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Characterization of pathogen responsible for infection in bhindi plant by using phytoplasma specific universal primers

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Infection in bhindi plant (*Abelmoschus esculentus,* Family-*Malvaceae*) is very common in Gorakhpur district of Eastern U.P. region of India. To detect the pathogen, genomic DNA was isolated from the infected bhindi plant sample and amplified through polymerase chain reaction (PCR) by using P1 forward primer and Tint reverse primer, which were phytoplasma specific universal primers. Obtained PCR amplicons were cloned and sequenced. BLAST analysis discovered their 91% identity with the members of 'Uncultured bacterium'. Phylogenetic tree also make their relationship with uncultured bacterium. Obtained nucleotide sequence was submitted to NCBI through accession number KF663567. To the best of our knowledge, this is the first report of "uncultured bacterium" (473 bp), from Gorakhpur district of Eastern U.P. region of India, associated with infection in bhindi plant.

Key words: *Abelmoschus esculentus*, Gorakhpur district, Uncultured bacterium, Acc. No. KF663567.

INTRODUCTION

Abelmoschus esculentus (L.) (Moench) belonging to the family *Malvaceae*, usually known as bhindi, is one of the important vegetable crops grown in tropical, subtropical and warm regions (Charier, 1984). This is a rich source of iodine. The states, Uttar Pradesh, Assam, Bihar, Orissa, Maharashtra, West Bengal and Karnataka are the major producers of this vegetable (Prakasha et al., 2010). bhindi, exported from India as a fresh vegetable, comprises 70% of the total fresh vegetable earnings, apart from onion (Anonymous, 2000). There are several pathogens, which causes 20 to 30% total loss of this vegetable (Hamer and Thompson, 1957).

Plants infected by phytoplasma shows a variety of symptoms (Bertaccini, 2007; Bertaccini and Duduk, 2009). During survey period, we observed symptoms of leaf distortion, leaf curling and overall stunting of infected bhindi plant which make suspicion for phytoplasmal infection. This infection adversely harms the bhindi pods production and their quality. So, in the present study, we tried to identify pathogen causing leaf distortion, leaf

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Table 1. PCR Components used in present study.

PCR components (concentration)	Volume (µl)
Template DNA	5.0
P1 forward primer (10 µM)	1.0
Tint reverse primer (10 µM)	1.0
10X PCR buffer	2.5
50 mM $MgCl_2$	0.75
10 mM dNTP mixture	0.5
Taq DNA Polymerase (5 U/µl)	0.2
Sterile distilled water	14.05

Table 2. PCR conditions during the present study.

Temperature (°C)	Time (min)	Cycles
95	4	1
94	1	35
56	1	
72	1	
72	10	1
4	∞	

curling and overall stunting of bhindi plants of Gorakhpur district of U.P., India.

To our knowledge, there is little work done on molecular characterization of pathogen responsible for infection in bhindi plant of Gorakhpur district of U.P., India.

MATERIALS AND METHODS

Plant samples

Infected bhindi (*A. esculentus*) plant specimens, showing possible symptoms of phytoplasamal infection, that is, leaf distortion, leaf curling and overall stunting were collected from Gorakhpur district.

DNA extraction

There are series of different extraction procedures performed for phytoplasmal DNA. Each procedure of extractions involved the collection of sufficient plant material to perform the DNA extraction. Here, we followed procedure published by Ahrens and Seemüller (1992) and included a phytoplasma enrichment step. An amount of 1.5 g of infected plant material was incubated for 10 min in 8 ml of phytoplasma grinding buffer in a mortar maintained on ice, and then finely crushed with a pestle, adding 5 ml of PGB. The homogenate was then centrifuged for 5 min at 2,500 *g*. The supernatant of each sample was transferred to clean tubes and centrifuged for 25 min at 18,000 *g*. The pellet was dissolved in 1 ml CTAB buffer. After 1 h incubation at 60°C, the nucleic acids were purified by chloroform-isoamyl alcohol (24:1), and centrifuged at 12,000 *g* for 10 min. An equal volume of cold isopropanol was added to the drawn aqueous phase, and then incubated in ice for 1 h and then centrifuged at 12,000 *g* for 10 min. After centrifugation, 1 ml 70% ethanol was added and centrifuged at 12,000 *g* for 10 min. Supernatant was decanted and the pellet dried at 37°C for 30 min. DNA was dissolved in 30 µl of sterile water.

