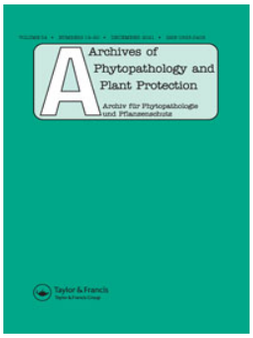

Archives of Phytopathology and Plant Protection

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/gapp20

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To cite this article: Selvi Helina, Titik Nur Aeny & Ivayani Ivayani (2021) Simultaneous detection of virus infecting tobacco plant using multiplex-PCR in Klaten, Indonesia, Archives of Phytopathology and Plant Protection, 54:19-20, 1838-1847, DOI: 10.1080/03235408.2021.1950502

To link to this article: https://doi.org/10.1080/03235408.2021.1950502

Published online: 15 Aug 2021.

ARCHIVES OF PHYTOPATHOLOGY AND PLANT PROTECTION
2021, VOL. 54, NOS. 19–20, 1838–1847
https://doi.org/10.1080/03235408.2021.1950502

Simultaneous detection of virus infecting tobacco plant using multiplex-PCR in Klaten, Indonesia

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ABSTRACT

Tobacco is one of the important plantation commodities in Indonesia. The multiple virus attack in the field causes crop damage to crop failure. Therefore, detection is needed quickly and accurately as a basis for control strategies. This study aimed to determine the viruses that attack tobacco plants in Klaten, Indonesia through simultaneous detection using multiplex-PCR. The study was conducted by observation in the field then detection in the Laboratory. Based on Multiplex-PCR detection results using primers of MJ 1/MJ 2, Krusty/Homer, and CMV P1/P2 showed that tobacco plants infected with several viruses from the genus *Potyvirus, Begomovirus* and *Cucumovirus*. Based on the results of the nucleotide sequencing blast, it was found that the tobacco isolate was positively infected by the *Tomato yellow leaf curl Kanchanaburi virus (*TYLCV), *Cucumber mosaic virus* (CMV) and *Potato virus (*PVY).

ARTICLE HISTORY
Received 17 March 2021
Revised 8 June 2021
Accepted 25 June 2021

KEYWORDS
CMV;
PVY;
tobacco;
TYLCV;
multiplex-PCR

Introduction

Tobacco plants are one of the important plantation commodities in Indonesia. This is because tobacco plays a role in the country's economy, especially the provision of jobs, sources of income for farmers and the foreign exchange. Indonesia is one of the world's tobacco producers with tobacco plantation area reaching 198,054 thousand hectares with production of 164,851 thousand tons in 2007 (Rachmat and Aldillah 2016). Some regions in Indonesia are centers of tobacco cultivation, one of which is the Klaten district in Central Java. BPS (2015) reported that the total tobacco planting area in Klaten in 2015 was 369,598 Ha with a dry production of around 609 Tons. Despite this, efforts to increase

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tobacco production experienced obstacles including a virus attack. The virus attack causes a decrease in tobacco production to crop failure.

Several viruses that attack tobacco plants have been widely reported such as *Cucumber Mosaic Virus*, *Tobacco Mosaic Virus* (Wahyuni et al. 2008; Khamphirapaeng et al. 2017), *Potato virus Y* (Ghosh and Bapat 2006), *Tobacco leaf curl virus* (Jing et al. 2016) and other viruses (Akinyemi et al. 2016). Tobacco diseases caused viruses are not only one type of virus, but also there have been multiple virus attacks (Otsuki and Takebe 1976; Wahyuni et al. 2008). It is because vector insects are able to associate with several types of viruses in tobacco plants such as aphids that transmit *Potyvirus* and *Cucumovirus* (Hull 2002; Pirone and Peny 2002; Wang et al. 2002), and whitefly that transmits *Begomovirus* and *Crinivirus* (Hull 2002). The existence of this attack causes damage to plants that are severe and cannot be produced. Based on the various reports, early detection is needed to find out the presence of viruses on tobacco plant.

The importance of accurate diagnosis is used as a basis for managing plant diseases and predicting yield losses (Aboul-Ata et al. 2011). Many kinds of techniques are available now and are developing for the diagnostic purpose (Jeong et al. 2014) such as PCR (Webster et al. 2004). PCR is a fast detection method that has high accuracy and sensitivity because it uses DNA as an information center (Jeong et al. 2014). In addition, two or more DNA or RNA targets can be detected simultaneously in one reaction using multiplex PCR techniques (Webster et al. 2004; Lopez et al. 2009). Several reports have shown about the simultaneous detection of several viruses such as *Begomovirus* and *Crinivirus* (Bhat and Siju 2007). This study aimed to detect viruses simultaneously on tobacco plants in Klaten, Indonesia. In this way it is expected to make it easier in decisions for control measures.

