RESEARCH PAPER

Distribution and molecular characterization of *Squash mosaic virus* on cucumber in Gianyar, Bali

Listihani[1], Ni Putu Pandawani[1], Tri Asmira Damayanti[2], Mimi Sutrawati[3], Dewa Gede Wiryangga Selangga[4], Ketut Ayu Yuliadhi[5], Trisna Agung Phabiola[5], & Gusti Ngurah Alit Susanta Wirya[5]

ABSTRACT

Squash mosaic virus (SqMV) has been reported to infect cucumber plants in Java and cause a decrease in fruit quality and quantity. Until now, there is no information regarding Sqmv infection in Cucurbitaceae in Bali. In a preliminary research conducted during 2020, we found mosaic symptoms in Gianyar, Bali. SqMV was inferred to be the possible cause of the virus-disease-like symptoms. The study aimed to determine the precence of SqMV in Gianyar, Bali and to obtain information related to its molecular character. This research method includes surveys, field observations, virus identification by RT-PCR, and DNA analysis. Severe disease incidence caused by SqMV was observed on cucumber cultivation in Gianyar that was in the range of 5.81–66.87%. PCR using specific primer for coat protein (CP) gene of SqMV was successfully amplified the DNA fragments of ± 582 bp on samples from Payangan, Tegallalang, Ubud, Sukawati, Blahbatuh, and Gianyar districts, except for samples from Tampaksiring. This indicates that the presence of SqMV is widespread in Java and Gianyar.The SqMV isolate from Bali had the highest nucleotide homology at 91.9–93.4% and amino acids 94.0–94.5 and was closely related to the Brazil isolate (KT923125) and had lower isolate homology than other countries (China, Trinidad, Spain, Japan, Arizona, Cekoslowakia, and Australia). It proved that those mosaic symptoms on cucumber is associated with SqMV infection. SqMV is classified as a quarantine organism of category A2, so it is necessary to prevent its spread to other areas.

Key words: coat protein, disease incidence, mosaic symptoms, polymerase chain reaction

INTRODUCTION

Squash mosaic virus (SqMV) infection causes greenish-yellow mosaic symptoms on cucumber leaves. The leaves become malformations with narrow leaf size, and stunted plants. Cucumber plants infected with SqMV showed shrinkage of the fruit base and fruit malformations. The infected fruit can change in taste and nutritional content, seed germination, fruit weight, and the amount of fruit production (Lestari & Nurhayati, 2014). According to Ali et al. (2012), *Cucumis sativus*, *C. melo, Cucurbita pepo*, *C. moschata*, and *C. maxima* plants infected with SqMV showed mosaic symptoms. In advanced symptoms, SqMV causes a decrease in fruit production and malformations.

SqMV is a seed-borne virus in cucurbit plants and can spread during planting and at harvest after primary infection of infected seeds (Şevik & Toksöz, 2008). SqMV can also be transmitted mechanically or via insect vectors (Maina et al., 2017). SqMV infection in the field occurs with the help of vector insects, namely beetles from the Chrysomelidae family (*Acalymma thiemei*, *Diabrotica* sp., and *Aulacophora similis*) and Coccinelidae (*Epilachna chrysomelina*) (Ali et al., 2012).

Regulation of the minister of agriculture of the republic of Indonesia number 25 year 2020 concerning types of quarantined plant destruction organisms (OPTK) stipulates the status of SqMV as OPTK category A2 group 1 which is still limited in West Java and it's that cannot released from carrier media (Kepmentan, 2020). Lestari & Nurhayati (2014) reported that

Corresponding author:
Gusti Ngurah Alit Susanta Wirya (susantawirya@unud.ac.id)

