Schemes for the production of healthy plants for planting
Schémas pour la production de végétaux sains destines à la plantation

Pathogen-tested 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 1996-2009.
Revised in 2005-09.

The certification scheme for pathogen-tested trees and rootstocks of olive (*Olea europea*) 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 on a heated bed. Plant material produced according to this certification scheme is derived from nuclear-stock plants that have been tested and found free from the following pathogens: *Arabis mosaic nepovirus* (ArMV), *Cucumber mosaic cucumovirus* (CMV), *Strawberry latent ringspot sadwavirus* (SLRSV), Olive leaf-yellowing associated closterovirus (OLYaV), *Cherry leaf roll nepovirus* (CLRV), 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 (OEPP/EPPO, 1992).

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: individual plants of each variety and rootstock to be taken into the scheme are selected.

2 Production of nuclear stock: candidate nuclear-stock plants are propagated by seedlings, cuttings or grafts. For grafted candidate nuclear-stock plants, rootstocks of nuclear-stock status should be used. The candidate plants are kept isolated from the nuclear stock. The candidate nuclear stock is tested and kept under conditions ensuring freedom from infection. Only candidate nuclear-stock 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 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 of at least propagation-stock standard should be used.

Throughout the whole procedure, care should be taken to maintain the pomological characters of the originally selected plants. Checks should be built in for possible mutations especially for varieties.

The scheme is represented diagrammatically in Fig. 1. 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 (2)). 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.

