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MUNGBEAN NECROSIS DISEASE CAUSED BY A STRAIN OF *GROUNDNUT BUD NECROSIS VIRUS*

Ho Xuan Thien[1], A.I. Bhat[2] and R.K. Jain[1]

ABSTRACT

Tospovirus infection on mungbean (up to 70%) was noticed at Indian Agricultural Research Institute experimental farm, New Delhi, in different varietal trials. Symptoms under field conditions included necrosis of plant parts such as leaves, stems, petioles, growing points, buds and pods. Symptomatic mungbean plants showed positive reaction with Groundnut bud necrosis virus (GBNV) and Watermelon silver mottle virus (WSMV) antisera in direct antigen-coated enzyme-linked immunosorbent assay. Mungbean Tospovirus was easily transmitted to cowpea cv. Pusa Komal, which could be used as a diagnostic host. The virus was also mechanically transmitted to different plant species belonging to families Fabaceae, Cucurbitaceae and Solanaceae. The nucleocapsid (N) gene of the virus was amplified, cloned and sequenced (GenBank Accession number AF515818). The sequenced region contained an ORF of 831 nucleotides that could potentially code for N protein of 276 amino acids. Comparative sequence analyses revealed that the N gene shared 97% and 99% sequence identity with GBNV at nucleotide and amino acid levels respectively, suggesting the Tospovirus isolate from mungbean to be a strain of GBNV.

Key words: *Tospovirus*, mungbean, nucleocapsid gene, *Groundnut bud necrosis virus*

INTRODUCTION

Low productivity of mungbean (0.425 ton per hectare) (Asthana and Chaturvedi 1999) in India could be attributed to biotic stresses including viruses. Among viruses, *Tospoviruses* have been recognized as emerging pathogens that can cause significant yield reduction in different crops (Bhat *et al.* 2001, 2002).

Natural infection of *Tospovirus* on mungbean (*Vigna radiata* (L.) Wilczek) and other legumes such as urdbean (*Vigna mungo* (L.) Hepper), cowpea (*V. unguiculata* (L.) Walp) and soybean (*Glycine max* (L.) Merr.) was recently recorded from Delhi (Bhat *et al*. 2001). Mungbean *Tospovirus* was serologically related to *Groundnut bud necrosis* (GBNV) and *Watermelon silver mottle* (WSMV) viruses in direct antigen-coated enzyme-linked immunosorbent assay. However, the exact taxonomic status of the virus remained unaddressed. The present study was thus undertaken to evaluate whether mungbean *Tospovirus* isolate is a strain of GBNV or a distinct virus species.

MATERIALS AND METHODS

Virus isolate

Mungbean fields at Indian Agricultural Research Institute (IARI) experimental farm, New Delhi were visited at different stages of crop growth for *Tospovirus* infection and disease incidence was recorded in Initial Varietal (IVT), Advanced Varietal (AVT) and Released Varieties (RVT) trials. Symptomatic mungbean plants showing leaf chlorosis/necrosis, stem necrosis and bud necrosis were collected and were subjected to bio- and immuno-assays.

The virus was rub-inoculated on to cowpea (*Vigna unguiculata* cv. Pusa Komal) at primary leaf stage in a glasshouse using sterilized and chilled pestle and mortar and 0.1 M phosphate buffer (pH 7.2) containing

[1] Unit of Virology, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi 110 012, India
[2] Indian Institute of Spices Research, Calicut 673 012, India

0.1% β-mercaptoethanol. For immunoassay, standard direct antigen-coated enzyme-linked immunosorbent assay (DAC-ELISA) was performed following the procedure of Clark and Bar-Joseph (1984). Polyclonal antisera directed against nucleocapsid (N) protein of GBNV and WSMV received as gifts from Drs. D.V.R. Reddy, ICRISAT, Hyderabad and S.D. Yeh, NCHU, Taichung, Taiwan, respectively, were used.

