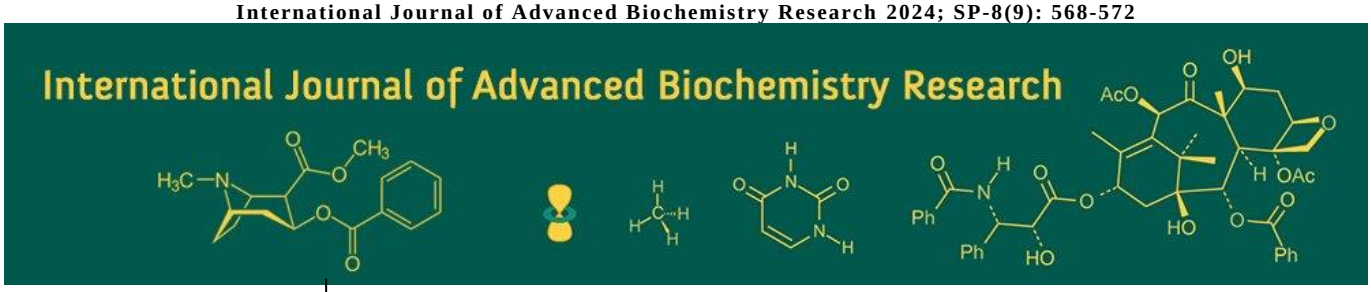

ISSN Print: 2617-4693
ISSN Online: 2617-4707
IJABR 2024; SP-8(9): 568-572
www.biochemjournal.com
Received: 02-07-2024
Accepted: 06-08-2024

Rajamanickam S
Department of Microbial Biotechnology, Bharathiar University, Coimbatore, Tamil Nadu, India

Angayarkanni J
Department of Microbial Biotechnology, Bharathiar University, Coimbatore, Tamil Nadu, India

Corresponding Author:
Rajamanickam S
Department of Microbial Biotechnology, Bharathiar University, Coimbatore, Tamil Nadu, India

Molecular characterization of groundnut bud necrosis virus (GBNV) infecting tomato and its phylogenetic relationship

Rajamanickam S and Angayarkanni J

DOI: https://doi.org/10.33545/26174693.2024.v8.i9Sg.2175

Abstract
Tomato is a prime vegetable cum spice crop and it is infected by Orthotospovirus, becoming a serious threat to cultivation worldwide. A survey was conducted in tomato-growing areas of Tamil Nadu and found the incidence of *tospovirus* based on necrotic symptoms on the plants. The samples with typical symptoms of necrosis have successfully established the chlorotic and necrotic lesions in the assay host, cowpea upon artificial inoculation as the characteristic of *tospovirus*. Further, an expected cDNA fragment sequence of about 840 bp was positively amplified using PCR, confirming the presence of groundnut bud necrosis virus (GBNV) in tomato. The nucleotide and amino acid sequence of CP gene of GBNV showed high sequence similarity with exiting isolates up to 97.78 percent. The nucleotide profiling of CP gene of GBNV provides the knowledge on properties of the CP gene and its structural relationships, which could pave the way to employ the development of a resistance strategy in tomato.

