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ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA

AGRONÓMICA ALIMENTARIA Y DE BIOSISTEMAS

EPIDEMIOLOGÍA Y CONTROL DE LA PODREDUMBRE PARDA

DEL MELOCOTÓN EN POSTCOSECHA

TESIS DOCTORAL

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RESUMEN

*EPIDEMIOLOGÍA Y CONTROL DE LA PODREDUMBRE PARDA DEL
MELOCOTÓN EN POSTCOSECHA*

La podredumbre parda es la principal enfermedad de la fruta de hueso que afecta a melocotones y nectarinas. Los agentes causales de la enfermedad en España son tres especies fúngicas del género *Monilinia*: *M. fructicola* (Winter) Honey, *M. fructigena* (Aderhold and Ruhland) y *M. laxa* (Aderhold and Ruhland) Honey. La enfermedad provoca pérdidas tanto durante la pre-cosecha, causando marchitez de yemas y flores y podredumbre de frutos, como durante la post-cosecha, donde la incidencia de la enfermedad tiene una mayor importancia y causa de media entre un 10 y un 30 % de podredumbre pudiendo alcanzar más del 80 % de pérdidas en aquellos años favorables al desarrollo de la enfermedad y en variedades tardías. Se han estudiado en profundidad la etiología, la epidemiología y el control de la enfermedad durante la pre-cosecha, pero no durante la post-cosecha. El propósito de esta tesis es estudiar la podredumbre parda durante la post-cosecha incidiendo en el estudio de las infecciones latentes en este periodo, que son una de las causas principales de la incidencia de la enfermedad en post-cosecha.

La infección de la fruta comienza cuando las conidias germinan en la superficie de la fruta dando lugar a tubos germinativos y/o apresorios que luego penetran el fruto. Se sabe que la fruta se vuelve más susceptible a medida que madura, sin embargo la relación entre los procesos de infección, la madurez de la fruta y las condiciones ambientales no está bien definida. Por ello se investigó como afectaban a la formación de tubos germinativos y apresorios de *M. fructicola* y *M. laxa*: (a) extractos de piel de melocotón maduro e inmaduro, (b) diferentes temperaturas y (c) diferentes humedades relativas; prestando especial atención a las condiciones ambientales presentes durante la post-cosecha. Se encontró que el mayor número de tubos germinativos se producía al incubar *M. laxa* o *M. fructicola* en medios de cultivo que contenían extractos de piel de melocotón maduro. Mientras que el mayor número de apresorios aparecía al incubarlos en medios de cultivo que contenían extractos de piel de melocotón inmaduro. Las conidias de *M. laxa* germinaron mejor que las de *M. fructicola* a bajas temperaturas. La formación de tubos germinativos y apresorios por *Monilinia* spp. se vio influenciada por los procesos de post-cosecha. *M. laxa* formaba un mayor número de tubos germinativos que *M.*

fructicola cuando las conidias se incubaron a 100 % de humedad relativa en las condiciones de post-cosecha, este número aumentaba tras 3 días consecutivos de refrigeración. El número de apresorios formados por *M. fructicola* y por *M. laxa* también aumentaba tras 3 días consecutivos de refrigeración. La germinación tanto de *M. fructicola* como de *M. laxa* durante las condiciones de post-cosecha a 60 % de humedad relativa fue insignificante. Se concluye que las diferentes capacidades de germinación y formación de apresorios de *M. fructicola* y *M. laxa* a bajas temperaturas, confieren a *M. laxa* una ventaja competitiva sobre *M. fructicola* durante la refrigeración y almacenamiento en post-cosecha.

Tras la germinación de las conidias, *Monilinia* spp. penetra la superficie del fruto y coloniza los tejidos del mismo, la mayoría de los hongos necrótrofos se ayudan de fitotoxinas y/o enzimas líticas para facilitar estos procesos. Existe muy poca información sobre las enzimas líticas de *Monilinia* y ninguna sobre sus fitotoxinas, por lo que se decidió identificar y caracterizar las fitotoxinas y enzimas líticas producidas por las tres especies de *Monilinia*. Se detectó actividad fitotóxica sobre círculos de nectarina en los extractos con butanol del zumo de nectarinas infectadas con *M. fructicola*, *M. fructigena* o *M. laxa* y también en el extracto con acetato de etilo del zumo de nectarinas infectadas con *M. fructicola*. Los extractos con actividad fitotóxica se fraccionaron mediante HPLC para delimitar las moléculas fitotóxicas observando que únicamente una de las fracciones (tiempo de retención de entre 3,5 y 4,5 min) tenía actividad fitotóxica sobre círculos de nectarina. Al analizar los metabolitos fitotóxicos existentes entre esos tiempos de retención con un espectrómetro de masas, se determinó que tenían un peso molecular comprendido entre 329 y 387 g mol⁻¹. Las tres especies de *Monilinia* presentaron actividad enzimática de α -glucosidasas, pectin-lisas, polygalacturonasas, proteasas y xilanasas en medio de cultivo. Adicionalmente, *M. fructicola* y *M. laxa* presentaron actividad enzimática de cutinasas en medio de cultivo. Ninguna de las especies tenía actividad enzimática de β -glucosidasas, necesaria para degradar hemicelulosa y callosa en medio de cultivo. Se concluye que *Monilinia* spp. utiliza tanto fitotoxinas como enzimas líticas para penetrar y colonizar los frutos. Se

precisan estudios adicionales para determinar la estructura química de las fitotoxinas identificadas y determinar el papel que tienen cada una de las actividades enzimáticas en la virulencia de *Monilinia* spp.

Tras la penetración de la superficie de los frutos se sabe que el micelio de *Monilinia* coloniza los tejidos del fruto. Sin embargo, no existe mucha información histológica acerca de cómo se produce esta colonización tanto en infecciones visibles como en infecciones latentes. Por ello se estudió la microanatomía de nectarinas con infecciones latentes y visibles causadas por *M. fructicola*. Para estudiar las infecciones visibles se incubaron nectarinas maduras inoculadas a 25 °C durante 0, 24, 48, 72 y 96 h en oscuridad. Para estudiar infecciones latentes se incubaron nectarinas maduras inoculadas primero a 25 °C durante 24 h y después, tras una desinfección superficial, a 4 °C durante 72, 144, 218 y 288 h en oscuridad. Al terminar los distintos periodos de incubación se diseccionaron muestras de tejidos de las zonas de inoculación para su examen con el microscopio óptico de campo claro y con el microscopio electrónico de transmisión. No se observaron síntomas superficiales en los frutos con infecciones latentes durante las 288 horas de incubación. Durante la infección latente *M. fructicola* primero colonizaba las cavidades subestomáticas y después se expandía colonizando lateralmente las células subepidérmicas y causando un colapso progresivo de las células epidérmicas sin extenderse al mesocarpo. En nectarinas con infección visible aparecían síntomas de podredumbre parda tras 24 h de incubación y a las 48 horas las capas superiores de células epidérmicas empezaban a colapsarse. Tras lo cual la enfermedad progresaba mostrando (a) colonización inter e intracelular de células epidérmicas y mesocárpicas con hifas finas y gruesas, (b) colapso y rotura de células epidérmicas y mesocárpicas, (c) aparición de cavidades lisogénicas en las células del mesocarpo, (d) degradación de la cutícula y la epidermis, y (e) esporulación de *M. fructicola*. Es de especial interés haber demostrado que las infecciones latentes de *Monilinia* permanecen activas, dado que se produce una colonización lenta y progresiva de las células subcuticulares.

Las técnicas de genética molecular, como la transformación fúngica permiten profundizar y ampliar el conocimiento de la patogénesis tanto a nivel molecular como histológico. Por ello se probaron tres métodos de transformación (CaCl_2 /Polietilenglicol, electroporación y acetato de litio) en dos aislados de *M. fructicola* y dos aislados de *M. laxa* y se construyeron vectores de transformación que contenían el gen de resistencia a fleomicina como gen de selección y el gen de una proteína fluorescente (GFP o mCherry). Ninguno de los protocolos dio lugar a transformantes en *Monilinia* spp., pero sí que se logró transformar *P. rubens* con los vectores de transformación contruidos. Las conidias, hifas y esporas de *M. fructicola* y *M. laxa* tienen un número variable de núcleos, que parecen tener cierto grado de heterocariosis lo que dificulta la obtención de transformantes y mutantes homocarióticos.

Para llevar a cabo una buena estrategia de control es necesario conocer la incidencia de podredumbre parda en precosecha, el riesgo de infección en los 30 días previos a la cosecha, los tratamientos de control aplicados en ese periodo y la incidencia de infecciones latentes en los frutos recogidos. Todo lo anterior se puede obtener de manera rápida y sencilla, salvo la incidencia de infecciones latentes. El método de congelación durante la noche (ONFIT por sus siglas en inglés Overnight Freezing Incubation Technique) es el más utilizado para detectar infecciones latentes de podredumbre parda, pero se requieren de 7 a 9 días para su detección. Para resolver este problema se aplicó un método basado en qPCR con objeto de (i) detectar infecciones latentes de podredumbre parda en flores y frutos de nectarino y (ii) identificar las especies de *Monilinia* en dichas infecciones. Para aplicar este método se indujeron infecciones latentes de forma artificial en flores y frutos de nectarino con 10 aislados de *M. fructicola*, 8 aislados de *M. fructigena* y 10 aislados de *M. laxa*. Las flores con infecciones latentes artificiales causadas por *M. fructicola* tenían mayor concentración de ADN fúngico que las que se infectaron con *M. fructigena* y *M. laxa*. Sin embargo había mayor concentración de ADN en el mesocarpio de frutos con infecciones latentes artificiales causadas por *M. laxa* que en aquellos en los que éstas estaban causadas por *M. fructicola*. En conclusión el método basado en qPCR era más sensible, fidedigno y rápido que el método ONFIT para la detección de infecciones latentes de

podredumbre parda, lo que demuestra su utilidad para la detección temprana del patógeno, tanto para su aplicación en estrategias de control como para la detección de las distintas especies de *Monilinia* en los países donde alguna de ellas sea un patógeno de cuarentena.

Para asegurar la reproducibilidad del método de detección de infecciones latentes mediante qPCR, se llevó a cabo un ensayo internacional colaborativo. En este ensayo se determinó la sensibilidad y especificidad del método qPCR de detección de pequeñas concentraciones de ADN (infecciones latentes) en diferentes termocicladores de tiempo real, en 5 laboratorios diferentes y con una metodología común. La validación incluyó un análisis de la precisión, especificidad analítica, sensibilidad analítica y reproducibilidad, de acuerdo a las definiciones de la Organización Europea de Protección de Plantas (EPPO) PM7/98. Todos los equipos de qPCR detectaron infecciones latentes y muestras de micelio de *Monilinia* con las dos sondas hidrolíticas (que distinguen entre *M. fructicola* y *M. fructigena/M. laxa*), mientras que las muestras de flores y frutos sanos no fueron detectadas. La especificidad del método fue consistente entre los distintos laboratorios, pese a los diferentes equipos, sin que ningún laboratorio obtuviese valores “Z” en la zona inaceptable. Las muestras de infecciones latentes causadas por *M. fructicola* fueron correctamente detectadas por todos los laboratorios, pero en algunas de las causadas por *M. laxa* se produjo detección cruzada como si se tratase de muestras de *M. fructicola*. La detección cruzada de *M. laxa* se pudo compensar mediante la implementación de un paso de discriminación alélica, que permite diferenciar entre muestras de *M. fructicola* y de *M. laxa*. En conclusión, el ensayo colaborativo demostró la robustez del método y confirmó los resultados obtenidos en la validación propia. Por lo que el método puede usarse para detectar infecciones latentes en flores y frutos asintomáticos de nectarino. Sin embargo, es necesario seguir trabajando en los cebadores y sondas del método para evitar las detecciones cruzadas.

Los tratamientos de control durante la post-cosecha están muy restringidos por la normativa Europea. Además, se desconoce el efecto que tienen sobre las infecciones latentes los tratamientos químicos,

físicos y biológicos aplicados durante la post-cosecha. Por ello se decidió estudiar e identificar los puntos críticos de activación o promoción del desarrollo de las infecciones latentes durante el proceso de post-cosecha. Para ello, nectarinas desinfectadas superficialmente y congeladas durante 48 h a -20 °C se sometieron a varias combinaciones de: (a) volcado en agua (sí o no), (b) duración del almacenamiento en frío a 4 °C (0, 1 o 3 días antes del volcado y 0, 3 o 10 días después del volcado) y (c) humedad relativa (75 o 100 %). La activación de las infecciones latentes se producía durante los primeros 9 días cuando las nectarinas se incuban a 25 °C. Tanto el volcado en agua como la duración del almacenamiento en frío influyeron sobre la activación de las infecciones latentes, mientras que la humedad relativa no tuvo ningún efecto sobre ella. La activación de las infecciones latentes causadas por *Monilinia* se vio reducida por el volcado en agua y de forma combinada cuando el almacenamiento en frío era inferior a 11 días. Las combinaciones de post-cosecha afectaron a las infecciones latentes causadas por *M. fructicola* y por *M. laxa* en la misma medida. Se concluye que tanto el volcado en agua, como el acortamiento del almacenamiento en frío de los frutos son factores clave para limitar la incidencia de las infecciones latentes durante la post-cosecha.

ABSTRACT

*EPIDEMIOLOGÍA Y CONTROL DE LA PODREDUMBRE PARDA DEL
MELOCOTÓN EN POSTCOSECHA*

Brown rot is the main disease of stone fruit, affecting peaches and nectarines. In Spain the disease is caused by three fungal species of the genus *Monilinia*: *M. fructicola* (Winter) Honey, *M. fructigena* (Aderhold and Ruhland), and *Monilinia laxa* (Aderhold and Ruhland) Honey. Brown rot causes losses both before harvest, infecting buds and causing blossom blight and fruit rot, and during postharvest, when the brown rot incidence is most important. In average the disease causes 10 to 30 % of the harvest to be lost, and in extreme cases when the weather conditions are favorable for the disease in late cultivars the losses may reach more than 80 % of the harvest. Disease etiology, epidemiology and control before harvest had been thoroughly studied, but there is little information of those aspects during postharvest. The aim of this doctoral thesis is to study the epidemiology and control of *Monilinia* spp. latent infections during postharvest as the main causal agent of brown rot incidence in these conditions.

The fungal infection process begins when fungal conidia germinate on the fruit surface to produce germ tubes and/or appressoria. The incidence of brown rot increases as fruit approaches maturity. However, the interaction between the fungal infection process, peach maturity, and the environmental conditions is not well understood. Thus, the effects of exposing *M. laxa* and *M. fructicola* to: (a) peach skin from mature and immature fruit, (b) several temperatures and (c) several relative humidities (RHs), over their germ tube and appressorial formation were studied, taking special attention to the postharvest environmental conditions. The biggest number of germ tubes was found when *M. laxa* or *M. fructicola* was incubated in culture medium which contained a skin extract of mature peaches. In contrast, the biggest number of appressoria was found when *M. laxa* or *M. fructicola* was incubated in culture medium which contained a skin extract of immature peaches. *M. laxa* conidia germinated better than *M. fructicola* conidia at low temperatures. Germ tube and appressorial formation by *Monilinia* spp. were influenced by fruit postharvest handling. The number of germ tubes that were formed by *M. laxa* conidia during postharvest was significantly bigger than that for *M. fructicola* when the conidia were incubated at 100 % RH, and this number increased after 3 consecutive days of refrigeration. The

number of appressoria that were formed by both *Monilinia* spp. also increased after 3 consecutive days of refrigeration. Negligible or no germination of *M. fructicola* and *M. laxa* conidia occurred when the RH was 60 %. We concluded that the dissimilar abilities of *M. laxa* and *M. fructicola* to germinate and form appressoria at low temperatures conferred a competitive advantage to *M. laxa* to survive during fruit postharvest refrigeration and cold storage.

After conidial germination *Monilinia* spp. penetrate the fruit epidermis and colonize the fruit tissues, most necrotrophic fungi use phytotoxins and/or degrading enzymes to facilitate these processes. There is little information about the secreted degrading enzymes by *Monilinia* spp. and no information at all about their phytotoxins. Hence, secreted phytotoxins and degrading enzymes by the three *Monilinia* species were identified and characterized. The butanol extract from juice of nectarines inoculated with *M. fructicola*, *M. fructigena*, or *M. laxa*; and the ethyl acetate extract from juice of nectarines inoculated with *M. fructicola* presented phytotoxic activity on nectarine circles. The extracts with phytotoxic activities were fractioned by HPLC to identify the molecules with phytotoxic activity inside those extracts. Only one (retention time between 3.5 and 4.5 min) out of the total number of fractions of each *Monilinia* spp. inoculated nectarine juice produced toxic activity on nectarine circles. Phytotoxic metabolites in this retention time were analyzed with the mass spectrometer and they presented a molecular weight between 329 and 387 g mol⁻¹. All three *Monilinia* species had α -glucosidase, pectin-lyase, polygalacturonase, protease, and xylanase enzymatic activities on culture media. Additionally, *M. fructicola* and *M. laxa* had cutinase enzymatic activities on culture media. None of the *Monilinia* species had β -glucosidase enzymatic activities that serve to degrade hemicellulose and callose on culture media. We conclude that *Monilinia* spp. uses both phytotoxins and degrading enzymes to penetrate the fruit surface and colonize fruit tissues. Further studies are required to elucidate the complete chemical structure of the toxins from *Monilinia* spp. and determine the virulence capacity of each enzyme produced by *Monilinia* spp.

It is known that *Monilinia* mycelia colonize fruit tissues after penetrating the fruit surface. However, little is known about the histologic features of a latent and a visible *Monilinia* fruit infection. Thus, the microanatomy of nectarines with latent and visible *M. fructicola* infections was analyzed. Mature nectarines were inoculated with a *M. fructicola* isolate and incubated at 25 °C for 0, 24, 48, 72, and 96 h in the dark to analyze visible infections. For investigating the latent infection process, the inoculated nectarines were first incubated at 25 °C for 24 h in the dark and then, after a fruit surface disinfection, incubated at 4 °C for 72, 144, 216, and 288 h in the dark. At the end of the incubation period, samples of nectarine tissue were excised from the inoculation points and prepared for light and transmission electron microscopic examinations. No signs of disease were seen on the surface of nectarines with a latent infection over the 288 h incubation period. When the tissue samples of nectarines with latent infections were microscopically examined, *M. fructicola* colonized the stomata and this stomatal colonization progressively increased over time and was associated with gradual collapse of the epidermal cells and colonization of the subepidermis. In nectarines with visible brown rot, the disease usually appeared after 24 h on the surface and in the uppermost layers of epidermal cells, which began to collapse after 48 h. Subsequently, the diseased tissues of the nectarines displayed (a) colonization of the epidermis and mesocarp by *M. fructicola* with thin and thick hyphae, (b) collapse and disruption of epidermal and mesocarpic cells, (c) lysogenic cavities in the mesocarp, (d) degradation of the cuticle and epidermis, and (e) *M. fructicola* sporulation. We found that *M. fructicola* is active during latent infections because slow and progressive colonization of nectarine subcuticular cells by the fungus occurs.

Molecular tools such as fungal transformation, allow furthering the molecular and histologic pathogenic studies. For that reason it was decided to test three different fungal transformation methods (CaCl₂/Poly ethylene glycol, electroporation, and lithium acetate) in two *M. fructicola* and two *M. laxa* isolates. Expression vectors with the phleomycin resistance gene, as the selection marker, and one fluorescence protein (GFP or mCherry) were constructed. No *Monilinia* spp. transformants were

obtained by any of the tested methods, but *P. rubens* was transformed with the expression vectors. *M. fructicola* and *M. laxa* conidia, hyphae, and protoplasts have a variable number of nuclei and are heterokaryotic, which hinders the fungal transformation of *Monilinia* spp.

A correct postharvest disease control strategy must be based on knowledge obtained before harvest: incidence of brown rot during pre-harvest, risk of infection in the 30 days prior to harvest, control treatments applied during this period, and incidence of latent infection. All easily obtainable except for the incidence of latent infection. The overnight freezing-incubation technique (ONFIT) is a well-established method for detecting latent brown rot infections, but it takes between 7 to 9 days. To solve this issue a qPCR-based method was applied to (i) detect a latent brown rot infection in the blossoms and fruit of nectarine trees 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 *M. fructicola* isolates, 8 *M. fructigena* isolates, and 10 *M. 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, reliable, and quicker than ONFIT for detecting a latent brown rot infection, and could be useful both for control strategies and also in those countries where *Monilinia* spp. are classified as quarantine pathogens.

The *Monilinia* latent infection detection qPCR method was tested in an inter-laboratory trial (ring test) to ensure its reproducibility. A test performance study involving five laboratories was conducted to validate the sensitivity and specificity of several real-time PCR platforms and laboratories for the detection of low concentration of *Monilinia* DNA (latent infections), using a common protocol. Validation included test performance in terms of accuracy, analytical specificity, analytical sensitivity, repeatability, and reproducibility as defined by European Plant Protection Organization (EPPO) standard PM7/98. All qPCR platforms detected *Monilinia* latent infections and mycelia samples with

the two hydrolysis probes (that distinguish between *Monilinia fructicola* and *M. fructigena*/ *M. laxa*.), while healthy flowers and fruit samples remained undetected. The method specificity was consistent between different laboratories despite different equipment used, and there were no laboratories with z-scores in the unacceptable region. *M. fructicola* latent infection samples were correctly detected by all laboratories, but some *M. laxa* samples were cross-detected as if they were *M. fructicola*. *M. laxa* cross-detection could be compensated by including an allelic discrimination step in the qPCR run, which permitted differentiating between *M. fructicola* and *M. laxa* samples. In conclusion, the inter-laboratory comparison showed the robustness of the developed method and confirmed the in-house validation data. Thus, the method could be used to detect latent infections of *Monilinia* in asymptomatic nectarine's fruit and flowers. However, further development of qPCR primers and probes should be undertaken in order to solve the cross-detection problem.

Due to European regulation postharvest control treatments are limited. Furthermore, the effect of the chemical, physical and biological control methods over *Monilinia* latent infections remains unknown. Thus the critical processes inducing latent infection activation during postharvest on the processing plants and fruit distribution have been determined. Disinfected nectarines frozen at -20 °C for 48 h were subject to different combinations of: (a) dump in water (yes or no), cold storage duration at 4 °C (0, 1, or 3 days before and 0, 3, or 10 days after dump in water), and (c) relative humidity (75 or 100 % RH). At room temperature latent infection activation takes places during the first 9 days of incubation. Both dump in water and cold storage duration influenced *Monilinia* spp. latent infection activation, whereas RH had no effect over latent infection activation. Dump in water and combined cold storage durations of less than 11 days reduced latent infection activation. Postharvest handling combinations affected *M. fructicola* and *M. laxa* latent infections to the same extent. We conclude that both fruit dump in water and short postharvest handling periods are key factors on reducing *Monilinia* spp. latent infection activation rates on nectarines.

1. INTRODUCTION

*EPIDEMIOLOGÍA Y CONTROL DE LA PODREDUMBRE PARDA DEL
MELOCOTÓN EN POSTCOSECHA*

1.1. PRODUCTION OF NECTARINES AND PEACHES

1.1.1. Economic importance and geographical distribution of peaches and nectarines production

Peaches (*Prunus persica* L. Batsch) and nectarines (*P. persica* var. *nucipersica* (Suckow) C. K. Schneid) are the ninth largest fruit species produced in the world, with a global production of 1.25×10^{14} t in 2014 (FAO, 2017). The main world producers of peaches and nectarines in 2014 were: China (1.25×10^{14} t), Spain (1.57×10^6 t), Italy (1.38×10^6 t), Greece (9.63×10^5 t), U.S.A. (9.6×10^5 t), Turkey (6.09×10^5 t), Islamic republic of Iran (5.75×10^5 t), Chile (3.56×10^5 t), Argentina (2.91×10^5 t), and Egypt (2.90×10^5 t) (Fig. 1) (FAO, 2017). In Europe the principal producers of peaches and nectarines in 2014 were the Mediterranean countries (Spain, Italy, and Greece) that produced 87 % of the total European production (FAO, 2017).

Fig. 1. Production quantity of the 10 principal peaches and nectarines world producers in 2014 (FAO, 2017).

Spain is the second world producer of peaches and nectarines and the first European producer (FAO, 2017). Spain had a combined production of 1,573,435 t of nectarines (642, 573 t) and peaches (930,862 t) in 2014, 99.47 % (1,565,113 t) of which was destined for exportation (MAGRAMA, 2017a). Spanish peach-tree cultivated area and production had remained stable (approximately 50,000 ha and 0.80×10^6 t) since 2007 when the Spanish peach cultivated area and production dropped because until 2006 peaches and nectarines were considered as only one production by the Spanish government (Fig. 2) (MAGRAMA, 2017a). While Spanish nectarine-tree cultivated area and production had been steadily increasing since 2004, with a cultivated area of approximately 35,450 ha and a production of approximately 0.64×10^6 t in 2014 (Fig. 2) (MAGRAMA, 2017a). The main production area of both peaches and nectarines in Spain is the Ebro Valley (51 % of Spanish production), in the autonomous communities of Aragón (25 % of the Spanish production) and Cataluña (26 % of the Spanish production) (MAGRAMA, 2017a). Other important production areas of peaches and nectarines in Spain are the autonomous communities of Murcia, whose importance as a producer had been rapidly increasing over the last decade (22 % of the Spanish production), Extremadura (10 % of the Spanish production) and Andalucía (10 % of the Spanish production) (MAGRAMA, 2017a).

Fig. 2. Spanish historical evolution of peaches (orange lines) and nectarines (red lines) cultivated area (solid line) and production quantity (dotted line) from 2004 to 2014 (MAGRAMA, 2017a).

1.1.2. Taxonomy, morphology, cultivars and phenological stages of peaches

1.1.2.1. Peach taxonomy

The peach is a tree native to the Northwest region of China, between the Tarim Basin and the north slopes of the Kunlun Shan mountains, where its domestication occurred as early as 6,000 BC (Faust and Timon, 2010; Zheng et al., 2014). They were transported to Persia through the mountain trade routes where they got to be known as Persian fruit, causing its scientific name (Faust and Timon, 2010). Around 330 BC peaches were introduced in Greece and during the Middle Age to the rest of Europe (Faust and Timon, 2010; Vaughan and Geissler, 2009). According to their taxonomy peaches belong to the kingdom plantae, phylum Tracheophyta, class Magnoliopsida, order Rosales, family Rosaceae, genus *Prunus* and specie *Prunus persica* L. Batsch.

1.1.2.2. Peach morphology

The peach is a small deciduous tree; it can grow to 6 m in height and 15 cm in diameter or remain at the size of a bush (Durán, 1993). It has superficial highly dendritic roots that do not mix between tree stands (Durán, 1993). It has lanceolate leaves, 7.5 to 15 cm long, 2 to 3.5 cm broad, pinnately veined, with a lightly serrated margin and a petiole 1 to 1.5 cm long (Fig. 3A) (Fideghelli, 1995). The flowers blossom before the leaves in early-spring; they are solitary or paired pink to red in color and have five petals (Fig. 3B). The fruit is a drupe, 3 to 10 cm in diameter, with a thin skin either velvety (peaches) or smooth (nectarines); it has yellow or whitish fleshy mesocarp adhere (clingstones) or not (freestones) to the stone; its lignified endocarp (stone or pit) contains the seed, which is formed by the embryo, 2 cotyledons and a wood-like husk (Fig. 3C). Peaches are developed from a single ovary, only one of the 2 ova gets fertilized, and produces the seed; this unbalanced fertilization produces one half of the fruit to be slightly larger than the other (Calderón, 1998).

Fig. 3. Peach morphological characteristics: lanceolate peach leaves (A), pink flowers with 5 petals (B), and freestone nectarine fruit, with thin skin and yellow flesh (C).

1.1.2.3. Peach cultivars

The peach trees have the highest cultivar dynamism among the fruit-tree species with hundreds of different cultivars, for example more than 500 new cultivars appeared between 1990 and 1999 (Fideghelli et al., 1998). Its highly cultivar diversity allow peach trees to adapt to different growing conditions, and consumer preferences (Badenes et al., 1999). Peach cultivars can be divided in four different categories according to their morphological characteristics (Felipe, 2002):

- 1) Freestone-peaches (*P. persica* L. Batsch): those whose flesh separates readily from the pit, had yellow or whitish flesh and velvety skin (e.g. Baby Gold, Plácido, Rojo Septiembre, Roig d'Albesa, or Calanda).
- 2) Clingstone-peaches (*P. persica* L. Batsch): those whose flesh clings tightly to the pit, had mostly yellow flesh and velvety skin, and are frequently destined for industry (e.g. Romea, or Tirrenia).
- 3) Nectarines (*P. persica* L. Batsch var. *nucipersica* Suckow): they are peaches with yellow or whitish flesh, whose most characteristic feature is their soft skin instead of velvety skin (e.g. Fantasia, Venus, Autumm Free, Big Top, or Caldesi 20-20)
- 4) Saturn or flat peaches (*P. persica* L. Batsch var. *platycarpa*): they are smaller and flatter than regular peaches that usually have fuzzy red and yellow skin and whitish flesh (e.g. Ufo 3, Ufo 4, Flat Petty, or Sweet Cap).

1.1.2.4. Peach cultivation and phenological stages

Peaches and nectarines had a vegetative development that can be divided in four phases: (i) sprouting or bud development, (ii) shoot development, (iii) leaf fall (it takes approximately 7 months from leaf emergence to leaf fall), and (iv) dormancy period (Calderón, 1998; Rom, 1988). Four different phases are also present during their reproductive development: (i) flowering (that occurs prior to leaf development), (ii) pollination, (iii) fertilization (it takes approximately 2 weeks for the grain of pollen to reach the ovary and fertilize the ovum, and (iv) fruit development (Calderón, 1998; Rom, 1988). Peaches and nectarines grow in fairly limited ranged climates, they need dry, continental or temperate climates due to climatic constraints during their development. The three principal constraints are: (i) a chilling requirement (more than 500 h at temperatures below 7 °C for most cultivars) during their dormancy phase, (ii) susceptibility to spring frost (due to the early-spring flowering), and (iii) summer heat with mean temperatures of the hottest month between 20 to 30 °C (required for fruit development).

Knowing the phenological stages evolution of a crop is an essential tool for pathogen control. It determines the susceptibility during different stages to different pests and permits to create risk assessments predictions to determine when to treat the crop (Meier et al., 1994). The phenological stages used in this doctoral thesis were determined by Gil-Albert (1995a) and Meier et al. (1994) (Fig. 4). The notation used in this doctoral thesis is the BBCH (general scale from Biologische Bundesanstalt, Bundessortenamt and Chemische Industrie, Germany (Meier et al., 1994)) that divided the development in 10 different stages (Table 1). Phenological stages 1 through 5 refer to the vegetative growth, while phenological stages 6 through 10 are related to flowering and fruit development (phenological stages 2 and 4 are not present in fruit trees) (Meier et al., 1994).

1. INTRODUCTION

Table 1. Phenological growth stages and BBCH-identification keys of stone fruit.

Code	Description
Principal growth stage 0: Sprouting/Bud development	
00	Dormancy: leaf buds and the thicker inflorescence buds closed and covered by dark brown scales
01	Beginning of bud swelling (leaf buds); light brown scales visible, scales with light colored edges
03	End of leaf bud swelling: scales separated, light green bud sections visible
09	Green leaf tips visible: brown scales fallen, buds enclosed by light green scales
Principal growth stage 1: Leaf development	
10	First leaves separating: green scales slightly open, leaves emerging
11	First leaves unfolded, axis of developing shoot visible
19	First leaves fully expanded
Principal growth stage 3: Shoot development 1	
31	Beginning of shoot growth: axes of developing shoots visible
32	Shoots about 20 % of final length
33	Shoots about 30 % of final length
3#	Stages continuous till . . .
39	Shoots about 90 % of final length
Principal growth stage 5: Inflorescence emergence	
51	Inflorescence buds swelling: buds closed, light brown scales visible
53	Bud burst: scales separated, light green bud sections visible
54	Inflorescence enclosed by light green scales, if such scales are formed (not all cultivars)
55	Single flower buds visible (still closed) borne on short stalks, green scales slightly open
56	Flower pedicel elongating; sepals closed; single flowers separating
57	Sepals open: petal tips visible; single flowers with white or pink petals (still closed)
59	Most flowers with petals forming a hollow ball
Principal growth stage 6: Flowering	
60	First flowers open
61	Beginning of flowering: about 10 % of flowers open
62	About 20 % of flowers open
63	About 30 % of flowers open
64	About 40 % of flowers open
65	Full flowering: at least 50 % of flowers open, first petals falling
67	Flowers fading: majority of petals fallen
69	End of flowering: all petals fallen
Principal growth stage 7: Development of fruit	
71	Ovary growing; fruit fall after flowering
72	Green ovary surrounded by dying sepal crown, sepals beginning to fall
73	Second fruit fall
75	Fruit about half final size
76	Fruit about 60 % of final size
77	Fruit about 70 % of final size
78	Fruit about 80 % of final size
79	Fruit about 90 % of final size
Principal growth stage 8: Maturity of fruit and seed	
81	Beginning of fruit coloring
85	Coloring advanced
87	Fruit ripe for picking
89	Fruit ripe for consumption: fruit have typical taste and firmness
Principal growth stage 9: Senescence, beginning of dormancy	
91	Shoot growth completed; foliage still fully green
92	Leaves begin to discolor
93	Beginning of leaf fall
95	50 % of leaves discolored or fallen
97	All leaves fallen
99	Harvested product

Fig. 4. Peaches and nectarines phenological stages evolution BBCH. Principal growth stage 0, sprouting/bud development: 00 (A), 01 (B), 03 (C), and 09 (D); principal growth stage 1, leaf development: 11 (E), and 19 (F); principal growth stage 5, inflorescence emergence: 51 (G), 53 (H); 54 (I), 55 (J), 56 (K), and 59 (L); principal growth stage 6, flowering: 60 (M), 61 (N), 65 (O), and 69 (P); principal growth stage 7, development of fruit: 72 (Q), 75 (R), and 77 (S); and principal growth stage 8, maturity of fruit and seed: 87 (T).

1.1.3. Postharvest processes of peaches and nectarines

Peaches and nectarines are harvested through 7 months, from April (early cultivars produced in Seville) to October (late cultivars produced in Calanda) (Felipe, 2002).

Peaches must be sufficiently developed so they can withstand handling and transport when they are harvested, and also meet consumer demands. Harvesting date is often determined by coloring changes on the fruit skin, and fruit flesh firmness. Optimum ripening for harvest is reached when flesh firmness withstands bruising by an 8 mm-needle penetrometer. This is an essential characteristic to reduce postharvest diseases. Peaches and nectarines harvest can be done either manually, semi-mechanically or mechanically (vibrating belt machine) (Durán, 1993; Gil-Albert, 1995b). Mechanical harvest reduces harvesting cost by 50 %, but it also increases the number of damaged fruit by 10 to 30 % and hence the susceptibility of them to new disease infections (Torregrosa et al., 2003).

Peaches and nectarines are climacteric fruit with a high ethylene production. Their high ethylene production shortens their ripening and decaying periods once they have been harvested. Even under refrigerated storage, the commercial life is less than 5 weeks for most peach cultivars and less than 7 weeks for most nectarine cultivars. Peaches and nectarines commercial life can be increased by handling temperature, relative humidity (RH), and controlling the atmosphere inside the storage chambers (Kenneth et al., 2016). The optimum storage temperature is between -1 and 0 °C. Since minimum respiration rate (2 to 3 mL kg⁻¹ h⁻¹) and minimum ethylene production (0.01 to 5 µL kg⁻¹ h⁻¹) are achieved when fruit is stored at 0 °C, and fruit freezing temperature normally oscillates between -3 to -2.5 °C depending on the soluble solid content of the fruit (Fideghelli, 1987). The optimum storage RH is between 90 to 95 %, with an air velocity of 1.5 m³ min⁻¹ (Fideghelli, 1987). Controlling the atmosphere during transport and storage helps maintain the fruit firmness and color. The recommended

atmosphere conditions to reduce internal decay of peaches are 6 % oxygen and 17 % carbon dioxide, however they could vary with cultivar, pre-harvest conditions, and time of storage (Durán, 1993).

Inadequate postharvest handling of peaches and nectarines can produce physiopathies on the fruit: cold damage, dark coloring, or cracks on the skin; and the apparition of brown rot caused by *Monilinia* spp. reducing their value.

In a stone fruit processing plants, nectarines and other stone fruit are subjected to the handling processes represented in Fig. 5. After harvest, fruit is transported to the processing plant, once there, fruit temperature is lower either by air or by water and then stored on the reception chambers for a few hours or up to 3 days at 0 °C (Fig. 5 phase 1). Once fruit can be processed it is dumped in the confection line either with a water interface or in dry (Fig. 5 phase 2). In the confection line fruit is classified by size and diameter and placed in boxes, which in turn are placed in pallets, in a process that takes between 1 to 6 h at a temperature between 15 to 25 °C (Fig. 5 phase 3). The pallets are cooled and stored in the expedition chambers at 0 °C until distribution by refrigerated transports taking between 1 and 10 days for fruit to get to the stores and markets (Fig. 5 phase 4). Finally, fruit is commercialized in the stores remaining a maximum of 5 days at ambient temperature (25 °C) (Fig. 5 phase 5).

Fig. 5. Stone fruit processing plant flow chart.

1.1.4. Peaches and nectarines postharvest diseases

1.1.4.1. Common diseases during postharvest of peaches and nectarines

Brown rot is the main disease of stone fruit both during the crop season and during postharvest. It determines the viability of the crop in some Spanish production areas when high precipitations occur during critical moments (Hong et al., 1997; Larena et al., 2005). But there are other rots affecting stone fruits during postharvest:

- Thread-like mucoralean molds (*Rhizopus stolonifer* (Ehrenb.:Fr.) Linder and *Mucor* spp.) produce the second most important disease of stone fruit during postharvest. Both fungi cause

a fast soft fruit rot that liquefies the flesh of the fruit. They produce a white filamentous mycelial net over the infected area crowned by black globular pinhead-like sporangia. In Spanish conditions *Rhizopus stolonifer* is most common than *Mucor* spp. Initially they can only colonize wounded fruit, but after infecting the first fruit, they break the fruit skin suppurating a characteristic sour smelling juice that contains degrading enzymes allowing them to colonize other unwounded-fruit by destroying their epidermis, this causes a niche effect. The pathogen survives on orchard grounds over vegetal debris, on wounded fruit both in the lower branches of the trees and on the orchard ground. They can also survive adhere to the wooden boxes and inside refrigeration chambers, treatments with water are the main secondary inoculum inside the stone fruit processing plants. Symptomatic fruit can be observed on the orchards, but it is during postharvest when the highest incidence is detected (Usall et al., 2000; 2017).

- Blue mold is frequent in apples, pears, cherries and plums and it is present in peaches and nectarines, but it is of less importance. It is caused by several *Penicillium* spp., but *P. expansum* Thom is the most common species causing the disease and the one causing most postharvest losses. The fungus can only infect wounded fruit, but the infection can take place even at 0 °C, although its optimal temperature growth is 25 °C. It has slow growth at cold temperatures during storage, but when fruit is moved to warmer temperatures it spreads quickly. The first symptoms appear as little circular soft light-brown spots, which are later covered by whitish mycelia producing bluish conidia that eventually cover the whole fruit. *Penicillium* spp. can survive in infected harvest boxes and on the walls of the storage chambers, and most fruit are infected through wounds produced during harvest and fruit handling, when they are treated with water containing conidia. The disease gains importance when consistent rain occurs during harvest season (Usall et al., 2000).
- Gray mold is caused by *Botrytis cinerea* and has little incidence on stone fruit during postharvest. *B. cinerea* can only infect wounded fruit causing hard lesions on immature fruit

and soft lesions on mature fruit. If high relative humidity conditions are present it forms a whitish mycelia on the surface of the affected tissues that sporulates sparingly on refrigeration conditions and copiously at room temperature, acquiring grayish tones. It is normally observed acting as a saprophyte on decomposing mater. Dispersal on postharvest processing plants can be by air and by water, and once a fruit gets infected it spreads quickly to adjacent healthy fruit, causing a niche effect (Usall et al., 2000).

- *Alternaria alternata* (Fr.) Keissler, can infect peaches and nectarines either before harvest or during postharvest. It can produce latent infections that remain quiescent until fruit is harvested developing symptoms during postharvest. Normally *A. alternata* infects the fruit through wounds or weakened tissues. Symptoms begin with round dry hard brown to blackish lesions, occasionally covered by dark olive green mycelia. Rot spots soften as the disease progresses. In refrigeration conditions lesions have a slow development (less than 25 mm in diameter after 5 months) (Usall et al., 2000).
- Other sporadic rot causal agents are: *Cladosporium herbarum* (Pers.) Link, *Aspergillus niger* Tiegh. Non. Cons., and *Trichothecium roseum* (Pers.) Link (Usall et al., 2000).

1.1.4.2. Economic losses due to brown rot

Brown rot is a fungal disease caused by phytopatogenic species of the genus *Monilinia* that affects stone and pome fruit: peaches (*P. persica* L. Batsch) (Fig. 6A), nectarines (*P. persica* L. Batsch var. *nucipersica* Suckow), apricots (*P. armeniaca* L.), cherries (*P. avium* L.) (Fig. 6B), plums (*P. domestica* L.) (Fig. 6C), almonds (*P. amygdalus* L.), apples (*Mallus pumilla* Mill.), pears (*Pyrus communis* L.), or quinces (*Cydonia vulgaris* Pers.).

Fig. 6. Brown rot symptoms in typical affected fruit: peaches (A), cherries (B), and plums (C).

Peaches and nectarines brown rot affects all the Spanish growing areas (De Cal and Melgarejo, 2000). Postharvest losses due to brown rot are typically greater than pre-harvest losses, and routinely occur during handling, storage, and transport (Hong et al., 1997). In extreme cases in late cultivar-orchards when disease favorable meteorological conditions occur, losses can reach 80 or 100 % of the harvest, while in average years, losses oscillate between 10 to 30 % of the harvest (Ahmadi et al., 1999; Elmer and Gaunt, 1993; Gell et al., 2008; Hong et al., 1997; Larena et al., 2005; Penrose et al., 1979; 1985). This represents a 77.25 to 231.75 million euros loss per year in the Spanish production, according to average prices perceive by the growers (MAGRAMA, 2017a). Additional losses during postharvest are produced due to latent infections, where asymptomatic fruit develops brown rot and can act as secondary inoculum. The incidence of latent *Monilinia* infections in harvested fruit ranges from 0 to 30 % and may even be as high as 50 % (Emery et al., 2000; Luo and Michailides, 2001; 2003; Luo et al., 2001a). The incidence of latent infection and that of postharvest brown rot are positively correlated: 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). Most stone fruit with a latent *Monilinia* infection do not develop visible signs of disease until the arrival of fruit at the consumer markets or the consumer's homes. Thus, additional economical losses are suffered due

to confidence loss by consumers and the distribution chain because of this sudden apparition of brown rot symptoms.