Host range

The test plant species belonging to six families were used for host range studies (Table 1). Seedlings of each test plant species were raised in earthen pots (5 per pot) and were rub-inoculated at 2-3 leaf stage. The inoculated seedlings were observed for symptom development up to 4-6 weeks and also tested for the presence of virus in DAC-ELISA.

Table 1. Host range of mungbean *Tospovirus* isolate

Plant species	No. plants infected /inoculated	Observation		
		Visual*		Serological (A405 nm)**
		Local	Systemic	
Fabaceae				
Arachis hypogaea (Groundnut)	3/9	1, 2,3	4, 7, 8, 10	0.65 (0.26)
Cajanus cajan (Redgram)	6/20	1	-	0.51 (0.12)
Cicer arietinum (Chickpea)	14/15	1, 2	7, 8, 10	0.37 (0.18)
Glycine max (Soybean)	3/10	1	-	0.53 (0.14)
Phaseolus vulgaris (French bean)	4/10	1, 2	4	0.57 (0.20)
Pisum sativum (Pea)	5/17	1, 2	7, 8, 10	2.29 (0.29)
Vigna radiata (Mungbean)	14/15	1, 2	5, 6, 7, 8, 9, 10	1.09 (0.21)
Vigna unguiculata (Cowpea)	19/19	1, 2, 3	4, 5, 6, 7, 8, 9, 10	0.53 (0.11)
Cucurbitaceae				
Citrulus vulgaris (Tinda)	6/10	1, 2, 3	-	0.26 (0.13)
Cucumis melo (Musk Melon)	0/10	-	-	0.20 (0.11)
Cucumis sativus (Cucumber)	5/11	1, 2, 3	-	0.22 (0.11)
Lagenaria siceraria (Bottle gourd)	2/10	1	-	0.38 (0.19)
Solanaceae				
Nicotiana benthamiana	7/8	1, 2, 3	4, 5, 6, 7, 10	0.71 (0.03)
Physalis floridana (Physalis)	7/7	1, 2, 3	4, 5, 6, 7, 10	0.41 (0.00)
Solanum tuberosum (Potato)	0/7	-	-	0.18 (0.17)
Malvaceae				
Abelmoschus esculentus (Bhindi)	0/9	-	-	0.11 (0.09)
Gossypium hirsutum (Cotton)	3/10	1	-	0.37 (0.16)
Compositae				
Helianthus annuus (Sunflower)	5/14	1, 2, 3	-	0.26 (0.13)
Gramineae				
Zea mays (Maize)	0/15	-	-	0.20 (0.18)

* *1= chlorotic lesion; 2= necrotic lesion; 3= ringspot; 4= leaf yellowing; 5= veinal necrosis; 6= leaf distortion; 7= bud necrosis; 8= stunting; 9= stem necrosis; 10= wilting; - = no infection*

** *Average reading of two wells each after 1 h of substrate reaction. Values in parentheses are of healthy control*

RNA isolation

Total RNA from the infected tissues (ca.10 mg) was extracted using RNeasy Kit (Qiagen Inc., Chatsworth, CA, USA) according to the manufacturer's instructions and was used as a template in the reverse transcription and polymerase chain reaction (RT-PCR).

PCR amplification

Reverse transcription and amplification (RT-PCR) were performed based on the procedure described by Pappu and his associates (1993). The template was first incubated at 76°C for 5 min and snap-cooled on wet ice for 2 min. A set of specific primers derived from the first

and last 21 bases of the coding region of the N gene of GBNV (Satyanarayana *et al*. 1996) and WSMV (Yeh and Chang 1995) were used to prime the amplification (Fig. 1). RT-PCR was performed in a single tube in an automated thermal cycler (Power Block 11, Ericomp Inc., San Diego, CA, USA) programmed for one cycle of 42°C for 45 min for cDNA synthesis and 40 cycles of amplification with 30s of denaturation at 94°C, 1 min of annealing at 56°C and 1 min of extension at 72°C followed by one cycle of final extension for 60 min at 72°C. Following PCR, amplicons were analyzed by 1% agarose gel electrophoresis in Tris-acetate EDTA (TAE) containing ethidium bromide (Sambrook and Russell 2001). The gel was observed under ultraviolet trans-illuminator and photographed on the thermal paper.

