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Characterization and transmission of *Columnea latent viroid* in tomato

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Abstract *Columnea latent viroid* (CLVd) causes yield loss in tomatoes. Its transmission data are critical in determining how to reduce the risk of disease spread. The current study characterized and studied CLVd transmission in tomato plants. CLVd crude sap was diluted and incubated at 100 ℃ or room temperature. CLVd was mechanically inoculated onto the tomato cultivars Seedathip 4 and Cherry 154. Total RNA was extracted from seeds or leaves from CLVd-infected tomato plants, and the presence of CLVd was confirmed using reverse-transcription polymerase chain reaction (RT-PCR), biological assays, and sequencing. When the crude sap was diluted with phosphate buffer to 10^{-1} and 10^{-2}, viroid infectivity remained constant. In addition, viroid infectivity was lost after 30 minutes of heating at 100 ℃. The dried viroid crude sap was infected in 2 days after incubation at room temperature. CLVd could be transmitted via mechanical means in seed but not via root transmission. The fruit shape, color, and size varied when tomato plants were inoculated at various stages of development. Yield loss may happen more severe in Seedathip 4 tomato plants inoculated at the seedling and flowering stages. The symptoms caused by CLVd were also found in the columella area in fruits. The rate of seed transmission was 0.25%. CLVd lost its infectivity after crude sap, which was treated using various methods. It found that CLVd did not carry through root transmission. While the rate of seed transmission was low, the seeds may carry viroid disease.

Keywords: *Columnea latent viroid*, Root transmission, Seed transmission, Tomato, Viroid

Introduction

About 50 years after the discovery of viroids (Diener, 1971; Raymer and Diener, 1969), these smallest and simplest RNA replicons have ever been discovered and captivated many researchers' interests due to their humble

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properties. They were first described as self-replicating, non-coding RNAs capable of causing various disease symptoms in vulnerable host plants, with latent symptoms appearing in the majority of ornamental plants and the severity varying among vegetative and agricultural plants (Diener, 1971). Their structures are single-stranded, organized, circular RNA with sizes extending from 246 to 463 nucleotides; they have been called "living fossils of a precellular RNA world" (Diener, 1989). *Columnea latent viroid* (CLVd), one of the members of the *Pospiviroidae* family, was first isolated from asymptomatic *Columnea erythrophae* (lipstick vine) leaves in Beltsville, Maryland, the USA when its nucleic acid was transferred to susceptible Rutgers tomato plants (Hammond *et al.*, 1989; Owens *et al.*, 1978). These 370 nucleotides of rod-like structural RNA with extensive base-pairing are the results of intra-cellular recombination between *Hop stunt viroid* (HSVd)-type, and one or more of the *Potato spindle tuber viroid* (PSTVd)-type replicating on the same plant (Hammond *et al*., 1989). CLVd infection causes yield loss in tomato and other vegetable crops, such as potato and pepper. Economic yield losses up to 80% had been observed in the UK, Canada, Germany, and France (Nixon *et al*., 2010; Singh, 1983; Singh *et al*., 1992; Spieker, 1996; Steyer *et al*., 2010). The symptoms commonly found in susceptible tomato plants infected by CLVd are growth stunting, leaf rugosity, and necrosis on the leaf vein, stem, and petiole (Tangkanchanapas *et al*., 2021). CLVd can be transmitted via vegetative propagation. Most cases have been reported in ornamental plants with no symptoms (Verhoeven *et al*., 2008). In Thailand, CLVd was first reported in 2007, and it became one of Thailand's quarantine pests with critical status.

