First Report of Tobacco Mosaic Virus on Cucumber [Cucumis sativus (L.)] in Java, Indonesia

Listihani1, S H Hidayat1, S Wiyono1 and T A Damayanti1*

1Department of Plant Protection, Faculty of Agriculture, Bogor Agricultural University, Indonesia

*E-mail: triadys@apps.ipb.ac.id

Abstract. A mosaic and mottle symptom was observed on several cucumber cultivations in Java, Indonesia. The study was aimed to characterize the causal virus of typical Tobamovirus-like symptoms. Symptomatic leaves were collected randomly from each location in Nganjuk, Kediri, Tulungagung (East Java), Brebes and Klaten (Central Java), Kulon Progo (Yogyakarta), and Subang, Indramayu, Bogor (West Java). The diseases frequency was determined by dot blot immuno binding assays (DIBA) test using specific antiserum of Cucumber green mottle mosaic virus (CGMMV). Furthermore, the Tobamovirus was detected by RT-PCR, then cloned into TA-cloning vector, and the DNA was sequenced. The disease frequency of CGMMV in East Java, Central Java, Yogyakarta, and West Java was up to 24.44%, 9.09%, 0%, and 5.49%, respectively. CGMMV was unable to be amplified by specific primer, however it was able to be amplified by universal primer of Tobamovirus with sized ± 800 bp. Nucleotide sequence analysis of samples which gave positive reaction against CGMMV antisera were identified as Tobacco mosaic virus (TMV). The homology of nucleotide and amino acid sequences analysis ranged from 95.0-97.7% and 98.1-99.4%, respectively. This is the first report of TMV infection on cucumber in Indonesia.

Keywords: CGMMV, cucumber, TMV, Tobamovirus

1. Introduction
Cucumber is one of horticulture crop that widely cultivated in Indonesia. Many factors affected its production. On 2012-2016 the cultivation area was contribute 5.45% of total of vegetables cultivation area and its production was contribute 7.25% of total production of vegetables in Indonesia [1]. Based on present data its production fluctuated and tends to decrease every year. In addition, in the field cucumber always show unhealthy because of the infection by much type of pathogens, including virus.

There are 32 species of viruses reported that can infected cucumber, including one of Tobamovirus member, Cucumber green mottle mosaic virus (CGMMV) [2,3]. Tobamoviruses have a very wide host range and can cause serious economic impact in many crops, i.e. cucurbits, brassicas, solanaceous, and ornamental plants, for instance chrysanthemums, impatiens, and petunia [2,4,5]. Infected plants showed different type of symptoms, i.e. mosaic, malformation, mottle, and stunting. Previously, infection of one member of Tobamovirus, Tobacco mosaic virus (TMV) on several crops in Iran, i.e. tomato, cucumber,
and pepper have been reported causing up to 59% and 90% of yield loses, respectively [5-7]. Generally, *Tobamovirus* can be easily transmitted by mechanically, seed, contact between plants, but not transmitted by vector [5] and the debris can become the most important sources of inoculums in the fields [8].

Recently on 2016, we conducted a field survey to collected cucumber’s leaves with typical symptom of virus-like disease from several cultivation areas in Java. Symptomatic samples showed vary such as mosaic, yellow mosaic, yellowing, yellowing with green veinal, etc. It was difficult to recognize the causal virus only based on phenotypic symptoms, because multiple infection of viruses are common phenomena in the fields. Based on serological test by dot blot immunobinding assays (DIBA) with several antisera, we found some samples react positively against CGMMV antisera. CGMMV is one of plant quarantine pest target which is considerate not present yet in Indonesia. Here, we reported the biological, physical and molecular characters of samples, which were react positively against CGMMV antisera to confirm its existence in Java, Indonesia.

2. Materials and methods

2.1. Samples collection
Samples were collected from several cucumber cultivations in Java, i.e. West Java (Bogor-Bgr, Subang-Sbg, and Indramayu-Idr regions), Central Java (Brebes-Brbs and Klaten-Kla regions), Yogyakarta (Kulon Progo-KP region) and East Java (Nganjuk-Ngi, Kediri-Kdr, and Tulungagung-Tlg regions). A total of 543 leaves samples were collected by purposive sampling method.

2.2. Serological etection
The disease frequency of virus was determined based on dot blot immunobinding assay (DIBA) test using *Cucumber green mottle mosaic virus* (CGMMV) antiserum. The DIBA test was conducted according to protocol provided by Towbin [9].

