



Pyrus (Pear)

Post-Entry Quarantine Testing Manual



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Pyrus Post-Entry Quarantine Testing Manual

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1. SCOPE

The scope of this manual is limited to nursery stock (dormant cuttings and plants in tissue culture), seed for sowing and pollen of *Pyrus* species permitted entry into New Zealand as listed in the Ministry for Primary Industries's (MPI) Plants Biosecurity Index (see: <http://www1.maf.govt.nz/cgi-bin/bioindex/bioindex.pl>). At the date of publication of this manual, these species were as follows:

<i>Pyrus betulaefolia</i>	<i>Pyrus pashia</i>
<i>Pyrus bretschneideri</i>	<i>Pyrus phaeocarpa</i>
<i>Pyrus calleryana</i>	<i>Pyrus pyrifolia</i>
<i>Pyrus communis</i>	<i>Pyrus salicifolia</i>
<i>Pyrus</i> × <i>lecontei</i>	<i>Pyrus serotina</i>
<i>Pyrus nivalis</i>	<i>Pyrus ussuriensis</i>

This manual describes the testing protocols specified in the import health standards for these commodities. The manual also provides an introduction to the crop and guidance on the establishment and maintenance of healthy plants in quarantine.

2. INTRODUCTION

The genus *Pyrus* belongs to the family Rosaceae and is composed of over 20 species. The natural habitat of *Pyrus* covers a large arc from Europe through Asia to Japan. The major groupings are the European pears, principally *P. communis*, and the Oriental or Asian pears e.g. *P. pyrifolia*. Additionally, there are hybrids between species e.g. some of the perry pears from England and France are thought to be hybrids between *P. communis* and *P. nivalis*, and the major commercial pears in China are thought to be derived from *P. pyrifolia*, *P. ussuriensis* and *P. × bretschneideri* (Bell, 1991). Most pears are deciduous, rarely evergreen and produce a medium sized tree, often with a tall narrow, crown, although some shrubby and weeping forms have been described. Fruit shape in many species is globose but can vary from round to the typical bulbous form of European pears.

The cultivation of pears has been known for at least 3,000 years, so selection of specific cultivars has occurred over a long period of domestication. Current world production of pears is almost 20 million tonnes, which comprises 60% Asian and 40% European pears. China is by far the world's largest producer with 11.6 million tonnes, followed by Italy with 0.85 million tonnes and USA with 0.77 million tonnes (World Pear Review, 2006). Most pears are consumed fresh, although there is an important canning industry, largely based on the European pear 'William's Bon Chrétien' (syn. 'Bartlett'), and smaller quantities of pears are used for juice, which may be fermented to produce perry.

Although many pear cultivars in New Zealand are commonly propagated on clonal quince (*Cydonia oblonga*) rootstocks, they are also propagated onto seedlings of *P. calleryana* and *P. betulaefolia* (syn. *P. betulifolia*). This rather unusual choice of rootstocks for pears arises from the fact that quince rootstocks offer the grower good tree size control and precocity, with ease of rooting on the stoolbed. *Pyrus* rootstocks are more difficult to root vegetatively, hence the use of seedlings as a means of propagation. Pear trees on *Pyrus* rootstocks are more vigorous in growth and less precocious than when worked on quince rootstocks. However, many pear scion cultivars are not graft compatible with quince, so in order to gain the advantages offered by quince as a rootstock, an interstem of a graft compatible scion e.g. 'Buerré Hardy' is used between the rootstock and the incompatible scion. Common quince

rootstocks are ‘Quince C’, normally abbreviated to QC, and ‘Quince BA29’. The most common commercially planted European pear in New Zealand is ‘Doyenné du Comice’ or its sport ‘Taylor’s Gold’, both of which are graft compatible with quince, whereas ‘Buerré Bosc’, New Zealand’s next most important export cultivar, requires an interstem. All Asian pears require an interstem with quince rootstocks.

Currently, the pear industry in New Zealand is small compared with the apple industry, with export earnings of approximately \$8 million, largely from European pears. Although the Asian or nashi pear industry was increasing in the early 1990s, it has since declined and most pears currently exported from New Zealand are European types. Nashi is the Japanese word for pear and is commonly used in New Zealand to describe Japanese pear cultivars e.g. ‘Hosui’ or ‘Nijisseiki’. There have, however, been some recent plantings of nashi pears to supply the growing New Zealand Asian population. The current area of European pears is approximately 900 hectares and nashi pears approximately 120 hectares.

There is an active pear breeding programme in New Zealand that is seeking to produce improved scion cultivars of European and Asian pears and interspecific hybrids. The largest breeding effort is into interspecific hybrids that have crisp, juicy, highly flavoured fruit. Strong directional selection pressure is placed on fruit quality, especially for crisp juicy textures, aromatic flavours, attractive and clean skin colour, pyriform fruit shape, and long shelf life.

3. IMPORT REQUIREMENTS

The import requirements for nursery stock (dormant cuttings and plants in tissue culture) and pollen of *Pyrus* are set out in MPI’s import health standard “Importation of Nursery Stock” (see: <http://www.biosecurity.govt.nz/files/imports/plants/standards/155-02-06.pdf>). Imported cuttings and tissue culture plants must meet the general requirements (sections 1-3) and the additional specific requirements detailed in the “*Pyrus*” schedule. This schedule is not available electronically but can be obtained by writing to MPI (plantimports@mpi.govt.nz). In summary, an import permit is required and a phytosanitary certificate must accompany all consignments certifying that the nursery stock has been inspected and found to be free of any visually detectable regulated pests, and has been treated for regulated insects and mites (cuttings only). On arrival in New Zealand, the nursery stock from non-accredited facilities must be grown for a minimum period of 24 months in a post-entry quarantine facility where it will be inspected, treated and/or tested for regulated pests.

Pyrus nursery stock imported from a MPI-accredited facility overseas undergoes a reduced post-entry quarantine requirement following arrival in New Zealand. A list of these facilities is available at <http://www.biosecurity.govt.nz/imports/plants/offshore-accredited-facilities.htm>. At the date of publication of this manual, there are no offshore accredited facilities for *Pyrus*.

The import requirements for *Pyrus* seed for sowing are set out in MPI’s import health standard “Importation of Seed for Sowing” (see: <http://www.biosecurity.govt.nz/files/imports/plants/standards/155-02-05.pdf>). Imported seed must meet the general requirements (sections 1-2) and the specific requirements detailed in the “*Pyrus*” schedule in section 3. In summary, an import permit is required and a phytosanitary certificate must accompany all consignments certifying that the seeds have been inspected and found free of any visually detectable regulated pests. On arrival in New

Zealand, the seed must be grown for a minimum period of 6 months in a Level 3 post-entry quarantine facility where it will be inspected, treated and/or tested for regulated pests.

4. PESTS

The following section lists regulated pests of *Pyrus* nursery stock and seed for sowing for which generic or specific measures are required or optional. A complete list of regulated and non-regulated pests of *Pyrus* can be found in the nursery stock import health standard (see section 3).