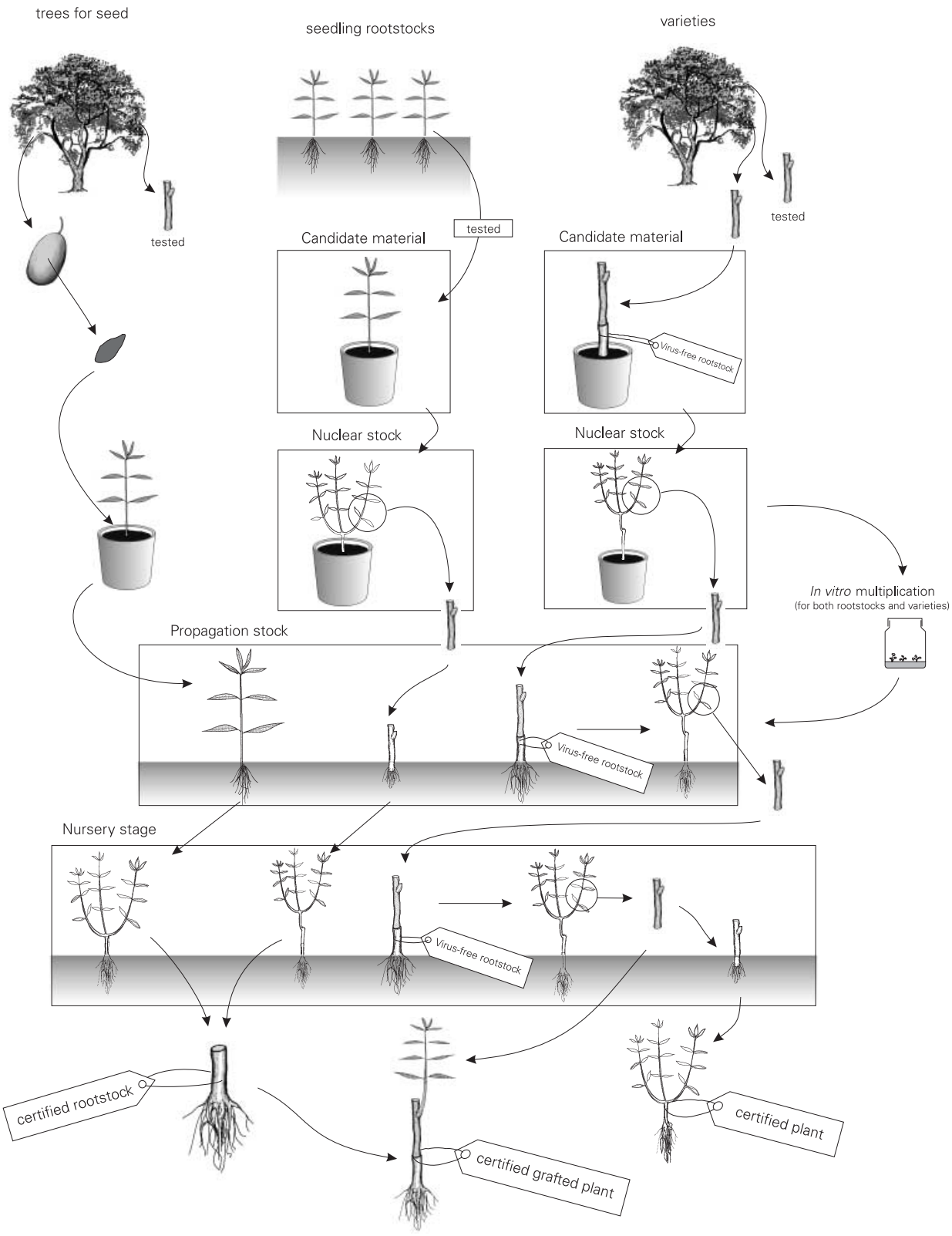

Fig. 1 Diagram of the stages in the olive certification scheme.

Table 1 Viruses occurring in olive

Virus	Geographical distribution	Frequency	References
Strawberry latent ringspot sadwavirus (SLRSV)	Italy, Spain, Portugal, Turkey	Widespread	Bertolini *et al*. (1998) Caglayan *et al*. (2004) Henriques *et al*. (1990, 1992) Marte *et al*. (1986) Savino *et al*. (1979)
Olive latent ringspot nepovirus (OLRSV)	Italy, Portugal	Rare	Rei *et al*. (1993) Savino *et al*. (1983)
Arabis mosaic nepovirus (ArMV)	Italy, Portugal, Turkey	Widespread	Caglayan *et al*. (2004) Savino *et al*. (1979)
Cherry leafroll nepovirus (CLRV)	Italy, Spain, Portugal	Widespread	Bertolini *et al*. (2001) Caglayan *et al*. (2004) Rei *et al*. (1993) Savino & Gallitelli (1981)
Cucumber mosaic cucumovirus (CMV)	Italy, Spain, Portugal, Turkey	Widespread	Bertolini *et al*. (2001) Caglayan *et al*. (2004) Rei *et al*. (1993) Savino & Gallitelli (1983)
Olive latent necrovirus 1 (OLV-1)	Italy, Jordan	Rare	Gallitelli & Savino (1985) Martelli *et al*. (1995)
Olive latent oleavirus 2 (OLV-2)	Italy	Rare	Savino *et al*. (1984)
Olive leaf yellowing associated closterovirus (OLYaV)	Italy	Widespread	Sbanadzovic *et al*. (1996) Savino *et al*. (1996)
Olive vein yellowing associated potexvirus (OVYaV)	Italy	Rare	Faggioli & Barba (1995)
Tobacco mosaic tobamovirus (TMV)	Italy	Rare	Triolo *et al*. (1996)
Olive semilatent virus (OSLV)	Italy	Rare	Materazzi *et al*. (1996)
Olive yellow mottling and decline associated virus (OYMDaV)	Italy	Rare	Savino *et al*. (1996)
Tobacco necrosis necrovirus (TNV)	Portugal	Rare	Felix & Clara (2002)

1. Selection of candidates for nuclear stock

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) should be selected in different orchards and/or pomological trial fields.

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.

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 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.

2. Production of nuclear stock

Propagation material should be collected from the pomologically selected trees. This material should be budded or grafted onto pathogen-tested rootstocks, or one-year-old young shoots should be rooted in a heated vermiculite bed supplied with 100% humidity in an isolated, suitably designed, aphid-proof house, separately from the nuclear stock (in quarantine). Well rooted shoots (3 months are generally enough to obtain rooted plantlets) should be transplanted into suitable containers. The plants should be grown in sterilized growing medium, in containers isolated from the soil to avoid any type of contamination, and tested for viruses and other pests that can be transmitted on propagation material, as specified in Table 1 by the methods given in Appendix I. To date, 13 viruses, of 7 genera, have been isolated from olive trees (Table 1). Plants giving negative results in all tests can be promoted to nuclear

Table 2 Viruses and other pathogens covered by the scheme

Pest	Geographical distribution	Transmission
Arabis mosaic nepovirus (ArMV)	Europe, Japan, New Zealand (isolated records elsewhere)	*Xiphinema diversicaudatum*
Cucumber mosaic cucumovirus (CMV)	Worldwide	Aphids
Strawberry latent ringspot sadwavirus (SLRSV)	Europe (isolated records elsewhere)	*Xiphinema diversicaudatum*
Olive leaf yellowing associated closterovirus (OLYaV)	Europe	Pseudococcid*s*
Cherry leaf roll nepovirus (CLRV)	Europe	Pollen, seed
Verticillium dahliae	Worldwide	
Pseudomonas syringae ssp. *savastanoi*	Worldwide	
Euzophera pinguis	Bulgaria, Spain, Tunisia	
Saissetia oleae	Worldwide	

stock and transplanted into the nuclear stock plot, or nuclear stock material can be propagated from it.

