SHORT COMMUNICATION

OCCURRENCE, PREVALENCE AND DISTRIBUTION OF CITRUS VIROID IN URUGUAY

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SUMMARY

The occurrence of Citrus exocortis viroid (CEVd), Citrus bent leaf viroid (CBLVd), Hop stunt viroid (HSVd), Citrus dwarfing viroid (CDVd), Citrus bark cracking viroid (CBCVd) and Citrus viroid VI (CVd-VI) in Citrus spp. cultivated in six provinces of Uruguay was surveyed in 2008-2009 and 2009-2010 growing seasons using Northern blot hybridization. Sixty two per cent of surveyed trees were infected with either single or mixed viroid inocula, the latter being more abundant. CBCVd and CDVd were not detected in the samples analyzed. HSVd showed the highest prevalence (92%) among infected plants followed by CDVd (50%), CEVd (23%) and CBLVd (19%). The most frequently observed viroid combination was HSVd+CDVd. Our results showed that CEVd, CBLVd, HSVd, CDVd are widespread throughout citrus orchards in Uruguay and highlight the presence of mixed viroid infections.

Key words: survey, citrus, CEVd, CBLVd, HSVd, CDVd, Northern blot hybridization.

Seven viroids reported to infect Citrus spp. belong to four genera of the family Pospiviroidae (Duran Vila et al., 1988; Ashulin et al., 1991; Ito et al., 2001; Duran-Vila and Semancik, 2003; Serra et al., 2008a): Citrus exocortis viroid (CEVd) (genus Pospiviroid), Hop stunt viroid (HSVd) (genus Hostuviroid), Citrus bark cracking viroid (CBCVd) (genus Cocaduviroid), and Citrus bent leaf viroid (CBLVd), Citrus dwarfing viroid (CDVd), Citrus viroid V (CVd-V) and Citrus viroid VI (CVd-VI) (genus Apscaviroid). CEVd is the causal agent of the exocortis disease. HSVd includes variants that induce the cachexia disease as well as non-cachexia inducing variants (Semancik et al., 1988; Duran-Vila and Semancik, 2003). The other viroids cause minor effects (Vernière et al., 2004) with complex interactions when co-infecting the same tree (Vernière et al., 2006). CEVd, CBLVd, HSVd and CDVd are widely distributed worldwide (Singh et al., 2003) whereas CBCVd has limited distribution in citrus-growing areas (Malfitano et al., 2005; Mohamed et al., 2009; Murcia et al., 2009; Cao et al., 2010). CVd-V and CVd-VI are two newly reported viroid species (Owen et al., 2011). CVd-V has been reported in California (USA) and Japan (Serra et al., 2008b; Bani-Hashemian et al., 2010; Ito and Ohta, 2010; Cao et al., 2010) and recently in Pakistan (Cao et al., 2013), whereas CVd-VI seems to be restricted to Japan (Ito et al., 2003).

Bark cracking and scaling followed by severe dwarfing and yield losses occur in viroid-infected citrus plants when commercial species are grafted on sensitive rootstocks such as trifoliate orange (Poncirus trifoliata (L.) Raf.), Rangpur lime (Citrus limonia Osb.) and citranges (C. sinensis x P. trifoliata) and strain severity is high (Bani-Hashemian et al., 2009). Considering that more than 90% of citrus plants in Uruguay are grafted on either trifoliate orange or citranges, local citrus production may be at risk (Bisio et al., 2004).

A procedure for viroid detection from field-grown citrus trees originally described by Murcia et al. (2009) and successfully applied elsewhere (Mohamed et al., 2009) has been found to be highly sensitive, specific and an efficient alternative to biological indexing procedures because the time required for diagnosis is markedly shortened. Briefly, RNA preparations extracted from bark tissues of field-grown citrus trees were subjected to polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions and Northern blot hybridization analysis with viroid-specific digoxigenin (DIG)-labelled probes.

This work reports the results of a survey on the occurrence, prevalence and distribution of CEVd, CBLVd, HSVd, CDVd, CBCVd and CVd-VI in commercial citrus species in Uruguay using PAGE-Northern blot hybridization analysis.

