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# DETECTION AND PARTIAL CHARACTERIZATION OF *GROUNDNUT BUD NECROSIS VIRUS* IN CHILLI

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**ABSTRACT**
Plants showing chlorotic and necrotic concentric ring spot on leaves of chilli were collected from field. After mechanical inoculation on a local lesion host such as cowpea, a host range test was performed. The virus induced chlorotic local lesions in *Chenopodium amaranticolor*, necrotic local lesions followed by systemic necrosis in four Nicotiana species and leaf distortion in *Datura stramonium*, and necrotic local leasions in *Ipomoea marginata* and *Petunia hybrid* 4-5 days after inoculation. *Arachis hyopogaea*, *Phaseolus vulgaris*, *Dolichos lablab*, *Glycine max*, *Pisum sativa*, *Citrullus lanatus*, *Solanum tuberosum* and *Tagetes* spp showed systemic symptoms like mosaic with chlorotic ring spot yellowing and bud necrosis symptoms. DAC-ELISA was performed to all infected plants including chilli samples shows positive reaction to the GBNV antisera. Electron microscopic observations of ultra thin sections from infected chilli leaves showed the presence of spherical, membrane-bound particles of typical tospovirus species. RT-PCR was performed after total RNA extraction from infected chilli leaves. A fragment of approximately 0.8kbp corresponding to the N gene was amplified, cloned and sequenced. The nucleotide and amino acid sequence of N protein from the chilli isolates had highest identity of 94.5% to 98.7% with the reported GBNV isolates of cowpea (JX843805), groundnut (U27809) and mungbean (AY871098).

**KEYWORDS:** Inoculation, DAC-ELISA, RT-PCR, RNA extraction, amplification.

## INTRODUCTION

Chilli (*Capsicum annuum* L.), an important spice crop in India and is grown for its pungency, color and flovor. *Capsicum* fruits are used in a wide range of fresh and processed food products and are an important source of vitamins *viz.*, A and C and essential nutrients. *Capsicum* extracts are also used in pharmaceutical and cosmetic industries. Chilli belongs to the genus *Capsicum* and family Solanaceae, is herbaceous or semi-woody annual or perennial. *C. annuum* and *C. frutescens* are commercially cultivated species. There are approximately 25 species within the genus *Capsicum*, five of which have been domesticated. The most widely grown and economically important species is *Capsicum annuum*, and the other four domesticated species are *C. baccatum, C. pubescens, C. chinense* and *C. frutescens;* originated in the tropical Americas (Bosland and Votava, 2000). Classification of *Capsicum* below the species level is largely based on fruit types and uses. A wide diversity of fruit shapes exists both within and between species (De Wit and Bosland, 1996).

India is the world's largest producer of chilli with an annual production of 14.92 million metric tonnes from 7.75 million hectares representing 25% to the world production (FAO statistics, 2015) and contributes significantly to foreign exchange through exports. Now a day's viral diseases are the major production constraints for the production of chilli. It is highly susceptible to a large number of viruses through natural infection and is known to be affected by 42 viruses worldwide which cause a great economic loss. In India a few viruses are detrimental affecting chilli crop, subsequently the economy of the country. The diseases caused by tospoviruses (family Bunyaviridae; genus Tospovirus) are emerging as a significant limiting factor for the sustainable production of chilli in India. Recently *Groundnut bud necrosis virus* (GBNV) (Satyanarayana *et al*., 1996a, b; Hemalatha *et al*., 2008, Anjaneya Reddy *et al.*, 2008) and *Capsicum chlorosis virus* (CaCV) (Krishnareddy *et al*., 2008) have been reported in India. The geographical expansion and 90 to 100% incidences of tospovirus infections in chilli/ peppers has caused significant concern to farmers in Khammam and Warangal districts of the then Andhra Pradesh (Ravi *et al*., 2007), Karnataka, Tamil Nadu and Maharashtra States. Outbreaks of disease caused by GBNV in different tomato growing regions in Maharashtra, Karnataka and Andhra Pradesh are common showing up to 100% disease incidence (Kunkalikar *et al.*, 2011). GBNV is currently recognized as the most economically important tospovirus, losses due to GBNV alone have been estimated at more than US$89 million per annum in Asia (Reddy *et al*., 1995). GBNV caused 70 to 90% loss of groundnut in India. Serious outbreak of GBNV was reported in different tomato growing regions in Maharashtra, Karnataka and Andhra Pradesh, where up to 100% disease incidence was recorded during 2003 to 2006 (Kunkalikar *et al*., 2011; Mandal *et al*., 2012).

