

SHORT COMMUNICATION

Genetic Diversity of *Cucumber Mosaic Virus* Strain Soybean from Several Areas

TRI ASMIRA DAMAYANTI* AND SURYO WIYONO

*Department of Plant Protection, Faculty of Agriculture, Institut Pertanian Bogor,
Jalan Kamper, Kampus IPB, Darmaga Bogor 16680. Indonesia*

Cucumber mosaic virus strain soybean (CMV-S) is one of economically important virus infecting soybean in Indonesia. However, it is very few information related with the CMV-S Indonesia isolates. Thus, the aim of present work is to detect and identify CMV-S isolates from different origin. Leaf samples collected from six different soybean fields in Java and North Sumatera. Molecular detection was carried out by reverse transcription polymerase chain reaction (RT-PCR) using specific primer of the coat protein (CP) gene and DNA sequencing. RT-PCR were successfully amplified the entire CP gene size ~657 bp from all samples and encoded 218 amino acids. Analysis of nucleotide and amino acid sequences showed high homology among isolates ranging from 99.5% and 100%. However isolate from Sindang Laut either nucleotides or amino acids showed lower homology to that of five isolates ranging from 89.4-89.6% and 91.7%, respectively. The differences between CMV-S with other non legume strain found in six amino acid positions in the CP gene, suggesting the differences might related with host specificity. Phylogenetic analysis of amino acid of six isolates to other CMV strain showed that the five CMV-S isolates were in the same cluster, while Sindang Laut isolate was in different cluster together with the non-legume CMV from India. It is indicating the present of genetic variability among CMV-S isolates in Java.

Key words: CMV strain S, genetic variability, soybean

Cucumber mosaic virus strain soybean (CMV-S) adalah salah satu virus penting secara ekonomi yang menginfeksi kedelai di Indonesia. Namun, sangat sedikit sekali informasi terkait CMV-S isolate Indonesia. Oleh karena itu, tujuan penelitian ini untuk mendeteksi dan mengidentifikasi beberapa isolat CMV-S dari daerah yang berbeda. Sampel daun dikoleksi dari enam pertanaman kedelai yang berbeda di Jawa dan Sumatera Utara. Deteksi molekuler dilakukan dengan *reverse transcription polymerase chain reaction* (RT-PCR) menggunakan sepasang primer spesifik gen protein selubung (*coat protein*) dan peruntukan sekuen DNA. RT-PCR berhasil mengamplifikasi seluruh gen CP yang berukuran ~657 pb dan mengkode 218 asam amino dari semua sampel. Analisis sekuen nukleotida dan asam amino menunjukkan homologi yang tinggi antar isolat berkisar dari 99,5% dan 100%. Namun homologi sekuen nukleotida dan asam amino isolat Sindang laut menunjukkan homologi yang lebih rendah terhadap lima isolat lainnya berkisar 89,4-89,6% dan 91,7%. Perbedaan CMV-S dengan strain non legum ditemukan dalam enam posisi asam amino dalam gen protein selubung. Perbedaan ini diduga terkait spesifisitas inang. Analisis filogenetik asam amino enam isolat CMV-S terhadap strain CMV lainnya menunjukkan lima isolat CMV-S berada dalam satu kluster, sedangkan isolat Sindang Laut berada dalam kluster yang berbeda bersama dengan CMV strain non legum dari India. Hal ini menunjukkan ada variasi genetik pada isolat-isolat CMV-S di Jawa.

Kata kunci : CMV strain S, kedelai, variasi genetik

Cucumber Mosaic Virus (CMV) is one of *Cucumovirus* genus member which well know has widely host range. The CMV genome is tripartite with ssRNA consisting of RNA1 and RNA 2 which encoded protein 1a and 2a for replication, and RNA 3 which play role as movement protein and coat protein (Palukaitis and Garcia-Arenal 2003). There are several strain of CMV distributed worldwide. At least 15 strains of CMV whose nucleotide sequences have been

determined were used for a complete phylogenetic analysis of the virus. CMV strain classified on 2 groups based on the similarities sequences such as group I (IA and IB) and group II (Roossinck 2002).

