SHORT COMMUNICATION

COMPLEX MIXTURES OF VIROIDS IDENTIFIED IN THE TWO MAIN CITRUS GROWING AREAS OF IRAN

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SUMMARY

The citrus industry in Iran is based on graft propagation of local and imported cultivars on a limited number of rootstocks such as sour orange, Mexican lime and Bakraee, a local natural hybrid. Infection of the available cultivars with graft-transmissible agents, viroids in particular, may have deleterious effects on the productivity of citrus and, more importantly, limits the choice of rootstocks. Rootstocks other than sour orange and Mexican lime will be needed as a consequence of the introduction and spread of Citrus tristeza virus (CTV) in the country. In the present study, two cv. Sanguinello sweet orange and two cv. Duncan grapefruit trees were selected on the basis of the bark scaling symptoms observed on the trifoliate orange and Troyer citrange rootstocks. Analysis by sPAGE and Northern blot hybridization of nucleic acid preparations of Etrog citrons graft-inoculated with these sources revealed the presence of several viroids: Citrus exocortis viroid (CEVd) and Hop stunt viroid (HSVd), the causal agents of exocortis and cachexia, respectively, as well as Citrus bent leaf viroid (CBLVd), Citrus dwarfing viroid (CDVd) and Citrus bark cracking viroid (CBCVd). These viroids were sequenced and compared with the corresponding reference sequences. To extend these results, samples from different citrus cultivars were collected in the two main citrus growing regions and analyzed for viroids. Of the 49 samples tested, 22 were viroid-positive, witnessing the occurrence of cultivars infected with CEVd, HSVd, CBLVd, CDVd, or CBCVd as mixed infections in the surveyed regions. One sample was also co-infected with the newly described Citrus viroid V (CVD-V).

The Islamic Republic of Iran has been a citrus-growing country for about 2500 years, citron (Citrus medicina L.) being the first cultivated species in the country. After the Arab conquest, other citrus species such as sour orange, lemon and lime were introduced in the 10th century. Much later, many other species reached the country, first introduced probably from India, and between 1933 and 1977, from Turkey, Italy, Lebanon, USA, Australia, Japan, Morocco, France and the former USSR, without much concern about the sanitary status of the imported budwood. For many centuries, citrus were seed-propagated and, since most virus and virus-like pathogens are not seed-borne, the citrus industry remained essentially free from these virus and virus-like diseases. However, the heavy losses due to Phytophthora root rot prompted the growers to switch from seedling trees to graft-propagated trees (Bové, 1995).

Presently there are two major citrus growing areas: (i) The Caspian Sea Belt which includes Golestan, Mazadaran and Guilan provinces, with Mediterranean climate, mainly producing early maturing cultivars; (ii) The southern region, that comprises Fars, Kerman, Ilam, Khuzestan, Khoikhuie and Boyerahmad provinces and the Southern Coastal Belt of the Persian Gulf and the Sea of Oman, which includes Hormozgan and Bushehr provinces with tropical and subtropical climate. Citrus is graft-propagated on a limited number of rootstocks, sour orange (Citrus aurantiunum L.) being the major one in the Caspian Sea area. In the southern areas, Mexican lime (Citrus aurantifolia Christm. Swing.) and Bakraee, a local natural hybrid between mandarin and lime, are the major rootstocks because of their drought tolerance.

Citrus tristeza virus (CTV) was introduced in 1969 in Mazadaran province through the importation of over 40,000 Satsuma trees grafted on trifoliate orange (Poncirus trifoliate L.) Raf.) from Japan, where tristeza is endemic (Ebrahim-Nesbat and Nienhaus, 1978; Bové, 1995). Even though the natural spread of CTV was initially low, the virus was eventually moved by Aphis gossypii (Rhamian et al., 2000) throughout the Caspian Sea Belt where sour orange is the major rootstock. The southern region was
assumed to be tristeza-free. However, surveys conducted in 1996 in Fars and Bushehr provinces revealed the presence of CTV (Shafiee and Izadpanah, 1996). Further characterization of the CTV strains from the southern areas indicated that they were of different origin than those in the North (Izadpanah et al., 2002).

