SUMMARY

A dot-blot hybridization protocol using digoxigenin-labelled riboprobes was finalized for the detection of *Citrus psorosis virus* (CPsV) and *Citrus variegation virus* (CVV). Both viruses were readily identified in different organs of screenhouse-grown and, throughout a 9-month period, in-field-grown citrus plants. With CPsV, strong hybridization signals were obtained by dot blot hybridization from flowers (ovaries) and young leaves and, with CVV, from young leaves and shoots. In tissues prints, CPsV was satisfactorily detected from ovaries and CVV from petioles, ovaries, young leaves and tender shoots. The sensitivity threshold of the assay was determined to be 75 pg of *in vitro* transcripts for both CPsV and CVV, allowing detection of both viruses even when their titres decreased in plant tissues and ELISA tests failed.

Key words: Citrus, total RNA, riboprobes, molecular hybridization, ELISA, diagnosis.

INTRODUCTION

*Citrus psorosis virus* (CPsV) and *Citrus variegation virus* (CVV) are the causal agents of relevant and widespread diseases of citrus (Gonsalves and Garnsey, 1974, 1975a, 1975b; da Graça et al., 1991; Martín et al., 2004).

CPsV, the type species of the genus *Ophiovirus* (Vaira et al., 2005), has a genome consisting of three single-stranded RNAs of negative polarity that have been totally sequenced (Barthe et al., 1998; Sanchez de la Torre et al., 1998, 2002; Naum-Onganía et al., 2003; Martín et al., 2005). The complementary strand (vcRNA) of CPsV RNA-1 contains two ORFs potentially encoding a 24 kDa protein with unknown function and the ~49 kDa coat protein (CP) (Sanchez de la Torre et al., 1998), respectively.

CVV, a definitive species of the genus *Ilarvirus*, family *Bromoviridae* (Roossinck et al., 2005) has a tripartite, positive-sense single-stranded RNA genome. RNA-1 and RNA-2 (Li et al., 2008) are monocistronic and bicistronic, respectively, and encode the replication-associated proteins. RNA-3 (Scott and Ge, 1995) is bicistronic, with ORF1 coding for the movement protein and ORF-2 for the viral coat protein (CP), which is expressed from a subgenomic RNA (RNA-4) (Gonsalves and Garnsey, 1975b).

Routine detection of CPsV and CVV is mainly based on ELISA or direct tissue blot immunoassay (DTBIA) using polyclonal antisera or specific monoclonal antibodies (MAb) (Garnsey, 1974; Davino and Garnsey, 1984; García et al., 1997; Alioto et al., 1999; Potere et al., 1999; D’Onghia et al., 2001; Martín et al., 2002; Loconsole et al., 2006). However, the use of these techniques is limited by the low titre and the uneven distribution of both viruses in infected plants and, for CVV, by the narrow period of application during the year, detection being reliable only in young tissues from spring to early summer (Davino et al., 1988; Roistacher, 1993). Thus, molecular assays, based on RT-PCR and hybridization, have been developed in the last few years (Bennani et al., 2002; Martín et al., 2004; Roy et al., 2005; Rosa et al., 2007). In this paper we report the development of a specific and sensitive dot-blot hybridization assay for routine detection of CPsV and CVV by digoxigenin-labelled riboprobes and its validation in the field as diagnostic tool for large-scale surveys.

MATERIALS AND METHODS

Plant material, cDNA cloning and synthesis of riboprobes. Total RNAs were extracted from 100 mg of citrus leaf tissues using RNeasy Plant Mini kit (Qiagen, Italy) according to the manufacturer’s instructions. Tissues were from two citrus accessions, infected respectively by an Apulian CPsV isolate denoted ps101 and a Sicilian CVV isolate denoted cvv300, both maintained in a collection of the University of Bari (Table 1). Isolate ps101 was
previously identified by Potere et al. (1999), whereas cvv300 was identified by indexing and TAS-ELISA using a commercial kit (Direction des Domaines Agricoles, Morocco). CPsV- and CVV-specific probes were produced by RT-PCR using primers CPV-1 and CPV-2 (Barthe et al., 1998; accession No. AF036338), and cvv187-FW (5’ TGGAGAAGTCTATCCAGGTCAGC 3’) and cvv187-Rv (5’ GCCATATCCATTGGATTGC 3’), designed on the CP sequence of an American CVV isolate [Scott and Ge (1995), accession No. U17389]. For

