Detection of Latent *Monilinia* Infections in Nectarine Flowers and Fruit by qPCR

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Abstract

Most stone fruit with a latent brown rot infection caused by *Monilinia* do not develop visible signs of disease until the arrival of fruit at the markets or the consumer's homes. The overnight freezing-incubation technique (ONFIT) is a well-established method for detecting latent brown rot infections, but it takes between 7 to 9 days. In this report, we inform on the advantages of applying a qPCR-based method to (i) detect a latent brown rot infection in the blossoms and fruit of nectarine trees (*Prunus persica* var. *nucipersica*) and (ii) distinguish between the *Monilinia* spp. in them. For applying this qPCR-based method, artificial latent

infections were established in nectarine flowers and fruit using 10 *Monilinia fructicola* isolates, 8 *M. fructigena* isolates, and 10 *M. laxa* isolates. We detected greater amounts of *M. fructicola* DNA than *M. laxa* and *M. fructigena* DNA in latently infected flowers using qPCR. However, greater DNA amounts of *M. laxa* than *M. fructicola* were detected in the mesocarp of latently infected nectarines. We found that the qPCR-based method is more sensitive, reliable, and quicker than ONFIT for detecting a latent brown rot infection, and could be very useful in those countries where *Monilinia* spp. are classified as quarantine pathogens.

Monilinia spp. are fungal causal agents of fruit brown rot, which is an economically important fungal disease of nectarines (*Prunus persica* [L.] Batsch var. *nucipersica* [Suckow] C. K. Schneid), peaches (*P. persica*), and other stone fruit species, and is responsible for substantial preharvest and postharvest losses (Byrde and Willetts 1977). Until 2006, two *Monilinia* spp., namely *M. fructigena* Honey and *M. laxa* (Aderh. & Ruhland) Honey, were identified as the causal fungi of brown rot in peaches and nectarines in Spain (De Cal et al. 2009). In 2006, *M. fructicola* (G. Winter) Honey, a European and Mediterranean Plant Protection Organization (EPPO) A1 quarantine pest until 2004 and now classified as an EPPO A2 (EPPO 2009) quarantine pest, was identified for the first time in peach and nectarine orchards in northeastern Spain (De Cal et al. 2009). Since this identification, *M. fructicola* has completely displaced *M. fructigena* as a cause of brown rot, and *M. fructicola* and *M. laxa* now have the same frequency of occurrence in these peach and nectarine orchards (Villarino et al. 2013). *M. fructicola* has also been identified in several EPPO member countries: France (Lichou et al. 2002), the Czech Republic (Duchoslavová et al. 2007), Italy (Pellegrino et al. 2009), Germany (Grabke et al. 2011), Slovenia (Munda and Marn 2010), Serbia (Vasić et al. 2012), and Greece (Papavasileiou et al. 2015).

Monilinia infection occurs when the fungus penetrates fruit through natural openings, such as stomata, or surface injuries when weather conditions are favorable for fungal growth and development (Biggs and Northover 1988; Phillips 1984). When weather conditions are unfavorable, a latent *Monilinia* infection may occur (Byrde and Willetts 1977). *M. fructicola* and *M. laxa* have been isolated from peaches, nectarines, and plums with latent infections (Emery et al. 2000; Fourie and Holz 2003a, b; Gell et al. 2008; Luo et al. 2001; Northover and Cerkauskas 1994). The incidence of latent *Monilinia* infections in harvested fruit ranges from 0 to 30% and may even be as high as 50% (Emery et al. 2000; Luo et al. 2001; Luo and Michailides 2001, 2003). Most stone fruit with a latent *Monilinia* infection do not develop visible signs of disease until the arrival of fruit at the consumer markets or the consumer's homes. Hence, fruit with a latent *Monilinia* infection is a silent reservoir of the pathogenic fungus. Moreover, detecting a latent *Monilinia* infection in stone fruit is

crucial for preventing spread of the pathogenic fungus to countries and/or regions free from pathogens.

The overnight freezing-incubation technique (ONFIT) is a well-established method for detecting latent *Monilinia* infections (Luo and Michailides 2003). However, one of ONFIT's disadvantages is its duration: it takes 7 to 9 days to detect the latent infection, which is activated by causing senescence in epidermal cells by freezing the fruit at -20°C for 48 h (Luo and Michailides 2003; Villarino et al. 2012). Once the epidermis is damaged, the fruit is first incubated for 5 to 7 days at 25°C and a high relative humidity (RH) and then examined for signs of brown rot.

