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Molecular characterization of tomato leaf curl New Delhi virus and its transmission by whitefly *Bemisia tabaci* in bitter gourd

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Abstract
Bitter gourd is an important vegetable crop, which is well known for its nutritive and medicinal value. For the last five year, bitter gourd cultivable area is gradually decreasing by a devastating yellow mosaic disease. The disease is caused by two important Begomoviruses *viz.*, Tomato leaf curl New Delhi virus (ToLCNDV) and Bitter gourd yellow mosaic virus (BgYMV). In which, ToLCNDV is more prevalent in Coimbatore district of Tamil Nadu. The survey indicated that the disease incidence was from 58.1 to 89.3% in five locations *viz.*, Agarapatti, Narasipuram, Pappampatti, Thondamuthur and Vellimalaipattinam. The incidence was high at Pappampatti village with 89.3% with high whitefly population of 8.75/plant. The PCR analysis confirmed the presence of ToLCNDV in all the five locations by using specific primers for specific for the AV1 and AC3 regions. The Pappampatti isolate of ToLCNDV (ToLCNDV BGPP; Accession number-OR051905) was sequenced and BLAST search showed 98.01% similarity with ToLCNDV isolate of tomato (MT890778) and bitter gourd isolates (MT966792) from Karnataka. The ToLCNDV BGPP isolate was successfully transmitted by a group of 20 whiteflies with 93.33% transmission efficiency. The ToLCNDV BGPP was detected in whiteflies after 24h AAP and 24h IAP through PCR. Out of 15 plants transmitted with whiteflies, five symptomatic and nine asymptomatic plants were positive for ToLCNDV specific primers.

Keywords: Begomovirus, ToLCNDV, *Bemisia tabaci*, PCR, transmission

Introduction
Bitter gourd (*Momordica charantia* L) belongs to the family *Cucurbitaceae*, regarded as an essential home gardening vegetable. Tropical Asia is believed to be its origin, where it is widely spread in Pakistan, China, India, Bangladesh, Malaysia and Tropical Africa (Abdullah Khan *et al.*, 2022) [7]. In India, bitter gourd is cultivated in 109 ha with 1330 MT production. Madhya Pradesh stands first in production of bitter gourd 229.91 tonnes followed by Chattisgarh and Tamil Nadu. In Tamil Nadu the production of bitter gourd about 128.91 tonnes (National Horticultural Board 2021-2022). Bitter melon is traditionally used as a medicinal food in different system of pharmaceutical. It has significant importance in providing basic nutrients and prevention of various ailments. It contains a variety of bioactive compounds including alkaloids, polypeptides, vitamins, and minerals. A diversity of bioactive compounds comprises two classes of saponins such as oleanane and cucurbitane- type triterpenoids (Vidhu Aeri *et al.*, 2020). The productivity of bitter gourd is highly attributed to several diseases such as downy mildew, powdery mildew, fusarium wilt, white rot, damping off and root rots, bacterial wilt and leaf spot, mosaic, watermelon bud necrosis, leaf curl and leaf distortion virus. The tomato leaf curl New Delhi virus is one of the most important viral diseases in bitter gourd. The incidence of disease was significant and the symptom was yellow mosaic, upward curling of leaves with reduction of fruit size. ToLCNDV, was first reported from tomato in India in 1994. Tomato leaf curl New Delhi virus is an emerging problem for agricultural crops in India and neighbouring countries. ToLCNDV is widely distributed throughout India (Reddy *et al.*, 2005) [2] and other neighbouring countries likes Pakistan (Tahir & Haider 2005; Hussain *et al.*, 2005) [13, 4], Bangladesh (Maruthi *et al.*, 2005) [9] and Thailand (AF102276, Ito *et al.*, 2008) [5] *Tomato leaf curl New Delhi virus* (ToLCNDV) is a bipartite begomovirus (family *Geminiviridae*) transmitted by the whitefly *Bemisia tabaci*. The present study attempted to investigate the causal agent of yellow mosaic disease of bitter gourd through molecular characterization, symptomology and its transmission.

