



# *Corylus* (Hazelnut)

## Post-Entry Quarantine Testing Manual



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# *Corylus* Post-Entry Quarantine Testing Manual

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## 1. SCOPE

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

<i>Corylus avellana</i>	<i>Corylus heterophylla</i>
<i>Corylus colurna</i>	<i>Corylus maxima</i>
<i>Corylus cornuta</i>	<i>Corylus sieboldiana</i>
<i>Corylus ferox</i>	<i>Corylus tibetica</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 *Corylus* consists of deciduous species which naturally occur in temperate forest areas in Europe, the Middle East, Asia and North America. As many as 25 species have been described, but 9 species are generally recognised (Thompson *et al.*, 1996). The shrub species are *C. avellana* (includes *C. maxima*), *C. americana*, *C. cornuta*, *C. heterophylla* and *C. sieboldiana*. These shrub species are commonly multi-stemmed suckering shrubs 2 to 10 metres high. Four species, *C. colurna*, *C. chinensis*, *C. ferox* (syn. *C. tibetica*), and *C. jacquemontii* are deciduous trees up to 30 metres in height (Mehlenbacher, 1991). All species bear edible nuts.

The commercial hazelnut industry is based on selections of the European hazel, *Corylus avellana*. Alternative names for hazelnuts, "cobnut" and "filbert", are used in some localities. In the United Kingdom, "cobnuts" is used to refer to round nuts with an open husk, except for the Kentish Cob which is a long nut with a long clasping husk, and "filbert" is used to refer to nuts with a long clasping husk. Most of these "filbert" cultivars were originally placed in a separate species, *C. maxima*. In the United States of America, "filbert" is often used to cover all commercial hazelnuts of *C. avellana* origin.

Hazelnuts will grow in a wide range of soil types from acidic mountain soils to basic soils derived from limestone. The plants grow best in mild, humid climates without extremes of heat or cold. However, buds, leaves, catkins, and female flowers are tolerant of frosts down to -7 °C. Hazelnuts are wind pollinated and flowering occurs during winter although fertilisation is delayed until the middle of spring. Hazelnut cultivars are self-incompatible and often cross-incompatible in many combinations (Mehlenbacher, 1991). Therefore commercial cultivars must be carefully matched with appropriate pollinisers.

World hazelnut production is dominated by Turkey, with about 67% of world production (average 2001-2004), with other significant production areas in Italy (16.5%), Spain (3%), and the USA (3.9%). Smaller producers are China, Iran, Georgia, and Azerbaijan (Kilic & Alkan 2006). Commercial hazelnut production is being trialled in many countries with suitable climates, including Australia, Chile, France, Korea and New Zealand.

Mean global hazelnut production for the period 2001-2004 was approximately 797,000 tonnes per annum. Hazelnuts make up approximately 35% of the world hard shell nut consumption. Approximately 80% are used in the chocolate sector; 15% in cakes, biscuits, and sweets, and 5% as appetisers (e.g. salted nuts) (Kilic & Alkan, 2006). The American hazelnut, *C. americana* also has attractive red, autumnal foliage and may have potential use as an ornamental plant.

Hazelnut production in New Zealand is dominated by the cultivar ‘Whiteheart’ which makes up at least 85% of all trees planted. The variety was selected from three imported hazelnut varieties which were originally sold under the name ‘Waterloo’. In New Zealand, harvest dates range from February (North Island, early cultivars) through to late April (South Island).

Production in New Zealand is concentrated in the Canterbury area where the existence of a large processing factory, set up by a grower’s co-operative (HazelzNZ), has stimulated the planting of large orchards. Smaller plantings exist in most parts of New Zealand. Nearly all orchards are on lifestyle blocks. New Zealand plantings are estimated at approximately 150 hectares ([www.hazelnut-growers.org.nz](http://www.hazelnut-growers.org.nz), 2006). Most of this area has been planted since 1998 (HGANZ, 2002). Grower support is provided by the Hazelnut Growers Association of New Zealand and the New Zealand Tree Crops Association.

Most species have been imported into New Zealand as seed. The last known importations of commercial *C. avellana* cultivars occurred in the 1980’s. Characteristics that the New Zealand hazelnut industry would like to import include: high yields, precocious nut-bearing, low chilling requirements, suckerless trunks, and resistance to Eastern Filbert Blight (*Anisogramma anomala*) and hazel blight (*Xanthomonas aboricola* pv. *corylina*).

