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

CERTIFICATION SCHEME

Pathogen-tested material of grapevine varieties and rootstocks

Specific scope

This standard describes the production of certified pathogen-tested material of grapevine varieties and rootstocks.

Specific approval and amendment

First approved in September 1993 and revised in 2008.

The certification scheme for grapevine (*Vitis* spp.) provides detailed guidance on the production of pathogen-tested material of grafted grapevine varieties and rootstocks. Plant material produced according to this certification scheme is derived from nuclear-stock plants that have been tested and found free from the pathogens listed in Table 1, and produced under conditions minimizing infection from other major pathogens of the species concerned. Certified grapevine 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 grapevine varieties and rootstocks the following successive steps should be taken.

1 Selection for pomological and enological quality: individual plants of each variety and rootstock to be taken into the scheme are selected. Selected plants should not show evident viral symptoms: serological and/or molecular tests performed at this stage could highly reduce the risk to put into the scheme plants that not fulfill sanitary requirements. In the case that no one single clone of the selected variety has been found free from the considered pathogens, those affected with a minor number of viruses or by viruses more easily eliminated by sanitation techniques could be selected.

2 Production of nuclear stock: candidate nuclear-stock plants are propagated by cuttings. 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 under screenhouse. Alternatively, virus-free plants (candidate nuclear stock) can be produced by heat treatment and/or meristem-tip (shoot-tip) culture followed by testing. 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 retesting 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 (grafted varieties, vegetatively-propagated rootstocks) are produced in nurseries from the propagation stock. For grafted plants, rootstocks of at least propagation stock standard should be used.

Throughout the whole procedure, care should be taken to maintain the pomological and enological 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). 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.

1. Selection of candidates for nuclear stock

Select vines of each variety or rootstock according to procedures which ensure trueness-to-type and high pomological and

Table 1 Pathogens, transmissibility, geographical distribution and vectors for the diseases of grapevine occurring in the EPPO region

Virus	Geographical distribution	Vector
A. *Grapevine degeneration complex* (all mechanically transmissible)		
1. *Arabis mosaic virus* (*Nepovirus*, ArMV)	Europe, Japan, USA (California)	*Xiphinema diversicaudatum*
2. Grapevine chrome mosaic virus (*Nepovirus*, GCMV)	Hungary, former-Yugoslavia, Slovakia, Austria	Unknown
3. *Grapevine fanleaf virus* (*Nepovirus*, GFLV)	Worldwide	*Xiphinema index* *Xiphinema italiae* *Xiphinema vuittenezi*
4. *Raspberry ringspot virus* (*Nepovirus*, RpRSV)	Germany	*Paralongidorus maximus* *Longidorus macrosoma* *Longidorus elongatus*
5. *Strawberry latent ringspot virus* (*Sadwavirus*, SLRV)	Germany, Italy, Turkey	*Xiphinema diversicaudatum*
6. *Tomato black ring virus* (*Nepovirus*, TBRV)	Canada (Ontario), Germany, Hungary	*Longidorus attenuatus* *Longidorus elongatus*
B. *Grapevine leafroll complex*		
7. *Grapevine leafroll-associated virus 1* (*Ampelovirus*, GLRaV 1)	Worldwide	*Heliococcus bohemicus,* *Neopulvinaria innumerabilis,* *Phenacoccus aceris,* *Parthenolecanium corni*
8. *Grapevine leafroll-associated virus 2* (*Closterovirus*, GLRaV 2)	Worldwide	Unknown
9. *Grapevine leafroll-associated virus 3* (*Ampelovirus*, GLRaV 3)	Worldwide	*Planococcus citri,* *Planococcus ficus,* *Pseudococcus affinis,* *Pseudococcus calceolariae,* *Pseudococcus comstocki,* *Pseudococcus longispinus,* *Pseudococcus maritimus,* *Pseudococcus viburni,* *Pulvinaria vitis*
10. Grapevine leafroll-associated virus 4 (*Ampelovirus*, GLRaV 4)	Mediterranean, USA	Unknown
11. *Grapevine leafroll-associated virus 5* (*Ampelovirus*, GLRaV 5)	Mediterranean, USA	*Pseudococcus longispinus*
12. Grapevine leafroll-associated virus 6 (*Ampelovirus*, GLRaV 6)	Europe	Unknown
13. Grapevine leafroll-associated virus 7 (GLRaV 7)	Italy, Greece, Albania	Unknown
14. Grapevine leafroll-associated virus 8 (Ampelovirus, GLRaV 8)	USA	Unknown
15. Grapevine leafroll-associated virus 9 (GLRaV 9)	USA, Australia	*Pseudococcus longispinus*
C. *Grapevine rugose wood complex* (only grapevine closterovirus A is mechanically transmissible)		
16. *Grapevine virus A* (*Vitivirus*, GVA)	Worldwide	*Heliococcus bohemicus, Neopulvinaria innumerabilis* *Planococcus citri* *Planococcus ficus* *Pseudococcus affinis* *Pseudococcus comstocki* *Pseudococcus longispinus*
17. *Grapevine virus B* (*Vitivirus*, GVB)	Europe, Mediterranean Basin, USA, Australia	*Planococcus ficus* *Pseudococcus affinis* *Pseudococcus longispinus*
18. Grapevine rupestris stem pitting associated virus (*Foveavirus*, GRSPV)	Worldwide	Pollen (suspected)
D. *Grapevine fleck disease*		
19. *Grapevine fleck virus* (*Maculovirus*, GFkV)	Worldwide	Unknown
E. *Grapevine diseases caused by phytoplasmas*		
20. Grapevine flavescence dorée phytoplasma	Europe	*Scaphoideus titanus*
21. Grapevine bois noir and other yellows phytoplasmas	Europe, Chile, Israel, New Zealand, Australia	*Hyalestes obsoletus* (bois noir) *Oncopsis alni* (Palatinate yellows)