1.2. BROWN ROT OF PEACHES AND NECTARINES

1.2.1. Disease symptoms on peaches and nectarines

Monilinia spp. is a necrotrophic pathogen that affects peach- and nectarine-tree shoots, flowers and fruit. Typical symptoms include leaf buds necrosis, inflorescence buds necrosis, shoot cankers, and fruit rot (De Cal and Melgarejo, 2000; Ogawa et al., 1995). Flower necrosis (blossom blight) takes place during spring, first symptoms appear on the anthers and advances through pistil and the ovary. Infected flowers die and remain attached to the shoot in the form of a gummy mass, high fungal sporulation appear over the flowers when humid meteorological conditions occur (Fig. 7A) (Ogawa et al., 1995). Shoots and buds are generally infected from an infected flower or shoot. The leaves of the infected shoots dry out due to lack of water intake and remain adhered to the shoot (Fig. 7B and C). Leaf infection is not common and when it occurs productivity remains unaffected. Over the dead tissues new fungal spores are formed infecting new shoots, flowering buds, or fruit.

Infected buds and shoots form fusiform or elliptical cankers with massive rubber production (Fig. 7D) (Biggs and Northover, 1988). Cankers can surround small shoots and cause their death, or be surrounded by callused tissue in bigger branches (Byrde and Willetts, 1977). Over the years cankers can recede or keep developing increasing their size. When cankers remain on trees they cause important losses since they serve as an entry point for other diseases. Cankers are also an inoculum source with abundant fungal sporulation along several years (Biggs and Northover, 1988; Byrde and Willetts, 1977).

Brown rot on mature fruit typically develops as a rapidly spreading, firm, brown decay. Under optimum conditions, the decay of ripe infected peach and nectarine fruit may become visible within 48 to 72 h of infection (Byrde and Willetts, 1977). Additionally, it can take 36 h after the first symptoms for conidia to appear as smooth grey spots over the decaying tissue (Fig. 7E), and less than 5 days for the fruit to be completely colonize, rotten, and full of spores. It is normal for infected fruit to remain adhered to the tree, because peduncle abscission does not occur on infected fruit. Infected fruit losses its water content over time becoming mummified, serving as an inoculum reservoir.

Fig. 7. Brown rot symptoms: flower necrosis (A), dried leaves due to shoot infection (B and C), shoot canker with massive rubber production (D), infected fruit with conidial sporulation on the tree (E), and infected fruit with conidial sporulation on the ground (F).

1.2.2. Etiology of brown rot

1.2.2.1. Fungal causal agents of brown rot

Monilinia spp. are fungal causal agents of peach- and nectarine-fruit brown rot. Until 2006 two *Monilinia* spp., namely *M. fructigena* (Aderhold & Ruhland) Honey and *M. laxa* (Aderhold & Ruhland) Honey, were identified as the causal agents of brown rot in peaches and nectarines in Spain

(De Cal et al., 2009a). In 2006 a third *Monilinia* specie, *M. fructicola* (G. Winter) Honey, was identified causing brown rot in peach and nectarine orchards in North-Eastern Spain (De Cal et al., 2009a). The genus *Monilinia* belongs to the phylum Ascomycota, subphylum Pezizomycotina, class Leotiomycetes, subclass Leotiomycetidae, order Helotiales, and family Sclerotiniaceae (Kirk et al., 2001; Lumbsch and Huhndorf, 2007).

1.2.2.2. *Monilinia* spp. morphology

The genus anamorph name is *Monilia*. When they are culture on media, first they develop a hyaline mycelia and a dark irregular stroma, containing large amounts of melanin, as the culture gets old (De Cal and Melgarejo, 1994). Conidia are produced on single or dichotomically branched moliniform chains (Fig. 8A and B), which are grouped in sporodochium (Byrde and Willetts, 1977). These conidia are unicellular, hyaline, and alimonate, whose dimensions 11.5 to 21 μm length and 8 to 13 μm broad may vary with temperature and culture media (Fig. 8C) (Biggs and Northover, 1988). Germinated conidia can form either a germination tube (Fig. 8C and D) or an appressorium (Fig. 8E), depending on the environmental conditions (Cruickshank and Wade, 1992; Lee and Bostock, 2006b). Additionally, the genus produces round microconidia 2 μm in diameter on conidiophores in the form of a bottle (phialides) located in many cases in fruiting bodies similar to the pycnidia (pseudo-pycnidia) (Fig. 8F) (Byrde and Willetts, 1977). Microconidia do not germinate, but they are metabolically active and capable to express cutinases (Lee et al., 2010), additionally they appear to possess a spermatid function for the formation of apothecia (Byrde and Willetts, 1977).

Fig. 8. Microscopic details of *Monilinia* spp.: conidia chains (A and B) (scale bar = 100 μ m), conidia (C) (scale bar = 100 μ m), germination tubes (C and D) (scale bar = 100 μ m), appressorial (E) (scale bar = 50 μ m), and pseudo-pycnidia forming microconidia (F) (scale bar = 20 μ m). (Figure F was obtained from the University of Maine).

The teleomorph state is characterized by the formation of sexual spores (ascospores) inside fruiting bodies (apothecia) (Fig. 9). *M. fructigena* and *M. laxa* rarely form apothecia on field conditions or on culture medium, and have never been seen on Spain (Byrde and Willetts, 1977; Villarino et al., 2012a; b). *M. fructicola* apothecia are formed on mummified fruit on the ground (Fig. 9A and B) both under field and under controlled conditions (Byrde and Willetts, 1977; De Cal et al., 2014; Holtz et al., 1998). Apothecia are orange in the form of a cup 5 to 20 mm in diameter (Fig. 9A, B and C). Asci are 102 to 215 μ m length and 4 to 8 μ m broad (Fig. 9D), and contain 8 ascospores, which are unicellular, ovoid in form, and 6 to 15 μ m length and 4 to 8 μ m broad (Fig. 9E).

Fig. 9. *M. fructicola* apothecia characteristics: apothecia around 3 buried mummified fruit (A), apothecia on top of peach mummy (B), apothecia extracted from the ground and the peach mummy (C), microscopic view of the asci on the periphery of the apothecia (D), and microscopic detail of the asci with 8 ascospores each (E).

1.2.2.3. Detection and identification of *Monilinia* spp.

Even though there are morphological differences between the three brown rot *Monilinia* species, it can be difficult to distinguish between them, especially by analyzing their symptomatology. One way to differentiate them is by growing them on Potato Dextrose Agar (PDA). On PDA *M. fructicola* has a quick growth and quick sporulation forming concentric rings, while *M. fructigena* and *M. laxa* have a slow growth, and *M. laxa* has wavy perimeter colonies (Fig. 10) (Byrde and Willetts, 1977; De Cal and Melgarejo, 1999; Villarino et al., 2016b). A clear difference between *M. laxa* and the other two

Monilinia species is the length of its germination tubes until the first ramification when they are grown on water-agar (2 %) medium, which is smaller than 60 μm , while that of both *M. fructicola* and *M. fructigena* is of at least 200 μm (De Cal and Melgarejo, 1999). *M. fructigena* further differentiates from *M. fructicola* because their colonies grow less than 8 mm in PDA with a 16 h photoperiod under black lighting (320 to 380 nm) at 22 °C and 8 h of dark at 18 °C (De Cal and Melgarejo, 1999).

Fig. 10. PDA grown cultures of *M. fructigena* (A), *M. laxa* (B), and *M. fructicola* (C).

An identification protocol for *Monilinia* species based on their morphological differences was designed by De Cal and Melgarejo (1999). However, with the rise of the molecular technologies and due to the long time requirements of this method, new protocols such as, immunoprecipitation (Banks et al., 1997), SDS-PAGE profiles of proteins (Belisario et al., 1999), or Polymerase Chain Reaction (PCR)-based methods e.g. (Fulton et al., 1999; Gell et al., 2007; Hughes et al., 2000), have been developed to speed up the identification of these species. The first DNA-based molecular method for detecting *M. fructicola* was based on amplification of the group 1 introns in the nuclear small subunit ribosomal RNA (SSU rDNA) gene by polymerase chain reaction (PCR) (Fulton and Brown, 1997). Since this method was only able to identify *M. fructicola*, new PCR-based methods, which amplify other specific regions of DNA of *M. fructicola*, *M. fructigena*, and *M. laxa*, were developed (Boehm et al., 2001; Côté et al., 2004; Förster and Adaskaveg, 2000; Gell et al., 2007; Hughes et al., 2000; Ioos and Frey, 2000; Ma et al., 2003). Luo et al. (2007) developed the first qPCR-based method using

SYBR GreenTM (Thermo Fisher Scientific Inc., Waltham, MA) to detect *M. fructicola*, but this method has since been validated only for *M. laxa*. Van Brouwershaven et al. (2010) developed a qPCR-based method that uses hydrolysis probes to differentiate between *M. fructicola* and the three other causal agents of brown rot, *M. fructigena*, *M. laxa* and *Monilia polystroma* (G. C. M. van Leeuwen) Kohn. Moreover, Guinet et al. (2016) developed a multiplex real-time PCR assay that detects and differentiates between *M. fructicola*, *M. fructigena*, and *M. laxa* in just one-step on both *Prunus* and *Malus* species.

1.2.3. *Monilinia* spp. distribution

Until recently, *Monilinia* spp. world distribution was considered dichotomous since it was normal to encounter only two species coexisting in the same geographical area (CABI, 2015; 2016a; b; Keske et al., 2011; May-De Mio et al., 2011). *M. fructigena* and *M. laxa* coexisted in Europe, *M. fructicola* and *M. laxa* coexisted in America and Oceania, and *M. fructicola* and *M. fructigena* coexisted in Asia. Two main issues had change *Monilinia* dichotomous-distribution view: (i) introduction of new *Monilinia* spp. in areas where they were not present before, and (ii) the discovery of three new *Monilinia* species able to cause brown rot in *Prunus* species.

Due to the global economy, pathogens are transported alongside merchandise from one continent to another. There are quarantine measures to prevent the spread of quarantine diseases, but sometimes they are insufficient. For example, *M. fructicola* was considered an European and Mediterranean Plant Protection Organization (EPPO) A1 quarantine pest until 2004 and now it is classified as an EPPO A2 (EPPO, 2009) quarantine pest due to its identification inside several EPPO member countries: France (Lichou et al., 2002), the Czech Republic (Duchoslavova et al., 2007), Italy (Pellegrino et al., 2009), Spain (De Cal et al., 2009a), Germany (Grabke et al., 2011), Slovenia (Munda and Marn, 2010), Serbia (Vasic et al., 2012), and Greece (Papavasileiou et al., 2015) (Fig. 11). Furthermore, since 2014 *M.*

fruticola is no longer considered as a quarantine organism in the European Union (European Commission, 2014). Determining the origin of the European *M. fruticola* population is complex, but studies show that it could have come from two different places in the U.S.A., one from the East Coast and another from the West Coast area (Weger et al., 2011).

Fig. 11. World distribution of *M. fruticola*. Orange areas indicate presence of the pathogen (EPPO, 2016a).

Due to the extended usage of molecular tools in disease diagnostic, it has been discovered that there are at least six species causing brown rot on *Prunus* spp., three more species than the initially described (*M. fruticola*, *M. fructigena*, and *M. laxa*). Peaches and other stone fruit from the *Prunus* genus were originated in occidental regions of China (Li, 1984). In addition of being the origin of the domesticated *Prunus* spp., those regions are also considered the evolutionary origin of pathogens that cause diseases to this genus (Hu et al., 2011). *Monilia polystroma* (G. C. M. van Leeuwen) Kohn, was the first new *Monilinia* species described, it was previously considered *M. fructigena* and it was originated on Japan (van Leeuwen et al., 2000; 2002). It has been isolated from *Malus* spp., *Pyrus* spp., and some *Prunus* spp., but not from peaches (Zhu and Guo, 2010). Recently *M. polystroma* isolates had been detected

in several European regions; Hungary (Petróczy and Palkovics, 2009), Czech Republic, Serbia (Vasic et al., 2013), Croatia (Di Francesco et al., 2015), and Italy (Martini et al., 2015). Up to this moment its presence had not been describe in Spain. The other two new species that infect peaches in Asia: *M. mumecola* (Harada et al., 2004; Hu et al., 2011), and *M. yunnannensis* (Hu et al., 2011) had not been detected in Spain either.

1.2.4. Pre-harvest *Monilinia* spp. epidemiology and control

1.2.4.1. *Monilinia* spp. life cycle

In Spain, mycelia present in mummified fruit found both on trees and the orchard ground, and in the infected twigs of the three *Monilinia* species (*M. fruticola*, *M. fructigena*, and *M. laxa*), acts as overwintering inoculum (Fig. 12) (Gell et al., 2009; Villarino et al., 2010). When meteorological conditions are favorable for the disease, they produce primary inoculum conidia infecting new flowering buds, leaf buds, twigs, and blossoms (Fig. 12) (Byrde and Willetts, 1977; Ogawa et al., 1985). Each structure have a different frequency of infection: mummified fruit on trees caused 70 % of new infections, mummified fruit on the ground 14 % of them, and infected twigs 10 % of the new infections of peaches in Spain (Villarino et al., 2010). Furthermore the number of mummified fruit on trees can explain 65 % of the incidence of brown rot during postharvest (Villarino et al., 2010). However, the relative importance of different inoculum sources may vary between orchards and host species. For example, small-scale clustering of cherry fruit with brown rot within the canopy volume suggests that twig canker may serve as the most important source of inoculum (Everhart et al., 2011). In addition, *M. fruticola* can present a teleomorph or sexual state (Holtz et al., 1998; Landgraf and Zehr, 1982). During early spring fruiting bodies (apothecia), containing asci with 8 ascospores each, are produced on *M. fruticola* mummified fruit found on the orchards ground, (Fig. 12) (Biggs and Northover, 1985; Byrde and Willetts, 1977). *Monilinia* spp. conidia can be dispersed in the air, in the

rain, or by some insects (Kable, 1965; Luo et al., 2001b). There is a positive correlation between the number of conidia of *Monilinia* spp. per m³ of air and the number of mummified fruit and infected branches present on the orchard ground (Villarino et al., 2010).

The primary inoculum conidia infect blossoms and twigs during periods of moderate temperature and humid weather (Gell et al., 2009; Koball et al., 1997). For blossom blight, stamens and stigma are the usual disease entry points, although high humidity facilitates entry via all floral parts except for sepals (Weaver, 1950). Blossom blight symptoms appear quickly causing flower death, while twig canker appears more slowly. Numerous *Monilinia* spp. conidial sporodochia appear at periods of humid weather, with new conidia that serve as secondary inoculum (Fig. 12) (Ogawa et al., 1995). *Monilinia* species do not directly infect leaves or tree bark. Fruit can be infected at any developmental stage but their disease susceptibility increases as they mature (Gell et al., 2008; Lee and Bostock, 2006b; 2007; Villarino et al., 2011; Xu et al., 2007). For fungal infections of fruit, conidia must first be deposited on and adhere to the fruit surface before penetrating the surface through the natural openings and/or injured areas of the fruit surface (Biggs and Northover, 1988; Phillips, 1984). A quick fungal colonization of the infected fruit takes place under disease-favorable meteorological conditions. The colonization produces conidial pustules on top of the degraded tissues as it progresses, approximately after 5 days of infection, acting as a secondary inoculum (Fig. 12) (Byrde and Willetts, 1977). Infected fruit may (i) remain adhere to the tree dehydrating and forming a mummy, which can either stay on the tree (where the conidial viability is maintained for more than one year) (Casals et al., 2015), or (ii) they may fall directly to the ground where they are decompose (Fig. 12) (Ogawa et al., 1985). In summary infected flowers, shoots, and fruit can act as a source of secondary inoculum (Fig. 12).

If environmental conditions are unfavorable for the disease once fruit has been infected, a latent infection may form. Fruit with latent infection do not develop symptoms until the fruit matures, causing fruit brown rot during postharvest (Byrde and Willetts, 1977). There is a positive correlation between

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the number of *Monilinia* spp. latent infections and the incidence of brown rot during postharvest (Emery et al., 2000; Gell et al., 2008; Luo et al., 2001a). The incidence of brown rot can be predicted once the first latent infection is produced and the first conidia appear in the air and in the fruit surface (Villarino et al., 2012b). Incidence of latent infection caused by *Monilinia* spp. can explain 54.6 % of the incidence of brown rot during postharvest (Gell et al., 2008). Landgraf and Zehr (1982) hypothesized that the main inoculum sources during postharvest were latent infection and new infections produced by already infected fruit at harvest acting as secondary inoculum. In north-eastern Spanish orchards it has been determined that 72 % of the incidence of brown rot during postharvest is caused by infections produced before harvest (Villarino et al., 2012b).

Fig. 12. *Monilinia* spp. life cycle. The grey colored area on the left corresponds to the sexual phase of *M. fructicola*. (Obtained from *The American Phytopathological Society*).

1.2.4.2. Brown rot infection process

The fungal infection process can be divided into three phases: pre-penetration or adhesion, penetration, and colonization.

The infection process begins when *Monilinia* conidia germinate on the flower or fruit surface to produce germ tubes and/or appressoria. Conidial germination of *Monilinia* spp. is dependent on ambient temperature, RH and water availability (water activity: a_w) (Luo et al., 2001a; Tamm and Flückiger, 1993; Xu et al., 2001). *M. laxa* conidia can germinate at temperatures as low as $-4\text{ }^{\circ}\text{C}$ on fruit (Tian and Bertolini, 1999). The conidia of *M. laxa*, *M. fructicola*, and *M. fructigena* can germinate at $0\text{ }^{\circ}\text{C}$ (Casals et al., 2010a). No germination of *M. laxa*, *M. fructicola*, and *M. fructigena* conidia occurred at $38\text{ }^{\circ}\text{C}$ nor below $0.87\text{ }a_w$ (Casals et al., 2010a). Eighty percent of viable conidia of *M. fructicola* and *M. fructigena* can germinate within 2 h and those of *M. laxa* can germinate within 4 h at $25\text{ }^{\circ}\text{C}$ and $0.99\text{ }a_w$ on culture media (Casals et al., 2010a).

Monilinia spp. penetration can be done by germination tubes or appressoria depending on the prevailing conditions (Lee and Bostock, 2006b). *M. laxa* can infect through cuticles on healthy blossom and fruit or via tiny injuries on the fruit surface caused by insects, natural splitting, or fruit pickers during harvest (Byrde and Willetts, 1977). Most studies on appressorial formation have focused on *M. fructicola*, the surface properties of the fruit, the stage of fruit maturity, and the signaling pathways that are required for surface attachment and appressorial formation (Cruickshank and Wade, 1992; Lee and Bostock, 2006b).

Mycelial growth of *Monilinia* spp. is dependent on ambient temperature, RH, and water availability (Luo et al., 2001a; Tamm and Flückiger, 1993; Xu et al., 2001). The mycelial growth of *M. laxa* is better than that of *M. fructicola* isolates at low temperatures on blossoms and fruit (Papavasileiou et

al., 2015; Tamm et al., 1995). Additionally, mycelial growth of *M. laxa* can still occur at 0 °C (Tamm and Flückiger, 1993).

1.2.4.3. Brown rot pre-harvest epidemiology

There are two distinctive phases of the disease, one when flowers are affected and another when fruit is affected. Additionally fruit infection can present different phases depending on fruit maturity.

The infection of flowers and the incidence of blossom blight are dependent on: (i) amount of primary inoculum on the orchard (mainly mummified fruit on trees), (ii) phenological state of the flower, and (iii) meteorological conditions, especially temperature and wetness duration (Holb, 2008; Luo et al., 2001b). Flower infection can either cause blossom blight (flower necrosis and death), or remain as a latent infection (Luo et al., 2005). Blossom blight due to *Monilinia* spp. is infrequent in Spanish orchards, but it has been observed in recent years (Villarino et al., 2012b). Studies in cherry orchards with *M. fructicola* have shown that flower infection can only occur at temperatures ranging from 10 to 30 °C, when there is a wetness duration of at least 4 h (Luo et al., 2001b). Luo et al., (2001b) have developed a risk assessment model, by which the occurrence of flower infection increases with wetness duration and initial primary inoculum, and decreases with flower maturation.

Fruit infection is dependent on the same factors: inoculum density, fruit phenological stage, and meteorological conditions (Gell et al., 2008; Hong et al., 1998; Luo and Michailides, 2003; Wade and Cruickshank, 1992). It is well known that fruit are more susceptible to *Monilinia* spp. infection as they mature (Biggs and Northover, 1988; De Cal et al., 2013; Gell et al., 2008; Lee and Bostock, 2006b; 2007; Luo and Michailides, 2003; Villarino et al., 2011; Xu et al., 2007). Differences in fruit susceptibility can be related to differences in the chemical content of fruit and its epidermis, which can influence the first steps of the infection process (adhesion and penetration). For example, the content of volatile and phenolic compounds and organic acids in fruit and/or the fruit epidermis changes

substantially as fruit approaches maturity. The matrix glycans in the walls of peach mesocarp cells are gradually depolymerized and the polymers are deglycosylated during maturation, and the chelator-soluble polyuronides are solubilized and depolymerized after the initiation of ripening (Brummell et al., 2004). Villarino et al. (2011) reported that the susceptibility of peaches to *M. laxa* infection was greatest when the pericarp was completely formed, and the concentrations of chlorogenic and neochlorogenic acid in the pericarp are low. The production of melanin by *M. laxa*, is inhibited when the concentrations of chlorogenic and neochlorogenic acid in the pericarp are high and melanin is essential for penetration of the pericarp by *Monilinia* spp. (Villarino et al., 2011). The presence of phenolic acids, such as chlorogenic and caffeic acids, can inhibit the cutinase and polygalacturonase activity of *M. fructicola* in immature nectarines and peaches (De Cal et al., 2013). Furthermore, this inhibition is related to changes in the electrochemical redox potentials of *M. fructicola* cultures and alters the intracellular levels of the cellular antioxidant, glutathione (Bostock et al., 1999; Lee and Bostock, 2006a). The malic/citric acid ratio in the mesocarp has two maxima during peach maturation. The first maximum occurs in early immature fruit and the second maximum occurs in mature fruit when sucrose levels are at their highest and quinic acid levels are at their lowest (Chapman et al., 1991). Temperature is also the most important factor for brown rot development (Tamm and Flückiger, 1993). During harvest *M. fructicola* optimum temperature for peach infection ranges from 22.5 to 25 °C, but fruit can be infected at temperatures between 5 and 30 °C (Tamm and Flückiger, 1993). Furthermore, fungal development at this stage is favored by warm temperatures (15 to 25 °C), while cold temperatures slow brown rot development (Tamm and Flückiger, 1993; Tian and Bertolini, 1999).

Pests can also promote *Monilinia* spp. infections (Gell, 2007). Pests normally cause wounds on flowers, twigs, and fruit, which can be used by *Monilinia* spp. to penetrate and infect the tissues. Additionally, pests weaken the host, increasing its susceptibility to other diseases and pests. The most frequent pests of peach and nectarine crops are: (i) moths such as the peach twig borer (*Anarsia lineatella* Zell), or the oriental fruit moth or peach moth (*Cydia molesta* Busk), (ii) aphids such as the

black peach aphid (*Brachycaudus persicae* Pass.), mealy plum aphid (*Hyalopterus pruni* Geooff), green peach aphid (*Myzus persicae* Sulz), peach-clematis aphid (*Myzus varians* Davids), and (iii) hemipterous insect like the San Jose scale (*Quadraspidiotus perniciosus* Comst) (Gell, 2007).

Similar to what happens with flowers, *Monilinia* spp. infecting fruit, can either cause brown rot or remain as a latent infection until a later period. Immature fruit before the pit hardening and before harvest is mostly susceptible to latent infections, whereas mature fruit close to harvest and during postharvest is mostly susceptible to brown rot (Gell et al., 2008; Luo and Michailides, 2003; Villarino et al., 2012b). Furthermore, infection of intact fruit generally leads to latent infections (Fourie and Holz, 2006; Szôdi et al., 2008; Villani and Cox, 2010; Xu et al., 2007). Temperature and wetness duration are also key aspects on *Monilinia* spp. fruit penetration and infection, both in brown rot infections and latent infections (Gell et al., 2008). These two factors can explain 90 % of the incidence of latent infections in immature fruit (Gell et al., 2008). Incidence of *M. fructicola* latent infections increases exponentially as the wetness duration increases (Luo and Michailides, 2001). During pit hardening optimum temperature for latent infections of *M. fructicola* ranges from 14 to 18 °C, however it could be lower if the wetness duration is higher than 8 h (Luo and Michailides, 2001). In Spain latent infection development requires warm temperatures and at least 7 h of wetness duration, values easily reached at the end of spring and at the end of summer (Gell et al., 2008). Latent infections follow a typical pattern of subcuticular infection of immature fruit followed by rapid cessation of growth of the pathogen (Rungjindamai et al., 2014). As the fruit matures, fungal growth resumes and results in postharvest brown rot.

1.2.4.4. Stone fruit latent infections

Latent infections due to *Monilinia* spp. have been detected in nectarines and plums (Fourie and Holz, 2003a; b), and a high correlation between brown rot and latent infections exists in some stone fruit,

such as peaches, plums, and cherries (Emery et al., 2000; Gell et al., 2008; Luo and Michailides, 2001; Northover and Cerkauskas, 1994; Xu et al., 2007). Latent infections have been described as a dynamic equilibrium between the host, pathogen, and environment without any visible macroscopic signs of disease (Gell et al., 2008; Luo and Michailides, 2001), or as an invisible infection that is chronic and in which a host and parasite relationship has been established (Holliday, 2001). Other authors differentiate between latent (non-visible) and dormant or quiescent (visible) infections in which a host-parasite relationship has been established (Ahimera et al., 2003). Although a pathogen has a low metabolic level in a latent infection, pathogenicity factors may be activated to end the period of latent infection when conditions for disease development become conducive (Luo and Michailides, 2001).

Fungal pathogens usually develop structures, such as appressoria, germ tubes, or hyphae, for penetrating and causing a latent infection in a host (Prusky, 1996). Intercellular hyphae have been described in a latent infection of (a) mangos with an *Alternaria alternata* infection (Prusky et al., 1983), (b) mangos (Verhoeff, 1974), avocados (Binyamin and Schiffma, 1972), citrus fruit (Brown, 1975), and blueberries (Daykin and Milholland, 1984) with a *Colletotrichum gloeosporioides* infection, and (c) soybeans (Impullitti and Malvick, 2014) with a *Phialophora gregata* infection. Appressoria have been described in a latent infection of (a) unripe bananas (Muirhead and Deverall, 1981) with a *C. musae* infection, (b) tangerines (Brown, 1975; 1977) and papayas (Stanghellini and Aragaki, 1966) with a *C. gloeosporioides* infection, and (c) plums (Janisiewicz et al., 2011) with a *M. fructicola* infection. *Botrytis cinerea* uses an infection peg which develops from a germinated appressorium to penetrate the nectarine or plum cuticle in a latent infection (Fourie and Holz, 1995).

1.2.4.5. Disease control during the growing season

According to current regulations (EC 1107/2009, RD 1311/2012) disease control must be achieved with integrated pest management (IPM) schemes, combining cultural practices (orchards hygiene), use

of resistant varieties, biological control agents (BCAs), physical, and chemical control. Traditional brown rot control of peaches included cultural practices, physical control, and chemical control (Zehr, 1982). Nowadays, brown rot is still mainly control by cultural sanitary measures and chemical control with fungicides (Peris, 2013).

1.2.4.5.1. Cultural methods

Cultural methods pretend to reduce or eradicate primary and secondary inoculum sources (Villarino et al., 2010; 2012b). Mummified fruit and canker twigs on trees and orchard grounds constitute *Monilinia* spp. overwintering inoculum (Gell et al., 2009; Villarino et al., 2010). Mummified fruit can continue to produce conidia 2 to 3 years after inoculation, and they produce a number of conidia 10 times higher than that produced on newly infected blossoms (Holb, 2008). Furthermore the conidia produced in overwintered fruit are viable during most of the next crop season, especially those that remain on trees (Casals et al., 2015). For these reasons it is especially important to remove whenever possible mummified fruit on trees and the orchard ground, or at least to drop the ones on the trees to the orchard ground (Villarino et al., 2010). To avoid mummified fruit and twig cankers as primary inoculum on the next crop season, they should be removed completely during autumn or early spring (Byrde and Willetts, 1977; van Leeuwen et al., 2000; 2002). They also need to be removed as soon as they are detected to reduce secondary inoculum during the present crop season, especially fruit with brown rot symptoms 10 to 15 days before harvest (Villarino et al., 2010).

Both peaches and nectarines trees are highly susceptible to root asphyxia, needing well drained soils (Gell, 2007). Furthermore, they are also highly sensible to herbicides, depending on mechanical methods to control weeds (Majek and Welker, 1990). These two factors make the use of rotavator labors a common periodical practice on peaches and nectarines orchards during the vegetative phase in order to control weeds and drainage (Holtz et al., 1998). Using a rotavator destroys and/or buries

the sources of inoculum, reducing it for the next crop season. The extended use of this practice can explain the absence of apothecia sightings on the mummified fruit on orchard grounds (Villarino et al., 2010).

1.2.4.5.2. Intrinsic host resistance

Host resistance can be intrinsic or induced and it is influenced by biotic and abiotic factors. Induced host resistance is normally related with other control-methods, such as biological, physical or chemical control and as such it is treated in the subsequent subsections. Even though there is great cultivar diversity for peaches and nectarines and *Monilinia* spp. is an economically important disease of them, few breeding programs had focus on obtaining cultivars resistant to the disease. For example, there are no peach cultivar highly resistant to *Monilinia* spp. infection, although some cultivars like cv. “Bolinha” are partially resistant to brown rot (Feliciano et al., 1987). The few efforts made into obtaining host resistance to brown rot have focused on resistance to *M. fructicola*, and have discovered that resistance is usually related with a higher content of phenolic compounds, thicker epidermis, and higher content of wax and other components normally related with fruit immaturity in the cuticle of fruit (Gradziel et al., 1998). Recently, the genome of various *P. persica* cultivars has been sequenced, opening new opportunities for breeding, based on QTLs (Quantitative Trait Loci) studies (Ahmad et al., 2011; Martinez-Garcia et al., 2013; Verde et al., 2013). Additionally, new methods to determine cultivar susceptibility to *Monilinia* spp. have been recently published (Giménez Soro et al., 2013; Obi et al., 2013). Additionally, early cultivars are somehow brown rot resistant, since they usually escape brown rot attack, while late cultivars are exposed to high inoculum pressures at environmentally-disease-favorable periods (warm temperatures, and high RH) (Biggs and Northover, 1988; Gell et al., 2008).

1.2.4.5.3. Chemical control

Fungicide applications to blossom (in early spring) and fruit is still the main method for managing brown rot during the crop season. However due to European regulations (EC 1107/2009) the number of chemical phytosanitary products have been reduced from 917 active ingredients available in 1993 to less than 250 at the end of 2008 (Jiménez Díaz, 2009). In Spain only 26 active ingredients are authorized for the control of brown rot (Table 2) (MAGRAMA, 2017b), presented by themselves or combined in different percentages between each other in a total of 84 formulations.

Table 2. Classification of the fungicides active ingredients authorized for *Monilinia* spp. control in Spain (MAGRAMA, 2017b).

Fungicide type	Group name	Chemical group	Active ingredient common name
Contact fungicides	inorganic	inorganic	copper (different salts) sulphur
	dithio-carbamates and relatives	dithio-carbamates and relatives	mancozeb metiran thiram ziram
	phthalimides	phthalimides	captan folpet
	SDHI (Succinate dehydrogenase inhibitors)	pyridine-carboxamides	boscalid
	PP-fungicides (PhenylPyrroles)	phenylpyrroles	fludioxonil
	diverse	diverse	potassium bicarbonate
Systemic fungicides	MBC-fungicides (Methyl Benzimidazole Carbamates)	thiophanate	thiophanate-methyl
	QoI-fungicides (Quinone outside Inhibitors)	methoxy-carbamates	pyraclostrobin
	chloronitriles (phthalonitriles)	chloronitriles (phthalonitriles)	chlorothalonil
	SDHI (Succinate dehydrogenase inhibitors)	pyridinyl-ethyl-benzamides	Fluopyram
	AP-fungicides (Anilino-Pyrimidines)	anilino-pyrimidines	cyprodinil
	dicarboximides	dicarboximides	iprodione procymidone
	microbial (<i>Bacillus</i> spp.)	<i>Bacillus</i> spp. and the fungicidal lipopeptides produced	<i>Bacillus subtilis</i> syn.
	DMI-fungicides (DeMethylation inhibitors)	Triazoles	cyproconazole difenoconazole fenbuconazole myclobutanil tebuconazole
	SBI (Sterol Biosynthesis In membranes): Class III	hydroxylanilides amino-pyrazolinone	fenhexamid fenpyrazamine

The principal chemical strategy for brown rot control consists on calendar-based fungicide applications. With application of fungicides to blossoms in highly humid springs, and to fruit from 30 to 45 days before harvest until harvest, due to the high disease susceptibility during these periods

(Rüegg et al., 1999; Zehr et al., 1999). In the Ebro Valley fungicides are applied 3 to 5 times during the crop season, depending on the weather conditions (Usall et al., 2010). Brown rot chemical control in Spain is based on the use of combined or single application of contact fungicides (aniline-pyrimidines, dicarboximides, and triazoles) (Usall et al., 2013). Mixtures of fludioxonil and ciprodinil or boscalid and piraclostrobin, tebuconazole and fenhexamide, present good controlling efficacies (Usall et al., 2013).

However, fungicide-based methods are not sustainable in the long term because of the emergence and subsequent spread of *M. fructicola* and *M. laxa* strains with reduced sensitivity to fungicides (Egüen et al., 2015; 2016; Ma et al., 2005; Malandrakis et al., 2013; Thomidis et al., 2009; Weger et al., 2011; Zhu et al., 2010). Recent studies in the Ebro Valley have detected a *M. fructicola* population with isolates that were single resistant, double resistant, or even triple resistant to tiophanate-methyl, iprodione and tebuconazole (Egüen et al., 2015). And *M. laxa* populations single, or double resistant to tiophanate-methyl, and iprodione (Egüen et al., 2016).

1.2.4.5.4. *Biological control*

Biological control agents (BCAs) are an alternative to physical and chemical controls. Their mode of action may involve: (i) competition for nutrients and space, (ii) production of antibiotics, (iii) direct parasitism, (iv) lysis, and (v) induced resistance (Sharma et al., 2009). BCAs are more likely to compete with the pathogen through a combination of mechanisms of inhibition, which will defer generation of resistance in fungi.

The pre-harvest phyllosphere environment is considered a harsh and hostile environment for microbes because the plant surfaces are exposed to rapid fluctuations of meteorological conditions as well as limited nutrient availability (Magan, 2006). The capability to tolerate such harsh conditions is an important trait for a successful BCA, and thus the epiphyte microflora on healthy plant materials has

long been considered as a good source of BCAs (Janisiewicz and Buyer, 2010; Janisiewicz et al., 2013). The survival potential of BCAs may be improved by different formulations (Burges and Jones, 1998; Schisler et al., 2004) increasing the likelihood of pre-harvest biocontrol working in field conditions.

A lot of work has been done describing *Bacillus* spp. and their potential as BCAs against several pathogens (Arrebola et al., 2010). They are usually bioactive through the production and secretion of lipopeptides and other inhibitory substances. Pioneering work on the B-3 strain of *B. subtilis* showed that the isolate was capable of inhibiting *M. fructigena* in vitro (Pusey and Wilson, 1984), and now *B. subtilis* is the active ingredient of the only commercial biocontrol agent authorized in Spain to control *Monilinia* spp. (Serenade Max (15.67 %) (5.13×10^{10} CFU g⁻¹) [WP] P/P; Bayer CropScience). Serenade max active ingredient is formed by several *B. subtilis* strains (QST 713, FZB24, MBI600 y D747) and its mode of action is based on their capacity to secrete lipopeptides from the iturin and fengycin families. Other *Bacillus* spp. strains continue to demonstrate their *Monilinia* spp. controlling efficiency on the field: *Bacillus* spp. OSU142 reduced blossom blight of apricots (Altindag et al., 2006), *B. amyloliquefaciens* C06 that produces and extracellular mucilage (γ -poliglutamic acid) that increases its survivability and plant surface colonization (Liu et al., 2011), or *B. subtilis* CPA-8 that mainly secretes fengycin family lipopeptides (Yanez-Mendizabal et al., 2012).

Yeasts or yeast-like fungi are also attractive candidates as BCAs, as fruit surfaces usually support the growth of large quantities of these organisms. The main genera present on stone fruit are: *Aureobasidium*, *Cryptococcus*, *Rhodotorula*, and *Sporidiobolus* (Janisiewicz et al., 2010). These organisms do not secrete inhibitory chemicals, but rely on competition for nutrients as their main mechanism of inhibition of plant pathogens. *Cryptococcus laurentii* has shown great survivability capacity during the crop season and postharvest of stone fruit (Tian et al., 2004). *Aureobasidium pullulans* has been frequently described as a BCA under different conditions (Mari et al., 2012),

specifically against *M. laxa* on sweet cherries (Schena et al., 2003), on plums and peaches during postharvest (Zhang et al., 2010a; b), or reducing sporulation produced by *M. fructigena* plum and cherry mummies (Rungjindamai et al., 2014). There is a commercial formulated product of two *A. pullulans* strains (DSM 14940 and DSM 14941), ‘BoniProtect’, against *M. fructigena* authorized in some European countries, but not in Spain.

Penicillium frequentans (Pf909) and *Epicoccum nigrum* (282) are filamentous fungal BCAs against *Monilinia* spp. (De Cal et al., 2009b; Guijarro et al., 2008b; Larena et al., 2005; 2010; Madrigal et al., 1994). They were initially isolated from the surface of peach healthy shoots (Melgarejo et al., 1986) and their control mechanism is based on competition and secretion of inhibitory chemicals such as antibiotics (Melgarejo et al., 2007). Both of them are able to survive in field conditions (De Cal et al., 2009b; 2012; Guijarro et al., 2008b) and are tolerant to some of the fungicide treatments applied to stone fruit (De Cal et al., 1990; Madrigal et al., 1994). Their population dynamics have been studied in commercial orchards where they were able to significantly control brown rot (De Cal et al., 2009b; Guijarro et al., 2008b). Their active ingredient have been improved with additives and Pf909 is been prepared for its registration as a BCA (De Cal et al., 2002; Guijarro et al., 2006; 2007a; b; 2008a; Larena et al., 2003; 2004; 2007; 2010). Their use in combination with different fungicide treatments reduced blossom blight and brown rot of peaches (De Cal et al., 1990; Larena et al., 2005; Madrigal et al., 1994; Melgarejo et al., 1986). Additionally, applications of *A. pullulans* and *E. nigrum* to cherry blossoms reduced the incidence of latent infections by *M. fructicola* (Wittig et al., 1997). Another filamentous fungus BCA is *Trichotecium roseum*, which reduced brown rot on Brazilian orchards after multiple applications during the crop season (Moreira et al., 2014).

1.2.5. Postharvest *Monilinia* spp. epidemiology and control

1.2.5.1. Origin of *Monilinia* spp. postharvest infections

Brown rot incidence during postharvest is the sum of the infections already present when the fruit is harvested, either active or latent infections, and those produced between fruit harvest and the point of consumption (Landgraf and Zehr, 1982). Recent studies have shown that most *Monilinia* spp. infections during postharvest have been caused in the orchards during pre-harvest. In general, postharvest handling processes have little incidence on the apparition of new infections (Villarino et al., 2012b). Seventy-two percent of the incidence of brown rot during postharvest can be explained by the incidence of brown rot when the peaches are harvested (Villarino et al., 2012b). Relative importance of each of these infection types over incidence of postharvest brown rot, varies depending on the harvest period and also between years (Usall et al., 2017).

1.2.5.2. Infection process during postharvest

The infection processes for those infections occurring on pre-harvest but developing during postharvest have already been described in subsection “1.2.4.2. Brown rot infection process”. Here we look at the *de novo*-infections produced during postharvest.

The influence of new infections produced during postharvest is unknown, but it is assumed to have little effect on the total postharvest brown rot incidence due to the time constraints during this period. Still, they are a part of the postharvest incidence of brown rot. During postharvest there are situations that influence conidia survivability, infection processes, and fruit colonization, such as temperature changes, water condensation, mechanical wounding, or contact between fruit and water.

Secondary inoculum may appear anywhere in infected tissues whose moisture content is sufficient for the pathogen to sporulate (Landgraf and Zehr, 1982). In theory, fruit rot caused by degrading enzymes and toxins secreted by *Monilinia* spp., areas with water condensation, and fruit water cooling and dumping to the confection lines can produce sufficient moisture content for *Monilinia* to sporulate. The generated conidia can be transferred to other uninfected fruit by the air movement in the storage chambers, with the dump in water, or by direct contact with other fruit, infecting new fruit. Furthermore, conidia from all *Monilinia* spp. present in Spanish orchards (*M. fructicola*, *M. fructigena*, and *M. laxa*) can germinate and their mycelia can grow at 0 °C (Casals et al., 2010a). Especially *M. laxa* mycelia that has a better growth than that of *M. fructicola* at low temperatures (Papavasileiou et al., 2015; Tamm and Flückiger, 1993). However, there is no knowledge about the infection structures (appressoria) generated in these conditions, or about the interaction between environmental conditions and fruit ripening state.

Little is known about the role that each postharvest process (cold storage in the reception chamber duration, dump in water, fruit processing duration, cold storage in the expedition chamber duration, and display in markets duration) has over *Monilinia* spp. infection processes, inoculum activation, and brown rot incidence during postharvest. To further this knowledge in **objective 1** of this doctoral thesis **we study the interaction of fruit maturity and environmental conditions on conidial germination and appressorial formation in a *Monilinia* spp. infection, with particular interest on the environmental conditions present during postharvest.**

Monilinia species like other necrotrophic fungi use degrading enzymes and toxins to cause cell death and to penetrate the intact surface of fruit (Byrde and Willetts, 1977). However, little is known about pathogenic mechanisms in *Monilinia* species and what little is known has been studied in *M. fructicola*. At the end of the twentieth century, some studies were made revealing that *M. fructicola* secretes endopolygalacturonases and pectin esterases (Hall, 1971; 1972; Wade and Cruickshank, 1992) and

that it produces at least four cutinase isozymes when grown on cutin as a carbon source (Wang et al., 2000). Furthermore, *M. fructicola* is able to penetrate intact tissue and it has been proved that the penetration of intact tissue is enhanced by the production of cutinases (Wang et al., 2000; 2002). More recent studies, revealed that MFCUT1 the main cutinase of *M. fructicola* is a virulence factor, required to penetrate plant tissues (Lee and Bostock, 2006a; Lee et al., 2010). Additionally 5 different endopolygalacturonases, have been detected and the most abundantly expressed of them, MfPG1 has an impact in *M. fructicola* virulence (Chou et al., 2015). These last developments have been possible due to genomic advances (*M. fructicola* genome sequencing and *Agrobacterium tumefaciens*- and protoplast CaCl₂/poly ethylene glycol-mediated transformation of *M. fructicola*) (Chou et al., 2015; Lee and Bostock, 2006a). However, *M. fructigena* and *M. laxa* genomes are still unsequenced, and the one from *M. fructicola* is not readily available to the scientific community, difficulting further advancements through this avenue.

Knowing the phytotoxins and degrading enzymes produced by *Monilinia* spp. would help discern the infection processes of fruit during both postharvest, when most brown rot occurs, and pre-harvest. Thus, **the main phytotoxins and degrading enzymes produced by *M. fructicola*, *M. fructigena*, and *M. laxa*** have been studied as part of **objective 2**.