### Table 1. Symptoms from field observation and biological indexing are reported in comparison with ELISA and hybridization assays for 44 CPsV isolates from the collection of the University of Bari. In bold the isolate used for synthesizing CPsV riboprobe.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Origin</th>
<th>Field symptoms</th>
<th>Symptoms on indicator plants</th>
<th>DAS-ELISA Spring</th>
<th>Winter</th>
<th>Hybridization Spring</th>
<th>Winter</th>
</tr>
</thead>
<tbody>
<tr>
<td>40-X</td>
<td></td>
<td>OLP, LS, BS</td>
<td>OLP, LS, S</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>194-X</td>
<td></td>
<td>OLP, LS</td>
<td>LS, OLP, S</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>195-X</td>
<td></td>
<td>OLP, LS</td>
<td>OLP, LS, RS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>197-X</td>
<td></td>
<td>OLP, LS</td>
<td>LS</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>203-X</td>
<td></td>
<td>OLP, LS</td>
<td>OLP, LS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>204-X</td>
<td></td>
<td>OLP, LS</td>
<td>OLP, LS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>207-X</td>
<td></td>
<td>LS, BS, S</td>
<td>RS, LS</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>208-X</td>
<td></td>
<td>OLP, LS</td>
<td>OLP, LS</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>321-X</td>
<td></td>
<td>RS</td>
<td>LS, S</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>365-X</td>
<td></td>
<td>OLP</td>
<td>LS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>367-X</td>
<td></td>
<td>LS</td>
<td>LS, S</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>372-X</td>
<td>Southern Italy</td>
<td>LS, S</td>
<td>LS, BS, S</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>373-X</td>
<td></td>
<td>LS, LV</td>
<td>LV, LS</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>381-X</td>
<td></td>
<td>LS, OLP</td>
<td>OLP, LS</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>391-X</td>
<td></td>
<td>LS</td>
<td>LS</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>394-X</td>
<td></td>
<td>LS</td>
<td>LS</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5V</td>
<td></td>
<td>LS</td>
<td>LS</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6V</td>
<td></td>
<td>LS, OLP</td>
<td>OLP, LS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7V</td>
<td></td>
<td>LS, OLP</td>
<td>LS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>36V</td>
<td></td>
<td>LS</td>
<td>OLP</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>68V</td>
<td></td>
<td>LS</td>
<td>LS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>72V</td>
<td></td>
<td>LS, OLP</td>
<td>LS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>74V</td>
<td></td>
<td>LS, OLP</td>
<td>LS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>77V</td>
<td></td>
<td>LS, OLP</td>
<td>LS, OLP</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>86V</td>
<td></td>
<td>LS</td>
<td>LS</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>243-X</td>
<td></td>
<td>BS</td>
<td>LS, S</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>244-X</td>
<td></td>
<td>LS</td>
<td>LS, S</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>245-X</td>
<td></td>
<td>LS</td>
<td>LS, S</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>246-X</td>
<td></td>
<td>LS</td>
<td>LS, S</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>247-X</td>
<td></td>
<td>BS</td>
<td>LS, S</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>248-X</td>
<td></td>
<td>LS</td>
<td>S</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>249-X</td>
<td></td>
<td>BS</td>
<td>LS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>250-X</td>
<td></td>
<td>RS</td>
<td>LS, RS, S</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>251-X</td>
<td></td>
<td>LS</td>
<td>LS, S</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>252-X</td>
<td></td>
<td>LS</td>
<td>LS, S</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>253-X</td>
<td></td>
<td>LS</td>
<td>LS, S</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>254-X</td>
<td></td>
<td>LS</td>
<td>LS, S</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>304-X</td>
<td></td>
<td>RS</td>
<td>LS, RS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>316-X</td>
<td></td>
<td>LS</td>
<td>LS, OLP</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>317-X</td>
<td></td>
<td>BS, LS</td>
<td>LS, RS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>318-X</td>
<td></td>
<td>BS, LS</td>
<td>LS, RS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9N36 (2)</td>
<td>Spain</td>
<td>OLP</td>
<td>LS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9N56(1)</td>
<td>Lebanon</td>
<td>OLP</td>
<td>LS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Dweet tangor and Sweet Orange cv. Madam Vinous were used as indicator plants.

