Molecular detection of olive viruses*

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Olive hosts 13 viruses belonging in seven different genera. Additional non-mechanically transmissible viruses probably infect olive in nature, as suggested by the widespread occurrence of double-stranded RNAs (dsRNAs) in trees from which no viruses can be recovered by manual inoculation. Because sanitary selection appears to be the only measure for restraining virus dissemination through propagating material, detection methods are needed which are more sensitive and reliable than those currently available (biological and serological). The following molecular techniques have therefore been used and their efficiency compared: (1) dsRNA analysis; (2) dot-blot hybridization with digoxigenin-labelled riboprobes in separate reactions or in mixture; and (3) reverse transcription-polymerase chain reaction (RT-PCR). It was found that: (1) dsRNAs were detected in 210 out of 286 olive accessions (73.4%) coming from six different Italian regions; (2) one-step RT-PCR yielded much better results using TNA extracts than crude sap; and (3) dot-blot hybridization of denatured dsRNAs with digoxigenin-labelled virus-specific riboprobes was the most reliable detection method available.

Introduction

Italian nursery production of olive is subject to quality and phytosanitary norms embodied in EU Directive 93/48 of 23.06.93 *'Conformitas Agraria Communitatis'* (CAC), concerning propagative materials of fruit trees, and in a Decree regulating voluntary certification, issued in 1993-06 by the Italian Ministry of Agricultural Policy. These laws make assessment of the sanitary status of propagating material compulsory. According to CAC, plants produced in nurseries must be true to type and free from all viruses, whereas the virus-tested category of the Italian certification scheme requires freedom from a selected number of viruses only. However, detection and identification of olive viruses, now numbering 13 (Felix & Clara, 2000; Martelli, 2000) (Table 1), is impaired by a number of constraints: (1) virus infections are quite often symptomless; (2) no differential woody indicators are available; (3) mechanical transmission to herbaceous hosts is erratic and has a low intrinsic sensitivity; and (4) DAS-ELISA is also erratic because of the low antigen titre in olive tissues.

It has recently been found that double-stranded RNAs (dsRNAs) are frequent in olive trees (Martelli *et al.*, 1995a,b) from whose tissues they can readily be extracted (Sabanadzovic *et al.*, 1999). This suggested that dsRNAs could serve as infection markers, based on the notion that these molecules are of viral origin, even when they are apparently endogenous to plants (Gibbs *et al.* 2000). dsRNAs have been used both for discriminating between healthy and infected plants, and as templates for virus identification by dot-blot hybridization with virus-specific probes.

Materials and methods

dsRNAs were recovered by phenol extraction and chromatography on a cellulose CF11 column (Morris & Dodds, 1979) from cortical scrapings from young olive shoots sampled from the quadrant of each tree. Tissue samples (5–10 g) were ground in liquid nitrogen, the powder was transferred to 250-mL Nalgene bottles and suspended in cool extraction buffer (30 mL STE 2×, 5.9 mL 10% SDS, 0.7 mL 45 mg mL^{-1} bentonite, 0.7 mL 2-mercaptoethanol). After a brief incubation at room temperature, 24 mL of phenol–chloroform (1:1 v/v) was added, the mixture was shaken for 30 min then centrifuged at 10 000 rev min^{-1} for 10 min. The aqueous phase was recovered and shaken slowly for 1 h at room temperature after the addition of 7.5 mL absolute alcohol and 1 g Whatman CF11 cellulose. The cellulose was sedimented by centrifuging at 4500 rev min^{-1} for 15 min, resuspended in 17% ethanol-STE 1×, and transferred to a disposable syringe plugged with a Miracloth disk. The cellulose column was washed twice with 25 mL of 17% ethanol-STE 1×, dsRNA was eluted with 12.5 mL of STE 1× and precipitated with 30 mL cold absolute alcohol and 1.2 mL of 3 M sodium acetate pH 5.5 for 2 h at −70°C. The extract was digested with RNase A and RNase-free DNase, electrophoresed in polyacrylamide gel (PAGE) and stained with silver nitrate (Saldarelli *et al.*, 1994). Nuclease digestion was not

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Table 1 Olive viruses and their geographical distribution

Virus	Genus	Geographical distribution
Strawberry latent ringspot (SLRSV)	*Nepovirus*	Italy, Portugal, Spain,
Arabis mosaic (ArMV)	*Nepovirus*	Italy
Cherry leafroll (CLRV)	*Nepovirus*	Italy, Portugal, Spain,
Cucumber mosaic (CMV)	*Cucumovirus*	Italy, Portugal, Spain,
Olive latent ringspot (OLRSV)	*Nepovirus*	Italy, Portugal
Olive latent 1 (OLV-1)	*Necrovirus*	Italy, Jordan, Turkey
Olive latent 2 (OLV-2)	*Oleavirus*	Italy, Lebanon
Olive vein yellowing-associated (OVYaV)	*Potexvirus*	Italy
Olive yellow mottling and decline-associated (OYMDaV)	Undetermined	Italy
Tobacco mosaic (TMV)	*Tobamovirus*	Italy
Olive semi-latent (OSLV)	Undetermined	Italy
Olive leaf yellowing-associated (OLYaV)	*Closterovirus*	Italy
Tobacco necrosis (TNV)	*Necrovirus*	Portugal

carried out if dsRNA was used as substrate for molecular hybridization.

