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Research article

Biological and molecular characterization of citrus bent leaf viroid

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A R T I C L E I N F O

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Host range

A B S T R A C T

Background and aim: Citrus bent leaf viroid (CBLVd) is one of the emerging and widely distributed viroids in citrus-growing areas of the world, including Pakistan. Previously, CBLVd has been reported in Pakistan for the first time in 2009. Therefore, characterization of CBLVd is required to monitor the viroid status in the citrus orchards concerning citrus decline.
Methods: Biological and molecular characterization of CBLVd was studied through biological indexing and confirmation through RT-PCR, followed by phylogenetic analysis of selected CBLVd isolates. Among four citrus cultivars viz., Kinnow (*Citrus nobilis* × *Citrus deliciosa*), Mosambi (*C. sinensis*), Futrell's Early (*C. reticulata*) and Lemon (*C. medica*) used as indicator plants for two transmission trials viz., graft inoculation and mechanical inoculation. Graft inoculation was more efficient than mechanical inoculation.
Results: Symptoms such as mild mosaic, slight backward leaf bending, and leaf curling were observed after eight months' post-inoculation. *Citrus nobilis* × *Citrus deliciosa*, *C. reticulata* and *C. sinensis* were more sensitive to CBLVd as compared to *C. medica.* Inoculated plants were reconfirmed through RT-PCR amplicons of 233 bp. The phylogenetic tree of submitted sequences showed more than 90% relevance of CBLVd in Pakistan compared to the rest of the world.
Conclusions: There was slight genetic variability, but more than 90% relevance was found among the submitted and already reported CBLVd isolate from Pakistan. Scanty literature is available regarding the biological and molecular studies of CBLVd in Pakistan. Therefore, the transmission and molecular characterization of CBLVd in Pakistan were studied for the first time.

1. Introduction

Virus and virus-like pathogens in citrus have different modes of transmission: insect, mechanical, and vegetative propagation (grafting and budding). Vegetative propagation is very useful in transmitting virus and virus-like pathogens from diseased to healthy plants in citrus [1,2]. Like citrus viruses, viroids are transmitted through grafting, cuttings and tubers. The cambium union of rootstock

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and scion occurs in grafting [3]. The pollen and seeds are the other means of transmission in members of the family of *pospiviroidae*. Pruning tools like knives, blades and human hands have high efficacy in the mechanical transmission of viroids [4–6]. More than 30 citrus viruses and viroids are graft-transmissible [7]. Like other citrus viroids, transmission trials of Citrus bent leaf viroid (CBLVd) through the sap, bud-wood, and grafting on indicator plant Ertog citron (Citrus *medica*) were carried out [8,9]. CBLVd belongs to the genus *Apscaviroid* and has shown its transmissibility through pruning tools and grafting [6,10]. CBLVd infects all the citrus cultivars regardless of rootstock [11,12]. Symptoms due to viroids infection are bark cracking, backward leaf bending, yellowing of leaves, dwarfing and stunting, which lead to citrus decline 13–15. Advanced detection assays have been established for the indexing of citrus viroids. Routine and regular detection of viroids is done through reverse transcriptase polymerase chain reaction (RT-PCR), which is sensitive and time-saving [16]. Multiplex RT-PCR has been found to quickly and simultaneously detects the number of viroids [17]. However, RT-PCR and its variants are sensitive but sometimes fail to detect lower concentrations of viroids in the host plants [18,19]. Therefore, biological indexing and RT-PCR followed by sequential polyacrylamide gel electrophoresis (sPAGE) give best results [20]. sPAGE has been used for the nucleic acid analysis of different CEVd and CBLVd [21,22]. The sPAGE confirmed the mobility of the circular RNA while detecting the citrus viroids [23]. CBLVd has been detected through biological indexing and sPAGE to characterize isolates [23,24]. Sometimes, additional smaller RNA is detectable through sPAGE [25]. Biological indexing of CBLVd is reliable but is time-consuming, while RT-PCR and sPAGE are quick, sensitive and time-saving [19,26,27]. The various isolates of CBLVd have been characterized through biological indexing and sPAGE [13,28,29]. After the first report of CBLVd in Pakistan [11], the characterization of CBLVd is the dire need of time to monitor the prevalence of CBLVd in declining citrus orchards. Therefore, this research was carried out to characterize the CBLVd biologically on different citrus indicators and to evaluate the sensitivity of different citrus cultivars in Pakistan. This study helps develop quick and accurate detection techniques for early intervention. In Pakistan, scanty literature and very limited work on the molecular and biological characterization of citrus bent leaf viroid through sPAGE is available.