CTV infection of trees grafted on sour orange, Mexican lime or Bakraee will eventually cause tree decline, and alternative rootstocks will be needed. The choice of rootstocks to replace the existing ones depends on the presence of graft-transmissible agents in general, and viroids in particular, in the species and cultivars to be propagated. Since no information on viroid infection in citrus grown in Iran is available, we now report the identification of citrus viroids in a selection of cultivars collected in different areas.

Two declining cv. Sanguinello sweet orange (Fig. 1A) and two dwarfed cv. Duncan grapefruit (Fig. 1C) trees were selected on the basis of the severe (Fig. 1B) and mild (Fig. 1D) bark scaling symptoms observed in the trifoliate orange and Troyer citrange rootstocks, respectively. These cultivars had been introduced from Italy in the 1930s and were still available at the Citrus Research Institute at Kotra Station, Caspian Sea belt.

Samples from these sources were biologically indexed by graft-transmission to the sensitive selection 861-S1 of Etrog citron (C. medica L). Analysis of nucleic acid preparations of the inoculated citrus by sequential polyacrylamide gel electrophoresis (sPAGE) and silver staining (Rivera-Bustamante et al., 1986; Duran-Vila et al., 1993) revealed the presence of three up to seven bands with the mobilities of the circular forms of viroids (Fig. 2A). To confirm the viroid nature of the bands viewed in sPAGE analysis, the RNAs of these samples and the corresponding controls electroblotted in a tank (400 mA for 2 h) to positively-charged nylon membranes using TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA) were hybridized with digoxigenin (DIG)-labeled viroid specific probes generated by PCR (Palacio et al., 1999). The citrus viroids identified were: *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd) and *Citrus dwarfing viroid* (CDVd) (former *Citrus viroid III*, CVd-III) in all four samples tested (Fig. 2A, 2B: lanes 1 to 4); *Citrus bent leaf viroid* (CBLVd) in the two cv. Sanguinello sweet orange

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**Fig. 1.** A. Declining cv. Sanguinella sweet orange tree grafted on trifoliate orange at Kotra Station, Caspian Sea Belt; B. Severe bark scaling symptoms in the rootstock of the same tree; C. Dwarfed cv. Duncan grapefruit tree grafted on Troyer citrange at Kotra Station, Caspian Sea Belt; D. Mild bark scaling symptoms in the rootstock of the same tree.
sources and in one of the cv. Duncan grapefruit sources (Fig. 2A, 2B: lanes 1, 2 and 4); and Citrus bark cracking viroid (CBCVd) (former Citrus viroid IV, CVd-IV) in the two cv. Sanguinello sweet orange sources (Fig. 2A, 2B: lanes 1 and 2). All plants tested negative for Citrus viroid VI (former CVd-OS) (Ito et al., 2001) and Citrus viroid V (CVd-V) (Serra et al., 2008a) (Fig. 2A, 2B: lanes 1 to 4). sPAGE and Northern hybridization analysis showed that two of the samples (Fig. 2A, 2B, lanes 2 and 4) contained two distinct HSVd bands whereas the other two samples presented a single HSVd band (Fig. 2A, 2B: lanes 1 and 3). Similarly, two of the samples contained several CDVd bands (Fig. 2A, 2B: lanes 1 and 2).

The nucleic acid preparations from these four citron plants were subjected to reverse transcription and PCR amplification (RT-PCR) using viroid-specific primers (Bernad and Duran-Vila, 2006) yielding amplicons of the expected sizes for CEVd, CBLVd, HSVd, CDVd and CBCVd. The amplicons were sequenced either directly or after cloning into the pGEM-T vector (Promega, USA), using an ABI PRISM DNA sequencer 377 (Perkin Elmer, USA). The sequences were aligned with reference sequences of the corresponding viroids using the Clustal W program (Thompson et al., 1994).

