

**Original Research Article**

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**Prevalence of Bud Blight of Tomato Caused by *Groundnut bud necrosis virus* in Tamil Nadu, India**

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A field survey was conducted in major tomato growing districts of Tamil Nadu viz., Coimbatore, Dharmapuri, Krishnagiri, Salem and Madurai during 2015-2016. *Groundnut bud necrosis virus* (GBNV) infection on tomato exhibited the symptoms viz., chlorotic and necrotic spots on leaves, necrosis of growing young buds (bud blight), bronzing, wilting and stunting. The per cent disease incidence of GBNV ranged from 18.6% to 35.3% in the above districts. A high per cent disease incidence of 35.3% was recorded in Thondamuthur village of Coimbatore district. Cowpea cv. CO5 was used as local lesion host for maintenance of GBNV isolates separately. A DNA fragment of around 800bp corresponding to a part of replicase gene was amplified using the tospovirus universal primer pair in all the samples. The DNA fragments were cloned into pGEMT vector and sequenced. The sequence analysis revealed higher similarity among the present isolates. However, they had an identity of 92.5% to 99.3% with the other isolates in NCBI. The results revealed that the isolates of GBNV is not divergent throughout Tamil Nadu.

**Introduction**

Tomato (*Solanum lycopersicum* L.) is the most popular widely cultivated vegetable crop, belongs to the family *Solanaceae*. It is a vital source of minerals, vitamin A and C. According to Euro fresh distribution magazine the tomato production is around 130 million tons during 2016 globally. India tops in the world's tomato production. According to Indian statistics, the total production of tomato is around 19.7 million tons during 2016-2017.

In India, the state of Tamil Nadu is reported as one of the leading producers of tomato.

Among the viral diseases reported in the past two decades (Anon., 1983), Tomato leaf curl and Tospoviruses are the most significant diseases observed in tomato crop. Umamaheswaran *et al.*, (2003) have reported the tospovirus infection in tomato as bud blight, and were identified as the *Groundnut bud necrosis virus* (GBNV). GBNV belongs to the genus *Orthotospovirus* (Family:

*Tospoviridae*; Order: *Bunyaviridales*). In India, GBNV has been reported in states like Karnataka (Sastry, 1982), Tamil Nadu (Doraiswamy *et al.*, 1984), Andhra Pradesh (Prasad Rao *et al.*, 1980) and Maharashtra (Joi and Summanvar, 1986). GBNV is a major limiting factor in crop production in India and in addition to tomato it infects in groundnut (Ghanekar *et al.*, 1979), peas (Prasad Rao *et al.*, 1985), blackgram and greengram (Amin *et al.*, 1985), soybean (Bhat *et al.*, 2002), cowpea, brinjal, chilli (Prasad Rao *et al.*, 1987), cluster bean (Krishna Reddy and Verma, 1990), potato (Khurana *et al.*, 1989), ridge gourd (Mandal *et al.*, 2003), cucumber (Jain *et al.*, 2007), jute (Sivaprasad *et al.*, 2011), taro (Sivaprasad *et al.*, 2011), onion (Sujitha *et al.*, 2012), bitter gourd (Nagendran *et al.*, 2018) and rajmash or french bean (Akram *et al.*, 2013). Raja and Jain (2006) observed symptomatic plants of tomato were characterized by severe necrosis of buds, petiole and leaves with chlorotic rings. GBNV infected tomato fruit exhibit circular concentric rings of pale yellow and broken red rings (Todd *et al.*, 1975). The tospovirus particles are spherical, 80-110nm diameter and enveloped with tripartite genome. Tospoviruses have three linear ssRNAs: L RNA, M RNA and S RNA. L RNA is negative sense and encoded for virus replicase protein size of 337 K. M RNA is ambisense (i.e., part of it is negative sense) encoded for glycoproteins (34K) and movement protein (127K). S RNA is also ambisense, encoded for non-structural small protein (34K) and nucleocapsid protein (28K).

## Materials and Methods

### Survey and collection of plant samples

A systematic field survey was conducted in major tomato growing districts of Tamil Nadu viz., Coimbatore, Krishnagiri, Dharmapuri, Salem and Madurai to know the prevalence of

*Groundnut bud necrosis virus* in tomato during the 2015-2016 season. The survey was conducted to record the incidence of GBNV under field conditions. During the survey, information on crop stage, per cent disease incidence was recorded along with GPS coordinates. The percentage of disease incidence was recorded by counting the number of plants showing disease symptoms and the total number of plants observed by using the formula.

$$\text{Disease incidence} = \frac{\text{Number of diseased plants}}{\text{Total number of plants examined}} \times 100$$

$$\text{Per cent of GBNV}$$

During the survey characteristics symptoms of GBNV were observed on tomato plants. They exhibited necrotic ring spots on leaves and necrotic patches on stems. Necrosis on young growing buds, bronzing of leaves with brown necrotic lesions and followed by wilting and stunting of the plants in severe cases. Ripened fruits were exhibiting chlorotic or yellow circular markings with concentric rings on fruits. The infected plant samples were collected and brought to laboratory at the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore. The samples were stored at -80°C until further studies.

