



# *Fragaria* (Strawberry)

## Post-Entry Quarantine Testing Manual



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# *Fragaria* Post-Entry Quarantine Testing Manual

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## 1. SCOPE

The scope of this manual is limited to nursery stock (cuttings [runner tips, stem cuttings] and plants in tissue culture only), seed for sowing and pollen of *Fragaria* species permitted entry into New Zealand as listed in the Ministry of Agriculture and Forestry's (MAF) 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:

*Fragaria chiloensis*  
*Fragaria moschata*  
*Fragaria vesca*  
*Fragaria virginiana*  
*Fragaria* × *ananassa*

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 strawberry is a member of the Rosaceae family and belongs to the genus *Fragaria* (derived from the Latin, *fragare*, meaning pleasant aroma). While there are over 20 named species, most cultivated strawberries have originated from six main species from Europe, North America and Chile. The main *Fragaria* species include *vesca* (with 4 subspecies), *viridis*, *moschata*, *virginiana* (4 subspecies) and *chiloensis* (4 subspecies). Most cultivated strawberries are descendents of *F. chiloensis* and *F. virginiana* (*F.* × *ananassa*) and are octoploids ( $2n=56$ ). *F. vesca*, the woodland strawberry used as a virus indicator, is diploid ( $2n=14$ ). Tetraploids and hexaploids are also known (Staudt, 1962).

Up until the late 1700s, most strawberries were selections of individual species with fruit around 10 mm or smaller in diameter. By 1759, hybridisation between *F. chiloensis* and *F. virginiana* resulted in increased fruit size and quality. Most modern strawberries are categorised as *Fragaria* × *ananassa*, this name reflecting the mix of multiple species.

The strawberry is a perennial herb. The crown of the plant is a very short stem, with leaves and axillary buds borne in a restricted area of the apex. Leaves are usually trifoliate but vary in number from 1 to 5. Buds formed in the axils of the leaves may develop into branch crowns (leafy shoots) or stolons (runners). The distal end of the runner normally develops into a runner plant, or daughter plant, with roots on the underside and leaves at the tip. The strawberry is not a true fruit. It is actually an enlarged fleshy receptacle upon which many hard single-seeded achenes are borne. The size of the fruit is a reflection of the number of achenes (Dana, 1980).

Strawberries are probably the most popular berryfruit grown worldwide. They are mostly eaten fresh but there is a substantial international trade in frozen fruit, with the fruit used in a wide range of products including jams, dairy food and bakery products. Strawberries are cultivated in most places throughout the world from the Arctic Circle to the tropics. World production is estimated at 2.8 million tonnes per year (<http://www.fas.usda.gov/htp/Hort.circular/2006/03-06%20strawberry%20article.pdf>). The USA, China and Spain are the biggest producers worldwide, with New Zealand producing very small quantities in world terms, about 6000 tonnes per year (Strawberry Growers NZ estimates). This is grown on about 200 ha producing about 30 t/ha. The

major production sites are based in the Auckland and Waikato regions but there are strawberry growers located in most areas of New Zealand.

There has been recent interest in the tetraploid musk strawberry *F. moschata*, reported to be the most aromatic strawberry of all. Known as ‘hautbois’ in France, and ‘hautboy’ in England, the musk strawberry has mottled brownish-red or rose-violet skin and tender white flesh. The fruit has a spicy floral aroma and the flavour is complex with hints of honey, musk and wine (<http://www.smithsonianmagazine.com/issues/2006/july/strawberry.php>). There are also decorative types grown for their ornamental value as well as ground cover types. Pink-flowered ornamental types grown from seed with edible fruit are also now available (Bentvelsen & Bouw, 2006). *Duchesnea indica* (formerly *Fragaria indica*) is a species with golden-yellow flowers and round, red insipid fruit with hard seeds. This species is seen in New Zealand gardens where it is used as a ground cover but is also considered a weed by some gardeners.

There are two main strawberry types cultivated for fruit production in New Zealand, short-day (June-bearing) and day-neutral (ever-bearing). The terms relate to the conditions required for flower initiation, with short-day types initiating flowers during periods of short days (autumn to spring), while day-neutrals initiate flowers at any time when temperatures allow growth.

Up until 2006, most cultivars present in New Zealand came from the University of California breeding programme. ‘Camarosa’ was the most popular with ‘Gaviota’, ‘Pajaro’ and ‘Ventana’, also important. These are all short-day types. In the South Island, day-neutral types dominate with ‘Seascape’, ‘Aromas’ and ‘Sunset’ being the most popular.

There is no breeding programme currently underway in New Zealand. Importation is primarily targeted at potential cultivars for direct commercial use. Characteristics of importance are flavour, shelf life, fruit size, pest and disease resistance, weather tolerance, and yield.

### 3. IMPORT REQUIREMENTS

The import requirements for nursery stock (cuttings and plants in tissue culture) and pollen of *Fragaria* are set out in MAF’s import health standard “Importation of Nursery Stock” (see: <http://www.biosecurity.govt.nz/files/ihs/155-02-06.pdf>). Imported cuttings and plants in tissue culture must meet the general requirements (sections 1-3) and the additional specific requirements detailed in the “*Fragaria*” schedule. 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, nursery stock must be dipped in 1% sodium hypochlorite for 2 minutes at an approved treatment facility (cuttings only). All material from non-accredited facilities must be grown for a minimum period of 16 months in a Level 3 post-entry quarantine facility where it will be inspected, treated and/or tested for regulated pests.

The import requirements for *Fragaria* seed for sowing are set out in MAF’s import health standard “Importation of Seed for Sowing” (see: <http://www.biosecurity.govt.nz/files/ihs/155-02-05.pdf>). Imported seed must meet the general requirements (sections 1-2) and the specific requirements detailed in the “*Fragaria*” 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 to be free of any visually detectable regulated pests. On arrival in New Zealand, the seed must be grown for a minimum period of 1 growing season in a Level 3 post-entry quarantine facility where it will be inspected, treated and/or tested for regulated pests.

The import requirements for pollen are stated in section 2.2.3 in MAF's import health standard "Importation of Nursery Stock" (see: <http://www.biosecurity.govt.nz/files/ihs/155-02-06.pdf>) and further details can be found in section 7.3 of this manual.

## 4. PESTS

The following section lists regulated pests of *Fragaria* nursery stock, seed for sowing and pollen that require generic or specific measures. A complete list of regulated pests of *Fragaria* can be found in the nursery stock and seed for sowing import health standards (see section 3).

### 4.1 Regulated pests for which generic measures are required

#### Insects, nematodes & fungi:

Refer to import health standard.

#### Bacteria or bacteria-like organisms:

<i>Ralstonia solanacearum</i> (Race 2)	[Nursery stock only]
Strawberry rickettsia yellows	[Nursery stock only]

### 4.2 Regulated pests for which specific tests are required

#### Mites:

<i>Diptacus fragarifoliae</i>	[Plants in tissue culture (Nursery stock) only]
<i>Tetranychus kanzawai</i>	[Plants in tissue culture (Nursery stock) only]
<i>Tetranychus lobustus</i>	[Plants in tissue culture (Nursery stock) only]
<i>Tetranychus neocalendonicus</i>	[Plants in tissue culture (Nursery stock) only]
<i>Tetranychus pacificus</i>	[Plants in tissue culture (Nursery stock) only]

#### Bacteria or bacteria-like organisms:

" <i>Candidatus</i> <i>Phlomobacter fragariae</i> " (Strawberry marginal chlorosis)	[Nursery stock only]	Figure 1.1
<i>Xanthomonas arboricola</i> pv. <i>fragariae</i>	[Nursery stock only]	Figure 1.2
<i>Xanthomonas fragariae</i>	[Nursery stock only]	Figure 1.3
<i>Xylella fastidiosa</i> ( <i>Fragaria vesca</i> only)	[Nursery stock only]	

