Citrus Viroids

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Viroids are unique plant pathogens that are smaller than viruses and consist of a short single-stranded circular RNA without a protein coat (Diener, 1971).

These pathogens cause damage to a number of economic important crops. Traditional detection methods for viroids include hybridization methods, return gel PAGE, dot blot hybridization, RT-PCR, and Next Generation Sequencing.
Citrus Viroids

- Exocortis
- Cachexia (caused by variants of CVd-II)
- CVd-I
- CVd-III
- CVd-IV
- CVd-V
- CVd-VI
- Variants are common within groups and mixed infections of several groups occur
### TABLE 2. Symptoms Induced by Citrus Viroids on Etrog citron Arizona 861-S1 (*Citrus medica* L.)

<table>
<thead>
<tr>
<th>Viroid</th>
<th>Stunting</th>
<th>Leaf Epinasty</th>
<th>Necrosis</th>
<th>Leaf Tip</th>
<th>Petiole</th>
<th>Mid-Vein</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEVd (exocortis)</td>
<td>Severe</td>
<td>Severe</td>
<td>General</td>
<td>±</td>
<td>±</td>
<td></td>
</tr>
<tr>
<td>CVd-I (CBLVd)</td>
<td>Mild</td>
<td>Random</td>
<td>Local</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CVd-II (HSVd)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>±</td>
<td></td>
</tr>
<tr>
<td>(cachexia)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>±</td>
</tr>
<tr>
<td>CVd-III</td>
<td>Moderate</td>
<td>General</td>
<td>General</td>
<td>±</td>
<td>±</td>
<td></td>
</tr>
<tr>
<td>CVd-IV</td>
<td>Moderate</td>
<td>Random</td>
<td>General</td>
<td>±</td>
<td>±</td>
<td></td>
</tr>
</tbody>
</table>

*See Plate 96.*
History of Viroids

• In the 1920s, symptoms of a previously unknown potato disease were seen in New York & New Jersey. The tubers on affected plants were elongated and misshapen, hence the name potato spindle tuber disease.

• The symptoms appeared on plants onto which pieces from affected plants had been budded—indicating the disease was caused by a transmissible pathogenic agent. However, a fungi or bacteria could not be found consistently associated and therefore, it was assumed the disease was caused by a virus.

• Numerous attempts over the years to isolate and purify the assumed virus, using increasingly sophisticated methods, these were unsuccessful.

• In 1971 T. O. Diener showed that the agent was not a virus, but a totally unexpected novel type of pathogen, 1/80th the size of typical viruses. He proposed the term "viroid".

• Semancik & Weathers in 1972 isolated & characterized Citrus exocortis viroid
• Basic research elucidated the ‘viroids' physical, chemical, and macromolecular properties. Shown to consist of short stretches of single-stranded RNA without a protein coat.

• Viroids are small in size, ranging from 246 to 467 bp. In comparison, the genomes of the smallest known viruses capable of causing an infection by themselves are around 2,000 bp long.

• In 1976, Sänger et al. presented evidence that potato spindle tuber viroid is a "single-stranded, covalently closed, circular RNA molecule, existing as a highly base-paired rod-like structure"—believed to be the first such molecule described.”
Citrus Viroids

• Citrus Exocortis Viroid (CeVd)
• Hop stunt viroid (HSVd); cachexia, xyloporosis
• Citrus bent leaf viroid (CBLVd)
• Citrus dwarfing viroid (CDVd)
• Citrus bark cracking viroid (CBCVd)
• The above have been recognized as true viroid species
• More recently, two additional viroids, Citrus viroid VI (CVd-VI) (former Citrus viroid OS) and Citrus viroid V (CVd-V) have also been reported
• All viroids are very infective and very stable. Carried on grafting tools
## Citrus Viroids and their Grouping

