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An integrated approach for understanding the high infection rates of olive viruses in Tunisia

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Abstract This study was conducted to understand the high incidence of viruses previously reported in Tunisian olive groves. Forty three cultivars, native and introduced, under different cultivation regimes, were sampled in Tunisia, one of the countries with the highest levels of olive viruses. All olive-infecting viruses were tested. Total nucleic acids were extracted from 280 olive trees and their use as template for RT-PCR resulted in several non-specific products. Alternatively, dsRNAs were also extracted and 229 sampled trees showed dsRNAs molecules, suggesting the presence of viruses. However, RT-PCR tests using dsRNA as template, allowed to identify viruses in 209 samples. *Olive mild mosaic virus* (OMMV), *Olive latent virus 1* (OLV-1), *Olive leaf yellow associated virus* (OLYaV) and *Tobacco necrosis virus* D (TNV-D) were detected. OLYaV was the most prevalent (63.0%), followed by OLV-1 (42.9%), TNV-D (21.80%) and OMMV (9.50%). The highest infection levels were observed in the northwest and centre east regions of Tunisia. Native cultivars presented higher virus incidences, mostly due to the most widespread cultivars in Tunisia: Chemlali (87%), Chetoui (94%), Meski (94%). Among the introduced cultivars, Picholine, Arbequina and Koroneiki presented the highest virus levels (88%, 79% and 64%, respectively). Considerable differences in virus presence were observed in samples from olive trees under different modes of management where levels were significantly ($p > 0.05$) higher in intensive orchards, regardless of the region and cultivar. To our knowledge, this was the first time that OMMV and TNV-D were detected in Tunisia and that such a complete analysis was performed.

Keywords Mode of management · Olive cultivar · dsRNA · RT-PCR

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Introduction

Olive is among the most important fruit trees in the Mediterranean countries, including Tunisia. Just behind the European Union, the Tunisian average production reached, in the last 6 years (2010–2016), 202,500 tons, with a maximum of 366,000 tons in 2015 produced on nearly 18,000 km^2 (IOC 2016) which represents 20% of the world olive growing area. Tunisia is also one of the most important producers of olive oil in the world with a 6.1% share and an important exporter of the Southern Mediterranean region (IOC 2016).

The varietal assortment of olive cultivars in Tunisia is rich and diversified; with over 50 main Tunisian native varieties (Trigui and Msallem 2002) and about 10 introduced varieties in intensive cultivation (Allalout and Zarrouk 2015). Native cultivars account for over 90% of the total olive production (Mohamed et al. 2017). The two main olive cultivars in Tunisia are the native Chemlali and Chétoui, together they account for near the total of olive oil production from native cultivars (Trigui and Msallem 2002). Other native cultivars include Meski, Ouslati, Gerboui, Zalmati, Barouni, Chamchali Gafsa. The most important varieties used in intensive systems are the introduced cultivars Arbequina (Spain), Picholine (France), Arbosana (Spain) and Koroneiki (Greece) (IOC 2000; FAOSTAT 2015).

Olive trees are susceptible to several pathogens. Until now, 15 viruses have been identified in olive plants (Felix et al. 2012), eight of them in Tunisia (Alabdullah et al. 2009; El Air et al. 2011). Some viruses have been recovered from asymptomatic trees and others with symptoms that include bumpy fruits, chlorosis, weakness or defoliation and reduction of yield and oil quality (Marte et al. 1986; Castellano et al. 1987; Alabdullah et al. 2009; Cardoso et al. 2009; Godena et al. 2012).

Virus surveys carried in the countries of the Mediterranean basin confirmed by the presence of double-stranded RNA showed high levels of infection and a general degraded status of olive orchards (Martelli et al. 1995; Saponari et al. 2002). More recently, RT-PCR has demonstrated to be the most rapid, sensitive and reliable technique (Varanda et al. 2010; Albanese et al. 2012). Different templates such as TNA, total RNA and dsRNA have been used as viral targets for RT-PCR. The use of dsRNA as a template reduces the constraint caused by the common low viral concentration and uneven distribution in trees, by using large amounts of sample and, at the same time, allowing the elimination of virus non-related nucleic acids that interfere in viral genome amplification (Bertolini et al. 2003; Varanda et al. 2010; Luigi et al. 2011; Varanda et al. 2014).

Considering the importance of olive crops in Tunisia, a need for high-quality nursery plants is highly desirable to obtain and provide certified propagative material to be used by producers worldwide. For this purpose and with the aim of improving national olive production, Tunisia adopted a voluntary program for certification of propagative material based on the Italian law (DM 20/11/2006) concerning two sanitary status: 'virus-free' (free from all olive infecting viruses) and 'virus-tested' (free from the most important olive viruses such as *Strawberry latent ringspot virus* (SLRSV), *Arabis mosaic virus* (ArMV), *Cherry leafroll virus* (CLRV), *Olive leaf yellow associated virus* (OLYaV) and *Olive latent virus 1* (OLV-1)). This has allowed the determination of virus levels as high as 86%, greater than the ones found in other Mediterranean countries that range from 25% in Croatia to 50.7% in Syria (Al Abdullah et al. 2005; Fadel et al. 2005; Faggioli et al. 2005; El Air et al. 2011; Luigi et al. 2011).

