New natural host of *Tobacco mosaic virus* on three cucurbits in Java, Indonesia

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Abstract. *Tobacco mosaic virus* (TMV) is a newly emerging virus on cucumber in Java. Regardless the importance of TMV on Cucurbitaceae, little is known about the existence of TMV in Cucurbitaceae plants. The study aimed to detect and identify TMV on bitter gourd, ridged gourd, and watermelon. Symptomatic leaves were collected from several cucurbit cultivations at 5 regencies in West Java, Central Java and East Java provinces. The frequency of virus on samples were determined serologically by DIBA method using specific antisera to 6 viruses (CMV, CABYV, PRSV, SqMV, TMV, and ZYMV). Nucleic acid detection was conducted by RT-PCR using universal primer for *Tobamovirus* followed by DNA sequencing to confirm virus identity. The results revealed that samples were infected by 6 viruses with frequency varied and multiple infections occurred. The frequency of TMV infection ranged from 5.5 to 24.4%. RT-PCR successfully amplified DNA fragment with size ± 800 bp from bitter gourd, ridged gourd, and watermelon. The similarity of coat protein gene among isolates reached up to 100%. These TMV isolates showed highest nucleotide and amino acid similarity with cucumber isolate from Brebes regency and other isolates infecting several different crops from other countries. This is a new report on the existence of TMV on different cucurbit species in the fields.

1. Introduction

*Tobamovirus* members are well known as devastating viruses infecting many crops such Solanaceae, Brassicaceae, Cucurbitaceae and ornamental plants [1-4]. *Tobacco mosaic virus* (TMV) is a typical member of the *Tobamovirus* in the family Virgaviridae. TMV is one of the most infective plant viruses. TMV can survive in crop debris for months and the infection occurs mainly by contact between roots and infected soils, and by workers’ contact during cultural practice activity. Its spread can occur by leaf contact and seed transmission [5]. The virion is a rigid rod particle with a size of about 18 nm x 300 nm containing a single strand RNA [6, 7].

Recently, we found natural multiple infections of TMV with other viruses in cucumber cultivation areas in Java. The frequency varied based on serological tests ranging from 5.5 to 24.4%. The identity of TMV cucumber isolates from Java was closed to corresponding isolates from China [8]. TMV was detected almost in all cucumber sampling areas in Java and it presumably has potential as an essential emerging disease not only on cucumber but also on other crops in the future. A previous study reported that TMV infected cucurbits, non-cucurbits crops and weeds in Iran [9]. These raise a
curiosity to conduct further observation of TMV infection on other cucurbits species as its natural alternative hosts in the fields.

2. Methods

2.1. Observation of virus-like symptom in the fields and samples collection

Virus-like symptomatic samples were collected from 5 regencies i.e. Bogor and Subang (West Java province), Brebes and Klaten (Central Java province) and Nganjuk (East Java province). A total of 35 samples of bitter gourd leaves was collected from Bogor and Subang, 63 samples of ridged gourd from Klaten, and 220 samples of watermelon from Brebes and Nganjuk. All samples were stored in a deep freezer at -80°C until used.

2.2. Detection of virus and its frequency by dot immunobinding assays (DIBA)

To determine the frequency of viruses, samples were detected serologically by DIBA method using antisera to Cucumber mosaic virus (CMV, DSMZ), Cucurbit aphid-borne yellows virus (CABYV, DSMZ), Papaya ringspot virus (PRSV, DSMZ), Squash mosaic virus (SqMV-Agdia), Tobacco mosaic virus (TMV, DSMZ) and Zucchini yellow mosaic virus (ZYMV, DSMZ).

A total of 0.1 g of each sample was ground in a small plastic bag with 1 mL of TBS buffer. The sap was blotted on nitrocellulose membrane and allowed to air dried. Subsequently, the DIBA was conducted according protocol provided [10].

