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# Impact of Olive leaf yellowing associated virus on olive (*Olea europaea* L.) oil

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## Summary

Among the fifteen virus species detected in olive trees, Olive leaf yellowing associated virus (OLYaV) was found with high incidence and frequency especially in Southern Italy. Effects of OLYaV on virgin olive oil yield and quality of 'Leccino' and 'Ottobratica' cultivars in Calabria region (Southern Italy) were analysed. Oil yield, free acidity, number of peroxides, spectrophotometric indexes, total content of chlorophylls and carotenoids, total phenol content, composition of the fatty acids, total tocopherols and total sterols content were determined on oil obtained from olive fruits collected in healthy/virus-free and OLYaV-infected trees. Almost all analysed oil parameters were not statistically different between the two oil types with some exceptions. Oils derived from 'Ottobratica' OLYaV-infected plants had free acidity significantly lower than oil from healthy plants. K<sub>232</sub> of oil from virus-free 'Leccino' trees was significantly lower than those of oil from infected trees. Even though some quality parameter differences between healthy and OLYaV-infected oils were found, it is important to highlight that all oils can be considered in extra virgin olive oil category, within the UE range accepted maximum limit. Results suggest that, except for K<sub>232</sub> values, OLYaV does not interfere negatively in oil yield and quality, while surprisingly does it positively in free acidity parameter.

**Practical applications:** Based on the evidence that OLYaV does not interfere negatively in oil yield and quality parameters, it seems appropriate that the European Union Council Directives (2014/96/EU, 2014/97/EU, 2014/98/EU) introduced in the last olive certification scheme only ArMV, CLRV and SLRSV. The application of compulsory EU directive, in Italy as in other countries, will improve the facilities of olive plants commercialization, guaranteeing enough their sanitary status. The findings here reported support the suggestion that the Italian Agriculture Ministry could be less restrictive in voluntary Italian regulation (D.M. 20/11/2006).

**Keywords:** olive viruses / OLYaV / virgin olive oil / oil quality

## 1 Introduction

Among the fifteen virus species belonging to nine genera found in olive [1], the largest number of them have been detected in Italy, Portugal and Spain, and more recently also in other countries. High incidences of virus infections as 97.9% in California [2], 86.3% in Tunisia [3], from 32.8% [4] to 73.4% [5] in Italy, 51% in Siria [6], and 34% in Lebanon [7] were found; while a low incidence of

19.4 % was detected in Croatian Istria [8]. Some olive viruses have been identified with a frequency of 33.3% and 29.2% for *Cherry leaf roll virus* (CLRV) and *Strawberry latent ring spot virus* (SLRSV), respectively [9], 34.3% for *Olive latent virus-1* (OLV-1), 25.7% or 34.7% for *Cucumber mosaic virus* (CMV) [2, 3].

*Olive leaf yellowing associated virus* (OLYaV) is the virus that has been found with the highest incidence of 93.8% in California [2]. In Southern regions of Italy many OLYaV-infected olive trees have also been detected (60% in Sicily and 86% in Calabria) and in a large number of cultivars (35/50 and 18/25 positive/tested, respectively) [4, 10]. It worth to highlight that in all examples above reported OLYaV-infected trees were asymptomatic.

Since olive sanitation and sanitary selection, are the only effective strategies to prevent the dissemination of viruses, but they are very expensive, need long times to be performed, require a good virologists team and an efficient sanitary certification program [11], to know the real damages determined by each virus is very important.

Even if direct and indirect damages associated with viral infections as lower yield, reduction in quality products, or market value due to defects in visual attraction (changes in size, shape, and fruit color), reduced consumer appeal (grading, taste, texture, and composition) are reported [12], virus interference on olive grown and production have been rarely studied.

In particular, since OLYaV is the only virus reported in so many cultivars grown in Southern Italy, sanitary selections conducted up today in that area have been very difficult in finding some cultivar free of it. OLYaV is a member of the family *Closteroviridae* [13]; but it is not allocated to any of the genera composing this family because more biological and molecular data are needed for unequivocal classification. This virus has a monopartite positive-sense single-stranded RNA; only part of the viral genome, comprising 4,605 nucleotides from ORFs 1b (RdRp), 2 (21kDa), 3 (7kDa), 4 (HSP70h), and the 5' end of ORF 5 (HSP90h) has been sequenced [14].

Effects evaluation of the OLYaV infections on olive propagation (cuttings' rooting and grafting capacity) have been reported by Roschetti et al. [15] (2009). This work aimed to improve the knowledge on effects of OLYaV on olive trees evaluating if it interferes in oil yield and quality.