4.1 Regulated pests for which generic measures are required

Insects, mites & fungi: Refer to import health standard.

Bacteria:

<i>Agrobacterium rhizogenes</i>	[Nursery stock only]
<i>Pseudomonas syringae</i> pv. <i>papulans</i>	[Nursery stock only]

Diseases of unknown aetiology:

Pear bark measles*	[Nursery stock & seed for sowing]
Pear bark split	[Nursery stock only]
Pear concentric ring pattern	[Nursery stock only]
Pear mild mosaic	[Nursery stock only]
Pear russet ring*	[Nursery stock only]
Quince yellow blotch*	[Nursery stock only]

***Note:** The current import health standard for nursery stock requires that these pests are tested by ELISA. No antisera are available for these pathogens; therefore growing season inspection is the only requirement.

4.2 Regulated pests for which specific tests are required

Bacterium:

<i>Spiroplasma citri</i>	[Nursery stock only]
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Phytoplasma:

Pear decline phytoplasma (‘ <i>Candidatus</i> Phytoplasma pyri’)	[Nursery stock only]	Figure 1.1
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Viruses:

<i>Tobacco necrosis virus</i>	[Nursery stock only]
<i>Tomato bushy stunt virus</i>	[Seed for sowing only]

Viroid:

<i>Apple scar skin viroid</i>	[Seed for sowing only]
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Diseases of unknown aetiology:

Pear bark necrosis	[Nursery stock only]
Pear bud drop	[Nursery stock only]
Pear corky pit	[Nursery stock only]
Pear freckle pit	[Nursery stock only]
Pear rough bark	[Nursery stock only]

4.3 Regulated pests for which specific tests are optional

Bacterium:

Xylella fastidiosa [Nursery stock only] Figure 1.2
(causal agent of Pear leaf scorch)

Viruses:

Pear latent virus [Nursery stock only] Figure 1.3
Tomato bushy stunt virus [Nursery stock only]

Viroids:

Apple dimple fruit viroid [Nursery stock only]
Apple scar skin viroid [Nursery stock only]
Pear blister canker viroid [Nursery stock only] Figure 1.4

5. PROPAGATION, CARE AND MAINTENANCE IN POST-ENTRY QUARANTINE

Plants must be maintained in a healthy, vigorous state. Normally the nutrients contained within the planting media will be sufficient for the first three months of growth, after which regular repotting should provide an adequate supply of nutrients. It is important that pruning and cutting tools used on the imported cuttings are disinfected between each plant. Alternatively, disposable razor blades may be used.

Pyrus plants in quarantine may exhibit symptoms associated with abiotic stresses such as water and nutrient deficiencies. Common nutrient deficiencies are from nitrogen, magnesium, zinc and manganese. Photographs of the symptoms caused by some of these nutrient deficiencies can be found in Appendix 2. Nitrogen deficiency is seen as a general yellowing of the foliage; magnesium deficiency shows as an inter-veinal yellowing of the older leaves; zinc deficiency results in small, narrow, stunted leaves, often in rosettes, while manganese deficiency shows as an inter-veinal yellowing over the whole plant. Further illustrations of nutrient deficiencies can be found on the American Phytopathological Society's CD-ROM 'Nutrient Deficiencies and Toxicities of Plants' (www.aps.org) or at <http://www.luminet.net/~wenonah/min-def/>.

5.1 Nursery stock

Pyrus nursery stock may be imported as dormant cuttings or as plants in tissue culture.

5.1.1 Dormant cuttings

It is best to import *Pyrus* cuttings between December to February (from the northern hemisphere), and June to July (from the southern hemisphere). All imported cutting material must be free of fruit, foliage, roots or soil to minimise the introduction of disease.

Most *Pyrus* germplasm is difficult to root as hardwood cuttings, so it is advisable to graft onto a seedling rootstock of *P. calleryana* or *P. betulaefolia*. Trees of both of these species are present in New Zealand and locally collected seed is available. See section 5.2 for details on germinating *Pyrus* seed. Both of these species are considered tolerant of dry and wet soils, useful for trees in pots.

Budding of dormant buds onto actively growing *Pyrus* seedlings can be performed either by chip budding or by T budding. The dormant budwood can be maintained in a coolstore until the seedlings are actively growing. Alternatively, budding can be performed during winter or early spring onto dormant seedlings; growth of the bud begins when the grafted tree emerges from dormancy. Both chip and T budding make better use of the available material than conventional grafting, as each bud can be used on a separate seedling rootstock. Chip budding can be used over a longer period of the growing season as it does not require the bark to be lifted to insert the bud, as with T budding. It is also easier to match the two cambia around the rim of the cut surface with chip budding. Tying with grafting tape or polyethylene strips must be done soon after matching the bud to the stock to avoid drying out of the tissue.

Various conventional grafting techniques can be used with 2 bud splice grafts (also known as a whip graft), whip and tongue grafts or cleft grafts being well suited. Ideally grafting requires careful matching up of the diameters of the scion and rootstock material to ensure that the cambia of both tissues are in intimate contact. If the diameters do not match then ensure cambial contact on one side of the graft. Tie the graft firmly with grafting tape and paint the top of the graft with Bacseal[®] Super to prevent drying out. Slit the tape when the graft is growing and when the base of the graft has swollen.

Extensive details on grafting are given by Garner (1993) and on the following websites:

- http://www.rhs.org.uk/advice/profiles0802/chip_budding.asp
- http://www.aces.uiuc.edu/vista/pdf_pubs/BUDDING.PDF
- <http://www.fao.org/DOCREP/006/AD224E/AD224E14.htm>

5.1.2 Plants in tissue culture

Tissue culture plants should ideally be imported in winter (June to July in the southern hemisphere) to allow them to become established prior to summer.

Pyrus tissue culture plantlets to be tested must be deflasked and grown in a Level 3 post-entry quarantine facility. The plantlets are washed to remove any remaining agar and planted into pots of pasteurised planting media containing 1:1 (v/v) peat:pumice or 1:1 (v/v) peat:perlite with a medium-term, slow-release fertiliser (e.g. Osmocote[®]). The plantlets must be protected from evaporation, for approximately 3 weeks, by covering with plastic (tub or bag) or misted regularly to keep the planting media just moist and maintain a high relative humidity. The plantlets also need protection from high light using 70% shade during this weaning period. After this period, any coverings are gradually removed and the plants moved to higher light intensity under normal glasshouse conditions.

Any remaining tissue culture plantlets that are not deflasked may remain as a back up in a Level 3 quarantine tissue culture laboratory but cannot be released without testing. Tissue culture plantlets can be sub-cultured after arrival by excising the growing tips and placing into new tissue culture vessels with fresh nutrient media for growth and rooting.

5.2 Seed for sowing

Pyrus seed is best stored dry. For short-term storage, seeds should be kept inside an airtight container or plastic bag (e.g. Ziploc[®] bags) in a refrigerator (approximately 4°C). For long-term seed storage, plastic bags containing seed should be placed in a freezer at -20° to -40°C.

