Detection of olive tree viruses in Egypt by one-step RT-PCR

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Abstract

Olive (*Olea Europaea* L.) is a major and economically important crop for the new reclamation land in Egypt. The estimated acreage of cultivated olive trees in Egypt is above one hundred and thirty five thousand Feddens of which total production is five hundred thousand tons per year (Ministry of Agriculture statistics, 2007). Olive trees are affected by several viruses and virus-like diseases. To date, 15 viruses in eight genera have been isolated from olive trees. In a preliminary study for the assessment of the sanitary status of olive trees in five locations in Egypt, shoots from 300 trees of 9 cultivars were collected. Using virus-specific primers, a one-step RT-PCR assay was used to detect and identify each of the eight viruses most commonly found in olives. Namely, *Cucumber mosaic virus* (CMV), *Olive latent ringspot virus* (OLRSV), *Olive latent virus*-1 (OLV-1), *Olive latent virus*-2 (OLV-2), *Olive leaf yellowing-associated virus* (OLYaV), *Strawberry latent ringspot virus* (SLRSV), *Cherry leaf roll virus* (CLRV), and *Arabis mosaic virus* (ArMV). Among the eight viruses assayed, OLRSV (6.7 %), OLV-1 (5.7 %), CLRV (4.7 %), OLV-2 (2.7 %), SLRSV (2.3 %), OLYaV (1.3 %) and ArMV (0.7%) were detected. The most common virus detected was CMV which prevailed with a high incidence of 24.7 % in olive orchards. The use of one step RT-PCR was efficient and reliable to detect the eight olive viruses found in Egypt. Surprisingly, the infection rate found is lower than expected, if we take into consideration previous surveys conducted in the Mediterranean area. This technique is useful for detection of olive viruses for production of certified plant propagative material in certification programs.

Keywords: Olive cultivars, olive virus detection, olive viruses in Egypt, one step RT-PCR.

Introduction

Olive (*Olea europaea* L.) trees are hosts to number of diseases caused by viruses, phytoplasmas, bacteria, fungi, and agents of diseases of unknown etiology which disseminate by propagating material, and are the object of certification programs in many countries, including Egypt. Sensitive and reliable detection methods of olive tree viruses are needed in these programs. Enzyme-linked immunosorbent (ELISA) is routinely used in certification as it allows sensitive, specific, and simultaneous analysis of many samples (Garnsey and Cambra, 1991). However, molecular methods based on polymerase chain reaction (PCR) amplification of the pathogen nucleic acid (Olmos et al., 1999) enable greater sensitivity especially when the target is in low concentration, or the pathogen has uneven distribution as in asymptomatic olive trees. Among molecular methods, RT-PCR has proved to be the most rapid, sensitive and reliable technique for detecting RNA of the target in infected plants (Hadidi and Candresse, 2001). Thus, the use of PCR technology is an important step to optimize and speed up olive tree virus diagnosis. In this study, we applied a one-step RT-PCR protocol to detect the eight most common olive tree virus species that belong to five genera: *Cucumovirus*. *Cucumber mosaic virus* (CMV) (Savino and Gallitelli, 1983); *Sadwavirus*, *Strawberry latent ringspot virus* (SLRSV); *Nepovirus*, *Arabis mosaic virus* (ArMV) (Savino et al., 1979), *Olive latent ringspot virus* (OLRSV), and *Cherry leaf roll virus* (CLRV) (Savino and Gallitelli, 1981); *Necrovirus*, *Olive latent virus*-1(OLV-1) (Gallitelli and Savino, 1985); *Oleavirus*, *Olive latent virus*-2 (OLV-2) (Savino et al., 1984), and *Closteroviridae*, *Olive leaf yellowing associated virus* (OLYaV) (Savino et al., 1996).

Materials and methods

Source of plant material: Shoots from 300 symptomatic or asymptomatic olive trees were collected from five different locations in Egypt (Giza, Fauom, Nubaria, Behera and Ismailia). The trees represented 9 different cultivars, covering two native cultivars (Aggazi and Maraki) and seven imported cultivars (Dolce, Kalamata, Koronaki, Koratina, Manzanello, Picual and Sorany).

