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Diagnostics

PM 7/18 (3) *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 and in 2019-07.

1. 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*, has been reported on several occasions in Europe on apricot, peach, apple and pear (Martini *et al*., 2015). Its geographical distribution is given in the EPPO Global Database (EPPO, 2018) and it is thought to be of probable Japanese origin (van Leeuwen *et al*., 2002).

For geographical distribution of *M. fructicola* see the EPPO Global Database (EPPO, 2018) and EFSA (2011). Further spread of this pest in the EPPO region 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). A flow diagram describing the detection/identification of *Monilinia fructicola* is given in Fig. 1.

[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.

2. Identity

Name: *Monilinia fructicola* (Winter) Honey.
Synonym: *Sclerotinia fructicola* (Winter) Rehm, *Monilia fructicola* Batra.
Taxonomic position: Fungi: Ascomycota: Helotiales.
EPPO Code: MONIFC.
Phytosanitary categorization: EPPO A2 list: no. 153.

3. Detection

3.1. Disease symptoms

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. A canker can be observed, usually sunken, with sharp edges. Infected leaves show brown dead spots 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 2A,B.

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

3.2. Requirements for laboratory sample

Symptomatic rosaceous fruits, flowers or twigs should be provided to the laboratory; mycelium present on plant material can also be collected.

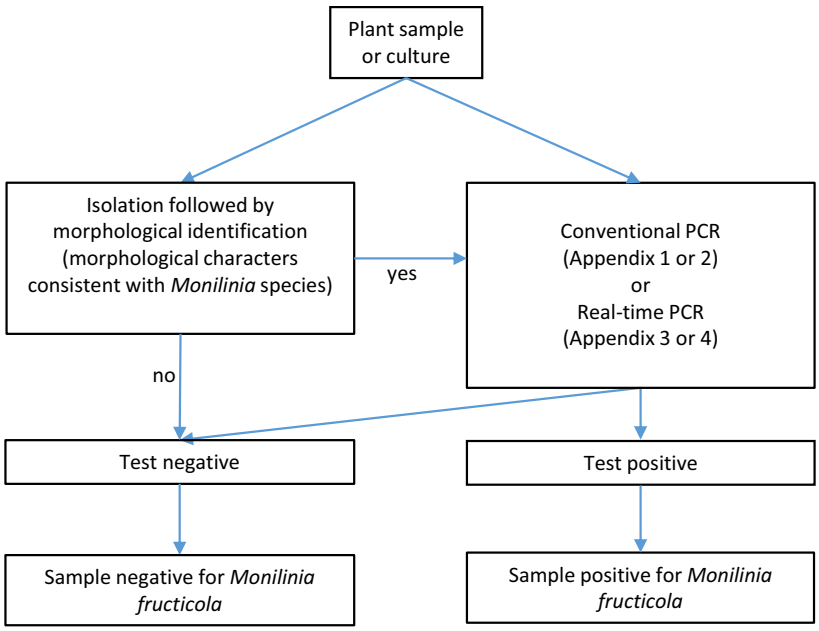

Fig. 1 Flow diagram for the diagnostic procedure for *Monilinia fructicola*. [Colour figure can be viewed at wileyonlinelibrary.com]

3.3. Isolation

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

3.3.1. Culture media

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

3.3.2. 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. 2C), with the sporogenous tissue hazel (red/brown to green/brown) to isabelline (pale grey-yellow) in colour (not yellowish/beige, pale orange/yellow, greenish yellow or yellow/cream). The colony margin should be mostly entire (uniform radial colony growth) 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).

3.3.3. 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 2 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*.

