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EPPO STANDARD ON SCHEMES FOR THE PRODUCTION OF HEALTHY PLANTS FOR PLANTING

PM 4/17 (3) Certification scheme for olive trees and rootstocks

Specific scope: This Standard describes the production of certified pathogen-tested olive trees and rootstocks.
Specific approval and amendment: First approved in 1996–09.

Revised in 2005–09 and in 2022–09.

Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

The certification scheme for pathogen-tested trees and rootstocks of olive (*Olea europaea*) provides detailed guidance on the production of propagated varieties to be grown on their own roots, of vegetatively propagated or seedling rootstocks, and of grafted trees. Although the production of grafted plants is covered in this scheme, it should be noted that, in practice, olive plants are mainly produced by rooting shoots in appropriate rooting conditions. Plant material produced according to this certification scheme is derived from nuclear-stock plants that have been tested and found to be free from the following pathogens: *Arabis mosaic virus* (ArMV), *Cherry leaf roll virus* (CLRV), *Strawberry latent ringspot virus* (SLRSV), *Verticillium dahliae* and *Xylella fastidiosa* and produced under conditions minimizing infestation by other pests.

Certified olive material for export should in any case satisfy the phytosanitary regulations of importing countries, especially with respect to any of the pathogens covered by the scheme which are also quarantine pests. The scheme is presented according to the general sequence proposed by the EPPO Panel on Certification of Fruit Crops and adopted by EPPO Council (EPPO, 1992).

Definitions such as '*candidate nuclear stock*', '*certification scheme*', '*certified material*', '*nuclear stock*' and '*propagation stock*' are included in the general introduction to the Series PM 4 (EPPO, 2009a). Other terms are used as defined in ISPM 5 (IPPC, 2021), such as '*field*', '*free from*', '*inspection*', '*lot*', '*official*', '*pest*', '*plants*', '*quarantine pest*', '*regulated non-quarantine pest*', '*test*'.

2 | OUTLINE OF THE SCHEME

For the production of certified olive varieties and rootstocks the following successive steps should be taken.

1. Selection for pomological quality or other specific desired traits: individual plants of each variety and rootstock to be taken into the scheme are selected.
2. Production of nuclear stock: candidates for nuclear-stock plants are propagated by seedlings, cuttings or grafts. For grafted candidates, rootstocks of nuclear-stock status should be used. The candidate plants are kept isolated from the nuclear stock. The candidate plant is tested. The candidate for nuclear stock is kept under conditions ensuring freedom from infestation. Only candidate plants that have met all requirements are promoted to nuclear-stock plants.
3. Maintenance of nuclear stock: nuclear-stock plants are maintained under conditions ensuring freedom from infection, with re-testing as appropriate. The plants should be grown in containers of sterilized or pest-free growing medium, isolated from the soil.
4. Production of propagation stock: propagation stock is produced from nuclear-stock material in as few steps as possible under conditions ensuring freedom from infection.
5. Production of certified plants: certified plants (varieties, rootstocks or grafted trees) are produced in nurseries from the propagation stock. For grafted trees, rootstocks fulfilling conditions of at least propagation-stock should be used.

Throughout the whole procedure, care should be taken to maintain the pomological characters of the originally selected plants.

The scheme is represented diagrammatically in Fig. 1.

In the scheme, a specific terminology has been used for the successive stages of multiplication and certification: 'candidate nuclear stock', 'nuclear stock' and 'propagation stock'. These terms have been defined for all EPPO certification schemes (EPPO, 2009a) and can be related as follows to alternative terms used by e.g. the EU (EU, 2014): candidate nuclear stock corresponds to

