Diagnostics
Diagnostic

Monilinia fructicola

Specific scope

This standard describes a diagnostic protocol for *Monilinia fructicola*[1].

Specific approval and amendment

First approved in 2002–09.
Revised in 2009–09

Introduction

Monilinia fructicola is an extremely destructive disease mainly of stone fruits, which can also affect other rosaceous fruit trees (e.g. *Malus* and *Pyrus*). The disease may destroy or seriously reduce a crop by killing blossoms or by rotting mature fruits, either on the tree or after harvest. Leaves and shoots are also attacked. The severity of the disease is determined largely by the weather. Blossom blight can be expected in humid or showery weather with mild daytime temperatures (20–25°C) and cool nights. Rotting of mature fruits proceeds rapidly with high humidity and high temperatures. Three *Monilinia* species and one *Monilinia* anamorph (*Monilia* sp.) may cause brown rot, of which two (*Monilinia fructigena* and *Monilinia laxa*) have long been present in Europe. *Monilia polystroma*, an anamorph species closely related to *M. fructigena*, is only known from Japan (van Leeuwen *et al.*, 2002). *Monilinia fructicola* occurs in North and South America, Japan and Australia (EPPO/CABI, 1997). It was detected in France during surveys carried out in 2001 (OEPP/EPPO, 2002). Isolated outbreaks have been reported in Spain (OEPP/EPPO, 2006) and in a survey conducted in 2006 in the Czech Republic (OEPP/EPPO, 2008). Further spread in Europe would lead to increased crop losses, especially in peach, nectarine and apricot. Costs of control would increase and resistance to fungicides may develop (van Leeuwen *et al.*, 2001).

Identity

Name: *Monilinia fructicola* (Winter) Honey
Synonym: *Sclerotinia fructicola* (Winter) Rehm
Anamorph: *Monilia fructicola* Batra

[1]Use of names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

Taxonomic position: Fungi: Ascomycota: Helotiales
EPPO code: MONIFC.
Phytosanitary categorization: EPPO A2 list: no. 153; EU Annex designation: I/A1.

Detection

Infected blossoms turn brown and die and, if humid or wet weather continues, tufts of fungal spores are produced on the dead tissue. Shoot infection commonly follows blossom blight as the fungus grows from blighted blossoms into the adjacent twig tissue. Here it causes an area of dying bark, usually sunken, with sharp edges. Infected leaves show more or less circular brown dead areas that may later fall away to give a 'shot hole' appearance, or the entire leaf may be killed. The developing fruits can be attacked at any stage, but generally the disease does not become serious until the fruits approach maturity. Infected fruits either fall to the ground or remain attached to the tree. They become dried-out and shrivelled and are then known as mummies. Conidial sporodochia occur on all infected organs. Characteristic disease symptoms are shown in Fig. 1A,B.

The commodities that are most likely to be responsible for international spread of the pathogen are rooted plants and fresh fruits.

Identification

The *Monilinia* (*Monilia*) species causing brown rot of fruit crops are difficult to distinguish from each other. Identification is possible by combining culture characteristics, such as growth rate, growth pattern and colour, with morphological data such as the conidial dimensions and the length of the germ tube (van Leeuwen & van Kesteren, 1998; De Cal & Melgarejo, 1999). Most of

