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Priya Y Gawande
Department of Plant Pathology, TNAU, Coimbatore, Tamil Nadu, India

Dr. M Karthikeyan
Department of Plant Pathology, TNAU, Coimbatore, Tamil Nadu, India

Dr. I Johnson
Department of Plant Pathology, TNAU, Coimbatore, Tamil Nadu, India

Dr. R Swarnapriya
Floriculture Research Station, Thovalai, Tamil Nadu, India

Dr. N Manikanda Boopathi
Department of Plant Biotechnology, TNAU, Coimbatore, Tamil Nadu, India

Corresponding Author:
Priya Y Gawande
Department of Plant Pathology, TNAU, Coimbatore, Tamil Nadu, India

Overview of little leaf disease in eggplant in Tamil Nadu

Priya Y Gawande, Dr. M Karthikeyan, Dr. I Johnson, Dr. R Swarnapriya and Dr. N Manikanda Boopathi

Abstract
Phytoplasma is one of the devastating pathogens responsible for significant crop losses in eggplant globally that causes little leaf of brinjal. This study focuses on survey, disease incidence and molecular characterization of BLL (Brinjal little leaf) phytoplasma. In 2021-2022, surveys were carried out in nine districts of Tamil Nadu where brinjal is grown, and 9 BLL samples were collected for phytoplasma identification. Disease incidence is calculated on the basis of symptoms exhibited by phytoplasma infected plant in three seasons. Collected samples exhibited little leaf, phyllody, and witches' broom symptoms. Coimbatore region showed highest disease incidence (Kharif- 32.40%, Rabi- 27.56%, Summer- 35.16%) and Tenkasi with lowest (Kharif- 17.42%, Rabi- 17.02%, Summer- 19.33%). Phytoplasma-specific primer pairs P1/P6 (amplify 16S rRNA region) R16F2n/R16R2 (amplify internal region of the 16S rRNA region) were used in the nested PCR to detect and characterize the of phytoplasmas present in each of the nine BLL samples. Sequence analysis discovered that the *Candidatus* Phytoplasma trifolii was associated with BLL with 98.54% percent and identity, with other isolates.

Keywords: Phytoplasma, brinjal, little leaf, molecular characterization, *Candidatus* phytoplasma trifolii

Introduction
Solanum melongena L., often known as brinjal or eggplant, is an important solanaceous vegetable crop grown all over the world. India stands second (22.58%) in the production of brinjal next to China 60% in 2020 [7]. It is a widely grown, indigenous, and significant vegetable crop that has been grown in India for more than 4,000 years, with the exception of higher altitudes. Brinjal is grown over an area of 27.41 thousand hectares with an annual production of 325.97 MT during 2021-22 [1] in Tamil Nadu. Brinjal is a perennial plant native to the tropics, and its fruit is a trove of nutrients including soluble carbohydrates, vitamins, minerals, and various proteins [8]. The therapeutic effects of brinjal fruits have been found to be advantageous for diabetics and those with liver diseases [21]. In addition to its limited genetic diversity, the extreme vulnerability of brinjal to biotic and abiotic stresses is a significant factor limiting its output. It is impacted by a number of diseases, with little leaf disease caused by phytoplasma being one of the most significant factors and inflicting substantial economic losses [16, 19]. Thomas and Krishnaswami, (1939) [23] were the first to report this disease in India.

The lack of a cell wall and a pleiomorphic or filamentous shape measuring 200–800 nm defines phytoplasma, which are microscopic bacteria that reside in the phloem sieve elements of infected plants and insects. The short genomes of phytoplasmas range in size from 530 to 1350 kb. Phytoplasma are solely host dependent due to the isotonic conditions that are provided by plant phloem and insect hemolymph for their growth and reproduction [4, 11]. Phytoplasma belonged to the Mollicutes class and was more akin to *Acholeplasma* species than to *Spiroplasma* species or animal mycoplasmas [17]. Six distinct phytoplasma subgroups continue to infect the crop now all throughout the world *viz*., 16SrI (Japan, Bangladesh and India); 16SrII-D (Egypt); 16SrIII-J & 16SrIII-U (Brazil); 16SrVI-A &D (Turkey & India); 16SrIX-C (Iran) and 16SrXII-A (Russia) [18]. The phytoplasma-related eggplant diseases often appear one to two months after the crop is transplanted and then spread like wildfire

Infected plants display a wide range of symptoms brought on by phytoplasmas, and these symptoms might vary depending on the strain, host, disease stage, time since infection, age of the plant at the time of infection, and environmental conditions [3, 15, 20]. symptoms such as virescence, phyllody, yellowing, witches' broom, leaf roll, and decline [5], auxiliary bud

proliferation, proliferation of secondary roots, enlarged stipules and other abnormalities of flower and fruits, off-season growth, and brown discoloration of phloem tissues [2, 12, 13, 14].