Keywords: Tomato, *Tospovirus*, CP gene sequences; phylogenetic analysis

Introduction
The most frequently grown and well-known vegetable crop in the Solanaceae family is the tomato (Lycopersicon esculentum Mill.) and it is an essential source of vitamin A and C as well as other minerals. Lycopene is a unique flavonoid antioxidant phytochemical found in tomatoes (Agarwal and Rao, 2000) [1]. In India, an estimated 0.86 million hectares are used for tomato cultivation, yielding a production of 21.81 million tonnes. Tamil Nadu is one of the top tomato-growing regions in India, covering an estimated 46 thousand hectares overall and producing 1.49 million tonnes of tomatoes annually (Horticulture Statistics, 2021; Karthikeyan *et al*., 2024) [8, 10]. Tomato is affected by a large number of viruses including groundnut bud necrosis virus (GBNV) and it is an economically important virus with a broad host range and widespread distribution throughout the world (Vanthana *et al*., 2019) [23]. In India, vegetable necrosis disease is caused by a group of viruses known as the orthotospovirus group. A novel orthotospovirus known as groundnut bud necrosis virus (GBNV) emerged in late 2000 and severely reduced India's vegetable crop yield. The virus was first recorded on groundnuts during 1968 (Reddy *et al*., 1968) [16]. It is now a significant barrier to the cultivation of several solanaceous and leguminous hosts, including tomatoes, potatoes, and chilli (Bhat *et al*., 2002; Jain *et al*., 2004; Pavithra *et al*., 2016) [9, 14]. It is known that many thrips species persistently and preoperatively spread the tospoviruses. Within plant cells, tospoviruses encapsulate three nucleocapsids within a lipid envelope that has two surface glycoprotein projections. The particles are pleomorphic and spherical. The virus consists of a tripartite genome with S, M and L RNA segments.
There are few investigations focused on the molecular characterization on different viruses infecting tomato under field conditions (Vanthana *et al*., 2019; Karthikeyan *et al*., 2024) [23, 10]. Strategies for the management of viral diseases normally include control of vector population using insecticides, use of virus free propagating material, appropriate cultural practices and use of resistant cultivars. Recently, plant virologists have focused their attention to develop transgenic or enhanced host plant resistance against plant viruses through molecular approaches worldwide. The enhancement of host plant resistance appears as one of the best options because it offers an easy, cheap, and sustainable technology to farmers, i.e., improved cultivars.

Under these circumstances, proper diagnosis of viral disease and understanding the genetic makeup of virus makes the possible approach for the development of a management strategy. In this prospects, field observation of characteristic virus symptoms are the primary detection techniques for the virus infection followed by the different serological and molecular tools for further endorsement. Hence, the present study was aimed at molecular characterization and phylogenetic relationship of GBNV as they represent the most important genus in Orthotospovirus.

Materials and Methods

Survey and collection of virus infected samples

Field survey was conducted during the growing seasons by covering major tomato growing areas *viz*., Coimbatore, Dharapuram, Pollachi and Udumalapet of Tamil Nadu state to document the virus diseases. The percent disease incidence was recorded by counting the total number of plants and virus infected plants in each and every field of different areas. The plant samples showing characteristic *tospovirus-like* symptoms were collected separately for further analyses. Ten samples from each field in each place were collected in zip-loc bags and labeled. Samples were transported to the laboratory on ice and kept at 4°C. All samples were processed within 24 hrs of collection.

Inoculums preparation and mechanical transmission

The samples of *tospovirus* were used for transmission studies in cowpea by mechanical sap inoculation. Cowpea cv. C152 was used as a propagating host for the virus. As cowpea produces typical chlorotic and necrotic lesions within 3-4 days of inoculation. The plants were raised in the glass house and maintained under insect-proof conditions. The virus extract was prepared by macerating infected tomato plant tissue with 0.1M sodium phosphate buffer pH 7.0 containing 0.1per cent β-mercaptoethanol, using an ice tray. Inoculation was carried out by gentle rubbing with inoculum using the broad end of the pestle on the cotyledonary leaves of six day old cowpea plants, which were previously dusted with 600 mesh carborundum powder. After few min, the excess inoculum was washed with a jet of sterile distilled water using a wash bottle. The inoculated plants were kept under observation for 3 - 7 days for cowpea for symptom expression (Subramanian and Narayanasamy, 1973) [21].

Total RNA extraction, cDNA synthesis and amplification of coat protein (CP) genes

Total RNA approximately, 50 µg/µl were extracted from 100 mg leaves of infected tomato using Trizol plant extraction kit (Genei, Bangalore) according to the manufacturers protocol and resuspended in 50 µl nuclease free water. The total RNA isolated from the virus infected field tomato samples were subjected to PCR in 50µl reaction volume containing cDNA and 2 units of enzyme mix and with primers specific to GBNV (CPF - ATGTCTAACGTYAAGCAGCTC; GBNV CPR - TTACAACTCTAGCGAAGGAC) corresponding to coat protein gene of GBNV, to amplify the complete coding region of CP genes. The PCR settings comprised of 35 cycles of amplification including denaturation at 94°C for 2 min, annealing at 52°C for 30 s, extension at 72°C for 1 min and a final extension at 72°C for 10 min. PCR reaction was carried out in Eppendorf Mastercycler Gradient ES. The amplified products were analysed on 1% agarose gel, stained with ethidium bromide and photographed under UV-gel doc system (Alpha imager).

