Simultaneous Detection of the Three Iarviruses Affecting Stone Fruit Trees by Nonisotopic Molecular Hybridization and Multiplex Reverse-Transcription Polymerase Chain Reaction

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ABSTRACT

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The three most economically damaging ilarviruses affecting stone fruit trees on a worldwide scale are the related *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV), and *Apple mosaic virus* (ApMV). Nonisotopic molecular hybridization and multiplex reverse-transcription polymerase chain reaction (RT-PCR) methodologies were developed that could detect all these viruses simultaneously. The latter technique was advantageous because it was discriminatory. For RT-PCR, a degenerate antisense primer was designed which was used in conjunction with three virus-specific sense primers. The amplification efficiencies for the de-

tection of the three viruses in the multiplex RT-PCR reaction were identical to those obtained in the single RT-PCR reactions for individual viruses. This cocktail of primers was able to amplify sequences from all of the PNRSV, ApMV, and PDV isolates tested in five *Prunus* spp. hosts (almond, apricot, cherry, peach, and plum) occurring naturally in single or multiple infections. For ApMV isolates, differences in the electrophoretic mobilities of the PCR products were observed. The nucleotide sequence of the amplified products of two representative ApMV isolates was determined, and comparative analysis revealed the existence of a 28-nucleotide deletion in the sequence of isolates showing the faster electrophoretic mobility. To our knowledge, this is the first report on the simultaneous detection of three plant viruses by multiplex RT-PCR in woody hosts. This multiplex RT-PCR could be a useful time and cost saving method for indexing these three ilarviruses, which damage stone fruit tree yields, and for the analysis of mother plants in certification programs.

Stone fruit trees are affected by a large number of diseases of viral etiology that can cause substantial economic losses (6,15,30). The most important ilarviruses affecting stone fruits are *Apple mosaic virus* (ApMV), *Prune dwarf virus* (PDV), and *Prunus necrotic ringspot virus* (PNRSV). These viruses, alone or in combination, can affect fruit yield, fruit maturity, and tree growth (30). In addition, PNRSV and PDV are pollen and seed-borne transmitted (2,14) which contributes to their rapid spread. These three ilarviruses are phylogenetically closely related (26) and they show at least 65% of sequence similarity at the coat protein level.

To date, the only effective way to prevent virus spread in woody crops is through the use of healthy material. Therefore, great efforts have been made during recent years to improve the sensitivity and speed of molecular diagnostic methods. Both molecular hybridization-based methods (5,17,19,23,27) and polymerase chain reaction (PCR)-based methods (8,19–21,23,29) detect these viruses, even at low concentration limits. As a next step toward development of faster methods, attempts have been made to simultaneously detect at least two viruses. This was achieved by nonisotopic molecular hybridization, for instance, in the detection of viruses affecting tomato (22) or carnation (24) plants. Regarding PCR-based techniques, there are still few examples of simul-

taneous detection of more than two plant viruses in a single assay (3,7). For stone fruit viruses, PNRSV and *Plum pox virus* (PPV) (11) or *Apple chlorotic leaf spot virus* (ACLSV) and PPV (16) were detected in a single assay by multiplex reverse transcription (RT)-PCR with the same sensitivity as that observed during individual RT-PCR analyses. Additionally, a PCR-enzyme-linked immunosorbent assay (ELISA) procedure was reported for the simultaneous detection of ApMV and PNRSV (4) but, in this case, an extra probe-capture step was required to differentiate between the two viruses.

In this study, we describe the simultaneous detection of the three ilarviruses affecting stone fruit trees (PNRSV, PDV, and ApMV) by nonisotopic molecular hybridization and a modified multiplex RT-PCR. For the latter technique, we took advantage of the high sequence similarity at the 3'-untranslated region (3'-UTR) of the RNA 3 of these viruses, to design a degenerate antisense primer, which in conjunction with virus-specific sense primers, allowed the simultaneous detection and identification of the three viruses in a single assay. To our knowledge, this is the first report detecting three plant viruses simultaneously through a multiplex RT-PCR from woody host tissue.

