Importance and Genetic Diversity of Vegetable-Infecting Tospoviruses in India

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ABSTRACT

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A survey for *Peanut bud necrosis virus* (PBNV), Watermelon bud necrosis virus (WBNV), *Capsicum chlorosis virus* (CaCV), and *Iris yellow spot virus* (IYSV) was conducted between 2002 and 2009 in the major vegetable-growing areas in India. PBNV was documented widely in tomato and chili peppers in 14 states representing southern, north-western, north-eastern, and central regions and WBNV was predominantly detected in watermelons and cucurbits in all except north-eastern

regions. In addition, the expanded host range of PBNV to watermelons and other cucurbits and WBNV to tomato and chili peppers was observed leading to natural mixed infection of the two viruses. IYSV was found in onion in southern, central, and north-eastern regions and CaCV in tomato and chili peppers in northern and southern regions, respectively. Phylogenetic analysis of the nucleocapsid gene revealed segregation of field isolates of PBNV and WBNV into two distinct subclades, whereas isolates of CaCV and IYSV each clustered into a single clade. A proposal for establishing WBNV as a distinct tospovirus species is made based on the molecular characterization of small- (S) and medium- (M) RNA segments.

Additional keyword: sero-diagnosis.

India is the world's second largest producer of vegetables with an annual production of 87.53 million tonnes from 5.86 million hectares representing 14.4% to the world production (FAO statistics, 2009). A variety of vegetables are grown under field conditions in the diverse agroclimatic zones of the country making it possible to grow almost all varieties of fresh vegetables year round to meet the increased demand for dietary requirements of vegetables in both rural and urban areas. In India, tomato, chili pepper, okra, potato, and onion are economically important and occupy nearly 50% of the total area under vegetable production. Chili pepper, onion, and tomato are cultivated in an area of 9.3, 4.54, and 0.35 million ha with a production of 8.0, 6.3, and 5.3 million metric tons, respectively, and contribute significantly to India's agricultural exports.

Diseases caused by tospoviruses (family *Bunyaviridae*; genus *Tospovirus*) are emerging as a significant limiting factor for the sustainable production of vegetables in India. To date, *Peanut bud necrosis virus* (PBNV) (10,32), *Peanut yellow spot virus* (PYSV) (31), *Watermelon bud necrosis virus* (WBNV) (14), *Iris yellow spot virus* (IYSV) (25), and *Capsicum chlorosis virus* (CaCV) (18,19) have been identified in the country. PBNV, CaCV, and IYSV represent definitive members and PYSV and WBNV are considered tentative species of the genus *Tospovirus* (7). PBNV, WBNV, CaCV, and IYSV have been detected in different vegetables, whereas the natural host range of PYSV is currently not known. In recent years, the geographical expansion of PBNV,

WBNV, CaCV, and IYSV have been reported in the country due to increased area of production of different vegetable crops. However, reliable information regarding the distribution of these four tospoviruses in different vegetable-growing regions is lacking.

Until the mid-2000, the occurrence of PBNV was limited to a few regions. Beginning in 2001, epidemics of PBNV were reported in several tomato-growing regions of India, including Hoskote and Nasik regions of Karnataka and Maharashtra States, respectively, that are the major tomato-producing areas. Consequently, PBNV has become a major constraint (1,26), there by limiting the sustainability of tomato that contributes nearly 8% of the total production of vegetables in India (www.indiabudget.nic.in). Meanwhile, 90 to 100% incidence of tospovirus infections in chili peppers has caused significant concern to farmers in Khammam and Warangal districts of Andhra Pradesh State (26). Currently, PBNV is recognized as the most wide spread and damaging tospovirus in India, causing production problems to both legumes and solanaceous crops (38).

WBNV is an economically important pathogen of watermelon [*Citrullus lanatus* (Thumb.) Matsun and Nakai], with the disease incidence in some areas reaching 100% leading to total loss of watermelons. The virus has also been reported in ridge gourd (*Luffa acutangula* Roxb.) (20), cucumber (*Cucumis sativus* L.) (13), and bitter melon (*Momordica charantia* L.). In 2005, an IYSV epidemic was reported in major bulb and seed onions in Maharashtra state (25). IYSV has a direct bearing on the export potential of onions because India ranks second in global onion production and onion is the fourth most important commercial vegetable crop, accounting for 5% share in the vegetable production. CaCV was reported for the first time in tomato in northern India during 2007 post-rainy season (December to May) and subsequently in chili peppers in southern India (17–19). Previ-

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*The e-Xtra logo stands for "electronic extra" and indicates that Figure 3 appears in color online.

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ously, CaCV was reported in chili pepper in Australia (15,21), tomato in Thailand (4,16), and peanut in China (2). PYSV is considered of less economic importance to vegetables as its incidence is sporadic and confined to peanut.

Different tospoviruses are known to produce similar, though not identical, symptoms in a given crop making symptoms-based diagnosis difficult (36). Therefore, an understanding of the prevalence and genetic diversity of natural populations of tospoviruses will help in developing sustainable disease management strategies. This is especially critical due to the lack of genetic sources of resistance to PBNV and WBNV in vegetables. In this study, samples were collected from vegetable crops suspected for tospovirus infection from major vegetable-growing regions of India between 2002 and 2009 and tested for PBNV, WBNV, CaCV, and IYSV using virus-specific diagnostic assays. In addition, sequence analysis of the nucleocapsid gene of field isolates revealed diversity among these viruses with isolates of PBNV and WBNV segregating into two distinct subclades and isolates of CaCV and IYSV each forming a single clade. The results also revealed an expanded host range of PBNV and WBNV that may facilitate their natural mixed infections in tomatoes and watermelons. A proposal for establishing WBNV as a distinct tospovirus species

is made based on the molecular characterization of small- (S) and medium- (M) RNA segments.