Multiple sequence alignment and phylogenetic analysis of *tospovirus*

The amplicon of CP gene fragment was purified using QIAGEN gel extraction kit (Qiagen Inc., Chatsworth, CA, USA), cloned into pGEM-T Easy vector and transformed into *Escherichia coli* DH5α following standard protocol (Sambrook *et al*., 1989) [20]. Plasmid DNA from positive clones was sequenced, edited using the BIOEDIT Software (Hall, 1999) [6] and the Basic Alignment Search Tool (BLAST) search program was used for sequence similarity. The nucleotide and amino acid sequences were aligned with selected sequences using MEGA 7 (Larkin *et al*., 2007; Saitou and Nei, 1987) [12, 19] (Table 1). A phylogenetic tree was constructed using the neighbour-joining method with a bootstrap value of 1,000 replicates (Tamura *et al*., 2011) [22]. *Tomato zonate spot virus* was used as a reference out-group member of the genus *tospovirus* for rooting the phylogenetic tree.

Results

Virus isolates and diagnosis

During the survey, the highest percent disease incidence of *tospovirus* was recorded in Madampatti area of Coimbatore district of Tamil Nadu state. The severe disease symptom of *tospovirus* infection was observed under field conditions in young plants, before the flowering stage. During the field survey for recording the incidences of *tospovirus*, the plant showed the diagnostic symptoms of virus infections were chlorotic and necrotic ring spots on the leaves and streaks on the stems. The plant samples were collected from symptomatic and asymptomatic (apparently healthy) plants and brought to the laboratory under ice-cold conditions for further analysis.

Biological assay for proliferation of virus

The tomato showing characteristic symptoms of *tospovirus* were collected from the field was propagated on cowpea cv. C152 plants separately through mechanical sap inoculation. The assay host plant cowpea cv. C152 expressed distinct local chlorotic lesions on 4 days after post-inoculation (dpi). The inoculated cowpea cotyledonary leaves developed necrotic lesions and then the systemic veinal necrosis occurred. The veinal necrosis resulted in severe stem necrosis and led to the death of inoculated plants.

Nucleotide sequences and identification of CP genes

The cDNA derived from the extracted RNA of tomato samples collected from the field was used for PCR amplification of CP genes of *tospovirus*. The amplified PCR products from infected samples with an amplicon size of approximately 840 bp to CP gene GBNV (figure 1), were excised and cloned into pGEM-T easy vector. The independent clones were selected and confirmed through restriction analysis using EcoRI. The independent clones were sequenced in both orientations. The gene sequence was edited using BIOEDIT software and the full length nucleotide sequence was obtained. The nucleotide sequence analysis using NCBI BLAST confirmed the association of groundnut bud necrosis virus (GBNV). The CP gene

sequence of GBNV isolates was submitted in NCBI Genbank database bearing Accession No. PQ117735.

Molecular phylogenetic analysis of CP genes

Analysis of CP gene sequence of GBNV isolate was compared to the reference sequence with other tospovirus isolates from different crops using EBI Tool: ClustalW at the nucleotide and amino acid sequence levels. The GBNV sequences of our isolates exhibit cent percent similarity between the isolates. However, the nucleotide sequence recorded a maximum of 97.78% nucleotide homology with tomato GBNV of Indian isolate (AY463968). This was followed by a sequence of our isolates that showed 97.41%, 96.67% and 96.67% nucleotide homology with GBNV from chilli, cowpea and brinjal of Indian isolates (AY882002, KX244330 and AF515819), respectively. Multiple nucleotide sequence alignment and phylogenetic analysis revealed very high homologies between the GBNV isolates, separately. The *tospoviruses* formed a single cluster (figure 2). Where, Further, sequences showed near-perfect homology between the nucleotide sequence of chilli, groundnut, brinjal, cowpea and tomato isolates, and sequences of GBNV in the group further confirm the placement of isolates in a single clade.

