^0C and flicking was done. The DNA was precipitated with 30 µl of 3M solution acetate and $1/10^{th}$ volume of 95 per cent of ethanol. The tube was kept on ice-for 10-20 min. The tube was centrifuged for 5 min with 12000 rpm. The ethyl alcohol was poured off. The pellet was dried for 40 min at 37^0C. The pellet was dissolved in 70-100 µl of double distilled water, reheated 45-50^0C.

2.6. DNA extraction by using membrane bard nucleic acid technique (Baranwal, Gupta and Singh, 2007)

Take 100 mg of petiole and midrib of leaves tissue from cla infected plant were homogenized in 1 ml of alkaline solution of NaOH. The resulted extract were incubated at room temperature (24-32^0C) for 15 min or centrifuged at 1200 g for 10 min. 5 µl of sap were spotted on untreated NCM5 (BAS 85, poresize 0.45 µm Scwicher and Schuee, Kece, N.H.) that were dried for 30 min at 24-32^0C. Individual spot (4.0 mm) for each sample were cut out with paper hole punch (Kangaro industries, Ludhiana, India) and eluted in 30 µl of sterile distilled water by incubation at 80^0C for 10 min on a heat block. The liquid was collected by centrifugation (termed NCM eluted extract). Volume of 2.5, 5, 10 and 20 µl were used for detection of cla ANAs by PCR.

2.6.1. Primer synthesis

Pair of primer from conserved region of ribosomal β-operon gene and ribosomal DNA was synthesized and used to study the detection of citrus greening bacterium in PCR system (CG3450F) primer (Table 1).

2.6.2. Ploymerase enzyme

Polymerase enzymes (Taq polymerase) were evaluated for their efficiency to amplify and it was used with primer to compare their efficacy.

2.6.3. PCR amplification

The amplification was performed in thermal cycle using primer and polymerase enzyme (Taq) for comparing their efficiency in amplification of DNA of greening bacterium based on the number of sample amplified and intensity of amplified DNA band. The composition of various request and their volume used in PCR with their thermo cycling profile.

The conditions followed for PCR are as follows. Initial denaturation was given at 94° C for 5 minutes 1 cycle followed by denaturation at 94° for 30 seconds, annealing at 60° C for 30 seconds and extension of 72° C for 1 minute and the final extension was given at 72° C for 10 minutes 30 cycles.

Table 1
Specific primers used for amplification of ribosomal β-operon gene and 16s ribosomal DNA of citrus greening bacterium.

Primers	Primer sequence	Annealing temperature	Amplicon size
A	5'GCGCGTATCCAATACGAGCGGCA3' 5'GCCTCGCGACTTCGCAACCCAT3'	62^{0}C	1160bp
B	5'TATAAAGGTTGACCTTTCGAGTTT3' 5'ACAAAAGCAGAAATAGCAAACA3'	58^{0}C	703bp
C	5'TGGGTGGTTTACCATTCAGTG3' 5'CGCGACTTTCGCAACCCATTG'	58^{0}C	450bp

2.7. Analysis of PCR product by electrophoresis

Following PCR, amplicon were analyzed in 1 per cent agarose and electrophoresed in Tris-acetate EDTA (TAE) buffer containing ethidium bromide 0.5 g agarose was melted in 50 ml 1x TAE running buffer and 2µl ethidium bromide was added to it after cooling to around 50^{0}C and poured into a casting tray for polymerization placing the 12 well combs. The comb was removed after polymerization and the gel was then placed on electrophoresis tray filled with 1 x PAE buffer 20 µl each of PCR product mixed with 2 µl of 6 x loading dye was loaded into the well and was run at 60 volt for 30 min. An aliquot of 1 kb DNA ladder 4 µl was named with dye similarly and electrophoresed to serve as molecular weight marker. After the run the gel was observed under ultraviolet (UV) transillumina for and photographed on thermal paper using gel documentation system.

2.8. Validation of PCR for detection of greening bacterium

Following the standardization of DNA extract method primer and enzyme the technology was validated by taking 40 random sample collections from the apparently infected mandarin tree in orchard.

3. Results

3.1. Confirmation of the presence of greening in infected trees

The trees identified during survey and tested for the presence of greening by PCR. It was found that the pathogen was amplified from all the trees. The details of the PCR will be given in subsequent results. The leaves from these trees were collected and used in further experiments.

