



Research Article

Detection and Analysis of Protein Profile on Stunt Virus-Infected Rice Plant with Different Severity Level on Ciherang and Situ Bagendit Varieties

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ABSTRACT

Rice stunt virus is one of the limiting factors in the decline of rice production in Indonesia. The virus consists of rice grassy stunt virus (RGSV) and rice ragged stunt virus (RRSV) that is transmitted by brown planthopper (WBC) in a persistent propagative manner. This study aimed to determine the presence of rice stunt virus in Bantul, Yogyakarta through fast detection using RT-PCR. It also aimed to learn the pattern of total protein profile of healthy and infected rice plants by the virus on different severity level in the field. The results showed that rice varieties of Ciherang and Situ Bagendit in Bantul were infected with RGSV and RRSV. They were classified as mild, moderate, severe, and failure in severity level. Homology analysis using BioEdit showed that the nucleotide sequence of RGSV in Bantul isolate had the highest percentage of nucleic acids similarity with Klaten isolate (98.1%). Meanwhile, RRSV of Bantul isolate had the highest percentage of nucleic acids similarity to Philippines isolate (99.5%). Analysis of protein profiles using SDS-PAGE showed a pattern of protein profiles formed on rice infected with the virus at different severity levels which was not found in healthy rice. These proteins presumably were nonstructural p5 and nucleocapsid protein (NCP) of RGSV with a molecular weight of ~22 and 34-35 kDa; and viral spike protein and protein capsid (S8) of RRSV with MW ~39 and ~43 kDa.

Keywords: brown planthopper, rice grassy stunt virus, rice ragged stunt virus, RT-PCR, SDS-PAGE

INTRODUCTION

Rice is the staple food commodity in Indonesia. Aside from being a food source, rice has a major role in Indonesian economy, i.e contributing to national gross domestic product (GDP), providing jobs and increasing household income (Suryana *et al.*, 2009). Therefore, the efforts to increase rice production are constantly being developed. One of the obstacles that may reduce rice production is the attack of brown planthopper (BPH), *Nilaparvata lugens* (Stal) (Homoptera: Delphacidae) (Baehaki, 2011). BPH acts as a pest and vector of the rice stunt virus diseases: rice grassy stunt virus (RGSV) and rice ragged stunt virus (RRSV) (Hibino *et al.*, 1985; Hibino, 1996; Toriyama *et al.*, 1997; Chomchan *et al.*, 2003). Both viruses are transmitted persistently. They multiply inside the vector body and stay inside, even after molting. However, it does not pass on to the next generation through eggs (Hibino, 1986; Milne & Ling, 1982). Ditlin (2010) reports that the total damage of BPH attack as a pest and a vector in the period of 2001–2010 in Indonesia reached 35,748 ha with 11,354 ha of failure. In 2011, the

area of BPH attack almost doubled compared to the area attacked a year earlier (173,890 ha with 22,613 ha of failure) (Ditlin, 2011).

Bantul is the second producing rice center in the Special Region of Yogyakarta with the rice fields reaching 14,535 Ha in 2009–2013 (BPS, 2014). Ciherang and Situ Bagendit varieties are the most commonly grown variety in Bantul. BPH attack followed by a stunt virus will certainly reduce rice production. Rahmawati *et al.* (2015) states that the viral infections cause different symptoms and affect the severity of the disease; hence, it will have an impact on the growth and production of rice. Plants with mild severity are still able to produce even though they continue to decline. However, plants with high severity will cause crop failure. Therefore, it is necessary to detect the presence of the disease at various specific severities in the field. RT-PCR is one of the sensitive detection methods as it is able to determine the presence of viruses in a low concentration (Endarsih *et al.*, 2017). In addition, rice plants infected with stunt viruses have different protein profiles than healthy rice. Protein profile analysis was carried

out using Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) technique (Hemes, 1998). This technique is suitable to identify differences and/or similarities in total protein band profile between healthy rice plants and rice plants infected with stunt virus. This study aimed to determine the presence of stunt viruses in rice in Bantul, D.I. Yogyakarta through rapid detection using RT-PCR and to determine healthy and symptomatic total protein profiles pattern of rice plants based on the severity in the field.

