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Determination of the Dominant Variants of *Hop Stunt Viroid* in Two Different Cachexia Isolates from North and South of Iran

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ABSTRACT

Citrus plants are hosts of several viroid species, among which, pathogenic variants of *Hop Stunt Viroid* (HSVd) induce citrus cachexia disease. Stunting, chlorosis, gumming of the bark, stem pitting and decline are symptoms of cachexia in mandarins and their hybrids as susceptible hosts. Based on the pathogenic properties on citrus, *HSVd* variants are divided in two distinct groups: those that are symptomless on sensitive citrus host species and those that induce cachexia disease. In this study, two cachexia isolates were selected and biological indexing was performed in a controlled temperature greenhouse (40ºC day and 28ºC night) using Etrog citron *(Citrus medica*) grafted on Rough lemon (*C. jhambiri*), as a common indicator for citrus viroids. The plants were inoculated with the inocula from a severe symptomatic tree of a newly declining orchard of Jiroft, Kerman province and a mild symptomatic tree from Mazandaran province. Presence of *HSVd* was confirmed with sPAGE, Hybridization by DIG-labeled probes and RT-PCR using specific primers of *HSVd*. Primary and secondary structures of the isolates were studied. The consensus sequence of RT-PCR amplicons of the severe isolate (JX430796) presented 97% identity with the reference sequence of a IIb variant of *HSVd* (AF213501) and an Iranian isolate of the viroid (GQ923783) deposited in the gene bank. The mild isolate (JX430798) presented 100% homology with the *HSVd-IIc* variant previously reported from Iran (GQ923784). Both isolates were shown to be cachexia inducing according to their sizes, sequences and lack of "non-cachexia expression motif" structures.

Keywords: Biological indexing, Cachexia, Citrus, *HSVd*, Viroid.

INTRODUCTION

Viroids are small molecules of single strand, covalently closed RNA and their genome size varies between 246 to 401 nucleotides. They belong to two families, the *Pospiviroidae* and the *Avsunviroidae* (King, *et al.* 2012). Citrus plants are natural hosts of several viroids, all belonging to the *Pospiviroidae* family: *Citrus Exocortis Viroid* (CEVd), *Hop Stunt Viroid* (HSVd), *Citrus Bent Leaf Viroid* (CBLVd), *Citrus Dwarfing Viroid* (CDVd), *Citrus Bark Cracking Viroid* (CBCVd), *Citrus Viroid V* (CVd-V) and *Citrus Viroid VI* (CVd-VI) (Eiras *et al*., 2013). Among the important citrus diseases, *CEVd* and pathogenic variants of *HSVd* are the causal agents of exocortis and cachexia, respectively. They induce symptoms in susceptible host species whereas others remain symptomless. Stunting, chlorosis, gumming of the bark, stem pitting and decline are symptoms of cachexia that appear in mandarins (*Citrus reticulata*) and their hybrids as cachexia susceptible hosts. Severely affected trees are stunted and may even die (Duran-Vila *et al*., 2000).

Cachexia was first described in 1950 as a disease of Orlando tangelo (Childs, 1950). *Hop Stunt Viroid* (HSVd) with a size of 295–

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303 nucleotides, the only member of the genus *Hostuvioid* within the family *Pospiviroidae* (Flores *et al*., 2005), was later identified as the causal agent of cachexia (Semancik *et al*., 1988). Five structural domains termed as central, variable, pathogenic, terminal left and right were characterized in the rod-like secondary structure of the viroids (Keese and Symons, 1985). There are three *HSVd* variants in citrus, variant IIa is non pathogenic, IIb and IIc variants, are pathogenic (Sano *et al*., 1988; Semancik *et al*., 1988; Levy and Hadidi, 1993). Five nucleotides in the cachexia-expression motif located in the variable domain, have been demonstrated to differentiate between pathogenic and non-pathogenic variants of *HSVd* (Palacio-Bielsa *et al*., 2004; Reanwarakorn and Semancik, 1998). Since the beginning of 2010, a widespread disease with decline symptoms appeared in citrus trees of Jiroft region in Kerman province. Several cases of declining Minneola Tangelo (*C. paradisi* X *C. reticulata*) trees with typical cachexia symptoms were noticed during surveys and the present research was conducted as a part of etiological studies (Banihashemian and Bani Hashemian, 2012).

