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# Multiplex RT-PCR for detection and identification of three necroviruses that infect olive trees

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**Abstract** An optimized multiplex RT-PCR assay was developed to discriminate three necrovirus (*Olive latent virus 1* (OLV-1), *Tobacco necrosis virus D* (TNV-D) and *Olive mild mosaic virus* (OMMV)) that infect olive trees. An olive orchard consisting of 54 trees of cv. "Galega vulgar" in the south of Portugal was surveyed. dsRNA fraction was used as template and revealed the 3 viruses, singly or in multiple infections, present in 17 out of 54 trees in the orchard. OMMV was the most frequent occurring in 15 trees, followed by OLV-1 in 12 and TNV-D in 4 plants. The results obtained showed that necrovirus- specific dsRNAs do exist in infected tissues in amounts below the resolution permitted by gel electrophoresis analysis and that the developed multiplex PCR based assay is of much higher sensitivity. The design of the specific primers described enabled, for the first time, to discriminate between OMMV and TNV-D by means of RT-PCR assays, an indispensable tool in identification, epidemiology and survey studies.

**Keywords** Multiplex RT-PCR · Olive necrovirus · Primer design · Viral detection

**Abreviations**

| | |
|---|---|
| % | percentage |
| bp | base pair |
| ca. | *circa* |
| cDNA | complementar DNA |
| CP | coat protein |
| cv. | cultivar |
| dsRNA | double stranded RNA |
| Fig | Figure |
| g | gram |
| Kb | Kilobase |
| OLV-1 | *Olive latent virus 1* |
| OMMV | *Olive mild mosaic virus* |
| ORF | Open reading frame |
| RdRp | RNA-dependent RNA polymerase |
| RT-PCR | Reverse transcription—Polymerase chain reaction |
| TNV-A | *Tobacco necrosis virus A* |
| TNV-D | *Tobacco necrosis virus D* |

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*Tobacco necrosis virus* (TNV) was first described infecting *Nicotiana tabacum* L. plants by Smith and Bald (1935). Since then, many viruses causing

necrosis on host plants, were recovered from woody and herbaceous hosts, soils, lake and river waters, and were "identified" as TNV. Strains were then distinguished by host range and capacity to support satellite virus replication (Kassanis 1970). The generalized use of serological techniques led to grouping TNVs into a number of serotypes based on coat protein (CP) epitopes. With the advent of molecular biology that allowed full viral genome sequencing and gene products identification, TNV-A (type member) and TNV-D are now recognized as members of the genus *Necrovirus* (family *Tombusviridae*), together with *Olive mild mosaic virus* (OMMV) and *Olive latent virus 1* (OLV-1). The genus is characterized by possessing small icosahedral viruses ca. 28 nm in diameter and a single stranded positive-sense RNA genome of approximately 3.8 Kb long (Van Regenmortel et al. 2000) containing 5 open reading frames (ORF). Recently, the complete genome sequence of a TNV-D isolate (TNV-$D^P$) obtained from olive was for the first time determined (Cardoso et al. 2009). OMMV, the most recent member of that genus, was found for the first time in olive trees in Portugal (Cardoso et al. 2005) and new molecular data, submitted to Genbank (Accession numbers: EF201608, EF201607, EF201606, EF201605), places this virus as the causal agent of Augusta disease of tulips, in Netherlands (Kassanis 1949). Sequence analysis of the OMMV genome revealed that its CP shares 86.2% of amino acid identity with that of TNV-D and its RNA-dependent RNA polymerase (RdRp) shares 91.2% with that of OLV-1 (Cardoso et al. 2005) leading the authors to suggest that OMMV have originated from genomic recombination events occurred during simultaneous replication of TNV-D and OLV-1 in a host cell. The high identity of CP gene of both viruses makes ambiguous all identifications of TNV-D based either on serology or on PCR amplification of that gene. In fact, the primers designed by Cardoso et al. (2004), within the CP region of TNV-D genome detect indistinctively TNV-D and OMMV in RT-PCR assays. A previous survey conducted in an olive collection of clones of the cultivar "Negrinha de Freixo" using this technique, revealed 22% infections which, in light of the present knowledge, may be ascribed to either TNV-D, OMMV, or both (Varanda et al. 2006).

Here we report on the design of two sets of specific primers, for each OMMV and TNV-D and on their use together with the primers specific for OLV-1 (Martelli et al. 1996) (Table 1) in a multiplex PCR, aiming at the simultaneous detection and differentiation of necroviruses in olive. Due to the likely recombinant nature of OMMV between TNV-D and OLV-1, caution was taken to ensure both specificity and compatibility of the primers for each of the 3 necrovirus genome.

Specific primers with similar annealing temperatures were designed after multiple alignment, using BioEdit (version 7.0.9.0) (Hall 1999), of TNV-D and OMMV genomic published sequences, NC_003487 and NC_006939, respectively, retrieved using the Nucleotide Sequence Search program located in the Entrez Browser (http://www.ncbi.nlm.nih.gov/Entrez). The set of primers designed for OMMV detection hybridize in the RdRp (OMMVd5′) and in the CP gene (OMMVd3′) regions whereas those specific for TNV-D are complementary to regions within the RdRp gene (TNVDd5′ and TNVDd3′), originating amplicons sized 934 bp and 278 bp, respectively, in PCR based tests. The use of these primers in monospecific PCR assays using as template dsRNA extracted from *Nicotiana benthamiana* plants infected with OLV-1 (GM6 olive isolate, Félix et al. 2005), OMMV (GP olive isolate, Cardoso et al. 2005) and *Chenopodium murale* plants infected with TNV-D (TNV-$D^P$ olive isolate, Cardoso et al. 2009), resulted in the amplification of cDNA fragments sized *ca*. 747 bp, 934 bp and 278 bp, respectively, as expected (data not shown).