### 3. IMPORT REQUIREMENTS

The import requirements for nursery stock (whole plants, cuttings and tissue culture) and pollen of *Corylus* are set out in MPI’s import health standard “Importation of Nursery Stock” (<http://www.biosecurity.govt.nz/files/imports/plants/standards/155-02-06.pdf>). Imported nursery stock must meet the basic requirements (sections 1-2) and the specific requirements detailed in the “*Corylus*” schedule in section 3. 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 (whole plants and cuttings). On arrival in New Zealand, the nursery stock must be grown for a minimum period of 3 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 *Corylus* seed for sowing are set out in MPI’s import health standard “Importation of Seed for Sowing” (<http://www.biosecurity.govt.nz/files/ihs/155-02-05.pdf>). Imported seed is only required to meet the general requirements (sections 1-2) and there are no specific requirements for the genus. An import permit is not required and seed meeting the import requirements is given biosecurity clearance at the border without the need for post-entry quarantine.

The import requirements for pollen is stated in section 2.2.3 in MPI’s import health standard “Importation of Nursery Stock” (<http://www.biosecurity.govt.nz/files/ihs/155-02-06.pdf>). An import permit is required, however there are no regulated pests affecting pollen of *Corylus*,

and pollen meeting the import requirements is given biosecurity clearance at the border without the need for post-entry quarantine.

#### **4. PESTS**

A list of the regulated pests of *Corylus* can be found in the nursery stock import health standard (see section 3).

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

###### **Insects:**

*Curculio nucum*

*Cydia latiferreana*

###### **Fungi:**

*Anisogramma anomala*

[Fig. 1]

*Armillaria mellea*

*Monilinia fructigena*

*Pseudomonas avellanae*

*Pseudomonas syringae* pv. *coryli*

*Phytophthora ramorum*

*Taphrina coryli*

*Xanthomonas arboricola* pv. *corylina*

[Fig. 2]

[formerly *Xanthomonas campestris* pv. *corylina*]

##### **4.2 Regulated pests for which specific tests are optional**

###### **Fungi:**

*Monilinia fructigena*

*Phytophthora ramorum*

###### **Phytoplasma:**

‘*Candidatus* Phytoplasma mali’

[casual agent of Hazelnut yellows]

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

Plants must be maintained in a healthy, vigorous state. *Corylus* plants are susceptible to wilting and should not be subjected to hot, dry conditions. Plants in quarantine may exhibit symptoms associated with abiotic stress, such as water and nutrient deficiencies. Some species may have particular requirements and may need specific nutrient additions (Erdogan & Mehlenbacher, 2000). 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 boron deficiency are illustrated in Appendix 2, and on the American Phytopathological Society’s CD-ROMs ‘Diseases of Orchard Fruit and Nut Crops’ and ‘Nutrient Deficiencies and Toxicities of Plants’ ([www.aps.org](http://www.aps.org)).

## 5.1 Nursery stock

*Corylus* nursery stock may be imported as whole plants, dormant or non-dormant cuttings, or as plants in tissue culture. It is best to import *Corylus* cuttings between December to February (from northern hemisphere), and June to July (from southern hemisphere). All imported cutting material must be free of fruit, foliage, roots or soil to minimise the introduction of disease. Tissue culture plants should ideally be imported around June to July (southern hemisphere winter) to allow them to become established prior to summer.

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.

### 5.1.1 Whole plants

Rooted plants should be potted up immediately after arrival. On arrival, plants should be examined for damage. Damaged roots should be trimmed and damaged tops cut back to the closest live bud below the damage. Plants should be potted in sterilised potting mix containing slow release fertiliser at label rates. Fertiliser may have to be topped up if quarantine extends beyond one growing season. Nutrient deficiencies that occur can be corrected with appropriate foliar or solid fertilisers.

### 5.1.2 Non-dormant and dormant cuttings

The success rates for propagating hazels from cuttings can vary depending on cultivar, time of cutting selection, and age of the parent tree. Softwood cuttings strike well but have a high proportion of non-viable buds that fail to open in the spring. Hardwood cuttings have low strike rates for most cultivars. The success rate can be improved by selecting etiolated sucker material.

Propagation is best achieved with semi-hardwood cuttings taken from young non-producing trees. In the southern hemisphere, cuttings should be collected in mid-summer (January-early February) when the trees are still actively growing and prior to the formation of dormant terminal buds (Smith & McNeil, 1996).

