DISTRIBUTION OF OLIVE TREE VIRUSES IN ITALY AS REVEALED BY ONE-STEP RT-PCR

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SUMMARY

We have used a one-step RT-PCR protocol to detect and identify each of the eight viruses most commonly found in olive trees namely: *Arabis mosaic virus* (ArMV), *Cherry leaf roll virus* (CLRV), *Cucumber mosaic virus* (CMV), *Olive leaf yellowing associated virus* (OLYaV), *Olive latent ring spot virus* (OLRSV), *Olive latent virus-1* (OLV-1), *Olive latent virus-2* (OLV-2), and *Strawberry latent ring spot virus* (SLRSV). We have assayed 345 samples collected from olive fields in Italian areas in which national and local olive tree cultivars and selections are grown. The average percentage virus infection of olive trees was 32.8%. Among the eight viruses assayed, only OLYaV (20.9%), SLRSV (7.8%) and CLRV (4.9%) were detected. The most common virus was OLYaV, which was found at a high percentage in southern Italy. SLRSV was the main virus in central Italy whereas CLRV was detected in five samples from Latium, Umbria and Sicily and in all analyzed trees from Abruzzo.

Key words: CLRV, olive trees, OLYaV, SLRSV.

INTRODUCTION

Olive (*Olea europaea* L.) is a very important crop in Italy. The estimated acreage of cultivated olive trees in Italy is above 2 million hectares. Italy ranks second in the world in olive oil production (more than 600,000 tons per year) and first in olive oil consumption. The great importance of the olive trees in Italy is also indicated by the wide range of cultivars grown. In addition to the national and international cultivars (i.e. 'Frantoio', 'Leccino', 'Moraiolo'), there are many local cultivars that are assuming a fundamental role in increasing oil quality. The current tendency in olive tree cultivation is towards the use of local cultivars to produce oil of special quality (DOP, monovarietal oil) that is typical of particular geographical areas. For this reason, regional administrations have supported studies and activities aimed at the characterization and recovery of local and old cultivars to establish germplasm collections that limit genetic erosion. Thus, an evaluation of the sanitary status of olive germplasm, aimed at the most common olive cultivars and olive cultivars of local importance, is necessary due to the recent European Union Community rules on the commercialization and the quality of plant propagative material (CAC) and the Italian voluntary certification.

Olive trees are affected by several viruses and virus-like diseases. To date, 13 viruses that belong to eight genera have been isolated from olive trees (Martelli, 1999; Felix and Clara, 2002). Most of these viruses were isolated from symptomless trees and/or reported only in one or a very limited number of trees [i.e., *Olive latent ring spot virus* (OLRSV) (Savino *et al*., 1983); *Olive vein yellowing associated virus* (OVYaV) (Faggioli and Barba, 1995); Olive semilatent virus (OSLV) (Materazzi *et al*., 1996)]. *Strawberry latent ring spot virus* (SLRSV) in olive trees in Italy is well investigated. It was reported for the first time in 1979 in central Italy (Savino *et al*., 1979) and its role in causing 'bumpy fruit' disease in cv Ascolana tenera was ascertained (Marte *et al*., 1986). Moreover, recently its effect on some morphological parameters of cv Raggiola has been clearly demostrated (Ferretti *et al*., 2002), confirming its pathological and economic importance among olive tree viruses. Other olive tree viruses have been found to be associated with particular symptoms, i.e. OVYaV (Faggioli and Barba, 1995), Olive yellow mottling and decline associated virus (OYMDaV) and *Olive leaf yellowing associated virus* (OLYaV) (Savino *et al*., 1996) were associated with leaf-yellowing complex disease. OSLV was associated with vein clearing, and *Tobacco mosaic virus* (TMV) was associated with vein banding (Triolo *et al*., 1996). There is no clear evidence, however, of the etiological involvement of these viruses in the diseases.

The difficulty of recognizing and/or diagnosing virus-infected olive trees during field surveys is due to a lack of disease symptoms, the absence of differential

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woody indicator plants for bioassays and/or the unreliability of serological tests (ELISA). All these factors have made olive tree virus diagnosis very problematic.