The advent of molecular methods has enabled the rapid detection, identification, and differentiation of *Monilinia* spp. The first molecular method for detecting *M. fructicola* was based on amplification of the group 1 introns in the nuclear small subunit ribosomal RNA (SSU rDNA) gene by PCR (Fulton and Brown 1997). Since this method was only able to identify *M. fructicola*, new PCR-based methods, which amplify other specific regions of DNA of *M. fructicola*, *M. fructigena*, and *M. laxa*, were developed (Boehm et al. 2001; Côté et al. 2004; Förster and Adaskaveg 2000; Gell et al. 2007; Hughes et al. 2000; Ioos and Frey 2000; Ma et al. 2003). Luo et al. (2007) developed the first qPCR-based method using SYBR Green (Thermo Fisher Scientific Inc., Waltham, MA) to detect *M. fructicola*, and this method has since been validated for only *M. laxa*. van Brouwershaven et al. (2010) developed a qPCR-based method that uses hydrolysis probes to differentiate between *M. fructicola* and the three other principal causal agents of brown rot, *M. fructigena*, *M. laxa*, and *M. polystroma* (G. C. M. van Leeuwen) Kohn. Moreover Guinet et al. (2016) developed a multiplex real-time PCR assay that detects and differentiates between *M. fructicola*, *M. fructigena*, and *M. laxa* in just one step on both *Prunus* and *Malus* species.

No molecular methods and none of the existing qPCR-based methods have been developed for detecting a latent *Monilinia* infection in nectarines. Hence, the main objective of this investigation was to determine whether the qPCR-based method that was developed by van Brouwershaven et al. (2010) could be applied to rapidly, reliably, and reproducibly (i) detect a latent *Monilinia* infection in the blossoms and fruit of nectarine trees, and (ii) distinguish between *Monilinia* spp.

Materials and Methods

Fungal isolates and preparation of conidial suspensions.

Twenty-eight *Monilinia* isolates (10 *M. fructicola* isolates, 8 *M. fructigena* isolates, and 10 *M. laxa* isolates) from the culture collection of Plant Protection Department of Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Madrid, Spain, were used in the different experiments (Table 1). The *Monilinia* isolates of the collection were confirmed by morphological identification and PCR

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*The e-Xtra logo stands for "electronic extra" and indicates that two supplementary tables and two supplementary figures are available online.

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assays according to the method developed by Gell et al. (2007). The *M. fructicola* and *M. laxa* isolates were stored either as a conidial suspension in 20% glycerol at -80°C for long-term storage or as a culture on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) at 4°C for short-term storage. The *M. fructicola* isolates were grown on PDA plates at 20 to 25°C in the dark for 7 days for conidial production. The *M. laxa* isolates were first grown on PDA plates in the dark at 20 to 25°C for 10 days and then at 4°C for 5 days for conidial production. The *M. fructigena* isolates were maintained on PDA slants at 4°C . For conidial production of *M. fructigena* isolates, surface-disinfected nectarines were inoculated with 3-mm diameter mycelial plugs from 1-week-old cultures that were grown on PDA at 25°C in the dark. Nectarines were surface-disinfected by immersion for 5 min in a 1% sodium hypochlorite solution, immersion for 1 min in a 70% ethanol solution, two 1-min washes in sterilized distilled water (SDW), and drying for 2 h in a laminar flow hood (Sauer and Burroughs 1986). The fruit were then incubated in a humidity chamber that was lined with sterilized moist filter paper at 20 to 25°C under fluorescent lighting ($100\ \mu\text{E m}^{-2}\ \text{s}^{-1}$ with a 16 h photoperiod) for 7 to 10 days.

The conidial suspensions were prepared using conidia that were harvested from the PDA plates (*M. fructicola* and *M. laxa*) or from fruit (*M. fructigena*) by scratching the surface with a sterilized disposable scalpel after adding SDW. The harvested conidia and mycelia were filtered through glass wool in order to remove the mycelia after a 30 s sonication in an ultrasonic bath (J.P. Selecta S.A., Barcelona, Spain). The filtrate was adjusted to the desired conidial concentration using SDW after counting the number of conidia using a hemocytometer and a light microscope (Zeiss Axioskop 2; Carl Zeiss, Inc., Oberkochen, Germany).

Establishment of an artificial latent *Monilinia* infection in nectarine flowers. Due to the almost nonexistent occurrence of natural flower latent infections by *Monilinia* in Spanish conditions (Villarino et al. 2012), artificial latent infections were conducted to

ensure *Monilinia* detection. Two hundred and ninety nectarine flowers without any visible signs of brown rot were collected from a 'Romea' nectarine tree in the Jerte Valley, Caceres, Spain, in 2014. After their collection, the flowers were first surface-disinfected (previously described on *M. fructigena* conidial production). After drying, the flowers were randomly divided in groups of 10. Each nectarine flower in each group was inoculated placing inside the calyx a $30\ \mu\text{l}$ conidial suspension (10^6 conidia ml^{-1}) drop of one of the *M. fructicola*, *M. fructigena*, and *M. laxa* isolates using a micropipette (approximately 780 pg DNA). After inoculation, the flowers were incubated in sterile humidity chambers, i.e., polystyrene boxes, which were lined with moist sterilized filter paper, at 25°C and 100% RH for 24 h in the dark. At the end of the incubation period, the flowers were again surface-disinfected and air-dried in a laminar flow hood, as previously described. After drying, the flowers were incubated in humidity chambers at 4°C and 100% RH for 5 days in the dark. The control group consisted of 10 flowers, each of which was inoculated with $30\ \mu\text{l}$ SDW.