MATERIALS AND METHODS

Collection of samples. Samples were obtained from surveys conducted between 2002 and 2009 during rainy (June to November) and post-rainy (December to May) cropping seasons in major vegetable growing regions of India. A total of 914 plant samples suspected for tospovirus infection were collected from southern (Andhra Pradesh [AP], Karnataka [KAR], Tamil Nadu [TN], and Goa [GOA]), north-western (Gujarat [GUJ], Rajasthan [RAJ] and Delhi [DEL]), north-eastern (Chattisgarh [CHA], Uttar Pradesh [UP], Haryana [HAR], West Bengal [WB]), and central (Maharashtra [MAH], and Madhya Pradesh [MP]) states of India (Fig. 1).

Serological testing of samples. The samples were tested for PBNV, WBNV, and CaCV by direct antigen coating-enzyme-linked immunosorbent assay (DAC-ELISA) using polyclonal antibodies (PABs) to the N protein of PBNV isolate infecting peanut (obtained from the International Crops Research Institute for the Semi-Arid Tropics, Hyderabad, India). Onion samples were tested

Fig. 1. Geographical map of India indicating locations from where vegetable-infecting tospoviruses viz. *Peanut bud necrosis virus* (PBNV), *Watermelon bud necrosis virus* (WBNV), *Capsicum chlorosis virus* (CaCV), and *Iris yellow spot virus* (IYSV) samples were collected: 76 isolates of PBNV (□), 28 isolates of WBNV (■), 3 isolates of CaCV (●), and 28 isolates of IYSV (○) were characterized for the N gene sequence. The locations of the isolates are marked: AP-Andhra Pradesh, KAR-Karnataka, TN-Tamil Nadu, GJ-Gujrat, GOA-Goa, RAJ-Rajasthan, DEL-Delhi, CH-Chattisgarh, UP-Uttar Pradesh, WB-West Bengal, MAH-Maharashtra, and MP-Madhya Pradesh.

by double antibody sandwich-ELISA (DAS-ELISA) using polyclonal antibodies to the N protein of IYSV (obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH [DSMZ], Germany). In addition, samples were tested using PABs raised against the N protein of *Watermelon silver mottle virus* (WSMoV), *Tomato spotted wilt virus* (TSWV), IYSV, *Tomato yellow fruit ring virus* (TYFRV), and CaCV (obtained from DSMZ, Germany). Monoclonal antibodies (MAbs) specific to N and nonstructural protein (NSs) of PBNV and WSMoV were also used to test samples by triple antibody sandwich-ELISA (TAS-ELISA) (3) (Table 1). Absorbance values at 405_{nm} that were threefold or more higher than the negative control were considered to be positive.

Cloning and sequencing of N gene. Total RNA was extracted from ELISA-positive samples of representative virus isolates using the RNeasy Plant Mini kit (Qiagen Inc., Chatsworth, CA). Fifty nanograms of total RNA was used as template in reverse transcription-polymerase chain reaction (RT-PCR) assay consisting of 1× AMV/Tfl buffer, 2.5 μM each of dNTPs, 2 mM MgSO₄, 10 picomoles of each primer, 2.5 U of AMV reverse transcriptase and 2.5 U Tfl DNA polymerase in a 25 μl reaction volume (Access RT-PCR kit, Promega Corp., Madison, WI). A pair of degenerate primers (Mh-F and Mh-R) was designed based on the N gene sequence of PBNV, WBNV, CaCV, and WSMoV available in GenBank and used to amplify the N gene sequence (Table 2). RT-PCR was carried out in GeneAmp 9700 PCR machine (Applied Biosystems) at 42°C for 45 min followed by denaturation at 94°C for 3 min, and 35 cycles of PCR with each cycle consisting of 94°C for 1 min, 57°C for 45 s and 72°C for 1 min. Final extension was carried out at 72°C for 10 min. The amplified products were cloned into pGEM-T Easy vector (Promega Corp.) following the manufacturer's instructions and sequenced using BigDye terminator cycle sequencing kit (Applied Biosystems) using an automated DNA sequencing system (3130 Genetic Analyzer-Applied Biosystems) (29). Two independent clones for each isolate were sequenced in both orientations by using universal M13 reverse and forward primers.