In recent years, the application of molecular diagnostic techniques (dsRNA, molecular hybridization, reverse transcription-polymerase chain reaction (RT-PCR), for virus detection has appeared more promising than the traditional detection methods (Grieco *et al*., 2000; Bertolini *et al*., 2001a; Faggioli *et al*., 2002). Their reliability has promoted an increase in investigations of the distribution of olive tree viruses by surveys in different geographical areas (Albanese *et al*., 2003; Bertolini *et al*., 2001a,b; Faggioli *et al*., 2002; Saponari *et al*., 2002a).

Among molecular methods, RT-PCR has proved to be the most rapid, sensitive and reliable technique for detecting an RNA target in infected plants (Hadidi and Yang, 1990; Hadidi and Candresse, 2001). Thus, the use of PCR technology is an important step in work to optimize and speed up olive tree virus diagnosis. In this work, we applied a one-step RT-PCR protocol to detect the eight most common olive tree viruses that belong to five genera: *Cucumovirus*, *Cucumber mosaic virus* (CMV) (Savino and Gallitelli, 1983); *Sadwavirus*, SLRSV (formerly a tentative nepovirus); *Nepovirus*, *Arabis mosaic virus* (ArMV) (Savino *et al*., 1979), 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 one family: *Closteroviridae*, OLYaV (Savino *et al*., 1996).

The method has been assessed for the detection of the eight above mentioned viruses in samples collected from olive fields in regions of Italy in which both national and local cultivars of olive trees are grown.

MATERIALS AND METHODS

Source of material. Shoots from 345 symptomatic or asymptomatic olive trees were collected from nine Italian regions (Abruzzo, Calabria, Campania, Friuli Venezia Giulia, Latium, Marche, Sicily, Tuscany and Umbria). Olive shoot samples were of 141 cultivars and 6 selections. RNA extracted either from naturally infected olive trees or inoculated herbaceous indicator plants was used for positive controls. Specifically, olive shoots naturally infected with CLRV, OLV-2 or OLYaV, were kindly supplied by Prof. V. Savino (University of Bari, Italy); an isolate of OLV-1 from *Nicotiana benthamiana* Domin. was kindly supplied by Dr. S. Kanematsu (NARCT, Morioka, Japan); OLRSV, ArMV, SLRSV and CMV controls were obtained from the virus collection of Istituto Sperimentale per la Patologia Vegetale (ISPaVe, Rome, Italy). All tested samples are listed in Table 1.

Design of primers. Specific primers for ArMV, OLRSV, OLV-1 and OLYaV were designed by computer analysis with PILEUP, FASTA and PRIME programs (Wisconsin Package Version 10.0; Genetic Computer Group 'GCG', Madison, WI, USA), using sequence data available in EMBL and Genbank databases. Each primer pair can be used to amplify a specific fragment of the pertinent virus coat protein gene (Table 2). To verify that the obtained amplified products were from the expected viruses, the sequences of amplicons from the positive controls were compared with those of ArMV, OLRSV, OLV-1 and OLYaV retrieved from the database. CLRV, CMV, OLV-2 and SLRSV were detected using primer pairs designed in previous work (Table 2).

Viral target RNA preparation and one-step RT-PCR amplification. Phloem tissue, scraped from ten shoots (two-year old) per tree collected in spring and/or autumn of 2001-2003, was powdered in liquid nitrogen. Then, 0.1 g of each sample was used for total RNA extraction using the RNeasy Plant Mini Kit, according to the manufacturer's protocol (Qiagen GmbH, Hilden, Germany). RNA was finally eluted with 50 µl of RNase-free water.

A one-step protocol (Faggioli *et al*., 2002) was used for the reverse-transcription and amplification of target RNA. In particular, 1.2 µl of target RNA solution, ten-fold diluted, was added to 28.8 µl of the reaction mixture containing 10 mM Tris-HCl, pH 9.0; 50 mM KCl; 0.1% Triton X-100 (w/v); 1.5 mM $MgCl_2$, 125 µM each dNTPs, 0.2 µM specific sense and antisense primers (Table 2); 2.5 units of avian myeloblastosis virus (AMV)-RT (Promega, Madison, WI, USA); 20 units of RNase Out (Invitrogen Corporation, Paisley, Scotland, UK); 1.5 units of Taq polymerase (Promega, Madison, WI, USA). Synthesis of cDNA was performed at 46°C for 30 min, followed by denaturation at 95°C for 3 min.

