Monoclonal antibodies to the recombinant nucleocapsid protein of a groundnut bud necrosis virus infecting tomato in Karnataka and their use in profiling the epitopes of Indian tospovirus isolates

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A tospovirus infecting tomato in the fields of Karnataka, India, was propagated in greenhouse-grown *Nicotiana benthamiana* plants by mechanical inoculation. The viral RNA was extracted from purified virus and used for amplification of *N* and *NSs* genes by RT–PCR using appropriate primers. The *N* and *NSs* PCR products were cloned into a pRSET vector and sequenced. The *N* gene of tomato tospovirus showed 98% identity with that of *Groundnut bud necrosis virus* (GBNV), alternate name *Peanut bud necrosis virus* (PBNV). Interestingly, though the virus was isolated from tomato plants, it showed only 82% identity with the *N* gene of GBNV-To isolate from Taiwan. The *NSs* gene of the virus under study showed 98% identity with GBNV. These results suggest that the tomato tospovirus in Karnataka is a strain of GBNV and is henceforth designated as GBNV-To (K). The *N* gene was overexpressed in *Escherichia coli* and the recombinant N protein was purified using Ni–NTA agarose affinity chromatography. The purified protein was used for the generation of poly- and monoclonal antibodies (mAbs). The polyclonal antiserum thus obtained had a dilution end-point >1 : 32,000 and nine unique mAbs were also obtained. These mAbs were used for epitope profiling of the tospovirus isolates from South India and for developing detection methods. The results showed that there are distinct GBNV strains in South India. A simple dot-blot assay was developed for detection of GBNV from infected field samples.

Keywords: *Groundnut bud necrosis virus*, monoclonal antibodies, tomato, tospovirus.

IN recent years, thrips and tospoviruses have become a serious problem in various Leguminosae, Solanaceae and Cucurbitaceae crops all over India. The genus *Tospovirus*, family Bunyaviridae, includes viruses with enveloped, quasi-spherical particles of 80–120 nm diameter and a

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tripartite, single-stranded, ambi-sense RNA genome. The RNAs are named L (large), M (medium) and S (small), and have a size of approximately 8.9, 4.8 and 2.9 kb respectively. They are bound by a nucleocapsid (N) protein[1]. The L-RNA codes for the RNA-dependent RNA polymerase and is translated from the viral complementary sense RNA (VC). The M-RNA encodes a nonstructural (NSm) protein in the viral (V) sense and the precursor for the glycoproteins G1 and G2 in the VC sense. The S-RNA encodes NSs protein in the V sense and N protein in the VC sense.

Several tospoviruses have been reported in tomato (*Lycopersicon esculentum*), chilli (*Capsicum anuum*), cowpea (*Vigna unguiculata*), brinjal (*Solanum melongena*), pea (*Pisum sativum*), peanut/groundnut (*Arachis hypogaea*), potato (*Solanum tuberosum*), soyabean (*Glycine max*) and watermelon (*Citrullus vulgaris*) from India[2–9]. The ability of tospoviruses to cause severe losses on a broad range of crops, places them amongst the most economically important plant pathogens in the world at present.

Among the tospoviruses reported from India, *Groundnut bud necrosis virus* (GBNV), also called *Peanut bud necrosis virus* (PBNV), has been characterized[5,10] and the three L, M and S-RNA genomes have been cloned and sequenced[11–13]. The *Peanut yellow spot virus* (PYSV) S-RNA has been completely sequenced and the sequence comparisons demonstrated that it is a distinct species, different from GBNV[14]. The *Watermelon bud necrosis virus* (WBNV) *N* gene has been cloned and sequenced, and its sequence was found to be different from those of PYSV and GBNV[15]. All these viruses are closely related and belong to serogroup IV of tospoviruses[7,8,10,15,16]. More recently, the *N* and *NSm* genes of several isolates from various Leguminosae and Solanaceae sources from different locations in India have been sequenced, and the results show that they are all strains of GBNV[4,8,17,18].