1.2.5.3. Brown rot and latent infections during postharvest

During postharvest fruit is mainly susceptible to brown rot. Brown rot can be developed on fruit infected with active or latent infections before harvest and those infected between harvest and fruit consumption.

Fruit with active *Monilinia* spp. infections at the beginning of postharvest, have to had been infected a few days before harvest or even during harvest, because they do not have noticeable symptoms when they are harvested. During this short period just before harvest peaches and nectarines are almost ripe,

and most susceptible to infection (Byrde and Willetts, 1977). During harvest there is a high conidia concentration in the air as a result of the movement of machinery and the tree canopies, (Villarino et al., 2012b); at the same time fruit may suffer injuries due to fruit harvest, fruit knocking, careless packaging, etc. All of these factors are known to increase the incidence of brown rot and are good conditions for new infections to develop just before postharvest (Gell et al., 2008; Hong et al., 1998; Luo and Michailides, 2003; Wade and Cruickshank, 1992).

Monilinia spp. latent infections are those produced during the crop season when the environmental conditions were not favorable for disease development. They can remain latent during the crop season due to unfavorable meteorological conditions, nutrient deficits, or high levels of fungal growth inhibitory compounds (Gell et al., 2008; Wade and Cruickshank, 1992). A positive correlation between the number of latent infections and the incidence of postharvest brown rot in peaches has been established for *M. fructicola* (Emery et al., 2000; Luo and Michailides, 2001), and for *M. fructigena* and *M. laxa* (Gell et al., 2008). Luo et al. (2005) reported that 96 % of the incidence of postharvest brown rot in plums caused by *M. fructicola* in California was due to latent infections that were present during the pit hardening stage of their development.

The first symptoms of the disease are small brown stains over the fruit surface that quickly develop soft rot and are later covered by grey pustules of *Monilinia* spp. conidia. Symptoms can appear in apparently healthy fruit after 3 to 4 days of incubation at room temperature. In a recent study Bernat et al. (2017) have determined the effects of temperatures on *M. fructicola* and *M. laxa* fruit decay and mycelium development and the subsequent sporulation on peaches and nectarines. The rates of decay and mycelium development increased with temperature from 0 to 25 °C for both *Monilinia* species (Bernat et al., 2017). At 0 °C, both *M. laxa* decay and mycelium development, were faster than those of *M. fructicola* and only *M. laxa* could develop sporodochia (Bernat et al., 2017). While *M. fructicola* decayed the fruit and developed mycelia faster than *M. laxa* at 33 °C, and at this temperature only *M.*

fructicola could develop sporodochia (Bernat et al., 2017). No differences on decay and mycelium development were observed between both species at temperatures between 4 and 20 °C (Bernat et al., 2017).

It has been hypothesized that latent infections follow a subcuticular infection followed by rapid cessation of growth of the pathogen, based on the observed symptoms (Byrde and Willetts, 1977; Luo et al., 2001b; Prusky et al., 2003). However, those hypotheses only refer to latent infections due to immature fruit, not those due to adverse climatic conditions for pathogen development. Furthermore, the fungal structure used by *Monilinia* spp. to cause a latent infection in peaches and nectarines, and how *Monilinia* spp. transitions between latent infection and brown rot are unknown. For these reasons the **histological features, microanatomy and evolution of latent infections** due to adverse environmental conditions for pathogen development (cold temperatures) **and those of a visible brown rot infection on nectarines are studied and compared in objective 3** of this doctoral thesis.

Molecular tools such as fungal transformation, can help the understanding of the infection process, by studying the germination, penetration and colonization of fruit by fluorescence isolates expressing the Green Fluorescent Protein (GFP) or another fluorescent protein like mCherry. Furthermore, being able to obtain transformants can be used to determine which enzymes and metabolic factors are essential for the infection processes of fruit. Some *M. fructicola* isolates have been transformed mainly by *Agrobacterium tumefaciens*-mediated transformation (Chen et al., 2017; Dai et al., 2003; Lee and Bostock, 2006a; Lee et al., 2010) but also by CaCl₂/poly ethylene glycol-mediated transformation (Chou et al., 2015). However, the yield of transformants in these studies was low and there are no studies of *M. laxa* transformation. Thus, **transformation of *M. fructicola*, and *M. laxa* is further studied in objective 4.**

1.2.5.4. Disease control during postharvest

Recent studies have shown that most *Monilinia* spp. infections during postharvest have been caused in the orchards during pre-harvest. In general, postharvest handling processes have little incidence on the apparition of new infections (Usall et al., 2017). Since *Monilinia* spp. growth and development are slowed under storage conditions and usual postharvest durations are less than 2 weeks. Thus, postharvest control should focus on reducing and controlling already existing infections by curative methods.

Taking this into account, a correct postharvest disease control strategy must be based on previous knowledge obtained before harvest, such as fruit incidence of brown rot, risk of infection in the 30 days before harvest, treatments applied during this period, and incidence of latent infection (Villarino et al., 2012b). This knowledge would allow to divide fruit in function of its risk level and treat it accordingly, e.g. fruit with low risk levels could remain untreated, while those with moderate to high risk of infection should be carefully monitored and treated.

Most of the information required is easy to obtain. However, the incidence of latent infection is normally evaluated by the Over Night Freezing Incubation Technique (ONFIT) (Luo and Michailides, 2003) which is a slow process that takes 7 to 9 days. ONFIT activates latent infections causing senescence in epidermal cells by freezing the fruit at -20 °C for 48 h and then incubating fruit for 5 to 7 days at 25 °C and a high RH while examining fruit for signs of brown rot (Luo and Michailides, 2003; Villarino et al., 2012b). A faster method is required to detect latent infections in order to improve brown rot control strategies. Rapid detection, identification, and differentiation of *Monilinia* spp. isolated from culture or symptomatic plant samples have been obtained with PCR (Boehm et al., 2001; Côté et al., 2004; Förster and Adaskaveg, 2000; Fulton and Brown, 1997; Gell et al., 2007; Hughes et al., 2000; Ioos and Frey, 2000; Ma et al., 2003). Due to the nature of latent infections where a small fungal infection has arrested its development, a highly sensible method would be required to detect

them. 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 methods for detecting infectious microorganisms with a low titer and an uneven distribution in plants, such as apple proliferation phytoplasma (Baric et al., 2006). Three different groups have developed qPCR methods for detection of *Monilinia* spp. from fungal cells collected from symptomatic plant material (Guinet et al., 2016; Luo et al., 2007; van Brouwershaven et al., 2010). But they have not been used to detect latent infection. **In objectives 5 and 6 we have studied: (i) the viability of a qPCR-based method to detect and identify *Monilinia* spp. on latent infections of nectarines (objective 5), and (ii) its reproducibility (objective 6).**

1.2.5.4.1. Cultural methods

The first cultural practices during postharvest starts during harvest, when wounding the fruit should be avoided. Common practices to achieve it are: (i) placing the fruit on clean boxes, (ii) avoiding fruit knocking, and (iii) careful but quick transportation (Casals and Usall, 2015). In the best of cases fruit will arrive without infection or latent infection to the processing plant, but they could have inoculum on their surface due to the movement produced during harvest. The possible presence of latent infections and inoculum on fruit surface, makes important to lower fruit temperature as soon as possible, to slow development of latent infections and new fungal colonization (Casals and Usall, 2015; Kenneth et al., 2016).

1.2.5.4.2. Chemical control

Postharvest fungicide treatments are not commonly used in the European Union. However, in the last years the use of fludioxonil and pirimetanil have been authorized in accordance with article 53 of the Norm (EC) 1107/2009 for emergency phytosanitary situations. However, due to the negative perception of residues presence on fruit by the consumers, distribution chains have established their own regulations further limiting the number of active substances and the quantity of residues that can be present on fruit, further difficulting a rational management of the disease. Nonetheless, chemical substances still are an essential tool to achieve disease control in most of the situations.

Alternative chemical compounds unrelated to the traditional synthesis fungicides have been tested on stone fruit to control brown rot. Some examples of those alternative chemical compounds are: peracetic acid and chlorine dioxide (Mari et al., 1999), lime sulfur (Holb and Schnabel, 2008), food additives (Karaca et al., 2014), synthetic and glucosinolate-derived isothiocyanates (Mari et al., 2008), salicylic acid and methyl jasmonate (Yao and Tian, 2005a; b), berberine (Hou et al., 2010), essential oils (Lazar-Baker et al., 2011; Ziedan and Farrag, 2008), or fungal volatile compounds (Li et al., 2015). However, none of these have been used for commercial stone fruit production yet.

1.2.5.4.3. Physical and biological control

Almost all postharvest control is based on physical treatments due to the European regulatory restrictions on the use of fungicides during this period. Mechanisms underlying physical treatments include: (i) reducing fruit respiration, (ii) killing pathogens on fruit surface, (iii) suppressing/eradicating latent infection, and (iv) stimulating induced resistance.

Lowering fruit temperature is the first physical control measure on peach and nectarines processing plants (Kenneth et al., 2016; Usall et al., 2010). Cold storage of fruit slows their maturity and increases

their shelf-life (Robertson et al., 1990). ISO regulations for peaches and nectarines (ISO 873:1980), assume that storing peaches and nectarines at temperatures between 1 and 2 °C allow most of commercial cultivars a shelf-life between 2 to 4 weeks. Some authors consider that *Monilinia* spp. growth ceases at temperatures below 0 °C (Barkai-Golan, 2001; Sommer, 1985). Others considered that cold storage only serves to slow *Monilinia* spp. growth, but it does not cease (Usall et al., 2010). Lowering fruit temperature as soon as possible and storing it at cold temperatures during postharvest are essential, but insufficient, measures to control brown rot during postharvest (Sisquella et al., 2014).

Hot water dipping (HWD) and curing treatments have shown promising results on artificially inoculated fruit (Casals et al., 2010b; Karabulut et al., 2010; Zhang et al., 2010a). The principles behind hot water treatments are: (i) direct inhibition of *Monilinia* spp. (accumulation of reactive oxygen species, collapse of mitochondrial membrane potential and a decrease in intracellular ATP), and (ii) induction of host resistance (induction of defense-related genes including chitinase, β -1,3-glucanase and phenylalanine ammonia lyase) (Liu et al., 2012). HWD at 55 and 60 °C for 60 and 30 s, respectively reduced disease incidence and lesion diameter on peaches, nectarines, and plums artificially inoculated with *M. fructicola*, but heating injury occurred when fruit were treated at temperatures of 65 °C or above (Karabulut et al., 2010). HWD at 50 and 60 °C for more than 40 or 10 s, respectively prevented conidial germination and germ tube elongation of *M. laxa* (Zhang et al., 2010a). When artificially-inoculated peaches and nectarines were HWD-treated at 48 °C for 12 min, 24 h after inoculation, the incidence of brown rot was significantly reduced (Jemric et al., 2011). *M. fructicola* was shown to have a greater tolerance to HWD than *M. fructigena* and *M. laxa*, but conidial germination of the 3 species was prevented by HWD at 60 °C for 60 s without visual damage to fruit in commercial trials (Spadoni et al., 2013). Curing treatments at 50 °C for 2 h was enough to control 95 % of brown rot (Casals et al., 2010c). However, a later study probed that curing treatment efficiency diminishes both with commercial fruit maturity (only 65 % efficient) and time transpired between *Monilinia* spp. infection and treatment (64 % of efficiency 48 h after inoculation) (Casals et al., 2010b).

Controlled atmospheres with low oxygen (O₂) or high carbon dioxide (CO₂) concentrations have been investigated to control *Monilinia* spp. on sweet cherries (Akbudak et al., 2009; Tian et al., 2001) and apples (Balla et al., 2008) with promising results. Atmospheres containing 30 % CO₂ at 0 °C completely prevented brown rot disease on sweet cherries (Tian et al., 2001). Additionally the incidence of *M. fructicola* on sweet cherries was reduced from 24 % to 4 % after 60 days of storage in a 20 % CO₂ and 5 % O₂ atmosphere (Akbudak et al., 2009). On apples ultra-low oxygen conditions (less than 1 kPa), nearly eliminated brown rot caused by *M. fructigena*, compared to the 18 to 35 % incidence under conventional storage conditions (Balla et al., 2008).

Radio frequency (RF) treatments can be used to increase temperatures within fruit and control brown rot. The interest in RF as a physical control method arises from the necessity to obtain an effective and fast thermic treatment (Sisquella et al., 2013b). In this aspect RF and Microwave treatments allow to reach the same temperatures in a shorter time period or higher temperatures for the same time periods than hot water treatments (Tang et al., 2000). A RF treatment of 27.12 MHz for 18 min generated a sufficient temperature rise within fruit to completely inhibit natural infections by *Monilinia* spp. and led to reduction in the brown rot incidence on artificially *M. fructicola*-inoculated peaches, but not on nectarines (Casals et al., 2010d; Sisquella et al., 2011). In a subsequent study by Sisquella et al. (2013b) the combine application of RF and fruit immersion in 20 °C water was able to control brown rot on nectarines and peaches after a 9 min treatment. Microwaves have also been used to reduce brown rot incidence in peaches and nectarines but internal fruit damage was reported (Sisquella et al., 2013a).

Biocontrol agents have more difficulty controlling the disease during postharvest than during pre-harvest, since most postharvest infections come from the field and are at different developmental states. Thus biocontrol agents should have some curative effect to be more efficient during postharvest. Nonetheless, some studies have had success using biological control on postharvest diseases of fruit (Janisiewicz et al., 2013; Nunes, 2012; Wisniewski et al., 2007). Besides, postharvest conditions have

some benefits for BCAs, because of the stable storage conditions, long interaction time between BCAs and pathogens, and an accessible host surface (facilitating good coverage by the BCA) (Magan, 2006). The effectivity of using a *B. subtilis* strain CPA-8 against *M. laxa* depended on storage temperature (Casals et al., 2010e). Postharvest treatment of nectarines incubated at 20 °C with *A. pullulans* led to significant reduction in *Monilinia* infection after 7 days (Mari et al., 2012). Fresh *P. frequentans* conidia treatments during postharvest of plum fruit reduced the incidence and lesion diameter of brown rot caused by *M. laxa* (De Cal et al., 2002; Larena et al., 2004). Postharvest nectarine treatments of *E. nigrum* were effective in reducing the incidence of postharvest brown rot (Mari et al., 2007).

Furthermore, biological and physical methods can complement each other increasing their efficiency, e.g the incidence of *M. laxa* on peaches was further reduced by the combination of an *A. pullulans* strain and HWD (Zhang et al., 2010a).

Commercial use of the previous physical controlling techniques has been limited due to several factors: (i) physical and heating injuries, (ii) time constraints during postharvest, and (iii) labor costs (Casals et al., 2010d; Karabulut et al., 2010). Furthermore, the effect of the chemical, physical and biological control methods over *Monilinia* latent infections remains unknown. Thus, in **objective 7** of this doctoral thesis, the **critical processes inducing latent infection activation during postharvest on the processing plants and fruit distribution** have been determined.

2. OBJECTIVES

*EPIDEMIOLOGÍA Y CONTROL DE LA PODREDUMBRE PARDA DEL
MELOCOTÓN EN POSTCOSECHA*

Brown rot etiology and epidemiology have been thoroughly studied during the crop season, but there is an important gap of knowledge about brown rot during postharvest. The aim of this doctoral thesis is to study the epidemiology and control of *Monilinia* spp. latent infections during postharvest as the main causal agent of brown rot incidence in these conditions. This aim was achieved through the following seven objectives:

1. To determine the **effect of the maturity state and postharvest environmental conditions over the pre-penetration stages of *Monilinia* spp.**

This is achieved by (i) investigating the effect of mature and immature peach skin extract components, temperature, and relative humidity on germ tube and appressorial formation by *M. laxa* and *M. fructicola*, and (ii) studying the effects of postharvest environmental conditions on germ tube and appressorial formation by *M. laxa* and *M. fructicola*.

2. To determine the **phytotoxins and degrading enzymatic activities produced by *Monilinia* spp.**

This is achieved by (i) detecting and characterizing phytotoxins produced by *M. fructicola*, *M. fructigena*, and *M. laxa* that participate on the infection of nectarine fruit by HPLC, and (ii) determining the degrading enzymatic activities of the three *Monilinia* species that facilitates fruit penetration and colonization with growth assays in amended media.

3. To determine how *Monilinia* latent infections develop and transit to visible infections, by **microscopic analysis of latent and visible *Monilinia fructicola* infections in nectarines**

This is achieved by analyzing and comparing the microanatomy of nectarines with (i) latent and (ii) visible *M. fructicola* infections using optical and transmission electron microscopy.

4. To transform the brown rot pathogens *M. fructicola* and *M. laxa*

This is achieved by testing several transformation methods (CaCl₂/poly ethylene glycol-mediated transformation, electroporation, and lithium acetate-mediated transformation) on *M. fructicola* and *M. laxa* in order to introduce by heterologous or homologous recombination a

plasmid containing the phleomicyn resistance gene *bleo* and either the green fluorescence protein (GFP) or the red fluorescence protein (mCherry).

5. To detect latent *Monilinia* spp. infections on nectarine flowers and fruit by qPCR

This is achieved by determining whether the qPCR-based method that was developed by van Brouwershaven et al. (2010) could be applied to rapidly and reliably (i) detect a latent *Monilinia* infection in the blossoms and fruit of nectarine trees, and (ii) distinguish between *Monilinia* spp.

6. To determine the proficiency of a latent *Monilinia* spp. infection detection by Real-Time PCR in nectarine flowers and fruit

This is achieved by comparing the sensitivity and specificity of several real-time PCR platforms and laboratories for the detection of low concentration of *Monilinia* DNA (latent infections), inside plant material matrices, using a common protocol. Thus, determining the transferability of a real-time PCR method for detection of *Monilinia* latent infections based on the work of van Brouwershaven et al. (2010) and modified by Garcia-Benitez et al. (2017b) as a tool for *Monilinia* latent infection detection. This evaluation was done through an international inter-laboratory testing.

7. To determine *Monilinia* spp. latent infection activation during postharvest handling conditions in nectarines

This is achieved by looking for critical points in actual postharvest handling practices that activate or favor *Monilinia* latent infections, specifically, temperatures, relative humidity and duration of each process.

3. MATERIALS AND METHODS

*EPIDEMIOLOGÍA Y CONTROL DE LA PODREDUMBRE PARDA DEL
MELOCOTÓN EN POSTCOSECHA*

3.1. EFFECT OF THE MATURITY STATE AND POSTHARVEST ENVIRONMENTAL CONDITIONS OVER THE PRE-PENETRATION STAGES OF *Monilinia* spp.

3.1.1. Fungal culture and conidial production

Six 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 investigation (Table 3). All isolates were originally collected from several commercial peach and nectarine orchards in the Ebro Valley, Lleida, Spain. All isolates were stored as conidial suspensions in 20 % glycerol at -80°C for long-term storage or as cultures on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) at 4°C for short-term storage. For conidial production, (a) the three *M. fructicola* isolates were grown on PDA plates at 20 to 25°C for 7 days in the dark, and (b) the three *M. laxa* isolates were first grown on PDA plates at 20 to 25°C for 10 days and then for 5 days at 4°C in the dark.

Table 3. The host, isolation tissue and year of isolation of the six *Monilinia laxa* and *M. fructicola* isolates that were used to study the effects of fruit maturity and postharvest environmental conditions on the pre-penetration stages of *Monilinia* infection in peaches.

Isolate name	Collection name	<i>Monilinia</i> species	Host	Isolation tissue	Year of isolation
Ml1	FLOR2009SUD5.1	<i>M. laxa</i>	Nectarine	Flower	2009
Ml2	2014COSFANTASIA8	<i>M. laxa</i>	Nectarine	Fruit	2014
Ml3	2014COSFANTASIA9	<i>M. laxa</i>	Nectarine	Fruit	2014
Mf1	ALF2009COS5R7	<i>M. fructicola</i>	Peach	Fruit	2009
Mf2	ALF2009MOA4	<i>M. fructicola</i>	Peach	Mummified fruit on trees	2009
Mf3	SUD2009COS2R6	<i>M. fructicola</i>	Nectarine	Fruit	2009

Conidial suspensions of all isolates were prepared using conidia that were harvested from the PDA plates and then transferring the harvested conidia into Czapek-Dox broth (Difco Laboratories, Detroit,

MI) or sterile distilled water (SDW). After a 30 s sonication of the conidia in an ultrasonic bath (J.P. Selecta s.a., Barcelona, Spain), the conidial concentration of the suspensions was first determined using a hemocytometer and a light microscope, and then adjusted to a final concentration of 10^4 conidia mL⁻¹.

3.1.2. Bioassays

Germ tube and appressorial formation were measured using previously described protocols (De Cal et al., 1988) with some modifications. Briefly, three sterile glass slides and three moistened filter papers were placed inside 150 mm diameter sterile Petri dishes. A 15 µL-aliquot of an *M. fructicola* or *M. laxa* conidial suspension was mixed with a 30 µL-aliquot of culture medium on each glass slide. Germinated *Monilinia* conidia can form either germ tubes or appressoria. A germ tube was considered to be formed when a slender hypha with pointed ends, which was longer than the minor diameter of the conidium, was formed. An appressorium was considered to be formed when swollen structures, that were mostly oval-shaped, were formed instead of slender hyphae with pointed ends (Cruickshank and Wade, 1992; Lee and Bostock, 2006b). For the bioassays which are described below, three replicates of each condition were done on 50 randomly selected conidia in each replicate and each bioassay was repeated at least twice with each studied isolate.

To determine the effects of temperature, RH, and fruit maturity on germ tube and appressorial formation, conidial suspensions in Czapeck-Dox broth were incubated with a skin extract from immature or mature peaches at 4, 10, 25, and 35 °C for 24, 48 and 72 h in the dark at an RH of 60 %, 80 %, and 100 %. For preparing the peach skin extracts, the skins of fungicide-free ‘Rojo Albesa’ peaches were collected using a previously described protocol (Tomas-Barberan et al., 2001). The skins were first manually peeled with a razor blade, and then grinded into powder in liquid nitrogen with a sterilized pestle and mortar. The resultant powders were transferred to a 50 mL Falcon tube, weighed,

and mixed with ethanol, MilliQ water, and beach sand (3.6 g of powder was mixed with 5.76 mL 100 % ethanol, 1.44 mL MilliQ water, and 2 g of beach sand). The mixture was then homogenized twice at a speed of 6.0 m s^{-1} for 60 s using a high-speed benchtop tissue homogenizer (FastPrep®-24 Instrument, MP Biomedicals, Solon, Ohio). The homogenates were then transferred to sterile tubes which were centrifuged at 10,000 rpm for 20 min at 4 °C in a Sorvall RC-5C Plus centrifuge (Kendro Laboratory Products, Newton, Connecticut). When the centrifugation was complete, the supernatant was carefully collected, transferred to a new sterile 50 mL Falcon tube, and stored at -20 °C until required. Immature peaches were collected at BBCH = 79, 76 days before harvest (4 July) and mature peaches at BBCH = 89 harvest date (18 September) (BBCH, general scale from Biologische Bundesantalt, Bundessortenamt and Chemische Industrie, Germany (Meier et al., 1994). The three RHs were created by preparing different mixtures of distilled water (DW) and glycerol using a previously described protocol (Forney and Brandl, 1992). Briefly, DW without glycerol was used to create the 100 % RH environment and 104.3 mL or 209.3 mL of glycerol were mixed with 1 L DW (final volume) to create the 80 % RH and 60 % RH environments, respectively.

The environmental conditions under which harvested fruit are processed and stored vary in different fruit processing and storage centers. To determine the effects of those environmental conditions on germ tube and appressorial formation, a mixture of Czapek-Dox broth and the *M. fructicola* (Mf1) or *M. laxa* (Ml1) (Table 3) conidial suspensions in SDW was first incubated at 4 °C for 0, 1, or 3 days in the dark at an RH of either 60 % or 100 % to simulate refrigeration before fruit processing. The mixtures were then incubated for 20 min at 15 °C to simulate fruit processing conditions at an RH of either 60 % or 100 %. At the end of the incubation, the mixtures were incubated at 4 °C for 0, 3, or 10 days in the dark at an RH of either 60 % or 100 % to simulate the cold storage conditions until distribution to the wholesalers and markets.

3.1.3. Data analysis

Data on the effects of temperature, RH, fruit maturity, and the duration of refrigeration and cold storage of harvested fruit at 4 °C on germ tube and appressorial formation were analyzed by a multiway analysis of variance (Snedecor and Cochran, 1980). Data were arcsine transformed before the analysis in order to improve the homogeneity of variance. When F-test was significant at $P \leq 0.05$, the means were compared using the Student-Newman-Keuls multiple range test.

The linear regression models, $f(T, RH, it)$, germ tube formation (%), and appressorial formation (%) were used to investigate the relationship between germ tube or appressorial formation and temperature (T), RH, and incubation period (it) for each culture medium which contained the skin extracts of mature and immature peaches and fungal species. In these models, $f(T, RH, it)$ is a linear function of $T, RH, it, TRH, Tit, T^2, RHit, RH^2, it^2, TRHit, T^3, T^2RH, T^2it, RH^3, TRH^2, RH^2it, it^3, Tit^2$, and $RHit^2$, whose values were estimated by the regression analysis. Data were fitted to the model using the Regression Model Selection procedure of Statgraphics Centurion XVI for Windows, Version 16.1.03 (StatPoint Technologies, Inc., Herndon, VA). Selection of the final model was performed according to the significance of the estimated parameters and the adjusted coefficient of determination.

3.2. ISOLATION AND PARTIAL CHARACTERIZATION OF PHYTOTOXINS AND DETERMINATION OF THE DEGRADING ENZYMATIC ACTIVITIES PRODUCED BY *Monilinia* spp.

3.2.1. Fungal culture

Twenty-nine isolates, 10 *M. fructicola*, 9 *M. fructigena*, 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 investigation (Table 4). All isolates were originally collected from commercial stone fruit and pome fruit orchards in the Ebro Valley, Lleida, Spain. *M. fructicola*, and *M. laxa* isolates were stored either as conidial suspensions in 20 % glycerol at –80 °C for long-term storage or as cultures on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) at 4 °C for short-term storage. *M. fructigena* isolates were maintained on PDA slants at 4 °C.

Table 4. Identification of the 29 isolates (10 *M. fruticola* isolates, 9 *M. fructigena* isolates, and 10 *M. laxa* isolates) that were used in the different phytotoxin and degrading enzymes identification experiments.

Collection name	<i>Monilinia</i> species	Host	Plant material	Year of isolation
ALB2010PB5R14	<i>M. fruticola</i>	Peach	Twig	2010
ALB2009COS10R1	<i>M. fruticola</i>	Peach	Fruit	2009
ALF2009COS5R7	<i>M. fruticola</i>	Peach	Fruit	2009
ALB2009IL14D4R9	<i>M. fruticola</i>	Peach	Li ^a	2009
ALF2009MOA4	<i>M. fruticola</i>	Peach	MFT ^b	2009
SUD2009COS2R6	<i>M. fruticola</i>	Nectarine	Fruit	2009
ALB2009PB5R14	<i>M. fruticola</i>	Peach	Twig	2009
SUD2009COSLER100.1	<i>M. fruticola</i>	Nectarine	Fruit	2009
ALF2009COS10R8	<i>M. fruticola</i>	Peach	Fruit	2009
ALF2009COSLER35.3	<i>M. fruticola</i>	Peach	Fruit	2009
BOLAÑO1	<i>M. fructigena</i>	Apple	Fruit	2010
BOLAÑO8	<i>M. fructigena</i>	Apple	Fruit	2010
BOLAÑO10	<i>M. fructigena</i>	Apple	Fruit	2010
BOLAÑO12	<i>M. fructigena</i>	Apple	Fruit	2010
BOLAÑO13	<i>M. fructigena</i>	Apple	Fruit	2010
71	<i>M. fructigena</i>	Apple	Fruit	2010
199	<i>M. fructigena</i>	Apple	Fruit	2010
FRANZA2012.2	<i>M. fructigena</i>	Apple	Fruit	2012
D-45	<i>M. fructigena</i>	Apple	Fruit	2009
ALB20097DIL10R10	<i>M. laxa</i>	Peach	Li ^a	2009
BRM2009ALB2.3	<i>M. laxa</i>	Peach	Twig	2009
FLOR2009SUD5.1	<i>M. laxa</i>	Nectarine	Flower	2009
SUD2009IL21D8R2	<i>M. laxa</i>	Nectarine	Li ^a	2009
SUD2009IL14D4R1	<i>M. laxa</i>	Nectarine	Li ^a	2009
ALB2009ILCOS6R6	<i>M. laxa</i>	Peach	Li ^a	2009
MS2009ALF10	<i>M. laxa</i>	Peach	MFO ^c	2009
SUD2009IL7D5R1	<i>M. laxa</i>	Nectarine	Li ^a	2009
MOA2009SUD3	<i>M. laxa</i>	Nectarine	MFT ^b	2009
ALF2009COSLER107.1	<i>M. laxa</i>	Peach	Fruit	2009

^a Tissue with latent infection.

^b Mummified fruit from a tree.

^c Mummified fruit on orchard bed.

3.2.2. Plant material

Commercial nectarines (*Prunus persica* L. Batsch var. *nucipersica* Suckow), with yellow flesh and soft skin, were used to test the different HPLC extracts and collected fractions. All the nectarines used on these experiments had a BBCH = 89 phenological stage (BBCH: general scale from Biologische

Bundesanalt, Bundessortenamt and Chemische Industrie, Germany (Meier et al., 1994)) and did not have apparent brown rot symptoms.

3.2.3. Toxin characterization

Twelve nectarines, 3 inoculated with *M. fructicola*, 3 with *M. fructigena*, 3 with *M. laxa*, and 3 uninoculated nectarines; were incubated in the dark at 25 °C and 100 % RH for 7 days separately in sterile humidity chambers, i.e. polystyrene boxes, which were lined with moist sterilized filter paper. After the incubation period, the juice from each nectarine was obtained by squeezing the decayed fruit flesh through a Miracloth (Merck-Millipore, Darmstadt, Germany). Juice samples were kept at –20 °C until required for analytical procedure. The initial extraction of compounds from the culture filtrate followed a solvent fractioning procedure as follows; 10 mL of the nectarine juice were first partitioned in a separation funnel with 3x10 mL of n-hexane. The aqueous layers were collected and extracted with 3x10 mL of dichloromethane. Subsequently the aqueous layers were collected and extracted with 3x10 mL of ethyl acetate. The last extraction of the aqueous phase was performed with butanol (Fig. 13). Each organic extract was then evaporated to dryness with a vacuum concentrator (Eppendorf, Hamburg, Germany) and reconstituted in 2 mL water. In order to detect necrotic activity, each aqueous solution was tested in wounded nectarine circles 1.5 cm in diameter. Nectarine circles were obtained from surface disinfected nectarines (1 min wash with 1 % NaClO, followed by 5 min wash with 70 % ethanol, and two 1 min washes with sterile deionized water (Sauer and Burroughs, 1986)) by boring holds with a bore-hole and then dissecting the first 1.5 mm with the aid of a sterile disposable scalpel blade. Nectarine circles were placed in disinfected humid chambers and wounded with sterile needles. A drop of 30 µL of each of the reconstituted organic extracts was placed on top of the wound. Three replicates of each reconstituted organic extract and *Monilinia* specie were made and experiments were repeated at least twice. Pictures of each of the nectarine circles were taken after 2 and 4 days of

incubation at 25 °C with a Leica M80 modular stereo microscope equipped with a Leica DC 500 camera (Leica microsystems, Wetzlar, Germany). Pictures were analyzed and the necrotic areas surrounding nectarine circle wounds were measured in ImageJ (Schneider et al., 2012). In order to detect positive toxic response, the necrotic areas surrounding the wound of the aqueous solutions from nectarines infected with *Monilinia* spp. juices were compared with those areas from a blank sample, i.e. a filtrated juice without fungus inoculation subjected to the same extraction procedure and tested in parallel to the samples.

Fig. 13. Procedure for liquid-liquid partition extraction of nectarine juices.

The next step was to compare HPLC chromatograms of organic extracts that gave a positive toxic response (butanol of *M. laxa*, butanol of *M. fructigena* and ethyl acetate of *M. fructicola*) with chromatograms of the blank extracts in order to find compounds responsible of toxicity. Extracts were analyzed by an HPLC (1200 HPLC system, Agilent Technologies, Santa Clara, CA) coupled with a diodoarray and a mass spectrometer detector. HPLC is equipped with a quaternary solvent delivery system, an autosampler, a column oven, vacuum degasser and a fraction collector. Chromatographic

separation was achieved with a particle diameter of 3 μm using a Waters Atlantis Atlantis® T3 (4.6 x 150 mm) with a dC18 guard column. The mobile phase was a mixture of water 0.1 % of formic acid (A) and acetonitrile (B). The gradient elution was as follows: 0-60 min, 5-55 % (B); 60-61 min, 55 % (B); 61-80 min, 55-95 % (B). The flow rate was 0.7 mL min^{-1} and the injection volume was 80 μL . Detection was performed on an Agilent 6420 series MS/MS with an ESI interface operated in the positive mode and a photodiode array detector (DAD). The ESI source conditions were: fragmentor 100, gas temperature 350 $^{\circ}\text{C}$, drying gas flow rate 10 L min^{-1} , nebulizer pressure 40 psi, capillary voltage 4000 V, N_2 gas was used as nebulizer gas. LC-MS/MS accurate mass spectra were recorded across the range of m/z 20-1000.

The comparison of chromatograms of organic extracts with those of blank samples permitted to identify fractions with different profiles. Afterwards, the selected fractions were isolated with the fraction collector. Twenty-nine fractions were collected of *M. fructicola* ethyl acetate extract, 14 fractions of *M. fructigena* butanol extract and 15 fractions of *M. laxa* butanol extract. Each isolated fraction was evaporated to dryness and reconstituted with 1 mL of ultrapure water. Each of these fractions was once again tested on wounded nectarine circles in order to find the fraction/s responsible for the necrosis (process described above).

3.2.4. Enzymatic activity

To test enzymatic activity 1-week-old mycelial plugs (0.7 cm in diameter) of each *Monilinia* spp. isolate grown on PDA were placed on minimum media plates. The minimum media plates contained 25 mL of water with 2 % European bacteriological agar (Laboratorios CONDA, Madrid, Spain), yeast extract (Laboratorios CONDA) and 1 % of one of the following substrates: callose, casein hydrolysate (acid hydrolyzed) for microbiology (Merck-Millipore, Darmstadt, Germany), cutin, methylcellulose viscosity: 25 cP (Sigma-Aldrich Co., St. Louis, MO), pectin from apple (Sigma-Aldrich Co.),

polygalacturonic acid sodium salt (Sigma-Aldrich Co.), D(+)-Sucrose for analysis, ACS (AppliChem GmbH, Darmstadt, Germany), and xylan from birch wood (Sigma-Aldrich Co.). Cutin was prepared from apple fruit peels as described by Bostock et al. (1999). The control minimum plates had no substrates on them. Three replicates of each amended medium and *Monilinia* isolate were incubated for 28 days in the dark at 25 °C. Each week data on: (i) colonies expansion (% of the plate covered), (ii) density (from 0 the medium can be seen as if nothing was growing on them to 5 neither the medium nor the light can be seen through the mycelia), and (iii) sporulation (% of the colony sporulated) was recorded for each plate. The experiments with each amended media were repeated at least twice.

3.2.5. Data analysis

Data on the necrosis area around the wounds of nectarine circles was analyzed pairwise between necrosis area generated by extracts from juice of nectarines inoculated with *Monilinia* spp. and the blank extract, using analysis of variance (Snedecor and Cochran, 1980). When F-test was significant at $P \leq 0.05$, the means were compared using the Student-Newman-Keuls multiple range test in the statistical software Statgraphics Centurion XVI for Windows, Version 16.1.03 (StatPoint Technologies, Inc., Herndon, VA).

Data on the colonies expansion, density and sporulation on each amended medium was calculated as percentages and arcsine transformed prior to statistical analysis. The colony expansion was calculated as a percentage by dividing the colonies diameter by the petri plate diameter. The density was measured by giving a value between 0 (the medium was perfectly visible across the fungal colony) and 5 (neither the medium, nor the light can be seen through the colony) to each colony, which was then converted into percentages. Colony sporulation was calculated as a percentage by dividing the sporulated area of the colony by the total area of the colony. Colonies expansion, density and sporulation of each *Monilinia* species on each amended medium were analyzed by analysis of variance (Snedecor and

Cochran, 1980). When F-test was significant at $P \leq 0.05$, the means were compared using the Student-Newman-Keuls multiple range test in the statistical software Statgraphics Centurion XVI for Windows, Version 16.1.03 (StatPoint Technologies).

3.3. MICROSCOPIC ANALYSIS OF LATENT AND VISIBLE *M. fructicola* INFECTIONS IN NECTARINES

3.3.1. Fungal cultures and conidial production

An isolate of *M. fructicola* (ALF 2009 COS 5R7), which was originally collected from a harvested nectarine in Alfarras, Lleida, Spain in 2009, was used in the investigation. The isolate was grown on potato dextrose agar (PDA) plates (Difco Laboratories, Detroit, MI) at 20-25 °C in the dark for seven days for conidial production. The isolate was stored either as a culture on PDA at 4 °C for short-term storage or as a conidial suspension (see later for preparation) in 20 % glycerol at –80 °C for long-term storage.

Conidial suspensions were prepared using conidia that were harvested from the PDA plates 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 hemocytometer and a light microscope (Zeiss Axioskop 2; Carl Zeiss, Inc., Oberkochen, Germany).

3.3.2. Conidial germination on the nectarine's epidermis

Samples of the epidermis (about 0.5 cm² and 0.5 to 1 mm thick) of mature nectarines were obtained from surface-disinfected fruit using a sterilized disposable scalpel. To this end, the fruit surface was disinfected by first immersing the fruit in a 0.1 % sodium hypochlorite solution for 5 min, followed by immersion in a 70 % ethanol solution for 1 min, and finally two rinses of the fruit in SDW (Sauer and Burroughs, 1986). Samples of the epidermis were placed on the surface of 2 % water agar plates (Laboratorios Conda S.A., Torrejón de Ardoz, Madrid, Spain). An aliquot (5 µL) of the conidial suspension (3x10³ conidia mL⁻¹) was then deposited on each sample, and the samples were then incubated at 25 °C or 4 °C for four or 24 h in the dark. At the end of the incubation, the samples were cleared by immersion in a 3:1 (v/v) mixture of ethanol and chloroform which contained 0.15 % (w/v) trichloroacetic acid for 15 min at 70 °C, then immersed in a solution of phenol:water:glycerine:lactic acid (1:1:2:1 v/v) for 2 min, and then stained with lactophenol blue (Sigma-Aldrich Quimica SL., Madrid, Spain). After staining, each sample was mounted on glass slides in a solution of phenol:water:glycerine:lactic acid (1:1:2:1 v/v). Images of the specimens were examined after their capture by a Leica DFC550 camera, which was attached to a Leica DMRE microscope (Leica Microsystems, Wetzlar, Germany). Three samples were made for each temperature and incubation time. The controls were samples of the epidermis from uninoculated nectarines and the entire assay was repeated twice.

3.3.3. Visible infection studies: fruit inoculation and incubation

Mature nectarines were used to investigate the visible infection process. For this purpose, the surface of the nectarines was first disinfected, as previously described, and then dried for 2 h in a laminar flow cabinet. Once dried, four 30-µL drops of a conidial suspension (10⁶ conidia mL⁻¹) were placed at

different points on the surface close to the insertion point of the peduncle. The inoculated fruit were kept in the laminar flow cabinet until the drops evaporated. The fruit were then transferred to dry plastic plates in a humidity chamber at 100 % relative humidity (RH) and incubated at 25 °C for 0, 24, 48, 72, or 96 h in the dark. One fruit was used for each incubation time. The controls were fruit which were inoculated with SDW and the entire assay was repeated twice.

3.3.4. Latent infection studies: fruit inoculation and incubation

Mature nectarines were used to investigate the latent infection process. For this purpose, the surface of the nectarines was first disinfected, as previously described, and then dried for 2 h in a laminar flow cabinet. Once dried, four 30- μ L drops of a conidial suspension (10^6 conidia mL⁻¹) were placed at different points on the surface close to the insertion point of the peduncle. The inoculated fruit were then transferred to plates in polystyrene boxes which were lined with sterilized filter paper that was moistened with SDW and incubated at 25 °C for 24 h in the dark. After completion of the incubation, the surface of the inoculated nectarines was again disinfected, as previously described, and then dried for 2 h in a laminar flow cabinet. Once dried, the fruit were transferred to dry plastic plates in a humidity chamber at 100 % RH and incubated at 4 °C for 72, 144, 216, and 288 h in the dark. The controls were uninoculated fruit and the entire assay was repeated twice.

3.3.5. Light microscopic analysis of nectarines with a visible or latent *M. fructicola* infection

At the end of the incubation, a sample of nectarine tissue (0.1 cm x 1 cm x 1 cm) from each of the four inoculation points was excised from the inoculated nectarines for examination by light microscopy. To this end, each sample was fixed by immersion in a solution of formaldehyde-acetic acid-alcohol

(ethanol: acetic acid: formaldehyde: water, 50:10:35:5, v/v/v/v). To facilitate fixative infiltration, the samples were first degassed by maintaining them in a mild vacuum for 2 h and then overnight incubation in a formaldehyde-acetic acid-alcohol solution at 4 °C. Following the overnight incubation, the samples were first gradually dehydrated in graded ethanol (50, 70, 80, and 100 % - once for 60 min at each step) and then soaked in a graded series of ethanol:Histoclear II (National Diagnostics, Atlanta, GA) (3:1, 1:1, 1:3, 0:1 v/v - once for 120 min at each step) to remove the alcohol. The samples were then embedded in paraffin by first immersing each sample in a graded series of molten paraffin: Histoclear II at 60 °C (Histo-comp, Casa Alvarez, Madrid, Spain) (1:3, 1:1, 3:1 v/v - once for 60 min at each step) and then in molten paraffin for at least 48 h. Five sections (15-µm thick) of each sample were prepared using a microtome (Microtome pfm medical ag, Köln, Germany), mounted on glass slides, and then stained with Safranin O-Fast-green stain using a previously described protocol (Peterson, 1969) or Calcofluor White fluorescent stain (Sigma-Aldrich Co., St. Louis, MO) according to the manufacturer's instructions. Images of each microsection were examined after their capture by a Leica DFC550 camera, which was attached to a Leica DMRE microscope (Leica Microsystems, Wetzlar, Germany).

