Partial Characterization of a Badnavirus Associated with Citrus Yellow Mosaic Disease in India

Y. S. Ahlawat, A. Varma, R. P. Pant, A. Shukla, and B. E. L. Lockhart

ABSTRACT. A previously undescribed badnavirus was found to be associated with a yellow mosaic disease occurring commonly in citrus orchard trees and nursery plants in India. The virus was graft and dodder transmitted to 14 of 15 citrus species or cultivars, and was also transmitted mechanically from citrus to citrus. A protocol for the purification of citrus yellow mosaic virus was developed. By electron microscopy, the virus particles were found to have the bacilliform structure typical of badnaviruses. In ultrathin sections, the virions were observed in the cytoplasm of diseased tissue but not in the nucleus. Electrophoresis of enzyme-digested viral nucleic acid preparation revealed it to be DNA. The virus was also shown to have a dsDNA genome by PCR amplification using degenerate oligonucleotide primers based on conserved badnavirus genome sequences. The virus was named citrus yellow mosaic bacilliform virus (CYMBV). A homologous antiserum specifically reacted to CYMBV antigen in DAS-ELISA and IEM tests. In IEM tests, CYMBV was found to be related serologically to sugarcane bacilliform badnavirus and to eight other badnaviruses. Mixed infections of CYMBV, tristeza and Indian citrus ringspot were common in orchard trees.

In India mosaic disease of citrus is of common occurrence, especially in sweet orange (2, 16). The incidence of mosaic disease ranges from 10 to 70% in Satgudi and Chini sweet orange orchards in South India. In 1992, a 10-yr old pummelo tree was observed with bright yellow mosaic and vein banding symptoms in Bangalore. Later, similar symptoms were observed in several Satgudi and Chini sweet orange trees in Karnataka and Andhra Pradesh and in three grapefruit trees at Delhi. Leaf and budwood samples were collected from affected trees to investigate the etiology of the disease. It was found that the disease was graft-transmitted, and bacilliform virions were observed by electron microscopy (EM) in both field samples and samples from inoculated glasshouse plants (3). The losses caused by the mosaic disease were apparent in Satgudi sweet orange orchards because several orchards with trees 4 to 10 yr old were abandoned since they were no longer productive. In view of this, studies were conducted to identify and characterize the virus associated with citrus yellow mosaic disease in India so that suitable diagnostic reagents could be developed.

MATERIALS AND METHODS

Virus isolate. The citrus yellow mosaic isolate used in these studies was obtained by graft inoculation as described earlier from the original isolate on pummelo (3). This isolate was multiplied by wedge grafting on plants of Mosambi sweet orange and pummelo and maintained in the glasshouse.

Transmission. Dodder transmission tests were conducted using *Cuscuta reflexa* as described earlier (1). Insect transmission was attempted using aphids, *Myzus persicae* Sulz., *Aphis gossypii* Glover, *A. spiraecola* Patch (syn. *A. citricola* van der Goot), *A. craccivora* Koch., and mealybugs, *Planococcus citri* Russo, *Brevennia rehi* Lind. and *Saccharicoccus sacchari* Cock. from colonies maintained in an insectary.

The aphid transmission tests were done in a non-persistent manner; whereas mealybugs were tested for persistent transmission. Mechanical inoculations were done from the original source of virus collected

from the field. Inoculum was prepared from young, fully expanded symptomatic leaves of pummelo. The leaves were macerated in liquid nitrogen in a sterilized pestle and mortar. The leaf powder was then homogenized in 0.05 M Tris buffer (pH 7.4) in a ratio of ½ (w/v). The extract was squeezed through double layer of muslin cloth and used as inoculum. Leaves of test plants were immediately inoculated using carborundum as an abrasive. The test plants used in these experiments were 3-mo. old seedlings of pummelo, grapefruit, Satgudi sweet orange, Rangpur lime and *Citrus decumana*. Ten plants of each test species were inoculated and maintained in the greenhouse for observation.

Host range. For these studies, available citrus species were inoculated by wedge grafting from mechanically infected plants. Five 1-yr-old seedlings of each test species were inoculated.