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ATGTCTAACGTTAAGCAGCTCACCGAGAAGAAAATCAAGGAACTTTTGGCTGGTGGCTCT      60
M  S  N  V  K  Q  L  T  E  K  K  I  K  E  L  L  A  G  G  S       20

GCAGATGTTGAAATTGAAACAGAAGATTCCACTCCCGGATTTAGTTTTAAAGCTTTCTAT     120
A  D  V  E  I  E  T  E  D  S  T  P  G  F  S  F  K  A  F  Y       40

GACACTAACAAAAATATTGAAATAACTTTTACAAACTGTTTGAATATTTTGAAGTGCAGG     180
D  T  N  K  N  I  E  I  T  F  T  N  C  L  N  I  L  K  C  R       60

AAGCAGATCTTTGCTGCTTGCAAAAGTGGTAAGTATGTTTTTTGTGGTAAAACTATTGTT     240
K  Q  I  F  A  A  C  K  S  G  K  Y  V  F  C  G  K  T  I  V       80

GCTACAAATACTGACGTAGGACCAGATGACTGGACCTTCAAAAGGACAGAAGCTTTCATC     300
A  T  N  T  D  V  G  P  D  D  W  T  F  K  R  T  E  A  F  I      100

AGAACCAAAATGGCTAGTATGGTTGAAAAGAGCAAGAATGATGCTGCTAAGCAGGAGATG     360
R  T  K  M  A  S  M  V  E  K  S  K  N  D  A  A  K  Q  E  M      120

TACAATAAAATAATGGAATTGCCATTAGTGGCAGCCTATGGATTAAATGTTCCTGCATCT     420
Y  N  K  I  M  E  L  P  L  V  A  A  Y  G  L  N  V  P  A  S      140

TTCGATACATGTGCTTTGAGGATGATGCTCTGCATTGGAGGTCCTCTGCCTCTCTTGTCT     480
F  D  T  C  A  L  R  M  M  L  C  I  G  G  P  L  P  L  L  S      160

AGCATGACAGGTCTGGCACCAATCATATTCCCTCTGGCTTATTATCAAAATGTGAAGAAA     540
S  M  T  G  L  A  P  I  I  F  P  L  A  Y  Y  Q  N  V  K  K      180

GAGAAATTAGGAGTTAAAAACTTTTCTACTTATGAACAGGTTTGCAAAGTAGCTAAAGTA     600
E  K  L  G  V  K  N  F  S  T  Y  E  Q  V  C  K  V  A  K  V      200

CTTTCTGCTTCACAGATTGAATTCAAAAATGAACTAGAGGAAATGTTTAAATCAGCTGTA     660
L  S  A  S  Q  I  E  F  K  N  E  L  E  E  M  F  K  S  A  V      220

AAGCTATTGAGTGAGAGTAACCCTGGAACAGCCAGCTCTATCTCACTTAAGAAATATGAT     720
K  L  L  S  E  S  N  P  G  T  A  S  S  I  S  L  K  K  Y  D      240

GAACAGGTCAAATATATGGACAAAGCTTTCAGTGCCAGTCTCTCAATGGATGATTATGGT     780
E  Q  V  K  Y  M  D  K  A  F  S  A  S  L  S  M  D  D  Y  G      260

GAACATTCTAAGAAGAAGAGTTCAAAGGCTGGTCCTTCGCTGGAATTGTAA     831
E  H  S  K  K  K  S  S  K  A  G  P  S  L  E  L  <       276
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Figure 1. Nucleotide (shown as DNA) and deduced amino acid sequences of the nucleocapsid (N) gene of mungbean *Tospovirus* isolate. Primer sequences used for the amplification are underlined