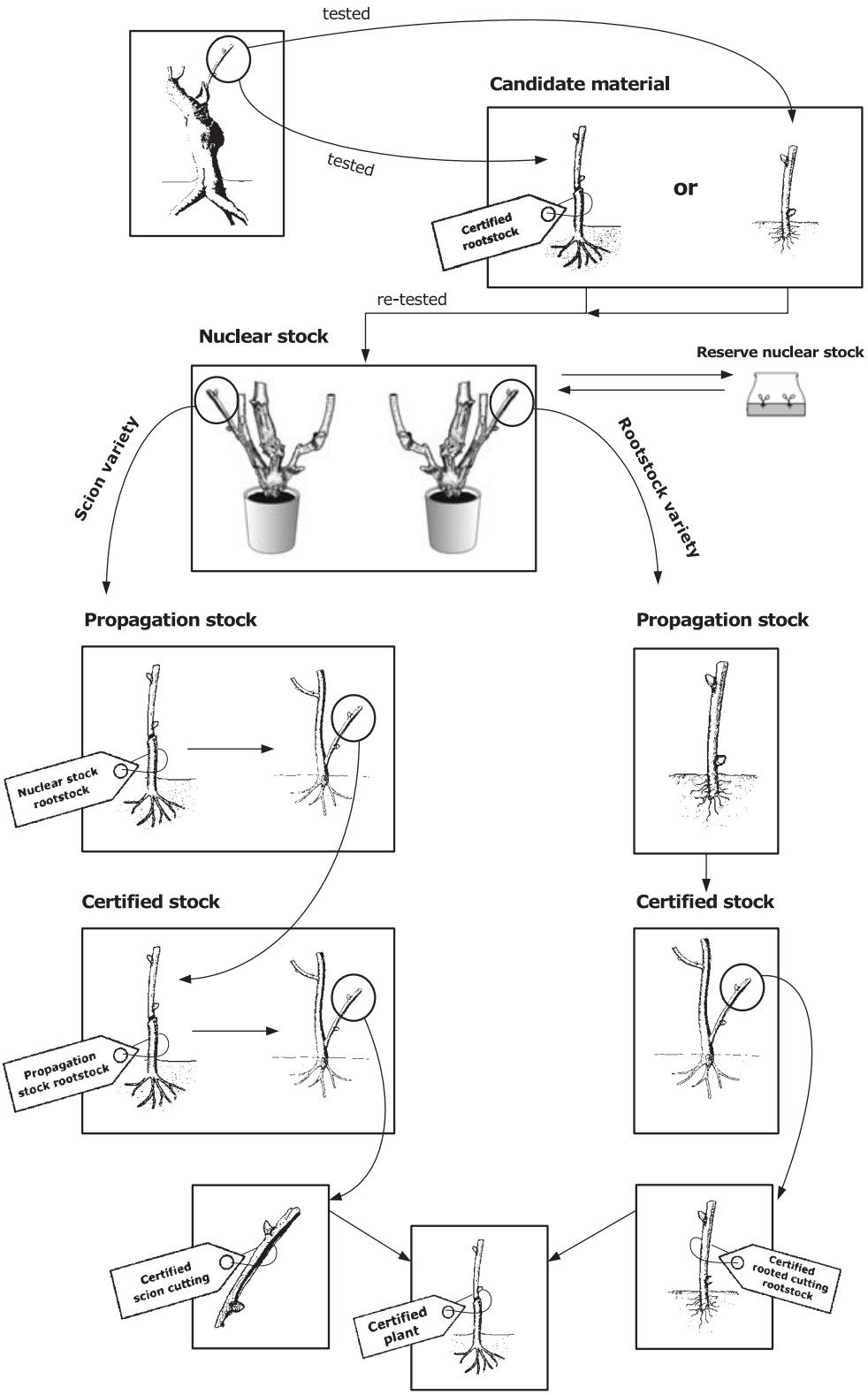

Fig. 1 Diagram of the stages in the grapevine certification scheme.

Table 2 Main indicators for virus and virus-like diseases of grapevine*

Indicator	Disease identified
1. *Vitis rupestris* St George	Degeneration†, fleck, rupestris stem pitting
2. *Vitis vinifera* Cabernet franc, Pinot noir and other red-berried cultivars	Leafroll‡
3. Kober 5BB (*Vitis berlandieri* × *Vitis riparia*)	Kober stem grooving
4. LN 33 (Couderc 1613 × *Vitis berlandieri*)	Corky bark, enation, LN33 stem grooving
5. *Vitis riparia* Gloire de Montpellier	Vein mosaic§
6. 110 R (*Vitis rupestris* × *V. berlandieri*)	Vein necrosis§

*Appendix 1 provides full details of the conditions for the tests and suggests some alternative indicators.
†In countries where degeneration is also caused by nepoviruses other than grapevine fanleaf nepovirus, Siegfriedrebe (FS4 201/39) may be used as an indicator.
‡The choice of the most suitable indicator for leafroll depends on climatic conditions of the region where the testing is done.
§As noted in Appendix 1, these are optional.

enological quality. Selected plants should not show evident viral symptoms: serological and/or molecular tests performed at this stage could highly reduce the risk of including plants in the scheme that do not fulfill sanitary requirements. In the case that no one single clone of the selected variety has been found free from the considered pathogens, those affected with a minor number of viruses or by viruses more easily eliminated by sanitation techniques could be selected.

Collect cuttings from the selected vines and cold-store (e.g. at 2–4°C) until use. If conditions are favourable (e.g. sandy soils, low levels of infestation by phylloxera, *Viteus vitifoliae*), force cuttings of scion varieties or rootstocks to root in a glasshouse and transplant rooted cuttings as such in the field. Alternatively, for scion varieties, graft buds or bud sticks from selected vines onto vegetatively propagated certified rootstocks. Prior to use, cuttings and rootstocks should be tested for freedom from the harmful diseases and pathogens specified in Tables 1 and 2.