The importation of *Corylus* plant material as softwood or semi-hardwood cuttings has not been attempted in the past. The plant material would have to be kept cool (4°C) and moist throughout the shipment period and then processed immediately upon arrival. The use of semi-hardwood cuttings is recommended for the propagation of hazels while in quarantine.

Cuttings should be made at a node, and each cutting should be approximately 10-15 cm long. The base of the cutting should be wounded once with a sharp blade and the cutting then soaked for 5 minutes in fungicide (e.g. Benlate, 2.0 g in 5 litres of water). After blotting the base of the cutting dry, the cutting can be dipped into Indole-3-butyric acid (IBA) solution (4000 ppm concentration) for 5 seconds. The cuttings can then be planted at a depth of around 5 cm deep into rooting medium. Trays containing cuttings should be placed in a misting unit with bottom heat set to 22°C. Bottom heat should be maintained until roots begin to form. It is best to keep the trays in a misting unit for 2 weeks after the bottom heat is turned off, after which the trays can then be moved to a misted shade house. It is important that a high level of humidity is maintained at all times (Smith & McNeil, 1996).

Woody *Corylus* material imported as hardwood scions should be collected as ripened wood during dormancy, and grafted onto seedling rootstocks. To minimise the risk of mould

growing during storage, it is recommended that the scions are washed in dilute (2.6%) sodium hypochlorite (or similar disinfecting solution) and rinsed in clean tap water before storing in damp sphagnum or filter paper at 4°C.

Hazels do not callus readily at normal room temperatures. Acceptable success rates can be achieved by exposing the grafts to temperatures of 25 to 27°C. The use of heated grafting tables or tubes is preferable to using hot boxes or rooms (Achim *et al.*, 2001). Hazels can be grafted on to any vigorous rootstock. For quarantine purposes, seedlings should be grown in a sterile medium in quarantine glasshouse conditions and be free of any diseases. Seedlings with a similar diameter to the scion wood should be grafted when buds on the rootstock seedlings are starting to swell in early spring (August/September). Both whip-and-tongue and cleft grafts using standard techniques give good success rates under heat treatment. Grafts should be kept heated for 4 weeks then gradually acclimatised to glasshouse conditions. Remove any growths that emerge below the graft. Tapes should be cut or removed as soon as the graft union appears to be well callused.

### **5.1.3 Plants in tissue culture**

Hazels are not easy to propagate by tissue culture, and bulking up by this method in quarantine is unlikely until the technology is refined, however, tissue culture plantlets offer an ideal method of importing clean plant material.

Plantlets to be tested are carefully excised from the tissue culture vessel and washed to remove any remaining agar and planted into pots of planting media containing 50:50 (v/v) pasturised peat:perlite or 50:50 (v/v) peat:vermiculite. The plantlets must be protected from evaporation for approximately 4 to 6 weeks by covering initially with a vented plastic tub or bag. Alternatively, the plants can be misted regularly to keep the planting media moist, and to maintain a high relative humidity. Pots should be placed in bright light, but not direct sunlight during the 4-6 weeks. After this period, remove covering and allow the plants to gradually acclimatise to normal glasshouse conditions (Remmick, 2005).

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. These plantlets must be maintained in a Level 3 quarantine tissue culture laboratory and do not require testing.

## **5.2 Seed for sowing**

Fresh nuts should be stored in a cool moist environment to maintain viability. Drying causes loss of viability. Nuts should be stored for no longer than 12 months.

Prior to stratification, nuts should be surface sterilised by soaking in 2.6% sodium hypochlorite for 1 min. Stratify in trays of sterilised vermiculite at 5°C for 2 months (Erdogan & Mehlenbacher, 2000). Germination of thick shelled nuts is aided by partially cracking the nut. The shell should be cracked but remain intact. The trays should be examined periodically during stratification for the presence of mouldy nuts. These should be removed and destroyed. Following stratification, the trays should be removed from the cooler and kept at room temperature for 1 week. Any sprouting nuts should be potted up in pasturised potting mix and the trays returned to the cooler for further stratification for another 3 weeks. Check the trays weekly for sprouting nuts. After 3 weeks, hold the trays at room temperature again and repeat the process. Most nuts should have germinated after 6 months

but some can take up to 2 years to germinate. Gibberellic acid treatment is an alternative method to stratification. Nuts should be cracked and the kernels treated with gibberellic acid (GA3) at 50 mg per litre for 48 hours. Place the kernels between 2 layers of moist filter paper in plastic boxes. Check every 7 to 14 days and pot out into seedling mix as radicles emerge (Thompson *et al.*, 1996). Grow on sprouted nuts using standard nursery practice.

