

Construction of Infectious Clones and Confirmation of Pathogenicity of *Tomato leaf curl New Delhi virus* and *Chickpea chlorotic dwarf virus*

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Abstract

Geminiviruses are a group of single stranded DNA viruses that are responsible for severe disease in many crops, especially in tropical and subtropical regions. The development of infectious clones for plant viruses has proven to be a powerful tool to study viral genes, and it facilitates the genetic screening of germplasms for virus resistance. In the present study, rolling circle amplification as well as restriction digestion techniques have been successfully employed to develop tandem repeats and subsequently infectious clones of two economically important geminiviruses, i.e. *Tomato leaf curl New Delhi virus* (ToLCNDV) and *Chickpea chlorotic dwarf virus* (CpCDV). The pathogenicity of these infectious clones has been tested in several plant species. Infected plants were evaluated for symptoms development and infection was confirmed by PCR. PCR data showed that 77.5% and 81.7% of all inoculated plant species were infected with ToLCNDV-JO and CpCDV-JO, respectively.

Keywords: CpCDV-JO, ToLCNDV-JO, Geminiviruses, *Orosius albicinctus*, *Bemisia tabaci*.

1. Introduction

According to the Food and Agriculture Organization, the total share of the agriculture sector in the Gross Domestic Product (GDP) is approximately 5 % (FAO, 2025). In addition, it has an essential role in the economic and social system of rural communities (Hindi, 2009). Given the economic importance of agriculture in Jordan and globally, it is utmost important to study the incidence of plant diseases, particularly those caused by bacteria, fungi, and viruses, that cause severe yield losses to vegetable crops.

Plant diseases caused by viruses are considered among the most important diseases that affect many agricultural crops worldwide (Tatineni and Hein, 2023). In general, these diseases are not easy to control (Gómez *et al.*, 2009). In nature, plant viruses that belong to the *Geminiviridae* family are widely spread and cause great crop damages (Bhattacharjee and Hallan, 2022). Members of this family have monopartite circular ssDNA genomes, except begomoviruses, which have either monopartite (DNA-A) or bipartite (DNA-A and DNA-B) genomes. DNA-A encodes for genes necessary for virus replication and packaging, vector transmission, and viral suppressors (Prasad *et al.*, 2020), while DNA-B encodes genes required for virus movement (Materatski *et al.*, 2021). Since monopartite viruses lack DNA-B, their mobility is controlled by the coat protein and pre-coat protein genes (Prasad *et al.*, 2020).

Begomoviruses are causing economically significant diseases in major vegetable crops such as tomatoes, beans,

cassava, squash, and cotton (Saad *et al.*, 2021). These viruses are considered as the most important limiting factors for vegetable production in most tropical and subtropical regions (Roumagnac *et al.*, 2022). Plants infected with begomoviruses show growth reduction, yellowing, leaf rolling, and necrosis (Varma and Malathi, 2003). In nature, begomoviruses are transmitted by the whiteflies (*Bemisia tabaci*) (Tabein and Behjatnia, 2023). Another group of plant viruses that cause severe yield reduction belongs to the genus *Mastrevirus*. *Chickpea chlorotic dwarf virus* (CpCDV), a member of the genus *Mastrevirus*, is transmitted by leafhoppers such as *Orosius albicinctus* and *O. orientalis* (Akhtar *et al.*, 2011). The virus was reported for the first time in India to affect chickpea plants (Horn *et al.*, 1993). In addition to chickpea, CpCDV infects plants in the *Fabaceae*, *Cucurbitaceae* and *Solanaceae* families (Kraberger *et al.*, 2013). Yield losses due to CpCDV infection was reported to reach 100% (Horn *et al.*, 1995).

The development of infectious clones of plant viruses is crucial for studying virus-virus interactions, functional genomics, replication, host range, expression of viral proteins, as well as understanding host-virus interactions and testing plants germplasm for disease resistance (Brewer *et al.*, 2018; Pasin *et al.*, 2019; Arif *et al.*, 2021).

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2. Materials and Methods

2.1. Virus sources

In a previous work, Jordanian isolates of ToLCNDV (hereafter ToLCDV-JO) and CpCDV (hereafter CpCDV-JO) have been isolated, characterized and cloned into pCambia2300 and pBINPLUS plasmid vectors, respectively (Unpublished data). The sequences of these

two viruses have been deposited in the GenBank under accession numbers (MW407988 for CpCDV) and (OM455497, OM455498 for ToLCNDV-JO).