3.2. Graft transmission

Of the Six-PCR positive trees, grafted on mandarin plants showed typical greening symptoms in 3-6 months after grafting. The new leaves showed yellowing followed by mottling of leaves. Of the grafted plant, plants showed amplification of greening pathogen in PCR when tested after 6 months. However, since the studies were planned with a view to develop PCR diagnostic from field trees, enough leaf material was collected from individual PCR positive trees in the fields and stored at –80^{0}C in deep freeze and was used in subsequent studies.

3.3. DNA isolation from citrus tissue

Results (Table 2) revealed that the average DNA yield of three samples isolated by sodium sulphite method was 1216.16 ng/ µl. It was slightly higher in nucleic acid membrane method with average of 1429.16 ng/ µl. However, yield of DNA by kit method was only 159 ng/µl.

3.4. Determination of quality of DNA isolated by three methods

Results (Table 3) indicated that average value of DNA extracted by sodium sulphite method was 1.50 and by commercial kit was 1.54 while it was only 1.14 with DNA extractable by CTAB method.

Table 2
The quantity of DNA obtained from three infected sample by 3 method of DNA isolation.

Samples	Sodium sulphite (ng/ µl)	Commercial kit (ng/ µl)	Nucleic acid (ng/µl)
1	1531.0	123.25	535.0
2.	2864.0	201.00	1056.0
3.	986.5	165.00	2113
4.	796.0	124.75	1053
5.	552.0	207.50	2212
6.	568.0	136.50	1606
Average	1216.16	159.60	1429.16

Table 3
Quality comparison of three methods (A_{260}/A_{280}).

Samples	Sodium sulphite	Commercial kit	Nucleic acid membrane
1	1.06	1.05	1.14
2	1.53	2.86	1.02
3	1.13	1.05	1.01
4	2.54	1.44	0.99
5	1.76	1.60	1.55
6	1.01	1.27	1.15

4. Discussion

The presence of greening disease in India was suggested by Fraser *et al.* (1966). Since then, the disease has been reported from various parts of the country (Ahlawat, 1997). In the absence of reliable diagnostic reagents and tools like electron microscope, the actual incidence and distribution of citrus greening bacterium (CGB) in India could not achieved till 1991. During the period from 1991 to 1996, 51, 339 trees from 98 orchards were tested which were collected from 8 different states of India and presence of CGB was confirmed by electron microscopy, immunoflorescence and DNA-DNA hybridization technique (Bove *et al.*, 1993, Ahlawat and Pant, 2003). Subsequently, the PCR technology was developed for detection of CGB (Harakava *et al.*, 2000; Hocquellet *et al.*, 2000; Tian *et al.*, 1996; Bove *et al.*, 1993; Jagouiex *et al.*, 1996). However this PCR technique was used for first time to detect greening disease in citrus in India by Ahlawat *et al.*, (2003). Serodiagnosis was not found effective as it requires a panel of strain-specific monoclonal antibodies and due to sacrifice of animals in production of monoclonal antibodies and due to sacrifice of animals in production of monoclonal antibody, it is now being discouraged all over the world. The nucleic acid hybridization technique required radioactive material and is not advisable if other efficient and reliable techniques like PCR are available. Therefore, during the present investigation, the information has been developed on suitability and reliability of PCR technique for detection of greening bacterium as a routine procedure of indexing. There was not information available of characterization of Asian greening bacterium by amplification, cloning and sequencing although reports are three of African greening bacterium (Planet *et al.*, 1995). During present study, trees of mandarin of the age of 3-10 years were periodically surveyed at the orchards of Marathwada region. A very high incidence of 65.00 per cent was observed. The greening incidence information in these investigation, was determined on the basis of typical symptoms of greening disease in field trees and the analysis of six candidate trees by polymerase chain reaction (PCR) using protocol. Six trees were identified based on PCR reaction for these studies. The leaves from these trees were collected and preserved at 80^0C in deep freeze for the use in various experiments. The graft transmission was also obtained from this tree on Mosambi seedling after 3-6 months of grafting. However, only 4 plants out of six grafted, showed positive amplification in PCR. This may happen because the scions grafted on these two PCR negative plants may not be having the required quantity of pathogen required for graft transmission. It is known that the distribution of greening bacterium is erratic in the plant tissues (Varma *et al.*, 1993; Bove *et al.*, 1993) and this could be the reason of non transmission in plants by grafting. Since the studies were planned with a view to

standardize and validate PCR diagnostics in field trees, the materials collected from six identified greening positive trees were used in these studies.

