Plant Viruses ©2007 Global Science Books

Biological and Molecular Characterization of *Olive latent virus 1*

Maria do Rosário Félix[1,3*] • Joana M. S. Cardoso[2,3] • Solange Oliveira[2,3] • Maria Ivone E. Clara[1,3]

[1] Departamento de Sanidade Animal e Vegetal, Universidade de Évora, Apartado 94, 7002-554 Évora, Portugal
[2] Departamento de Biologia, Universidade de Évora, Apartado 94, 7002-554 Évora, Portugal
[3] ICAM, Instituto das Ciências Agrárias Mediterrâneas, Universidade de Évora, Apartado 94, 7002-554 Évora, Portugal

Corresponding author: * **mrff@uevora.pt**

ABSTRACT

Olive latent virus 1 (OLV-1) belongs to the *Necrovirus* genus, *Tombusviridae* family and is pathogenic to olive, citrus and tulip plants. It is easily mechanically transmissible to indicator plants causing necrotic lesions and can be transmitted through the soil into the plant roots in the absence of biological vectors. Infected cells contain virus aggregates, inclusions made up of excess of viral coded peptides and extensive vesiculation in the cytoplasm. The virions are isometric with *ca*. 30 nm, possess a monopartite single-stranded positive-sense RNA genome sized 3700 nt with 5 open reading frames (ORFs) and small inter cistronic regions. ORF 1 encodes a polypeptide with a molecular weight of 23 kDa and the read through of its amber stop codon results in ORF 1 RT that encodes the virus RNA dependent RNA polymerase with 82 kDa. ORF2 and ORF3 encode two small peptides, with 8 kDa and 6 kDa, respectively, which appear to be involved in the virus cell-to-cell movement. ORF 4 is located in the 3′-terminal and encodes a protein with 30 kDa identified as the viral coat protein. The complete genomic sequences of two well characterized OLV-1 isolates (obtained from citrus and olive) are similar, revealing an overall nucleotide sequence identity of 95%. The electrophoretic profile of the dsRNAs recovered from infected tissues exhibits three major species with *ca*. 3.7, 1.5, and 1.3 kbp. Application of molecular techniques based on PCR and on dot blot hybridization has been successfully used for routine diagnosis of OLV-1 infections.

Keywords: OLV-1, olive, molecular characterization, necrovirus, virus diagnosis
Abbreviations: bp, base pair; **CP**, coat protein; **dsRNA**, double stranded RNA; **ELISA**, Enzyme linked immunosorbent assay; **kb**, kilo base; **nt**, nucleotide; **OMMV**, Olive mild mosaic virus; **ORF**, open reading frame; **PCR**, polymerase chain reaction; **RdRp**, RNA dependent RNA polymerase; **RT-PCR**, reverse transcription polymerase chain reaction; **ssRNA**, single stranded RNA; **TNV-A**, *Tobacco necrosis virus A*; **TNV-D**, *Tobacco necrosis virus D*

CONTENTS

INTRODUCTION

Olive latent virus 1 has an apparently restricted natural host range. It was first detected in olive (*Olea europaea* L.) trees growing in Southern Italy that showed either no symptoms or occasional fasciation and bifurcation of stems (Gallitelli and Savino 1985). The virus was recovered by means of mechanical inoculation of olive flower extracts onto herbaceous indicator plants where it caused, mainly, local lesions (**Table 1**). Following virus purification and characterization it was revealed that it had physico-chemical properties similar to those of necroviruses but differed serologically from them. Thus, it was proposed as a new species of the *Necrovirus* genus (Gallitelli and Savino 1985; Martelli *et al*. 1996). In 1995, similar isolates were found in olive in Jordan, in Portugal (Félix and Clara 2002) and successively in other countries of the Middle East, being likely that the virus is widely distributed wherever the crop is grown (**Table 1**). However, its economic importance is yet to be ascertained.

In addition to olive, in Turkey OLV-1 was detected in several citrus species where it appeared symptom less, as well as, in many plants affected by the 'chlorotic dwarf disease', that is characterized by leaf malformation, chlorotic flecking in the interveinal areas and oak leaf patterns (Çinar *et al*. 1993; Martelli *et al*. 1996). In Portugal several isolates were obtained from Galega vulgar, Cordovil de Serpa and Verdeal Alentejana cultivars showing low vigor, leaf chlorosis (**Fig. 1A**) and, occasionally, no symptoms. Recently, a virus recovered from tulips, in Japan, exhibiting mottling

Received: 5 July, 2007. Accepted: 12 October, 2007.

Invited Review

Table 1 Host range, symptoms and world distribution of OLV-1.

Natural hosts		Experimental hosts		Geographical distribution (first report)
Plant	**Associated symptoms**	**Plant species**	**Induced symptoms**	
Olive (several cultivars)	Tree low vigour, stem fasciation and bifurcation, leaf chlorosis; no symptoms	*Nicotiana benthamiana*	Leaf local necrosis and systemic mosaic or necrotic local lesions only	Italy (Gallitelli and Savino 1985) Jordan (Martelli *et al*. 1995) Turkey (Martelli *et al.* 1996)
Citrus (lemon, lime, tangelo, grapefruit and orange)	Leaf deformation and chlorotic flecking; no symptoms	*Celosia cristata*, *Chenopodium amaranticolor*, *Ch. quinoa*, *Cucumis sativus*, *Cucurbita pepo*, *Datura stramonium*, *Gomphrena globosa*, *Momordica balsamina*, *Nicotiana*	Local lesions	Portugal (Félix and Clara 2002) Japan (Kanematsu *et al.* 2001) Lebanon (Fadel *et al.* 2005) Syria (Al Abdullah *et al.* 2005)
Tulip	Leaf mottling and yellow streaking	*cavicola*, *N. clevelandii*, *N. glutinosa*, *N. megalosiphon*, *N. occidentalis*, *N. rotundifolia*, *N. rustica, N. tabacum*, *Ocymum basilicum*, *Petunia hybrida*, *Phaseolus aureus*, *Ph. vulgaris* and *Vigna unguiculata*		

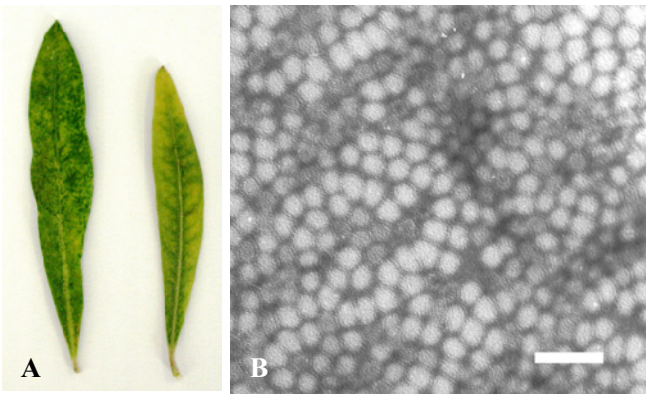


Fig. 1 Symptoms exhibited by leaves of an OLV-1 infected olive tree showing mild to intense chlorosis (**A**), electron micrograph of a purified preparation of OLV-1 (GM6 olive isolate) negatively stained in 2% aqueous uranyl acetate, Bar = 100 nm (**B**).

and yellow streak leaf symptoms (Kanematsu *et al*. 2001) was identified as an OLV-1 isolate (Pare P) based on a notably high homology of an amplified viral coat protein (CP) genomic sequence with that of the citrus isolate, and on the lack of serological relationship with other necroviruses.

