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RESEARCH ARTICLE

Occurrence, Distribution and Molecular Characterization of Phytoplasma Infecting Chickpea (*Cicer arietinum* L.) from Tamil Nadu

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ABSTRACT

Phytoplasma is an obligate prokaryote infecting a wide array of crops such as urdbean, sesame, brinjal and many other vegetable crops in India. Several workers have noticed the recent outbreak of phytoplasma disease in several pulse crops. A study was conducted to identify the phytoplasma infection in chickpea from Tamil Nadu. The average disease incidence due to phytoplasma was recorded with a range of 5-20% under field condition. The infected plants produced symptoms including stunting of plants, bushy appearance, reduced leaf size, chlorosis and reddening of infected leaves. The association of phytoplasma with these symptoms was confirmed by nested PCR assay using the universal primers P1/P7 and R16F2n/R16R2. The infected samples were amplified with an amplicon size of 1.2 kb and sequence analysis showed more than 99% similarity with phytoplasma belongs to *Candidatus Phytoplasma aurantifolia*. Phylogenetic analysis of nucleotide sequences confirmed the chickpea phyllody phytoplasma forms a single subgroup with other Indian isolates of various crops.

Keywords: *Chickpea phytoplasma; Occurrence; Nested PCR; Phylogenetic analysis*

INTRODUCTION

Chickpea (*Cicer arietinum* L.), one of the important pulse crops, belongs to the family fabaceae grown in several countries, accounting total share of 50% of the total pulse crops. India represents the largest producer of chickpea, accounting for around 70% of the global production. It contains a rich source of protein and is consumed as dal by people worldwide. The crop is cultivated with an area of 9.93 Mha and a productivity of 935.34 kg/ha in India (Merga and Haji, 2019). The major chickpea growing states are Madhya Pradesh (MP), Rajasthan, Maharashtra,Uttar Pradesh (UP), Andhra Pradesh (AP), Karnataka, Chhattisgarh, Bihar, Tamil Nadu and Jharkhand.These states contribute more than 95% of the chickpea production in India (Pradipa *et al*., 2018; Gurivi Reddy *et al*., 2021).In Tamil Nadu, the crop is cultivated in districts of Coimbatore, Tiruppur, Dindigul, Thiruvallur, Kancheepuram, Erode, Salem, Namakkal, Perambalur, Tiruchirappali, Madurai, Virudhunagar, Ramanathapuram, Thoothukkudi, Tirunelveli and Dharmapuri (Pradipa *et al*., 2018). The crop loss due to biotic and abiotic stress has been reported for several years. Among the biotic factors, the crop is infected by several fungal and bacterial diseases, including dry root rot, *Fusarium* wilt, *Colletotrichum* stem blight and *Botrytis* grey mold. In addition, phytoplasma disease expressing symptoms of bushy appearance, reduced leaf size and reddening were found to be an emerging disease of chickpea in Tamil Nadu. Venkataraman (1959) reported the phyllody of chickpea for the first time in Tamil Nadu as a phytoplasma disease based on symptomatology. In the early 1990s, the occurrence of phytoplasma disease in other pulses has also been reported from Tamil Nadu (Lakshmanan *et al*., 1988; Ghanekar *et al*., 1988). However, the occurrence and characterization of phytoplasmas in chickpea notably has not been adequately reported in Tamil Nadu. The chief symptoms reported by several workers associated with phytoplasma disease include stunting of plants, dwarfing, bushy appearance and leaf reddening. The plants infected in the early stages, it produced severe stunting symptoms (Shreenath *et al*., 2020; Gurivi Reddy *et al*., 2021).

Phytoplasmas are intracellular obligate prokaryotes that lack cell walls and their genome is very small (680-1,600 kb). Phytoplasmas have been reported as vital pathogens infecting wide range of cultivated crops such as grapevine, sugarcane, and coconut causing serious epidemics (Weintraub and Beanland, 2006; Akram *et al*., 2016) since from the first report of this organism by Doi *et al*. (1967). In several plant species, phytoplasmas cause complex syndromes with symptoms such as