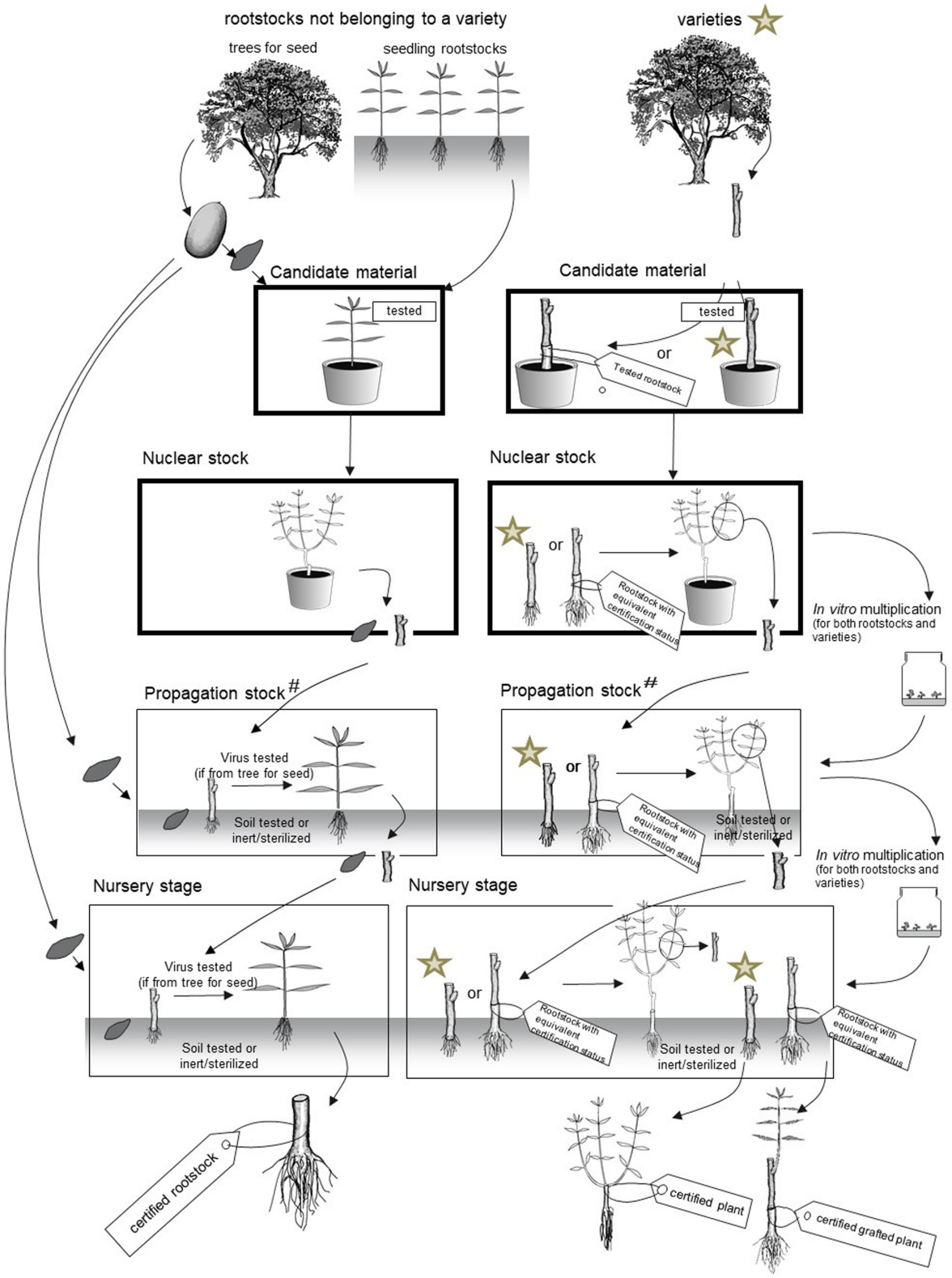

FIGURE 1 Diagram of the stages in the olive certification scheme.
☆: main line for production of olive plant reproductive material is through rooting shoots in appropriate rooting conditions.
#: although this is not illustrated in the diagram, propagation stock can also be grown in pots. Frame in bold: always under insect proof conditions.
Remarks: Propagation stock can be obtained from nuclear stock material or by multiplication from other propagation stock mother plant. Certified mother plants can be obtained from nuclear stock material or from propagation stock material. The nursery stage corresponds to 'certified mother plants' and 'certified materials'.

'candidate pre-basic mother plant', nuclear stock corresponds to 'pre-basic mother plants and pre-basic materials' and propagation stock corresponds to 'basic mother plants and basic materials'.

The certification scheme should be carried out by an official organization or by an officially registered, specialized nursery or laboratory satisfying defined criteria (see EPPO Standard PM 4/7 *Nursery requirements - recommended requirements for establishments participating in certification of fruit or ornamental crops*). All tests and inspections during production should be recorded. If the stages of the certification scheme are conducted by a registered nursery, certification will be granted by the official organization on the basis of the records of the tests and inspections performed during production, and of visual inspections to verify the apparent health of the stock.

3 | SELECTION OF CANDIDATES FOR NUCLEAR STOCK

3.1 | Varieties

A number of productive trees bearing typical characters of each variety or clone to be taken into the scheme (i.e. true to type), and showing the same genetic profile (when available), should be selected in different orchards and/or pomological trial fields.

3.2 | Seedling rootstocks

Healthy-looking, vigorous, uniformly growing, and well rooted individual plants of each rootstock type to be taken into the scheme should be selected in different germination beds.

3.3 | Trees for production of seeds for seedling rootstocks

Vigorous productive trees of each rootstock type to be taken into the scheme should be selected in different orchards or plantations. Selected trees should show no apparent symptoms of virus infection, and be affected as little as possible by other pathogens causing infectious graft-transmissible diseases. The selected trees should be known, as far as possible, to produce uniformly growing and true-to-type progeny, or else this should be checked.

