Proficiency of real-time PCR detection of latent Monilinia spp. infection in nectarine flowers and fruit

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Summary. Rapid and reliable detection of Monilinia latent infections is needed to prevent and control dispersion of Monilinia spp. in infected localities and non-infected countries. A fast multiplex quantitative real-time PCR method (qPCR) for the detection and identification of Monilinia spp. latent infections in blossoms and fruit of nectarine trees (Prunus persica var. nucipersica) was tested in an inter-laboratory trial. The test performance study involving five laboratories was conducted to validate the sensitivity and specificity of several real-time PCR platforms for the detection of low amounts of Monilinia DNA (latent infections), using a common protocol, and to identify possible difficulties when these tests were implemented by diagnostic laboratories or national reference centres. The method has two hydrolysis probes distinguishing between Monilinia fructicola and M. fructigena/M. laxa. Validation included test performance accuracy, analytical specificity and sensitivity, repeatability, and reproducibility, as defined by standard PM7/98 of the European Plant Protection Organization (EPPO). All qPCR platforms detected Monilinia latent infections and mycelium samples with both hydrolysis probes, and healthy flowers and fruit samples gave negative results. The method specificity was consistent between different laboratories, despite different equipment used, and there were no laboratories with z-scores in the unacceptable region. Monilinia fructicola latent infection samples were correctly detected by all laboratories, but some M. laxa samples were cross-detected as if they were M. fructicola. Monilinia laxa cross-detection could be compensated by including the allelic discrimination step in qPCR runs, which permitted differentiating between M. fructicola and M. laxa samples. The inter-laboratory comparison demonstrated the robustness of the developed method and confirmed in-house validation data. This method could be used to detect latent infections of Monilinia in asymptomatic nectarine fruit and flowers.

Key words: brown rot, qPCR, inter-laboratory validation, performance assessment, sensitivity, specificity.

Introduction

Brown rot is an economically important disease of stone fruit, responsible for substantial pre-harvest and post-harvest losses caused by the fungi Monilinia fructicola, M. fructigena, and M. laxa (Byrde and Willetts, 1977). Monilinia fructicola is not considered a quarantine pathogen in the European Union since 2014 (European Commission, 2014), but is still considered as an A2 quarantine pest by the rest of the European and Mediterranean Plant Protection Organization (EPPO, 2009). Detection of the different Monilinia spp. is important for eradication and sur-
Monilinia spp. can produce visible infections when ambient conditions are favourable for the infection, or the fungi remain latent until favourable conditions are present (Byrde and Willetts, 1977). *Monilinia fructicola* and *M. laxa* have been detected as latent infections in fruit of peach, nectarine and plum (Northover and Cerkauskas, 1994; Emery et al., 2000; Fourie and Holz, 2003a, 2003b; Gell et al., 2008). Tissues of mature nectarine fruit with latent *M. fructicola* infections are characterized by the presence of subcuticular intercellular hyphae (Garcia-Benitez et al., 2016). The incidence of latent *Monilinia* infections in harvested fruit ranges from 0 to 30%, and may be as high as 50% (Emery et al., 2000; Luo et al., 2001; Luo and Michailides, 2001, 2003). Most stone fruit with latent *Monilinia* infections do not develop visible signs of disease until they arrive at consumer markets or after sale. Hence, latent infections constitute a source for the diffusion of brown rot (Gell et al., 2008, 2009; Villarino et al., 2013), and detecting a latent *Monilinia* infections in stone fruit is crucial for preventing spread of these pathogens to countries and/or regions.

There are several PCR-based methods for diagnosis of brown rot and/or detecting the different *Monilinia* species, but these methods require the macroscopic presence of fungal structures (Fulton and Brown, 1997; Förster and Adaskaveg, 2000; Hughes et al., 2000; Ioos and Frey, 2000; Boehm et al., 2001; Ma et al., 2003; Côté et al., 2004; Gell et al., 2007). There are three real-time PCR based methods used for asymptomatic samples. One uses the SYBR™ green intercalation agent and is only validated for *M. fructicola* (Luo et al., 2007), the second uses hydrolysis probes in a duplex detection to differentiate between *M. fructicola* and other *Monilinia* species (van Brouwershaven et al., 2010), and the third uses hydrolysis probes in a tetraplex assay that enables the simultaneous detection of *M. fructicola*, *M. fructigena*, and *M. laxa* (Guinet et al., 2016). In the EPPO Bulletin (2009), the use of the PCR method developed by Ioos and Frey (2000) or the real-time PCR method developed by van Brouwershaven et al. (2010) are suggested for distinguishing *Monilinia* spp. However, neither the PCR nor the real-time PCR-based methods have been used to detect latent *Monilinia* infections.