MATERIALS AND METHODS

Observation and Sampling of Rice Leaf in Bantul Isolate

Observation and sampling of rice leaves were carried out in two locations: rice field of Situ Bagendit variety with an area of 3225 m² in Sumberagung Village, Jetis District, Bantul Regency, D.I. Yogyakarta and rice field of Ciherang variety with an area of 1270 m² in Seloharjo Village, Pundong District, Bantul Regency, D.I. Yogyakarta. In the fields, the rice was observed diagonally with different severity of the disease. The sampled rice plants had typical symptoms of rice stunt that represented all the symptoms found in both locations. Samples were classified according to their severity level: healthy, mild, moderate, severe, and failure using scoring method (Table 1).

Molecular Detection of Rice Stunt Virus Based on Severity Level with RT-PCR DNA Extraction

Rice leaves with symptoms of both Ciherang and Situ Bagendit varieties in each category were used to detect the presence of rice stunt viruses. The total RNA extraction was performed using the RNeasy

Plant RNA mini kit as a procedure of Qiagen Sample & Essay Technology.

Making Complementary-DNA (cDNA)

RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction) method was performed to create cDNA using TOYOBO products. The microtube was labeled and 4 µl 5x RT Buffer (containing 25mM Mg 2+), 1 µl *Primer oligo* (dT) 20 (10 pmol/µl), 2 µl dNTP mixture (10 mM) 1 µl *Primer oligo* (dT) 20 (10 pmol/µl), and 2 µl dNTP mixture (10 mM), 1 µl ReverTra Acc, 1 µl RNase Inhibitor (10 U/µl) and 8 µl RNase-free H₂O were inserted into the tube. The extracted RNA was pipetted into 3 µl PCR tube and homogenized using a vortex machine. The sample was placed into a PCR machine with an incubation temperature at 42°C for 20 minutes, 99°C for 5 minutes and 4°C for 20 minutes.

cDNA amplification

cDNA resulted from RT-PCR in the previous stage was amplified using RGSV (F1:5'-GGCTTAT GATAGTCTGTGATTTG-3'/R:5'GTGTAAGAT GGGTAAAGTGCA-3') and RRSV (F3:5'-GAC TAGGGATGTGCGTTC-3'/B3:5'-TGTAATCGAC GTTCGCTC-3') specific primers. PCR amplification was performed using My Taq HS RedMix method with the composition of 4 µl free water (ddH₂O), 8 µl mix PCR RedMix, 2 µl for each RRSV F and RRSV in the concentration of 5 pmol R specific primers, and 2 µl templates. Thus, the total volume was 20 µl. PCR was carried out with an initial denaturation cycle at 95°C for 1 minute, denaturation at 95°C for 15 seconds, attachment at 50°C for 15 seconds, elongation at 72°C for 10 seconds, and final elongation at 72°C for 5 minutes. The PCR products were further identified by DNA visualization using electrophoresis.

Table 1. Severity categories of rice stunt virus disease

Category	Description
Healthy	No definite symptoms
Mild	Slightly shortened, sometimes with serrated edges, panicles yielded are not as much as healthy plants
Moderate	A bit stunting, profuse number of tillering, grows stiff and short, leaves are in dark green color with serrated and twisted edges, leaf blades and sheaths develop swelling and gall, delayed flowering and empty grains
Severe	Stunting, little number of tillering, grows stiff and short, narrow leaves, serrated edges with twist at the tip, leaf blades and sheaths develop swelling, partially exerted panicles, malformed panicles, empty grains or does not even yield panicles
Failure	Stunting, little number of tillering, grows stiff and short, narrow and yellow leaves with mottled spots, does not yield panicles

DNA Visualization

DNA of the rice stunt virus was analyzed through electrophoresis using 1% 15 ml TBE 1x and 0.15 gram agarose gel in a beaker. The DNA was measured using 4 µl of 100 kb ladder marker and 5 µl of sample prepared. Each sample was added into wells of gel using micropipette. Electrophoresis was performed at 50 V DC voltage for 50 minutes. After the electrophoresis process was complete, staining was conducted using ethidium bromide while visualization used UV transilluminator. Photos for documentation were captured using digital camera.

