Final Report

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Project Title (Acronym)
Development and validation of molecular tools for detection and identification of European Monilinia species (DIMO)

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2. Executive Summary

Project Summary

Brown rot caused by *Monilinia laxa* (Aderhold and Ruhland) Honey, *M. fructicola* (Winter) Honey (an EPPO A2 quarantine organism), or *M. fructigena* (Aderhold and Ruhland) is a serious fungal disease of peaches. Post-harvest losses are typically more severe than pre-harvest losses, and routinely occur during storage and transport, in some cases even affecting fruit at the processing stage. When the climatic conditions are unfavourable, Monilinia infections may remain latent until the conditions for disease development become favourable or the fruit matures when its susceptibility to disease increases. Latent infections have been described as an asymptomatic infection in which a host-parasite relationship has been established or as a dynamic equilibrium between the host, pathogen, and environment without any visible signs of disease. The incidence of latent infections ranges between 0 to 30% or even 50% of harvested fruit and most of this latent infections are produced the days preceding the harvest. However, most of the latent infections remain asymptomatic until fruit arrive to the markets, which is of especial importance in long distance exports.

Although several molecular methods have been developed to identify and distinguish among Monilinia species on the visible presence of the fungus (van Brouwershaven *et al*. 2010). But none of these methods has been used to detect brown rot latent infections which detection consist on facilitating latent infection activation causing epidermis senescence either with paraquat, an herbicide, or by freezing the fruit at -20ºC for 48 hours (on-fit method) before 5-7 days of incubation to observe the pathogen.

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 *Monilinia fructigena* isolates, and 10 *Monilinia laxa* isolates. We detected bigger amounts of *M. fructicola* DNA than *M. laxa* and *M. fructigena* DNA in latently infected flowers using qPCR. However, bigger 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 and 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.

Second objective of this project was to implement new molecular methods to be applied on symptomatic plant material for Monilinia/Monilia species identification (*M. laxa* and *M. fructigena*, the most common species in Europe, the recently introduced *M. fructicola* and the emerging *M. polystroma*). The new methods were based on the Real-Time PCR and Lamp technology.

Third objective was to develop a detection system based on qPCR combined with a staining dye that allows differentiation between dead and viable conidia from different plant materials. Specifically, the evaluation of the different standard curves

and optimization of the PMA-qPCR conditions were carried out before validation of PMA-qPCR methodology in artificially infected samples and quantification of viable conidia of *M. fructicola* in naturally infected samples. The methodology developed in this work showed a possible strategy to distinguish *M. fructicola* from other *Monilinia* spp. or other microorganisms together with accurate quantification of viable conidia of this pathogen. It should be an ideal method to study the epidemiology and ecology of this phytopathogenic fungus.

The last objective of this project was to determine the viability, practicality, and robustness of a real-time PCR method for detection of Monilinia latent infections a tool for Monilinia latent infection risk quantification on imported or/and exported fruit. This evaluation was done through an international ring test. To ensure homogeneity and avoid quarantine organism manipulation it was decided that sample preparation and DNA extraction would be done by the scheme provider laboratory and then shipped to the rest of the participant laboratories with the rest of the reagents inside boxes with dry ice via fast courier. A common-protocol to storage and handling of samples and reagents, and a data-sheet to record the results was also used. Collaborating laboratories (partners 1, 2, 4, and 5) were asked to record the CT value and the standard deviation of each sample with each probe in the results-data-sheet and send it to the scheme provider besides the raw data of the real-time PCR assay. 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* mycelial samples yielded a positive signal with their corresponding probe. We limited the use of the z-scores to identify those laboratories producing results out of line. The z-scores are calculated to assess the results of each sample for each participant.

3. Report

3.1. Introduction

Brown rot caused by *Monilinia* spp. is an economically important fungal disease of stone and pome fruit and the disease is responsible for substantial pre-harvest and postharvest losses (De Cal and Melgarejo, 1999). Brown rot incidence caused by *Monilinia* spp after harvest is related to the infections, either active or latent, present in fruit at harvest (Villarino et al., 2012) and infections that occur during the harvest period.

Until 2002, brown rot in Europe was only caused by either *M. laxa* (Aderh et Rulh) Honey or *M. fructigena* Honey in Whetzel (De Cal and Melgarejo 1999; Gell *et al*., 2009). Of the two species, *M. laxa* was the most prevalent (85-90%) (Larena *et al*., 2005). In 2002, *M. fructicola* was detected in peach orchards in France (Lichou *et al*., 2002), then it spread in many other countries, like Czech Republic, Spain, Italy, Slovenia, and Germany, until the last detection in Serbia (Hrustić *et al*., 2013). *M. fructicola* is still listed on European and Mediterranean Plant Protection Organization list (EPPO's A2 list). A fourth Monilinia specie, *M. polystroma*, a close relative of *M. fructigena*, was only known from Japan (van Leeuwen *et al*., 2002a), but lately it has also been reported from Hungary, Czech Republic and Switzerland (Petróczy and Palkovics, 2009, Hilber-Bodmer, 2011). *M. fructicola* was now displacing *M. fructigena* and co-existed with the same relative frequency as *M. laxa* in some European peach orchards (Villarino *et al*. 2013). Early, accurate detection and identification of *Monilinia* spp would be essential for effective plant disease management (Lievens 2006).

Traditionally, *Monilinia* spp. are differentiated based on morphological and cultural traits, which require at least 9-10 days (De Cal and Melgarejo, 1999; van Leeuwen and van Kesteren, 1998). The major drawbacks of these methods, although fundamental to plant pathogen diagnostics, are the reliance on the ability of the organism to be cultured, the time consuming and labour intensive nature, and the requirement for skilled taxonomical expertise. Furthermore, visual identification is not always unambiguous due to qualitative, partly shared morphological characteristics among Monilinia species, so that identification has to be conducted under standardized conditions and on pure cultures (van Leeuwen and van Kesteren, 1998). Molecular techniques can overcome many of the shortcomings of the conventional assays, especially if they make use of the polymerase chain reaction (PCR). In general, these methods are more sensitive, more accurate, more specific, and much faster than conventional techniques. Currently, specific primers are available to detect on infected fruit Monilinia species by conventional PCR (Fulton *et al*., 1999; Hughes *et al*., 2000; Ioos and Frey, 2000; Coté *et al*., 2004; Gell *et al*., 2007). However, the PCR assay based on a group I intron in the 18S rDNA (SSU) (Fulton and Brown, 1997; Snyder and Jones, 1999) was not reliable, as some isolates of *M. fructicola* lack this intron (Fulton *et al*., 1999). The analytical sensitivity (detection limit) of these conventional PCR assays is sufficiently low when using pure cultures of *Monilinia* spp., but is too high for routine detection of *M. fructicola* on fruit samples, presumably because of the presence of PCR inhibitors in the samples.

In addition to detection and identification, pathogen quantification is an important aspect with respect to plant disease management, since it provides the information required for determining the necessity, and the extent, of appropriate control strategies. The development of real-time PCR has been a powerful development with

regard to pathogen quantification, elevate detection sensitivity, reduce analysis time and increase automation capability (Baric *et al*., 2006). Increasingly, real-time PCR is being used for plant pathogen diagnosis, including brown rot (Brouwershave *et al*. 2010). However, many of them have not been validated for all species or only been on symptomatic plant tissue and even with extensive fungal growth.
The objective of this study will be: i) to implement a real-time PCR (qPCR) for *Monilinia/Monilia* spp. detection in symptomatic and symptomless plant material, and for conidial quantification of *Monilinia/Monilia* spp.; ii) to validate detection protocols combined with an automated DNA isolation method enabling quick and reliable diagnosis; and iii) to developed a detection system based on qPCR combined with a staining dye that will allow differentiation between dead and viable conidia from different plant materials.

3.2. **Objectives and tasks of the project** (as stated in the proposal, with degree of achievement)

DIMO project workplan was the implementation of real-time PCR for *Monilinia/Monilia* and protocol validations is performed in this project and organised in 4 work packages with the followed achievement:

Work Package	Partner	Degree of achivement
WP 1: Developing a real-time PCR for *Monilinia/Monilia* spp. detection in symptomless plant material.	1 (ES-INIA)	Successfully achieved. One manuscript has been submitted for publication and at present are under revision. One paper to disseminate are in progress
WP 2: Developing new molecular methods for *Monilinia/Monilia* spp. detection in symptomatic plant material.	2 (IT-CREA) and 4 (TR-GDAR)	CREA-PAV, due to the unavailability of funds, delayed the work on the development of Lamp methods to detect *M. fructicola* in symtomatic plant tissues
WP 3: Developing a real- time PCR for quantification of viable conidia of Monilinia/Monilia spp on plant material	3 (ES-IRTA)	Successfully achieved. One manuscript has been submitted for publication and at present are under revision. One paper to disseminate are in progress
WP 4: Validate detection protocols combined with an automated DNA isolation method enabling quick and reliable diagnosis	1 (ES-INIA) ,2 (IT-CRA), 4 (TR-GDAR), 5 (FR-DGAl), and 6 (LT-MoA)	Successfully achieved. One manuscript is being written for publication at present

All the tasks planned in WP1, WP3, and WP4 have been successfully achieved and no incidence along the development of these objectives has occurred. WP2 were delayed due to lack of expected funds. The collaboration with the private company Enbiotech srl made possible to start and carry out part of the programmed research activity.

3.3. Methods used and Results

WP 1: Developing a real-time PCR for *Monilinia/Monilia* spp. detection in symptomless plant material.

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. The *Monilinia* isolates of the collection were confirmed by morphological identification and PCR assays. 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, USA) at 4°C for short-term storage. The *M. fructicola* isolates were grown on PDA plates at 20 to 25ºC 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 25ºC for 10 days and then at 4ºC for 5 days for conidial production. The *M. fructigena* isolates were maintained on PDA slants at 4ºC. 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 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 µE m^{-2} 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 sterilized distilled water (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 haemocytometer and a light microscope (Zeiss Axioskop 2; Carl Zeiss, Inc., Oberkochem, Germany).

Establishment of an artificial latent *Monilinia* infection in nectarine flowers. Due to the almost non-existent occurrence of natural flower latent infections by *Monilinia* in Spanish conditions (Villarino *et al*. 2012); artificial latent infections were generated to ensure *Monilinia* detection such as described in García-Benitez *et al*. (in revision). Two hundred and ninety nectarine flowers without any visible signs of brown rot were collected from a Romea variety nectarine tree in the Jerte Valley, Caceres, Spain in 2014. Flowers were surface-disinfected, dried and inoculated. At the end of the incubation period, the flowers were again surface-disinfected and dried.

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 number homogeneity 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 such as described in García-Benitez et al (in revision).

Detection of latent Monilinia infection by qPCR and ONFIT. Five flowers inoculated with each *Monilinia* isolate and 5 control-uninoculated flowers were stored at -80ºC and then lyophilized in a laboratory freeze dryer (Cryodos -50, Azbil Telstar Technologies, SLU, Terrassa, Spain). Each lyophilized flower was placed on 2 mL micro-centrifuge 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, Ohio, USA). 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 3 nectarines inoculated with each isolate and 3 control-uninoculated nectarines from each year were excised using a sterile disposal scalpel at the end of the 5-day incubation period at 4ºC. The mesocarp was further divided into external mesocarp (from the epidermis to a depth of 1 cm), and the 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 ºC, and lyophilized and homogenized inside each 50 mL centrifuge tube in the conditions previously described for flowers. Genomic DNA was extracted from 5 randomly selected 20 mg samples of each homogenate (nectarine epidermis, external mesocarp, or internal mesocarp) using the DNeasy Plant Mini Kit, 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 labelled with different reporter dyes and quenchers and all primers and probes were obtained from Integrated DNA Technologies Inc. (Coralville, IA, USA). 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 (P2_fgn/lx/ps). Genomic DNA from the flowers (20 ng) or the 3 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, USA) sealed with a clear adhesive. The 20-µL reaction mixture contained 1x 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 or P2_fgn/lx/ps. Thermal cycling was done using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the same conditions and using the same thresholds described by van Brouwershaven *et al*. (2010) with slight modifications: polymerase activation at 95ºC for 10 min, followed by 40 amplification cycles at 95ºC for 15 s and 60ºC for 1 min. Emission was measured at the annealing-extension step. The threshold value was set at fluorescence (ΔRn) of 0.1. 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.