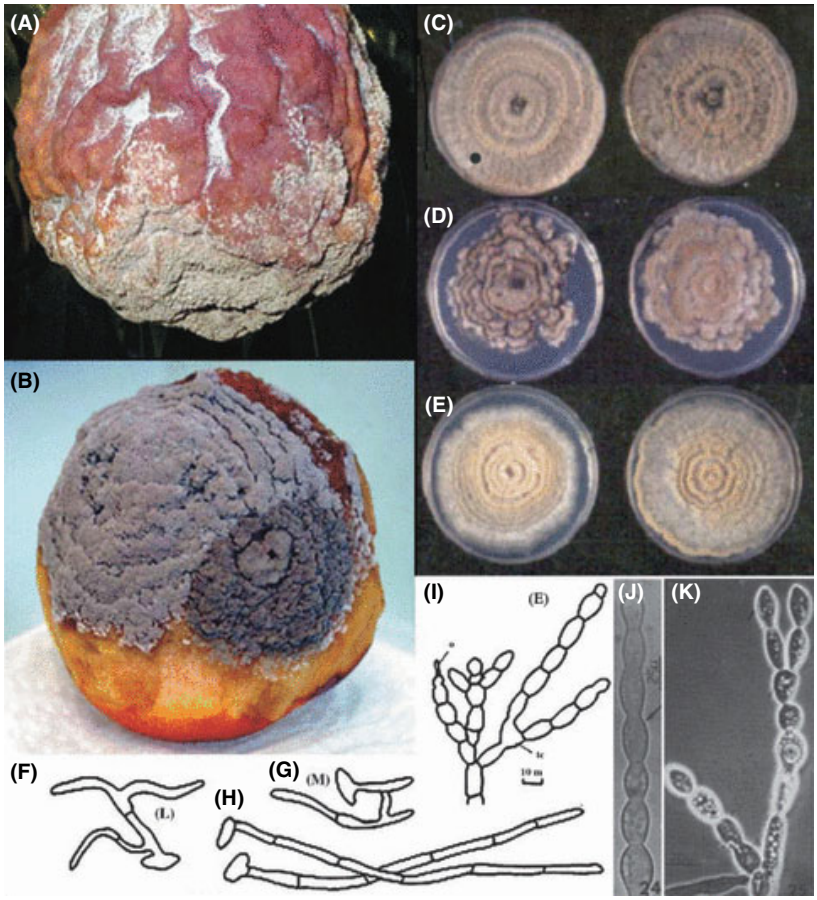

Fig. 2 Disease symptoms caused by *Monilinia fructicola* (A, B), cultural characteristics of *M. fructicola*, *M. laxa* and *M. fructigena* (C–E), mode of conidial germination in these three species (F–H), and conidial chains (I–K). (A) Sporodochia on a naturally infected and mummified peach. (B) Sporodochia 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).

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

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

4. 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 (see section 3.3), 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 these characteristics 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. The present diagnostic protocol recommends analysis by conventional or real-time PCR directly on the sample (host tissue or mycelium), or after isolation of *Monilinia* spp. from the host. The procedures for the identification of *M. fructicola* are described in the flow diagram in Fig. 1.

4.1. Morphological identification

4.1.1. Morphological characteristics

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. 2I,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-yellowish/beige 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. 2H). 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.

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

4.1.2. 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*.

4.2. Molecular methods

Several molecular tests have been developed for *M. fructicola*. The first tests were based on the use of SSU rDNA group I intron (Fulton & Brown, 1997; Snyder & Jones, 1999). Subsequent studies showed that these tests 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 PCR primers were developed by Hughes *et al*. (2000), Ioos & Frey (2000), Côté *et al*. (2004) and 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* have been 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) and 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*.

Real-time PCR tests have been developed by Luo *et al*. (2007), van Brouwershaven *et al*. (2010) and Guinet *et al*. (2016). The first test uses a SYBR Green dye and has only been validated against *M. laxa*. The two other methods use a hydrolysis probe and are validated against three or all four brown rot causing *Monilinia* spp.

The conventional PCR methods of Ioos & Frey (2000) and Côté *et al*. (2004) are described in full in Appendices 1 and 2.

The real-time PCR tests by van Brouwershaven *et al*. (2010) and Guinet *et al*. (2016) are described in full in Appendices 3 and 4.

5. Reference material

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

6. Reporting and documentation

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

7. Performance criteria

When performance criteria are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended that this database is consulted as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8. Further information

Further information on this organism can be obtained from:

Lane CR, Fera Science Limited, Sand Hutton, York Y041 1LZ (GB).

van Leeuwen GCM, National Reference Laboratory (NRL), PPS-Wageningen (NL).