Phytoplasma grinding buffer (PGB)

The phytoplasma grinding buffer contained 100 mM K_2HPO_4, 30 mM KH_2PO_4, 10% sucrose, 0.15% bovine serum albumin fraction, 2% polyvinylpyrrolidone-10 and 25 mM ascorbic acid.

CTAB buffer

The CTAB buffer contained 2% CTAB, 100 mM Tris pH 8, 1.4 M NaCl and 20 mM EDTA.

Target gene

Target gene was 16s-23s rRNA spacer regions.

Primers used in the study

Primers used in the study were universal phytoplasma specific primer pair, which were P1 forward primer: 5'AAGAGTTTGATCCTGGCTCAGGATT3' and Tint reverse primer: 5'TCAGGCGTGTGCTCTAACCAGC3'.

PCR setup

Genomic DNA from the test samples was PCR amplified using the PCR components as mentioned in Table 1. The reactions were cycled using a 2720 thermal cycler (Applied Biosystems) according to the PCR conditions mentioned in Table 2.

Agarose gel electrophoresis of PCR products for confirmation of PCR amplification

After PCR is completed, the PCR products were checked on 1% Agarose by Agarose Gel Electrophoresis and amplicon size was compared using reference Ladder. 1% agarose gel spiked with ethidium bromide at a final concentration of 0.5 µg/ml was prepared using agarose (LE, Analytical Grade, Promega Corp., Madison, WI 53711 USA) in 0.5X TBE buffer. 5.0 µl of PCR product was mixed with 1 µl of 6X Gel tracking dye. 5 µl of g Scale 1000 bp size standard (geneOmbio technologies, India) was loaded in one lane for confirmation of size of the amplicon using reference ladder. The DNA molecules were resolved at 5 V/cm until the tracking dye was 2/3 distance away from the lane within the gel. Bands were detected under a UV trans illuminator. Gel images were recorded using BIO-RAD GelDocXR gel documentation system. The PCR product of size 1500 bp was generated through this reaction.

Cloning of PCR products

PCR product obtained from PCR analysis of bhindi plant was gel eluted using Invitrogen Gel DNA purification kit as per the manufacturer's instructions. This product was then ligated to TOPO vector and cloned in TOP 10 *Escherichia coli* ultra competent cells. The transformants were selected by following a blue white screening procedure. The putative recombinant clones were confirmed by colony PCR using M13 PCR primers. The recombinant clone confirmed by having the insert from the phytoplasma PCR was subjected to sequencing. The sequencing was performed using Tint Primer.

