Pest Management in Horticultural Ecosystems
Vol. 28, No.1 pp 161-167 (2022)

DOI number : 10.5958/0974-4541.2022.00030.3

Molecular characterization of *Peanut Bud Necrosis Virus* infecting tomato in Tamil Nadu

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ABSTRACT: Tomato bud blight caused by *Peanut bud necrosis virus* (PBNV) is an economically important disease affecting tomato causing great yield loss to the farming community. In the present study, the PBNV virus isolate, (GBNV CBE T02) was purified from the infected plant through mechanical transmission on cowpea cv. CO7, which produced chlorotic and necrotic local lesions on 4th days post inoculation. The pathogenicity of the virus on tomato cv. PKM1 was also proven via sap transmission. Transmission electron microscopy of infected tomato samples revealed spherical virus particle with diameter of 58 to 82 nm at 80000 X magnification. Amplification of the various genomic components of PBNV in the infected leaf samples through RT-PCR using gene specific primers produced an amplicon of 800 bp, 903 bp, 613 bp, 831 bp and 790 bp for RdRp, NSm, Gn/Gc, NP and NSs respectively. Sequence analysis of movement protein (NSm) (Gen Bank Acc. No. ON 529555) and Non-structural small protein (NSs) (Gen Bank Acc. No. ON529556) revealed that the isolate shared 98.98 % and 98.04 % identity respectively with other isolates in the GenBank database.

Keywords: Tomato, PBNV, TEM, RT-PCR, Sequence, Phylogeny

INTRODUCTION

Tomato (*Solanum lycopersicum* L., Family-*Solanaceae*), is one of the most important and popular vegetable crops grown throughout the world. It is rich in vitamins A & C, minerals, organic acids and is the richest source of the dietary antioxidant lycopene. Edible portion (100 g) of tomato contains 3.9 % carbohydrates, 0.9 % proteins, 2.6 % sugar, 1.2 % fibre, 0.2 % fat, 18.9 mg vitamin C, and 12.58 mg lycopene (Hanif *et al.,* 2006). In India, tomato is being cultivated in an area of 851.69 thousand ha with a production of 21002.81 thousand tonnes and productivity of 24.66 MT/ha. In Tamil Nadu, tomato is cultivated in an area of 82.98 thousand ha with the production of 2370.58 thousand tonnes and productivity of 28.57 MT/ ha (Indiastat, 2021). Biotic and abiotic stresses are the major limiting factors in tomato cultivation throughout the world (Abu Qamar *et al.,* 2013). Tomato is affected by various diseases, among which *Tospoviruses* are a major constraint creating huge economic loss in commercial cultivation (Scholthof *et al.,* 2011). More than 20 *Tospoviruses* have been reported globally (Zhu *et al.,* 2019), among which *Peanut bud necrosis virus* (syn. *Groundnut bud necrosis orthotospovirus,* GBNV) infecting tomato cause severe destruction to the crop (Mandal *et al.*, 2012).

Peanut bud necrosis virus belongs to the genus *Orthotospovirus* (Maes *et al.,* 2018), family *Tospoviridae* and order *Bunyavirales*, has tripartite ss RNA in their genome (Pappu *et al.,* 2009). They are transmitted by thrips in a circulative propagative manner (Gopal *et al.,* 2011). PBNV is characterized with enveloped isometric virus particle of diameter 80-120 nm (Radhakrishnan *et al.,* 2016). The genome includes negative sense large RNA (L-8.9 kb) encoding for virus replicase protein (RNA dependent RNA polymerase- RdRp) of 337 kDa (Gowda *et al.,* 1998); ambisense medium RNA (MP-34kDA kb) encoding movement protein (NSm or MP) of 127 kDa in sense orientation and glycoproteins (Gn/Gc) of 127 kDa in antisense orientation (Saritha and Jain, 2007) ; ambisense small RNA (S-3.05 kb) encoding non-structural small protein (NSs) of 49.5 kDa in sense orientation and nucleocapsid protein (N or CP) of 30.6 kDa in antisense orientation (Satyanarayana *et al.,* 1996).

