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**Research Article**

# Double Infections of *Rehmannia mosaic virus* and *Potato virus Y* on Tobacco Plants in Central Java and Special Region of Yogyakarta

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## ABSTRACT

Various viruses can cause mosaic disease on tobacco plants. Proper detection of the cause of mosaic diseases, helps determine effective control. The purpose of this study was to detect the presence of double infection of *Rehmannia mosaic virus* (ReMV) with *Potato virus Y* (PVY) using multiplex RT-PCR in tobacco plants from Central Java and Special Region of Yogyakarta. The viral suspension was inoculated on *Chenopodium amaranticolor* to obtain one viral colony from one local lesion. The multiplex RT-PCR method using Tobamovirus primers (TobRT-up1 and TobRT-do2) and Potyvirus primers (MJ1 and MJ2) can detect double infection caused by ReMV with PVY in tobacco plants distributed in Central Java and Special Region of Yogyakarta. The multiplex RT-PCR product showed that tobacco samples with mosaic symptoms from Temanggung, Klaten, Bantul, and Kalasan were positive ReMV. Multiplex RT-PCR has successfully detected double infection of ReMV and PVY on tobacco samples from Klaten and Kalasan. ReMV Bantul, Kalasan, and Klaten were homolog to ReMV USA isolate and ReMV Temanggung was homolog to ReMV Japanese isolate. PVY Klaten was homolog to PVY Turkey isolate, and PVY Kalasan was homolog to PVY Iran.

Keywords: double infection; multiplex RT-PCR; *Nicotiana tabacum*; *Potato virus Y*; *Rehmannia mosaic virus*

## INTRODUCTION

Tobacco (*Nicotiana tabacum*, L.) is one of the commodities grown in Indonesia and is classified as an annual crop. According to the *Direktorat Jenderal Perkebunan* (2021), tobacco production in the Central Java in 2020 has increased by 724 tons compared to 2019. However, the province of Special Region of Yogyakarta in 2020 sustained a decrease of 46 tons in tobacco production compared to the previous year. Viruses that cause disease on tobacco plants include *Tobacco mosaic virus* (TMV), *Tomato spotted wilt virus* (TSWV), *Tobacco leaf curl virus* (TLCV), *Potato virus Y* (PVY), *Cucumber mosaic virus* (CMV), *Alfalfa mosaic virus* (AMV), *Tobacco rattle virus* (TRV), *Artichoke yellow ringspot virus* (AYRV), *Eggplant mottled dwarf virus* (EMDV), and *Rehmannia mosaic virus* (ReMV) (Dalmadiyo *et al.* 1997; Chatzivassiliou *et al*., 2004; Kubota *et al.,* 2012).

Various viruses can cause mosaic disease on tobacco in Indonesia. CMV (Genus: Cucumovirus) causes systemic necrosis, mosaic, chlorosis, and stunting in tobacco plants (Mochizuki & Ohki, 2012). TMV (Family: Virgaviridae, Genus: Tobamovirus) causes mosaic disease in tobacco (Hanadyo *et al.,* 2013). ReMV (Family: Virgaviridae, Genus: Tobamovirus) causes the mosaic disease; showed high similarity to the nucleotide sequence of TMV RNA. ReMV was first discovered on the Rehmannia plants (*Rehmannia glutinosa* L.) in the Henan Province, China (Zhang *et al.,* 2004). The development of symptoms on plants infected by ReMV showed that they developed prominent leaf necrosis, severe leaf fall, and followed by stem necrosis and fruit distortion (Kubota *et al*., 2012). In addition, PVY (Family: Potyviridae, Genus: Potyvirus) causes mosaic curl and veinal necrosis in tobacco. PVY can cause yield

losses of 39–75% in tobacco (Tian *et al*., 2011; Tsedaley, 2015; Korbecka-Glinka *et al*., 2017).

Two different viruses can infect tobacco plants. The synergistic interaction of the two viruses in double infection can increase disease severity in plants (Otsuki & Takebe, 1976). Currently, a fast, accurate, and cost-effective method for detecting double infection in plants caused by two different viruses has been found, i.e., multiplex RT-PCR. Pathogen detection technique with multiplex RT-PCR can simultaneously detect several pathogens in one sample with several sets of primers. The multiplex RT-PCR method in Indonesia has been carried out to detect viral RNA that causes mosaic symptoms in sugarcane and Crinivirus in tomato plants (Asharo *et al.,* 2017; Ningrum *et al*., 2019).