Nucleotide Sequence Analysis

Nucleotide sequence analysis was performed using the ABI-Prism 3100-Avant Genetic Analyzer sequencer in Research and Development Center of PT Genetics Science, Indonesia. The DNA sequences were analyzed by the Basic Local Assessment Search Tool (BLAST) program on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov) to compare the target virus sequences with the registered-nucleotide sequences of viruses from other countries in Genbank. Nucleotide and amino acid homology levels were obtained by the ClustalW multiple alignment and Sequence Identity Matrix program using Bioedit 7.05 software.

Protein Profile Analysis using SDS-PAGE Gel Electrophoresis

The analysis of the total protein band profile was carried out using SDS-PAGE gel electrophoresis technique based on the Leammli method (Gall *et al.*, 1980). The infected and healthy rice leaf were weighed (0.1 g), added the phosphate buffer and then crushed well. The sap was put into a 1.5 ml centrifuge microtube, added 300 µl extraction buffer (Tris HCl 0.5 M pH 6.8; glycerol; SDS 10%; deionized water) and 2% mercaptoethanol. The extract was incubated for 5 minutes at 95°C and centrifuged at 8000 g for 5 minutes. The supernatant was transferred to a new microtube and added with 0.5% bromophenol blue with a ratio of 1:5 of the sample volume. Protein electrophoresis was conducted using SDS-PAGE solution with a concentration of 12.5%. Electrophoresis ran between a constant voltage of 20 mA, 100 Volt, and 5 Watt until the marker (bromophenol blue) close to the lower limit of the gel. The gel was soaked in 12.5% glacial acetic acid for 5 minutes then stained for 24 hours using 0.25% Coomassie brilliant blue

solution. The process continued with destaining the gel using a mix solution of methanol, acetic acid, and distilled water while beating vigorously until the protein bands appeared. The total protein electrophoresis was documented in digital photos.

RESULTS AND DISCUSSION

Symptoms of Rice Stunt Virus

The observation of rice stunt symptoms in Bantul, D.I. Yogyakarta showed that Ciherang and Situ Bagendit varieties were infected with two rice stunt viruses (double infection), rice grassy stunt virus (RGSV) and rice ragged stunt virus (RRSV) with following symptoms: stunting, yellowing at the leaf tip, no panicle, stiff tillering, serrated, gall, and twisted leaf tip, especially in young leaves (Figure 1). Rice plants were sampled then categorized based on the severity in the field (Figure 2).

Symptoms caused by two stunt viruses showed more severe symptoms than a single infection. This is similar to the study reported by Dini *et al.* (2015) and Du *et al.* (2005) that double infection of rice plants caused leaves became dark green leaves, shorter, and thinner; small tillering; young leaves become browner and yellowing in the old leaves. In this study, although the incidence of stunt disease in the field reached 80%, the severity of rice stunt disease in these varieties varied. In the category of mild severity to failure, variations in symptoms were clearly seen in the rice plants (Figure 2). Rice plants with mild to moderate severity were generally still able to produce panicles. However, panicles were not produced in failure category.

Molecular Detection of Rice Stunt Viruses and Nucleotide Sequence Analysis

Detection of stunt symptom of each severity category by RT-PCR using RGSV F1/R and RRSV F3/B3 specific primers showed positive infection by RGSV and RRSV with visible DNA bands of 450 bp and 210 bp (Figures 3 and 4). F1/R and F3/B3 specific primers may be used to amplify nucleotides encode RGSV protein nucleocapsid gene and to encode the formation of structural proteins S8 RRSV, respectively (Nam *et al.*, 2007; Le *et al.*, 2010) hence the primer are suitable to be used for identification. Mild, moderate, severe, and failure severities showed that sampled rice planted were both positively infected with stunt viruses. This indicates that the

virus existed since the vegetative stage, considering that the mild category of rice plants had no definite symptoms. A similar study reported by Rahmawati *et al.* (2015) states that viral infection causes various symptoms and affects the severity of the disease and it may effect on the growth and production of rice plants. Therefore, early detection is necessary to conduct the control efforts as early as possible.

