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## Molecular detection of *Candidatus phytoplasma* associated with little leaf disease in Brinjal from Southern Gujarat region of India

**RK Kalaria, Sunita Ghanghas and AI Patel**

### Abstract

Brinjal little leaf (BLL) is a common phytoplasma etiology disease in India that causes serious financial losses. The sample was collected from the south Gujarat region of India during March, 2019 showing severe symptoms of stunting and profuse branching with little leaf were collected for phytoplasma identification and detection. Direct PCR assays using phytoplasma-specific primary pairs P1/P7 were performed from the leaf DNA of diseased plant. Amplified ~900bp fragment was further compared and a 16S rRNA gene sequence of BLL phytoplasma strains was verified as *Candidatus Phytoplasma* spp. strain NAU gujarat 16S ribosomal RNA gene (MK942854) belonging to the 16SrVI category of phytoplasma clover proliferation. To our understanding, this *Candidatus Phytoplasma* spp. first report incidence of brinjal in Gujarat, India.

**Keywords:** *Solanum melongena* L., Brinjal little leaf, *Candidatus phytoplasma*, 16sRNA, PCR and mycoplasma

### Introduction

The Brinjal (*Solanum melongena* L.) belongs to the *Solanaceae* family and is one of the primary crops grown in the world's tropics and subtropics. Recently, one of the major factors limiting its small genetic base susceptibility to different biotic and abiotic stresses. It is impacted by various diseases in which the small leaf disease of Brinjal (BLL) caused by phytoplasma, significant financial losses (Rao *et al.*, 2011) [17]. Phytoplasma also damages other plant species globally of comprehensive economic losses (Siddique *et al.*, 2001; Rao *et al.*, 2011; Kumar *et al.*, 2012; Maejima *et al.*, 2014) [18, 17, 8, 10]. In India, one of the main vegetable plants is eggplant or brinjal. After China, India is the world's second-largest brinjal producer with an average output of 17.42 Mt / ha (Kumar *et al.*, 2017) [9]. Little leaf with stunting development, leaf yellowing, stunting and profuse branching, axillary flowers proliferation, large bud of this disease are the most common symptoms. (Doi *et al.* 1967; Rao *et al.*, 2011) [3, 17]. Phytoplasma connected with white leaf diseases were first recorded on Bermuda grass in Taiwan in 1972. (Marcone *et al.*, 1997) [11], and in 2004, allocated into a separated taxon as '*Ca. P. cynodontis*' (Marcone *et al.* 2004) [12]. In some northern India, the association of Phytoplasma with Brinjal as a small-leaf causative agent is recorded in many cases. (Mello *et al.*, 2011; Azadvar and Baranwal, 2012; Kumar *et al.*, 2012; Naik *et al.*, 2018a; Yadav *et al.*, 2015a) [13, 2, 8, 14, 21].

The term phytoplasma relates to a group of mycoplasma-like organisms that are microscopic, pleomorphic, obligate parasites, plant pathogenic bacteria present in plant sieve components, and spread through insect vectors that feed on phloems. Usually, phytoplasma are present in phloem sieve tubes of plants and in the salivary glands of insect vectors like leaf hopper and plant hopper. As phytoplasmas are replicates in the phloem, very less is known about its physiology and also difficult to cultivate them invitro. Therefore, they are mostly detected by molecular techniques (Rathnam and Patil, 2016) [16]. The PCR assays to characterize phytoplasma primarily by targeting the conserved 16S rRNA region and the spacer region between the 16S and 23S rRNA genes using universal primers obtained from conserved 16S rRNA gene sequences [1]. New taxon designation '*Candidatus Phytoplasma*' has been suggested (IRPCM, 2004) [6] and classified further into several groups and subgroups based on 16SrRNA sequences (Hodgetts *et al.*, 2008) [5]. New species '*Candidatus Phytoplasma*' is nomenclature based on 16SrRNA gene nucleotide sequence resemblance (kumar *et al.*, 2017;

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(kumar *et al.*, 2017; Naik *et al.*, 2018b; Venkataravanappa *et al.*, 2017) <sup>[9, 15, 20]</sup>.

### Materials and Methods

The infected plant leaves with typical disease symptoms are subjected to DNA isolation using CTAB methods followed by PCR under controlled condition using P1/P7 universal primers derived from conserved 16sRNA region (Gunderson and Lee, 1996) <sup>[4]</sup>. Amplified fragment was separated on an agarose gel later eluted for purification and sequencing (Kalaria *et al.*, 2013) <sup>[7]</sup>. The retrieved sequence was later compared in NCBI for phylogenetic analysis using the neighbor-joining method of MEGA 6 (Tamura *et al.*, 2007) <sup>[19]</sup> and classified in specific groups.

