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

Certification scheme for strawberry

Specific scope

This standard describes the production of certified pathogen-tested material of strawberry.

Specific approval and amendment

First approved in September 1994 and revised in 2008.

The certification scheme for pathogen-tested material of strawberry (*Fragaria* × *ananassa*) provides detailed guidance on the production of vegetatively propagated strawberry plants. The certification scheme has the aim of providing strawberry plants which are true-to-type, free from virus diseases and substantially free from other pests. 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 infestation by other 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 pathogen-tested strawberry plants, the following successive steps should be taken:

1 Selection for pomological quality: individual plants of each cultivar to be taken into the scheme are selected
2 Production of nuclear stock: candidate nuclear stock plants are tested, or submitted to heat treatment or an *in vitro* sanitation method, followed by testing. Only candidate nuclear stock plants that have met all requirements are promoted to nuclear stock plants
3 Maintenance of the nuclear stock: nuclear stock plants are maintained under conditions ensuring freedom from infection via pollen, aerial or soil vectors, with re-testing as appropriate
4 Production of propagation stock: propagation stock is produced from nuclear stock material in one or more phases (propagation stock), under conditions ensuring freedom from infection, with retesting as appropriate
5 Production of certified material: runners taken from propagation stock are grown under conditions minimizing infections to produce certified plants (runners finally distributed for fruit production).

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.

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

New or existing cultivars of strawberry (*Fragaria* × *ananassa*) may be selected as candidate material. The starting material should be selected visually on the basis of trueness-to-type, vigour, quality and absence of symptoms of pests. Alternatively, starting material may be obtained from existing certification schemes in other EPPO countries. Material imported from outside the EPPO region should also be tested for all other viruses occurring naturally in strawberry in the region of origin.

2. Production of nuclear stock

The candidate material for nuclear stock status should be kept under quarantine in an isolated suitably designed insect-proof glasshouse or gauzehouse, separately from the nuclear stock. All plants should be grown in individual pots in a sterilized growing medium with precautions against infection by pests. Particular care should be taken to prevent infection by any of the pests listed in Appendix 1. The general status of the plants with respect to these pests, and to other diseases or

Table 1. Recommended methods of detection and identification of strawberry viruses and virus-like agents. Adapted from Converse (1987)

Pathogen	Symptoms in cvs*	Mechanical inoculation to herbaceous hosts	Indicators for leaf graft transmission†	Alternative tests (see Notes 1 and 2)
Virus and virus-like agents occurring in the EPPO region and which are tested for in this scheme				
Aphid-borne				
Strawberry crinkle virus (*Cytorhabdovirus*, SCV)	–	–	4, 5	Petal streak RT-PCR
Strawberry mild yellow edge virus (*Potexvirus*, SMYEV)	–	–	4, 5	UC-6 latent, RT-PCR
Strawberry mottle virus (*Sadwavirus,* SMoM*)*	–	–	4, 5	by Cf RT-PCR
Strawberry veinbanding virus (*Caulimovirus*, SVBV)	–	–	6, 12	10, 11 latent PCR
Leafhopper-borne				
Strawberry green petal phytoplasma	+	–	–	Distinguish on herbaceous hosts, PCR
Nematode-borne				
Arabis mosaic virus (*Nepovirus*, ArMV)	–	+	–	
Raspberry ringspot virus (*Nepovirus*, RpRSV)	–	+	–	
Strawberry latent ringspot virus (*Sadwavirus*, SLRV)	–	+	–	
Tomato black ring virus (*Nepovirus*, TBRV)	–	+	–	
Virus and virus-like agents not present in the EPPO region or of minor importance which may optionally be tested				
Aphid-borne				
Strawberry pseudo mild yellow-edge virus (*Carlavirus*, SPMYEV)	–	–	4, 12, Alp	10, 11 latent
Strawberry latent C	–	–	5, EMC	
Leafhopper-borne				
Aster yellows phytoplasma	+	–	–	PCR
Lethal decline	+	–	–	
Phytoplasma yellows	+	–	–	
Rickettsia yellows	+	–		
Nematode-borne				
Tomato ringspot virus (*Nepovirus*, TRSV)	–	+	4, 5, Alp	
Vector unknown				
Strawberry chlorotic fleck virus (*Closterovirus*, StCFV)	–	–	EMB, EMK	RT-PCR
Leafroll	+	–	5	
Witches' broom	+	–	4, 5	
Multiplier plant	+	–	–	
Feather-leaf	+	–	Alp, 4, 1	
Strawberry associated pallidosis virus (*Crinivirus*, SPaV)	–	–	10, 11	RT-PCR
Strawberry necrotic shock virus (*Ilarvirus*, SNSV)	–	+	Alp, 4	RT-PCR
Beet pseudo-yellows virus (*Crinivirus*, BPYV*)*	–	–	–	RT-PCR, ELISA
Fragaria chiloensis cryptic virus (FClCV)	–	–	–	RT-PCR
Fragaria chiloensis latent virus (*Ilarvirus*, ClLV)	–	–	–	RT-PCR
Apple mosaic virus (*Ilarvirus*, ApMV)	–	–	–	RT-PCR

