Partial characterization of citrus mosaic virus

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ABSTRACT: Citrus mosaic disease is of common occurrence in India. A bacilliform virus belonging to badnavirus group is associated with the mosaic disease. The virus was transmitted by wedge grafting and mechanical inoculations from citrus to citrus but not by three species of mealybugs and aphids. Out of 15 cultivars inoculated with the virus 14 were infected. The particles associated with citrus mosaic are non-enveloped bacilliform, measuring 125-130\(\times\)25-30 nm in size. The virus was serologically related to sugarcane bacilliform virus (ScBV) and 8 other badnaviruses. A polyclonal antiserum has been developed and used for detection of the virus in Enzyme-Linked immunosorbent assay (ELISA) and Immuno-sorbent electron microscopy (IEM).

Keywords: Citrus mosaic virus, badnavirus, transmission, genome, serology

Citrus crop has significant importance in fruit economy of the country and is the second largest industry in India. In India total area under citrus is about 3.86 lakh ha with a production of about 28.22 lakh tonnes of fruits.

During the last two decades, a number of virus and virus like diseases have been recorded from citrus trees in India. (Ahlawat, 1989; Ahlawat et al., 1993 and 1996). Citrus mosaic is an important disease especially in citrus trees in Southern and North-eastern states of India (Ahlawat et al., 1985; Reddy et al., 1972). The incidence of the disease has been reported from 10-77% (Reddy and Murti 1985). A badnavirus has been reported to be associated with mosaic disease of citrus (Ahlawat et al., 1996). This paper communicates additional information generated on the citrus mosaic virus in India.

MATERIALS AND METHODS

Virus isolate

The virus isolate used in these studies was originally collected from Andhra Pradesh (Hindupur) during 1992 and maintained on seedlings of mosambi sweet orange and pummelo. From these plants the mosaic virus was mechanically transmitted to mosambi plants (Fig. 1) and the plants thus infected were free from other viruses as evidenced by electron microscopy and immuno-electron microscopy. However, graft inoculated plants from original isolate when indexed were found to have a mixture of citrus tristeza, ringspot and mosaic viruses (Fig. 2), indicating that the original host had the mixed infection.

Mechanical transmission

Symptomatic leaves of sap inoculated mosambi plants were macerated in liquid nitrogen in sterilized pestle and mortar. The leaf powder thus obtained was mixed with 0.05M Tris-citrate buffer (pH-7.4) in a ratio fo 1:2 (W/V). The extract was squeezed through double layer muslin cloth and
used as inoculum. Celite (about 2%) was added to the inoculum as an abrasive and the leaves of one year-old citrus seedlings were inoculated with forefinger and the inoculated plants were maintained in insect proof glasshouse. Ten healthy seedlings each of six citrus cultivars, mosambi, decumana, galgal, kinnow, Nagpur mandarin and kagzi lime were inoculated.

Insect transmission

Three aphid species, *Aphis gossypii* Glover *A. citricola* Patch and *A. craccivora* Koch and three mealybug species, *Planococcus citri* Risso, *Brevennia rehi* Lind, *Saccharicoccus sacchari*Cook were used for virus transmission. These insects were maintained in the insectry at the Division of Plant Pathology. The aphid species were tested in non-persistant way whereas mealy bug species were tested in persistent manner. Ten insects of each species per plant were used for inoculation ten healthy seedlings of mosambi.

Host range

To study host-range, both citrus and non- citrus hosts were inoculated. Among non-citrus hosts, five plants of following species were inoculated by sap inoculation: *Chenopodium amaranticolor* Coste and Reyn; *C. murale* L.; *C. quinoa* Willd; *Nicotiana tabacum* L., *N. glutinosa* L., *Lycopersicum esculentum* L., *Solanum nigrum* L., *Oryza sativa* L., and *Saccharum officinarum*. The host-range of the virus in *Citrus* spp. was studied by wedge grafting of 5 plants each of *C. limonia*, *C. volkameriana*, *C. jambhiri*, *C. sinensis* cvs sathgudi, mosambi and chini, *C. reticulata* cv. Nagpur orange, *C. limettiodes*, *C. aurantifolia*, *C. grandis*, *C. paradisi* cv. duncan, *C. medica*, *C. aurantium*, *C. mitis*, and *C. decumana*.

