

SHORT COMMUNICATION  
DEVELOPMENT OF A NESTED PCR PROTOCOL FOR DETECTION  
OF OLIVE-INFECTING VIRUSES IN CRUDE EXTRACTS

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SUMMARY

A reverse transcription nested polymerase chain reaction (RT-n-PCR) was developed and applied to the detection of *Arabis mosaic virus* (ArMV), *Cherry leaf roll virus* (CLRV), *Olive latent virus-1* (OLV-1), *Olive latent virus-2* (OLV-2), *Olive latent ringspot virus* (OLRSV) and *Strawberry latent ringspot virus* (SLRV) in naturally infected olive cuttings.

The method was more sensitive and reliable than conventional RT-PCR. This increased sensitivity allowed virus detection in samples that were negative to the first round of RT-PCR amplification. Coupled with simple sample preparation from cortical scrapings of olive cuttings, this protocol should be useful in routine testing for olive viruses.

*Key words:* olive-infecting viruses, nested-PCR, detection, crude extract.

In the last twenty years 13 different viruses of seven different genera have been identified in olive trees (*Olea europea* L.). (Martelli, 1999; Felix and Clara, 2000). Some are well-characterised ubiquitous pathogens that infect many crops, whereas others appear exclusive to olive and have been characterised only in part. Analysis of double-stranded RNA (dsRNA) profiles from asymptomatic plants suggests that a number of other undescribed viruses also infect olive. These viruses have escaped detection as they probably occur in low concentration and may not be mechanically transmissible.

According to legislation enforced by the European Union Directive 93/48 on the Conformitas Agraria Communitatis (CAC), olive nurseries should be free from a number of detrimental organisms, including viruses. Due to widespread occurrence of latent viral infections, visual inspection is virtually useless, and field observations should be complemented by quick, sensi-

tive and reliable laboratory tests. Reverse transcriptase-polymerase chain reaction (RT-PCR) has been reported as one of the most sensitive methods for detection/identification of RNA viruses that infect olive (Grieco *et al.*, 2000). However, the last's reliability is affected by a number of little known factors (*e.g.* virus distribution/concentration within the plant, time of sampling), which can lead to variable results (F. Grieco, personal communication).

In this paper we report development of a reverse transcription and nested (or heminested) PCR (RT-n-PCR) protocol for detection of *Arabis mosaic virus* (ArMV), *Cherry leaf-roll virus* (CLRV), *Olive latent virus-1* (OLV-1), *Olive latent virus-2* (OLV-2), *Olive latent ringspot virus* (OLRSV) and *Strawberry latent ringspot virus* (SLRV), in naturally infected olive cuttings.

Olive plants single infected by ArMV, CLRV, OLV-1, OLV-2, and SLRV came from a collection our Department collection, whereas OLRSV-infected material was kindly provided by Dr. M. Barba (Istituto Sperimentale per la Patologia Vegetale, Rome, Italy).

Samples were collected in winter and consisted of 1- or 2-year old cuttings, approx. 30 cm long. Cortical shavings (*ca* 200 mg) were crushed in a mortar with liquid nitrogen. The powder was vortexed with 300 µl of a mixture containing 0.1% Triton X-100, 0.1% n-laurylsarcosine, 40 mM dithiothreitol (DTT) (Grieco and Gallitelli, 1999) and the slurry incubated at 70°C for 5 min. After a brief centrifugation, 2 µl of the supernatant were recovered and diluted 50-fold in RNase-free water (crude extract). Other extraction procedures involved the preparation of total nucleic acid (TNA) with the silica-capture extraction method (Foissac *et al.*, 2000) or with the aid of the Plant RNeasy Qiagen columns (Qiagen, Germany) according to manufacturer's instructions.

Two microliters of the crude extract of each sample were mixed with 25 pmol of the specific reverse primer (Table 1), in a final volume of 10 µl of 0.01% Triton X-100, denatured for 3 min at 95°C and cooled on ice. This mixture was used for reverse transcription at 37°C for 90 min with 200 units of M-MLV reverse transcriptase (Gibco-BRL, USA) in 1x RT buffer containing

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**Table 1.** Primers used in RT-PCR.