Eight primer pairs specific to each of the viruses comprised in the Italian voluntary certification scheme (CMV, SLRSV, ArMV, CLRV, OLRSV, OLV-2, OLV-1 and OLYaV) were used for reverse transcription-polymerase chain reaction (RT-PCR). These primers (Table 2) were designed on genomic sequences retrieved from the EMBL database (CMV, SLRSV, ArMV and CLRV) or determined in our laboratory: OLV-2, Grieco *et al.* (1995); OLV-1, Grieco *et al.* (1996); OLYaV, Sabanadzovic *et al.* (1999); and OLRSV, Alkowni *et al.* (2000). Total nucleic acid (TNA) extracted from olive cortical tissues with the RNeasy Plant Extraction kit (Quiagen) or, alternatively, sap from olive leaves prepared according to Grieco & Gallitelli (1999) was used as templates for RT-PCR (Martelli et al., 1996). Briefly, 1 μL TNA was mixed with 150 ng of random hexamers (Boehringer Mannheim), denatured for 10 min at 70°C and kept on ice. RT reaction was for 1 h at 42°C in a 20-μL volume of 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTPs, 200 units of Moloney Murine leukaemia virus reverse transcriptase (Bethesda Research Laboratories). Next, 3 μL of the mixture was subjected to PCR amplification in 1× *Taq* polymerase buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 mM of both primers and 2.5 units of *Taq* polymerase in a final volume of 50 μL. cDNAs were amplified in a Perkin Elmer Cetus Thermal Cycler apparatus with 35 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 1 min. In the last cycle, extension at 72 °C was for 7 min. Amplification products were analysed in 1.2% agarose gel electrophoresis (Sambrook *et al.*, 1989).

dsDNA fragments specific for the eight above viruses were cloned in the *Eco*RI/*Hind*III sites of a pSPT64 vector and the recombinant plasmids used to transform Escherichia coli DH5α. Specific digoxigenin-labelled riboprobes (Dig-riboprobes) were obtained by *in vitro* transcription (DIG-RNA Labelling Kit, Boehringer) of 1 μg of plasmid DNA digested with the appropriate restriction enzyme (Table 3).

Table 2 Primers for RT-PCR of olive-infecting viruses

Virus	Amplicon length	Amplified region	Detection limit	Primer sequences
OLV-1	747 nt	3′-terminal	c. 10 fg	5′-CTCACCCATCGTTGTGTGG-3′ 5′-TTTCACCCCACCAAATGGC-3′
SLRV	525 nt	coat protein	c. 100 fg	5′-TCAAGGAGAATATCCCTGGCCC-3′ 5′-CTAAGTGCCAGAACTAAACC-3′
CMV	513 nt	RdRp	c. 10 fg	5′-TAACCTCCCAGTTCTCACCGT-3′ 5′-CCATCACCTTAGCTTCCATGT-3′
ArMV	504 nt	coat protein	c. 100 fg	5′-TTGGTTAGTGAATGGAACGG-3′ 5′-TCAACTCACCCTCCAAATCCC-3′
OLRSV	492 nt	3′-terminal	c. 100 fg	5′-CTGCAAAACTAGTGCCAGAGG-3′ 5′-TGCATAAGGCTCACAGGAG-3′
CLRV	431 nt	coat protein	c. 100 fg	5′-TTGGCGACCGTGTAACGGCA 5′-GTCGGAAAGATTACGTAAAAGG-3′
OLV-2	390 nt	RdRp	c. 10 fg	5′-ACGTGTTAGTCGCTGTGGTACC 5′-TATGTTTGACGCACCGGAGCG-3′
OLYaV	383 nt	hsp70-like protein	c. 10 fg	5′-CGAAGAGAGCGGCTGAAGGCTC-3′ 5′-GGGACGGTTACGGTCGAGAGG-3′