Purification. The virus could not be purified to a desired level using previous methods (4, 11). Therefore, purification process was further investigated. One hundred grams of symptomatic leaves were powdered in liquid nitrogen and then extracted with two volumes of 50mM Tris citrate buffer (pH 7.4). Chloroform (25% v/v) was added to the extract and stirred for 10 min at 4°C. The extract was centrifuged at 10,000 *g* for 10 min., the virus was pelleted by centrifugation at 136,000 *g* for 60 min and the pellet was dissolved in 10mM phosphate buffer (pH 7.2) and centrifuged at 10,000 *g* for 10 min. The supernatant was passed through 30% sucrose cushion at 136,000 *g* for 60 min. Resulting pellet was resuspended in 10mM phosphate buffer (pH 7.2) and layered on a 0 to 30% CsCl density gradient and centrifuged at 116,000 *g* for 4.5 hr. The virus band was collected and dialysed in phosphate buffer for 8 hr with three changes. The OD values of the purified preparation were measured in a spectrophotometer. A similar protocol was used for partial purification of the virus from leaves of affected field trees to see whether they were contaminated with other known viral infections.

Electron microscopy (EM). Leaf samples were examined by EM using the usual leaf dip method. The preparations were negatively stained using 2% sodium phosphotungstate (PTA) or 2% aqueous uranyl acetate (UA). The amount of virions present was also examined by EM at various stages of purification and in the final preparation. For ultrathin sections, pieces of leaf tissue (2.5 × 2 mm) were removed from healthy and mechanically inoculated Satgudi sweet orange plants. Samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0 for 24 hr, rinsed and post-fixed in 0.2% osmium tetroxide in the same buffer for 24 hr. After dehydration through an alcohol series, samples were passed through propylene oxide for 10 min and then embedded in Emix resin (Emscope Laboratories Ltd, U.K.). Sections of 90 nm thickness were cut with a diamond knife on an LKB IV ultramicrotome. Sections were collected on formvar-coated grids, doubly stained with 5% UA for 10 min and 5% lead citrate for 5 min, and examined in a JEOL 10OCX II EM.

Serology. Polyclonal antibodies against the virus were prepared in a white albino rabbit. Four weekly intramuscular injections were given with 750 µl of purified preparation emulsified with an equal amount of Freund's incomplete adjuvant. The blood was collected 10 days after the last injection, serum was collected by the usual method and stored at 4°C after adding 0.02% sodium azide. The antiserum was evaluated for yellow mosaic virus detection by double-antibody sandwich-(DAS) ELISA and immunoelectron microscopy (IEM).

To examine serological relationships, antisera of the following badnaviruses were used in IEM tests: banana streak virus (BSV) (Morocco, Rwanda, and Mysore isolates), sugarcane bacilliform virus (SCBV) (4MX and SB isolates), kalanchoë top spotting virus (KTSV), piper yellow mottle virus (PYMV), dioscorea bacilliform virus (DBV), cacao swollen shoot virus (CSSV) and commelina yellow mottle virus (CoYMV). These antisera were obtained from B. E. L. Lockhart. Homologous antiserum was also tested for its specificity and comparative efficacy of IEM tests with other badnaviruses.

Viral DNA extraction and PCR amplification. Genomic nucleic acid was extracted from purified virions as described earlier (13) and digested with RNase, and DNase and electrophoresed along with markers. For PCR, nucleic acid was extracted from partially purified preparations of CYMBV as described earlier (12). This included pre-digestion of the samples with DNase and RNase prior to extraction of virion nucleic acid to eliminate non-encapsidated nucleic acids. Nucleic acid extracted from virions was amplified by PCR using degenerate oligonucleotide primers based on consensus sequences located in the three conserved regions of ORF III of the badnavirus genome (12). Products generated by PCR amplification were analyzed by electrophoresis on agarose gels stained with ethidium bromide (12).