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stunting, proliferating auxiliary shoots, development of sterile deformed flowers, virescence, and phyllody (Lee *et al*., 2000; Bhaskara Reddy *et al*., 2014; Gurivi Reddy *et al*., 2021). Association of phytoplasma in chickpea has been reported from Pakistan, Australia, Myanmar and Sudan (Nasir *et al*., 2017).The phytoplasma disease is transmitted through leaf hopper under field conditions as well as side wedge grafting under experimental plots (Lakshmanan *et al*., 1988). The association of phytoplasma disease in plants was recently identified using an electron microscope, serological and molecular approaches such as PCR and restriction fragment analysis (T-RFLP) (Hodgetts *et al*., 2007; Oberhansli *et al*., 2011; Vijay Kumar Naik *et al*., 2018a). Gundersen *et al*. (1996) designed the new universal primer R16mF2 /R1 and R16mF2/ R2 for the detection of phytoplasma associated with plant tissue using nested PCR as a molecular tool. The proper identification of phytoplasma disease in chickpea through molecular characterization and its proper genetic makeup provides clues to develop management strategies for the betterment of the farming community. In this study, we attempted to document the occurrence, distribution and molecular detection of phytoplasma in chickpea from Tamil Nadu.

MATERIAL AND METHODS

Sample collection

The survey was conducted in chickpea growing areas of Coimbatore, Dharapuram, Pollachi, Udumalpet and experimental plots of Pulse Department, TNAU, Coimbatore during the year 2018-2019. The incidence of phyllody was recorded and per cent disease incidence was calculated. Two fields were selected in each village and three plots were randomly selected in each field for calculating the mean disease incidence. The pathogen infection was expressed as per cent disease incidence for each field.

DNA extraction from chickpea leaf sample

The DNA was isolated from infected chickpea samples using the modified CTAB method to detect phytoplasma as per the protocol described by Warokka *et al*. (2006). The symptomatic infected leaf sample of chickpea (0.4 g) was ground in liquid nitrogen with a precooled mortar and pestle, then poured with 2 mL of Phytoplasma Grinding Buffer (PGB) in a centrifuge tube and incubated for 10 min. The mixture was centrifuged at 5000 rpm for 5 min,supernatant was transferred to new eppendorf tubes and again centrifuged at 10000 rpm for 25 min. The pellet was resuspended in 1 mL CTAB buffer, incubated at 65°C for 1 h and added with equal volume of chloroform-isoamyl alcohol (24:1) for further purification of the mixture. Then, mixture was centrifuged at 10000 rpm for 10 min at 4°C. The supernatant was collected in a new eppendorf tubes and added with ice-cold isopropanol to separe the DNA from the drawn aqueous phase and then incubated on ice for 1 h.The DNA pellet was gained through centrifugation at 10000 rpm for 10 min and then purified using 3 M sodium acetete. The purified pellet was washed with 80 % ethanol, finally resuspended in 35 µL of TE buffer and purity of isolated DNA was analysed through 0.8 % agarose gel electrophoresis.

Figure 1. Symptoms of phytoplasma on chickpea with stunting and bushy appearance of infected plants (a)and healthy plants (b) under field cultivation.

Amplification of 16S rRNA gene of phytoplasma by nested PCR

The specificity in amplifying the 16S rRNA gene of phytoplasma in nested PCR method was used to detect the phytoplasma present in disease-infected chickpea samples. For the first-round amplification, 1 µL of DNA was used to amplify the phtoplasma with primer pairs P1/P7. Subsequently, 0.5 µL of the PCR product from first round reaction was used as template in nested-PCR without dilution using phytoplasma specific primers R16F2n/R16R2.The PCR assays were performed in a thermal cycler (Eppendorf, Germany) with the following thermal programme: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min., annealing at 54°C for 1 min and extension at 72°C for 2 min and final extension of 72°C for 10 min.The PCR products were analyzed on 1.2 % agarose by gel electrophoresis and stained with ethidium bromide (0.5 µg/mL). The gel was photographed in the gel documentation method after visualising under UV transilluminator (Alpha Innotech Corporation, USA).

Sequencing and analysis of 16S rRNA gene sequences of phytoplasma

The amplified PCR products were submitted to Barcode Biosciences Pvt. Ltd., Bangalore, for

sequencing. The resulted sequences were edited using BIOEDIT software to obtain full length sequence of 16S rRNA of phytoplasma.