*The cultivar itself develops symptoms that enable the causal agent to be identified.

†Abbreviations for strawberry indicators. Numbers = UC indicator clones 1–12; Alp = *F. vesca* var. *semperflorens* 'Alpine'; EMB, EMV, EMK = various clones of *F. vesca* 'East Malling clone'; Cq = *Chenopodium quinoa*; Cf = *Chaetosiphon fragaefolii* (for further details, see Converse, 1987).

Note 1: ELISA tests can be used for confirmation of infection if identification to species is required. Some nepoviruses (RpRSV, TBRV, TRSV and SNSV) are quite variable and a single antiserum may not detect all isolates. This is especially true if one is using monoclonal antibodies. A summary of all strawberry viruses and detection methods is provided in Martin & Tzanetakis (2006).

Note 2: Pathogen diagnosis based on PCR has undergone a rapid development over the past decade. This includes nucleic acid extraction technology from almost any plant tissue enabling subsequent enzymatic reactions. As a result, PCR detection is generally available for pathogens whenever their genomes have been characterized. However, it should be kept in mind that PCR tests cannot be regarded as reliable unless knowledge is available on the variability of individual pathogens and some experience has been gained on the specific crop. For the characterized viruses in strawberry, the situation for PCR detection is at different level of development. Therefore, PCR detection is only mentioned when the Panel had knowledge that the tests were of equal or superior quality to other recommended methods in Table 1. It can be expected that additional PCR tests will become available before the existing scheme may be updated.