Phytoplasmas are regarded being one of the most molecularly enigmatic genera of plant diseases due to inability to culture them effectively *in vitro* and lack of knowledge of their host-pathogen-vector interactions. Sensitive approaches must be used to keep track of the existence and progress of phytoplasma diseases. The most versatile technique for identifying phytoplasma in host plants and their vectors is polymerase chain reaction (PCR) [22]. Use of 16S rRNA gene seems to be the most popular choice of many researchers working with detection of this pathogen aided by PCR experiments using universal primers followed by a group specific primer pair. The main objective of the current investigation was to study disease incidence, detection, characterization, and learning the phylogeny of phytoplasma associated with little leaf of brinjal in Tamil Nadu.

Materials and Methods

Sample collection

In 2021-2022, field survey was carried out in Coimbatore, Tiruppur, Pudukkottai, Tiruvannamalai, Madurai, Tenkasi, Dindigul, Theni and Tiruchirappalli districts of Tamil Nadu to gather brinjal samples that had been infected by phytoplasma in order to identify the causative agent or to record the incidence of phytoplasma disease in brinjal field. The brinjal crops in every region visited were examined for characteristic phytoplasma disease symptoms, and the disease incidence was calculated. Tender, distorted leaves, deformed flowers, and immature shoot buds were removed from symptomatic plants during sampling and stored in sampling bags. A representative healthy brinjal sample from each region was also taken in order to distinguish phytoplasma-infected samples from healthy samples. Samples collected from each region were preserved in sampling bags and further used for molecular characterization. After being kept in sampling bags, the samples gathered from each area were used for molecular characterization. After being kept in sampling bags, the samples gathered from each area were used for molecular characterization.

Disease incidence

Using the following calculation, the percent incidence for all the fields was calculated to determine the severity of the LLB disease;

$$\text{Disease Incidence (percent)} = \frac{\text{Number of infected plants}}{\text{Total plants}} \text{X } 100$$

Based on the morphological symptoms that infected brinjal plants showed, the prevalence of LLB disease was calculated.

Molecular characterization

Total DNA was extracted using the Cetyl trimethyl ammonium bromide (CTAB) technique from collected infected samples of symptomatic brinjal. From each sample 1 g of plant material (leaf midrib) was washed with distilled water, dried and grounded with liquid N_2 to make fine powder. This fine powder was taken in micro-centrifuge tube and 750 µl of CTAB buffer (DNA extraction buffer) was added in each tube separately followed by incubation at 65 °C in water bath for about 30 min with intermittent shaking. The mixture was centrifuged at 12,000 rpm/min for 10 min at 4°C to form pellet. An equal volume of phenol, chloroform, and isoamyl alcohol (25:24:1) was added to the supernatant in a new micro centrifuge tube, and the mixture was slowly inverted for 2-3 minutes after that. The mixture was once more centrifuged for 10 minutes at 4 °C at 12,000 rpm. The aqueous supernatant was transferred to a new tube, 600 µl of isopropanol was added, and the mixture was incubated at – 20 °C overnight. It was again centrifuged at 12,000 rpm/min for 10 minutes after being incubated for the overnight. The supernatant was discarded and the pellet was washed with 70% ethanol. The pellet was dissolved in 30 µl of TE buffer and same is used for PCR amplification.

PCR Amplification

For amplification of phytoplasma ribosomal DNA phytoplasma universal primer pairs F P1(5′-AAGAGTTTGATCCTGGCTCAGGATT-3′) and R P6 (5′-CGGTAGGGATACCTTGTTACGACTTA-3′) [6] were used followed by nested primer pairs F R16F2n (5′-GAAACGACTGCTAAGACTGG-3′) and R R16R2 (5′-TGACGGGCGGTGTGTACAAACCCCG-3′) [9]. The universal primers amplify 16S rRNA region and specific primer pair amplify the internal region of 16S rRNA. The PCR was done with a 20 µl reaction consisting of 7.5 µl Takara Emerald Amp® GT PCT Master Mix and 1 µl of each primer, 2 µl of DNA and 3.5 µl of sterile water. Mastercycler® Nexus gradient X2 PCR cycler (MA, USA) is used to carry out all PCR reaction with following parameters: Initial denaturation at 95 °C (2 min), DNA denaturation at 94 °C (1 min), annealing at 55 °C (1 min), extension at 72 °C (90 sec) and final extension at 72 °C (10 min) for 1st set of PCR and Initial denaturation at 95 °C (2 min), DNA denaturation at 94 °C (1 min), annealing at 60 °C **(**1 min), extension at 72 °C (90 sec) and final extension at 72 °C (10 min). The product obtained from the first round of PCR assay was diluted 1:20 with double sterilized distilled water and 2 µl was used as template in nested PCR assay. 5 µl of nested PCR product was subjected to electrophoresis in a 1.0% (w/v) agarose gel, stained with ethidium bromide and observed under UVTEC Gel doc EZ Imaging system.