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, which comprised DNA (10 pg) from the different *M. fructicola*, *M. fructigena*, and *M. laxa* isolates in order to monitor reaction performance and efficiency of the qPCR. 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, 8 standard curves, one for each 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 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. 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).

ONFIT (Luo and Michailides 2003) was used to detect latent *Monilinia* infections in the remaining 5 flowers and 3 nectarines of each group. Briefly, the nectarines and flowers were first frozen at -20ºC for 48 h to induce tissue senescence. The senescent fruit and flowers were transferred to plates in humidity chambers (previously described) and incubated at 25ºC 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 numbers 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 3 replicates of the 5 flowers and 5 samples of each of the 3 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 nectarines 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 flowers or at least one of the 5 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, USA) was used to statistically analyse the data. Because some of the qPCR results were negative, there was no homogeneity of variance among treatments. 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) at the 95% confidence level, 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 1). A low limit of detection (less than 30 fg of DNA) was achieved with the different probes for all the different matrixes (Table 1). With the exception of the standard curve for *Monilinia 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 1). Additionally, the fit of the generated data to the regression line was high, as measured by value of the R^2 correlation coefficient that was greater than 0.99 for all curves (Table 1).

Detection of a latent Monilinia infection in nectarine flowers by qPCR. The qPCR detection method was tested in latently infected nectarine flowers by *M. fructicola*, *M. fructigena* and *M. laxa* isolates. The results of these tests were used to compare the latent infections capacities of each species on flowers.
DNA from the *M. fructicola*, *M. fructigena*, and *M. laxa* isolates was not detected in the 5 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 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 between 0.01 ± 0.01 pg and 3.00 ± 1.28 pg, and those from *M. laxa* isolates ranged between pg 0.004 ± 0.006 and 0.57 ± 0.22 pg (Figure 1).

Detection of latent Monilinia infection in nectarines by qPCR. The qPCR detection method was used in latently infected nectarines by *M. fructicola* and *M. laxa*. The results of these tests were used to delimit the depth of the latent infection on nectarines and compare the latent infection capacities of both species. The nectarines were divided in three areas (epidermis, external mesocarp and internal mesocarp) to study the differences on the DNA concentrations of each tissue infected with each *Monilinia* species.
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 2 out of the 3 control-uninoculated nectarines from 2014 and the 3 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 are shown in Figure 2. For the *M. fructicola* and *M. laxa* isolates, the amounts of their DNA detected in the epidermis were bigger than those in the external and internal

mesocarp of latently infected nectarines; except for Mlx2 and Mlx8 (Figure 2B). 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 (Figure 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 (Figure 2). There were no significant differences between external and internal mesocarp median DNA amounts; except in the latently infections caused by Mfc9, Mlx3, Mlx4, Mlx5 and Mlx6 isolates were external mesocarp *Monilinia* DNA amount was bigger than the internal mesocarp *Monilinia* DNA amount.

Comparison between ONFIT and qPCR for detecting a latent Monilinia infection. The *Monilinia*-latent-infection-detection-qPCR-method was compared with ONFIT, the commonly used latent infection detection method, to assess its efficiency and sensitivity. Figure 3 summarises 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, 4 *M. fructicola*, and 1 *M. laxa* isolates, were detected by ONFIT, whereas qPCR detected all *Monilinia* isolates latently infecting flowers, except for isolate Mlx10 (Figure 3). Eighteen of the isolates were detected in all infected flowers by qPCR (100%), while only isolate Mfc8 was detected in all latently infected flowers by ONFIT (Figure 3).

The ONFIT-method only detected 9 *M. fructicola* and none *M. laxa* isolates latently infecting nectarines whereas the qPCR-method detected all 10 *M. fructicola* and 10 *M. laxa* isolates latently infecting nectarines (Figure 3). Furthermore, qPCR detected the latent infections in all infected nectarines from all *M. fructicola* isolates and 9 *M. laxa* isolates, while ONFIT only detected 100% latent infections in 5 of the *M. fructicola* isolates.

Therefore, qPCR detected 67% more latent infections than ONFIT in both flowers and nectarines.

Table 1. Characteristics of the 6 standard curves for detecting and quantifying DNA by qPCR in nectarine flowers and fruit with a latent *Monilinia* infection.

Species	Sample matrix	Limit of Detection (fg)	*y*-intercept	Slope	E (%)	R^2
M. fructicola	Water	8.95± 2.29	32.25 ± 0.09	-3.47 ± 2.9x10^{-2}	94 ± 0.87	0,994
	Flower	9.62± 3.86	30.72 ± 0.10	-3.67 ± 0.8x10^{-2}	87 ± 0.24	0.996
	Epidermis	4.63 ± 0.74	30.04 ± 0.08	-3.57 ± 2.9x10^{-2}	91 ± 0.98	0.999
	Mesocarp	4.11 ± 1.27	28.43 ± 0.13	-3.30 ± 4.6x10^{-2}	100 ± 1.96	0.998
M. laxa/ M. fructigena	Water	26.77 ± 3.76	33.12 ± 0.06	-3.59 ± 0.8x10^{-2}	90% ± 0.28	0.999
	Flower	11.22 ± 0.54	31.16 ± 0.03	-3.60 ± 1.0x10^{-2}	90 ± 0.33	0.996
	Epidermis	3.51 ± 0.19	31.18 ± 0.12	-3.29 ± 4.0x10^{-2}	100 ± 1.67	0.993
	Mesocarp	4.81 ± 0.41	31.19 ± 0.03	-3.57 ± 2.1x10^{-2}	91%± 0.75	0.999

Values are displayed as mean ± standard error.

Figure 1 (left). Amounts of DNA from 10 *M. fructicola* (◆), 8 *M. fructigena* (▲), and 10 *M. laxa* (●) isolates in latently infected flowers that were detected by qPCR. Values are displayed as mean DNA amount ± standard error of 10 inoculated flowers and 3 replicates per repetition.

Figure 2 (right). Amounts of DNA from 10 *M. laxa* isolates A) and *M. fructicola* isolates B) from the epidermis (◆), external mesocarp (▲), and internal mesocarp (●) of 3 latently infected nectarines that were detected by qPCR. Values are displayed as mean DNA amount ± standard error of 3 different inoculated fruit, 5 DNA extractions and 3 replicates.

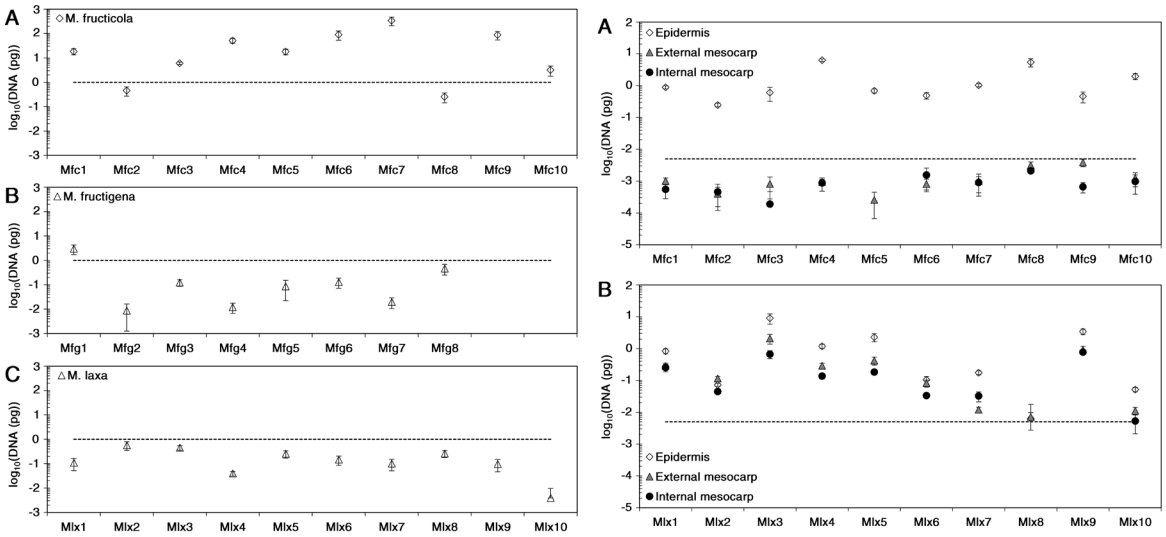
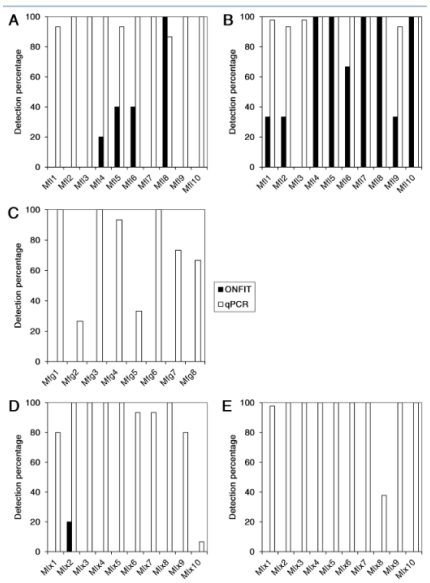


Figure 3. The abilities of ONFIT (■) and qPCR (□) to detect a latent infection in nectarine flowers (A, C, and D) and the epidermis of nectarines (B and E) that was caused by 10 *M. fructicola* isolates (A and B), 8 *M. fructigena* isolates (C), and 10 *M. laxa* isolates (D and E). Data are expressed as a percentage of the number of inoculated flowers (10) and fruit (3) or the number of DNA extractions of flowers (10) and fruit epidermis (15).



WP 2: Developing molecular methods for Monilinia/Monilia spp. detection in symptom plant material

CREA-PAV, due to the unavailability of funds, delayed the work on the development of Lamp methods to detect *M. fructicola* in symtomatic plant tissues. The Lamp technology is an isothermal DNA amplification method very rapid and sensitive that could be very useful “in field” (country border included) application.
The activity, run in collaboration with Italian private company Enbiotech S.r.l., included at the moment:

- Collection of strains and DNA extraction;
- Design of primers for the Loop-Mediated Isothermal Amplification for the following targets *M. fructicola* and *M. fructigena*;
- Verification of the primers against *M. fructicola* and *M. fructigena*: the reaction takes place in a single tube containing Isothermal Lamp Mix, target genomic DNA (extracted from mycelium) and primers. The tube was incubated at 65°C. The amplified product is detected by a portable instrument provided by the private company;
- Evaluation of performances of the test, determining the specificity, sensitivity, repeatability and reproducibility with genomic DNA from mycelium cultures and from symptomatic plant material, is in progress.