2. Materials and methods

2.1. Biological indexing and mechanical transmission

These experiments continued a previous study regarding the survey for detecting citrus bent leaf viroid in citrus growing areas of Sargodha, Pakistan. The samples were collected from different regions of the Sargodha district, a citrus hub in Punjab, Pakistan. Symptomology was the basic criterion, such as backward leaf rolling, pin holing stunting and dwarfing. A total of 150 samples/area were collected to confirm the CBLVd. The sampling methods and acquisition of samples have been described in a previously published article [30]. PCR-confirmed samples were used for the transmission trials. The transmission trials were carried out to monitor the reactivity of citrus varieties towards CBLVd. Four citrus cultivars viz., Kinnow (*Citrus nobilis* × *Citrus deliciosa*), Musambi (*C. sinensis*), Feutrall's early (*Citrus Reticulata*), and lemon (*C. limon*) were used as indictor plants for graft inoculation of infected citrus samples [27,31]. The indicator plants were RT-PCR confirmed CBLVd free. Fifteen plants of each variety were used during the transmission trial through vegetative propagation. Twelve plants of each variety were grafted and inoculated from already RT-PCR confirmed infected bud-wood [30], whereas three plants were grafted from healthy scion and kept as control (Fig. 1A and B). The infected CBLVd source was taken from mandarin 'Kinnow' samples. All the indicator plants were maintained in an insect-free, temperature-controlled greenhouse at 28–32 °C for symptom development. A separate trial for mechanical transmission was carried out of the same number of indicator plants, and conditions followed in the vegetative propagation trail. Crude sap from the CBLVd infected samples was extracted

Fig. 1. Graft inoculation of CBLVd on citrus cultivars (A and B).

in 0.02 M phosphate buffer [32] followed by the mechanical inoculation on indicator plants. RT-PCR using the specific primers reconfirmed the presence of CBLVd [30].

2.2. Sample acquisition and RT-PCR conditions

The infected budwoods from RT-PCR confirmed CBLVd infected mandarin trees were collected [23]. The bud wood with plenty of sap and two to three buds was used for vegetative propagation. The leaves from each citrus variety were used for the RT-PCR assay after the appearance of symptoms. The RNA from positive samples was extracted using the method of Nakahara et al. [33], with slight modification as described by Iftikhar et al. [42]. The primers from the previous study were used for this experiment [30]. The RT-PCR conditions were as follows with slight modification [23]: denaturation was done at 94 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 30 s. Then, the temperature was lowered to 60 °C for 60 s before annealing at 60 °C for 10 s. The primers were extended at 72 °C for the first 10 s and then for 5 min. PCR products were amplified on 2% agarose gel in 1x TBE buffer at 100 V for 50 min. The gel was stained with ethidium bromide for 10 min, followed by washing it with distilled water for 5 min. The 100 bp DNA ladder was used to identify bands during all the PCR analyses.

2.3. Phylogenetic analysis

The PCR amplicons were sent for sequencing directly, and after the initial NCBI blast of all PCR positive amplicons, the selected positive isolates MW183820 (CBLVd14) and MW183821 *(CBLVd15)* sequences were submitted to the GeneBank. The phylogenetic analysis of submitted sequences was carried out using the maximum-likelihood method in the MEGA 6.1 program [34].

