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RESEARCH PAPERS

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-

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veillance programmes, both in infected areas and in countries free of the pathogen to control dispersion of *Monilinia* spp.

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 symptomatic 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 (C_q = 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. Eightteen 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® RealPlex^2 (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 (C_q) 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 C_q value and the standard deviation of each sample with each hydrolysis probe in the results datasheets 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 (C_q < 40) and negative detection (C_q 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.

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

  The qualitative results of the qPCR-method were compared against those obtained with the ONFIT-method, following EPPO bulletin (2014). This method is normally used to compare a new method against a validated one. Even though the ONFIT method in not validated, it is routinely used in the laboratories for latent infection detection. The comparison of both methods is based in the positive agreement (P_a), negative agreement (N_a), positive deviation (P_d), and negative deviation (N_d) between results. The studied parameters were as follows:
- Relative accuracy (A) of the method, which represents the correlation between the results obtained with ONFIT and those obtained with qPCR. This was calculated by using $A = (P_a + N_a) / (P_a + P_d + N_d + N_a)$.
- Diagnostic specificity (S_p) of the method, which provides an estimation of the ability of the qPCR to not detect the target when it is not detected by ONFIT. This was calculated by $Sp = N_a / (N_a + P_d)$.
- Diagnostic sensitivity (S_e) of the method, which provides an estimation of the ability of the qPCR to detect the target when it is detected by ONFIT. This was calculated by $S_e = P_a / (P_a + N_d)$.

To assess the proficiency of the method, "The International Harmonized Protocol for the proficiency testing of analytical chemistry laboratories" (IUPAC Technical Report) (Thompson *et al.*, 2006) was followed. The use of the *z*-scores was limited to identify those laboratories producing results out of line. The *z*-scores are calculated to assess the results of each sample for each participant. The *z*-score was calculated by $z = (x - x_a) / \sigma_p$, where x is the result obtained by the participant, x_a is the "assigned value" for that sample and σ_p is the fitness-for-purpose basis "standard deviation for proficiency assessment". The assigned value for each analysed sample was determined by the consensus of the participants using the Hubert robust mean, and the robust standard deviation of the participants' results were used as σ_p.

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-

Table 1. Qualitative results of the qPCR assays of blind *Monilinia* and "healthy" samples, performed by five participating laboratories with different real-time PCR platforms for each hydrolysis probe.

Sample	Expected results		PCR platform										ONFIT
			ABI 7500 FAST		CFX96 Touch		LC 480		Mastercycler RealPlex^2		Rotor-Gene Q		
	P1[c]	P2[d]	P1[c]	P2[d]	P1[c]	P2[d]	P1[c]	P2[d]	P1[c]	P2[d]	P1[c]	P2[d]	
M. fructicola mycelia	+	-	+	-	+	-	+	-	+	-	+	+	+
M. fructicola LIF[a]	+	-	+	-	+	-	+	-	+	-	+	+	+
M. fructicola LIN[b]	+	-	+	-	+	-	+	-	+	-	+	+	+
M. laxa mycelia	-	+	+[e]	+	+[e]	+	-	+	+	+	+	+	+
M. laxa LIF[a]	-	+	+[e]	+	+[e]	+	-	-	+	+	+	+	-
M. laxa LIN[b]	-	+	+[e]	+	+[e]	+	-	+	+	+	+	+	-
Healthy flower	-	-	-	-	-	-	-	-	-	-	-	-	-
Healthy nectarine	-	-	-	-	-	-	-	-	-	-	-	-	-
Mixed-mycelia	+	+	+	+	+	+	+	+	+	+	+	+	+
Nuclease-free water	-	-	-	-	-	-	-	-	-	-	-	-	

[a] LIF: latently infected flower.
[b] LIN: latently infected nectarine.
[c] P1/P_fc Hydrolysis probe with a FAM reporter and a ZEN / Iowa Black FQ quencher for *M. fructicola* detection.
[d] P2/P2_fgn/lx/ps Hydrolysis probe with a HEX reporter and a ZEN / Iowa Black FQ quencher for *M. fructigena*/*M. laxa*/*M. polystroma* detection.
[e] Negative after examining the allelic discrimination results.

