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Research Article

An Uncultured Bacterium Associated with Infection in Capsicum annuum in India

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ABSTRACT

We make an extensive survey of Gorakhpur district of Eastern U.P. region of India from 2011-2013, for vegetable plant samples showing visible symptoms of possible phytoplasmal infection. We observed symptoms such as reduction in leaf size, curling of leaf, etc. in infected chilli plants (*Capsicum annuum* L., Family *Solanaceae*), growing in the fields of Gorakhpur district. By using universal phytoplasma specific P1 and Tint primers, we perform their PCR analysis. The PCR product of size 1600 bp was generated which were further purified and sequenced. Nucleotide sequence obtained in present study was deposited in GenBank through accession number KF298062 and named as "Uncultured Pseudomonas sp.' with/ clone=RCGKP1". BLAST analysis and Phylogenetic tree established their association with '*Pseudomonas*' group. In our knowledge, this is a new uncultured bacterium (359 bp), associated with infection in *Capsicum annuum* plant in Gorakhpur district of Eastern U.P. region of India.

INTRODUCTION

Phytoplasmas are very small sized, specialized bacteria. Mostly, they live in phloem cells of host plants and transmitted by insect vectors (Arocha et al., 2005; Weintraub and Beanland, 2006; Phyllis, 2007). First time they were discovered in 1967 and called as Mycoplasma-Like Organisms (Doi et al., 1967). They cannot be cultured in vitro (Jones, 2002), so diagnosis of phytoplasmal diseases is difficult. Phytoplasmas are responsible for causing a large number of diseases in several plant species worldwide (McCoy et al., 1989; Lee et al., 2000; Liefting et al., 2004). Several phytoplasmal diseases have been also reported in various vegetable plants (Chang et al., 1995; Marcone et al., 1997; Singh and Singh, 2000; Ing-Ming Lee et al., 2006; Nenad Trkulja et al., 2011; Raul Tapia-Tussell et al., 2012).

Plants infected by phytoplasmas shows several visible symptoms of infection for example leaf curling, little leaf, phyllody, yellowing, witches' broom, severe stunting of plant, etc. (Bertaccini et al., 2005).

Due to advancement in molecular techniques, several phytoplasma has been identified from several diseased plants (Bertaccini, 2007; Bertaccini and Duduk, 2009).

Chilli (*Capsicum annuum* L.) belonging to *Solanaceae* family, is a very frequent, delicious vegetable and spice of food menu. Chilli is a rich source of vitamin C and Capsaicin. Capsaicin is useful for treatment of pain, arthritis, blood sugar levels, cancer and several others (http://en.wikipedia.org/wiki/Capsaicin).

A large number of diseases are responsible for reducing chilli production and their quality, both (http://aces.nmsu.edu/pubs/_circulars/circ549.html; Skaggs et al., 2000; Singh and Singh, 2000; Khan and Raj, 2006).

During survey period, we have find that infection in chilli plants were very common in Gorakhpur district of Eastern U.P. region of India and this also causes vast loss of chilli productions.

In India, Singh and Singh (2000) reported that chilli little leaf disease is associated with a phytoplasma and Khan and Raj, (2006) identified an Aster yellows phytoplasma ('*Candidatus* Phytoplasma asteris') (Accession No. DQ343288), infecting chilli plant in India.

In our knowledge, little work has been done for characterization and ultimate identification of phytoplasmas responsible for infection in chilli plants of Gorakhpur district of U.P., India.

So in present work, we make an attempt to identify and characterize the phytoplasma responsible for infection in chilli plants of Gorakhpur district of U.P., India.

MATERIALS AND METHODS

(A) Plant samples: Infected Chilli (*C. annuum* L.) plant samples showing reduction in leaf size, curling of leaf, etc. (Figure 1B) and healthy chilli plant samples (Figure 1A), were collected from Gorakhpur district, during the course of survey in their growing seasons 2011-2012 and 2012-2013.

Figure 1 A: Healthy leaf of Chilli plant.

Figure 1 B: Infected leaf of Chilli plant.