In the present study, a tospovirus infecting tomato in Karnataka, India was purified and by sequence analysis of the *N* and *NSs* genes shown to be a strain of GBNV. With a view to develop sensitive detection methods with minimal host protein background, the recombinant N protein (rNP) was purified and used in the production of polyclonal antibodies (pAbs) and monoclonal antibodies (mAbs). The results presented demonstrate their use in tospovirus diagnosis.

Sixteen virus isolates from infected tomato (*L. esculentum*), chilli (*C. anuum*), watermelon (*C. lanatus*), cowpea (*V. unguiculata*), brinjal (*S. melongena*), pumpkin (*Cucurbita pepo*), cucumber (*Cucumis sativus*), gherkin (*Cucumis anguria*), *Acanthospermum hispidum*, lady's finger (*Abelmoschus esculentus*), muskmelon (*Cucumis melo*) used in this study were collected from different places in Maharashtra, Andhra Pradesh, Karnataka and Tamil Nadu, based on symptoms like chlorotic and necrotic ring spots on leaves, necrosis of the growing tip, tip wilting and yellowing symptoms induced by tospoviruses. These samples were confirmed to be tospovirus isolates by ELISA using polyclonal antibodies to GBNV. One of the tomato tospovirus isolates collected from the tomato-growing fields around Bangalore was maintained in the greenhouse by mechanical inoculation to *Nicotiana benthamiana* plants. The sample (1 : 2 w/v) was ground in 50 mM potassium phosphate buffer, pH 7.5 containing 0.02 M mercaptoethanol using mortar and pestle, and used to inoculate on celite (600 nm mesh size)-dusted 15-days-old *N. benthamiana* seedlings.

Tospovirus-infected *N. benthamiana* leaves were used routinely for purification of the virus as described earlier[10]. The purity of the virus was checked by SDS–PAGE and authenticated by Western blot analysis using GBNV antibodies (DSMZ, Germany).

The viral RNA was extracted by phenol chloroform method[19] and the size of the RNAs was estimated by formaldehyde agarose gel electrophoresis[20]. Primers corresponding to the N and C termini of the *N* gene were designed based on available sequence information of GBNV[12] as follows:

Sense primer: 5′CGCGCTAGCCATATGATGTCTAA/CCGTC/TAAGCAA/GCTC3′

Antisense primer: 5′CAGCGGATCCTTACAA/CTTCCAA/GA/CGAAG/T3′.

The *N* gene was amplified by RT–PCR using the following protocol. Viral RNA 2 µl (1–5 µg) and 1 µl (10 pmol) of antisense primer were added to 6 µl of diethyl pyrocarbonate (DEPC)-treated distilled water and heated for 2 min at 90°C. To this, 11 µl of reverse transcription mix (2 µl of 5× M-MLV buffer, 1 µl of DTT, 1 µl of 10 mM dNTPs, 0.5 µl of RNAse inhibitor and 0.5 µl of reverse transcriptase) and 6 µl of autoclaved milli-Q water were added. This reaction mix was incubated for 1–1.5 h at 42°C. Three microlitres of RT reaction sample was added to the PCR reaction mix (2.5 µl of 10× Thermopol buffer, 1 µl (5 pmol) each of sense and antisense primers, 0.5 µl (5 units) of Deep vent polymerase and made up to 25 µl with distilled water). The mixture was subjected to thermal cycling: 94°C for 4 min, followed by 30 cycles of 94°C for 30 s, 52°C for 1 min, 72°C for 1 min and final elongation at 72°C for 10 min. The amplified PCR product of the *N* gene was cloned into pRSET A vector at *Nhe*I/*Bam*HI sites. The *N* gene was sequenced completely and compared with those of other tospovirus isolates using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Similarly, the *NSs* gene was amplified by RT–PCR using sense and antisense primers corresponding to the N and C termini of the GBNV NSs protein[12]. The primers are:

Sense primer: 5′CTAGCTAGCCATATGTCA/TACC/TGCAAA/GGAA/GTGC3′

Antisense primer: 5′CCCTCGAGGGTTAC/TTCTGG/CC/TTTCACAAC/TA/GAAA/GTG3′.

