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OPTIMIZATION OF PCR CONDITIONS AND DETECTION OF VIRUS AND VIROIDS AS DISEASE CAUSING AGENTS IN CITRUS CULTIVARS BY RT-PCR WITH SPECIFIC PRIMERS

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ABSTRACT

Citrus is one of the most important fruit crops in Sri Lanka. Yellowing of leaf vein and the downsized leaves in trees are the main problems associated with citrus cultivation in the country. These symptoms were suspected to be a result of zinc deficiency, lack of essential elements, infection with virus and viroid-like diseases or combination of above factors. This study was thus, conducted to investigate whether these symptoms are associated with virus and viroids by Reverse Transcription Polymerase Chain Reaction (RT-PCR). Downsized leaves with yellowing symptoms in leaf veins were randomly sampled from four citrus species namely, *Citrus reticulata* (Commercial Mandarin), *C. reticulata* (Heennaran), *C. sinensis* (Sweet orange) and *C. grandis* (Pumello), from the experimental fields of Fruit Crop Research and Development Centre, Horana, Sri Lanka. Samples were tested for the presence of *Citrus exocortis* viroid (CEVd), Citrus bent leaf viroid (CBLVd), Hop stunt viroid (HSVd), Citrus viroid III (CVd-III), Citrus viroid IV (CVd-IV), Citrus viroid OS (CVd-OS), a distinct variant of CBLVd (CVd-I-LSS) and Citrus tatter leaf virus (CTLV) using specific primers, which have been designed for each viroid. The amplified products of HSVd, CBLVd and CVd-I-LSS were obtained with RT-PCR at optimized annealing temperature of 55°C, using specific primers. According to the results, three viroids namely, HSVd, CBLVd and CVd-I-LSS were detected in more than 50% of the samples in all 4 species confirming their association with yellowing of leaf vein and downsized leaf symptoms.

KEYWORDS: Citrus cultivars, RT-PCR, viruses, viroids.

INTRODUCTION

Citrus (family: *Rutaceae*) is an evergreen tree grown widely in the tropical and subtropical regions in the world. Being the first fruit crop in international trade in terms of value, the world citrus production has increased significantly due to its high consumer demand resulting from beneficial health and nutritive properties. Most of the total citrus production is utilized for processing and fresh fruit consumption. In addition, essential oils and citrus pulp are also recovered from the fruit.

In Sri Lanka, Monaragala, Rathnapura, Kandy, Nuwara Eliya and Bibile are the most desirable places to grow citrus. Although Sri Lanka is having its own citrus cultivation, 8,347 mt of orange and lemon were imported in 2007 (Anon, 2008), due to the low fruit production as a result of many reasons including the susceptibility to pest and diseases.

Citrus canker disease (Broadbent *et al.*, 1992), greening disease of citrus (Jagoueix *et al.*, 1995; Jagoueix *et al.*, 1994; Gonzales, 1989), *citrus tristeza virus* (Bar-Joseph *et al.*, 1979; Powell and Pelosi, 1993), aphid and rust mite are the major economic pest and disease problems associated in Sri Lanka and worldwide. In addition, yellowing of leaf vein and downsized leaves in citrus trees are the recently reported main problem in citrus cultivation in the country. This condition is severe in Rahangala, Horana, Gannoruwa and Bibile research stations of the Department of Agriculture, Sri Lanka. In order to increase the productivity and yield of citrus, identification of causal organism and application of appropriate disease management programmes are essential.

Scientists suspect that the yellowing of leaf vein (N. Omori, Ehime Prefectural International Center, Japan) is caused due to zinc deficiency, lack of essential elements, infection of virus and viroid-like disease, and/or combination of these factors. Among them viruses and viroids diseases are the most critical agents, which could be easily transmitted causing unprecedented damage. There are no control measures available for virus and viroid diseases, other than eradication of the infected plants. Therefore, before the spread of the above diseases to other fields, it is imperative to identify whether yellowing of leaf vein and downsized leaves in trees are associated with virus and viroid-like diseases.