A total of 84 mature plants of the genus Citrus and Fortunella and trifoliate orange seedling trees were surveyed in commercial orchards located in six provinces.
of Uruguay during the 2008-2009 and 2009-2010 growing seasons (Fig. 1; Table 1). In each orchard, plants were randomly selected and sampling included both symptom- and symptomless trees. Arizona 861-S1 Etrog citrons (C. *medica* L.) grafted onto rough lemon (C. *jambhiri* Lush.) and infected with different combinations of four viroid species (CEVd, CBLVd, HSVd, CDVd) were used as a source of leaf/bark tissue for positive controls. Uninfect- ed and micrografted citron plants were used as negative controls. Tissues or nucleic acid preparations, kindly pro- vided by colleagues, were used as additional negative and positive controls. As a whole, 17 positive and 6 negative control plants were included in this study. In order to get high viroid titers, infected citron plants, were maintained at 28-32°C at 16 h light/8 h dark photoperiod. CBCVd and CVd-VI controls were obtained as monomeric transcripts from cDNA sequences cloned into TS4.7 and S10S plasmids according to Umaña (2010).

Five grams of bark tissues per tree, collected from shoots 30-50 cm long and 0.5-1.0 cm in diameter were processed following the nucleic acid extraction proto- col previously described by Semancik *et al.* (1975) with minimal modifications reported by Murcia *et al.* (2009). DIG-labelling of viroid-specific cDNA probes of CEVd, CBLVd, HSVd, CDVd and Cvd-VI were carried out by PCR. In some instances the reverse transcription PCR (RT-PCR) protocol described by Bernard and Du- ran-Vila (2006) was used as an additional procedure to confirm the presence of viroids in nucleic acid samples.

Aliquots (20 μl) of nucleic acid preparations (about 333 mg of fresh weight tissue) were electrophoresed in 5% non-denaturing polyacrylamide gel for 3 h at 60 mA as described by Murcia *et al.* (2009). Following migration, a gel section (2 cm wide) corresponding to the region where the signal recognition particle 7S RNA migrates, was sliced and electrotransferred to a positively-charged nylon mem- brane (Roche Applied Science, Germany) using 1× TBE buffer (90 mM Tris, 90 mM boric acid, and 2 mM EDTA) at 400 mA for 1.5 h. After transfer, gel slices were silver- stained to verify that RNAs had been completely electro- transferred. For nucleic acid fixation, membranes were exposed to 70,000 μJoules/cm² using an UV-crosslinking oven (Hoefer-Uvc500, Amersham Biosciences, USA). Pre-hybridization (42°C for 2 h) and hybridization with viroid-specific DIG-labelled probes (60°C overnight) were performed as previously described (Murcia *et al.*, 2009). Chemiluminescence signals from DIG-labelled hybrids were detected by autoradiography using anti-DIG-alkaline phosphatase conjugate and the CSPD substrate (Roche Applied Science, Germany).

An example of the results obtained by Northern hy- bridization analyses is shown in Fig. 2, in which RNA preparations from bark tissues of six citrus varieties were independently hybridized with four DIG-labelled probes, each specific for a single viroid. This autoradiography provided evidence of two-viroid mixed infections in lanes 3 (CBLVd+HSVd) and 4 (HSVd+CDVd) and four-viroid

**Table 1.** Citrus type, variety and sampling location (province) of the 84 citrus plants surveyed for viroid infection during 2008-2009 and 2009-2010 growing seasons in Uruguay.