Viroids spread quickly in the field, primarily through mechanical means. Grafting infected-vegetative planting materials is a common occurrence (Verhoeven *et al.*, 2008). Tools used to cut viroid-infected plants can unintentionally carry viroid disease (Diener, 1987), such as knife cutting, stem slashing with a razor blade, or even rubbing by hand. One report suggested that viroid transmission through roots is possible, as was previously suggested for *Hop stunt viroid* (HSVd) and *Apple scar skin viroid* (ASSVd) (Hadidi, 2003). There are reports of viroid transmission via an insect through pollination activities, such as for *Tomato chlorotic mottle viroid* (TCDVd) (Matsuura *et al.*, 2009) and *Potato spindle tuber viroid* (PSTVd) encapsidated with *Potato leafroll luteovirus* (PLRV) (Syller *et al*., 1997). On the other hand, there has not been reported of insect and root transmission of CLVd, and though such routes may be possible, it should be viewed as spread via mechanical means. Transmission of CLVd naturally occurs in pollen to ovules (horizontal transmission) to seed (vertical transmission) (Bhuvitarkorn and Reanwarakorn, 2019; Hammond *et al*., 1989; Hull, 2004). A little known about CLVd biology,

the current study aimed to investigate the physical properties and transmission of CLVd in tomato plants, which should provide useful information for determining the best method for preventing, managing, and eradicating viroids that come into seed contact and for developing seed transmission detection technology for seed import and export.

Materials and methods

Plant material and viroid sources

The CLVd isolate NK-KUKPS1 accession no. KY235369 was multiplied using Rutgers tomato. The dried leaves of CLVd-infected Rutgers tomato were used as the 1st inoculum for mechanical inoculation by grinding with 0.1 M phosphate buffer (pH 9.0) at the ratio 1g:10 mL. Then, the prepared mixture was rubbed onto Rutgers tomato leaves at 4 weeks post sowing. The inoculated plants were maintained in an insect-proof greenhouse with a temperature range of 25–32 ℃ and a natural lighting period of 12–14 hours. Then, the infected plants were confirmed using RT-PCR and used as an inoculum source for further experiments in this study.

Infectivity of diluted-viroid crude sap

The mixture of viroid inoculum freshly prepared from the CLVd-infected tomato leaves as previously described, was serially diluted with phosphate buffer to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}, respectively. Carborundum powder was added into each mixture and chilled on ice before rubbing on the young tomato leaves (cv. Rutgers at 4 weeks post-germination) and left for 2–3 minutes before being rinsed with distilled water. Control plants were mock-inoculated as above using the buffer without viroid inoculum. All plants were kept in an insect-proof greenhouse under natural light and monitored weekly for 8 weeks, and viroid infection was confirmed using RT-PCR.

Infectivity of viroid crude sap after incubation at 100 ℃

The mixture of viroid inoculum freshly prepared from the CLVd-infected tomato leaves was incubated at 100 ℃ for 10, 20, 30, 40, and 50 minutes, respectively. After each incubation period, the viroid inoculum was used to inoculate Rutgers tomato plants at 4 wps (weeks post sowing) based on the method described above.

Infectivity of viroid crude sap after incubation at room temperature

The mixture of viroid inoculum was incubated at room temperature for 1, 2, 3, 4, 5, 6 and 7 days, respectively. Then, viroid crude sap was used to inoculate Rutgers tomato plants at 4 wps based on the method described above.

CLVd root transmission assay

The CLVd root transmission study used Seedathip 3, Seedathip 4, Cherry 154 and Rutgers tomato plants at the 4 wps stage. Plants were separated into different combinations using a clear polycarbonate screen, with no leaf contact within and between other treatments. One plant from each treatment was inoculated with CLVd-infected leaves using the method described above. Symptoms were monitored weekly for 10 weeks when RT-PCR was carried out. To confirm no leaf contact, the leaves of uninoculated plants in the same pot as an inoculated one were used to inoculate Rutgers tomato plants as indicator plants.

Symptom development of CLVd in tomato plants

CLVd-infected leaves were used to inoculate tomato plants (cv. Seedathip 4 and Cherry 154) at three different stages: pre-flowering (4 wps), flowering when 50% of the tested plants were flowering), and fruiting (when 50% of the test plants were fruit setting. The symptoms, plant height, fruit weight and size were observed for 12 weeks. RT-PCR was used to confirm the presence of CLVd.

CLVd seed transmission assays

CLVd was inoculated onto tomato plants at two different stages: flowering and fruiting. The symptoms were weekly monitored, and all tomato fruit were harvested, and their seeds incubated inside plastic bags for 12–24 hours, washed out of the slurry layer using dH_2O and strained using a metal sieve. The strained seeds were spread out on filter paper (Whatman no. 1) and allowed to dry for 2–3 days before packing in zip bags for further tests.