RT-PCR visualization showed various DNA band thickness in each severity category (Figures 3 and 4). Ciherang variety was positively infected with RRSV and RGSV hence DNA bands in the failure and severe categories were thicker than that in moderate and mild categories (Figure 4). it was due to the accumulation of high concentration of virus in the failure and severe categories than that in moderate and mild categories. The higher the concentration

of the virus in the plant, the higher the severity (Subekti *et al.*, 2006). Low or high concentrations of viruses in plants are caused by the presence of the virus in the vector body, the susceptibility of plants to the virus, and environmental factors that support the development of virus and plant susceptibility. According to Chen *et al.* (1997), the stunt virus is persistently propagative in the body of BPH. The virus will multiply in BPH body and its concentration will increase. The higher the concentration of the virus, the greater the symptoms. In addition, BPH has preference of feeding more frequently on the plants at severe and failure severities. Thus, the viral concentration is accumulated in rice plants. Hibino *et al.* (1977) states that the longer the period of vector inoculation and acquisition in plants, the higher the virus concentration in the vector or the plant becomes.

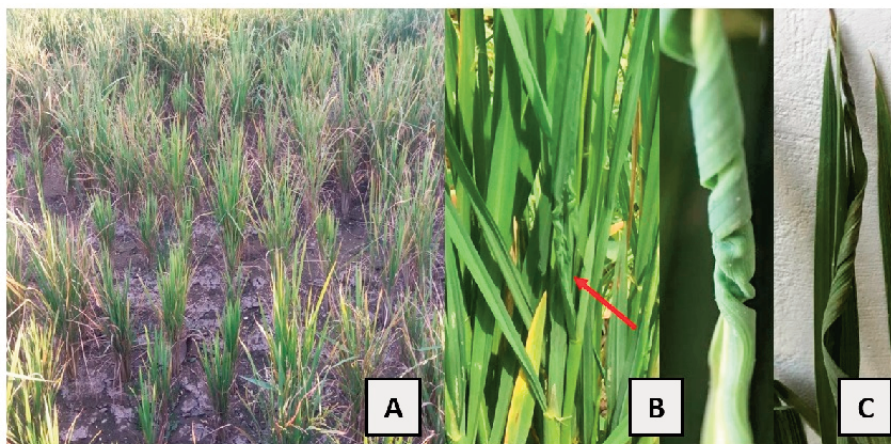


Figure 1. Symptoms of rice plants infected with rice stunt virus in Sumberagung Village, Jetis District, Bantul Regency; (A) showing a stunt with stiff tillering, (B) gall at base of rice stems, (C) twisted at base and tip of leaves

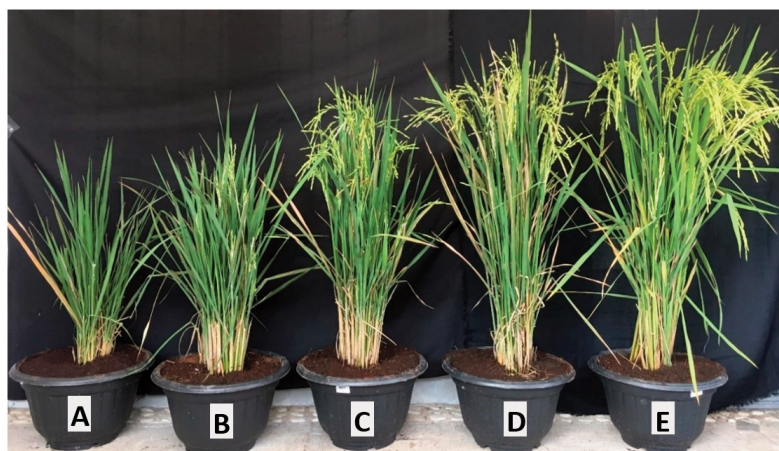


Figure 2. The severity of rice plants infected with rice stunt virus; failure (A), severe (B), moderate (C), mild (D), and healthy (E)