4 | PRODUCTION OF NUCLEAR STOCK

Propagation material should be collected from the authenticated selected trees. This material should be budded or grafted onto pathogen-tested rootstocks, or one-year-old young shoots should be rooted in a substrate (either inert or sterilized) under 100% humidity conditions, in an isolated, suitably designed, aphid proof house fulfilling the requirements of EPPO Standard PM 5/8 *Guidelines on the phytosanitary measure* 'Plants grown under physical isolation', and separately from the nuclear stock. When produced in an area where *X. fastidiosa* is present, conditions in Appendix 1 should be fulfilled (including a 5-m wide surrounding zone around the structure kept free from any vegetation). Well rooted shoots (2 months are generally enough to obtain rooted plantlets) should be transplanted into suitable containers. The plants should be grown in inert or sterilized growing medium, in containers isolated from the soil to avoid any type of contamination. To date, 17 viruses have been detected from olive trees (Appendix 2). Testing is recommended for the following pathogens that can be transmitted on propagation material: ArMV, CLRV, SLRSV, *V. dahliae* and *X. fastidiosa*. (Table 1). Tests are specified in Appendices 3 and 4. For *X. fastidiosa* see EPPO Standard PM 7/24 *Xylella fastidiosa* (EPPO, 2019). Plants giving negative results in all tests can be promoted to nuclear stock and transplanted into the nuclear stock plot. Nuclear stock material and propagation material can be propagated from the nuclear stock plants.

Note: Most of the viruses in Appendix 1 have been isolated from symptomless trees and reported only in one or a very limited number of trees (e.g. Olive latent ringspot virus (OLRSV), Olive semilatent virus (OSLV)).

Only the viruses which were assessed as fulfilling the minimum criteria for listing as regulated non-quarantine pests (RNQPs) are recommended for testing in this certification scheme: SLRSV in olive trees has been well investigated. It was reported for the first time in 1979 in Central Italy and then its role in causing 'bumpy fruit' disease on cv. 'Ascolana tenera' was ascertained. In addition, its effect on some morphological parameters of cv. 'Raggiola' has been clearly demonstrated, confirming its pathological and economic importance among olive viruses. Even though CLRV is asymptomatic in olive, infected olives of cv. 'Frantoio' were shown to produce a lower oil yield and maturity index; and the quality of the virgin oil obtained was reduced in terms of richness in o-diphenols, and oleic/linoleic (the latter could lower the oxidative stability during the oil storage) (Godena et al., 2012). It should be noted that, such results would need confirmation on a broader scale and in other olive varieties (same impact was not observed in cv. 'Ascolana tenera'). SLRSV, CLRV and ArMV are reported to have an economic impact in other host plants (e.g. *Juglans*, *Vitis*, *Rubus*) which may be produced at the same place of production, and be infected via tools and machinery.

Other olive viruses have been found associated with specific symptoms. For example, *Olive vein yellowing associated virus* (OVYaV), *Olive yellow mottling and decline associated virus* (OYMDaV) and *Olive leaf yellowing associated closterovirus* (OLYaV) have been associated

TABLE 1 Pests covered by the scheme

Pest	Categorization	Geographical distribution	Main transmission modes (in addition to plants for planting)
Bacteria			
Pseudomonas savastanoi pv. *savastanoi*[a]		Worldwide	Wind driven rain, agricultural practices (incl. machinery)
Xylella fastidiosa	EPPO A2 List	The Americas, Europe, Iran, Taiwan, Israel	Insect vectors (xylem feeders)
Fungi			
Verticillium dahliae		Worldwide	Soil (incl. Soil in irrigation water or on agricultural machinery)
Venturia oleaginea[a]		Worldwide	Rain droplets, wind driven rain and insects
Insects			
Euzophera pinguis[a]		Bulgaria, Spain, Tunisia	
Saissetia oleae[a]		Worldwide	
Nematodes			
Meloidogyne arenaria		Worldwide	Soil
Meloidogyne incognita		Worldwide	Soil
Meloidogyne javanica		Worldwide	Soil
Pratylenchus vulnus		Worldwide	Soil
Xiphinema diversicaudatum		Europe, New-Zealand, South Africa, USA	Soil
Viruses			
Arabis mosaic virus (ArMV)		Europe, Egypt, Lebanon, Syria, USA	*Xiphinema diversicaudatum*, seed?[b]
Cherry leaf roll virus (CLRV)		Europe, Egypt, Lebanon, Syria, Tunisia, USA	Pollen, seed
Olive leaf yellowing associated virus (OLYaV)[a]		Europe, Egypt, Lebanon, Slovenia Syria, Tunisia, USA	Pseudococcid*s*
Olive vein yellowing-associated virus (OVYaV)[a]		Italy	
Olive yellow mottling and decline associated virus (OYMDaV)[a]		Italy	
Strawberry latent ringspot virus (SLRSV)		Europe, Egypt, Lebanon, Syria, Tunisia, USA	*Xiphinema diversicaudatum*, seed?[b]

[a]Pest only covered in the scheme by visual examination and/or conditions minimizing infestation.