Viroids are single stranded, non translated, circular RNA molecules and not encapsulated in a protein coat. Therefore, viroids cannot be detected by serological methods but can be identified and characterized by methods based on molecular detection techniques such as nucleic acid hybridization, Polymerase Chain Reaction (PCR) and DNA micro arrays. Hence, it was considered important to develop a detection system for citrus viroid identification in Sri Lanka, by modifying the already published DNA methodologies. The objectives of this study were to develop and optimize the RT-PCR system conditions for detection of citrus viruses and viroids and detection of citrus viroids and Citrus tatter leaf virus (CTLV) in symptomatic plants using specific primers.

MATERIALS AND METHODS

The study was conducted at the Plant Virus Indexing Center (PVIC), Gabadawatta, Homagama, from February to October 2009.

Optimization of reverse transcription polymerase chain reaction (RT-PCR) system conditions

The purified virus samples were obtained from Japan (The Ehime Prefectural International Center) and taken as positive controls for each test.

Virus/viroid sample No. 1 was taken as the negative control for viroid No. 2 sample, and *vice versa* (Table 1). The dilution used was 1:10 (positive control: sterilized distilled water; N. Omori, Ehime prefectural International Center, Japan – personal communication).

Table 1. Purified viral/ viroid RNA and tube numbers imported from Japan

Tube	*Virus/viroid*	*Amount*
No. 1	CTLV, CEVd	60µL
No. 2	CBLVd, CVd-I-LSS, HSVd, CVd-III, CVd-IV, CVd-OS	60µL

Reverse transcription and cDNA synthesis

In each viroid and citrus tatter leaf virus (CTLV), the antisense primer (D 11, E 2, E-4, E 6, E 8, E 10, F 1 and D 9) was used for cDNA synthesis from viral RNA (Table 2). Reverse transcription reaction mixture contained 4µl of 5 × Reverse transcriptase buffer (M-MuLV), 2 µl of dNTPs (10mM), 1µl antisense primer, 0.1µl of RNAsin Ribonuclease inhibitor (Promega), 0.25µl of M-MuLV Reverse Transcriptase (Promega), 4.65 µl of deionized water and 8 µl of RNA template. In the thermocycler, the first strand synthesis was done at 37°C for 45 min followed by inactivation at 95°C for 15 min.

Calculation of annealing temperature

The annealing temperature for each primer was theoretically calculated (http://www.sabina.anzlovar.com).

Amplification of cDNA by polymerase chain reaction (PCR)

Citrus viroids and CTLV virus cDNA were amplified using 8 primer pairs, which were specific for each viroid and CTLV, respectively (Ito *et al.*, 2002b) (Table 2). For each virus and viroid, 7 PCRs were carried out separately with different annealing temperatures, starting from 52°C (N. Omori, Ehime prefectural International Center, Japan – personal communication), 53°C, 54°C, 55°C, 56°C, 57°C and 58°C (calculated value: http: //www.sabina.anzlovar.com).

The PCR mixture consisted of 5 µl of 5 x PCR buffer, 1.5 µl of $MgCl_2$ (25 mM), 1.0 µl of each dNTPs (10 mM), 1.0 µl of 10 mM sense primer (E 1, E 3, E 5, E 7, E 9, E 11, F 2 and D 10), 1.0µl of 10 mM antisense primer (D 11, E 2, E 4, E 6, E 8, E 10, F 1 and D 9) (Table 2), 0.25 units of *Taq* polymerase (5u/µL) (Promega), 3 µl of cDNA template and deionized water in a total volume of 25 µl.

The thermocycler conditions were as follows; initial denaturation 94^{0}C for 30 seconds, followed by 35 cycles, denaturation at 94^{0}C for 10 seconds, annealing at T_a°C (52°C, 53°C, 54°C, 55°C, 56°C, 57°C or 58°C) for 30 sec, extension 72°C for 1 min with final extension at 72°C for 10 min (N. Omori, Ehime prefectural International Center, Japan – personal communication).

Table 2. Specific primers for detection of citrus viroids and CTLV, annealing temperatures and the size of the amplified products