2.3. Source of inoculum and host range test
To obtain the source of inoculum, symptomatic leaves from Bogor (Bgr) was mechanically inoculated on indicator plant *Nicotiana glutinosa* for three times serially. The lesion local necrotic (LLN) was collected, then used as inoculum to propagate the virus on *N. tabacum*.

Host range test was conducted using *Tobamovirus* Bogor isolate. Symptomatic leaves were ground in 0.025 M phosphate buffer pH 7.0 containing 1% β-mercaptoethanol using mortar and pistil. Sap was mechanically inoculated on the test plants. The test plants consisted of 23 plants species from 10 families, i.e. Cucurbitaceae (*Cucumis sativus, Cucumis melo, Cucurbita moschata, Momordica charantia, Luffa acutangulata*), Amaranthaceae (*Gomphrena globosa, Amaranthus viridis*), Chenopodiaceae (*Chenopodium amaranticolor*), Compositae (*Ageratum conyzoides*), Solanaceae (*Solanum lycopersicum, Capsicum annum, Solanum melongena, Nicotiana tabacum, Nicotiana glutinosa, Physalis angulata*), Fabaceae (*Arachis hypogaea, Vigna radiata, Vigna sesquipedalis, Glycine max*), Portulacaceae (*Talinum paniculatum*), Oxalidaceae (*Oxalis barrelieri*), Onagraceae (*Ludwigia hyssopifolia*), and Asteraceae (*Widelia trilobata*). The incubation period and type of symptom were observed daily for at least up to 30 days post inoculation. DIBA test was conducted especially to confirm the virus infection on symptomless test plants.

2.4. Electron microscopy
The morphology and size of viral particles were examined by transmission electron microscopy (TEM) (JEM 1010 JEOL, Tokyo, Japan) at PT Eijkman, Jakarta, Indonesia. Virus particles from leaves crude extract was prepared as described by Love [10].
2.5. RT-PCR
Total RNA was extracted from symptomatic plants using CTAB method with minor modification [11]. The cDNA was synthesized from total RNA using Moloney Murine Leukimia Virus (M-MuLV) according to protocol provided by Thermoscientific, USA. RT-PCR reactions was carried out in a 25 µl mixture containing 12.5 µl Go Taq Green (Thermoscientific, USA), 2 µl cDNA template, 1 µl of the primers (10 µM) for each, and 9.5 µl ddH₂O. Amplification of the cDNA was carried out using either a pair of primer specific to CP gene of CGMMV or universal primer for Tobamovirus. The expected size of RT-PCR product was ±830 bp and ±800 bp [3]. Amplification products were analyzed on 1% agarose gel electrophoresis in 0.5X TBE buffer containing nucleic acid staining dye FlouroVue TM (Smobio, Taiwan).

2.6. DNA cloning and sequences analysis
PCR products were cloned into pTZ57R/T easy vector system based on the protocol provided by Thermo Scientific, USA. Plasmid DNA recombinant was sequenced and analyzed. The nucleotide sequences of the gene were aligned with those of corresponding virus sequences deposited in GenBank database by using software Clustal-W [12]. Sequences homology analysis of the gene were performed using Bio Edit version 7.05 software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and phylogenetic tree was constructed using MEGA 6.0 software with the neighbor-joining algorithm and 1.000 bootstrap replications [13].

3. Results

3.1. Symptoms on infected cucumber
Infected leaves of cucumber in the field showed mosaic, mottle, yellowing, and chlorosis symptoms. The symptom variation was depending on the cucumber cultivar cultivated in each location. Mosaic and mottle symptoms on the leaves were dominantly observed in all area, while chlorosis was observed dominantly in Subang. Propagation of single lesion local on tobacco showed typical systemic mosaic (figure 1.a,b).

Figure 1. Typical symptoms of Tobamovirus-like symptom on infected cucumber found in the fields (a) and symptom on propagation plant Nicotiana tabacum (b)
3.2. Serological test
Based on serological test using several antisera, there are nine viruses detected on samples with various disease frequencies and showed that multiple infection of two up to nine viruses occurred naturally (data not shown). Among them, CGMMV detected up to 24.44% from samples in East Java, 9.09% in Central Java, 0% in Yogyakarta, and 5.49% in West Java, respectively. Since CGMMV was consider as quarantine pest, further characterization of biological, physical and nucleic acid characters are necessary to identify to confirm its existence in Java, Indonesia as described bellows.