[b]These viruses are known to be often transmitted through infected seeds (EFSA PLH, 2013), but seed transmission of ArMV and SRLSV in olive plants has not been demonstrated (Albanese et al., 2012; Lister & Murant, 1967).

with leaf-yellowing complex disease. OLYaV is most often asymptomatic and the presence of OVYaV as well as OYMDaV in olive is very scarce. These yellowing viruses are not considered to cause any unacceptable economic impact in olive. OSLV has been associated with vein clearing and *Tobacco mosaic virus* (TMV) with vein banding. There is no clear evidence, however, of the etiological involvement of these viruses with the diseases.

5 | MAINTENANCE OF NUCLEAR STOCK

Nuclear-stock plants should be kept in a suitably designed insect proof house fulfilling the requirements of Standard PM 5/8 and containing only nuclear-stock plants. They should be maintained under the same conditions as candidate nuclear stock, with the exception that soil can be tested for nematodes in Table 1 and for *V. dahliae* instead of using sterilized or inert soil. A limited number of nuclear-stock plants (at least 2) should be maintained for each source of each variety, rootstock type or tree for seed production included in the scheme and checked for trueness to type. Initially, all the sources of each variety, rootstock type or tree for seed production should be maintained. However, the number of sources may be reduced when the pomological comparisons have been made and the best sources are known. Each nuclear-stock plant should be routinely tested for *V. dahliae* and *X. fastidiosa* (each plant should be tested at least every 10 years, with some plants tested every year, for testing for *X. fastidiosa* see PM 7/24). In

addition, all plants should be inspected for symptoms associated to these pests (see EPPO Standard PM 3/82 for *X. fastidiosa*) and to OLYaV, OVYaV, OYMDaV, SLRSV[1] *Venturia oleaginea*, *Pseudomonas savastanoi* pv. *savastanoi*, and for infestations by *Euzophera pinguis* and *Saissetia oleae*.

6 | PRODUCTION OF PROPAGATION STOCK

The nuclear stock is multiplied in as few steps as possible to obtain the required quantity of propagation stock:

- For rooting shoots and grafted plants only one multiplication step should be allowed.
- For grafted plants, nuclear-stock material should be budded or grafted onto rootstocks of equivalent certification status or onto seedling rootstocks produced under propagation stock conditions.
- Multiplication in vitro beginning with axillary buds from nuclear stock plants can also be used (Appendix 6) for both varieties and rootstocks. To avoid mutations, in vitro multiplication of chimeric clones is not allowed. Care should be taken to limit the number of propagation steps (e.g. maximum of 10 subcultures for the propagation phase). The total duration of propagation steps should not exceed 4 years and cold storage is not allowed for more than 12 months. Care should also be taken to prevent the formation of callus and any in vitro cultures showing morphological abnormalities (e.g. fasciations) should be eliminated.

The propagation material is kept either under insect proof conditions or outdoors. In areas where *X. fastidiosa* is present, the propagation material is kept under insect proof conditions fulfilling the requirements of Standard PM 5/8 for vectors of *X. fastidiosa* (see Appendix 1, including a surrounding zone of 5 m wide around the structure kept free from any vegetation). The propagation material is kept either in containers of sterilized growing medium or in soil that has been tested and found free from *Xiphinema diversicaudatum* (see EPPO Standard PM 4/35), *Meloidogyne arenaria, M. incognita, M. javanica, Pratylenchus vulnus* and *V. dahliae* (Appendix 5). In the field, the material should be separated by at least 20 m from other olive material, and reasonably isolated from sources of infection (e.g. irrigation water should be filtered and a surrounding zone 2 m wide around the plot should be kept free from any vegetation). Propagation stock should be kept under continuous surveillance and sprayed regularly with appropriate plant protection products, to control the normal pests of olive. General precautions against infection should be maintained, and appropriate control measures should be taken if any pests are observed.

The propagation stock should be inspected each year for virus symptoms and for the other pests mentioned above (see EPPO Standard PM 3/82 for *X. fastidiosa*). Any symptomatic plant should be removed. If there is an indication that infestation may have derived from the previous generation, it is recommended to remove all the plants in the lot and to retest the possible source plant. For quarantine pests (e.g. *X. fastidiosa*), eradication measures should be applied.

For the production of rootstock not belonging to a variety, coming from seed, and intended to be propagation stock, the plants growing from the seeds should be tested for viruses (see Table 1).

Trueness to type must be checked by examining morphological characteristics or testing the genetic profile.

7 | PRODUCTION OF CERTIFIED PLANTS

For the production of certified grafted olive trees, the scion material should be grafted or budded onto rootstocks of equivalent (i.e. propagation-stock) or higher certification status only.

Certified mother plants and plants to be certified should be kept in nurseries in soil which has been tested and found free from *Xiphinema diversicaudatum, Meloidogyne arenaria, M. incognita, M. javanica, Pratylenchus vulnus* and *Verticillium dahliae* (or which has been fumigated and retested to ensure freedom), separated by at least 4 m from other olive material, and reasonably isolated from sources of infection (e.g. irrigation water should be filtered and a surrounding zone 2 m wide around the plot should be kept free from any vegetation). In areas where *X. fastidiosa* is present, the propagation material should be kept under insect proof conditions fulfilling the requirements of Standard PM 5/8 for vectors of *X. fastidiosa* (see Appendix 1, including a surrounding zone of 5 m wide around the structure kept free from any vegetation).