CMV infecting soybean previously known as Soybean stunt virus (SSV), later it known as CMV strain soybean (CMV-S) (Hong *et al.* 2003). It is one of major virus infecting soybean in Indonesia. Infected young soybean will produce seed transmitted virus up to 74.1% (Suseno *et al.* 1992). Apparent symptom varied, depend on host variety, plant age when infection occur, and environment condition. The

*Corresponding author; Phone : +62-251-8629364, Fax: +62-251-8629362, E-mail : triasmiradamayanti@gmail.com

symptom on soybean is systemic mosaic occurred at 7-10 days after mechanical inoculation. Infected plants become stunted, leaf curling with mosaic, with fewer pod number, smaller seeds with brown mottling (Hartman *et al.* 1999).

Recent field observation on 2013, the incidence of mosaic symptoms quite high in several soybean fields in Java and identification of virus(es) associated with the mosaic symptoms is under progress (YF Rahim 2014, personal communication). The study on biological characteristics of CMV-S (SSV) have been studied extensively, however there is no available sequence information of CMV soybean strain (Hong *et al.* 2003; Vo Phan *et al.* 2014), especially for sequence information of Indonesian isolates and its diversity still not available yet until present. The biological as well as molecular characteristic is an important to determine appropriate management strategies of viral disease. Also, it will be useful for breeder to produce tolerant or resistant cultivar against the virus. Thus, the objective of the research was detect and identify the symptomatic leaves which may associated with CMV soybean strain (CMV-S) from several area in Indonesia to obtain the gene information and its genetic variability.

Samples of soybean leaves showing mosaic symptom randomly were collected from various area such as Samosir (North Sumatera), Bogor, Cirebon (Plumbon and Sindang Laut) (West Java), Magelang (Central Java), and Jember (East Java).

Total RNA was extracted from 0.1 g leaves with simple direct tube (SDT) extraction method as described previously by Suehiro *et al.* (2005) with minor modification in ratio of buffer and leaf samples. Samples were grinded in buffer PBST 1x (Phosphate buffer saline tween) with ratio 1:5 (w/v). Plant sap as 50 μ l was incubated in PCR tube for 15 min for total protein immobilization (plant and viruses). PCR tubes were washed 3-4 times by buffer PBST until no debris remain in the tube. Total RNA was extracted by providing 30 μ l DEPC water containing 15U RNase Inhibitor (Ribolock, Thermo), then PCR tubes heated at 95 °C for 1 min and cooling down immediately on ice for at least 5 min.

Nucleic acid was detected by One-Step RT-PCR (reverse transcription PCR). Reagent composition used in one-step RT-PCR including; 2 μ l of total RNA, 2.5 μ l of 10x buffer PCR, 1 μ l of 10 mM dNTP, 2 μ l 25 mM MgCl₂, 1.25 μ l 50 mM DTT, 1.25 μ l of 10 μ M primer forward and reverse each, 0.05 μ l of M-MuLV (200 U μ L⁻¹) (Revertaid, Thermo), 0.1 μ l of RNase

Inhibitor (40 U μ L⁻¹) (Ribolock, Thermo), 0.25 μ l of Taq polymerase enzyme (5 U μ L⁻¹) (Hot start maxima, Thermo) and nuclease-free water up to total volume 25 μ l. Premix one-step RT-PCR was mixed and was carried out on PCR machine Gene Amp 9700. PCR condition was applied as follows; RT reaction at 42 °C for 60 min, 94 °C for 2 min, continued by 1 cycle at 95 °C for 3 min, 45 °C for 1 min, 72 °C for 1 min and followed by 35 cycles at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min and final extension at 72 °C for 10 min.

RT-PCR was carried out by using specific primer for CMV CP gene (forward primer CMVcp-F 5'-GCATTCTAGATGGACAAATCTGAATC-3') and reverse primer (CMVcp-R 5'-GCATGGTACCTCAAACCTGGGAGCAC-3'). The RT-PCR product size is approximately 650 bp. The DNA product was analyzed on 1% TBE (Tris Boric EDTA) agarose gel containing 0.5 μ g/ μ L Ethidium bromide. Electrophoresis was conducted at 100 V for 20 min. The DNA observed under UV transilluminator and documented digitally.

DNA from RT-PCR was subjected to nucleotides sequencing at Charoen Pokphand Tbk Indonesia, Jakarta. The DNA sequences were analyzed by using CLUSTAL W (Thompson *et al.*, 1994) (BioEdit version 7.05: <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) to obtain identity matrix of nucleotide and amino acid homology in compared to that of CMV deposited in the GenBank. Phylogenetic tree was constructed by using MEGA 6.0 software (Tamura *et al.* 2013) with neighbor-joining method and p-distances model to estimated the distances, and bootstrap supported was estimated with 1 000 replicates. The CP gene of *Peanut stunt virus* (AY775057) was used as out group.

