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Molecular Detection and Characterization of Little Leaf Disease in Brinjal

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A nested Polymerase Chain Reaction (PCR) was optimised to detect the phytoplasma pathogen of brinjal showing little leaf symptoms. The second round primers amplicon was then cloned with pGEMT vector and fully sequenced. A 1200 bp DNA fragment encoding the portion from 16S rRNA gene of brinjal phytoplasma consistently amplified by universal primers and 890 bp from second round specific primers. The amplicon was cloned and sequenced. Nucleotide analyses of the fragment (GQ184436) showed that the gene was closely related to members of *candidatus* phytoplasma. The sequence identity similarity with members of the clover proliferation group (16Sr VI) was >99%, while the sequence identity with members of other groups (16Sr VI Elm yellows group) showed 97-98 % similarity. On the basis of sequence identity and phylogenetic relationship studies, it was concluded that the phytoplasma infecting brinjal in Tamil Nadu, India belonged to the clover proliferation group.

Key words: 16S rRNA Gene of phytoplasma, Brinjal, Little leaf disease, Nested PCR

Brinjal or eggplant (*Solanum melongena*), is an important vegetable in Indian cuisine. Brinjal crop in field is infected by a number of diseases. Among these, the little leaf disease caused by phytoplasma is very important because, it causes extensive loss to yield. This pathogen is transmitted by the leaf hopper *Cestius* (*Hishimonus*) *phycitis* and *Amrasca biguttula* (Munyaneza *et al.,* 2006; Choueiri *et al.*, 2007; Sertkaya *et al.,* 2007).

The leaves of the infected plants in the early stages are light yellow in colour. The plants shows chlorosis in young leaves followed by axillary bud proliferation. The affected plants show reduction in the above ground plant parts such as leaves and nodes, giving a bushy appearance. In severe infections, the plants become sterile and flower and fruit setting is negligible. The affected plants are severely stunted.

The disease is caused by Phytoplasma: wall-less prokaryotes, living as obligate parasites that are among the smallest and simplest self replicating organisms. It is a phloem-inhabiting organism at low concentrations and has an uneven distribution in woody plants. They cannot be cultured *in vitro* in cell-free media or isolated from the relatively small amount of phloem tissue in plants (Weintraub and Beanland, 2006). So detection and characterization of phytoplasmas is very complicated (Seemuller, 1998). The molecular tools are very important for detection of phytoplams. Group-specific primers have been applied to detect mixed-phytoplasma infections in a single host plant. A modified PCR named recycled PCR, was also developed to enable the detection of mollicute-specific DNA fragments and phytoplasma-specific or group-specific DNA fragments as multiple bands. Nested-PCR assays show higher specificity and increased over 10-fold detection sensitivity allowing ready detection of phytoplasmas from all infected woody hosts and insect vectors (Lee *et al.,* 2000). In some other crops, the nested PCR assay was used for detection of phytoplasmas (Botti and Bertaccini, 2007; Rao *et al*., 2008).

The objective of the present study was to detect the phytoplasma in little leaf disease infected brinjal in Tamil Nadu, India through molecular methods.

Materials and Methods

Sample collection

Malformed leaves were collected from symptomatic plants of little leaf infected brinjal in Tamil Nadu, India. In addition, healthy plants were collected from the same location to serve as a negative control.

DNA extraction from brinjal leaf samples

Modified CTAB method (Warokka *et al.,* 2006) was used for the extraction of total DNA from leaf samples of brinjal for detecting phytoplasma.

An infected sample (0.4 g) was incubated for 10 min in 2 ml of Phytoplasma Grinding Buffer (PGB) (100 mM K_2HPO_4, 30 mM KH_2PO_4, 10 % sucrose, 0.15 % bovine serum albumin fraction V, 2 %

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polyvinyl pyrrolidone, 25 mM ascorbic acid) in a mortar which was maintained in ice and then finely crushed with a pestle, adding 1.25 ml more of PGB. The homogenate was centrifuged for 5 min at 2,500 rpm. The supernatant of each sample was transferred into clean tubes and centrifuged for 15 min at 15,000 rpm. The pellet was dissolved in 1 ml CTAB buffer (2 % CTAB, 100 mM Tris pH 8.0, 1.4 M NaCl, 20 mM EDTA). After one-hour incubation at 65°C, the nucleic acids were purified by chloroform-isoamyl alcohol (24:1) extraction and an equal volume of ice cold isopropanol was added to the drawn aqueous phase and then incubated in ice for 1 h. After centrifugation at 10,000 rpm for 10 min, the pellet was dissolved in 400 ml of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) to which 40 ml of 3 M sodium acetate and 0.9 ml of 95 % ethanol was added. After incubation for 2 h at -20°C, the mixture was centrifuged for 10 min at 10,000 rpm. Once the supernatant was eliminated and the tubes were washed with 80 % ethanol, the pellet containing the DNA was dissolved in 35 µl of sterile water. The integrity of DNA was checked by using 0.8 % agarose gel electrophoresis.