To determine seed infection in each infected plant, total RNA was extracted from randomly selected seeds (5 seeds/plant), and the presence of CLVd was detected using the RT-PCR technique as described. All seeds collected from the CLVd-infected tomato plants (both at inoculation and during the flowering and fruiting stages) were sown using the top-of-paper

germination technique (Rao *et al.*, 2006) to determine whether CLVd had been transmitted via the seed or via seedborne transmission. After 10 days of germination, the seed coat, seedlings, and roots were separated and bulked for CLVd detection (20 seeds/batch).

To diagnose the seedborne transmission of CLVd, random seeds from CLVd-infected tomato plants were directly germinated and grown under the conditions mentioned above in a grow-out test. The young leaves of the test plants were collected and tested for the presence of CLVd monthly for 3 months.

Biological indexing

To determine viroid infectivity, total RNA extracted from infected-tomato leaves and viroid-free tomato leaves was mechanically inoculated onto tomato seedlings (cv. Rutgers) at 3 weeks after sowing, with leaves collected and extracted 4 four weeks for a total of 12 weeks to confirm CLVd using RT-PCR.

RNA extraction

Plant RNA was prepared from 100 mg samples of young tomato leaves using a modified CTAB method (Li *et al.*, 2008; Reanwarakorn *et al.*, 2011), using a CTAB extraction buffer (100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1.0% Na_2SO_3, and 2.0% PVP-40). The final RNA pellet was dissolved in 50 µL of RNase-free dH_2O and stored at -20 ℃ until use.

Detection of CLVd by reverse transcription-polymerase chain reaction

CLVd infection was confirmed using CLVd-specific primers (cCL-P2: CTGCAGCCATGCAAAGA and hCL-P2: GGTCAGGTGTGAACCAC) (Marach, 2008). Total RNA was used to synthesize cDNA using Thermo Scientific RevertAid Reverse Transcriptase (RT) (Thermo Scientific^{TM}). PCR reactions were performed using the Taq DNA polymerase (Thermo Scientific^{TM}) under conditions as follows: 96 ℃ for 40 s, 54 ℃ for 40 s, 72 ℃ for 40 s, and a final extension at 72 ℃ for 7 min. All steps were performed according to the manufacturer's protocols in a Biometra T1 model thermocycler (Biometra GmbH). The RT-PCR amplification products were analyzed using gel electrophoresis with 2% agarose gel, with RedSafe^{TM} Nucleic Acid Staining Solution (iNtRON Biotechnology) as the staining dye. The results were visualized under UV light using a Gel Documentation UV-transilluminator (SYNGENE: Genesis^3, United Kingdom).

Construction of infectious cDNA clone and sequence analysis

According to the manufacturer's instructions, RT-PCR products synthesized from the diseased tomato plants were cleaned and diluted using a FavorPrepTM Gel/PCR purification kit (Favorgen, Taiwan). Then, purified cDNA was cloned into pGEM-T Easy (Promega) using the T4 DNA ligase enzyme. The recombinant DNA was transformed into *E. coli* (DH5α). Cloned samples carrying the inserted DNA were selected based on their ampicillin-resistance. *E. coli* was grown in liquid cultures in Lauria Broth (LB) medium containing the appropriate antibiotics at 37℃ with vigorous shaking. The plasmid was extracted using a plasmid extraction kit (Favorgen, Taiwan) and kept at -20℃ until use. An automated DNA sequencer carried out sequence analysis of cloned viroid plasmid. The resulting sequences were compared with viroid sequences in the NCBI Genbank using the BLAST system, and multiple alignments of related sequences were created using Clustal Omega and MEGA7 ver.7.0.26.

Results

CLVd properties

At 3 weeks post-inoculation, tomato plants inoculated with 10^{-1} dilution started to show necrosis symptoms on the left vein, and the leaves started to tilt downward, while at 4 weeks post-inoculation, more stunting was observed. The other samples were asymptomatic. RT-PCR was used to confirm the presence of CLVd at 8 weeks post-inoculation, with 1 out of 10 tomato plants inoculated with a 10^{-2} dilution showing positive results. The viroid lost its infectivity after it was diluted to 10^{-3} (Table 1).