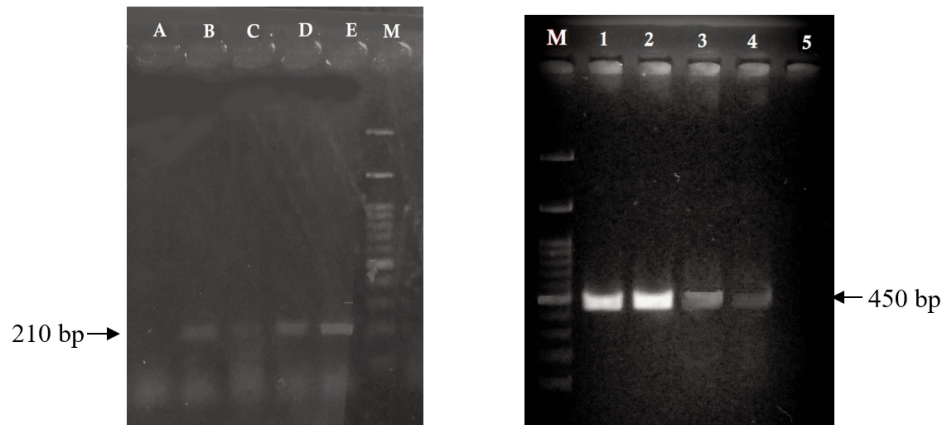


Figure 3. RT-PCR visualization using RRSV F3/B3 primer and 1.5% agarose gel of Ciherang variety at severity level of: healthy (A), mild (B), moderate (C), heavy (D), failure (E), and 100 pb Marker; RT-PCR visualization using RGSV F1/R primer and 1.5% agarose gel at severity level of: failure (1), severe (2), moderate (3), mild (4), and healthy rice isolates (5)

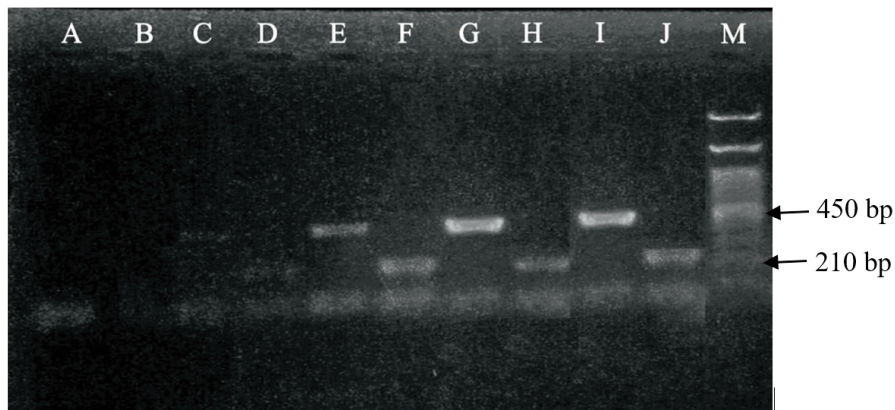


Figure 4. RT-PCR visualization using RRSV F3/B3 primer and RGSV F1/R and 1.5% agarose gel of Situ Bagendit variety at severity level of: healthy (A and B); mild RGSV (C), mild RRSV (D), moderate RGSV (E), moderate RRSV (F), severe RGSV (G), severe RRSV (H), failure RGSV (I), failure RRSV (J), and Marker 100 pb (M)

UV visualization conducted on Situ Bagendit was similar to RT-PCR in the variations of DNA bands thickness (Figure 4). Severe to failure categories showed thicker DNA bands than that in other categories. This explained that the high concentration of viruses accumulates along the high severity of plants in the field.