### Results and Discussions

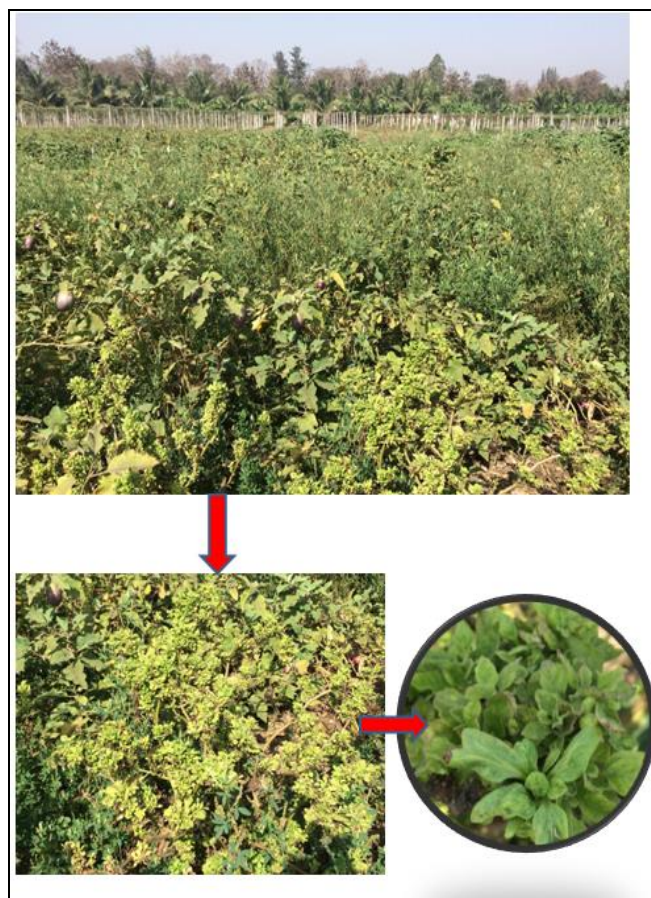
During a small survey in March 2019, severe little leaf symptoms were observed on brinjal near ASPEE Horticulture and Forestry Farm, Navsari agricultural university, Navsari (20° 92' 49.342" N) and 72° 90' 79.144" E) south Gujarat region in India. The causal pathogen was suspected to be a Phytoplasma spp. with severe little leaf by stunting growth, Leaf yellowing with phyllody of flowers observed in few leaves of brinjal in the field (Fig. 1). Randomly few plant samples were collected as suspected to associated Phytoplasma spp. for further detection. In the home gardens of the Nemmara Village of Kerala region of India, similar symptoms have also been noted (Yadav *et al.*, 2015a) <sup>[21]</sup>. In the areas of Bihar, India, Kumar *et al.* (2012) <sup>[8]</sup> also noted similar small leaf symptoms on brinjal crops. Kumar *et al.* (2017) <sup>[9]</sup> also gathered infected brinjal samples from various areas of India from small leaf disease.

In order to detect and recognize BLL-infected brinjal crops, a total of five leaf samples were subjected using modified CTAB for full genomic DNA isolation (Fig.2) and the polymerase chain reaction (PCR) was conducted directly using a couple of phytoplasma P1/P7 universal primers (Gunderson and Lee, 1996) <sup>[4]</sup>. PCRs was set up in a 25 µL reaction included with slight modification, initial denaturation at 94°C for 3min. and 30 cycles of denaturation at 94°C for 1min., annealing at 55°C for 1min. and extension at 72°C for 2min. followed by a final extension at 72°C for 10min (Yadav *et al.*, 2015a; Yadav *et al.*, 2015b; Rathnamma and Patil, 2016; Naik *et al.*, 2018a) <sup>[21, 22, 16, 15]</sup>. Kumar *et al.* (2012) <sup>[8]</sup> also using the same PCR primers and above-mentioned Phytoplasma spp. detection conditions.