Table 1. Infectivity of diluted viroid crude sap

Dilution of CLVd inoculum[1/]	**RT-PCR**	**Infected test plants/ total test plants**
No dilution	+	9/10
10^{-1}	+	8/10
10^{-2}	+	1/10
10^{-3}	-	0/10
10^{-4}	-	0/10
10^{-5}	-	0/10
10^{-6}	-	0/10
10^{-7}	-	0/10
Mock-inoculation	-	0/10

1/: CLVd inoculum was diluted with phosphate buffer (pH 7.0)

The high temperature affects viroid infectivity. CLVd inoculum samples were separately incubated at 100℃ for different periods. At 3 weeks after inoculation, tomato plants inoculated with no-incubation crude sap had necrotic symptoms on the leaf vein and stem and leaf deformation. Tomato plants inoculated with crude sap for 10 and 20 minutes of incubation started to express symptoms of curled leaves 1 week later. The test showed that CLVd crude sap could be inactivated for 30 minutes at 100℃ (Table 2).

Table 2. Infectivity of viroid crude sap after incubation at 100°C

Treatment time[1/] (min)	**RT-PCR**	**Infected test plants/ total test plants**
No treatment	+	10/10
10	+	10/10
20	+	6/10
30	-	0/10
40	-	0/10
50	-	0/10
Mock-inoculation	-	0/10

1/: CLVd inocula were incubated at 100℃ for the times stated

The results of the CLVd infectivity experiment after incubation at room temperature revealed a low chance of infection, with 4 out of 10 plants inoculated with viroid crude sap incubated for 1 day testing positive for RT-PCR. Only 1 out of 10 plants inoculated with viroid crude sap incubated for 2 days tested positive. The results indicated that the dried viroid crude sap was infectious for 2 days after incubation (Table 3).

Table 3. Infectivity of viroid crude sap after incubation at room temperature

Treatment period[1/] (days)	**RT-PCR**	**Infected test plants/ total test plants**
0	+	10/10
1	+	4/10
2	+	1/10
3	-	0/10
4	-	0/10
5	-	0/10
6	-	0/10
7	-	0/10
Mock-inoculation	-	0/10

1/: Inocula of CLVd were incubated at room temperature for specified times

Root transmission

Tomato plants inoculated with CLVd-infected leaves started to express symptoms at 2–3 weeks post-inoculation. The symptoms included leaf size

reduction, stunting, leaves curling downward, leaves yellowing, and necrosis of the leaf vein and petiole in Seedathip 4 tomato plants. No symptoms were observed in uninoculated tomato plants in the same pot as an inoculated one nor in Rutgers tomato plants used as indicator plants (Table 4).

Table 4. Symptoms on inoculated tomato plants and RT-PCR results for root transmission assay

Treatment[1/]	**Symptoms on CLVd-inoculated plants**	**RT-PCR**[2/]
Seedathip 3/ Seedathip 3	Deformed leaves	+/-
Seedathip 3/ Seedathip 4	Deformed leaves, stunting	+/-
Seedathip 3/ Cherry 154	Deformed leaves	+/-
Seedathip 3/ Rutgers	Deformed, curled leaves	+/-
Seedathip 4/ Seedathip 3	Deformed, wilted leaves	+/-
Seedathip 4/ Seedathip 4	Deformed leaves	+/-
Seedathip 4/ Cherry 154	Deformed leaves, stunting	+/-
Seedathip 4/ Rutgers	Deformed leaves	+/-
Cherry 154/ Seedathip 3	Deformed leaves	+/-
Cherry 154/ Seedathip 4	Deformed leaves	+/-
Cherry 154/ Cherry 154	Deformed, curled leaves	+/-
Cherry 154/ Rutgers	Deformed leaves	+/-
Rutgers/ Seedathip 3	Deformed leaves, stunting	+/-
Rutgers/ Seedathip 4	Deformed leaves	+/-
Rutgers/ Cherry 154	Deformed leaves	+/-
Rutgers/ Rutgers	Deformed leaves, stunt	+/-