Virus	Forward reverse	Sequence	Expected size	Amplified region
OLV-1 Acc.# NC001721.1	JX25 <sup>3</sup> JX23 <sup>2</sup>	5'-GTGGACTGCGCTCGAATGGA-3' 5'-CTCACCATCGTTGTGTGG-3'	~230 nt	CP gene
OLV-2 Acc.# X94347.1	OLV2-A <sup>2</sup> OLV2-B <sup>2</sup>	5'-CCGTTCTGTGGCCTTTGAGA-3' 5'-AACACGATCCTCACCC-3'	~222 nt	RdRp
OLRSV Acc.# AJ277435	OLRSV-1 <sup>1</sup> OLRSV-2 <sup>1</sup>	5'-AAGAATTCTGCAAACTAGTGCCAGAGG-3' 5'-AAAAGCTTGATAAGGCTCACAGGAG-3'	~492 nt	3' terminal RNA2
ArMV Acc.# X55460, X81814, X81815	AP1 <sup>2</sup> AP2 <sup>2</sup>	5'-AATACCCCGGGTGTACATCG-3' 5'-CATTAACCTAAGATCAAGGATTC-3'	~421 nt	CP gene
SLRV Acc.# X75165, X77466	SLRV-1 <sup>1</sup> SLRV-2 <sup>1</sup>	5'-AAAAGCTTCAAGGAGAATATCCCTGGCCC-3' 5'-AAGGATCCTAAGTGCCAGAATAAACC-3'	~525 nt	CP gene
CLRV Acc.# 563537, 584125, U24694	CLRV-1 <sup>1</sup> CLRV-2 <sup>1</sup>	5'-AAAAGCTTGGCGACCGTGTAACGGCA-3' 5'-AAGAATTCGTCCGAAAGATTACGTAAAAGG-3'	~431 nt	non coding region 3'-terminal RNA2

<sup>1</sup> Grieco *et al.*, 2000.<sup>2</sup> Newly designed.<sup>3</sup> Grieco *et al.*, 1996.

10 mmol of each of the four dNTPs, 20 mmol of DTT and 5 units of human placental ribonuclease inhibitor (HPRI) (Amersham-Pharmacia Biotech, Sweden). Five microlitres of the RT mixture were submitted to the first round of amplification in a final volume of 50 µl in the presence of 1x EZ PCR buffer (GeneAmp-Perkin Elmer, USA) containing 5 pmol of the reverse primer, 10 pmol of the forward primer (Table 1), 10 mmol of each of the four dNTPs, 50 mmol of MgCl<sub>2</sub> and 2.5 units of AmpliTaq DNA Polymerase (Perkin Elmer, USA). Amplification was carried out in a Hybaid PCR express apparatus with a denaturation phase of 2 min at 94°C, followed by 35 cycles of amplification with temperature profiles of 30 s at 94°C, 30 s at 50°C (55°C in the case of OLV-2) and 1 min at 72°C. In the last cycle, time extension was 7 min at 72°C.

To optimize conditions for the nested (or heminested) amplification, 3 µl of products from the first amplification were added to a reaction volume of 50 µl 1x EZ PCR buffer containing different amounts of each specific nested primer (Table 2) and MgCl<sub>2</sub>, 10 mmol of each of the four dNTPs and 2.5 units of AmpliTaq DNA Polymerase. The temperature profile was as before.