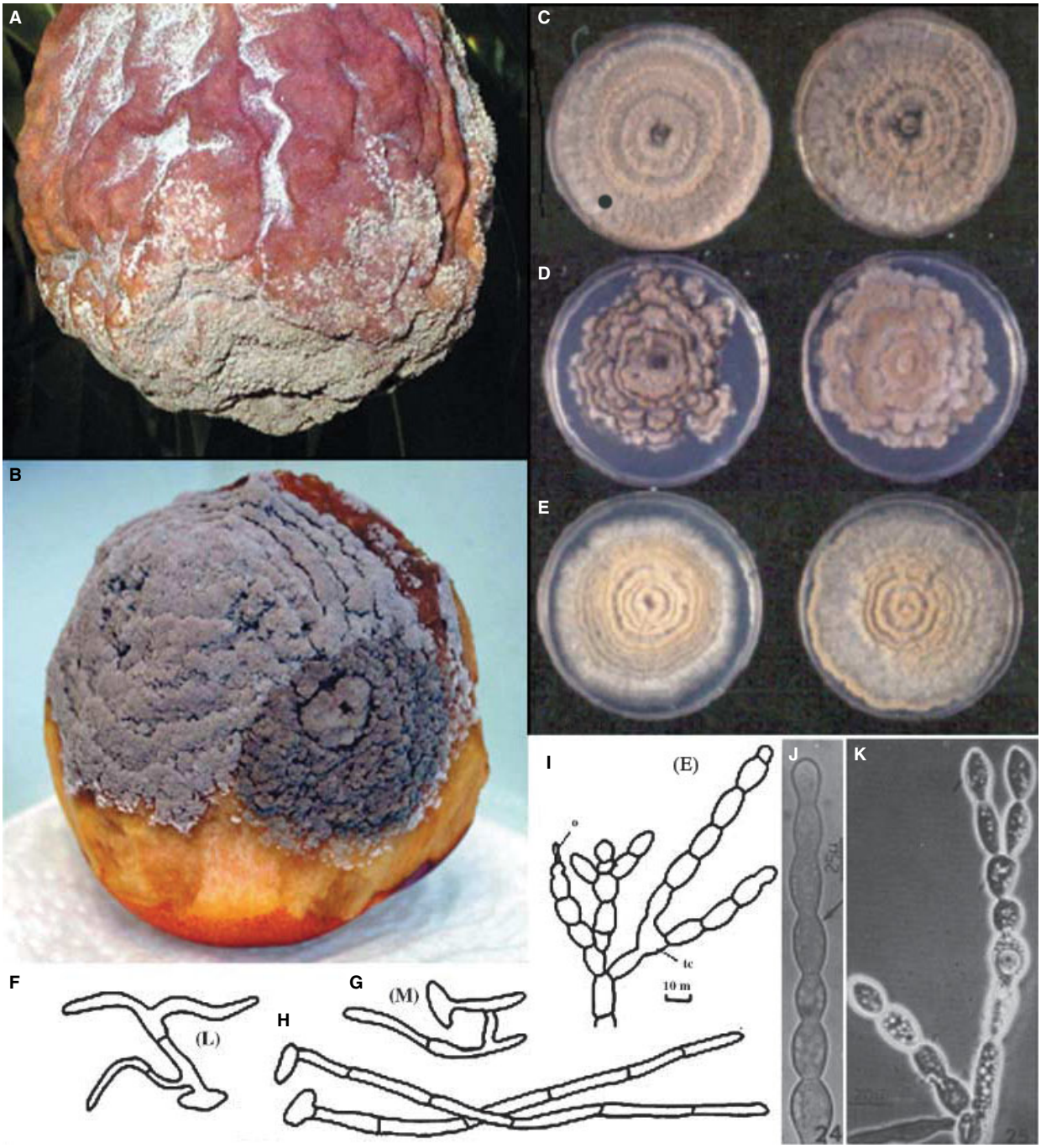

Fig. 1 Disease symptoms caused by *Monilinia fructicola* (A,B), cultural characteristics of *M. fructicola*, *M. laxa* and *M. fructigena* (C–E), mode of conidial germination in these three species (F–H), and conidial chains (I–K). (A) Sporodochia on a naturally infected and mummified peach. (B) Sporodochia on an artificially infected peach. (C) PDA cultures of *M. fructicola* have concentric rings. (D) PDA cultures of *M. laxa* produce lobed rosettes. (E) PDA colonies of *M. fructigena* do not produce rosettes and are creamy yellow rather than greyish. (F, G) Typical conidial germination of *M. laxa*. (H) Typical conidial germination of *M. fructigena* and *M. fructicola*. (I, J) Conidial chains of *M. fructicola*. *M. fructigena* (K), and *M. laxa* (not shown) look exactly like each other. A–B: Courtesy of V Mercier, Avignon (FR). C–E: Courtesy of GCM. van Leeuwen, Wageningen (NL). F–I: Byrde & Willetts (1977). (J,K): Batra (1991).

these characters are quantitative and overlap, so that identification has to be conducted under standardized conditions and starting from pure cultures. Even so, atypical isolates of *M. fructicola* may be misidentified as *M. laxa* and vice versa (van Leeuwen & van Kesteren, 1998). Consequently, classical methods alone are not adequate for phytosanitary diagnosis, which requires speed and reliability, and particularly for perishable soft fruit consignments.

