

SYMPTOMS VARIATION OF VIRAL INFECTIONS IN YARDLONG BEAN

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ABSTRACT

Symptoms variation of viral infections in yardlong bean. Yardlong bean (*Vigna unguiculata* subsp. *sesquipedalis* L.) is an important horticultural commodity in Indonesia. During the last ten years, yardlong bean planting areas in Indonesia especially in Java often suffered from epidemics of viral disease showing typical symptoms of yellow mosaic on their leaves. Some researchers have separately reported that the symptoms associated with some viral infections. This research aimed to examine viruses associated with the symptoms of yellow mosaic on yardlong bean in endemic areas in Sukoharjo, Central Java, Indonesia. Yardlong bean leaves were taken from the district by purposive sampling method, then the virus was detected by nucleic acid amplification using specific primers for Begomovirus, *Bean common mosaic virus* (BCMV) and *Cucumber mosaic virus* (CMV). The results showed that one sample of yardlong bean could be infected by more than one virus. Infection of two kind of virus in a yardlong bean showed symptoms of each virus plus a synergistic effect where the symptoms become more severe.

Key words: BCMV, CMV, detection, Geminivirus, Potyvirus

INTRODUCTION

Yardlong bean (*Vigna unguiculata* subsp. *sesquipedalis* L.) is an important horticultural commodity in Indonesia. Besides being easily cultivated in various land conditions, this plant is also a cheap source of protein. It also has ability to fertilizes the soil because it belongs to the Leguminosae family (Duriat, 1998; Sastro, 2016). Till date, the production of yardlong bean in Indonesia has not been maximized, one of the causal is a viral infection (Taufik, 2015).

Various viruses were reported to infect yardlong bean. The presence of yellow mosaic disease in yardlong bean in Java was first reported in 2008 (Damayanti *et al.*, 2009). This disease was associated with infection of Begomovirus, *Bean common mosaic virus* (BCMV) and *Cucumber mosaic virus* (CMV) (Damayanti *et al.*, 2009; Tsai *et al.*, 2013). In Indonesia, other viruses that were found to infect yardlong bean are *Bean yellow mosaic virus* (BYMV), *Cowpea chlorotic mottle virus* (CCMV), *Cowpea golden mosaic virus* (CGMV), *Cowpea aphid-borne mosaic virus* (CabMV), and *Southern bean mosaic virus* (SBMV) (Semangun, 2004; Triharso *et al.*, 1998). These viruses can infect singly (individually) or double/multiple infections (infection with more than one type of virus).

Procedurally, the diagnosis of viral disease in plants begins with observation of symptoms. Some viral diseases in plants have been easily diagnosed based only on observations of the symptoms. Double or multiple infections besides worsen the disease, they also obscure the symptom of each infecting virus and make it difficult to diagnose.

This research aimed to detect the presence of viruses in some sample of yardlong bean leaves showing a certain symptoms. Detection was carried out by viral genome amplification method. The relationship between viruses infecting yardlong bean and their symptoms are described in this study. Some detected viruses in this study were also analyzed for their nucleic acids genomes, therefore the relationship to their closely related viruses were revealed.

MATERIALS AND METHODS

Research Site. The research was conducted in yardlong bean centers in Sukoharjo Regency, Central Java Province, Indonesia. Samples of yardlong bean leaves were collected from endemic areas of viral diseases in the district.

Collection of Yardlong Bean Samples. Yardlong bean leaves showing symptoms of viral disease in vegetative phase were selected by purposive sampling method. Fresh leaves samples were detected for the presence of virus/(s) and the rest were stored as virus isolates collection in the laboratory at -20 °C.