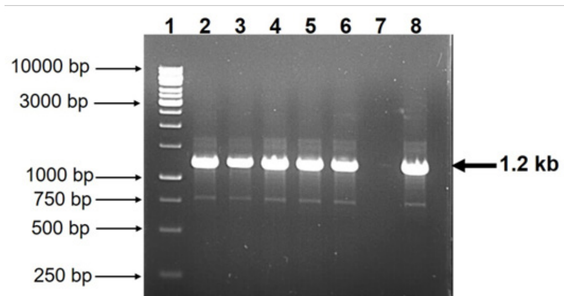


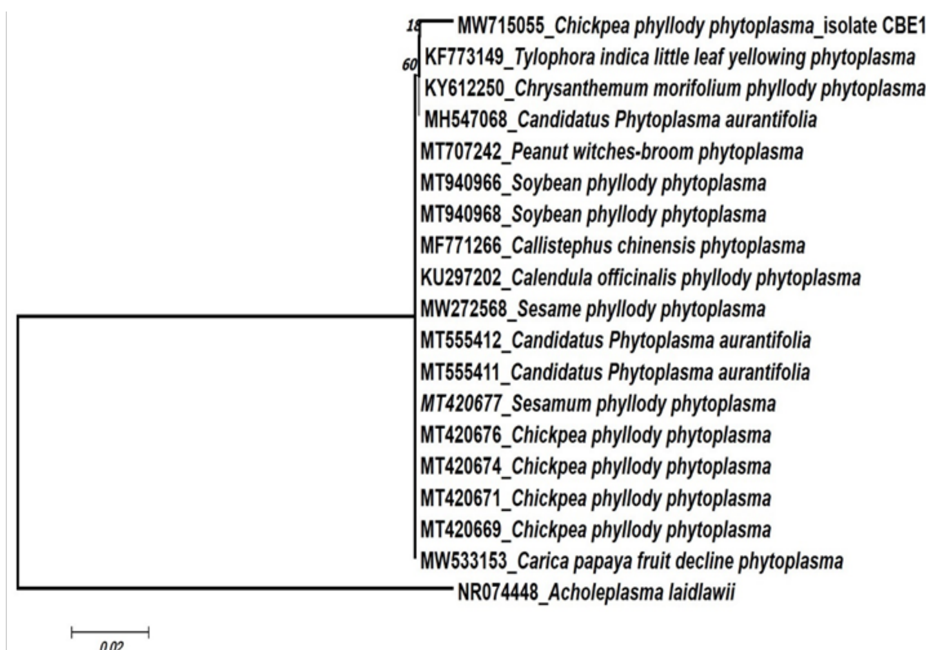
Figure 2. PCR amplification of 16S rRNA gene from phytoplasma naturally infected in chickpea. Lane 1 - 1 kb ladder; Lanes 2 to 6 - Amplified DNA fragment from infected samples; Lane 7- DNA from healthy sample; Lane - Positive control

The nucleotide sequences were searched for sequence homology using BLAST search against Genbank database(http://www.ncbi.nih.gov/BLAST). The related phytoplasma 16S rRNA gene sequences retrieved from the GenBank database wereused for phylogenetic analysis. The phylogenetic tree was constructed with bootstrap for 1000 times using the neighbor-joining method (Saitou and Nei, 1987; Tamura *et al*., 2011) and compared.

RESULTS AND DISCUSSION

The field survey revealed the occurrence of phytoplasma incidence in farmer fields at Coimbatore, Dharapuram, Pollachi and Udumalpet. The phytoplasma incidence was recorded with the range of 5-20% from chickpea growing areas of Tamil Nadu. The maximum incidence of phytoplasma was recorded in the research plot of Pulse Department, TNAU, Coimbatore, which accounted for 20% of infection. This was followed by Dharapuram area accounted 17% of infection.The infected plants expressed the symptoms including stunting of plants, bushy appearance, reduced leaf size, chlorosis and reddening of infected leaves (Fig 1). A similar type of symptoms were observed by Akhtar *et al*. (2008), who reported that phytoplasma infected chickpea produced small leaves, bushy appearance and flowers modified into leaf-like structure. The infected chickpea samples produced a variety of symptoms, including pale green leaves, bushy appearance and excessive axillary proliferation (Vijay Kumar Naik *et al*., 2018b). Gurupada Balol *et al*. (2019) observed the symptoms of phytoplasma infection in chickpea with the change of leaves into pale green leaves, bushy appearance and excessive axillary proliferation. Similarly, Pallavi *et al*. (2012) reported the symptoms of phytoplasma associated with chickpea in India. The infected plants showed symptoms *viz.,* pale green leaves, bushy appearance due to excessive stunting of shoots, decreased internodal length and phyllody like excessive axillary proliferation in infected plants.

Figure 3. Neighbour joining phylogenetic tree based on the nucleotide sequences of 16S rRNA of *Chickpea phyllody phytoplasma* and *Acholeplasma laidlawii* is kept as out group.



