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Effect of Initial Inoculum Level of *Cowpea mild mottle Carlavirus* on Mottle Disease Development and Yield of Soybean

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ABSTRACT

Cowpea mild mottle virus (CPMMV) was reported causing systemic mottling, chlorotic blotch, and leaf malformation on cowpea from several tropical regions of Africa, Asia, and South America. Recently, CPMMV infection was reported from several soybean growing areas in Indonesia with high incidence. The disease tends to spread and develop very rapidly. A field experiment at Bogor was conducted to study disease development caused by CPMMV and its impact on soybean cv yield. Wilis. Soybean field was infested by CPMVV at different infection levels, i.e., 0%, 10%, 30%, and 50%. Virus indexing was done at two weeks interval by symptom observation and leaves collection for virus detection using dot immunobinding assay. Mild mottle symptoms were first shown 14 days after planting. The infection rate decreased with the increasing number of initial inoculums, whereas disease incidence increased with the growing number of initial inoculums. The infection of CPMMV caused a reduction of pods number and seed number, i.e., up to 18.81% and 30%, respectively.

Keywords: *Bemisia tabaci, disease incidence, disease rate, dot immunobinding assay*

1. INTRODUCTION

Soybean is one of the essential food crops in Indonesia. Soybean has an economic value as the source of protein, the raw material for industry, and animal feed. Soybean demand increase from year to year, but has not been fulfilled by domestic production. The domestic production in Indonesia is 700.000-800.000 tons per year, while the requirement is 2.2-2.3 million tons. Thus it is 30% of domestic output, and the remaining at 70% fulfilled from import. One of the diseases that can decrease soybean production is Cowpea mild mottle virus (CPMMV).

Cowpea mild mottle virus (CPMMV) was first reported infecting soybean in Indonesia by Iwaki et al. [1]. The infection of CPMMV caused endemic disease on soybean in Java and Sumatera. We conducted a field survey in 2016 and detected infection of CPMMV on several soybean growing areas in West Java, Central Java, East Java, South Sumatera, and Southeast Sulawesi (unpublished data). A high incidence of CPMMV infection was recently reported from several Indonesia regions as soybean growing areas are increased. This situation shows the potency of more comprehensive and rapid disease spread caused by CPMMV. This is especially true since CPMMV can be transmitted by its insect vector, *Bemisia tabaci* [2]. The transmission of CPMMV in the seed is still a controversial subject because some authors reported seed transmission of CPMMV in soybean, while others failed to produce such transmission [3]. CPMMV is known as seed-transmitted in *Vigna unguiculata*, *Glycine max*, and *Phaseolus vulgaris* [4]. Brito et al. [5] show that CPMMV can be transmitted from infected yardlong bean plants to seeds from the plant. From 231 sources harvested from previously infected plants, 222 seedlings were obtained and evaluated up to 45 days emergence, and 40% of the plants developed symptoms of mottling, mild mosaic, and slight leaf deformation. However, Horn et al. [6] reported that in Indonesia, CPMMV was not detected in soybean seeds and peanuts trough serological detection with enzyme-linked immunosorbent assay (ELISA). Tavassoli et al. [3] show that plants that CPMMV is not transmitted through seeds on Clark variety soybeans in Iran.

CPMMV has become a severe problem in Sudan [7]. Offei and Albrechtsen [8] reported that infection of CPMMV on Bambara groundnut (*Vigna subterranea* L.) caused a reduction in the number of pods, seeds, and seed weight. In Indonesia, the condition of CPMMV on soybean caused seed weight loss of 15.5% to 53.5%, and abnormal seed shape reached 7.6% to 54.35% [9]. The incidence of CPMMV infection and its impact on soybean production is critical for the effectiveness of the soybean intensification program launched recently by The Government of the Republic of Indonesia through the Ministry of Agriculture.

The use of healthy seed source and disease management in the field is significant to reduce yield loss caused by the disease. Management of viral infections in the soybean field usually relies on insecticide applications to control insect vector. Pedersen et al. [10] showed the effectiveness of insecticide application to control soybean aphid, which positively affected the suppression of *Soybean mosaic virus* infection. The amount of initial inoculum influences disease development in the field and population of the insect vector. In a case of seed-borne viruses, the primary condition is present immediately upon the germination or planting of the infected plants in the field. Movement and feeding behavior of insect vector will then spread the virus widely. This paper reported the effect of different initial infection levels of CPMMV on disease development and soybean yield.