Materials and Methods

Survey and documentation of begomovirus infecting bitter gourd

A survey was conducted in bitter gourd fields of Agarapatti, Narasipuram, Papampatti, Thondamuthur and Vellimalaipattinam villages of Coimbatore district of Tamil Nadu for the documentation of begomovirus infection and whitefly population during the period of February to May in 2023. The data on the symptom type, percent disease incidence and whitefly population were assessed and percent disease incidence of begomovirus was calculated by using the following formula.

$$\text{Percent disease incidence} = \frac{\text{Number of infected plants}}{\text{Total numbers of plants observed}} \text{ X } 100$$

The incidence was recorded by roving survey in hundred plants per field. The leaf samples of bitter gourd plants showing typical begomovirus symptoms were collected from all locations. A representative of two samples for each location was used for DNA isolation. Total DNA was isolated from the symptomatic leaf samples using GEM-CTAB protocol given by Rouhibakhsh *et al.*, 2008 [12].

Establishment of whitefly culture

The whitefly egg masses were collected from bitter gourd plants and placed on brinjal plants and kept in the insect-proof rearing chamber for multiplication. The pure population of the whitefly *Bemisia tabaci was* continuously maintained on brinjal plants in the insect proof chamber.

Molecular characterization of ToLCNDV through PCR

For molecular confirmation of the begomovirus, DNA extracted from leaf samples were subjected to PCR using a degenerate primer pair "PARIv772 5'GGNAARATHTGGATGGA 3' and PALIc1960 5'ACNGGNAARACNATGTGGGC 3' designed by Roja's (1993) [11], which gives an expected amplicon of 1.2kb. Those samples which were positive for Roja's primers were then subjected to PCR with ToLCNDV specific primes. The primer pair of F-BM794 (492-509)-5'CCTTGTAAGGTGCAGTCC3' and R-BM795 (1194-1175)-5'AACCC AGGTCCTTAAGT ACC3' specific for AV1 and AC3 region of ToLCNDV with an expected amplicon size of 702bp were used for detection. PCR was performed with 10 µl reaction mixture containing 5µl of master mix, 2µl of water, 1µl of forward primer, 1 µl of reverse primer and 1µl of DNA and incubated at following temperature cycle for successful amplification. Initial denaturation of DNA at 94 ^{0}C for 3 min followed by 35 cycles of denaturation at 94 ^{0}C for 30 sec, annealing at 56 ^{0}C for 45 sec, extension at 72 ^{0}C for 1 minute and final extension 72 ^{0}C for 10 minutes. Finally, PCR products were visualized by 1% agarose gel electrophoresis for 1h at 80V. The gel was observed in gel documentation unit (Uvitec Cambridge – UK) for the presence of DNA band and photographed. 1 kb molecular weight marker was used to find out the exact size of DNA band.The PCR product of Agarapatti sample was sequenced through outsourcing (Syngenome Pvt Ltd.). The sequence obtained was analysed through BLAST search in NCBI data base.

Transmission of ToLCNDV in bitter gourd through whitefly

The ToLCNDV infected samples from Vellimalaipattinam were used as inoculum source for the transmission studied. The PALEE F1 hybrid seeds of bitter gourd were raised in pots and the plants were checked for ToLCNDV through PCR and the virus free plants were used for whitefly transmission. A total of fifteen plants were maintained in five pots, each with three plants and were kept in insect proof chamber for transmission studies. Whiteflies (*Bemisia tabaci*) were collected from rearing chamber by using aspirator in clip cages. Collected whiteflies (20 nos) were allowed to feed on the ToLCNDV infected bitter gourd leaves (ToLCNDV BGPP isolate) to acquire the virus with 24h of acquisition access period (AAP). The viruliferous whiteflies with 24h of AAP were carefully removed from the infected source and allowed to feed on 10 days old healthy bitter gourd plants (15 plants) to inoculate the virus with 24h of inoculation access period (IAP). After 24hrs of AAP, 10 whiteflies were taken to confirm the acquisition of ToLCNDV through PCR using ToLCNDV. After 24h of IAP, whiteflies were killed by spraying systemic insecticide (Imidacloprid 200SL @ 0.4 ml/l). Finally, the plants were maintained in insect proof cages for symptom expression. Five plants were maintained without transmission for comparison. The observations on symptom expression, days taken to express symptoms were recorded and leaves were collected from both the symptomatic and asymptomatic plants and subjected to PCR for the detection of ToLCNDV using the specific primers.

