Asian Journal of Plant Pathology

ISSN 1819-1541

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS Asian Journal of Plant Pathology

ISSN 1819-1541
DOI: 10.3923/ajppaj.2017.28.34

Research Article
Sanitary Selection of Olive (*Olea europaea* L.) Cultivars for Worldwide Distribution

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Abstract

Background: Olive growing is an important agriculture branch worldwide and especially in Italy, where to avoid spread of the most dangerous olive pathogens several decrees regulate the certification program of plant propagating materials. Specifically, *Verticillium dahliae*, *Pseudomonas savastanoi*, phytoplasmas and viruses, due to their biological (systemic pathogens) and pathological (they induce severe diseases or are latent in olive but dangerous for other crops) characteristics are considered regulated pathogens. In order to divulge this positive experience this study described the way of adding new olive varieties to the certification program in Italy. **Materials and Methods:** Seventy two of the main olive cultivars grown in various Italian regions (Central and Southern Italy) were selected, observed and analyzed for the presence of *Verticillium dahliae*, *Pseudomonas savastanoi*, phytoplasmas and viruses. Specifically, all olive trees were analyzed by visual inspection for the presence of characteristic symptoms of verticillium wilt, olive knot, bumpy fruit and vegetative disorder in general, isolation for the fungus and molecular amplification for phytoplasmas and viruses, in different seasons through 2 years of activity. **Results:** The conducted survey allowed 10 cultivars from Calabria, 18 from Campania, 8 from Lazio and 10 from Umbria to be included in the Italian certification program. **Conclusion:** The use of these certified materials reduces risks of pathogen dissemination, ensure the commercialization of high quality olive plants and it is strongly recommended in the introduction of olive plants in a new area. The Italian certification program, validated also for other European countries can be easily adopted to any areas suited to the olive growing in order to avoid the risk of spreading dangerous pathogens and to improve olive oil production.

Key words: CLRV, SLRSV, ArMV, OLYaV, certification program

Received: March 23, 2016 Accepted: May 30, 2016 Published: December 15, 2016

Citation: F. Faggioli, A. Roschetti, M. Luigi, G. Leo and G. Albanese, 2017. Sanitary selection of olive (*Olea europaea* L.) cultivars for worldwide distribution. Asian J. Plant Pathol., 11: 28-34.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The continuous search for alternative sources of genetic variants in order to increase production to improve quality and to acquire new sources of resistance has re-launched the role of local varieties of the Italian olive germplasm in recent decades. The local cultivars can provide high quality oils and the production of mono-varietal oils is beginning to give an important prospect of revenue for many growers[1]. Moreover, the increasing expansion of olive crops in new areas of the world is stimulating the international demand and the exchange of olive germplasm, leading to the adoption at European and international level of harmonised certification programs to reduce risk of pathogen dissemination and to ensure the commercialization of high quality propagation material[2].

Olive trees could be infected by viruses, phytoplasmas, bacteria and fungi. Fifteen viruses were described infecting the olive trees, some of them are symptomless and identified only on few trees[3] but others, especially the ones included in the Italian certification program caused severe symptoms on olive trees like 'Bumpy fruit' by SLRSV[4] or on other plant species like CLRV causal agent of 'Black line' on walnut[5].

The first phytoplasmas were identified in olive in 1995, starting from it, they have been identified in Italy, Spain, Iran and classified in three groups: 16S-IB (Aster yellows group), 16S-VA (Elm yellows group) and 16S-XIIA (Stolbur group) always associated to foliage disorder[6-8].

The bacterium *Pseudomonas savastanoi* pv. *savastanoi* causes the most frequent disorder occurring in olive plants known as olive knot disease. This bacterial disease is present in all areas of the world where olive plants are cultivated. This is due to the ability of its causal agent to colonize the phylloplane of the tree.

The fungus *Verticillium dahliae* is a soil-borne pathogen that attacks olive trees (as well as over a hundred woody and herbaceous species), particularly when their roots are stressed. It causes the most severe disease suffered by olive plants, named verticillium wilt that induces yellow leaves, defoliation and death due to the fungus attacking the plants' vascular system. After the first report of verticillium wilt in Italy, it has later been detected in Algeria, Arizona, California, Egypt, France, Greece, Iran, Malta, Marocco, Syria, Spain and Turkey[9].