Since year 2000, OLV-1 is recognized by the International Committee on Taxonomy of Viruses (van Regenmortel *et al*. 2000), as a distinct and definitive species within the *Necrovirus* genus of the *Tombusviridae* family.

Here, an up-to-date overview is provided focusing on OLV-1 properties, cytology, molecular genome organization and expression strategies in comparison with that of other members of *Tombusviridae*, using the isolates from citrus (Grieco *et al*. 1996) and from the olive cultivar 'Galega vulgar', in Portugal, (GM6) (Félix *et al*. 2005b) as models, since they are the only ones studied in sufficient detail.

VIRUS PROPERTIES

Biological and physico-chemical properties

OLV-1 can infect a range of herbaceous hosts (**Table 1**) where it causes local lesions ranging from chlorotic to necrotic or reddish, as in *Gomphrena globosa*, except in *N. benthamiana*, where it is reported to also induce systemic mosaic (Gallitelli and Savino 1985; Merciega *et al*. 1996). In our work with the Portuguese olive isolate, GM6, systemic leaf mosaic seldom occurs but preliminary evidence shows that mixed inoculation with Olive mild mosaic virus (OMMV) results in systemic invasion of *N. benthamiana* by both necroviruses (Félix *et al*. 2006b). OLV-1 inoculation of young tobaccos at 4 to 6 leaf stage, employing medium-high inocula, leads to a complete necrosis with the plant dying prematurely within a few days.

The virus is easily purified by means of butanol and chloroform clarification of infected plant extracts, followed by polyethylene glycol precipitation and recovery through differential centrifugation cycles. Partial purified virus suspensions, when ultracentrifuged in sucrose density gradients, sediment as a single component, with *ca*. 111 S, which is infectious (Gallitelli and Savino 1985). Transmission electron microscopy of uranyl acetate treated purified preparations shows a homogeneous population of isometric particles with a 30 nm diameter and an outline that appears rounded to slightly angular depending on whether the particles are free or closely packed (**Fig. 1B**). Similar size and morphology are also shared by the necrovirus TNV-A, TNV-D (Merciega *et al*. 1996) and OMMV (Cardoso *et al*. 2004).

Gel electrophoresis analysis of the viral capsid dissociated in the presence of SDS and mercaptoethanol shows it to be made of a major polypeptide with an apparent molecular mass of 32 kDa. The virus is moderately immunogenic. Serological analysis by Ouchterlony's gel double diffusion, using polyclonal antibodies raised against the OLV-1 strain (olive, Italian) showed it to be unrelated to several tombusvirus tested (Gallitelli and Savino 1985) but indistinguishable from the citrus Turkish isolate and from a few others from Italian, Jordanian and Portuguese olives (Merciega *et al*. 1996; Félix and Clara 2002). These observations indicate that the virus capsid is a conserved feature among isolates present in geographically distant regions. On the other hand, ELISA tests using OLV-1 GM6 antigen did not react with OMMV specific antibodies nor with a commercial mixture of specific immunoglobulins for eleven different TNV serotypes (Loewe Phytodiagnostic, Germany), demonstrating a total lack of serological relationship with those necroviruses.

Analysis of the nucleic acid fraction extracted from OLV-1 preparations by a standard SDS-phenol treatment revealed it to consist of a single stranded RNA molecule *ca*. 1.4×10^6 Da (*ca*. 3.7 kb) infectious to *N. benthamiana*. On the other hand, dsRNA fractions, extracted from infected tissues by means of phenol, cellulose chromatography of the aqueous phase and serial elutions with appropriate buffers, were found to contain three major classes of dsRNA. Their sizes, 2.6×10^6, 1.05×10^6 and 0.94×10^6 Da, indicate that they are the replicative forms of the full length viral RNA and of two smaller subgenomic RNAs, respectively (Merciega *et al*. 1996; Pantaleo *et al*. 1999). Comparative analysis of the OLV-1 RNAs related with those of TNV-A, TNV-D (Merciega *et al*. 1996) and OMMV (Cardoso *et al*. 2004) shows them to be similar in number and size.

Cytopathology

Significant ultrastructural changes occur in cells of OLV-1

infected hosts as compared with that of non-inoculated control plants, when observed under electron microscope. Major alterations are: i) presence of electron dense rounded particles, sized about 22 nm in diameter, interpreted as virions. They occur in the cytoplasm of mesophyll parenchyma cells and in conducting tissues, either scattered or in ordered paracrystaline arrays, and often in association with filaments of proteinaceous nature; ii) extensive vesiculation of the cell membrane system, from which numerous vesicles appear to derive, with variable sizes up to 100 nm in diameter. These can be observed within the nuclear envelope likely originated from the inner and/or the outer membrane of the nucleus, freely scattered in the cytoplasm, aggregated near the dyctiosomes, and inside the vacuole appearing to protrude from the tonoplast. The most part of such abundant vesicular structures contain a fine network of fibrils, usually interpreted as nucleic acid folded strands; iii) cytoplasmic inclusions made up of parallel filaments with a criss-cross pattern or helical structure, often mingled with virus-like particles. Occasionally, these filaments were also seen inside the cell nucleous; iv) small electron dense amorphous inclusions scattered throughout the cytoplasm; v) anomalous thickening of the cell wall due to deposition of electron lucent callose-like material between that structure and the cell plasma membrane (Castellano *et al*. 1987).

Generally, the described cell alterations are seen in both locally and systemically infected *N. benthamiana* as well as in *C. quinoa*. For the most part, the role of the newly induced structures in the infection process or in cell defence, if any, is not known. However, recent immunocytochemical studies using gold labelled antibodies against an *Escherichia coli* expressed movement protein of the virus, designated p8, revealed their association (identification) with the fibrous cytoplasmic inclusions made up of the thin filaments above mentioned. Thus, it is suggested that these inclusions may represent a form of accumulation of the OLV-1 encoded p8 (Castellano *et al*. 2005). Further application of the gold immunolabeling technology permitted the identification of the electron dense amorphous inclusions as excess of viral CP accumulating in the cell cytoplasm (Pantaleo *et al*. 2006).