The present diagnostic protocol recommends isolation of *Monilinia* spp. from the host, followed by species-specific PCR. When mycelium is present on the examined material, direct PCR is also possible.

When PCR technology is not available, cultures suspected to be *M. fructicola* should be sent to a laboratory where the test can be reliably performed, and morphology studied in detail.

Morphological identification

Isolation

For isolation, the standard procedure is to place pieces of infected material (with or without surface sterilization) on slightly acid agar media (pH 4–4.5). Presence of mixed infections of *M. fructicola* with other *Monilinia* spp. are reported, consequently different parts of the fruit should be selected for isolation.

Culture media

Potato dextrose agar (PDA) (van Leeuwen & van Kesteren, 1998; De Cal & Melgarejo, 1999).

Growth characteristics in culture

Reported growth rates for *M. fructicola* on PDA at 22°C under 12–16 h near-UV light (320–380 nm) are 9–20 mm per 24 h (De Cal & Melgarejo, 1999), with an average of approximately 13 mm per 24 h (van Leeuwen & van Kesteren, 1998). Plugs (4 mm diameter) from the edge of a 4-day-old culture grown on PDA at 22°C in the dark should be placed in the centre of two duplicate plates, and incubated for 10 days at 22°C in 12 h light/12 h dark (colonies of *M. fructicola* will fill a 9-cm-diameter plate in 6–7 days). Alternatively, 10 plates can be incubated for 5 days and the growth rate calculated from the change in diameter between 3 and 5 days. Sporulation should be profuse, in concentric rings (Fig. 1C), with the sporogenous tissue hazel to isabelline in colour (not buff, pale luteous or yellow/cream). The colony margin should be mostly entire and the colony surface even (no rosettes with black arcs). Irregular stromatal crusts and discoid sclerotia may develop on the agar surface or within the medium as colonies age. Abundant microconidia may be apparent macroscopically as black raised areas, particularly at the edge of the Petri dish. Colours of cultures should be assessed according to Rayner (1970).

Comparison with similar species

Monilinia fructigena: Colonies of *M. fructigena* have lower growth rates (about half that of *M. fructicola*) under the conditions mentioned above. The colony colour of *M. fructigena* is cream/yellow while the colony colour of *M. fructicola* is distinctly 'not cream/yellow' but hazel/isabelline ('greyish'). *M. fructigena* sporulates sparsely.

Monilinia laxa: Colonies of *M. laxa* have lower growth rates (about half that of *M. fructicola*) under the conditions mentioned above. *M. laxa* has a markedly lobed colony margin and the colonies are rosetted. Characteristic black rings/arcs are associated with the petals of the rosettes in the colony. The bottom of the dish shows black arcs or rings associated with the 'petals' (black dotted areas or brown arcs or rings can be ignored). Rosetted colonies (with the appearance of an opened flower, i.e. mycelium in distinct layers on top of each referred to as 'petals') can be recognized from above or below. Sporulation is sparse.

Monilia polystroma: Colonies of *M. polystroma* are similar to those of *M. fructigena*, except for intense formation of black stromatal plates after 10–12 days of incubation.

Table 1 summarizes results for the four species grown under standard conditions. Figure 1 illustrates the differences in cultural morphology (C–E) and conidial germination (F–H). The synoptic key of Lane (2002), based on colony characters, can be used to distinguish the three species *Monilinia fructicola*, *M. fructigena* and *M. laxa.*

Cultures grown on 4% PDA at 22°C under 12 h dark/12 h near-UV light (320–380 nm).