#### Phytoplasmas:

Aster yellows phytoplasma	[Nursery stock only]	Figure 1.4
Clover phyllody phytoplasma	[Nursery stock only]	
Clover proliferation phytoplasma	[Nursery stock only]	
Clover yellow edge phytoplasma	[Nursery stock only]	
Stolbur phytoplasma	[Nursery stock only]	
STRAWB1 phytoplasma	[Nursery stock only]	
STRAWB2 phytoplasma	[Nursery stock only]	
Strawberry green petal phytoplasma	[Nursery stock only]	Figure 1.5
Strawberry leafy fruit phytoplasma	[Nursery stock only]	
Strawberry multicipita phytoplasma	[Nursery stock only]	
Strawberry multiplier phytoplasma	[Nursery stock only]	Figure 1.6
Strawberry phylloid fruit phytoplasma	[Nursery stock only]	
Strawberry yellows phytoplasma	[Nursery stock only]	Figure 1.7

## Viruses:

<i>Fragaria chiloensis latent virus</i>	[Nursery stock, seed for sowing & pollen]	
<i>Raspberry ringspot virus</i>	[Nursery stock & seed for sowing]	
<i>Strawberry chlorotic fleck virus</i>	[Nursery stock only]	
<i>Strawberry latent C virus</i>	[Nursery stock only]	Figure 1.8
<i>Strawberry latent ringspot virus</i> (strains not in New Zealand)	[Nursery stock & seed for sowing]	
<i>Strawberry mild yellow edge-associated virus</i>	[Nursery stock only]	Figure 1.9
<i>Strawberry pallidosis-associated virus</i>	[Nursery stock only]	Figure 1.10
<i>Strawberry pseudo mild yellow edge virus</i>	[Nursery stock only]	Figure 1.11
<i>Strawberry vein banding virus</i>	[Nursery stock only]	Figure 1.12
<i>Tobacco necrosis virus</i> (strains not in New Zealand)	[Nursery stock only]	
<i>Tobacco rattle virus</i> (strains not in New Zealand)	[Seed for sowing only]	
<i>Tobacco streak virus</i> (strains not in New Zealand)	[Nursery stock only]	
<i>Tomato black ring virus</i>	[Nursery stock, seed for sowing & pollen]	
<i>Tomato bushy stunt virus</i>	[Nursery stock only]	
<i>Tomato ringspot virus</i> (strains not in New Zealand)	[Nursery stock, seed for sowing & pollen]	Figure 1.13

## Diseases of unknown aetiology:

Strawberry feather leaf disease	[Nursery stock only]	
Strawberry lethal decline disease	[Nursery stock only]	Figure 1.14

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

Plants must be maintained in a healthy, vigorous state. *Fragaria* plants in quarantine may exhibit symptoms associated with abiotic stresses such as water and nutrient deficiencies. Potting up the plants to allow large root volumes and foliar feeding can help avoid these problems, as can regular re-potting into fresh media. Symptoms of the most common nutrient deficiencies of *Fragaria* are illustrated in Appendix 2. Further illustrations of nutrient deficiencies can be found on the American Phytopathological Society's CD-ROMs 'Diseases of Small Fruits' and 'Nutrient Deficiencies and Toxicities of Plants' ([www.aps.org](http://www.aps.org)).

### 5.1 Nursery stock

*Fragaria* nursery stock may be imported as cuttings or as plants in tissue culture.

It is important that pruning and cutting tools used on the imported plants are disinfected between each plant. Alternatively, disposable razor blades may be used.

#### 5.1.1 Cuttings

It is best to import *Fragaria* cuttings between May and June from the northern hemisphere, and from August to October from the southern hemisphere. All imported cutting material must be free of fruit, foliage, roots or soil to minimise the introduction of disease.

Runner tips (unrooted runners) and stem cuttings are planted into 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<sup>®</sup>) and placed under mist at 16-20°C until roots develop. Roots form quickly and weaning from the mist can start after 2-3 weeks, depending on light, temperature and the initial size of the plantlets.

### 5.1.2 Plants in tissue culture

Tissue culture is commonly used for the distribution of new cultivars worldwide and is also used commercially for producing parent-stock plants of poor runner producers, especially some of the day-neutral types. It is also a useful way of overcoming the seasonal differences between hemispheres. Tissue-cultured plants are able to be managed at virtually any time of year, although New Zealand autumn and early winter times are more difficult and best avoided. Tissue-cultured stock is naturally vegetative and will produce runners as soon as the plants are large enough (about 6 weeks after establishment).

Carefully remove plants from the tissue culture container, wash the plantlets to remove any remaining agar and transplant into an artificial media, such as pumice, perlite or vermiculite. Water gently and then place under a mist or fog system, or keep covered in plastic for about 3 weeks while plants establish. Gradually expose the plants to normal glasshouse conditions. Bottom heat of around 20°C will speed up root development. Once plants have become established, they should be re-potted into 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<sup>®</sup>).

Any remaining tissue culture plantlets that are not deflasked remain as back up in a Level 3 quarantine tissue culture laboratory and 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

Seed is used for propagation of types that breed true from seed such as some forms of *F. vesca*. More recently, seed lines have been developed for ornamental use (Bentvelsen & Bouw, 2006) where plants of consistent shape and appearance can be produced. Plants raised from seed of hybrid types will usually show considerable variability when propagated by seed. For propagation of some types, scarification of the seed is necessary to simulate the process of passing through a bird's digestive system. Scarification is achieved by soaking the seed in concentrated H<sub>2</sub>SO<sub>4</sub> for 12 minutes. The seed and acid mix are then poured into a large volume of ice water (never add water to the acid), to minimise heat generation and to dilute the acid. After thoroughly rinsing the seeds with water, they are allowed to dry on blotting paper and then immediately sown on top of a peat/sand potting mix. Germination under mist will take about 3 days and can be removed from the mist after about 10 days. Seedlings can be pricked out when seedlings have a forked root system.

### 5.3 Pollen

Pollen can be stored for up to 20 months at -18°C (Aslantas & Pirlak, 2002). As strawberry plants can be easily manipulated to flower at any time of the year, long-term storage is not normally required and 2 months storage at 4°C is usually effective.

Pollen should be collected, and air-dried before being sealed in small, clean airtight containers. Care should be taken to avoid contamination with other flower parts. Allow containers to warm to room temperature before use.

Imported pollen is used to fertilise mother plants of New Zealand origin held in a Level 3 post-entry quarantine facility. The resulting seed is sown as described in section 5.2 and tested for the pathogens of concern described in section 7.3.

#### **5.4 Propagation of established plants**

In order to promote runner production and to increase the virus titre of plants imported as tissue culture, strawberry plants must go through a period of dormancy. Days shorter than 14 hours and temperatures below 7°C will induce dormancy in strawberry plants. Plants are exposed to these conditions for about 4 weeks after which they are trimmed down to a few leaves and then stored in a cold room at 3-4°C for 8-10 weeks. Plants that are poor runner producers and day-neutral types in particular will often need extra chilling to achieve runner production.

Once the chilling requirement has been met, plants will initially flower and once day-lengths no longer favour flower initiation (>10 h), plants will produce runners. This can be induced earlier by providing chilling in excess of that required for fruiting and initial growth. Most types of strawberry produce runners. Daughter plantlets develop at the second node on the runner from the parent. Primary root initials (peg roots) develop from their base once the first trifoliate leaf has emerged. Additional primary roots arise once the first roots have anchored the plant. Once branching of these roots has occurred, about two weeks after the establishment of the initial anchor roots, the plant is usually capable of independent growth. Once a plant is in runner mode (growing vigorously and producing runners), it will continue to produce more plants as will subsequent daughters, with one parent plant capable of producing up to 400 daughters in a single eight-month growing season.

Plantlets will establish well once separated from mothers if placed under mist at 16-20°C once plantlets have 1-3 compound leaves and have developed root initials (Crawford *et al.*, 2001). Roots form quickly and weaning from the mist can start after 2-3 weeks, depending on light, temperature and the initial size of the plantlets.

Alternatively, plants can be trained directly into separate pots or into bins containing standard-type potting mixes. Plants will fill a 0.45 L pot with roots in about 2 months, and will grow satisfactorily in a pot this size for up to 4-6 months. A 450 mm × 300 mm × 100 mm tray will support up to 20 plants for 4-6 months. Optimum conditions for growth are diurnal temperatures of 12°C and 25°C (Wang & Camp, 2000).

