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# Polymerase chain reaction detection of *candidatus liberibacter* asiatic associated with citrus huanglonbing

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ABSTRACT

Polymerase chain reaction diagnosis of *Candidatus liberibacter* asiatic associated with citrus Huanglonbing disease is molecular technique which is used for detection of disease when pathogen present is very low concentration in disease sample. Among these three DNA isolation methods *viz*., commercial kit method, sodium sulphite method and membrane bard nucleic acid technique, sodium sulphite method is cost effective for commercial use. In nucleic acid membrane method for DNA extraction isolation there is no use of liquid nitrogen. Polymerase chain reaction detection of disease is based on principal of thermal cycling in which PCR instrument allow to run generally 60-65 thermal cycle, during PCR operation it allow different stages of cycle at different temperatures for different period of time i.e. initiation ($94^0$C), denaturation ($94^0$C), primer annealing ($60^0$C), extension/elongation step ($72^0$C), final elongation ($72^0$C) and holding temperature ($4^0$C). PCR based diagnosis system is developed for detection of greening bacteria. The comparative cost of detection by various combinations of reagent and sampling time was determined and cost effective technology was standardized and validated.



## 1. Introduction

Citrus belongs to family Rutaceae and has approximately 150 genera and 1600 species, which are found in tropical, temperate and arid region of the world. In India, total area under cultivation is 165.5 million ha, of which 15.3 million ha is under fruits and vegetables. The citrus is third largest fruit crop in India which occupies an area of around 0.56 million ha with production of 4.58 million tonnes. The Indian productivity of citrus is 8-9 tonnes per hectare, which is very low when compared with world average productivity of 25-30 tonnes per hectare (Economic survey, 2003-04). Disease is known as one of the important factors in low productivity of citrus fruits in India (Ahlawat *et al.*, 1996).

Among the diseases of citrus, viral diseases cause heavy economic losses in varying proportion. Around 30 viral diseases are known to infect citrus worldwide. In India, the major pathogens of economic importance in citrus are *Citrus tristeza* (CTV), Citrus yellow mosaic badna virus (CYMV), Indian citrus ring spot virus (ICRSV), viroids disease like citrus exocortis viroid and a fastidious prokaryote causing citrus greening disease (Ahlawat, 1997). In India, the greening disease was first identified by Fisher in 1965 and its wide spread occurrence was confirmed by Varma *et al.* (1993), Ahlawat and Pant (2003). With the discovery of mycoplasma like organisms (MLOs), the microorganism was later thought to be mycoplasma like organism (MLO) but subsequent studies provided it to be bacterial in nature (Garnier and Bove, 1993) and called as bacteria like organisms (BLO).

Biological indexing is a time consuming procedure and temperature dependent. It requires a well-equipped glass house 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 in Polymerase Chain Reaction (PCR) and its application on large scale. During the present study, diagnostic procedure PCR was developed for detection of citrus greening bacterium and it was standardized for improving sensitivity and reliability than known before.

## 2. Materials and methods

Symptoms can occur throughout the tree especially if the infection occurs during or soon after propagation (McClean, 1974). In general, leaf symptoms are of two types. Primary symptom is characterized by yellowing of normal mixed leaves along the vein and somchime, by the development of blotchy mottle (Schneider, 1968) with secondary symptom. In India using the diagnostic technique of electron microscopy ELISA and DNA-DNA hybridization it has been established that molting of leaves is authentic symptoms of greening disease (Varma *et al.*, 1993). Infected fruit are small lopsided and have a bitter taste (McClean, 1974) probably because of higher acidity and lower sugar.

### 2.1. DNA extraction from citrus tissue

The DNA was isolated from midrib and petiole of symptomatic leaves of infected field tree. Three method of DNA extraction *viz.* DNA-easy plant mini kit (QIAGEN, Germany), Sodium sulphide (Baranwal *et al.*, 2003) and membrane base nucleic acid technique.