Cloning and sequencing

The PCR products were ligated into PGEM-T Easy vector (Promega, Madison, WI, USA) and competent *Escherichia coli* cells (strain DH 5α) were transformed by following standard molecular biology procedures (Sambrook and Russell 2001). Selected recombinant clones with an insert of N gene (~800 bp) of mungbean *Tospovirus* isolate were sequenced at the automatic DNA sequencing facility at Department of Biochemistry, South Campus, University of Delhi, Delhi, India. Sequence data were initially compiled using SeqAid II™ version

3.60 (Rhoads and Roufa 1985). Multiple sequence alignments were generated using CLUSTAL W (Thompson *et al*. 1994). Sequence phylograms were constructed using TREEVIEW software (Bootstrap analysis with 1000 replicates) (Page 1996). N gene sequences of other known *Tospoviruses* (Table 2) were collected from GenBank (Benson *et al*. 1999). Both nucleotide and amino acid sequences of N gene of different *Tospovirus* species were compared and the corresponding phylogenetic trees were generated.

Table 2. Source of nucleocapsid (N) gene nucleotide and amino acid sequences used for comparison

Virus	Designation used	GenBank accession number
TSWV serogroup		
Groundnut ringspot virus	GRSV	S54327
Tomato chlorotic spot virus	TCSV	AF282982
Tomato spotted wilt virus	TSWV	AF048716
WSMV serogroup		
Groundnut bud necrosis virus	GBNV	U27809
Groundnut bud necrosis virus	**GBNV-Mb**	**AF515818 (This study)**
Watermelon bud necrosis virus	WBNV	AF045067
Watermelon silver mottle virus	WSMV	U78734
Serologically unrelated		
Chrysanthemum stem necrosis virus	CNSV	AF067068
Groundnut chlorotic fan-spot virus	GCFV	AF080526
Groundnut yellow spot virus	GYSV	AF013994
Impatiens necrotic spot virus	INSV	D00914
Iris yellow spot virus	IYSV	AF001387
Melon yellow spot virus	MYSV	AB024332
Physalis severe mottle virus	PSMV	AF067151
Zucchini lethal chlorosis virus	ZLCV	AF067069

RESULTS AND DISCUSSION

Under field conditions, symptoms on mungbean included necrosis of all plant parts including leaves, stems, petioles, growing points, buds and pods. Early infected plants were severely stunted with reduced internodal length and many axillary shoots. Maximum *Tospovirus* infection was observed in RVT followed by AVT and IVT. In RVT, disease incidence was maximum in Pusa 2072 (71%), followed by Pusa Bold (63%), Pusa 105 (46%), and Pusa Vishal (20%). Incidence of *Tospovirus* infection in IVT and AVT ranged from 14-38% and 19-44%, respectively.

The *Tospovirus* from symptomatic mungbean plants was mechanically transmitted on to cowpea (*Vigna unguiculata* cv. Pusa Komal), a diagnostic assay host for *Tospovirus* (Bhat *et al*. 2001). Both localized as well as systemic infections on cowpea were observed. In localized infection, inoculated leaves showed chlorotic lesions three days after inoculation, which turned necrotic two days later. This was followed by veinal necrosis of the inoculated leaves, which finally became chlorotic or pale yellow in color before senescence. Newly emerging leaves showed systemic infection symptoms which consisted mild mosaic, concentric chlorotic ringspots and necrotic spots. Necrosis affected plants reacted with the polyclonal antisera directed against nucleocapsid protein of GBNV (A_{405} = 0.75) and WSMV (A_{405} = 0.40), suggesting that the mungbean *Tospovirus* belongs to WSMV serogroup (Moyer 1999).