Research on the epidemiology of viruses infecting olive trees will help to understand why such high levels of virus occur in olive orchards, representing an essential contribution to improve the sanitary status of the crops. The most studied viruses in terms of transmission modes are the olive-infecting necroviruses (OLV-1, *Tobacco necrosis virus D* (TNV-D) and *Olive mild mosaic virus* (OMMV)) that have shown efficient means of dissemination (Varanda et al. 2011a, b; Felix et al. 2012; Varanda et al. 2015). However, little is known concerning their transmission in olive, except for OLV-1 seed transmission (Saponari et al. 2002). Apart from OLYaV, all olive infecting viruses are mechanically transmitted (Van Regenmortel et al. 2000).

The wide geographic distribution of olive viruses seems to suggest that olive viruses mostly originate from rooted cuttings of infected mother plants used for propagation. To understand, not only the source but also the dissemination of the viruses in olive orchards, it is crucial to know what cultural operations, or other conditions, are contributing to such successful spread of the viruses in order to take the most effective measures to reduce virus infections. With this purpose, a spatial sampling was conducted to determine the virus incidence in olive trees from different cultivars, native or introduced, cultivated under different modes of

management, and grown in several regions in Tunisia. In addition, although not a previous defined goal for this study, different nucleic acid extraction techniques were used and compared. All olive-infecting viruses with a known genetic sequence, were tested in this study. In addition, *Tobacco necrosis virus A* (TNV-A) was included due to its high sequence similarity to the olive-infecting OMMV, OLV-1 and TNV-D.

Material and methods

Field samplings

Sampling was performed from October 2013 to April 2014. A total of 280 samples were collected from symptomatic or asymptomatic olive trees located in several regions covering the whole of Tunisia and divided as follows: 75 samples were collected in northeast, 28 in northwest, 55 in centre-east, 39 in centre-west, 72 in southeast and 11 in southwest. Samples consisting of 10 cuttings of ca. 20 cm in length were collected from 2-year stems from each quadrant of the canopy of each tree and stored in plastic bags at 4 °C. Sampled trees belonged to 33 different native cultivars (160 trees) and 10 different cultivars introduced from abroad (120 trees), growing in 56 orchards under different types of management: 33 traditional (80 trees), two semi-intensive (60 trees) and 21 intensive (140 trees), covering a total area of 276,27 km^2. Crops with a density of less than 100 trees per hectare were considered traditional crops, irrigated crops with densities between 100 and 200 trees per hectare were considered semi-intensive crops, while irrigated cropping systems of density greater than 200 trees per hectare were considered intensive (Duarte et al. 2006).

Data provided by the Tunisian Ministry of Agriculture and its regional institutions concerning the olive-producing area, management types of olive orchards and the geographical distribution of the cultivars, were used to obtain a highly representative sampling.

Nucleic acids extraction and mechanical transmission

Total nucleic acids (TNAs) were extracted from 0.2 g of cortical scrapings of twigs by adsorption on silica columns (Foissac et al. 2001). Silica was collected by centrifugation and 150 µl of sample was transferred to a tube and stored at −20 °C.

Double stranded RNAs (dsRNAs) were extracted from 10 g of cortical scrapings in the presence of phenol chloroform, followed by separation through chromatography on cellulose CF11 column and ethanol precipitation (Morris and Dodds 1979). DsRNA were visualized by agarose 0,8% gel electrophoresis prior to denaturation by heating at 100 °C for 5 min followed by 15 min on ice.

300 mg of cortical scrapings of olive twigs were ground in 0.1 M phosphate buffer pH 7.2 and then rubbed onto leaves of *Nicotiana benthamiana* and *Chenopodium murale*. Inoculated plants were maintained for 2–3 weeks in a growth chamber with a 14 h photoperiod at 25 °C and observed for the development of symptoms. Leaves of symptomatic plants were macerated in liquid nitrogen and total RNA was extracted using the commercial RNeasy Plant Mini Kit (Qiagen) in accordance with manufacturer's instructions and used as template for RT-PCR as described previously.

RT-PCR

For cDNA synthesis, 1 µg of TNA, or denatured dsRNA, was used in a 20 µL reaction containing 200 U of RevertAid H Minus Reverse Transcriptase (ThermoScientific™) in the presence of 1.5 µg of random hexamers (Promega) and 1× first reaction buffer (ThermoScientific™), in accordance with the manufacturer's instructions.

OMMV, OLV-1 and TNV-D dsRNAs provided by the laboratory of Plant Virology of the University of Évora, were used as positive controls.

Samples were tested for the presence of twelve olive viruses (OMMV, TNV-D, OLYaV, SLRSV, ArMV, CLRV, OLV-1, *Olive latent virus 2* (OLV-2), *Olive latent virus 3* (OLV-3), OLRSV, *Cucumber mosaic virus* (CMV), *Tobacco mosaic virus* (TMV)), and TNV-A, using reliable sets of primers (Table 1). Primers for TNV-A were designed here, after multiple alignment of TNV-A genomic published sequences, NC_001777 and AY546104, retrieved using the Nucleotide Sequence Search program located in the Entrez Browser (http://www.ncbi.nlm.nih.gov/Entrez), using BioEdit (Hall 1999). Both primers (TNV-A5' and TNV-A3') are complementary to regions within the coat protein (CP), originating an amplicon of 552 bp in PCR tests.