BS: bark scaling; LS: chlorotic flecking or spotting in young leaves; OLP: oak leaf pattern; RS: ringspot in leaves; S: shock reaction (leaf shedding and necrosis of young shoots); LV: variegation in young leaves.
cDNA synthesis, 2.5 µl of total RNAs extract (500 ng) and 0.5 µl of random primers (0.5 µg/µl) were denatured for 5 min at 95°C and chilled on ice. 1x M-MLV-RT buffer (Invitrogen, USA), 1.25 µl of 2.5 mM dNTPs, 1 µl RNasin (40 U/µl) and 0.4 µl M-MLV-RT (200 U/µl) were added and the samples were incubated for 1 h at 42°C. Five µl of cDNA were added to 25 µl of a PCR cocktail containing 1x PCR buffer (Promega, USA), 1.25 µl of dNTPs 2.5 mM, 1.25 µl of each forward and reverse primer (6 µM stock) and 1 U of Tag polymerase. PCR conditions for CPsV were: 1 cycle of 5 min at 94°C; 35 cycles of 30 sec at 94°C, 30 sec at 58°C, 30 sec at 72°C; finally 1 cycle of 7 min at 72°C. CVV amplification differed only in the annealing temperature (60°C). Amplification products, detected by 1% agarose electrophoresis and ethidium bromide staining, were purified using QIAquick PCR Purification Kit (Qiagen, Italy), ligated to pGEM-T easy vector (Promega, USA) and transformed into Escherichia coli TOP 10. Clones (Ps101 and CVV300) were automatically sequenced (MWG Biotech, Germany). Nucleotide sequences were compared with the existent sequences of CPsV and CVV isolates using FASTA (Altschul et al., 1997) and deposited in the EMBL database under accession No. AM409317 and AJ508381, respectively. Synthesis of RNA probes was performed according to the manufacturer's instructions. Briefly, 1 mg of Ps101 and CVV300 plasmids was linearized with PstI and NcoI and transcribed with T7 or SP6 RNA polymerase, respectively.

Total RNA extraction and dot-blot hybridization. Different procedures of sample preparation were compared to optimise CPsV and CVV detection in citrus by molecular hybridization: (i) 100 µg of healthy or CPsV- or CVV-infected tissues were homogenized in 1 ml extraction buffer (100 mM NaCl, 100 mM Tris-HCl pH 8.0, 5 mM EDTA, 2% sodium meta bisulphite), mixed with 150 µl of 10% SDS at 70°C and then with potassium acetate (KOAc) in ice (De Paula and Powell, 1995). The precipitate was removed by centrifugation and the supernatant containing nucleic acids was subjected to a phenol-chloroform extraction and ethanol precipitation; (ii) extraction by capture on silica particles according to Foissac et al. (2000); (iii) crude sap extraction in 50 mM NaOH, 2.5 mM EDTA (100 mg tissue per 1 µl of solution), followed by incubation at room temperature for 5 min (Gallichitelli and Saldarelli, 1996). In all protocols, citrus tissues were homogenized with a Mill300 mixer (Qiagen, Italy).

Total RNAs extracted following protocols (i) and (ii) were resuspended in DEPC-treated water and aliquots corresponding to 20 µg of tissue were denatured in 1 vol of 100 mM NaOH and 5 mM EDTA solution for 5 min at room temperature and immediately spotted onto nylon membrane (Hybond-N+, Amersham, UK). As to protocol (iii), 5 µl of crude sap were directly spotted on a 2x SSC-soaked membrane. Nucleic acids were fixed on the membrane by UV cross-linking.

Prehybridization and hybridization were done following manufacturer's conditions (Roche, Germany) for DIG-labelled riboprobes. The hybridization solution contained 100 ng/ml of the probe. Hybridization was carried out overnight at 56°C. Membranes were washed twice (15 min each) in 2x SSC, 0.1% SDS at room temperature and twice (15 min each) in 0.1x SSC, 0.1% SDS at 68°C. Blots were incubated for 30 min in 2x SSC containing 1 µg/ml RNase A (Sigma-Aldrich, USA). Hybridization was detected with the Anti-Digoxigenin-AP Fab fragments and the chemiluminescent substrate Disodium 2-chloro-3-(4-methoxyspiro[1,2-dioxetane-3,2’-(5’-chloro) tricyclo[3.3.1.13,7] decan]-4-yl)-1-phenyl phosphate (CDP-star) as recommended by the manufacturer (Roche, Germany) and exposed overnight to X-ray films. To determine whether some inhibitors of the plant extracts affected the sensitivities of the assays, unlabeled viral transcripts were produced by in vitro transcription (Roche, Germany) on Ps101 and CVV300 plasmids, were serially diluted in total RNA extracted from 100 mg of healthy leaves by silica capture, and spotted on the membrane.

