Association of cucumovirus and potyvirus with betelvine (Piper betle L.) as evidenced by ELISA and RT-PCR

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Betelvine (Piper betle L.) is an important cash crop in Asia. India has a trade of worth Rs. 700 crores. Singh in 1987 recorded the natural infection of Tobacco mosaic virus (TMV) on betelvine for the first time in India. Johri et al. (1990) also reported a Potyvirus on betelvine after purification of the virus and examination by electron microscope. In present study, an attempt has been made to investigate the association of different viruses in betelvine. Enzyme linked immuno-sorbent assay (ELISA) was conducted for detection of Cucumber mosaic virus (CMV) and Bean yellow mosaic virus (BYMV) using antibodies to the respective viruses. We also attempted RT-PCR for amplification of cDNA fragments of both the viruses separately using RNA extracted from betelvine samples and CMV/potyvirus specific primers for providing molecular based evidence for their natural occurrence on betelvine.

A survey of betelvine fields was conducted at Mahoba and Banthara research stations, India. Betelvine leaf samples were collected from plants that apparently showed various symptoms of virus diseases. Enzyme linked immuno-sorbent assay (ELISA) for Cucumber mosaic virus (CMV) and Bean yellow mosaic virus (BYMV) were performed as described earlier. DAC-ELISA was performed for 10 samples collected from Banthara and the green house at National Botanical Research Institute, Lucknow, India. The healthy leaf tissue of betelvine was taken as negative control. For DAC-ELISA, antigen was prepared by crushing leaf tissue (25 mg) in extraction buffer containing PVP (2%) in PBS-T (20 mM, sodium phosphate; 150 mM, sodium chloride, pH 7.4 + 0.05% Tween 20) and serial dilutions (1: 100; 1:500; 1:1000) of antigens were made. The wells of ELISA plate were coated with various dilutions of antigens and incubated at 4°C overnight. The wells were washed two times with water and three times with PBS-T, blocked with 5% milk prepared in PBS-T and kept for half an hour at room temperature. Antisera of CMV (PVAS 242a, purchased from ATCC, USA) and of BYMV (raised earlier in our lab) were diluted (1:1000) in PBS-TO (0.2% Ovalbumin) used for coating the wells. The antibodies were added in the respective wells and incubated at 37°C. The wells were washed three times with water and three times with PBS-T. Anti-rabbit IgG alkaline phosphatase conjugate, dilution (1:5000) in PBS-TPO was added and left for incubation for 2 hr at room temperature. The wells were washed three times with PBS-T and the substrate (p-nitro phenyl phosphate) in 10% diethanolamine buffer (pH 9.5) was added. The plate was left for 1 hr in dark to develop yellow color and reaction was stopped with NaOH (4 N). Readings were taken in a Bio-Rad ELISA reader at 405 nm.

RT-PCR was conducted separately with 6 samples collected from Mahoba Research Station, India, which showed various types of virus disease symptoms (Table 1). For isolation of nucleic acid, 100 mg of betelvine leaf tissue were ground in 500 µl extraction buffer (20 mM tris, pH 8.3, 5 mM magnesium chloride, 0.1% Tween 20, 100 mM potassium chloride, 0.02% gelatin) containing 2-beta mercaptoethanol (BME; 1%). To the sap added equal volume of phenol:chloroform (1:1), mixed and centrifuged (10,000 rpm) for 5 min at room temperature. The
supernatant was transferred to a fresh tube and added ethanol (2 vol.). It was then kept at -20°C for precipitation and centrifuged (10,000 rpm) again at 4°C for 20 min. The nucleic acid obtained was washed with 70% alcohol, dried and the pellet re-suspended in RNAse free sterile water.