Recently, a qPCR-based method has been reported (Garcia-Benitez et al., 2017) that is able to detect latent *Monilinia* infections in the blossoms and fruits of nectarine (*Prunus persica* var. *nucipersica*), and to distinguish among the *Monilinia* spp. in the infections. The method was based on the real-time PCR method previously described and tested by van Brouwershaven et al. (2010) for *Monilinia* identification on fruit with visible infections, and was adapted for detecting latent infections. Artificial latent infections were established in nectarine flowers and fruit for development of this method. The frequencies of latent *Monilinia* infections in the flowers and fruit detected using qPCR, and using the overnight freezing-incubation technique (ONFIT), were compared. The qPCR-based method was more sensitive, reliable and rapid than ONFIT for detecting latent *Monilinia* infections (Garcia-Benitez et al., 2017).

The aim of the present study was to compare the sensitivity and specificity of several real-time PCR platforms for the detection of low concentrations of *Monilinia* DNA (latent infections), on diverse plant material, using a common protocol. This is crucial for determining the transferability of a real-time PCR protocol for detection of *Monilinia* latent infections, based on the research of van Brouwershaven et al. (2010) and modified by Garcia-Benitez et al. (2017), as a tool for *Monilinia* latent infection risk quantification on imported or/and exported fruit. This evaluation was done through international inter-laboratory evaluation.

**Materials and methods**

**Design of the study**

The qPCR-based method proposed by Garcia-Benitez et al. (2017) for the detection of *Monilinia* spp. latent infections in fruit and flowers was tested across five different laboratories in five countries (France, Italy, Lithuania, Spain, and Turkey). Each laboratory analysed ten identical blind samples following pre-specified working protocols and data collection procedures. This “ring test” was carried between September 2015 and September 2016, from sample preparation to data statistical analysis and final report.

To ensure homogeneity and avoid quarantine organism manipulation, sample preparation and DNA extraction was carried out in the scheme organizer.
laboratory. Samples were then shipped by fast courier with the rest of the required reagents inside boxes with dry ice, to the other participant laboratories. A working-protocol and a data sheet to record results were sent by e-mail to the participant laboratories.

Sample preparation

Shipped samples contained DNA from: uninfected nectarine fruit, uninfected nectarine flower, nectarine fruit with a latent infection by *M. fructicola*, nectarine flower with a latent infection by *M. fructicola*, nectarine fruit with a latent infection by *M. laxa*, nectarine flower with a latent infection by *M. laxa*, *M. fructicola* mycelia, *M. laxa* mycelia, and a mixture of *M. fructicola* and *M. laxa* mycelia. Samples were designed to give low or high quantification cycle values (Cq = 17 or 35, respectively). Latent infections on flowers and fruit were artificially induced with cold storage following a previously described protocol (Garcia-Benitez et al., 2016).

Genomic DNA from whole lyophilised flowers or 20 mg of pooled-lyophilised fruit epidermis (1 mm thick), was extracted using the DNeasy® Plant Mini Kit (Qiagen GmbH) in accordance with the manufacturer’s instructions. Eighteen μL DNA aliquots were prepared and then lyophilized in a laboratory freeze dryer (Cryodos -50, Azbil Telstar Technologies, SLU).

Other items supplied by the organizer laboratory

The reagents needed for the qPCR assay included: nuclease-free water, 2 × GoTaq® probe qPCR Master Mix (Promega Corporation), Mon139F and Mon139R primers, and P_fc and P2_fgn/lx/ps hydrolysis probes (van Brouwershaven et al., 2010) obtained from Integrated DNA Technologies Inc. The hydrolysis probes were labelled with different reporters and quenchers from those used by van Brouwershaven et al. (2010). A FAM reporter and a ZEN / Iowa Black FQ quencher were used for *M. fructicola* probe (P_fc) instead of a FAM-TAMRA, and a HEX reporter with a ZEN / Iowa Black FQ quencher was used instead of a VIC-TAMRA for *M. fructigena*, *M. laxa*, and *M. polystroma* probe (P2_fgn/lx/ps).

qPCR conditions

Genomic DNA from the samples (10 ng) was amplified in 20 μL reaction mixture, which contained 1× GoTaq® probe qPCR Master Mix, 200 nM of each of the primers (Mon139F and Mon 139R), and 200 nM of each of the probes (P_fc and P2_fgn/lx/ps). Thermal cycling was carried out using the real-time PCR platform of each laboratory. These platforms were: Applied Biosystems® 7500 Fast Real-Time PCR (Thermo Fisher Scientific); CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.); LightCycler® 480 Real-Time PCR System (F. Hoffmann-La Roche AG); Mastercycler® RealPlex² (Eppendorf AG); and Rotor-Gene Q (Qiagen GmbH). The cycle conditions conditions were: polymerase activation at 95°C for 10 min, followed by 40 amplification cycles at 95°C for 15 s and 60°C for 1 min. Emission was measured at the annealing-extension step. The threshold value was set at a fluorescence (ΔRn) of 23,000 or automatically for those qPCR platforms for which that value was too high. A quantification threshold (Cq) value below 40 was scored as a positive detection. Additionally, due to cross-detection of *M. laxa* when using the P_fc probe detected in initial testing, an allelic discrimination step was added when the real-time PCR platform permitted, to distinguish between *M. fructicola* and *M. laxa* isolates, and to identify mixtures of the *Monilinia* isolates.

