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Characteristics of Virus Symptoms in Chili Plants (*Capsicum frutescens*) Based on RGB Image Analysis

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ABSTRACT

Virus infection in chili plants may cause various symptoms. The complexity of the symptoms and human vision ability often become limiting factors during disease investigations. Digital image analysis is expected to become a method to assist in comprehensively describing the symptoms of plant viruses. A disease survey was conducted on cayenne pepper fields in Southeast Sulawesi Province to observe symptoms of virus infection virtually and to record the symptomatic plant using an RGB camera. The split-channel method is used to process images, followed by multidimensional scaling statistical analysis. Later on, viruses associated with plants were detected serologically. Single or mixed infection of *Tobacco mosaic virus*, *Cucumber mosaic virus*, *Chili veinal mottle virus*, and *Pepper mottle virus* was confirmed by plant leaves showing yellow-mosaic and mottle symptoms. The digital image analysis method could show variations in the characteristics of symptoms based on digital numbers in that cannot be recognized based on the observation of visual symptoms. A new approach to study the interactions between plant infecting viruses and their effects based on image analysis has also been developed during this research. This method needs to be further validated through testing under controlled conditions, such as inoculating plants with a predetermined type of virus.

INTRODUCTION

Virus infection in chili plants causes various symptoms as a manifestation of disorders in host cell physiology catalyzed by viral replication (Osterbaan & Fuchs, 2019). Disease symptoms such as chlorosis, mosaic, vein clearing, or vein banding occur due to degradation of chlorophyll pigment, changes in chloroplast structure, a decrease of chlorophyll content, changes in electron transport rate, photoinhibition, and reduction of CO_2 fixation (Zanini et al., 2021; Zhao, Zhang, Hong, & Liu, 2016) causing a decrease in the rate and efficiency of photosynthesis (Lei, Jiang, Hu, Yan, & Zhu, 2017; Soni et al., 2022). Furthermore, Ananthu & Umamaheswaran (2019) reported that virus-infected plants contained less chlorophyll and carbohydrates than uninfected plants.

Observation of external symptoms visually with the naked eye is a conventional method in recognizing and assessing virus infection in the field. This method is preferred because it is fast and easy to perform and does not require special equipment (Bock, Poole, Parker, & Gottwald, 2010). However, there are several limiting factors in this method, among others are (a) infection by different viruses may cause similar visual symptoms in the same plant (Hull, 2014), (b) interactions between

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different viruses when infecting plants can affect the expression of visual symptoms (Hull, 2014; Singhal, Un Nabi, Yadav, & Dubey, 2020), and (c) mild symptoms are often invisible (Paudel & Sanfaçon, 2018). In addition, the human eye's tendency to be less sensitive to differences in color brightness (Dey, Sharma, & Meshram, 2016) and the presence of color blindness (Nilsson, 1995) is another limiting factor.

Since aerial photography of plant diseases was reported by Neblette in the 1920s (Bock, Poole, Parker, & Gottwald, 2010), photography and image analysis techniques, especially digital images, have been used for the diagnosis, assessment, and study of various plant diseases such as bacterial leaf blight, blast, brown spot, and tungro virus in rice (Asfarian, Herdiyeni, Rauf, & Mutaqin, 2014), downy mildew in cucumbers (Ma, Du, Zheng, Zhang, & Sun, 2019), bacterial rot in pomegranates (Sharath, Akhilesh, Kumar, Rohan, & Prathap, 2019), soybean rust and potato late blight (Alves et al., 2022), banana leaf disease (Krishnan, Deepa, Rao, Divya, & Kaviarasan, 2022), gray mold in strawberry (Bhujel et al., 2022), or major diseases in turmeric plant (Devisurya, Devi Priya, & Anitha, 2022). Saranya, Karthick, & Thulasiyammal (2014) explained that digital image analysis is a non-destructive process that can examine and obtain information from images using currently available image collection equipment such as cameras (photography), computers, scanners, and image processing applications.