Table 3 Riboprobes for identification of olive-infecting viruses

Virus	Clone Name	R.E. cut	Promoter	Transcript length	Transcribed region
ArMV	pSPArMV	*Hin*dIII	T7	490nt	CP gene
CLRV	pSPCLRV	*Hin*dIII	T7	420nt	3′-terminal
SLRSV	pSPSLRSV	*Hin*dIII	T7	520nt	CP gene
OLYaV	pSPOLYV	*Hin*dIII	T7	530nt	hsp70-like protein
CMV	pCMVS3	*Bam*HI	T7	1500nt	RNA polymerase
OLV-1	pSPTOLV	*Eco*RI	T7	1050nt	CP gene
OLV-2	pSPOLV2	*Hin*dIII	T7	1280nt	MP gene
OLRSV	pSPOLRSV	*Hin*dIII	T7	1100nt	3′-terminal

Dot-blot hybridization with Dig-riboprobes was carried out as described (Saldarelli *et al.*, 1996). For determining the best substrate for molecular hybridization, dsRNA, TNA and sap extracted from the same amount (500 mg) of cortical scrapings from olive trees infected by single viruses were spotted on a nylon membrane (Hybond N+, Amersham) and hybridized with Dig-riboprobes. Prior to hybridization, dsRNA preparations were denatured by treating with 50 mM NaOH.

Results

The dsRNA extraction protocol of Morris & Dodds (1979) was successfully adapted to olive virus detection. Leaves, roots and cortical scrapings were compared as starting material for dsRNA recovery. Cortical scrapings proved to be the best source, yielding very clean dsRNA preparations from as little as 5 g of tissue. Under our experimental conditions, a single operator was able to run up to 12 dsRNA extractions per day.

Of 286 trees from six Italian regions (Puglia, Umbria, Sardegna, Toscana, Lazio, Abruzzo) tested, 210 (73.4%) contained dsRNAs (Table 3).

The eight selected primer pairs successfully amplified their specific target RNA (Fig. 1) from purified virions and from total RNAs extracted from infected cortical tissues with the RNeasy Plant Extraction kit. The identity of the amplified products was ascertained by hybridization with Dig-riboprobes. PCR amplification from plant sap was erratic, except for olive samples infected by OLV-1 and OLYaV.

Denatured dsRNAs were the only substrate giving consistent and reproducible signals in molecular hybridization assays. The sensitivity of the eight Dig-riboprobes was very high, as they could detect up to 1 pg of viral RNA, and gave no signal with heterologous nucleic acids (e.g. TNA from healthy olives) (Fig. 2). When used as a mixture in a single hybridization reaction, the eight riboprobes did not interfere with one another, thus allowing simultaneous detection of the eight viruses.

Discussion

It is no longer possible to dismiss viral problems of olive as negligible. Symptomless field infection has been found to be widespread, and this conflicts with EU specifications on quality of nursery production, the implementation of the Italian voluntary certification scheme and the increasing international demand for propagation material of high sanitary standard. To meet these requirements, it is essential to

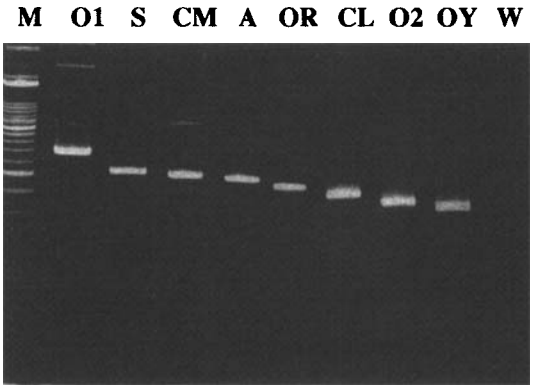

Fig. 1 Polyacrylamide gel electrophoresis of RT-PCR fragments specific to the following viruses: 01, olive latent 1; S, strawberry latent ringspot; CM, cucumber mosaic; A, arabis mosaic; OR, olive latent ringspot; CL, cherry leafroll; O2, olive latent 2; OY, olive leaf yellowing. M, DNA marker XIV (Boehringer, Mannheim). W, water control.

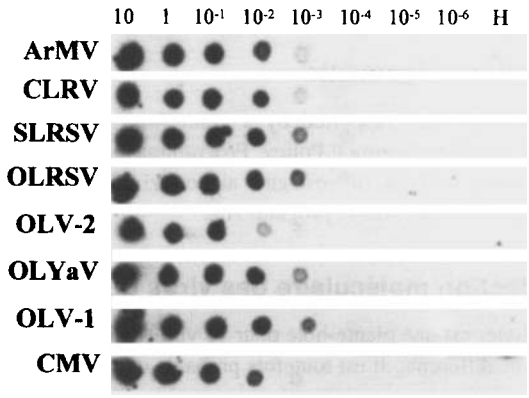

Fig. 2 Chemiluminescent detection of 10-fold dilutions of eight different viral RNAs in ng amounts by dot-spot hybridization. H, total nucleic acid extract from healthy olive.

assess the sanitary status of the olive crop. Up to now, this has not been possible because of the latency of viral infections and unavailability of efficient and sensitive diagnostic tools.