Hazel plants that have been forced all winter in greenhouses will cease growth whereas those exposed to chilling temperatures for several months before transplanting resume growth (Thompson *et al.*, 1996). Seed imported from the Northern Hemisphere will be harvested in September/October and achieving a reasonable sized plant before winter may be difficult using cold stratification. Gibberellic acid treatment may be a better option than dry storage of the nuts for 6-9 months prior to stratification and spring sowing.

### 5.3 Pollen

Anthers can be collected from mature but unopened flowers and dried in warm, light conditions. Following this drying period, pollen should be collected into a centrifuge vial or into gel capsules and stored at 4°C in a sealed container in the presence of a strong desiccant such as calcium chloride.

## 6. INSPECTION

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

Photographs of symptoms caused by significant regulated diseases can be found in Appendix 1.

## 7. TESTING

The following tests are optional unless specified in the import permit.

*Monilina fructigena* can spread to young hazel twigs via infected fruit. The presence of the fungus can result in local cankers, discolouration of the outer bark and necrosis of the underlying tissue (Mackie, 2000). If *M. fructigena* infection is suspected during inspection of imported plant material, or during quarantine, the affected tissue can be tested for the presence of *M. fructigena* by PCR, isolation on media, or a combination of both (refer to sections 7.1.1.1.1 and 7.1.2.1).

*P. ramorum* can cause bleeding cankers with dark-stained wood under the bark. If *P. ramorum* is suspected during inspection of imported plant material, or during quarantine, the affected tissue can be directly tested for the presence of *P. ramorum* by PCR, or by isolating the fungus onto media and testing by PCR (refer to sections 7.1.1.1.2 and 7.1.2.2).

If phytoplasma testing is to be carried out, this should be done at the end of the summer. Detection of phytoplasma requires testing of mid-veins from at least two fully expanded leaves. The leaves should be sampled from each of two different branches of the main stem, one a younger leaf and one an older leaf. The mid-veins from the sampled leaves from each plant should be bulked together and tested as soon as possible after removal from the host.

If leaf samples have to be stored before testing, the plant material 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.

**Table 1: Summary of the regulated pests for *Corylus* indicating the specific tests that are optional (★)**

Organism type	PCR	Isolation on media
<b>Fungi</b>		
<i>Monilinia fructigena</i>	★	★
<i>Phytophthora ramorum</i>	★	★
<b>Phytoplasma</b>		
' <i>Candidatus</i> Phytoplasma mali'	★	

## 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 Polymerase chain reaction (PCR)

PCR primers used to detect fungi and phytoplasma infecting *Corylus* are listed in Table 2, along with plant internal control primers for 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 Gd1/Berg54 primers amplify the 16S rRNA gene from chloroplasts. The COX primers amplify the constitutive cytochrome oxidase 1 gene found in plant mitochondria.

Please note that the primers listed in Table 2 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, Ministry for Primary Industries. Alternative reagents may give similar results but will require validation.