In this study, the recombinant nature of these plasmids has been confirmed using the alkaline lysis method as described by Green and Sambrook (2016) with minor modifications. After that, plasmids were used as templates in PCR tests using virus-specific primer pairs (Table 1).

Table 1. Oligonucleotides used in this study

Viruses	Primer Name	Primer Sequence (5'-3')	Cyclic Conditions	bp
CpCDV-JO	CpCDVCP527F	AGGGAATCGTTGCAGGTGG	1 cycle:94°C/3min;35 cycles: 94°C/50sec; 60 °C/1min;	619
	CpCDVCP1145R	CGGACTTGAAGTACACTCGGA	72°C/1min; 1 cycle:72°C/10min	
ToLCNDV-JO	ToLCNDVF1	TCTAGAACGTCTCCGTCTTTGTGTCGATG	1 cycle:94°C/3min;30 cycles: 94°C/1min; 55 °C/30sec;	1321
	ToLCNDV R2	GCAGGCCACTTCCGTAAGACTTGATAAC	72°C/2min; 1 cycle:72°C/10min	
	ToLCNDVCP1	CTCCAAGAGAATTGAGAAGTCC	1 cycle:94°C/3min;40 cycles: 94°C/50sec; 55 °C/1min;	1049
DNA-A	ToLCNDVCP2	TCTGGACGGGCTTACGCCCT	72°C/2min; 1 cycle:72°C/10min	
DNA-B	ND-B-BamHI921F	TTTGGATCCGTGAATGCCTGC	1 cycle:94°C/3min;35 cycles: 94°C/30sec; 57 °C/30sec;	498
	ND-B-ClaI1419R	AATCGATCCAGGTCCATCGGCA	72°C/2min; 1 cycle:72°C/10min	
	ND-B-BamHI933R*	CACGGATCCAAACATTTCACAAAAGG	1 cycle:94°C/3min;35 cycles: 94°C/30sec; 57 °C/30sec;	2206
	ND-B-ClaI1410F	TGGATCGATTGCCGAATCATCTAATC	72°C/2min; 1 cycle:72°C/10min	

*Parrella *et al.*, 2018

2.2. Development of Chickpea chlorotic dwarf virus infectious clone

In brief, a recombinant pBINPLUS bearing the full-length genome of CpCDV-JO (pBINPLUS-2.5-CpCDV) was extracted and subjected to PCR analysis using the CpCDV-JO specific primer pair CpCDVCP527F/CpCDVCP1145R (Table 1). After that, pBINPLUS-2.5-CpCDV was subjected to restriction digestion using *EcoRI* enzyme and the obtained ~2.5 kb fragment was self-ligated and subjected to Rolling circle amplification (RCA) using Φ 29 DNA polymerase (Thermo Scientific, USA) according to the manufacturer's instructions. RCA products were partially digested with *KpnI* enzyme to generate a dimer (~5 kb) of virus genome. The fragment of the expected size was gel-eluted and cloned into the binary pBINPLUS cloning vector to develop pBINPLUS-5-CpCDV-JO. As a final step, the GV3101 strain of *A. tumefaciens* was transformed with pBINPLUS-5-CpCDV.

2.3. Development of Tomato leaf curl New Delhi virus infectious clone

The dimeric molecule of viral DNA-A was generated by digestion of the recombinant pCambia2300 carrying the full-length DNA-A with *BamHI*. After self-ligation, DNA-A was subjected to RCA and the reaction products were partially digested with *BamHI* to produce the dimeric molecule. The obtained ~5.4 kb fragment was cloned into the *BamHI* site of pBINPLUS to generate pBINPLUS-5.4-ToLCNDV-DNA-A and transformed into *A. tumefaciens* and stored at -80°C. The dimeric molecule of viral DNA-B was generated by digestion of the recombinant pCambia2300 carrying the full-length DNA-B with *BamHI*. After self-ligation, DNA-B was subjected to RCA and the amplified RCA products were partially digested with *Acc65I* to produce the dimeric molecule. The obtained ~5.4 kb fragment was cloned into the *Acc65I* site of pBINPLUS to generate pBINPLUS-5.4-ToLCNDV-DNA-B and transformed into *A. tumefaciens* and stored at -80°C.