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 C_q 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 C_q 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 C_q 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

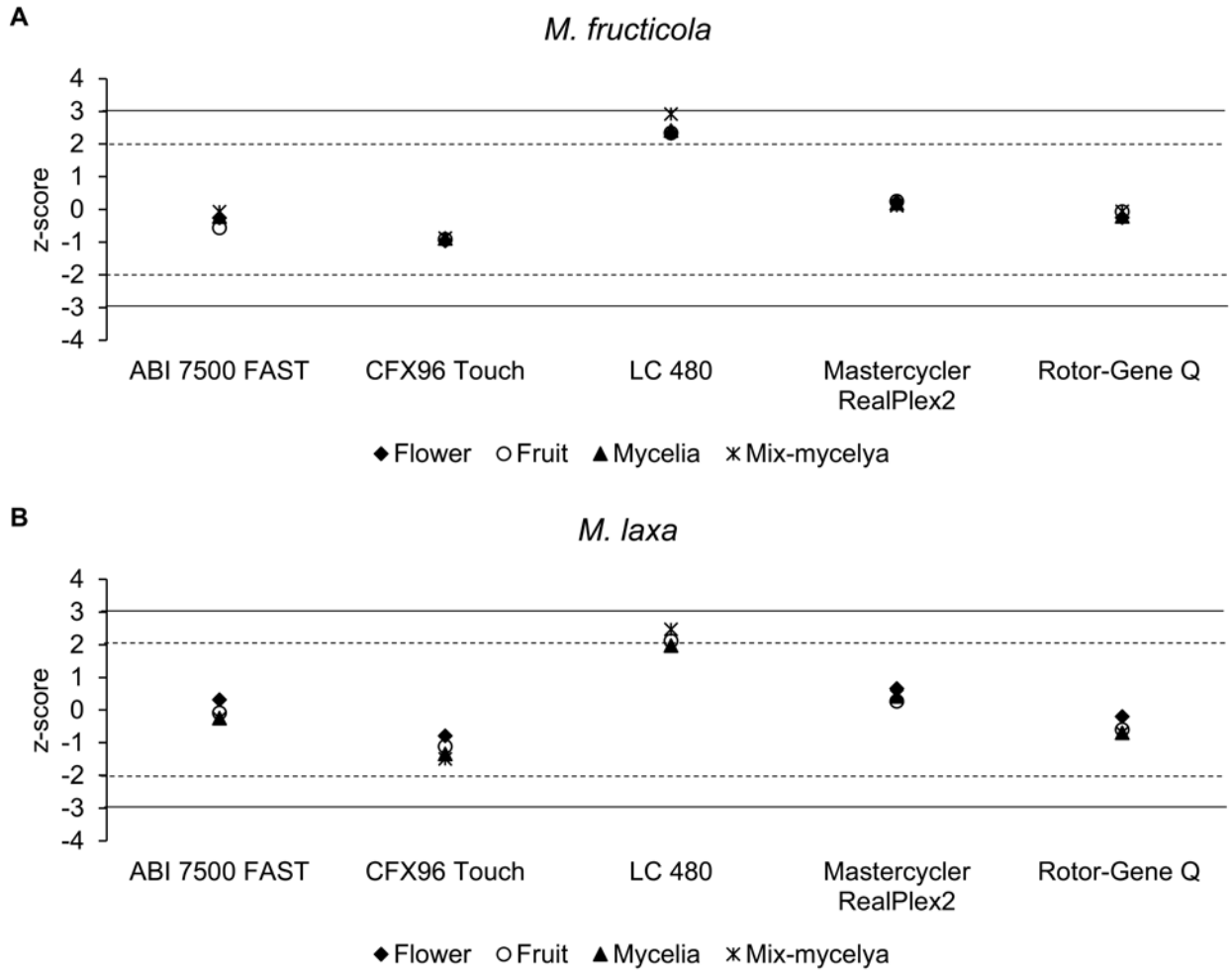

Figure 1. *Z*-scores for the detection of different *Monilinia fructicola* (A) and *M. laxa* (B) samples with P_fc and P2_fgn/lx/ps hydrolysis probes, for each of five laboratories. *Z*-scores between 2 and -2 are acceptable (dotted line), those greater than 3 or lower than -3 are unacceptable (solid line) and those between the lines need to be reviewed.

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

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 Örnemark, 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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Literature cited

Agren J., R.A. Hamidjaja, T. Hansen, R. Ruuls, S. Thierry, H. Vigre, I. Janse, A. Sundstrom, B. Segerman, M. Koene, C. Lofstrom, B. Van Rotterdam and S. Derzelle, 2013. In silico and in vitro evaluation of PCR-based assays for the detection of *Bacillus anthracis* chromosomal signature sequences. *Virulence* 4, 671–685.

AOAC INTERNATIONAL, 2012. *Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces*, 19th ed, AOAC INTERNATIONAL, Rockville, MD, USA.