Amplification was carried out for 35 cycles under the following conditions: denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, extension at 72°C for 45 sec, followed by a final extension for 7 min at 72°C.

The OLYaV detection protocol was modified as follows: target RNA solution was not diluted before its addition to the reaction mixture; the annealing and extension steps were performed at 50°C for 30 sec and 72°C for 1 min respectively.

Healthy, positive and water controls were used for all tests.

Analysis of amplified products. Amplified products were analyzed by electrophoresis in 1.5% agarose gel and stained with ethidium bromide.

RESULTS

By using the one-step RT-PCR analysis, virus infected olive trees were found in all investigated regions except

Friuli Venezia Giulia and Tuscany. The average percentage of virus infection was 32.8%. Among the eight analyzed viruses, only OLYaV, SLRSV, and CLRV were detected in the investigated olive trees. The average incidence of infection by each of these viruses was 20.9% for OLYaV, 7.8% for SLRSV, and 4.9% for CLRV (Fig. 1).

Table 1. List of the analyzed olive samples and results obtained by one-step RT-PCR assay for the detection of ArMV, CLRV, CMV, OLRSV, OLV-1, OLV-2, OLYaV, and SLRSV.

Geographical origin (from north to south)	Cultivar or selection [a]	Detected viruses and No. of infected trees	No. of tested trees	Infection %
Friuli-Venezia Giulia	Bianchera (3), Campeglio (1), Fiaschetti (1), Gorgazzo (1), Medeazza (2), Plominka (1), Rocca Bernarda (2), San Rocco (2), Simiaka (1)	None-0	14	0.0
Tuscany	Apollo (1), Argo (1), Diana (1), Frantoio (2), Leccino (10), Maurino (2), Minerva (1), Moraiolo (2), Pendolino (2), Toscanina (1), Urano (3), Zeus (1)	None-0	27	0.0
Marche	Ascolana dura (1), Ascolana tenera (1), Canino (1), Capolga (1), Carboncella (1), Carmelitana (3), Cornetta (1), Coroncina (1), Dritta (1), **Frantoio (4/7)**, Lea (1), Leccio dal corno (1), Maurino (1), Mignola (2), Mignolone (1), Nebbia (1), Orbetana (1), Piantone Falerone (1), Piantone Mogliano (1), Raggia (1), **Raggiola (8/8)**, Rosciola Colli Esini (1), Sargano di Fermo (1), Sargano di San Benedetto (1)	SLRSV-12	40	30.0
Umbria	Selection ANT (1), selection ANT 15A (1), **unknown (1/9)**, **Ascolana tenera (1)**, Borgiona (3), selection CMS (1), selection CMS 16/A (1), **Corniolo (7/8)**, Dolce Agogia (1), **Frantoio (1/10)**, Leccino (13), **Moraiolo (1/10)**, **Nostrale 4/A (1)**, Pocciolo (3), Raia1 (1), **San Felice (2/16)**, Tendellone (1), Vocio (1)	OLYaV-1 SLRSV-11 SLRSV+ CLRV-1	82	15.9
Latium	**Selection BTL (1/3)**, selection PRT (3), **unknown (1)**, Blasi (1), Borgiona (1), **Canino (1)**, Canino mutato (1), **Capena (1)**, Carboncella di Palombara (1), Carboncella Pianacce (1), Itrana Passamacera (1), Itrana Raino II (1), Itrana Valle Quercia (1), Leccino (2), Marina (1), Moraiolo (1), Nostrale (1), Nostrale di Fiano (1), Pocciolo (1), Reale (1), Rosciola Col Todino (1), Salviana (1), Valanella (1)	CLRV-1 SLRSV-3	28	14.3
Abruzzo	**Gentile di Chieti (3/3)**, **Intosso (3/3)**, **Rustica (3/3)**, **Toccolana (3/3)**	CLRV-12	12	100.0
Campania	Carpellese (1), Ogliarola (1), Ortice (1), Ravece (2), **Rotondella (1)**	OLYaV-1	6	16.7
Calabria	Borgese (1), Brandofino (1), Calatina (1), **Carolea (4/17)**, **Cassanese (1)**, Ciciarello (1), Geracese (1), Mafra (1), **Moresca (1)**, Nera di Cantinelle (1), Nocellara del Belice (1), Nocellara etnea (1), Nocellara messinese (1), Nostrana (1), **Ogliarola messinese (1)**, Olivo di Mandanici (1), **Ottobratica (1)**, **Rossanese (1)**, Sinopolese (1), Tonda di Filadelfia (1), Tonda di Filogaso (1), Tonda iblea (1), Tondina (1), **Zaituna (1)**	OLYaV-10	40	25.0
Sicily	**Abunara (1)**, **Aitana (1)**, **Baddara (1)**, **Biancolilla (2/3)**, **Biancolilla Caltabellotta (3/3)**, Biancolilla napoletana (1), Biancolilla siracusana (1), Biancolilla Pantelleria (1), **Biancolilla di Marco (1)**, **Bottone di Gallo (1)**, **Brandofino (1/2)**, **Calatina (1)**, Carbucia (1), **Carolea (2/6)**, **Castricianella rapparina (1)**, **Castriciana (1)**, **Cavalieri (1/2)**, **Cerasola (1)**, **Crastu (1)**, **Erbano (1)**, **Galatina (1)**, Giarfara (1), **Giarraffa (2/4)**, **Lumiaro (1)**, Mantonica (1), **Manzanilla (1)**, Marfia (1), **Marmorina (1)**, Minuta (2), **Moresca (4/4)**, **Morghetana (2/2)**, **Morghigna (1)**, Nasitana frutto grosso (1), **Nerba (1/2)**, **Nocellara del Belice (1/3)**, **Nocellara Etnea (8/8)**, **Nocellara etnea ovale (1)**, Nocellara messinese (1), **Oglialora messinese (3/3)**, **Olivo di castiglione (1/2)**, **Olivo di Mandanici (2/4)**, Palermitana (1), Passulunara (1), Piricuddara (1), **Pirunara (1)**, **S. Benedetto (2/2)**, **Sant'Agatese (1/2)**, T**onda iblea (2/3)**, Verdello (1), **Zaituna (7/8)**	CLRV-1 CLRV+ OLYaV-2 OLYaV-58	96	64.6
Total		113	345	32.8