3. Results

Biological indexing and mechanical transmission of Citrus bent leaf viroid (CBLVd) were observed in four citrus cultivars: Kinnow, Mosambi, Feutrell's early and lemon. Graft and sap inoculation showed different transmission rate. CBLVd was transmitted through grafting and mechanical inoculation in all the citrus cultivars. Different symptoms such as slight leaf backward bending, mild mosaic and curling of leaves were observed after eight months post-inoculation in the 6-months inoculated citrus plants (Fig. 2A–D). Graft inoculation was more efficient than mechanical inoculation (Tables 1 and 2). In mechanical transmission among all the citrus cultivars, Kinnow showed the highest transmission rate at 66.66%, at par with Mosambi. Lemon showed the lowest transmission rate at 25% (Table 1). A similar trend of transmission of CBLVd was observed in graft inoculation but at a higher rate. The CBLVd was transmitted at 91.66% in Kinnow, followed by Mosambi at 75%. Lemon also showed 41% CBLVd transmission (Table 2). Based on the results, Kinnow and Mosambi were more susceptible to CBLVd, while lemon was found to be least susceptible to CBLVd infection in nature. All the citrus plants in transmission trials were also subjected to PCR for confirmation after the production of symptoms (Fig. 3). The samples were subjected to RT-PCR and found the specific bands using primers to detect CBLVd. The specific amplified fragments to the CBLVd were obtained from the citrus samples of mandarin, Kinnow orange and Mosambi because these are two widely cultivated species in the citrus orchards of Pakistan and in the Asian region as well. The phylogenetic analysis showed that the two submitted

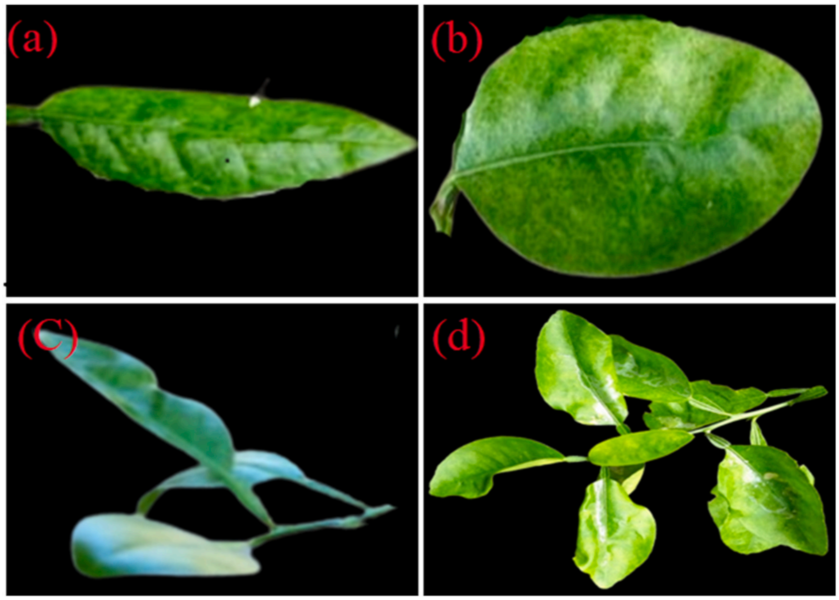

Fig. 2. Citrus cultivars showed the symptoms of Mild mosaic (A and B); backward leaf bending (C); Leaf curling (D).

Table 1
Mechanical inoculation of CBLVd on different citrus cultivars.