1/: Combination of inoculated/uninoculated tomato cultivar
2/: RT-PCR results from inoculated/uninoculated tomato plants

Plant development, symptomatology, and seed transmission

The first effects of CLVd infection in Seedathip 4 tomato occurred at 2 weeks post-inoculation. Symptoms included a bunched top, necrosis on leaves, veins, and petioles, chlorosis, leaves curling downward, shortened internodes, leaf, flower, and fruit size reduction, and fruit deformation and discoloration. The Cherry 154 tomato plants expressed symptoms similar to Seedathip 4, with the exception that the marbling pattern was not as distinct as in Seedathip 4. Fading of fruit color was also observed. Tomato plants inoculated at pre-flowering stages produced the smallest fruit size (Figure 1). Tomato yield losses were 94.96, 75.67, and 57.05% when Seedathip 4 tomato plants were inoculated at the pre-flowering, flowering, and fruiting stages, respectively, while Cherry 154 tomato plants lost 88.87, 63.50, and 19.89%, respectively. Furthermore, there were no significant differences in fruit yield or fruit weight between inoculated and uninoculated plants when plants were inoculated at the

fruiting stage (Table 5). Because no seeds were obtained from Cherry 154 tomato plants, Seedathip 4 tomato plants were used in subsequent experiments.

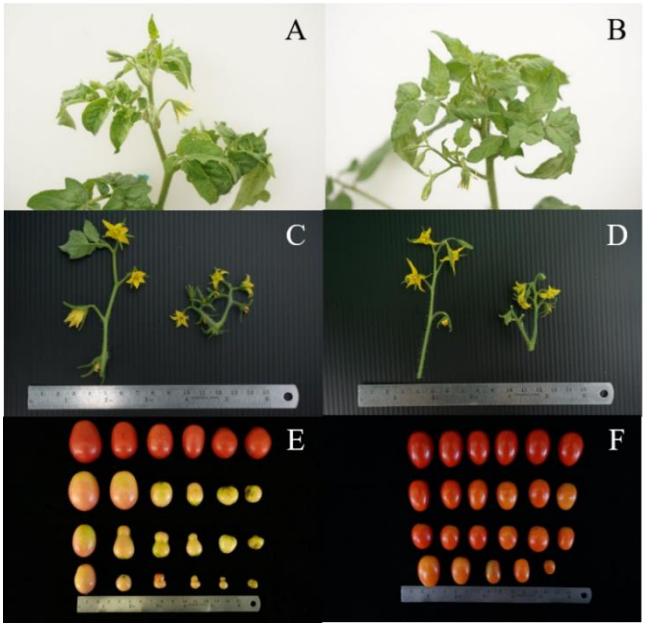

Figure 1. Symptoms of Seedathip 4 and Cherry 154 tomato plants inoculated with CLVd: A, C, E, Tomato cv. Seedathip 4; B, D, F, Tomato cv. Cherry 154. A, B, Bunched top symptoms and leaf reduction; C, D, Twisted inflorescence and size reduction of CLVd-infected plant (right) compared to healthy tomato (left); E, F, Fruit harvested from healthy tomato (top row) compared with fruit harvested from plants inoculated at fruiting, flowering and pre-flowering stages, respectively, (lower 3 rows respectively)

Fruits were harvested from 11 out of 40 plants in the flowering inoculation stage and 30 out of 40 plants in the fruiting stage of Seedathip 4 tomato plants. The viroid infection also affected the ripening, color, and shape of the fruit. In the control plants, the fruit was an oval shape, and a gradual coloring from green to red occurred. For the CLVd-infected tomato plants, a marbling pattern with a malformed fruit shape was observed in the fruit when they were not fully ripe. Tomato fruit harvested from tomato plants inoculated at the flowering stage ripened more quickly than uninoculated plants and the fruiting inoculation stage. Fruit deformation characterized as one of eight shapes was notable in tomato plants inoculated at the flowering stage. Oval tomato fruits with marbling patterns were observed when tomato plants were inoculated at the fruiting stage. Fruits harvested from tomato plants inoculated

at the fruiting stage had a similar marbling pattern (Figure 2). A cross-section of tomato fruit was also observed. The fruits collected from tomato plants inoculated during the flowering stage displayed necrosis in the columella area (31.7–36.5%). When tomato plants were inoculated at the fruiting stage, the symptoms were reduced (5.5–9.9%), as shown in Figure 3 and Table 6. The presence of CLVd in the columella area was confirmed using RT-PCR, with 13 out of 14 samples (92.86%) being positive (data not shown).