Note

Most of the viruses concerned have been isolated from symptomless trees and reported only in one or a very limited number of trees (e.g. *Olive latent ringspot nepovirus* (OLRSV), Olive semi-latent virus (OSLV). *Strawberry latent ringspot sadwavirus* (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. Other olive viruses have been found associated with specific symptoms. For example, *Arabis mosaic nepovirus* (ArMV), *Cherry leaf roll nepovirus* (CLRV), *Olive vein yellowing associated potexvirus* (OVYaV), Olive yellow mottling and decline associated virus (OYMDaV) and Olive leaf yellowing associated closterovirus (OLYaV) have been associated with leaf-yellowing complex disease. OSLV has been associated with vein clearing and *Tobacco mosaic tobamovirus* (TMV) with vein banding. There is no clear evidence, however, of the etiological involvement of these viruses with the diseases.

3. Maintenance of nuclear stock

Nuclear-stock plants should be kept in a suitably designed insect-proof house containing only nuclear-stock plants. They should be maintained under the same conditions and with the same checks on pathogen freedom as candidate nuclear stock. 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 all viruses listed in Table 2 (each plant should be tested at least every 7 years), and visually inspected for infection by *Verticillium dahliae, Spilocaea oleagina*, *Pseudomonas syringae* ssp. *savastanoi*, and for infestations by *Euzophera pinguis*, *Saissetia oleae*. The plants should also be visually inspected each year for possible mutations.

4. Production of propagation stock

The nuclear stock is multiplied in as few steps as possible to obtain the required quantity of propagation stock. For grafted plants, nuclear-stock material should be budded or grafted onto rootstocks of equivalent certification status or onto seedling rootstocks produced under nuclear stock conditions. Multiplication *in vitro* beginning with axillary buds from nuclear stock plants can also be used (Appendix III) 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). 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 in a gauzehouse or outdoors, either in containers of sterilized growing medium or in soil that has been tested and found free from *Xiphinema diversicaudatum, Meloidogyne arenaria, Pratylenchus vulnus* (see EPPO Standard PM 4/34, in preparation) and *Verticillium dahliae* (Appendix II). In the field, the material should be separated by at least 20 m from other olive material, and reasonably isolated from infected sources (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 visually each year for virus symptoms and for the other pests mentioned above. Any infected plant should be removed. If there is an indication that infection may have derived from the previous generation, it is advisable to remove all the plants in the lot and to retest the possible source plant. The plants should be inspected visually

for possible mutations and a pomological assessment on olive fruits can also be made.

5. 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. Plants to be certified should be kept in nurseries in soil which has been tested and found free from *Xiphinema diversicaudatum, Meloidogyne arenaria, 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).

To be certified, the plants should be inspected by the official organization for symptoms of viruses, virus-like disease or any of the pests mentioned above. Any plants showing symptoms should be removed and certification may be granted to the remainder.

6. Administration of the certification scheme

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. Otherwise, certification will not be granted and/or the plants concerned will not be permitted to continue in the certification scheme.

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 check 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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Herbaceous hosts	ArMV	SLRV	CMV	CLRV
Chenopodium quinoa	ChLL, SM	ChLL, SM	–	NLL, ChLL, SM
Chenopodium amaranticolor	ChLL, SM	ChLL, SM	–	ChLL, SMottle
Nicotiana glutinosa	–	ChLL	–	–
Nicotiana benthamiana	–	ChLL	SM	–
Nicotiana tabacum	–	–	–	ChLL, NLL, SChR
Cucurbita pepo	–	–	SM	–

Table 3 Main herbaceous indicators of olive viruses listed in Table 1 and the symptoms produced by different viruses

ChLL = chlorotic local lesions; SM = systemic mosaic; NLL = necrotic local lesions; SChR = systemic chlorotic rings; – = no infection.

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Werner R, Mühlbach HP & Büttner C (1997) Detection of *Cherry leaf roll nepovirus* (CLRV) in birch, beech and petunia by immuno-capture RT-PCR using a conserved primer pair. *European Journal of Forest Pathology* **27**, 309–318.