The consensus sequences of CEVd isolates from samples 1 and 3 (Sanguinello sweet orange and Duncan grapefruit, respectively) obtained by sequencing the RT-PCR amplicons, showed identities of 99.7% and 100% with the predominant sequence variant of our type strain (Gandía et al., 2005) (Table 1) and had a composition of the P (pathogenicity) domain characteristic of severe strains of CEVd as defined by Visvader and Symons (1986) on tomato and therefore may (or may not) express similar phenotypes in citrus hosts. Most of the nucleotide changes identified in these two isolates were clustered in a loop of the V (variable) domain and did not affect the predicted viroid secondary structure (data not shown). The two CEVd isolates from samples 2 and 4 (Sanguinello sweet orange and Duncan grapefruit, respectively) presented high identities with a new class of CEVd variants identified originally in the Sultanate of Oman (Bernad et al., 2005) (Table 1).

The consensus sequences of the three CBLVd isolates (samples 1, 2 and 4) (two Sanguinello sweet orange samples and one Duncan grapefruit sample) obtained by sequencing the RT-PCR amplicons, had a size of 318 nt and presented the highest identities with CBLVd (variant CVd-Ib) (Hataya et al., 1998) (Table 1).

Since the pathogenic properties of HSVd in citrus depends on the presence of either the “cachexia expression motif” in strains that induce cachexia disease or the “non-cachexia expression motif” in strains that do not induce cachexia (Reanwarakorn and Semancik, 1998), the four HSVd isolates were characterized by cloning, sequencing and multiple alignments with the reference sequences of CVd-IIa-117 (non-cachexia inducing variant), CVd-IIb and CVd-IIc (cachexia inducing variants) (Reanwarakorn and Semancik, 1999; Palacio-Bielsa et al., 2004). The HSVd isolate in sample 1 (Sanguinello sweet orange) contained a mixture of variants of 300 and 299 nt. The 300 nt variant contained the sequence and structure of the “non-cachexia expression motif” and presented a high identity (98.3%) with the CVd-IIa-117 reference sequence, whereas de 299 nt variant contained the sequence and structure of the “cachexia expression motif” and presented the highest identity with the CVd-Ib reference sequence (Table 1). The relatively low sequence identity of 94.3% (Table 1) was the consequence of 13 changes, two deletions and one addition affecting the upper and lower strands of the regions flanking the central conserved region (CCR), as well as the HSVd secondary structure. This variant had a high identity (99.7%) with a cachexia inducing variant previously described in Japan (Table 1) (Ito et al., 2002).

