

## Updates on hosts and distribution of pepper yellow leaf curl Indonesia virus and squash leaf curl China virus in Central Java Province, Indonesia

### Pembaruan inang dan sebaran *pepper yellow leaf curl Indonesia virus* dan *squash leaf curl China virus* di Provinsi Jawa Tengah, Indonesia

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##### ABSTRACT

Chili pepper, tomato, and squash cultivated in Magelang Regency, Central Java Province, Indonesia showed severe viral symptoms. Three samples were taken then molecularly tested against begomovirus, potyvirus, tobamovirus, and polerovirus. However, PCR test confirmed only begomovirus infections. BLAST analysis concluded that the chili pepper and tomato isolates were pepper yellow leaf curl Indonesia virus (PepYLCIV) while the squash isolate was squash leaf curl China virus (SLCCNV). The 552 bp partial AV1 gene sequences of the three isolates were given NCBI GenBank acc nos. OR924278-80. PepYLCIV OR924279 and OR924280 formed a subgroup with LC542629 from Bali in the phylogenetic tree constructed using MEGA11, and shared 95.8 – 96.7% identities at nucleotide (nt) and 98.4 – 99.5% at amino acid (aa) levels according to Sequence Demarcation Tool v1.2 software. Meanwhile, SLCCNV OR924278 was clustered, and shared 95.8 – 96.7% nt and 98.4 – 99.5% aa identities with three Malaysian isolates (MW248685, MW248687, and MW248689). Different plant species inoculated with SLCCNV OR924278 remained symptomless up to four weeks observation, suggesting that the isolate is not mechanically transmissible. This study contributed additional knowledge on molecular variation while expanding hosts and distribution of PepYLCIV and SLCCNV in Central Java Province.

##### ABSTRAK

Cabai, tomat, dan labu yang dibudidayakan di Kabupaten Magelang, Provinsi Jawa Tengah, Indonesia menunjukkan gejala virus yang parah. Tiga sampel diambil kemudian diuji secara molekuler terhadap begomovirus, potyvirus, tobamovirus, dan polerovirus. Namun, tes PCR hanya mengkonfirmasi adanya infeksi begomovirus. Analisis BLAST menyimpulkan bahwa isolat cabai dan tomat merupakan pepper yellow leaf curl Indonesia virus (PepYLCIV) sedangkan isolat labu merupakan squash leaf curl China virus (SLCCNV). Urutan 552 bp parsial gen AV1 dari ketiga isolat diberikan NCBI GenBank acc nos. OR924278-80. PepYLCIV OR924279 dan OR924280 membentuk subgrup dengan LC542629 dari Bali dalam pohon filogenetik yang dibuat menggunakan MEGA11, dan memiliki 95.8 – 96.7% identitas pada nukleotida (nt) dan 98.4 – 99.5% pada asam amino (aa) menurut Sequence Demarcation Tool v1.2 software. Sementara itu, SLCCNV OR924278 dikelompokkan, dan berbagi 95.8 – 96.7% nt dan 98.4 – 99.5% aa identitas dengan tiga isolat Malaysia (MW248685, MW248687, dan MW248689). Spesies tanaman berbeda yang diinokulasi dengan SLCCNV OR924278 tetap tidak menunjukkan gejala apapun hingga empat minggu pengamatan, menunjukkan bahwa isolat tersebut tidak dapat ditularkan secara mekanis. Penelitian ini memberikan kontribusi pengetahuan tambahan mengenai variasi molekuler sekaligus memperluas inang dan distribusi PepYLCIV dan SLCCNV di Provinsi Jawa Tengah.

