Agr. Nat. Resour. 58 (2024) 419–426

AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Detection of mixed infections among three begomoviruses in pumpkin using multiplex polymerase chain reaction technique

Salit Supakitthanakorn[a,*], Thanakit Suksodkhiaw[a], Sunittra Aupanun[b], Anyamanee Auvuchanon[c]

[a] *Department of Plant Pathology, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand*

[b] *Department of Entomology, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand*

[c] *Department of Horticulture, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand*

Article Info

Article history:
Received 27 March 2024
Revised 24 May 2024
Accepted 11 June 2024
Available online 30 August 2024

Keywords:
Cucurbita moschata,
Cucurbits,
ToLCNDV,
TYLCKaV,
TYLCTHV

Abstract

Importance of the Work: Begomoviruses limit pumpkin cultivation. Precise virus detection is essential for understanding disease etiology and planning disease management strategies.

Objectives: To investigate multiple infections of three begomoviruses in pumpkins using multiplex polymerase chain reaction (PCR) assay.

Materials and Methods: Total DNA was extracted from pumpkin leaves collected in Nakhon Pathom and Chiang Mai provinces, Thailand. ToLCNDV-, TYLCKaV- and TYLCTHV-specific primers were used to optimize the multiplex PCR amplification. Sensitivity and specificity of the multiplex PCR assay were assessed. Then, the assay was performed to investigate mixed *Begomovirus* infections in pumpkins.

Results: The multiplex PCR assay successfully detected amplicons of ToLCNDV (400 bp), TYLCTHV (770 bp) and TYLCKaV (831 bp). The limit of detection was 1×10^{-4} diluted DNA, with no cross-reactivity among the three begomoviruses observed. The investigation of mixed infections revealed that ToLCNDV and TYLCTHV were the most frequently co-infected (76.00%), followed by multiple infections of all three begomoviruses (8.00%). Single infections of TYLCKaV, TYLCTHV and ToLCNDV were 6.00%, 4.00% and 2.00%, respectively. In summary, multiple *Begomovirus* infections were common in pumpkins.

Main finding: Multiplex PCR assay detected mixed infections of begomoviruses in pumpkins for the first time. ToLCNDV and TYLCTHV were commonly detected together and could serve as targets for breeding resistant pumpkin cultivars to enhance disease control. Therefore, multiplex PCR could be used routinely to investigate mixed infections of begomoviruses in pumpkins.

* Corresponding author.
E-mail address: fagrslsu@ku.ac.th (S. Supakitthanakorn)

https://doi.org/10.34044/j.anres.2024.58.4.01

Introduction

Pumpkin (*Cucurbita moschata* Duch.) is an important vegetable crop in tropical and subtropical regions that is cultivated throughout Thailand (Ketsakul et al., 2020). Pumpkin fruits are rich sources of alpha- and beta-carotenes, along with other carotenoids, providing a nutritious and health-beneficial vegetable for consumption (Usha et al., 2010). The production of pumpkins faces challenges, particularly diseases caused by viruses. For example, among plant viruses, members of the genus *Begomovirus* are considered the most devastating viruses affecting cucurbits (Jaiswal et al., 2012; Radouane et al., 2021).

Begomovirus, belonging to the family *Geminiviridae*, is the largest genus of plant-infecting viruses, containing 388 species reported by the International Committee on Taxonomy of Viruses (Fiallo-Olivé et al., 2021). The virus genome is single stranded, closed and circular DNA encapsidated in non-enveloped, icosahedral, twinned particles; there are two types of begomoviruses, consisting of a monopartite genome called DNA-A and those with a bipartite genome referred to as DNA-A and DNA-B (Nawaz-ul-Rehman and Fauquet, 2009).

Begomoviruses are transmitted by the whitefly *Bemisia tabaci* cryptic species complex, belonging to the family *Aleyrodidae*, order *Hemiptera*, in a persistent, circulative, but non-propagative manner (Rosen et al., 2015). Globally, whiteflies have long been economically important agricultural pests, transmitting more than 300 known plant viruses to over 1,000 plant species worldwide (Li et al., 2021). Furthermore, warm (25–30°C) and humid (70% relative humidity) conditions favor the multiplication of the vector whitefly, leading to increased disease severity and loss of production (Nagendran et al., 2017; Wu et al., 2023).

Many begomoviruses are of major importance to cucurbitaceous crops in the tropics and subtropics of Asia, Northern Africa and Southern Europe and can cause yield losses of up to 100% (Kenyon et al., 2014). Typical characteristic symptoms caused by begomoviruses in cucurbits are chlorotic mosaic, green-yellow mosaic/mottle, interveinal yellowing, yellow spots, and vein swelling and mottling accompanied by leaf curling and stunted plant growth (Inoue-Nagata et al., 2016).

Mixed infection refers to the coexistence of more than one virus (different or related viruses) within a single host plant, resulting in varied symptoms simultaneously. The presence of multiple viruses often complicates the understanding of disease etiology (Singhal et al., 2020). In cases of mixed virus infections, viruses may interact with each other in various ways, ranging from neutralism to synergism to antagonism. These interactions directly impact the host plant by either increasing (synergism) or reducing (antagonism) symptom severities (Alazem et al., 2023).