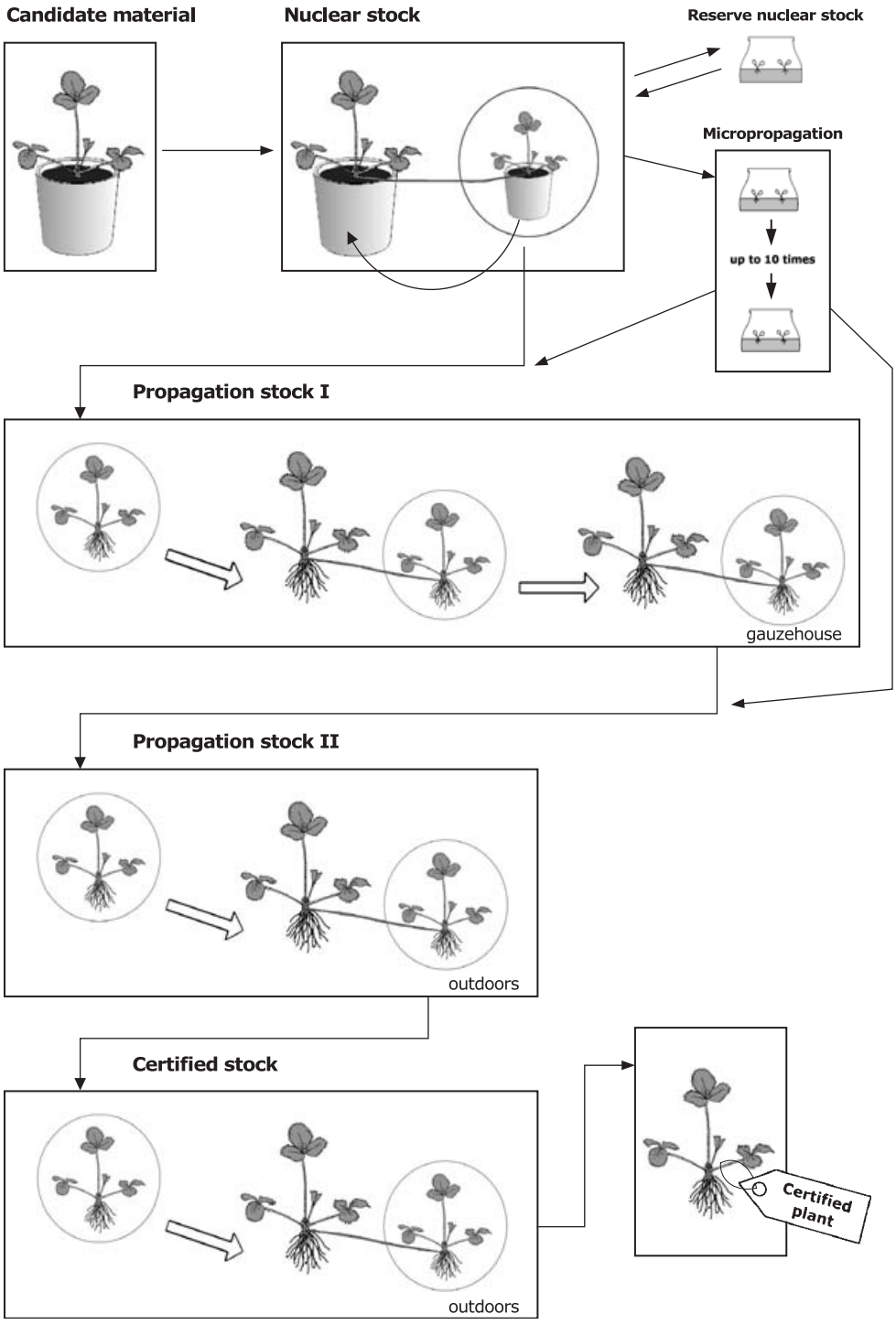

Fig. 1 Diagram of the strawberry certification scheme.

Table 2 Suggested tolerances in visual inspection for strawberry pests at different stages of certification

		% plants			
		NS	PSI	PSII	CM
Viruses	Listed for strawberry in Table 1	0	0	0	2*
Phytoplasmas	Listed for strawberry in Table 1	0	0	0	1
Bacteria	*Xanthomonas fragariae*	0	0	0	0
Oomycetes	*Phytophthora cactorum*	0	0	0	1
	Phytophthora fragariae var. *fragariae*	0	0	0	0
Fungi	*Colletotrichum acutatum*	0	0	0	0
	Verticillium dahliae & *V. albo-atrum*	0	0	0	2
	Rhizoctonia fragariae	0	0	0	1
Arthropods	*Chaetosiphon fragaefolii*	0	0	1	1
	Phytonemus pallidus fragariae	0	0	0	0.1†
Nematodes	*Aphelenchoides* spp.	0	0	0	0
	Ditylenchus dipsaci	0	0	0	0
Other pests and diseases	e.g. *Tetranychus urticae*, *Sphaerotheca alchemillae*	Substantially free	Substantially free	Substantially free	Substantially free

*With the exception of quarantine viruses in the production country for which a zero tolerance applies.
†Tolerance applies to the presence of live mites not of symptoms.
NS = nuclear stock; PSI = propagation stock I; PSII = propagation stock II; CM = certified material.
Note: a new disease, leaf marginal chlorosis, has recently been observed in Spain and in France. It has been described in France and is caused by a phloem-limited bacterium. Tolerances have not yet been established.

unknown symptoms, should be regularly checked by visual inspection.

All plants are individually tested (according to Appendix 1) for the following virus diseases: *Arabis mosaic virus (Nepovirus)*, *Raspberry ringspot virus (Nepovirus)*, *Strawberry crinkle virus (Cytorhabdovirus)*, strawberry green petal phytoplasma, *Strawberry latent ringspot virus (Sadwavirus)*, *Strawberry mild yellow edge virus (Potexvirus)*, *Strawberry mottle virus (Sadwavirus)*, *Strawberry vein banding virus (Caulimovirus)*, *Tomato black ring virus (Nepovirus).* All plants are also individually tested for *Phytophthora fragariae* var. *fragariae*, *Xanthomonas fragariae*, *Phytophthora cactorum*, *Colletotrichum acutatum*, *Ditylenchus dipsaci* and the following species of *Aphelenchoides*: *A. besseyi*, *A. blastophthorus*, *A. fragariae* and *A. ritzemabosi*. The recommended test methods are given in Appendix 1. Plants giving negative results in all tests are promoted to nuclear stock plant status and should be transferred to a separate gauzehouse of similar standard (see Section 3). Plants giving positive results in any test should be removed immediately.