Establishment of an artificial latent *Monilinia* infection in nectarines. Natural fruit latent infections by *Monilinia* have a higher frequency of occurrence than those of flowers, but they are highly variable in number (Gell et al. 2008; Villarino et al. 2012). Inducing latent infections with a high pathogen inoculum concentration ensures presence and homogeneous numbers of latent infections in nectarines, thus reducing the effects over the results of the sporadic natural latent infections. Two nectarine varieties of similar susceptibility, Alba Red and Big Top, which had been routinely used in the INIA lab over the years, were selected and used for the establishment of artificial latent infections. One hundred thirty-two nectarines of similar phenological status (BBCH = 89, at harvest; BBCH: general scale from Biologische Bundesanstalt, Bundessortenamt and Chemische Industrie, Germany [Meier et al. 1994]) without any visible signs of brown rot were collected from orchards in the Ebro Valley,

Table 1. Identification of the 29 isolates (10 *Monilinia fructicola* [Mfc] isolates, 8 *M. fructigena* [Mfg] isolates, and 11 *M. laxa* [Mlx] isolates) that were used in the different experiments

ID	Collection name	<i>Monilinia</i> species	Host	Plant material	Isolation year
Mfc1	2014COSALBARED36	<i>M. fructicola</i>	Nectarine	Fruit	2014
Mfc2	2014COSFANTASIA10	<i>M. fructicola</i>	Nectarine	Fruit	2014
Mfc3	ALF2009COS5R7	<i>M. fructicola</i>	Peach	Fruit	2009
Mfc4	ALB2009IL14D4R9	<i>M. fructicola</i>	Peach	LI ^a	2009
Mfc5	ALF2009MOA4	<i>M. fructicola</i>	Peach	MFT ^b	2009
Mfc6	SUD2009COS2R6	<i>M. fructicola</i>	Nectarine	Fruit	2009
Mfc7	ALB2009PB5R14	<i>M. fructicola</i>	Peach	Shoot	2009
Mfc8	ALC2013FRPC13	<i>M. fructicola</i>	Peach	Fruit	2013
Mfc9	SUD2013FRPC68	<i>M. fructicola</i>	Nectarine	Fruit	2013
Mfc10	SUD2012MS4.2	<i>M. fructicola</i>	Nectarine	MFO ^c	2012
Mfg1	71	<i>M. fructigena</i>	Apple	Fruit	2010
Mfg2	199	<i>M. fructigena</i>	Apple	Fruit	2010
Mfg3	BOLAÑO1	<i>M. fructigena</i>	Apple	Fruit	2010
Mfg4	BOLAÑO13	<i>M. fructigena</i>	Apple	Fruit	2010
Mfg5	BOLAÑO12	<i>M. fructigena</i>	Apple	Fruit	2010
Mfg6	BOLAÑO8	<i>M. fructigena</i>	Apple	Fruit	2009
Mfg7	FRANZA2012.2	<i>M. fructigena</i>	Apple	Fruit	2012
Mfg8	D-45	<i>M. fructigena</i>	Apple	Fruit	2009
Mlx1	2014COSFANTASIA13	<i>M. laxa</i>	Nectarine	Fruit	2014
Mlx2	BRM2009ALB2.3	<i>M. laxa</i>	Peach	Shoot	2009
Mlx3	FLOR2009SUD5.1	<i>M. laxa</i>	Nectarine	Flower	2009
Mlx4	SUD2009IL21D8R2	<i>M. laxa</i>	Nectarine	LI	2009
Mlx5	SUD2009IL14D4R1	<i>M. laxa</i>	Nectarine	LI	2009
Mlx6	ALB2009ILCOS6R6(1)	<i>M. laxa</i>	Peach	LI	2009
Mlx7	MS2009ALF10	<i>M. laxa</i>	Peach	MFO	2009
Mlx8	SUD2009IL7D5R1	<i>M. laxa</i>	Nectarine	LI	2009
Mlx9	2014COSFANTASIA9	<i>M. laxa</i>	Nectarine	Fruit	2014
Mlx10	ALF2009COSLER107.1	<i>M. laxa</i>	Peach	Fruit	2009
Mlx11	ALB20097DIL10R10	<i>M. laxa</i>	Peach	Fruit	2009

^a Tissue with latent infection.