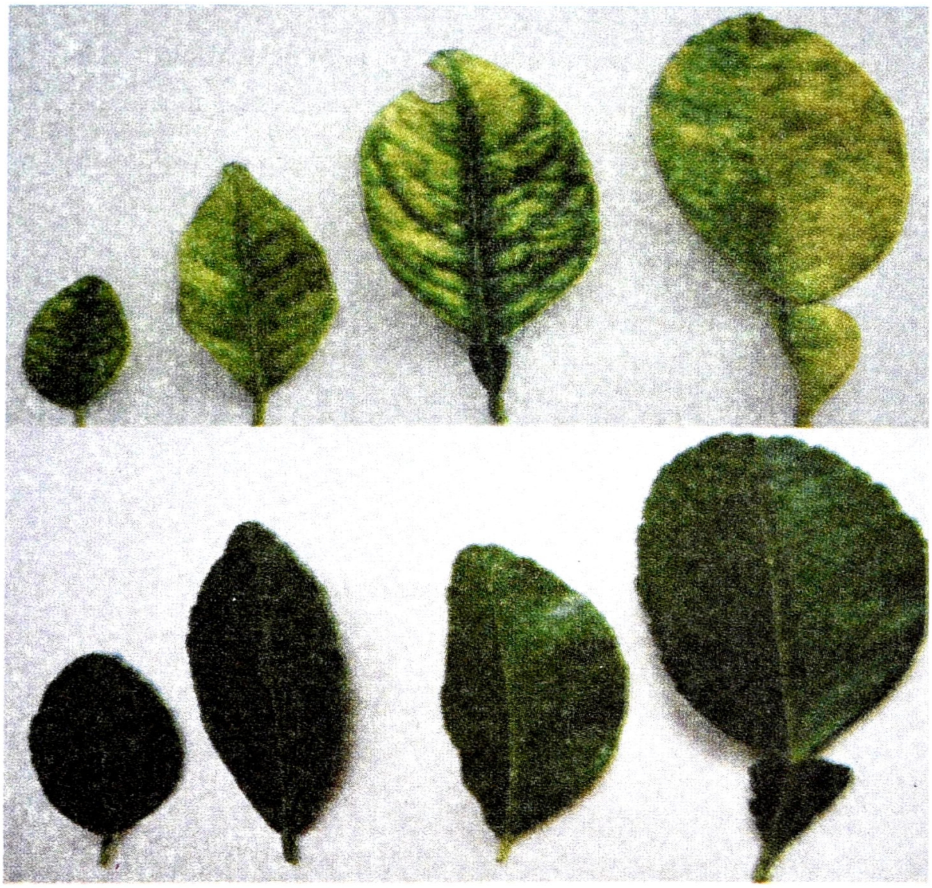
Viroid/ Virus	*Primer*	*Primer sequences (5'-3')*	*Annealing Temperature °C*	*Product size*
CEVd	D 11	CCGGGGATCCCTGAAGGACTT	58.038	371 bp
	E 1	GGAAACCTGGAGGAAGTCGAG	58.090	
CBLVd	E 2	TCGACGACGACCAGTCAGCT	58.080	233 bp
	E 3	TCCCCTTCACCCGAGCGCTGC	58.033	
CVd-I-	E 4	ACGACCGCTCAGTCTCCTCT	58.080	247 bp
LSS	E 5	CTGTAACCGGACCGGTCTCCTTC	58.057	
HSVd	E 6	CCGGGGCTCCTTTCTCAGGTAAGT	58.000	302 bp
	E 7	GGCAACTCTTCTCAGAATCCAGC	58.008	
CVd-	E 8	TCACCAACTTAGCTGCCTTCGTC	58.008	271 bp
III	E 9	CTCCGCTAGTCGGAAAGACTCCGC	58.041	
CVd-	E 10	TCTATCTCAGGTCGCGAAGGAAGAAGC	58.011	209 bp
IV	E 11	TCTGGGGAATTTCTCTGCGGGACC	58.000	
CVd-	F 1	GTCCGCTCGACTAGCGGCAGAGACC	58.024	166 bp
OS	F 2	CGTCGACGAAGGCATGTGAGCTT	58.052	
CTLV	D 9	TAGAAAAACCACACTAACCCGGAAATGC	58.043	456 bp
	D 10	CCTGAATTGAAAACCTTTGCTGCCACTT	58.044	

Detection of PCR products

The PCR products were analyzed on 1% agarose gel in TAE buffer (0.04M Tris acetate, 0.001M EDTA; pH 8) and visualized by ethidium bromide staining under UV transilluminator and photographed using a digital camera.