Leaf samples collecting from several area of soybean fields showed symptom variations such as mild mosaic to severe mosaic, and leaf curling with mosaic (Fig 1a-d). Symptom of infected plants from Sindang laut varied such as mild mosaic to yellow mosaic (data not shown). Multiple virus infection and the differences of variety cultivated in each sampling area might contribute to the symptom variation in the fields.

Molecular detection by RT-PCR was amplified a ~650 base pair (bp) DNA fragment from all six samples with specific primer for CMV CP gene (Fig 2). It indicated that the symptomatic leaves collected from all area were infected by CMV.

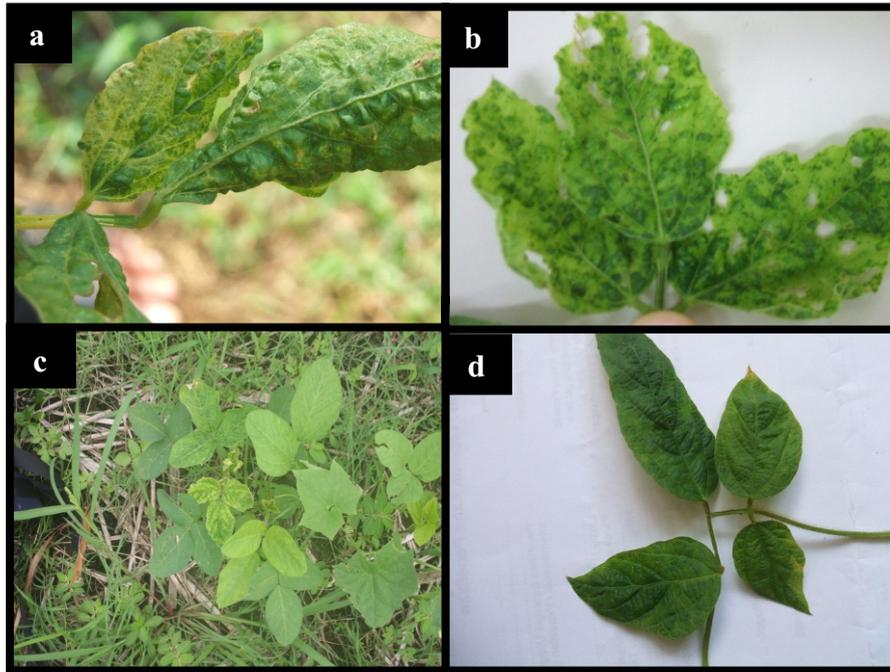


Fig 1 Variation of mosaic symptoms which caused by CMV-S infection or multiple infection of virus collected from the fields.

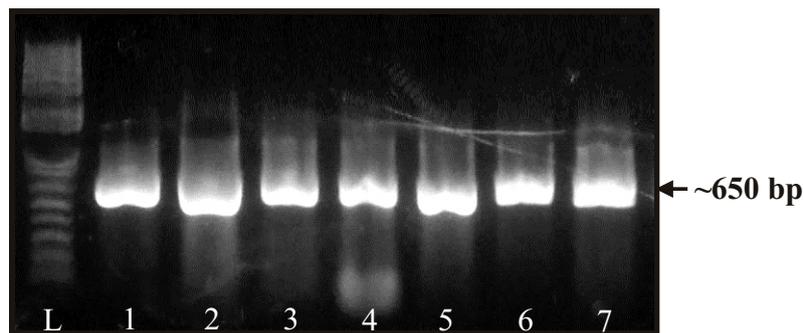


Fig 2 RT-PCR of CP gene of CMV-S isolates. L, DNA ladder 1 kb plus (Invitrogen), (1) positive control, (2) CMV-S Bogor isolate, (3) Plumbon isolate, (4) Sindang laut isolate, (5) Magelang isolate, (6) Jember isolate, and (7) Samosir isolate.