## 2 Materials and methods

Two-hundred-twenty-two trees of 'Leccino' and 'Ottobratica' olive cultivar in an olive orchard located in Calabria region (Southern Italy) were analyzed during a tree years surveying to evaluate their health status. Diagnostic tests for viruses (*Arabis mosaic virus* - ArMV, CLRV, SLRV, CMV, OLV-1, *Olive latent virus-2* - OLV-2, OLYaV and *Tobacco necrotic virus* - TNV), required to be assent in Italian voluntary olive sanitary certification scheme, were RT-PCR using protocols and primers reported in Loconsole et al. [16] with some modifications. In particular, cuttings ca. 25-30 cm long were collected from 1- to 2-year-old twigs from the quadrant of each tree canopy. Total RNA (RNA<sub>t</sub>) extraction was performed using Rneasy Plant Mini Kit (Qiagen GmbH, Germany) according to protocol supplied by the manufacturer. Reactions were performed in 1X GoTaq buffer (Promega, USA, Medison) in a final volume of 25 µl containing 500 µM of dNTPs, 0.2 µM of each primer, 1.25 U of GoTaq DNA polymerase (Promega), 5 mM DTT, 5U of AMV (Promega), 20 U of RNase inhibitor and 2 µl of RNA<sub>t</sub> solution. PCR condition were common for all viruses, and consisted of 30 min incubation at 46°C followed by a denaturation step 95°C for 3 min; 35 cycles: 30 s at 94°C, 45 s

at 55°C, 45 s at 72°C. Reaction products were finally incubated at 72°C for 7 min. PCR products were detected with 1.5% agarose gel electrophoresis, using SYBR SAFE DNA gel stain (Invitrogen, USA).

To be sure about the plants sanitary condition, RT-PCR analyses were repeated two times (in spring and autumn) for three consecutive years. OLYaV was the only virus detected in 26% of tested trees, while other viruses were never found. Among tested plants six trees of 'Leccino' (*L*) and six of 'Ottobratica' (*O*) olive cultivar, homogeneous for ages (twelve year-old) and agronomical conditions, were selected for their sanitary status [three healthy/virus-free (H) and three OYLaV-infected (I)].

The genetic identity of each tree was accomplished by comparing 10 microsatellite markers preselected and used in molecular characterization of Italian olive germplasm [17].

Olive fruits were harvested from *L/O* and *H/I* trees when mature status was achieved and leaved separate for each tree. The oil extraction from 'Leccino' drupes was made an Oliomio (Toscana Enologica Mori, Tavarnelle Val di Pesa, Firenze, Italy) mill machine, and from 'Ottobratica' by a small olive oil press mill of the Company AgrimecValpesana, Calzaiolo, San Casciano (Florence-Italy) and then oil was centrifuged to eliminate water, filtered and stored in dark bottles without headspace at room temperature. The oil yield was determined as oil weight out of fresh drupes weight (%).

The main analyses on the oil quality, represented by the free acidity, the number of peroxides, the spectrophotometric indexes, were accomplished according to the methods contained in the Official Regulation of the European Community [18, 19]; the total content of chlorophylls and carotenoids as reported by Minguez-Mosquera et al. [20]; and the total phenol content as suggested by Baiano et al. [21].

The composition of the fatty acids, the total tocopherols and total sterols content were determined by FT-NIR (MPA, Bruker srl Italia) following the method described in Azizian et al. [22] and using previously developed calibration curves.

Each analysis was carried out in duplicate and the data, grouped by variety (*L/O*) and by sanitary status of plants (*H/I*), were statistically elaborated with *one-way* variance analysis and *post-hoc* test by Duncan using SPSS software version 11.

### 3 Results and discussion

The data obtained in 'Leccino' oils showed that oil yields (13.7% H vs 14.7% I) as well as the greater part of qualitative oil parameters were not statistically different in healthy and infected trees. Only the value of K<sub>232</sub> was significantly lower in oil from virus-free than from infected plants (1.29 H vs 1.35 I) (Table 1).

The oil yield values of 'Ottobratica' olives harvested from healthy and OLYaV affected plants did not have significant difference from each other (10.2% H vs 9.9% I). Almost all qualitative parameters were not statistically different between the oils obtained from plants H and I. In this cultivar, however, only the free acidity in oil of OLYaV-infected trees was significantly lower than oil of healthy plants (0.25% I vs 0.35% H) (Table 2).

Although only one significant difference has been found in qualitative characteristics between healthy and OLYaV-infected oils of both *L* and *O* cultivars, it is important to underline that all the oils are within the limits set by the EU Regulation for extra virgin olive oils. In fact, all samples showed

#### **4 Conclusions**

Based on the above-described findings and in particular on the evidence that, except for K<sub>232</sub> values, OYLaV does not interfere negatively in oil quality while surprisingly does it positively in free acidity, it seems appropriate that the European Union Council Directives (2014/96/EU, 2014/97/EU, 2014/98/EU) introduced in the last olive certification scheme only ArMV, CLRV and SLRSV as requiring laboratory-based diagnosis. In compliance with the aforementioned EU Directives, the mandatory sanitary certification scheme was amended in Italy (D.M. 06/12/2016), much less restrictive than voluntary Italian regulation (D.M. 20/11/2006) [11]. The application of compulsory EU directive, in Italy as in other countries, will improve the facilities of olive plants commercialization, guaranteeing enough their sanitary status. The findings here reported support the suggestion that the Italian Agriculture Ministry could be less restrictive in voluntary Italian regulation (D.M. 20/11/2006).

