The percentage of Bean Common Mosaic Virus (BCMV) carried by seeds and detection of virus position inside long beans (*Vigna sinensis* L.) seeds in Bali

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ABSTRACT

The percentage of Bean Common Mosaic Virus (BCMV) carried by seeds and detection of virus position inside long beans (*Vigna sinensis* L.) seeds in Bali

Long bean (*Vigna sinensis* L.) is a horticulture crop with an essential economic value in Indonesia and Bali. Long beans productivity in Bali reached 6023 tons in 2013, and decreased to 5157 tons in 2014. Cases of disease that lowers the production of long beans in Bali were caused by Bean Common Mosaic Virus (BCMV) infection by 56.06%. BCMV can be brought by the seeds (seed-borne disease) and can infect long beans plants since the early stage of development. This research aims to evaluate the percentage of BCMV transmission by commercial long beans seeds, commonly used by farmers in Bali, and observe the location of BCMV inside the seeds. The morphological observation was used to evaluate the percentage of BCMV brought by the seeds by observing BCMV specific symptoms appear on two weeks old long beans seedlings. To observe the location of BCMV inside the long bean seeds, harvested seeds from an infected plant were separated to collect the cotyledon and embryo. The RT-PCR technique was used as a method in this research. The result showed the percentage of BCMV brought by long beans seeds used by farmers in Bali categorized as high. The percentage of BCMV brought by seeds from the highest to the lowest were; variety A 48.39%, variety B 46.66%, variety C 43.59%, variety D 37.83%, variety E 22.86%, and variety F 22.72%. The variation of the symptoms was mosaic vein banding, malformation, and dwarf plant with wrinkled leaves. The BCMV brought by long bean seeds was resides on the embryo.

Keywords: BCMV, Long beans, RT-PCR, Seeds

1. Introduction

Long bean (*Vigna sinensis* L.) is a horticulture crop that can be a beneficial commodity in the future and become an alternative to a source of economic growth (Pitojo, 2006). Long beans cultivation can be relied on as an agricultural business to increase farmers' income (Suryadi *et al*., 2003). This crop is demanded in Indonesia

because long beans can proceed into many food variations, sources of vitamins, and minerals that can be used to raise nutrition (Haryanto *et al*., 2007). Long beans contain vitamins A, B, and C and minerals; meanwhile, long beans seeds contain protein, fat, and carbohydrate.

Even though long beans are one of the essential vegetables consumed by the people, long beans in Indonesia decrease from year to year (Kariada *et al*., 2003). Based on the Indonesian Central Bureau of Statistics in 2013, the production of long beans in Indonesia in 2010 reached 489,449 tons and in 2013 decreased to 450,859 tons. Several factors, including plant diseases, cause the decrease in quality and quantity of the long bean production. Damayanti *et al*. (2009) reported that in 2008-2009, the incident of long beans' yellow mosaic disease spread out in several areas of the northern coast of West Java. The infected crops showed several symptoms like a yellow mosaic, growth inhibition, and leave malformation. The infection to young crops leads the plant to infertile, and if the infection goes terrible, it causes the early death of the crops.

Diseases caused by viruses make an enormous contribution to the decrease of long beans production in Bali, and it is known commonly brought by the seeds. BCMV is one of the diseases categorized into family of potyviridae, genus *potyvirus* (Purwaningsih, 2015). Some members of *potyvirus* reported attacking crops that produce beans which are economically very important. The infection is through the seeds and naturally spreads out through insect vectors non-persistently (Morales & Bos, 1988). The characteristics of BCMV that can be infected through the seeds can widen the infection of long bean mosaic disease.

Mosaic symptoms that appear on long beans affected by BCMV are blisters, yellow and green patterns on leaves, and leaves malformation (Setyastuti, 2008). Some control actions related to BCMV infection, i.e., crop rotation, and controlling vector that causes the disease (leaves fleas). Even though those control actions have been done, BCMV can still survive because it is also transmitted through the seeds (seed-borne virus). A study done by Phabiola (2016) examined that BCMV transmission brought by the seeds will be the primary inoculum in the field. However, there is no research about the percentage of BCMV carried by the commercial long beans seeds in Bali and where the virus is located inside the seeds. Therefore, further research is needed to find out the percentage of BCMV transmission brought by long beans seeds in Bali and investigating the location of BCMV inside the seeds.