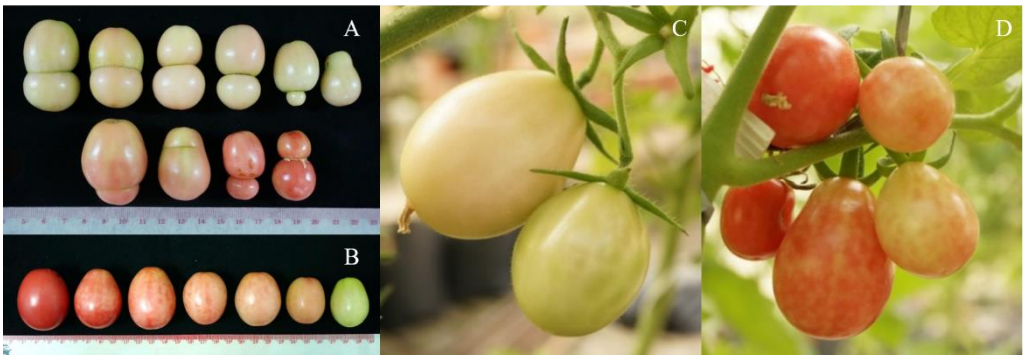

Figure 2. Symptoms caused by CLVd in tomato fruit: A, Fruit deformation in tomato plants inoculated at the flowering stage; B, marbling pattern on tomato fruit compared to healthy tomato fruit (far left); C, D, marbling pattern in unripe and ripe tomato fruit, respectively

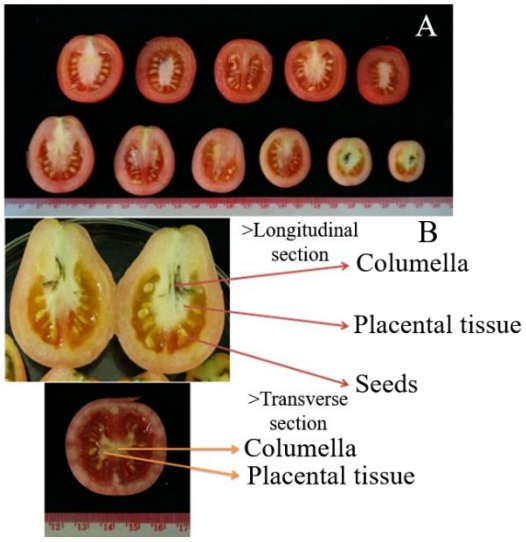

Figure 3. Necrotic symptoms inside tomato fruit (Seedathip 4) caused by CLVd: A, Symptoms caused by CLVd (bottom row) compared to healthy tomatoes (upper row); B, Longitudinal and transverse sections

Table 5. Average fruit weight and yield loss of CLVd-inoculated plants at pre-flowering, flowering, and fruiting stages

Tomato cultivar	Stage of CLVd inoculation	Average fruit weight per plant[1/] (g)	Yield loss compared with healthy tomato (%)	Pr>F
Seedathip 4	Healthy tomato	11.71a	0	1.08^{-12}
	Pre-flowering stage	0.59d	94.96	
	Flowering stage	2.85c	75.67	
	Fruiting stage	5.03b	57.05	
Cherry 154	Healthy tomato	6.29a	0	3.61^{-8}
	Pre-flowering stage	0.70c	88.87	
	Flowering stage	2.30b	63.50	
	Fruiting stage	5.04a	19.89	

1/: values in columns followed by the same lowercase letter are not significantly different based on the least significant difference (LSD) at 95 percent of confidence level