VIRAL DISSEMINATION

It is generally accepted that olive viruses are disseminated through infected vegetative material, that is, rooted stem cuttings used for plant propagation. Recent studies have shown that OLV-1 is detectable by sensitive RT-PCR analysis in whole olive flowers (Lobão *et al*. 2002), pollen (Saponari *et al*. 2002), fruit pulp (Félix, unpublished) as well as in a high percentage (over 80%) of seedlings originated from seeds of an infected olive tree (Saponari *et al*. 2002), demonstrating the virus seed transmission. Epidemiologically these data are relevant as they indicate that OLV-1 may be spreading among field olive plants by means of ovule fertilization with infected pollen and by means of grafting olive cultivars that are 'recalcitrant' to rooting onto seedlings originated from infected seeds, a practice in use in some olive growing regions.

Laboratory experiments using *N. benthamiana* as plant model showed that leaf inoculation with OLV-1 (the Portuguese GM6 isolate) results in local lesions, as expected, and in downwards symptom less invasion of plant roots followed by virus release into surrounding soil. The virus is then capable of invading healthy roots of new *N. benthamiana* plants grown in that soil or in a liquid substrate containing the virus (Félix *et al*. 2006b), in the absence of any biological vector. However, in such cases, OLV-1 infection is restricted to the root system, rarely extending to the tobacco lower leaf, as clearly shown by ELISA and RT-PCR (Félix *et al*. 2004). It is likely that this mode of dissemination also occurs in olive fields and especially in nurseries, where thousands of stem cuttings are closely packed in a rooting substrate, under a high moisture environment, for *ca*. two months before transplantation to individual containers. Plant root proximity and moisture flow are conditions highly conducive to virus spread.

So far there are no indications that biological vectors, other than plant parts, are involved in OLV-1 transmission. Thus, effective avoidance of virus spreading relies mainly upon the use of virus free vegetative material and of non contaminated plant growth substrates in the nurseries.

GENOME ORGANIZATION AND EXPRESSION

The OLV-1 genome consists of a single molecule of ssRNA of messenger sense with *ca*. 3.7 kb. The genome of two isolates, citrus and GM6 olive, have been fully sequenced and characterized (Grieco *et al*. 1996; Félix *et al*. 2005b). It is not capped at the 5′ terminus nor polyadenylated at the 3′ terminus.

The viral RNA contains five open reading frames (ORF) and the strategies employed for genome expression includes stop codon suppression, overlapping of reading frames and subgenomic messenger RNAs. In the olive GM6 isolate ORF 1, which follows a non coding leader sequence of 60 nt, begins at the first AUG codon in position 61 and extends to the UAG stop codon in position 666, encoding a 23 kDa (p23) peptide. The read through of that leaky amber termination codon generates an 82 kDa (p82) product encoded by ORF 1 RT completely overlapping that of ORF 1. Located in the central region of the virus genome ORF 2 and ORF 3 encode two small peptides with 8 kDa (p8) and 6 kDa (p6), respectively, whereas ORF 4 occupies the 3′ end of the genome and codes for a 30 kDa protein. The schematic representation of the viral RNA organization is shown in **Fig. 2** and the relative position of the five ORFs and other genomic features of the two OLV-1 isolates are compared in **Table 2**.

The read through portion of the 82 kDa peptide contains the GDD (glycine-aspartic acid-aspartic acid) motif and other typical polymerase sequences suggesting that p82 is the virus RdRp (Poch *et al*. 1989). Both p23 and p82 are expressed directly from the virus genome and are indispensable for its replication, as inferred from site directed mutagenesis (Pantaleo *et al*. 1999). The p8 and p6 products, expressed from a bicistronic subgenomic RNA (sgRNA1) 1519 nt long, are involved in facilitating the virus cell-to-cell movement. Additionally, p6 contains a predicted transmembrane motif that may have a membrane docking function (Castellano *et al*. 2005). The p30 is the viral CP which is translated from a 1237 nt sgRNA2. Besides its role in the capsid formation p30 appears to assist the citrus isolate in its systemic spread in infected *N. benthamiana* plants.

In the CP 'shell' domain of three OLV-1 isolates (citrus, GM6 olive and tulip) the 'S' signature consensus pattern, consisting of 26 amino acids and conserved among small icosahedral viruses is easily recognized (Falquet *et al*. 2002). It was found that in all those isolates the amino acid leucine occupies the 17th position in the signature sequence, contrary to that exhibited by other necrovirus species. Thus the sequence is now proposed to be enunciated as follows: [FYW]-x-[PSTA]-x(7)-G-x-[LIVM]-x-[LIVM]-x-[FYWI**L**]-x(2)-D-x(5)-P (Félix *et al*. 2005a).

Single strand conformation polymorphism (SSCP) analysis of a *ca*. 750 nt sequence comprising about 90% of the CP gene, which was generated through the application of specific RT-PCR (**Fig. 3A**) (see Diagnosis) to a set of 14 olive OLV-1 isolates, showed 5 distinct electrophoretic profiles consisting of 1, 2 or 3 bands corresponding to different conformations of the amplicon molecules, four of which are shown in **Fig. 3B** (Félix *et al*. 2006a). SSCP analysis is considered adequate to reveal genetic diversity as it detects a single nucleotide change in DNA fragments sized up to *ca*. 700 nt (Rubio *et al*. 1996). The variability observed within that amplified sequence suggests the occurrence of mixed infections, in some cases. That finding is not surprising given the century long life span of the olive host that allows plenty of opportunity for mutation and genetic exchange to take place within the virus natural population andaccumu-

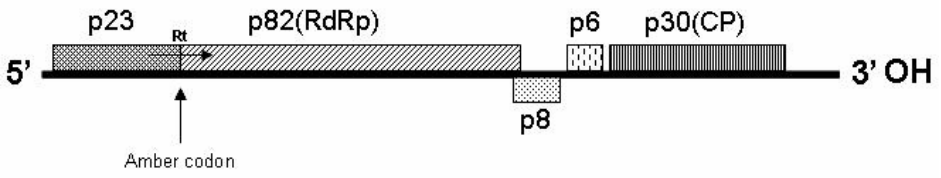


Fig. 2 Schematic representation of the genomic organization of OLV-1. The thick line represent the (+) strand RNA genome. The boxes represent the ORFs encoding proteins p23, p82, p8, p6 and p30 kDa. Rt – read through, RdRp – RNA dependent RNA polymerase, CP – coat protein.

Table 2 Comparative genome organization features of OLV-1 isolates.