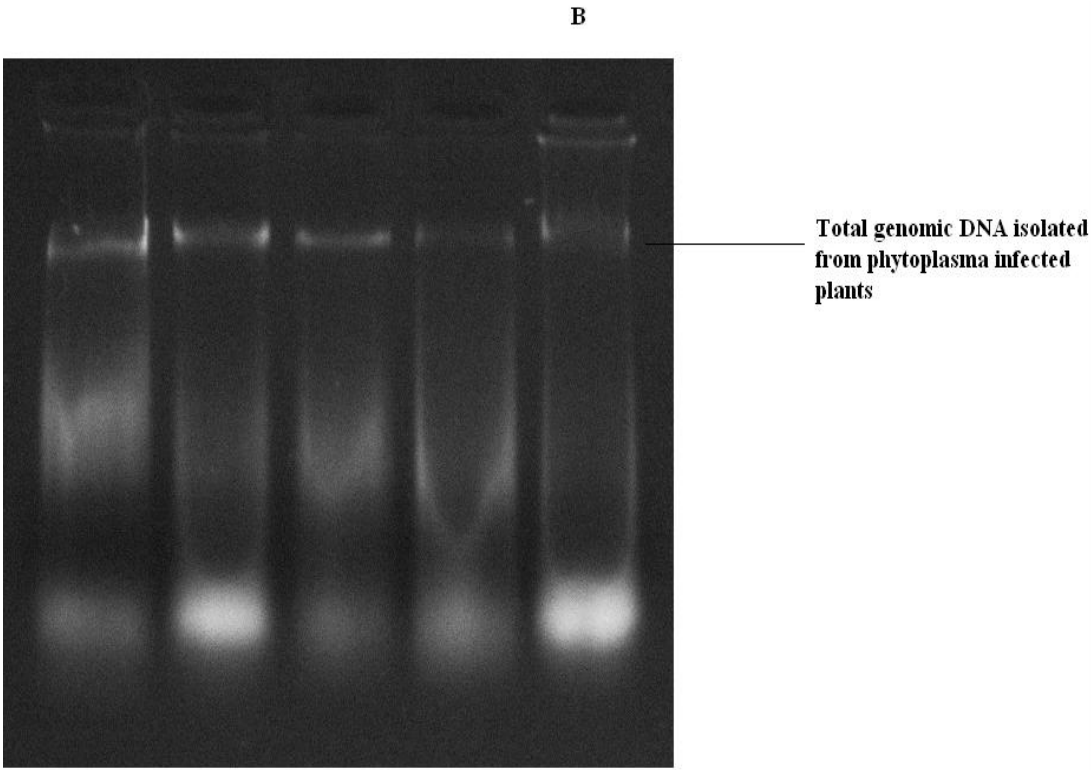

Figure 1. Genomic DNA QC image (1% agarose (w/v) gel electrophoresis). Well No. B, bhindi infected.

DNA sequencing

Using the gene specific sequencing primers and ABI BigDye® Terminator v3.1 Cycle Sequencing reaction kit (Applied Biosystems, USA), the insert DNA was sequenced.

BLAST analysis

BLAST analysis was conducted on the finally obtained sequence at http://blast.ncbi.nlm.nih.gov/Blast.cgi by using BLASTN 2.2.28+ program (Stephen et al., 1997). Sequence alignment was performed by sing clustalW sequence alignment tool available at http://www.genome.jp/tools/clustalw/.

Phylogenetic analysis

Genetic distance in the tree was calculated by default using Blast tree viewer. Finally, the obtained sequence was submitted to GenBank. The sequence generated from the present study and reference strains sequence retrieved from GenBank were used for phylogenetic analysis.

RESULTS AND DISCUSSION

During the course of survey for suspected phytoplasmal diseases in vegetable plants of different parts of Gorakhpur District, we were observed several plants. Among them, maximum suspected plant samples were collected for further characterization of their causative pathogen. PCR reactions with P1 and tint primer pairs resulted in the production of the PCR product of size approximately 1500 bp (Figures 1 and 2), which were further cloned and sequenced: Finally obtained nucleotide sequence (473bp) was deposited into NCBI (Acc. No. KF663567).

Total 473 bp: 1 gtcgtcagct cgtcccgtga ggtgttaggt taagtcctat aacgagggca acccctgttt; 61 ttagttgcca gcacgtaatg ttgggaactt taacaagact tccggtgaaa actgtgagga; 121 agggggggat gaggtcaaat tatcacggcc cttacgtcct gggccacaca cgtggtacaa; 181 taggcggtcc agagagcagc tacctagtga taggatggga atctataaaa ccgttctcag; 241 tttggatcgg agtttgcaac ttgactccgt gaagctggaa ttgctagtaa taggatatca; 301 cccatgatcc ggggaatggg ttcccgggcc ttgtaaaccc cgcccgtcaa accgtggaag; 361 ctggggttac cagaagtcgg tgaccgcaag gagtttccta gggtaaaact ggtaactagg; 421 gataagttta aacaagtaac tagtaccgga aggagcgcct ggaacacctc ctt.

Detected organism having 473 bp from total amplified PCR product (1500bp) indicates that amplifiable gentic materials were presented into them, but in smaller fragments.