PBNV infected tomato, show chlorotic and necrotic spots on young leaf, stem, petioles followed by drying of young bud (Umamaheshwaran *et al.,* 2003). Early infection in plants results in yellowing of leaves, stunted growth, and death. The infected plants set fruits with chlorotic concentric rings and reduced size. More than 80 percent yield loss has been reported due to PBNV in India (Dasgupta *et al.,* 2003). Now-a-days PBNV infection has become a serious disease in Tamil Nadu, Karnataka and Andhra Pradesh (Mandal *et al.,* 2017). The disease eventually causes death of the plant which

Table 1. Primers sequence used for detection of PBNV

RNA Segment	Gene	Sequence	Amplicon size
L RNA	RdRp	F: 5' CCTTTAACAGTDGAAACAT 3'	800 bp
		R: 5' CATDGCRCAAGARTGRTARACAGA 3'	
M RNA	NSm	F: 5' ATGTCTCGCTTDTCTAAHGTB 3'	903 bp
		R: 5' TTATATTTCAAGAAGATTATC 3'	
M RNA	Gn/Gc	F: 5' TGGATTAAAGTCAGTTTGCGCC 3'	613 bp
		R: 5' CTTTCTGTAAGGTTGTCTCGTG 3'	
S RNA	NSs	F: 5'CTAGCTAGCCATATGTCAACTGCAAAGAATGC 3'	790 bp
		R:5' CCCTCGAGGGTTATTCTGCTTTCACAATGAAGTG3'	
S RNA	N	F: 5' ATGTCTAACGT(C/T)AAGCA(A/G)CTC 3'	831 bp
		R: 5' TTACAATTCCAGCGAAGGACC 3'	

later affects the market value of tomato (Kunkalikar *et al.,* 2011). Accurate identification of this virus disease in the early stage of the crop is highly essential which will help to overcome losses in the later stage. Virus recombination occurs frequently under field conditions and hence sequencing of the various genomic components is highly essential. Based on this, in the present study, PBNV infecting tomato at Coimbatore district of Tamil Nadu was characterized through molecular methods.

MATERIALS AND METHODS

Survey and sample collection

A field survey was conducted in different locations of Coimbatore district, Tamil Nadu during 2022 to document the spotted wilt symptoms in tomato. At each stage, totally 60 plants were observed in each field and the percent disease incidence was calculated using