MATERIALS AND METHODS

Plant Materials and HSVd Sources

Two *HSVd* isolates were collected from a severe (HH3) and a mild (HI3) cachexia symptomatic Minneola tangelo tree from Kerman and Mazandaran provinces respectively (Figure 1). Biological indexing was performed using Etrog citron 861-S1 (*C. medica*) grafted on Rough lemon (*C. jambhiri*) rootstock, as the common indicator for citrus viroids. An Italian source of cachexia (HG3) containing three *HSVd* variants (kindly provided by Dr. K. Djelouah, IAMB, Italy) was used for inoculation of positive controls. Blocks of five seedlings were singly-inoculated with two graft patches from each viroid isolate and grown under greenhouse conditions (40ºC day and 28ºC night) for nine months (Banihashemian and Bani Hashemian, 2012). Non-inoculated plants were used as negative controls.

RNA Extraction Methods

SDS-potassium acetate method (Bernard and Duran-Vila, 2006) was used as the RNA extraction method for Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Briefly, tissue samples (500 mg of leaf and bark) were placed in sealed plastic bags in the presence of 5 ml of extraction buffer (0.1M Tris-HCl, pH 8.0; 50 mM EDTA; 0.5M NaCl; 10 mM mercaptoethanol) and were homogenized using a pestle. The homogenate was subjected to alkaline denaturation. Standard viroid extraction designed to yield high viroid titers (Bernard and Duran-Vila, 2006), was applied for other detection methods. Tissue samples (5 g of young leaves and

Figure 1. Severe symptoms of cachexia, including gumming and pitting of bark and wood (A) of declining Minneola tangelo orchard from Jiroft, Kerman Province (B) in comparison with mild symptoms of cachexia in the same variety from Mazandaran Province (C).

barks) from Etrog plants were homogenized in 20 ml of extraction medium containing 15 ml phenol and 5 ml buffer [0.4M Tris-HCl, pH 8.9; 1% (w/v) Sodium Dodecyl Sulfate (SDS); 5 mMEDTA, pH 7.0; 4% (v/v) mercaptoethanol]. The total nucleic acids were partitioned in 2M LiCl, and the soluble fraction was concentrated by ethanol precipitation and resuspended in TKM buffer [10 mM Tris-HCl; pH 7.4; 10 mM KCl, 0.1 mM MgCl2]. Standard extracts from citrons infected *HSVd, CEVd, CDVd, CBCVd* and *HSVd-IIa* variants (kindly provided by Dr. N. Duran-Vila, IVIA, Spain) were used for hybridization and electrophoresis.

Sequential Polyacrylamide Gel Electrophoresis (sPAGE)

Twenty µl of the nucleic acid preparations from standard extraction method were subjected to two consecutive rounds of polyacrylamide gel electrophoresis in 5% gels using a vertical electrophoresis system, first gel under non-denaturing and the second under denaturing conditions (Rivera-Bustamante *et al*., 1986). The circular forms of the viroids were viewed by silver staining (Igloi, 1983).

Northern Blot Hybridization

The RNAs separated by sPAGE were electroblotted (313 mA for 2 hours) in a transfer system to positively charged nylon membranes (Roche Applied Science) using TBE buffer (90 mM Tris, 90 mM boric acid, and 2 mM EDTA, pH 8.0) and immobilized 2 hours at 80ºC in oven. Prehybridization (60°C for 2h) and hybridization (50°C overnight) were performed (Murcia *et al*., 2009) using digoxigenin (DIG)-labeled viroid-specific probes of *HSVd, CEVd, CBCVd* and *CDVd* generated by PCR from plasmids containing the full-length viroid sequence kindly provided by Dr. N. Duran-Vila. The reaction was detected using a Dig-detection kit (Roche Applied Science).

