European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes

**Phytosanitary procedures**
**Procédures phytosanitaires**

# Post-entry quarantine for potato

## Specific scope

This standard describes inspection and test methods for detection of viroids, viruses, bacteria and phytoplasmas in *Solanum* species or hybrids imported for germplasm conservation, breeding or research purposes, by testing and observation for symptoms of disease in post-entry quarantine. It satisfies the requirements of EPPO Standard PM 8(1) Commodity Standard for potato.

## Specific approval and amendment

First approved in 1983-09.
Revised version approved in 2004-09.
(Editorial corrections in 2006-03).

## Introduction

This phytosanitary procedure for testing potato material in post-entry quarantine should be used by NPPOs in order to prevent the entry and spread of quarantine pests of potato into and within the EPPO region. Although directed primarily at quarantine pests, the testing procedure is designed to detect most potato pathogens. In principle, infected material should not be released from quarantine. However, countries may individually lay down special measures to be taken, depending on the pest and the purpose, to release material for use under confinement.

The Standard takes into account recommendations made in FAO/IPGRI Guidelines for Potato (Jeffries, 1998), the requirements of EU Commission Directive 97/46 (EU, 1998), previous EPPO documents on testing in post-entry quarantine (OEPP/EPPO, 1983; EPPO/CABI, 1996) and methods used by various EPPO member countries.

The procedure is applied to potato breeding material: *Solanum tuberosum* and other cultivated *Solanum* spp.; wild stolon- and tuber-forming *Solanum* spp.; closely related *Solanum* spp. that hybridize with potato and may not develop stolons or tubers. It covers both vegetative material and true potato seeds.

## Specific definitions

*Accession*: a sample of seeds within a gene bank given a unique accession number
*Candidate material*: material received for testing under quarantine, i.e. tubers, microplants, true potato seeds
*Complete vegetative cycle*: the cycle of growth from seed or microplant through to mature plant and the natural onset of senescence
*Microplants of potato*: plants (including tubers) in tissue culture of tuber-forming *Solanum* spp
*Unit*: a single microplant, single tuber or single true potato seed
*Vegetative material*: material submitted for testing under quarantine in the form of tubers or plants (including microplants)

**Outline of the procedure**

The phytosanitary procedure involves:

- establishing candidate material *in vitro* or *in vivo*[1]
- growing candidate material (or plants derived from it) under confinement[2] (e.g. *in vitro* or in insect-proof glasshouses, screenhouses or growth rooms[3])
- testing each unit of the candidate material (or plants derived from it) for specified pathogens by specified methods (as indicated) at a stage of plant growth which optimizes the detection of any pathogen, including those with uneven distribution or low concentrations
- growing the candidate material (or plants derived from it) in the glasshouse, usually through a complete vegetative cycle, and inspecting for symptoms of disease, with appropriate investigation
- growing and maintaining the tested material using procedures which minimize the risk of cross infection or contamination and accidental release of a pathogen
- destroying material found to be infected unless, for material infected with viruses, virus elimination is attempted following FAO/IPGRI recommended procedures (Jeffries, 1998). In general, virus elimination should be started only after testing of microplants for freedom from at least viroids and regulated bacteria. After viruses have been eliminated, the material is subject to the full phytosanitary procedure. Following FAO/IPGRI recommended procedures (Jeffries, 1998), elimination of viroids and phytoplasmas can also be attempted but plants infected with other regulated bacteria should be destroyed.
- for material that passes the tests described, release with a Germplasm Health Statement that specifies the tests done and the results.

**Requirements for growing conditions**

*In vitro*

The recommended procedure for post-entry quarantine involves establishing each unit of candidate material as a single plant *in vitro* (the Mother Plant) (Appendix 1, Fig. 1). A suitable medium for growth of microplants is Murashige and Skoog Medium without growth regulators (Sigma Cat. No M-5519) and with 30 g $L^{-1}$ sucrose and 5-8 g $L^{-1}$ Oxoid No.3 Agar. Microplants should be incubated at 18-22°C, with 14-16 h daylight, under cool white fluorescent tubes. The Mother Plant should be subcultured to produce microplants for testing and for growing in the glasshouse for visual inspection and for further testing.

*Glasshouse*

Plants derived from candidate material should be grown in the glasshouse, over a full vegetative cycle at 18-25°C and with at least a 14-h photoperiod. Plants should be slightly shaded if necessary to aid symptom development. To aid establishment of plants taken directly from *in vitro* culture, these should be allowed to adapt to the glasshouse environment (e.g. using mist to retain high humidity) for the first few weeks.

Suggested propagation and testing programmes for candidate material received as microplants, tubers and true potato seeds are described in Appendix I.

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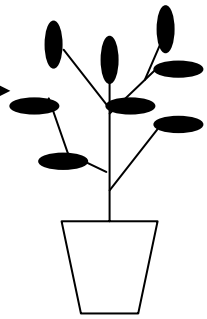
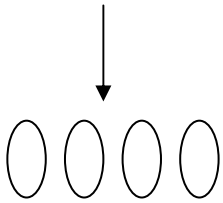
[1] EPPO recommends *in vitro* propagation as a phytosanitary confinement procedure for potato quarantine. This complements the recommendations of EPPO Standard PM 4/28 Certification scheme for seed potatoes, and is in line with the recommendations of the IPGRI. However, *in vivo* quarantine procedures that give equivalent phytosanitary security may also be used. An example of such a procedure is described in Appendix II.

[2] See draft EPPO Standard PM 3/64 Guidelines for intentional import of live organisms that are potential plant pests. This includes a description of confinement conditions. Confinement conditions are also described in the EU's "Scientific Work Directive" (95/44/EC ) (EU, 1995).

[3] Hereafter only glasshouses will be referred to in the text but screenhouses and growth rooms may also be used.

**Fig. 1** Example of propagation pathway for material received as microplants (see Figure 2 for extra detail).

| | **Microplants *in vitro*** | **Plant in glasshouse** | **Tests on plants grown in glasshouse** |
|---|---|---|---|
| **Stage 1**<br>Receipt of microplants for testing and optional destructive testing for viroids, *C. m. sepedonicus* and *R. solanacearum*<br><br>After subculture of mother plant test stem base of mother plant for *C. m. sepedonicus* and *R. solanacearum* | Reception<br>Select plant for quarantine testing<br>**Mother plant**<br>subculture | | |
| **Stage 2**<br>Test microplant subcultures for viroids, phytoplasmas and specified viruses. Plant GH1 in glasshouse | **GH1**<br>+ subcultures<br>subculture |  | Inspect weekly, test for viroids, phytoplasmas and viruses |
| **Stage 3**<br>GH2 only planted in glasshouse if GH1 dies or if required for investigation | **GH2**<br>subculture |  | Cut, inspect.<br>Test for *C. m. sepedonicus* and *R. solanacearum* |
| **Stage 4** | **Microplants stored at 8°C**<br>Maintenance of line during testing<br>Release once material has passed quarantine testing | Destroy | |

**Requirements for pathogen testing**

If candidate material is received as tubers, preliminary testing of tuber sap or sprouts may be useful to detect viroids and viruses at an early stage in the quarantine period, in order to avoid unnecessary propagation of infected material. These tests are optional and therefore any of the tests described may be used.

