Comparative analysis of ELISA, nonradioactive molecular hybridization and PCR for the detection of prunus necrotic ringspot virus in herbaceous and Prunus hosts

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Three methods were compared for the detection of prunus necrotic ringspot virus in herbaceous and woody plants: DAS-ELISA, nonisotopic dot-blot hybridization and reverse transcriptional polymerase chain reaction (RT-PCR). When purified virus preparations were used, the detection limit of the RT-PCR technique was 1·28 pg mL⁻¹ whereas nonisotopic molecular hybridization and DAS-ELISA allowed detection of 0·8 ng mL⁻¹ and 4 ng mL⁻¹, respectively. Several sample processing procedures were evaluated for virus detection by the nonisotopic molecular hybridization technique. When a very short and simple sample processing method was used, the detection limit of the nonisotopic molecular hybridization technique was 25 times higher than that of DAS-ELISA and 625 times lower than that of RT-PCR. A comparison of the level of virus accumulation in mature fruits and in leaf tissue showed that, on average, 125 times more virus was found in the fruits.

Introduction

Prunus necrotic ringspot virus (PNRSV) is found worldwide infecting a wide range of Prunus species. It belongs to the Ilarvirus genus, the members of which are characterized by having a tripartite genome and quasi-isometric particles (Francki, 1985). PNRSV is phylogenetically closely related to prune dwarf virus (PDV) and apple mosaic virus (ApMV) (Sánchez-Navarro & Pallás, 1997). Alone or in combination with other viruses it affects fruit yield, fruit maturity and tree growth (see Uyemoto & Scott, 1992 and references therein). The virus is pollen and seedborne, contributing to a rapid spread in stone fruit trees (M ink, 1992).

Serological procedures have been used more than others for the detection of PNRSV (Halk et al., 1984; Mink & Aichele, 1984; Scott et al., 1989; Uyemoto et al., 1989). However, erratic ELISA results were reported for shoot and leaf collections made early in May when day temperatures exceeded 38°C over a 12-day period (Uyemoto et al., 1989). In addition, Scott et al. (1992) noted that, when ELISA was used, PNRSV was detectable in peach tissues only until the cessation of stem elongation. Radioactive molecular hybridization allowed discrimination between healthy and virus-infected material throughout the growing season (Scott et al., 1992) and was suitable for detecting PNRSV serotypes that reacted poorly in ELISA (Crosslin et al., 1992). However, the use of radioactive precursors made this approach unsuitable for routine diagnosis. Although a nonisotopic riboprobe was used to monitor PNRSV infection after in vitro micrografting (Heuss-LaRosa et al., 1995) no sensitivity limit of this technique was reported. More recently, several variations of the PCR technique were applied for the detection of PNRSV (Rowhani et al., 1995; Spiegel et al., 1996). However, no efforts appear to have been made to determine the detection limit of these three techniques from the same starting material.

In this work a comparative analysis of DAS-ELISA, nonradioactive molecular hybridization and PCR methods for detection of PNRSV in herbaceous and Prunus hosts is presented. Several sample processing procedures were compared for virus detection by the nonisotopic molecular hybridization technique. A comparison was made of the level of virus accumulation within mature fruits versus leaf tissue.

Materials and Methods

Plant and virus source

Petals from a field-grown PNRSV-infected nectarine (Prunus persica) tree were homogenized and served as inoculum for cucumber (Cucumis sativus cv. National Pickling) plants. After several passages, virus was

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purified following essentially the method reported by Hall & Fulton (1978) for PDV. Leaves from healthy and infected nectarine field trees were collected in June from various parts of the tree to minimize effects of uneven virus distribution. Leaf tissues from systemically-infected cucumber were obtained 7–10 days after inoculation. Samples were cut into small strips, which were then randomly divided into three groups for ELISA, nonradioactive molecular hybridization and PCR analysis.

**RNA extraction**

Viral RNA was extracted from virus particles by addition of a dissociation buffer containing 1% SDS and 4 μg mL⁻¹ of proteinase K in 0·01 M Tris-HCl, pH 7·5. After incubation for 1 h at 37°C, the viral RNA was extracted with phenol-chloroform, ethanol precipitated and resuspended in sterile water.