The 16S rDNA sequences obtained from amplified and cloned products were analyzed by multiple sequence alignment with nucleotide sequences of other 16S rDNA

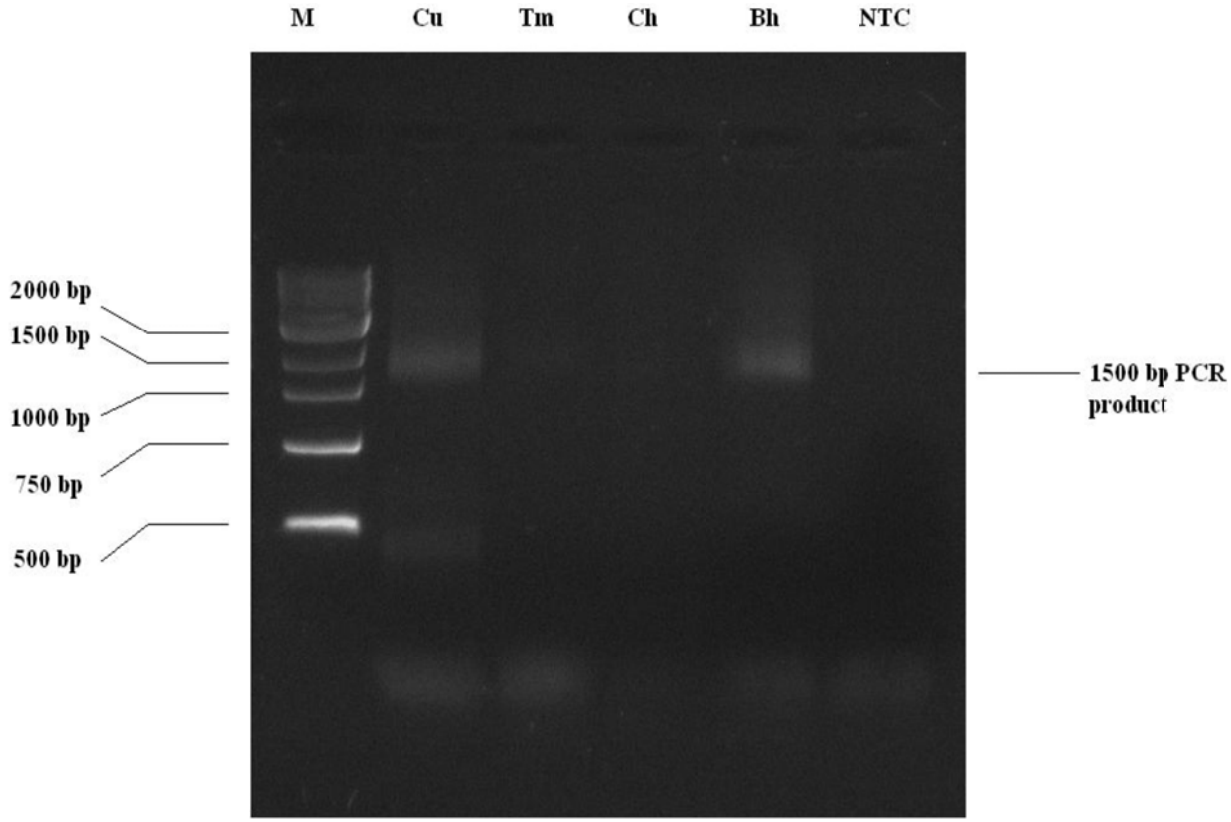

Figure 2. Representative data for PCR amplification: 1% Agarose (w/v) gel electrophoresis of 16S rRNA gene PCR products. Lane M, 1000 bp ladder; Lane Bh, bhindi plant; Lane NTC, negative control.

sequences from microorganism available at GenBank database of NCBI using BLASTN 2.2.28+ program (Stephen et al., 1997).