RESULTS

Transmission. CYMBV was transmitted from infected sweet orange to 14 of 15 citrus species or cultivars by wedge grafting and dodder (Table 1). No symptoms were observed one year after inoculation in plants inoculated by aphids or mealybugs, and no virus was detected in the plants by EM examination. The disease was also transmitted by mechanical inoculation to, pummelo (6/10), *C. decumana* (10/10) and Satgudi sweet orange (7/10),

TABLE 1
HOST RANGE OF CITRUS YELLOW MOSAIC BACILLIFORM VIRUS

Plant species	No. of plants infected out of five inoculated by grafting	Symptoms[z]
Rangpur lime	5	CS, VB, MM
Volkamer lemon	5	YS, YB,YM
Rough lemon	4	VF, M, YS
Sweet orange cvs		
Satgudi	3	M, YR
Mosambi	5	M, YR
Chini	5	CS, YM
Mandarin cv Nagpur	3	VB
Sweet lime	1	MM
Mexican lime	0	—
Pummelo	5	YM, SL
Grapefruit cv Duncan	2	VF, YM
Citron	1	VY
Sour orange	5	CS,YS,YM
Citrus mitis	5	YR, SL
C. decumana	5	YM,SL,CS

[z]CS = chlorotic spot; VB = Vein banding; MM = mild mosaic; YS = yellow spots; YB = yellow blotches; YM = yellow mosaic; VF = vein flecking; M = mosaic; YR = yellow rings; SL = small leaves; VY = vein yellowing