2. Material and Methods

2.1 Research materials

The seeds used in this research were commercial seeds which commonly cultivated by the farmer in Bali. Six seeds, namely; variety A, B, C, D, E, and F, were investigated. Then silica gel, label papers, buffer, H_2O, HCl, NaOH, Liquid nitrogen, Thermo scientific GeneJET, Total RNA Mini Kit Plant (Geneaid), BCMV specific primer, and alcohol were used for the RT-PCR test. The utensils were used in this

research are tweezers, seedling tray, erlenmeyer, becker glass, micro straw, icebox, digital camera, mortar and pestle, test tube, PCR machine, and electrophoresis machine.

2.2 Research implementation

This research includes several steps; 1) Observation of long bean varieties which the farmer in Bali commonly cultivates, 2) Long bean seeds cultivation, 3) The observation of 2 weeks old long bean seedlings to determine the percentage of BCMV brought by the seeds and molecular test using RT-PCR technique to the symptomatic sample plant and 4) Observation the location of BCMV inside the long bean seeds (the embryo and cotyledon) using RT-PCR technique.

2.3 Long bean cultivation

Six varieties of long bean seeds were cultivated for two weeks for morphological observation purposes. Fifty seeds of each variety were randomly chosen for this experiment. The samples seeds were soaked in water and then grown in the seedling tray, which contained mixed media, soil, and charcoal husk in the ratio of 1:2. After two weeks of cultivation, the seedling was observed. The seedlings that showed BCMV symptoms such as mosaic vein banding, chlorosis, leaf malformation, and dwarf plants with wrinkled leaves were categorized as BCMV infected seeds. Further analysis with the molecular-based investigation was performed to ensure that the symptom appeared were caused by BCMV. The first growing perfect leaves of three long bean seedlings will be used as samples through RT-PCR using BCMV specific primer. The percentage of BCMV infected the long bean seedling was calculated based on the equation from (Zadocks & Schein, 1979); the percentage of crops with virus symptoms = (Number of crops with virus symptoms) / (Crops population) x 100%.

2.4 Confirmation of BCMV infection on long beans seeds through RT-PCR technique

Molecular testing using RT-PCR was done to ensure that the symptoms on the long beans sample were caused by BCMV infection. The molecular testing in this research including viral RNA extraction, synthesis of complementary DNA (cDNA), DNA amplification, and visualization of RT-PCR results. In detail, the workflow was done in this research mention as follows.

For the RNA extraction, the symptomatic and healthy seedlings were collected. The symptom that appeared in the sample plant was mosaic vein banding, leaves malformation and dwarf plant with wrinkled leaf. The seedling without those symptoms was categorized as a healthy sample plant. The seedling without symptom was used as a negative control, and the seedling with symptom was used as a positive control. Each 100 mg fresh long bean leaves sample was cut off and crushed into powder using liquid nitrogen. Then, the samples were extracted to get the total RNA following the instruction from Geneaid-Taiwan, by using Total RNA Mini Kit (Plant). The kit used contains RB Buffer, PRB Buffer, DNase I1, DNase I Reaction Buffer,

W1 Buffer, Wash Buffer2, RNase-free Water, Filter Columns, RB Columns, and 2 ml Collection Tubes.

The total RNA as a result of the extraction previously was used in the reverse transcription to become cDNA (complementary DNA) by SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase. The method was done following the manual instruction from Invitrogen Life Technologies-California. The SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase is designed for the sensitive, reproducible, end-point detection and analysis of RNA molecules by RT-PCR. The specific BCMV primer used in this research is BIC-cpF (5'- TCA GGA ACT GGG CAG CCG CAA C -3') and BIC-CPR (5'- CTG CGG GGA ACC CAT GCC AAG -3'). PCR amplification was started by early denaturation at the temperature of 95°C for 5 minutes, continued by 35 cycles of denaturation 94°C for 2 minutes, annealing 66°C for 1 minute and DNA synthesis 72°C for 1 minute, and the final extension 72°C for 10 minutes, the cycles end with a temperature 4°C. Furthermore, electrophoresis was used to visualize the result of RT-PCR.

2.5 Observation of BCMV inside the long beans seeds using Reverse Transcription-polymerase Chain Reaction (RT-PCR)

The virus brought by the long bean seeds was detected using the sample of seeds from BCMV infected mother plants cultivated in Baturiti village, Baturiti district, Tabanan regency. The comparison of healthy and infected mother plants can be seen in figure 1. The seeds samples were divided into two parts, which are embryos and cotyledons (figure 2). All molecular observation stages in this study were similar to the previous study when confirming the BCMV on the symptomatic sample plants in 2.4.