The amplified PCR product of the *NSs* gene was cloned into the pRSET C vector at the *Nhe*I/*Xho*I site and sequenced.

BL21 (DE3) pLysS *E. coli* cells were transformed with the pRSET A N clone. A single colony was inoculated to a 25 ml Luria Broth culture and grown overnight. This overnight culture was used for inoculating 500 ml terrific broth[20] culture containing 50 µg/ml ampicillin. The overexpression of N protein was induced by adding 0.3 mM IPTG when the cell density had reached A_{600} of 0.6. The induction was continued for 4 h and the cells were harvested. The cell pellet was resuspended in 20 mM Tris buffer containing 300 mM NaCl and 10 mM imidazole, pH 8 and disrupted by pulses of sonication (amplitude 70) using a macroprobe (Vibra cell sonicator, Sonics and Materials, Inc, USA) for 10 min in an ice-bath. The sonicated slurry was subjected to centrifugation at 10,000 rpm for 10 min. The rNP was purified from the soluble fraction using Ni–NTA agarose affinity chromatography (Quiagen Inc, Chatsworth, CA), according to the manufacturer's protocol.

Purified rNP was used as an antigen to produce polyclonal antibodies in a 12-week-old New Zealand white rabbit. Pre-immune serum was collected one week before immunization. The purified rNP (250 µg) was emulsified in 250 µl of Freund's complete adjuvant for the first injection and this was followed by two injections of 150 µg protein in Freund's incomplete adjuvant. The injections were given intramuscularly at 21 days interval. Ten days after the second booster, the rabbit was bled and checked for the antibody titre. The serum titre and specificity were determined by direct antigen coating–ELISA (DAC–ELISA)[21] using purified rNP (1 µg/100 µl) as an antigen.

A group of three female BALB/c (8–12-weeks-old) mice were injected with rNP emulsified in Freund's complete adjuvant at 150 µg protein/animal, subcutaneously. The first and second boosters were given at 21 days intervals in Freund's incomplete adjuvant with half of the antigen concentration as mentioned above. After ten days, the mice were bled and checked for the titre at different antibody dilutions by DAC–ELISA. The mouse that had the highest antibody titre was allowed to rest for a month and given a final booster 3 days before fusion, intraperitoneally in saline. The mouse was sacrificed and the spleen was harvested aseptically. Splenocyte suspension was prepared from immunized mouse and fused with myeloma cells[22]. After the fusion, cells were resuspended and cultured in Iscoves modified Dulbeccos medium supplemented with 20% Foetal bovine serum, 10^{-1} mM hypoxanthine, 4×10^{-4} mM aminopterin, 1.6×10^{-2} mM thymidine, 25 mM HEPES and 20 µM 2-mercaptoethanol and seeded in 96 well culture plates. Culture medium without aminopterin was replaced on the seventh day and 10–12 days after fusion, hybridoma colonies were tested for the presence of N protein-specific antibodies. The monoclonals were obtained by limiting dilution method[23]. Out of 96 clones, 64 were found to secrete rNP-specific antibodies in primary screening. Twenty-three of the high-titre primary clones were cultured to 5 ml stage and nine of them were taken through limiting dilution for obtaining the mAbs.

ELISA (direct)[21], dot immuno binding assay (DIBA)[24] and Western blotting[25] were standardized for the detection of tospoviruses using the mAbs. The standardizations included antigen/antibody dilutions, extraction conditions of virus samples, etc.

For the dot blot immunobinding assay, nitrocellulose strips (Hybond-C super, Amersham) were coated with rNP (3 ul) and leaf extracts (5 ul) of infected *N. benthamiana* prepared in 50 mM PBS, pH 7.5 (1 g/5 ml). Nine mAbs (1 : 100) were used as primary antibodies and HRP-labelled goat anti-mouse IgG was used as secondary antibody (Genei, India). The colour was developed by incubating the membrane with 0.05 M sodium citrate buffer (pH 4.8) containing 0.01–0.03% hydrogen peroxide and diamino benzidine (DAB).