3.3. Host range test
Host range test on twenty three plant species showed that the virus infected locally on G. globosa, C. amaranticolor, S. melongena, C. moschata, M. charantia, G. max, O. barrelieri, L. hyssopifolia, A. conyzoides, and W. trilobata. Systemic infection was occurred on S. lycopersicum, N. tabacum, N. glutinosa, C. annum, P. angulata, C. sativus, C. melo, A. hypogaeae, and V. sesquipedalis. There were no obvious symptoms on A. viridis, L. acutangulata, V. radiata, and T. paniculatum, but confirmed serologically (table 1).

3.4. Electron microscopy
Electron microscopy confirmed the presence of Tobamovirus-like particle, with rigid rod shape morphology and about 330 nm in length and 30 nm in diameter (figure 2), and similar particle was reported previously by Love [10].

![Figure 2. Transmission electron micrograph of Tobamovirus-like particle from crude leaf extract. The particle was stained negatively with 2% uranyl acetate with 60,000 x magnification. Bar: 100 nm.](image)

3.5. Amplification and nucleotide sequences analysis
Amplification of cDNA using specific primer for CGMMV was unable to amplified the DNA (data not shown). However, amplification using universal primer for Tobamovirus successfully amplified the DNA with sized ± 800 bp (figure 3). It is suggested that the causal virus was not CGMMV, but other species of Tobamovirus member. There were two Tobamovirus member were recognized in Indonesia i.e Tobacco mosaic virus (TMV) and odontoglossum mosaic virus (ORSV), but TMV has distantly related to ORSV [14]. Further, we test the samples serologically by ELISA method, and the result gave positive reaction against TMV antisera (data not shown), indicating the cross reaction of CGMMV antisera to the samples infected by TMV on previous DIBA test.
Based on nucleotide sequences analysis of samples which gave positive reaction against CGMMV antisera were identified as TMV. The homology of either nucleotide or amino acid sequences of the CP gene TMV among isolates was ranged 86.2 to 97.7% and 90.8 to 99.4%. The nucleotide and amino acid sequences of the CP gene of Java isolates had highest identity with isolate of TMV from China (JX993906) ranged from 90.7% - 95.1% and 93.6% - 97.4%, respectively (table 2). Phylogenetic tree analysis showed that TMV Java isolates were in the same cluster separated from other TMV isolates deposited in GenBank (figure 4).

Table 1. Host range of the virus.

| Plant family/species | Incubation period (days) | Symptoms I/S | DIBA I/S |
|----------------------|--------------------------|--------------|----------|
| **Amarantaceae**     |                          |              |          |
| Gomphrena globosa    | 2                        | CLL/-        | +/-      |
| Amaranthus viridis   | -                        | +/-          | +/-      |
| **Chenopodiaceae**   |                          |              |          |
| Chenopodium amaranticolor | 2                   | CL/-         | +/-      |
| **Solanaceae**       |                          |              |          |
| Solanum lycopersicum | 4                        | M/M, Mf      | +/-      |
| NICOTIANA tabacum    | 4                        | LN/M         | +/-      |
| N. glutinosa         | 2                        | LN/LN        | +/-      |
| Capsicum annum       | 3                        | M/M, Mf      | +/-      |
| Solanum melongena    | 5                        | CS/-         | +/-      |
| Physalis angulata    | 4                        | M/M, Mf      | +/-      |
| **Cucurbitaceae**    |                          |              |          |
| Cucumis sativus      | 10                       | CL/M         | +/-      |
| Cucumis melo         | 7                        | CL/M         | +/-      |
| Cucurbita moschata   | 6                        | CS/-         | +/-      |
| Momordica charantia  | 6                        | CS/-         | +/-      |
| Luffa acutangulata   | -                        | +/-          | +/-      |
| **Fabaceae**         |                          |              |          |
| Arachis hypogaea     | 7                        | CL/CL        | +/-      |
| Vigna radiata        | -                        | +/-          | +/-      |
| Vigna sesquipedalis  | 9                        | M/M          | +/-      |
| Glycine max          | 8                        | CL/-         | +/-      |
| **Oxalidaceae**      |                          |              |          |
| Oxalis barrelieri    | 6                        | CL/-         | +/-      |
| **Onagraceae**       |                          |              |          |
| Ludwigia hyssopifolia | 3                     | ML/-         | +/-      |
| **Compositae**       |                          |              |          |
| Ageratum conyzoides  | 22                       | CL/-         | +/-      |
| **Asteraceae**       |                          |              |          |
| Wedelia trilobata    | 16                       | CL/-         | +/-      |
| Portulacaceae        | -                        | +/-          | +/-      |
| Talinum paniculatum  | -                        | +/-          | +/-      |

I: inoculated leaves, S. systemic leaves; CLL. chlorotic local lesions, CL.chlorotic local, CS.chlorotic spot, ML.mosaic local, Mf.malformation, SM.systemic mosaic, NL. Necrotic lesions -.no symptom. +.virus was detectable by DIBA, -.virus unable detected by DIBA
Figure 3. RT-PCR product of DNA *Tobamovirus*. Isolate Ngjk-Nganjuk, Kdr-Kediri, Bgr-Bogor, KP-Kulon Progo, Brbs-Brebes, Idr-Indramayu, Tlg-Tulungagung, Sbg-Subang, and Kltn-Klaten; M. Marker of 1 kb DNA (Thermo Scientific, USA).