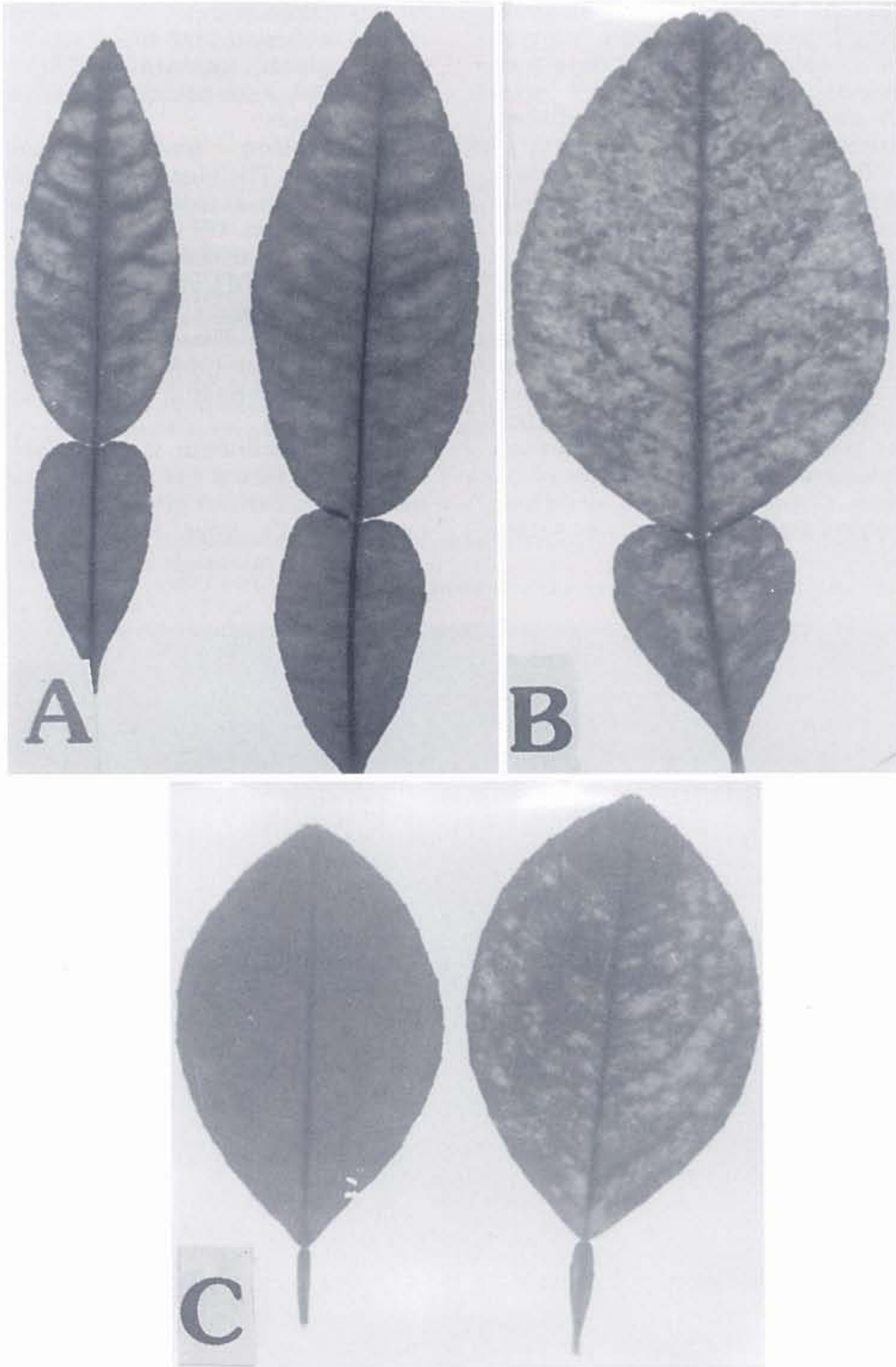

Fig. 1A-C. Foliar symptoms associated with citrus yellow mosaic bacilliform virus infection. Fig. 1A. *Citrus decumana* leaves from mechanically inoculated plant. Fig. 1B. Pummelo leaf showing mosaic symptoms upon inoculation from pure culture. Fig. 1C. Healthy and mosaic infected leaf of mosambi sweet orange.

but not to grapefruit or Rangpur lime. Infected plants developed typical mosaic symptoms 90 days post-inoculation (Fig. 1a) and were shown by EM to contain bacilliform particles.

Symptoms. The characteristic symptoms of the disease in field-infected Satgudi and pummelo trees consisted of bright yellow mottling of the leaves and yellow flecking along the veins. Glasshouse-inoculated plants also developed the same symptoms (Fig. 1b, Fig. 1c). Symptoms that were produced in various citrus species upon graft inoculation are given in Table 1. Except Mexican lime, graft-inoculated plants of all other *Citrus* spp. tested developed varying degrees of symptoms within 70 days after inoculation. Indexing by EM confirmed that all symptomatic test plants contained bacilliform virions that were not detectable in healthy plants.

Purification and electron microscopy. The virus was purified by the protocol described in Materials and Methods. UV absorption values of the purified preparations showed 0.694 OD at 260 nm and 0.542 at 280 nm. The A260/A280 ratio was 1.28. These results suggested that the preparation contained nucleoproteins similar to badnaviruses.

Typical bacilliform virions measuring 130 × 30 nm were observed in leaf dip and purified preparations of samples both from field-infected