A pair of upstream (P7: 5'-AACAATGGACAAA-TCTGAAATCAACCAG-3') and downstream (P8: 5'-TCAAAATCGGGACACCCAGACG-3') primers was designed to amplify the complete coat protein gene of CMV including initiation and termination codons. The primers (P7 and P8) could yield a product of 657 bp. The potyvirus primers described by Gibbs & Mackenzie were used to amplify 1.6 kb and ~600 bp of cDNA fragments. Both the sets of primers were synthesized by Genset Singapore Biotech Pvt. Ltd., Singapore. RT-PCR reactions were carried out separately for BYMV and CMV as described earlier. To 10 μl of RNA template, 1 μl of downstream primer was added. The mix was heated for 1 min in boiling water and cooled at room temperature. To this mix then added RT buffer (5 X), RNA guard (20 U), dNTPs (2 mM each) and RT (10 U) and the volume made up to 20 μl. cDNA synthesis was carried out at 42°C for 90 min after which the nucleic acid was precipitated for overnight in ethanol containing 0.3 M sodium acetate, pH 5.2.

The precipitated cDNA was centrifuged for 15 min at 4°C at 12,000 rpm, washed with alcohol (70%), dried and resuspended in 30 μl of sterile water. cDNA (5 μl) was subjected to PCR. PCR mix containing (Taq buffer 10×, 25 pmole each of downstream and upstream primers, 20 μM each of dNTPs, 3 U Taq DNA polymerase and 1.5 mM of MgCl₂) components apart from cDNA in a total volume of 50 μl. Amplification was performed in Perkin-Elmer 9600 Gene amplification PCR system and was initiated by denaturing for 5 min at 94°C, 1 cycle, followed by 30 cycles of 1 min each at 94°C, 1 min at 52°C and 1 min at 72°C and finally completed with 1 cycle of 5 min at 72°C. Reaction products were resolved by electrophoresis on 1.2% (w/v) agarose gels as described earlier, stained with ethidium bromide (0.5 μg/ml) and viewed on UV transilluminator.

Ten leaf samples of betelvine collected from Banthara and the green house at NBRI, India, showed mild symptoms of virus infection. Six samples collected from Mahoba Research Station showed severe symptoms of yellow mosaic, leaf curling, severe

![Fig. 1](image-url)
mosaic, yellow vein, mosaic accompanied with pinhead necrotic lesions and tip and marginal necrosis.

DAC-ELISA was conducted with the leaf sap extracted from 10 samples collected from Banthara. OD at 405 nm of 10 betelvine samples obtained by DAC-ELISA using antibodies to CMV have been plotted in Fig.1. Among three dilutions of antigen (leaf sap) used, the dilution 1:100 was found most suitable for obtaining best results as indicated in Fig. 1. Results of DAC-ELISA indicated presence of CMV in 7 samples (no. 1, 2, 3, 5, 6, 7 and 8) out of 10 based on OD > 0.15. Rest of the samples (no. 5, 9 and 10) indicated OD less than 0.15 as compared to the above samples, but more than the healthy and buffer samples taken as negative control. Concentration of the virus was high in 1, 2, 5 and 6 based on OD > 0.2 and sample 3, 7 and 8 were < 0.2, but > 0.15, so they were considered as moderate, whereas sample no. 9 and 10 were showing OD < 0.15 but were > 0.1, they were considered as weak signals with low concentration of CMV. Sample no.4 had OD of about 0.11, but more than negative control and therefore considered as non-specific reaction and not virus infection. DAC-ELISA using BYMV antibodies did not indicate presence of potyvirus in any of the 10 samples tested, as OD was very low, < 0.15 (data not shown).

RT-PCR was conducted in 6 samples collected from Mahoba Research Station, India, which showed severe symptoms of viral disease. Results of RT-PCR using CMV and potyvirus specific primers have been shown in Fig. 2A and B respectively and also summarized in Table 1. RT-PCR using specific primers to CMV indicated the presence of CMV in 4 samples out of 6 tested, based on presence of amplification product of ~700 bp (657 bp precisely) as observed on agarose gel during electrophoresis of RT-PCR products (Fig. 2A). Intensity of ~700 bp band in sample 3 showing severe mosaic symptoms was prominent, while in 4, 5 and 6 showing yellow vein, tip and margin necrosis and pinhead necrotic lesion respectively were moderate. No CMV could be detected in samples 1 and 2 showing yellow mosaic and leaf curl symptoms respectively (Fig. 2A, Table 1).