The soil should be free from nematode vectors and vines should be carefully protected from aerial vectors (mealybugs and leafhoppers) in areas where these may occur. For the repository, a safety distance from commercial vineyards or mother-vine plots is not strictly necessary. Contiguity between repository and other grapevine stands should, however, be avoided.

2. Production of nuclear stock

To become nuclear stock plants, selected vines should undergo testing for the harmful diseases and pathogens occurring in the EPPO region specified in Tables 1–2 and/or sanitation according to the procedures of Appendices 1 and 2. Material imported from outside the EPPO region should also be tested by EPPO-recommended methods for all other viruses or virus-like pathogens occurring naturally in *Vitis* in the region of origin. Testing on the woody indicators specified in Table 2 is essential for material to be classified as nuclear stock, but the other procedures given in the disease-detection summaries of Appendix 1 may be useful for preliminary screening or for retesting. Regardless of the type of treatment used, sanitized vines should be retested. Plant rooted explants from heat chamber or tissue culture into separate pots, and grow them in a glasshouse or screenhouse to ensure freedom from aerial vectors. A duplicate of nuclear stock plants should be kept *in vitro* or in cryopreservation. If vigorous growth is obtained, serological or molecular tests and testing on woody indicators can be performed within a year after transplanting. If candidate nuclear stock plants of a given variety or rootstock give negative results in all tests, it can be promoted to a nuclear stock plant and moved to the nuclear stock repository.

If, for a given variety or rootstock, 100% virus infection can be expected, it is advisable not to waste time with the first testing, but to proceed directly with sanitation.