Virus purification

Four protocols were followed for the purification of citrus mosaic virus (Lockhart 1986, Lockhart and Autrey 1988, Omura *et al.* 1983 and Cabauatan and Hibino 1988). However, a modified protocol of Lockhart (1986) as detailed below proved to be the best out of four tried.

Protocol for purification of citrus mosaic virus

- 100 g of symptomatic mosambi leaves washed thoroughly and dried and powdered in liquid nitrogen.
- mix the leaf powder in 2 vol. of 50mM Tris-citrate buffer (pH-7.4) and the homogenate was filtered through double layer muslin cloth.
- Filtrate was clarified by low speed centrifugation (10,000g for 10 min. after adding 25% chloroform (v/v) and allow the extract for 10 min. at 4°C while stirring.
- Virus was pelletised by high speed centrifugation at 136,000g for 60 min.
- Pellet was dissolved in10mM phosphate buffer, pH-7.2 and again clarified by low speed centrifugation at 10,000g for 10 min.
- Supernatant was passed through 30% sucrose cushion at 136,000g for 60 min.
- Pellet resuspended in 10 mM phosphate buffer, pH-7.2 and put on 0-30% cesium chloride (CsCl) density gradient and centrifuged at 116,000g for 4.5 h.
- Virus peak was collected by ISCO density gradient fractionator.
- Virus fractions were concentrated at 136,000g for 60 min.
- Virus pellet of each tube was dissolved in 200µl of 10mM phosphate buffer (pH-7.2), pooled and dialysed for 8h in phosphate buffer giving three changes.
- Final preparation was examined inTEM (JEOL 100 CX II) and stored at 4°C and used for various studies.

Electron microscopy

The virus preparation as obtained above was
Table 1. Host range of Citrus mosaic virus in the family Aurantioideae.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>No. plant infected out of 10 inoculated</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. limonia</td>
<td>9</td>
<td>CS, VB, MM</td>
</tr>
<tr>
<td>C. volkameriana</td>
<td>8</td>
<td>CS, VB, M</td>
</tr>
<tr>
<td>C. jambhiri</td>
<td>8</td>
<td>VF, M, CS</td>
</tr>
<tr>
<td>C. sinensis cv. sathgudi</td>
<td>6</td>
<td>M, CS</td>
</tr>
<tr>
<td>mosambi</td>
<td>10</td>
<td>M, CS</td>
</tr>
<tr>
<td>chini</td>
<td>10</td>
<td>CS, M</td>
</tr>
<tr>
<td>C. reticulata cv. Nagpur orange</td>
<td>6</td>
<td>VB,M</td>
</tr>
<tr>
<td>C. limettiodes</td>
<td>2</td>
<td>MM</td>
</tr>
<tr>
<td>C. aurantifolia</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>C. grandis</td>
<td>10</td>
<td>M, SL</td>
</tr>
<tr>
<td>C. paradisi cv. duncun</td>
<td>4</td>
<td>VF, M</td>
</tr>
<tr>
<td>C. medica</td>
<td>2</td>
<td>VF, M</td>
</tr>
<tr>
<td>C. aurantium</td>
<td>10</td>
<td>CS, SM</td>
</tr>
<tr>
<td>C. mitis</td>
<td>10</td>
<td>CR, SL, M</td>
</tr>
<tr>
<td>C. decumana</td>
<td>10</td>
<td>M, CS, SL</td>
</tr>
</tbody>
</table>

CS=Chlorotic Spot
VB=Vein banding
MM=Mild mosaic
VF=Vein flecking
M=Mosaic
SL=Smalling of leaves

stained with 2% uranyl acetate and examined in EM for virion size and morphology. The modal length of particle was calculated by the following formula.

\[
\text{Size of virus particles in } \mathrm{nm} = \frac{\text{Measured size in mm} \times 10,000,000}{\text{Magnification}}
\]

UV absorption profile

The OD value of the purified virus was measured at 260 and 280 nm in Pye Unichem Sp-8-400 UV/VIS spectrophotometer. The A260/A280 ratio was calculated.