MATERIALS AND METHODS

Plant material and RNA extraction. Single infections of ApMV and PDV in dormant cuttings of almond (*Prunus amygdalus*, isolates AL015 and AL016, respectively) and PNRSV-infected cuttings of plum (*Prunus salicina*, isolate PL44)

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were obtained from a virus collection at the Istituto Agronomico Mediterraneo (Bari, Italy). The infected cuttings were forced to grow at room temperature, and leaves from these cuttings were used for molecular hybridization and preliminary RT-PCR studies. In addition, leaf samples from 42 trees of *Prunus* spp. (almond, apricot, cherry, peach, and plum), that were grown in the field and naturally infected by one, two, or three Ilarviruses, were used to evaluate the effectiveness of the nonisotopic molecular hybridization and multiplex RT-PCR techniques. These samples were originating mainly from southern Italy (32 samples), the United States (3 samples), or the Czech Republic (7 samples).

Total RNA was extracted from 0.4 g of fresh leaf tissue as described previously (23), except for samples from cherry trees in which a treatment to eliminate polysaccharides was incorporated (18). Alternatively, 0.2 g of tissue per sample was homogenized in a plastic bag with 10 vol of sodium citrate buffer (50 mM sodium citrate and 5 mM EDTA at pH 8.5) (23). The homogenates were clarified by centrifugation at $5,000 \times g$ for 1 min and the supernatants were applied to nylon membranes (Boehringer GmbH, Mannheim, Germany) and analyzed by nonisotopic molecular hybridization (17).

In vitro transcription of digoxigenin-labeled riboprobes. An ApMV clone containing the CP gene was provided by P. H. Berger and P. J. Shield (University of Idaho) (28). A PDV clone containing the CP gene and 3'-noncoding region was provided by S. W. Scott (Clemson University, SC) (27), and a PNRSV clone containing the RNA 4 sequence (25,26) was used to synthesize riboprobes. One microgram of plasmid was linearized with *Bam*HI, *Hind*III, and *Pst*I for the ApMV, PDV, and PNRSV clones, respectively. Linearized plasmids were made blunt-ended with T4 DNA polymerase. After linearization and blunt-ending, the plasmids were purified with phenol-chloroform, ethanol-precipitated, and resuspended in sterile water. The synthesis of the digoxigenin-labeled RNA probes was conducted as described previously (13,17).

Dot blot hybridization. Total RNA or clarified sap preparations were diluted five-fold with healthy extracts, and 3.5 μ l of each dilution was directly applied to nylon membranes (GmbH). Membranes were air dried and the nucleic acids were cross-linked to the membrane by exposure to UV irradiation from a transilluminator for 3 min. Prehybridizations and hybridizations with single or mixed probes were carried out as previously described (17,24), except that the hybridization temperature was raised to 70°C for the clarified sap extracts. Chemiluminescent detection with CSPD reagent as substrate was performed as recommended by the manufacturer (GmbH). Films were exposed for 10 to 60 min.

Primers and multiplex RT-PCR. The primers used for single or multiplex RT-PCR are shown in Table 1. A degenerate antisense primer (VP77) was used for the reverse transcription reaction. First strand cDNAs were synthesized in a 20- μ l reaction containing 2 μ l of total RNA (≈ 3 to 5 μ g), 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM spermidine, 1 mM each of dNTP, 40 units of ribonuclease inhibitor (Amersham International, Cleveland), 8 units of Avian myeloblastosis virus-RT (Promega Corp., Madison, WI), and 100 pmol of VP77 antisense primer. To perform specific PCR amplifications for each virus, the universal antisense VP77 and the virus-specific sense primers (VP79 for ApMV, VP80 for PDV, and VP78 for PNRSV), identical to a region in the corresponding CP genes (Table 1) were used. Multiplex PCR was carried out by a mixture of four primers in a single tube. PCR cocktail included: 5 μ l of the reverse transcription products, 10 μ l of 10 $\times$ buffer (166 mM (NH₄)₂SO₄, 670 mM Tris-HCl at pH 8.8, and 0.1% Tween 20), 3 mM MgCl₂, 0.2 mM each of dNTP, and 60 pmol of each primer in a total volume of 50 μ l. Prior to thermal cycling, 1 unit of *Eco*Taq DNA polymerase (Ecogen SRL, London) was added to the cocktail and overlaid with mineral oil (Sigma Chemical Co., St. Louis). PCR was carried out in a thermal cycler (Perkin-Elmer