Non-runnering forms are propagated by crown division. Plants that have multiple crowns can be split lengthwise ensuring that a section of roots adheres to each division. Each division is then potted into a suitably sized container. Crown sections without roots will also sometimes take root when propagated under mist.

## **6. INSPECTION**

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

Photographs of symptoms caused by some of the significant regulated pests can be found in Appendix 1. It is important to be aware that pot-grown strawberry plants can be prone to nutrient deficiencies if not adequately fertilised and nutrient deficiencies can resemble virus infection, e.g.,

chlorosis and necrosis. Photographs of the common nutrient deficiencies of *Fragaria* can be found in Appendix 2.

## 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. This testing is required to detect latent infections.

For each *Fragaria* plant, at least two young fully-expanded leaves must be sampled from the apical crown region. 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.

Graft and herbaceous indexing must be done in the early spring using young, vigorous indicator plants for graft assays. Laboratory tests for viruses (ELISA and RT-PCR) must be carried out in the spring using the new flush of spring growth. Laboratory tests for phytoplasmas and bacteria (PCR) must be carried out at the end of the summer. Tissue culture plantlets must go through a period of dormancy before virus testing to increase the virus titre. For plants that have had dormancy induced out-of-season, testing can be performed at times that are equivalent to spring and summer.

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

**A. Nursery stock**

Organism type	Graft indexing	Herbaceous indexing	ELISA	PCR	Microscope inspection
<b>Mites (Plants in tissue culture only)</b>					
<i>Diptacus fragarifoliae</i>					■
<i>Tetranychus kanzawai</i>					■
<i>Tetranychus lobustus</i>					■
<i>Tetranychus neocalendonicus</i>					■
<i>Tetranychus pacificus</i>					■
<b>Bacteria or bacteria-like organisms</b>					
“ <i>Candidatus</i> Phlomobacter fragariae” (Strawberry marginal chlorosis)				■	
<i>Xanthomonas arboricola</i> pv. <i>fragariae</i>				■ <sup>3</sup>	
<i>Xanthomonas fragariae</i>				■ <sup>4</sup>	
<i>Xylella fastidiosa</i> <sup>1</sup>				■ <sup>4</sup>	
<b>Phytoplasmas</b>					
Aster yellows phytoplasma				■	
Clover phyllody phytoplasma				■	
Clover proliferation phytoplasma				■	
Clover yellow edge phytoplasma				■	
Stolbur phytoplasma				■	
STRAWB1 phytoplasma				■	
STRAWB2 phytoplasma				■	
Strawberry green petal phytoplasma				■	
Strawberry leafy fruit phytoplasma				■	
Strawberry multicipita phytoplasma				■	
Strawberry multiplier phytoplasma				■	
Strawberry phylloid fruit phytoplasma				■	
Strawberry yellows phytoplasma				■	
<b>Viruses</b>					
<i>Fragaria chiloensis</i> latent virus		■			
<i>Raspberry ringspot virus</i>		■	□	□	
<i>Strawberry chlorotic fleck virus</i>	■				
<i>Strawberry latent C virus</i>	■				
<i>Strawberry latent ringspot virus</i> <sup>2</sup>		■	□	□	
<i>Strawberry mild yellow edge-associated virus</i>	■				
<i>Strawberry pallidosis-associated virus</i>	■				
<i>Strawberry pseudo mild yellow edge virus</i>	■				
<i>Strawberry vein banding virus</i>	■			■	
<i>Tobacco necrosis virus</i> <sup>2</sup>		■	■		
<i>Tobacco streak virus</i> <sup>2</sup>		■			
<i>Tomato black ring virus</i>		■	□	□ <sup>3</sup>	
<i>Tomato bushy stunt virus</i>		■			
<i>Tomato ringspot virus</i> <sup>2</sup>		■	□	□	
<b>Diseases of unknown aetiology</b>					
Strawberry feather leaf disease	■				
Strawberry lethal decline disease	■				

<sup>1</sup> PCR testing is only required for *Fragaria vesca*.

<sup>2</sup> Strains not in New Zealand.

<sup>3</sup> Real-time PCR only.

<sup>4</sup> Real-time and conventional PCRs are available.

## B. Seed for sowing

Organism type	Herbaceous indexing	ELISA	PCR
<b>Virus</b>			
<i>Fragaria chiloensis latent virus</i>	■		
<i>Raspberry ringspot virus</i>	■	□	□
<i>Strawberry latent ringspot virus</i> <sup>1</sup>	■	□	□
<i>Tobacco streak virus</i>	■		
<i>Tomato black ring virus</i>	■	■	
<i>Tomato ringspot virus</i> <sup>1</sup>	■	□	□

<sup>1</sup>Strains not in New Zealand.

### 7.1 Specific tests for nursery stock

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

- the plants are derived from a single imported cutting which was split into separate cuttings upon arrival in New Zealand, in accordance with the requirements of the permit to import; or
- in the case of tissue culture where clonal plants are derived from the same mother plant, and this is confirmed by evidence from the national plant protection organisation in the exporting country

#### 7.1.1 Graft indexing

Each *Fragaria* plant must be tested by leaf-grafting onto two replicate indicator cultivars as listed in Table 2. The minimum number of indicators can be achieved by using the following cultivars: *Fragaria vesca* 'EMB' or 'EMK', 'UC-5', 'Alpine' and *Fragaria virginiana* 'UC-10' or 'UC-11'.

**Table 2: Recommended *Fragaria* indicator cultivars for graft indexing**

Target organism	<i>Fragaria</i> indicator cultivar
<b>Viruses</b>	
<i>Strawberry chlorotic fleck virus</i>	<i>Fragaria vesca</i> 'EMB' or 'EMK'
<i>Strawberry latent C virus</i>	<i>Fragaria vesca</i> 'EMC' or 'UC-5'
<i>Strawberry mild yellow edge-associated virus</i>	Two of the following indicators: <i>Fragaria vesca</i> 'UC-4' or 'UC-5' or 'Alpine'
<i>Strawberry pallidosis-associated virus</i>	<i>Fragaria virginiana</i> 'UC-10' or 'UC-11'
<i>Strawberry pseudo mild yellow edge virus</i>	<i>Fragaria vesca</i> 'UC-4' or 'Alpine' or <i>Fragaria virginiana</i> 'UC-12'
<i>Strawberry vein banding virus</i>	Two of the following indicators: <i>Fragaria vesca</i> 'UC-5' or 'UC-6' or 'Alpine' or <i>Fragaria virginiana</i> 'UC-12'
<b>Diseases of unknown aetiology</b>	
Strawberry feather leaf disease	<i>Fragaria vesca</i> 'UC-1' or 'UC-4' or 'Alpine'
Strawberry lethal decline disease	<i>Fragaria vesca</i> 'Alpine'

It is best to grow the indicator plants from young cuttings as seeds of most strawberry cultivars do not breed true to type. Non-runnering forms such as *Fragaria vesca* 'Alpine' will need to be propagated from seed or by crown division. See section 5 for details of these propagation methods. The indicator plants are ready for grafting when they have two or more fully expanded leaves. The indicator plants must be maintained in a vigorous state of growth before and after grafting and must be grown under moderate temperatures and light intensities.

To avoid cross-contamination of plants during the grafting process, use a sterile scalpel for each *Fragaria* plant to be tested.

