Chapter 6

Phytosanitary Certification

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Additional information is available at the end of the chapter

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1. Introduction

Olive plants are among the most ancient cultivated fruit trees. Over the centuries, propagation occurred mainly vegetatively. The longevity of trees and the latency of most of the virus infections allowed the dissemination through the propagative material of hidden viruses, which were not detected until recently, when the advent of novel diagnostic tools surprisingly revealed that virus infections are in fact widespread.

In the past, the selection of high value olive germplasm has been mainly based on the agronomic and pomological traits of the plants and on the quality and yield of the olive-derived product (oil). Specifically, investigation on the sanitary status of the selected ecotypes were done mainly by visually inspections. This fact leaded to the propagation and spread of systemic pathogens harbored either in a latent form or in the form of specific symptoms that initially have been confused with the phenotypic expression of the plant (as in the case of symptoms caused by infection of *Strawberry latent ringspot virus* (SLRSV) in the cultivar 'Raggiola')

In order to prevent the spread of dangerous pathogens (*Verticillium dahliae, Pseudomonas savastanoi* pv. *savastanoi)* and viruses in particular, remedies rely mainly on preventive measures such as the use of pathogen-tested propagative material. The main approach used to obtain, propagate and commercialize plants free from harmful pathogens is through phytosanitary selection and certification programs, which also encompass pomological selection for trueness to type and superior quality traits. In order to obtain pathogen-free material from infected trees, sanitation treatments such as heat therapy, meristem tip culture and micrografting, although still limited for their application for virus elimination in olive plants, can be applied.

A certification program is a procedure whereby single well-analysed candidate mother plants (nuclear stock plants) are used as sources of propagation material with a process of filiation. In this way, it is possible to provide growers with high quality (genetic and

sanitary) material. The certification scheme in general, and phytosanitary in particular, can be adopted either for worldwide spread varieties or for those locally distributed.

Each step of the propagation (descendent filiation) (Pre-basic, Basic and Certified material) must comply with the requirements that are intended to produce and maintain the selected material in the best growing conditions as specified by the enforced phytosanitary regulations. In particular, the sanitary status must be assessed following the officially recognized technical procedures, regarding the list of the target pathogens, type of sample, period of sampling and protocol for testing.

Phytosanitary selection requires the use of appropriate diagnostics protocols for pathogen detection. The difficulty in recognising and/or diagnosing virus-infected olive trees during field surveys imposes the use of laboratory tests in order to assess the absence of the target pathogens. Due to the lack of indicators for the biological assays and the unreliability of the ELISA test in olive, the application of molecular diagnostic techniques for viruses, fungi and bacteria detection became, in the recent past, critical for the assessment of the sanitary status of a given selected ecotype. These sensitive and reliable methods are absolutely necessary as they are at the basis of efficient and valid certification programs.

The increasing international demand for olive products, and therefore the expansion of olive crops, is stimulating the exchange of olive germplasm in new areas of the world, prompting for the adoption at European and International level of harmonised Certification Programs that reduce risks of pathogen dissemination and ensure the commercialisation of high quality propagative material and, consequently, guarantee high quality olive productions.

2. Systemic pathogens transmissible with the propagative material

To date, 8 virus-like diseases have been described, and fifteen different viruses (Tab. 1) and five phytoplasmas have been identified in olive plants. The actual Italian olive certification law (DM 20/11/2006) imposes the absence, in the propagation material, of some of the abovementioned pathogens as well as the most dangerous fungus and the most widespread bacterium in olive crops (Tab. 2). An appraisal of other ways of transmission than vegetative propagation if known, the susceptible hosts, effects and diseases caused by these pathogens and Countries where they have been detected in olive is reported below.

Arabis mosaic virus (ArMV) is a member of the genus *Nepovirus*, family *Secoviridae* (Sanfaçon et al., 2011). It is transmitted by the longidorid nematode *Xiphinema diversicaudatum,* but there is no evidence of its transmission to olive plants by this vector. The main hosts of this virus are strawberry, hop, *Vitis* spp., raspberry (*Rubus idaeus*), *Rheum* spp., *Sambucus nigra*, sugarbeet, celery, gladiolus, horseradish and lettuce. The most common symptoms induced by ArMV are leaf mottling and flecking, stunting and several forms of deformation including enations. Because of the serious damages caused on some crops this virus is inserted among the "harmful organisms known to occur in the community and relevant for the entire community" in Directive 2000/29/EC and its absence must be determined on plant material of *Fragaria* and *Rubus.* The symptoms vary depending on the host plant but also on

the virus isolate, cultivar, season and year. Many plant species infected with ArMV, including olive trees, do not show any symptoms (Martelli et al., 2002). The virus has been reported in olive trees from Italy (Savino et al., 1979), Portugal (Martelli, 2011) Egypt, USA (Saponari & Savino, 2003), Turkey (Çağlayan et al., 2004), Syria (Alabdullah et al., 2005) and Lebanon (Fadel et al., 2005).

Cherry leaf roll virus (CLRV) belongs to the family *Secoviridae*, genus *Nepovirus,* subgroup c. Even if it is classified as a *Nepovirus*, its transmission by nematodes has not yet been demonstrated to date, whereas it effectively occurs by pollen and, in some hosts, very efficiently by seed too. In olive plants, its transmission by means of pollen has not been demonstrated, but has been ascertained by seeds at the rate of 41% (Saponari et al., 2002). CLRV infects many herbaceous, shrubs and woody plants of genera: *Betula, Celtis, Cornus, Fagus, Juglans, Ligustrum, Olea, Populus, Ulmus, Rubus, Sambucus* and *Rheum*. The virus often induces symptoms in ash, birch, cherry, elderberry and walnut including delayed leaf development, chlorotic leaf streaks or spots, as well as dieback of branches or whole trees but it is symptomless in olive trees (Savino & Gallitelli, 1981). Its presence in olive trees was reported in Italy, Portugal, Spain (Martelli, 1999), then in Egypt, USA (Saponari & Savino, 2003), Turkey (Çağlayan et al., 2004), Syria (Alabdullah et al., 2005), Lebanon (Fadel et al., 2005) and recently in Croatia (Luigi et al., 2011), where it has been shown to have a negative impact on olive fruit and virgin oil quality (Godena et al., 2012).