### Virus isolates and maintenance of inoculum

Tomato plants showing characteristics symptoms of *Groundnut bud necrosis virus* (GBNV) were collected from the naturally infected field and used as the source of the virus isolates. The leaf samples collected from Coimbatore, Krishnagiri, Dharmapuri, Salem and Madurai were inoculated separately on cowpea. The cowpea plants cv. CO5 plants were used for propagating the virus since they produce characteristics local lesion symptoms within 3-4 days of inoculation. The cowpea

plants were raised in glasshouse under insect-proof condition. The virus extract was prepared by macerating GBNV infected fresh leaf samples with 0.1 M sodium phosphate buffer pH 7.0 at the ratio of 1:2 (infected leaf tissue: buffer volume) using ice tray. Inoculation was carried out by gentle rubbing with inoculum using broad end of the pestle on the cotyledonary leaves of six-day-old cowpea plants previously incubated in the dark for 12 hrs and dusted with 600 mesh carborundum powder. The excess inoculum was washed with a jet of sterile distilled water using a wash bottle. The inoculated plants were kept under observation for 4-5 days for the expression of symptoms (Subramanian and Narayansamy, 1973). The inoculum of different isolates of GBNV collected from different places were maintained separately on cowpea cv. CO5 plants throughout the study under insect-proof glasshouse.

#### **RNA isolation and cDNA synthesis**

The total RNAs were extracted from the 100mg of cowpea leaf sample showing local lesions of GBNV using the Trizole method by TRI reagent developed by Chomczynski and Sacchi (1987). The plant sample showing symptom along with healthy samples was homogenized separately using liquid nitrogen. One ml of Trizol reagent (Sigma- Aldrich now Merck) was added, transferred to a 1.5ml centrifuge tube and incubated at under room temperature for 2 min and then centrifuged @ 12,000 rpm for 10 min at 4°C. The supernatant was transferred to a new 1.5ml centrifuge tube, and 250 µl of chloroform was added, mixed well and the centrifuged @ 13000 rpm for 15 min. the aqueous layer was transferred to another new tube and 250 µl of isopropanol and 250 µl NaCl was added. Then it was incubated over the ice for 10 min and then centrifuged @ 13000 rpm for 15 min. the supernatant was discarded and the pellets retained in the tube were washed with 75%

ethanol. The pellets were air dried and dissolved in 30 µl of RNase free water under the sterile condition and store at -80°C. First strand cDNA synthesis was carried out by using first strand cDNA synthesis kit (Thermo scientific RevertAid first strand cDNA synthesis kit, USA) as per manufacturer's instruction. The reaction was performed at 42°C for 60 min followed by incubation at 70°C for 5 min.

#### **Molecular detection of GBNV infection**

Detection was done through Reverse transcription- polymerase chain reaction (RT-PCR). RT-PCR was carried out with the tospovirus universal primer pair, gL3637/F (CCTTTAACAGTDGAAACAT) and gL4435/R (CATDGCRCAAGARTGRTARA CAGA) (Chu *et al.*, 2001) corresponding to partial L RNA segment of tospovirus. The PCR was carried out with the master mix (Smart prime, India) in 50µl reaction volume containing master mix- 25µl; gL3637/F- 5 µl; gL4435/R- 5µl; distilled water- 10µl; cDNA- 5µl. The PCR setting comprised with an initial denaturation for 94°C for 2 min, 35 cycles of denaturation for 94°C for 1 min, annealing for 50°C for 1 min, the extension for 72°C for 1 min and a final extension of 72°C for 10 min. The RT-PCR product was analysed on 1.2% agarose gel, stained with ethidium bromide and viewed under transilluminator. The amplicon of partial replicase genes was purified using GenJET PCR purification kit (Thermo Fischer Scientific Inc.,) and ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA) at 4°C following the manufacturer's instructions. The ligated product was transformed into *Escherichia coli* DH5α competent cells by following standard molecular biology procedures (Sambrook, 1989). Plasmid DNA was isolated from the potential recombinant clones using GenJET plasmid Midiprep kit (Thermo Fischer Scientific Inc.,) according to the

manufacturer's protocol. Potential recombinant clones were identified by restriction digestion analysis using *EcoRI* enzyme. Two independent clones were sequenced in both the orientations by using universal sequencing primers M13 (forward and reverse) and sequencing was done with M/s Chromous Biotech Pvt. Ltd., Bangalore. The PCR products were sequenced and the sequence results were analysed in the BLASTn search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