Camera sensors with the ability to record changes in leaf spectral reflectance due to disease infection can be used as a non-destructive method for the early detection of plant diseases (Cordon, Andrade, Barbara, & Romero, 2022; Kuska et al., 2022; Nguyen et al., 2021). Hasan, Widodo, Mutaqin, Taufik, & Hidayat (2021) reported that using cameras and digital image analysis techniques, namely SI-NDVI (Single Image-Normalized Difference Vegetation Index), diseased plants can be recognized at 13 days before the symptoms are visible. Furthermore, Dey, Sharma, & Meshram (2016) explained that digital image analysis techniques based on trichromatic colors, namely red, green, and blue (RGB), can be used to measure leaf chlorophyll as an important indicator of plant health index.

This digital image analysis method has been widely developed as an alternative method for sensing or recognizing diseased plants with or without symptoms (Peng et al., 2022; Sankaran, Mishra, Ehsani, & Davis, 2010). Nevertheless, knowledge and specific information about digital image-based methods for plant diseases caused by viruses are still very limited. Therefore, it is necessary to study the potential of digital image analysis as an alternative method to describe characteristics and assess the condition of plant disease symptoms caused by infection with various types of viruses.

MATERIALS AND METHODS

Field Surveys

Disease surveys were carried out in July 2018 at cayenne pepper (*Capsicum frutescens*) fields owned by farmers in the South Konawe Regency, Southeast Sulawesi, Indonesia; at location coordinate of 4°23'46.6"S 122°02'56.2" E and with an altitude of 122.7 masl. The activities during the surveys involved visual symptom observation, plant symptom images recording, and plant samples collection for virus detection in the laboratory.

Symptoms Observation and Image Recording

Image recording of symptomatic chili plants was carried out between 08:00 – 08:31 am UTC +8. During images recording, the intensity of sunlight is relatively low due to cloudy sky conditions. The target of image recording are plants showing different symptoms of virus infection. The plant image was recorded using a Canon EOS 750D DSLR Camera + EF-S18-55 mm f/3.5-5.6 IS II kit lens with a focal length of 30.0 mm (autofocus), and intelligent auto scene as the camera's recording mode. The camera was positioned above the surface of the plant canopy with an approximate distance of 40 cm.

Image Processing and Data Analysis

The recorded image was processed using Fiji–ImageJ 1.52k application (Schindelin et al., 2012). First, plant leaf images (6000 × 4000 pixels) were prepared, then 30 regions of interest (ROI) were determined as replicates for each plant leaf image using the rectangle tool with sizes varying from 68 × 72 pixels to 210 × 222 pixels due to adjusting shape and size of the leaves and avoid taking the stem, flower, and fruit areas as well (Fig. 1). All ROIs were then resized to 512 × 512 pixels using the resize tool (interpolation = bicubic); followed by converting the RGB image into separate red, green, and blue channels using the split channels tool. Conversion to Grayscale image was also carried out using the image tool and continued with extracting the average digital number from each converted image using the measurement tool.

The average digital value of each symptomatic plant image ROI was analyzed by descriptive and multidimensional scaling (MDS) analysis with the Euclidean method for distance measure using STAR application version 2.0.1. The ROI samples of symptomatic leaf images were visualized in false color to observe areas of leaves that reflect and absorb light, especially red and blue.

Confirmation of Virus Infection

Leaves from symptomatic plants selected as recording targets were collected, i.e., 5–7 leaves per plant. Leaf samples were put into plastic bags and then stored in a cooler containing ice gel to maintain the freshness of the leaf samples until the samples arrived in the laboratory. Virus detection was carried out by tissue blot immunobinding assay (Chen, Buss, & Tolin, 2004; Damayanti & Mahar, 2015; Lin, Hsu, & Hsu, 1990) using specific antibodies for *Tobacco mosaic virus* (TMV), *Cucumber mosaic virus* (CMV), *Chili veinal mottle virus* (ChiVMV), and *Pepper mottle virus* (PepMoV). Virus detection was conducted at the Plant Virology Laboratory, Department of Plant Protection, IPB University.

