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INCIDENCE OF SOYBEAN MOSAIC DISEASE IN EAST JAVA PROVINCE

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ABSTRACT

The objectives of this study were: 1) to identify the mosaic symptom severity and the incidence the virus and relate these to soybean yield reduction is four regions of East Java; 2) diagnostic of the symptom using Indirect ELISA, RT-PCR and electron microscope observation. Results from experiments indicated that soybean plants infected with SMV and CMMV, alone or in combination produced mosaic symptom. Incidence of the virus, as judged by symptomatology, ranged from mild to severe infection the percentage of plants being from 13.42–30.10%. Soybean plants with mosaic symptom caused SMV from an early stage of development (14-28 days after planting). *Soybean mosaic virus* belongs to the virus family *Potyviridae.* Specific DNA fragment of 1687 bp was successfully amplified from soybean infected by SMV isolate Ngawi, Madiun, Magetan, and Ponorogo. Specific DNA fragment of 1385 bp was successfully amplified from SMV by CI coding region. The mosaic symptom on soybean plant (28-42 days after planting) caused CMMV. Flexious virus particle 650 nm in length was observed on electron microscope. It caused local lesions on *Chenopodium amaranticolor*, but not detected by I ELISA with antiserum SMV and RT-PCR with universal primer.

Key words: SMV, CMMV, I.ELISA, RT-PCR.

INTRODUCTION

Soybean is a valuable economic commodity with great potential for future production in Indonesia and imports of soybean have risen to 1.6 million of ton in 2010 (2). Soybean mosaic disease is a major problem associated with production and induces major economic losses in commercial soybean production (Hobbs *et al*., 2003; Astuti, 2006).

The disease can be caused by two viruses, *soybean mosaic virus* (SMV, Genus Potyvirus and *cowpea mild mottle virus* (CMMV, genus Carlavirus). The genus *Potyvirus* (family *Potyviridae*), which constitutes the largest and economically most important group of plant viruses (Chen *et al*.,2001). SMV is seed transmitted (Giesler and Ziems, 2006; Iwai *et al*., 1985; Semangun, 2004), but CMMV is not transmitted through the soybean seed (Baliadi and Saleh, 1990; Horn *et al.,* 1991). It has been shown that insect vectors (*Aphis glycines* and *Bemisia tabaci*) may play an important role in spreading the disease caused by SMV and CMMV (Burhanuddin and Hasanuddin, 1993; Muniyappa and Reddy, 1983).

The temporal and spatial distributions of viruses can change from year to year and also in response to vector pressure and with proximity to sources of inoculums (Albrechtsen, 1989) and these factors may affect the development of epidemics. In the field, infected seed can be the primary source of SMV inoculum. Up to 30% or more of the seeds of diseases plants can be infected of SMV (Hull, 2002). The virus may survive in seed for two years (Matthews, 1991;Sinclair and Backman, 1993) and soybean plants infected with SMV may produce mottled seed with a range of seedcoat mottling or abnormal pigmentation. Seedcoat mottling is also under genetic control and is most visible when the gene combination result in a colored hilum (Iwai *et al*., 1985). Leguminous weeds and other crops are probably more important as primary sources of CMMV infection (Baliadi and Saleh, 1990). The presense of the CMMV in the seed coat, however, is not likely to result in seed transmission (Horn et al., 1991). Seed health

and seed quality in global trade are becoming an important issue due to the potential of seed to disseminate a range of seed-borne diseases.

The research objectives of this study were to determine: (i) the etiology of mosaic disease (SMV and CMMV) of soybean crops in the East Java; (ii) the level of SMV accumulation in the seed; and (iii) the incidence the virus and relate this to soybean yield reduction. This information is a critical first step towards the formulation of an integrated approach for effectively controlling mosaic disease in soybean and, thereby, reducing the economic losses in the East Java Province.

MATERIALS AND METHODS

Sources of inoculums. Materials studied were soybean plants and seeds of the variety 'Wilis' growing in Ngawi, Ponorogo, Madiun and Magetan, East Java, Indonesia at 14-28 and 28-42 day after planting.

Survey of soybean crops. Samples were collected during February 2008 from 32 fields in four major soybean growing areas including Ngawi (8 fields), Ponorogo (8 fields), Madiun (8 fields) and Magetan (8 fields). In each area, plants were randomly evaluated for virus-like symptoms such as chlorotic mosaics, cupping and stunted growth due to shortened petioles and internodes, vein clearing and ripper leaves that were rugose and downward curled.