**Table 2: PCR primers used for the detection of regulated pests of *Corylus* and plant internal control**

Target organism	Primer name	Sequence (5'-3')	Tm (°C)	Size (bp)	Reference
<b>Fungi</b>					
<i>Monilinia fructigena</i>	ITS1	TCCGTAGGTGAACCTGCGG	59	460	Hughes <i>et al.</i> , 2000
	Mfg-R2	GGTCAACCATAGAAAATTGGT			
<i>Phytophthora ramorum</i>	Phyto 1	CATGGCGAGCGCTTGA	62	687	Hayden <i>et al.</i> , 2004
	Phyto 4	GAAGCCGCCAACACAAG			
	Pram114F	TCATGGCGAGCGCTTGA	60	76	Hughes <i>et al.</i> , 2006
	Pram 190R	AGTATATTCAGTATTTAGGAATGGGT TAAAAAGT			
Pram P <sup>1</sup>	FAM-TTCGGGTCTGAGCTAGTAG- NFQ <sup>2</sup>				
<b>Phytoplasma</b>					
Universal phytoplasma	P1	AAGAGTTTGATCCTGGCTCAGGATT	53	1,800	Deng & Hiruki, 1991 Schneider <i>et al.</i> , 1995
	P7	CGTCCTTCATCGGCTCTT			
	R16F2	ACGACTGCTAAGACTGG	50	1,248	Lee <i>et al.</i> , 1995
	R16R2	TGACGGGCGGTGTGTACAAACCCCG			
	Phyto-F	CGTACGCAAGTATGAAACTTAAAGGA	60	75	Christensen <i>et al.</i> , 2004
	Phyto-R	TCTTCGAATTAACAACATGATCCA			
Phyto P <sup>1</sup>	FAM-TGACGGGACTCCGCACAAGCG- NFQ <sup>2</sup>				
<b>Internal control</b>					
Plant DNA control	Gd1	ACGGAGAGTTTGATCCTG	50-62	1,500	Andersen <i>et al.</i> , 1998
	Berg54	AAAGGAGGTGATCCAGCCGCACCTTC			
Plant NA control	COX-F	CGTCGCATTCCAGATTATCCA	60	74	Weller <i>et al.</i> , 2000
	COX-R	CAACTACGGATATATAAGAGCCAAA ACTG			
	COX-P <sup>1</sup>	FAM-TGCTTACGCTGGATGGAATG CCCT- NFQ <sup>2</sup>			

<sup>1</sup>Real-time probe; <sup>2</sup>NFQ (Non-Fluorescent Quencher)

### 7.1.1.1 Fungal PCR

#### Recommended method: conventional PCR

1. Extract total DNA from symptomatic bark scrapings, or from mycelium isolated from bark tissue, or selective media. Successful PCR amplification can be achieved with DNA extracted using the DNeasy<sup>®</sup> Plant Mini Kit (Qiagen Cat. No. 69104). Alternatively, for suspected *M. fructigena*, mycelial threads that are visible in the plant tissue can be placed directly into the PCR mix; this has been shown to give quick reliable diagnosis (PM 4/31, EPPO Bulletin, 2003). An alternative DNA extraction method may also be used after validation.

2. Optional: Perform a PCR on the purified DNA with the Gd1/Berg54 internal control primers (Table 2) using the components and concentrations listed in Table 3 and cycle under the conditions listed in Table 4.
3. Perform a PCR on the purified DNA with the fungal-specific primers (Table 2) using the components and concentrations listed in Table 3 and cycle under the conditions listed in Table 4. The following controls must be included for each set of PCR reactions:
  - (a) positive control: DNA from the appropriate fungus. If the internal control primers are not used, then the fungal DNA must be mixed with healthy *Corylus* 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 *Corylus* tissue) is included.
4. Analyse the PCR products by agarose gel electrophoresis.

### Interpretation of results for conventional PCR

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

- (a) the positive control produces the correct sized product as indicated in Table 2; 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 (healthy) 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 diluting the DNA.

**Table 3: Conventional PCR reaction components**

Reagent	Volume per reaction (µl)
Sterile deionised H <sub>2</sub> O	12.8
10 × PCR buffer (Invitrogen)	2.0
50 mM MgCl <sub>2</sub>	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
<b>Total</b>	<b>20.0</b>

**Table 4: Generic cycling conditions for conventional PCR**

Step	Temperature	Time	No. of cycles
Initial denaturation	94°C	5 min	1
Denaturation	94°C	30 sec	40
Annealing	See Table 3	30 sec	
Elongation	72°C	1 min	
Final elongation	72°C	10 min	1

### Recommended method: real-time PCR

1. Extract total DNA from symptomatic bark scrapings or from mycelium isolated from bark tissue or from selective media, according to a standard protocol (as described above).
2. Set-up the PCR using pathogen-specific primers (Table 2) and the components and concentrations listed in Table 5 and cycle under the conditions listed in Table 6. Please note that reaction and cycling conditions can be changed depending on the real-time machine used, but this would require validation.
3. Optional: Perform PCR on the nucleic acid using the COX internal control primers (Table 2), and using the components and concentrations listed in Table 5 and cycle under the conditions listed in Table 6. The following controls must be included for each set of reactions:
  - (a) positive control: DNA from the appropriate fungus. If the internal control primers are not used, then the fungal DNA must be mixed with healthy *Corylus* 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 *Corylus* tissue) is included.