Cloning and sequencing of S- and M-RNA of WBNV. An isolate of WBNV (WBNV-Wm-Jal) collected from a watermelon field near Jalna, Maharashtra State, was used for cloning and sequencing of S- and M-RNA segments. A single lesion isolate obtained after two successive inoculations on *Chenopodium quinoa* was inoculated onto *N. benthamiana* and viral double-stranded RNA (dsRNA) extracted as described by Yeh et al. (39). The dsRNA was subsequently used as template for amplification of S- and M-RNA segments by RT-PCR. Forward and reverse primers to amplify S-RNA were designed based on the conserved nucleotide (nt) sequences in the S-RNA of PBNV (NC_003619, AY871098), WSMoV (NC_003843, AY864852), and CaCV (NC_008301, DQ256123). Primers for amplifying the M-RNA segment of WBNV were designed based on the conserved nucleotide (nt) sequences in the M-RNA of PBNV (NC_003620,

AY871097), WSMoV (NC_003841), and CaCV (NC_008303). The primer sequences and their location in the S- and M-RNA of WBNV are listed in Table 2. RT-PCR was carried out in a GeneAmp 9700 PCR machine (Applied Biosystems) with 1 cycle for cDNA synthesis at 42°C for 45 min followed by 35 cycles of PCR with each cycle consisting of 94°C for 30 s, 55°C for 45 s, and 72°C for 90 s and a final extension step at 72°C for 15 min. Cloning and sequencing of the amplified fragments was carried out as described above.

Sequence analysis. Nucleotide sequences were analyzed using the Contig-Express and AlignX tools available in Vector NTI software version 6.0 (Invitrogen). Amino acid sequences were deduced from the primary nt sequence data either by using the same software or using translation program in Bio-Edit Sequence

TABLE 2. Nucleotide sequences of forward and reverse primers used for polymerase chain reaction to amplify N gene of *Peanut bud necrosis virus* (PBNV-R), *Capsicum chlorosis virus* (CaCV-R), and M RNA of Watermelon bud necrosis virus (WBNV-R)

Primer name	Sequence	Position
N gene		
Mh-F	ATGTCTAMCGTYARGCAACTTAC	–
Mh-R	CCCTYACAMTTCHAKAGAWGDRCTAG	–
PBNV-R	GGGTGCTTTCACATCAATAGCTT	–
WBNV-R	CCATAGTCATCCATGGAAAGA	–
CaCV-R	CATAACATCATCTGAGAGCACATG	–
S and M RNA of WBNV		
S RNA		
SN1-F	AGAGCAATCGGGGCATCTAA	1–20 ^a
SN1-R	AGATGAGAATCAACGT	990–974
SN2-F	TACGCAAAAACTGTTG	908–924
SN2-R	GCCTCTTTCGAGGTC	1651–1637
SN3-F	ATGAAAGTTAATAAAG	1584–1599
SN3-R	GACTATGGTGAGCATCC	2567–2551
SN4-F	TTACACTTCCAGAGAAG	2511–2519
SN4-R	ATGTCTAACGTTAAAGCAAC	3338–3319
SN5-F	GCTGAGCAACTCAACAGCTG	2666–2686
SN5-R	AGAGCAATCGAGGCGC	3405–3390
M RNA		
SM1-F	AGAGCAATCGGTGCGCCAATTA	1–22
SM1-R	TCACGAATTTCCAAGTAAGTTAT	983–961
SM2-F	GAAGCTTACAAAAGTTGACAGA	880–901
SM2-R	TATAGAGTCTATGGAATCTAATG	1517–1495
SM3-F	CTAGATGTCTAAAAGAGAAATCATAA	1409–1433
SM3-R	TTGTACAAACATATATGATCTATC	2624–2601
SM4-F	GCAGATCTCTGCCGACACATGAC	2546–2568
SM4-R	ATCAATTCTCATCTCATATATACC	3512–3489
SM5-F	CCAAACTTATCAGTTGTCAGAT	3399–3420
SM5-R	GTACTGTGTCATTAAAGGTGT	4493–4473
SM6-F	TATGTTTGAAGAGCTGCTTATGC	4397–4419
SM-6-R	AGAGCAATCAGTGCACCAATTTAATAC	4794–4771

^a Nucleotide positions correspond to the S and M RNA of WBNV-Wm-Jal isolate (EU249351, FJ694963).

TABLE 1. Serological cross reaction of *Peanut bud necrosis virus* (PBNV), *Watermelon bud necrosis virus* (WBNV), *Capsicum chlorosis virus* (CaCV), and *Iris yellow spot virus* (IYSV) to antisera in enzyme-linked immunosorbent assay

Sl no.	Antisera ^a	Absorbance (A _{450 nm})						
		PBNV-To-Hsk	WBNV-Wm-Jal	CaCV-To-Var	IYSV-On-Nas	Healthy tomato	Healthy watermelon	Healthy onion
1	PBNV-NP PAb	1.852	1.625	0.895	0.095	0.121	0.095	0.092
2	PBNV-NP MAb	0.995	0.979	0.458	0.085	0.115	0.090	0.089
3	WSMoV-NP MAb	2.971	1.741	1.821	0.118	0.113	0.119	0.117
4	WSMoV-NSs MAb	1.578	0.531	0.528	0.082	0.112	0.085	0.083
5	CaCV-NP PAb	0.381	0.319	0.985	0.153	0.130	0.152	0.119
6	IYSV-NP PABs	0.103	0.115	0.118	0.945	0.121	0.114	0.112
7	TYFRV-NP PABs	0.105	0.104	0.130	0.105	0.123	0.115	0.118
8	TSWV-NP PABs	0.122	0.122	0.116	0.119	0.105	0.128	0.131
9	PYSV-NP PABs	0.132	0.123	0.132	0.123	0.109	0.140	0.128

^a WSMoV, *Watermelon silver mottle virus*; TYFRV, *Tomato yellow fruit ring virus*; TSWV, *Tomato spotted wilt virus*; and PYSV, *Peanut yellow spot virus*.