Strawberry latent ringspot virus (SLRSV) is an unassigned species in the *Secoviridae* family. It is transmitted by the nematode *X. diversicaudatum* and by seed in several species (Cooper, 1986), but in olive plants, these kinds of means of transmission have not been demonstrated. SLRSV infects strawberry and raspberry, mostly without symptoms but resulting in various degrees of mottle and decline in some cultivars. The virus was isolated for the first time from olive in cv. 'Corregiolo' in Italy (Savino et al., 1979) and later in Portugal (Henriques et al., 1992), Spain (Bertolini et al., 1998), Egypt, USA (Saponari & Savino, 2003), Turkey (Çağlayan et al., 2004), Lebanon (Fadel et al., 2005), Syria (Alabdullah et al., 2005), Croatia (Bjelis et al., 2007), Tunisia (Martelli, 2011) and Albania (Luigi et al., 2009). Small, pear-shaped, puckered fruits with deformed kernels (bumpy fruits), narrow and twisted leaves, bushy growth and reduced crop were described in olive trees of cv. 'Ascolana tenera' affected by SLRSV (Marte et al., 1986). Similar symptoms were observed in cvs 'Negrinha' and 'Galega' in Portugal, associated with a severe reduced rooting ability of the cuttings (Henriques et al., 1992). Among 15 different olive cultivars reporting plants being affected by SLRSV in Portugal, only some showed symptoms (Henriques et al., 1992) in agreement with what was observed in Italy (Savino et al., 1979; Marte et al., 1986); no symptoms are apparently associated with SLRSV infections in Spain (Bertolini et al., 1998). Very interesting is the fact that previously, the 'Raggiola' and 'Frantoio' were considered different olive varieties due to morphological and agronomical dissimilarities. A relatively recent study showed that the two cultivars are genetically identical and that their differentiations are due to the constant presence of SLRSV in 'Raggiola' and the repeated SLRSV absence in 'Frantoio' (Fig. 1) (Ferreti et al., 2002). Rooting trials conducted to compare SLRSV-infected 'Raggiola' with virus-free 'Frantoio' showed that the virus does not influence the rooting

rate of olive cuttings (Roschetti et al., 2009), contrary to what had been previously reported in Portugal.

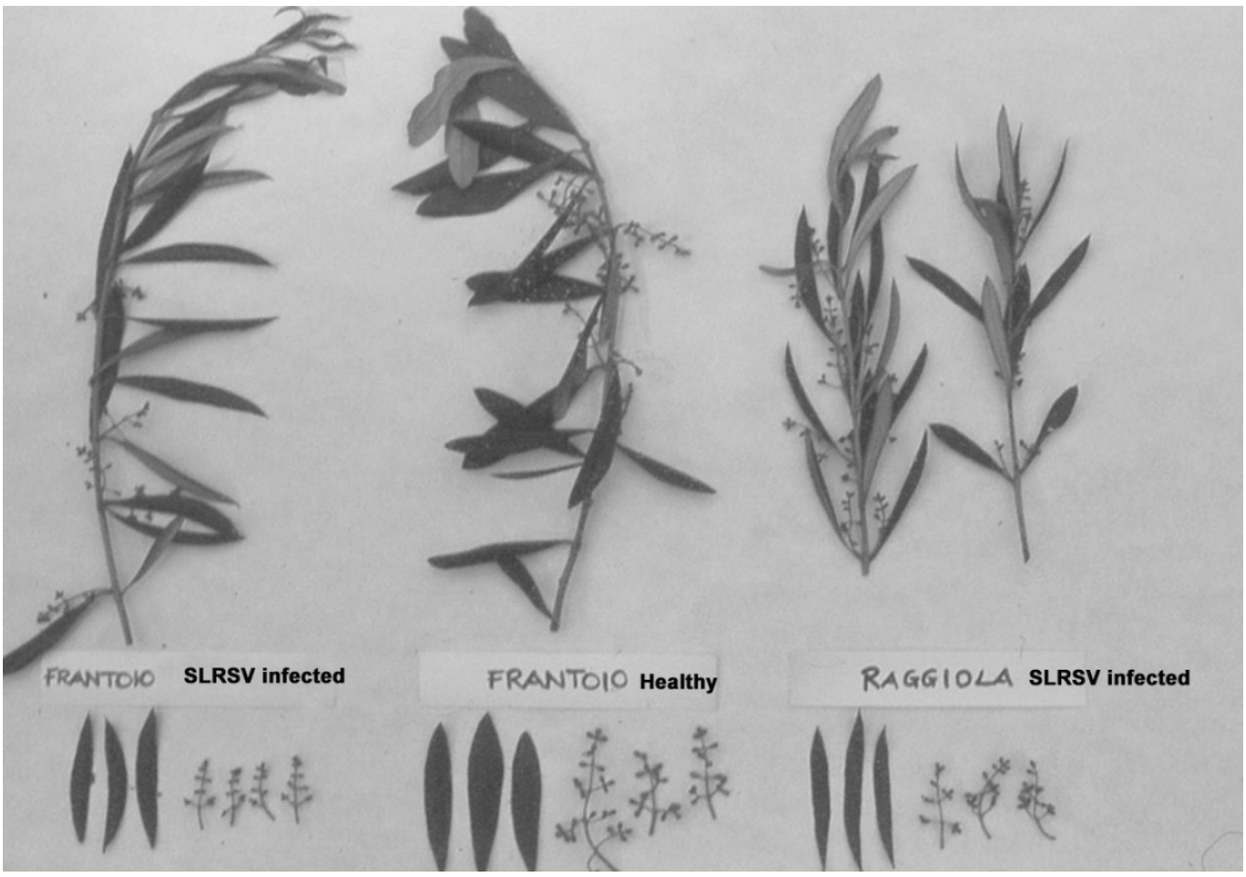

Figure 1. Phenotypic expression of olive cultivars 'Frantoio' SLRSV-affected, healthy 'Frantoio' and 'Raggiola' SLRSV-affected. See as morphological aspect of 'Frantoio' SLRSV-affected and 'Raggiola' SLRSV-affected are identical (narrow leaves and small inflorescences)

Cucumber mosaic virus (CMV) belongs to the genus *Cucumovirus*, family *Bromoviridae*. It is one of the most dangerous virus affecting vegetable plants (about 800 wild and cultivated plant species are its hosts). CMV induces important vegetative and productive reductions (up to 100% in plants such as tomato and pepper). When this virus affects herbaceous plants, it is transmitted very efficiently by 75 different aphid species and with varying efficiency by seed. CMV infection in olive is symptomless and its transmission by aphid vectors to/from olive has not yet been proven. It was isolated the first time from olive trees by Savino and Gallitelli (1983) in Italy. This report was confirmed in Portugal by Rei et al. (1993) who detected CMV alone, as well as together with SLRSV. CMV in olive trees was also found in Spain (Bertolini et al., 1998), Turkey (Çağlayan et al., 2004), Syria (Alabdullah et al., 2005), Croatia (Bjelis et al., 2007), Tunisia (Martelli, 2011) and recently in California (Al Rwahnih et al., 2011).