**Table 5: Real-time PCR reaction components for fungi and phytoplasma**

Reagent	Volume per reaction (µl)
Sterile H <sub>2</sub> O	4.4
2 × Reaction Mix <sup>1</sup>	10.0
10 µg/µl Bovine Albumin Serum (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	2.0
<b>Total</b>	<b>20</b>

<sup>1</sup>Platinum<sup>®</sup> qPCR SuperMix-UDG (Cat. No. 11730-017)

**Table 6: Generic cycling conditions for real-time PCR**

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 sec	40
Annealing	See Table 3	45 sec	

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

The real-time PCR 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 (healthy) 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 DNA extraction has failed or compounds inhibitory to PCR are present in the DNA extract or the DNA has degraded.

### **Fungal positive controls for PCR**

Plasmid DNA of *M. fructigena* and *P. ramorum* 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.

#### **7.1.1.1.1 *Monilinia fructigena***

It is optional to test plants for *M. fructigena* by PCR using the conventional primer pair ITS1/Mfg-R2 (Table 2). See section 7.1.1.1 for details of test methods and interpretation of results.

#### **7.1.1.1.2 *Phytophthora ramorum***

It is optional to test plants for *P. ramorum* by PCR using the conventional primer pair Phyto1/Phyto4 or real-time primer pair Pram 114F/Pram 190R (Table 2). See section 7.1.1.1 for details of test methods and interpretation of results.

### **7.1.1.2 Phytoplasma PCR**

#### **Recommended method: conventional PCR**

1. Extract total DNA from the vascular tissue (mid-veins) of leaves. 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.An alternative method may also be used after validation.
2. Optional: Perform a PCR on the purified DNA with the Gd1/Berg54 internal control primers (Table 2) using the components and concentrations listed in Table 3 and cycle under the conditions listed in Table 4.
3. Perform a nested PCR on the purified DNA using the universal phytoplasma primer pair, P1/P7 (Table 2), for first-stage PCR, followed by the R16F2/R16R2 primer pair for the second-stage PCR (Table 2).
4. Set up the first-stage and second-stage PCR reactions using the components and concentrations listed in Table 3 and cycle under the conditions listed in Table 4. 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 *Corylus* 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 *Corylus* tissue) is included.
5. Analyse the products from the first and second-stage PCRs by agarose gel electrophoresis.

#### **Recommended method: real-time PCR**

Extract total DNA from the vascular tissue (mid-veins) of leaves according to a standard protocol as described above. Refer to section 7.1.1.1 for details of setting-up real-time PCR reactions.

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

See section 7.1.1.1 for details of interpretation of results.

## **Phytoplasma positive control for PCR**

Phytoplasma DNA 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.

### **7.1.1.2.1 ‘*Candidatus Phytoplasma mali*’**

It is optional to test plants for ‘*Candidatus Phytoplasma mali*’ by nested-PCR using the universal primers P1/P7 and R16F2/R16R2, or by real-time PCR using the primer pair Phyto-F/Phyto-R (Table 2). See section 7.1.1.2 for details of test methods and interpretation of results.

## **7.1.2 Fungal isolation on media**

Isolation of regulated fungi from plants is an optional test. Plants should be tested separately. Aseptic techniques should be used throughout the test procedure.

### **7.1.2.1 *Monilinia fructigena***

#### **Recommended method**

*M. fructigena* is easily cultured onto standard media such as prune extract agar (PEA) or potato dextrose agar (PDA) from shoots/branches. The fungus can be induced to sporulate by placing sections of suspected infected tissue into a humidity chamber. Spores can be examined under the microscope (to aid identification) and transferred directly to the culture medium using a sterile needle or inoculation loop.

Alternatively, the fungus can be directly isolated from suspected infected plant material. The plant tissue should initially be surface sterilised by soaking in 1 % sodium hypochlorite for 3 to 10 minutes (the thicker the tissue the longer the soaking) and rinsing in sterile water three times. Small sections (2 to 3 mm<sup>3</sup>) of tissue should be cut out from the margin between the healthy and infected area, using a sterile scalpel, and placed onto the culture medium.

Plates from the direct spore transfers or tissue isolations should be incubated at 22°C for five days in 12hr light/12hr dark, then examined for the presence of *Monilinia* spp. Cultural characteristics can be used to determine the species of *Monilinia*, however, as many characters overlap between species (Table 7), it is recommended to confirm species identity by PCR.

Alternatively, PCR can be carried out on DNA extracted from plate cultures, or directly on mycelial threads from culture plates or suspected infected tissue. (see section 7.1.1.1 for details of test method).