Properties	GM6 (olive) isolate	Citrus isolate
Genome full length	3702	3699
5′ NCR[1] base composition (60 nt)	36.7% A, 23.3% C, 18.3% G, 21.7% U	35% A, 20% C, 21.6% G, 23.4% U
ORF 1	61 to 666 nt	61 to 666 nt
ORF 1 RT	666 to 2232 nt	666 to 2232 nt
ORF 2	2219 to 2437 nt	2216 to 2443 nt
ORF 3	2443 to 2610 nt	2443 to 2613 nt
ORF 4	2635 to 3444 nt	2635 to3447 nt
ICR[1] (ORF 2 – ORF 3)	5 nt	2 nt
ICR (ORF 3 – ORF 4)	24 nt	21 nt
Size overlap ORF 1 RT – ORF 2	14 nt	17 nt
3′ NCR	258 nt	253 nt

[1] ICR – Inter cistronic region; NCR – Non coding region

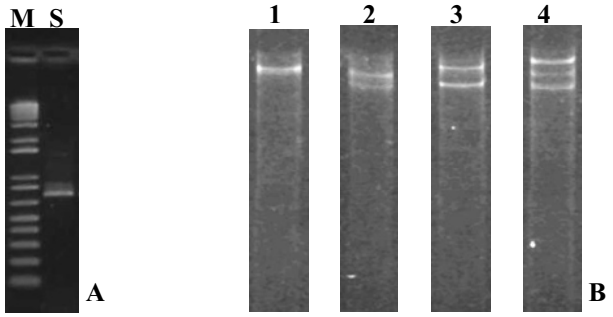


Fig. 3 Agarose gel electrophoretic analysis of the 750 nt amplicon obtained by RT-PCR applied to olive stem tissues using OLV-1 specific primers. M - 1kb Plus DNA Ladder (Invitrogen), S – OLV-1 infected sample (**A**). Electrophoretic SSCP profiles of the 750 nt amplicon obtained by RT-PCR applied to several naturally OLV-1 infected Portuguese olive cultivars. Numbers above the lanes indicate four different SSCP patterns (**B**).

lation of new variants within the tree. Concerning the above 14 isolates no correlation was found between the SSCP electrophoretic pattern observed and neither the host location nor the type of olive cultivar from which the viral isolates were derived (Félix *et al*. 2006a). Interestingly, the SSCP electrophoretic profile corresponding to the GM6 isolate showed only one band indicating, on one hand, that its viral population is homogeneous in the part of the genome analysed and, on the other hand, that different (complementary) sequences can exhibit stable conformations with identical mobility in the gel.

PHYLOGENETIC ANALYSIS

Multiple alignment and phylogenetic analysis of the RdRp sequences from OLV-1 isolates revealed significantly greater sequence similarity with that of OMMV and TNV-A isolates than with that of TNV-D isolates and other *Tombusviridae* analysed, indicating that the RdRps of necrovirus form two distinct clusters (**Fig. 4A** and **Table 3**). On the other hand, similar analysis of CP sequences from the same viruses showed a very high sequence similarity among the three OLV-1 isolates, in contrast with that of other necrovirus, namely OMMV, although the necrovirus CPs appear as a single cluster (**Fig. 4B** and **Table 3**). Concerning the OLV-1 GM6 isolate small proteins, p6 and p8, it is remarkable the extensive sequence similarity with those of other OLV-1 isolates, of OMMV and of TNV-A isolates, as opposed to that of other necro- and carmovirus analysed (**Fig. 5** and **Table 4**).

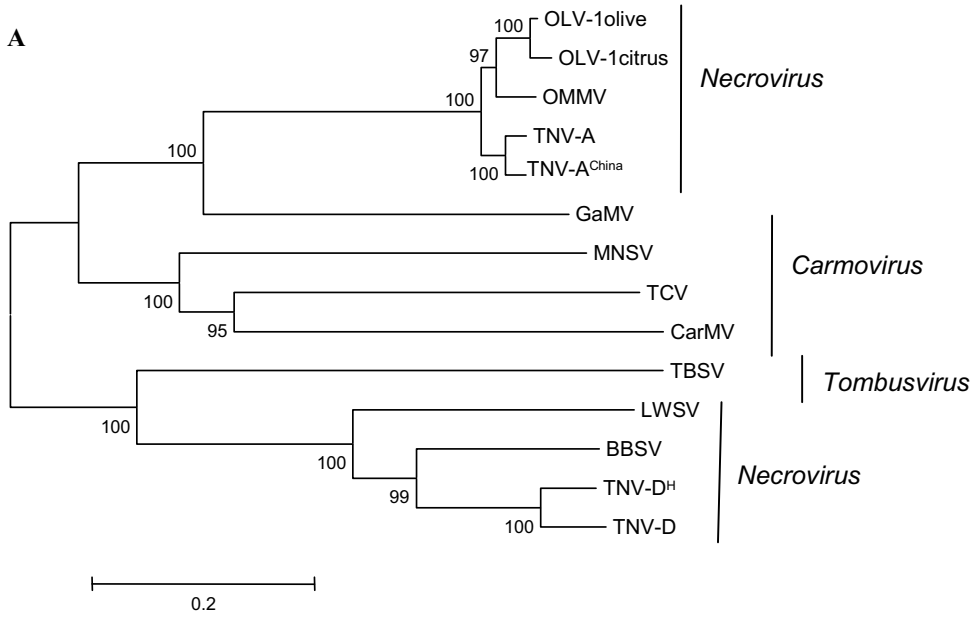
In this respect it is interesting to note the case of OMMV that originated from RNA recombination events, probably via a mechanism of replicase-driven template switching mode, where TNV-D RNA function as 'donor' and OLV-1 RNA as 'acceptor' template. OMMV shares high homology in about two thirds (RdRp, p6 and p8) and one third (CP), with OLV-1 and TNV-D, respectively (Cardoso *et al*. 2005). OMMV was originally recovered from an olive tree of the cultivar 'Galega vulgar', in Portugal, and initially identified as a TNV-D isolate (designated GP) based on ELISA reactions and on the nucleotide sequence of the CP gene (Félix and Clara 2002; Cardoso *et al*. 2004; Louro 2004), but was later recognized as a distinct necrovirus species based on the complete genome organization, sequencing and comparison with those of other small plant RNA viruses (Cardoso *et al*. 2004). More recently OMMV was found in Holland appearing to be involved in the Augusta complex disease of tulips (Pham *et al*., unpublished).

Besides OMMV, also GaMV appears to have derived from recombination occurred between a necro- and a tombusvirus (Ciuffreda *et al*. 1998), which may explain its relative position in the phylogenetic trees here presented (**Figs. 4**, **5**).

These and other data give support to the idea that the necrovirus is not a homogeneous genus and that some of its species need more research for a better understanding of its taxonomic standing.