Results

Survey and documentation of begomovirus infecting bitter gourd: A field survey conducted in bitter gourd fields of five locations of Coimbatore district exhibited various pattern of symptoms like mosaic, curling, blistering, puckering, severe stunting, reduction in leaf lamina and bushy (Fig 1a, b & c). The disease incidence was from 58.1 to 89.3%. The highest disease incidence was recorded in Pappampatti with 89.3% and whitefly population was also found to be high with (8.75 / plant) in PALEE F1 hybrid. It was followed by Agarapatti (85.70%), Vellimalaipattinam (82.3%), Narasipuram (70.5%) and Thondamuthur (58.1%). The minimum whitefly population was recorded in Thondamuthur with 2.10/plant (Table 1).

a. Mild mosaic, b. Severe mosaic and upward curling, c. blistering, d. Clustering

Fig 1a-d: Symptom expressed by begomovirus infection in the field

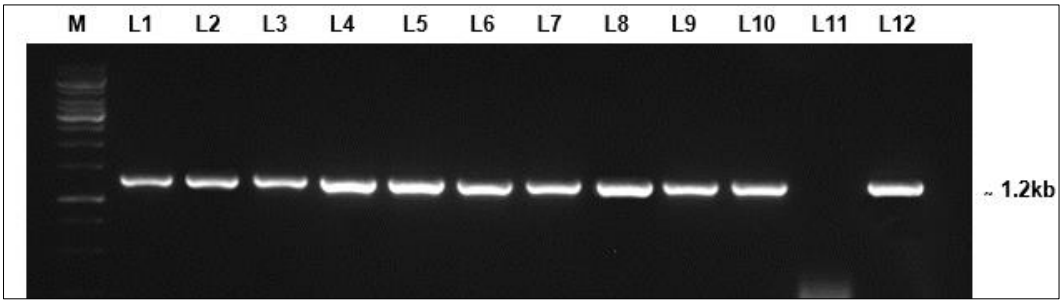
Table 1: Survey and documentation of yellow mosaic disease incidence and whitefly

S. No	Location/ Hybrid	GPS Co ordinates		Stage of the crop infected	Symptom type	Percent disease incidence	Number of whitefly per plant
		Latitude	Longitude				
1	Agarapatti (Rukshan)	10.41703°	78.698588°	Fruit set	Mosaic, puckering and clustering	85.7	8.46
2	Narasipuram (Rukshan)	10.988138°	76.776239°	Fruit set	Chlorosis Mosaic	70.5	6.02
3	Pappampatti (Palee F1)	10.943923°	77.112716°	Flowering	Chlorosis Leaf curl	89.3	8.75
4	Thondamuthur (Rukshan)	10.99788°	76.828107°	Fruit set	Chlorosis Leaf curl	58.1	2.10
5	Vellimalaipattinam (Rukshan)	10.995972°	76.787269°	Flowering	Chlorosis Mosaic	82.3	7.44

Population in Coimbatore district (February – May 2023)

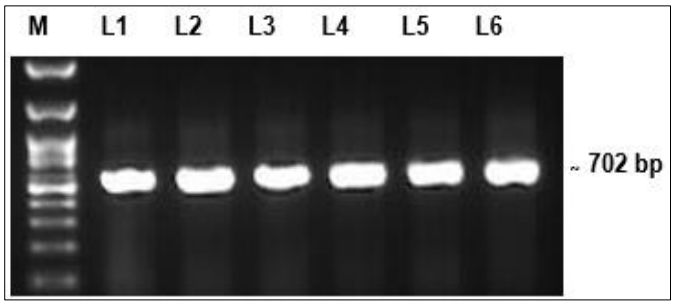
Molecular confirmation of begomovirus through PCR

The PCR performed with Roja's primers amplified an expected amplicon of 1.2kb in all the ten samples from five locations. Further, one positive sample per location was analyzed for ToLCNDV using specific primers (Fig.2). All the five samples were found to be positive with an expected amplicon of ~702 bp. The nucleotide sequence of Pappampatti isolate (ToLCNDV BGPP) was analysed through BLAST search in NCBI data base and the virus involved was identified as tomato leaf curl New Delhi virus. It showed 98.01% similarity with ToLCNDV isolate of tomato (MT890778) and bitter gourd isolate (MT966792) from Karnataka. The sequence was submitted in NCBI data base with accession number OR051905 (Fig.3).