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus</th>
<th>Former Name</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pospiviroidae</td>
<td>Pospiviroid</td>
<td>CEVd</td>
<td><em>Citrus exocortis viroid</em> (CEVd)</td>
</tr>
<tr>
<td></td>
<td>Hostuviroid</td>
<td>CVd-II</td>
<td><em>Hop stunt viroid</em> (HSVd)</td>
</tr>
<tr>
<td></td>
<td>Cocadviroid</td>
<td>CVd-IV</td>
<td><em>Citrus bark cracking viroid</em> (CBCVd)</td>
</tr>
<tr>
<td></td>
<td>Apscaviroid</td>
<td>CVd-I</td>
<td><em>Citrus bent leaf viroid</em> (CBLVd)</td>
</tr>
<tr>
<td></td>
<td>Apscaviroid</td>
<td>CVd-III</td>
<td><em>Citrus dwarfing viroid</em> (CDVd)</td>
</tr>
<tr>
<td></td>
<td>Apscaviroid</td>
<td>CVd-V</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Apscaviroid</td>
<td>CVd-VI</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Apscaviroid</td>
<td>CVd-VI</td>
<td></td>
</tr>
</tbody>
</table>

*Table 1. Citrus viroids and viroid species.*
Figure 4. Characteristic symptoms as a result of viroid infection in plants grafted on trifoliate orange: Bark scaling induced by CEVd in trifoliate orange (A); bark cracking induced by *Citrus bark cracking viroid* (CBCVd) in trifoliate orange (B); wood pitting and gum deposits in the clementine scion caused by cachexia strains of HSVd (C); green streaks under the cracks induced by CBCVd in trifoliate orange (D). Lesions in the roots caused by CEVd in Citrange carrizo roots (E,F).
Citrus Exocortis Viroid

• In 1948 Exocortis disease of citrus was first reported and described by (Fawcett and Klotz) as a bark-shelling disorder of trifoliate orange rootstock.

• It may have occurred previously in Australia as early as the 1930s (Benton et al.)

• Semancik & Weathers, 1972: CEVd causal agent was isolated and characterized as a 371 nucleotide RNA molecule.

• Gross et al., 1982: Nucleotide sequence and secondary structure of citrus exocortis and chrysanthemum stunt viroid

• Visvader et al., 1982: Citrus exocortis viroid: nucleotide sequence and secondary structure of an Australian isolate
Symptoms of Citrus Exocortis

• It can cause stunted growth and reduced yields in affected plants. The disease is also sometimes called "scalybutt".

• Present on trifoliate and trifoliate hybrid rootstocks; however Carrizo is often resistant to most isolates

• Symptoms are gum-impregnated pits or pockets in the trifoliate rootstock, which can be seen on the wood surface if the bark is removed. Gum impregnated areas range from few to numerous and from small to large ones

• CEVd can also infect tomato plants; tomato bunchy top disease.
• Extremely tolerant to heat and have not been successfully eliminated from budwood by thermotherapy but can be eliminated by shoot tip grafting.
• Early detection by inoculating indicator plants such as Etrog citron and gynura.
• Now detected by PCR
Hop Stunt Viroid (HSVd)

- Causes cachexia or xyloporosis disease
- Xyloporosis, a disease that has severe effects on citrus trees grafted onto certain citrus rootstocks
- Cachexia, synonymous with xyloporosis, a disease that mainly affects mandarin trees.
- HSVd infections are mostly symptomless in numerous Near East and Western Mediterranean fruit trees and grapevines
- 3 essential phases of xyloporosis. 1. small depressions appear on the stem bark of the rootstock with small conoid pits with interfacing brownish pegs in the inner part of the bark.
- Symptoms often appear within 1 year of grafting and are often close to the bud union.
- In 2nd stage the wood becomes discolored and, typically, the young tree becomes bent over and its leaves show symptoms typical of root rot. In the 3rd stage, there is blackish discoloration on the bark, the bark splits and leaves are small and yellow. Eventually, the branches wilt and die.
Cachexia
Symptoms on Mandarin
Lemon
Citrus leaf bent viroid

• Synonymous with Citrus viroid I, causes leaf bending on Etrog citron and point necrosis on the leaf midribs. *Citrus bent leaf viroid* (318 nt) is a species in the *Apscaviroid* genus.