To be certified, the plants should be inspected by the official organization for symptoms of nematodes, viruses, virus-like disease or any of the pests mentioned above (see EPPO Standard PM 3/82 for *X. fastidiosa*). Any plants showing symptoms should be removed and certification may be granted to the remainder. For the production of rootstock not belonging to a variety, coming from seed, and intended to be certified plant, the plants growing from the seeds should be tested for viruses (see Table 1).

Trueness to type must be checked by examining morphological characteristics or testing the genetic profile.

[1]No inspection for symptom is recommended for ArMV and CLRV, because these viruses are reported to be asymptomatic in olive plants (Albanese et al., 2012).

8 | ADMINISTRATION OF THE CERTIFICATION SCHEME

8.1 | Monitoring of the scheme

An official organization should be responsible for the administration and monitoring of the scheme. If officially registered nurseries carry out the different stages of the scheme, the official organization should confirm that all necessary tests and inspections have been performed during production, and should verify the general health status of the plants in the scheme by visual inspections. If these requirements are not met, certification will not be granted and/or the plants concerned will not be permitted to continue in the certification scheme.

8.2 | Control on the use and status of certified material

Throughout the certification scheme, the origin of each plant should be known so that any problems of health or trueness to-type may be traced. The use of propagation material in nurseries to produce certified plants should be checked by an official or officially authorized organization which controls the health, origin and amount of such material on the basis of field inspections and of the records and documents presented by the nursery. The nursery plant protection programme and the inspections should also take account of other important pests that can affect quality, so that the certified plants delivered to the olive tree grower are substantially free from these pests. Certified material for export should in any case satisfy the phytosanitary regulations of importing countries. Certified plants leaving the scheme should carry an official certificate (which may be a label) indicating the certifying authority, the plant producer and the certification status of the plants.

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APPENDIX 1 - INSECT PROOF MAINTENANCE CONDITIONS IN AREAS WHERE *X. FASTIDIOSA* IS PRESENT

The following conditions are based on recommendations provided during task 9.4 of the XF-Actors Project (EU H2020 research project on *X. fastidiosa*)

Description of the structure

- Materials can be either rigid (e.g. glass, Plexiglas) or flexible (plastic, insect proof nets).
- Fixation between the soil and the structure, as well as between flexible and rigid parts should be airtight.
- The insect proof facilities should have a double door entrance. Dimensions of this entrance should be compatible with the size of the largest machinery to be used.

Size net and properties

- Insect proof nets should either cover the whole production site or be installed at the vents of structures, including windows.
- The nets used should have a suitable mesh size to exclude the potential vectors, and a sufficient resistance to wind.
- It is required that the mesh size is not larger than 1 mm.
- Nets should be knitted.

Entrance, dedicated clothes and controlled access (movement controls)

- Any windows and doors should be locked shut when not in use. The two doors should never be open at the same time.
- Considering the risk of entry and movement of vector(s) with the personnel working in the structure, dedicated outerwear should be used.

Other plants/material entering the insect proof facility

- Any plant for planting that enters the structure (even if it is not a known host plant for *X. fastidiosa*) should be treated (if possible) against the vectors and found to be free of the vectors.
- Freedom of vectors should be verified prior to introduction.

Vector control

- Examination for the presence of vectors in the insect proof facilities should preferably be performed visually. These inspections should be recorded.
- In case of the finding of a known vector of *X. fastidiosa* in the insect proof structure, an inspection of the structure should be carried out, the entire protected production site should be treated against the vector and the need for additional corrective actions should be considered. The vector should be tested to know whether it was infected with *X. fastidiosa* (see EPPO Standard PM 7/24 (EPPO, 2019)).
- In case of the finding of an unexpected insect (including other xylem-feeding species), an inspection of the structure should be carried out and the need for other corrective actions should be considered (including treatment).

Buffer zone

It is recommended to control plants (including weeds) over a distance of at least 5 m around the protected production site, to prevent the entry of non-adult vectors which are considered to be less mobile.

Specific traceability requirements:

A register for personal should be made available at the entrance of the insect proof structure (name, date).