The summary of testing requirements is shown in Table 1 and the lists of pathogens for specific testing in Tables 2 (EPPO quarantine pests) and 3 (other pathogens for specific testing). Certain other pathogens, reported to infect potato naturally (Jeffries, 1998), are recommended only for non-specific testing using bioassay (Table 4). For other pathogens, no testing is recommended: *Erwinia carotovora* subsp. *atroseptica, Erwinia chrysanthemi.* The same applies to certain viruses and diseases which are not mechanically transmitted: *Beet curly top virus* (BCTV), potato deforming mosaic virus (PDMV Brazil), *Solanum* apical leaf curling virus and the uncharacterized virus-like diseases potato deforming mosaic disease (Argentina) and Saq'O (Jeffries, 1998). Observation of growing plants is sufficient in these cases.

For vegetative material, the general requirement is that plants derived from candidate material are tested twice, by the methods described in Tables 2 and 3. For quarantine based on *in vitro* propagation, the first test (Test A in Tables 1, 2 and 3) should be done on *in vitro* plants or on glasshouse-grown plants and the second test (Test B) on glasshouse-grown plants. For quarantine based on *in vivo* propagation, Tests A and B should be done on glasshouse-grown plants.

For true potato seeds, the general requirement is that plants derived from candidate material are tested once. For quarantine based on *in vitro* propagation, *in vitro* plants should be tested using Test A, or alternatively glasshouse-grown plants should be tested using Test A or B (use Test A for viroids) (Tables 1, 2 and 3). For quarantine based on *in vivo* propagation, glasshouse-grown plants should be tested using Test A or B (use Test A for viroids). It is in any case preferable that both tests A and B should be done for quarantine procedures based on either *in vitro* or *in vivo* propagation. At least 20 seedlings per accession should be tested to avoid in-breeding depression and genetic drift (Hawkes, 1990).

Except in the case of bacteria, Test B need not be done if plants give a positive result in Test A. However, further testing may be needed to confirm the identity of the pathogen, particularly if the result is likely to be challenged (see later: Administration of the procedure).

Glasshouse plants should be inspected at least once a week for symptoms of disease. At each inspection, records should be made of the plants which have been inspected and of symptoms, if present. At the end of the testing programme, the overall result should be recorded.

**Table 1** Summary of testing requirements (Test A, Test B) for quarantine procedures based on *in vitro* and *in vivo* quarantine propagation[1]

| Type of plant material | | *In vitro* propagation | | *In vivo* propagation |
|---|---|---|---|---|
| | | Microplant | Glasshouse plant | Glasshouse plant |
| Vegetative (for all pathogens other than viroids) | Option 1 | A | B | A+B |
| | Option 2 | | A+B | |
| Vegetative (for viroids) | Option 1 | A | Preferably B | A + preferably B |
| | Option 2 | | A + preferably B | |
| True potato seeds (for all pathogens other than viroids) | Option 1 | A | Preferably B | A or B, but preferably A+B |
| | Option 2 | | A or B but preferably A+B | |
| True potato seeds (for viroids) | Option 1 | A | Preferably B | A but preferably A+B |
| | Option 2 | | A but preferably A+B | |

[1] For details of Test A and Test B, see Tables 2 and 3

**Table 2** List of pathogens to be tested on *in vitro* and glasshouse plants, and test method: EPPO quarantine pests for specific testing

| | | | Stage of testing | | |
|---|---|---|---|---|---|
| | | | **Plants *in vitro* and/or glasshouse-grown plants** | **Glasshouse-grown plants** | |
| **Pathogen** | **Genus** | **Acronym** | **Test A**[1] | **Test B**[2] | Comment |
| **Viroid** | | | | | |
| *Potato spindle tuber viroid*** | *Pospiviroid* | PSTVd | rPAGE or DIG | rPAGE, DIG, or RT-PCR[4] | rPAGE will detect all Pospiviroids that may infect potato and DIG will detect many of them. A different test method should preferably be used for Test A and Test B |
| **Viruses** | | | | | |
| *Andean potato latent virus*** | *Tymovirus* | APLV | ELISA | Bioassay | Great serological variability. |
| *Andean potato mottle virus* | *Comovirus* | APMoV | ELISA | Bioassay | |
| *Potato black ringspot virus** | *Nepovirus* | PBRSV | ELISA | Bioassay | |
| *Potato virus T*** | *Trichovirus* | PVT | ELISA | Bioassay | |
| Potato yellow vein virus | *Crinivirus* | PYVV | RT-PCR (Salazar *et al*. 2000); Offei, *et al*. (2003). | RT-PCR | Not mechanically transmitted. Until reliability of RT-PCR methods is established, observation of growing plants. |
| *Tomato spotted wilt virus* | *Tospovirus* | TSWV | ELISA | Bioassay | |
| **Bacteria** | | | | | |
| *Clavibacter michiganensis* subsp. *sepedonicus* | | | IF, MTNA, PCR FISH or bioassay [3] | IF, MTNA, PCR, FISH, or bioassay[3] | Use a different test method for Test A and Test B, e.g. IF and PCR. |
| *Ralstonia solanacearum* | | | IF, SMSA, PCR, FISH or bioassay [3] | IF, SMSA, PCR, FISH or bioassay[3] | Use a different test method for Test A and Test B, e.g. SMSA and PCR. |
| **Phytoplasma** | | | | | |
| Stolbur | | | Universal primers | Universal primers | Use a different primer pair for Test A and Test B. |

** For plants derived from true potato seeds, test only for these pathogens

[1] Test A to be done on either plants *in vitro* or glasshouse-grown plants if plants *in vitro* have not been tested previously.
[2] Test B to be done on glasshouse-grown plants.
[3] Testing for *C. m. sepedonicus* and *R. solanacearum* should normally be done on tubers, either candidate tubers or, if material was received as microplants, daughter tubers grown from microplants. However, if tubers are not produced, stem bases of glasshouse-grown plants should be tested. For all bioassays, further tests should be done if no symptoms are observed (see text). Detection of *C. m. sepedonicus* using MTNA selective isolation may be adversely affected by background populations of saprophytes. Although, in infected tissue, competing saprophytes can usually be diluted out while the pathogen remains, the test should preferably be used only on 'clean' material, e.g. microplants.
[4] Also includes real-time PCR.