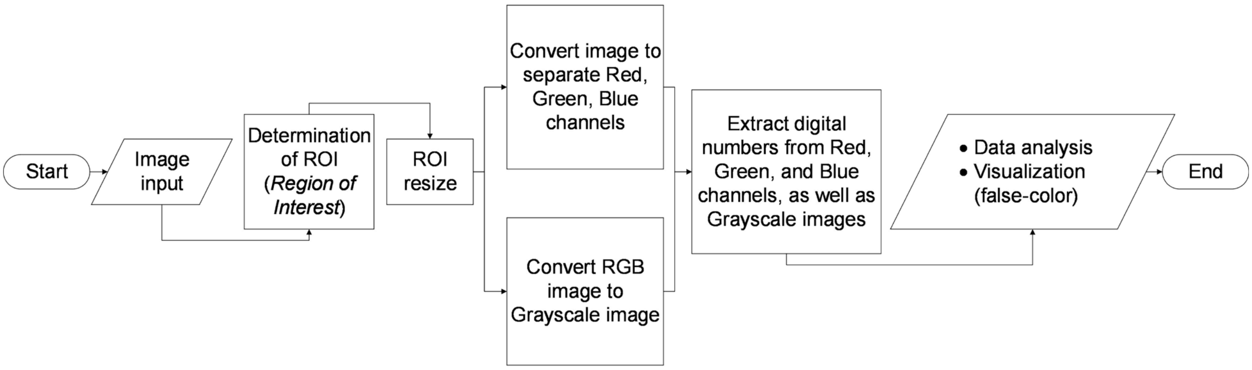

Fig. 1. Digital image processing workflow

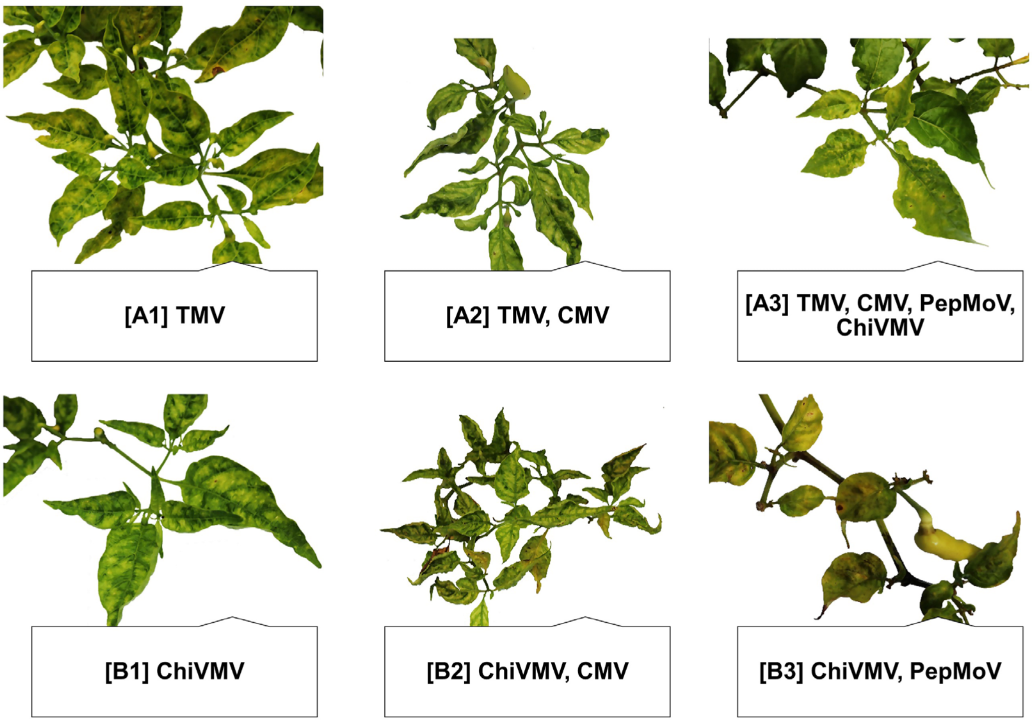

Fig. 2. Variation of virus symptoms infecting cayenne pepper in the field

RESULTS AND DISCUSSION

Main Viruses Infecting Chili Plants

Symptoms of viral infection are very obvious in the field, and not even one chili plant was found to be free from virus symptoms. The main symptoms are yellow mosaic and mottle (Fig. 2). Virus detection using the TBIA method showed that TMV and CMV were the most