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Original Article

Novel variants of citrus exocortis viroid from southern of Iran

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Abstract

Background and Aims: Citrus exocortis disease is naturally limited to citrus plants and causes a serious disease on sensitive rootstocks such as trifoliate orange. The aim of this study was to sample new variants of citrus exocortis viroid from Fars trees with yellowing and suberization symptoms and comparison with other isolates of this viroid.

Materials and Methods: A number of symptomatic or symptomless citrus trees were sampled and studied for viroid detection. After RNA extraction, reverse transcription-polymerase chain reaction was conducted and the full length genome of viroids were cloned and sequenced. Three CEVd isolates from Fars were selected and used for comparison with four CEVd isolates from Mazandaran and a number of other sequences from GenBank.

Results: It was shown that on the basis of homology, three CEVd isolates from Mazandaran and all three CEVd isolates from Fars clustered into one group while one of the Mazandaran sequences fell apart.

Conclusions: Therefore we proposed that Iranian CEVd isolates except one isolate from north have the same origin.

Keywords: Viroid; CEVd; phylogeny; citrus viroids; citrus exocortis viroid.

Introduction

Viroids are the smallest single-stranded, circular plant pathogenic RNAs with a length of 246 to 401 nucleotides, rod-like secondary structure and no coat-protein (1& 2). So far there have been several reports of citrus viroids which belong to Pospiviroidae family and are represented in genuses including Pospiviroid, Aspcaviroid, Hostuviroid and Cocaviroid. CEVd is one member of the genus Pospiviroid and causes citrus exocortis disease. Infected trees grown on the trifoliate orange rootstocks represent stunting and bark scaling symptoms (3). Most species and varieties of citrus infected by CEVd do not show explicit macroscopic signs, only susceptible ones express mild to severe symptoms [Olson, 1968]. Researchers observed bark scaling and stunting symptoms by graft transmission of the disease from infected Washington navel orange tree (Citrus sinensis) which had no disease signs to the trifoliate rootstocks (5).

In 1988 citrus samples suspected to have exocortis disease in Mazandaran province was reported (6). However this report was perceptional and wasn't proved by Koch principle. In 1996 pathogenicity of citrus viroids in Khuzestan was confirmed (7). Montazeri et al., reported a pseudo exocortis viroid in Mazandaran and proved its pathogenicity. They transmitted the viroids to the benchmark plants including clematis, Citrus medica, Etrog citrons, Gynura sarmentosa and tomato. The visible signs in the plants, except clematis, were epinasty, short distance veins and reduction in plant growth. There were pale spots on the surface of leaves of clematis that led to necrosis in discoloring parts. Moreover, the infected leaves became shorter than the normal ones (8). In Iran,

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molecular detection of citrus exocortis viroid was performed in 2006 (9). There are also reports suggesting that citrus trees despite being infected with Hop dwarfism viroid had no symptoms (10 & 11). In this study, three new variants of citrus exocortis viroid were sampled from Fars trees with yellowing and suberization symptoms and compared with other isolates of this viroid.

Methods

Sampling. Recently, different gardens of citrus trees in Jahrom city were visited and 40 orange trees with yellowing and suberization symptoms or symptomless were sampled in summer. Some young foliage was taken from each tree and after freezing in liquid nitrogen they were maintained at -70OC for further experiments.

RNA extraction from plant. RNA extraction from plants was implemented according Yang et al., (1992) method with slight changes. All RNA extraction steps were performed under sterile conditions and all utilized solutions were prepared by sterile water treated by DEPC. 5 µl of extracted RNA were utilized in a 25 µl volume polymerase chain reaction.