<table>
<thead>
<tr>
<th>Citrus type (species)</th>
<th>Cultivar</th>
<th>Plants analyzed&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Location&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orange <em>Citrus sinensis</em></td>
<td>Washington Navel</td>
<td>19</td>
<td>CA, MO, SJ, SA</td>
</tr>
<tr>
<td></td>
<td>Valencia late</td>
<td>10</td>
<td>CA, CO, SA</td>
</tr>
<tr>
<td>Mandarin</td>
<td>Murcott</td>
<td>3</td>
<td>CA, SA</td>
</tr>
<tr>
<td></td>
<td>Ellendale</td>
<td>2</td>
<td>SA</td>
</tr>
<tr>
<td></td>
<td>Common</td>
<td>7</td>
<td>CA, SA</td>
</tr>
<tr>
<td></td>
<td>Satsuma</td>
<td>14</td>
<td>CO, RN, SA</td>
</tr>
<tr>
<td>Lemon <em>Citrus limon</em></td>
<td>Lisbon</td>
<td>16</td>
<td>CA, CO, MO, RN, SJ</td>
</tr>
<tr>
<td>Grapefruit <em>Citrus paradisi</em></td>
<td>Marsh Seedless</td>
<td>4</td>
<td>CA</td>
</tr>
<tr>
<td></td>
<td>Duncan</td>
<td>1</td>
<td>CA</td>
</tr>
<tr>
<td></td>
<td>Star Ruby</td>
<td>2</td>
<td>CO, RN, SA</td>
</tr>
<tr>
<td>Kumquat <em>Fortunella margarita</em></td>
<td>Nagami</td>
<td>2</td>
<td>CO, MO</td>
</tr>
<tr>
<td>Volkamer lemon <em>Poncirus trifoliata</em></td>
<td>2</td>
<td>SA</td>
<td></td>
</tr>
<tr>
<td>Trifoliate orange</td>
<td>2</td>
<td>SA</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>number of plants.

mixed infections (CEVd+CBLVd+HSVd+CDVd) in lane 5. In addition to lane 1 (negative control), viroids were undetectable in the remaining three lanes. No single viroid infection was found in the samples shown in this figure. The results were confirmed by RT-PCR for some field-grown samples (results not shown).

From the 84 field-grown citrus trees analyzed, 62% (52/84) were infected with at least one viroid or combinations of CEVd, CBLVd, HSVd and CDVd (Table 2). CBCVd and CDV-VI were not detected (Umaña, 2010). Positive samples comprised either single (23/84) or mixed (29/84) viroid infections. Single viroid infections were only observed for CBLVd and HSVd, with HSVd being the most commonly detected (40% of infected plants). Mixed infections with two, three and four viroids were found in 19%, 10% and 5% of the tested plants, respectively. In the case of mixed infections with either two or three viroids, different viroid combinations were detected. The most frequent mixed infection was HSVd+CDVd (14/84) followed by CEVd+HSVd+CDVd (4/84) and CBLVd+HSVd+CDVd (3/84). HSVd was the most frequently detected viroid either in single or mixed infections with 92% (48/52) of the total number of infected plants, while CDVd, CEVd and CBLVd were detected in 50% (26/52), 23% (12/52) and 21% (11/52) of the positive samples, respectively. Noteworthy, HSVd+CDVd were detected in 27% (14/52) of infected plants.

With the exception of grapefruit cv. Star Ruby, viroid infection was found in all the commercial varieties tested: lemon (81%), grapefruit (71%), mandarine (62%) and sweet orange (59%). Viroids were also detected in trifoliate orange but neither in Volkamer lemon (C. volkameriana Ten. & Pasq.) nor in Nagami kumquat (Table 2). Since the sample size for some of these citrus types was rather small, further sampling efforts would be necessary to confirm these findings.

This survey complements previous data regarding viroid occurrence, prevalence and distribution in commercial

Table 2. Detection of single infection of HSVd or CBLVd and mixed infections of CEVd, CDVd, HSVd and/or CBLVd in different citrus species from commercial orchards in Uruguay by Northern-blot hybridization analysis.