SDS–PAGE analysis of the virus purified from tospovirus-infected *N. benthamiana* leaves showed the presence of bands corresponding to G1, G2 and N proteins as expected of purified tospoviruses (data not shown). No other non-specific band was observed. RT–PCR using *N* gene-specific primers with the RNA isolated from purified virus resulted in a product of expected size, 831 bp. The PCR product was cloned into pRSET A vector and sequenced.

The *N* gene (AY184354) of the present tospovirus infecting tomato showed 98% identity with the GBNV *N* gene[12]. Interestingly, the GBNV-To isolate from Taiwan[26] isolated from tomato plants showed only 82% identity with the tospovirus infecting tomato from Karnataka. Recently, the *N* gene sequences of several tospoviruses from India (Table 1) have been reported in the GenBank, which clearly show that all of them are strains of GBNV, with 93–100% identity. The complete *NSs* gene (DQ328318) also showed 98% identity with GBNV *NSs* gene (PBU27809). These results suggest that the tospovirus tomato isolate (Karnataka) is a strain of GBNV. Based on these results the present Karnataka isolate of GBNV has been named GBNV-To (K). The rNP was cloned, expressed and purified as described earlier. The purified rNP was subjected to 12% SDS–PAGE[27] and as shown in Figure 1, the purified rNP had the expected M_r of 32 kDa. The yield of purified rNP was 10–15 mg/l of the culture broth.

The pAbs were obtained against purified rNP as described earlier. They reacted specifically with rNP when subjected to Western blot analysis (Figure 1). Antibody dilution curves obtained by DAC–ELISA showed a dilution end-point of 1 : 32,000 when 1 : 5 diluted crude sap was used as antigen (data not shown). Recently, polyclonal antisera to rNP of GBNV-cowpea isolate was obtained

and shown to be capable of detecting the virus in the field samples. However, some cross-reactivity with healthy plant proteins was observed[6]. Further, it would not be possible to differentiate the virus strains using pAbs Hence, mAbs to GBNV-To (K) were developed as described earlier.

Table 1. *N* gene sequences of several tospoviruses reported in the GenBank

Accession number	Details of the isolate	Maximum identity (%)
AY184354	GBNV (tomato, Karnataka)	100
U27809	PBNV (peanut)	98
AY727923	GBNV (tomato)	98
AF515820	PBNV Madhya Pradesh (potato)	98
AY512652	PBNV (tomato)	98
DQ375809	PBNV (tomato)	98
AY512650	PBNV (black gram)	98
AY957610	GBNV (tomato)	98
AY512648	PBNV (brinjal)	98
DQ375811	PBNV (brinjal)	97
AY882006	PBNV (soybean)	97
AY510132	PBNV (tomato)	97
AF515821	PBNV Rajasthan (potato)	97
AY882000	PBNV (tomato)	97
AY173043	PBNV Bangalore (tomato)	97
AY529713	PBNV (mungbean)	97
AY734235	GBNV (tomato)	97
AY618563	PBNV (tomato)	97
AF467289	PBNV Delhi (soybean)	96
AY882003	PBNV (chilli)	96
DQ058078	PBNV (cowpea)	94
AY463968	GBNV (tomato)	94
AY472081	PBNV isolate Coimbatore (tomato)	93
AF515817	PBNV (tomato)	93
AY426317	GBNV (cotton)	93
AY882002	PBNV (chilli)	93
AY510138	PBNV (tomato)	93