**Table 3** List of pathogens to be tested on *in vitro* and glasshouse plants, and test method: other pathogens for specific testing*

| | | | Stage of testing | | |
|---|---|---|---|---|---|
| | | | **Plants *in vitro* and/or glasshouse-grown plants** | **Glasshouse-grown plants** | |
| **Pathogen** | **Genus** | **Acronym** | **Test A**[1] | **Test B**[2] | **Comment** |
| **Viruses** | | | | | |
| Arracacha virus B - oca strain** | ?*Nepovirus* | AVB-O | ELISA | Bioassay | |
| Potato latent virus | *Carlavirus* | PotLV | ELISA | Bioassay or ELISA | Bioassay may not be reliable. |
| *Potato leafroll virus* | *Polerovirus* | PLRV | ELISA | ELISA | Not mechanically transmitted. |
| *Potato mop-top virus* | *Pomovirus* | PMTV | ELISA | Bioassay or ELISA | Virus unevenly distributed. Testing of microplants and young glasshouse plants may be more reliable than testing older glasshouse plants. ELISA may be more reliable than bioassay. |
| Potato rough dwarf virus | *?Carlavirus* | PRDV | ELISA | Bioassay | Polyclonal antibodies to PVP will detect PRDV also. |
| *Potato virus A* | *Potyvirus* | PVA | ELISA | Bioassay | |
| *Potato virus M* | *Carlavirus* | PVM | ELISA | Bioassay | |
| Potato virus P | ?*Carlavirus* | PVP | ELISA | Bioassay | Polyclonal antibodies to PRDV will detect PVP also. |
| *Potato virus S* | *Carlavirus* | PVS | ELISA | Bioassay | |
| *Potato virus V* | *Potyvirus* | PVV | ELISA | Bioassay | |
| *Potato virus X* | *Potexvirus* | PVX | ELISA | Bioassay | |
| *Potato virus Y* | *Potyvirus* | PVY | ELISA· | Bioassay | |
| Potato yellowing virus** | *?Alfamovirus* | PYV | ELISA | Bioassay or ELISA | Bioassay may not be reliable. Isolates serologically variable. |
| *Tobacco rattle virus* | *Tobravirus* | TRV | RT-PCR (Robinson, 1992) | Bioassay or RT-PCR | NM types detected unreliably by bioassay. |
| **Phytoplasmas** | | | | | |
| Other phytoplasmas *** | | | Universal primers | Universal primers | |

* EU Directive 2000/29 (EU, 2000) lists AVB-O and non-European isolates of PLRV, PVA, PVM, PVS, PVV, PVX, PVY as quarantine pests. In addition, Directive 97/46 (EU, 1997) lists PYV for quarantine testing.
** For plants derived from true potato seeds, test only for these pathogens
*** e.g. potato marginal flavescence, potato phyllody, potato purple toproll, potato purple top wilt, potato witches' broom
[1] Test A to be done on either plants *in vitro* or glasshouse-grown plants if plants *in vitro* have not been tested previously
[2] Test B to be done on glasshouse-grown plants

*Viroids*

The viroid of principal concern is the *Pospiviroid Potato spindle tuber viroid* (PSTVd) since it is the only viroid known to infect cultivated species of potato naturally. However, *Mexican papita viroid* has been found infecting the wild potato species *Solanum cardiophyllum* (papita guera, cimantli) (Martinez-Soriano *et al*., 1996) and other viroid species in the Genus *Pospiviroid,* e.g. *Citrus exocortis viroid* (Semancik *et al*. 1973; Verhoeven *et al*. 2004), *Columnea latent viroid* (Verhoeven *et al*., 2004), *Tomato chlorotic dwarf viroid* (Singh *et al*., 1999), and *Tomato planta macho viroid* (Galindo *et al.,* 1982), have been shown to infect cultivated potato experimentally.

For viroid testing, Test A should be done, and preferably also Test B. The method used for test B should preferably be different from that used for Test A. The methods for Test A are return polyacrylamide gel electrophoresis (rPAGE) or a digoxigenin-labelled cRNA probe (DIG). Test B also includes the option of testing by RT-PCR (including real time PCR) (see EPPO Standard PM 7/33 Diagnostic protocol for PSTVd). rPAGE detects all the viroids referred to above and DIG probe detects many if not all of them. Since none of these methods specifically diagnose PSTVd, sequencing of the PCR product should be done if required.

Testing should be done on *in vitro* plants with good growth (4-6 weeks old and with stems of at least 5 cm length). Glasshouse-grown plants should be tested when about 25 cm tall, but prior to flowering and pollen production. Samples should be taken from a fully expanded leaflet at the top of a stem of the plant. Plants should be tested either individually, or several combined ('bulked'), provided that the specific 'bulking rate' used has been adequately validated. The weight of the positive control should be 1/10 of the weight of tissue normally used for each sample. If this amount is not detected, the test should be repeated.

*Viruses*

Testing should be done on *in vitro* subcultures of the Mother Plant with good growth (4-6 weeks old and with stems of at least 5 cm length). Cultures of each line should be tested separately. Plants giving a positive test result should not be planted in the glasshouse unless they are required for further investigation.

Glasshouse-grown plants should be tested when about 25 cm tall, at or near flowering. Samples should be taken from at least two positions on each plant, including a young, fully expanded terminal leaflet at the top of a stem and an older terminal leaflet from a midway position. Leaves from different plants should not be "bulked" together for ELISA unless the specific bulking rate has been validated. Leaves from up to 5 plants may be 'bulked' together for bioassay for plants belonging to the same line.

Other methods, e.g. nucleic acid hybridization or RT-PCR, may be used to confirm the presence or identity of the virus. Electron microscopy may be used to confirm the presence of virus particles.

ELISA

The sensitivity and specificity of ELISA depends on the antibodies used. The user should be aware of any known serotypes or strains of the virus (Jeffries, 1998). For many of the viruses listed in Tables 2 and 3, antibodies are available commercially. These should be used according to the manufacturers' instructions.

Bioassay

The bioassay procedure is described in Appendix III. The recommended list of indicator plants (Table 5) is based on the first version of this Standard, but other indicators may be used if they have been shown by the NPPO to give the same broad coverage of detection as the recommended indicators. Broad coverage is essential in order to detect unknown potato viruses which may possibly be present.

The first version of this Standard listed, for vegetative material, *Chenopodium amaranticolor*, *Chenopodium murale, Chenopodium quinoa*, *Datura metel*, *Nicotiana clevelandii*, *Nicotiana tabacum* (or clone A 6) and for true potato seeds *C. amaranticolor, C. murale* and *C. quinoa*, although these may not reliably detect potato yellowing virus (PYV) and some other mechanically transmitted viruses, e.g. potato latent virus and potato rough dwarf virus. For true potato seeds, *N. occidentalis*-P1 and *N. hesperis*-67A can be used (Verhoeven & Roenhorst, 2000) and recently the three-indicator set *N. occidentalis*-P1, *N. hesperis*-67A and *C. quinoa* has been proposed as sufficient to detect most of the viruses of concern in this Standard (Verhoeven & Roenhorst, 2003). Since bioassay may be influenced by a number of variables including indicator plant accession (Van Dijk & Cuperus, 1986) and environmental conditions, each laboratory should validate each indicator plant against each virus of concern before use.