<table>
<thead>
<tr>
<th>Citrus species</th>
<th>CEVd</th>
<th>CBLVd</th>
<th>HSVd</th>
<th>CDVd</th>
<th>CEVd+CBLVd</th>
<th>CEVd+HSVd</th>
<th>CEVd+CDVd</th>
<th>CBLVd+HSVd</th>
<th>CEVd+CBLVd+HSVd</th>
<th>CEVd+CBLVd+CDVd</th>
<th>CBLVd+HSVd+CDVd</th>
<th>CEVd+CBLVd+HSVd+CDVd</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orange</td>
<td>29</td>
<td>14</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>32 (38.0)</td>
</tr>
<tr>
<td>Mandarine</td>
<td>26</td>
<td>10</td>
<td>10</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10 (38.0)</td>
</tr>
<tr>
<td>Lemon</td>
<td>16</td>
<td>3</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>5 (29.0)</td>
</tr>
<tr>
<td>Grapefruit</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>7 (29.0)</td>
</tr>
<tr>
<td>Volkamer lemon</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (29.0)</td>
</tr>
<tr>
<td>Trifoliate</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (29.0)</td>
</tr>
<tr>
<td>Fortunella sp.</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (29.0)</td>
</tr>
<tr>
<td>Total (%)</td>
<td>84</td>
<td>32</td>
<td>21</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>14</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>84 (100)</td>
</tr>
</tbody>
</table>

* total number of sampled plants.

* number of plants with non-detectable infection.

* in parentheses: number of infected plants/total number of sampled plants x 100.
citrus orchards in Uruguay (Pagliano et al., 1998) as also reported in other citrus-growing countries (Japan, Italy Cuba, Brazil, Spain and Sudan) (Malfitano et al., 2005; Murcia et al., 2009; Mohamed et al., 2009). Our results point out that viroid infections occur (62%) in many citrus orchards in Uruguay, affecting all the commercial varieties tested and confirm the occurrence of CEVd, CBLVd, HSvd and CVDv. These viroids appear to be present throughout the six provinces sampled in agreement with previous preliminary surveys conducted in Uruguay (Pagliano et al., 1998; Pagliano, 2000). It is also confirmed in other reports that there is no presence of CBCVd and CVD-VI in any of the samples analyzed (Umaña, 2010). The failure to detect CB-CVd and CVd-VI, confirms other reports indicating a more restricted distribution worldwide (Duran-Vila et al., 1988; Ito et al., 2001). HSvd was the most frequently detected viroid, which is in agreement with previous reports and confirms its high prevalence and worldwide distribution (Ito et al., 2002). HSvd was found at a slightly lower frequency in single than mixed infections and it was detected not only in plants older than 40 years but also in young (three-year-old) symptomless trees.

No attempts were made in this study to differentiate between cachexia and non-cachexia variants of the viroid. The occurrence of mixed viroid infections was 20% higher than single infections (Table 2). Malfitano et al. (2005) also reported a high frequency of mixed viroid infections in surveys conducted in southern Italy and suggested that a high frequency of single and mixed infections in propagation material probably occurred long time ago and that the use of infected budwood, contaminated tools and top-grafting may have been responsible for viroid spread and accumulation in individual trees. Overall, the prevalence of viroid-infected citrus trees in commercial orchards grafted onto susceptible rootstocks in Uruguay poses a threat to the current and future citrus production in the country. Therefore, actions against further viroid dissemination to new plantations should be undertaken by propagating viroid-free materials under appropriate certification programs.

Northern blot hybridization analysis has been recently highlighted as a useful method for viroid detection due to its high specificity, sensitivity, repeatability and diagnostic efficiency (Mohamed et al., 2009; Murcia et al., 2009). In the present work, we were able to survey viroid occurrence and distribution from a large number of sampled field-grown citrus trees in a short time (three days). Moreover, the results reported here expand those of earlier surveys performed by using other techniques (Pagliano et al., 1998; Pagliano, 2000) and update the occurrence and distribution of citrus viroids in the most important citrus-growing areas of Uruguay. Northern blot hybridization allowed the analysis of large numbers of samples that could not be handled using more laborious, time-consuming analysis and/or biological indexing methods. Consequently, as a molecular diagnostic tool, the Northern blot hybridization analysis meet diagnostic requirements for quarantine and sanitation programs for propagating materials in Uruguay, either as a primary screening method or as an additional tool to further support results from indexing tests on indicator plants.

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