Detection of citrus viorids and CTLV in symptomatic plants

Samples were collected from the experimental blocks of a citrus field at the Fruit Crops Research and Development Centre (FCRDC), Kananwila, Sri Lanka. The downsized leaves with yellowing symptoms in leaf vein (Figure 1) were randomly sampled from four different citrus species namely, *Citrus reticulata* (Commercial Mandarin), *C. sinensis* (Sweet orange), *C. reticulata* (Heennaran) and *C. grandis* (Pumello). From each species, sampling was done in 5 trees (Table 3), with two samples per tree.



A B C D

Figure 1. Symptoms of yellowing of leaf vein and downsized leaves, compared to healthy leaves (bottom); A: *Citrus crenatifolia* (Heennaran), B: *Citrus reticulata* (Mandarin), C: *Citrus sinensis* (Sweet orange), D: *Citrus grandis* (Pumello)

RNA extraction

The 'Size fractionated silica extraction method' (Gunasinghe *et al.*, 2009) was used to extract and purify the viral RNA for RT-PCR assays. A symptomatic leaf lamina tissue of 100 mg was crushed with 1ml of lysis buffer (40 mM Tris-HCl pH 6.4, 17 mM EDTA, 4 M guanidine thiocyanate, 1% Triton X – 100 ml) using mortar and pestle. Then the lysate was transferred to an Eppendorf tube and centrifuged at 840g for 5 min. After centrifugation, the supernatant was saved and 10 µl of fractionated silica was added. Then the mixture was vortexed thoroughly, kept for 5 min and vortexed again. The mixture was centrifuged at 840g for 1 min and the supernatant was discarded. Then 1 ml of wash buffer (50% absolute ethanol 50 ml, 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 50 mM NaCl) was added, the tube was vortexed, centrifuged at 840g for 1 min, and the supernatant was discarded.

Table 3. Symptoms of test samples collected from FCRDC field

Species	Sample	Plant No:	Yellowing of leaf vein	Yellowing of whole leaf	Down sized leaves
Citrus reticulata (Commercial Mandarin)	CR-25	66	✓	x	✓
	HOCR-29	58	x	✓	✓
	HOCR-23	37	x	✓	✓
	HOCR-22	26	✓	x	✓
	HOCR-19	07	✓	x	✓
Citrus reticulata (Heennaran)	CC-08	03	✓	x	✓
	CC-11	04	x	✓	x
	CC-06	01	✓	x	✓
	CC-04	03	x	✓	✓
	CC-07	01	✓	x	x
Citrus grandis (Pumello)	CG-07	01	x	✓	✓
	CG-06	02	x	✓	x
	CG-05	02	✓	x	x
	CG-03	01	x	✓	✓
	CG-02	01	x	✓	✓
Citrus sinensis (Sweet orange)	VA-02	02	✓	x	✓
	VA-01	02	✓	x	✓
	AR-01	03	✓	x	x
	BI -01	01	✓	x	✓
	BI -03	01	✓	x	✓

✓ = Symptom present; x = Symptom absent

The wash buffer was added again to the tube, and was centrifuged at 840g for 1 min. The supernatant was discarded and 500 µl sterile distilled water was added, inverted several times and centrifuged at 840g for 1 min. The water was removed and the tube was centrifuged at 840g for 1 min to remove any remaining water. Then 20 µL of sterile distilled water was added to the tube and incubated at 56°C for 8 min. Thereafter, the tube was centrifuged at 840g for 3 min and 14 µL of RNA extract was removed to a PCR tube.

Reverse transcription and cDNA synthesis

The cDNA synthesis from viral RNA was done as described previously in this paper.

Amplification of cDNA by PCR

Citrus viroids and CTLV cDNA were amplified with 8 specific primer pairs as described previously in this paper. The PCR conditions were - initial denaturation at 94°C for 30 sec, followed by 35 cycles, denaturation at 94°C for 10 sec, annealing at 55°C (optimized) for 30 sec, and extension at 72°C for 1min with final extension at 72°C for 10 min.

Analysis of PCR products

The PCR products were analyzed as described previously in this paper.

Testing for reproducibility

Samples, which produced amplified products in the previous experiments, were tested with the procedure described in this paper to determine the reproducibility.

RESULTS AND DISCUSSION

Product amplification at different annealing temperatures

The amplified products for purified viral RNA were obtained only at the annealing temperature of 55°C. Positive results were obtained for CBLVd (233 bp), when purified viroid samples were amplified at 55°C as its annealing temperature. Its negative control did not give an amplified product. Purified viroid samples gave negative results, when it was amplified at annealing temperature 52°C, while its negative control also gave negative results for CBLVd (Figure 2).