[1]Study Program of Agrotechnology, Faculty of Agriculture and Business, University of Mahasaraswati, Jl. Kamboja No.11A, Denpasar, Bali, Denpasar, Indonesia 80233
[2]Department of Plant Protection, Faculty of Agriculture, IPB University, Jl. Raya Dramaga, Bogor, West Java, Indonesia 16680
[3]Faculty of Agriculture, Bengkulu University, Jl. W.R Supratman, Kandang Limun, Bengkulu 38371A
[4]Faculty of Agriculture, Warmadewa University, Jl. Terompong No. 24, Tanjung Bungkak, Denpasar, Bali 80239
[5]Study Program of Agroecotechnology, Faculty of Agriculture, Udayana University, Jl. P.B. Sudirman, Denpasar, Bali 80234, Indonesia

SqMV had infected 100% of cucumber seeds. SqMV has been detected in five cucumber varieties in Indonesia and has been detected in Java (Purba et al., 2017; Listihani, 2019). Several factors that can trigger an increase in disease severity in an area are changes in climatic conditions, plant cultivation techniques, and traffic of plant material between regions (Ahanger et al., 2013; Hanssen et al., 2010). *Tobacco mosaic virus* (TMV) increases its multiplication when temperature reaches 34–36 °C (Ahanger et al., 2013). Given the nature of SqMV, it is possible that the source of SqMV infection in Indonesia comes from imported vegetable seeds.

Until now, there have no reports of SqMV infection in cucumber plants in Bali. Therefore, this study aimed to determine the presence of SqMV in cucumber plants in Bali. This information is very important because there is no information regarding the detection and presence of SqMV in Bali.

MATERIALS AND METHODS

Collection and Disease Incidence. The survey and sampling of cucumber cultivations were carried out in Gianyar Regency, Bali Province (Payangan, Tegallalang, Tampaksiring, Ubud, Sukawati, Blahbatuh, and Gianyar Districts). 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. Calculation of disease incidence by random sampling with each location as many as 50 plants. The disease incidence (DI) in the field is calculated by:

$$DI = \frac{\text{The number of symptomatic samples}}{\text{Total samples in the field}} \times 100\%$$

Total RNA Extraction from Symptomatic Leaves and RT-PCR Amplification. Total RNA was extracted from symptomatic plant leaf tissue using the CTAB method. Total viral RNA was isolated from infected leaf with a procedure described by Doyle & Doyle (1990). Fresh tissue (0.1 g) grinded with liquid nitrogen and added 500 µL of 10% CTAB buffer (cetyl-trimethyl-ammonium bromide, 0.1 M Tris-HCl pH 8, 0.05 M EDTA, 0.5 M NaCl, 1% β-mercaptoethanol). Then it transferred to 1.5 mL micro tubes and incubated in a water bath at 65 °C for 30 min, then the micro tubes were inverted every 10 min. After 30 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 500 µL of the Chloroform: Is-

oamyl alcohol mixture with a ratio of 24: 1 (v:v). The mixture was vortexed for 5 min until well mixed, then centrifuged (Microlitre Centrifuge Z 207M, Hermle, Germany) at a speed of 14,000 rpm for 15 min. A total of 450 µL of supernatant was taken and transferred into a new micro tube, then 3 M of sodium acetate was added. The mixture was vortexed and incubated at -80 ºC for 2 hours or -20 ºC 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 µL of 70% ethanol, then centrifuged again at 8000 rpm for 5 min, pellets were dried. After drying, the pellets containing total RNA were dissolved in 50 to 100 µL of TE buffer (pH 8) (10mM Tris-HCl, 8 mM EDTA) and it was stored at -20 ºC until ready for use.

The composition of the reverse transcription (RT) PCR mixture consisted of 1 µL oligo dNTP 10 mM, 2 µL total RNA, and 2.75 µL dH2O. All reagents were vortex gently and incubated at 65 ºC for 5 min in water bath, then immediately cooled in ice. Next to the reactants were added 2 µL of RT buffer, 1 µL dNTP 10 mM, 0.5 µL DTT 50 mM, 0.5 µL RNase inhibitor (RiboLock RNase Inhibitor 20 units/µL), 0.25 µL MmuLV (Revertaid 200 units/µL) (Thermo Fisher Scientific, Waltham, MA, USA) to a total volume of 10 µL. The reverse transcription reaction was carried out at 42 ºC for 60 min followed by 70 ºC for 10 min in order to deactivate the enzyme in the PCR machine. The cDNA product can then be used as a template for amplification.