**Table 4** Other pathogens, reported to infect potato naturally (Jeffries, 1998), for non-specific testing using bioassay (test glasshouse-grown plants once)

| **Viruses** | Genus | Acronym |
|---|---|---|
| *Alfalfa mosaic virus* | *Alfamovirus* | AMV |
| *Cucumber mosaic virus* | *Cucumovirus* | CMV |
| *Eggplant mottled dwarf virus* | *Nucleorhabdovirus* | EMDV |
| *Impatiens necrotic spot virus* | *Tospovirus* | INSV |
| *Potato aucuba mosaic virus* | *Potexvirus* | PAMV |
| *Potato virus U* | *Nepovirus* | PVU |
| *Potato yellow dwarf virus* | *Nucleorhabdovirus* | PYDV |
| *Potato yellow mosaic virus* | *Begomovirus* | PYMV |
| *Sowbane mosaic virus* | *Sobemovirus* | SoMV |
| *Tobacco mosaic virus* | *Tobamovirus* | TMV |
| *Tobacco necrosis virus* | *Necrovirus* | TNV |
| *Tobacco streak virus* | *Ilarvirus* | TSV |
| *Tomato black ring virus* | *Nepovirus* | TBRV |
| *Tomato mosaic virus* | *Tobamovirus* | ToMV |
| *Wild potato mosaic virus* | *Potyvirus* | WPMV |

*Bacteria*

Tests for *Clavibacter michiganensis* subsp. *sepedonicus* are immunofluorescence (IF), fluorescent *in-situ* hybridization (FISH), polymerase chain reaction (PCR), MTNA selective isolation medium and bioassay to aubergine (*Solanum melongena* cv. Black Beauty)[4]. Tests for *Ralstonia solanacearum* are IF, FISH, PCR, SMSA selective isolation medium and bioassay to tomato (*Lycopersicum esculentum* cv. Moneymaker). One of these should be chosen for Test A and a different method for Test B (e.g. IF and PCR for *C. m.* s*epedonicus* and SMSA and PCR for *R. solanacearum*). If either test gives a positive result, further investigation should be done, for example by inoculation to aubergine for *C. m. sepedonicus* or tomato for *R. solanacearum*. For all bioassays, further tests should be done if no symptoms are observed. For *C. m. sepedonicus*, if no symptoms are observed after 4 weeks, IF or PCR

[4] For *C. m. sepedonicus* IF and bioassay methods are described in EPPO Standard PM 3/25. For *R. solanacearum* IF, SMSA and bioassay are described in EU Directive 98/57/EEC (EU, 1998) or EPPO Standard 3/26 (under revision). All methods including new PCR methods and the FISH methods will be included in the revised EU ring rot and brown rot directives. The use of the two test methods is different in this post-entry quarantine procedure and in the EU Directives. For post-entry quarantine, use of two test methods increases stringency of the testing, whereas in the EU Directives use of two test methods reduces the number of false positives.

should be done on a composite sample of 1-cm stem sections of each test plant taken above the inoculation site. For *R. solanacearum*, if no symptoms are observed after 3 weeks, IF, PCR or isolation on SMSA should be done on a composite sample of 1-cm stem sections of each test plant taken above the inoculation site.

*Phytoplasmas*

A general test for phytoplasmas[5] should be used, with universal phytoplasma primers[6]. Nucleic acid should be extracted from whole *in vitro* plants or from leaf midribs of glasshouse-grown plants, when about 25 cm tall but prior to flowering and pollen production (phytoplasma enrichment) (e.g. Lee *et al*., 1991; Ahrens & Seemüller, 1992), using the methods described in these papers or commercial nucleic acid extraction kits. Primer pairs that may be used for detecting potato phytoplasmas are: R16F2/R16R2 (Lee *et al*., 1993), fU5/rU3 (Lorenz *et al*., 1995) or P1/Tint (Smart *et al*., 1996). The primer set NPA2F/R (Heinrich *et al*., 2001) has also been used successfully to detect several phytoplasmas infecting solanaceous plants i.e. brinjal little leaf, potato purple top, Solanum big bud, stolbur and tomato big bud (J.T.J. Verhoeven, Dutch NPPO, pers. comm.). One of these primer pairs should be used for Test A and a different one for Test B. Differentiation of phytoplasmas may be attempted using RFLP or sequence analysis of 16SrDNA (Lee *et al.*, 1993; Schneider *et al.*, 1993; Seemüller *et al.*, 1994; Davis *et al.*, 1997).

**Confinement procedures**

Stringent confinement procedures should be applied within quarantine since, if infected material is also present, there is a risk of cross infection of uninfected material that may escape detection. Confinement procedures should therefore be designed to prevent cross infection, with rules to allow holding and re-testing of material, or even destruction, if sufficient guarantees cannot be given that cross infection has not occurred. All procedures should be documented (see below). Examples of confinement procedures are described elsewhere, but basic procedures include the following:

- for material in tissue culture, actions in handling germplasm should be chronologically recorded, so that material can, if necessary, easily be checked for cross infection if infected material is detected later. Stringent aseptic techniques and procedures should be applied, including autoclaving instruments or using a glass-bead sterilizer between units of each line (flame sterilization using methanol/ethanol may not always be effective but may be used within each unit) and cutting over a sterile disposable surface
- in the glasshouse, buffer zones should be established between the glasshouse entrance and the compartments where the plants to be tested are grown. Access should be restricted to persons directly involved in growing and testing of the plants and staff should use protective clothing. Potential vectors should be monitored by regular inspection of plants and sticky traps. To prevent potential contact/mechanical transmission between each line, screens (height up to 60 cm) for separation should be used and, during handling of plants, sterilized instruments and new disposable gloves should be used between each unit of a line. Glasshouse procedures are particularly important for quarantine based on *in vivo* procedures where plant material may not have received testing before being planted into the glasshouse and material is released from the glasshouse at the end of the quarantine procedure.

[5] Since true potato seeds do not transmit phytoplasmas, plants derived from true potato seeds should not be tested.

[6] PCR has been shown to be the most sensitive and reliable method of detecting phytoplasmas in a wide range of crops, but little work has been done on the reliability of detection and differentiation of the 6 phytoplasmas described so far as affecting potato (Jeffries, 1998).