3.3.6. Transmission electron microscopic (TEM) analysis of nectarines with a visible *M. fructicola* infection

At the end of the incubation, four samples (2 cm x 2 mm) of the dermal and ground tissues from infected and uninfected nectarines were collected using a sterilized disposable scalpel for examination by TEM. To this end, each samples was first fixed by immersion in modified Karnovsky's fixative solution which contained 2.5 % (v/v) glutaraldehyde and 4 % (v/v) paraformaldehyde in sodium phosphate buffer (pH 7.0) at 4 °C for 6 h, and then washed four times for 10 min in Karnovsky's fixative solution. Each sample was postfixed in a 1 % (w/v) aqueous osmium tetroxide solution at

20 °C for 2 h, and then washed three times in SDW. Each sample was gradually dehydrated in graded acetone (30 %, 50 %, 70 %, 80 %, and 95 % - once for 15 min at each step), twice in 100 % acetone for 15 min, embedded in Spurr resin, and polymerized at 65 °C for 48 h according to a previously described protocol (Fourie and Holz, 1995). Ultrathin sections (15-µm thick) of each sample was prepared using an ultramicrotome with a diamond knife (Ultracut Reichert, Vienna, Austria) and then mounted on copper grids. The grids were counterstained with 2 % (w/v) uranyl acetate for 20 min and with Reynolds' lead citrate for 4 min before being examined using a TEM (JEM-1010, LEOL Ltd., Tokyo) which was operated at 80 kV. At least four embedded blocks were prepared for each incubation time, and more than ten ultrathin sections per block were examined by TEM.

3.4. TRANSFORMATION OF THE BROWN ROT FUNGAL PATHOGENS *M. fructicola* AND *M. laxa*

3.4.1. Fungal culture, conidial production and bacterial strains

Four single-spore 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 investigation (Table 5). All isolates were originally collected from several commercial peach and nectarine orchards in the Ebro Valley, Lleida, Spain. All isolates were stored as conidial suspensions in 20 % glycerol at -80 °C for long-term storage or as cultures on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) at 4 °C for short-term storage. For conidial production, (a) the two *M. fructicola* isolates were grown on PDA plates at 20 to 25 °C for 7 days in the dark, and (b) the two *M. laxa* isolates were grown on PDA plates amended with tomato puree (250 mL per liter of medium) at 20 to 25 °C for 10 days in the dark.

Table 5. The host, isolation tissue and year of isolation of the four *Monilinia laxa* and *M. fructicola* isolates used in the fungal transformation experiments.

Isolate name	Collection name	<i>Monilinia</i> species	Host	Isolation tissue	Year of isolation
MI1	SUD2009IL21D8R2	<i>M. laxa</i>	Nectarine	Fruit	2009
MI2	MS2009ALF10	<i>M. laxa</i>	Peach	Mummified fruit on the orchard ground	2009
Mfc1	ALF2009MOA4	<i>M. fructicola</i>	Peach	Mummified fruit on trees	2009
Mfc2	2014COSALBARED36	<i>M. fructicola</i>	Nectarine	Fruit	2014

The plasmids were propagated in *Escherichia coli* strain DH5 α (Meselson and Yuan, 1968). The *E. coli* strain has an F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ 80d*lacZ* Δ M15 Δ (*lacZYA-argF*)U169 hsdR17 (rk- mk+) λ - genotype and was stored in DMSO at -80 °C.

3.4.2. Auxotrophic spontaneous mutant induction

To avoid introducing an antibiotic resistance-gene for the selection of the *Monilinia* spp. transformants, a search for spontaneous *M. fructicola* and *M. laxa* mutants was carried out in two metabolic pathways: i) in nitrate metabolism through disruption of the nitrate reductase activity (mutations mapping in the homologous *niaD* gene) and ii) the essential pyrimidine metabolic pathway involving the orotidine 5'-phosphate decarboxylase activity (mutations affecting the homologous *pyrG* gene). Respective auxotrophic mutants would be unable to either use nitrate as a nitrogen source or grow without uracil- and uridine-amended growth medium. These auxotrophies could be complemented by introducing the corresponding homologue gene by fungal transformation techniques.

3.4.2.1. Mutants in the nitrate reductase pathway

The *M. fructicola* and *M. laxa* isolates were cultivated on minimal medium amended with different nitrogen sources: sodium nitrate, sodium nitrite, urea, proline, glutamine, asparagine, and arginine to determine (i) if they were able to utilize nitrate and nitrite, and (ii) alternative nitrogen sources by analysing colonial radial growth. Solid minimal medium contained: 1 % D-glucose, 1x concentrated salt solution, 2.5/5/10 mM of each Nitrogen source, and 1.5 % agar; the minimal medium pH was adjusted to 6.5 with sodium hydroxide. The 50x concentrated salt solution contained: potassium chloride (26 g L⁻¹), magnesium sulphate heptahydrate (26 g L⁻¹), potassium dihydrogen phosphate (76 g L⁻¹), sodium tetraborate decahydrate (2 mg L⁻¹), copper chloride dehydrate (20 mg L⁻¹), iron chloride (40 mg L⁻¹), zinc chloride (0.4 g L⁻¹), and magnesium chloride tetrahydrate (40 mg L⁻¹). Once determined that both *M. fructicola* and *M. laxa* were able to use nitrate as a nitrogen source, the susceptibility of *Monilinia* spp. to potassium chlorate was tested by adding 0, 10, 25, 50, 150 and 500 mM potassium chlorate to minimal medium with 5 mM urea as nitrogen source. When the nitrate reductase pathway is active chlorate is transformed into chlorite which is toxic for fungal growth. After establishing 150 mM potassium chlorate as the growth inhibitory concentration, conidia were inoculated in zigzag onto 75, 100, and 125 mM potassium chlorate selective media and incubated for 14 days. Conidia from those colonies and sectors able to grow on the selective medium passed through three additional selective rounds of selection for tolerance to chlorate toxicity before testing their ability to use nitrate as a nitrogen source.

3.4.2.2. Mutants on the orotidine 5'-phosphate decarboxylase

Monilinia spp. isolates were grown on potato dextrose agar (PDA) with different 5-fluoroorotic acid (5-FOA) concentrations: 0.5, 1, 1.5, 2, and 2.5 mg mL⁻¹; to identify the toxic range. Deficiencies on *pyrG* or *pyrF* homologous genes enables the fungus to grow with 5-fluoroorotic acid, since it is not

metabolize. However, deficiencies in this pathway are lethal unless media is amended with uracil (5 mM final concentration) and uridine (5 mM final concentration, UU). Those colonies that formed sectors or survived when the *Monilinia* spp. isolates were grown on PDA UU plates with 1.5 mg mL⁻¹ of 5-FOA, were transferred to new PDA UU plates with 1.5 mg mL⁻¹ of 5-FOA, at least 4 times before testing their growth on PDA plates without uracil and uridine. Since we are interested in *pyrG* mutants and not *pyrF* mutants, and 5-FOA is able to generate mutants in both genes, those colonies unable to grow without uracil and uridine were analyzed to select only those carrying loss of function mutations in the *pyrG* gene.

3.4.3. Determination of the nuclei number in the conidia, hyphae and protoplast of *Monilinia* spp.

The number of nuclei present in the conidia, hyphae, and protoplasts of *M. fructicola* and *M. laxa* isolates were determined by staining their nucleic acids with DAPI (4',6-diamidino-2-phenylindole) (Sigma-Aldrich Chemie GmbH, Munich, Germany).

In order to observe the distribution and determine the number of nuclei present inside the conidia, conidia were harvested from sporulated PDA cultures and placed in a 1.5 mL mini-centrifuge tube containing 1 mL phosphate buffer solution (PBS) (150 mM NaCl, 200 mM Na₂HPO₄, 5 mM NaH₂PO₄), 0.01 % Triton X-100, and 60 µg mL⁻¹ of DAPI and incubated for 10 min at room temperature (22 °C) in a vacuum chamber. After the 10 min incubation period conidial suspension were centrifuged at 13.000 rpm for 3 min and washed for 1 min in 1 mL of PBS. This washing step was repeated 3 times. DAPI stained cells were observed under an epifluorescence microscope Nikon Eclipse 80i (Nikon Instruments Europe BV, Amsterdam, Netherlands) equipped with a GFP-3035B filter (Semrock, Inc., Rochester, New York), a TXRED-4040B filter (Semrock, Inc.), and a DAPI-

1160B filter (Semrock, Inc.), and analyzed with the MetaMorph® Microscopy Automation & Image Analysis Software (Molecular Devices, LLC., Sunnyvale, CA).

For observation of nuclei in hyphae, conidia were grown in watch minimal medium (0.1 % D-glucose, 1x concentrated salt solution, 5 mM ammonium tartrate, and 25 mM of sodium dihydrogen phosphate) overnight at 25 °C in the dark. After extracting the media, germinated conidia were incubated for 10 min at room temperature in a vacuum chamber in a DAPI PBS solution with 0.01 % triton X-100. Cells were washed of excess of DAPI solution by adding 1 mL of PBS and incubating the germinated conidia on it for 1 min, this step was repeated three times. Subsequently, stained cells were observed using an epifluorescence microscope Nikon Eclipse 80i (Nikon Instruments Europe BV).

The number of nuclei inside the protoplast was determined by staining *Monilinia* spp. protoplasts with DAPI (120 µg mL⁻¹) diluted in protoplast solution 6 (1 M sorbitol and 10 mM Tris HCl buffer pH 7.5) with 0.01 % Triton X-100 for 10 min before observing them under the epifluorescence microscope Nikon Eclipse 80i (Nikon Instruments Europe BV).

3.4.4. Transformation vector

Transforming vectors for the expression of the phleomycin resistance gene (*bleo*) and either the green fluorescence protein (GFP) or the red fluorescence protein (mCherry) were constructed by introducing expression cassettes in the pGEM®-T Easy Vector (Promega Corporation, Madison, WI) (Fig. 16). The expression cassettes were constructed by fragment-fusion PCR as described by Yang et al. (2004) using the PrimeSTAR® HS DNA Polymerase (Takara Bio Inc. Shiga, Japan). In the fragment-fusion PCR the primers of the adjoining fragments have a 20 to 30 bp extension complementary to the adjoining fragment, which promotes the fusion of the fragments. All fragment-fusion PCRs were done in 0.2 µL centrifuge tubes containing 10 µL of MgCl₂ 5x buffer, 4 µL of dNTPs solution (200 µM), 3 µL of each primer (0.5 µM), 0.3 µL of PrimeSTAR® HS DNA Polymerase, 0.5 µg of each DNA

fragment, and the rest of the volume up to 50 μ L of water. The fragment-fusion PCR conditions were: 98 $^{\circ}$ C for 2 min, followed by 10 cycles of 98 $^{\circ}$ C for 10 s, 55 $^{\circ}$ C for 5 s and 72 $^{\circ}$ C for 2 min + 1 min for each kilobase of final fragment length, 15 cycles of 98 $^{\circ}$ C 10 s, 58 $^{\circ}$ C 5 s and 72 $^{\circ}$ C for 2 min + 1 min for each kilobase of final fragment length + 20 additional seconds per cycle, and 72 $^{\circ}$ C for 4 min + 1 min for each kilobase of final fragment length. Oligonucleotide primers used in this paper were obtained from Sigma-Aldrich Chemie GmbH and are given in Table 6.

Fig. 14. Diagram of the *Monilinia* expression cassette (MEC) inside the pGEM®-T Easy Vector, for the heterologous recombination, indicating the SpeI restriction site, where the vector was opened to insert the sequence of *M. fructicola* cutinase1 gene.

The phleomycin resistance cassette (DNA sequence of *bleo* flanked with the promoter of the glyceraldehyde-3-phosphate dehydrogenase of *Aspergillus nidullans* (*gpdA*) and the *CYC1* terminator from the Cytochrome c, isoform 1) was PCR amplified from a pBS-K+ plasmid (p.1030) using primers

MEC7 and MEC8 (Table 6). Coding sequences for GFP and mCherry tags were obtained by PCR amplification using primers MEC3' and MEC4' (Table 6) from two different plasmids. Finally, the sequences of the promoter and the terminator of the *Botrytis cinerea* protein homologous to GPDA were PCR amplified with primers MEC1 and MEC2, and MEC5' and MEC6' respectively (Table 6).

The PCR-amplified sequences of *B. cinerea* GPDA promoter and terminator were fused by PCR to either coding sequences for GFP or mCherry tags by using primers MEC 1 and MEC 6 (Table 6). Resultant PCR fragments were fused to the phleomycin resistance cassette by PCR using primers MEC1 and MEC8 (Table 6). Both PCR fused fragments (one with the GFP tag and another with the mCherry tag) were introduced in the expression vector pGEM®-T Easy Vector (Promega).

Additionally, the sequence of *M. fructicola* cutinase 1 gene (CUT1), obtained by PCR amplification with Cut1_1 and Cut1_2 primers (Table 6), was inserted into the expression vectors for homologous recombination by using restriction sites for enzyme Spe I (New England Biolabs, Ipswich, MA) present at the polylinker and at both sides of CUT1 PCR fragment.

Table 6. Oligonucleotide sequences of the primers used in the construction of the *Monilinia* expression cassette (MEC).

Identifier	Oligonucleotide sequence (5'-3')
MEC1	CATTCGTCAGCAGGTACATCGACACCG
MEC2	CATTGTTGAATCAATTTTACGGTAGCGC
MEC3	GCGCTACCGTAAAATTGATTCAACAATGGGAGCTGGTGCAGGCGCTGGAGCC
MEC3'	GGAGCTGGTGCAGGCGCTGGAGCC
MEC4	CTTCCTAAAGACTGAGCAAAAATCTCTGGTCAATCACTGGTAACTCCACGG
MEC4'	TGGTCAATCACTGGTAACTCCACGG
MEC5	GACCAGAGATTTTTGCTCAGTCTTTAGG
MEC5'	AGAGATTTTTGCTCAGTCTTTAGG
MEC6	GGATCAGAAGAGGGTTGGTGTACCTTGGATATCATAGCGAGAGCG
MEC6'	CTTGGATATCATAGCGAGAGCG
MEC7	GATATCCAAGGTACACCAACCCTCTTCTGATCC
MEC8	AGCTTGCAAATTAAAGCCTTCGAGCGTCCC
Cut1_2 ^a	AGGaCtaGTAAGTTCCCTATACTCTCTGG
Cut1_3 ^a	CCActaGTCCCAGTTGCCAGTGTTCAGTTGCC

^a Lowercase letters indicate changes on the original sequence to introduce Spe I restriction sites.

3.4.5. *Monilinia* transformation

Along this work a number of transformation procedures have been analyzed for suitability of use with *Monilinia* species. Two transformation protocols based on CaCl₂/polyethylene glycol (PEG)-mediated transformation were tested on *M. fructicola* and *M. laxa* isolates. An isolate of *Penicillium rubens* served as a control for these fungal transformation procedures. One of these protocols was developed for *A. nidulans* transformation by Tilburn et al. (1983) and the second was developed for *M. fructicola* by Chou et al. (2015). In both procedures a mycelial culture was the starting material. Five million *Monilinia* spp. conidia suspended in 20 mL 0.0001 % tween solution were used to inoculate 400 mL of liquid minimal medium (1 % D-glucose, 1x concentrated salt solution, and 5 mM ammonium tartrate; minimal medium pH was adjusted to 6.5) inside a 2 L Erlenmeyer flask. After 16 h incubation at 25 °C and 140 rpm, mycelium was harvested by filtration and 1 g was used for protoplast formation. Both protoplast formation and transformation were done following each protocol, with the exception that 200 mg of Vinoflow® Max (Lamothe-Abiet, Bordeaux, France) lytic enzymes for each gram of mycelia were used for protoplast formation.

In addition, two lithium acetate (LiAc)-mediated transformation protocols were tested on *M. fructicola* and *M. laxa* isolates, one developed for yeast transformation by Ito et al. (1983), and modified by Schiestl and Gietz (1989), Hill et al. (1991), and Gietz et al. (1992) and another developed for the transformation of *Beauveria bassiana* (Jiang et al., 2007). For the initial fungal culture approximately one sixth of a sporulated *Monilinia* spp. plate was scratched and incubated in 50 mL of liquid minimal medium overnight (16 h) at 25 °C and 140 rpm.

The protocol developed by Sánchez and Aguirre (1996) and Brown et al. (1998) and modified by Marchand et al. (2007) for genetic transformation by electroporation of *Pseudozyma antarctica*, a basidiomycetous yeast-like fungus, was tested on *M. fructicola* and *M. laxa* isolates. To test this

protocol 50 mL of liquid minimal medium containing 10^6 conidia mL^{-1} of *Monilinia* spp. was incubated at 25 °C and 140 rpm until conidial germination reached 50 % (approximately 4 h). For the electroporation process, a Gene Pulser II with Pulse Controller (Bio-Rad Laboratories, Inc. Hercules, CA) was used. Two combinations of voltage (1.40 kV) and resistance (400 or 800 Ω) were tested. Capacitance was held constant at 25 μF with one pulse. After the pulse, germinated conidia were incubated in potato dextrose broth (Difco Laboratories), and later on transferred to PDA plates.

As a general rule, all fungal transformation protocols included a control transformation vial subjected to the complete transformation process but lacking of DNA transformation vector. Also, the viability of fungal cells (protoplasts) after each transformation method was evaluated by plating part of the control transformation (no DNA) onto regeneration medium and absence of contaminants by plating other part onto selective medium. Possible transformants were selected by plating transformation mixtures onto selective medium. The regeneration and selection plates of the CaCl_2 /PEG-mediated transformation were made with regeneration minimal medium (1 % D-glucose, 1x concentrated salt solution, 1 M sucrose, 5 mM ammonium tartrate, and 1 % agar; the regeneration minimal medium pH was adjusted to 8). Those of the LiAc-mediated transformation and electroporation transformation were made with PDA adjusted to pH 8. The plates were incubated for 16 h at 25° C in the dark before adding a medium cover (regeneration medium with only 0.4 % agar) to the plates, which contained 200 μg of phleomycin as the selective pressure. The cover medium of the regeneration plates lacked of phleomycin. Transformantion plates were incubated at 25 °C in the dark for 5 days.

3.5. DETECTION OF LATENT *Monilinia* INFECTIONS IN NECTARINE FLOWERS AND FRUIT BY qPCR

3.5.1. Fungal isolates and preparation of conidial suspensions

Twenty-eight *Monilinia* isolates (10 *M. fructicola* isolates, 8 *M. fructigena* isolates, and 10 *M. laxa* isolates) from the culture collection of Plant Protection Department of Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Madrid, Spain were used in the different experiments (Table 7). The *Monilinia* isolates of the collection were confirmed by morphological identification and PCR assays according to the method developed by Gel et al. (2007). The *M. fructicola* and *M. laxa* isolates were stored either as a conidial suspension in 20 % glycerol at -80 °C for long-term storage or as a culture on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) at 4 °C for short-term storage. The *M. fructicola* isolates were grown on PDA plates at 20 to 25 °C in the dark for 7 days for conidial production. The *M. laxa* isolates were first grown on PDA plates in the dark at 20 to 25 °C for 10 days and then at 4 °C for 5 days for conidial production. The *M. fructigena* isolates were maintained on PDA slants at 4 °C. For conidial production of *M. fructigena* isolates, surface-disinfected nectarines were inoculated with 3-mm diameter mycelial plugs from 1-week-old cultures that were grown on PDA at 25 °C in the dark. Nectarines were surface-desinfected by immersion for 5 min in a 1 % sodium hypochlorite solution, immersion for 1 min in a 70 % ethanol solution, two 1-min washes in sterilized distilled water (SDW), and drying for 2 h in a laminar flow hood (Sauer and Burroughs, 1986). The fruit were then incubated in a humidity chamber that was lined with sterilized moist filter paper at 20 to 25 °C under fluorescent lighting ($100 \mu\text{E m}^{-2} \text{s}^{-1}$ with a 16 h photoperiod) for 7 to 10 days.

Table 7. Identification of the 29 isolates (10 *M. fructicola* [Mfc] isolates, 8 *M. fructigena* [Mfg] isolates, and 11 *M. laxa* [Mlx] isolates) that were used in the different qPCR detection experiments.

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

^a Tissue with latent infection.

^b Mummified fruit from a tree.

^c Mummified fruit on orchard bed.

The conidial suspensions were prepared using conidia that were harvested from the PDA plates (*M.*

fructicola and *M. laxa*) or from fruit (*M. fructigena*) by scratching the surface with a sterilized

disposable scalpel after adding SDW. The harvested conidia and mycelia were filtered through glass wool in order to remove the mycelia after a 30 s sonication in an ultrasonic bath (J.P. Selecta S.A., Barcelona, Spain). The filtrate was adjusted to the desired conidial concentration using SDW after counting the number of conidia using a haemocytometer and a light microscope (Zeiss Axioskop 2; Carl Zeiss, Inc., Oberkochen, Germany).

3.5.2. 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., 2012b), artificial latent infections were conducted to ensure *Monilinia* detection. Two hundred and ninety nectarine flowers without any visible signs of brown rot were collected from a 'Romea' nectarine tree in the Jerte Valley, Caceres, Spain in 2014. After their collection, the flowers were first surface-disinfected (previously described on *M. fructigena* conidial production). After drying, the flowers were randomly divided in groups of 10. Each nectarine flower in each group was inoculated placing inside the calix a 30 μ L conidial suspension (10^6 conidia mL^{-1}) drop of one of the *M. fructicola*, *M. fructigena* and *M. laxa* isolates using a micropipette (approximately 780 pg DNA). After inoculation, the flowers were incubated in sterile humidity chambers, i.e. polystyrene boxes, which were lined with moist sterilized filter paper, at 25 °C and 100 % RH for 24 h in the dark. At the end of the incubation period, the flowers were again surface-disinfected and air-dried in a laminar flow hood, as previously described. After drying, the flowers were incubated in humidity chambers at 4 °C and 100 % RH for 5 days in the dark. The control group consisted of 10 flowers each of which was inoculated with 30 μ L SDW.

3.5.3. 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., 2012b). Inducing latent infections with a high pathogen inoculum concentration ensures presence and homogeneous numbers of latent infections in nectarines, thus reducing the effects over the results of the sporadic natural latent infections. Two nectarine varieties of similar susceptibility, ‘Alba Red’ and ‘Big Top’, which had been routinely used in the INIA lab over the years, were selected and used for the establishment of artificial latent infections. One hundred thirty-two nectarines of similar phenological status (BBCH = 89, at harvest; BBCH: general scale from Biologische Bundesanstalt, Bundessortenamt and Chemische Industrie, Germany (Meier et al., 1994)) without any visible signs of brown rot were collected from orchards in the Ebro Valley, Lleida, Spain. After their collection, the nectarines were surface-disinfected and dried in a laminar flow hood, as previously described on *M. fructigena* conidial production. After drying, the nectarines were randomly divided in groups of 6, and each nectarine of each group was inoculated by a 30 s immersion in a conidial suspension (approximately 10^5 conidia mL⁻¹). Sixty-six ‘Alba Red’ nectarines were inoculated with *M. fructicola* isolates in 2014 and 66 ‘Big Top’ nectarines with *M. laxa* isolates in 2015. After inoculation, the nectarines were incubated in humidity chambers (previously described) at 25 °C and 100 % RH for 24 h in the dark. At the end of the incubation period, the nectarines were again surface-disinfected and dried in a laminar flow hood, as previously described. After drying, the nectarines were incubated in humidity chambers (previously described) at 4 °C and 100 % RH for 5 days in the dark. A control group was made each year with 6 nectarines, each of which was immersed in SDW for 30 s.

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

Five flowers inoculated with each *Monilinia* isolate and 5 control-uninoculated flowers were stored at -80 °C and then lyophilized in a laboratory freeze dryer (Cryodos -50, Azbil Telstar Technologies, SLU, Terrassa, Spain). Each lyophilized flower was placed on 2 mL micro-centrifuge tubes and homogenized for 60 s at a speed setting of 4.0 m s⁻¹ using a high-speed benchtop tissue homogenizer (FastPrep-24 Instrument, MP Biomedicals, Solon, OH). Genomic DNA from each flower was extracted using the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's instructions except that DNA was eluted in a final volume of 100 µL.

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

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

Latent *Monilinia* infections in the flowers and fruit were detected applying the qPCR-based method and hydrolysis probes of van Brouwershaven et al (2010) with modifications as follows. The hydrolysis probes were labelled with different reporter dyes and quenchers and all primers and probes were obtained from Integrated DNA Technologies Inc. (Coralville, IA). Specifically, we used a FAM reporter dye and a ZEN / Iowa Black FQ quencher for *M. fructicola* probe (P_fc) instead of a FAM-TAMRA, and a HEX reporter dye with a ZEN / Iowa Black FQ quencher instead of a VIC-TAMRA quencher for *M. fructigena* and *M. laxa* probe (P2_fgn/lx/ps). Genomic DNA from the flowers (20 ng) or the 3 fruit areas (10 ng) was amplified in 20- μ L reaction mixture in each well of a 96-well clear optical reaction plate (Applied Biosystems, Foster City, CA) sealed with a clear adhesive. The 20- μ L 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 and P2_fgn/lx/ps). Thermal cycling was done using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) under the same conditions and using the same thresholds described by van Brouwershaven et al. (2010) with slight modifications: polymerase activation at 95 °C for 10 min, followed by 40 amplification cycles at 95 °C for 15 s and 60 °C for 1 min. Emission was measured at the annealing-extension step. The threshold value was set at fluorescence (ΔR_n) of 23,000. A quantification cycle (C_q) value below 40 was scored as a positive detection. Additionally, an allelic discrimination step was added due to cross-detection of *M. laxa* and *M. fructigena* when using the P_fc probe. Allelic discrimination allowed us to distinguish between the *M. fructicola* isolates, the *M. fructigena* isolates, the *M. laxa* isolates, and mixtures of the *Monilinia* isolates with absolute certainty. The allelic discrimination step measures fluorescence in each well of the qPCR-plate before and after qPCR amplification, and places each sample in an X-Y graph according to these results. The *M. fructicola* samples are placed on a line parallel to the Y-axis on top of the vertex formed by the negative controls while the *M. fructigena*/ *M. laxa* samples are placed on a line parallel to the X-axis and to the

right of the vertex formed by the negative controls, and the mixtures of *M. fructicola* and *M. fructigena*/*M. laxa* appear between both axes with the bisectrix indicating mixtures of equal amounts.

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

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 -20 °C for 48 h to induce tissue senescence. The senescent fruit and flowers were transferred to plates in

humidity chambers (as previously described) and incubated at 25 °C and 100 % RH for 1 week in the dark. At the end of the incubation, the fruit and the flowers were examined for visible signs of a *Monilinia* infection and the number of rotten plant parts was recorded for each *Monilinia* isolate. The frequencies of latently infected flowers and fruit that were detected by ONFIT and qPCR were compared.

3.5.5. 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 flower 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) was used to statistically analyse the data. Because some of the qPCR results were negative for all technical replicates within a sample, heterogeneity of variance among treatments was detected. To compensate the lack of homogeneity of variance, the Kruskal-Wallis test was used to compare the medians of the amount of *Monilinia* DNA detected in the flowers and fruit with a latent infection. When a result of the Kruskal-Wallis test was significant ($P < 0.05$), the group medians of the samples were compared.

3.6. PROFICIENCY OF A LATENT *Monilinia* spp. INFECTION DETECTION BY REAL-TIME PCR IN NECTARINE FLOWERS AND FRUIT

3.6.1. Design of the study

The qPCR-based method proposed by Garcia-Benitez et al. (2017b) for the detection of *Monilinia* spp. latent infections in nectarine fruit and flowers was tested across five different laboratories in five different countries (France, Italy, Lithuania, Spain, and Turkey). Each laboratory analyzed 10 identical blinded samples following the working protocols and data collection sheets provided. The ring test was carried between Sept. 2015 and Sept. 2016 from sample preparation to data statistical analysis and final report.

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 working-protocol and a data-sheet to record the results were sent by e-mail to the different laboratories.

3.6.2. Sample preparation

Shipped samples contained DNA from: uninfected nectarine fruit, uninfected nectarine flower, nectarine fruit with a latent infection by *M. fructicola*, nectarine flower with a latent infection by *M. fructicola*, nectarine fruit with a latent infection by *M. laxa*, nectarine flower with a latent infection by *M. laxa*, *M. fructicola* mycelia, *M. laxa* mycelia, and a mixture of *M. fructicola* and *M. laxa* mycelia.

Samples were designed to give both low and high C_q values (17 and 35 respectively). Latent infections on flowers and fruit 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 min in a 1 % sodium hypochlorite solution, immersion for 1 min in a 70 % ethanol solution, two one-minute washes in sterile distilled water (SDW), and drying for 2 h in a laminar flow hood (Sauer and Burroughs, 1986). After drying, nectarine flowers were inoculated with a 30 μ L 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 s of either a *M. fructicola* or a *M. laxa* isolate. After inoculation, the flowers and fruit were incubated for 24 h in humidity chambers at 25 °C and 100 % relative humidity (RH) in the dark. At the end of the incubation period, they were subjected to a second surface-disinfection (previously described) and then the flowers and fruit were incubated at 4 °C and 100 % RH in the dark for 5 days.

Genomic DNA from whole lyophilized flowers or 20 mg of pooled-lyophilized-fruit-epidermis (1 mm thick), was extracted using the DNeasy® Plant Mini Kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's instructions. The DNA from the flowers was eluted to a final volume of 100 μ L, and the DNA from the fruit was eluted to a final volume of 50 μ L. 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, 18 μ L aliquots were prepared and then lyophilized in a laboratory freeze dryer (Cryodos -50, Azbil Telstar Technologies, SLU, Terrassa, Spain).

3.6.3. Other items supplied by the organizer

The reagents needed for the qPCR assay included: nuclease free-water, 2 x GoTaq® probe qPCR Master Mix (Promega Corporation, Madison, WI), Mon139F and Mon139R primers, and P_fc and

P2_fgn/lx/ps hydrolysis probes (van Brouwershaven et al., 2010) obtained from Integrated DNA Technologies Inc. (Coralville, IA). The hydrolysis probes were labelled with different reporters and quenchers than those used by van Brouwershaven et al. (2010). 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*, *M. laxa*, and *M. polystroma* probe (P2_fgn/lx/ps).

3.6.4. qPCR conditions

Genomic DNA from the samples (10 ng) 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 platform of each laboratory (Applied Biosystems® 7500 Fast Real-Time PCR (Thermo Fisher Scientific, Waltham, MA), CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., California), LightCycler® 480 Real-Time PCR System (F. Hoffmann-La Roche AG, Basel, Switzerland), Mastercycler® RealPlex² (Eppendorf AG, Hamburg, Germany), and Rotor-Gene Q (Qiagen GmbH, Hilden, Germany)) under the following conditions: polymerase activation at 95 °C for 10 min, followed by 40 amplification cycles at 95 °C for 15 s and 60 °C for 1 min. Emission was measured at the annealing-extension step. The threshold value was set at a fluorescence (ΔR_n) of 23,000 or automatically for those qPCR platforms for which that value was too high. A quantification threshold (C_q) value below 40 was scored as a positive detection. Additionally, due to cross-detection of *M. laxa* when using the P_fc probe detected in initial testing an allelic discrimination step was added when the real-time PCR platform permitted it, to distinguish between *M. fructicola* and *M. laxa* isolates, and to identify a mixture of the *Monilinia* isolates.

3.6.5. Data collection and analysis

Collaborating laboratories were asked to record the C_q value and the standard deviation of each sample with each hydrolysis probe in the results-data-sheet and send it with the raw data to the scheme provider. 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. The results were transformed into qualitative results, detection ($C_q < 40$) and negative detection (C_q undetermined) to compare between laboratories and techniques.

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

- False positive rate is the number of misclassified known positive samples over the total number of known positive samples.
- False negative rate is the number of misclassified known negative samples over the total number of known negative samples.

The qualitative results of the qPCR-method were compared against those obtained with the ONFIT-method, following the EPPO bulletin (2014). This method normally is used to compare a new method against a validated one. Even though the ONFIT method is not validated, it is routinely used in the laboratories for latent infection detection. The comparison of both methods is based in the positive agreement (P_a), negative agreement (N_a), positive deviation (P_d), and negative deviation (N_d) between results; and the studied parameters were as follows:

- Relative accuracy (A) of the method, which represents the correlation between the results obtained with ONFIT and those obtained with qPCR. It is calculated by $A = (P_a + N_a) / (P_a + P_d + N_d + N_a)$.
- Diagnostic specificity (S_p) of the method, which provides an estimation of the ability of the qPCR not to detect the target when it is not detected by ONFIT. It is calculated by $S_p = N_a / (N_a + P_d)$.
- Diagnostic sensitivity (S_e) of the method, which provides an estimation of the ability of the qPCR to detect the target when it is detected by ONFIT. It is calculated by $S_e = P_a / (P_a + N_d)$.

To assess the proficiency of the method, “The International Harmonized Protocol for the proficiency testing of analytical chemistry laboratories” (IUPAC Technical Report) (Thompson et al., 2006) was followed. 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 were used as σ_p .

3.7. *Monilinia* spp. LATENT INFECTION ACTIVATION DURING POSTHARVEST HANDLING CONDITIONS IN NECTARINES

3.7.1. Fruit samples

The investigation was conducted during 3 years, from 2012 to 2014. Each year nectarines of equivalent phenological status (BBCH = 89, at harvest; BBCH: general scale from Biologische Bundesanstalt, Bundessortenamt and Chemische Industrie, Germany (Meier et al., 1994)) and without apparent brown rot symptoms were harvested. A different cultivar was used each year: ‘Autumn Free’ nectarines in 2012, ‘Red Jim’ nectarines in 2013 and ‘Alba Red’ nectarines in 2014; nectarines were harvested from orchards located in Sudanell (2012 and 2013) and Ivars de Noguera (2014), Lerida (Spain). At the end of the assay the *Monilinia* spp. infections were identified morphologically (De Cal and Melgarejo, 1999) and when in doubt by PCR (Gell et al., 2007).

3.7.2. Brown rot and latent infection incidence after harvest

Brown rot incidence was determined each year by placing 30 nectarines in a humidity chamber that was lined with sterilized moist filter paper and incubating them at 25 °C under fluorescent lighting ($100 \mu\text{E m}^{-2} \text{s}^{-1}$ with a 16 h photoperiod) for 7 days. At the end of the incubation period the incidence of brown rot was calculated as the number of *Monilinia* spp. infected nectarines over the total number of nectarines in percentage.

Initial latent infection incidence was calculated each year by placing 30 surface-disinfected-frozen-nectarines (see below) in a humidity chamber that was lined with sterilized moist filter paper and incubating them at 25 °C under fluorescent lighting ($100 \mu\text{E m}^{-2} \text{s}^{-1}$ with a 16 h photoperiod) for 7 days.

At the end of the incubation period the incidence of latent infection was calculated as the number of *Monilinia* spp. infected nectarines over the total number of nectarines in percentage.

3.7.3. Latent infections after postharvest process

Upon reception on the lab, 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 placed in sterile paper bags and frozen at -20 °C for 48 h to damage the epidermis and favor latent infection detection (Luo and Michailides, 2003). After freezing nectarines were placed over sterile Petri dishes on sterile trays with sterile humidified filter paper at the bottom. Trays of nectarines were then randomly selected and subjected to different postharvest treatments. Postharvest treatments combined: (i) fruit dump in water, (ii) different durations of cold storage, and iii) different break durations during cold storage for fruit confection. Breaks during cold storage were only studied in 2012. Nectarines with activated latent infections were recorded and removed daily in order to prevent secondary infections during their incubation at 25 °C.

In the 2012 assay, 196 healthy nectarines were subjected to surface-disinfection, and frozen for 48 h. The nectarines were then divided in 32 treatments (6 nectarines per treatment). The variables analyzed were: (i) days of cold storage at 4 °C in the reception (0, 1, or 3 days) and in the expedition chamber (0, 1, or 10 days), (ii) dump in water (yes or no), and (iii) hours of break during cold storage for fruit confection (0, 1, or 6 h) both at 15 and 25 °C. All treatments were then placed at 25 °C during 5 days (Fig. 15). All the treatments were done at 100 % RH. Controls were nectarines placed in the combination with 0 days of cold storage at 4 °C in the reception and expedition chambers, without dump in water and 0 h of break during cold storage for fruit confection.

After the results of the year 2012 assay, a reduced set of postharvest handling combinations were selected for the assays carried out on the years 2013 and 2014 with 600 nectarines each year. Nine different combinations (Table 8) of days of cold storage on the reception chamber at 4 °C (0, 1, or 3 days), dump in water, and days of cold storage in the expedition chamber at 4 °C (0, 3, or 10 days) were studied both at 75 % RH and 100 % RH (Fig. 15). After their treatment nectarines were incubated at 25 °C under fluorescent lighting ($100 \mu\text{E m}^{-2} \text{s}^{-1}$ with a 16 h photoperiod) until the total postharvest period reached 20 days (Fig. 15). Two groups of nectarines were incubated constantly at 25 °C under fluorescent lighting ($100 \mu\text{E m}^{-2} \text{s}^{-1}$ with a 16 h photoperiod), one at 75 % RH and another at 100 % RH. Data from these groups after 5 days of incubation was used as a baseline control to compare latent infection activation rates. Chamber relative humidity was checked using an OM73 Temperature/humidity data logger (OMEGA®, Stamford, Connecticut). Each treatment and control had 3 replicates of 10 nectarines in 2013 and 6 replicates of 5 nectarines in 2014.

Fig. 15. Flow chart of the different postharvest treatments carried out in this study (^a only 2012 assay, and ^b only 2013/2014 assays).

Table 8. Different combinations of storage duration on the reception and expedition chambers, dump in water, and display in market duration, in the different postharvest treatments studied on 2013 and 2014.

Treatment	Storage duration in the reception chamber at 4 °C (days)	Dump in water	Storage duration on the expedition chamber at 4 °C (days)	Display in markets at 25 °C duration (days)
Control	0	No	0	5
1	0	Yes	0	5
2	0	Yes	3	5
3	0	Yes	10	5
4	1	Yes	0	5
5	1	Yes	3	5
6	1	Yes	10	5
7	3	Yes	0	5
8	3	Yes	3	5
9	3	Yes	10	5

3.7.4. Data analysis

Latent infection activation rates were calculated with the following equation $r = (\text{Nap Log } [100 / (100 - ILI_T)] - \text{Nap Log } [100 / (100 - ILI_C)]) / (t_T - t_C)$, where ILI_C is the control treatment incidence of latent infection at the time t_C and $ILIT$ is the incidence of latent infection each treatment at a later time t_T . Median latent infection activation rates were compared by Krustal-Wallis test with the statistical software Statgraphics Centurion XVI for Windows, Version 16.1.03 (StatPoint Technologies, Inc., Herndon, VA) to determine significance of the different parameters: cold storage in the reception chamber duration, fruit dump in water, cold storage in the expedition chamber duration, and RH. Analysis of the relation between postharvest processes studied in 2012 (accumulated number of cold storage days, break of cold storage for fruit confection, and dump in water of fruit) and the activation of infections was carried out using principal components analysis (PCA) with the statistical software Statgraphics Centurion XVI.

4. RESULTS AND DISCUSSION

*EPIDEMIOLOGÍA Y CONTROL DE LA PODREDUMBRE PARDA DEL
MELOCOTÓN EN POSTCOSECHA*

4.1. EFFECT OF THE MATURITY STATE AND POSTHARVEST ENVIRONMENTAL CONDITIONS OVER THE PRE-PENETRATION STAGES OF *Monilinia* spp.

4.1.1. Results

4.1.1.1. The effects of temperature, RH, and fruit maturity on germ tube and appressorial formation

M. fructicola and *M. laxa* conidia emitted germ tubes and/or produced appressoria at different temperatures and RHs. The greatest effects of temperature, RH, and fruit maturity on germ tube and appressorial formation by *M. fructicola* and *M. laxa* isolates were seen after a 48 h incubation period (Fig. 16).

The number of germ tubes and appressoria were the same when *M. fructicola* conidia were incubated at 4 °C for 48 h at 60 % and 80 % RH (Fig. 16A and B). The number of germ tubes was greater than the number of appressoria when *M. fructicola* conidia were incubated at 10 or 25 °C for 48 h at all tested RHs (Fig. 16A and B). For *M. laxa* the number of germ tubes was bigger than the number of appressoria at all tested temperatures, except when the conidia were incubated in culture medium which contained a skin extract of immature peaches at 10 °C for 48 h at 80 % or 100 % RH, and at 25 °C for 48 h at 60 % RH (Fig. 16C and D). Maximum germ tube formation occurred for both species at 25 °C and 100 % RH when the conidia were incubated in culture medium that contained a skin extracts from either mature or immature peaches (Fig. 16A and C). Little or no germ tube formation by *M. fructicola* conidia was recorded for 48 h of incubation at (a) 4 °C and 60 % or 80 % RH, (b) 10 °C and 60 % RH, and (c) 35 °C and 60 % RH (Fig. 16A). Little or no germ tube formation by *M. laxa* conidia was recorded at (a) 4 °C and 60 % RH, and (b) 35 °C at all tested RHs (Fig. 16C).

Fig. 16. Germ tube (A and C) and appressorial (B and D) formation by *M. fructicola* (A and B) and *M. laxa* (C and D) in culture medium which contained a skin extract of immature (■) or mature (□) peaches after a 48 h incubation period at different temperatures (4, 10, 25, and 35 °C) and relative humidities (60 %, 80 %, and 100 %). Data on the number of germ tubes and appressoria are displayed as a mean percentage of 50 randomly selected conidia of each of the three *M. fructicola* and *M. laxa* isolates. Groups of columns with an asterisk (*) represent statistically significant differences at a 95 % confidence level according to the results of the Student-Newman-Keuls multiple range test.

When *M. fructicola* and *M. laxa* conidia were incubated in culture medium that contained a skin extract of mature peaches, the number of germ tubes was greater than that formed when the conidia of both species were incubated in culture medium that contained a skin extract of immature peaches (Fig. 16A and C). Specifically, statistically significant differences in the number of germ tubes were found after

a 48 h incubation at (a) 4 °C and 100 % RH, (b) 10 °C and 80 % RH, and (c) 25 °C and 80 % RH for *M. fructicola* (Fig. 16A), and at (a) 4 °C and 80 % or 100 % RH, (b) 10 °C and 80 % or 100 % RH, and (c) 25 °C and 60 % RH for *M. laxa* (Fig. 16C).

We found that the numbers of appressoria that were produced by *M. fructicola* and *M. laxa* conidia (Fig. 16B and D) were lower than the numbers of germ tubes that were emitted by *M. fructicola* and *M. laxa* conidia (Fig. 16A and C). We also found that the number of appressoria that were produced by *M. laxa* was bigger than that for *M. fructicola* conidia at (a) 10 °C for 48 h at 80 % and 100 % RH, and (b) 25 °C for 48 h at 60 % RH (Fig. 16B and D). The maximum number of appressoria formation that were produced by *M. fructicola* and *M. laxa* occurred when the temperature and RH were not optimal for germ tube formation, namely at 10 °C for 48 h at 60 % RH (Fig. 16). The number of appressoria that was produced by *M. fructicola* conidia was significantly bigger when the conidia were incubated in culture medium which contained a skin extract of immature peaches than that which contained a skin extract of mature peaches. Specifically, this difference was found after a 48 h incubation at (a) 10 °C and 100 % RH and (b) 25 °C and 60 % RH (Fig. 16B). The number of appressoria that were produced by *M. laxa* conidia when the conidia were incubated in culture medium which contained a skin extract of immature peaches was significantly bigger than when the conidia were incubated in culture medium which contained a skin extract of mature peaches. Specifically, this difference was found at (a) 10 °C and 100 % RH and (b) 35 °C and 80 % RH (Fig. 16D).

4.1.1.2. Linear regression models of germ tube formation by *M. fructicola* and *M. laxa*

The linear regression models for germ tube formation by *Monilinia* spp. at different temperatures (T), RHs, and incubation periods (it) for the two types of skin extracts and the two fungal species are shown on Table 9. Linear regression models for appressorial formation were not generated.