Editor (version 7.2) and then compared with corresponding sequences available in GenBank. Multiple sequence alignments were generated by ClustalW and sequence identities determined using Bio-Edit Sequence Editor. Phylogenetic trees were constructed by N-J method using MEGA 3.1 (34) and with 1,000 bootstrap replications.

Multiplex RT-PCR. Multiplex RT-PCR was carried to detect CaCV, PBNV, and WBNV in mixed virus infections. Degenerate forward primer (Mh-F) and species-specific reverse primer (PBNV-R, WBNV-R, and CaCV-R) were designed to amplify 494, 647-, and 764-bp fragment specific to CaCV, PBNV, and WBNV, respectively (Table 2). The conditions for multiplex RT-PCR included RT for 45 min at 42°C followed by 35 cycles of PCR (30 s at 94°C, 45 s at 57°C, and 1 min at 72°C) and 10 min extension at 72°C.

RESULTS

Serological detection of PBNV, WBNV, CaCV, and IYSV. A panel of PABs and MABs produced against different tospoviruses for their ability to detect PBNV, WBNV, CaCV, and IYSV was tested. The results (Table 1) showed that samples infected with PBNV, WBNV, and CaCV gave higher A_{405} values (>1.5) with MABs raised against the N protein of WSMoV indicating a common epitope present in the N protein of these tospoviruses. Conversely, both PABs and MABs raised against the N protein of PBNV gave higher A_{405} values with WBNV- but not CaCV-infected samples, indicating that WBNV is serologically more closely related to PBNV than to CaCV. The PBNV samples gave higher A_{405} values with a MAB raised against the NSs protein of WSMoV, whereas WBNV- and CaCV-infected samples gave lower A_{405} values indicating that PBNV and WSMoV shared common epitopes in their NSs protein. In contrast, PABs raised against the N protein of CaCV gave lower A_{405} values with PBNV and WBNV infected samples further indicating that CaCV is serologically distantly related to both PBNV and WBNV. The onion samples infected with IYSV gave positive A_{405} values with PABs to the N gene of IYSV and negative results with antibodies to PBNV, CaCV, and WSMoV. None of the samples tested positive with antibodies specific to TSWV and TYFRV. Based on these results, we selected PABs and MABs raised against the N protein of PBNV and WSMoV, respectively, for documenting PBNV, WBNV, and CaCV in different vegetables and PABs raised against the N protein of IYSV for testing onion samples collected from disease surveys.

Multiplex reverse transcription-polymerase chain reaction. Due to serological cross-reactions mentioned a multiplex reverse transcription-polymerase chain reaction (RT-PCR) was optimized for the detection of CaCV, PBNV, and WBNV using a degenerate upstream primer (Mh-F) and species-specific downstream reverse primers (PBNV-R, WBNV-R, and CaCV-R). The 494-, 647-, and 764-bp fragments specific to CaCV, PBNV, and WBNV, respectively, were amplified in single and mixed virus infections. The specificity of virus-specific amplicons was ascertained by cloning and sequencing and comparing with corresponding sequences of PBNV, WBNV, and CaCV (data not shown).

Distribution and incidence of PBNV, WBNV, CaCV, and IYSV. A combination of ELISA and RT-PCR was used to detect PBNV, WBNV, CaCV, and IYSV in samples collected during surveys conducted over a period of 8 years between 2002 and 2009.

PBNV was detected in 650 samples collected from vegetable crops tomato, chili pepper, brinjal, and carrot from the southern (TN, KAR, AP, and GOA), central (MAH and MP), north-western (GUJ and RAJ), and north (UP, HAR, DEL, and CH) and north-eastern (WB) states of India (Fig. 1). Nearly 40% of these samples came from tomato fields. PBNV was found at a low incidence in eggplant (*Solanum melongena* L.) in MAH, KAR, AP, and DEL states, chili pepper in MAH, KAR, AP, UP, and CH states, fababean, mungbean, and soybean in MAH, pumpkin in KAR and blackgram in AP, watermelon in MAH and HAR states, and carrot in AP state. The incidence of PBNV in different tomato cultivars and hybrids grown in farmers' fields in different locations was highly variable, ranging from 1 to 85%. Severe outbreaks of PBNV were observed in the Nasik region of MAH (80% incidence) during 2004, Hoskote, Bangalore, and Ranibennur regions of KAR (90 to 95% incidence) in 2006, and Madanapalli and Khammam regions of AP (90 to 100% incidence) in 2003 and 2006. A broad range of disease symptoms such as chlorotic, necrotic spots on leaves, and necrotic streaks on petioles, stems, and buds were observed. Chlorotic concentric rings, yellow blotches and reduced fruit size were commonly observed in several varieties and hybrids (Fig. 2). No definite correlation was observed between symptoms in different cultivars/hybrids, seasons and/or geographic locations. During winter (December to March) cropping in northern India, bronzing of the canopy was one of the dominant symptoms of PBNV in tomato. These results indicated widespread distribution of PBNV in vegetables with higher level of incidence in tomato.

