

NATURAL INFECTION OF *Tobacco mosaic virus* ON BUTTERNUT SQUASH IN BALI, INDONESIA

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ABSTRACT

Natural infection of Tobacco mosaic virus on butternut squash in Bali, Indonesia. *Tobacco mosaic virus* (TMV) was a newly emerging virus infecting cucumbers in Indonesia since 2017. The mosaic disease caused by TMV potentially caused yield loss cucumber in Java. In 2019, mosaic symptoms were observed in butternut squash plants in Bali and molecular detection using universal primer of *Tobamovirus* indicated the presence of TMV infection. Further research was conducted to determine molecular characteristics of TMV on butternut squash plants in Bali. Specific DNA bands of *Tobamovirus* were amplified using reverse transcription polymerase chain reaction method, followed by DNA sequencing. The DNA were successfully amplified from CP *Tobamovirus* using universal primers from several butternut squash samples, i.e. Denpasar, Gianyar, Buleleng, and Karangasem Districts. The homology analysis of nucleotide and amino acid sequences of TMV among isolates from Denpasar, Gianyar, Buleleng, and Karangasem Districts was ranged between 95.6 – 97.7% and 98.1 – 99.4%, respectively. This indicated that low genetic diversity of TMV among Bali isolates. The highest homology of corresponding sequences of TMV isolates from Denpasar, Gianyar, Buleleng, and Karangasem Districts was closely related to TMV Kediri-Indonesia isolate on cucumber plant. Correspondingly, the phylogenetic analysis showed that TMV Bali isolates were categorized into same cluster with Kediri-Indonesia isolates. This was the first report of TMV on butternut squash in Indonesia.

Key words: genetic diversity, nucleotide sequences, phylogenetic analysis, *Tobamovirus*

INTRODUCTION

Butternut squash (*Cucurbita moschata* Durh) is one of the beneficial plants for human health. It has a high economic value due to its high price that can reach IDR 35,000 per kg. Butternut squash from Indonesia have been exported to Singapore and are able to compete with products from Australia and Thailand. Butternut squash that are cultivated in Bali Province experiences problems in overcoming plant-disturbing organisms. Therefore, in the field butternut squash always appear unhealthy due to the infection of various pathogens, including virus (Kurniati *et al.*, 2018).

There are many species of viruses that able to infect Cucurbitaceae, including one of *Tobamovirus* member, *Tobacco mosaic virus* (TMV) (Letschert *et al.*, 2002; Farahani *et al.*, 2014). *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 (Letschert *et al.*, 2002; Nassar *et al.*, 2012; Alishiri *et al.*, 2013). Infected plants showed different symptoms, i.e. mosaic, malformation, mottle, and stunting. Previously, infection TMV on several crops in Iran, i.e. tomato, cucumber, and pepper have been reported causing up to 59–90% of yield losses (Alishiri *et al.*, 2013; Vinayarani *et al.*, 2011; Chitra *et al.*, 2002). Generally, *Tobamovirus* can be easily transmitted by mechanical, seed, contact between plants, but not transmitted by vector and the debris can become the most important sources of inoculums in the fields (Alishiri *et al.*, 2013; Massumi *et al.*, 2009). The first report of TMV on cucumbers in Java, Indonesia in 2017 with a disease incidence of 24.44% (Listihani *et al.*, 2018). In 2019, TMV was reported infecting other Cucurbitaceae such as bitter gourd, ridged gourd, and watermelon on the Java Island (Damayanti *et al.*, 2019).

Recently on 2019, we conducted a field survey to collect butternut squash leaves with typical symptom of virus-like disease from several cultivation areas in Bali. The samples showed various symptoms such as mosaic and yellow mosaic. It was difficult to recognize the causal virus only based on phenotypic symptoms, because multiple infection of viruses are common phenomena in the fields. The symptoms found were similar with TMV infection in Cucurbitaceae plants in Java. Here, we reported the molecular characters of samples with mosaic symptoms to confirm its existence in Bali, Indonesia.