Table 2. Homology of nucleotide (nt) and amino acid (aa) of TMV Java Isolates with other isolates from other countries established in GenBank

| Isolate (country) | Kediri nt | Kediri aa | Bogor nt | Bogor aa | Kulon Progo nt | Kulon Progo aa | Indramayu nt | Indramayu aa | Accession number |
|-------------------|-----------|-----------|----------|----------|----------------|----------------|--------------|--------------|-----------------|
| IDN - Ngjk        | 100       | 100       | 96.9     | 98.1     | 97.7           | 99.4           | 96.3         | 98.1         | LC311787        |
| IDN - Kdr         | 96.9      | 98.1      | 100      | 100      | 97.7           | 99.4           | 95.0         | 98.1         | LC311785        |
| IDN - Kdr         | 97.7      | 99.4      | 97.7     | 99.4     | 100            | 100            | 95.4         | 98.1         | LC311788        |
| IDN - Indramayu   | 96.3      | 98.1      | 95.0     | 98.1     | 95.4           | 98.1           | 100          | 100          | LC311786        |
| China             | 92.3      | 94.9      | 95.1     | 97.4     | 93.1           | 94.9           | 90.7         | 93.6         | JC993906        |
| China             | 92.1      | 95.8      | 94.9     | 97.9     | 92.8           | 94.9           | 90.5         | 94.3         | HE818421        |
| China             | 91.9      | 94.6      | 94.6     | 97.0     | 92.6           | 94.2           | 90.2         | 94.2         | AF395128        |
| Korea             | 92.2      | 94.9      | 95.0     | 97.4     | 92.9           | 94.9           | 90.6         | 93.6         | X68110          |
| Spain             | 92.3      | 94.9      | 95.1     | 97.4     | 93.1           | 94.9           | 90.7         | 93.6         | KF972435        |
| Africa            | 92.2      | 94.9      | 95.0     | 97.4     | 92.9           | 94.9           | 90.6         | 93.6         | AY360447        |
| Japan             | 92.6      | 94.6      | 95.4     | 97.6     | 93.3           | 95.0           | 90.9         | 93.6         | V01408          |
| Japan             | 88.1      | 92.4      | 90.9     | 94.9     | 88.8           | 92.4           | 86.5         | 91.1         | D63809          |
| Japan - ORSV*     | 87.8      | 92.0      | 90.5     | 94.3     | 88.2           | 92.0           | 86.2         | 90.8         | HQ593620        |

*ORSV: *Odontoglossum ringspot virus* Japan isolate as out group; nt (nucleotide) and aa (amino acid); IDN: Indonesia

4. Discussion

In Indonesia, TMV infected tobacco in Jember [15], chili pepper in Malang and Lampung [16,17], and orchids in Yogyakarta, Java, and Bali [18,19]. However, there is no report that TMV infected cucumber in Indonesia. In recent years, there have been reports of increasing importance of virus diseases in cucumber crops in Indonesia [20,21]. In the earlier reports, distribution and genetic diversity of viruses infecting cucumber crops were studied by serological and molecular methods, but none of these were
TMV. Information on frequency, alternative host, and genetic diversity of TMV on cucumber crops have never been reported previously.

Widespread prevalence of TMV in most cucumber crops areas of Java would implicate its major impact on cucumber production. TMV isolate from Bogor had broad host range in compare to Iran isolate. Experimentally, TMV isolate Brbs showed able to infect systemically *S. lycopersicum, N. tabacum, N. glutinosa, C. annuum, P. angulata, C. sativus, C. melo, A. hypogaeae, and V. sesquipedalis*. Natural infection of TMV on *P. angulata, C. sativus, C. melo, A. hypogaeae, and V. sesquipedalis* did not reported yet occur in Indonesia. Since by mechanical inoculation in green house showed that those hosts can infect systemically, those host might can act as reservoir plants in the field if infection occurs naturally. In addition, the TMV particle stability is high, the virus abundance in leaf tissues, easily to transmit by sap of diseased plants and contact between plants [22] and also can survive for many years in dead, dried plant material, it will implicate on the difficulty to inactivate TMV [23], especially in the fields.