Data collection and analyses

Collaborating laboratories were asked to record the Cq value and the standard deviation of each sample with each hydrolysis probe in the results data-sheets and send these with the raw data to the organizer laboratory. For validation of the qPCR assay, the following conditions had to be met: the negative control (DNase- and RNase-free water) yielded no target signal and the *M. fructicola* and *M. laxa* mycelium samples yielded positive signals with their corresponding probes. The results were transformed into qualitative results, detection (Cq < 40) and negative detection (Cq undetermined) to compare between laboratories and techniques.

Results of the assay specificity were calculated as a percentage of false positive and false negative results for each of the hydrolysis probes (P_fc and P2_fgn/lx/ps) (Broeders et al., 2014). The false positive and negative rates are calculated as follows:

- False positive rate is the number of misclassified known positive samples divided by the total number of known positive samples.

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Results

All participating laboratories results were transformed into qualitative results (Table 1). All qPCR platforms detected Monilinia in latent infection and mycelium samples with both hydrolysis probes, but not on healthy flowers and fruit (Table 1).

Monilinia fructicola samples were correctly detected by all five laboratories and qPCR platforms with hydrolysis probe P_fc (Table 1). However, the M. fructicola samples were also cross-detected by the hydrolysis probe P2_fgn/lx/ps, as if they were M. laxa, when using the Rotor-Gene Q platform (Table 1). Monilinia laxa samples were detected with the P2_fgn/lx/ps probe by all qPCR platforms, except for the low-concentration M. laxa latently infected flower sample that was not detected by the LightCycler® 480 Real-Time PCR System (Table 1). With the exception of the LightCycler® 480 Real-Time PCR System, the other qPCR platforms cross-detected M. laxa samples with the probe P_fc, as if they were M. fructicola (Table 1). Two of the four platforms, Applied Biosystems® 7500 Fast Real-Time PCR and CFX96 Touch™ Real-Time PCR Detection System, cross-detecting M. laxa as M. fructicola could add an allelic discrimination step to the qPCR assay, differentiating between M. fructicola and M. laxa samples (Table 1).

The specificity of the qPCR results was tested using the qualitative data, calculating the percentages of false positive and false negative results. Neither the P_fc probe, nor the P2_fgn/lx/ps probe were specific, since the false positive rates (respectively 0% and 5%), and/ or the false negative rates (respectively, 20% and 10%) were greater than 0%. The greatest bias came from the Rotor-Gene Q platform, which was not able to differentiate between M. fructicola and M. laxa with either hydrolysis probe adding 10% to each false negative rate. The P_fc probe was specific for LightCycler® 480 Real-Time PCR System and for Applied Biosystems® 7500 Fast Real-Time PCR and CFX96 Touch™ Real-Time PCR Detection System, when the allelic discrimination step was incorporated into the qPCR assay. However, the LightCycler® 480 Real-Time PCR System was not able to detect the M. laxa latently infected flower sample with low concentration of M. laxa DNA, adding a 5% false positive rate for the P2_fgn/lx/ps probe.

A comparison between qPCR detection and ONFIT detection was made with the data provided by the laboratories, obtaining the computed values for diagnostic sensitivity (100±2%), diagnostic specificity (72±13%), and relative accuracy (82±17%). The qPCR method was as sensitive as the ONFIT method, since there was no negative deviation between meth-
ods. However, because the qPCR method detected 15 more positive samples than the ONFIT method, both the relative accuracy and diagnostic specificity of the qPCR-method were less than 100%.

Z-scores were calculated from the data to determine the qPCR platforms producing out of line results with respect to the rest (Figure 1). Only the LightCycler® 480 Real-Time PCR System produced results with z-scores between 2 and 3, and therefore subject to revision (Figure 1). This slight deviation of the z-scores for the LightCycler® 480 system was because the sample Cq values were consistently greater than those obtained with the rest of the platforms. This also explains why the M. laxa low DNA concentration sample (latently infected flower) was not detected, because this Cq was out of range. The rest of the platforms scored z-scores in the acceptable region between -2 and 2 (Figure 1). There were no laboratories with z-scores in the unacceptable region.