Boehm E.W.A., Z. Ma and T.J. Michailides, 2001. Species-specific detection of *Monilinia fructicola* from California stone fruits and flowers. *Phytopathology* 91, 428–439.

Braun-Kiewnick A., N. Viaene, L. Folcher, F. Ollivier, G. Anthoine, B. Niere, M. Sapp, B. van de Vossenberg, H. Toktay and S. Kiewnick, 2016. Assessment of a new qPCR tool for the detection and identification of the root-knot nematode *Meloidogyne enterolobii* by an international test performance study. *European Journal of Plant Pathology* 144, 97–108.

Broeders S., I. Huber, L. Grohmann, G. Berben, I. Taverniers, M. Mazzara, N. Roosens and D. Morisset, 2014. Guidelines for validation of qualitative real-time PCR methods. *Trends in Food Science & Technology* 37, 115–126.

Byrde R.J. and H.J. Willetts, 1977. *The Brown Rot Fungi of Fruit: their Biology and Control*, Pergamon Press, Oxford, UK.

Côté M.J., M.C. Tardif and A.J. Meldrum, 2004. Identification of *Monilinia fructigena*, *M. fructicola*, *M. laxa*, and *Monilia*

polystroma on inoculated and naturally infected fruit using multiplex PCR. *Plant Disease* 88, 1219–1225.

Ebentier D.L., K.T. Hanley, Y. Cao, B.D. Badgley, A.B. Boehm, J.S. Ervin, K.D. Goodwin, M. Gourmelon, J.F. Griffith, P.A. Holden, C.A. Kelty, S. Lozach, C. McGee, L.A. Peed, M. Raith, H. Ryu, M.J. Sadowsky, E.A. Scott, J.S. Domingo, A. Schriewer, C.D. Sinigalliano, O.C. Shanks, L.C. Van De Werfhorst, D. Wang, S. Wuertz and J.A. Jay, 2013. Evaluation of the repeatability and reproducibility of a suite of qPCR-based microbial source tracking methods. *Water Research* 47, 6839–6848.

Emery K.M., T.J. Michailides and H. Scherm, 2000. Incidence of latent infection of immature peach fruit by *Monilinia fructicola* and relationship to brown rot in Georgia. *Plant Disease* 84, 853–857.

EPPO, 2009. PM 7/18 (2) *Monilinia fructicola*. *Bulletin OEPP/EPPO Bulletin* 39, 337–343.

EPPO, 2014. PM 7/98 (2) Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity. *Bulletin OEPP/EPPO Bulletin* 44, 117–147.

EPPO, 2016. Euphresco. http://www.euphresco.net/projects/portfolio.

European Commission. 2014. Commission Implementing Directive 2014/78/EU on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community. Official Journal of the European Union L 183/23. Available at: http:// eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32014L0078 Accessed 2014-06-24.

European Network of GMO Laboratories, 2011. Verification of analytical methods for GMO testing when implementing interlaboratory validated methods EUR24790-EN. Luxembourg: Publications Office of the European Union, 23 pp. doi:10.2788/88038

Förster H. and J.E. Adaskaveg, 2000. Early brown rot infections in sweet cherry fruit are detected by *Monilinia*-specific DNA primers. *Phytopathology* 90, 171–178.

Fourie P.H. and G. Holz, 2003a. Germination of dry, airborne conidia of *Monilinia laxa* and disease expression on nectarine fruit. *Australasian Plant Pathology* 32, 9–18.

Fourie P.H. and G. Holz, 2003b. Germination of dry, airborne conidia of *Monilinia laxa* and disease expression on plum fruit. *Australasian Plant Pathology* 32, 19–25.

Fulton C.E. and A.E. Brown, 1997. Use of SSU rDNA group-I intron to distinguish *Monilinia fructicola* from *M. laxa* and *M. fructigena*. *FEMS Microbiology Letters* 157, 307–312.

Garcia-Benitez C., P. Melgarejo and A. De Cal, 2017. Detection of latent *Monilinia* infections in nectarine flowers and fruit by qPCR. *Plant Disease* 101, 1002–1008.

Garcia-Benitez C., P. Melgarejo, A. De Cal and B. Fontaniella, 2016. Microscopic analyses of latent and visible *Monilinia fructicola* infections in nectarines. *PloS one* 11, e0160675.

Gell I., J. Cubero and P. Melgarejo, 2007. Two different PCR approaches for universal diagnosis of brown rot and identification of *Monilinia* spp. in stone fruit trees. *Journal of Applied Microbiology* 103, 2629–2637.