Reverse transcription and polymerase chain reaction. These two reactions were conducted applying a specific primer pair of citrus exocortis viroid (12) consisting of reverse primer (5′- CCGGGGATCCCTGAAGGA-3′) and forward primer (5′-GGAAACCTGGAGGAAGTCG-3′) which reproduce about 370 nucleotide pairs. The requirements for the reverse transcription reaction was a 20 µl volume containing 4µl of extracted RNA from viroid, 1µM of reverse primer, 1 µM of each of four deoxynucleotide triphosphates, 10 mM of dithiothreitol, 4.5µl of MMuLV specific buffer (5X) and 1µl of MMuLV-Reverse transcriptase enzyme (200 U/µl). The mixture of RNA and reverse primer was firstly kept at a temperature of 70OC in a thermocycler with a cover temperature of 105 OC for ten minutes after RNA denaturation it was placed in ice water for three minutes. After centrifuging for several seconds, the remaining material was added to the micro-tube. The mixture was gently pipetted for 2 or 3 times and then was centrifuged again for a few seconds, after that the mixture was maintained in the thermocycler at a temperature of 42 OC for two hours. Thereupon, it was kept at 94 OC for ten minutes and then quickly cooled on ice for three minutes. Five µl of the mixture was utilized in 25ml polymerase chain reaction with one µl of each of primers (10 µM), 0.5 µl of the four deoxynucleotide triphosphates (10 mM), one µl of dithiothreitol (10 mM), 0.75 ml MgCl2 (50mM), 2.5 ml of Taq DNA polymerase specific buffer (10 X) and 0.25 ml of Taq DNA polymerase enzyme (5U/ml).

The chemicals used in the reverse transcription reaction and the polymerase chain reaction were totally prepared by Fermentas and CinaGen companies.

Thermal cycle of the polymerase chain reaction consisted of a cyclic program including 94 OC for 30 seconds and a 35 cycles program of 94 OC for 30 seconds, 60 OC for 30 seconds and 72 OC for one minute. After the final cycle, the mixture was maintained at a temperature of 72 OC for five minutes.

The analysis of PCR product was performed using electrophoresis in agarose gel 1% in TBE buffer (10.8 gr Tris, 5.5 gr boric acid and 0.73 gr EDTA in 1000 ml distilled water, pH=8.3). Being colored with ethidium bromide, the gel was pictured by gel documentation equipment and nucleic acid bands were perceived by the UV trans-illuminator device.

Cloning. By the use of High Pure PCR Product Purification Kit (Roche) according to the company's guidelines the PCR product was refined and it was placed in pTZ57R/T plasmid vector as the Fermetas company guides. To this end the mixture of plasmid and PCR product was kept at 22 OC for one night. The mixture contained three µl pTZ57R/T plasmid vector, four µl of PCR product, three µl of Ligation buffer 10 X, three µl PEG solution 4000 (50w/v), one µl of T4 DNA Ligase (five U/µl) and 16 µl distilled sterile water for inducting cDNA into the plasmid vector.

As the guidelines of the manufacturer transition of the recombinant plasmid was occurred to the E. coli bacteria using Ins T/A

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Clone PCR Product Cloning Kit (Fermentas). After a night of cultivation in a solid LB medium white colonies containing recombinant plasmid were picked up and cultured separately in 2-5 ml of LB liquid medium and kept in a shaker incubator at a temperature of 37 OC. The solid mixture contained 100 µg/ml ampicillin antibiotics, 20µg/ml of IPTG and 20 µg/ml of X-gal and the liquid medium included 100 µg/ml ampicillin. The recombinant plasmid DNAs were extracted from the bacteria cells by

Table 1: List of CEVd isolates used in this study and their characteristics.

Isolate	Accession no.	Host	Origin	Number of nucleotides
CEVd-1	FJ626863	Citrus	Iran (Fars)	370
CEVd-2	FJ626864	Citrus	Iran (Fars)	370
CEVd-3	FJ626866	Citrus	Iran (Fars)	370
CEVd-4	EF126048	Citrus	Iran (Mazandaran)	370
CEVd-5	EF186990	Citrus	Iran (Mazandaran)	370
CEVd-6	EF186991	Citrus	Iran (Mazandaran)	370
CEVd-7	EF126047	Citrus	Iran (Mazandaran)	371
CEVd-Aus	M34917	Gynura	Australia	371
CEVd-A	M30868	Gynura	Australia	371
CEVd-Gynura	J02053	Gynura	Germany	371
CEVd-54-E-18 Uy	AF428064	Grapefruit	Uruguay	371
CEVd-g	Y00328	Grape	Spain	369
CEVd-AD	AB054592	Citron	Japan	371
CEVd-bro	S79831	Common bean	Spain	373
CEVd-tom	X53716	Tomato	India	372
CEVd-g-Severe	AF298177	Gynura	USA	371
CEVd-g-Mild	AF298178	Gynura	USA	370
HSVd-sb (in phylogenetic tree)	FJ465507	Citrus	Iran (Fars)	299

