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Abirami R
Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Manoranjitham SK
Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Rajasree V
Department of Vegetable Science, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Mohankumar S
Department of Biotechnology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Karthikeyan G
Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Corresponding Author:
Karthikeyan G
Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Occurrence and documentation of clover proliferation and witched broom subgroup members of Phytoplasma on brinjal in Tamil Nadu

Abirami R, Manoranjitham SK, Rajasree V, Mohankumar S and Karthikeyan G

Abstract
An intensive fixed plot survey was conducted in TNAU Research farm during in all cropping season of the year 2019-2020 to document and investigate the incidence of little leaf disease in brinjal. There was no incidence of little leaf till 75 days post transplanting, later the incidence was started and progressed gradually to more than 50% at the end of the cropping season. Infected samples were subjected for Nested PCR using 16-23S ribosomal universal primer (P1 & P7) to amplifying 16S rRNA region followed by amplifying the internal region of 16SrRNA region using nested primer (R16F2n & R16Frn) to detect and characterize the phyoplasma infecting brinjal. Upon sequence analysis the brinjal crop in TNAU research farm was found to be infected with two important species of phytoplasma *viz.*, *Candidatus Phytoplasma trifolii* (*Ca.* P. trifolli) and *Candidatus* Phytoplasma aurentifolia (*Ca.* P. aurentifolia) with 99 and 99.5% in identity with other isolates in BLASTn analysis. Phylogenetic analysis by neighbor joining tree method revealed that the two isolates (LL TNB1 and LL TNB2) clustered among their subgroups using. The nucleotide sequences of phytoplasma were analyzed in *i*phy classifier online search tool to find the subgroups of the phytoplasma. LL TNB1 was found to be clover proliferation group belongs to 16Sr VID sub-group and LL TNB2 was belongs to witches broom group of 16Sr IIB.

Keywords: Brinjal little leaf, Nested PCR, Candidatus *Phytoplasma trifolii*, Candidatus Phytoplasma aurentifolia

1. Introduction
India is the second largest producer of brinjal all over the world next to China. The crop act as important vegetable in dinning as it contains high nutrition and dietary fiber and the crop is widely cultivated in tropical and sub-tropical regions. One of the oldest pathogen, phytoplasma act as barrier to brinjal production under natural conditions since 1939 (Thomas and Krishnaswamy 1939) [11] and creates huge loss till today. Until today, the crop is infected by six different subgroups of phytoplasma around 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) (Rao and Kumar 2017) [4]. The crop in India is facing the mixed infection of viruses and phytoplasmas *viz.*, (ToLCV) and *Candidatus* Phytoplasma (Singh *et al.* 2015) [8] and PVY, PVX and phytoplasma (Kumar *et al.* 2016) [3]. The co-infection and mixed infection of phytoplasma and virus makes the crop more vulnerable for brinjal production in the country. To study the intensity of little leaf disease through the cropping season the study is dealt and the data provided in the study helps in better understanding of the epidemiology of little leaf with the subgroups of phytoplasma infecting brinjal.

2. Materials and Method
2.1 Fixed plot survey
Brinjal crop in TNAU, Research farm was contemplated to investigate the occurrence and distribution of little leaf disease throughout the cropping seasons of the year (2019-2020). In order to assess the phytoplasma occurrence in the entire cropping season of the year, a fixed plot survey was conducted in brinjal fields during the *Karif* and *Rabi* seasons. Observing the number of plants showing symptoms and the total number of plants in the field, the per cent phytoplasma disease incidence for each field was calculated at 15-day intervals.

The leaf samples were collected from diseased plants.

2.2 Detection of phytoplasma – Nested PCR

Cetyl trimethyl ammonium bromide (CTAB) method was used to extract the total DNA from symptomatic brinjal samples and the DNA was quantified using an Eppendorf Biospectrometer (Germany), which was uniformly diluted to a concentration of 2000 ng/l. Despite the fact that several primer sets for phytoplasma detection were available, the nested technique proposed by Gunderson and Lee (1996) [1] was used for accurate diagnostic evaluation. The 16S rRNA region of phytoplasma were amplified in the first round with universal primer pair P1 F 5'AAGAGTTTGATCCTGGCTCAGGATT 3' and R (P7) 5' GTCCTTCATCGGCTCTT 3' corresponding to the 16S rRNA region of phytoplasma and the second round of PCR to amplify the internal region of 16S rRNA (Lee *et al*., 1993) [5], R16F2n (F) 5' GAAACGACTGCTAAGACTGG 3' and R16R2 (R) 5' TGACGGGCGGTGTGTACAAACCCCG 3'. The PCR was done with a 25 µl mixture of 2X Promega Go Taq® green Master Mix and 1 µl of each primer, 1.5 ng of DNA and 2 µl of sterile water.