The test will be validated using two approaches: 1) validation as a new test by obtaining the minimum test performance criteria: analytical sensitivity, analytical specificity, repeatability, reproducibility. 2) Validation by comparison with the standard test, where the same samples were processed in parallel. All the validation procedures will be carried out as described in the EPPO Standard PM 7/98 (2) (EPPO, 2014).

Results
Analytical sensitivity was evaluated on pure mycelium and on pure mycelium spiked with healthy fruit tissues, peach for *M. fructicola*, apple for *M. fructigena*. Analytical specificity was evaluated on pure mycelium of *M. fructicola*, *M. fructigena*, *M. laxa* and *M. polystroma*.

Preliminar results are shown in **Table 2**

Performance criteria	*M. fructicola*	*M. fructigena*
Analytical sensitivity on pure mycelium (fresh tissue)	5 μg	50 μg
Analytical sensitivity on pure mycelium (fresh tissue) spiked with healthy fruit tissues	50 μg	50 μg
Analytical specificity	no cross reactions	no cross reactions
Repeatability	100%	100%
Reproducibility	100%	100%

WP 3: Developing a real- time PCR for quantification of viable conidia of *Monilinia/Monilia* spp on plant material

Nectarines, peaches and peach flowers were surface-sterilized by dipping them into a blench solution (1 % v/v) for 5 min, immersed in 70 % ethanol for 1 min, rinsed with sterilized water twice for 1 min and allowed to dry at room temperature. For fruit matrix preparation (nectarines or peaches), eight pieces of peel (16 mm diameter) were pooled from 5 fruits and mixed with 20 mL of water with 0.01 % Tween-80 (w/v) and pummelled in a Stomacher 400 set at normal speed for 90 s. The liquid was recovered in a 50 mL Falcon tube. For flower matrix preparation, ten peach flowers were pooled from 2 branches and mixed with 20 mL of water with 0.01 % Tween-80 (w/v) and pummelled in a Stomacher 400 set at normal speed for 90 s. The liquid was also recovered in a 50 mL Falcon tube.

The strain *M. fructicola* (CPMC1) used in this work was obtained from the collection of the Postharvest Pathology Program, IRTA Centre in Lleida (Spain). This strain was identified by the Department of Plant Protection, INIA (Madrid, Spain) and comes from an infected stone fruit. To prepare the conidial suspensions, the strain was grown on Petri dishes containing potato dextrose agar (PDA, Biokar Diagnostics, Allone, France) supplemented with 25 % tomato pulp at 25 ºC for 7 days. Conidia were collected by rubbing the surface of the agar with addition of sterile water with 0.01 % Tween-80 (w/v). Conidia were counted using a haemocytometer and diluted to the desired concentration.

M. fructicola genomic DNA was extracted using the protocol described by Crespo-Sempere *et al*. (2013) with modifications. Briefly, conidia from conidial suspensions were recovered after 10 min centrifugation at 19,060 x *g* and 300 µL of DNA extraction buffer were added (Tris-HCL 200 mM pH 8.5, NaCl 250 mM, EDTA 25 mM, SDS 0.5 %). Conidia were lysed by vortexing with several glass beads (425-600 µm) for 10 min. After centrifugation at 19.060 x *g* for 10 min, 150 µL of 3 M sodium acetate (pH 5.2) was added to the supernatant. The supernatant was stored at -20 ºC for 30 min and then centrifuged at 24.900 x *g* for 15 min. The supernatant was transferred to a new tube and nucleic acids were precipitated by the addition of one volume of isopropanol. After a 5 min incubation time at room temperature, the DNA suspension was centrifuged at 19.060 x *g* for 10 min. The DNA pellet was washed with 70 % ethanol to remove residual salts by vortexing for 10 min. Finally, the pellet was dried at room temperature and DNA resuspended in 25 µL of TE buffer (Tris-HCl 10 mM pH 8, EDTA 1 mM). The amount and purity of DNA samples were determined using a ND-1000 Nanodrop spectrophotometer (Thermo Scientific, DE, USA) and the overall quality verified by agarose gel electrophoresis using GelRed (Biotium Inc, Hayward, CA, USA) such as dye.

To quantify *M. fructicola* genomic DNA, the ABI-7500 qPCR Sequence Detection System (ABI, Applied Biosystems, Madrid, Spain) was used. Primers and TaqMan probes were selected from the published literature (van Brouwershaven *et al*., 2010) and were commercially synthesized by Applied Biosystems. Each reaction was run in triplicate in a final volume of 10 µL containing 1x PCR TaqMan Universal PCR Master Mix II, 200 nM of each primer, 200 nM of TaqMan MGB probe and 2 µL of extracted DNA. Cycling conditions were as follows: 2 min at 50 ºC, 10 min at 95 ºC, followed by 40 cycles at 15 s at 95 ºC and 1 min at 60 ºC. In all cases, a non-template control (NTC) was included using 2 µL of DNAse free water instead of the DNA sample. The quantification cycle (Cq) values represent the cycle number at

which the fluorescence generated within a reaction crosses the defined fluorescence threshold.

Several standard curves were calculated in order to quantify conidia from *M. fructicola*: (1) ten-fold serial dilutions of extracted DNA from *M. fructicola* in TE (used range from 3.2 x 10^4 to 3.2 x 10^{-2} conidia/reaction); (2) DNA extracted from ten-fold serial dilutions of *M. fructicola* conidial suspensions in water with 0.01 % Tween-80 (w/v) (used range from 1.6 x 10^4 to 1.6 x 10^0 conidia/reaction); (3) DNA extracted from ten-fold serial dilutions of *M. fructicola* conidial suspensions in nectarine matrix (used range from 1.3 x 10^4 to 1.3 x 10^{-1} conidia/reaction). qPCR measurements were analysed in triplicate in three independent experiments. Standard curves were created by plotting the number of conidia from each reaction against the Cq values exported from the qPCR machine. Amplification efficiency was calculated from the slope of the standard curve (E = 10-1/slope; Efficiency = (E-1) x 100 (Kubista *et al*., 2006)).

qPCR sensitivity in *M. fructicola* quantification was evaluated in different inoculated matrices. A higher concentration of *M. fructicola* (measured using haemocytometer to approx. 10^7 conidia/mL) was used to prepare several conidial suspensions in water with 0.01 % Tween-80 from 1 x 10^7 to 1 x 10^3 conidia/mL and in nectarine matrix from 9.1 x 10^5 to 9.1 x 10^0 conidia/mL. After DNA extraction and 10 or 100-fold dilution (depending on the purity and quality of the samples) in DNAse free water, samples were analysed by qPCR. Obtained Cq values from these samples were extrapolated using the standard curves described in section 2.5.

Similar methodology was followed to prepare several conidial suspensions in flower matrix from 1 x 10^6 to 1 x 10^2 conidia/mL. After DNA extraction and 10 or 100-fold dilution (depending on the purity and quality of the samples) in DNAse free water, samples were analysed by qPCR. Obtained Cq values from these samples were extrapolated using DNA standard curve. Artificially inoculated samples were also quantified by plating on PDA media and incubated for 48 h at 25 ºC to compare with qPCR values. All reactions were performed in triplicate and three biological replicates were analysed for each experimental condition.

To develop the PMA-qPCR methodology it was necessary to distinguish between viable and dead conidia. Different methodologies (waterbath (Elizaquível *et al*., 2014) and isopropanol addition (Nocker *et al*., 2007)) were tested to obtain a suspension of 10^6 conidia/mL of dead conidia. *M. fructicola* conidial suspensions in water with 0.01 % Tween-80 were treated in a water bath at different time-temperature conditions or adding different volumes of isopropanol for different times. In the case of the isopropanol treatment, cells were removed from isopropanol by centrifugation at 19.060 x *g* for 10 min. After removing the supernatant, pellets of killed conidia were allowed to dry at room temperature and then suspended in 500 µL of water with 0.01 % Tween-80 (w/v). Conidia viability was tested by plating on PDA media and incubation for 48 h at 25 ºC and DNA concentration recovery was checked using a Nanodrop spectrophotometer. To check the penetration of promidium monoazide (PMA), viable and dead conidia (waterbath and isopropanol) were treated with 50, 60, 70 and 100 µM of ready to-use PMA (Biotium Inc, Hayward, CA, USA) for 20 min of incubation in the dark at room temperature with constant agitation at 200 rpm (VMR mini shaker). Thereafter, PMA-treated conidial suspensions were exposed to light using a photo-activation system during 10 min in a closed box with refectory walls and blue wavelength light-emitting diodes (Soto-Muñoz *et al*., 2014). PMA permeability was observed under light microscope using and UV filter from both viable and dead cells.

At the beginning of this experiment, PMA toxicity was tested to establish the maximum PMA concentration available without toxicity problems. Aliquots containing 500 µL of 10^6 conidia/mL were treated with different PMA concentrations (from 50 to 70 mM) and plated on PDA media with incubation at 25 ºC for 48-72 h to record viable conidia.

To select the optimal concentration of PMA, different concentrations were added to 500 µL of 10^6 conidia/mL in nectarine matrix to obtain a final reagent concentration of 60, 80 and 100 µM. After the addition of the reagent, samples were incubated for 20 min in the dark and 10 min of LED exposition as described in the above section.

To optimize the PMA incubation time and after the addition of the reagent to obtain a concentration of 60 µM, samples were incubated for different periods (20, 30, 40 and 50 min) in the dark and 10 min LED exposition.

After LED exposition, samples were used to DNA extraction. Extracted DNA was diluted 10 or 100-fold and samples were analysed by qPCR as described in section 2.4. Obtained Cq values from these samples were extrapolated using DNA standard curve. All reactions were performed in triplicate and three biological replicates were analysed for each experimental condition.

Once the PMA-qPCR methodology was optimized, the protocol was validated using different artificially inoculated samples. Aliquots of 500 µL of nectarine matrix (1 x 10^6 conidia/mL) and flower matrix (1 x 10^6 and 1 x 10^4 conidia/mL) of dead or viable conidia were divided in two different groups. In one group, three aliquots were treated with 60 µM of PMA, incubated in the dark for 20 min at room temperature with agitation at 200 rpm and 10 min of LED exposition; in the other one three aliquots were non-PMA treated.

M. fructicola viable conidial suspension was diluted at different concentrations (10^6, 10^5 and 10^4 conidia/mL) in peach and flower matrix. Viable and isopropanol-killed conidial suspensions were mixed to obtain three different ratios of viable:killed conidia of 10^4:10^6, 10^5:10^6 and 10^6:10^6. For each mixture, 500 µL of these conidial suspensions were PMA treated as described above.

Extracted DNA was diluted 10 or 100-fold and samples were analysed by qPCR. Obtained Cq values from these samples were extrapolated using DNA standard curve. Moreover, conidia viability was also tested by plating on PDA media and incubation for 48 h at 25 ºC. All reactions were performed in triplicate and three biological replicates were analysed for each experimental condition.

Mummies and flowers were obtained from a commercial orchards of stone fruits in Fraga (Aragón, Spain) and in Alcarrás (Cataluña, Spain), respectively and used immediately after harvest.

The quantification of *M. fructicola* on naturally infected samples was carried out following three different methodologies: haemocytometer, dilution plating and PMA-qPCR. Different phenotypes of mummified peaches were both pulp and peel extracted and mixed with 20 mL of potato dextrose broth (PDB, Scharlau Microbiology, Sentmenat, Spain) media in a Stomacher set at normal speed for 90 s. In the case of naturally infected flowers, twenty flowers with symptoms of infection from 2 different branches were mixed with 20 mL of PDB in a similar manner. In both cases, the liquid suspension was recovered in a 50 mL Falcon tube. Each suspension was analysed in triplicate for haemocytometer counting, dilution plating and PMA-qPCR following the methodologies described in the above sections. Each sample was analysed individually due to important differences detected among them.