Two µl of cDNA were used in PCR carried out in 1X DreamTaq Buffer (Thermo Scientific™), 0,2 mM dNTPs, 0,5 µM of each primer and 2.5 U of DreamTaq

Table 1 Primers used in RT-PCR assays for the diagnosis of olive infecting viruses

Virus	Primers	Sequence 5′- 3’	Amplicon (bp)	Amplified region	Reference
OMMV	OMMVd5’ OMMVd3’	CCGTGCCAAACACAATCTC CCTAGATCTTCTGGGCTAAGC	934	RdRp and CP	(Varanda et al. 2010)
TNV-D	TNVDd5’ TNVDd3’	GTAGGTGACAAGGACGCTGA GGATAGCGACTTTTTAGCCGCT	278	RdRp	
OLV1	PB PA	TTTCACCCCACCAAATGGC CTCACCCATCGTTGTGTGG	747	3’terminal	(Grieco et al. 2002)
ARMV	ArMV-5A ArMV-3A	TACTATAAGAAACCGCTCCC CATCAAAACTCATAACCCAC	302	CP	(Faggioli et al. 2005)
TNV-A	TNV-A5’ TNV-A3’	CCAGTGGATCAATTGCCATG CAGGGTCTGCCAAGGTCTTGTG	552	CP	This work
SLRSV	SLRSV-5D SLRSV-3D	CCCTTGGTTACTTTTACCTCCTCATTGTCC AGGCTCAAGAAAACACAC	293	CP	(Faggioli et al. 2002)
CMV	CMV-CPN5 CMV-CPN3	ACTCTTAACCACCCAACCTT AACATAGCAGAGATGGCGG	280	RdRp	(Lumia et al. 2001)
CLRV	CLRV-5 CLRV-3	TTGGCGACCGTGTAACGGCA GTCGGAAAGATTACGTAAAAGG	416	CP	(Werner et al. 1997)
OLRSV	OLRSV5’ OLRSV3’	CTGCAAAACTAGTGCCAGAG TGCATAAGGCTCACAGGAG	492	3′-terminal	(Alkowni et al. 2000)
OLYAV	OLYAV5’ OLYAV3’	CGAAGAGAGCGGCTGAAGGCTC GGGACGGTTACGGTCGAGAGG	346	Hsp70-like protein gene	(Sabanadzovic et al. 1999)
OLV-2	OLV2-H OLV2-C	GAAGGTGGCTCGCCTAGAG GCCAGGAGTTTGAGCTTTG	206	RdRp	(Bertolini et al. 2001)
OLV-3	OLV-3f OLV-3r	CCCGTTGAGCAAGTTGTCTTCC GCAGTGGCTGGAGAGCATGGAG	176	RdRp	(Alabdullah et al. 2009)
TMV	TMV5’ TMV3’	CGACATCAGCCGATGCAGC ACCGTTTTCGAACCGAGACT	880	replicase	(Kumar et al. 2011)

DNA Polymerase (Thermo Scientific™) in a total volume of 50 μl. Amplifications were carried out in a Thermal Cycler (Bio-Rad) at 95 °C for 1 min, 35 cycles at 95 °C for 30 s, and 54 °C, 58 °C or 60 °C for 1 min. PCR optimizations revealed the best annealing conditions of 1 min at 54 °C for TNV-A, OMMV, ARMV, TNV-D, OLV1, SLRV, CMV and OLV3; 58 °C for OLRSV and OLYAV; 60 °C for CLRV, OLV2 and TMV) and 72 °C for 1 min, and a final extension step of 72 °C for 10 min. Amplified products were analysed by electrophoresis in 1% agarose gel and three amplicons from each virus were randomly selected for confirmation through sequencing.

Sequencing

PCR products were purified using DNA Clean and Concentrator Kit (ZYMO RESEARCH), or zymoclean gel DNA recovery kit in accordance with the manufacturer's instructions. DNA sequencing reactions were performed on both strands, by Macrogen (The Netherlands). The search for homologous sequences was done using BLAST. Multiple sequence alignment was performed with BioEdit 7.1.3.0 (Hall 1999).

Data analysis

Virus abundances (number of trees with each virus within a group of ten trees sampled), were calculated using the virus dataset from each "Origin of cultivars" and "Type of management". A two-way permutational analysis of variance (PERMANOVA) was applied to test the hypothesis that significant differences existed between "Origin of cultivars" and "Type of management". The PERMANOVA analysis was carried out following the two-factor design: "Origin of cultivars": Native and Introduced (two levels, fixed); "Type of management": Intensive, Traditional and Semi-Intensive (three levels, random).

Virus abundances data were square root transformed to scale down the importance of highly abundant viruses and therefore increase the importance of the less abundant ones in analysis of similarity between viruses. The PERMANOVA analysis was conducted on a Bray-Curtis similarity matrix (Clarke and Green 1988). The null hypothesis was rejected at a significance level < 0.05 (if the number of permutations was lower than 150, the Monte Carlo permutation p was used). Whenever significant interactions in effects of the factors were detected, these were examined using a posteriori pairwise comparisons, using 9999 permutations under a reduced model. The similarity of the viruses within 'Origin of cultivars' (Native and Introduced), 'Type of management' (Intensive, Traditional and Semi-Intensive) were plotted by Principal coordinates analysis (PCO) using the Bray-Curtis similarity measure. The relative contribution of each virus to the similarity and dissimilarity within origin of cultivars (Native and Introduced) was calculated using one-way-crossed similarity percentage analysis (SIMPER; cut-off percentage 100%).