2. MATERIALS AND METHODS

A field experiment was conducted from June to October 2016 at IPB University Farm in Cikabayan, Bogor, West Java. Virus detection was performed at the Laboratory of Plant Virology, Department of Plant Protection, Faculty of Agriculture, IPB University.

2.1 Experimental design and field observation

A field experiment wasconducted from June to October 2016 at IPB University Farm in Cikabayan, Bogor, West Java. The experiments were arranged in a randomized complete block design with three initial inoculum levels, i.e., 10%, 30%, and 50%, whereas 0% initial inoculum was treated as a negative check. The experiments were performed on three main plots as three replications, and each main plot consisted of 3 levels of initial inoculum. Each experimental plot's size was 2 x 3 m with space among plants was 25 cm, and the total number of plants was 88 plants per plot.

One week after planting, virus inoculation was conducted in the field following the mechanical inoculation method described above. The number of plants inoculated in each plot was adjusted to each treatment. Therefore, 10%, 30%, and 50% of plants, i.e., 9, 29, and 44 plants were inoculated at the plot with 10%, 30%, and 50% initial inoculum level, respectively. One week after inoculation, leaf samples were collected for virus infection confirmation using the DIBA method.

2.2 Virus inoculation

Virus inoculum was propagated by inoculating young soybean plants cv. Wilis with CPMMV isolate from Cianjur (CR16) in the screen house. The inoculum was prepared by grinding leaves from infected soybean plants in 0.1 M phosphate buffer (pH 7.0) with a ratio of 1:5 (w:v). Carborundum (600 mesh) was incorporated in the inoculum as an abrasive. The inoculum was gently rubbed over the leaf, from its base to the top using forefinger. After inoculation, the leaves were rinsed with water.

2.3 Detection of CPMMV by dot immunobinding assay (DIBA) method

Detection using DIBA was conducted following the method described by Mahmood et al. [11]. Firstly, plant sap was prepared by grinding soybean leaves in tris buffer saline (TBS) (pH 7.5) containing Tris-HCl 0.02 M and NaCl 0.15M with a ratio of 1:10 (w:v).The sap was dotted to a nitrocellulose membrane, i.e., two µL for each sample. The membranes were air-dried, then incubated in 10 ml blocking solution (2% skim milk and 2% Triton X-100 in TBS). After incubation, the membranes were washed five times in dH_2O, 5 min per wash. The membranes were then incubated overnight at 4°C in a 1:3000 dilution of alkaline phosphatase-conjugated CPMMV antibody using TBS containing 2% skim milk. The membranes were washed in TBS, which includes 0.05% Tween (TBST) (five washes, 5 min per wash). The membranes were thenincubated in substrate solution containing 45 µL of nitro blue tetrazolium (NBT) and 35 µL of Bromochloroindolylphosphate (BCIP) in 10 mL alkaline phosphate (AP) buffer (Tris-HCl 0.1 M, NaCl 0.1 M, $MgCl_2$ 5 mM, and aqua dest; pH 9.3). Positive reactions are characterized by development of purple color on the nitrocellulose membrane that has been dotted with plant sap; the reaction was stopped by soaking the membranes in dH_2O.

2.4 Fields Observation

The observation was conducted on several parameters, i.e., type of symptoms, virus incubation period, disease incidence, whitefly population, number of pods, and number of seeds.Virus development was observed at two weeks interval based on symptom observation, and at the same time, leaf samples were collected for virus detection using DIBA. The incidence calculated the ratio of the number of infected plants and the total number of tested plants based on DIBA results. Five yellow sticky traps (20 cm x 15 cm) were placed diagonally on the field started four weeks after planting (WAP) for 24 hr to monitor the whitefly population.

Data for plant height and number of leaves were recorded at a 2-week interval throughout 7 WAP. Yield components such as number of pods, number of 100 g of seed, dry weight of grain (g) were taken at harvest time. Harvested pods were dried, and the value was calculated using a digital compact scale.