Prior to germination, the seeds are surface sterilised using a 1% sodium hypochlorite solution for 15 minutes, and rinsed thoroughly with tap water using a sieve (this is important to reduce fungal development). Seeds need to be moist stratified and can be placed in a plastic bag containing damp but not wet sterilised peat or fine vermiculite, then held in a cool store or refrigerator at approximately 4°C for 60 days before planting.

If the peat in the plastic bag is too wet, seed rotting will occur. If the seeds and peat used for seed storage are not adequately pasteurised, fungal infection can occur.

Following moist stratification, seeds can be directly sown into individual pots filled with planting media consisting of 1:1 (v/v) peat:pumice or 1:1 (v/v) peat:perlite, and medium-term slow release fertiliser. The seeds should be covered with a light layer of sowing media no deeper than 1 cm. Alternatively, seeds can be sown directly onto trays filled with pasteurised sowing media, consisting of 1:1 (v/v) peat:pumice or 1:1 (v/v) peat:perlite. The seeds are covered with a very light layer of sowing media, no deeper than 1 cm. Fine vermiculite may also be used. The seed tray is placed in bright light within a glasshouse and watered to keep the media moist. Seeds germinate more readily when placed in a temperature range of 15-20°C rather than >25°C. Germinated seedlings can then be carefully transplanted into individual pots of planting media containing medium-term, slow release fertiliser (e.g. Osmocote[®]) when they are approximately 5-7 cm tall.

5.3 Pollen

Pollen needs to be kept in a cool and low moisture environment to maintain and maximise its viability. Pollen should be stored in a container over dry silica gel crystals and held in a refrigerator (approximately 4°C) for storage of up to 1 year. Regular drying of the silica gel crystals is required to ensure there is no moisture build up. For long-term storage, pollen should be enclosed in a container over dry silica gel then frozen at -20°C to -40°C. When removed from the freezer, pollen should be thawed slowly in a refrigerator before use. Pollen containers removed from the refrigerator for use in the field or glasshouse should be kept cool.

Pollen viability can be determined by the fluorochromatic reaction as described by Heslop-Harrison & Heslop-Harrison (1970) or by germination on Petri plates of agar containing dextrose and boric acid. Pollen is germinated for 24 hours at approximately 20°C and then killed by dropping 2 ml of an ethanol-based stain onto each plate. Germination counts are determined with a compound microscope.

There are no regulated pests affecting pollen of *Pyrus* spp., and pollen may be imported without specific testing or the need for post-entry quarantine.

6. INSPECTION

The inspection requirements for the operator of the facility are set out in the “MPI Biosecurity Authority Standard PBC-NZ-TRA-PQCON” (see: <http://www.biosecurity.govt.nz/border/transitional-facilities/plants/psc-nz-tra-pqcon.htm>).

Photographs of symptoms caused by some of the significant regulated pests can be found in Appendix 1.

7. TESTING

Each of the specific tests required in the import health standard (as described in section 4 and summarised in Table 1) must be done irrespective of whether plants exhibit symptoms.

Table 1: Summary of the regulated pests for *Pyrus* nursery stock (A) or seed for sowing (B) indicating the specific tests that are required (■), alternative (□) or optional (★)

A. Nursery stock

Organism type	Woody indexing	Herbaceous indexing	ELISA	PCR
Bacteria				
<i>Spiroplasma citri</i>				■
<i>Xylella fastidiosa</i> ¹				★
Phytoplasma				
Pear decline phytoplasma				■
Viruses				
<i>Pear latent virus</i> ¹		★		★
<i>Tobacco necrosis virus</i> ²		■	■	
<i>Tomato bushy stunt virus</i> ¹		★	★	★
Viroids				
<i>Apple dimple fruit viroid</i> ¹				★
<i>Apple scar skin viroid</i> ¹				★
<i>Pear blister canker viroid</i> ¹				★
Diseases of unknown aetiology				
Pear bark necrosis	■			
Pear bud drop	■			
Pear corky pit	■			
Pear freckle pit	■			
Pear rough bark	■			

¹These pests are not on the import health standard; therefore testing for these is optional.

²Strains not in New Zealand.

B. Seed for sowing

Organism type	Herbaceous indexing	ELISA	PCR
Virus			
<i>Tomato bushy stunt virus</i>	■	□	□
Viroid			
<i>Apple scar skin viroid</i>			■

Herbaceous indexing and laboratory tests (ELISA and RT-PCR) for viruses and viroids must be carried out in the spring using the new flush of spring growth. Laboratory tests (PCR) for bacteria and phytoplasma must be carried out at the end of summer on mature plants that have good development of phloem and xylem. The replication of *Xylella*, spiroplasmas and phytoplasmas are restricted to the vascular tissue of infected plants, therefore detection of these pathogens requires testing leaf petioles and mid-veins.

For each of the herbaceous indexing and laboratory tests, *Pyrus* plants must be sampled from at least two positions including a young, fully expanded leaf at the top of the plant and an older leaf from a midway position. The samples from each plant must be bulked together and tested as soon as possible after removal from the host. If samples have to be stored before

testing, the leaves must be kept whole, all surface water removed and the material stored in a plastic bag at 4°C for no more than 7 days. Samples that become partially decayed or mouldy should not be tested, and further samples must be collected.

Woody indexing should ideally be carried out in a Level 1 quarantine facility as it relies on the development of fruit and bark symptoms. These tests can only be performed outdoors once all the herbaceous indexing and laboratory tests have been completed. Inoculations are usually performed from late spring to mid summer.

7.1 Specific tests for nursery stock

Each plant must be tested separately with the exception that samples from up to 5 plants may be bulked for testing provided that either:

- (a) the plants are derived from a single imported dormant cutting which was split into separate cuttings upon arrival in New Zealand, in the presence of a MPI inspector; or
- (b) in the case of tissue culture where plants are clonal, and this is confirmed by evidence from the national plant protection organisation in the exporting country.

7.1.1 Woody indexing

Each *Pyrus* plant must be tested by woody indexing onto two indicator cultivars each of *Pyrus communis* ‘Beurré Hardy’ and ‘Beurré Bosc’. This is ideally performed in a Level 1 quarantine facility using indicators that are of fruit-bearing age.

Recommended method

1. Indicator scions of ‘Beurré Hardy’ and ‘Beurré Bosc’ are grafted onto rootstocks 2-3 years ahead of inoculation. Prune and train for fruit production.
2. Inoculations are usually performed from late spring to mid summer when the indicator tree is in active growth and when the bark readily separates from the wood (known as slipping).
3. Four inoculum chips from the test material are grafted onto each indicator tree. They can be budded anywhere on the tree but the smaller branches are easier. At least two of the inoculum chips should have buds on them. Ensure that the cambial tissue is aligned and the inoculum is firmly attached to the indicator by ties or tape. Do not inoculate trees that are stunted or show unusual symptoms.
4. At least two trees per row must be left ungrafted as the negative controls. These plants must be subjected to the same horticultural practices as the inoculated plants.
5. Optional: The indicators may be budded with a positive control. The positive control is a disease of unknown aetiology that produces distinctive bark or fruit symptoms, e.g. Pear stony pit (non-regulated). Please note that approval from a Chief Technical Officer is required for propagation of unwanted organisms.
6. Approximately 2-3 weeks after grafting, check that the graft union has successfully healed and that the inoculum is still alive. All graft inoculations must have survived; they must be repeated if the inoculum is dead. The following year, it is optional to cut back the growth from the inoculum buds although it can be distracting for symptom observation.
7. Observe leaves, fruit and bark for symptoms at least 4 times per year for at least 2 growing seasons. Two fruit crops must be observed.

