Omonrice 10: 60-66 (2002)

Serological Studies and Polyclonal Antiserum Production against the Nucleocapsid Protein of Mungbean *Tospovirus* from New Delhi

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

ABSTRACT

Natural infection of *Tospovirus* on mungbean was recorded in New Delhi, India. Symptomatic mungbean samples reacted positively with *Groundnut bud necrosis virus* (GBNV) and *Watermelon silver mottle virus* (WSMV) antisera in direct antigen-coated enzyme-linked immunosorbent assay. The virus was maintained in cowpea which served as a propagation host. The nucleocapsid (N) protein of the virus was purified. The polyclonal antiserum against the N protein with homologous titer 1:512 was produced. The antiserum could detect *Tospovirus* isolates from cowpea, potato and tomato colleted from different locations.

Key words: mungbean, *Tospovirus*, DAC-ELISA, antiserum production

INTRODUCTION

Tospoviruses, ascribed to the family '*Bunyaviridae'* (Francki *et al*., 1991; van Regenmortel *et al*., 2000), are serious viral pathogens affecting many important agricultural crops (Moyer, 1999). Annual losses due to tospoviruses are estimated at over US$ 1 billion (Prins and Goldbach, 1998). In Indian subcontinent, tospoviruses are fast spreading and expected to become a major constraint in realizing the full yield potential of various crops in this millennium.

At present, meagre information is available on the naturally occurring tospoviruses in India as they have received sporadic attention. So far, three distinct tospoviruses, *Groundnut bud necrosis* (GBNV) (Reddy *et al*., 1992) and *Groundnut yellow spot* (GYSV) (Satyanarayana *et al*., 1998) from groundnut, and *Watermelon bud necrosis* (WBNV) (Jain *et al*., 1998) from watermelon, have been identified. Besides groundnut and watermelon, *Tospovirus* infections have also been recorded from a large number of vegetable crops (Prasada Rao *et al*., 1980, 1985, 1987; Thakur *et al*., 1998), but their exact identification is lacking. Recently, *Tospovirus* infections on different grain legume crops, e.g. urdbean, cowpea and soybean from Delhi have been identified by bio- and immuno-assays (Bhat *et al*., 2001). The precise aim of the present investigation was thus to evaluate the *Tospovirus* isolate from mungbean at serological level and to produce polyclonal antiserum against the viral nucleocapsid (*N*) protein.

MATERIALS AND METHODS

Mungbean plants showing leaf chlorosis/necrosis were collected (Figure 1). Infected areas on the leaves were harvested, weighed and macerated using sterilized and chilled pestle and mortar adding 0.01 M phosphate buffer (pH 7.2, 1:1, w/v) containing 0.1% β-mercaptoethanol. The extracted sap was used as standard extract for inoculation. Cowpea (*Vigna unguiculata* cv. Pusa Komal) leaves (2-3 leaf stage) were dusted with celite (diatomaceous earth), which served as an abrasive. The standard extract was then applied directly by rubbing gently onto the leaves with the chilled mortar to exert uniform pressure. The seedlings were briefly washed with water and kept in an insect-proof

[1] Cuu Long Delta Rice Research Institute, O Mon, Cantho, Vietnam

[2] Advanced Center for Plant Virology, Division of Plant Pathology Indian Agricultural Research Institute, New Delhi 110 012, India

glasshouse for observation. Local lesions developed at 4-5 days post-inoculation were used for further inoculation to establish pure virus culture. The virus culture was then maintained in insect-proof glasshouse. The virus isolate was also preserved at –80°C for purification.

Figure 1: Chlorotic and necrotic lesions on mungbean (*Vigna radiata*) induced by mungbean *Tospovirus* isolate.

Immunoassay

Standard direct antigen-coated enzyme-linked immunosorbent assay (DAC-ELISA) (Clark and Bar-Joseph, 1984) test was performed to detect the association of *Tospovirus* with mungbean samples. Polyclonal antisera directed against nucleocapsid (N) protein of different *Tospovirus* species were used to check the serological affinity of the mungbean *Tospovirus* isolate. Antisera were collected from different sources (Table 1).