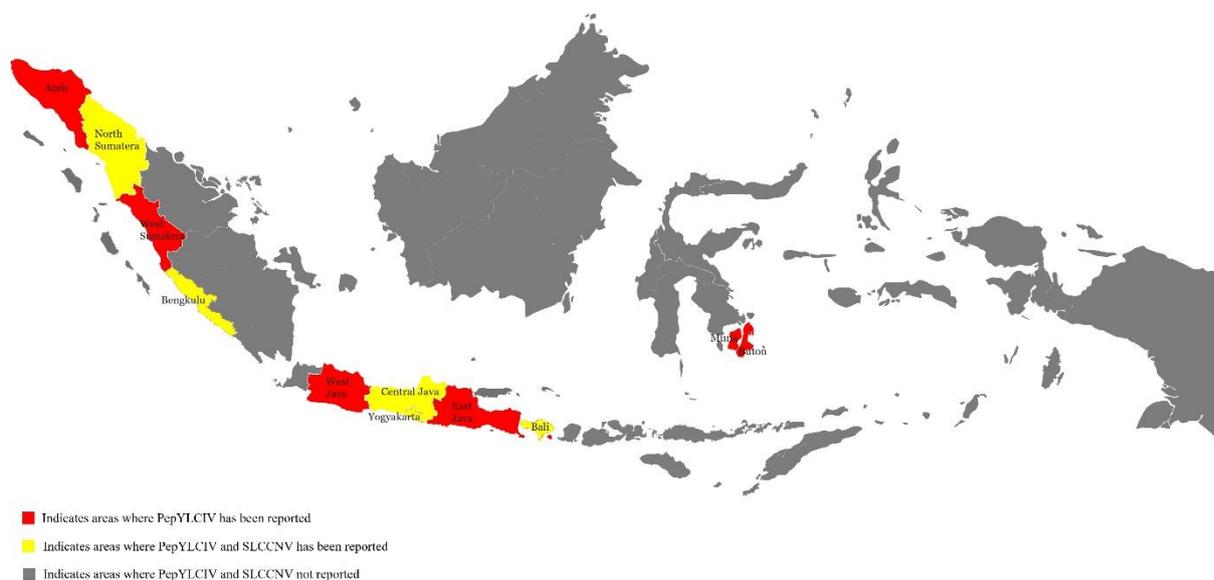
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## INTRODUCTION

Members of Solanaceae family: chili pepper (*Capsicum annuum*) and tomato (*Solanum lycopersicum*), and Cucurbitaceae family: squash (*Cucurbita pepo*) are among the most valuable and widely planted horticultural commodities in many regions of Indonesia. Tomato cultivation in the country is considered profitable thus productivity has been increased from 15.31 in 2016 to 18.93 ton/ha in 2020 (FAO, 2024). Combination of bio-fertilizer was suggested for further improvement (Khairi et al., 2023) but it is actually very challenging due to other limitations. One of major constraints in the cultivation of chili pepper, tomato, and squash in the country is yellowing disease caused by begomoviruses (Santosa & Somowiyarjo, 2023; Wahyono et al., 2023). Species of *Begomovirus* have monopartite or bipartite single stranded DNA genomes, and transmitted by whitefly (*Bemisia tabaci*) and some also by seeds (Brown et al., 2015).

Pepper yellow leaf curl Indonesia virus (PepYLCIV) was recently reported to be the dominant Begomovirus species in Java Island by infecting 329 of the collected 360 chili pepper samples (91%). Tomato yellow leaf curl Kanchanaburi virus (TYLCKaV) and tomato leaf curl New Delhi virus (ToLCNDV) were also detected with small infection rates of 11% and 3%, respectively, in the same observation (Wahyono et al., 2023). Other recent papers confirmed PepYLCIV presence in chili pepper in Bali (Selangga et al., 2021), Nusa Penida (Selangga & Listihani, 2021), Sumatera (Sipriyadi et al., 2022), Buton, and Muna (Widodo et al., 2023) Islands thus revealed the truly expansive nature of the seedborne virus (Fadhila et al., 2020).



**Figure 1.** Current distribution of pepper yellow leaf curl Indonesia virus (PepYLCIV) and squash leaf curl China virus (SLCCNV) in Indonesia.

Another *Begomovirus* species, squash leaf curl China virus (SLCCNV), is rapidly spreading among cucurbits in Indonesia. The virus might be first recorded infecting cucumber (*Cucumis sativus*) in Bali Island (Wiratama et al., 2015). In Java Island, SLCCNV was found in melon (*Cucumis melo*) in Sleman and Bantul Regencies of Special Region of Yogyakarta, and in watermelon (*Citrullus lanatus*) in Purworejo Regency of Central Java Province (Subiastuti et al., 2019). Expansion of the virus to Sumatera Island has been documented in cucumber and squash in North Sumatra (Kesumawati et al., 2020) and Bengkulu Provinces (Sutrawati et al., 2022). SLCCNV was recently shown to also naturally infect tomato (Qiu et al., 2022), further complicating management of begomoviruses. The current distribution of PepYLCIV and SLCCNV in Indonesia is presented in Figure 1. However, the real span of SLCCNV in the country needs to be well understood as the virus was reported to be widespread in China (Wu et al., 2020), India (Venkataravanappa et al., 2021b), and South East Asian countries such as Malaysia (Chen et al., 2021), Thailand (Piyatasee & Hongprayoon., 2021), and the Philippines (Neoh et al., 2023).