Positive control for woody indexing

Positive control material for Pear stony pit may be obtained from the Investigation and Diagnostic Centre and Response – Tamaki, MPI (see the Contact Point, section 8). A charge may be imposed to recover costs.

Interpretation of results

The woody indexing results will only be considered valid if no symptoms are produced on each of the negative controls (ungrafted plants). If the optional positive control (disease of unknown aetiology) was used, then the correct symptoms must be produced on the indicator cultivar. If Pear stony pit was used as the positive control, the following symptoms will be produced on the woody indicators:

- ‘Beurré Bosc’ – Fruit distortion and development of dark green flecks in the flesh.
- ‘Beurré Hardy’ – Development of dark brown internal pits in the fruit.

The symptoms produced by each of the regulated pathogens on woody indicators are described below.

Pear bark necrosis:

- ‘Beurré Hardy’ – Two types of bark symptoms can be produced. In one type, closely packed ring-like and twisting cracks appear on the bark that spread rapidly both on old and younger branches resulting in death of the bark and wood. The other type causes browning then purpling of large areas of bark that is at least one year old. Leaves wilt and the cambium dies in branches that still appear healthy. By the end of the first vegetation period following the appearance of the symptoms, the tree declines.

Pear bud drop:

- ‘Beurré Hardy’ – Leaf epinasty and reduced tree growth first appear in the summer after infection and some trees die by the end of the vegetation period. Buds of surviving trees begin to drop by early winter. Necrosis develops at the bud base and buds easily break off. Most trees stay bare in the following spring and eventually die. If connecting tissue still survives below affected buds, buds begin to grow, but the leaves wilt and dry out. Rosette formation can be observed on some trees.

Pear corky pit:

- ‘Beurré Bosc’ – Fruits develop shallow pits or grooves. Under the depressions the flesh contains brown, cork-like areas, which sometimes form individual knots under the skin, but more often spread throughout the entire flesh of the fruit. Leaves and bark develop no diagnostic symptoms in most pear cultivars.

Pear freckle pit:

- ‘Beurré Bosc’ – Symptoms only become visible one month before harvest. At that time, dark green, sunken pits develop on the fruit surface. Dark green strands develop in the fruit flesh. Effects are more concentrated and deeper at the calyx end. As the fruit ripens, the colour of pits lightens while the network of strands in the flesh turn brown. Leaves and bark develop no diagnostic symptoms.

Pear rough bark:

- ‘Beurré Bosc’ – The first distinct symptoms are pronounced splits and furrows, which form on both old branches and younger shoots. The bark of these affected areas later becomes thick and coarse.

The ‘Beurré Hardy’ and ‘Beurré Bosc’ woody indicators will also detect Pear bark split (a pathogen not on the import health standard). The symptoms produced by this pathogen are as follows:

- The bark becomes cracked at the lower parts of the new shoots by the end of summer. In the following year, the cracks deepen and extend into the bases of young shoots.

Subsequently, the bark around affected areas becomes rough and scabby. Diseased trees experience reduced growth and young trees may decline.

7.1.2 Herbaceous indexing

Each *Pyrus* plant must be tested by mechanical inoculation onto two indicator plants each of *Chenopodium quinoa* and *Nicotiana clevelandii*. Mechanical inoculation onto *Nicotiana benthamiana* to detect *Pear latent virus* is optional.

It is important that the pre- and post-inoculation growing conditions of the herbaceous indicator plants promote their susceptibility. Plants must be grown at 18-25°C before and after inoculation. Select indicator plants at the four- to six-leaf stage of growth with at least two leaves fully expanded.

Recommended method

1. Place indicator plants in the dark for approximately 24 hours prior to inoculation to increase susceptibility.
2. Grind leaf tissue (approximately 1/4; w/v) in 0.1 M sodium phosphate buffer (pH 7.5), containing 5% (w/v) polyvinylpyrrolidone (PVP-40) and 0.12% (w/v) sodium sulphite (Na₂SO₃). A negative (inoculation buffer only) and a positive control must be included on each day that the inoculations are performed. The positive control is a non-regulated virus which is moderately transmissible and produces clear symptoms on the herbaceous indicators, e.g. *Arabis mosaic virus*. Sap expressed from *Pyrus* plants contains phenolic compounds that can inhibit virus transmission. If the positive control is not *Pyrus* tissue, we therefore recommend mixing healthy *Pyrus* leaf material close to the same physiological stage as the test plants with the positive control (approximately 3:1 w/w) prior to carrying out the inoculations. The plants must be inoculated in the following order:
 - (a) negative control (inoculation buffer only); then
 - (b) test plants; then
 - (c) positive control (non-regulated virus).
3. Select two young fully expanded leaves, preferably opposite leaves, to be inoculated on each indicator plant and mark them by piercing holes with a pipette tip (or similar).
4. Lightly dust the leaves with Celite or carborundum powder (wear a mask or perform in a fume hood). Alternatively, a small amount of Celite or carborundum powder may be mixed with the sap extract.
5. Using a gloved finger gently apply the sap to the marked leaves of the indicator plants, stroking from the petiole towards the leaf tip while supporting the leaf below with the other hand.
6. After 3-5 minutes rinse inoculated leaves with water.
7. Maintain inoculated plants for a minimum of 4 weeks. Inspect and record plants twice per week for symptoms of virus infection.

Positive control for herbaceous indexing

The *Arabis mosaic virus* positive control may be obtained from:

1. ATCC Cat. No. PV-192, PV-589, PV-590 (<http://www.atcc.org>).
2. DSMZ Cat. No. PV-0045, PV-0046, PV-0215, PV-0216, PV-0217, PV-0230, PV-0232 (<http://www.dsmz.de>).
3. The Investigation and Diagnostic Centre and Response – Tamaki, MPI (see the Contact Point, section 8) (available as freeze-dried leaf material). A charge may be imposed to recover costs.

Interpretation of results

The herbaceous indexing results will only be considered valid if:

- (a) no symptoms are produced on the indicator hosts with the negative control (inoculation buffer only); and
- (b) the correct symptoms are produced on the indicator hosts with the positive control (non-regulated virus). If *Arabidopsis mosaic virus* was used as the positive control, the following symptoms will be produced on the herbaceous indicators:
 - *C. quinoa* – local lesions, systemic chlorotic mottling.
 - *N. clevelandii* – local lesions, systemic chlorotic spots, rings and lines.