Table 1: Antisera used and their sources

Antisera	Source
Groundnut bud necrosis virus (GBNV)	Dr. D.V.R. Reddy, ICRISAT, Hyderabad, India
Groundnut chlorotic fan-spot virus (GCFV)	Dr. A.C. de Avila, EMBRAPA, Brazil
Groundnut ringspot virus (GRSV)	"
Impatiens necrotic spot virus (INSV)	"
Tomato chlorotic spot virus (TCSV)	"
Tomato spotted wilt virus (TSWV)	"
Watermelon silver mottle virus (WSMV)	Dr. S.D. Yeh, NCHU, Taichung, Taiwan

Polyclonal antiserum production

The N protein of the virus was purified from symptomatic cowpea leaves by following the protocol of Satyanarayana and his associates (1996) (Box 1). Freshly purified N protein preparation was used for the production of polyclonal antibodies. The viral N protein suspension emulsified with Freud's incomplete adjuvant (1:1, v/v) was injected intramuscularly into New Zealand white albino rabbit. Four such injections were given at weekly interval. The test bleed (approximately 1 ml) was taken one week after the last

injection. The blood was allowed to coagulate at room temperature for 1 h and then kept at 4°C overnight. The clean antiserum was decanted and clarified at 6,600 g for 5 min. The antiserum (supernatant) was mixed with sterile glycerol (1:1 ratio, v/v) and stored at –20°C in small aliquots (200 µl). The antiserum titer was determined by DAC-ELISA test. The first bleed (approximately 10 ml) was taken at 2 weeks after the last injection and antiserum titer was determined.

Box 1. Virus purification protocol of Satyanarayana and his associates (1996) – Flow chart

Symptomatic cowpea leaf tissue

↓ *homogenized with 4 volumes (w/v) of 0.1 M potassium phosphate buffer (pH 7.6) containing 0.01 M Na_2SO_3 and 0.01 M Na-EDTA (pH 8.0)*

Homogenate

↓ *filtered through muslin cloth and centrifuged at 800 g for 10 min*

Supernatant

↓ *centrifuged at 50,000 g for 40 min*

Pellets

↓ *suspended in 50 ml 0.01 M potassium phosphate buffer (pH 7.6) containing 0.01 M Na_2SO_3 and 0.01 M Na-EDTA [PSE buffer] and stirred for 30 min prior to centrifugation at 6,000 g for 10 min*

Supernatant

↓ *layered onto 15 ml cushion of 30% sucrose dissolved in PSE buffer and centrifuged at 100,000 g for 75 min*

Pellets

↓ *re-suspended in 1 ml PSE buffer*

Supernatant

↓ *layered onto 20-40% continuous sucrose gradient prepared in PSE buffer and centrifuged at 140,000 g for 2.5 h*

Three light scattering zones

↓ *diluted in PSE buffer and pelletized at 100,000 g for 2 h*

Virus nucleocapsid protein pellet

↓ *dissolved in 500 µl 0.01 M potassium phosphate buffer (pH 7.0)*

RESULTS AND DISCUSSION

The virus was easily mechanically transmitted from field-infected mungbean onto cowpea (cv. Pusa Komal), a diagnostic host for *Tospovirus* infection. Both localized as well as systemic infections on cowpea were observed and thus reconfirming the *Tospovirus* infection on mungbean plants (Figure 2).

Figure 2: Different types of symptoms of tospovirus infection on cowpea (*Vigna unguiculata*) cv. Pusa Komal induced by mungbean *Tospovirus* isolate.

Immunoassay

Serodiagnosis is currently the method of choice for detection of tospoviruses in plants and thrips (German *et al*., 1992; Mumford *et al*., 1996). On the basis of nucleocapsid (N) protein serology, tospoviruses have been classified into TSWV and WSMV serogroups and a group containing serologically unrelated viruses (Moyer, 1999). Of the seven polyclonal antisera directed against nucleocapsid (N) protein of different tospoviruses tested, *Groundnut bud necrosis virus* (GBNV) and *Watermelon silver mottle virus* (WSMV) antisera showed positive reaction with mungbean *Tospovirus* isolate in direct antigen-coated enzyme-linked immunosorbent assay (DAC ELISA) (Table 2), suggesting the association of a *Tospovirus* antigenically related to GBNV/WSMV and belonged to WSMV serogroup. GBNV antiserum reacted stronger than WSMV antiserum. No reaction was observed with antisera of other five tospoviruses tested.

Different plant parts from *Tospovirus* affected mungbean plants were also checked for the presence of virus by DAC-ELISA. Plant parts such as leaf, stem, bud and pod reacted positively with GBNV antiserum (Table 3). The maximum absorbance (0.98-1.01) was observed with leaf samples.