DIAGNOSIS

The economic importance of OLV-1 is not yet clearly known. That virus infection is associated with leaf mild mosaic and low vigour of olive trees (**Fig. 1A**), chlorosis and dwarfing of citrus (Martelli *et al*. 1996), leaf mottle and yellow streaks in tulips (Kanematsu *et al*. 2001) but OLV-1 also occurs asymptomatic in these hosts (**Table 1**). The increasing international demand for olive plants and legislation enacted by the European Union, as the Directive 93/48 dated 23-06-93 *Conformitas Agraria Communitatis*, require that all propagative material of olive 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, particularly in Italy, Portugal and Spain. Reliable virus detection is also needed in epidemiological studies and in establishing strategies for virus disease control.



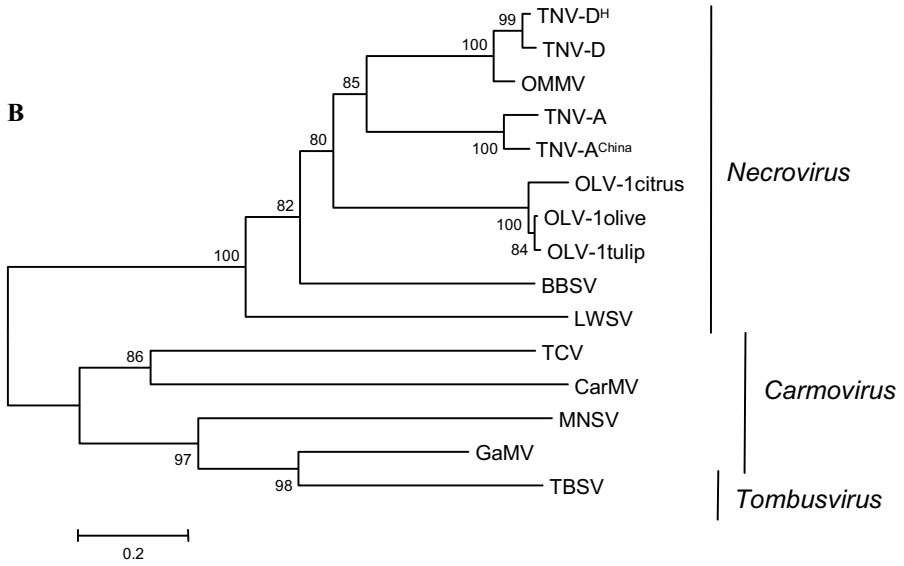


Fig. 4 Phylogenetic tree generated from multiple alignment of deduced amino acid sequence of RdRp (**A**), and of CP (**B**), encoded by OLV-1 isolates, with their respective counterparts encoded by other *Tombusviridae* members , using the Neighbour-Joining method (Saitou and Nei 1987).

Table 3 Amino acid sequence identity of RdRp and CP encoded by OLV-1 GM6 genome with their counterparts from other Tombusviridae.

Virus / isolate	Sequence identity of proteins (%)	
	RdRp	CP
OLV-1citrus	97.3	87.7
OLV-1tulip	na	98.5
OMMV	92.1	43.3
TNV-A	90.7	41.5
TNV-A^{China}	90.4	41.1
TNV-D	34.1	42.5
TNV-D^{H}	35.3	42.2
LWSV	33.3	28.2
BBSV	34.8	36.3
GaMV	51.1	13.9
MNSV	37.8	14.6
TCV	36.3	14.6
CarMV	32.0	13.4
TBSV	31.5	14.6

na- not available

NCBI of proteins accession number: OLV-1 (GM6 olive) p8 (AAZ43262) and p6 (AAZ43263); OLV-1citrus p8 (NP_043909) and p6 (NP_043910); OMMV p8 (YP_224018) and p6 (YP_224019); TNV-A p8 (NP_056826) and p6 (NP_056827); TNV-A^{Ch} – China isolate p7 (AAT69239) and p5 (AAT69240); TNV-D^{H} – Hungary isolate p7$_1$ (NP_608313), p7a (NP_608314) and p7b (NP_608315); TNV-D p7$_1$ (BAA00787), p7a (BAA00788) and p7b (BAA00789); BBSV p5 (NP_758812), p7a (NP_758813) and p7b (NP_758814); LWSV p11 (NP_044742) and p6 (NP_044743); MNSV p7a (NP_041229) and p7b (NP_041230); TCV p8 (NP_620722); GaMV p8 (NP_044734) and p7 (NP_044735).

Table 4 Amino acid sequence identity among the small peptides p8 and p6 encoded by the OLV-1 GM6 genome with their counterparts from other *Tombusviridae*.

Virus/isolate	Peptide	Identity (%)	Virus/isolate	Peptide	Identity (%)
OLV-1olive	p8	100	OLV-1olive	p6	100
OLV-1citrus	p8	93.2	OLV-1citrino	p6	100
OMMV	p8	87.6	OMMV	p6	100
TNV-A	p8	89.0	TNV-A	p6	98.2
TNV-A^{Ch}	p7	91.7	TNV-A^{Ch}	p5	98.2
TNV-D^{H}	p7$_1$	10.5	TNV-D^{H}	p7a	7.6
TNV-D^{H}	p7a	20.5	TNV-D^{H}	p7b	16.4
TNV-D	p7$_1$	6.7	TNV-D	p7a	6.1
TNV-D	p7a	20.5	TNV-D	p7b	17.9
BBSV	p5	12.3	BBSV	p7a	7.9
BBSV	p7a	23.2	BBSV	p7b	20.0
LWSV	p11	13.0	LWSV	p6	21.0
MNSV	p7a	28.7	MNSV	p7a	9.2
TCV	p8	15.7	MNSV	p7b	27.4
GaMV	p8	43.2	GaMV	p7	36.2

NCBI of proteins accession number: OLV-1 (GM6 olive) p8 (AAZ43262) and p6 (AAZ43263); OLV-1citrus p8 (NP_043909) and p6 (NP_043910); OMMV p8 (YP_224018) and p6 (YP_224019); TNV-A p8 (NP_056826) and p6 (NP_056827); TNV-A^{Ch} – China isolate p7 (AAT69239) and p5 (AAT69240); TNV-D^{H} – Hungary isolate p7$_1$ (NP_608313), p7a (NP_608314) and p7b (NP_608315); TNV-D p7$_1$ (BAA00787), p7a (BAA00788) and p7b (BAA00789); BBSV p5 (NP_758812), p7a (NP_758813) and p7b (NP_758814); LWSV p11 (NP_044742) and p6 (NP_044743); MNSV p7a (NP_041229) and p7b (NP_041230); TCV p8 (NP_620722); GaMV p8 (NP_044734) and p7 (NP_044735).