The infectivity of the CpCDV-JO and ToLCNDV-JO clones has been tested on different plants species (Table 2) by infiltrating the lower side of the leaves with the bacterial suspension using a syringe without needle.

Table 2. Response of different plant species inoculated with ToLCNDV-JO and CpCDV-JO clones.

Clones	Plant Species	No. of inoculated plants	No. of symptomatic plants	PCR +ve/ total
ToLCNDV-JO	<i>Solanum lycopersicum</i> cv. Baladiah	20	1	13/20
	<i>Nicotiana benthamiana</i>	10	10	10/10
	<i>Cucurbita pepo</i> cv. Black Beauty	6	6	6/6
	<i>Cucurbita pepo</i> cv. JAZYF1	8	8	8/8
	<i>Citrullus lanatus</i> cv. Sugar Baby	8	1	6/8
	<i>Cucumis melo</i> var cv. Melon Piel de Sapo	7	7	7/7
	<i>Capsicum annuum</i> cv. Peperone Corno di Toro	7	0	4/7
	<i>Capsicum annuum</i> cv. Peperone Yolo Wonder	7	0	2/7
	<i>Solanum melongena</i> cv. Melanzana BIANCA	7	0	6/7
CpCDV-JO	<i>Nicotiana benthamiana</i>	10	10	10/10
	<i>Solanum lycopersicum</i> cv. Baladiah	20	19	19/20
	<i>Cucurbita pepo</i> cv. Black Beauty	6	0	6/6
	<i>Citrullus lanatus</i> cv. Sugar Baby	9	0	8/9
	<i>Cucumis melo</i> var cv. Melon Piel de Sapo	6	6	6/6
	<i>Capsicum annuum</i> cv. Peperone Corno di Toro	20	7	20/20
	<i>Capsicum annuum</i> cv. peperone Yolo Wonder	7	0	7/7
	<i>Solanum melongena</i> cv. Melanzana BIANCA	15	0	0/15

3. Results

3.1. Development of Chickpea chlorotic dwarf virus infectious clone and pathogenicity test

Before plants were inoculated with the recombinant bacterium and to confirm that the dimeric molecule of CpCDV-JO has been correctly transformed into *A. tumefaciens*, pBINPLUS-5-CpCDV-JO has been extracted from *A. tumefaciens* cells and subjected to double digestion with *SacI* and *SmaI* restriction enzymes. The expected size (~5kb) of CpCDV-JO dimer could be released (not shown).

Data presented in Table 2 summarize the response of different plant species to CpCDV-JO infection. Three plant species including pepper, melon and *N. benthamiana* developed disease symptoms 28 days post infection with CpCDV-JO (Fig. 1), and infection has been confirmed by PCR. On the other hand, all tomato plants were symptomless 28 days post inoculation and disease symptoms developed 60 days post inoculation and with time plants recovered. Plant species that were symptomless throughout the experiment were watermelon, zucchini, pepper (Peperone Yolo Wonder) and eggplant. When tested with PCR, the virus could be detected in 8 out of 9 inoculated watermelon plants, and all zucchini and pepper (Peperone Yolo Wonder) plants were found infected with the virus. All attempts to detect CpCDV-JO in inoculated eggplant plants failed. PCR results showed that 81.7% of all inoculated plants were infected with CpCDV-JO.