Ioos R & Guinet C., ANSES, Unit of Mycology of the Plant Health Laboratory, Domaine de Pixérécourt,

CS40009, F-54220 Malzéville (FR). Email: renaud.ioos@anses.fr, cecile.guinet@anses.fr.

9. Feedback on this diagnostic protocol

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share, please contact diagnostics@eppo.int.

10. Protocol revision

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website. When errata and corrigenda are in press, this will also be marked on the website.

Acknowledgements

This protocol was originally drafted by Baayen RP, Pieters R & van Leeuwen GCM, Dutch Plant Protection Service, Wageningen, NL, and Hughes KJD & Lane CR, Central Science Laboratory, DEFRA, GB. It was first revised by Kox LFF & van Leeuwen GCM, National Reference Laboratory, NRL, Plant Protection Service, NL and by Schenck N. , ANSES, FR. The second revision was led by Ioos R. and Riccioni L. The protocol was reviewed by the Panel on Diagnostics in Mycology.

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Appendix 1 – Identification of *Monilinia fructicola* by conventional PCR (Ioos & Frey, 2000 with adaptions and validation data from Ioos & Iancu, 2008)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1 This conventional PCR is used for the detection/identification of *Monilinia fructicola* on symptomatic rosaceous fruits or a culture of a fungus.

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

1.3 The PCR primers are selected in the ITS region of the rDNA gene (sequences of the ITS region for *M. fructicola* may be retrieved from Genbank, accession numbers Z73777, Z 73778, U21815, AF010500 to AF010502).

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 used.

Forward primer ITS1Mfc1*	5′-TAT GCT CGC CAG AGG ATA ATT-3′
Reverse primer ITS4Mfc1*	5′-TGG GTT TTG GCA GAA GCA CAC T-3′

*Care should be taken that the primers are purified.

1.7 PCR reactions were initially developed with a Hybaid thermal cycle model Omn-E and Life Technologies Taq polymerase and then performed on a GeneAmp PCR System 9700 (Applied Biosystem) with Hotgoldstar Taq polymerase (Eurogentec).

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 transferring into a 2 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, Hilden, Germany, or equivalent). The samples may also be ground in a mortar by a pestle with liquid nitrogen, or by 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). Commercial DNA extraction kits are also suitable (Ioos R., pers. comm.).

2.1.3 DNA should preferably be stored at approximately −20°C or kept at 2–8°C for immediate use.

2.2 Polymerase Chain Reaction (PCR)

2.2.1 Master Mix

Reagent	Working concentration*	Volume per reaction (µL)	Final concentration
Molecular grade water*	NA	12.148	NA
Buffer (Hotgoldstar Eurogentec)	10×	2.0	1×
MgCl2	25 mM	1.6	2.0 mM
Primer ITS1MFcl	10 µM	0.4	0.2 µM
Primer ITS4MFcl	10 µM	0.4	0.2 µM
dNTPs Bovine Serum Albumin (BSA)	25 mM each 10 mg/mL	0.121.2	0.15 mM each 0.6 µg/µL
DNA polymerase (Hotgoldstar Eurogentec)	5 U/µL	0.132	0.033 U/µL
Subtotal		18	
DNA extract		2	
Total		20	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.22-µm filtered) and nuclease-free water.
NA, not applicable.

2.2.2 PCR conditions

3–10 min (depending on the type of DNA polymerase used) at 95°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. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction

and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.

- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of the nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls can be used to monitor each individual sample separately. Positive internal controls can be genes either present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample. 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 a separate PCR with the universal 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 (White *et al*., 1990), 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.
- Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism or DNA fragment (size different from the target size) amplified by the primers, introduced into the Master Mix.

3.2. Interpretation of results: in order to assign results from the PCR-based test the following criteria should be checked:

Verification of the controls

- NIC and NAC should produce no amplicon.
- PIC and PAC should produce an amplicon of 356 bp.
- If present IC, or amplification or co-amplification of a universal fungal/plant gene should produce an amplicon of the expected size (the presence of the amplicon is required only for negative *M. fructicola* results).