**ELISA test**

Double-antibody sandwich ELISA was performed essentially as described by Clark & Adams (1977), using a commercially available PNRSV IgG and alkaline phosphatase-conjugated PNRSV IgG (Loewe). Polystyrene microtitre plates were coated for 2 h at 37°C with 200 μL per well of coating IgG in 50 mM carbonate buffer, pH 9·6, with 0·02% sodium azide. The plates were then washed six times with PBST (10 mM phosphate buffer, pH 7·2, containing 0·8% NaCl, 0·02% KCl and 0·05% Tween-20). Healthy and PNRSV-infected cucumber samples were triturated in 10 volumes (w/v) of PBST buffer, pH 7·3 containing 2% (w/v) polyvinyl pyrrolidone and 0·2% bovine serum albumin (BSA) (sample buffer) and serially diluted (fivefold dilution) in the same buffer. When healthy and PNRSV-infected nectarine trees were used, 1 g of leaf tissue was triturated in 20 mL of sample buffer. A purified PNRSV preparation, the concentration of which was determined spectrophotometrically, assuming that 1 mg mL⁻¹ of virus has an OD 260nm of 5, was used to determine the sensitivity limit of the DAS-ELISA technique. Aliquots of 100 μL of prepared samples were added to each well and the plates were incubated overnight at 4°C. Plates were then washed, incubated for 2 h at 37°C with 200 μL per well of alkaline phosphatase-conjugated PNRSV IgG diluted in PBST plus 1% BSA, washed again and incubated for 60–90 min with p-nitrophenylphosphate (0·7 mg mL⁻¹) in 10% diethanolamine, pH 9·8. The reactions were read at 405 nm in a microtitre plate reader LP 300 (Sanofi Diagnostic Pasteur, France). ELISA readings were considered positive when the absorbance of sample wells was at least two times greater than the mean absorbance reading of three healthy controls.

**Construction of a PNRSV specific clone and in vitro transcription of digoxigenin-labelled RNA probe**

Previous results (Sánchez-Navarro & Pallás, unpublished data) showed that RNA 3 and 4 accumulated to a higher level than RNA 1 and 2 in leaf tissue. After partial genome characterization (Sánchez-Navarro & Pallás, 1994, 1997; Pallás et al., 1995), a clone bearing the full sequence of RNA 4 was considered as optimum for diagnosis purposes. To obtain it, 1 μg of PNRSV RNA 4 was heated at 90°C for 3 min in the presence of the 32-mer oligonucleotide VP 4 (5'-TTTAAAAGGCTCGCC-3'), the 3' 20 nt being complementary to the 3'-terminus of PNRSV RNA 4, and encompassing the unique endonuclease restriction site Sac I, underlined and sequentially cooled to room temperature. The mixture was then subjected to reverse transcription (RT) with avian myeloblastosis virus (AMV) reverse transcriptase (Promega) at 42°C for 45 min, in a 20 μL volume. A 1/10 volume of the RT product was subjected to PCR amplification in a 50 μL reaction volume in the presence of oligonucleotide VP 4 and the 29-mer VP no. 3 (5'-GTTAACGGTGACCGTTT-TCTTTTCTTCCG-3'), which contains the first 17 nt of PNRSV RNA 4, a Kpn I site, underlined, and a further six additional unrelated residues at its 5' end. PCR parameters were: 30 cycles of 40 s at 94°C, 30 s at 55°C and 2 min at 72°C followed by 10 min at 72°C to finish the amplification. The amplified product was digested with Kpn I and Sac I restriction enzymes and cloned into the plasmid vector Bluescript KS+ (Stratagene Inc.) to yield pPN4-890. Of the plasmid 100 ng were linearized with Kpn I, made blunt-ended with T4 polymerase, purified with phenol-chloroform, ethanol precipitated and resuspended in sterile water. The synthesis of the digoxigenin-labelled RNA probe was carried out as described (Astruc et al., 1996; Pallás et al., 1998). Transcript RNA was recovered by ethanol precipitation and resuspended in sterile water.