Serological assay. Indirect ELISA was performed using the method of Koening (l981). Sap was extracted from leaf and seed samples in plastic bags by rolling a pestle over the bags. The sap was diluted 1:5 (w/v) with 0.05 M sodium arbonate coating buffer at pH 7.4. Wells of microtitre plates were coated with 150 µl aliquots of tissue extracts and incubated for 4 h at 27^{o}C. The wells were then rinsed with phosphate buffered saline (PBS, pH 7.4) containing 0.05% Tween 20. 150 µl of specific antiserum SMV (diluted 1:1000 (v/v) was added to each well. The plates were incubated for 24 h at 4–6^{o}C. After incubation, the wells were rinsed with PBS as before and then 150 µl aliquots of goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase (diluted at 1:1000 dilution in IEB) was added to the wells and incubated for 4 h at 4–6^{o}C. The wells were again rinsed with PBS containing Tween 20 and bound enzyme conjugate was detected 30–60 min after addition of diethalonamine buffer at 150 µl/well. The absorbance of the solution in the wells was determined at 405 nm using an ELISA Reader 680 XR. Samples were considered to be positive when A_{405} values exceeded the mean of the virus-free samples by at least a factor of two.

Local lesion host assay. Sap from infected soybean leaves was inoculated to *Chenopodium amaranticolor* by carborundum 400 mesh. *Chenopodium amaranticolor* was mechanically inoculated in an attempt to result local lesion host.

Electron microscopy. In leaf dip method preparation of young mosaic symptomatic leaves on soybean plant (28-42 days after planting) examined by electron microscope. Sap from infected leaves was coated on carbon-formvar coated 38 µm (400 mesh) grids, negatively stained with 2% phosphotungstate (PTA, pH 6.5) and examined with a JEOL 100S electron microscope (EM) (JEOL Ltd, Tokyo, Japan).

RNA extraction, Reverse transcription polymerase chain reaction (RT-PCR). Total RNA from infected fresh (100 mg/sample) or freeze dried (20 mg/sample) leaves was extracted after grinding in liquid nitrogen with an RNeasy Plant Minirep kit (Qiagen). First strand cDNA was produced following the method of Suehiro *et al.* (2005). A synthesis kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) was used for the synthesis of cDNA using an oligo dT primer and universal primer designed against the coat protein (CP) region of each virus was amplified, using the KOD-Plus Neo DNA polymerase (TOYOBO, Japan). Reverse transcription products were separated by electrophoresis in 5% PAGE and detected using ethidium bromide.

Molecular detection of the virus used to methods by Kim *et al*., (2004) modification. The test followed are: (1) Total RNAs were extracted from infected tissues using Isogen. Cloroform and isopropanol was added to produce pellet. The pellet was then washed with ethanol, which was then immediately added TE buffer (Tris-HCl 10 mM, EDTA 1 mM); (2) First strand cDNA synthesised by reverse transcriptase and oligo dT primer. The tubes were heated at 70°C for 15 min.; (3) Cylindrical Inclusion Coding region was used to amplify by RT-PCR. The primers used to amplify the resulting fragment were C15' (5'-GCATTCAACTGTGCGCTTAAAGAAT-

3') and (5'- TTGAGCTGCAAAAATTTACTCACTT-3'). The tubes were heated at 94^{o}C for 3 min and then subjected to 40 cycles of amplification using the following regime: 45 s at 95^{o}C for denaturation, 45 s at 58^{o}C for annealing and 60 s at 70^{o}C for extension, followed by 10 min at 70^{o}C for a final extension. The amplified products were separated by 1.2% agarose gel electrophoresis and visualized with an UV transluminator.

Location of SMV on seed parts. To investigate the virus distribution pattern in various tissues, seed coat, cotyledon, embryo in SMV infected soybean plant were carefully and cleanly dissected into each part sample for ELISA**.** These parts were then surface decontaminated by washing in running tap water for 15 min, followed by three times of rinse in distilated water.

Design and statistical analysis. Data obtained was analyzed using statistical program of SPSS. Analysis of variance was performed on the incidence of mosaic disease. The effect of primary source of infection and production were determined by linear regression.