Data analysis. All data were subjected to the analysis of variance (ANOVA) with Statistical analysis software(SAS) version 9.4, and means were separated by Duncan’s multiple range tests (DMRT) $P \leq 0.05$. Correlation analysis was used to quantify the relationships between the number of initial inoculums in the field and the number of seeds per plant.

3. RESULTS AND DISCUSSION

3.1 Development of Mottle Disease on Several Initial Inoculum Level

The development of the disease can be determined through the increase of disease incidence (%), rate of disease development (r), and value of AUDPC (area under disease progress curve). Initial inoculum of 10%, 30%, and 50% could cause relatively similar disease incidence, approximately 82 - 83% at the end of the observation, which is 8 WAP (Figure 1).

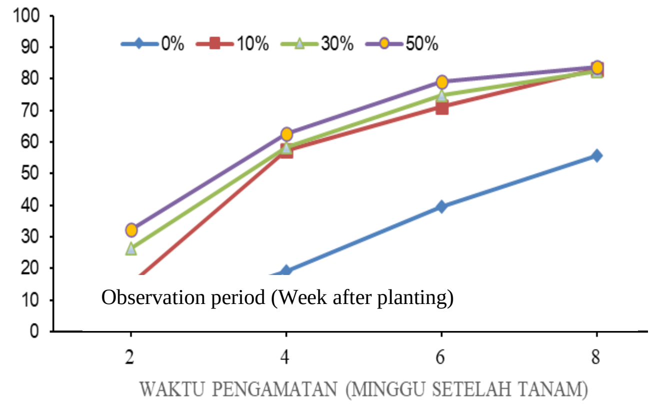

Figure 1 Development of mottle disease on 'Wilis' variety of soybean between 2 to 8 WAP at several initial inoculums of CPMMV (0%, 10%, 30%, and 50%).

The incidences of mottle disease at an initial inoculum of 10%, 30%, and 50% were significantly different against the control (0%). Higher initial inoculum resulted in higher disease incidence at the end of observation (Table 1). the incidence showed a significant increase, especially started from 2 WAP until 6 WAP. The rise of disease incidences was not significantly different afterward. The incidence was related to the plant growth stage because, from 6 WAP, soybean began to start the generative phase, forming a flower. Thus it would not produce new leaves (determinate). At the same time, the feeding activity of whitefly depends on the nutrients inside young leaves. Whitefly moves from one crop to another which provide nutrient of young leaves.

The development of disease incidence is positively related to the population of whitefly. The spread of CPMMV is supported by the presence of whitefly as its vector and can spread CPMMV so that the incidence of mottle disease can reach 82% in the field. The initial inoculum level of 10-50% caused the same disease incidence at the end of the observation and decreased the soybean yield by 32.23%. The whitefly's statement at the yellow trap on 4 WAP showed a population of 22-45 whiteflies per catch. The economic injury level of *B. tabaci* is seven nymph per leaf or 11 adult per field [12]. Though the whitefly population during the experiment was lower than the economic injury level, whiteflies have high efficiency in transmitting CPMMV Muniyappa dan Reddy [13] and Iwaki [1] reported that a single insect of whitefly imago was successfully transmitted CPMMV with a 30% rate, while the maximum transmission of 90% was obtained using 5 insects. Therefore, the 22-45 insect population on the yellow sticky trap was considered very high and capable to rapidly transmit CPMMV to healthy crops surrounding the inoculum resource.

Table 1 Incidence of mottle disease (2 to 8 WAP) on cv. 'Wilis' of soybean with various levels of initial inoculum of CPMMV.