All data were analysed for significant differences using analysis of variance (ANOVA) with the JMP 8 (SAS Institute Inc., Cary, USA) statistical package. Statistical significance was defined as $P<0.05$; when the analysis was statistically significant, Tukey's test for the separation of means was performed.

Results

To evaluate the efficiency of the primers and the efficiency of DNA extraction, different standard curves were generated. Figure 4 shows the obtained standard curves from three different experiments: (A) ten-fold dilutions of genomic DNA; (B) ten-fold dilutions of DNA extracted from measured conidial suspensions in water; and (C) ten-fold dilutions of DNA extracted from measured conidial suspensions in nectarine matrix. All of them showed a strong linear relationship (R^2 = 0.99) between log conidia/reaction and obtained Cq values. To obtain the slope of the curve, Cq values were plotted against the conidia concentration. The standard curve obtained from the DNA dilution, the slope was -3.45 which represents efficiency (E) of 94.8 %. In the case of conidial suspensions in water and in nectarine matrix, the slope of the curve was -3.99 and -3.48, respectively, which correspond to a good efficiency in the case of conidial suspensions from nectarine matrix (93.8 %), but not high enough in the case of conidial suspensions in water (78.2 %).

Additionally, the DNA standard curve was used to observe the specificity of the primers. Higher DNA concentrations than those extracted from 3.2 x 10^4 conidia/reaction resulted in a partial PCR inhibition and lower DNA concentrations than those extracted from 3.2 x 10^{-2} conidia/reaction, resulting in Cq values next to the limit of quantification (LOQ = 40 cycles) (Fig. 4A). Standard curves obtained from dilutions of conidial suspensions were used to determine the specificity of DNA extraction. In both cases higher concentrations than 1.3-1.6 x 10^4 conidia/reaction resulted in a partial PCR inhibition. However, conidial suspensions in nectarine matrix had a higher LOQ (1.3x10^{-1} conidia/reaction) than conidial suspensions in water (1.6 conidia/reaction) (Fig. 4B-4C).

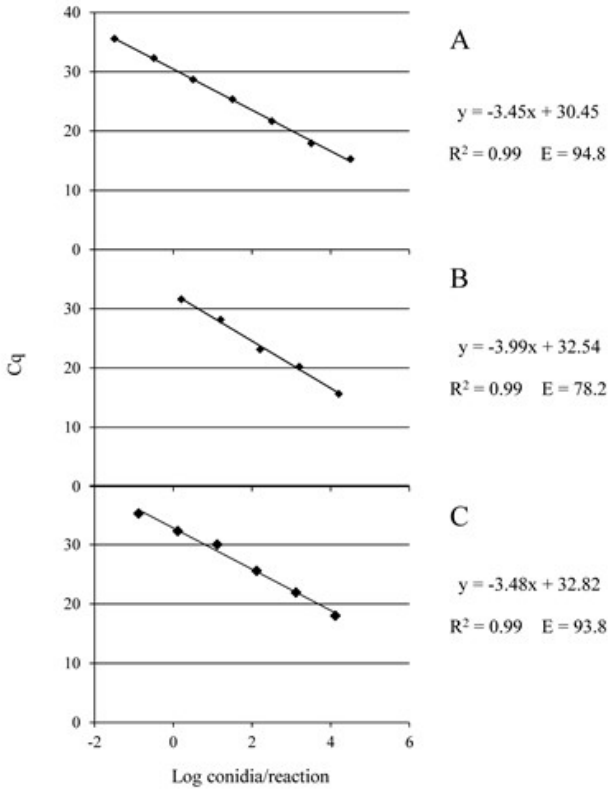


Figure 4. Standar curves for *Monilinia fructicola* used specific primers and TaqMan probes.

qPCR sensitivity to quantify *M. fructicola* conidia in artificially inoculated samples

Cq values obtained from spiked samples were quantified using the above three standard curves to analyse which one showed more accurate quantification (Table 3). Despite the three obtained standard curves had different efficiencies, all of them resulted in an accurate quantification. The general pattern of both spiked samples (*M. fructicola* conidial suspensions in water or in nectarine matrix) resulted approximately in a

reduction less than 0.5 log when standard curve DNA was used for quantification. However, in the case of quantification derived from standard curve of conidial suspensions, especially in nectarine matrix, an overestimated quantification was observed. For this reason and taking into account the resulted efficiency, the standard curve DNA was the one selected to quantify the samples in the next experiments. For that, the standard curve DNA was included in each qPCR run.

Sample	Haemocytometer (conidia mL^{-1})	St curve DNA (conidia $mL^{-1} \pm SD$)	St curve CSW (conidia $mL^{-1} \pm SD$)	St curve CSM (conidia $mL^{-1} \pm SD$)
Conida in water	1.0×10^7	$1.2 \times 10^7 \pm 1.5 \times 10^6$	$1.2 \times 10^7 \pm 1.4 \times 10^6$	$2.4 \times 10^7 \pm 2.9 \times 10^6$
	1.0×10^6	$7.3 \times 10^5 \pm 2.0 \times 10^5$	$7.0 \times 10^5 \pm 1.6 \times 10^5$	$1.4 \times 10^6 \pm 3.3 \times 10^5$
	1.0×10^5	$1.2 \times 10^5 \pm 6.7 \times 10^5$	ND	ND
	1.0×10^4	$5.3 \times 10^3 \pm 1.0 \times 10^3$	ND	ND
	1.0×10^3	$8.3 \times 10^2 \pm 6.8 \times 10^2$	$1.4 \times 10^3 \pm 7.2 \times 10^2$	$2.7 \times 10^3 \pm 1.4 \times 10^3$
Conida in nectarine matrix	9.1×10^5	$4.8 \times 10^5 \pm 1.2 \times 10^5$	$1.3 \times 10^6 \pm 8.8 \times 10^5$	$1.5 \times 10^6 \pm 3.8 \times 10^5$
	9.1×10^4	$4.5 \times 10^4 \pm 1.5 \times 10^4$	$1.9 \times 10^5 \pm 1.6 \times 10^5$	$1.5 \times 10^5 \pm 2.9 \times 10^4$
	9.1×10^3	$3.4 \times 10^3 \pm 1.4 \times 10^3$	ND	ND
	9.1×10^2	$3.5 \times 10^2 \pm 2.3 \times 10^3$	$2.4 \times 10^3 \pm 4.9 \times 10^3$	$1.3 \times 10^3 \pm 8.9 \times 10^3$
	9.1×10^1	$4.5 \times 10^1 \pm 3.7 \times 10^1$	$2.0 \times 10^2 \pm 3.7 \times 10^1$	$1.3 \times 10^2 \pm 1.0 \times 10^1$
	9.1×10^0	$1.0 \times 10^0 \pm 1.9 \times 10^1$	$1.2 \times 10^1 \pm 8.3 \times 10^1$	$5.4 \times 10^0 \pm 1.3 \times 10^2$

Table 3: Conidia concentration from standard curves obtained from spiked samples

The sensitivity of the qPCR to quantify *M. fructicola* conidial suspensions in flower matrix was analysed and compared to plate count methodology (Fig. 5). The range of assayed concentrations was from 10^6 to 10^2 conidia/mL. In all tested concentrations no significant differences were found between the quantification using qPCR and plate count, except for 10^2 conidia/mL which was under LOQ for plate count methodology.

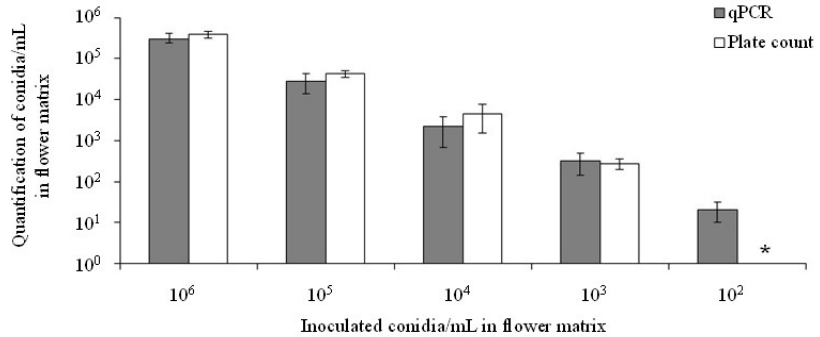


Figure 5. Sensitivity of qPCR to quantify *Monilinia fructicola* conidial suspensions.

For the development of PMA-qPCR methodology to quantify viable *M. fructicola* conidia the first step was to be able to discriminate between live and dead conidia. In our study we used first the water bath as a methodology to kill conidia but despite this technique was useful to kill the conidia, we observed an important decrease in DNA quantity during extraction (Fig. 6A). Moreover, the DNA reductions were greater at higher temperature (60-70 ºC) or longer contact times (10-20 min). Our results showed that the selected temperature was a key factor in the penetration of PMA into

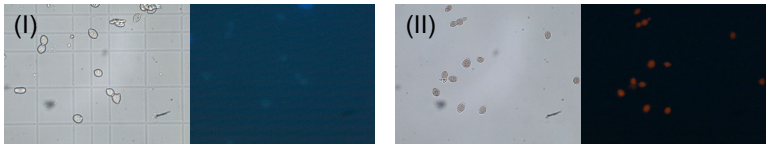
the conidia: at 50 ºC the PMA just penetrated into around 50 % conidia while at 70 ºC the PMA penetrated into all observed conidia. Due to the decrease in DNA concentration using waterbath-killed conidia, isopropanol methodology was assayed. Different volumes of isopropanol were added to conidial suspensions at different contact times (Fig. 6A). Both assayed volumes resulted in 100 % of both died conidia and PMA penetration. However, ½ volume of isopropanol was selected to kill conidia due to less than 5 % of DNA yield losses were obtained. Using light microscopy, isopropanol-killed conidia showed a red colour while live conidia were blue under UV filter which means that PMA treatment was only able to penetrate into isopropanol-killed conidia (Fig. 6B). Different concentrations of PMA (50, 60, 70 and 100 µM) were used to treat the samples and no toxicity was observed at any assayed concentration (data not shown).

A

(i) (ii)

Waterbath-killed conidia			Isopropanol-killed conidia		
Time	Temperature		Time	Isopropanol quantity	
	70-60 ºC	50 ºC		2 volums	½ volum
1-5 min	100 % died conidia	100 % died conidia	5 min	100 % died conidia	100 % died conidia
	80 % reduction in DNA concentration	30 % reduction in DNA concentration		10 % reduction in DNA concentration	< 5 % reduction in DNA concentration
	100 % died conidia with PMA	50 % died conidia with PMA		100 % died conidia with PMA	100 % died conidia with PMA
10-20 min	100 % died conidia	100 % died conidia	10 min	100 % died conidia	100 % died conidia
	85 % reduction in DNA concentration	60 % reduction in DNA concentration		10 % reduction in DNA concentration	< 5 % reduction in DNA concentration
	100 % died conidia with PMA	50 % died conidia with PMA		100 % died conidia with PMA	100 % died conidia with PMA

B



Live conidia Dead conidia

Figure 6. Procedures assayed to obtain killed conidia (A): (i) water bath and (ii) isopropanol. Visualization of PMA penetration into conidia (B) using bright field (I) or UV filter (II) microscopy on live and dead conidia.

