Journal of **BIOLOGICAL RESEARCHES**

ISSN: 08526834 | E-ISSN:2337-389X

Volume 21| No. 2| June | 2016

Original Article

FOLIAR SYMPTOMS RECOVERY: DEVELOPING SCORING TECHNIQUE FOR ASSESSMENT OF SOYBEAN RESISTANCE TO CPMMV (COWPEA MILD MOTTLE VIRUS)

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ABSTRACT

Soybean is a commodity that has an important role as the source of protein, but its production is affected by various factors including disease. CpMMV (Cowpea mild mottle virus) is one of the most damaging viruses that cause soybean disease. CpMMVs belong to the group of Carlavirus that are transmitted by whitefly (*Bemisia tabaci*). The use of CpMMV resistant plants as biological control can prevent viral diseases. Indonesia has many soybean germplasm from many regions and introduction from other countries that need to be evaluated for CpMMV. The assessment technique for soybean resistance to CpMMV is not available yet, but it is still based on other virus diseases. The specific assessment of plant resistance is important because some diseases can cause certain symptoms, depends on the resistance and the kinds of plants that are infected. This paper presents the assessment technique for soybean resistance to CpMMV infection, which can be used for various purposes and studies. One of the benefits is to find out soybean resistance to CpMMV or other objectives. This resistance assessment is not only based on leaves symptoms, but also based on the phenomenon of foliar symptoms recovery.

Keywords: CpMMV, foliar symptoms recovery, scoring technique, soybean resistance.

INTRODUCTION

Soybean is one of the major food crops in Indonesia besides rice and corn. Soybean has an important role as the source of protein, animal feed, as well as raw materials for small or household industrial scale to large scale. Soybean demand continues to increase from year to year, but has not been fulfilled by domestic production. According to BPS (2015), the production in 2014 is 953.96 thousand tons, increased by 173.96 thousand tons (22.30%) compared with 2013. The average production of Indonesian soybean is 700-800 thousand tons per year, while the requirement is 2.2-2.3 million tons, and the remaining 1.4-1.5 million tons are supplied from import. Thus, it is approximately 30% from domestic production and at least 70% from import. Even if the soybean production increases every year, soybean demand has not been fulfilled due to various factors, such as limited agricultural land and diseases infestation. One of the diseases that can decrease soybean yield is caused by CpMMV (cowpea mild mottle virus).

CpMMV is a member of the genus of *Carlavirus*. Currently, this virus is classified in family *Betaflexiviridae*, which is characterized by flexuous filamentous particles of approximately 650 nm in length. CpMMV is transmitted in a non-persistent manner by the whitefly *Bemisia tabaci* G. (Tavasoli *et al*., 2009). It is also distributed by mechanical transmission (Salaudeen & Aguguom, 2014). However, the transmission of CpMMV in seed is still a controversial subject because some authors reported seed transmission of CpMMV in soybean, while others failed to produce such transmission (Tavasoli *et al*., 2009). CpMMV was first observed causing chlorotic blotches, systemic mottling, and leaf malformations in cowpea (*Vigna unguiculata* L.) at 1973 in Ghana (Brito *et al*., 2012). Since then, it has been subsequently reported as the limiting factor of soybean production in many countries such as Argentina, Egypt, Nigeria, Yemen, Israel, Kenya, Brazil, Thailand, Malaysia, and others including Indonesia (Buchen-Osmond, 2002). CpMMV is also known infectis the other hosts systemically with clear symptoms in *Chenopodium amaranticolor*, peanut, kidney bean, beans, tomatoes, eggplants, grasses, and various other plants (Buchen-Osmond, 2002; Pardina *et al*., 2004). According to Iwaki *et al*. (1986) sometimes CpMMV infection in some host plants is symptomless and the symptoms will appear if inoculated to the indicator plant.

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CpMMV causes blotchy yellow leaf, mosaic or rough mosaic, wrinkled, chlorosis, necrosis of apical and malformation of leaves symptoms depending on the infected soybean cultivars (Kameya, 2001; Buchen-Osmond, 2002). The yield of CpMMV-infected soybean may be decreased up to 90% depending on the plant age when infected, virus strain, and environmental condition (Sinclair, 1993). In moderate resistant varieties, generally it show chlorotic spots, mild mottle, and medium wrinkled leaves symptoms, but plant growth is not much affected. However, in susceptible varieties, infection at the beginning of growth resulting in stunted plants, small leaves, and produces little pods (Sinclair, 1993). Based on the symptoms, in India CpMMV can be identified into two strains, i.e. CpMMV-S that causes severe symptoms and CpMMV-M that causes mild symptoms (Naidu *et al*., 1998).