Morphology

Hyphae: primary hyphae thin-walled, frequently over 250 µm long and 7–10 µm wide with one or more branches initiated before the first septum. Secondary and subsequent branches are often much narrower.

Conidia: blastic, formed in chains (Fig. 1I,J) with the youngest spore at the distal end, or occasionally arthric, ellipsoid, ovoid or limniform often with truncate ends, 8–28 × 5–19 µm (mostly 12–16 × 8–11 µm), hyaline (greyish-buff in mass). On tap water agar (18 h at 25°C), most conidia form a single long unbranched germ tube of 750–900 µm (Fig. 1H). However, this may be more variable with conidia taken directly from fruit. A phialidic spermatial (microconidial) state is usually present and frequently becomes conspicuous in old colonies.

Sclerotia: discrete sclerotia are not normally formed, but infected fruits develop dry substratal stromata in which stromatic layers replace most of the pericarp.

Table 1 Comparison of the colony characters of *Monilinia* spp. from pome and stone fruits

	M. fructicola	*M. laxa*	*M. fructigena*	*M. polystroma*
Colony colour	Hazel/isabelline ('grey')	Hazel/isabelline ('grey')	Yellow/cream	Yellow/cream
Growth in 24 h	9–20 mm	2–11 mm	0–12 mm	4–9 mm
Sporulation	Abundant	Sparse	Sparse	Sparse
Concentric ring of spores	Yes	No	Sometimes	Sometimes
Colony margin lobed	No	Yes	No	No
Colony rosetted	No (rare)	Yes	No	No
Rosettes with black arcs	No	Yes	No	No

Apothecia: these are erratically formed on fallen mummified fruits in spring.

Comparison with similar species

Monilinia fructigena has larger conidia (mostly 17–21 × 10–13 µm), and often forms two germs tubes per conidium.

Monilinia laxa has conidia similar in size to that in *M. fructicola*, germ tubes are single but short (150–350 µm) and twisted.

Monilia polystroma has a similar morphology than *M. fructigena*, except that conidia are slightly smaller (13–17 × 9–11 µm) than in *M. fructigena*, and fall in the same range as those of *M. fructicola*.

Molecular methods

Several molecular diagnostic methods have been developed for *M. fructicola*. The first methods were based on the use of SSU rDNA group I intron (Fulton & Brown, 1997; Snyder & Jones, 1999). Subsequent studies showed that these methods were not reliable because some isolates of *M. fructicola* lack a group I intron in their nuclear rDNA small subunit (Fulton *et al.*, 1999). Reliable new PCR primers were developed by Hughes *et al.* (2000), Ioos & Frey (2000), Côté *et al.* (2004), Gell *et al.* (2007). Their protocols distinguish *M. fructicola*, *M. fructigena* and *M. laxa* from each other. Other PCR primers and protocols for *M. fructicola* were published by Förster & Adaskaveg (2000), Boehm *et al.* (2001) and Ma *et al.* (2003). However, these methods discriminate *M. fructicola* from *M. laxa* but have not been validated for distinguishing *M. fructicola* from *M. fructigena*. According to the authors, the PCR method of Hughes *et al.* (2000), Ioos & Frey (2000) & Côté *et al.* (2004) have been shown not to give cross-reaction with *M. polystroma*. None of the other methods have been validated for distinguishing *M. fructicola* from *M. polystroma*; this may not be a problem in the EPPO region because the latter species is only known to occur in Japan.

Real-time PCR methods have been developed by Luo *et al.* (2007) and van Brouwershaven *et al.* (2009). The first method is a SYBR Green assay, and has only been validated against *M. laxa*. The other method is a TaqMan assay validated against all four brown rot-causing *Monilinia* spp.

The PCR methods of Ioos & Frey (2000) and van Brouwershaven *et al.* are described in full in Appendices 1 and 2. When mycelium is present on the examined material, direct PCR is also possible.

Reference material

ATCC, 12301 Parklane Drive, Rockville, MD 20852-1776, USA. Fax +1 301 231 5826. Centraalbureau voor Schimmelcultures (CBS), Uppsalalaan 8, 3584 CT Utrecht (NL). Fax +31 30 251 2097.