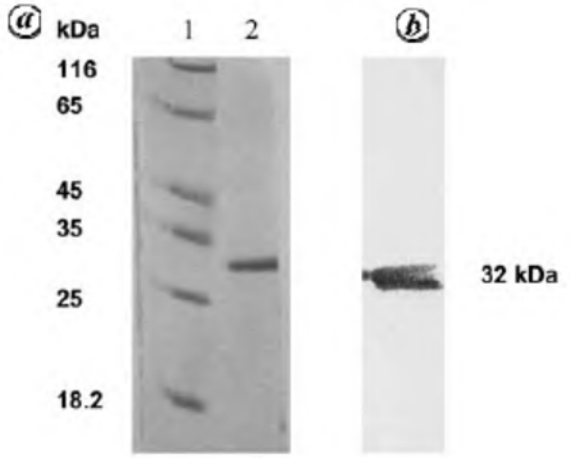

Figure 1. Electrophoresis and Western analysis of expressed rNP in *Escherichia coli*. The Bl21 (DE3) pLys S *E. coli* cells harbouring pRSET-N were induced with 0.3 mM IPTG. The expressed protein was purified using Ni–NTA column and analysed by SDS–PAGE. ***a***, Lane 1, Molecular weight markers and lane 2, Purified rNP (6 ug). ***b***, Western blot of rNP duplicate of lane 2 carried out using GBNV polyclonal antibodies obtained in the present study.

The antigen and antibody dilution curves for five of these mAbs in culture supernatants are shown in Figure 2 *a* and *b* respectively. Fifty per cent binding could be detected with 15–30 ng of purified protein and the dilution end-point was at 1 : 1024. Figure 2 *c* shows the sensitivity of the mAbs to detect the virus in crude extracts. The virus could be detected in as little as 1 : 200 dilution of the crude extract prepared using 1 g of leaf material/5 ml of buffer. All the nine mAbs gave a strong reaction when used for Western blot analysis (data not shown). Thus all