RT-PCR Protocol and Viroid Characterization

RT-PCR was performed in two steps using Revert Aid Kit (Fermentas) and *HSVd* specific primers HSVd-RT (5'GTGTTGCCCCGGGGCTCCTTTCTCT GG-3'), HSVd-F1 (5'GGGGCAACTCTTCTCAGAATCC-3') and HSVd-R1 (5'-GGGGCTCCTTTCTCAGGTAAGTC-3') (Bernard and Duran-Vila, 2006). The viroid template obtained from SDS potassium acetate extracts and the HSVd-RT primer were denatured at 95ºC for 5 minutes. The reaction mixture (20 µl final volume) was incubated at 42ºC for 1 hour. PCR amplification was performed with the *HSVd* specific primers (HSVd-F1 and HSVd-R1) using a PCR Master Mix (Fermentas).

Sequence Analysis and Determination of Secondary Structures

The RT-PCR products of *HSVd* isolates were sequenced and compared with other *HSVd* sequences from the gene bank. Multiple alignments of *HSVd* sequences were obtained using Clustal W (Thompson *et al*., 1994). The most stable secondary structure was obtained with the RNA structure software (version 4.6).

RESULTS AND DISCUSSION

Symptom expression of typical epinasty (Figure 2-A) and petiole browning (Figure 2-B) were observed in citrons inoculated with the positive control, HG3, after nine months. A mild leaf epinasty (Figures 2-C and -D) was noticed in the plants inoculated with two cachexia isolates HH3 and HI3 (Banihashemian and Bani Hashemian, 2012). Biological indexing has been

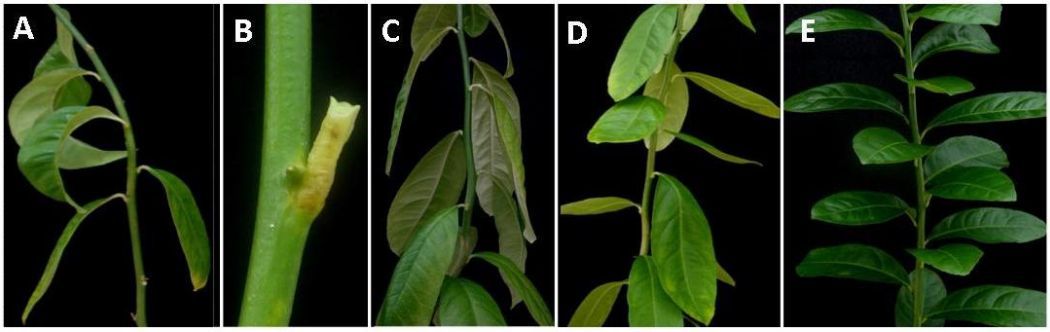

Figure 2. Symptoms of Etrog citron indicator plant inoculated with HG3 (A and B), HH3 (C), HI3 (D) isolates compared with the healthy control plant (E).

performed for many years for detection of citrus viroids. However it is used as an important step in detection of viroid infections, but is not always practical because the technique needs greenhouse facilities (Banihashemian *et al*., 2012). Parsons Special Mandarin, the specific cachexia indicator requires a long incubation period under special environmental conditions for symptom development (Pina *et al*., 1991) and for this reason it was not applied in this study. Etrog citron is the common indicator plant for detection of citrus viroids (Roistacher, 1991). Although citrus viroids other than *CEVd* do not produce specific symptoms (Roistacher, 1991) but it is clear that all citrus viroids can well multiplicate on Etrog Citron (Bani Hashemian *et al*., 2015). Therefore a combination of biological indexing using Etrog citron and molecular methods (Duran-Vila *et al*., 1988 and 1993) was used for detection of citrus viroids and *HSVd* characterization of the study. Epinasty, a symptom related to *CEVd* (Roistacher, 1991), were observed in the Etrog plants inoculated with HG3. The presence of *CEVd* in this isolate was confirmed by hybridization (Figure 3-C).

sPAGE analysis of the citron plants inoculated with HG3 demonstrated that the source contained several viroids (data non shown). Presence of *CEVd* and *HSVd* but not *CDVd* and *CBCVd* was confirmed by hybridization in the same isolate (Figures 3-B, -C, -D and -E). Because of similar sequence of variable and terminal left domains of *CEVd* and *CBCVd* (Puchta *et al*.,