Several PMA concentrations (Fig. 7A) and incubation times (Fig. 7B) were tested to optimize the PMA-qPCR methodology for quantification of viable conidia of *M. fructicola* in artificially inoculated nectarine matrix. No significant differences among assayed PMA concentrations were observed both in signal reduction of viable conidia and isopropanol-killed conidia. In agreement with this result, the PMA concentration chosen for PMA-qPCR optimization was 60 µM which gave a signal reduction of 7.31 cycles between live non-PMA treated and isopropanol PMA-treated conidia in nectarine matrix. Another relevant factor to optimize this methodology was the incubation time of samples with PMA. Different periods of incubation were tested (20, 30, 40 and 50 min) but not significant differences in signal reduction among them were observed (5.10, 4.92, 5.25 and 4.85 respectively). For this reason, an incubation time of 20 min was selected to be used in next experiments.

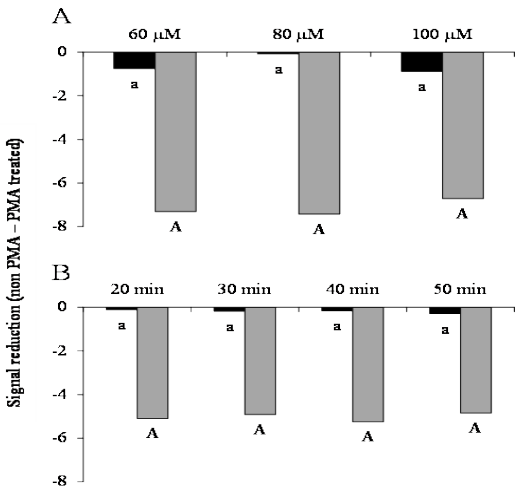


Figure 7. Effect of PMA concentration (A) and incubation time (B) on PMA-qPCR signals of live (black columns) and isopropanol-killed (grey columns) *Monilinia fructicola* conidia in nectarine matrix.

The PMA-qPCR methodology obtained before (60 µM PMA, 20 min incubation period and 10 min LED exposure) was validated in nectarine and flower artificially inoculated (Fig. 8). In nectarine matrix inoculated with 1 x 10^6 conidia/mL, no significant differences were found between the viable conidia detected using plate count (2.07 x 10^5 conidia/mL) or qPCR methodology (3.84 x 10^5 conidia/mL). However, when PMA was added to dead conidia, a decrease of approximately 1.6 log was detected (7.71 x 10^3 conidia/mL) (Fig. 8A). No differences were observed between live conidia treated or not with PMA. However, a slight decrease was observed in PMA untreated dead conidia.

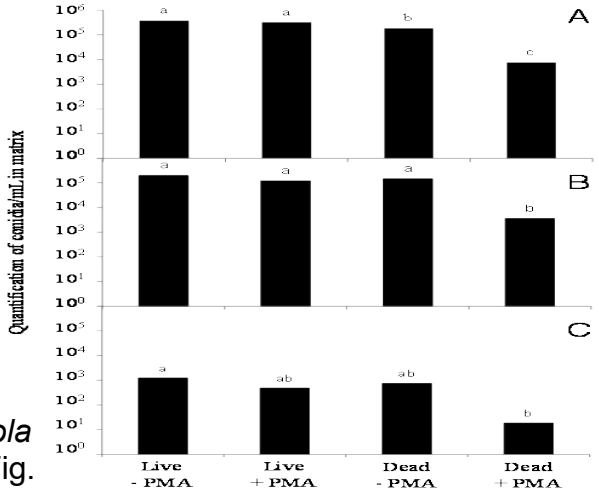
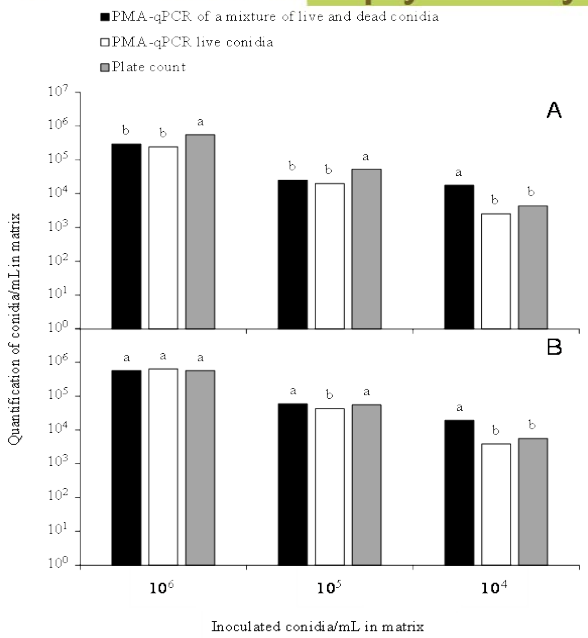


Figure 8. qPCR quantification of live and dead conidia of *Monilinia fructicola* without (-PMA) and with PMA (+PMA) treatment, at 10^6 conidia mL^{-1} inoculum concentrations in nectarine (A) and flower (B) matrix, and at 10^4 conidia mL^{-1} inoculum concentration in flower matrix (C).

In the case of flower matrix two different concentrations of *M. fructicola* were inoculated (10^6 or 10^4 conidia/mL, Fig. 8B and 8C respectively). No significant differences between the viable conidia quantified using plate count or qPCR methodology were found in flower matrix inoculated with 10^6 conidia/mL (5.11 x 10^5 and 1.98 x 10^5 conidia/mL, respectively) or with 10^4 conidia/mL (3.05 x 10^3 and 1.23 x 10^3 conidia/mL, respectively). In flower matrix inoculated with 10^6 conidia/mL, differences between the conidial concentrations in untreated live conidia (1.98 x 10^5 conidia/mL) and PMA-treated dead conidia (3.6 x 10^3 conidia/mL) using PMA-qPCR methodology resulted in approximately 1.7 log; in flower matrix inoculated with 10^4 conidia/mL the differences between both treatments resulted in approximately 1.8 log (1.23 x 10^3 conidia/mL and 1.87 x 10^1 conidia/mL, respectively).

The effect of high concentrations of dead conidia in the accuracy of PMA-qPCR quantification of live conidia was evaluated in peach (Fig. 9A) and flower (Fig. 9B) matrices. Live and dead conidia were mixed at different ratios (10^6:10^6, 10^5:10^6 and 10^4:10^6, respectively) in peach or flower matrices and treated with PMA before quantification. Equal quantities of live and dead conidia did not show differences in concentration compared to live conidia in both peach and flower matrices. Moreover, the most important interferences of dead conidia were observed in the mixture which contains the minimum concentration of live conidia (10^4 conidia/mL). In this case, the overestimation of live conidia by PMA-qPCR represented 0.8 and 0.7 log in peach and flower matrices, respectively.

Figure 9. Quantification of *Monilinia fructicola* using the PMA-qPCR and plate count methodologies containing a variable concentrations of a mixture of live and dead conidia (10^6 conidia/mL) and live conidia in peach (A) and flower matrix (B).



M. fructicola viable conidia were quantified in different phenotypes of naturally infected samples by: haemocytomer, plate count and PMA-qPCR (Fig. 10). Fig. 10A shows the different phenotype of the analysed naturally infected samples (1) one brown mummy, (2) two black mummies and (3) naturally infected flowers. Using haemocytometer and plate count techniques was rather difficult to distinguish *M. fructicola* conidia from other coexistent microorganisms with similar morphology (Fig. 10B and 10C, respectively). Moreover, overestimation of haemocytometer and plate count techniques in comparison to PMA-qPCR was due to the presence of *M. laxa* in brown mummies (Fig. 10D). Despite all mummy samples were harvested in February, a high concentration of *M. fructicola* viable conidia was detected in brown mummies although the concentration among similar phenotype was quite different. In black mummies and flowers, *M. fructicola* was not detected using PMA-qPCR (Table 4). The conidia counted using haemocytometer or plate count methodologies were not effective to quantify *M. fructicola* due to the difficulty of distinguish in the same sample *M fructicola* from *M. laxa*. However, viable conidia of *Monilinia* spp.in black mummies were detected in a range of 4.3 x 10^4 to 1.1 x 10^5 conidia/mL, and in flowers in a range of 8.0 to 10^3-3.8 x 10^4 conidia/mL.

Figure 10. Different phenotypes of naturally infected samples analysed (A) such as brown (1) and black mummies (2) and flowers (3). Images of conidia using a haemocytometer (B) and colony formed units on plate count (C) from naturally infected samples. Quantification of conidia by PMA-qPCR (white columns), haemocytometer (black columns) and plate count (grey columns) in different brown mummies (D).

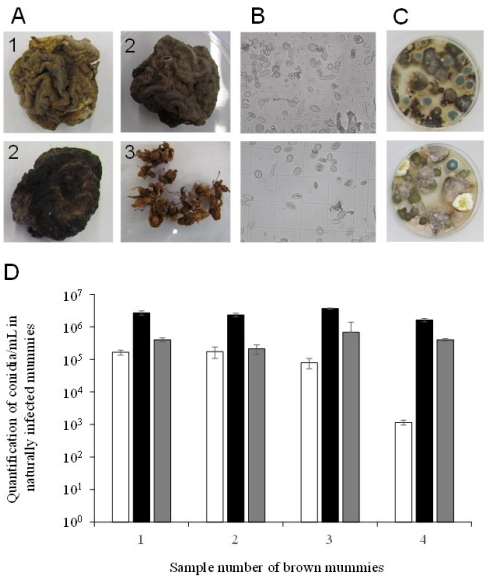


Table 4. Quantification of *Monilinia fructicola* in naturally infected samples (black mummies and flowers) using three different methodologies: PMA-qPCR, haemocytometer and plate count.

Naturally infected samples	Sample number	Quantification metodology		
		PMA-qPCR (conidia mL^{-1} ± SD)	Haemocytometer (conidia mL^{-1} ± SD)	Plate count (conidia mL^{-1} ± SD)
Black mummies	1	< detection limit	$1.5 \times 10^5 \pm 7.1 \times 10^4$	$5.3 \times 10^4 \pm 2.3 \times 10^4$
	2	< detection limit	$2.5 \times 10^5 \pm 7.1 \times 10^4$	$5.7 \times 10^4 \pm 5.8 \times 10^3$
	3	< detection limit	$1.6 \times 10^6 \pm 6.7 \times 10^5$	$1.1 \times 10^5 \pm 5.8 \times 10^3$
	4	< detection limit	$1.0 \times 10^6 \pm 3.5 \times 10^5$	$4.3 \times 10^4 \pm 1.5 \times 10^4$
Flowers	1	< detection limit	$8.0 \times 10^4 \pm 5.0 \times 10^3$	$3.5 \times 10^4 \pm 1.3 \times 10^4$
	2	< detection limit	$3.7 \times 10^5 \pm 1.7 \times 10^4$	$3.8 \times 10^4 \pm 7.5 \times 10^3$
	3	< detection limit	$5.2 \times 10^4 \pm 7.6 \times 10^3$	$1.5 \times 10^4 \pm 3.1 \times 10^3$
	4	< detection limit	$1.0 \times 10^5 \pm 1.8 \times 10^4$	$3.5 \times 10^4 \pm 1.3 \times 10^4$
	5	< detection limit	$2.0 \times 10^5 \pm 1.1 \times 10^4$	$3.2 \times 10^4 \pm 2.1 \times 10^3$

WP 4: Validate detection protocols combined with an automated DNA isolation method enabling quick and reliable diagnosis

The qPCR -based method proposed by García-Benitez *et al*., (in revision) for the detection of *Monilinia* spp. latent infection in fruit and flowers was tested across five different laboratories (Table 5). Each laboratory analysed 10 identical blinded samples following the working protocols and data collection sheets provided. The ring test was carried between September 2015 and September 2016 from sample preparation to data statistical analysis and final report.