Appendix I Guidelines on testing procedures

Testing on olive indicators

No information is available at the moment on the use of woody indicators to detect the viruses listed in Table 2.

Inoculation on herbaceous hosts

The use of herbaceous indicators allows the detection of all mechanically transmissible viruses of olive. In fact, all are sap-transmitted to herbaceous hosts from pollen and flowers. The main herbaceous indicators are listed in Table 3. It should be noted that the reactions on indicator plants may vary and each testing laboratory should validate the tests for its local conditions.

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

In recent years the application of molecular diagnostic techniques (dsRNA, molecular hybridization, reverse transcription-polymerase chain reaction: RT-PCR) for virus detection has appeared more promising than the traditional detection methods (Grieco *et al*., 2000; Bertolini *et al*., 2001; Faggioli *et al*., 2002; Bertolini *et al*., 2003). Their reliability allowed increased investigations on distribution of olive viruses by conducting several surveys in different geographical areas. Among molecular methods, RT-PCR has proved to be the most rapid, sensitive and reliable technique for detecting RNA target from infected plants (Hadidi & Yang, 1990; Hadidi & Candresse, 2001; Faggioli *et al*., 2005). Thus RT-PCR technology, using specific primers (Table 4) represents an important step in optimizing and speeding up virus diagnosis in olive. Best 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 4 Sequences of specific primers used for the detection of olive tree viruses

Designation	Size of product	*Primer sequence*	References
ArMV-5 A	302	5′-TACTATAAGAAACCGCTCCC-3′ sense	Faggioli *et al*. (2005)
ArMV-3 A		5′-CATCAAAACTCATAACCCAC3′ antisense	
CMV-CPN5	280	5′-ACTCTTAACCACCCAACCTT-3′ sense	Lumia *et al*. (2001)
CMV-CPN3		5′-AACATAGCAGAGATGGCGG-3′ antisense	
SLRSV-5D	293	5′-CCCTTGGTTACTTTTACCTCCTCATTGTCC-3′ sense	Faggioli *et al*. (2002)
SLRSV-3D		5′-AGGCTCAAGAAAACACAC-3′ antisense	
OLYaV-H	346	5′- ACTACTTTCGCGCAGAGACG -3′ sense	Faggioli *et al*. (2005)
OLYaV-C		5′- CCCAAAGACCATTGACTGTGAC-3′ antisense	
CLRV-5	416	5′-TGGCGACCGTGTAACGGCA-3′ sense	Werner *et al*. (1997)
CLRV-3		5′-GTCGGAAAGATTACGTAAAAGG-3′ antisense	

Appendix II Guidelines on soil sampling for Verticillium dahliae analysis

Soil samples are air-dried for 1–2 weeks at 25°C, passed through a 2-mm sieve and mixed well, then 10-g subsamples are taken for further processing. The subsamples are washed through sieves with 125-mm and 37-mm pore size. The residue on the 37-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 ethanol-streptomycin-agar medium prepared by adding 1.6 g agar to 200 mL of distilled water and autoclaving the mixture for 20 min (Nadakavukaren & Horner, 1959). After the agar mixture has cooled to 50°C, 27 mg of streptomycin sulphate (75%) is added. This mixture, combined with the water agar, is poured onto 10 dishes. 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. For enumeration of *Verticillium* populations in soil, the sum of colonies counted on each set of agar dishes should equal the number of microsclerotia in 10 g of soil. (Butterfield & De Vay, 1977; Goud & Termorshuizen, 2003).

Appendix III *In vitro* maintenance and multiplication of olive

Two disinfection methods can be used: the method described by Rugini & Fontanazza (1981); dipping the samples in 70% ethanol (1 min), Mercryl (1 min) and 2% calcium hypochlorite (3 min). Both disinfection procedures are followed by three 10-min washes with sterile distilled water.

Shoot growth is induced from single-node explants using cultivation on Rugini basal medium (Rugini, 1984), with 3% sucrose, autoclaved 20 min at 121°C. Growth regulators (BA, NAA, ZR) are added as necessary (Sghir *et al*., 2005).

Cultures are maintained under fluorescent light (40 µmoles m^{-2} s^{-1}) following a 16 h-photoperiod with a matching 25/22°C thermoperiod. *In vitro* shoots are rooted in OM medium (Rugini, 1984) containing 5.37 µM NAA or 24.6 µM IBA (single phase) or with 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 medium.