When these conditions are met

- A test will be considered positive if 356 bp PCR amplicons are produced.
- A test will be considered negative if no band or a band of a different size is produced.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

4.1 Analytical sensitivity data

The analytical sensitivity of the test has been published in Riccioni & Valente (2015) and is 0.5 pg of the target per PCR tube. For this evaluation, the authors have replaced the BSA by water as described in the original paper of Ioos & Frey (2000).

4.2 Analytical specificity data

The specificity was evaluated by Ioos & Frey (2000) with 39 *Monilinia* isolates: *Monilinia laxa* (17), *M. fructicola* (6) and *M. fructigena* (16), and by Riccioni & Valente (2015) with a verification of the performance criteria using 14 isolates of *M. fructicola*, 6 isolates of *M. fructigena*, 7 isolates of *M. laxa* and 1 single isolate of *M. polystroma* and using 4 different host plant tissues (apple, pear, plum and peach). DNA from all *M. fructicola* isolates was amplified and no cross-reaction was observed with DNA from the non-target species. The primers were also tested on total DNA extracted from *Botrytis cinerea* and *Sclerotinia sclerotiorum*, two fungi genetically close to the genus *Monilinia*, and from 12 other fungal pathogens commonly associated with brown rot on trees or fruits; no PCR amplification was obtained with any of the fungal species tested. For this evaluation, the authors also used water instead of BSA.

4.3 Other data

This test underwent a test performance study (TPS) 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 diagnostic accuracy, diagnostics sensitivity and diagnostic specificity of the test were all 100%. The accordance and concordance (i.e. qualitative repeatability and reproducibility) of the test were also both estimated as 100% (Ioos & Iancu, 2008). In addition, a TPS was performed for this test, following the method described in Ioos &

Frey (2000) involving 4 Italian laboratories (Riccioni & Valente, 2015). In order to evaluate the robustness of the PCR protocols, all the participants used their laboratories' own reagents. The collaborative study showed that the diagnostic accuracy, diagnostic sensitivity and diagnostic specificity were 99%, 97% and 100%, respectively. The accordance and concordance were 98% and 97%, respectively.

Appendix 2 – Identification of *Monilinia fructicola* by conventional PCR (adapted from Côté *et al.*, 2004)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1 This conventional PCR is used for the detection/identification of *Monilinia fructicola* on fruits or cultures. It was initially developed by Côté *et al*. (2004) as a multiplex PCR targeting *M. laxa, M. fructigena* and/or *M. polystroma*. It has been validated by Riccioni & Valente (2015).

1.2 The PCR primers were designed from the sequence of a DNA fragment amplified by RAPD (SCAR) from *M. fructigena*. The sequence of this DNA fragment may be retrieved from Genbank, accession number AF506701.

1.3 The amplicon for *M. fructicola* covers a region spanning bases 64–83 to bases 584–603 of the RAPD fragment.

1.4 Amplicon size is 535 bp for *M. fructicola* (the test uses a mixture of four primers and could also produce amplicons of sizes 402 bp for *M. fructigena*, 535 bp, 351 bp product for *M. laxa* and 425 bp for *Monilia polystroma*).

1.5 Oligonucleotides used

Forward primer specific for *M. fructicola* MO368-10R	5′-AAG ATT GTC ACC ATG GTT GA-3′
Forward primer specific for *M. fructigena* and *M. polystroma* MO368-8R	5′-AGA TCA AAC ATC GTC CAT CT-3′
Forward primer specific for *M. laxa* Laxa-R2	5′-TGC ACA TCA TAT CCC TCG AC-3′
Reverse primer for *M. fructicola, M. fructigena, M. laxa* and *M. polystroma* MO368-5	5′-GCA AGG TGT CAA AAC TTC CA-3′

1.6 PCR reactions were carried out in a PTC-200 DNA engine thermocycler (MJ Research, Watertown, MA, USA) or a PerkinElmer 9600 thermocycler (Applied Biosystems)

2. Methods

2.1 Nucleic Acid Extraction and Purification

2.1.1 Tissues source mycelia or symptomatic fruits. Mycelium (30–60 mg dry material or a corresponding amount of fresh weight) or approximately the equivalent of 100 µL taken from the potentially infected fruit tissue in a 1.5 mL tube with 550 µL of extraction buffer (100 mM Tris-HCL, pH 8.0, 10 mM EDTA, 2% sodium dodecyl sulphate). The material is then crushed with a disposable pestle.