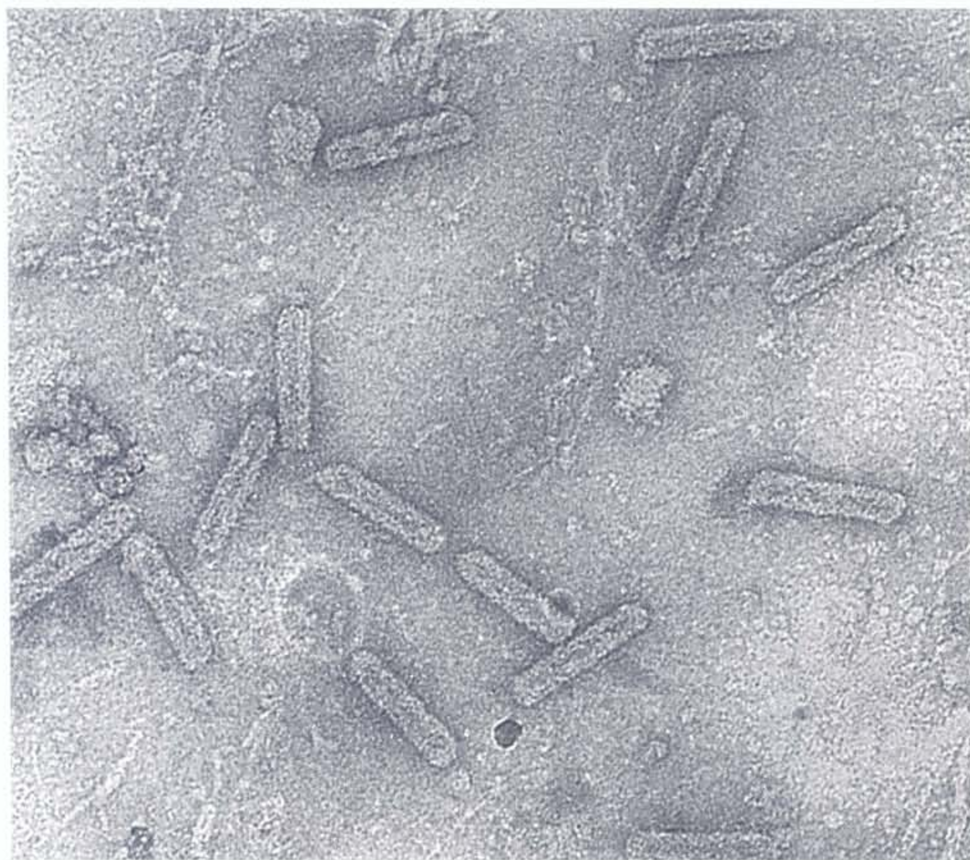

Fig. 2. Bacilliform particles purified from mechanically inoculated plants of pummelo.

trees as well as from inoculated glasshouse plants (Fig. 2). No virions were observed in similar preparations from uninoculated control plants. However, partially purified preparations from field infected trees showed a mixed infection of tristeza virus, Indian citrus ringspot virus (5) and CYMBV especially in Hindupur region of Andhra Pradesh (Fig. 3). In ultrathin sections, large numbers of bacilliform particles were observed in the cytoplasm of tissue from CMBV infected plants (Fig. 4) but no virions were observed in the nuclei of CYMBV affected cells or in tissue of healthy plants.

Serology. The polyclonal antiserum prepared for CYMBV reacted specifically in EM-trapping and decoration tests (Fig. 5a). A dilution of 1:500 was optimum for trapping and a dilution of 1:50 was best for decoration tests. In DAS-ELISA, the following dilutions were optimum: antigen 1:10, specific antibodies 1:1,000, conjugate (goat antirabbit conjugated with alkaline phosphatase) 1:2,000 and substrate (p-nitrophenol-phosphate) 0.5 mg/ml. Out of 136 field samples from South India evaluated, 86 showed the presence of CYMBV in DAS-ELISA. The samples which showed poor absorbance values at 405 nm (<0.12) in ELISA were further tested by IEM but none of the negative samples in ELISA were positive by IEM, sug-