Viral RNA preparation and one-step RT-PCR amplification: Phloem tissue from young shoots were scraped and powdered in liquid nitrogen. About 100 mg of each sample was used for total RNA extraction using the Plant Total RNA Mini Kit, according to the manufacturer's protocol (Real Biotech, Corp., Taiwan). RNA was finally eluted with 50 µl of RNase- free water, and stored at -20 ºC until used. RT-PCR was carried out on RNA preparations with Reverse-iT™One-Step RT-PCR Kit (ABgene®UK). This allows RT and amplification to be performed sequentially in the same

tube. In particular, 2.5 µl of total RNA containing the target RNA was mixed with 12.5 µl 2x RT-PCR Master mix containing 1.25U/50µl Thermoprime Plus DNA Polymerase; 1.5mM $MgCl_2$, 0.2 mM each dNTPs, 10µM specific forward and reverse primers (Table 1); 0.5 µl Reverse-iT™ RTase Blend (50U/µl); RNase/DNase-free water to a volume of 25 µl. Synthesis of cDNA was performed at 47 ºCfor 30 min, followed by denaturation at 94 ºC for 2 min. Amplification was carried out for 35 cycles under the following conditions: denaturation at 94 ºC for 30 sec, annealing at 50 °C for 45sec (55 ºC in case of OLV-2), extension at 72 ºC for 60 sec, followed by a final extension for 7 min at 72 ºC. Amplified products were detected by 1-1.5 % agarose gel electrophoresis in TBE buffer, stained with ethidium bromide and visualized by gel documentation system (Bio-Rad, USA).

Tab. 1 Sequence of specific primers used for the detection of olive tree viruses.

Virus	Primer sequence	Expected size	Amplification region
OLV-1F	5'-GTGGACTGCGCTCGAATGGA-3'	230 nt	CP gene
OLV-1R	5'-CTCACCATCGTTGTGTGG-3'		
OLV-2F	5'-CCGTTCTGTGGCCTTTGAGA-3'	220nt	RdRp
OLV-2R	5'-AACACGATCCTCACCC-3'		
OLYaVF	5'-ACTACTTTCGCGCAGAGACG-3'	346nt	
OLYaVR	5'-CCCAAAGACCATTGACTGTGAC-3'		
OLRSVF	5'-AAGAATTCTGCAAAACTAGTGCCAGAGG-3'	492nt	3'terminal
OLRSVR	5'-AAAAGCTTGCATAAGGCTCACAGGAG-3'		
SLRVF	5'-AAAAGCTTCAAGGAGAATATCCCTGGCCC-3'	525nt	CP gne
SLRVR	5'-AAGGATCCTAAGTGCCAGAACTAAACC-3'		
CLRVF	5'-AAAAGCTTGGCGACCGTGTAACGGCA-3'	431nt	non coding region
CLRVR	5'-AAGAATTCGTCGGAAAGATTACGTAAAA-3'		
ArMVF	5'-TTGGCCCAGATATAGCGTAAAAAT-3'	519 nt	
ArMVR	5'-CAGCGGATTGGGAGTTCGT-3'		
CMVF	5'-GCCGTAAGCTGGATGGACAA-3'	~499nt	CP gene
CMVR	5'-TATGATAAGAAGCTTGTTTCGCG-3'		

Results and discussion

Natural infections of olive tree with viruses are mostly symptomless; those viruses that elicit symptoms in certain cultivars (e.g. SLRSV, OLYaV) are latent in others. Thus it is difficult to base diagnosis on symptoms expression. Biological indexing of olive viruses on woody differential indicators is not done as they are currently not available. The diagnostic bioassay that has been used extensively up to a recent past is mechanical transmission. This assay, however, is unreliable because of the low intrinsic sensitivity (Felix et al., 2001).

To detect viruses using PCR, it is important that nucleic acid samples extracted from the original source of plant material is free of appreciable amounts of oil, polysaccharides, phenolic compounds, and other PCR Inhibitors (Wilson, 1997). These inhibitors are present in olive tissue extracts (Amiot et al., 1989; De Niro et al., 1997). They must be removed in order to detect the viral RNA targets by PCR. The choice of an extraction technique that can be used for routine testing of a large number of samples must take into account simplicity of use and rapidity of execution. The results of this study showed that RNA extraction procedure developed is suitable for routine use in diagnostic laboratories. One step RT-PCR analysis was simple and fast; it allowed testing of hundreds of samples to be done in a relatively short time. Virus infected olive trees were found in all investigated locations and cultivars in Egypt. Each pair of the eight selected primer pairs amplified successfully its specific target RNA from total RNAs extracted from infected tissues with the Plant Total RNA Mini Extraction kit.(Figure 1). Noticeably, the majority of olive viruses were isolated from symptomless trees. The average incidence of infection for each virus tested was 24.7 % for CMV, 6.7 % for OLRSV, 5.7 % for OLV-1, 4.7 % for CLRV, 2.7 % for OLV-2, 2.3 % for SLRSV, 1.3 % for OLYaV and 0.7 % for ArMV (Table 2; Figure 2). Mixed infection was observed in large number of tested trees. The reliability of this detection method allowed increased investigations on distribution of olive viruses by conducting several surveys in different geographical areas in Egypt.