Inspection for other pests

Besides the diseases and pathogens specifically considered above, all material should also be checked for the presence of other pests which can be carried on propagation material and affect its quality. In particular, this should be done for freedom from crown gall (*Agrobacterium tumefaciens*, *A. vitis*), *Xylophilus ampelinus*, and fungal type diseases (*Phomopsis viticola*, *Eutypa* spp., *Phaeoacremonium aleophilum*, *Fomitoporia punctata*, *Phaeomoniella chlamydospora*), and mites (*Calepitrimerus vitis*, *Panonychus ulmi*, *Eotetranychus carpini*). It should be noted that latent infections of *A. tumefaciens* and *A. vitis* may occur. Other graft-transmissible diseases known to occur in the EPPO region are tolerated for the moment, but every effort should be made to eliminate them.

3. Maintenance of nuclear stock

Pot individually a limited number (2–5) of nuclear stock plants of each source (clone) of each variety or rootstock type taken into the scheme, and grow them under conditions ensuring freedom from re-infection by aerial or soil vectors. For this purpose, double-door entry, insect-proof screenhouses with gravel floor, heavy plastic or tarpaulin or any other material preventing contact of the roots with soil are suitable. Nuclear stock plants should be kept under continuous surveillance and be sprayed regularly with appropriate pesticides, to control the normal quality pests of grapevine.

In vitro storage of a duplicate set of each nuclear stock plant can be envisaged when reliable procedures for *in vitro* maintenance of *Vitis* germplasm become available.

Vines in the repository should be checked each year for virus symptoms and other pathogenic disorders. Retesting is advisable if new and better detection techniques, antisera, molecular tools or indicators become available, or whenever visual inspections suggest tests should be carried out.

4. Production of propagation stock

The nuclear stock should be multiplied in as few steps as possible to obtain the required quantity of propagation stock. Propagation stock should be established, preferably, in soils with no grapevine history, or in soils that have not hosted grapevines for at least 6 years and, in any case, are found free from virus-transmitting nematodes. These fields should have a safety distance of 15–20 m from any vineyard made up of material of lower category (certified), but this may be reduced if the soil in the adjacent fields (vineyards or orchards) has been found to be free from virus-transmitting nematodes.

(a) Rootstocks. Place cuttings from each rootstock type in the nuclear stock in a glasshouse for rooting, and plant rooted cuttings directly in the field, each source in a separate plot, or row, and labelled so as to be readily distinguished from one another.

(b) Varieties. Graft each variety taken into the scheme onto rootstocks of the same certification level, and transplant the grafted vines into the field.

Propagation stock is visually inspected each year for symptoms of graft-transmissible diseases, and vines showing suspect symptoms should be tested immediately. During production of propagation material, some random tests on virus status should also be performed using available short-time testing methods (e.g. ELISA test, green grafting). This random testing scheme may be initiated when plants are 5-years old, and 10% of the plants are tested every year so that after 10 years all plants have been tested at least once.

5. Production of certified plants

(a) *Varieties*. Scion material from propagation stock is distributed to nurseries. For the production of mother-vine plants from which certified propagative material is to be derived, propagation stock scion material should be grafted by the nurseries onto rootstocks of the propagation stock category only.

(b) *Rootstocks*. Rooted cuttings, intended for the establishment of mother-vine plants for the production of certified cuttings or rooted cuttings, are distributed to nurseries. Mother vines for the production of certified material (bud sticks of scion varieties, rooted cuttings of rootstocks, grafted vines) should be established in plots at a minimum distance of 8–10 m from other vineyards or orchards and in soils free from virus-transmitting nematodes. To this effect, the nurseries should produce a certificate of nematological analysis issued by an official, or officially authorized, organization.

During production of certified material in nurseries, some random tests on virus status should also be performed using available short-time testing methods (e.g. ELISA test, green grafting).

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 grapevine growers 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, the certification status of the plants, and the name of the variety and the rootstock.

Appendix 1 Guidelines on testing procedures

1. Testing on *Vitis* indicators

The use of *Vitis* indicators is still a compulsory step in any grapevine certification programme. It cannot be excluded because there are some diseases (leafroll and rugose complex) which cannot be identified except on woody differential hosts. Testing is performed by grafting on the indicators listed in Table 2. Since at least three replicates of any variety or rootstock type taken into the scheme are grafted on each indicator, a total of 6–8 grafts is required for each candidate vine. Various grafting techniques can be used:

(a) Whip or cleft grafting in the field
(b) Machine grafting
(c) Green grafting (Walter *et al*., 1990).