RT-PCR using primers of potyvirus resulted in PCR amplicons of about ~600 bp and ~1.6 kb in 5 out of 6 betelvine samples tested. Intensity of PCR amplicons was prominent in the sample numbers 1 and 6, which showed yellow mosaic and mosaic accompanied by pinhead necrotic lesions respectively (Fig. 2B). Intensity of the amplicons in the samples number 3, 4 and 5 showing symptoms of severe mosaic, yellow vein and tip and margin necrosis respectively was moderate. However, no potyvirus infection could be detected in sample number 2 (Fig. 2B, Table 1).

Results of RT-PCR summarized in Table 1 also indicated mixed infection of CMV and BYMV in samples 3, 4, 5 and 6 showing yellow mosaic, yellow vein, tip and margin necrosis, and mosaic accompanied with pinhead necrotic lesions respectively. Presence of CMV and BYMV in these betelvine samples may be due to mixed infection of both the viruses at

![Fig. 2 — Agarose gel electrophoresis of RT-PCR products from leaf extracts of betelvine using primers specific for (A) CMV; (lane M: Marker Lambda DNA EcoRI / HindIII double digest (Bangalore Genei Pvt. Ltd); lane C- PCR product from CMV-A inoculated Nicotiana rustica,(+) ve control; lane 1-6: PCR products from betelvine samples showing an amplification of ~700bp of CMV-CP), and (B) Potyvirus; (lane M: Marker Lambda DNA/ EcoRI digest (Bangalore Genei Pvt. Ltd); lane 2- PCR product from BYMV infected Amarathus, (+) ve control; lanes (2-6)-PCR products from betelvine samples (1-6) showing amplification of ~600 bp and 1.6Kb).

### Table 1 — RT-PCR detection using CMV or BYMV specific primers

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Symptoms observed</th>
<th>RT-PCR</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>CMV</td>
</tr>
<tr>
<td>1</td>
<td>Yellow mosaic</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>Leaf curling</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>Mosaic</td>
<td>+++</td>
</tr>
<tr>
<td>4</td>
<td>Yellow vein</td>
<td>++</td>
</tr>
<tr>
<td>5</td>
<td>Tip &amp; marginal necrosis</td>
<td>++</td>
</tr>
<tr>
<td>6</td>
<td>Mosaic with pinhead lesions</td>
<td>++</td>
</tr>
<tr>
<td>7</td>
<td>CMV infected Amarathus*</td>
<td>++++</td>
</tr>
</tbody>
</table>

*Samples taken as positive control for CMV, NT= not tested.
Mahoba fields from where the samples were collected. BYMV infection alone could be detected in only one sample (no.1), which showed yellow mosaic symptoms.

RT-PCR using specific primers for detection of CMV or BYMV failed to detect both the viruses from sample number 2 showing leaf curl symptoms (Fig. 2A, B). The geminivirus infection in betelvine showing typical leaf curl symptoms may be suspected, and need to be confirmed by whitefly transmission and/or PCR amplification with geminivirus specific primers.

Natural occurrence of TMV and a potyvirus have been recorded earlier on betelvine. Johri, et al. have observed two bands after density gradient centrifugation during their particle purification of the virus from betelvine, the upper band contained flexuous rods (approx. 720 nm size) which they have identified as a potyvirus but no such particle could be detected in the lower one, hence, presence of another type of virus has been suspected. Present report of a potyvirus infection in betelvine supported the earlier findings. However, association of CMV with betelvine seems to be the first report of its natural occurrence.

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References
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