Materials and methods

Research site

This research was conducted on tobacco plantations in Klaten, Central Java. The observation results were taken to the Virology Laboratory, Faculty of Agriculture, Gadjah Mada University for simultaneous detection. The study was conducted from October to December 2020.

Observation of viruses in tobacco plants

Observation was conducted by looking at the symptoms of plant were attacked by virus. Tobacco plants that induced mosaic, yellowing, wrinkle

and dwarf symptoms were picked up and taken to the Laboratory for molecular detection using Multiplex-PCR.

DNA and RNA extraction of plant viruses

Total RNA extraction was carried out using *RNeasy Plant RNA mini kit* with procedures according to *Qiagen Sample & Assay Technology*. While DNA extraction uses *Total Plant DNA mini kit (Plant)* with procedures according to *Geneaid*'s recommendation.

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 25 mM 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 H_2O were inserted into the tube. Then the extracted RNA was put into the PCR tube as much as 3 µl and homogenised using a vortex machine. The sample is then placed into a PCR machine for RT-PCR with an incubation temperature of 42° C for 20 minutes, heat 99° C for 5 minutes and 4° C for 20 minutes.

cDNA amplification

cDNA results from RT-PCR were previously mixed with several reagents before being amplified using a PCR machine. Each sample will be amplified using several primers in one reaction (Table 1). PCR amplification uses the *My Taq HS RedMix* method with a 5 µl free water (ddH_2O) composition, 10 ml of RedMix mix PCR, each primer with a 5 pmol concentration of 0.5 µl with each template 1 µl, so that the total volume reaches 20 µl. then do PCR with an initial denaturation cycle

Table 1. Primer used for simultaneous detection using multiplex PCR on viruses infecting tobacco plants.

Targets	Primary codes	Primary base order (5′-3′)	Product sizes	Sources
Begomovirus	Krusty	CCNMRDGGHTGTGARGGNCC	580 bp	Revill et al. (2003)
	Homer	SVDGCRTGVGTRCANGCCAT		
Potyvirus	MJ 1	ATGGTHTGGTGYATHGAR	320 bp	Marie-Jeanne et al. (2000)
	MJ 2	TGCTGCKGCYTTCATYTG		
CMV	CP-F	ATGGACAAATCTGAATCAAC	650 bp	Bhat and Siju (2007)
	CP-R	TCAAACTGGGAGCACCC		
CMV	P1	GCCGTAAGCTGGATGGACAA	500 bp	Wylie et al. (1993)
	P2	TATGATAAGAAGCTTGTTTCGCG		

of 95° C for 3 minutes, denaturation of 95° C for 45 seconds, pasting 53° C for 45 seconds, elongation of 72° C for 1 minute and a final elongation of 72° C for 5 minutes. Visualisation of the amplification product was carried out using 1% agarose gel in 1 × TBE (Tris-borate EDTA) with electrophoresis for 50 minutes at 50 Volts.

Analysis of nucleotide sequences

Analysis of nucleotide sequences using *sequencer ABI-Prism 3100-Avant Genetic Analyzer* in the Research and Development Center laboratory of PT. Genetics Science, Indonesia. The DNA sequence results were analysed with *the Basic Local Alaigment 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 nucleotide virus sequences from other countries registered with Genbank. The level of homology of nucleotides and amino acids was obtained by *the ClustalW multiple alignment* dan *Sequences Identity Matrix* using Bioedit 7.05 software.

Results

Tobacco plants with symptoms of mosaic, wrinkle and dwarf found in Sub Districts of Karanganom dan Bayat in Klaten regency showed the presence of multiple infections from several viruses (Figure 1). In addition, was found some vector insects on the surface of the leaves, namely Aphids and whitefly.

Several DNA bands were successfully amplified in tobacco leaf samples (Figures 2 and 3). This showed that tobacco plants in Klaten Regency, Indonesia were infected with several viruses of the genus *Potyvirus, Begomovirus* and *Cucumovirus. Potyvirus* using MJ 1/MJ 2 primers was

Figure 1. Symptoms of mosaics in tobacco plants; (A) Tobacco plants in Karanganom sub-district, (B) Tobacco plants in Bayat sub-district.