A Principal Component Analysis (PCA) of the virus database was performed to explore patterns in multidimensional data by reducing the number of dimensions with minimal loss of information. The PCA ordination was based on the values of each virus measured in 'Origin of cultivars' (Native and Introduced) and 'Type of management' (Intensive, Traditional and Semi-Intensive). Prior to the calculation of the virus' resemblance matrix based on Euclidean distances, data were checked for uniform distribution, and if necessary were log $(X + 1)$ transformed and normalized (subtracting the mean and dividing by the standard deviation, for each variable) prior to analysis.

The statistical analyses were performed using the PRIMER v6 software (Clarke and Warwick 2001) with the permutational analysis of variance (PERMANOVA) add-on package (Anderson et al. 2008).

Results

Samples were collected from symptomatic and asymptomatic olive trees. Symptoms observed in the field possibly related to viral origin, included: leaf yellowing, deformed branches and fruits, and low vigour. Other symptoms related to bacterial or fungal origin as leaf spots (*Spilocaea oleagina* Cast.), canker and olive tuberculosis (*Pseudomonas* spp.) were also observed. Several non-specific RT-PCR products were obtained in each of the 280 samples when TNA was used as template (data not shown). Optimizations of RT-PCR protocol, namely different cycling temperatures and times, did not allow the elimination of many non-specific products. Nearly all samples presented products with the expected size for each virus, however, sequencing of all these RT-PCR products did not reveal the

presence of any virus. Amplicons of expected sizes showed high homology to sequences matching bacteria (*Cellvibrio* spp., *Saccharophagus degradans*, among other unidentified and uncultured bacteria) or olive. For this reason, alternatively, dsRNA extracts were used as template for RT-PCR. However, prior to RT-PCR, dsRNA molecules were observed through gel electrophoresis and a dsRNA pattern of two bands with ca. 1.5 and 1.3 kbp, suggestive of the presence of RNA viruses was found in 229 (about 81.80%) out of the 280 samples analysed (data not shown).

RT-PCR tests using dsRNA as template clearly demonstrated that, of the 280 samples tested, 209 (74.6%) were infected with at least one virus. From the 13 viruses tested, only four were detected. The final PCR amplified products, ca. 934 bp, 747 bp, 346 bp and 278 bp in size, suggested OMMV, OLV-1, OLYaV and TNV-D infections, respectively (data not shown). Sequencing of the amplicons confirmed the identity of each virus.

OLYaV was the most prevalent (63.0%), followed by OLV-1 (42.9%), TNV-D (21.80%) and OMMV (9.50%). Mixed infections with two, three and four viruses were detected in 35.21%, 11.26% and 2.34% of samples, respectively.

The four viruses were found in olive trees from all Tunisian regions. Total infection levels ranged from 60% (centre west) to 90% (northwest). OMMV and TNV-D showed the highest infection levels in the centre east region, with 14.3% and 42.8%, respectively, whereas OLV-1 and OLYaV reached their highest levels in the northwest, with 52.4% and 76.2%, respectively.

High infection rates were observed in the most widespread cultivars in Tunisia, Chemlali (87%), Chetoui (94%), Meski (94%), Picholine (88%), Arbequina (79%) and Koroneiki (64%). Native cultivars showed an infection of 81% and introduced cultivars showed an infection of 63%. However, PERMANOVA analysis revealed no significant differences between native and introduced cultivars ($p > 0.05$). These results are also supported by PCO ordination plot that did not reflect a distinct pattern between native and introduced cultivars (Fig. 1).

All viruses showed higher infection rates in native cultivars than in introduced. In native cultivars, the higher mean abundance (number of trees with each virus within a group of ten trees sampled), ±SE was obtained with OLYaV (6.93 ± 0.53) followed by OLV-1 (4.28 ± 0.67), TNV-D (2.93 ± 0.58) and OMMV (1.13 ± 0.32). In introduced cultivars, the higher mean abundance ±SE was also obtained with OLYaV (5.73 ± 0.82) followed by OLV-1 (4.50 ± 0.98), TNV-D (1.60 ± 0.66) and OMMV (0.85 ± 0.27) (Fig. 2).

The SIMPER analysis showed how the viruses contributed to the similarity and dissimilarity of a priori defined groups, native and introduced cultivars. In native cultivars, OLYaV presented the highest contribution to the similarity, followed by OLV-1, TNV-D and OMMV. In introduced cultivars, OLYaV presented the highest contribution to the similarity, followed by OLV-1, OMMV and TNV-D. The virus that presented the highest contribution to the dissimilarity between native and introduced cultivars was TNV-D followed by OLV-1, OMMV and OLYaV (Table 2).

Concerning the mode of management, PERMANOVA analysis showed that there were significant differences ($p < 0.05$) between the intensive and the traditional and semi intensive orchards. No significant differences ($p > 0.05$) were found between the traditional and semi intensive modes. The highest infection rates were observed in intensive orchards (81%). In traditional and semi intensive orchards, infection rates were 69% and 68%, respectively. All the four viruses were detected in trees under the three types of management, except for OMMV and TNV-D that were not found in trees under semi-intensive orchards. TNV-D and OMMV showed higher infection rates in trees under intensive management, 25% and 11%, respectively and OLYaV and OLV-1 showed higher infection rates in trees under semi-intensive management, 67% and 42%, respectively. Infection rates under traditional type of management were 59% for OLYaV, 41% for OLV-1, 24% for TNV-D and 10% for OMMV. Infection rates for OLYaV and OLV-1 under intensive management were 52% and 36%, respectively.