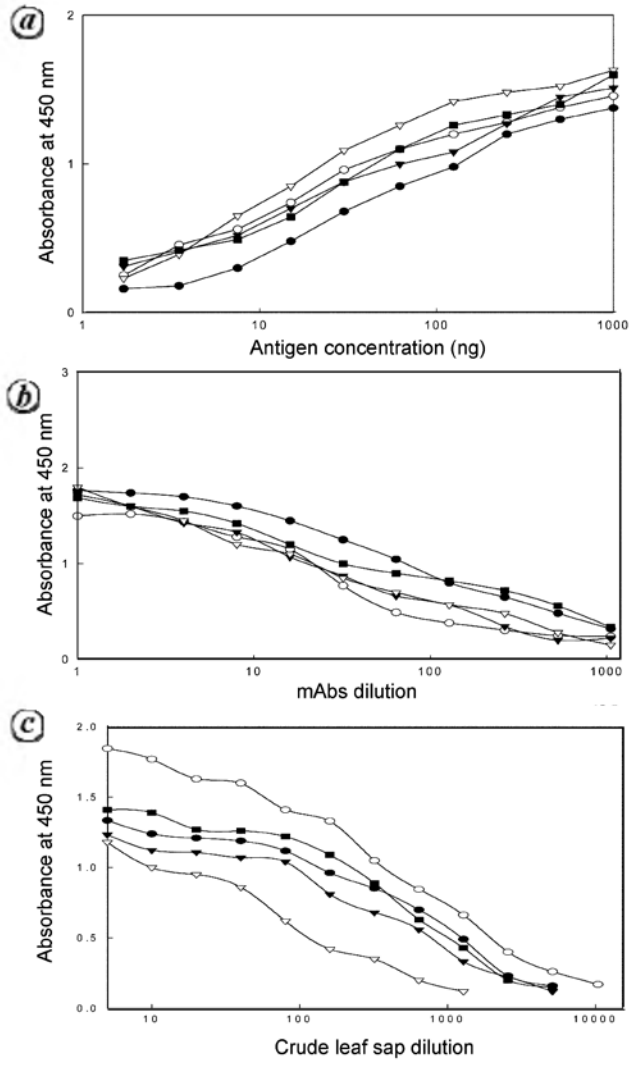

Figure 2. ***a***, Antigen dilution curves for mAbs. Serial double-dilutions of rNP (1 µg–2 ng) were used in DAC–ELISA with the mAbs A10D10 (•), B8D12 (■), C10A7 (▽), E6D8 (▼) and F7C8 (○) as primary antibodies. Anti-mouse IgG coupled to HRP was used as the detecting antibody. The reaction was monitored at 450 nm. ***b***, Antibody dilution curves for mAbs. The wells were coated with rNP (250 ng) and analysed by DAC–ELISA. Serial double-dilutions of the mAbs A10D10 (•), B8D12 (■), C10A7 (▽), E6D8 (▼) and F7C8 (○) in the hybridoma culture supernatants were used. The intensity of the product developed was read at 450 nm. ***c***, Crude sap dilution curves for mAbs. Serial double-dilutions of crude sap (infected tomato; 1 : 5–1 : 10,240) were used in DAC–ELISA with the mAbs A10D10 (•), B8D12 (■), C10A7 (▽), E6D8 (▼) and F7C8 (○) at 1 : 5 dilution as primary antibody.

Table 2. Epitope profiling. The nine mAbs were used for profiling different tospovirus isolates. DAC–ELISA was done for different isolates from South India. (These experiments were performed three times and the results are consistent every time.) 1 : 5 diluted infected leaf crude saps were used for coating the ELISA plates. mAbs culture supernatant 1–50 diluted was used as primary antibody

	Different monoclonal antibodies								
GBNV isolate	F7C8	A10D10	B8D12	C10A7	E6D8	A4F12	B12E4	D3C6	H7A3
Tomato – Bangalore	++++	+++	++++	+++	+++	+++	+++	+++	+++
Tomato – Tamil Nadu	++++	++++	++++	++++	++++	+	++++	++++	+
Tomato – Maharashtra	+++	+++	+++	++	++	+	+++	++	++
Cowpea – Tamil Nadu	+++	+++	+++	+++	++	+++	+++	+++	+++
Chilli – Andhra Pradesh	++++	++++	++++	+++	++++	+++	+++	+++	++
Chilli – Dharwad	++++	++	++++	++++	++++	+++	+++	++++	++
Chilli – Maharashtra	++	+	++	+	+	+	+	++	+
Chilli – Bangalore	+++	++	+++	++	+++	+	++	++	+
Watermelon – Bangalore	++	+++	++++	+++	+	+	+	+	++
Pumpkin – Bangalore	+	+	+	+	+	+	---	+	+
Gherkin – Tamil Nadu	++	+	++	+	++	---	+	+	+
Cucumber – Bangalore	++	+	++	++	+	---	+	+	---
A. hispidum – Bangalore	+++	+++	+	+++	++	+++	+	++	+

A_{450} value. 0.100, Negative threshold; 2.2, Positive threshold (rNp); 0.110–0.350, +; 0.351–0.500, ++; 0.501–1.000, +++; >1, ++++.

