Single-step multiplex reverse transcription-polymerase chain reaction (m-RT-PCR) for simultaneous detection of five RNA viruses affecting stone fruit trees

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A single-step multiplex reverse transcription-polymerase chain reaction (m-RT-PCR) was developed for the simultaneous detection and discrimination among five RNA viruses namely *Apple cholorotic leaf spot virus* (ACLSV), *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV), *Plum pox virus* (PPV) and *Tomato ringspot virus* (ToRSV) which considered the most economically damaging viruses of stone fruit trees in Egypt and worldwide. Five compatible primer sets for one-step RT-PCR amplification were used in a single closed tube to detect the presence of virus infection. Five fragments with different sizes corresponding to the different viral targets were amplified from infected samples, i.e., 677, 172, 300, 220 and 490 bp for ACLSV, PDV, PNRSV, PPV and ToRSV respectively. Results showed that multiplex PCR products of the expected sizes were obtained for all virus isolates assayed when they were tested either alone or in combination in an artificial mixture with no interference. This method saves time and reagent costs compared with monospecific RT-PCR which needs several reactions for the same number of tests. Using this multiplex method, many samples can be simultaneously analyzed and it can be used routinely for sanitary and certification programmes.

Key words: Stone fruit viruses, Detection, ELISA, RT-PCR and m-RT-PCR

INTRODUCTION

Plant pathogenic viruses are responsible for increasing economic losses worldwide. They can cause a large range of symptoms in most cultivated plants, which can be affected in their different parts with various agronomic impact. Viruses cause plant diseases that are difficult to control because of the lack of efficient products for chemical treatment under field conditions.

Consequently, preventive measures to avoid planting of contaminated material are of the highest importance in the context of an integrated approach to control. Among such measures, testing of planting material for

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status is an important, although not exclusive method for controlling viral disease of plants **(Janes and Wennekcr, 2002; Martin *et al.*, 2000).**

Stone fruit trees are affected by a large number of viral diseases that can cause substantial economic losses **(Desvignes, 1999; Nemeth, 1986; Uyemoto and Scott, 1992).** The most important viruses affecting stone fruit are *Apple cholorotic leaf spot virus* (ACLSV), *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV), *Plum pox virus* (PPV) and *Tomato ringspot virus* (ToRSV). These viruses, alone or in combination, can affect fruit yield, fruit maturity and tree growth **(Uyemoto and Scott, 1992).** In addition, PNRSV, PDV and ToRSV are pollen and seed transmitted **(Aparicio *et al.*, 1999; Mink, 1992)** which contributes to their rapid spread. 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. PCR based methods detect the viruses, even at low concentration limits **(Pallas *et al.*, 1998; Rowhani *et al.*, 1995; Spiegel *et al.*, 1996; Youssef *et al.*, 2002).** Multiplex reverse transcription-polymerase chain reaction (m-RT-PCR) was developed for plant virus detection in 1994 with advantage of ability to discriminate two or more targets simultaneously, but there are still few examples of simultaneous detection of more than two plant viruses in a single assay **(Bariana *et al.*, 1994; Grieco and Gallitelli, 1999).** For stone fruit viruses, PNRSV and PPV **(Kolber *et al.*, 1998),** **ACLSV and PPV (Nemchinov *et al.*, 1995),** PPV and PDV **(Youssef *et al.*, 2002)** were detect in a single assay by m-RT-PCR with the same sensitivity as that observed during individual RT-PCR analysis. In this study we describe the development and optimization of the simultaneous detection of five viruses affecting stone fruit trees (ACLSV, PDV, PNRSV, PPV and ToRSV) in a single reaction.

MATERIALS AND METHODS

Source of plant material.

Naturally infected apricot and peach leaves showing necrotic and chlorotic spots on the leaves dwarfing, shorten internodes yellowing, line pattern and fru

deformation were collected from different stone fruit orchards in different locations in Egypt i.e., Munofia, Fayoum, Qualubia and Behera. Infected leaves were homogenized separately in 1 ml of sample extraction buffer 2.40g Tris-hydroxymethyl, 8g NaCl, 20g PVP (Polyvinylpyrrolidone- MW 2400), 0.20g KCl, 0.50g Tween-20 and 0.20g NaN_3 in 1 liter distilled water (pH 7.4). From each sample, an aliquot of 200 µl was used for ELISA.