DNA sequencing results successfully identified the full-length of CMV-S gene from all samples. The size of the CP gene is 657 nucleotides, and it encodes 218 amino acids. Analysis of nucleotide (nt) and amino acid (aa) sequences of the CMV-S CP gene of those isolates (isolates Bogor-Bgr, Plumbon-Plbn, Magelang-Mgl, Jember-Jmbr, and Samosir-Smsr) showed high homology among isolates, ranging 99.5-100% (nt) and 99-100% (aa) (Table 1). However, isolate Sindang laut (Sdl) showed lower homology of either nucleotide (89.4-89.6%) or amino acid (91.7%) to that of five isolates, respectively (Table 1).

Pair wise comparison of these sequences with corresponding nucleotide sequences in the GenBank showed that the five isolates (Bgr, Plbn, Mgl, Jmbr,

Smsr) had homology ranging 88.8-91.7% (nt) and 90.3-92.6% (aa) with CMV non legume strain. However, CMV-S isolate Sdl showed closely homology in nucleotides (91.9-94.5%) and amino acid (94.4-98.1%) to that of CMV non legume strain (Table 2). It is indicating the genetic variability among CMV-S isolates in the CP gene.

Comparison of amino acid sequences between five CMV-S isolates (Bgr, Plbn, Mgl, Jmbr, Smsr) and Sdl isolate showed the amino acid differences in 14 positions such as S12N, P25S, N31T, I32F, G34V, L53I, K65R, D98E, V142M, A144S, K180R, V192A, M196T and I200L (first symbols are amino acids of five isolates of CMV-S, last symbols are amino acids of CMV-S isolate Sdl, number: the position of amino

Table 1 Homology of nucleotides and amino acids of CMV-S CP gene among isolates

CMV-S Isolates	Idn-Bgr*		Idn-Plbn		Idn-Sdl		Idn-Mgl		Idn-Jmbr		Idn-Smsr	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
Bogor			99.5	99.0	89.6	91.7	99.5	99.0	99.5	99.0	99.5	99.0
Plumbon					89.4	91.7	100.0	100.0	100.0	100.0	100.0	100.0
S. Laut							89.4	91.7	89.4	91.7	89.4	91.7
Magelang									100.0	100.0	100.0	100.0
Jember											100.0	100.0
Samosir												

*CMV-S isolate from Bgr-Bogor, Plbn-Plumbon, Sdl-Sindang laut, Jmbr-Jember, Smsr-Samosir

Table 2 Homology of nucleotides and amino acids of CMV-S CP with corresponding CMV in GenBank

CMV-S Isolates	Idn-Bgr		Idn - Plbn		Idn-S. Laut		Idn-Mgl		Idn- Jmbr		Idn-Smsr	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
California	91.6	92.6	91.4	92.6	93.6	98.1	91.4	92.6	91.4	92.6	91.4	92.6
India-Luc	88.8	92.6	88.7	92.6	94.5	98.1	88.7	92.6	88.7	92.6	88.7	92.6
India-Ama	91.7	90.3	91.6	90.3	93.7	97.2	91.6	90.3	91.6	90.3	91.6	90.3
Italy	91.4	92.6	91.3	92.6	94.0	98.1	91.3	92.6	91.3	92.6	91.3	92.6
Taiwan	90.8	90.3	90.7	90.3	91.9	94.4	90.7	90.3	90.7	90.3	90.7	90.3
Thailand	89.6	90.8	89.4	90.8	93.9	98.1	89.4	90.8	89.4	90.8	89.4	90.8

*CMV-California/tomato (AF523340.1), CMV-India lucknow/tomato (EF153734.2), CMV-India/amaryllis (EF187825.1), CMV-Italy/tomato (Y16926.1), CMV-Taiwan/yard long bean (D49496.1), CMV Thailand/cucumber (AJ810264.1)

acids in CP gene). The differences of amino acid might related to the lower nucleotide homology of isolate Sdl to that of other five isolates. Comparison of amino acid sequences of CMV-S to other strain of CMV showed differ in 6 amino acids at position of L123V, S132A, T141A, E147A, I160V, and P171A. These might be related to strain differences and host specificity, however it needs further investigation.

Phylogenetic analysis of nucleotide sequences showed that all five isolates of CMV-S were in one cluster distantly related to those other CMV strains. However CMV-S Sdl isolate more closely related to CMV ordinary strain from other countries (data not shown). Similarly, phylogenetic based on amino acid sequences show that the five CMV-S isolates are located in one same cluster distantly related to other CMV ordinary strain, while Sdl isolate separated far

from others CMV-S isolates and closely to CMV infecting tomato from India (Fig 3).