[a] Cultivars or selections in bold were virus infected. Numbers in brackets indicate the number of tested trees per cultivar or selection (n) or the number of infected trees out of the total number of tested trees per cultivar or selection (n/N).

Table 2. Sequences of specific primers used for the detection of olive tree viruses.

Designation	Size of product	Primer sequence	References
SLRSV-5D	293	5'-CCCTTGGTTACTTTTACCTCCTCATTGTCC-3' sense	Faggioli *et al*., 2002
SLRSV-3D		5'-AGGCTCAAGAAAACACAC-3' antisense	
CLRV-5	416	5'-TGGCGACCGTGTAACGGCA-3' sense	Werner *et al.*, 1997
CLRV-3		5'-GTCGGAAAGATTACGTAAAAGG-3' antisense	
ArMV-5A	302	5'-TACTATAAGAAACCGCTCCC-3' sense	This work
ArMV-3A		5'-CATCAAAACTCATAACCCAC3' antisense	
OLRSV-R1	356	5'-GATTGCCAAGGAATATGCTG-3' sense	This work
OLRSV-R2		5'-CTCCCAACAAATGATTGCTG-3' antisense	
CMV-CPN5	280	5'-ACTCTTAACCACCCAACCTT-3' sense	Lumia *et al*., 2001
CMV-CPN3		5'-AACATAGCAGAGATGGCGG-3' antisense	
OLV1-HA	299	5'-ACACAGAAATCATAAGTGCC-3' sense	This work
OLV1-CA		5'-CCATAGCACCATCATACC-3' antisense	
OLV2-H	206	5'-GAAGGTGGCTCGCCTAGAG-3' sense	Bertolini *et al*., 2001a
OLV2-C		5'-GCCAGGAGTTTGAGCTTTG-3' antisense	
OLYaV-H	346	5'- ACTACTTTCGCGCAGAGACG -3' sense	This work
OLYaV-C		5'- CCCAAAGACCATTGACTGTGAC-3' antisense	

In the wide range of olive germplasm analyzed, an average of 39% (55/141), including both national and local cultivars, and 16.7% (1/6) of olive selections were found to be infected by one or two viruses (Table 1).