DAS-ELISA

Double antibody sandwich (DAS) ELISA **(Clark and Adams, 1977)** was carried out by coating the plates with polyclonal IgG, diluted in coating buffer {1.59g Na_2CO_3, 2.93g $NaHCO_3$ and 0.20g NaN_3 in 1 liter distilled water (pH. 6)}. The prepared samples were incubated with specific polyclonal IgG overnight at 4°C. The positive reaction was revealed by alkaline phosphatase-linked goat anti-mouse (IgG-AP) conjugate and relative substrate which is p-nitrophenyl phosphate (pNPP) in diethanolamine buffer **(Cambra *et al.*, 1994).** Two wells for positive, negative control and buffer controls per plate were used. Antisera to the following viruses were utilized: *Apple cholorotic leaf spot virus*, *Prune dwarf virus*, *Prunus necrotic ringspot virus*, *Plum pox virus* and *Tomato ringspot virus*. Serological reactants were commercial kits from Loewe, Germany. The reading of optical density (OD) against buffer control after 1 hour incubation at room temperature of substrate was used to calculate the average of OD for each sample. The average OD value of the buffer control was subtracted from the OD values of samples. The positive samples (OD> 0.2) were noticed as "+", the negative values (OD<0.05) were considered as "-".

RNA Extraction.

RNA was extracted from fresh apricot and peach leaf samples using Plant Total RNA Mini Kit (RBC, Inc). Fifty mg of tissue were ground under liquid nitrogen to a fine powder using a mortar, and then transferred to a sterile microfuge tube. A 500 µl of RB buffer were added, vortexed vigorously and incubated at room temperature for 5 min. These lysates were applied to the filter spin column sitting in a 2-ml collection tube, and centrifuged for 2 min at maximum speed. The flow-through fraction was transferred from filter column to a new tube without disturbing the cell debris pellet in the collection tube, 0.5 volumes of ethanol (96-

100%) were added to the cleared lysate and mixed well by pipetting. These samples were applied, including any precipitate which may have formed, onto an RB column sitting in a 2-ml collection tube, then centrifuged for 2 min at maximum speed, 400 µl of buffer WI were pipetted onto the column and centrifuged for 1 min at maximum speed, then the flow-through and collection tube were discarded. RB columns were transferred into a new 2-ml collection tube. 500 µl wash buffer were pipetted onto the RB column, and centrifuged for 1 min at maximum speed, the flow-through were discarded. The RB column was centrifuged for 2 min at maximum speed to dry the membrane. RB column were transferred into a new 1.5-ml collection tube, and 30~50 µl of RNase-free water were added directly onto the RB membrane and centrifuged for 1 min at maximum speed to elute the RNA.

Table 1. Oligonucleotide used for reverse transcription-polymerase chain reaction (RT-PCR) detection of five viruses of stone fruits

Virus[a] -[b]	Sequence (5'-3')[c]	Authors	Expected size of amplicon[d]
ACLSV-f	5'-AAGTCTACAGGCTATTTATTATAAGTCAA-3'	Menzel *et al.*, 2002	677 bp
ACLSV-r	5'-TTCATGGAAAGACAGGGGCAA-3'		
PDV-f	5'-ATG GAT GGG ATG GAT AAA ATA AT- 3'	Parakh *et al.*, 1995	172 bp
PDV-r	5'-TAG TGC AGG TTA ACC AAA AGG AT- 3'		
PNRSV-f	5'-AGACGTCGTGACAGACGTCGAAG-3'	Hammond and Crosslin, 1995	300bp
PNRSV-r	5'-TTCTGTACCTGCCAATATCCTACTTCG-3'		
PPV-f	5'-GTA GTG GTC TCG GTA TCT ATC ATA-3'	Hadidi and Levy, 1994	220 bp
PPV-r	5'-GTC TCT TGC ACA AGA ACT ATA ACC-3'		
ToRSV-f	5'-GGACGCGTTTGGTCGTTATGATTCG-3'	Rott *et al.*, 1991	490 bp
ToRSV-r	5'-CGAGCCCTGGAAAAAACGCAATAAG-3'		

[a] ACLSV= *Apple chlorotic leaf spot virus*, PDV= *Prune dwarf virus*, PNRSV= *Prunus necrotic ringspot virus*, PPV= *Plum pox virus*, ToRSV= *Tomato ringspot virus*.

[b] f= forward primer, r= reverse primer.