2.3 Sequencing and computational analysis

Resulted amplicons were purified using Sigma - GenElute™ kit and, sequenced at both the orientations with M/s Barcode Biosciences, Bangalore. The sequences were first blasted in the NCBI database (www.ncbi.nlm.nih.gov/) and similar sequences were retrieved for further analysis based on identity. CLUSTALW was used to align the sequences (www.ebi.ac.uk). BioEdit sequence alignment editor version 7.0 was used to align several nucleotide (nt) and amino acid (aa) sequences. The Neighbor-joining tree method with 1000 bootstrap replication was used to draw phylogenies using the MEGA-X version.

2.4 Grouping of phytoplasma through i Phy Classifier

The consensus nucleotide sequences of phytoplasma were subjected to *i*phyClassifier (an online tool) for classifying the group/sub-group of phytoplasma.

3. Results and Discussion

3.1 Documentation and incidence

From the 15th day of post transplanting (DPT) at 15 days interval, the crop was intensively monitored for the expression of the little leaf symptoms. The symptom was not noticed till 70 DPT after 75 DPT but afterwards when the crop reaches the end of its vegetative stage, the little leaf symptom emerges. In both the seasons, the crop exhibited partial little leaf, complete little leaf, emergence of side shoots, reduced internodes, stunted growth, partial phyllody and complete phyllody symptoms. The crop had 2% disease incidence initially, but as the crop becomes older, the incidence rises more than 50% was documented after 170 DPT (Table 1; Fig. 1.). Until 1939, the little leaf disease on brinjal was not reported in the country, it was first recorded by Thomas and Krishnaswamy (1939) [11] in Coimbatore, Tamil Nadu. The authors suggested that pathogen could be the virus but the infected crop was witnessed with 100% sterility. Earlier, Smith (1937) [9] mentioned the disease was not caused by the viral pathogen. Since then the pathogen threats the brinjal production in the country. The disease progressively increased from 2% of incidence at 90 DPT and reaches more than 50% at end of the cropping period. If the incidence occurs in the early stage of the crop 100% yield loss was estimated.

3.2 Species identification and molecular characterization of *Cadidatus* Phytoplasma

Partial gene sequencing of phytoplasma nucleotide using 16SrRNA gene exemplified 99% identity with *Ca.* P. trifolii in one isolate (LL TNB1) and another showed 99.5% identity with *Ca.* P. aurentifolia (LL TNB2) upon NCBI-BLASTn analysis. Pairwise alignment revealed 95 and 90% identity between LL TNB1 and LL TNB2 isolates. Both the isolates showed least per cent identity with *Ca.* P. Australasia (45%) that causes big bud disease. The respective sequences were deposited in NCBI database (Accession No- SK880125 and SK880126). According existing evidences, all four subgroups of brinjal little leaf phytoplasma in India were 16SrI, 16SrIID, 16SrV, and 16SrVI-D, which were caused by *Ca.* P. asteris, *Ca.* P. australasia, *Ca.* P. ulmi, and *Ca.* P. trifolii (Kumar *et al*. 2012; Maheshwari *et al*. 2017 and Snehi *et al*. 2021). Kumar *et al*. (2017) [2, 6, 10, 4] also identified that the subgroup 16Sr IIA was detected only in Uttar Pradesh. Otherwise there is no reports of 16Sr IIA subgroup infection in brinjal on Tamil Nadu. However, the two subgroups 16Sr VI-D caused by *Ca*. P. trifolii belongs to clover proliferation groups and 16Sr IIB was wide spread in crop plants, weeds and many species of hoppers (Maheshwari *et al*. 2017) [6]. Neighbor joining tree of phylogenetic analysis expressed the study isolat *Ca.* P. trifolii clustered along the other *Ca.* P. trifolii isolates and similarly

Ca. P. aurentifolia clustered with the other *Ca.* P. aurentifolia isolates and both the study isolates formed two different clusters (Fig. 2.). The study isolates were segregated to their respective groups based the online search tool *i*phy classifier. *Ca.* P. trifolii was belongs to 16SrVI-D subgroup that causes clover proliferation group phytoplasma and *Ca.* P. aurentifolia was sub-grouped in 16SrIIB of witches broom group phytoplasma. Further, the species of phytoplasma can be studied with RFLP to know the diversified occurrence of the phytoplasma. Initially, the crop was infected only with one species of phyoplasma on 90 DPT that is *Ca.* P. trifolii later the symptomatic samples were detected with another species of phytoplasma *Ca.* P. aurentifolia. In some of the samples mixed infection of phytoplamas where also observed.