• CBLVd has three variants which are CVd-Ia (327 - 329 nucleotides), CVd-Ib (315 - 319 nucleotides) and CVd-I-LSS (325 - 330 nucleotides). They are distributed within the main citrus producing countries such as China, Costa Rica, Iran, Japan, Pakistan, Spain and USA. Viroid pathogenicity is significantly attributed to the T1 loop and the T2 loop of CBLVd. Moreover, CBLVd fostered leaf epinasty on Etrog citron, and caused stunting and reduced fruit yield on citrus plants. Citrus plants are the only host for CBLVd. In addition, CBLVd is transmitted via sap and mechanical tools in citrus. RT-PCR and multiplex RT-PCR are predominantly employed in detection of CBLVd. Regulatory and cultural practices are imposed to control and eradicate CBLVd.
Citrus bent leaf viroid
Citrus bent leaf viroid symptoms
Citrus dwarfing viroid

- Citrus dwarfing viroids, synonymous with Citrus viroid III,
- Cause dwarfing on *P. trifoliata*, citrange, and citrumelo rootstocks. Citrus viroid IIIa (297 nt) and Citrus viroid IIIb (294 nt) are species in the *Apscaviroid* genus.
Citrus bark cracking viroid

• Formerly Citrus viroid IV causes leaf drooping on Etrog citron indicator plants, and general necrosis of the leaf midribs. Citrus bark cracking viroid (284 nt) is a species in the Cocadviroid genus.

• A minor pathogen of citrus plants causing bark cracking in trifoliate orange (Poncirus trifoliata). does not have a negative effect on growth and yield in citrus. However, when co-infected with HSVd, a synergistic effect has been observed with yield reduction

• Also causes bark cracking in hops and stunting in Slovenia; called severe hop stunt disease
Viroid species associated with the bark-cracking phenotype of 'Tahiti' acid lime in the State of São Paulo, Brazil. 2010. Marcelo Eiras et al.

CEVd, HSVd and CDVd were found in different combinations.

FIGURE 1 - Different severity of bark-cracking symptoms on acid lime ‘Tahiti’ clone “Quebra-galho”. A. Mild symptoms; B. Moderate symptoms; C. Severe symptoms.
Additional Citrus Viroids

• Citrus viroid V produces mild symptoms in Etrog citron. Citrus viroid V (294 nt) is a species in the *Apscaviroid* genus (Serra et al., 2007).

*Citrus viroid V* induced mild characteristic symptoms of very small necrotic lesions and cracks, sometimes filled with gum, in the stems of the viroid indicator plant, Etrog citron. However, CVd-V reacted synergistically when Etrog citrus was co-infected with either *citrus bent leaf viroid* (CBLVd) or *Citrus dwarfing viroid* (CDVd), and showed severe stunting and epinasty with multiple lesions in the midvein. Plants co-infected with CBLVd and CVd-V exhibited severe stem cracking characteristic of CBLVd, but without gum exudates, whereas plant co-infected with CDVd showed necrotic lesions (Serra et al., 2008a). Symptoms induced by CVd-V alone in commercial species and varieties are presently not known.
Citrus Viroid V

Symptoms of viroid infection in Etrog citron plants (B) Bending of the leaves of CBLVd-infected plants. (C) Local midvein necrosis on the underside of the leaf blade of CBLVd-infected plants. (D) Cracks releasing gum exudates in CBLVd-infected plants. (E) Branching pattern and gum exudates in CBLVd-infected plants. (F) Petiole and midvein necrosis of CVd-III-infected plants. (G) Cracks in the stem of CVd-V-infected plants.
Mixed viroid infections

Symptoms of viroid infection in Etrog citron plants (B) Bending of the leaves of CBLVd-infected plants. (C) Local midvein necrosis on the underside of the leaf blade of CBLVd-infected plants. (D) Cracks releasing gum exudates in CBLVd-infected plants. (E) Branching pattern and gum exudates in CBLVd-infected plants. (F) Petiole and midvein necrosis of CVd-III-infected plants. (G) Cracks in the stem of CVd-V-infected plants. (H) Leaf curling of plants co-infected with CVd-V and CBLVd (left), or with CVd-V and CVd-III (right). (I) Lesions in the midvein of plants co-infected with CVd-V and CBLVd, or with CVd-V and CVd-III. (J) Severe cracks devoid of gum in plants co-infected with CVd-V and CBLVd.
Multiple Viroids/viruses in the Same Plant
Citrus tree size control with viroids
Labeling Nucleic Acids

• Traditional *In situ* Hybridization
  • Previously used
  • Low signal amplification
  • Long process

• Newer methods enhance signal selective for target sequences

• Single-stranded RNA detected in citrus