If no plants of a cultivar or clone prove to be free from these pathogens, heat treatment may be applied to eliminate infection. The plants resulting from heat treatment are considered again as candidate material and must be re-tested for the viruses above and re-assessed for agronomic and varietal characters.

Alternatively, meristem culture may be used to eliminate infection (Appendix 3). In this case, the resulting nuclear stock plants are normally kept in tissue culture and explants should be re-tested for the viruses above. It should be noted that meristem culture is also very effective in eliminating fungal and bacterial diseases of strawberry, and foliage nematodes.

If no plants of a cultivar or clone prove to be free from the stem and foliage nematodes *D. dipsaci*, *A. fragariae*, *A. besseyi*, *A. blastophthorus* or *A. ritzemabosi*, the plants may be treated according to the methods described in Appendix 3 (Elimination of stem and foliage nematodes) and subsequently re-tested.

3. Maintenance of the nuclear stock

Nuclear stock plants should be kept in a suitably designed insect-proof gauzehouse, containing only nuclear stock plants. They should be maintained under the same conditions and with the same checks on pest freedom as candidate nuclear stock plants. Nuclear stock plants of strawberry are normally propagated once a year by the first method mentioned in 4.1: some runners from each plant are retained to become the following year's nuclear stock plants, if grown under the same conditions and individually tested[1] at least for *P. fragariae* var. *fragariae* and for the aphid-transmitted viruses; other runners are taken as samples to test for stem and foliage nematodes; the remainder will normally be used to produce propagation stock (Section 4). After propagation, the mother plant is removed (and may also usefully be tested for stem and foliage nematodes). In general, any plant giving a positive result in a test or showing symptoms of any disease (fungal, bacterial, viral) in Table 2 should be eliminated.

[1]The possibility of infection by the other pests for which the candidate nuclear stock was tested should be considered; occasional re-testing is advised.

Reserve nuclear stock may be maintained *in vitro* on a growing medium without hormones, at 2°C for 3–4 years without any sub-cultures, or for a longer period with sub-cultures every 2 years. If such reserve material is to be removed from *in vitro* conditions and used for further propagation, it must be checked for trueness-to-type.

4. Production of propagation stock

4.1. Propagation stock I

Two methods for producing propagation stock I can be used:

Method 1. Runner tips from nuclear stock plants are pinned down in separate pots of sterilized growing medium. The pots in which runner tips are rooted are kept at a higher level than the nuclear stock pots to avoid transmission of soil or root pathogens through watering. When the runners have rooted, they are separated from the parent nuclear stock plants. These plantlets become the first stage of propagation stock I and are transferred to a separate insect-proof gauzehouse where they may be planted in runner beds of sterilized growing medium to act as mother plants for a second generation of propagation stock I (i.e. a maximum of two generations of propagation stock I)

Method 2. Multiplication entirely *in vitro*, beginning with meristems, apical tips or axillary buds (Appendix 4) from nuclear stock plants. The number of reproduction cycles should be, at most, 10 (see Appendix 4). The rooted plants transplanted out of the *in vitro* conditions become propagation stock I or II, depending on the demand for the number of plants. All plants produced by *in vitro* multiplication must be clearly designated as such. However, the progeny of such plants need not carry the designation, as trueness-to-type can be adequately determined within one multiplication step, provided an appropriate number of plants produces fruit.

Propagation stock I plants are randomly tested for *Phytophthora cactorum*, *Phytophthora fragariae*, *Colletotrichum acutatum*, and *Xanthomonas fragariae* var. *fragariae*.

General precautions against pests should be maintained but plant protection products known to mask symptoms of *Phytophthora* species or *Verticillium albo-atrum* and *Verticillium dahliae* should be avoided. Any plant showing symptoms of any disease or infested with any pest listed in Appendix 2 should be eliminated.

For stem and foliage nematodes, visual inspection of plants in glasshouses may not be sufficient to detect their presence, as symptoms are not very pronounced under these conditions. Tests for these nematodes may be conducted on the mother plants at the stage when the progeny plants have been separated from the mother plants (see Section 3 and Appendix 1).