2.1.2 Nucleic acid extraction: DNA is extracted with a hexadecyltrimethylammonium bromide (CTAB)/proteinase K method (see Côté *et al*., (2004) for details) or using the DNeasy Plant Mini Kit (Qiagen) or the Nucleospin II Plant kit (Macherey-Nagel).

2.1.3 The purified DNA obtained from the Nucleospin II Plant kit, when used as template for PCR, should be diluted at least 1:10 (Riccioni & Valente, 2015).

2.1.4 Extracted DNA should be stored at 2–8°C for immediate use or at less than −20°C until analysis.

2.2 Polymerase Chain Reaction (PCR)

2.2.1 Master Mix

Reagent	Working concentration*	Volume per reaction (µL)	Final concentration
Molecular grade water*	NA	5375	NA
10x Mg2+-free DyNAzyme** buffer	10×	1	1×
$MgCl_2$	50 mM	0.5	2.5 mM
Primer MO368-10R	10 µM	0.2	0.2 µM
Primer MO368-5	10 µM	0.2	0.2 µM
Primer MO 368-8R	10 µM	0.2	0.2 µM
Primer Laxa-R2	10 µM each	0.2	0.2 µM
dNTPs	10 mM	0.2	0.2 mM each
DNA polymerase **(Dynazyme II, Finnzymes)	2 U/µL	0.125	0.025 U/µL
Subtotal		8	
DNA extract		2	
Total		10	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.22-µm filtered) and nuclease-free water.
**These enzymes were used in Riccioni & Valente, 2015 (not in Côté *et al*., 2004).
NA, not applicable.

2.2.2 PCR conditions

2 min at 95°C followed by 35 cycles of denaturation (95°C, 15 s), annealing (60°C, 15 s) and extension (72°C, 60 s), with a final extension (72°C, 3 min).

3. Essential procedural information

3.1. PCR products are separated on 1.5% agarose gel in 1× TBE buffer, stained with ethidium bromide and visualized under ultraviolet (UV) light.

3.2 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample.
- Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.3. Interpretation of results: in order to assign results from the PCR-based test the following criteria should be checked:

Verification of the controls

- NIC and NAC should produce no amplicon.
- PIC and PAC should produce an amplicon of 535 bp.

When these conditions are met

- A test will be considered positive if a 535 PCR amplicon is produced.
- A test will be considered negative if no band or a band of a different size is produced (for *Monilinia* species: 402 bp for *M. fructigena*, 351 bp for *M. laxa* and 425 bp for *M. polystroma*).
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

4.1 Analytical sensitivity data

The analytical sensitivity of the test has been determined in Riccioni & Valente (2015) and is 25 pg of the target per PCR tube.

4.2 Analytical specificity data

The specificity was evaluated by Côté *et al*., (2004) with 17 isolates of *Monilinia fructicola*, with 34 isolates of 7 other species of *Monilinia* (including *M. laxa, M. fructigena* and *M. polystroma*) and with isolates of *Botrytis cinerea* and *Sclerotinia sclerotiorum*; 28 *Monilinia* spp. were also tested by Riccioni & Valente (2015) using 4 different host plant tissues (apples, pears, plums and peaches). DNA from all isolates of *M. fructicola* was amplified and no cross-reactions were observed with DNA from the other species.

4.3 Data on repeatability

The repeatability was evaluated by Riccioni & Valente (2015) analysing *M. fructicola* DNA at three concentration levels, low, medium and high: the test showed 100% repeatability.

4.4 Data on reproducibility

As for the repeatability, the reproducibility was determined in Riccioni & Valente (2015) analysing *M. fructicola* DNA at three concentration levels, low, medium and high, in different conditions: the test showed 100% reproducibility.