The frequency of each virus infection was determined using a formula:

\[
\text{Virus frequency (\%)} = \left( \frac{\text{Number of positive samples}}{\text{Total samples tested}} \right) \times 100\%
\]

2.3. Total RNA extraction and cDNA synthesis

Total RNAs of 0.1 g TMV positive samples representative from each location were conducted using cetyl trimethyl ammonium bromide (CTAB) method [11]. Total RNA was diluted in 50 µL nuclease-free water and stored at -20°C.

The cDNA synthesis was carried out in a total volume of 10 µL. The reaction consisted of 2 µL, 0.25 µL RNAse Inhibitor (Ribolock, Thermo Fisher Scientific Waltham, MA, USA), 10 mM 0.5 µL dNTP, 1.0 µL of 10 µM d(T), 2.0 µL 50 mM DTT, 0.5 µL MMuLV (Revertaid, Thermo Fisher Scientific, USA) and nuclease-free water until total volume 10 µL. The procedure of cDNA synthesis was conducted according to protocol provided by Thermo Fisher Scientific (Waltham, MA, USA).

2.4. RT-PCR

RT-PCR was carried out using universal primer for Tobamovirus, i.e. Tob1 and Tob2, with an expected size of DNA amplicon ± 800 bp [12]. RT-PCR was conducted in a 25 µL premix mixture containing 12.5 µL Go Taq Green (Thermo Fisher Scientific, Waltham, MA, USA), 1 µL cDNA template, 1 µL of 10 µM of each primer, and 9.5 µL nuclease-free water.

The DNA product was analysed on 1% agarose gel electrophoresis in 0.5X TBE buffer containing nucleic acid staining dye FlouroVue TM (Smobio, Taiwan) at 100 V for 30 min. The DNA was visualized under UV transilluminator and documented by digital camera.

2.5. DNA sequencing and analysis

DNA from PCR product was gel purified and directly sequenced. The sequences identity matrix of TMV CP gene was compared to corresponding isolates from other countries available in the GenBank database using Bio Edit v7.05 program. The phylogenetic tree analysis was constructed by MEGA v6.0 software with the neighbor-joining algorithm and bootstrap value 1,000 replicates [13].
3. Results and discussion

3.1. Virus infection symptoms in the fields
Samples collected from the fields showed varying symptoms depends on plant species. The symptoms on bitter gourd involved mosaic, mosaic with vein banding, chlorosis to yellowing with vein banding (Fig. 1a-d); virus infection on ridged gourd induced chlorosis mosaic, chlorosis to yellowing with green vein banding (Fig. 1e-h); while on watermelon showed mosaic with vein banding, chlorosis with leaf curl and necrosis (Fig. 1i-l).

It was difficult to differentiate viruses based on phenotypic symptoms, since multiple infections of more than two viruses was found very frequently. Furthermore, symptom expressions depend on environmental conditions, type of virus, virus strain and plant variety.

![Symptomatic samples collected from bitter gourd (a-b, Bogor and c-d, Subang), ridged gourd (e-h, Klaten) and watermelon (i-j, Nganjuk and k-l, Brebes). The type of symptoms involved: mosaic (a,e,j), mosaic with vein banding (i), mosaic with leaf curl and vein banding (b), chlorosis mosaic with vein banding and necrosis (f), leaf curl, chlorosis and necrosis (k), chlorosis with vein banding (c,d,g,h), leaf curl (l) and leaf deformation (b, f, k, l).](image)

3.2. Virus frequency
The identity of viruses on collected samples showed almost all samples infected by multiple viruses. Based on DIBA, the frequency of TMV ranged from 5.88 to 89.58%, PRSV ranged from 11.76 to 87.50%, CAYBV ranged from 23.83 to 83.33%, CMV ranged from 8.72 to 88.89%, SqMV ranged from 8.33 to 39.69%, and ZYMV ranged from 8.33 to 61.11%, respectively (Fig. 2). The frequency of TMV infection showed about 61.11% on bitter gourd in Bogor, 17.46% on ridged gourd in Klaten and the highest frequency about 89.58% on watermelon in Nganjuk.