Sr#	Variety	Number of Inoculated Plants	Number of plants with successful inoculation followed by RT-PCR confirmation	Number of plants with no symptoms or died	Symptoms observed	Infection %
1	Kinnow	15 (12 + 3 control)	8	3 died +4 with no symptoms including control	Mild mosaic and mid vein necrosis, Slight leaf bending	66.66
2	Mosambi	15 (12 + 3 control)	8	2 died+ 5 with no symptoms including control	Mild mosaic and mid vein necrosis	66.66
3	Feutrell's Early	15 (12 + 3 control)	6	9 with no symptoms including control	Mild mosaic and leaf curling	50
4	Lemon	15 (12 + 3 control)	3	12 plants showed no symptoms including control	Mild mosaic	25

Table 2
Graft inoculation of CBLVd on different citrus cultivars.

Sr#	Variety	Number of Inoculated Plants	Number of plants with successful inoculation followed by PCR confirmation	Number of plants with no symptoms or died	Symptoms observed	Infection %
1	Kinnow	15 (12 + 3 control)	11	4, including control showed no symptoms	Mild mosaic and slight leaf bending	91.66
2	Mosambi	15 (12 + 3 control)	9	6, including control showed no symptoms	Mild mosaic and slight leaf bending	75
3	Feutrell's Early	15 (12 + 3 control)	7	8, with no symptoms including control	Mild mosaic	58.33
4	Lemon	15 (12 + 3 control)	5	10, with no symptoms including control	Mild mosaic	41.66

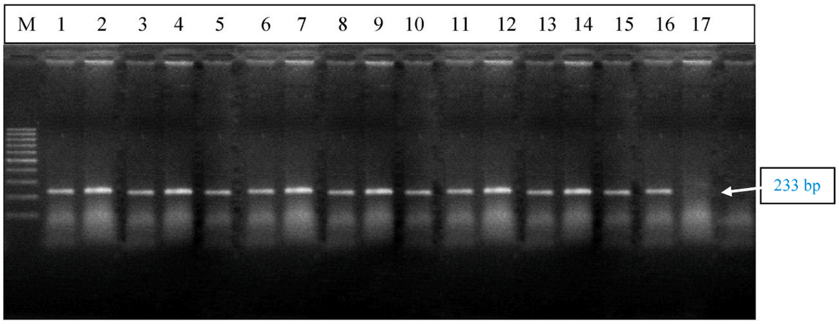

Fig. 3. RT-PCR confirmed the presence CBLVd in inoculated citrus samples. Lane M: Marker (100 bp DNA ladder); Lane 1–4: CBLVd in Kinnow; Lane 5–8: CBLVd in Mosambi; Lane 9–12: CBLVd in Feutrell's early; Lane 13–16: CBLVd in Lemon; Lane17: Negative Control; Lane 18: Healthy sample.

isolates were 100% similar and had a more than 90% sequence similarity with the CBLVd isolates in different parts of the world. It was also interesting to find that the sequences of submitted CBLVd isolated had slight genetic variability from the already reported isolate from Pakistan. The genetic relevance among those was more than 90 % (90–92%).

4. Discussion

The PCR confirmed the presence of viroids by producing the amplicons of 233bp [17]. The sequenced clones were 100% alike; therefore, mandarin 'kinnow' was used as an infected source on all the indicator plants and symptoms were observed (Tables 1 and 2). In mechanical transmission, the mild mosaic was observed in all four varieties viz., Kinnow, Mosambi, Feutrell's early and lemon. Whereas mid vein necrosis and slight leaf bending in Kinnow were also observed. Mosambi and Feutrell's early showed mid-vein necrosis, leaf curling, and mild mosaic. Similarly, in transmission through vegetative propagation, mild mosaic and slight leaf bending were observed in Kinnow and Mosambi. Meanwhile, Feutrell's early and lemon showed a mild mosaic only. Like other citrus viroids, CBLVd is widely distributed and can deteriorate the quality of citrus. CBLVd alone or in combination is leading the citrus orchards toward decline [30]. Our results were in close conformity with previous literature. Results regarding transmission and symptomology in our study are in accordance with the results of Cao et al. [11], Mathioudakis et al. [13] and Pagliano et al. [35]. The expression of symptoms in response of CBLVd infection in our study was similar to the symptoms reported by Iftikhar et al. [42]. Citrus viroids induce characteristic symptoms like dwarfing, leaf bending, mid-vein, and petiole necrosis [24]. The slight leaf-bending symptoms on the inoculated calamondins correspond to the symptoms induced by CBLVd infections in citrus regardless of varieties, as reported in Etrog citron [36].