## Results and Discussion

The GBNV disease incidences were recorded under field conditions in major tomato growing districts of Tamil Nadu during 2015-2016 and the results are given in Table 1. A maximum disease incidence of 35.3% was observed in Thondamuthur village of Coimbatore district. However, the disease incidence on tomato in other places ranged from 18.6% to 34.8%. The present study finds the natural distribution and occurrence of GBNV on tomato. GBNV produces chlorotic

and necrotic ring spots on leaves (Figure 1 and 2) followed by necrotic streaks or patches on stems and petioles (Figure 3). Chlorotic rings were appeared on infected fruits (Figure 4). Necrosis on young leaves leads to bud blight necrosis (Figure 5). Early infection on plants leads to stunting and wilting of the entire plant (Figure 6). The occurrence of disease was observed during all stages starting from young stage to flowering stages. Ruth (2016) reported that GBNV infected tomato samples in Andhra Pradesh exhibited symptoms like chlorotic and necrotic lesions on leaves, bronzing on the stem and ring spots on fruits.

In Bangladesh, the prevalence of GBNV on tomato was reported with symptoms like terminal bud necrosis, mottling of leaves, necrotic streaks on veins, shortened internodes, terminal bud necrosis and concentric ring on fruits (Akhter *et al.*, 2012). In India, the presence of TSWV in chrysanthemum was first reported by Renukadevi *et al.*, (2015) and recorded chlorotic and necrotic spots on leaves.

**Table.1** Prevalence of bud blight disease of tomato in Tamil Nadu during 2015-2016

| District    | Location            | Global Positioning System (Latitude and longitude coordinates) |           | Per cent bud blight incidence |      |
|-------------|---------------------|--|-----------|-------------------------------|------|
|             |                     | Latitude   | Longitude | 2015                          | 2016 |
| Coimbatore  | TNAU, Research farm | 11.009   | 11.009    | 26.6                          | 23.4 |
|             | Thondamuthur        | 10.997   | 10.997    | 35.3                          | 34.8 |
|             | Valayapalayam       | 11.534   | 11.534    | 27.1                          | 24.5 |
| Krishnagiri | Paiyur              | 12.378   | 12.378    | 28.5                          | 26.5 |
|             | Denkanikottai       | 12.528   | 12.528    | 27.3                          | 29.8 |
| Dharmapuri  | Pappireddipatti     | 11.913   | 11.913    | 27.8                          | 25.3 |
|             | Karimangalam        | 12.378   | 12.378    | 29.2                          | 25.8 |
| Salem       | Gopalapuram         | 11.568   | 11.568    | 19.1                          | 18.6 |
|             | Kallanatham         | 11.604   | 11.604    | 21.3                          | 19.8 |
| Madurai     | TNAU, Research farm | 9.969  | 9.969     | 21.8                          | 22.7 |
|             | Thirumangalam       | 9.941  | 9.941     | 23.6                          | 21.8 |

**Table.2** Per cent nucleotide sequence identities of the partial replicase protein gene of different isolates of GBNV

| Isolate                   | MH754140<br>(CBE) | MH754141<br>(MDU) | MH754142<br>(KGI) | MH754143<br>(DMP) | FJ177300 | KY940037 | KX950791 | KX965704 |
|---------------------------|-------------------|-------------------|-------------------|-------------------|----------|----------|----------|----------|
| <b>MH754140<br/>(CBE)</b> | 100               | 95.30             | 98.90             | 98.50             | 97.90    | 97.00    | 96.10    | 95.80    |
| <b>MH754141<br/>(MDU)</b> | 95.30             | 100               | 94.50             | 94.20             | 93.80    | 93.40    | 92.50    | 93.00    |
| <b>MH754142<br/>(KGI)</b> | 98.90             | 94.50             | 100               | 99.30             | 98.40    | 96.70    | 96.80    | 96.50    |
| <b>MH754143<br/>(DMP)</b> | 98.50             | 94.20             | 99.30             | 100               | 98.50    | 96.40    | 96.50    | 96.30    |
| <b>FJ177300</b>           | 97.90             | 93.80             | 98.40             | 98.50             | 100      | 96.70    | 96.30    | 96.60    |
| <b>KY940037</b>           | 97.00             | 93.40             | 96.70             | 96.40             | 96.70    | 100      | 95.50    | 94.90    |
| <b>KX950791</b>           | 96.10             | 92.50             | 96.80             | 96.50             | 96.30    | 95.50    | 100      | 95.20    |
| <b>KX965704</b>           | 95.80             | 93.00             | 96.50             | 96.30             | 96.60    | 94.90    | 95.20    | 100      |