dominant viruses infecting chili plants in the field, either occurring as a single or mixed infection with other viruses (PepMoV and ChiVMV).

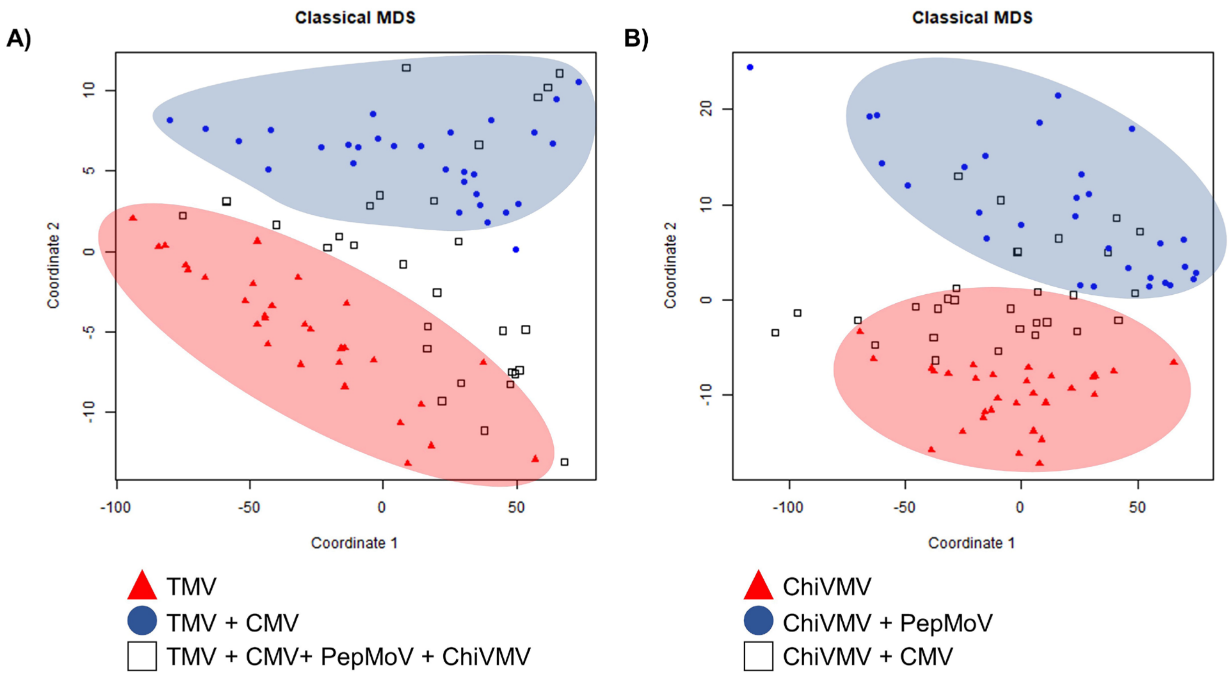

Fig. 3. MDS plot of the digital number images of symptoms in cayenne pepper caused by a single and mixed viral infection. Single infection of TMV, mixed infection of TMV and CMV, and mixed infection of TMV, CMV, PepMoV and ChiVMV (A); Single infection of ChiVMV, mixed infection of ChiVMV and PepMV, and mixed infection of ChiVMV and CMV (B)