Indonesia has many soybean germplasm from many regions and introduction from other countries, and many of them have not been evaluated for CpMMV yet. The assessment of resistance to the specific disease in plant is needed, because the virus may not show the same symptoms in different host plants. Tavassoli *et al*. (2009) and Rodrigues *et al*. (2014) reported that CpMMV did not show any visible symptoms in some host plants like *Vigna radiata*, *V. aconitifolia*, and tomato, although they were infected and gave positive results in the ELISA test for the virus. It seem that the expression of symptoms in infected plants could difference that will make assessment of the disease severity and incidence and its management very difficult. There are many reasons why estimating or measuring disease effect on plant is important, particularly for decision-makers where disease must be related to yield loss; for disease management decisions such as applying pesticides to control disease epidemics; in plant breeding where various germplasm, varieties and/or cultivars need to be scored; and also for understanding fundamental processes in biology, including plant disease epidemiology and coevolution (Burdon *et al*., 2006; Bock *et al*., 2010).

The existence of a reference scoring of soybean resistance to CpMMV is necessary. Various publications about determination of scoring on soybean resistance against other viruses such as yellow mosaic virus (Usharani *et al*., 2004) and southern bean mosaic virus (Gumedzoe *et al*., 1997) cannot be applied for CpMMV because the virus is different. Hopefully, by the reference of scoring determination on soybean plant resistance to CpMMV is expected to be used for assess specifically. Previously, Zubaidah *et al*. (2006) have developed an assessment for soybean resistance to CpMMV which done at the age of approximately 35 days after planting (DAP). This assessment is very helpful on several related studies. However, in the observation during the development before 35 DAP, we discovered the phenomenon of foliar symptoms recovery. The symptoms changes gradually as the soybean age increases that may affect the category of disease severity on leaves symptoms, the scores and the criteria of plant resistance. Hence, we develop the assessment of soybean resistance to CpMMV based on foliar symptoms recovery. In this study the assessment of resistance is conducted three times i.e. on 21, 28, and 35 DAP.

The cause of the recovery phenomenon of CpMMV-infected soybean leaves is still unknown. For other plants, the recovery appears to be induced by different factors and it can be correlated to various biological factors. These include the induction of systemic acquired resistance (SAR), the activity of particular substances or plant secondary metabolites, the presence and dominance of hypovirulent strains of the pathogens, and the presence of antagonists or phytoplasma parasitoids (Romanazzi *et al*., 2009a).

METHODS

The experiment was conducted in Indonesian Legumes and Tuber Crops Research Institute, Indonesian Agency for Agricultural Research and Development, Malang. The research material consisted of 100 genotypes of soybean germplasm. Each genotype were planted in four polybags, each of which consists of four plants, then reduced became two plants so the total plants were 800 plants. The CpMMV inoculums were supplied by providing 40 infected soybean plants. These inoculums plants were placed around the tested soybean germplasm. This technique allowed CpMMV inoculation from CpMMV inoculums to the tested plants by the vector of CpMMV (*Bemisia tabaci*). Inoculation was applied from 7 to 14 days after planting. It was more effective than mechanical inoculation using extract of disease leaf containing the viruses (Saleh et al., 2005). After inoculation, the inoculated plants were placed in the screen house as well as the control plants.

The existence of viruses in plants was determined by ACP-ELISA (antigen-coated plate enzyme-linked immunosorbent assay) serological techniques using CpMMV antiserum, with manual procedures in accordance with the manufacturer (DSMZ, Germany). Quantification of the virus was determined by the absorbance value using the ELISA reader at a wavelength of 405 nm. The test was declared positive if the sample absorbance value is two times than healthy control.

The assessment resistance was performed using ordinal scale which was developed by Zubaidah *et al*. (2006), at 21 days after planting (DAP) (the first scoring), 28 DAP (the second scoring), and 35 DAP (the third scoring). The symptoms categories with a score of 1-5 are shown in Table 1 and the symptoms visualization are in Figure 1-5. The symptoms category showed the disease severity, that is the area (relative or absolute) of the sampling unit (leaf, fruit, etc.) showing symptoms of disease (Bock *et al*., 2010).