Recently, there have been reports about a new member of the Tobamovirus genus that infects tobacco plants, i.e., *Rehmannia mosaic virus* (ReMV). The existence of ReMV still requires more research regarding the current identification of the distribution of various types of tobacco plants in multiple locations, especially in Indonesia. Therefore, this study aims to detect double infection of ReMV and PVY in tobacco in Central Java and Special Region of Yogyakarta.

## MATERIALS AND METHODS

### Research Site

This research was carried out in 2020–2021 in Tobacco Land (Temanggung, Klaten, Bantul, Kalasan) with a sampling technique on plants with mosaic symptoms, i.e., simple random sampling. Mechanical inoculation and multiplex & singleplex RT-PCR testing were implemented at the Plant Diseases Science Laboratory, Plant Virology Sub-Laboratory, Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta.

### Inoculation in Indicator Plants for Obtaining One Viral Colony from One Local Lesion

One g samples of tobacco leaves with mosaic symptoms (Temanggung, Klaten, Bantul, Kalasan) were added to 10 ml of 0.01 M phosphate buffer (pH 7) (w:v = 1:10). The sap was filtered using cotton and transferred to a small petri dish. The inoculation was carried out at the top of the leaf *Chenopodium amaranticolor* mechanically using carborundum and sap. After 5 minutes, the plants were sprayed with water to wash the excess sap leaf extract. The symptoms that appeared on the *C. amaranticolor* plant were observed.

### RNA Extraction

RNA extraction was carried out using RNeasy Plant Mini Kit (Qiagen) procedure. Then, synthesis of cDNA in this extracted samples using ReverTra Ace -α- kit (Toyobo, Japan) in a total volume of 10 µl with a composition of 2 µl of RNA samples; 3.5 µl RNase Free $H_2O$; 2 µl 5x RT Buffer; 1 µl dNTP Mixture; 0.5 µl Oligo primer (dt) 20 to detect Tobamovirus and Potyvirus & 0.5 µl random primers for CMV; 0.5 µl RNase Inhibitor; and 0.5 µl of ReverTraAce. RT-PCR cycles were done according to the protocol of the ReverTra Ace -α- kit (Toyobo, Japan).

### DNA Amplification

Two kinds of primers were used to amplify the coat protein-coding gene for Potyvirus & CMV and the movement protein-coding gene for Tobamovirus. Detection of members of the Tobamovirus genus using common primers, namely TobRT-up1 (5'-GARTAYSCIGCI YTICARAC-3') and TobRT-do2 (5'-BGCYTCRAARTTCCA-3') with a target size 568 bp (Dovas *et al*., 2004). Members of the genus Potyvirus were detected using a common primer designed by Marie Jeanne *et al.* (2000), namely MJ1 (5'-TGGTHTGGTGYATHGARAA YGG-3') and MJ2 (5'-TGCTGCKGCYTTCAT YTG-3') with a target size of 320 bp. Detection for CMV using specific primers, CMV-P1 (5'-GCCG TAAGCTGGATGGACAA-3') and CMV-P2 (5'-TATGATAAGAAGCTT GTTTCGCG-3') with a target size 500 bp (Wylie *et al.,* 1993). DNA amplification reaction for Tobamovirus and Potyvirus were conducted by the multiplex RT-PCR method with a total reaction volume of 25 µl, consisting of 12.5 µl MyTaqTM HS Red Mix, 1 µl TobRT-up1 primer, 1 µl TobRT-do2 primer, 1 µl MJ1 primer, 1 µl MJ2 primer, 6.5 µl $dH_2O$ and 2 µl template DNA. The multiplex RT-PCR program was carried out with 35 cycles, 94°C pre denaturation for 3 minutes, denaturation 94°C for

30 seconds, primer attachment 48°C for 1 minute, 72°C elongation for 1 minute, and elongation final 72°C for 10 minutes. DNA amplification reaction to detect CMV was conducted by the singleplex RT-PCR method with a total reaction volume of 10 µl, consisting of 5 µl MyTaqTM HS Red Mix, 1 µl DNA template, 3 µl $dH_2O$, 0.5 µl CMV-P1 primer, and 0.5 µl CMV-P2. The singleplex RT-PCR program according to Wylie *et al.* (1993) with 35 cycles, 94°C pre denaturation for 3 minutes, denaturation 94°C for 1 minute, primer attachment 51°C for 30 seconds, 72°C elongation for 1 minute, and elongation final 72°C for 5 minutes. PCR product was visualized using 1% agarose gel in 1x TBE with an electrophoresis program at 50 volts for 50 minutes.