4. RESULTS AND DISCUSSION

Table 9. Linear regression models which describe the relationship between temperature (T), relative humidity (RH) and incubation period (it) and germ tube formation (y) by *M. fructicola* and *M. laxa* conidia that were incubated in culture medium which contained a skin extract of either immature or mature peaches.

<i>Monilinia</i> specie	Fruit extract maturity stage	Equation	R ² values
<i>M. fructicola</i>	Immature peach	$y = -45.213 + 0.381 \text{ RH} + 0.099 \text{ Tit} + 0.211 \text{ T}^2 - 0.003 \text{ T}^2 \text{it} - 0.006 \text{ T}^3$	0.62
	Mature peach	$y = -50.206 + 0.490 \text{ RH} + 0.119 \text{ Tit} + 0.202 \text{ T}^2 - 0.003 \text{ T}^2 \text{it} - 0.005 \text{ T}^3$	0.64
<i>M. laxa</i>	Immature peach	$y = -39.638 + 0.407 \text{ RH} + 0.109 \text{ Tit} + 0.139 \text{ T}^2 - 0.003 \text{ T}^2 \text{it} - 0.004 \text{ T}^3$	0.55
	Mature peach	$y = 5.340 - 2.110 \text{ it} + 0.213 \text{ Tit} + 0.025 \text{ RHit} - 0.005 \text{ T}^2 \text{it} - 0.0006 \text{ TRHit}$	0.61

The regression models were obtained from the number of germ tubes that were formed by 50 randomly selected conidia of each of the three *M. fructicola* and *M. laxa* isolates.

The parameters of the equations are given in the following units: temperature (°C), RH (%), and incubation period (h).

4.1.1.3. Effects of the environmental conditions of harvested fruit on germ tube and appressorial formation by the *M. fructicola* (Mf1) and *M. laxa* (Ml1) isolates

Tables Table 10 and Table 11 summarize the effects of the environmental conditions of harvested fruit on germ tube and appressorial formation by the Mf1 and Ml1 isolates. Although Mf1 and Ml1 conidia were able to germinate in Czapek-Dox broth under all simulated refrigerated conditions, the number of germ tubes that were formed by Ml1 conidia was significantly bigger than that for Mf1 conidia when the conidia were incubated at 100 % RH (Table 10). Negligible or no germination of Mf1 and Ml1 conidia occurred when the RH was 60 % (Table 10). We also found that (a) the number of germ tubes that were formed by Ml1 conidia increased after 3 days of refrigeration when the RH was 100 % and (b) reached its maximum after 10 days of cold storage (Table 10). The numbers of appressoria produced by Mf1 and Ml1 at an RH of 100 % were bigger than the number that were produced by these two isolates at an RH of 60 %. The number of appressoria that were formed by Mf1 increased after 3 consecutive days of refrigeration for each cold storage duration (Table 11), and the numbers of appressoria were always bigger than those for Ml1.

Table 10. The effect of refrigeration and cold storage on germ tube formation by *Monilinia fructicola* and *M. laxa* isolates.

Specie	RH (%)	Duration of refrigeration before processing (days)	Duration of cold storage after processing (days)		
			0	3	10
<i>M. fructicola</i>	60	0	2.0 a	2.0 a	1.3 ab
		1	2.0 a	2.7 a	0.0 a
		3	0.7 a	1.3 a	0.0 a
	100	0	2.0 a	0.7 a	3.3 ab
		1	0.0 a	2.0 a	0.0 a
		3	2.0 a	1.3 a	1.3 ab
<i>M. laxa</i>	60	0	0.0 a	0.7 a	6.7 ab
		1	0.7 a	0.7 a	9.3 b
		3	0.7 a	3.3 a	2.0 ab
	100	0	0.0 a	53.3 b	72.0 c
		1	0.7 a	64.0 bc	87.3 d
		3	60.0 b	73.3 c	87.3 d
MSE ^Z			(27.5)	(31.5)	(28.2)

The two postharvest environmental conditions that were tested are refrigeration for 0, 1, or 3 days before fruit processing and cold storage for 0, 3, or 10 days at 4 °C in the dark at a relative humidity (RH) of either 60 % or 100 %.

Data on the number of germ tubes are expressed as a percentage of 50 randomly selected conidia of one *Monilinia fructicola* isolate (Mf1) or one *Monilinia laxa* isolate (Ml1) (Table 1) which formed a germ tube and are the mean of three replicates. Experiments were repeated at least twice.

^y Means with the same letter in each column are not significantly different from each other according to the results of the Student-Newman-Keuls multiple range test ($P < 0.05$). Data were arcsine transformed before analysis.

^z MSE = mean square error

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Table 11. The effect of refrigeration and cold storage on appressorial formation by *Monilinia fructicola* and *M. laxa* isolates.

Specie	RH (%)	Duration of refrigeration before processing (days)	Duration of cold storage after processing (days)		
			0	3	10
<i>M. fructicola</i>	60	0	0.0 a	0.0 a	1.3 a
		1	0.7 a	2.0 a	4.0 a
		3	0.0 a	4.7 ab	5.3 a
	100	0	0.0 a	2.0 a	38.0 c
		1	2.0 a	10.7 ab	33.0 c
		3	13.3 c	30.0 c	70.0 d
<i>M. laxa</i>	60	0	0.0 a	0.0 a	0.0 a
		1	0.0 a	0.0 a	0.0 a
		3	0.0 a	0.0 a	0.0 a
	100	0	0.0 a	9.3 ab	24.0 b
		1	0.0 a	4.0 ab	11.3 a
		3	8.0 b	18.0 b	11.3 a
MSE ^z			(1.8)	(11.8)	(8.6)

The two postharvest environmental conditions that were tested are refrigeration for 0, 1, or 3 days before fruit processing and cold storage for 0, 3, or 10 days at 4 °C in the dark at a relative humidity (RH) of either 60 % or 100 %.

Data on the number of appressoria are expressed as a mean percentage of 50 randomly selected conidia of one *Monilinia fructicola* isolate (Mf1) or one *Monilinia laxa* isolate (Ml1) (Table 1) which formed an appressorium and are the mean of three replicates. Experiments were repeated at least twice.

^y Means with the same letter in each column are not significantly different from each other according to the results of the Student-Newman-Keuls multiple range test ($P < 0.05$). Data were arcsine transformed before analysis.

^z MSE = mean square error

4.1.2. Discussion

In this investigation, we found that germ tube and appressorial formation by *Monilinia* spp. are influenced by temperature, RH, and the maturity stage of the fruit. The biggest number of germ tubes was found when *M. fructicola* and *M. laxa* conidia were incubated in culture medium which contained a skin extract of mature peaches. These finding is different to that for appressorial formation: the biggest number of appressoria was found when *M. fructicola* and *M. laxa* conidia were incubated in culture medium which contained a skin extract of immature peaches. Although the optimal environmental conditions for germ tube formation (25 °C and 80 to 100 % HR) by *M. fructicola* and *M. laxa* are similar, we found that the number of germ tubes that are formed by *M. fructicola* conidia was greater than that for *M. laxa* conidia under these conditions. Casals et al. (2010a) reported that the maximum germination of *M. laxa* and *M. fructicola* conidia occurred when temperature was 25 °C and RH was 99 %. However, no conidial germination was recorded by these authors when RH was lower than 87 %. Our results are in agreement with those of Papavasileiou et al. (2015) who found that maximum germination of *M. laxa* and *M. fructicola* conidia occurred when the isolates were grown on PDA and the temperature was between 10 and 25 °C. Tamm and Flückiger (1993) have also reported that maximum germination of *M. laxa* conidia occurred when temperature was between 15 and 25 °C and RH was 100 %.

We found that (a) *M. laxa* conidia emits germ tubes when the temperature is 4 °C and the RH is between 80 % and 100 %, and (b) *M. fructicola* conidia are not able to emit germ tubes under these conditions. These results are in accordance with those that were reported by Tamm and Flückiger (1993) and Tian and Bertolini (1999), who found that *M. laxa* conidia are able to germinate when the temperature is as low as -4 °C. The ability of *M. laxa* conidia to germinate at low temperatures has also been reported by Watson et al. (2002). Casals et al. (2010a) and Papavasileiou et al. (2015) reported that *M. fructicola* conidia can germinate at low temperatures, namely 0 and 5 °C. Papavasileiou et al. (2015) measured

conidial germination of five different *M. fructicola* and *M. laxa* isolates, 6 days after their plating on PDA. They found that (a) germination of the conidia of the two *Monilinia* species was greater than 70 %, and (b) mycelial growth of the *M. laxa* isolates was greater than that of the *M. fructicola* isolates after a 6 day incubation period at 5 °C. Accordingly, we surmise that our short incubation periods could account for higher levels of germination of *M. laxa* conidia than *M. fructicola* conidia at low temperatures.

We found that no or little germ tube and appressorial formation occurred at 35 °C for both *Monilinia* spp. These results were similar to those of Papavasileiou et al. (2015) who reported that the conidia of five *M. laxa* isolates and five *M. fructicola* isolates did not germinate when they were grown on PDA at 35 °C. However, Casals et al. (2010a) reported that the germination of conidia of three different *Monilinia* isolates, an *M. laxa* isolate, an *M. fructigena* isolate, and an *M. fructicola* isolate, occurred at 35 °C, but not at 38 °C. A possible reason for the difference between these three sets of results could be due to the use of only one *Monilinia* isolate of each species.

We found that the environmental conditions of harvested fruit before, during, and after their processing did not prevent germ tube and appressorial formation by *M. fructicola* and *M. laxa*. High RH in the refrigeration chambers is required to maintain fruit quality. We found that a high RH increased the germ tube formation by *M. laxa* and *M. fructicola* conidia, and that this increase in germ tube formation at a high RH was very evident for *M. laxa*. The ambient temperature in washing and sorting units is between 15 and 25 °C and the washing and sorting of harvested fruit takes between 1 and 6 h. We found that a cold stop in fruit refrigeration could reduce appressorial formation by *M. fructicola* conidia, which needs 3 consecutive days of refrigeration or cold storage.

Germ tube formation by *M. laxa* and appressorial formation by *M. fructicola* occurs during the first stage of the infection process. We found that appressorial formation by *M. fructicola* increased when the optimal temperatures and/or RHs for conidial germination were not present. These results differ

from those on appressorial formation by other phytopathogenic ascomycetes, such as *Colletotrichum acutatum*. In this species, the formation of melanised appressoria decreased when the optimal temperatures and/or RHs for conidial germination were not present (Miles et al., 2013). This difference between the results of our study and those from Miles' study suggests that pathogenic *C. acutatum* requires an appressorium to penetrate its hosts, and that *M. fructicola* need an appressorium to penetrate fruit only when the environmental conditions are unfavorable. Lee and Bostock (2006b) proposed that the appressoria of *M. fructicola* facilitates its penetration into immature fruit and could even be a resting or a non-pathogenic phase of the pathogen when unfavorable environmental conditions for infection were present. We also found that the rate of appressorial formation by *M. laxa* was higher than that of *M. fructicola* when we investigated the effects of temperature, RH, and fruit maturity on appressorial formation. Although we did not detect such a difference when we investigated the effects of the environmental conditions of harvested fruit on *M. laxa* appressorial formation before, during, and after fruit processing, we attribute this difference in the rates of appressorial formation to the utilization of the nutrients in the Czapeck-Dox broth by *M. laxa* and *M. fructicola* conidia. Lee and Bostock (2006b) reported that the composition of nutrient media, such as CV8 medium, can induce the germination of already-formed appressoria.

When our results are appraised together with those of Papavasileiou et al. (2015), we concluded that *M. laxa* and *M. fructicola* have their own competitive edge for conidial germination and mycelial growth in different environmental conditions. We also surmise that the competitive edge of each species in different environmental conditions accounts for the coexistence of *M. laxa* and *M. fructicola* in Spanish peach and nectarine orchards. This competitive edge becomes advantageous for *M. fructicola* when temperature is between 10 and 25 °C and when temperature is between 4 and 10 °C for *M. laxa*. These individual competitive advantages could explain (a) the incomplete and slow supplanting of *M. laxa* by *M. fructicola*, even though *M. fructicola* is more aggressive and grows faster

than *M. laxa* under optimum growing conditions (25 °C and 100 % RH) (Villarino et al., 2016b), and (b) the different adaptabilities to postharvest conditions of *M. fructicola* and *M. laxa*.

Accordingly, we suggest that the incidence of brown rot in harvested peaches could be lowered by lessening the period between fruit arrival times and their processing at fruit processing units, reducing RH in the refrigeration chambers, and shortening the duration of cold storage. Incorporating these measures and practices into the modus operandi of the handling of harvested peaches should create the environmental conditions that constrain conidial germination of *Monilinia* spp. Reducing RH in the refrigeration chambers could potentially reduce peach quality by causing fruit dehydration. Hence, further investigations are needed to determine the optimum RH for reducing conidial germination of *Monilinia* spp. without adversely affecting fruit quality.

4.2. ISOLATION AND PARTIAL CHARACTERIZATION OF PHYTOTOXINS AND DETERMINATION OF THE DEGRADING ENZYMATIC ACTIVITIES PRODUCED BY *Monilinia* spp.

4.2.1. Results

4.2.1.1. Toxin characterization

No toxin activity was detected in any of the three *Monilinia* species in n-hexane and dichloromethane fraction, neither in *M. fructigena* nor in *M. laxa* ethyl acetate fractions (Table 12).

Table 12. Evaluation of toxicity of culture filtrates to nectarine circles separated using linear separation systems.

Fraction	Response of Toxicity			
	<i>M. fructicola</i>	<i>M. fructigena</i>	<i>M. laxa</i>	Control
Butanol	+	+	+	-
Ethyl Acetate	+	-	-	-
Dichloromethane	-	-	-	-
n-Hexane	-	-	-	-
Distilled Water	-	-	-	-

^a the “+” signs indicates that symptoms were produced, and the “-“ indicates that no symptoms were developed.

Based on differences in the profiles of blank samples and the organic extract chromatograms with a positive response (Fig. 17), various fractions were selected to be collected: 29 fractions of ethyl acetate from juice of nectarines inoculated with *M. fructicola*, 14 fractions of butanol from juice of nectarines inoculated with *M. fructigena*, and 15 fractions of butanol from juice of nectarines inoculated with *M. laxa*. In parallel fractions at the same retention time intervals of juice from uninoculated nectarines of the ethyl acetate and butanol extracts were collected (Fig. 18), to compare their necrotic areas on nectarine circles.

Fig. 17. HPLC chromatograms of ethyl acetate fraction from juice of nectarine inoculated with: *M. fructicola* (A), and of butanol fraction from juice of nectarine inoculated with *M. fructigena* (B) and *M. laxa* (C).

Fig. 18. HPLC chromatograms of ethyl acetate fraction from juice of uninoculated nectarine (A), and of butanol fraction from juice of uninoculated nectarines (B) and (C).

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Only one out of the total number of fractions of each *Monilinia* spp. inoculated nectarine juice produced toxic activity (Fig. 19).

Fig. 19. Comparison between necrotic areas formed by the collected fractions from juice of nectarines inoculated with *M. fruticicola* (A), *M. fructigena* (B), or *M. laxa* (C), and those formed by the collected fractions from juice of uninoculated nectarines.

The collected fraction that produced toxic activity had a retention time between 3.5 and 4.5 min in the three species. Even though each *Monilinia* specie had slightly different peaks in this region, all chromatograms presented a peak at a retention time of approximately 4 min (Fig. 20). Phytotoxic metabolites in this retention time were analyzed with the mass spectrometer and presented a molecular weight between 329 and 387 g mol⁻¹.

Fig. 20. Detailed amplification between 3.5 and 4.5 min retention times of the HPLC chromatograms of ethyl acetate fraction from juice of nectarine inoculated with: *M. fructicola* (A), and of butanol fraction from juice of nectarine inoculated with *M. fructigena* (B) and *M. laxa* (C).

The symptoms produced on the nectarine circles by the collected fractions with phytotoxic activity of all three *Monilinia* species were similar to those produced by the fungus after 24 h of incubation (Fig. 21). They all produced a necrotic area around the inoculation point (Fig. 21A, B and, C); while the fractions from the blanks did not produce symptoms (Fig. 21D, E, and F).

Fig. 21. Arrows point towards the perimeter of the necrotic areas produced on a wounded nectarine circle by the fraction of ethyl acetate from juice of nectarine inoculated with *M. fructicola* (A), and of butanol from the juice of nectarines inoculated with *M. fructigena* (B) and *M. laxa* (C) after 2 days of incubation at 25 °C. Their controls did not present any apparent symptoms after 2 days of incubation at 25 °C (D, E, and F). Pictures A, B, D, and E are at 1.6 x while pictures C and F are at 2.5 x).

4.2.1.2. Enzymatic activity determination

The growth of *M. fructicola*, *M. fructigena*, and *M. laxa* isolates on different amended yeast extract-agar media can be seen in Fig. 22. In the minimal medium without adding any substrate *M. fructicola*, *M. fructigena*, and *M. laxa* isolates had high mycelial expansion (72.81, 84.38, and 86.67 % respectively), low mycelial density (39.17, 24.54, and 37.08 % respectively) and low sporulation (0.69, 0.00, and 0.00 % respectively) (Table 13). None of the three *Monilinia* species was able to utilize callose and methylcellulose as substrates, which seem to have a pernicious effect on mycelial

expansion, density, and sporulation since lower values were scored on the amended medium than those for the minimal medium without substrates (Table 13). Additionally *M. fructigena* isolates were not able to utilize cutin as a substrate since the values of mycelial expansion, density and sporulation on the cutin-amended minimal medium were statistically equal or lower than those from the minimal medium without substrate (Table 13).

Fig. 22. Mycelial expansion, density, and sporulation of some *M. fructicola*, *M. fructigena*, and *M. laxa* isolates grown on minimal medium (yeast extract), and minimal medium amended with xylan, peptin and cutin.

When *M. fructicola* isolates were grown on: casein, pectin, or xylan-amended minimal medium, its mycelial expansion, density and sporulation were higher than those produced on minimal medium (Table 13). Additionally, *M. fructicola* isolates had a higher mycelial density and sporulation when grown on minimal medium amended with sucrose and polygalacturonic acid than when grown on just minimal medium (Table 13). Finally, *M. fructicola* isolates had higher sporulation on minimal medium amended with cutin, than on minimal medium (Table 13). The increases in mycelial growth and sporulation indicate that *M. fructicola* isolates have cutinase, α -glucosidase, pectin-lyase, polygalacturonase, protease, and xylanase enzymatic activities.

When *M. fructigena* isolates were grown on minimal medium amended with: casein, pectin, polygalacturonic acid, sucrose, and xylan; their mycelial density values were higher than those obtained when *M. fructigena* isolates were grown on minimal medium without substrates (Table 13). Additionally, when *M. fructigena* isolates were grown on pectin amended minimal medium, their sporulation was higher than when isolates were grown on minimal medium (Table 13). Thus, *M. fructigena* isolates have α -glucosidase, pectin-lyase, polygalacturonase, protease, and xylanase enzymatic activities.

M. laxa isolates grown on minimal medium amended with: casein, cutin, pectin, or polygalacturonic acid had a higher mycelial density than when they were grown on minimal medium without any substrate (Table 13). Additionally *M. laxa* isolates had greater mycelial density and sporulation when they were grown on sucrose and greater mycelial expansion and density on xylan, than when they were grown on minimal medium (Table 13). The increases in mycelial growth and sporulation indicate that *M. laxa* isolates have cutinase, α -glucosidase, pectin-lyase, polygalacturonase, protease, and xylanase enzymatic activities.

Table 13. Mycelial growth, density and sporulation of the different *Monilinia* species grown on different amended minimum media.

<i>Monilinia</i> specie	Substrate	Mycelial expansion (%)	Mycelial density (%)	Sporulation (%)
<i>M. fructicola</i>	Without substrate	72.81 d	39.17 b	0.69 a
	Callose	39.17 b	0.83 a	0.00 a
	Casein	78.75 e	100.00 e	65.00 c
	Cutin	25.63 a	38.33 b	14.99 b
	Methylcellulose	66.09 d	0.00 a	0.00 a
	Pectin	97.92 g	83.33 d	53.89 c
	Polygalacturonic acid	54.99 c	53.33 c	51.07 c
	Sucrose	66.25 d	100.00 e	100.00 e
	Xylan	90.00 f	80.83 d	81.09 d
<i>M. fructigena</i>	Without substrate	84.38 e	24.54 b	0.00 a
	Callose	30.09 b	20.37 b	0.00 a
	Casein	87.04 e	83.33 e	0.00 a
	Cutin	25.49 a	34.31 bc	0.65 a
	Methylcellulose	75.00 d	0.00 a	0.00 a
	Pectin	90.69 e	58.82 d	2.94 b
	Polygalacturonic acid	51.10 c	41.66 c	0.00 a
	Sucrose	88.89 e	96.30 f	0.00 a
	Xylan	95.10 e	76.46 e	1.31 ab
<i>M. laxa</i>	Without substrate	86.67 ef	37.08 b	0.00 a
	Callose	24.58 a	0.00 a	0.00 a
	Casein	77.08 de	95.00 f	0.00 a
	Cutin	40.42 b	51.67 c	2.22 a
	Methyl cellulose	77.50 d	0.00 a	0.00 a
	Pectin	85.63 ef	83.75 e	1.11 a
	Polygalacturonic acid	59.42 c	61.61 c	0.00 a
	Sucrose	92.92 fg	100.00 f	7.78 b
	Xylan	95.63 g	70.83 d	0.84 a

^a letters indicate statistically significant differences between different amended mediums for each *Monilinia* species, according to the Student-Newman-Keuls test with a confident level of at least 95 %.

4.2.2. Discussion

Phytotoxins and degrading enzyme activities have been identified from *Monilinia* spp. isolates. The production of phytotoxins and degrading enzymes is extended throughout all necrotrophic fungi that used them to damage plant tissues and facilitate colonization. *Monilinia* closely related necrotrophic

ascomycetes such as *Botrytis cinerea* and *Sclerotinia sclerotiorum* produced phytotoxins and degrading enzymes (Choquer et al., 2007; Pedras and Ahiahonu, 2004; Rebordinos et al., 1996; Yeon et al., 2004).

Phytotoxic activity appeared in the butanol extracts of all *Monilinia* species and in the ethyl acetate extract of *M. fructicola*. Additionally, collected fractions with phytotoxic activity corresponded with the first collected fraction in the HPLC elution process, which is the one with the highest polarity. When comparing with other fungus toxic metabolites reported in the literature, the isolated toxin of *S. sclerotiorum*, sclerin, had a lower polarity than those of *Monilinia* since it could only be isolated in the ethyl acetate fraction and not in the butanol fraction (Pedras and Ahiahonu, 2004). *B. cinerea* toxin metabolites botrydial and botcinolide, and their derivatives were also extracted in the ethyl acetate fraction, having relatively less polarity than the toxins of *Monilinia* spp. (Reino et al., 2004).

The collected fractions with phytotoxic activity of the three *Monilinia* species have similar retention times between 3.5 and 4.5 min, indicating that toxic compounds could be the same. Compounds that were present in those collected fractions had a molecular weight between 329 and 387 g mol⁻¹. The toxins from other necrotrophic fungi have lower molecular weights than those estimated for the three *Monilinia* species. However, their molecular weights were similar to each other, sclerin 234.25 g mol⁻¹ (Pedras and Ahiahonu, 2004), botrydial and its derivatives have molecular weight ranging from 232 to 311 g mol⁻¹ (Collado et al., 1996), or helminthosporoside a host specific toxin of *Helminthosporium sacchari* that have a molecular weight of 236 g mol⁻¹ (Steiner and Strobel, 1971). All these toxins were mainly formed by carbon, hydrogen and oxygen and have a ring structure (Collado et al., 1996; Pedras and Ahiahonu, 2004; Steiner and Strobel, 1971). The molecular weight of *B. cinerea* second group of toxins botcinolide derives from the botcinic acid, which has a molecular weight of 402.84 g mol⁻¹ and a ramified structure (Dalmais et al., 2011; Reino et al., 2004), is similar to those estimated for *Monilinia* in the present study. Some secondary metabolites with antimicrobial and phytotoxic activities

produced by *M. fructicola* benomil-resistant strains, salicylaldehyde-type octaketides, named monilidiol (312 mol g⁻¹) and dechloromonilidiol (296 mol g⁻¹) had slightly lower molecular weights than the toxins described in the present study (Sassa et al., 1983). The higher molecular weight of *Monilinia* spp. toxins, with respect to sclerin, botrydial, helminstosporoside and their derivatives, shows that these metabolites are different compounds though their chemical structure could be similar. In order to elucidate the complete chemical structure of the toxins from *Monilinia* spp. additional studies such as quadrupole time-of-flight (Q-Tof) Mass Spectrometry (MS) and nuclear magnetic resonance (NMR) are required.

M. fructicola, *M. fructigena*, and *M. laxa* have α -glucosidase, pectin-lyase, polygalacturonase, protease, and xylanase enzymatic activities. Additionally *M. fructicola* and *M. laxa* have cutinase enzymatic activities. Most of these enzymatic activities correspond to cell wall-degrading enzymes, which are produced in a wide array by necrotrophic fungi and some are critical for virulence in phytopathogenic fungi (Reignault et al., 2008).

Cutinases are extracellular enzymes that degrades the protected cuticle of aerial plant organs composed of an insoluble polymeric structural compound, cutin, which is a polyester composed of hydroxy and hydroxyepoxy fatty acids (Ettinger et al., 1987). Cutin degradation facilitates fungus penetration through the cuticle, and its inhibition can prevent fungal infection through intact cuticles. *M. fructigena* was the only *Monilinia* studied that did not have cutinase activities on culture media. Additionally, is the less pathogenic of the three species (Villarino et al., 2016b) and it has been reported that *M. fructigena* requires wounding of sweet cherries and apples in order to infect them (Xu and Robinson, 2000; Xu et al., 2007). The lack of cutinases in *M. fructigena* can explain its lesser pathogenicity and its impossibility to infect intact cuticles of some fruit. It has been determined that *M. fructicola* has at least four cutinases (Wang et al., 2000), and the most abundant one MfCUT1 contributes to its virulence (Lee et al., 2010). Additionally, cutinases have been purified from a number of

phytopathogenic fungi, such as *Aternaria brassicicola* (Trail and Köller, 1990), *B. cinerea* (Gindro and Pezet, 1999), *Colletotrichum gloeosporioides* (Dickman et al., 1982), *Fusarium solani* f. sp. *pisi* (Li et al., 2002), *Magnaporthe grisea* (Sweigard et al., 1992), and *Venturia inaequalis* (Parker and Köller, 1998).

β -glucosidases are extracellular enzymes required to degrade cellulose or callose, polymers composed of beta-1,4-linked glucosyl residues. These enzymes are needed to degrade plant cell walls by pathogens and other organisms consuming plant biomass. In this study, we have not seen β -glucosidase enzymatic activity in any of the three *Monilinia* species grown on culture media, only α -glucosidase activities degrading sucrose. This finding is surprising, since related fungi such as *B. cinerea* (González-Fernández et al., 2014) and *S. sclerotiorum* (Riou et al., 1991; Waksman, 1988) both have β -glucosidases. However, not all the components of the cell wall have to be degraded in order to penetrate plant cells, which can explain this enzymatic deficit presented by *Monilinia* species.

Pectin is a major component of the middle lamella and primary cell walls and are particularly abundant in the non-woody parts of plants. Pectin consists of a complex set of polysaccharides rich in galacturonic acid. Pectin-lyases and polygalacturonases are two of the extracellular enzymes required to degrade this complex set of polysaccharides, of these two types of enzymes polygalacturonases had been studied in depth due to their relation with virulence. *M. fructicola* has 5 genes coding for endo-polygalacturonase enzymes, and a strain overexpressing MfPG1, the most highly expressed of them, produced smaller lesions and an increase in reactive oxygen species on the petals of peach and rose flowers, indicating a virulence function (Chou et al., 2015). The relative expression of MfPG2 and MfPG3 increased 12-fold and 6-fold, respectively, when the ambient pH was lowered from 4.5 to 3.6 suggesting the importance of ambient pH for the secretion of pathogenicity factors by *M. fructicola* (De Cal et al., 2013). In *B. cinerea* 6 different endo-polygalacturonases had been described all with

virulence function (Wubben et al., 2000) and in *S. sclerotiorum* 4 endo-polygalacturonases, also involved in virulence, had been described (Li et al., 2004).

Xylanases are the extracellular enzymes that degrade the linear polysaccharide beta-1,4-xylan into xylose, thus breaking down hemicellulose, one of the major components of plant cell walls. *M. fructicola*, *M. fructigena* and *M. laxa* had xylanases as do other related phytopathogenic fungi *B. cinerea* (González-Fernández et al., 2014) and *S. sclerotiorum* (Waksman, 1988).

Most of the studies concerning enzymatic activities were done in the late 1980s or early 1990s. But recently thanks to new technologies and molecular tools available: DNA sequencing, fungal transformation and proteomics techniques, a more in depth study of the extracellular degrading enzymes secreted by fungal pathogens have been done, opening a new avenue of enzymatic studies (Chou et al., 2015; González-Fernández et al., 2014; Lee et al., 2010; Li et al., 2004).

The present study serves as a first building block into the identification and characterization of the different phytotoxins and degrading enzymes present on *Monilinia* spp.

4.3. MICROSCOPIC ANALYSIS OF LATENT AND VISIBLE *M. fructicola* INFECTIONS IN NECTARINES

4.3.1. Results

4.3.1.1. Conidial germination and hyphal growth on the nectarine's epidermis

Fig. 23 contains representative light microscopic images of *M. fructicola* germination and hyphal development on the nectarine's epidermis after a 4 h and 24 h incubation at 25 °C or 4 °C. Clusters of *M. fructicola* conidia were observed around the guard cells of the stomata and some of these conidia

had germinated 4 h after inoculation when the nectarines were incubated at 25 °C (Fig. 23A and B). The germ tubes grew progressively into long hyphae on the surface between a 4 h, a 16 h (Fig. 23C and D), and a 24 h (Fig. 23E) incubation at 25 °C (Table 14), and the hyphae penetrated the surface mostly through stomata (Fig. 23B and F). A lower number of conidia were observed in areas without stomata (Fig. 23A and D). When the inoculated nectarines were incubated at 4 °C, thick ramified hyphae were observed on the surface after a 24 h incubation (Fig. 23F). Appressoria were not seen on the surface or in the tissues after a 4 h and 24 h incubation at 25 °C or 4 °C.

Fig. 23. Germination on and penetration of the surface of mature nectarines by *M. fructicola* conidia and hyphae. The nectarine's tissues and fungal structures were stained with lactophenol blue (A, B, E and F) or calcofluor white fluorescent stain (C and D). A and B: Clusters of non-germinated (blue) and germinated (arrow) *M. fructicola* conidia (c) around the guard cells of stomata (s) on the surface of mature fruit after a 4 h incubation period at 25 °C. C and D: Conidia and long germ tubes (h) on the surface of mature fruit after a 16 h incubation period at 25 °C. Scale bar = 50 µm. E: Long germ tubes

on the surface of mature fruit after a 24 h incubation period at 25 °C. F: thick ramified hyphae (rh) on the surface of mature fruit after a 24 h incubation period at 4 °C.

Table 14 summarizes the timeline of tissue changes in nectarines with a latent or a visible *M. fructicola* infection according to the results of the light microscopic and TEM analysis. For nectarines with visible brown rot, the signs of brown rot appeared within 48 h on the surface of infected nectarines as brown spots on the surface at the inoculation sites and then became smooth grey spots after 72 h. Thereafter, the disease developed over the surface and in the parenchyma. For nectarines with a latent infection, no signs of disease were seen on the nectarine's surface over the 288 h incubation period at 4 °C.

4. RESULTS AND DISCUSSION

Table 14. Timeline of tissue changes in nectarines with a visible and a latent *M. fructicola* infection

		Initial observations of the infection process 24 h after inoculation at 25 °C (t=0)	Times of appearance of signs of visible infection (hours after t=0, at 25 °C)			Times of appearance of signs of latent infection (hours after t=0, at 4 °C)			
			24	48	72	72	144	216	288
Visible signs of disease	<i>Epidermal discoloration</i>	-	+	+	+	-	-	-	-
	<i>Brown spots and brown rot</i>	-	+	+	+	-	-	-	-
	<i>Sporulation</i>	-	-	-	+	-	-	-	-
	<i>Diameter of macroscopic lesion (cm)</i>	-	1	2.5	5	-	-	-	-
Fungal infection structures and their location	<i>Conidial germination on fruit surface</i>	+	+	+	+	-	-	-	-
	<i>Germ tube growth</i>	+	+	+	+	-	-	-	-
	<i>Thin hyphae in the substomatal cavity</i>	+	+	+	+	+	+	+	+
	<i>Thin hyphae in the epidermis</i>	-	+	+	+	-	-	+	+
	<i>Thin hyphae in the mesocarp</i>	-	-	+	+	-	-	-	-
	<i>Thick hyphae in the epidermis and mesocarp</i>	-	-	-	+	-	-	-	-
	<i>Sporulation</i>	-	-	-	+	-	-	-	-
Light microscopic analysis	<i>Partial cuticular degradation</i>	-	+	+	+	-	-	-	-
	<i>Cell degradation in the substomatal cavity</i>	+	+	+	+	-	+	+	+
	<i>Collapse and disruption of epidermal cells</i>	-	+	+	+	-	-	+	+
	<i>Formation of lysogenic cavities at subepidermal level</i>	-	-	+	+	-	-	-	-
	<i>Collapse and disruption of mesocarpic cells</i>	-	-	+	+	-	-	-	-
	<i>Formation of lysogenic cavities at mesocarpic level</i>	-	-	+	+	-	-	-	-
	<i>Cellular degradation and cuticular breakdown</i>	-	-	-	+	-	-	-	-
Transmission electron microscopic analysis	<i>Germination and adherence of conidia to fruit surface</i>	+		+					
	<i>Thin intramural hyphae in epidermal cells; cell wall degradation</i>	+		+					
	<i>Thick intercellular and intracellular hyphae in mesocarpic cells; cell wall degradation and cell death</i>	-		+					

4.3.1.2. Light microscopic analysis of tissues from nectarines with a visible *M. fructicola* infection

Fig. 24A and B display representative light microscopic images of epidermal, subepidermal, and mesocarp cells from uninoculated nectarines and Fig. 24C through J display representative images of diseased tissue from nectarines with visible signs of a *M. fructicola* infection. In nectarines with visible infection, signs of the disease usually appeared after 24 h on the surface, preferentially in areas which surround stomata but they also appeared in stomata-free areas (Fig. 24C and D; Table 14). The brown rot became evident in the uppermost layers of epidermal cells below the cuticle and these epidermal cells began to collapse after 48 h (Fig. 24E and F; Table 14). The number of collapsed epidermal cells increased after 72 h and this cellular collapse was accompanied by (a) extensive colonization of the deep subdermal tissues by *M. fructicola*, and (b) apparition of lysogenic cavities in the mesocarp (Fig. 24G and H; Table 14). After 96 h, the diseased tissues of the nectarines with visible brown rot displayed (a) colonization of the epidermis and mesocarp by *M. fructicola* with thin and thick hyphae, (b) collapse and disruption of epidermal and mesocarpic cells, (c) lysogenic cavities in the subepidermis and mesocarp, (d) degradation of the cuticle and epidermis, and (e) *M. fructicola* sporulation (Fig. 24I and J; Table 14).

4.3.1.3. Light microscopic analysis of tissues from nectarines with a latent *M. fructicola* infection

Fig. 24K displays representative light microscopic images of epidermal, subepidermal, and mesocarp cells from uninoculated nectarines and Fig. 24L through O display representative images of the histological changes on the surface and parenchyma of nectarines with a latent *M. fructicola* infection. Although no visible signs of infection were seen in nectarines with a latent infection after a 288 h incubation at 4 °C, microscopic signs of the infection became apparent over time. *M. fructicola* colonized the stomata of inoculated fruit after 72 h (Fig. 24L; Table 14) and the extent of this stomatal colonization had increased after 144 h (Fig. 24M; Table 14). Colonization of the subdermal tissues by

4. RESULTS AND DISCUSSION

M. fructicola with the collapse of the epidermal cells was observed after 216 h (Fig. 24N; Table 14). After 288 h, most of the epidermal cells had collapsed and the subepidermis of the inoculated fruit had become colonized by *M. fructicola* (Fig. 24O; Table 14).

Fig. 24. Light microscopic analysis of tissues from nectarines with a visible or latent *M. fructicola* infection. The nectarine's tissues were stained with Safranin O-Fast Green stain and the unaffected walls of the nectarine's cells are blue and the affected nectarine's cells, and the hyphae and conidia of *M. fructicola* are red: A, B and K: representative images of epidermal, subepidermal and mesocarp cells from uninoculated nectarines; C and D: signs of *M. fructicola* infection in areas which surround the stomata and in stomata-free areas of nectarines with a visible infection after a 24 h incubation at 25 °C. E and F: invasion and collapse of the uppermost layer of epidermal cells of nectarines with a visible infection after a 48 h inoculation at 25 °C. G and H: Collapse of epidermal cells, apparition of lysogenic cavities in the mesocarp, and extensive colonization of the deep subdermal tissues by *M. fructicola* of nectarines with a visible infection after a 72 h incubation at 25 °C. I and J: Colonization of the epidermis and mesocarp by *M. fructicola* with thin and thick hyphae, collapse and disruption of the nectarine's epidermal and mesocarpic cells, lysogenic cavities in the subepidermis, degradation of the cuticle and epidermis, and *M. fructicola* sporulation after a 96 h incubation at 25 °C. L: Colonization of the stomata of nectarines with a latent *M. fructicola* infection after a 72 h incubation at 4 °C. M: Increased colonization of the stomata of nectarines with a latent *M. fructicola* infection after a 144 h incubation at 4 °C. N: Colonization of the subdermal tissues by *M. fructicola* with the collapse of the epidermal cells was observed after 216 h incubation at 4 °C. O: Collapse of the epidermal cells and colonization of the subepidermis by *M. fructicola* of nectarines with a latent *M. fructicola* infection after a 288 h incubation at 4 °C. Scale bar = 50 µm.

4.3.1.4. TEM analysis of tissues from uninfected nectarines and nectarines with a visible *M. fructicola* infection

Fig. 25 and Fig. 26 display representative micrographs of the ultrastructure of healthy nectarines and nectarines with a visible *M. fructicola* infection. The cuticle of uninfected fruit had a variable thickness and was covered a layer of epidermal cells (Fig. 25A). The epidermal and hypodermal cells of healthy nectarines have a thick cell wall of variable thickness and a dense cytoplasm with intact organelles (Fig. 25A and B). The mesocarpic cells of uninfected nectarines were large with a thin cell wall and the intercellular spaces between the mesocarpic cells were also large. In these cells, the cytoplasm was almost completely occupied by a central vacuole (Fig. 26A).

Fig. 25. Ultrastructure of tissues from healthy nectarines and nectarines with a visible *M. fructicola* infection after a 24 h incubation at 25 °C. A and B: The cuticle (cu), cell wall (cw) and ultrastructure of epidermal and hypodermal cells of healthy nectarines, scale bar = 5000 nm. C-E: Conidial germination (co) and germ tubes (gt) of *M. fructicola* on the surface of the cuticle of nectarines with a

visible infection, scale bars = 2000 nm (C and D) and 500 nm (E). F and G: Inter cellular hyphae (h) of *M. fructicola*, scale bar = 2000 nm.

In nectarines with a visible infection, conidia and germ tubes began to adhere to the nectarine's cuticle after 24 h (Fig. 25C to E). The conidia had a two-layered cell wall, a plasmalemma, and their cytoplasmic matrix was dense with nuclei, mitochondria, and vacuoles (Fig. 25C and D). Partial degradation and/or dissolution of cuticle and cell wall were observed under germinated *M. fructicola* conidia (Fig. 25C to E). Long branching germ tubes were present on the cuticular surface and some of these tubes had penetrated the cuticle presumably through the stomatal cavities into epidermal cells (Fig. 25E). Thin infective hypha that grew intercellularly (Fig. 25F) or intracellularly (Fig. 25G) in epidermal cells were observed after 24 h. Substantial dissolution of the cell walls and degeneration of cytoplasmic organelles were evident in infected epidermal and hypodermal cells (Fig. 25F and G). Hyphal invasion, cytoplasmic necrosis, and degeneration of cytoplasmic organelles in mesocarpic cells were observed after 72 h (Fig. 26B to F). The mesocarpic cells were colonized by large intercellular (Fig. 26B to F) and intracellular hyphae (Fig. 26B to D, and F). Intracellular hyphae, which were close to the cell wall, contain numerous organelles in their cytoplasm, have a double cell wall layer, and are surrounded by vesicles (Fig. 26B). The matrix of the mesocarpic cell walls, which were close to intracellular hyphae, appeared fibrillary and disrupted, and groups of vesicles were present near the mesocarpic cell walls (Fig. 26B and C). The intercellular hyphae, which had invaded mesocarpic cells, were ultrastructurally different from the intracellular hyphae: their cytoplasm contained no vesicles and their cell walls were thick (Fig. 26D and E).

Fig. 26. Ultrastructure of tissues from healthy nectarines and nectarines with a visible *M. fructicola* infection after a 72 h incubation at 25 °C. A: The parenchyma of an uninfected nectarine, scale bar = 5000 nm. B, C, and F: Intracellular hyphae of *M. fructicola* in a mesocarpic cell of a nectarine with a visible infection, scale bars = 2000 nm (B and F) and 500 nm. D: The intracellular and intercellular hyphae of *M. fructicola* in a mesocarpic cell and in the space around a mesocarpic cell of a nectarine with a visible infection, scale bar = 5000 nm. E: The intercellular hypha of *M. fructicola* in a mesocarpic cell of a nectarine with a visible infection, scale bar = 2000 nm

4.3.2. Discussion

The aim of this investigation was to analyze the microanatomy of nectarines with a latent and a visible *M. fructicola* infection using light microscopy and TEM. Tissues of mature nectarines with latent *M. fructicola* infections are characterized by the presence of intercellular hyphae at the subcuticular level. Although these intercellular hyphae do not penetrate more than the first two subcuticular cell-layers of nectarines with latent infections, they do not remain totally dormant but slowly colonize the tissue. At the same time, the tissues of mature nectarines with visible *M. fructicola* infections are characterized by extensive colonization of the deep subdermal tissues by the hyphae of *M. fructicola*. The tissues and the cells were colonized inter- and intracellularly and this colonization was accompanied by increasing degradation of the cell walls, a typical colonization of necrotrophic fungi.