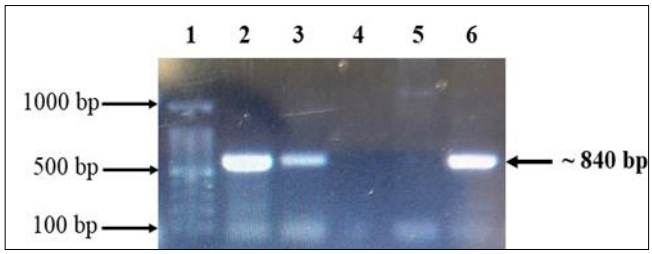

Fig 1: Amplification GBNV from infected tomato samples. Where, Lane 1 - 100 bp ladder; Lane 2&3 - Infected samples; Lane 4&5 - Healthy control; Lane 6 - Positive control

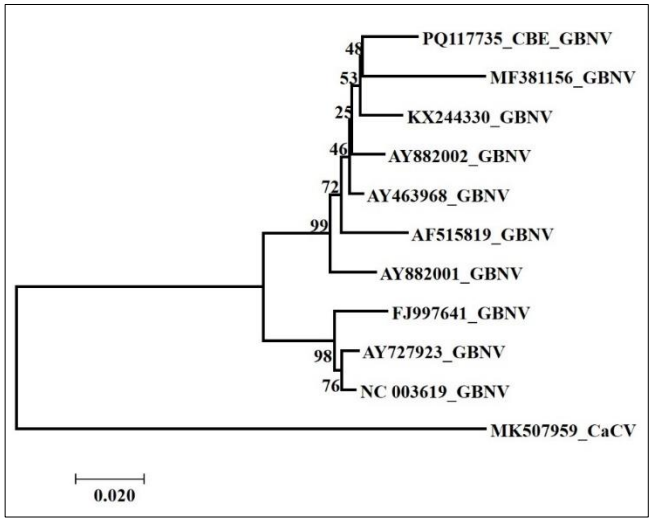

Fig 2: Neighbour joining phylogenetic tree based on the nucleotide sequences of CP gene of GBNV and *Capsicum chlorosis virus* (CaCV) as a for out group

Discussion

Studies on the Orthotospovirus infecting tomato in Tamil Nadu state are highly significant because they offer a foundational understanding of the variety of symptoms caused by tospoviruses and enable the diagnosis of disease and the analysis of the genetic makeup of the virus. The infected tomato samples showed symptoms of *tospovirus* including necrosis on leaves and streaks on stems. Initially, small concentric rings appeared on the leaves, which later coalesced to produce complete necrosis on older leaves. In some plants, circular necrotic rings were first noticed on the immature leaves of certain plants, and as they developed into terminal buds, they led to total drying in the field. Necrotic streaks were seen on the tender section of the stem at an early stage, resembling the GBNV found in infected vegetable crops. A similar type of symptom was reported by Manjunatha *et al.* (2010) [13] who found naturally infected tomato plants produced symptoms of tospovirus including yellowing, wilting, tip drying, ring patches on leaves, necrosis on newly developing stems, and finally plant death. On the fruit stalks, stem, and petiole of leaves, brown necrotic streaks have occasionally been noticed. Infected tomato from Tamil Nadu also produced symptoms such as necrotic ring spots on leaves, necrotic streaks on stems and growing buds (Gayathri *et al.*, 2024) [5]. The tomato exhibiting typical symptoms of *tospovirus* were inoculated on cowpea cv. C 152 plants, resulted in the production of typical circular chlorotic lesions on inoculated primary leaves on 4 dpi; the advanced stage of infection leads to the death of plants under glasshouse conditions. The development of circular chlorotic and necrotic lesions on cowpea are the characteristic symptoms of *tospovirus* by mechanical inoculation (Ramiah *et al*., 2001; Haokip *et al.*, 2016) [15, 7]. This result was supported by Kunkalikar *et al*., (2010) [11] who reported that tospovirus produced symptoms of cholorotic and necrotic lesions in many plant species belongs to Solanaceae, Amaranthaceae, Leguminaceae and Fabaceae. The mechanical transmission of sap obtained from infected tomato successfully produced local lesion symptoms on cowpea within 4-5 days of inoculation (Manjunatha *et al.*, 2010) [13]. The sap inoculated cowpea plants produced chlorotic and necrotic lesion upon mechanical inoculation after 6-7 days of inoculation as the diagnostic host for tospovirus (Balol and Patil, 2014) [2]. In addition, when tomato plants were artificially inoculated with GBNV, they developed concentric necrotic lesions on the infected leaves, which later coalesced to form enormous patches and stunted the plants. This resulted in both local and systemic infection, suggesting the possible occurrence of GBNV as the causative agent of necrosis in tomato.