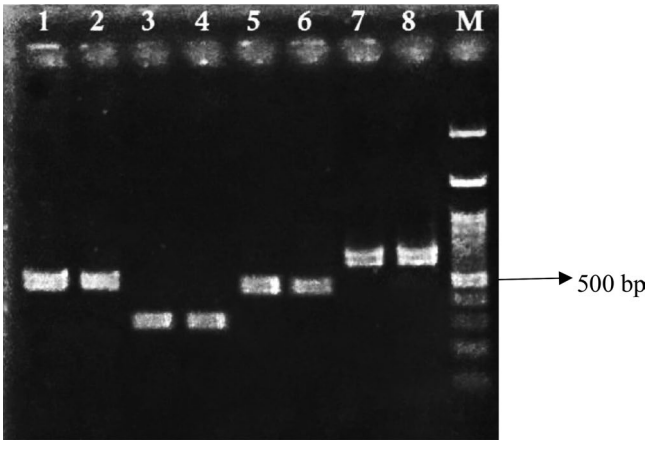

Figure 2. Visualisation of PCR with primers on agarose gels of 1.5%; Krusty/Hommer Primer in tobacco plants in Karanganom (1), Krusty/Hommer Primer in tobacco plants in Bayat (2), MJ1/MJ2 Primers in tobacco plants in Karanganom (3), MJ1/MJ2 Primers in tobacco plants in Bayat (4); CMV P1/P2 primers in tobacco plants in Karanganom (5), CMV P1/P2 primers in tobacco plants in Bayat; (6): Primary CMV CP F/R in tobacco plants in Karanganom (7), Primary CMV CP F/R in tobacco plants in Bayat and (8), Marker 100 bp (M).

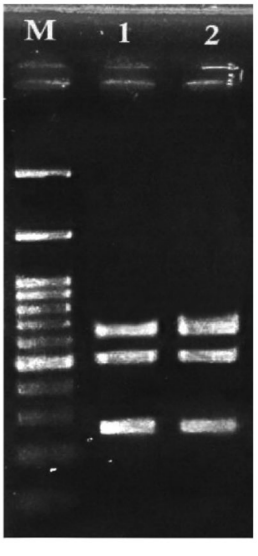

Figure 3. Visualisation results of Multiplex PCR with CMV CP F/R primers (650 bp), Krusty/Hommer (580 bp), and MJ1/MJ2 (320 bp) on 1.5% agarose gel in tobacco samples; Marker 100 bp (M), Tobacco samples in Karanganom (1), Tobacco samples in Bayat (2).

successfully amplified on DNA bands around 320 bp, *Begomovirus* using Krusty/Homer primers at 580 bp DNA band size, and *Cucumovirus* using specific CMV CP and CMV P1/P2 primers on DNA band sizes 650 bp and 500 bp.

Based on the results of the nucleotide sequencing blast, it was found that klaten isolate amplified on the 320 bp DNA band was the *Tomato*

yellow leaf curl Kanchanaburi virus. The results of grouping isolates based on nucleotides in part of the *Begomovirus* coat protein sequence showed that Klaten isolates were one group with KF446675_TYLCVKaV isolates from Indonesia and close to KU569596_TYLCVKaV isolates from Thailand (Figure 4). While the results of grouping isolates based on nucleotides in part of the CMV protein coat sequence showed that Klaten isolates were close to CMV isolates from China and Ecuador (Figure 5). Meanwhile, the results of the nucleotide blast sequencing of some PVY protein coat sequences indicate that Klaten isolate is close to the PVY isolate from Turkey (Figure 6).

Based on the homology level of nucleotide sequences of several viruses on tobacco plants in Klaten showed that TYLCVKaV Klaten isolates had the highest homology with TYLCVKaV isolates from Indonesia. While CMV isolates from Klaten had the highest homology with CMV isolates from China and Equador, whereas PVY isolates from Klaten had the highest homology with PVY isolates from Turkey (Tables 2–4).