Different means of transmission for CBLVd, such as through cell sap and grafting, have been well studied [8,9]. Symptoms expression in citrus viroid infection varies depending upon the host reaction [36]. Characteristic symptoms of single viroid infection have been observed on *Ertog citron* which is considered a biological indicator of CBLVd [10,37]. Successful transmission of citrus viroid, including CBLVd, through mechanical and propagative means has also been reported [6,38,39]. However, mechanical inoculation by stem perforation or leaf friction is commonly used [3,40]. The transmission mode of citrus viroids should be monitored to avoid contact loss due to viroid [41]. Previously described results have evidence of mechanical and graft transmissibility of citrus viroids on a few indicator plants.

Moreover, complete viroid sequencing is needed to characterize the CBLVd isolate and its impact on the expression of symptoms in the host. Therefore, our study will be a link to investigate the host reaction towards different citrus viroids concerning CBLVd. To our knowledge, this is the first transmission study on CBLVd transmission in Pakistan. The sequences from our study had high similarity with the isolates from Uruguay, Iran, China, Malaysia, Japan, and Pakistan [10, 11, 16, 33, 42]. Phylogenetic analysis also revealed that all the Asian isolates of CBLVd are more similar. Still, slight genetic variation occurs with already known isolates [11] from Pakistan (Fig. 4). This slight variation may be due to our study's partial genome sequencing. It showed that the two submitted isolates had a high sequence similarity from 90 to 98% with the CBLVd isolates in the different parts of the world. The sequences from our study had high similarity to Uruguay, Iran, Chinese, Malaysian, Japanese and Pakistan isolates. However, the sequences of these isolates had a slight variation from previously reported Pakistani isolates compared to the isolates from the rest of the world. This is more likely due to the partially sequenced CBLVd genome in our study.