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boiling method (13). One µl of the **extracted** DNA of the recombinant plasmid was added to the PCR reaction mixture, based on the mentioned conditions, then the outcomes were estimated by electrophoresis in order to verify the transmission of the segments to the plasmid.

Nucleotide sequencing. After verifying the cloned pieces, the bacteria having recombinant plasmid was cultured again, then by using High Pure Plasmid Isolation Kit (Fermentas) according to the company's guidelines, its plasmid was extracted and investigated with the enzymatic digestion method. All cases, after screening for each cloned product containing the desired components were delivered to Macrogen Company in South Korea for sequencing with the primer of citrus exocortis viroid (12), and they were sequenced in two terms. The obtained sequence(s) was (were) compared with the NCBI sequences in GenBank, using BLAST software.

Analysis of nucleotide sequences. The multiple alignments of the nucleotide sequence were compared and the amount of genetic variations among the CEVd isolates from Iran and other parts of the world (Table 1) were calculated using the Vector NTI 9 software (InforMax, Bethesda, MD). Using DNAMAN software (version 4.0.1.1), the phylogenetic analysis was conducted. The phylogenetic tree was depicted by the neighbor-joining method (14) with 10000 replicates.

The comparison of nucleotide combination and depiction of secondary structure of the studied isolates were performed by BioEdit (version 5.0.9) (15) and RNAstructure (version 4.6), respectively.

Results and Discussion

Reverse transcription polymerase chain reaction (RT-PCR). A segment of about 370 nucleotides was proliferated in eight orange trees from Jahrom due to the reverse transcription polymerase chain reaction with a specific primer of exocortis viroid in citrus. The emerged segments were successfully cloned and sequenced in two terms. The results demonstrated that the nucleotide sequences in primers' joining locations of the sequenced clones were similar to the applied primers. Their similarity with different variants of citrus exocortis viroids was proved by comparing the resulting sequence with the GenBank database through BLAST software. There was no viroid band of citrus exocortis detected in the remaining studied trees.

Nucleotide sequence analysis and RNA secondary structure. All amplified segments were about 370 nucleotides and among the eight infected samples, only three individual sequences were identified and established in Genbank, EMBL and DDBJ databases with accession number of FJ626863, FJ626864 and FJ626866 in the name of CEVd-cit1, CEVd-cit2 and CEVd-cit3, respectively (Fig. 1, lanes three to five).

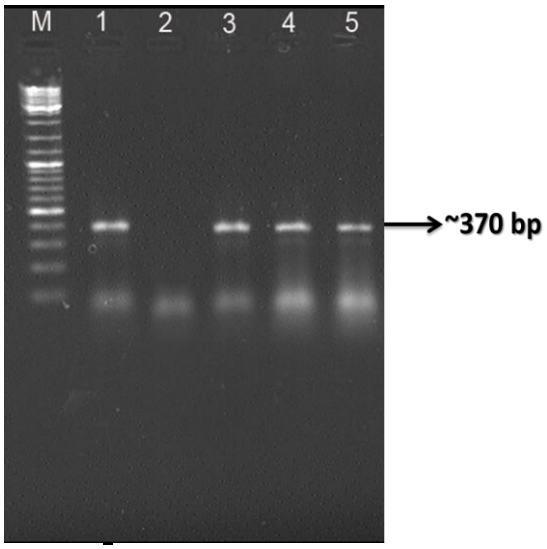


Fig. 1. Electrophoresis pattern of DNA fragments amplified in RT-PCR with a specific citrus exocortis viroid primer pair. Lane 1: Positive control. Lanes 3-5: Sweet orange trees from Jahrom. Lane 2: Healthy control. M: Marker.