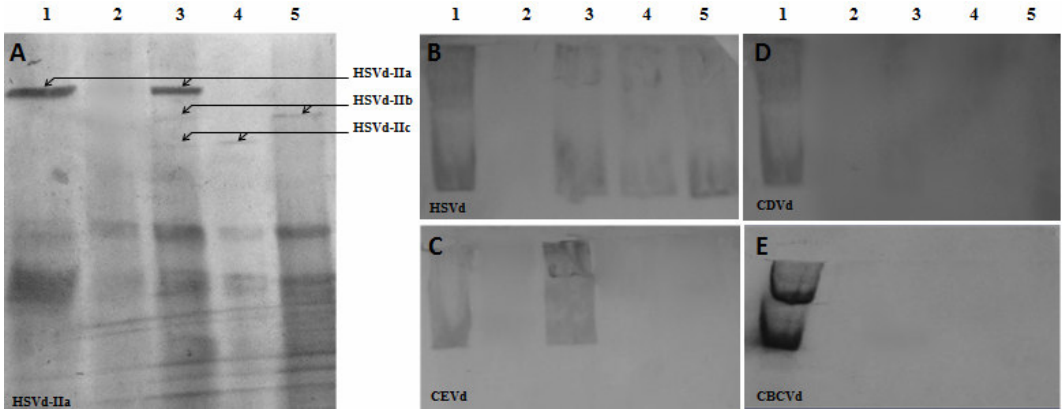

Figure 3. Stained polyacrylamide gel (A) and hybridization with viroid specific probes of *HSVd* (B), *CEVd* (C), *CDVd* (D) and *CBCVd* (E) containing extracts of positive control (Lane 1), viroid free sample (Lane 2), HG3 (Lane 3), HI3 (Lane 4) and HH3 (Lane 5). Extracts from citrons infected with *HSVd-IIa, HSVd, CEVd, CDVd* and *CBCVd* were used respectively as positive controls of electrophoresis and hybridization.

1991), two bands in hybridization analysis with specific probe of *CBCVd* were seen (Figure 3-E). It shows that the positive extract contains *CEVd* in addition to *CBCVd*. Electrophoresis also revealed presence of three bands related to distinct *HSVd* variants (Figure 3-A). The highest intensive band with the equal mobility of the positive extract of IIa is related to the presence of *HSVd-IIa* in HG3 as a dominant variant. According to negative reaction to *CDVd* probe, two lower bands in electrophoresis of HG3 should be associated with infection to *HSVd-IIb* and *IIc* variants (Duran-vila *et al*., 1988; 1993). Due to the characteristic mobility of three *HSVd* variants in HG3, the single RNA band found in electrophoresis of HH3 and HI3 (Figure 3-A), could be respectively related to *HSVd-IIb* and *HSVd-IIc* variants. The nucleic acid preparations from Etrog citron inoculated with HG3, HH3 or HI3 had a positive reaction in RT-PCR with *HSVd* specific primers. PCR amplification produced amplicons of ~300bp and no fragments were detected from healthy control samples (Figure 4). Extracts from citrons infected with *HSVd-IIa, HSVd, CEVd, CDVd* and *CBCVd* were used respectively as positive controls of electrophoresis and hybridization.

Cachexia is an economically important disease that is widespread in great parts of citrus growing areas of the world (Duran-Vila *et al*., 2000). *HSVd* had been identified as a viroid with a broad host range and two distinct groups of variants in citrus (Loconsole *et al*., 2013). Based on their pathogenic properties on citrus, variants that induce citrus cachexia disease in the sensitive hosts were shown to be pathogenic (IIb and IIc variants) while those that did not induce symptoms in the same hosts were named as non-pathogenic (IIa variant) (Palacio-Bielsa *et al*., 2004). Cachexia was first reported from Kerman and Mazandaran provinces of Iran (Habashi and Rahimian, 1984). Two *HSVd* isolates of this study were selected from the mentioned provinces. The severe cachexia isolate (HH3) with typical symptoms of the disease including stunting, chlorosis, gumming of the bark and stem pitting obtained from declining tangelo trees of Kerman province were compared with a mild symptomatic isolate (HI3) from the same variety of Mazandaran province. Since the elevated temperatures favor viroid replication, it has been accepted that warm temperature is very important for maximum expression of cachexia symptoms (Semancik *et al*., 1988). Hence the indicator plants under index for cachexia and other citrus