The aim of the present study was to increase knowledge on the incidence of olive viruses in Italy and to optimize nucleic acid-based assays for their sensitive detection and identification. Substantial advances were made towards the achievement of both goals. The dsRNA assay we developed is simple, cheap, relatively quick and appears to be extremely efficient not only for determining the sanitary condition of olive plants (presence or absence of infection), but also for detecting unknown viruses which cannot be distinguished and identified by traditional means (Sabanadzovic *et al.*, 1999). Using this diagnostic approach, we confirmed an alarming frequency of viral infections in some of the main Italian olive-growing regions. The fact that these infections are mostly latent makes sanitary selection based only on visual examination impossible, hence the necessity for modern sensitive laboratory tests. RT-PCR detected OLV-1 and OLYaV directly from plant sap, but none of the other viruses (ArMV, SLRV, CLRV, OLRSV, OLV-2, CMV), whose genome could be amplified only if TNAs from olive were used as a template. Although the sensitivity of our RT-PCR assay tallied with that reported in the literature for viruses and viroids of woody crops (Minafra & Hadidi, 1994; Olmos *et al.*, 1997; Wan Chow Wah & Symons, 1997), use of the method is limited by the time needed for template preparation.

All Dig-riboprobes showed a high level of specificity to their target RNAs, had no affinity for host nucleic acids, and had a sensitivity equalling that reported in the literature for comparable non-radioactive probes (Crosslin *et al.*, 1992; Mas *et al.*, 1993). dsRNAs dot-blot hybridization assays give highly reproducible results and, when used in mixture, detected multiple infections with a level of sensitivity comparable to that of single hybridization reactions. While waiting for the optimization of nested PCR protocols, now being investigated in our laboratory, hybridization of denatured dsRNAs with virus-specific probes seems the most reliable procedure for molecular identification of olive-infecting viruses.

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Détection moléculaire des virus de l'olivier

L'olivier est une plante-hôte pour 13 virus appartenant à sept genres différents. Il est toutefois probable que d'autres virus, qui ne sont pas transmis mécaniquement, infectent l'olivier dans la nature, comme suggéré par la présence fréquente d'ARN bicaténaire (ARNds) dans des arbres sur lesquels aucun virus n'a été récupéré par inoculation manuelle. La sélection sanitaire semble être la seule mesure permettant de limiter la dissémination des virus par le matériel de multiplication et des méthodes de détection plus sensibles et plus fiables que celles disponibles pour le moment (biologiques et sérologiques) sont donc nécessaires. Les techniques moléculaires suivantes ont donc été utilisées et leur efficacité comparée: (i) analyse de l'ARNds; (ii) hybridation dot-blot avec des ribosondes marquées à la digoxygénine dans des réactions séparées ou en mélange; (iii) RT-PCR. Il a été trouvé que: (i) de l'ARNds a été détecté dans 210 des 286 accessions d'olivier (73,4 %) provenant de six régions italiennes; (ii) la RT-PCR en une étape donne de bien meilleurs résultats en utilisant des extraits d'acide nucléique total plutôt que de la sève brute; (iii) l'hybridation dot-blot d'ARNds dénaturé avec des ribosondes marquées à la digoxygénine spécifiques au virus est la méthode de détection la plus fiable disponible actuellement.

Молекулярное выявление вирусов маслины

Маслина служит растением-хозяином для 13 вирусов, принадлежащих семи различным родам. В природе существуют еще и другие вирусы, передающиеся немеханическим путем, которые, по-видимому, заражают маслину, что можно предположить на основе широко распространенного присутствия двунитевых рибонуклеиновых кислот (dsRNAs) на деревьях, от которых вирусы не могут быть получены с помощью ручной инокуляции. Поскольку фитосанитарный контроль представляется единственной мерой, ограничивающей распространение вируса через посадочный материал, необходимы такие методы выявления, которые являются более чувствительными и надежными, нежели те, которые доступны в настоящее время (биологические и серологические). Поэтому использовались следующие молекулярные методы и сравнивалась их эффективность: (i) анализ dsRNA; (ii) дот-блот гибридизация с рибозондами, маркированным дигоксигенином в отдельных реакциях или в их сочетании: (iii) RT-PCR. Было найдено, что: (i) dsRNAs были обнаружены в 210 из 286 растений маслины (73,4%) из шести различных областей Италии; (ii) одноступенчатый анализ RT-PCR показал намного лучшие результаты на основе экстрактов TNA, нежели сырого сока; (iii) дот-блот гибридизация денатурированной dsRNAs с рибозондами маркированным дигоксигенином, специфичным к вирусам, был наиболее надежным методом выявления из доступных в настоящее время.

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