Amplification of cDNA total using machine PCR thermal cycler (MultiGene OptiMax Thermal Cycler 230V Model, Labnet, USA). The primer pair used to amplify SqMV was SqMV RNA2-fl (5'-GGTG-CAGCAGCTTGGAACTTATAATCCAATTTGG-3')/ SqMV RNA2-r1 (5'-TGGGAAAGAAGCCACAA-CAAAACCC-3'), with target amplicon sizes ± 582 bp (Chinnaraja et al., 2015). The composition of the amplification reaction for a total volume of 25 µL was 12.5 µL Go Taq green 2x (Thermo scientific, USA), 1 µL 10 µM reverse primer, 1 µL 10 µM forward primer, 9.5 µL nuclease-free water, and 1 µL cDNA.

DNA was electrophoresis on 1% agarose gel [0.3 g of agarose dissolved in 0.5x TBE buffer (45 mM Tris-borat, 1 mM EDTA) up to 30 mL]. The agarose gel solution was cooled to 50 ºC for 15 min, then fluoroVuo TM nucleic acid dye (Smobio, Taiwan) was added. Electrophoresis was carried out at a voltage of 100 V for 20 min. The results of the electrophoresis were then visualized under ultraviolet transilluminator and documented with a digital camera.

Identification of SqMV Based on DNA Sequence Analysis. The amplified DNA fragments were sent to 1st Base Malaysia for the sequencing. The sequence was analyzed using BLAST (Basic Local Alignment Search Tool) program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) aims to see the level of similarity to the nucleotide sequences of related species found in Gen-Bank. Matrix homology identities were analyzed using BioEdit software 7.0.9 for Windows 95/98/NT/2000/XP (Hall, 2007) and phylogenetic trees were analyzed using MEGA v6.0 software (Tamura et al., 2013) using bootstrap 1000 times replication.

RESULTS AND DISCUSSION

Disease Symptoms on Cucumber Plants. During field surveys in several cucumber cultivation areas in Gianyar Regency, we found varying virus-like symptoms on cucumbers such as mosaic, yellowing, vein banding, vein clearing, and leaf malformation, and mottle (Table 1). Mosaic symptoms were found in all districts, except in Tampaksiring. Mosaic symptoms observed in the field were seen on young leaves (Figure 1A) and on fruit showed malformations accompanied by mosaic symptoms (Figure 1B). Symptoms like these are typical symptoms of SqMV infection in Cucurbitaceae plants (Aulia, 2004; Ali et al., 2012; Firmansyah et al., 2017).

Diseases Incidence of Virus like Symptoms. The incidence of disease in the field ranged from 5.81% to 66.87% (Table 1). The disease incidence was more than 50% in Ubud and Blahbatuh Districts, while the incidence of disease is less than 10% in Sukawati District. Good sanitation practices and vectors of rare insects on cucumber plantations in Sukawati lead to low incidence. According to Aulia (2004) showed that the percentage of SqMV infection in Cucurbitaceae plants in Bogor was the highest compared to other viruses

Table 1. Symptoms and incidence of disease on cucumber plants in Gianyar Regency, Bali Province

Districs	Plant age (DAP)*	Disease symptoms	Disease incidence (%)
Payangan	33–40	Mosaic, yellowing, leaf malformation and mottle	33.97 (123/362)
Tegallalang	24–42	Mosaic, vein banding, yellowing, leaf malformation	37.83 (213/563)
Tampaksiring	28–37	Vein banding, yellowing, leaf malformation	19.11 (134/701)
Ubud	35–42	Mosaic, yellowing, vein banding, and mottle	66.87 (523/782)
Sukawati	20–33	Mosaic and yellowing	5.81 (42/739)
Blahbatuh	30–43	Mosaic, vein clearing, vein banding, and mottle	63.80 (520/815)
Gianyar	33–46	Mosaic, yellowing, vein banding, vein clearing, and leaf malformation	31.67 (134/428)