Discussion

A real-time PCR method for detection of Monilinia latent infections was tested through an international inter-laboratory trial, where all qPCR platforms detected Monilinia latent infections and mycelia on nectarine flower and fruit samples. The qPCR method performed as expected by in house validation. The assay was sensitive, and results were consistent, even when tested under different conditions (time, equipment, location, analyst), and was therefore reproducible between different laboratories. No qualitative results and data interpretation differences were observed between five different qPCR platforms used, even though Cq values differed. In addition, the assay was simple to use and can be performed by any plant pathology laboratory equipped with a real-time PCR platform.

The qPCR method was at least as sensitive as the ONFIT. It detected all the latent infections detected in the samples tested.
qPCR detection of Monilinia latent infection

by ONFIT and 15 additional infections that remained undetected by ONFIT. The same was observed in the previous study of Garcia-Benitez et al. (2017), where qPCR detected 67% more latent infections than ONFIT. Garcia-Benitez et al. (2017) also showed that the latent infection detection qPCR-based method is more consistent than ONFIT since the number of replicates scoring positive detections was greater, especially when it was used for detecting latent infections caused by M. fructigena and M. laxa. This makes qPCR a good method to detect latent infections and/or low DNA concentrations of Monilinia spp. Furthermore, the time required to detect the fungal pathogens in latently infected flowers and fruit using this qPCR-based method is between 24 and 48 h, whereas the ONFIT method required 7 to 9 d of sample preparation and incubation, plus additional time to identify the specific Monilinia spp. using PCR or another molecular method (Garcia-Benitez et al., 2017). The rapid detection of latent fungal infections is very important for predicting outbreaks of brown rot in fruit after harvest and/or after storage (Thomidis and Michailides, 2010).

No unacceptable z-scores for any of the qPCR platforms were obtained, but not all the tested qPCR platforms performed as expected. Cross-detection of M. fructicola occurred in the Rotor-Gene Q system, while cross-detection of M. laxa appeared in Figure 1. Z-scores for the detection of different Monilinia fructicola (A) and M. laxa (B) samples with P_fc and P2_fgn/1x/ps hydrolysis probes, for each of five laboratories. Z-scores between 2 and -2 are acceptable (dotted line), those greater than 3 or lower than -3 are unacceptable (solid line) and those between the lines need to be reviewed.
four of the five qPCR platforms, the exception being the LightCycler® 480 Real-Time PCR System. Other studies have reported some differences between qPCR platforms while studying virus or bacterial infections (Kamihira et al., 2010; Ebentier et al., 2013). However, it is more common to observe the same detection results in different platforms. For example, Agren et al. (2013) tested five methods for detection of Bacillus anthracis in five qPCR platforms without finding differences between the results of ABI 7500 Fast Real-Time PCR and LightCycler® 480 Real-Time PCR systems. Little variability between results obtained with those two systems was also observed by Braun-Kiewnick et al. (2016) while testing a Meloidogyne enterolobii qPCR detection method across seven laboratories. These results indicate that the influence of the qPCR platforms depends on: (i) the region of amplification, primer, and probe DNA sequences; (ii) extraction matrices; (iii) organism; or other factors. This highlights the need to test qPCR methods in several platforms to determine their reproducibility. Monilinia laxa cross-detection was compensated with an allelic discrimination step. However, allelic discrimination is not a feature common to all qPCR platforms, and is normally used for other purposes. Thus, we consider that the P_fc hydrolysis probe should be modified, to avoid this cross-detection.

Several inter-laboratory trials evaluating detection and diagnostic methods for plant pathogens using molecular methods, such as conventional PCR, qPCR, or LAMP, have been carried out or are on-going inside the Euphresco initiative, which encourages cooperation among European diagnostic laboratories for method testing (EPPO, 2016). Inter-laboratory trials are considered essential across several biological and chemical scientific disciplines for method validation, and determination of reproducibility and robustness (ISO, 2005; Thompson et al., 2006; European Network of GMO Laboratories, 2011; AOAC INTERNATIONAL, 2012; Broeders et al., 2014; Magnusson and Ornemark, 2014). To facilitate this validation, the present study only tested one DNA extraction method and one qPCR method, and all reagents for the qPCR were provided by the organizer laboratory to the rest of the participants. In addition, a previous test performance study on M. fructicola and M. laxa mycelium detection by real-time PCR was carried out to provide the range of C_q values for the qPCR method and the limit of detection.

In conclusion, this inter-laboratory comparison confirmed the in-house validation of the qPCR-method developed by van Brouwershaven et al. (2010) and modified by Garcia-Benitez et al. (2017) for detection of Monilinia spp. latent infections in asymptomatic nectarine fruit and flowers. This method could be used as a tool for quantifying Monilinia spp. latent infection risk on imported and/or exported fruit, implementation of phytosanitary measures, and surveys or monitoring studies for brown rot pathogen distribution, spread, and survival. Additional investigation of new primers and probes for Monilinia species identification should be conducted to make identification more transferable among qPCR platforms and laboratories.

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