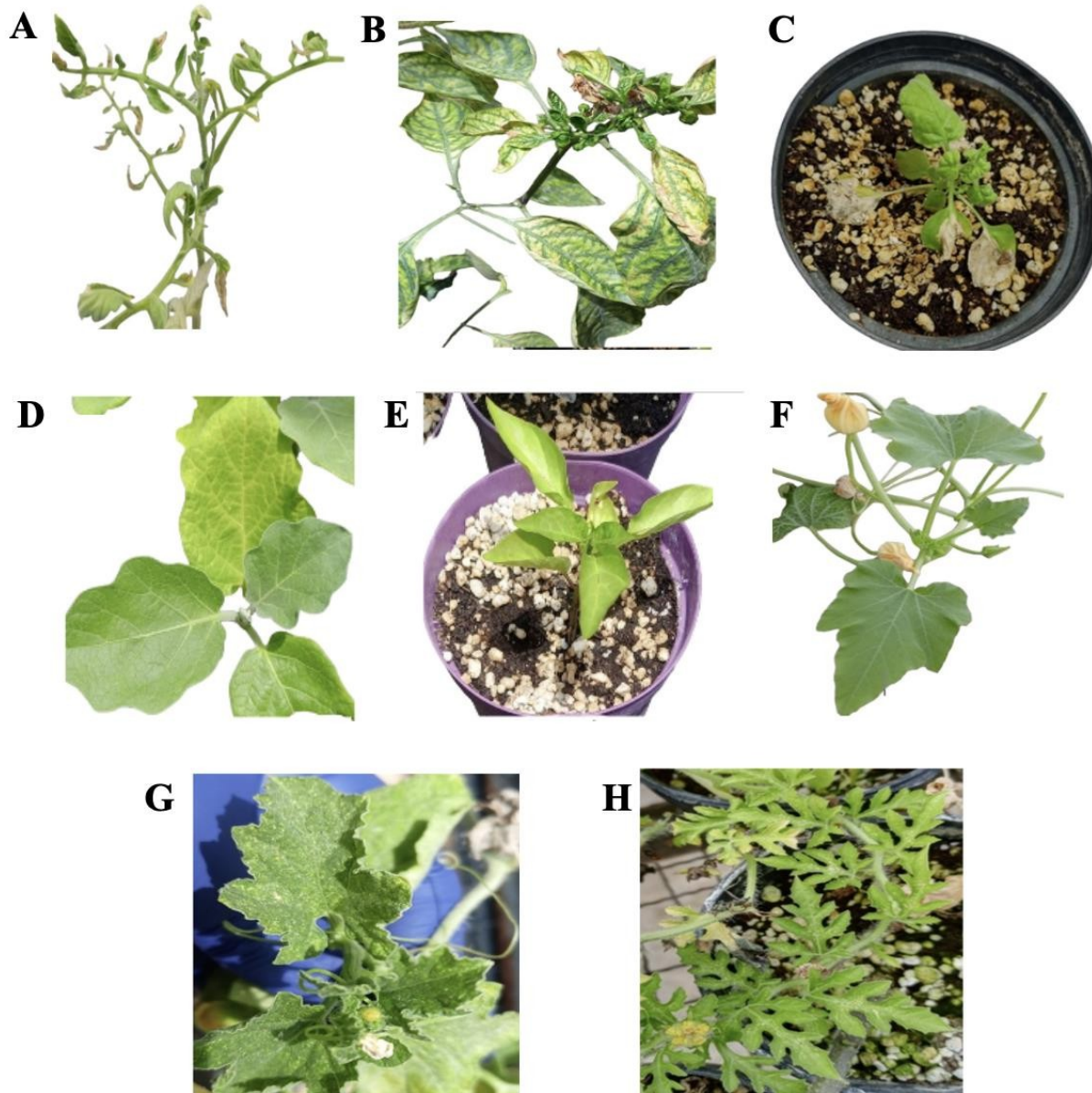


Figure 1. Plant species inoculated with CpCDV-JO infectious clone. A: tomato (Balady), B: pepper (Peperone Corno di Toro Rosso), C: *N. benthamiana*, D: eggplant (Melanzana BIANCA A UOVO), E: pepper (Peperone yolo wonder), F: zucchini (Black Beauty), G: melon (Melon piel de sapo) and H: watermelon (Sugar Baby).

3.2. Development of Tomato leaf curl New Delhi virus infectious clone and pathogenicity test

Before the ToLCNDV-JO clone was used to inoculate test plants, pBINPLUS-5.4-ToLCNDV-DNA-A and pBINPLUS-5.4-ToLCNDV-DNA-B plasmids were subjected to PCR using ToLCNDV-F2/ToLCNDV-R1 and ND-B-Clal1419R/ND-B-BamHI921F specific primer pairs, respectively (Table 1). Figure (2) shows the specificity of the recombinant plasmids. Plants belonging to *Solanaceae* and *Cucurbitaceae* families were inoculated with ToLCNDV-JO. All *N. benthamiana*, zucchini and melon plants developed disease symptoms, and only one tomato

and watermelon plant was symptomatic (Fig. 3). Symptoms of chlorotic mosaic and curling developed on tomato plant cv. Balady, while severe leaf curling (Fig. 3, A), leaf yellowing and leaf malformation were observed on zucchini cv Black Beauty plants (Fig. 3, E). Melon plants showed chlorotic lesions, downward leaf curling and yellowing and *N. benthamiana* plants developed leaf curling, yellowing and mottling symptoms (Fig. 3. I). On the other hand, all eggplant and pepper plants remained symptomless throughout the period of the experiment (Fig. 3 H and I).