### 2.2. DNA extraction protocol by commercial kit method (The protocol of QUAGEN was followed)

150 mg tissue from leaves of greening infected midriff were ground in liquid nitrogen in sterilized pestle and mortar. The tissue power was transferred in 1 ml eppendorf tube. 400 µl of buffer AP1 and 4µl of RNAase a stock solution (100 mg 1 ml) to a maximum of 100 mg of ground plant tissue was added and vortexed vigoursly. The mixture was incubated for 10 minute at $65^0$C and was thoroughly mixed 2-3 times during incubation by inverting the tube. 130µl of buffer AP2 was added to the lysate, mixed and incubated for 5 minute of ice. The tube was centrifuged for 5 minutes at 12000 x g. 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 volume of buffer AP3 was added to the cleared lysate and mixed by pipetting. 650µl 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 through was discarded. The DNase column was placed in new 2 ml

collection tube and 500 µl buffer 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 membrane. DNeasy column was transferred to 1.5 ml micro centrifuge tube and 50 µl of preheated $65^0$C 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.

**2.3. DNA extraction by sodium sulphite method**

150 mg midribs of leaves were ground in liquid nitrogen. 10 ml extraction buffer was prepared (Appendix). 10 ml extraction buffer was heated to $65^0$C 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 $95^0$C 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 $37^0$C for 20 min.480 µl of isoporpanol was added to it and mixed by gentle rocking. The tube was centrifuge 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 $50^0$C and flicking was done. The DNA was precipitated with 30µl of 3 M sodium 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. 400 µl of 70 per cent ethanol was added and vortexing was done. Centrifugation for 3 min at 12000 rpm was carried out. The supernatant was poured off. The pellet was dried for 40 min at $37^0$C. The pellet was dissolved in 70-100 µl of double distilled water; pre heated 45-$50^0$C (Baranwal *et al.*, 2003).

**2.4. DNA extraction by using membrane bard nucleic acid technique**

Take 100 mg of petiole and midrib of leaves tissue from cla infected plant were homogenized in 1 ml of alkaline solution of NaOH ELISA. The resulted extract were incubated at room temperature (24-$32^0$C) 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-$32^0$C. Individual spot (4.0 mm) for each sample were cut out with paper hole punch (Kangaro industries, Ludiana, India) and eluted in 30 µl of sterile distilled water by incubation at $80^0$C 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 (Baranwal, Gupta and Singh, 2007).

**2.5. Quantification of DNA**

The purified total DNA was diluted in double distilled water 5 µl DNA of each sample was diluted in 495 µl of double distilled water for recording uv absorption at 260 nm and 280 nm. Recording were recorded and average value of sample was calculated. The yield of DNA per µl was calculated using formula.

$$\text{ng of DNA per µl} = \frac{\text{UV absorption at 260} \times \text{dilution factor} \times 50}{1000} \times 100$$

Quality of DNA was evaluated based on A260/A280 ratio for each sample and average value was calculated.

**2.6. Approximately cost evaluation of each method of DNA extract**

The cost per sample was calculated of all the three method based on the chemical used by each method except the commercial kit. The cost per sample from the kit was calculated on the basis of total cost of the kit.

**2.7. 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.

**2.8. Ploymerase enzyme**

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

**2.9. PCR amplification**

The amplification was performed in thermal cycle using primer and polymerase enzyme (Tag) 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 requests and their volume used in PCR with their thermo cycling profile. Volume required (1 sample) for amplification mixture used for PCR with tag enzyme are Distilled water 24.5µl, 10x buffer 5µl, 10 mm dNTPs 1µl, (CG 3450 F) Primer F 2µl, (CG 3450 R) primer R 2µl, Taq polymerase enzyme 0.5µl and DNA 15µl.

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

**2.10. Analysis of PCR product by electrophoresis**

Following PCR, amplicon were analyzed in 1 per cent agarose and electrophoresis 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 comb. 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 loaded into the well and was run at 60 volt for 30 min. A aliquot of 1 kb DNA ladder 4 µl was named with dye similarly and electrophoreses to serve as molecular weight marker. After the run the gel was observed under ultraviolet (UV) transillumina and photographed on thermal paper using gel documental system.