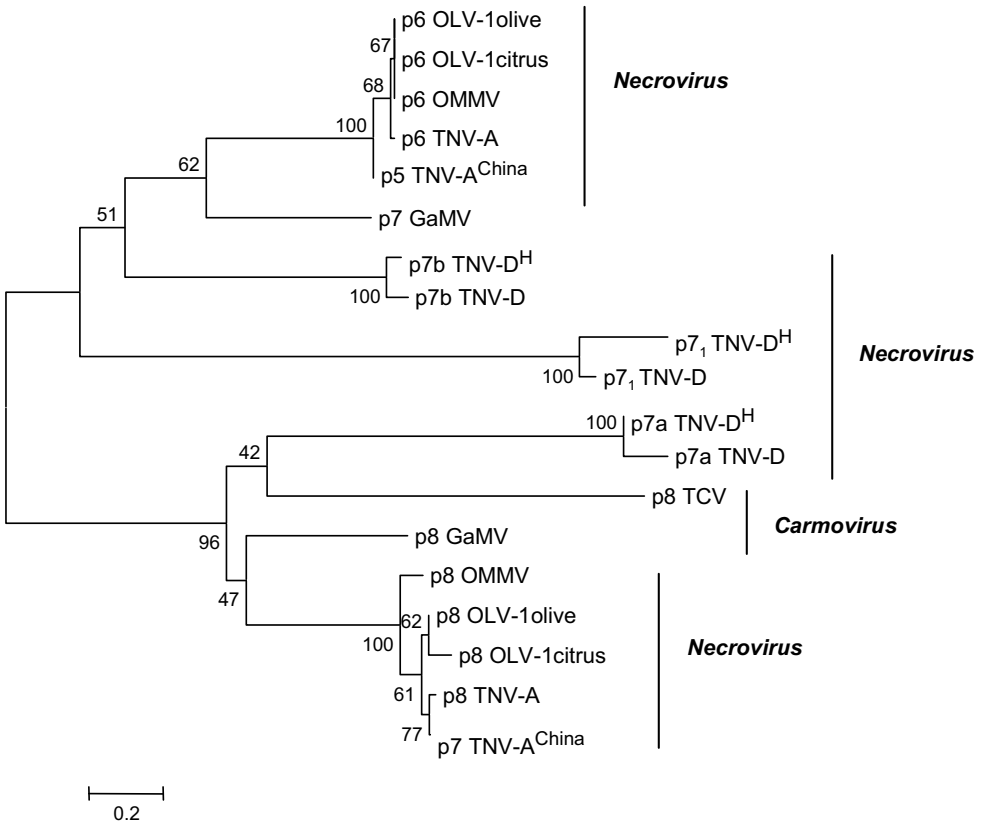


Fig. 5 Phylogenetic tree generated from multiple alignment of deduced amino acid sequences of the small peptides, p8 and p6, encoded by OLV-1 isolates, with their counterparts encoded by other *Tombusviridae* members, using the Neighbour-Joining method (Saitou and Nei 1987).

Table 5 Primers and probes used in molecular tests for diagnosis of OLV-1.

Type of test	Primer/ Probe	Sequence 5′ – 3′	Amplicon length (nt)	Amplification/ Hybridization region	Reference
RT-PCR	Fwd	CTCACCCATCGTTGTGTGG	747	3′ terminal	Martelli *et al.* 1996
	Rev	TTTCACCCCACCAAATGGC			
	Fwd	ACACAGAAATCATAAGTGCC	299	CP	Faggioli *et al.* 2005
	Rev	CCATAGCACCATCATACC			
RT-n-PCR	Fwd*	GTGGACTGCGCTCGAATGGA	230	CP	Pantaleo *et al.* 2001
	Rev*	CTCACCATCGTTGTGTGG			
	Fwd**	AATGTTACCCTGGCCACC	167	CP	
	Rev**	TGTGGTTACAAATTGAC			
Dot blot hybridization	Probe	CCCGTTGCTGGTGGAGTGATTTCTACAGAAGGGCACGTTC	--	CP	Bertolini *et al.* 2001

-- not applicable; CP, coat protein; Fwd, forward; Rev, reverse; *, **, primers used in the first and second PCR amplification reactions, respectively.

Main difficulties faced when deciding on a suitable detection technique reside on the irregular distribution of the virus within the canopy, specially in woody crops as olive and citrus, the low antigen titre and the presence of compounds i.e. phenols, polysaccharides, oils and pigments in certain tissues. In addition, all these factors vary throughout the year season interfering with the outcome of some detection tests. Thus time of sampling and type of tissue to be selected are very important in diagnosis. Concerning the olive plant it was found that scrapings of cortical tissues taken from two year stems, in the spring, are very good OLV-1 sources, although fruits and shoots collected in the autumn are also adequate (Faggioli *et al*. 2005; Varanda 2005).

Recently, dot blot hybridization and various formats of RT-PCR tests were successfully optimized for the direct detection of OLV-1, in single or in mixed infections of olive tissues.

Dot blot hybridization

In these tests the viral target is spotted onto nylon membranes and, under appropriate conditions, hybridized to OLV-1 specific riboprobes, designed complementary to a region of the virus genome (**Table 5**) and labelled with a non-radioactive marker, as digoxigenin (dig), which is later detected by chemiluminescence or by ELISA (Grieco *et al*. 2000). Of the several substrates tested, crude olive leaf extracts, total nucleic acids recovered from olive cortical tissues with the RNeasy Plant Extraction Kit (Qiagen), and denatured dsRNA preparations, the latter proved to be the only one allowing successful detection of OLV-1. The sensitivity of the dig-riboprobes used in these reactions was shown to be very high, detecting up to a 1 pg of the RNA target, revealing them very specific and reliable, with potential to be routinely used in plant sanitary selection programmes.

A similar hybridization technique is applicable to the final reaction product of RT-PCR assay (described below) specific for OLV-1. In this case, the final amplicon is denatured, spotted onto a nylon membrane and hybridized with 3′ dig-labelled probes internal to that PCR product. The label is then colourimetrically evidenced by anti-dig-alkaline phosphatase conjugated antibody, using nitroblue tetrazolium and bromochloroindolyl phosphate as the enzyme substrate (Bertollini *et al*. 2001). This procedure has a sensitivity 10 times higher than that of monospecific RT-PCR, and it works best when the viral target is the total RNA fraction recovered from infected tissues through the use of RNeasy Plant mini kit. Although more laborious this

procedure has the advantage of avoiding use of the toxic ethidium bromide employed in gel electrophoresis to reveal the presence of the viral related amplicon.

Reverse Transcription–Polymerase Chain Reaction

RT-PCR in its various formats, conventional two-step or single step, monospecific or multiplex or nested PCR (RT-n-PCR) have been successfully applied to diagnose OLV-1 in single and mixed infections aiming at the certification of propagative material. The type of substrates suitable to detect OLV-1 in olive tissues include crude plant sap, total RNA preparations obtained from cortical stem tissues or from fruits by using commercial extraction kits, and dsRNA fractions obtained from cortical tissues or fruits (Grieco *et al*. 2000; Bertollini *et al*. 2001; Varanda *et al*. 2006). Crude plant sap is not always adequate as a substrate to use in RT-n-PCR assays whereas total RNA extracted with the commercial kit is the best and easiest method to prepare the target to obtain reliable results.