The HSVd isolate in sample 2 (Sanguinello sweet orange) contained a mixture of variants of 302 and 298 nt. The 302 nt variant contained the sequence and structure of the “non-cachexia expression motif” and presented the highest identity with the CVd-IIa-117 reference sequence, whereas the 298 nt variant contained the sequence and structure of the “cachexia expression motif” and presented the highest identity with CVd-Ib reference sequence (Table 1). HSVd isolate in sample 3 (Duncan grapefruit) contained a 300 nt variant with the sequence and structure of the “non-cachexia expression motif” and a high identity with the CVd-IIa-117 reference sequence. HSVd isolate in...
Table 1. Molecular characteristics of the citrus viroids identified in four isolates from Iran.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Viroid</th>
<th>Accession No.</th>
<th>Size (nt)</th>
<th>Sequence identity</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>CEVd</td>
<td>GQ260196</td>
<td>371</td>
<td>99.7% (CEVd, E117V1)</td>
<td>Gandía et al., 2005</td>
</tr>
<tr>
<td></td>
<td>CBLVd</td>
<td>GQ260200</td>
<td>318</td>
<td>98.1% (CVd-Ib)</td>
<td>Hataya et al., 1998</td>
</tr>
<tr>
<td></td>
<td>HSVd</td>
<td>GQ260207</td>
<td>300</td>
<td>98.3% (CVd-IIa, 117)</td>
<td>Palacio-Bielsa et al., 2004</td>
</tr>
<tr>
<td></td>
<td>HSVd</td>
<td>GQ260206</td>
<td>299</td>
<td>94.3% (CVd-IIb, X-715-1)</td>
<td>Palacio-Bielsa et al., 2004</td>
</tr>
<tr>
<td></td>
<td>CDVd</td>
<td>GQ260212</td>
<td>294</td>
<td>100% (CVd-IIIb)</td>
<td>Rakowski et al., 1994</td>
</tr>
<tr>
<td></td>
<td>CDVd</td>
<td>GQ260214</td>
<td>292</td>
<td>98.6% (CVd-IIIb)</td>
<td>Rakowski et al., 1994</td>
</tr>
<tr>
<td></td>
<td>CBCVd</td>
<td>GQ260216</td>
<td>284</td>
<td>100% (CVd-IV)</td>
<td>Puchta et al., 1991</td>
</tr>
<tr>
<td>2</td>
<td>CEVd</td>
<td>GQ260199</td>
<td>370</td>
<td>100% (CEVd, gb4)</td>
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<tr>
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<td>99.0% (CVd-IIa-117)</td>
<td>Palacio-Bielsa et al., 2004</td>
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<tr>
<td></td>
<td>HSVd</td>
<td>GQ260205</td>
<td>298</td>
<td>97.3% (CVd-IIb, X-715-1)</td>
<td>Palacio-Bielsa et al., 2004</td>
</tr>
<tr>
<td></td>
<td>CDVd</td>
<td>GQ260210</td>
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<td>96.0% (CVd-IIIa)</td>
<td>Rakowski et al., 1994</td>
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<td></td>
<td>CDVd</td>
<td>GQ260215</td>
<td>293</td>
<td>98.6% (CDVdSO)</td>
<td>Murcia et al., 2009a</td>
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<td>CBCVd</td>
<td>GQ260216</td>
<td>284</td>
<td>100% (CVd-IV)</td>
<td>Puchta et al., 1991</td>
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<td>CEVd</td>
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<td>371</td>
<td>100% (CEVd, E117V1)</td>
<td>Gandía et al., 2005</td>
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<td></td>
<td>HSVd</td>
<td>GQ260208</td>
<td>300</td>
<td>98.7% (CVd-IIa, 117)</td>
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<td>99.7% (CVd-IIIb)</td>
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<td>297</td>
<td>99.7% (CVd-IIIa)</td>
<td>Rakowski et al., 1994</td>
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</table>

Sample 4 (Duncan grapefruit) contained a mixture of variants of 302 and 295 nt. The 302 nt variant contained the sequence and structure of the “non-cachexia expression motif” and presented a high identity with the CVd-IIa-117 reference sequence, whereas the 295 nt variant contained the sequence and structure of the “cachexia expression motif” and presented 100% identity with CVd-IIc reference sequence (Table 1).

Given the large number of variants with different pathogenic properties described (Murcia et al., 2009a), the four CDVd isolates were also characterized by cloning, sequencing and multiple alignments with the reference sequences of CVd-IIIa, CVd-IIIb and CVd-IIIc (Rakowski et al., 1994; Semancik et al., 1997). The CDVd isolate in sample 1 (Sanguinello sweet orange) contained a mixture of variants of 294 and 292 nt both with high identity with the reference variant CVd-IIIb (Table 1). The 292 nt variant differed in three changes that affected the organization of the secondary structure of the T1 domain found in the 292 nt variant from sample 1. The CDVd isolate from sample 3 (Duncan grapefruit) contained a 294 nt variant with a consensus sequence that presented a high identity with the reference sequence CVd-IIIb, whereas the isolate from sample 4 (Duncan grapefruit) contained a 297 variant with a consensus sequence with the highest identity with the reference sequence CVd-IIIa.