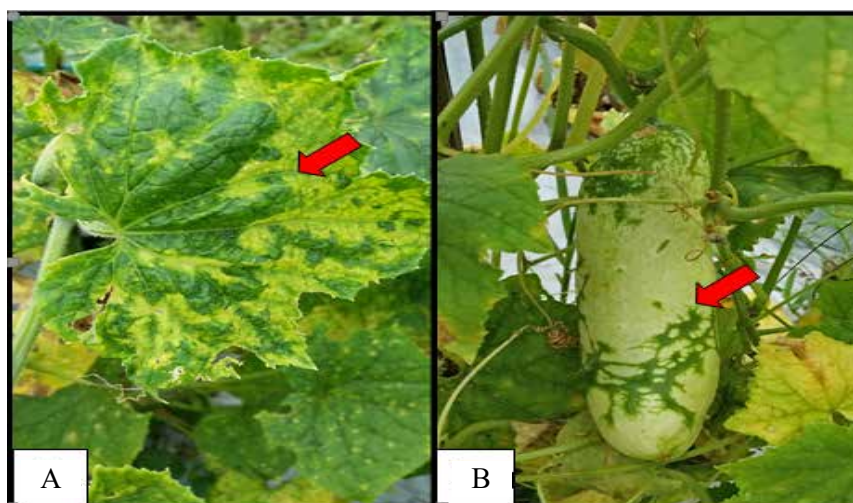*= Days after planting (DAP).

Figure 1. Symptoms of SqMV infection on cucumber in Bali; (A) Mosaic and curly on leaf; (B) Mosaic and malformation on fruit.

(CMV, PRSV, ZYMV). Ali et al. (2012) reported that the percentage of Cucurbitaceae plants infected with SqMV was only 3.8%. Meanwhile, Şevik & Toksöz (2008) reported that SqMV infection in pumpkins (*C. moschata* and *C. maxima*) was 21.11% and in zucchini as much as 20%, while Dikova & Hristova (2002) stated that seed-borne SqMV in Cucurbitaceae plants reached 91%. The spread of SqMV in the field is due to the inoculum sources that are continuously available in the field due to the unavailability of virus-free seeds.

Virus Identification by RT-PCR, Sequencing and Sequence Analysis. Specific DNA band SqMV measuring ±582 bp was successfully amplified in several samples showing mosaic symptoms from Payangan, Tegallalang, Ubud, Sukawati, Blahbatuh, and Gianyar Districts, except for samples from Tampaksiring (Figure 2). No mosaic symptoms were found on cucumbers in Tampaksiring and no SqMV was detected by RT-PCR, it was suspected that there were other viruses that infect cucumber plants. Chinnaraja et al. (2015) succeeded in detecting SqMV from watermelon and pumpkin plant samples in Trinidad with an amplicon size of ±582 bp using the same primer.

The homology of SqMV nucleotides and amino acids between isolates in other countries was 87.2–96.5% and 89.8–96.1%, respectively. These data indicated that the samples from Bali which were aligned with the sequence from GenBank were SqMV isolates. The nucleotide sequence similarity of the CP SqMV gene ranged from 81.1–99.6% for the same virus species (Haudenshield & Palukaitis, 1998). The SqMV isolate from Bali had the highest nucleotide homology at 91.9–93.4% and amino acids 94.0–94.5% and was closely related to the Brazil isolate (KT923125) and had lower isolate homology than other countries. The relationship between isolates based on phylogenetic analysis showed that SqMV isolates from Bali were in the same group as SqMV watermelon isolates from Brazil and separated from SqMV isolates from other countries (China, Trinidad, Spain, Japan, Arizona, Cekoslowakia, and Australia) (Figure 3). In this study, the differences in these groups are not due to geographic location and hosts (watermelon, squash, crookneck pumpkin).