^b Mummified fruit from a tree.

^c Mummified fruit on orchard bed.

Lleida, Spain. After their collection, the nectarines were surface-disinfected and dried in a laminar flow hood, as previously described on *M. fructigena* conidial production. After drying, the nectarines were randomly divided in groups of six, and each nectarine of each group was inoculated by a 30 s immersion in a conidial suspension (approximately 10^5 conidia mL^{-1}). Sixty-six Alba Red nectarines were inoculated with *M. fructicola* isolates in 2014 and 66 Big Top nectarines with *M. laxa* isolates in 2015. After inoculation, the nectarines were incubated in humidity chambers (previously described) at 25°C and 100% RH for 24 h in the dark. At the end of the incubation period, the nectarines were again surface-disinfected and dried in a laminar flow hood, as previously described. After drying, the nectarines were incubated in humidity chambers (previously described) at 4°C and 100% RH for 5 days in the dark. A control group was made each year with six nectarines, each of which was immersed in SDW for 30 s.

Detection of latent *Monilinia* infection by qPCR and ONFIT.

Five flowers inoculated with each *Monilinia* isolate and five control-uninoculated flowers were stored at -80°C and then lyophilized in a laboratory freeze dryer (Cryodos -50, Azbil Telstar Technologies, SLU, Terrassa, Spain). Each lyophilized flower was placed on 2 ml microcentrifuge tubes and homogenized for 60 s at a speed setting of 4.0 m/s using a high-speed benchtop tissue homogenizer (FastPrep-24 Instrument, MP Biomedicals, Solon, OH). Genomic DNA from each flower was extracted using the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's instructions except that DNA was eluted in a final volume of 100 μL .

The epidermis and the mesocarp of three nectarines inoculated with each isolate and three control-uninoculated nectarines from each year were excised using a sterile disposal scalpel at the end of the 5-day incubation period at 4°C. The mesocarp was further divided into external mesocarp (from the epidermis to a depth of 1 cm), and internal mesocarp (the rest of the mesocarp until the stone) so that the depth of the latent infection in each nectarine could be delimited. The different areas of each fruit were placed separately into 50 ml centrifuge tubes, stored at -80°C, and lyophilized and homogenized inside each 50 ml centrifuge tube in the conditions previously described for flowers. Genomic DNA was extracted from five randomly selected 20 mg samples of each homogenate (nectarine epidermis, external mesocarp, or internal mesocarp) using the DNeasy Plant Mini Kit (Qiagen GmbH), in accordance with the manufacturer's instructions, with the exception that the DNA was eluted in a final volume of 50 μL .

DNA amount and purity in the flower and fruit specimens were determined using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, Germany). The DNA concentrations were adjusted to 4 ng μL^{-1} for flowers and 2 ng μL^{-1} for nectarines using sterile Milli-Q water for the qPCRs.

Latent *Monilinia* infections in the flowers and fruit were detected applying the qPCR-based method and hydrolysis probes of van Brouwershaven et al. (2010) with modifications as follows. The hydrolysis probes were labeled with different reporter dyes and quenchers and all primers and probes were obtained from Integrated DNA Technologies Inc. (Coralville, IA). Specifically, we used a FAM reporter dye and a ZEN/Iowa Black FQ quencher for *M. fructicola* probe (P_{fc}) instead of a FAM-TAMRA, and a HEX reporter dye with a ZEN/Iowa Black FQ quencher instead of a VIC-TAMRA quencher for *M. fructigena* and *M. laxa* probe (P_{2_fgn/lx/ps}). Genomic DNA from the flowers (20 ng) or the three fruit areas (10 ng) was amplified in 20- μL reaction mixture in each well of a 96-well clear optical reaction plate (Applied Biosystems, Foster City, CA) sealed with a clear adhesive. The 20- μL reaction mixture contained 1 $\times$ GoTaq probe qPCR Master Mix (Promega Corporation, Madison, WI), 200 nM of each of the primers (Mon139F and Mon 139R), and 200 nM of each of the probes (P_{fc} and P_{2_fgn/lx/ps}). Thermal cycling was done using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) under the same conditions and using the same thresholds described by van Brouwershaven et al. (2010) with slight modifications: 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 fluorescence (ΔRn) of 23,000. A quantification cycle (C_q) value below 40 was scored as a positive detection. Additionally, an allelic discrimination step was added due to cross-detection of *M. laxa* and *M. fructigena* when using the P_{fc} probe. Allelic discrimination allowed us to distinguish between the *M. fructicola* isolates, the *M. fructigena* isolates, the *M. laxa* isolates, and mixtures of the *Monilinia* isolates with absolute certainty. The allelic discrimination step measures fluorescence in each well of the qPCR plate before and after qPCR amplification, and places each sample in an X-Y graph according to these results. The *M. fructicola* samples are placed on a line parallel to the Y-axis on top of the vertex formed by the negative controls while the *M. fructigena*/*M. laxa* samples are placed on a line parallel to the X-axis and to the right of the vertex formed by the negative controls, and the mixtures of *M. fructicola* and *M. fructigena*/*M. laxa* appear between both axes with the bisectrix indicating mixtures of equal amounts.