### **Recommended method**

1. Prune the indicator plants to two young actively growing trifoliate leaves.
2. Using a scalpel blade, remove the centre leaflet of each leaf and make a 1-2 cm vertical split down the middle of the petiole.
3. Remove young actively growing trifoliate leaves from the imported plant to be tested and trim away the two outside leaflets, leaving only the centre leaflet and the petiole. Cut the petiole into a wedge shape, trim away about half of the leaflet blade and insert in the split of the indicator plant petiole. Bind the graft firmly with self-adhesive medical tape (e.g. stericrepe) or similar.
4. A single plant of each indicator cultivar must be left ungrafted as a negative control each day that grafting is performed. These plants must be subjected to the same horticultural practices and environmental conditions as the inoculated plants.
5. Optional: Each indicator cultivar may be graft-inoculated with a positive control. The positive control is a non-regulated virus of *Fragaria*.
6. Hold the grafted plants in a mist bed or create a humid atmosphere by covering with a plastic bag or tub for about 1-2 weeks until a graft union has formed.
7. Approximately two weeks after grafting, check that the graft union has successfully healed and that the inoculum is still alive. All graft inoculations must have survived; the graft inoculation must be repeated if the inoculum is dead.
8. Examine grafted plants regularly for symptoms over a 3 month period.

### **Interpretation of results**

The graft 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 (non-regulated virus) was used, then the correct symptoms must be produced on the indicator cultivar.

The symptoms produced by each of the regulated pathogens on the *Fragaria* indicator cultivars are described below.

#### *Strawberry chlorotic fleck virus:*

- ‘EMB’ and ‘EMK’ – distortion and down-curling of the young leaves. Vein clearing followed by the appearance of small chlorotic spots is sometimes evident.

#### *Strawberry feather leaf disease:*

- ‘Alpine’, ‘UC-1’ and ‘UC-4’ – feather-leaf symptoms including dwarfing, leaves narrowed, strap-like, somewhat rugose, with deeply serrated margins, and leaflets that may be fused at the base. Other symptoms include vein clearing or fasciation and translucent spots on young leaves.

#### *Strawberry latent C virus:*

- ‘EMC’ and ‘UC-5’ – severe epinasty of young leaflets followed by moderate to severe dwarfing without epinasty, mottling or distortion (Figure 1.8).

#### *Strawberry lethal decline disease:*

- ‘Alpine’ – development of bronzed, wilted leaves.

#### *Strawberry mild yellow edge-associated virus:*

- ‘Alpine’, ‘UC-4’ and ‘UC-5’ – leaflets cupped, chlorotic margins and vigour reduced.

*Strawberry pallidosis-associated virus:*

- UC-10 and UC-11 – small chlorotic leaves; runners may be shortened; severe strains can be lethal (Figure 1.9).

*Strawberry pseudo mild yellow edge virus:*

- ‘Alpine’ and ‘UC-4’ – mottled discolouration (yellow to red) followed by premature necrosis (Figure 1.10).
- ‘UC-12’ – yellow to reddish colouration with necrotic areas in older leaves.

*Strawberry vein banding virus:*

- All *F. vesca* and *F. virginiana* indicators – vein banding, leaf curl or necrosis depending on the virus isolate (Figure 1.11). Vein banding symptoms, seen as chlorotic banding along the primary and secondary veins, are most intense in the first few leaves that develop after grafting. Leaves that develop later may show discontinuous streaks or mild chlorosis along the veins, or no symptoms. Symptoms of necrosis may develop on mature leaves. The net veins may become necrotic, followed by necrosis of the interveinal tissues.

### 7.1.2 Herbaceous indexing

Each *Fragaria* plant must be tested by mechanical inoculation on two indicator plants each of *Chenopodium quinoa* and *Cucumis sativus*.

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 of the leaves being fully expanded. *C. sativus* seedlings should have expanded cotyledons but the first true leaves should be no bigger than 1-2 mm.

#### Recommended method

1. Place indicator plants in dark for 24 hours prior to inoculation to increase susceptibility. Ensure that the *C. sativus* seedlings are dry prior to dark treatment or mildew will develop after inoculation.
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 (Na<sub>2</sub>SO<sub>3</sub>). A negative (inoculation buffer only) and a positive control must be included in each batch of inoculations. The positive control is a non-regulated virus which is moderately transmissible and produces clear symptoms on the herbaceous indicators, e.g. *Arabidopsis mosaic virus*. 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 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.

## 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.
  - *C. sativus* – local lesions, systemic chlorosis.

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

*Fragaria chiloensis latent virus*:

- *C. quinoa* and *C. sativus* – systemic mottle, chlorotic patches and stunting.

*Raspberry ringspot virus*:

- *C. quinoa* – chlorotic or necrotic local lesions, systemic chlorotic mottle or apical necrosis.

*Strawberry latent ringspot virus*:

- *C. quinoa* – chlorotic or necrotic local lesions, systemic chlorosis and deformation, necrosis or faint chlorotic mottle.
- *C. sativus* – chlorotic local lesions or none, systemic interveinal chlorosis or necrosis, recovery in summer, in winter symptoms may persist. Some isolates induce enations.

*Tobacco necrosis virus*:

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

*Tobacco rattle virus*:

- *C. quinoa* and *C. sativus* – necrotic and chlorotic local lesions; no systemic infection.

*Tobacco streak virus*:

- *C. quinoa* – necrotic local lesions, systemic necrosis.
- *C. sativus* – chlorotic local lesions.

*Tomato black ring virus*:

- *C. quinoa* – necrotic local lesions, systemic chlorotic mottling, necrosis.
- *C. sativus* – necrotic local lesions, systemic mottling, necrosis.

*Tomato bushy stunt virus*:

- *C. quinoa* – chlorotic local lesions, rarely systemic.

*Tomato ringspot virus*:

- *C. quinoa* – chlorotic local lesions on inoculated leaves followed by systemic apical necrosis.
- *C. sativus* – yellow local lesions on inoculated cotyledons followed by necrosis and withering of cotyledons, mosaic, distortion, necrosis, and stunting of the leaves.

### 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 MAF Investigation and Diagnostic Centre – Tamaki (see the Contact Point, section 8) (available as freeze-dried leaf material). A charge may be imposed to recover costs.

### 7.1.3 Serological and molecular assays

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

- *Tobacco necrosis virus*

ELISA **OR** PCR **MUST** be carried out for the following viruses:

- *Raspberry ringspot virus*
- *Strawberry latent ringspot virus*
- *Tomato black ring virus*
- *Tomato ringspot virus*

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

- “*Candidatus Phlomobacter fragariae*”
- *Xanthomonas arboricola* pv. *fragariae*
- *Xanthomonas fragariae*
- *Xylella fastidiosa* (*Fragaria vesca* only)
- Phytoplasmas
- *Strawberry vein banding virus*

#### 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 3); and
  - (b) negative control: *Fragaria* 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.

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>).
- Neogen Europe Ltd (<http://www.neogeneurope.com>).

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

**Table 3: Source of antisera and positive controls for ELISA**

<b>Virus</b>	<b>Antisera<sup>1</sup></b>	<b>Positive control<sup>2</sup></b>
<i>Raspberry ringspot virus</i>	Loewe Cat. No. 07141C	Loewe Cat. No. 07144PC
<i>Strawberry latent ringspot virus</i>	Agdia Cat. No. SRA 14000 Loewe Cat. No. 07115C	Agdia Cat. No. LMC 14000 Loewe Cat. No. 07115PC
<i>Tobacco necrosis virus</i>	Bioreba Cat. No. 152465 Loewe Cat. No. 07036C	Bioreba Cat. No. 152453 Loewe Cat. No. 07046PC
<i>Tomato black ring virus</i>	Neogen Cat. No. 1068-09 (Identikit96)	Neogen Cat. No. 1068-11
<i>Tomato ringspot virus</i>	Agdia Cat. No. SRA 22000	Agdia Cat. No. LPC 22000

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

<sup>2</sup>The positive control is included if the complete reagent set for antisera is purchased.

### 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, phytoplasmas and bacteria of *Fragaria* are listed in Table 4, 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 most prokaryotes as well as from chloroplasts. The COX primers amplify the constitutive cytochrome oxidase I gene found in plant mitochondria.

Please note that the primers listed in Table 4 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 MAF Investigation and Diagnostic Centre – Tamaki. Alternative reagents may give similar results but will require validation. Bovine Serum Albumin (BSA) is included in the PCR reactions to overcome inhibitors that are present in *Fragaria* tissue.