Ngablak Subdistrict is one of the production cores that supply horticultural commodities to Magelang Regency and surrounding areas in Central Java Province. Our investigation revealed severe viral symptoms that may hamper cultivation of chili pepper, tomato, and squash there but the exact virus species was unknown. As part of the subdistrict's plant virus survey, samples were gathered and molecularly examined. The gathered data might potentially contribute significantly to our understanding of the distribution and phylogeny of plant viruses in Indonesia.

## MATERIALS & METHODS

### *Samples collection*

Viral symptomatic chili pepper, tomato, and squash samples were taken from fields close to each other in Ngablak Subdistrict, Magelang Regency, Central Java Province, Indonesia during a visit on 16 September 2023. Samples were collected into separate plastic bags then brought to Plant Virology Room, Universitas Gadjah Mada and stored at -4 °C until further testing several days later.

### *Nucleic acid extraction and PCR*

Total DNA and RNA were extracted from the collected samples using 'Genomic DNA Mini Kit (Plant)' and 'Total RNA Mini Kit (Plant)' (Geneaid Biotech Ltd., Taiwan), following standard procedures for respective kits. Immediately after RNA extraction, cDNA was synthesized using ReverTra Ace kit (Toyobo, Japan) in a reaction volume of 10  $\mu\text{L}$ : 2  $\mu\text{L}$  RNA, 2  $\mu\text{L}$  5x RT Buffer, 1  $\mu\text{L}$  dNTP, 0.5  $\mu\text{L}$  (10 pmol  $\mu\text{L}^{-1}$ ) Oligo(dT) primer, 0.5  $\mu\text{L}$  RNase inhibitor, 0.5  $\mu\text{L}$  ReverTraAce<sup>®</sup>, and 3.5  $\mu\text{L}$  nuclease-free water. The reverse transcription was programmed at 42 °C for 20 min and then 99 °C for 5 min.

The subsequent standard PCRs were performed using universal primer pairs for detection of some genera of plant virus commonly found in *Solanaceae* and *Cucurbitaceae* (Table 1), each PCR reaction was in a volume of 40  $\mu\text{L}$ : 20  $\mu\text{L}$  of MyTaq HS Red Mix (Bioline, Germany), 2  $\mu\text{L}$  (10 pmol/ $\mu\text{L}$ ) each of reverse primer and forward primer, 4  $\mu\text{L}$  of DNA or cDNA, and 12  $\mu\text{L}$  of PCR-grade water. The thermal cycler program was 95 °C for 3 min as a pre-denaturation, 35 cycles of denaturation at 95 °C for 1 min, annealing (see Table 1), and elongation at 72 °C for 1 min, followed by 72 °C for 10 min as a final elongation.

The appearance of the targeted band with specific size on 1% agarose gel stained with Florosafe DNA Staining (1st BASE, Malaysia) was observed under a UV transilluminator (Optima Inc., Japan) after electrophoresis for 50 min at 100 V. Positive PCR products were submitted to 1st BASE (Malaysia) to be sequenced bidirectionally using Sanger method. Isolates identification against NCBI GenBank database was determined using nucleotide BLAST online software (<https://blast.ncbi.nlm.nih.gov>). Nucleotide sequences of the novel isolates were then deposited to NCBI GenBank.

### *Construction of phylogenetic tree, and identity study*

Sequences of isolates in NCBI GenBank selected based on different percentage identities, origins, and hosts were aligned with and then trimmed according to the lengths of sequences of the newly obtained isolates using ClustalW suits in MEGA11 software (Tamura et al., 2021). Phylogenetic tree was built using MEGA11 with Maximum Likelihood (ML) statistical method and Tamura-3 as the best suited parameter model (Tamura, 1992). Statistical significance of each branch was tested with 1000 bootstrap replicates. The nucleotide (nt) and amino acid (aa) percentage identities among tested isolates were calculated using Sequence Demarcation Tool (SDT v1.2) software (Muhire et al., 2014).