OLYaV was the most common virus especially in southern Italy. It was detected at a very high percentage (64.6%) in Sicily, where the majority of tested cultivars were infected, at lower percentages in Calabria (25%) and Campania (16.7%) and only in one tree of unknown cultivar in Umbria (1.2%). Two Carolea plants located in Calabria (Figg. 2A and 2B) and the unknown variety collected in Umbria showed yellowing symptoms in the foliage, associated with OLYaV; all other OLYaV-infected cultivars were symptomless.

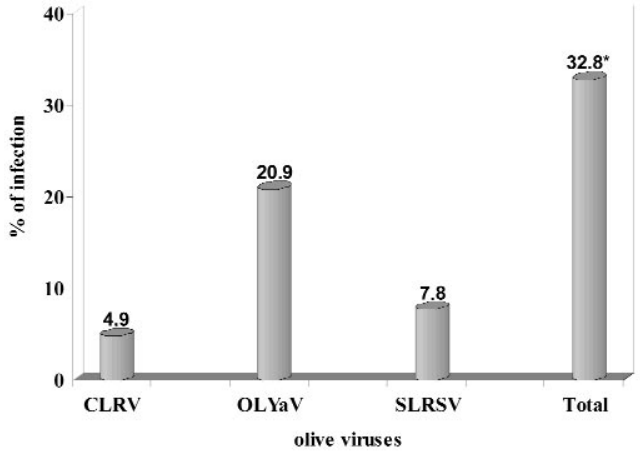

Fig. 1. Percentage of infection of the three detected viruses (CLRV, OLYaV and SLRSV) obtained in this investigation. The total percentage of infection has been obtained considering the mixed infections.

SLRSV was the main virus in central Italy where it was detected at different percentages in different regions: Latium (10.7%), Marche (30.0%) and Umbria (14.6%). Specifically, SLRSV was found in all symptomatic trees (narrow and twisted leaves, deformed fruits, bunchy growth, and reduced crop yield) of cultivars Ascolana tenera, Corniolo, San Felice, Raggiola (Fig. 2C) and Frantoio (Fig. 2D) and also in 9 symptomless samples.

CLRV was detected in a tree of 'Canino' from Latium, one of 'Corniolo' from Umbria and three plants of 'Biancolilla Caltabellotta', 'Pirunara' and 'Sant'Agatese' from Sicily and in all analyzed trees of Abruzzo varieties (Table 1); all CLRV-infected olive trees were symptomless. For the first time, mixed infections were found, of CLRV and SLRSV in a 'Corniolo' sample collected in Umbria and of CLRV and OLYaV in the 'Biancolilla Caltabellotta' and 'Pirunara' samples from Sicily (Table 1).

Even though samples were found to be infected only by OLV-1, OLV-2, OLRSV, ArMV or CMV, the PCR conditions and all primers were nonetheless tested prior to the experiments and were shown to be reliable for all the viruses involved in the survey (Fig. 3).

Sequencing of amplified products from control samples infected with ArMV, OLRSV, OLV-1 and OLYaV and their comparison with sequences retrieved from database gave very high percentage of identity (from 92 to 95%, data not shown), demonstrating that the primers designed in this work allowed the amplification of a specific coat protein gene fragment for each virus of interest. Amplification products obtained from all positive controls are shown in Fig. 3.