The symptoms produced by each of the regulated viruses on herbaceous indicators are described below.

Pear latent virus:

- *N. benthamiana* – chlorotic local lesions followed by deformation of the leaves, chlorotic mottling, generalised systemic yellowing, withering and sometimes death of the plant.

Tobacco necrosis virus:

- *C. quinoa* and *N. clevelandii* – necrotic local lesions, usually no systemic spread.

Tomato bushy stunt virus:

- *C. quinoa* – chlorotic/necrotic local lesions surrounded by chlorotic haloes.
- *N. clevelandii* – chlorotic local lesions in inoculated leaves followed by systemic mottling and severe distortion of the leaves and finally apical necrosis and death.

7.1.3 Serological and molecular assays

ELISA **MUST** be carried out for the following virus:

- *Tobacco necrosis virus*

PCR **MUST** be carried out for the following pathogens:

- *Spiroplasma citri*
- Pear decline phytoplasma

ELISA **OR** PCR is **OPTIONAL** for the following virus:

- *Tomato bushy stunt virus*

PCR is **OPTIONAL** for the following pathogens:

- *Xylella fastidiosa*
- *Pear latent virus*
- *Apple fruit crinkle viroid*
- *Apple scar skin viroid*
- *Pear blister canker viroid*

7.1.3.1 Enzyme-linked immunosorbent assay (ELISA)

Recommended method

1. Perform the ELISA according to the manufacturer's instructions. The following controls must be included on each ELISA plate:
 - (a) positive control: infected leaf tissue or equivalent (Table 2); and
 - (b) negative control: *Pyrus* leaf tissue that is known to be healthy; and

- (c) buffer control: extraction buffer only.
2. Add each of the samples and controls to the ELISA plate as duplicate wells. It is not recommended to perform ELISA with plant samples or sap that has been frozen, unless it has been demonstrated that it does not affect the performance of the test.
 3. Measure the optical density 60 minutes after addition of the substrate or as recommended in the manufacturer's instructions.

Table 2: Source of antisera and positive controls for ELISA

Virus	Antisera¹	Positive control²
<i>Tobacco necrosis virus</i>	Bioreba Cat. No. 152465 Loewe Cat. No. 07036C	Bioreba Cat. No. 152453 Loewe Cat. No. 07046PC
<i>Tomato bushy stunt virus</i>	Agdia Cat. No. SRA 45400 Loewe Cat. No. 07067C	Agdia Cat. No. LPC 45400 Loewe Cat. No. 07067PC

¹The catalogue numbers for the complete reagent sets are given; the reagents can also be purchased separately.

²The positive control is included if the complete reagent set for antisera is purchased.

Further information on the suppliers listed in Table 3 can be found on their websites:

- Agdia Incorporated, USA (<http://www.agdia.com>).
- Bioreba AG, Switzerland (<http://www.bioreba.com>).
- Loewe Biochemica GmbH, Germany (<http://www.loewe-info.com>).

The antisera listed have been tested by the Investigation and Diagnostic Centre and Response – Tamaki, MPI. Alternative antisera and positive controls are available from other manufacturers.

Interpretation of results

A result is considered positive if the mean absorbance of the two replicate wells is greater than 2 times the mean absorbance of the negative control. The test will only be considered valid if:

- (a) the absorbances for the positive and negative controls are within the acceptable range specified by the manufacturer; and
- (b) the coefficient of variation (standard deviation / mean × 100), between the duplicate wells is less than 20%.

If the test is invalid, it must be repeated with freshly-extracted sample. Samples that are close to the cut-off must be retested or tested using an alternative method recommended in the import health standard if available (see Table 1).

7.1.3.2 Polymerase chain reaction (PCR)

PCR primers used to detect viruses, viroids, phytoplasma and bacteria of *Pyrus* are listed in Table 3, along with plant internal control primers for RNA and DNA. The inclusion of an internal control assay is recommended to eliminate the possibility of PCR false negatives due to extraction failure, nucleic acid degradation or the presence of PCR inhibitors. The *Nad5* primers amplify mRNA from plant mitochondria and the Gd1/Berg54 primers amplify the 16S rRNA gene from chloroplasts.

Please note that the primers listed in Table 3 have not been optimised for use in multiplex PCR. We recommend that the pathogen-specific PCR and the internal control PCR are performed simultaneously in separate tubes, unless the PCR has been optimised as a multiplex.

The PCR reagents listed for the methods described in this section have been tested by the Investigation and Diagnostic Centre and Response – Tamaki, MPI. Alternative reagents may give similar results but will require validation.

Table 3: PCR primers used for the detection of regulated pests of *Pyrus* and plant internal controls

Target organism	Primer name	Sequence (5'-3')	Tm (°C)	Size (bp)	Reference
Viruses					
<i>Pear latent virus & Tomato bushy stunt virus</i>	Gral F1	AAGGGTAAGGATGGTGAGGA	55	587	Harris <i>et al.</i> , 2007
	Gral R1	TTTGGTAGGTTGTGGAGTGC			
Viroids					
<i>Apple dimple fruit viroid</i>	AD-38	CCTTTGAGACTTGACCGGTTCTC	55	254	Di Serio <i>et al.</i> , 2002
	ADAS-36	GCCTTCGTCGACGACGACAG			
<i>Apple scar skin viroid</i>	AS-37	CGGTGACAAAGGAGCTGCCAG	55	330	Di Serio <i>et al.</i> , 2002
	ADAS-36	GCCTTCGTCGACGACGACAG			
<i>Pear blister canker viroid</i>	C166	GCTGGTTTTCTTCTCCAAAGGAGCGAT TACTCACAG	55	315	Loreti <i>et al.</i> , 1997
	H167	GTTGCTTCCTGCCTGAGCCTCGTCTTC TGTCCCG			
Phytoplasma					
Universal phytoplasma	P1	AAGAGTTTGATCCTGGCTCAGGATT	53	1,800	Deng & Hiruki, 1991 Schneider <i>et al.</i> , 1995
	P7	CGTCCTTCATCGGCTCTT			
Universal phytoplasma	R16F2	ACGACTGCTAAGACTGG	50	1,248	Lee <i>et al.</i> , 1995
	R16R2	TGACGGGCGGTGTGTACAAACCCCG			
Bacteria					
<i>Spiroplasma citri</i>	P58-6f	GCGGACAAATTAAGTAATAAAAAGAGC	56	450	Yokomi <i>et al.</i> , 2008
	P58-4r	GCACAGCATTGGCCAACACTACA			
<i>Xylella fastidiosa</i>	PLS-F	TGGACGTTGTGGTATCGGTG	56	416	C. C. Su, pers. comm.
	PLS-R	TTGAAGTTGACGTGTGGCTG			
Internal control					
Plant RNA control	<i>Nad5-F</i>	GATGCTTCTTGGGGCTTCTTGTT	50-60	181	Menzel <i>et al.</i> , 2002
	<i>Nad5-R</i>	CTCCAGTCACCAACATTGGCATAA			
Plant DNA control	Gd1	ACGGAGAGTTTGATCCTG	50-62	1,500	Andersen <i>et al.</i> , 1998
	Berg54	AAAGGAGGTGATCCAGCCGCACCTTC			