Olive latent virus 1 (OLV-1) is a member of the genus *Necrovirus*, family *Tombusviridae*. The virus is one of the few viruses detected in olive trees that is transmitted by seed (at a rate of 82%) (Saponari et al., 2002). It was detected in olive trees in Italy (Gallitelli & Savino, 1985),

Jordan (Martelli et al., 1995), Portugal (Felix & Clara, 2002), Egypt, USA (Saponari & Savino, 2003), Lebanon (Fadel et al., 2005), Syria (Alabdullah et al., 2005), Tunisia (Martelli, 2011) and Turkey (Serce et al., 2007). Several OLV-1 isolates have been obtained from symptomless or weakened trees. Since this virus has also been isolated from citrus in Turkey and Italy (Martelli et al., 1996) and from tulips in Japan (Kanematsu et al., 2001) it is reasonable to assume that it, as well as other olive viruses, may also have a larger host range.

Olive latent virus 2 (OLV-2) is the type species of the monotypic genus *Oleavirus*, family *Bromoviridae* (Grieco & Martelli, 1997). OLV-2 was isolated by mechanical inoculation from symptomless olive trees in Apulia, Southern Italy (Savino et al., 1984). It has subsequently been identified in Lebanon (Fadel et al., 2005), Syria (Alabdullah et al., 2005), Croatia (Bjelis et al., 2007) and Tunisia (Martelli, 2011) from symptomless olive cultivars. The host range of OLV-2 was limited to olive trees until castor beans (*Ricinus communis* L.), showing yellowish vein netting and systemic mottling on leaves, were reported in Greece to be infected with this virus (Grieco et al., 2002).

Olive latent virus 3 (*OLV-3*) is classified as a tentative member of the genus *Marafivirus,* family *Tymoviridae*. The virus is not mechanically transmitted. Search of possible vectors, *Euphyllura olivina* and *Saissetia oleae,* was not successful even if OLV- 3 was detected by RT-PCR in the psyllid. A survey conducted in the Mediterranean region showed the OLV-3 presence in Italy, Syria, Malta, Tunisia, Portugal, Turkey, Lebanon and Greece with an infection rate average of 30% always in symptomless olive trees *(*Alabdullah et al., 2010).

Olive latent ringspot virus (*OLRSV*) is an approved species of the genus *Nepovirus,* family *Secoviridae*. The virus is transmitted by mechanical inoculation and the existence of a natural vector is unknown. OLRSV is latent in olive trees, but it causes some symptoms on diagnostically susceptible hosts, such as apical necrosis on *Chenopodium quinoa* and *C. amaranticolor,* and red-rimmed local lesions and malformation on tip leaves of *Gomphrena globosa.* The virus was isolated from asymptomatic olive trees in Lazio, Central Italy (Savino et al., 1983), in Portugal in 1990, in Syria (Alabdullah et al., 2005) and then in Tunisia (Martelli, 2011).

Olive leaf yellowing associated virus (OLYaV) is an unassigned species in the family *Closteroviridae.* Various studies have been published (Sabanadzovic et al., 1999; Essakhi et al., 2006; Luigi et al., 2010) and are still in progress to define its taxonomic position. OLYaV presence in psyllid *E. olivina* and unidentified mealybugs of genus *Pseudococcus* gave the indication that transmission by these vectors could be possible (Sabanazdovic et al., 1999). The olive leaf yellowing (OLY) disease was recorded for the first time in Italy on cv. 'Biancolilla' (Savino et al., 1996) and it is characterized by a bright leaf yellow discoloration (Fig. 2). A survey conducted in Italy showed that, in old OLYaV-affected olive trees, leaf yellowing symptom is frequently absent (Albanese et al., 2003). The OLY syndrome, consisting of poor fruit set, bright yellow discoloration of the foliage, mottling, necrosis, extensive defoliation and dieback, has been associated to other viruses such as *Olive vein yellowing associated virus (OVYaV)* (Faggioli & Barba, 1995) and *Olive yellow mottle and decline associated virus (OYMDaV)*

(Savino et al., 1996), but their presence on olive trees was very rare. On the other hand, OLYaV seems to be one of the most widespread olive viruses: in Italy it infects more than 60% of southern Italy olive cultivars (Faggioli et al., 2005) and it has also been reported in high percentages in Israel (Martelli, 2011), Egypt, USA (Saponari & Savino, 2003), Lebanon (Fadel et al., 2005), Spain (Martelli, 2011), Syria (Alabdullah et al., 2005), Albania (Luigi et al., 2009), Croatia (Bjelis et al., 2007), Tunisia (Martelli, 2011) and California (Al Rwahnih *et al.,* 2011). A study on the rooting and grafting capacity of OLYaV-infected 'Carolea' and its respective healthy controls showed that the virus does not influence the rate of rooting of the cuttings and does not interfere with the grafting success rate; positive significant effects in grafting ability were observed on infected material only during a temperature stress, probably due to the reduced water need of infected shoots (Roschetti et al., 2009). Significant difference in vegetative growth was observed between virus-free and OLYaV-infected young olive plants, demonstrating negative OLYaV interference (Cutuli et al., 2011). To date, no other hosts have been found for this virus.

(a) (b)

Figure 2. Yellowing symptoms in an olive cultivar 'Carolea' (a) which tested positive for OLYaV and detail of yellow leaves of the same tree (b)

Olive semilatent virus (OSLV) is still unclassified. It was transmitted mechanically from olive tree to *Nicotiana benthamiana* (Materazzi *et al.*, 1996). The main symptom observed in Italy on OSLV-affected olive trees was a very mild chlorotic vein clearing of the leaves, but there is not enough evidence of the etiological involvement of this virus in the disease (Martelli, 1999).

Tobacco mosaic virus (TMV) belongs to the genus *Tobamovirus,* family *Virgaviridae*. Mechanical transmission to herbaceous indicator plants was possible, but not easily. It was isolated in central Italy from olive trees showing vein banding, discolorations along the main veins, severe defoliation and decline (Triolo et al., 1996). However, there is no conclusive evidence that TMV is agent of these symptoms.

Tobacco necrosis virus (TNV). Viruses with properties similar to those of TNV were first detected in symptomless olive trees by Félix & Clara (2002) in Portugal. One isolate was studied further, revealing its identity as TNV-D species (Cardoso et al., 2004). However, further genomic characterization of this isolate led to its classification as a new species in the *Necrovirus* genus named *Olive mild mosaic virus (OMMV)* (Cardoso et al., 2005). To date, it is not clear if TNV-D can be considered among the viruses isolated from olive trees, even if recent data shows the presence of this species in olive trees (Cardoso and colleagues deposited the complete genome sequence of a TNV-D isolate from olive trees in the Gene Bank, accession number FJ666328). Virions are readily transmitted by mechanical inoculation and naturally by the fungus *Olpidium brassicacae.* TNV has a wide host range that includes monocotyledonous and dicotyledonous plants, which frequently cause necrotic lesions on the roots and leaves.