Lane M-1kb ladder; L1, L2 – Agarapatti sample; L3, L4 – Narasipuram sample; L5, L6 – Pappampatti sample; L7, L8 – Thondamuthur sample; L9, L10 – Vellimalaipattinam sample; L11 – Healthy control; L12 – Positive control (ICMV infected leaf sample)

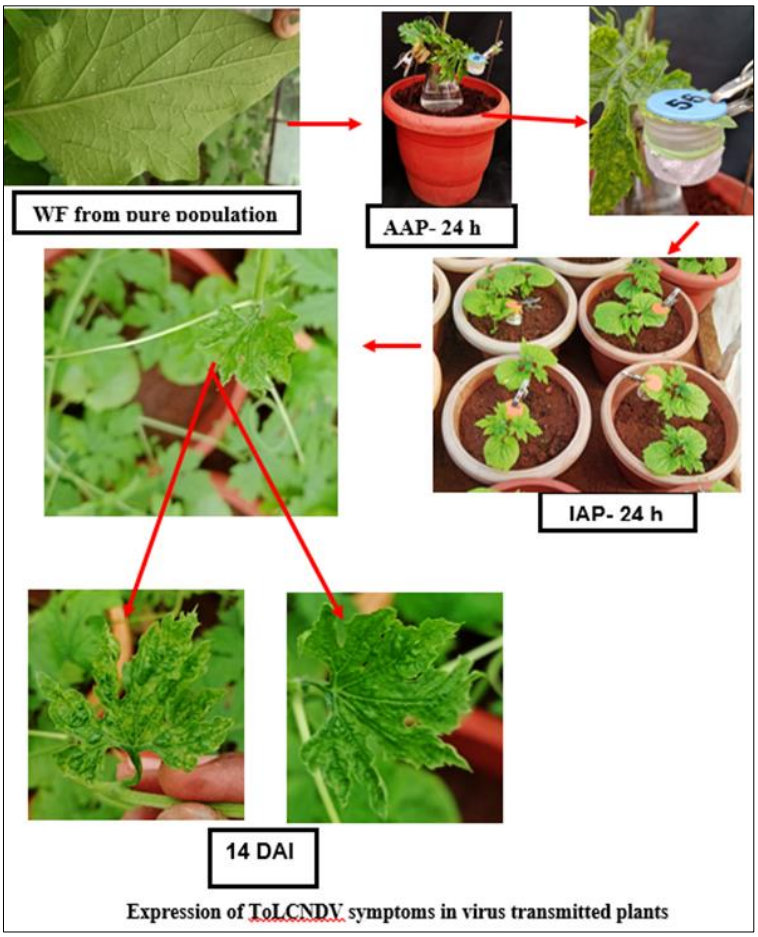
Fig 2: Agarose gel electrophoresis of PCR products from begomovirus infected bitter gourd leaves from different locations using Roja's primers



Lane M – 1kb ladder; L1 – Agarapatti isolate; L2 – Narasipuram isolate; L3 – Pappampatti isolate; L4 – Thondamuthur isolate; L5 – Vellimalaipattinam isolate; L6 – Positive control

Fig 3: Agarose gel electrophoresis of PCR products from begomovirus infected bitter gourd leaves from different locations using Roja's primers

Transmission of ToLCNDV in bitter gourd through whitefly: The successful transmission of ToLCNDV was recorded through whitefly *Bemisia tabaci*. A characteristic yellow mosaic symptom was seen 14 days after inoculation in five out of 15 plants and the remaining plants didn't produce any symptoms (Table 2; Fig.4). PCR analysis of whiteflies after 24hrs of AAP and 24h IAP for ToLCNDV specific primers revealed the successful acquisition ToLCNDV with positive amplicon of 702bp (Fig.5). Leaf samples from all the fifteen plants (Both symptomatic and asymptomatic) were analysed for ToLCNDV using specific primers. Out of 15 plants, 14 were PCR positive with an expected amplicon of 702bp (Fig.6.). This result revealed that in addition to the symptomatic plants (five plants), nine asymptomatic plants were also found to be positive for ToLCNDV. The transmission efficiency was 93.33%.