The current research work aimed to characterize the GBNV precisely from tomato using molecular characterization of the CP gene. The results of our study showed the amplification of approximately 840 bp corresponding to CP gene of GBNV using specific primers and the GBNV isolates shared high homologies, shared 97.78% identities with Indian isolate (AY463968) from tomato. The results were in line with those obtained earlier by Balol and Patil (2014) [2] and they also amplified the CP gene of GBNV from infected tomato plants collected from different parts of India using RT-PCR. Similarly, the presence of GBNV from infected tomato collected from the farmers field were confirmed through the characterization of CP gene, the results revealed more than 96 percent sequence similarity and showed relatedness with Indian isolates of GBNV (Karthikeyan *et al.*, 2024) [10]. Renuka *et al.*, (2020) [17] have collected the virus-infected vegetable crops including tomato to characterize the GBNV. The result revealed that the samples amplified the amplicon size of 0.8kb corresponding to the N gene of GBNV and showed

the highest nucleotide sequence similarities of 93-99 percent with GBNV from blackgram, tomato, chilli, peas, and groundnut and potato. In our study, a phylogenetic tree constructed by NJ algorithm using *tospoviruses* revealed the formation of a single cluster. A similar type of results was obtained by Ruth *et al.* (2013) [18] who successfully amplified the coat protein gene of GBNV and sequence shared 93-99 percent homology with the other GBNV isolates. Further, the cluster dendrogram revealed the GBNV from Andhra Pradesh produced a single cluster and results depicting the nucleotide similarity among the isolates showed that the GBNV isolate of tomato is more related to GBNV isolates from groundnut, brinjal, chilli, soybean, potato and tomato. This suggests that phylogenic analysis of genes can reveal the correlation between and among viruses. The phylogenetic analysis enabled the establishment of a close serological relationship between sunflower necrosis virus strains by generating a distinct cluster and their gradual evolution in comparison with the members of nine virus genera (Chavhan *et al.*, 2018) [4]. This study concludes the occurrence of GBNV in tomato at molecular levels through CP gene profiling. This result on sequence relationships of *tospovirus* could be useful in developing novel virus control strategies targeting the CP gene residues.

Acknowledgements
The authors gratefully acknowledge the UGC for the financial support of the project under Dr.D.S.Kothari Post Doctoral Fellowship (Letter No.202122-BL/20-21/0045 from UGC, New Delhi, dated 14.09.2021). Also, acknowledge Bharathiar University, Coimbatore for providing infrastructure facilities.