To assess reproducibility, preliminary experiments on tissues of *Nicotiana benthamiana* infected with OLV-1 were carried out. Consistent amplification of a product of 0.23 kbp was obtained in all tests with either TNA prepared by the silica-capture extraction method from systemically infected leaves (Fig. 1, lane b) or with

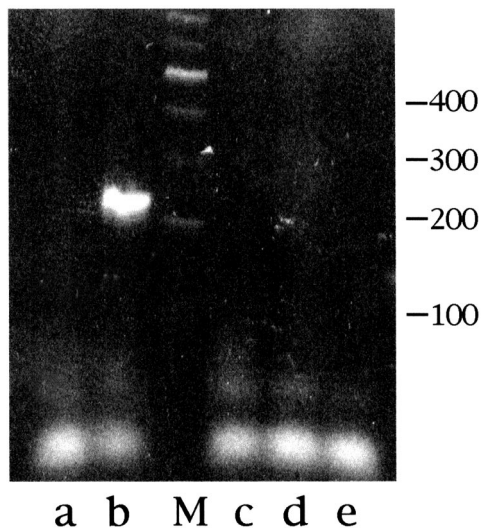
the Plant RNeasy Qiagen columns (not shown). However, we failed to amplify the expected product in three out of four tests, each carried out with a different preparation of either TNA (Fig. 1, lane d) or crude extract (Fig. 2, lane a in panel 3) obtained from cortical scrapings of OLV-1 infected olive cuttings. Similar results were obtained with all the viruses under study, when the RT-PCR was performed on crude extracts (Fig. 2, lane a in all panels; Table 3).

The identity of the amplified products was confirmed by Southern hybridisation (not shown) with the specific digoxigenin-labelled riboprobes against OLRSV, OLV-2, OLV-1, SLRV, CLRV and ArMV, according to the protocol described by Grieco *et al.* (2000).

These samples were therefore subjected to the nested reaction. To optimise the second round of amplification with OLV-1, we used various amounts of both nested primers and first amplification mixture. The procedure which provided the best results and reproducibility involved: (i) purification of the first-round mixture through Quantum Prep PCR Kleen Spin Columns (Bio-Rad, USA) using 50 pmol of each oligonucleotide; (ii) use of 3 ml of the first amplification mixture and 50 pmol of nested primers in a final volume of 50 ml containing 50 nmol of MgCl<sub>2</sub>. When the protocol set up for OLV-1 was applied to the other five viruses, the amplicon of expected size was obtained in all instances (Fig. 2, lane b in all panels; Table 3).

**Table 2.** Nested primers.

Virus	Name	Sequence	Expected size
OLV-1	OLV1-n1	5'-AATGTTACCCTGGCCACC-3'	~167 nt
Acc.# NC001721.1	OLV1-n2	5'-TGTGGTTACAAATTGAC-3'	
OLV-2	OLV2-n1	5'-CGTCCGAGATTATCTCTGA-3'	~175 nt
Acc.# X94347.1	OLV2-n2	5'-TGACTCTGTTTCAGAAGTAG-3'	
OLRSV	OLRSV-n1	5'-GTGGTGACGTGCTCTATCC-3'	~163 nt
Acc.# AJ277435	OLRSV-n2	5'-GGAGTCTAGGAATTGAAAACA-3'	
ArMV	AP2	see table 1	~184 nt
Acc.# X55460, X81814, X81815	ArMV-n1	5'-CCCCAATGATTATTTCTATGG-3'	
SLRV	SLRV2	see table 1	~396 nt
Acc.# X75165, X77466	SLRV-n1	5'-CATTGTCCATGTGTTGAGGCT-3'	
CLRV	CLRV-n1	5'-CCCAAGAATTTAGGGGG-3'	~170 nt
Acc.# 563537, 584125, U24694	CLRV-n2	5'-AAACTCTAAAAGTAAA-3'	