Multiple infection of 2 to 6 viruses occurred among samples with various frequency. The highest frequency of multiple infection was found for 3 viruses (TMV+PRSV+CAYBV), i.e. 45.83% on watermelon in Nganjuk regency; while the lowest multiple infection was found for 5 viruses
(TMV+PRSV+CABYV+SqMV+ZYMV) and (TMV+PRSV+CABYV+CMV+ZYMV), i.e. 0.58% on watermelon in Brebes regency. Multiple infection caused more severe symptoms than single infection and also caused more difficult to identify the causal viruses.

![Figure 2](image)

**Figure 2.** Frequency of 6 viruses from bitter gourd, ridged gourd and watermelon detected serologically by DIBA method. Bgr, Bogor; Sb, Subang; KL, Klaten; Ngj, Nganjuk.

### 3.3. RT-PCR

RT-PCR using universal primer of *Tobamovirus* successfully amplified the DNA with size ± 800 bp (Fig. 3) only from 3 samples, i.e. bitter gourd with mosaic symptoms from Bogor (Fig. 1a), ridged gourd with vein banding and necrosis symptoms from Klaten (Fig. 1f) and watermelon with mosaic and vein banding symptoms from Nganjuk (Fig. 1i). The amplicon was obtained from samples with strong reaction on DIBA. The amplified DNA covers 480 bp of the CP region and partial sequences of movement protein gene at 5' and flanking regions of the CP gene at 3'.

![Figure 3](image)

**Figure 3.** Amplified DNA by RT-PCR using universal primer for *Tobamovirus*. The target DNA size is ± 800 bp. BG, Bitter gourd; RL, Ridged gourd; WM, Watermelon, M. Ladder DNA 1kb (Thermo Fisher Scientific, Waltham, MA, USA).

### 3.4. DNA analysis

DNA sequencing successfully revealed full-length size of 480 nucleotides, encoded 159 amino acids. BLAST analysis confirmed the identity of TMV from those 3 samples. The sequences of 3 TMV isolates were deposited in GenBank database with accession number LC413506, LC390164, and LC385824 for bitter gourd, ridged gourd and watermelon isolates, respectively.

The similarity among 3 isolates is up to 100% for both nucleotide and amino acid level. The 3 isolates closed to cucumber isolates from Brebes, Kulon Progo, Kediri and Indramayu, Indonesia. However, those isolates less closed to tobacco isolates (LC390329) from Jember, Indonesia (Table 1).

Phylogenetic tree analysis revealed that there are 5 different clades. Clade I consisted of isolates from different countries from Asia, Africa, America, and Europe; clade II consisted of isolates from
China. The TMV isolates from bitter gourd, ridged gourd and watermelon was in the same clades III along with cucumber isolates from Java; while isolates from Iran and Japan was in clades IV and V, respectively (Fig. 4).

**Table 1.** Comparison of TMV CP gene sequences of bitter gourd, ridged gourd and watermelon isolates to corresponding sequences in GenBank database.