**Determination of viral genome**

**Extraction of nucleic acid**

Nucleic acid was extracted from purified virus preparation by the method of Lot et al. (1991). The virions were treated with proteinase K (50μg/ml in 10 mM Tris HCL, pH-8.0, 5mM EDTA) in the presence of 0.5% SDS at 65°C for 1h. The suspension was extracted three times with phenol-chloroform 1:1 (V/V) and once with chloroform alone. The nucleic acid was finally precipitated by adding 2.5 vol. of ethanol in the presence of 0.03M sodium acetate. The precipitated nucleic acid was first washed with 70% ethanol and then with absolute ethanol. Finally the preparation was sus-
Table 2. Optimum conc. of antibody and antigen in DAC-ELISA for citrus mosaic virus

<table>
<thead>
<tr>
<th>Antibody dilution</th>
<th>Buffer</th>
<th>Healthy</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:2</td>
<td>1:20</td>
<td>1:2</td>
</tr>
<tr>
<td>1:100</td>
<td>0.03</td>
<td>0.08</td>
<td>0.0</td>
</tr>
<tr>
<td>1:200</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>1:300</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>1:400</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>1:500</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Electrophoresis

Electrophoresis was done in 1% agarose gel. 5μl of nucleic acid was mixed with 2μl of buffer, 1 μl RNase (Promega chemical co. USA) and 12 ul of distilled water separately in eppendorf tubes. The preparation was incubated at 37°C for 3-4 h for digestion. After the incubation, 2μl of loading dye was added and spun for short time. Electrophoresis was carried out for 1h at 80 volts and electrophoresed gel was stained with ethidium bromide.

Production of antiserum

The purified virus preparation was injected to white albino rabbit, at weekly intervals. Four weekly injections were given with 750μl of purified virus (1mg/m) mixed with equal amount of Freund's incomplete adjuvant. Ten days after the last injection, the rabbit was bled and antiserum was obtained by usual decantation method. The optimum titre of the antiserum was determined for ELISA and IEM.

Serological relationship

To determine serological relationship of citrus mosaic virus with other badnaviruses, antisera of following badnaviruses were obtained (courtesy Prof. BEL Lockhart, Department of Plant Pathology, Univ. of Minnesota, St. Paul USA): banana streak virus (BSV), Morrocco, Rwanda and mysore (Trinidad) isolates, sugarcane bacilliform virus (ScBV), kalanchoe top spotting virus (KTShV), discorea bacilliform virus (DBV), cocoa swollen shoot virus (CSSV) and commelina yellow mottle virus (CoYMV). These antisera were used in IEM at a dilution of 1:50 with the antigen of citrus mosaic virus.

RESULTS

Symptoms

The virus was mechanically transmitted to all the ten inoculated plants of mosambi (Fig. 1), decumana and galgal but not to kinnnow, Nagpur mandarin and kagzi lime. Infected plants showed typical bright yellow mosaic symptoms after 80 days of inoculation. Presence of the virus in plants showing symptoms was confirmed by electron microscopy. However, plants inoculated by three aphid and three mealybug species neither developed any symptoms nor the virions were observed even after 6 months of inoculation.

Host range

Variable symptoms of the disease were observed in 14 citrus species/cultivars out of 15 inoculated (Table-I). However, C. aurantifolia did not show any disease symptoms and results of back inoculations were also negative.
Fig. 1. Typical mosaic symptoms on the leaves of mosambi sweet orange plant. Leaf at left is from healthy plant.

Fig. 2. Electron micrograph of a purified preparation of sample collected from mosaic infected sathgudi sweet orange tree from Bangalore showing CRSV, CTV and CMBV particles. Mag. 80,000. Bar=100nm
1. CRSV, 2. CTV, 3. CRSV
Purification and electron microscopy

The virus was successfully purified by the protocol as mentioned in Materials and Methods. Purified preparation when examined in EM showed large number of virus particles which were non-enveloped and bacilliform in shape measuring 125-130nm in length and 25-30nm in width (Fig. 3a).

The UV absorption profile of purified virus showed OD values at 260 nm as 0.694 and at 280 nm as 0.542. The $A_{260}/A_{280}$ ratio was 1.28 suggesting the virus preparation as nucleoprotein in nature and close to that of badnaviruses (Lockhart, 1994).