All amplifications included the following controls: (i) a negative control (DNase- and RNase-free water) in order to check for DNA contamination, and (ii) a positive control, consisting of separate DNA samples (10 pg) from the different *M. fructicola*, *M. fructigena*, and *M. laxa* isolates in order to monitor reaction performance and efficiency of the qPCR. A proposed workflow to use this qPCR method as a diagnostic tool for latent *Monilinia* spp. infections can be found in Supplementary Figure S1. Supplementary Tables S1 and S2 show the qPCR plate layout for the latently infected flowers and nectarines. The results of the qPCR were considered reliable when all controls in the series gave the expected results (Kox et al. 2005, 2007).

For quantification of the DNA amount in each specimen, eight standard curves, one for each tested combination of *Monilinia* species (*M. fructicola* and *M. fructigena*/*M. laxa*) and plant material (flowers, epidermis, and mesocarp), were generated. The standard curves were generated using a 10-fold dilution series, ranging from 4 ng to 4 fg of genomic DNA (approximately from 154,000 to 0.15 *M. laxa* conidia) from each fungal species, and after spiking the samples with nectarine DNA, 20 ng for the flowers and 10 ng for the nectarine's epidermis and mesocarp. The genomic DNA used for the standard curves was extracted from purified cultures of isolates Mfc3 and Mlx11, and its concentration was determined using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.). Each dilution series was done in triplicate for each standard curve. The essential parameters of the qPCR assay performance (PCR efficiency, limit of detection, and precision) were calculated according to Bustin et al. (2009). Equivalences between number of cells or conidia and DNA quantity are calculated using the haploid genome size of 0.04 pg obtained for one *M. laxa* isolate sequenced by our group (unpublished data) and an average number of 6.6 nuclei (with a range from 4 to 10) per cell (Hall 1963; Lee and Bostock 2006).

ONFIT (Luo and Michailides 2003) was used to detect latent *Monilinia* infections in the remaining five flowers and three nectarines of each group. Briefly, the nectarines and flowers were first frozen at -20°C for 48 h to induce tissue senescence. The senescent fruit and flowers were transferred to plates in humidity chambers (as previously described) and incubated at 25°C and 100% RH for 1 week in the dark. At the end of the incubation, the fruit and the flowers were examined for visible signs of a *Monilinia* infection and the number of rotten plant parts was recorded for each *Monilinia* isolate. The frequencies of latently infected flowers and fruit that were detected by ONFIT and qPCR were compared.

Data analysis. The results of the qPCR amplifications were transformed into DNA amount for each of the three replicates of the five flowers and five samples of each of the three fruit areas individually, using the standard curves previously obtained, prior to any statistical analysis. DNA amounts were compared among each *Monilinia* species for the flower samples and nectarine samples. The amount of DNA of each nectarine area (epidermis, external mesocarp, and internal mesocarp) was compared for each of the *Monilinia* isolates. The results of the qPCR were transformed into qualitative results

(positive detection or negative detection) in order to compare them with the results obtained with ONFIT. A positive detection was considered when a flower or at least one of the five nectarine epidermis samples of each nectarine scored a C_q lower than 40. Statgraphics Centurion XVI for Windows, Version 16.1.03 (StatPoint Technologies, Inc., Herndon, VA) was used to statistically analyze the data. Because some of the qPCR results were negative for all technical replicates within a sample, heterogeneity of variance among treatments was detected. To compensate the lack of homogeneity of variance, the Kruskal-Wallis test was used to compare the medians of the amount of *Monilinia* DNA detected in the flowers and fruit with a latent infection. When a result of the Kruskal-Wallis test was significant ($P < 0.05$), the group medians of the samples were compared.

Results

Standard curves characteristics. Standard curves for the two hydrolysis probes (P_fc [*M. fructicola*] and P2_fgn/lx/ps [*M. fructigena*, *M. laxa*, and *M. polystroma*]) were done to assess the efficiency of the qPCR method in each matrix (milli-Q water, nectarine flower DNA, nectarine fruit epidermis DNA, and nectarine fruit mesocarp DNA) (Table 2). A low limit of detection (less than 27 fg of DNA [approximately 1 *M. laxa* cell]) was achieved with the different

probes for all the different matrices (Table 2). With the exception of the standard curve for *M. fructicola* in flowers that have an amplification efficiency of 87%, the rest of the standard curves had optimal amplification efficiencies between 90 and 100% (Table 2). Additionally, the fit of the generated data to the regression line was high, as measured by value of the R^2 coefficient that was greater than 0.99 for all curves (Table 2).