Discussion

Viral attacks on tobacco plants caused a decrease in tobacco production, especially in Klaten, Indonesia. Moreover, multiple viruses attack caused tobacco plants cannot be produced. The existence of multiple viruses

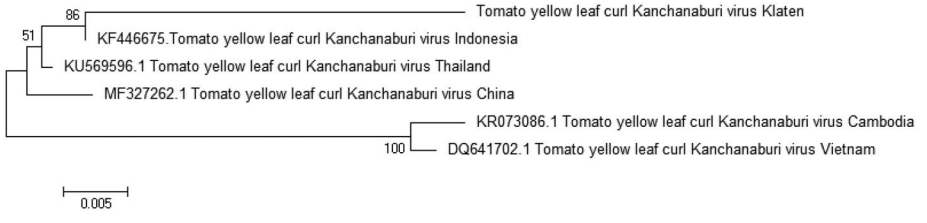

Figure 4. Dendogram of TYLCKancV genetic relationship from Klaten isolate and several TYLCKancV isolates published in the NCBI database.

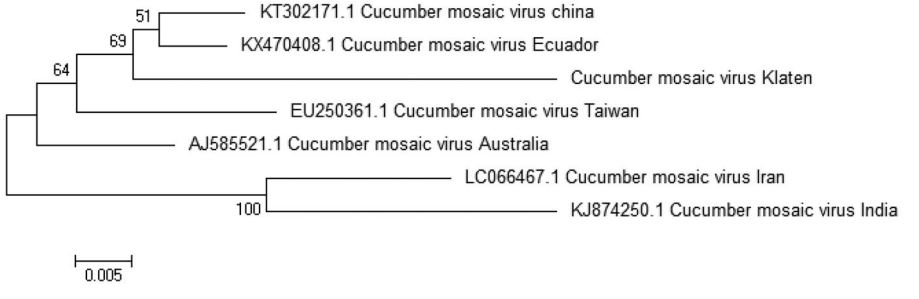

Figure 5. Dendogram of CMV genetic relationship from Klaten isolate and several other CMV isolates that have been published in the NCBI database.

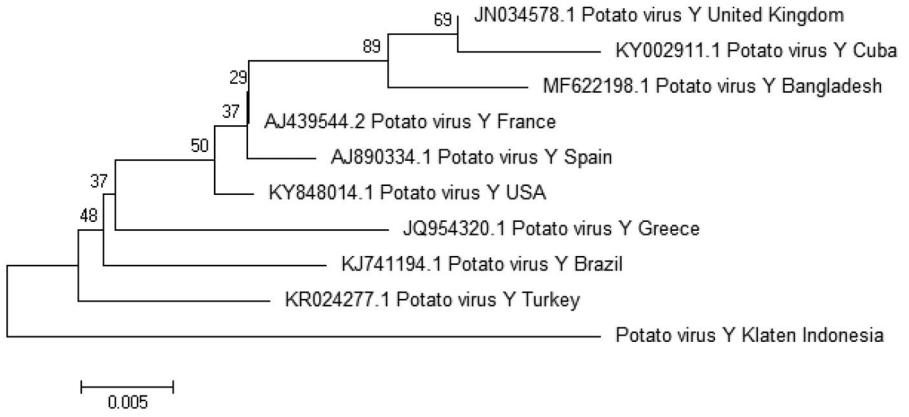

Figure 6. Dendogram of PVY genetic relationship from Klaten isolate and several other PVY isolates that have been published in the NCBI database.

Table 2. The homology of the NCP TYLCV gene nucleotide sequence in the sample and several other TYLCV isolates obtained from the NCBI GenBank.

	Isolates	1	2	3	4	5	6
1	*Tomato yellow leaf curl Kanchanaburi virus* Klaten	ID					
2	KF446675.*Tomato yellow leaf curl Kanchanaburi virus* Indonesia	97.1	ID				
3	KU569596.1 *Tomato yellow leaf curl Kanchanaburi virus* Thailand	96.7	99.6	ID			
4	MF327262.1 *Tomato yellow leaf curl Kanchanaburi virus* China	96	99	99.4	ID		
5	KR073086.1 *Tomato yellow leaf curl Kanchanaburi virus* Cambodia	92.9	96	96	95.8	ID	
6	DQ641702.1 *Tomato yellow leaf curl Kanchanaburi virus* Vietnam	93.1	96.2	96.2	96	99.4	ID

Remarks: ID = Identical.

Table 3. The homology of the NCP CMV gene nucleotide sequence in the sample and several other CMV isolates obtained from the NCBI GenBank.