APPENDIX 2 - VIRUSES OCCURRING IN OLIVE (ALBANESE ET AL., 2012; MARTELLI, 2011)

Virus	Countries where the virus is reported in olive	Frequency in olive	References
Arabis mosaic virus (ArMV)	Egypt, Greece, Italy, Lebanon, Portugal, Syria, Turkey, USA	Widely distributed	Caglayan et al. (2004), Savino et al. (1979)
Cherry leafroll virus (CLRV)	Croatia, Egypt, Greece, Italy, Lebanon, Portugal, Spain, Syria, Tunisia, Turkey, USA	Widely distributed	Bertolini et al. (2001), Caglayan et al. (2004), Mathioudakis et al. (2020), Rei et al. (1993), Savino and Gallitelli (1981)
Cucumber mosaic virus (CMV)	Croatia, Greece, Italy, Portugal, Slovenia, Spain, Syria, Tunisia, Turkey, USA	Widely distributed	Bertolini et al. (2001), Caglayan et al. (2004), Mathioudakis et al. (2020), Rei et al. (1993), Savino and Gallitelli (1983), Viršček Marn and Mavrič Pleško (2018), Xylogianni et al. (2021)
Olive latent ringspot virus (OLRSV)	Italy, Portugal, Syria, Tunisia	At low prevalence	Rei et al. (1993), Savino et al. (1983)
Olive latent virus 1 (OLV-1)	Egypt, Italy, Jordan, Lebanon, Portugal, Syria, Turkey, Tunisia, USA	At low prevalence	Gallitelli and Savino (1985), Martelli et al. (1995)
Olive latent virus 2 (OLV-2)	Italy	At low prevalence	Savino et al. (1984)
Olive latent virus 3 (OLV-3)	Italy	At low prevalence	Alabdullah et al. (2010)
Olive leaf yellowing-associated virus (OLYaV)	Albania, Croatia, Egypt, Greece, Israel, Italy, Lebanon, Slovenia, Spain, Syria, Tunisia, USA	Widely distributed	Campos et al. (2019), Savino et al. (1996), Sbanadzovic et al. (1999), Mathioudakis et al. (2020), Viršček Marn and Mavrič Pleško (2018)
Olive mild mosaic virus (OMMV)	Portugal, Tunisia	At low prevalence	Campos et al. (2019), Cardoso et al. (2005)
Olive semilatent virus (OSLV)	Italy	At low prevalence	Materazzi et al. (1996)
Olive vein yellowing associated virus (OVYaV)[a]	Italy	At low prevalence	Faggioli and Barba (1995)
Olive virus T (OlVT)	Greece	At low prevalence	Xylogianni et al. (2021)
Olive yellow mottling and decline associated virus (OYMDaV)[a]	Italy	At low prevalence	Savino et al. (1996)
Strawberry latent ringspot virus (SLRSV)	Albania, Croatia, Egypt, Greece, Italy, Lebanon, Portugal, Spain, Syria, Tunisia, Turkey, USA,	At low prevalence	Bertolini et al. (1998), Caglayan et al. (2004), Henriques et al. (1990, 1992), Marte et al. (1986), Mathioudakis et al. (2020), Savino et al. (1979)
Tobacco mosaic virus (TMV)	Italy	At low prevalence	Triolo et al. (1996)
Tobacco necrosis virus (TNV)	Portugal	At low prevalence	Felix and Clara (2002)
Tobacco necrosis virus D (TNV-D)	Portugal, Tunisia	At low prevalence	Campos et al. (2019), Cardoso et al. (2004)

[a] OYMDaV and OVYaV are not confirmed by the International Committee of Taxonomy of Viruses (ICTV).

APPENDIX 3 - GUIDELINES ON TESTING PROCEDURES FOR VIRUSES

Testing on olive indicators

No information is available at the moment on the use of woody indicators to detect the viruses listed in Table 1.

Inoculation on herbaceous hosts

Bioassays on herbaceous hosts were used in the past before the introduction of PCR-based tests for the identification of plant viruses but are no longer used since the introduction of PCR-based tests and are consequently not described in this Standard.

ELISA testing

Suitable antisera and ELISA procedures are not currently available for olive. According to Martelli (1999) and Bertolini et al. (2001), serological tests were not considered reliable when olive material was analysed by ELISA.

Molecular testing

RT-PCR tests (in two-step or one-step format) using specific primers (Table A1) are used for virus detection in olive (Loconsole et al., 2010). Optimal results are obtained when phloem tissues from 1- to 2-year-old twigs, collected from late winter to early spring (end of February – end of April), are tested.

TABLE A1 Examples of sequences of specific primers which may be used for the detection of olive tree viruses