7.1.3.2.1 Virus and viroid reverse transcription-PCR (RT-PCR)

Recommended method

1. Extract total RNA according to a standard protocol. Successful RT-PCR amplification can be achieved using RNA that has been extracted using the following methods:
 - (a) RNeasy[®] Plant Mini Kit (Qiagen Cat. No. 74904); or
 - (b) RNeasy[®] Plant Mini Kit (Qiagen Cat. No. 74904) with a modified lysis buffer as described by MacKenzie *et al.* (1997); or
 - (c) silica-based method as described by Menzel *et al.* (2002).
 See Appendix 3 for details of the latter two extraction methods. Alternative methods may also be used after validation.
2. Optional: Perform a one-step RT-PCR on the RNA with the *Nad5* internal control primers (Table 3) using the components and concentrations listed in Table 4 or Table 5 and cycle under the conditions listed in Table 6.
3. Perform a one-step RT-PCR on the RNA with the pathogen-specific primers (Table 3) using the components and concentrations listed in Table 4 or Table 5 and cycle under the

conditions listed in Table 6. The Invitrogen one-step RT-PCR kit described in Table 5 is more sensitive than using the individual reagents listed in Table 4. The following controls must be included for each set of RT-PCR reactions:

(a) positive control: RNA of the appropriate virus or viroid extracted from any host tissue or a cDNA clone. If the internal control primers are not used, then the RNA or cDNA clone must be mixed with healthy *Pyrus* RNA to rule out the presence of PCR inhibitors.

(b) no template control: water is added instead of RNA template.

When setting up the test initially, it is advised that a negative control (RNA extracted from healthy *Pyrus* leaf tissue) is included. Please note that the *Nad5* internal control primers do not reliably amplify a product from RNA extracted from freeze-dried material. We therefore recommend mixing fresh healthy *Pyrus* leaf material with freeze-dried positive control material (3:1 w/w) prior to carrying out the extraction.

4. Analyse the PCR products by agarose gel electrophoresis.

Virus and viroid positive controls for PCR

1. *Tomato bushy stunt virus* may be obtained from:

(a) ATCC Cat. No. PV-90, PV-163, PV-483, PV-500 (<http://www.atcc.org>).

(b) DSMZ Cat No. PV-0268, PV-0269 (<http://www.dsmz.de>).

(c) the commercial sources listed in Table 2.

2. *Apple dimple fruit viroid*, *Apple scar skin viroid* and *Pear blister canker viroid* may be obtained from the Investigation and Diagnostic Centre – Tamaki, MPI Biosecurity New Zealand (see the Contact Point, section 8). A charge may be imposed to recover costs.

Positive control material for *Pear latent virus* is currently un-obtainable; it has therefore not been possible to validate the PCR for this virus.

Table 4: Generic one-step RT-PCR reaction components

Reagent	Volume per reaction (µl)
Sterile deionised H ₂ O	12.7
10 × PCR buffer (Invitrogen)	2.0
50 mM MgCl ₂	0.6
10 mM dNTPs	0.4
5 µM Forward primer	1.0
5 µM Reverse primer	1.0
0.1 M DTT	1.0
5 U/µl Platinum [®] Taq DNA polymerase (Invitrogen 10966-026)	0.1
40 U/µl RNasin [®] Plus RNase Inhibitor (Promega N261A)	0.15
200 U/µl SuperScript [™] II Reverse Transcriptase (Invitrogen 18064-014)	0.05
RNA template	1.0
Total	20.0

Table 5: Generic one-step RT-PCR reaction components using the SuperScript[™] III One-Step RT-PCR System with Platinum[®] Taq DNA Polymerase

Reagent	Volume per reaction (µl)	
Sterile deionised H ₂ O	2.6	6.2
2 × Reaction mix (Invitrogen)	5.0	10.0
5 µM Forward primer	0.5	1.0
5 µM Reverse primer	0.5	1.0
SuperScript [™] III RT / Platinum [®] Taq Mix (Invitrogen 12574-026)	0.4	0.8
RNA template	1.0	1.0
Total	10.0	20.0

Table 6: Generic PCR cycling conditions

Step	Temperature	Time	No. of cycles
cDNA synthesis (RT-PCR only)	50°C	30 min	1
Initial denaturation	94°C	5 min	1
Denaturation	94°C	30 s	35
Annealing	See Table 3	30 s	
Elongation	72°C	45 s (viruses, viroids) 1 min (phytoplasmas, bacteria)	
Final elongation	72°C	10 min	1

Interpretation of results

The RT-PCR or PCR test will only be considered valid if:

- (a) the positive control produces the correct size product as indicated in Table 3; and
- (b) no bands are produced in the negative control (if used) and the no template control.

If the *Nad5* internal control primers are also used, then the negative control (if used), positive control and each of the test samples must produce a 181 bp band. Failure of the samples to amplify with the internal control primers suggests that the RNA extraction has failed, compounds inhibitory to PCR are present in the RNA extract or the RNA has degraded. The effect of inhibitors may be overcome by adding Bovine Serum Albumin (BSA) to the PCR reaction at a final concentration of 0.5 µg/µl.

7.1.3.2.1.1 *Apple dimple fruit viroid*

It is optional to test plants for *Apple dimple fruit viroid* by PCR using the primer pair AD-38/ADAS-36 (Table 3). See section 7.1.3.2.1 for details of test methods and interpretation of results.

7.1.3.2.1.2 *Apple scar skin viroid*

It is optional to test plants for *Apple scar skin viroid* by PCR using the primer pair AS-37/ADAS-36 (Table 3). See section 7.1.3.2.1 for details of test methods and interpretation of results.

7.1.3.2.1.3 *Pear blister canker viroid*

It is optional to test plants for *Pear blister canker viroid* by PCR using the primer pair C166/H167 (Table 3). See section 7.1.3.2.1 for details of test methods and interpretation of results.

7.1.3.2.1.4 *Pear latent virus*

It is optional to test plants for *Pear latent virus* by PCR using the primer pair Gra1 F1/Gra1 R1 (Table 3). Please note that as a suitable positive control is not commercially available for *Pear latent virus*, it has not been possible to validate the PCR with this virus. Sequence analysis indicates that the generic *Tombusvirus* primers, Gra1 F1/Gra1 R1, will detect this virus. See section 7.1.3.2.1 for details of test methods and interpretation of results.

7.1.3.2.1.5 *Tomato bushy stunt virus*

It is optional to test plants for *Tomato bushy stunt virus* by PCR using the primer pair Gra1 F1/Gra1 R1 (Table 3). See section 7.1.3.2.1 for details of test methods and interpretation of results.