Table 1: Seasonal disease incidence of little leaf on brinjal in Tamil Nadu

***Kharif* (2018-2019)**				***Rabi* (2019-2020)**			
DAT	**Little leaf disease incidence (%)**	**Symptom (s) observed**	**Phytoplasma detected**	**DAT**	**Little leaf disease incidence (%)**	**Symptom observed**	**Phytoplasma detected**
15	-	-	-	15	-	-	-
30	-	-	-	30	-	-	-
45	-	-	-	45	-	-	-
60	-	-	-	60	-	-	-

75	-	-	-	75	-	-	-
90	2	Emergence of little leaf on top leaves	*Ca.* P. trifollli	90	3	Emergence of little leaf on top leaves	*Ca.* P. trifollli
115	8	Emergence of little leaf on top leaves	*Ca.* P. trifollli	115	11	Emergence of little leaf on top leaves	*Ca.* P. trifollli
130	14	Little leaf & emergence of phyllody	*Ca.* P trifollli and *Ca.* P. aurentifolia	130	15	Little leaf & emergence of phyllody	*Ca.* P trifollli and *Ca.* P. aurentifolia
145	20	Little leaf & phyllody	*Ca.* P trifollli and *Ca.* P. aurentifolia	145	19	Little leaf & phyllody	*Ca.* P trifollli and *Ca.* P. aurentifolia
160	31	Side shoot little leaf initiation along with little leaf & phyllody	*Ca.* P trifollli and *Ca.* P. aurentifolia	160	27	Side shoot little leaf initiation along with little leaf & phyllody	*Ca.* P trifollli and *Ca.* P. aurentifolia
175	50	Complete phyllody	*Ca.* P trifollli and *Ca.* P. aurentifolia	175	56	Complete phyllody	*Ca.* P trifollli and *Ca.* P. aurentifolia

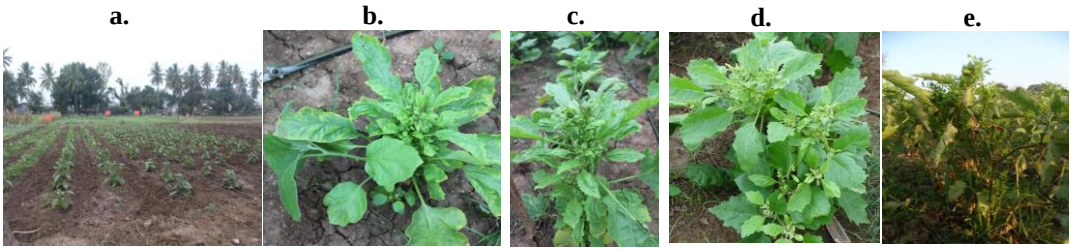

Fig 1: Incidence & symptomatology of little leaf of brinjal. a. fixed plot survey, b & c. little leaf disease at 80 & 90 days old crop, d. partial phyllody at 120 days old crop and e. complete phyllody at 150 days old crop

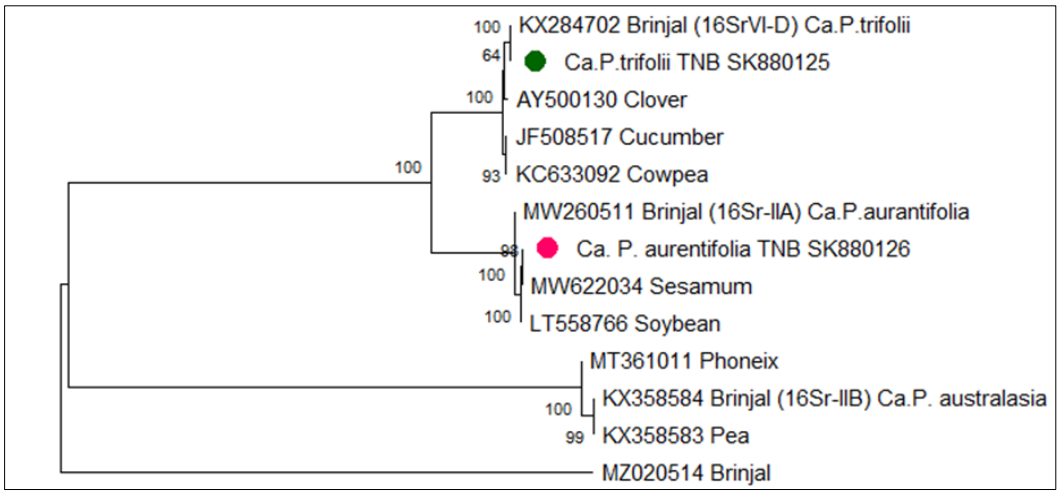

Fig 2: Phylogenetic analysis of *Ca.* P. trifolli and *Ca.* P. aurentifolia of LL TNB1 and LL TNB2 isolates of brinjal with other species of Ca. P. isolates. The evolutionary history was inferred from neighbor joining method with 1000 bootstrap replication. GBNV infecting brinjal was included as out group

4. Conclusion

The present study delivers the incidence and severity of the two major sub-groups of phytoplasma species that infecting brinjal crop in Tamil Nadu during the complete cropping seasons all around the year. The mode of transmission, genetic variability, and the epidemiology of both the mollicute have to be studied in detail for the better management of the pathogen and to reduces the crop losses.

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