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

S. N. Banihashemian¹, S. M. Bani Hashemian²*, and S. M. Ashkan¹

**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 (40°C day and 28°C night) using Etrog citron (*Citrus medica*) grafted on Rough lemon (*C. jambhiri*), 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–
303 nucleotides, the only member of the genus *Hostuvioeid* 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 Ila 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 (40°C day and 28°C 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 mM EDTA, 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 citrus 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 80°C 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'GTGTGCCCCGCGGCTCTTCTCTTGG-3'), HSVd-F1 (5'GGGGCAACTTCTTCAGAATCC-3') and HSVd-R1 (5'-GGGGCTCTTCTTCTAGTAAAGTC-3') (Bernard and Duran-Vila, 2006). The viroid template obtained from SDS potassium acetate extracts and the HSVd-RT primer were denatured at 95°C for 5 minutes. The reaction mixture (20 µl final volume) was incubated at 42°C 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...
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.,...
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 HSVd. 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](image-url)
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).

**ACKNOWLEDGEMENTS**

The research was carried out as part of an MSc. thesis project and by funding of Citrus and Subtropical Fruits Research Center (CSRC). The authors would like to acknowledge Dr. Musa Najafi Nia, agricultural research center of Jiroft, for helping in visiting the orchards of the region, Dr. Hossein Taheri (CSRC) for reading the manuscript, Leila Ebrahimi-Moghaddam and Iman Jourbanian for technical assistance.

**REFERENCES**


---

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

<table>
<thead>
<tr>
<th>HSVd source</th>
<th>Genbank accession</th>
<th>Structure of expression motif</th>
<th>Reference expression motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>HG3</td>
<td>JX430797</td>
<td>![structure diagram]</td>
<td>![structure diagram]</td>
</tr>
<tr>
<td>HH3</td>
<td>JX430796</td>
<td>![structure diagram]</td>
<td>![structure diagram]</td>
</tr>
<tr>
<td>HI3</td>
<td>JX430798</td>
<td>![structure diagram]</td>
<td>![structure diagram]</td>
</tr>
</tbody>
</table>


**Dominant Variants of Hop Stunt Viroid**

تصوّر والیهای غالب ویروئید کوتولگی رازک در دو جدایی مختلف کاکسیا از شمال و جنوب ایران

س.د. بی‌هاشمیان، س. م. بی‌هاشمیان، و. س. م. اشکان

چکیده

مربکت ویروئید سیترس (C. medica) و گروه دیگر ویروئید کوتولگی رازک (Hop stunt viroid, HSVd) باعث کاهش کیفیت و کاهش تولید محصولات سبزیجات می‌شود. این ویروئید‌ها به عنوان میزان‌های حساس ظاهر می‌گردند و ایجاد می‌کنند. در ایالت هرمزگان در طی سالهای اخیر، آن‌هایی که در ارتفاع ارتفاعات کاکسیا بی‌حاشمیان، جنوب غربی ایران بودند، علائم فصل‌های مختلف HSVd و HSVd که مربک داشتند، مشاهده شدند. این امر، احتمال انتقال HSVd و HSVd در آن‌ها و افزایش این ویروئید در کاکسیا بی‌هاشمیان را می‌تواند نشان دهد. مطالعه جامعه کاکسیا بی‌هاشمیان در این منطقه، آینده اگر می‌تواند نشان دهد که HSVd و HSVd می‌توانند در تولید محصولات سبزیجات کاکسیا بی‌هاشمیان در این منطقه ایجاد کنند.