Reporting and documentation

Guidance on reporting and documentation is given in EPPO Standard PM 7/77 (1) *Documentation and reporting on a diagnosis.*

Further information

Further information on this organism can be obtained from: CR Lane, Central Science Laboratory (DEFRA), Sand Hutton, York Y041 1LZ (GB); GCM van Leeuwen, National Reference Laboratory (NRL), PPS-Wageningen (NL).

Acknowledgements

This protocol was originally drafted by: RP Baayen, R Pieters and GCM van Leeuwen, Dutch Plant Protection Service, Wageningen (NL). KJD Hughes and CR Lane, Central Science Laboratory (DEFRA), Sand Hutton, York YO41 1LZ (GB). It was revised by LFF Kox and GCM van Leeuwen, National Reference Laboratory (NRL), Plant Protection Service, PO Box 9102, 6700 HC Wageningen (NL). Email: g.c.m.van.leeuwen@minlnv.nl

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Appendix 1 – Identification of *Monilinia fructicola* by conventional PCR (Ioos & Frey, 2000)

This protocol initially developed by Ioos & Frey (2000) was further improved and validated by a European collaborative study (Ioos & Iancu, 2008).

This test was ring-tested according to EN ISO 16140 in 2007 by 13 European laboratories through the European mycological network (EMN) regarding the molecular part of the test, excluding the extraction. The collaborative study showed that the accuracy, sensitivity and selectivity of the test were 100%, 100% and 100%, respectively. The accordance and concordance (i.e. qualitative repeatability and reproducibility) of the test were also estimated to 100% each (Ioos & Iancu, 2008).

Species specific primers have been designed. The specificity of the primers was tested with a large collection of *Monilinia* species consisting of 17 isolates of *M. laxa*, 16 isolates of *M. fructigena* and 6 isolates of *M. fructicola.* The primers have also been tested with *Botrytis cinerea* and *Sclerotinia sclerotiorum,* two fungi close to the genus *Monilinia,* and with DNA extracted from other pathogens commonly associated with brown rot on trees or fruits. No amplification was obtained with any of the fungal species tested.

1. General Information
   - 1.1. This protocol has been developed by Ioos & Frey (2000)
   - 1.2. DNA is extracted (A) from cultures or (B) from suspect fruits.
   - 1.3. The target regions are two polymorphic regions of the internal transcribed spacer region (ITS).
   - 1.4. The amplicon covers a region spanning from bases 88–108 (ITS 1) to bases 422–443(ITS 2).
   - 1.5. Amplicon size is 356 bp.
   - 1.6. Oligonucleotides: forward primer 5′-TAT-GCTCGCCAGAGGATAATT-3′ (ITS1-Mfc1), reverse primer 5′-TGGGTTT TGGCAGAAGCAC-ACT-3′ (ITS4Mfc1). Care should be taken that the primers are purified.
   - 1.7. Taq DNA polymerase at a concentration of 5 U μL^{-1}
   - 1.8. Nucleotide concentration: 0.2 μM for the oligonucleotides, 150 μM each for the dNTPs
   - 1.9. Taq polymerase buffer 20 mM Tris–HCl pH 8.4, 50 mM KCl
   - 1.10. TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH8)
   - 1.11. PCR grade water is used for all reactions
   - 1.12. PCR reactions were initially developed with a Hybaid thermal cycle model Omn-E, but other thermocyclers are suitable.
2. Methods
   - 2.1. Nucleic acid extraction and purification
   - 2.1.1. DNA is extracted (A) from cultures from a 1-cm^2 plug taken from a culture of the fungi or (B) from suspect fruits by cutting approximately 1 cm^2 of suspect tissue with a scalpel and transferred into a mL microcentrifuge tube. The sample is then ground for 2 min with two 3-mm steel or tungsten carbide beads and 400 μL of the lysis buffer, at a frequency of 30 Hz with a bead beater (Tissuelyser®; Qiagen, les Ullis, FR, or equivalent). The samples may also be ground in a mortar by a pestle with liquid nitrogen, or using other efficient grinding techniques.
   - 2.1.2. Nucleic acid extraction: DNA is extracted with the hexadecyltrimethylammonium bromide (CTAB)/proteinase K method (Henrion *et al.*, 1994). Other commercial DNA extraction kits are also suitable (Ioos, pers. comm.) and should preferably used since they require less toxic reagents.
   - 2.1.3. Fungal or infected plant tissue DNA samples are stored at less than −17°C until analysis
   - 2.2. Polymerase chain reaction – PCR (final concentration)
   - 2.2.1. Total reaction volume of a single PCR reaction: 20 μL
   - 2.2.2. PCR buffer : 1×