Table 5. Laboratory name, country and real-time PCR system

Laboratory	Country	Real-time PCR system
ANSES-Plant Health Laboratory	France	Rotor-Gene Q (Qiagen GmbH, Hilden, Germany)
CREA–Plant Pathology Research Centre (CREA-PAV)	Italy	CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., California, USA)
The State Plant Service under the Ministry of Agriculture (SPS-MoA)	Lithuania	Mastercycler® RealPlex^2 (Eppendorf AG, Hamburg, Germany)
Department of Plant Protection, National Agricultural Research Institute (INIA)	Spain	Applied Biosystems® 7500 Fast Real-Time PCR (Thermo Fisher Scientific, Waltham, MA USA)
General Directorate of Agricultural Research (GDAR) Plant Protection Central Research Institute	Turkey	LightCycler® 480 Real-Time PCR System (F. Hoffmann-La Roche AG, Basel, Switzerland)

To ensure homogeneity and avoid quarantine organism manipulation it was decided that sample preparation and DNA extraction would be done by the scheme provider laboratory and then shipped to the rest of the participant laboratories with the rest of the reagents inside boxes with dry ice via fast courier. A common-protocol to storage and handling of samples and reagents, and a data-sheet to record the results was also used.

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 both low and high C_T values (17 and 35 respectively). Latent infections on flowers and fruits were artificially induced with cold storage following a previously described protocol (Garcia-Benitez et al. 2016). Briefly, Flowers and fruit were first surface disinfected by immersion for 5 minutes in a 1% sodium hypochlorite solution, immersion for 1 minute in a 70% ethanol solution, two 1 minute washes in sterile distilled water (SDW), and drying for 2 hours in a laminar flow hood (Sauer and Burroughs 1986). After drying, nectarine flowers were inoculated with a 30 µL drop of a conidial suspension (10^6 conidia mL^{-1}) and fruit were inoculated by immersion in a conidial suspension (10^5 conidia mL^{-1}) for 30 seconds of either a *M. fructicola* or a *M. laxa* isolate. After inoculation, the flowers and fruit were incubated for 24 hours at 25 ºC and 100% relative humidity (RH) in the dark. At the end of the incubation, they were subjected to a second surface disinfection (previously described) and dried in a laminar flow hood for 2 hours. After drying, the flowers and fruit were incubated at 4 ºC and 100% RH in the dark for 5 days.

Genomic DNA from asymptomatic flowers and fruit was extracted such as described García-Benitez et al. (in revision) using the DNeasy® Plant Mini Kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's instructions. The DNA from the flowers was eluted in 100 µL elution buffer, which is included in the kit, and the DNA from the fruit was eluted in 50 µL elution buffer. DNA concentration was measured with a Nanodrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, Germany) and adjusted to 2 ng μL^{-1} using sterile milli-Q water aliquots of 18 µL were prepared and then lyophilized in a laboratory freeze dryer (Cryodos -50, Azbil Telstar Technologies, SLU, Terrassa, Spain).

The reagents needed for the qPCR assay included: nuclease free-water, 2 x GoTaq® probe qPCR Master Mix (Promega Corporation, Madison, WI, USA), Mon 139F and Mon139R primers, and TaqMan™ probes P_fc and P2_fgn/lx/ps probes (van Brouwershaven et al. 2010) obtained from Integrated DNA Technologies Inc., Coralville, IA, USA. The TaqMan™ probes were labelled with different reporters and quenchers we used a FAM reporter and a ZEN / Iowa Black FQ quencher for *M. fructicola* probe (P_fc) instead of a FAM-TAMRA and a HEX reporter with a ZEN / Iowa Black FQ quencher instead of a VIC-TAMRA for *M. fructigena* and *M. laxa* probe (P2_fgn/lx/ps).

Latent *Monilinia* infections in the nectarine flowers and fruit were detected using the qPCR-based method with TaqMan™ probes that were developed by van Brouwershaven et al (2010) with modifications. Specifically, genomic DNA from the samples (10 ng/5 µL) was amplified in 20-µL reaction mixture, which contained 1x 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 done using the real-time PCR System of each lab (Table 1) under the following conditions: polymerase activation at 95 ºC for 10 minutes, followed by 40 amplification cycles at 95 ºC for 15 seconds and 60 ºC for 1 minute. Emission was measured at the annealing-extension step. The threshold value was set at fluorescence (ΔRn) of 0.2 (0.02 Lab France). A cycle threshold (Ct) 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, when the real-time PCR system permitted it, an allelic discrimination step was added to distinguish between *M. fructicola* and *M. laxa* isolates, and to identify a mixture of the *Monilinia* isolates.

Collaborating laboratories were asked to record the C_T value and the standard deviation of each sample with each Probe in the results-data-sheet and send it to the scheme provider besides the raw data of the real-time PCR assay. 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* mycelial samples yielded a positive signal with their corresponding probe.
To assess the proficiency of the method, "The International Harmonized Protocol for the proficiency testing of analytical chemistry laboratories" (IUPAC Technical Report) (Thompson, Ellison and Wood, 2006) was followed. We limited the use of the *z*-scores 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 is 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-bases "standard deviation for proficiency assessment". The assigned value for each analyzed sample was determined by the consensus of the participants using the Hubert Robust Mean and the robust standard deviation of the participants' results was used as σ_p.

Results

Qualitative detection of latent infections has been reported by qPCR in every laboratory.
Figure 11 show the C_q values for *M. fructicola* and *M. laxa* DNA detection with their respective hydrolysis probes (P_fc and P2_fgn/lx/ps). There is great variability across laboratories, approximately 10 C_q between the extremes for both *M. fructicola* and *M. laxa* (Fig. 11). However, there is less than 5 C_q differences between the 25% and 75% quartiles, except for the flower latently infected with *M. fructicola* sample (Fig. 11).

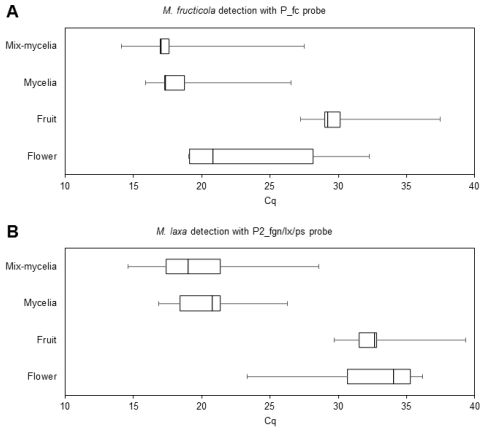


Figure 11. Box-plot of the cycle quantification for *M. fructicola* detected with P_fc hydrolysis probe (A) and *M. laxa* detected with P2_fgn/lx/ps hydrolysis probe (B) in the different DNA samples (flowers and fruits latently infected with either *M. fructicola* (A) or *M. laxa* (B), mycelia from *M. fructicola* (A) or *M. laxa* (B), and mycelia from both *M. fructicola* and *M. laxa* (A and B)) across the five laboratories.

Cross-detection of *M. fructicola* with the P2_fgn/lx/ps hydrolysis probe occurred in 1 of the laboratories (Fig. 12 A), while cross-detection of *M. laxa* with the P_fc probe was more general and appeared in 4 of the 5 laboratories (Fig. 12 B).

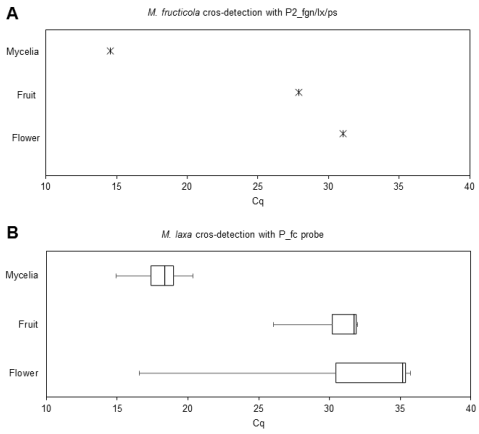


Figure 12. Box-plot of the cycle quantification for *M. fructicola* cross-detected with P2_fgn/lx/ps hydrolysis probe (A) and *M. laxa* cross-detected with P_fc hydrolysis probe (B) in the different DNA samples (flowers and fruits latently infected with either *M. fructicola* (A) or *M. laxa* (B), and mycelia from *M. fructicola* (A) or *M. laxa* (B)), across the five laboratories. Includes data

excluded with the allelic discrimination additional step.
The specificity of the qPCR was tested using the false positive and false negative rate for each of the probes with the data collected from the 5 participating laboratories (Table 6). Neither the P_fc probe, nor the P2_fgn/lx/ps probe were specific, since the false positive rates and/ or the false negative rates were greater than 0%. Four laboratories detected all the *M. laxa* samples with the P_fc hydrolysis probe; however, two of those laboratories were able to differentiate between *M. fructicola* and *M. laxa* by implementing and additional allelic discrimination step to the qPCR assay (Table 6). One laboratory also detected all the *M. fructicola* samples with the P2_fgn/lx/ps hydrolysis probe (Table 6). Finally, one of the laboratories was not able to detect 1 of the 4 *M. laxa* samples with P2_fgn/lx/ps hydrolysis probe, giving a 5% false positive rate as a result (Table 6).

Table 6. Specificity of the qPCR assay across the 5 participating laboratories.

	P_fc probe	**P2_fgn/lx/ps probe**
False positive rate	0%	5%
False negative rate	40% / 20%*	10%

*Two of the four laboratories that identified *M. laxa* as *M. fructicola* were able to distinguish between them using the allelic discrimination step reducing the False negative rate.

A comparison between qPCR detection and ONFIT detection was made with the data provided by the laboratories, obtaining the computed values for diagnostic sensitivity, diagnostic specificity and relative accuracy were obtained (Table 7). The qPCR method was as sensible as the ONFIT since there was no negative deviation between methods. However, both the relative accuracy and diagnostic specificity of the qPCR-method were lower than 100% because of the positive deviation, where 15 more positive-samples were obtained by qPCR than ONFIT.

Table 7. Results of positive agreement (PA), positive deviation (PD), negative deviation (ND), negative agreement (NA), relative accuracy (AC), diagnostic specificity (SP) and diagnostic sensitivity (SE) of the qPCR when compared with ONFIT.

PA	PD	ND	NA	AC (%)[a]	SP (%)[a]	SE (%)[a]
30	15	0	39	82 ± 17	72 ± 13	100 ±

[a]Values are expressed as value ± 95% CI.

Z-scores were calculated with the data to determine those laboratories producing results out of line with respect to the rest of the participating laboratories (Fig. 3). Only one laboratory produced results with *z*-scores between 2 and 3, and then subject to revision (Fig. 13). The rest of the laboratories scored z-scores on the acceptable region between -2 and 2. And there were no laboratories with *z*-scores in the unacceptable region.