Among various viral detection techniques, polymerase chain reaction (PCR)—used to amplify viral gene segments using sequence-specific primers—is considered the most accurate, highly sensitive and technically facile technique (Roy et al., 2005). However, single PCR can detect only one target, which may not be suitable for detecting mixed infections of viruses (Deb et al., 2023). Reductions in the time and cost of detection have been main objectives pursued in recent years to increase the feasibility of routine use. Therefore, techniques that can simultaneously detect several viruses in a single assay with high sensitivity and specificity are strongly recommended (Pallas et al., 2018).

Multiplex PCR is a technique for amplifying several targets in a single assay through the combination of individual specific primers, which reduces the cost and time required for disease surveillance, making it more feasible for routine monitoring and large-scale application (Chun et al., 2007). This technique successfully detected 10 wheat-infecting viruses (Deb et al., 2023), 3 lily-infecting viruses (Xu and Ming, 2022) and 7 cucurbit-infecting viruses (Kwon et al., 2014). Although several viruses can concurrently infect pumpkins, there remains a deficiency in available information of mixed infections, especially for begomoviruses. Therefore, the current study aimed to detect mixed infections of three begomoviruses in pumpkins, specifically ToLCNDV, TYLCKaV and TYLCTHV, using a newly improved multiplex PCR assay to clarify whether single or mixed infections and their related symptoms could be used as a guideline for field inspection. Additionally, the dominant virus could be a target for further breeding of virus-resistant pumpkin cultivars.

Materials and Methods

Sample collection and DNA extraction

Pumpkin leaf samples were collected in 2023 from fields in Nakhon Pathom province, Thailand at the Department of Horticulture, Faculty of Agriculture at Kamphaeng Saen (14°01’41.7”N 99°58’17.2”E), at the Tropical Vegetable Research Center (14°01’58.8”N 99°57’51.1”E) and in Chiang Mai province, Thailand (18°26’14.3”N 98°41’58.1”E,

18°42'01.2"N 98°54'31.2"E and 18°51'24.6"N 98°45'35.9"E). Total DNA samples were extracted from the pumpkin leaves using a GF-1 Plant DNA Extraction Kit (Vivantis; Selangor Darul Ehsan, Malaysia) according to the manufacturer's instruction. The quality and quantity of extracted DNA were determined using gel electrophoresis and a NanoDrop 2000c spectrophotometer (Thermo Fisher; Waltham, MA, USA), respectively. The DNA was kept at -20°C until use.

Polymerase chain reaction detection

The single PCR detection was performed using 2X PCR SuperMix (Bio-Helix, New Taipei City, Taiwan). In total, 25 µL of PCR cocktail was used, made up of 12.5 µL of PCR master mix, 1 µL (5 µM) of forward primer, 1 µL (5 µM) of reverse primer (Table 1) and 1 µL of target DNA, with DEPC-treated water added to make up the final volume. The PCR reaction was set at 94°C for 4 min, 40 cycles at 94°C for 30 s, 55–65°C for 30 s and 72°C for 45 s, followed by a final extension at 72°C for 7 min. The PCR products were visualized in 1.5% agarose gel electrophoresis staining with RedSafe™ Nucleic Acid Staining Solution (iNiTron; Gyeonggi-do, Republic of South Korea).

The PCR products were purified using PCR Clean-Up and Gel Extraction Kits (Bio-Helix; New Taipei City, Taiwan) according to the manufacturer's instructions and then analyzed using fluorescent dye-terminator sequencing on ABI Prism™ 3730xl DNA sequencers (Applied Biosystems; Foster City, CA, USA). The obtained sequences were aligned using Molecular Evolutionary Genetics Analysis (MEGA) version X (Kumar et al., 2018), and subsequently analyzed using the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Optimization of multiplex polymerase chain reaction assay

The multiplex PCR detection was performed using 2X PCR SuperMix (Bio-Helix; New Taipei City, Taiwan). In total, 25 µL of PCR cocktail was used, made up of 12.5 µL of PCR master mix, 0.5 µL (2.5 µM) of each of the ToLCNDV, TYLCKaV and TYLCTHV-specific forward primers, 0.5 µL (2.5 µM) of each of the ToLCNDV, TYLCKaV and TYLCTHV-specific reverse primers (Table 1) and 1 µL of target DNA, with DEPC-treated water added to make up the final volume. The PCR reaction was set at 94°C for 4 min, 40 cycles at 94°C for 30 s, 50, 55, 60 and 65°C for 30 s and 72°C for 45 s, followed by a final extension at 72°C for 7 min. PCR products were visualized in 2.0% agarose gel electrophoresis staining using RedSafe™ Nucleic Acid Staining Solution (iNiTron; Gyeonggi-do, Republic of South Korea).