RESULTS

The results of ELISA showed that all of the tested plants were positive, while control plants were negative. It seems that the preparation technique of the inoculums source and naturally viral inoculation from the inoculums source to the tested genotype by the vector was effective. Transmission of the virus naturally by the vector *B. tabaci* was closer to the actual conditions than mechanically artificial inoculation. The natural technique can also be used to screen large numbers genotypes. However, these techniques has the disadvantage in which the effectiveness depends on the population of insect vectors of *B. tabaci* and the possibility of another viral infection which is transmitted by *B. tabaci* or others, because the insects vector *B. tabaci* were originated from around the experiment site allowed containing various CpMMV strains or other diseases.

The symptoms found to be vary from the lightest with leaves look healthy, no mottle or mild yellow blotches; until the most severe symptoms with clear yellow blotches, wrinkles, obviously mosaic, necrosis on the leaf vein, malformation, smaller leaves, curved to downward or upward. Some genotypes showed stunt or dwarf. These symptoms were similar to the study of Thouvenel *et al*. (1982) in plants that were mechanically infected using CpMMV. We also found some variation patterns of plant resistance based on observation conducted three times as shown in Table 3.

Table 1. The Leaf Symptoms Category

Score	Symptoms
1	Look healthy, no mottle or mild yellow blotches
2	Yellow blotches, no wrinkles
3	Yellow blotches, slightly wrinkled, slightly mosaic
4	Yellow blotches, wrinkles, obviously mosaic, no necrosis
5	Yellow blotches, wrinkles, obviously mosaic, necrosis on the leaf vein, malformation, smaller leaves, curved to downward or upward, plant dwarf

Table 2. The Criteria of Soybean Resistance to CpMMV

Disease Severity	Soybean Resistance
$S \leq 25\%$	Resistant
$25\% < S \leq 50\%$	Medium resistant
$50\% < S \leq 75\%$	Medium susceptible
$75\% < S$	Susceptible

Table 3. The Patterns of Soybean Resistance Variation Based on Observation Conducted Three Times

First Scoring (21 DAP)	Second Scoring (28 DAP)	Third Scoring (35 DAP)	Condition
Medium resistant	Medium susceptible	Medium susceptible/ susceptible	Not recovery
Medium resistant	Medium resistant	Medium resistant/ Medium Resistant	Not recovery
Resistant	Resistant	Medium resistant/ Medium susceptible	Not recovery
Resistant	Resistant	Resistant	Recovery/Not recovery
Medium resistant	Medium resistant	Medium resistant/ Resistant	Recovery
Medium resistant	Medium susceptible	Resistant	Recovery
Medium susceptible	Medium susceptible	Medium resistant	Recovery

Figure 1. Symptoms of Score 1: look healthy, no mottle or mild yellow blotches.

Figure 2. Symptoms of Score 2: Yellow blotches, no wrinkles.

Figure 3. Symptoms of Score 3: Yellow blotches, slightly wrinkled, slightly mosaic.

Figure 4. Symptoms of Score 4: Yellow blotches, wrinkles, obviously mosaic, no necrosis.

Figure 5. Symptoms of Score 5: Yellow blotches, wrinkles, obviously mosaic, necrosis on the leaf vein, malformation, smaller leaves, curved to downward or upward.

$$S = \frac{\sum(n \cdot v)}{N \cdot Z} \times 100\%$$

Description:
S : disease severity per plant (%)
n : the number of affected leaves on certain category per plant
v : the score category of certain infected leaf
N : the number of observed leaves per plant
Z : the value of the highest category

DISCUSSION

We used ordinal scale to score because it is still quite widely acceptable for specific diseases, particularly for scoring some virus diseases where symptoms are not easy to measure quantitatively (Madden *et al*. 2007). Ordinal scoring scales have some advantages, i.e. easy to learn and use, provide a rapid way for assessing large numbers of plants, as might sometimes be the need in plant breeding programs, and particularly that they often describe the disease development as the symptoms become increasingly severe and thus can be interpreted by the rater (Bock *et al*. 2010). Ordinal data should be analyzed using non-parametric tests, which are becoming more sophisticated and powerful for these types of data (Madden *et al*. 2007).

The assessment of soybean resistance to CpMMV in this study was performed only based on the rater eyes and without tools. According to Bock *et al*. (2010), visually assessed disease has some advantages, where the process can be quick, relatively easy to recognize and differentiate multiple diseases with some training, and no equipment required. Besides, visually assessment has some disadvantages where substantial inter- and intra-rater variability (subjectivity) were found, raters may tire and lose concentration, thus decreasing their accuracy, raters are prone to various illusions (for example, area infected), and visual scoring can be destructive if samples are collected in the field for assessment later in laboratory. Hence, training of raters before assessment is needed. To maintain the rater quality, the training should be repeated periodically.