[c] Oligonuclutide sequence of primers used in RT-PCR reaction.

[d] Expected size in base pair of PCR products.

RT-PCR.

The oligonucleotide primers used for single or multiplex RT-PCR are shown in Table 1. Reverse transcription RT-PCR was done using Reverse-iT™1-Step Kit (ABgene, Inc.). RT and PCR are carried out sequentially in the same tube. All components required for both reactions were added during setup, and there was no need to add additional components once the reaction has been started. This kit has been specifically designed to streamline the RT-PCR process. The kit is able to detect RNA from as little as 100 femto grams. Optimal reaction conditions, such as incubation times and temperatures during PCR amplification, will vary and need to be determined.

Multiplex RT-PCR

The multiplex RT-PCR assays were optimized according to **Henegariu** *et al.* (1997). Several parameters such as primer concentrations (from 0.1 to 1 µM), magnesium chloride concentration (from 1.5 to 4 mM), and elongation time (from 1 to 5 min) were analysed to establish the best conditions for the simultaneous amplification targets of all five tested stone fruit tree viruses. The optimum cocktail mixture was prepared in a thin-walled 0.2 µl PCR tube, by combining of 25 µl 2x RT-PCR master mix (containing Thermo prime Plus, $MgCl_2$ and dNTPs). In addition, 0.5mM $MgCl_2$, 0.3 µM ToRSV and PPV primers, 0.6 µM PDV, ACLSV and PNRSV primers, 1 µl Reverse-iT™ RTase Blend, and 5 µl of total RNA in a total volume of 50 µl. First strand cDNA synthesis was done using the following parameters: 47°C for 30 min at 1 cycle to allow reverse transcription to be performed. Second strand cDNA synthesis and PCR amplification were done in the same tube using DNA Thermal Cycler with the following parameters: 94°C for 2 min at 1 cycle. The amplification parameters: denaturation at 94°C for 30 sec, primer annealing at 50°C for 1min, and extension at 72°C for 2 min, for 30 cycles with a final extension at 72°C for 7 min.

Analysis of RT-PCR products

Five-microliter aliquots of RT-PCR were analyzed on 2% agarose gels (6 x 8 cm), in TBE buffer (89 mM Tris-HCL, 89 mM boric acid, 2.5 mM EDTA, pH 8.5) at 120 volt. Gels were stained with ethidium bromide 10 µg / ml, visualized by examination under a UV transilluminator and

photographed using Gel Documentation System (AlphaInnotech Corporation). **(Sambrook *et al.*, 1989).** 100bp DNA size markers (RBCs, Inc.) were used to determine the size of RT-PCR products. The PCR products were cloned into the pGEM-T-Easy (Promega) vector and resultant recombinants were selected for nucleotide sequence analysis.

RESULTS AND DISCUSSION

ELISA:

A total of 480 leaf samples were collected (180 apricot and 300 peach) and tested using double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) to detect the presence of virus infection. Of these, 119 were shown to be infected with one or more viruses. Apricot (30%) being more affected with *Apple cholorotic leaf spot virus, Prune dwarf virus, Prunus necrotic ringspot virus, Plum pox virus* and *Tomato ringspot virus* than peach (22%). Data presented in Table 2, indicated that single and mixed natural viral infections could be detected using ELISA. The most common method for the detection of plant viruses is ELISA because it is rapid, inexpensive, specific and robust. However, the serological methods have the drawbacks of limited availability of antisera and questions regarding the specificity of antisera produced from preparations containing virus mixtures. Raising antisera is also time consuming. RT-PCR has become an important method for pathogen detection **(Petrunak *et al.* 1991).** By contrast, a diagnostic technique using RT-PCR can be rapidly implemented in independent laboratories after the basic protocol and primer sequences are made available. However, monospecific RT-PCR assays requiring separate amplification of each virus of interest are potentially expensive and resource intensive. To overcome theses limitations, there has been a keen interest in the development of new nucleic acid-based assays. M-RT-PCR permits simultaneous amplification of several viruses in a single reaction **(James *et al.* 2006).**

Table 2. Viruses of stone fruit detected by ELISA

Tested hosts	No. of tested samples	Infected samples		Single infection					Mixed infection	
		No.	%	PDV	PPV	PNRSV	ToRSV	ACLSV	2 viruses	3 viruses
Apricot	180	54	30	3	6	8	2	25	10	-
Peach	300	65	22	4	19	10	6	13	11	2
Total	480	119	24.8	7	25	18	8	38	21	2