**Table 5** Recommended indicators for potato viruses (after Jeffries, 1998)

| **Virus** | ***Chenopodium amaranticolor*** | ***Chenopodium murale*** | ***Chenopodium quinoa*** | ***Nicotiana benthamiana*** | ***Nicotiana bigelovii*** | ***Nicotiana clevelandii*** | ***Nicotiana debneyi*** | ***Nicotiana hesperis* –67A** | ***Nicotiana occidentalis*-P1** | ***Nicotiana tabacum* 'White Burley'** | **Virus** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| AMV | LS | | LS | S | | | | LS | LS | S | AMV |
| APLV | LS | L | L?S | L?S | LS[1] | S | S | LS | LS | | APLV |
| APMoV | | | | S | S | S | S | LS | LS | | APMoV |
| AVB-O | LSR? | S | S | | | | | LS | LS | | AVB-O |
| BCTV * | | | | | | | | | | | BCTV * |
| CMV | L | | L | | | | | S | S | | CMV |
| EDMV[3] | | | | LS | | | | | | L?S | EDMV[3] |
| INSV | | | | | | | | LS? | LS | | INSV |
| PAMV | | | | | | | | LS | LS | | PAMV |
| PBRSV | LS | | LS | LS | | | | LS | LS | L?S | PBRSV |
| PDMV (Brazil) * | | | | | | | | | | | PDMV (Brazil) * |
| PLRV * | | | | | | | | | | | PLRV * |
| PotLV | | L? | | | S? | | S? | S? | S? | | PotLV |
| PMTV | L | | L | S | | | S | L?S? | S? | | PMTV |
| PRDV | L | | LS? | | | | S | | S? | | PRDV |
| PVA | | | | S | | S | S | S | S | S | PVA |
| PVM | | L | | | | S | L | L?S | S | | PVM |
| PVP | L | | L | | | | S | | S | | PVP |
| PVS | LS[2] | L | LS[2] | | | | S? | | S | | PVS |
| PVT | L?S | S | L?S | S | | | S? | S | S | | PVT |
| PVU | LS | | LS | | | | | | | SR | PVU |
| PVV | | | | S | | S | LS | LS | LS | S | PVV |
| PVX | L | L | L | LS | | S | S | LS? | LS | LS | PVX |
| PVY | | | | S | | S | S | L?S | L?S | S | PVY |
| PYDV | | | | | | LS | | | L?S | | PYDV |
| PYMV | | | | LS | | | | | | | PYMV |
| PYVV * | | | | | | | | | | | PYVV * |
| PYV | L | L | L | | | | L | S | S? | L | PYV |
| SALCV * | | | | | | | | | | | SALCV * |
| SoMV | LS | LS | LS | | | | | | | | SoMV |
| TMV | | | L | S | | | | S | S | | TMV |
| TNV | L | | L | | | L | | L | L | L | TNV |
| TRV | L? | | L? | S? | | L?S? | L?S? | LS? | LS? | L?S? | TRV |
| TSV | | | S | S | | | | LS | LS | LSR? | TSV |
| TBRV | LS | | LS | | | LS | | L?S | LS | LSR? | TBRV |
| ToMV | LS? | LS | LS? | S | | | | LS | LS | | ToMV |
| TSWV | | | LS | LS | | | | LS | LS | LS? | TSWV |
| WPMV | | | | S | S | S | | L?S | S | | WPMV |

Note: Only symptoms with diagnostic characteristics are considered. Susceptibility without symptoms or non-susceptibility of a host to virus infection are not considered. L) Local symptoms, S) Systemic symptoms, R) Symptoms may be followed by recovery so that no symptoms are seen. ?) Infection or symptoms may be absent, and may depend on virus isolate. For PRDV and PVP, *N. bigelovii* needs to be left approximately 40 days for symptoms to develop. Production of symptoms may depend on many factors and users are recommended to ensure that the viruses can be detected under local conditions.

* Virus not mechanically transmitted

[1] Symptoms best in winter in Northern Europe

[2] Only PVS Andean isolates systemic

[3] Also reported as potato chlorotic stunt virus

**Administration of the procedure**

The NPPO should be responsible for administration and monitoring of the procedure. The post-entry quarantine facilities, and all testing in post-entry quarantine, should be subject to its control or supervision. All procedures used in post-entry quarantine testing should be documented and records kept for at least 10 years of all tests done on the material and results, in a manner ensuring traceability. For potentially controversial positive results, isolates or material should be kept for at least 1 year after reporting the results, to allow for further or independent investigation if requested by the NPPO of the exporting country.

Material subject to the procedure should be imported only with an import permit. It is not the responsibility of the NPPO of the importing country to verify the provenance or identity of the material. Importers should be informed that all material is received on the understanding that the line is as stated on the import permit[7].

Material should be released from quarantine only if is derived from plants which have given negative results in the tests specified and has been inspected and found free from symptoms of disease during a complete vegetative cycle. For quarantine based on *in vitro* propagation, only microplants should be released. For procedures based on *in vivo* propagation, glasshouse-grown plants or tubers are released. The released material should be accompanied by a Germplasm Health Statement that specifies the tests done and the results.

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[7] There is also a small risk that imported vegetative material may not be true to phenotype and that mutations may arise during micropropagation. Importers should be made aware of this and advised to reduce the risk of producing solely aberrant material by importing more than one "clone" of each line for quarantine testing.

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## Appendix I

### Example of propagation programmes based on tissue culture for material received as microplants, tubers and true potato seeds

The propagation pathways are designed to minimize the risk of failing to detect pathogens, particularly bacteria, because of uneven distribution or low concentration.

In general:

- test each unit of candidate material (100% testing)
- adopt strict confinement procedures
- surface-sterilize tubers, seeds and sprouts using, for example, 2.5% sodium hypochlorite (sodium hypochlorite is equivalent to 8-10% active chlorine) for 15-30 min, followed by washing in sterile distilled water
- use a suitable medium for growth of microplants, e.g. Murashige and Skoog Medium without growth regulators
- incubate microplants at 18-22°C, with 14-16 h daylight, under cool white fluorescent tubes.

*Material received as microplants*

Request up to 5 microplants (derived from the same mother plant) for each line (1 microplant per tube). On receipt, inspect microplants closely for the absence of fungi, bacteria and arthropod pests, particularly mites and thrips. Destroy infested material, although material contaminated with saprophytic bacteria may be tested at the discretion of the NPPO. Before subculturing, destructively test 1 plant from each line for viroids and 1-2 plants for *C. m. sepedonicus* and *R. solanacearum*. These tests are optional, but testing at this stage reduces the risk of propagating infected material. Any of the test methods described may be used.

If the above tests are negative, select one of the remaining microplants (the Mother Plant, STAGE 1 in Figs 1 and 2) for full testing. Subculture the Mother Plant to produce plants for pathogen testing, planting in the glasshouse (GH1 and GH2) and for storage or release following the propagation pathway shown in Figs 1 and 2. Destructively test the Mother Plant for *C. m. sepedonicus* and *R. solanacearum* and the STAGE 2 cultures (Figs 1, 2) for viroids, viruses and phytoplasmas (Test A in Tables 2 and 3).