Specificity and sensitivity assays

To determine the possible cross-reactivity among begomoviruses, the mixed DNA of three viruses and DNA of individual viruses were used as positive controls, and the DNA from a healthy pumpkin was used as a negative control. The multiplex PCR reaction was performed under optimal conditions and the PCR amplicons were visualized using agarose gel electrophoresis.

To evaluate the limit of detection (LOD) of the multiplex PCR assay, DNA was prepared using a 10-fold serial dilution method (1×10^{0} to 1×10^{-10}) and the DNA concentration was measured using the NanoDrop 2000c spectrophotometer. Then, the multiplex PCR assay was performed under optimal conditions and the PCR amplicons were visualized using agarose gel electrophoresis.

Evaluation of multiplex polymerase chain reaction assay

The performance was investigated of multiplex PCR assay for detecting multiple infections of begomoviruses by comparing single and multiplex PCR detection. Hence, the 50 samples of pumpkin leaves that had been used for the single PCR detection of each of the ToLCNDV, TYLKaV and TYLCTHV viruses were used in the multiplex PCR detection. The multiplex PCR reaction was performed under the optimal conditions described previously.

Table 1 Primers used for polymerase chain reaction (PCR) and multiplex PCR in this study

Virus	Primer name	Sequence (5'-3')	Ta (°C)	Amplicon (bp)	Reference
ToLCNDV	AV105-F	CCCATCTCTCGTGAAGCTCTC	55	400	Sivalingam and Varma (2007)
	AV107-R	AGTCTATTTAAAGGACCCC			
TYLCKaV	TYKa-F	TACATAATCAGCTGCGCGTATTACA	65	831	Kesumawati et al. (2020)
	TYKa-R	CATAGGAATKGTRATTGAAGGTGAATCT			
TYLCTHV	CPA2	TTAATTCGTCACTGAGTCAT	55	770	Ieamkhang et al. (2005)
	CPA5	ATGTCGAAGCGTCCAGCAGA			

ToLCNDV = *Tomato leaf curl New Delhi virus*; TYLCKaV = *Tomato yellow leaf curl Kanchanaburi virus*; TYLCTHV = *Tomato yellow leaf curl Thailand virus*; Ta = annealing temperature.

Results

Polymerase chain rection detection

The single PCR detection of ToLCNDV from the 50 pumpkin leaf samples revealed that 41 samples (82.00%) were ToLCNDV-positive, displaying detected 400 bp PCR amplicons (Fig. 1A).

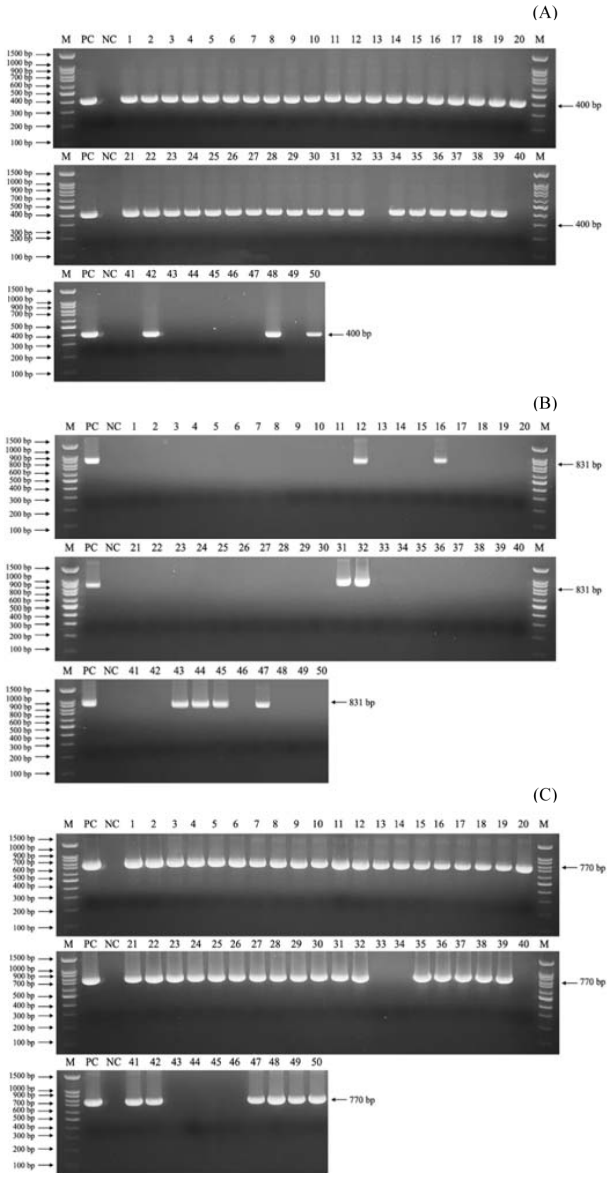

Fig. 1 Polymerase chain reaction (PCR) detection of ToLCNDV, TYLCKaV and TYLCTHV from pumpkin leaf samples (lanes 1–50), with lanes 1–35 from Kamphaeng Saen (KPS) district, Nakhon Pathom province, Thailand; lanes 36–40 from Bang Len (BL) district, Nakhon Pathom province, Thailand; and lanes 41–50 from Chiang Mai province (CM), Thailand: (A) ToLCNDV detection with 400 bp PCR amplicons; (B) TYLCKaV detection with 831 bp PCR amplicons; (C)TYLCTHV detection with 770 bp PCR amplicons, where M = DNA marker, PC = positive control and NC = negative control (DEPC-treated water).