| TMV Isolates (country)** | % Similarity of nucleotide (nt) and amino acids (aa) | Host | Accession |
|--------------------------|---------------------------------------------------|------|-----------|
|                          | Bogor     | Klaten | Nganjuk | Host         | Accession |
|                          | nt         | aa    | nt     | aa          |           |
| IDN-Bogor                | 100       | 100   | 100    | 100         | Bitter gourd | LC413506 |
| Klaten                   | 100       | 100   | 100    | 100         | Ridged gourd | LC390164 |
| Nganjuk                  | 100       | 100   | 100    | 100         | Watermelon  | LC385824 |
| Brebes                   | 100       | 100   | 100    | 100         | Cucumber    | LC311785 |
| Kulon Progo              | 98        | 96    | 98     | 96          | Cucumber    | LC311788 |
| Kediri                   | 97        | 94    | 97     | 94          | Cucumber    | LC311787 |
| Indramayu                | 96        | 92    | 96     | 96          | Cucumber    | LC311786 |
| Jember                   | 92        | 96    | 92     | 96          | Tobacco     | LC390329 |
| DEU                      | 97        | 99    | 97     | 99          | Tobacco     | AJ429078 |
| KOR                      | 97        | 99    | 97     | 99          | Tobacco     | X68110 |
| USA                      | 97        | 99    | 97     | 99          | Tobacco     | V01408 |
| CAF                      | 97        | 99    | 97     | 99          | Eggplant    | AY360447 |
| IND                      | 97        | 99    | 97     | 99          | Soybean     | JQ895560 |
| CHN                      | 96        | 98    | 97     | 98          | Tobacco     | AF395128 |
| ESP                      | 96        | 99    | 97     | 99          | Arabidopsis | KF972435 |
| SVN                      | 96        | 99    | 97     | 99          | Tomato      | KY810785 |
| JPN                      | 93        | 96    | 93     | 96          | Tobacco     | D63809 |
| IRN                      | 91        | 99    | 91     | 91          | Watermelon  | HQ593620 |
| KGMMV-IDN                | 51        | 51    | 51     | 51          | Melon       | AB162006 |
| CGMMV-JPN                | 47        | 49    | 47     | 49          | Watermelon  | AB015146 |

**a** KGMMV Indonesian isolate and CGMMV Jepang isolate as outgroups, IDN: Indonesia, DEU: Germany, IRN: Iran, KOR: Korea, USA:United State of America, CAF: Africa, IND: India, CHN: China, ESP: Spain, SVN: Slovenia, JPN: Japan, nt: nucleotide, aa: amino acid.

A previous study reported that cucumber was found for the first time as natural host of TMV in Java [8]. Experimentally, TMV was able to infect melon, watermelon, pumpkin, yard long bean, French bean and soybean as systemic hosts [14]. This study revealed that bitter gourd, ridged gourd, and watermelon as a new natural host of TMV in the fields. TMV and other *Tobamovirus* members has broad host ranges and transmit easily by contact between infected to healthy plants, mechanically via culture practice tools, persist in soil as plant debris, and contaminate seeds produced by infected plants. Based on those characters, the expansion of TMV to other hosts will increase the difficulty on virus management and will bring an adverse impact on cucurbit production in Indonesia.

In the future, TMV may cause a significant yield loss on crops production as an emerging disease in Indonesia. The spread of *Tobamovirus* worldwide is facilitated by global seed trading and its adverse effect on it, will cause an emerging disease in new areas or countries [15]. Utilizing virus-free seeds and restrained seed introduction from other countries will become a rational strategy to mitigate the virus and its distribution in Indonesia.

The existence of *Tobamovirus* species including TMV on vegetables, ornamental plants, and weeds from different areas in Indonesia is necessary to study. The study will provide more data related to the *Tobamovirus* species identities, their biological and genetic diversity and strategies to manage the virus.
Figure 4. Phylogenetic tree of CP gene sequences of TMV isolate bitter gourd (LC413506), ridged gourd (LC390164) and watermelon (LC38524) from Java against 15 corresponding isolates in GenBank database. Cucumber green mottle mosaic virus (CGMMV) and Kyuri green mottle mosaic virus (KGMMV) are used as outgroups. The phylogenetic tree was constructed by neighbour-joining method using MEGA v6.0 software. Bootstrap values expressed as a percentage of 1,000 replicates, that is greater than 70 are shown on tree branches.

4. Conclusion
This study describes the new finding on natural host of TMV on 3 different species of cucurbits in Java. The existence of TMV bitter gourd, ridged gourd, and watermelon isolates are confirmed genetically and showed in the same clade III along with cucumber isolates and separated from other isolates from different countries. Furthermore, multiple infections of several viruses found as common phenomena on those three cucurbit species.

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