Extraction of Total RNA. Total RNA was isolated from infected leaf following a procedure described by Doyle & Doyle (1987) with minor modification. Fresh tissue (0.1 g) was ground in liquid Nitrogen to powder followed by addition of 500 µL of CTAB buffer (10% Cetyl-trimethyl-ammonium bromide, 0.1 M Tris-HCl pH 8.0, 0.05 M EDTA, 0.5 M NaCl, 1% β-mercapto-ethanol). Lipid and protein then separated by incubation in a water bath at 65 °C for 30 min, followed by shaking every 10 min. The debris was pelleted by addition of 500 µL of chloroform/isoamyl alcohol (24:1, v/v), vortexed for 5 min and centrifuged at 10,000 rpm for 15 min. RNA was precipitated by addition of 3 M ammonium acetate and isopropanol then incubated for 30 min at -80 °C, followed by centrifugation at 10,000 rpm for 7 min. RNA pellet was dissolved in 50 µL of TE buffer 1x (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and was ready for Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

Potyvirus Detection by RT-PCR. Total RNA was used as template for one-step Reverse Transcription-Polymerase Chain Reaction (RT-PCR). One-step RT-PCR reaction contains 2 µL RNA total, 12.5 µL premix *Go Taq Green* (Promega Japan), 1 µL each of primer MJ1 (5'-ATGGTHTGGTGYATHGARAAYGG-3') and MJ2 (5'-TGCTGCKGICYTTCATYTG-3') (Marie-Jeanne *et al.*, 2000), 2 µL DTT (dithiothreitol) 50 mM, 0.1 µL M-MuLV (200 U/µL), 0.1 µL RNase inhibitor (40 U/µL), 0.5 µL 25 mM MgCl₂, and the reaction was adjusted to 25 µL with nuclease free water. One-step RT-PCR reaction was performed in automated thermal cycler (GeneAmp PCR System 9700; Applied Biosystem, US) with 60 min at 42 °C and 3 min at 94 °C for RT reaction in 1 cycle, followed by 35 cycles of DNA amplification with 30 sec 94 °C, 1 min at 50 °C and 1 min at 72 °C. The amplicons was electrophoresed on 1% agarose gel in 0.5 x TBE (Tris-Boric acid-EDTA) buffer, stained using EtBr, and visualized under UV transilluminator.

CMV Detection by RT-PCR. Total RNA was used as template for one-step RT-PCR as in CMV detection above, using primers pair 1F (5'-AGCTAACCATGGAC

AAATCTGGATGTCCCAAT-3') and 1R (5'-AGCAT TCCATGGGGCAGTTTGAAGCAATACTACC-3') (Namba *et al.*, 1991). The reactions were 60 min at 42 °C and 3 min at 93.5 °C for RT reaction in 1 cycle, followed by 35 cycles of DNA amplification with 45 sec 93.5 °C, 45 sec at 55 °C, 1 min at 72 °C. The amplicons was electrophoresed on 1% agarose gel in 0.5 x TBE (Tris-Boric acid-EDTA) buffer, stained using EtBr, and visualized under UV transilluminator.

Extraction of Total DNA. Basically, total DNA extraction was the same as the total RNA extraction explained above. The difference was after pelleting the debris by the addition of 500 µL of chloroform/ isoamyl alcohol (24:1, v/v), the preparation was centrifuged at 14,000 rpm for 15 min. DNA was then precipitated by the addition of 3 M ammonium acetate and isopropanol, then incubated overnight at -20 °C, followed by centrifugation at 12,000 rpm for 10 min. DNA pellet was dissolved in 50 µL of TE buffer 1x (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and was ready for amplification.

Geminivirus Detection by PCR. Geminivirus detection was performed by DNA amplification using specific primers pair for Begomovirus, SPG1 (5'-CCC CKGTGCGWRAATCCAT-3') and SPG2 (5'-ATCCVA AYWTYCAGGGAGCTAA-3') (Li *et al.*, 2004). PCR reaction was conducted in 25 µL premix *Go Taq Green* (Promega, Japan) using Automated Thermal Cycler (GeneAmp PCR System 9700; Applied Biosystem, US). The reactions were 35 cycles (1 min at 94 °C of denaturation, 1 min at 50 °C of annealing, and 7 min at 72 °C of extension). The amplicons was electrophoresed on 1% agarose gel in 0.5 x TBE (Tris-Boric acid-EDTA) buffer, stained using EtBr, and visualized under UV transilluminator.