The primary structure and comparison of multiple alignments of the genome sequence; CEVd-cit1, CEVd-cit2 and CEVd-cit3 with other isolates of CEVd (Table 1) are illustrated in Fig. 2, and the comparison of nucleotide combination and the numbers and the types of constituent nucleotides of CEVd-cit1, CEVd-cit2 and CEVd-cit3 are represented in Table 2.

The primary structure and comparison of multiple alignments of the genome sequence; CEVd-cit1, CEVd-cit2 and CEVd-cit3 with

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other isolates of CEVd (Table 1) are illustrated in Fig. 2, and the comparison of nucleotide combination and the numbers and the types of constituent nucleotides of CEVd-cit1, CEVd-cit2 and CEVd-cit3 are represented in Table 2. CEVd-cit1, CEVd-cit2 and CEVd-cit3 are fairly similar to each other in terms of nucleotide composition, number and types of

Table 2. Comparison of nucleotide composition, number and type of nucleotides and molecular weights of CEVd-cit 1 and CEVd-cit 2 using BioEdit (version 5.0.9) progrom (Hall, 1999).

Viroid	Number of nucleotides and proportion								Ratio		Ratio		Molecular Weight estimation	
	A		U		C		G		A+U	G+C	A/U	G/C	Single stranded	Double stranded
	no.	%	no.	%	no.	%	no.	%	%	%	%	%	Daltons	Daltons
CEVd-cit1	70	18.92	74	20.00	112	30.27	114	30.81	38.92	61.08	0.95	1.02	118806	237214
CEVd-cit2	69	18.65	74	20.00	112	30.27	115	31.08	38.65	61.35	0.93	1.03	118782	237229
CEVd-cit3	70	18.92	75	20.27	111	30.00	114	30.81	39.19	60.81	0.93	1.03	118767	237191