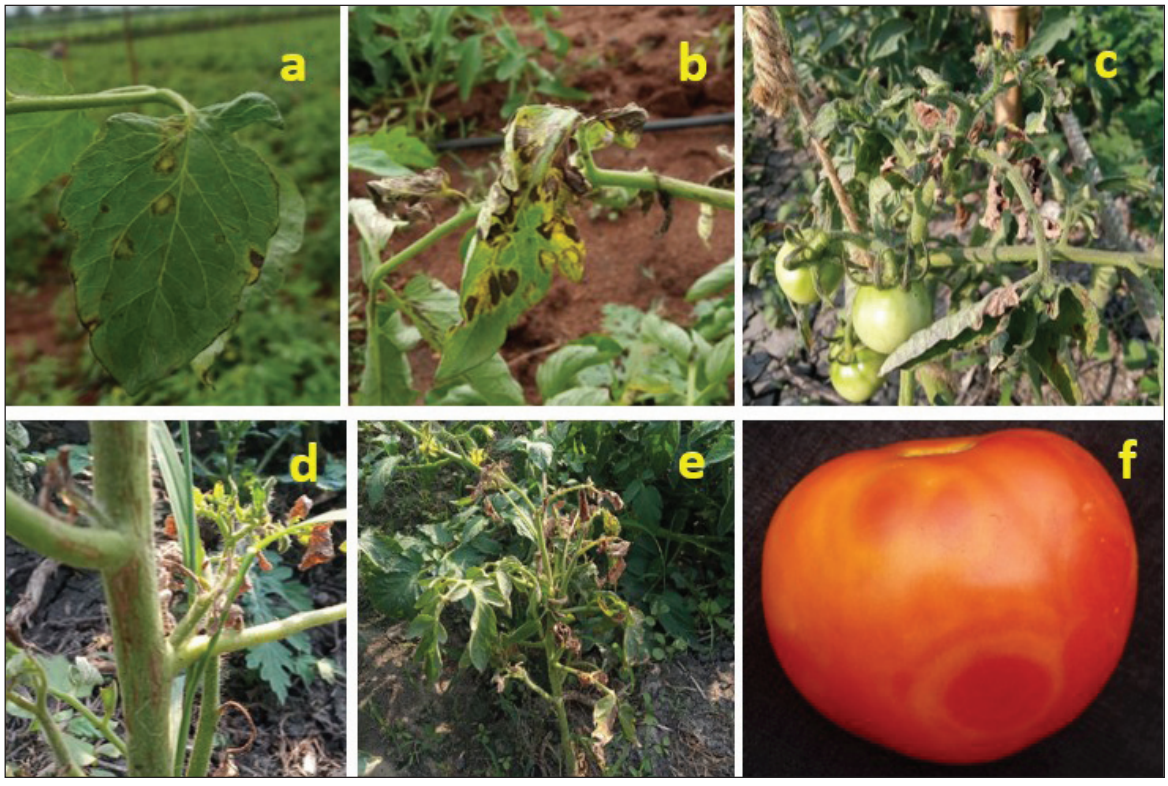

Fig. 1. Symptoms of PBNV in tomato observed in the field: a. Chlorotic ring spots on tomato leaves, b. Necrotic ring spots on tomato leaves which eventually coalesce leading to drying of leaves, c. Bud necrosis symptom, d. Stem necrosis, e. Stunting and wilting of young plant, f. Chlorotic ring spots on tomato fruit

Table 2. Tomato Spotted wilt incidence in Coimbatore district, Tamil Nadu

Location	Latitude	Longitude	Bud blight incidence (%)
Devarayapuram	10.7798° N,	77.0068° E	18.33
Madampetti	10.9698° N	76.8598° E	26.67
Narasipuram	10.9880° N	76.7740° E	8.33
Thenkarai	10.9366° N	76.8433° E	33.33
Thondamuthur	10.9899° N	76.8409° E	30.00
TNAU Orchard	11.0069° N	76.9309° E	11.67
Viraliyur	10.9978° N	76.7843° E	36.67

the *formula (Reddy *et al.,* 2008). The tomato plants exhibiting symptoms of PBNV *viz*., bronzing of leaves, chlorotic and necrotic lesions in the stem were collected from the field. About four to five symptomatic leaves were collected per plant. The samples were stored at -80°C and used for further studies.

$$\text{Percent disease incidence} = \frac{\text{Number of infected plants}}{\text{Total no. of plants observed}} \times 100$$

Sap inoculation and pathogenicity test

The infected samples collected from the field were maintained in cowpea plants (*Vigna ungiculata* cv. CO7) through mechanical inoculation since cowpea leaves produce characteristic local lesion symptoms within 3-4 days after inoculation (Manjunatha *et al.,* 2010). The seeds of cowpea cv. CO7 were raised in PL480 glasshouse, Department of Plant Pathology, TNAU, Coimbatore under insect-proof condition. The sap transmission of GBNV was carried out by extraction of crude sap from infected tomato leaves using 0.01 M Sodium phosphate buffer (pH- 7.2) with 0.1% ß- merca ptoethanol in pre-chilled pestle and mortar. Seven days old cowpea plants were pre-dusted with carborundum powder (600 mesh) and the sap was gently rubbed on the leaves. The inoculated leaves were washed with distilled water and kept for observation. Similarly, the virus was inoculated in 25 days old tomato plants (*Solanum lycopercicum* cv. PKM1) for proving the pathogenicity of the virus.

Characterization of PBNV through Transmission Electron Microscopy (TEM)

TEM of the viral particle was obtained with the partially purified GBNV samples ground with phosphate buffer amended with 2 % polyvinylpyrroli done and 0.2 % sodium sulfite (Zechmann and Zellnig, 2009). Carbon coated copper grid was subjected to positive staining with a few drops of 0.05 M of potassium phosphate buffer for 10 min followed by dipping with partially purified samples for 15 min, allowing binding of the virus. Then the sample coated grid was subjected to negative staining with 2 % uranyl acetate for 5 min. After negative staining, the grid was washed three times with sterile distilled water and the copper grid was dried in desiccator to remove excess moisture. The sample coated copper grid was visualized under transmission electron microscope (Tecnai Sprit G2, FEI, Netherlands) at the Department of Nano Science and Technology, TNAU, Coimbatore.