## MATERIALS & METHODS

### Collection of virus isolate

Survey was conducted in tospovirus affected areas of chilli in Karnataka. Symptomatic leaf samples showing mosaic, mottle, yellowing, chlorotic or necrotic ringspot were collected. The samples were immediately placed in plastic bags, transported in cold boxes and stored at 4°C until testing by direct-antigen coating enzyme-linked immunosorbent assay (DAC-ELISA).

### Maintenance of virus isolates inoculation and ELISA

The collected samples were inoculated on 8-10 days old cowpea seedlings for purification and to avoid mixed infection. 4-5days after post inoculation chlorotic local lesion was developed on cowpea plants and it was used for further maintenance on chilli plants. 20-25 days old healthy seedlings of chilli or the seedlings with fully expanded cotyledons and two true leaves stage were used for sap inoculation. The inoculated plants were maintained at 25°C and a 12-hour photoperiod for a week then transfer to the glass house and symptom expressions recorded periodically. The inoculated plants were tested by ELISA to confirm the presence of virus.

### Host range studies

Studies on host range of the GBNV isolate were conducted to determine the host plant susceptibility. For determination of host range, virus was inoculated to different hosts *viz., Vigna mungo, Vigna radiate, Arachis hyopogia, Cajanus cajana, Cicer arietinum, Vigna unguiculata, Phaseolus vulgaris, Pisum sativam* and *Glycine max* host plants belonging to Leguminosae family *Cucumis sativus, Benincosa hispida, Citrullus lanatus* and *Memordica charantia* belonging to Cucurbitaceae family *Ipomoea marginata* belongs to Convolvulaceae family, *Amaranthus viridis* and *Gomphrena globosa* belongs Amaranthaceae family, *Nicotiana tabacum, Nicotiana glutinosa, Nicotiana benthamiana Lycopersicon esculantum, Solanum tuberosum, Solanum nigrum, Petunia hybrid, Datura stramonium* and *Solanum melongena* belongs to Solanaceae family, *Chenopodium amaranticolor* belongs to Chenopodiaceae family and *Tagetes* spp belongs to Asteraceae family was evaluated.

### Electron microscopy

The final viral suspension that was obtained by purification was taken for electron microscopic studies. The formavar coated grids were floated on purified viral suspension for 10 minutes. Then, stained the grids with 2 per cent phosphotungstic acid (PTA) for five minutes the excess stain was drained by touching a blotting paper strip to the edge of the grid. The grids were dried for 15-30 min in dessicator and examined under JOEL 100 S transmission electron microscope at various magnifications and taken the picture of viral particles.

### Total RNA extraction from infected plant samples

RNA was isolated from infected leaf samples as well as healthy samples used as controls. Total RNA extracted from the above samples using RNAeasy Plant Mini Kit according to manufacturer's instructions and it was used as a template in the reverse transcription-polymerase chain reaction (RT-PCR).

### Cloning and sequencing of N gene

Reverse transcription was carried out in eppendorf thermo cycler. RT reaction was set up for virus by adopting manufacturer's instructions. Amplification was performed by using programmed for one cycle of 40ºC for 60 min for cDNA synthesis. The PCR amplification was carried out by using SGF (ATGTCTACCGTYAAGCAACTYAC) and SGR (CCATRTAYTTRACYTGNTCATC) with the following conditions; initial denaturation at 94°C for 3 min followed by 35 cycles having the following parameters 45 sec of denaturation at 94°C, 1 min of annealing at 55°C and extension for 1.30 min at 72ºC followed by a final extension for 20 min at 72ºC. Amplified DNA fragments were electrophoresed in 0.8 per cent agarose gel. 800-bp amplified products corresponding to the complete N gene of chilli was cloned into pTZ57R/T cloning vector by following standard molecular biology procedures (Sambrook and Russell, 2001). After successful confirmation for the presence of expected insert in the clone, the plasmid DNA was isolated in large scale using plasmid extraction kit (Cat# 12123, QIAGEN GmbH, Hilden, Germany) and sequenced using the automated sequencing facility at Chromous Biotech Pvt. Ltd., Bengaluru. Sequencing was done in both directions using M 13 forward and reverse primes in an ABI Prism 377 DNA sequence.