2.2.3. 2 mM $MgCl_2$
2.2.4. 150 µM of each dNTPs
2.2.5. 0.0375 U per µL of Taq polymerase
2.2.6. 0.2 µM forward primer
2.2.7. 0.2 µM reverse primer
2.2.8. 1–3 ng per µL of template DNA.
2.2.9. PCR grade water is added to 20 µL
2.2.10. PCR cycling parameters: 3 min at 94°C followed by 35 cycles of denaturation (94°C, 30 s), annealing (63°C, 30 s), and extension (72°C, 60 s), with a final extension (72°C, 10 min).

3. Essential Procedural Information
3.1. PCR products are separated on 1% agarose gel in 0.5× TBE buffer.
3.2. A negative control (no target DNA) should be included to ensure the absence of contamination, as well as a positive control corresponding to the limit of detection (diluted genomic DNA from a reference strain of *M. fructicola*, or diluted subcloned *M. fructicola* ITS1Mfcl/ITS4Mfcl PCR product). In addition, since two other European *Monilinia* species, namely *M. fructigena* and *M. laxa* may be present in the same ecological niche and are phylogenetically very close to *M. fructicola*, genomic DNA from reference strains of both species may be used in each PCR run as specificity controls for the PCR reaction.
3.3. The quality of the DNA extract may be assessed for instance by using an ad hoc internal amplification control or by testing the extract in PCR with the universal fungal ribosomal genes primers ITS1 and ITS4 (White *et al.,* 1990). In the latter case, the PCR conditions are those described above, simply replacing the ITS1Mfcl/ITS4Mfcl primers with ITS1 and ITS4 primers, and decreasing the annealing temperature to 50°C. A positive signal following this test would mean that the DNA extract was amplifiable: DNA was successfully extracted and a sufficiently low level of inhibitory compounds was co-extracted.
3.4. Plant samples whose DNA extract yields a 356-bp fragment should be considered as infected by *M. fructicola*. Samples whose DNA extract does not yield the expected fragment should be considered as negative only providing that the DNA extract was amplifiable and that there was no significant inhibitory effect.

Appendix 2 – Identification of *Monilinia fructicola* by real-time PCR (van Brouwershaven *et al.*)

1. General Information
1.1. Protocol developed by Van Brouwershaven *et al.* (in press.)
1.2. Nucleic acid source: mycelium.
1.3. The assay is designed to internal transcribed spacer (ITS) sequences producing an amplicon of 140 bp.
1.4. Oligonucleotides used: Forward primer Mon139F(5′-CAC CCT TGT GTA TYA TTA CTT TGT TGC TT-3′), reverse primer Mon139R (5′-CAA GAG ATC CGT TGT TGA AAG TTT TAA-3′) and dual-labelled (Taqman) probes P_fc (FAM-TAT GCT CGC CAG AGG ATA ATT-MGBNFQ and P_2_fg/lx/ps (5′-VIC-AGT TTG RTT ATT CTC TGG CGA-MGBNFQ)
1.5. Molecular grade water (MGW) is used to make up reaction mixes; this should be purified (deionised or distilled), sterile (autoclaved or 0.45 µm filtered) and nuclease-free.
1.6. 2X TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, US), containing Taq polymerase, reaction buffer containing $MgCl_2$ and nucleotides (Applied Biosystems), is used for PCR.
1.7. Amplification is performed using a real-time PCR thermal cycler, e.g. 7900 Sequence Detector (Applied Biosystems).
1.8. The analytical sensitivity (detection limit) of the assay: 0.6 pg DNA.
1.9. The analytical specificity of the assay was assessed using 11 isolates of *M. fructicola*, 10 isolates of *M. fructigena*, 6 isolates of *M. laxa*, 5 isolates of *M. polystroma* and 14 isolates of related species (*Botrytis cinerea* and *Sclerotinia sclerotium*) and fungi that can be present on stone and pome fruit. All *M. fructicola* isolates reacted positive. No cross-reactions with other species were observed