MATERIALS AND METHODS

Research Site. This research was conducted from October 2020 to March 2021 at the Molecular Biotechnology Laboratory, Udayana University. Symptomatic samples of butternut squash were collected from butternut squash planting area in Bali.

Sample Collection and Observation of Disease Incidence. The survey and sampling of butternut squash plants were carried out of nine districts in Bali (Denpasar, Gianyar, Badung, Buleleng, Tabanan, Klungkung, Karangasem, Jembrana, and Bangli). Sampling was carried out by purposive sampling method, as many as 10 symptomatic samples were taken from each location. The total samples taken were 90 samples, then used as material for virus detection. The disease incidence (DI) based on symptoms of viral infection in the field was calculated by:

$$DI = \frac{n}{N} \times 100\%$$

DI = the incidence of disease (%),
n = number of symptomatic samples,
N = total samples in the fields.

RNA Extraction. Total RNA was extracted from symptomatic plant leaf tissue using the CTAB method. Total viral RNA was isolated from infected leaf with procedure described by Doyle & Doyle (1987). Fresh tissue (0.1 g) was grinded with liquid nitrogen and added with 500 μ L of 10% CTAB buffer (cetyl-trimethylammonium bromide, 0.1 M Tris-HCl pH 8, 0.05 M EDTA, 0.5 M NaCl, 1% β -mercaptoethanol). Then it was transferred to 1.5 mL micro tubes and incubated in a water bath at 65 $^{\circ}$ C for 60 min (30 min for total RNA extraction), the micro tubes were turned back and forth every 10 min to separate the lipids and proteins. After incubated 60 min the micro tube containing the mixture

was taken from a water bath and allowed to stand for 2 min at room temperature, then added with 500 μ L of the Chloroform: Isoamylalcohol mixture with a ratio of 24: 1 (v:v). The mixture was vortexed for 5 min until well mixed, then centrifuged (Gyrozen 1730R) at 14,000 rpm for 15 min. A total of 450 μ L of supernatant was taken and transferred into a new micro tube, then 3 mM of sodium acetate was added from the volume of the supernatant. The mixture was vortexed and incubated at -80 $^{\circ}$ C for 2 h or -20 $^{\circ}$ C for one night. After incubation, the nucleic acid mixture was centrifuged at 12,000 rpm for 10 min to precipitate the nucleic acids. The nucleic acid pellets were washed by 500 μ L of 70% ethanol, then centrifuged again at 8000 rpm for 5 min, pellets were air-dried. After drying, the pellets containing total RNA were dissolved in 50–100 μ L of nucleic free water or TE buffer (10 mM Tris·Cl, pH 8.0; 1 mM EDTA) and it was stored at -20 $^{\circ}$ C until ready for to use.

cDNA Synthesis. The total RNA was used as a template for cDNA synthesis. The composition of the reverse transcription (RT) consisted of 1 μ L oligo dNTP 10 mM, 2 μ L total RNA, and 3.75 μ L dH₂O. All reagents were vortex gently and incubated at 65 $^{\circ}$ C for 5 min, then immediately cooled in ice. Next to the reactants were added 2 μ L of RT buffer, 1 μ L dNTP 10 mM, 0.5 μ L DTT 50 mM, 0.5 μ L RNase inhibitor (RiboLock RNase Inhibitor 20 units/ μ L, Thermo scientific), 0.5 μ L MmuLV (Revertaid 200 units/ μ L, Thermo Scientific) to a total volume of 10 μ L. The reverse transcription reaction was carried out at 42 $^{\circ}$ C for 60 min followed by 70 $^{\circ}$ C for 10 min in order to deactivate the enzyme. The cDNA product can then be used as a template for amplification.