Sequence information generated by sequencing a clone from both the directions was assembled using the software BIOEDIT version 7.0 programs or DNASTAR program (DNAStar Inc, USA). The sequence homology of the gene sequences and multiple alignment was carried out by using Bio-Edit sequence Editor Software. Database searches with Tospovirus sequences were carried out by NCBI-BLAST program (http://blast.ncbi. nlm.nih.gov). Nucleotide (nt) and amino acid (aa) sequence alignments was performed using CLUSTALW program using Mac Vector software (v11.1.2; MacVector Inc., USA). These data was used for phylogenetic analysis using MEGA version 4.0 (Tamura *et al*., 2007). A phylogenetic tree was constructed using the neighbor-joining method with 500 bootstrap replications using tospovirus sequences from the GenBank.

## RESULTs & DISCUSSION

### Host range and symptoms

The test plant species inoculated with the crude sap, *Arachis hyopogaea, Phaseolus vulgaris, Dolichos lablab, Glycine max, Pisum sativa, Citrullus lanatus, Solanum tuberosum* and *Tagetes* spp showed systemic symptoms like mosaic with chlorotic ring spot yellowing and bud necrosis symptoms after ten days of inoculation. Localized chlorotic lesions followed necrotic lesions were observed on leaves of *Vigna unguiculata*, different species of Tobacco, *Datura stramonium, Ipomoea marginata, Petunia hybrida* and *Chenopodium amaranticolor* four to five days after inoculation. All the inoculated weed plants are the excellent host to GBNV and the virus produced chlorotic and necrotic local lesion on the *Cassia tora, Crotalaria juncea* and *Nicandra physalodes*. Similar result was obtained by Singh (2005), several host range of tospovirus which was produced systemic and local lesion on several plant species belonging to Amaranthaceae, Boraginaceae, Chenopodiaceae, Compositae, Cucurbitaceae, Leguminosae and Solanaceae families. Similarly many authors reported that GBNV infects the members of families Amaranthaceae, Asteraceae, Chenopodiaceae, Cucurbitaceae, Compositae, Fabaceae,

Solanaceae and Malvaceae (Raja, 2005; Saritha and Jain, 2007; Mandal *et al.*, 2012). Compositae and Solanaceae have largest range of host plants susceptible to GBNV (Prins *et al*., 1996).

**Electron microscopic study**

The electron microscopic observation of the purified preparation of diseased samples showed the presence of spherical virus (80-120 nm in diameter) in diseased samples of chilli which were not found in healthy samples. These particles have morphology similar to those of tospoviruses (Sivaprasad and Gubba, 2006). Each viral particle consists of a granular core of nucleocapsids, bounded by a lipid envelope (5nm thick), which is covered with surface projections. A few particles appeared distorted giving a characteristic 'dumbbell' or germinate form. Similarly spheroidal particles of GBNV approximately 50 mμ diameter, grouped inside a capsule or envelope to give easily flattened and distorted particles of twice that diameter under electron microscope was observed by Best and Palk (1964), Sivaprasad and Gubba (2006), Krishnareddy *et al*. (2008) and Yin *et al.* (2014).

**DAC-ELISA**

This technique was standardized and used for the detection of GBNV in chilli. Infected samples were mechanically inoculated different host were tested by the direct antigen coating enzyme linked immunosorbent assay (DAC-ELISA). The results revealed that the virus showed positive reaction to tospovirus antiserum which was later confirmed as *groundnut bud necrosis virus* through amplification, cloning and sequencing. DAC-ELISA was also performed for identification of other tospoviruses like MYSV, CaCV, TNRV and WSMoV ((Knierim *et al*., 2006; Chiemsombat *et al*., 2008; Anurag, 2012).