The filiation of the plants should be recorded so that each propagation stock I plant is known to be derived from nuclear stock by not more than a fixed number of generations (i.e. two generations in production method 1 above or ten generations in method 2). Additional generations may be permitted, by method 1, for cultivars that do not runner freely.

4.2. Propagation stock II

Plants of propagation stock II are produced by runnering from propagation stock I in as few generations as possible. The plants are produced under conditions which reduce the risk of aphid vectors and the soil should be tested for nematodes (see Appendix 1). The site should only be used for the production of propagation stock plants if found substantially free or if those found are shown to be free from virus (see EPPO Standard PM4/35, in preparation).

The plants are propagated on plots isolated by at least 50 m from non-certified material of strawberry. Precautions should be taken against aerial borne vectors and soil borne diseases. The presence of other alternative hosts of *C. acutatum* should be taken into account.

General precautions against pests should be maintained but plant protection products known to mask symptoms of *V. albo-atrum* and *P. fragariae* should be avoided. Propagation stock II plants should be inspected regularly and should conform with the recommended certification standards in Appendix 2.

5. Production of certified material

Strawberry plants from which certified material will be taken (runners then grown for fruit production), are produced by runnering from propagation stock II. These plants are kept on plots isolated by at least 50 m from non-certified material of strawberry. Precautions should be taken against aerial borne vectors and soil borne diseases.

The soil should be tested for nematodes (see Appendix 1) and the site should only be used for the production of propagation stock plants if found substantially free or if those found are shown to be free from virus (see EPPO Standard PM4/35, in preparation).

Precautions should be taken to avoid, as much as possible, infestation by aphid vectors of strawberry viruses and by *Meloidogyne hapla* (e.g. treatment at an appropriate time with a suitable plant protection product).

General precautions against pests should be maintained and the plants should be inspected regularly and be found to conform with the recommended certification standards in Appendix 2. It may be useful to allow such plants to fruit. Any plant showing symptoms of pests which are listed in Table 2 should be eliminated.

Plants should be substantially free from *Sphaerotheca alchemillae* and *Tetranychus urticae*. Certified material (runners) may be held for a certain period before sale under refrigeration or in waiting beds under the same conditions as their mother plants.

Throughout the production of propagation stock and certified plants, checks should be made on varietal purity and on possible mutations or back mutations and on June yellows. Special care should be taken in the case of material from *in vitro* multiplication.

Inspection for the granting of certificates should be performed in early summer.

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 strawberry 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 and the certification status of the plants.

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Appendix 1 Guidelines on testing procedures

Testing for viruses on indicator plants

Viruses occurring specifically in strawberry are tested for by means of mechanical inoculation onto suitable sensitive herbaceous indicators or foliage grafting on *Fragaria* indicators. A compilation of well tested indicators and instructions on use of the foliage-grafting technique can be found in Converse (1987) (from which Table 1 has been adapted).

In general, all strawberry viruses occurring in the EPPO region can be detected by leaf-grafting to the hybrid clones UC4 or UC5, *Fragaria vesca* EMC or *Fragaria vesca* var. *semperflorens* Alpine (for aphid-borne viruses). Nepoviruses can be detected by mechanical inoculation to *Chenopodium quinoa* or *Chenopodium amaranticolor*. For the identification of these viruses, serological and molecular methods or other indicators shown in Table 1 can be used. Phytoplasmas can be detected by PCR or visual observation of symptoms.

Xanthomonas fragariae

Testing procedures for *X. fragariae* can be found in the EPPO diagnostic protocol (EPPO Standard PM7/65(1), 2006).

Phytophthora fragariae

All detection methods in use are based on the bait test originally developed by Duncan (1980). Root tips (2.0–2.5 cm long) are cut from the plants for test and mixed with a soil-free compost (1:3 sand/peat, plus fertilizers, trace elements and lime; final pH 5.5). The mixture is put into 12.5-cm plastic pots and each pot is planted with 5 plants, about 7.5–10.0 cm tall of *F. vesca* var. *semperflorens* cv. Baron Solemacher, grown from seed or of a susceptible runnering hybrid clone (e.g. UC5). The seedlings should have been transplanted into trays of compost 2–3 weeks after sowing and grown for 4–6 weeks in a growth cabinet at 20°C under continuous light (8000 lux). If using rooted runners, these should be rooted for 10–14 days prior to use in bait tests. The pots are placed on a bench designed to collect all pot drainage water to prevent local contamination. Glasshouse temperature is maintained at 15°C. Plants are watered by mist irrigation for 15 min every 6 h or by careful direct watering. After 5 weeks the roots of each bait plants are examined for the typical red steles and oospores. Control plants grown in compost alone should be placed at random among test plants to detect the possibility of cross contamination.