These fragments were purified by GFX PCR DNA and Gel band purification kit (GE Healthcare) and cloned into pGEM-T® Easy Vector (Promega, Madison, WI, USA). Their sequencing confirmed both the expected size and the corresponding genomic region sequence.

Thereafter, a single multiplex procedure was optimized using the primers here designed together with those specific for OLV-1 detection (Martelli et al. 1996) using as template dsRNA fractions obtained from a *Nicotiana benthamiana* plant multiple infected with OLV-1, TNV-D and OMMV. The expected PCR products were concomitantly produced and easily distinguished by agarose gel electrophoresis (data not shown).

dsRNAs extracted from green fruits of 54 sampled trees, harvested in October and from 2 year stems collected in May from olive trees were analysed by gel electrophoresis but no molecules were visible after

**Table 1** Primers used in RT-PCR assays for diagnosis of olive infecting necrovirus

| Virus | Primer | Position in the genome | Sequence 5′ - 3′ | Amplicon (bp) |
|---|---|---|---|---|
| OLV-1 | PB | 2720–2738 | TTTCACCCCACCAAATGGC | 747 |
| | PA | 3448–3466 | CTCACCCATCGTTGTGTGG | |
| OMMV | OMMVd5′ | 1857–1875 | CCGTGCCAAACACAATCTC | 934 |
| | OMMVd3′ | 2771–2791 | CCTAGATCTTCTGGGCTAAGC | |
| TNV-D | TNVDd5′ | 303–322 | GTAGGTGACAAGGACGCTGA | 278 |
| | TNVDd3′ | 560–581 | GGATAGCGACTTTTTAGCCGCT | |

staining with ethidium bromide (data not shown), suggesting no RNA virus infection. However RT-PCR tests using these dsRNA fractions as templates and the specific primers above mentioned, clearly demonstrated that 17 trees were necrovirus infected. The final PCR amplified products, sized *ca*. 934 bp in 15 trees, 747 bp in 12 trees and of 278 bp in 4 trees, revealed OMMV, OLV-1 and TNV-D infections, respectively (Fig. 1).

OMMV occurred either alone in 5 trees or in mixed infections with OLV-1 and with TNV-D. OLV-1 was also found either singly in 2 trees or together with the other olive necroviruses. TNV-D was detected in 4 trees all of which were co-infected with both OMMV and OLV-1. Double infections of OMMV and OLV-1 were demonstrated in six olive trees.

The findings here reported show that necrovirus infection reached 31%. Despite the small number of samples tested, the prevalence of OMMV over TNV-D, which did not occur singly, suggests that OMMV is either better adapted to the host or has some advantage over TNV-D in natural dissemination.

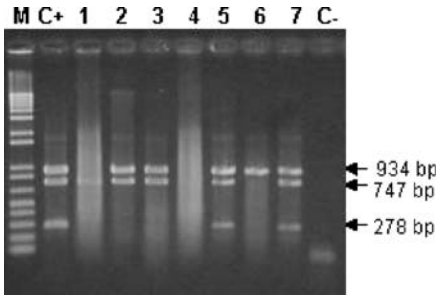


**Fig. 1** Products of multiplex RT-PCR applied to dsRNA fraction extracted from olive trees using specific primers for OMMV, OLV-1 and TNV-D, separated on a 1% agarose gel. Lane M: 1 Kb plus DNA ladder (Invitrogen); Lane C+: positive control obtained by using dsRNA extracted from an herbaceous plant multiple infected with OMMV, OLV-1 and TNV-D; Lane 1–7: trees tested; Lane C-: olive uninfected control. Size of generated amplicons are indicated on the right

This work shows that dsRNA analysis, by itself, is not sensitive enough to detect necroviruses, in accordance to other data (Varanda et al. 2006) which may be due to the low concentration of replicating virus molecules in olive infected tissues, contrary to that observed in infected herbaceous hosts. The use of dsRNA fractions in PCR amplifications is often a useful strategy as their isolation procedure eliminates much of virus non related nucleic acids present in the plant tissues as well as other components that may interfere with viral genome amplification (Saldarelli et al. 1994; Nolasco et al. 2000). Additionally, the process of extracting dsRNA as the advantage of using a large sample (*ca*. 10 g) thus increasing the possibility of detecting viruses unevenly distributed in plant tissues (Bertolini et al. 2003), as compared to the much smaller sample size (100 mg) processed when total RNA fraction is used as template.

The specificity of the primers here described for identification of OMMV and of TNV-D and their compatibility with those specific for OLV-1 allows their reliable use in multiplex RT-PCR assays, which is relevant for virus-free certification programmes of *O. europaea*, as required by EU directive 93/48 concerning the *Conformitas Agraria Communitatis*. Additionally,,for the first time, it is posible to accurately diagnose those viruses; and to obtain and interpret data on mutual virus interaction, geographical distribution and means of field dissemination, which to a large extent remain unclear.

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