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Detection of *Ca. Liberibacter* associated with Huanglongbing (HLB) in citrus plants-*Citrus Medica* (Lemon), *Citrus Indica* (Orange), *Citrus Reticulata* (Narangi)

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Abstract

This study aimed at the detection of *Candidatus liberibacter asiaticus* in the citrus plants viz *Citrus Medica* (Lemon), *Citrus Indica* (Orange), *Citrus Reticulata* (Narangi). The infected plant samples *Citrus Medica* (Lemon), *Citrus Indica* (Orange), *Citrus Reticulata* (Narangi) were collected from the nurseries of Kozhikode (Kerala), Bangalore and Jaunpur (U.P) respectively. The sample processing was then done by a series of washing of roots with 70% ethanol, 8% sodium hypochlorite and sterile distilled water. Aliquots from five consecutive washing was collected and spread onto TYA media. Bacteria were identified using various biochemical tests i.e., Oxidase test, Nitrate reductase test, McConkey test and Gram staining. The culture was then used for bacterial DNA isolation. The qualitative and quantitative determination of the extracted DNA was then done. The amplified product was then sequenced and the sequence obtained was further analyzed using BLAST (www.ncbi.nlm.nih.gov/BLAST/)and CLUSTALW2 (www.ebi.ac.uk) tool. The study helped to identify the Ca.Liberibacter bacteria in the samples from the infected plants. Thus study can be used to derive that yellowing of leaves in citrus plants viz., *Citrus Medica* (Lemon), *Citrus Indica* (Orange), *Citrus Reticulata* (Narangi) are caused by the Asian form species i.e *Candidatus Liberibacter Asiaticus.*

Keywords: *Amplification,alignment,Ca.liberibacter, citrius plants, huanglongbing, phylogenetic tree.*

1. Introduction

Huanglongbing (HLB), ex citrus greening, is one of the most serious diseases that occur in citrus. The symptoms include leaf mottling, yellowish shoots, and, frequently, small and lopsided fruit. It is caused by a phloem-limited, gram-negative; nonculturable bacterium designated "*Candidatus Liberibacter*", a member of the alpha- subdivision of the phylum Proteobacteria. Two forms of HLB disease are known, African and Asian [1]. The African form is caused by a heat-sensitive and Trioza erytreae–vectored bacterium designated *"Ca. L. africanus"*, whereas the Asian form is caused by a heat-tolerant and Diaphorina citri vectored bacteria named "*Ca. L . asiaticus*". Both insect vectors can naturally transmit their respective form of bacteria, which infect all Citrus spp. and cultivars. The disease causes severe losses in the production of sweet orange (Citrus

sinensis), mandarin (C. reticulata), lemon (C. limon), grapefruit (C. paradisi), and other economically important citrus species [2]. Various DNA amplification methods, including conventional polymerase chain reaction (PCR), real-time PCR, nested PCR, and loop-mediated isothermal amplification, have been used to detect greening-infected plants [3][4], Conventional PCR is the preferred method because it is inexpensive and easy to perform. Improved conditions and Las-specific primers for conventional PCR are often reported and applied. In particular, conventional PCR using the Las-specific primer set Las606/LSS, which targets a specific part of Las 16S ribosomal DNA, is a highly sensitive and robust method for Las detection [5]. Polymerase chain reaction (PCR) diagnosis is the more reliable and sensitive diagnostic tool for detecting the greening bacterium, (Candidatus Liberibacter) than other conventional approaches like DNA-DNA hybridization, electron microscopy, and immunofluorescence for the detection of citrus greening. Results revealed that sodium sulphite method of DNA isolation provides highest yield and better quality DNA than other previous methods [6]. Many genes involved in the key ecological processes such as carbon fixation, nitrogen cycling, metal homeostasis, phosphorus utilization, were significantly greater in health than in the 'Ca. L. asiaticus' infected citrus rhizosphere. Their results showed that the microbial community of 'Ca. L. asiaticus' infected citrus rhizosphere has shifted away from using there more easily degraded sources of carbon to more recalcitrant forms. Overall, their study provides evidence that the change in plant physiology is mediated by 'Ca. L. asiaticus' infection which could elicit shifts in the composition and functional potential of the rhizosphere microbial communities. These fluctuations might have important implications for the productivity and sustainability of citrus producing agro ecosystems [7].

2. Materials and Methods:

Plant materials:

Roots of citrus plants showing HLB symptoms were collected in different geographic regions of Kozhikode (Kerala), Bangalore and Jaunpur (U.P.).