### *Hosts indexing*

To confirm RT-PCR results, ability of the virus to be transmitted mechanically was tested in an inoculation experiment with different plant species: *Cucurbitaceae*: squash, cucumber, melon, and bitter melon (*Momordica charantia*); *Solanaceae*: chili pepper and tomato; *Fabaceae*: yardlong bean (*Vigna unguiculata* subsp. *sesquipedalis*), and *Amaranthaceae*: *Chenopodium amaranticolor*. The symptomatic squash leaves collected in Ngablak Subdistrict and tested positive for SLCCNV were ground thoroughly using a mortar and pestle. During the grinding process, 5 ml 0.01 M potassium phosphate buffer pH 7.0 per 1 g leaf tissue was gradually added to the homogenate. The sap was filtered

through cheese cloth to remove large debris and then put in a glass Petri dish. Leaves of the tested plants were dusted with carborundum (400 mesh) before rubbed with the sap. Control plants were rubbed solely using buffer. Ten minutes after mechanical inoculation, the plants were rinsed with distilled water to remove buffer and carborundum. Plants were maintained at 25 – 27 °C and observed for symptoms expression for a period of four to five weeks (Santosa et al., 2018).

**Table 1.** List of universal primers used in this study for detection of different genera of plant virus

Virus	Target gene	Sequence (5'-3')	Annealing temp. and time	Expected product size (bp)	References
Begomovirus	Partial CP	F-CCNMRDGGHTGTGARGGNCC	55 °C for 30 s	580	Revill et al., 2003
		R-SVDGCRTGVGTRCANGCCAT			
Potyvirus	Partial CP + 3'-end	F-CCGGAATTCATGRTITGGTGYATIGAIAYGG	56 °C for 1 min	600 - 800	Langeveld et al., 1991
		R-GGATCCCGGGTTTTTTTTTTTTTTTTTV			
Tobamovirus	Partial RdRp	F-GARTAYSCIGCIYTICARAC	48 °C for 1 min	568	Dovas et al., 2004
		R-BGCYTCRAARTCCA			
Polerovirus	Partial CP	F-CCCGGATCCATGAATACGGYCGCTAG	55 °C for 1 min	600	Cheewachaiwit et al., 2017
		R-TTCCTGCAGCTATTTCCGGTTCTGGA			

## RESULTS & DISCUSSIONS

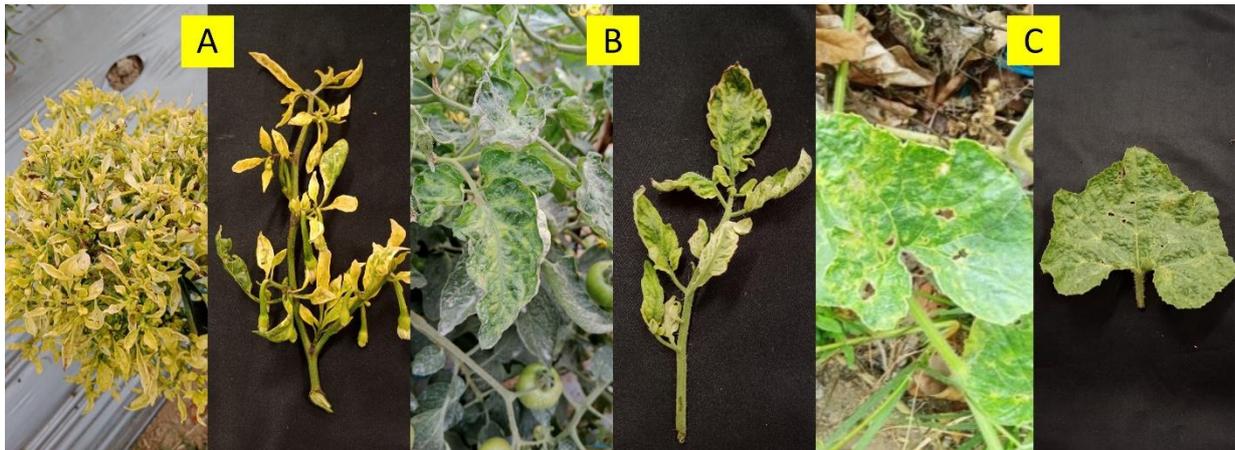
### *Molecular identification of virus species*