Germinated conidia with long germ tubes have been described in mature nectarines with brown rot due to *M. fructicola* and this germination is associated with a low stomatal density, reduced hydrophobicity of the cuticle, numerous cuticular cracks and fissures, and the accumulation of volatile compounds (Lee and Bostock, 2006b). In our study, we observed long germ tubes of *M. fructicola* around the guard cells of stomata on the surface of mature fruit mainly when samples of the epidermis from inoculated nectarines were incubated at 25 °C. For *Monilinia* spp., conidial germination, sporulation, growth, and propagule formation on fruit surfaces are dependent on T, RH, and water availability (Lalancette and McFarland, 2015; Luo et al., 2001a; Magan and Lacey, 1988; Tamm and Flückiger, 1993; Tamm et al., 1995; Xu et al., 2001). We found germination of *M. fructicola* conidia and hyphal formation on the nectarine surface following a 4 h incubation at 25 °C and a 24 h incubation at 4 °C. We also found that this germination and hyphal formation were more abundant in areas around the stomata than in areas without stomata. Our results on *M. fructicola* germination on the fruit surface are similar to those of other investigators who reported that *Monilinia* spp. can penetrate stone fruit

through the stomata, the cuticle of the intact surface, the base of the surface hairs and trichomes, and surface wounds (Curtis, 1928; Smith, 1936; Wade and Cruickshank, 1992; Xu et al., 2007).

We did not detect appressoria on the surface or inside epidermal cells of mature nectarines with either a visible or latent *M. fructicola* infection. Other investigators have reported the presence of appressoria on immature fruit and flowers. For example, Lee and Bostock (2006b) described appressorial formation by *M. fructicola* on immature nectarine surfaces. *M. fructicola* forms non-melanized appressoria for penetrating *Prunus* spp. petals and immature fruit, and appresoria has been proposed as resting structures of latent infections in immature fruit (Lee and Bostock, 2007). Other factors, such as unidentified volatile and nonvolatile compounds in the cuticle and/or cell wall, have been implicated for stimulating of appressorial formation by fungal pathogens (Grambow and Grambow, 1978; Grambow and Riedel, 1977).

Although visible evidence of brown rot was not seen on the surface of nectarine with a latent infection, we detected fungal hyphae in the epidermal cells after 144 h of incubation at 4 °C when the tissues of these nectarines were microscopically examined. Rungjindamai et al. (2014) suggested that latent infections follow a typical pattern of subcuticular infection of immature fruit followed by rapid cessation of growth of the pathogen. In this investigation, we found slow and continuous growth of the pathogen in nectarine subcuticular tissues with a latent infection under conditions of cold storage. Although no macroscopic brown rot symptoms are observed, *Monilinia* hyphae were visible at subcuticular level and the latent infection could be considered a quiescent infection under optical microscopy conditions according to the definition of Ahimera et al. (2003). It has been reported that activation of a latent infection can be facilitated by large gene families of cell wall-degrading enzymes (Prusky et al., 2013). Additionally other pathogenic fungi use tissue acidification to activate a latent infection and stimulate the transition of a latent infection to a visible infection (Prusky et al., 2013). It has also been reported that *M. fructicola* can acidify the tissues of the host and hence up-regulate the

expression of polygalacturonase genes, which is controlled by acidic pH as the infected fruit matures (De Cal et al., 2013). We found cell wall degradation in the epidermal and mesocarpic cells in the nectarines with a visible *M. fructicola* infection. We also found (a) partial degradation or dissolution of cuticle and cell wall areas under germinated conidia, (b) collapse of epidermis cells, and (c) formation of lysogenic cavities on the nectarine mesocarp 72 h after inoculation from visible *M. fructicola* infections. It has been reported that *M. fructicola* produces cell degrading enzymes, such as cutinase (Wang et al., 2000), and that this cutinase is important for *M. fructicola* virulence during fungal growth and the development of brown rot lesions in nectarines (Lee et al., 2010). It has also been reported that the redox state of nectarines can influence appressorial formation and expression of the *M. fructicola* cutinase *Mfcut1* and polygalacturonase *Mfpg1* genes (Lee and Bostock, 2007). Interestingly, Wade and Cruickshank (Wade and Cruickshank, 1992) reported a thick mechanical barrier around the infection point, suberized cell walls of the surrounding living cells, and intracellular accumulation of phenolic compounds in apricots with a latent *M. fructicola* infection.

We detected that *M. fructicola* only colonizes the intercellular spaces at subcuticular level in nectarines with a latent infection, whereas hyphae colonize inter- and intracellularly in nectarines with a visible infection. These findings are similar to those which have been reported for corn: the hyphae of *F. moniliforme* and *F. verticillioides* colonize the intercellular spaces in latent infections, whereas their hyphae colonize intracellularly in visible infections (Bacon and Hinton, 1996; Oren et al., 2003; Yates et al., 1997). Other pathogenic fungi, such as *Phialophora gregata* in soybean (Impullitti and Malvick, 2014), or *Colletotrichum acutatum* in their solanaceous hosts (Horowitz et al., 2002), have been reported to colonize and survive intercellularly.

A latent brown rot infection is a well-known but poorly understood phenomenon. In this investigation, we found that (a) intercellular hyphal colonization was restricted to the epidermal and two subdermal cell layers in nectarines with a latent *M. fructicola* infection, and (b) no macroscopic evidence of

disease was detected in latently infected nectarines even after 216 h of incubation at 4 °C. We also found that intra- and intercellular hyphae colonized all mesocarp cells in nectarines with visible brown rot. Finally, we also found that *M. fructicola* is active during latent infections because slow and progressive colonization of nectarine cells by the fungus occurs.

4.4. TRANSFORMATION OF THE BROWN ROT FUNGAL PATHOGENS *M. fructicola* AND *M. laxa*

4.4.1. Results

4.4.1.1. *Monilinia* mutants in the nitrate reductase pathway

M. fructicola and *M. laxa* isolates were able to use sodium nitrate and sodium nitrite as nitrogen sources. Isolates from both species grew better on sodium nitrate and sodium nitrite than in minimal medium lacking of a principal nitrogen source (Fig. 27 and Fig. 28). However, colonies grown on sodium nitrate and sodium nitrite amended minimal medium had smaller diameter than colonies grown on media containing urea, proline, glutamine, asparagine, and arginine as main nitrogen sources (Fig. 27 and Fig. 28).

Fig. 27. *M. fructicola* isolates Mfc1 and Mfc2 growth on potato dextrose agar (PDA), minimal medium without nitrogen source (MM), and minimal medium with different nitrogen sources (sodium nitrate (NaNO_3), sodium nitrite (NaNO_2), urea, proline, glutamine, asparagine, and arginine) at different concentrations 2.5 mM (top sector), 5 mM (left sector), and 10 mM (right sector) after 8 days of incubation at 25 °C in the dark.

Fig. 28. *M. laxa* isolates Mlx1 and Mlx2 growth on potato dextrose agar (PDA), minimal medium without nitrogen source (MM), and minimal medium with different nitrogen sources (sodium nitrate (NaNO_3), sodium nitrite (NaNO_2), urea, proline, glutamine, asparagine, and arginine) at different concentrations 2.5 mM (top sector), 5 mM (left sector), and 10 mM (right sector) after 8 days of incubation at 25 °C in the dark.

M. fructicola and *M. laxa* isolates were unable to grow with potassium chlorate concentrations of 150 mM or higher in minimal medium. Thus, possible chlorate-resistant mutant colonies in the nitrate reductase pathway were isolated from minimal media amended with 75, 100, or 125 mM of potassium chlorate (Fig. 29).

Fig. 29. *M. fructicola* and *M. laxa* isolates Mfc1 (A), Mfc2 (B), Mlx1 (C), and Mlx2 (D), growth on minimal medium with 5 mM of urea as a nitrogen source and 0, 75, 100, or 125 mM of potassium chlorate after 7 days of incubation at 25 °C in the dark.

All putative *Monilinia* mutants (>20 resistant colonies) for the nitrate reductase activity selected after three selection rounds on potassium chlorate medium were able to grow on the presence of potassium chlorate but also able to grow on minimal medium with 5 mM of sodium nitrate as the only nitrogen source. Hence, no homokaryotic *Monilinia* mutants in the nitrate reductase pathway were obtained.

4.1.1.2. Search for *Monilinia* mutants in the orotidine 5'-phosphate decarboxylase activity

Growth tests determined that *M. fructicola* and *M. laxa* isolates were able to tolerate the presence of at least 1 mg mL⁻¹ of 5-FOA in PDA. Thus, selective conditions for the isolation of possible *Monilinia* mutants in the orotidine 5'-phosphate decarboxylase pathway were adjusted to PDA containing 1.5 and 2 mg mL⁻¹ of 5-FOA. After five rounds of growth through selective medium, all candidate mutants (17 5-FOA resistant colonies) were able to grow on PDA without addition of uracil and uridine (Fig. 30A). Furthermore, the selected mutants recovered their normal growth capabilities on non-amended PDA after plating them twice on this medium (Fig. 30B). Hence, no stable homokaryotic *Monilinia* mutants were obtained on the pyrimidine biosynthetic pathway.

Fig. 30. Comparison of the growth on potato dextrose agar (PDA), and PDA amended with uracil and uridine (UU) of four *M. fructicola* Mfc2 candidate mutant colonies after 5 rounds through selective medium (PDA with 1.5 mg mL⁻¹ of 5-fluoroorotic acid (5-FOA)) on unselective medium for the first time (A) and for the second time (B).

4.1.1.3. Determination of the number of nuclei present in *Monilinia* spp. conidia, hyphae and protoplast

Both *M. fructicola* and *M. laxa* presented a variable number of nuclei in their conidia (Fig. 31), their hyphae (Fig. 32 and Fig. 33) and their protoplasts (Fig. 34). *M. fructicola* conidia and hyphae were more permeable to DAPI than those of *M. laxa*, while the opposite was true for their protoplasts. However, both species presented similar characteristics: average nuclei diameter of 1.5 μ m, average conidial diameter of 13 μ m, hyphal diameter of 3 μ m, and average distance between nuclei in their

hyphae of 9 μm . *Monilinia* conidia had a number of nuclei ranging between 3 and 11 (Fig. 31). Additionally some *Monilinia* conidia were not stained with DAPI suggesting that either stain could not enter those cells or these conidia were depleted of nuclei (Fig. 31). The number of nuclei in the apical hyphal segments correlated with the number of nuclei inside the conidia (Fig. 32), but there could be a difference of 1 or 2 nuclei between hyphal segments of older colonies (Fig. 33). A higher variability in the number of nuclei was observed in protoplasts formed by *Monilinia* spp. ranging between 1 and more than 11 (Fig. 34).

Fig. 31. *M. fructicola* conidia (A and B) and conidia chains (C to F) stained with DAPI and observed under differential interference contrast (DIC) microscopy (A, C, and E) and under ultra violet fluorescence light (B, D, and F). Nuclei appear as bright white dots on the fluorescence pictures (arrow on picture B).

Fig. 32. Germinated *M. fructicola* conidia stained with DAPI an observed under differential interference contrast (DIC) microscopy (A, C, and E) and under ultra violet fluorescence light (B, D, and F). Nuclei appear as bright white dots on the fluorescence pictures.

Fig. 33. *M. fructicola* hyphae stained with DAPI and observed under differential interference contrast (DIC) microscopy (A, C, E, and G) and under ultra violet fluorescence light (B, D, F, and H). Nuclei appear as bright white dots on the fluorescence pictures. Arrows point towards hyphal segment divisions, septa.

Fig. 34. *M. laxa* protoplasts stained with DAPI an observed under differential interference contrast (DIC) microscopy (A, C, E, and G) and under ultra violet fluorescence light (B, D, F, and H). Nuclei appear as bright white dots on the fluorescence pictures.

4.1.1.4. *Monilinia* transformation

No *Monilinia* spp. transformants were obtained with any of the studied transformation protocols. *M. fructicola* and *M. laxa* were able to form viable protoplasts and grow on the regeneration plates using both CaCl₂/PEG-mediated transformation protocols. However, the few colonies that were able to grow on the selection plates did not express any fluorescence protein. The CaCl₂/PEG-mediated transformation protocols worked on *P. rubens* (Fig. 35), and the *P. rubens* transformants were able to express GFP or mCherry fluorescence proteins under the epifluorescence microscope (Fig. 36). Few *Monilinia* spp. colonies grew on the regeneration plates after LiAc- and electroporation-transformation. No differences were observed between the heterologous and the homologous recombination vector transformation efficiencies.

Fig. 35. *P. rubens* CaCl_2 /poly ethylene glycol-mediated transformation results using *Monilinia* expression cassette vector with GFP (A) and with mCherry (B).

Fig. 36. *P. rubens* transformant colonies expressing mCherry (A to D) and GFP (E and F) observed under differential interference contrast (DIC) microscopy (A, C, and E) and epifluorescence microscopy (B, D, and F).

4.4.2. Discussion

Nowadays, functional genomics, especially targeted gene disruption, are the main tool to elucidate the different metabolic and regulatory pathways (He et al., 2016; Michielse et al., 2005b; Ruiz-Díez, 2002). Many functional genomics tools have been developed for the yeast *Saccharomyces cerevisiae* such as, computational annotation of gene function transcriptional profiling, and protein profiling (Bader et al., 2003). Most of these tools have been adapted for filamentous fungi mainly for *Aspergillus* spp. (Meyer et al., 2003; Michielse et al., 2005a; b; Sims et al., 2004) and *Neurospora crassa* (Mannhaupt et al., 2003). In the genus *Monilinia* some initial attempts at functional genomics have been made with the species *M. fructicola*. The role and expression of three different genes have been studied in *M. fructicola*: the cutinase gene *MfCUT1* (Lee et al., 2010), the endopolygalacturonase gene *MfPG1* (Chou et al., 2015), and the genetic element ‘Mona’ associated with fungicide resistance (Chen et al., 2017). However, there are no studies in any of the other *Monilinia* species due to the lack of an effective transformation protocol.

In this study, we show that both *M. fructicola* and *M. laxa* have a variable number of nuclei in their hyphae, conidia and protoplasts. Lee and Bostock (2006a) reported that the number of nuclei in *M. fructicola* hyphae varies depending on the media they are grown on between 3 nuclei in minimum medium and more than 8 nuclei on complete medium (clarified V8 agar or YPD). Furthermore, Wylletts and Calonge (1969) indicated that *M. fructicola* conidia were also multinucleated, and both Margosan and Phillips (1985) and Phillips et al. (1989) described a variable number of nuclei inside the conidia depending on *M. fructicola* isolates, growing medium and temperatures. In the present study both *M. fructicola* and *M. laxa* conidial nuclei number oscillated between 4 and 11 when they were harvested from PDA plates grown at 25 °C. Margosan and Phillips (1985) reported similar conidial nuclei number under similar conditions, *M. fructicola* isolate A with an average nuclear number of 5.4 nuclei and isolate B with 6 nuclei per conidia. The number of nuclei inside *Monilinia*

spp. protoplasts has not been studied. Some studies assumed that the number of nuclei inside the protoplast were lower than that of hyphae and conidia and tried to obtain homokaryotic mutants or transformants by protoplast generation (Lee and Bostock, 2006a; Lee et al., 2010). The present study shows that protoplasts have a variable number of nuclei, which can be higher than that present on hyphal cells and conidia.

We were unable to obtain homokaryotic mutants in the nitrate reductase and the orotidine 5'-phosphate decarboxylase pathways on *M. fructicola* and *M. laxa* isolates. Although, pyrimidine (pyr-) auxotrophic mutants of the ascomycetes *P. rubens* were previously generated by this method (Villarino et al., 2016a). Other studies had pointed to the multinucleate nature of *M. fructicola* and the difficulty to obtain homokaryotic gene disruptants even after single-spore, hyphal tip, or protoplast purification (Chen et al., 2017; Chou et al., 2015; Dai et al., 2003; Lee and Bostock, 2006a; Lee et al., 2010). The impossibility to obtain homokaryotic mutants or gene disruptants after several rounds of selection might indicate that viable conidia and hyphae of *M. fructicola* and *M. laxa* must be heterokaryotic. Thus, construction of transformants in these strains would require the simultaneous integration of transformation cassettes in diverse and complementary nuclei. Other fungus also have a variable number of nuclei inside their conidia, for example, many strains of *Heterobadium annosum* produced conidia containing one to five nuclei (Ramsdale and Rayner, 1994). The distribution of numbers of nuclei in conidia produced by any one *H. annosum* strain was relatively stable throughout repeated subculturing, and was not affected by the age of the colony from which the spores were taken. In homokaryons of *H. annosum*, predominantly binucleate conidia were produced and uninucleate conidia germinated less readily than others (Ramsdale and Rayner, 1994).

It was not possible to obtain transformants of the *M. fructicola* and *M. laxa* isolates used in this study by using CaCl₂/PEG-mediated transformation protocol. However, validity of plasmids and transformation protocol were validated by using *P. rubens* as transformation model. This

transformation method is one of the most used across laboratories working with filamentous fungi, including species from different zygomycetes, ascomycetes and basidiomycetes (He et al., 2016; Ruiz-Díez, 2002). It has even been used to transform *M. fructicola* isolate MUK-1; however, it yielded a low number of transformants, 5 and 7 transformants in each of their experiments (Chou et al., 2015). Alternative transformation methods have been approached to compensate low efficiencies in the CaCl₂/PEG-mediated transformation in other species, e.g. electroporation transformation and *Agrobacterium tumefaciens*-mediated transformation of *Pseudozyma antarctica*, a basidiomycetous yeast-like fungus, rendering a higher yield of transformants than CaCl₂/PEG-mediated transformation (Marchand et al., 2007). Electroporation and *Agrobacterium tumefaciens* mediated transformations are the principal alternative transformation methods to protoplast CaCl₂/PEG-mediated transformation and have solve transformation problems in a number of species such as *Agaricus bisporus* and *Sclerotinia sclerotiorum* (Minz and Sharon, 2010). Nevertheless, there are other alternative methods for fungal transformation such as lithium acetate-, biolistics-, liposome-, viral vector-, or nanomaterial-mediated transformation (He et al., 2016). In this study, two alternative methods were tested: electroporation and lithium acetate-mediated transformation, but no *M. fructicola* nor *M. laxa* transformants were obtained with them. Other groups have managed to transform four *M. fructicola* isolates (DL25W, MUK-1, Bmpc7, and HG3) by *Agrobacterium tumefaciens*-mediated transformation (Chen et al., 2017; Dai et al., 2003; Lee and Bostock, 2006a; Lee et al., 2010). This transformation method also yielded a low number of transformants in *M. fructicola*, although it was higher than that yielded by CaCl₂/PEG-mediated transformation, 13 transformants per 10⁵ conidia in homologous recombination (Lee and Bostock, 2006a) or 1 transformant per 10⁸ conidia in heterologous recombination (Dai et al., 2003). Further studies should be made in order to find a reliable transformation method for *Monilinia* spp.

The heterokaryotic and variable number of nuclei inside the conidia and hyphae of *Monilinia* spp. hinders the transformation of this fungus. Nonetheless, due to the importance of genetic transformation

as a tool for pathogenesis studies, further studies are required to find a suitable method for *Monilinia* spp. transformation extendable to any isolate and specie regardless of their nuclei number.

4.5. DETECTION OF LATENT *Monilinia* INFECTIONS IN NECTARINE FLOWERS AND FRUIT BY qPCR

4.5.1. Results

4.5.1.1. Standard curves characteristics

Standard curves for the two hydrolysis probes (P_fc (*M. fruticola*) 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 15). A low limit of detection (less than 27 fg of DNA (approximately 1 *M. laxa* cell)) was achieved with the different probes for all the different matrices (Table 15). With the exception of the standard curve for *M. fruticola* in flowers that have an amplification efficiency of 87 %, the rest of the standard curves had optimal amplification efficiencies between 90 and 100 % (Table 15). Additionally, the fit of the generated data to the regression line was high, as measured by value of the R^2 coefficient that was greater than 0.99 for all curves (Table 15).

4. RESULTS AND DISCUSSION

Table 15. Characteristics of the 8 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
<i>M. fructicola</i>	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
<i>M. laxa/ M. fructigena</i>	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.

4.5.1.2. Detection of a latent *Monilinia* infection in nectarine flowers by qPCR

DNA from the *M. fructicola*, *M. fructigena*, and *M. laxa* isolates was not detected in the 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 Fig. 37. The median amounts of DNA from *M. fructigena* and *M. laxa* species were similar, 0.10 ± 0.05 pg (median ± standard error) and 0.13 ± 0.06 pg, respectively and lower than the median amount of DNA from *M. fructicola* 18.32 ± 8.47 pg. The median amount of DNA from *M. fructicola* isolates in the latently infected flowers was bigger than 1 pg in 8 out of 10 isolates whereas those from *M. fructigena* and *M. laxa* isolates were less than 1 pg, in 7 out of 8 and 10 out of 10 isolates respectively. The median amount of DNA of *M. fructicola* isolates ranged between 0.25 ± 0.11 pg and 338.73 ± 128.01 pg, the ones from *M. fructigena* isolates ranged between 0.01 ± 0.01 pg and 3.00 ± 1.28 pg, and those from *M. laxa* isolates ranged between 0.004 ± 0.006 pg and 0.57 ± 0.22 pg (Fig. 37).

Fig. 37. Amounts of DNA from 10 *M. fructicola* (A), 8 *M. fructigena* (B), and 10 *M. laxa* (C) isolates in latently infected flowers that were detected by qPCR. Values are displayed as median DNA amount $\pm$ standard error of 5 inoculated flowers and 3 pseudo-replicates per repetition. Dotted lines at 1 pg DNA divide the graphs to highlight the differences, and facilitate the comparison between graphs.

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

Latent brown rot infections in the epidermis and the mesocarp of inoculated nectarines that were caused by *M. fructicola* and *M. laxa* isolates were successfully detected by qPCR. DNA from the *M. fructicola*, and *M. laxa* isolates was not detected in the epidermis and the mesocarp of 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 were similar inside each specie and those corresponding to isolate Mfc3 and Mlx4 are shown on Fig. 38; additionally, the values for all of the isolates are shown in Fig. 39. 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 (B). The amounts of DNA from the *M. fructicola* in the epidermis of latently infected nectarines ranged between 0.25 ± 0.03 pg and 6.33 ± 0.60 pg; and were similar to those from the *M. laxa* isolates that ranged between 0.01 ± 0.004 pg and 9.10 ± 3.26 pg (Fig. 38 and Fig. 39). In contrast, the amount of DNA from the *M. fructicola* isolates in the mesocarp of latently infected nectarines ranged between 0.0002 ± 0.0003 pg and 0.004 ± 0.001 pg; and was significantly lower than that of the *M. laxa* isolates that ranged between 0.005 ± 0.01 pg and 2.10 ± 0.72 pg (Fig. 38 and Fig. 39). 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 where external mesocarp *Monilinia* DNA amount was bigger than the internal mesocarp *Monilinia* DNA amount.

Fig. 38. Amounts of DNA from *M. fructicola* isolate Mfc3(■) and *M. laxa* isolate Mlx4 (□) from the epidermis, external mesocarp, and internal mesocarp of 3 latently infected nectarines that were detected by qPCR. Values are displayed as median DNA amount $\pm$ standard error of 3 different inoculated fruit, 5 DNA extractions and 3 pseudo-replicates.

Fig. 39. Amounts of DNA from 10 *M. fructicola* isolates (A) and 10 *M. laxa* isolates (B) from the epidermis ($\diamond$), external mesocarp ($\triangle$), and internal mesocarp ($\bullet$) of 3 latently infected nectarines that were detected by qPCR. Values are displayed as median DNA amount $\pm$ standard error of 3 different inoculated fruit, 5 DNA extractions and 3 pseudo-replicates. Dotted lines at 0.005 pg DNA divide the graphs to highlight the differences, and facilitate the comparison between graphs.

4.5.1.4. Comparison between ONFIT and qPCR for detecting a latent *Monilinia* infection

The qPCR-based method for the detection of *Monilinia* latent infections was compared with ONFIT, the commonly used latent infection detection method, to assess its efficiency and sensitivity. Table 16 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 (Table 16). 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 (Table 16). 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 (Table 16). 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.

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

Table 16. Detection percentages of *Monilinia* isolates artificial latent infections of flowers and nectarines by ONFIT and qPCR.

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

^a Percentages are based on 5 artificially latent infected nectarine flowers.

^b Percentages are based on 3 artificially latent infected nectarines.

^c Positive detection was considered when *Monilinia* DNA was amplified in one of the flowers or at least one of the 5 DNA extractions of each nectarine epidermis.

4.5.2. Discussion

In this study, a qPCR-based method was used to detect latent brown rot infections in nectarine flowers and fruits caused by *M. fructicola*, *M. fructigena* and *M. laxa*. It takes between 24 and 48 h to detect the fungal pathogen in latently infected flowers and fruit using qPCR, while the ONFIT method required a much longer time to detect latent brown rot infections; 7 to 9 days of sample preparation and

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 intended for long-term storage or transported to distant markets, as suggested by Sanzani et al. (2012) for the control of *Botrytis cinerea* in table grapes.

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

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

The advantages of the qPCR-based method for detecting a latent *Monilinia* infection in nectarines are its high sensitivity, its ease and rapidity of execution, the low number of handling steps, and reduced personnel costs. The disadvantages of the qPCR-based method for detecting a latent *Monilinia*

infection in nectarines are the high cost of consumables and reagents, which are much greater than those of ONFIT, and the occurrence of false positives due to detection of 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).

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

4.6. PROFICIENCY OF A LATENT *Monilinia* spp. INFECTION DETECTION BY REAL-TIME PCR IN NECTARINE FLOWERS AND FRUIT

4.6.1. Results

4.6.1.1. Sample detection by the different qPCR platforms

All participating laboratories results were transformed into qualitative results (Table 17). All qPCR platforms detected *Monilinia* in latent infection and mycelia samples with both hydrolysis probes, but not on healthy flowers and fruit (Table 17).

M. fructicola samples were correctly detected by all laboratories and qPCR platforms with hydrolysis probe P_fc (Table 17). However, the *M. fructicola* samples were also cross-detected by the hydrolysis probe P2_fgn/lx/ps, as if they were *M. laxa*, when using the Rotor-Gene Q platform (Table 17). *M. laxa* samples were detected with the P2_fgn/lx/ps by all qPCR platforms, except for the low-concentration-*M. laxa*-latently-infected-flower-sample that was not detected by the LightCycler® 480 Real-Time PCR System (Table 17). With the exception of the LightCycler® 480 Real-Time PCR System, the rest of the qPCR platforms cross-detected *M. laxa* samples with the probe P_fc, as if they were *M. fructicola* (Table 17). Two of the four platforms, Applied Biosystems® 7500 Fast Real-Time PCR and CFX96 Touch™ Real-Time PCR Detection System, cross-detecting *M. laxa* as *M. fructicola* could add an allelic discrimination step to the qPCR assay, differentiating between *M. fructicola* and *M. laxa* samples (Table 17).

4. RESULTS AND DISCUSSION

Table 17. Qualitative results of the qPCR assay performed by the participating laboratories with different real-time PCR platforms of all the blind samples with each hydrolysis probe.

Sample	qPCR Platforms used											
	Expected results		ABI 7500 FAST		CFX96 Touch		LC 480		Mastercycler RealPlex ²		Rotor-Gene Q	
	P1 ^c	P2 ^d	P1 ^c	P2 ^d	P1 ^c	P2 ^d	P1 ^c	P2 ^d	P1 ^c	P2 ^d	P1 ^c	P2 ^d
<i>M. fructicola</i> mycelia	+	-	+	-	+	-	+	-	+	-	+	+
<i>M. fructicola</i> LIN ^a	+	-	+	-	+	-	+	-	+	-	+	+
<i>M. fructicola</i> LIF ^b	+	-	+	-	+	-	+	-	+	-	+	+
<i>M. laxa</i> mycelia	-	+	+ ^e	+	+ ^e	+	-	+	+	+	+	+
<i>M. laxa</i> LIF ^b	-	+	+ ^e	+	+ ^e	+	-	-	+	+	+	+
<i>M. laxa</i> LIN ^a	-	+	+ ^e	+	+ ^e	+	-	+	+	+	+	+
Mix-mycelia	+	+	+	+	+	+	+	+	+	+	+	+
Healthy flower	-	-	-	-	-	-	-	-	-	-	-	-
Healthy nectarine	-	-	-	-	-	-	-	-	-	-	-	-
Nuclease-free water	-	-	-	-	-	-	-	-	-	-	-	-

^a LIN: latently infected nectarine.

^b LIF: latently infected flower.

^c P1/P_{fc} Hydrolysis probe with a FAM reporter and a ZEN / Iowa Black FQ quencher for *M. fructicola* detection.

^d P2/P2_{fgn/lx/ps} Hydrolysis probe with a HEX reporter and a ZEN / Iowa Black FQ quencher for *M. fructigena*/*M. laxa*/*M. polystroma* detection.

^e Negative after examining the allelic discrimination results.

4.6.1.2. Specificity of the qPCR assay

The specificity of the qPCR results was tested using the qualitative data calculating the percentages of false positive and false negative results (Table 18). 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 % (Table 18). The greater bias came from the Rotor-Gene Q platform, which was not able to differentiate

between *M. fructicola* and *M. laxa* with either hydrolysis probe adding 10 % to each false negative rate (Table 18). P_fc probe was specific for LightCycler® 480 Real-Time PCR System and also for Applied Biosystems® 7500 Fast Real-Time PCR and CFX96 Touch™ Real-Time PCR Detection System when the allelic discrimination step was incorporated into the qPCR assay. However, LightCycler® 480 Real-Time PCR System was not able to detect the *M. laxa* latently infected flower sample with low concentration of *M. laxa* DNA, adding a 5 % false positive rate to the P2_fgn/lx/ps probe (Table 18).

Table 18. 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 qPCR platforms were able to reduce the false negative rate of P_fc with the allelic discrimination step.

4.6.1.3. Comparison between qPCR and ONFIT results

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 (Table 19). The qPCR method was as sensible as the ONFIT since there was no negative deviation between methods. However, because the qPCR method was able to detect 15 more positive-samples than the ONFIT, both the relative accuracy and diagnostic specificity of the qPCR-method were lower than 100 % (Table 19).

Table 19 Comparison between qPCR and ONFIT to obtain the relative accuracy (*A*), diagnostic specificity (*S_p*) and diagnostic sensitivity (*S_e*), according to positive agreement (*P_a*), positive deviation (*P_d*), negative deviation (*N_d*), and negative agreement (*N_a*) of the results.

qPCR vs ONFIT						
<i>P_a</i>	<i>P_d</i>	<i>N_d</i>	<i>N_a</i>	<i>A</i> (%) ^a	<i>S_p</i> (%) ^a	<i>S_e</i> (%) ^a
30	15	0	39	82 ± 17	72 ± 13	100 ± 2

^a Values are expressed as value ± 95 % CI.

4.6.1.4. Proficiency of the qPCR method

Z-scores were calculated with the data to determine those qPCR platforms producing results out of line with respect to the rest (Fig. 40). Only the LightCycler® 480 Real-Time PCR System produced results with z -scores between 2 and 3, and therefore subject to revision (Fig. 40). This slight deviation on the z -scores of the LightCycler® 480 system was because the samples' C_q values were consistently higher than those obtained with the rest of the platforms, which also explains why the *M. laxa* latently infected flower sample with low DNA concentration, was not detected, because its C_q was out of range. The rest of the platforms scored z -scores on the acceptable region between -2 and 2 (Fig. 40). And there were no laboratories with z -scores in the unacceptable region.

Fig. 40. 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.

4.6.2. Discussion

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

The qPCR method was at least as sensible as the ONFIT, because it detected all the latent infections detected by ONFIT. Additionally, the qPCR method detected 15 more positive samples than ONFIT. The same was observed on the previous study by Garcia-Benitez et al. (2017b), where qPCR detected 67 % more latent infections than ONFIT. Garcia-Benitez et al. (2017b) also detected that the qPCR-based method is more consistent than ONFIT because 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*. This makes qPCR a good method to detect latent infections and/or low DNA concentrations of *Monilinia* spp. Furthermore, the time required to detect the fungal pathogen in latently infected flowers and fruit using this qPCR-based method is between 24 and 48 h whereas the ONFIT method required 7 to 9 days of sample preparation and incubation plus additional time to identify the specific *Monilinia* spp. by PCR or another molecular method (Garcia-Benitez et al., 2017b). 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).

No unacceptable z -scores values for any of the qPCR platforms have been obtained, but not all the tested qPCR platforms performed as expected. Cross-detection of *M. fructicola* occurred in the Rotor-Gene Q system, while cross-detection of *M. laxa* appeared in four of the five qPCR platforms, all except LightCycler® 480 Real-Time PCR System. Different detection results between platforms is not always observed, for example, Agren et al. (2013) tested five qPCR platforms and methods to detect *Bacillus anthracis*, and they did not found differences between the results of ABI 7500 Fast Real-Time PCR and LightCycler® 480 Real-Time PCR Systems on their study. Little variability between results obtained with those two systems was also observed by Braun-Kiewnick et al. (2016) while testing a *Meloidogyne enterolobii* qPCR detection method across seven laboratories. However, other studies have reported some differences between qPCR platforms while studying virus or bacteria (Ebentier et al., 2013; Kamihiro et al., 2010). All these results seem to indicate that the influence of the qPCR platforms varies depending on the DNA (region of amplification, primers, and probes sequences; extraction matrices; organism; etc.), and makes patently important the need to test qPCR methods, in several platforms to determine their reproducibility. *M. laxa* cross-detection could be compensated with an allelic discrimination step. However, this is not a feature common to all qPCR platforms and it is normally not used for this purpose. Thus, we consider that the P_{fc} hydrolysis probe should be modified, to avoid this common cross-detection altogether.

Several inter-laboratory trials evaluating detection and diagnostic methods for plant pathogens, using molecular methods such as conventional PCR, qPCR or LAMP; have been done or are on-going inside the Euphresco initiative which encourages the cooperation among European diagnostic laboratories for method testing (EPPO, 2016b). Inter-laboratory trials are considered essential across several biological and chemical scientific disciplines for method validation, reproducibility determination, and robustness determination (Broeders et al., 2014; Brunelle et al., 2012; European Network of GMO Laboratories (ENGL), 2011; ISO, 2005; Magnusson and Örnemark, 2014; Thompson et al., 2006). In order to facilitate this validation, the present study only tested one DNA extraction method and one

qPCR method, and all reagents for the qPCR were provided by the scheme provider laboratory to the rest of the participants. In addition, a previous test performance study on *M. fructicola* and *M. laxa* mycelia detection by real-time PCR was carried out to provide the range of C_q-values for the qPCR method and the limit of detection (LOD).

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

4.7. *Monilinia* spp. LATENT INFECTION ACTIVATION DURING POSTHARVEST HANDLING CONDITIONS IN NECTARINES

4.7.1. Results

4.7.1.1. Brown rot and latent infection incidence after harvest

Both brown rot and latent infection incidence, and the frequency of *Monilinia* spp. isolated on fruit varied among cultivars, origin and years (Table 20). The highest incidence of brown rot (58.1 %) was recorded in 2012, followed by 2014 (43.3 %) and 2013 (26.6 %) (Table 20). Furthermore, the highest incidence of latent infection (20.8 %) was also recorded in 2012, followed by 2013 (10.0 %) and 2014 (6.7 %) (Table 20). *M. laxa* was the main isolated species in 2012 (99.0 % of the isolates) and 2014

4. RESULTS AND DISCUSSION

(89.3 % of the isolates), while *M. fructicola* was the main isolated species in 2013 (97.4 % of the isolates) (Table 20).

Table 20. Brown rot and latent infection incidence after harvest, and frequency of *Monilinia* spp. isolated on nectarine fruit from 2012, 2013 and 2014.

Year	Nectarine cultivar	Orchard location	Brown rot incidence (%)	Latent infection incidence (%)	Frequency of <i>M. fructicola</i> isolates (%)	Frequency of <i>M. laxa</i> isolates (%)
2012	Autumn Free	Sudanell	58.1	20.8	1.0	99.0
2013	Red Jim	Sudanell	26.6	10.0	97.4	2.6
2014	Alba Red	Ivars de Noguera	43.3	6.7	10.7	89.3

Fig. 41 shows the 2014 latent infection curve for fruit incubated at 25 °C for 20 days in a 16 h light photoperiod. The increase of latent infection incidence during the first 9 days of incubation at 25 °C was steeper than that observed in the following days of incubation at 25 °C.

Fig. 41. Latent infection incidence (%) throughout 20 days of fruit storage at 25 °C in a 16 h light photoperiod during the 2014 experiment.

4.7.1.2. Latent infections rates after postharvest process

4.7.1.2.1. Fruit confection effect on latent infection at postharvest

Thirty-two different postharvest handling combinations were tested in 2012, where the incidence of latent infection varied between 20 to 100 % and daily latent infection activation rate (%/d) between -48.96 to 6.22 (Table 21). While storage in both reception and expedition chamber at 4 °C and fruit dump in water had an effect over the incidence of latent infection, confection duration and temperature did not seem to have any influence on the incidence of latent infection (Table 21).

Table 21. Latent infection activation (%/d) of the 32 different postharvest handling combinations in 2012.

Storage on reception chamber at 4 °C (days)	Fruit dump in water	Confection duration (h)	Confection temperature (°C)	Storage on expedition chamber at 4 °C (days)	Latent infection activation rate (%/d)
0	No		-	0	0.00
		0	-	1	6.22
			-	10	0.62
		1	15	0	-6.89
		6	15	0	-2.04
	Yes	0	-	0	-48.96
1	No	0	-	0	1.09
	Yes	0		0	5.91
			15	1	0.53
				10	0.56
				0	0.38
			25	1	3.03
		6		10	0.08
				0	0.32
			15	1	0.48
				10	0.55
				0	0.32
			25	1	0.18
3	No	0	-	0	0.13
	Yes	0		0	0.00
			15	1	0.10
				10	-0.02
				0	-0.15
			25	1	0.10
		6		10	0.08
				0	0.12
			15	1	0.26
				10	0.08
				0	0.34
			25	1	0.00
				10	0.07

The PCA analysis confirmed that latent infection activation was only dependent on accumulated number of cold storage days and dump in water of fruit during postharvest (Fig. 42). Both accumulated number of cold storage days, and dump in water of fruit were on the positive side (Fig. 42). All components accounted for 100 % of variation. Components 1, 2 and 3 accounted for 43.96, 35.056, and 20.986 % of the variation, respectively (Fig. 42). No significant effect on latent infection activation was observed by break of cold storage for fruit confection.

Fig. 42. Principal component analysis of the total variation of brown rot latent infection activation rate (%/d) on fruit, and postharvest processes (accumulated number of cold storage days, break of cold storage for fruit confection, and dump in water of fruit) in 2012. All components accounted for 100 % of variation. Components 1, 2 and 3 accounted for 43.96, 35.056, and 20.986 % of the variation, respectively.

4.7.1.2.2. Dump in water of fruit and days of cold storage effects on latent infection during postharvest

The postharvest handling combinations studied in 2013 and 2014 are represented in combination with the progression of the latent infection of fruit constantly incubated at 25 °C for 20 days in Fig. 43. Nectarines dump in water showed a reduction in latent infection incidence (Fig. 43) [treatment 1]. Cold storage at 4 °C also reduces the incidence of latent infections for the first 10 days (Fig. 43) [treatments 2, 3, 4, 5, 7, and 8]. When the combined duration of cold storage between the storage in the reception chamber and the storage in the expedition chamber is equal to or greater than 11 days no differences

on the incidence of latent infection were observed between the postharvest treatments and fruit stored constantly at 25 °C (Fig. 43) [treatments 6 and 9].

Fig. 43. Latent infection incidence (%) comparison between incubating fruit continuously at 25 °C in a 16 h photoperiod (line), and the 9 different postharvest handling combinations and control (Table 8) (bars).

Latent infection activation rates (Table 22) shown that dumping the nectarines in water significantly reduced the latent infection activation rate. Additionally, postharvest treatments 1, 2, 3, 4, and 8 at both 75 and 100 % RH, treatment 7 at 75 % RH, and treatments 5 and 9 at 100 % RH had a significantly reduced activation rates than the control treatments (Table 22). Whereas in the rest of the treatments no significant differences were observed between the latent infection rates of the treatments and their controls (Table 22). No significant differences appear between equivalent treatments at different RH (Table 22). There was a relationship between total number of days of cold storage (sum of the days of cold storage on the reception chamber and the expedition chamber) and the latent infection activation rate, the higher the total number of days in cold storage the higher the latent infection activation rate (Table 22).

Table 22. Latent infection rates depending on the different postharvest treatments in 2013 and 2014.

RH (%)	Treatment ID	Dump in water	Days of cold storage on the reception chamber	Days of cold storage on the expedition chamber	Latent infection rate (%/day)	
75	Control	No	0	0	0.00	d
	1	Yes	0	0	-3.31 ± 0.0	a
	2			3	-0.02 ± 0.0	c
	3			10	-0.01 ± 0.0	c
	4		1	0	-0.07 ± 0.0	b
	5			3	$-0.008 \pm 4.3 \times 10^{-3}$	cd
	6			10	$-0.003 \pm 1.5 \times 10^{-3}$	cd
	7		3	0	-0.02 ± 0.0	c
	8			3	$-0.006 \pm 2.9 \times 10^{-3}$	c
	9			10	$0.01 \pm 8.5 \times 10^{-3}$	cd
100	Control	No	0	0	0.000	d
	1	Yes	0	0	-3.3 ± 0.0	a
	2			3	-0.02 ± 0.0	c
	3			10	$-0.003 \pm 1.7 \times 10^{-3}$	c
	4		1	0	-0.07 ± 0.0	b
	5			3	-0.02 ± 0.0	c
	6			10	$-0.003 \pm 1.6 \times 10^{-3}$	cd
	7		3	0	$-0.01 \pm 5.7 \times 10^{-3}$	cd
	8			3	-0.01 ± 0.0	c
	9			10	-0.005 ± 0.0	c

Values are given as the median latent infection rate $\pm$ standard error. Values followed by a different letter are statistically different with a 95 % confidence according to the Krustal-Wallis test.

4.7.2. Discussion

When the fruit is kept at room temperature, the activation of latent infections on nectarines occurred mainly during the first 9 days after harvest. Latent infections due to *Monilinia* spp. have been detected in nectarines, and a high correlation between postharvest brown rot and latent infection exists in fruit at pre-harvest (Gell et al., 2008; Villarino et al., 2012b). However, to our knowledge, this is the first time that the activation of latent infections in postharvest conditions has been described.

Postharvest handling can reduce the activation of *Monilinia* latent infections, especially dump in water of fruit and cold storage. Dumping the nectarines in water could wash *Monilinia* conidia on fruit

surface, but considering that the nectarines used in this study were surface-disinfected, this could not be the case since there were no *Monilinia* in the surface to be washed away with the water. Additionally, fruit water dumping increases wetness and water availability, both factors reported to favor conidial germination and increase latent infection (Luo et al., 2001a; Tamm and Flückiger, 1993). However, during latent infections, *Monilinia fructicola* has a slowed subepidermal hyphal growth (Garcia-Benitez et al., 2016), and submerging the fruit on water will reduce the oxygen available for the fungal cells and increase the fruit cells turgor, hindering mycelial growth. Fruit cold storage has been described as the best method to reduce the growth of several fruit pathogens. Significant reduction on brown rot latent infection incidence was observed for those postharvest handling combinations where the combined storage duration was 10 days or shorter, but no reduction was observed for those combinations with a combined storage duration longer than 10 days. In a previous in vitro study we also reported that *Monilinia* germination increased with the period between fruit arrival times and their processing at fruit processing units (Garcia-Benitez et al., 2017a).