The virus was easily sap-transmissible to the members of *Fabaceae* and *Solanaceae* and both localized and systemic infection was observed. Symptoms exhibited by different hosts included chlorotic/necrotic lesions and ringspots, followed by leaf yellowing, veinal necrosis, leaf deformation. Other symptoms were stunting, bud necrosis, stem necrosis and wilting. Only localized infection (chlorosis, necrosis, ringspot) was observed in plant species belonging to *Cucurbitaceae*,

Malvaceae and *Compositae* (Table 1). ELISA reactions were consistent with visual observation. Intensity of ELISA reactions varied with plant species. Members of *Fabaceae* and *Solanaceae* showed higher absorbance values (0.37-2.29), whereas those of *Cucurbitaceae*, *Compositae* and *Malvaceae* showed lower absorbance values (0.22-0.38). These host species could serve as potential reservoirs for mungbean *Tospovirus*. Host range studies suggested that the mungbean *Tospovirus* was closely related to GBNV.

The nucleotide and the translated amino acid sequences of the N gene of the mungbean *Tospovirus* revealed that the sequenced region contained a single open reading frame (ORF) of 831 nucleotides that could potentially code for a protein of 276 amino acids (Figure 1). The nucleotide sequence data were submitted to the GenBank under accession number AF515818.

The N gene sequence of mungbean *Tospovirus* isolate was compared with corresponding genes from other recognized *Tospovirus* species at nucleotide and amino acid levels. Cluster dendrograms revealed that mungbean *Tospovirus* isolate was most closely related to GBNV, forming one cluster (Figure 2). Comparative sequence analyses also revealed that mungbean *Tospovirus* isolate shared maximum sequence identity with GBNV at nucleotide (97%) as well as amino acid (99%) levels (Table 3). In contrast, 79-81% nucleotide sequence identity was observed with N genes of other members of WSMV serogroup such as WBNV and WSMV. Nucleotide identities with eleven other *Tospovirus* species were in the range of 41-64%. Similarly, comparison of amino acid sequences of N genes revealed that the N gene of mungbean *Tospovirus* shared 82-84% sequence identity with other members of WSMV serogroup, in contrast to 16-58% identity with other *Tospoviruses* (Table 3). Isolates in the *Tospovirus* genus with greater than 90% N gene sequence identity are classified as strains of the same virus (Moyer 1999). Since the biological characteristics and N gene sequences of mungbean *Tospovirus* were similar to GBNV, it is proposed that the mungbean *Tospovirus* should be regarded as a strain of GBNV belonging to WSMV serogroup, henceforth designated as GBNV-Mb. This is the first report of the presence of GBNV on mungbean under natural condition.

Considering that GBNV has long been endemic to India on groundnut and has a wide host range (Ghanekar *et al.* 1979), it is possible that GBNV has spread from groundnut to mungbean. Recently, soybean was also identified as natural host of GBNV (Bhat *et al*. 2002) and GBNV-Sb shared 97% amino acids sequence identity with GBNV-Mb. Thus, natural infection of GBNV on other crops should also be monitored.

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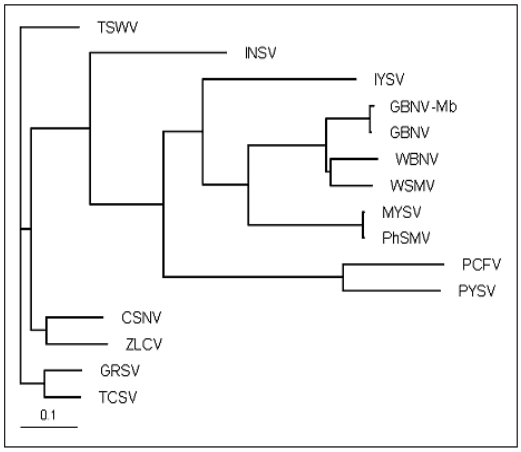