**2.11. 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 mosambi tree in orchard.

**3. Results and Discussion**

Results revealed that the total DNA was successfully extracted by all the three method. But quality of DNA was different by each method. The quantification (Table 1) of isolated DNA was done by formula mentioned in the material and method. The average yield of all three method of DNA extraction was taken into consideration.

**3.1. Determination of quality of DNA isolated by three methods**

Results (Table 2) indicated that the quality of DNA extracted by each method was tested by calculating A260/A280 ratio of each sample. The average value of DNA extracted by sodium sulphite method was 1.50 and by commercial kit was 1.54 while 1.14 with DNA extracted by nucleic acid membrane method.

**3.2. Cost comparison**

The cost was approximately calculated as shown in Table 3 that the cost of isolation of DNA per sample was only Rs. 0.78 by sodium sulphite, Rs. 1.25 per sample by nucleic acid membrane method and Rs. 140 per sample by commercial kit.

**Table 1**
Quantity of DNA obtained from infected sample by 3 method of DNA isolation.

| Sample | Sodium sulphite (ng/µl) | Commercial Kit (ng/µl) | Nucleic acid membrane method |
|---|---|---|---|
| 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 | 2312 |
| 6 | 568.0 | 136.50 | 1608 |
| Total | 12165.16 | 159.60 | 1429.16 |

**Table 2**
Quality comparison of three methods (A260/A280).

| Sample | Sodium sulphite (ng/µl) | Commercial Kit (ng/µl) | Nucleic acid membrane method |
|---|---|---|---|
| 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 | 1.55 |
| Total | 1.50 | 1.54 | 1.14 |

**Table 3**
Approximately cost involved in DNA isolation per sample by 3 method.

| Sodium sulphite method | | Nucleic acid membrane method | Commercial kit method |
|---|---|---|---|
| Chemical | Cost | | |
| Extension buffer | 0.14 | Untreated NCM NaOH EDTa | |
| Isopropanol | 0.10 | | |
| 3 m NaoAc | 0.14 | | |
| Ethanol (600 µl) | 0.20 | | |
| RNase (5 µl) | 0.20 | | |
| Total | 0.78 | 1.25 | 140 |

### 3.3. Validation of standardized PCR amplification technique for greening disease

It has been established through earlier experiment that following combination did the best diagnosis of citrus greening disease. 2 µl of template DNA extracted by sodium sulphate method and nucleic acid membrane. Use of Tag enzyme at rate of 0.25 µl/25 µl reaction mixture. Primer (CG 3450 F) at the rate of 0.5 µl per 25 µl reaction mixture.

The presence of greening disease in India was suggested by Fraser *et al.*, (1966). Since, then, 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 be achieved till 1991. With the establishment of Advanced Centre for Plant Virology at the Indian Agricultural Research Institute (IARI), New Delhi, the serological and molecular techniques for detection of greening bacterium were developed.

During the period from 1991 to 1996, 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; Hung *et al.*, 2004). 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 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 no information available on characterization of Asian greening bacterium by amplification, cloning and sequencing although reports are three for African greening bacterium (Planet *et al.*, 1995).

During present study trees of Mosambi sweet orange of the age of 3-10 years were very high incidence of 65 per cent was observed. The high incidence of greening disease has also been reported by Ahlawat and Sardar