The epidemiology of most olive tree viruses is still unknown. Although some of these viruses are soil-borne *Strawberry latent ringspot virus* (SLRSV), *Arabis mosaic virus* (ArMV) and *Tobacco necrosis virus* (TNV) others can be transmitted mechanically *Tobacco necrosis virus* (TNV) by seed *Cherry leafroll virus* (CLRV) and *Olive latent virus* (OLV-1)[10], by aphids *Cucumber mosaic virus* (CMV) or only by mechanical inoculation and grafting *Olive latent virus*-2 (OLV-2) and *Olive latent ringspot virus* (OLRSV). Also regarding the epidemiology of phytoplasmas on olive trees the information was not adequate. Generally, infections by *Pseudomonas savastanoi* pv. *savastanoi* remain localized, resulting in gall formation at the infection site. Secondary galls, although rare can be initiated by bacterial movement within the xylem vessels of the olive. These secondary galls typically form in close proximity to the primary gall and the potential for a plant to support secondary gall development can vary by cultivar. Verticillium wilt can be transmitted through soil agents as water, manure etc.

All these pathogens however, expected to be transmitted by vegetative propagation of infected olive plants. The movement of the propagation materials, if not carefully managed, might have a very negative effect upon the sanitary state of the olive crop. The only way to guarantee the growing of healthy plants is: (i) To ensure that the planting material is certified from a sanitary point of view and (ii) To eliminate the factors that could contribute to field contamination, like viruses that harbored by the olive trees can cause serious diseases to other crops[11,12], so the distribution of infected olive material represents a potential threat to other crops or new agriculture areas. For these reasons, several Italian decrees regulate certification programs, either mandatory (DM 14/04/1997) or voluntary (DM 4/05/2006, DM 20/11/2006) of plant propagating material (Table 1). "Healthy" mother plants must be identified through sanitary and clonal selection performed within the framework of a certification program[2]. These laws also establish that trees should be submitted to visual inspection. If no symptoms referable to the pathogens listed in Table 1 are present, they are suitable for the next steps. Particularly, yellow leaves, leaf and fruit deformation or other alterations imputable to viruses or virus-like agents[11], tubercles attributable to *Pseudomonas savastanoi* pv. *savastanoi*, defoliation associated with a dark reddish-brown streak on the wood of branches or trunks, clearly due to *Verticillium dahliae* and/or typical phytoplasma symptoms like shoots proliferation, witche's brooms, leaf rolling and yellowing, hypertrophied inflorescences, dwarfing or decline, led to the olive trees not being considered eligible for the certification program. Then the virus presence must be ascertained by one-step RT-PCR. The selected molecular methods were validated in a ring test performed by eight Italian laboratories[13].

In recent years many efforts have been conducted to bring the highest number of Italian olive cultivars to the genetic and sanitary status suitable for their inclusion in the

Table 1: List of pathogens covered by certification program

Pathogen	Diagnosis	Sanitary status	
		Virus-free (VF)	Virus-tested (VT)
Viruses			
Arabis mosaic virus (ArMV)	RT-PCR or molecular hybridization	X	X
Cherry leafroll virus (CLRV)		X	X
Strawberry latent ringspot virus (SLRV)		X	X
Cucumber mosaic virus (CMV)		X	
Olive latent virus-1 (OLV-1)		X	X
Olive latent virus-2 (OLV-2)		X	
Olive leaf yellowing associated virus (OLYaV)		X	X
Tobacco necrosis virus (TNV)		X	
Phytoplasmas	Visual observation or PCR	X	X
Fungi			
Verticillium dahliae	Visual observation, isolation or PCR	X	X
Bacteria			
Pseudomonas savastanoi pv. *savastanoi*	Visual observation	X	X

X: The absence must be ascertained

Italian and European certification programs. Within this study, the most important olive cultivars of Calabria, Campania, Lazio and Umbria (Central-Southern Italy) were analyzed and results obtained are reported.

MATERIALS AND METHODS

Plant materials: From 1-4 olive trees for each of 19 varieties (already ascertained for their genetic trueness) from Campania, 17 from Lazio, 11 from Umbria and 25 from Calabria were analyzed in this study. Plants were singled out and selected either in germplasm collection fields and/or productive orchards for a total of 189 plants (Table 2).