Figure 2. Cluster dendrogram illustrating phylogenetic relationships based on the multiple alignments of the nucleocapsid (N) protein amino acid sequences of 14 known *Tospovirus* species and mungbean *Tospovirus* isolate (GBNV-Mb). Sequences for comparisons were obtained from GenBank and designation given to each of the isolates and their GenBank accession numbers are given in Table 2

Table 3. Percent nucleotide sequence (above the diagonal line) and amino acid sequence (below the diagonal line) identity of nucleocapsid (N) gene between mungbean *Tospovirus* (GBNV-Mb) and other *Tospovirus* isolates

	TSWV	GRSV	TCSV	WSMV	WBNV	GBNV	GBNV-Mb	PSMV	IYSV	MYSV	ZLCV	CSNV	GCFV	INSV	GYSV
TSWV	**100**	77	77	44	44	44	44	43	46	43	73	75	42	57	43
GRSV	77	**100**	82	45	446	47	47	46	48	46	74	72	45	58	45
TCSV	79	84	**100**	44	45	45	48	45	46	45	74	73	44	58	42
WSMV	27	27	27	**100**	**79**	**80**	**80**	64	53	63	46	46	43	45	41
WBNV	25	26	25	**84**	**100**	**81**	**81**	63	52	63	46	46	42	44	44
GBNV	27	26	27	**84**	**82**	**100**	**97**	64	53	64	45	45	41	44	41
GBNV-Mb	26	25	26	**84**	**82**	**99**	**100**	64	53	64	45	45	41	43	42
PSMV	26	27	24	56	57	58	58	**100**	58	97	47	46	43	44	41
IYSV	31	28	29	38	38	42	41	44	**100**	58	47	47	42	45	42
MYSV	26	27	24	56	57	58	58	99	44	**100**	46	46	43	43	41
ZLCV	74	75	74	25	25	26	25	24	28	24	**100**	77	40	57	44
CSNV	75	73	74	25	24	25	25	26	29	26	79	**100**	44	58	45
GCFV	15	16	14	14	17	18	17	16	15	16	14	16	**100**	45	67
INSV	53	52	52	26	26	26	26	24	25	24	51	53	15	**100**	44
GYSV	16	18	12	18	16	16	16	17	17	17	13	13	65	17	**100**

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SUMMARY IN VIETNAMESE

Groundnut bud necrosis tospovirus gây bệnh hoại tử trên đậu xanh

Các ruộng thí nghiệm giống đậu xanh tại Viện Nghiên Cứu Nông Nghiệp Ấn Độ, New Delhi, bị nhiễm bệnh do *Tospovirus* gây ra với tỉ lệ bệnh lên đến 70%. Các triệu chứng bệnh ngoài đồng thường là hoại tử của các bộ phận như lá, thân, cuống lá, các điểm sinh trưởng, chồi và trái. Các mẫu bệnh mang triệu chứng đã cho phản ứng dương tính với các kháng huyết thanh của *Groundnut bud necrosis virus* (GBNV) và *Watermelon silver mottle virus* (WSMV) trong phản ứng ELISA trực tiếp (DAC ELISA). Chủng *Tospovirus* đậu xanh được truyền dễ dàng sang đậu bò giống Pusa Komal và giống này có thể được sử dụng làm thực vật chẩn đoán virus này. Virus cũng được truyền sang các loài thực vật khác thuộc các họ *Fabaceae*, *Cucurbitaceae* và *Solanaceae*. Gene cấu tạo vỏ protein của virus (N) đã được nhân bản và đọc mã (mã số truy cập GenBank AF515818). Đoạn gene được giải chứa một ORF dài 831 nucleotide và có thể mã hoá cho một protein vỏ virus dài 276 amino acid. Các phân tích so sánh chuỗi cho thấy đoạn gene cấu tạo vỏ virus này tương đồng với gene N của GBNV 97% ở mức độ nucleotide và 99% ở mức độ amino acid. Điều này cho thấy chủng *Tospovirus* đậu xanh của New Delhi là một nòi của GBNV.