The incidence of WBNV was up to 90% in watermelon fields in MH, KAR, RAJ, and UP states of India (Fig. 1). About 54 leaf and stem samples from symptomatic watermelons tested positive in DAC-ELISA with PABs specific to the N protein of PBNV, since previous studies have shown that PABs to PBNV can detect WBNV (27). In 2004, high incidence (up to 80%) was recorded in Jalna district of MAH, 70% incidence in Dharwad district of KAR, and 55% in Dharmapuri district of TN states. However, variation in symptoms on watermelon leaves, stem and fruits was observed in different locations. The predominant symptoms include mosaic, mottling, necrotic spots on leaves, necrosis of lateral and flower buds; necrotic spots and streaks on stem, upward bending of growing tips of infected plants, and shortened internodes leading to stunted growth. Early-infected plants produced no or small fruits, while late infection resulted in the production of malformed fruits with chlorotic and/or necrotic spots and/or rings on fruit surface (Fig. 3).

CaCV was observed in symptomatic tomato and chili pepper collected from Indian states of Haryana and Uttar Pradesh in 2008. However, the incidence of CaCV was low when compared with the incidence of PBNV. Infected tomato produced necrotic rings on leaves and chlorotic lesions on fruits, whereas chili pepper and bell pepper produced chlorotic to necrotic rings on leaves and yellow blotches on fruits with reduced size (Fig. 3).

Fig. 2. A, Neighbor-joining tree of N protein sequences of 76 isolates of *Peanut bud necrosis virus* (PBNV), 28 isolates of *Watermelon bud necrosis virus* (WBNV), 3 isolates of *Capsicum chlorosis virus* (CaCV), and 28 isolates of *Iris yellow spot virus* (IYSV) collected from vegetable-growing regions of India during the study period 2002 to 2009. Each representative isolate was designated using virus name (four letters) followed by host (two letters) and location (three letters). Analysis was done with MEGA 3.1 with 1,000 replicates of bootstrapping. **B,** Neighbor-joining tree of N protein sequences of 22 reported tospoviruses viz., *Gloxinia ringspot virus* (GloxRSV) AAY312061, (CaCV) HM021139, *Tomato necrosis virus* (ToNV) AAT68025, (PBNV) U27809, (WBNV) AF045067, *Watermelon silver mottle virus* (WSMoV) X78556, *Tomato zonate spot virus* (TZSV) NC010489, *Calla lily chlorotic spot virus* (CCSV) AY867502, *Melon yellow spot virus* (MYSV) AB038343, *Physalis severe mosaic virus* (PhySMV) AAD34201, *Polygonum ringspot virus* (PolRSV) EF445397, *Tomato yellow ring virus* (TYRV) DQ462163, (IYSV) AAF75556, *Chrysanthemum stem necrosis virus* (CSNV) AB438998, *Zucchini lethal chlorosis virus* (ZLCV) AF067069, *Groundnut ring spot virus* (GRSV) S54327, *Tomato chlorotic spot virus* (TCSV) AF282982, *Tomato spotted wilt virus* (TSWV) DQ777613, *Impatiens necrotic spot virus* (INSV) DQ523597, *Peanut chlorotic fanspot virus* (PCFV) AF080526, *Peanut yellow spot virus* (PYSV) AF013994, and *Alstroemeria necrotic streak virus* (ANSV) GQ478668. Analysis was done with MEGA 4 with 1,000 replicates of bootstrapping.

These symptoms were similar to those produced by PBNV making it difficult to differentiate CaCV from PBNV under field conditions based on symptoms.

Onion samples suspected for IYSV infection were collected from 17 districts in MAH, KAR, TN, GUJ, UP, and MP states. Virus-infected plants showed a wide range of symptoms, consisting of chlorotic spindle-shaped lesions, chlorotic spindle-shaped concentric rings, long yellow stripes, and bending of scapes. Among these symptoms, spindle shaped lesions were predominant in most of the samples. Since 2008, symptoms with yellow stripes running from top to bottom of scapes were also observed in samples collected from GUJ and MAH states. In many cases, two to three types of symptoms were observed on a single plant (Fig. 3). The disease was more prominent on seed crop than the bulb crop. The disease incidence varied from 50 to 85% in different locations, with Nasik district of MAH state recording 85% incidence.

Expanded host range of WBNV to cucurbit and solanaceous crops. A muskmelon (*Cucumis melo* L.) field in Dharmapuri district of TN was observed with 45% disease incidence and leaves of infected plants showed mosaic mottling and necrosis. In DAC-ELISA, symptomatic leaves tested positive with PABs to the N protein of PBNV. In order to confirm whether the virus was PBNV or WBNV, the N gene was amplified by RT-PCR, cloned, and sequenced. A comparison of the N gene sequence with corresponding sequences of other tospoviruses revealed 93.5 and 96.3% identity at nucleotide and amino acid level, respectively, with the N gene of WBNV. Similarly, presence of WBNV in a pumpkin field showing 40% disease incidence in Tonk district of RAJ state and in a cucumber field showing 25% incidence in Kurukshetra district of HAR state was confirmed by N gene sequence analysis. In these fields, pumpkin and cucumber were grown as intercrop with watermelon. In 2007, plants in eight tomato and three chili pepper fields in Kurukshetra district of

Fig. 3. Peanut bud necrosis virus symptoms; **A**, necrotic spots/rings on leaves; **B**, necrotic streaks on petiole; **C**, chlorotic spots on fruit; and **D**, chlorotic blotches on fruit. Watermelon bud necrosis virus symptoms: **E**, chlorosis and upward curling of plants; **F**, chlorotic lesions on fruit; and **G**, necrotic spots and reduced size of fruits. Capsicum chlorosis virus symptoms: **H**, necrotic rings on tomato leaf; **I**, chlorotic rings on tomato; **J**, chlorotic and necrotic rings on leaf; and **K**, reduced size of bell pepper fruits with blotches. Iris yellow spots virus symptoms: **L**, chlorotic lesions on infected plant; **M**, chlorotic rings on spathe; **N**, chlorotic stripes on spathe; and **O**, **P**, and **Q**, oval/spindle shaped elongated lesions on spathe.