TYLCKaV single detection from 50 pumpkin leaf samples indicated that 8 samples (16.00%) were TYLCKaV-positive, displaying detected 831 bp PCR amplicons (Fig. 1B). For TYLCTHV, the single PCR detection from the same samples showed that 43 samples (86.00%) were TYLCTHV-positive, displaying detected 770 bp PCR amplicons (Fig. 1C).

Sequence analysis of selected samples showed that five ToLCNDV sequences shared 99.5–99.8% identity with previous ToLCNDV isolates retrieved from GenBank. Similarly, five TYLCKaV sequences shared 98.6–99.4% identity with previous TYLCKaV isolates and five TYLCTHV sequences shared 99.0–99.6% identity with previous TYLCTHV isolates from GenBank (data not shown).

Inspection of the infected pumpkins revealed that in the case of single virus infection, most ToLCNDV-detected samples exhibited leaf curling and vein banding symptoms (Fig. 2A), while most TYLCTHV-detected samples exhibited green-yellow mosaic, yellow spots and mottle symptoms (Figs. 2B and 2C).

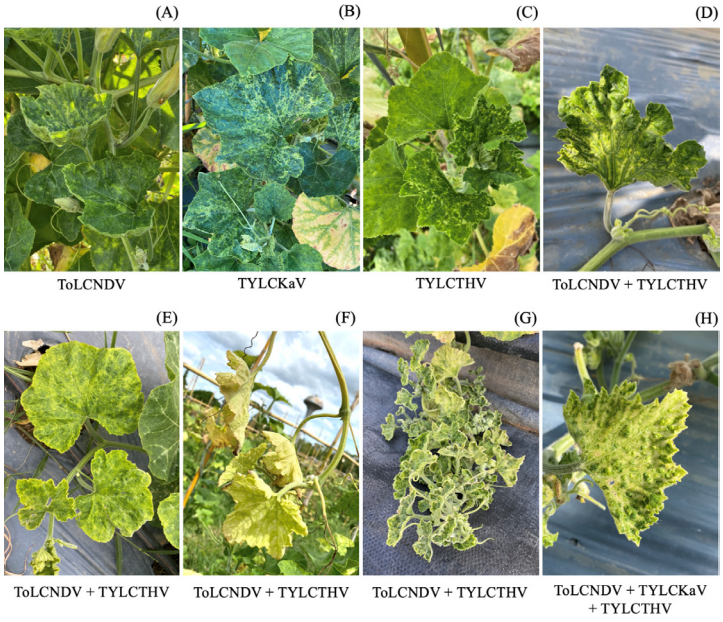

Fig. 2 *Begomovirus*-associated symptoms on pumpkin leaves verified using multiplex polymerase chain reaction detection: (A) leaf curling + vein banding from a single ToLCNDV-detected sample; (B) green yellow mosaic from a single TYLCKaV-detected sample; (C) yellow spot + mottle from a single TYLCTHV-detected sample; (D) leaf curling + vein banding + malformation from a ToLCNDV + TYLCTHV-detected sample; (E) yellow mosaic from a ToLCNDV + TYLCTHV-detected sample; (F) yellowing + chlorosis from a ToLCNDV + TYLCTHV-detected sample; (G) severe shoot leaf curling from a ToLCNDV + TYLCTHV-detected sample; (H) yellowing + bubbling + vein banding from a ToLCNDV + TYLCKaV + TYLCTHV-detected sample

Optimization of multiplex polymerase chain reaction assay

Multiplex PCR detection, using a mixed DNA template of ToLCNDV, TYLCKaV and TYLCTHV from naturally infected samples, revealed that all amplicons (ToLCNDV, 400 bp; TYLCKaV, 831 bp; TYLCTHV, 770 bp) were successfully amplified at annealing temperatures of 50–55°C. The amplicon of ToLCNDV was faint when annealed at the higher temperature of 60°C and was not observed when annealed at 65°C (Fig. 3A). Therefore, the most optimal annealing temperature for amplifying all amplicons using multiplex PCR was 55°C, based on this temperature producing the highest intensity of detected amplicons for ToLCNDV, TYLCKaV and TYLCTHV (Fig. 3A).

Specificity and sensitivity assays

The specificity assay demonstrated no cross-reactivity among the three begomoviruses. The multiplex PCR successfully detected all amplicons of ToLCNDV (400 bp), TYLCKaV (831 bp) and TYLCTHV (770 bp) together in the multiplex lane (Fig. 3B). When the multiplex PCR was conducted on individual positive DNA samples, each specific amplicon was detected only in the presence of ToLCNDV, TYLCKaV or TYLCTHV, with no amplicons detected from the negative control (DEPC-treated water) or healthy pumpkin (Fig. 3B).