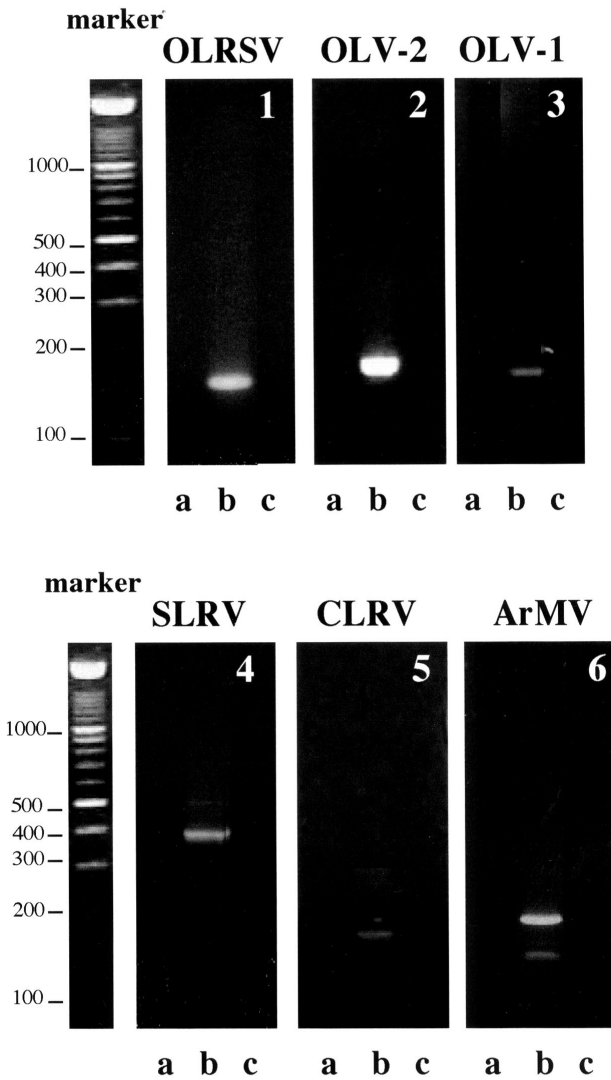
**Fig. 1.** 2% agarose gel showing OLV-1 amplicons obtained by RT-PCR performed on TNA prepared with the silica-capture extraction procedure from healthy *N. benthamiana* (a), OLV-1 infected *N. benthamiana* (b), healthy olive tree (c), OLV-1 infected olive tree (d) and water control (e). M = 100 bp.

The results of this preliminary study suggest that failure to consistently detect viruses from cortical olive tissues with standard RT-PCR may not be due to plant inhibitors since the amplified product also remained undetectable using TNA preparations obtained with the silica-capture extraction procedure (Foissac *et al.*, 2000) or with the aid of the Plant RNeasy Qiagen columns.

Rather it may be due to an irregular distribution/concentration of viral sequences in cortical scrapings of different cuttings from the same olive tree. The RT-n-PCR protocol and the set of nested (or heminested) primers described here combine specificity and sensitivity and may help in overcoming these problems. It should be kept in mind, however that n-PCR is a very sensitive test, and the slightest contamination may lead to false positive results. Work is in progress to adapt the test to the newly proposed automated PCR-based assays that allow real time identification of amplified DNA through emission of a fluorescent signal, and reduce risk of contamination between samples (Tyagi and Kramer, 1996; Nazarenko *et al.*, 1997; Whitcombe *et al.*, 1999). RT-n-PCR in a single closed tube, developed by Cambra *et al.* (2000) could also be useful, although it will probably need a specific set up for olive extracts. In conclusion, the procedure presents risks of contamination, but it will dramatically reduce both costs and time in routine testings.

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**Fig. 2.** 2% agarose gels showing amplicons obtained by RT-PCR (lane a in all panels) and nested PCR (lane b in all panels) from crude extracts prepared from cortical scrapings of infected olive cuttings for each of the viruses analysed. Negative control of RT-n-PCR (crude extract from healthy *N. benthamiana*) (lane c in all panels).

**Table 3.** Results of a series of experiments of RT-PCR and n-PCR on crude extracts prepared from cortical scrapings each obtained from a different infected olive cutting.

Virus	Positive/total experiments	
	RT-PCR	Nested PCR
OLV1	1/4	4/4
OLV-2	0/3	3/3
OLRSV	0/1	1/1
ArMV	1/4	4/4
SLRV	1/4	4/4
CLRV	0/2	2/2

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