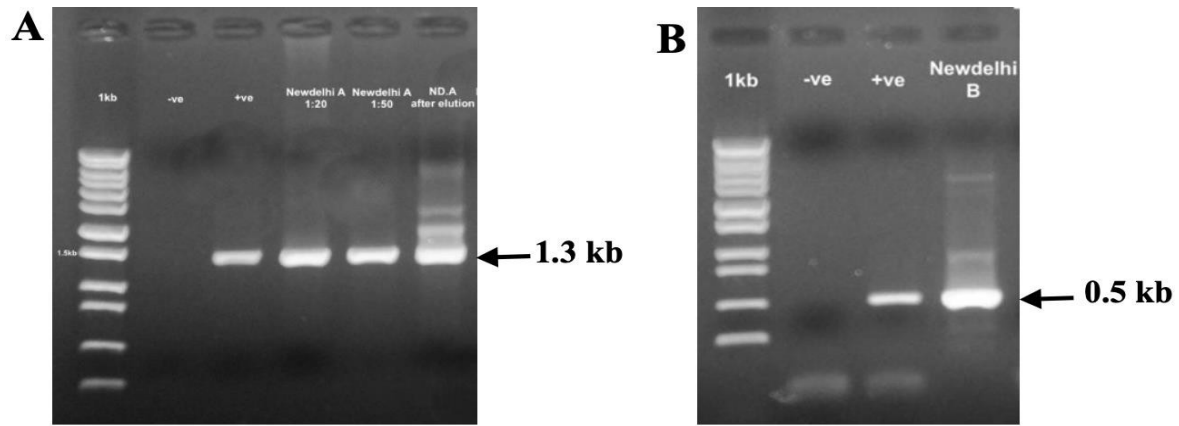


Figure 2. Confirmation of pCambia2300-5.4-ToLCNDV-DNA-A and pCambia2300-5.4-ToLCNDV-DNA-B identities by PCR. A: pCambia2300-5.4-ToLCNDV-DNA-A amplified using primer pair ToLCNDVF1/ToLCNDV R2; B: pCambia2300-5.4-ToLCNDV-DNA-B amplified using ND-B-ClaI1419R/ND-B-BamHI921F primer pair. 1kb: 1 kb DNA ladder; -ve: negative control (PCR mix components without template); +ve: positive control (DNA-A or DNA-B of ToLCNDV-JO).