Table 6. Occurrence of necrosis in columella area for Seedthip 4 tomato plants

Stage of inoculation	Number of tomato fruits showing necrosis in columella area			
	1st Harvesting	2nd Harvesting	3rd Harvesting	4th Harvesting
Flowering stage	40/126 (31.7%)	64/175 (36.5%)	-	-
Fruiting stage	1/18 (5.5%)	17/171 (9.9%)	5/130 (3.8%)	16/190 (8.4%)

- no fruit was produced

Presence of CLVd on seeds, seed coat, and seedlings

The CLVd detection of tomato seeds (5 seeds/plant) produced 100% positive results. The remaining seeds (1,6161 seeds) were divided into two groups for the top-of-paper germination and grow-out tests. The first sets of seeds (20 seeds/batch) were used to test for CLVd using the top-of-paper germination technique. In total, 400 seed batches were germinated. Following germination, the seed coat, seedlings, and root parts were collected and tested for viroid infection. The seed coat and root samples were negative. However, one batch of seedlings tested positive for CLVd. According to the findings, the seed transmission rate was 0.25%. The second set of 8,000 seeds was used in the grow-out experiment over 3 months, with none showing the presence of CLVd.

To exclude the failure of seed transmission due to a lack of infectivity of CLVd, total RNA extracted from infected seedlings was inoculated onto susceptible Rutgers tomato plants. CLVd was detected in the inoculated Rutgers tomato plants at 8 weeks post-inoculation.

Sequence analysis

PCR products were synthesized from a seedling that was positive for CLVd, stock CLVd, and seed that were cloned and sent for sequencing. The obtained data were compared to the sequences in GenBank, and the results showed the similarity of the CLVd isolate to CLVd accession no. JF742635.1 (Tangkanchanapas *et al.*, 2005). Multiple sequence alignment was analyzed using Clustal Omega and MEGA7 ver.7.0.26, with similarities of 93.5, 94.04, and 93.77%, respectively.

Discussion

Viroids are the smallest plant pathogen known to cause various diseases in many crops; however, due to their complexity, their infectivity has been of great interest to many researchers (Carbonell *et al*., 2008; Gross *et al*., 1982; Loss *et al*., 1991). Different viroid strains can cause different symptoms in the same host, ranging from latent to severe. On the other hand, the same viroid may cause different symptoms on a different host cultivar, or the same host cultivar grown under different conditions or factors (Adkar-Purushothama and Perreault, 2020; Kovalskaya and Hammond, 2014). CLVd showed no symptoms on lipstick vine, but it was detectable when the same preparation was used to inoculate tomato plants (Owens *et al.,* 1978). The current study investigated infectivity when the CLVd-crude sap was prepared differently. The infectivity of CLVd crude sap remained at 10^{-2} dilution, whereas *Tomato chlorotic dwarf viroid* (TCDVd) could be diluted up to 10^{-6} in another study (Matsushita *et al.,* 2009), and even lower when pollen-infected *Tomato planta macho viroid* (TPMVd) and *Potato spindle tuber viroid* (PSTVd) were used, which retained infectivity at 10^{-1} (Yanagisawa *et al.,* 2019). To date, no successful method has been published to eradicate viroid diseases. However, numerous methods have been tested, including the use of heat inactivation (Barba *et al*., 2017). We incubated CLVd-crude sap at 100 ℃ and found that the heat inactivation time for the CLVd crude sap was 30 minutes, which was less than the 40 minutes of incubation for TCDVd (Matsushita *et al.,* 2009). Heat treatment may reduce viroid infectivity by promoting the formation of nuclease activity that cleaves double-stranded RNA (Matoušek *et al.,* 1995), but it may be reversible under normal conditions (Matoušek *et al*., 2001).