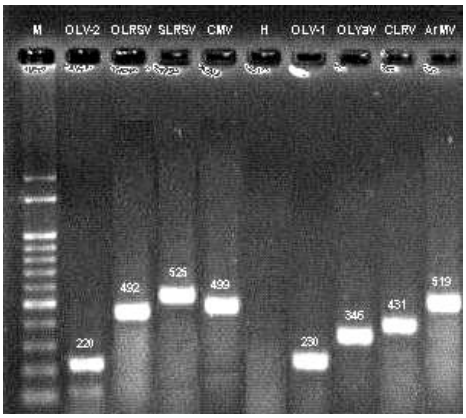

Fig. 1 Agarose gel electrophoresis analysis of amplified products obtained by one step RT-PCR using primers for the eight olive tree viruses (Table 1). Lane M, 100 bp DNA marker

Tab. 2 List of analyzed cultivars (Number of infected/number of tested) of olive trees and percentage of infection obtained by one-step RT-PCR assay for the detection of CMV, OLRSV, OLV-1, CLRV, OLV-2, SLRSV, OLYaV, and ArMV.

Cultivars	**Virus Tested**							
	CMV	**OLRSV**	**OLV-1**	**CLRV**	**OLV-2**	**SLRSV**	**OLYaV**	**ArMV**
Kalamata	8/38	6/38	0/38	2/38	2/38	0/38	1/38	0/38
Koronaki	10/48	4/48	8/48	5/48	1/48	0/48	0/48	1/48
Koratina	9/39	4/39	0/39	1/39	1/39	2/39	0/39	0/39
Picual	15/46	1/46	1/46	3/46	4/46	0/46	0/46	0/46
Manzanello	12/46	2/46	5/46	3/46	0/46	4/46	3/46	1/46
Dolce	4/10	2/10	0/10	0/10	0/10	0/10	0/10	0/10
Sorany	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Aggazi	8/31	0/31	1/31	0/31	0/31	1/31	0/31	0/31
Maraki	7/37	1/37	2/37	0/37	0/37	0/37	0/37	0/37
% of infection	24.7	6.7	5.7	4.7	2.7	2.3	1.3	0.7

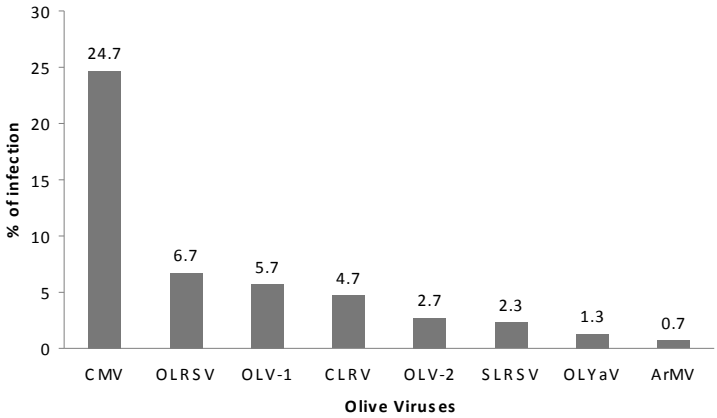

Fig. 2 Percentage of infection of the eight detected viruses (CMV, OLRSV, OLV-1, CLRV, OLV-2, SLRSV, OLYaV and ArMV) obtained in this investigation.

Theses results coupled with the sensitivity and the absence of contamination risks (since the assay is done in a single tube), made this technique very suitable for large-scale investigation (Bertolini et al., 2001; Ragozzino et al., 2004). The one-step RT-PCR protocol confirmed, for all tested olive tree viruses, its rapidity and reliability (Faggioli et al., 2005). Since the genomic sequences of the majority of viruses, including the somewhat rare OLV-1, OLV-2 and OLRSV, are known (Grieco et al., 1995, 1996a,b), the design and use of adequate PCR primers is now possible, as exemplified by the successful identification of CMV, CLRV, ArMV, SLRSV, OLYaV, OLV-1 and OLV-2 by single step RT-PCR (Sabanadzovic et al., 1999; Grieco et al.,2000; Bertolini et al., 1998, 2001).

Throughout the increasing international demand for olive plants and legislation enacted require that all olive propagative material produced in nurseries must be free of all viruses. This led to the development of sensitive diagnosis techniques to assist in selection, improvement and sanitary certification of olive planting material. Reliable virus detection is also needed in epidemiological studies and in-establishing strategies for control and certification programs.

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