Detection of a latent *Monilinia* infection in nectarine flowers by qPCR. DNA from the *M. fructicola*, *M. fructigena*, and *M. laxa* isolates was not detected in the five control-uninoculated flowers. The median amounts of DNA of each *M. fructicola*, *M. fructigena*, and *M. laxa* isolate in the latently infected flowers are displayed in Figure 1. The median amounts of DNA from *M. fructigena* and *M. laxa* species were similar, 0.10 ± 0.05 pg (median $\pm$ standard error) and 0.13 ± 0.06 pg, respectively, and lower than the median amount of DNA from *M. fructicola* 18.32 ± 8.47 pg. The median amount of DNA from *M. fructicola* isolates in the latently infected flowers was bigger than 1 pg in 8 out of 10 isolates whereas those from *M. fructigena* and *M. laxa* isolates were less than 1 pg, in 7 out of 8 and 10 out of 10 isolates, respectively. The median amount of DNA of *M. fructicola* isolates ranged between 0.25 ± 0.11 pg and 338.73 ± 128.01 pg, the ones from *M. fructigena* isolates ranged

Table 2. Characteristics of the eight standard curves for detecting and quantifying DNA by qPCR in nectarine flowers and fruit with a latent *Monilinia* infection^a

Species	Sample matrix	Limit of detection (fg)	Y-intercept	Slope	E (%)	R ²
<i>M. fructicola</i>	Water	8.95 ± 2.29	32.25 ± 0.09	$-3.47 \pm 2.9 \times 10^{-2}$	94 ± 0.87	0.994
	Flower	9.62 ± 3.86	30.72 ± 0.10	$-3.67 \pm 0.8 \times 10^{-2}$	87 ± 0.24	0.996
	Epidermis	4.63 ± 0.74	30.04 ± 0.08	$-3.57 \pm 2.9 \times 10^{-2}$	91 ± 0.98	0.999
	Mesocarp	4.11 ± 1.27	28.43 ± 0.13	$-3.30 \pm 4.6 \times 10^{-2}$	100 ± 1.96	0.998
<i>M. laxa/M. fructigena</i>	Water	26.77 ± 3.76	33.12 ± 0.06	$-3.59 \pm 0.8 \times 10^{-2}$	90 ± 0.28	0.999
	Flower	11.22 ± 0.54	31.16 ± 0.03	$-3.60 \pm 1.0 \times 10^{-2}$	90 ± 0.33	0.996
	Epidermis	3.51 ± 0.19	31.18 ± 0.12	$-3.29 \pm 4.0 \times 10^{-2}$	100 ± 1.67	0.993
	Mesocarp	4.81 ± 0.41	31.19 ± 0.03	$-3.57 \pm 2.1 \times 10^{-2}$	91 ± 0.75	0.999

^a Values are displayed as means $\pm$ standard error.

Fig. 1. Amounts of DNA from 10 *Monilinia fructicola* (A), 8 *M. fructigena* (B), and 10 *M. laxa* (C) isolates in latently infected flowers that were detected by qPCR. Values are displayed as median DNA amount $\pm$ standard error of five inoculated flowers and three pseudo-replicates per repetition. Dotted lines at 1 pg DNA divide the graphs to highlight the differences, and facilitate the comparison between graphs.

between 0.01 ± 0.01 pg and 3.00 ± 1.28 pg, and those from *M. laxa* isolates ranged between 0.004 ± 0.006 pg and 0.57 ± 0.22 pg (Fig. 1).

Detection of latent *Monilinia* infection in nectarines by qPCR.

Latent brown rot infections in the epidermis and the mesocarp of inoculated nectarines that were caused by *M. fructicola* and *M. laxa* isolates were successfully detected by qPCR. DNA from the *M. fructicola* and *M. laxa* isolates was not detected in the epidermis and the mesocarp of two out of the three control-uninoculated nectarines from 2014 and the three control-uninoculated nectarines from 2015. The third control-uninoculated nectarine from 2014 had a natural latent *Monilinia* infection because the qPCR detected *M. fructicola* DNA in its epidermis (2.27 ± 0.23 pg DNA). The median amounts of DNA from each *M. fructicola* and *M. laxa* isolate in the epidermis, the external mesocarp, and the internal mesocarp of latently infected nectarines where similar inside each specie and those corresponding to isolate Mfc3 and Mlx4 are shown on Figure 2; additionally, the values for all of the isolates are shown in Supplementary Figure S2. For the *M. fructicola* and *M. laxa* isolates, the amounts of their DNA detected in the epidermis were greater than those in the external and internal mesocarp of latently infected nectarines, except for Mlx2 and Mlx8. The amounts of DNA from the *M. fructicola* in the epidermis of latently infected nectarines ranged between 0.25 ± 0.03 pg and 6.33 ± 0.60 pg, and were similar to those from the *M. laxa* isolates that ranged between 0.01 ± 0.004 pg and 9.10 ± 3.26 pg (Fig. 2). In contrast, the amount of DNA from the *M. fructicola* isolates in the mesocarp of latently infected nectarines ranged between 0.0002 ± 0.0003 pg and 0.004 ± 0.001 pg, and was significantly lower than that of the *M. laxa* isolates that ranged between 0.005 ± 0.01 pg and 2.10 ± 0.72 pg (Fig. 2). There were no significant differences between external and internal mesocarp median DNA amounts, except in the latent infections caused by Mfc9, Mlx3, Mlx4, Mlx5, and Mlx6 isolates, external mesocarp *Monilinia* DNA amount was greater than the internal mesocarp *Monilinia* DNA amount.