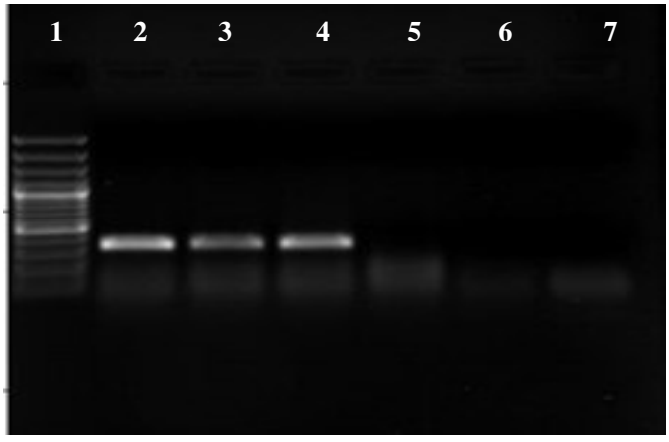

Figure 4. Electrophoresis of RT-PCR products of *HSVd* in 1% agaros gel from Etrog citron indicator plant. Lane 1: 100 bp Ladder; Lane 2: HG3; Lane 3: HH3; Lane 4: HI3, Lane 5: Negative control (-) included RT-PCR analysis of viroid free samples; Lane 6: RT control without RNA template, and Lane 7: PCR control without cDNA template.

viroids should be grown in an environment as warm and practical as possible (Roistacher, 1991). Tangelos are among susceptible varieties to cachexia. Expression of typical symptoms of cachexia in the source orchard of HH3 isolate and subsequent detection of *HSVd-IIb* as a pathogenic variant of the viroid, can justify the cause of decline in the trees. Infection of citrus varieties of Iran to different viroids, including *HSVd* was demonstrated before (Bani Hashemian *et al*., 2013; Amiri Mazhar *et al*., 2014). The mild isolate of the present study, presented 100% homology with an *HSVd-IIc* variant (GQ923784) previously reported from Iran (Bani Hashemian *et al*., 2013).

The RT-PCR products of *HSVd* isolates were sequenced and compared with the reference sequences of *HSVd* variants deposited in the gene bank (Figure 5). HG3 (JX430797) with 303 nt. and the five nucleotides (108, 110, 116, 189 and 194) characteristic of "non-cachexia expression motif" structures of *HSVd-IIa* variant (Palacio-Bielsa *et al*., 2004), showed the same homology of reference sequence of this variant (AF213503). The consensus