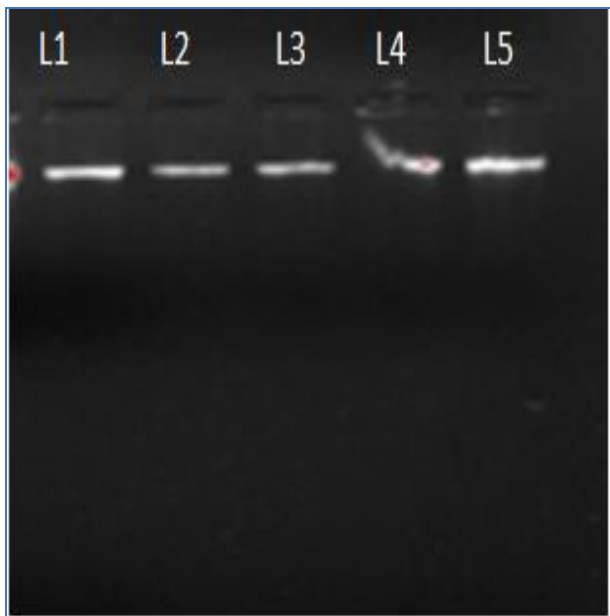
The PCR product of ~900bp size was identified in symptomatic plants and further distinguished by agarose gel, eluted (Fig.3), purified and sequenced by the Sanger sequencing technique (Kalaria *et al.*, 2013) <sup>[7]</sup>. Amplified 16S rDNA sequence was compared using nBLAST search for sequence homology with other database reference. The n-BLAST analysis revealed that the sequence showed 92% query cover and 99.15% similarity with Brinjal little leaf phytoplasma 16S ribosomal RNA gene (EU168777.1) (Table 1). The sequence was named as Candidatus Phytoplasma spp. strain NAU gujarat 16S ribosomal RNA gene and deposited in the GenBank with the accession number MK942854. Yadav *et al.* (2015a) <sup>[21]</sup> also had 97% resemblance to ' *Candidatus Phytoplasma trifolii* ' (JX104336.1). Kumar *et al.* (2012) <sup>[8]</sup> also discovered 98% of *Candidatus Phytoplasma asteris* sequence identity. Kumar *et al.* (2017) <sup>[9]</sup> also

demonstrated a maximum of 99 percent 16SrDNA sequence identity with clover proliferation group phytoplasma strains (16SrVI).

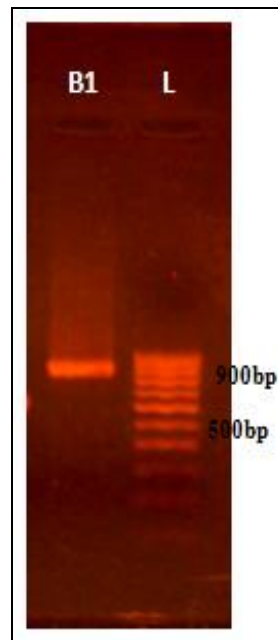
16SrRNA genes sequence (MK942854) compared with other NCBI sequences for multiple sequence alignment using Clustal\_X version 2.0 Software and neighbor-joining method with 1000 bootstrap replications followed a phylogenetic tree was constructed using the neighbor-joining method of MEGA 6 (Tamura *et al.*, 2007) <sup>[19]</sup>. Phylogenetic analysis (Fig. 4) showed that Candidatus Phytoplasma spp. strain NAU gujarat 16S ribosomal RNA gene showed closest connections with isolates Brinjal little leaf phytoplasma 16S ribosomal RNA gene, partial sequence EU168777, EU168776 and EU168778, which belonged to the 16Sr-VI group of phytoplasma clover proliferation. Similar findings were acquired from Azadvar and Baranwal (2012) <sup>[2]</sup> during the phylogenetic analysis of *Candidatus Phytoplasma trifolii* with the same universal first pairs in New Delhi's brinjal small leaf phytoplasma disease. Similarly, little leaf disease of Datura innoxia by Rao *et al.* (2011) <sup>[17]</sup> and Kumar *et al.* (2012) <sup>[8]</sup> reported little leaf disease of brinjal caused by '*Candidatus Phytoplasma asteris*' at brinjal field of Bihar, India. Rathnamma and Patil in (2016) <sup>[16]</sup> also reported 16S rRNA gene of brinjal little leaf phytoplasma belonging to the phytoplasma clover proliferation group (16SrVI) '*Ca. Phytoplasma trifolii*'. Infected Little Leaf Brinjal samples from Mysore and Dharwad belong to 16SrVI-D also reported by Yadav *et al.* (2015a) <sup>[21]</sup>.