**Preparation of tissue extracts and dot-blot hybridization**

In order to obtain optimum conditions for dot-blot analysis, two extraction buffers and four sample processing procedures were tested, depending on the purification level of the sample. Buffer 1 (50 mM sodium citrate pH 8·5) was used to detect carnation mottle virus (CarMV) and cherry leaf roll virus (CLRV) by non-isotopic molecular hybridization (Más et al., 1993; Sánchez-Navarro et al., 1996) and buffer 2 (200 mM potassium phosphate containing 0·1% Triton X-100, 5 mM dithiothreitol, and 10 mM 2-mercaptoethanol) to detect PNRSV in infected tissue by radioactive dot-blot hybridization (Crossin et al., 1992). To determine the sensitivity limit of the technique, purified PNRSV was serially diluted (fivefold dilution) in buffer 1, or in healthy extracts made up in buffer 1. Healthy and PNRSV-infected cucumber plants were homogenized in 10 volumes of buffer 1 and serially diluted in the same buffer. Samples were clarified by centrifugation at 5000 g for 5 min at 4°C and 4 μL of the supernatant were directly applied to nylon membranes (Boehringer
Leaves from healthy and PNRSV-infected nectarine trees were divided into two aliquots and these were homogenized in a plastic bag with 10 volumes (w/v) of buffers 1 and 2 simultaneously. After clarifying the samples by centrifugation at 5000 g for 5 min, the resultant supernatants were analysed by four different processing techniques: (a) the supernatant was serially diluted in the same sample buffer and directly applied to nylon membranes; (b) 50 μL of supernatant were mixed with 50 μL of denaturation solution (8×SSC + 10% formaldehyde) and heated at 60°C for 15 min, then serial fivefold dilutions were applied to the membrane; (c) 100 μL of supernatant were purified by phenol–chloroform extraction, ethanol precipitated, resuspended in 100 μL of sterile water and serial dilutions were applied as above and (d) 50 μL of undiluted purified material was mixed with 50 μL of denaturation solution, heated at 60°C for 15 min and the last fivefold dilutions were made in 8×SSC. The first spot of each procedure corresponded to a dilution of 1:20 (w/v) of the original tissue. Membranes were air-dried and the nucleic acid bound using UV irradiation from a transilluminator for 3 min. Pre-hybridization and hybridization were carried out as previously described (Más & Pallás, 1995; Pallás et al., 1998). Chemiluminescent detection using CSPD® reagent as substrate was carried out as recommended by the manufacturer (Boehringer Mannheim). Films were exposed to the membranes for 10–60 min and analysed with a densitometer (Shimadzu CS-9000).

Reverse transcription and PCR amplification (RT-PCR)

Viral RNA, PNRSV-infected cucumber plants and nectarine trees were analysed by RT-PCR. Samples (1 g) of cotyledons (cucumber plants) or leaves (nectarine trees) were homogenized in a plastic bag with 10 volumes of buffer 2 (see dot-blot hybridization section) and centrifuged at 5000 g to clarify the extract. The supernatant was purified by phenol–chloroform extraction, the nucleic acids ethanol precipitated and resuspended in sterile water. RT-PCR analysis of this partially purified nucleic acid preparation and the viral RNAs were carried out by using primers VP C and VP D (Sánchez-Navarro et al., 1997). PCR parameters were: one cycle at 94°C for 3 min followed by four cycles of 94°C for 30 s, 45°C for 30 s and 72°C for 40 s, followed by 30 cycles where the annealing temperature was increased to 60°C and extension finished by incubation at 72°C for 5 min. Amplified products (10 μL each) were electrophoresed in 1% agarose gels in TAE (40 mM...
Tris-acetate, 1 mM EDTA pH 8.0) and stained with ethidium bromide.