**Table 4: PCR primers used for the detection of regulated pests of *Fragaria* and plant internal controls**

Target organism	Primer name	Sequence (5'-3')	Tm (°C)	Size (bp)	Reference
<b>Viruses</b>					
<i>Raspberry ringspot virus</i>	RpRSV-F1	TGTGTCTGGCTTTTGATGCT	61	385	Ochoa-Corona <i>et al.</i> , 2005
	RpRSV-R1	GAGTGCATAGGGGCTGTT			
<i>Strawberry latent ringspot virus</i>	SLRSV-F	CCTCTCCAACCTGCTAGACT	55	497	Postman <i>et al.</i> , 2004
	SLRSV-R	AAGCGCATGAAGGTGTAAC			
<i>Strawberry vein banding virus</i> <sup>1</sup>	SVBVdeta	AGTAAGACTGTTGGTAATGCCA	55	422	Thompson <i>et al.</i> , 2004
	SVBVdetb	TTTCTCCATGTAGGCTTTGA			
<i>Tomato black ring virus</i>	TBRV-70F	GCTCGTAACAGTTGCGGAGATAT	62	72	Harper <i>et al.</i> , 2011
	TBRV-70R	TGTCCACACTGTCATGGGA			
	TBRV-70P <sup>2</sup>	FAM-TGCATAGGCTCACTCCTTGGGA-NFQ <sup>3</sup>			
<i>Tomato ringspot virus</i>	U1	GACGAAGTTATCAATGGCAGC	55	449	Griesbach, 1995
	D1	TCCGTCCAATCACGGAATA			
<b>Phytoplasmas</b>					
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			
<b>Bacteria</b>					
“ <i>Candidatus</i> Phlomobacter fragariae”	Fra4b	CTCCTCTATCTCTAAAGG	52	550	Danet <i>et al.</i> , 2003
	Fra5b	AGCAATTGACRTTAGCGA			
<i>Xanthomonas arboricola</i> pv. <i>fragariae</i>	Xaf pep-F	GCGTGCCGCAGCCGC	60	64	Weller <i>et al.</i> , 2007
	Xaf pep-R	CCGGTGGGCTTGGCGCCG			
	Xaf pep-P <sup>2</sup>	FAM-CCGGAAACCGGCAAGAAGGCA-NFQ <sup>3</sup>			
<i>Xanthomonas fragariae</i>	Xf gyrB-F	CCGCAGCGACGCTGATC	60	129	Weller <i>et al.</i> , 2007
	Xf gyrB-R	ACGCCATTGGCAACACTTGA			
	Xf gyrB-P <sup>2</sup>	FAM-TCCGCAGGCACATGGGCGAAGAATTC-NFQ <sup>3</sup>			
	245A	CGCGTGCCAGTGGAGATCC	68	300	Pooler <i>et al.</i> , 1996
	245B	CGCGTGCCAGAAGTAGCAG			
<i>Xylella fastidiosa</i>	RST31	GCGTTAATTTTCGAAGTGATTTCGA	55	733	Minsavage <i>et al.</i> , 1994
	RST33	CACCATTTCGTATCCCGGTG			
	XF-F	CACGGCTGGTAACGGAAGA	62	70	Harper <i>et al.</i> , 2010
	XF-R	GGGTTGCGTGGTGAAATCAAG			
	XF-P <sup>2</sup>	FAM-TCGCATCCCGTGGCTCAGCC-NFQ <sup>3</sup>			
<b>Internal control</b>					
Plant RNA control	Nad5-F	GATGCTTCTTGGGGCTTCTTGTT	50-60	181	Menzel <i>et al.</i> , 2002
	Nad5-R	CTCCAGTCACCAACATTGGCATAA			
Plant DNA control	Gd1	ACGGAGAGTTTGATCCTG	50-62	1,500	Andersen <i>et al.</i> , 1998
	Berg54	AAAGGAGGTGATCCAGCCGCACCTTC			
Plant DNA control	COX-F	CGTCGCATTCCAGATTATCCA	60	74	Weller <i>et al.</i> , 2000
	COX-R	CAACTACGGATATATAAGAGCCAAAAGT			
	COX-P <sup>2</sup>	FAM-TGCTTACGCTGGATGGAATGCCCT-NFQ <sup>3</sup>			

<sup>1</sup>*Strawberry vein banding virus* is a dsDNA *Caulimovirus*.

<sup>2</sup>Real-time probe.

<sup>3</sup>NFQ = Non-fluorescent quencher.

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

#### Recommended method for RNA viruses: Conventional RT-PCR

1. Extract total RNA from leaf tissue according to a standard protocol. Successful RT-PCR amplification can be achieved using RNA that has been extracted using the following methods:
  - (a) RNeasy<sup>®</sup> Plant Mini Kit (Qiagen Cat. No. 74904); or
  - (b) RNeasy<sup>®</sup> 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 4) using the components and concentrations listed in Table 5 and cycle under the conditions listed in Table 7.
3. Perform a one-step RT-PCR on the RNA with the pathogen-specific primers (Table 4) using the components and concentrations listed in Table 5 and cycle under the conditions listed in Table 7. The following controls must be included for each set of RT-PCR reactions:
  - (a) positive control: RNA of the appropriate virus extracted from any host tissue. A cloned fragment of the virus may also be used. If the internal control primers are not used, then the RNA or cDNA clone must be mixed with healthy *Fragaria* 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 *Fragaria* 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 strawberry 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.

#### Recommended method for *Strawberry vein-banding virus* (DNA virus): Conventional PCR

1. Extract total DNA from leaf tissue according to a standard protocol. Successful PCR amplification can be achieved using DNA extracted with the Qiagen DNeasy<sup>®</sup> Plant Mini Kit (Qiagen Cat. No. 69104). Alternative methods may also be used after validation.
2. Optional: Perform a PCR on the DNA with the Gd1/Berg54 internal control primers (Table 4) using the components and concentrations listed in Table 6 and cycle under the conditions listed in Table 7.
3. Perform a PCR on the DNA with the pathogen-specific primers (Table 4) using the components and concentrations listed in Table 6 and cycle under the conditions listed in Table 7. The following controls must be included for each set of PCR reactions:
  - (a) positive control: DNA of the appropriate virus extracted from any host tissue. A cloned fragment of the virus may also be used. If the internal control primers are not used, then the DNA or clone must be mixed with healthy *Fragaria* RNA 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 *Fragaria* leaf tissue) is included.
4. Analyse the PCR products by agarose gel electrophoresis.

**Table 5: Generic conventional one-step RT-PCR reaction components for RNA templates using the SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase**

Reagent	Volume per reaction (µl)
Sterile deionised H <sub>2</sub> O	4.2
2 × Reaction mix (Invitrogen 12574-026)	10.0
10 mg/ml BSA (Sigma A7888)	1.0
5 µM Forward primer	1.0
5 µM Reverse primer	1.0
SuperScript™ III RT / Platinum® Taq Mix	0.8
RNA template	2.0
<b>Total</b>	<b>20.0</b>

**Table 6: Generic conventional PCR reaction components for DNA templates using GoTaq® Green Master Mix**

Reagent	Volume per reaction (µl)
Sterile H <sub>2</sub> O	5.0
GoTaq® Green Master Mix (Promega M7122)	10.0
10 mg/ml BSA (Sigma A7888)	1.0
5 µM Forward primer	1.0
5 µM Reverse primer	1.0
DNA template	2.0
<b>Total</b>	<b>20.0</b>

**Table 7: Generic conventional PCR cycling conditions**

Step	Temperature	Time	No. of cycles
Reverse transcription (RT-PCR only)	50°C	30 min	1
Initial denaturation	94°C	5 min	1
Denaturation	94°C	30 s	40
Annealing	See Table 4	30 s	
Elongation	72°C	45 s (viruses) 1 min (phytoplasmas, bacteria)	
Final elongation	72°C	10 min	1

### Interpretation of results for conventional PCR

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 4; and
- (b) no bands are produced in the negative control (if used) and the no template control.

If the *Nad5* or 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 181 bp band (*Nad5*) or a 1,500 bp band (Gd1/Berg54). Failure of the samples to amplify with the internal control primers suggests that the RNA/DNA extraction has failed, compounds inhibitory to PCR are present in the RNA/DNA extract or the RNA/DNA has degraded.