Gell I., A. De Cal, R. Torres, J. Usall and P. Melgarejo, 2008. Relationship between the incidence of latent infections caused by *Monilinia* spp. and the incidence of brown rot of peach fruit: factors affecting latent infection. *European Journal of Plant Pathology* 121, 487–498.

Gell I., A. De Cal, R. Torres, J. Usall and P. Melgarejo, 2009. Conidial density of *Monilinia* spp. on peach fruit surfaces in relation to the incidences of latent infections and brown rot. *European Journal of Plant Pathology* 123, 415–424.

Guinet C., C. Fourrier-Jeandel, I. Cerf-Wendling and R. Ioos, 2016. One-step detection of *Monilinia fructicola*, *M. fructigena*, and *M. laxa* on *Prunus* and *Malus* by a multiplex real-time PCR assay. *Plant Disease* 100, 2465–2474.

Hughes K.J.D., C.E. Fulton, D. McReynold and C.R. Lane, 2000. Development of new PCR primers for identification of *Monilinia* species. *Bulletin OEPP/EPPO Bulletin* 30, 507–511.

Ioos R. and P. Frey, 2000. Genomic variation within *Monilinia laxa*, *M. fructigena* and *M. fructicola*, and application to species identification by PCR. *European Journal of Plant Pathology* 106, 373–378.

ISO, 2005. General requirements for the competence of testing and calibration laboratories. *International Organization for Standardization*. www.iso.org.

Kamihira S., Y. Yamano, M. Iwanaga, D. Sasaki, M. Satake, A. Okayama, K. Umeki, R. Kubota, S. Izumo, K. Yamaguchi and T. Watanabe, 2010. Intra- and inter-laboratory variability in human T-cell leukemia virus type-1 proviral load quantification using real-time polymerase chain reaction assays: A multi-center study. *Cancer Science* 101, 2361-2367.

Luo Y. and T.J. Michailides, 2001. Factors affecting latent infection of prune fruit by *Monilinia fructicola*. *Phytopathology* 91, 864–872.

Luo Y. and T.J. Michailides, 2003. Threshold conditions that lead latent infection to prune fruit rot caused by *Monilinia fructicola*. *Phytopathology* 93, 102–111.

Luo Y., Z. Ma and T.J. Michailides, 2001. Analysis of factors affecting latent infection and sporulation of *Monilinia fructicola* on prune fruit. *Plant Disease* 85, 999-1003.

Luo Y., Z. Ma, H.C. Reyes, D. Morgan and T.J. Michailides, 2007. Quantification of airborne spores of *Monilinia fructicola* in stone fruit orchards of California using real-time PCR. *European Journal of Plant Pathology* 118, 145–154.

Ma Z., Y. Luo and T.J. Michailides, 2003. Nested PCR assays for detection of *Monilinia fructicola* in stone fruit orchards and *Botryosphaeria dothidea* from pistachios in California. *Journal of Phytopathology-Phytopathologische Zeitschrift* 151, 312–322.

Magnusson B. and U. Örnemark, 2014. Eurachem Guide: The fitness for purpose of analytical methods – A laboratory guide to method validation and related topics. *Eurachem Guide*. http://www.eurachem.org.

Northover J. and R.F. Cerkauskas, 1994. Detection and significance of symptomless latent infections of *Monilinia fructicola* in plums. *Canadian Journal of Plant Pathology-Revue Canadienne de Phytopathologie* 16, 30–36.

Sauer D.B. and R. Burroughs, 1986. Disinfection of seed surfaces with sodium-hypochlorite. *Phytopathology* 76, 745–749.

Thomidis T. and T.J. Michailides, 2010. Development and implementation of cost-effective strategies to manage brown rot of peach trees in Imathia, Greece. *European Journal of*

Plant Pathology 126, 575–582.
Thompson M., S.L.R. Ellison and R. Wood, 2006. The international harmonized protocol for the proficiency testing of analytical chemistry laboratories (IUPAC technical report). *Pure and Applied Chemistry* 78, 145–196.
van Brouwershaven I.R., M.L. Bruil, G.C.M. van Leeuwen and L.F.F. Kox, 2010. A real-time (TaqMan) PCR assay to differentiate *Monilinia fructicola* from other brown rot fungi of fruit crops. *Plant Pathology* 59, 548–555.
Villarino M., B. Egüen, N. Lamarca, J. Segarra, J. Usall, P. Melgarejo and A. De Cal, 2013. Occurrence of *Monilinia laxa* and *M. fructigena* after introduction of *M. fructicola* in peach orchards in Spain. *European Journal of Plant Pathology* 137, 835–845.

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