RNA isolation

The total RNA was extracted from cowpea plants exhibiting local lesions of GBNV using the Trizol method (Chomczynski and Sacchi, 1987). Leaf samples (100 mg) showing symptoms of PBNV along with healthy samples was homogenized using liquid nitrogen. One

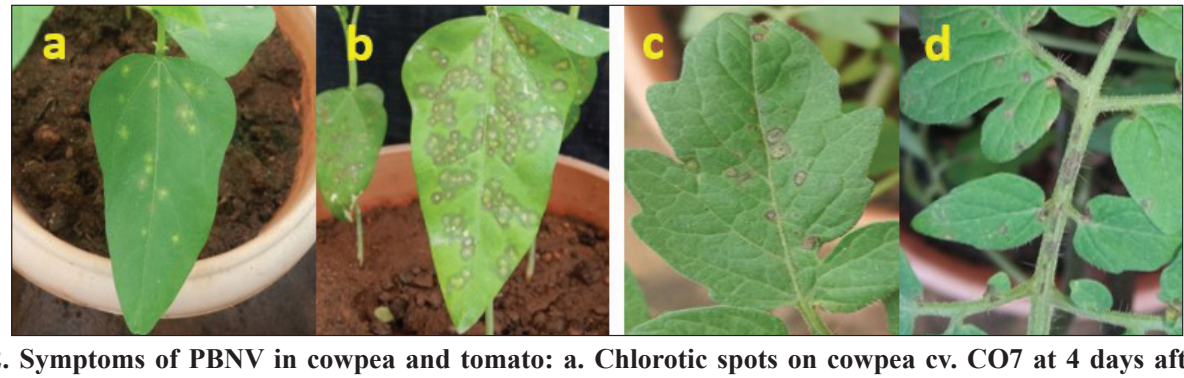

Fig. 2. Symptoms of PBNV in cowpea and tomato: a. Chlorotic spots on cowpea cv. CO7 at 4 days after sap inoculation with PBNV, b. Necrotic spots on cowpea at 8 days after virus inoculation, c. Chlorotic spots on tomato var. PKM 1 at 6 days after sap inoculation with PBNV, d. Necrotic streaks on stem of tomato plants 21 days after virus inoculation.

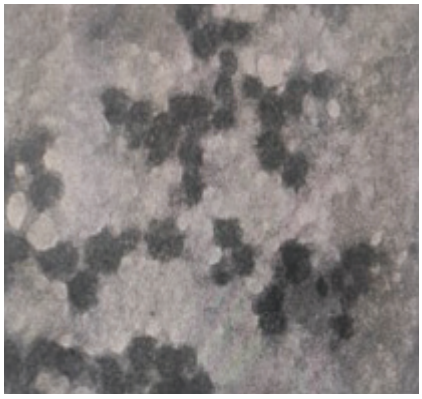

Fig. 3.Transmission electron microscopy (TEM) of virus: Spherical virus particle with diameter 58 to 82 nm observed in infected tomato samples at 80000X nm magnification

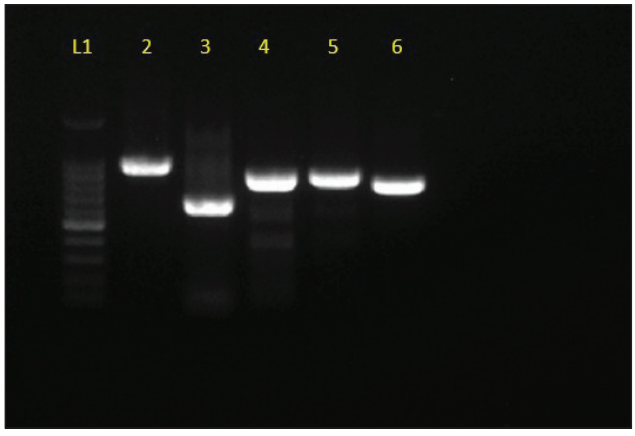

Fig. 4. Agarose gel electrophoresis of RT-PCR analysis with gene specificprimers. L1- 100bp ladder, 2- Nsm (903bp), 3- Gn/Gc (613bp), 4- RdRp (800bp), 5- N (831bp), 6- NSs (790bp)

ml of Trizol reagent was added, transferred to a 1.5ml centrifuge tube and incubated under room temperature for 2 min and then centrifuged at 12,000 rpm for 15 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 centrifuged at 12000 rpm for 15 min. The aqueous layer was transferred to another new tube and 250 µl of ice cold isopropanol, 250 µl 5M NaCl were added. Then it was incubated over the ice for 15 min and then centrifuged at 12000 rpm for 15 min. The supernatant was discarded and the pellets retained in the tube were washed with 70 % ethanol. Excess ethanol was removed and the pellet was dissolved in 30 µl RNA ase free water.