The sensitivity of the multiplex PCR was evaluated using a ten-fold serial dilution ranging from 1×10^{0} to 1×10^{-10} of mixed DNA of ToLCNDV, TYLCKaV and TYLCTHV. The results showed that the limit of detection (LOD) of multiplex PCR was up to 1×10^{-4} of diluted DNA, where all amplicons were detected (Fig. 3C). At a dilution of 1×10^{-5}, the amplicons of TYLCKaV and TYLCTHV were faint, with none detected at a dilution of 1×10^{-6}. However, the amplicon of ToLCNDV was still strongly detected at 1×10^{-6} dilution. Furthermore, at greater dilution (1×10^{-7} to 1×10^{-10}), no amplicons were detected (Fig. 3C).

Evaluation of multiplex polymerase chain reaction assay

The multiplex PCR detection results were mostly the same as for the single PCR detection of individual ToLCNDV, TYLCKaV and TYLCTHV. Samples that tested positive based on single PCR detection for ToLCNDV, TYLCKaV and TYLCTHV had the same amplicons in multiplex PCR detection (Fig. 4). Out of the 50 pumpkin leaf samples, mixed double infection of ToLCNDV and TYLCTHV was the most frequently detected at 76.00% (38/50 samples), followed by mixed multiple infection of all viruses at 8.00% (4/50 samples), single infection of TYLCKaV at 6.00% (3/50 samples) and mixed double infection of TYLCKaV and TYLCTHV at 2.00% (1/50 samples), as shown in Figs. 4 and 5. However, amplicons other than the specific ones for ToLCNDV (400 bp), TYLCKaV (831 bp) and TYLCTHV (770 bp) were considered unexpected amplicons.

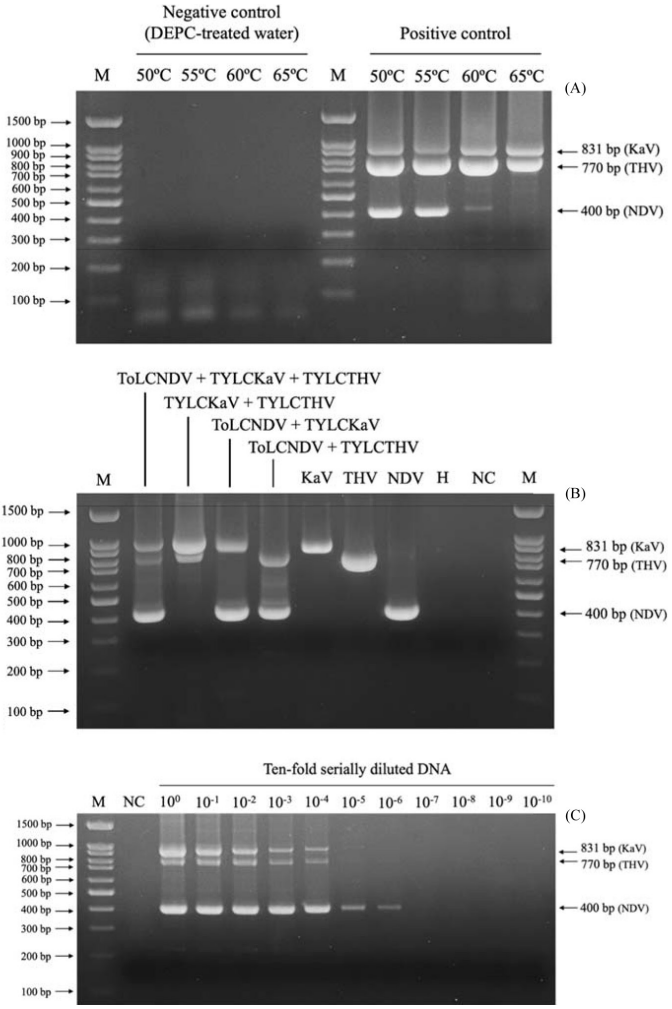

Fig. 3 Optimization of multiplex polymerase chain reaction assay for simultaneous detection of ToLCNDV (NDV; 400 bp), TYLCKaV (KaV; 831 bp) and TYLCTHV (THV; 770 bp) from pumpkin: (A) optimization of annealing temperature (50°C, 55°C, 60°C, 65°C) for simultaneous detection of ToLCNDV, TYLCKaV and TYLCTHV; (B) specificity assay; (C) t sensitivity assay using serial ten-fold diluted DNA with the limit of detection at 1×10^{-4} of diluted DNA, where M = 100 bp + 1.5 kb DNA ladder and NC = negative control (DEPC-treated water)