Certification flowchart and pathogens detection: The best tree per for cultivar, chosen as a candidate to become nuclear stock material and applying the flowchart of sanitary procedures reported in Fig. 1 was assessed for its phytosanitary status. The visual monitoring was conducted every spring and autumn for the projects time-lapse on all plants chosen in this sanitary selection and when the symptoms described above were detected, the trees were discarded. Plants were also tested for presence/absence of different pathogens. Olive plants detected as pathogen-free for the first 3 years of analysis were monitored seasonally for two more years to confirm their phytosanitary status so they could be suitable to become nuclear stock plants.

The trees were analyzed for the presence of viruses listed in Table 1 using the RT-PCR one-step protocol already published and validated[13]. Briefly, 2 µL of TNA (extracting using RNeasy plant mini kit, Qiagen) were added to a 23 µL of mixture containing: GoTaqG2 buffer 1X (Promega), 125 µM each dNTPs, 5 mM DTT, 0.2 µM specific sense and antisense primers[3] 2.5 U of avian myeloblastosis virus (AMV)-RT (Promega), 20 U of RNase out (Life technologies) and 1.25 U GoTaq polymerase (Promega).

Synthesis of cDNA was performed at 46°C for 45 min, followed by denaturation at 95°C for 30 sec. Amplification was carried out for 35 cycles under the following conditions: Denaturation at 95°C for 30 sec, annealing at 55°C for 45 sec, extension at 72°C for 45 sec, followed by a final extension for 7 min at 72°C.

Specifically, at least two different subsets of samples for each tree (6-8 woody 1 year old branches) were collected in the autumn of the 1st year, in the spring and autumn of the 2nd year and in the spring of the 3rd year of investigation. Since in Calabria many plants were found to be virus-infected, the number of trees analyzed for each variety was increased up to 4, 5 or 12.

Diagnosis of *V. dahliae* was performed through an accurate search for foliar symptoms and vascular browning. Foliar chlorosis and necrosis could be due to other causes such as root rot diseases, for this reason the conclusive detection was attempted by isolating the fungus on agar media from suspected olive tissues.

The plants negative for *V. dahliae* and viruses were tested for phytoplasmas at the end of 3rd year, through nested-PCR on total DNA extracted from olive plants using a CTAB protocol[14]. Gene amplification was performed using a direct PCR with primers P1/P7, followed by a nested-PCR with primers R16/F2[15-17].

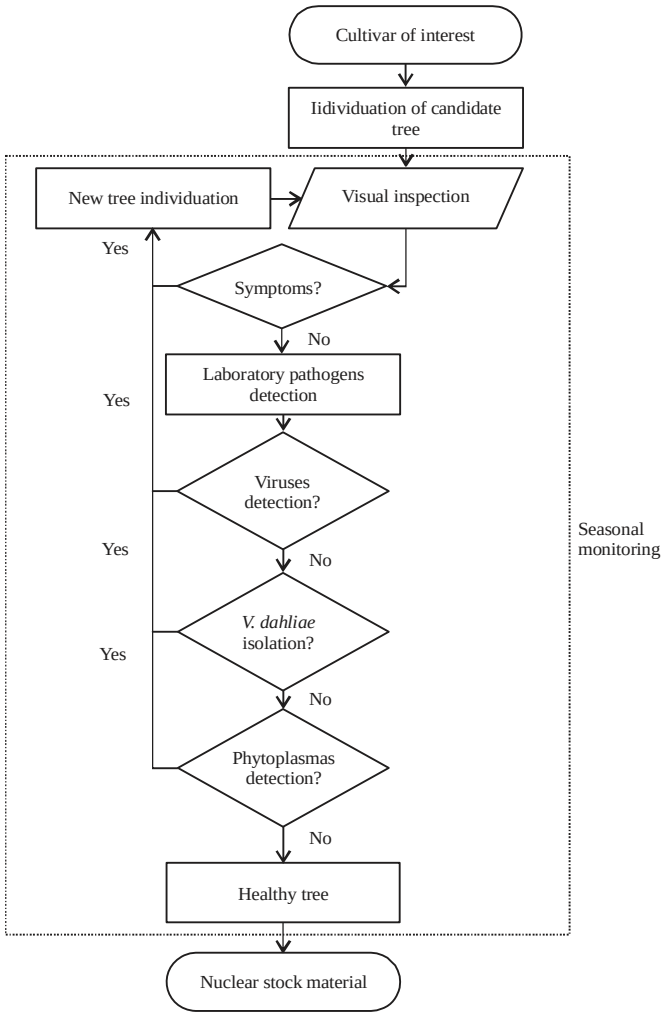

Fig. 1: Flowchart of sanitary procedures to allow candidate olive tree to become nuclear-stock material, No: Symptoms and/or laboratory analysis are negative for the searched pathogen, Yes: Symptoms and/or laboratory analysis are positive for the searched pathogen