PERMANOVA analysis revealed no significant interactions between the factors "Origin of cultivars" and "Type of management" ($p > 0.05$). In native cultivars, the individual pairwise comparisons revealed significant differences in the type of management within intensive and semi-intensive ($p < 0.02$) and within traditional and semi-intensive ($p < 0.01$). In addition, in introduced cultivars the individual pairwise comparisons only revealed significant differences in the type of management within intensive and semi-intensive ($p < 0.03$).

The PCA ordination of the viruses showed that the first two components (PC1, 51.4% and PC2, 45.9%) accounted for 97.3% of the variability of the data (Fig. 3).

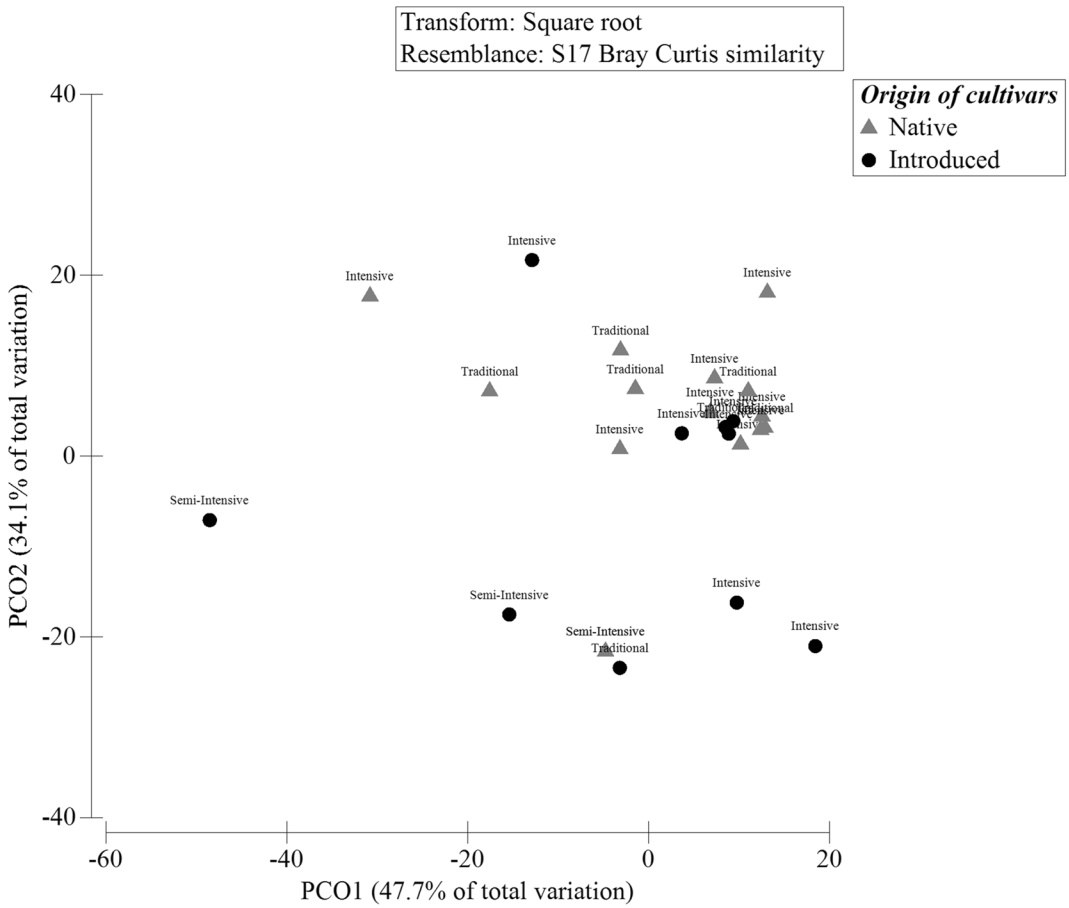

Fig. 1 Principal Coordinates Analysis (PCO) based on the virus dataset "Native and Introduced cultivars" (origin of cultivars, 2 levels, fixed) and "Intensive", "Traditional" and "Semi-intensive" (type of management, 3 levels, random). PCO1 = 47.7%, PCO2 = 34.1%

PCA ordination separated the samples; Introduced (Traditional) and Native (Semi-intensive) with the viruses OLYaV and OLV-1, from the samples from Native (Intensive and Traditional), Introduced (Intensive) with the viruses OMMV and TNV-D. In addition, the samples from introduced cultivars (Semi-intensive) were clearly separated from the others. The PCA (Fig. 3) shows clearly that OMMV and TNV-D were prevalent in intensive orchards and native cultivars, and were not detected in semi intensive orchards. On the other hand, OLV-1 and OLYaV appear near the semi-intensive/native factors, as expected, as they were the only viruses detected in semi-intensive orchards, where most trees belong to native cultivars.

Inoculation of host plants with cortical scrapings of twigs of 209 positive RT-PCR samples caused necrotic lesions both in *Nicotiana benthamiana* and *Chenopodium murale* only when using two OMMV-positive samples (data not shown). No other positive samples caused lesions in the indicator plants.

The expected amplification product for OMMV was detected after RT-PCR using RNA extracted from symptomatic leaves of both samples and both hosts. Sequencing results of the amplicons confirmed the presence of OMMV in both samples.