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Table 1. Results of tested parameters carried out on olive oils extracted from healthy (H) and OLYaV-infected (I) trees of Leccino cultivar

<b>Tested olive oil parameters</b>	<b>H</b>	<b>I</b>	<b>Sign</b>
Free acidity (% oleic acid)	0,23±0,04	0,20±0,07	n.s.
PV (mEq O <sub>2</sub> /kg)	6,76±0,94	4,54±4,00	n.s.
K <sub>232</sub>	1,29±0,12	1,35±0,04	**
K <sub>270</sub>	0,12±0,02	0,15±0,01	n.s.
<b>Total pigments</b>			
Total chlorophylls (mg/kg)	19,63±7,24	21,93±0,91	n.s.
Total carotenoids (mg/kg)	10,58±3,18	12,45±0,55	n.s.
<b>Fatty acid composition</b>			
C 14:0 (%)	0,01±0,00	0,01±0,00	n.s.
C16:0 (%)	11,32±0,14	11,52±0,19	n.s.
C16:1 (%)	0,79±0,11	0,87±0,14	n.s.
C17:0 (%)	0,02±0,01	0,02±0,00	n.s.
C17:1 (%)	0,03±0,01	0,03±0,00	n.s.
C18:0 (%)	2,21±0,12	2,28±0,07	n.s.
C18:1 (%)	79,92±0,35	79,98±0,73	n.s.
C18:2 (%)	4,07±0,65	3,97±0,47	n.s.
C18:3 (%)	0,68±0,02	0,51±0,12	n.s.
C20:0 (%)	0,45±0,02	0,62±0,13	n.s.
C20:1 (%)	0,21±0,08	0,22±0,05	n.s.
C22:0 (%)	0,10±0,00	0,10±0,00	n.s.
C24:0 (%)	0,02±0,03	0,03±0,01	n.s.
Total tocopherols (%)	74,80±63,70	46,87±20,07	n.s.
Total sterols (mg/kg)	1060±131	1067±22	n.s.
Total phenols (mg/kg)	367±20	396±24	n.s.
Oil yield (% f.w.)	13,7±0,10	14,7±0,10	n.s.

*Each value is the average of 6 analyses (duplicate analyses for three H/I plants) ± standard deviation; n.s.: not significant difference; \*\*Significance for  $p < 0,05$*



Table 2. Results of tested parameters carried out on olive oils extracted from healthy (H) and OLYaV-infected (I) trees of Ottobratica cultivar

<b>Tested olive oil parameters</b>	<b>H</b>	<b>I</b>	<b>Sign.</b>
Free acidity (% oleic acid)	0,35±0,01	0,25±0,04	**
PV (mEq O <sub>2</sub> /kg)	4,38±0,20	4,29±0,60	n.s.
K <sub>232</sub>	1,31±0,04	1,40±0,14	n.s.
K <sub>270</sub>	0,14±0,01	0,17±0,05	n.s.
<b>Total pigments</b>			
Total chlorophylls (mg/kg)	3,79±3,41	4,13±2,23	n.s.
Total carotenoids (mg/kg)	2,72±1,56	3,16±1,20	n.s.
<b>Fatty acid composition</b>			
C 14:0 (%)	0,01±0,00	0,01±0,00	n.s.
C16:0 (%)	11,33±0,42	11,45±0,14	n.s.
C16:1 (%)	1,01±0,26	1,11±0,16	n.s.
C17:0 (%)	0,02±0,00	0,02±0,00	n.s.
C17:1 (%)	0,04±0,01	0,04±0,01	n.s.
C18:0 (%)	2,55±0,11	2,49±0,09	n.s.
C18:1 (%)	77,65±1,24	77,15±0,51	n.s.
C18:2 (%)	6,51±0,69	6,80±0,23	n.s.
C18:3 (%)	0,88±0,10	0,88±0,05	n.s.
C20:0 (%)	0,45±0,01	0,45±0,01	n.s.
C20:1 (%)	0,21±0,03	0,20±0,04	n.s.
C22:0 (%)	0,10±0,01	0,10±0,01	n.s.
C24:0 (%)	0,00±0,00	0,00±0,00	n.s.
Total tocopherols (%)	214±50	190±44	n.s.
Total sterols (mg/kg)	1139±110	1186±14	n.s.
Total phenols (mg/kg)	702±190	703±86	n.s.
Oil yield (% f.w.)	10,2±0,9	9,9±1,1	n.s.

*Each value is the average of 6 analyses (duplicate analyses for three H/I plants) ± standard deviation; n.s.: not significant difference; \*\*Significance for  $p < 0,05$*