### Sequencing and Analysis

The sequencing process was conducted using positive results of the PCR product with a total volume of 50 µl. Sequencing method provided by PT Genetika Science Indonesia. Sequencing results were analyzed using the Bioedit program (7.0.5.3) (Hall, 1999). Analysis of the phylogenetic tree and sample identity using the Mega 7.0 program based on Neighbor-joining with the bootstrap test method (1000 replicates) (Kumar *et al.*, 2016). Sequencing results were compared with other countries that have been registered in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). Homology analysis of sample sequences were analyzed using multiple sequence alignment with CLUSTAL W program (http://www.ebi.ac.uk/clustalw/).

## RESULTS AND DISCUSSION

### Symptom Characteristics of Tobacco Samples

Tobacco plants with mosaic symptoms were found in Temanggung, Bantul, Klaten, and Kalasan. Tobacco samples from Temanggung and Bantul (Figure 1A and Figure 1B) showed mosaic symptoms, namely irregular green and yellow color differences in tobacco leaves that can be caused by ReMV infection. The Klaten and Kalasan tobacco samples showed mosaic and light yellow spots on the leaves; this could be due to the presence of double infection in the samples (Figure 1C and Figure 1D). In a previous study, ReMV caused mosaic symptoms in the leaves of *Rehmannia glutinosa, Nicotiana glutinosa, N. tabacum var. Xanthi, N. rustica, Datura stramonium, Chenopodium amaranticolor, Nicotiana benthamiana*, and *Capsicum annum* (Zhang *et al.*, 2004; Kubota *et al.*, 2012). In addition, mosaic symptoms in tobacco can be caused by TMV, CMV, and PVY. TMV was transmitted by contact and not through insect vectors, while CMV and PVY can be transmitted non-persistently and non-circulative by *Myzus persicae* (Hanadyo *et al.*, 2013; Tsedaley, 2015; Shi *et al.,* 2016)

### Mechanical Transmission using *Chenopodium amaranticolor*

The inoculation method was used as an initial screening to diagnose the cause of mosaic disease in tobacco samples—inoculation of infected plants for mechanical transmission to the healthy leaf of *C. amaranticolor*. The local lesion appeared five days after inoculation. These results indicated that the cause of mosaic symptoms could be transmitted mechanically through wounds (Figure 2).

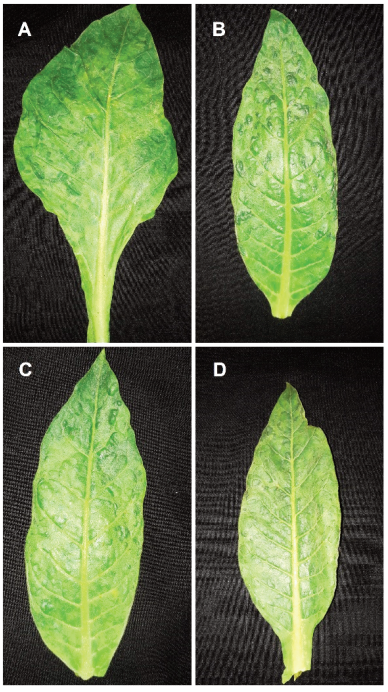


Figure 1. Symptoms of mosaic disease in tobacco samples; (A) Temanggung, (B) Bantul, (C) Klaten, (D) Kalasan