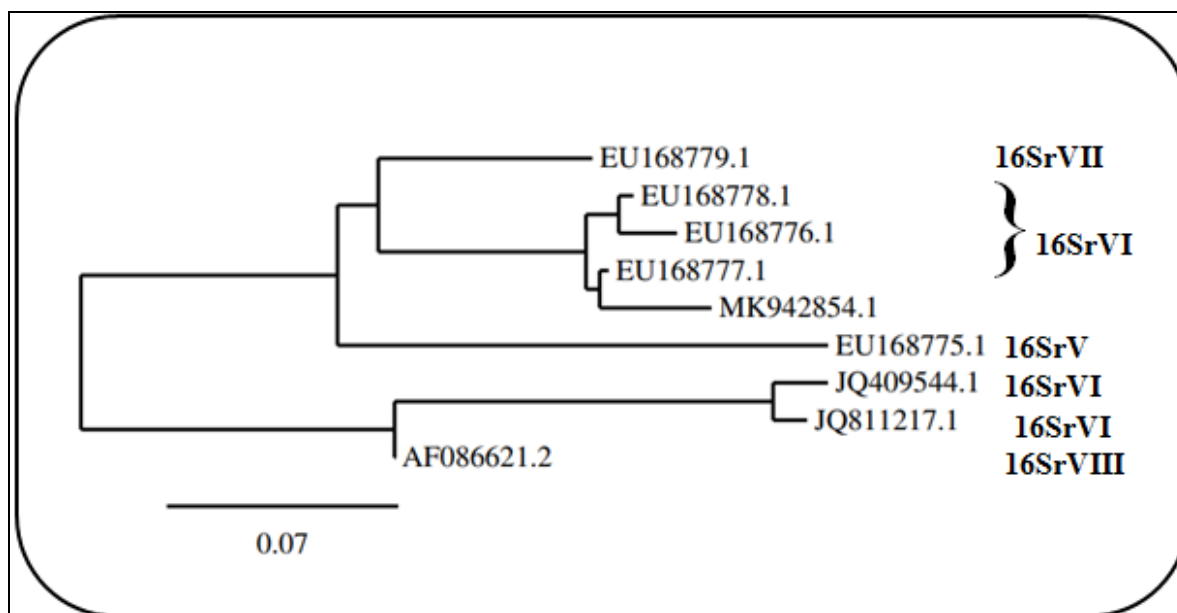
**Fig 1:** Phytoplasma spp. infected Brinjal plants exhibiting severe leaf curl, blistering reduction of leaf size and stunting of whole plant



**Fig 2:** Total Genomic DNA isolation from *Phytoplasma* spp. infected Brinjal plants



**Fig 3:** Amplification of 16S rRNA of *Phytoplasma* spp.



**Fig 4:** Phylogenetic tree showing the genetic relationship of *Candidatus phytoplasma* spp. [MK942854.1] to other phytoplasmas based on 16S rDNA sequences

**Table 1:** Percent Identities (Nucleotide) between 16S rRNA of *Candidatus phytoplasma* spp. [MK942854.1] with BLASTN of other *CANDIDATUS* sp. reported worldwide.

No.	Accession no.	Name of organism	Query cover	E value	Percentage Identity
1.	EU168777.1	Brinjal little leaf phytoplasma 16S ribosomal RNA gene, partial sequence;	92%	0.0	99.15%
2.	EU168776.1	Potato witches'-broom phytoplasma 16S ribosomal RNA gene, partial sequence;	92%	0.0	97.47%
3.	EU168778.1	<i>Candidatus phyllody</i> phytoplasma 16S Ribosomal RNA gene partial sequence	92%	0.0	96.46%
4.	EU168779.1	<i>Candidatus phytoplasma fraxini</i> 16S Ribosomal RNA gene partial sequence	92%	0.0	90.30%
5.	AF086621.2	Loofah witches broom phytoplasma t RNA –Ile gene, complete sequence	88%	0.0	88.81%
6.	JQ811217.1	Tomato big bud phytoplasma isolate ZYT-3 23S ribosomal RNA gene,	58%	0.0	99.66%
7.	JQ409544.1	Brinjal little leaf phytoplasma 23S Ribosomal RNA gene complete sequence	58%	0.0	98.45%
8.	EU168775.1	Elm witches'-broom phytoplasma 16S ribosomal RNA gene, partial sequence;	83%	0.0	83.31%

**Conclusion**

Brinjal little leaf (BLL) is a prevalent phytoplasma etiology disease causing severe economic losses in India. During March 2019, samples were gathered from the southern Gujarat region of India showing serious signs of stunting and

abundant branching for phytoplasma identification and detection with little leaf. Direct PCR assays were conducted from the leaf DNA of the diseased plant. A 900bp sequence of 16S rRNA gene sequence of BLL phytoplasma strains was verified as *Candidatus phytoplasma* spp. strain NAU Gujarat

16S ribosomal RNA gene (MK942854) belonging to the 16SrVI category of phytoplasma clover proliferation. This is the first report of a 16Sr-VI group *Candidatus phytoplasma* spp. in Gujarat, India, to our understanding. However, in order to explore phytoplasma diseases, host-vector interactions and their epidemiology, further molecular-based studies are needed to formulate novel Brinjal Little Leaf disease management approaches.

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