Sample Processing:

Sample processing was done using three different solvents of different percentage. 70% ethanol, 8% Sodium hypochlorite and Sterile Water were used for the processing of roots infected with HLB disease.

Biochemical Tests:

Different Biochemical tests were performed to identify the bacterial culture. Oxidase test, McConkey test, Nitrate Reductase test, Kligler's Iron Agar Test, Motility agar test were performed for this study on the cultures obtained on Tryptone Yeast Agar. Gram staining was also done for morphological study of the bacteria.

DNA extraction and Quantification:

DNA extraction was done from the bacterial culture broth (Nutrient broth) using phenol:chloroform, Tris-HCl, detergent Sodium dodecyl Sulphate, Tis-EDTA.

A 0.8% agarose gel stained with ethidium bromide was used to visualize DNA. Quantification of extracted DNA was done spectrophotometrically at 260/280 nm ratio.

PCR Amplification:

Extracted DNA were amplified by PCR using primer pairs:
forward, 5´CCTTGAACAGGTGGAGGCCAG-3´, reverse, 5´-GCGGTGAGAGTGGGGTGGAG-3´, The PCR reaction (total volume 20 µl) was launched with a mixture containing 5µl genomic DNA, 5µl dNTPs, 1µl of each primer and 0.5µl Taq DNA polymerase which was added into 3µl of PCR buffer and added the double distilled water to make up the total volume. PCR conditions were as follows: Initial denaturation step at 94 °C for 5 min, 30 cycles at 94 °C for 30 s denaturation temperatures, 62 °C for 45s annealing temperature, and at 72 °C for 45 s extension temperature. The final extension step was performed at 72 °C for 5 min.

Sequencing and Sequence Analysis:

The amplified PCR product was then sequenced using Sanger's Method. The percent sequence identity was determined using BLAST (www.ncbi.nlm.nih.gov/BLAST/) and phylogenetic tree construction done by using the ClustalW2 program (www.ebi.ac.uk/Tools/msa/clustalw2/).

3. Result & Discussion

The infected plant samples Citrus Medica (Lemon), Citrus Indica (Orange), Citrus Reticulata (Narangi) were collected from the nurseries of Kozhikode (Kerala), Bangalore and Jaunpur (UP) respectively.

Fig 1: Citrus medica plant showing symptoms of Yellowing of Leaves (b) Citrus indica plant showing leaves in which diseased part is feeded by Trioza

In the present study, the processing was done using successive washing with ethanol, sodium hypochlorite and sterile distilled water.

Fig 2: Roots of infected plant (a) after running tap water wash (b) immersed in 8% Sodium hypochlorite

The culture obtained on TSA media was used for biochemical tests. The isolated bacterial species shown positive result for Mac Conkey Test, hence all the bacterial species which are gram negative grew on the media. In case of oxidase test, change of color from blue to purple occurred due to production of cytochrome oxidase. Red color ring form, reduction of nitrate to Nitrite gav a positive test for nitrate reductase test. Positive test of kliger iron test as red slants were seen. Positive result for Motility agar test as diffused zone of Growth was observed. Gram staining showed pink stained bacteria i.e. Gram negative bacteria.

The bands of DNA were observed on 0.8% agarose gel. And band for amplified PCR product were observed on 1.2% gel in gel documentation system.

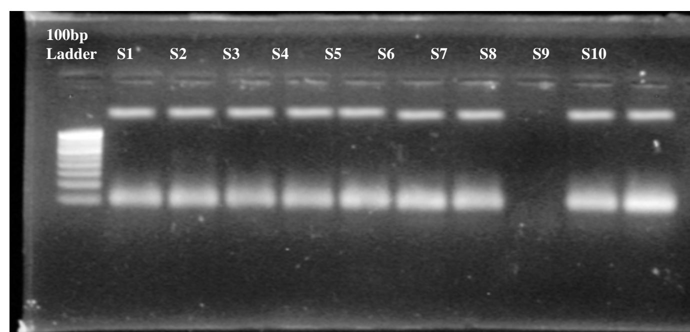

Fig 3: DNA isolated from bacterial culture run on a 0.8% gel stained with ethidium bromoide and analyzed in Gel Doc System. S1-10: bacterial culture samples

The PCR product was sequenced and the sequence received was analyzed to construct the phylogenetic tree and calculate the maximum identity with the query sequence from database sequences.