#### **Interpretation of results**

The three morphologically similar species of *Monilinia* can be identified using the synoptic key described by Lane (2002).

**Table 7: Comparison of the colony and other morphological characteristics of four *Monilinia* species**

Characteristic	<i>M. fructigena</i> <sup>1</sup>	<i>M. fruticola</i> <sup>1</sup>	<i>M. laxa</i> <sup>1</sup>
Colony	Yellow/cream	Hazel/isabelline (greyish/yellow)	Hazel/isabelline (greyish/yellow)
Growth in 24 h	0-12 mm	9-20 mm	2-11 mm
Sporulation	Sparse	Abundant	Sparse
Concentric ring of spores	Sometimes	Yes	No
Colony margin lobed	No	No	Yes
Colony with rosette pattern	No	No (rare)	Yes
Rosettes with black arcs	No	No	Yes
Conidia dimension	12-34 x 9-15 µm (mean 22 x 13 µm)	8-28 x 6-19 µm	8-23 x 7-16 µm (mean 19 x 13.5 µm)
Germ tube branching	Long germ tube before branching	Long unbranched germ tube	Germ tube branches close to conidium

<sup>1</sup>Cultures grown on PDA at 22°C under 12 h dark / 12 h near UV light (320-380 nm) (Byrde & Willetts, 1977; Leeuwen *et al.*, 2002).

#### 7.1.2.2 *Phytophthora ramorum*

##### Recommended method

If plant material is suspected to be infected with *P. ramorum*, the tissue can initially be examined under the microscope. Sporangia can sometimes be seen directly on infected tissue. If no sporangia are observed, the fungus can be induced to sporulate by incubating infected plant material for 3-5 days in a humidity box.

To isolate the fungus, plant material should be surface sterilised by wiping with a tissue soaked in a 1% sodium hypochlorite solution. Small pieces of tissue (0.5-1.0 cm<sup>2</sup>) can then be taken from the junction of healthy and infected tissue. The pieces of tissue should then be rinsed in sterile distilled water for 10 seconds, followed by a wash in 50% ethanol for 10 seconds, and a final wash in sterile distilled water for 10 seconds. The tissue can then be plated on media.

P<sub>5</sub>ARP [H] media (Appendix 3), is a semi-selective media that is commonly used for *Phytophthora* spp., and on which characteristic features of *P. ramorum* can be observed. At least 4 pieces of decontaminated tissue per sample should be placed onto the media using a sterile scalpel. Plates should be incubated at between 20-25°C on a bench top, or at 20-22°C in an incubator.

##### Intepretation of results

Identification of *Phytophthora* spp. using cultural characteristics and morphological features can be difficult and time consuming. For rapid and accurate identification of *P. ramorum*, the infected tissue or isolated mycelium should be tested by PCR, (see section 7.1.1.1.2). The morphology of the fungus can be used to corroborate the PCR result.

On P<sub>5</sub>ARP [H], characters can be observed after 4-6 days at 20°C. The growth characteristics on agar and morphological features are described in detail in Werres *et al.* (2001). *P.*

*ramorum* has unique morphological features. The most distinguishing feature is the presence of large (22-72 µm) chlamydospores that are mainly terminal. The chlamydospores change from hyaline to cinnamon-brown as they mature. Other characteristic features are shown in table 8 (Smith & Cunnington, 2006).

**Table 8: Growth characteristics on *P. ramorum* on a semi-selective medium**

Character	Description of character
<b>Colony</b>	Weakly coralloid, grows within agar, little or no superficial growth
<b>Growth in 24 h</b>	2 mm (slow growing)
<b>Sporulation</b>	Sporangia are ellipsoid and in small clusters. Relatively narrow initial sporangium commonly producing secondary smaller sporangia. The sporangia are abundant on the agar surface. They are semi-papillate, caduceus, with short or no stalk. Size 20-32 x 40-80 µm.
<b>Chlamydospores</b>	More common in older colonies (7-10 days). Very large (up to 80 µm in diameter), hyaline turning to brown.