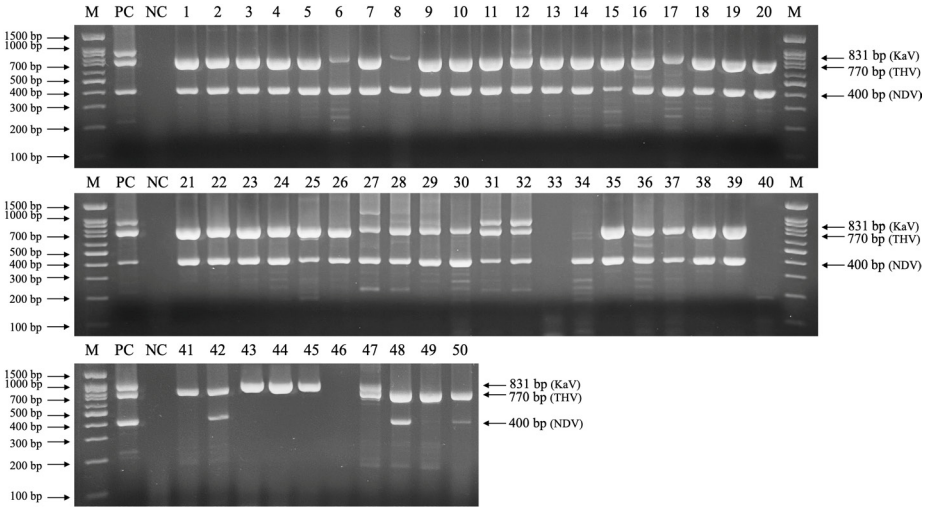

Fig. 4 Simultaneous detection using multiplex polymerase chain reaction assay of ToLCNDV (NDV; 400 bp), TYLCKaV (KaV; 831 bp) and TYLCTHV (THV; 770 bp) from pumpkin leaf samples (lanes 1–50), with lanes 1–35 from Kamphaeng Saen (KPS) district, Nakhon Pathom province; lanes 36–40 from Bang Len (BL) district, Nakhon Pathom province; and lanes 41–50 from Chiang Mai province (CM), where M = DNA marker, PC = positive control and NC = negative control (DEPC-treated water)

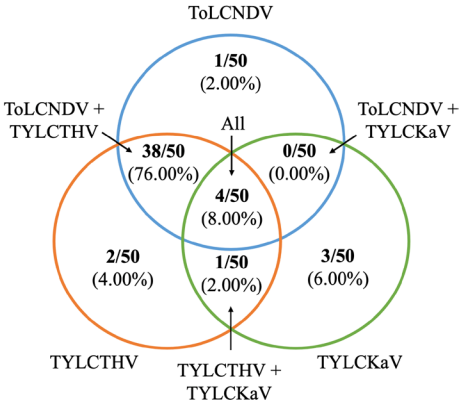

Fig. 5 Ratio of single, double and multiple infections of ToLCNDV, TYLCKaV and TYLCTHV in pumpkin detected using multiplex polymerase chain reaction assay

There were diverse symptoms on the pumpkins infected with multiple viruses. Mixed double infection of ToLCNDV and TYLCTHV exhibited symptoms such as leaf curling, vein banding, malformation, yellow mosaic, yellowing, chlorosis and severe shoot leaf curling (Figure 2D–G). Most samples with mixed multiple virus infections of ToLCNDV, TYLCKaV and TYLCTHV exhibited yellowing, bubbling and vein banding symptoms (Fig. 2H).

Discussion

The most common symptoms observed on leaves of pumpkin, squash, and gourds in commercial fields and greenhouses are light- and dark-green mosaic, vein banding, vein clearing, puckering and malformation, caused by several viruses such as Cucumber mosaic virus, Papaya ringspot virus, Squash mosaic virus, Tobacco ringspot virus, Tomato ringspot virus, Watermelon mosaic virus and Zucchini yellow mosaic virus (Jossey and Babadoost, 2008).

The success of virus disease detection and management relies on diagnostic methods and early virus detection (Radouane et al., 2021). Identifying characteristic symptoms induced by viruses on diseased plants is the initial step in diagnosis, but it becomes challenging due to mixed infections by more than one virus (whether from related or different genera) occurring on a diseased plant simultaneously (Devendran et al., 2022). In the case of pumpkins, certain symptoms observed in *Begomovirus*-detected samples resembled those caused by other viruses, such as melon yellow spot virus, a member of the genus *Orthotospovirus*, which induces yellow spot symptoms on pumpkins in Thailand (Supakitthanakorn et al., 2018).

The current results of multiplex PCR detection indicated that the relationship between begomoviruses in pumpkin could be characterized as synergistic. This was inferred from the increased variation and severity of symptoms observed in

multiple virus infections compared to single virus infections. These findings aligned with the results reported by Gil-Salas et al. (2012), who found that cucumbers co-infected with cucumber vein yellowing virus and cucurbit yellow stunting disorder virus exhibited a synergistic increase in disease severity compared to individual virus infections.

Multiplex PCR is a valuable tool in many biological studies, but it is a complex procedure that requires thorough planning and optimization to achieve robust and meaningful results. The adjustment of primer concentrations is crucial to ensure even amplification of all targeted DNA fragments (Sint et al., 2012). The current study used one-half concentrations (0.25 µM) of primers compared to the single PCR assay (0.5 µM), a commonly used concentration for multiplex PCR assays. This approach aimed to ensure proper complementation of all primers to their targets and reduce primer-dimer formation (Kwon et al., 2014).