**Fig.1** Appearance of chlorotic and necrotic spots on tomato leaves



**Fig.2** Necrotic spots are coalesced leads to drying of leaves



**Fig.3** Appearance of necrotic streaks on stem of infected plant



**Fig.4** Chlorotic rings on fruits



**Fig.5** Bud blight/ bud necrosis of infected plant



**Fig.6** Stunting and wilting of infected plant

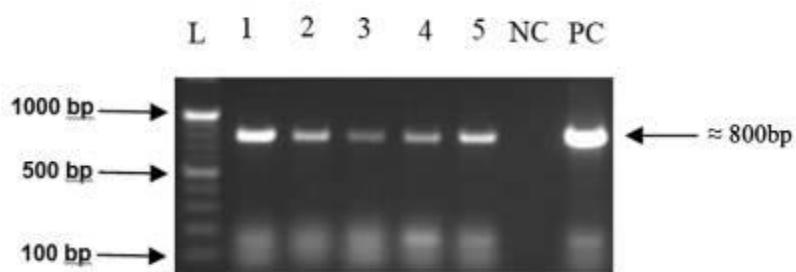


**Fig.7** GBNV inoculated cowpea Cv. CO5 with chlorotic spots at 4 days after post inoculation



**Fig.8** Necrotic spots on cowpea 6 days after post inoculation

**Fig.9** RT-PCR analysis with the primer pair gl3617/F (5'-CCTTTAACAGTDGAAACAT-3') gl4435c/R (5'-CATDGCRCAAGARTGRT ARACAGA-3') corresponding to the partial replicase gene of GBNV. A DNA fragment of approximately 800 base pairs specific to the partial replicase gene of GBNV were amplified in RNA extracted from GBNV infected cowpea leaves (Lane 1-5), while no amplification was observed in RNA extracted from cowpea sample inoculated with healthy tomato (Lane NC). L- 100bp Ladder; 1. Dharmapuri (DMP); 2. Coimbatore (CBE); 3. Krishnagiri (KGI); 4. Salem (SLM); 5. Madurai (MDU); PC- Positive control



## Characterization of GBNV

The GBNV infected tomato leaf samples collected from different parts of Tamil Nadu were inoculated on cowpea plants separately in an insect proof glasshouse and inocula were maintained separately throughout the study period. The mechanically inoculated cow pea leaves exhibited circular to irregular chlorotic on 4<sup>th</sup> day and necrotic spots 6<sup>th</sup> day of inoculation (Figure 7 and 8). There was no difference in the symptoms (local lesion) and time taken for symptom expression among the isolates. RNA extracted from symptomatic cowpea leaves and healthy leaves were subjected for RT-PCR assay with *Tospovirus* universal primers, gl3617/F and gl4435c/R. The symptomatic leaves resulted in the amplification of approximately 800bp amplicon and there was no amplification from non-symptomatic leaves, which confirm the presence of *Tospovirus* in the inoculated cowpea leaves (Figure 9). Though samples were collected from both Dharmapuri (DMP) and Salem (SLM) districts, they were considered one isolate as Dharmapuri (DMP) for sequencing, since these districts are very closer and has similar agroclimatic conditions.

Amplified products were cloned, sequenced and analysed. The BLAST analysis of present four isolates viz., Coimbatore (CBE), Krishnagiri (KGI), Dharmapuri (DMP) and Madurai (MDU) of GBNV nucleotide sequences (Acc. MH754140 (CBE), MH754141 (MDU), MH754142 (KGI), MH754143 (DMP) shared a similarity percentage of 94.5% to 99.3% among themselves. These sequences had 93.8% to 99.5% per cent identity with FJ177300 (Peanut) isolate of Indonesia (Table 2). L RNA encoded for RNA dependent RNA polymerase shared a nucleotide sequence identity with 85.9% with *Watermelon silver mottle virus* belongs to *Tospovirus* (Meng et

al., 2015). The present results indicated that the isolates of GBNV on tomato are not divergent throughout Tamil Nadu.

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