References

1. Agarwal S, Rao AV. Tomato lycopene and its role in human health and chronic diseases. Canadian Medical Association Journal. 2000;163:739-744.
2. Balol BG, Patil MS. Biological characterization and detection of groundnut bud necrosis virus (GBNV) in different parts of tomato. Journal of Pure and Applied Microbiology. 2014;8(1):1-4.
3. Bhat AI, Jain RK, Varma A, Lal SK. Nucleocapsid protein gene sequence studies suggest that soybean bud blight is caused by a strain of groundnut bud necrosis virus. Current Science. 2002;82:1389-1392.
4. Chavhan RL, Verma MK, Hinge VR, Kadam US, Kokane SB, Chakrabarty PK. Sequence analysis of coat protein and molecular profiling of sunflower necrosis virus (SNV) strains from Indian subcontinent. Journal of Plant Biochemistry and Biotechnology. 2018;27:28-35.
5. Gayathri M, Sharanya R, Renukadevi P, Nakkeeran S, Saranya N, Varanavasiappan S, Raveendran M, Suhail A, Alkahtani S. Deciphering the antiviral nature of endophytic *Bacillus* spp. against groundnut bud necrosis virus in cowpea and tomato. Frontiers in Microbiology. 2024;15:1410677.
6. Hall TA. Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series. 1999;41:95-98.
7. Haokip BD, Alice D, Nagendran K, Renukadevi P, Karthikeyan G. Detection of *Capsicum chlorosis virus* (CaCV), an emerging virus infecting chilli in Tamil Nadu, India. Vegetos. 2016;29:1-4.
8. Horticulture Statistics. Horticulture statistics at a glance. 2021; Retrieved from https://www.agriwelfare.gov.in/Documents/Horticultural_Statistics_at_Glance_2021.pdf.
9. Jain RK, Paul Khurana SM, Bhat AI, Chaudhary V. Nucleocapsid protein gene sequence studies confirm that potato stem necrosis is caused by a strain of groundnut bud necrosis virus. Indian Phytopathology. 2004;57:169-173.
10. Karthikeyan G, Suganyadevi M, Rajamanickam S. Replicase gene-mediated RNA silencing mechanism confers resistance against groundnut bud necrosis virus in tomato (*Lycopersicon esculentum* Mill.). European Journal of Plant Pathology. 2024. https://doi.org/10.1007/s10658-024-02908-y.
11. Kunkalikar SR, Sudarsana P, Rajagopalan P, Zehr UB, Ravi KS. Biological and molecular characterization of *Capsicum chlorosis virus* infecting chilli and tomato in India. Archives of Virology. 2010. https://doi.org/10.1007/s00705-010-0681-5.
12. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, *et al*. Clustal W and Clustal X version 2.0. Bioinformatics. 2007;23:2947-2948.
13. Manjunatha L, Patil MS, Kavitha TR, Vanitha LS, Vijaya Mahantesha SR. Mechanical transmission of groundnut bud necrosis virus. Environment & Ecology. 2010;28(4A):2456-2458.
14. Pavithra BS, Krishnareddy M, Rangaswamy KT. Detection and partial characterization of groundnut bud necrosis virus in chilli. International Journal of Science and Nature. 2016;7(4):843-847.
15. Ramiah M, Bhat AI, Jain RK, Pant RP, Ahlawat YS, Prabhakar K, Verma A. Partial characterization of an isometric virus causing sunflower necrosis disease. Indian Phytopathology. 2001;54:246-250.
16. Reddy M, Reddy DVR, Appa Rao A. A new record of virus disease on peanut. Plant Disease Reporter. 1968;52:494-495.
17. Renuka HM, Ganesh Naik R, Krishnareddy M. Biological and molecular characterization of GBNV infecting solanaceous vegetable crops. International Journal of Current Microbiology and Applied Sciences. 2020;9(4):2085-2092.
18. Ruth C, Subba Rao M, Krishna Murthy KVM, Raghavaiah G, Srinivasa Rao V, Bhaskar Reddy BV. Molecular characterization of bud necrosis virus isolates infecting tomato (*Lycopersicon esculentum* Mill.) by coat protein gene analysis in Andhra Pradesh. Archives of Phytopathology and Plant Protection. 2013;46(1):7-18.
19. Saitou N, Nei M. The neighbour-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution. 1987;4:406-425.
20. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, New York: Cold Spring Harbor; 1989. p. 405-413.
21. Subramanian KS, Narayanasamy P. Mechanical transmission of Whitefly-borne yellow mosaic virus of *Lablab niger* Medikus. Current Science. 1973;47:92-93.

22. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution. 2011;28:2731-2739.
23. Vanthana M, Nakkeeran S, Malathi VG, Renukadevi P, Vinodkumar S. Induction of *in planta* resistance by flagellin (Flg) and elongation factor-TU (EF-Tu) of *Bacillus amyloliquefaciens* (VB7) against groundnut bud necrosis virus in tomato. Microbial Pathogenesis; c2019. https://doi.org/10.1016/j.micpath.2019.103757.