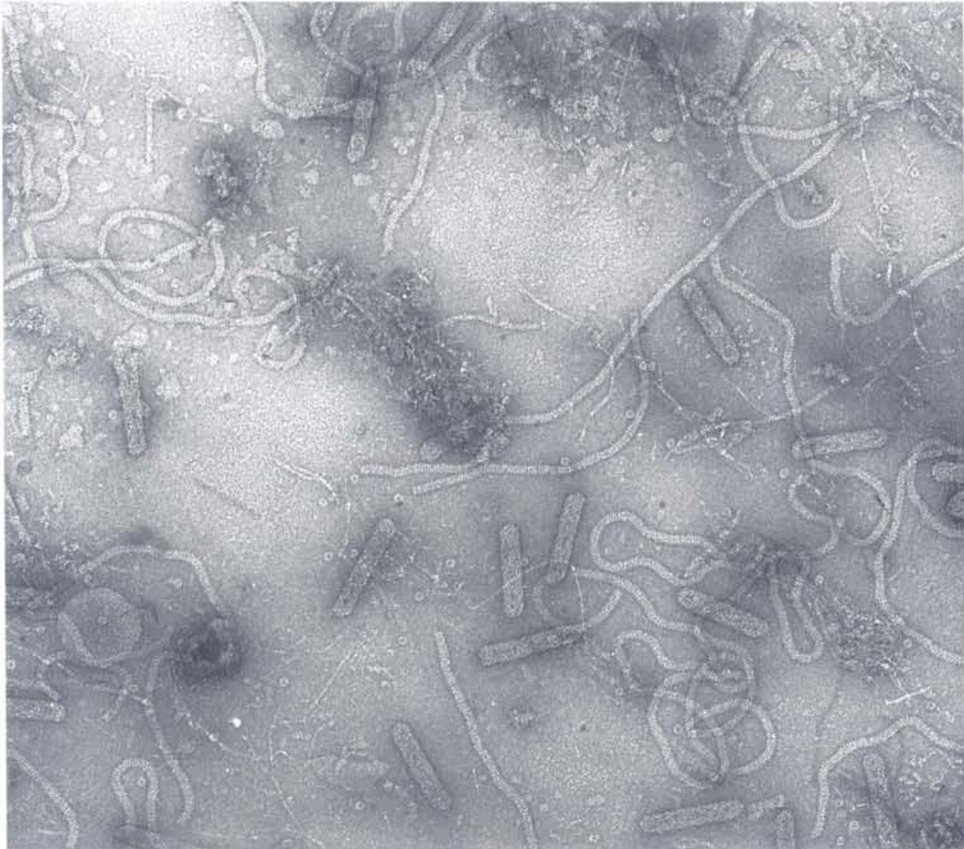

Fig. 3. Purification of virions from field affected trees showing particles of CYMBV, CTV and Indian CRSV.

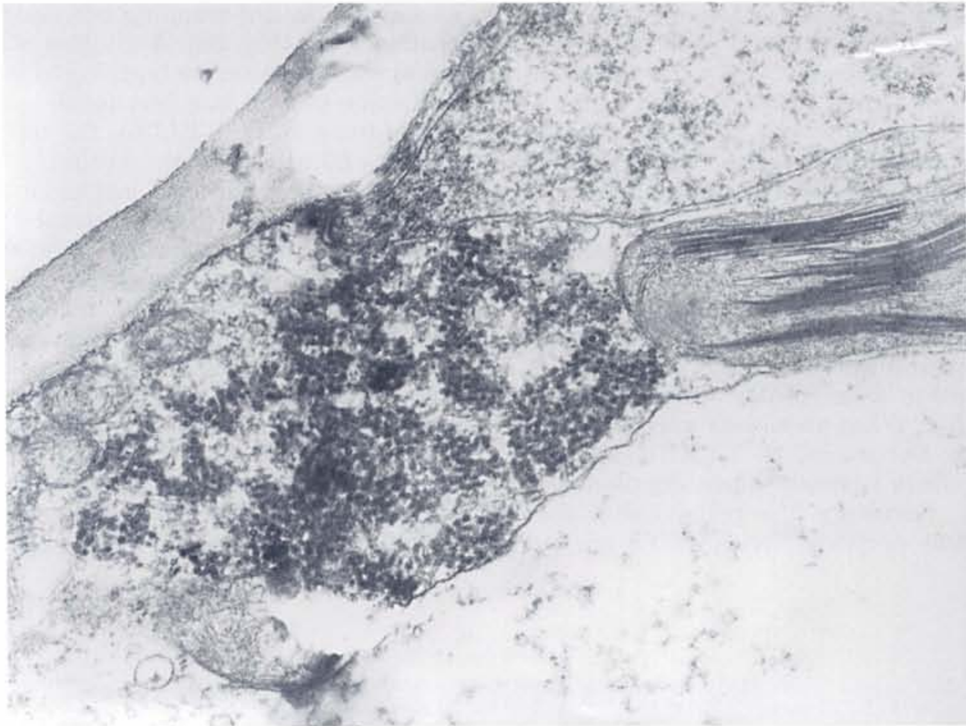

Fig. 4. Bacilliform particles in cells from CYMBV inoculated plants in ultrathin section.

gesting that the detection of CYMBV was specific.