It is recognized that green grafting has distinct advantages over other techniques. Therefore, an effort should be made to encourage its use.

2. ELISA testing

The use of ELISA is recommended for *Grapevine fanleaf virus* and other European nepoviruses where they occur, for *Closteroviridae* (GLRaV 1 to 9), vitiviruses (GVA and GVB), and maculavirus (GFkV) for which antisera are available. Sources of antigens for ELISA tests can be grapevine buds, roots, leaves and wood shavings. Wood shavings, however, are advantageous because: (i) they can be used throughout the year without apparent loss of efficiency due to the seasonable variation of antigen titre in vegetative organs; (ii) give low and consistent background readings; (iii) are much more reliable for identification of viruses belonging to family *Closteroviridae* in American rootstocks, especially *Vitis rupestris* and its hybrids. Use of ELISA testing is regarded as a complement to, but not as a substitute for, other diagnostic procedures. It may be useful, for example, for preliminary screening or for random testing.

3. Molecular testing

In recent years, PCR has gained popularity due to his higher sensitivity over bioassay and ELISA. It is particularly useful if antisera for ELISA are not available or not suitable. It has been demonstrated that the use of RT-PCR can increase (at least by 10%) the number of positive samples, especially for GFLV and GFkV detection (Faggioli & La Starza, 2006). For this reason the use of RT-PCR, in single or multiplex reaction is strongly suggested on ELISA negative samples. This procedure will reduce the number of indicator graftings with the consequence that almost 90% of candidate clones will give negative results in biological tests. Multiplex RT-PCR (mRT-PCR) protocols have been developed for detection of several viruses in a single reaction, thus providing quick, reliable and cost-effective routine diagnosis (Faggioli & La Starza, 2006; Gambino & Gribaudo, 2006).

Phloem scraped from mature canes collected during winter pruning is the best tissue to use for RNA extraction. Different protocols for total RNA extraction can be applied. Several primer sets for virus detection can be used in single or multiplex combination (Faggioli & La Starza, 2006; Gambino & Gribaudo, 2006). Molecular testing is helpful in early screening of tissue culture material, in the framework of sanitation programs, because it requires a small amount of fresh *in vitro* grown tissue (200 mg).

4. Detection of individual diseases

Several diseases and viruses (such as enation, vein necrosis, vein mosaic, *Artichoke Italian latent virus*, *Grapevine Bulgarian latent virus*, *Grapevine Tunisian ringspot virus*) were included in the first version of this EPPO Certification scheme but they have been deleted because of their scarcity and low importance.

Fanleaf (GFLV)
Graft transmission
Indicator: *Vitis rupestris* St George
No. plants per test: 3–5 rooted cuttings
Inoculum: Wood chips, single buds, bud sticks, shoot tips
Temperature: 22–24°C
Symptoms: (a) Acute phase (shock) symptoms. Chlorotic spots, rings and lines, localized necrosis 3–4 weeks after grafting (chip-bud or green grafting)
(b) Chronic symptoms. Reduced growth, severely deformed leaves with prominent teeth (distorting strains), bright yellow discolorations and mild deformation of the leaves (yellow mosaic strains)
Recommended tests: Serology (ELISA), Molecular (RT-PCR, probes).

Grapevine degeneration (European nepoviruses)
Graft transmission
Indicators: Several *Vitis vinifera* cultivars: Pinot noir, Jubileum 75 (GCMV); Siegfriedrebe (ArMV, RRV, TBRV)
No. plants per test: 3–5 rooted cuttings
Inoculum: Wood chips, single buds, bud sticks, shoot tips
Temperature: 22–24°C
Symptoms: Severe stunting and necrosis of the apex of Pinot noir in second year of vegetation (GCMV); foliar discolorations and cane deformations of Siegfriedrebe within the first year after inoculation.
Recommended tests: Serology (ELISA), Molecular (RT-PCR, probes).

Leafroll (GLRaV's) compulsory to check at least: GLRaV 1, 2, 3
Graft transmission
Indicators: Several cultivars of red-fruited *V. vinifera* (Pinot noir, Cabernet franc, Merlot, Barbera, Mission)
No. plants per test: 3–5 rooted cuttings
Inoculum: Wood chips, single buds, bud sticks, shoot tips
Temperature: 22°C (green grafting)
Symptoms: Rolling and reddening of the leaves in 4–6 weeks (green grafting) or 6–8 months to 2 years (field testing)
Recommended tests: Serology (ELISA), Molecular (RT-PCR, probes).