CMV-S from various area of soybean fields in Indonesia are distinct strain to CMV ordinary strain based on nucleotide and amino acid analysis of the CP gene except CMV-S Sindang laut isolate. These results supported the previous report for CMV-S from Japan (Hong *et al.* 2003). CMV-S Sindang laut isolate is locates in the same cluster to the CMV non legume strains. It is indicating the genetic variability of CMV-S in Java based on CP gene sequences. All Indonesian isolates of CMV-S were a member of sub group IB (Fig 3). The difference between CMV-S and CMV common/ordinary strain such CMV-Y that, common strain could not infect soybean event locally. CMV-S can not systemically infect cucumber, whereas common strain of CMV can. Similar strain of CMV-S

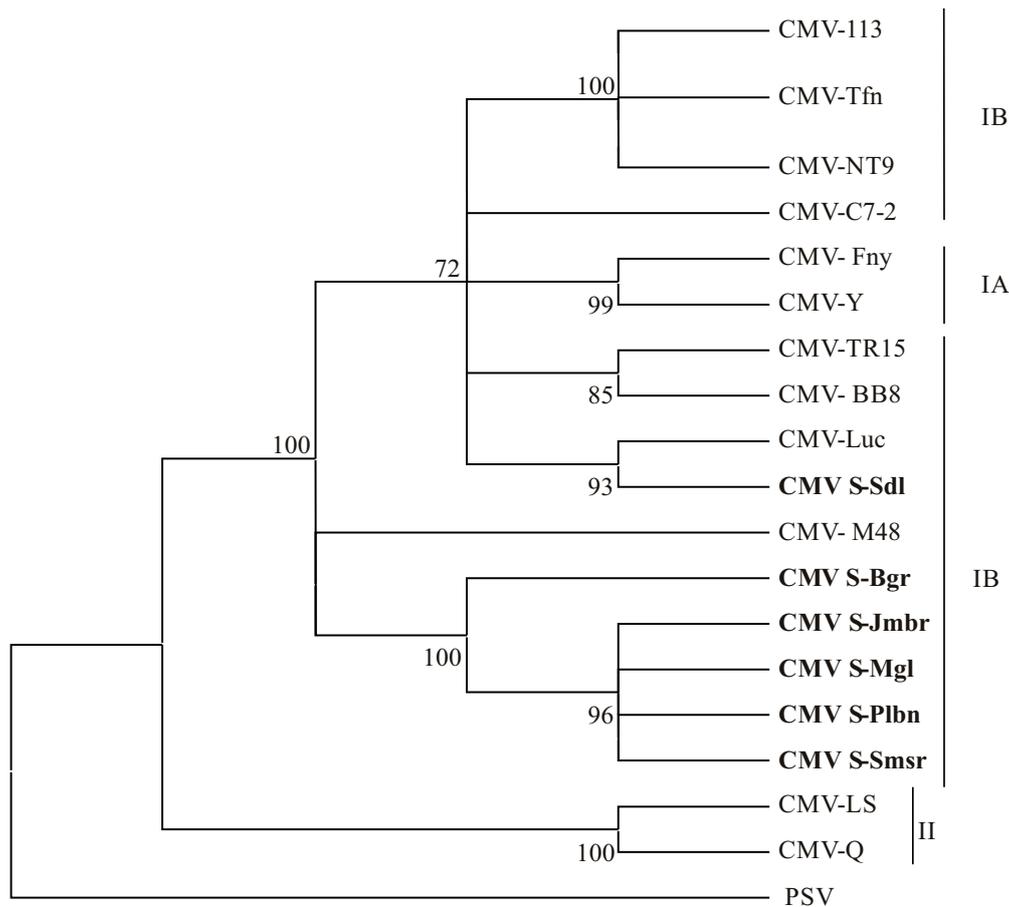


Fig 3 Phylogeny of the CP gene amino acid of CMV-S isolates from several area in Indonesia (Idn). The CMV-S isolates are highlighted in grey. The tree were inferred by the Neighbor-Joining method using molecular evolutionary genetics analysis (MEGA) software version 6.0 based on the ClustalW alignment sequences from Indonesia, India, Italy, Taiwan, Thailand and USA. The tree was rooted using *Peanut stunt virus* (PSV) as out groups. Bootstrap values expressed as percentage of 1 000 replicates that greater than 70 are shown on the tree branches.

(CMV-209) from Korea is only infects systemically on wild soybean and pea, but not infect the other legume species (Vo Phan *et al.* 2014). The ability to infect its host was related with the movement protein as host determinant of CMV (Hong *et al.* 2007).