Virions of CYMBV were successfully trapped with antisera to BSV, CoYMV, PYMV, CSSV, DBV, KTSV and ScBV in IEM tests (Fig. 5b). However, these tests showed that CYMBV had the closest serological relationship to ScBV.

PCR amplification and CYMBV genomic nucleic acid. One percent non-denaturing agarose gel-electrophoresis of nuclease digested virus nucleic acid preparations showed two bands at 23.1 and 6.6 kb after RNase treatment but no band after DNase treatment (Fig. 6) indicating that the genome of CYMBV was DNA.

Two regions of the nucleic acid extracted from CYMBV virions were successfully amplified by PCR using two pairs of degenerate oligonucleotide primers known to prime amplification of badnavirus DNA. The badnavirus specific PCR products, approximately 1.5 kb and 1.0 kb in size, corresponded to similar PCR products obtained using other badnavirus genomic templates (12).

DISCUSSION

Mosaic diseases in citrus have been reported from India (2, 6) and Japan (9). Isometric particles were reported to be associated with Japanese citrus mosaic disease (17). However, we have reported bacilliform virions associated with the disease, in question, (3) distinguishing it from other mosaic diseases. The bacilliform virions were considered as particles of rhabdovirus on the basis of preliminary EM studies but detailed studies revealed that the