RT-PCR detection

The oligonucleotide primers used in RT-PCR detection amplified specifically a 172 bp, 220 bp, 300 bp, 490 bp and 677 bp products when used to assay plants infected with PDV, PPV, PNRSV, ToRSV and ACLSV respectively. Fig. 1 shows individual detection of the five viruses (lanes 1, 2, 3, 4, and 5). The five viruses could be easily detected by the RT–PCR with the single pair of primers and the same PCR condition when total nucleic acids from infected plants were used as templates. Reliable detection was obtained when using either infected leaves. The total RNA extracted from all uninfected controls gave negative results when tested by RT–PCR. Reverse transcription-polymerase chain reaction (RT-PCR) has the potential to be an extremely sensitive alternative to ELISA, providing a means for detecting viruses in woody plants throughout the year (**Kawasaki, 1990**), even during season of low titer. The design of nucleic acid primers for RT-PCR is a demanding task, and primer sequences may require revision in the advent of evolved strains of a particular virus (**Mathews *et al.*1997; Rowhani *et al.* 1993**).

Sequence analysis:

The amplified products of the five viruses were cloned into the pGEM-T-Easy vector and their nucleotide sequences were determined. Determined nucleotide sequence results aligned with the sequence of ACLSV, PDV, PNRSV, PPV and ToRSV revealed that the amplified DNA products were the corresponding regions of each virus (data not shown).Highly conserved regions were determined from sequence alignments of all available sequences from the GenBank of the National Center for Biotechnology Information for each virus. Sequencing results also confirmed the viral origin of

the amplified products and revealed some differences between the Egyptian isolates and the respective reference sequences (GenBank accession numbers (*Apple chlorotic leaf spot virus*: ACCESSION AB326225 **(Yaegashi *et al.*, 2007)**, *Prune dwarf virus*: ACCESSION EU170007 **(Komorowska, 2007)**, *Prunus necrotic ring spot virus*: ACCESSION EU368738 **(Sala-Rejczak *et al.*, 2007)**, *Plum Pox Virus*: ACCESSION X56258 **(Wetzel *et al.*,1991)** and *Tomato ringspot virus*: ACCESSION NP-733971 **(Buckley *et al.*, 1993)**.

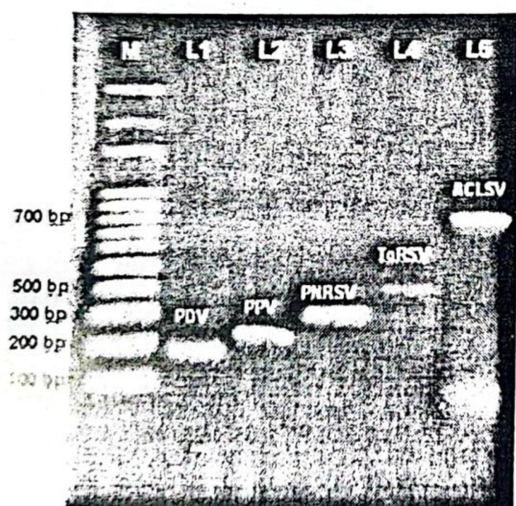

Fig.1. Agarose gel electrophoresis of RT-PCR products obtained from total RNA extracted from apricot and peach leaves individually infected with *Prune dwarf virus* (PDV, lane 1), *Plum pox virus* (PPV, lane 2), Prunus necrotic ringspot virus(PNRSV, lane 3), *Tomato ringspot virus* (ToRSV, lane 4), and *Apple cholorotic leaf spot virus* (ACLSV, lane 5). The virus specific amplified bands corresponding to 172bp for PDV, 220bp for PPV, 300bp for PNRSV, 490bp for ToRSV, and 677bp for ACLSV. Lane M, 100bp ladder marker.

Multiplex RT-PCR

Positive results were obtained by multiplex RT-PCR on infected woody samples with ACLSV, PDV, PNRSV, PPV and ToRSV. Simultaneous amplification of a 677 bp fragment of the ACLSV genome, a 172 bp fragment of the PDV genome, a 300 bp fragment of the PNRSV genome, a 220 bp fragment of the PPV genome and a 490 bp fragment of the ToRSV genome were observed. Fig. 2 shows simultaneous detection in infected samples. The test was found to be very sensitive and stringent techniques were required to prevent cross contamination as seen in Fig. 2. The optimum conditions of the variables assessed for simultaneous

detection of these viruses included the following: 2 mM MgCl2, 50°C annealing temperature, 2 min extension time, and 30 cycles.