Reverse transcription

RNA was quantified using Nanodrop and the quality of RNA was assessed by A260/A280 ratio. First strand cDNA synthesis was carried out using first strand cDNA synthesis kit (Thermo scientific RevertAid first strand cDNA synthesis kit, USA). The reaction mixture contains Reaction buffer - 4 µl, dNTPs - 2 µl, random primer – 1 µl, reverse transcriptase - 1 µl, RNase inhibitor - 1 µl and total RNA- 3 µl (1800 ng) which was made up to 20 µl with DEPC treated water. The contents were incubated at 45 ºC for 60 min followed by 70 ºC for 5 min. The resultant cDNA was used to amplify the various genomic components of PBNV.

Amplification of genomic components of PBNV through RT-PCR

Amplification of the genomic components of PBNV was carried out through Reverse transcription- polymerase chain reaction (RT-PCR) using gene specific primers (Balol and Patil, 2016; Saritha and Jain, 2007. Table 1). The PCR reaction contains Master mix- 20 µl, 4 µl each of forward and reverse primers (5 µM), nuclease free water- 8 µl and cDNA- 4 µl. The PCR was carried out in a thermal cycler (C1000 Biorad Ltd.) using the following PCR conditions : initial denaturation of 94°C for 5 min, 35 cycles of denaturation for 94°C for 1 min, annealing for 50°C for 1 min, 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 gel documentation unit.

Sequence analysis

Partial sequencing of the amplified products of PBNV was done in Biokart Pvt. Ltd., Bangalore, India, analysed and submitted in the NCBI Genbank database and accession numbers were obtained. Nucleotide sequences were aligned using CLUSTAL X 1.81 and phylogenetic analysis of the sequence was carried out by comparing the reference sequence retrieved from the Genbank database with 1,000 bootstrap replicates using MEGA 11 software (Tamura *et al.*, 2021).

RESULTS AND DISCUSSIONS

Survey and sample collection

A survey was carried out in tomato fields in the Coimbatore district, Tamil Nadu during 2021and the results are presented in Table 2. The disease incidence ranged from 8.33 % (Narasipuram village) to a maximum of 36.67 % (Viraliyur village) (Table 2). The disease occurred in all the stages of the crop from young stage to flowering stage. The survey implies the natural distribution and symptomatology of PBNV in tomato. PBNV initially produced chlorotic ring spots (Figure 1a) which later turned to necrotic ring spots (Figure 1b) on the leaves. Severe infection on young shoots lead to bud

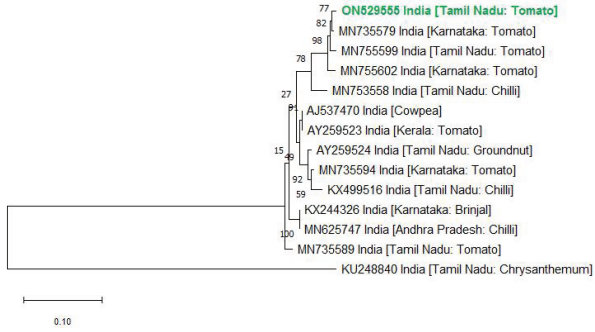

Fig. 5. Phylogenetic relationship of GBNV isolate (ON529555) from Tamil Nadu with other GBNV isolates reported based on movement protein gene. Phylogenetic tree generated using MEGA 11 software using maximum likelihood tree with 1000 replicates and TSWV (KU248840) isolate used as out group.

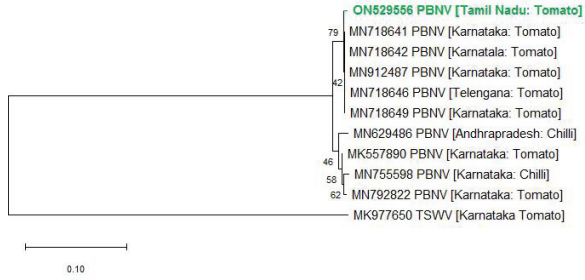

Fig. 6. Phylogenetic relationship of GBNV isolate (ON529556) from Tamil Nadu with other GBNV isolates reported based on Non structural small protein gene. Phylogenetic tree generated using MEGA 11 software using maximum likelihood tree method with 1000 replicates

blight necrosis (Figure 1c). On stem and petioles, PBNV caused necrotic streaks (Figure 1d). PBNV infection on early crop stage caused wilting and stunting of the whole plant (Figure 1e). On the fruit, it caused chlorotic ring symptom (Figure 1f). In Tamil Nadu, the prevalence of PBNV on Tomato was reported with the symptoms of chlorotic and necrotic ringspots on leaves followed by necrotic streaks on stems, petioles and chlorotic rings on infected fruits (Suganyadevi *et al.,* 2018).