2400, Perkin-Elmer Corp., Norwalk, CT) programmed for one cycle at 94°C for 2 min followed by 30 cycles of 94°C for 35 s, 52°C for 35 s, and 72°C for 35 s, and finally with an extension temperature at 72°C for 5 min. For multiplex PCR on naturally infected cherry samples collected in the field, the same RT-PCR conditions were used, except 1% polyvinylpyrrolidone was added to the PCR reaction mixture to avoid inhibitory effects of phenolic compounds (12). Amplified products (5 μ l each) were electrophoresed in 2% agarose or 5% polyacrylamide gels in 40 mM Tris-acetate and 1 mM EDTA at pH 8.0 (TAE) buffer and stained with ethidium bromide. For Southern blot analysis, 2 μ l of the PCR products were electrophoresed, in triplicate, 5% polyacrylamide gels with TAE buffer. Prior to transfer, gels were washed for 10 min in 0.5 M NaOH/1 M NaCl and 10 min in 0.5 M Tris-HCl (pH 7.5)/1.5 M NaCl and electrotransferred in TAE buffer onto nylon membranes. Hybridizations were performed as described by dot blot analysis, except hybridization temperature was 60°C. For sequencing of the amplified fragments of some ApMV isolates, the PCR products were extracted from 2% agarose gels and directly sequenced in both orientations by VP77 and VP79 primers with an automated DNA sequencer (ABI PRISM 377, Perkin-Elmer).

RESULTS

Multiple nonisotopic molecular hybridization detection. Total RNA prepared from singly infected *Prunus* hosts with ApMV, PDV, or PNRSV and their five-fold serial dilutions were applied onto four nylon membranes (Fig. 1A). The first three membranes were hybridized separately with specific digoxigenin-labeled riboprobes, whereas the remaining membrane was treated with a mixture of the three riboprobes (multiple hybridization).

In single hybridizations, specific signals were clearly observed through dilutions of 5⁻⁶ for PDV and 5⁻⁵ for ApMV and PNRSV, corresponding to the equivalent of 0.5 and 0.1 μ g of infected leaf tissue, respectively. No signal was detected in the nonrelated infected extracts (Fig. 1A). The simultaneous detection of the three ilarviruses when the riboprobes were mixed within the hybridization solution showed the same sensitivity limit as observed in the single hybridizations (Fig. 1A). Therefore, based on these results, multiple hybridization procedure could be used for the detection of any of these three viruses in a *Prunus* host.

In order to simplify the extraction procedure, tissue extracts from the same infected samples used in Figure 1A were prepared by a very fast method as previously described for PNRSV and other plant viruses (23). Although the specificity of the procedure was as good as total nucleic acids extraction method, the sensitivity was decreased up to 125 times (Fig. 1B). However, regardless of the sample preparation method used, the end point dilutions obtained in the multiple hybridizations were similar to those obtained in the single hybridizations, indicating the suitability of this easy procedure for the routine simultaneous detection of the three viruses.

Multiplex RT-PCR. With the aim of setting up a procedure that would allow the simultaneous detection of the three Ilarviruses affecting stone fruit trees by a PCR-based method, an antisense degenerate primer was designed to the 3' end of their RNAs 3 and 4 (Table 1). This degenerate primer was designed to avoid the need to include three different primers in the reverse transcription reaction. Virus specific sense primers were made to be used for the unequivocal identification of each of the three viruses (Table 1).

As a first step in determining the versatility of the degenerate primer, the same total RNA extracts used in the molecular hybridization experiments obtained from *Prunus* hosts individually infected with ApMV, PDV, or PNRSV were analyzed by RT-PCR. PCR products of the expected size were obtained for PNRSV (356 bp) and PDV (517 bp), whereas for ApMV a PCR product of a slightly smaller size than the expected one (390 bp vs. 417 bp)

was obtained (Fig. 2). Both molecular hybridization experiments and Southern blot analysis confirmed the validity of the amplified segment to be of an isolate of ApMV (described below). No interfering bands were obtained indicating that the degenerate antisense primer was able to recognize the genome of all three ilarviruses tested. No PCR products were observed in the healthy control sample (Fig. 2, lane 4).