There are 14 amino acids difference between five CMV-S isolates and Sdl isolate on the CP gene, however the Sdl isolate infects soybean systemically as well as other CMV-S isolates. It is support the previous report that host determinant of CMV rely on the movement protein rather than in the CP gene. The Sdl isolate may different in several amino acid in the CP gene, but it may not contribute to its ability to infect soybean, suggesting the movement protein gene of Sdl isolate may similar to those other CMV-S isolates. It needs further sequences analysis of the movement protein gene that is was not covered in these analysis. Among 14 amino acids difference between five isolates of CMV-S and Sdl isolate, one of it at position 25 is serine, while those other five isolates maintain

amino acid proline at the position. The substitution of P25S on Sdl isolate may imply a substantial structural change in protein. The amino acids at position 25, 76 and 214 are reported affect to transmission efficiency by aphid if substitution occurs at those positions (Moury 2004).

Based on phylogenetic analysis it shown that CMV-S is CMV strain different to other CMV (non-legume). SSV-In (SSV from Indonesia) is one of divergent isolate compared to SSV isolate B, C, D and AE from Hokkaido, Japan, isolate from China and isolates WR from Russia. SSV-In do not cause systemic infection on various wild type of soybean in Japan. SSV-In is supposed to be adapted specifically to local Indonesian soybean, which not influenced by gene flow from wild soybean (Hong *et al.* 2003). The variability of the CP gene of CMV-S may leads to the differences of biological characteristics that need further investigation.

ACKNOWLEDGEMENT

The research is conducted by financial support of Competitive Research Grant of Directorate General of Higher Education through DIPA IPB with Contract No 05/I3.24.4/SPP/PHB/2011 on March 28, 2011.

REFERENCES

- Hartman GL, Sinclair JB, Rupe JC. 1999. Compendium of Soybean Disease. 4th Ed. APS Press.
- Hong JS, Ohnishi S, Masuta C, Choi JK, Ryu KH. 2007. Infection of soybean by *cucumber mosaic virus* as determined by viral movement protein. Arch Virol. 152: 321-328. doi: 10.1007/s00705-006-0847-3.
- Hong JS, Masuta C, Nakano M, Abe J, Uyeda I. 2003. Adaptation of *cucumber mosaic virus* soybean strain (SSVs) to cultivated and wild soybeans. Theor Appl Genet. 107: 49-53. doi: 10.1007/s00122-003-1222-3.
- Moury B. 2004. Differential selection of genes of *Cucumber mosaic virus* Subgroups. Mol Biol Evol. 21(8):1602-1611. doi:10.1093/molbev/msh164.
- Palukaitis P, García-Arenal F. 2003. Cucumoviruses. Adv Virus Res. 62:241-323.
- Roossinck MJ. 2002. Evolutionary history of *cucumber mosaic virus* by deduced by phylogenetic analyses. J Virol. 76:3382-3387. doi: 10.1128/JVI.76.7.3382-3387.2002.
- Suehiro N, Matsuda K, Okuda S, Natsuaki T. 2005. A simplified method for obtaining plant viral RNA for RT-PCR. J Virol Methods. 12:67-73. doi:10.1016/j.jviromet.2005.01.002.
- Suseno R, Suastika G, Kusumah YM. 1992. Studi beberapa aspek pengendalian virus terbawa benih kedelai yang ditularkan kutudaun, virus bantut kedelai (*Soybean stunt virus/SSV*) dan virus mosaik kedelai (SMV). Laporan akhir penelitian pendukung PHT dalam rangka pelaksanaan program nasional pengendalian hama terpadu. Bogor; Kerjasama proyek prasarana fisik Bappenas dengan Fakultas pertanian, Institut Pertanian Bogor.
- Tamura K, Stecher G, Peterson D, Filipinski A, Kumar S. 2013. MEGA 6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 30(12):2725-2729. doi: 10.1093/molbev/mst197.
- Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acid Res. 22: 4673-4680.
- Vo Phan MS, Seo JK, Choi HS, Lee SH, Kim KH. 2014. Molecular and biological characterization of an isolate of *Cucumber mosaic virus* from *Glycine soja* by generating its infectious full-genome cDNA clones. Plant Pathol J. 30(2):159-167. doi: 10.5423/PPJ.OA.02.2014.0014.