Detection of citrus viroids and CTLV in symptomatic plants

All the samples tested did not give amplified products for CEVd, CVd-III, CVd-IV, CVd-OS and CTLV. Positive results were observed only for CBLVd, CVd-I-LSS and HSVd, where more than 50 % of the samples in all 4 citrus species gave amplified products for these 3 viroids (Table 4). The gel photographs of the amplified products (CBLVd, CVd-I-LSS and HSVd) viewed under UV transilluminater are shown in Figure 3, 4 and 5).

In the present study, already published systems were utilized with several modifications as annealing temperature 60°C for 1 min (Ito *et al.*, 2002b) and 52°C for 15 seconds (N. Omori, Ehime prefectural International Center, Japan – personal communication). Finally conditions were optimized using positive purified viral RNA imported from Japan into, 55°C for 30 sec in primer annealing. Extraction of RNA is extremely difficult due to its high degradable nature as a result of the abundance of RNase enzyme in the environment. As reported by Barbarossa *et al.* (2007), the RNA was extracted in this study by adsorption of RNA on to the silica particles after guanidinium buffer treatment, followed by protocol of Gunasinghe *et al.* (2009). Apart from reducing the cost of extraction, the protocol proposed by Gunasinghe *et al.* (2009) extracts RNA within an hour, which allowed indexing more samples during a shorter period of time.

Table 4. Results of product amplification, non amplification of 7 viroids and CTLV in symptomatic plants (10 samples were tested from each species)

Species	*Amplified or not amplified*	*CEVd*	*CBL Vd*	*CVd-I-LSS*	*HSVd*	*CVd-III*	*CVd-IV*	*CVd-OS*	*CTLV*
Citrus reticulata (Commercial Mandarin)	amplified	-	80%	60%	50%	-	-	-	-
	NA	100%	20%	40%	50%	100%	100%	100%	100%
Citrus reticulata (Heennaran)	amplified	-	100%	-	40%	-	-	-	-
	NA	100%	-	100%	60%	100%	100%	100%	100%
Citrus grandis (Pumello)	amplified	-	50%	50%	-	-	-	-	-
	NA	100%	50%	50%	100%	100%	100%	100%	100%
Citrus sinensis (Sweet orange)	amplified	-	40%	40%	60%	-	-	-	-
	NA	100%	60%	60%	40%	100%	100%	100%	100%

NA – not amplified

According to Ito and Namba (2002), HSVd and CVd -III were the first and second most frequently detected viroids in Japan, and CEVd, CBLVd, CVd-IV and CTLV were detected occasionally. However, in the present study the presence of CVd-III was not reported, where occasionally detectable CBLVd in Japan was present frequently. Although Malfitano *et al.* (2005) and Ito *et al.* (2002a) reported that CEVd, HSVd and CVd-III were the most widely detected viroids, CEVd was not detected in this study. Therefore, it can be assumed that most citrus species grown commercially, such as sweet orange, grapefruit and Commercial Mandarin are tolerant to CEVd (Malfitano *et al.*, 2005). Similar to the reports from Japan (Ito *et al.*, 2002a; Ito *et al.*, 2002b), CVd-I-LSS was found in citrus species in Sri Lanka during this study. Although CTLV was reported in Japan using RT-PCR (Ito *et al.*, 2002a; Ito *et al.*, 2002b; Ito and Namba, 2002) it was not found in the tested Sri Lankan samples.

Absence of amplification of other viroids except HSVd, CBLVd and CVd-I-LSS, may be due to many reasons. In the present study, the number of samples was limited to 40 from 20 trees due to limited time and facilities, and the chemicals used in RT-PCR are expensive. Cost of screening for one sample is approximately Sri Lankan Rs. 1000 and hence a minimum number of samples was screened in this study. If the sample size is increased, it may have been possible to identify other viroids, too. In addition, sample collection from many citrus growing areas may help to identify more diseases in citrus cultivations. If genetic variants of viroids were present in citrus species in Sri

Lanka, with respect to CVd-III, CVd-IV, CEVd, CVd-OS and CTLV, these may have got amplified with the specific primers designed for original viroids.