Acronym	Virus species	Genus	Geographical distribution
OLV 1	*Olive latent virus 1*	*Necrovirus*	Italy, Jordan, Portugal, Egypt, USA , Lebanon, Syria, Turkey, Tunisia
OLV 2	*Olive latent virus 2*	*Oleavirus*	Italy, Syria, Croatia, Lebanon, Tunisia
OLV 3	*Olive latent virus 3*	*Marafivirus*	Greece, Italy, Lebanon Malta, Portugal, Syria, Tunisia, Turkey
OLRSV	*Olive latent ringspot virus*	*Nepovirus*	Italy, Portugal, Syria, Tunisia
OVYaV	*Olive vein yelloving associated virus*	*Potexvirus*	Italy
OYMDaV	*Olive yellov mottling and decline associated virus*	Unclassified	Italy
OLYaV	*Olive leaf yellowing associated virus*	*Closteroviridae,* unassigned species	Albania, Croatia, Egypt, Italy, Israel, Lebanon, Spain, Syria, Tunisia, USA
OSLV	*Olive semilatent virus*	Unclassified	Italy
OMMV	*Olive mild mosaic virus*	*Necrovirus*	Portugal
SLRSV	*Strawberry latent ringspot virus*	*Secoviridae,* unassigned species	Croatia, Egypt, Italy, Lebanon, Portugal, Spain, Syria, Tunisia, Turkey, USA
CLRV	*Cherry leafroll virus*	*Nepovirus*	Croatia, Egypt, Italy, Lebanon, Portugal, Spain, Syria, Tunisia, Turkey, USA
ArMV	*Arabis mosaic virus*	*Nepovirus*	Egypt, Italy, Lebanon, Portugal, Syria, Turkey, USA
CMV	*Cucumber mosaic virus*	*Cucumovirus*	Croatia, Italy, Portugal, Spain, Syria, Tunisia, Turkey, USA
TMV	*Tobacco mosaic virus*	*Tobamovirus*	Italy
TNV	*Tobacco necrosis virus*	*Necrovirus*	Portugal

Table 1. Viruses identified in olive trees and their geographical distribution (Martelli, 2011; Çağlayan et al., 2009)

Phytoplasmas constitute a monophyletic clade within the *Mollicutes* class. Their classification has been possible through the use of restriction fragment length polymorphism (RFLP) analysis and sequencing of the conserved 16S rRNA gene (Lee et al., 1998; Semüller et al., 1998). A variable range of symptoms in olive trees such as shoot proliferation, shortening of internodes, witches'-brooms, little leaves (Fig. 3a), leaf rolling and yellowing, leaf bronzing, phyllody, flower abortion, hypertrophied inflorescences (Fig. 3b), fasciation, erect growth, dwarfing, decline and die-back have been frequently associated with the presence of phytoplasma in Spain, Italy and Iran (Ahangara et al., 2006; Bertaccini et al., 2002; Font et al., 1998; Pasquini et al., 2000). Identification of phytoplasmas detected in olive plants showed they were members of the 16S-IB (Aster yellow), 16S-IC (Clover phyllody), 16Sr-III (Peach X disease), 16S-VA (Elm yellow) or 16S-XIIA (Stolbur) groups and subgroups. The failure to detect phytoplasmas in many symptomatic olive trees leads to doubts on whether these type of alterations could be associated with other causes (Barba, 1993; Camele et al., 1999). Nevertheless, phytoplasmas detected in olive plants are agents causing very well known and severe diseases in other hosts. These include aster yellow, clover phyllody, peach X disease, elm yellow and stolbur in solanaceous plants, as well as grapevine yellow (= Bois Noir). Even if their transmission by leaf-hopper vectors has been proven for some of them (among various host plants but not yet in and from olive plants) their presence in olive plants indicates a serious potential threat for other important crops.

(a) (b)

Figure 3. Shortened internodes, witches'-brooms and little leaves (a); hypertrophied inflorescences (b) on olive trees affected by phytoplasmas

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 (Fig. 4) and death due to the fungus attacking the plants' vascular system. Internally, a dark reddish brown streak on the wood occurs in most plants. This is

visible on branches when the bark peeled off. If the cross-section of infected branches or trunks is examined, the brown woody coloration may appear as a ring. Although some plants may die quickly, more commonly trees with only a few wilted branches during a growing season become more severely infected the following year. 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 (Bubici & Cirulli, 2011). Another species, *V. albo-atrum*, may occasionally cause the same disease in olive plants.

Figure 4. Yellow leaves, defoliation and wilt of olive caused by *V. dahliae* (photo by Antonio Ippolito)

The bacterium *Pseudomonas savastanoi* pv. *savastanoi* causes the most frequent disorder occurring in olive plants known as olive knot disease. The disease manifests itself through the growth of tubercles (Fig. 5) , which either appear individually, or in clusters on any part of the plant, but most commonly on twigs, young branches and around wounds on the main trunk. Knots can damage the stem structure and can deform the scaffold of the tree if infection is severe during the early stages of the tree. This may become a serious problem in nurseries that grow olive plantlets for marketing. *P. savastanoi* causes a similar disease in other plants as oleander, ash, jasmine, Japanese privet, *Forsythia* spp., *Phyllirea* spp., *Retama sphaerocarpa, Rhamnus alathernus* and myrtle (Surico & Marchi, 2011). 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.

Figure 5. Olive knots: rough galls and swellings on twigs and branches caused by *P. savastanoi* pv. *savastanoi*

Harmful organisms	Sanitary status		
	Acronym	Virus-free (VF)	Virus-tested (VT)
VIRUSES:			
Arabis mosaic	**ArMV**	**X**	**X**
Cherry leafroll	**CLRV**	**X**	**X**
Strawberry latent ringspot	**SLRSV**	**X**	**X**
Cucumber mosaic	**CMV**	**X**	
Olive latent 1	**OLV-1**	**X**	**X**
Olive latent 2	**OLV-2**	**X**	
Olive leaf yellowing associated	**OLYaV**	**X**	**X**
Tobacco necrosis	**TNV**	**X**	
PHYTOPLASMAS		**X**	**X**
FUNGI:			
Verticillium dahliae		**X**	**X**
BACTERIA:			
Pseudomonas savastanoi pv. *savastanoi*		**X**	**X**
NEMATODES:			
Meloidogyne incognita		**X**	**X**
Meloidogyne javanica		**X**	**X**
Pratylenchus vulnus		**X**	**X**
Xiphinema diversicaudatum		**X**	**X**

X = the absence of this organism must be ascertained

Table 2. Pathogens and pests that must be absent in order to obtain the "virus–free" or " virus–tested" sanitary status according to Italian olive certification law (DM 20/11/2006)

3. Strategies to control invasive olive pathogens and the importance of phytosanitary certification

Pathogens associated with olive propagative material may be systemic (viruses and phytoplasmas and probably *P. savastanoi* pv. *savastanoi*) or associated with the vascular system (*V. dahliae*) and they are unlikely to be eliminated during the vegetative propagation of an infected source. Accordingly, local and long-distance spread of these pathogens through the movement of infected propagative material has caused a highly threatening worldwide distribution of infectious diseases. The symptomless nature of several olive virus infections may also contribute to the inadvertent propagation and distribution of infected material.