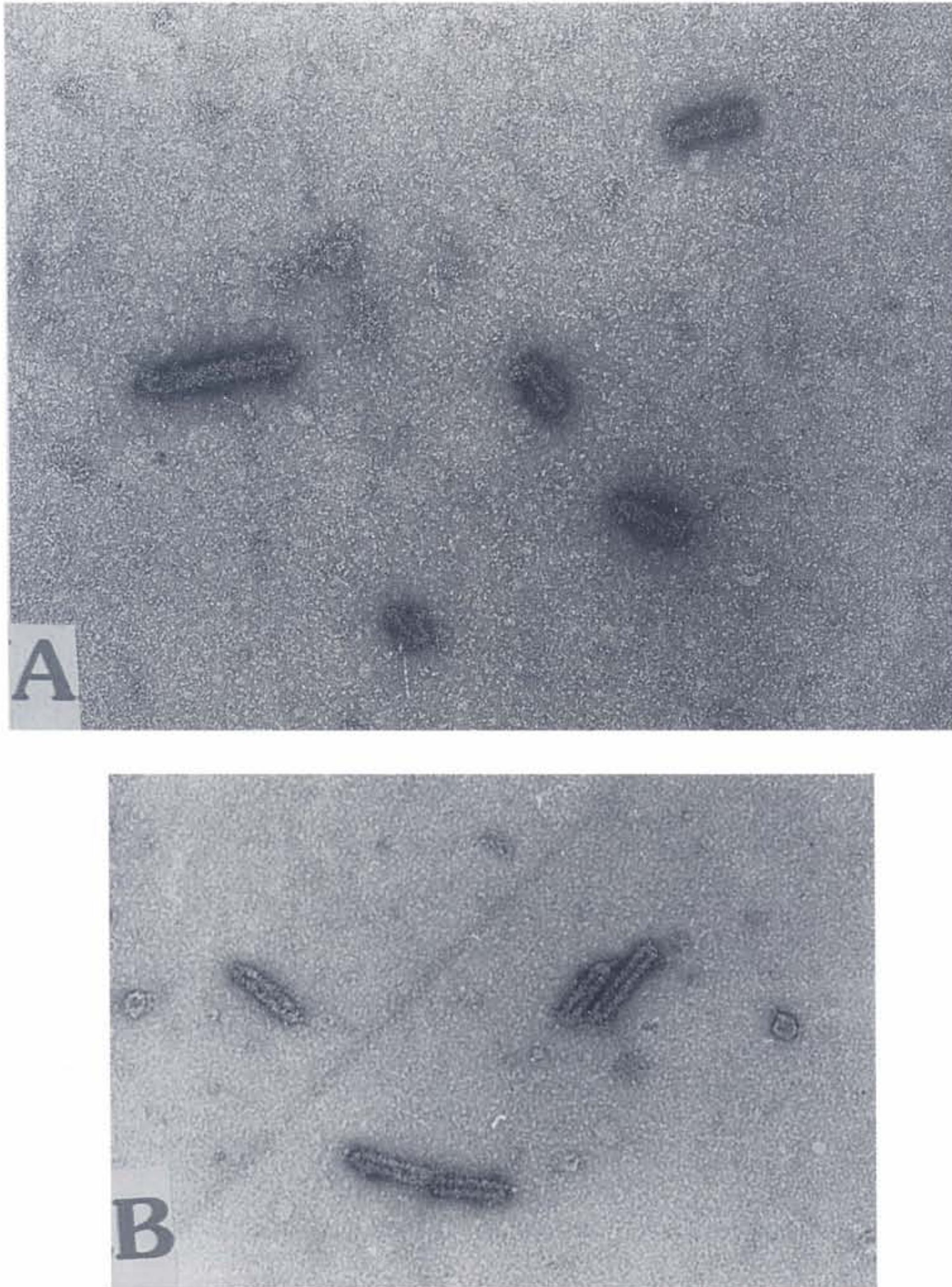

Fig. 5. A. Decoration of CYMBV particles with homologous antiserum. Fig. 5B. Decoration of particles with sugarcane badnavirus antiserum.

bacilliform virions associated with citrus yellow mosaic disease were, in fact, the particles of badnavirus group as determined by serological tests and PCR amplification. Part of these studies has been reported elsewhere (4). A bacilliform virus has been reported to be associated with citrus leprosis disease in Brazil (10). However, the bacilliform virions

associated with citrus leprosis occur in the nucleus (10) unlike those of badanviruses, which occur only in cytoplasm (7, 8, 14, 15).

Citrus yellow mosaic disease is widely distributed in India and is of great economic importance to the citrus industry. The presence of the disease in commercial nurseries suggested inadvertent spread of the disease through contaminated budwood. CYMBV, as indicated in Table 1, is capable of infecting the major commercial citrus cultivars and rootstocks used in India. Mechanical transmission of CYMBV will help in separating the virus from mixed infections which are quite common in orchard trees.

Positive reaction of CYMBV in IEM tests with antisera to a range of badnaviruses, presence of bacilliform virion in cytoplasm of infected cells, digestion of virus nucleic acid with DNase and PCR amplification of CYMBV genomic nucleic acid with badnavirus-specific oligonucleotide primers confirm that CYMBV is a badnavirus. This is the first report of a badnavirus infection in citrus.

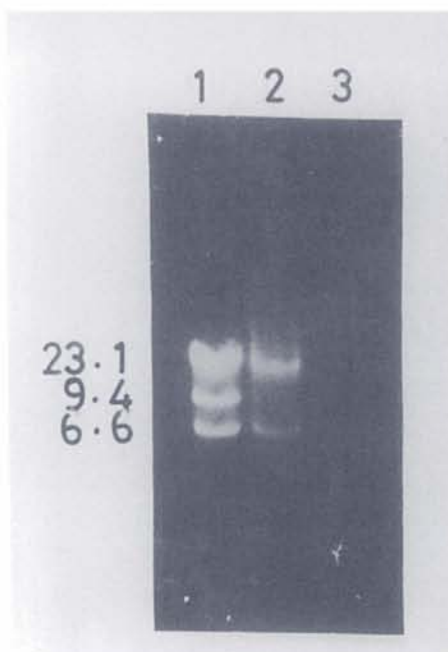

Fig. 6. Electrophoresis of nucleic acid from a purified virus preparation after treatment with nucleases. Lane 1: Marker (DNA/Hind III cut, Bangalore Genei Pvt. Ltd. India). Lane 2: treated with RNase showing two bands at 23.1 and 6.6 Kb. Lane 3: treated with DNase showing no bands.

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