Phytoplasma diseases in pulses have been reported by earlier workers using the nested PCR, RFLP and other molecular approaches. In our study, leaf samples showing the typical symptoms of phytoplasma from infected plants of chickpea were tested through nested PCR using phytoplasma-

specific primers. The first round of PCR was performed using the universal primers (p1/p7) and nested PCR was performed with a specific primer (R16F2n/R16R2) to characterize the 16S rRNA gene of chickpea phytoplasma. The samples were PCR amplified with a DNA fragment size of ~1.8 kb and ~1.2 kb from DNA extracted from infected plants but not from the DNA of healthy leaves (Fig 2). The PCR products were sequenced on both the orientations and the consensus sequences were deposited in GenBank(Accession No.MW715055). Further, the nucleotide sequences pertaining to 16S rRNA region of phytoplasma reported from different crops were selected from GenBank using the tool NCBI BLAST for phylogenetic analysis. The analysis revealed that the nucleotide sequence of phytoplasma infecting chickpea had more than 99% similarity with other isolates from NCBI, confirms the association of phytoplasma from chickpea. The chickpea nucleotide sequence also had 99.10% identity among the sequences from three host crops *viz., Chrysanthemum morifolium, Tylophora indica* and bamboo (Accession Nos. MH547068; KY612250 and KF773149), especially with 16S rRNA gene sequences of 16SrI (*Candidatus Phytoplasma aurantifolia*) phytoplasmas infecting bamboo and chickpea from India (Table 1).

Table 1. Nucleotide (nt) identities of 16S rRNA gene of phytoplasma from chickpea with corresponding sequences of selected isolates from NCBI.

Crop/Isolate	Country	Per cent nucleotide similarity
Bambusa vulgaris	India	99.10
Callistephus chinensis	India	99.00
Chrysanthemum morifolium	India	99.10
Tylophora indica	India	99.10
Calendula officinalis	Iran	99.00
Sesamum indicum	Iran	99.00
Croton bonplandianus	India	99.00
Croton bonplandianus	India	99.00
Sesamum indicum	India	99.00
Cicer arietinum	India	99.00
Cicer arietinum	India	99.00
Cicer arietinum	India	99.00
Cicer arietinum	India	99.00
Carica papaya	India	99.00
Glycine max	India	99.00
Glycine max	India	99.00
Crossandra	India	99.00

The phylogenetic analysis of these sequences revealed that nucleotide sequences from chickpea isolate formed a single cluster with other isolates confirmed the high sequence similarity with other isolates of various crops (Fig 3). Similar results were obtained by Pallavi *et al*. (2012), who isolated the DNA from infected chickpea samples and amplified the fragments with a size of 1.8 kb and 1.2 kb using phytoplasmas 16S rDNA primers P1/P7 and R16F2n/R16R2, respectively. The BLAST analysis showed 99% similarity with 16SrII group belongs to *Candidatus Phytoplasma aurantifolia*. The *Candidatus phytoplasma asteris* and *Candidatus phytoplasma aurantifolia* are the major groups belong to 16SrI and 16SrII group of phytoplasmas associated with pulse crop in India (Vijay Kumar Naik *et al*., 2018a).

Similarly, Gurivi Reddy *et al*. (2021) conducted the nested PCR for the infected chickpea plants collected from difenent parts of India *viz.,* Karnataka, Andhra Pradesh, Telangana, Madhya Pradesh, Uttar Pradesh and New Delhi. The phytoplasma infected samples amplified the fragments corresponding to 16S rRNA gene and five multi locus genes, which confirmed the association of 16SrII-C and 16SrII-D subgroups of phytoplasmas strain with chickpea. Vijay Kumar Naik *et al*. (2018b) found the association of phytoplasma disease in chickpea collected from the fields in Kurnool district, Andhra Pradesh. The DNA isolated from infected chickpea produced amplicon size of 1.8 kb and 1.2 kb using the universal primers P1/P7 and R16F2n/R16R2 corresponding to 16S rRNA region. The sequence analysis showed that nucleotide sequence shared the highest homology of 98% with Sesame phyllody phytoplasma 16SrII-D belongs to *'Candidatus Phytoplasma aurantifolia'* of the 16SrII-D group infecting chickpea from Andhra Pradesh.

CONCLUSION

The association of Phytoplasma in chickpea was confirmed through symptomatology and molecular assay using nested PCR analysis. The infected chickpea plants showing typical phytoplasma symptoms of stunting, dwarfing and bushy appearance under field conditions were collected and analysed which confirmed the association of phytoplasma by specific amplification with anamplicon size of around 1.2 kb through nested PCR. Further studies on the vector involved in transmission will help in developing management strategies.

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