To detect *P. fragariae*, the following tests can also be used (Bonants *et al*., 2004):
- Water bait test followed by visual examination for red steles and oospores
- Nested PCR on water from a water bait test
- Water bait test combined with PCR of bait roots.

The water bait test can be used alone or in combination with a PCR test of the bait plants and of water from the water bait test. Direct PCR on smaller samples is also possible but for larger samples the water bait test is recommended.

Phytophthora cactorum

Three leaf bases are removed from each of the plants to be tested and are covered with tap water or sterile soil water in a Petri dish, or transferred to a suitable growing medium. After incubation in the light at ambient laboratory temperature (at approximately 20°C), they are examined each day for sporangia of *P. cactorum*, and discarded after 3 days.

Colletotrichum acutatum

Paraquat test

Strawberry plants may harbour this pathogen in a quiescent state in the form of inconspicuous lesions on any part of the plant and infection cannot be detected reliably by visual examination. The pathogen is found most frequently at the bases of older leaf stalks. Sporulation can be stimulated by treatment with paraquat herbicide before incubation (Cook, 1993), as follows. A sample for testing is obtained by removing the oldest still living leaf stalk (petiole) from each plant. The basal 2 cm of each petiole, including stipules, is excised. These are washed, surface-sterilized, and washed again. The petiole bases are then immersed for 1 min in a solution of paraquat diluted 1:40 with water. They are then washed and incubated on damp paper in plastic boxes at 25°C in constant light for 6 days. The incubated petiole bases are then examined microscopically for acervuli bearing characteristic conidiophores and conidia.

Other tests can be used to detect *C. acutatum*, combining paraquat treatments with ELISA and PCR (see EPPO Standard PM7/25, 2004).

Stem nematodes (*Ditylenchus dipsaci*) and leaf and bud nematodes (*Aphelenchoides besseyi*, *A. blastophthorus*, *A. fragariae* and *A. ritzemabosi*)

Young folded leaves and buds are collected from the crown of the plant to be tested, and also the tips and nodes of runners. If the plant can be destructively sampled, all such tissue is removed; otherwise (if the tested plant is to be retained, as in the case of candidate nuclear stock plants), approximately 50% of the appropriate tissue is collected. The tissue is cut up with a pair of scissors and distributed on muslin or nylon sieve, which is placed in a funnel of water optionally containing 0.15% H_2O_2 so that the tissue is just immersed. A piece of rubber tube is attached to the stem of the funnel and is closed with a spring clamp. Any nematodes present will leave the tissue and gather in the stem of the funnel; they can be collected after 2–5 days by releasing a small amount of water from the tube be means of the clamp. This method is known as the Baermann funnel technique.

Examination of the sample can be performed with a dissecting microscope at 50× and any nematodes found should be mounted on a microscope slide and identified at a higher magnification. Identification to species can only be done by a trained taxonomist or otherwise qualified personnel.

Soil testing for virus-vector nematodes

Soil in which propagation stock II and plants producing certified material is to be planted should be sampled and the samples found substantially free from the following species of nematode vector: *Xiphinema diversicaudatum* (the vector of *Arabis mosaic virus* and *Strawberry latent ringspot virus*), *Longidorus macrosoma* (*Raspberry ringspot virus*), *L. attenuatus* (*Tomato black ring virus*) and *L. elongatus* (*Raspberry ringspot virus* and *Tomato black ring virus*). The nematodes can be tested directly for the presence of virus (see EPPO Standard PM4/35, in preparation). Alternatively a bait test can be carried out on a duplicate sample of soil taken from the field (Taylor & Brown, 1976).

Appendix 2 Recommended certification standards for strawberry

Nuclear stock

Records must show that all nuclear stock plants were negative when tested for all listed viruses and virus-like agents, for *Xanthomonas fragariae*, *Phytophthora fragariae* var. *fragariae*, *P. cactorum*, *Colletotrichum acutatum*, *Aphelenchoides* spp. and *Ditylenchus dipsaci*. No plant may show any symptom of fungal, bacterial or viral disease, or of infestation by any pests in Table 2. All plants should also be substantially free from other pests. If these conditions are not met at the time of the certification inspection, certification will be refused.