Typically, a RT-PCR uses 100 mg to 1 g of tissue powdered in liquid nitrogen of which a 0.1 g sample is taken and further extracted with the RNeasy Plant mini kit to obtain total RNA preparation (Faggioli *et al*. 2005). Several primers specific to OLV-1 (**Table 5**) have been designed to amplify particular sequences of the genome and used routinely in RT-PCR tests to diagnose the virus directly in tissues from olive trees (**Fig. 5A**). In addition to high specificity, RT-PCR also exhibits high sensitivity detecting as little as 10 fg of OLV-1 RNA (Grieco *et al*. 2000).

This technique has been successfully applied in virus surveys to detect single and multiple targets. For instance, OLV-1 was the 2nd and the 5th most prevalent olive virus in Lebanon and in Syria, respectively, and at one site in each country, its presence reached 20 % of the sampled trees (Al Abdullah *et al*. 2005; Fadel *et al*. 2005). In Portugal, RT-PCR analysis of a collection of mechanically transmitted virus from naturally infected olive tissues revealed that 60% were OLV-1, which is most prevalent in the south (Félix, unpublished).

The development of a multiplex RT-PCR to detect the 3 necrovirus infectious to olive, OLV-1, TNV-D and OMMV, using either total RNA or ds RNA fractions extracted from stems or fruits, allowed the viral survey of a field collection of olive clones in the northeast of Portugal. Data obtained showed that only one tree had OLV-1 among the 35 that were necrovirus infected (Félix *et al*. 2003; Varanda *et al*. 2006). In this limited survey it was not possible to distinguish between TNV-D and OMMV infections as the primers used in the PCR hybridize within the CP domain and both viruses share extensive identity (*ca*. 77%) in that gene (Cardoso *et al*. 2005).

The RT-n-PCR technique is much more sensitive than RT-PCR but the experimental procedure presents high risks of contamination, as it involves two separate rounds of PCR reactions, rendering it difficult to be routinely used (Pantaleo *et al*. 2001). To overcome these Bertollini *et al*. (2003) adapted a device developed by Olmos *et al*. (1999) that is a microtube containing a specially adjusted plastic tip inside that allows the complete RT-n-PCR reaction to be carried out in a single closed tube. This avoids or greatly reduces the risk of outside contaminations. The application of such procedure was successful in simultaneously diagnosing four different olive viruses and its sensitivity proved to be at least 100 times higher than that of multiplex RT-PCR (Bertollini *et al*. 2003).

CONCLUDING REMARKS

OLV-1 is a typical necrovirus as shown by its biological and biochemical properties, genome organization and gene expression strategy. It infects two major socio economic crops, olive and citrus, and is present in many groves of Mediterranean and Middle East countries. The virus is often associated to olive decline and citrus dwarfism but its impact in the yield and/or quality of plant products is not known and should be investigated as it is very widespread.

OLV-1 is a plant virus exhibiting several features that contribute for its success in nature, namely its capacity to multiply in different plant genera and to remain viable in the soil, subsequently infecting healthy hosts via their root system. Lacking known fungus, nematode or arthropode vectors, OLV-1 relies for survival on the natural spreading or human trading of infected seeds, rootstocks, scions or bulbs. This emphasizes the importance of developing and use of sensitive OLV-1 diagnostic techniques to assist in plant breeding and sanitary selection programmes to prevent or greatly restrict viral dissemination, as required by European Union legislation.

An interesting feature of the OLV-1 genome is its potential ability to act as a 'parental' molecule, together with that of TNV-D, to give rise to a new recombinant virus as is the case of OMMV. The recent finding of a virus infecting tulips showing extensive genomic identity with that of OMMV reveals that this virus species is more widespread than anticipated and that additional hosts may emerge. Whether this is a result of selection pressure favouring genetic recombination between the two necrovirus species is speculative at this point but very interesting to investigate further.

ACKNOWLEDGEMENTS

The authors thank to the graduate students working in our Plant Virus Laboratory (ICAM/University of Évora) whose efforts contributed to our research in this field. The second author is recipient of a PhD fellowship from Fundação para a Ciência e a Tecnologia - FCT (SFRH/BD/16673/2004). This work was partially supported by research grants from the Portuguese Ministries of Agriculture and of Science, Technology and Higher Education.

REFERENCES

Al Abdullah A, El Beaino T, Saponari M, Hallak H, Digiaro M (2005) Preliminary evaluation of the status of olive-infecting viruses in Syria. *Bulletin OEPP/EPPO* **35**, 249-252

Bertolini E, Olmos A, Martínez MC, Gorris MT, Cambra M (2001) Single-step multiplex RT-PCR for simultaneous and clourimetric detection of six RNA viruses in olive trees. *Journal of Virological Methods* **96**, 33-41

Bertolini E, Olmos A, López MM, Cambra M (2003) Multiplex nested reverse transcription-polymerase chain reaction in a single tube for sensitive and simultaneous detection of four RNA viruses and *Pseudomonas savastanoi* pv. *savastanoi* in olive trees. *Phytopathology* **93**, 286-292

Cardoso JMS, Félix MR, Clara MIE, Oliveira S (2005) The complete genome sequence of a new necrovirus isolated from *Olea europaea* L. *Archives of Virology* **150**, 815-823

Cardoso JMS, Félix MR, Oliveira S, Clara MIE (2004) A *Tobacco necrosis virus D* isolate from *Olea europaea* L.: Viral characterization and coat protein sequence analysis. *Archives of Virology* **149**, 1129-1138

Castellano MA, Di Franco A, Martelli GP (1987) Electron microscopy of two olive viruses in host tissues. *Journal of Submicroscopic Cytology* **19**, 495-508

Castellano MA, Loconsole G, Grieco F, Di Sansebastiano GP, Martelli GP (2005) Subcellular localization and immunodetection of movement proteins of *Olive latent virus 1*. *Archives of Virology* **150**, 1369-1381

Çinar A, Kersting U, Onelge N, Kormaz S, Sas G (1993) Citrus virus and virus-like diseases in Eastern Mediterranean region of Turkyie. *Proceedings 12th Conference IOCV, Riverside 1993, USA*, pp 397-400

Ciuffreda P, Rubino L, Russo M (1998) Molecular cloning and complete nucleotide sequence of Galinsoga mosaic genomic RNA. *Archives of Virology* **143**, 173-180

Fadel C, Digiaro M, Choueiri E, El Beaino T, Saponari M, Savino V, Martelli GP (2005) On the presence and distribution of olive viruses in Lebanon. *Bulletin OEPP/EPPO* **35**, 33-36