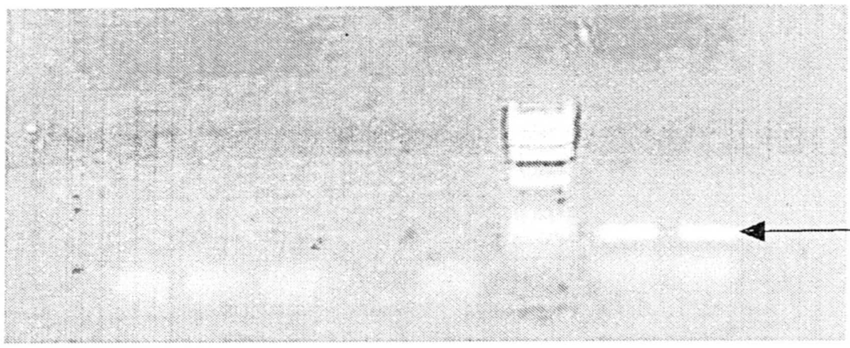


Figure 2. Amplified products of CBLVd, using primer pairs E02 and E03. Lane 1, 2: purified viral RNA (annealing temperature 52°C), Lane 3: negative control (annealing temperature 52°C), Lane 4: not loaded, Lane 5: negative control (annealing temperature 55°C), Lane 6: Molecular size marker (100bp DNA Ladder, Gene Script Coop), Lane 7, 8: purified viral RNA (annealing temperature 55°C)

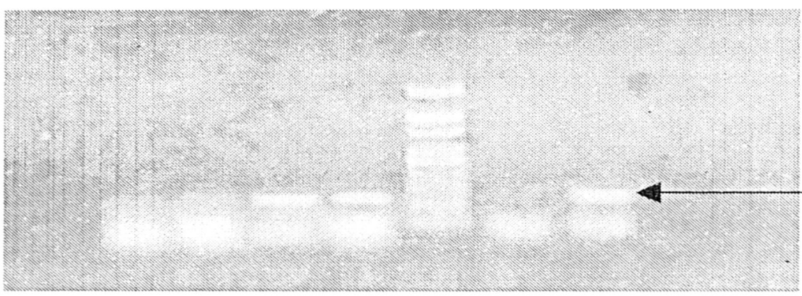


Figure 3. Amplified product of CBLVd, using primer pairs E 2 and E 3. Lane 1: CG-3 (*Citrus grandis* (Pumello)), Lane 2: VA-2 (*Citrus sinensis* (Sweet orange)), Lane 3: HOCR-23 (*Citrus reticulata* (Commercial Mandarin)), Lane 4; VA-1 (*Citrus sinensis* (Sweet orange)), Lane 5: Molecular size marker (100 bp DNA Ladder, Gene Script Corp), Lane 6: negative control, Lane 7: positive control

When reproducibility testing was conducted, previously amplified viroids were not present in some samples. As revealed by Guardo *et al.* (2005), viroid detection in field-grown trees can be influenced by climatic conditions and host species. Guardo *et al.* (2005) also reported that identification of CEVd by RT-PCR in field samples can be affected by place of collection and temperature, and that other viroids can be detected consistently only in warmer months. In the present study, the first batch of samples were collected in a drier season where the second batch of samples in order to test the reproducibility of the protocol, was collected after a heavy rainfall. These factors may have affected the results of reproducibility in HSVd amplification.