RESULTS AND DISCUSSION

Field survey result showed that incidence of the virus, as judged by symptomatology, ranged from mild to severe with the percentage of plants being infected ranging from 13.4–30.1% . The disease incidence was related to the time of infection and the development stage of the soybean plant when infection occurred in the different region. Infected plants exhibited predominantly chlorotic mosaics (60%) and cupping (11%) and stunted growth due to shortened petioles and internodes (29%). The virus also caused vein clearing and plants where the ripper leaves were rugose and downward curled. Younger leaves the showed distortions and yellow mottling. The infected seed was found to have either abnormal (mottled, malformed, small) and normal seed. Incidence of the virus, as judged by symptomatology, ranged from mild to severe with the percentage of plants being infected ranging from 13.4–30.1% (Table 1).

The development and severity of symptoms of the soybean mosaic disease was related to the time of infection and the development stage of the soybean plant when infection occurred by visual observation. Regression analysis of the data as a whole showed that as the incidence of seedling infection increased, soybean production decreased by linear regression (Figure 1).

Visual symptoms were confirmed by indirect ELISA and RT-PCR. The detection has proved that SMV has infected soybean plants in East Java. Results from experiments indicated that soybean plants infected with SMV produced mosaic symptom. Soybean plants with mosaic symptom contained SMV from an early stage of development (14−28 days after planting), as was shown by positive reaction in indirect ELISA with SMV antiserum (Table 2).

The extent of seedcoat mottling was not related to the accumulation of SMV in the seed coat, as SMV was detected in both mottled and non-mottled seed coats, but using the germination seed and ELISA, SMV was found in each sampled seed coat, from both mottled and non-mottled seed from SMV infected plants (Table 3).

SMV, which causes soybean mosaic disease, is a member of the genus Potyvirus in the family Potyviridae. Specific DNA fragment of 1687 bp (the 5'CP and 3' Non coding region) was successfully amplified from soybean infected by SMV isolate Ngawi, Madiun, Magetan, and Ponorogo (Figure 2).

Table 1. The percentage incidence of mosaic disease

Locations	% Incidence (weeks after planting)				
	2	3	4	5	6
Ponorogo	22.57 b	22.47 b	23.39 b	25.50 b	26.48 ab
Madiun	16.05 c	16.32 bc	17.79 bc	19.17 bc	19.40 bc
Magetan	13.42 c	13.65 c	14.53 c	15.73 c	16.14 c
Ngawi	30.10 a	32.09 a	33.49 a	35.20 a	35.34 a
LSD 0.95	3.87	8.32	9.58	8.32	8.87

Remarks= similar letters in each column indicate no significan difference to Least significant difference (LSD) test at 5% level

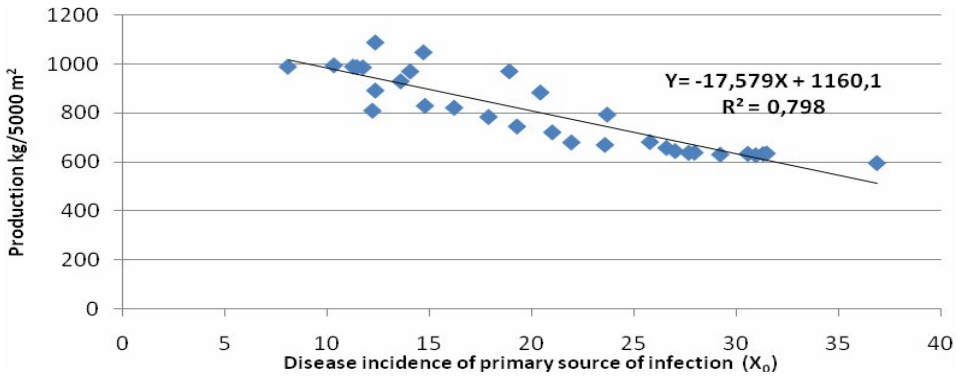

Figure 1. The relationship between incidence of seedling infection and soybean production.