(B) DNA extraction: During present study, we follow the procedure published by Ahrens and Seemüller (1992) and include 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 more 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 one-hour incubation at 60℃, the nucleic acids were purified by chloroform- isoamyl alcohol (24:1), and centrifuge 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 hour. Then centrifuge at 12,000 g for 10 min. After centrifugation, add 1 ml 70% ethanol and centrifuge at 12,000 g for 10 min. Decant supernatant and dry the pellet at 37℃ for 30 min. Dissolve DNA in 30 µl of sterile water.

Phytoplasma Grinding Buffer (PGB): 100mM K_2HPO_4; 30 mM KH_2PO_4; 10% Sucrose; 0.15% Bovine serum albumin fraction V; 2% Polyvinylpyrrolidone-10; 25 mM Ascorbic acid.

CTAB buffer: 2% CTAB; 100 mM Tris pH 8; 1.4 M NaCl; 20 mM EDTA.

(C) Primers used in the study: The primers used for PCR were specific to phytoplasma and for sequencing we also used gene specific sequencing primers.

P1 forward primer:
AAGAGTTTGATCCTGGCTCAGGATT
Tint reverse primer:
TCAGGCGTGTGCTCTAACCAGC
Target gene: 16s-23s rRNA spacer regions.

(D) PCR setup: Genomic DNA from the test samples was PCR amplified (Figure-2; 3) 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.

Table-1: PCR Components used in present study.

PCR Components (Conc.)	Volume
25µl Reaction volume contains:	
Template DNA	5.0 µl
P1 forward primer (10µM)	1.0 µl
Tint reverse primer (10µM)	1.0 µl
10X PCR buffer	2.5 µl
50mM $MgCl_2$	0.75 µl
10mM dNTP mixture	0.5 µl
Taq DNA Polymerase (5U/µl)	0.2 µl
Sterile distilled water	14.05µl

Table-2: PCR conditions during present study.

95℃	4 min	
94℃	1 min	35 Cycles
56℃	1 min	
72℃	1 min	
72℃	10 min	
4℃	∞	

(E) Sequencing:

(a) 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 1000bp 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 5V/cm until the tracking dye is 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 1600 bp was generated through this reaction (Figure-3).

(b) Purification of PCR products: Sequencing uses one primer, while PCR utilizes two. If we try to sequence with two primers present, we'll get the two sequences back, superimposed on each other and completely unreadable. Hence it is necessary to purify a PCR product prior to sequencing. PCR products were purified using geneO-Spin PCR purification Kit (geneOmbio technologies, India). The PCR products were eluted in final volume of 20.0 µl.

(c) Agarose gel electrophoresis of purified PCR products: The protocol mentioned above, was used for checking of purified PCR products on 1% agarose gel and determination of approximate concentration of DNA.

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

(F) BLAST Analysis: BLAST analysis was conducted on the 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 using clustalW sequence alignment tool available at http://www.genome.jp/tools/clustalw/

Finally 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.

Phylogenetic tree (Figure-4) was generated using Fast Minimum Method with max. sequence difference value set at 0.75. Genetic distance in the tree was calculated by default using Blast tree viewer.

(G) Instruments used:

PCR: ABI 2700 Thermal Cycler (Applied Biosystems, USA)
DNA Sequencing: ABI 3130 Genetic Analyser (Applied Biosystems, USA)
Imaging: BioRAD Laboratories GelDoc-XR documentation system for photographing electrophoresis gel.

RESULTS AND DISCUSSION

(A) Survey: During the course of survey of phytoplasmal infection in vegetable plants of different locations of Gorakhpur District, a variety of suspected symptoms like reduction in leaf size, leaf curling, leaf twisting, witches' broom, etc. were observed in fields.

Suspected samples of chilli plant showing symptoms of reduction in leaf size, leaf curling was collected and used for further identification and characterization of their causative phytoplasma.

(B) PCR analysis: PCR analysis with P1 and tint primers produced 1600 bp PCR products (Figure-3), which were further purified and then sequenced.