GACGGGTGAGTAACACATATTGGCAGTCCTCGAGAGAC
TAAGGTTTTTCTACGGGATAACGCATGGAAACGTGTGC
TAATACCGTATACGCCCCATTGGAGGAAAGATTTTATT
GGAGAGAGATGAGCCTGCGTTGGATTAGCTAGTTGGTA
GGGTAAGAGCCTACCAAGGCTACGATCTATAGCTGGTC
TGAGGGGACGATCAGCCACACTGGGACTGAGACACGG
CCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGG
ACAATGGGGGCAACCCTGATCCAGCCATGCCCATATAT
AAAAGGGTCCTCTCTCTTTGGGAAATTCTCTCTCCGGGT
GTAAAGCTCTTTCGCCGGGGAAGATAATGACGGTATTC
GGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGC
GGTAATACGAAGGGGGCGAGCGTTGTTCGGAATAACTG

GGCGTAAAGGGCGCGTAGGCGGGCGATTAAGTTAGAG
GTGAAATCCCAGGGCTCAACCTAACGGATGGAACTGCC
TTTAATACTGGTTGTCTAGAGTTTAGGAGAGGTGAGTG
GAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGG
AGGAACACCGGTGGCGAAGGCGGCTCACTGGCCTGATA
CTGACGCTGAGAACTTCGGCTGCTGGTAATTCCCTACA
CCCTACAAATTCCGGGGCAGCTCAGAAGTCCACGCCGT
AAACGATGAGTGCTAGCTGTTGGGTGGTTTACCATTCA
GTGGCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAG
TACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGG
GCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATG
CAACGCGCAGAACCTTACCAGCCCTTGACATGTATAGG
ACGATATCAGAGATGGTATTTTCTTTTCGGAGACCTTTA
CACAGGTGCTGCATGGCTGTCGTTTGGGTACTTTATAGG
GACTGCCGGTGATAAGCCGGAGGAAGGTGGGGATGAC
GTCAAGTCCTCATGGCCCTTATGGGCTGGGCTACACAC
GTGCTACAATGGTGGTTACA

A BLAST search for homologies revealed that the given sample sequence exhibited 92% identity with *Ca. Liberibacter africanus* (GU991650.1).To further characterize the "*Ca. Liberibacter*" the nucleotide sequences obtained were aligned with the *maximum identity hit* sequences of "*Ca. Liberibacter* africanus" isolates. A phylogenetic tree was constructed using the maximum-parsimony method. Interestingly, the group was divided into three subgroups corresponding to the two "*Ca. Liberibacter*" species.

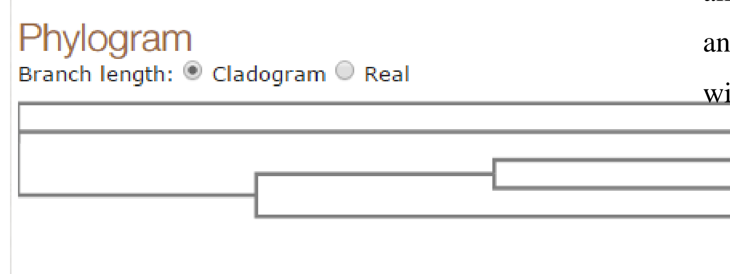

Fig 4: A phylogenetic tree constructed using CLUSTALW2 showing relationship among Ca. Liberibacter species

4. Conclusion:

The objective of this study was the detection of the bacterium *Candidatus Liberibacter asiaticus* in the Citrus plants viz *Citrus Medica (Lemon), Citrus Indica (Orange), Citrus Reticulata (Narangi).* From the earlier studies, it was observed that this bacterium is responsible for the yellowing, notching and mottling of leaves, seed abortion, fruit colour alteration in citrus plants. For the present study, we took the extracts from roots of the three infected citrus species i.e. Medica, Indica and Reticulata. The infected plant amples Citrus Medica (Lemon), Citrus Indica (Orange), Citrus Reticulata (Narangi) were collected from the nurseries of (Kozhikode) Kerala, Bangalore and Jaunpur (U.P) respectively. The sample processing was done and spread onto TYA media. The plates were grown overnight at 28ºC for 72 hrs. The samples gave positive results for all the tests performed. A gram negative bacteria was observed. The culture was then used for bacterial DNA isolation and bands were observed on 0.8% gel. The amplified product was then sequenced, analysis was done using BLAST and multiple sequence alignment using CLUSTALW2 tool, the alignment and tree construction showed a maximum identity with *Ca.Liberibacter asiaticus*.

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