We addressed the question of using a cocktail of all four primers to check the method's efficiency confronted with the three possible single infections, three simulated double infections, and one simulated triple infection. Virus-specific bands that did not vary in concentration or in expected size with respect to the prod-

ucts obtained in the case of single infections (Fig. 3, lanes 1 to 3), were clearly detected in all combinations. A large molecular weight product was obtained from healthy samples (Fig. 3, lane 8) and was absent in the infected samples, but this did not interfere with the interpretation of the results.

Detection limits for the cocktail of four primers in both single and simulated triple infections were determined by making five-fold serial dilutions of the corresponding total RNA extracts. In single infections, ApMV and PNRSV specific bands were detected up to 5⁻⁸ dilution, whereas the PDV specific product was detected up to 5⁻¹⁰ dilution, corresponding to 20 and 0.8 ng of infected leaf tissue, respectively (data not shown). The same de-

Fig. 1. Detection of *Prunus necrotic ringspot virus* (PNRSV), *Apple mosaic virus* (ApMV), and *Prune dwarf virus* (PDV) in infected tissue by nonisotopic molecular hybridization in single (top six membranes) or simultaneous (bottom, mixed probes) assays. **A**, Total RNA and **B**, clarified sap extracts from stone fruit leaves individually infected with ApMV, PDV, or PNRSV and their five-fold serial dilutions were applied onto nylon membranes in the rows indicated at the left. The membranes were hybridized by an individual or a mixture of the three RNA probes (mixed-riboprobes) in the hybridization solution. Numbers at the bottom represent the dilutions performed on the original undiluted sample.

TABLE 1. Sequence of degenerate antisense and virus-specific sense primers used in the multiplex reverse-transcription polymerase chain reaction

Degenerate antisense primer	Specific sense primers				Amplified product (bp)
	Sequence	Virus ^a	Code	Location in the RNA 3	
VP77 ^b					
T TT AG	5'-CGTCGAGGAAGTTTAGGTTG-3'	ApMV	VP79	1638–1656	417
5'-GCCTCC T GGGGCATC-3'	5'-CAACGTAGGAAGTTCACAG-3'	PDV	VP80	1610–1628	517
A CA TT	5'-GAACCTCCTTCCGATTTAG- 3'	PNRSV	VP78	1586–1599	356

^a *Apple mosaic virus* (ApMV), *Prune dwarf virus* (PDV), and *Prunus necrotic ringspot virus* (PNRSV).
^b VP77 primer corresponds to the complementary sequence of the last 19 nucleotides (nt) of PNRSV, ApMV, and PDV.

tection limits were obtained when the three viruses were amplified in the multiplex RT-PCR reaction (Fig. 4). According to these results, the technique was at least 125 times more sensitive than multiplex nonisotopic molecular hybridization.

To further evaluate the efficiency of this multiplex RT-PCR technique, leaf samples were collected from the trees grown in the field, which were suspected to be naturally infected with one or a combination of these three viruses. Total RNAs were extracted from leaves of 42 trees including 5 *Prunus* spp. (almond, apricot, cherry, peach, and plum) and subjected to the multiplex RT-PCR procedure. Some of the results are shown in Figure 5, representing most of the potential combinations. Single infections are shown in eight lanes. Interestingly, two PCR bands of different size account for the detection of ApMV, the one described to represent the reference virus isolate in this work (Fig. 5A, lanes 1 and 2) and the one expected from the previously characterized isolate (417 bp; Fig. 5A, lane 3) (28). To verify that the PCR products obtained were from the expected viruses, representative samples of the different combinations, including the two ApMV PCR bands differing in size (Fig. 5A, lanes 2 and 11 vs 6 and 17) were analyzed by Southern blot hybridization by the corresponding virus-specific riboprobes (Fig. 5B). Hybridization signals were obtained in the expected lanes, including those of the ApMV isolates that gave remarkable size heterogeneity. To study the origin of this size heterogeneity, these two PCR products were gel purified and their primary structures determined. As seen in Figure 5C, a 28-nt deletion was present in the sequence of isolates, which gave a band with faster electrophoretic mobility. Double infections of the type PDV-PNRSV (Fig. 5A, lanes 4, 5, 7, 8, 9, and 13) and ApMV-PNRSV (Fig. 5A, lanes 11 and 17) were clearly discriminated. A natural triple infection was also detected (lane 6). These results indicated that the technique described here is quite reliable and could be used for testing field samples. The detection limit for the triple-infected tree sample was 5^{-3} for PNRSV and ApMV and 5^{-2} for PDV (data not shown). These limits were clearly lower than those observed in the simulated triple infection (Fig. 4). More triple infections need to be analyzed to know the real detection limits of this technique in these exceptional cases. It is important

to note that when total RNA from cherry samples were used, no PCR bands were detected unless a specific treatment for eliminating polysaccharides (18) was carried out and PVP was incorporated in the PCR buffer (12) (data not shown).