Nucleotide Sequence Analysis. The nucleotide sequence analysis was carried out in The DNA Sequencing BioSM Laboratories (Malaysia). The DNA sequence results were analyzed using the Basic Local Alignment Search Tool (BLASTN) program on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov) to compare the sequences of targeted virus to the close related viral nucleotide sequences deposited in GenBank Data base. Nucleotide homology and Identity Matrix Sequences were analyzed using Clustal W multiple alignment program and Bioedit 7.05 software (Hall, 1999), respectively.

RESULTS AND DISCUSSION

Samples of yardlong bean leaves were collected from endemic areas of viral diseases in yardlong bean centers in Sukoharjo District, Central Java Province, Indonesia. The samples of yardlong bean leaves were then grouped into 12 groups based on their symptoms on two grouping criteria i.e. leaf discoloration and leaf malformation relative to healthy leaves (Figure 1–5).

The 12 sample groups were obtained as follows: 4 sample groups were Geminivirus single-infected (Figure 1), 4 sample groups were Potyvirus single-infected (Figure 2), One sample group was CMV single-infected (Figure 3), one sample group was Geminivirus-Potyvirus double-infected (Figure 4), and one sample group was Potyvirus-CMV double-infected (Figure 5). Nurulita *et al.* (2015) reported that from 15 samples of

yardlong bean leaves collected from 6 districts in Central Java, West Java, and Special Region of Yogyakarta, 7 samples were single infected with Geminivirus, 2 samples were single infected with Potyvirus, no sample was infected with CMV, 4 samples were double infected with Geminivirus and Potyvirus, and 2 samples were not infected with any of the virus.

Yardlong bean leaves which were single infected with Geminivirus, showed a variety of symptoms ranging from discoloration (changes in color) to malformation (changes in shape). However, in general it can be described that the symptoms of yardlong bean leaves infected with Geminivirus were striking yellow color (yellow mosaic), curved (cupping), and dwarf (Figure 1). The Geminivirus infection is shown by the presence of a 912 bp band of nucleic acid as a result of amplification using a primers pair specific to Geminivirus