To avoid disease and/or pathogen dissemination through vegetative propagation, possible remedies include mainly preventive strategies based on the use and propagation of "healthy" mother plants. In fact, in order to attain sanitary improvements of any crop, a system of preventive, protective and often of sanitation measures has to be established and implemented, encompassing a complex series of interventions currently referred to as "phytosanitary selection and certification".

In the framework of a phytosanitary and clonal improvement program, the main activities include: (i) field surveys for the selection of olive trees with no apparent disease symptoms and fulfilling the pomological traits of the cultivar; (ii) samples collection for laboratory tests, both for pathogen detection and DNA marker analysis; (iii) molecular tests (RT-PCR, dot blot hybridization and dsRNA analysis) for the detection of viruses included in the certification program; (iv) genetic characterisation using SSR markers; (v) sanitation by heat therapy, meristem tip culture and micrografting in case of no healthy trees being detected for one or more cultivars; (vi) propagation of the candidate nuclear stocks under conditions that ensure freedom from re-infections, usually in insect-proof greenhouses.

Field surveys should be carried out in the main olive-growing areas for the specific cultivar undergoing the clonal and sanitary selection program. Usually mature trees are selected (i.e. 25-year-old) based on visual inspection during spring and autumn. Samples for virus testing consist of 10-15 cuttings collected from 1- to 2-year-old twigs or young leaves for DNA extraction and SSR marker analysis.

Despite limited information being available on the application and effectiveness of sanitation protocols on olive plants, *in vivo* and *in vitro* heat therapy, *in vitro* shoot tip culture and micrografting have all been applied in attempts to regenerate OLYaV- and CLRV-free material and some successful results have been obtained (Bottalico et al, 2002). For *in vivo* heat therapy, plants can be grown at 38°C for 3 to 12 months. During heat therapy, 2 to 2.5 cm long shoot tips are excised no earlier than three months from the beginning of the treatment. After surface-sterilization in 0,05% mercuric hydrochloride for 10 min, the shoot tips are placed *in vitro* in petri dishes on different media according to the cultivar [OM (Rugini, 1984); MSM media (Leva et al., 1994)] and grown at 24°C with a 16 h photoperiod.

Regenerated explants are subcultured 3 or 4 times every four weeks using the proliferation medium reported by Rugini (1984), prior to transplanting in jiffy pots. For *in vitro* heat therapy, 2 to 3 cm long in vitro plantlets are exposed for 1 to 3 weeks to 38°C. After heat treatment and subcultures, the surviving plantlets are transplanted in jiffy pots.

For shoot tip culture, apexes are excised from well-established *in vitro* cultures maintained on OM or MSM media. Regenerated apexes are subcultered on the same media prior to being transplanted in jiffy pots.

Olive seedlings for micrografting are recovered from seeds soaked for a few seconds in 70% alcohol before the endocarp removal. Kernels are then soaked for 10 min in 0.05% mercuric hydrochloride solution, rinsed three times in sterile water, and placed on wet sterilized paper in petri dishes at 25° in the dark. After 2-3 months, the regenerated seedlings are cut, leaving about 1.0 cm of the epicotyl. Shoot apexes, excised from *in vitro* grown plantlets, are then grafted on the top of decapitated seedlings. After grafting, plants are cultured in rooting medium (Rugini, 1984). Generally less than 70% of the grafts are successful, and only 10% of the plants survive after transplantation in the soil.

Although phytoplasma diseases may be cured by treatment with certain classes of antibiotics and by heat water therapy, such approaches have not been applied to olive plants in order to obtain sanitation from these pathogens. This is because olive trees affected by phytoplasmas are very rare, and during a phytosanitary selection, phytoplasma-free plants can be easily found. Since their transmission by leaf-hopper vectors is ascertained for other crops, growing nuclear stock plants in insect-proof greenhouses ensures also freedom of infection from phytoplasmas.

The detection of *V. dahliae*-free plants must be carried out with great care. A visual diagnosis is in fact insufficient in guaranteeing the absence of this fungus and have recommended the use of new and sensitive diagnostic tools that are now available (see paragraph 4).

In order to prevent infections by *P. savastanoi* pv. *savastanoi* selected materials must be free from symptoms of the disease, and before propagation material is harvested, mother plants must be sprayed with a copper-based treatment to reduce risk of infections by the epiphytic bacterial population.

The candidate nuclear-stock material obtained through the field selection and/or sanitation treatments describe above can enter the certification program upon official approval (see paragraph 5), and genetically and sanitary certified propagative material will be available to growers.

Demand for olive products is constantly increasing in local and foreign markets, stimulating the expansion of olive crops and encouraging the exchange of olive germplasm at an international level. The activation of a selection and certification program is thus crucial to guarantee the quality of the propagative material and reduce risks for pathogen dissemination.

4. Identification of olive pathogens: updates on diagnostic tools

Sanitary certification programs require reliable and sensitive diagnostic tests in order to allow for the identification of pathogen-free trees and the assessment of their overall plant production processes. Due to the latency of several infections caused for example by viruses, visual inspections are not reliable and laboratory tests must be performed to certify virus-free or virus-tested materials. Biological tests and serological assays, widely used to detect pathogens affecting other crops like stone fruits, grapes, pome fruits, result ineffective in olive plants due to the absence of differential woody indicators for the bioassays and the low viral titre and/or to the interference by some contaminants. All these factors have made olive tree virus diagnosis very problematic. Luckily, in the last decade several molecular approaches have been developed and improved to detect olive viruses, bacteria and fungi in the propagating materials. Different molecular techniques such as RT-PCR in single/double step or nested, PCR, real time PCR, dot blot hybridization and dsRNA analysis, have been implemented in recent years and drastically improved sensitivity and specificity of olive-infecting pathogens' diagnosis. Recently, molecular technology has been successfully applied for routine and large scale detection and could easily be transferred to those Countries that intend to develop their olive crops through production, maintenance and distribution of healthy (virus-free or virus-tested) planting material.