Haryana state and northern part of Delhi showed symptoms resembling those caused by tospovirus infection. These samples tested positive to PABs against the N protein of PBNV in DAC-ELISA and the N gene of isolates from tomato (WBNV-To-Del, WBNV-To-Har) and chili pepper (WBNV-Ch-Pan) revealed 93.5 to 93.9% and 97.8 to 98.1% identity, respectively, with WBNV N gene sequences. These results indicated expanded host range of WBNV under field conditions to different cucurbit and solanaceous crops. To our knowledge, this is the first report of the occurrence of WBNV in tomato and chili pepper.

Mixed infections of PBNV and WBNV. During a survey of watermelon fields in Jalna area of MAH state in June 2006, severe mosaic mottling on leaves, necrotic streaks on stem, petiole and growing buds, and chlorotic and/or necrotic rings on malformed fruits was observed in two watermelon fields. Samples from two individual plants were tested positive for both PBNV and WBNV in multiplex RT-PCR assay (data not shown). To ascertain their specificity to each virus, the amplicons were cloned and four independent clones were sequenced. Nucleotide sequences from two clones shared 97.8 to 97.9% and 98.9% identity at the nucleotide and amino acid level, respectively, with PBNV (NC_003619, AY871098) and the other two clones shared 92.8 to 93.8% and 96.0 to 96.3% identity at nucleotide and amino acid level, respectively, with WBNV (EU373795 to 98). These results indicated presence of PBNV and WBNV sequences in a single plant and, to our knowledge, this is the first report of natural mixed infection of PBNV and WBNV in watermelons.

Sequence diversity and phylogenetic clustering. The nucleotide sequence of the N gene of 76 field isolates of PBNV, 28 field isolates of WBNV and 28 field isolates of IYSV was determined (Table 3). In pairwise comparisons, the 76 N gene sequences of PBNV showed identities ranging between 91.3 and 100% at the amino acid level. The identities among WBNV N gene sequences ranged between 94.1 and 100%, among CaCV N gene sequences ranged between 97.0 and 98.9%, and IYSV N gene sequences ranged between 94.8 and 100% at the amino acid level. These

values are within the limits of species demarcation criteria for members of the genus *Tospovirus*, where isolates with 90% or more amino acid sequence similarity are considered strains of the same virus (7). Based on these results, it can be concluded that divergent strains of PBNV, WBNV, CaCV, and IYSV are present in different vegetable crops in India. The N gene-based phylogenetic relationships of PBNV, WBNV, and IYSV isolates were compared among themselves and with corresponding sequences available in GenBank. The results (Fig. 2A) showed segregation of PBNV, WBNV, and CaCV each into two clades, with greater number of PBNV and CaCV sequences clustering within one clade and WBNV isolates segregating more or less equally between two clades. The mean genetic distances between PBNV, WBNV, and CaCV clades are 0.161, 0.170, and 0.222, respectively. In contrast, IYSV sequences formed a monophyletic cluster single lineage. These results may indicate propensity of the occurrence of distinct variants of PBNV, WBNV, and CaCV, but not IYSV, in India. The N gene-based phylogenetic relationships of PBNV, WBNV, and IYSV isolates were compared among themselves and with other tospovirus sequences available in GenBank (Fig. 2B).

Molecular characterization of S- and M-RNA of WBNV.

The full-length S-RNA segment of WBNV-Wm-Jal isolate consisted of 3,405 nts with two open reading frames (ORFs) in ambisense arrangement. The first ORF (67 to 1,386 nts) encoded the NSs protein with a predicted Mr of 49.6 kDa in sense direction, whereas the second ORF (2,511 to 3,339 nts) encoded N protein with a predicted Mr of 30 kDa in antisense direction. The A/T rich intergenic region consisted of 1,124 nts. The 5' and 3' untranslated regions (UTRs) consisted of 66 and 67 nts, respectively, with high potential to form a genomic panhandle pseudocircle.

The sequence identity of WBNV-Wm-Jal isolate with other tospoviruses is shown in Table 4. The intergenic region combining NSs and N genes was 351 nts longer than PBNV S-RNA and 131 nts shorter than WSMoV (NC_003843) and CaCV (FJ011449) S-RNAs. Structural analysis of S-RNA using