Table 1. Digital number average of red (R), green (G), blue (B), and combined [(R+G+B)/3] channels on cayenne pepper plant symptom images

[Images Code] Virus Types	Symptoms Variation (based on …)				
	Visual	Digital Number (average pixel ± sd) on … Channels			
		Red (R)	Green (G)	Blue (B)	Grayscale (combined)
[A1] TMV *	ym	73.043 ± 21.50	89.080 ± 24.90	9.763 ± 2.80	57.295 ± 16.20
[A2] TMV, CMV **	ym	94.788 ± 24.10	115.929 ± 26.10	27.125 ± 6.90	79.281 ± 18.80
[A3] TMV, CMV, Pep-MoV, ChiVMV ***	ym, m	97.756 ± 25.20	119.953 ± 23.20	21.159 ± 8.20	79.623 ± 17.90
[B1] ChiVMV *	ym, m	83.783 ± 18.90	113.957 ± 19.10	17.535 ± 2.60	71.758 ± 13.30
[B2] ChiVMV, CMV **	ym, m	94.940 ± 23.90	110.134 ± 27.00	19.830 ± 4.60	74.968 ± 18.20
[B3] ChiVMV, PepMoV **	ym, m	85.183 ± 34.50	87.579 ± 28.30	14.605 ± 4.10	62.449 ± 22.10

Remarks: * (single virus infection); ** (mixed infection of 2 types of viruses); *** (mixed infection > 2 types of virus); ym (yellow mosaic); m (mottle); sd (standard of deviation)

These results complement to previous studies which reported various types of viruses associated with chili plants in Indonesia, such as *Pepper yellow leaf curl Indonesia virus* (PYLCIV) and *Pepper yellow leaf curl Aceh virus* (PYLCAV) (Hidayat, Haryadi, & Nurhayati, 2009; Kesumawati et al., 2019; Koeda et al., 2022; Pohan et al., 2022), *Polerovirus* (Suastika, Hartono, Nyana, & Natsuaki, 2012), *Potato virus Y* (PVY) (Damiri, 2014), CMV and ChiVMV (Subekti, Hidayat, Nurhayati, & Sujiprihati, 2006), as well as TMV (Taufik, Khaeruni, & Rombe, 2011).

Digital Numbers of RGB Images Approach to Study Variations in Symptoms Severity and Relationships Between Viruses

Virus infection can cause degradation of chlorophyll pigment due to chloroplast damage (Senesi et al., 2022; Soni et al., 2022), resulting in disruption of chlorophyll function in the absorption of sunlight for photosynthesis. The active chlorophyll can be evaluated based on the reflectance of red, green, and blue light (Red-Green-Blue) (Guendouz, Bendada, & Benniou, 2022; Huang et al., 2022) because the chlorophyll in chloroplasts will absorb more red light (630-680 nm) and blue light (450-520 nm); on the contrary, green light (520-600 nm) is absorbed less. This causes the reflectance of red and blue light to be lower than that of green light. With the help of a digital camera sensor that produces a photographic image, the reflectance of the three lights can be assessed (Kitchen & Goulding, 2001). Assessment can be carried out because digital images store numeric or digital numbers as a representation of light radiation captured by the sensor (Shankar, 2017).