Discussion

This study was conducted to determine and understand the high virus incidence in olive trees in Tunisia. Olive trees from different cultivars, native or introduced, under different modes of management and grown in several regions in the country, were sampled. All olive-

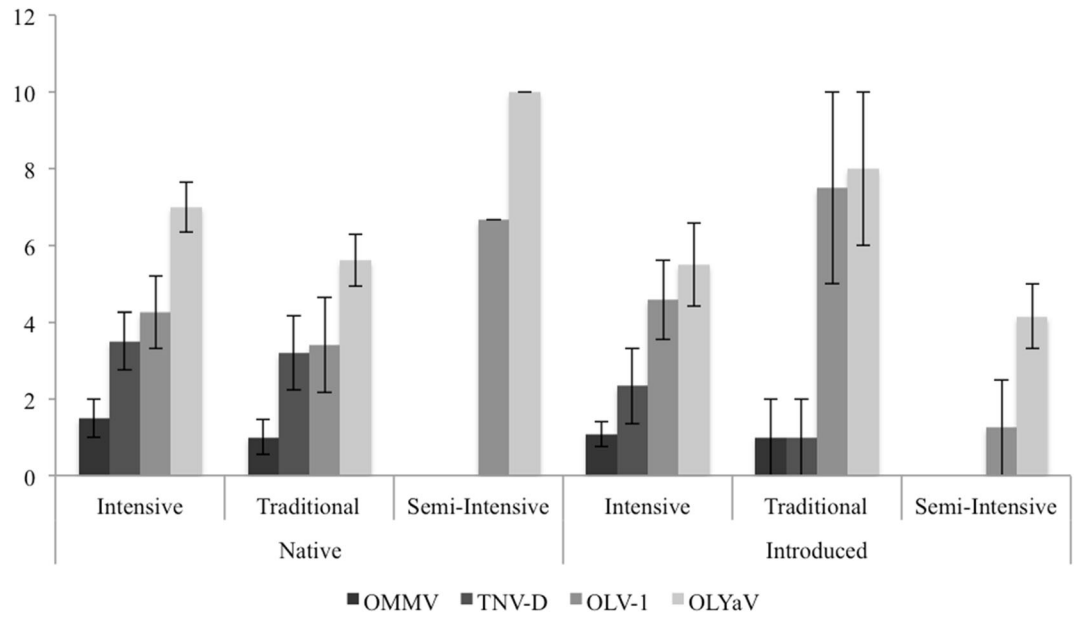

Fig. 2 Mean abundance ± standard error (SE) of viruses on each origin of cultivars (Native and Introduced) and type of management (Intensive, Traditional, Semi-intensive)

infecting viruses with a known genetic sequence were tested. Olive vein yellowing-associated virus (OVYaV), Olive yellow mottling and decline associated virus (OYMDaV) and Olive semi-latent virus (OSLV) do not have a known sequence and because of that they were not included in this study. In addition, TNV-A was included due to its high similarity to OMMV, OLV-1 and TNV-D. To our knowledge, this was the first time that twelve olive infecting viruses were molecularly tested in the same study. It was also the first time that OMMV, TNV-D, TMV and TNV-A were included and that such a complete and integrated study was performed, in olive trees.

No relation was established after comparing symptoms (or lack of symptoms) of olive trees with the presence of viruses. The difficulty to attribute viral infections to field symptoms in olive is well-known, in addition, natural virus infections are many times symptomless; and higher levels of infection are frequently obtained in symptomless trees when compared to symptomatic trees (Luigi et al. 2011).

The inoculation of *Nicotiana benthamiana* and *Chenopodium murale* with the cortical scrapings of twigs of the 209 positive RT-PCR samples caused necrotic lesions in host plants only with two samples. Both samples revealed to be positive for OMMV. In addition to using different host plant species, plants from different ages were also used and several repetitions were performed. This shows the well-known difficulties of mechanical transmission of olive viruses, which are frequently attributed to the low concentration of viruses in olive tissues, irregular distribution, and presence of inhibitors (Martelli 1999; Saponari et al. 2002).

The use of TNA as template for RT-PCR resulted in the amplification of several unspecific products, including products of the expected sizes for most viruses tested. Sequencing of such RT-PCR products did not reveal any sequences of viral origin, most products were identified as bacteria or olive. This may be due to the fact that, TNA extraction resulted in many different nucleic acids from very distinct organisms, and so the possibility of amplification of non-viral target sequences was extremely high. This result is extremely important

Table 2 Viruses that contribute most to the similarities and dissimilarities between Native and Introduced cultivars as identified by SIMPER analysis

	Native	Introduced	Native vs. Introduced
Virus	Similarity		Dissimilarity
	76.25%	65.36%	29.24%
OLYaV	46.41%	54.14%	17.55%
OLV-1	27.44%	30.93%	29.89%
TNV-D	19.7%	6.59%	32.43%
OMMV	6.46%	8.34%	20.13%