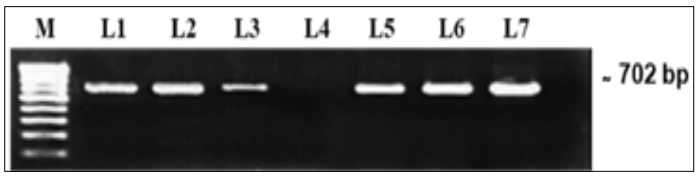


Expression of ToLCNDV symptoms in virus transmitted plants

Fig 4: Transmission of ToLCNDV in bitter gourd through whitefly

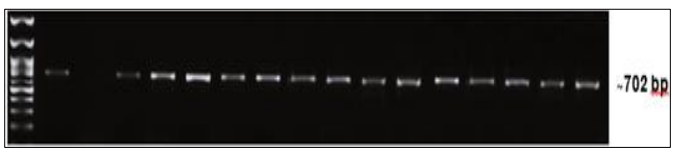
Table 2: Assessment of ToLCNDV transmission efficiency of whiteflies in bitter gourd

Days taken to express symptoms	No of plants expressed symptoms/ Total no. of plants inoculated	Types of symptoms expressed	Number of samples positive in PCR	No. of plants asymptomatic/ Total no of plants inoculated	Transmission efficiency (%)
14	5/15	Chlorosis, Mosaic, Leaf curl, Enation and Vein thickening	14/15	9 /15	93.33



Lane M-100bp ladder; 20 white files per sample L1, L2 & L3– Viruliferous whiteflies after 24h; AAP L4 – Aviruliferous healthy whiteflies; L5, L6 – Whiteflies after 24 IAP; L7 – Positive control

Fig 5: Agarose gel electrophoresis of PCR products of ToLCNDV from white files after 24h AAP and IAP through PCR using ToLCNDV



Lane M - 100 bp ladder; L1 to L15 – Whitefly mediated virus transmitted leaf samples; L 16- Positive control

Fig 6: Agarose gel electrophoresis of PCR products of ToLCNDV transmitted bitter gourd leaf samples with ToLCNDV specific primers

Discussion

Globally tomato leaf curl New Delhi virus (ToLCNDV) is a serious threat to cultivation of vegetable crops. It is currently widespread in India and has also been reported in countries like Pakistan, Bangladesh, and Thailand. The emergence of new strains of ToLCNDV in various crops and non-crop plants suggests that the virus has become more potent in the field, posing a substantial threat particularly in Southeast Asia. While ToLCNDV has been known to infect tomatoes for over a decade, its prevalence has increased significantly in the past five years. In the future, ToLCNDV could seriously hinder horticultural crop production due to its highly virulent strains that can infect a wide range of hosts. Tiwari *et al.* (2010) [14] made the first report of ToLCNDV infection in a *Momordica charantia* L. plants associated with yellow mosaic disease in Eastern Uttar Pradesh, India. Manivannan *et al.* (2019) [8] reported a severe yellow mosaic disease in bitter gourd crop in and around Coimbatore district with percent disease incidence of 58 to 100%. In a study conducted by Naik *et al.* in 2019 [10], they observed varying levels of Disease Incidence (DI) and Vulnerability Index (VI) during the Kharif season of 2015-16 in Tumakuru and Mandya

districts. The DI ranged from 19.25% to 40.67%, and the VI ranged from 12.83% to 27.11%, while the whitefly population per plant varied between 2.74 and 5.81. The present study also showed that the percent disease incidence was as high as 89.3% in Pappampatti and whiteflies number per plant. This indicated that the disease incidence is positively correlated with whitefly population in the field. Janssen *et al.*, 2022 reported that when the tomato plants were fed with groups of 5, 20, and 50 viruliferous *B. Tabaci* resulted in 15, 30, and 100% of symptomatic and virus positive tomato plants, respectively. Similarly in the present study also, a group of twenty whiteflies transmitted ToLCNDV in bitter gourd with 93.33% transmission efficiency. Gomathi Devi *et al.*, 2023 [3] reported that the seed serves as a potential source for the spread of ToLCNDV by *Bemisia tabaci*. They recorded the transmission of ToLCNDV from both symptomatic and asymptomatic bitter gourd plants by using a group of 20 whiteflies. The whiteflies transmitted ToLCNDV even from asymptomatic plants, which were PCR positive for ToLCNDV. This study strongly envisaged that ToLCNDV is predominantly present in bitter gourd crop and is efficiently transmitted by *Bemisia tabaci*. In future, attempts have to be made to develop a diagnostic kits for the detection of virus in seeds and also an integrated approach for managing both whitefly vector and ToLCNDV.

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