Amplification of 16S rRNA gene of phytoplasma by nested PCR

The nested PCR approach was followed to detect the phytoplasma present in the disease affected brinjal samples. All the nested-PCR reactions were performed with a uniform concentration of ingredients to a total volume of 20 ml in 0.2 ml PCR tube. The mixture containing DNA 25 ng/ml, dNTPs (2.5 mM), forward and reverse primer (0.5 µM), 10X assay buffer, *Taq* polymerase (3 units), magnesium chloride (1.5 mM) and sterile distilled water. All the PCR assays were performed in a thermal cycler (Eppendorf gradient master cycler, Germany) with the following conditions in nested PCR with R16F2n/R16R2 primers (95ºC for 5 min followed 35 cycles of initial denaturation 95ºC for 1 min, annealing 60ºC for 2 min, extension 72ºC for 1min and final extension of 72ºC for 10 min) followed by fU5/rU3 (92ºC for 1min 30 sec followed by 35 cycles of initial denaturation 92ºC for 30 sec, annealing 57ºC for 30 sec, extension 72ºC for 50 sec and final extension of 72ºC for 10 min) (Lorenz *et al*., 1995). An aliquot of 20 µl PCR products were analyzed by horizontal gel electrophoresis through 1.5% agarose with ethidium bromide (0.5 µg/ml) using TAE buffer as the running buffer. The PCR samples mixed with 2 µl of gel loading buffer (0.25 % Bromophenol blue + 40 % Sucrose in 100 ml of water) were then loaded in gel slots. The gel was visualized on a UV trans illuminator and photographed in the gel documentation system.

Cloning of PCR products

The amplified 16S rRNA gene was purified using spin columns (Quiagen). The purified products were ligated into pGEMT ®vector (Promega) following manufacturer instructions. Five microliters of the ligation mixture was transformed to high efficiency competent cells of *Escherichia coli* (DH 5á) and plated on Luria-Bertani (LB) medium containing ampicillin (Amp) (100 ìg/ml), 5-bromo-4-chloro-3-indolyl â-D-galactopyranoside (X-Gal) (20 mg/ml) and isopropyl â-D-thiogalactopyranoside (IPTG) (10 mg/ml). The transformants with insert (white colonies) were selected on LB+Amp plates and plasmid purified by the miniprep method using a Plasmid extraction kit (Invitrogen). The presence of the insert was confirmed by restriction digestion with EcoR1 (Fermentas, USA), which released the insert DNA from plasmid.

Sequencing and analysis of 16S rRNA sequences of phytoplasma

The plasmids DNA of several samples were submitted to the 1 stBase laboratory (Malaysia) for sequencing. The resulted sequences, after correction using Bioedit software, were searched for sequence homology using BLAST search against the GenBank Database (http://www.ncbi.nih.gov/BLAST). The related phytoplasma 16S rRNA gene sequences retrieved from genbank database were used for phylogenetic analysis. Using nearly the full length of the sequence, the phylogenetic tree was constructed using the neighbor-joining method, and the tree was evaluated by performing boot-strap values at 1000 times. The validation of the phylogenetic tree was reconfirmed by constructing the tree using the maximum likelihood and parsimony methods with mega 4 software (Kumar *et al.,* 2004).