Differences on *Monilinia* latent activation rates were not observed between different durations of cold storage-breaks for fruit confection. In a previous in vitro study we observed that stopping the cold storage for fruit processing had an increasing effect over *Monilinia* conidia germination, but only when conidia were stored in the expedition chamber for at least 3 days (Garcia-Benitez et al., 2017a). This effect was not present on latent infections on in vivo conditions.

Reduction of latent infection activation during postharvest handling could depend on the value of latent infection at harvest. Latent infection incidence has varied between years. The values observed during this study were within the range established in previous studies (0 to 30 %) for incidence of *Monilinia* latent infection (Emery et al., 2000; Luo and Michailides, 2001; 2003; Luo et al., 2001a). However, the highest latent infection incidence values from 2012 represented higher reduction effects of fruit dump in water and lower reduction effects of cold storage, than those from 2013 and 2014.

The postharvest handling combinations affected latent infections activations of both *Monilinia* species to the same extent. No significant differences have been observed between 2013 and 2014, although *M. fructicola* was the predominant species on 2013, and *M. laxa* on 2014. Conidial germination, growth, and sporulation of *Monilinia* spp. are dependent on ambient temperature, relative humidity (RH), and water availability (water activity: aw) (Luo et al., 2001a; Tamm and Flückiger, 1993; Xu et al., 2001). *M. laxa* and *M. fructicola* have their own competitive edge for conidial germination and mycelial growth in different environmental conditions (Garcia-Benitez et al., 2017a). This competitive edge becomes advantageous for *M. fructicola* when temperature is between 10 and 25 °C and for *M. laxa* when temperature is between 4 and 10 °C (Garcia-Benitez et al., 2017a).

In this study no statistically significant differences were found between *Monilinia* latent infection activation rates, when nectarines were stored at different RH conditions (75 % and 100 %). With the results of a previous in vitro study we hypothesized that reducing the RH in the refrigeration chambers could reduce the incidence of brown rot in harvested peaches and nectarines (Garcia-Benitez et al., 2017a). The previous study looked at the *Monilinia* spp. germination and appressorial formation capacities, while the present study is centered on latent infections. We have also reported that during latent infections *Monilinia* mycelia is located below the nectarine's epidermis, and mycelial growth takes place when nectarines are incubated at 4 °C (Garcia-Benitez et al., 2016). While growing inside the nectarine's epidermis, *Monilinia* mycelia is growing in a high RH environment and protected from the storage chamber RH, which would only affect the conidial germination and mycelial growth of the *Monilinia* cells on the epidermis surface.

Both fruit dump in water and short postharvest handling periods are key factors on reducing *Monilinia* spp. latent infection activation rates on nectarines. With the results of a previous in vitro study we hypothesized that reducing the duration of cold storage could reduce the incidence of brown rot in harvested peaches and nectarines (Garcia-Benitez et al., 2017a). This means that the period between

fruit arrival times and their expedition to markets affects both the latent infection activation rates and incidence, and *Monilinia* germination. Hence the shorter the storage in cold chambers the better control of brown rot during postharvest. Taking these factors into account it could be interesting setting a method to detect incidence of latent infection at fruit arrival to allocate those fruit with higher latent infections to the shortest postharvest handling combinations to minimize fruit losses due to latent infection activation.

5. CONCLUSIONS

*EPIDEMIOLOGÍA Y CONTROL DE LA PODREDUMBRE PARDA DEL
MELOCOTÓN EN POSTCOSECHA*

1. Thanks to the in vitro study on the effect of the maturity state and environmental conditions over the pre-penetration stages of *Monilinia* spp. infections, it is concluded that:

- Both *M. fructicola* and *M. laxa* can germinate and develop under postharvest environmental conditions.
- *M. laxa* has a competitive advantage over *M. fructicola* during postharvest cold storage conditions, while, while *M. fructicola* germinates better than *M. laxa* at their optimal growing temperature (25 °C).
- *Monilinia* spp. form appressoria to face adverse environmental conditions such as: fruit immaturity, temperatures lower than 25 °C, and RH lower than 80 %.
- In order to reduce *Monilinia* spp. new infections during postharvest the period between fruit arrival and their processing at fruit processing units, the RH in the refrigeration chambers, and the total postharvest duration, had to be reduced.

2. By studying the phytotoxins and degrading enzymatic activities of *M. fructicola*, *M. fructigena*, and *M. laxa* it is concluded that:

- *Monilinia* species can produce phytotoxins and litic enzymes to penetrate and colonize stone fruit.
- Phytotoxic activity appeared in butanol extracts of juice of nectarines infected with all *Monilinia* species and in ethyl acetate extract of juiced of nectarines infected with *M. fructicola*.
- Phytotoxic fungal metabolites of *M. fructicola*, *M. fructigena*, and *M. laxa* present on those extracts have molecular weights between 329 and 387 g mol⁻¹.
- The three *Monilinia* species have α -glucosidase, pectin-lyase, polygalacturonase, protease, and xylanase enzymatic activities in culture media; *M. fructicola* and *M. laxa* also have cutinase activity in those conditions.

3. Thanks to the microscopic analysis of latent and visible *M. fructicola* latent infections it is concluded that:

- *M. fructicola* conidia form long germ tubes around the guard cells of stomata on the surface of mature fruit.
- *M. fructicola* does not form appressoria on the surface or inside epidermal cells of mature nectarines.
- *M. fructicola* is active during latent infections because slow and progressive colonization of nectarine cells by the fungus occurs.
- However, not all of its metabolic functions are active since its colonization was limited to the intercellular spaces of the epidermis and the first two subcuticular cell layers, whereas during visible infections *M. fructicola* colonize inter- and intracellularly epidermal and deep subdermal tissues.

4. After the *M. fructicola* and *M. laxa* fungal transformation efforts it is concluded that:

- *M. fructicola* and *M. laxa* conidia have a variable number of nuclei, ranging from 4 to 7, with an average diameter of 1.5 μm .
- The multinucleated and heterokaryotic nature of *Monilinia* spp. conidia, hyphae and protoplast, difficults fungal transformation, using either CaCl_2/PEG , electroporation or lithium acetate.
- Even though transformation of *Monilinia* spp. isolates was not possible by heterologous nor by homologous recombination. It was possible to transform *Penicillium rubens* with the vectors generated for the heterologous recombination of *Monilinia*. *P. rubens* transformants were phleomycin resistant and expressed one of the fluorescence proteins GFP or mCherry.

5. After developing the qPCR-based method to detect and identify latent brown rot infections in nectarine flowers and fruit caused by *M. fructicola*, *M. fructigena* and *M. laxa* it is concluded that:

- The qPCR method can detect the pathogen in artificial and natural latent brown rot infections on nectarine's flowers and fruit. The method can be used for early detection of the pathogen, both on brown rot control strategies and in those countries where *Monilinia* spp. are classified as quarantine fungal pathogens.
- *M. fructicola* induced a higher number of latent infections in nectarine flowers than *M. fructigena* and *M. laxa*.
- *M. laxa* is able to colonize epidermal and mesocarpic tissues of nectarines during latent infection while, *M. fructicola* mainly remains colonizing epidermal tissues.
- The qPCR method is more reliable and consistent, and faster than ONFIT, and it has a low limit of detection, less than 27 fg of DNA (approximately 1 *M. laxa* cell).

6. After studying the proficiency of the latent *Monilinia* spp. infection detection qPCR-based method in an international collaborative assay it is concluded that:

- All tested qPCR platforms detect *Monilinia* latent infections and mycelia on nectarine flower and fruit samples.
- The hydrolysis probes of the method were not specific across all the qPCR platforms, but these specificity problem could be compensated with an allelic discrimination step to the qPCR run.

7. By studying the activation of *Monilinia* spp. latent infections during postharvest conditions in nectarines it is concluded that:

- The activation of latent infections on nectarines stored at room temperature occurs during the first 9 days after harvest.

5. CONCLUSIONS

- Postharvest handling reduces the activation of *M. fructicola* and *M. laxa* latent infections to the same extent.
- Fruit dump in water to the confection line and cold storage durations shorter than 11 days reduce brown rot latent infection activation.
- However, no differences on *Monilinia* latent infection activation rates were seen between 75 and 100 % RH conditions in nectarines.

6. CONCLUSIONES

*EPIDEMIOLOGÍA Y CONTROL DE LA PODREDUMBRE PARDA DEL
MELOCOTÓN EN POSTCOSECHA*

1. Tras estudiar in vitro el efecto de la madurez de la fruta y de las condiciones ambientales sobre los estadios de pre-penetración de las infecciones de *Monilinia* se concluye que:

- Tanto *M. fructicola* como *M. laxa* son capaces de germinar y desarrollarse bajo las condiciones ambientales de postcosecha.
- *M. laxa* tiene una ventaja competitiva sobre *M. fructicola* durante la postcosecha ya que germina mejor a temperaturas bajas (4 °C), mientras que *M. fructicola* germina mejor que *M. laxa* a temperaturas óptimas (25 °C).
- Las especies de *Monilinia* forman apresorios para enfrentarse a condiciones ambientales adversas como son la inmadurez de la fruta, las temperaturas inferiores a 25 °C y las humedades relativas inferiores al 80 %.
- Para disminuir el número de nuevas infecciones producidas durante la postcosecha hay que reducir: el periodo comprendido entre la llegada de la fruta a la central hortofrutícola y su procesamiento, la humedad relativa de las cámaras de refrigeración y la duración total del periodo de postcosecha.

2. Tras estudiar las fitotoxinas y enzimas líticas de *M. fructicola*, *M. fructigena* y *M. laxa* se concluye que:

- Las especies de *Monilinia* pueden producir tanto fitotoxinas como enzimas líticas para penetrar y colonizar la fruta de hueso.
- Los extractos de butanol del zumo de nectarinas infectadas con las tres especies de *Monilinia* y el extracto de acetato de etilo del zumo de nectarinas infectadas con *M. fructicola* tienen actividad fitotóxica.
- Los metabolitos fitotóxicos de *M. fructicola*, *M. fructigena* y *M. laxa* en dichos extractos tienen un peso molecular comprendido entre 329 y 387 g mol⁻¹.

- Las tres especies de *Monilinia* presentan en medio de cultivo actividad enzimática α -glucosidasa, pectin-liasa, polygalacturonasa, proteasa y xilanasas, adicionalmente *M. fructicola* y *M. laxa* presentan actividad enzimática cutinasa en estas condiciones.

3. Tras los estudios de microscopía óptica y electrónica de transmisión de las infecciones latentes y visibles de *M. fructicola* se concluye que:

- Las conidias de *M. fructicola* forman largos tubos germinativos alrededor de las células guarda de los estomas de nectarinas maduras.
- *M. fructicola* no forma apresorios en las nectarinas maduras, ni en la superficie ni en el interior de las células epidérmicas de las mismas.
- *M. fructicola* permanece activa en las infecciones latentes, colonizando de forma lenta pero progresiva las células epidérmicas y subepidérmicas de las nectarinas.
- Sin embargo, no todas sus funciones metabólicas están activas, ya que solo coloniza intercelularmente dichas células, mientras que en las infecciones visibles *M. fructicola* coloniza inter e intracelularmente tanto células subepidérmicas como células del mesocarpo.

4. Tras los intentos de transformación de *M. fructicola* y *M. laxa* se concluye que:

- Las especies de *Monilinia* cuentan con un número variable de núcleos en sus conidias, entre 4 y 11, que tienen un diámetro medio de 1,5 μm .
- La naturaleza multinucleada y heterocarionte de las conidias, hifas y protoplastos de *M. fructicola* y de *M. laxa* dificulta la transformación de estos hongos, ya sea mediante el uso de CaCl_2/PEG , electroporación o acetato de litio.
- No se consiguió transformar *Monilinia* ni por recombinación homóloga, ni por recombinación heteróloga. Sin embargo, si fue posible transformar *Penicillium rubens*, con los plásmidos generados para la recombinación heteróloga de *Monilinia*; consiguiéndose transformantes que

expresaban tanto resistencia a fleomicina como una de las dos proteínas fluorescentes, GFP o mCherry.

5. Tras poner a punto un método de PCR en tiempo real para la detección de infecciones latentes de *Monilinia* tanto en flores como en frutos de nectarino, y la identificación de la especie de *Monilinia* causante de dichas infecciones se concluye que:

- El método de qPCR es capaz de detectar infecciones latentes artificiales y naturales causadas por *Monilinia* en flores y frutos de nectarino. Este método puede aplicarse para la detección temprana del patógeno, tanto en estrategias de control de podredumbre parda como en la detección del mismo en controles fronterizos donde las especies de *Monilinia* sean consideradas patógenos de cuarentena.
- *M. fructicola* induce mayor número de infecciones latentes en flores de nectarino que *M. fructigena* y *M. laxa*.
- *M. laxa* coloniza la epidermis y el mesocarpo de las nectarinas durante las infecciones latentes, mientras que *M. fructicola* coloniza principalmente la epidermis.
- El método de detección de infecciones latentes mediante qPCR es más fiable, consistente y rápido que el método ONFIT y tiene un límite de detección inferior a 27 pg de ADN, lo que corresponde aproximadamente a una célula de *M. laxa*.

6. Tras estudiar la reproducibilidad del método qPCR de detección de infecciones latentes de *Monilinia* se concluye que:

- Las cinco plataformas de PCR en tiempo real utilizadas detectan las infecciones latentes causadas por *Monilinia* en flores y frutos de nectarino.
- Las sondas hidrolíticas del método de qPCR no son específicas en algunas de las plataformas de PCR en tiempo real utilizadas, pero esto se puede compensar mediante la discriminación alélica.

7. Tras estudiar la activación de las infecciones latentes de *Monilinia* en nectarinas durante la postcosecha se concluye que:

- En las nectarinas almacenadas tras su recolección a temperatura ambiente, se activan la mayor parte de las infecciones latentes de *Monilinia* durante los primeros 9 días de incubación.
- El manejo durante la postcosecha reduce la activación de las infecciones latentes de *M. fructicola* y *M. laxa* en la misma medida.
- El volcado en agua a la línea de confección, y los periodos de refrigeración de la fruta inferiores a 11 días reducen la activación de las infecciones latentes.
- Sin embargo, no se observa ninguna reducción en la activación de infecciones latentes al reducir la humedad relativa en las cámaras de refrigeración de un 100 % a un 75 %.

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ANNEX 1: MICROSCOPIC ANALYSES OF LATENT AND VISIBLE *Monilinia fructicola* INFECTIONS IN NECTARINES

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RESEARCH ARTICLE

Microscopic Analyses of Latent and Visible *Monilinia fructicola* Infections in Nectarines

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Abstract

Little is known about the histologic features of a latent *Monilinia fructicola* infection and brown rot in infected fruit. This report informs on the results of an investigation whose aim was to analyze the microanatomy of nectarines with a latent and visible *M. fructicola* infection. Mature nectarines were inoculated with an *M. fructicola* isolate and incubated at 25°C for 0, 24, 48, 72, or 96 hours in the dark. For investigating the latent infection process, the inoculated nectarines were first incubated at 25°C for 24 hours in the dark and then incubated at 4°C for 72, 144, 216, and 288 hours in the dark. At the end of the incubation, samples of nectarine tissue were excised from the inoculation points and prepared for light and transmission electron microscopic examinations. No signs of disease were seen on the surface of nectarines with a latent infection over the 288-hour incubation period. When the tissue samples were microscopically examined, *M. fructicola* colonized the stomata and this stomatal colonization progressively increased over time and was associated with gradual collapse of the epidermal cells and colonization of the subepidermis. In nectarines with visible brown rot, the disease usually appeared after 24 hours on the surface and in the uppermost layers of epidermal cells, which began to collapse after 48 hours. Subsequently, the diseased tissues of the nectarines displayed (a) colonization of the epidermis and mesocarp by *M. fructicola* with thin and thick hyphae, (b) collapse and disruption of epidermal and mesocarpic cells, (c) lysogenic cavities in the subepidermis and mesocarp, (d) degradation of the cuticle and epidermis, and (e) *M. fructicola* sporulation. *M. fructicola* is active during latent infections because slow and progressive colonization of nectarine subcuticular cells by the fungus occurs.

Introduction

Brown rot is an economically important fungal disease of peaches (*Prunus persica* (L.) Batch) and nectarines (*P. persica* var. nectarina (Aiton) Maxim), and is responsible for substantial pre-harvest and post-harvest losses [1]. In Spain, brown rot is caused by three *Monilinia* spp.: *M. fructicola* (G Winter) Honey, *M. fructigena* (Aderhold & Ruhland) Honey, and *M. laxa* (Aderhold & Ruhland) Honey [2], and of these three fungi, *M. fructicola* has the fastest growth rate and is the most aggressive one [3]. Although fruit can be infected with *Monilinia* spp. at

any stage of its development, disease incidence increases and the index of disease severity becomes greater with fruit ripening [4–6]. Brown rot on ripening or mature fruit typically develops as a rapidly spreading, firm, brown decay. Under optimum conditions, the decay of ripe infected peach and nectarine fruit may become visible within 48–72 hours of infection [2].

The fruit surface is a natural barrier against infection and must be penetrated before a pathogen can cause infection. For fungal infections of fruit, conidia must first be deposited on and adhere to the fruit surface before penetrating the surface through the natural openings and/or injured areas of the fruit surface [7,8]. For *Monilinia* spp., airborne conidia are first deposited on the fruit surface and penetration is implemented by germ tubes and/or appressoria [2] through stomata and/or natural cracks on fully mature (late stage III) fruit [9]. When the climatic conditions are unfavorable, *Monilinia* infections may remain latent until the conditions for disease development become favorable [2,10,11]. Latent infections have been described as a dynamic equilibrium between the host, pathogen, and environment without any visible signs of disease [5,12], or as an invisible infection that is chronic and in which a host and parasite relationship has been established [13]. Other authors differentiate between latent (non-visible) and dormant and quiescent (visible) infections in which a host-parasite relationship has been established [14]. Although a pathogen has a low metabolic level in a latent infection, pathogenicity factors may be activated to end the period of latent infection when conditions for disease development become conducive.

Fungal pathogens usually develop structures, such as appressoria, germ tubes, or hyphae, for penetrating and causing a latent infection in a host [15]. Intercellular hyphae have been described in a latent infection of (a) mangos with an *Alternaria alternate* infection [16], (b) mangos [17], avocados [18], citrus fruit [19], and blueberries [20] with a *Colletotrichum gloeosporioides* infection, and (c) soybeans [21] with a *Phialophora gregata* infection. Appressoria have been described in a latent infection of (a) unripe bananas [22] with a *C. musae* infection, (b) tangerines [19,23] and papayas [24] with a *C. gloeosporioides* infection, and (c) plums [25] with an *M. fructicola* infection. *Botrytis cinerea* uses an infection peg, which develops from a germinated appressorium to penetrate the nectarine or plum cuticle in a latent infection [26].

Latent infections due to *Monilinia* spp. have been detected in nectarines and plums [27,28], and a high correlation between post-harvest brown rot and latent infection exists in some stone fruit, such as peaches, plums, and cherries [5,6,12,29,30]. We have previously reported that the incidence of latent infection and that of post-harvest brown rot are positively correlated increasing both of them along the crop season: the average incidence of latent infection during the crop season in Spanish peach orchards explains 55% of the total variation in the incidence of post-harvest brown rot [5]. Gell et al. [5] have also developed a model of latent *Monilinia* infection in which temperature (T) and wetness duration (W) can be used for predicting disease outbreaks. Results of this multiple regression analysis indicate that T and W account for 83% of the total variation in the incidence of latent infection.

Little is known on the infection process during its transition from a latent *M. fructicola* infection to visible brown rot caused by *M. fructicola* in nectarines. Therefore, we conducted an investigation whose aim was to analyze the microanatomy of nectarines with a latent and visible *M. fructicola* infection using light and transmission electron microscopy.

Materials and Methods

Fungal cultures and conidial production

An isolate of *M. fructicola* (ALF 2009 COS 5R7), which was originally collected from a harvested nectarine in Alfarras, Lleida, Spain in 2009, was used in the investigation. The isolate was grown on potato dextrose agar (PDA) plates (Difco Laboratories, Detroit, MI, USA) at 20–

25°C in the dark for seven days for conidial production. The isolate was stored either as a culture on PDA at 4°C for short-term storage or as a conidial suspension (see later for preparation) in 20% glycerol at -80°C for long-term storage.

Conidial suspensions were prepared using conidia that were harvested from the PDA plates 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-second sonication in an ultrasonic bath (J.P. Selecta S.A., Barcelona, Spain). The filtrate was adjusted to the desired conidial concentration using SDW after counting the number of conidia using a hemocytometer and a light microscope (Zeiss Axioskop 2; Carl Zeiss, Inc., Oberkochen, Germany).

Conidial germination on the nectarine's epidermis

Samples of the epidermis (about 0.5 cm² and 0.5–1 mm thick) of mature nectarines were obtained from surface-disinfected fruit using a sterilized disposable scalpel. To this end, the fruit surface was disinfected by first immersing the fruit in a 0.1% sodium hypochlorite solution for five minutes, followed by immersion in a 70% ethanol solution for one minute, and finally two rinses of the fruit in SDW. Samples of the epidermis were placed on the surface of 2% water agar plates (Laboratorios Conda S.A., Torrejón de Ardoz, Madrid, Spain). An aliquot (5 µl) of the conidial suspension (3×10^5 conidia ml⁻¹) was then deposited on each sample, and the samples were then incubated at 25°C or 4°C for four or 24 hours in the dark. At the end of the incubation, the samples were cleared by immersion in a 3:1 (v/v) mixture of ethanol and chloroform which contained 0.15% (w/v) trichloroacetic acid for 15 minutes at 70°C, then immersed in a solution of phenol:water:glycerine:lactic acid (1:1:2:1 v/v) for two minutes, and then stained with lactophenol blue (Sigma-Aldrich Química SL, Madrid, Spain). After staining, each sample was mounted on glass slides in a solution of phenol:water:glycerine:lactic acid (1:1:2:1 v/v). Images of the specimens were examined after their capture by a Leica DFC550 camera, which was attached to a Leica DMRE microscope (Leica Microsystems, Wetzlar, Germany). Three samples were made for each temperature and incubation time. The controls were samples of the epidermis from uninoculated nectarines and the entire assay was repeated twice.

Visible infection studies: fruit inoculation and incubation

Mature nectarines were used to investigate the visible infection process. For this purpose, the surface of the nectarines was first disinfected, as previously described, and then dried for two hours in a laminar flow cabinet. Once dried, four 30-µl drops of a conidial suspension (10^6 conidia ml⁻¹) were placed at different points on the surface close to the insertion point of the peduncle. The inoculated fruit were kept in the laminar flow cabinet until the drops evaporated. The fruit were then transferred to dry plastic plates in a humidity chamber at 100% relative humidity (RH) and incubated at 25°C for 0, 24, 48, 72, or 96 hours in the dark. One fruit was used for each incubation time. The controls were fruit which were inoculated with SDW and the entire assay was repeated twice.

Latent infection studies: fruit inoculation and incubation

Mature nectarines were used to investigate the latent infection process. For this purpose, the surface of the nectarines was first disinfected, as previously described, and then dried for two hours in a laminar flow cabinet. Once dried, four 30-µl drops of a conidial suspension (10^6 conidia ml⁻¹) were placed at different points on the surface close to the insertion point of the peduncle. The inoculated fruit were then transferred to plates in polystyrene boxes which were lined with sterilized filter paper that was moistened with SDW and incubated at 25°C for 24

hours in the dark. After completion of the incubation, the surface of the inoculated nectarines was again disinfected, as previously described, and then dried for two hours in a laminar flow cabinet. Once dried, the fruit were transferred to dry plastic plates in a humidity chamber at 100% RH and incubated at 4°C for 72, 144, 216, and 288 hours in the dark. The controls were uninoculated fruit and the entire assay was repeated twice.

Light microscopic analysis of nectarines with a visible or latent *M. fructicola* infection

At the end of the incubation, a sample of nectarine tissue (0.1 cm x 1 cm x 1 cm) from each of the four inoculation points was excised from the inoculated nectarines for examination by light microscopy. To this end, each sample was fixed by immersion in a solution of formaldehyde-acetic acid-alcohol (ethanol:acetic acid:formaldehyde:water, 50:10:35:5, v/v/v/v). To facilitate fixative infiltration, the samples were first degassed by maintaining them in a mild vacuum for two hours and then overnight incubation in a formaldehyde-acetic acid-alcohol solution at 4°C. Following the overnight incubation, the samples were first gradually dehydrated in graded ethanol (50, 70, 80, and 100%—once for 60 minutes at each step) and then soaked in a graded series of ethanol:HistoClear II (National Diagnostics, Atlanta, GA, USA) (3:1, 1:1, 1:3, 0:1 v/v—once for 120 minutes at each step) to remove the alcohol. The samples were then embedded in paraffin by first immersing each sample in a graded series of molten paraffin: HistoClear II at 60°C (Histo-comp, Casa Alvarez, Madrid, Spain) (1:3, 1:1, 3:1 v/v—once for 60 minutes at each step) and then in molten paraffin for at least 48 hours. Five sections (15-μm thick) of each sample were prepared using a microtome (Microtome pfm medical ag, Köln, Germany), mounted on glass slides, and then stained with Safranin O-Fast-green stain using a previously described protocol [31] or Calcofluor White fluorescent stain (Sigma-Aldrich Co., St. Louis, MO, USA) according to the manufacturer's instructions. Images of each microsection were examined after their capture by a Leica DFC550 camera, which was attached to a Leica DMRE microscope (Leica Microsystems, Wetzlar, Germany).

Transmission electron microscopic (TEM) analysis of nectarines with a visible *M. fructicola* infection

At the end of the incubation, four samples (2 cm x 2 mm) of the dermal and ground tissues from infected and uninfected nectarines were collected using a sterilized disposable scalpel for examination by TEM. To this end, each sample was first fixed by immersion in modified Karnovsky's fixative solution which contained 2.5% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde in sodium phosphate buffer (pH 7.0) at 4°C for six hours, and then washed four times for ten minutes in Karnovsky's fixative solution. Each sample was postfixed in a 1% (w/v) aqueous osmium tetroxide solution at 20°C for two hours, and then washed three times in SDW. Each sample was gradually dehydrated in graded acetone (30%, 50%, 70%, 80%, and 95%—once for 15 minutes at each step), twice in 100% acetone for 15 minutes, embedded in Spurr resin, and polymerized at 65°C for 48 hours according to a previously described protocol [26]. Ultrathin sections (15-μm thick) of each sample was prepared using an ultramicrotome with a diamond knife (Ultracut Reichert, Vienna, Austria) and then mounted on copper grids. The grids were counterstained with 2% (w/v) uranyl acetate for 20 minutes and with Reynolds' lead citrate for four minutes before being examined using a TEM (JEM-1010, LEOL Ltd., Tokyo) which was operated at 80 kV. At least four embedded blocks were prepared for each incubation time, and more than ten ultrathin sections per block were examined by TEM.

Results

Conidial germination and hyphal growth on the nectarine's epidermis

Fig 1 contains representative light microscopic images of *M. fructicola* germination and hyphal development on the nectarine's epidermis after a 4-hour and 24-hour incubation at 25°C or 4°C. Clusters of *M. fructicola* conidia were observed around the guard cells of the stomata and some of these conidia had germinated four hours after inoculation when the nectarines were incubated at 25°C (Fig 1A and 1B). The germ tubes grew progressively into long hyphae on the surface between a 4-hour, a 16-hour (Fig 1C and 1D), and a 24-hour (Fig 1E) incubation at 25°C (Table 1), and the hyphae penetrated the surface mostly through stomata (Fig 1B and 1F). A lower number of conidia were observed in areas without stomata (Fig 1A and 1D). When the

Fig 1. Germination on and penetration of the surface of mature nectarines by *M. fructicola* conidia and hyphae. The nectarine's tissues and fungal structures were stained with lactophenol blue (A, B, E and F) or Calcofluor White fluorescent stain (C and D). A–B: Clusters of non-germinated (blue) and germinated (arrow) *M. fructicola* conidia (c) around the guard cells of stomata (s) on the surface of mature fruit after a 4-hour incubation at 25°C. C and D: Conidia and long germ tubes (h) on the surface of mature fruit after a 16-hour incubation at 25°C. Scale bar = 50 µm. E: Long germ tubes on the surface of mature fruit after a 24-hour incubation at 25°C. F: thick ramified hyphae (rh) on the surface of mature fruit after a 24-hour incubation at 4°C.

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Table 1. Timeline of tissue changes in nectarines with a visible and a latent *M. fructicola* infection.

		Initial observations of the infection process 24 hours after inoculation at 25°C (t = 0)	Times of appearance of signs of visible infection (hours after t = 0, at 25°C)			Times of appearance of signs of latent infection (hours after t = 0, at 4°C)			
			24	48	72	72	144	216	288
Visible signs of disease	Epidermal discoloration	-	+	+	+	-	-	-	-
	Brown spots and brown rot	-	+	+	+	-	-	-	-
	Sporulation	-	-	-	+	-	-	-	-
	Diameter of macroscopic lesion (cm)	-	1	2.5	5	-	-	-	-
Fungal infection structures and their location	Conidial germination on fruit surface	+	+	+	+	-	-	-	-
	Germ tube growth	+	+	+	+	-	-	-	-
	Thin hyphae in the substomatal cavity	+	+	+	+	+	+	+	+
	Thin hyphae in the epidermis	-	+	+	+	-	-	+	+
	Thin hyphae in the mesocarp	-	-	+	+	-	-	-	-
	Thick hyphae in the epidermis and mesocarp	-	-	-	+	-	-	-	-
	Sporulation	-	-	-	+	-	-	-	-
Light microscopic analysis	Partial cuticular degradation	-	+	+	+	-	-	-	-
	Cell degradation in the substomatal cavity	+	+	+	+	-	+	+	+
	Collapse and disruption of epidermal cells	-	+	+	+	-	-	+	+
	Formation of lysogenic cavities at subepidermal level	-	-	+	+	-	-	-	-
	Collapse and disruption of mesocarpic cells	-	-	+	+	-	-	-	-
	Formation of lysogenic cavities at mesocarpic level	-	-	+	+	-	-	-	-
	Cellular degradation and cuticular breakdown	-	-	-	+	-	-	-	-
Transmission electron microscopic analysis	Germination and adherence of conidia to fruit surface	+	+						
	Thin intramural hyphae in epidermal cells; cell wall degradation	+	+						
	Thick intercellular and intracellular hyphae in mesocarpic cells; cell wall degradation and cell death	-	+						

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inoculated nectarines were incubated at 4°C, thick ramified hyphae were observed on the surface after a 24-hour incubation (Fig 1E). Appressoria were not seen on the surface or in the tissues after a 4-hour and 24-hour incubation at 25°C or 4°C.

Table 1 summarizes the timeline of tissue changes in nectarines with a latent or a visible *M. fructicola* infection according to the results of the light microscopic and TEM analyses. For nectarines with visible brown rot, the signs of brown rot appeared within 48 hours on the surface of infected nectarines as brown spots on the surface at the inoculation sites and then became smooth grey spots after 72 hours. Thereafter, the disease developed over the surface and in the parenchyma. For nectarines with a latent infection, no signs of disease were seen on the nectarine's surface over the 288-hour incubation period at 4°C.

Light microscopic analysis of tissues from nectarines with a visible *M. fructicola* infection

Fig 2A and 2B display representative light microscopic images of epidermal, subepidermal, and mesocarp cells from uninoculated nectarines and Fig 2C–2J display representative images of diseased tissue from nectarines with visible signs of a *M. fructicola* infection. In nectarines with visible infection, signs of the disease usually appeared after 24 hours on the surface, preferentially in areas which surround stomata but they also appeared in stomata-free areas (Fig 2C and 2D; Table 1). The brown rot became evident in the uppermost layers of epidermal cells below the cuticle and these epidermal cells began to collapse after 48 hours (Fig 2E and 2F;

Fig 2. Light microscopic analysis of tissues from nectarines with a visible or latent *M. fructicola* infection. The nectarine's tissues were stained with Safranin O-fast Green stain and the unaffected walls of the nectarine's cells are blue and the affected nectarine's cells, and the hyphae and conidia of *M.*

fructicola are red: A, B and K: representative images of epidermal, subepidermal and mesocarp cells from uninoculated nectarines; C and D: signs of *M. fructicola* infection in areas which surround the stomata and in stomata-free areas of nectarines with a visible infection after a 24-hour incubation at 25°C. E and F: invasion and collapse of the uppermost layer of epidermal cells of nectarines with a visible infection after a 48-hour incubation at 25°C. G and H: Collapse of epidermal cells, apparition of lysogenic cavities in the mesocarp, and extensive colonization of the deep subdermal tissues by *M. fructicola* of nectarines with a visible infection after a 72-hour incubation at 25°C. I and J: Colonization of the epidermis and mesocarp by *M. fructicola* with thin and thick hyphae, collapse and disruption of the nectarine's epidermal and mesocarpic cells, lysogenic cavities in the subepidermis, degradation of the cuticle and epidermis, and *M. fructicola* sporulation after a 96-hour incubation at 25°C. L: Colonization of the stomata of nectarines with a latent *M. fructicola* infection after a 72-hour incubation at 4°C. M: Increased colonization of the stomata of nectarines with a latent *M. fructicola* infection after a 144-hour incubation at 4°C. N: Colonization of the subdermal tissues by *M. fructicola* with the collapse of the epidermal cells was observed after 216-hour incubation at 4°C. O: Collapse of the epidermal cells and colonization of the subepidermis by *M. fructicola* of nectarines with a latent *M. fructicola* infection after a 288-hour incubation at 4°C. Scale bar = 50 µm.

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Table 1). The number of collapsed epidermal cells increased after 72 hours and this cellular collapse was accompanied by (a) extensive colonization of the deep subdermal tissues by *M. fructicola*, and (b) apparition of lysogenic cavities in the mesocarp (Fig 2G and 2H; Table 1). After 96 hours, the diseased tissues of the nectarines with visible brown rot displayed (a) colonization of the epidermis and mesocarp by *M. fructicola* with thin and thick hyphae, (b) collapse and disruption of epidermal and mesocarpic cells, (c) lysogenic cavities in the subepidermis and mesocarp, (d) degradation of the cuticle and epidermis, and (e) *M. fructicola* sporulation (Fig 2I and 2J; Table 1).

Light microscopic analysis of tissues from nectarines with a latent *M. fructicola* infection

Fig 2K displays representative light microscopic images of epidermal, subepidermal, and mesocarp cells from uninoculated nectarines and Fig 2L–2O display representative images of the histological changes on the surface and parenchyma of nectarines with a latent *M. fructicola* infection. Although no visible signs of infection were seen in nectarines with a latent infection after a 288-hour incubation at 4°C, microscopic signs of the infection became apparent over time. *M. fructicola* colonized the stomata of inoculated fruit after 72 hours (Fig 2L; Table 1) and the extent of this stomatal colonization had increased after 144 hours (Fig 2M; Table 1). Colonization of the subdermal tissues by *M. fructicola* with the collapse of the epidermal cells was observed after 216 hours (Fig 2N; Table 1). After 288 hours, most of the epidermal cells had collapsed and the subepidermis of the inoculated fruit had become colonized by *M. fructicola* (Fig 2O; Table 1).

TEM analysis of tissues from uninfected nectarines and nectarines with a visible *M. fructicola* infection

Figs 3 and 4 display representative micrographs of the ultrastructure of healthy nectarines and nectarines with a visible *M. fructicola* infection. The cuticle of uninfected fruit had a variable thickness and covered a layer of epidermal cells (Fig 3A). The epidermal and hypodermal cells of healthy nectarines have a thick cell wall of variable thickness and a dense cytoplasm with intact organelles (Fig 3A and 3B). The mesocarpic cells of uninfected nectarines were large with a thin cell wall and the intercellular spaces between the mesocarpic cells were also large. In these cells, the cytoplasm was almost completely occupied by a central vacuole (Fig 4A).

In nectarines with a visible infection, conidia and germ tubes began to adhere to the nectarine's cuticle after 24 hours (Fig 3C–3E). The conidia had a two-layered cell wall, a plasma-lemma, and their cytoplasmic matrix was dense with nuclei, mitochondria, and vacuoles (Fig 3C and 3D). Partial degradation and/or dissolution of cuticle and cell wall were observed under germinated *M. fructicola* conidia (Fig 3C–3E). Long branching germ tubes were present on the cuticular surface and some of these tubes had penetrated the cuticle presumably through the

Fig 3. Ultrastructure of tissues from healthy nectarines and nectarines with a visible *M. fructicola* infection after a 24-hour incubation at 25°C. A and B: The cuticle (cu), cell wall (cw) and ultrastructure of epidermal and hypodermal cells of healthy nectarines, scale bar = 5000 nm. C–E: Conidial germination (co) and germ tubes (gt) of *M. fructicola* on the surface of the cuticle of nectarines with a visible infection, scale bars = 2000 nm (C and D) and 500 nm (E). F and G: Inter-cellular hyphae (h) of *M. fructicola*, scale bar = 2000 nm.

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stomatal cavities into epidermal cells (Fig 3E). Thin infective hypha that grew intercellularly (Fig 3F) or intracellularly (Fig 3G) in epidermal cells were observed after 24 hours. Substantial dissolution of the cell walls and degeneration of cytoplasmic organelles were evident in infected epidermal and hypodermal cells (Fig 3F and 3G). Hyphal invasion, cytoplasmic necrosis, and degeneration of cytoplasmic organelles in mesocarpic cells were observed after 72 hours (Fig 4B–4F). The mesocarpic cells were colonized by large intercellular (Fig 4B–4F) and intracellular hyphae (Fig 4B–4D and 4F). Intracellular hyphae, which were close to the cell wall, contain numerous organelles in their cytoplasm, have a double cell wall layer, and are surrounded by vesicles (Fig 4B). The matrix of the mesocarpic cell walls, which were close to intracellular hyphae, appeared fibrillary and disrupted, and groups of vesicles were present near the mesocarpic cell walls (Fig 4B and 4C). The intercellular hyphae, which had invaded mesocarpic cells, were ultrastructurally different from the intracellular hyphae: their cytoplasm contained no vesicles and their cell walls were thick (Fig 4D and 4E).

Discussion

The aim of this investigation was to analyze the microanatomy of nectarines with a latent and a visible *M. fructicola* infection using light microscopy and TEM. Tissues of mature nectarines with latent *M. fructicola* infections are characterized by the presence of intercellular hyphae at the subcuticular level. Although these intercellular hyphae do not penetrate more than the first two subcuticular cell-layers of nectarines with latent infections, they do not remain totally dormant but slowly colonize the tissue. At the same time, the tissues of mature nectarines with visible *M. fructicola* infections are characterized by extensive colonization of the deep subdermal tissues by the hyphae of *M. fructicola*. The tissues and the cells were colonized inter- and intracellularly and this colonization was accompanied by increasing degradation of the cell walls, a typical colonization of necrotrophic fungi.

Germinated conidia with long germ tubes have been described in mature nectarines with brown rot due to *M. fructicola* and this germination is associated with a low stomatal density, reduced hydrophobicity of the cuticle, numerous cuticular cracks and fissures, and the accumulation of volatile compounds [9]. In our study, we observed long germ tubes of *M. fructicola* around the guard cells of stomata on the surface of mature fruit mainly when samples of the epidermis from inoculated nectarines were incubated at 25°C. For *Monilinia* spp., conidial germination, sporulation, growth, and propagule formation on fruit surfaces are dependent on T, RH, and water availability [11,32–36]. We found germination of *M. fructicola* conidia and hyphal formation on the nectarine surface following a 4-hour incubation at 25°C and a 24-hour incubation at 4°C. We also found that this germination and hyphal formation were more abundant in areas around the stomata than in areas without stomata. Our results on *M. fructicola* germination on the fruit surface are similar to those of other investigators who reported that *Monilinia* spp. can penetrate stone fruit through the stomata, the cuticle of the intact surface, the base of the surface hairs and trichomes, and surface wounds [6,37–39].

We did not detect appressoria on the surface or inside epidermal cells of mature nectarines with either a visible or latent *M. fructicola* infection. Other investigators have reported the presence of appressoria on immature fruit and flowers. For example, Lee and Bostock [9] described appressorial formation by *M. fructicola* on immature nectarine surfaces. *M. fructicola* forms

Fig 4. Ultrastructure of tissues from healthy nectarines and nectarines with a visible *M. fructicola* infection after a 72-hour incubation at 25°C. A: The parenchyma of an uninfected nectarine, scale bar = 5000 nm. B, C, and F: Intracellular hyphae of *M. fructicola* in a mesocarpic cell of a nectarine with a visible infection, scale bars = 2000 nm (B and F) and 500 nm. D: The intracellular and intercellular hyphae of an *M. fructicola* in a mesocarpic cell and in the space around a mesocarpic cell of a nectarine with a visible infection, scale bar = 5000 nm. E: The intercellular hypha of *M. fructicola* in a mesocarpic cell of a nectarine with a visible infection, scale bar = 2000 nm.

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non-melanized appressoria for penetrating *Prunus* spp. petals and immature fruit, and appressoria has been proposed as resting structures of latent infections in immature fruit [40]. Other factors, such as unidentified volatile and nonvolatile compounds in the cuticle and/or cell wall, have been implicated for stimulating of appressorial formation by fungal pathogens [41,42].

Although visible evidence of brown rot was not seen on the surface of nectarine with a latent infection, we detected fungal hyphae in the epidermal cells after 144 hours of incubation at 4°C when the tissues of these nectarines were microscopically examined. Rungjindamai et al. [43] suggested that latent infections follow a typical pattern of subcuticular infection of immature fruit followed by rapid cessation of growth of the pathogen. In this investigation, we found slow and continuous growth of the pathogen in nectarine subcuticular tissues with a latent infection under conditions of cold storage. Although no macroscopic brown rot symptoms are observed, *Monilinia* hyphae were visible at subcuticular level and the latent infection could be considered a quiescent infection under optical microscopy conditions according to the definition of Ahimera et al. [14]. It has been reported that activation of a latent infection can be facilitated by large gene families of cell wall-degrading enzymes [44]. Additionally other pathogenic fungi use tissue acidification to activate a latent infection and stimulate the transition of a latent infection to a visible infection [44]. It has also been reported that *M. fructicola* can acidify the tissues of the host and hence up-regulate the expression of polygalacturonase genes, which is controlled by acidic pH as the infected fruit matures [45]. We found cell wall degradation in the epidermal and mesocarpic cells in the nectarines with a visible *M. fructicola* infection. We also found (a) partial degradation or dissolution of cuticle and cell wall areas under germinated conidia, (b) collapse of epidermis cells, and (c) formation of lysogenic cavities on the nectarine mesocarp 72 hours after inoculation from visible *M. fructicola* infections. It has been reported that *M. fructicola* produces cell degrading enzymes, such as cutinase [46], and that this cutinase is important for *M. fructicola* virulence during fungal growth and the development of brown rot lesions in nectarines [47]. It has also been reported that the redox state of nectarines can influence appressorial formation and expression of the *M. fructicola* cutinase *Mfcut1* and polygalacturonase *Mfpg1* genes [40]. Interestingly, Wade and Cruickshank [37] reported a thick mechanical barrier around the infection point, suberized cell walls of the surrounding living cells, and intracellular accumulation of phenolic compounds in apricots with a latent *M. fructicola* infection.