Sequencing of 16S rRNA gene

The amplified PCR product was sent to Eurofins Genomics India Pvt Ltd, Bangalore, India for sequencing at both the orientations. Using the Bio Edit sequence alignment editor tool, forward and reverse direction sequences were aligned to provide assembled and consensus sequences [10]. Obtained sequence was blasted in NCBI database (www.ncbi.nlm.nih.gov) and for further analysis similar sequences were retrieved. The aligned sequences were deposited in the GenBank database and accession no is obtained.

Results and Discussion

Disease incidence and symptomatology

In this study, field survey was conducted in 9 brinjal growing areas of Tamil Nadu state of India viz, Coimbatore, Tiruppur, Pudukkottai, Tiruvannamalai, Madurai, Tenkasi, Dindigul, Theni and Tiruchirappalli. Infected plants were identified based on physical appearance of symptoms on the plant. The characteristics symptoms of phytoplasma infected plant mainly comprised of drastic reduction in leaf size and excessive growth of axillary shoots which give plants bushy

appearance, sterility and absence floral parts. Such plants rarely produce fruit, if produced, they were smaller in size, hard and inedible. Other symptoms include necrosis, malformed flower, phyllody, side shoot little leaf initiation along with little leaf, little leaf with mosaic (Figure 1).

When the disease incidence was assessed throughout all infected fields, it was discovered that it ranged from 24% to 41% in Coimbatore, 14% to 27% in Tiruppur, 15% to 29% in Pudukkottai, 12% to 25% in Tiruvannamalai, 19% to 34% in Madurai, 9% to 23% in Tenkasi, 20% to 37% in Dindigul, 10% to 23% in Theni and 18% to 31% in Tiruchirappalli over all season. It is also confirmed that disease incidence in summer (27.05%) is more followed by kharif (25.08%) and rabi season (22.07%) (Table 1).

Molecular characterization and Species identification:

The 16S rRNA regions were amplified with the universal primers P1/P6 followed by nested primer pair R16F2n and R16R2 to validate the initial identification. All nine isolates were amplified with approx. 1200 bp which has confirmed it as *Ca. Phytoplasma* (Figure 2). The amplified BLLP-1 isolate were sequenced by sangar dideoxy sequencing in NCBI. Partial gene sequencing of phytoplasma nucleotide showed 98.54% similarity with *Ca. P. trifolii* Gene sequence in one isolate (BLLP-1) with BLASTn analysis. Pairwise sequence comparison revealed 96.7% similarity between BLLP-1. The sequence results obtained were submitted in NCBI GenBank, and the isolate BLLP-1was assigned with the accession number as ON870804.

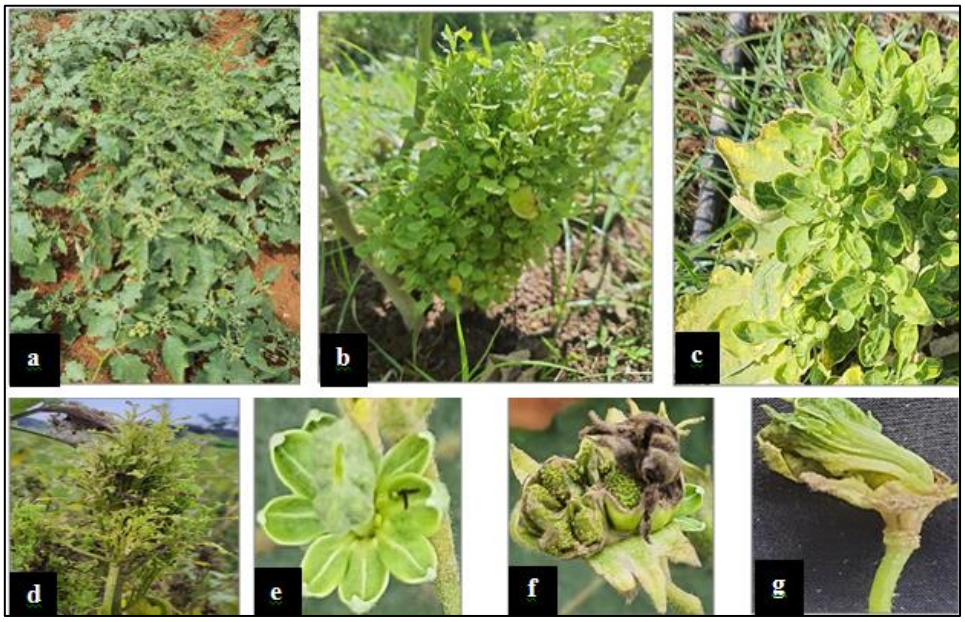

Fig 1: Typical phytoplasma affected brinjal samples collected from Tamil Nadu: a. Initiation of little leaf, b. Side shoot little leaf initiation along with little leaf, c. little leaf with mosaic symptom, d. necrosis, e. virescence, f. malformed flower, g. phyllody