Amplification of RNA by RT-PCR. The nucleotide pair used to amplify Tobamovirus was Tob-Uni 1 (5'-ATTTAAGTGGA SGGAAA VCACT-3')/Tob-Uni 2 (5'-GTYGTTGATGAGTTCRTGGA-3'), with target amplicon sizes \pm 800 bp (Letschert *et al.*, 2002). The composition of the amplification reaction for a total volume of 25 μ L was 12.5 μ L Go Taq green 2 \times (Thermo scientific), 1 μ L Tob-Uni 1, 1 μ L Tob-Uni 2, 9.5 μ L nuclease-free water, and 1 μ L cDNA.

DNA Visualization. DNA was electrophoreted on 1% agarose gel (0.3 g of agarose dissolved in 0.5 \times TBE buffer 30 mL). The agarose gel solution was cooled to 50 $^{\circ}$ C for 15 min, then fluoroVuo TM nucleic acid dye was added (Smobio, Taiwan). Electrophoresis was carried out at a voltage of 100 V for 20 min. The results

of the electrophoresis were then visualized under ultraviolet transilluminator (Accuris E3000) and documented with a digital camera (Canon EOS M50).

DNA Analysis. The amplified DNA fragments were sent to 1st Base Malaysia for the nucleotide tracing process. The results of nucleotide tracing were sent to BLAST (Basic Local Alignment Search Tool) program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to reveal the level of similarity to the nucleotide sequences found in GenBank. Matrix homology identities were analyzed using BioEdit software V.7.0.5 software program (Geneious), and phylogenetic trees were analyzed using MEGA v6.0 software (Tamura *et al.*, 2013) using bootstrap 1000 times.

RESULTS AND DISCUSSION

Symptoms on Infected Butternut Squash. The butternut squash plants found in the field showed symptoms of mosaic (Figure 1). Symptoms of TMV infection in butternut squash were similar with TMV infection on cucumber plants (Listihani *et al.*, 2019). Symptoms of TMV in Solanaceae showed typical

symptoms, while those in the Cucurbitaceae showed mild symptoms (Listihani *et al.*, 2019). Cucurbitaceae infected by TMV will show mosaic symptoms (Damayanti *et al.*, 2019).

Diseases Incidence of TMV. The distribution of *Tobamovirus* in butternut squash plants in Bali was spread in Denpasar, Buleleng, Karangasem, and Gianyar Districts. The results showed that the disease incidence of *Tobamovirus* was more than 50% in two of four districts, namely Buleleng and Denpasar Districts (Table 1). Labu Madu F1 and Bulldog F1 cultivar were not resistant to *Tobamovirus* infection. In previous studies, the biological and molecular characteristics of TMV of cucumber isolates were different from those of tobacco isolates, furthermore characterization of nucleic characters was needed to identify their presence in Bali, Indonesia.

Amplification of *Tobamovirus* DNA by RT-PCR. The PCR results showed that DNA with amplicon size ± 800 bp was detected in samples from Denpasar, Gianyar, Buleleng, and Karangasem Districts (Figure 2). However, the DNA sample from other five

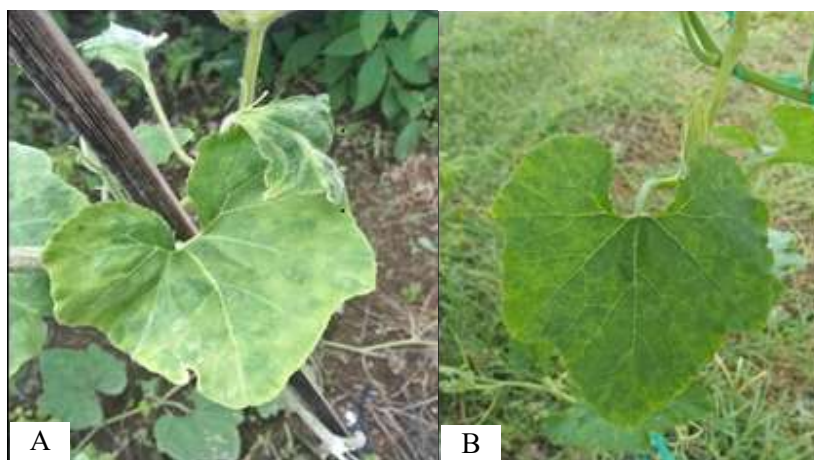


Figure 1. Symptoms of TMV infection on butternut squash in Bali. (A) Mosaic and leaf curly; (B) Mosaic.