Blast analysis of obtained gene sequence (Acc. No. KF663567) showed 91% identity with uncultured bacterium sp. and uncultured flavobacterium sp., for example uncultured bacterium partial 16S rRNA gene, clone E130 (Acc. No. AM500800.1), Uncultured bacterium partial 16S rRNA gene, clone SMA4 (Acc. No. AM183001.1), etc. Their identities were furthered confirmed by phylogenetic analysis (Figure 3).

Bacteria are single-celled microorganisms which may be plant pathogens (causing disease), plant asymptomatic bacteria (have no evident effects), and plant growth-promoting bacteria (PGPB) (push up plant growth) (Vidaver and Lambrecht, 2004).

PGPB may be advantageous to plants by several ways such as auxin production, nitrogen fixation (Compant et al., 2005; Watanabe et al., 1979). They may be rhizospheric bacteria (live at root surface), symbiotic bacteria and endophytic bacteria (live inside the plant) (Bacon and White, 2000; Bacon and Hinton, 2006). Bacterial endophytes were first discovered in Germany in 1903 (Freeman, 1903; Tan and Zou, 2001) and defined as “microorganisms that colonize healthy plant tissue without causing obvious symptoms or producing obvious injuries to the host” (Bacon and White, 2000; Bacon and Hinton, 2006). So, that bacterium is not considered as endophyte which causes infections and produces symptoms for disease. By phylogenetic view, endophytic bacteria placed between saprophytic bacteria and plant pathogens (Hallmann et al., 1997).

In infected plants, phytoplasmas colonize sieve cells of phloem tissue and characteristically induce disease symptoms by disturbing their normal metabolic pathways (Chang, 1998; Curkovic´-Perica et al., 2007). They are unique bacteria, as they inhibit insects and plants (Xiaodong et al., 2006). Phytoplasma falls into class Mollicutes (soft-skinned bacteria), due to absence of an outer cell wall and generally have small genomes, low G-C content and essential metabolic activities (Bove, 1997). Mollicutes are directly associated with low G-C, Gram-positive bacteria for example Bacillus, Clostridium and Streptococcus species (Weisburg et al., 1989; Woese, 1987).

Phylogenetic investigation shows that phytoplasma come down from gram-positive, walled bacteria but way by which first phytoplasma originate still unknown (Wei et al., 2008). They are pleomorphic bacteria which fall from an acholeplasma-like ancestor and have small, AT-rich