Figure 1. The comparison of healthy and infected long beans mother plants; 1. The healthy mother plant, 2. Infected mother plant

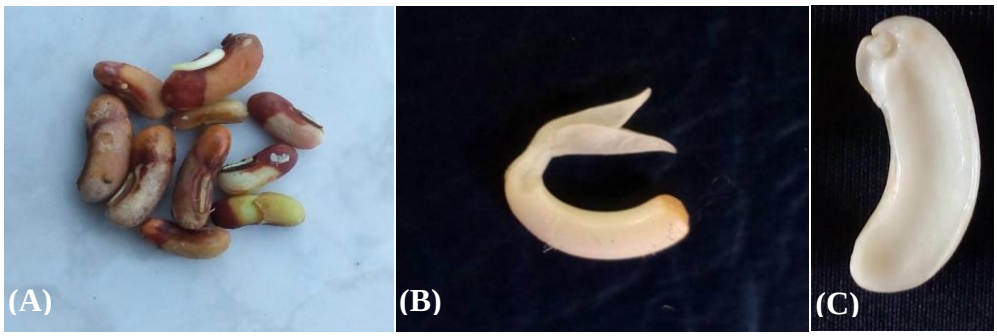

Figure 2. Long bean seeds for the detection of BCMV location inside the seeds. A. The seeds from BCMV infected mother plant, B. Long bean seed embryo, C. Long bean seed cotyledon

3. Results and Discussion

3.1 The percentage of BCMV brought by long beans (Vigna sinensis L.) seeds in Bali

Based on the data collected from this research, the percentage of BCMV brought by the long bean seeds in Bali is categorized as high. The observation of BCMV related symptoms on two-week-old long bean seedlings was done to obtain the percentage data. The symptoms observed were notable symptoms of BCMV, i.e., mosaic vein banding leaves malformation and dwarf plants with wrinkled leaves. The symptoms that were observed and used as an indicator in this research can be seen in figure 3.

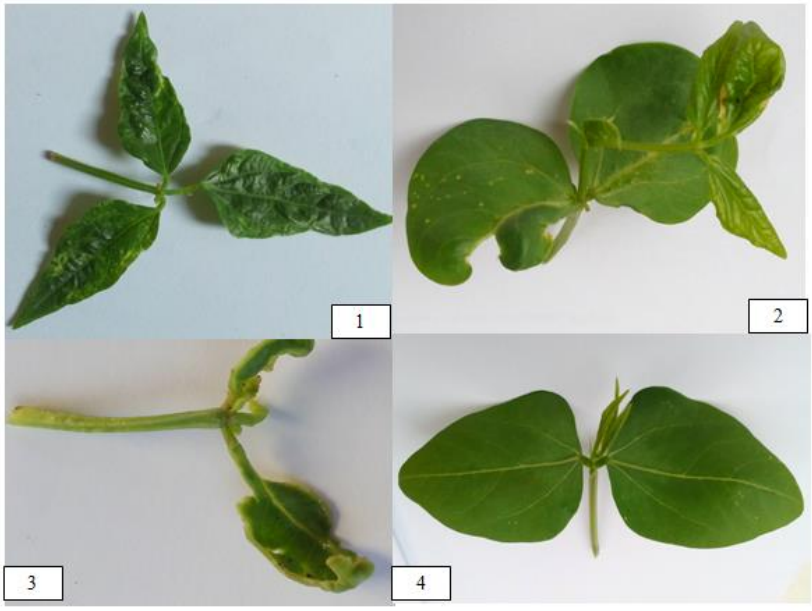

Figure 3. Two weeks old of long bean seedling with and without BCMV infected symptom. A. Mosaic vein banding, 2. Leaves malformation, 3. Dwarf plant with wrinkled leaves, 4. Healthy plant

The percentage of seeds infected BCMV on the commercial long bean varieties in Bali; variety A, variety B, variety C, variety D, variety E, and variety F, used as research samples, can be seen in Table 1.