Chili pepper, tomato, and squash samples were all positive for begomovirus infection by forming a single 580 bp band on agarose gel upon PCR using universal primer pairs. PCR assay also showed that the three samples were negative for infection by potyvirus, tobamovirus, and polerovirus. The three begomovirus isolates were sequenced. BLAST analysis on the obtained sequences indicated that squash sample was infected by SLCCNV while chili pepper and tomato samples by PepYLCIV. GenBank acc. no. OR924278 was assigned to the new SLCCNV isolate, and OR924279 and OR924280 to the two new PepYLCIV isolates.

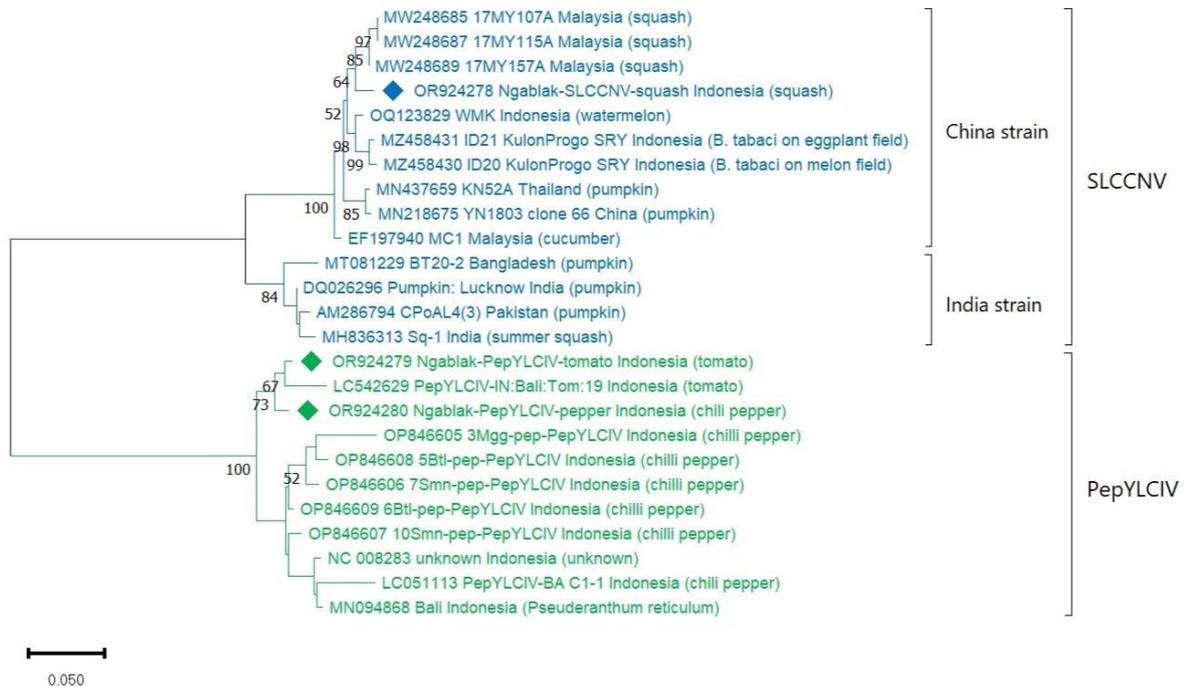
The PepYLCIV infected chili pepper growth was severely stunted, and all of its leaves showed chlorosis completely. Tomato plant with PepYLCIV appeared to grow relatively well and produced decent fruits, but its leaves displayed rather severe mosaic and chlorosis. Systemic mosaic and chlorosis appeared on leaves of squash infected by SLCCNV (Figure 2). Begomoviruses have caused significant problems for plant farmers due to their fast dissemination via the vector whitefly and, in certain cases, seeds. In line with that, different horticultural species cultivated close to each other in a relatively small Ngablak Subdistrict were found to be infected by two distinct species of *Begomovirus*: PepYLCIV and SLCCNV. All of the tested samples exhibited severe symptoms such as chlorosis and stunting which were likely caused by early infections, further emphasizing the importance of healthy seedlings preparation. Vector management in the surveyed fields was observed to be actually quite well but still highly depend on routine application of synthetic chemicals.