Target virus	Primer name	F/R/P[a]	Size of product	Primer sequence	Test	References
ArMV	ArMV-5 A	F	302	5′-TACTATAAGAAACCGCTCCC-3′	One step RT-PCR	Faggioli et al. (2005)
	ArMV-3 A	R		5′-CATCAAAACTCATAACCCAC-3′		
	ArMV1	F	340	5′-CGGATTGGGAGTTCGTTGTCG-3′	Multiplex Nested RT-PCR in a Single Tube	Bertolini et al. (2003)
	ArMV 2	R		5′-CCGTTCCATTCACTAACAACTC-3′		
	ArMV i1	F	203	5′-AATTATATGCTGAGTTTGAG-3′		
	ArMV i2	R		5′-AAAATTATACACCTTATGAGTA-3′		
	ArMV[b]	P		5′-ATCCCACCACTGGAATATGACTTAAG TGCAACCAG-3′		
	ArMV-F	F	504	5′-TTGGTTAGTGAATGGAACGG-3′	RT-PCR	Grieco et al. (2000)
	ArMV-R	R		5′-TCAACTCACCCTCCAAATCCC-3′		
CLRV	CLRV-5	F	416	5′-TGGCGACCGTGTAACGGCA-3′	One step RT-PCR	Faggioli et al. (2005) (primers Werner et al. (1997))
	CLRV-3	R		5′-GTCGGAAAGATTACGTAAAAGG-3′		
	CLRV 1	F	283	5′-CATTTCCATGCGACCGGTCTT-3′	Multiplex Nested RT-PCR in a single tube	Bertolini et al. (2003)
	CLRV 2	R		5′-AGTCCGACACTCATACAATAAGC		
	CLRV i1	F	171	5′-GTTAACGAATATCTACTGC-3′		
	CLRV i2	R		5′-CAAATATTGCTAAACAACC-3′		
	CLRV[b]	P		5′-AAGCCCAAGAATTTAGGGGGTTATGT GGGTAGATAGCGTT-3′		
	CLRV-F	F	431	5′-TTGGCGACCGTGTAACGGCA-3′	RT-PCR	Grieco et al. (2000)
	CLRV-R	R		5′-GTCGGAAAGA′ITACGTAAAAGG-3′		
SLRSV	SLRSV-5D	F	293	5′-CCCTTGGTTACTTTTACCTCCTCATT GTCC-3′	One step RT-PCR	Faggioli et al. (2005)
	SLRSV-3D	R		5′-AGGCTCAAGAAAACACAC-3′		
	SLRSV 1	F	181	5′-GTTACTTTTACCTCCTCATTGTCCATG TGTTG-3′	Multiplex nested RT-PCR in a single tube	Bertolini et al. (2003)
	SLRSV 2	R		5′-GACTATCGTACGGTCTACAAGCGTGT GGCGTC-3′		
	SLRSV i1	F	109	5′-TGGCCTTTATTGGTTGGAT-3′		
	SLRSV i2	R		5′-ATCTGCCACTGATTCTCAC-3′		
	SLRSV[b]	P		5′-AGTAAGCAGCCGCTAGCGTTCTGGA WTTCCAGGCAYAGTG-3′		
	SLRSV-F	F	525	5′-TCAAGGAGAATATCCCTGGCCC-3	RT-PCR	Grieco et al. (2000)
	SLRSV-R	R		5′-CTAAGTGCCAGAACTAAACC-3′		

[a]F = Forward; R = Reverse, P = Probe.
[b]Indicates fluorescent-labelled probe.

APPENDIX 4 - GUIDELINES ON TESTING PROCEDURES FOR *VERTICILLIUM DAHLIAE*

The pathogen is detected using real-time PCR, or nested PCR, with variations regarding the primers or the cycling process used (Atallah et al., 2007; Markakis et al., 2009; Mercado-Blanco et al., 2001, 2002, 2003; Trapero et al., 2018; Table A2).

APPENDIX 5 - GUIDELINES ON SOIL SAMPLING FOR *VERTICILLIUM DAHLIAE* ANALYSIS

To test whether *V. dahliae* is present in the soil, several subsamples should be taken. Subsamples are mixed and bulked, then air-dried for 1–2 weeks at 25°C, passed through a 2-mm sieve and mixed well, then 10-g subsamples

TABLE A2 Examples of sequences of specific primers used for the detection of *V. dahliae*

Primer name	F/R[a]	Size of product	Primer sequence	Test	References
VertBt	F	115	5′-AACAACAGTCCGATGGATAATTC-3′	Multiplex real-time PCR	Atallah et al. (2007)
VertBt	R		5′-GTACCGGGCTCGAGATCG-3′		
NDf	F	1410	5′-ATCAGGGGATACTGGTACGAGA-3′	Nested PCR	Mercado-Blanco et al. (2001); Mercado-Blanco et al. (2002)
NDr	R		5′-GAGTATTGCCGATAAGAACATG-3′		
INTNDf	F	1163	5′-CCACCGCCAAGCGACAAGAC-3′		
INTNDr	R		5′-TAAAACTCCTTGGGGCCAGC-3′		
INTND2f	F	824	5′-CTCTTCGTACATGGCCATAGATGTGC-3′		
INTND2r	R		5′-CAATGACAATGTCCTGGGTGTGCCA-3′		
ITS1-F	F	347	5′-CCGCCGGTCCATCAGTCTCTCTGTTTATAC-3′	Real-time PCR	Markakis et al. (2009)
ITS2-R	R		5′-CGCCTGCGGGACTCCGATGCGAGCTGTAAC-3′		
OLG 70		NA	5′-CAGCGAAACGCGATATGTAG-3′	Real-time PCR	Trapero et al. (2018) primers described in Eynck et al. (2007),
OLG 71			5′-GGCTTGTAGGGGGTTTAGAv		