4.5 Other data

A test performance study was performed involving 4 Italian laboratories (Riccioni & Valente, 2015). In order to evaluate the robustness of the PCR protocols, all the participants used their laboratories' own reagents. The collaborative study showed that the diagnostic accuracy, diagnostic sensitivity and diagnostic specificity were 98%, 96% and 100%, respectively. The accordance and concordance were both 96%.

Appendix 3 – Identification of *Monilinia fructicola* by real-time PCR (van Brouwershaven *et al.*, 2010)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

This real-time PCR is used for the identification of *Monilinia fructicola* from mycelium from pure cultures or fruits. A pair of primers and two probes (the first targeting *M. fructicola* and the second targeting *M. fructigena*, *M. laxa* or *M. polystroma*) have been developed for this duplex real-time PCR.

1.1 The test was developed by van Brouwershaven *et al.* (2010).

1.2 The PCR primers and probes are selected in the ITS region of the rDNA gene.

1.3 The PCR primers produce 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′
Dual-labelled (Taqman MGB) probe P_fc	5′-FAM-TAT GCT CGC CAG AGG ATA ATT-MGBNFQ-3′
Dual-labelled (Taqman MGB) probe P_2_fgn/lx/ps	5′-VIC-AGT TTG RTT ATT CTC TGG CGA-MGBNFQ-3′

1.5 Amplification is performed using a real-time PCR thermal cycler, e.g. 7900 Sequence Detector (Applied Biosystems).

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 flat-top cap (e.g. Superlock tubes, BIOzym TC, Landgraaf, the Netherlands) 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 albumin) The tube is placed in a bead mill (e.g. Mixer Mill MM300, Retsch, Eragny sur Oise, France) 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, Finland) 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 column 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, or 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).

Reagent	Working concentration*	Volume per reaction (µL)	Final concentration
Molecular grade water*	NA	6.7	NA
1XTaqMan Universal PCR Master Mix (Applied Biosystems)	2 X	12.5	1 X
Primer Mon139F	25 µM	0.2	0.2 µM
Primer Mon139R	25 µM	0.2	0.2 µM
Probe P_fc	25µM	0.2	0.2 µM
Probe P_2_fgn/lx/ps	25 µM	0.2	0.2 µM
Subtotal		20	
DNA		5	
Total		25	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.22-µm filtered) and nuclease-free water.
NA, not applicable.

2.2.2 PCR conditions

95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/elongation 60°C for 1 min.

3. Essential procedural information

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolation and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor cross-reactions with the host tissue and/or contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of PCR grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

As alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA).
- Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract (NA). 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.

3.2 Interpretation of results

Verification of the controls

- The PIC and PAC amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met:

- A test will be considered positive for *M. fructicola* if, with the P_Fc probe, it produces an exponential amplification curve. An exponential amplification curve with probe P2_fgn/lx/ps indicates the presence of *M. fructigena, M. laxa* or *M. polystroma*.
- A test will be considered negative for *M. fructicola* if it produces no exponential amplification curve with the P_Fc probe.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

4.1 Analytical sensitivity data

The test can detect 0.6 pg of DNA from *Monilinia fructicola* (P_Fc) and of *M. fructigena* (P2 fgn/lx/ps).

4.2 Analytical specificity data

The analytical specificity of the test was assessed using 12 isolates of *M. fructicola*, 10 isolates of *M. fructigena*, 6 isolates of *M. laxa*, 5 isolates of *M. polystroma* and 13 isolates of related species (*Botrytis cinerea* and *Sclerotinia sclerotium*) and fungi that can be present on stone and pome fruit. All *M. fructicola* isolates tested positive. No cross-reactions with DNA from the other species were observed.

4.3 Data on repeatability

No data available.

4.4 Data on reproducibility

No data available.

4.5 Data on robustness

No data available.

Appendix 4 – Identification of *Monilinia fructicola* by real-time PCR (Guinet *et al.*, 2016)

The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1 This real-time PCR has been established and validated for the identification of *Monilinia fructicola*, *M. laxa* and *M. fructigena* from pure cultures (mycelium) and different types of plant tissues. Four pairs of primers and 4 probes are used for this quadruplex real-time PCR that includes an 18S Uni universal test to check the quality of the DNA template. Using the test in monoplex mode (targeting *M. fructicola*) or in duplex mode (targeting *M. fructicola* and 18S Uni loci) is possible but validation data are not available.