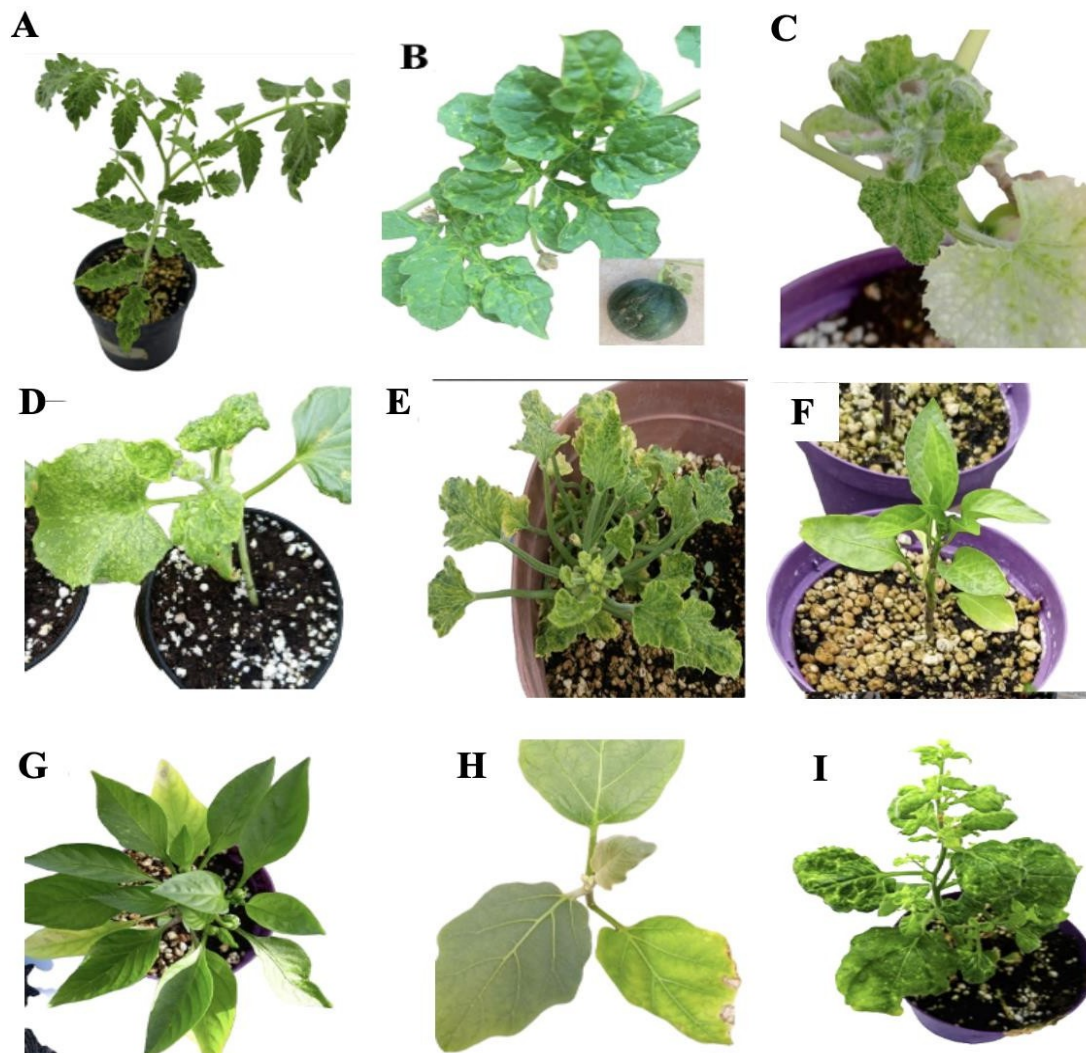


Figure 3. Plant species inoculated with ToLCNDV-JO infectious clone. A: tomato (Balady); B: watermelon (Sugar baby); C: squash cv. JAZYF1; D: melon cv. piel de sapo; E: zucchini cv. Black Beauty; F: pepper (Peperone Corno di Toro Rosso); G: pepper cv. peperone yolo wonder; H: eggplant cv. Melanzana BIANCA; I: *N. benthamiana*.

It is important to note that when asymptomatic plants were tested for ToLCNDV-JO infection by PCR, many plants were found positive for the virus (Table 2). For example, one tomato plant showed disease symptoms while 13 other asymptomatic plants out of 20 plants were found positive for the virus by PCR. Similarly, one

watermelon plant showed chlorotic mosaic and curling, while 6 plants were asymptomatic but were positive for the virus as confirmed by PCR. PCR results demonstrated that the infection rate of ToLCNDV-JO was 77.5%.

4. Discussion

The primary goal of this study was to develop infectious clones of two plant viruses belonging to the *Geminiviridae* family and to investigate their pathogenicity in host plants. This goal is accomplished by inserting multimers of virus genomes into binary vectors, which are subsequently introduced into an *Agrobacterium* and used to infect host plants (Saad *et al.*, 2021).

The first infectious clone developed in this study was CpCDV-JO. Previous studies examined the pathogenicity of CpCDV as a control on *N. benthamiana* plants in the presence of betasatellite of CLCuMV (CLCuMβ). The results and symptoms development were in accordance with results obtained in this study; however, the pathogenicity of the CpCDV-JO infectious clone has been investigated without the co-infection with beta-satellites. In a previous study, Khalid *et al.* (2017) tested the pathogenicity of a CpCDV infectious clone on tomato plants. Infected plants showed yellow lesions and curling of newly developed leaves, and plants were severely stunted. Similar disease symptoms were also observed on tomato plants inoculated with CpCDV-JO two months post inoculation. Possible explanations of symptoms delay include low number of CpCDV-JO particles (titer) introduced into tomato cells and the number of tomato cells that were successfully infiltrated with the bacterial suspension. Furthermore, environmental conditions such as temperature, light and humidity prevailed in our greenhouses may also played a role in symptoms expression (Pares *et al.*, 1996).