SqMV is an exotic virus because it includes quarantine plant pest organisms (OPTK) category A2 group 1. Prior to 2015, SqMV was included in quarantine plant pest organism category A1 (OPTK A1) whose presence had not been reported in Indonesia. In 2015 until now, SqMV was reported in Indonesia on cucumber plants in Bogor (Kementan, 2020), so that in 2015, SqMV changed its status from OPTK A1 to OPTK A2, namely OPTK that already exists in Indonesia, but its existence is still limited. in some areas only.

SqMV may have infected Cucurbitaceae plants in Indonesia for a long time, but not many have reported its molecular character. SqMV has been reported to infect cucumber plants in Bogor based on serological detection (Firmansyah et al., 2017). In addition, the molecular character of SqMV isolates from Java has been reported by Listihani (2019). The presence of SqMV infection in cucumber plants in Gianyar in this study indicated that the distribution was increasing and it was difficult to control SqMV in the field.

This is the first report of SqMV infection in cucumber plants in Bali. The absence of an appropriate detection method and clean sanitation in the field, the present of insect vectors all year round in the field, as well as the use of non-virus-free seeds are the causes of the rapid spread of SqMV distribution in the field (Firmansyah et al., 2017). Several factors that can trigger the emergence of new diseases in an area are plant cultivation techniques and traffic of plant material between regions as well as changes in climatic conditions
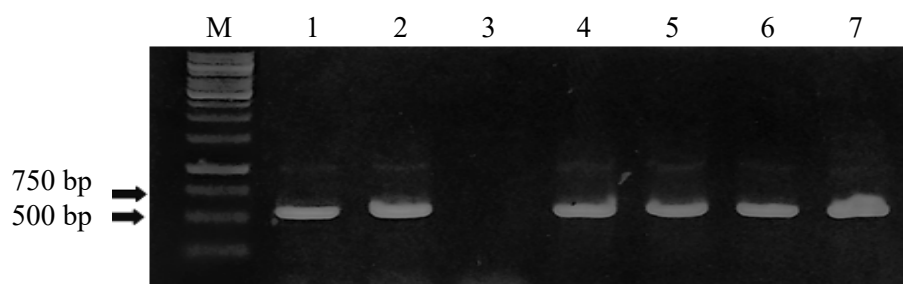