Three steps are important for detection of pathogen in PCR. They are: a) Nucleic acid isolation and its quantity and quality b) Primer designing synthesis and its evaluation c) Evaluation of polymerase enzymes for PCR amplification. During the present studies all the three steps were standardized. The DNA was isolated by three methods, sodium sulphate (Baranwal *et al.*, 2003). CTAB method (Murray and Thompson, 1980) and commercial kit obtained from QIAGEN Germany. The leaf material collected from 6 PCR positive trees was used in most of the experiments. The quantity of the DNA obtained from six samples was more CTAB method followed by Sodium sulphite method. Similar results were obtained. Commercial kit and CTAB method while working with potato and a cherry virus and Baranwal *et al.* (2003) with citrus yellow mosaic virus. However, the yield of DNA by commercial kit (159.60 ng/ml) was much less as against sodium sulphate method (1216.16 ng/ml). The quality of DNA obtained by all the three methods was assessed by calculating A260/A280 ratio and it was observed that quality obtained by Sodium Sulphite method and commercial kit was almost at par (1.50 and 1.54, respectively). Although the best quality of DNA is known with the A_{260}/A_{280} of 1.8. However, this ratio has not been achieved with the any of the methods. But the satisfactory amplification was obtained even of the DNA extracted by sodium sulphite method with the A_{260}/A_{280} ratio of 1.50. That is why this method was used in the experiment too. Since the quantity and quality of DNA isolated by sodium sulphite method was found to be better, this method was preferred over the methods of commercial kit and nucleic acid membrane method.

Although during the present studies, primers were not designed, but they were synthesized based on the published sequence data by Harakava *et al.*, (2000). Three parts of primers from conversed region of ribosomal β operon gene and 16 S rDNA were got synthesized by Sigma, Germany. These three pairs of primers were designated as A, B, C which provides amplicon size as 1160 bp, 703 bp and 450 bp, respectively. These three sets of primers were evaluated with different dilution of the greening bacterial DNA. The comparative studies show that C sets of primer (450 bp) was superior than A and B as it could amplify the DNA upto 100 pg as against only 100 ng and 10 ng dilution of DNA with A and B sets of primers. The evaluation was also done using Taq and Klen Taq enzymes and Klen Taq as was expected gave better intensity of the bands obtained in 1 per cent agarose gel. Similar evaluation for primer has also been used by some worker with different viruses (Singh and Nie, 2003; Harakava *et al.*, 2000).

During the present study, the DNA extraction method by sodium sulphite, Klen Taq enzyme and 450 bp set of primers were identified to give best amplification in PCR system of diagnosis. Using these protocols, samples from mandarin tree collected in March, 2010 were analyzed in PCR using combination of all the three standardized steps. It was found that the amplification of DNA isolated from out of twenty samples, 15 and 19 were PCR positive during the month of October and March, respectively. During the survey of greening diseases 65.00 per cent of incidence of disease was observed while testing trees for the presence of greening bacterium in PCR, 15 were positive during October and 19 in March indicating much higher incidence of disease with PCR testing. The results suggested that the PCR is reliable technique for detection of greening bacterium as against the diagnosis based on visible symptoms. It was therefore evident that protocols developed during present studies can detect greening bacterium in field trees irrespective of the season and the concentration of pathogen in the host. However, since more samples showed the presence of greening bacterium during March, the samples for detection of the bacterium should be taken in March to get maximum detection.

Detection of viruses and virus like pathogens as by PCR technique are gaining importance over the other technique as it is comparatively sensitive and reliable and can detect the pathogen even at a very low concentration upto 0.1 pg. The concentration of greening bacterium is very low in sieve tubes and distribution is also uneven (Varma *et al.*, 1993) and it is important that the samples are taken from different direction of the tree. The technology of PCR detection of greening bacterium developed during present investigation is new and validated for the first time in India. It will be very useful for field diagnosis of greening disease in planting material. The technology developed is cost effective and highly reliable for indexing bud wood certification programmes in citrus in India elsewhere. These studies will also be useful in plant quarantine for export and important of citrus germplasm.

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