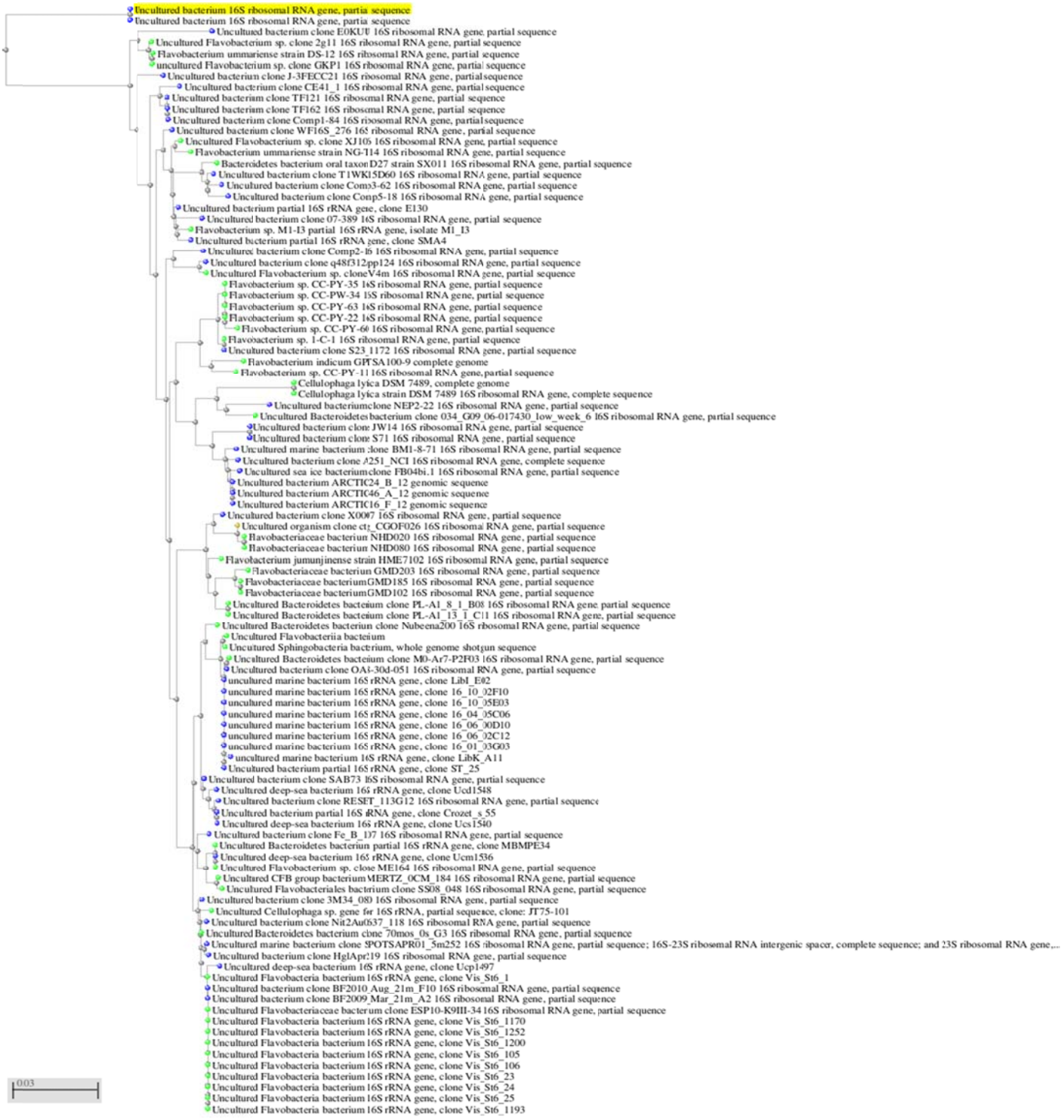

Figure 3. Phylogenetic tree (neighbor joining).

genomes through which they can live in two hosts and act as pathogen (Gundersen et al., 1994; Lee et al., 2000).

Phytoplasmal genomes made up by repeated genes, structured in units of nearly 20 kb, called PMUs (Potential Mobile Units) which involved in phytoplasma genome instability and recombination (Dickinson, 2010). Although PMU is a mobile unit, but it may engage in phase-variation mechanism by which phytoplasma can live in plant and vector (Dickinson, 2010). Phytoplasmal genomes

are special due to their unique structural design, having genes repetitively clustered in non-randomly distributed segments called “Sequence Variable Mosaics” (SVMs) that were formed through repeated, targeted attacks by mobile elements (Jomantiene and Davis, 2006; Jomantiene et al., 2007, Wei et al., 2008).

Wei et al. (2008) discovered that ‘cryptic prophases’ or prophage genome remnants form important structural constituent of phytoplasmal genomes and phage-mediated gene exchange which allow them to live into plant and insect host, for infection and to start events that initiate evolution of phytoplasma clade.

Phytoplasma posses extremely reduced genomes in comparison to other mollicutes, which is responsible for their unique metabolism by which they cannot artificially cultured (Xiaodong et al., 2006).

Phytoplasma produces several symptoms such as witches' broom, phyllody, generalized yellowing, decline and stunting of plants which indicates that they inhibit normal plant development (Hogenhout et al., 2008).

In present study, we also observed symptoms of leaf distortion, leaf curling and overall stunting of plant which makes suspicion that causative pathogen may have some relation with phytoplasma.

Conclusion

Here, we identified an “uncultured bacterium sp.” responsible for infection in bhindi plant (*A. esculentus*) of Gorakhpur district of Eastern U.P. region of India. Their nucleotide sequence deposited in GenBank has accession number KF663567. On the basis of visible symptoms of infection and positive PCR amplification with universal phytoplasma specific primers (P1/Tint), we can believe that the identified organism has some phytoplasmal nature.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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