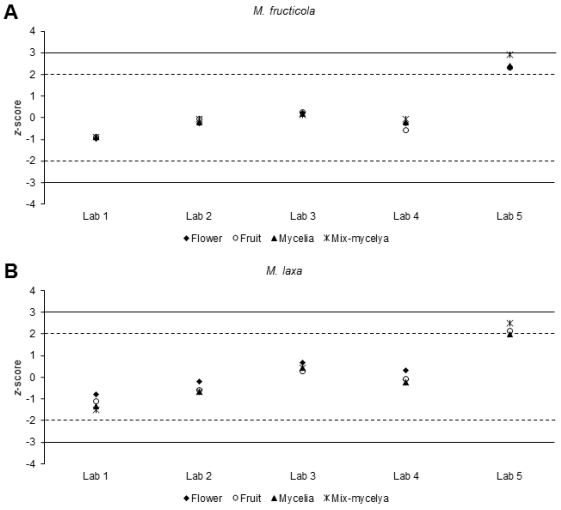


Figure 13. *z*-scores of the detection of the

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

3.4. Discussion of results and their reliability

In this report, we apply a qPCR-based method to detect latent brown rot infections in nectarine flowers and nectarines caused by *M. fructicola*, *M. fructigena* and *M. laxa* isolates. 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 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 pre-harvest control methods. Furthermore, the early, rapid, and accurate detection of latent brown rot infections might help producers and wholesalers choose fruit which, are destined to 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 infections that were caused by *M. fructigena* and *M. laxa*. The growth rate of *M. fructicola* over the nectarine's surface is faster than that of *M. laxa* (Villarino *et al*. 2016), and this difference in growth rate could explain the varying ability of ONFIT to detect latent brown rot infections caused by *M. fructicola*, *M. fructigena*, and *M. laxa*. However, many authors have reported that qPCR detects all DNA, including DNA from non-viable isolates, and this undiscriminating 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 bigger amounts of *M. fructicola* DNA than *M. laxa* and *M. fructigena* DNA in latently infected flowers using qPCR. We found that latent *M. laxa* infections had bigger 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).
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 that of ONFIT, and the occurrence of false positives due to detection of non-viable 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).
The specificity of the primers measured by the standard curve of DNA resulted in a high efficiency (E=94.8) and strong linearity (R^2=0.99). The sensitivity of the DNA extraction of conidial suspensions in nectarine matrix was also very high (efficiency of 93.8 %) however, that obtained in conidial suspensions in water was not high enough (Postollec *et al*., 2011). The LOQ obtained using the DNA standard curve and the conidial suspensions in nectarine matrix standard curve were very accurate (3.2×10^{-2} and 1.3×10^{-1} conidia/reaction, respectively). Moreover, using the standard curve from conidial suspensions it is possible to analyse the sensitivity of the DNA extraction protocol using a nectarine matrix (Postollec *et al*., 2011). Despite these differences among efficiencies and LOQs, all standard curves showed a good correlation when were used to plot the artificially inoculated samples.
Quantification of flower matrix spiked samples using qPCR resulted more sensitive than the plate count methodology at lower inoculum concentrations (10^2 conidia/mL) and in nectarine matrix spiked samples was possible to quantify even 9.1 conidia/mL. That results represent that qPCR methodology reduce the time analysis and quantify lower concentrations than traditional methods.

To optimize the PMA-qPCR methodology one of the first steps to define was the protocol to kill the conidia before PMA treatment. In this study two different methodologies were compared to kill *M. fructicola* conidia using physical (heat) or chemical (isopropanol) treatments. In this study, important DNA losses were observed when heat was used to kill conidia, after and before PMA treatment, even at low temperatures (50 ºC) in which the PMA only penetrated in around 50 % died conidia (data not shown). Our results could be attributed because we used conidial suspensions and the DNA dissolved in water could be eliminated during DNA extraction. Isopropanol was the selected methodology to kill *M. fructicola* conidia before PMA treatments; and a complete PMA penetration into dead conidia without yield DNA losses during DNA extraction was obtained.

PMA concentration is one of the first tested parameters to evaluate the cytotoxic effect in live conidia and an efficient uptake in dead conidia. However, these parameters are highly variable depending on the microorganisms and the food matrices or buffers that are containing them. In this study, no cytotoxic effect was observed in live conidia at any assayed concentrations, even when conidial suspensions were in water or fruit matrices.

No differences in signal reduction were also obtained using different PMA concentrations or different incubation times; for this reason the minimum concentration and time was selected: 60 µM, 20 min incubation time and 10 min LED. These results demonstrated that increasing dye concentrations or increasing exposure time was not possible to increase the signal reduction from 7.31 cycles. The difference between live without PMA treated samples and dead with PMA treated samples resulted in 1.6 to 1.8 log reduction in nectarine or flower matrices.

To evaluate the capacity of PMA in distinguishing live conidia in the presence of dead conidia, different ratios of dead:live conidia were evaluated in different matrices using the PMA-qPCR methodology. Different studies showed that high concentrations of dead conidia could overestimate the live conidia quantification (Pan and Breidt, 2007, Elizaquível *et al*., 2012, Varma *et al*., 2009). They attributed this overestimation in part to the presence of 'ghost' bacteria (intact cell-wall/membrane but metabolically inactive cell), but in our case the low overestimation is more probably due to the incomplete signal exclusion of dead conidia. The present study demonstrated that the PMA-qPCR methodology accurately measured low concentration of *M. fructicola* viable conidia (10^4 conidia/mL) without overestimation of dead conidia (10^6 conidia/mL).

Different phenotypes of naturally infected samples were quantified using different methodologies (haemocytometer, plate count and PMA-qPCR). Looking the phenotypes of the mummies and flowers it was not possible to know the presence of *M. fructicola* conidia. Using the traditional methodologies (haemocytometer and plate count) *M. fructicola* viable conidia in brown mummies were overestimated due to the presence of *M. laxa* (equal morphology especially in haemocytometer). In the case of black mummies and flowers, *M. fructicola* was not detected. Previous work using traditional methodologies have showed the presence of viable conidia of *Monilinia* spp on fruit mummies in the field as a primary inoculum source (Zhong *et al*., 2008, Casals *et al*., 2015). Our experiment showed that there were important shortcomings to distinguish among species and, therefore, an overestimates of the population in naturally infected samples occurred using haemocytometer and also by plate count. Additionally, plate count may sub estimate the populations due to some of viable conidia could not grow in culturing media.

Qualitative detection of latent infections has been reported by qPCR in every laboratory. The qPCR method was as sensible as the ONFIT although 15 more positive-samples were obtained by qPCR than ONFIT. There is great variability across laboratories, although there were no laboratories with z-scores in the unacceptable region. Only two of those laboratories were able to differentiate between *M. fructicola* and *M. laxa* by implementing and additional allelic discrimination step to the qPCR assay. The method modified from Van Leeuwen *et al*. is not suitable using the RotorGene Q system, unless discrimination allelic can be done and with a value of threshold line biggest than 0,02 units of fluorescence.

3.5. Main conclusions

The expected benefits and usability of results (technology transfer)

- The aim of the research was to realize an easy, rapid and cheap tool to control and monitor the spread of Monilinia/Monilia species, giving practical implications

in the integrated management of orchards, reducing impacts of incursions of exotic pests by rapid detection and identification at the border and post-border

- qPCR-based method could be used for detecting latent *Monilinia* infections in stone fruits with latent infections
- qPCR-to detect latent infection has been validated by a ring test in DIMO project
- quantification of viable conidia of this pathogen
- a possible strategy to distinguish *M. fructicola* from other *Monilinia* spp. or other microorganisms together

Implication for stakeholders and policy

- qPCR-based method will also be very useful for detecting latent *Monilinia* spp. infections in those countries where *Monilinia* spp. are classified as quarantine pests.
- Invasive diseases are increasingly points of contention between stakeholders nationally and internationally, resulting in quarantines and trade restrictions: surveillance methods based on early detection and control of the disease, preferring those that are statistically validated, represent essential tools for National and International Regulatory agencies
- to evaluate the efficacy of fungicides applied to reduce overwintering inoculum in the orchards
- to study the epidemiology and ecology of phytopathogenic fungus

Recommendations for future work (on the activities or other steps that may be taken to further develop, disseminate or to uptake the results of the project)

- Future works should be aimed to study in deep the population biology of these pathogens for a better understanding the genetic diversity and pathogenicity, defining the fungal evolution in agricultural environments in order to develop rational control strategies.

4. Papers, other publications and dissemination activities done.

- C. Garcia-Benitez, P. Melgarejo, A. De Cal “Detection of latent Monilinia infections in nectarine flowers and fruit by qPCR” Plant Disease. Submitted: 30/09/2016
- Laura Vilanova; Josep Usall; Neus Teixidó; Rosario Torres "Assessment of viable conidia of *Monilinia fructicola* in flower and stone fruit combining propidium monoazide (PMA) and qPCR" Plant Pathology. Submitted: 16/09/2016
- C. Garcia-Benitez, P. Melgarejo, A. Beniusis, C. Guinet, S. Özben, M.T. Valente, L. Riccioni, A. De Cal. “Proficiency of a latent Monilinia spp. infection detection by Real-Time PCR in nectarine flowers and fruit” (in progress)
- Project ID: Development and validation of molecular tools for detection and identification of European Monilinia species (DIMO). Real-time PCR for the detection of latent infections and the assessment of inocula viability of Monilinia spp. in EUPHRESCO Successful story. September 2016. (www.euphresco.net)
- Garcia-Benitez, C., **A. De Cal. 2015.** *Monilinia* spp. fruit and flower latent infection detection by qPCR. International Workshop in Monilinia Lleida-Fruitcentre 25-26 Noviembre 2015.
- De Cal A, Usall J. (2014). Proyecto DIMO: “Development and validation of molecular tools for detection and identification of European Monilinia species”. Taller de seguimiento de proyectos Euphresco. 16 December 2014 Madrid, España

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6. References

1. Baric, S., Kerschbamer, C., and Dalla Via, J. 2006. TaqMan real-time PCR versus four conventional PCR assays for detection of apple proliferation phytoplasma. Plant Mol Biol Rep 24 2:169-184.
2. Biggs, A. R., and Northover, J. 1988. Early and late-season susceptibility of peach fruits to Monilinia fructicola. Plant Dis 72 12:1070-1074.
3. Boehm, E. W. A., Ma, Z., and Michailides, T. J. 2001. Species-specific detection of Monilinia fructicola from California stone fruits and flowers. Phytopathology 91 5:428-439.
4. Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., and Wittwer, C. T. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55 4:611-622.
5. Byrde, R. J., and Willetts, H. J. 1977. The brown rot fungi of fruit: their biology and control. Pergamon Press, Oxford, UK.
6. Casals C, Segarra J, De Cal A, Lamarca N, Usall J, 2015. Overwintering of Monilinia spp. on mummified stone fruit. Journal of Phytopathology 163, 160-167.
7. Côté, M. J., Tardif, M. C., and Meldrum, A. J. 2004. Identification of Monilinia fructigena, M. fructicola, M. laxa, and Monilia polystroma on inoculated and naturally infected fruit using multiplex PCR. Plant Dis 88 11:1219-1225.
8. Crespo-Sempere A, Estiarte N, Marín S, Sanchis V, Ramos AJ, 2013. Propidium monoazide combined with real-time quantitative PCR to quantify viable *Alternaria* spp. contamination in tomato products. *International Journal of Food Microbiology* **165**, 214-220.
9. De Cal, A., Gell, I., Usall, J., Viñas, I., and Melgarejo, P. 2009. First report of brown rot caused by Monilinia fructicola in peach orchards in Ebro valley, Spain. Plant Dis 93 7:763-763.
10. Duchoslavova, J., Siruckova, I., Zapletalova, E., Navratil, M., and Safarova, D. 2007. First report of brown rot caused by Monilinia fructicola on various stone and pome fruits in the Czech Republic. Plant Dis 91 7:907-907.
11. Elizaquível P, Aznar R, Sánchez G, 2014. Recent developments in the use of viability dyes and quantitative PCR in the food microbiology field. *Journal of Applied Microbiology* **116**, 1-13.
12. Elizaquível P, Sánchez G, Aznar R, 2012. Quantitative detection of viable foodborne *E. coli* O157:H7, *Listeria monocytogenes* and *Salmonella* in fresh-cut vegetables combining propidium monoazide and real-time PCR. *Food Control* **25**, 704-708.
13. Emery, K. M., Michailides, T. J., and Scherm, H. 2000. Incidence of latent infection of immature peach fruit by Monilinia fructicola and relationship to brown rot in Georgia. Plant Dis 84 8:853-857.
14. EPPO. 2009. PM 7/18 (2) Monilinia fructicola. EPPO Bulletin 39:337-343.
15. Fittipaldi, M., Nocker, A., and Codony, F. 2012. Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. J Microbiol Methods 91 2:276-289.
16. Förster, H., and Adaskaveg, J. E. 2000. Early brown rot infections in sweet cherry fruit are detected by Monilinia-specific DNA primers. Phytopathology 90 2:171-178.
17. Fourie, P. H., and Holz, G. 2003a. Germination of dry, airborne conidia of Monilinia laxa and disease expression on nectarine fruit. Australas Plant Pathol 32 1:9-18.