Comparison between ONFIT and qPCR for detecting a latent *Monilinia* infection. The qPCR-based method for the detection of *Monilinia* latent infections was compared with ONFIT, the commonly used latent infection detection method, to assess its efficiency and sensitivity. Table 3 summarizes the percentages of the latent infections detected in the flowers and nectarines by ONFIT and qPCR.

Five out of 28 *Monilinia* isolates latently infecting flowers, four *M. fructicola*, and one *M. laxa* isolates, were detected by ONFIT, whereas qPCR detected all *Monilinia* isolates latently infecting flowers, except for isolate Mlx10 (Table 3). Eighteen of the isolates were detected in all infected flowers by qPCR (100%), while only

Fig. 2. Amounts of DNA from *Monilinia fructicola* isolate Mfc3 (■) and *M. laxa* isolate Mlx4 (□) from the epidermis, external mesocarp, and internal mesocarp of three latently infected nectarines that were detected by qPCR. Values are displayed as median DNA amount $\pm$ standard error of three different inoculated fruit, five DNA extractions, and three pseudo-replicates.

isolate Mfc8 was detected in all latently infected flowers by ONFIT (Table 3).

The ONFIT method only detected nine *M. fructicola* and no *M. laxa* isolates latently infecting nectarines whereas the qPCR-method detected all 10 *M. fructicola* and 10 *M. laxa* isolates latently infecting nectarines (Table 3). Furthermore, qPCR detected the latent infections in all infected nectarines from all *M. fructicola* isolates and nine *M. laxa* isolates, while ONFIT only detected 100% latent infections in five of the *M. fructicola* isolates.

Overall, qPCR detected 67% more latent infections than ONFIT in both flowers and nectarines as estimated from the above detection frequencies in both methods.

Discussion

In this study, a qPCR-based method was used to detect latent brown rot infections in nectarine flowers and fruits caused by *M. fructicola*, *M. fructigena*, and *M. laxa*. It takes between 24 and 48 h to detect the fungal pathogen in latently infected flowers and fruit using qPCR, while the ONFIT method required a much longer time to detect latent brown rot infections: 7 to 9 days of sample preparation an incubation plus additional time to identify the specific *Monilinia* spp. by PCR or another molecular method. The rapid detection of latent fungal infections is very important for predicting an outbreak of brown rot in fruit after their harvest and/or after their storage (Thomidis and Michailides 2010). We have previously reported that the average incidence of latent infection during the crop season in Spanish peach orchards explains 55% of the total variation in the incidence of postharvest brown rot (Gell et al. 2008). Therefore, the early, rapid, and accurate detection of latent brown rot infections in the field could be useful for developing disease prediction models and improving the timing of application and efficacy of preharvest

Table 3. Detection percentages of *Monilinia* isolates artificial latent infections of flowers and nectarines by ONFIT and qPCR

ID	Flowers ^a		Nectarines ^b	
	ONFIT (%)	qPCR (%) ^c	ONFIT (%)	qPCR (%) ^c
Mfc1	0	100	33	100
Mfc2	0	100	33	100
Mfc3	0	100	0	100
Mfc4	20	100	100	100
Mfc5	40	100	100	100
Mfc6	40	100	67	100
Mfc7	0	100	100	100
Mfc8	100	80	100	100
Mfc9	0	100	33	100
Mfc10	0	100	100	100
Mfg1	0	100	-	-
Mfg2	0	40	-	-
Mfg3	0	100	-	-
Mfg4	0	80	-	-
Mfg5	0	40	-	-
Mfg6	0	100	-	-
Mfg7	0	80	-	-
Mfg8	0	60	-	-
Mlx1	0	80	0	100
Mlx2	20	100	0	100
Mlx3	0	100	0	100
Mlx4	0	100	0	100
Mlx5	0	100	0	100
Mlx6	0	100	0	100
Mlx7	0	80	0	100
Mlx8	0	100	0	33
Mlx9	0	80	0	100
Mlx10	0	0	0	100

^a Percentages are based on five artificially latent infected nectarine flowers.