Based on the alignment of DNA sequencing from Bantul isolate, the isolate was positively infected with RGSV and RRSV (double infection). BLAST through NCBI showed that Bantul isolate (Indonesia) had higher homology than the isolates from other countries in the GeneBank database (Tables 2 and 3).

Furthermore, homology analysis using BioEdit showed that the RGSV and RRSV nucleotide sequence

of Bantul isolate had the highest nucleic acid similarity percentage to Klaten isolate (Indonesia) (98.1%) (Table 2) and Philippine isolate (99.5%) (Table 3), respectively. These results were similar to the study conducted by Suprihanto *et al.* (2015) stating that isolates from several regions in Indonesia have high similarity to Philippines isolate of more than 97%. Therefore, the rice virus isolates in Asia have a close relationship. King *et al.* (2012) states that a virus has a close relationship if it has > 89% nucleotide sequence homology. Based on the analysis of homology, the spread of rice grass stunt disease (RGSV) started from Thailand (Chiengwattana, 2010), Vietnam (Rattanakarn & Pattawun, 2010), Philippines and China with a yield loss of 80% in

2010. In Indonesia (Klaten), those viruses still cause damage to this day. This might be caused by the high ability of BPH migration over long distances. According to Baehaki & Mejaya (2014), BPH has higher ability to migrate within subtropical and temperate countries, e.g. Vietnam, the Philippines, China, and Thailand compared to the tropical countries.

Protein Bands Profile Analysis of Rice Plants Infected with the Rice Stunt Virus

Protein isolation of healthy rice leaf and those infected with stunt virus were confirmed using the SDS-PAGE method with a concentration of 12.5%. SDS-PAGE analysis showed that the presence of several protein banding patterns appeared in Ciherang and Situ Bagendit varieties (Figure 5). The analysis of protein profile patterns using SDS-PAGE showed that healthy and infected plants with different severity had similar profile of proteins that appeared at the molecular weight (MW) of ~50 kDa (Figure 5) caused by glutelin protein in rice (Phongthai *et al.*, 2017). Glutelin is the main fraction of protein in rice with 22.7–40.25% of the total protein contained in the rice bran (Cao *et al.*, 2009).

Sample of rice infected with rice stunt virus showed a pattern of protein bands with MW of 22 kDa. This protein was assumed as a protein encoded

by the viral genome RNA 5 (vRNA 5) p5 RGSV. It acts as a viral suppressor for RNA Silencing and interacts with p3 nonstructural proteins (Chomchan *et al.*, 2003; Zhang *et al.*, 2015). Based on the results of visualization, protein bands with a molecular weight of 34 kD were detected in the infected rice in severe and failure categories on Ciherang and Bagendit varieties and 33 kDa and 43 kDa in failure category on both varieties. These protein bands were not detected in healthy rice samples. It proved that the band is a protein of the rice stunt virus. This result was similar to Upadhyaya *et al.* (1996) and Jia *et al.* (2012) report saying that rice plants infected with the stunt virus show a profile of a specific protein band with a molecular weight approaching 43kDa, which was suspected to be the major capsid protein RRSV encoded by segment 8 genome (S8).

Protein profile patterns close to 39 kDa were also detected in failure category on Ciherang varieties; and moderate, severe, and failure categories on Situ Bagendit varieties. According to Guoying *et al.* (1999), an MW protein of 39 kDa is a viral spike protein or one of the protein components has a role in the viral attachment process encoded by the genome segment 9 (S9) to

Table 2. The homology of the NCP RGSV gene nucleotide sequence homology of the rice plants and RGSV isolates obtained from the NCBI GeneBank

No.	Origin of Isolate	Homology (%)					
		1	2	3	4	5	6
1	2878682_RGSV_Bantul	ID					
2	KF438691.RGSV_Indonesia:Klaten	98.1	ID				
3	KF438757.RGSV_Thailand:Pathumthani	97.7	99.1	ID			
4	KF438715.RGSV_Thailand:Suphanburi	97.4	99.3	99.3	ID		
5	KF438733.RGSV_VietNam:DongThap	97.7	99.5	99.5	99.8	ID	
6	KF438721.RGSV_VietNam:LongAn	97.7	99.5	99.5	99.8	100	ID