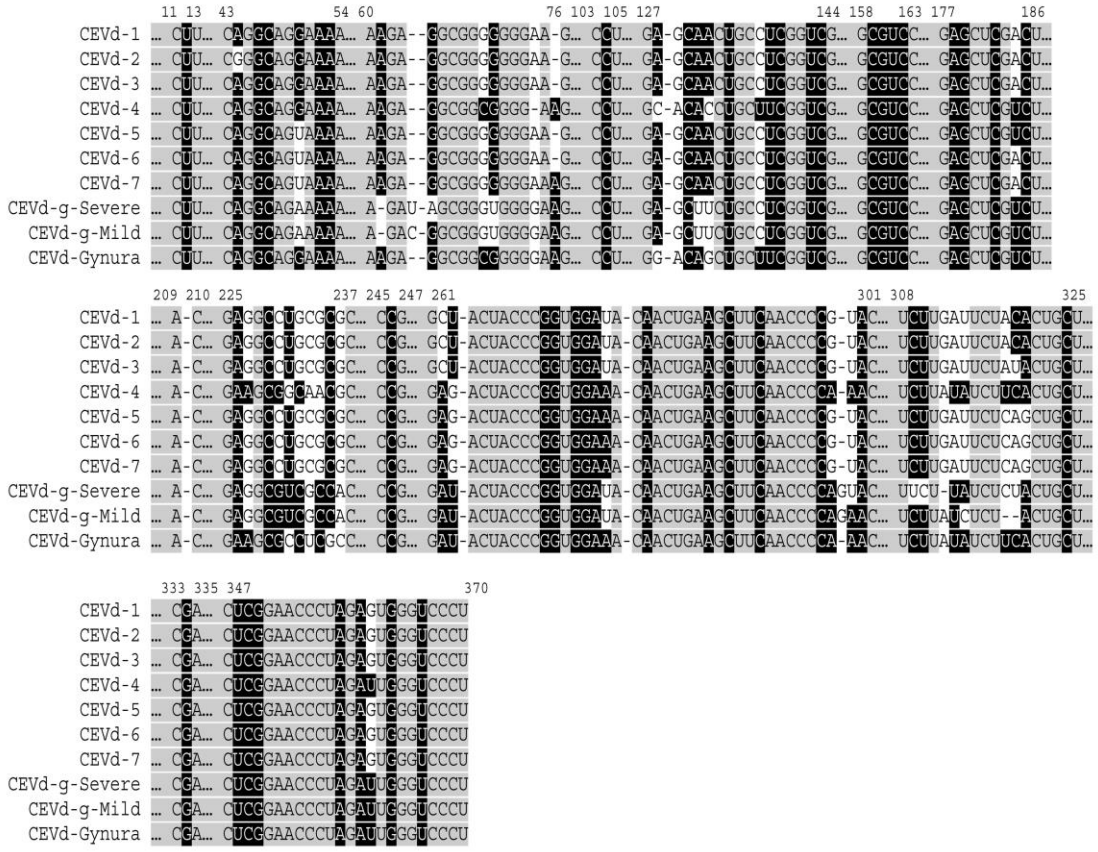


Fig. 2. Primary structures of CEVd variants from various sources. Sequences are aligned for maximum homology. Nucleotides: Conserved sequences; Nucleotides: Identical sequences; -: lack of nucleotide.

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constituent nucleotides and estimated RNA molecular weight (Table 2). These isolates are G+C rich (Table 2) and their nucleotide

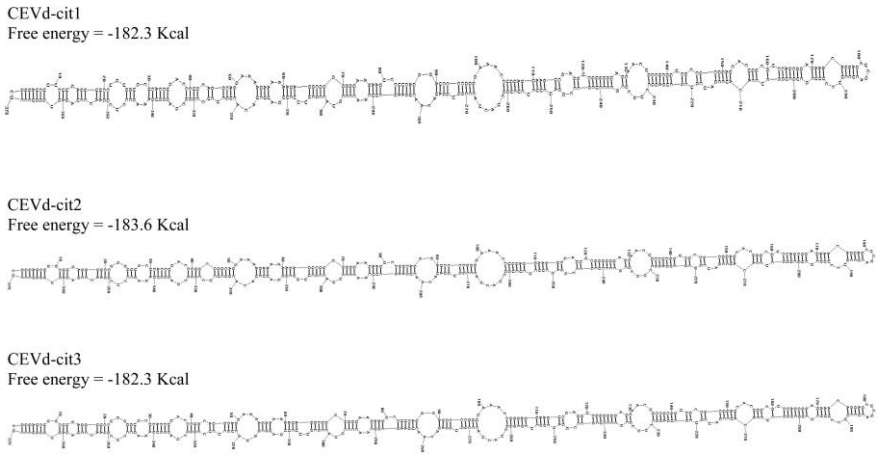


Fig. 3. Comparison of secondary structures and minimum free energy of CEVd-cit1, CEVd-cit2 and CEVd-cit3.

Table 3. Percent sequence identity between selected CEVd isolates used in this study.

	CEVd-1	CEVd-2	CEVd-3	CEVd-4	CEVd-5	CEVd-6	CEVd-7	CEVd-g-Severe	CEVd-g-Mild	CEVd-ycv	CEVd-A	CEVd-Aus	CEVd-AD	CEVd-54-E-18Uy	CEVd-g	CEVd-Gynura	CEVd-tom	CEVd-bro
CEVd-1	100	100	100	94	98	98	98	93	94	92	93	93	94	94	88	93	88	92
CEVd-2		100	99	93	98	98	98	93	93	92	93	93	94	93	87	93	88	92
CEVd-3			100	93	98	98	98	93	94	92	93	93	94	93	87	93	88	92
CEVd-4				100	94	94	94	93	94	96	98	98	96	97	91	98	90	96
CEVd-5					100	100	99	93	93	92	94	94	92	92	88	93	89	91
CEVd-6						100	100	93	93	91	93	93	92	92	88	93	89	91
CEVd-7							100	93	93	92	94	94	92	92	88	93	89	91
CEVd-g-Severe								100	98	91	93	93	93	93	89	94	89	92
CEVd-g-Mild									100	92	94	94	94	94	90	95	89	93
CEVd-ycv										100	97	97	96	97	90	97	88	96
CEVd-A											100	100	97	98	92	99	90	97
CEVd-Aus												100	97	98	92	99	90	97
CEVd-AD													100	98	92	97	89	96
CEVd-54-E-18Uy														100	92	99	89	98
CEVd-g															100	92	86	91
CEVd-Gynura																100	90	97
CEVd-tom																	100	88
CEVd-bro																		100