Tissue-print hybridization. Different tissues and organs were compared for virus detection. Transversely cut tender shoots and rolled-up aged and young leaf blades, whole fruits, fruit peel and flavedo, were gently pressed onto nylon membranes, previously soaked in 50 mM NaOH, 2.5 mM EDTA and dried at room temperature. Prints were allowed to dry for 30 min at room temperature. Membranes were exposed to UV and submitted to hybridization as above.

ELISA. A DAS-ELISA (Clark and Adams, 1977) protocol, based on a cocktail of 14 monoclonal antibodies for plate coating and on the broad-spectrum conjugated Mab Ps-29 (Alioto et al., 1999; Potere et al., 1999; Djelouah et al., 2000), was used to detect CPsV. These Mabs were able to recognize 40 different CPsV isolates from America, Italy, Spain, and Lebanon (Djelouah et al., 2000; D’Onghia et al., 1998).

A TAS-ELISA protocol (Cambra et al., 1995) was used to detect CVV with a Moroccan commercial ELISA kit as previously described (Djelouah et al., 2002). A polyclonal antiserum and a mix of four monoclonal antibodies were used at a dilution of 1:1000 for plate coating and 1:500 as secondary antibody, respectively. Alkaline-phosphatase-conjugated goat antirabbit IgGs, diluted in a 1:1000 conjugate buffer, was added and plates were incubated for 2 h at 37°C before the addition of 1 mg/ml p-nitrophenyl phosphate.

In ELISA for both CPsV and CVV, citrus leaf tissues (100 mg) were homogenized in 1 ml of extraction buffer (20 g/l PVP-24000, 0.5 ml/l Tween-20, 2% non-fat dry
milk, in PBS 1x, pH 7.4) and extracts were incubated for 12 h at 4°C. A reaction was considered positive when the mean absorbance value was twice the mean value of healthy controls.

**CVV RT-PCR.** Total RNAs were extracted from citrus leaves following protocol (ii), whereas cDNA synthesis and PCR were done following the procedure described above. CVV was detected using primers cvv249fw (5' TACCATTGCCTACATGACCC 3', positions 1592-1611 on RNA3) and cvv249rev (5' GCCTTCATCGGAACCGTG 3', positions 1819-1840 on RNA3), designed on a conserved region identified by multiple alignment of the following CP sequences [Scott and Ge (1995), accession No. U17389; Bennani et al., 2002, accession No. AF434922.1; AF434914.1; AF434915.1; AF434921.1; AF434919.1; AF434918.1; AF434913.1; AF434920.1; AF434917.1; AF434916.1; AF434911.1; AF434912.1]. CVV amplification differed only in the annealing temperature (58°C). Amplification products were detected by 1% agarose electrophoresis and ethidium bromide staining. CVV amplicons were purified using QIAquick PCR Purification Kit (Qiagen, Italy) and sequenced (Primm, Italy). Nucleotide sequences were searched in the EMBL database of CVV isolates using FASTA (Altschul et al., 1997) and deposited in the EMBL database under accession No. FJ228139; FJ228141; FJ228143; FJ228142.

**RESULTS**

**Cloning and dot-blot hybridization.** RT-PCR of total RNA extracts from citrus leaves infected by CPsV ps101 and CVV cvv300 (Table 1) yielded amplicons of 600 and 187 bp respectively, which were cloned and sequenced. Identity values of 81-98% at the nucleotide level were found between the partial CP sequence of CPsV ps101 and comparable sequences reported in the EMBL database (Barthe et al., 1998; Alioto et al., 2003; Martin et al., 2006), whereas cvv300 sequence had 96-97% identity at the nucleotide level with the sequences of the CVV isolates studied by Scott and Ge (1995) and Bennani et al. (2002).

Both viruses gave the best hybridization signals with total RNAs extracted from citrus leaves infected by CPsV ps101 and CVV cvv300 (Table 1) yielded amplicons of 600 and 187 bp respectively, which were cloned and sequenced. Identity values of 81-98% at the nucleotide level were found between the partial CP sequence of CPsV ps101 and comparable sequences reported in the EMBL database (Barthe et al., 1998; Alioto et al., 2003; Martin et al., 2006), whereas cvv300 sequence had 96-97% identity at the nucleotide level with the sequences of the CVV isolates studied by Scott and Ge (1995) and Bennani et al. (2002).