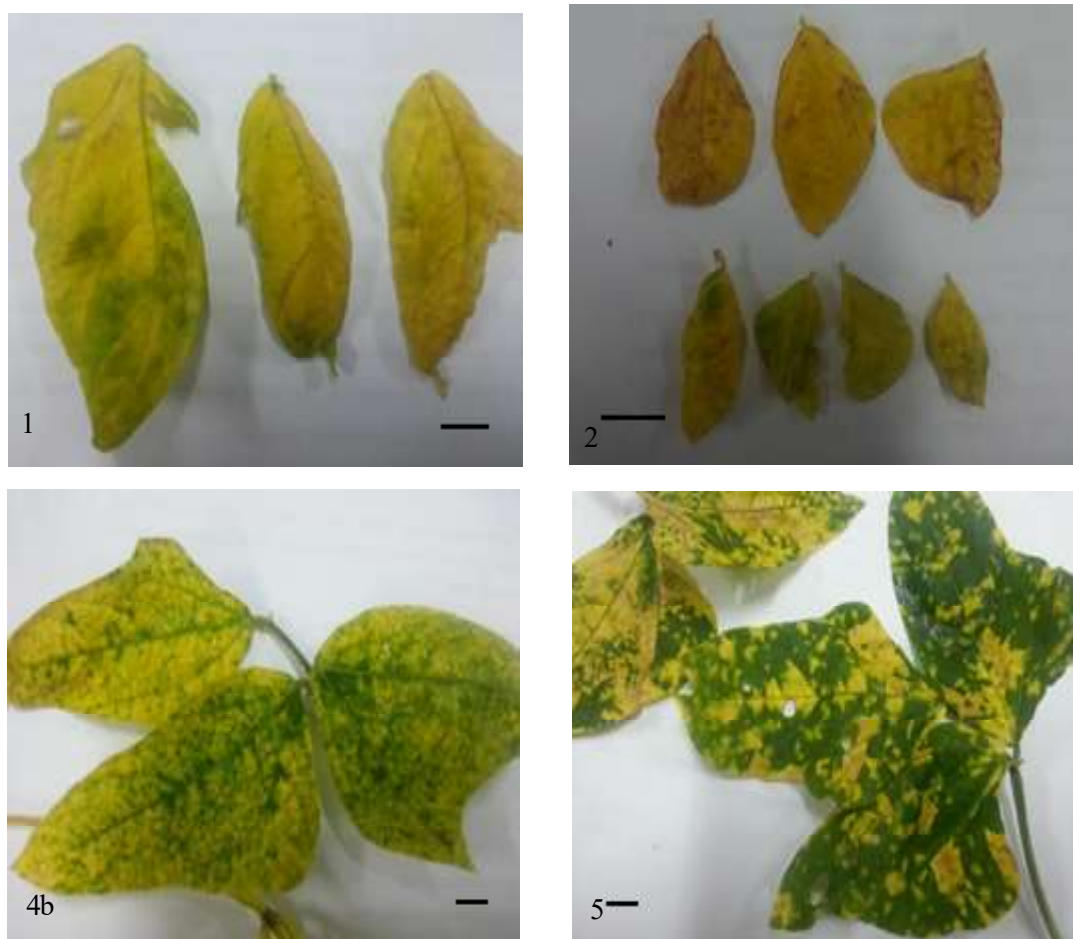


Figure 1. Symptoms variation of yardlong bean leaves infected with Geminivirus (leaves are striking yellow in color/yellow mosaic, curving/cupping, and dwarfing). (1) Leaves have striking yellow color with a little mosaic, cupping, leaves size are smaller; (2) Leaves have striking yellow color almost orange, cupping, leaves size are smaller; (4b) Leaves have striking yellow mosaic following the pattern of leaf veins, leaf veins are green, dominant in yellow; (5) Leaves have striking yellow mosaic following the pattern of leaf veins, leaf veins are green. The number refers to the sample group. Bar = 1 cm.

(Figure 6). Nurulita *et al.* (2015) reported that a single infection of Geminivirus in yardlong bean showed a variety of symptoms of leaves yellowing and yellow mosaic.

Yardlong bean leaves which were single infected with Potyvirus, showed a variety of symptoms ranging from discoloration to malformation. However, in general it can be described that the symptoms of yardlong bean



Figure 2. Symptoms variation of yardlong bean leaves infected with Potyvirus (leaves are light green mosaic/chlorosis, cupping/curling, and dwarfing). (6) Leaves have light green mosaic following the pattern of leaf veins, leaf veins are thick and hard; (9) Leaves have dark green color with a light yellow mosaic on the tip/edge of the leaf, leaf curl/roll; (10) Leaves have light green mosaic following the pattern of leaf veins, leaf veins are thick and hard and have yellow mosaic; (12) Leaves have light green color with yellow mosaic on the leaf veins. The number refers to the sample group. Bar = 1 cm.



Figure 3. Symptoms of yardlong bean leaves infected with CMV (leaves were mosaic, chlorosis, without/minimal changes in leaf shape). (8) Leaves have light green color with light yellow mosaic. Leaf veins are thick and hard. The number refers to the sample group. Bar = 1 cm.

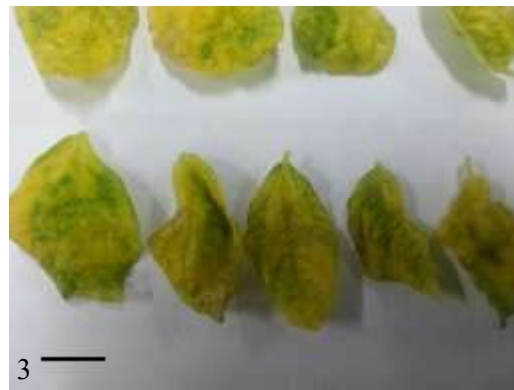


Figure 4. Symptoms variation of yardlong bean leaves double infected with Geminivirus and Potyvirus (leaves are dominant striking yellow color/yellow mosaic, cupping/curling, and dwarfing). (3) Leaves have striking light yellow color with light green mosaic around the leaf veins, cupping, leaves are small. The number refers to the sample group. Bar = 1 cm.



Figure 5. Symptoms of yardlong bean leaves double infected with Potyvirus and CMV (leaves are mosaic chlorosis, curling/dwarfing). (7) Leaves have green color with light yellow mosaic, curling, leaves are small. The number refers to the sample group. Bar = 1 cm.