Concerning viruses, RT-PCR assay has proved to be the most rapid, sensitive and reliable technique for detecting an RNA target in infected plants, and in recent years, different protocols have been developed for olive viruses detection (Grieco et al., 2000; Bertolini et al., 2001a, 2003; Pantaleo et al., 2001; Faggioli et al., 2002, 2005). Recently, a one step RT-PCR protocol has been set up and validated in an inter-laboratory ring test (Loconsole et al., 2010) for the diagnosis of the eight most important olive viruses. This should be a starting point for anyone wishing to approach the sanitary selection of olive plants. New and improved diagnostic techniques (e.g. Real Time RT-PCR, multiplex RT-PCR, polyprobe for molecular hybridization) will be continuously developed as the knowledge on the genetics and biology of olive-infecting pathogens advances.

Phytoplasma detection is now accomplished through nested-PCR on total DNA extracted from olive plants using the protocol of Barba et al. (1998). Gene amplification is performed using a direct PCR with primers P1/P7 (Deng & Hiruki, 1991; Schneider et al., 1995), followed by a nested-PCR with primers R16F2/R2 (Lee et al., 1993). The use of this analysis allows to determine whether plants are affected by phytoplasma, but does not give information about the identity of the pathogen. Identification of phytoplasma can be achieved through restriction fragment length polymorphism (RFLP) analysis, but it is not required for sanitary certification since the certified olive material must be free from all phytoplasmas. In recent years, the diagnostic technique has also been developed and improved for phytoplasmas. Real time PCR protocols for the identification and group characterization of phytoplasmas are now available. Whilst not yet applied to the diagnosis of olive phytoplasmas, these techniques have all the necessary features for this purpose, and there is therefore potential for their use in the near future (Christensen et al., 2004; Hodgetts et al., 2009).

Diagnosis of *V. dahliae* is preliminarily 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, whereas browning on cross section of stems was sometimes not found. Conclusive detection is attempted by isolating the fungus on agar media from olive tissues and possibly using PCR or nested PCR with *V. dahliae* specific primers (Nigro et al., 2002; Mercado-Blanco et al., 2002) or by Real Time Scorpion PCR (Schena et al., 2004).

Identification of *P. savastanoi* pv. *savastanoi* is very easy when the typical knots are present on plants. Nevertheless, its presence in latent and systemic form has been reported by Penyalver et al., (2006). The presence of *P. savastanoi* pv. *savastanoi* both as epiphytic and entophytic agent makes its control in the certified material absolutely compulsory; moreover, for a more sensitive and reliable diagnosis, molecular techniques are needed. Recently, molecular protocols of nested-PCR have been set up to obtain reliable diagnoses of latent infections (Bertolini et al., 2001b). This is also possible (and has been done) simultaneously with four other olive viruses (Bertolini et al., 2003).

5. Phytosanitary certification program: The Italian experience in the last twenty years

The production of healthy, high-quality olive products depend to a large extent on the quality of the plant material used for olive cultivation. In general, the production of "healthy" plants for planting occurs through defined certification procedures by which a particular cultivated selection, whose health status and trueness to type have been officially attested, is propagated following specific requirements. In a typical certification scheme, the certified material is descended by a defined number of propagation steps from individual plants, found to be free from pathogens and pests, maintained and propagated under rigorous conditions that exclude recontamination.

To this end, appropriate regulations are necessary to ensure the production, marketing and movement of certified plant propagation material with high standards and free from harmful pathogens (Annex I). Over the past twenty years, through globalisation and the expansion of several crops in new areas, concerns were raised about new disease emergencies transmitted by infected propagating material. In an attempt to limit the potential impact of the spread of pests and pathogens through the movement of infected plants, specific laws have been issued at regional, national and European levels.

The Council Directive 2008/90/EEC of 29 September 2008 (recast version of Directive 92/34/EEC) on the marketing of fruit plant (including olive) propagating material and fruit plants intended for fruit production established a harmonized Community regime which ensures that growers throughout the Community receive propagating material and fruit plants which are healthy and of good quality. This applies to fruit plant propagating material and fruit plants of genera and species listed in Directive Annex I, which may only be marketed if they are either CAC (*Conformitas Agraria Communitatis*), Pre-basic, Basic or Certified material.

To be classified as such, material must comply with the criteria of quality, plant health, testing methods and procedures, propagation systems and varietal aspects and must have been recognised following official inspections. In addition, propagating material or fruit plants may, in most circumstances, only be marketed by accredited suppliers, whose production methods and facilities meet the requirements of the Directive. Propagating material and fruit plants from Countries outside of the European Union (EU) may only be marketed within the Community if they offer the same guarantees as materials produced in the Community complying with Council Directive 92/34/EEC. Each EU Member State adopts its own enforcement and implementation policies using the EU Directive as a guide.

In Italy, it is compulsory that the production and marketing of olive propagating material fulfils the requirements established by the Italian Decree amended on 14/04/1997 in compliance with the aforementioned EU Directive. According to this law, assessment of true to type and certification of sanitary status of olive propagating materials are compulsory and plants are certified as CAC. In this kind of certification plants must be free from *Euzophera pinguis*, *Meloidogine* spp., *S. oleae*, *P. savastanoi* pv. *savastanoi*, *V. dahliae* and all known viruses (15 to date). However, several aspects concerning for example the procedures for inspections and controls are not well defined, leading to misinterpretations and heterogeneous application of the law in the different Italian regions by the regional Phytosanitary Services. Besides this compulsory system, which relies on the propagation of olive mother plants identified by the single nurseryman and found free from the target pathogens, a voluntary certification system has been activated since 1987 at the national level by the Ministry of Agriculture. During the last 10 years, the legislation has been revised, and in late 2006 the revision process was completed with the publication of 2 decrees concerning the revised organisation of the system (DM 24/7/2003, DM 4/5/2006) and 4 decrees concerning the updated official technical operations for the certification of pommes, stone fruit, olive and strawberry (DM 20/11/06).

The DM 20/11/06 provides detailed guidance on the production of olive trees and rootstocks. Plant material produced according to this certification scheme is derived from nuclear-stock plants (also identified as Primary source) officially recognised and registered in the database of certified accessions by the Ministry of Agriculture. The material deriving from the first multiplication of the nuclear-stock material enters in the certification process as Pre-basic material. Once the nuclear-stock has been registered, the breeders or Institutions or Research Centres responsible for its production and selection must keep the material under conditions that minimise recontamination risks.