Both viruses gave the best hybridization signals with total RNAs extracted from citrus leaves infected by CPsV ps101 and CVV cvv300 (Table 1) yielded amplicons of 600 and 187 bp respectively, which were cloned and sequenced. Identity values of 81-98% at the nucleotide level were found between the partial CP sequence of CPsV ps101 and comparable sequences reported in the EMBL database (Barthe et al., 1998; Alioto et al., 2003; Martin et al., 2006), whereas cvv300 sequence had 96-97% identity at the nucleotide level with the sequences of the CVV isolates studied by Scott and Ge (1995) and Bennani et al. (2002).

Based on these results, procedure (ii) was selected since it allowed detection of CPsV and CVV using total RNAs extracted in the same way. The sensitivity of the system was evaluated by hybridizing serial dilutions (7.5 ng, 0.75 ng, 75 pg, 7.5 pg) of in vitro transcripts of both viruses. Results (Fig. 2) showed that a detectable signal was obtained up to 75 pg with both CPsV and CVV riboprobes.
Detection of CPsV and CVV in screenhouse and field samples. Fifty-three citrus accessions were assayed by dot blot hybridization and routine ELISA using young and aged leaves collected in spring and winter (Table 1 and 2). These plants, grafted on sour orange, are maintained in an insect-proof screenhouse at University of Bari and originated from Southern Italy, Spain, Lebanon and USA. All were previously characterized by biological indexing and ELISA and found to be infected by CPsV (44 isolates) and CVV (9 isolates) (D’Onghia et al., 1998; D’Onghia, 1998; Djelouah and D’Onghia, 1998; Djelouah et al., 2000, 2002).

As shown in Table 1 and 2, during spring dot blot hybridization detected all CPsV and CVV isolates whereas ELISA gave the same results for CPsV but identified only two out of the 9 CVV isolates. The higher sensitivity of the molecular with respect to the serological assay was more evident in aged leaves tested in winter, since CPsV and CVV were detected in 40 and 5 samples, respectively. By comparison, ELISA was able to detect only 22 and 0 samples for CPsV and CVV, respectively.

The dot blot assay using ps101 and cvv300 riboprobes, was validated in field surveys conducted in Apulia (southern Italy) during spring and early summer 2006, testing a total of 1000 plants from 15- to 30-year-old commercial groves of different species (sour and sweet orange, clementine, Satsuma Myiagawa, and lemon). Sampling was carried out at random regardless of whether the plants showed symptoms or not, along the longest diagonal in a grove of 1 ha, collecting flowers and young leaves from the quadrant of the canopy (Barnett, 1986; D’Ongia et al., 1998; Scott et al., 1999). Results (Table 3) showed that CPsV was detected by molecular hybridization in 92 samples, 84 of which were positive in ELISA and 70 showed symptoms. By converse, molecular detection of CVV was positive in 19 samples, with only 4 reacting in ELISA and 15 showing symptoms.

Table 2. Symptoms from field observation and indexing in comparison with ELISA and hybridization assays for CVV and CPsV isolates from the collection of the University of Bari.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Origin</th>
<th>Field symptoms</th>
<th>Symptoms on indicator plants*</th>
<th>Hybridization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ELISA TAS-ELISA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Spring</td>
<td>Winter</td>
</tr>
<tr>
<td>cvv300</td>
<td>CL, C, V</td>
<td>CL, C, LD</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>cvv302</td>
<td>CL</td>
<td>CL, C, LD</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>60-X</td>
<td>LD, C</td>
<td>CL, C</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>185-X</td>
<td>Southern Italy</td>
<td>LD</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>209-X</td>
<td>symptomless</td>
<td>CL, C, LD</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>314-X</td>
<td>C</td>
<td>CL, C, LD</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>324-X</td>
<td>CL, V</td>
<td>CL, C, LD</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>326-X</td>
<td>CL</td>
<td>CL, C, LD</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>327-X</td>
<td>USA</td>
<td>CL, C</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Eureka lemon was used as indicator.

CL: crinkly leaf; C: chlorotic leaves; V: variegation on young leaves; LD: leaf deformation.