Results and Discussion

In the present study, the work was mainly concentrated on the detection of phytoplasma at early stages of infection on brinjal plants using the nested PCR approach. The PCR product amplified from universal primers (R16F2n/R16R2) was used as template for the nested PCR using the primers fU5/rU3. The PCR amplified a product of universal primer at 1200 kb and nested PCR second round primer at 890 bp from little leaf infected brinjal leaves

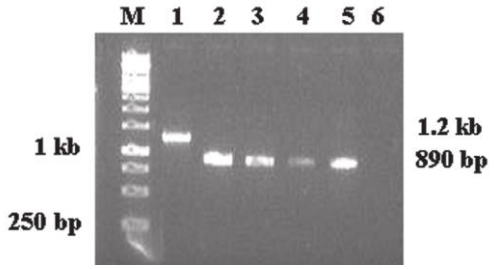

Fig. 1. Nested PCR amplification of 16S rRNA gene of phytoplasma from naturally infected brinjal plants

Lane: M- 1 Kb ladder, Lane 1: Little leaf infected plant sample (First round PCR), Lanes 2-5: Little leaf infected plant sample (Second round Nested PCR), Lanes 6: negative control (Healthy plant sample)

(Fig 1). There was no amplification obtained from the healthy brinjal leaf samples.

The most important requirement for the effective diagnosis of phytoplasma is a quick, reliable and cost-effective protocol. A number of methods have been developed to detect a wide variety of phytoplasmas. The comparative ease to use, speed and sensitivity makes them versatile tools and allows many applications to study phytoplasma. For sensitive detection of phytoplasma, primers based on mollicutes 16S rRNA genes have been used to selectively amplify the phytoplasma DNA from mixtures with host DNA (Myrie *et al.*, 2007).

Sometimes single amplification is difficult or impossible to detect in phytoplasma from woody hosts such as fruit and ornamental tree species (Wei *et al*., 2007; Hodgetts *et al.,* 2009). The availability of highly sensitive molecular biology techniques, such as nested PCR enables efficient detection of phytoplasma in host tissues (Ammar *et al.,* 2005). The organism can be identified by using initially amplified DNA segments as a template for further PCRs (*i.e.* nested PCR) using primers that amplify DNA from only particular phytoplasma.

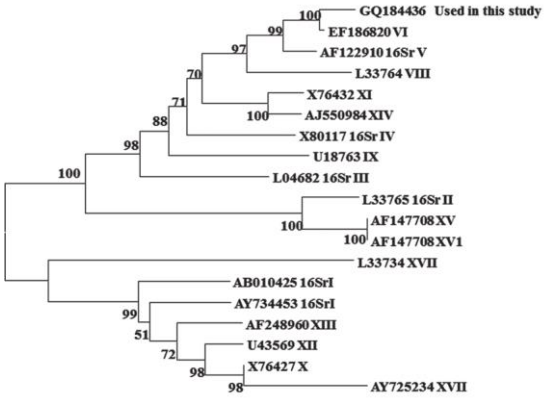

Fig. 2. Dendrogram constructed by the neighbor-joining method, showing the phylogenetic relationships between the brinjal little leaf phytoplasma with other groups phytoplasmas constructed based on 16S rRNA samplings. 16S rRNA groupings are based on the classification system of Wei *et al*. (2007)

The use of nested PCR enables the detection of secondary phytoplasma or cryptic phytoplasma in a given species in cases of mixed infection. In the absence of evidence for mixed infection study, it is safe to conclude that yellowing is caused by a single phytoplasma (Botti *et al.,* 2007). Nested PCR assay proved to be more specific than universal PCR and permitted detection of phytoplasmas in some trees before the onset of noticeable symptoms. Some DNA samples tested negative by universal PCR and then positive by nested PCR, may be an indication of the presence of contamination with other components at concentration inhibitory to PCR specificity of the oligo nucleotide primers, which are important to avoid the false positive results. Nested PCR employing primer pairs derived from ribosomal or non-ribosomal DNA sequences have been used to detect the little leaf of *Withania somnifera* phytoplasma from leaves (Khan *et al.,* 2006).

The 16S rRNA gene of little leaf phytoplasma from brinjal leaves was cloned into the T/A vector of pGEMT®and transformed into *Escherchia coli* strain DH5á.The PCR products obtained from the little leaf affected brinjal leaves were cloned and sequenced on both orientations. The elucidated sequence of the little leaf phytoplasma were deposited in NCBI with accession number (GQ184436).

The nucleotide sequence analysis of the 16S rRNA gene revealed that brinjal little leaf phytoplasma is very closely related to the brinjal little leaf phytoplasma (Group VI) (Fig 2). The current brinjal little leaf phytoplasma sequence GQ184436 and other brinjal little leaf phytoplasma (EF186820) shared a 16S rRNA sequence similarity of 99 per cent. The DNA gene sequence levels of brinjal little leaf phytoplasma from Tamil Nadu, India are placed under the clover proliferation group. This technique could be effectively used for detection, characterization and diagnosis of little leaf phytoplasma of brinjal.

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