No	Isolat	1	2	3	4	5	6	7
1.	*Cucumber mosaic virus* Klaten	ID						
2.	KT302171.1 *Cucumber mosaic virus* China	95.1	ID					
3.	KX470408.1 *Cucumber mosaic virus* Ecuador	95.6	98.6	ID				
4.	AJ585521.1 *Cucumber mosaic virus* Australia	93.8	97.5	97	ID			
5.	EU250361.1 *Cucumber mosaic virus* Taiwan	94.1	96.8	96.7	96.5	ID		
6.	LC066467.1 *Cucumber mosaic virus* Iran	91	94.1	93.8	94.6	93.8	ID	
7.	KJ874250.1_*Cucumber_mosaic_virus*_India	90.5	92.8	92.8	93.6	92.8	95.8	ID

Remarks: ID = Identical.

is caused by the ability of several vectors that can transmit more than one virus to the host plant. Besides that, tobacco is a plant that is preferred by several types of vector insects, thus increasing the presence of viruses in tobacco plants. More than 550 virus species transmitted by vectors were recorded, namely 55% aphids, 11% by leafhoppers, 11% by beetles, 9% by whiteflies, 7% by nematodes, 5% by fungi and

Table 4. The homology of the NCP PVY gene nucleotide sequence in the sample and several other PVY isolates obtained from the NCBI GenBank.

No	Isolates	1	2	3	4	5	6	7	8	9	10
1	*Potato virus Y* Klaten Indonesia	ID									
2	KR024277.1 *Potato virus Y* Turkey	95.4	ID								
3	KJ741194.1 *Potato virus Y* Brazil	94.9	97.7	ID							
4	AJ439544.2 *Potato virus Y* France	95.4	98.1	98.1	ID						
5	KY848014.1 *Potato virus Y* USA	95	97.7	97.7	99.6	ID					
6	JN034578.1 *Potato virus Y* United Kingdom	95	96.9	96.9	98.9	98.5	ID				
7	AJ890334.1 *Potato virus Y* Spain	95.4	97.7	97.7	99.6	99.2	98.5	ID			
8	MF622198.1 *Potato virus Y* Bangladesh	94.6	96.5	96.5	98.5	98.1	98.9	98.1	ID		
9	JQ954320.1 *Potato virus Y* Greece	94.5	97.3	97.3	97.7	98.1	96.5	97.3	96.1	ID	
10	KY002911.1 *Potato virus Y* Cuba	94.1	96.1	96.1	98.1	97.7	99.2	97.7	98.1	96	ID

Remarks: ID = Identical.

plasmodiophorids, and the remaining 2% by thrips, mites, mirid, or white lice (Astier et al. 2001).

Based on observations, tobacco attacked by multiple viruses were caused by vector insects that were mutually associated such as the aphids and whitefly. Aphids are capable of transmitting more than 130 types of viruses such as *Potyvirus* and *Cucumovirus* transmitted non-persistently (Zitter and Murphy 2009), while *Begomovirus* was transmitted by whitefly with persistent circulative non-propagative circulatives (Andret-Link and Fuchs 2005). In this case the interaction between viruses and vectors was important for virus identification so that they can develop appropriate control strategies. This strategy is designed to reduce losses caused by viruses with reducing the source of infection and limiting the spread by vectors (Lecoq et al. 2004). Control in the field needs to be done to prevent the spread of the virus to a wider area. Moreover, the climate in Indonesia is suitable for breeding aphids and whitefly as a virus vector in the field. By doing early detection quickly and accurately, helping agricultural actors to carry out appropriate control strategies so that yield losses caused by viruses can be minimised.

Virus management in tobacco plants should be carried out by prioritising effective and efficient methods. Thus far, curative control methods cannot be applied to tobacco plants that have been infected with the virus. As reported by Hutapea et al. (2014) disease management that is suitable for use is by planting virus-resistant tobacco varieties, preventing viruses by providing the nutrients needed by plants and managing virus vectors.

Conclusion

Based on the simultaneous detection results using multiplex PCR, it showed that the multiple virus attack on tobacco plantations in Klaten,

Indonesia was caused by the *Tomato yellow leaf curl Kanchanaburi virus* (TYLCV), *Cucumber mosaic virus* (CMV), and *Potato virus* Y (PVY).

Acknowledgements

We would like to thank the headman of Klaten regency for permitting take the tobacco samples and the anonymous reviewers for their suggestions that improved the manuscript.

Disclosure statement

The authors declare that they have no conflict of interest.

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