Although current identification works are focused mostly on chili pepper, PepYLCIV has been known to also infect tomato and *Ageratum conyzoides* weed in West Java and Bali Provinces since a long time ago (Tsai et al., 2006; Sakata et al., 2008; Neriya et al., 2020). Results of the current study did not change the distribution of PepYLCIV and SLCCNV since both viruses had been reported in Central Java Province (Figure 1). However, tomato as PepYLCIV host in Ngablak, Magelang Regency was recorded for the first time in Central Java Province. SLCCNV hosts in Central Java Province were also expanded to include squash in Magelang Regency as previously it was only identified in watermelon in Purworejo Regency (Subiastuti et al., 2019). Therefore, this study has also complemented an earlier report on the presence of RNA

viruses from *Potyvirus* and *Carlavirus* genera in the neighboring Kajoran Subdistrict of Magelang Regency (Santosa et al., 2024).



**Figure 2.** Samples with clear viral symptoms collected from fields in Ngablak, Magelang Regency, Central Java Province of Indonesia. A. Chili pepper infected with PepYLCIV isolate no. OR924280 showing complete chlorosis and stunted growth; B. Tomato with PepYLCIV isolate no. OR924279 infection showing severe mosaic and chlorosis on leaves; C. Squash infected with SLCCNV isolate no. OR924278 exhibiting systemic mosaic and chlorosis on leaves.



**Figure 3.** Phylogenetic tree was constructed using MEGA11 software based on partial sequences of AV1 gene of begomovirus (552 bp) encoding coat protein. Tamura-3 was implemented as a parameter model in Maximum Likelihood (ML) statistical method with 1000 bootstrap replicates to statistically test significance of each branch. Only > 50% bootstrap values were shown. Diamond signs indicated the two new pepper yellow leaf curl Indonesia virus (PepYLCIV) and one squash leaf curl China virus (SLCCNV) isolates described in this study.

Phylogenetic study

The obtained sequences of three isolates were 552 bp in length, covering partial AV1 gene that encodes coat protein. The new SLCCNV isolate was aligned and compared with nine China strain and four India strain isolates while the two new PepYLCIV isolates were tested against nine other isolates in GenBank. Phylogenetic tree demonstrated that OR924279 and OR924280 isolates were very closely related, and shared a common basal node with LC542629, a PepYLCIV tomato isolate from Bali, Indonesia. Meanwhile, five isolates from Magelang, Bantul, and Sleman Regencies (OP846605 – OP846609) formed a separate subgroup with an isolate from North Sumatera Province (LC051113) despite their geographical proximity to Ngablak (Figure 3). In SLCCNV tree, the OR924278 isolate together with three Malaysian squash isolates (MW248685, MW248687, and MW248689) constituted a subgroup which distinct to another subgroup consists of three Indonesian isolates (MZ458430, MZ458431, and OQ123829). The OR924279 and OR924280 isolates were shown to be members of China strain which genetically distant to India strain (Figure 3).

SLCCNV phylogeny based on full sequence of DNA-A divides isolates according to their origins into China and India strains (Venkataravanappa et al., 2021a). However, analysis on the partial AV1 gene, which encodes coat protein, in this study was indicated to be enough to separate both SLCCNV strains (Figure 3). SLCCNV isolate no. OR924278 as well as isolates from China and South East Asian countries: Indonesia, Thailand, and Malaysia belong to the China strain in the constructed phylogenetic tree. The phylogenetic tree also revealed that SLCCNV isolates in Indonesia were genetically diverse as OR924278 related closer to three Malaysian isolates than other isolates from Indonesia. Thus, suggesting a potential cross-border transmission. Similarly, the two novel PepYLCIV isolates were clustered with isolates from Bali Island instead of with other isolates from Magelang as well as those from nearby Sleman and Bantul Regencies in Java Island.