In general, there are no significant differences of visual symptoms caused by single or mixed virus infections. However, analysis of the recorded image of symptomatic leaves was able to show different characteristics of digital numbers (Table 1). For example, a single infection of TMV (A1) and mixed infection of TMV and CMV (A2) showed the same yellow mosaic symptom but had different digital numbers. The digital number of single TMV-infected leaf (A1) images was lower than that of mixed TMV and CMV (A2) infection in the red, green, blue, and grayscale channels. Similarly, a single infection of ChiVMV (B1) showed the same symptoms with a mixed infection of ChiVMV and CMV (B2) or ChiVMV and PepMoV (B3), i.e., yellow mosaic and mottle. However, the digital number of the image of leaves infected by ChiVMV and CMV (B2) was higher than those of ChiVMV single infection (B1) and mixed infection of ChiVMV and PepMoV (B3), especially in green, blue, and their combination (grayscale) channels. The higher the digital number of symptomatic leaf images, the greater the amount of red, green, and blue light reflected by the leaf and recorded by the camera sensor. At the same time, it provides an overview of the severity of viral infection due to a decrease in the ability of symptomatic leaves to absorb light, especially the red and blue for photosynthesis.

Characteristics of symptom severity in plants infected by a single virus or different combinations of viruses can be mapped based on MDS analysis. The MDS plot shows that the digital image numbers of A1 and A2 form separate groups. Meanwhile, some of the digital image numbers of A3 form a separate group, and some belong into the same group with A1 and A2 (Fig. 3A). Likewise, the digital image numbers of B1 and B3 form separate groups; while some of the digital image numbers of B2 form separate groups, and some are in the same group with the B3 (Fig. 3B). The MDS plot indicated that digital image numbers of RGB can be used to distinguish the severity of symptoms caused by a single infection from a mixed viral infection.

Not only has the potential to be used for describing variations in the symptoms severity, digital image analysis of symptomatic leaves also has the potential to explain the relationship between viruses that infect the same plants. Interactions between viruses can be divided into three categories, i.e., neutral interactions if the symptoms caused by single and mixed viral infections look the same, and synergistic and antagonistic interactions if there are differences (Moreno & López-Moya, 2020; Tatineni, Alexander, & Qu, 2022; Xu, Ghanim, & Liu, 2022). Other researchers classify interactions between viruses based on their functions, i.e., facilitation (positive interaction) and competition or interference (negative interaction), also known as synergistic and antagonistic interactions, respectively (Alcaide, Rabadán, Moreno-Pérez, & Gómez, 2020). The occurrence of more severe symptoms in a mixed infection compared to a single infection indicated a synergistic interaction, while infection with one virus that suppresses infection of the other virus is an antagonistic interaction (Singhal, Un Nabi, Yadav, & Dubey, 2020).

Interactions between viruses that infect the same plant are generally classified based on observations on fruit weight (Subekti, Hidayat, Nurhayati, & Sujiprihati, 2006); plant height, plant fresh weight, and visual symptoms (Murphy & Bowen, 2006); production of phytohormones (Alazem & Lin, 2015); next-generation sequencing (NGS) and metagenomic analysis (Mascia & Gallitelli, 2016); and viral titers (Singhal, Un Nabi, Yadav, & Dubey, 2020). However, in this study, we propose an RGB digital image analysis method as a new approach to classifying the relationship between these viruses. The results of digital image analysis show that the average digital number in the image of symptoms developed by a mixed infection of TMV and CMV (A2) which is higher than that of TMV (A1) infection is an indication of a synergistic interaction. On the other hand, the average digital number in the image of symptom caused by ChiVMV (B1) infection which is higher than that of mixed ChiVMV and PepMoV (B3) infection is an indication of antagonistic interactions between different virus types (Table 1).

Visualization of the Impact of Virus Infection on Plant

Damage to the leaf area due to a virus infection which can cause a decrease in the ability of the leaves to absorb light, especially red and blue light which is very important in the photosynthesis process, can be identified through the digital image analysis approach. Visualization of symptomatic leaves in the form of false-color images can facilitate human vision to identify leaf areas that have the highest to lowest digital numbers as a representation of the amount of light that is not absorbed (reflected) by the leaves with virus symptoms and recorded by the RGB digital camera sensor. The combined red and blue channel image [(R + B)/2] provides visualization of more contrast between the areas that absorb and reflect red and blue light than the red and blue channel images separately (Fig. 4). Visualization of the results of symptomatic leaf image analysis can help humans to better understand the characteristics of virus symptoms. Leaf areas that reflect more red and blue light as markers of impaired chlorophyll function are easier to identify and study.