Sap inoculation and Pathogenicity test

The virus sample collected from the field was maintained through sap inoculation on cowpea plants. The same was done in tomato plants for proving pathogenicity of PBNV. On cowpea, the virus produced chlorotic spots at 4 days after inoculation (Figure 2a) and necrotic ring spots (Figure 2b) at 8 days after inoculation. On tomato, the virus produced chlorotic spots on leaves (Figure 2c) on 6 days after sap inoculation and necrotic streaks on the stem (Figure 2d) proving the Koch postulates. Vanthana *et al.* (2019) has reported that the PBNV sap inoculated cowpea plants (CO7) exhibited chlorotic ring spots which later turn to necrotic spots within 4-5 dpi and tomato plants inoculated with PBNV has shown necrotic rings on leaves and necrotic streaks on stem similar to field symptoms.

Characterization of virus by TEM

Characterisation of the virus particles through TEM analysis revealed that the virions possess spherical virus particle with diameter 58 to 82 nm in infected tomato samples at 8000 X magnification (Figure 3). Pant *et al.* (2019) alsoreported the presence of tospovirus-like particles measuring 80-110 nm in diameter in PBNV infected samples through TEM analysis.

Molecular detection of PBNV in infected plants

RNA was extracted from the infected plants and RT-PCR was carried out using PBNV gene specific primers. The isolate, GBNV CBET 02 could amplify all the genomic components of PBNV. RT-PCR amplified different genes with a product size of 800 bp, 903 bp, 613 bp, 831 bp, and 790 bp for RdRp, NSm, Gn/Gc, N and NSs gene respectively (Figure 4). Suganyadevi *et al.* (2018) also reported that the replicase gene of PBNV produced an amplicon of 800 bpin all the infected samples. The results was in accordance with Sangeetha *et al.,* (2020) and Rajamanickam *et al.,* (2020), where the coat protein gene of PBNV was amplified with a size of 830 bp. Similarly, Amplified products of NSm and NSs genes were partially sequenced and analyzed. The sequences are available in NCBI database under accession numbers ON529555 and ON529556 respectively. The BLASTn search revealed 98.98 % identity for movement protein and 98.06 % for Non-structural small protein with the available PBNV sequences in the Genbank database.

Phylogenetic analysis

The nucleotide sequence of PBNV NSm gene available under accession number ON529555 was compared with other isolates of PBNV infecting wide range of hosts which revealed three groups (Figure 5). The study isolate was closely related to PBNV isolated from tomato at Karnataka (Accession no: MN35579), tomato at Tamil Nadu (Accession no: MN55599) and tomato at Karnataka (Accession no: MN755602) whereas *tomato spotted wilt virus* tree of partial segment of NSs gene (Accession number: ON529556) is closely related to the isolates MN718641 at Karnataka and MN718642 at Karnataka (Figure 6). The comparison of PBNV nucleotides revealed that PBNV isolates are not divergent among different host throughout India.

CONCLUSION

In this study, PBNV infecting tomato in Western region of Tamil Nadu was characterized. The pathogenicity of the virus isolate was proved through sap inoculation. The virus was purified and the infected virions were characterized through TEM analysis. Besides, amplification of the five genomic components of PBNV was carried out through RT-PCR. Sequencing of the NSm gene and NSs gene showed its close identity with the PBNV isolate from Tamil Nadu and Karnataka thus confirming the presence of the pathogen.

ACKNOWLEDGEMENT

The authors are grateful to Professor and Head, Department of Plant Pathology, TNAU, Coimbatore for the support provided and DST-SERB No: CRG/2020/004589 for providing financial assistance for conducting the experiments.

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MS Received: 10 March 2022
MS Accepted: 25 May 2022