Results

Detection of PNRSV by serological methods

Purified PNRSV and PNRSV-infected cucumber plants and nectarine trees were used to determine the sensitivity of the DAS-ELISA test. For the purified preparation, the detection limit was 4 ng mL\(^{-1}\) (Fig. 1a) corresponding to a minimum amount of virus detected of 0.4 ng. When infected cucumber cotyledons were used, the dilution endpoint was 1:625 (dilution 5\(^{-4}\)), corresponding to an equivalent of 16 mg of fresh weight tissue (or 160 mg mL\(^{-1}\)). For infected nectarine leaves the dilution endpoint was 1:25 (dilution 5\(^{-2}\)), corresponding to 200 µg of fresh weight tissue (or 2 mg mL\(^{-1}\)) (Fig. 1b). The same tissue material was used for direct comparison with both nonradioactive molecular hybridization and RT-PCR methods.

PNRSV detection by nonradioactive dot-blot hybridization method

Detection by dot-blot hybridization was performed using a digoxigenin-labelled RNA 4 probe complementary to both viral RNA 3 and 4 (Sánchez-Navarro & Pallás, 1994, 1997). Fivefold dilutions of purified PNRSV were prepared in buffer 1 or in healthy extracts made with the same buffer, and the detection limit of the method was determined to be 0.8 ng mL\(^{-1}\), corresponding to a minimum amount of virus detected of 3.2 pg (Fig. 2a).

No differences in the detection limit were observed when healthy extracts instead of buffer were used to dilute the samples (data not shown). When infected cucumber plants were used, the virus was detected in extracts diluted 5\(^{-6}\), corresponding to a concentration of 6.4 µg mL\(^{-1}\) or an equivalent of 25.6 ng of leaf tissue, whereas no signal was observed in mock-inoculated cucumber plants (Fig. 2b). In Prunus hosts the virus accumulates to significantly lower levels and these hosts have more compounds that may interfere with viral RNA detection (e.g. polysaccharides, phenolic compounds, etc.). When alternative purification techniques were used to test the presence of the virus in nectarine leaves, similar results were obtained when the samples were extracted with buffer 1 without any more manipulation (Fig. 3, row a) or with buffer 2 after extraction in phenol–chloroform and ethanol precipitation (Fig. 3, row g). In both cases, the detection limit was 1:625 (dilution 5\(^{-4}\)), representing the equivalent of 25.6 ng of leaf tissue, whereas no signal was observed in mock-inoculated cucumber plants (Fig. 2b).

PNRSV detection by polymerase chain reaction

RT-PCR was carried out with two specific primers within the CP gene, whose sequence is represented in both RNA 3 and 4. These two primers were selected to
Discussion

PNRSV, an economically important virus of Prunus spp., has been detected, by biological assay, serological methods (Halk et al., 1984; Mink & Aichele, 1984; Scott et al., 1989), molecular hybridization (Scott et al., 1992; Crosslin et al., 1992) including nonradioactive riboprobes (Heuss-LaRosa et al., 1995) and RT-PCR (Rowhani et al., 1995; Spiegel et al., 1996). However, no attempts appear to have been made to use the same material and compare the three methods simultaneously.

In the present work the sensitivities of three detection methods are compared, using purified virus preparations and herbaceous (Cucumis sativus) and woody (Prunus persica) hosts.

Data obtained using a purified virus preparation revealed that the nonradioactive dot hybridization method was sensitive enough to detect PNRSV at a concentration of 0·6 ng mL$^{-1}$, i.e. five times higher than DAS-ELISA and 625 times lower than RT-PCR techniques (Table 1). Considering the total amount of virus used in the diagnosis procedure, the detection limit of nonradioactive molecular hybridization was 3·2 pg of virus, being then 125 times more sensitive than DAS-ELISA (0·4 ng, detection limit) but 125 times less sensitive than RT-PCR (25·6 fg limit). The limit of detection by RT-PCR is similar to that described for another isolate of PNRSV (20 fg; Rowhani et al., 1995) and in terms of the detection limit per mL, eight times more sensitive than the detection of plum pox virus (PPV, 10 pg mL$^{-1}$; Wetzel et al., 1991). When nonradioactive RNA probes were used, the sensitivity of the assay was similar to that obtained for purified CarMV (Sánchez-Navarro et al., 1996) and higher than that for other viruses (Roy et al., 1988; Fouly et al., 1992; Dietzgen et al., 1994). This level of sensitivity was higher than that described by Crosslin et al. (1992) for the same virus, using radioactive labelling of riboprobes. The different types of probes used could explain this result. Although Crosslin et al. (1992) provided no information.