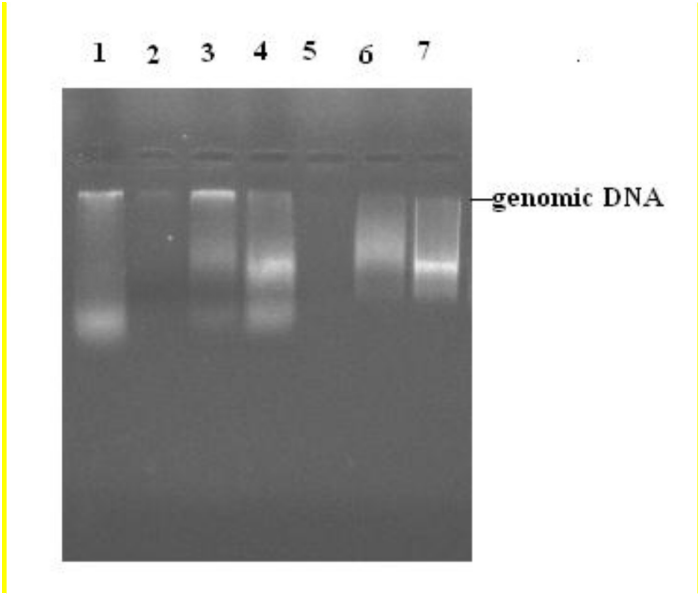

Figure-2: Genomic DNA QC image.

- Representative data for genomic DNA: 1% Agarose (w/v) gel electrophoresis.
- Well No. 1: Chilli Infected; Well No. 5: Blank; Well no. 6: Chilli Healthy.

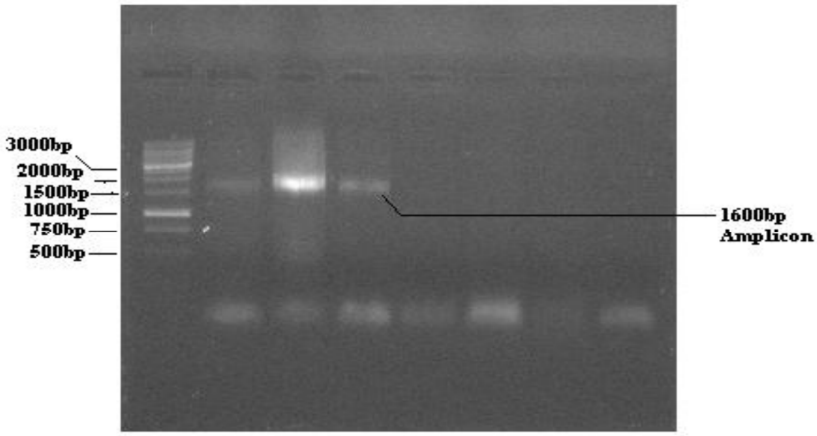

Figure-3: Phytoplasma PCR QC-1% Agarose gel with 1kb DNA marker.

- Representative data for PCR amplification: 1% Agarose (w/v) gel electrophoresis of 16S rRNA gene PCR products.
- Lane 1: 1000bp Ladder; Lane 2: Chilli healthy plant; Lane 3: Chilli infected plant; Lane 8: Negative Control.

(C) Sequencing result:

359 bp; DNA linear; BASE COUNT: 108 a 65 c 106 g 80 t

1	**ggtacagagg**	**gtggccaaat**	**tgcgaggggg**	**ggttaatcac**	**ataaaaacga**	**tagtagtcgg**
61	**aattacagtc**	**aaaaactcga**	**ttgtgagaag**	**ttggaatcga**	**tagtaatcga**	**gaatcagaag**
121	**ttcacggaga**	**ataagatcct**	**ggaccttata**	**cacaccgccc**	**gtcacaccat**	**gggagtgggt**
181	**tgctccagaa**	**gtagctagtt**	**taaccttcgg**	**gaggacggtt**	**accaggggggt**	**gattcatgac**
241	**aggggcgaag**	**tcgtaacaag**	**gtagtcatag**	**gggaacttta**	**gggtagatca**	**gctcctgtat**
301	**cgaagctgtc**	**agctggttga**	**caagcttcca**	**caagaattgc**	**ttgattcagg**	**gtagaagac**

(D) Sequence Comparison and Phylogenetic Analysis: Above obtained sequence was deposited in Gene Bank, which allotted him accession number KF298062 and named as "Uncultured Pseudomonas sp.' with/clone=RCGKP1".