Figure 2. Visualization of DNA amplification of SqMV with primer pair of SqMV RNA2-f1/RNA2-r1 on 1% agarose gel. (M) DNA marker (1 kb ladder) (Thermo Scientific), (1 to 7) DNA samples from: (1) Payangan; (2) Tegallalang; (3) Tampaksiring; (4) Ubud; (5) Sukawati; (6) Blahbatuh; (7) Gianyar.
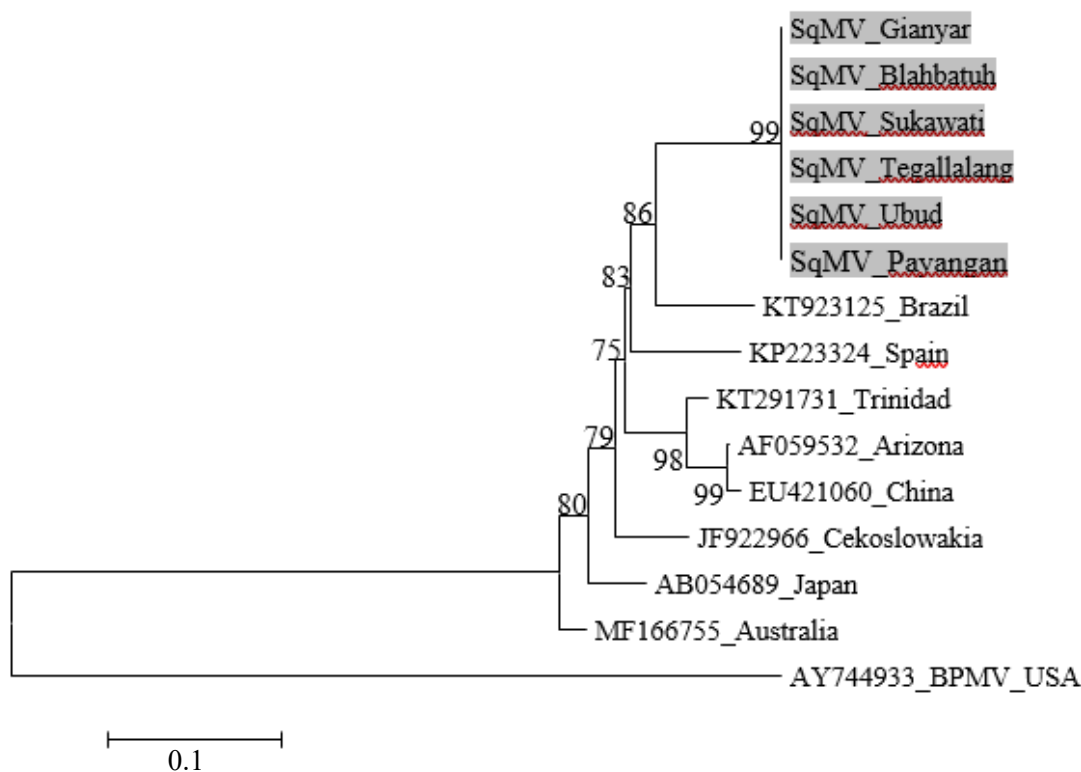
Figure 3. Phylogeny tree of nucleotide sequences of CP SqMV Bali Isolates using MEGA 6.0 (Neighbour Joining with bootstrap 1000x) with SqMV from other countries that deposited in GenBank. *Bean pod mottle virus* (BPMV) from America is used as out groups. The highlight mark is a SqMV isolates from Bali.

(Jones, 2016). Nancarrow et al. (2014), studied the effects of elevated (10–21 ºC, night/day) or ambient (5–16 ºC, night/day) temperature winter growing season regimes on wheat plants infected with BYDV. Infected plants grown under elevated temperature were larger, developed virus symptoms earlier, and had higher virus titers than plants grown at ambient temperature. SqMV can infect cucumber seeds up to 100% (Firmansyah et al., 2017). This does not rule out the possibility that the source of SqMV infection in Indonesia comes from imported seeds.

CONCLUSION

Variations in symptoms were found in all sampling locations, but mosaic symptoms were not found in Tampaksiring. based on RT-PCR, plants showing mosaic symptoms were positive for SqMV. Therefore, cucumber plantings at all locations in Gianyar were positive for SqMV infection, except Tampaksiring. This study is the first report of SqMV infection in Bali. The existence of SqMV in Gianyar, Bali shows the increasing number of sources of inoculum in the field.

Therefore, it is necessary to prevent the distribution of seeds and seedlings to other areas that have not been infected with SqMV.

AUTHORS' CONTRIBUTIONS

I, NPP, DGWS, KAY, TAP, and GNASW carried out the survey and sampling including observation of disease symptoms on cucumber cultivations in Gianyar, Bali. TAD and MS supported the research materials. I, DGWS, KAY, and GNAWS performed molecular work and data analysis. I, DGWS, NPP, KAY, TAP, GNAWS, TAD, and MS prepared the manuscript. The author provides feedback and comments on the flow

of research, data analysis and interpretation as well as shape of the manuscript. All the authors have read and approved the final manuscript.

COMPETING INTEREST

Authors declare no competing interest such as financial or non-financial interests, professional or personal relationships that are directly or indirectly connected to the work submitted for publication.

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