### Recommended method for RNA viruses: Real-time RT-PCR

1. Extract total RNA from leaf tissue according to a standard protocol (as described above).
2. Optional: Perform a PCR on the nucleic acid with the COX internal control primers (Table 4) using the components and concentrations listed in Table 8 and cycle under the conditions listed in Table 9.
3. Set-up the real-time PCR using pathogen-specific primers (Table 4) and the components and concentrations listed in Table 8 and cycle under the conditions listed in Table 9. The reaction

and cycling conditions can be changed depending on the real-time reagents and machine used, but this would require validation. The following controls must be included for each set of reactions:

- (a) positive control: RNA of the appropriate virus extracted from any host tissue. A cloned fragment of the virus may also be used. If the internal control primers are not used, then the RNA or cDNA clone must be mixed with healthy *Fragaria* 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 *Fragaria* leaf tissue) is included.

4. Analyse real-time amplification data according to the real-time thermocycler manufacturer's instructions.

**Table 8: Generic real-time RT-PCR reaction components for RNA templates using the Invitrogen Superscript III One-step qRT PCR system**

Reagent	Volume per reaction (µl)
Sterile H <sub>2</sub> O	3.9
2 × Reaction Mix (Invitrogen 11732-020)	10.0
50 mM MgCl <sub>2</sub>	0.4
10 µg/µl BSA (Sigma A7888)	0.5
5 µM Forward primer	1.2
5 µM Reverse primer	1.2
5 µM Dual-labelled fluorogenic probe	0.4
SuperScript™ III RT / Platinum® Taq Mix	0.4
RNA	2.0
<b>Total</b>	<b>20.0</b>

**Table 9: Generic cycling conditions for real-time RT-PCR**

Step	Temperature	Time	No. of cycles
Reverse transcription	50°C	30 min	1
Initial denaturation	95°C	2 min	1
Denaturation	95°C	10 sec	40
Annealing and extension	See Table 4	40 sec	

### Interpretation of results for real-time RT-PCR

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

- (a) the positive control produces an amplification curve with the pathogen-specific primers; and
- (b) no amplification curve is seen (i.e. cycle threshold [C<sub>T</sub>] value is 40) with the negative control (if used) and the no template control.

If the COX internal control primers are also used, then the negative control (if used), positive control and each of the test samples must produce an amplification curve. Failure of the samples to produce an amplification plot with the internal control primers suggests that the nucleic acid extraction has failed or compounds inhibitory to PCR are present in the nucleic acid extract or the nucleic acid has degraded.

### Virus positive controls for PCR

1. The following viruses may be obtained from the American Type Culture Collection (ATCC; <http://www.atcc.org>):
  - (a) *Strawberry latent ringspot virus*; Cat. No. PV-1069, PV-1070.
  - (b) *Tomato ringspot virus*; Cat. No. PV-100, PV-174, PV-239.

2. The following viruses may be obtained from the German Resource Centre for Biological Material (DSMZ; <http://www.dsmz.de>):
  - (a) *Raspberry ringspot virus*; Cat. No. PV-0307.
  - (b) *Strawberry latent ringspot virus*; Cat. No. PV-0247.
  - (c) *Tomato ringspot virus*; Cat. No. PV-0049, PV-0381, PV-0380.
3. *Raspberry ringspot virus*, *Strawberry latent ringspot virus* and *Tomato ringspot virus* may be obtained from the commercial sources listed in Table 3.
4. *Strawberry vein banding virus* may be obtained from the MAF Investigation and Diagnostic Centre – Tamaki (see the Contact Point, section 8). A charge may be imposed to recover costs.

#### **7.1.3.2.1.1 *Raspberry ringspot virus***

Plants can be tested for *Raspberry ringspot virus* by PCR using the primer pair RpRSV-F1/RpRSV-R1 (Table 4). See section 7.1.3.2.1 for details of test methods and interpretation of results.

#### **7.1.3.2.1.2 *Strawberry latent ringspot virus***

Plants can be tested for *Strawberry latent ringspot virus* by PCR using the primer pair SLRSV-F/SLRSV-R (Table 4). See section 7.1.3.2.1 for details of test methods and interpretation of results.

#### **7.1.3.2.1.3 *Strawberry vein banding virus***

Plants must be tested for *Strawberry vein banding virus* by PCR using the primer pair SVBVdeta/SVBVdetb (Table 4). See section 7.1.3.2.1 for details of test methods and interpretation of results.

#### **7.1.3.2.1.4 *Tomato black ring virus***

Plants can be tested for *Tomato black ring virus* by real-time RT-PCR using the primers and probe shown in Table 4. See section 7.1.3.2.1 for details of test methods and interpretation of results.

#### **7.1.3.2.1.5 *Tomato ringspot virus***

Plants can be tested for *Tomato ringspot virus* by PCR using the primer pair U1/D1 (Table 4). 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, leaf mid-veins and/or stolons. Successful PCR amplification can be achieved using DNA extracted by the following methods:
  - (a) DNeasy<sup>®</sup> 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 4) using the components and concentrations listed in Table 6 and cycle under the conditions listed in Table 7.
3. Perform a nested PCR on the purified DNA using the universal phytoplasma primer pair, P1/P7 (Table 4), for first-stage PCR followed by the R16F2/R16R2 primer pair for the second-stage PCR (Table 4).

4. Set up the first-stage and second-stage PCR reactions using the components and concentrations listed in Table 6 and cycle under the conditions listed in Table 7. 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. A cloned fragment of the phytoplasma may also be used. If the internal control primers are not used, then the phytoplasma DNA must be mixed with healthy *Fragaria* 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 strawberry tissue) is included.
5. Analyse the products from the first and second-stage PCRs by agarose gel electrophoresis.

### 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 4; 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. An effective method to further purify the DNA is by using MicroSpin™ S-300 HR columns (GE Healthcare Cat. No. 27-5130-01).

### Phytoplasma positive controls for PCR

DNA of Aster yellows, Clover phyllody and Stolbur phytoplasmas may be obtained from the MAF Investigation and Diagnostic Centre – Tamaki (see the Contact Point, section 8). A charge may be imposed to recover costs.

#### 7.1.3.2.1 All phytoplasmas

Plants must be tested for the phytoplasmas listed in Table 1 by nested-PCR using the universal primers listed in Table 4. See section 7.1.3.2.2 for details of test methods and interpretation of results.

Strawberry lethal yellows (“*Candidatus* Phytoplasma australiense”) is a non-regulated phytoplasma of *Fragaria* that has currently been reported only from Australia and New Zealand. If a positive PCR result is obtained from any of the test plants, the phytoplasma can be identified by sequencing the R16F2/R16R2 amplicon. Isolates of “*Ca. P. australiense*” will share >99.6% sequence homology. Please note that two phytoplasma diseases (GenBank accession numbers AY903951 and AY725212) not from Australia and New Zealand have been incorrectly classified as “*Ca. P. australiense*”.

#### 7.1.3.2.3 Bacteria PCR

##### Recommended method: Conventional PCR

1. Extract total DNA from leaf petioles, leaf midveins and/or stolons. Successful PCR amplification can be achieved using DNA extracted with 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 4) using the components and concentrations listed in Table 6 and cycle under the conditions listed in Table 7.

3. Perform a PCR with the bacteria-specific primers (Table 4) on the purified DNA using the components and concentrations listed in Table 6 and cycle under the conditions listed in Table 7. The following controls must be included for each set of PCR reactions:
  - (a) positive control: total DNA or a cloned fragment from the appropriate bacteria. If the internal control primers are not used, then the DNA must be mixed with healthy *Fragaria* 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 *Fragaria* leaf tissue) is included.
4. Analyse the PCR products by agarose gel electrophoresis.

### Interpretation of results for conventional PCR

See section 7.1.3.2.2 for details of interpretation of results for conventional PCR.