Multiplex polymerase chain reaction (m-PCR) accommodates several pairs of primers in one reaction, resulting in reduced material costs and time when compared to several individual PCR reactions. For example, Chamberlain and **Chamberlain (1994)** reported simultaneous amplification of nine different DNA templates by m-PCR. In contrast, amplification of RNA templates, requiring reverse transcription (RT) prior to amplification by PCR (m-RT-PCR), have been limited to five viruses using purified RNA **(Bariana *et al.*, 1994)** and only two viruses using total nucleic acid extract **(Singh *et al.*, 1996; Jacobi *et al.*, 1998)**. Furthermore, during RT ascertaining the desired concentrations of various reagents and primer combinations, which significantly affect the subsequent amplification process, is a lengthy process **(Singh *et al.*, 2000)**. Therefore, in cDNA preparation, a common primer for RT would be faster and more economical by avoiding the complexities of adjusting the antisense primer ratio (**Singh *et al.*, 2000**).

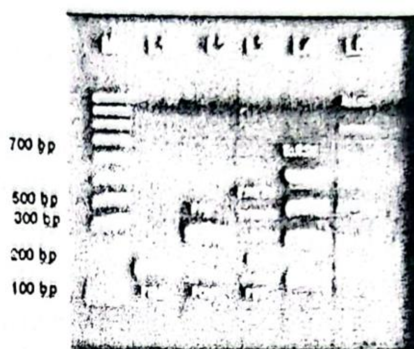

Fig. 2. Detection of *Prune dwarf virus*, *Plum pox virus*, *Prunus necrotic ringspot virus*, *Tomato ringspot virus*, and *Apple cholorotic leaf spot virus* simultaneously by multiplex RT-PCR. The amplified products were separated on a 2% agarose gel, stained with ethidium bromide and visualized under UV light. Lane M, 100bp DNA markers (RBCs, Inc.). Expected sizes for the five viruses amplicons are: 172bp for PDV, 220bp for PPV, 300bp for PNRSV, 490bp for ToRSV and 677bp for ACLSV Lane 1: individual PDV, L2: mixture of PDV+PPV, L3: mixture of PDV+PPV+PNRSV, L4: mixture of PDV+PPV+PNRSV+ToR

SV and Lane5: PDV+PPV+PNRSV+ToRSV+ ACLSV

The m-RT-PCR approach was chosen rather than RT-PCR tests because of (i) high sensitivity and specificity due to the combination of virus specific primer and their specificity; and (ii) virus detection directly in crude plant extracts rather than in partially or highly purified nucleic acid preparations. This paper described the development and optimization of the simultaneous detection of five economically important and widespread viruses affecting stone fruit trees in a single reaction. Methods other than multiplex RT-PCR can be used for detecting these viruses, but they are labor-intensive, expensive and tedious (woody indicators) or not suitable (ELISA) **(Nemchinov *et al.*, 1995; MacKenzie *et al.*, 1997)**. Multiplex RT–PCR was effective for simultaneous detection of *Apple stem grooving virus* and *Cucumber mosaic virus*. The extension time, and the concentrations of dNTP, primer and MgCl2 affected the efficiency of multiplex detection which agrees with the findings of **Seoh *et al.* (1998)**. The RT–PCR or the multiplex RT–PCR approach described in this study simplifies detection and reduces the cost by eliminating the need for costly and unpredictable RNA extraction prior to reverse transcription and PCR amplification. Use of a system that allows simultaneous detection of multiple viruses will contribute significantly to cost reduction and increase the feasibility of using PCR analysis as a routine diagnostic tool for virus testing in plants. The techniques described, with the appropriate optimization, may be suitable for the detection of other fruit tree viruses

In summary, the multiplex RT-PCR assays described here are reliable and suitable tool for indexing apricot and peach plants for *Apple cholorotic leaf spot virus*, *Prune dwarf virus*, *Prunus necrotic ringspot virus*, *Plum pox virus* and *Tomato ringspot virus* and may replace the presently used less advantageous techniques like ELISA or woody indicators for the indexing of these pathogens.

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