CpCDV-JO was able to induce upward leaf curling and yellowing symptoms on pepper plants cv. Peperone Corno di Toro Rosso. Similar results were reported by Akhtar *et al.* (2011). It is worth mentioning that the infection rate of CpCDV-JO was low (7 symptomatic plants out of 20 infected plants). This may be due to low viral load (Kumar *et al.*, 2022). It is important to note that although all CpCDV-JO infected pepper, watermelon and zucchini plants did not develop disease symptoms; however, the virus could be detected in these plants by PCR. These results suggest that asymptomatic plants may possess high level of resistance against CpCDV-JO infection. From epidemiological point of view, asymptomatic host plants infected with plant viruses pose high risk especially in the presence of insect vectors.

CpCDV was reported to infect cucurbit plants including watermelon and squash. In Egypt, squash plants with severe leaf curling, yellowing, stunting and reduced fruit size were observed in different fields in Giza governorate. PCR results showed that plants were mixed infected with CpCDV and *Squash leaf curl virus* (SLCV). However, authors did not show whether the observed disease symptoms were due to SLCV or CpCDV infections (Fahmy *et al.*, 2015). In another study, Zaagueri *et al.* (2017) reported that CpCDV is the causal agent of hard fruit syndrome of watermelon cv. Crimson in Tunisia. On the light of this study, it can be speculated that watermelon plants inoculated with CpCDV-JO may show disease symptoms on fruits. This may be true since 8 out of 9 plants were PCR positive for CpCDV-JO infection. In addition, further attempts must be done to inoculate other watermelon cultivars to investigate the susceptibility of these cultivars to CpCDV-JO infection.

ToLCNDV-JO was recently detected on cucurbits, tomato and pepper crops in Jordan (unpublished data). In this study, an infectious clone of the virus has successfully been developed. The infectivity assay test showed high (100%) infection rate of ToLCNDV-JO clone. Data presented in this study demonstrated that ToLCNDV-JO clone could infect melon, watermelon, tomato, squash, zucchini and *N. benthamiana* plants. A large body of literature showed similar results obtained by different research groups (Cifuentes *et al.*, 2017; Mei *et al.*, 2023; Vo *et al.*, 2023). It is worth noting that only one ToLCNDV-JO infected tomato plant cv. Balady out of 20 plants showed mild vein-yellowing symptoms. In agreement with these data, Yamamoto *et al.* (2021) reported that most tomato plants inoculated with ToLCNDV-[ES-Alm-Cuc-16] were symptomless and only few plants developed slight vein yellowing symptoms. In the present study, latent infections with ToLCNDV-JO were observed in pepper and eggplants. No disease symptoms could be detected on these plants; however, PCR results showed that most of eggplants were positive for ToLCNDV-JO infection. It is important to note that only 2 out of 7 pepper plants were found infected with ToLCNDV-JO. A possible explanation may be that the used pepper cultivars were not susceptible to the virus. Therefore, more trials must be conducted to inoculate other pepper cultivars, especially those commonly grown in Jordan. Against our results, Mnari-Hattab and co-authors reported that pepper plants were severely infected with ToLCNDV (Mnari-Hattab *et al.*, 2022). Data presented in this study also showed that eggplant is a symptomless host of the ToLCNDV-JO. However, eggplants infected an Italian isolate of the virus developed severe disease symptoms (Parrella *et al.*, 2018). More studies are necessary to evaluate the response of other host plants, commonly grown in Jordan, to CpCDV-JO and ToLCNDV-JO infections.

5. Conclusions

This is the first study in Jordan that demonstrates the development of infectious clones to CpCDV and ToLCNDV. These clones should be available to other scientists in Jordanian universities who are doing research in the field of plant virology. Furthermore, the outcomes of this study can be useful to seed companies who are interested in developing germplasm resistant to plant viruses.

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