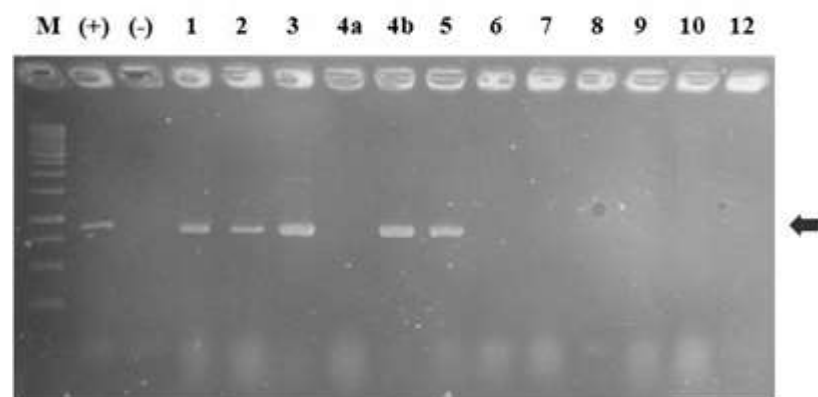


Figure 6. The results of the molecular detection of Geminivirus. Marker used in this study was 1 kb Plus DNA ladder (Invitrogen); (+) Positive standard using Geminivirus isolate of Laboratory of Virology, Bogor Agricultural University; (-) negative standard using virus-free yardlong bean leaves. The arrow indicates the bands of 912 bp. The lane numbers at the top refer to the sample group explained in the Results and Discussion and also in Figure 1–5.

leaves infected with Potyvirus were light green mosaic (chlorosis), (cupping/curling), and dwarfing (Figure 2). The Potyvirus infection is shown by the presence of a 328 bp band of nucleic acid as a result of amplification using a primers pair specific to Potyvirus (Figure 7). Nurulita *et al.* (2015) reported that a single infection of Potyvirus in yardlong bean showed symptoms of mosaic vein banding.

Yardlong bean leaves which were single infected with CMV, showed symptoms of light green leaf with a light yellow mosaic, veins were thick and hard. More specifically it can be described that the symptoms of yardlong bean leaves infected with CMV were mosaic,

chlorosis, without/minimal changes in leaf shape (Figure 3). The CMV infection is proven by the presence of a 320 bp band of nucleic acid as a result of amplification using a primers pair specific to CMV (Figure 8).

Yardlong bean leaves which were double-infected with Geminivirus-Potyvirus, showed the combined symptoms of each infecting virus plus symptoms that were a result of synergistic effect of the two viruses infection (Figure 1, 2, and 4). In more detail, it can be described that the symptoms of yardlong bean leaves double infected with Geminivirus-Potyvirus were dominant striking yellow leaves (yellow mosaic), cupping/curling, and dwarfing. Nurulita *et al.* (2015)

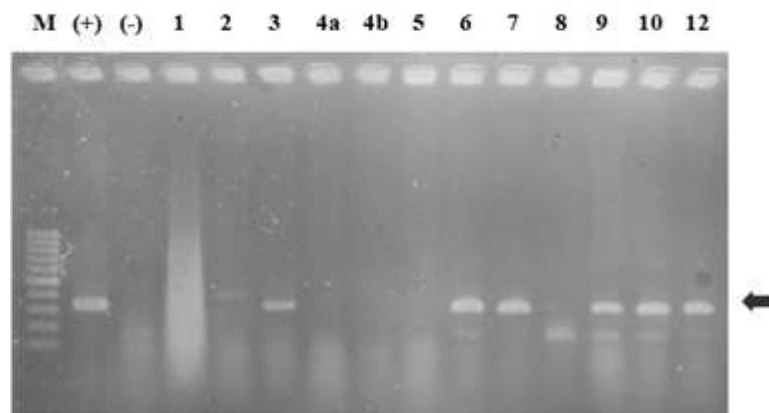


Figure 7. The results of the molecular detection of Potyvirus. Marker used in this study was 1 kb Plus DNA ladder (Invitrogen); (+) Positive standard using Potyvirus isolate of Laboratory of Virology, Bogor Agricultural University; (-) Negative standard using virus-free yardlong bean leaves. The arrow indicates the bands of 328 bp. The lane numbers at the top refer to the sample group explained in the Results and Discussion and also in Figure 1–5.