Fig. 3a. Bacilliform virions isolated from mosaic inoculated plants of mosambi sweet orange. Mag. 80,000. Bar=100nm

Fig. 3b. Decoration of CMBV with homologous antiserum at dilution of 1:50. Mag. 80,000. Bar=100nm
Determination of viral genome

The results of electrophoresis of nucleic acid on 1% agarose gel are given in Fig. 4. It is evident from Fig. 4 that lane 2 treated with RNase showed two bands of DNA comprising 23.1 Kb and 6.6 Kb. The slow migrating band of 23.1 Kb represented circular form of DNA whereas the faster migrating band of 6.6 Kb represented the linear molecules arising during DNA extraction. However, no band was visible in lane 3 which was treated with DNase indicating that the viral DNA was completely digested with DNase. This confirms that genome of citrus mosaic virus is a DNA.

Enzyme-linked immunosorbent assay and immuno-electron microscopy

Direct antigen coating ELISA (DAC-ELISA) was performed using whole serum at various dilutions. The optimum virus detection was obtained with antigen dilution of 1:2, antibody dilution of 1:500 and conjugate dilution of 1:2000 (Table-2). For IEM tests, the optimum dilution for trapping was 1:200 while for decoration it was 1:50 (Fig. 3b). Incubation played an important role for efficient trapping of virus particles. In this case maximum number of particles were trapped when antibody coated grids were stored at 4°C overnight on diseased sap.

Serological relationship

It was found that all the eight antisera of badnaviruses viz: banana streak virus (BSV), Morocco, Rwanda and Mysore (Trinidad) isolates, Sugarcane bacilliform virus (ScBV), kalanchoe top spotting virus (KTSV), dioscoria bacilliform virus (DBV), cocoa swollen shoot virus (CSSV) and commelina yellow mottle virus (CoYMV) decorated the virions of citrus mosaic in IEM but to a varying degree. However maximum decoration was obtained with homologous antiserum followed by sugarcane bacilliform virus showing close serological relationship of citrus mosaic virus with ScBV.

DISCUSSION

Mosaic diseases of citrus have been reported from India and Japan. (Reddy et al., 1972; Murthy and Reddy, 1975; Ahlawat et al., 1985; Ishigai and Jinno, 1958; and Kishi, 1967). The present disease is similar in symptomatology to the citrus mosaic disease reported from India but different from the Japanese citrus mosaic in host range and also in virion morphology. The Indian citrus mosaic has bacilliform particles as against spherical virions associated with the Japanese citrus mosaic (Tanaka and Yamada, 1972).

The present virus isolate was transmitted by graft and also by mechanical inoculations to C. decumana, C. sinensis and C. limon and thus obtained pure culture of the virus which would have been otherwise difficult as the original source of the virus showed a mixed infection of three viruses.

The mosaic virus of citrus reported earlier
(Ahlawat et al., 1985, Reddy et al., 1972) was transmissible by aphids, *Myzus persicae*, *Aphis citricola* and *Toxoptera citricidus*. However, during our studies these aphids failed to transmit the disease agent suggesting that this isolate may be a different strain of the mosaic virus.

This isolate was not transmitted to any non-citrus host but was transmitted to 14 out of 15 citrus species/cultivars inoculated. *C. aurantifolia* was not infected by citrus mosaic virus in repeated tests and hence was considered to be resistant to mosaic infection.

The present virus had bacilliform particles measuring 125-130nm × 25-30nm. A bacilliform virus (100-120×40nm) is associated with citrus leprosis disease reported from Brazil (Kitazima et al., 1972). However, these two viruses belong to two different groups of viruses i.e. leprosis to rhabdovirus group and mosaic to badnavirus group, showing clear distinction between them.

The badnavirus like nature of the Indian citrus mosaic virus was confirmed by electron microscopy, nuclease digestion, electrophoresis and serological relationship. It was serologically related to Sugarcane bacilliform virus (ScBV) and eight other badnaviruses. The virion morphology, genomic studies and serological relationship have provided enough evidence to the effect that the Indian citrus mosaic virus is a definite member of badnavirus group which is a new record.

REFERENCES