Table 1. The disease incidence of *Tobamovirus* in butternut squash plants in Bali based on RT-PCR

Districs	Cultivar	Plant age (HST)	Disease incidence (%)
Denpasar	Labu Madu F1	35	55.66
Badung	Labu Madu F1	37	0
Bangli	Labu Madu F1	30	0
Buleleng	Bulldog F1	44	62.36
Tabanan	Bulldog F1	40	0
Karangasem	Labu Madu F1	34	47.89
Klungkung	Labu Madu F1	28	0
Gianyar	Labu Madu F1	37	27.27
Jembrana	Labu Madu F1	43	0

districts showed no amplification using *Tobamovirus* universal primers. There were two *Tobamovirus* member recognized in Indonesia i.e TMV and *Odontoglossum mosaic virus* (ORSV), but TMV had distantly related to ORSV (Lakani *et al.*, 2010).

Molecular Characterization of TMV. The homology of TMV nucleotides and amino acids between isolates from other countries (China, South Korea, USA, Spain,

Japan, and Iran) was 86.2–95.4% and 90.8–97.6%, respectively (Table 2). These data indicated that the samples from Bali which were aligned with the sequence from GenBank were TMV isolates. The TMV isolate from Bali had the highest nucleotide homology at 95.1–96.0% and amino acids 97.3–98.1% and was closely related to the Kediri-Indonesia isolate infected cucumber plants (LC311787), and had lower isolate homology than other countries. The relationship between

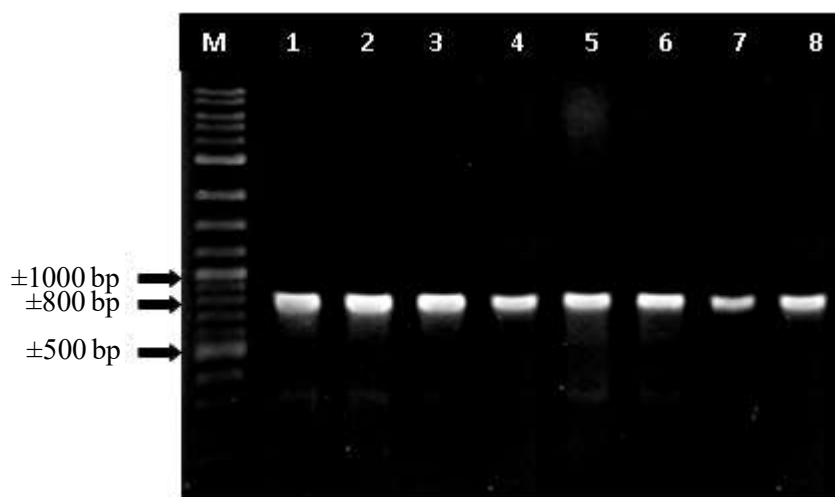


Figure 2. Visualization of DNA amplification of TMV. Sample from Denpasar District (1–2); Gianyar District (3–4); Buleleng Districts (5–6); Karangasem District (7–8); M. DNA marker 1 kb (Smobio, Taiwan).