To confirm the specificity of hybridization signals obtained with ps101 and cvv300 riboprobes from the 22 psorosis-infected and the 4 infectious variegation-infected but symptomless trees, RT-PCR was performed on total nucleic acid extracted with silica from these plants. CPsV was detected in all 22 symptomless samples, yielding the expected amplicon of 600 pb. A positive response to CVV was also obtained from the four symptomless trees and the 249 bp amplicon was sequenced for further confirmation. Sequences of the four Italian isolates (UBAcvv981, UBAcvv983, UBAcvv994, UBAcvv999) showed 91-97% and 93-98% identity at the nucleotide and amino acid level, respectively, with

![Fig. 3. Dot blot hybridization, with ps101 (panel A) and cvv300 (panel B) riboprobes, of total RNA extracted by silica capture from young leaves/flowers collected from different citrus trees. Total RNA extracts from healthy citrus and from plants infected by ps101 and cvv300 isolates were included as negative (-) and positive (+) controls.](image)
the comparable sequences reported by Bennani et al. (2002) and Scott and Ge (1995).

Five plants (two sour oranges, one clementine, one Satsuma Myiagawa and one naveline) infected by CPsV and five plants infected by CVV (four clementine and one naveline) identified during the field survey, were selected to test the reliability of dot-blot hybridization.

RNAs were extracted monthly (except for July-September) from aged and young leaves, flavedo, flowers, juice and seeds collected from these plants, and tested. Table 4 shows that molecular detection of both CPsV and CVV was possible throughout the year from one organ/tissue or the other, although the highest detection rates were in spring from flowers and young leaves.

### Table 3. Comparison of different diagnostic procedures (hybridization, ELISA and symptoms) for the detection of CPsV and CVV in field-grown plants.

<table>
<thead>
<tr>
<th>Diagnostic procedure</th>
<th>Response pattern</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Citrus psorosis virus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Psorosis-like symptoms (OLP, RS, CL, BS) in the field</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>DAS-ELISA</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Hybridization (probe ps101)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>No. of trees showing each response pattern</td>
<td>66</td>
<td>18</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Total positive trees to CPsV</td>
<td>92</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Citrus variegation virus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infectious variegation-like symptoms (CL, V, C, LD) in the field</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>TAS-ELISA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Hybridization (probe cvv300)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>No. of trees showing each response pattern</td>
<td>2</td>
<td>2</td>
<td>13</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Total positive trees to CVV</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BS: bark scaling; CL: chlorotic flecking; OLP: oak leaf pattern; RS: ringspot; CL: crinkly leaf; C: chlorotic leaves; V: variegation on young leaves; LD: leaf deformation.

**Fig. 4.** Results of tissue-print hybridization. Panel A. CPsV detection with ps101 riboprobe: from ovaries (rows 1), young leaves (rows 2) and flavedo (rows 3). Panel B. CVV detection with cvv300 riboprobe: from ovaries (row 1), tender shoots (row 2), young leaves (row 3) and old leaves (row 4). In both panels, tissue prints of from healthy citrus plants in lane H. S = infected samples.
Tissue-print hybridization. The efficiency of this technique was evaluated in spring on field-grown CPsV- and CVV-infected plants. Effective and reliable detection of CPsV was obtained with prints from ovaries, whereas other tissues gave weak or non specific signals. By contrast, CVV was satisfactorily detected from ovaries, tender shoots and young leaves (Fig. 4).

**DISCUSSION**

Routine detection of CPsV and CVV is mainly based on ELISA protocols that are rapid, sensitive and cost-affordable. However, serological tests are limited by the supply and quality of the reagents (Davino and Garnsey, 1984; Davino et al., 1988; Garcia et al., 1997; Alito et al., 1999; Potere et al., 1999; D’Onghia et al., 2001; Loconsole et al., 2006), whose obtention is made difficult by the scarce immunogenicity and instability of the antigens and their low titre in host tissues (Garnsey, 1974; Davino et al., 1988; Roistacher, 1993; Martín et al., 2002). RT-PCR and hybridization-based techniques for the detection of CPsV and CVV have been recently developed (Bennani et al., 2002; Martín et al., 2004; Roy et al., 2005; Rosa et al., 2007), but apparently were not applied for large scale testing in field surveys. Moreover, molecular techniques for the detection of CVV were never compared with serological assays.