Identity studies

The compared 11 PepYLCIV isolates had 90.6 – 98.9% and 96.7 – 100% identities at nt and aa levels, respectively. The new PepYLCIV isolates (OR924279 and OR924280) shared the highest percentage identities with LC542629: 95.8 – 96.7% at nt and 98.4 – 99.5% at aa levels. Fourteen SLCCNV isolates tested in this study showed 88 – 100% nt and 95.7 – 100% aa identities among them. OR924278 isolate shared 95.8 – 96.7% nt and 98.4 – 99.5% aa identities with the three Malaysian isolates (MW248685, MW248687, and MW248689) (Figure 4). Results of percentage identity analysis further supported the notion of relatively high diversity among Indonesian PepYLCIV and SLCCNV isolates.

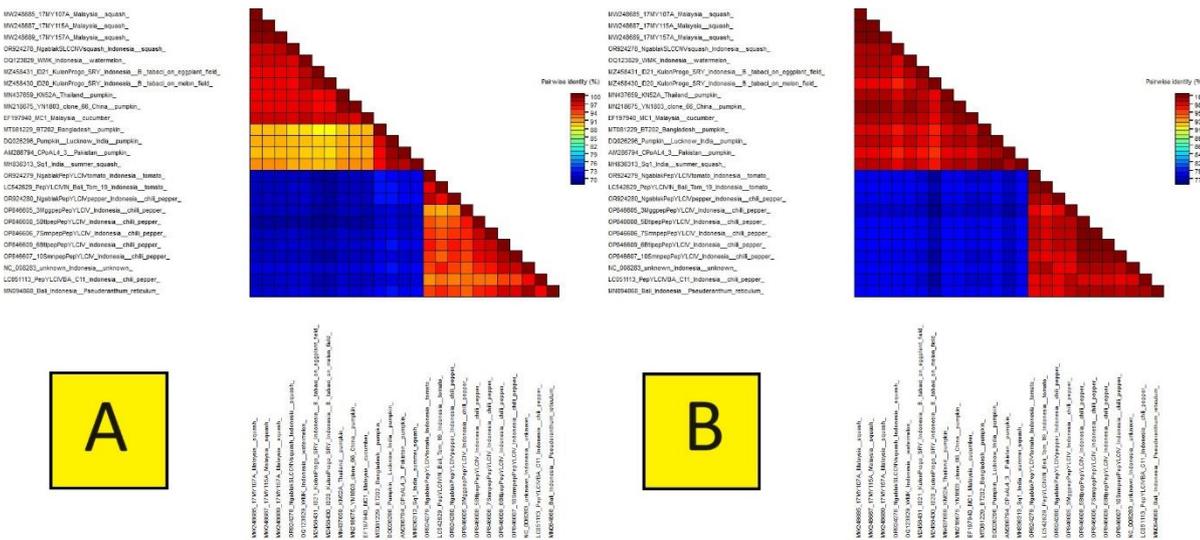
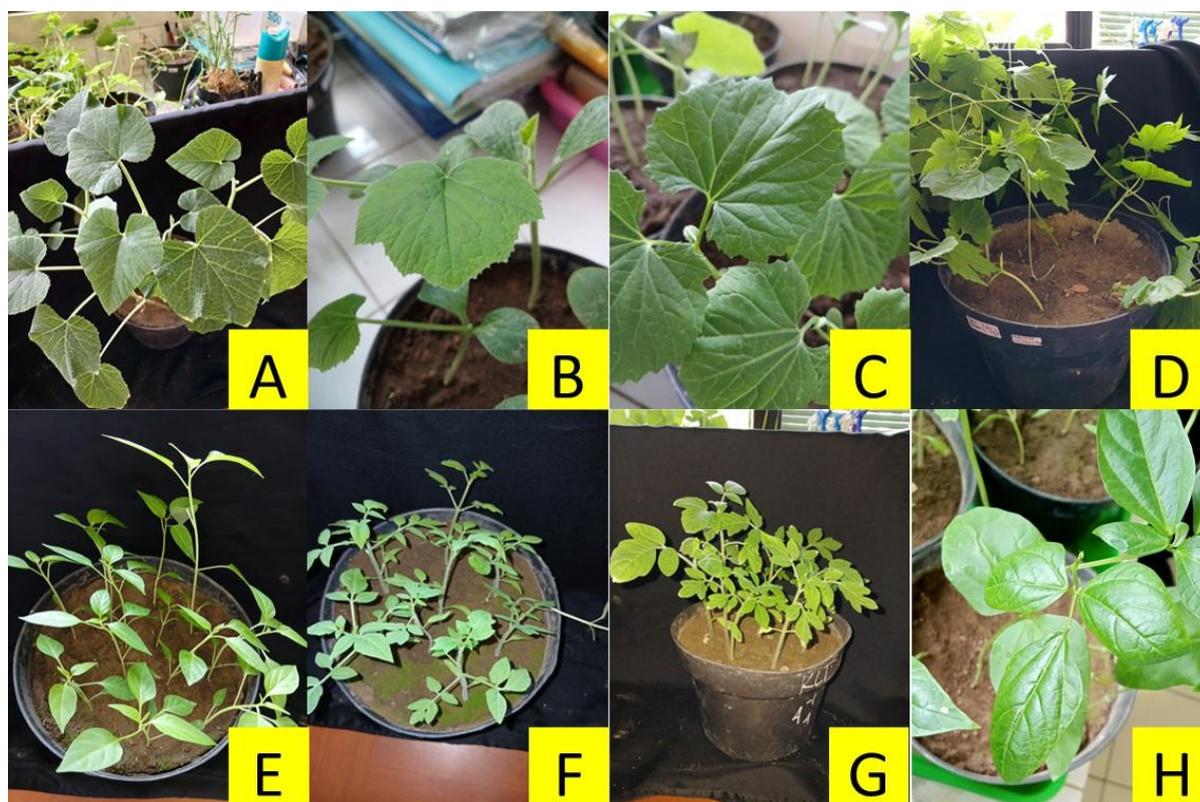