RESULTS AND DISCUSSION

Verticillium dahliae was never isolated from the suspected samples as well as phytoplasma infections were never found in the trees analyzed.

Virus detection showed a differing situation according to the differing origin of the trees. From Campania, Lazio and Umbria 100 plants were analyzed and 10 were found infected. More specifically, 3 trees tested positive to *Olive leaf yellowing associated virus* (OLYaV), 6 to CLRV and 1 to ArMV (Table 2). In Calabria, 79 out of a total of 89 plants tested positive to OLYaV, whereas no other viruses were detected. Worthy of attention is the fact that in the region where virus infection was high, the detection of the infected plants takes more time, showing only two OLYaV-positive out of 25 samples tested (8%) during the first diagnosis, whereas 14/25 (56%) and 44/51 (86%) in 2nd and 3rd analyses, respectively. These inconsistency of the analyses conducted have been due to different factors: First, they confirmed the necessity to repeat tests for several subsequent spring and autumn seasons because of the erratic distribution of the viruses, above all OLYaV in olive plants as already reported[13], second that some vectors are present in the orchard and contribute to the transmission of the virus, in this case probably the presence of mealy bugs[18].

The high percentage of OLYaV-infected trees detected in this study in the Southern part of Italy and clearly shown in Fig. 2 was found also in previous studies[3,19-22]. The detection of a high number of virus-infected plants

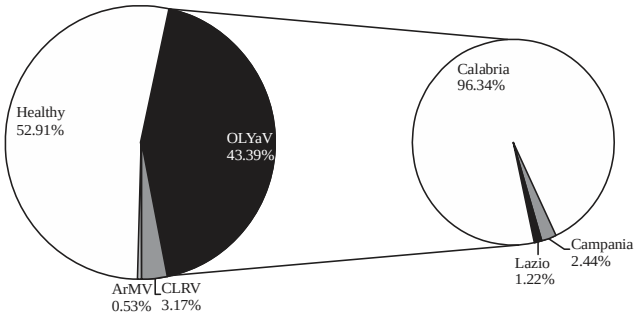

Fig. 2: On the left are reported the various percentages of infected trees found. On the right is highlighted the unequal distribution of OLYaV among the Italian regions

Table 2: Results obtained in analyzing the olive plants from Calabria, Campania, Lazio and Umbria regions