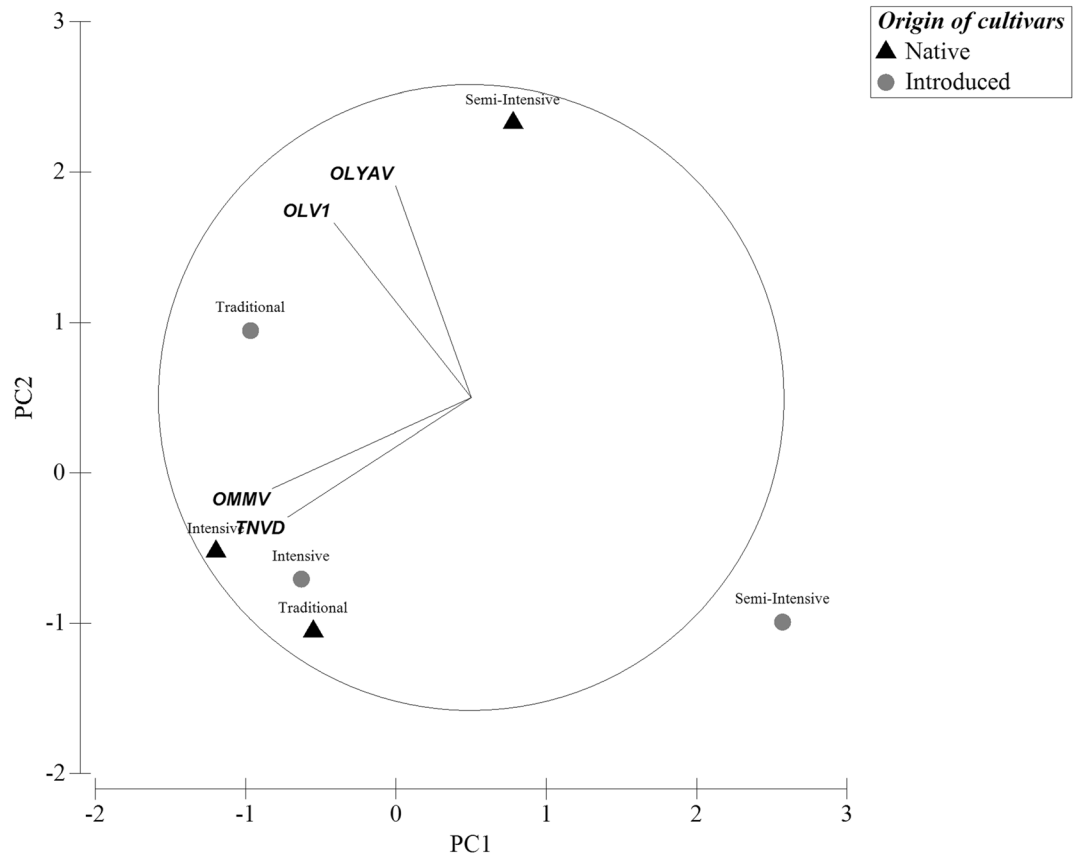

Fig. 3 Principal Component Analysis (PCA) plot based on the virus dataset measured at each "Native and Introduced cultivars" (origin of cultivars, two levels, fixed) and "Intensive", "Traditional" and "Semi-intensive" (type of management, three levels, random). PC1 = 51.4%, PC2 = 45.9%

as TNA has been widely used as template for RT-PCR in olive surveys and shows the importance of sequencing. Despite the fact that TNA extracted by using commercial kits show better results (Loconsole et al. 2010), the use of dsRNA as template in RT-PCR has previously shown to give more accurate results (Felix et al. 2012), because it allows the use of a larger and more representative sample, reducing the constraints of low viral concentration and uneven distribution (Varanda et al. 2010, 2014). In addition it allows the elimination of virus non-related nucleic acids that interfere in the viral genome amplifications (Bertolini et al. 2003; Luigi et al. 2011) which revealed to be a problem when TNA was used as template in RT-PCR in this study. Therefore, in this study we proceed by extracting dsRNA. Prior to the use of dsRNA as RT-PCR template, dsRNA fragments were visualized through gel electrophoresis in 229 of the 280 samples tested (81.8%). The use of dsRNA as template in RT-PCR showed that only 209 of the 280 samples (74.6%) presented at least one of the viruses tested. The higher number of positive dsRNA-tested samples over RT-PCR-tested samples has been previously reported (Saponari et al. 2002) and suggests that other RNA or dsRNA viruses remain to be identified and need to be further investigated. It is also important to notice that there is a huge variety of viruses that present dsRNA, such as the ones that infect arthropods or fungi that may be present in the olive trees. Although the quantity of such viruses is likely low, if present they could make the analysis complex and overestimate olive infecting virus levels. On the other hand, 54 of the RT-PCR positive samples did not reveal any dsRNA fragments, showing that dsRNA analysis by itself is not sensitive enough to detect olive viruses.

The level of infection presented in this study, determined by RT-PCR (74.6%), is near to the 86.3% found in a previous study in Tunisia (El Air et al. 2011), but is much higher than infection levels found in Italy

(32.8%), Syria (50.7%) and Croatia (25%), where the main eight olive-infecting viruses were surveyed (SLRSV, CLRV, ArMV, OLRSV, CMV, OLYaV, OLV-1 and OLV-2). In Lebanon, a previous study on the incidence of SLRSV, CLRV, ArMV, OLYaV and OLV-1 also showed lower infection levels (31.3%) and in Portugal a survey for the detection of necroviruses revealed 31% infection (Al Abdullah et al. 2005; Fadel et al. 2005; Faggioli et al. 2005; Varanda et al. 2010; Luigi et al. 2011).