If the above tests are negative, plant GH1 in the glasshouse for growing through a complete vegetative cycle. Inspect regularly for symptoms of disease and test for viroids, viruses and

phytoplasmas (Test B in Tables 2 and 3). At the end of the growing cycle, harvest tubers, inspect and test for *C. m. sepedonicus* and *R. solanacearum*. Cut a proportion of tubers and check for symptoms of disease e.g. brown rot, ring rot and spraing.

Complete the record sheets. The person responsible for post-entry quarantine attests by signature that the material has passed or failed the tests. If material passes the tests, maintain only microplants derived from GH2. Release stored microplants. Destroy all other microplants and the glasshouse material. Supply a Germplasm Health Statement.

*Material received as tubers*
For each line, receive up to 5 tubers, surface-sterilize, place in suitable containers and incubate in the dark at 15-20°C. Test each tuber for *C. m. sepedonicus* and *R. solanacearum* using Test A in Table 2 (e.g. IF for *C. m. sepedonicus*; SMSA for *R. solanacearum*). Test B may be also completed at this time or done on microplants later in the programme. Test each tuber for viroid by taking eye-plug cores. This test is optional and any of the methods described may be used. Once tubers have sprouted, test a sprout from each tuber for viruses by ELISA. This test is optional.

Once the tubers have sprouted, select one tuber from each line, remove the sprout(s), surface-sterilize them, and excise at least 5 nodes from the sprout(s) and plant onto M & S Medium. Once the sprout node cultures have established, select the "best" culture (the Mother Plant in STAGE 1 of Fig. 3) and subculture it to produce plants for pathogen testing, planting in the glasshouse (GH1 and GH2) and for storage or release, following the propagation pathway shown in Fig. 3. From this point on, the procedure is the same as for microplants, starting at 'Destructively test the Mother Plant for bacteria…'.

*Material received as true potato seeds*
For true potato seeds, the general requirement is that plants derived from candidate material are tested once. Therefore *in vitro* plants should be tested using Test A (Tables 2 and 3) or glasshouse-grown plants should be tested using Test A or B for viruses and Test A for viroids. In this example, testing using tests A and B is described for viruses and viroids.

On receipt, inspect true potato seeds closely for the absence of arthropod pests. Treat infested material by storage at –20°C for 7 days. For each accession, surface-sterilize 30-50 seeds. Aseptically transfer each surface-sterilized seed to a tube containing M & S medium. Use the same instruments within each accession. Select at least 20 seedlings at random for full testing. These are the mother plants in STAGE 1 (Fig. 4). Subculture the mother plants to produce plants for ELISA testing, planting in the glasshouse (GH1 and GH2) and for storage or release following the propagation pathway shown in Fig. 4.

After regrowth of the Mother Plant, destructively test it for viroids (using Test A in Table 2) and test subcultures for virus freedom (Test A in Tables 2 and 3). If the viroid and virus tests are negative, plant GH1 in the glasshouse for growing through a complete vegetative cycle. Inspect regularly for symptoms of disease and test for viroids and viruses (using Test B in Tables 2 and 3). From this point on, the procedure is the same as for microplants, starting at "If material passes the tests, maintain …"

## Appendix II

### Example of propagation programmes based on *in vivo* procedures for material received as microplants, tubers and true potato seeds

In general:

- grow candidate material in the glasshouse in pest-free compost, test each unit (100% testing) and, if it passes the tests, release the plants or progeny tubers.
- adopt strict confinement procedures.

*Material received as microplants*

Request up to 5 microplants (derived from the same mother plant) for each line (1 microplant per tube). Destructively test 1 plant from each line for viroids and 1-2 plants for bacteria. These tests are optional, but testing at this stage reduces the risk of propagating infected material. Any of the test methods described may be used. Select 2 microplants and plant each microplant separately in a small pot in pest-free compost under humid conditions in the glasshouse. After about 10 days, lower the humidity to normal glasshouse conditions. When the plants have rooted well, transplant into larger pots (e.g. 13 cm x 13 cm). For each plant, allow only one stem to grow. Remove all lateral shoots from the main stem.

Select one of the two plants for full quarantine testing. Complete Tests A and B (Tables 2 and 3) when the plant has reached a length of about 25 cm (the stage at which first flowers are formed or will be formed soon). After the viroid, virus and phytoplasma tests have been completed and some tubers have formed, test the stem of the plant for *C. m*. s*epedonicus* and *R. solanacearum*. Optionally, test any remaining plants that have not been subject to full quarantine testing for viroids, *C. m. sepedonicus* and *R. solanacearum* and destroy if no longer required.

Complete the record sheets. The person responsible for post-entry quarantine attests by signature that the material has passed or failed the tests. If the plant passes the tests (and there are no concerns about cross infection), release all the tubers with a Germplasm Health Statement.

*Material received as tubers*

For each line, import 2-5 tubers (one tuber will be fully quarantine-tested, the others will be partially processed and held in reserve should they be required). Dormancy may be broken by storing the tubers at 4°C for several weeks and then by raising temperatures to 18-20°C. Test each tuber for *C. m. sepedonicus* and *R. solanacearum* (Test A in Table 2). Test B may also be done at this time or done on stems later in the programme. It may also be useful to conduct preliminary testing of tuber sap or sprouts for other pathogens in order to avoid unnecessary propagation of infected material.

From each tuber take an eye-plug, soak in a solution of 2 µg $mL^{-1}$ gibberellic acid for 15 min and then plant in a pot (e.g. 11 x 11 cm). For each plant, allow only one stem to grow. Remove all lateral shoots from the main stem.

Select one plant for full quarantine testing. Complete Tests A and B (Tables 2 and 3) when the plant has reached a length of about 25 cm (the stage at which first flowers are formed or will be formed soon.) After the viroid, virus and phytoplasma tests have been completed and some tubers have formed, test the stem of the plant using Test B for *C. m. sepedonicus* and *R. solanacearum* if not done earlier. Optionally, test any remaining plants that have not been

subject to full quarantine testing for viroids, *C. m. sepedonicus* and *R. solanacearum* (Test A in Table 2) and destroy if no longer required.

If the plant passes the tests (and there are no concerns about cross infection), release the plants or tubers with a Germplasm Health Statement.

*Material received as true potato seeds*
For each accession, sow the seeds individually in a row in pest-free compost. When the seedlings have 2-4 leaves, transplant at least 20 seedlings individually into pots (e.g. 11 x 11 cm). These will be subject to full quarantine testing.

For each seedling allow only one stem to grow. Remove all lateral shoots from the main stem. Complete Tests A or B (Tables 2 and 3) when the plants have reached a length of about 25 cm (the stage at which first flowers are formed or will be formed soon). Optional tests include testing excess seedlings which are not to be fully quarantine-tested for viroids.