[a]F = Forward; R = reverse.

are taken for further processing. The subsamples are washed through sieves with 150-mm and 35-mm pore size. The residue on the 35-mm sieve is surface-disinfected for 10 s in 0.525% NaOCl, rinsed, and washed into a 50-mL beaker (total volume of residue and water 15–20 mL). Using a spoon, this slurry is distributed onto the surface of 10 Petri dishes (10 cm diameter) containing modified sodium polipectate agar (Butterfield & De Vay, 1977; Nadakavukaren & Horner, 1959). After 7–10 days of incubation at 22°C in darkness, soil is washed from the agar surface with water. Plates are incubated for 2–3 additional days before verifying the identity of the fungal colonies.

APPENDIX 6 - IN VITRO MAINTENANCE AND MULTIPLICATION OF OLIVE

Multiplication and rooting are generally performed under aseptic conditions, adding different growth regulators to the agar nutrient medium, and adjusting the concentrations to the specific needs of the variety (Lambardi & Rugini, 2003). It should be noted that the plant genotype greatly influences the protocol for in vitro multiplication of olive plant and a protocol valid for each case cannot be recommended. Table 1 of Lambardi and Rugini (2003) provides a summary of best culture conditions for micropropagation of specific olive cultivars.

Step 1 – in vitro establishment

To establish in vitro cultures, explants (nodal segments of approximately 1 cm) should be recovered from source plants grown under controlled conditions in order to reduce contaminations that often occur when using field-grown plants (Lambardi & Rugini, 2003). Explants are cut from tender twigs.

Explants are surface sterilized using one of the following procedures:

(i) Explants are dipped sequentially in 70% ethanol (1 min), Mercryl (1 min) and 2% calcium hypochlorite (3 min) and washed with sterile distilled water (Rugini & Fontanazza, 1981);
(ii) Explants are dipped for 3–5 min, at low pressure created by a vacuum pump, in a solution of 13% sodium hypochlorite, 350 mg/L mercuric chloride, and a drop of Tween® 20; and finally rinsed in sterile water for 30 min (Mencuccini, 1995);
(iii) Explants are dipped for 5 min in 0.05–0.1% mercuric chloride and rinsed in sterile water. They are then dipped in a solution of 15% sodium hypochlorite, and finally rinsed three times in sterile water (Martino et al., 1999);
(iv) Explants are dipped in a solution of sodium hypochlorite (7–9% Cl active) for 20 min followed by three washes in sterile water for 1 to 2 minutes.

Shoot growth is induced from single-node explants cultivated on one of the following suggested media:

(i) Rugini olive medium (Rugini, 1984), supplemented with 3% sucrose.
(ii) Rugini olive medium (Rugini, 1984) supplemented with zeatin 1.0 mg/L and mannitol 36 g/L (Miazzi et al., 2020). This media was reported to be successful for different olive varieties.

(iii) Rugini olive medium (Rugini, 1984) supplemented with growth regulators (BA, NAA, Zeatin Riboside) as necessary (Sghir et al., 2005).

Media are autoclaved 20 min at 121°C.

Explants are maintained under fluorescent light (40 µmoles m^{-2} s^{-1}) following a 16 h-photoperiod with a thermal regime between 21 and 23 ± 2°C constant in 24 h.

Step 2 - in vitro multiplication

After approximately 40 days from the settlement of the explants, the shoots sprouted from the axillary buds are removed and transferred to a fresh preparation of the same medium, maintained under the same conditions as the above step.

During the multiplication phase both tissues with morpho-physiological abnormalities and callus should be removed.

Step 3 - in vitro/in vivo rooting

The in vitro rooting of the shoots is obtained by one of the following procedure:

(i) rooted in Rugini olive medium (Rugini, 1984) containing 5.37 µM Naphthalene acetic acid (NAA) or 24.6 µM indole-3-butyric acid (IBA) (single phase)

(ii) a two-phase protocol (after Druart, 1997) with a 5-day induction phase in liquid 24.6 µM IBA solution in the dark with further cultivation on regulator-free Rugini olive medium. Mencuccini (2003) reported that darkening the rooting medium (adding 100–200 mg/L of dye) significantly enhanced root formation.

Rooting is often conducted in vivo; the most vigorous shoots being obtained at the end of the multiplication. When the rooting is performed in vivo, the multiplication is the last phase conducted under aseptic conditions. The shoots are used as 'cuttings' and subjected to hormonal treatment, before being directly transplanted in soil or mix of soil, in which they will develop functional roots which are already able to support the function of water absorption and nutrients. The advantage of in vivo rooting is the reduction of the time required for acclimation and recovery of photosynthetic capacity.

Recording of activities

Growing containers should be maintained in a specific and clearly identified laboratory compartment and individually labelled. The label should report the date, the sub-culture progressive number and the growing stage.

Transplanting operations, as well as the elimination of containers and development of contamination, should be daily recorded.