It is also necessary to vary the annealing temperature for multiplex PCR because different primers have different ranges for their annealing temperature; it is essential to adjust to the optimal annealing temperature for the successful amplification all individual specific amplicons when combining them together (Hosokawa et al., 2007; Pallas et al., 2018). The current study used an optimal annealing temperature of 55°C because this temperature produced PCR products with the highest intensity for all amplicons. However, even though the recommended annealing temperature for tomato yellow leaf curl Kanchanaburi virus (TYLCKaV) was 65°C (Kesumawati et al., 2020), annealing at 55°C still yielded satisfactory results.

In conclusion, the PCR conditions optimized in the current study were applicable for the detection of multiple viral infections in pumpkin fields, especially in Thailand where the viruses were found. Multiplex PCR detection of mixed *Begomovirus* infections revealed that ToLCNDV and TYLCTHV commonly co-infected pumpkins. Therefore, targeting these two viruses in breeding and screening programs for resistant pumpkin cultivars could greatly enhance disease control strategies. Multiplex PCR could be applied for the routine detection of begomoviruses in pumpkin and other cucurbitaceous plants, offering high sensitivity, accuracy and the capability of simultaneous detection. This technique should be an essential tool for monitoring and quarantine purposes.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The Tropical Vegetable Research Center (TVRC), Kasetsart University, Kamphaeng Sean Campus, Nakhon Pathom province, Thailand and the farmers in Chiang Mai province, Thailand for providing the field crops for the surveys and sample collection.

References

Alazem, M., Bwalya, J., Hsuan, P., Yu, J., Chu, H.C., Burch-Smith, T., Kim, K.-H. 2023. Viral synergism suppresses R gene-mediated resistance by impairing downstream defense mechanisms in soybean. Plant Physiol. 192: 3088–3105. doi.org/10.1093/plphys/kiad255

Chun, J.Y., Kim, K.J., Hwang, I.T., Kim, Y.J., Lee, D.H., Lee, I.K., Kim, J.K. 2007. Dual priming oligonucleotide system for the multiplex detection of respiratory viruses and SNP genotyping of CYP2C19 gene. Nucleic Acids Res. 35: e40. doi.org/10.1093/nar/gkm051

Deb, M., Anderson, J.M., Scofield, S.R. 2023. Development of a multiplex RT-PCR assay for simultaneous detection of ten major viral pathogens of wheat. Agronomy 13: 833. doi.org/10.3390/agronomy13030833

Devendran, R., Kumar, M., Ghosh, D., Yogindran, D., Karim, M.J., Chakraborty, S. 2022. Capsicum-infecting begomoviruses as global pathogens: Host-virus interplay, pathogenesis and management. Trends Microbiol. 30: 170–184. doi.org/10.1016/j.tim.2021.05.007

Fiallo-Olivé, E., Lett, J.-M., Martin, D.P., Roumagnac, P., Varsani, A., Zerbini, F.M., Navas-Castillo, J. 2021. ICTV virus taxonomy profile: *Geminiviridae* 2021. J. Gen. Virol. 98: 131–133. doi.org/10.1099/jgv.0.001696

Gil-Salas, F.M., Peters, J., Boonham, N., Cuadrado, I.M., Janssen, D. 2012. Co-infection with *Cucumber vein yellowing virus* and *Cucurbit yellow stunting disorder virus* leading to synergism in cucumber. Plant Pathol. 61: 468–478. doi.org/10.1111/j.1365-3059.2011.02545.x

Hosokawa, M., Shiba, H., Kawabe, T., Nakashima, A., Yazawa, S. 2007. A simple and simultaneous detection method for two different viroids infecting chrysanthemum by multiplex direct RT-PCR. Hort. J. 76: 60–65. doi.org/10.2503/jjshs.76.60

Ieamkhang, S., Riangwong, L., Chatchawankanphanich, O. 2005. Detection of *Tomato yellow leaf curl Thailand virus* by PCR without DNA extraction. Mol. Biotechnol. 31: 233–238. doi.org/10.1385/MB:31:3:233

Inoue-Nagata, A.K., Lima, M.F., Gilbertson, R.L. 2016. A review of geminivirus diseases in vegetables and other crops in Brazil: Current status and approaches for management. Hortic. Bras. 34: 8–18. doi.org/10.1590/S0102-053620160000100002

Jaiswal, N., Saritha, R.K., Datta, D., Singh, M., Dubey, R.S., Rai, A.B., Rai, M. 2012. Mixed infections of begomoviruses in pumpkins with yellow vein mosaic disease in north India. Arch. Phytopathol. Pflanzenschutz 45: 938–941. doi.org/10.1080/03235408.2011.646670

Jossey, S., Babadoost, M. 2008. Occurrence and distribution of pumpkin and squash viruses in Illinois. Plant Dis. 92: 61–68. doi.org/10.1094/PDIS-92-1-0061

Kenyon, L., Tsai, W.-S., Shih, S.-L., Lee, L.-M. 2014. Emergence and diversity of begomoviruses infecting solanaceous crops in East and Southeast Asia. Virus Res. 186: 104–113.