Figure 4. Percentage identities among the compared PepYLCIV and SLCCNV isolates at A. nucleotide and B. amino acid levels were estimated using Sequence Demarcation Tool (SDT) v1.2.

### Host indexing

Up to four weeks after inoculation, the inoculated and control plants showed normal growth, and none of the inoculated plants exhibited clear viral symptoms when compared to control plants (Figure 5). This was a strong indication that the virus infecting squash was not mechanically transmissible. The results also supported the results of molecular analysis that most likely the tested samples each have only a single infection of either PepYLCIV or SLCCNV. Begomoviruses are mainly transmitted by whitefly vector. Some are also seed-borne, and only few are mechanically transmissible with difficulties (Rojas et al., 2005). PepYLCIV transmission to healthy chili pepper plants could be experimentally established using *Agrobacterium* inoculation technique (Agroinoculation) (Koeda et al., 2018). Furthermore, PepYLCIV presence in embryo of chili pepper seeds had been proven thus the virus is potentially seed transmissible (Fadhila et al., 2020), which may explain its rapid expansion across Indonesia. Similarly, the transmission of SLCCNV in China is facilitated by *B. tabaci*, with a 95% transmission rate to melon plants (Wu et al., 2020). Subsequently, another research confirmed that SLCCNV is transmitted by whitefly, but is not seed-borne (Venkataravanappa et al., 2021b).



**Figure 5.** Different tested plant species did not exhibit visible viral symptoms four weeks post inoculation with SLCCNV isolate no. OR924278. *Cucurbitaceae*: A. squash, B. cucumber, C. melon, D. bitter gourd; *Solanaceae*: E. chili pepper, F. tomato cultivar Gustavi, G. tomato cultivar Servo; *Fabaceae*: H. yardlong bean.

### CONCLUSION

This research contributed to the understanding of molecular variation, hosts, and distribution of PepYLCIV and SLCCNV isolates in Central Java Province, Indonesia. The close relationship between PepYLCIV Ngablak and Bali isolates as well as the distinct grouping of SLCCNV Ngablak with Malaysian isolates indicated complex patterns of viral transmission and evolution that may not strictly follow geographical boundaries. The phylogenetic analysis emphasizes the necessity for comprehensive sequencing of Indonesian isolates that may lead to suitable management in the country. Moving forward, the comprehensive genomic analysis of isolates from various plant hosts is essential to determine the distinct characteristics of PepYLCIV and SLCCNV strains.

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