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

Isolates (country)	Homology (%)								Accession number
	Denpasar		Gianyar		Buleleng		Karangasem		
	nt	aa	nt	aa	nt	aa	nt	aa	
IDN - Dps	100	100	96.9	98.1	97.7	99.4	96.3	98.1	-
IDN-Gia	96.9	98.1	100	100	97.7	99.4	95.6	98.1	-
IDN-Bull	97.7	99.4	97.7	99.4	100	100	95.8	98.1	-
IDN-Krg	96.3	98.1	95.6	98.1	95.8	98.1	100	100	-
IDN-Kediri	96.0	98.1	95.1	97.3	95.4	97.3	95.4	97.3	LC311787
China	92.3	94.9	95.1	97.4	93.1	94.9	90.7	93.6	JX993906
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
USA	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
Iran	87.8	92.0	90.5	94.3	88.2	92.0	86.2	90.8	HQ593620
Japan-ORSV*	59.6	61.7	60.8	64.1	59.8	61.7	58.2	60.4	E04305

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

isolates based on phylogenetic analysis showed that TMV isolates from Bali were in the same group as TMV cucurbitaceae isolates from Kediri-Indonesia and separated from TMV isolates that infected Solanaceae from other countries (China, South Korea, USA, Spain, Japan, Thailand, Serbia, Africa, and Iran) (Figure 3). Phylogenetic tree analysis that the TMV was divided into 6 groups, namely groups I (China, South Korea, USA, Spain, Africa), II (Thailand, Serbia, China), III (Bali-Indonesia, Kediri-Indonesia), IV (Serbia), V (Japan), and VI (Iran). The differences in these groups were not due to geographic location, but to different types of hosts.

The results of molecular identification showed that *Tobamovirus* was detected in butternut squash plants in Bali Islands. *Tobamovirus* infection in butternut squash plants in Bali was the first report in this study and the distribution of *Tobamovirus* in Indonesia. The incidence of *Tobamovirus* was highest in Buleleng,

presumably because of many butternut squash cultivations appeared unhealthy, high temperatures ranging from 25–40 °C, and there were no butternut squash varieties that were resistant to *Tobamovirus*. The high incidence of disease was influenced by the use of hybrid seeds throughout the growing season and the monoculture cultivation system (Maruthi *et al.*, 2014).

In Indonesia, TMV infected tobacco in Jember, chili pepper in Malang and Lampung, while TMV infected orchids in Yogyakarta, Java, and Bali, and cucumber, bitter melon, ridged gourd, and watermelon in Java (Wahyuni *et al.*, 2008; Akin & Nurdin, 2003; Kusumawati *et al.*, 2013; Muharam *et al.*, 2013; Somowiyarjo *et al.*, 2016; Listihani *et al.*, 2018; Listihani *et al.*, 2019, Damayanti *et al.*, 2019). The information on frequency, alternative host, and genetic diversity of TMV on cucumber crops had been previously reported. However, there was no report of TMV infection in butternut squash in Indonesia.