Rugose wood complex (GVA, GVB)
Graft transmission
Indicators: Kober 5BB
No. plants per test: 3–5 rooted cuttings
Inoculum: Wood chips or single buds (recommended for rupestris stem pitting), bud sticks
Temperature: 22°C (green grafting)
Symptoms: Stem grooving (Kober stem grooving)
Recommended tests: Serology (ELISA), Molecular (RT-PCR, probes).

***Fleck (GFkV)*[1]**
Graft transmission
Indicator: *Vitis rupestris* St George
No. plants per test: 3–5 rooted cuttings
Inoculum: Wood chips, single buds, bud sticks, shoot tips
Temperature: 22°C (green grafting or growth chamber)
Symptoms: Clearing of veinlets in 4–6 weeks according to growing conditions
Recommended tests: Serology (ELISA), Molecular (RT-PCR, probes).

Grapevine yellows *(Flavescence dorée, Bois noir and other European yellows diseases)*
Symptoms: Symptom observation in the field during summer. Leaves turn yellow or red depending on the cultivar. They roll downward and become brittle. The interveinal areas of leaves may become necrotic. Shoots show incomplete lignification and rows of black pustules develop on the green bark along the diseased branches; they are thin, rubbery and hang pendulously.
Recommended tests: Molecular (PCR), details can be found in the EPPO diagnostic protocol (EPPO Standard PM 7/79, 2007).

Appendix 2 Guidelines on sanitation procedures

1. Heat treatment

All known graft-transmissible infectious agents of grapevine, except viroids, can be eliminated from parts of infected plants with varying levels of efficiency by heat therapy. Heat treatment can be performed in several ways but, regardless of the procedure used, testing of the treated material for assessment of its health status should follow. A sufficient interval between sanitation of the material and the conclusion of virus testing is necessary in order to avoid false negatives. The use of RT-PCR technique will help in selecting the successfully sanitized material, avoiding the false negative recovery due to the very low virus concentration after the first step of sanitation.

Hot-air treatment
(a) Place pot-grown vegetative vines (e.g. rooted cuttings 2-years-old or older) of each variety or rootstock type to be taken into the scheme into a heat cabinet and maintain at constant temperature of 38 ± 1°C and 16–18 h artificial illumination. Collect tips 0.5–1 cm long from vegetative shoots after 4 weeks or more (up to 300 days if the vines survive) from the beginning of the treatment, and root in a heated (25°C) sand bench under mist or, after surface sterilization, in agarized nutrient medium under sterile conditions. Pot rooted explants and let them grow in a glasshouse until ready for testing. For further details, see Goheen & Luhn (1973), Martelli (2002), Ottenwaelter *et al*. (1973), Stellmach (1980).

[1]It must be absent only from rootstocks.

Hot-water treatment
Hot water treatment of dormant canes is successfully used to eliminate phytoplasmas, partially *Agrobacterium vitis* and several other pests. The dormant material is immersed in agitated water at 50°C and treated for 45 minutes according to the method of Caudwell *et al*. (1991). The hot water treatment should be done immediately before grafting, at the end of storage in a specially designed equipment maintaining exactly the required temperature throughout the plant material by an efficient mixing system (Burr *et al*., 1996; Boudon-Padieu & Grenan, 2002).

2. Shoot (meristem) tip culture in vitro

Collect shoot tips or auxiliary buds from vines grown at 36–38°C, surface-sterilize by dipping explants for 20 min in a 5% solution of commercial sodium hypochlorite and 0.1% Tween 20. Rinse thoroughly with 2–3 changes (10 min each) of sterile distilled water.

Dissect 0.4–0.6 mm-long explants comprising the meristematic dome and the first pair of leaf primordia and transfer to sterile test tubes in agarized Murashige and Skoog medium supplemented with 0.5 ppm benzylaminopurine. Allow explants to grow for 45 days at 25°C in a cabinet with 16 h artificial illumination (about 4000 lux).

Separate actively growing shoots and transfer them individually to a medium containing 1 ppm benzylaminopurine for 45–50 days for elongation. Transfer elongated shoots (3 nodes long or more) individually to a medium containing 0.5–1 ppm indolbutyric acid for root production. Transfer rooted explants to small pots containing vermiculite under saturated humidity conditions, then to pots with soil compost and protect plantlets with a polyethylene bag for as long as necessary (usually 2–3 weeks). Grow plantlets in a glasshouse until ready for testing.

For further details, see Barlass *et al*. (1982).

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