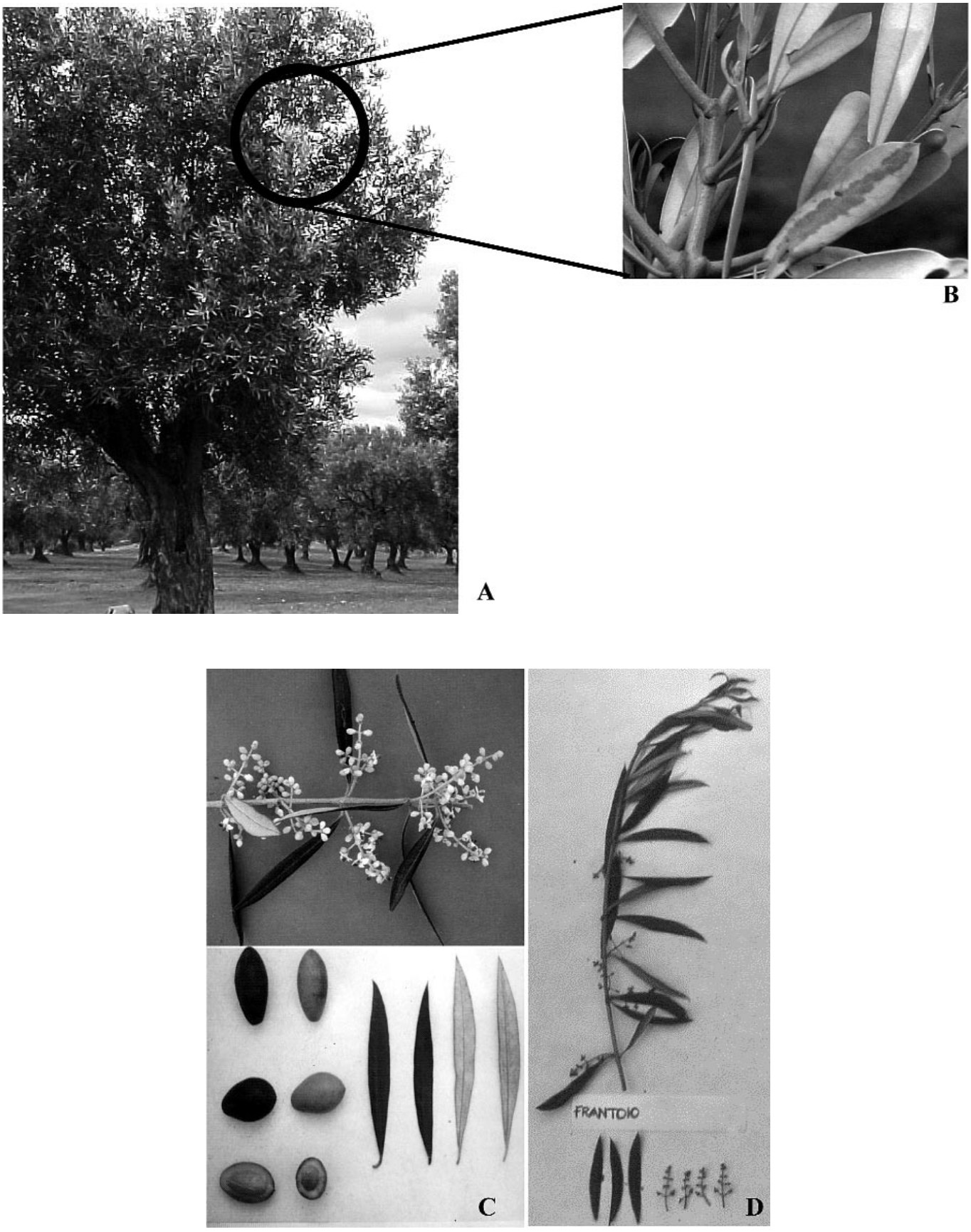

Fig. 2. Yellowing symptoms in an olive tree cv Carolea located in Calabria (**A**) which tested positive for OLYaV. Detail of leaves from the same tree (**B**). Shoots, leaves, fruits and flowers of olive trees cv Raggiola (**C**) and cv Frantoio (**D**) that tested positive for SLRSV showing narrow leaves and deformed fruits.