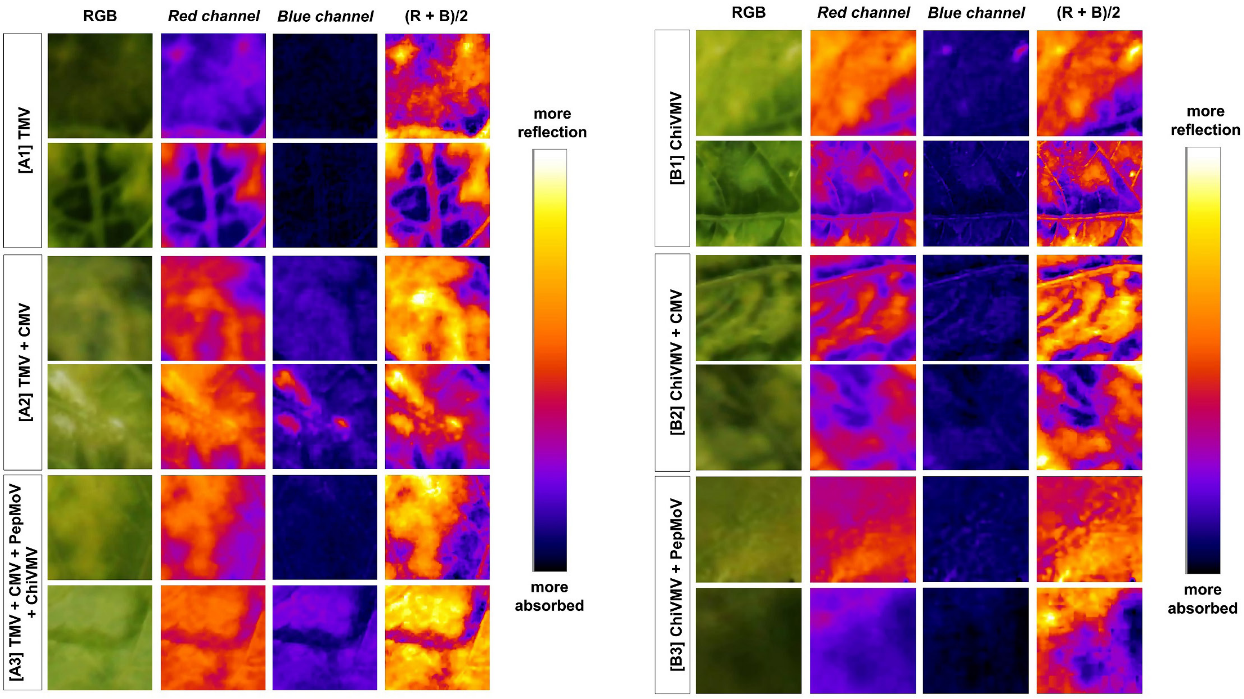

Fig. 4. ROI sample of virus symptoms image in chili pepper (RGB, *Red Green Blue*; *Red channel*, red channel; *Blue channel*, blue channel; R+B /2, a combination of red and blue channels)

CONCLUSION

Different virus infections often cause similar visual symptoms but can be distinguished based on RGB image analysis. Furthermore, RGB image analysis has the potential to distinguish the symptoms caused by single and mixed viral infections. In addition, it can also prove synergistic and antagonistic interactions between different types of viruses that infect plants, and it can visualize the impact of viral infections on leaves. This method needs to be further validated through testing under controlled conditions, such as inoculating a number of plants with a predetermined type of virus.

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