Because of their compact folding structure, viroids have a very stable secondary structure (Flores *et al*., 2012). Handling infected plant debris may unintentionally spread the viroid disease. The effectiveness of CLVd crude sap incubated at room temperature was tested. The infectivity of CLVd was lost

after 2 days of incubation, which was similar to the findings of Matsushita *et al.* (2009), who reported that TCDVd remained infectious for 3 days. (Matsushita *et al.,* 2009). The infectivity of a viroid could be reduced when the crude sap was applied directly, as demonstrated by the findings of Mackie *et al.* (2015), who tested PSTVd on various surfaces. They discovered that PSTVd only lasted 24 hours (Mackie *et al*., 2015). Our findings have the potential to be used in greenhouse routine experimental assays. Furthermore, the findings suggested that CLVd can be easily transmitted unintentionally through contaminated tools, hands, machinery, and plant materials, necessitating strict hygiene procedures, crop handling, and the maintenance of a viroid-free environment.

In this study, no root transmission was observed, indicating that CLVd may not horizontally travel to the root organs. *Tomato apical stunt viroid* (TASVd) was detected in the root by Antignus *et al*. (2007); however, they reported that it could not transmit to healthy tomato plants when exposed to naturally infested soil. When PSTVd inoculum was added to the rooting soil, no viroid transmission occurred, according to the findings of Seigner *et al.* (2008). However, the root system might be damaged by natural or mechanical means, such as root grafting. In such cases, viroid transmission may occur, as demonstrated in a report using *Citrus exocortis viroid* (CEVd) for root transmission assay (Broadbent *et al.,* 1988).

The mechanical inoculation to test the effect of CLVd infection on tomato plants at different stages of development was investigated. No seed from infected tomato plants was produced by Cherry 154 tomato, and so the transmission of seeds by Seedathip 4 was investigated. When these plants were infected at an early plant growth stage, there was a significant yield loss. On the other hand, plants infected at the fruiting stage showed no significant difference. CLVd infection did not affect fruit development in either of the tomato cultivars used in this study, indicating that the similarity of genetic information is responsible. The reduction in fruit size and the numbers of fruit were the main causes of the significant yield losses, which agreed with other studies (Aviña-Padilla *et al.*, 2018; Singh *et al*., 2003). According to the current study, the time of viroid infection in tomato plants affects necrotic symptoms on the fruit, with fruit from tomato plants inoculated during the flowering stage having a higher prevalence of necrosis in the columella area, which could have been due to ATP synthase that scavenges ATP from the host (Martinelli *et al.,* 2012).

Furthermore, CLVd that caused significant critical yield loss in the current study using tomatoes is more harmful to potato yields (Verhoeven *et al.,* 2004). The current study recorded a low seed transmission rate of CLVd (0.25%), which agreed with a previous report that showed various low seed

transmission rates between 5.3 and 100% using tomato S-1, S-4, and M-1 cultivars (Matsushita and Tsuda, 2016). Dall *et al.* (2019) tested sample sizes of 3,000 and 9,400 seeds and found viroid contamination seed rates of 15% and 42%, respectively, at the 95% confidence level, with a low level of CLVd detection of 6%. (Dall *et al*., 2019). In a report by Singh and Dilworth (2009), TCDVd was inoculated onto plants at the seedlings stage, and the seed transmission rate was as high as 80% (Singh and Dilworth, 2009). The pattern of CLVd distribution was carried out based on our previous study, demonstrating the long-distance movement of CLVd throughout the entire plant within 1 month (data not shown). Because of the high structural contact base-pairing feature of viroid nucleotides, their nucleotides play an important role. To cause disease or to demonstrate the ability to transmit through seeds, their structure may need to attach to host plants to form a proper function in plants (Ding, 2009). As a result, the seed transmission rate may be affected by the tomato cultivar, plant growth stage, fruit physiology, and viroid strain. Each year, large quantities of vegetable seeds are produced and exported to a variety of countries, necessitating the use of viroid-free seeds.

CLVd may readily cause severe damage to tomato crops, possibly due to its rapid spread and ease of transmission via mechanical, seed, and pollen vectors. Preventing the introduction of CLVd into the field would appear the most efficient way to eradicate or reduce its level of seriousness. However, because no root transmission was observed, one benefit concerns the growing plants in the same soil bed which is safety shown as the plants are spaced apart. The seed transmission appears to be the primary method of spreading CLVd across countries, pre-and post-testing for CLVd-infested seed batches would be concerned.

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