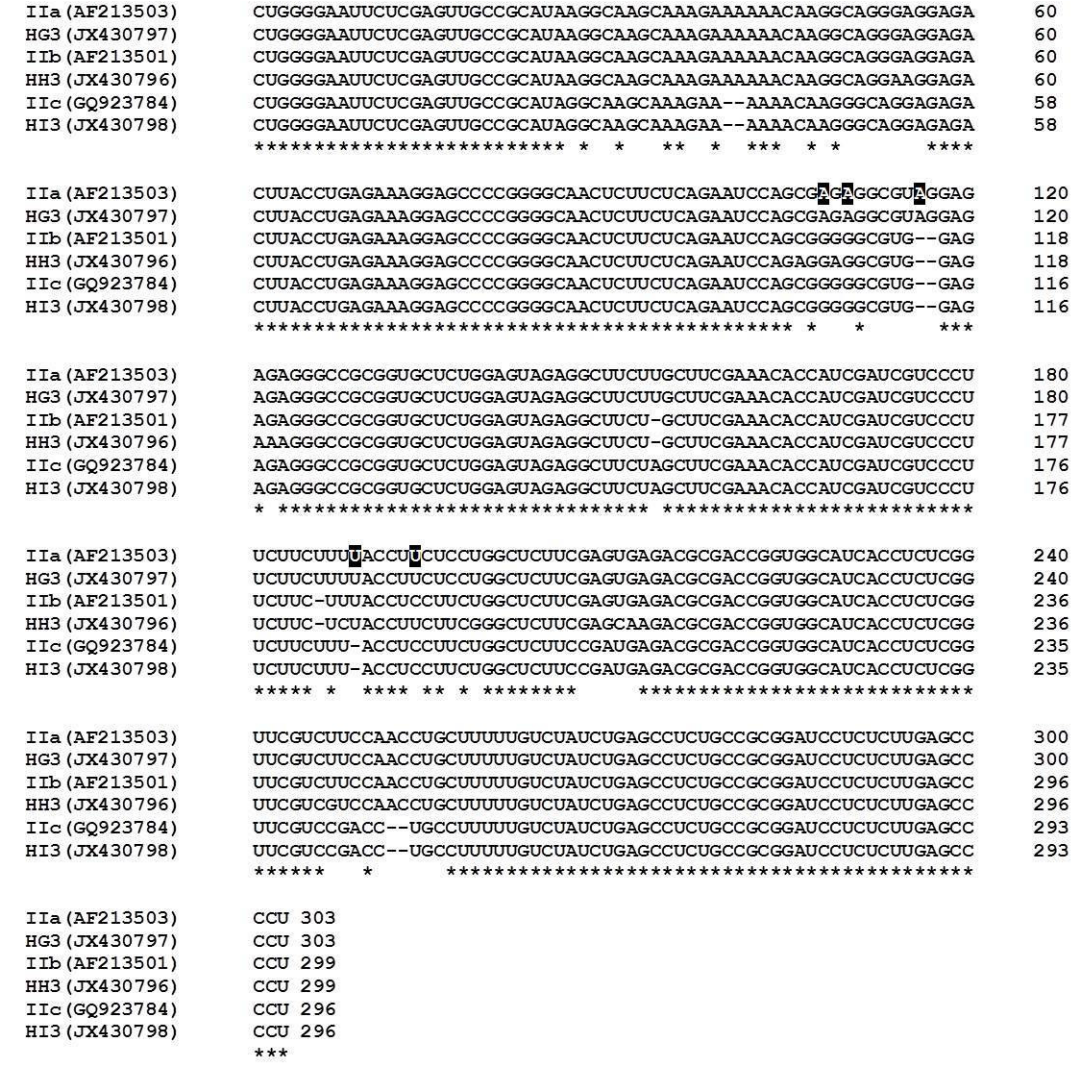

Figure 5. Sequence alignment of three *HSVd* variants of the study, HG3, HI3 and HH3, compared with the reference sequences of *HSVd* variants deposited in the gene bank. Five nucleotides discriminating non-cachexia sequences are shaded (Reanwarakorn and Semancik, 1998).

sequence of the HH3 (JX430796) had a size of 299 nt and presented 97% identity with the reference sequence of IIb variant of *HSVd* (AF213501) and an Iranian isolate of this viroid (GQ923783). HI3 (JX430798) with 296 nt, presented 100% homology with IIc variant previously reported from Iran (GQ923784). Five determined nucleotides of *HSVd-IIa* were not found in the secondary structures of HH3 and HI3 (Figure 5). Based on similarity in size (299 and 296) and sequence with the reference variants, these isolates were considered as cachexia induced variants (Figure 5, and Table 1). Differentiation of *HSVd* variants by using viroid specific primers and probes still remains as a challenge to replace sequence analysis probably due to existence of intermediate variants (Mohamed *et al*., 2009; Loconsole *et al*., 2013). For this reason, the characterization of dominant variants of *HSVd* was carried out by sequence analysis. The expression motif located in the variable domain plays a major role in inciting cachexia symptoms (Palacio-Bielsa *et al*., 2004). A five nucleotide in this region allows discrimination between non-pathogenic and pathogenic variants (Palacio-Bielsa *et al*., 2004; Reanwarakorn and Semancik, 1998). HG3 with the five determinant nucleotides characteristic of "non-cachexia expression motif" showed that it was an HSVd-IIa variant. HH3 and HI3 isolates were cachexia inducing viroids according to their sizes, sequences and lack of "non-cachexia expression motif" structures (Figure 5, and Table 1). However further work is needed to determine the relationship between the type of pathogenic variant of *HSVd* (IIb or IIc) and the expected symptoms in the host, it was demonstrated that any changes within the "cachexia expression motif", affect symptom severity and may even suppress symptoms expression (Serra *et al*., 2008).