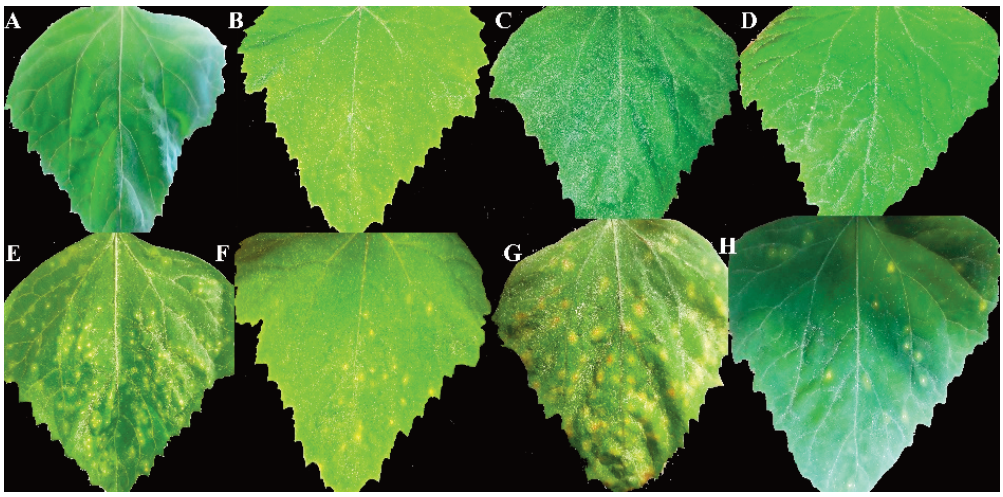


Figure 2. Leaves of Chenopodium amaranticolor were inoculated with samples from various regions in Central Java and Yogyakarta; Temanggung, Klaten, Bantul, Kalasan control treatment (A, B, C, D) and 5 days after inoculated with samples of Temanggung, Klaten, Bantul, Kalasan (E, F, G, H)

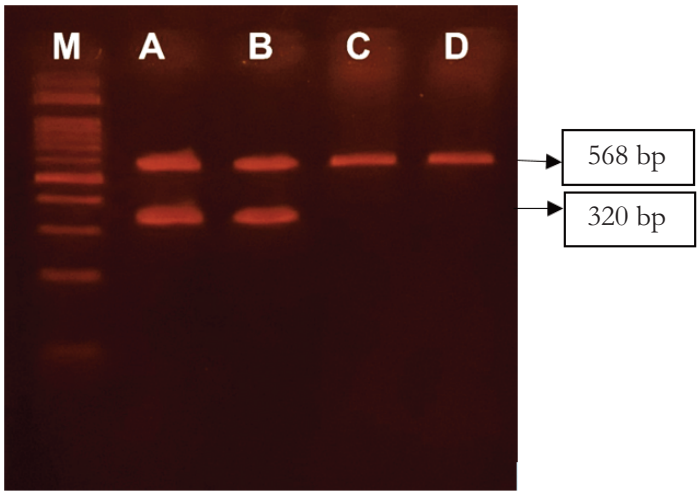


Figure 3. Visualization of multiplex RT-PCR amplification of tobacco samples using TobRT-up1 and TobRT-do2 & MJ1 and MJ2 primers to detect the presence of ReMV and PVY with 1% agarose gel in 1× TBE; (M) 100 bp markers. Sample from (A) Klaten; (B) Kalasan; (C) Temanggung; and (D) Bantul

**Detection of Pathogens with Multiplex & Singleplex RT-PCR**

The purpose of using multiplex and singleplex RT-PCR as detection methods is to get an accurate result. The analysis of PCR products using the multiplex RT-PCR technique (Figure 3) explained the presence of double bands in Klaten and Kalasan tobacco samples detected 568 bp for Tobamovirus and 320 bp for Potyvirus. This result proved that ReMV and PVY double infected both samples of tobacco after analyzed sequencing. The studies on double infection of tobacco plants by TMV and PVX, or with CMV and PVX that has been discovered. The two different viruses can infect tobacco plants and multiply within the same cell. The two or more different viruses interact synergistically or antagonistically depending on the combination of viruses (Otsuki & Takebe, 1976).

The synergistic interaction of two viruses (ReMV & PVY) in double infection increased disease severity in the plant according to the appearance of symptoms in tobacco samples from Klaten and Kalasan. According to Balogun *et al.* (2002), double infection by the combination of two viruses affects the concentration of one virus. Temanggung and Bantul tobacco samples contained a single band

detected 568 bp, which indicated that both samples were infected with ReMV and not infected with PVY because farmers in Bantul and Temanggung prevented the spread of PVY vector with insecticide. Based on PCR product using the singleplex RT-PCR method, CMV was not detected in tobacco samples because insect vector of CMV (aphids) was not found there (Figure 4).