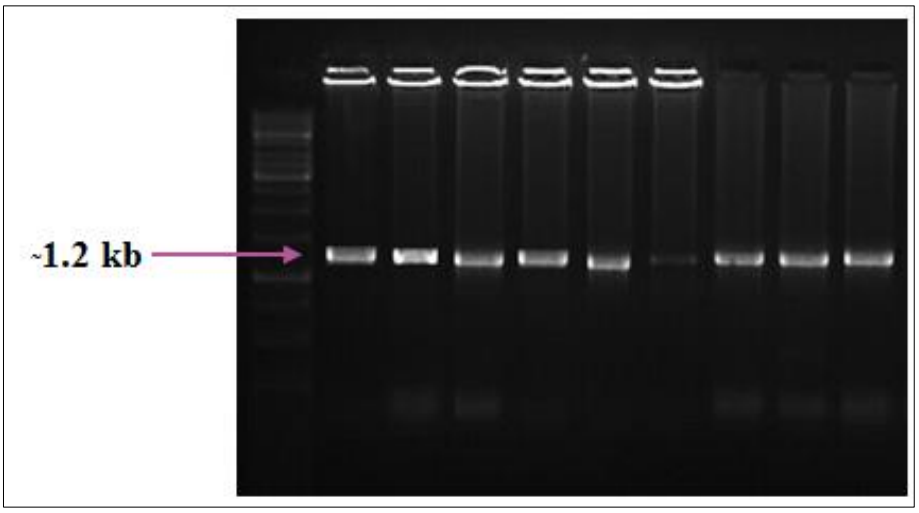

Fig 2: Nested PCR assay results of phytoplasma DNA amplification from brinjal little leaf and phyllody plants with primer pair RI6F2n/RI6R2n; Lane 1: Ladder, Lane 2: BLLP-1, Lane 3: BLLP-2, Lane 4: BLLP-3, Lane 5: BLLP-4, Lane 6: BLLP-5, Lane 7: BLLP-6, Lane 8: BLLP-7, Lane 9: BLLP-8, Lane 10: BLLP-9

Table 1: Incidence of brinjal little leaf in various district Tamil Nadu during 2021-22.

	Source	*Percent incidence					
		Kharif 2021		Rabi 2021-2022		Summer 2022	
		Incidence Range	Average	Incidence Range	Average	Incidence Range	Average
1	Coimbatore	27-35	32.40 [a] (34.69)	24-30	27.56 [a] (31.66)	29-41	35.16 [a] (36.36)
2	Tiruppur	16-25	23.63[f] (29.08)	14-21	20.03 [f] (26.58)	18-27	25.87 [f] (30.57)
3	Pudukkottai	19-27	25.38 [e] (30.25)	15-24	22.97 [e] (28.63)	20-29	27.53 [e] (31.64)
4	Tiruvannamalai	14-23	20.62 [g] (27)	12-20	18.57 [g] (25.52)	15-25	21.84 [g] (27.86)
5	Tenkasi	10- 20	17.42 [i] (24.66)	9-18	17.02 [i] (24.36)	12-23	19.33 [i] (26.08)
6	Madurai	21-31	28.27 [c] (32.12)	19-27	25.43 [c] (30.28)	25-34	31.56 [c] (34.17)
7	Dindigul	23-33	30.99 [b] (33.82)	20-29	26.07 [b] (30.70)	27-37	32.19 [b] (34.56)
8	Theni	11-21	19.86 [h] (26.46)	10-19	17.97 [h] (25.08)	13-23	20.54 [h] (26.94)
9	Tiruchirappalli	20-29	27.15 [d] (31.4)	18-26	23.01 [d] (28.64)	22-31	29.46 [d] (32.87)
	Average		25.08 (29.94)		22.07 (27.94)		27.05 (31.23)

*Mean of three replications
In a column, means followed by a common letter is not significantly different at 5% level by DMRT
Figures in parenthesis are Arcsine transformed value

Conclusion

The present study delivers the BLL incidence in regions of Tamil Nadu. It has been found that Coimbatore has highest incidence and Tenkasi the lowest in summer followed by kharif and rabi season. Based on the findings, it has been inferred that *Candidatus* Phytoplasma trifolii is associated with BLL.

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