Cultivars/local synonyms	Region	No. of virus-infected/ tested trees	Cultivars/local synonyms	Region	No. of virus-infected/ tested trees
Agristigna	Calabria	4/4 (OLYaV)	Ravece	Campania	0/2
Borghese	Calabria	4/4 (OLYaV)	Ortice	Campania	0/2
Carolea/Becco di corvo	Calabria	3/4 (OLYaV)	Rotondella	Campania	0/2
Cassanese/Grossa di Cassano	Calabria	4/5 (OLYaV)	Ritonnella	Campania	1/3 (CLRV)
Ciciarello	Calabria	0/1	Biancolilla	Campania	0/3
Corniola/Cornale/Farisana	Calabria	0/1	Pisciottana	Campania	0/2
Dolce di Cerchiara	Calabria	4/4 (OLYaV)	Oliva Da Olio	Campania	1/2 (CLRV)
Grossa di Gerace/Geracese	Calabria	4/4 (OLYaV)	Ruvella	Campania	0/2
Imperiale	Calabria	4/4 (OLYaV)	Marina	Lazio	1/2 (ArMV)
Mafra	Calabria	4/4 (OLYaV)	Maurino	Lazio	0/2
Nera di Catinelle	Calabria	0/1	Olivastro	Lazio	1/2 (CLRV)
Nostrana/Nostrale	Calabria	4/4 (OLYaV)	Olivella	Lazio	0/2
Oliva di Cerchiara/Olivella di Cerchiara	Calabria	4/4 (OLYaV)	Raja	Lazio	0/2
Ottobratica	Calabria	12/12 (OLYaV)	Reale	Lazio	0/2
Pennulara	Calabria	4/4 (OLYaV)	Vallanella	Lazio	1/2 (OLYaV)
Romanella/Rotondello	Calabria	0/1	Pendolino	Lazio	0/2
Rossanese/Dolce di Rossano	Calabria	4/5 (OLYaV)	Rosciola	Lazio	0/2
Sinopolese	Calabria	0/1	Salviana	Lazio	0/2
Tombarello/Tummarello	Calabria	4/4 (OLYaV)	Canino	Lazio	0/2
Tonda di Filadelfia	Calabria	0/1	Carboncella	Lazio	0/2
Tonda di Filogaso	Calabria	4/4 (OLYaV)	Frantoio	Lazio	0/2
Tonda di Strongoli	Calabria	4/4 (OLYaV)	Itrana Precoce	Lazio	0/2
Tonda dolce	Calabria	4/4 (OLYaV)	Itrana S4	Lazio	0/2
Tondina/Roggianella	Calabria	0/1	Leccino	Lazio	0/2
Zuzufarica/Zinzifarica	Calabria	4/4 (OLYaV)	Moraiolo	Lazio	0/2
Tenacella	Campania	0/3	Borgiona	Umbria	0/2
Asprinia	Campania	1/4 (OLYaV)	Dolce Agogia	Umbria	0/2
Pampagliosa	Campania	0/2	Frantoio	Umbria	0/2
Cornia	Campania	2/3 (OLYaV, CLRV)	Leccino 3	Umbria	0/2
Salella	Campania	0/2	Moraiolo	Umbria	0/2
Raccioppella	Campania	0/2	Nebbia/Bianchella di Umbertide	Umbria	0/2
Caiazzana	Campania	0/2	Nostrale di Rigali/Giove	Umbria	0/2
Ortolana	Campania	1/2 (CLRV)	Raio	Umbria	0/2
Ogliarola	Campania	0/2	Rosciola (di Panigale)	Umbria	0/2
Carpellese	Campania	0/2	Tendellone/Fecciano	Umbria	1/2 (CLRV)
Tonda	Campania	0/2	San Felice	Umbria	0/2
Total					89/189 (47%)

In brackets the virus identified

highlighted the need to implement the olive sanitation and sanitary selection, being the only effective strategies to prevent the dissemination of olive systemic pathogens.

CONCLUSION

The finding of 57 virus-free olive cultivars out of 72 tested, together with their morphological and genetic

characterizations obtained in a parallel study, allowed them to be introduced in the certification program adopted in Italy. This ensures health, genetic trueness-to-type and uniformity, since the certified plants are obtained through subsequent clonal propagation steps, starting from a single registered nuclear-stock. After obtaining the registration approval from the Ministry of Agriculture, the pre-basic materials, derived directly from the propagation of the nuclear-stock materials, were produced and now they are maintained in insect-proof green-houses (at the conservation for pre-multiplication repository).

Production of valuable virus-free and true-to-type primary sources belonging to the most widespread or local varieties registered and propagated through the certification system, make the adoption of this scheme available to the growers (after the 2006 revision DM 20/11/2006), encouraging the carrying on of the certification program in Italy. Furthermore, the increase of the long distance movement of plant propagation material and of the expansion of olive crops in new areas may hopefully promote the use of common and harmonized certification procedures applied in all countries interested in olive growing.

SIGNIFICANT STATEMENT

This study describes a sanitary selection of olive cultivars carried out in order to avoid the risk of spreading dangerous pathogens and to qualify olive oil production. Specifically, 46 olive varieties have been selected and included in the Italian certification program. The use of high quality olive plants is strongly recommended for the introduction of olive plants in a new area. Moreover, it is highlighted also the importance in the monitoring on the presence of the main olive viruses during seasons to better control the phytosanitary state of trees.

ACKNOWLEDGMENTS

This study was supported by 'Progetto interregionale Qualificazione del vivaismo olivicolo-OLVIVA' and by 'Progetto PON Ricerca e Competitività 2007-2013 – PON03PE_00090_2'.

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