Remarks: ID = Identical

Table 3. The homology of the S8 RRSV gene nucleotide sequence of the rice plants and RGSV isolates obtained from the NCBI GeneBank

No.	Origin of Isolate	Homology (%)			
		1	2	3	4
1	2878684_RRSV_Bantul	ID			
2	AF486811.RRSV_Philippines	99.5	ID		
3	HM125546.RRSV_China:Fujian	98.9	99.5	ID	
4	HM125556.RRSV_China:Guangdong	98.4	98.9	99.5	ID

Remarks: ID = Identical

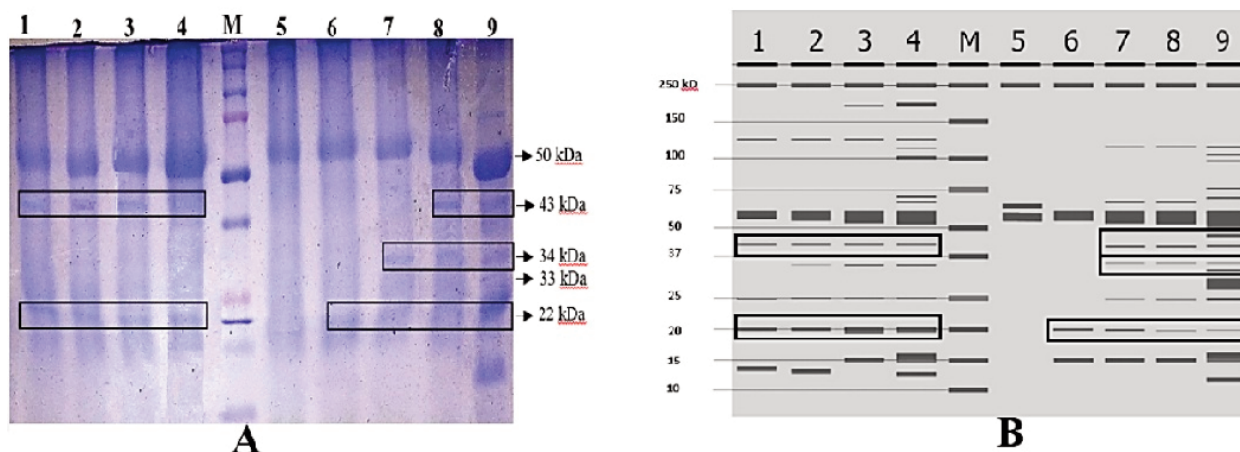


Figure 5. Protein band pattern of healthy rice plants and those infected with stunt virus at different severity level using SDS-PAGE gel electrophoresis; 1–4: mild to failure severity on Ciherang variety; M: Marker; 5: healthy; 6–9: mild-failure severity on Situ Bagendit variety (A) and replicas of protein banding patterns using Paint Shop Pro 5 software (B)

inhibit the virus transmission by insect vectors. Besides the proteins of RRSV, there are also some proteins of RGSV, e.g. nucleocapsid (NCP) RGSV protein with MW of 34–35 kDa. The observations also showed differences in the pattern of protein profiles between healthy and infected plants, especially in failure category of the two varieties which formed lots of protein profiles. Suwarno *et al.* (2013) states that this is caused by the detected-pattern of protein bands bringing additional protein coat from the virus; hence, the expression of protein banding patterns has various level of thickness. This result indicated that the failure severity has lots of protein found in Ciherang and Situ Bagendit varieties, presumably from RGSV and RRSV expressed in rice plants.

CONCLUSION

Detection with RT-PCR showed that Ciherang and Situ Bagendit varieties in Bantul Regency, D.I. Yogyakarta area were infected with RRSV and RGSV. The protein profile analysis using SDS-PAGE showed a protein band pattern of infected rice plants at different severity of 34, 35, 39, and 43 kDa with molecule weight (MW), i.e. nucleocapsid proteins, viral spikes, and capsids which were not found in healthy rice plants.

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