As stated by Bock *et al*. (2010), recovery can be complete or partial, temporary or permanent, and common or rare. Consequently, it can be practically significant or not for an infected plant. We did not study whether the recovery of CpMMV infected soybean showing complete or partial and temporary or permanent, but in grapevine and apple with severe symptoms for several years showing a complete remission of the symptoms, associated to the disappearance of the phytoplasms from the crown (Carraro *et al*. 2004). Recovery appears to be induced by different factors, such as the presence and dominance of hypovirulent strains of the pathogens, the presence of antagonists or phytoplasma parasitoids, the activity of particular substances or plant secondary metabolites, and the induction of systemic acquired resistance (SAR). From various references, Romanazzi *et al*. (2009b) explained that in grapevines, this natural phenomenon has been observed in different varieties and viticultural regions. In grapevines, the recovery phenomenon depends on some factors such as phytoplasma identity, host-plant variety (Bellomo *et al.* 2007), rootstock combination (Romanazzi & Murolo, 2008), environmental conditions (Braccini & Nasca, 2008), and agronomic practices such as pruning or transplanting. In recovered plants, molecular analysis of some grapevine varieties leaf veins has failed to reveal the presence of phytoplasms in several Italian and German areas (Romanazzi & Murolo, 2008).

The physiological basis of plant recovery from symptoms is not yet completely known. Thakur (2007) stated that various factors could influence resistance expression such as the inoculum density, crop maturity, plant habitat, weather variables, interplot interference, agronomic practices, etc. The expression of resistance genes often get modified by the action of other genes. There are some reports about recovery in plant disease as stated further. It has been observed that in grapevine, in apple, and in apricot, recovery from phytoplasma-associated diseases was related by an overproduction of hydrogen peroxide localised in the phloem tissues (Musetti *et al*. 2007). In pepGMV (Pepper golden mosaic virus)-infected pepper plants, the concentrations of viral nucleic acid in recovered leaves were reduced compared to severely symptomatic leaves (Carillo *et al*. 2007; Rodriguez *et al*. 2009). The recovery process has been associated with transcriptional and post-transcriptional gene silencing mechanisms (Rodriguez *et al*. 2009).

Regarding to the presence of recovery phenomenon, we found that based on observation conducted three times (21, 28, and 35 DAP), the CpMMV resistance assessment should be applied as follows:

a. If the assessment is done once, the scoring could be carried out at 35 DAP or more because it has undergone a process of recovery or not recovery (limited to the results of this study). In this case, we can use the assessment technique that has been previously published (Zubaidah *et al*. 2006), as shown in Table 1 and Table 2.
b. For other purposes especially to track the processes which are associated with a resistance and susceptibility, it is better if the scoring is carried out more than once, at least three times at 21, 28, and 35 DAP. It is supposed to be done more than one, using the later techniques as explained further. The scoring is done several times using the category in Table 1. On the first scoring, the second, or before the final scoring, we only look up the disease severity. After all scorings are done, we use the recovery data and determine the criteria of soybean resistance as shown in Table 4.

Table 4. The Criteria of Resistance to CpMMV Based Soybean Foliar Symptoms Recovery

Disease Severity	Resistance Criteria
S ≤ 25%, not recovery	Very resistant
S ≤ 25%, recovery	Resistant
25% < S ≤ 50%, recovery	Medium resistant
25% < S ≤ 50%, not recovery	Medium susceptible
50% < S ≤ 75%, recovery	Medium susceptible
50% < S ≤ 75%, not recovery	Susceptible
75% < S	Very susceptible

The technique and formula that developed for determination of soybean resistance to CpMMV are still need to be completed by observations scoring of the dwarf as supporting data, where if there are two plants have the same disease severity but the one more dwarf, it is considered to be more susceptible. However, so far we have not found such cases yet. Apparently, the technique and the formula is still need to be developed by observing the level of plants dwarfism and other aspects.

ACKNOWLEDGEMENT

We thank to The Directorate General of Higher Education (Dikti), Ministry of National Education, Indonesia which funded this research through Fundamental Research Project No: 323/UN32.10/PL/ 2011 and to Indonesian Legume and Tuber Crops Research Institute for providing the soybean germplasm.

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