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Table 1. Comparison of expression motif of *HSVd* variants of the study with the references.

HSVd source	Genebank accession	Structure of expression motif	Refrence expression motif
HG3	JX430797	AGCGAGAGGCGUAGGAGA UC CUCUUC CAUUUUCU	AGCGAGAGGCGUAGGAGA UC CUCUUC CAUUUUCU
HH3	JX430796	C→A C→A AGAGGAGGCGUGGA CUUCUUC CAUCU -U C→U C→A	104 AG C GGGGGCGUGGA 117 195 UC U UCCUCCAUUU 183 Reference CVd-IIb (AF213501)
HI3	JX430798	102 AG C GGGGGCGUGGA 115 194 UC U UCCUCCAUUU 182	102 AG C GGGGGCGUGGA 115 194 UC U UCCUCCAUUU 182

Nucleotide Sequence and Structural Features of *Hop Stunt Viroid* and *Citrus Bent Leaf Viroid* Variants from Blighted Citrus Plants in Kohgiluyeh–Boyerahmad Province of Iran. *J. Agr. Sci. Tech.,* **16:** 657-665.

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تعیین واریانت غالب ویروئید کوتولگی رازک در دو جدایه مختلف کاککسیا از شمال و جنوب ایران

س. ن. بنی‌هاشمیان، س. م. بنی‌هاشمیان، و س. م. اشکان

چکیده

مرکبات میزبان چندین گونه ویروئید است که از آن میان، بیماری کاککسیای مرکبات به‌وسیله‌ی واریانت‌های بیماری‌زای ویروئید کوتولگی رازک (*Hop stunt viroid*, HSVd)، ایجاد می‌شود. کوتولگی، زردی، صمغ زیر پوست، ساقه آبله‌ای و زوال، علایم کاککسیا هستند که در ارقام نارنگی و دورگ‌های آن به عنوان میزبان‌های حساس ظاهر می‌گردد. بر اساس خصوصیات بیماری‌زایی در مرکبات، دو گروه مجزا از واریانت‌هایHSVd وجود دارد. آن‌هایی که در ارقام حساس مرکبات بدون علایم هستند و گروهی که بیماری کاککسیا ایجاد می‌کنند. در این مطالعه، دو جدایه کاککسیا انتخاب شد و نموده سازی بیولوژیکی در گلخانه تحت شرایط کنترل شده دمایی (روز ۴۰ درجه سانتی-گراد و شب ۲۸ درجه سانتی‌گراد) با استفاده از گیاه بالنگ اتراگ (*C. medica*) پیوند شده روی پایه

رافلمون (*C. jambhiri*)، به عنوان گیاه محک عمومی ویروئیدهای مرکبات، انجام شد. گیاهان با منبع آلودگی از درختی با علایم شدید از باغ‌های در حال زوال منطقه جیرفت استان کرمان و درختی با علایم خفیف از استان مازندران، مایه زنی شدند. حضور HSVd با سه روش مولکولی sPAGE، هیبریداسیون با پروب‌های نشاندار شده و RT-PCR با استفاده از پرایمرهای اختصاصی HSVd تأیید گردید. ساختارهای اولیه و ثانویه جدایه‌ها بررسی شد. توالی فرآورده نهایی RT-PCR از جدایه شدید (JX430796)، با توالی مرجع واریانت HSVd-IIb (AF213501) و یک جدایه ایرانی از ویروئید (GQ923783) گزارش شده از بانک ژن، ۹۷ درصد تشابه نشان داد. جدایه خفیف (JX430798) با واریانت HSVd-IIc که قبلاً از ایران گزارش گردید (GQ923784) شباهت کامل داشت. بر اساس اندازه، توالی و فقدان ساختار مشخصه واریانت غیر کاککسیا، هر دو جدایه مورد بررسی به عنوان واریانت‌های مولد کاککسیا تشخیص داده شدند.