Gel picture showing amplification of N gene in GBNV infected chilli sample
Lane M: Lambda DNA/ECOR1+HindIII Marker Lane 1-2: Water control Lane 3-6: infected chilli

**N gene cloning and sequencing**

The total RNA was isolated and used for reverse transcription the cDNA obtained in RT-PCR step was used as template for converting double stranded DNA by using tospovirus specific forward and reverse primers in the presence of taq DNA polymerase. A DNA fragment with approximately 830 base pairs (bp), corresponding to the nucleocapsid (N) gene obtained was cloned into pTZ57R/T and completely sequenced. The N gene from the chilli isolate has 830 nucleotides, with the potential to code for a 277 amino acid protein.

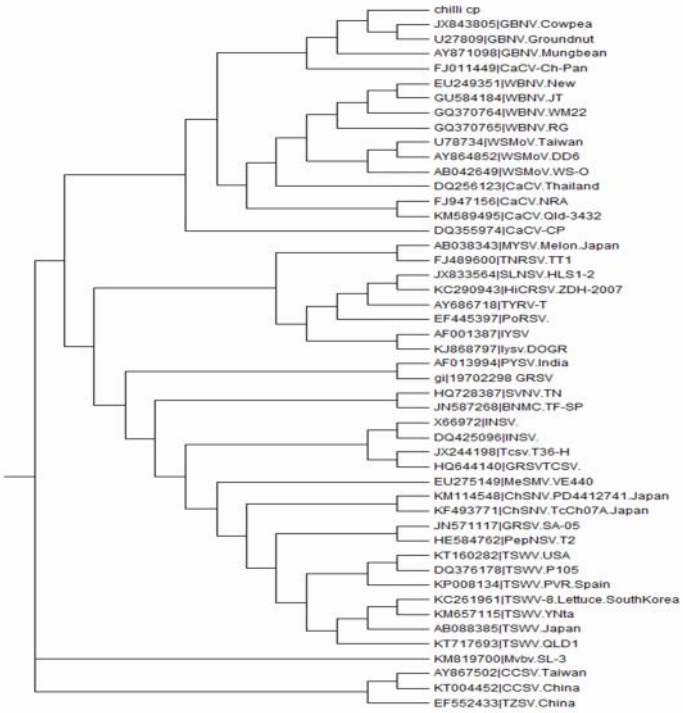


**FIGURE1.** Dendrogram illustrating nucleoprotein (N) nucleotide sequence relationships of tospovirus species. Abbreviations are Watermelon silver mottle virus (WSMoV), Capsicum chlorosis virus (CaCV), Groundnut bud necrosis virus (GBNV), Watermelon bud necrosis virus (WBNV), Calla lily chlorotic spot virus (CCSV), Tomato zonate spot virus (TZSV), Tomato necrotic ringspot virus (TNRV), Melon yellow spot virus (MYSV), Tomato yellow ring virus (TYRV), Iris yellow spot virus (IYSV), Impatiens necrotic spotvirus (INSV),Chrysanthemum stem necrosis virus (CSNV), Zucchini lethal chlorosis virus (ZLCV), Tomato spotted wilt virus (TSWV),Tomato chlorotic spot virus(TCSV), Groundnut ringspot virus (GRSV), Groundnut yellow spot virus (GYSV) and Groundnut chlorotic fan-spot virus (GCFSV).

The comparative sequence analysis of tospoviruses of the N protein gene chilli isolates showed highest identities of 93.2 % nucleotide and 97.4 % amino acid identity with GBNV isolates of cowpea, groundnut and mungbean. The phylogenetic tree generated based on nucleoprotein of both nucleotide and amino acid sequences showed grouping of chilli tospo isolates along with GBNV as subgroup within the major WSMoV serogroup clade. The GBNV chilli isolates N protein aa sequence showed highest identity (97.4 to 99.6%) to that of the previously reported cowpea, groundnut, mungbean, onion, okra, pea, potato, soybean and tomato isolates ( Bhat *et al.,* 2002; Hemalatha *et al.,* 2008; Jain *et al.,* 2004; Umamaheswaran *et al.,* 2003). The N protein of GBNV shares the highest homology of 85.5 % with that of *Capsicum chlorosis virus* (CaCV), 79.5 to 81.0% with *Watermelon bud necrosis virus* (WBNV) and *Watermelon silver mottle virus* (WSMoV) isolates and 55.5-65.5 % to the rest of the members of the WSMoV serogroup.

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