DISCUSSION

The current work demonstrates the success obtained in simultaneously detecting three ilarviruses (PNRSV, PDV, and ApMV) in tissue from fruit trees through nonisotopic molecular hybridization

Fig. 3. Evaluation of multiplex reverse-transcription polymerase chain reaction technique carried out with a cocktail of the four primers (one degenerated antisense primer and three virus-specific sense primers). Total RNA extracted from the individually infected samples and mixtures of these extracts simulating the three double infections and the triple infection were amplified with the four primers cocktail and the products obtained were analyzed by 5% polyacrylamide gel electrophoresis. Arrows indicate the virus-specific bands. Lane M, 1-kb ladder marker; lane 1, *Prunus necrotic ringspot virus* (PNRSV)-infected sample; lane 2, *Apple mosaic virus* (ApMV)-infected sample; Lane 3, *Prune dwarf virus* (PDV)-infected sample; lane 4: simulated PNRSV-ApMV-infected sample; lane 5, simulated PNRSV-PDV-infected sample; lane 6, simulated ApMV-PDV-infected sample; lane 7, simulated PNRSV-ApMV-PDV-infected sample; lane 8, healthy sample; and lane 9, water control.

Fig. 2. Reverse-transcription polymerase chain reaction (RT-PCR) products obtained from total RNA extracted from stone fruit leaves individually infected with *Prunus necrotic ringspot virus* (PNRSV, lane 1), *Apple mosaic virus* (ApMV, lane 2), or *Prune dwarf virus* (PDV, lane 3). The RT-PCR reactions were performed with the common antisense degenerate primer and the specific sense primer for each ilarvirus and electrophoresed in 2% agarose gel. Arrows indicate the virus-specific amplified bands corresponding to 358 bp for PNRSV, 417 bp for ApMV, and 517 bp for PDV. Lane M, 1-kb ladder marker; and lane 4, RT-PCR products obtained from total RNA extracted from healthy sample.

Fig. 4. Sensitivity limits of multiplex reverse-transcription polymerase chain reaction technique in simulated triple ilarvirus infection. Fivefold serial dilutions of a mixture of total RNA from individually infected samples with *Apple mosaic virus* (ApMV), *Prune dwarf virus* (PDV), or *Prunus necrotic ringspot virus* (PNRSV) were made with healthy extract analyzed in the last lane and amplified by single reactions with the four primers cocktail. The products obtained were analyzed by 5% polyacrylamide gel electrophoresis. The specific bands were detected up to 5^{-8} dilution for ApMV and PNRSV and 5^{-10} dilution for PDV. Numbers at the top of figure represent the dilution performed on the original undiluted sample. Lane M, 1-kb ladder marker; and lane H, healthy sample.

and a modified multiplex RT-PCR procedure, the latter having the additional advantage of allowing the identification of each virus in a single test.

When total nucleic acid extracts from simulated triple infections were analyzed by nonisotopic molecular hybridization, it was observed that the sensitivity limits were identical to those obtained in the corresponding single analyses. This result was consistent with previous reports in which this strategy has been successfully applied: to the phytosanitary certification of tomato plants (22) and to the large scale evaluation of the sanitary status

of carnation mother plants (24). Although the use of sap extracts in the simultaneous detection of the three ilarviruses resulted in a 5 to 25 times reduction in sensitivity limits, this easier extraction procedure could facilitate handling of a significantly larger number of samples and should be considered in routine diagnostic procedures.