TABLE 3. Comparison of N gene amino acid identity of tospoviruses

Sl. no	Tospovirus ^a	Amino acids	N gene amino acid identity (%)			
			PBNV (this study)	WBNV (this study)	CaCV (this study)	IYSV (this study)
1	PBNV (this study)	276	91.3–100	–	–	–
2	WBNV (this study)	275	81.1–86.2	94.1–100	–	–
3	CaCV (this study)	275	82.2–86.2	78.5–82.1	97.0–98.9	–
4	IYSV (this study)	273	42.2–45.5	41.5–44.0	44.0–45.5	94.8–100
5	PBNV-NC_003619	276	98.9	85.5	85.1	44.8
6	WBNV-AF045067	275	84.4	97.0	81.8	43.0
7	CaCV-AY036057	275	83.6	78.9	96.7	45.1
8	CaCV-EF625227	275	83.6	79.2	97.0	45.1
9	CaCV-EF625228	275	83.6	79.2	97.0	45.1
10	IYSV-DQ838590	273	43.7	42.2	43.3	90.8
11	IYSV-DQ150107	273	44.0	42.6	43.7	90.8
12	IYSV-AF067070	273	43.7	42.6	43.3	90.8
13	IYSV-DQ233478	273	43.7	42.2	43.3	90.4
14	WSMoV-AY514629	275	84.7	82.1	84.3	43.3
15	MYSV-AY673635	279	58.0	57.3	56.9	48.2
16	TYFRV-DQ810195	274	43.0	41.9	43.3	73.3
17	TYFRV-DQ788694	274	42.6	41.9	43.0	72.9
18	ZLCV-AF067069	260	27.8	26.8	28.3	28.1
19	GRSV-AF251271	258	28.5	28.3	29.7	30.2
20	TCSV-AF282982	258	27.1	26.8	28.6	29.1
21	TSWV-DQ777470	258	27.8	27.5	29.7	30.9
22	INSV-DQ523598	262	26.8	25.8	28.0	26.0
23	PCFSV-AF080526	270	20.5	19.1	20.9	18.1
24	PYSV-AY529714	246	18.2	18.2	21.1	18.7
25	TZSV-NC_010489	278	62.5	62.5	61.1	43.4

^a PBNV: *Peanut bud necrosis virus*; WBNV: *Watermelon bud necrosis virus*; CaCV: *Capsicum chlorosis virus*; IYSV: *Iris yellow spot virus*; WSMoV: *Watermelon silver mottle virus*; MYSV: *Melon yellow spot virus*; TYFRV: *Tomato yellow fruit ring virus*; ZLCV: *Zucchini lethal chlorosis virus*; GRSV: *Groundnut ring spot virus*; TCSV: *Tomato chlorotic spot virus*; TSWV: *Tomato spotted wilt virus*; INSV: *Impatiens necrotic spot virus*; PCFV: *Peanut chlorotic fan spot virus*; PYSV: *Peanut yellow spot virus*; and TZSV: *Tomato zonate spot virus*.

NetNGlyc site indicated the potential to form two hairpin structures as reported in WSMoV. The nucleotide sequence comparison of 3' and 5' UTRs revealed that 46% of nucleotides are complementary to each other, which is less compared with 85% identity with WSMoV (NC_003843) and 80% with TSWV (D13926). The terminal 7 nts of 5' UTR were complementary to 7 nts of 3' UTR.

The M-RNA segment of WBNV-Wm-Jal isolate consisted of 4,794 nts with two ORFs in ambisense arrangement similar to other tospoviruses. The ORF (55 to 975 nts) encoded the putative NSm protein with a predicted Mr of 34.1 kDa in sense direction and the second ORF (1,388 to 4,750 nts) encoded Gn/Gc protein with a predicted Mr of 127.5 kDa in antisense direction. The A/T rich intergenic region combining NSm and Gn/Gc consisted of 412 nts. The 5' and 3' UTRs consisted of 54 to 44 nts, respectively, with high potential tendency to form genomic panhandle pseudocircle (Table 4).

DISCUSSION

In India, tospoviruses have emerged as a serious threat to vegetable crops (38) during the last 10 to 15 years. However, only a few tospovirus sequences have been reported from vegetables grown in different parts of the country (13,17,26,31,32). Therefore, the present study represents a comprehensive analysis of genetic diversity of N gene sequences of field isolates of PBNV, WBNV, CaCV, and IYSV collected over a period of 8 years. The information expands our current knowledge on distribution of vegetable-infecting tospoviruses across the country and the information on diversity of tospoviruses offers a prelude to initiating management strategies for mitigating their negative impacts on vegetable production (4,8,22–24).

In this study, the three distinct tospoviruses of the WSMoV serogroup were detected in different vegetable crops and the multiplex RT-PCR protocols described provided reliable diagnostic tools for their discrimination in single and mixed virus

infections. Such a capability is critical for accurate detection of a specific virus given the high cross-reactivity of PABs and MABs used in this study (Table 1). In almost all previous studies (5,9,11,12,27,28,35), ELISA tests were used to survey vegetables for tospoviruses and positive ELISA readings with antibodies to the N protein of PBNV were interpreted as PBNV infections by overlooking high cross-reactivity of polyclonal antibodies. This could be a reason why reliable information on mixed infections of PBNV and WBNV in different vegetables, the expanded host range of WBNV to tomato and chili peppers and the distribution of CaCV has until now been overlooked. In this context, our findings of expanded host range of WBNV and mixed infections of PBNV and WBNV will be substantially informative and offers new information that has practical importance to vegetable crop improvement against tospoviruses.

Molecular analysis of the complete S- and M-RNA segments of WBNV indicated that the coding strategy is identical to that of other tospoviruses (6,30,37). Comparison of N protein amino acid sequences and serological relations revealed that WBNV was most closely related to PBNV in WSMoV serogroup. However, the identities of S- and M-RNA sequences with other tospoviruses within this serogroup were less than 86%. It has been proposed that any tospovirus with an N protein sequence showing less than 90% identity with that of any other tospoviruses should be considered a distinct species (7). Therefore, based on molecular characterization of S- and M-RNA of WBNV and in conjunction with previous reports (14,33), we conclude that WBNV should be confirmed as distinct species in the genus *Tospovirus*.