Detection of PNRSV in fruit samples

When fruits from nectarine trees were tested together with their corresponding leaves and harvested at the same time, (Fig. 5), the results clearly showed that PNRSV reached a concentration 125 times higher in mature fruits than in leaves. This result was consistently repeated in the 10 different sets of samples examined.

Table 1 Comparison of the sensitivity limits for the detection of PNRSV using DAS-ELISA, nonradioactive molecular hybridization and RT-PCR

<table>
<thead>
<tr>
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<th>DAS-ELISA</th>
<th>Molecular hybridization</th>
<th>RT-PCR</th>
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<tbody>
<tr>
<td>Purified PNRSV</td>
<td>4 ng mL$^{-1}$</td>
<td>&lt; 0·8 ng mL$^{-1}$</td>
<td>1·28 pg mL$^{-1}$</td>
</tr>
<tr>
<td>Infected nectarine</td>
<td>2 mg mL$^{-1}$</td>
<td>&lt; 80 μg mL$^{-1}$</td>
<td>128 ng mL$^{-1}$</td>
</tr>
<tr>
<td>Infected cucumber</td>
<td>160 μg mL$^{-1}$</td>
<td>&lt; 6·4 μg mL$^{-1}$</td>
<td>10·2 ng mL$^{-1}$</td>
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* Relative sensitivities appear in rows below the sensitivity limits and refer to the immediately preceding value

about the region recognized by the probe, it seemed to be specific to RNA 3, whereas with the riboprobe used in the present work both RNA 3 and 4 were recognized.

Data obtained using herbaceous and woody hosts also revealed that the nonisotopic molecular hybridization technique was 25 times more sensitive than DAS-ELISA and 625 times less sensitive than RT-PCR (Table 1). However, considering the minimum amount of tissue required to give a positive result, the nonisotopic molecular hybridization technique was 500 times more sensitive than DAS-ELISA.

In order to reduce and optimize handling of samples for dot hybridization analysis, several protocols were compared for processing nectarine tree leaves. Direct application of samples ground in sodium citrate buffer allowed detection of 80 μg mL⁻¹ PNRSV (corresponding to 320 ng of tissue), similar to the amount detected using the nucleic acid extracts (Fig. 3). Unlike the results obtained for other viruses (Wetzel et al., 1991; M ás et al., 1993), a denaturation step was not necessary to increase the detection limit of the dot hybridization assay, which sometimes decreased after this treatment (Fig. 3, rows b, d, and h). This easy and short extraction procedure, previously described for the detection of PPV (Varveri et al., 1987), CLRV (M ás et al., 1993) and CarMV (Sánchez-Navarro et al., 1996), constitutes a good alternative for routine diagnosis of PNRSV, even with woody hosts. It has been applied to large-scale analysis of apricot samples and, when used in the nonisotopic molecular hybridization assay, resulted in 10% more samples being scored as positive than when DAS-ELISA was used (Dominguez et al., 1997).

Previous reports indicate that serological methods may provide erratic results in infected shoot and leaf collections during the summer (Uyemoto et al., 1989) and that PNRSV may be detectable in peach tissues only during stem elongation (Scott et al., 1992). The high sensitivity obtained by the nonisotopic molecular hybridization as well as RT-PCR techniques can overcome these problems. The combined reliability and ease of sample preparation and the durability of nonradioactive probes make nonisotopic molecular hybridization a good alternative to serological methods for the detection of PNRSV in certification schemes. The most sensitive, but also the less affordable and time-consuming, RT-PCR technique might be limited to virus indexing for sanitation purposes. Finally, the observation that PNRSV reached a 125-times higher concentration in mature fruits than in leaves implies that, whenever possible, fruit samples should be analysed to guarantee the highest sensitivity in the detection of the virus.

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References