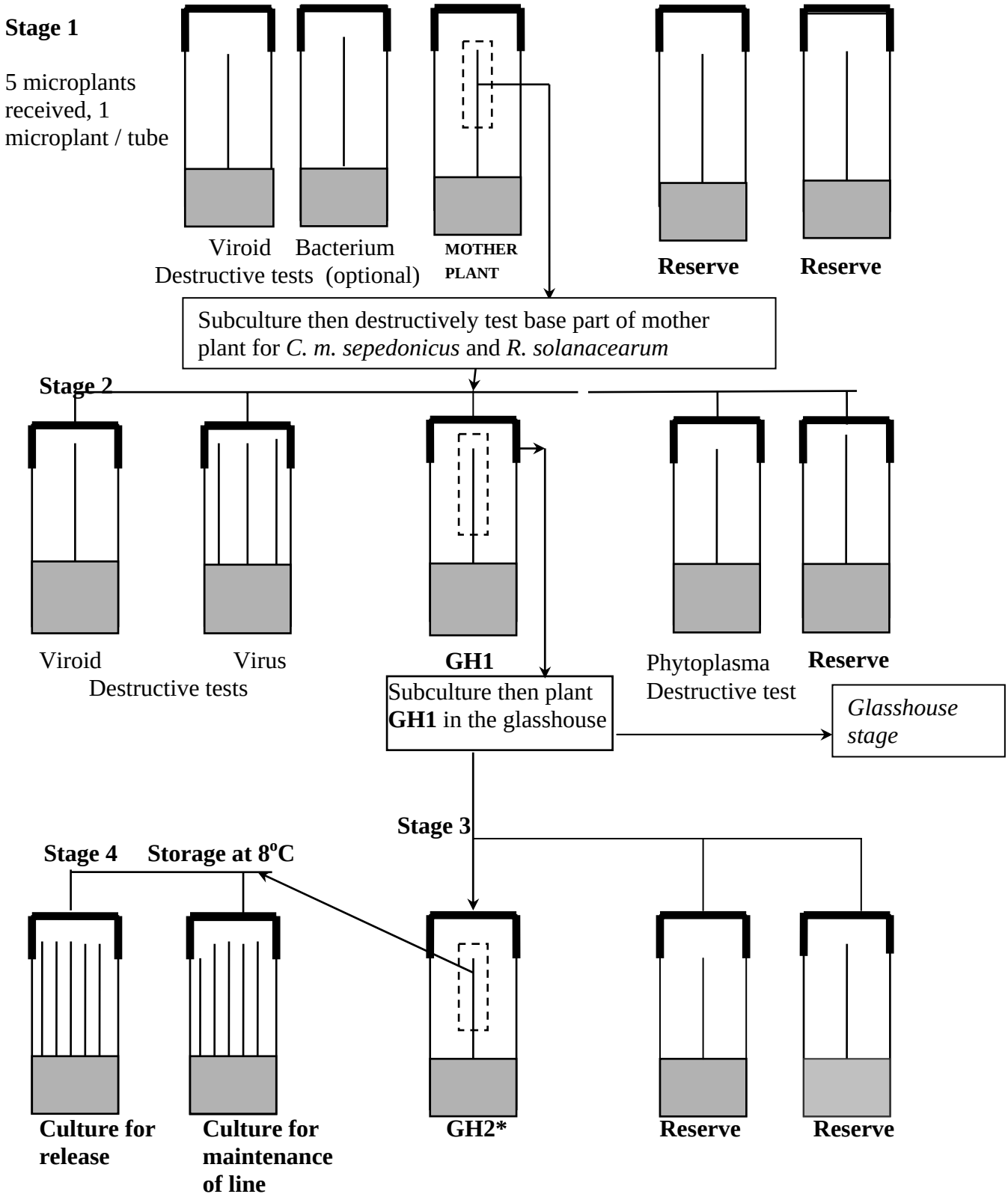
If material passes the tests (including no concerns about cross infection), release all the tested plants or tubers for the accession with a Germplasm Health Statement.

## Appendix III

### Virus bioassay

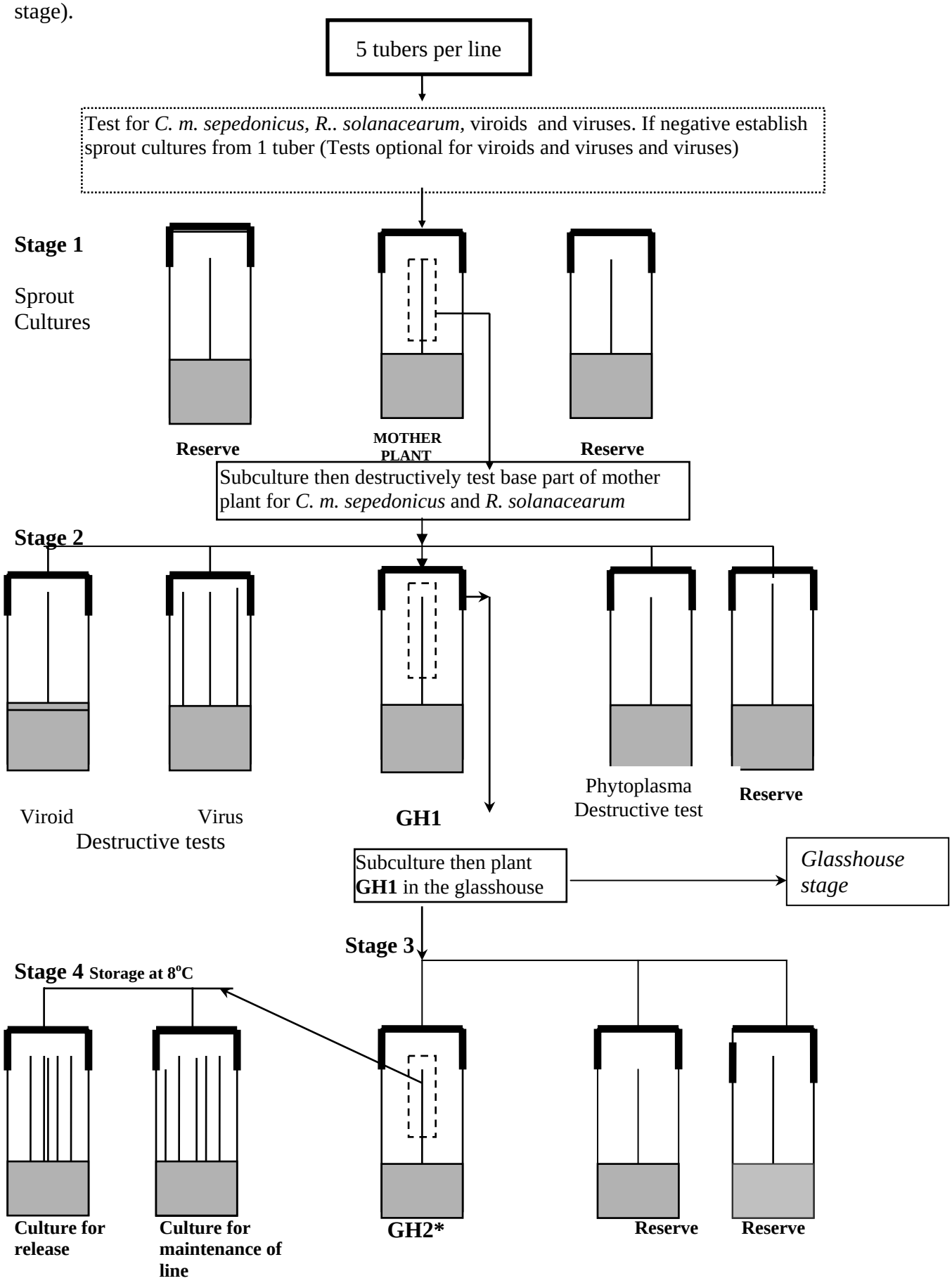
Prepare inoculum by grinding leaf tissue in phosphate inoculation buffer, e.g. 0.02 M phosphate buffer ($Na_2HPO_4$ 2.5 g $L^{-1}$, $NaH_2PO_4$ 0.8 g $L^{-1}$) pH 7.4 + 2% w/v polyvinylpyrrolidone (PVP, MW 10000). If another buffer is used, it should be tested for suitability against all viruses listed for testing. Use at least 2 plants from each indicator plant species. Inoculate young (3-6 leaf stage), soft, actively growing test plants by rubbing plant sap on leaves lightly dusted with carborundum (400-600 mesh). After inoculation, rinse the sap from the leaves with water and grow the plants at 18-25 °C for at least 3-4 weeks under slight shade. The shorter growing time may be used only if the specific environmental conditions used in the glasshouse or growth room, or the species of indicator plant used, have been shown and have been documented as allowing reliable detection of the listed viruses within 3 weeks. Shading (e.g. by covering with paper) prior to and after inoculation may enhance susceptibility and symptom development. Inspect plants at least once a week for symptoms and record that the plants have been inspected and symptoms if present. Suitable indicator plants for each virus are indicated in Table 5.