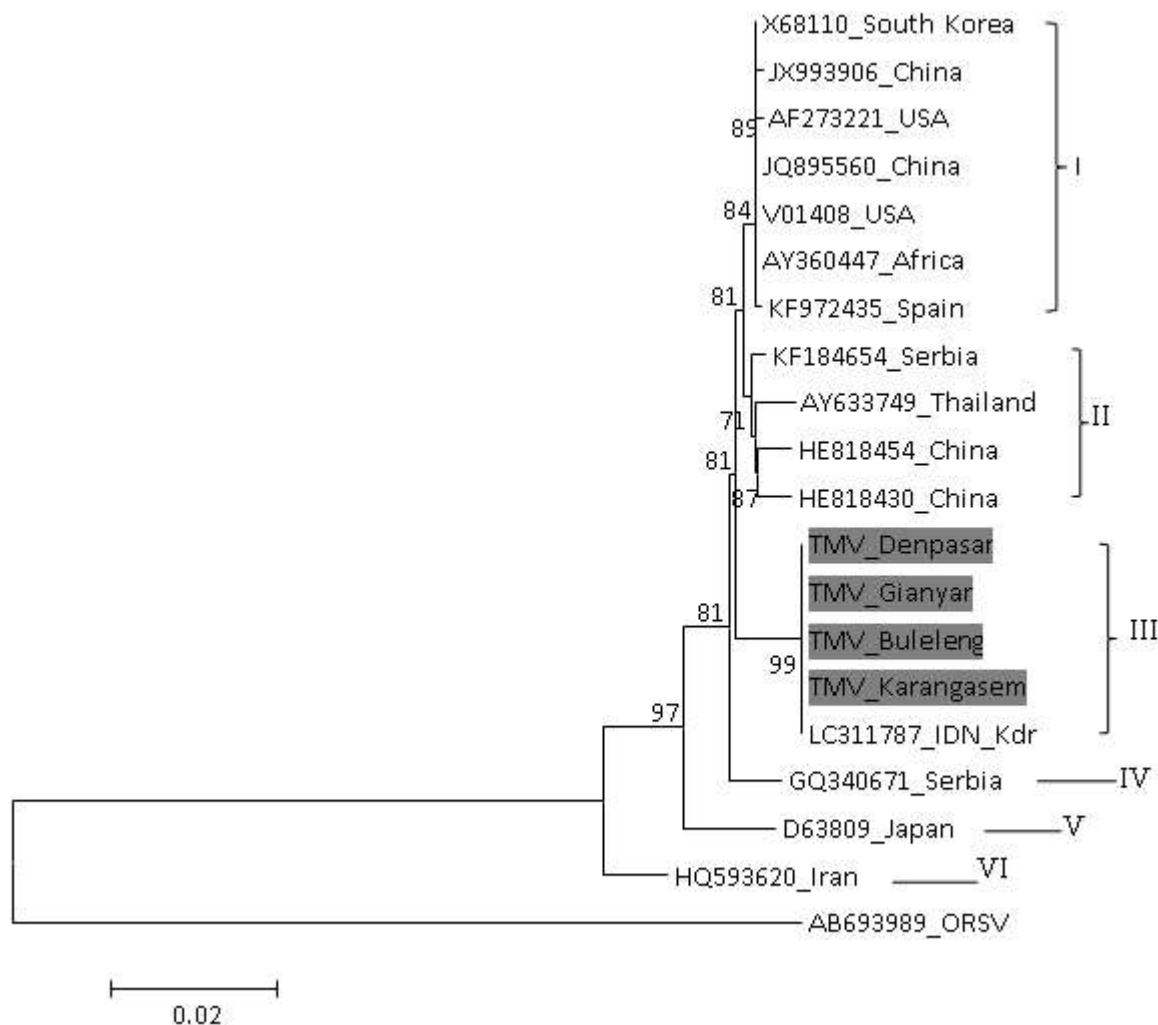


Figure 3. Phylogenetic tree of nucleotide sequences of TMV Bali Isolates using MEGA 6.0 (Neighbour Joining method with bootstrap 1000x). *Odontoglossum ringspot virus* (ORSV) is used as out groups.

Previously, *Tobamoviruses* were mostly distributed on Solanaceous crops with incidence up to 12.3%, whereas the incidence on Cucurbitaceous crops was up to 8.1% in Iran (Alishiri *et al.*, 2013). TMV Bali isolates belongs to strain *vulgare* showed highest similarity to Java isolate which infected cucumber. The highly homology of CP gene of either nucleotide or amino acid among Bali isolates indicated the low genetic variation. TMV Bali isolates showed in the same cluster separately from other isolates, suggesting that TMV Bali isolates differ from other country's isolates. The differences might because of genetic variation among TMV strain present in different host and environmental condition.

CONCLUSION

Tobacco mosaic virus had infected butternut squash in Bali Island, particularly Denpasar, Gianyar, Buleleng, and Karangasem District. The TMV butternut squash isolate from Bali was closely related to the Kediri-Indonesia isolate which infected cucumber plants (LC311787), and had lower isolate homology than other countries. The relationship between isolates based on phylogenetic analysis showed that TMV isolates from Bali were in the same group as TMV Cucurbitaceae isolates from Kediri-Indonesia and was separated from TMV isolates that infected Solanaceae from other countries.

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