1.2 The protocol was established in 2016 (Guinet *et al.*, 2016).

1.3 The PCR primers and probes are selected from a DNA amplicon generated by RAPD (MO168, Côté *et al.*, 2004). The sequences of this DNA amplicon for the different species of *Monilinia* may be retrieved from Genbank, accession numbers AY456197 and KU343247 to KU343287.

1.4 Amplicon size for *M. fructicola* is 151 bp. The sizes are 151 and 159 bp for *M. fructigena* and *M. laxa,* respectively.

1.5 Oligonucleotides used

Primers and hydrolysis probe for *M. fructicola*	
Forward primer Mfcl368-F	5′-ACT AAA CGA CGC GGT AAT GG-3′
Reverse primer Mfcl368-R	5′-CTT TTA ACT TCT TAG CCG CTC CA-3′
Probe Mfcl368-P	5′-FAM-CAC GAA TGT CGT GAA AGG ATA ATG GA-BHQ1-3′
Primers and hydrolysis probe for *M. fructigena*	
Forward primer Mfgn368-F	5′-AGC ACA GCG AGT ACA ATA AGC-3′
Reverse primer Mfgn368-R	5′-TAC CCA GAC ACC ACC TCC TC-3′
Probe Mfgn368-P	5′-Cy5-TGC TCC GTA GGC AAT CGG TAA AGA-BHQ2-3′
Primers and hydrolysis probe for *M. laxa*	
Forward primer Mlx368-F	5′-CCA AGG GCT CCG TAG GTA A-3′
Reverse primer Mlx368-R	5′-TCC ACA CCG TCG AAC AAT AA-3′
Probe Mlx368-P	5′-ROX-CAG ATC GTG AAG GGC GTG AGG T-BHQ2-3′
Primers and hydrolysis probe for 18S Uni	
Forward primer 18S-UniF	5′-GCA AGG CTG AAA CTT AAA GGA A-3′
Reverse primer18S-UniR	5′-CCA CCA CCC ATA GAA TCA AGA-3′
Probe18S-UniP	5′-JOE-ACG GAA GGG CAC CAC CAG GAG T-BHQ1-3′

1.6 PCR reactions were carried out in a Rotor-Gene 6500 (Corbett Research).

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Mycelium: fungal DNA is extracted by transferring mycelium into a 2-mL microtube filled with 400 µL of 1-mm glass beads and 400 µL of AP1 Lysis buffer and 4 µL of RNase A provided with the DNeasy Plant minikit (Qiagen). The sample is homogenized (grinding step) for 30 s at 6.5 units with the FastPrep 24 instrument (MP Biomedicals), then incubated for 15 min at 65°C, and the DNA extraction is carried out following the manufacturer's instructions. Plant material: fruit skin samples (fresh fruit or mummies) are excised using, for example, a 5-mm diameter sterile cork borer. For naturally infected fruits or mummies, an entire 5-mm skin disc can be used and transferred into a 2-mL Lysing Matrix A tube (MP Biomedicals) containing one 6-mm ceramic sphere and garnet matrix. Symptomatic flowers can be used entirely and transferred individually into a 2-mL Lysing Matrix A tube. For necrotic twigs, fragments of 5 mm^2 of symptomatic woody tissue are excised and cut in smaller parts with a sterile scalpel blade, then all the fragments are transferred into a 2-mL Lysing Matrix A tube. Total DNA is extracted following the same protocol as described above for fungal DNA, except that the grinding step is extended to 2 min.

2.1.2 Nucleic acid extraction: DNA is extracted with the DNeasy Plant Minikit (Qiagen) following the manufacturer's instructions.

2.1.3 Extracted DNA should then be stored at 2–8°C for immediate use or at −20°C or less until analysis.

2.2. Polymerase chain reaction

2.2.1. Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	NA	6.8	NA
qPCR Mastermix No Rox (Eurogentec)	2×	10	1×
Forward primers** (each of the 4 forward primers)	40 µM each	0.1 × 4	0.2 µM
Reverse primers** (each of the 4 reverse primers)	40 µM each	0.1 × 4	0.2 µM
Probes** (each of the 4 probes)	10 µM each	0.1 × 4	0.05 µM
Subtotal		18	
DNA		2	
Total		20	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.22-µm filtered) and nuclease-free water.