Previously, Tobamoviruses were distributed mostly on solanaceous crops with incidence up to 12.3%, whereas the incidence on cucurbitaceous crops was up to 8.1% in Iran [5]. TMV Java isolates belongs to strain vulgarе and showed lower identity to Iran isolate which infect cucumber. The highly homology of

---

**Figure 4.** Phylogeny tree of nucleotide sequences of TMV Java Isolates. *Odontoglossum ringspot virus* (ORSV) is used as out groups.
CP gene of either nucleotide or amino acid among Java isolates indicating the low genetic variation. TMV Java isolates showed in the same cluster separately from other isolates, suggesting that TMV Java isolates differ from other country’s isolates. The differences might because of genetic variation among TMV strain present in different host and environmental condition.

5. Conclusion
TMV was found in cucumber cultivations in Java. TMV can be transmitted mechanically on some type of test plants with different symptoms, and the symptoms are lesions local and lesions systemic. Rigid rod structure of 30 nm in diameter of virus particle was obtained. TMV Java, Indonesia isolates showed in the same cluster with China isolate.

Acknowledgments
The research was funded by PMDSU program provided by Ministry of Research, Technology and Higher Education of Indonesia on 2016 for the research team.

References
[1] [BPS] Central Bureau of Statistics 2017 Vegetable Crop Production in Indonesia 2012-2016 (Jakarta: Central Bureau of Statistics)
[2] Farahani A A, Rakshandehroo F and Shahraeen N 2014 J. Plant Pathol. 96(4) 113-131
[3] Letchert B, Gunter A, Lesemann D E, Willingmann P and Heinze C 2002 Virological Method. 106 1-10
[4] Nassar E A, El-Dougoud K A, Osman M E, Dawoud R A and Kinawy A H 2012 Int. J. Virol. 8 14-26
[5] Alishiri A, Rakshandehroo F, Zamanizadeh H R and Palukaitis P 2013 Plant. Pathol. J. 29:260-273
[6] Cherian S and Muniyappa V 1998 Ind. J. Virol. 14 65–69
[7] Chitra T R, Prakash H S, Albrechtsen S E, Shetty H S and Mathur S B 2002 Ind. Phytopathol. 55 84–86
[8] Massumi H, Shaabanian M, Hosseini Pour A, Heydarnajad J and Rahimian H 2009 Plant Dis. 93 67–72
[9] Towbin H, Staehelin T and Gordon J 1979 Proc. Natl. Acad. Sci. USA 76 4350-54
[10] Love A J, Makarov V V, Sinitisyna O V, Shaw J, Yaminsky I V, Kalinin N O and Taliansky M E 2015 Frontiers in Plant Science 6 1-10
[11] Doyle J J and Doyle J L 1987 Phytochemic. Bulletin. 19 11-15
[12] Thompson J D, Higgins D G and Gibson T J 1994 Nucleic Acids Res. 22 4673-80
[13] Tamura K, Stecher G, Peterson D, Filipski A and Kumar S 2013 Mol. Biol. Evol. 30(12) 2725-29
[14] Lakani I, Suastika G, Mattijk N and Damayanti T A 2010 Hayati J. Biosci. 17(2) 101-104
[15] Wahyuni W S, Trisusilowati E B and Sulistianto D 1999 Indonesia J. Plant Protec. 5(2) 100-107
[16] Akin H M and Nurdin M 2003 J Trop. Plant. Pests. Dis. 3(1) 10-12
[17] Kusumawati D E, Hadisaton T and Marosudiro M 2013 J. Pests Plant Dis. 1(1) 66-79
[18] Muharam A, Sulyo Y, Rahardjo I B, Diningsih E and Suryanah 2013 J. Hort. 23(1) 56-64
[19] Somowiyarjo S, Hartono S, Sulandari S and Putri S U 2016 Indonesia J. Phytopathol. 12(2) 69-73
[20] Haerunisa R, Suastika G and Damayanti T A 2016 Indonesian J. Hort. 7 9-20
[21] Lestari S M and Nurhayati E 2014 Indonesian J. Phytopathol. 10(3) 81-86
[22] Lewandowski D J, Hayes A J and Adkins S 2010 Plant Dis. 94 542–550
[23] Broadbent L H 1965 Ann. Appl. Biol. 56 177–205