The main outlines of the Italian certification scheme (Tab. 3 and Fig. 6) are the following:

a. *Registration of nuclear-stock material*: Breeders or researchers that intend to introduce a new accession for a specific variety in the certification system must provide detailed information about the trueness to type and sanitary controls performed by filling the official forms defined in the DM 20/11/06. The evaluation and eligibility of the registration request is carried out by a technical certification committee authorised by the Ministry of Agriculture.

b. *Maintenance and propagation of Pre-basic material*: Pre-basic olive material derives directly from the propagation of the nuclear-stock; the Pre-basic plants must be maintained in insect-proof green-houses (at the Conservation for Premultiplication Repository) with at least two replications. The plants are grown in *V. dahliae* and *X. diversicaudatum* -free soil mixture and periodically tested for viruses using molecular tools (10% of the plants each year, starting from the 5th year). Molecular tests should be also performed if, after visual inspection, plants show symptoms of *V. dahliae* or phytoplasmas. Cuttings and seeds collected from the Pre-basic material is used to produce, in the same facilities, the Basic plants for the establishment of the Premultiplication Repository.
c. *Maintenance and propagation of Basic material:* Basic olive planting material is the propagation material that is obtained from Pre-basic material, maintained in open field (Repository for the Premultiplication) in a variable replication number (2 minimum) depending on the importance of the cultivar. Premultiplication field plots must be tested and found free from *V. dahliae* and *X. diversicaudatum*, and have a 20 meters non-cultivated border. Basic plants must be periodically inspected and tested as defined for the Pre-basic material. Cuttings and seeds collected from the Basic material are used to produce the certified mother plants for the establishment of the Multiplication mother blocks.
d. *Maintenance and propagation of Certified material:* Certified mother plants obtained from the propagation of the Basic material represent the source for nursery certified olive plant production. Mother plants are grown in open fields in variable replication numbers depending on the market of the specific cultivar. Plants are visually inspected at least once a year, while each plant must undergo laboratory tests for virus detection at least once within a 30-year period.
e. *Certified nursery productions*: Production takes place in authorized nurseries that join the certification program. The nursery production must comply with the requirements established by the DM 20/11/06 in terms of: (i) soil mixture (free from *V. dahliae*, *X. diversicaudatum, Meloidogyne incognita, M. javanica and Pratylenchus vulnus*), (ii) location of the certifiable olive blocks; (iii) maintenance of a farm business registry. The regional phytosanitary service, following visual inspections and examination of the documentation, is in charge of releasing the official certification (blue label) for every single plant or seedling.

In order to facilitate the certification and the availability of certified material for new olive cultivars or clones, the Premultiplication and the Multiplication blocks may be created directly using planting material deriving from the first multiplication of the nuclear-stock. In this way, the timeframe between the approval of a new accession in the system and the availability of certified plants in the nurseries is effectively reduced.

This certification program has been supported until now mainly by public funds that cover the costs for the management of the Conservation for the Premultiplication and Premultiplication repositories; starting in 2012 the program should shift to a self-sustaining system, in which taxes recovered on each released certification label will make up for the costs of the repository management.

Steps	Plant category	Facilities	Current active Repository for olive certified material in Italy	Controls and certification released by
Selection of Nuclear-stock	Nuclear-stock	Screen-houses	Several Research Centers	Regional Phytosanitary Services
Conservation for Premultiplication	Pre-basic	Screen-houses	- CRA-PAV Rome - University of Bari - Azienda Agricola Sperimentale "Improsta"	
Premultiplication	Basic	Open field	- CRA-PAV Rome - CRSA Basile Caramia, Bari - Azienda sperimentale di Santa Paolina, Follonica - CAV Tebano	
Multiplication	Certified	Open field	ConsorzioVivaisticoPugliese	
		Nurseries	--	

Table 3. Organization of the Certification program for olive propagating material

As aforementioned, the Italian voluntary certification program involves several woody crop species. In most cases, the main reason prompting for the certification of such accessions is the presence of pathogens that can cause detrimental effects on the affected plants (i.e. quarantine pests for stone fruit or citrus). Contrastingly, in the case of olive plants, the main aspect that promoted the adoption of this program has been the high level of genetic and phenotypic variability within each cultivar, which could results in heterogeneous plants and misidentification of such cultivars.

The certification scheme adopted in Italy ensures trueness-to-type and uniformity, since the certified plants are obtained through subsequent clonal propagation steps from a single registered accession.