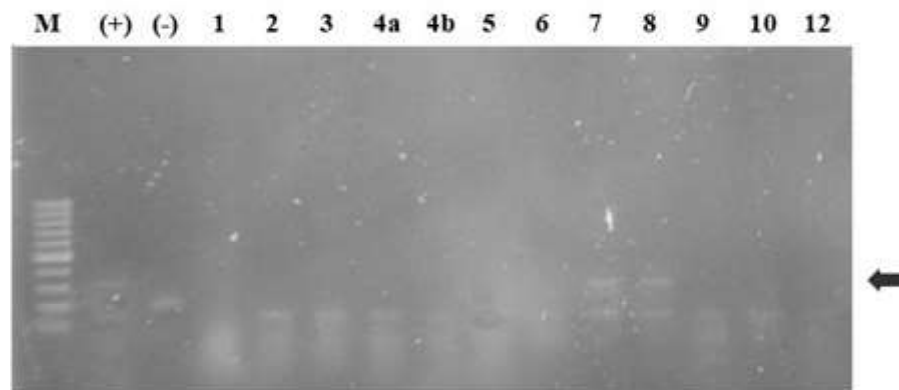


Figure 8. The results of the molecular detection of CMV. M, 1 kb Plus DNA ladder, Invitrogen; (+) Positive standard using CMV isolate of Laboratory of Virology, Bogor Agricultural University; (-) Negative standard using virus-free yardlong bean leaves. The arrow indicates the bands of 320 bp. The lane numbers at the top refer to the sample group explained in the Results and Discussion and also in Figure 1–5.

reported that double infection of Geminivirus and Potyvirus in yardlong bean showed symptom variation of yellow mosaic and yellow.

Yardlong bean leaves which were double-infected with Potyvirus-CMV also showed the combined symptoms of each infecting virus plus symptoms that were a result of synergistic effect of the two viruses infection (Figure 2, 3, and 5). It can be described that the Potyvirus-CMV double infection of yardlong bean leaves showed mosaic chlorosis and curling/dwarfing. In Indonesia, double infection of Potyvirus and CMV in yardlong bean was first reported by Damayanti *et al.* (2009) in the Bogor area. The symptoms were heavy yellow mosaic on leaves, deformation and mosaic in pods. Damayanti *et al.* (2010) also reported the presence of double infection of Potyvirus and CMV with symptoms of severe mosaic to strong yellow mosaic, and vein clearing.

Then four leaves samples were chosen to analyze the viral nucleic acid. The four samples were sample number 4b (single infected Geminivirus), sample number 6 (single infected Potyvirus), sample number 3 (double infected Geminivirus-Potyvirus), and sample number 7 (double infected Potyvirus-CMV).

The results of BLASTN analysis through NCBI of DNA sequences from samples number 4b and 3 showed that the infecting virus was Geminivirus, and had a high homology to several Geminivirus isolates from Indonesia which were deposited in the GeneBank database. The Geminivirus nucleotide sequences from

samples number 4b and 3 have the highest percentage of nucleotide similarity to isolate of Indonesian *Mungbean yellow mosaic India virus* (MYMIV) from Brebes which are 98% and 99%, respectively (Table 1). The existence of MYMIV in Indonesia was first reported by Nurulita *et al.* (2015). The authors collected yardlong bean diseased leaves samples from various locations in Java including Tegal, Klaten, Magelang, Subang and Bogor which were all positively infected with MYMIV. Based on the results of nucleic acid analysis, it was revealed that the virus was a group with MYMIV from Bangladesh, India, Pakistan and Nepal.