**The primers and probes targeting *M. fructicola* may be used alone, simply replacing the other primers and probe with PCR grade water.

NA, not applicable.

2.2.2 PCR conditions

95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/elongation 60°C for 1 min.

3. Essential procedural information

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolation and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor cross-reactions with the host tissue and/or contamination during

nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of PCR grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection (LOD).

As alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls can be used to monitor each individual sample separately. Positive internal controls can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:
- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA). This is the case in the quadruplex reaction described above (18S Uni primers/probe combination).
- Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls
- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. In the quadruplex reaction described the 18S Uni universal test is used to check the quality of the DNA template and the absence of inhibitory effects. In Guinet *et al*. (2016), a cut-off (Ct) value for the 18S Uni test was preliminarily determined for the apple fruit matrix, beyond which DNA was deemed unsuitable for PCR analysis (presence of inhibitory compounds, poor DNA yield, etc.). It was determined by computing the mean and standard deviation (SD) of the 18S Uni Ct values obtained with DNA from 30 samples; a DNA was considered as suitable for amplification when its 18S Uni Ct value was below the mean + 2SD.

3.2 Interpretation of results

Verification of the controls
- The PIC and PAC amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met
- A test will be considered positive for *M. fructicola* if, with the Mfcl-368F/R/P primers and probe, it produces an exponential amplification curve.
- A test will be considered negative for *M. fructicola* if, with the Mfcl-368F/R/P primers and probe, it produces no exponential amplification curve. In this case, the 18S Uni test should be positive, if not the quadruplex test should be repeated with a 1/10 or 1/100 dilution of the DNA extract.
- Tests should be repeated if any contradictory or unclear results are obtained.

For *M. fructigena* and *M. laxa*, the same procedure should be followed for the interpretation of results.

4. Performance criteria available

These performance criteria have been assessed with the quadruplex real-time PCR.

4.1 Analytical sensitivity data

The test can detect as little as 438 plasmidic copies (pc) of the target DNA for *Monilinia fructicola*. The analytical sensitivity is 480 and 474 pc per PCR tube for *M. fructigena* and *M. laxa*, respectively.

4.2 Analytical specificity data

The analytical specificity of the assay was assessed using 42 isolates of *Monilinia* spp., including 5 isolates of *M. polystroma*. It was also tested on 13 isolates of fungi genetically close to *Monilinia* or frequently found on fruits. The assay proved to be 100% specific for the 3 species-specific primers/probe combinations.

4.3 Data on repeatability

Using target plasmidic DNA set at concentrations ranging from 10 times to 1000 times the limit of detection, intraassay coefficients of variation (CV) ranged from 0.49 to 1.50, 2.76 to 5.08 and 0.78 to 1.32% for *M. fructicola, M. laxa* and *M. fructigena*, respectively.

4.4 Data on reproducibility

Using target plasmidic DNA set at concentrations ranging from 10 times to 1000 times the limit of detection, interassay coefficients of variation (CV) ranged from 1.91 to 1.94, 5.02 to 6.32 and 1.40 to 1.68% for *M. fructicola, M. laxa* and *M. fructigena*, respectively.

4.5 Data on robustness

The robustness of the assay was assessed with cocktails of target DNA with concentrations close to the LOD: (a) by varying the reaction volume (±10%), the largest difference between mean Ct values was still inferior to

0.79, (b) with very few exceptions, increasing or decreasing the volume of DNA template by 10% did not significantly affect the mean Ct value, regardless of the target species and concentration, (c) with a 2°C variation of the hybridization and polymerization temperature, the mean Ct variation never exceeded 2.23, which meant that each pathogen would be still detected, even at the lowest concentration level, (d) in the least stringent conditions (i.e. –10% reaction volume, +10% increase of template DNA volume, or −2°C decrease of hybridization temperature) no cross-reactions were observed with DNA from non-target species.