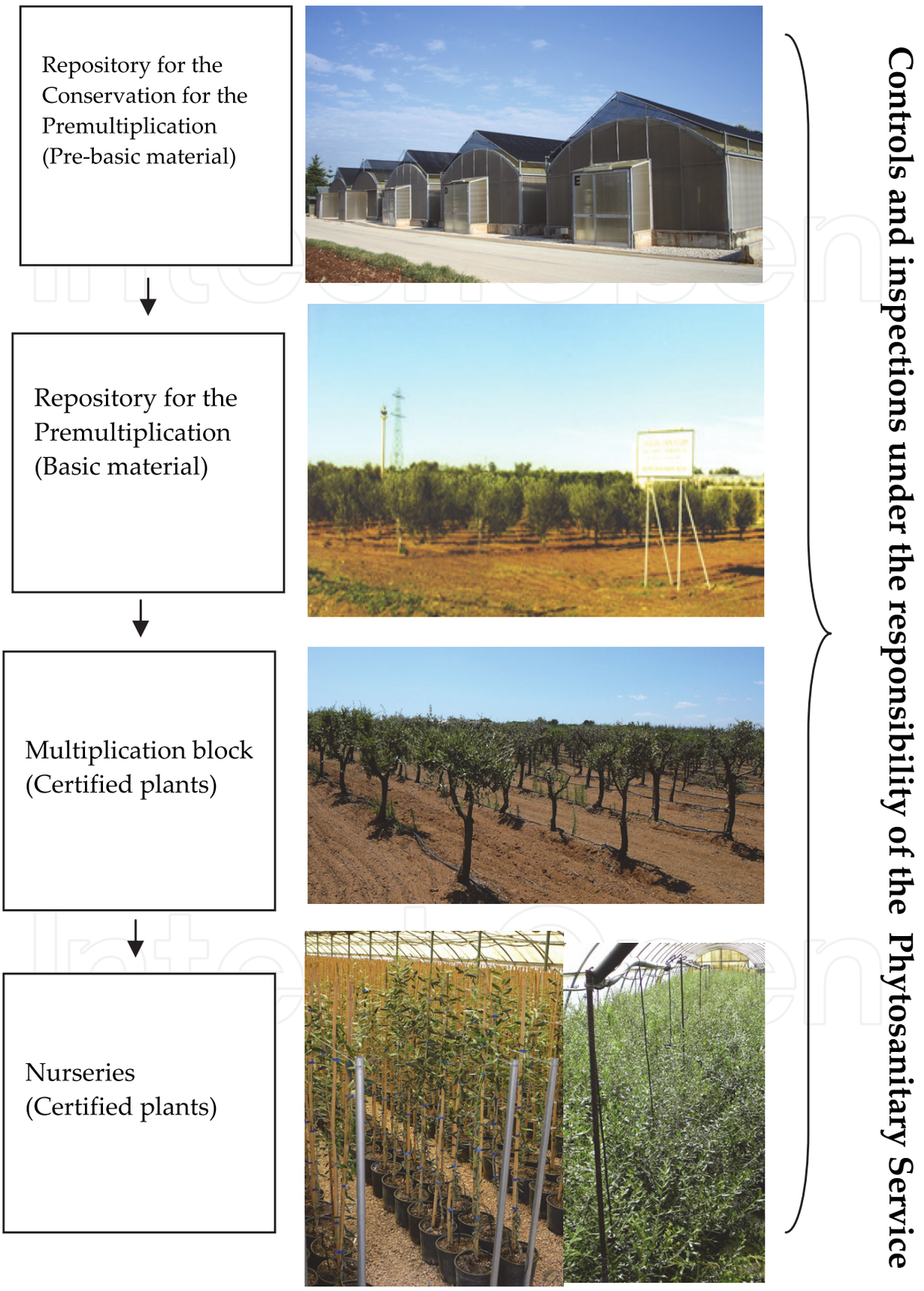

Figure 6. Outline of the general certification steps and facilities

Annex I. – List of the reference European Union (EU) Directives and Italian national regulations

Basic EU Directive

- *Council Directive 92/34/EEC* of 28 April 1992 on the marketing of fruit plant propagating material and fruit plants intended for fruit production
- *Council Directive 2008/90/EC* of 29 September 2008 (Recast version of Directive 92/34/EEC) on the marketing of fruit plant propagating material and fruit plants intended for fruit production

Implementing measures of Directive 92/34/EEC :

- *Commission Directive 93/48/EEC* of 23 June 1993 setting out the schedule indicating the conditions to be met by fruit plant propagating material and fruit plants intended for fruit production, pursuant to Council Directive 92/34/EEC
- *Commission Directive 93/64/EEC* of 5 July 1993 setting out the implementing measures concerning the supervision and monitoring of suppliers and establishments pursuant to Council Directive 92/34/EEC on the marketing of fruit plant propagating material and fruit plants intended for fruit production
- *Commission Directive 93/79/EEC* of 21 September 1993 setting out additional implementing provisions for lists of varieties of fruit plant propagating material and fruit plants, as kept by suppliers under Council Directive 92/34/EEC

Basic Italian Regulations

- *DM 14 aprile 1997* Recepimento delle direttive delle Commissione n. 93/48/CEE del 23 giugno 1993, n. 93/64/CEE del 5 luglio 1993 e n. 93/79/CEE del 21 settembre 1993, relative alle norme tecniche sulla commercializzazione dei materiali di moltiplicazione delle piante da frutto e delle piante da frutto destinate alla produzione di frutto
- *DM 24 luglio 2003* Organizzazione del servizio nazionale di certificazione volontaria del materiale di propagazione vegetale delle piante da frutto
- *DM 4 maggio 2006* Disposizioni generali per la produzione di materiale di moltiplicazione delle specie arbustive ed arboree da frutto, nonché delle specie erbacee a moltiplicazione agamica
- *DM 20 novembre 2006* norme tecniche per la produzione di materiali di moltiplicazione certificati di Agrumi, Fragola, Olivo, Pomoidee, Prunoidee (supplemento ordinario alla Gazzetta Ufficiale n. 141 del 20 giugno 2007)
- *Decreto Legislativo 25 giugno 2010, n.124* Attuazione della direttiva 2008/90 relativa alla commercializzazione dei materiali di moltiplicazione delle piante da frutto destinate alla produzione di frutti (refusione) (pubblicato nella Gazzetta Ufficiale n. 180 del 4 agosto 2010)

6. Conclusion

In this chapter an overview on the olive graft-transmissible pathogens and on the latest phytosanitary directives embodied by the EU and by the Italian Ministry of Agriculture is given.

As remarked, olive has always been considered a very resistant species to diseases caused by different pathogens; however, several pathogens, mainly systemic, can affect the trees and, in some case, invalidate the production. Recent advances in plant pathology and molecular biology, significantly contributed to the discover of new olive pathogens, to characterize their genome, biology and epidemiology.

Italy has been amongst the first Countries to adopt an effective certification system for the production of plant propagation material with high quality standards. After 10 years from its promulgation the Italian Regulation has been revised with the support of a technical committee, in order to improve the program and meet the quality standards amended in the late '90 by the EU, which are mandatory for all member States. In 2006 a revised national Regulation was issued (DM 20/11/2006), updating the list of pathogens that need to be checked and implementing the protocols for their identification. In the last 5 years several valuable virus-free and true-to-type primary sources, belonging to the most widespread or local Italian varieties, have been registered, propagated through the certification system, and made available to the growers. Although, the EU directives and the Italian regulations concerning the production of olive propagation material have been critically revised and implemented, it is necessary to continuously update the list of the pathogens and the diagnostic protocols, including the latest tools for genetic and phytosanitary assessment. It should be considered for example that some specific olive viruses such as OLV-1, OLV-2 and OLRSV are rare, infections are symptomless on olive plants, and there are no evidences about their threat to other crops. OLYaV is currently included in the list of the harmful pathogen for the Italian phytosanitary regulation, but even if OLYaV-infected trees are widespread, there are very few plants showing symptoms of yellowing, and more importantly the association of this virus with the OLY disease has not been clearly demonstrated. Regarding CMV and TNV, although these viruses are polyphagous and very damaging to other crops, they in olive are rare (CMV) or present only in a restricted geographical area (TNV- Portugal). On the basis of these data, the list of the viruses to be included in the phytosanitary certification program could be restricted to the following: SLRSV, CLRV (both are associated to manifest diseases either in olive plants or in other crops), ArMV (one of the harmful pathogens for *Fragaria*, *Rubus* and other crops) and perhaps TNV. Whereas, it is important to ensure that the certified olive material is free from phytoplasmas, *V. dahliae* and *P. savastanoi* pv. *savastanoi*.

Long distance movement of plant propagation material and the expansion of olive crops in new areas impose the use of common and harmonized certification procedures which are crucial to restrict the spread of harmful pathogens and pests.

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