## 8. CONTACT POINT

This manual was developed by:

Dr Lisa Ward

Plant Health & Environment Laboratory

Investigation and Diagnostic Centre and Response – Tamaki

Ministry for Primary Industries

231 Morrin Road

St Johns

PO Box 2095

Auckland

Tel: +64 9 909 3015

Fax: +64 9 909 5739

Email: [peqtesting@mpi.govt.nz](mailto:peqtesting@mpi.govt.nz)

Website: <http://www.biosecurity.govt.nz/imports/plants/standards/high-value-crops>

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## Appendix 1: Symptoms of significant regulated pests of *Corylus*

### 1.1 *Anisogramma anomala*



Eastern filbert blight cankers containing rows of elliptical stromata of *Anisogramma anomala* (Courtesy J.K. Stone, reproduced with permission from the Diseases of Orchard Fruit and Nut Crops CD-ROM, 2002, APS, St Paul MN, USA).

### 1.2 *Xanthomonas arboricola* pv. *corylina*



Brown lesions on the stem of a young hazelnut tree infected with *Xanthomonas arboricola* pv. *corylina* (Courtesy M. Redpath, Waitrara Forest Farm, New Zealand).

## Appendix 2: Symptoms of nutrient deficiencies in *Corylus*

### 2.1 Boron Toxicity



Hazelnut leaves showing Boron Toxicity (Courtesy J. Olsen, reproduced with permission from the Diseases of Orchard Fruit and Nut Crops CD-ROM, 2002, APS, St Paul MN, USA).

## Appendix 3: Protocols referenced in the manual

### 3.1 Silica-milk RNA extraction protocol (Menzel *et al.*, 2002)

1. Grind 0.2-0.5 g leaf tissue (1/10; w/v) in RNA 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 micro-centrifuge 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 micro-centrifuge 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 (10mM 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 micro-centrifuge tube, being careful not to disturb the pellet. Store at -80°C.

### 3.2 Phytoplasma DNA enrichment CTAB extraction protocol (Kirkpatrick *et al.*, 1987 and modified by Ahrens & Seemüller, 1992)

1. Grind approximately 0.3 g tissue (petioles, veins) in 3 ml 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 mM EDTA (pH 8.0), 10% (w/v) sucrose, 2% (w/v) PVP-40; Adjust pH to 7.6 and filter sterilise. Just prior to use add 0.15% (w/v) Bovine Serum Albumin (BSA) and 1 mM ascorbic acid).
2. Transfer crude sap to a cold 2 ml micro-centrifuge tube.
3. Centrifuge at 4°C for 5 min at 4500 rpm.
4. Transfer supernatant into a clean 2 ml micro-centrifuge tube.
5. Centrifuge at 4°C for 15 min at 13000 rpm.
6. Discard the supernatant.
7. Resuspend the pellet in 750 µl of 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). The pellet is easier to resuspend in a smaller volume of CTAB buffer (e.g. 100 µl) then the remaining volume of CTAB buffer is added (e.g. 650 µl).
8. Incubate tubes at 55 °C for 30 min with intermittent shaking.
9. Cool the tubes on ice for 30 sec.

10. Add 750 µl chloroform:octanol (24:1 v/v) and vortex thoroughly.
11. Centrifuge at 4°C or at room temperature for 4 min at 13000 rpm.
12. Carefully remove upper aqueous layer into a clean 1.5 ml micro-centrifuge tube.
13. Add 1 volume ice-cold isopropanol and vortex thoroughly.
14. Incubate on ice for 4 min.
15. Centrifuge at 4°C or at room temperature for 10 min at 13000 rpm.
16. Discard supernatant.
17. Wash DNA pellets with 500 µl ice-cold 70% (v/v) ethanol, centrifuge at 4°C or at room temperature for 10 min at 13000 rpm.
18. Dry DNA pellets in the DNA concentrator or air-dry.
19. Resuspend in 20 µl sterile distilled water. Incubating the tubes at 55°C for 10 min can aid DNA resuspension.
20. Store DNA at -20°C for short-term storage or -80°C for long-term storage.

### **3.3 P<sub>5</sub>ARP[H] media for culturing *Phytophthora ramorum* (Jeffers & Martin, 1986)**

For 1 litre of media add 17 g of cornmeal agar. Autoclave, then cool to 50°C in a waterbath. In 10 ml of sterile distilled water, dissolve (to a final concentration) 5 mg pimaricin; 250 mg ampicillin (Na salt); 10 mg rifampicin (dissolved in 1 ml of 95% ethanol); 100 mg PCNB; 22.5 mg hymexazol. Add to cooled media before pouring. Store plates at 4°C in the dark and use within 5 days of preparation.