18. Fourie, P. H., and Holz, G. 2003b. Germination of dry, airborne conidia of Monilinia laxa and disease expression on plum fruit. Australas Plant Pathol 32 1:19-25.
19. Fulton, C. E., and Brown, A. E. 1997. Use of SSU rDNA group-I intron to distinguish Monilinia fructicola from M. laxa and M. fructigena. FEMS Microbiol Lett 157 2:307-312.
20. Gell, I., Cubero, J., and Melgarejo, P. 2007. Two different PCR approaches for universal diagnosis of brown rot and identification of Monilinia spp. in stone fruit trees. J Appl Microbiol 103 6:2629-2637.
21. Gell, I., De Cal, A., Torres, R., Usall, J., and Melgarejo, P. 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. Eur J Plant Pathol 121 4:487-498.
22. Grabke, A., Hu, M. J., Luo, C. X., Bryson, P. K., and Schnabel, G. 2011. First report of brown rot of apple caused by Monilinia fructicola in Germany. Plant Dis 95 6:772-772.
23. Guinet, C., Fourrier-Jeandel, C., Cerf-Wendling, I., and Ioos, R. 2016. One-step detection of Monilinia fructicola, M. fructigena, and M. laxa on Prunus and Malus by a multiplex real-time PCR assay. Plant Dis 100 12:2465-2474.
24. Hughes, K. J. D., Fulton, C. E., McReynold, D., and Lane, C. R. 2000. Development of new PCR primers for identification of Monilinia species. EPPO Bulletin 30:507-511.
25. Ioos, R., and Frey, P. 2000. Genomic variation within Monilinia laxa, M. fructigena and M. fructicola, and application to species identification by PCR. Eur J Plant Pathol 106 4:373-378.
26. Ippolito, A., Schena, L., Nigro, F., Soleti Ligorio, V., and Yaseen, T. 2004. Real-time detection of Phytophthora nicotianae and P. citrophthora in citrus roots and soil. Eur J Plant Pathol 110 8:833-843.
27. Kox, L. F. F., Boxman, I. L. A., Jansen, C. C. C., and Roenhorst, J. W. 2005. Reliability of nucleic acid amplification techniques: modified target RNA as exogenous internal standard for a real-time RT-PCR for Potato spindle tuber pospiviroid. EPPO Bulletin 35:117-124.
28. Kox, L. F. F., van Brouwershaven, I. R., van de Vossenberg, B. T. L. H., van den Beld, H. E., Bonants, P. J. M., and de Gruyter, J. 2007. Diagnostic values and utility of immunological, morphological, and molecular methods for in planta detection of Phytophthora ramorum. Phytopathology 97 9:1119-1129.
29. Kubista M, Andrade JM, Bengtsson M*, et al.*, 2006. The real-time polymerase chain reaction. *Molecular aspects of medicine* **27**, 95-125.
30. Lichou, J., Mandrin, J. F., Bréniaux, D., Mercier, V., Giauque, P., Desbrus, D., Blanc, P., and Belluau, E. 2002. Une nouvelle moniliose. Monilia fructicola s'attaque aux arbres fruitiers à noyaux. Phytoma la Défense des Végétaux 547:22-25.
31. Lievens, B., Brouwer, M., Vanachter, A. C. R. C., Cammue, B. P. A., and Thomma, B. P. H. J. 2006. Real-time PCR for detection and quantification of fungal and oomycete tomato pathogens in plant and soil samples. Plant Sci 171 1:155-165.
32. Luo, Y., and Michailides, T. J. 2001. Factors affecting latent infection of prune fruit by Monilinia fructicola. Phytopathology 91 9:864-872.
33. Luo, Y., and Michailides, T. J. 2003. Threshold conditions that lead latent infection to prune fruit rot caused by Monilinia fructicola. Phytopathology 93 1:102-111.

34. Luo, Y., Ma, Z., and Michailides, T. J. 2001. Analysis of factors affecting latent infection and sporulation of Monilinia fructicola on prune fruit. Plant Dis 85 9:999-1003.
35. Luo, Y., Ma, Z., Reyes, H. C., Morgan, D., and Michailides, T. J. 2007. Quantification of airborne spores of Monilinia fructicola in stone fruit orchards of California using real-time PCR. Eur J Plant Pathol 118 2:145-154.
36. Ma, Z., Luo, Y., and Michailides, T. J. 2003. Nested PCR assays for detection of Monilinia fructicola in stone fruit orchards and Botryosphaeria dothidea from pistachios in California. J Phytopathol -Phytopathol Z 151 6:312-322.
37. Meier, U., Graf, H., Hess, M., Kennel, W., Klose, R., Mappes, D., Seipp, D., Stauss, R., Streif, J., and van den Boom, T. 1994. Phänologische Entwicklungsstadien des Kernobstes (Malus domestica Borkh. und Pyrus communis L.), des Steinobstes (Prunus-Arten), der Johannisbeere (Ribes-Arten) und der Erdbeere (Fragaria×ananassa Duch.). Nachrichtenbl. Deut Pflanzenschutzd, 46: 141–153.
38. Munda, A., and Marn, M. V. 2010. First report of brown rot caused by Monilinia fructicola affecting peach orchards in Slovenia. Plant Dis 94 9:1166-1166.
39. Nocker A, Sossa-Fernandez P, Burr MD, Camper AK, 2007. Use of propidium monoazide for live/dead distinction in microbial ecology. *Applied and Environmental Microbiology* **73**, 5111-5117.
40. Northover, J., and Cerkauskas, R. F. 1994. Detection and significance of symptomless latent infections of Monilinia fructicola in plums. Can J Plant Pathol - Rev Can Phytopathol 16 1:30-36.
41. Pan Y, Breidt F, 2007. Enumeration of viable *Listeria monocytogenes* cells by real-time PCR with propidium monoazide and ethidium monoazide in the presence of dead cells. *Applied and Environmental Microbiology* **73**, 8028-8031.
42. Papavasileiou, A., Testempasis, S., Michailides, T. J., and Karaoglanidis, G. S. 2015. Frequency of brown rot fungi on blossoms and fruit in stone fruit orchards in Greece. Plant Pathol 64 2:416-424.
43. Pellegrino, C., Gullino, M. L., Garibaldi, A., and Spadaro, D. 2009. First report of brown rot of stone fruit caused by Monilinia fructicola in Italy. Plant Dis 93 6:668-668.
44. Phillips, D. J. 1984. Effect of temperature on Monilinia fructicola conidia produced on fresh stone fruits. Plant Dis 68 7:610-612.
45. Postollec F, Falentin H, Pavan S, Combrisson J, Sohier D, 2011. Recent advances in quantitative PCR (qPCR) applications in food microbiology. *Food Microbiology* **28**, 848-861.
46. Sanzani, S. M., Schena, L., De Cicco, V., and Ippolito, A. 2012. Early detection of Botrytis cinerea latent infections as a tool to improve postharvest quality of table grapes. Postharvest Biol Technol 68:64-71.
47. Sauer, D. B., and Burroughs, R. 1986. Disinfection of seed surfaces with sodium-hypochlorite. Phytopathology 76 7:745-749.
48. Schena, L., Li Destri Nicosia, M. G., Sanzani, S. M., Faedda, R., Ippolito, A., and Cacciola, S. O. 2013. Development of quantitative PCR detection methods for phytopathogenic fungi and oomycetes. J Plant Pathol 95 1:7-24.
49. Schena, L., Nigro, F., Ippolito, A., and Gallitelli, D. 2004. Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. Eur J Plant Pathol 110 9:893-908.
50. Soto-Muñoz L, Teixidó N, Usall J, Viñas I, Crespo-Sempere A, Torres R, 2014. Development of PMA real-time PCR method to quantify viable cells of *Pantoea*

agglomerans CPA-2, an antagonist to control the major postharvest diseases on oranges. *International Journal of Food Microbiology* **180**, 49-55.
51. Thomidis, T., and Michailides, T. J. 2010. Development and implementation of cost-effective strategies to manage brown rot of peach trees in Imathia, Greece. Eur J Plant Pathol 126 4:575-582.
52. van Brouwershaven, I. R., Bruil, M. L., van Leeuwen, G. C. M., and Kox, L. F. F. 2010. A real-time (TaqMan) PCR assay to differentiate Monilinia fructicola from other brown rot fungi of fruit crops. Plant Pathol 59 3:548-555.
53. Varma M, Field R, Stinson M, Rukovets B, Wymer L, Hauglanda R, 2009. Quantitative real-time PCR analysis of total and propidium monoazide-resistant fecal indicator bacteria in wastewater. *Water Research* **43**, 4790-4801.
54. Vasic, M., Duduk, N., Ivanovic, M. M., Obradovic, A., and Ivanovic, M. S. 2012. First report of brown rot caused by Monilinia fructicola on stored apple in Serbia. Plant Dis 96 3:456-456.
55. Villarino, M., Egüen, B., Lamarca, N., Segarra, J., Usall, J., Melgarejo, P., and De Cal, A. 2013. Occurrence of Monilinia laxa and M. fructigena after introduction of M. fructicola in peach orchards in Spain. Eur J Plant Pathol 137 4:835-845.
56. Villarino, M., Melgarejo, P., and De Cal, A. 2016. Growth and aggressiveness factors affecting Monilinia spp. survival peaches. Int J Food Microbiol 227:6-12.
57. Villarino, M., Melgarejo, P., Usall, J., Segarra, J., Lamarca, N., and De Cal, A. 2012. Secondary inoculum dynamics of Monilinia spp. and relationship to the incidence of postharvest brown rot in peaches and the weather conditions during the growing season. Eur J Plant Pathol 133 3:585-598.
58. Wang, S. S., and Levin, R. E. 2006. Discrimination of viable Vibrio vulnificus cells from dead cells in real-time PCR. J Microbiol Methods 64 1:1-8.
59. Zhong YF, Zhang YW, Chen XY, Luo Y, Guo LY, 2008. Overwintering of *Monilinia fructicola* in stone fruit orchards in Northern China. *Journal of Phytopathology* **156**, 229-235.

7. Problems encountered delays and corrective actions taken (if any), publications and (planned) dissemination activities, and possibly terms and definitions, abbreviations, protocols.

Activities of WP2 were delayed due to lack of expected funds. The collaboration with the private company Enbiotech srl made possible to start and carry out part of the programmed research activity.

Partner 1 will prepare a publication to disseminate the methodology developed in WP1 in Spanish professional journal as ‘Agricola Vergel’ or similar.

Partner 3 will prepare a publication to disseminate the methodology developed in WP3 in Spanish professional journal as ‘Fructicultura profesional’ or similar