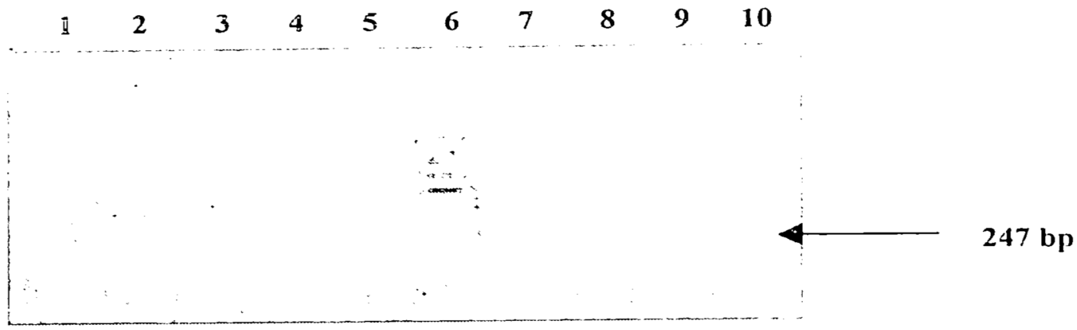


Figure 4. **Amplified products of CVd-I-LSS, using primer pairs E 4 and E 5. Lane 1: CC-8 (*Citrus reticulata* (Heennaran)), Lane 2: CG-2 (*Citrus grandis* - Pumello), Lane 3: CG-5 (*Citrus grandis* - Pumello), Lane 4: BI-1 (*Citrus sinensis* - Sweet orange), Lane 5; BI-3 (*Citrus sinensis* - Sweet orange), Lane 6: Molecular size marker (100 bp DNA Ladder, Gene Script Corp), Lane 7: CR-25 (*Citrus reticulate* - Commercial Mandarin), Lane 8: HOCR-19 (*Citrus reticulata* - Commercial Mandarin), Lane 9: negative control, Lane 10: positive control**

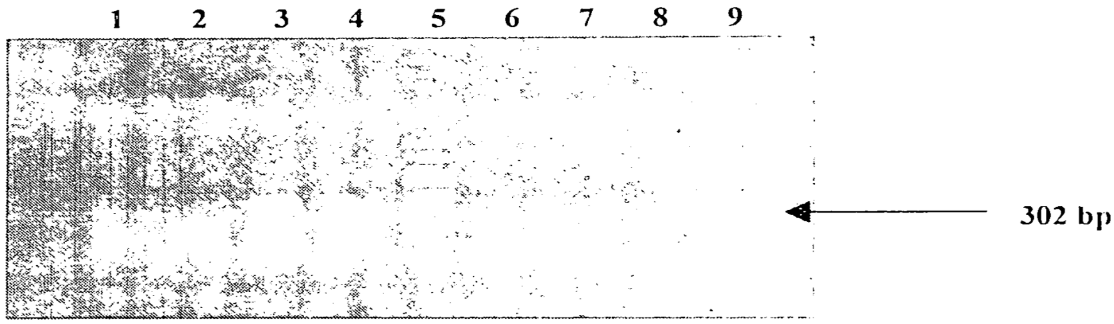


Figure 5. **Amplified products of HSVd using primer pairs E 6 and E 7. Lane 1: HOCR-29 (*Citrus reticulate* - Commercial Mandarin), Lane 2: CC-11 (*Citrus reticulata* - Heennaran), Lane 3: BI-1 (*Citrus sinensis* - Sweet orange), Lane 4: BI-3 (*Citrus sinensis* - Sweet orange), Lane 5: Molecular size marker (100 bp DNA Ladder, Gene Script Corp), Lane 6: CC-7 (*Citrus reticulata* - Heennaran), Lane 7: CG-3: (*Citrus grandis* - Pumello), Lane 8: negative control, Lane 9: positive control**

Generally, symptoms of HSVd and its variants are discoloration, gumming, browning of phloem tissues and bark cracking (Frison and Taher, 1991). However, the symptoms observed in Sri Lanka were yellowing of leaves. According to Ito *et al.* (2002a), mixed citrus viroid infections can be broadly present in a single tree. Malfitano *et al.* (2005) reported that most of the citrus trees (82%) are infected with more than one viroid and symptom expression may be influenced by synergistic or inhibitory interactions. This factor is more supportive for the modulating of symptoms in the present study. Similar to Ito *et al.* (2002a), the present study revealed the presence of non-specific bands together with positive products in some amplicons. However, no such fragments have been detected in healthy samples by Ito *et al.* (2002a).

The confirmation of detection of HSVD, CBLVD and CVD-I-LSS should be further supported by more information from sequential analyses in order to establish virus- and viroid-free citrus cultivation, to promote the citrus industry in Sri Lanka.

CONCLUSIONS

Amplified products of the viroids HSVd, CBLVd and CVd-I-LSS were obtained by RT-PCR, at an optimized annealing temperature of 55°C, using specific primers. Further these three viroids were detected in more than 50% of the samples in all 4 citrus species, *i.e. Citrus reticulata* (Heennaran), *C. grandis* (Pumello), *C. sinensis* (Sweet orange) and *C. reticulata* (Commercial Mandarin). The results confirm the association of these three viroids with the symptoms of yellowing of leaf vein and downsized leafves in citrus cultivars of Sri Lanka.

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