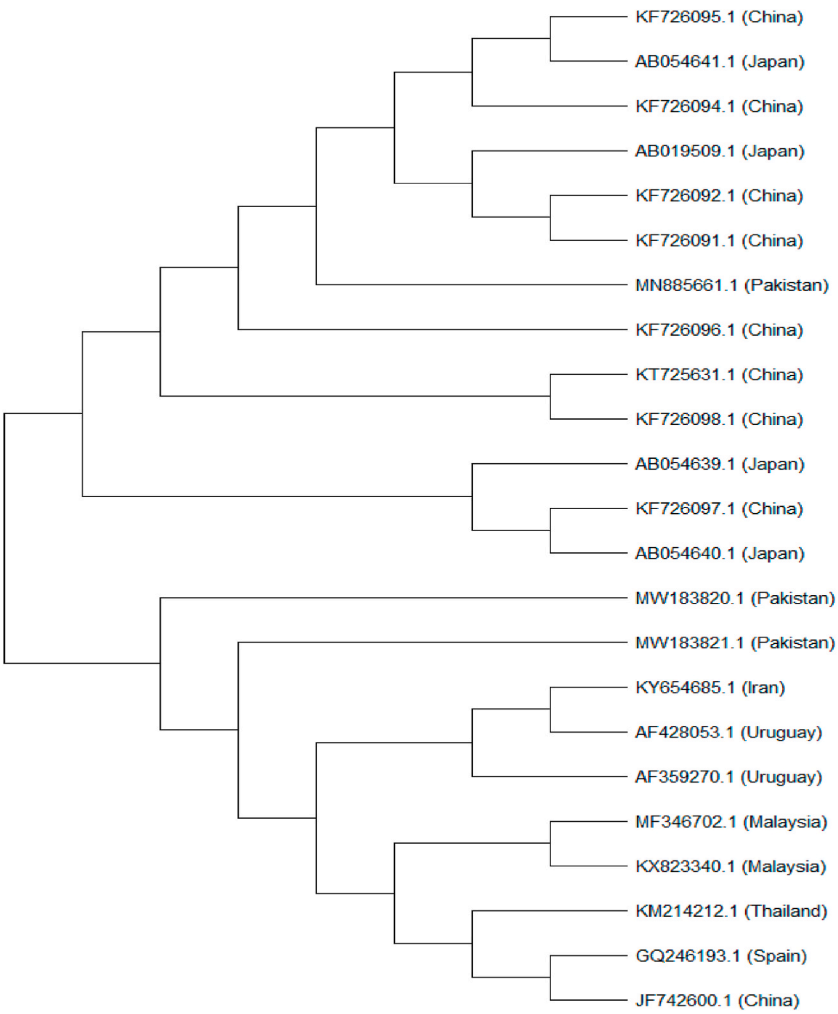

Fig. 4. Phylogenetic analysis of Citrus bent leaf viroid isolates *MW183820.1* and *MW183821.1* from *Citrus reticulata* and *C. sinensis* from Punjab, Pakistan.

Moreover, CBLVd has three isolates [12]; therefore, the exact characterization and determination of CBLVd isolates is possible upon complete genome sequencing. The complete genome sequencing and characterization lead to the future direction of research related to CBLVd in Pakistan. A distinct CBLVd isolates CVd-I-LSS size of 325–330 nt had a similarity in sequence of 82–85% with CVd-I [23]. Our results of biological indexing and sequencing also support the first report of CBLVd in Pakistan [11]. CBLVd like RNA molecules were successfully characterized and separated from the other citrus viroids through sPAGE [23]. Based on our results, the study concluded that biological indexing, mechanical transmission, RT-PCR, and sequence analysis provided the identity of CBLVd from the citrus samples.

5. Conclusions

It is concluded that CBLVd infects different commercially available citrus cultivars in citrus-growing areas of Punjab, Pakistan. All citrus cultivars used in the study were found to be susceptible to CBLVd with different symptoms in different citrus cultivars. The transmission rate was maximum in mandarin and sweet orange as these are the two major cultivated varieties in citrus growing areas of the Punjab, Pakistan. An emerging viroid's infection and transmission rate can be lethal to citrus quality. Lemon has been found with the minimum transmission rate. The transmission trials showed that grafting is an efficient transmission mode of citrus viroids. The phylogenetic analysis revealed the genetic variation among the CBLVd from Pakistan and the rest of the world. Characterization of different CBLVd isolates is a dire need to sequence the complete genome of the isolates present in Pakistan.

Limitations

Biological and molecular characteristics are essential for efficient management of CBLVd. It's challenging to differentiate from other viroids that produce comparable symptoms. It is necessary to use sensitive molecular methods like RT-PCR. As there is no known control, management is achieved by resistance cultivars and hygienic practices. Questions remain unanswered about long-term effects, resistance development potential, and particular transmission routes.

Data availability statement

Data will be made available on request.

Human and animal's rights

No human or animal participants were involved in this research.

CRediT authorship contribution statement

Mustansar Mubeen: Writing – review & editing, Writing – original draft, Visualization, Validation, Data curation. **Faheema Bakhtawar:** Writing – review & editing, Methodology, Investigation, Data curation. **Yasir Iftikhar:** Writing – review & editing, Supervision, Conceptualization. **Qaiser Shakeel:** Writing – review & editing, Formal analysis. **Ashara Sajid:** Writing – review & editing, Methodology, Investigation. **Rashid Iqbal:** Writing – review & editing, Software. **Reem M. Aljowaie:** Writing – review & editing, Project administration. **Talha Chaudhary:** Writing – review & editing, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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