Table 2: Serological reaction of mungbean *Tospovirus* isolate with different *Tospovirus* antisera in direct antigen-coated enzyme-linked immunosorbent assay

Antiserum against	Absorption values at 405 nm*
Tomato spotted wilt virus serogroup	
Groundnut ringspot virus (GRSV)	0.20 (0.15)
Tomato chlorotic spot virus (TCSV)	0.19 (0.13)
Tomato spotted wilt virus (TSWV)	0.16 (0.12)
Watermelon silver mottle virus serogroup	
Groundnut bud necrosis virus (GBNV)	0.75 (0.26)
Watermelon silver mottle virus (WSMV)	0.40 (0.17)
Serologically unrelated	
Groundnut chlorotic fan-spot virus (GCFV)	0.23 (0.21)
Impatiens necrotic spot virus (INSV)	0.14 (0.15)

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

Table 3: Serological reaction of symptomatic mungbean plant parts with *Groundnut bud necrosis virus* antiserum in direct antigen-coated enzyme-linked immunosorbent assay

Symptom type	Absorption values at 405 nm*
Leaf chlorosis	1.01
Leaf necrosis	0.98
Stem necrosis	0.71
Bud necrosis	0.72
Pod necrosis	0.69
Healthy control	0.32

** Average reading of two wells each after 1 h of substrate reaction*

Nucleocapsid purification and polyclonal antiserum production

The N protein of mungbean *Tospovirus* was purified and the polyclonal antiserum against it was raised. The antiserum titer was 1:512 in DAC-ELISA (Table 4). The antiserum not only reacted with the homologous antigen, but also with *Tospovirus* isolates from cowpea, potato and tomato collected from different locations in India. This suggests that mungbean, cowpea, potato and tomato *Tospovirus* isolates are antigenically related and it would be difficult to segregate them by immuno-assays (Table 5).

Although *Tospovirus* identification based on N protein serology and sequence homology of N gene are consistent, yet sequence homology is accepted as a measure of overall relatedness (Moyer, 1999). Isolates in the *Tospovirus* genus with greater than 90% nucleocapsid sequence homology are considered as strains of the same virus (Goldbach and Kuo, 1996; Moyer, 1999). In view of this, further attempts should be made to clone and sequence the N gene of mungbean *Tospovirus* in order to identify the virus up to species level. Further, identification of nucleocapsid gene would facilitate its use as transgene for conferring resistance in mungbean as nucleocapsid gene-mediated transgenic resistance against Tospoviruses has been well documented in different crops (Pappu, 1997; Prins and Goldbach, 1998; Herrero *et al*., 2000).

Table 4: Homologous titer determination of polyclonal antiserum against nucleocapsid (N) protein of mungbean *Tospovirus* isolate in direct antigen-coated enzyme-linked immunosorbent assay.

Titer	Absorption values at 405 nm*
1:256	1.370 (0.110)
1:512	1.020 (0.053)
1:1024	0.508 (0.006)
1:2048	0.323 (0.040)
1:4096	0.140 (0.010)
1:8192	0.076 (0.001)

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

Table 5: Serological relationship of different *Tospovirus* isolates with mungbean *Tospovirus* isolate

Tospovirus isolate	Place of collection	Absorption values at 405 nm*
Mungbean (*Vigna radiata*)	Delhi	0.700
Tomato (*Lycopersicon esculentum)*	Kerala	0.320
Potato (*Solanum tuberosum)*	Gujarat	0.740
Cowpea (*Vigna unguiculata)*	Kerala	0.490
Healthy control (Cowpea)		0.070

Average reading of two wells each after 1 h of substrate reaction.

REFERENCES

Bhat AI, RK Jain, A Varma, N Chandra, and SK Lal, (2001). *Tospovirus*(es) infecting grain legumes in Delhi – their identification by serology and nucleic acid hybridization. *Indian Phytopathol*. **54** (1): 112-116.

Clark M.F. and Bar-Joseph (1984). Enzyme immunosorbent assays in plant virology. In: *Methods In Virology*, Vol VII (Eds. Maramorsch, K. and Koprowski, H.). pp. 51-85. Academic Press, New York.

Francki RIB, CM Fauquet, DL Knudson, and F Brown, eds. (1991). *Classification and Nomenclature of viruses. Rep. ICTV Arch. Virol., 5th*, Suppl. 2. 450 pp. Springer Verlag, New York.

German TL., DE Ullman, and JW Moyer (1992). Tospoviruses: Diagnosis, molecular biology, phylogeny and vector relationships. *Annu. Rev. Phythopathol*. **30**: 315-348.

Goldbach R and G Kuo (1996). Tospoviruses and thrips. *Acta Horticulturae* **431**: 16-43.