We detected that *M. fructicola* only colonizes the intercellular spaces at subcuticular level in nectarines with a latent infection, whereas hyphae colonize inter- and intracellularly in nectarines with a visible infection. These findings are similar to those which have been reported for corn: the hyphae of *F. moniliforme* and *F. verticillioides* colonize the intercellular spaces in latent infections, whereas their hyphae colonize intracellularly in visible infections [48–50]. Other pathogenic fungi, such as *Phialophora gregata* in soybean [21], or *Colletotrichum acutatum* in their solanaceous hosts [51], have been reported to colonize and survive intercellularly.

A latent brown rot infection is a well-known but poorly understood phenomenon. In this investigation, we found that (a) intercellular hyphal colonization was restricted to the epidermal and two subdermal cell layers in nectarines with a latent *M. fructicola* infection, and (b) no macroscopic evidence of disease was detected in latently infected nectarines even after 216 hours of incubation at 4°C. We also found that intra- and intercellular hyphae colonized all

mesocarp cells in nectarines with visible brown rot. Finally, we also found that *M. fructicola* is active during latent infections because slow and progressive colonization of nectarine cells by the fungus occurs.

Supporting Information

S1 Fig. Germination on and penetration of the surface of mature nectarines by *M. fructicola* after 4 hours of incubation at 25°C.
(TIF)

S2 Fig. Germination on and penetration of the surface of mature nectarines by *M. fructicola* after 24 hours of incubation at 25°C.
(TIF)

S3 Fig. Germination on and penetration of the surface of mature nectarines by *M. fructicola* after 24 hours of incubation at 4°C.
(TIF)

S4 Fig. Light microscopic analysis of tissues from nectarines without *M. fructicola* infection.
(TIF)

S5 Fig. Light microscopic analysis of tissues from nectarines with a visible *M. fructicola* infection after 24 hours of incubation at 25°C.
(TIF)

S6 Fig. Light microscopic analysis of tissues from nectarines with a visible *M. fructicola* infection after 48 hours of incubation at 25°C.
(TIF)

S7 Fig. Light microscopic analysis of tissues from nectarines with a visible *M. fructicola* infection after 72 hours of incubation at 25°C.
(TIF)

S8 Fig. Light microscopic analysis of tissues from nectarines with a visible *M. fructicola* infection after 96 hours of incubation at 25°C.
(TIF)

S9 Fig. Light microscopic analysis of tissues from nectarines with a latent *M. fructicola* infection after 144 hours of incubation at 4°C.
(PDF)

S10 Fig. Light microscopic analysis of tissues from nectarines with a latent *M. fructicola* infection after 256 hours of incubation at 4°C.
(PDF)

S11 Fig. Light microscopic analysis of tissues from nectarines with a latent *M. fructicola* infection after 288 hours of incubation at 4°C.
(PDF)

S12 Fig. Ultrastructure of tissues from healthy nectarines.
(TIF)

S13 Fig. Ultrastructure of tissues from nectarines with a visible *M. fructicola* infection after a 72-hour incubation at 25°C.
(TIF)

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ANNEX 2: FRUIT MATURITY AND POST-HARVEST ENVIRONMENTAL CONDITIONS INFLUENCE THE PRE-PENETRATION STAGES OF *Monilinia* INFECTIONS IN PEACHES

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Fruit maturity and post-harvest environmental conditions influence the pre-penetration stages of *Monilinia* infections in peaches

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ABSTRACT

Brown rot caused by the fungi *Monilinia laxa* (Aderhold and Ruhland) Honey, *M. fructicola* (Winter) Honey, or *M. fructigena* (Aderhold and Ruhland) is a serious fungal disease of peaches. The fungal infection process begins when fungal conidia germinate on the fruit surface to produce germ tubes and/or appressoria, and the incidence of brown rot increases as fruit approaches maturity. The interaction between the fungal infection process, peach maturity, and the environmental conditions is not well understood. Accordingly, the objectives of this investigation were to investigate germ tube and appressorial formation by *M. laxa* and *M. fructicola* when they were exposed to peach skin from mature and immature fruit at various temperatures and relative humidities (RHs). The greatest number of germ tubes was found when *M. laxa* or *M. fructicola* was incubated in culture medium which contained a skin extract of mature peaches. In contrast, the greatest number of appressoria was found when *M. laxa* or *M. fructicola* was incubated in culture medium which contained a skin extract of immature peaches. Although *M. fructicola* produced the same number of germ tubes and appressoria at 4 °C, *M. fructicola* produced more germ tubes than appressoria at temperatures higher than 10 °C, *M. laxa* produced more germ tubes than appressoria at any temperature, except when it was incubated for 48 h on culture medium which contained a skin extract of immature peaches at 10 °C at 80% or 100% RH, or at 25 °C at 60% RH. *M. laxa* conidia germinated better than *M. fructicola* conidia at low temperatures. Germ tube and appressorial formation by *Monilinia* spp. were influenced by fruit postharvest handling. The number of germ tubes that were formed by *M. laxa* conidia was significantly greater than that for *M. fructicola* when the conidia were incubated at 100% RH, and this number increased after 3 days of refrigeration. The number of appressoria that were formed by both *Monilinia* spp. also increased after 3 consecutive days of refrigeration. Negligible or no germination of *M. fructicola* and *M. laxa* conidia occurred when the RH was 60%. We concluded that the dissimilar abilities of *M. laxa* and *M. fructicola* to germinate and form appressoria at low temperatures conferred a competitive advantage to *M. laxa* to survive during fruit postharvest refrigeration and cold storage at 4 °C.

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1. Introduction

Brown rot caused by *Monilinia laxa* (Aderhold and Ruhland) Honey, *M. fructicola* (Winter) Honey, or *M. fructigena* (Aderhold and Ruhland) is a serious fungal disease of peaches (Hong et al., 1997; Mari et al., 2008; Ogawa et al., 1995). Postharvest losses due to brown rot are typically greater than preharvest losses, and routinely occur during handling, storage, and transport (Hong et al., 1997). Since its detection in 2006 in Spain, *M. fructicola* has supplanted *M. fructigena* as the main cause of brown rot and now exists at the same frequency of occurrence as *M. laxa* (Villarino et al., 2013).

The fungal infection process can be divided into three phases: pre-penetration or adhesion, penetration, and colonization. The infection process begins when *Monilinia* conidia germinate on the

fruit surface to produce germ tubes and/or appressoria, which then penetrate the fruit surface depending on the prevailing environmental conditions (Lee and Bostock, 2006). Most studies on appressorial formation by *M. fructicola* have focused on the surface properties of the fruit, the stage of fruit maturity, and the signaling pathways that are required for surface attachment and appressorial formation (Cruickshank and Wade, 1992; Lee and Bostock, 2006). The incidence of brown rot increases as fruit approaches maturity (Gell et al., 2008; Lee and Bostock, 2007; Villarino et al., 2011); immature fruit are more resistant to infection than mature fruit (Gell et al., 2008; Lee and Bostock, 2006, 2007; Xu et al., 2007).

The chemical content of fruit and its epidermis can also influence the first step of the infection process. For example, the content of volatile and phenolic compounds and organic acids in fruit and/or the fruit epidermis changes substantially as fruit approaches maturity. The matrix glycans in the walls of peach mesocarp cells are gradually depolymerized and the polymers are deglycosylated during

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maturation, and the chelator-soluble polyuronides are solubilized and depolymerized after the initiation of ripening (Brummell et al., 2004). Villarino et al. (2011) reported that the susceptibility of peaches to *M. laxa* infection was greatest when the pericarp was completely formed, and the concentrations of chlorogenic and neochlorogenic acid in the pericarp are low. Melanin production by *M. laxa* is inhibited when the concentrations of chlorogenic and neochlorogenic acid in the pericarp are high and melanin is essential for penetration of the pericarp by *Monilinia* spp. (Villarino et al., 2011). The presence of phenolic acids, such as chlorogenic and caffeic acids can inhibit the cutinase and polygalacturonase activity of *M. fructicola* in immature nectarines and peaches. Furthermore, this inhibition is related to changes in the electrochemical redox potentials of *M. fructicola* cultures and alters the intracellular levels of the cellular antioxidant, glutathione (Bostock et al., 1999; Lee and Bostock, 2006). The malic/citric acid ratio in the mesocarp has two maxima during peach maturation. The first maximum occurs in very immature fruit and the second maximum occurs in mature fruit when sucrose levels are at their highest and quinic acid levels are at their lowest (Chapman et al., 1991).

Conidial germination, growth, and sporulation of *Monilinia* spp. are also dependent on ambient temperature, relative humidity (RH), and water availability (water activity: a_w) (Luo et al., 2001; Tamm and Flückiger, 1993; Xu et al., 2001). Casals et al. (2010) reported that more than 80% of viable conidia of *M. fructicola* and *M. fructigena* can germinate within 2 h and those of *M. laxa* can germinate within 4 h at 25 °C and 0.99 a_w on culture media. They also reported that (a) no germination of *M. laxa*, *M. fructicola*, and *M. fructigena* conidia occurred at 38 °C, (b) the conidia of *M. laxa*, *M. fructicola*, and *M. fructigena* can germinate at 0 °C, and (c) the conidia of the three *Monilinia* spp. do not germinate at 0.87 a_w . It has also been reported that (a) the mycelial growth of *M. laxa* is better than that of *M. fructicola* isolates at low temperatures on blossoms and fruit (Papavasileiou et al., 2015; Tamm et al., 1995), (b) *M. laxa* conidia can germinate at temperatures as low as –4 °C on fruit (Tian and Bertolini, 1999), and (c) mycelial growth of *M. laxa* can still occur at 0 °C (Tamm and Flückiger, 1993).

The interaction of fruit maturity and environmental conditions on germ tube and appressorial formation in a *Monilinia* infection is not well understood. Accordingly, the objectives in this investigation were to (i) investigate the effect of mature and immature peach skin extract components, T, and RH on germ tube and appressorial formation by *M. laxa* and *M. fructicola*, and (ii) study the effects of postharvest environmental conditions on germ tube and appressorial formation by *M. laxa* and *M. fructicola*.

2. Materials and methods

2.1. Fungal culture and conidial production

Six 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 investigation (Table 1). All isolates

Table 1
The host, isolation tissue and year of isolation of the six *Monilinia laxa* and *M. fructicola* isolates that were used to study the effects of fruit maturity and post-harvest environmental conditions on the pre-penetration stages of *Monilinia* infection in peaches.

Isolate name	Collection name	<i>Monilinia</i> species	Host	Isolation tissue	Year of isolation
MI1	FLOR2009SUD5.1	<i>M. laxa</i>	Nectarine	Flower	2009
MI2	2014COSFANTASIA8	<i>M. laxa</i>	Nectarine	Fruit	2014
MI3	2014COSFANTASIA9	<i>M. laxa</i>	Nectarine	Fruit	2014
MF1	ALF2009COSSR7	<i>M. fructicola</i>	Peach	Fruit	2009
MF2	ALF2009MOA4	<i>M. fructicola</i>	Peach	Mummified fruit on trees	2009
MF3	SUD2009COS2R6	<i>M. fructicola</i>	Nectarine	Fruit	2009

were originally collected from several commercial peach and nectarine orchards in the Ebro Valley, Lleida, Spain. All isolates were stored as conidial suspensions in 20% glycerol at –80 °C for long-term storage or as cultures on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI, U.S.A.) at 4 °C for short-term storage. For conidial production, (a) the three *M. fructicola* isolates were grown on PDA plates at 20 to 25 °C for 7 days in the dark, and (b) the three *M. laxa* isolates were first grown on PDA plates at 20 to 25 °C for 10 days and then for 5 days at 4 °C in the dark.

Conidial suspensions of all isolates were prepared using conidia that were harvested from the PDA plates and then transferring the harvested conidia into Czapek-Dox broth (Difco Laboratories, Detroit, MI, U.S.A.) or sterile distilled water (SDW). After a 30 s sonication of the conidia in an ultrasonic bath (J.P. Selecta s.a., Barcelona, Spain), the conidial concentration of the suspensions was first determined using a hemocytometer and a light microscope, and then adjusted to a final concentration of 10^4 conidia/mL.

2.2. Bioassays

Germ tube and appressorial formation were measured using previously described protocols (De Cal et al., 1988) with some modifications. Briefly, three sterile glass slides and three moistened filter papers were placed inside 150 mm diameter sterile Petri dishes. A 15 μ L aliquot of an *M. fructicola* or *M. laxa* conidial suspension was mixed with a 30 μ L aliquot of culture medium on each glass slide. Germinated *Monilinia* conidia can form either germ tubes or appressoria. A germ tube was considered to be formed when a slender hypha with pointed ends, which was longer than the minor diameter of the conidium, was formed. An appressorium was considered to be formed when swollen structures, that were mostly oval-shaped, were formed instead of slender hyphae with pointed ends (Cruickshank and Wade, 1992; Lee and Bostock, 2006). For the bioassays which are described below, three replicates of each condition were done on 50 randomly selected conidia in each replicate and each bioassay was repeated at least twice with each studied isolate.

To determine the effects of temperature, RH, and fruit maturity on germ tube and appressorial formation, conidial suspensions in Czapek-Dox broth were incubated with a skin extract from immature or mature peaches at 4, 10, 25, and 35 °C for 24, 48 and 72 h in the dark at an RH of 60%, 80%, and 100%. For preparing the peach skin extracts, the skins of fungicide-free 'Rojo Albesa' peaches were collected using a previously described protocol (Tomas-Barberan et al., 2001). The skins were first manually peeled with a razor blade, and then ground into powder in liquid nitrogen with a sterilized pestle and mortar. The resultant powders were transferred to a 50 mL Falcon tube, weighed, and mixed with ethanol, MilliQ water, and beach sand (3.6 g of powder was mixed with 5.76 mL 100% ethanol, 1.44 mL MilliQ water, and 2 g of beach sand). The mixture was then homogenized twice at a speed of 6.0 m/s for 60 s using a high-speed benchtop tissue homogenizer (FastPrep®-24 Instrument, MP Biomedicals, Solon, Ohio, U.S.A.). The homogenates were then transferred to sterile tubes which were centrifuged at 10,000 rpm for 20 min at 4 °C in a Sorvall RC-5C Plus centrifuge (Kendro Laboratory Products, Newton, Connecticut, U.S.A.). When the centrifugation was complete, the supernatant was carefully collected, transferred to a new sterile 50 mL Falcon tube, and stored at –20 °C until required. Immature peaches were collected at BBCH = 79, 76 days before harvest (4 July) and mature peaches at BBCH = 89 harvest date (18 September) (BBCH, general scale from Biologische Bundesanstalt, Bundessortenamt and Chemische Industrie, Germany; Meier et al., 1994). The three RHs were created by preparing different mixtures of distilled water (DW) and glycerol using a previously described protocol (Forney and Brandl, 1992). Briefly, DW without glycerol was used to create the 100% RH environment and 104.3 mL or 209.3 mL of glycerol were mixed with 1 L DW (final volume) to create the 80% RH and 60% RH environments, respectively.

The environmental conditions under which harvested fruit are processed and stored vary in different fruit processing and storage centers. To determine the effects of those environmental conditions on germ tube and appressorial formation, a mixture of Czapek-Dox broth and the *M. fructicola* (Mf1) or *M. laxa* (Ml1) (Table 1) conidial suspensions in SDW was first incubated at 4 °C for 0, 1, or 3 days in the dark at an RH of either 60% or 100% to simulate refrigeration before fruit processing. The mixtures were then incubated for 20 min at 15 °C to simulate fruit processing conditions at an RH of either 60% or 100%. At the end of the incubation, the mixtures were incubated at 4 °C for 0, 3, or 10 days in the dark at an RH of either 60% or 100% to simulate the cold storage conditions until distribution to the wholesalers and markets.

2.3. Data analysis

Data on the effects of temperature, RH, fruit maturity, and the duration of refrigeration and cold storage of harvested fruit at 4 °C on germ tube and appressorial formation were analyzed by a multiway analysis of variance (Snedecor and Cochran, 1980). Data were arcsine transformed before the analysis in order to improve the homogeneity of variance. When F-test was significant at $P \leq 0.05$, the means were compared using the Student-Newman-Keuls multiple range test.

The linear regression models, $f(T, RH, it)$, germ tube formation (%), and appressorial formation (%) were used to investigate the relationship between germ tube or appressorial formation and temperature (T), RH, and incubation period (it) for each culture medium which contained the skin extracts of mature and immature peaches and fungal species. In these models, $f(T, RH, it)$ is a linear function of T, RH, it, TRH , Tit , T^2 , RH^2 , it^2 , $TRHit$, T^3 , T^2RH , T^2it , RH^3 , TRH^2 , RH^2it , it^3 , Tit^2 , and $RHit^2$, whose values were estimated by the regression analysis. Data were fitted to the model using the Regression Model Selection procedure of Statgraphics Centurion XVI for Windows, Version 16.1.03 (StatPoint Technologies, Inc., Herndon, VA, U.S.A.). Selection of the final model was performed according to the significance of the estimated parameters and the adjusted coefficient of determination.

3. Results

3.1. The effects of temperature, RH, and fruit maturity on germ tube and appressorial formation

M. fructicola and *M. laxa* conidia emitted germ tubes and/or produced appressoria at different temperatures and RHs. The greatest effects of temperature, RH, and fruit maturity on germ tube and appressorial formation by *M. fructicola* and *M. laxa* isolates were seen after a 48 h incubation period (Fig. 1).

The number of germ tubes and appressoria were the same when *M. fructicola* conidia were incubated at 4 °C for 48 h at 60% and 80% RH (Fig. 1a and b). The number of germ tubes was greater than the number of appressoria when *M. fructicola* conidia were incubated at 10 or 25 °C for 48 h at all tested RHs (Fig. 1a and b). For *M. laxa* the number of germ tubes was greater than the number of appressoria at all tested temperatures, except when the conidia were incubated in culture medium which contained a skin extract of immature peaches at 10 °C for 48 h at 80% or 100% RH, and at 25 °C for 48 h at 60% RH (Fig. 1c and d). Maximum germ tube formation occurred for both species at 25 °C and 100% RH when the conidia were incubated in culture medium that contained a skin extract from either mature or immature peaches (Fig. 1a and c). Little or no germ tube formation by *M. fructicola* conidia was recorded for 48 h of incubation at (a) 4 °C and 60% or 80% RH, (b) 10 °C and 60% RH, and (c) 35 °C and 60% RH (Fig. 1a). Little or no germ tube formation by *M. laxa* conidia was recorded at (a) 4 °C and 60% RH, and (b) 35 °C at all tested RHs (Fig. 1c).

When *M. fructicola* and *M. laxa* conidia were incubated in culture medium that contained a skin extract of mature peaches, the number

of germ tubes was greater than that formed when the conidia of both species were incubated in culture medium that contained a skin extract of immature peaches (Fig. 1a and c). Specifically, statistically significant differences in the number of germ tubes were found after a 48 h incubation at (a) 4 °C and 100% RH, (b) 10 °C and 80% RH, and (c) 25 °C and 80% RH for *M. fructicola* (Fig. 1a), and at (a) 4 °C and 80% or 100% RH, (b) 10 °C and 80% or 100% RH, and (c) 25 °C and 60% RH for *M. laxa* (Fig. 1c).

We found that the numbers of appressoria that were produced by *M. fructicola* and *M. laxa* conidia (Fig. 1b and d) were lower than the numbers of germ tubes that were emitted by *M. fructicola* and *M. laxa* conidia (Fig. 1a and c). We also found that the number of appressoria that were produced by *M. laxa* was greater than that for *M. fructicola* conidia at (a) 10 °C for 48 h at 80% and 100% RH, and (b) 25 °C for 48 h at 60% RH (Fig. 1b and d). The maximum number of appressoria that were produced by *M. fructicola* and *M. laxa* occurred when the temperature and RH were not optimal for germ tube formation, namely at 10 °C for 48 h at 60% RH (Fig. 1). The number of appressoria that were produced by *M. fructicola* conidia was significantly greater when the conidia were incubated in culture medium which contained a skin extract of immature peaches than one containing a skin extract of mature peaches. Specifically, this difference was found after a 48 h incubation at (a) 10 °C and 100% RH and (b) 25 °C and 60% RH (Fig. 1b). The number of appressoria that were produced by *M. laxa* conidia when the conidia were incubated in culture medium which contained a skin extract of immature peaches was significantly greater than when the conidia were incubated in culture medium which contained a skin extract of mature peaches. Specifically, this difference was found at (a) 10 °C and 100% RH and (b) 35 °C and 80% RH (Fig. 1d).

3.2. Linear regression models of germ tube formation by *M. fructicola* and *M. laxa*

The linear regression models for germ tube formation by *Monilinia* spp. at different temperatures (T), RHs, and incubation periods (it) for the two types of skin extracts and the two fungal species are shown in Table 2. Linear regression models for appressorial formation were not generated.

3.3. Effects of the environmental conditions of harvested fruit on germ tube and appressorial formation by the *M. fructicola* (Mf1) and *M. laxa* (Ml1) isolates

Tables 3 and 4 summarize the effects of the environmental conditions of harvested fruit on germ tube and appressorial formation by the Mf1 and Ml1 isolates. Although Mf1 and Ml1 conidia were able to germinate in Czapek-Dox broth under all simulated refrigerated conditions, the number of germ tubes that were formed by Ml1 conidia was significantly bigger than that for Mf1 conidia when the conidia were incubated at 100% RH (Table 3). Negligible or no germination of Mf1 and Ml1 conidia occurred when the RH was 60% (Table 3). We also found that (a) the number of germ tubes that were formed by Ml1 conidia increased after 3 days of refrigeration when the RH was 100% and (b) reached its maximum after 10 days of cold storage (Table 3). The numbers of appressoria produced by Mf1 and Ml1 at an RH of 100% were greater than the number that were produced by these two isolates at an RH of 60%. The number of appressoria that were formed by Mf1 increased after 3 consecutive days of refrigeration for each cold storage duration (Table 4), and the numbers of appressoria were always greater than those for Ml1.

4. Discussion

In this investigation, we found that germ tube and appressorial formation by *Monilinia* spp. are influenced by temperature, RH, and the maturity stage of the fruit. The greatest number of germ tubes was

Fig. 1. Germ tube and appressorial formation by *Monilinia* spp. which were incubated for 48 h at different temperatures and relative humidities in culture medium which contained a skin extract of either immature or mature peaches. Germ tube (a and c) and appressorial (b and d) formation by *M. fructicola* (a and b) and *M. laxa* (c and d) in culture medium which contained a skin extract of immature (■) or mature (□) peaches after a 48-hour incubation at different temperatures (4, 10, 25, and 35 °C) and relative humidities (60%, 80%, and 100%). Data on the number of germ tubes and appressoria are displayed as a mean percentage of 50 randomly selected conidia of each of the three *M. fructicola* and *M. laxa* isolates. Groups of columns with an asterisk (*) represent statistically significant differences at a 95% confidence level according to the results of the Student-Newman-Keuls multiple range test.

found when *M. fructicola* and *M. laxa* conidia were incubated in culture medium that contained a skin extract of mature peaches. This finding is different from that for appressorial formation: the greatest number of appressoria was found when *M. fructicola* and *M. laxa* conidia were incubated in culture medium that contained a skin extract of immature peaches. Although the optimal environmental conditions for germ tube formation (25 °C and 80 to 100% RH) by *M. fructicola* and *M. laxa* are similar, we found that the number of germ tubes formed by

M. fructicola conidia was greater than that for *M. laxa* conidia under these conditions. Casals et al. (2010) reported that the maximum germination of *M. laxa* and *M. fructicola* conidia occurred when temperature was 25 °C and RH was 99%. However, no conidial germination was recorded by these authors when RH was lower than 87%. Our results are in agreement with those of Papavasileiou et al. (2015) who found that maximum germination of *M. laxa* and *M. fructicola* conidia occurred when the isolates were grown on PDA and the temperature was

Table 2

Linear regression models which describe the relationship between temperature (T), relative humidity (RH) and incubation period (it) and germ tube formation (y) by *M. fructicola* and *M. laxa* conidia that were incubated in culture medium which contained a skin extract of either immature or mature peaches.

<i>Monilinia</i> specie	Type of peach skin extract	Equation	R ² values
<i>M. fructicola</i>	Immature peach	$y = -45.213 + 0.381 RH + 0.099 T + 0.211 T^2 - 0.003 T^3 - 0.006 T^4$	0.62
	Mature peach	$y = -50.206 + 0.490 RH + 0.119 T + 0.202 T^2 - 0.003 T^3 - 0.005 T^4$	0.64
<i>M. laxa</i>	Immature peach	$y = -39.638 + 0.407 RH + 0.109 T + 0.139 T^2 - 0.003 T^3 - 0.004 T^4$	0.55
	Mature peach	$y = 5.340 - 2.110 T + 0.213 T^2 + 0.025 RH - 0.005 T^3 - 0.0006 TRH$	0.61

The regression models were obtained from the number of germ tubes that were formed by 50 randomly selected conidia of each of the three *M. fructicola* and *M. laxa* isolates. The parameters of the equations are given in the following units: temperature (°C), RH (%), and incubation period (hours).

Table 3

The effect of refrigeration and cold storage on germ tube formation by *Monilinia fructicola* and *M. laxa* isolates^a.

Specie	RH (%)	Duration of refrigeration before processing (days)	Duration of cold storage after processing (days)		
			0	3	10
<i>M. fructicola</i>	60	0	2.0 a	2.0 a	1.3 ab
		1	2.0 a	2.7 a	0.0 a
		3	0.7 a	1.3 a	0.0 a
	100	0	2.0 a	0.7 a	3.3 ab
		1	0.0 a	2.0 a	0.0 a
		3	2.0 a	1.3 a	1.3 ab
<i>M. laxa</i>	60	0	0.0 a	0.7 a	6.7 ab
		1	0.7 a	0.7 a	9.3 b
		3	0.7 a	3.3 a	2.0 ab
	100	0	0.0 a	53.3 b	72.0 c
		1	0.7 a	64.0 bc	87.3 d
		3	60.0 b	73.3 c	87.3 d
MSE ^c			(27.5)	(31.5)	(28.2)

The two post-harvest environmental conditions that were tested are refrigeration for 0, 1, or 3 days before fruit processing and cold storage for 0, 3, or 10 days at 4 °C in the dark at a relative humidity (RH) of either 60% or 100%.

Data on the number of germ tubes are expressed as a percentage of 50 randomly selected conidia of one *Monilinia fructicola* isolate (Mf1) or one *Monilinia laxa* isolate (Ml1) (Table 1) which formed a germ tube and are the mean of three replicates. Experiments were repeated at least twice.

^a Means with the same letter in each column are not significantly different from each other according to the results of the Student–Newman–Keuls multiple range test ($P < 0.05$). Data were arcsine transformed before analysis.

^c MSE = mean square error.

between 10 and 25 °C. Tamm and Flückiger (1993) have also reported that maximum germination of *M. laxa* conidia occurred when temperature was between 15 and 25 °C and RH was 100%.

We found that (a) *M. laxa* conidia emit germ tubes when the temperature is 4 °C and the RH is between 80% and 100%, and (b) *M. fructicola* conidia are not able to emit germ tubes under these conditions. These results are in accordance with those that were reported by Tamm and Flückiger (1993) and Tian and Bertolini (1999), who found that *M. laxa* conidia can germinate when the temperature is as low as −4 °C. The ability of *M. laxa* conidia to germinate at low

temperatures has also been reported by Watson et al. (2002), Casals et al. (2010) and Papavasileiou et al. (2015) who reported that *M. fructicola* conidia can germinate at low temperatures, namely 0 and 5 °C. Papavasileiou et al. (2015) measured conidial germination of five different *M. fructicola* and *M. laxa* isolates 6 days after plating on PDA. They found that (a) germination of the conidia of the two *Monilinia* species was greater than 70%, and (b) mycelial growth of the *M. laxa* isolates was greater than that of the *M. fructicola* isolates after a 6 day incubation period at 5 °C. Accordingly, we surmise that our short incubation periods could account for higher levels of germination of *M. laxa* conidia than *M. fructicola* conidia at low temperatures.

We found that little or no germ tube and appressorial formation occurred at 35 °C for both *Monilinia* spp. These results were similar to those of Papavasileiou et al. (2015) who reported that the conidia of five *M. laxa* isolates and five *M. fructicola* isolates did not germinate on PDA at 35 °C. However, Casals et al. (2010) reported that the germination of conidia of three different *Monilinia* isolates, one *M. laxa* isolate, one *M. fructigena* isolate, and one *M. fructicola* isolate, occurred at 35 °C, but not at 38 °C. A possible reason for the difference between these three sets of results could be due to the use of only one *Monilinia* isolate of each species.

We found that the environmental conditions of harvested fruit before, during, and after their processing did not prevent germ tube and appressorial formation by *M. fructicola* and *M. laxa*. High RH in the refrigeration chambers is required to maintain fruit quality. We found that a high RH increased the germ tube formation by *M. laxa* and *M. fructicola* conidia, and that this increase in germ tube formation at a high RH was very evident for *M. laxa*. The ambient temperature in washing and sorting units is between 15 and 25 °C and the washing and sorting of harvested fruit takes between 1 and 6 h. We found that a cold stop in fruit refrigeration could reduce appressorial formation by *M. fructicola* conidia, which needs 3 consecutive days of refrigeration or cold storage.

Germ tube formation by *M. laxa* and appressorial formation by *M. fructicola* occurs during the first stage of the infection process. We found that appressorial formation by *M. fructicola* increased when the temperatures and/or RHs for conidial germination were not optimal. These results differ from those on appressorial formation by other phytopathogenic ascomycetes, such as *Colletotrichum acutatum*. In this species, the formation of melanised appressoria decreased when temperatures and/or RHs for conidial germination were sub-optimal (Miles et al., 2013). This difference between the results of our study and those from Miles et al.'s study suggests that pathogenic *C. acutatum* requires an appressorium to penetrate its hosts, and that *M. fructicola* need an appressorium to penetrate fruit only when the environmental conditions are unfavorable. Lee and Bostock (2006) proposed that the appressoria of *M. fructicola* facilitates its penetration into immature fruit and could even be a resting or a non-pathogenic phase of the pathogen when unfavorable environmental conditions for infection were present. We also found that the rate of appressorial formation by *M. laxa* was higher than that of *M. fructicola* when we investigated the effects of temperature, RH, and fruit maturity on appressorial formation. Although we did not detect such a difference when we investigated the effects of the environmental conditions of harvested fruit on *M. laxa* appressorial formation before, during, and after fruit processing, we attribute this difference in the rates of appressorial formation to the utilization of the nutrients in the Czapeck–Dox broth by *M. laxa* and *M. fructicola* conidia. Lee and Bostock (2006) reported that the composition of nutrient media, such as CV8 medium, can induce the germination of already-formed appressoria.

When our results are appraised together with those of Papavasileiou et al. (2015), we concluded that *M. laxa* and *M. fructicola* have their own competitive edge for conidial germination and mycelial growth in different environmental conditions. We also surmise that the competitive edge of each species in different environmental conditions accounts for the coexistence of *M. laxa* and *M. fructicola* in Spanish peach and

Table 4

The effect of refrigeration and cold storage on appressorial formation by *Monilinia fructicola* and *M. laxa* isolates^a.

Specie	RH (%)	Duration of refrigeration before processing (days)	Duration of cold storage after processing (days)		
			0	3	10
<i>M. fructicola</i>	60	0	0.0 a	0.0 a	1.3 a
		1	0.7 a	2.0 a	4.0 a
		3	0.0 a	4.7 ab	5.3 a
	100	0	0.0 a	2.0 a	38.0 c
		1	2.0 a	10.7 ab	33.0 c
		3	13.3 c	30.0 c	70.0 d
<i>M. laxa</i>	60	0	0.0 a	0.0 a	0.0 a
		1	0.0 a	0.0 a	0.0 a
		3	0.0 a	0.0 a	0.0 a
	100	0	0.0 a	9.3 ab	24.0 b
		1	0.0 a	4.0 ab	11.3 a
		3	8.0 b	18.0 b	11.3 a
MSE ^b			(1.8)	(11.8)	(8.6)

The two post-harvest environmental conditions that were tested are refrigeration for 0, 1, or 3 days before fruit processing and cold storage for 0, 3, or 10 days at 4 °C in the dark at a relative humidity (RH) of either 60% or 100%.

Data on the number of appressoria are expressed as a mean percentage of 50 randomly selected conidia of one *Monilinia fructicola* isolate (Mf1) or one *Monilinia laxa* isolate (Ml1) (Table 1) which formed an appressorium and are the mean of three replicates. Experiments were repeated at least twice.

^a Means with the same letter in each column are not significantly different from each other according to the results of the Student–Newman–Keuls multiple range test ($P < 0.05$). Data were arcsine transformed before analysis.

^c MSE = mean square error.

nectarine orchards. This competitive edge becomes advantageous for *M. fructicola* when temperature is between 10 and to 25 °C and when temperature is between 4 and 10 °C for *M. laxa*. These individual competitive advantages could explain (a) the incomplete and slow supplanting of *M. laxa* by *M. fructicola*, even though *M. fructicola* is more aggressive and grows faster than *M. laxa* under optimum growing conditions (25 °C and 100% RH) (Villarino et al., 2016), and (b) the different adaptabilities to postharvest conditions of *M. fructicola* and *M. laxa*.

Accordingly, we suggest that the incidence of brown rot in harvested peaches could be reduced by shortening the period between fruit arrival times and their processing at fruit processing units, reducing RH in the refrigeration chambers, and shortening the duration of cold storage. Incorporating these measures and practices into the *modus operandi* of the handling of harvested peaches should create the environmental conditions that constrain conidial germination of *Monilinia* spp. Reducing RH in the refrigeration chambers could potentially reduce peach quality by causing fruit dehydration. Hence, further investigations are needed to determine the optimum RH for reducing conidial germination of *Monilinia* spp. without adversely affecting fruit quality.

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ANNEX 3: DETECTION OF LATENT

***Monilinia* INFECTIONS IN**

NECTARINE FLOWERS AND FRUIT

BY QPCR

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*EPIDEMIOLOGÍA Y CONTROL DE LA PODREDUMBRE PARDA DEL
MELOCOTÓN EN POSTCOSECHA*

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

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Abstract

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

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

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

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

Moreover, detecting a latent *Monilinia* infection in stone fruit is crucial for preventing spread of the pathogenic fungus to countries and/or regions free from pathogens.

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

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

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

Materials and Methods

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

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

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

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

Establishment of an artificial latent *Monilinia* infection in nectarine flowers. Due to the almost nonexistent occurrence of natural flower latent infections by *Monilinia* in Spanish conditions

(Villarino et al. 2012), artificial latent infections were conducted to ensure *Monilinia* detection. Two hundred and ninety nectarine flowers without any visible signs of brown rot were collected from a 'Romea' nectarine tree in the Jerte Valley, Cáceres, Spain, in 2014. After their collection, the flowers were first surface-disinfected (previously described on *M. fructigena* conidial production). After drying, the flowers were randomly divided in groups of 10. Each nectarine flower in each group was inoculated placing inside the calyx a $30\ \mu\text{l}$ conidial suspension (10^6 conidia ml^{-1}) drop of one of the *M. fructicola*, *M. fructigena*, and *M. laxa* isolates using a micropipette (approximately 780 pg DNA). After inoculation, the flowers were incubated in sterile humidity chambers, i.e., polystyrene boxes, which were lined with moist sterilized filter paper, at 25°C and 100% RH for 24 h in the dark. At the end of the incubation period, the flowers were again surface-disinfected and air-dried in a laminar flow hood, as previously described. After drying, the flowers were incubated in humidity chambers at 4°C and 100% RH for 5 days in the dark. The control group consisted of 10 flowers, each of which was inoculated with $30\ \mu\text{l}$ SDW.

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

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

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

^a Tissue with latent infection.

^b Mummified fruit from a tree.

^c Mummified fruit on orchard bed.

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

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

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

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

Latent *Monilinia* infections in the flowers and fruit were detected applying the qPCR-based method and hydrolysis probes of van Brouwershaven et al. (2010) with modifications as follows. The hydrolysis probes were labeled with different reporter dyes and quenchers and all primers and probes were obtained from Integrated DNA Technologies Inc. (Coralville, IA). Specifically, we used a FAM reporter dye and a ZEN/Iowa Black FQ quencher for *M. fructicola* probe (P_{fc}) instead of a FAM-TAMRA, and a HEX reporter dye with a ZEN/Iowa Black FQ quencher instead of a VIC-TAMRA quencher for *M. fructigena* and *M. laxa* probe (P2_{fgn/lx/ps}). Genomic DNA from the flowers (20 ng) or the three fruit areas (10 ng) was amplified in 20- μl reaction mixture in each well of a 96-well clear optical reaction plate (Applied Biosystems, Foster City, CA) sealed with a clear adhesive. The 20- μl reaction mixture contained 1 $\times$ GoTaq probe qPCR Master Mix (Promega Corporation, Madison, WI), 200 nM of each of the primers (Mon139F and Mon 139R), and 200 nM of each of the probes (P_{fc} and P2_{fgn/lx/ps}). Thermal cycling was done using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) under the same conditions and using the same thresholds described by van Brouwershaven et al. (2010) with slight

modifications: polymerase activation at 95°C for 10 min, followed by 40 amplification cycles at 95°C for 15 s and 60°C for 1 min. Emission was measured at the annealing-extension step. The threshold value was set at fluorescence (ΔRn) of 23,000. A quantification cycle (C_q) value below 40 was scored as a positive detection. Additionally, an allelic discrimination step was added due to cross-detection of *M. laxa* and *M. fructigena* when using the P_{fc} probe. Allelic discrimination allowed us to distinguish between the *M. fructicola* isolates, the *M. fructigena* isolates, the *M. laxa* isolates, and mixtures of the *Monilinia* isolates with absolute certainty. The allelic discrimination step measures fluorescence in each well of the qPCR plate before and after qPCR amplification, and places each sample in an X-Y graph according to these results. The *M. fructicola* samples are placed on a line parallel to the Y-axis on top of the vertex formed by the negative controls while the *M. fructigena*/*M. laxa* samples are placed on a line parallel to the X-axis and to the right of the vertex formed by the negative controls, and the mixtures of *M. fructicola* and *M. fructigena*/*M. laxa* appear between both axes with the bisectrix indicating mixtures of equal amounts.

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

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

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

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

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

Results

Standard curves characteristics. Standard curves for the two hydrolysis probes (P_fc [*M. fruticola*] and P2_fgn/lx/ps [*M. fructigena*, *M. laxa*, and *M. polystroma*]) were done to assess the efficiency of the qPCR method in each matrix (milli-Q water, nectarine flower DNA, nectarine fruit epidermis DNA, and nectarine fruit mesocarp DNA) (Table 2). A low limit of detection (less than 27 fg of DNA [approximately 1 *M. laxa* cell]) was achieved with the different probes for all the different matrices (Table 2). With the exception of the standard curve for *M. fruticola* in flowers that have an amplification efficiency of 87%, the rest of the standard curves had optimal amplification efficiencies between 90 and 100% (Table 2). Additionally, the fit of the generated data to the regression line was high, as measured by value of the R^2 coefficient that was greater than 0.99 for all curves (Table 2).

Detection of a latent *Monilinia* infection in nectarine flowers by qPCR. DNA from the *M. fruticola*, *M. fructigena*, and *M. laxa* isolates was not detected in the five control-uninoculated flowers. The median amounts of DNA of each *M. fruticola*, *M. fructigena*, and *M. laxa* isolate in the latently infected flowers are displayed in Figure 1. The median amounts of DNA from *M. fructigena* and

M. laxa species were similar, 0.10 ± 0.05 pg (median $\pm$ standard error) and 0.13 ± 0.06 pg, respectively, and lower than the median amount of DNA from *M. fruticola* 18.32 ± 8.47 pg. The median amount of DNA from *M. fruticola* 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. fruticola* 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 0.004 ± 0.006 pg and 0.57 ± 0.22 pg (Fig. 1).

Detection of latent *Monilinia* infection in nectarines by qPCR. Latent brown rot infections in the epidermis and the mesocarp of inoculated nectarines that were caused by *M. fruticola* and *M. laxa* isolates were successfully detected by qPCR. DNA from the *M. fruticola* and *M. laxa* isolates was not detected in the epidermis and the mesocarp of two out of the three control-uninoculated nectarines from 2014 and the three control-uninoculated nectarines from 2015. The third control-uninoculated nectarine from 2014 had a natural latent *Monilinia* infection because the qPCR detected *M. fruticola* DNA in its epidermis (2.27 ± 0.23 pg DNA). The median amounts of DNA from each *M. fruticola* and *M. laxa* isolate in the epidermis, the external mesocarp, and the internal mesocarp of latently infected nectarines where similar inside each specie and those corresponding to isolate Mfc3 and Mlx4 are shown on Figure 2; additionally, the values for all of the isolates are shown in Supplementary Figure S2. For the *M. fruticola* and *M. laxa* isolates, the amounts of their DNA detected in the epidermis were greater than those in the external and internal mesocarp of latently infected nectarines, except for Mlx2 and Mlx8. The amounts of DNA from the *M. fruticola* in the epidermis of latently infected nectarines ranged between 0.25 ± 0.03 pg and 6.33 ± 0.60 pg, and were similar to those from the *M. laxa* isolates that ranged between 0.01 ± 0.004 pg and 9.10 ± 3.26 pg (Fig. 2). In contrast, the amount of DNA from the *M. fruticola* isolates in the mesocarp of latently infected nectarines ranged between 0.0002 ± 0.0003 pg and 0.004 ± 0.001 pg, and

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

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

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

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

was significantly lower than that of the *M. laxa* isolates that ranged between 0.005 ± 0.01 pg and 2.10 ± 0.72 pg (Fig. 2). There were no significant differences between external and internal mesocarp median DNA amounts, except in the latent infections caused by Mfc9, Mlx3, Mlx4, Mlx5, and Mlx6 isolates, external mesocarp *Monilinia* DNA amount was greater than the internal mesocarp *Monilinia* DNA amount.

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

Five out of 28 *Monilinia* isolates latently infecting flowers, four *M. fructicola*, and one *M. laxa* isolates, were detected by ONFIT, whereas qPCR detected all *Monilinia* isolates latently infecting flowers, except for isolate Mlx10 (Table 3). Eighteen of the isolates were detected in all infected flowers by qPCR (100%), while only isolate Mfc8 was detected in all latently infected flowers by ONFIT (Table 3).

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

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

Discussion

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

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

early, rapid, and accurate detection of latent brown rot infections in the field could be useful for developing disease prediction models and improving the timing of application and efficacy of preharvest control methods. Furthermore, the early, rapid, and accurate detection of latent brown rot infections might help producers and wholesalers choose fruit intended for long-term storage or transported to distant markets, as suggested by Sanzani et al. (2012) for the control of *Botrytis cinerea* in table grapes.

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

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

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

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

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

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

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; Iosif and Frey 2000; van Brouwershaven et al. 2010). We found that the qPCR-based method can detect the pathogen in artificial and natural latent brown rot infections. Many authors have reported that qPCR has a higher sensitivity and test specificity than conventional PCR for detecting and quantifying the DNA of soil-borne fungi, oomycetes, bacteria, nematodes, viruses, and phytoplasmas (Baric et al. 2006; Ippolito et al. 2004; Lievens et al. 2006; Schena et al. 2004, 2013). PCR-based methods are also considered the most effective method for detecting infectious microorganisms with a low titer and an uneven distribution in plants, such as apple proliferation phytoplasma (Baric et al. 2006).

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

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

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

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