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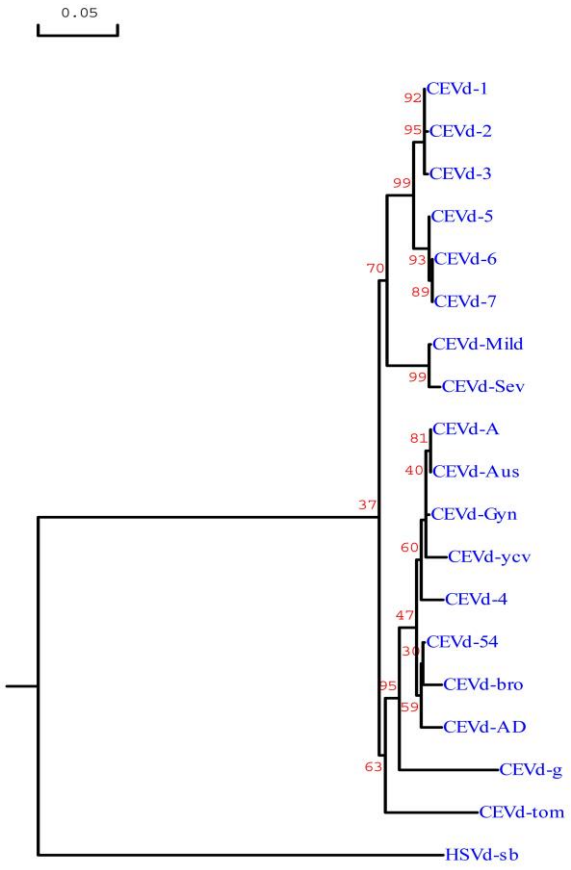


Fig. 4. Phylogenetic tree constructed from the alignment of nucleotide sequences of 17 CEVd isolates using the neighbor-joining method based on 10 000 replicates. The numbers indicate bootstrap percentage. See Table 1 for viroid accession numbers.

composition presents an RNA with a strong, heat resistant structure and low molecular weight, which are characteristic of viroids.
The most stable secondary structures of these three isolates are rod-shaped and with small loops like the structures of other viroids of these isolates.
Comparison of secondary structures and free energies of CEVd-cit1, CEVd-cit2 and CEVd-cit3 demonstrated great similarities. (Fig.3)
Comparing the multiple alignments of nucleotide sequence of the mentioned isolates with other 17 isolates of CEVd revealed that the homogeny of citrus exocortis viroids isolates are always more than 90% (Table 3). CEVd-cit1, CEVd-cit2 and CEVd-cit3 among the studied isolates, have the least similarity (%87) with CEVd-g (reported from Spain) and the most similarity (%98) with the isolates from northern of Iran (CEVd-cit4, 5, 6, 7). However, generally and with respect to reported isolates from other parts of the world, the isolates from Mazandaran province are much closer to the reported isolates from Australia (CEVd-Aus and CEVd-A) and the isolates of Fars province are similar to reported isolates from Japan (CEVd-AD) (Table 3).
Dendrogram obtained from phylogenetic studies demonstrates that the investigated CEVd isolates belong to six different groups; Iranian isolates are placed in a same group and in two subgroups, illustrating the isolates of Fars and Mazandaran provinces, respectively (Fig. 4).
Although CEVd isolates from Fars or Mazandaran provinces do not differ extremely introducing of these isolates as new variants of citrus exocortis viroid is crucial, considering the huge impact of any changes even in one nucleotide (16& 17).
CEVd-cit 1 , 2 and 3 didn't induce any exclusive symptoms since the symptomatic samples without the mentioned viroids confirm this fact. Probably, only susceptible species represent disease symptoms (4). It is possible that transplanting the assumed trees on sensitive rootstocks (like trifoliate orange) lead to the emergence of disease symptoms (5).