Kesumawati, E., Okabe, S., Khalil, M., Alfan, G., Bahagia, P., Pohan, N., Zakaria, S., Koeda, S. 2020. Molecular characterization of begomoviruses associated with yellow leaf curl disease in *Solanaceae* and *Cucurbitaceae* crops from Northern Sumatra, Indonesia. Hort. J. 89: 410–416. doi.org/10.2503/hortj.UTD-175

Ketsakul, S., Imsabai, W., Tangtrakulwanich, K., Auvuchanon, A. 2020. Identification of genes controlling fruit shape in Thai pumpkin (*Cucurbita moschata* Duch.). Int. J. Agric. Technol. 16: 629–640.

Kumar, S., Li, G., Stecher, M., Knyaz, C., Tamura, K. 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35: 1547–1549. doi.org/10.1093/molbev/msy096

Kwon, J.Y., Hong, J.S., Kim, M.J., Choi, S.H., Min, B.E., Song, E.G., Kim, H.H., Ryu, K.H. 2014. Simultaneous multiplex PCR detection of seven cucurbit-infecting viruses. J. Virol. Methods 206: 133–139. doi.org/10.1016/j.jviromet.2014.06.009

Li, Y., Mbata, G.N., Punnuri, S., Simmons, A.M., Shapiro-Ilan, D.I. 2021. *Bemisia tabaci* on vegetables in the Southern United States: Incidence, impact, and management. Insects 12: 198. doi.org/10.3390/insects12030198

Nagendran, K., Mohankumar, S., Aravintharaj, R., et al. 2017. The occurrence and distribution of major viruses infecting cucurbits in Tamil Nadu state, India. Crop Prot. 99: 10–16. doi.org/10.1016/j.cropro.2017.05.006

Nawaz-ul-Rehman, M.S., Fauquet, C.M. 2009. Evolution of geminiviruses and their satellites. FEBS Lett. 583: 1825–1832. doi.org/10.1016/j.febslet.2009.05.045

Pallas, V., Sanchez-Navarro, J.A., James, D. 2018. Recent advances on the multiplex molecular detection of plant viruses and viroids. Front. Microbiol. 9: 2087. doi.org/10.3389/fmicb.2018.02087

Radouane, N., Ezrari, S., Belabess, Z., et al. 2021. Viruses of cucurbit crops: Current status in the Mediterranean Region. Phytopathol. Mediterr. 60: 493–519. doi: 10.36253/phyto-12340

Rosen, R., Kanakala, S., Kliot, A., et al. 2015. Persistent, circulative transmission of begomoviruses by whitefly vectors. Curr. Opin. Virol. 15: 1–8. doi.org/10.1016/j.coviro.2015.06.008

Roy, A., Fayad, A., Barthe, G., Brlansky, R.H. 2005. A multiplex polymerase chain reaction method for reliable, sensitive, and simultaneous detection of multiple viruses in citrus trees. J. Virol. Methods 129: 47–55. doi.org/10.1016/j.jviromet.2005.05.008

Singhal, P., Nabi, S.U., Yadav, M.K., Dubey, A. 2020. Mixed infection of plant viruses: Diagnostics, interactions and impact on host. J. Plant Dis. Prot. 128: 353–368. doi.org/10.1007/s41348-020-00384-0

Sint, D., Raso, L., Traugott, M. 2012. Advances in multiplex PCR: Balancing primer efficiencies and improving detection success. Methods Ecol. Evol. 3: 898–905. doi.org/10.1111/j.2041-210X.2012.00215.x

Sivalingam, P.N., Varma, A. 2007. PCR based diagnosis of Begomoviruses associated with tomato leaf curl disease in India. J. Plant Biochem. Biotechnol. 16: 17–22. doi.org/10.1007/BF03321923

Supakitthanakorn, S., Akarapisan, A., Ruangwong, O.-U. 2018. First record of melon yellow spot virus in pumpkin and its occurrence in cucurbitaceous crops in Thailand. Australas. Plant Dis. Notes 13: 32. doi.org/10.1007/s13314-018-0314-5

Usha, R., Lakshmi, M., Ranjani, M. 2010. Nutritional, sensory and physical analysis of pumpkin flour incorporated into weaning mix. Malays. J. Nutr. 16: 379–387.

Wu, H.-Y., Li, W.-H., Weng, S.-H., Tsai, W.-S., Tsai, C.-W. 2023. Differential effects of two tomato begomoviruses on the life history and feeding preference of *Bemisia tabaci*. Insects 14: 870. doi.org/10.3390/insects14110870

Xu, L., Ming, J. 2022. Development of a multiplex RT-PCR assay for simultaneous detection of *Lily symptomless virus, Lily mottle virus, Cucumber mosaic virus*, and *Plantago asiatica mosaic virus* in Lilies. Virol. J. 19: 219. doi.org/10.1186/s12985-022-01947-3