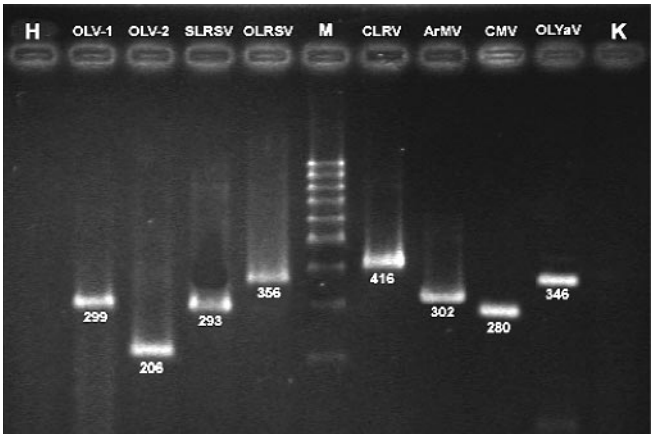

Fig. 3. Agarose gel electrophoretic analysis of amplified products obtained by one step RT-PCR using primers for the eight olive tree viruses (Table 2). Lane H, healthy olive; lane K, water control; lane M, DNA size markers (Gene Ruler™ 100 bpDNA Ladder, Fermentas, Vilnius, Lithuania). The number under each of the amplified product indicates the size (bp) of the amplicon.

DISCUSSION

Our results showed that one step RT-PCR analysis was simple and fast; it allowed testing of hundreds of samples to be done in a relatively short time. These 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.*, 2001a; Ragozzino *et al*., 2004).

The one-step RT-PCR protocol confirmed, for all tested olive tree viruses, its rapidity and reliability. RNA extraction of 24 samples required only 30 min; the preparation of reaction mixtures, enzymatic transcription and amplification in a single tube and visualization of results for 48 extracts took only 5 h per virus.

The detection of viruses in olive trees was very reliable, even though the percentage of infected trees was low. Infection rates with CLRV and SLRSV, were 8.6% (14/162) or 16.7%, (25/162) respectively in the four regions of central Italy, CLRV was also found in three samples from Sicily and the closterovirus OLYaV was found in 50% (71/142) of samples from southern Italy. In previous work, when similar investigations were done using molecular diagnostic methods, the virus infection rates were CMV (3%), CLRV (4.1%) and SLRSV (4.1%) in Spain (Bertolini *et al*., 2001b); SLRSV (15%) (Faggioli *et al.*, 2002) in central Italy, ArMV (50%), CLRV (33.3%), SLRSV (29.2%), OLYaV (41.7%), OLV-1 (8%) (Saponari *et al*., 2002a) and OLYaV (45.4%) (Albanese *et al*., 2003) in southern Italy, agreeing in part with our results. The high percentage of OLYaV infections has been confirmed by Albanese *et al.* (2003) and Saponari *et al.* (2002a); the percentage infection by CLRV was almost the same as that reported by Bertolini *et al.* (2001b) in Spain, whereas there is a discrepancy between our detection of no infection with ArMV compared to the previously reported 50% infection level (Saponari *et al.*, 2002a).

The fact that the PCR conditons and new designed primers resulted reliable in preliminary experiments would eliminate any suggestions that the PCR protocol and or primers could have influenced the sensitivity and outcome of the assay as showed in Fig. 3.

In the future, to further simplify the protocol, a multiplex RT-PCR could be assessed to make easier and faster the detection of the olive viruses considered.

A better understanding of the distribution of olive tree viruses will be possible only by increasing our knowledge of their epidemiology. Nematodes are the putative vectors of olive nepoviruses. The possibility of pollen- and seed-transmission for CLRV and OLV-1 has also been discussed (Saponari *et al*., 2002b). The field transmission of these viruses, however, appears unlikely because of the low incidence of infections and the occasional and erratic detection of some of these viruses in one or a few infected trees. The high percentage of OLYaV infection in southern Italy could be explained by possible transmission by olive psylla *Euphyllura olivina* (Costa) and unidentified mealybugs of genus *Pseudococcus* (Sabanazdovic *et al*., 1999). However, as with other woody crops, infected propagative material and the use of different methods of propagation (self-rooting, grafting) might well be the main means of virus dissemination.

In the light of the increasing international demand for olive propagative material, the European Union specifications on quality (CAC) and the high qualitative standards required by the Italian voluntary certification scheme, more controls of the sanitary status in the nursery for olive tree productions are necessary. Moreover, in order to improve olive growing in DOP and marginal areas, sanitary controls are also of potential value for local cultivars.

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