From the 13 viruses tested, only OLYaV, OLV-1, TNV-D and OMMV were detected. The six viruses ArMV, CLRV, SLRSV, CMV, OLRSV and OLV-2, included in the Italian and Tunisian certification scheme together with OLV-1 and OLYaV, frequently detected in most Mediterranean olive virus surveys, were not detected in this study. In addition, TMV and TNV-A were not detected. The type of olive orchards selected, which did not include nurseries and mother plant fields might even increase the levels of infection to, or over, the levels found in Tunisia (El Air et al. 2011).

The highest levels of virus infection were obtained in the northwest region of Tunisia, where 90% of the trees presented at least one virus and where OLYaV and OLV-1 were the most accountable for that. In addition, the cultivar Chétoui, one of the most infected cultivars, is grown mainly in the north of Tunisia and may have contributed to the high levels of infection in that region.

OMMV and TNV-D showed the highest infection levels in the centre east region of Tunisia. Interestingly, these viruses are very common in vegetable crops, which are increasing in the centre east region and extending to the olive-growing areas, promoting viral transmission between vegetable crops and olive trees and may be contributing to the increase of these viruses in olive. In addition, the highest levels of infection of both OMMV and TNV-D were found in the cultivar Chemlali, which is mainly found in the centre of the country.

The higher number of intensive orchards sampled in these regions in comparison to traditional ones may also have contributed to the high infection levels. The average density of olive trees in Tunisia is closely related to rainfall and decreases from the north to the south of Tunisia and the climate becomes drier. In the north of the country, agricultural production benefits from the presence of vast irrigated perimeters and consequently, the plantation density of olive trees is higher (IOC 2016). Olive trees under intensive mode of management showed significant higher infection levels (trees infected with at least one virus) when compared to trees under traditional modes. In general, the higher stress that olive trees grown under intensive modes are subjected to may make them more susceptible to viruses. Also, most olive viruses are mechanically transmitted and the proximity of the trees contributes to their dissemination. However, the fact that OLYaV is not mechanically transmitted and is the most prevalent virus suggest efficient means of transmission that should be further investigated.

High levels of OLYaV have also been observed in previous studies where either RNA or TNA were used as template in RT-PCR assays in Tunisia (49.1%), Italy (20.91%), Lebanon (23.7%), Syria (14.3%) and Croatia (Saponari et al. 2002; Al Abdullah et al. 2005; Fadel et al. 2005; Faggioli et al. 2005; El Air et al. 2011; Luigi et al. 2011). OLV-1 has shown higher levels than previously reported in Tunisia (34.3%), Portugal (22%) and much higher than the ones found in other Mediterranean countries (<8.3%) (Saponari et al. 2002; Al Abdullah et al. 2005; Fadel et al. 2005; Faggioli et al. 2005; Varanda et al. 2010; El Air et al. 2011; Luigi et al. 2011).

To our knowledge, it was the first time that TNV-D and OMMV were detected in Tunisia. TNV-D presented a higher infection level (21.8%) when comparing to the 7% found in Portuguese orchards in opposition to OMMV that presented a lower infection level (9.5%) than the 28% found in Portugal (Varanda et al. 2010).

The main olive cultivars in Tunisia (the native cultivars Chemlali, Chétoui, Meski and the introduced cultivars Picholine, Koroneiki, Arbequina) presented the highest viral levels, ranging from 64% in Chétoui to 94% in Meski. In all viruses detected, olive trees belonging to these cultivars accounted for more than half of the total infection of each virus tested, with the native ones (Chemlali, Chétoui, Meski) contributing a little more for that than the introduced, although with no significant differences. The longer presence of native cultivars in Tunisian fields (over 70 years) may help to explain the high infection levels. Similar results were obtained in a study conducted in Tunisia, where the main cultivars showed infection levels higher than 63% and native cultivars showed higher infection levels when compared to introduced ones (El Air et al. 2011). Despite being predominant in native cultivars, the highest levels of OLV-1 infection were found in cv. Arbequina (90.9%), a cultivar that is rapidly becoming one of the main olive cultivars in the world and that is cultivated mostly under highly intensive management.

It is crucial to understand what activities are contributing for the high levels of olive viruses in Tunisia. It is possible that the main viral source is the propagating material, however, the higher infection levels in native cultivars than in introduced ones suggest that most infections are well established in Tunisian olive orchards.

In addition, the extremely high number of olive cultivars cultivated in this country may make it possible for the most virus-susceptible cultivars to contribute to the accumulation and adaptation of some viral species to Tunisian climatic conditions; these will then, to a greater extent, infect other cultivars that otherwise (with smaller inoculum) would not become infected. In addition, most olive field operations in Tunisia, such as pruning and harvesting, are done in a very traditional manner and may also contribute to a great dissemination of viruses.

Our results confirm the degraded status of Tunisian olive orchards surveyed randomly and stresses the need for accurate diagnosis. In this study, it is shown that the use of TNAs as template for RT-PCR, vastly used in many Mediterranean surveys, may lead to inaccurate results and that dsRNA used as template is a suitable alternative for detecting olive viruses.

It is also shown here that differences in virus infection levels were only significant among the trees under different modes of management, higher in intensive orchards, regardless of the cultivar and geographic conditions. This is extremely important as intensive and super-intensive orchards are increasing and there is an urgent need to implement strict regulations. Virus free stock materials should be used to start an effective certification system. The high levels of TNV-D and OMMV found in this study makes imperative the need to include these viruses in the 'virus tested' category of the program for certification of propagative material.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This article does not contain any studies involving human participants or animals.

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