(1975) from Darjeeling hills, Ahlawat (1997) and Verma *et al.* (1993) from other citrus growing states. The greening incidence information in these investigations was determined on the basis of typical symptoms (Plate 1) of greening diseases in field trees and the analysis of candidate trees by Polymerise Chain Reaction (PCR) using protocol of Ahlawat *et al.*, (2003). Trees were identified based on PCR reaction for these studies. The leaves from these trees were collected and preserved at $80^0$C in deep freeze for the use in various experiments. However, only some plants showed positive amplification in PCR. It is known that the distribution of greening bacterium is erratic in the plant tissue (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 diagnostic in field trees, the materials collected from 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 sulphite (Baranwal *et al.*, 2003). Nucleic acid membrane method and commercial kit obtained from QIAGEN Germany. The leaf material from PCR positive trees was used in most of the experiments. Similar results were obtained by Singh *et al.*, (2003) of commercial kit and 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 it (159.60 ng/ml) was much less as against Sodium sulphite method (1216.16 ng/ml). The quality of DNA obtained by all the three methods was also 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 $A_{260}/A_{280}$ 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 membrane based nucleic acid extraction. Further, an approximate cost per sample was calculated of all the 3 methods of DNA isolation and it was interesting to know that the cost per sample by sodium sulphite method was only Rs. 0.75 as against Rs. 1.25 per sample by nucleic acid membrane method and Rs. 140 by commercial kit. Therefore, the protocol developed for DNA isolation by sodium sulphite method was not only superior but also very cost effective. Such comparative studies have not been reported as yet. Two polymerase enzymes, Tag and Klen Tag were also compared and evaluated using DNA template obtained by three methods described earlier and it was apparent from experiment. The per cent amplification was obtained by Tag as it is a mixture of two DNA polymerase with proof reading activity. Therefore, its sensitivity was more than Tag and this also does not require addition of $MgCl_2$ separately in the amplification reaction mixture like Tag polymerase.

During the present study, the DNA extraction method by sodium sulphite, Klen Tag enzyme and 450 bp set of primers were identified to give best amplification in PCR system of diagnosis. Using these protocols, samples from 40 more Mosambi tree collected once in January, 2010 and another 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 40 samples, 32 and 38 were PCR positive during the month of January and March, respectively. During the survey of greening diseases, 65 per cent of incidence of disease was observed while testing 40 trees for the presence of green bacterium in PCR, 15 were positive during January 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 protocol 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 by PCR technique are gaining importance over the other techniques as it is comparatively sensitive and reliable and can detect the pathogen even at a very low concentration upto 10 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 and elsewhere. These studies will also be useful in plant quarantine for export and important of citrus germplasm.

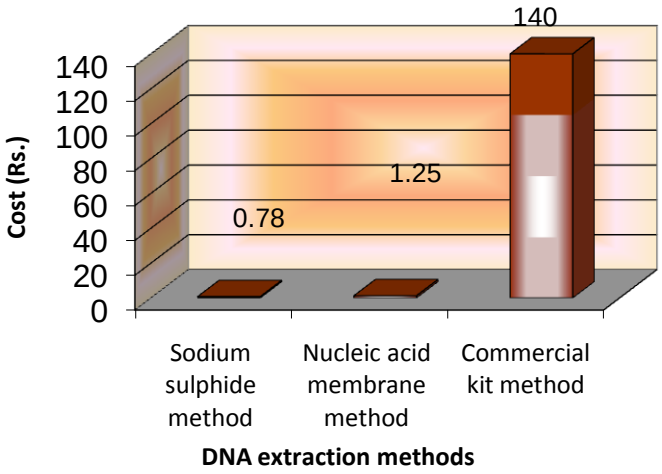


**Fig. 1.** Cost required for the different DNA extraction method.

**Plate 1.** Confirmation of presence of citrus greening in infected trees.

**4. Conclusion**

Citrus greening disease caused by Candidatus Liberibacter asiaticus, a fastidious bacterium, is one of the important diseases of citrus in India and elsewhere. The polymerase Chain Reaction (PCR) diagnosis is more reliable and sensitive diagnostic tool for greening bacterium than other conventional approaches like Electron Microsocpy, DNA-DNA hybridization, and immunofluorescence (IF) for detection of citrus greening. Confirmation that a citrus tree is affected by greening has up to now relied on the electron microscopial 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). Among these three DNA isolation method is sodium sulphide is cost effective for commercial use. The comparative cost of detection by various combinations of reagents and sampling time was determined and cost effective technology was standardized and validated.

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