This work analyzed the genetic diversity of five tospoviruses collected over a period of 8 years and their phylogenetic analysis revealed that all the IYSV isolates studied clustered together and PBNV, WBNV, and CaCV segregated into two clades with no geographic structuring. A possible explanation for this might be that IYSV isolates were obtained only from onion and transmission specificity of IYSV by *Thrips tabaci* might be contributing to purifying selection. In contrast, variability among PBNV, WBNV,

TABLE 4. Percent nucleotide sequence identities of nonstructural protein (NSs) and N genes of S RNA and NSm and Gn/Gc of M RNA of Watermelon bud necrosis virus Jalna isolate (WBNV-Wm-Jal) with other tospoviruses

Virus ^a	S RNA						M RNA					
	Accession Number	Percent identity				Total length	Accession Number	Percent identity				Total length
		NSs		N				NSm		Gn/Gc		
		nt	aa	nt	aa			nt	aa	nt	aa	
WBNV	EU249351	100.0	100.0	100.0	100.0	3,405	FJ694963	100.0	100.0	100.0	100.0	4,794
PBNV-Pn	NC003619	80.6	85.6	79.9	85.1	3,057	NC003620	80.0	84.3	76.6	65.3	4,801
PBNV-Mb	AY871098	79.9	84.9	78.9	85.1	3,057	AY871097	80.1	84.6	75.9	67.7	4,815
WSMoV	NC003843	80.9	85.4	78.1	84.0	3,524	NC003841	75.0	78.5	74.0	66.3	4,880
CaCV-India	FJ011449	75.9	80.8	77.6	80.3	3,105	FJ011450	77.3	81.1	73.3	65.5	4,821
CaCV-Thi	DQ256123	75.9	80.6	77.2	80.0	3,477	NC008303	76.3	82.1	73.4	65.8	4,823
TZSV	NC010489	63.3	59.0	64.8	63.3	3,279	NC010490	69.8	69.9	66.3	54.9	4,945
MYSV	AB038343	54.5	45.2	62.6	59.1	3,232	NC008307	62.8	61.7	61.9	50.6	4,815
IYSV	AF001387	55.0	49.8	52.5	42.0	3,105	AF214014	66.6	64.8	59.5	46.8	4,838
TSWV	D13926	36.4	17.2	40.2	27.2	2,837	NC002050	45.4	33.9	43.3	25.0	4,821
INSV	NC003624	37.5	17.9	41.6	25.1	2,992	NC003616	49.0	35.1	44.9	24.8	4,972
GRSV	L12048	35.8	16.8	41.3	27.2	3,049	ND	ND	ND	ND	ND	ND
GRSV-NSm	ND	ND	ND	ND	ND	ND	AF213673	51.9	38.7	ND	ND	1,024
GRSV-Gn/GC	ND	ND	ND	ND	ND	ND	AY574055	ND	ND	43.6	24.6	3,817
TCSV-NSm	ND	ND	ND	ND	ND	ND	AF213674	51.1	38.7	ND	ND	1,024
TCSV-Gn/Gc	ND	ND	ND	ND	ND	ND	AY574054	ND	ND	43.8	24.6	3,822
CSNV	AF067068	ND	ND	46.3	28.3	783	AF213675	51.6	37.4	ND	ND	1,025
CCSV	AY867502	61.6	60.6	63.4	65.3	3,172	ND	ND	ND	ND	ND	ND
PCFV	AF080526	33.4	14.3	39.2	18.1	2,833	ND	ND	ND	ND	ND	ND
PYSV	AF013994	32.6	11.2	36.0	17.2	2,970	ND	ND	ND	ND	ND	ND
TYFRV	DQ462163	57.8	52.1	53.6	42.4	3,006	ND	ND	ND	ND	ND	ND

^a Legends: nt: nucleotide; aa: amino acid; ND: not determined; WBNV: Watermelon bud necrosis virus Jalna isolate; PBNV-Pn: *Peanut bud necrosis virus* peanut isolate; PBNV-Mb: *Peanut bud necrosis virus* mungbean isolate; WSMoV: *Watermelon silver mottle virus*; MYSV: *Melon yellow spot virus*; CaCV: *Capsicum chlorosis virus*; CCSV: *Callalilly chlorotic spot virus*; IYSV: *Iris yellow spot virus*; TSWV: *Tomato spotted wilt virus*; INSV: *Impatiens necrotic spot virus*; GRSV: *Groundnut ring spot virus*; PCFV: *Peanut chlorotic fan spot virus*; PYSV: *Peanut yellow spot virus*; TZSV: *Tomato zonate spot virus*; TCSV: *Tomato chlorotic spot virus*; CSNV: *Chrysanthemum stem necrosis virus*; and TYFRV: *Tomato yellow fruit ring virus*.

and CaCV could be due to their origin from different host species, although they all may be transmissible by the same vector *T. palmi*. The lack of geographic structuring or host specificity of different clades would suggest polyphyletic nature of virus variants that are circulating in vegetable cropping systems across the country possibly due to movement of thrips vector. However, further studies are required to understand the geographic distribution of tospoviruses and their variants and transmission efficiency of the thrips vectors in India.

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