The sequences obtained from amplified PCR product were analysed by multiple sequence alignment with other nucleotide sequences available at GenBank database of NCBI using BLASTN 2.2.28+ program (Stephen, et al., 1997).

Sequence alignment was performed using clustalW sequence alignment tool available at http://www.genome.jp/tools/clustalw/.

Phylogenetic tree (Figure-4) was generated using the online BLAST pair wise alignment. BLAST computes a pair wise alignment between a query and the database sequences searched. The tree was generated using Fast Minimum Method with maximum sequence difference value set at 0.75. Genetic distance in the tree was calculated by default using Blast tree viewer.

Figure-4: Phylogenetic Tree.

(E) BLAST Analysis: Sequence alignments showed the maximum identity (90%) of obtained sequence with those of *Pseudomonas fulva 12-X*, complete genome (Acc. No. CP002727.1); *Pseudomonas protegens CHAO*, complete genome (Acc. No. CP003190.1); *Pseudomonas mendocina NK-01*, complete genome (Acc. No. CP002620.1); *Uncultured Pseudomonadales bacterium HF0500_12O04* genomic sequence (Acc. No. GU474920.1); *Uncultured bacterium HF0500_12004* genomic sequence (Acc. No. EU795190.1); *Pseudomonas mendocina ymp*, complete genome (Acc. No. CP000680.1); *Pseudomonas protegens Pf-5*, complete genome (Acc. No. CP000076.1); *Pseudomonas stutzeri CCUG 29243*, complete genome (Acc. No. CP003677.1); *Pseudomonas poae RE*1-1-14*, complete genome (Acc. No. CP004045.1); *Pseudomonas fluorescens A506*, complete genome (Acc. No. CP003041.1); *Pseudomonas sp. UW4*, complete genome (Acc. No. CP003880.1); *Pseudomonas fluorescens F113*, complete genome (Acc. No. CP003150.1); *Pseudomonas brassicacearum subsp. Brassicacearum NFM421*, complete genome (Acc. No. CP002585.1); *Pseudomonas fluorescens Pf0-1,* complete genome (Acc. No. CP000094.2); *Pseudomonas fluorescens SBW25*, complete genome (Acc. No. AM181176.4); *Pseudomonas fluorescens strain LMG 5329 16S ribosomal RNA gene*, partial sequence (Acc. No. JQ974027.1); *Pseudomonas fluorescens Pf29Arpcontig024*, whole genome shotgun sequence (Acc. No. ANOR01000024.1); *Pseudomonas fluorescens ribosomal RNA operon rrnB*, complete sequence (Acc. No. AF134704.1); *Pseudomonas syringae pv. Syringae B728a*, complete genome (Acc. No. CP000075.1); *Pseudomonas stutzeri DSM 10701*, complete genome (Acc. No. CP003725.1); *Pseudomonas syringae pv. Phaseolicola 1448A*, complete genome (Acc. No. CP000058.1); *Pseudomonas syringae pv. Tomato str. DC3000*, complete genome (Acc. No. AE016853.1) and *Pseudomonas resinovorans NBRC 106553 DNA*, complete genome (Acc. No. AP013068.1).

Thus identification and characterization of phytoplasma responsible for infection in chilli plant of Gorakhpur district of Eastern U.P. region of India was done in the present study. On the basis of maximum sequence identities (90%) and their close phylogenetic relationship, which was named as "Uncultured Pseudomonas sp.' with/clone=RCGKP1".

CONCLUSION

Thus, we can conclude that infection in chilli plants (*Capsicum annuum*) of Gorakhpur district of Eastern Uttar Pradesh region of India, as observed during present study is due to "Uncultured Pseudomonas sp.' with/clone=RCGKP1" and their GenBank accession number for their nucleotide sequence is KF298062.

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