Treatment	Disease Incidence (%)*
Level of Initial Inoculum	
0% (Negative control)	29.26a
10%	56.63b
30%	60.60b
50%	64.39b
Observation period	
2 WAP	19.14a
4 WAP	49.29b
6 WAP	66.24c
8 WAP	76.21d

Remarks: *mean value followed by the same letter at the same column indicates no significant difference (Duncan multiple range tests, α=5%)

Generally, soybean varieties grown in Indonesia have a determinate type of growth, namely the development of young stems and leaves stalled when the plant enters the flowering phase. The increase in the incidence of mottle disease is very high when the plant is in the vegetative growth phase, which is 2 to 6 weeks after planting because, during that phase, the formation of young leaves occurs. The increase in whitefly populations is also closely related to the stage of plant growth. The whitefly population is high in the vegetative growth phase and decreases when the plant enters the generative phase. Latif and Akhter [14] report that imago of *Bemisia tabaci* tends to lay eggs on young leaves, making it easier for the offspring to pierce to suck young leaf tissue than old leaf tissue. Young leaves have softer leaf tissue than older leaves. Soybean leaves have started to turn yellow and fall before harvest during the pod maturation phase so that the nutrient content needed by *B. tabaci* is already reduced in the host plant. This causes the image to move to look for new sources of feed.

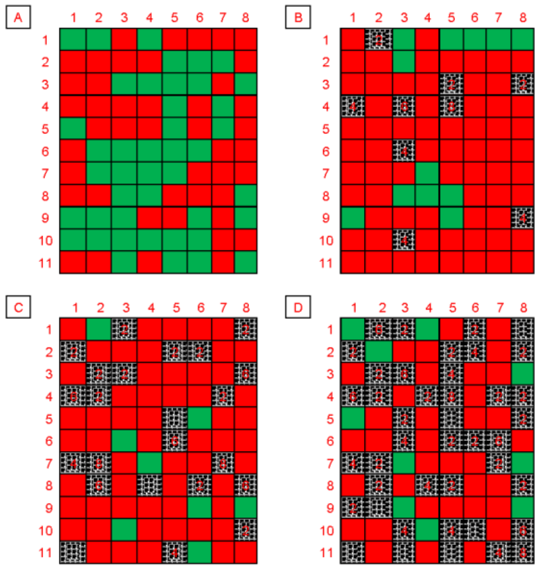

Figure 2 Distribution of CPMMV in Field Soybean cv. 'Wilis' in 4 times of observation period (Number inside the box indicates observation period [WAP] of CPMMV infection; Box with line pattern are the spots of initial inoculum of the Block; red boxes are the CPMMV infected crop, and green boxes are healthy crop without CPMMV infection).

In Figure 2., the green boxes indicate crops that were mechanically inoculated with CPMMV but were not found infected, or CPMMV was not detected using the DIBA detection method. Block of negative control without initial inoculum (A), Block with 10% of initial inoculum (B), Block with 30% initial inoculum (C), block with 50% initial inoculum (D). Number 1 to 8 at the top of the box are the plant row, 1 to 11 on the left side of the box are the plant row. Based on the map in Figure 2., CPMMV was generally transmitted to the nearest crop surrounding the inoculum resource. It is suggested to be related to the feeding activity of whitefly that moved in between the closest crops. Block with the number of initial inoculum from 10% to 50% showed a higher incidence and higher initial inoculum. Hence, if the CPMMV infected seeds were used as the planting materials, it has a higher potency to be the initial inoculum that could be transmitted to other healthy crops by the whitefly vector's help. The whitefly population increased during the vegetative due to the higher production of young leaves during this growth stage, which is the nutrient resource and suitable habitat for the whitefly. The presence of CPMMV at the negative control block on 4 WAP was due to the transmission from another Block with the incidence of CPMMV by whiteflies' help. This suggested that utilization of soybean variety with no CPMMV seed transmission potency, e.g., 'Wilis,' should result in no incidence of mottle disease, regardless of the presence of whiteflies.

Table 2 Plant height and number of leaves ofsoybean cv. 'Wilis' at several CPMMV initial inoculum level based on the observation period.

Treatment		Plant height (cm)	Number of leaves
Levels of initial inoculum (%)			
0		31.10±1.19a	17.89±0.58a
10		31.87±1.74a	17.01±0.43a
30		30.69±1.12a	17.68±0.59a
50		30.15±1.66a	17.68±0.36a
Observation time (Week after planting)			
3		20.98±0.92a	12.47±0.24a
5		31.42±0.36b	17.43±0.38b
r7		40.47±2.01c	22.79±0.86c

Remarks: Data was taken at 3, 5, and 7 weeks after planting; Means within a column followed by the same letter are not significantly different at 0.05 level in a DMRT, α = 5%

Plant height and number of leaves on 3-7 WAP was not significantly different between each treatment (Table 2). Weekly observation of plant height and number of leaves showed a significant increase. Thus, it has proved that the infection of CPMMV on soybean cv. 'Wilis' did not affect the plant height and number of leaves. This is different from CPMMV infection in the groundnut in Sudan, which caused stunting, reduction of internodes length, and leaf [7].