Table 1. Observation of the Percentage of BCMV Brought by Seeds

Seeds Variety	Number of Samples	Growing Capability	Symptomatic Plant	Symptoms	BCMV Brought by Seeds (%)
Variety A	50	31	15	Mvb, mf, d	48.39
Variety B	50	45	21	Mvb, mf, d	46.66
Variety C	50	39	17	Mvb, mf, d	43.59
Variety D	50	37	14	Mvb, mf, d	37.83
Variety E	50	35	8	Mvb, mf	22.86
Variety F	50	44	10	Mvb, mf	22.72

Note: mvb: mosaic vein banding, mf: malformation, d: dwarf

Based on the data in Table 1 shows that the percentage of BCMV brought by seeds from the highest to the lowest is Variety A, B, C, D, E, and F, with the percentage of 48.39%, 46.66%, 43.59%, 37.83%, 22.86%, and 22.72% respectively. The variation of symptoms was mosaic vein banding, malformation, and a dwarf with wrinkled leaves. Based on the result, the symptom observed was vein banding on the young first trifoliate leaves. Followed with the changing leaves colour turned yellowish. The other symptom was leaves malformation, wrinkled veins, and curled leaves. In the healthy plant, those symptoms did not appear on the seedling.

Further molecular analysis was conducted to ensure that the observed symptoms on the two-weeks-old long bean seedling were infected or caused by BCMV. This research was done using the RT-PCR technique with a specific primer to detect the coat protein gene of BCMV in a size of 850 bp. The samples used were leaves with mosaic vein banding symptoms, leaves malformation, and a dwarf with wrinkled leaves. Long bean leaves infected by BCMV were used as the positive control; meanwhile, long beans' leaves from the healthy plants were used as the negative control.

Based on the RT-PCR observation DNA band was amplified on 850 bp, which was matched with the primer used to detect the coat protein gene of BCMV. Therefore, it could signify that the previously observed symptoms appear on the samples plant was infected by BCMV brought by seeds. Although the cultivation condition was customized as a free BCMV vector area, the percentage data is still not 100% reliable to showed the BCMV brought by the seed cases. Some errors might occur due to the cultivation and observation. Nevertheless, the data assume that the commercial seeds used by Balinese farmers are not free from BCMV. This is also a sign that we need to be careful when choosing seeds variety to cultivate in broad areas. Considering that

the virus can be brought by the seeds and can also become a primary inoculum of BCMV in the field.

3.2 The observation of BCMV inside the long bean seeds

To broaden our knowledge about the virus brought by the seeds, we observe the location of BCMV inside the long bean seeds in this research. The seeds sample used was gained from the mother plant infected BCMV from the early stage of development. The mother plant showing severe BCMV symptoms but still can produce a limited amount of seeds.

Based on the molecular observation, the DNA band was observed at around 850 bp. It suited the specific BCMV primer that was used in this research. Sample 1 was the cotyledon; it showed no band appeared after electrophoresis. Sample 2 was the seed's embryo, establishing the presence of the DNA band around 850 bp. Positive control came from long bean leaves with symptoms from unhealthy plants infected by BCMV, and normal plant leaves were used as a negative control. Thus, this study suggested that the BCMV virus position inside the seeds is detected in the embryo tissue.

The presence of viruses inside the seeds was highly supported when the virus infected the mother plant in the early stage of development. The long beans infected by the virus after four weeks old (critical phase) could produce the seeds that contain the virus. Once the plant is infected, the virus will replicate and moving from one cell intracellularly through plasmodesmata. The virus replication happened continuously following the metabolism of the primary cell until getting into the xylem vein system through nutrient flow until at the tip of leaves. In the following step, the virus would follow photosynthesis flow through the phloem vein until the root area, spreading systematically to all plants through the xylem vein. When the virus spreading out systematically before the flower formation process has a high potential to spread reaching the seeds.

4. Conclusion

The percentage of BCMV brought by six of the commercial long bean seeds in Bali from the highest to the lowest was the Variety A, B, C, D, E, and F with the percentage of 48.39, 46.66, 43.59, 37.83, 22.86, and 22.72% respectively. The variation of symptoms observed was mosaic vein banding, malformation, and a dwarf with wrinkled leaves. Moreover, the location of BCMV brought by long bean seeds in the sample used in this research was observed on the embryo. The result gained based on this research have several limitations to consider. For the percentage result, it just used only 50 seeds sample each variety. This sampling technique might not be enough to represent the whole number of seedlings on every package. The percentage value data is also not 100% guaranteed that BCMV caused the symptoms that appeared on the long bean seedling. It was because the whole sample was not checked through a molecular-based technique for confirmation. Hereinafter, when the cultivation

preparation, the seeds were not through surface sterilization. It might allow the other viruses and pathogens like bacteria or fungi that influence the growth of the seedlings. Moreover, the observation of symptoms was done using the eye. As we know, that method was not reliable enough with the high subjectivity from the researcher. For the location of the virus, in this research, we only used one mother plant to collect the seeds for this study. For more reliable results, further research with a plentiful number of samples is needed.

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