7.1.3.2.2 **Phytoplasma PCR**

Recommended method

1. Extract total DNA from leaf petioles and mid-veins, or buds and bark scrapings if no leaves are present. Successful PCR amplification can be achieved using DNA extracted by the following methods:
 - (a) DNeasy[®] Plant Mini Kit (Qiagen Cat. No. 69104); or
 - (b) a phytoplasma enrichment procedure as described by Kirkpatrick *et al.* (1987) and modified by Ahrens & Seemüller (1992). See Appendix 3 for details of this extraction method.

Alternative methods may also be used after validation.

2. Optional: Perform a PCR with the Gd1/Berg54 internal control primers (Table 3) using the components and concentrations listed in Table 7 and cycle under the conditions listed in Table 6.
3. Perform a nested PCR on the purified DNA using the universal phytoplasma primer pair, P1/P7 (Table 3), for first-stage PCR followed by the R16F2/R16R2 primer pair for the second-stage PCR (Table 3).
4. Set up the first-stage and second-stage PCR reactions using the components and concentrations listed in Table 7 and cycle under the conditions listed in Table 6. The first-stage PCR products, including the controls, are diluted 1:25 (v/v) in water and 2 µl used as template in the second-stage PCR. The following controls must be included for each set of PCR reactions:
 - (a) positive control: DNA from any phytoplasma extracted from any host tissue. If the internal control primers are not used, then the phytoplasma DNA must be mixed with healthy *Pyrus* DNA to rule out the presence of PCR inhibitors.
 - (b) no template control: water is added instead of DNA template. An additional no template control is included in the second-stage PCR.

When setting up the test initially, it is advised that a negative control (DNA extracted from healthy *Pyrus* tissue) is included.

5. Analyse the products from the first and second-stage PCRs by agarose gel electrophoresis.

Table 7: PCR reaction components for phytoplasmas and bacteria

Reagent	Volume per reaction (µl)
Sterile deionised H ₂ O	12.8
10 × PCR buffer (Invitrogen)	2.0
50 mM MgCl ₂	0.6
10 mM dNTPs	0.4
5 µM Forward primer	1.0
5 µM Reverse primer	1.0
5 U/µl Platinum [®] <i>Taq</i> DNA polymerase (Invitrogen 10966-026)	0.2
DNA template	2.0
Total	20.0

Phytoplasma positive control for PCR

DNA of Pear decline phytoplasma may be obtained from the Investigation and Diagnostic Centre and Response – Tamaki, MPI (see the Contact Point, section 8). A charge may be imposed to recover costs.

Interpretation of results

The pathogen-specific PCR test will only be considered valid if:

- (a) the positive control produces the correct size product as indicated in Table 3; and
- (b) no bands are produced in the negative control (if used) and the no template control.

If the Gd1/Berg54 internal control primers are also used, then the negative control (if used), positive control and each of the test samples must produce a 1,500 bp band. Failure of the samples to amplify with the control primers suggests that the DNA extraction has failed, compounds inhibitory to PCR are present in the DNA extract or the DNA has degraded. The effect of inhibitors may be overcome by passing the DNA through a MicroSpin™ S-300 HR column (GE Healthcare Cat. No. 27-5130-01) or by adding Bovine Serum Albumin (BSA) to the PCR reaction at a final concentration of 0.5 µg/µl.

7.1.3.2.2.1 Pear decline phytoplasma

Plants must be tested for Pear decline phytoplasma by nested-PCR using the universal primers listed in Table 3. See section 7.1.3.2.2 for details of test methods and interpretation of results.

7.1.3.2.3 Bacteria PCR

Recommended method

1. Extract total DNA from leaf petioles and mid-veins. Successful PCR amplification can be achieved using DNA extracted using the DNeasy® Plant Mini Kit (Qiagen Cat. No. 69104). An alternative method may also be used after validation.
2. Optional: Perform a PCR with the Gd1/Berg54 internal control primers (Table 3) using the components and concentrations listed in Table 7 and cycle under the conditions listed in Table 6.
3. Perform a PCR with bacteria-specific primers on the purified DNA using the components and concentrations listed in Table 7 and cycle under the conditions listed in Table 6. The following controls must be included for each set of PCR reactions:
 - (a) positive control: DNA from the appropriate bacteria. If the internal control primers are not used, then the bacteria DNA must be mixed with healthy *Pyrus* DNA to rule out the presence of PCR inhibitors.
 - (b) no template control: water is added instead of DNA template.When setting up the test initially, it is advised that a negative control (DNA extracted from healthy pear leaf tissue) is included.
4. Analyse the PCR products by agarose gel electrophoresis.

Bacteria positive controls for PCR

1. *Spiroplasma citri* lyophilised positive controls may be obtained from Agdia Incorporated, USA (<http://www.agdia.com>); Cat. No. LPC 14700.
2. DNA of *Spiroplasma citri* and *Xylella fastidiosa* (Pear leaf scorch strain) may be obtained from the Investigation and Diagnostic Centre and Response – Tamaki, MPI (see the Contact Point, section 8). A charge may be imposed to recover costs.

Interpretation of results

See section 7.1.3.2.2 for details of interpretation of results.

7.1.3.2.3.1 *Spiroplasma citri*

Plants must be tested for *Spiroplasma citri* by PCR using the primer pair P58-6f/P58-4r (Table 3). See section 7.1.3.2.3 for details of test methods and interpretation of results.

7.1.3.2.3.2 *Xylella fastidiosa*

It is optional to test plants for *Xylella fastidiosa* by PCR using the primer pair PLS-F/PLS-R (Table 3). See section 7.1.3.2.3 for details of test methods and interpretation of results. Please note that the RST31/RST33 primers, the most widely used primers for *X. fastidiosa* detection, do not detect the Pear leaf scorch strains.

7.2 Specific tests for seed for sowing

Tests are to be carried out on plants germinated from imported seeds. Samples from up to 5 plants may be bulked for testing provided that the plants were derived from the same mother plant and this is confirmed by evidence from the national plant protection organisation in the exporting country.

Seed imported for sowing must be tested for the following pathogens:

- *Apple scar skin viroid*
- *Tomato bushy stunt virus*

See Table 1B for the required or alternative tests for these pathogens and the relevant section for details of the test methods and interpretation of results.