Herrero, S., Culbreath, A.K., Csinos, A.S., Pappu, H.R., Rufty, R.C. and Daub, M.E. (2000). Nucleocapsid gene-mediated transgenic resistance provides protection against *Tomato spotted wilt virus* epidemics in the field. *Phytopathol*. **90**: 139-147.

Jain RK., HR Pappu, SS Pappu, MK Reddy and A Vani. (1998). *Watermelon bud necrosis tospovirus* is a distinct virus species belonging to serogroup IV. *Archives of Virology* **143**: 1637-1644.

Moyer JW (1999). Tospoviruses (*Bunyaviridae*). In: *Encyclopedia of Virology* (Eds. Granoff, A. and Webster, R.G.), pp. 1803-1807. Academic Press, San Diego.

Mumford RA., I Barker and KR Wood (1996b). The biology of tospoviruses. *Annals of Applied Biology* **128**: 159-183.

Pappu HR (1997). Managing tospoviruses through biotechnology: progress and prospects. *Biotechnology and Development Monitor*, No. 32, p. 1417.

Prasada Rao RDVJ, SK Chakraborthy, R Rajeswari and AS Reddy. (1987). Occurrence of *Tomato spotted wilt virus* on cowpea (*Vigna unguiculata* (L.) Walp.), chili (*Capsicum annuum* L.) and brinjal (*Solanum melongena* L.). *Indian J. Plant Prot*. **15**: 117-119.

Prasada Rao RDVJ, N Izuna, V Ragunathan and NC Joshi. (1980). Occurrence of *Tomato spotted wilt virus* on tomato in Andhra Pradesh. *Indian Phytopathol*. **33**: 436-439.

Prasada Rao RDVJ, R Rajeswari, MVB Rao, V Ragunathan and NC Joshi, (1985). Spotted wilt of pea in India. *India Phytopathol*. **38**: 90-93.

Prins M and R Goldbach. (1998). The emerging problem of *Tospovirus* infection and nonconventional methods of control. *Trends in Microbiology* **6**: 31-35.

Prins M and R Goldbach. (1998). The emerging problem of *Tospovirus* infection and nonconventional methods of control. *Trends in Microbiology* **6**: 31-35.

Reddy DVR, AS Ratna, MR Sudarshana, F Poul, and I Kirankumar, (1992). Serological relationships and purification of bud necrosis virus, a tospovirus occurring in peanut (*Arachis hypogaea* L.) in India. *Ann. Appl. Biol*. **120**: 279-286.

Satyanarayana T, S Gowda, K Lakshminarayana Reddy, SE Mitchell, DE Dawson, and DVR Reddy, (1998). *Peanut yellow spot virus* is a member of serogroup V of *Tospovirus* genus based on small (S) RNA sequence and organization. *Arch. Virol*. **143**: 353-364.

Satyanarayana T, SE Michell, DVR Reddy, S Brown, S Kresovich, R Jarret, RA Naidu, and JW Demski. (1996). *Peanut bud necrosis tospovirus* S-RNA: complete nucleotide sequence, genome organization and homology to other tospoviruses. *Arch. Virol*. **141**: 85-98.

Thakur MP, KP Verma, and KC Agrawal. (1998). Characterization and management of bud blight disease of soybean in India. **44**: 87-92.

van Regenmortel MHV, CM Fauquet, DHL Bishop, EB Carstens, MK Ester, SM Leman, J Maniloff, MA Mayo, DJ McGeoch, DJ Pringle, and RB Wickner, (2000). *Virus Taxonomy*. *Classification And Nomenclature Of Viruses*. Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego.

SUMMARY IN VIETNAMESE

Nghiên cứu huyết thanh học và sản xuất kháng thể đa dòng kháng nucleocapsid protein của chủng Tospovirus trên đậu xanh tại New Delhi

Tospovirus được ghi nhận xâm nhiễm trong tự nhiên trên cây đậu xanh tại New Delhi, Ấn Độ. Bằng kỹ thuật DAC-ELISA, các mẫu bệnh mang triệu chứng đã cho phản ứng dương tính với các kháng thể kháng *Groundnut bud necrosis virus* (GBNV) và *Watermelon silver mottle virus* (WSMV). Đậu bò được dùng làm ký chủ nhân mật số của virus này. *Nucleocapsid* (N) protein của virus đã được tinh chế. Kháng thể đa dòng kháng N protein có độ pha loãng chuẩn là 1:512 đã được sản xuất. Kháng thể đa dòng này có thể dùng để phát hiện các chủng *Tospovirus* trên đậu bò, khoai tây và cà chua thu thập từ các vùng khác nhau.