**Fig. 2** Detail of propagation pathway for material received as microplants (up to glasshouse stage).



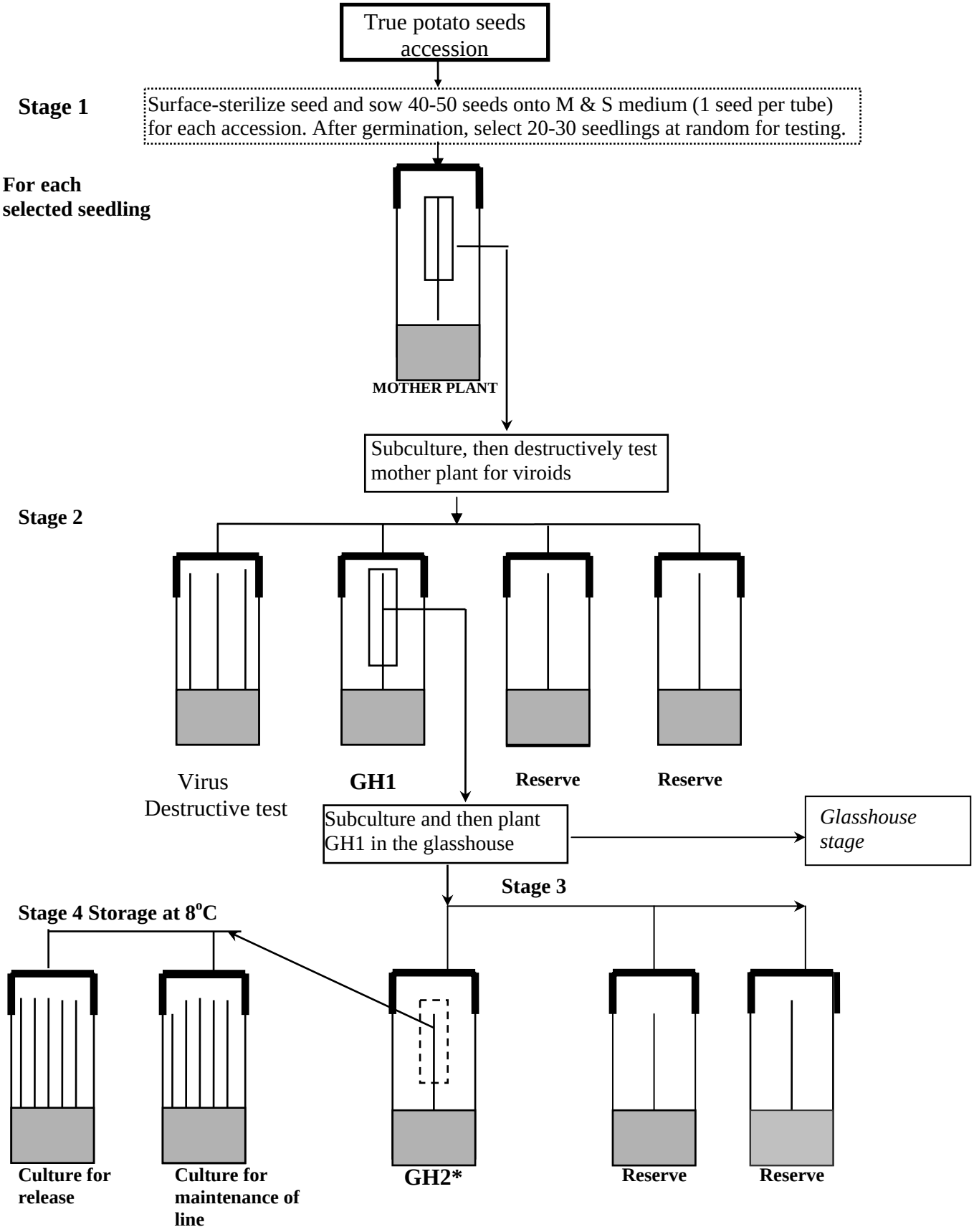

***GH2** for use as backup, if **GH1** dies or symptoms in **GH1** require further investigation.

**Fig. 3** Example of propagation pathway for material received as tubers (up to glasshouse stage).




***GH2** for use as backup, if GH1 dies or symptoms in **GH 1** require further investigation.

**Fig. 4** Example of propagation pathway for material received as true potato seeds (up to glasshouse stage).




***GH2** for use as backup, if **GH1** dies or symptoms in **GH1** require further investigation.