The results of BLASTN analysis through NCBI of DNA sequences from samples number 6 and 7 showed that the infecting virus was Potyvirus, and had a high homology with several Potyvirus isolates from abroad which were deposited in the GeneBank database. The Potyvirus nucleotide sequences from samples number 6 and 7 have the highest percentage of nucleotide similarity to isolate of *Bean common mosaic virus* (BCMV) from Cina of 92% and 90%, respectively (Table 1). Melinda *et al.* (2015) have reported the presence of BCMV associated with yardlong bean yellow mosaic disease in West and Central Java. From the samples collected from the two regions, it was revealed that 4 isolates (Cangkurawok, Subang, Solo and Sleman) had the highest homology and had a close relationship to BCMV-BIC from Taiwan. Whereas 2 isolates (Cirebon and Tegal) had the highest homology to BCMV-NL1 from the UK, and had close relationships

Table 1. The results of BLASTN analysis of nucleotide sequence of viruses detected in yardlong bean leaves and their homology values to the viral isolates from *GeneBank Data base*

Sample group number	Primer pairs	Nucleotide size (bp)	Closely related with	Homology (%)	Accession number
3	Universal primers for Geminivirus (SPG1 & SPG2). Amplicons size 912 bp	909	<i>Mungbean yellow mosaic India virus Indonesia</i> (Brebes isolate)	99	JN368436.1
4b	Universal primers for Geminivirus (SPG1 & SPG2). Amplicons size 912 bp	908	<i>Mungbean yellow mosaic India virus Indonesia</i> (Brebes isolate)	98	JN368436.1
6	Universal primers for Potyvirus (MJ1 & MJ2). Amplicons size 328 bp	333	<i>Bean common mosaic virus</i> (China)	92	KC832502.1
7	Universal primers for Potyvirus (MJ1 & MJ2). Amplicons size 328 bp	334	<i>Bean common mosaic virus</i> (China)	90	KJ807819.1

to the BCMV-NL1 from the UK and BCMV-BIC Y from China.

Based on the results of the analysis, it could be said that Geminivirus and Potyvirus isolates infecting yardlong bean in the Soloraya areas are closely related to the Geminivirus and Potyvirus isolates that have been reported by the previous researchers (Nurulita *et al.*, 2015; Melinda *et al.*, 2015). Both viruses are transmitted by whitefly and a group of aphids which were found abundantly on the Island of Java. Whitefly, *Bemisia tabaci* (Homoptera: Aleyrodidae) has been reported exist in Indonesia in 1938 (Kalshoven, 1981). The group of aphids also has long been reported exist in Indonesia (Kalshoven, 1981).

In the field, MYMIV transmission occurs through the help of insects vector (whitefly) and via mechanically, although rarely (Fauquet *et al.*, 2005). BCMV in the field also could be transmitted by insect vectors. But the vector for this virus is Aphid in non-persistent manner. Other than that, BCMV also could be transmitted by seeds (Fauquet *et al.*, 2005). Similar with BCMV, CMV in the field could be transmitted through the help of Aphids in a non-persistent manner (Fauquet *et al.*, 2005).

Based on the way of transmission in the field, the three viruses are transmitted in more or less the same way, i.e. through insect vectors, through seeds, and also by mechanical means. This gives an implication to the control strategy of the viruses. Thus, the strategy to control the three viruses can be formulated that is control of insect vectors, the use of virus-free seeds, and careful handling of plants.

CONCLUSION

There are three kinds of viruses were detected infecting yardlong bean in Solo Raya. The three viruses were *Mungbean yellow mosaic geminivirus* (MYMIV), *Bean common mosaic Potyvirus* (BCMV), and *Cucumber mosaic virus* (CMV). These viruses could infect yardlong bean both individually or doubly. Infection of two kinds of virus in a yardlong bean showed symptoms of each virus plus a synergistic effect where the symptoms become more severe. Geminivirus and Potyvirus infecting yardlong bean in the Soloraya are closely related (have a high nucleotides similarity) to Geminivirus and Potyvirus isolates that have been reported by the Indonesian previous researchers.

The implication of the findings obtained from this study on disease management strategies is that both MYMIV, BCMV, and CMV have the same mode of transmission through seeds, insects vector, and

mechanics. Therefore, the control can be done in the same strategy that is using healthy seeds, pest control (especially piercing-sucking insect) and handling plants carefully.

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