### Recommended method: Real-time PCR

1. Extract total DNA from leaf petioles, leaf midveins and/or stolons (as described above).
2. Optional: Perform a PCR with the COX internal control primers and probe (Table 4) using the components and concentrations listed in Table 10 and cycle under the conditions listed in Table 11.
3. Perform a PCR with the bacteria-specific primers and probe (Table 4) using the components and concentrations listed in Table 10 and cycle under the conditions listed in Table 11. Please note that reaction and cycling conditions can be changed depending on the real-time machine used, but this would require validation.
4. The following controls must be included for each set of reactions:
  - (a) positive control: total DNA or a cloned fragment from the appropriate bacteria. If the internal control primers are not used, then the DNA must be mixed with healthy *Fragaria* 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 *Fragaria* leaf tissue) is included.
5. Analyse real-time amplification data according to the manufacturer's instructions accompanying the real-time PCR machine.

**Table 10: Generic real-time PCR reaction components for DNA templates using Invitrogen Platinum<sup>®</sup> qPCR SuperMix-UDG**

Reagent	Volume per reaction (µl)
Sterile H <sub>2</sub> O	4.4
2 × Reaction Mix (Invitrogen 11730-017)	10.0
10 mg/ml BSA (Sigma A7888)	0.8
5 µM Forward primer	1.2
5 µM Reverse primer	1.2
5 µM Dual-labelled fluorogenic probe	0.4
DNA template	2.0
<b>Total</b>	<b>20.0</b>

**Table 11: Generic real-time PCR cycling conditions**

Step	Temperature	Time	No. of cycles
UDG incubation hold	50°C	2 min	1
Initial denaturation	95°C	2 min	1
Denaturation	95°C	10 s	40
Annealing and elongation	See Table 4	45 s	

## Interpretation of results for real-time PCR

See section 7.1.3.2.1 for details of interpretation of results for real-time PCR.

### Bacteria positive controls for PCR

1. Cultures may be obtained from the Landcare Research International Collection of Microorganisms from Plants (ICMP); <http://www.landcareresearch.co.nz/research/biodiversity/fungiprogram/icmp.asp>) for:
  - (a) *Xanthomonas arboricola* pv. *fragariae*; ICMP No. 17064, 17065.
  - (b) *Xanthomonas fragariae*; ICMP No. 6269, 11939, 11944 (plus others).
  - (c) *Xylella fastidiosa*; ICMP No. 6575, 6576, 8729-8745, 8693, 8694.
2. DNA of “*Candidatus Phlomobacter fragariae*”, *Xanthomonas arboricola* pv. *fragariae*, *Xanthomonas fragariae* and *Xylella fastidiosa* may be obtained from the MAF Investigation and Diagnostic Centre – Tamaki (see the Contact Point, section 8). A charge may be imposed to recover costs.

#### 7.1.3.2.3.1 “*Candidatus Phlomobacter fragariae*”

Plants must be tested for “*Candidatus Phlomobacter fragariae*” by PCR using the primers listed in Table 4. See section 7.1.3.2.3 for details of test methods and interpretation of results.

#### 7.1.3.2.3.2 *Xanthomonas arboricola* pv. *fragariae*

Plants must be tested for *Xanthomonas arboricola* pv. *fragariae* by real-time PCR using the primers and probe listed in Table 4. See section 7.1.3.2.3 for details of test methods and interpretation of results.

#### 7.1.3.2.3.3 *Xanthomonas fragariae*

Plants must be tested for *Xanthomonas fragariae* by conventional or real-time PCR using the primers and probe (for real-time PCR) listed in Table 4. See section 7.1.3.2.3 for details of test methods and interpretation of results. Please note that conventional PCR should be set-up and cycled as shown in Tables 12 and 13, respectively.

**Table 12: Conventional PCR reaction components for *Xanthomonas fragariae***

Reagent	Volume per reaction (µl)
Sterile H <sub>2</sub> O	18.2
10 × HotMaster <i>Taq</i> buffer with 25 mM Mg <sup>2+</sup> (Eppendorf)	3.0
10 × Triton X-100 and gelatin solution <sup>1</sup>	3.0
10 mM dNTPs	0.6
10 µM 245A primer	1.0
10 µM 245B primer	1.0
5 U/µl HotMaster <i>Taq</i> DNA polymerase (Eppendorf)	0.2
DNA template	3.0
<b>Total</b>	<b>30.0</b>

<sup>1</sup>This solution is prepared by dissolving 10% (v/v) Triton X-100 and 1% (w/v) gelatine in warm water.

**Table 13: Conventional PCR cycling conditions for *Xanthomonas fragariae***

Step	Temperature	Time	No. of cycles
Initial denaturation	94°C	4 min	1
Denaturation	94°C	1 min	40
Annealing	68°C	1 min	
Elongation	72°C	1 min	
Final elongation	72°C	2 min	1

#### 7.1.3.2.3.4 *Xylella fastidiosa*

*Fragaria vesca* plants must be tested for *Xylella fastidiosa* by conventional or real-time PCR using the primers and probe (for real-time PCR) listed in Table 4. See section 7.1.3.2.3 for details of test methods and interpretation of results. Please note that the real-time PCR must be set-up as shown in Table 14 and cycled as shown in Table 11.

**Table 14: Real-time PCR reaction components for *Xylella fastidiosa* using the Invitrogen Platinum<sup>®</sup> qPCR SuperMix-UDG**

Reagent	Volume per reaction (µl)
Sterile H <sub>2</sub> O	4.2
2 × Reaction Mix (Invitrogen 11730-017)	10.0
10 mg/ml BSA (Sigma A7888)	0.6
50 mM MgCl <sub>2</sub>	0.4
5 µM Forward primer	1.2
5 µM Reverse primer	1.2
5 µM Dual-labelled fluorogenic probe	0.4
DNA template	2.0
<b>Total</b>	<b>20.0</b>

#### 7.1.4 Microscopic inspection for mites

*Fragaria* plants in tissue culture must be examined for regulated mites.

##### Recommended method

- For each plant, use a hand lens to inspect the underside of the 3 youngest leaves for mite eggs, nymphs, adults and symptoms of mite presence. If mites are present the following symptoms may be observed on the underside of leaves: webbing, distinct small yellow spots (which get larger over time), leaf browning and heavily infested leaves may shrivel up and die. Overall, plant vigour and growth may be affected. Mites of the *Diptacus* genus are usually vagrant on the underside of leaves, form only small populations and cause no apparent damage to host plants.
- If mites are observed or mite damage is suspected, collect infested leaves for further examination using a binocular microscope.
- For species identification, adults of both sexes must be slide mounted and examined under a microscope. Adult males are smaller than the females for all *Diptacus* and *Tetranychus* species. Male mites should be mounted laterally onto a microscope slide and female mites should be mounted dorsally to expose the diagnostic characters. To improve transparency, the mites can be cleared in lactic acid under a table lamp prior to mounting.
- Identify the specimens using one or more of the following methods:
  - expertise/experience
  - keys and detailed morphological descriptions
  - comparison with diagnostic images
  - comparison with reference specimens

## 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 viruses:

- *Fragaria chiloensis latent virus*
- *Raspberry ringspot virus*
- *Strawberry latent ringspot virus* (strains not in New Zealand)
- *Tobacco streak virus* (strains not in New Zealand)
- *Tomato black ring virus*
- *Tomato ringspot virus* (strains not in New Zealand)

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.

## 7.3 Specific tests for pollen

Seed resulting from fertilisation of a mother plant with imported pollen must be tested in the same way as for seed for sowing (see section 7.2). Tests are to be carried out on plants germinated from the seed. Samples from up to 5 plants may be bulked for testing provided that the plants were derived from the same batch of pollen.

Tests for the following viruses must be carried out:

- *Fragaria chiloensis latent virus*
- *Raspberry ringspot virus*
- *Tobacco streak virus* (strains not in New Zealand)
- *Tomato black ring virus*
- *Tomato ringspot virus* (strains not in New Zealand)

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

## 8. CONTACT POINT

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The validation experiments for phytoplasmas and “*Candidatus Phlomobacter fragariae*”, and *Xanthomonas fragariae* (conventional PCR) and *Xylella fastidiosa* were performed by Mr Mark Andersen and Dr Joel Vanneste, respectively.