PCR-based technology is several-fold more sensitive than ELISA and nucleic acid hybridization (10,19). In addition, this technology allows the simultaneous amplification of more than one locus in the same reaction (9). There are still few examples

Fig. 5. A, Representative results obtained by multiplex reverse-transcription polymerase chain reaction (RT-PCR) technique for ilarvirus detection in field tree samples. Total RNA from 42 stone fruit *Prunus* spp. that were thought to be naturally infected with one or more of the three ilarviruses were analyzed with the four primers cocktail and electrophoresed in a polyacrylamide 5% gel. M, 1-kb ladder marker; C, simulated triple infection used as positive control; H, healthy sample; and lanes 1 to 17, representative results obtained from 42 *Prunus* spp. samples analyzed. **B**, Some of the samples analyzed in **A** covering the triple (lane 6), double (lanes 7 and 11), and single (lanes 2, 14, and 15) infections were electrophoresed in triplicate and analyzed by Southern blot hybridization with virus-specific riboprobes. In the case of *Apple mosaic virus* (ApMV), samples that gave different PCR bands with different electrophoretic mobilities (described in text) were included in this analysis. **C**, The nucleotide sequence of the PCR products corresponding to ApMV isolates of lanes 2 and 17 was determined and compared with the reference isolate (ApMV-A; 28). The region causing the differences in the electrophoretic mobilities of ApMV isolates is boxed. Nucleotide residues that can be folded in a very stable stem-loop structure are in italic. The stop codon for the coat protein cistron is in bold. Dots indicate identical residues, and dashes denote gaps in the sequence.

showing its application to simultaneously detect more than two viruses. Some of them include the detection of five seedborne legume viruses (3) and of three viruses infecting globe artichoke (7). In regard to stone fruit viruses, a maximum of two viruses have been detected by variations of the multiplex RT-PCR. Examples include the simultaneous detection of ACLSV and PPV (16), PNRSV and PPV (11), or PNRSV and ApMV (4). This strategy has also been applied to differentiate two biologically distinct groups of PNRSV isolates from cherry (8). In our study, we have shown that an antisense degenerate primer and three virus-specific sense primers can be used to simultaneously detect (and discriminate between) the three characterized ilarviruses affecting stone fruit trees. To design the PCR-primers we took advantage of the high degree of sequence identity at the 3' end of RNA 3 in the three viruses (26). In primer design, the 3' end is more important than the 5' end and, although the antisense primer has five degeneracies, the last eight bases at the 3' end were identical in all three viruses. When using a multiplex PCR, there are several critical parameters that significantly influence the yield and quality of the loci (9). In general, raising the buffer concentration to 2 $\times$ improves the efficiency of the multiplex reaction. In the case of PDV, however, this modification led to the almost total disappearance of the product (data not shown), probably due to the fact that longer products are harder to denature. In addition, in contrast to previous recommendation (9), lowering the annealing temperature by 4 to 6°C in the multiplex mixtures was not necessary for amplification of the three targets. It was assumed that, in our case, the established conditions for single RT-PCR worked well enough for the multiplex RT-PCR. This can be explained by: (i) only one antisense degenerate primer was required, (ii) the virus-specific sense primers were designed in such a way that they have identical melting temperature, and (iii) the size differences of the amplified products were not so significant as to require any modification of the critical parameters.

Another important feature of the multiplex RT-PCR procedure described here is that it works well with all the isolates tested in this work. In the case of PNRSV, the sense primer was designed within a region that was strictly conserved in the 15 PNRSV isolates previously characterized (1). Concerning PDV and ApMV, no sequence variability data were available to use in designing sense primers, but the primers amplified all the field isolates tested and verified their efficiency. In this respect, from the results obtained in the testing of field samples, it can be concluded that ApMV isolates can be present in significant size variability at the last 400 nt of their RNAs 3 and 4. Two main PCR products were observed among all the ApMV isolates analyzed in this work. Sequence analysis allowed us to determine that the isolates that gave a faster migrating PCR product presented a 28-nt deletion in their 3'-UTRs. Interestingly, secondary structure analysis of the deleted sequence revealed that the deleted sequence could be folded in a very stable stem-loop structure.

In summary, the multiplex RT-PCR procedure described here could be a very useful method for saving time and costs during indexing of the three characterized ilarviruses that affect stone fruit trees. It may also be used in the testing of mother plants for these viruses in certification programs and particularly for testing Conformitas Agraria Communitatis propagating material, which is already compulsory for European Union countries.

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