In the present work, we report the development of a dot blot and tissue print hybridization techniques for the detection of CPsV and CVV in citrus plants, using two DIG-labelled riboprobes produced on the CP gene sequences of the Apulian CPsV isolate ps101 and the Sicilian CVV isolate cvv300. We showed that high-quality nucleic acids suitable for dot blot hybridization were obtained by microchromatography on silica particles and the detection limit of the method was comparable to already reported hybridization assays on silica particles and the detection limit of the method was comparable to already reported hybridization assays for other viruses (Sanchez-Navarro et al., 1996; Pallas et al., 1998). Furthermore the test was optimized for routine diagnosis using different citrus species either grown in screenhouses or in the field.

In screenhouse analysis, CPsV detection rate in young leaves/flowers was similar by ELISA and dot blot

---

**Table 4.** Results of monthly hybridization tests on aged leaves, young leaves, flowers, fruit peel and flavedo, juice and seeds, collected from field-grown CPsV- and CVV-infected plants.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aged leaves</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPsV</td>
<td>4/5(a)</td>
<td>3/5</td>
<td>5/5</td>
<td>5/5</td>
<td>3/5</td>
<td>3/5</td>
<td>3/5</td>
<td>3/5</td>
<td>3/5</td>
<td>3/5</td>
<td>4/5</td>
<td></td>
</tr>
<tr>
<td>CVV</td>
<td>4/5</td>
<td>4/5</td>
<td>2/3</td>
<td>2/3</td>
<td>3/5</td>
<td>2/5</td>
<td>3/5</td>
<td>3/5</td>
<td>2/5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Young leaves</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPsV</td>
<td>nt</td>
<td>3/3</td>
<td>5/5</td>
<td>5/5</td>
<td>3/4</td>
<td>5/5</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CVV</td>
<td>nt</td>
<td>nt</td>
<td>5/5</td>
<td>5/5</td>
<td>4/4</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Flowers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPsV</td>
<td>nt</td>
<td>nt</td>
<td>4/4</td>
<td>5/4</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CVV</td>
<td>nt</td>
<td>nt</td>
<td>5/5</td>
<td>5/5</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fruit peel and flavedo</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPsV</td>
<td>4/4</td>
<td>3/4</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>2/3</td>
<td>2/3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CVV</td>
<td>1/5</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>1/3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Juice</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPsV</td>
<td>4/4</td>
<td>3/4</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>3/4</td>
<td>2/3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CVV</td>
<td>1/5</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>1/3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Seeds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPsV</td>
<td>0/1</td>
<td>1/1</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>1/2</td>
<td>2/2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CVV</td>
<td>4/5</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>3/3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(a) Infected samples/tested samples; nt = not tested.
hybridization during spring, whereas in winter, using aged leaves, ELISA response decreased by nearly 50% (22 viz. 40 samples, Table 1). With CVV (Table 2), although less infected samples were found by both techniques, as compared with CPsV, the detection level was higher with hybridization than ELISA. In fact, no sample was ELISA-positive in winter. The higher sensitivity of both techniques during spring is likely to depend on the higher virus concentration in flowers and young leaves as observed by other authors (Davino et al., 1988; Martin et al., 2002).

Results from field testing in spring (Table 3), confirmed the higher sensitivity of dot blot hybridization which detected 8 and 15 more samples for CPsV and CVV, respectively, in comparison with ELISA. The improvement was especially significant in the case of CVV.

Moreover the presence of both viruses in symptomless field trees (respectively 22 and 4 plants), detected by hybridization using ps101 and cvv300 riboprobes, was confirmed by RT-PCR and sequencing. The higher efficiency of the dot blot hybridization assay over ELISA was confirmed by the results obtained over a 9-month period, testing different tissues from selected field-grown CPsV and CVV infected plants (Table 4).

Furthermore, ps101 and cvv300 riboprobes were reliably used for CPsV and CVV detection by tissue-print hybridization, respectively, in ovaries and young tissues (ovaries, shoots, leaves) collected from field-grown plants. Test allows large scale analysis of both viruses since no sample processing is required and tissue prints can be prepared directly during field surveys. Printed membranes can be stored for long periods before processing and sent anywhere for processing.

Based on the outcome of the present study, it can be concluded that dot blot hybridization is more sensitive than ELISA and can be usefully applied for routine large-scale diagnosis, thus constituting an useful alternative to serological assays.

ACKNOWLEDGEMENTS

Work supported by MiPAF Project “Ricerche e Sperimentazione nell’Agrumicoltura Italiana”. Grateful thanks are expressed to Dr. P. Saldarelli for useful suggestions and comments and to Prof. Martelli for revising the manuscript.

REFERENCES