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Appendix 1. Symptoms of significant regulated pests of *Pyrus*

1.1 Pear decline phytoplasma



Two types of symptoms are caused by Pear decline phytoplasma depending on the rootstock. Quick decline (left photo) is characterised by leaves suddenly wilting, becoming dry, and turning dark. Trees die within a few days or weeks. Slow decline (right photo) can be seen as a reduction in apical growth with pale, sparse foliage, few blossoms and little or no fruit. Infected trees die before the next spring. (Both images courtesy of J. K. Clark, University of California, USA)

1.2 *Xylella fastidiosa* (Pear leaf scorch strain)



Different degrees of scorch symptoms on pear leaves (left photo) and symptoms on the twigs of a diseased pear tree caused by Pear leaf scorch (right photo). (Both images courtesy of C. C. Su, Department of Pesticide Application, Agricultural Chemical and Toxic Substances Research Institute, Taiwan)

1.3 *Pear latent virus*



Chlorotic leaves (top photo) and premature shedding of leaves (right photo) caused by *Pear latent virus*. (Both images courtesy of C. Vovlas, Università degli Studi and Istituto di Virologia Vegetale del CNR, Sezione di Bari, Italy)



1.4 *Pear blister canker viroid*



Typical bark blister cankers induced by *Pear blister canker viroid* (isolate P2098T) approximately 3 years after inoculation to the pear indicator 'A20'. (Courtesy R. Flores, Descriptions of Plant Viruses, <http://www.dpvweb.net/dpv/showdpv.php?dpvno=365>)

Appendix 2. Symptoms of nutrient deficiencies in *Pyrus*

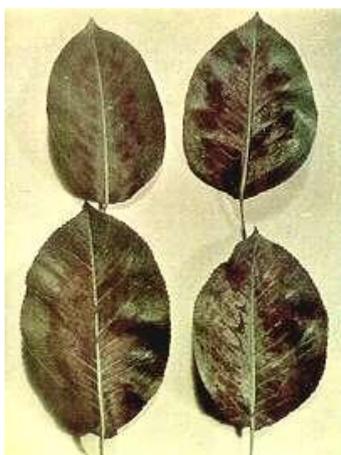
Source: Wallace T (1943) *The diagnosis of mineral deficiencies in plants by visual symptoms*. His Majesty's Stationary Office; London; 116 pp; (<http://www.luminet.net/~wenonah/min-def/>).

2.1 Nitrogen deficiency



Pear leaves showing general yellowing caused by nitrogen deficiency.

2.2 Magnesium deficiency



Central interveinal necrosis of pear leaves caused by magnesium deficiency.

2.3 Manganese deficiency



Manganese deficiency in pear showing interveinal chlorosis of the leaves that begins near the margins.

Appendix 3. Protocols referenced in the manual

3.1 Modified Qiagen RNA extraction method (MacKenzie *et al.*, 1997)

1. Grind 0.1 g leaf tissue (1/10; w/v) in extraction buffer (4 M guanidine isothiocyanate, 0.2 M sodium acetate [pH 5.0], 25 mM EDTA, 2.5% [w/v] PVP-40).
2. Transfer the homogenised extract to a microcentrifuge tube containing 100 µl of 20% (w/v) sarkosyl per ml of homogenate.
3. Incubate at 70°C for 10 minutes with intermittent shaking.
4. Then follow steps 4-11 of the protocol for “Isolation of Total RNA from Plant Cells and Tissues and Filamentous Fungi” as described in the RNeasy® Plant Mini Kit (Qiagen Cat. No. 74904) instruction handbook.

3.2 Silica-based RNA extraction method (Menzel *et al.*, 2002)

1. Grind 0.2-0.5 g leaf tissue (1/10; w/v) in extraction buffer (6 M guanidine hydrochloride, 0.2 M sodium acetate, 25 mM EDTA, 2.5% [w/v] PVP-40 adjusted to pH 5 with acetic acid).
2. Transfer 500 µl of the homogenised extract to a microcentrifuge tube containing 100 µl of 10% (w/v) SDS.
3. Incubate at 70°C for 10 minutes with intermittent shaking, and then place on ice for 5 minutes.
4. Centrifuge at 13,000 rpm for 10 minutes.
5. Transfer 300 µl supernatant to a new microcentrifuge tube and add 300 µl high salt buffer (6 M sodium iodide, 0.15 M sodium sulphite), 150 µl absolute ethanol and 25 µl silica milk (1 g/ml silicon dioxide, 1-5 µm size particles, suspended in 100 mM glycine, 100 mM NaCl, 100 mM HCl, pH 2).
6. Incubate at room temperature for 10 minutes with intermittent shaking.
7. Centrifuge at 3,000 rpm for 1 minute and discard the supernatant.
8. Resuspend the pellet in 500 µl of wash buffer (10 mM Tris-HCl pH 7.5, 0.05 mM EDTA, 50 mM NaCl, 50% [v/v] absolute ethanol), centrifuge at 3,000 rpm for 1 minute and discard the supernatant. Repeat this wash step.
9. Centrifuge at 3,000 rpm for 1 minute and remove any remaining wash buffer from the pellet.
10. Resuspend the pellet in TE buffer (10 mM Tris-HCl pH 7.5, 0.05 mM EDTA).
11. Incubate at 70°C for 4 minutes then centrifuge at 13,000 rpm for 5 minutes.
12. Transfer 100 µl of the supernatant to a sterile nuclease-free microcentrifuge tube, being careful not to disturb the pellet. Store at -80°C.

3.3 Phytoplasma enrichment extraction method (Kirkpatrick *et al.*, 1987 and modified by Ahrens & Seemüller, 1992)

1. Grind 0.3 g leaf petioles and midveins or buds and bark scrapings (1/10; w/v) in ice-cold isolation buffer (0.1 M Na₂HPO₄, 0.03 M NaH₂PO₄, 10% [w/v] sucrose, 2% [w/v] PVP-40, 10 mM EDTA, pH 7.6 to which 0.15% [w/v] bovine serum albumin and 1 mM ascorbic acid were added just before use).
2. Transfer ground sample to a cold 2 ml microcentrifuge tube and centrifuge at 4°C for 5 min at 4,500 rpm.
3. Transfer supernatant into a new 2 ml microcentrifuge tube and centrifuge at 4°C for 15 min at 13,000 rpm.
4. Discard the supernatant.

5. Resuspend the pellet in 750 μ l hot (55°C) CTAB buffer (2% [w/v] CTAB, 100 mM Tris-HCl [pH 8.0], 20 mM EDTA [pH 8.0], 1.4 M NaCl, 1% [w/v] PVP-40). It is easier to resuspend the pellet by first adding a small volume of CTAB buffer (e.g. 100 μ l) then the remaining volume of CTAB buffer is added once the pellet has been resuspended.
6. Incubate at 55°C for 30 min with intermittent shaking then cool on ice for 30 seconds.
7. Add 750 μ l chloroform:octanol (24:1 v/v), vortex thoroughly and centrifuge at 4°C or at room temperature for 4 min at 13,000 rpm.
8. Carefully remove upper aqueous layer into a new 1.5 ml microcentrifuge tube.
9. Add 1 volume ice-cold isopropanol, vortex thoroughly and incubate on ice for 4 min. Centrifuge at 4°C or at room temperature for 10 min at 13,000 rpm. Discard supernatant.
10. Wash DNA pellet with 500 μ l ice-cold 70% (v/v) ethanol, centrifuge at 4°C or at room temperature for 10 min at 13,000 rpm.
11. Dry DNA pellet in a DNA concentrator or air-dry.
12. Resuspend in 20 μ l sterile distilled water. Incubating the tubes at 55°C for 10 min can aid DNA resuspension.
13. Store DNA at -20°C for short term storage or -80°C for long term storage.