The consensus sequences of the two CBCVd isolates (samples 1 and 2) (Sanguinello sweet orange) obtained by sequencing the RT-PCR amplicons, had a size of 284 nt and both presented 100% identity with the CBLVd reference sequence (Puchta et al., 1991). The analysis of these four samples provided an indication that at least five of the seven citrus viroids known to infect citrus worldwide are present in Iran. With the availability of a procedure sensitive enough to detect viroids directly in commercial citrus species and cultivars, without the need of an amplification step in citron (Murcia et al., 2009b; Serra et al., 2008b), samples from the field collection maintained at the Iran Citrus Research Institute in Ramsar as well as from Research Stations, nurseries and commercial field plots from the Caspian Sea Belt and the Southern region, were tested for the presence of viroids. Briefly, bark samples were powdered in liquid nitrogen,
Table 2. Viroids detected by northern hybridization in citrus samples collected in different locations of Iran.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Scion</th>
<th>Rootstock</th>
<th>Location</th>
<th>Region</th>
<th>Original Source</th>
<th>symptom</th>
<th>CEVd</th>
<th>HSVd</th>
<th>CBLVd</th>
<th>CDVd</th>
<th>CBCVd</th>
<th>CVd-V</th>
<th>CVd-VI</th>
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<td>Marrs navel sweet orange</td>
<td>Sour orange</td>
<td>Ramsar station</td>
<td>Caspian sea</td>
<td>California</td>
<td>-</td>
<td>-</td>
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<td>Trifoliate orange</td>
<td>Ramsar station</td>
<td>Caspian sea</td>
<td>California</td>
<td>-</td>
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<td>-</td>
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<td>4</td>
<td>Okitsu satsuma</td>
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<td>Ramsar station</td>
<td>Caspian sea</td>
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<td>-</td>
<td>-</td>
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<td>Clausellina satsuma</td>
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<td>Ramsar station</td>
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<td>Sugiyama satsuma</td>
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<td>Ramsar station</td>
<td>Caspian sea</td>
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<td>Ramsar station</td>
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<td>Ramsar station</td>
<td>Caspian sea</td>
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<td>Pag mandarin</td>
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<td>Ramsar station</td>
<td>Caspian sea</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<td>Duncan Grapefruit</td>
<td>Trifoliate orange</td>
<td>Khoram Abad station</td>
<td>Caspian sea</td>
<td>Unknown</td>
<td>+</td>
<td>+</td>
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1 (+) Bark scaling symptoms were observed in the rootstock of the trees from which samples were collected.
and the nucleic acids, recovered in the aqueous phase of the phenol-containing extraction medium, were partitioned in 2 M LiCl and concentrated by ethanol precipitation. The RNAs separated by 5% PAGE (60 mA, 2h) were electroblotted to Nylon membranes and hybridized with viroid-specific DIG-labelled DNA probes. The results (Table 2) can be summarized as follows.

Of the 49 samples tested, 27 were viroid-free. The viroid-free samples covered a range of cultivars (sweet orange, satsuma, clementine, mandarin, tangelo, grapefruit, lemon, Persian lime and Palestine sweet lime), which had been introduced from California (USA), Corsica (France) and Morocco (Table 2). The remaining 22 samples were all co-infected with several viroids, with 16 of them (Page mandarin, 2 sources of Duncan grapefruit, 3 sources of Moro blood orange, Redblush grapefruit, Eureka lemon, Sanguigno blood orange, Sanguinello blood orange, 2 sources of Thompson navel sweet orange, 2 sources of local sweet orange, Valencia sweet orange and Pera shape sweet orange) containing CEVd, the causal agent of the exocortis disease. Seven of the CEVd containing samples (Table 2: samples 17, 18, 20, 22, 27, 35 and 36) were collected from trees grafted on trifoliate rootstocks that showed the characteristic exocortis bark scaling symptoms (Fig. 1 and 3A). Sample 17 was taken from a Page mandarin tree grafted on a trifoliate rootstock which, according to the information provided by the grower, was a Swingle citrumelo. Tolerance of Swingle citrumelo to citrus viroids is still controversial (Bello et al., 2000; Davino et al., 2005) and should be further evaluated. Other CEVd containing trees grafted on sour orange or Mexican lime rootstocks (samples 24, 25, 33, 39, 42, 43, 45, 46 and 47) had no rootstock symptoms. HSVd, CDVd, CBLVd, and CBCVd were widely spread in the co-infected samples (found respectively in 21, 19, 12 and 11 out of the 49 samples tested). The newly described CVD-V was identified in a single