**Phylogenetic Analysis and Sample Identity**

The phylogenetic tree is an analytical method used to determine the relationship between the sequences obtained in this study and the sequence data from Genbank at NCBI (Abdullah *et al.*, 2019). Based on the phylogenetic tree, the cause of mosaic symptoms in Central Java and Special Region of Yogyakarta was ReMV. Therefore, the phylogenetic tree of tobacco samples has a different cluster from TMV. The partial alignment of ReMV DNA sequences from several isolates in Central Java (ReMV Bantul, ReMV Kalasan, and ReMV Klaten) has a homology nucleotide of more than 98%. However, if these three isolates were compared to ReMV Temanggung, the nucleotide sequence homology was over 94%. (Figure 5).

Analysis of the percentage of nucleotide sequence homology of ReMV species from Central

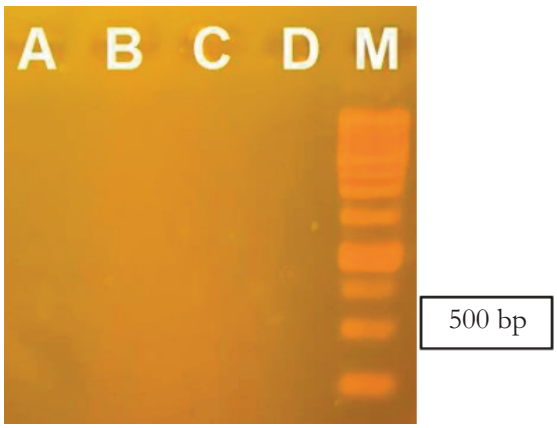


Figure 4. Visualization of singleplex RT-PCR amplification of tobacco samples using CMV-P1 and CMV-P2 primers to detect the presence of CMV with 1% agarose gel in 1× TBE; (M) 1 kb markers. Sample from (A) Klaten; (B) Kalasan; (C) Temanggung and; (D) Bantul

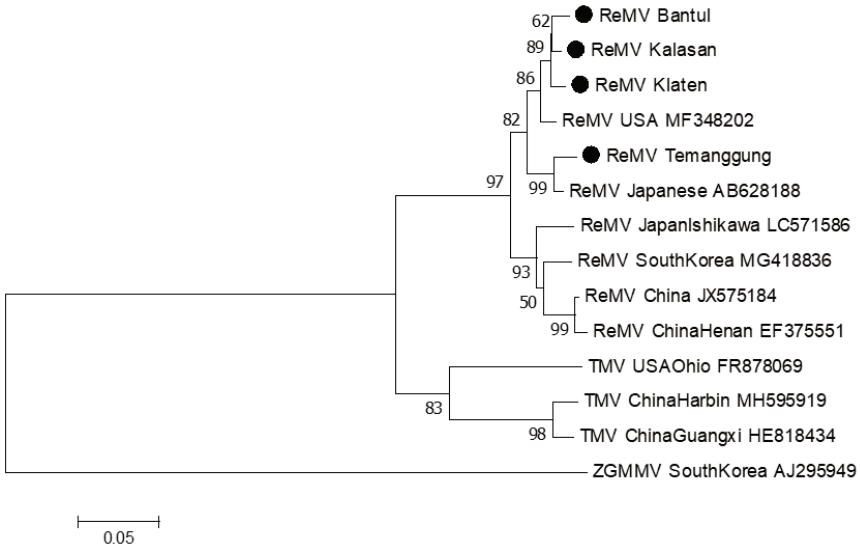


Figure 5. Phylogenetic tree of ReMV isolates that infect tobacco in Central Java and DIY based on partial nucleotide sequence alignment of DNA using MEGA7.0 program (Neighbor-Joining method with 1000 bootstrap replicates)