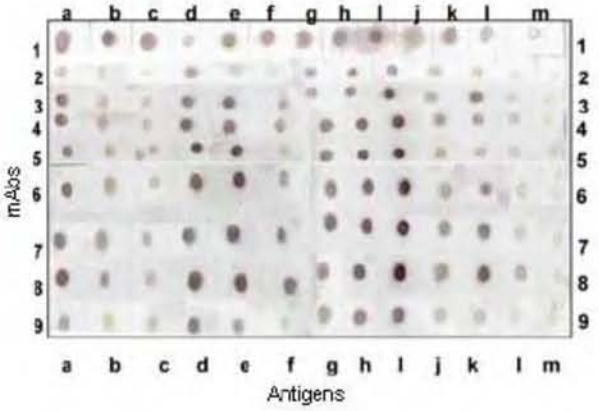

Figure 3. Reactivity of GBNV-To (K) mAbs to various plant extracts. The dot-blot assay was performed with various tospovirus-infected plant extracts (3 ul 1 : 5 diluted) using GBNV-To (K) mAbs. Panels 1–9 represent mAbs F7C8, A10D10, B8D12, C10A7, E6D8, A4F12, B12E4, D3C6 and H7A3 (1 : 10 diluted) respectively. Lane a, Tomato (*L. esculentum*), Bangalore; lane b, *A. hispidum*, Bangalore; lane c, Lady's finger (*A. esculentus*), Maharashtra; lane d, Muskmelon (*C. melo*), Bangalore; lane e, Chilli (*C. anuum*), Andhra Pradesh; lane f, Brinjal (*S. melongena*), Andhra Pradesh; lane g, Gherkin (*C. anguria*), Tamil Nadu; lane h, Tomato (Maharashtra); lane i, Tomato (Tamil Nadu); lane j, Weed species; lane k, Cucumber (*C. sativus*), Bangalore; lane l, Pumpkin (*C. pepo*), Bangalore and lane m, Healthy tomato.

the mAbs were of high affinity and could be used in the detection and profiling of the GBNV strains. Monoclonal antibodies have been produced against purified TSWV earlier and used in ELISA for detection and comparison of tospoviruses[28,29]. Serological comparison of 96 tospovirus isolates obtained from all over the world was carried out earlier by DAS–ELISA with ten polyclonal antisera and by TAS–ELISA with 33 mAbs. Based on these results various isolates could be classified into serogroups I, II, III and IV[30]. GBNV belongs to serogroup IV and in the present investigation, the possibility of using the mAbs raised against GBNV-To (K) for strain discrimination was tested.

An epitope profile of different isolates of GBNV collected from various parts of South India (Table 2) by the authors and supplied by V. G. Malathi (IARI, New Delhi; kind gift) based on symptoms was obtained using the nine mAbs. The isolates collected based on the symptoms were confirmed to be tospovirus infections by ELISA using GBNV pAbs (data not shown). Table 2 depicts the reactivity of the nine mAbs to different isolates. The profile of the isolates with different mAbs is not similar. Even though the sequence similarity of GBNV strains is high and it varies from 93 to 100%, the mAbs could differentiate them. Among the nine mAbs, F7C8, A10D10, C10A7, E6D8 and D3C6 gave a strong reaction for tomato (Bangalore, Tamil Nadu, Maharashtra), chilli (Andhra Pradesh, Bangalore, Dharwad), *A. hispidum* (Bangalore) and cowpea (Tamil Nadu), whereas weaker reaction was observed with infected leaf material from chilli (Maharashtra), gherkin (Tamil Nadu), cucumber (Bangalore), watermelon (Bangalore) and pumpkin (Bangalore). In general pumpkin (Bangalore) gave a weak reaction with all the mAbs. This could be due to the difference in the virus concentration. However, the fact that reactivity of the mAbs is different for different isolates suggests that they are indeed antigenically different strains. For example, the epitope profile of chilli (Bangalore) differed from the profile of chilli (Dharwad), though the reactivity of all the mAbs to these isolates was reasonably high.

DIBA has been shown to be eight times more sensitive than ELISA in detecting the virus in the crude sap[24,26]. The sensitivity of the nine mAbs to detect tospoviruses was tested by DIBA as described earlier at different dilutions of both rNP and the infected leaf crude sap. In the case of rNP, the detection was possible even at a concentration as low as 3.5 ng (data not shown). Similarly, in the crude sap the virus could be detected at a dilution of 1 : 160 and there was no reaction with the healthy sap in the case of all the mAbs (data not shown).

DIBA was also performed as described earlier with the tospovirus-infected leaves collected from different locations in South India on the basis of the symptoms produced. As can be seen from Figure 3, the virus could be detected in all the infected field samples. The mAb F7C8 could detect GBNV in all the samples, whereas the other mAbs showed differential reactivity. The results demonstrate that DIBA could be used conveniently to detect the virus in infected field samples. None of these mAbs reacted with the healthy crude sap.

Thus the results presented here clearly demonstrate that all the nine mAbs and the pAbs raised against the rNP of GBNV-To (K) are of high affinity and can dectect the native virus from the infected field samples. The virus could be detected at 1 : 160 dilution in crude extracts in both dot-blot assays and DAC–ELISA. Thus mAbs can be employed for the detection of the virus by dot-blot assays at extension centres that may not have instrumentation facilities.

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