Conclusions

Due to the increasing importance of viroids as the factors of reducing citrus growth, investigation of these pathogens specially in southern parts of the country by constructing their infectious clones, conducting pathogenicity tests, observing the symptoms and preventive measures for preventing mechanical transmission or withdrawal and transfer of pathogens from infected areas by grafts would be necessary.

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References

1. Gora-Sochacka A. Viroids: Unusual small pathogenic RNAs. Acta Biochim Pol. 2004; 51: 587-607.
2. Avina-Padilla K, Martinez de la Vega O, Rivera-Bustamante R, Martinez-Soriano JP, Owens RA, Hammond RW, Vielle-Calzada J. In silico prediction and validation of potential gene targets for pospiviroid-derived small RNAs during tomato infection. Gene. 2015; 564: 197–205.
3. Duran-Vila N, Semancik JS, Broadbent. Viroid diseases, cachexia, and exocortis. Compendium of Citrus Diseases. 2nd ed. The American Phytopathological Society, St. Paul, MN. 2000; P51-54.
4. Olson EO. Review of recent research on exocortis disease. In: Proc. 4th Conf. Intl. Org. Citrus Virol. (IOCV) (J F L Child, Ed.), University Florida Press, Gainesville. 1968; 92-96.
5. Benton RJ, Bowman FT, Fraser L, Kebby RC. Stunting and scaly butt of citrus associated with Poncirus trifoliata root stock. Agric gaz NSW. 1950; 61: 20-22.
6. Habashi M. Survey of citrus exocortis disease in northern of Iran. Project Annual Report. Plant Pests and Diseases Research Laboratory of Mazandaran. 1988.
7. Toodeh-Fallah M. Survey of citrus exocortis disease in Khuzestan province. MS thesis in Plant Pathology; Shahid Chamran University of Ahvaz. 1996; 62.
8. Montazeri M, Rahimina H, Hamdollah-Zadeh A. Study on citrus exocortis-like viroid in Mazandaran. Iranian Journal of Plant Pathology 1997; 33: 47-57.
9. Alavi SM, Hashemi-Sahi H, Ahoonmanesh A, Rahimian H. Molecular detection of citrus exocortis viroid and biological indexing of exocortis - like affected citrus trees in Mazandaran province. Iran J Plant Pathol. 2006; 48: 269-275.
10. Ito T, Ieki H, Ozaki K, Iwanami T, Nakahara K, Hataya T, Ito T, Isaka M, Kano T. Multiple citrus viroids in citrus from Japan and their ability to produce exocortis-like symptoms in citron. Phytopathology. 2002; 92: 542-547.
11. Su X, Fu S, Qian Y, Xu Y, Zhou X. Identification of Hop stunt viroid infecting Citrus limon in China using small RNAs deep sequencing approach. Virol. J. 2015; 12: 103.
12. Gross H.J, Krupp G, Domdey H, Raba M, Jank P, Lossow C, Alberty H, Ramm K, Sanger HL. Nucleotide sequence and secondary structure of citrus exocortis and chrysanthemum stunt viroid. Eur J Biochem. 1982; 121: 249-257.
13. Holmes DS, Quigley M. A rapid boiling method for the preparation of bacterial plasmids. Anal Biochem. 1981; 114: 193.
14. Saitou N and Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987; 4: 406–425.
15. Hall TA. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser. 1999; 41: 95-98.
16. Serra P, Gago S, Duran-Vila N. A single nucleotide change in Hop stunt viroid modulates citrus cachexia symptoms. Virus Res. 2008; 138: 130–134.
17. Katsarou K, Rao ALN, Tsagris M, Kalantidis K. Infectious long non-coding RNAs. Biochimie. 2015; 117: 37-47.

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