2. Methods
2.1. Nucleic Acid Extraction and Purification
2.1.1. DNA is extracted from mycelium dissected from the fruit or grown on agar plates. The mycelium (approximately 1 cm^2) is transferred to a 1.5-mL micro centrifuge tube with a secure fitting flattop cap (e.g. Superlock tubes; BIOzym TC, Landgraaf, NL) containing 1 stainless steel bead (3.97 mm in diameter) and 300 µL extraction buffer (0.02 M PBS, 0.05% Tween T25, 2% polyvinylpyrrolidone, 0.2% bovine serum albumine) The tube is placed in a bead mill (e.g. Mixer Mill MM300; Retsch, Eragny Sur Oise, FR) for 80 s at 1800 beats per min. The mixture is centrifuged for 5 s at maximum speed in a microcentrifuge (16 100 ***g***) and 75 µL of the resulting supernatant is used for DNA extraction.
2.1.2. DNA can be extracted using commercially available DNA extraction kits, e.g. DNeasy Plant Kit (Qiagen) or QuickPick Plant DNA kit (Bionobile, Parainen, FI) according to the manufacturer's instructions. The final volume of the DNA solution is 50 µL.
2.1.3. A DNA purification using spin columns filled with polyvinylpyrolidone (PVPP) is necessary for DNA isolated using the DNeasy Plant kit. The columns are prepared by filling Axygen Multi-Spin columns (Dispolab, Asten, The Netherlands) with 0.5 cm PVPP, placing it on an empty reaction tube, and washing twice with 250 µL MGW by centrifuging the col-

umn for 5 min at 4000 ***g***. The DNA suspension is applied to a PVPP column and centrifuged for 5 min at 4000 ***g***. The flow through fraction is used as input for the PCR. For DNA isolated using the QuickPick kit no DNA purification is necessary.

2.1.4. Either use extracted DNA immediately, store overnight at 4°C or at −20°C for longer periods.

2.2. Polymerase Chain Reaction

2.2.1. Master mix (concentration per 25 µL single reaction).

1X TaqMan Universal PCR Master Mix (Applied Biosystems)

0.2 µM of each primer

0.2 µM of each TaqMan probe Molecular grade water is added to 20 µL

5.0 µL extracted DNA obtained as described above.

2.2.2. PCR cycling parameters: 95°C for 10 min

40 cycles of 95°C for 15 s and 60°C for 1 min.

3. Essential Procedural information

3.1. A negative control (no DNA target) should be included in every experiment to test for contamination as well as a positive control (DNA from a reference strain of the pathogen).

3.2. It is recommended to include an extraction inhibition control used to monitor for the co-extraction of assay inhibitors. This can include testing extracted NA with a PCR-based assay known to amplify a non-target specific sequence (e.g. a conserved host gene or a 'universal' ITS gene). Alternatively, where available, a synthetic Internal Amplification Control can be used.

3.3. It is recommended to include an extraction contamination control for every batch of samples tested. This consists of performing a NA extraction using a known 'blank' sample that does not include target NA (e.g. uninfected plant material or clean extraction buffer).

3.4. A cycle threshold (Ct) value <40 with probe P_fc indicates the presence of *M. fructicola* DNA. A cycle threshold (Ct) value <40 with probe P2_fg/lx/ps indicates the presence of *M. fructigena, M. laxa* or *M. polystroma* DNA.