Fig. 3. A. Severe bark scaling symptoms in the rootstock of a Page mandarin tree grafted on a trifoliate rootstock which was considered by the grower as Swingle citrumelo (Table 2: sample 17) at Ramsar Station, Caspian Sea Belt; B. Absence of cachexia symptoms in the bark above the bud union of the same tree after scraping; C. Nucleotide changes of the structural motif of HSVd isolated from the same tree compared with the reference motif; D. Mild gum impregnation in Mineola tangelo (Table 2: sample 32) at Kotra Station, Caspian Sea Belt; E. Gum impregnation observed after removing the bark in the same tree; D. Structural motif of HSVd isolated from the same tree compared with the reference motif.
cv. Moro blood orange tree of unknown origin (Table 2: sample 42).

In an attempt to associate HSVd infection with the presence of cachexia symptoms in cachexia sensitive cultivars, a Page mandarin and a Mineola tangelo (Table 2: samples 17, 32) were chosen for further evaluation. Scraping the bark of the scion revealed the presence of gum-impregnated pits in Mineola tangelo (Fig. 3D) but not in Page mandarin (Fig. 3B). The cachexia symptoms in Mineola tangelo were further confirmed by removing the bark that presented gum exudates (Fig. 3E). The nucleic preparations from these trees were subjected to RT-PCR, cloned and sequenced as describe above. Multiple alignments with the reference sequences showed that HSVd from Page mandarin had a consensus sequence (GenBank GQ923783) of 302 nt and 98.7% identity with the CVD-IIb reference sequence (GenBank AF213501) with two insertions (+U107 and +G195) and a substitution (U186→C) affecting the structure of the “cachexia expression motif” (Fig. 3C). As recently reported, small changes in this region may affect infectivity and symptom expression (Serra et al., 2008c) and the biological significance of the changes identified must be further evaluated. The HSVd from Mineola tangelo had a consensus sequence (GenBank GQ923784) of 296 nt and 100% identity with the CVD-IIc reference sequence (GenBank AF131250) with the “cachexia expression motif” (Fig. 3F) characteristic of cachexia inducing variants. The mild symptoms of the affected Mineola tangelo tree are probably due to the relatively mild climatic conditions of the Caspian Sea Belt region from where the sample was taken (Bani Hashemian et al., 2009).

Given the present situation of the citrus industry in Iran, with sour orange and Mexican lime as the major rootstocks, the spread of CTV (Izadpanah et al., 2002; Barzegar et al., 2006; Ahmadi et al., 2007) will endanger citrus production unless these two rootstocks are replaced by rootstock species giving CTV-tolerant combinations with the scion. Therefore, cultivars that carry viroids cannot be grafted on viroid-sensitive rootstocks such as tangelo (C. reticulata X C. paradisi), alewoc (C. macrophylla), Rangpur lime (C. limonia), trifoliolate orange and Troyer and Carrizo citrange (P. trifoliata X C. sinensis). In addition, cachexia inducing variants of HSVd not only affect tangelos used as rootstocks, but also tangelo, mandarin, satsuma, and clementine, when they are used as scions, regardless of rootstocks. Since the control of tristeza disease is based on planting rootstock/scion combinations tolerant to CTV, the choice of rootstock species will strongly depend on the sanitary status of the commercial scion cultivars available in Iran.

The worldwide problem of CTV-induced tristeza-quick decline has been solved long ago by using adequate rootstocks to replace sour orange. The problem of viroids affecting certain rootstocks as well as certain scions has also been solved in many countries by producing viroid-free cultivars. In Iran, these problems must also find a quick solution, so much more rapidly as two new diseases seriously affect the southern citrus belt: witches’ broom disease of lime and huanglongbing.

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REFERENCES