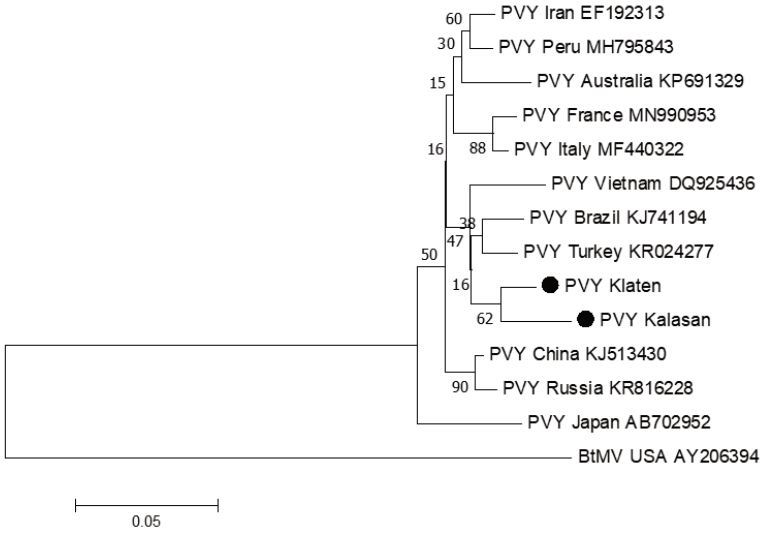


Figure 6. Phylogenetic tree of PVY species from Klaten and Kalasan isolates with isolates in the Genbank Database using MEGA 7.0 analysis (Neighbor-Joining method with 1000 bootstrap replicates)

Java compared with other isolates published in the Genbank Database at NCBI showed that ReMV Bantul, Kalasan, and Klaten were close relationship with ReMV USA isolates (MF348202), which was above 97%. ReMV Temanggung isolates was the same (97.9%) with ReMV Japanese (AB628188). According to the International Committee on Taxonomy of Viruses [ICTV] (2021a), the homology similarity of more than 90% can be considered to characterize the strain of the same species. The homology of tobacco samples with TMV nucleotides from the Genbank Database was 77.75%; this indicates that the tobacco samples were indeed classified as ReMV. The nucleotide sequence of the Tobamovirus genus can be classified into different species if it has a sequence identity of less than 90%. The ReMV genome found to infect *Rehmannia glutinosa* plants has the highest nucleotide homology similarity to TMV (*Tobacco mosaic virus*), which is 84%; thereby, ReMV is classified as a new species of the Tobamovirus genus (Zhang *et al*., 2008). Each of the ReMV and PVY isolates in this study had nucleotide differences (nt), with the comparison isolates from the Genbank Database as 66 nt and 41 nt. Based on sequence homology, this data between ReMV nucleotide samples and ReMV from the Genbank Database they classified in the same strain based on ICTV.

PVY Kalasan was similar to PVY Klaten, although these isolates originally came from a distant location. The nucleotide sequence homology of PVY Klaten and PVY Kalasan was 96.2%. The PVY Klaten was 97.6% homolog to PVY Turkey KR024277 and classified in the same strain. The nucleotide homology of PVY Kalasan isolate with PVY Iran EF192313 was 95.5%. Therefore, PVY Kalasan isolate and PVY Iran were classified in the same strain (Figure 6). The Potyviridae species demarcation criteria in the ICTV (2021b) that the complete ORF were <76%. The optimal species demarcation criteria for the coat protein were 76–77% nucleotides, while between strains of PVY were 90–99% (average 95%) (Shukla & Ward, 1988).

Based on the result, ReMV has spread in Indonesia, USA, Japan, China, and South Korea. PVY has spread in Indonesia, Iran, Peru, Australia, France, Italy, Vietnam, Brazil, Turkey, China, Russia and Japan. The widespread of ReMV and PVY in these countries were through trade activities by seed contamination. Based on the farmer observation, ReMV and PVY in tobacco plants affect the quality and quantity of tobacco. The decrease in quality and quantity of Temanggung tobacco (Temanggung) affects the production of cigarettes. In addition, Kedusili tobacco (Bantul) and Bligon tobacco (Kalasan) are the Special Region of Yogyakarta tobacco types for making *kretek* cigarettes.

Kedusili and Bligon tobacco are still usable if infected by ReMV and PVY. However, the poor quality of Vorstenlanden tobacco (Klaten) for cigars causes a severe problem for farmers because unused anymore.

## CONCLUSION

The mosaic symptoms in tobacco samples from Temanggung, Klaten, Kalasan, and Bantul were caused by the *Rehmannia mosaic virus* (ReMV). The nucleotide sequence analysis showed that the ReMV isolates from Bantul, Kalasan, and Klaten were classified with the same strain of ReMV USA isolate; ReMV Temanggung isolate classified with the same strain of ReMV Japanese isolate. PVY Klaten was classified with the same strain as PVY Turkey. PVY Kalasan was classified with the same strain as PVY Iran. This study was the first report discovering that double infection of ReMV and PVY in Klaten and Kalasan tobacco samples can be detected with multiplex RT-PCR.

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