Propagation stock I

Random testing for symptomless infection of *Phytophthora fragariae* var. *fragariae*, *Colletotrichum acutatum* and *Phytophthora cactorum* should be performed. If any plant gives a positive test result, certification will be refused to the whole lot. No plant may show any symptom of infestation by any pest in Table 2. All plants must also be substantially free from other pests. If these conditions are not met at the time of the certification inspection, certification will be refused to the whole lot.

Propagation stock II

Infestation by various pests should not exceed the limits indicated in Table 2 at the time of the certification inspection.

If the limits are exceeded, certification will be refused to the whole lot.

Certified material

At the certification inspection, infestation by various pests should not exceed the limits indicated in Table 2. If the limits are exceeded, certification will be refused to the whole lot.

Appendix 3 Guidelines on sanitation procedures

Elimination of viruses, bacteria and fungi

Viruses and virus-like organisms, and also fungi and bacteria, are readily removed by a combination of hot air treatment and tissue culture. To this end, plants in containers are exposed to high temperatures (37–38°C) for a period of 4 to 8 weeks. Runners then are collected, and runner tips (0.2–10 mm) are rooted and cultured in a sterile culture medium.

Alternatively, meristems (preferably apical) of 0.1–0.3 mm are collected and cultured. The microplants obtained from meristem culture are then transferred to a proliferation medium containing growth regulators (indole butyric acid, benzylaminopurine, gibberellic acid). Subsequently, explants are pricked out on a medium without benzylaminopurine, but containing indole butyric acid to favour rooting.

When the plants derived from either method reach 3–4 cm and are well rooted, they are transplanted into peat blocks in the glasshouse at high relative humidity.

Plants produced in this way are considered to be candidate plants, and should always be retested individually according to the methods described in section 2 (Production of nuclear stock). When all tests mentioned above have shown to give negative results, the material can be promoted to nuclear stock.

Elimination of leaf and stem nematodes

If no plants of a cultivar or clone prove to be free from the leaf and stem nematodes *Ditylenchus dipsaci*, *Aphelenchoides fragariae*, *A. besseyi*, *A. blastophthorus* or *A. ritzemabosi*, the following treatment can be applied: remove the plant from its pot and wash the roots clean of soil or growing medium; cut through the crown (i.e. the compressed stem) below the level of all green tissue; discard the upper part and replant the roots and crown base in fresh growing medium; maintain at 20°C for 4 weeks to allow regeneration of the leaves and runners.

Appendix 4 Guidelines on *in vitro* multiplication of strawberry

In vitro multiplication of strawberry includes four stages, of which the first is normally 'regeneration', i.e. elimination of viruses, and the last three are micropropagation as such. The following is an example of how such propagation can be performed.

1. Culture establishment

Mother plants are selected in production fields. Meristems of 0.1–0.3 mm are collected, disinfected and cultured. The aim is to exclude pathogens such as viruses, phytoplasmas, fungi and nematodes. Apical tips (larger than 0.3 mm) or axillary buds can also be cultured by the same methods but will not exclude stem and foliage nematodes and so must be taken from nuclear stock plants tested for leaf and stem nematodes. In principle, a pathogen-free nuclear stock plant can also be put into meristem-tip culture by the same method.

2. Multiplication

The microplant obtained from meristem culture is transferred to a proliferation medium containing growth regulators (indole butyric acid, benzylaminopurine, gibberellic acid). Up to 10 multiplication steps can be achieved, but this figure should not be exceeded. In any case, stocks are renewed at least every 2 years.

3. Rooting

Explants are pricked out on a medium without benzylaminopurine, but containing indole butyric acid to favour rooting.

4. Planting out

When the plants reach 3–4 cm and are well rooted, they are transplanted into peat blocks in the glasshouse at high relative humidity. Such plants are considered as *in vitro* propagation stock. The success rate at the different stages is variable, but can be 90–95%. *In vitro* culture of strawberry can present problems (e.g. loss of trueness-to-type, abnormal behaviour of the micropropagated plants). For this reason, it is important to use culture media with relatively low hormone content, to limit the number of propagation steps and to prevent the formation of callus. With these precautions, *in vitro* multiplication can be perfectly satisfactory to produce certified material.