Table 3 The number of pods, seeds, seed weight of soybean cv. 'Wilis' in the field as influenced by initial inoculum levels

I(%)	Number of pods per plant	Seeds per plant	Number of seeds per 100 g	Number of abnormal seeds per 100g*
0	43.48±9.97a	133.42±1.15a	1154.3±153.00a	123.00±72.02a
10	37.92±9.02ab	133.08±0.76a	1105.0±168.02a	133.00±85.47a
30	35.31±5.84b	90.42±1.07b	1058.3±81.51a	124.67±55.97a
50	35.30±5.23b	93.42±1.53b	1079.7±49.65a	185.33±25.16a

Remarks: Means within a column followed by the same letter are not significantly different at 0.05 level in a DMRT, α = 5%

*Abnormal seed was indicated by discoloration and malformation of seed (black and wrinkled seed)

Generally, the condition of CPMMV on soybean caused a systemic mottle disease and leaves' malformation, but not stunting. Thus the height of the plant is normal, as well as the number of leaves. 'Wilis' soybeans that were infected by CPMMV had a standard number of leaves; however, the mottle symptoms on the leaf caused by the damages on chlorophyll affected photosynthate production. Hence, infection of CPMMV more likely affected the inhibition of pods formation and filling due to the decreasing of photosynthate production.

Table 4 Detection of CPMMV on the seed of 'Wilis' variety of soybean collected from crop infected on a different period

Age of infected crop (MST)	Germination rate (%)[1]		The efficiency of Seed transmission (%)[2]	
	Symptomless seeds	Wrinkled and Black	Symptomless seed	Wrinkled and black
2	100	38	0	0
4	100	56	2	0
6	98	54	0	0
8	100	64	0	0

[1]Number of germinated seed/number of planted seed x 100
[2]Detection of CPMMV based on DIBA; the number of positive sample/total sample x 100%

The infection of CPMMV reduced the number of pods and the number of seed at 30-50% of the initial inoculum level (Table 3). The high rate of initial inoculum caused the decrease of 'Wilis' soybean productivity up to 8.2 pods per crop, or as much as 18.81%, and decrease the number of seed up to39.98-42.98 seeds per yield, or as much as 32.23%. The production loss is more rad high, even though the cultivated crop showed a mild mottle symptom, but its effect was relatively high. This can be a disadvantage for farmers due to the mild mottle symptom, sometimes symptomless, but the potency to cause production loss was very high. The farmers might ignore those mild symptoms, thus lead to no eradication attempt.

However, the number of seed and abnormal seed within 100 g seed was not different on every initial inoculum level (Table 3). More number of seeds per 100 g seed means a smaller size of the grain. The level of initial inoculum tends not to affect the size of the seed. Thus, the infected crop did not cause the seed to shrink, yet the number of pods was decreased. The abnormal seed in this research was wrinkled and showed black coloration. The number of abnormal seed was not significantly different between the negative control plot and the infected crop plots with every initial inoculum level.

One of the factors that affect the incidence of pathogens on the seed is the crop stage of growth when infection occurs. Therefore, in this research, seeds of the infected crop were separated based on the infection period, i.e., 2, 4, 6, and 8, WAP. Detection of the virus on seed from various infection time are shown in Table 4. infection of CPMMV in 'Wilis' soybean was not seed transmitted, except on the 4 WAP that was detected on 2 out of 100 seeds.

4. CONCLUSION

The level of CPMMV infestation in the early stage of plant growth will affect disease development. Disease incidence and yield loss increased significantly when the initial infestation of CPMMV reached 30% or more. Therefore, it is essential to develop a level of tolerance in the seed health certification program. Soybean variety with low viral seed transmission efficiency, such as var 'Wilis,' is recommended to suppress disease development.

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