^b Percentages are based on three artificially latent infected nectarines.

^c Positive detection was considered when *Monilinia* DNA was amplified in one of the flowers or at least one of the five DNA extractions of each nectarine epidermis.

control methods. Furthermore, the early, rapid, and accurate detection of latent brown rot infections might help producers and wholesalers choose fruit intended for long-term storage or transported to distant markets, as suggested by Sanzani et al. (2012) for the control of *Botrytis cinerea* in table grapes.

We found that the qPCR-based method is more reliable and consistent than ONFIT because the number of positive detections and the number of replicates scoring positive detections was higher, especially when it was used for detecting latent brown rot infections that were caused by *M. fructigena* and *M. laxa*. The growth rate of *M. fructicola* over the nectarine surface is faster than that of *M. laxa* (Villarino et al. 2016), and this difference in growth rate could cause *M. fructigena* and *M. laxa* isolate presence to be lower than their disease thresholds after the second surface-disinfection and hence not being detected by ONFIT. However, many authors have reported that qPCR detects all DNA, including DNA from nonviable isolates, and this indiscriminating ability of qPCR could give false positives and an overestimation of the number of positive detections (Fittipaldi et al. 2012; Wang and Levin 2006). We detected greater amounts of *M. fructicola* than *M. laxa* DNA and *M. fructigena* DNA in latently infected flowers using qPCR. We found that latent *M. laxa* infections had greater DNA amounts in the mesocarp of latently infected nectarines than *M. fructicola*, which could indicate *M. laxa* has a higher colonization of the mesocarp during latent infections than *M. fructicola*. This deeper colonization of the nectarines by *M. laxa* could increase the time needed for brown rot symptoms to appear in *M. laxa* latently infected nectarines, explaining the low ONFIT detection scores for *M. laxa*.

Although conventional PCR and qPCR-based methods have already been developed for identifying and discriminating *Monilinia* species, these methods rely on sampling plant material with visible disease (Côté et al. 2004; Gell et al. 2007; Guinet et al. 2016; Hughes et al. 2000; Ioos and Frey 2000; van Brouwershaven et al. 2010). We found that the qPCR-based method can detect the pathogen in artificial and natural latent brown rot infections. Many authors have reported that qPCR has a higher sensitivity and test specificity than conventional PCR for detecting and quantifying the DNA of soil-borne fungi, oomycetes, bacteria, nematodes, viruses, and phytoplasmas (Baric et al. 2006; Ippolito et al. 2004; Lievens et al. 2006; Schena et al. 2004, 2013). PCR-based methods are also considered the most effective method for detecting infectious microorganisms with a low titer and an uneven distribution in plants, such as apple proliferation phytoplasma (Baric et al. 2006).

This study proves that the qPCR method developed by van Brouwershaven et al. (2010) could be modified and used to detect latent *Monilinia* infections. Both studies presented good amplification efficiencies and R^2 values; however, the limits of detection shown in the present study (lower than 27 fg) are smaller than those presented in the study by van Brouwershaven et al. (2010) (600 fg). Moreover, cross detection of *M. fructigena* and *M. laxa* was found when using the P_{fc} probe in this study and an additional allelic discrimination step had to be added to distinguish between *M. fructicola* and *M. fructigena*/*M. laxa* while van Brouwershaven et al. (2010) did not detect any cross identification. These differences both in limit of detection and cross-identification can be explained by either the different probe and primer manufacturers or the different qPCR platform used.

The advantages of the qPCR-based method for detecting a latent *Monilinia* infection in nectarines are its high sensitivity, its ease and rapidity of execution, the low number of handling steps, and reduced personnel costs. The disadvantages of the qPCR-based method for detecting a latent *Monilinia* infection in nectarines are the high cost of consumables and reagents, which are much greater than those of ONFIT, and the occurrence of false positives due to detection of nonviable fungal DNA. The number of false positive detections could be reduced by using RNA instead of DNA for qPCR amplification or fluorescent photo affinity labels (photo reactive DNA binding dyes), such as ethidium monoazide or propidium monoazide (Fittipaldi et al. 2012).

To conclude, we propose that the qPCR-based method could be used for detecting latent *Monilinia* infections in stone fruits with latent infections, although specific experiments on peaches, plums, and cherries should be done (Emery et al. 2000; Luo and Michailides 2001; Northover and Cerkaskas 1994). We also conclude that the qPCR-based method will also be very useful for detecting latent *Monilinia* spp. infections in those countries where *Monilinia* spp. are classified as quarantine fungal pathogens.

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