Table 2. Mosaic disease of soybean crops as detected SMV by indirect ELISA

Locations	Symptom	Average of absorbance value ($A_{405\ nm}$)*)	Reaction of ELISA
Ponorogo	no symptom	0.246	−
	mosaic	0.850	+
Madiun	no symptom	0.337	−
	mosaic	0.932	+
Magetan	no symptom	0.281	−
	mosaic	0.963	+
Ngawi	no symptom	0.453	−
	mosaic	0.989	+
Control −**)		0.231	
Buffer		0.219	

Table 3. Transmission of SMV by mature seeds collected from infected soybean by Indirect ELISA

Symptom of seeds	Parts of seeds	Average	Reaction
Non mottled *)	Sc	0.987	+
	Co	1.052	+
	Em	1.047	+
	Fms	0.832	+
Mottled **)	Sc	0.993	+
	Co	0.972	+
	Em	0.547	+
	Fms	1.024	+
Control − ***)		0.094	−
Buffer		0.092	−

Remarks =Sc ,Seed coat; Co, Cotyledon; Em, Embryo; Fms, Full maturity stage of the seed

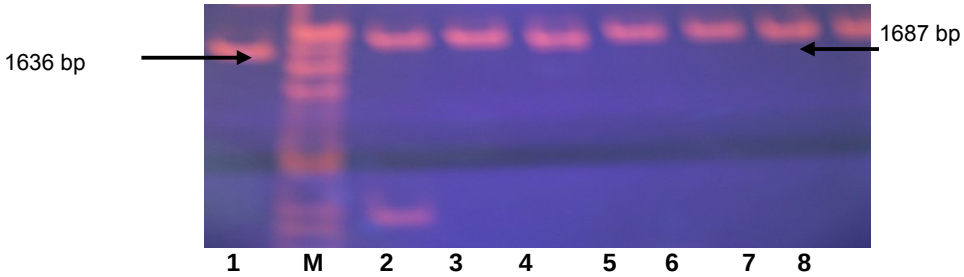

Figure 2. Universal primer detect SMV of *Potyviridae*. 1−2 SMV of Magetan; 3−4 isolate of Ngawi; 5−6 isolate of Madiun; 7−8 isolate of Ponorogo; M: DNA Ladder 1 kb as marker

Primer CI 5560 R and CI 4176 F was used to amplify by RT-PCR. The RT-PCR was successfully from soybean infected at 14−28 day after planting to associate with SMV. Under the optimal RT-PCR condition, we detected an array of amplication products of expected size 1385 bp fragment of CI gene from soybean leaves infected in former fields (Figure 3). The result of amplification products of expected size have similarity to demonstrated with Kim *et al.* (2004).

The virus caused mosaic disease on soybean plant sample (28-42days after planting) to consist of flexuous particle 650 nm in length (Figure 4). *Cowpea mild mottle virus* also caused local lesions on *Chenopodium amaranticolor*

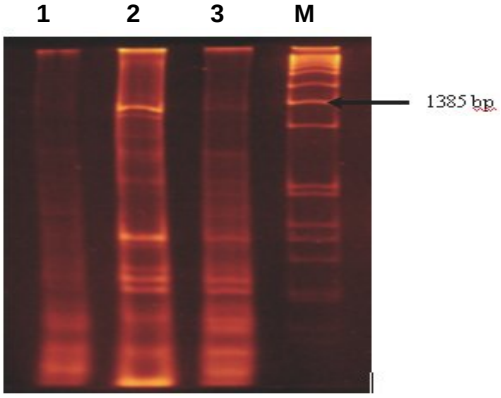

Figure 3. Reverse transcription (RT)-PCR products was used CI 5560 R and CI 4176 F primer

Figure 4. Particle of CMMV. The bar in the electron micrograph is 100 nm

CONCLUSIONS AND SUGGESTIONS

Incidence of soybean mosaic disease ranged from 13.4–30.1%. The development and severity of symptoms of the soybean mosaic disease is related to the time of infection and the development stage of the soybean plant when infection occurred. The synergistic interaction of the viruses infecting the plant could impact yield. Soybean mosaic disease has spread tremendouly due to increased primary sources and population of its vector.

Soybean mosaic virus is the most prevalent and dominant virus infecting soybean crops in Madiun, Magetan, Ponorogo, Ngawi. Ability of virus transmission from soybean seed to seedling are influenced by the location of SMV particles on soybean seed. The mosaic symptom on soybean plant (28-42 days after planting) was caused CMMV. Soybean mosaic virus is seed transmitted, CMMV but not transmitted through the soybean seed. *Soybean mosaic virus* and CMMV, alone or in combination produced mosaic symptom.

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