Faggioli F, Ferretti L, Albanese G, Sciarroni R, Pasquini G, Lumia V, Barba M (2005) Distribution of olive tree viruses in Italy as revealed by one-step RT-PCR. *Journal of Plant Pathology* **87**, 49-55

Falquet L, Pagni M, Bucher P, Hulo N, Sigrist CJ, Hofman K, Bairoch A (2002) The PROSITE database, its status in 2002. *Nucleic Acids Research* **30**, 235-238

Félix MR, Cardoso JMS, Clara MIE (2006b) Single Strand Conformation Polymorphism analysis of *Olive latent virus 1* isolates from *Olea europaea* L.. *Proceedings of the Olivebioteq 2006 - Second International Seminar "Biotechnology and Quality of Olive Tree Products around the Mediterranean*

Basin", Sicily, Italy, Vol II, pp 243-246

Félix MR, Cardoso JMS, Oliveira S, Clara MIE (2003) Detection of *Tobacco necrosis virus* and *Olive latent virus 1* in *Olea europaea* L. by multiplex RT-PCR. *Phytopathologia Mediterranea* **42**, 298

Félix MR, Cardoso JMS, Oliveira S, Clara MIE (2005a) Viral properties, primary structure and phylogenetic analysis of the coat protein of *Olive latent virus 1* isolate from *Olea europaea* L. *Virus Research* **108**, 195-198

Félix MR, Cardoso JMS, Varanda CMR, Oliveira S, Clara MIE (2005b) Complete nucleotide sequence of an *Olive latent virus 1* isolate from olive trees. *Archives of Virology* **150**, 2403-2406

Félix MR, Clara MIE (2002) Two Necrovirus isolates with properties of *Olive latent virus 1* and of *Tobacco necrosis virus* from olive in Portugal. *Acta Horticulturae* **586**, 725-728

Félix MR, Varanda C, Cardoso JMS, Clara MIE (2004) Soil transmission of an olive isolate of *Olive latent virus 1*. *Proceedings of the 15th International Plant Protection Congress*, Beijing, China, 447

Félix MR, Varanda C, Cardoso JMS, Clara MIE (2006a) Plant root uptake of *Olive latent virus 1* and *Olive mild mosaic virus* in single and mixed infections. *Proceedings of the 12th Congress of the Mediterranean Phytopathological Union*, Rhodes, Greece, pp 516-517

Gallitelli D, Savino V (1985) *Olive latent virus 1*, an isometric virus with a single RNA species isolated from olive in Apulia, Southern Italy. *Annals of Applied Biology* **106**, 295-303

Grieco F, Alkowni R, Saponari M, Savino V, Martelli GP (2000) Molecular detection of olive viruses. *Bulletin OEPP/EPPO* **30**, 469-473

Grieco F, Savino V, Martelli GP (1996) Nucleotide sequence of the genome of a citrus isolate of *Olive latent virus 1*. *Archives of Virology* **141**, 825-838

Kanematsu S, Taga Y, Morikawa T (2001) Isolation of *Olive latent virus 1* from tulip in Toyama Prefecture. *Journal of General Plant Pathology* **67**, 333-334

Lobão DL, Félix MR, Clara MIE, Oliveira S, Leitão FA, Serrano JF (2002) Detection of *Olive latent virus 1* in *Olea europaea* L. tissues by reverse transcription-polymerase chain reaction. *XIII Congresso Nacional de Bioquimíca*, Lisboa, Portugal, p 102

Louro TJFM (2004) Caracterização de um isolado viral de *Olea europaea* L., produção de anticorpos policlonais específicos, e aplicação de métodos de diagnóstico. MSc Thesis, Universidade de Évora, Évora, Portugal, 91 pp

Martelli GP, Sabanadzovic S, Savino V, Abu-Zurayk AR, Masannat M (1995) Virus-like disease and viruses of olive in Jordan. *Phytopathologia Mediterranea* **34**, 133-136

Martelli GP, Yilmaz MA, Savino V, Baloglu S, Grieco F, Güldür ME, Greco N, Lafortezza R (1996) Properties of a citrus isolate of *Olive latent virus 1*, a new necrovirus. *European Journal of Plant Pathology* **102**, 527-536

Merciega V, Boscia D, Savino V (1996) Comparison of five isolates of *Olive latent virus 1*. *Phytopathologia Mediterranea* **35**, 1-8

Olmos A, Cambra M, Esteban O, Gorris MT, Terrada E (1999) New device and method for capture, reverse transcription and nested PCR in a single closed-tube. *Nucleic Acids Research* **27**, 1564-1565

Pantaleo V, Grieco F, Castellano MA, Martelli GP (1999) Synthesis of infectious transcripts of *Olive latent virus 1*: genes required for RNA replication and virus movement. *Archives of Virology* **144**, 1071-1079

Pantaleo V, Saponari M, Gallitelli D (2001) Development of a nested PCR protocol for detection of olive-infecting virus in crude extracts. *Journal of Plant Pathology* **83**, 143-146

Pantaleo V, Grieco F, Di Franco A, Martelli GP (2006) The role of the C-terminal region of *Olive latent virus 1* coat protein in the host systemic infection. *Archives of Virology* **151**, 1973-1983

Poch O, Sauvaget I, Delarue M, Tordo N (1989) Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. *The EMBO Journal* **8**, 3867-3874

Rubio L, Ayllón MA, Gerri J, Pappu H, Niblett C, Moreno P (1996) Differentiation of *Citrus tristeza Closterovirus* (CTV) isolates by single-strand conformation polymorphism analysis of the coat protein gene. *Annals of Applied Biology* **129**, 479-489

Saitou N, Nei N (1987) The neighbour-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406-442

Saponari M, Savino V, Martelli GP (2002b) Trasmissioni per seme dei virus dell'olivo. *Frutticoltura* **4**, 103-105

van Regenmortel MHV, Fauquet CM, Bishop DHL, Carstens EB, Estes MK, Lemon SM, Maniloff J, Mayo MA, McGeoch DJ, Pringle CR, Wickner RB (2000) *Virus Taxonomy*, Academic Press, San Diego, California, USA, 1162 pp

Varanda C (2005) Avaliação das técnicas de diagnóstico viral baseadas em isolamento de dsRNA e RT-PCR, para certificação de uma colecção de clones da cv. 'Negrinha de Freixo' (D.O.) de *Olea europaea* L. MSc Thesis, Universidade de Évora, Évora, Portugal, 65 pp

Varanda C, Félix MR, Leitão F, Sismeiro R, Clara MIE (2006) Application of reverse transcription – polymerase chain reaction to screen a collection of clones of *Olea europaea* L. for the presence of necrovirus (*Tombusviridae*). *8th Conference of the European Foundation for Plant Pathology & British Society for Plant Pathology Presidental Meeting 2006*. *Sustainable Disease Management: The European perspective*, Copenhagen, Denmark, p 21