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## Appendix 1. Symptoms of significant regulated pests of *Fragaria*

### 1.1 “*Candidatus Phlomobacter fragariae*”



Small, cup-shaped leaves with chlorotic margins caused by marginal chlorosis disease. In nursery plants symptoms also include red discoloration of leaflets (right photo). (Left image courtesy of J.G. Nourrisseau; reproduced, with permission, from the Diseases of Small Fruits CD-Rom, 2000, APS, St Paul, MN, USA. Right image courtesy of X. Foissac)

### 1.2 *Xanthomonas arboricola* pv. *fragariae*



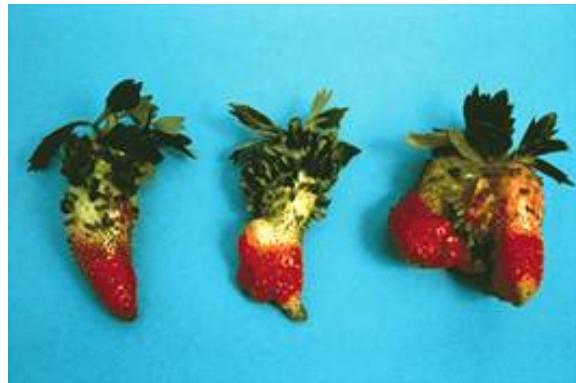
Large, brown, V-shaped lesions surrounded by a yellow halo on the upper side of strawberry leaves (left photo) and small, reddish-brown lesions on the lower side of strawberry leaves (right photo) caused by the bacterial leaf blight pathogen, *Xanthomonas arboricola* pv. *fragariae*. (Both images courtesy of M. Scortichini; [www.atlasplantpathogenicbacteria.it](http://www.atlasplantpathogenicbacteria.it))

### 1.3 *Xanthomonas fragariae*



Initial symptoms of small angular water soaked spots (left photo) that progress along the main veins of the leaf (right photo) caused by the bacterial angular leaf spot of strawberry, *Xanthomonas fragariae*. (Both images courtesy of R. Gozzi; [www.atlasplantpathogenicbacteria.it](http://www.atlasplantpathogenicbacteria.it))

### 1.4 Aster yellows phytoplasma



Phyllody of achenes (left photo) and fruit distortion (right photo) in ‘Marmolada’, caused by strawberry aster yellows phytoplasma. (Both images courtesy of S. Guerrini; reproduced, with permission, from the Diseases of Small Fruits CD-Rom, 2000, APS, St Paul, MN, USA)

### 1.5 Strawberry green petal phytoplasma



Green flower petals, small, hard, green fruit and dwarfed, cupped, chlorotic leaves caused by strawberry green petal. (Courtesy USDA; reproduced from the Diseases of Small Fruits CD-Rom, 2000, APS, St Paul, MN, USA)

### 1.6 Strawberry multiplier phytoplasma



Symptoms of multiplier disease (left plant) with numerous crowns produced along the runners; the plant on the right is healthy. (Courtesy M. F. Clark; reproduced, with permission, from the Diseases of Small Fruits CD-Rom, 2000, APS, St Paul, MN, USA)

### 1.7 Strawberry yellows phytoplasma



General chlorosis and stunting of leaves in 'Redlands Crimson', caused by phytoplasma yellows. (Courtesy USDA; reproduced from the Diseases of Small Fruits CD-Rom, 2000, APS, St Paul, MN, USA)

### 1.8 Strawberry latent C virus



Severe epinasty of young leaves of *Fragaria vesca* 'EMK', caused by *Strawberry latent C virus*. (Courtesy USDA; reproduced from the Diseases of Small Fruits CD-Rom, 2000, APS, St Paul, MN, USA)

### 1.9 *Strawberry pallidosis-associated virus*



Leaves of *Fragaria virginiana* 'UC-10' infected with *Strawberry pallidosis-associated virus* (left and centre) and a healthy leaf (right). (Courtesy USDA; reproduced from the Diseases of Small Fruits CD-Rom, 2000, APS, St Paul, MN, USA)

### 1.10 *Strawberry pseudo mild yellow edge virus*



Stippled pattern of discoloration on an older leaf of *Fragaria vesca* 'Alpine', caused by *Strawberry pseudo mild yellow edge virus*. (Courtesy USDA; reproduced from the Diseases of Small Fruits CD-Rom, 2000, APS, St Paul, MN, USA)

### 1.11 *Strawberry vein banding virus*



Chlorosis along veins in *Fragaria vesca* 'UC-5', a diagnostic symptom of *Strawberry vein banding virus*. (Courtesy R. R. Martin & S. Spiegel; reproduced, with permission, from the Diseases of Small Fruits CD-Rom, 2000, APS, St Paul, MN, USA)

### 1.12 *Tobacco necrosis virus*



Necrosis of older leaves of *Fragaria vesca* 'Alpine' infected with *Tobacco necrosis virus*. (Courtesy USDA; reproduced from the Diseases of Small Fruits CD-Rom, 2000, APS, St Paul, MN, USA)

### 1.13 *Tomato ringspot virus*



Red, necrotic lesions on *Fragaria virginiana* 'UC-11' graft-inoculated with *Tomato ringspot virus*. (Courtesy R. R. Martin & S. Spiegel; reproduced, with permission, from the Diseases of Small Fruits CD-Rom, 2000, APS, St Paul, MN, USA)

### 1.14 Strawberry lethal decline disease



Bronzing of older leaves and cupping of younger leaves in 'Hood' due to strawberry lethal decline. (Courtesy USDA; reproduced from the Diseases of Small Fruits CD-Rom, 2000, APS, St Paul, MN, USA)

## Appendix 2. Symptoms of nutrient deficiencies in *Fragaria*

### 2.1 Nitrogen deficiency



Red colouration of a strawberry leaf caused by severe nitrogen deficiency. (Courtesy G. May & M. P. Pritts; reproduced, with permission, from the Diseases of Small Fruits CD-Rom, 2000, APS, St Paul, MN, USA)

### 2.2 Magnesium deficiency



Progression of magnesium deficiency symptoms in strawberry leaves starting with initial chlorosis (left leaf) eventually becoming necrotic (middle and right leaves). (Courtesy G. May & M. P. Pritts; reproduced, with permission, from the Diseases of Small Fruits CD-Rom, 2000, APS, St Paul, MN, USA)

### 2.3 Phosphorus deficiency



Reddish-purple tint on an older strawberry leaf due to phosphorus deficiency. (Courtesy G. May & M. P. Pritts; reproduced, with permission, from the Diseases of Small Fruits CD-Rom, 2000, APS, St Paul, MN, USA)

### 2.4 Potassium deficiency



Dark, dehydrated mid-veins and leaf blades of strawberry leaves caused by potassium deficiency. (Courtesy G. May & M. P. Pritts; reproduced, with permission, from the Diseases of Small Fruits CD-Rom, 2000, APS, St Paul, MN, USA)

## 2.5 Manganese deficiency



Severe manganese deficiency symptoms of interveinal chlorosis in strawberry leaves (upper right). (Courtesy F. Johanson; reproduced, with permission, from the Diseases of Small Fruits CD-Rom, 2000, APS, St Paul, MN, USA)

## 2.6 Iron deficiency



Yellowing and green-veining of young mature strawberry leaves caused by iron deficiency. (Courtesy G. May & M. P. Pritts; reproduced, with permission, from the Diseases of Small Fruits CD-Rom, 2000, APS, St Paul, MN, USA)

## 2.7 Zinc deficiency



Basal narrowing of leaflets, greening of veins and green halo around the serrated leaf margins of strawberry caused by zinc deficiency. (Courtesy F. Johanson; reproduced, with permission, from the Diseases of Small Fruits CD-Rom, 2000, APS, St Paul, MN, USA)

### 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<sup>®</sup> 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 Na<sub>2</sub>HPO<sub>4</sub>, 0.03 M NaH<sub>2</sub>PO<sub>4</sub>, 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 µl 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 µl) 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 µl 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 µl 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 µl 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.